Signal transduction through JAK1: the effect of a mutant on the induction of Class II HLA by Interferon-gamma, antiviral responses, and the response to dsRNA.

Timothy Mark Williams.

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Biochemical Regulatory Mechanisms Laboratory
Imperial Cancer Research Fund
44 Lincoln's Inn Field
London
WC2A 3PX

University College London
Gower Street
London
WC1E 6BT
In memory of my parents.
ABSTRACT.

The protein tyrosine kinase JAK1 is integral to signal transduction in response to the Interferons (IFNs), many cytokines and a number of growth factors. A recombinant mutant of JAK1 (JAK1ΔB) with an interesting dominant-negative phenotype has been characterised. Stable expression in human cells of the mutant JAK1 has no effect on the activation of the JAK/STAT pathway by IFNγ but results in selective inhibition of the induction of Class II HLA and the antiviral response. There is little effect on the response to α/β IFNs.

Consistent with the inhibition of the Class II response, IFNγ induction of the transcription factor CIITA (which is essential for expression of Class II) is inhibited, as is expression from reporter constructs driven by the IFNγ-inducible promoter of CIITA. There is no detectable effect on factors known to interact with the promoter, suggesting a possible inhibition by JAK1ΔB of a novel JAK1-dependent signal(s) regulating the assembly or function of higher order transcription complexes.

Despite the relative selectivity of the effects on CIITA and Class II HLA, expression profiling using DNA oligonucleotide arrays indicated that as many as 10% of the IFNγ-induced genes may be affected by the mutant JAK1.

JAK1ΔB inhibits the induction of genes in response to dsRNA, reflecting an inhibition of both NFκB activation and IRF3 function.

Although IFNγ primes the induction of IFNβ and, for example, the activation of p38 MAP kinase in response to dsRNA, the results of a more detailed analysis argue against a major role for IFNγ priming of dsRNA induction of Type I IFNs or IFN inducible genes in the primary antiviral response.
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Life is a journey. Travel it well.
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Interferons (IFNs) were originally identified as factors secreted by cells in response to viral infection (94). They are powerful regulators of cellular activities, and exposure to IFNs induces several types of response. Foremost amongst these is the development of an antiviral state which inhibits the replication of many viruses, but IFNs also regulate antiproliferative and immunomodulatory responses, and the development of immune cells. Analysis of IFN signalling led to the identification of a novel signal transduction pathway involving the Janus receptor-associated tyrosine kinases (Jaks) and their substrates, a family of transcription factors called STATs (Signal Transducer and Activator of Transcription). The role of Jak/STAT pathways in transducing signals from a wide range of ligand receptors is now well characterised and the focus of research is broadening to address the nature of additional signals which modulate responses activated by these pathways.

Interferons.

The IFNs are a family of proteins with distinctive biological activities (reviewed in (47)). IFNs are classified into two groups: each group uses a discrete receptor complex, but all members of the family utilise Jak/STAT pathways to effect signal transduction to the nucleus. Expression of IFNs is strongly induced by virus infection, bacterial endotoxin (LPS), double-stranded RNA (dsRNA) and immunostimulatory cytokines such as interleukin-1 (IL-1), IL-2 and tumour necrosis factor α (TNFα).

The ability to competitively bind to the Type I receptor characterises the Type I or α/β IFNs, of which there are three subgroups. In humans, there are 14 non-allelic
functional genes and one pseudogene for the α⁺-IFN group. IFNω is the only expressed member in the α⁺-IFN group, which also contains an additional 5 pseudogenes. IFNβ is the lone member of the third group of Type I IFNs. The secreted forms of the Type I IFNs are of 165 or 166 amino acid residues (except IFNω which has 172 residues), are glycosylated, and bind to the Type I or α/β receptor as monomers. The genes of the Type I IFNs are intronless, and are all clustered on the short arm of chromosome 9 in humans.

The significance of so many IFNα-subtypes is not defined. Their biological activity per molecule is variable, such that IFN-α8 and IFNβ elicit a stronger antiviral response than the other α/βIFNs (61), but the basis of these differences - mediated through binding to the same receptor - remains unknown.

In contrast to the diversity of α/β IFNs, IFNγ is the only Type II IFN and utilises the Type II or IFNγ receptor. Interestingly, no sequence or evolutionary relationship between IFNγ and the Type I IFNs has been shown. IFNγ is present as a single copy gene on chromosome 12, and is a 143 residue glycosylated protein, which homodimerises to form the active molecule.

IFNs are produced by different cells in response to a variety of stimuli. This contributes to functional compartmentalisation of responses to the different IFNs. Broadly speaking, it is believed that all cells can produce α/β IFNs when challenged with virus or dsRNA. IFNα is produced by a sub-type of leukocytes, and is also known as leukocyte IFN, and IFNβ is produced by fibroblasts. Production of IFNγ, or immune IFN, is restricted to lymphocytes, and is not induced by viral infection.
Cellular responses to IFNs.

Responses to IFNs can be placed into three broad and overlapping categories - antiviral, antiproliferative and immunomodulatory (reviewed in (99)). Within these categories the different IFNs induce specific, but overlapping sets of genes which contribute to the overall phenotypes.

Antiviral responses.

The classical response to IFNs is the transition of the cell to an antiviral state in which virus replication is inhibited by a variety of mechanisms (195, 208). IFNs “prime” cells by stimulating production of antiviral proteins such as dsRNA-dependent protein kinase (PKR) and dsRNA-dependent 2’-5’ oligoadenylate synthetase (2-5 OAS). Priming increases the capacity of a cell to respond to the stimuli associated with virus infection, namely viral proteins or double-stranded RNA. Other major proteins induced by IFNs which are believed to have antiviral effects include GBP, the Mx proteins and 9-27, the roles of which are described later. The α/β IFNs are the main stimulators of antiviral responses and are themselves directly induced by virus, producing a signal to induce an antiviral state in surrounding tissues. IFNγ has antiviral properties too, but because its expression is restricted and it is not induced by virus it plays a more minor role in countering viral infections. In this way, IFNα/β receptor knockouts have more serious effects in mice infected with virus than IFNγ receptor knockouts (241).
Antiproliferative responses.

Another type of response to IFNs is the antiproliferative response (reviewed in (34)), which is increasingly linked to STAT1, activation of which by IFNs is reportedly defective in many tumours. Beyond this, the mechanism of the antiproliferative effect(s) of IFNs is unresolved, and is probably complex, but seems to involve inhibition of either the G0/G1 transition or the initiation of S-phase (10, 138). Several cell cycle regulatory proteins are reported to be modulated by IFNs (reviewed in (105)). Examples of this include the induction of CDK inhibitors such as p15/16 and p21\textsuperscript{WAF1/Cip} by IFNs (28, 147, 198, 230) or the decrease in levels of cyclin D and cdc25A (234). IFNs also affect the expression of proto-oncogenes (e.g. c-myc, c-fos and c-ras). The antiproliferative response may also reflect aspects of the antiviral response, through inhibition of protein synthesis and the general reduction in cellular metabolism.

In addition to influencing cell-cycle progression, IFNs can induce both pro-apoptotic and anti-apoptotic responses in several cell types, in conjunction with other stimuli such as TNFα (116), LPS and dsRNA (51). As with the antiproliferative effects, STAT1 seems to be important for IFN regulation of apoptosis, although IRF1 may be important too (197). Again, it is possible that this type of response is related to the antiviral response, being mediated by antiviral proteins (275). The death of a cell infected by virus before the full cycle of virus replication reaches completion would be advantageous to the organism. There is evidence of a co-operative interaction between IRF1 and p53 (232) leading to cell-cycle arrest but not apoptosis. Novel IFN-regulated genes encoding death associated proteins (DAPs) have also been identified which are necessary for IFN-induced apoptosis in HeLa cells (49, 92, 130).
Immunomodulation.

In addition to their central role in the antiviral response IFNs also regulate other aspects of immune responses on several levels (reviewed in (17, 241)). IFNs are strong inducers of antigen presentation, upregulating both MHC Class I and Class II proteins. Both types of IFN induce Class I which presents endogenously-derived peptides. The effect of this is to stimulate cytotoxic responses by CD8 positive cells against intracellular pathogens such as virus and parasites. IFNγ also induces Class II which presents exogenous peptides, normally on professional antigen presenting cells (APCs) that stimulate the CD4 positive T cell response. The nature of the peptides presented is also regulated by IFNs which can alter the subunit composition and therefore the activity of proteasomes, perhaps biasing specificity towards the presentation of viral proteins.

The IFNs also regulate the immune system by moderating the activities of immune cells. The IFNs contribute to macrophage activation, and are an important inducer of responses to intracellular parasites through the induction of genes such as inducible nitric oxide synthase (iNOS) and indoleamine 2,3-dioxygenase (IDO). IFNγ also regulates the immune response by influencing the development of T cells. In conjunction with IL-12 it drives helper T cell differentiation towards the TH1 rather than TH2 subtype. TH1 cells in turn produce IFNγ which stimulates macrophages and natural killer cells. In this way IFNγ favours the cell-mediated rather than humoral response, but IFNs also regulate B cells directly, leading to immunoglobulin class-switching.

Regulation of IFN-responsive genes.

Regulation of genes by IFNs (reviewed by Stark et al. (224)) depends broadly on four groups of proteins - receptor chains, Jaks, STATs and the IFN regulatory
factors (IRFs) which are a family of transcription regulatory proteins induced by IFNs. IFN-responsive genes can be grouped into several categories. Primary genes are stimulated directly following ligand-receptor binding, being regulated by pre-existing transcription factors i.e. the STATs. IFN regulatory factor 1 (IRF1) and guanylate-binding protein (GBP) are both induced in this way. Secondary genes like MHC proteins and CIITA require protein synthesis to occur and are dependent on the products of primary genes such as IRF1. For others, such as 9-27, transcription does not require protein synthesis but is enhanced by it. Additionally there are genes inhibited by IFN, such as myc.

The effects of cytokines and growth factors are generally dependent on de novo protein synthesis, and result from regulated expression of genes via the Jak/STAT pathway. Estimates of the number of genes induced in response to IFNs vary. A recent review listed over ~200 IFNγ inducible genes (17). Analysis of gene expression using oligonucleotide microarrays identified over 100 IFN-induced genes and 20 that were repressed (52). The recent completion of a draft of the human genome has allowed informed estimates of the number of genes present therein and these vary from ~35,000 (57, 189) to ~120,000 depending on the method of estimation. Given that there were approximately 6800 genes represented on the microarray, a conservative estimate of the number of genes regulated by IFNs is around 600. Clearly the IFNs are powerful pleiotropic agents.

The initial identification of IFN-responsive genetic elements in the promoters of ISGs facilitated identification of the components of the IFN signalling pathways. IFN-responsive DNA-binding factors were purified isolating the first of a family of transcription factors called the STATs (Signal Transducers and Activators of Transcription). Identification of the balance of the proteins required for signalling in response to IFN was through two genetic approaches based on the complementation of cell lines mutagenised to be unresponsive to IFN. These approaches, described below, revealed the role of the Jak family of tyrosine kinases in the ligand-dependent activation
of the STATs. The cell lines generated were defective in 8 proteins involved in signal transduction regulating IFN-responsive genetic elements, and these cell lines have been invaluable in subsequent work to analyse the interactions and regulation of the individual proteins.

**IFN response elements.**

Two types of element characterise the majority of IFN-stimulated genes (40, 200). The IFN-stimulated response element (ISRE) is typical of genes responsive to α/β IFN, and the gamma-activated sequence (GAS) is typical of IFNγ responsive genes, but as discussed below, this distinction is not rigid. The characteristic ISRE is a direct repeat of GAAA with two or three intervening bases giving a consensus along the lines of GAAANN(N)GAAA, and confers inducibility by IFNα/β on a heterologous promoter (129). The major regulator of ISREs is the ISGF3 complex activated by α/βIFNs, but additional factors which bind include IRFs induced by IFNs or activated by virus or dsRNA.

GAS elements are loosely palindromic and the consensus often cited by reviewers is TTNNNNNAA, with two pairs of A's or T's flanking five intervening bases. The symmetrical or palindromic nature of the GAS and ISRE would be consistent with dimeric STATs binding to a particular site - presumably each monomer binding to a half site.

**Genetic analysis of signalling in response to IFNs.**

The mutagenesis of cells to produce lines defective in responses to IFNs involved two approaches (reviewed in (40, 179)).

In the 2fTGH system (178) a construct containing the bacterial gene for guanosine phosphoribosyltransferase (gpt), under the control of the IFNα/β inducible
6-16 promoter, was stably transfected into HPRT HT1080 cells to produce the 2fTGH cell line. Expression of gpt - the bacterial homologue of HPRT - confers susceptibility to the drug 6-thioguanine (6TG). So cells which respond to IFNα/β express gpt, and in the presence of 6TG are unable to grow, allowing selection of cells unresponsive to IFN. Importantly, HPRT cells cannot recycle guanine nucleotides. As a consequence, 2fTGH cells cultured in the presence of aminopterin - an inhibitor of de novo purine synthesis - fail to grow. If hypoxanthine and thymidine are present, and the cells are maintained in IFN (inducing expression of gpt) this permits purine synthesis via the salvage pathway, restoring growth. Thus, the 2fTGH-derived cells could be selected on the basis of expression or lack of expression of gpt when cultured in the presence of IFNα/β.

The 2C4 system (254) used a cell line stably transfected with the cell-surface CD2 marker regulated by the IFNγ-inducible 9-27 promoter. These 2C4 cells could be selected for high or low expression of both CD2 and also endogenous HLAs when treated with IFNγ. Again this allowed the selection of cells that were unresponsive or responsive to IFNγ.

The 2fTGH and 2C4 cells were mutagenised with the frame shift mutagen ICR 191, and cells unresponsive to IFNα/β or γ were selected. These unresponsive clones were then complemented, re-selecting on the basis of restored IFN responsiveness. The complemented mutants fell into eight complementation groups, with each mutation affecting signalling from one or both of the IFN receptor complexes. These are listed in the Table 1.1., together with the proteins able to complement the signalling defects.

The mutants have proven invaluable in three ways -

- showing that the Jaks and STATs are essential for responses to IFNs,
- identifying a role for the Jaks,
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- as a negative background for the analysis of Jak/STAT signalling.

For example, the importance of serine and tyrosine phosphorylation of STAT1 for transcriptional responses was shown using U3 cells complemented with STAT1 defective for either one of these phosphorylation events (88). The analysis of signalling in response to other cytokines which use the Jak/STAT pathway has also benefited from the mutants, which were used, for example, to show the importance of Jak2 but not Jak1 for signalling in response to growth hormone.

**Jak/STAT pathways.**

**The Jaks.**

The Jaks were first identified by screens for novel kinases (258). Complementation of cells in the U1 group demonstrated the role of Jak family kinases in IFN signalling (244). There are four known members of the human Jak family: Jak1, 2 and 3, and Tyk2. Expression of Jak3 is restricted to haematopoietic cells, but the other Jaks are ubiquitously expressed. They range in mass from 120kDa to 140kDa, and contain seven regions of close homology, referred to as JH1-7 (80) (**Figure 1.1**). JH1 is the C-terminal tyrosine kinase domain and adjacent to this is a pseudokinase domain homologous to JH1, designated JH2. The pseudokinase domain lacks critical residues within catalytically important motifs (258), and its role remains unclear but it may be involved in intra-molecular regulation. Point mutations in the JH2 domain of Jak2 and Tyk2 lead to a constitutively active kinase (144, 265). JH3-7 are believed to mediate interactions with receptor chains based on work with chimeras using the N and C-terminal regions from different Jaks (111).

Until recently, with the exception of the Jak homology domains mentioned above, no other structural motifs which might mediate protein-protein interactions such as SH2, SH3, PTB or pleckstrin homology (PH) domains had been positively identified in the Jaks. However, a close structural homology to the FERM (4.1/ezrin-
radixin-moesin) domain (reviewed in (29)) was identified in the N-terminal half of Jaks (65), congruent with JH7-4. Other proteins containing a FERM-domain are known to interact with transmembrane proteins or signalling proteins involved in the membrane proximal Rho pathway, and a Jak FERM domain may target Jaks to the plasma membrane or mediate interactions with the receptor complex. This would be consistent with the function already ascribed to the part of the Jaks containing the FERM domain, namely localising Jaks to the receptor. The identification of the FERM domain provides a focus of the analysis of Jak interaction with other signalling proteins, and functional analysis should prove enlightening.

