REGULATION OF THE HUMAN BETA-INTERFERON PROMOTER

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Abstract

Beta-interferon (IFN-β) is secreted from virus-infected cells to protect neighbouring cells from subsequent infection. In uninduced cells, IFN-β production is repressed, whereas upon induction expression reaches high levels. Expression of IFN-β is controlled at the level of transcriptional initiation. The changes in transcription rates are governed by a promoter, the complexity of which reflects its stringent regulation. Previous studies have indicated that the preinduction repression of the IFN-β promoter is maintained by sequence-specific transcriptional repressors, although their identity has remained elusive. In contrast, several proteins have been identified that produce increased transcriptional rates upon induction.

In this work, we have studied several aspects in regulation of the IFN-β promoter, with the emphasis on factors contributing to preinduction repression. We have identified candidate proteins that have properties of preinduction repressors: They bind to the genetically defined negative regulatory elements, and their DNA affinities are decreased upon induction. The transcription factor Oct-1 is one such candidate repressor. We have investigated the modifications that may lead to its affinity decrease. We have established that the Oct-1 protein levels remain unchanged upon induction. The DNA binding domain of Oct-1 can be phosphorylated by nuclear kinases, specifically by protein kinase A; however, this phosphorylation alone cannot account for the decrease in DNA affinity. We have established a transient transfection system, which allows the detection of preinduction repression of the promoter and, using this, studied the consequences of the modulation of intracellular levels of the Oct-1 protein. While we have obtained evidence that Oct-1 can have a repressive effect on the IFN-β promoter, our analysis has also revealed an unexpected degree of complexity in the regulatory properties of Oct-1. The Un1 and Un2 complexes are further candidates for preinduction repressors. We have studied their DNA binding specificity and shown that their DNA binding can be regulated by their phosphorylation status. The results of the purification of the polypeptide components of Un1 and Un2 are presented. Finally, an analysis of the positive regulatory domain IV and an investigation into the involvement of protein kinase A in the signalling pathways leading to the induction of the promoter, are presented.
DEDICATION

This thesis is dedicated to
My Mother

Tämä kirja on omistettu
Äidilleni
Acknowledgements

- There is more to life than books
  but not much more. -

I am grateful to my supervisor Steve Goodbourn for his scientific wisdom, for allowing me the privilege of using my own brains in addition to my own hands, for guidance through the jungle of international bureaucracy, for teaching me a few more useful survival techniques, and for his friendship.

I thank Cath for challenging as well as appreciating me as a scientist, for sharing many precious moments in Soho, for understanding the reasons for my ongoing personal and scientific frustration, and above all, for her love.

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I thank Chriz and Melanie for contributing to my survival, more than they know.

I thank multiple other individuals I have encountered in this alien country for having a drink, a conversation, an argument, a trip of any kind, or various combinations of these with me. These include many girls (Frances, Charlotte, Bea, Maria, Arlene) and many boys (Jaz, Patrik, Ned, Jim, Jan, Volker, Simon, Peter, Ken) and many inbetweens.

I dedicate my thesis and the other few achievements in my life to my best friend, My Mother, for thousands of reasons, the most important of which is that I love her. Kiitos Aizy.

I will always remain envious of the width of scientific perspective of Prof. Olli Halkka in the Department of Genetics, at the University of Helsinki.

I warmly acknowledge the enormous amount of continuous encouragement provided by Prof. Kari Cantell. If I ever equal his benevolence combined with scientific excellence, I will have achieved far more than this degree.

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I thank the Academy of Finland, Imperial Cancer Research Fund and St. George's Hospital Medical School for an opportunity.

I thank anyone who has ever liked me, even if only transiently.

- And if you must go to work tomorrow,
  well, if I were you, I would not bother.
  For there are brighter sides to life.
  And I should know, because I have seen them.
  But not very often. -

(I thank S.P.Morrissey for the quotations.)
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<tr>
<td>ATP</td>
<td>adenosine 5'-triphosphate</td>
</tr>
<tr>
<td>β-gal</td>
<td>beta-galactosidase</td>
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<tr>
<td>bp</td>
<td>base pair(s)</td>
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<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>bZIP</td>
<td>basic zipper</td>
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<tr>
<td>cAMP</td>
<td>adenosine 3':5'-cyclic monophosphate</td>
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<tr>
<td>CAT</td>
<td>chloramphenicol acetyltransferase</td>
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<tr>
<td>cDNA</td>
<td>complementary DNA</td>
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<tr>
<td>CIP</td>
<td>calf intestinal phosphatase</td>
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<td>CKII</td>
<td>casein kinase II</td>
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<td>CTP</td>
<td>cytidine 5'-triphosphate</td>
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<td>dATP</td>
<td>2'-deoxyadenosine 5'-triphosphate</td>
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<td>db-cAMP</td>
<td>dibutyl cAMP</td>
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<td>dCTP</td>
<td>2'-deoxycytidine 5'-triphosphate</td>
</tr>
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<td>dGTP</td>
<td>2'-deoxyguanosine 5'-triphosphate</td>
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<tr>
<td>DMSO</td>
<td>dimethylsulfoxide</td>
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<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
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<tr>
<td>dsRNA</td>
<td>double-stranded RNA</td>
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<td>DTT</td>
<td>dithiothreitol</td>
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<tr>
<td>dTTP</td>
<td>2'-deoxythymidine 5'-triphosphate</td>
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<tr>
<td>ECL</td>
<td>enhanced chemiluminescence</td>
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<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
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<td>EMSA</td>
<td>electrophoretic mobility shift assay</td>
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<tr>
<td>FCS</td>
<td>foetal calf serum</td>
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<tr>
<td>GR</td>
<td>glucocorticoid receptor; a transcription factor</td>
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<td>GTP</td>
<td>guanosine 5'-triphosphate</td>
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<td>H89</td>
<td>N-[2-(p-bromocinnamylamino)ethyl]-5-isouquinolinesulfonamide</td>
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<td>HD-</td>
<td>a mutated homeodomain (see the text for details)</td>
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<tr>
<td>Hepes</td>
<td>4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid</td>
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<td>His</td>
<td>histidine</td>
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<td>HMG</td>
<td>high mobility group; a family of DNA binding proteins</td>
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<td>HSV</td>
<td>herpes simplex virus</td>
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<td>ICSBP</td>
<td>IFN consensus sequence binding protein</td>
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<td>IFN</td>
<td>interferon</td>
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<tr>
<td>I-POU</td>
<td>inhibitory POU; a transcriptional repressor protein</td>
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<tr>
<td>Acronym</td>
<td>Description</td>
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<tr>
<td>ISGF</td>
<td>interferon stimulated gene factor</td>
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<tr>
<td>kD</td>
<td>kilodalton(s)</td>
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<td>MMTV</td>
<td>mouse mammary tumor virus</td>
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<td>NLS</td>
<td>nuclear localization signal</td>
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<td>Nonidet P-40</td>
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<td>NRD</td>
<td>negative regulatory domain</td>
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<td>Oct</td>
<td>octamer binding protein; a family of DNA binding proteins</td>
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<td>PAGE</td>
<td>polyacrylamide gel electrophoresis</td>
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<td>PBS</td>
<td>phosphate-buffered saline</td>
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<td>PKA</td>
<td>protein kinase A; cAMP-activated</td>
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<td>PKR</td>
<td>protein kinase R; dsRNA-activated</td>
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<td>PMSF</td>
<td>phenylmethylsulphonylfluoride</td>
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<tr>
<td>poly(I)-poly(C)</td>
<td>polyinosinic-polycytidylic acid (dsRNA)</td>
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<td>PRD</td>
<td>positive regulatory domain</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
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<td>SAR</td>
<td>scaffold attachment region</td>
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<td>SDS</td>
<td>sodium dodecyl sulphate</td>
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<td>SRF</td>
<td>serum response factor; a transcription factor</td>
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<td>STAT</td>
<td>signal transducer and activator of transcription; a transcription factor</td>
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<tr>
<td>TE (x:y)</td>
<td>(x) mM Tris, (y) mM EDTA</td>
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<td>TFM</td>
<td>Transfectam</td>
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</tr>
<tr>
<td>Tris</td>
<td>Tris (hydroxymethyl) aminomethane</td>
</tr>
<tr>
<td>Tween-20</td>
<td>polyoxyethylene sorbitan monolaurate</td>
</tr>
<tr>
<td>UTP</td>
<td>uridine 5'-triphosphate</td>
</tr>
<tr>
<td>VSV</td>
<td>vesicular stomatitis virus</td>
</tr>
<tr>
<td>YY1</td>
<td>yin and yang 1; a transcription factor</td>
</tr>
</tbody>
</table>

1) bHLH | basic helix-loop-helix

2) PIC  | preinitiation complex
Chapter 1: Introduction

1.1. Initiation of Transcription as a Regulatory Decision

The correctly coordinated expression of specific genes governs the temporal and spatial identity of eukaryotic cells. Gene expression can be controlled at any stage of the processing of genetic information. For most genes, initiation of transcription is the primary regulatory point in the control of their expression. The advantages of gene control at the level of transcriptional initiation are obvious when one considers aspects of cellular economy: By only initiating the synthesis of transcripts for necessary protein products, the energy required for the later stages of transcription, as well as for pre-mRNA processing, for possible RNA editing, for transportation of a mature mRNA from a nucleus to cytoplasm, for various steps of mRNA translation, and for post-translational modifications of a protein product, is not wasted.

1.1.1. Basal Transcription Machinery

Transcription of protein-encoding genes by RNA polymerase II is regulated by specific DNA elements that associate with proteinaceous transcription factors. These are classified as basal or regulatory transcription factors. Basal transcription factors assemble as a preinitiation complex over the two defined core promoter elements, referred to as the TATA box and the Inr (initiator) element. An RNA polymerase II-dependent promoter can contain either one or both of these core elements. Together with RNA polymerase II, basal transcription factors are capable of maintaining low basal levels of transcription; regulatory transcription factors then increase (activators), or antagonize (repressors) this basal transcription by contacting the basal transcription machinery either directly, or indirectly via intermediary proteins. Six basal transcription factors (TFIIA, -B, -D, -E, -F, -H) have been identified, and complementary DNAs encoding most of their subunit polypeptides cloned. This has allowed in vitro studies of transcription in a controllable system largely reconstituted from recombinant products of such basal factors. For a more thorough discussion on various aspects of the mechanisms of basal transcription, see Appendix II (Eloranta and Goodbourn 1995).

Multiple targets for effective interactions by regulatory transcription factors have been identified within a preinitiation complex. The most studied of these targets are the components of the TATA box binding complex TFIID: TBP (TATA box binding protein)
and TAFs (TBP associated factors). TBP is an essential component of the RNA polymerase II (as well as RNA polymerase I and III) transcription machinery, and required for both TATA-containing and TATA-less promoters. Although direct contacts between TBP and activator proteins are well documented, and bound to contribute to the transcriptional effect, in \textit{in vitro} transcription assays, TBP can only sustain basal levels of transcription. To respond to activator proteins a complex forming on a TATA box must contain TAFs stably associated with TBP. Many TAFs appear to provide additional or alternative protein surfaces for the regulatory factors to target. In addition to the components of the TFIID complex, TFIIB, and more recently TFIIH and TFIIF, have also been shown to be targets for such interactions. Furthermore, in both yeast and higher eukaryotes, specific adapter proteins have been identified, the purpose of which is to mediate signals from the regulatory transcription factors to the basal machinery. The implications of having such a multitude of potential targets for transcriptional regulatory proteins are further discussed in section 1.1.3., in connection with the phenomenon of transcriptional synergy, and thoroughly discussed in Eloranta and Goodbourn (1995).

While the distinction between regulatory and basal transcription factors is clear, it should be stressed that the basal machinery itself appears inherently regulated. Repressor proteins, such as Dr1, Dr2, and ADI, have been identified which stably associate within, and interact with the components of, a preinitiation complex. One of the major roles of the basal transcription factor TFIIA appears to be to reverse this inherent inhibition of the function of preinitiation complexes by excluding their association with the repressor activities (Eloranta and Goodbourn 1995).

1.1.2. Regulated Transcription

The spectrum of regulatory transcription factors primarily determines which genes may be transcribed in a particular cell type. Typical regulatory factors are tethered to the regulatory regions of their target genes by specific DNA sequence recognition. In their simplest forms, transcriptional regulators contain a DNA binding domain and one or more activation and/or repression domains on a single polypeptide. They can usually be seen to be assembled from evolutionarily conserved domains in a modular manner, so that the DNA binding and the transcriptional effector (activator/repressor) functions are separable. In addition, there are proteins that bind DNA but do not contain effector regions and thus can only regulate transcription when complexed with a second protein that provides the effector function. To complement this picture there must also be proteins that do not efficiently bind DNA but provide the activation/repression potential when tethered to a specific DNA site through another protein. An example for this kind of regulatory pair is provided by the
complex between Oct-1 (DNA binding component) and viral protein VP16 (activating component), which will be discussed in more detail below. It should be emphasized that proteins such as VP16 do not fit into the category of adapter proteins, since they act on, and are tethered to, specific DNA elements, whereas the proteins of the adapter class are not considered to exhibit any DNA sequence specificity of action. Yet another class of regulatory factors that affect transcriptional initiation only because of the modulation of higher-order nucleoprotein structures by virtue of their DNA binding function is discussed in section 1.1.2.3.

Transcription factors often function as dimers, and in many cases the exact subunit composition of such dimers determines their DNA binding specificity or affinity, and the transcriptional regulatory activity. The dimerization potential allows more diversity in a function of any such factor. If distinct signal transduction pathways affect different partners in a heterodimer, dimerization can also be seen to provide a way to coordinate the simultaneous responses of a single transcriptional effector entity to multiple signalling pathways. For example, a target of the cyclic AMP/protein kinase A signalling pathway, CREB (cyclic AMP response element binding protein), can associate with targets for the protein kinase C pathway, Fos and Jun (Masquilier and Sassone-Corsi 1992). The control of muscle differentiation provides an example where alternative heterodimerization of myogenic transcription factors of the bHLH family determines the biological consequences (reviewed in Mohun et al 1992): To direct myoblast differentiation the myogenic factors bind to their target sites as heterodimers with the members of the E-protein family, while cell proliferation antagonizes differentiation when the myogenic factors associate with c-Jun. The involvement of a particular factor in different dimers can often target it to distinct DNA sites: For example, a Jun-Fos heterodimer binds to AP1 target sites, whereas Jun-CREB or Jun-ATF2 heterodimers prefer CRE-like sequences (Benbrook and Jones 1994). In addition to many transcription factors that function as dimers, a yeast transcription factor has recently been described that contains three subunits all essential for DNA binding (McNabb et al 1995); it remains to be determined whether the division of the DNA binding activity into three different polypeptides that form a heterotrimeric complex provides any further regulatory significance.

According to their DNA binding or dimerization folds, transcription factors belong to families, such as basic leucine zipper (bZIP), basic helix-loop-helix (bHLH), zinc finger, POU domain, homeodomain, paired domain, and winged helix families (reviewed in Harrison 1991, Pabo and Sauer 1992, Nelson 1995). Certain regulatory factors contain combinations of more than one of such conserved protein motifs in a single polypeptide chain; for example the Myc and Max proteins (reviewed in Amati and Land 1994) possess
basic DNA binding domains adjacent to both leucine zipper and helix-loop-helix dimerization interfaces.

The function of many transcription factors are dictated by such parameters as the exact promoter context, a specific cell type, or variations in the concentration of the factors themselves.

Transcription factors belonging to the evolutionarily conserved homeodomain family provide an example where heterologous and differential protein-protein interactions modulate the sequence specificity of action. While it is easy to envisage how combinations of transcription factors with different and clearly defined DNA binding specificities could cooperate to achieve appropriate regulation of specific target genes, homeodomain proteins pose a problem: they exhibit strikingly similar DNA binding specificities in vitro. Despite this they possess distinct in vivo functions, and a possibility has emerged that the discrepancy could be explained by variable protein-protein interactions in which these proteins are involved (reviewed in White 1994). An illustrative example is provided by the Saccharomyces cerevisiae mating type genes that encode homeodomain proteins a1 and a2, both of which recognize similar DNA elements. In diploid yeast cells they form heterodimers that repress the transcription of a haploid-specific set of genes, whereas in haploid cells of the α mating type the homodimeric α2 represses α-specific genes in association with the MCM1 protein. The target specificity appears to derive from the different constraints imposed by different protein-protein interactions on the homeodomain proteins - as a result the preferred target sites for the two distinct dimeric homeodomain complexes contain different spacings between the two actual homeodomain contact points (Smith and Johnson 1992). Similarly, in both nematodes and Drosophila, interactions between homeodomain proteins have been shown to affect their specificities.

Promoter context-dependent functional variation could derive from the exact sequence of the factor binding site: on different DNA element variants, the factor could be forced to assume distinct conformations, thus exposing different functional domains for the other components of the transcriptional machinery, for example. This kind of dependence of function on the variant nature of a response element appears to take place on certain high affinity binding sites for glucocorticoid receptor (Drouin et al 1993). In accord with this, the conformation of glucocorticoid receptors has indeed been suggested to be allosterically modulated by a DNA binding site (Lefstin et al 1994). Also, the protease sensitivity and activation properties of the dimers of Rel-family factor p50 appear to vary according to the alterations in their binding sites, suggesting a correlation between the DNA element-induced conformational changes and functional potentials (Fujita et al 1992, Hay and
Furthermore, the nature of the immediately flanking DNA sequence, not necessarily required for the DNA binding affinity of a transcription factor, can promote a functionally distinct conformational effect on a DNA-bound protein. An example of this is provided by the selective interaction of Oct-1 and VP16 on TAATGArAT motifs (r=purine; Walker et al 1994). While the GArAT portion of the element is not necessary for Oct-1 to bind the DNA element, it nevertheless induces a specific protein conformation in Oct-1, most likely by virtue of its characteristic DNA secondary structure. The altered conformation allows physical contacts with VP16, ternary complex formation and transcriptional activation. Another example was recently reported, where the transcription activation potential \textit{in vivo} and \textit{in vitro}, but not the DNA binding affinity, of an as yet unidentified transcription factor is stimulated by the degree of intrinsic DNA bending (Kim et al 1995).

The functioning of a transcription factor as an activator or repressor may depend on its intranuclear concentration. An example of this is provided by a \textit{Drosophila} zinc finger type transcription factor Krüppel. The Krüppel protein can have either an activating or repressive effect on transcription through a single DNA binding site, depending on the specific concentration of the Krüppel protein (Sauer and Jäckle 1993). At low concentrations, a monomeric form of Krüppel is a transcriptional activator; however, at adequately high concentrations, the Krüppel protein forms homodimers which actively repress transcription through a protein region distinct from the activation domain, although through binding to the same target sequence on DNA.

Several activator-repressor pairs appear to be generated through alternative splicing of primary transcripts (for example, Foulkes et al 1991, Treacy et al 1992) or alternative usage of translation initiation codons (for example, Descombes and Schibler 1991, Delmas et al 1992). In many cases it has been proposed that generation of functionally distinct products from a single locus is subject to tissue-specific, developmental, or specific signal-dependent control.

\textbf{1.1.2.1. Transcriptional Activation}

Transcriptional activation domains have been loosely classified according to the preponderance of certain amino acids: for example, acidic, proline-rich, serine- and threonine-rich, glutamine-rich and isoleucine-rich domains have been identified (Triezenberg 1995). A large number of interactions between activation domains and components of the basal transcription machinery have been documented; these are summarized and discussed in more detail in Appendix II (Eloranta and Goodbourn 1995).
It was originally hypothesized that certain types of activation domains would contact distinct classes of targets in the basal transcription machinery. However, there does not seem to be a simple one-to-one relationship: A single activator can contact more than one component of the transcriptional machinery, and conversely, some of the target proteins appear to be capable of interacting with multiple activators with different overall structures. Judged by the varying arrays of interactions they can make with the basal transcription machinery (Eloranta and Goodbourn 1995), all the activation domains of a certain type are clearly not functionally equivalent, and there are likely to be further determinants to refining the three-dimensional protein surfaces in activation domains that can interact with the basal transcription machinery.

1.1.2.2. Transcriptional Repression

In its earlier stages, the study of eukaryotic transcription concentrated on the identification and description of positively acting transcriptional activators. However, it has since become obvious that negative regulation of transcription is an equally important process (reviewed in Goodbourn 1990, Clark and Docherty 1993, Herschbach and Johnson 1993). Inhibition of transcription can be brought about by several mechanisms, summarized in figure 1.1., and discussed in further detail in the following sections. The repression mechanisms are not mutually exclusive.

1.1.2.2.1. Active repressors

Those regulatory transcription factors capable of active repression function in an analogous manner to transcriptional activator proteins. It is believed that active repression is effected by specific repression domains, which productively interact with a preinitiation complex, either directly, or through signal-mediating intermediary proteins (reviewed in Eloranta and Goodbourn 1995). Occupancy of a DNA recognition element is not sufficient for active repression. Like activation domains, repressor domains that are transferable to heterologous DNA binding subunits have been identified in several proteins. The properties of the repression domains are not well defined, but to the extent to which they have been examined, they are often characterized by a high content of alanine (Licht et al 1990, Han and Manley 1993a, b), proline (Han and Manley 1993a), or basic residues (Saha et al 1993). In other cases where a repressor domain has been delineated, no obvious consensus sequence motifs have been identified (Cowell et al 1992, Gashler et al 1993). The nature and consequences of the interactions between repression domains and basal transcription machinery are less well understood than those involving activator proteins. The negatively acting interactions may lead to the prevention of a preinitiation complex formation, the
Figure 1.1. Mechanisms of transcriptional repression.

A: Active repression. A repression domain in a sequence-specific repressor protein contacts the preinitiation complex either directly (as shown in the figure) or indirectly through negative adapter proteins.

B: Competition for overlapping binding sites. A sequence-specific repressor protein sterically inhibits DNA binding of an activator protein.

C: Inactive Heterodimerization. A repressor protein heterodimerizes with an activator protein and thus competes for heterodimers between two activator proteins. The inactive heterodimers can either be DNA binding defective or remain competent for DNA binding.

D: Quenching. A repressor protein that either binds DNA at an adjacent site or does not directly interact with DNA masks the activation potential of an activator protein.

E: Squelching. An excess of activator proteins sequesters either a specific adapter protein (as shown in the figure) or a target component of the preinitiation complex into a nonpromoter location.

Of the five mechanisms, three (B, C, D) cannot decrease the basal level of transcription, but rather inhibit the action of activator proteins. The mechanism A leads to the repression of basal transcription. The mechanism E leads to a decrease in basal transcription only if the sequestered target within the basal transcription machinery is an essential one.

key:

- a preinitiation complex
- a repressor protein
- an activator protein
- an adapter protein
destabilization of such preformed complexes, or repress their function without affecting the extent of initiation complex assembly. Since there are integrated negative activities, such as the Dr factors mentioned above, in the basal transcription machinery, the repressor proteins could also act by enhancing their integration into the preinitiation complex.

The thyroid hormone receptor (TR) is a potent hormone-dependent transactivator protein. In the absence of a ligand, TR can still bind DNA, but acts as a transcriptional repressor. The silencing function of TR, as well as its oncogenic counterpart v-ErbA, is transferable to a heterologous DNA binding domain (Banahmad et al 1992). Deletion analyses have suggested an important role for basic and hydrophilic amino acid stretches within this particular repression domain. Utilizing the in vitro transcription assay, it has been shown that the basal transcription machinery is the target for the repression mediated by unliganded TR (Fondell et al 1993). TR affects an early step in preinitiation complex formation, and fully formed PICs are refractory to repression. A specific physical interaction between TFIIB and TR has been observed, implying this basal transcription factor as a target for the TR-mediated repression (Banahmad et al 1993, Fondell et al 1993). Two distinct regions of TFIIB are targeted by two distinct regions of TR, one of the two regions in TR overlaps with its ligand binding domain. Accordingly, the appropriate ligand thyroid hormone, which converts TR into an activator significantly decreases the interaction between TR and TFIIB, perhaps by inducing a conformational change in the ligand binding domain.

1.1.2.2.2. Competition for Overlapping Binding Sites on a Promoter

When a factor bearing no activation potential in a particular promoter context binds to a sequence overlapping, or closely adjacent to, the binding site for an activator protein, it may prevent the binding of, or displace, this activator. This competition mechanism is essentially passive, and depends on both the concentrations of the competing proteins within a cell, and their relative affinities for the regulatory DNA element. The transcription factors belonging to the same family often exhibit very similar or identical sequence specificity, and are thus often involved in executing such regulation by competition.

A divergent homeodomain protein Gtx (glial- and testis-specific homeobox gene) exhibits high affinity binding for the known response element for the serum response factor-related proteins (Komuro et al 1993). Gtx can efficiently compete with one such protein RSRF (related to serum response factor) for binding to the DNA element; furthermore, cotransfection of gtx cDNA together with a serum-inducible RSRF binding site-dependent
reporter construct efficiently leads to the prevention of the serum-induced activation of the reporter gene.

A specialized case of competition involves the prevention of the binding of TBP to a TATA box, and is termed TATA box occlusion, and is discussed in connection with our results section 3.9.2.3.4. The TATA box occlusion mechanism has some resemblance to the most frequently observed repression mechanism in prokaryotes, in which other DNA binding proteins compete with basal transcription factors for DNA occupancy.

1.1.2.2.3. Formation of Inactive Heterodimers

Like all the proneural gene products that are required for the development of the peripheral nervous system in Drosophila, Extramacrochaete (Emc) contains a helix-loop-helix (HLH) dimerization motif. However, Emc lacks the adjacent basic domain that is required for specific DNA binding by the positive proneural regulators, such as the Achaete and Daughterless proteins. Thus Emc can negatively regulate the latter group of activator proteins by forming DNA binding defective dimers with them and thereby sequestering the functional Achaete/Daughterless dimers from DNA (Van Doren et al 1991, Martinez et al 1993).

A member of the mammalian helix-loop-helix family of proteins, Id, serves as an inhibitor of MyoD, a transcriptional activator involved in muscle cell development (Benezra et al 1990). In Id, substitution of certain conserved residues by prolines renders its basic domain defective in binding DNA, attenuating the ability of MyoD to bind DNA as dimers with other proteins.

A developmentally regulated nuclear protein CHOP (C/EBP homologous protein) negatively modulates the activity of C/EBP-like (CCAAT/enhancer binding protein) proteins in certain terminally differentiated cells (Ron and Habener 1992). CHOP has strong sequence similarity to C/EBP-like proteins within the bZIP region corresponding to the dimerization/DNA binding domain; however, CHOP contains two proline-substitutions in the basic region, which is critical for DNA contacts. Thus, since a single DNA-contacting surface is insufficient to allow the "scissor-grip" configuration of the dimeric DNA-binding form of a bZIP protein (Vinson et al 1989), heterodimerization with CHOP renders C/EBP-like proteins unable to bind their cognate DNA enhancer elements.

In Drosophila, another well elucidated example of repression of transcription by the formation of DNA binding defective heterodimers is provided by a factor referred to as I-
POU (inhibitory POU). I-POU lacks two basic amino acids in the highly conserved cluster at the amino-terminal portion of the POU homeodomain, rendering it incapable of DNA binding (Treacy et al 1991). Thus, I-POU cannot bind DNA but forms POU domain mediated heterodimers with another POU domain factor Drifter, which is expressed in the same cells of the *Drosophila* developing nervous system. The heterodimer formation inhibits the ability of Drifter to bind and activate the regulatory regions of the dopa decarboxylase gene. Interestingly, the I-POU locus can also generate a protein referred to as Twin of I-POU, which does contain the two basic residues absent in I-POU (Treacy et al 1992). Twin of I-POU cannot dimerize with Drifter, but acts as a positive transcription factor on targets distinct from those regulated by Drifter.

Clearly, the repression mechanism involving heterodimerization depends strongly on the relative concentrations in solution of the interacting proteins, and the repressor protein needs to be in a functional excess of the inhibited protein for this mechanism to be effective. The functionally effective ratio is not only determined by the actual relative concentrations of proteins, but also by the relative affinities of the inhibited activator proteins for a specific DNA binding site and for a repressive heterodimerization partner.

A negatively acting heterodimerization partner can also act by taking part in formation of inactive heterodimers that do remain competent for DNA binding. This mechanism could in some cases be seen as a combination of two mechanisms: competition for DNA binding sites and formation of inactive heterodimers. An example of this is provided by bHLHLZ factors Myc and Max (reviewed in Amati and Land 1994). The Myc protein requires dimerization with Max in order to efficiently bind a specific DNA target sequence CACGTG, and thus to activate transcription. However, when in excess over Myc, the Max protein can form homodimers that can still specifically bind DNA, but are unable to activate transcription. To increase the complexity, alternative heterodimerization partners, referred to as Mad and Mx1, for the Max protein exist. The Mad-Max and Mx1-Max heterodimers behave like Max-Max homodimers, in that they can bind DNA, but are incompetent for activation. Thus they are negative regulators of Myc that act by both recruiting its heterodimerization partner and competing for its binding site, both of which are required for transcriptional effects by the Myc protein.

Another case of negative regulation, where repression by heterodimerization and competition over DNA binding can operate interchangeably is provided by negative regulation of promoters that confer liver-specific expression. NF-IL6 is a bZIP transcription factor of the C/EBP family contributing to the tissue specificity of liver-specific promoters (Descombes et al 1990). Homodimers of the liver inhibitory protein
(LIP), which can be translated from the NF-IL6 mRNA by an internal translation initiation event, function as a competitive inhibitor of NF-IL6 (Descombes and Schibler 1991). However, it has not been excluded that it also functions by forming less potent heterodimers with NF-IL6.

1.1.2.2.4. Neutralization of Activators or Quenching

Transcription can also be negatively regulated by masking the activation domains of DNA binding activators by either other DNA binding proteins that bind to non-overlapping elements or non-DNA binding factors. This prevents the activator proteins from making appropriate contacts with preinitiation complexes. A classical example is provided by GAL80, a regulator of galactose metabolism in yeast, which itself does not bind DNA, but physically masks the activation domain of a DNA-bound activator protein GAL4 (Ma and Ptashne 1988).

1.1.2.2.5. Squelching

Excessive amounts of transcriptional activators suppress the level of transcription, a phenomenon referred to as squelching. This phenomenon is thought to be due to sequestration of a limiting component of transcriptional machinery into a nonproductive location (for discussion see Eloranta and Goodbourn 1995). While squelching has made the identification of true repressor proteins more difficult, it has also been of elementary importance in the genetic identification of certain yeast adapter proteins (Berger et al 1990). Furthermore, it remains possible that a squelching-like mechanism is a naturally existing process for regulation of transcription, although no such examples have been reported yet. To be a physiologically significant regulatory mechanism, squelching would require the concentrations of "squelchable" targets to be limiting.

Naturally, a squelching-like titration mechanism could also serve to activate a promoter by inhibiting the action of DNA bound repressors. This model would require the existence of specific "negative adapters", which have not yet been shown to unequivocally exist (but see Eloranta and Goodbourn 1995). These negative adapters, the function of which would be to specifically mediate the signal from active DNA-bound repressors to the basal transcription machinery, could be quantitatively recruited to inactive complexes by the high expression of proteins that possess surfaces resembling putative repression domains. To our knowledge, such an activation mechanism by "negative squelching" has not been reported even in experimental systems that rely on overexpression of repressor proteins.
beyond physiological levels. This possibility will be discussed further in connection with our own experiments in section 3.9.2.1.

1.1.2.3. Architectural Transcription Factors

The DNA helix is straight only under idealized conditions. Bends in DNA may result from thermal forces, special sequence patterns, such as repeated A tracts, and from stress induced by DNA binding proteins. The chief mechanism in vivo to cause DNA to bend is protein-induced, as first observed on prokaryotic systems (see, for example, Prentki et al 1987, DeVargas et al 1989, Rojo et al 1990). In eukaryotes, histones influence the DNA architecture by producing polynucleosomal fibers. Subsequently, it has been shown that many eukaryotic sequence-specific regulatory transcription factors, such as Drosophila heat shock transcription factor (Shuey and Parker 1986), also induce DNA bending at specific promoter sites. It has become obvious that in both prokaryotic and eukaryotic systems, bending is not a mere side effect of DNA-protein complex formation, but can be of functional significance in biological processes, such as transcription, replication and recombination. Bending at promoter sites may facilitate unwinding of the DNA necessary for the initiation at the transcriptional start site. Another simple mechanism by which DNA bending can influence transcription is through facilitation of binding of other proteins that favour the altered DNA conformation, or through facilitation of physical interactions between other DNA proteins by decreasing the effective distance between them. This could lead to a synergistic effect on transcription without the requirement for the direct interaction between the cooperating factors.

Although site-specific delivery of activation or repression domains is unquestionably an important role for most DNA binding domains, many regulatory transcription factors that possess such effector domains also bend DNA and can thus exhibit alternative ways to regulate transcription, operative either simultaneously or in a manner dependent on the exact promoter context. Certain factors lacking all intrinsic activation or repression potential utilize only their DNA binding function to achieve the transcriptional effect by facilitating the assembly of higher-order nucleoprotein complexes (reviewed in Grosschedl et al 1994). LEF-1, a protein shown to play an important role in the activation of T cell receptor gene expression (Giese et al 1992), is one such factor. LEF-1 contains an HMG domain, binds to the minor groove of the DNA helix, and is not capable of activating transcription on its own. Rather, it induces a sharp bend at a DNA site some distance away from other elements of the T cell receptor gene enhancer, and thus facilitates interactions between proteins bound to these elements.
A ubiquitously expressed DNA binding protein YY1 (yin and yang) has been shown to repress the activity of the c-fos promoter through an effect on DNA structure (Natesan and Gilman 1993). YY1 binds to, and induces a phased bend at, three sites on the c-fos promoter; this is thought to regulate the contacts between other promoter-bound factors.

The reported role for the DNA binding protein HMGI(Y) as a factor organizing the spatial arrangement on the IFN-ß promoter will be discussed in section 1.2.2.4.5. Furthermore, the possible role for Oct-1 as such an architectural factor of the human IFN-ß promoter will be discussed in connection with our results (section 3.2.).

As discussed in the next section, position of nucleosomes at specific sites on certain promoters can also potentiate transcription by architectural mechanisms.

1.1.2.4. Chromatin Structure in Transcriptional Regulation

1.1.2.4.1. Nucleosomal Structure May Influence Factor Binding

The DNA in eukaryotic cells is organized in an hierarchical series of nucleoprotein structures, generically referred to as chromatin. It is believed that the 10nm polynucleosome fiber is the actual template for RNA polymerases. Indeed, transcriptionally active genes exhibit generalized DNAase sensitivity, which may reflect the decondensation of the 30nm solenoidal fiber to a simple polynucleosome array. The 10nm fiber consists of nucleosomes - structures in which 160bp of DNA is wrapped in two turns around an octamer core (H2A/H2B/H3/H4)2 of histones. Access of a transcription factor to its binding site on DNA can be significantly impeded by wrapping of the target site into nucleosomes. Furthermore, DNA structure in a nucleosome becomes extremely distorted; thus, the binding site on deproteinized DNA may look rather different from that present on a nucleosome surface. The competence for transcriptional initiation may be critically dependent on the disruption or displacement of nucleosomes along the promoter regions (reviewed in Felsenfeld 1992, Wallrath et al 1994, Wolff 1994a), but once initiated, RNA chain elongation can take place through histone-covered templates. Genetic evidence for a role of nucleosomes in transcriptional regulation has been obtained in S. cerevisiae. In these studies, altering the stoichiometry of core histones alters transcription patterns (Clark-Adams et al 1988) and depletion of histone H4 leads to nucleosome loss and concomitant activation of particular genes (Han et al 1988).

Depending on the changes in their local chromatin structure at the promoters, the inducible genes fall loosely into two categories: remodelled and preset. The promoter regions of...
remodelled genes are packaged into nucleosomes in their uninduced state, and the nucleosomal arrangement must be perturbed in response to a stimulatory signal, in order to the regulatory factors to gain access to their DNA recognition elements. A well-studied example of the remodelling process takes place on the promoter region of the MMTV (mouse mammary tumor virus), which is organized on a precisely phased array of six nucleosomes. A DNA binding protein, glucocorticoid receptor (GR), when activated and translocated to the nucleus upon hormonal stimulation, is able to bind without apparent interference to its target sequence wrapped around a particular nucleosome (Pina et al 1990, Archer et al 1991). On the other hand, another DNA binding protein, NF1/CTF, is excluded from its nucleosomal binding site. Thus, the organization of DNA sequences into highly structured nucleoprotein templates can result in differential access of transcriptional regulatory proteins to their target sites, although it is not clear whether the difference in binding reflects distinct properties of the factors rather than the location of nucleosomes with respect to the binding sites. It is possible that some sequence specific factors (such as GR) that can bind DNA on the nucleosomal surface function as "structure opening" factors, which then allow the binding of secondary factors (such as NF1/CTF) when appropriate. This mechanism appears to be operative also on the promoter of the rat TAT gene, where hormone-induced binding of the glucocorticoid receptor alters the chromatin structure to allow a distinct liver-specific factor HNF5 to bind to the same sequence (Rigaud et al 1991).

Preset genes are those in which the binding of regulatory factors are accessible prior to activation. An illustrative example is provided by the Drosophila hsp26 gene (Lu et al 1993), transcription of which is induced within minutes upon heat shock. Prior to heat shock, one nucleosome is specifically positioned between the two functional response elements 5' of the transcription start site. Upon heat shock stimulation, the only change in the nucleoprotein organization of the promoter is the binding of heat shock factor to the promoter, while no major changes in chromatin structure take place.

It is clear that nucleosomes on the promoter regions can affect the function of certain regulatory transcription factors. Vice versa, sequence-specific transcription factors can also influence folding of the promoter regions into nucleosomal structures. It has been shown that in addition to interacting with the basal transcription machinery, the activation domains of regulatory factors can stimulate transcription by relieving nucleosomal repression by displacing histone octamers from the template, both in vitro (Workman et al 1991, Croston et al 1992) and in vivo (Pham et al 1991, Axelrod and Majors 1993).
Chromatin structure does not only have a repressive effect on transcription but can also potentiate it. Similar to the action of some of the architectural transcription factor discussed in section 1.1.2.3., the folding of DNA as a result of nucleosome assembly between two distant regulatory elements will bring these, and the proteins bound to them, closer together in space. This may serve to enhance transcription by increasing the local concentration of transcription factors, thus facilitating communication between them. For example, in the case of the *Xenopus* vitellogenin B1 gene, a single positioned nucleosome brings into juxtaposition an oestrogen-responsive DNA element and liver specific response elements located almost 200bp apart (Schild et al 1993). This leads to severalfold stimulation of transcription in vitro, as a result of the formation of a specific chromatin structure. Similarly, on the *Drosophila hsp26* promoter, the binding sites for the heat shock transcription factor and the GAGA factor are 200bp apart on linear DNA, but are brought into proximity with each other and the preinitiation complex by the wrapping of DNA around a specifically positioned histone octamer (Lu et al 1993).

### 1.1.2.4.2. Position Effects and Silencing

Within the eukaryotic nucleus, chromatin components serve to divide the genome into structurally and functionally independent domains. Depending on whether it is positioned adjacent to an active or inactive chromatin domain, the expression of a given gene can be affected (reviewed in Karpen 1994, Rivier and Pillas 1994, Wolffe 1994b). A striking example of generalized repression at the level of chromatin organization is dosage compensation in mammals. In female mammals, one of the two X chromosomes condenses into an inactive heterochromatic structure, and transcription of genes along the inactive X chromosome is repressed. It appears that genes can exist in at least two distinct transcriptional states: genes in inactive domains are not accessible to the regulation mediated by their promoters, while genes residing in active chromatin are accessible to the full extent of regulation, both activation and repression.

A well studied example of the position effect in yeast is transcriptional silencing of the *S.cerevisiae HML* and *HMR* loci, encoding the cryptic mating type loci, and also of the yeast telomeres (Aparicio et al 1991, Laurensen and Rine 1992, Sandell and Zakian 1992). Genes positioned within, or sufficiently close to, these domains exhibit transcriptional repression. Several proteins, including SIR2, SIR3, SIR4, and histone H4, have been identified by genetic criteria as important for silencing (Laurensen and Rine 1992). SIR factors are believed to be either structural components of transcriptionally repressed chromatin, or factors contributing to the assembly or modification of such inactive templates; for example, SIR2 appears to promote hypoacetylation of histones (Braunstein et
A locus control region (LCR) is responsible for the tissue- and stage-specific expression of the vertebrate β-like globin genes (reviewed in Felsenfeld 1992). The β-globin LCR functions as a dominant transcriptional enhancer over a distance of 75kbp. In transfection and transgenic assays, the LCR confers tissue-specific, high-level and position-independent expression to cis-linked genes. The LCR appears to have a role in creating and maintaining a region either free of nucleosomes or with an altered nucleosome structure over the promoters in the β-globin domain.

Insulating elements, such as constitutively hypersensitive scs (specialized chromatin structure) sites at the ends of the Drosophila heat shock locus (Kellum and Schedl 1992), have been identified that can serve as boundaries between chromosomal domains of different transcriptional activity. Unlike LCRs, which are active participants in the maintenance of position independence, insulator elements have no regulatory activities of their own, but rather act passively to block the functional interaction of enhancers and promoters between which they are interposed. How the insulation effect is achieved has not yet been established. It may prove important that a nucleoprotein complex with similar properties to scs elements has been defined in Drosophila. This complex consists of the gypsy transposable element and the suppressor of Hairy wing [su(Hw)] protein (Holdridge and Dorsett 1991, Geyer and Corces 1992). Formation of such a nucleoprotein complex between an enhancer and a promoter can block enhancer activity, and when placed at the boundaries of a DNA fragment containing a transgene, this piece of DNA is protected from the repressive effects of heterochromatin.

In higher eukaryotes chromosomal DNA is organized into loops of 30-100kb, which are attached to a proteinaceous scaffold structure that lies along the chromosomal axis through unique DNA elements referred to as scaffold attachment regions (SARs). It has been hypothesized that the general activity state of a particular loop can be controlled.
independently of the neighbouring ones. This seems to be the case, since certain SARs can function as insulators, or definers, of the functionally distinct chromosomal domains (Bonifer et al 1990). The relationship between SARs and other elements that provide boundary functions remains to be established.

1.1.2.5. Extracellular Signals Modulate Transcription

Specific programs of gene expression in eukaryotic cells are modulated in response to changes in their microenvironment. Rapid responses to external signals are often mediated by post-translational modifications of transcription factors, as opposed to their de novo synthesis.

Specific phosphorylation and dephosphorylation events, mediated by protein kinases and protein phosphatases, respectively, have been shown to modulate the activity of a variety of transcription factors within the cell (Hunter and Karin 1992, Whiteside and Goodbourn 1993, Hill and Treisman 1995). Quantitative and qualitative changes in the phosphorylation pattern of transcription factors can affect various properties of theirs, such as DNA binding activity, the potency of effector domains, and subcellular localization, separately or simultaneously. The phosphorylation can either induce allosteric conformational changes in, or alter the electrostatic properties of, a transcription factor.

The initiation of a signal transduction pathway from the cell membrane to the nucleus often involves the generation of a second messenger. For example, a peptide hormone forskolin activates adenylyl cyclase upon its binding to an appropriate receptor; this increases the intracellular concentration of cyclic AMP (cAMP). Cyclic AMP functions as a second messenger, which can bind to, and dissociate, the regulatory subunit of the protein kinase A (PKA), leading to the release and nuclear localization of the active catalytic PKA subunit. The activated kinase can then directly phosphorylate the Ser133 residue of the transcription factor CREB (cyclic AMP response element binding protein), which increases the ability of this transcription factor to stimulate transcription (Gonzales and Montminy 1989). The signal from the phosphorylated activation domain of CREB is transferred to the basal transcription machinery by the adapter protein CBP (Chrivia et al 1993, Arias et al 1994, Kwok et al 1994). The phosphatases regulating the properties of transcription factors have been less well elucidated than the kinases. However, it has been shown that transcripational attenuation following cAMP induction requires dephosphorylation of the CREB residue Ser133 by protein phosphatases (Hagiwara et al 1992).
In many cases, signals that are initiated from the cell surface are transmitted through the cytoplasm to the nucleus as kinase cascades. An illustrative example from simpler eukaryotes is the yeast mating pheromone response (reviewed in Bardwell et al. 1994, Herskowitz 1995, Schultz et al. 1995). When a pheromone interacts with its cognate cell surface receptor of the "seven transmembrane segments" family, it triggers the dissociation of an associated heterotrimeric G (guanine nucleotide binding) protein. The released G\(_{\beta\gamma}\) subunit then activates a pathway involving at least four protein kinases, which by genetic analysis have been ordered into a cascade. The kinases at the end of the cascade can eventually phosphorylate a transcription factor STE12. The signal responsive transcription factor STE12 induces both G1 arrest and morphological changes required for cellular and nuclear fusion. Structural and functional analogs of the components of the yeast pheromone response pathway have been identified in the signalling systems of multicellular eukaryotes. The advantages of such cascades are that they allow signal amplification, signal dissemination and integration by incoming and outgoing branchpoints along the cascade, and signal modulation by positive or negative feedback loops along the cascade.

Investigation of the function of the leucine zipper proteins Fos and Jun, components of the transcription factor AP-1, in the regulation of cell proliferation has yielded much information about the signalling pathways that control the activities of transcription factors. Phorbol ester stimulation or the activation by certain growth factors, cytokines, or neurotransmitters, of their cognate receptor tyrosine kinases, trigger a complex signal transduction pathway that involves multiple protein-protein interactions, generation of GTP-bound Ras, and activation of a kinase cascade. Finally this results in the alterations of the phosphorylation state of the Fos and Jun proteins, and in many cases, these signal-dependent changes in phosphorylation correlate with alterations in the transcriptional activity of AP-1. The c-Jun protein can be phosphorylated on at least five residues, two within its amino-terminal activation domain and three clustered next to its carboxy-terminal DNA binding domain. Treatment of cells with the phorbol ester TPA (12-O-tetradecanoylphorbol-13-acetate) in the presence of an activated Ha-Ras protein leads to the rapid dephosphorylation of sites adjacent to the DNA binding region, resulting in an increased DNA binding activity, whereas the expression of Ha-Ras alone stimulates phosphorylation of the activation domain increasing c-Jun transactivation potential (Binétruy et al. 1991, Smeal et al. 1991). Similarly to CREB, the amino-terminal activation domain of c-Jun can bind to an adapter protein CBP in a phosphorylation dependent manner (Chrivia et al. 1993, Arias et al. 1994). CKII (casein kinase II) phosphorylates c-Jun on sites that inhibit DNA binding, and microinjection of peptides that inhibit CKII activates AP-1 activity in living cells (Lin et al. 1992). Furthermore, it has been suggested that certain c-Jun stimulatory signals can phosphorylate a repressor of c-Jun; once phosphorylated, the
repressor is suggested to dissociate from c-Jun (Baichwal et al 1991). In addition to modifying its own transcriptional properties, the TPA-induced phosphorylation of the c-Jun protein also decreases its c-Fos trans-degradative properties, and thus regulates the stability of c-Fos protein in response to signals (Papavassiliou et al 1992).

A novel mechanism to control the activity of NF-IL6 was recently discovered (Kowentz-Leutz et al 1994). The activation of signalling pathways results in phosphorylation of its inhibitory domains by MAP kinase, thus abolishing inhibition by unmasking the transactivating functions, either intramolecularly or intermolecularly between two NF-IL6 molecules.

The Rel family of transcription factors provide currently the most vigorously studied example of cytoplasmic retention of a transactivator protein that is regulated by extracellular signals (Beg and Baldwin 1993, Gilmore and Morin 1993, Baueerle and Henkel 1994). The Rel proteins are retained in the cytoplasm by interactions with inhibitory IxB factors. Dissociation of these interactions, and the subsequent migration of Rel factors to a nucleus, is mainly controlled by phosphorylation and subsequent degradation of IxB factors. The activation mechanisms of latent Rel subunit complexes in mammalian cells are discussed in more detail in section 1.2.2.4.3. A Rel-family member, referred to as Dorsal, has also been identified in Drosophila. While Dorsal is ubiquitously present in the cells of an early embryo, it is located in the nucleus in only those cells, whose fate is to become dorsalized (Govind and Steward 1991). The nuclear distribution of Dorsal is controlled by the Cactus protein, a functional homolog of the mammalian IxBs. The inhibitory activity of Cactus is in turn regulated by a signalling cascade that initiates at the cell surface, by the interaction of the Toll receptor with its ligand Spätzle (Morisato and Anderson 1994). The cascade ends at a direct phosphorylation of the Cactus protein. The Dorsal-Cactus system shows that the regulation of the subcellular localization of a transcription factor can bring about major developmental consequences, and is a mechanism conserved in evolution from Drosophila to human.