The major function of the Jaks is as receptor associated signal transducing kinases. The Jaks interact with the juxta-membrane cytoplasmic region of receptor chains. These receptor chains often contain two motifs called Box 1 and Box 2 in a membrane proximal area. Ligand-binding is believed to induce reciprocal phosphorylation of Jaks associated with the receptor chains. This activates the Jaks which can transduce the signal through additional proteins recruited to the activated receptor complex, most commonly the STATs. Jaks also interact with receptors having their own intrinsic tyrosine kinase activity such as the receptors for PDGF (246) and EGF (45, 122). These receptors also activate STATs but the Jaks do not appear to be required for this, and their role is not known.

The determinants of substrate specificity of the Jaks are unclear but it seems that recruitment of substrate to the specific receptor complex with which a Jak associates controls substrate phosphorylation, rather than a specificity per se of the Jak. This was shown using a chimeric receptor in which the moiety which recruits STAT3 to the gp130 receptor was found to recruit it to the erythropoietin receptor (222) leading to EPO-dependent STAT3 activation.

In addition to functioning as activators of STATs, the Jaks are involved in an increasing number of ligand-dependent functions, described later.
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The STATs.

STATs were first identified by purifying proteins which bound to IFN-responsive DNA elements (202). Seven mammalian STAT genes have been identified—designated as STATs 1-6 including STAT5a and STAT5b. The STAT proteins range in size between 84 kDa and 113 kDa. STATs 1, 2, 3 and 6 are expressed widely, STAT4 predominantly in the testis and thymus, and STAT5 is restricted to the mammary gland and other tissues. There are two splice variants of STAT1, the 91 kDa STAT1α and the 84 kDa STAT1β which has a different carboxyl terminus and lacks the transactivation domain and phosphorylatable serine.

A number of common features have been identified structurally and functionally (Figure 1.1)(reviewed in (26, 213)), and the crystal structure of a STAT1 homodimer bound to DNA has confirmed the importance of several of these (26).  

- Within the C-terminal region they contain a highly conserved tyrosine which is phosphorylated during STAT activation. This is located in an SH2 domain which is involved in protein:protein interactions. The SH2 domain is required for recruitment of STATs to the phosphorylated receptor chain and STAT dimerisation leading to DNA-binding.  
- C-terminal of this SH2 domain is a domain important for transactivation. The splice variant of STAT1 called STAT1β lacks the C-terminal 38 amino acids present in STAT1α and does not activate transcription (161). With the exception of STAT2 and STAT6 this C-terminal region contains a conserved serine which is inducibly phosphorylated, and is required for maximum transactivation of many promoters (reviewed in (48)). Binding studies have also shown that the C-terminal is involved in interactions with histone acetyltransferase proteins such as CBP and p300 (15, 272).
• The N-terminal half of the STATs contains a region which may be involved in DNA-binding and also a putative coiled coil region which may mediate protein:protein interactions between STATs and transcriptional partners such as p48 or Jun. The very N-terminal region has been implicated in interactions between adjacent STAT dimers (247) bound to tandem STAT-binding promoter elements such as in the IFNγ promoter (260). This N-terminal region is also thought to mediate interactions with CBP and p300 (272).

• In the centre of the STATs a linker region between the SH2 domain and the N-terminal half of the protein contains an SH3-like domain, and has been shown to be essential for transactivation in STAT1 (263).

• Recent analysis of STAT1 has revealed three functional leucine-rich nuclear export signals (NESs). Two are located in the coiled coil domain, encoded within residues 197-205 (158) and 308-315 (13), tagging of which results in accumulation of STAT1 in the nucleus. The third lies in the DNA-binding domain (154). The function of these NESs is sensitive to the drug leptomycin B (LMB), which is known to suppress nuclear export by inhibiting binding of Crm1 to leucine-rich export signals.

Interestingly, no classical nuclear localisation signal (NLS) has been reported in STATs. One explanation is that STAT dimerisation is functionally equivalent to an NLS, allowing interaction with nuclear import proteins. Alternatively, STATs may interact with chaperones which mediate their nuclear import.

The standard model of STAT activation is that they are recruited via their conserved SH2 domain to phosphotyrosine containing moieties on activated receptor complexes. This is corroborated by STAT SH2-domain swap experiments in which recombinant STAT2 containing the STAT1 SH2 domain was recruited to the IFNγ receptor (84). Once recruited, a STAT is then phosphorylated by a receptor-associated
Jak tyrosine kinase. But there are exceptions to this, such as the recruitment of STAT5 by Jak2 itself at the gp130 subunit of the IL-6 receptor (63).

Although it appears that the IFN system is restricted to mammals, the Jak/STAT pathway is more widespread. A Jak/STAT-related pathway operates in *Drosophila*, where it is involved in developmental patterning in embryos (262). The Jak homologue is called hopscotch and the STAT homologue called D-STAT, or Marelle. DNA-binding by D-STAT is dependent on tyrosine phosphorylation, and it binds to a similar DNA sequence to mammalian STATs (reviewed in (271)). These proteins are known to regulate proliferation and developmental events, and a dominant mutant of hopscotch called Tumorus-lethal acts in an analogous fashion to an oncogene, leading to abnormal proliferation and differentiation (143). Interestingly this mutant is the product of a single amino acid substitution which produces a hyperphosphorylated protein, and the corresponding mutation in Jak2 produces an analogous phenotype (144).

Even *Dictyostileum* has a STAT-like protein (102) which homodimerises through reciprocal SH2-phosphotyrosine interactions and binds to ISRE-like elements, although a Jak-like partner has yet to be identified.

**Jak/STAT signalling through the Type I receptor.**

The Type I receptor is bound by all the different α/βIFN sub-types. The receptor contains two chains designated as IFNα/β receptor 1 and 2 (IFNAR1 and 2), both of which are required for full responses to Type I IFNs. IFNAR2 is expressed as three splice variants, yielding one soluble (IFNAR2a) and two transmembrane proteins (IFNAR2b and c) (167). Only IFNAR2c is able to complement U5A cells which are unresponsive to Type I IFNs (145). Tyk2 associates with IFNAR1 (35, 36) and phosphorylates it *in vitro*, and Jak1 associates with IFNAR2.

Monomeric Type I IFN binds to both receptor chains, oligomerising the two chains to initiate signalling (reviewed in (224)). Receptor-associated Jak1 and Tyk2
cross-phosphorylate leading to their activation, and consequently the tyrosine phosphorylation of IFNAR1 and 2c. IFNAR1 phosphorylation leads to recruitment of STAT2 through the SH2 domain of the transcription factor. It is thought that STAT2 initially interacts with IFNAR2c via a non-SH2-phosphotyrosyl interaction, but translocates to IFNAR1 following ligand binding and subsequent oligomerisation of IFNAR1 and 2 (261). STAT1 is believed to interact with the receptor via STAT2 (91, 128), and also translocates concomitantly. Both STAT1 and STAT2 are rapidly phosphorylated on a conserved tyrosine residue (at positions 701 and 690 respectively) following ligand binding to the Type I receptor. They heterodimerise and translocate to the nucleus where they bind to IFNα/β-responsive ISREs in conjunction with a protein called p48 - a member of the IRF family of proteins described later. This complex of STAT1, STAT2 and p48 is called ISGF3 (IFN-stimulated gene factor 3).

Not all transcriptional responses to Type I IFNs are mediated by ISGF3. STAT1 homodimers and STAT1-STAT2 heterodimers are also formed and can induce transcription through GAS elements more typically found in the promoters of IFNγ-regulated genes. For example, IFNα induction of IRF1 is mediated through the GAS element in the promoter, which also regulates IFNγ induction of the same gene (76, 132).

**Activation of the Jak/STAT pathway by IFNγ.**

The Type II receptor (reviewed in (9, 59)) is ubiquitously expressed and consists of a 90-kDa α chain, also known as IFNGR1 and a 62-kDa β chain, known also as IFNGR2 or accessory factor 1 (AF1). IFNγ binds to its receptor as a homodimer, interacting with IFNGR1, suggesting that two IFNGR1 chains homodimerise, each bringing with it an associated IFNGR2 chain (59). Jak1 is associated with IFNGR1, and Jak2 with IFNGR2 (113) and, as for the Type I
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receptor, these associations appear to be constitutive. Receptor chain dimerisation induces trans-phosphorylation of the Jaks, and subsequent phosphorylation of IFNGR1 on tyrosine residue 440 by the activated Jaks (70, 89). IFNGR2 does not appear to undergo tyrosine phosphorylation. Experiments with kinase-negative mutants of Jak1 and Jak2 indicate that Jak2 plays the key role in the phosphorylation events at the receptor (18). A kinase negative Jak1 (Jak1K>E) expressed in Jak1 negative U4A cells is sufficient for inducible phosphorylation of both Jak2 and Jak1, whilst there is no phosphorylation at all in the reciprocal experiment with kinase negative Jak2K>E in Jak2 negative γ2A cells. A role for Jak1 is indicated by the observation that IFNGR1 phosphorylation and possibly STAT1α activation are less efficient in U4A/Jak1K>E. Nevertheless, physically Jak1 is absolutely required for the IFNγ response, indicating a structural as well as enzymatic role.

STAT1α is recruited to the phosphorylated receptor complex via the tyrosine 440 containing motif of activated IFNGR1 (71), and is itself phosphorylated at tyrosine 701 (203, 214, 215). This allows homodimerisation of STAT1 (40) to form a GAF, or IFNγ-activated factor, and subsequently translocation to the nucleus in a RAN/TC4 dependent fashion (207). In the nucleus the GAF binds to GAS sites in the promoters of IFNγ-responsive genes and participates in initiation of transcription. The promoters of a limited number of IFNγ responsive genes also contain ISREs, and STAT1 homodimers can also form a complex with p48 which is analogous to the ISGF3 complex and interacts with these IFNγ-stimulated ISREs, as happens with 9-27 (187).

Transcriptional regulation by the STATs.

STATs are able to regulate transcription in a variety of ways. The paradigm is that of the STAT1-STAT1 dimer or GAF which forms in response to IFNγ. This dimer
is able to bind to DNA, and presumably recruits other factors, leading to assembly of the transcription initiation complex. It seems likely that the STATs must interact with a variety of other proteins to facilitate these processes, and interactions (mostly involving STAT1) have been shown with other transcription factors, transcription co-activators and additional proteins. These interactions with STATs and their possible function are discussed later.

The interaction of STATs with IRFs is particularly interesting given the role of IRFs in IFN responses. The interaction of STAT1 and 2 with p48/IRF9 to form ISGF3 is the most obvious example. The STAT1:STAT2 heterodimer cannot bind to an ISRE without p48, which primarily interacts with STAT2. p48 provides the DNA-binding function and STAT2 is thought to be responsible for transactivation, since ISGF3 formed from STAT1β which lacks the transactivating C-terminal of STAT1α is still fully functional (161).

In addition to its interaction with p48 as part of ISGF3, STAT1 co-operates with at least one other IRF - IRF1 - to regulate constitutive transcription at the composite LMP2 promoter (25), which contains overlapping IRF-binding and GAS elements. It is important to note that tyrosine phosphorylation of STAT1 is not required for this interaction, suggesting that here STAT1 does not require tyrosine phosphorylation to translocate to the nucleus.

Direct and indirect STAT1 interactions with other transcription factors mediate the synergistic effects of cytokines. These interactions usually occur between transcription factors bound at closely spaced binding sites in promoters. NFκB is a common partner, being involved in IFNγ/TNFα induction of ICAM1 (169, 180), or IFNγ/LPS induction of iNOS and IP-10 (168). Other transcription factors with which STATs co-operate include Jun, SPI and the API heterodimer.
The mechanism by which DNA-bound STATs mediate transactivation is becoming clearer with the characterisation of STAT interactions with the histone acetyltransferase co-activators. The interaction of STAT1 and STAT2 (15) with CBP/p300 may facilitate chromatin remodelling leading to enhanced access for the transcriptional apparatus. The importance of CBP/p300 is emphasised because it is targeted by viral proteins, resulting in attenuation of IFN-dependent signalling. Furthermore, STAT1 and STAT5 interact with N-myc interacting protein (Nmi) which enhances STAT-dependent transcription (277) possibly through recruitment of CBP/p300. Finally, immunodepletion of pCIP, a protein which is known to interact with CBP, inhibited GAS-dependent transcription (112), emphasising that CBP may be required to recruit additional factors to enhance transcription.

BRCA1 was recently shown to be a co-factor which mediates differential regulation of gene expression by STAT1 in response to IFNγ (171). The function of BRCA1, which is reported to physically interact with STAT1, is not clear. It does not directly contact DNA, but interactions with CBP/p300 have been reported, and BRCA1 may enhance the recruitment of histone deacetylases to specific promoters under certain conditions. MCM5 was identified in a screen for novel proteins which interact with the transactivating C-terminal domain of STAT1 (273). MCM5 is a member of the minichromosome maintenance family of proteins involved in DNA-replication, but it is believed that MCM5 enhances STAT1-dependent transcription through an unknown mechanism - the level of nuclear MCM5 correlating with the response of a STAT1-dependent reporter.

It is interesting to speculate that the multiple NESs which have been identified in STAT1 might correlate with multiple modes of transactivation which would require masking of these NESs through different protein:protein interactions or regulatory modifications of the STATs. Irrespective of this, the importance of recruitment of additional proteins to promoters by DNA-bound STATs is now established. The nature of these additional proteins seems to be an important regulator of transcriptional
responses and may contribute to the specificity of cytokine-dependent transcriptional responses.

**Down-regulation of the Jak/STAT pathway.**

Transcription of IFN-induced genes usually peaks after a few hours and then diminishes. The decrease is sensitive to inhibition of protein synthesis, suggesting that IFN-induced factors mediate the down-regulation of transcription (120). The most immediate mechanism for the attenuation of the Jak/STAT pathway is the action of pre-existing phosphatases. Treatment of resting cells with a phosphatase inhibitor such as pervanadate can even activate the Jak/STAT pathway, confirming the involvement of phosphatases (44, 75). The SH2-containing phosphatases SHP-1 and SHP-2 are cytokine receptor-associated tyrosine phosphatases known to dephosphorylate specific Jaks and STATs (41, 78). A mutation of SHP-1 leads to elevated Jak1 and STAT1 activation following IFNα treatment (41). The importance of SHP-1 is demonstrated by the motheaten mouse (216) which lacks SHP-1 and displays multiple haematopoietic and immunological abnormalities. However, the function of both SHP-1 and 2 appears to be complex and they may also play a positive role in signalling (41, 269). In addition to these cytoplasmic phosphatases, a nuclear phosphatase activity dephosphorylates activated STATs (42, 82).

This basic mechanism of down-regulation by phosphatases is augmented by a family of ligand-inducible Jak/STAT inhibitors (reviewed in (225)). The first to be identified was termed cytokine-inducible SH2-containing protein (CIS), an inhibitor of EPO signalling. The next member of the family identified was cloned independently by three groups, and was variously identified as JAB (Jak-binding protein) (56), SOCS (suppressor of cytokine signalling) (226) and STAT-inducible STAT inhibitor (SSI), but subsequently confirmed as being the same protein, called SOCS1.
Now recognised as a family, the SOCS proteins (as they are now known) contain eight members to date - SOCS1-7 and CIS - and are characterised by the SOCS box domain, a 40 residue motif at the carboxyl-end of the protein, and an adjacent SH2 domain in the middle of the protein. However, up to twelve additional proteins have been identified which contain the SOCS box (268), although the precise function of the SOCS box itself has yet to be determined. mRNA for the SOCS proteins is rapidly induced by a variety of cytokines in response to STAT activation and constitutive expression of SOCS1 inhibits signalling in response to a variety of ligands confirming the role of the SOCS proteins as modulators of cytokine responses.

Two different mechanisms have been suggested for the action of SOCS proteins whereby they participate in an elegant negative feedback loop by inhibiting further receptor-mediated STAT-activation. Several studies suggest that SOCS1 inhibits signalling by directly interacting with Jaks and inhibiting substrate phosphorylation (56, 162, 226), and it has been hypothesised that a region in SOCS1 and SOCS3 contains a homology to the Jak activation loop with which a Jak will interact, rather than interacting with legitimate substrates. SOCS proteins may also recruit phosphatases to bring about dephosphorylation of Jaks, or trigger their degradation (see below).

An alternative mechanism is that some SOCS, such as CIS, interact with the activated receptor, preventing the interaction of Jak substrates with the receptor-associated Jaks (153, 268). It is interesting to note that SOCS proteins induced by one cytokine will inhibit the response to subsequent ligands. For example, IL-10 inhibits IFN signalling because it induces SOCS3 (95), and this type of cross-talk is probably widespread.

Distinct from the down-regulation of activated receptor components by SOCS proteins, another family of proteins interact with activated STATs to suppress their activity. These proteins of the PIAS (protein inhibitor of activated STAT) family are constitutively expressed, and specifically interact with individual STATs - PIAS1 with
STAT1 and PIAS3 with STAT3 - and this interaction is thought to require phosphorylation of the STAT (31).

Finally, internalisation and destruction of the cytokine receptor complex could represent another activation-dependent mechanism of attenuation of the Jak/STAT pathway. Specific destruction of particular proteins is also a possibility, with the demonstration that STAT1 is the target of ubiquitin/proteasome-mediated degradation (104) whilst proteasome inhibitors are reported to prolong the ligand-dependent activation of the Jak/STAT pathway (21). There is evidence that the SOCS proteins are also ubiquitinated and degraded by the proteasome, and it has been suggested that the SOCS proteins may target associated proteins for degradation too, adding an additional level of control. Interestingly, virus-dependent degradation of components of the Jak/STAT pathway has also been reported (156).

**Activation of Jaks and STATs by other ligands.**

Cytokine receptors can be categorised into four subgroups - Classes I-IV - on the basis of conserved structural features. Although first identified through their participation in IFN signalling, the Jaks and STATs are involved in a wider range of signalling through cytokines whose receptors fall into Classes I and II (reviewed in (127)). The IFN and IL-10 receptors form the Class II cytokine receptor superfamily. The Class I cytokine receptor superfamily includes receptors for other Interleukins, the gp130 cytokine family (IL-6, LIF, OSM) and several growth factors (e.g. growth hormone, GM-CSF) (reviewed in (90, 97)). In keeping with the paradigm of IFN signalling, ligand binding to cytokine receptors leads to oligomerisation or a conformational change of the receptor chains. This brings about Jak activation, receptor phosphorylation, and the recruitment and activation of STAT proteins (Figure 1.2).
Table 1.2. taken from (77) lists examples of those cytokines and growth factors which utilise the Jak/STAT pathway. Clearly, the Jaks and STATs play a major role in mediating cell-cell signalling and a range of immune, developmental and proliferative processes which require the co-ordinate activities of cells. Much research has focused on the ways in which specificity is retained when one particular system is so frequently used. Specificity can be exerted at several levels, through the cell-type specific expression of receptors to a particular ligand, or the accessibility of the promoter or enhancer within a cell (often governed by epigenetic factors), and ultimately the combination of factors activated by a particular ligand in addition to the Jak/STAT pathway, which may be crucial to whether upregulation occurs or not. At whatever level the specificity occurs it must be tightly controlled and co-ordinated, otherwise aberrant responses will lead to a variety of disease states, from immune disfunction to cancer.

Additional pathways regulating responses to IFNs.

The activation of STATs by so many different ligands implies overlap between the responses to these ligands, and that there are additional ligand-specific signals which regulate transcriptional responses (103). Moreover, it is now apparent that Jaks are not the only activators of STATs, and STATs are not the only substrate of Jaks. It is no surprise that particular ligands activate more than one signal using distinct pathways. For example, in HeLa cells PDGF and EGF can induce ISGs through activation of STAT1α (192). But PDGF activation of c-fos transcription requires other PDGF-induced transcription factors in addition to STATs (85). Thus, activation of specific additional signalling pathways contributes to specificity from ligands which use the Jak/STAT pathway. One intriguing example of this is the inhibition of c-myc expression by IFNγ in mouse embryo fibroblasts, whereas the same ligand induces c-myc RNA rapidly and transiently in STAT1 knockout cells (185). The STAT1-mediated
inhibition requires a GAS site in the c-myc promoter and STAT1 serine phosphorylation - the implication being that STAT1 acts as an inhibitor in this context, and that IFNγ sends both an inhibitory and stimulatory signal at the same time.

The Interferon Regulatory Factors.

The IRFs are an expanding family of transcription factors (reviewed in (146)) which play a major role in regulating responses to IFNs and in the induction of IFN expression. The IRFs are characterised by homologies in their DNA-binding domain, which contains a repeat motif of five tryptophan residues separated by between ten and eighteen residues. Many IRFs regulate transcription through ISRE type elements found in the promoters of most α/βIFN-inducible genes. For example IRF1 induced by IFNα regulates transcription control mediated by ISRE sequences (174) sustaining the expression of genes initially regulated by ISGF3. In addition IRFs bind to the IRE (IFN response element) (79) such as the one in the MHC Class I promoter, and the interferon regulatory element (IRF-E) or positive regulatory domains (PRDs) I and III in the IFNβ promoter (181).

IRF1 is the archetypal IRF, and plays a major role in gene expression in response to IFNs. STAT activation by both Type I and II IFNs leads to expression of IRF1 which is required for many of the responses to IFNs, such as the antiviral response and regulation of MHC proteins. Other cytokines which use the Jak/STAT pathway, in particular IL-6 (81), induce IRF1 weakly but it is not believed to play a major role here. The importance of IRF1 in IFN responses was underlined in knock-out mice (106, 107). IRF1 is thought to be regulated by serine phosphorylation. The putative phosphoacceptor residues are important for transactivation by IRF1 and may be modified by casein kinase II (16, 136). Alternatively, induction of GBP by IFNγ, which requires IRF1, seems to require PKR at some level (118).
IRF2 was originally identified as antagonistic to IRF1 (79), regulating the activity of IRF1 by binding to the same promoter elements without activating transcription, but has since been shown to function as a transcriptional activator too (242). IRF2 is not induced by IFNs and it may function primarily as a constitutive transcriptional repressor, regulating basal transcription until levels of IRF1 increase beyond a threshold, triggering induction.

p48/ISGF3γ is also known as IRF9 and modulates the DNA-binding specificity of ISGF3. IRF4/Pip and IRF8/ICSBP are expressed in a tissue specific manner and are involved in regulation of the immune system. IRF4 regulates both T and B-cell development, and IRF4 deficiency results in severe immunodeficiency. IRF8 regulates myeloid development and proliferation. Tyrosine phosphorylation of IRF8 at residues conserved in other IRFs (211) suggests a potential role for tyrosine phosphorylation in the regulation of IRF activity. IRF3 and IRF7 play major roles in the induction of the IFN genes and ISGs in response to virus infection or double-stranded RNA, as described below. Both are regulated by activator-dependent phosphorylation.

Similar to the STATs, IRFs recruit additional factors to promoters to facilitate transcriptional upregulation. Again CBP/p300 appears to be a common co-factor for various IRFs (55, 133, 253, 255), and it is intriguing that different transcription factors activated by the same ligands may utilise the same pool of co-activating proteins.

**Additional Jak1-dependent pathways.**

Several Jak-dependent pathways have been characterised which are activated in response to different ligands. IFNα induces the release of arachadonic acid, the result of Jak1-dependent activation of cytosolic phospholipase A₂ (cPLA₂) (60). Inhibitors of cPLA₂ block the IFNα activation of ISGF3 confirming the role of cPLA₂ in the response to IFNα, but no role in the IFNγ-response has been shown. Further analysis
of this pathway implicates p38 MAPK in activation of cPLA$_2$, and also in the serine phosphorylation of STAT1 (68).

The Jak1-dependent activation of Raf-1 was demonstrated in response to both IFN$\gamma$ (194), IFN$\beta$ and OSM (223), and shown to be independent of p21$^{ras}$ in the case of IFN$\gamma$. This may be a mechanism of IFN activation of p38 MAPK, although p38 MAPK requires both threonine and tyrosine phosphorylation for activation, suggesting a direct role for Jak1 too.

Linked to the activation of Raf-1, the Jaks are also involved in the activation of insulin receptor substrate 1 (IRS1) by Type I IFNs, OSM and IL-4 (19) and the activation of phosphatidylinositol 3'-kinase (PI3 kinase), which potentially feeds into a wide spectrum of signalling pathways. Recently it was shown that specific inhibition of PI3 kinase in HeLa cells reduces IFN$\gamma$-dependent STAT1 serine phosphorylation, whilst constitutively active PI3 kinase augments STAT1-dependent transcription (Nguyen, H., and Stark, G.R., pers. com). This points to an important role for PI3 kinase signalling in IFN$\gamma$-induced STAT signalling. The activation of this pathway may be mediated by an adaptor protein called Cbl which was shown to be inducibly phosphorylated by IFN$\gamma$ (4) and may recruit other proteins such as p59fyn (238).

In addition to the pathways mentioned above, new Jak-interacting proteins are gradually being identified, and these will increase the variety of pathways activated by cytokines.

**Serine phosphorylation of STATs.**

The regulation of STAT serine phosphorylation has a major modulatory effect on STAT-dependent transcriptional responses. The issue of serine phosphorylation of the STATs is complex, but provides a link to other pathways activated in responses to cytokines. Several candidate kinases have been identified, and it is likely that the nature
of the activating ligand and the cell-type probably determine which kinase or pathway is responsible (reviewed (48)) in a given situation.

Historically, p38 has been a prime candidate, but the role of p38 MAPK in the IFN response remains controversial. Some data supports activation of p38 MAPK in response to Type I IFNs (237), and is consistent with p38 MAPK being responsible for STAT1 serine 727 phosphorylation in response to stress, but not IFNγ, which failed to activate p38 MAPK (115). The phosphorylatable serine residue lies within a MAPK family consensus recognition site (5, 199) which would be consistent with a MAPK-related protein being responsible for the phosphorylation, and the reported \textit{in vitro} interaction between STAT1 and ERK2 reinforces this possibility (43). The Ras/MAPK pathway is required for full transcriptional activation of STAT1α and STAT3 (257). Although the IFNs do not activate this pathway directly, activation of ERK2 is mediated by interaction with the IFNα/β receptor (43). Perhaps the real significance of MAP kinases in responses to IFN is indicated by a report of p38 MAPK-dependent phosphorylation of histones in the PML gene promoter in response to IFN (236).

\textbf{PKR.}

The IFN-induced dsRNA dependent protein kinase (PKR) whose role in the antiviral response is discussed below, is involved in non-dsRNA-dependent responses to extracellular signals too, such as TNFα, PDGF and IFN (259). Ligand-dependent activation of PKR is reported, and a role for PKR in the regulation of STAT serine phosphorylation (185) and also in the activation of IRF1 (118) is implied by the observation that these are deficient in PKR knock-out cells. PKR is also reported to regulate the activation of NFκB (118) and the phosphorylation of IκBβ in response to TNFα and double-stranded RNA (117). Thus, in addition to IFN activated proteins, IFN-induced proteins may also modulate the transcriptional responses to other ligands.
Finally, it is important to highlight the possible role of inducible inhibitors of cytokine signalling (discussed previously). The activation of the Jak/STAT pathway by one cytokine can lead to the induction of inhibitory factors which negatively regulate this stimulatory signal, but can also interfere with the signalling response to a different ligand (95).

The accumulating evidence is consistent with cross-talk between the Jak/STAT pathway and various other signal-transduction systems. It seems likely that this cross-talk may modulate events at the receptor and in the nucleus, contributing to specificity of transcriptional responses through a common signal transduction pathway. The apparent cell-type specificity of many of the additional ligand-dependent pathways ensures that the correct response is induced by the activated STATs in different physiological compartments.

**Cellular responses to virus infection.**

Virus infection triggers a complex set of intracellular responses which interfere with the replication of the virus and ensure priming of neighbouring cells against subsequent infection (Figure 1.3). One of the key triggers which elicits these responses is double-stranded RNA (dsRNA) (73) produced in the course of replication of RNA viruses. This virus dsRNA can be simulated with duplex polyinosinic-polycytosinic acid (poly(I)-poly(C)), which provides a useful tool for the study of responses to dsRNA. The three aspects of the response to dsRNA are - (i) the direct activation of antiviral enzymes, (ii) the induction of α/βIFNs leading to upregulation of ISGs, and (iii) the direct induction of a subset of ISGs.
Chapter 1. Introduction.

PKR and the 2-5 oligoadenylate system.

Various cellular proteins recognise and bind to dsRNA, activating antiviral proteins and shutting down general translation to slow replication of virus (73, 195). A family of dsRNA-dependent enzymes called 2'-5' oligoadenylate synthetases (2-5 OAS) are activated by dsRNA and respond by synthesising short 2'-5' oligoadenylate molecules. These specifically bind to and activate dormant cytoplasmic RNase L, which cleaves single-stranded RNA substrates. These probably include both viral and cellular RNAs, including the ribosomal RNAs (166, 217). Cleavage of the ribosomal RNA would result in inhibition of protein synthesis, and cleavage of specific cellular mRNAs encoding labile proteins may relieve inhibition of pathways, contributing to the antiviral response.

The dsRNA-dependent protein kinase (PKR) is an IFN-induced serine-threonine kinase which is also activated by binding to dsRNA (reviewed in (259)). A major substrate for the activated PKR is eukaryotic translation initiation factor eIF2α. PKR serine phosphorylation of eIF2α interferes with the initiation of translation, disrupting protein synthesis (46, 196). In addition to this, PKR is believed to act as a signal transducing kinase, being implicated in the activation of NFκB (117) and the regulatory serine phosphorylation of STAT1 (185). Regulation of these two transcription factors contributes respectively to induction of α/βIFN in response to virus, and subsequent autocrine responses.

Transcriptional responses to dsRNA.

Whilst the inhibition of protein synthesis by PKR and the 2-5 OAS system slows virus replication, latent transcription factors are also activated by dsRNA. There are two elements to the transcriptional response to dsRNA. The dsRNA-direct mechanism involves the induction of ISGs independently of IFN-stimulated signal transduction (12, 251). The ISGs induced this way participate in the cell's response to
infecting virus. Concomitant with this there is transcription of IFNs as well, and IFN produced by infected cells primes other cells against virus infection by directly inducing antiviral proteins and inducing transcription of different IFN subtypes to amplify the signal (148). Thus, IFNβ activates the Type I receptor, stimulating transcription of IRF-7 which participates in transcription of Type I IFN following phosphorylation by an inducer in a similar way to IRF3. The IRF3-IFNβ-IRF7 system forms an elegant amplification circuit to enhance the response to viral infection.

The βIFN enhanceosome is a well-characterised dsRNA-responsive system (108, 155, 253, 266). The βIFN promoter contains four discrete DNA elements called Positive Regulatory Domains (PRDs). PRD I and III are binding sites for IRFs, and PRD II and IV are binding sites for NFκB and AP-1. The enhanceosome assembles in response to dsRNA following virus infection, and contains the DNA-binding proteins of the IRF-family, NFκB and ATF-2/c-Jun, all of which are phosphorylated in response to dsRNA or virus. In addition to these transcription factors, the complex contains one of the histone acetyltransferases CBP or p300, and a chromatin remodelling factor of the HMG family of proteins. The kinase required for the activation of the latent transcription factors remains to be identified, and there may be additional signals which regulate enhanceosome assembly.

The direct induction of α/βIFN-responsive genes by dsRNA is mediated through ISREs, but is independent of the induction of βIFN since it occurs in the presence of cycloheximide (11, 252). Furthermore, induction through these ISREs is not mediated by ISGF3 as in the response to α/βIFNs, but instead by specific dsRNA-activated factors (DRAFs) (39). The DRAFs are activated independently of protein synthesis, and contain at least two proteins - IRF3 and the histone acetyltransferase
CBP or 300. Genes induced by DRAFs include ISG15 and RANTES (8, 134). It is apparent that the DRAFs and the βIFN enhanceosome are related but distinct, and the two complexes probably integrate different subsets of regulatory signals (209).

**Responses independent of dsRNA.**

In addition to the dsRNA-responsive mechanisms, several proteins have been identified which directly interfere with virus replication. The Mx proteins are IFN-induced GTPases whose antiviral function is unclear, although it has been reported that Mx A interacts with nucleo-capsids of specific infecting viruses, and may also inhibit viral polymerases (109, 110, 256). Guanylate Binding Protein (GBP) is an IFN-induced protein whose antiviral effects have been demonstrated using antisense and over-expression approaches (6), although the mechanism of its antiviral effect is unknown. Similarly, the IFN-inducible 9-27 protein inhibits VSV replication (3), and nitric oxide synthase (iNOS) inhibits several viruses infecting macrophages (100), although the mechanisms are not understood.