A more global kind of regulation of transcription may be achieved by phosphorylation of carboxy-terminal domain (CTD) of the large subunit of RNA polymerase II, which has multiple heptad repeats that can be phosphorylated at multiple sites (reviewed in Eloranta and Goodbourn 1995). Hyperphosphorylated forms predominate in transcribing RNA polymerase II complexes, and only the unphosphorylated form can enter the initiation pathway. It seems that the CTD phosphorylation is needed to trigger conversion of a preinitiation complex into a form competent for elongation. The link from transcriptional regulatory proteins to the polymerase may be provided by two basal transcription factors
known to form target surfaces: TFIIH and TBP. TFIIH possesses a kinase activity capable of modifying CTD; this kinase activity is stimulated upon formation of a complete preinitiation complex. TBP directly contacts the unphosphorylated CTD, and this association becomes blocked by phosphorylation of the CTD repeats.

1.1.3. Combinatorial Control and Transcriptional Synergy

The transcription of many genes is controlled by a mosaic arrangement of several response elements, and their respective binding factors. A large array of physical interactions between different types of transcription factors in specific promoter contexts have been described; for example, NF-κB has been shown to associate with NF-IL6 (LeClair et al 1992), ATF-2 (Du et al 1993, Kaszubska et al 1993), Spl (Perkins et al 1993) and AP-1 (Stein et al 1993). The advantages of such combinatorial control are multiple.

Combinatorial interactions may reflect evolutionary selection for the economical use of transcription factors: By achieving a distinct transcriptional effect by distinct combinations of factors, there is no need to generate a specific factor for each particular transcriptional event, and thus synthesis of only limited number of such factors is required.

Because of the risk of unwanted endogenous squelching, there may be a limit to the strength of an activation (or repression) domain that can be safely accommodated in a cell. A combinatorial arrangement of factor binding sites may serve to avoid the problem: Activators that are sufficiently weak not to cause squelching could still activate transcription to a high level when brought together on a promoter, whereas a single potent activator could have deleterious effects. While considering this model, it is noteworthy, that perhaps the strongest activator described, VP16 (Sadowski et al 1988), is produced by herpes simplex virus during lytic growth when the maintenance of the health of the host cell is not of particular importance. Another option to avoid squelching problem by creating the requirement for constructing multiprotein complexes on a promoter could be to divide the activating potential of a strong activator between two proteins, which could only form a complex when bound to DNA. This model could include such dimers that form in a manner dependent on a specific DNA sequence, or factors that strongly enhance or stabilize each other's DNA binding.

Combinatorial organization of promoters also provides a way to achieve specificity in the response to extracellular signals. For example, a single promoter may possess recognition elements for multiple signal regulated factors, each of them required for activation. Those stimuli that can simultaneously activate all the elements will activate transcription, while
signals that efficiently activate only a subset of such transcription factors would not. This mechanism may be operative on the \(c-fos\) promoter: Both IFN-\(\alpha\) and platelet derived growth factor (PDGF) can efficiently induce the binding of STAT (signal transducer and activator of transcription) factors to the promoter; however, only PDGF can activate the other cooperating promoter elements required for efficient transcription (Hannigan and Williams 1992). This issue of specificity achieved by combinatorial nature of a promoter is further discussed in connection with the introduction of the human IFN-\(\beta\) promoter, in section 1.2.2.6.

Promoters containing multiple binding sites for regulatory factors may be more active than promoters with single sites simply, because distributing DNA binding proteins over the whole promoter length may efficiently contribute to the disruption of the nucleosomal organization. In this regard, it has been suggested that synergistic enhancement of transcription may depend on the number of proteins bound to the promoter, rather than on the number of activation domains (Oliviero and Struhl 1991). Nevertheless, as discussed above (section 1.1.2.4.1.), there is a considerable amount of evidence that activation domains of regulatory factors can also participate in nucleosome disruption by mechanisms separate from their DNA binding function.

Protein-protein interactions between members of the same or different families of transcription factors have been implicated in the transcriptional regulation of many genes. The direct interactions may mediate transcriptional synergy, which, in the case of activation, means that the stimulatory effect by two or more activators is greater than the sum of the effects by them individually (discussed in Eloranta and Goodbourn 1995). Synergistic activation may be a consequence of cooperative DNA binding of transcription factors; however, certain activators can also work cooperatively under conditions at which their DNA binding sites are saturated. Functional cooperation between transcription elements without cooperative DNA binding has become more rationalizable along with the findings that several components of the transcriptional machinery - or several regions in a single component - can serve as targets for activators. Synergy follows when multiple individual signals merge on a single transcriptional event. Sometimes artificial or natural test promoters containing multiple binding sites for a single activator can be synergistically activated, for example, Sp1 is capable of synergistic activation of the promoters containing multiple Sp1 sites, even if the DNA binding does not appear cooperative (Pascal and Tjian 1991). This may be explained by the activator under study having the capacity to contact multiple targets. The \textit{in vivo} synergy on promoters consisting of binding sites for several distinct activators appears to depend on the extent of "cooperation compatibility" between factors, and some response elements seem to cooperate nearly universally, whereas others
exhibit strong selectivity (Wang and Gralla 1991). It will be of great interest to determine whether this in any way correlates with the variety of contacts that the regulatory factors under examination can make with the adapters and/or the basal transcription machinery. For example, perhaps only those factors capable of hitting distinct targets can cooperate, and perhaps the factors that can productively interact with several distinct members of the transcription machinery function as the "nearly universal cooperators".

1.2. Regulation of Human Beta-Interferon Gene Expression

As evident from the introduction above, regulation of transcriptional initiation can utilize several mechanisms. The aim of our laboratory has been to investigate, which of these mechanisms are operative on the complex promoter regulating the human beta-interferon (IFN-β) gene. To put the study of the IFN-β gene induction into an appropriate biological context, I will first introduce the interferon system, before proceeding to discuss the main theme in this thesis: the regulation of the human IFN-β promoter.

1.2.1. Overview of the Interferon System

1.2.1.1. Interferon Proteins And Genes

Interferons (IFNs) belong to the large family of cytokines, a class of soluble mediators involved in cell-cell communications. They were discovered in 1957 (Isaacs and Lindenmann 1957), by demonstrating that when one kind of virus colonized cells in animals or on a culture dish, the invasion interfered with the ability of other, unrelated viruses to establish infections at the same time. IFNs were shown to be polypeptides secreted by infected cells that are able to protect vertebrate cells against subsequent viral infection in a species-specific manner (reviewed in Pestka et al 1987, Sen and Lengyel 1992, Johnson et al 1994). While an IFN-producing cell is often killed as a result of viral infection, the secreted material activates a protective antiviral state in neighbouring, noninfected cells in an altruistic fashion. IFNs are capable of impairing various steps of a viral life cycle, including viral penetration into a cell, uncoating the viral particle, translation of viral mRNAs, and the assembly of progeny viruses (reviewed in Gresser 1990, Sen and Lengyel 1992). In addition to their antiviral activity, IFNs are also potent antiproliferative agents possessing antitumor activity on a variety of human malignancies; thus IFNs and their positive regulatory proteins/effecter proteins are putative tumor suppressor gene products, and the proteins that have a negative effect on the IFN system could have oncogenic properties (Gresser 1990, Lengyel 1993). Due to their antiviral and
antiproliferative properties IFNs have been examined as potential therapeutic agents for treating certain human virally transmitted diseases and malignancies (Baron et al. 1991). At present, IFNs have been approved for treating several diseases, for example Kaposi's sarcoma, hairy cell leukemia, hepatitis virus B and C infections, and genital warts caused by papillomaviruses; furthermore, they appear promising in therapy of patients suffering from non-Hodgkin's lymphoma, malignant melanoma and chronic myelogenous leukemia. Also, many encouraging reports have documented potent antiviral effects of IFNs in restriction of HIV replication in T cell lines and blood leukocytes (Bednarik et al. 1989, Vieillard et al. 1994, Su et al. 1995). Application of IFN therapy is complicated by its rapid clearance from the blood, its toxicity, and the possible emergence of hyporesponsiveness with continued use.

Two structurally and functionally distinct classes of IFNs have been defined: Virally inducible type I IFNs are acid- and heat-stable, whilst type II IFNs are induced by mitogens, and cannot tolerate low pH or heating. Type I IFNs are further subdivided into α- and β-IFNs - also referred to as leukocyte-IFN and fibroblast-IFN, respectively - that are antigenically distinct. Two novel classes, IFN-α and IFN-τ, have been discovered, the members of which closely resemble the IFN-α variants but are slightly larger. IFN-τ is produced exclusively by the trophoblast during the peri-implantation stage of pregnancy in ruminant ungulate species, and this trophoblast-specific type I IFN has been implicated in the process of maternal recognition and maintenance of early pregnancy in ruminants (Cross and Roberts 1991, Roberts et al. 1992).

A secreted IFN-α pool is a mixture of several different proteins, encoded by at least 20 distinct intronless IFN-α genes in humans (reviewed in Weissmann and Weber 1986); these subtypes share approximately 92% homology. In addition to the functional genes, there are several IFN-α pseudogenes in the human genome. All the human IFN-α genes are located in a distinct region of the short arm of the chromosome 9. IFN-α can be produced \textit{ex vivo} from a peripheral blood leucocyte (PBL) cell population, which consists of several cell types. The entire variety of cell types capable of producing IFN-α is not known; macrophages, T cells, B cells, and fibroblasts are all IFN-α producing cells. Whether all of the cell types are capable of producing all the IFN-α-subtypes, or whether any of these are restricted to any particular cell type(s) remains undetermined. Also, it is unclear, how much the multiple IFN-α genes differ functionally. It is possible, that different subtypes elicit heterogeneous antiviral states. One such example has been reported (Bell et al. 1983), where the IFN pool secreted by purified human macrophages infected with the respiratory syncytial virus (RSV) was more effective in blocking the RSV growth in target cells than was the IFN-pool secreted by cells challenged with influenza virus. The replication of the
influenza virus in target cells appeared equally sensitive to the IFN-pool induced by either RSV or the influenza virus itself.

In contrast to the large IFN-α family, only one human IFN-β gene has been isolated (Gross et al 1981, Houghton et al 1981, Lawn et al 1981, Tavernier et al 1981). Like IFN-α genes, the IFN-β gene is intronless, and is located in the same region of chromosome 9; furthermore, it shows limited sequence homology to the IFN-α genes. It seems likely that all the type I IFN genes are derived from a single ancestral gene as a result of multiple intrachromosomal duplications. Most mammals are like human in that they have large IFN-α families but only a single IFN-β gene (reviewed in Weissmann and Weber 1986). IFN-β is produced in both non-lymphoid and lymphoid cells and is the major species of IFN secreted by the former.

The type II IFN, also known as IFN-γ, is not homologous to type I IFNs, and the gene encoding human IFN-γ is located on chromosome 12 and contains three introns (Gray and Goeddel 1982). While the native quaternary structure of type I IFNs is as single polypeptides, the active IFN-γ exists as a homodimer. Although IFN-γ also induces antiviral activity, it is induced by stimulation with mitogens or superantigens, and not by viral infection per se. While essentially all cells can produce one or another type I IFN, only two cell types, T lymphocytes and natural killer cells, release IFN-γ. IFN-γ has prominent modulatory functions in the lymphokine network, e.g. it stimulates lymphokines involved in immune response.

1.2.1.2. Interferon Signalling Pathway

Type I IFNs interact with multisubunit high-affinity receptors on cell surfaces. The interferon-receptor interaction triggers a signal transduction pathway that leads to the rapid and protein synthesis-independent transcriptional activation of a large number of cellular genes that participate in executing the functions of the pleiotropic interferon response (reviewed in Stark and Kerr 1992, Pellegrini and Schindler 1993, Shuai 1994). The activation of these genes is largely mediated by the components of the regulatory transcription factor complex ISGF3 (IFN stimulated gene factor 3), the subunits of which pre-exist in an inactive monomeric form, but becomes assembled within minutes by binding of IFN to receptors on cell surface. Activation following the binding of the type I IFNs to their cognate receptors involves the phosphorylation of cytoplasmic ISGF3α components Stat2 (signal transducer and activator of transcription) and Stat1α/Stat1β (two proteins produced from alternatively spliced mRNAs) by IFN receptor-associated tyrosine kinases of the JAK (Janus kinase, just another kinase) family (Ziemiecki et al 1994, Ihle
and Kerr 1995). Phosphorylated ISGF3α components associate with the DNA binding component of the ISGF3 complex, ISGF3γ, which is a 48kD protein of the IRF (IFN regulatory factor) family. The entire complex is then translocated into the nucleus, where it recognizes the conserved IFN-stimulated response element (ISRE, 14-18bp) in the 5' regulatory sequences of type I IFN-inducible genes. The signal transduction pathways of IFNs do not thus primarily depend on second messengers, such as cAMP, diacylglycerol, or Ca²⁺, but are more direct.

In addition to the independent signalling pathway involving Stat factors, IFNs appear to exert their antiproliferative action by interfering with the growth factor-stimulated Ras/MAP kinase signalling pathway (Xu et al 1994). Whether the upstream events of the former pathway, that is the activation of JAK kinases, are involved in this cross-interference between the two signalling cascades, remains to be investigated.

1.2.1.3. Mediators of Interferon Action

Interferon-inducible genes encode mediators of the antiviral and growth-inhibitory effects of IFNs (Kerr and Stark 1992, Sen and Lengyel 1992, Lengyel 1993). More than 30 IFN-inducible proteins are known; these include protein kinase R (PKR) and 2'-5' oligoadenylate synthetase (2'-5' OAS). In contrast to these, IFNs can also inhibit the expression of other proteins, such as ornithine decarboxylase (Sreevalsan et al 1979) and several mitochondrial proteins (Shan et al 1990).

PKR (previously referred to as DAI, p68 kinase, dsI) is a serine/threonine protein kinase activatable by dsRNA (Hovanessian 1989, 1991). The mRNA encoding the PKR is strongly induced by treatment of mouse or human cells with interferons, in a dose-dependent manner; furthermore, the PKR protein levels correlate with the extent of induction. The increase in PKR mRNA by interferons is a direct transcriptional event, since it is not affected by inhibition of protein synthesis (Meurs et al 1990). Naturally occurring cellular or virally derived double-stranded RNAs, or single-stranded RNAs with sufficiently long and accessible double-stranded secondary structures, can activate PKR. Enhanced phosphorylation and activation of PKR have been observed in virally infected cells, consistent with its proposed role in the antiviral response. PKR is activated by low but inhibited by high concentrations of dsRNA, which may reflect the presence of distinct high and low affinity binding sites for dsRNA (Galabru et al 1989). A more complex explanation has been suggested, according to which the balance between activation and inhibition depends on the length as well as abundance of a particular dsRNA species - RNA duplexes extensive enough to be able to bind the both of the dsRNA binding sites in PKR.
may be required for activation (Manche et al 1992). Upon activation, the kinase first becomes autophosphorylated on several serine and threonine residues; it is not clear whether the reaction takes places intramolecularly or intermolecularly between two molecules of PKR, or both. A transdominant negative action by an inactive PKR variant implies that PKR exists as a dimer (Koromilas et al 1992). PKR activated by autophosphorylation can subsequently catalyze dsRNA-independent phosphorylation of exogenous substrates. The most studied of these is the α-subunit of eukaryotic initiation factor 2 (eIF2), the phosphorylation of the Ser51 residue of which prevents the recycling of eIF2α-GDP, and thus the formation of a ternary complex Met-tRNA-eIF2-GTP, leading to the inhibition of protein synthesis (Proud 1986). Physiological substrates other than eIF2α have not been confirmed; however, the inhibitory subunit of the NF-κB system, IκB (Kumar et al 1994; K.Mellits and S.Goodbourn, pers. comm.), as well as the HIV protein TAT (B.Williams, pers.comm.), can serve as substrates for PKR *in vitro*.

Several viruses have evolved mechanisms to overcome the antiviral processes elicited by IFNs by inhibiting the PKR action (reviewed in Katze 1992). For example, the highly structured adenovirus VAI (Mathews and Shenk 1991) and Epstein-Barr virus EBER-1 (Clarke et al 1991, Sharp et al 1993) RNAs are able to bind to PKR and prevent its activation. Poliovirus infection in turn leads to dramatic degradation of PKR by a cellular protease (Black et al 1993). Several putative cellular inhibitors of PKR suggested to modulate the physiological kinase function in uninfected cells have also been identified; one of the best characterized of these is a small mouse protein dRF (dsI regulatory factor), which has been suggested to inhibit the autophosphorylation by preventing the dsRNA-dependent binding of ATP to PKR by direct effect on the enzyme (Judware and Petryshyn 1992), although such a direct interaction has not been documented. It is conceivable that PKR can be regulated by protein phosphatases (see, for example, Szyszka et al 1989), but these remain poorly characterized. Furthermore, proteins that bind and/or unwind dsRNA molecules may inhibit the activation of PKR by sequestering or inactivating inducer dsRNAs; the La autoimmune antigen appears to be one such cellular RNA-binding protein (Xiao et al 1994).

Expression of a functionally defective mutant of human PKR in NIH3T3 cells results in malignant transformation, suggesting that PKR acts as an inhibitor of cell proliferation and exerts a tumor suppressor function (Koromilas et al 1992, Meurs et al 1993). It was suggested that the tumor suppression may not be mediated by the eIF2α phosphorylation, since the phosphorylation status of this substrate was not affected by the presence of the dominant negative mutant PKRs. However, in contrast to this, changing the serine in the PKR recognition site of eIF2α into a nonphosphorylatable residue leads to tumorigenicity.
Expression of wild type PKR in yeast has an inhibitory effect on cell growth (Chong et al 1992), suggesting that the antiproliferative action by PKR is conserved across the eukaryotic lineage.

One other IFN-induced enzyme, 2'-5' OAS, is also activatable by dsRNA (Hovanessian 1991). After activation, 2'-5' OAS converts ATP into 2'-5' oligoadenylate oligomers, which in turn bind to, and activate, a latent endoribonuclease RNAase L, capable of degrading cellular and viral RNAs 3' of UpUp and UpAp. This system is thought to work at localized sites of virus multiplication, where the 2'-5' OAS binds to viral dsRNAs, leading to the activation of RNAase L by 2'-5' A oligomers at the localized sites. Overexpression of a dominant negative mutant of the RNAase L suppresses type I IFN induced inhibition of EMCV replication (Hassel et al 1993), as expected if 2'-5' OAS system is indeed one of the mediators of antiviral response. The 2'-5' OAS system appears to have a role in cellular physiology, in addition to participating in the mediation of interferon response: A strong negative correlation between the cellular 2'-5' A content and the rate of cell proliferation is apparent, suggesting a general role in the regulation of cell growth.

The dsRNA binding domains of 2'-5' OAS and PKR do not have any apparent sequence homology (Patel and Sen 1992), and there is a profound difference between the two enzymes with respect to the structural features of the dsRNA that can activate them (Desai et al 1995). The small adenoviral VAI RNA species can bind to PKR (see above), but it inhibits rather than activates it. In contrast, VAI RNA can both bind to, and activate, 2'-5' OAS.

Another interferon inducible protein RBP9-27 is an RNA binding protein that can bind and functionally antagonize the Rev responsive element (RRE) of HIV-1 (Constantoulakis et al 1993). RRE is the site of interaction of the HIV-1 Rev protein with the viral mRNA; this interaction is required for the expression of structural proteins and thus viral particle formation. The observed inhibition of HIV-1 expression in vivo by RBP9-27 may be explained by its ability to bind RRE.

The six or more related proteins encoded by a gene cluster in the murine chromosome 1 are also inducible by IFN. A particular member of the cluster, the Ifi202 gene, encodes a 52kD nuclear phosphoprotein, p202, that can associate with the well-studied negative growth regulator protein RB (retinoblastoma protein) (Choubey and Lengyel 1995). The hypophosphorylated form of the RB protein, which also associates with p202, retains the cells in the G0/G1 phase of the cell cycle (Cobrinik et al 1992). It is thus conceivable that...
the interaction with p202 could impair the inactivatory phosphorylation of the RB protein, thus maintaining its ability to suppress cell cycle progression. Consistent with this model, IFN can inhibit the growth of certain types of cultured cells by arresting the cell cycle at the G0/G1 phase (Einat et al 1985).

1.2.2. Induction of Beta-Interferon Transcription

The study of the induction of type I IFN genes has been greatly facilitated by the fact that their promoter regions are appropriately regulated when transfected into cultured cell lines (Canaani and Berg 1982, Hauser et al 1982, Mantei and Weissmann 1982, Ohno and Taniguchi 1982, Pitha et al 1982, Zinn et al 1982).

1.2.2.1. Nature of Inducing Signals

The synthesis of type I IFNs is not detectable in normally growing cells, but reaches high levels after induction. In vivo, almost all viruses can act as inducers, whether their genome consists of DNA, single-stranded RNA, or double-stranded RNA. Furthermore, many viruses can induce IFNs ex vivo in isolated tissues and cell suspensions, or in vitro in primary fibroblast cultures, and in many established fibroblastoid and lymphoblastoid cell lines. However, several viruses that are efficient IFN-inducers in vivo, are poor inducers, or not inducers at all, of cultured cells.

In addition to viral infection, IFN-β can be induced in vitro by treatment of cells with double-stranded RNAs, such as synthetic poly(I)-poly(C). Poly(I)-poly(C), though an effective inducer in vitro, is a rather poor inducer in vivo, due to RNA-degrading enzymes in serum. The exact structural features of dsRNA molecules important for induction are not clear, presumably uninterrupted double-stranded stretches of certain length are necessary (Marcus 1983).

It has been believed that the viral induction of IFN-β gene is also mediated by dsRNA that either forms the viral genome, or is generated from it as an intermediate at some stage of a viral replication cycle (Marcus 1984). However, the induction pathways by viruses and dsRNA are clearly not identical, and at least some viruses seem to provide an inducing factor, or elicit a cellular signal transduction pathway, that is different from, or additional to, those provided by dsRNA. For example, certain ssRNA viruses can induce IFN under conditions non-permissive for replication, and certain replication-defective mutants of reovirus do not induce IFN, even if their genome is dsRNA (Lai and Joklik 1973). Furthermore, the viral induction of the IFN-α genes does not appear to be mediated solely
by dsRNA, since they are inducible by NDV but not by poly(I)-poly(C) in primary human fibroblasts, whereas the IFN-β gene can be induced by both agents (Havell et al 1978). In addition, partial induction of otherwise priming-dependent cell lines, can be reached by Sendai virus [a paramyxovirus; genome (-)ssRNA] without the need to pretreat cells with IFN (King and Goodbourn 1994). A difference between Sendai virus and dsRNA has also been reported at the level of the DNA binding factors that bind to the DNA elements within the IFN-β promoter: in differentiated mouse embryonal carcinoma cells, Sendai can induce the PRD II binding activity NF-κB, whilst dsRNA cannot (Ellis and Goodbourn 1994). The nature of the non dsRNA component(s) provided by the Sendai virus is not known, although it has been suggested that the viral protein C could function as an efficient IFN-β inducer (Taira et al 1987).

The signal pathway generated by dsRNA, and leading to specific gene activation, has not been well elucidated. It should be emphasized that the induction of IFN-β transcription does not require de novo protein synthesis, suggesting that the effect on the transcription factors is posttranslational, perhaps mediated by specific phosphorylation events, known to modulate the activity of many DNA binding proteins. Inhibition of IFN-β induction can be achieved by the purine analog 2-aminopurine (Marcus and Sekellick 1988, Zinn et al 1988), a rather nonspecific kinase inhibitor, known to inhibit PKR among other kinases. It is interesting that PKR, a kinase induced by IFNs and implicated as a mediator of IFN response, is activated by dsRNA - thus it can be an effector molecule functioning at more than one level of the IFN system. It has indeed been shown that by virtue of its phosphorylation activity, PKR can activate in vitro one transcription factor, NF-κB, important for the regulation of the IFN-β promoter (Kumar et al 1994). Also, the selective ablation of the PKR mRNAs in HeLa cells inhibits the dsRNA mediated activation of NF-κB (Maran et al 1994). It remains to be investigated whether the other DNA binding regulators of the IFN-β promoter could be targets for regulatory phosphorylations by PKR, either as direct substrates or at the end of a signal cascade where PKR would be an upstream effector.

It has also been suggested that under some circumstances the accumulation of naturally occurring cellular double stranded RNAs can induce IFN production (Belhumeur et al 1993). If this kind of endogenous induction machinery exists, it would have to be tightly regulated to prevent inappropriate IFN expression, which would inhibit cell proliferation. Cellular RNA unwindases could be such regulators.

1.2.2.2. Induction Cycle
The induction of IFN-β has been shown to occur primarily at the level of transcriptional initiation (figure 1.2.; Raj and Pitha 1983, Nir et al 1984). In uninduced cells the IFN-β mRNA is undetectable. The induction cycle begins with a lag period after the introduction of an inducer. This period does not appear to result from the delayed entry of an inducer into the cell (Hauser et al 1982), nor does it reflect a need for synthesis of poly(I)-poly(C)-induced proteins, since IFN-β mRNA is inducible in the presence of protein synthesis inhibitors. Rather, the lag period is likely to reflect the time required to derepress the promoter to allow efficient transcription. The lag period is followed by an IFN synthesis phase peaking 6-12 hours after the cells have encountered an inducer, during which a substantial proportion of the newly synthesized mRNA is IFN-β-specific - that is, several thousand transcripts per cell. The induction of the IFN-β is transient, and at the final postinduction turn-off stage, the IFN production rapidly decreases back to undetectable levels. The lengths of different phases vary depending on the cell type and inducer.

The dramatic changes in the IFN-β expression during the induction cycle reflect the biological properties of IFN-β - the potent cytostatic effects would make expression in uninduced cells incompatible with cellular growth, while overproduction in induced cells serves to minimize the spread of viral infection.

Induction can occur without the requirement for de novo protein synthesis, indicating that all the factors necessary for these events pre-exist in the cell in some form (Cavalieri et al 1977). In fact, simultaneous treatment of dsRNA-induced cells with metabolic inhibitors of protein synthesis, such as cycloheximide which blocks the elongation of a peptide chain, causes an enhancement in the degree of IFN-β induction, a phenomenon referred to as superinduction. As discussed in section 1.2.2.5., in many cell lines, the inhibition of protein synthesis interferes with the postinduction shutoff of the promoter.

In some cultured cell lines IFN-β induction can be strongly increased by pretreating cells with IFN before induction, a phenomenon known as priming. Furthermore, the kinetics of induction accelerated by priming (Abreu et al 1989, Content et al 1980, Fujita and Kohno 1981). The basis of the priming phenomenon remains somewhat unclear, but has been shown to operate at the level of transcription (Nir et al 1985). Within the IFN-β promoter, the priming effect cannot be localized to any specific sequence element, suggesting that a cellular function is induced that allows the inducer dsRNA to activate independent cellular targets (King and Goodbourn 1994). On the basis of complementation in cell fusion experiments, it seems that priming provides an IFN-inducible factor required for dsRNA-induction that is constitutively present in priming-independent cells (Enoch et al 1986), but
Figure 1.2. Induction kinetics of IFN-β expression.

HeLa cells were induced with dsRNA for varying periods as indicated. One set of cells was primed, that is, treated with interferon 16 hours prior to induction, whereas the other set was not. Cytoplasmic RNA was isolated and specific mRNA levels quantitated by RNAase protection assay. The probes to map endogenous mRNAs specific for human IFN-β and γ-actin (internal control) are described in Materials and Methods. The panel on the left shows the priming dependence for induction of HeLa cells. The panel on the right illustrates that the induction of the IFN-β occurs at the level of transcriptional initiation and shows the three phases of induction: a lag phase, a production phase and a postinduction shut-off phase.

This figure is adapted from King and Goodbourn (1994).
the nature of such an activity remains unelucidated. Priming-dependent cells lack this IFN-inducible component essential to transduce a signal in response to dsRNA. Unlike the induction process itself, priming appears to require protein synthesis (Enoch et al 1986). Certain cell lines, such as HeLa cells (figure 1.2; King and Goodbourn 1994) and differentiated mouse embryonal carcinoma cells (Ellis and Goodbourn 1994), are conditionally inducible by dsRNA, so that priming is an absolute prerequisite for induction. Although induction in response to dsRNA is completely dependent upon priming in these cells, unprimed cells can be induced by Sendai virus to a certain degree, further implying there to be different pathways mediating the effects of the two inducers (Matsuyama et al 1993, Ellis and Goodbourn 1994, King and Goodbourn 1994).

Priming may be important in amplifying and accelerating the IFN production in vivo. It is conceivable, that IFN produced by the first virally infected cells could prime the neighbouring cells, in order to increase the amount of IFN synthesized by them upon their subsequent encounter with a virus. Thus, in living organisms, even if the primary infection would not induce the affected cells to produce sufficient amounts of IFNs to combat viral invasion, the cytokines would then themselves, in an autocrine manner, further enhance the secondary IFN response, so that high enough IFN titres would be achieved to quench the spread of infection.

It should be noted that cells respond to the IFN-β inducers in a heterogeneous manner, so that only a proportion of cells are stimulated to produce IFN-β mRNA (Enoch et al 1986). The reasons for this heterogeneity in cellular response are not known, but it does not appear to reflect the ability of cells to respond to an inducer at only certain stages of the cell cycle (S. Goodbourn, T. Enoch and T. Maniatis, pers. comm.).

1.2.2.3. The Beta-Interferon Gene Promoter

The transcriptional regulation of the IFN-β gene has been extensively studied as a paradigm for the regulation of highly inducible promoters. The requirement for stringent positive and negative transcriptional control of the IFN-β promoter at different stages of the induction cycle is reflected in the complexity of its organization (reviewed in Taniguchi 1989, Goodbourn 1990b).

Transcription from the human IFN-β gene can be transiently induced in many cell types by viral infection or treatment with synthetic double-stranded RNA (dsRNA) (Stewart 1979). The induction can occur in the presence of protein synthesis inhibitors, implying that the regulatory transcription factors required pre-exist in uninduced cells, and their
transcriptional activity or access to their respective response elements is modified upon induction. Genetic analysis utilizing various deletions expressed in host cells has revealed that the approximately 200bp 5'-flanking regulatory sequence responsible for transcriptional activation consists of multiple distinct positive and negative DNA response elements, referred to as PRDs (positive regulatory domains) and NRDs (negative regulatory domains) (figure 1.3.; reviewed in Goodbourn 1990b). The murine IFN-β promoter contains significant homology to the human one (Dirks et al 1989). The regulatory regions of the IFN-β promoters can confer inducibility to a heterologous promoter, which has greatly facilitated the analyses of the induction events.

The degree of dependence of maximal induction of the IFN-β gene upon distinct regulatory DNA elements appears to differ, depending on the exact cell line used in transfection analyses. Different cell lines may utilize different arrays of transcription factors that recognize the cis-acting regulatory elements of the gene. In murine L929 cells, the level of induction by Newcastle disease virus diminished dramatically as the 5' deletion extended from -105 to -91 (Fujita et al 1985). Further deletion from -91 to -78 rendered the promoter uninducible. Also in HeLa cells, efficient inducibility requires sequences upstream of -91 (Du and Maniatis 1992, King and Goodbourn 1994). On the other hand, it has been shown that the critical boundary for induction resides between -77 and -73 in mouse C127 fibroblasts transformed by episomal bovine papilloma virus vectors carrying various deletion mutants of the IFN-β gene (Zinn et al 1983). However, while this was the requirement for substantial inducibility, the sequences upstream of -77 exhibited a modulatory effect. Specifically, the extensions of the promoter to -91 and -104 both caused further two-fold increases in inducibility. Extending the promoter even further to -210 actually decreased both the basal and induced transcription levels, suggesting that a negative regulatory element lies in these upstream sequences. A similar pattern of changes in the inducibility of the promoter variants in C127 cells was observed either in transient transfection assays, or when the deletion mutants were stably introduced into the host chromosomes; although in the latter of these systems, the effect of the sequence between -104 and -91 was more pronounced (Goodbourn et al 1985).

The IFN-β promoter elements can also confer inducibility to a heterologous promoter: in C127 cells the region between -77 and -36 is sufficient for this (Goodbourn et al 1985), while in L929 cells the further sequences in the 5' direction are also required (Fujita et al 1985).

1.2.2.3.1. DNA Sequence Elements that Mediate Preinduction Repression
Figure 1.3. A schematic representation of the human IFN-β promoter.

Nucleotide positions are relative to the cap site.
PRD = positive regulatory domain
NRD = negative regulatory domain
TATA = a consensus TATA box
The IFN-β promoter is tightly repressed in uninduced cells, which is necessary because of the cytotoxic properties of the protein product. The repression of the IFN-β promoter is complex and does not appear to be mediated by a single sequence element. Two negative regulatory domains (NRDs) have thus far been identified. As judged by the effect on transcription levels of progressive 5′ promoter deletions, the NRD II element is located just upstream of the nucleotide position -100 (Zinn et al 1983, King and Goodbourn 1994). Deletion of the NRD I region located at the 3′ side of PRD II (see below) also leads to elevated basal activity (Goodbourn et al 1986). The precise 5′ end point of NRD I has been difficult to define, since the element overlaps with a positive regulatory domain, PRD II (see below), and point mutations across this region of overlap can thus affect both basal and inducible levels of transcription. However, certain single base substitutions within PRD II cause an elevated basal expression without increasing the induced levels of expression (Goodbourn and Maniatis 1988), suggesting that the phenotype is produced by creating a weakened binding site for a preinduction repressor protein rather than a stronger binding site for an activator protein. For further discussion on the definition of NRD I, see section 3.9.1.1.

Besides being a virus-inducible element (see below), the PRD I element also acts as an inhibitory DNA element for enhancers, such as the viral SV40 enhancer, when these are positioned upstream (Kuhl et al 1987). This implies that the element is capable of binding active transcriptional repressor proteins, and suggests that preinduction repressors of the IFN-β promoter that can act through the PRD I element exist.

1.2.2.3.2. DNA Sequence Elements that Mediate High Levels of Induced Transcription

Upon induction of the IFN-β promoter the repression is relieved and a number of PRD sequence elements combine to stimulate expression. With the exception of PRD I and PRD III, PRDs are not related in sequence, further suggesting there to be more than one downstream target for inducer-triggered cellular processes. The relative contribution of the various PRDs differs between cell lines, and in some cases a particular PRD is dispensable. In isolation as single copies, none of the four identified PRDs (I-IV) can function as virus-inducible elements. However, they have been shown to be inducible, either in the context of the native IFN-β promoter (figure 1.3), or in artificial constructs in certain combinations of more than one such element (Fan and Maniatis 1989, LeBlanc et al 1990). In undifferentiated mouse embryonal carcinoma cells, in which the IFN-β promoter does not respond to inducers, all multimers of the individual PRD-elements remain uninducible (Ellis and Goodbourn 1994).
PRD I is a 14bp element GAGAAGTGAAAGT between -77 and -64. PRD III, located between -90 and -77, is highly homologous to PRD I at the DNA sequence level. PRD III appears necessary for efficient induction in HeLa cells (Burstein 1986) and L cells (Fujita et al 1985, 1987), but not in C127 cells.

The sequences between -66 and -57 of the IFN-6 promoter define the PRD II element. It seems that PRD II activation is a key event in the induction process (Goodbourn and Maniatis 1988, Lenardo et al 1989, Visvanathan and Goodbourn 1989). Hence, a recent demonstration that the IFN-6 promoter variants that lack PRD II sequences remain inducible in differentiated mouse embryonal carcinoma cells is rather surprising (Ellis and Goodbourn 1994).

Sequences located between -104 and -87 comprise the PRD IV element, and are required for induction in L929 cells (Fujita et al 1985), but not in C127 cells (Zinn et al 1983). In the case of the PRD IV element, a controversy remains: multimers of such an element have been shown to be inducible by others (Du and Maniatis 1992), but in our experimental systems they are uninducible (chapter 5).

1.2.2.4. Transcription Factors that Bind to the Human Beta-Interferon Promoter

Induction is thought to be brought about by a change in activity or availability of the DNA binding proteins that regulate the behaviour of the multiple elements within the IFN-6 promoter. This is supported by an in vivo footprinting analysis, which indicated that the DNAase cleavage patterns before and after induction differ significantly (Zinn and Maniatis 1986). Several experimental approaches have been taken to identify these proteins, including electrophoretic mobility shift assay (EMSA). Complementary DNAs encoding these DNA binding activities have also been cloned by screening expression libraries. A large number of proteins that can bind to each of the four PRDs have been characterized; in contrast, attempts to identify NRD I binding factors have not been equally successful, despite considerable effort. Some of the factors involved in the induction process may not be readily clonable in binding site screenings of bacterial libraries, since these factors may undergo certain posttranslational modifications during the induction cycle that confer on them the ability to bind DNA. As discussed, the independence of the induction process of new protein synthesis clearly suggests that this may be the case. Also, the cDNAs encoding proteins that require dimerization or oligomerization for DNA binding could not be retrieved from such expression libraries. The factors known to bind to the IFN-6 promoter
are summarized in figure 1.4. and will be discussed in connection with their respective DNA binding elements.

1.2.2.4.1. NRD I and NRD II Binding Factors

The transcription of the IFN-β gene can be induced to some degree by treatment of cells with the protein synthesis inhibitor cycloheximide alone (Enoch et al 1986, Ringold et al 1984), implying that the low basal level is maintained by labile negatively regulatory factors, or by labile factors that modulate such transcriptional repressors. The repressor proteins act through specific cis-acting NRDs, and also through some of the PRDs. Induction is brought about by a change in the abundance and/or activities of the negatively and positively acting DNA binding proteins.

No reports have been published about any identified mammalian DNA binding proteins that are capable of binding to the NRD I region - in this thesis we identify the ubiquitous factor Oct-1 as one such factor (chapter 3). Two HeLa cell factors of molecular weights 95 and 100kD were identified by Nourbakhsh et al (1993) that can bind to the NRD I; however, these have not been reported to have been characterized any further. Nevertheless, it is clear that neither of these are identical to the Oct-1-containing complex we have discovered, since they do not form on the same EMSA probes. An interesting observation was made by Lehming et al (1994) that a Drosophila protein DSP1 (Dorsal switch protein 1) can bind to the NRD I and inhibit NF-κB-mediated activation through PRD II (see below). DSP1 was cloned in a yeast screen for Dorsal corepressor factors, and is homologous to the proteins of the high mobility group (HMG) family. It should be emphasized, however, that no functional studies have indicated that DSP1 can decrease the level of transcription from the IFN-β promoter in uninduced cells, which should obviously be a necessary prerequisite for a preinduction repressor. Furthermore, NRD I can function as a negative regulatory element when isolated from the neighbouring PRD II, whereas the effect of DSP1 requires the native context. Also, DSP1 is a Drosophila protein, and no identification of functional mammalian homologs has been reported.

Similarly to NRD I, no factors capable of specific binding to the NRD II region have been previously reported. In this thesis, we identify Oct-1 (chapter 3) and Un1/Un2 (chapter 4) as such NRD II binding activities. It should further remembered that the 3' end of NRD II overlaps with the 5' portion of the PRD IV element, thus the factors shown to bind PRD IV (see below) should also be considered candidate NRD II binding proteins.
Figure 1.4. A summary of binding activities known to interact with the human IFN-β promoter.

The transcription factors are aligned with their specific binding site or sites on the promoter. The panel on the right indicates relative changes in the binding affinities of factors during the induction cycle. For references concerning individual binding activities, see the text.
<table>
<thead>
<tr>
<th>Factor</th>
<th>Uninduced</th>
<th>Induced</th>
<th>Induced + CHX</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oct-1</td>
<td></td>
<td>++</td>
<td></td>
</tr>
<tr>
<td>Un1 / Un2</td>
<td></td>
<td>++</td>
<td></td>
</tr>
<tr>
<td>DSP1</td>
<td>?</td>
<td>?</td>
<td>?</td>
</tr>
<tr>
<td>HMG1(Y)</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>ATF-2</td>
<td>-</td>
<td>?</td>
<td>+++</td>
</tr>
<tr>
<td>ATF-2/c-Jun</td>
<td>-</td>
<td>?</td>
<td>+++</td>
</tr>
<tr>
<td>IRF-2</td>
<td>+++</td>
<td>+++</td>
<td>+</td>
</tr>
<tr>
<td>IRF-2*</td>
<td>-</td>
<td>++</td>
<td>+++</td>
</tr>
<tr>
<td>IRF-1</td>
<td>-</td>
<td>+++</td>
<td>-</td>
</tr>
<tr>
<td>NF-kappaB</td>
<td>-</td>
<td>++</td>
<td>+++</td>
</tr>
</tbody>
</table>
In addition to the NRD elements, at least one of the PRD regions of the IFN-β promoter, namely PRD I, is involved in the preinduction repression of transcription. As discussed above, besides being a virus-inducible element, PRD I also acts as an inhibitory DNA element for enhancers, such as SV40 enhancer, when these are positioned upstream (Kuhl et al 1987). This implies that the PRD I element is capable of binding active transcriptional repressor proteins, which function as preinduction repressors of the IFN-β promoter. The silencing effect is reversed upon virus induction, implying that constitutive PRD I-binding repressor proteins are displaced by positive regulators during the process. In chapter 4, we present our analysis on the putative PRD I-specific repressive DNA binding complexes Un1 and Un2.

1.2.2.4.2. PRD I and PRD III Binding Factors

PRD I has been shown to bind transcription factors IRF-1 and IRF-2 (interferon regulatory factor 1 and 2). The mRNAs for both of these are expressed at low levels in unstimulated cells, but are strongly inducible by viral infection or IFN-stimulation (Harada et al 1989). The IRF-1 cDNA was isolated from a λgt11 library with a DNA probe containing multiple copies of a PRD I derivative (Miyamoto et al 1988). IRF-2 was subsequently isolated by cross-hybridization with an IRF-1 probe (Harada et al 1989), by virtue of the fact that the amino termini of the IRF factors are homologous (62% identity). It seems likely that IRF-1 and IRF-2 loci are derived from a single ancestral gene as a result of gene duplication, and subsequent sequence divergence. Although the amino-terminal regions of the IRFs lack strong homologies with other known DNA binding proteins, they are responsible for DNA binding. In keeping with the high degree of homology between the amino-termini of the two IRFs, the interaction of IRF-1 and IRF-2 with DNA exhibits virtually identical sequence preference (Tanaka et al 1993). They apparently compete for the same cis-acting sequences, but mediate different effects.

IRF-1 is a positive regulator of IFN-β transcription, as evidenced by experiments with a human fibroblast cell line constitutively overexpressing IRF-1 mRNA in either sense or antisense orientation (Reis et al 1992). Upon induction with dsRNA or NDV, cells harboring the sense IRF-1 message produced more IFN-β mRNA than the control cells, whereas cells expressing the antisense IRF-1 mRNA produced neither IFN-β mRNA nor protein. High-level expression of the IRF-1 cDNA in transfected monkey COS cells results in detectable, albeit distinctively low compared with the treatment of cells with a virus, induction of the endogenous IFN-β, as well as IFN-α, genes (Fujita et al 1989b). While it is clear that IRF-1 can act as a positive regulator of IFN-β promoter, it cannot be the primary, or essential, activator of the IFN-β gene in all cell types: Multimers of PRD I are
inducible in the absence of de novo protein synthesis; however, IRF-1 is undetectable in uninduced HeLa cells (Whiteside et al 1992). Indeed, both the intact IFN-β promoter (Pine et al 1991) and multimers of PRD I (Whiteside et al 1992) are inducible under conditions in which IRF-1 is not detectable. Furthermore, IFN-α and -β genes are both inducible by virus in murine embryonal stem cells, in which both IRF-1 alleles have been disrupted (Ruffner et al 1993).

The transcriptional activation domain of IRF-1 lies in its carboxy-terminus (Fujita et al 1989b), within which a region rich in serines and threonines can be found. The carboxy-terminus of IRF-2 shows only little homology (25%) with that of IRF-1; thus IRF-2 lacks the activation domain of IRF-1. In accord with this, IRF-2 does not stimulate transcription, but rather has a repressor-like activity on the repeated PRD I-like AAGTGA sequence (Harada et al 1989), and, in embryonal carcinoma cells, IRF-2 inhibits the activation mediated by IRF-1 of the IFN-α/β promoters (Harada et al 1990). Since overexpression of IRF-2 can thus block the transactivation effects of IRF-1, a model was proposed that IRF-2 could function as the preinduction repressor of PRD I activity, and that induction would somehow bring about a change in the relative activities or abundances of IRF-1 and -2. However, a targeted disruption, or a gene knock out, of IRF-2 failed to generate detectable IFN-β expression in uninduced cells (Matsuyama et al 1993). Furthermore, as discussed in chapter 4, in a mutagenesis study, the binding affinity of IRF-2 for PRD I variants did not correlate with repression prior to induction (Whiteside et al 1992). Our favoured candidates for preinduction repressors through PRD I are the two binding activities termed Un1 and Un2, and will be introduced in chapter 4.

IRF-2 is proteolytically processed during induction by dsRNA to leave an amino-terminal fragment missing approximately 185 amino acids, but still capable of binding DNA (Cohen and Hiscott 1992; Palombella and Maniatis 1992; Whiteside et al 1992, 1994). It appears that the truncation product has a higher affinity for a specific DNA binding site, and is also a more potent repressor of the IFN-β promoter, than the full-length precursor (Whiteside et al 1994). Furthermore, the kinetics of production of the cleavage product lags behind that of the activation of IFN-β transcription. It has thus been proposed that the truncated IRF-2 is a postinduction repressor generated by a proteolytic event upon induction. Indeed, supporting this, the only detectable effect of IRF-2 knock out is an inefficient turn-off of expression following induction (Matsuyama et al 1993).

It has been shown that the two IRF factors regulate cell growth in a mutually antagonistic manner, such that IRF-1 has tumor suppressive, and IRF-2 oncogenic, properties. Overexpression of IRF-2 in cultured NIH3T3 cells results in their transformation;
furthermore, the cells overexpressing IRF-2 are tumorigenic in nude mice (Harada et al 1993). Concomitant overexpression of IRF-1 reverses the transformed phenotype. Furthermore, embryonic fibroblasts from mice with a null mutation in the IRF-1 gene are predisposed to transformation by expression of an activated Ha-Ras protein, and the transformed phenotype can be suppressed by the expression of the IRF-1 cDNA (Tanaka et al 1994). A role of IRF-1 in the negative regulation of cell proliferation is further supported by the finding of deletions and inactivating rearrangements of the IRF-1 locus (5q31.1) in human leukemias and preleukemic myelodysplasias (Willman et al 1993). It remains to be determined whether the oncogenic and tumor suppressive effects of the IRF factors are associated with their function as transcriptional effectors; this could be proven by the inability of the IRFs defective in DNA binding or activation/repression function to exert the function. After confirming this, it will be interesting to learn what are the critical target genes. As discussed above, two of the IFN inducible products, PKR and 2'-5' OAS have been shown to have antiproliferative activities, suggesting that they may be candidates for IRF target genes. However, induction of these genes by IFNs in IRF-1+/− cells is not impaired (Matsuyama et al 1993, Ruffner et al 1993).

While screening expression libraries with a PRD I probe, Keller and Maniatis (1991) have obtained a cDNA clone that encodes a novel PRD I binding protein, PRD I-BFI, containing five zinc fingers. The PRD I-BFI gene is inducible by virus, and the peak in PRD I-BFI mRNA levels follows that of the IFN-β mRNA, suggesting that PRD I-BFI functions as a postinduction repressor of the IFN-β promoter. In agreement with this, overexpression of PRD I-BFI can block viral induction of either the intact IFN-β promoter or PRD I multimers.

In accord with the fact that PRD III is similar to PRD I, it binds an extensively overlapping set of transcription factors. A model could thus be imagined, in which the cells that require the PRD III element would contain insufficient levels of PRD I binding factor to activate a promoter containing only one binding site; cooperative binding of (a) factor(s) to the combined PRD I/PRD III sites may on the other hand be sufficient to activate transcription. It should be noted that two molecules of IRF-1 cannot bind simultaneously to the adjacent PRD I and PRD III elements (S.Goodbourn, pers. comm.), indicating that IRF-1 does not function cooperatively through the PRD I/PRD III elements.

1.2.2.4.3. PRD II Binding Factors

The sequence GGGAAATTC between -64 and -55 of the IFN-β promoter represents a consensus motif for the binding of the transcription factor NF-κB (Fujita et al 1989a,
Lenardo et al. 1989, Visvanathan and Goodbourn 1989), a transcription factor originally identified as a regulator of immunoglobulin \( \kappa \) light chain transcription, and shown to play a central role in the regulated expression of a number of immune and inflammatory response genes (reviewed in Grilli et al. 1993, Baeuerle and Henkel 1994). Point mutations that abolish the binding of a classical NF-\( \kappa \)B-dimer p65/p50 to PRD II result in considerable reduction in virus-induced expression (Goodbourn and Maniatis 1988, Thanos and Maniatis 1995a). NF-\( \kappa \)B makes contacts with the major groove at the GC-rich ends of the \( \kappa \)B site in the IFN-\( \beta \) promoter, while another protein, HMGI(Y), binds to the minor groove in the central (A+T)-rich region (Thanos and Maniatis 1992).