Given that IFNs themselves are expressed in response to virus infection, it is unsurprising that many of the gene-products induced by IFNs have antiviral properties. But there are many more ISGs whose functions have not been characterised, and although IFNs do not function solely to help fight off viral infections, it is reasonable and consistent with many findings that many antiviral mechanisms remain to be identified. Perhaps the most highly induced ISG is IFI56K, but the function of the protein itself is unknown. 9-27 is another protein highly induced by IFNs, but no function has been attributed to it. A protein with a very high homology to 9-27 was identified which binds to the rev-response element of the human immunodeficiency virus HIV-1, and inhibits rev-mediated gene expression (37). This suggests a novel mode of action for antiviral proteins, by targeting the regulation of viral transcription.
Indeed, IFNs have been shown to interfere with most aspects of the virus life cycle from entry to transcription, translation, maturation and release. But few proteins have been identified to account for all these effects with the exception of those affecting translation mentioned above.

Knock-out experiments suggest that there are additional factors involved in the response to virus, most compellingly because triply deficient mice knocked out for PKR, RNase L and Mx still show an enhanced antiviral response following treatment with IFN (276). Experiments in yeast have identified proteins which interact specifically with viral mRNAs to prevent their translation (14), as opposed to generally inhibiting translation, and at least one homologue has been identified in humans (183). Furthermore, there is evidence that virus infection can induce an apoptotic response in cells. It is postulated that labile repressors of members of the stress kinase family rapidly decay following dsRNA-dependent inhibition of protein synthesis during virus infection. This leads to activation of the stress kinases, which in turn feeds into an apoptotic response. In effect the cell commits suicide before the replication cycle of the virus is complete.

Virus-mediated inhibition of host responses.

Viruses have evolved multiple mechanisms to subvert antiviral responses of host cells, many of which involve the IFN system. Several viruses including poxvirus (221) and myxoma virus (239) produce soluble IFN receptor homologues which bind IFNs, inhibiting their action. Viruses also inhibit the signal transduction in response to IFNs in a variety of ways. CMV has been shown to target components of the Jak/STAT pathway for degradation, preventing ligand-dependent receptor activation (156). Several viruses including HHV-8 (20, 131, 279) encode homologues of IRFs. These vIRFs interact with cellular IRFs preventing their functional dimerisation essential for transactivation. Other viral proteins including adenoviral E1A and HPV E6 and E7 sequester cellular IRFs or their co-factors preventing the endogenous IRFs functionally
interacting (24, 98, 175). PKR and responses to dsRNA are also a major target for viral inhibitory mechanisms e.g. by HIV and Hepatitis C virus.

Thus viruses have evolved mechanisms to overcome host responses. These provide both an insight into the importance of recognised responses and a handle to identify unrecognised responses which undoubtedly remain to be elucidated. For example, many viruses encode anti-apoptotic gene products, which may emphasise the importance of apoptosis as an antiviral response.

**Complexity in IFN signalling.**

The picture of signalling in response to Type I IFNs remains incomplete. Although they bind to the same receptor complex, the Type I IFNs induce subtly different biological responses, a good example being the βIFN specific induction of β-R1 (186). The mechanism for the differences in responses is unclear; it is thought that it may reflect variations in the interaction between ligand and the receptor chains, which affect the interactions of the receptor associated Jaks and STATs. This might produce differences in the kinetics of interactions between the activated receptor and STATs or other proteins involved in signalling, and also in the duration of the response. Thus U1A cells which lack Tyk2 show no response to IFNα but are partially responsive to IFNβ, suggesting Tyk2 plays less of a role in signalling in response to IFNβ, although the IFNAR1 chain with which Tyk2 interacts is still required for signalling. Conversely, U4A cells which lack Jak1 do not respond to any Type I IFN. For a review, see (157).

There is strong evidence that, in addition to Jaks and STAT1, other factors are required for IFNγ signalling. The fact that GAS elements in Type II responsive genes are not regulated by GAFs generated in response to Type I IFNs suggests that an
additional signal generated by IFNγ is required. It is widely recognised that both types of IFN induce overlapping sets of genes, but there still remains a high degree of specificity, with genes induced by one type rather than another and this is probably a function of additional ligand-dependent signals.

New participants in IFNγ signalling are slowly being identified. The Fanconi Anaemia Protein FANCC was shown to bind STAT1, and is required for its recruitment to the IFNγ receptor, and STAT1 activation in response to IFN and other cytokines (172). IFNγ was recently shown to enhance expression regulated by a novel response element called γ-IFN-activated transcriptional element (GATE) which is dependent on CCAAT/enhancer-binding protein-β (C/EBP-β), also known as NF-IL6 (191).

The antiviral response to IFNγ is thought to require an unidentified factor(s) or signal(s) too. Mouse macrophages expressing both human Type II receptor chains respond to human IFNγ but do not acquire full protection from virus, which would indicate that an additional species-specific factor is also required (125). A similar experiment also indicates that although IFNGR1 and 2 are sufficient to reconstitute a response to human IFNγ, demonstrated by the induction of MHC Class I, an additional factor is required for the antiviral response to EMCV (218, 219).

In the case of U4A/Jak1K>E cells mentioned previously, although there is substantial gene induction in response to IFNγ the antiviral response is not reconstituted by the kinase-negative Jak1, and, when expressed in wild-type cells, a kinase negative Jak1 actually inhibits the antiviral response to IFNγ without a detectable inhibition of the Jak/STAT1 pathway. Similarly, mouse macrophages expressing both chains of the
human Type II receptor do not acquire the full protection from human IFNγ afforded them by murine IFNγ, again implying a requirement for an additional component to the signalling.

It is interesting to note that the genetic approach which successfully identified so many of the components involved in IFN signalling apparently failed to identify additional complementation groups. Does this mean that there are no other factors essential for the activation of the Jak/STAT pathway by IFNs or are additional components involved, the deletion of which is lethal? The strategy used relied on the two specific promoters, those of the 6-16 and 9-27 genes, which may have specific regulatory requirements, and the use of other promoters with different regulatory requirements might identify additional factors.
Summary.

Jaks and STATs are essential but not sufficient for the induction of the full response to a wide range of cytokines and growth factors. The activity of the STATs is regulated by tyrosine phosphorylation, but modulated by serine phosphorylation and by interactions with other transcription factors and co-activators. This allows a single event - ligand-binding - to produce a spectrum of effects and cell-specific responses.

Aims.

The overall objective was to analyse further the complexity of the IFNγ response and the role of the Jaks, particularly Jak1. The initial approach was through the analysis of the selective dominant negative effects of a mutant Jak1. When expressed as a dominant negative in wild type cells Jak1ΔB had no detectable effect on the IFNγ-mediated Jak/STAT1 pathway, but inhibited both the antiviral and the well-characterised HLA Class II responses. The initial objective was to identify the sites and mechanism of the Jak1ΔB-mediated inhibition of the HLA Class II response to reveal any novel Jak1-mediated signal(s) in addition to STAT1 which are required for this response. It transpired that Jak1ΔB also inhibits dsRNA-mediated responses. An analysis of this additional inhibition led in turn to an investigation of the potential importance of IFNγ-primed dsRNA responses in the IFNγ-mediated antiviral state.
Table 1.1. Complementation groups identified by genetic analysis of IFN signalling.

<table>
<thead>
<tr>
<th>Complementation group</th>
<th>Response to IFN</th>
<th>Complemented by</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>α</td>
<td>β</td>
</tr>
<tr>
<td>U1</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>U2</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>U3</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>U4</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>U5</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>U6</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>γ1</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>γ2</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>
The general structure of the Jak and STAT family proteins.
Figure 1.1.

Figure 1.1a. General structure of the Jak family proteins.

![Diagram of Jak family proteins]

Limits of putative FERM domain

Figure 1.1b. General structure of the STAT family proteins.

![Diagram of STAT family proteins]

Functionally significant regions of the STATs.
A minimal model for cytokine induction of gene-expression.

Binding of cytokine to its receptor activates receptor associated Jak family tyrosine kinases. These phosphorylate the receptor, allowing recruitment of cytoplasmic STAT transcription factors to the tyrosine-phosphorylated receptor chain motifs. The STATs are phosphorylated, dimerise, and translocate to the nucleus where they bind to cytokine-responsive promoter elements. In conjunction with putative additional cytokine-dependent signals, this results in the recruitment of co-activators and the transcriptional apparatus, leading to transcription.
Figure 1.2.

1. Cytokine binds cytokine receptor chain
2. Receptor activation
3. STATs recruited
4. Activated STATs localise to nucleus
5. STATs bind to promoter
6. Transcription apparatus recruited to promoter
Table 1.2. Signalling responses involving the JAK/STAT pathway.

<table>
<thead>
<tr>
<th>Ligand</th>
<th>Jaks activated</th>
<th>STATs activated</th>
</tr>
</thead>
<tbody>
<tr>
<td>IFN family</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IFNα/β</td>
<td>Jak1, Tyk2</td>
<td>STAT1, STAT2, STAT3</td>
</tr>
<tr>
<td>IFNγ</td>
<td>Jak1, Jak2</td>
<td>STAT1</td>
</tr>
<tr>
<td>IL-10</td>
<td>Jak1, Tyk2</td>
<td>STAT1, STAT3</td>
</tr>
<tr>
<td>gp130 family</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-6</td>
<td>Jak1, Jak2, Tyk2</td>
<td>STAT1, STAT3</td>
</tr>
<tr>
<td>IL-11</td>
<td>Jak1, Jak2</td>
<td>STAT1, STAT3</td>
</tr>
<tr>
<td>IL-12</td>
<td>Jak2, Tyk2</td>
<td>STAT3, STAT4</td>
</tr>
<tr>
<td>Leukaemia inhibitory factor</td>
<td>Jak1, Jak2</td>
<td>STAT1, STAT3</td>
</tr>
<tr>
<td>Oncostatin M</td>
<td>Jak1, Jak2</td>
<td>STAT1, STAT3</td>
</tr>
<tr>
<td>Ciliary neurotrophic factor</td>
<td>Jak1, Jak2, Tyk2?</td>
<td>STAT1, STAT3</td>
</tr>
<tr>
<td>Granulocyte colony-stimulating factor</td>
<td>Jak1, Jak2, Tyk2</td>
<td>STAT1, STAT3</td>
</tr>
<tr>
<td>Gamma c family</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-2</td>
<td>Jak1, Jak3</td>
<td>STAT1(?), STAT3, STAT5</td>
</tr>
<tr>
<td>IL-4</td>
<td>Jak1, Jak3</td>
<td>STAT5</td>
</tr>
<tr>
<td>IL-7</td>
<td>Jak1, Jak3</td>
<td>STAT5</td>
</tr>
<tr>
<td>IL-9</td>
<td>Jak1, Jak3</td>
<td>STAT5</td>
</tr>
<tr>
<td>IL-13</td>
<td>Jak1, Tyk2</td>
<td>STAT6</td>
</tr>
<tr>
<td>IL-15</td>
<td>Jak1, Jak2, Jak3</td>
<td>STAT5</td>
</tr>
<tr>
<td>gp140 family</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-3</td>
<td>Jak2</td>
<td>STAT5</td>
</tr>
<tr>
<td>IL-5</td>
<td>Jak2</td>
<td>STAT5</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>Jak2</td>
<td>STAT5</td>
</tr>
<tr>
<td>Growth Hormone family</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Growth hormone</td>
<td>Jak2</td>
<td>STAT1, STAT3, STAT5</td>
</tr>
<tr>
<td>Erythropoietin</td>
<td>Jak2</td>
<td>STAT5</td>
</tr>
<tr>
<td>Prolactin</td>
<td>Jak2</td>
<td>STAT1, STAT5</td>
</tr>
<tr>
<td>Receptor Tyrosine Kinase family</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Epidermal growth factor</td>
<td>Jak1, Jak2</td>
<td>STAT1, STAT3</td>
</tr>
<tr>
<td>Platelet-derived growth factor</td>
<td>Jak1, Jak2</td>
<td>STAT1, STAT3</td>
</tr>
<tr>
<td>Colony-stimulating factor-1</td>
<td>Jak1, Jak2</td>
<td>STAT1, STAT3</td>
</tr>
</tbody>
</table>
Cellular responses to dsRNA.

Virus infection leads to the production of viral dsRNA. This is known to directly activate at least two proteins - PKR and 2'-5' OAS. PKR phosphorylates eIF2α, leading to the inhibition of translation initiation and a reduction in protein synthesis. IκB, the cytoplasmic inhibitor of NFκB is also phosphorylated by a PKR dependent pathway, leading to degradation of the inhibitor and release of NFκB which participates in the induction of βIFN. 2'-5' OAS synthesises short 2'-5’ oligoadenylates which activate RNaseL, which digests both viral and cellular RNAs, including ribosomal RNA, again inhibiting translation.

An unidentified kinase is activated by dsRNA and phosphorylates the latent cytoplasmic transcription factor IRF3, leading to its nuclear accumulation. Activated IRF3 participates in induction of ISGs and, in conjunction with NFκB, the induction of βIFN.
Figure 1.3.

Virus

dsRNA

2'–5' OAS

oligoadenylates

Ribosome

PKR

eIF2-α

RNase L

IRF3

ISGs

IκB

NFκB

βIFN

ISGs

?
Chapter 2.

MATERIALS AND METHODS.

Chemicals.

Unless stated otherwise, chemicals were obtained from Sigma.

Tissue Culture.

Cells were cultured as a monolayer in Dulbecco’s Modified Eagles Medium (DMEM) containing penicillin and streptomycin, made and supplied by LCRF Media Production, supplemented with 10% (v/v) foetal calf serum (heat-inactivated at 56°C for 30 minutes) and 2mM glutamine. Cells carrying selectable resistance markers were grown in the presence of 250μg/ml Hygromycin B (Calbiochem), 700μg/ml G418/Neomycin (GIBCO) as appropriate for maintenance. Typically cells were passaged 1:10 as follows: monolayers were rinsed in versene (0.02% (w/v) EDTA in PBSA), detached in versene containing 1% trypsin, and resuspended in DMEM/10% serum for replating. Cells for experiments were grown and treated in the absence of selection drugs. All disposable plastic tissue culture plates were obtained from Falcon.

Cells were stored in liquid nitrogen frozen in DMEM/20% heat-inactivated serum (v/v) and 10% dimethyl sulphoxide (v/v). To revive frozen cells a vial was warmed to 37°C, and the contents placed into a sterile universal tube. DMEM/10%FCS was added drop-wise to the cell suspension and then the cells were pelleted by low speed centrifugation, resuspended in fresh DMEM/10%FCS and plated out as required.

**DNA manipulation, preparation and sequencing.**

Molecular biology procedures such as restriction digestions, ligations and polymerase chain reaction (PCR) were performed using enzymes, buffers and reagents from Boehringer Mannheim, Promega or New England Biolabs.

Plasmid DNA was propagated in the XL-1 Blue strain of *E. Coli*. Bacteria were transformed by electroporation using a BioRad electroporator, and positive clones selected and cultured in the presence of 50µg/ml Ampicillin.

Miniprep DNA from overnight cultures derived from a single colony was prepared using a Qiagen Biorobot 9600 workstation operated by ICRF Equipment Park. DNA was sequenced using the ABI Prism Dye Terminator Cycle Sequencing kit (Perkin Elmer). Sequence information was obtained using an ABI 377 sequencer, again operated by ICRF Equipment Park.

Large scale preparation of plasmid DNA was carried out using Qiagen Maxi-prep kits (Qiagen) following the manufacturer's instructions. For each prep a single colony from an agar plate, or a stab from a glycerol stock, was inoculated into 250 ml of LB medium containing 50µg/ml ampicillin and cultured at 37°C overnight.