NF-\( \kappa \)B is sequestered in the cytoplasm by association with an inhibitor termed I\( \kappa \)B, which masks the nuclear localization sequences of the Rel subunits (reviewed in Gilmore and Morin 1993, Beg and Baldwin 1993). NF-\( \kappa \)B is liberated from the inhibitor by dsRNA, but also by a variety of other inducers, such as TPA, IL-1, TNF, cAMP, viral transactivators, reactive oxygen intermediates, and energy-rich radiation. Nevertheless, the activation pathways of NF-\( \kappa \)B by dsRNA or TPA do not appear to be identical, since in mouse embryonal carcinoma cells, the former cannot induce NF-\( \kappa \)B binding to DNA while the latter can do so (Ellis and Goodbourn 1994). The mammalian NF-\( \kappa \)B activity can consist of homodimers or heterodimers of the subunits belonging to the Rel family, including p50 (NF-\( \kappa \)B-1), p65 (RelA), c-Rel, p49, and RelB. All these proteins share a conserved 300 amino acid domain (Rel homology domain) required for DNA binding, dimerization, I\( \kappa \)B binding and nuclear localization. Both the precise preferred DNA sequence for various dimers, and the regulatory consequences of binding particular forms of NF-\( \kappa \)B differ from each other. Several cDNA clones have been isolated which encode proteins with I\( \kappa \)B-like properties; these include MAD3 (I\( \kappa \)Ba), and the product of the \( bcl-3 \) proto-oncogene. Different I\( \kappa \)B activities have different targets for the inhibition: MAD3 prevents both the nuclear uptake and the DNA binding of p65 or c-Rel -containing NF-\( \kappa \)B-dimers, whereas Bcl-3 inhibits the DNA binding of the p50 homodimers. The NF-\( \kappa \)B subunits p50 and p49 are derived from the precursor proteins p105 and p97, respectively, through proteolytic processing. The carboxy-terminal regions of both of these precursors share a conserved putative protein-protein interaction domain, called the ankyrin repeat, with the I\( \kappa \)B proteins, and indeed, p105 exhibits an I\( \kappa \)B-like activity in that its \textit{trans}-inhibitory carboxy-terminus can block the nuclear localization or DNA binding of NF-\( \kappa \)B.

According to the current model (Mellits et al. 1993, Traenckner et al. 1994, Thanos and Maniatis 1995b) for the activation of a classical NF-\( \kappa \)B heterodimer, that is the p50/p65-complex, both the inhibitor MAD3 and the p50 precursor protein p105 become specifically phosphorylated upon induction with dsRNA, or the other inducers. The phosphorylation of
MAD3 is believed to mark it for its subsequent degradation, which then releases NF-κB. While many kinases (e.g. PKC, PKA, heme-regulated kinase, Raf-1) are capable of activating NF-κB-IkB complexes in cell free systems, the true *in vivo* IkB-kinases remain to be unequivocally determined. In the context of the IFN-β induction, the findings that the dsRNA-activated kinase PKR is capable of phosphorylating IkB and activating NF-κB *in vitro* (Kumar et al 1994), and that the blockage of PKR can inhibit NF-κB activation by dsRNA within living cells (Maran et al 1994), may prove important. The induction-specific phosphorylations are followed by two proteolytic events also necessary for NF-κB induction: the rapid degradation of MAD3, and the processing of p105 to p50 to remove the inhibitory carboxy-terminus (Mellits et al 1995). The p105 processing - and possibly also the MAD3 degradation - appears to be mediated by the ubiquitin-mediated protein degradative pathway (Palombella et al 1994). After escaping the proteolytically inactivated inhibitory activities, NF-κB translocates from cytoplasm to nucleus in order to associate with κB response elements.

1.2.2.4.4. PRD IV Binding Factors

The PRD IV elements contain a binding site for the ATF/CREB family of bZip transcription factors, and it has been suggested that ATF-2 mediates the virus-inducibility through PRD IV, either as homodimers or heterodimers with c-Jun (Du and Maniatis 1992, Du et al 1993). Accordingly, the overexpression of either ATF-2 or c-Jun antisense RNA in transfected HeLa cells decreases the inducibility of the native human IFN-β promoter encompassing all the identified PRDs and NRDs. Base substitutions that interfere with *in vitro* binding of the ATF factors to PRD IV decrease the level of virus induction in mouse L929 cells, and multiple copies of PRD IV have been reported to confer inducibility by both virus and cAMP treatment on a cotransfected heterologous promoter. While the entire PRD IV element appears to be required for viral induction, the flanking 5' and 3' A+T rich regions are dispensable for stimulation by cAMP. This is consistent with the suggestion that these A+T rich sequences interact with HMGI(Y) proteins, and that these protein-DNA interactions are necessary for viral induction (see below).

1.2.2.4.5. Contribution of HMGI(Y) Proteins to Induction

The HMGI(Y) protein has been reported to be required for the transcriptional activities of both NF-κB (Thanos and Maniatis 1992, 1995) and ATF-2 (Du et al 1993) in the context of the IFN-β promoter. HMGI(Y) is a basic, low-molecular weight protein that binds to double-stranded DNA with a limited sequence specificity for A tracts (reviewed in Bustin et al 1990). The mechanisms by which HMGI(Y) may act on the PRD elements II and IV
appear rather similar. The Maniatis group have reported HMGI(Y) to make minor groove contacts with both the central (A+T)-rich region of PRD II and the two (A+T)-rich regions flanking PRD IV. They further showed that the degree of HMGI(Y) binding to any of these sites correlates with the extent of the virus induction of the IFN-β gene, and that the overexpression of antisense HMGI(Y) RNA can block the virus induction (but see discussion in chapter 5). The mechanism of action of the HMGI(Y) protein appears to involve the bending of DNA upon its binding, which then leads to the enhancement of binding of both NF-κB and ATF-2 to their respective binding sites, as well as to the physical interaction between the DNA-bound NF-κB and ATF-2 proteins. Furthermore, even in the absence of DNA, HMGI(Y) can interact directly with both NF-κB and ATF-2.

In a cell-free assay in the presence of high concentrations of specific proteins, the association between HMGI(Y) and a particular splicing isoform of ATF-2 in solution triggers an equilibrium shift towards dimerization of this ATF-2 variant (Du and Maniatis 1994); this could contribute to the observed stimulation of the ATF-2 binding activity. In conclusion, the function of HMGI(Y) may be to contribute to the assembly of an inducible multiprotein complex on the IFN-β promoter, by facilitating both protein-DNA and protein-protein interactions. The role proposed for HMGI(Y) in the context of the IFN-β promoter clearly differs mechanistically from the one implicated by the early studies by Strauss and Varshavsky (1984). They proposed that by binding to discrete sites in the α-satellite repeats of African green monkey chromosomes, HMGI(Y) could determine certain patterns of nucleosome phasing. Furthermore, it has been suggested that by interacting with multiple sites in mouse satellite repeats, the HMGI(Y) proteins would influence the degree of heterochromatic condensation (Radic et al 1992). While all the proposed models of action by HMGI(Y) implicate a structural function, they would operate at different levels (promoter sequence-specific nucleoprotein structures vs. chromatin architecture). Obviously, these models are not exclusive, and they could all depend on the same functional domains of the HMGI(Y) proteins. The organization of the IFN-β promoter into nucleosomal structure(s), and the potential regulatory significance of this, has not been sufficiently investigated to propose or exclude the possibility that HMGI(Y) proteins (or any other proteins) can function at that level. In section 3.2., we speculate that Oct-1 could be another candidate protein to have such a regulatory role as an organizer of the IFN-β promoter into higher-order nucleoprotein structures.

1.2.2.5. Postinduction Repression

The postinduction repression of the IFN-β promoter also appears to occur at the level of transcription, and requires yet another distinct set of transcription factors. In mouse C127 cells, the postinduction shut-off is caused, in part, by changes in the rate of transcription
initiation (Whittemore and Maniatis 1990a,b). In transfection analyses, the virus-inducible IRE region from the IFN-β promoter, that contains the elements PRD I, PRD II, and NRD I, can be appropriately turned off when fused to a heterologous gene, thus this region must contain the regulatory elements involved in postinduction repression. NRD I is not a likely candidate for the postinduction repressor binding site, since the kinetics of the induction cycle are not affected by a deletion that removes most of the NRD I - thus factors involved in pre- and postinduction repression appear to be distinct. Interestingly, virus-inducible multimers of either PRD I or PRD II exhibit the characteristic postinduction repression, indicating that both elements can serve as binding sites for regulatory proteins capable of mediating the effect. Two PRD I-binding factors, IRF-2 and PRD I-BFI, have been proposed to mediate postinduction shutoff (see section 1.2.2.4.2.).

Although the IFN-β gene is turned off at the transcriptional level, rapid degradation of the transcript also contributes to the postinduction decrease in IFN-β mRNA (Whittemore and Maniatis 1990a). Analysis of fusion gene constructs in stably transfected mouse C127 cells indicates that there are two distinct destabilizers in the IFN-β transcript. One destabilizer is located in the 3’ untranslated region and is similar to the AU-rich motif found in many other highly inducible mRNAs with short half-lives, such as the c-myc, c-fos, and GM-CSF (granulocyte-macrophage colony-stimulating factor) mRNAs (for review, see Braverman 1989). The other destabilizer within the IFN-β mRNA is located 5’ to the translation stop codon and does not resemble AU-rich destabilizers. Rapid decay of IFN-β transcripts occurs constantly, and is not regulated during the induction cycle.

The postinduction shut-off in expression can be delayed in the presence of cycloheximide, so that the rate of transcription remains high up to 24 hours after induction (Whittemore and Maniatis 1990a), suggesting the existence of a mechanism dependent on protein synthesis. The postinduction repression of several other inducible promoters can similarly be interfered with by the inhibition of protein synthesis. For example, it has been shown that the inhibition of protein synthesis with cycloheximide- or puromycin-treatment following oestrogen withdrawal superinduces mRNA from the oestrogen-receptor-inducible apolipoprotein II (apoII) promoter without affecting the stability of the apoII mRNA (Sensel et al 1994).

1.2.2.6. The Complexity of the Beta-Interferon Promoter

The correct transcriptional regulation of the IFN-β promoter requires coordinated function of multiple response elements and regulatory transcription factors. The changes in the DNA binding activities at all the stages of the induction process are summarized in figure 1.5.
Figure 1.5. Induction cycle of the IFN-β promoter.

A schematic representation of the changes in the array of DNA binding factors that occur on the IFN-β promoter during the induction cycle.
Uninduced

Early induction

Late induction

Postinduction

- Preinduction repressors
- Primary activators
- Secondary activators
- Postinduction repressors
The ultimate challenge is to determine the roles of individual transcription factors during the different stages of the induction cycle. It seems likely that the promoter is organized into distinct stage-specific three-dimensional nucleoprotein structures of great complexity. Despite years of investigation by several groups on the IFN-β promoter, it still seems a formidable challenge to elucidate all the protein-DNA and protein-protein interactions mediating these changes in promoter architecture.

An induction response that requires multiple events is likely to serve to prevent fortuitous transcriptional activation of a gene encoding such a potent cytostatic protein. This becomes apparent when one considers the possibility that one or more of the regulatory elements may be affected by other signal transduction pathways. Indeed, in addition to dsRNA, NF-κB can be activated by a number of cellular signals (see section 1.2.2.4.3.), none of which appears to induce the IFN-β gene (Lenardo et al 1989, Lacoste et al 1990, Watanabe et al 1991). Also, as discussed above, PRD I is related to an element of other regulatory properties, termed ISRE (Interferon Stimulatable Response Element; reviewed in Williams 1991), which is sufficient to mediate induction of a heterologous gene by IFNs. In fact, reiterated AAGTGA, a PRD I-like element, also has the same property. However, it is important to bear in mind that the intact IFN-β promoter cannot be induced by interferons. This does not exclude the possibility that the induction can be enhanced by IFNs after the primary signal, such as dsRNA, has initiated the necessary events for induction. Perhaps the IFN-β produced from the gene itself further contributes to the induction in a positively autoregulatory manner. Nevertheless, the production of IFN cannot be obligatory, since protein synthesis is not required for induction (see above). Furthermore, it has been demonstrated that a transfected IFN-β gene is inducible when introduced into a cell line that lacks its own type I genes (Mosca and Pitha 1986).

Another interesting aspect of the induction process is the involvement of induction-specific proteolytic processing events affecting the factors that act on at least two separate regulatory elements: the truncation of a PRD I binding factor IRF-2 (Palombella and Maniatis 1992; Whiteside et al 1992, 1994), and the two proteolytic events required for NF-κB activation of the PRD II element. The two processing pathways are not identical, judged by their differential sensitivity to the specific protease inhibitors (Palombella et al 1994, Whiteside et al 1994). However, it is possible that while processing pathways diverge, or converge, at some stage, they may share the same components mediating either distal or proximal events. In this context, it may prove important that while certain protease inhibitors can indeed block IFN-β induction, they cannot block the proteolysis of IRF-2 (Whiteside 1992). In any case, it is intriguing to speculate that the induction process would involve multiple proteolytic events, which could be required in a temporally coordinated fashion.
during the induction cycle. Perhaps the essential derepression of the IFN-β promoter also involves specific degradation of preinduction repressor proteins early in the induction cycle; this has been investigated in the case of one of our candidates for a preinduction repressor, Oct-1 (section 3.5).

1.2.2.7. Regulation of Other Type I Interferon Promoters

Since the human IFN-β protein is encoded by a single gene, it is much more amenable for induction studies than the multimember IFN-α gene family. However, the transcriptional regulation of the IFN-α genes is believed to be dependent on very similar mechanisms. The 5' flanking regions that mediate the transcriptional activation are somewhat homologous for both IFN-α and IFN-β genes. Nevertheless, there are also differences between the IFN-α and IFN-β genes with respect to activation. For example, the virus-responsive regions of the IFN-α genes do not contain NF-κB binding sites (MacDonald et al 1990, Raj et al 1991). There are also differences in the virus-inducibilities between the individual members of the IFN-α gene family (Bisat et al 1988); in the case of mouse IFN-α4 and IFN-α6 genes, this is due to a difference in the sequence of two nucleotides in their regulatory regions (Raj et al 1991).

A role has been proposed for IRF-1 in the induction of IFN-α genes. The overexpression of IRF-1 in COS cells leads to the transcriptional activation of both the endogenous IFN-α and IFN-β genes (Fujita et al 1989b). In P19 embryonal carcinoma cells, a reporter gene under the control of an IFN-α promoter is activated by overexpression of IRF-1 as efficiently as one under the control of an IFN-β promoter (Harada et al 1990). Furthermore, transient overexpression of IRF-1 leads to stimulation of the cotransfected murine IFN-α4 and IFN-α6 promoters (Au et al 1992). However, similarly to the IFN-β promoter, IRF-1 cannot be ubiquitously essential for induction of IFN-α promoters, since these can be induced in murine embryonal carcinoma cells in which both the IRF-1 alleles have been targeted for gene disruption (Ruffner et al 1993).

The IFN-α promoters are also conditionally inducible in priming-dependent cell lines. In the case of IFN-α4 promoter, it has been shown that the cis-elements required for priming and virus-inducibility are identical (Rosztoczy and Pitha 1993), thus the effect appears to be mediated by factors binding to virus-inducible elements, similarly to the IFN-β promoter.

The promoter of the IFN-τ gene has not been thoroughly analyzed; however, the IFN-τ mRNA synthesis is also known to be increased by exposure to virus or dsRNA (Cross and
Roberts 1991), thus the transcriptional cues regulating its expression are likely to be somewhat similar to those operating on the other type I IFN genes.

1.2.2.8. Regulatory Similarities between Type I IFN and IFN-Inducible Promoters

As discussed above (section 1.2.1.2.), genes induced by type I IFNs contain in their promoter a conserved response element ISRE (consensus GGAAART$_{T/A}$GAAACTR, where R=purine). ISRE is very similar in sequence to PRD I - for example, the IRS of the H-2K$^b$ gene promoter differs by only one nucleotide from PRD I. By using EMSA, three major complexes have been shown to specifically interact with ISRE elements: ISGF1, -2, and -3 (IFN-stimulated gene factors 1, 2, and 3) (Levy et al 1988). The constitutive binding activity ISGF-1 appears to be homologous to the PRD I binding factor IRF-2. In contrast to ISGF1, the binding of ISGF2 and -3 is induced in response to type I IFN treatment, but only the induction of the ISGF2 depends on de novo protein synthesis. ISGF2 has subsequently been shown to be identical to IRF-1 (Pine et al 1990). The time course of ISGF3 binding activity (see section 1.2.1.2.) closely parallels the transcriptional activation of IFN-inducible genes, implying it as the primary positive activator (Levy et al 1989). Mutational analyses of the ISRE region have demonstrated the requirement of the entire 15bp sequence for both ISGF3 binding and IFN-stimulated induction of the ISRE-containing promoters, whereas only a core region of approximately 9bp appears sufficient to bind IRF-1 and -2. As is the case for the type I IFN promoters, IRF-1 cannot be essential for the transcriptional induction for the IFN-stimulated genes, since this can proceed in murine embryonal carcinoma cells devoid of any IRF-1 expression (Ruffner et al 1993). However, it is likely that IRF-1 has a role as a secondary activator to enhance the transcriptional response. Further supporting the importance of IRF-1 in the establishment of IFN-induced antiviral state, the inhibition of encephalomyocarditis virus (EMCV; picornavirus) replication is severely impaired in cells from mice whose both IRF-1 alleles have been disrupted by gene knockout events (Kimura et al 1994). Interestingly, the effect exhibits some selectivity with respect to the virus species, since the complete lack of endogenous IRF-1 did not considerably affect replication of two other types of viruses: vesicular stomatitis virus (rhabdovirus) and herpes simplex virus (herpesvirus). This result probably bears more physiological relevance than those by Pine (1992) who reported that overexpression of the IRF-1 cDNA induces an antiviral state against the members of three RNA virus families, namely VSV (rhabdovirus), EMCV (picornavirus) and Newcastle disease virus (paramyxovirus).
Another member of the IRF family, ICSBP (IFN consensus sequence binding protein), the expression of which is restricted to macrophages and lymphocytes, only weakly binds to an ISRE element. However, ICSBP is capable of forming a complex with either IRF-1, IRF-2, or ISGF3, both in vivo and in vitro, in the presence or absence of the specific DNA element (Bovolenta et al 1994). The association with IRFs appear to substantially increase the extent of interaction between DNA and ICSBP, which by itself is not a very potent DNA binding protein (Driggers et al 1990). In contrast, the interaction of ICSBP with ISGF3 leads to the inhibition of the DNA binding activity of the latter. In functional studies it has become apparent that ICSBP can repress the induction of transcription by either IRF-1 or IFN-α (Nelson et al 1993); this repression may be attributed to the formation of complexes between ICSBP and the IRF family factors.

In keeping with the observation that members of the IRF family can interact with regulatory elements of both the interferon genes and the IFN-inducible genes, synthetic promoters containing multiple copies of either PRD I or ISRE are induced by IFN-α/β and IFN-γ, as well as by poly(I)-poly(C) or virus (Fan and Maniatis 1989, MacDonald et al 1990). However, the intact IFN-β promoter is not IFN-inducible. In contrast, some, but not all, IFN-inducible genes are induced by dsRNA or virus (Vilcek 1989). The possibility that the activation of PRD I- or ISRE-containing promoter by viral infection was indirectly mediated by induced IFN in these experiments has been ruled out by performing the inductions in cells which lack IFN-α and IFN-β genes (Wathelet et al 1992). The differences in the activities of intact promoters, as opposed to those that contain PRD I or ISRE elements in isolation, may be the result of negative regulatory domains that preclude the binding of positive regulatory factors.

1.2.2.9. Similarities between the IFN-β Promoter and Other Inducible Promoters

It has recently become apparent that the promoters of many genes encoding other immunologically important proteins resemble that of the IFN-β gene in the degree of their complexity. These include several transiently inducible genes for cytokines and cell adhesion molecules. The transcription factor NF-κB is required for the expression of a number of these such as those encoding cytokines interleukin 2, interleukin 6, granulocyte colony stimulating factor (reviewed by Grilli et al 1993), and cell adhesion molecules E-selectin, ICAM, and VCAM-1 (Collins 1993).

One particular promoter region that has recently gained much experimental attention is that regulating the expression of the E-selectin gene. The transcription of E-selectin gene is
transiently induced in endothelial cells in response to cytokines such as interleukin-1 and tumor necrosis factor α. The organization of the E-selectin promoter bears striking resemblance to that of the IFN-β promoter, including both NF-κB and ATF sites, which appear to cooperate transcriptionally, leading to synergistic activation (Kaszubska et al 1993). Furthermore, the HMGI(Y) has been proposed to have a role in the E-selectin promoter activation by mediating NF-κB binding (Lewis et al 1994). Despite the many similarities between the E-selectin and IFN-β promoters, the inducer specificity is maintained: IFN-β transcription is not cytokine-inducible, and the E-selectin promoter is only very weakly induced by viral infection (T. Maniatis, pers. comm.).

Another inducible promoter which may rely on HMGI(Y)-stimulated protein-protein interactions of NF-κB family proteins is that of the IL2Ra (interleukin 2 receptor α chain) gene (John et al 1995). Interestingly, in T cells, the binding of HMGI(Y) to this promoter appears inducible by mitogens, whereas the HMGI(Y) binding to the IFN-β promoter in fibroblastic cells has been proposed to be constitutive.
Aims of the Thesis

The purpose of the project was to further investigate several aspects of the regulation of the IFN-β promoter. The main emphasis has been in elucidation of the factors involved in the preinduction repression of the promoter, which has remained less well characterized than the factors mediating the transcriptional induction.

In Chapter 3 we propose that the ubiquitous transcription factor Oct-1 may be one of the preinduction repressors of the IFN-β promoter. We present an in vitro analysis of both the DNA binding properties and posttranslational modifications of Oct-1. Also, our functional analysis on the role of Oct-1 in the regulation of the IFN-β promoter is presented. To enable us to perform these functional studies we established a transient transfection system in which the preinduction repression of the IFN-β promoter can be demonstrated.

In Chapter 4 we introduce two other DNA binding activities, referred to as Un1 and Un2, which are further candidates for the elusive preinduction repressors of the promoter. The results of the large-scale purification of these factors, as well as further analysis on their DNA binding properties are presented.

In Chapter 5 we present our analysis on the promoter element PRD IV.

In Chapter 6 we investigate whether protein kinase A is involved in the IFN-β induction process, and discuss the implications of our findings for the studies on the dsRNA-triggered signal transduction pathway.

In Appendix I we report the results of our comparison between the characteristics of two transient transfection methods: calcium phosphate coprecipitation and Transfectam methods.

Appendix II is a manuscript on the basic mechanisms of transcriptional regulation of RNA polymerase II promoters (Eloranta and Goodbourn 1995, in press).
Chapter 2: Materials and Methods

2.1. Basic DNA Manipulations

2.1.1. DNA Preparations

2.1.1.1. Large-Scale Plasmid Preparations

A fresh 2ml bacterial culture grown overnight in L-broth supplemented with 60μg/ml ampicillin was used to inoculate 200ml of brain-heart infusion. Cultures were incubated overnight with vigorous aeration, at 37°C. After pelleting the bacteria at 4200rpm in a J6 centrifuge for ten minutes, they were suspended in 10ml of solution I (50mM glucose; 25mM Tris, pH8.0; 10mM EDTA) at room temperature. 20ml of solution II (0.2M NaOH, 1% SDS) was added, followed by vigorous mixing. After 5 minutes at 4°C, 15ml of cold solution III (5M potassium acetate, pH4.8) was added, followed by mixing. After 10 minutes at 4°C, the bulk of chromosomal DNA and bacterial nucleic acids were allowed to precipitate for 5 minutes on ice, and pelleted by centrifugation, as above. The supernatant was further cleared by running it through cheesecloth, and 0.6 volumes of isopropanol added to it. Nucleic acids were allowed to precipitate for 5 minutes on ice, and pelleted by centrifugation, as above. The pellets were dissolved in 5ml TE(10:1) pH8.0, and 5ml of 5M LiCl added to precipitate the bulk of contaminating bacterial RNA. Contents of the tubes were mixed, and let stand on ice for 5 minutes, after which the centrifugation was repeated and supernatants recovered. Next, 25ml of ethanol (>99.7%) was added, tubes mixed, and incubated on ice for 5 minutes. After another centrifugation, the pellets were redissolved in 2.5ml TE(10:1) pH8.0. Into exactly 2.7ml of the DNA solution 4.2g of cesium chloride was added and dissolved. Next, 0.2ml of the intercalating dye ethidium bromide [stock 10mg/ml in TE(10:1) pH7.5] was added. The DNA-CsCl-EtBr solution was layered beneath 8ml of 55% CsCl in TE(10:1) (pH7.5), in Quick-Seal polyallomer tubes (Beckman). The filled tubes were balanced within 30mg, sealed and centrifuged for at least 16 hours at 50000rpm in a fixed-angle ultracentrifuge rotor. This allows separation of supercoiled plasmid DNA from the remaining contaminating chromosomal DNA and RNA species. The DNA was recovered from the gradient using a needle and a syringe. The ethidium bromide was carefully removed by multiple extractions with isobutyl alcohol saturated with 1M NaCl. Finally the DNA was ethanol precipitated twice, the final pellet dissolved in an appropriate volume of TE(10:1) (pH7.4), and concentrations of the plasmid preparations determined.

2.1.1.2. Small-Scale Plasmid Preparations
Selected colonies were used to inoculate 2ml brain-heart infusion containing 60μg/ml ampicillin, and these were incubated overnight in a shaker at 37°C. Bacteria were harvested by centrifugation and treated with the solutions I (100μl), II (200μl) and III (150μl), as in section 2.1.1.1. After this, 400μl equilibrated phenol was added to extract the proteins away from DNAs (see below), and extracted supernatants precipitated with ethanol (see below). Precipitated DNA pellets were dissolved in TE(10:1) (pH7.4) supplemented with 40μg/ml RNAase A.

2.1.1.3. Synthesis of Oligonucleotides

All oligonucleotides used in this study were provided by K.Hobbs and I.Goldsmith of the ICRF Oligonucleotide Service.

The following oligonucleotides were used in DNA-protein interaction assays, as well as in experiments involving intracellular sequestering of specific DNA binding proteins. They were all synthesized so that following annealing, they would have a single stranded 5' GATC overhang at either end.

IFN-β -108/-95 (NRD II) upper: GATCCAAAATGTAAATGACA
IFN-β -108/-95 (NRD II) lower: GATCTGTCATTTACATTTTG
IFN-β -104/-91 upper: GATCTGTAAATGACATAG
IFN-β -104/-91 lower: GATCCTATGTACATTTTG
IFN-β -55/-40 (NRD I) upper: GATCCTCTGAAATAGAGAG
IFN-β -55/-40 (NRD I) lower: GATCCCTCATATAAATAGGA
IFN-β -33/-20 (TATA) upper: GATCCCTCATATAAATAGGA
IFN-β -33/-20 (TATA) lower: GATCCCTCATATAAATAGGA
IFN-β -77/-64 (PRD I) upper: GATCCGAGAAGTGAAAGTGA
IFN-β -77/-64 (PRD I) lower: GATCCCTACTTTCACCTTCTC
IFN-β -64/-55 (PRD II) upper: GATCGGGAAATTCC
IFN-β -64/-55 (PRD II) lower: GATCGGAAATTCC
IFN-β -91/-78 (PRD III) upper: GATCGGGAATTTGAGAAGG
IFN-β -91/-78 (PRD III) lower: GATCCCTTCAGTTTCC
octamer upper: GATCCATGCAAATGAA
octamer lower: GATCCTTCATTTGCATG
hepoct+ upper: CGAGTGCTCATGAATATGCAAATCAATTGG
hepoct+ lower: TCGACCAATTTGAGCATATTCATGAGCACTCGAGCT
hepoct- upper: CGAGTGCTCATGAATATCAGTGCCTATTGG
hepoct- lower: TCGACCAATGGCGACTGATATTCATGAGCACTCGAGCT

collagenase API upper: GATCCGGCTGACGTCATCAAGCTA

collagenase API lower: GATCTAGCTTGATGACGTCAGCCG

somatostatin CRE upper: GATCCTGACGTCAGCCAAGGATC

somatostatin CRE lower: GATCGATCCTTGGCTGACGTCAG

SV40 API upper: GATCCTTGCTGACTAATTGAG

SV40 API lower: GATCCTCAATTAGTCAGCAA

Oligonucleotides used in subcloning procedures were as described in the text.

2.1.1.4. Measurement of DNA Concentration

DNA concentrations were determined by absorbance assuming 50µg dsDNA/A260 unit or 33µg ssDNA/A260 unit.

2.1.1.5. Phenol Extraction of DNA

Phenol extractions were performed to remove proteins from nucleic acid solutions. Prior to use phenol was equilibrated to pH>7.6 (Sambrook et al 1989), and an antioxidant hydroxyquinoline added to the final concentration of 0.1%. Equilibrated phenol was added to the DNA samples 1:1, the contents mixed and phases separated by centrifugation. The upper aqueous phase containing DNA was transferred to a clean tube for ethanol precipitation.

2.1.1.6. Ethanol Precipitation of DNA

To concentrate sample DNAs and/or change the buffer conditions of DNA preparations ethanol precipitations were performed. The concentration of monovalent cations in DNA samples was adjusted to either 0.25M sodium acetate (pH5.2) or 2.0M ammonium acetate, and two and a half volumes of cold ethanol was added to samples. DNA samples were stored on dry ice or in -20°C to allow the precipitates to form. The precipitates were pelleted by centrifugation at 4°C and washed with 70% ethanol followed by another centrifugation. Pellets were then air-dried and dissolved in an appropriate buffer.

DNA molecules under 200bp were precipitated in the presence of 10mM MgCl2. Whenever the amount of DNA to be precipitated was less than 500ng, either glycogen (20µg) or tRNA (20µg) was added to the sample prior to the addition of ethanol.
2.1.2. Agarose Gel Electrophoresis

Agarose gel electrophoresis was used to confirm the purity and concentrations of large-scale plasmid preparations, to verify the completion of restriction enzyme digestions, and to determine the sizes of the resulting fragments of restriction enzyme reactions. Electrophoresis was performed in horizontal gel boxes, in AGB buffer (40mM Tris-acetate/1mM EDTA). The agarose concentration of gels varied from 0.8% to 2%, depending on the expected sizes of the fragments to be resolved. The intercalating DNA dye ethidium bromide was added to the gels at the final concentration of 0.5μg/μl to enable the detection of DNA fragments upon illumination with an ultraviolet light source.

An aliquot of 1kb marker mixture (Gibco-BRL) was run in parallel in order to estimate the sizes of the DNA fragments in the samples to be separated.

2.1.3. Subcloning Procedures

2.1.3.1. Restriction Endonuclease Digestions

Restriction enzymes were obtained from Boehringer Mannheim, New England Biolabs, USB, or Promega, and used according to the manufacturer’s recommendations. All reactions were allowed to proceed to completion, unless otherwise stated.

2.1.3.2. Fill-in Reactions

The 3' recessed ends of digested DNA fragments were filled in with Klenow fragment of DNA polymerase I (Boehringer Mannheim) or AMV-reverse transcriptase (Life Sciences Inc.), to create blunt DNA termini. Reactions were performed under buffer conditions recommended by the manufacturers. Klenow (2 units/50μl) reactions were allowed to proceed for 5 minutes at room temperature, and reverse transcriptase (20 units/50μl) reactions for 30 minutes at 37°C.

2.1.3.3. Dephosphorylation of DNA Termini

Where appropriate, to prevent the circularization of the vector fragments with blunt termini during the incubation with DNA ligase, the phosphates from DNA termini were removed with calf intestinal alkaline phosphatase (CIP; Boehringer Mannheim). Dephosphorylation reactions were allowed to take place in volumes of 50μl, in the following buffer conditions: 50mM Tris (pH8.0), 100μM EDTA. The total incubation at 37°C was 1 hour, and the CIP
enzyme was added at two points: one unit in the beginning of an incubation and one unit after the first 30 minutes. Reactions were terminated by phenol extraction, and phenol-extracted DNAs precipitated with ethanol.

2.1.3.4. Phosphorylation of DNA Termini

The termini of double-stranded DNA oligonucleotide inserts were phosphorylated to enhance their ligation efficiency. The single stranded components of oligonucleotide probes used in SouthWestern were phosphorylated in the presence of 40μCi [γ-^32P]ATP/μg DNA in order to label them radioactively. Both phosphorylation reactions were performed by using T4 polynucleotide kinase (Boehringer Mannheim). The phosphorylation buffer contained 50mM Tris (pH8.2), 10mM MgCl₂, 100μM EDTA, 5mM DTT, 100μM spermidine.

2.1.3.5. Mung Bean Nuclease Reactions

To remove both 3' and 5' single-stranded extensions from DNA termini to leave ligatable blunt ends, mung bean nuclease (NEBiolabs) was used. The reaction buffer contained 30mM sodium acetate (pH4.6), 50mM NaCl, 1mM ZnCl₂. DNAs were digested with 50 units of MBN (Pharmacia) for 5 minutes at 16°C, after which the reactions were stopped by adding Tris (pH9.0) to 10mM, LiCl to 0.5M and SDS to 1%. Reactions were then extracted with phenol and precipitated with ethanol.

2.1.3.6. Bal31 Exonuclease Reactions

Bal31 nuclease degrades both 5' and 3' termini of duplex DNA without generating internal scissions, and was used for progressive shortening of double-stranded DNA. The reaction buffer contained 600mM NaCl, 12mM CaCl₂, 12mM MgCl₂, 20mM Tris (pH8.0) and 1mM EDTA. Reactions (40μl) were terminated by adding 60μl volume of TE(10:1)/20mM EGTA, which specifically chelates the essential cofactor Ca²⁺, together with 100μl phenol (pH8.0) and 20μg tRNA. Phenol extracted samples were then precipitated with ethanol.

2.1.3.7. Isolation of DNA Fragments

Native polyacrylamide gels (6-15%) were used to purify radioactively labelled oligonucleotides and closely migrating fragments less than 500bp; the electrophoresis was performed in vertical gel boxes, with TBE (90mM Tris-borate/2mM EDTA) as both gel and running buffers. DNA was located by ethidium bromide staining and UV-illumination, or
by autoradiography (if fragments were radioactively labelled). DNA fragments were eluted from gel slices in 400μl of the elution buffer (1M ammonium acetate, 1% SDS and 1mM EDTA) at 37°C overnight.

DNA fragments larger than 500bp were separated by agarose gel electrophoresis and eluted from LMP agarose (FMC Bioproducts) by adding at least five volumes of TE (20:1; pH8.0), heating the mixture at 70°C for 10 minutes, and phenol extracting the samples.

2.1.3.8. DNA Ligation Reactions

T4 DNA ligase catalyzes the formation of a phosphodiester bond between juxtaposed 5' phosphate and 3' hydroxyl termini in duplex DNA, and was used to join restriction fragments together. The reaction conditions were: 66mM Tris (pH7.5), 5mM MgCl₂, 1mM DTT, 1mM ATP, and 0.1-0.5 units/μl T4 DNA ligase (Boehringer Mannheim). Where applicable, the vector:insert-ratio was varied between 1:3 and 1:5. Ligation reactions were allowed to take place at the optimal temperature 16°C for 4-16 hours.

For ligation reactions involving blunt DNA termini, the reaction conditions were modified, so that the final ATP concentration was 50μM. This is believed to enhance the ligation efficiency of blunt termini.

2.1.3.9. DNA Transformation into Competent Escherichia coli Strains

Cells of the *E. coli* strain SCS-1 (F⁻, endA1, gyrA96, thi-1, hsdR17 [rK-mK+], supE44, recA1, relA1, λ⁺) (Stratagene) were made competent by the method of Hanahan (1985). A single colony of the SCS-1 strain was used to inoculate an overnight culture of 2ml in L-broth. This was used to further inoculate 400ml L-broth, which was split into four 1 liter sterile flasks. The cultures were grown with vigorous shaking at 37°C until their optical density (λ=550nm) was within the range 0.45-0.55. The cultures were cooled on ice, and pelleted by a centrifugation at 4°C with minimal force. Thoroughly drained bacterial pellets were suspended in 120ml of cold RF1 (100mM RbCl, 45mM MnCl₂, 35mM potassium acetate, 10mM CaCl₂, 5mM MgCl₂, 0.5mM LiCl, 15%(w/v) sucrose; pH5.8) and left on ice for 15 minutes. Cells were pelleted and drained as above, after which they were resuspended in 30ml of cold RF2 (10mM MOPS, 10mM RbCl, 75mM CaCl₂, 15%(w/v) glycerol; pH 6.8) and left on ice for 15 minutes. Aliquots of this suspension were dispensed into Eppendorf tubes and quickly frozen in liquid nitrogen. By this method, transformation competences exceeding 10⁷cfu/μg DNA were routinely achieved. An 100-200μl aliquot of competent bacteria were used for each transformation event.
To transform competent bacteria a ligation mixture or pure plasmid DNA was added to them, and left on ice for 20 minutes. The transformants were then heat shocked in a 45°C waterbath for 2 minutes. After this 1ml of L-broth was added and transformants incubated at 37°C for one hour. The transformation mixtures were finally plated out on LB-agar (1.5%) plates containing 100µg/ml ampicillin.

2.1.4. DNA Dideoxy Sequencing

DNA dideoxy sequencing reactions were performed using Sequenase or Taquence kits (United States Biochemicals), according to the manufacturer's instructions. The radioactive nucleotide used was either [α³²P]dATP or [α³⁵S]dATP. Aliquots of the reactions were electrophoresed in 6% denaturing gels at 32W, after which the gels were fixed (10% methanol, 10% acetic acid), dried in a gel dryer, and subjected to autoradiography.

2.1.5. Autoradiography of Radioactively Labelled Macromolecules

Gels and membranes containing radioactively labeled macromolecules were exposed to Kodak XAR films, for an appropriate period of time. Generally, the exposure to detect ³⁵S-labeled products was performed at room temperature, whereas autoradiographic exposures to assay molecules carrying ³²P were done at -70°C with an intensifying screen.

2.2. RNA Procedures

All solutions used in RNA handling were treated with DEPC (diethylpyrocarbonate), except Tris solutions, which were prepared by dissolving RNAase-free Tris in DEPC-treated water. DEPC-treatments of solutions were performed by adding 0.1% (v/v) diethylpyrocarbonate (Sigma) for at least 6 hours at room temperature, prior to autoclaving.

2.2.1. Isolation of Cytoplasmic RNA from Tissue Culture Cells

Cells on ø9cm dishes were washed twice with cold PBS. Washed cells were scraped into 1ml of cold PBS and pelleted in a microfuge at 4°C for 15 seconds. Pelleted cells were resuspended in 375µl ice-cold lysis buffer (50mM Tris, pH8.0; 100mM NaCl; 5mM MgCl₂, 0.5% NP40) and left on ice for 5 minutes. Nuclei and cell debris were spun out in microfuge at 4°C for 2 minutes. Next, 4µl 20%SDS and 4µl 10mg/ml of a freshly prepared proteinase K solution were added to samples, which were then mixed and incubated at
37°C for 15 minutes. After extraction with phenol:chloroform (1:1) and ethanol precipitation the RNAs were finally dissolved in TES, and quantitated by measuring the absorbance at the wavelength 260nm (1 A$_{260}$ unit = 40μg/ml RNA).

2.2.2. Preparation of Radiolabelled RNA Probes

Template plasmids, in which specific transcription is directed by the SP6 promoter, were used for the preparation of radiolabelled RNA probes to detect the expression levels of the endogenous IFN-β (the probe pSP65'IF described in Goodbourn et al 1985) or γ-actin (internal quantitation control, described in Enoch et al 1986) mRNAs. To ensure that the two protected species could be comparatively quantitated on the same autoradiographic exposures, the γ-actin probe was prepared at a tenfold lower specific activity than the IFN-β probe.

The RNA probe synthesis reaction was set up at room temperature to avoid precipitation of the DNA template by the spermidine in the transcription buffer. A typical 10μl reaction produced enough probe for approximately 100 RNAase protection reactions, and contained 40mM Tris (pH 7.5), 6mM MgCl$_2$, 2mM spermidine, 10mM DTT, 500μM ATP, 500μM UTP, 500μM CTP, 5μl [α$^{32}$P]GTP (Amersham, 400Ci/mmol), 10 units of RNAase inhibitor (from bovine pancreas, EC 3.1.27.5, DNAase-free; Boehringer Mannheim), and 5 units of SP6 RNA polymerase (EC 2.7.7.6; Boehringer Mannheim), together with 0.5μg of linearized template plasmid. Reactions were allowed to proceed for 2 hours at 37°C, and then for 15 more minutes after adding 20 units of DNAaseI (from bovine pancreas, EC 3.1.21.1, RNAase-free; Boehringer Mannheim) in order to digest the template. Reactions were stopped by adding 100μl TES (TE + 0.1% SDS), phenol extracted, and precipitated, by adding ammonium acetate to 2M and 2.5 volumes of ethanol, together with 20μg of carrier yeast tRNA. A precipitated probe was dissolved in 10μl of RNAase-free formamide loading buffer, and run on a 6% denaturing polyacrylamide gel to purify the full-length probe from partial, or degradation, products. After electrophoresis, the probe was located by autoradiography, cut out of the gel and eluted 8-16h in RNAase-free gel elution solution (1M ammonium acetate, 1% SDS, 1mM EDTA) at 37°C. An eluted probe was ethanol precipitated and finally dissolved in an appropriate volume of TES.

2.2.3. RNAase Protection Assay

Steady state levels of RNA expressed from endogenous genes were quantitated by RNAase protection assay.
The RNA samples (between 5µg and 20µg of total cellular RNA) were ethanol precipitated as above, and the RNA pellets dissolved in 30µl of hybridization solution (80% deionized formamide; 40mM PIPES, pH6.4; 400mM NaCl; 1mM EDTA), including the radioactively labelled probes in 3µl TES. Samples were first heated to 85°C for 10 minutes, and then hybridization reactions were allowed to proceed for 12-16h at 45°C. 350µl of RNAase solution (10mM Tris, pH 7.5; 5mM EDTA; 300mM NaCl; 40µg/ml RNAase A; 2µg/ml RNAase T1) was added to each sample, which were then incubated at 37°C for 30 minutes to digest the unhybridized probe RNA. Next, 10µl of 20% SDS and 10µl of freshly prepared 5mg/ml proteinase K in TE (10:1; pH7.5) were added, and samples incubated for 15 minutes, to terminate the RNAase reaction. Samples were then phenol extracted, and exactly 350µl of the aqueous layer from each sample, together with 20µg carrier yeast tRNA, precipitated with ethanol as above. Precipitated RNA pellets were dissolved in 4µl formamide loading buffer, heated at 95°C for five minutes, and loaded onto 6% denaturing gels.

2.3. Mammalian Cell Culture Procedures

2.3.1. Maintenance of Cultured Cells

HeLaE cells (a gift from Dr. E.Laufer; referred elsewhere in the text simply as HeLa cells) were cultured as monolayers on plastic tissue-culture grade dishes (Nunc) in Dulbecco's modified Eagle's medium (DMEM; ICRF Cell Culture Media Services) supplemented with 10% foetal calf serum plus penicillin (100 units/ml) and streptomycin (100µg/ml).

Mouse fibroblast L929 cells (ATCC CCL1) were cultured in MEM-alpha medium (Gibco-BRL) supplemented with 10% foetal calf serum plus penicillin (100 units/ml) and streptomycin (100µg/ml).

Every two days cells of both lines were trypsinized and passaged at 1 in 10.

HelaS cells were provided by the Central Cell Services of the ICRF. They were grown in spinner flasks, and maintained at 3-6x10^5 cells per ml.

2.3.2. Transfections with Transfectam Reagent

All Transfectam (TFM) transfections were performed on cells that had reached 50-60% confluency, on Ø6cm dishes.
The Transfectam (TFM) stock solution was prepared by dissolving 1mg of lyophilized powder into 40μl 96% ethanol and adding 360μl sterile deionized water. DNA to be transfected was added to 100μl of 0.3M NaCl solution, and the TFM reagent to 100μl amount of deionized water. Prior to adding into the serum-free medium, the DNA and TFM solutions were mixed together. TFM-DNA complexes were left in contact with cells for 6 hours (HeLa) or 9 hours (L929), after which the medium was replaced by 10% FCS-containing one. After approximately 48 hours, the reporter enzymes were quantitated as described below.

Optimization of the TFM transfection method for the cell lines used, as well as a comparison between the TFM and calcium phosphate method, are presented in Appendix I.

Transfectam reagent was purchased from Northumbria Biotech Ltd or Promega.

2.3.3. Transfections with Calcium Phosphate Coprecipitation

Calcium phosphate coprecipitations on ø9cm dishes were performed as described in Sambrook et al 1989. Briefly, DNA to be transfected was first mixed with 500μl 2xHBS [15mM Na₂HPO₄, 280mM NaCl, 10mM KCl, 12mM dextrose, 50mM HEPES (pH7.05)]. An equal volume of 0.25M CaCl² was slowly added to the DNA solution with vigorous mixing. Precipitates were allowed to form for 10 minutes, after which they were added to the cell media. Transfected cells were incubated with the precipitates for 6 hours, after which the media were replaced with 3ml of 20% glycerol in serum-free medium for exactly 2 minutes. Cells were then thoroughly washed with PBS, fresh medium added and incubation continued for approximately 48 hours before performing the reporter enzyme quantitations.

2.3.4. Priming and Induction of Cells

Mouse (L929, C127) and primed or unprimed human (HeLa) cells were grown to 70-80% confluence prior to inductions.

In order to prime human HeLa cells they were incubated for 16-24h prior to induction with 500U/ml Wellferon (a mixture of IFN-αs, Wellcome) in 5ml (ø6cm dishes) or 10ml (ø9cm dishes) DMEM + 10% FCS.

Inductions of human cells with dsRNA were carried out by incubating them with 100μg/ml poly(I)-poly(C) (Pharmacia) in 1.5ml (ø6cm dishes) or 3ml (ø9cm dishes) of serum-free
DMEM. Inductions of human cells with Sendai virus were carried out by diluting the virus stock (75000 hemagglutination units/ml) 1 to 10 in the same conditions. Human cell inductions were allowed to proceed for 4 hours. Where applicable, cycloheximide was added to a concentration of 50μg/ml, together with inducing agents.

Inductions of mouse cells with dsRNA were carried out by adding 25μg/ml poly(I)-poly(C)/600μg/ml DEAE-Dextran into 1.5ml (ø6cm dishes) or 3ml (ø9cm dishes) serum-free MEM. Inductions of mouse cells with Sendai virus were carried out as described for human cells. Mouse cell inductions were allowed to proceed for six hours.

2.4. Protein Methods

2.4.1. SDS Polyacrylamide Gel Electrophoresis

Protein samples were fractionated in a discontinuous electrophoretic system, in 7-12% polyacrylamide (29:1 mono:bis) separating gels containing SDS (Laemmli 1970). Fractionated proteins were analyzed either by Coomassie staining, or in the case of an analysis of ^35S-methionine labelled proteins, by autoradiography. All protein samples for SDS-gels were prepared by incubating them in SDS sample buffer [60mM Tris (pH6.8), 2% SDS, 10% (v/v) glycerol, 0.025% (w/v) bromphenol blue, 5% (v/v) 2-mercaptoethanol] at 85°C for 10 minutes.

In order to estimate the size of polypeptides in samples, an aliquot of either Rainbow or radioactive Rainbow protein markers (Amersham) were separated in parallel. The coloured proteins of the known molecular weights in the Rainbow mixtures were myosin (200kD), phosphorylase b (97.4kD), bovine serum albumin (69kD), carbonic anhydrase (30kD), trypsin inhibitor (21.5kD) and lysozyme (14.3kD).

2.4.2. Protein Quantitation and Detection

2.4.2.1. Bradford Assay

All protein concentration determinations were done by Bradford assay (BioRad). The dye concentrate was diluted 1:5, and the spectrophotometric measurement was performed at the wavelength 595nm. In each series of determinations, either bovine serum albumin (Sigma) or gammaglobulin (Sigma) was used to draw a standard curve to estimate the protein concentration of a given sample.
2.4.2. Coomassie Staining

Non-radioactive proteins separated on SDS-PAGE gels were visualized by staining with Coomassie Blue R-250 [0.25% (w/v) in 50% (v/v) methanol/10% (v/v) acetic acid] for 3 hours-overnight. Destaining was performed overnight by repeated changes of solution 10% (v/v) methanol/5% (v/v) acetic acid.

2.4.3. Western Blotting

Proteins separated by SDS-PAGE were transferred onto Immobilon membrane (Amicon) using a BioRad MiniTransBlot electrophoretic transfer cell. The transfer was performed according to the manufacturer's instructions with the buffer containing 25mM Tris, 192mM glycine, and 20% methanol (pH 8.0-8.3). The efficiency of transfer was visually monitored by the help of Rainbow coloured protein markers (Amersham).

Membranes were blocked for two hours at room temperature or overnight at 4°C in PBS supplemented with 5% dry milk and 0.2% Tween 20. After this filters were probed in the same solution containing rabbit primary antibodies (1:200 dilution) for one hour at room temperature. Two washes each for 30 minutes in the same solution were performed before incubation for one hour with a horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (1:1000 dilution). Filters were washed twice as above. Protein bands were detected by using an enhanced chemiluminescence detection system (Amersham), using the protocol provided by the manufacturer.

2.4.4. Preparation of Nuclear and Cytoplasmic Extracts

PBS-washed cells were gently scraped into 1ml (9cm tissue culture dishes) of ice-cold PBS, and transferred to an Eppendorf tube. Next, cells were pelleted by a brief (12 seconds) centrifugation, resuspended in 400µl of ice-cold buffer A supplemented with a protease inhibitor mixture (see 2.4.6.), and incubated on ice for 5 minutes. Then, 5µl of 10% NP40 was added, followed by immediate, but gentle mixing. Nuclei were pelleted by a centrifugation for 30 seconds, after which they were resuspended in 50µl buffer C supplemented with a protease inhibitor mixture. Soluble nuclear proteins were extracted by shaking at 4°C for from 30 minutes to one hour, the debris removed by a centrifugation, and the supernatants (nuclear extracts) either used immediately or frozen until used.

2.4.5. Expression and Purification of Recombinant Proteins
2.4.5.1. \textit{In vitro} Translation

Proteins were synthesized \textit{in vitro} in the presence of $^{35}$S-labeled methionine (15μCi in a 50μl reaction) by using a coupled transcription-translation wheat germ extract system (Promega) according to the manufacturer's instructions. Linearized expression plasmids were used for T7-directed expression. The success of the reactions was verified by an SDS-PAGE analysis (samples 1:50 of the total reaction), followed by autoradiography.

2.4.5.2. Bacterial Expression and Purification of His-Tagged Proteins

The coding sequences for proteins to be produced in bacteria were inserted into the pET21 vector series (Novagen), which fuse the carboxy-terminus of the protein to a stretch of six histidine residues (His-tag). A His-tag enables rapid and specific affinity purification of the overexpressed proteins, by virtue of its affinity to divalent nickel cations immobilized to agarose matrix (Qiagen).

The ligation reactions were transformed into \textit{E.coli} SCS-1 strain. For the expression of recombinant proteins, the selected subclones were retransformed into a λDE3 lysogen of the \textit{E.coli} BL21 strain (F', ompT, mB'), which harbours a stably integrated T7 RNA polymerase gene under the control of the lacUV5 promoter.

Bacterial cultures (typically in 100ml L-broth + ampicillin for an individual purification) were induced for 3 hours with IPTG (1mM) at mid-log phase (OD$_{600}$ 0.5-0.8), and the induced protein purified from endogenous bacterial proteins with a nickel-agarose column. Cells were harvested by centrifugation at 5000g for 5 minutes, and then suspended in 4ml of ice-cold binding buffer containing 5mM imidazole, 0.5M NaCl, 20mM Tris (pH7.9). After a brief sonication to degrade cells, the debris was removed by a centrifugation and the supernatants filtered through a 0.45μm membrane. The crude extracts were applied to a nickel agarose columns (1ml matrix per 100ml of bacterial culture), which were thoroughly prewashed with the binding buffer (see above). Under these conditions the His-tagged proteins associate with the matrix. The contaminating bacterial proteins were washed away with 5 column volumes of binding buffer and 5 column volumes of wash buffer [60mM imidazole, 0.5M NaCl, 20mM Tris (pH7.9)]. Finally the specific proteins were eluted from the affinity matrix with the buffer containing 1M imidazole, 0.5M NaCl, 20mM Tris (pH7.9).

2.4.6. Other Common Buffers Used in Protein Handling
A: 10mM Hepes-NaOH (pH7.9), 1.5mM MgCl₂, 10mM KCl, 0.5mM DTT
C: 20mM Hepes-NaOH (pH7.9), 0.42M KCl, 1.5mM MgCl₂, 0.2mM EDTA, 0.5mM DTT, 25% (v/v) glycerol
D: 20mM Hepes-NaOH (pH7.9), 100mM KCl, 0.2mM EDTA, 0.5mM DTT, 20% (v/v) glycerol

At all times, buffers A, C, and D were supplemented with protease inhibitors (purchased from Sigma). Their final concentrations were 0.5mM phenylmethylsulfonyl fluoride (serine protease inhibitor), 1.0mM benzamidine (serine protease inhibitor), 30µg/ml leupeptin (thiol protease inhibitor), 5µg/ml aprotinin (serine protease inhibitor), and 5µg/ml pepstatin A (acid protease inhibitor).

2.5. Protein-DNA Interaction Assays

2.5.1. Electrophoretic Mobility Shift Assay

EMSA probes were labeled by filling the ends of double stranded oligonucleotides with [α³²P]dATP and the Klenow enzyme. The reactions (10µl) included 50ng of an oligonucleotide, 0.25mM of dCTP, dGTP, and dTTP, 50-70µCi [α³²P]dATP, 1 unit of Klenow polymerase, and the buffer environment was 10mM Tris (pH7.5), 10mM MgCl₂, 50mM NaCl, 1mM dithioerythritol. Reactions were incubated at room temperature for 5 minutes, after which 5µl of orange loading dye was added. The labelled probes were then separated from unincorporated deoxyribonucleotides on a 15% native polyacrylamide gel. Using an autoradiograph for the alignment, the gel slices containing the radioactive probes were cut out of the gel and eluted in 400µl gel elution buffer (1M ammonium acetate/1% SDS/1mM EDTA) overnight at 37°C. Eluted probes were recovered by ethanol precipitation in the presence of 20mM MgCl₂ and 20µg of glycogen, and the precipitated probes redissolved in 50-100µl TE(10:1), pH7.4.

To assay protein-DNA interactions, 20µl reactions were set up as follows: 10µg of nuclear extract, or an indicated amount of recombinant or purified protein, was added into reaction mixtures containing 20mM Tris (pH8.0), 60mM KCl, 2mM MgCl₂, 15% glycerol and 0.3mM DTT. Next, 1.75µg of carrier DNA poly(dI)-(dC) (Pharmacia) was added to sequester non-specific DNA binding proteins from associating with the oligonucleotide probes. After 5 minutes at room temperature 5x10⁴ cpm (0.1-1ng) of an end-labeled oligonucleotide probe was added, together with unlabelled competitor oligonucleotides where applicable. After allowing the association between DNA and proteins for 10 minutes at 30°C, the samples were loaded onto a pre-electrophoresed native 5% polyacrylamide
(30:1 mono:bis) gel and separated at 200V in 0.5xTBE. Finally the gels were dried down onto Whatman DE81 paper and exposed to an X-ray film at -70°C.

In antibody shift assays, 1µl of antiserum (or control preimmune serum) was added prior to addition of the probe, and incubated on ice for 0.5-1 hour.

In competition EMSAs, unlabelled double-stranded oligonucleotide competitors were added together with the carrier DNA, prior to addition of the probe.

2.5.2. SouthWestern Blotting

For SouthWestern blotting the sample proteins were first separated on an SDS-PAGE and transferred onto Immobilon membrane as described above. After the transfer, the membranes were immersed in solution containing 5mM Tris (pH7.5), 50mM NaCl, 1mM EDTA, 1mM DTT, and 5%(w/v) dry milk, for one hour with gentle shaking. After this, the membranes were washed twice for 5 minutes in TNE-50 [10mM Tris (pH7.5), 50mM NaCl, 1mM EDTA, 1mM DTT]. The membranes were then incubated in TNE-50 containing 10^6 cpm/ml ^32P-labeled double stranded nucleotides, and 10µg/ml poly(dI-dC). After two hours at room temperature with gentle shaking, the membranes were washed three times for 30 minutes with TNE-50. The membranes were finally dried and exposed to autoradiography.

2.6. Protein-Protein Interaction Assays

2.6.1. Immunoprecipitation

Protein A-Sepharose (PAS) CL-4B (Pharmacia) was prepared by suspending the beads 1:1 with Tris-buffered saline (TBS).

To pre-clear the samples from proteins that non-specifically associate with either PAS beads or rabbit serum components, 15µl of PAS was added to the proteinaceous samples in RIPA buffer [50mM NaCl, 25mM Tris (pH8.2), 0.5% NP40, 0.5% sodium deoxycholate, 0.1% SDS] together with 10µl of a serum from non-immunized rabbits. Samples were incubated in a rotating wheel at 4°C for 15-30 minutes, and centrifuged for 10 minutes at 4°C at 12000g in a microfuge.

For immunoprecipitation, 20µl of PAS and 10µl of a specific antiserum were added to the precleared supernatant. Samples were incubated in a rotating wheel at 4°C for 4h-
overnight, after which a centrifugation for 10 minutes was performed. Immunocomplexes associated with PAS beads were subjected to five successive wash cycles with 1.4ml of ice-cold RIPA buffer, each time the washed beads were collected by centrifugation. The washed immunocomplexes were eluted from the PAS beads by incubating in Laemmli sample buffer at 85°C for 10 minutes, centrifuged briefly, and analyzed in SDS-PAGE.

2.6.2. WildWestern Blotting

The method for WildWestern blotting was modified from Lindon (1994). Separation of proteins and their transfer to a membrane were performed as for Western blotting. After this, the membranes were incubated overnight with a primary probe, that is, an unlabelled protein of interest, whose interactions are to be investigated. The primary probe solution contained 2µg of specific protein in 1ml of buffer D, supplemented with 20µg BSA as a carrier protein. After this, membranes were washed three times with buffer D. As a secondary probe, an antiserum raised against the primary protein probe was used (1:200 antibody dilution in buffer D) for one hour at room temperature. Two washes each for 30 minutes in buffer D were then performed, before incubation for one hour with a horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (1:1000 dilution). Membranes were washed twice as above. The protein sandwiches were finally detected using an enhanced chemiluminescence assay, as described for Western blotting.

2.7. Detection of Reporter Enzymes in Extracts Prepared from Transfected Cells

Cells for reporter gene assays were harvested 42-55 h after transfections.

2.7.1. Luciferase Assay

Extracts for the quantitation of luciferase activity were prepared by lysing cells on plates in 100µl of LucA buffer (25mM Tris Phosphate, pH7.8; 8mM MgCl₂; 1mM DTT; 1mM EDTA; 1%(v/v) Triton X-100). Lysates were transferred into 1.5ml Eppendorf tubes and an equal volume of LucB buffer (25mM Tris Phosphate, pH7.8; 8mM MgCl₂; 1mM DTT; 1mM EDTA; 0.8mM ATP; 1%(v/v) Triton X-100; 30%(v/v) glycerol; 2%(w/v) bovine serum albumin) added to each tube. Samples were thoroughly mixed and debris precipitated by centrifugation in a microfuge at 14000rpm for 2 minutes.

100µl of each sample was assayed for the luciferase activity. The total sample volume was adjusted to 350µl by adding 250µl of LucA:LucB (1:1) reaction buffer. An LKB1251
luminometer was used to detect emission following the injection of 33μl of 1.5mM luciferin (Sigma) into a sample. The peak values were recorded.

As a background control to be deducted from the sample values, an extract prepared from untransfected cells was assayed.

2.7.2. CAT Assay

For a combined luciferase/CAT assay, cells were lysed into the LucA/LucB buffer system as described above. For a combined CAT/β-galactosidase assay, cells were lysed in 150μl of a different lysis buffer (10mM Tris, pH8.0; 1mM EDTA; 150mM NaCl, 0.65% NP40), before removing the cell debris by centrifugation.

CAT activities were assayed using the procedure of Sleigh (1986). Chloramphenicol acetyltransferase activities in extracts prepared from transfected cells were determined by the transfer of [14C]-acetyl groups from labeled acetyl coenzyme A to unlabeled chloramphenicol. Prior to reactions, the extracts were first heated to 65°C to remove cellular activities that degrade acetyl coenzyme A. A 30μl aliquot of each extract was assayed in the total volume of 100μl, in the presence of 1.6mM chloramphenicol, 0.1mM acetyl coenzyme A (containing 100nCi of the [14C]-labeled derivative), and 75mM Tris, pH7.8. Reactions were incubated at 37°C for an appropriate period, so that the values were on a linear scale. Samples were then cooled on ice, 130μl of cold ethyl acetate added to them, mixed, centrifuged for one minute, and 100μl of the upper phase, which contains the acetylated chloramphenicol, transferred into 5ml of liquid scintillant. Labelled reaction products were quantitated by liquid scintillation counting. As a background control to be deducted from the sample values, an extract prepared from untransfected cells was assayed.

2.7.3. Beta-Galactosidase Assay

To prepare cells for the β-galactosidase assay, transfected cells were lysed with the combined CAT/β-galactosidase lysis buffer.

To 100μl of sample extracts, 400μl of LacZ buffer (60mM Na2HPO4, 40mM NaH2PO4, 10mM KCl, 1mM MgSO4, 0.27% β-mercaptoethanol) and 100μl of ONPG were added. Reactions were incubated at 37°C until yellow, after which 250μl of 1M Na2CO3 was added to terminate the reactions. An aliquot of 400μl from each sample was spectrophotometrically measured at the wavelength 420nm. As a background control to be deducted from the sample values, an extract prepared from untransfected cells was assayed.
Chapter 3: The Role of Oct-1 in the Regulation of the Beta-Interferon Promoter

3.1. Oct-1 Binds to Multiple Regions within the Beta-Interferon Promoter

In early EMSA studies in our laboratory, a binding activity was identified that was able to form a complex on a probe -55/-40, which is derived from the NRD I region (K.V. Visvanathan, and S. Goodbourn, unpublished). In competition EMSA experiments, it further became apparent that a complex possessing an identical binding specificity also formed on probes from the NRD II (-108/-95), and TATA box (-33/-20) regions of the human IFN-ß promoter. It was surprising to observe that these probes are not strongly homologous in sequence. A suggestion about the identity of the DNA binding component of the complex was provided by an EMSA analysis using TATA box probes that contained single point mutations. An observation that the mutation -28A>G, which creates a stronger binding site (ATGTAAAT) for the complex than the wild type TATA box (ATATAAAT), also creates a more complete match with the consensus site (ATGCAAAT; see below) for the previously identified transcription factor Oct-1, prompted us to test the possibility that this complex indeed contains Oct-1.

To confirm that the three probes derived from the IFN-ß promoter (NRD II, NRD I, TATA) can interact with Oct-1, an antibody-supershift EMSA was performed. Aliquots (1μl) of an Oct-1-specific polyclonal antiserum (a gift from Dr. P.O’Hare) or a preimmune serum were incubated with the nuclear extract samples prepared from HeLa cells prior to the addition of a particular radioactively labelled probe, and running the samples on an EMSA gel. While the preimmune sera had no effect on the binding of the activity on any of the IFN-ß probes, the Oct-1-specific antisera raised against the Oct-1 DNA binding domain (the POU domain) completely abolished the complex formation (figure 3.1.). As a positive control, the antiserum against Oct-1 also abolished complex formation on a probe containing a consensus octamer binding site (see below). It was thus concluded that the binding activity under study contains, or is indistinguishable from, Oct-1. To further verify this, recombinant Oct-1 was translated in wheat germ extract using mRNA transcribed in vitro from a plasmid containing Oct-1 cDNA under a T7 bacteriophage promoter. When this recombinant product was allowed to associate with the Oct-1 binding site probes, a complex of approximately the same size as the endogenous activity was detected (data not shown), further confirming the identity of the binding factor.
Figure 3.1. A complex forming on multiple probes from the IFN-β promoter reacts with an antiserum raised against the Oct-1 POU-domain.

An EMSA analysis of the binding activities present in HeLacell nuclear extracts on three probes derived from the IFN-β promoter [NRD II (-108/-95), TATA (-33/-20), NRD I (-55/-40)] as well as on a probe containing a perfect octamer motif ("octa"). The lanes in the panel on the right are from the same autoradiographic exposure of the same gel, while the figure on the left is from a different experiment. The specificity of the anti-POU antiserum for Oct-1 is evidenced by its lack of reactivity with the two higher mobility complexes (Un1 and Un2, see chapter 4) that form on the NRD II probe.
Oct-1 (figure 3.2.) belongs to a family of proteins, which share a highly homologous DNA-binding motif, referred to as the POU-domain (for mammalian proteins Pit-1, Oct-1 and -2, and a nematode protein involved in neural cell lineage determination: Unc-86), according to the proteins in which it was first discovered (Herr et al 1988). Over 20 members of the POU family have been identified in various organisms from Drosophila to mammals (reviewed in Ruvkun and Finney 1991, Verrijzer and van der Vliet 1993). Many POU proteins are expressed in a tissue-restricted manner, for example, Pit-1 is expressed in the pituitary gland and regulates the transcription of prolactin and growth hormone genes (Nelson et al 1988). In contrast, Oct-1 has a widespread pattern of expression, and has been implicated as having a role in transcription of housekeeping genes, such as small nuclear RNA genes and the histone H2B gene (reviewed in Kemler and Schaffner 1990). The consensus DNA recognition site for Oct-1, as well as for the tissue-restricted members of the Oct-protein family, is ATGCAAAAt, known as the octamer motif. A selection for an optimal Oct-1 binding site also identified the ATGCAAAAt motif, however it also revealed an equal preference for adenine or thymine in position 5 (Verrijzer et al 1992a). Since adenine appears to be more common at this position in naturally occurring octamer sites in cellular promoters, there may be further constraints for the in vivo functioning of the octamer element.

As judged by an EMSA assay with the four different probes, the relative binding affinity of the Oct-1-containing complex for the three different binding sites in the IFN-β promoter and for a perfect octamer motif (octa) is [octa > NRD II > TATA > NRD I] (figure 3.3.). This correlates very well with the degree of sequence similarity between the octamer consensus site and a particular binding site in the IFN-β promoter. There is no sequence within the NRD I probe that resembles the octamer motif sufficiently to directly deduce the actual Oct-1-DNA contact site; the alignment of the NRD I region with the stronger Oct-1 binding sites in figure 3.3. is derived from an analysis on the effect of point mutations across the NRD I region on the specific binding affinity of Oct-1 (S.Goodbourn, pers.comm). In any case, it is apparent from this that Oct-1 is able to bind to very degenerate octamer motifs, as previously noted by others (Baumruker et al 1988).

The Oct-1-containing complex appears to be the only specific octamer binding complex we can detect in HeLa cells (see, for example, figures 3.3., 3.9. and 3.10.).

3.2. DNA Binding Properties of the Recombinant POU Domain
Figure 3.2. A schematic representation of the Oct-1 protein.

The protein product encoded by the Oct-1 cDNA used throughout this thesis. The 5’ end of Oct-1 mRNA remains uncloned, and thus the endogenous Oct-1 polypeptide contains more amino-terminal amino acid residues than indicated here. In the figure, the DNA binding domain, referred to as the POU domain, and putative glutamine- and serine/threonine-rich activation domains are indicated.
Figure 3.3. The relative binding affinity of the complex containing endogenous Oct-1 to the three different binding sites in the IFN-β promoter and to the perfect octamer motif.

A) An EMSA analysis using HeLa cell nuclear extracts on the three probes (NRD II, TATA and NRD I) derived from the IFN-β promoter, as well as on the probe containing a perfect octamer motif. The photograph is from one autoradiographic exposure of a single gel.

B) An alignment of the Oct-1 binding sequences and the relative affinity of Oct-1 to these.
relative affinity of Oct1:

octa > NRDII > TATA > NRDI
The POU domain is a 155-162 amino acid region composed of two distinct, independently folding, DNA-binding structures, both of which make specific contacts with DNA, and thus contribute to effective DNA binding (Sturm and Herr 1988, Aurora and Herr 1992, Botfield et al 1992). The more carboxy-terminal subdomain, the POU-homeodomain (60 amino acids), is similar to the DNA binding domain of the previously characterized members of the homeodomain family of transcription factors, while the more amino-terminal POU-specific domain (74-82 amino acids) is characteristic of POU-proteins (reviewed in Verrijzer and Van der Vliet 1993). Homeodomains contain a helix-turn-helix DNA binding motif (Laughon et al 1984), of the kind which were first identified in bacterial DNA binding repressor proteins (Sauer et al 1982). Although the amino acid sequence of the POU homeodomain is quite divergent from the homeodomains in non-POU proteins, both the overall structure and mode of DNA recognition of POU homeodomains are very similar to the previously characterized ones, such as the Engrailed and MATα2 homeodomains (Klemm et al 1994). Recent NMR studies have demonstrated that the POU-specific domain also contains a helix-turn-helix motif, topologically similar to the DNA-binding domain of the phage λ repressor (Assa-Munt et al 1993, Dekker et al 1993), although in many POU proteins, including Oct-1, the turn region is longer. A POU-homeodomain and a POU-specific domain are covalently linked by a short (15-27 amino acids, 24 amino acids in Oct-1) and structurally disordered tether, which is nonconserved in sequence between different POU-family proteins. For example, Oct-1 and Oct-2 have nearly identical POU-specific and POU homeodomains - accordingly rendering their DNA binding specificity identical - however, their tether regions are very different (Herr et al 1988). Supporting the proposed flexible nature of the tether region, it is readily accessible to proteases (Botfield et al 1992). It is interesting to note that homeodomains are notoriously promiscuous in the sequence specificity of their DNA binding (figure 3.3.; reviewed by Laughon 1991); thus the POU-specific domain can be seen to function to supplement this limitation. A similar covalent association of two DNA binding folds has been utilized in Paired domain proteins to enhance target specificity. An interesting contrast to these is provided by a subclass of LIM proteins (Sánchez-Gárcia and Rabbitts 1994), which possess a homeodomain adjacent to the cysteine-rich zinc finger-like LIM domain. In these proteins, the DNA binding activity of the homeodomain appears to be inhibited by the neighbouring LIM domain (Sánchez-Gárcia et al 1993, Xue et al 1993).

The POU homeodomain has been shown to contact the 3' half (AAAT) of the consensus octamer target site ATGCAAAT, while the POU-specific domain associates with the 5' ATGC portion (Verrijzer et al 1990, Verrijzer et al 1992a, Klemm et al 1994) - the half sites lie on opposite sides of the DNA double helix. Biochemical studies have indicated that while the isolated Oct-1 homeodomain binds DNA reasonably well, the isolated POU-
specific domain binds only poorly. In addition, binding site selection and DNA binding competition studies indicate that Oct-1 prefers an arrangement in which the POU-specific binding half site and the POU homeodomain binding half site are juxtaposed (Verrizjer et al 1994). The POU-specific domain and POU homeodomain do not directly contact each other, despite that in the crystal structure analyses have revealed that they make contacts with overlapping phosphates (Klemm et al 1994). Even in the absence of direct protein-protein contacts between the domains, they may interact through subtle alterations in the structure or flexibility of DNA. This would be consistent with a biochemical study on chimaeric POU domains carrying corresponding protein segments from different POU proteins, which has suggested that the two subdomains influence each other's DNA binding specificity (Aurora and Herr 1992).

We have investigated the DNA binding properties of the Oct-1 POU domain in isolation, separated from the amino- and carboxy-terminal regions of the Oct-1 protein. To allow the production of recombinant POU domains, subclones for cell-free translation (pT7Oct1POUHD+ and pT7Oct1POUHD-, in which the production of the POU mRNA variants is governed by bacteriophage T7 promoter) and bacterial expression (pETOctlPOU) were created (see the legend to figure 3.4.). In these plasmid constructions we utilized the Oct-1 cDNA insert in the parental plasmids pCGOct1 and pCGOct1HD- (see figure 3.19.; a gift from W.Herr, Cold Spring Harbor).

The POU domains from T7 clones were synthesized in coupled in vitro transcription/translation extracts from wheat germ (figure 3.4.A.). The POU HD+ clone encodes the wild type Oct-1 POU domain, whereas the product of the POU HD- clone contains a triple alanine substitution (WFC>AAA) created in the DNA recognition helix of the POU homeodomain.

The pETOctlPOU plasmid was designed so that the POU encoding region is in frame with a 3' vector sequence encoding a stretch of six histidine residues. The presence of the Histag in the fusion protein allows the purification of the recombinant POU protein by virtue of the affinity of the polyhistidine stretch for nickel-ions. The bacterial expression and subsequent purification were performed as described in the Materials and Methods. A Coomassie-stained SDS-PAGE with the samples from the fractions collected during purification is shown in figure 3.4. The retrieval and elution of the overexpressed POU domain was very efficient; furthermore, no contaminating bacterial proteins can be detected in the selected elution fraction.
Figure 3.4. Production of the recombinant POU domains.

A) Analysis of the products of an in vitro translation reaction of the POU HD+ and POU HD- domains.

An aliquot of 1\mu l of each 50\mu l translation reaction was run on a 10% SDS-polyacrylamide gel. As a positive control for efficient translation 1\mu l of the control protein reaction (a set of RNAs encoding BMV proteins of known sizes, provided by the manufacturer) was run in parallel. The recombinant proteins were detected by autoradiography, by virtue of the inclusion of \(^{35}\text{S}\)-labeled methionine in the translation reactions. The constructions of the pT7Oct1POUHD+ and pT7Oct1POUHD- plasmids for in vitro translation of the POU HD+ and POU HD- domains, respectively, were carried out as follows. The pCGOct1 and pCGOct1HD- (a gift from W.Herr, Cold Spring Harbor; see figure 3.19) plasmids were first digested with PflMI (nucleotide 1324 in the Oct-1 cDNA), and the single-stranded 3' extensions removed with mung bean nuclease. The linearized plasmids were then digested with HincII (nucleotide 806 in the Oct-1 cDNA), and the DNA fragments encoding the POU domains isolated. The POU-encoding fragments were ligated into a pT7link vector plasmid (a gift from R.Treisman, ICRF) that had been digested at its polylinker region with EcoRI, filled in at the 3' recessed ends, and treated with calf intestinal alkaline phosphatase. The resulting pT7Oct1POU plasmids encode the amino acid sequences 270-440 of the Oct-1 protein, except for the three alanine substitutions at the amino acid positions 428-430 in the POU HD- protein product (see the text).

B) Purification of the bacterially produced His-tagged Oct-1 POU domain on a nickel-agarose column.

15\mu l aliquots of the prepurification material, of four wash fractions, and of the five elution fractions were loaded into a 10% SDS-polyacrylamide gel, and the proteins detected by Coomassie staining. The construction of the pETOct1POU plasmid for bacterial expression of the Oct-1 POU domain was carried out as follows. Briefly, pCGOct1 was first digested with PflMI (nucleotide 1324 in the Oct-1 cDNA), and the single-stranded 3' extension removed with mung bean nuclease. The linearized plasmid was then digested with HincII (nucleotide 806 in the Oct-1 cDNA), and the DNA fragment encoding the POU domain isolated. The fragment was ligated into a pET21b vector plasmid (Novagen) that had been digested with Nhel, filled in at the 3' recessed end, digested with Sall, and purified from the excised polylinker region. The resulting pETOct1POU plasmid encodes the amino acid sequences 270-441 of the Oct-1 protein.
We then tested the binding activity of the isolated POU domain in EMSA assays. In accord with the earlier studies (Sturm et al 1988, Clerc et al 1988), Oct-1 POU-domain appeared sufficient for sequence-specific DNA binding (figures 3.5. and 3.6.). The POU domain appears to bind to a single DNA recognition site as a monomer, since upon gradual increase of the [POU domain]/[probe]-ratio no shift to the formation of higher-order protein-DNA complexes can be observed (figure 3.5.). This has recently been verified by others (Verrijzer et al 1992a), and is consistent with the lack of symmetry in the octamer motif ATGCAAAT. In agreement with a previous study (Sturm and Herr 1988), the triple mutation in the POU HD\(^{-}\) variant completely abolished DNA binding, even when the amount of the POU HD\(^{-}\) added into an EMSA binding reaction was 1000-fold more than the minimum amount of POU HD\(^{+}\) that gave a clearly visible bandshift (data not shown).

The recombinant POU domains were tested for their relative binding affinity for the three Oct-1-binding probes derived from the IFN-8 promoter as well as for the perfect octamer motif. The binding preference of the isolated Oct-1 POU domain appears indistinguishable from that of the endogenous Oct-1 (figure 3.6., compared with figure 3.3.), indicating that the isolated Oct-1 POU domain contains all the information dictating the binding affinities of the Oct-1 protein to the different binding site variants.

As shown above, three binding sites for Oct-1 can be detected in the IFN-8 promoter in EMSA assays by using short probes (approximately 15mers) derived from the -110/-12 region. To investigate possible cooperativity in binding of more than one POU domains to a larger region of the IFN-8 promoter, a radioactive probe (-110/-12) that contains all of the three identified binding sites was used in EMSA. A constant but limiting amount of probe was added to the binding reactions together with increasing amount of \textit{in vitro} translated POU domain. Surprisingly, when the [POU domain]/[probe]-ratio was sufficiently high, binding of more than three POU domains could be achieved (figure 3.7.). In fact, higher order complexes consisting of up to seven POU domains together with the probe could be detected. One cannot deduce from the experiment, whether the additional POU domains participate in complex formation by virtue of their DNA binding contacts to cryptic sites, or whether they are held in the complexes purely by protein-protein interactions. In footprinting experiments, we have only observed protections over the two strongest POU binding sites (NRD II region and the IFN-8 TATA box), even in the highest-order complex containing seven POU domains (data not shown). This result implies that DNA binding activity is not required from the additional POU domains; however, it remains possible that they do indeed make DNA contacts too weak to be detected by such a protection assay. Consistent with this, no obvious protection could be seen over the weak POU-binding site in the NRD I region. Another way to investigate the contribution of the DNA-POU contacts
Figure 3.5. The Oct-1 POU domain binds a single DNA recognition element as a monomer.

An EMSA analysis in which the amount of radioactive octamer probe is varied (6x10$^3$, 1.7x10$^4$, 5x10$^4$, 1.5x10$^5$ and 4.5x10$^5$ cpm, from left to right), while the amount of the \textit{in vitro} translated POU domain is kept constant (1:500 of an \textit{in vitro} translation reaction).

Similar results were obtained by using the bacterially produced Oct-1 POU domain.
octamer probe

POU-DNA complex

free octamer probe
Figure 3.6. The isolated Oct-1 POU domain reproduces the binding specificity of the endogenous Oct-1 protein.

An EMSA analysis with the \textit{in vitro} translated POU domain and the three probes derived from the IFN-\(\beta\) promoter that contain a binding site for Oct-1 (TATA, NRD I, NRD II) together with the perfect octamer probe (octa).

unprogr. WGE: an 1\(\mu\)l aliquot of an unprogrammed wheat germ extract
1/50: an 1\(\mu\)l aliquot of a 50\(\mu\)l cell-free translation reaction programmed with the pT7Oct1POUHD\(^+\) plasmid
1/500: an 0.1\(\mu\)l aliquot of a 50\(\mu\)l cell-free translation reaction programmed with the pT7Oct1POUHD\(^+\) plasmid

Similar results were obtained by using the bacterially produced Oct-1 POU domain.
Figure 3.7. More than three Oct-1 DNA binding domains can cooperatively interact with the IFN-β promoter.

An EMSA analysis using a constant amount \((1 \times 10^4 \text{ cpm})\) of a probe (-110/-12, a radioactively labelled restriction fragment BgIII-NcoI of the pI5A8 plasmid) derived from the IFN-β promoter that spans the three binding sites and increasing amounts (0, 0.04, 0.4, 4, 40 and 400 ng, from left to right) of the bacterially produced Oct-1 POU domain. Short arrows indicate the POU-probe complexes.

Similar results were obtained by using the \textit{in vitro} translated Oct-1 POU domain.
to the higher-order complex formation could be to epitope-tag the DNA-binding defective POU HD*, mix this with untagged wild type POU domain, and see whether any of the higher order complexes could be supershifted with epitope-specific antibodies. However, it is not known whether the overall tertiary structure of the triple mutated POU HD* domain is sufficiently maintained that the assay described would truly measure the differences in the DNA binding ability of the two POU variants rather than, for example, the ability to interact with each other - although alanine residues are not believed to be likely to affect the backbone structure of a protein. Even so, because of these limitations, a negative result in either of the proposed assays could not be considered a definite answer.

It is also apparent from figure 3.7. that the in vitro binding of the Oct-1 POU domain to the multiple sites within the IFN-β promoter is cooperative, since higher order complexes begin to form before all the labeled probe is recruited to the previously highest-order complex. This is likely to indicate that the POU domains are capable of interacting with each other on DNA; alternatively, they could alter the conformation of the DNA probe so that the binding of additional POU domains is favoured. No additional proteins are required to achieve the cooperativity in DNA binding. The ability for POU domains to bind DNA cooperatively may result from their known ability to direct DNA bending upon binding (Verrijzer et al 1991).

In the absence of DNA recognition sites the Oct-1 POU domain has been suggested to mediate homologous interactions (Verrijzer et al 1992b); however, the detection of such DNA-independent POU dimers requires either the presence of cross-linking reagents or very large concentrations of recombinant POU domains, suggesting that the dimer formation is very transient and weak, at least in vitro. At physiological concentrations, the POU domain proteins exist in monomeric form in solution (see, for example, Ingraham et al 1990). In contrast to the experiments in solution, certain promoters have been identified on which the DNA contacts made by the POU proteins stabilize such POU-POU interactions. In the light of our results, it is interesting that the Oct-2 protein has been shown to bind cooperatively to adjacent consensus octamer sites (LeBowitz et al 1989). Furthermore, two Oct-1 or Oct-2 molecules can bind cooperatively to the octamer (ATGCAAAAT) and heptamer (CTCATGA) motifs found in the immunoglobulin heavy chain promoters (LeBowitz et al 1989, Poellinger and Roeder 1989, Poellinger et al 1989). The events on the immunoglobulin promoter may resemble those on the IFN-β promoter, in that a strong binding site (octamer) for a factor facilitates the recruitment of a factor to a nonconsensus site (heptamer) that would not independently serve as an effective binding site. The POU homeo (55 identical residues out of 62) and POU-specific (74 identical residues out of 75) domains of Oct-1 and Oct-2 are highly similar, it is thus not surprising
that the Oct-2 POU domain is sufficient for the cooperativity of DNA binding (LeBowitz et al 1989), just as we have shown for Oct-1. It has not been investigated whether Oct-1 and Oct-2 can interact with each other to exhibit binding cooperativity. While it is tempting to assume that the cooperative DNA binding by Oct-2 and Oct-1 is carried out by identical interactions, one should remember that a single amino acid divergence between the POU domains of Oct-1 and Oct-2 has been shown to diminish the avidity of Oct-2 for the viral transactivator VP16 by at least two orders of magnitude (Stern et al 1989, Lai et al 1992, Pomerantz et al 1992). Oct-1 can also associate with another tissue-specific POU protein, Pit-1, through POU-POU interactions, both on pituitary gland-specific elements on the rat prolactin promoter and also, weakly, in solution (Voss et al 1991). Coexpression of both of these POU-proteins has modest synergistic effects on both prolactin gene transcription and the isolated Pit-1 response element. Interestingly, no homologous association between two Oct-1 molecules on this promoter could be detected. Since our results indicate that several Oct-1 POU domains are capable of interacting on the IFN-β promoter, this suggests that these interactions are likely to depend on the exact DNA sequence. It is interesting to note that the interaction between Oct-1 POU domain and VP16 was recently shown to depend on a particular conformation of the POU domain induced by an intact "GArAT" sequence next to the octamer-like site (Walker et al 1994). It is likely that there exists a delicate interplay between the modulation of DNA conformation upon factor binding and the degree of cooperativity between the DNA binding factors. Specific DNA sequences may act as allosteric effectors to determine the activities of transcription factors (see discussion in section 1.1.2.).

It is very intriguing to speculate about the implications of the formation of higher-order POU complexes on the IFN-β promoter. If indeed seven or more Oct-1 molecules could bind in vivo to the promoter region of approximately 100bp, it would probably mean that the IFN-β promoter would be fully occupied. This would be likely to sterically inhibit the simultaneous binding by any other factor that has a binding site within this region. If, as discussed more extensively in section 3.9., Oct-1 functions as a repressor, this kind of complete occupation of the promoter achieved by cooperative binding could be a very effective way to prevent the activator proteins from binding to their response elements. Another possibility is that rather than being a repressor, Oct-1 could act as a potentiating factor for induction by effectively excluding the nucleosomes from the promoter and keeping it in a state competent to allow the subsequent binding of activator proteins in response to an inducing signal. In this model, the transcriptional role of the Oct-1 in the context of the IFN-β promoter would be only dependent on its DNA binding function. Such a chromatin disruptor function has been proposed for the glucocorticoid receptor (GR) in the liver-specific expression of the rat TAT gene (Rigaud et al 1991).
specific factor HNF5 recognizes the same DNA sequence as GR, but the occupation of such a site by the two proteins is mutually exclusive. However, the DNA binding by GR is a prerequisite for the HNF5 binding, and it appears that GR alters the chromatin structure so that HNF5 can subsequently interact with the DNA response element.

It should be noted that we have not shown that the full-length Oct-1 can form similar higher order complexes, and there is a possibility that the assembly of the substantially larger (>100kDa) full-length Oct-1 into a similar arrangement could be sterically impossible. Since the full-length Oct-1 is a very large protein, a standard EMSA assay would not be applicable for studying this. Recently, to investigate very large protein-DNA complexes, a Mg^{2+}-agarose gel electrophoresis system was described (Lieberman and Berk 1994) that could be usefully applied to studying the cooperative higher-order complexes by full-length Oct-1 proteins.

It is interesting to note the parallel between Oct-1 and a protein belonging to the high mobility group of DNA binding activities, HMGI(Y). Like Oct-1, HMGI(Y) can bind to multiple sites in the IFN-β promoter in a cooperative manner (Du et al 1993, T. Maniatis, pers. comm.). The HMGI(Y) protein appears to be required for the efficient induction of the transcription of the IFN-β promoter, and its function has been shown to be to stabilize the binding of "true" activator proteins to the promoter; perhaps the principle of the Oct-1 function is similar but opposite to that of HMGI(Y), in that it would help recruiting repressor proteins to the promoter. The binding sites for Oct-1 (this thesis) and HMGI(Y) (T. Maniatis, pers. comm.) largely overlap, raising the question of whether they are exclusive and alternative promoter organizer proteins in the uninduced and induced states, respectively.

### 3.3. DNA Binding Activity of Oct-1 Decreases upon Induction

The first reason to suspect a repressive role for Oct-1 in the regulation of the IFN-β promoter is that it binds to both of the two genetically defined negative regulatory regions, NRD I and NRD II. Oct-1 might also repress transcription by binding to the TATA box region of the IFN-β promoter, thus occluding the binding of the TFIID complex and preventing preinitiation complex formation (see section 3.9.2.3.4.).

In addition to binding to appropriate promoter sequences, our in vitro studies have revealed a further reason to believe that Oct-1 is involved in negative regulation of IFN-β transcription. In studies on HeLa cells, our laboratory determined that IFN-β
induction by dsRNA is absolutely dependent on priming (see section 1.2.2.2.; King and Goodbourn 1994), and furthermore, that induction can be substantially enhanced by the treatment of HeLa cells with the protein synthesis inhibitor cycloheximide. We have studied the ability of the endogenous Oct-1 in nuclear extracts prepared from HeLa cells to bind to the IFN-β promoter under these conditions. As shown in figure 3.8., the binding affinity of Oct-1 for the NRD II probe is specifically diminished under conditions of maximal dsRNA-induction. We note that the intensities of the Un1 and Un2 complexes that also form on the NRD II probe (see chapter 4) are similarly diminished. The loss in DNA binding activity of these proteins is not due to any general loss of DNA binding proteins in these nuclear extracts, since the fully induced extracts contain abundant levels of the NF-κB (see section 1.2.2.4.3.) DNA binding activity (data not shown). Furthermore, as discussed below (section 3.5.), the levels of nuclear Oct-1 protein are not altered by the induction process.

The decrease in Oct-1 binding activity to the NRD II probe can similarly be observed in EMSA studies with the NRD I and TATA box probes, as well as with the perfect octamer probe (figure 3.9.). The kinetics of the affinity reduction demonstrates that the loss of Oct-1 DNA binding activity precedes the induction of IFN-β mRNA (figure 3.10., compared with figure 1.2.). Levels of Oct-1 binding have begun to decrease one hour after the application of the inducer dsRNA, and the reduction is complete after two hours. This observation is consistent with a role for Oct-1 as a preinduction repressor of the IFN-β promoter.

3.4. The Affinity Reduction of Oct-1 Can be Mimicked by the POU Domain

To investigate the nature of the inducer-triggered affinity reduction of Oct-1 binding activity further, we aimed to determine whether the activities present in nuclear extracts prepared from primed/dsRNA-induced/CHX-treated HeLa cells could alter the DNA binding properties of recombinant DNA binding domain, or POU domain, of Oct-1.

When we mixed in vitro translated POU domains with nuclear extracts prepared from primed/dsRNA-induced/CHX-treated HeLa cells, prior to subjecting them to an EMSA analysis using the four Oct-1 binding site-containing probes (NRD II, NRD I, TATA, perfect octamer), we observed that the Oct-1 POU domains can mimic the induction-specific affinity reduction of the endogenous Oct-1 (figure 3.11.). Similar observations were made when using bacterially produced POU domains (data not shown). These experiments indicate that the effect is brought about by a posttranslational event, carried out
Figure 3.8. The binding activity of the endogenous Oct-1 decreases upon induction.

An EMSA analysis on the -108/-95 probe derived from the NRD II region of the IFN-β promoter. The nuclear extract samples were either untreated, dsRNA-induced/CHX-treated, primed/dsRNA-induced or primed/dsRNA-induced/CHX-treated HeLa cells. Two other specific complexes forming on the NRD II probe, Un1 and Un2, are also indicated.
Figure 3.9. The inducer-triggered DNA affinity reduction of Oct-1 can be observed on all of the four specific binding sites.

An EMSA analysis on the three probes containing the IFN-β promoter-derived binding sites for Oct-1 (NRD II, TATA, NRD I), as well as on the probe containing the perfect octamer motif. The nuclear extract samples were prepared from either untreated or primed/dsRNA-induced/CHX-treated HeLa cells.
Figure 3.10. The kinetics of the decrease in Oct-1 binding activity.

An EMSA analysis on the two probes derived from either the TATA-box region (-33/-20) or NRD I region (-55/-40) of the IFN-β promoter. The nuclear extract samples were either untreated HeLa cells or from the primed cells that have been induced by dsRNA and treated with CHX for 1, 2 or 4 hours.
Figure 3.11. Exogenously added recombinant POU domains can respond *in trans* to the affinity-reducing activity present in nuclear extracts prepared from induced HeLa cells.

An EMSA analysis on the four probes (octa, NRD II, TATA, NRD II) with nuclear extracts prepared from either untreated or primed/dsRNA-induced/CHX-treated HeLa cells.
by a soluble nuclear activity or activities. Furthermore, whatever the nature of the modification, it can be targeted to the DNA binding domain of Oct-1.

It should be noted that we have been unable to achieve the affinity reduction of either the endogenous Oct-1 or exogenously added POU domain by adding dsRNA directly to primed/uninduced/CHX-treated nuclear extracts (data not shown). Thus, the effect seems to require some signalling events that can only take place within the intact cellular infrastructure, or perhaps some cytoplasmic components that are translocated to nuclei following dsRNA treatment of cells.

The mechanisms by which the DNA affinity reduction could be achieved are discussed in the following sections.

3.5. Oct-1 Does Not Become Degraded upon Induction

There are several possible explanations for the observed reduction in the binding affinity of Oct-1. One possibility is that the specific concentration of the Oct-1 protein is reduced upon induction. The induction cycle of the IFN-β promoter involves several proteolytic processing events of transcription factors. It was thus important to establish whether such a regulated, inducer-triggered, degradation would be responsible for the decrease in the DNA binding of a candidate repressor protein Oct-1. As already shown, treatment of cells with cycloheximide does not lead to a significant decrease in Oct-1 binding activity, which suggests that this is not simply caused by the decrease in intracellular Oct-1 protein concentration following the shut-off of protein synthesis. By comparing nuclear extract samples prepared from either uninduced, primed/uninduced, primed/dsRNA-induced or primed/dsRNA-induced/CHX-treated HeLa cells subjected to Western analysis using antisera raised against the Oct-1 POU domain it was confirmed that the Oct-1 protein levels do not markedly change during induction (figure 3.12.).

Since exogenously added recombinant POU domain can respond to the signal that leads to the reduction in its DNA binding, it was also important to test whether the levels of recombinant proteins change. *In vitro* translated POU domains were mixed with nuclear extracts in EMSA buffer conditions, and then subjected to an SDS-PAGE analysis. It was confirmed that, like those of the endogenous Oct-1, the protein levels of the exogenous POU domain are not differentially affected by activities present in nuclear extracts prepared from either uninduced or induced HeLa cells (figure 3.13.).
Figure 3.12. The levels of the endogenous Oct-1 protein do not change upon induction.

A Western blot analysis on two sets on nuclear extracts (15μg) prepared from untreated, primed, primed/dsRNA-induced, or primed/dsRNA-induced/CHX-treated HeLa cells, using an antiserum raised against the Oct-1 POU domain. As a positive control a 1μl aliquot of the bacterially produced Oct-1 POU domain is included (the leftmost lane).
<table>
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<tr>
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<th>Nuclear extracts set 1</th>
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<tr>
<td>HisPOU</td>
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Figure 3.13. The integrity and recognition by specific antibodies, of recombinant POU domains, remains unchanged after mixing them with nuclear extracts from induced cells.

*In vitro* translated Oct-1 POU domains (1μl) were mixed with nuclear extracts (30μg) prepared from either untreated or primed/dsRNA-induced/CHX-treated HeLa cells prior to subjecting them to an SDS-PAGE analysis. Equal aliquots of recombinant POU domain not mixed with extracts were analyzed in parallel. Where indicated ("IP") the POU domains were subjected to immunoprecipitation with an antiserum raised against the Oct-1 POU domain, after the incubation with nuclear extracts. POU domains were detected by autoradiography by virtue of the fact that ^35^S-methionine replaced unlabeled methionine in the *in vitro* translation reaction.
We also subjected the recombinant POU domains to immunoprecipitation with the Oct-1 POU domain specific antiserum after mixing them with nuclear extracts, and analysed the immunoprecipitates in an SDS-PAGE. This was performed to investigate whether the proposed activity in induced extracts brings about a modification drastic enough to affect the recognition of the POU domain by the antibodies. The Western blotting analysis of the endogenous Oct-1 cannot provide an answer to this, since the proteins have undergone denaturation in an SDS-PAGE prior to probing with the antibodies, and may not have assumed a completely native conformation during the protein transfer and subsequent stages of the procedure. In contrast, immunoprecipitations are performed in native conditions, and might reveal possible gross induction-specific conformational changes in a protein. No induction-specific changes extensive enough to affect the ability of the antiserum to immunoprecipitate the POU domain were detected (figure 3.13.). It should, however, be borne in mind that a polyclonal antiserum was used; thus it is likely to contain antibody species specific for several different POU epitopes. Thus, even if the affinity of antibodies recognizing one particular epitope was influenced by a proposed modification, the bulk of antibodies with other specificities would still be effective in reacting with the POU domain, and the effect would not be detectable over this background. It could be possible to create a panel of monoclonal antibodies with specificities for distinct epitopes in the POU surface; as yet, we do not believe to have sufficient evidence to support the existence of an extensive induction-specific modification to perform the laborious creation of monoclonal antibodies.

In addition to IFN-β induction, regulated changes in DNA binding activity of the Oct-1 protein have also been observed in other biological contexts. A loss in Oct-1 binding activity has been reported to take place in the mouse mammary gland during the reductive remodelling process, or involution, after weaning (Marti et al 1994). Since Oct-1 mRNA is readily measurable in the involuting mammary gland on day four postweaning, a post-transcriptional mechanism appears to lead to the decrease of Oct-1. However, unlike upon induction by dsRNA, the decrease in Oct-1 DNA binding is accompanied by the decrease in the level of Oct-1 protein during the first four days of involution, as judged by Western analysis.