**Transient transfections.**

Transient transfection of expression constructs and/or reporter constructs was performed using Superfect transfection reagent from Qiagen. Briefly, plasmid was resuspended in 100 µl DMEM/µg plasmid and mixed with 6µl Superfect per µg plasmid, vortexed and left for ten minutes at room temperature. DMEM/10%FCS was added to give an appropriate volume (1.5 mls per 6cm dish or 2.5 mls per 10 cm dish) and the transfection mix warmed to 37°C. Monolayers of cells were incubated with the mix for up to four hours, then "washed" for ten minutes in DMEM/10%FCS three times, and left overnight to recover. The next morning cells were split as required for the experiment and allowed to adhere to dishes before stimulation and harvesting.
Expression Constructs.
Listed below are the expression constructs used in transient transfections.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Details</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>IRF3</td>
<td>In pFLAG-CMV-2 expression vector.</td>
<td>Dr John Hiscott, Lady Davis Institute For Medical Research, McGill University, Montreal.</td>
</tr>
<tr>
<td>IRF3(5D)</td>
<td>As above.</td>
<td>Dr John Hiscott</td>
</tr>
<tr>
<td>IRF3ΔN</td>
<td>As above</td>
<td>Dr John Hiscott</td>
</tr>
</tbody>
</table>

Construction of pIVmκB-Luciferase.
The plasmid pIV-Luciferase was used as a PCR template in conjunction with mutagenic oligonucleotide primers according to the strategy depicted in Figure 2.1. The NFκB site mutagenised sequence is that used by Demengeot et al. (50). Following the second round of PCR the fragment was purified, digested with KpnI and XbaI and inserted into pIVmκB-Luciferase digested with the same enzymes, and the sequence checked by sequencing. The oligonucleotide primers - with mutagenised sequence in bold type - used were as follows:

Primer 1 (Upstream)
5' -GGGAGGTACCAGCTCTTACGCG

Primer 2 (Top strand NFκB)
5' -CCCGGGGCCATTAACTCTCCCCG

Primer 3 (Bottom strand NFκB)
5' -CGGGAGAGTAAATGGCCCCCGG
Primer 4 (Downstream)

5'-CCAGCGGTTCATCTCTAGAGG

β-gal assay.

Reporter plasmids were co-transfected at a ratio of 10:1 with the plasmid pKS1β-gal containing a β-galactosidase reporter regulated by a CMV promoter, in order to control for transfection efficiencies between individual transfections. β-galactosidase activity was assayed from the same extract as the main reporter assay in a microtiter dish, using the yellow to red colour change of chlorophenored-β-D-galactoside (CDPG) (Calbiochem) which is catalysed by β-galactosidase. For each sample, 5 and 25μl of extract was assayed in 60 mM Na₂HPO₄·7H₂O, 40 mM NaH₂PO₄, 10 mM KCl, 1 mM MgSO₄, 2.7ml/l β-mercaptoethanol in the presence of 1 mg/ml CDPG. Samples were incubated for 30 minutes at 37°C, and then the absorbance measured at a wavelength of 595 nm using a plate reader.

Luciferase assay.

The Luciferase Assay System from Promega was used. Cells were lysed in 1x Reporter Lysis Buffer (as supplied), frozen on dry ice and thawed, vortexed briefly and then samples centrifuged for 2 minutes at 12000xg, and the supernatant assayed for Luciferase activity or stored at -70°C. To assay the Luciferase activity a 20μl aliquot of the extract was mixed with 100μl of Luciferase Assay Reagent using an injection luminometer. Light intensity was measured over a ten second period and averaged to give the relative light intensity for each sample.

Stable transfections.

Clones of cells stably expressing a construct were isolated by transfection and single cell dilution cloning. Initially, cells were transfected using the calcium phosphate
precipitation method. Typically, for the transfection of $3 \times 10^6$ cells, 10µg of plasmid DNA was linearised by restriction digestion with an appropriate enzyme, ethanol precipitated and resuspended in sterile deionised water. 4 hours prior to transfection the medium was changed on the cells. The volume of the solution of DNA was made up to 438 µl with water. 62 µl of CaCl₂ (260 mM CaCl₂, 0.87 mM Tris pH 7.2, 87µM EDTA) was added and then, drop-wise, 500µl 2xHBS (42 mM HEPES, 274 mM NaCl, 10 mM KCl, 1.4 mM Na₂HPO₄·2H₂O, pH 7.0), whilst vortexing the mixture to ensure the formation of a fine precipitate. The mixture was added to the cells and left overnight, then the medium was changed and the cells left for 24 hours before the addition of the appropriate selection drug. Once cell killing by the drug had taken place the surviving cells were cloned by limiting dilution into 96-well plates at 0.5 cells/well and over time expanded for screening to determine expression levels of the transfected construct.

**Cytokines and inducers.**

Details of the cytokines and other inducers used to elicit responses, and the concentrations used routinely are given below:

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>IFN α</td>
<td>Wellferon, a purified mixture of α IFNs - Welcome Research; 10³ IU/ml. (Specific activity 3x10³ IU/mg).</td>
</tr>
<tr>
<td>IFN γ</td>
<td>Recombinant human IFNγ from Boehringer Ingelheim, supplied by Dr. G.R. Adolf; 10³ IU/ml. (Specific activity 3x10⁷ IU/mg).</td>
</tr>
<tr>
<td>Poly (I)-poly(C)</td>
<td>Amersham Pharmacia; 100µg/ml.</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Material</th>
<th>Source</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNFα</td>
<td>Recombinant human TNFα - R&amp;D Systems</td>
<td>0.5 μg/ml</td>
</tr>
<tr>
<td>Oncostatin M</td>
<td>Recombinant human OSM - R&amp;D Systems</td>
<td>100 ng/ml</td>
</tr>
<tr>
<td>Interleukin 6</td>
<td>Human IL6 and its soluble receptor were gifts from Professor P.Heinrich, RWTH, Aachen.</td>
<td>IL6 was used at 2μg/ml and the soluble receptor at 500 ng/ml.</td>
</tr>
<tr>
<td>Anisomycin</td>
<td>A stress kinase agonist (83), used to activate p38 MAP kinase; 1μg/ml.</td>
<td></td>
</tr>
</tbody>
</table>

**Cytopathic antiviral assay.**

This assay was used to characterise the response of cells pre-treated with ligand and challenged with virus - in this case the picornavirus Encephalomyocarditis virus (EMCV) - assaying the ability of the ligand to reduce the cytopathic effect of the virus (58). Cells were plated at a density of 10⁴/well in 24-well plates and allowed to settle, after which the medium was changed with the addition of IFN where appropriate. Following overnight incubation the medium was removed and 0.5 plaque-forming units (pfu) of EMCV per cell was added. After 24 hours - at which stage the control, virus-infected, untreated cells had lysed and detached from the bottom of the wells - the plates were washed once with PBSA and fixed in formol saline for 30 minutes, then washed again in PBSA, and 0.00075% Giemsa added to the wells to stain those cells which were to have survived the virus infection.

**Virus yield assay.**

Cells were plated in 3 cm plates and allowed to adhere, treated with interferons where appropriate and infected with EMCV at 10 pfu/cell. 14 hours after infection the plates, together with the culture medium, were freeze-thawed three times at -80°C, scraped and vortexed, and frozen for subsequent analysis in aliquots.
To assay the relative amount of virus in the samples from the infected cells, murine L929 cells were used. 96-well plates were seeded at a density of $5 \times 10^4$ cells per well. After 24 hours, a doubling dilution series of the extracts containing the virus produced from the virus-infected cells was added to the plates to determine the relative amounts of virus from each infection. The L929 cells were left for 24 hours and then fixed and stained as described above for the cytopathic antiviral assay.

**Tritiated-uridine incorporation assay of viral replication.**

The assay was used to measure the incorporation of $^3$H-uridine into the RNA genome of replicating virus during an infection. $5 \times 10^5$ of the cells to be infected were plated on 3 cm plates. Prior to infection the cells were pre-treated for 1 hour with 2µg/ml actinomycin D to inhibit cellular RNA polymerase activity, then 5µCi $^3$H-uridine was added to the medium, and 1 hour later 10 pfu/cell of EMCV was added. At appropriate time points following this point individual plates were harvested. The medium was removed, the plate washed once with PBSA, and then filled with 5% TCA for 10 minutes. This was removed and fresh TCA added for a further 10 minutes. The plate was drained and 1 ml 0.1M NaOH was added to the plate for an hour to solubilise incorporated activity. An aliquot was dispersed in scintillation fluid and the activity was measured in a scintillation counter.

**Fluorescence activated cell scanner (FACS) analysis.**

Cells to be analysed were detached in versene, washed with PBSA, and resuspended in ice-cold DMEM. Subsequent steps were performed at 4°C to prevent aggregation or internalisation of antigen. The monoclonal antibodies used were phycoerythrin-conjugated anti HLA-DR (Becton-Dickinson), FITC-conjugated anti HLA-A, B, C (Harlan SeraLab) or anti-ICAM1 diluted 1 in 20 in DMEM. The cells
were stained for 45 minutes, washed twice with PBSA and then fixed in PBSA containing 1% paraformaldehyde in preparation for analysis. FACS analysis was carried out using Becton-Dickinson FACScan scanner and Cell Quest analysis software.

**Analysis of proteins expressed in cells.**

**Gel electrophoresis of proteins.**

Denaturing polyacrylamide gel electrophoresis (PAGE) of immunoprecipitated proteins or extracts was based on the method of Laemmli. The same gels were used to resolve immunoprecipitated proteins or with whole cell extracts. A stacking gel containing 5% (v/v) acrylamide (37.5:1 acrylamide:bis-acrylamide), 125 mM Tris-HCl pH 6.8 and 0.1% (w/v) SDS was cast over a separating gel of between 6% and 10% acrylamide (depending on the size of the proteins of interest) containing 0.375M Tris-HCl pH 8.8 and 0.1% SDS. The running buffer for gels contained 25 mM Tris, 186 mM Glycine and 0.1% SDS. Pre-stained markers run in parallel with proteins to monitor their size were High-range Molecular Weight Markers from Amersham Pharmacia.

**Preparation of cell extracts.**

Whole cell extracts for protein analysis were prepared in “Schindler” lysis buffer: 0.5% (v/v) NP 40, 50 mM Tris-HCl pH 8.0, 150 mM NaCl, 0.1 mM EDTA, 10% glycerol, 1 mM sodium vanadate, 0.4 mM phenylmethylsulphonyl fluoride (PMSF), 3μg/ml aprotonin, 1μg/ml leupeptin. Cells were washed in ice-cold PBSA, resuspended in lysis buffer, scraped and transferred to an Eppendorf tube, vortexed and incubated on ice for 20 minutes. Samples were centrifuged at 20,000xg for 5 minutes and the supernatant removed for analysis.
In order to isolate nuclear proteins whilst excluding cytoplasmic proteins the method of Schreiber et al. was used (204). Cells were resuspended in a hypotonic buffer (10 mM HEPES pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 0.5 mM PMSF) and allowed to swell on ice for 15 minutes. NP 40 was added to 0.5% (v/v), the samples vortexed and centrifuged for 30 seconds at 2000 rpm. Supernatant containing cytoplasmic proteins was removed and the pellet containing nuclei and membrane debris was resuspended in a hypertonic buffer (20 mM HEPES pH 7.9, 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1 mM PMSF, 3μg/ml aprotonin, 1μg/ml leupeptin). Samples were shaken for 30 minutes at 4°C, centrifuged at 20000g for 5 minutes, and supernatant removed for analysis.

The concentration of protein in extracts was determined using BioRad's DC protein assay, based on the Lowry method of protein determination.

For the analysis of p38 MAP kinase phosphorylation and phospho-specific analysis of STAT1 cells were lysed directly in SDS sample buffer: 62.5 mM Tris-HCl pH 6.8, 2% (w/v) SDS, 10% (v/v) glycerol and 50mM DTT. Lysate was sonicated, boiled and protein content determined before loading and running.

For analysing whole cell protein equal amounts of Schindler extract (in terms of the mass of cell protein) were mixed with an equal volume of 2xSDS-PAGE loading buffer (see below) and boiled before running.

**Immunoprecipitation.**

Matched Schindler extracts were incubated with 1μg of antibody for from 4 hours to overnight on a rotating wheel, with 50μl of a 50% suspension of protein G- and A-sepharose beads in 1x Schindler lysis buffer added for the last hour. Subsequently, the beads were washed twice with lysis buffer and resuspended in 1xSDS-PAGE loading buffer (10% glycerol, 4% SDS, 500 mM Tris-HCl pH 6.8, 100
**Western transfer of proteins.**

A transfer buffer containing 25 mM Tris, 186 mM Glycine and 10% (v/v) methanol was used. Gels were equilibrated in transfer buffer and transferred to PVDF membrane (Immobilon P, Millipore) using a wet blotter (IDEA Scientific Company) for 1 hour.

**Western blot analysis.**

The Table 2.2 below lists the antibodies used for Western-blot analysis of cell protein. Immediately following transfer membranes were equilibrated in TBST (10 mM Tris-HCl pH 7.4, 75 mM NaCl, 1 mM EDTA pH 8.0, 0.1% Tween-20). Membranes were blocked overnight in 5% (w/v) BSA dissolved in TBST containing 20 mM Na₃VO₄ and 0.01% sodium azide as preservative. To analyse tyrosine phosphorylation of proteins the primary antibody was diluted in 1% BSA/TBST/20 mM Na₃VO₄/0.01% sodium azide, whilst for other analysis vanadate was omitted. Primary antibodies were diluted 1:2000 unless stated below and incubated for 2 hours at room temperature. Between incubation with primary and secondary antibodies the membranes were washed three times in TBST for 5 minutes. Secondary antibody for phosphotyrosine analysis was diluted in 1% BSA in TBST, including vanadate but in the absence of azide, whilst secondary antibody for other primary antibodies was diluted in 5% (w/v) skimmed milk powder in TBST. Secondary antibodies were horse-radish peroxidase-conjugated (Amersham) anti-rabbit, mouse or goat, and were used at 1:2000 dilution, and incubated for 30-45 minutes at room temperature. After incubation with secondary antibody the membranes were washed extensively for at least 3 hours in TBST. Enhanced chemiluminescence (ECL) was used to visualise the proteins using solutions from Amersham. Following ECL and autoradiography, the membranes were stripped overnight in 2.5M glycine, pH 2.5 and then reprobed as required.
Separation of cytosol and total membrane protein.

Cells were harvested by low speed centrifugation and resuspended in Schindler lysis buffer without glycerol or NP-40. The suspension was added to a ball-bearing homogeniser which had been pre-chilled on ice and the cells were dounced twelve times and then centrifuged at 4°C at 14000 rpm. The supernatant was ultracentrifuged (50000 rpm, 4°C), and the new supernatant removed. The two pellets containing membrane-associated cell protein were solubilised in regular Schindler buffer, and the supernatant equilibrated to 10% glycerol, 0.5% NP40. Both samples were shaken for 1 hour at 4°C, and centrifuged at 14000 rpm. The supernatants were removed for analysis by SDS-PAGE and Western blotting.

PKR activity assay.

The activity of double-stranded RNA-dependent protein kinase (PKR) was measured by using an _in vitro_ autokinase assay based on the method provided by Dr Maryam Zadoush of the Cleveland Clinic Foundation.

Cells were treated with the appropriate ligand or inducer and then harvested in Schindler lysis buffer as for a standard immunoprecipitation. The protein levels in extracts were determined and equal amounts of protein incubated with 1µl of monoclonal antibody to PKR (provided by Dr Ara Hovanessian) for one hour. Antibody protein complexes were purified by the addition of Protein A sepharose beads for an hour, after which time the beads were washed twice with Schindler buffer and once with kinase buffer (20 mM Tris-HCl, pH 7.6, 80 mM KCl, 2 mM MgCl₂, 2 mM MnCl₂, 5 mM β-mercaptoethanol, 20% glycerol). The beads with purified PKR were incubated in kinase buffer containing 1µM ATP and 0.1µCi γ³²P-ATP per sample at 30°C for 15 minutes, after which time they were washed twice with kinase buffer. The beads were resuspended in 1xSDS-PAGE sample buffer and then boiled for 5 minutes.
and the samples loaded onto a 10% SDS-PAGE gel. After running, the gel was dried and $\gamma^{32}$P incorporated into PKR in the kinase reaction was visualised by autoradiography.

In parallel, an equal amount of protein from the original extract was resolved on a 10% SDS-PAGE gel and transferred to PVDF, and PKR protein was analysed by Western blotting of the membrane.