The DNA binding of Oct-1 has been shown to be strongly enhanced by the treatment of a human embryonal carcinoma cell line by two diverse signal-eliciting agents, interleukin 6 and retinoic acid (Hsu and Chen-Kiang 1993). Also in this case the increased Oct-1 binding correlates with increased specific protein levels. However, in contrast to the events in involuting mammary gland cells, the effect is transcriptional since the Oct-1 mRNA levels are also increased.
An octamer binding site in the histone H2B gene promoter is required for the cell cycle regulated transcription of the histone H2B promoter both in vivo (LaBella et al 1988) and in vitro (Fletcher et al 1987). Since Oct-1 is the only known ubiquitous octamer binding factor, it is believed to be responsible for the correct regulation of this housekeeping gene. Accordingly, the Oct-1 binding activity to the octamer motif from the human histone H2B promoter fluctuates during the cell cycle, so that mitotic extracts contain considerably less Oct-1 binding activity than present in any stage of the interphase (Segil et al 1991). In this case, the amount of Oct-1 protein does not undergo any obvious changes. This provides another example similar to our studies, where there is no clear correlation between the specific protein levels and signal-triggered changes in the specific DNA binding activity of Oct-1.

### 3.6. Nuclear Kinases Can Phosphorylate Oct-1 POU Domains

An obvious and widely utilized mechanism to achieve the affinity reduction of a DNA binding protein is to change its phosphorylation status. Specific phosphorylation and dephosphorylation events, mediated by protein kinases and protein phosphatases, respectively, have been shown to modulate the DNA affinity of a variety of transcription factors (Hunter and Karin 1992). For example, a CKII-like kinase activity phosphorylates c-Jun (Lin et al 1992) and c-Myb (Lüscher et al 1990) on sites that inhibit DNA binding. Similarly, the myogenic program is modulated by inhibition of DNA binding activity of myogenic HLH-transcription factors by fibroblast growth factor-induced and protein kinase C-mediated phosphorylation of their DNA binding domains (Li et al 1992). In connection with the studies on the role of Oct-1 in the cell cycle regulation of the histone H2B gene, it was shown by Roberts et al (1991) that Oct-1 is hyperphosphorylated when cells complete DNA synthesis and enter mitosis. The specific hyperphosphorylated status of Oct-1 is rapidly reversed as cells exit mitosis and enter the G1 phase of the cell cycle. Mitotic phosphorylation of Oct-1 correlates with the general inhibition of transcription that occurs during the mitotic stage. It thus appears that Oct-1 can indeed be phosphorylated within cells, and it remains possible that specific phosphorylation events modulate its function.

That Oct-1 can exist as a phosphoprotein has been previously suggested by others (Tanaka and Herr 1990, Roberts et al 1991). We have verified this and further shown that the Oct-1 POU domain can serve as a target region for phosphorylation by nuclear kinases, by mixing bacterially expressed POU domain with nuclear extracts in the presence of \([\gamma^{32}P]ATP\) (figure 3.14.). Phosphorylation reactions were allowed to take place in buffer
Figure 3.14. The Oct-1 POU domain is phosphorylated by nuclear kinases present in uninduced or induced HeLa cells.

Equal aliquots (400ng) of the bacterially produced Oct-1 POU domain were incubated with the nuclear extracts prepared from either untreated or primed/induced/CHX-treated HeLa cells, in the presence of 10μCi of [γ32P]ATP. To show the absence of any kinase activities capable of phosphorylating the POU domain in the purified recombinant POU preparation, an equal aliquot of the POU protein was incubated, in parallel, with [γ32P]ATP alone. The kinase reactions were performed in the EMSA sample conditions. After the phosphorylation reactions, the POU domains were first subjected to immunoprecipitation with an antiserum raised against the Oct-1 POU domain, and then to an SDS-PAGE. Radioactively labelled proteins were finally detected by autoradiography.
HisPOU + un/un NE
HisPOU + pr/dsRNA/CHX NE
HisPOU alone
conditions identical to the EMSA samples, prior to subjecting the samples to immunoprecipitation with an anti-POU antiserum. Nuclear extracts prepared from both uninduced cells and induced cells were tested. No quantitatively significant differences are apparent in the phosphorylation carried out by kinases present in uninduced nuclei as opposed to induced nuclei. However, it remains possible that different kinases are functional in different extracts, and they may consequently modify distinct residues within the POU domain. To investigate this one would have to subject the phosphorylated POU domains to phosphopeptide analysis. The purified POU preparation does not contain any contaminating kinases capable of modifying the POU domain, since upon addition of the [γ-32P]ATP alone, the POU domain does not become radioactively labelled.

3.7. Oct-1 POU Domain Is Phosphorylated by Protein Kinase A in vitro

Upon inspection of the amino acid sequence of the POU domain, two consensus sequences (BBXT/SA, where B=basic amino acid, X=any amino acid, T=threonine, S=serine, A=apolar amino acid) for recognition and modification by the cAMP-dependent protein kinase A (PKA) can be identified (PROSITE database, A.Bairoch, University of Geneva). These sites (T384, and S385) are located in the homeodomain aminoterminal to the first helix. In kinase assays, in the presence of [γ32P]ATP, we have shown that bacterially produced recombinant POU domain of Oct-1 can be phosphorylated by purified catalytic subunit of PKA in vitro (figure 3.15.). This phosphorylation can be inhibited by addition of potent and selective PKA inhibitor H89 into the kinase reaction, simultaneously with PKA. H89 inhibits PKA in a competitive fashion against ATP (Chijiwa et al 1990). We have not mapped the exact site(s) of the modification, but note that the recombinant POU domain we have used does contain the above mentioned consensus recognition sites for PKA. We speculate that these sites are used, but there may be alternative or additional sites elsewhere in the POU domain. We have not tested the ability of PKA to phosphorylate the full-length Oct-1; however, the relevant modification(s) affecting the Oct-1 DNA binding must be targeted to the POU domain since it can respond to the proposed activity in induced nuclear extracts by losing its DNA binding ability (see above). The catalytic subunit of PKA has been shown to be relocated from the cytoplasm to the nucleus upon activation, after dissociation from cAMP-bound regulatory subunit (Nigg et al 1985); thus it could be expected to be available to modulate nuclear Oct-1. Since the PRD II binding factor NF-κB is already known to be activated by PKA (Shirakawa and Mizel 1989), it is intriguing to speculate that the derepression by Oct-1 phosphorylation and activation of at least one positive regulator of the IFN-β promoter would be mechanistically integrated by the
Figure 3.15. The Oct-1 POU domain can be phosphorylated by purified protein kinase A.

All the kinase reactions (20μl) were allowed to take place in the EMSA buffer conditions, in the presence of 10μCi [γ³²P]ATP. As specificity controls, the three components [the bacterially produced POU domain (40ng), PKA (1 unit of catalytic subunits purified from bovine heart; purchased from Sigma), and H89 (0.4μg; purchased from LC laboratories)] were added into the reactions in all combinations (as indicated above the lanes). Only when the POU domain and PKA were added together, in the absence of H89, could the phosphorylation of the POU domain be achieved.
protein markers
POU
POU + PKA
POU + PKA + H89
POU + H89
PKA
PKA + H89
protein markers
21.5kDa
38kDa
46kDa
69kDa
involvement of the same kinase. We have investigated the involvement of PKA in the IFN-β induction process, and present the results in chapter 6.

The location of the potential sites for phosphorylation of the POU domain by PKA suggests that phosphorylation might influence the DNA binding activity of Oct-1. We have tested whether the phosphorylation of Oct-1 by PKA can bring about a reduction in its DNA binding affinity as observed upon induction. To investigate this we incubated both the nuclear extracts prepared from uninduced HeLa cells and bacterially produced POU domain, separately or together, with purified catalytic subunit of PKA and/or the PKA inhibitor H89. Samples were then subjected to an EMSA analysis with NRD II probe to monitor the effects of these reagents on DNA binding of the Oct-1 proteins. We failed to see any effect on the DNA affinity of either endogenous Oct-1 or the recombinant Oct-1 POU domain (figure 3.16.), and conclude that phosphorylation on the sites recognized by the catalytic subunit of PKA is not sufficient to bring about any changes in the DNA binding affinity of Oct-1. If such phosphorylations are at all involved in the observed reduction of Oct-1 binding affinity upon induction, additional modifications events are also necessary.

Similar studies on the influence on Oct-1 binding affinity of PKA phosphorylation have been performed by Segil et al (1991). By phosphoamino acid analysis they showed that Oct-1 modified in vitro by PKA exclusively contains phosphoserine, which may implicate the serine residue 385 as the target site. Since serine phosphorylation in vivo correlated with the observed loss in Oct-1 binding activity to the octamer site in EMSA analysis using nuclear extracts prepared from synchronized cultured cells that have entered mitosis, these authors speculated that PKA, or a related kinase, would be responsible for the reduction in affinity. Curiously, they further reported that a reduction can be achieved in the DNA binding affinity of the endogenous Oct-1 in HeLa cell nuclei, as well as of the recombinant POU domain, by incubation with purified catalytic subunits of PKA. We do not understand the differences between our results and those of Segil et al. It is possible that differential extraction of proteins during their nuclear extract preparation, or a different degree of purity of the bacterially produced POU domain, or of the PKA preparation, would explain the difference.

One other group have tackled the question of the consequences of the Oct-1 phosphorylation by PKA. Because of the correlation of the appearance of the PKA catalytic subunit in nuclei with the decrease in Oct-1 binding activity, it has been hypothesized that PKA is further involved in the loss of Oct-1 binding in involuting mammary gland epithelial cells (Marti et al 1994). Since Oct-1 protein levels also decreased during the
Figure 3.16. Specific phosphorylation of the endogenous Oct-1 or the recombinant POU domain by PKA does not result in changes in their binding affinity for a specific DNA element.

An EMSA analysis on the probe -108/-95 derived from the NRD II region of the IFN-β promoter. Where indicated, 20µg of nuclear extracts prepared from untreated HeLa cells and/or 4ng of bacterially produced Oct-1 POU domain were added. In addition, as indicated above the lanes, purified catalytic subunits of PKA (1 unit), PKA inhibitor H89 (0.4µg), and DMSO [equal volume (0.2µl) to H89], were added. DMSO serves as a specificity control for H89 action, since the kinase inhibitor is dissolved into DMSO.
process, it was suggested that phosphorylation by PKA could target Oct-1 for degradation, in a manner perhaps analogous to that suggested for the degradation of NF-κB inhibitor IκB (see 1.2.2.4.3.). The same authors also reported a loss of Oct-1 binding activity in EMSA in vitro, when purified PKA was added to the nuclear extracts prepared from lactating (that is pre-involuting) mammary gland cells; whether the addition of PKA in vitro also induces specific degradation of Oct-1 was not investigated. This is another result in apparent contrast with ours, since we cannot see any effect on Oct-1 binding activity by in vitro phosphorylation by PKA. Several explanations of the discrepancy can be envisaged. It remains possible that the specific phosphorylation by PKA does not directly modify the DNA binding properties of Oct-1. In this model, the mammary epithelial nuclei would contain other factors that become modified by PKA, in a manner that activate their ability to further modify Oct-1. Such factors might not be present in other cell types, such as HeLa cells. Perhaps the modification of Oct-1 by PKA is an essential event associated with the change in Oct-1 binding, but some other modifications, again brought about by cell-specific activities, are also required to cause the affinity reduction. As for the degradation of Oct-1 observed by Marti et al, if it indeed is triggered by PKA, it must also require factors not present in HeLa cells, or HeLa cells must contain activities inhibiting such degradation.

Another obvious candidate kinase to modify Oct-1 in response to dsRNA treatment of cells would be the dsRNA-activated protein kinase PKR, already known to activate PRD II binding activity NF-κB. Furthermore, amino-terminal to the Oct-1 POU domain there is an amino acid sequence LSRRR, which is identical to the peptide context around the serine (S) of the eIF2α protein modified by PKR (see section 1.2.1.3.). PKR was previously suggested to be a non-nuclear, ribosome-associated enzyme. However, recent investigations indicate that PKR may become localized to the nucleus (M.Clemens, pers. comm.), which means that it could be available to modify the nuclear protein Oct-1 in dsRNA-induced cells. Our multiple attempts to investigate the ability of PKR to phosphorylate the Oct-1 POU domain in vitro have been hindered by the presence of dsRNA-independent kinase activities in the purified PKR preparations tested by us. This dsRNA-independent phosphorylation of the Oct-1 POU domain generates rather a strong background signal, making quantitation of our results difficult (data not shown).

3.8. Attempts to Identify Cellular Proteins that Interact with Oct-1

In addition to the modulation of protein stability and/or post-translational modifications, the decrease in the DNA binding of Oct-1 upon induction might be associated with changes in
its involvement in protein-protein interactions. For example, such interactions could alter the intranuclear localization of Oct-1 in induced cells, so that it could be recruited away from DNA. It has been suggested that hormone-dependent repression of an octamer-dependent promoter by the glucocorticoid receptor is brought about by this mechanism (Kutoh et al. 1992). Oct-1 can be crosslinked, via its POU domain, with the glucocorticoid receptor, and it was reasoned that the down-regulation of the DNA binding activity of Oct-1 could be due to a formation of a transcriptionally abortive protein complex between the two transcription factors. Another example involving a POU domain-containing protein has been reported to take place in the cells of the developing Drosophila nervous system: the I-POU protein, which has a DNA binding defective POU domain, can interact with another POU domain protein Drifter, and thus prevent the latter from binding to specific sites on target promoters (Treacy et al. 1991). The POU homeodomains appeared to be sufficient for the interaction and to contain the residues which discriminate between different POU domains and thus confer dimerization specificity (Treacy et al. 1992).

In order to determine whether other nuclear proteins can interact with the Oct-1 POU domain, we have performed a WildWestern analysis. First, 30 µg aliquots of nuclear extract prepared from either uninduced or primed/dsRNA-induced/CHX-treated HeLa cells were fractionated on an SDS-PAGE, and separated proteins transferred to an Immobilon membrane. The membrane was then incubated with 2 µg/ml of the bacterially produced POU domain. After washing in mild conditions, the secondary probing with an anti-POU antiserum was performed. The entrapment of the anti-POU antibodies, and thus the location of those membrane-immobilized proteins that the recombinant POU domains had interacted with, was detected by enhanced chemiluminescence. The protein pattern detected (figure 3.17) was clearly more complex than recognized in a plain Western analysis using the same anti-POU antiserum (see figure 3.12.), suggesting that most of the proteins were indeed detected by virtue of their interaction with the Oct-1 POU domain. The proteins interacting with the POU domain were largely the same for nuclear proteins from both uninduced or induced cells, except for a very large (>200 kD) protein specifically detected in induced nuclei. We have not yet investigated the identity of this protein any further.

The incubation with the POU domain was also performed with or without 100 ng/ml of -110/-12 promoter fragment derived from the IFN-β promoter in order to distinguish between the potential DNA binding-dependent and -independent protein-protein interactions. No major differences in the resulting pattern were seen whether the promoter fragment was included or not (figure 3.17.), suggesting that all the interactions, specific or non-specific, were independent of DNA. The signal is considerably lower when the oligonucleotide is included, possibly indicating that the recombinant POU domain interacts
Figure 3.17. Interactions of the Oct-1 POU domain with nuclear proteins.

A WildWestern analysis with the recombinant POU domain and an anti-POU antiserum as probes. The proteins in HeLa nuclear extracts from either untreated cells or from primed/dsRNA-induced/CHX-treated cells, together with an aliquot of the bacterially produced Oct-1 POU domain as a positive control for the antibody-probing, were separated on an SDS-PAGE, and transferred onto an Immobilon membrane. The membranes were first probed with 2μg/ml of the bacterially produced POU domain, and then with an antiserum raised against the Oct-1 POU domain. The entrapment of Oct-1-specific antibodies was visualized by the enhanced chemiluminescence method. For the membrane on the right, 100ng/ml of the IFN-β promoter fragment -110/-12 (a restriction fragment BglII-NcoI of the pIFΔ8 plasmid) was included during the incubation with the recombinant POU domain.
with it during the incubation, and that its association with DNA excludes or strongly weakens the protein-protein interactions in a competitive manner.

All of the interactions Oct-1 is known to participate in, including the proposed interactions between two Oct-1 molecules (see section 3.9.3.), and those with VP16, TBP, and the B cell-specific cofactor, happen through the POU domain itself (see section 3.9.3.). Thus, high-affinity antibodies against the POU domain may competitively exclude interaction with some of the putative associated proteins, and the detection of such interacting proteins must depend on the relative affinity of the POU domain to the interacting nuclear proteins and to the antibody species in the antiserum. To get around this problem, the POU domain could be fused to a protein tag, and antibodies or affinity matrices against the tag used to trap the protein complexes. In fact, we have performed a set of experiments, in which histidine-tagged POU domain was mixed with metabolically labeled nuclear extracts from untreated or induced HeLa cells, and the mixture applied to a nickel-agarose column in non-denaturing conditions, in order to trap the POU domain together with possible interacting proteins. Unfortunately we were not successful in detecting any proteins interacting with the Oct-1 POU domains, including the >200kD protein seen in the WildWestern experiment (data not shown).

The potential interactions between Oct-1 and additional nuclear proteins have been also investigated by coimmunoprecipitation assay using metabolically labeled nuclear protein preparations. No POU-associated proteins could be coimmunoprecipitated from any nuclear extracts with anti-POU antiserum after mixing recombinant POU domain with nuclear extracts (data not shown). This analysis is also subject to the same problem of competition between the antibodies and POU-interacting proteins as the WildWestern experiment presented above. In addition, some of the parameters are different in the two assays: for example, in the WildWestern assay, although proteins are believed to renature in situ prior to protein probing of the membrane, they may not assume their complete native conformation, and are also separated from heterologous interacting proteins. Differences like this may explain why the POU-interacting proteins detected in WildWestern were not identified using the coimmunoprecipitation method.


3.9.1. The Repression of the Beta-Interferon Promoter Can Be Analysed in a Transient Transfection System
The two negative regulatory domains of the IFN-β promoter, NRD I and NRD II (see figure 1.3.), were originally identified using a BPV vector to stably transform mouse C127 cells (Zinn et al 1983, Goodbourn et al 1986, Goodbourn and Maniatis 1988). In these experiments, the transfected IFN-β promoter variants were carried on an extrachromosomal plasmid at a constant copy number. Subsequently it has been shown, that in stably transfected HeLa cells, deletion of NRD I caused an elevation in the basal level (Burstein 1986). In contrast, NRD mutations have not previously been shown to be able to alter the basal level of transcription in transient expression experiments, and it has been suggested that a specific chromatin structure could be required for NRD-dependent repression of the IFN-β promoter.

3.9.1.1 Characterization of the Negative Regulatory Domain I

In order to clarify whether the previous inability to define NRD I in transient transfection systems really reflects the state of establishment of the transfected DNA, and to find a system more amenable and less laborious to perform detailed studies on the repression of the IFN-β promoter than a stable transfection system, we have established a novel transient transfection system. As presented in the technical Appendix I, the properties of the Transfectam (TFM) transfection method are different from the previously utilized calcium phosphate system, and we thus decided to approach the question using the former system. Using the TFM reagent, we compared the behaviour of IFN-β test promoters either containing or lacking the NRD I element in transient transfections.

In these experiments we utilized a test plasmid that contains an inducible region (-91/+72) from the IFN-β promoter that encompasses the DNA elements PRD I, PRD II, PRD III and NRD I. In parallel with this, we assayed a test promoter that is otherwise identical to the -91/+72, but carries an internal deletion of the NRD I region (-55/-40) (described in Burstein 1986). In addition, we created novel promoter variants identical to the two mentioned above, except that the native IFN-β TATA boxes were replaced by those from the thymidine kinase (tk) promoter. For the details on the construction of the tk TATA-containing plasmids, see legend to figure 3.18.

All the test promoters are linked to cDNA encoding the luciferase reporter protein, since luciferase reporter system is highly sensitive - thus even subtle changes in the basal level of expression could be detected. The firefly (Photinus pyralis) luciferase cassette used in the reporter constructs is described in DeWet et al (1987). As an internal control for transfection efficiency, pBLCAT2 was used; it contains a chloramphenicol acetyltransferase (CAT) gene under the control of a herpes simplex virus thymidine kinase (tk) promoter.
Figure 3.18. NRD I-mediated repression can be detected in transient transfections.

A. HeLa cells.
B. L929 cells.

Both cell lines were transiently transfected using the Transfectam reagent. The cotransfected luciferase reporter plasmids (1μg) are indicated below the columns. In all the transfections, tkΔ(-105) promoter fused to a CAT cassette (pBLCAT2; 1μg) was cotransfected as normalization control for transfection efficiencies. The relative expression levels shown were obtained by dividing luciferase activities by CAT activities.

The expression plasmids pIF(-91/-37)tkΔ(-39)lucer and pIF(-91/-55)tkΔ(-39)lucer (indicated with “ΔNRD1” in the figure) were created as follows: First the parental plasmids pIF(-91/-55Δ-40/+72)lucer and pIF(-91/-39[linker]-40/+72)lucer (Burstein 1986) were both digested with BamHI and PvuI, and those of two resulting fragments in either case, which contain the IFN-β TATA box, removed. These were replaced with the BamHI-PvuI fragment derived from the ptkΔ(-39)lucer, which contains tk TATA box to create the plasmids pIF(-91/-55)tkΔ(-39)lucer and pIF(-91/-39)tkΔ(-39)lucer.
RELATIVE EXPRESSION LEVEL

tkΔ(-39)

pIF(-91/-37)tkΔ(-39)

-,,- ΔNRDI

pIF(-91/+72)

-,,- ΔNRDI
Relative expression levels were calculated by dividing the luciferase activities (from a test promoter) by the CAT activities (from the control promoter pBLCAT2).

The transient transfection analyses were performed in both human HeLa cells and murine L929 cells. In both cell lines the deletion of the DNA sequence encompassing the NRD I element caused an elevation in the basal transcription level (figure 3.18.A. and 3.18.B.). The effect of removing NRD I may appear modest (from 2- to 5-fold), but was very reproducible. Also, the effect could be observed whether the transcription was dependent on the native IFN-β TATA box or on the heterologous tk TATA box.

This result unequivocally demonstrates that stable establishment of the promoter within a transfected cell is not essential for NRD I to be appropriately regulated. Thus it is unlikely that a particular chromatin conformation is required for repression. The previous failure to observe repression in transient transfections would appear to be a function of the transfection procedure. As demonstrated in Appendix I, a far larger proportion of cells become transfected using TFM than by the calcium phosphate method; furthermore, the amount of exogenous DNA taken in by a given cell appears to be lower. Stable transfections similarly introduce less DNA per cell than conventional transient methods. It seems conceivable that the repressor protein(s), which interact with NRD I, may be present at low levels and may thus become sequestered by large excess of DNA in cells transfected by calcium phosphate coprecipitation.

3.9.2. Effects on Gene Expression by Modulation of the Intracellular Levels of the Oct-1 Protein

As described above, the DNA binding properties of Oct-1 would support the role of Oct-1 as a preinduction repressor of the human IFN-β promoter. We have obviously been interested in obtaining functional evidence to support, or contradict, the model, and, to that end, utilized a cDNA clone for Oct-1 (Sturm et al 1988; a gift from W. Herr, Cold Spring Harbor, New York) to perform a comprehensive series of transient transfection experiments in human HeLa cells. In these cells, the only specific octamer binding activity we can detect in our EMSAs is Oct-1 (see for example, figures 3.3. and 3.9.). We and others (Fletcher et al 1987, Sturm et al 1987) have not detected the existence of another, higher-mobility, octamer binding protein specific for cervical cells, reported to be expressed in HeLa cells by Dent et al (1991). However, these authors themselves noted a considerable variation with respect to the existence of this protein in different sublines of HeLa cells.
There is a caveat in performing transfection studies involving Oct-1. It is generally believed that the cloned cDNA encoding Oct-1 is not full-length, but lacks the stretch encoding at least 50 amino acids at its 5' terminus (Tanaka and Herr 1990). We (data not shown), and others (W.Herr, pers.comm., R.Sturm, pers.comm.) have attempted to retrieve the authentic 5' end of the Oct-1 cDNA, but without success. The possibility that the missing amino-terminus would contribute to the functional properties of Oct-1 must be taken into account; however, we stress that the cDNA used by us is identical to the one extensively utilized in reported studies by other investigators.

The plasmid vector carrying Oct-1 cDNA variants as inserts is pCG, which contains the human CMV promoter, HSV tk gene 5' untranslated leader and initiation codon, rabbit β-globin gene splicing and polyadenylation signals, and the replication origin of SV40 (figure 3.19; Tanaka and Herr 1990). The pCGOct1 plasmid contains the wild-type Oct-1 cDNA, whereas the Oct-1 cDNA in the pCGOct1HD expression vector encodes a variant protein carrying a triple alanine substitution (WFC>AAA) in the POU homeodomain. As discussed above, this mutation completely abolishes the DNA binding of Oct-1 in vitro. In addition, we constructed the plasmids pCGblunt and pCGOct1ΔBstXI for use as appropriate vector controls in transfection experiments. The pCGblunt plasmid retains all the other elements of the pCGOct1 expression plasmid, except the Oct-1 coding sequence. In pCGOct1ΔBstXI, a deletion in the 5'-terminal portion of the Oct-1 cDNA causes the rest of the Oct-1 cDNA (from the nt position 72 onwards) to be out of frame, and only a product of 39 amino acid (of which only 24 are authentic amino-terminal Oct-1 residues) can be produced from it. However, most of the DNA sequence of the Oct-1 cDNA is retained; thus indirect effects through it can be eliminated, or taken into account, in the normalization process of the results. As evident from figure 3.24., the behaviours of both pCGblunt and pCGOct1ΔBstXI are identically neutral, and are used in our studies interchangeably.

3.9.2.1. The Effect of Oct-1 Overexpression on the Proposed Internal Control Promoters

The correct interpretation of any transfection experiment depends upon the normalization of expression from the test promoter to a suitable internal control promoter. We thus tested the possible effect of Oct-1 on two such candidate promoters, the β-actin and the tk promoters, linked to reporter genes. pJATLACZ (Masson et al 1992) contains a β-galactosidase gene under the control of a rat β-actin promoter (from -340 to +10). pBLCAT2 contains the CAT reporter cassette under the control of HSV tk promoter (from -105 to +57). Neither of these test promoters have been reported to bind Oct-1; furthermore, upon inspection, no motifs
The plasmids pCGOct1 and pCGOct1HD vectors (Tanaka and Herr 1990) carry Oct-1 cDNA variants as inserts under the human CMV promoter. In addition, these plasmids contain the HSV tk gene 5' untranslated leader and initiation codon, rabbit β-globin gene splicing and polyadenylation signals, and the replication origin of SV40. The pCGOct1 plasmid contains the wild-type Oct-1 cDNA, whereas the Oct-1 cDNA in the pCGOct1HD expression vector encodes a variant protein carrying a triple alanine substitution (WFC>AAA) in the POU homeodomain.

To create pCGblunt, pCGOct1 was digested with XbaI and BamHI and resulting 5' extensions filled in with reverse transcriptase. The plasmid backbone was then separated from the Oct-1 cDNA insert and self-ligated.

pCGOct1ΔBstXI was created by digesting the pCGOct1 plasmid with BstXI, which recognizes two sites (nt positions 75 and 269) in the Oct-1 cDNA, removing the extending 3' termini with mung bean nuclease, separating the pCG vector from the small BstXI-BstXI fragment and self-ligating the plasmid backbone.

pCGΔNOct1 and pCGΔNOct1HD were created by digesting the plasmids pCGOct1 and pCGOct1HD, respectively, with XbaI and HincII, filling in the XbaI 3' recessed termini, removing the fragments encoding the Oct-1 amino-termini, and religating the vectors.
resembling the octamer consensus can be identified. We also constructed two hybrid promoters pJAT(-340/-39)tkA(-39) and pJAT(-340/-31)tkA(-39) containing the tk TATA box (downstream from and including -40), linked to the β-actin upstream sequences (either from -340 to -39 or from -340 to -31), which were fused to CAT cassette. The construction of these is described in figure legend 3.21. In the hybrid plasmids pJAT(-340/-39)tkA(-39)CAT and pJAT(-340/-31)tkA(-39)CAT, the β-actin and tk promoter sequences are in the same orientation and adjacent, only separated by a BamHI site.

As expected for a promoter lacking an octamer-like motif, no effect Oct-1 overexpression was detected on the expression level driven by the tkA(-105) promoter, whether this was fused to a CAT (figure 3.20.A) or β-galactosidase (the normalization construct in transfections presented in figures 3.20.A. and 3.22.) reporter cassette. Surprisingly, even if the β-actin test promoter does not contain octamer-like sequences, expression sustained by it was reproducibly stimulated by Oct-1 overexpression; the activation directly and positively correlated with the amount of Oct-1 expression plasmid transfected (figure 3.20.B.). Furthermore, transcription directed by the hybrid promoters carrying β-actin upstream sequences linked to the tk TATA box and CAT reporter cassette was similarly stimulated by Oct-1 overexpression (figure 3.22.), indicating that the targets for the activating effect of Oct-1 overexpression on the β-actin promoter are located in these upstream sequences.

Strikingly, the effect DNA binding defective Oct-1 HD- overexpression on β-actin promoter appears as strong as that by the wild type Oct-1 (figure 3.20.B.). Although it is virtually impossible to completely exclude the possibility that the Oct-1 HD- variant does not interact with DNA in vivo, we note that no residual binding was observed in EMSA analyses in vitro. Even if the Oct-1 HD- protein interacted with DNA within cells, perhaps recruited by other DNA binding activities, one would expect this association to be substantially weaker than that exhibited by the wild type Oct-1 protein, and thus the level of activation to be accordingly lower. This is not the case and we favour another explanation, which would be independent of the association of a Oct-1 protein with DNA. The puzzling effect on the β-actin promoter might be caused by "negative squelching", a reverse of the squelching phenomenon, which leads to repression by overexpression of activator proteins. In classical squelching, excess of an activation domain(s) sequesters specific target proteins (components of the preinitiation complex or adapter proteins) into nonproductive complexes away from DNA. The putative repressor domains in Oct-1 could similarly sequester targets specific for certain repression domains, and activation of transcription would be manifested. This model would require a few assumptions that are difficult to verify. First, endogenous levels of Oct-1 within cells would have to be
Figure 3.20. Oct-1 overexpression has no effect on the tk promoter, but activates the β-actin promoter.

A. A transient transfection analysis using the Transfectam reagent to investigate the effect of overexpression of Oct-1 or Oct-1 HD' on the thymidine kinase promoter linked to a CAT reporter gene [tkΔ(105)CAT = pBLCAT2, 1µg in each transfection]. The values for relative expression levels were obtained by dividing the CAT activities by β-galactosidase activities from cotransfected internal control plasmids (tkΔ(-105)LACZ, 1µg in each transfection).

B. A transient transfection analysis using the Transfectam reagent to investigate the effect of Oct-1 or Oct-1 HD' overexpression on a proposed control promoter derived from the regulatory region of the rat β-actin gene (downstream from -340), linked to a β-galactosidase cassette (pJATLACZ, 1µg in each transfection). The values for relative expression levels were obtained by dividing the β-galactosidase activities by the CAT activities from cotransfected internal control plasmids (pBLCAT2, 1µg in each transfection).

The amounts of effector plasmids ("Oct1" - pCGOct1, "HD-" - pCGOct1HD') transfected are indicated below the columns as micrograms. The total amounts of cotransfected pCG-plasmids were corrected to 2µg with the vector control pCGblunt.
Figure 3.21. Constructions of the β-actin/tk hybrid promoter-containing reporter plasmids.

The hybrid promoter constructs pJAT(-340/-39)tkΔ(-39)CAT and pJAT(-340/-31)tkΔ(-39)CAT were created as follows: First, pJATLACZ was digested with HindIII (position +10 of the β-actin promoter). Aliquots of the linearized plasmid were subjected to digestion with Bal31 nuclease for different periods to create 3' promoter deletions of appropriate lengths. The subaliquots of the deleted plasmids were digested with Avai, and the resulting fragments end-labeled with Klenow and [α^32P]dCTP. The radioactive fragments were separated in a denaturing gel, and detected by autoradiography (shown in the figure; the time points of the Bal31 reactions indicated above the lanes). This was used to determine the aliquots (8 min and 12 min timepoints; indicated with asterisks in the figure) of BamHI/Bal31-treated plasmids, in which the majority of the deletions had reached the sequences 5' of the β-actin TATA box. To these deletion variants blunt-ended BamHI-DNA linkers (CCGGATCCCG) were ligated. The ligation products were digested with BamHI, separated from the small linked fragments and religated. By sequence analysis two deletion plasmids were selected for further subcloning; these contain β-actin promoter sequences down to the position -31 and -39, adjacent to a BamHI linker. Finally, the one from the two BamHI-PvuI digestion fragments of each deletion plasmids that contains the β-actin promoter sequences was ligated into the PvuI-BamHI fragment of the plasmid tkΔ(-39)CAT which contains the tk TATA box and CAT reporter cassette, to create the plasmids pJAT(-340/-39)tkΔ(-39)CAT and pJAT(-340/-31)tkΔ(-39)CAT.
Figure 3.22. Oct-1 overexpression activates the β-actin promoter through sequences 5’ to the β-actin TATA box.

A transient transfection analysis using the Transfectam reagent to investigate the effect of Oct-1 or Oct-1 HD' overexpression on the synthetic hybrid promoter consisting of the β-actin upstream sequences and the tk TATA box [A. pJAT(-340/-39)tkΔ(-39)CAT, B. pJAT(-340/-31)tkΔ(-39)CAT; 1μg in each transfection]. The values for relative expression levels were obtained by dividing the CAT activities by β-galactosidase activities from cotransfected internal control plasmids (tkΔ(-105)LACZ, 1μg in each transfection).

The amounts of effector plasmids ("Oct1" - pCGOct1, "HD" - pCGOct1HD) transfected are indicated below the columns as micrograms. The total amounts of cotransfected pCG-plasmids were corrected to 2μg with the vector control pCGblunt.
saturatingly high, since overexpression of even the smallest amounts of exogenous Oct-1/Oct-1 HD- leads to activation. Secondly, in the context of this speculative model, it is interesting to note that the tk promoter does not respond to Oct-1 overexpression, whether linked to the CAT or β-gal reporter gene. This must mean that the same adapter protein or component of the preinitiation complex must be dispensable, or is not limiting, for the appropriate regulation of the tk promoter. Curiously enough, the further upstream sequences of the tk promoter not included in our test constructs have been reported to include a functional octamer site (Parslow et al 1987) - thus one might assume that the preinitiation complex on the tk promoter is potentially responsive to Oct-1 or other octamer binding factors, at least in some cells. Since the synthetic β-actin-tkTATA hybrid promoter must depend on the preinitiation complex formed on the tk TATA box, and still responds to the Oct-1/Oct-1 HD- overexpression we prefer to postulate the titration of the adapter rather than the titration of a component of the preinitiation complex. Presumably this adapter would be a target for a repressor protein that regulates the β-actin transcription by binding to a site in the upstream regions of the promoter. The model does not require that Oct-1 is a true regulator of the β-actin promoter, but rather that one of the true regulators of the β-actin promoter utilizes the squelchable target protein to mediate the effect on the preinitiation complex. We again emphasize that the rat β-actin promoter does not contain octamer-like motifs, and to the best of our knowledge, has not been reported to bind Oct-1.

3.9.2.2. Promoter-Independent Effect of Oct-1 on Luciferase Expression

A major limitation in functional experiments aiming at elucidating preinduction repression of the IFN-β promoter is that the basal level of expression from the endogenous IFN-β gene is too low to be detected. Therefore, in order to see any increased repression due to Oct-1 overexpression, it is necessary to introduce exogenous target promoters that have a detectable level of expression. Even in these experiments the low basal level of transcription can be reliably detected only by using a highly sensitive luciferase reporter system.

Before proceeding to test the effect of Oct-1 overexpression on various IFN-β promoter variants, we thought it necessary to investigate the possible influence by Oct-1 on the reporter gene cassette itself. In these experiments, the tkΔ(-105)lucer plasmid was transfected into HeLa cells together with a control reporter plasmid pBLCAT2. At the same time, Oct-1 expression plasmid variants were cotransfected.

To our surprise, the tk promoter-luciferase constructs were substantially and reproducibly activated by Oct-1 overexpression, and to a somewhat lesser degree by Oct-1 HD-
overexpression (figure 3.23.). Because expression from a control construct containing a CAT reporter gene controlled by the tk promoter is not affected by exogenous Oct-1 (also see results in section 3.9.2.1.), the activation cannot be specific for the tk promoter. As the most likely explanation, we believe that there are cryptic octamer binding sites within the luciferase reporter cassette. It is interesting to note that the effect of the DNA binding defective Oct-1 HD\textsuperscript{-} protein is less than that of the wild-type Oct-1. This could indicate that the DNA binding activity of Oct-1 is indeed a factor contributing to the activation phenomenon. Perhaps Oct-1 HD\textsuperscript{-} proteins are recruited to the promoter solely by protein-protein interactions with endogenous DNA binding competent Oct-1. While considering this possibility, the results of an EMSA analysis presented in figure 3.7. may prove important. As discussed, not more than three obvious binding sites can be identified within the -110/-12 probe, still complexes containing more than three POU domains can be detected. As speculated above, this may indicate the recruitment of Oct-1 proteins to a particular DNA site by their interactions with other proteins, independently of their DNA binding competence.

As an alternative approach we introduced antisense oligonucleotides complementary to the regions in the Oct-1 cDNA spanning the two possible initiation codons (AS1:ATTGTTCATTCTTGA/AS2:GTCCGCCATTTTGAA; the two putative methionine anticodons underlined). These oligonucleotides contain phosphorothioate linkages at both ends, which are known to protect the free DNA ends from attack by intracellular nucleases. As a control for nonspecific effect caused by the incubation of the cells with such nonphysiological oligonucleotides we used a sense phosphorothioate oligonucleotide of similar length that is derived from the same region of the Oct-1 gene as one of the antisense oligonucleotides (S1 TCAAGAATGAACAAT; the methionine codon underlined). Consistent with the activation of luciferase constructs by Oct-1 overexpression, the introduction of either one of the two antisense oligonucleotides led to the decrease in the expression of the tkΔ(-39)lucter (figure 3.23.B.).

To further investigate the dependence of the promoter-independent activation by Oct-1 upon its DNA binding activity, we performed cotransfections of Oct-1 variant cDNAs into HeLa cells together with both reporter plasmids [tkΔ(-105)lucter and pBLCAT2] and double-stranded oligonucleotides that either can (hepoc\textsuperscript{+}) or cannot (hepoc\textsuperscript{-}) serve as effective Oct-1 binding motifs \textit{in vitro} (Poellinger and Roeder 1989). Oct-1 is the only octamer-binding protein endogenously present in HeLa cells; thus, any effect by hepoc\textsuperscript{+} oligonucleotides is likely to reflect their ability to recruit either endogenous or exogenously expressed Oct-1 proteins. The cotransfection of hepoc\textsuperscript{+} oligonucleotides effectively decreased the expression from the tkΔ(-105)lucter reporter plasmids (figure 3.24). Since
Figure 3.23. Promoter-independent activation effect by Oct-1 on luciferase reporter gene containing constructs.

A transient transfection analysis in HeLa cells, using the Transfectam reagent. In all the cotransfections, 1μg of the internal control plasmid pBLCAT2 was also introduced into cells. The relative expression levels were obtained by dividing the luciferase activities by CAT activities.

A. The effect of overexpression of Oct-1 and Oct1 HD+ on the expression from tkΔ(-105)lucer construct. 1μg of the effector plasmids indicated below the columns were introduced into cells.

B. The effect of Oct-1 antisense phosphorothioate oligonucleotides on the expression from the tkΔ(-105)lucer construct. Prior to determining the reporter enzyme activities, the cells were incubated 2 days in the presence of 10μM of the phosphorothioate oligonucleotides (see the text) indicated below the columns.
RELATIVE EXPRESSION LEVEL

A.

pCGblunt 1

pCGOct1 3.1

pCGOct1HD- 1.7

pCGOct1ΔBstXI 1

B.

RELATIVE EXPRESSION LEVEL

SENSE 1

ANTI-SENSE 1 0.15

ANTI-SENSE 2 0.21
Figure 3.24. Oct-1 DNA binding activity is required for the promoter-independent activation of luciferase reporter constructs.

A transient transfection analysis in HeLa cells, using the Transfectam reagent, to investigate the effect of cotransfected Oct-1 binding site oligonucleotides on the tkΔ(-105)lucter plasmids (1μg in each transfection). In all the cotransfections, 1μg of the internal control plasmid pBLCAT2 was also introduced into cells. The relative expression levels were obtained by dividing the luciferase activities by CAT activities.

As indicated below the columns, the reporter plasmids were cotransfected with the pCG plasmids (0.5μg each), and also with 2μg of hepoc+ ("octa+") or hepoc- ("octa-") oligonucleotides (see the text).
the oligonucleotides that can recruit Oct-1 molecules, and presumably thus titrate them out from interacting with their other binding sites, can thus suppress the activation, it seems that the association of Oct-1 with DNA is indeed required for the effect. Interestingly, the activation by Oct-1 HD+ can be inhibited by oligonucleotides that are predicted to recruit Oct-1 within cells. One, and perhaps the most intriguing, interpretation of these results, is that the transcriptional activation effect by Oct-1 HD+ is dependent on endogenous Oct-1 which is capable of binding octamer sites. This would be consistent with a model, according to which the Oct-1 HD+ variants can indeed be recruited into the vicinity of DNA by cooperative interactions with wild type Oct-1 proteins possessing intact POU domains. As an interesting methodological point, we would like to draw attention to the fact that the activation effect by the exogenously introduced Oct-1 is completely inhibited by oligonucleotides containing Oct-1 binding sites. If this really reflects the intracellular recruitment of Oct-1 from cellular DNA, our approach should be a very efficient method to inhibit the action of specific transcription factors.

Whatever causes the result, it clearly poses a potential obstacle for performing the transient transfection studies on the IFN-β promoter constructs linked to the luciferase cassette. We tried to decrease or abolish the promoter-independent activation by Oct-1 by re-engineering our luciferase reporter constructs. One can identify 6/8 and 8/8 + 6/8 matches to the perfect octamer motif in the 5' and 3' untranslated regions of the luciferase gene, included in the reporter cassette. Since these regions are not part of the protein coding sequence and thus not necessary for the reporter activity, we decided to delete them from the tkΔ(-105)lucer plasmids. Also, it has been reported that the 5' untranslated region of the luciferase cassette can activate cryptic promoters within the non-promoter regions of the vector backbones (DeWet et al 1987). The replacement of the luciferase 5' untranslated region with the one from the β-globin gene (the resulting plasmid 3A/8AΔ5'ut), as well as the deletion of the 3' untranslated region (the resulting plasmid 3A/8AΔ3'ut) are described in figure 3.25. Furthermore, we replaced the reporter plasmid backbone, including the plasmid replication origin region by a different one derived from the pSP65 plasmid (Promega) (the resulting plasmid pSP3A/8A), in case a crucial cryptic octamer site is located in some of those regions (the construction of this plasmid is also presented in figure 3.25.).

The three novel engineered tk-luciferase constructs were tested in Transfectam transient transfection assays (figure 3.26.). Unfortunately, all three modified expression constructs were still activated by exogenously expressed Oct-1, suggesting that cryptic Oct-1-dependent enhancers are likely to be located within the luciferase coding sequence itself, and thus difficult to eliminate without losing the ability to produce a functional reporter enzyme. An interesting observation is that replacing the 5' untranslated region of the
Figure 3.25. Engineering of the tk-luciferase reporter constructs.

A. The parental plasmid 3A/8A = tkΔ(-105)lucter.

B.-D. The novel tk-luciferase plasmids.

B. A replacement of the plasmid backbone. The pBR-plasmid origin-containing NdeI (3' recessed end filled in)-PvuI fragment of the 3A/8A plasmid was replaced by the pUC-plasmid origin-containing PvuII-PvuI fragment of the pSP65 plasmid.

C. A replacement of the luciferase gene 5' untranslated region with that of the β-globin gene. The β-globin gene fragment was obtained from the πSVHSαβcap plasmid (Treisman et al 1983) as a BssHII-NcoI fragment. This fragment was ligated into the luciferase coding sequence-containing restriction fragment MluI-XbaI of the plasmid 3A/8A, with the help of the double-stranded "luclink" oligonucleotide in a three-way ligation reaction. The "luclink" oligonucleotide is derived from the 5'-most coding region of the luciferase gene (nucleotide positions +53 to +101, relative to the cap site) and carries a 5' NcoI compatible end and a 3' XbaI compatible end. The sequence of the "luclink" oligonucleotide is:

5' CATGGAAGAGCCAAAAACATATAAGAAAGCCCCGCGGCATTCTATCCTCTTCTGCGGTGTGGATTTCTCTTCCGGCCGCTGTAGATTAGGATCT 5'

D. A deletion of the luciferase gene 3' untranslated region. The Asp718 (3' recessed end filled in with Klenow enzyme)-PvuI fragment of the 3A/8A plasmid was ligated to the luciferase coding sequence-containing SspI-PvuI restriction fragment of the 3A/8A plasmid.
A. 

- Ampicillin resistance
- Duplicated SV40 terminator region
- 1k promoter
- 5' Firefly Luciferase cDNA → 3'
- Luciferase position 1841
- SV40 position 3723
- SV40 position 4286
- SV40 position 2066
- pBR322 position 4038/2704
- pBR322 position 2296
- Hpa site
- Ssp site
- Asp718 site
- pBR322 pos. 0

B. 

- pUCori
- Pvul
- pSP3A/8A
- Luc

C. 

- 3A/8AΔ5'ut
- Luc
- MseI
- BstEII
- Xbal
- 8-globin 5'ut

D. 

- Pvul
- 3A/8AΔ3'ut
- Luc
- Asp718
- SspI
Figure 3.26. The engineered tk-luciferase constructs remain activatable by Oct-1 or Oct-1 HD- overexpression.

A transient transfection analysis in HeLa cells, using the Transfectam reagent. One microgram of each of the luciferase test plasmids ["3A/8A" = tkΔ(-105)lucter, "Δ5" = 3A/8AΔ5'ut, "Δ3" = 3A/8AΔ3'ut, "pSP" = pSP3A/8A; see figure 3.25 for the plasmid descriptions] were introduced into cells. In all the cotransfections, 1μg of the internal control plasmid pBLCAT2 was also introduced into cells. The relative expression levels were obtained by dividing the luciferase activities by CAT activities.

Cotransfected effector plasmids, as indicated below the columns:
ΔBstXI = 1μg of the pCGOct1ΔBstXI plasmid
Oct1 = 1μg of the pCGOct1 plasmid
Oct1 HD- = 1μg of the pCGOct1HD- plasmid
RELATIVE EXPRESSION LEVEL

<table>
<thead>
<tr>
<th>3A/8A</th>
<th>( \Delta BstXI )</th>
<th>Oct1</th>
<th>Oct1HD-</th>
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</thead>
<tbody>
<tr>
<td>( +100 )</td>
<td>291</td>
<td>163</td>
<td></td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>( \Delta 5' )</th>
<th>( \Delta BstXI )</th>
<th>Oct1</th>
<th>Oct1HD-</th>
</tr>
</thead>
<tbody>
<tr>
<td>1244</td>
<td>444</td>
<td>608</td>
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</table>

<table>
<thead>
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<th>( \Delta 3' )</th>
<th>( \Delta BstXI )</th>
<th>Oct1</th>
<th>Oct1HD-</th>
</tr>
</thead>
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<tr>
<td>551</td>
<td>185</td>
<td>277</td>
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</tbody>
</table>

<table>
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<tr>
<th>( \text{pSP} )</th>
<th>( \Delta BstXI )</th>
<th>Oct1</th>
<th>Oct1HD-</th>
</tr>
</thead>
<tbody>
<tr>
<td>132</td>
<td>85</td>
<td>194</td>
<td></td>
</tr>
</tbody>
</table>
luciferase cDNA with a heterologous one from the β-globin gene led to a strong (approximately 4-fold) elevation of both the basal and Oct-1-stimulated activity of the luciferase reporter gene. This may indicate that the 5' untranslated sequences are inhibitory for the translation of the luciferase mRNA. Consistent with this, the deletion of this 5' region removes an upstream ATG codon which has the potential to initiate the translation of a short 13 amino acid peptide (de Wet et al 1987); such upstream ATG codons have been shown able to reduce the utilization of a downstream ATG codon for the initiation of protein synthesis (Johansen et al 1984, Kozak 1984).

We have also deleted the 5' end of the Oct-1 and Oct-1 HD" cDNAs in the context of the pCG backbone, so that the protein expressed in transfected cells lacks the amino-terminal region of Oct-1. The missing part of the Oct-1 protein contains glutamine-rich regions proposed to function as activation domains. Thus, the comparison of the behaviours of the amino-terminally deleted Oct-1 and DNA binding defective Oct-1 variants could reveal the involvement of a potential squelching effect caused by this putative activation domains. A clear difference between the effects of the overexpression of the full-length Oct-1 proteins and the deletion variants was observed on the activity of the tk-luciferase construct (figure 3.27). The DNA binding defective Oct-1 that lacks its amino-terminus cannot activate the construct any longer, but rather has a weak inhibitory effect. In contrast, the activation effect by the amino-terminally deficient Oct-1 with the wild type POU domain is clearly pronounced. Both observations suggest that the amino-terminal region of Oct-1 protein can indeed squelch when overexpressed in cells. In summary, the comparison of effects of the Oct-1 variants either including or missing the amino-termini reveals that there may be at least two different transcriptional effects carried out by Oct-1 occurring simultaneously: promoter-independent activation and squelching.

Above, we have assumed that the nonspecific activation effect of Oct-1 is transcriptional and direct. However, more indirect mechanisms, in which Oct-1 proteins induce the expression of other factors that actually execute the effect, or where the stability of the luciferase protein is affected cannot be completely excluded.

### 3.9.2.3. The Effect of Oct-1 on the Beta-Interferon Promoter

#### 3.9.2.3.1. Oct-1 Overexpression Represses the Full-Length IFN-β Promoter

To investigate the properties of Oct-1 with respect to the full-length IFN-β promoter we transfected the expression vector pCGOct1 into HeLa cells with the IFN-β promoter-luciferase reporter construct pIF(-210/+72)lucter. The luciferase reporter system
Figure 3.27. The amino-terminus of the Oct-1 protein can squelch the expression of luciferase reporter plasmids.

A transient transfection analysis in HeLa cells, using the Transfectam reagent, to investigate the effect of the deletion of the Oct-1 amino-terminus on the promoter-independent activation effect. In all the transfections, the test plasmids (1µg each) cotransfected were tkΔ(-105)lucter and the internal control pBLCAT2. The relative expression levels were obtained by dividing the luciferase activities by CAT activities.

The cotransfected effector plasmids (see figure 3.19. for the plasmid descriptions), as indicated below the columns:
- = 1µg of the pCGOct1ΔBstXI plasmid, the vector control
Oct1 = 1µg of the pCGOct1 plasmid
HD- = 1µg of the pCGOct1HD- plasmid
ΔNOct1 = 1µg of the pCGOct1ΔNOct1 plasmid
ΔNHD- = 1µg of the pCGOct1ΔNOct1HD- plasmid
RELATIVE EXPRESSION LEVEL

<p>| | |</p>
<table>
<thead>
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<th></th>
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<tbody>
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<td>Oct1</td>
<td>2.2</td>
</tr>
<tr>
<td>ND1</td>
<td>1.5</td>
</tr>
<tr>
<td>ΔND1/ΔND2</td>
<td>0.8</td>
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needs to be used in these experiments, since it is the only one sensitive enough to reliably
(or at all) detect the uninduced level of expression from the IFN-β promoter. The tk
promoter-CAT reporter plasmid, pBLCAT2, was cotransfected into cells as an internal
control to normalize for variations in transfection efficiency. DNA was introduced into cells
using Transfectam reagent, which was demonstrated above to enable the cis-repressive
effects of the NRD I element to be detected (section 3.9.1.1.). As we have determined
above, Oct-1 has a nonspecific activating effect on the luciferase reporter constructs. In
order to correct for this, we cotransfected the pCGOct1 plasmid with a tkΔ(-39)lucert
reporter, chosen because the tk promoter is not at all affected by Oct-1 overexpression (see
above). In this experiment, Oct-1 overexpression enhanced luciferase expression
(promoter-nonspecific) to a greater extent than that seen for the IFN-β promoter. By
correcting for the nonspecific effects of Oct-1 on the luciferase reporter cassette, we have
determined that a net repression of IFN-β promoter activity by Oct-1 overexpression can be
observed (figure 3.28.A.). While the effect was rather small (2.5-fold), one should bear in
mind that the recipient cells already contain high levels of endogenous Oct-1, which may
have a substantial effect on the test promoters. Permanent mammalian cell lines which
would lack Oct-1 have not been established, presumably because Oct-1 is known to
participate in the regulation of several genes that encode products important for cellular
physiology. This is supported by the fact that we have been unsuccessful in our attempts to
establish stably transfected cell lines overexpressing antisense Oct-1 mRNA (S.
Goodbourn, unpublished data). The possibility to stably incorporate antisense Oct-1
constructs under conditionally inducible promoters will be investigated in our forthcoming
studies.

3.9.2.3.2. Repression by Oct-1 Cannot Be Localized to a Single DNA Element

We next tried to locate the DNA element(s) mediating the modest repression of the IFN-β
promoter by Oct-1, by assaying test promoters carrying various regions of the promoter in
front of the luciferase cassette in transient Transfectam transfections. All the luciferase
reporter plasmids were cotransfected into HeLa cells with a control plasmid pBLCAT2.
After preparation of the cell lysates, luciferase activities were normalized to CAT values to
correct variations in transfection efficiency. Furthermore, the relative expression levels
from the IFN-β promoter-luciferase constructs were corrected for the promoter-nonspecific
effects of Oct-1 overexpression on the tkΔ(-39)lucert plasmid, as above.

It became obvious that the modest repression effect by Oct-1 could not be localized to a
single element within the IFN-β promoter (figure 3.28.B.). The impossibility of mapping
the effective response element within the IFN-β promoter that can mediate the observed
Figure 3.28. Oct-1 represses the IFN-β promoter

A. A transient transfection analysis to investigate the effect of Oct-1 overexpression on the pIF(-210/+72)lucer reporter construct. The pIF(-210/+72)lucer clone contains IFN-β sequences from -210 to +72 linked to the position -17 of the firefly luciferase gene (King and Goodbourn 1994).

B. A transient transfection analysis to investigate the effect of Oct-1 overexpression on luciferase reporter constructs carrying various regions derived from the IFN-β promoter. In the pIF(-x/+72) IFN-β promoter variants linked to the luciferase cassette, "-x" denotes the 5'-most base of the promoter included (the plasmid constructions as described in King and Goodbourn 1994, Ellis and Goodbourn 1994). In the pIF(-91/-69Δ-40/+72) and pIF(-91/-55Δ-40/+72) IFN-β promoter variants linked to the luciferase cassette, the promoter regions between [-69 and -40] and [-55 and -40], respectively, have been deleted (the plasmid constructions are unpublished, S.Goodbourn).

All transfections were performed in HeLa cells, using the Transfectam reagent. In both sets of experiments (A. and B.), the plasmid tkΔ(-39)lucer was also included to allow the normalization of the promoter-independent effect of Oct-1 overexpression. One microgram of each of the luciferase plasmid was transfected, together with 1μg of the pBLCAT2 plasmid to control for transfection efficiencies. In addition, every luciferase plasmid was cotransfected with 1μg pCGOct1 and 1μg pCGΔBstXI. To obtain the values in the figure, the relative expression levels were first determined by dividing the individual luciferase activities by the CAT activities. Next, the effect of Oct-1 overexpression on each luciferase test plasmid was determined by dividing the relative expression level affected by Oct-1 (pCGOct1) overexpression by that of the vector control (pCGΔBstXI) transfections. Finally, to normalize to the promoter-independent activation effect of Oct-1, the effects of Oct-1 overexpression on the expression levels of the individual luciferase constructs were divided by the effect of Oct-1 overexpression on the tkΔ(-39)lucer plasmid. Thus, the promoter-specific repression effect by Oct-1 on the tk promoter is 1, that is, tk promoter is not specifically affected by Oct-1 overexpression (as shown above).
A. repression by Oct1  
[normalized to tkΔ(-39)luc ter]

- tkΔ(-39)
- plF(-210/+72)

B. repression by Oct1  
[normalized to tkΔ(-39)luc ter]

- tkΔ(-39)
- plF(-40/+72)
- plF(-70/+72)
- plF(-78/+72)
- plF(-91/-69Δ-40/+72)
- plF(-91/-55Δ-40/+72)
- plF(-91/+72)
- plF(-104/+72)
- plF(-116/+72)
- plF(-210/+72)
repression indicates that Oct-1 is not likely carry out this function through the binding to a single response element within the promoter region. We cannot exclude the possibility that the effect is indirect, so that Oct-1 would not itself directly interact with the promoter at all, but would induce (a) factor(s) inhibiting the transcription through multiple DNA elements, or inhibit (a) factor(s) stimulating transcription through multiple DNA elements. By the methodology available, it is impossible to exclude indirect effects in such transfection assays.

Considering the most straightforward model, that the repression effect results from the specific association of Oct-1 with the IFN-β promoter, the implications of the absence of a single effective response element mediating the effect are intriguing. Firstly, the IFN-β TATA box is a strong binding site for Oct-1; nevertheless, the IFN-β TATA box alone cannot mediate repression. Second, as shown in figure 3.7., the binding of Oct-1 POU domains to several sites within the promoter is clearly cooperative. Third, in the same experiment, complexes were found to form that cannot solely depend on DNA sequences that serve as effective Oct-1 binding sites in isolation; and, as speculated, there may be context-dependent cryptic binding sites for Oct-1, to which POU domains may make contacts, if, and only if, recruited to the promoter by the cooperative action with another POU domain already bound on DNA. Taken together, it may be that one Oct-1 molecule strongly binds to the TATA box, but is unable to exhibit the repression. However, when this molecule cooperatively recruits more Oct-1 molecules to the promoter, the combined action leads to the observed repression. The requirement for more than one promoter-associated Oct-1 could be due to either a synergistic effect by multiple (as yet unelucidated) repression domains, or the ability of Oct-1 to function as an architectural factor promoting the repressive organization of the promoter. Consistent with this, the repression effect was the strongest on the most extended test promoter variant pIF(-210/+72).

3.9.2.3. Decrease in the Level of Intracellular Oct-1 Enhances IFN-β Transcription

An alternative approach we have taken is to introduce antisense phosphorothioate oligonucleotides targeted to specifically inhibit the translation of endogenous Oct-1 mRNAs. In accord with the observed repression of the IFN-β promoter by Oct-1 overexpression in transient transfection assays, the introduction of either one of the two antisense oligonucleotides leads to the modest (2-3-fold) increase in the expression of the IFN-β promoter after normalizing to the promoter-independent effect on the tkΔ(-39)luctor construct (figure 3.29.). Both the uninduced and induced expression levels of the IFN-β promoter are specifically increased, to a similar degree, by the application of antisense Oct-1 oligonucleotides.
Figure 3.29. Decrease in the level of intracellular Oct-1 enhances IFN-β transcription

A transient transfection analysis in HeLa cells, using the Transfectam reagent, to investigate the effect of the antisense Oct-1 oligonucleotides on the IFN-β promoter. Cells were transfected with either the tkΔ(-39)lucer or pIF(-210/+72)lucer luciferase reporter plasmids (1μg each). In all transfections, 1μg of the pBLCAT2 plasmid was cotransfected as a control for transfection efficiencies. Where indicated, the transfected cells were primed and induced with dsRNA prior to determining the reporter enzyme activities. Also, prior to reporter enzyme assays, the transfected cells were incubated for 2 days in the presence of 10μM of the phosphorothioate oligonucleotides (S-sense; AS1-antisense 1; AS2-antisense 2; described in section 3.9.2.2.).

To obtain the values in the figure, the relative expression levels were first determined by dividing the individual luciferase activities by the CAT activities. After this, to normalize to the promoter-independent effect of Oct-1 depletion on the luciferase constructs (see figure 3.23.B.), the relative expression levels of all three tkΔ(-39)lucer transfections were given the value 1, and the relative expression levels of the IFN-β promoter transfections corrected accordingly, so that the fold differences between all the relative expression levels affected by a particular oligonucleotide are maintained.

The fold activations of the IFN-β promoter by antisense oligonucleotides (compared with the sense oligonucleotides) are indicated above the columns.
RELATIVE EXPRESSION LEVEL
[NORMALIZED TO tkΔ(-39)lucter]
3.9.2.3.4. Investigation into the TATA Box Occlusion Model

The observation that a regulatory transcription factor can bind to the TATA box sequence of the IFN-β promoter is particularly interesting. It is intriguing to speculate that in uninduced cells Oct-1 could prevent the binding of the TATA box binding complex by steric occlusion, thus contributing to the repression by preventing the preinitiation complex assembly. Since the interaction of TFIIID with a TATA box is an essential, and often rate-limiting, step in transcription from TATA-containing RNA polymerase II promoters (Eloranta and Goodbourn 1995), the TATA box occlusion would provide a very efficient mechanism of repression. There is indeed some in vitro evidence from other promoter contexts that some negatively acting transcription factors may utilize this mechanism to repress their target promoters. For example, binding of the Engrailed homeodomain protein to the TATA box of a Drosophila gene hsp70 appears to inhibit transcription by competition with TFIIID (Ohkuma et al 1990). Drosophila P-element transposase is another sequence-specific DNA-binding protein that represses transcription from its target promoter by interfering with the TFIIID binding to the TATA box (Kaufman and Rio 1991). Similarly, it has been reported that HIV-1 promoter may be repressed by a cellular factor LBP-1 (Kato et al 1991), and human osteocalcin (Stromstedt et al 1991) and interleukin-6 (Ray et al 1990) promoters by glucocorticoid receptor through similar mechanisms involving blocking the TFIIID-TATA box interaction through overlapping binding sites.

To investigate this we have replaced the TATA box (consensus TATA\textsuperscript{T}/\textsubscript{A}ATA\textsuperscript{T}/\textsubscript{A}A) regions of the tk (CATAT\textsuperscript{T}/\textsubscript{A}AA) and rat β-actin (TATAAAAC) promoters with that of the IFN-β promoter (TATAAATA) (nonconsensus bases indicated with a smaller font). Both expression plasmids contain the IFN-β promoter sequences downstream of position -40, and upstream sequences from either the tk (from -105 to -39) or β-actin (from -340 to -40) promoter. The hybrid promoters were cotransfected together with Oct-1 expression constructs into recipient cells to see whether Oct-1 overexpression has any effect on these hybrid promoters. We failed to see any effects that would depend on the presence of the IFN-β TATA box (figure 3.30., compared with figure 3.20.). Again, however, this could be due to the saturating levels of the endogenous Oct-1. Furthermore, even if TATA box occlusion would be an operative mechanism, it might require other specific sequences of the IFN-β promoter, because of the possible auxiliary factors binding to them, or because of their local conformational effect on DNA.

It should be noted that the binding of Oct-1 and TFIIID to the IFN-β TATA box is not necessarily mutually exclusive. TBP makes only minor groove contacts (Lee et al 1991, Starr and Hawley 1991), and is predicted to only interact with one face of the DNA helix,
A transient transfection analysis in HeLa cells, using the Transfectam reagent. The test promoters were hybrid constructs (A. rat $\beta$-actin promoter sequences from -340 to -40, linked to the IFN-$\beta$ TATA box promoter sequences downstream from -40; B. tk promoter sequences from -105 to -39, linked to the IFN-$\beta$ TATA box promoter sequences downstream from -40), both linked to a CAT reporter cassette. In all transfections, the internal control reporter plasmid tk$\Delta$(-105)LACZ was included. The amount of each reporter constructs was 1µg plasmid/transfection. In addition, as indicated below the columns, 1µg of the effector plasmids (pCGOct1 or pCGOct1HD$^-$) or the vector control plasmid (pCGblunt) was cotransfected. The relative expression levels were obtained by dividing the CAT activities by the $\beta$-galactosidase activities.

A. To create the $\beta$-actin/IFN-$\beta$ TATA reporter plasmid, the tk TATA box-containing BamHI-BglII (the 5' extension at the BglII end filled in with Klenow enzyme) fragment of the pJAT(-340/−39)tk$\Delta$(-39)CAT plasmid (described in the legend to figure 3.21.) was replaced by a 34bp fragment containing the IFN TATA box, derived by digesting the pBVIFA(-40)fIRE plasmid (Goodbourn et al 1985) with HincII and BamHI.

B. To create the tk/IFN-$\beta$ TATA reporter plasmid, the tk TATA box-containing restriction fragment MluI (the 3' recessed end filled in)-BamHI was replaced by the tk/IFN-$\beta$ TATA-containing restriction fragment NcoI (3' recessed end filled in)-BamHI of the 318/2E plasmid (318/2E constructed by S.Goodbourn). A partial BamHI-digestion was performed to obtain this BamHI-NcoI fragment, since in addition to the BamHI site at the position -105 of the tk promoter, there is another BamHI recognition site separating the IFN TATA box and the tk promoter position -39 in the 318/2E plasmid.
RELATIVE EXPRESSION LEVEL

A.

- pcGblunt: 1
- pCGOct1: 2.7
- pCGOct1/HD+: 2.9

B.

- pcGblunt: 1
- pCGOct1: 1.1
- pCGOct1/HD+: 1.2
as judged by its recently determined crystal structure (Nikolov et al. 1992). Thus Oct-1 could bind the TATA box simultaneously with TFIID, and either positively or negatively affect its function. No evidence for this was observed in our transfection analyses. Alternatively, Oct-1 and TFIID might interact with the TATA element sequentially. If this is the case, one of the functions of Oct-1 could be to maintain the TATA box in an accessible state, before exchanging with TFIID upon induction of transcription. If this function of Oct-1 involves the exclusion of nucleosomes from the core promoter, it could not be detectable in transient transfection assays. Gross changes in nucleosomal organization of an exogenously introduced human IFN-6 promoter variant that has stably integrated into the host genome of cultured mouse cells have been observed upon induction with dsRNA (Bode et al. 1986), but the mechanisms how this is achieved have not been investigated.

3.9.2.3.5. Effect of Oct-1 on the Endogenous Beta-Interferon Promoter

We have utilized a transient transfection system to overexpress Oct-1, as well as the DNA binding defective variant Oct-1 HD- in HeLa cells, in order to monitor their effects on the expression of the endogenous IFN-6 promoter by RNAase mapping. To reduce the background signal from nontransfected cells, we cotransfected a vector expressing a selectable cell surface protein CD2 (the selection of CD2+ cells is described in detail in Appendix I). Also, unlike in the previous experiments, the calcium phosphate transfection method was used instead of Transfectam; this was because the Transfectam reagent is very expensive, and does not allow experiments on a large enough scale to obtain sufficient amounts of RNA from cotransfected selected cells for mapping. Furthermore, only the effect on the induced level of expression was investigated, since the uninduced level from the IFN-6 promoter is too low to be detected with the RNAase protection assay.

It appears that in this assay both Oct-1 and Oct-1 HD- strongly decreased the expression from the IFN-6 promoter (figure 3.31.). As shown above, Oct-1 HD- cannot bind DNA in vitro, so it could be assumed that the effect does not require the DNA binding activity of Oct-1. Again, however, one cannot exclude the possibility that even the HD- variant of Oct-1 is recruited to DNA in vivo. Nevertheless, we consider it more likely that the effect is caused by squelching by the Oct-1 activation domains. It might be difficult to see this fit into our previous results where we actually proposed that the overexpression of Oct-1 leads to "negative squelching" of the rat β-actin promoter. We emphasize, however, that there are clear inherent differences between the Transfectam and calcium phosphate transient transfection systems, as presented in the Appendix I. Furthermore, it can be envisaged that different promoters respond differently to variations in the intranuclear concentrations of specific transcription factors - this may be since their transcription depends on different
Figure 3.31. Overexpression of either Oct-1 or Oct-1 HD- decreases induced expression levels of the endogenous IFN-β promoter.

A transient transfection analysis in primed/dsRNA-induced/CHX-treated HeLa cells, using the calcium phosphate coprecipitation method, to investigate the effect of Oct-1 or Oct-1 HD- overexpression on induced expression level of the endogenous IFN-β gene. 5μg of either pCGOct1, pCGOct1HD-, or pCGOct1ΔBstXI (vector control) were transfected together with 5μg of the plasmid pKSCD2 (described in Appendix I, section I.II.), which encodes the human CD2 surface antigen. Prior to the selection of CD2-positive transfected cells, the cells were primed, and induced with dsRNA in the presence of cycloheximide. The selection of CD2-positive transfected cells was performed as described in Whiteside et al (1994). The amounts of the mRNAs specific for the human IFN-β gene and γ-actin (internal control) in 20μg aliquots of the cytoplasmic RNA pools prepared from the selected cell populations were quantitated by the RNAase protection method, with the probes described in section 2.2.2.
preinitiation complex variants, or on different adapter proteins. As seen in figure 3.31, the expression from the internal control promoter γ-actin remains largely unaffected by Oct-1/Oct-1 HD⁻ overexpression.

An interesting experiment to follow this would be to test the effects of the overexpression of amino-terminally deleted Oct-1 and Oct-1 HD⁻ variants in this assay, since, as shown above, they seem to be able to eliminate the effect of squelching by the amino-terminal region of Oct-1.

3.9.2.3.6. Transfected DNA Binding Sites for Transcription Factors Can Modulate the Expression from the Beta-Interferon Promoter

Another approach we took to investigate the effect of modulating intracellular Oct-1 protein available to be recruited to the endogenous IFN-6 promoter was to transflect concatamerized oligonucleotides containing binding sites for Oct-1, by calcium phosphate transfection method. For comparison, a concatamerized PRD II oligonucleotide derived from a non-Oct-1 binding region of the IFN-6 promoter was also included. The effects on the endogenous IFN-6 promoter, as well as on the internal control γ-actin promoter, in HeLa cells, were assayed by RNAase protection of specific transcripts.

In this experiment, only the induced transcription level of the IFN-6 promoter was assayed, since the transfection of any of the binding site oligonucleotides failed to increase basal expression levels to be detectable. The oligonucleotides bearing the IFN-6 TATA box region clearly inhibited the induced transcription (figure 3.32.), as expected if one assumes that they are effective in recruiting the preinitiation complex. It is interesting to note that constitutive transcription from the control γ-actin promoter remained unchanged, which might indicate the existence of promoter-specific complexes forming on the two TATA boxes. The PRD II oligonucleotides also inhibited induced IFN-6 transcription, consistent with the suggested role of this element in the induction of the promoter. However, neither the oligonucleotides representing perfect octamer motifs nor the NRD II elements (-108/-95) of the IFN-6 promoter had a substantial effect on the induced IFN-6 transcription. While, this result does not support an effective role for Oct-1 in the IFN-6 transcription, one should remember that only induced expression level was assayed, and the binding affinity of Oct-1 for specific DNA sites is reduced in induced HeLa cells.

3.9.3. Summary of the Studies on the Function of Oct-1 as a Transcription Factor
Figure 3.32. Transfected DNA binding sites for transcription factors can modulate the expression from the IFN-β promoter

An RNAase protection analysis to investigate the effect of the excess of oligonucleotides, which provide binding sites for various factors suggested to regulate the IFN-β promoter, on expression levels of the IFN-β promoter. HeLa cells were transfected with 20μg of the concatamerized, double-stranded oligonucleotides (monomers described in section 2.1.1.3.). After 48 hours, the transfected cells were primed, and the primed cells induced with dsRNA in the presence of cycloheximide. After inductions, the amounts of the mRNAs specific for the human IFN-β gene and γ-actin (internal control) in 20μg aliquots of the cytoplasmic RNA pools prepared from the transfected cell were quantitated by the RNAase protection method, with the probes described in section 2.2.2.
no oligo
octamer
TATA
PRDII
-108/-95
Although transcriptional activation domains have been defined in Oct-1 that can regulate certain classes of promoters (Tanaka et al 1988, Tanaka and Herr 1990), the dependence of the activation of RNA polymerase II promoters on these domains has remained unproven. It is not at all clear that Oct-1 falls into the category of classical transcription factors, which have an effector function separable from the DNA binding domain. The Oct-1 protein has been shown to be able to contact TBP in the basal transcription machinery, both \textit{in vitro} and \textit{in vivo}; however, this interaction is mediated by the POU domain rather than by the proposed activation domains (Zwilling et al 1994). The significance of this interaction has not been vigorously tested. Most controversial results have been reported in studies that involve transfections of Oct-1 expression vectors into cells. Tanaka and Herr (1990) reported that Oct-1 overexpression cannot affect the expression of a transfected \( \beta \)-globin reporter construct fused to multiple octamer binding sites. However, Voss et al (1991) could observe a substantial increase in the expression of luciferase reporter gene linked to an Oct-1 binding site-containing prolactin promoter. Both of these studies were performed using the calcium phosphate coprecipitation method. \textit{In vitro}, Oct-1 alone fails to detectably stimulate a promoter containing only a single octamer motif, although multiple octamer binding sites arranged in tandem do respond to Oct-1 in this assay (LeBowitz et al 1989, Annweiler et al 1993). Also, Oct-1 by itself does not activate transcription through the octamer-like TAATGArAT motifs found in the promoter regions of the herpes simplex virus immediate early genes. Instead, Oct-1 binds to these regulatory elements together with the viral protein VP16 and a host cell factor HCF (O'Hare and Goding 1988). In the ternary complex, it is the acidic region in the carboxy-terminus of VP16 protein that contains the strong activation potential (Triezenberg et al 1988, Greaves and O'Hare 1989). \textit{In vitro} transcription assays, VP16 can activate transcription in conjunction with the Oct-1 POU domain alone; other regions in Oct-1, including the proposed activation domains, are not required (Arnosti et al 1993).

Another case where activation by Oct-1, as well as Oct-2, through specific DNA octamer elements appears to strongly depend on a cell type specific cofactor is provided by the B cell specific transcription of octamer containing promoters (Luo et al 1992, Pfisterer et al 1994). The ability of the ubiquitous Oct-1 protein to utilize a B cell specific cofactor in order to carry out octamer-specific activation of transcription challenges the previous presumption that lymphoid-restricted Oct-2 would alone mediate tissue specific immunoglobulin promoter activity through the octamer motif (see, for example, Kemler and Schaffner 1990), but is consistent with the observations that either an absence (Corcoran et al 1993) or marked reduction (Feldhaus et al 1993) of Oct-2 in B cells has no effect on the amount of endogenous immunoglobulin transcription, whereas the mutation of the octamer sites in the immunoglobulin promoters does (Jenuwein and Grosschedl 1991).
A B cell-specific protein that can interact with the POU domains of Oct-1 and Oct-2, and possesses the appropriate functional properties to be the B cell specific cofactor for the two octamer binding proteins, has recently been cloned (Gstaiger et al 1995, Strubin et al 1995).

Another cell type-specific cofactor, termed Fx, implicated to function in connection with Oct-1 (and embryonal carcinoma cell specific octamer factor Oct-3) on the cell type specific FGF-4 promoter was recently reported to be expressed in F9 embryonal carcinoma cells (Dailey et al 1994). The ability of promoter variants to allow ternary complex formation between Oct-1/Oct-3 and Fx correlated with transcriptional activity. Since the cofactor function of the Fx protein is dependent on the DNA sequence flanking the octamer-like motif, it seems more likely that in its action it resembles VP16 more than the above discussed B cell specific, DNA-independent, cofactor.

Our various approaches to perform a functional analysis on the role of Oct-1 in the regulation of the IFN-β promoter revealed that the regulation by Oct-1 may be an unsuspectedly complex process and utilize several mechanisms. It is conceivable that several regulatory phenomena, physiologically relevant or not, could simultaneously contribute to our results in experiments, in which the levels of endogenous or exogenous Oct-1 variants have been modulated. These include self-squelching, negative self-squelching, squelching or negative squelching of the activity of heterologous transcription factors, site-specific activation or repression, and architectural potentiation or repression of transcription. If one assumes that these functions are separable, a careful mutational analysis combined with expression assays might then distinguish between the events. However, in transfection studies, the nuclear concentration of proteins, which is likely to influence the behaviour of a transcription factor with potential for multiple functions cannot be assayed with sufficient accuracy and reproducibility. High concentrations may drive a factor to take part in low-affinity protein-DNA or protein-protein interactions, as shown for the Drosophila factor Dorsal (Jiang and Levine 1993).

In light of our transient transfection experiments, it is interesting to note that a report has been published about the ability of Oct-1 to repress the human papillomavirus type 18 (HPV18) enhancer (Hoppe-Seyler et al 1991). The authors also utilized the luciferase reporter system, but their choice of transfection method was calcium phosphate coprecipitation. The HPV18 upstream regulatory region does contain a variant octamer motif (ATGCAATT), however, the repressive effect by Oct-1 overexpression was mapped to a promoter subregion of 135 base pairs, which does not include this octamer factor binding site. Furthermore, the Oct-1 variant mutated at the homeodomain region which is
identical to that used in our studies, and defective in *in vitro* DNA binding, was just as effective in repression as the wild type Oct-1. The authors concluded that this indicates that direct interaction between Oct-1 and DNA is not required; however, as our studies indicate, this interpretation may well be an oversimplification of the events that occur within cells. We believe the most likely single explanation for the results by Hoppe-Seyler et al to be that, in their system, activation regions of Oct-1 manifest squelching by titrating out the target protein for a positively acting transcription factor that acts on the mentioned 135bp promoter region. However, as indicated by our experiments, Oct-1 may carry out several transcriptional functions simultaneously.

The octamer motif-containing region is highly homologous between HPV types 18 and 16. The action by Oct-1 mediated by the latter has been investigated by Morris et al (1993). When they subcloned the HPV16 octamer site in front of the tk-promoter-derived TATA box linked to the CAT reporter cassette, and cotransfected this construct into HeLa cells or fibroblastoid BHK-21 cells together with Oct-1 expression plasmids, they observed a decrease in CAT expression. However, the octamer motif in its native HPV16 promoter context was not investigated, thus comparison of the results with those of Hoppe-Seyler et al is difficult. Also, Morris et al did not utilize a DNA binding defective Oct-1 variant to attempt the investigation of any possible contribution by squelching; however, the repressive effect appeared to depend on the presence of an intact octamer site.

It is interesting to note similarities between the properties of Oct-1 and a zinc finger transcription factor YY1. Both of these are ubiquitously expressed, show striking promiscuity in their DNA binding specificity, bend DNA, and, most importantly, have been implicated in both activation and repression of transcription. In the case of YY1, it has been suggested that both the repression and activation are manifestations of the same biochemical activity: the ability of YY1 to bend DNA, and thus organize the topology of a transcription complex forming on a particular promoter (Natesan and Gilman 1993), rather than directly influence the assembly. We interpret certain results of ours to suggest that one of the functions of Oct-1, at least in some promoter contexts, is also to organize such architectural aspects of a promoter.

To explain the multitude of results by us and others, one is tempted to conclude that Oct-1 is capable of both repressing and activating transcription, depending on parameters such as promoter context, concentration, and posttranslational modifications. A number of other mechanisms can be envisaged to explain that a particular transcription factor could function as both an activator and repressor of transcription. Such a factor could be differentially modified. While this mechanism could be functional for example in different cell types or at
different stages of differentiation, it is difficult to comprehend how such a mechanism could allow dual functioning of a factor within a single cell. Second, the exact sequence of a binding site could dictate the differences in function. In this model, different binding sites could force a transcription factor to assume different conformations, thus exposing different effector domains for the basal transcription complexes, for example. Third, according to the idea of combinatorial control, the interactions with other promoter binding proteins could be responsible for the context-dependent functional differences. Fourth, although the issue is still highly controversial, the preinitiation complexes that form on the core promoter elements of different promoters could contain different components, or expose the same components differentially, so that different targets for a particular regulatory factor would be available in different promoter contexts. Fifth, the functional outcome may depend on the concentration of a transcription factor. An example of this is provided by the concentration-dependent activation or repression by a *Drosophila* zinc finger type transcription factor Krüppel, through a single DNA binding site (Sauer and Jäckle 1993). At low concentrations, a monomeric form of Krüppel is a transcriptional activator; however, at adequately high concentrations, the Krüppel protein forms homodimers which actively repress transcription by binding to the same target sequence, but through a different protein effector domain.

Whether activation and/or repression by Oct-1 can take place under physiological conditions within cells, and the mechanisms by which these transcriptional effects are achieved remains inconclusive after our studies. As discussed in several of the previous sections, the main obstacle is probably the inability to perform the functional studies in an Oct-1-less cellular background. As an alternative approach one could attempt to utilize an *in vitro* transcription system. The feasibility of such an approach to investigate transcriptional events on the IFN-β promoter has only been superficially investigated (Cohen et al 1991). The advantage of this approach would be that it might enable studies in a background lacking endogenous Oct-1, since it should be easy to preclear the extracts from Oct-1 with specific antibodies. It remains to be seen whether other aspects of the regulation of the IFN-β promoter take place in an analogous enough manner in a cell-free system for the proposed analysis to be valid.

Finally, the Oct-1 protein produced from the cDNA used by us and other investigators lacks an undetermined number of amino-terminal residues, which may contribute to its function as a transcriptional regulator. Until the 5' end of the Oct-1 cDNA is successfully cloned, the effect of the missing residues will remain unclear. It is interesting to note that the amino acid sequence deduced from the portion of the longest cloned Oct-1 cDNA, immediately upstream of the currently 5’-most methionine codon, is distinctively alanine-
rich (9 alanines out of 18 residues). Similar regions of high alanine content have been delineated in previously identified transcriptional repression domains in other sequence-specific regulatory factors.
Chapter 4: Purification and Characterization of the DNA Binding Activities Un1 and Un2

4.1. Introduction to the DNA Binding Activities Un1 and Un2

In addition to the two identified negative regulatory domains, the positive regulatory domain PRD I has been implicated in the maintenance of preinduction repression of the IFN-β promoter. The SV40 enhancer placed upstream of the multimerized PRD I-derived hexamer AAGTGA fails to exert its transcriptional activation function, unless the recipient cells are induced by virus (Kuhl et al. 1987). This suggests that PRD I-like elements can bind repressor molecules in cells prior to induction. IRF-2 has been suggested as a repressor protein acting through PRD I (see section 1.2.2.4.2.), but we consider it an unlikely candidate for a preinduction repressor. As discussed above, targeted disruption of the IRF-2 gene fails to give detectable IFN-β expression in uninduced cells (Matsuyama et al. 1993). Furthermore, in a mutagenesis study, it has been observed that the binding of IRF-2 to the PRD I region does not correlate with transcriptional repression prior to application of an inducing agent (Whiteside et al. 1992). Our research group have previously identified several novel PRD I binding complexes, including two abundantly present in uninduced cells, referred to as Un1 and Un2 (Whiteside et al. 1992). In contrast to the behaviour of IRF-2, a good correlation is seen between the binding of the EMSA complexes Un1 and Un2 and the repression of synthetic promoters containing variants of PRD I (Whiteside et al. 1992). Furthermore, similarly to Oct-1, the Un1 and Un2 lose their affinity for specific DNA elements upon induction, in a manner enhanced by priming and treatment with cycloheximide (figure 4.1.) (Whiteside et al. 1992; and this thesis).

Interestingly, in competition EMSAs it has become obvious that Un1 and Un2 can also bind to oligonucleotide probes derived from other regions in the IFN-β promoter, namely PRD III (-91/-78) and NRD II (-108/-95) (figure 4.2.) (Whiteside 1992; and this thesis). It is interesting that all of the preinduction repressor complexes proposed by us, Oct-1 and Un1/Un2 share the property of possessing at least three binding sites within the IFN-β promoter.

4.2. Further Analysis on the DNA Binding Specificity of Un1 and Un2
Figure 4.1. The binding activities of Un1 and Un2 complexes decrease upon induction, in a manner dependent on priming the cells and cycloheximide treatment.

An EMSA analysis on the -108/-95 probe derived from the NRD II region of the IFN-β promoter. The nuclear extract samples were either untreated, dsRNA-induced/CHX-treated, primed/dsRNA-induced or primed/dsRNA-induced/CHX-treated HeLa cells.

This figure is identical to figure 3.8.
Figure 4.2. There are (at least) three binding sites for the Un1/Un2 complexes within the IFN-β promoter.

An EMSA competition analysis using a probe -91/-78 derived from the PRD III region of the IFN-β promoter. The fold excess of unlabelled competitor oligonucleotides (the sequences described in section 2.1.1.3.) is indicated above the lanes. The oligonucleotides -108/-95 (NRD II), PRD I and PRD III are efficient competitors for Un1/Un2 DNA binding, whereas the oligonucleotide derived from the PRD II region does not compete, and serves as a negative control.

This figure is adapted from Whiteside (1992).
<table>
<thead>
<tr>
<th></th>
<th>no competitor</th>
<th>5x(PRD) (-108/-95)</th>
<th>50x(PRD) (-108/-95)</th>
</tr>
</thead>
</table>

Legend:

- **Un1**
- **Un2**
- **free probe** PRDIII (-91/-78)
196

A probe -108/-95 (AAAATGTAAATGACA) from a region in the IFN-β promoter that spans sequences on both the NRD II and PRD IV elements, can bind both Oct-1 and Un1/Un2. However, the recognition sequences for these activities are clearly different. An overlapping but different probe -104/-91 (TGTAATGACATAG), which contains most of the PRD IV element, can bind Un1/Un2 with virtually unchanged affinity, but only very weakly interacts with Oct-1 (figure 4.3.). The residual binding of Oct-1 emphasizes the importance of either the adenine base at the position 1 of the octamer-like motif (ATGTAAAT) in the NRD II region, or of the flanking 5' region, for Oct-1 binding to this region.

Un1 and Un2 complexes exhibit identical sensitivity to mutations within the PRD I region (Whiteside et al 1992), suggesting that their DNA binding domains are highly homologous, or that they share a similar DNA binding subunit. To obtain further information about the DNA binding properties, and thus clues about the possible identity, of the Un1 and Un2 complexes, we have extended the competition analysis to include non-IFN-β promoter-derived oligonucleotides, in order to further delineate their DNA binding specificity. One of the binding sites for the Un1 and Un2 complexes within the IFN-β promoter is the NRD II-PRD IV region. Since PRD IV has been reported to serve as a binding site for certain factors of the bZIP family, we decided to test whether oligonucleotides containing binding sites for bZIP factors from other promoter contexts could be effective competitors for Un1/Un2-binding to the -104/-91 probe. Certain CRE and AP1 sites proved to be very efficient competitors of Un1/Un2 binding on the probe -104/-91 (figure 4.4.). The CRE consensus sequence TGACGTCA (Roesler et al 1988) is typically recognized by a large number of proteins (more than a dozen cDNAs encoding such factors cloned) belonging to the ATF/CREB family of bZIP factors (Habener 1990). The somatostatin CRE mediates cAMP-dependent stimulation of transcription in many cell types (Andrisani et al 1987). The AP-1 sites (consensus TGAC/GTCA), also referred to as TPA responsive elements (TRE's), function as signal-responsive transcription control elements in a number of viral and cellular promoters (reviewed in Kouzarides and Ziff 1989). The DNA binding competent and transcriptionally active forms of TRE binding protein complexes are dimers formed by particular combinations of two bZIP family members through a coiled-coil interaction, mediated by the leucine zippers.

Upon examination of the alignments one observes very little identity between the DNA sites that have proved to be effective Un1/Un2 binding elements (figure 4.5.). The only sequence determinant common to all of these appears to be the trinucleotide TGA. If one allows any five of the six oligonucleotides to share the conserved bases, a more extended "consensus motif" AANTGANA (N=any nucleotide) can be identified. However, a very
Figure 4.3. The binding of the Un1 and Un2 complexes to the NRD II region depends on different nucleotides than the binding of Oct-1.

An EMSA analysis, with HeLa nuclear extracts prepared from either untreated cells or primed/dsRNA-induced/CHX-treated cells, to compare the DNA-protein complexes forming on the labelled probes derived from the -108/-95 and -104/-91 regions of the IFN-β promoter.
Figure 4.4. The Un1 and Un2 complexes bind efficiently to certain CRE and AP1 oligonucleotides.

An EMSA competition analysis on the -104/-91 probe derived from the NRD II/PRD IV region of the IFN-8 promoter. The nuclear extracts were prepared from untreated HeLa cells. Sequences of the oligonucleotide competitors (all in 50-fold molar excess to the radioactive probe) are presented in section 2.1.1.3.

CRE oligonucleotide is derived from the regulatory region of the somatostatin gene, and AP1 sites from the regulatory regions of the SV40 enhancer and collagenase gene promoter.
Figure 4.5. A proposed alignment of identified Un1/Un2 binding sites.
GATCCAAAAATGTAAATGACAGATC -108/-95
GATCCTGTAAATGACATAGATC -104/-91
GATCCCGGAAAAACTGAAAGGAGATC -91/-78
GATCCGAGAAGTGAAAGTGAAGATC -77/-64

GATCCTTGACGTCAGCCAA or SMS CRE
TTGGCTGACGTCAGGATC

GATCCTTGCTGACTAATTGAGATC SV40 AP1
strong competitor SMS CRE only contains 5/8 match with this putative loose consensus. It should be borne in mind that such binding site comparisons are difficult to make, since there may be more than one critical contact motif for any given factor, thus different bases may contribute to efficient DNA binding on different sites. The DNA binding preferences of Un1 and Un2 should be revealed by a binding site selection experiment (Pollock and Treisman 1990); however, the performance of such a selection would first require identification and/or cloning of the polypeptide components of Un1 and Un2. As discussed above, a trinucleotide TGA is typically present in the recognition sites of bZIP DNA binding proteins, which are characterized by a 30 amino acid region rich in basic amino acid followed by a 30-40 amino acid leucine zipper motif. The leucine zipper mediates dimerization, juxtaposing two basic regions to form the DNA binding domain (reviewed in McKnight 1991). Since the DNA binding specificity of Un1/Un2 thus resembles that of the bZIP family factors we have carried out a supershift EMSA analysis with antisera against several bZIP factors (CREB, ATF-2, c-Jun, c-Fos, Fra, NF-IL6) available to us. None of these antisera could react with Un1 and Un2 (data not shown).

An interesting observation was made that the proteins in the most purified Un1/Un2 preparation (see below) could not form any detectable complexes on the -110/-12 probe (data not shown), which should contain three specific binding sites for them. This may indicate that the complex formation is sensitive to the exact conformation of the DNA, and that the conformation of the -110/-12 probe is unfavourable. Indeed, the conformation of this piece of DNA has been observed to be strongly bent (S.Goodbourn, pers. comm.).

4.3. Large-Scale Purification of Polypeptide Components in Un1 and Un2

4.3.1. Overview of Methods used to Purify Specific DNA Binding Proteins

To study the properties of sequence-specific transcription factors, it is often necessary to purify them to homogeneity. This would enable their biochemical characterization, facilitate the raising of antibodies, provide partial peptide sequences, and thus ultimately provide a means for cloning the genes encoding such factors. An alternative way to retrieve the cDNAs encoding DNA binding proteins is to screen prokaryotic expression libraries with radiolabelled DNA recognition sites as probes. However, this approach has some limitations: Those transcription factors that require posttranslational modifications of a eukaryotic type, or dimerization with a heterologous subunit, for effective DNA binding, cannot be cloned in such library screens using prokaryotic expression vectors. These
problems are not encountered, if an approach to purify the native forms of the proteins from cells that endogenously express them, is taken. A difficulty in obtaining homogenous preparations of regulatory transcription factors often derives from their low abundance. Furthermore, unstable proteins may not survive multistep purification procedures.

Sequence-specific DNA binding proteins can be purified by specific affinity chromatography with immobile resins or collectable beads that have proper DNA recognition sites attached to them. It has been observed by others (for example, Kadonaga and Tjian 1986) and us (data not shown) that partial purification and enrichment of transcription factors of low abundance by conventional separation methods is often necessary before performing specific affinity chromatography. Specific affinity chromatography using DNA recognition sites covalently coupled to Sepharose (Kadonaga and Tjian 1986) or latex particles (Kawaguchi et al 1989) with cyanogen bromide have been successfully used to purify low-abundance regulatory transcription factors. Magnetic solid phase technology (Gabrielsen et al 1989) used in our study provides a more rapid and powerful method for separating specific DNA-binding proteins from nonspecific contaminants.

4.3.2. Optimized Protocol to Purify Un1 and Un2

The optimized scheme for the purification of the polypeptide components of the Un1 and Un2 complexes is presented in figure 4.6., and includes two successive ion chromatography columns, followed by a specific affinity chromatography performed batchwise. The fractions collected throughout the purification were monitored for Un1/Un2 activity by EMSA analyses with the -108/-95 probe.

4.3.2.1. The First Step: Preparation of Nuclear Extracts

Nuclear extracts on a large scale (from 20 liters of HeLa spinner cells) were prepared as described for small scale extracts in Materials and Methods, but scaling the buffer volumes up appropriately.

4.3.2.2. The Second Step: BioRex Ion Exchange Chromatography

BioRex ion exchange chromatography was performed at 4°C. After a dialysis against buffer D the nuclear extract from HeLaS cells was applied to a BioRex70 (BioRad) column (40mg protein per ml of packed bed volume) equilibrated in buffer D. The flowthrough was collected, the column washed with two column volumes of buffer D, and step elution
Figure 4.6. The optimized procedure to purify polypeptides in the Un1 and Un2 complexes from large amounts of HeLaS cells.

For details of the procedure, see the text.
NUCLEAR EXTRACT

↓

BIO-REX 70

↓

0.3M KCl pool

DNA CELLULOSE

↓

0.3M KCl pool

STREPTAVIDIN-COATED MAGNETIC BEADS

+ 

BIOTINYLATED -108/-95 OLIGOS

final prep: 0.3M KCl eluate
performed with modified buffers D at KCl concentrations 0.3M, 0.6M, and 1.0M. The elution fraction volumes were 12ml. Both the protein concentrations and Un1/Un2 binding activities were assayed for the fractions. While there was virtually no Un1/Un2 activity in the flowthrough, and only trace amounts in the wash fractions, the bulk of the complexes eluted at 0.3M KCl (figure 4.7.). The two active fractions exhibiting Un1/Un2 binding were pooled to give the final volume of 24 ml, and dialyzed against buffer D overnight at 4°C.

4.3.2.3. The Third Step: DNA Cellulose Chromatography

The active BioRex fractions, which were pooled and dialyzed, were applied to a DNA cellulose column (10mg protein per ml of packed bed volume) equilibrated in buffer D. After thorough washing (three column volumes) with buffer D, proteins were step-eluted with buffer D variants containing 0.3M, 0.6M, and 1.0M KCl. The elution fraction volumes were 3ml. The Un1/Un2 components eluted at 0.3M KCl, while only slight amounts were present in flowthrough, wash fractions, and fractions eluted at higher salt concentration (figure 4.8). The four active fractions were pooled to give the final volume of 12ml and dialyzed overnight against buffer D at 4°C.

4.3.2.4. The Final Step: Specific Affinity Matrix

In the final step of purification the separation technique based on magnetic beads (Dynabeads™ M-280; monodisperse superparamagnetic polystyrene particles) was used. The beads were precoated with streptavidin protein. Specific double stranded DNA binding sites for Un1/Un2 carrying a biotin moiety were prepared by annealing an aliquot of single-stranded 5' end-biotinylated top strand (prepared on by K.Hobbs and I.Goldsmith, ICRF, by using biotin phosphoramidite on a DNA synthesizer) with an equal amount of the non-biotinylated lower strand. The biotin-streptavidin binding is extremely strong ($K_d \approx 10^{-15} \text{ M}$, Wilchek and Bayer 1988), enabling the efficient collection of the biotinylated oligonucleotides bound to specific DNA binding proteins.

During the optimization of the procedure it became apparent that it was necessary to let the reaction between the specific DNA binding sites and the enriched Un1/Un2 preparations from the DNA cellulose step to take place first, before the contact between the oligonucleotides and magnetic beads. This may be because of the steric hindrance between the beads and Un1/Un2 proteins, or because the binding of the oligonucleotides to the beads renders the conformation of the binding site unfavourable for the formation of the protein-DNA contacts. Furthermore, we were only successful when monomeric, but not
Figure 4.7. Un1/Un2 purification: BioRex column chromatography.

A protein concentration curve (A.) and an EMSA analysis on the -108/-95 probe (B.) for the fractions derived from the BioRex ion chromatography.

A. Protein concentrations for the fractions collected during the column purification were assayed with the Bradford reagent.

B. Equal relative volumes of the selected fractions (the fractions containing the most protein at each stage of the column purification, as determined in A) were assayed for Un1 and Un2 DNA binding activity.
A.

Protein concentration

Flowthrough +

0.1M KCl wash

0.3M KCl

0.6M KCl

1.0M KCl

B.

Load [KCl]

FT 0.1M 1.0M

Oct1

Un1

Un2
Figure 4.8. Un1/Un2 purification: DNA cellulose column chromatography.

A protein concentration curve (A.) and an EMSA analysis on the -108/-95 probe (B.) for the fractions derived from the DNA cellulose chromatography.

A. Protein concentrations for the fractions collected during the column purification were assayed with the Bradford reagent.

B. Equal relative volumes of the selected fractions (the fractions containing the most protein at each stage of the column purification, as determined in A) were assayed for Un1 and Un2 DNA binding activity.
concatamerized, binding sites were used; again, this may indicate that the covalently multimerized binding sites assume an unfavourable conformation, which excludes the binding of Un1 and Un2. We have not verified this hypothesis by performing a comprehensive EMSA analysis with -108/-95 oligomers of various lengths.