**Electrophoretic mobility shift assay (EMSA) analysis of DNA-binding protein complexes.**

The sequences of the oligonucleotides used in EMSA analysis are given below:

<table>
<thead>
<tr>
<th>Probe</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\beta$-Casein</td>
<td>5'GGATTTGAATTCTCTAGAAATCT3'</td>
</tr>
<tr>
<td>hSIE</td>
<td>5'GTCGACATTTCCGTAATC3'</td>
</tr>
<tr>
<td>ISG15 ISRE</td>
<td>5'GGGAAAGGAAACCCGAAACTGAGCC3'</td>
</tr>
<tr>
<td>NFkB</td>
<td>5'GGGGACTTCCC3'</td>
</tr>
<tr>
<td>NGE</td>
<td>5'GGCCAGGGCAGTGGGTGGATGCCACCTGCTGATAAGCAGCGTGG TGGCCACAG3'</td>
</tr>
</tbody>
</table>

**Probe labelling.**

200 ng of the appropriate oligonucleotide contain a protein binding sequence was incubated with 10 units of T4 Polynucleotide Kinase (New England Biolabs) in kinase buffer (70 mM Tris-HCl pH 7.6, 10 mM MgCl$_2$, 5 mM DTT) with 5µCi of $\gamma^{32}$P-ATP for 30 minutes at 37°C. 200 ng of complementary oligonucleotide and 100 mM NaCl were added to the reaction mixture, which was then heated to 95°C for 5 minutes and allowed to cool slowly in a water bath whilst annealing took place. NAP-5
Sephadex G-25 columns (Pharmacia Biotech) were used to purify the labelled double-stranded oligonucleotides from unincorporated label and unannealed oligo as follows. The column was washed with TE (10 mM Tris-HCl pH 7.5, 1 mM EDTA), and the probe was added to the column. The column was eluted with TE and eluate fractions collected. A scintillation counter was used to identify the first fraction with a high activity, containing the labelled probe.

**Mobility-shift assay.**

Extracts made in Schindler buffer were assayed in the presence of 2.5 mg/ml BSA, 500µg/ml tRNA, 4% (w/v) Ficoll (Pharmacia), 100 mM NaCl, 10 mM HEPES pH 7.9, 0.1 mM EGTA, 5% (v/v) glycerol, 0.5 mM DTT.

Nuclear extracts were assayed in 25 mM HEPES pH 7.9, 10% glycerol, 5 mM DTT, 200 mM NaCl.

In both cases matched extracts were preincubated with polydI:dC for 10 minutes at room temperature to eliminate non-specific DNA-binding. To supershift or disrupt specific DNA-binding complexes, antibody specific for proteins believed to be in a complex were preincubated with the extracts for an additional 15 minutes prior to the addition of the probe.

Extracts were incubated with probe for 20 minutes and then loaded onto 6% non-denaturing polyacrylamide gels (6% 37.5:1 acrylamide:bis-acrylamide, 1x TGE - 25 mM Tris, 250 mM Glycine, 1 mM EDTA) run at 200 volts. A marker of xylene cyanol blue was added to one lane, which was used to monitor the distance the samples had run, and once run far enough the gels were dried for autoradiography.
RNA preparation.

Cells were washed in ice-cold PBSA, resuspended in RNA extraction buffer (150 mM NaCl, 10 mM Tris-HCl pH 7.9, 1.5 mM MgCl₂, 0.65% (v/v)NP40), vortexed and incubated on ice for 5 minutes, then centrifuged at 20,000g for 5 minutes at 4°C. The supernatant was removed and adjusted to 0.5% (w/v) SDS, 1 mM EDTA, and proteins and DNA extracted using an equal volume of acid-buffered phenol (Tris pH 4.3) /chloroform/isoamyl alcohol (25:24:1), and then once more with chloroform/isoamyl alcohol (24:1). RNA was precipitated with 100% ethanol and 3M NaOAc pH 5.0, centrifuged for 30 minutes at 20,000g, washed with 70% ethanol and resuspended in deionised water. The OD at 260 nm was measured using a spectrophotometer, and converted to concentration on the basis that 1 OD unit = 40μg ssRNA.

Probe labelling.

Plasmid DNA containing template DNA for the probe was linearised with the appropriate restriction enzyme to cut at the distal end of the probe template. The digest was checked on an agarose gel, cleaned by phenol/chloroform extraction, ethanol precipitated and resuspended in deionised water.

1μg of template DNA was used in each labelling together with 100μCi α²³-UTP (Amersham, Bucks), 10 mM DTT, 15 mM GTP, ATP and CTP (Amersham Pharmacia Biotech), 1μl per reaction RNasin (Promega), 1x transcription buffer (Boehringer), and 1μl of the appropriate RNA polymerase (Promega). Reactions were incubated at 37°C for 2 hours, and then ribonuclease-free DNase (Boehringer) was added for 15 minutes. 80 μl of STE (10 mM Tris-HCl pH 7.2, 1 mM EDTA, 0.1% SDS (w/v) was added, and the mixture was extracted with phenol/chloroform/isoamyl alcohol. Probe was precipitated with 2.5M NH₄OAc, tRNA and 100% ethanol, resuspended in STE,
precipitated again and resuspended in 40 µl STE. RNase protection probes used are listed overleaf:

<table>
<thead>
<tr>
<th>Probe</th>
<th>Protected fragment</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Actin</td>
<td>130</td>
<td>(2)</td>
</tr>
<tr>
<td>9-27</td>
<td>160</td>
<td>(2)</td>
</tr>
<tr>
<td>6-16</td>
<td>190</td>
<td>(2)</td>
</tr>
<tr>
<td>IRF-1</td>
<td>175</td>
<td>(151)</td>
</tr>
<tr>
<td>p48</td>
<td>270</td>
<td>(243)</td>
</tr>
<tr>
<td>DRα</td>
<td>235</td>
<td>(124)</td>
</tr>
<tr>
<td>Invariant chain</td>
<td>300</td>
<td>(32)</td>
</tr>
<tr>
<td>IFI56K</td>
<td>155 or 245</td>
<td>(250)</td>
</tr>
<tr>
<td>STAT1α/β</td>
<td>400 and 322</td>
<td>(201)</td>
</tr>
<tr>
<td>βIFN</td>
<td>585</td>
<td>see text</td>
</tr>
<tr>
<td>CIITA</td>
<td>350</td>
<td>see text</td>
</tr>
<tr>
<td>Rabbit β-globin</td>
<td>240 or 170</td>
<td>see text</td>
</tr>
</tbody>
</table>

- The CIITA protection probe was a 350 bp HindIII fragment generated by PCR from a human CIITA cDNA (a gift from Dr Cheong-Hee Chang, Yale University) which was inserted into HindIII digested pGEM4, downstream of the T7 promoter. Clones were screened for orientation by sequencing using a T7 primer.

- The βIFN protection probe was a 585 bp BamHI-BglIII fragment generated by PCR from a βIFN cDNA. This was inserted into pcDNA3 linearised with BamHI and XbaI, downstream of the SP6 promoter. The construct was cloned by Dr Yoshi Watanabe.
The rabbit β-globin template was a linear double-stranded PCR product generated from the CIITA promoter IV-globin reporter construct. One primer contained a T7 promoter site in an overhang, and primed within the rabbit β-globin gene, and the other primer primed at position -155 upstream from the transcription start site within the promoter element in the reporter construct. The transcripts from a promoter IV-globin reporter would protect a 240 bp fragment of the probe, and those from a promoter III driven reporter just the 170 bp fragment of probe derived from the globin gene. The probe was designed by Dr Vermerulagesan Arulampalam.

**Hybridisation and mapping.**

13μg of RNA was dried in a speedvac and resuspended in 24 μl deionised formamide. A cocktail of probes and hybridisation buffer was made up and added to the RNA; final hybridisation conditions were 80% formamide, 40 nM PIPES pH 6.4, 400 mM NaCl and 1 mM EDTA. Samples were heated to 95°C for 5 minutes and placed at 47°C overnight to hybridise. Unprotected RNA was digested in 10 mM Tris-HCl pH 7.5, 5 mM EDTA, 300 mM NaCl, 0.5% SDS (w/v), 40μg/ml RNase A and 2μg/ml RNaseT1 (both Boehringer) at 37°C for 30 minutes. 45 μg/ml Proteinase K was added for 15 minutes and protein was extracted in phenol/chloroform/isoamyl alcohol. RNA was precipitated with tRNA and 100% isopropanol, and resuspended in 85% formamide, 0.1% (w/v) each of Bromophenol Blue and Xylene Cyanol. Samples were heated at 95°C for 5 minutes and run on a 6% denaturing gel (6% 37.5:1 acrylamide:bis-acrylamide, 0.5 xTBE - 45 mM Tris-borate, 1 mM EDTA - 7M urea), and dried for autoradiography.

**Expression profiling using oligonucleotide microarrays.**

Oligonucleotide microarrays can be used to simultaneously monitor the expression levels of large numbers of specific mRNAs in samples from cell-culture or
tissue (141). The Hu6800 GeneChip Arrays used here were manufactured by Affymetrix (Santa Clara, CA). They consist of high-density arrays of oligonucleotides covalently attached to a glass substrate, or chip, sealed in a plastic hybridisation chamber. Each array consists of four chips, A-D, each with ~1700 unique probe sets that together represent ~6800 human genes, and additional probe sets to control for differences between samples and chips. The oligonucleotides are synthesised in situ by a combination of photolithography and conventional oligonucleotide chemistry (reviewed (177)).

Sample mRNA is converted into cDNA and then in vitro transcribed in the presence of biotinylated nucleotides to generate a labelled “cRNA”. The sample “target” cRNA is probed by the oligonucleotide probes on the chips, which hybridise to complementary sequences in the cRNA. Following the hybridisation and washing, those probes annealed to cRNA are visualised by a streptavidin-phycoerythrin conjugate which binds to the biotin incorporated into the cRNA annealing to the probe oligonucleotide.

The methods for sample preparation used are those provided and recommended by Affymetrix. The cRNA samples were fragmented and hybridised by Doctor Leslie-Ann Hawthorn of the Cleveland Clinic Foundation (Cleveland, Ohio) where the chips were scanned and data analysed.

**Target preparation and hybridisation.**

100μg total RNA was isolated from appropriately cultured and stimulated cells using Trizol (Gibco). Poly(A)+ mRNA was purified from this using the Oligotex mRNA kit from Qiagen, and this was used to generate cDNA with the SuperScript Choice System (Life Technologies/Gibco BRL). First strand synthesis through reverse-transcription was primed using a special HPLC-purified oligo (dT)$_{24}$ primer (5’ GGCCAGTGAATTGTAATACGACTCACTATAGGGAGGCG G-(dT)$_{24}$ 3’) which contains a T7 RNA polymerase promoter site 5’ of the poly-A tract. Following second strand synthesis, the cDNA was used as template for in vitro transcription reaction (T7
MegaScript System, Ambion) in the presence of biotin-11-CTP and biotin-16-UTP (Enzo Diagnostics) utilising the T7 site incorporated during the reverse transcription reaction of first strand synthesis. The “copy RNA” (cRNA) labelled with biotinylated nucleotides was purified using RNAeasy spin columns (Qiagen) and quantified and checked by gel electrophoresis.

50μg of purified cRNA from each sample was fragmented at 94°C for 35 minutes in fragmentation buffer (40 mM Tris-acetate, pH 8.1, 100 mM potassium acetate, 30 mM magnesium acetate) and then adjusted to 1M NaCl, 10 mM Tris HCl, pH 7.6, 0.005% Triton X-100, 0.1 mg/ml herring sperm DNA. Control RNAs, or “spikes”, are added to allow comparison between hybridisations on individual chips and to allow the software to map and orientate the array during analysis. They are produced by in-vitro transcription (IVT) and are for bacterial and phage mRNAs. These are BioB, BioC, BioD and cre and are present at 1.5, 5, 25 and 100 pM respectively.

Samples were heated to 95°C for 5 minutes, cooled to 40°C and hybridised to the array at this temperature for 16 hours, rotating in an oven at 60 rpm. Following hybridisation the arrays were washed at 50°C in 6xSSPE (Affymetrix)/0.005% Triton, then 0.5%SSPE/0.005% Triton, stained with 10μg/ml streptavidin-phycoerythrin (Molecular Probes), 1 mg/ml BSA in 6xSSPE/0.005% Triton, washed, and scanned with a confocal microscope scanner (Molecular Dynamics/Affymetrix) using a 560 nm filter.

Data was analysed using GeneChip 3.1 software. An initial absolute analysis of the control sample was first carried out, and then comparative analysis based on this in which the data for the sample to be compared was adjusted on the basis of the relative intensities of control probe sets for GAPDH and Actin, and then normalised for all genes.
## Table 2.1. Promoter reporter constructs.

<table>
<thead>
<tr>
<th>Construct</th>
<th>Details</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>NFκB-Luciferase</td>
<td>3 copies of NFκB consensus element from the immunoglobulin κ-chain upstream of the ovalbumin core promoter (conA). (7)</td>
<td>Dr Ron Hay, St Andrews University.</td>
</tr>
<tr>
<td>IRF1 promoter-Luciferase</td>
<td>Fragment of IRF1 promoter from -138 to +7 upstream of β-globin 5'UTR + Luciferase.</td>
<td>Dr Steve Goodbourn, St George’s Hospital Medical School, London.</td>
</tr>
<tr>
<td>(Hex)$_4$-Luciferase</td>
<td>4 copies of IRF1-binding Hex element upstream from the thymidine kinase core promoter (tk) + Luciferase.</td>
<td>Dr Steve Goodbourn.</td>
</tr>
<tr>
<td>CIITA GAS-Luciferase</td>
<td>2 or 4 copies of an oligo containing the GAS site and the adjacent E-Box from CIITA promoter IV- (GATC)TCCACACGTGCT TTATCAGAAGTG - upstream of tk-Luciferase</td>
<td>Dr Steve Goodbourn.</td>
</tr>
<tr>
<td>IRF1 GAS-Luciferase</td>
<td>2 or 6 copies of an oligo containing the GAS site from the IRF1 promoter - (GATC)TGATTTC CCCGA ATG - upstream of tk-Luciferase.</td>
<td>Dr Steve Goodbourn.</td>
</tr>
<tr>
<td>pIV-Luciferase</td>
<td>Fragment of CIITA promoter IV from -346 to +50 inserted into pGL2 Basic.</td>
<td>Dr Jenny Ting, UNC Lineberger Comprehensive Cancer Centre, University of North Carolina at Chapel Hill, NC.</td>
</tr>
<tr>
<td>pIVmGAS-Luciferase</td>
<td>As above, but with GAS site altered to ggaagtcTAAA.</td>
<td>Dr Jenny Ting.</td>
</tr>
<tr>
<td>pIVmκB-Luciferase</td>
<td>As pIV-Luciferase but with κB site altered to attaACTCTCC.</td>
<td>See text.</td>
</tr>
<tr>
<td>pIV-globin</td>
<td>Fragment from CIITA promoter IV upstream of rabbit β-globin in pGEM-3Zf(+). (170)</td>
<td>Dr Bernard Mach, University of Geneva Medical School.</td>
</tr>
<tr>
<td>pKS1β-gal</td>
<td>CMV immediate early promoter driving constitutive expression of β-galactosidase.</td>
<td>Dr Katy Smith, ICRF.</td>
</tr>
</tbody>
</table>
Figure 2.1.

Strategy for the mutagenesis of the NFκB site in CIITA promoter IV.

Mutagenic PCR mismatch primers were designed incorporating the mutagenised NFκB site used by Demengeot et al. (50). Flanking primers were designed encompassing suitable restriction sites present in the plasmid containing the promoter IV-Luciferase construct, and the primers were used to prime PCR reactions as described.
First round: 2 reactions, primed with Primer 1&3, and 2&4 respectively. Template for both reactions is the plasmid pIV-luciferase. Primers 1 and 4 prime over the Kpn1 and Xba1 restriction sites flanking promoter IV in the plasmid. Primers 2 and 3 prime over the NFkappaB site and contain the mutated NFkappaB sequence flanked by non-mutagenic arms which anneal either side of the NFkappaB site. The mutagenic sequences loop-out, and are incorporated with the primer.

Second round: 1 reaction primed with Primers 1 and 4, using the products of the first round reactions as template, mixed 1:1.

Following the second round and clean up, the product is digested with Kpn1 and Xba1 and cloned into the vector - pIV luciferase - cut with the same enzymes.
Table 2.2. Antibodies used for immunoprecipitation and Western blot analysis, and also EMSA supershifts.