The binding of the oligonucleotides (40μg double-stranded -108/-95 oligonucleotide per a partially purified preparation starting from 20 litres of HeLa cells) with the proteins was allowed to proceed for 30 minutes on a rotating wheel in buffer D at room temperature; all the subsequent steps were performed at 4°C. The addition of nonspecific protein (bovine serum albumin, 20μg/ml) into the binding reaction was found to be necessary to enhance the binding of the specific proteins. The binding of the protein-DNA complexes with an excess of the magnetic beads [0.5ml of the bead suspension (3.3x10^8 beads) per a partially purified preparation starting from 20 litres of HeLa cells; the beads prewashed twice with buffer D] was allowed to proceed for one hour in a rotating wheel, after which the beads were collected with a magnet particle concentrator (Dynal MPC). During subsequent washes (five) the buffer volume was decreased to 1ml to concentrate the specific proteins. A large excess (50μg/ml) of nonspecific DNA (sonicated salmon sperm DNA) was added during the second wash to reduce the binding of remaining non-specific DNA binding proteins. The compositions of the elution buffers were as buffer D, except that the KCl concentrations were increased stepwise from 0.1M to 0.3M, 0.6M, and 1.0M. Two elutions with each of the buffers were performed, each in 1ml volume.

While a notable fraction of the total partially purified Un1/Un2 proteins remained in the unbound fraction after the magnetic separation, approximately 70% of the Un1/Un2 complexes became associated with the magnetic beads, and could be eluted off the beads with 0.3M KCl (figure 4.9.)

4.3.3. The Analysis of the Result of Un1/Un2 Purification

A description of a typical purification attempt from 20 litres of HeLaS cells is presented in figure 4.10. It should be emphasized that the estimations of enrichment at various stages of the purification are only crude approximations. Firstly, EMSA does not accurately reflect the amount of the specific protein in the preparation, but is rather prone to variations in the exact salt concentration and successive alterations in a protein environment of the sample. Secondly, in the last step, the total protein mass of a purified preparation is too low to be reliably determined by the Bradford assay.
Figure 4.9. Un1/Un2 purification: Specific affinity chromatography.

An EMSA analysis on the -108/-95 probe for the fractions derived from the specific affinity chromatography performed by using magnetic beads. Equal relative volumes of the fractions were assayed for Un1 and Un2 DNA binding activity. Two independent purification efforts are shown. The gel on the left is from the purification effort, for which the earlier steps are presented in figures 4.7 and 4.8.
unbound
2x washes
0.1M KCI
4x washes
0.2M KCI
2x elution
0.3M KCI
2x elution
0.6M KCI
2x elution
1.0M KCI

unbound
0.1M KCI
0.2M KCI
0.3M KCI
0.6M KCI
Figure 4.10. Un1/Un2 purification: A summary.

A summary of protein amounts and estimated degrees of enrichment of Un1 and Un2 complexes from a typical purification effort starting from 20 liters of HeLaS cells.
<table>
<thead>
<tr>
<th>Sample</th>
<th>Protein Mass</th>
<th>Estimated Enrichment vs. WCE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nuclear extract</td>
<td>800 mg</td>
<td>2 x</td>
</tr>
<tr>
<td>BioRex 0.3M KCl pool</td>
<td>117 mg</td>
<td>10 x</td>
</tr>
<tr>
<td>DNA cellulose 0.3M pool</td>
<td>10.5 mg</td>
<td>50 x</td>
</tr>
<tr>
<td>SA beads 0.3M eluate</td>
<td>130 μg</td>
<td>&gt;2000 x</td>
</tr>
</tbody>
</table>
To analyze the success of a purification attempt, as well as relative abundances of proteins in active fractions from different stages of purification, an SDS-PAGE analysis and Coomassie staining of gels were performed. While some persistent contaminants clearly copurify with Un1 and Un2 all the way to the last step, some polypeptides are clearly enriched along the purification process so that they are only visible in the final affinity purified fraction (figure 4.11). One such polypeptide is just under 46kD in size, while another is around 67kD. The pattern including these novel proteins has been reproducibly seen in several affinity purification attempts, and we believe that they represent good candidates for specific -108/-95 binding activities.

A SouthWestern analysis is a protein-DNA interaction assay, which may allow the molecular size of a specific DNA binding protein to be determined. In a SouthWestern analysis of HeLa cell nuclear extracts, an Un1/Un2 binding site probe -108/-95 produces a complex pattern of rather faint bands (figure 4.12.), which does not allow us to determine the molecular masses of specific proteins interacting with the -108/-95 probe. We tried to perform a SouthWestern analysis for a sample from the purified Un1/Un2 fraction, hoping that by eliminating the reaction with the bulk of contaminating nonspecific proteins, we could be able to verify the molecular masses of the DNA binding activities equal to the new protein species observed upon the Coomassie staining of a sample from the purified fraction. However, no signal was obtained with the purified sample (figure 4.12.). We see this as an indication that Un1 and Un2 consist of more than one polypeptide, at least two of which are required for efficient DNA binding. This idea is further supported by the fact that we had been previously unable to isolate any cDNA clones from a placental λgt11 or a HeLa cell λZAP expression library in a screen utilizing a radioactive DNA binding site probe (data not shown).

Even before the large-scale purification of Un1/Un2, we attempted to determine the molecular masses of their protein components by separating HeLa nuclear extracts on an SDS-PAGE, followed by excision of different size fractions from the gel. After elution and renaturation, no Un1/Un2 binding activity could be detected in EMSA in any size fractions, or in any combination of the size fractions. This is probably not due to unsuccessful renaturation of individual polypeptide components in the complexes, since we can recover some Un1/Un2 binding activity, when we renature a nuclear extract sample after subjecting it to a strong denaturant guanidinium hydrochloride (data not shown). If Un1 and Un2 indeed consist of more than one polypeptide, at least two of which are required for efficient DNA binding, the concentration of these subunits may be too low for the complex to be formed after the gel elution, or complex formation may only take place in the presence of other cellular proteins or cofactors. If a polypeptide in the Un1/Un2 complexes requires a
An SDS-PAGE analysis to present the protein content of selected samples at different stages of the purification. Shown is an example from one purification effort, but virtually identical protein patterns were reproducibly observed. The separated proteins were visualized by Coomassie staining.

The protein samples:
- crude nuclear extract: 10μg protein
- BioRex 0.3M KCl eluate pool: 10μg protein
- DNA cellulose 0.3M KCl eluate pool: 10μg protein
- 0.3M and 0.6M eluates from the specific affinity purification with streptavidin-coated magnetic beads: 1:40 (25μl) of the total volumes (1ml)
size markers

crude NE

BioRex 0.3M pool

DNA cellulose 0.3M pool

SA beads 0.3M eluate

SA beads 0.6M eluate
Figure 4.12. A SouthWestern analysis with the concatamerized -108/-95 probe.

The samples (10µg of a crude nuclear extract prepared from uninduced HeLa cells, 10µg of a crude nuclear extract from primed/dsRNA-induced/CHX-treated HeLa cells, 10µg of a crude nuclear extract from primed/Sendai-induced/CHX-treated HeLa cells, 25µl of the enriched affinity purified Un1/Un2 preparation) were first separated on an SDS-PAGE. After the protein transfer, the membrane was probed with radioactively labelled (as described in section 2.1.3.4.) -108/-95 oligonucleotides, that had been annealed and ligated to form concatamers.
protein markers
Un1/Un2 pur.
un/un
pr/dsRNA/CHX
pr/Sen/CHX
cofactor for proper folding or for efficient DNA binding, such a cofactor would probably be lost during an SDS-PAGE. We have tried adding zinc ions to the renaturation solution, since proteins of a zinc finger class have been shown to require coordinated zinc ions for the DNA binding-competent conformation (Coleman 1992), but the presence of this bivalent cation made no difference (data not shown). Also, varying the concentration of the reducing agent dithiotreitol in the EMSA samples fails to affect the DNA binding of the Un1 and Un2 complexes (data not shown), suggesting that the inability to recover the binding activity from the eluted gel fragments is not caused from critical alterations in their redox state.

Even if we have failed to provide further evidence that the novel polypeptides in our purified fractions represent components of the complexes Un1 and Un2, we believe that the reproducibility of the purification pattern justifies an attempt to obtain polyclonal antisera against these polypeptides by injecting the polypeptides eluted from the gels into rabbits, or by subcutaneously implanting excised gel slices into them, to trigger a specific immune response. The antibodies obtained would be first tested for specificity in EMSA, and if proved positive, used to screen expression libraries to retrieve the cDNAs encoding the polypeptides. Alternatively, the specific polypeptides eluted from a gel could be subjected to protein microsequencing, which may allow either their identification as already identified factors, or screening cDNA libraries by hybridization with a pool of degenerate oligonucleotide probes. After obtaining the cDNA clones, these factors could be subjected to biochemical and functional analyses, such as described for Oct-1 (chapter 3).

4.4. DNA Binding of Un1 and Un2 Is Affected by Their Phosphorylation Status

As discussed above (sections 1.1.2.5. and 3.6.), inducible phosphorylation or dephosphorylation of transcription factors is an important mechanism of signal-dependent gene regulation in eukaryotic cells. For c-Jun, treatment of cells with the phorbol ester TPA in the presence of activated Ha-Ras leads to rapid dephosphorylation of sites next to the DNA binding region, resulting in increased DNA binding activity. Conversely, CKII (casein kinase II) phosphorylates c-Jun on sites that inhibit DNA binding, and microinjection of peptides that inhibit CKII activates AP-1 activity in living cells (Lin et al 1992). The DNA binding activity of c-Myb is also inhibited by CKII; this inhibition can be relieved by protein phosphatase type 2A (Lüscher et al 1990). The myogenic program can similarly be modulated by inhibition of DNA binding activity of myogenic HLH-transcription factors by fibroblast growth factor-induced and protein kinase C-mediated phosphorylation of their DNA binding domains (Li et al 1992).
We have investigated whether the phosphorylation of Un1 and Un2 could affect their DNA binding affinity. Both crude nuclear extract aliquots from HeLa cells, and samples of the purified preparation (0.3M eluate from the magnetic bead step) were treated by varying concentrations of potato acid phosphatase (PAP) in the presence or absence of a mixture of phosphatase inhibitors, prior to subjecting them to standard EMSA on the -108/-95 probe. Two different concentrations of PAP (stock 60U/ml; Boehringer Mannheim) were used: "low" (0.2µl of 1:10 dilution) and "high" (0.2µl of undiluted enzyme). Prior to pipetting, the PAP samples were centrifuged briefly to remove the debris present in the enzyme preparation, and dialyzed against buffer D on Millipore 0.025µM filters to adjust the ion concentration to those used in standard EMSAs. A 4µl aliquot of the protein phosphatase inhibitor mix (5x concentrate) was included in 20µl EMSA samples where indicated. The concentrated phosphatase mixture contained 1:200 okadaic acid, 10mM NaPPi, 50mM sodium molybdate, 10mM sodium vanadate and 500mM sodium fluoride.

In the case of crude nuclear extracts, the treatment with either PAP concentration did not affect the DNA binding of Un1 and Un2 (figure 4.13.). However, the application of the inhibitor mix strongly reduced binding, both in the presence and absence of PAP. On the other hand, PAP treatment clearly enhanced the binding of purified Un1 and Un2, and at the high PAP concentration, when the DNA affinity was strongly increased, the protein phosphatase inhibitor cocktail could not reverse the effect. The result implies that the removal of phosphates from Un1 and Un2 enhances their DNA binding affinity. If one only examines the crude nuclear extract samples, one might suspect the effect of inhibitors to be nonspecific, and only result from an alteration in conditions such as ion concentrations. However, the fact that the same amount of inhibitors could not affect the enhanced binding by affinity purified Un1/Un2 treated with higher concentration of PAP strongly suggests that this is not the case. Rather it seems that the crude protein preparation contains endogenous protein phosphatases, which continuously dephosphorylate Un1 and Un2, if not artificially inhibited. Such phosphatases may be partially or completely eliminated during the multistep purification process, thus explaining the nonresponsiveness of purified "high" PAP-treated samples to inhibitors. Why then cannot PAP enhance the DNA binding of Un1/Un2 in crude extracts, if they can do so in the purified preparation? It is possible that the crude preparation contains so many heterologous substrates for the phosphatase activity, that the effect on Un1 and Un2 becomes titrated down. Another, not mutually exclusive, explanation for the enhanced PAP-response seen with purified Un1/Un2 is that these polypeptides become copurified with a specific kinase. The dependence of the application of PAP on the enzymatic activity has been verified by the
Figure 4.13. The DNA binding of the Un1 and Un2 complexes can be modulated by their phosphorylation status.

An EMSA analysis with either nuclear extracts prepared from uninduced HeLa cells, or 1:500 (2μl) of the affinity purified Un1/Un2 preparation, on the -108/-95 probe derived from the NRD II region of the IFN-β promoter. In addition "low" or "high" amounts of potato acid phosphatase ("PAP") and/or an aliquot of protein phosphatase inhibitors were added to the EMSA samples together with the carrier DNA.
crude NE

SA beads 0.3M

low PAP
low PAP + inh. mix
high PAP
high PAP + inh. mix
inh. mix alone
inability to achieve any effect on the DNA binding of Un1/Un2 by the boiled PAP sample (data not shown).

While the phosphatase experiment clearly demonstrates that the DNA affinity of Un1 and Un2 can be manipulated by phosphorylation and/or dephosphorylation events, it does not prove that such regulation takes place within cells, or that a specific phosphorylation is responsible for the decrease in the DNA binding activity upon induction. Indeed, we have failed to demonstrate the reversal by PAP treatment of the dsRNA-induced decrease in Un1 and Un2 binding in EMSA with crude nuclear extracts prepared from induced cells (data not shown). However, as discussed, this may be simply due to the presence of the excess of alternative substrates for the phosphatase in crude nuclear extracts. A similar analysis on the purified Un1/Un2 from induced cells has not been feasible because of the impossibility of purifying the polypeptides with the optimized procedure that relies on their DNA binding affinity, since induction itself renders them DNA binding defective. The clarification of this issue awaits the molecular cloning and recombinant production of the Un1/Un2 polypeptides.
Chapter 5: Analysis of the Positive Regulatory Domain IV

5.1. The Positive Regulatory Domain IV Is Uninducible

Sequences located between -104 and -87 comprise the PRD IV element, and are required for efficient induction in certain cell types, such as human HeLa (King and Goodbourn 1994) and mouse L929 cells (Fujita et al 1985), but not in others, such as mouse C127 cells (Zinn et al 1983). PRD IV contains a binding site for the ATF/CREB family of bZip transcription factors, and it has been suggested that ATF-2 mediates the virus-inducibility through PRD IV, either as homodimers or heterodimers with c-Jun (Du and Maniatis 1992, Du et al 1993). Accordingly, the overexpression of either ATF-2 or c-Jun antisense RNA in transfected HeLa cells can decrease the inducibility of the intact human IFN-β promoter. Base substitutions that interfere with in vitro binding of the ATF factors to PRD IV decrease the level of virus induction in mouse L929 cells, and multiple copies of PRD IV have been reported to confer inducibility by both virus and cAMP treatment on a cotransfected heterologous promoter. While the entire PRD IV element appears to be required for viral induction, the flanking 5' and 3' A+T rich regions are dispensable for stimulation by cAMP. This is consistent with the suggestion that these A+T rich sequences interact with HMGI(Y) proteins, and that the HMGI(Y)-DNA interactions are necessary for induction by viruses.

During the course of this work, we also have investigated the properties of the DNA element referred to as PRD IV. The generation of the reporter plasmid p(PRDIV)3tkΔ(-39)lucifer, which contains three PRD IV elements in tandem fused to the tk TATA box and luciferase cassette, is described in King and Goodbourn (1994). Using TFM transfection method, p(PRDIV)3tkΔ(-39) was cotransfected into L929 cells together with pBLCAT2, a control expression vector to monitor transfection efficiencies. As a positive control for induction by dsRNA and Sendai virus, the reporter plasmid pIF(-210/+72)lucifer containing the intact IFN-β promoter was used. The reporter plasmid tkΔ(-39)lucifer was cotransfected as a negative non-inducible control. To investigate the response of the test promoters to various inducing signals, the cells were treated with dsRNA, Sendai virus, 1mM db-cAMP (di(3′-5′)cAMP), or cotransfected with an expression plasmid pMtCa, which contains the mouse cDNA encoding the catalytic subunit of the protein kinase A under the control of metallothionein promoter (Mellon et al 1989).
As expected, the negative control promoter, the tk TATA box, did not respond to either dsRNA treatment or overexpression of the catalytic subunit of PKA (figure 5.1.). Also, in accord with the previous studies, the intact IFN-β promoter was clearly inducible by dsRNA and more potently by Sendai virus; in contrast, overexpression of the PKA catalytic subunit or treatment with the PKA inducer cAMP did not significantly alter the basal transcription level of the IFN-β promoter. In contrast with the results of Maniatis and coworkers, expression from the multimerized PRD IV element was not modulated by any of the inducers. Similar conclusions were drawn about the lack of inducibility of the PRD IV element by dsRNA in transiently cotransfected HeLa cells (figure 5.2.).

The lack of inducibility of PRD IV multimers is rather surprising, since in L929 and HeLa cells the efficient inducibility of the intact IFN-β promoter has been shown to require sequences upstream of -91, which do encompass the PRD IV element (Du and Maniatis 1992, King and Goodbourn 1994). We do not understand the reasons for the striking difference between our results and those of Du et al (1992), who have reported PRD IV multimers to be inducible by both virus and cAMP. It is possible that there are some profound differences between the sublines of particular established cell lines; the clarification of this would require the inducibility studies to be done in parallel with the exact HeLa and L929 sublines used in different laboratories. It should be noted that similar observations to ours, on the uninducibility of the multimerized PRD IV region, have been reported when using the mouse embryonal carcinoma cells as recipients (Ellis and Goodbourn 1994).

Du et al (1993) have reported that induction of L929 cells with dsRNA results in the formation of two inducible protein complexes on the PRD IV probes: One of the complexes contains an ATF-2 homodimer and HMGI(Y) while the other one consists of an ATF-2-c-Jun heterodimer together with HMGI(Y). In contrast, we cannot detect any inducible PRD IV activities in EMSA analysis, using an identical probe to theirs. An EMSA with HeLa cell nuclear extracts is shown in figure 4.3. (probe -104/-91); identical results were obtained using nuclear extracts prepared from L929 cells (data not shown). We note, however, that our studies (section 4.2.) on the binding specificity of the Un1 and Un2 complexes suggested a limited similarity to those of bZIP transcription factors - a protein family which ATF-2 and c-Jun also belong to. We see no complex corresponding to small HMGI(Y) proteins in extracts from either HeLa or L929 cells, whether uninduced or induced; Du et al could observe a high mobility complex in both uninduced and induced cells. The only specific complexes in both cell types that form on the PRD IV probe in our hands are Un1/Un2 and very weakly Oct-1; the binding affinities of all these decrease upon
Figure 5.1. A multimerized PRD IV element is uninducible.

A transient transfection analysis in L929 cells, using the Transfectam reagent to investigate the inducibility of the multimerized PRD IV region [contained in the plasmid p(PRDIV)3tkΔ(-39)lucter, “PRD IV” in the figure]. The luciferase test plasmids (1µg of each one transfected) are indicated below the columns. In all the transfections, the luciferase test plasmids were cotransfected with 1µg of the internal control plasmid pBLCAT2, to normalize to the variations in transfection efficiencies. The relative expression levels were obtained by dividing the luciferase activities by CAT activities. Where indicated (“cPKA”), 0.5µg of the plasmid encoding the catalytic subunit of PKA, pMtCa, was also cotransfected. Furthermore, where indicated below the columns, the transfected cells were induced with dsRNA, Sendai, or dibuturyl-cAMP. Sendai and dsRNA-inductions were as described in Materials and Methods, whereas cAMP-inductions were performed by supplementing the medium with 1mM db-cAMP for four hours, prior to determining the reporter enzyme activities.
RELATIVE EXPRESSION LEVEL

- 1
dsRNA 0.7
cPKA 0.6
- 3.7
dsRNA 3.7
SENDAI 5.7
cPKA 3.3
cAMP 2.6
- 3.9
dsRNA 2.7
SENDAI 430
cPKA 2
cAMP 2.6
A transient transfection analysis in HeLa cells, using the Transfectam reagent, to investigate the dsRNA-inducibility of the PRD IV region. The luciferase test plasmids (1µg of each one transfected) are described above the columns. In all the transfections, the luciferase test plasmids were cotransfected with 1µg of the internal control plasmid pBLCAT2, to normalize to the variations in transfection efficiencies. The relative expression levels were obtained by dividing the luciferase activities by CAT activities.

"un" = uninduced HeLa cells
"dsRNA" = primed and dsRNA-induced HeLa cells
induction, and polyclonal antisera raised against ATF-2 does not react with any of them (data not shown). As discussed in section 1.2.2.4.5., Maniatis and coworkers have suggested an essential role for the HMG-I(Y) protein in the induction of the IFN-β promoter. In their system, they can observe substantial inhibition of viral induction by overexpression of antisense HMG-I(Y) RNA in cells. We have also performed cotransfections using expression vectors producing either HMG-I(Y) sense or antisense RNA in both L929 and HeLa cells, and observed no effect whatsoever on the viral or dsRNA induction of the IFN-β promoter (data not shown). However, our conclusions remain unverified, since we have not managed to obtain efficient antisera against HMG-I(Y), with which we could have shown that our expression constructs and antisense oligonucleotides are indeed effective in modulating the intracellular HMG-I(Y) protein levels. It should be noted that in our experiments, the expression of the HMG-I(Y) RNAs was driven by the MLV promoter, whereas Maniatis and coworkers used CMV promoter-driven plasmids. It is thus possible that the difference in the expression levels of HMG-I(Y) RNAs explains the difference in our results.

5.2. The Basal Transcription Supported by the Multimerized PRD IV Element

We have made interesting further observations about the basal level of activity of the multimerized PRD IV element. While PRD IV construct is not inducible in HeLa cells, it has a very high constitutive activity when transiently transfected into these cells with the TFM reagent (figure 5.2.). Strikingly, in calcium phosphate transfections of HeLa cells, the activity of PRD IV remains low, compared with the relative expression levels supported by the intact IFN-β promoter or the tk TATA box (figure 5.3., compared with figure 5.2.). The most likely explanation is that the PRD IV multimer can indeed interact with an activator protein, which is already present and functional (at least on the multimerized element) in uninduced HeLa cells. We speculate that the effect cannot be seen when DNA is introduced into cells with the calcium phosphate method, because this causes larger intake of plasmids per transfected cell than TFM method (see Appendix I). Thus, the activator protein becomes titrated out by the excess DNA, and the enhancement of reporter gene transcription consequently quenched. As discussed in connection with our attempted functional analysis of Oct-1, this further emphasizes how susceptible transient transfection analyses are to technical variations that may not be informative in the context of transcriptional regulation under physiological conditions.

The nature of the constitutive activator of PRD IV remains unknown. In EMSA analysis, the pattern of binding activities that can specifically form on monomeric PRD IV region
Figure 5.3. The basal activity of the PRD IV multimer is low when transfected into HeLa cells by the calcium phosphate coprecipitation method.

A transient transfection analysis in HeLa cells, using the calcium phosphate coprecipitation method. The luciferase test plasmids (5μg of each one transfected) are described below the columns. In all the transfections, the luciferase test plasmids were cotransfected with 5μg of the internal control plasmid pBLCAT2, to normalize to the variations in transfection efficiencies. The relative expression levels were obtained by dividing the luciferase activities by CAT activities.
contains Un1 and Un2 together with only very weakly binding Oct-1 (see figure 4.3., probe -104/-91). Above, we have proposed a repressive role for all these factors in the context of the intact IFN-β promoter; it is, however, conceivable that their effect could be dependent on the promoter context. Even when one takes this into account, Oct-1 is a very unlikely candidate for activating transcription through multimerized PRD IV, since, the introduction into HeLa cells of the antisense phosphorothioate oligonucleotides complementary to the regions in the Oct-1 cDNA spanning the two possible initiation codons (see section 3.9.2.2.) actually strongly and specifically enhances the expression of a multimeric PRD IV element (figure 5.4.), suggesting that Oct-1 can repress, rather than activate, the multimeric PRD IV element. The strength of the effect is rather surprising since monomeric PRD IV only very weakly binds to Oct-1 in vitro. We note that the junctions between the three individual PRD IV elements do not create a motif resembling a consensus octamer motif, and do not thus believe that such sequences serve as effective binding sites for Oct-1. It is possible that even if the binding of Oct-1 to a monomeric PRD IV element is weak, the tandem arrangement of three such elements stabilizes the DNA binding. In any case, it appears that Oct-1 can indeed function as a transcriptional repressor, at least on the synthetic promoter consisting of the tk TATA box and three PRD IV elements. Alternatively, we cannot exclude that the effect by depletion of endogenous Oct-1 proteins takes place indirectly; this would require Oct-1 to be involved in either induction of a PRD IV-specific repressor, or repression of a PRD IV-specific activator protein.

It is interesting to note that mouse L929 cells do not appear to contain the activity responsible for the strong constitutive transcription, since the mRNA synthesis directed by a PRD IV construct is low in these cells, whether transfected by the TFM (figure 5.1.) or calcium phosphate method (data not shown). This is somewhat puzzling, since the same array of binding activities assembles on monomeric PRD IV probe, in EMSA analyses with nuclear extracts prepared from either uninduced L929 or HeLa cells. It is possible that multimeric arrangement favors the binding of cell type-specific proteins, which obviously would not be revealed by EMSA analyses using a PRD IV monomer probe. Also, DNA binding properties do not necessarily imply equal functional activities. Different cell types may allow the proteins to be modified differentially, or contain different cofactors, which may lead to differences in the actions of homologous DNA binding factors.
Figure 5.4. Oct-1 is a repressor of a multimerized PRD IV element.

A transient transfection analysis in HeLa cells, using the Transfectam reagent, to investigate the effect of the antisense Oct-1 oligonucleotides on the multimerized PRD IV element. Cells were transfected with either the tkΔ(-39)lucifer or p(PRDIV)3tkΔ(-39)lucifer luciferase reporter plasmids (1 μg each). In all transfections, 1 μg of the pBLCAT2 plasmid was cotransfected as a control for transfection efficiencies. Prior to reporter enzyme assays, the transfected cells were incubated for 2 days in the presence of 10 μM of the phosphorothioate oligonucleotides (S-sense; AS1-antisense 1; AS2-antisense 2; described in section 3.9.2.2.).

To obtain the values in the figure, the relative expression levels were first determined by dividing the individual luciferase activities by the CAT activities. After this, to normalize to the promoter-independent effect of Oct-1 depletion on the luciferase constructs (see figure 3.23.B.), the relative expression levels of all three tkΔ(-39)lucifer transfections were given the value 1, and the relative expression levels of the PRD IV plasmid transfections corrected accordingly, so that the fold differences between all the relative expression levels affected by a particular oligonucleotide are maintained.

The fold activations of the multimerized PRD IV element by antisense oligonucleotides (compared with the sense oligonucleotides) are indicated above the columns.
RELATIVE EXPRESSION LEVEL
[NORMALIZED TO tkΔ(-39)Lucer]
Chapter 6: Protein Kinase A Can Interfere with the Induction Process

The signal transduction pathway in response to dsRNA is poorly elucidated, and the possible second messengers and kinases involved have not been confirmed. The evidence that PKR, a protein kinase activated by dsRNA, could be involved in the induction process has been vigorously sought after. It has indeed been shown that 2-aminopurine, an inhibitor that suppresses the action of PKR, can inhibit IFN-β induction in some cell types (Marcus and Sekellick 1988, Zinn et al. 1988, Daigneault et al. 1992); however, 2-aminopurine is not exclusively specific for PKR, but also inhibits other kinases. In agreement with the proposed role for PKR, it has been shown to activate in vitro one transcription factor, NF-κB, important for the regulation of the IFN-β promoter (Kumar et al. 1994). Also, the selective ablation of the PKR mRNAs in HeLa cells inhibits the dsRNA-mediated activation of NF-κB (Maran et al. 1994). A confirmation that PKR is a participant in the signalling pathway that leads to the IFN-β gene activation awaits further studies.

Could another well characterized kinase, protein kinase A (PKA), also have a role along the signal transduction pathways converging at the IFN-β gene induction? Several observations prompted us to investigate this. First, as shown in section 3.7., PKA can phosphorylate the DNA binding domain of a putative repressor protein Oct-1. Second, as discussed in section 1.2.2.4.4. and chapter 5, the PRD IV element has been suggested to serve as a binding site for PKA-inducible transcription factors. Third, a PRD II binding factor NF-κB can be activated in vitro by PKA (Shirakawa and Mizel 1989). Fourth, in previous studies it has been shown that mismatched dsRNA species activate adenylate cyclase, thus increasing the level of cellular cAMP, a second messenger known to activate protein kinase A (Hubbell et al. 1991).

PKA is known to influence regulated transcription by virtue of its phosphorylating activity. The best characterized of such effects is the direct phosphorylation of the cyclic AMP response element binding protein (CREB) on serine 133 in the activation domain (Gonzalez and Montminy 1989). This phosphorylation event leads to the enhancement of the activation potential of CREB. Furthermore, the DNA binding activity of CREB to a subset of CREs can also be increased via direct phosphorylation by PKA (Nichols et al. 1992). It is interesting to note, that while many other factors of the CREB/ATF family interact with CREs, some of them, such as ATF-2, are not capable of responding to PKA activation.
CREB is not the only transcription factor whose properties are modulated by PKA. The nuclear localization of the bZIP transcription factor NF-IL6 is also affected by its phosphorylation in response to forskolin treatment; the change in localization accounts for the inducible binding of NF-IL6 to the serum response element of the \( c-fos \) gene (Metz and Ziff 1991). In addition, nuclear accumulation of c-Fos requires continual stimulation by serum growth factors in a cAMP-dependent manner (Roux et al 1990). Positive correlations have also been suggested between PKA activity and active nuclear import of SRF (Gauthier-Rouvière et al 1995), c-Rel (Mosialos et al 1991) and MyoD (Vandromme et al 1994). However, in the case of SRF and MyoD, it has been shown that direct phosphorylation of these factors by PKA is not required for the effect. In fact, the nuclear transport of both SV40-NLS-conjugated (NLS=nuclear localization signal) heterologous proteins and cyclin A can be impaired through inhibition of PKA activity (Gauthier-Rouvière 1995), suggesting a more general role for PKA in the control of nuclear translocation mechanisms. In this respect, it is interesting to note that two of the identified NLS-binding proteins (NBP70 and Nopp140), which allow transport through nuclear pore complexes in an ATP-dependent manner require phosphorylation \textit{in vivo} for their interaction with NLSs (Meier and Blobel 1992, Stochaj and Silver 1992).

We have investigated the effect of transient overexpression of the catalytic subunit of PKA on the induction process. The pMtCα plasmid (Mellon et al 1989) was used to cotransfect cells to direct the metallothioninein promoter-driven overexpression of the catalytic subunit of PKA. The reporter plasmids carrying the test promoters were cotransfected into murine L929 cells together with the expression plasmid pMtCα and the internal control plasmid pBLCAT2, by the TFM method. First, the effect of the overexpression of the catalytic subunit of PKA on the full-length IFN-β promoter fused to a luciferase cassette [pIF(-210/+72)lucter] was investigated. As shown in figure 6.1., PKA had a strong inhibitory effect on the Sendai virus-induced expression level of the IFN-β promoter in L929 cells, while the effect on the basal level of transcription was only minor. High levels of the catalytic subunit of PKA had no significant effect either on the control construct tkΔ(-39)lucter or on the normalization control pBLCAT2, confirming that the PKA response is a true property of the induced IFN-β promoter.

Next, we attempted to map the inhibitory effect of the PKA catalytic subunit to a particular virus-inducible region in the IFN-β promoter (figure 6.2.). The 5' deletions extending to -116 and -91, as well as synthetic promoters in which multimers of the individual PRDs, PRD I, PRD II and PRD IV were the only IFN-β-specific elements upstream of the tk TATA box, were tested as above. All of these promoter variants were fused to the
Figure 6.1. Overexpression of the catalytic subunit of PKA inhibits IFN-β induction by Sendai virus in L929 cells.

A transient transfection analysis in L929 cells, using the Transfectam reagent, to investigate the effect of overexpression of the catalytic subunit of PKA on the pIF(-210/+72) promoter in L929 cells. The luciferase test plasmids (1μg of each one transfected) are described below the columns. In all the transfections, the luciferase test plasmids were cotransfected with 1μg of the internal control plasmid pBLCAT2, to normalize to the variations in transfection efficiencies. The relative expression levels were obtained by dividing the luciferase activities by CAT activities. Where indicated, 0.5μg of the plasmid encoding the catalytic subunit of PKA, pMtCa, was also cotransfected. Furthermore, where indicated, the L929 cells were induced with Sendai virus prior to determining the reporter enzyme activities. The fold inhibitions of the induced expression levels from the luciferase expression constructs are indicated above the columns.
Figure 6.2. The inhibition of induction of the IFN-β promoter by overexpression of the catalytic subunit of PKA cannot be mapped to a specific promoter element.

A transient transfection analysis in L929 cells, using the Transfectam reagent. The luciferase test plasmids (1μg of each one transfected) are described below the columns. In all the transfections, the luciferase test plasmids were cotransfected with 1μg of the internal control plasmid pBLCAT2, to normalize to the variations in transfection efficiencies. The relative expression levels were obtained by dividing the luciferase activities by CAT activities. Where indicated, 0.5μg of the plasmid encoding the catalytic subunit of PKA, pMtCa, was also cotransfected. Furthermore, where indicated, the L929 cells were induced with Sendai virus prior to determining the reporter enzyme activities.

The plasmid p(PRDI)5tkΔ(-39)lucdc ("PRDI") contains five copies of the PRD I element fused to a tk TATA box and to the luciferase cassette (described in Visvanathan and Goodbourn 1989).

The plasmid p(PRDIV)3tkΔ(-39)lucdc ("PRDIV") contains three copies of the PRD IV element fused to the tk TATA box and to the luciferase cassette (described in King and Goodbourn 1994).

The pIF(-x/-k72)lucdc plasmids contain the IFN-β promoter sequences downstream from the position "x" linked to the luciferase cassette. See the legend to figure 3.28. for further details.
luciferase reporter gene. In chapter 5, the overexpression of the catalytic subunit of PKA was already shown to have no effect on the multimerized PRD IV element; similar observations were repeated in this experiment. On the other hand, the Sendai-induced transcription levels of all the virus-inducible promoter variants were substantially inhibited by high levels of intracellular PKA.

That the effect of modulation of intracellular levels of PKA cannot be mapped to any particular sequence element implies that PKA may not directly affect the DNA binding or transcriptional potency of any single transcription factor critical in induction. The pleiotropic nature of the interference by overexpressing exogenous PKA in L929 cells may suggest that a single upstream factor in the induction process is affected. As discussed earlier, PKA has been generally implicated in the cytonuclear translocation of proteins; the effect we see by modulation of PKA activity may result from interference of nuclear accumulation of one such upstream factor, or even of the whole array of individual transcription factors required for the induction process. We have not yet investigated the possible correlations between the inhibition of induction by PKA and the DNA binding of individual transcription factors.

One should pay particular attention to the fact that such inducible promoter variants that lack PRD II (the PRD I multimer) or PRD IV [pIF(-91/+72), as well as the PRD I and PRD II multimers] elements remain responsive to the inhibition by PKA, since earlier experiments have circumstantially implied these regulatory motifs as potential targets for PKA action. The PRD II binding activity NF-κB has been shown to be activated in vitro by PKA (Shirakawa and Mizel 1989), as well as by the IFN-β inducers (see section 1.2.2.4.3.). On the other hand, PRD IV has been suggested to serve as a binding site for certain PKA-inducible transcription factors.

Besides overexpressing the catalytic subunit of PKA, we also attempted to investigate the effect of inhibition of the endogenous PKA in L929 cells with a potent and selective PKA inhibitor H89. Cotransfections of cells were performed as above, but in addition, the transfected cells were treated with 30μM of H89 or an equal volume of DMSO (H89 solvent control), together with an inducer, prior to preparing extracts and determining the reporter enzyme activities. Perhaps surprisingly, the inhibition of endogenous PKA appeared to have the same inhibitory effect on virus-induced expression from the full-length IFN-β promoter as does high levels of the PKA catalytic subunit (figure 6.3.). In this experiment, inductions of the IFN-β promoter by dsRNA were also performed with similar effects to those on the Sendai-induced IFN-β promoter. This indicates that a
Figure 6.3. Both overexpression of the catalytic subunit of PKA and inhibition of the endogenous PKA inhibit induction of the IFN-β promoter in L929 cells.

A transient transfection analysis in L929 cells, using the Transfectam reagent. The luciferase test plasmids (1μg of each one transfected) are described below the columns. In all the transfections, the luciferase test plasmids were cotransfected with 1μg of the internal control plasmid pBLCAT2, to normalize to variations in transfection efficiencies. The relative expression levels were obtained by dividing the luciferase activities by CAT activities. Where indicated, 0.5μg of the plasmid encoding the catalytic subunit of PKA, pMtCα, was also cotransfected. Furthermore, where indicated, the L929 cells were induced with either Sendai virus or dsRNA prior to determining the reporter enzyme activities. For "H89"-marked and for "DMSO"-marked transfections the cells were treated with 30μM of the kinase inhibitor H89 and an equal volume of DMSO (solvent used for the H89 stock solution), respectively, for six hours, prior to determining the reporter enzyme activities.
component common to the induction pathways by both Sendai virus and dsRNA is affected by the modulation of levels of PKA in vivo.

The fact that both the nonphysiological overexpression of the catalytic PKA subunit and inhibition of the endogenous PKA can interfere with the induction may indicate that the activity of PKA is under delicate control within cells, so that both the complete lack and excessive activity of PKA are deleterious for the induction process. It may be that PKA both negatively and positively regulates the IFN-ß gene induction in response to viral infection or dsRNA; this dual functioning might be facilitated by PKA anchor proteins (Scott and McCartney 1994), which may make local PKA actions possible. While at the concentration used in these experiments, H89 has been shown to be selective in inhibiting PKA, as specifically opposed to protein kinase C, cGMP-dependent protein kinase, and casein kinases (Chijiwa et al 1990), it further remains possible that it modulates the activities of some other kinases that have not been tested.

We also tested the effect of the overexpression of the catalytic subunit of PKA on the full-length IFN-ß promoter in another cell line, namely HeLa cells. Interestingly, the transcription levels induced by either dsRNA or Sendai virus were not inhibited by elevated cellular contents of the PKA catalytic subunit, in HeLa cells (figure 6.4.). Rather, we observed that both the basal and induced levels were modestly and reproducibly increased by cotransfection of the pMtCa expression plasmid. This indicates that the inducer-triggered signalling pathways are not identical in all mammalian cell lines that are responsive to the induction. Similar observations have been made by Daigneault et al (1992), who reported an inhibition of type I IFN induction by the addition of a kinase inhibitor 2-aminopurine (see above), in L929 cells, but not in primary spleen cells.
Figure 6.4. Overexpression of the catalytic subunit of PKA cannot inhibit IFN-β induction in HeLa cells.

A transient transfection analysis in HeLa cells, using the Transfectam reagent to investigate the effect of the overexpression of the catalytic subunit of PKA on the IFN-β promoter [1μg of the pIF(-210/+72)lucer plasmid transfected]. In all the transfections, the luciferase test plasmids were cotransfected with 1μg of the internal control plasmid pBLCAT2, to normalize to variations in transfection efficiencies. The relative expression levels were obtained by dividing the luciferase activities by CAT activities. Where indicated, 0.5μg of the plasmid encoding the catalytic subunit of PKA, pMtCα, was also cotransfected. Furthermore, where indicated, the HeLa cells were primed and induced with either Sendai virus or dsRNA, prior to determining the reporter enzyme activities.
PKA PKA PKA
I_____________I_____________I_____________I
unprimed/ primed/ primed/
uninduced dsRNA induced Sendai induced

RELATIVE EXPRESSION LEVEL

- PKA - PKA - PKA
unprimed/ primed/ primed/
uninduced dsRNA induced Sendai induced

3.1 11 17 24 28
Appendix I: Analysis of the Transfectam Transient Transfection Method

The Transfectam (TFM) reagent is a synthetic, cationic lipopolyamine molecule, in which a spermine group is covalently attached through a peptide bond to the lipid moiety. The strong positive charge contributed by the spermine headgroup gives the molecule a high affinity for DNA ($10^5-10^6 \text{ M}^{-1}$), coating the DNA to be introduced into cells with a cationic bilayer, which facilitates binding to the cell membrane.

I.I. Optimization of the Transfectam Method

To investigate the effect of the amounts of exogenous DNA and the amount of the Transfectam reagent to be added to the transfection mixtures to achieve the optimal sensitivity of expression, we varied both of the parameters to transiently cotransfect HeLa cells. The expression plasmids used were pBLCAT2 and pJATLACZ (described above), equal amounts of each of which were added to each transfection mix, while the amount of total DNA added was varied. From table I.I. it is clear that the TFM:DNA ratio is the major determinant for the transfection efficiency. While it might thus be tempting to use very high amounts of the Transfectam reagent, it should be noted that this synthetic reagent is very expensive, and only commercially available. In the experiments described throughout this thesis, we decided to vary the amount of DNA between 2µg and 3µg and maintain the TFM:DNA ratio constantly as 5.

I.II. Comparison of the Transfectam and Calcium Phosphate Transfection Methods

We have investigated the properties of two transient transfection methods in two cultured cell lines, namely human HeLa and mouse L929 cells. We were specifically interested in comparing the percentage of transfected cells and the amount of DNA taken up by a transfected cell, in either of the two systems.
Table I.I. Optimization of the Transfectam transient transfection method for HeLa cells.

<table>
<thead>
<tr>
<th>total DNA (μg)</th>
<th>TFM (μl)</th>
<th>TFM/μg DNA</th>
<th>% β-gal/μg DNA</th>
<th>% CAT/μg DNA</th>
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Two duplicate dishes of HeLa or L929 cells per sample were each transfected with 1μg pKSCD2 and 0.5μg of both test plasmids [tkA(-105)lucter and pBLCAT2] using TFM, or 10μg pKSCD2 and 5μg of test plasmids when using CaPO₄ coprecipitation. pKSCD2, a plasmid that contains the human surface antigen CD2 cDNA under the control of a cytomegalovirus promoter-enhancer region, was obtained from Dr. K. Smith (ICRF). Transient expression of the CD2 surface antigen in otherwise CD2-negative cells allows the selection of cotransfected cells from those that have not taken up exogenous DNA (figure I.1). According to the principles of cotransfection, each plasmid in the transfection mixture is taken into most of the transfected cells together. Using enzyme markers, it has been established that the frequency of cotransfection with calcium phosphate coprecipitation is approximately 94% (S. Goodbourn, pers. comm.).

Approximately 48 hours after transfections, plates were washed twice with PBS, and cells harvested by scraping with a rubber policeman in a final volume of 5ml (CaPO₄) or 2.5ml (TFM) of ice-cold PBS per sample. The cell suspension was transferred to a 15ml tube, and 4 x 10⁷ magnetic beads conjugated to a monoclonal antibody specific for human CD2 surface antigen (see below) in a 100μl volume were added with vigorous mixing. Prior to mixing with the cells, the magnetic beads were prepared as follows; 10⁹ M450 Dynabeads coated with sheep anti-mouse IgG (Dynal) were vigorously mixed with 250μg of mouse monoclonal antibody OKT11 specific for the human CD2 gene product (a gift from D. Watling, ICRF), and then the binding reaction was continued on a rotating wheel at 4°C for 30 minutes. Beads were washed twice with ice-cold PBS, after which they were resuspended at 4 x 10⁵ beads per ml in PBS.

The mixtures of cells and pretreated magnetic beads were incubated on a rotating wheel at 4°C for 15 minutes. The CD2⁺ and CD2⁻ cell populations were then separated using multiple rounds of magnetization. During the first magnetization, a Dynal MPC-1 magnetic particle concentrator was used for the 15ml tubes. The enriched CD2⁺ population was then resuspended in 1ml ice-cold PBS and transferred to a 1.5ml microfuge tube, and subsequent magnetizations were performed using a Dynal MPC-E magnetic particle concentrator. The reduction in volume leads to substantially smaller cell losses (S. Goodbourn, pers. comm.). Rounds of magnetization were continued until the supernatants were clear of cells for the CD2⁺ population, and until no beads could be detected in the CD2⁻ population. Cells in both the CD2⁺ and pooled CD2⁻ populations were then pelleted, lysed in LucA/LucB buffer system, and reporter activities and protein concentrations determined.
Figure I.1. Schematic representation of the surface antigen selection of transiently cotransfected cells.

The cell selection procedure was modified from that described in Whiteside et al (1994).
transfect

40-44 hours

scrape

magnetic beads coated with mAb anti-CD2

CD2- cells

CD2+ cells

CD2- cells

CD2+ cells

magnet

make extracts and determine reporter enzyme activities and protein concentrations
The results are summarized in table I.II. Each figure is an average of the measurements from duplicate transfections. The protein concentrations measured for each sample were used to estimate the total cell mass in the selected population and the percentage of cells transfected calculated by the equation \[\left(\frac{\text{protein mass in CD2}^+ \text{ population}}{\text{protein mass in CD2}^+ \text{ population and CD2}^- \text{ population together}}\right)\]. Both luciferase and CAT activities were determined for all cell populations, and the values divided by protein mass used to evaluate the efficiency of DNA intake per cell. Although we are not directly detecting the copy numbers of plasmids per cell, we believe that the reporter enzyme activities do closely approximate this. A caveat with this approach is that we cannot exclude the possible effect of titration of transcription factors by the excess DNA taken by a cell, which may lead to reduction in reporter enzyme expression. One possibility to attempt the determination of actual plasmid copy numbers would be to perform a Southern blot on DNA samples from transfected cells. This would be technically rather difficult due to small sample volumes in some of the selected cell populations, and lower sensitivity of the proposed assay compared with the CAT and luciferase reporter enzyme assays. Furthermore, a Southern blot would be further prone to artefactual results by contaminating plasmids that associate with the cellular membranes without actually being taken in by the cells. Also, a nonfunctional compartmentalization of transfected plasmids would not be excluded by assaying at the DNA level, and we thus believe that determining reporter enzyme activities monitors the functional aspects more favourably.

After magnetic separation, 71-78% of the total reporter enzyme activities, both CAT and luciferase, of each individual transfection were associated with the selected CD2\(^+\) fractions. This figure is lower than the observed efficiency of cotransfection cited above. We do not believe that this reflects the lower frequency of cotransfection in this particular experiment. Rather, the magnetic separation in this experiment was performed in somewhat suboptimal conditions, so that exogenous protein BSA was not added to the PBS solution, since this could have made the subsequent determination of the total selected cell mass by Bradford assay unreliable. Under the optimized selection conditions, the carrier protein BSA prevents aggregation of the cells during the selection procedure. In the absence of BSA untransfected cells that do not themselves associate with magnetic beads become trapped into aggregates containing cotransfected CD2\(^+\) cells, and \textit{vice versa}. We believe that the lower retrieval of reporter activities with the selected population reflects the relative inefficiency of the selection of cells into the two populations due to the absence of carrier protein.
Table I.II. A summary of the comparison between Transfectam and calcium phosphate coprecipitation methods in transient transfections.

<table>
<thead>
<tr>
<th></th>
<th>% CD2⁺ cells</th>
<th>CAT/µg protein in CD2⁺ population</th>
<th>luciferase/µg protein in CD2⁺ population</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>HeLa</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CaPO₄</td>
<td>13</td>
<td>1166 units</td>
<td>23 units</td>
</tr>
<tr>
<td>TFM</td>
<td>46</td>
<td>428 units</td>
<td>14 units</td>
</tr>
<tr>
<td><strong>L929</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CaPO₄</td>
<td>6.7</td>
<td>1954 units</td>
<td>82 units</td>
</tr>
<tr>
<td>TFM</td>
<td>44</td>
<td>301 units</td>
<td>59 units</td>
</tr>
</tbody>
</table>
The results were very similar for both HeLa and L929 cells. However, striking differences were observed between the two transfection methods. While with the CaPO₄ method only a few cells become transfected, the TFM reagent causes many more cells to take in the transfected plasmids. By using the TFM transfection protocol optimized by us, very high transfection efficiencies of HeLa cells, exceeding 50% of the cells transfected, have also been observed by others (D.Watling, pers.comm.). By determining reporter activities per cell mass, it also became obvious that with the CaPO₄ method each cell takes in larger amounts of DNA, whereas in the TFM method the amount of transfected DNA per cell is lower. The latter may thus well represent a more physiological situation, and should thus be considered more suitable for functional transfection studies, which aim to serve as a model for authentic transcriptional regulation in vivo. The difference is more apparent with the CAT reporter enzyme than the luciferase. This probably reflects the fact that the luciferase enzyme is a more short-lived protein species than chloramphenicol acetyltransferase. Thus, while luciferase activity in transfected cells provides "a snapshot" over a short period of time, the CAT activity results from continuous accumulation of the enzyme.
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Appendix II

Manuscript in press

Eloranta, J.J., and Goodbourn, S.
Positive and Negative Regulation of RNA Polymerase II Transcription.