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Antibody (supplied by Santa Cruz unless stated.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phosphotyrosine</td>
<td>PY20 (Affiniti) and 4G10 (UBI) - both mouse monoclonal</td>
</tr>
<tr>
<td>STAT1</td>
<td>mouse monoclonal (Nova Castra Bio)</td>
</tr>
<tr>
<td>STAT1&lt;sup&gt;Phospho-serine 727&lt;/sup&gt;</td>
<td>rabbit polyclonal (Upstate Biotechnology)</td>
</tr>
<tr>
<td>STAT1&lt;sup&gt;Phospho-tyrosine 701&lt;/sup&gt;</td>
<td>rabbit polyclonal (New England BioLabs)</td>
</tr>
<tr>
<td>Jak1</td>
<td>M7 rabbit polyclonal raised against JH2 (Dr A Ziemicki) rabbit polyclonal (HR-785) 1:1000</td>
</tr>
<tr>
<td>Jak2</td>
<td>rabbit polyclonal (Dr A Ziemicki) rabbit polyclonal (HR-758)</td>
</tr>
<tr>
<td>IFNGR1</td>
<td>rabbit polyclonal (C-20) 1:1000</td>
</tr>
<tr>
<td>IRF1</td>
<td>rabbit (C-20) 1:200</td>
</tr>
<tr>
<td>IRF3</td>
<td>rabbit (FL-425) 1:200</td>
</tr>
<tr>
<td>p48</td>
<td>rabbit (C-20)</td>
</tr>
<tr>
<td>IκBβ</td>
<td>rabbit (C-19)</td>
</tr>
<tr>
<td>NFκB p50/105</td>
<td>rabbit (NLS) 1:1000</td>
</tr>
<tr>
<td>NFκB p50</td>
<td>rabbit (C-20) 1:1000</td>
</tr>
<tr>
<td>NFκB p65</td>
<td>rabbit (C-20) 1:1000</td>
</tr>
<tr>
<td>USF1</td>
<td>rabbit (C-20)</td>
</tr>
<tr>
<td>PKR</td>
<td>mouse monoclonal (Dr A. Hovanessien, Institut Pasteur) rabbit polyclonal (Dr B.R.G. Williams, CCF)</td>
</tr>
<tr>
<td>p38 MAPK</td>
<td>rabbit polyclonal (New England BioLabs)</td>
</tr>
<tr>
<td>Phospho-p38 MAPK</td>
<td>rabbit polyclonal (New England BioLabs)</td>
</tr>
</tbody>
</table>
### Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>6TG</td>
<td>6-thioguanine</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>bp</td>
<td>base pairs</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin (protease free)</td>
</tr>
<tr>
<td>CIITA</td>
<td>Class II Transactivator</td>
</tr>
<tr>
<td>CBP</td>
<td>CREB-binding protein</td>
</tr>
<tr>
<td>CMV</td>
<td>Cytomegalovirus</td>
</tr>
<tr>
<td>cpm</td>
<td>counts per minute</td>
</tr>
<tr>
<td>CTP</td>
<td>cytosine triphosphate</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethyl sulphoxide</td>
</tr>
<tr>
<td>dsRNA</td>
<td>double stranded RNA</td>
</tr>
<tr>
<td>DTT</td>
<td>dithiothreitol</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco's modified Eagle's medium</td>
</tr>
<tr>
<td>DRAF</td>
<td>dsRNA activated factor</td>
</tr>
<tr>
<td>ECL</td>
<td>enhanced chemi-luminescence</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediamine tetra-acetic acid</td>
</tr>
<tr>
<td>EGF</td>
<td>epidermal growth factor</td>
</tr>
<tr>
<td>EGTA</td>
<td>ethylene glycol-bis(b-aminoethyl ether) N, N', N'-tetra-acetic acid</td>
</tr>
<tr>
<td>eIF2α</td>
<td>eukaryotic initiation factor 2α</td>
</tr>
<tr>
<td>EMCV</td>
<td>encephalomyocarditis virus</td>
</tr>
<tr>
<td>EMSA</td>
<td>electrophoretic mobility shift assay</td>
</tr>
<tr>
<td>EPO</td>
<td>erythropoietin</td>
</tr>
<tr>
<td>ERK</td>
<td>extracellular regulated kinase</td>
</tr>
<tr>
<td>FACS</td>
<td>fluorescence activated cell scanner</td>
</tr>
<tr>
<td>FCS</td>
<td>foetal calf serum</td>
</tr>
<tr>
<td>FERM</td>
<td>4.1/ezrin radixin USFmoesin</td>
</tr>
<tr>
<td>Acronym</td>
<td>Definition</td>
</tr>
<tr>
<td>---------</td>
<td>------------</td>
</tr>
<tr>
<td>GAS</td>
<td>Interferon γ activated sequence</td>
</tr>
<tr>
<td>gpt</td>
<td>guanine phosphoribosyltransferase</td>
</tr>
<tr>
<td>GTP</td>
<td>guanosine triphosphate</td>
</tr>
<tr>
<td>HAT</td>
<td>hypoxanthine, aminopterin, thymidine medium</td>
</tr>
<tr>
<td>HEPES</td>
<td>N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid</td>
</tr>
<tr>
<td>HLA</td>
<td>human leukocyte antigen</td>
</tr>
<tr>
<td>HPLC</td>
<td>high performance liquid chromatography</td>
</tr>
<tr>
<td>HPRT</td>
<td>hypoxanthine phosphoribosyltransferase</td>
</tr>
<tr>
<td>HPV</td>
<td>human papiloma virus</td>
</tr>
<tr>
<td>hr</td>
<td>hours(s)</td>
</tr>
<tr>
<td>ICAM</td>
<td>intracellular adhesion molecule</td>
</tr>
<tr>
<td>IFN</td>
<td>Interferon</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>IRF</td>
<td>interferon regulatory factor</td>
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<td>IRS1</td>
<td>insulin receptor substrate 1</td>
</tr>
<tr>
<td>ISG</td>
<td>interferon stimulated gene</td>
</tr>
<tr>
<td>ISGF3</td>
<td>interferon stimulated gene factor 3</td>
</tr>
<tr>
<td>ISRE</td>
<td>interferon stimulated response element</td>
</tr>
<tr>
<td>IU</td>
<td>international units</td>
</tr>
<tr>
<td>Jak</td>
<td>Janus kinase/just another kinase</td>
</tr>
<tr>
<td>JH</td>
<td>Jak homology</td>
</tr>
<tr>
<td>kb</td>
<td>kilobase</td>
</tr>
<tr>
<td>LB</td>
<td>Luria Broth</td>
</tr>
<tr>
<td>LMB</td>
<td>Leptomycin B</td>
</tr>
<tr>
<td>LPS</td>
<td>lipopolysaccharide</td>
</tr>
<tr>
<td>m.o.i</td>
<td>multiplicity of infection</td>
</tr>
<tr>
<td>MAPK</td>
<td>mitogen activated protein kinase</td>
</tr>
<tr>
<td>MHC</td>
<td>major histocompatibility complex</td>
</tr>
<tr>
<td>NES</td>
<td>nuclear export sequence</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>NGE</td>
<td>NF-Gma GAS E box</td>
</tr>
<tr>
<td>NLS</td>
<td>nuclear localisation sequence</td>
</tr>
<tr>
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<td>Nonidet P40</td>
</tr>
<tr>
<td>OSM</td>
<td>Oncostatin M</td>
</tr>
<tr>
<td>PAGE</td>
<td>polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>PBSA</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>PCAF</td>
<td>p300/CBP associated protein</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>PDGF</td>
<td>platelet derived growth factor</td>
</tr>
<tr>
<td>pfu</td>
<td>plaque forming units</td>
</tr>
<tr>
<td>PI3K</td>
<td>phosphatidylinositol 3-kinase</td>
</tr>
<tr>
<td>PIPES</td>
<td>1, 4-piperazinediethansulphonic acid</td>
</tr>
<tr>
<td>PKR</td>
<td>dsRNA-dependent protein kinase</td>
</tr>
<tr>
<td>PMSF</td>
<td>phenylmethysulphonfluoride</td>
</tr>
<tr>
<td>PVDF</td>
<td>polyvinylidene difluoride</td>
</tr>
<tr>
<td>rpm</td>
<td>revolutions per minute</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulphate</td>
</tr>
<tr>
<td>SH</td>
<td>src homology domain</td>
</tr>
<tr>
<td>SIE</td>
<td>Serum Inducible Element</td>
</tr>
<tr>
<td>STAT</td>
<td>signal transducer and activator of transcription</td>
</tr>
<tr>
<td>TAE</td>
<td>Tris-acetate/EDTA buffer</td>
</tr>
<tr>
<td>TBE</td>
<td>Tris-borate/EDTA buffer</td>
</tr>
<tr>
<td>TBST</td>
<td>Tris-buffered saline with 0.1% Tween20</td>
</tr>
<tr>
<td>TE</td>
<td>10mM Tris/HCl, 1mM EDTA</td>
</tr>
<tr>
<td>TNF</td>
<td>tumour necrosis factor</td>
</tr>
<tr>
<td>Tris</td>
<td>2-amino-2(hydroxymethyl)-1,3-propanediol</td>
</tr>
<tr>
<td>tRNA</td>
<td>transfer RNA</td>
</tr>
<tr>
<td>USF</td>
<td>upstream stimulatory factor</td>
</tr>
<tr>
<td>UTP</td>
<td>uridine 5'-triphosphate</td>
</tr>
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</table>
Chapter 3.

THE IDENTIFICATION OF A JAK1 MUTANT WITH A DOMINANT INHIBITORY EFFECT ON IFN GAMMA ANTIVIRAL RESPONSE AND INDUCTION OF THE CLASS II TRANSACTIVATOR CIITA.

Introduction.

There is now persuasive evidence pointing to additional ligand-dependent signalling in response to IFNs which cannot be accounted for by the traditional Jak/STAT signal transduction pathway (125, 218, 219). Previous work in the lab analysed the effect of two kinase-inactivating mutations of Jak1, using a strategy based on motifs conserved across families of kinases (18, 72). One involved the substitution of lysine 912 for alanine (referred to as K>E) in a motif thought to bind ATP in JH1, and the other a substitution of aspartic acid 1019 with serine (referred to as SFG) in another conserved motif also in JH1. In both instances, when the kinase-negative Jak1 mutant is expressed in the Jak1-deficient U4A cell line, it is unable to mediate signalling in response to a variety of cytokines. In response to IFNγ, however, Jak/STAT activation and gene-induction is observed, but there is no antiviral response. Furthermore, when the kinase negative Jak1K>E is over-expressed in wild type cells there is no detectable effect on the Jak/STAT pathway and IFNγ-inducible gene expression, but the antiviral response to IFNγ is inhibited through a dominant-negative effect. The simplest possible interpretation of this data suggests a requirement for an
additional Jak1 function for the antiviral response, and any such function requires the kinase activity.

A panel of deletion mutants of Jak1 was generated to investigate whether any domain requirements of Jak1-mediated signalling could be defined. The mutant Jak1ΔB was identified as having a substantial dominant negative effect on the IFNγ antiviral and Class II HLA responses, with no apparent effect on ligand-dependent Jak/STAT activation. The degree of inhibition shows a good correlation with the expression level of Jak1ΔB itself.

Further characterisation revealed that Jak1ΔB also inhibits the IFNγ induction of CIITA mRNA with a concomitant inhibition of Class II HLA expression, and a small effect on Class I HLA, reflecting a limited requirement for CIITA for this response (149). Initial RNA protection analysis showed no detectable inhibition of other mRNAs, although subsequent expression profiling using oligonucleotide microarrays (Appendix) indicated that these results were misleading and that a substantial proportion of IFNγ-inducible genes might be affected.

The IFNα induction of Class I HLA was unaffected, but there was a small inhibition of the IFNα antiviral response.

**Results.**

**Inhibition of the antiviral response to IFNγ in cells expressing Jak1ΔB.**

Initial analysis of a series of recombinant deletion mutants of Jak1 expressed in wild type 2fTGH cells was through the cytopathic antiviral assay. A screen of the Jak1 mutants (illustrated in Figure 3.1) constructed by Dr Michele Popelierz yielded one, referred to as Jak1ΔB, with a similar but distinct phenotype to Jak1K>E, which was
potentially much more amenable to analysis. The mutant had a substantial dominant negative effect on the ability of IFNγ to induce an antiviral state in cells (Figure 3.2).

Following overnight pre-treatment with IFNα or γ at various concentrations, for wild type 2fTGH cells IFNα or γ each provide protection at 10 units/ml; in contrast, similarly pre-treated 2fJak1ΔB cells expressing the deletion mutant Jak1ΔB were not protected even at 10^3 units/ml of IFNγ. It should be noted that the response to IFNα is also slightly inhibited, but the effect is minor in comparison to that on IFNγ. Jak1ΔB was the only one of the mutants to demonstrate an effect in the assay. In view of this striking phenotype a more thorough analysis of cells expressing Jak1ΔB was undertaken.

Selective inhibition of the IFNγ induction of Class II HLA by Jak1ΔB.

It was important to assess to what extent Jak1ΔB inhibited responses to IFNγ – whether the inhibition was widespread or restricted to the induction of an antiviral state in response to IFNγ: another mutant of Jak1 with a dominant inhibitory phenotype (Jak1K>E) which had been characterised in the lab also inhibited the antiviral response, but was without an apparent effect on the activation of the Jak/STAT pathway or the induction of IFN-stimulated genes (ISGs) by IFNγ.

FACS analysis was used to assay the induction by IFNs of the Class I and Class II HLAs in cells expressing Jak1ΔB (Figure 3.3). The induction by IFNγ of Class II but not Class I HLAs is strongly inhibited by Jak1ΔB. IFNα induction of Class I HLAs is not measurably affected either.

The inhibition by Jak1ΔB of cell-surface expression of Class II HLA could be the result of a defect in the post-translational processing or transport of the protein,
rather than an effect on the expression *per se* of the genes themselves. Reporter assays were consistent with the inhibition of transcription (data not shown), and RNase protection analysis showed a strong induction of HLA-DRα mRNA in response to IFNγ in wild type cells but no induction in the presence of Jak1ΔB (Figure 3.4). No inhibition of the induction of IRF1, STAT1, 9-27 or p48 mRNA was seen after 6 and 18 hours of IFNγ treatment in the presence of Jak1ΔB. Of the genes assayed, DRα was the only one inhibited.

It would appear that Jak1ΔB selectively inhibits a subset of responses to IFNγ and has no major effect on responses to IFNα. This is in contrast with the Jak1K>E phenotype where no inhibition of the Class II response was seen, but inhibition of all responses to IFNα and other cytokines dependent on the Jak/STAT pathway, in addition to the antiviral response to IFNγ was evident (18). Thus, Jak1ΔB has a novel phenotype, although the effect on the antiviral responses seen in both cases may indicate some relationship between the two mechanisms.

**Inhibition by Jak1ΔB is dose-dependent.**

To ensure that the inhibition by Jak1ΔB was not the result of clonal variation, the response of two other clones of 2fTGH cells expressing Jak1ΔB was monitored. The original clone #5 showed a greater inhibition of Class II HLAs than clone #3, whilst the response of clone #6 was closer to wild type (Figure 3.5). Interestingly, the induction of Class I in response to IFNγ was now seen to be weakly inhibited. This observation became apparent through overlaying the profiles for the three clones, and the relative inhibition for the three clones was as seen for Class II (Figure 3.5).
The induction of mRNAs in response to IFNγ in the three clones was monitored (Figure 3.6). Consistent with the results of the FACS analysis the expression of the mRNAs for DRα (Class II HLA) and the co-regulated Invariant chain were substantially inhibited in clones #5 and #3, but not in clone #6. There was no significant inhibition of the other ISGs monitored (Figure 3.6).

The antiviral responses of the three clones were also compared. The same pattern again was evident, with the greatest inhibition in clone #5 and the least in clone #6 (Figure 3.7). The level of expression of the mutant Jak1ΔB protein in the three was monitored (Figure 3.8). Expression of Jak1ΔB was high in clones #5 and #3, but relatively low in clone #6. Thus, the degree of inhibition of the Class II HLA and antiviral responses correlates well with the level of expression of Jak1ΔB.

**Jak1ΔB inhibits the expression of class II transactivator (CIITA) in response to IFNγ.**

The regulation and co-ordinated expression of Class II HLAs has been extensively studied. Common cis-regulatory elements in the promoters of the genes for the class II α- and β-chains, and the Invariant chain (Ii) lead to co-regulation of the genes (reviewed in (66)). In addition to signals to these regulatory elements, a transcriptional regulatory protein called Class II transactivator (CIITA) has been identified as essential for expression of Class II genes (22, 227, 228). Although it does not directly bind to DNA, CIITA, which is itself strongly upregulated by IFNγ, is thought to act as a “transcriptional scaffold” which specifically integrates signals leading to transcription from the Class II promoters (278). The expression of CIITA in the three clones expressing Jak1ΔB was monitored following treatment with IFNγ (Figure 3.9). The results are consistent with an inhibition of the induction of CIITA by Jak1ΔB, and the degree of inhibition again correlates with the level of expression of the
mutant Jak1 - clone #5 the greatest and clone #6 the least. The inhibition of CIITA is also consistent with the slight inhibition of the Class I response to IFNγ seen, since CIITA, although not an essential factor, is known to contribute to the regulation of Class I (67, 149). Even after a prolonged treatment with IFNγ there was no significant induction of CIITA mRNA in 2fJak1ΔB (Figure 3.10), given that both zero time points appear slightly under-loaded based on actin levels.