In Eukaryotic Gene Transcription. Oxford University Press.
Chapter 1  Positive and negative regulation of RNA polymerase II transcription

JYRKI J. ELORANTA and STEPHEN GOODBOURN

1. Introduction

The correct temporal and spatial expression of specific genes governs the identity of eukaryotic cells. For most genes, the primary control point is the regulation of transcriptional initiation. The DNA sequences responsible for determining the exact start site and the level of mRNA synthesis are generically referred to as promoters. The core promoter elements (TATA boxes and initiator elements) determine, and reside immediately 5' to and overlap with, the mRNA startpoints. The minimal basal level of transcription can be supported by basal transcription machinery that assembles on the core promoter elements. In addition, promoters contain recognition sites for regulatory transcription factors, which are sequence-specific DNA binding proteins. Regulatory factors can influence transcription levels by either enhancing (activators) or antagonising (repressors) the assembly or activity of the basal transcription machinery. Typical regulatory factors are modular, so that the DNA binding domain is usually separable from the one mediating activation or repression (1). The functional analysis of various activator proteins has revealed that activation domains often appear to fall into distinct classes, such as acidic, glutamine-rich and proline-rich, according to the predominance of particular amino acid residues. Transcriptional repression domains have not been as well defined, although alanine-rich or basic regions found in some repressor proteins may serve as such negative effector domains (2-4).

One of the major goals of recent research has been the elucidation of the mechanisms by which regulatory transcription factors influence the rate of initiation by the basal transcription machinery. Influence on both the rate and extent of assembly of basal transcription machinery on core promoters have been suggested. As discussed in this
chapter, regulatory factors are believed to achieve their effect by protein-protein interactions with specific components of the basal transcription machinery.

2. The RNA polymerase II basal transcriptional machinery

The basal transcription factors (5-7) required for initiation by RNA polymerase II from most promoters were first identified in nuclear extracts as activities capable of supporting basal levels of transcription, and have been separated into biochemically defined fractions referred to as TFIIA, -B, -D, -E, -F, and -H. Subsequent cloning and biochemical analyses have shown that many of these factors are composed of multiple subunits. The basal factors can be assembled in a defined order on core promoters to form a pre-initiation complex (5-8) (Fig. 1). In most RNA polymerase II promoters, an A+T-rich sequence element known as the TATA box (consensus T A T A T/A A T/A A) is the critical determinant for the multistep assembly. Since TFIID is the only basal factor known to possess sequence-specific DNA binding activity, it is responsible for the initial template commitment. The TATA box is specifically recognised by a particular component of the TFIID complex, referred to as the TATA box binding protein (TBP). However, several footprinting studies have indicated that the DNA contacts made by the TFIID complex are more extensive than those by TBP alone (9-11). TBP alone typically generates a footprint of approximately 20 base pairs, centered around the TATA box, whereas the extended protected region by TFIID encompasses the transcriptional start site, as well as sequences further downstream. Other subunits of TFIID are thus likely to make additional DNA contacts. Indeed, a recently cloned subunit of the TFIID complex, a 150kD TBP-associated factor (dTAF150), has been shown to specifically bind to DNA sequences overlapping the start site of transcription and also to further 30 bp downstream (12). Together, TBP and dTAF150 seem to be largely responsible for the extended TFIID-footprint pattern.

Several RNA polymerase II genes do not contain discernible TATA boxes, yet they appear to be transcribed using the same basal machinery. The pre-initiation complex assembly on
TATA-less promoters is typically specified by initiator (Inr) elements that overlap the precise transcription start sites (13,14). The functional consensus sequence for Inr elements is rather loose (Py-Py-A(+1)-N-T/A-Py-Py, where Py is pyrimidine and N is any nucleotide; 15). The initial recognition of TATA-less promoters through these Inr elements probably utilises the dTAF150 component of TFIID (12,16,17). The specific binding of dTAF150 to the core promoter may explain how TFIID can become recruited and then orchestrate the transcriptional initiation events on TATA-less RNA polymerase II promoters that do not allow TBP-binding. It should be stressed that TBP has been shown to be required for TATA-less promoters, even if it does not function as the initial template-recognising factor (18). Interestingly, even when recruited to certain TATA-less promoters by protein-protein interactions, TBP makes DNA contacts in the -30 region, regardless of the exact DNA sequence (19). While the template recognition events between TATA-containing and TATA-less promoters are clearly distinct, the subsequent DNA-independent steps in the initiation process are likely to be highly similar for both promoter types (20).

Following template commitment, pre-initiation complex assembly continues with the association of TFIIA with TBP, an event which stabilises the TFIID-template interaction on TATA-containing promoters. TFIIA is believed to achieve this effect by counteracting the negative activities that are inhibitory to the TBP function (see below). It should be noted that TFIIA can also enter the assembly pathway at any later stage (21). TFIIIB is the next basal factor to enter the pre-initiation complex, and also interacts directly with TBP. Whereas TFIIA is capable of stabilising the interaction of TFIID with a TATA box, TFIIIB appears to have a similar effect on the TFIID-Inr interaction. The pre-initiation complex intermediate TFIID-TFIIA-TFIIIB serves as a nucleation site for the entry of the remaining basal transcription factors and RNA polymerase II. TFIIF mediates the entry of RNA polymerase II into the complex, and markedly decreases non-specific DNA binding of the polymerase. On eukaryotic RNA polymerase II-dependent promoters the assembly continues with the further association of TFIIE and TFIIH. In the presence of the four ribonucleoside triphosphates, the fully assembled complex is capable of initiating RNA
synthesis from specific start sites. On TATA-containing promoters these are typically located 25 to 30 nucleotides downstream of the TATA box.

The six basal transcription factors consist of more than 30 polypeptides, and the RNA polymerase itself of 10+-2 subunits; thus the potential for multiple protein-protein interactions within an assembled pre-initiation complex is vast. For example, RNA polymerase II interacts directly with TBP (22), TFIIB (23,24), TFIIF (25,26), TFIIE (26,27), and TFIIH (28). Recent studies suggest that many of the interactions involving basal factors are inductive, causing the interaction partners to assume new conformations or activities necessary to allow the subsequent steps in the assembly process (27,29-31).

The stepwise assembly model for the pre-initiation complex has recently been challenged by the purification of a largely pre-assembled complex, termed holozyme, from crude homogenates of the yeast Saccharomyces cerevisiae (32; see Fig.1). The holozyme contains TFIIB, TFIIF, and TFIIH together with RNA polymerase II, but no significant amounts of TFIID, TFIIA, or TFIIE. It will be interesting to learn whether similar partial pre-initiation complexes assemble in solution in the nuclei of higher eukaryotic cells.

All promoters do not appear to require the full set of basal transcription factors. For example, it has been shown that the immunoglobulin heavy chain (IgH) gene promoter can be accurately transcribed in vitro by RNA polymerase II in the presence of only TBP and TFIIB (33). This minimal requirement is dependent on the template DNA being negatively supercoiled, since transcription from the IgH promoter requires the entire array of basal factors when the template is linearized. This suggests that the free energy stored in DNA supercoils promotes the formation of an open complex by the minimal set of basal factors. In contrast to the IgH promoter, transcription from the adenovirus major late promoter requires the complete set of basal transcription factors, independent of the superhelical state of the template. The determinants for the differential requirements of different promoters for basal transcription factors remain somewhat unclear. The reason why TFIIE and TFIIH
appear dispensable for transcription of the immunoglobulin heavy chain (IgH) gene promoter may be that instead of being required for the formation or function of an initiation complex, their role is to convert a transcriptional initiation complex into an elongation complex, an event referred to as promoter clearance (27,34). Also, TFIIF has a helicase activity, believed to be responsible for the local unwinding of DNA around the initiation site; supercoiling may abrogate the requirement for TFIIF. While in vitro studies have been highly informative, the factor requirement in vivo might be different, due to the influence of chromatin components (see Chapter 2).

3. RNA polymerase I and III promoters

In addition to its requirement for basal transcription from both TATA-containing and TATA-less RNA polymerase II promoters, TBP is also required for transcription by RNA polymerases I and III (19,35,36). None of the RNA polymerase I promoters have TATA-like sequences, and the human RNA polymerase I-specific TBP-containing complex SL1 cannot bind independently to them, but needs to be tethered via protein-protein interactions with an upstream binding factor (37,38). Most RNA polymerase III promoters also have no TATA box and TBP is recruited to them via other proteins (39). Some RNA polymerase III promoters, such as that of the U6 snRNA gene, do contain a functional TATA box, which directs pre-initiation complex assembly to these promoters by binding TBP. Paradoxically, when the U6 TATA box is mutated, the promoter switches to be transcribed by RNA polymerase II (40,41). This provides an exceptional case, where the presence of a TATA box determines the selection of RNA polymerase III over RNA polymerase II.

4. TATA box binding protein

TBP is necessary for transcription by all three eukaryotic nuclear RNA polymerases, whether the promoters have identifiable TATA box sequences or not (19,35,36). TBP is thus an integral component of the transcription initiation complex in all three systems,
despite the differences in the modes of promoter recognition and biochemical properties of the three RNA polymerases. Its universal presence in transcription complexes suggests that TBP is likely to have been a member of an early initiation complex, that preceded the evolutionary divergence of the three eukaryotic RNA polymerases. In fact, the recent cloning of a TBP homologue from nucleless Archaea suggests that the role of TBP in transcription was established before the divergence of eukaryotic and Archaean lineages (42,43).

Little or no free TBP exists in solution in human or Drosophila cells, instead it is found in large multi-component complexes which contain additional polypeptides that are tightly bound to TBP, and are consequently referred to as TBP-associated factors, or TAFs (10,44,45; see below). The ability of TBP to stimulate transcription by each of the three RNA polymerases appears to be determined by its interactions with different arrays of TAFs. One of the future challenges is to learn the basis of channelling the newly synthesised TBP-pool into RNA polymerase I, II and III-specific transcription complexes, and to determine the degree of conservation in protein-protein interactions involving TBP in the different complexes. Three point mutations have been identified within the conserved carboxy-terminal region of TBP (see below) that abolish in vivo transcription by all three nuclear RNA polymerases, suggesting that the different classes of transcription machinery contact the same regions of the TBP polypeptide (46). However, two other point mutations produce specific effects: one prevents transcription by RNA polymerases II and III but allows transcription by RNA polymerase I; another abolishes TATA binding, but still functions on TATA-less RNA polymerase I- and III-dependent genes but not on a TATA-containing RNA polymerase II-dependent promoter (47). Therefore, components of the three different transcription systems are likely to utilise overlapping but distinct regions of TBP. Temperature-sensitive yeast strains that contain TBP specifically defective in RNA polymerase III-dependent transcription accumulate increased amounts of RNA polymerase II-dependent transcripts when grown at the non-permissive temperature; conversely, an RNA polymerase II-specific TBP mutant shows increased RNA polymerase III
transcription (48). This suggests that factors specific for the RNA polymerase II or RNA polymerase III system compete for limiting amounts of TBP in vivo; the competition could reflect overlapping recognition sites on the TBP surface for class-specific factors. Consistent with this, studies on the RNA polymerase I-specific complex SL1 indicate that the protein-protein interactions that associate TBP with either RNA polymerase I- or II-specific TAFs are mutually exclusive (49).

The carboxy-terminal 180 amino acids of TBP is highly conserved (19); for example, this domain shows 85% identity between human and yeast. Even the carboxy-terminus of TBP from Archaea is approximately 40% identical to human TBP, despite the large evolutionary distance (43). The conserved core domain of TBP has been shown to be sufficient to bind to TATA boxes, and to allow basal transcription (10,50,51). Furthermore, gene replacement studies indicate that the conserved domain of TBP is sufficient for supporting growth in yeast (52-55). The high conservation of sequence translates into functional conservation as human and yeast TBPs are interchangeable for both basal and activated transcription by RNA polymerase II in vitro (56); furthermore, human and yeast TBPs can support transcription in a cell-free Archaean transcription system (57). Surprisingly, while the S. pombe TBP can complement and support cell growth of a S. cerevisiae TBP null mutation (55,58), the human or Drosophila TBPs cannot (52,59). Nevertheless, it has been shown that the yeast TBP can mediate transcriptional activation by retinoic acid or GAL4-VP16 in some mammalian cell lines as efficiently as the human TBP (60). A study on the in vivo transcriptional activity of human TBP at different classes of yeast promoters indicates that the failure of human TBP to support yeast growth is not a consequence of its inability to generally support RNA polymerase II-dependent transcription (61).

TBP binds DNA as a monomer (62). Unlike most sequence-specific DNA binding proteins, TBP interacts primarily with the minor groove of DNA (63,64). The crystal structures of TBP from Arabidopsis thaliana (65) and the conserved carboxy-terminal domain of yeast TBP (66) have been solved. These analyses reveal a highly symmetrical,
evolutionarily conserved structure, in which the carboxy-terminal region of TBP forms a novel DNA-binding fold positioned on DNA in a saddle-like manner. The concave inner surface mediates contacts with DNA, whereas the convex outer surface provides an interaction interface for several proteins, such as TFIIA, certain TAFs and various activator proteins. The structure is in complete agreement with previous mutational analyses which identified regions in TBP as being involved in either DNA binding or protein interactions (19). Co-crystallization of TBP with DNA has confirmed that TBP induces an extremely strong bend in DNA (67,68), as previously suggested by gel mobility shift analysis using permuted binding sites as probes (69). TBP-induced DNA bending brings separate DNA sequences on linear DNA closer together in three-dimensional space, which may facilitate the interactions between the pre-initiation complex on a core promoter and regulatory factors on distant binding sites, and may also permit closer association between RNA polymerase II and the basal transcription factors. Interestingly, TBP bends the TATA element in an opposite direction to that seen in nucleosome-bound DNA, suggesting a mechanism for the observed competition between nucleosomes and the binding of TBP.

TFIID is likely to form a similar structure to TBP on the TATA box, although a high resolution structure has not yet been obtained.

5. TBP Associated Factors (TAFs)

TFIID can be purified from HeLa or Drosophila cells as a multi-protein complex composed of TBP and several stably associated proteins (eight in Drosophila), called TAFs, that range in size from 250kD to 30kD (10,44,45,70-73; see Fig.2). Several of the Drosophila and human TAFs have been shown to be structural homologues; furthermore, conservation of protein-protein interactions suggests conservation of function (Table I). It is therefore not surprising that the conserved TBP core domain is sufficient to allow association with TAFs (72). Whether the assembly of the multi-protein TFIID complex follows a precisely ordered pathway within cells is not known. However, the dependence on partially reconstituted
complexes for the incorporation of some TAFs into TFIID (74), and the vast number of mutual protein-protein interactions between the TFIID components would suggest that there are some assembly restrictions. For example, dTAF250 appears to be a "scaffold" protein that is needed for many other TAFs to be incorporated in TFIID. Similarly, dTAF40 incorporation requires dTAF60 to be pre-associated with the complex (see Fig.2). It is not known whether the assembly of the TFIID complex is a regulated process. However, in the absence of the other TAFs, dTAF250 specifically inhibits the binding activity of TBP to the TATA box (75,76) - neutralization of this inhibitory activity may be an important mechanism for transcriptional regulation.

Until recently, yeast TBP was believed to exist as an uncomplexed 27kD monomer (77-79). However, given the conservation of sequence and function of yeast, human and Drosophila TBPs, it has always seemed likely that yeast TBP would also be part of larger multi-protein complexes. Indeed, it has recently been reported that approximately 70% of yeast TBP is complexed with at least seven TAFs (80). Such a multi-subunit complex associated with the yeast TBP has been shown to be specifically required for activated RNA polymerase II transcription (81). Preliminary analysis of the cloned yeast TAFs has indicated that many of them represent homologues of the Drosophila and human TAFs (Table 1). The existence of a multi-subunit TFIID complex thus appears to be a universal phenomenon in eukaryotes. The observations that the yeast TBP core domain can assemble in vitro into a complete and functional TFIID complex together with the human TAFs (82), and that yeast TBP can mediate transcriptional activation in mammalian cells (60), suggest that the functionally relevant protein-protein interactions are conserved. For example, the previously identified essential yeast gene product TSM-1 is strikingly similar to dTAF150, suggesting that TSM-1 is a yeast TAF homologue (12). As expected for a true TAF, TSM-1 directly associates with the yeast TBP both in vivo and in vitro. Furthermore, like dTAF150, TSM-1 can interact with dTAF250, which further implies that yeast is also likely to contain a dTAF250 counterpart. Even if the in vitro interaction specificity between TSM-1 and dTAF150 thus appears to be conserved, the Drosophila protein is perhaps
surprisingly unable to rescue the TSM-1 mutant \textit{in vivo}. It is also interesting to note that while the activation domain of Sp1 can activate transcription with yeast TBP in human cells \textsuperscript{(83)}, it cannot function in yeast \textsuperscript{(84)}. Thus functional conservation is not absolute.

It is not clear whether there is only one type of TFIID-complex. The observation that a single major TFIID species is capable of supporting activation by a diverse class of activation domains suggests that this is the case \textsuperscript{(10)}. However, the existence of distinct low-abundance TFIID-complexes specifically involved in transcription of certain promoters cannot be excluded. The known TAFs listed in Table 1 are present in stoichiometric ratios in isolated TFIID complexes. However, there may be TAFs that are only present in certain tissues, or during certain developmental periods, that have escaped detection in studies utilising only a limited variety of extract sources.

As discussed above, TAFs are not restricted to the RNA polymerase II system. Transcription of vertebrate ribosomal rRNA by RNA polymerase I requires the selectivity factor SL1, which is composed of TBP and three RNA polymerase I-specific TAFs, all of which are essential for transcription \textsuperscript{(85)}. Reconstitution of transcription from the TATA-less RNA polymerase III promoters utilises yet another distinct protein complex, TFIIIB, which contains TBP together with specific TAFs \textsuperscript{(86,87)}. SNAPc (\textit{sn}RNA activating protein complex) is a complex consisting of TBP and at least three specific TAFs, and is specifically engaged in vertebrate snRNA (\textit{small nuclear RNA}) transcription of RNA polymerase III class \textsuperscript{(88)}. Depending on the presence of TATA sequences, some snRNA promoters are transcribed by RNA polymerase II, while others are transcribed by RNA polymerase III (see above); the SNAPc complex is involved in both cases. Unlike TFIID, SL1 and TFIIIB, which either recognise the TATA box or Inr, or have little or no affinity for specific DNA sequences, SNAPc binds specifically to a distinct DNA element located upstream of transcription initiation sites on snRNA promoters. Thus, the TAFs in SNAPc seem to reprogram the binding specificity of the TBP-containing complex, directing it to target a sequence that is unrelated to a TATA box. Reconstitution of \textit{in vitro} transcription
from a TATA-containing RNA polymerase III promoter requires TBP in addition to SNAPc, suggesting that this initiation complex contains at least two forms of TBP, one bound to the TATA box, and another as part of the SNAPc complex.

6. How do regulatory transcription factors work?

Transcriptional activators are thought to contact targets in the basal transcriptional machinery directly or indirectly via intermediary proteins. Once activator proteins are bound to specific DNA sequences, they interact with the basal transcription machinery, and either recruit it to DNA, stabilise components already recruited to DNA, or cause a productive conformational change or some other kind of modification (e.g. phosphorylation) in one or more components of the basal transcription machinery, and thereby initiates a cascade of events that leads to the increased initiation of transcription.

6.1. TBP as a target for activators

Because of the central role played by the TATA box binding protein (TBP) in the initiation of transcription, it is an obvious candidate to be one of the targets of upstream activator proteins. However, while TBP together with other RNA polymerase II basal transcription factors can perform basal transcription, other components of the TFIID complex (the TAFs) are thought to be required for efficient transcriptional enhancement by all classes of activator proteins (9,44,45,50,89,90). Since TBP alone is incapable of responding to activators, activator-TBP associations cannot completely explain the function of activation domains. Nevertheless, these interactions are believed to be important contributors to the efficacy of transcriptional stimulation.

Various groups have demonstrated direct physical interactions in vitro between human TBP and the activation domains of several viral transcriptional activators, including the acidic activation region of herpes simplex virus VP16 (Table 2). Subsequently, it has been shown
that activation domains of several cellular activators are also capable of directly interacting with TBP (Table 2). It is clear that the activation domains do not fall into any single defined structural class. Activator proteins have also been identified which interact with TBP through regions other than their activation domains (Table 2). This implies that at least some regulatory proteins are capable of hitting more than one target in the basal transcription machinery, since by definition their activation domains also generate a signal to activate transcription.

Evidence to support the importance of interactions between activation domains and TBP is provided by studies showing that mutations in these activation domains which decrease affinity for TBP in vitro also reduce transcriptional activation in vivo (91,92). The viral transactivator Zta stabilises the DNA binding of TFIID in vitro by decreasing the dissociation rate of DNA-bound TBP (93). As expected, the domain in Zta protein responsible for transactivation in vivo is required for the stable association with TBP. The stabilisation effect by Zta appears to be especially marked in the context of weak TATA boxes; thus association with TBP is more likely to be rate-limiting in promoters with a poor TATA consensus. It has been recently reported that Zta also increases the stability of the TFIIA-TFIID-promoter complex in vitro in a manner dependent on TAFs (94), although it is not clear that this effect is directly attributable to the activation domain-TBP-interaction, since there may be additional contacts between Zta and other polypeptides induced by the formation of ternary complex.

Some transcription factors, such as E1A (95), Sp1 (96), and p53 (97), show a preference for the interaction with human TBP over the yeast homologue, despite interacting with the conserved core domain of TBP. This effect may depend on the activator-specific contact residues on the surface of TBP, and is reflected in the transactivation potency of these factors. Other activators, like Oct-2, appear to interact equally strongly with the TBPs from either species (98). E1A, p53 and c-Rel are all capable of interacting with the TFIID complex as well as with isolated TBP, which indicates that the surface on TBP recognised
by these activators must be available for interaction, even though TBP is tightly associated with several TAFs (99-101).

6.2. TAFs as targets for activators

TAFs are required in addition to TBP for activator proteins to stimulate transcription, and they have also been postulated to provide protein surfaces for activator proteins to target.

Although the glutamine-rich activation domains of Sp1 can bind directly to TBP (see above), they also appear to interact with the TFIID complex by binding to dTAF110 (71). Interestingly, the interaction seems to occur between two glutamine-rich regions, since the amino-terminus of dTAF110 has a high content of glutamines. A positive correlation has been observed between the level of transcriptional activation by various GAL4-Sp1 mutants in *Drosophila* Schneider cells and the degree of binding of the GAL4-Sp1 derivatives to dTAF110 in the yeast two hybrid assay (102). Sp1 can mediate efficient transcriptional activation in *in vitro* reconstitution experiments with a partial TFIID complex containing TBP, dTAF250, dTAF150 and dTAF110, although it does not interact with the other two TAFs present (74). Another glutamine-rich activation domain, namely that of the cAMP-regulated transcription factor CREB (cyclic AMP response element binding protein) also interacts with dTAF110 (103). Interestingly, however, several other activation domains classified as glutamine-rich fail to associate with dTAF110 (71), implying that predominance of glutamines is not the only determinant for activation within this class of activators.

In *in vitro* protein binding assays dTAF40 binds directly and specifically to the VP16 acidic activation domain (104). Supporting the importance of this interaction, specific antibodies raised against dTAF40 inhibit VP16-mediated activation, while leaving basal transcription unaffected. TBP and dTAF40 appear to associate with different subdomains within the VP16 activation region, thus it is possible that VP16 interacts with the two components of
TFIID simultaneously. Another acidic activation domain, that of the p53 protein, can interact with two TAFs, dTAF40 and dTAF60; furthermore, the ability of this domain to bind both of the two TAFs correlates with its transcription activation potential (105). It is not known, whether the p53 activation region can contact the two TAFs alternatively or simultaneously. A reconstituted TFIID complex containing dTBP, dTAF250, and dTAF60, with or without dTAF40 can support activation by p53 activation domain; whether dTAF40 can function without dTAF60 cannot be tested, since it cannot be incorporated into the TFIID complex without the presence of dTAF60. The isoleucine-rich activation domain of the activator protein NTF-1 can similarly contact two TAFs, dTAF150 and dTAF60 (74). In this case, two distinct partial TFIID ternary complexes, TBP-dTAF250-dTAF150 and TBP-dTAF250-dTAF60, can support activation by NTF-1.

Sequence analysis of the largest human TAF, hTAF250 has revealed its identity to a gene CCG1, previously cloned as a DNA binding protein that overcomes the cell cycle arrest in late G1 phase in the temperature sensitive rodent cell line ts13 (106,107). It is intriguing to speculate that hTAF250 would somehow link transcriptional control to cell cycle, by being specifically involved in the regulation of genes important for cell cycle progression. The ts13 cells do not manifest a global defect in transcription at the non-permissive temperature, thus the cell cycle block may result from altered expression of a specific set of genes. In agreement with the hypothesis that TAFs are required for activated transcription, only activated but not basal transcription is temperature sensitive in ts13 extracts in vitro; as expected, the recombinant hTAF250 can restore transcriptional activation in vitro (108). While the link between cell cycle-linked transcription and hTAF250 is intriguing, a more universal role for this TAF has also been proposed. In mutational analysis of TBP, its general ability to respond to activators correlates with the nature of its direct interactions with hTAF250 (83).

Individual TAFs appear to serve as targets for distinct types of activators; however, in some cases there does not seem to be a simple one-to-one relationship. From the discussion
above it is clear that a single activator can contact more than one TAF; conversely, a human TAF, hTAF55, appears to be capable of interacting with multiple activators (Sp1, CTF, E1A, YY1 and USF) with different overall structures (109). Interestingly, this particular TAF is the only component of the human TFIID, for which a Drosophila homolog has not been identified.

6.3. TFIIB as a target for activators

Interactions have also been demonstrated between TFIIB and the activation domains of VP16, as well as other transactivation regions (110-114). Variant TFIIB proteins that cannot associate with the VP16 activation region support basal transcription at 80-100% of wild-type levels; however, these mutants are severely defective in supporting transcription directed by an acidic activation region (115). Conversely, a single amino-acid substitution mutant in VP16 that renders it defective in transactivation also fails to interact with TFIIB. The stable entry of TFIIB can indeed be a rate-limiting step in pre-initiation complex assembly in vitro (110). Since TFIIB dissociates from the pre-initiation complex following initiation, the activators could stimulate transcription by re-recruiting TFIIB to the promoter. In addition to quantitative recruitment to the pre-initiation complex, activators also qualitatively affect TFIIB, by inducing conformational changes such that pre-initiation complex assembly is driven forward (116). Whilst the enhancement of TFIIB recruitment by acidic activators requires the presence of TBP, TAFs are dispensable (112).

6.4. TFIIH as a target for activators

The carboxy-terminal domain (CTD) of the largest subunit of RNA polymerase II is highly conserved and consists of several repeats of the consensus heptapeptide Tyr-Ser-Pro-Thr-Ser-Pro-Ser (117). Due to variable phosphorylation of these heptapeptide repeats, RNA polymerase II exists as two forms: the extensively phosphorylated and the hypophosphorylated forms. Changes in CTD phosphorylation occur during the
transcription cycle, so that the hypophosphorylated enzyme preferentially associates with the pre-initiation complex, while actively elongating complexes mainly contain the phosphorylated RNA polymerase II (118-120; see Fig.1). The phosphorylation of CTD has thus been believed to be important in enhancing the escape of the RNA polymerase II from the promoter, in order to shift from the initiation to the elongation phase of transcription. The observations that the promoter-bound TBP and the putative promoter clearance factor TFIIIE can both selectively interact only with the hypophosphorylated RNA polymerase II are consistent with this model (22,27).

Although various other kinases in many organisms have been suggested to be capable of CTD phosphorylation in vitro, the strongest candidate for a true regulatory CTD kinase activity is contained in the multisubunit basal transcription factor TFIIH (121). The TFIIH kinase activity is enhanced in the context of the assembled pre-initiation complex. In particular, the presence of TFIIIE, which associates before TFIIH with the pre-initiation complex, strongly stimulates the kinase activity of the purified TFIIH in vitro (31). Interestingly, the TFIIH subunit possessing the kinase activity appears to be a previously identified cyclin-dependent kinase (122,123).

Two acidic activation domains, namely those of VP16 and p53, have recently been shown to directly interact with the 62kD subunit of TFIIH (124). Furthermore, point mutations in VP16 that reduce its activation potential also weaken its interaction with both the human and yeast TFIIH. Thus, transactivator proteins could directly affect the kinase activity of TFIIH to enhance transcriptional initiation.

6.5. TFIIF as a target for activators

TFIIF may also function in activated as well as basal transcription. It has recently been shown that TFIIF can relieve squelching (see below) by the activator protein SRF (125), and TFIIF can directly interact with SRF (126). Specifically, the larger of the two TFIIF
subunits, RAP74, can associate with the activation domain of SRF. There appears a good
correlation between binding of SRF to RAP74 and the ability of the RAP74 protein to
mediate transcriptional activation by SRF; however, the effect of SRF on the properties of
TFIIF remain unclear.

6.6. Adapters as targets for activators

Adapters are proteins that can transmit a signal from activators to the basal transcription
machinery without possessing independent DNA binding activity or being stably associated
with TBP (although they may well interact with it in a more transient manner than do
TAFs). The distinction between a TAF and an adapter is sometimes vague, and the
classification of a novel factor as one or the other may partly depend on whether it has been
identified by biochemical or genetic means.

Excessive amounts of transcriptional activators suppress the level of transcription, a
phenomenon referred to as squelching. This phenomenon is thought to be due to
sequestration of a limiting component of the transcriptional machinery into a non-
productive form. Overexpression of a GAL4-VP16 fusion protein in yeast squelches
expression and eventually proves toxic. The growth inhibition by high levels of GAL4-
VP16 requires the integrity of both the VP16 acidic activation domain and the GAL4 DNA
binding domain, implying that the effect is a consequence of the recruitment of basal
transcription factors into non-productive DNA-bound complexes to genomic GAL4 binding
sites (127).

Yeast selection screens have been carried out to isolate mutants that can resist the toxic
effect of GAL4-VP16. Such yeast strains might be expected to have alterations in limiting
components of the transcriptional machinery. Three genes, ada2, ada3 and gcn5
(alteration/deficiency in activation), have been isolated by this approach (128-130). The
ADA2, ADA3 and GCN5 products appears to act as transcriptional adapters only for
certain acidic activators, such as GAL4-VP16, but not for some others. Physical interaction has been demonstrated between ADA2 and the VP16 activation domain (131). ADA2, ADA3, and GCN5 form a trimeric complex (132), providing an explanation why double mutants in any two of the three genes do not any more severely affect yeast growth than any single mutant. The precise target within the pre-initiation complex for the adapter function of ADA2-ADA3-GCN5 complex has not been determined.

Adapters for activator function exist also in higher eukaryotes. Squelching experiments have identified groups of activation domains that cross-compete in transfection assays. For example, ectopic expression of the acidic transactivation domains of c-Jun itself, JunB, GAL4, and VP16 can repress c-Jun activity, whereas the activation regions belonging to different classes cannot (133). Three proteins could be co-immunoprecipitated with c-Jun in a transactivation domain-dependent manner; this association is sensitive to competition with the excess of VP16 protein, suggesting that these proteins are adapters.

A mammalian protein termed CBP (CREB-binding protein) has been identified that bears a 50 amino acid region of homology with the ADA2 gene product (134). CBP is a large protein of 250kD and also contains a bromodomain, a conserved structural motif found in Drosophila and yeast proteins involved in signal-dependent but not basal transcription and thought to be important for protein-protein interactions. CBP can specifically interact with the protein kinase A-phosphorylated, activated, form of CREB, and in transfection experiments enhances the CREB-mediated activation of reporter constructs in vivo in a manner that depends on the integrity and function of the protein kinase A phosphorylation site (135). Furthermore, microinjection of specific anti-CBP antibodies blocked the activity of a cAMP-regulated promoter in fibroblasts (136). Strikingly, anti-CBP antibodies also blocked activated transcription from other signal responsive promoters containing TREs (TPA responsive elements) or SREs (serum responsive elements), whilst leaving basal promoter activities unaffected. Like cAMP, the stimulating agents of TREs and SREs (phorbol esters and serum growth factors, respectively) also induce activatory
phosphorylations in specific transcription factors. CBP has since been shown to specifically interact with c-Jun that is phosphorylated on sites which are known to stimulate transcription in vitro, and which become phosphorylated upon mitogen-induction in vivo (136). Perhaps CBP, or a family of related proteins, has a role as an end point or integrator of various signal transduction pathways initiated at the cell surface, providing a link between signaling cascades and transcriptional effect.

As expected for an adapter protein, CBP activates transcription in a sequence-dependent manner when fused to a heterologous DNA binding domain (134, 135). CBP interacts with TFIIB in vitro (135). The CBP-interacting region in TFIIB partially overlaps the domain contacting VP16. CBP shares extensive sequence similarity with p300, a protein capable of associating with the adenovirus E1A protein (137,138). p300 appears to be another transcriptional adapter, whose function is negatively regulated by its association with E1A. The DNA binding factors that target p300 to specific promoters have yet to be identified; however, p300 has a cell cycle inhibitory function, and is likely to regulate the promoters for genes required for arrest in G0/G1. The similarity between CBP and p300 suggests that there may be a family of related, but perhaps functionally distinct, adapter proteins.

The recently cloned PC4 is a small (14kD) human protein that also has characteristics of an adapter protein. PC4 markedly enhances transcriptional stimulation in vitro by various types of activation domains - including acidic (VP16), proline-rich (CTF) and glutamine-rich (Sp1), but has only minor effects on basal transcription. TAFs need to be present for PC4 to exert its stimulatory function. PC4 is capable of directly interacting with both the VP16 activation domain and TFIIA, either in solution or as DNA-bound VP16-TFIIA-TBP complexes. The transcriptional potency of VP16 activation domain mutants correlate strongly with their binding affinity to PC4. The adapter activity of PC4 is regulated by the phosphorylation status of its amino-terminus, so that only the non-phosphorylated form is able to interact with VP16 and potentiate transcriptional activation (140,141).
6.7. Repressors within the pre-initiation complex: a role for TFIIA

Several negative activities specifically associated with TBP have been identified. These may also serve as targets for activator proteins, which may promote the release of such negative factors from a repressed pre-initiation complex, thus "antirepressing" the promoter in question. The net effect resulting from the presence of negative activities capable of repressing the basal core promoter activity is potentiation of the stimulatory effect of genespecific activators. Some transcriptional repressors may stabilise the association of the inhibitory factors with the pre-initiation complexes, or enhance their incorporation, although no such examples have been reported yet.

Human factor Dr1 (TFIID repressor 1) is a 19kD nuclear phosphoprotein that can associate with TBP and repress both basal and activated transcription (142,143). In a cotransfection assay, Dr1 can repress transcription directed by both TATA-containing and TATA-less promoters. When Dr1 associates with TBP as a monomer, it precludes the association of TFIIIB with TBP, presumably because both Dr1 and TFIIIB compete for binding to the same or an overlapping region in TBP. Dr1 can also associate with TBP as a dimer, trimer or tetramer, and as a tetramer Dr1 inhibits both TBP-TFIIIB and TBP-TFIIA interactions. In addition to RNA polymerase II transcription, Dr1 can also repress in vitro transcription from RNA polymerase III promoters, while leaving RNA polymerase I mediated transcription unaffected (144). In addition to its TBP-binding domain, Dr1 requires an alanine-rich region for transcriptional inhibition. Interestingly, this type of domain is also required for repression in many repressor proteins that bind DNA in a sequence-specific manner. However, when the TBP-binding domain in Dr1 is replaced by a heterologous DNA binding domain, the repressive activity cannot be restored. A subset of transcriptional activators, such as E1A, appear to be able to overcome Dr1-mediated repression in co-transfection analyses. The adenoviral transactivator E1A can disrupt the interaction of Dr1 with TBP, thus allowing the productive association of TBP with TFIIA (145). The domain
of E1A required for TATA box-dependent transcriptional activation is also needed for the
dissociation of Dr1 from the pre-initiation complex.

Dr2 activity has been purified to homogeneity and shown to be identical to human
topoiserase I (Topol) (146,147). Dr2/TopoI associates with TBP and prevents TFIID-
TFIIA-DNA-ternary complex formation. In the absence of transcriptional activators,
Dr2/TopoI represses basal transcription specifically from TATA box containing promoters
by associating with TBP. Topoisomerases isolated from other sources than human are
unable to repress the human in vitro transcription system, and the repression mechanism of
Dr2/TopoI remains unclear, since a TopoI mutant lacking the DNA unwinding activity is
still capable of repressing transcription. In addition to repressing basal transcription in the
absence of an activator, Dr2/TopoI can also effect a net increase in activation by a chimaeric
activator GAL4-AH; together the events acting on both basal and activated level lead to a
high activator-mediated transcriptional induction. The transcriptional activation by
Dr2/TopoI requires TAFs, whereas the repression effect can be seen with recombinant TBP
alone.

In the absence of an activator, TFIIA is capable of overcoming the repression imposed by
TBP-associated Dr2/TopoI (147). It has thus been hypothesised that the function of TFIIA
is to remove negative components present in the TFIID-complex. This could commit the
complex to a productive pathway, and render it further responsive to activators. The
observation that TFIIA is not required for those in vitro transcription systems that use
recombinant TBP (148), which - unlike the TFIID-complex purified from cell extracts - is
devoid of the negatively-acting factors, is in complete agreement with this model.

A TBP-inhibitory factor, ADI (ATP dependent inhibitor), has also been identified in yeast
that prevents the DNA binding of TBP in an ATP-dependent manner (149). ATP
hydrolysis seems to be required for the inhibition, since non-hydrolyzable ATP analogues
cannot substitute for ATP. TFIIA can counteract the effect of by ADI by stabilising the
binding of TBP to DNA; TFIIB also has the ability to prevent dissociation of TBP from DNA, but to a much lesser degree than TFIIA. Sequence analysis has revealed ADI to be the product of the mot1 (modifier of transcription 1) gene, which was originally isolated in a genetic yeast screen for mutants causing transcriptional derepression of several genes (150). The repression of transcription by ADI appears specific to RNA polymerase II system, and the presence of a TATA box is not sufficient to make a promoter regulatable by ADI. Recently, ADI has been demonstrated to be a yeast TAF, yTAF170, but does not appear to be part of the major yeast TFIID complex (151).

6.8. Action of sequence-specific transcriptional repressors

Perhaps the simplest means to repress transcription is to prevent promoter access by transcriptional activators, by steric occlusion or by cytoplasmic partitioning of the activator (152-154). However, it is becoming increasingly clear that repressors can also function by binding to promoters in a sequence-specific manner without impeding the access of other components of transcription machinery. By analogy with transcriptional activators, these factors presumably mediate repression by interfering with the activity or assembly of the basal transcription machinery, or perhaps by the enhancement of incorporation of inhibitory factors, such as Dr1, Dr2, and ADI. Accordingly, such factors possess specific repression domains (3,155,156).

The Even-skipped (Eve) protein belongs to the large family of homeodomain proteins that control the embryonic development of Drosophila (see Chapter 6). Eve can repress its target promoters, such as the Ubx promoter, by specifically binding to upstream sites and interfering with the assembly of a functional pre-initiation complex (157). Eve does not affect the kinetics of the assembly reaction but rather reduces the number of functional pre-initiation complexes that form on the promoters. Pre-initiation complexes become resistant to Eve repression early in the assembly pathway, which indicates that Eve affects one of the
first steps in their formation. The immediate physical target of Eve repression remains to be determined.

Utilising the in vitro transcription assay, it has been shown that basal transcription machinery is also the target for the repression mediated by unliganded thyroid hormone receptor (TR) (158). Like Eve, TR affects an early step in pre-initiation complex formation, and fully formed PICs are refractory to repression. In vitro, the transcriptional repression by TR appears independent of TAFs. A specific physical interaction between TFIIB and TR has been observed (158,159), the result being in agreement with the earlier observations that TFIIB is capable of contacting several other members of the steroid/nuclear receptor superfamily (160). Two distinct regions of TFIIB are targeted by two distinct regions of TR, one of the two regions in TR overlaps with its ligand binding domain. Accordingly, the appropriate ligand thyroid hormone, which converts TR into an activator significantly decreases the interaction between TR and TFIIB, perhaps by inducing a conformational change in the ligand binding domain.

In haploid α-mating type S. cerevisiae cells α-specific genes are repressed by the α2-MCM1 complex that binds to the conserved operator sequences upstream of each α-specific gene (161; see Chapter 5). The occupancy of the operators by α2-MCM1 in vivo is not sufficient for efficient repression, but rather requires two additional proteins known as SSN6 and TUP1. SSN6 and TUP1 associate to form a protein complex, involved in transcriptional repression of a diverse set of genes, including α-specific, haploid-specific and glucose-repressible genes (162). SSN6/TUP1 is thus proposed to function as a general repressor activity, targeted to specific promoters by interaction with DNA-bound complexes, such as α2-MCM1. This recruitment to DNA appears to be carried out by TUP1, which contains seven β-transducin repeats, capable of interacting with the α2 (163). SSN6/TUP1-complex appears to interfere directly with either the assembly or function of the basal transcription machinery, rather than to inhibit the function of transactivator proteins (164). All the genes naturally repressed by α2-MCM1 are transcribed by RNA
polymerase II. Nevertheless, it was recently shown that when α2-MCM1 operator is positioned upstream of a transcriptional initiation site in the context of RNA polymerase I promoters, α2-MCM1 could repress these in an SSN6/TUP1-dependent fashion (165). In contrast, no effect was seen on RNA polymerase III promoters. These results imply that the SSN6/TUP1 repressor complex may target, and maybe interact directly with, a component of the basal transcription machinery common to both RNA polymerase I and II systems. The putative SSN6/TUP1 target is either not a component of the RNA polymerase III machinery, or is not accessible to the repression effect in RNA polymerase III transcription complexes. Intriguingly, SSN6/TUP1 may provide an example of a negative adapter activity transferring a repressive signal to the basal transcription machinery. The SSN6/TUP1-complex lacks any detectable independent DNA binding activity, but as expected for a negatively-acting adapter, SSN6 can act as a LexA-operator-dependent and TUP1-dependent transcriptional repressor when brought to DNA as a fusion with the heterologous LexA DNA binding domain (161). In contrast, the repression by a LexA-TUP1-fusion protein is not dependent on the presence of SSN6 (166); thus, in addition to linking the repressor complex to DNA binding proteins, TUP1 exhibits partial independent repressor function. Indeed, TUP1 contains two alanine-rich regions, implicated in repressor function of many regulatory proteins. By use of a reconstituted oestrogen-responsive transcription system in yeast, it has been shown that SSN6 can repress transcriptional stimulation by the oestrogen receptor, suggesting that a mammalian counterpart to the SSN6 protein may exist (167).

7. Conclusions and implications for transcriptional synergy

Transcriptional activators appear to enhance transcriptional initiation by interacting favourably with adapters and/or members of the basal machinery, displacing general or specific negative regulators, or altering chromatin structure thus enabling themselves or other regulatory factors to engage certain critical promoter regions. In a similar manner, transcriptional repressors are thought to inhibit either the formation or the function of a pre-
initiation complex. The nature of the biochemical consequences of interactions between regulatory factors and basal transcriptional machinery remain largely unknown. An interaction may cause a conformational change or other type of modification in a target protein. This could possibly lead to the stabilisation of a pathway intermediate, or perhaps to novel or altered interactions between members of basal transcription machinery. Eventually signals from activators have to catalyse the switch to increased transcription; the phosphorylation of RNA polymerase II CTD, which facilitates its escape from the core promoter, might be the late event, where signals finally merge.

It is not yet clear, how limited the possible varieties of targets are for regulatory factors bearing a certain type of activation domain, but the list of interactions between certain members of the basal transcription machinery and specific activators is rapidly growing. For example, the well-studied acidic activation region of VP16, consisting of two subdomains, has six reported potential targets in mammalian cells: TBP, TFIIIB, dTAF40, TFIIH, PC4 and a possible mammalian homologue of the yeast ADA-complex (see Fig. 3). The p53 protein can interact with TBP, dTAF60, dTAF40 and TFIIH, whereas CREB can contact both dTAF110 and an adapter protein CBP. That activator proteins function at multiple steps is evidenced by the observation that raising the concentration of a single basal transcription factor does not overcome the requirement for activators (112). It remains to be seen whether the necessary steps towards the formation of a functional pre-initiation complex and transcription initiation are the same for every promoter, how many separable steps are necessary, and whether these steps have to take place in a strict temporal order.

Typical eukaryotic promoters contain a mosaic arrangement of DNA binding elements for several activators and/or repressors. The effect of multiple activators acting on the same promoter may lead to a synergistic effect on the stimulation of transcriptional initiation; in other words, two or more activators produce transcription levels greater than the sum of the levels resulting from the action of individual activators (168). Synergistic activation may be a consequence of co-operative DNA binding of transcription factors (169,170); however,
certain activators can also work co-operatively under conditions at which their DNA binding sites are saturated (171). The finding that several components of the transcriptional machinery can serve as targets for activators offers an alternative explanation for synergistic activation; in this model (see Fig. 4) activators bound to adjacent sites could contact different targets in the transcriptional machinery thus enhancing the rate of assembly or stability of this complex.

Artificial or natural test promoters containing multiple binding sites for a single activator can sometimes be synergistically activated, for example, Sp1 is capable of synergistic activation of the promoters containing multiple Sp1 sites, even if the DNA binding does not appear co-operative (172). This may be explained by the activator under study having the capacity to contact multiple targets. The in vivo synergy on promoters consisting of binding sites for several distinct activators appears to depend on the extent of "co-operation compatibility" between factors, and some response elements seem to co-operate nearly universally, whereas others exhibit strong selectivity (173). It will be of great interest to determine whether this in any way correlates with the variety of contacts that the regulatory factors under examination can make with the adapters and/or the basal transcription machinery.

Finally, the recently reported existence of partially pre-assembled pre-initiation complexes, holozymes (32), in yeast could simplify models for synergistic transcriptional activation. In the classical stepwise pathway for pre-initiation complex assembly, any of the successive stages is a potential target for activators. The pre-assembled factors could present a contiguous target surface for multiple activators and as a consequence the simultaneous action of these activators could recruit the complex to the TFIID-bound promoter DNA in a co-operative manner.

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Fig. 1. Stepwise assembly of the pre-initiation complex on an RNA polymerase II-dependent promoter. The template commitment step is illustrated as taking place through either TATA box or Inr element recognition by TFIIID. The subsequent individual assembly steps are described in the text. Once TFIIIE and TFIIH are recruited to the complex, the carboxy-terminal domain of the large subunit RNA polymerase II (CTD) becomes hyperphosphorylated, which leads to release of the RNA polymerase II from the promoter (an event referred to as promoter clearance) and initiation of transcription. The holozyme indicated at the right of the figure is a partially pre-assembled complex which has been identified in yeast; it is probable that this can be recruited to the template in a amplified assembly process.
Fig. 2. Components of TFIID. TATA box recognition is mediated by the TATA box binding protein (TBP) which also serves as a template for the assembly of other factors (TBP associated factors, or TAFs). Some of these factors are indicated as interacting directly with TBP (TAF30α, TAF40, TAF60, TAF150 and TAF250), consistent with published data (see Table 1), whereas others (TAF30β, TAF80 and TAF110) appear to require contacts with other TAFs for their incorporation into TFIID. TAF150 is thought to be required for Inr element recognition.
Fig. 3. The viral transactivator VP16 has multiple targets in the basal transcriptional apparatus. The identified interactions between VP16 and specific proteins in TFIID or other basal transcriptional components are indicated by connecting arrows. The TFIID components are represented in the same format as Fig. 2; the other TFII components have been superimposed onto this structure such that TAF50a and TAF50b are obscured. Only the identified targets are highlighted. In addition to TFII components, VP16 can interact with adaptor proteins, and these are indicated by the PC4 and the putative mammalian ADA-like complex interactions. The TFII targets for interactions of the adaptors are unknown, although PC4 has been shown to interact with TFIIA (see text).
Fig. 4. A model for transcriptional synergy. In the left-hand panel, two sequence-specific transcription factors, X and Y, are shown binding to upstream promoter elements. Each factor is capable of interacting with a different component of the basal transcription apparatus. In this hypothetical example, factor X interacts with TAF60 while factor Y interacts with TAF110; this representation is not meant to imply that interactions with other TFII components cannot participate in synergy. The consequence of this two-site interaction is the synergistic enhancement of recruitment, stabilisation, or modification of the properties of the basal transcriptional machinery. In the right-hand panel, the sequence-specific transcription factors, X and Y, are only capable of interacting with the same component of the basal transcription apparatus (shown here as TAF60) with a consequent inability to show co-operativity in transcriptional enhancement.
References