Constitutive expression of CIITA confers Class II HLA expression (but not an antiviral state) in Jak1ΔB cells.

Han et al. (74) showed that TNFα is able to suppress IFNγ induction of Class II HLA by destabilising CIITA mRNA. Stable transfection of a construct for constitutive expression of CIITA into 2fJak1ΔB clone #5 produced clones constitutively expressing Class II HLAs (Figure 3.11). This result argues against a major destabilising effect through Jak1ΔB on CIITA mRNA. The inhibition of expression from CIITA promoter/reporter constructs in the Jak1ΔB cells (Chapter 5) provides more definitive evidence that the effect of Jak1ΔB is upon transcription itself.

As expected, the constitutive expression of CIITA in the Jak1ΔB cells had no effect on the inhibition of the antiviral response (data not shown) (V. Arulampalam, unpublished data).
Figure 3.1.

Jak1 deletion mutants.

The figure depicts the deletion mutants of Jak1 (including Jak1ΔB) which were constructed by Dr Michelle Popelianz and screened using the cytopathic antiviral assay.
Figure 3.1.

JH7  JH6  JH5  JH4  JH3  JH2  JH1

N

wild-type Jak1

0  500  1000

amino acid residues

ΔBamHI

Jak1ΔB

ΔNco1-BstBI

Jak1ΔNB

ΔXhoI

Jak1ΔX

ΔAfl2-NheI

Jak1ΔN
Figure 3.2.

The effect of Jak1ΔB on the antiviral response of cells treated with IFNα and γ.

1x10^5 cells/well were seeded on a 24 well plate. IFN α and γ was added to cells as shown and left overnight. Medium was removed from the wells and replaced with 0.5 ml of medium containing 0.5 pfu/cell, except for control wells where medium alone was added. Cells were incubated for 24 hours, at which time the virus infected, non-IFN treated cells had died. Plates were fixed with formol saline and stained with Giemsa.
Figure 3.2.

<table>
<thead>
<tr>
<th>Units of IFN/ml</th>
<th>0</th>
<th>10</th>
<th>100</th>
<th>1000</th>
<th>10000</th>
</tr>
</thead>
<tbody>
<tr>
<td>No virus No IFN</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2fTGH IFN α</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2fTGH IFN γ</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2fJak1 ΔB IFN α</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2fJak1 ΔB IFN γ</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Figure 3.3.

FACS analysis of the effect of Jak1ΔB on the expression of Class I and II HLA proteins in response to IFNα and γ.

2fTGH and 2fJak1ΔB cells were treated with IFNα and γ for 72 hours. The cells were detached from culture plates and incubated with antibodies specific for either Class I or II HLA proteins, washed and fixed in 1% paraformaldehyde. The cell surface expression of HLA proteins was monitored by FACS analysis.
Figure 3.3.

Untreated
IFNα, 72 hours
IFNγ, 72 hours

Class I HLA

2fTGH

Class II HLA

2fJak1ΔB#5
Figure 3.4.

RNase protection analysis of the effect of Jak1ΔB on the expression of various genes in response to IFNγ.

2fTGH and 2fJak1ΔB cells were treated with IFNγ for 6 or 18 hours, and cytoplasmic RNA harvested, alongside that from untreated controls. RNase protection analysis was performed as described in the Material and Methods. All samples were probed for Actin, STAT1 and 9-27. In addition, 6 hour samples were probed for IRF1 and p48/ISGF3γ, 18 hour samples probed for HLA-DRα, and untreated control samples probed with all probes.
Figure 3.4.

<table>
<thead>
<tr>
<th></th>
<th>2fTGH</th>
<th>2fJak1ΔB</th>
</tr>
</thead>
<tbody>
<tr>
<td>- 6 18</td>
<td></td>
<td></td>
</tr>
<tr>
<td>- 6 18</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

hrs treatment with IFNγ

- Stat1α
- Stat1β
- p48/ISGF3γ
- DRα
- IRF1
- 9-27
- Actin
Figure 3.5.

FACS analysis of IFNγ induction of Class I and II HLA proteins in different clones stably expressing the mutant Jak1ΔB.

Three clones expressing Jak1ΔB were treated with IFNγ for 72 hours in parallel with wild type 2fTGH cells. Control untreated and treated cells were incubated with antibodies specific for either Class I or II HLA proteins, washed and fixed in 1% paraformaldehyde. The cell surface expression of HLA proteins was monitored by FACS analysis, and the profiles overlaid to show the relative levels of expression.
Figure 3.5.

**Expression of Class I HLA**

**Expression of Class II HLA**
Figure 3.6.

RNase protection analysis of the expression of Class II HLA mRNA in different clones of cells expressing Jak1ΔB.

Three clones expressing Jak1ΔB were treated with IFNα or γ for 18 hours in parallel with wild type 2fTGH cells. RNA was harvested from the cells, alongside that from untreated controls. RNase protection analysis was performed as described in the Material and Methods. Samples were probed for the expression of Actin, 9-27, IRF1, 6-16, Class II HLA (DRα) and Invariant chain (Inv).
Figure 3.6.

2fTGH  2fJak1ΔB
Clone 3  Clone 5  Clone 6

DRα
Inv
6-16
IRF-1
9-27
Actin

/ α γ / α γ / α γ / α γ / α γ

IFN Treatment
Chapter 3. Identification of a Jak1 mutant with a dominant inhibitory effect.

**Figure 3.7.**

The antiviral response to IFNγ of different clones of cells expressing Jak1ΔB.

Cells were seeded on a 24 well plate and IFNγ was added to cells as indicated and left overnight. Medium was removed from the wells and replaced with 0.5 ml of medium containing 0.5 pfu/cell, except for control wells where medium alone was added. Cells were incubated for 24 hours, at which time the virus infected, non-IFN treated cells had died. Plates were fixed, and stained with Giemsa.
Figure 3.7
Figure 3.8.

Western blot analysis of the level of expression of Jak1ΔB in different clones.

The three clones expressing Jak1ΔB were seeded onto plates in parallel with 2fTGH cells and harvested in Schindler lysis buffer the next day. The concentration of protein in the extracts was determined and equal amounts of protein run on a 6.5% SDS-PAGE gel. Proteins were transferred to PVDF and the membrane blocked, then Western blotted with anti-Jak1 antibody. After overnight stripping the membrane was reprobed with anti-STAT1 to control for the amount of protein present.
Figure 3.8.

Western blot

Anti Jak1

Anti STAT1

(40mg protein) (20mg protein)
Figure 3.9.

RNase protection analysis showing the IFNγ induction of CIITA mRNA in different clones expressing Jak1ΔB.

The three clones expressing Jak1ΔB were treated with IFNα or γ for 18 hours in parallel with wild type 2fTGH cells. RNA was harvested from the cells, alongside that from untreated controls. RNase protection analysis was performed as described in the Materials and Methods. Samples were probed for the expression of Actin, IRF1, 6-16 and CIITA.
Figure 3.9.

2fTGH  2fJak1∆B
clone3  clone5  clone6

Treatment:  -  α γ  -  α γ  -  α γ  -  α γ
Figure 3.10.

Time course of CIITA induction in 2fTGH and 2fJak1ΔB#5 cells treated with IFNγ.

2fTGH and 2fJak1ΔB#5 cells were treated with IFNγ for the times shown. RNA was harvested from the cells, alongside that from untreated controls. RNase protection analysis was performed using probes for Actin, 9-27, IRF1, 6-16, DRα and CIITA. The lanes corresponding to both 0 hour time points appear to be underloaded.
Figure 3.10.

2fTGH  2fJak1 ΔB

CIITA

DRα

6-16

IRF1

9-27

Actin

0 6 12 18 24 30 0 6 12 18 24 30 hours induction with IFNγ
Figure 3.11.

FACS analysis of the effect of stable expression of CIITA on HLA expression in cells expressing Jak1ΔB.

A clone of 2fJak1ΔB#5 stably transfected with an expression construct for CIITA, called 2fΔBCIITA#3.5, was plated out along with 2fJak1ΔB#5 and wild type 2fTGH cells. Each cell line was treated with IFNγ for 72 hours, the cells harvested, and incubated with antibodies specific for either Class I or II HLA proteins alongside untreated control cells. After incubation with anti-HLA antibodies, cells were washed and fixed in 1% paraformaldehyde. The cell surface expression of HLA proteins was monitored by FACS analysis as shown.
Figure 3.11.

**Expression of Class I HLA.**

2fTG1

2fJak1ΔB#5

2fΔBCIITA#3.5

**Expression of Class II HLA.**

Untreated

+IFNγ
Discussion.

Initial screening identified Jak1ΔB as having a profound effect on the IFNγ-induced antiviral response (Figure 3.2) without a similar effect on the response to IFNα. FACS analysis of HLA proteins induced in response to IFNα or γ showed a clear inhibition of IFNγ-induced Class II HLA expression in 2fJak1ΔB, but a slight inhibition of IFNγ-induced Class I HLA, and no inhibition of the response to IFNα as measured by expression of Class I (Figure 3.3). Inhibition of the Class II HLA response was shown to be at the mRNA level (Figure 3.4) and subsequently to be the result of inhibition of IFNγ-induced CIITA expression (Figure 3.9). Finally, the inhibitory effects of Jak1ΔB correlated with the level of expression (Figure 3.5-3.8).

There are a number of instances of specific inhibition of the IFNγ response. These include inhibition by virus (121), inhibition by other cytokines or growth factors such as βIFN (142) and IL-1β (190), and recently the inhibition by Chlamydia (274). Those more specifically affecting HLA Class II expression include those affecting CIITA function and expression.

For Jak1ΔB the mechanism for its effect is not apparent, and an overlap with one or more of these other mechanisms is a possibility. More specifically regarding the Jaks, the majority of Jak1 and Jak2 mutants have had no detectable phenotype in the available assays. Amongst the more informative have been point mutations in the motifs regulating kinase activity of Jak2 and Jak1 which produced kinase-dead proteins. Kinase-negative Jak2 does not support Jak/STAT activation in response to ligands, and acts as a dominant-negative with respect to IFNγ responses (18). On the other hand, a kinase-negative Jak1 mutant - Jak1K>E - can sustain STAT activation and significant
levels of gene expression, but not an antiviral response to IFNγ. In addition, when expressed as a dominant-negative it inhibits the antiviral response without a detectable effect on the Jak/STAT pathway or gene expression (18).

Comparisons of the mutant Jak1ΔB characterised here with the Jak1K>E are inevitable based upon the antiviral response, although the two mutants are distinct in the other assays used. The antiviral response is clearly a complex phenotype, whereas the inhibition of CIITA expression, which is unique to the Jak1ΔB mutant, is more amenable to analysis, being potentially mediated through a single promoter region. For this reason it was an obvious choice for further analysis of the mechanism of Jak1ΔB.
Chapter 4.

**JAKΔB HAS NO DETECTABLE EFFECT ON THE JAK/STAT PATHWAY.**

**Introduction.**

In view of the profound inhibition of the IFNγ antiviral response by JakΔB, the most obvious site of the inhibition was thought to be the Jak/STAT pathway, and thus a thorough analysis of it was undertaken. IFNγ binding leads to phosphorylation of Jak1, Jak2, IFNGR1 and STAT1: inhibition of any of these would affect the response to ligand binding. Jak2 is the essential kinase activity for IFNγ signalling, Jak1 kinase activity being essential only for the antiviral response, not for STAT1 activation (18). The phosphorylation of the receptor is required for STAT1 docking and subsequent tyrosine phosphorylation, which is obligatory for dimerisation and translocation to the nucleus for transcriptional regulation. There was no detectable inhibition by JakΔB of receptor complex activation or STAT1 activation, nor was any effect detectable on the serine phosphorylation of STAT1 which is required for full transcriptional activity. Proteins thought to regulate the serine phosphorylation pathway itself were also unaffected. Importantly, it was discovered that JakΔB was constitutively phosphorylated, presumably reflecting significant (auto)kinase activity. The introduction of a kinase-inactivating mutation into JakΔB disrupted this constitutive phosphorylation and resulted in the loss of the inhibitory phenotype. Analysis of proteins by cellular fractionation was consistent with JakΔB being cytosolic and having no association with the receptor complex. This was consistent
with there being no dominant negative effect on the Jak/STAT pathway and no ligand-dependent variation in the level of Jak1ΔB phosphorylation.

Results.

The Jak/STAT pathway is unaffected by Jak1ΔB.

Receptor events and the activation by IFNγ of the Jak/STAT1 pathway in the presence of Jak1ΔB were analysed by immunoprecipitation and anti-phosphotyrosine Western blot analysis. In the 2fJak1ΔB clone #5 cells analysed - assuming comparable antibody specificity - the mutant Jak is expressed to slightly higher levels than the endogenous Jak1 (Figure 3.8). Tyrosine phosphorylation of the IFNγ receptor 1 chain (IFNGR1), Jak1, Jak2 and STAT1 in response to IFNγ are unaffected by the presence of Jak1ΔB (Figure 4.1). However, the mutant Jak1ΔB is constitutively phosphorylated - in contrast to the inducible phosphorylation of wild type Jak1 - which may have implications for the mechanism of the inhibition.

Consistent with the absence of an effect on tyrosine phosphorylation of the Jaks or STAT1, EMSA analysis showed no difference between wild-type and Jak1ΔB-expressing cells in terms of the activation of STAT1 in response to IFNγ or IFNα (Figure 4.2). The activation of STAT1 and STAT3 by Oncostatin M was also unaffected (Figure 4.2).

Tyrosine phosphorylation is the essential step for STAT activation, but the transcriptional activity of several STATs is also modulated through phosphorylation of conserved serine residues. In the case of STAT1, phosphorylation of serine residue 727 is required for maximal transcriptional activity. There is increasing evidence that p38
Chapter 4. Jak1ΔB has no detectable effect on the Jak/STAT pathway.

MAP kinase (p38MAPK) can phosphorylate serine 727 and that it is activated in response to both α/β IFNs. More recently, IFNγ was shown to activate p38 MAPK and induce serine 727 phosphorylation (68), but there is conflicting evidence that it is p38 MAPK which is responsible for serine phosphorylation in response to IFNγ (48).

Western blot analysis of the serine phosphorylation of STAT1 in 2fTGH and 2fJak1ΔB cells shows an increase following IFNγ treatment (Figure 4.3). No inducible phosphorylation of p38 MAPK in response to IFNγ was observed in either cell line. Anisomycin - a potent activator of MAP kinases - did induce a strong autophosphorylation of p38 MAPK, with corresponding phosphorylation of serine 727 in both cell lines (Figure 4.3). Jak1ΔB had no obvious effect on this serine 727 phosphorylation or that observed in response to IFNγ (Figure 4.3). The same blot was reprobed, confirming the absence of any inhibition of the phosphorylation of tyrosine 701. Thus, Jak1ΔB appears to be without effect on either tyrosine or serine phosphorylation of STAT1 in response to IFNγ, and the observed activation of p38 MAPK.

Induction and activation of double-stranded RNA-dependent protein kinase (PKR) is not inhibited by Jak1ΔB.

Prolonged IFN treatment leads to induction of PKR, which is involved in the response to both α/β IFNs as well as IFNγ (118, 264). The autokinase activity of PKR immunoprecipitated from IFN-treated cell extracts was assayed. A low-level increase in activity in wild-type cells accompanied treatment with IFNγ, and this was not affected by Jak1ΔB (Figure 4.4c). The inducible expression of PKR protein was also unaffected (Figure 4.4b).
Chapter 4. Jak1ΔB has no detectable effect on the Jak/STAT pathway.

The inhibitory effect of Jak1ΔB is dependent on a functional kinase domain.

Western blotting repeatedly showed significant constitutive tyrosine phosphorylation of Jak1ΔB. It is recognised that overexpression of the Jak family kinases can lead to their autophosphorylation. For Jak1ΔB it was possible that the resultant kinase-activity could be a major factor in the observed dominant negative effects of the mutant. To address this issue, a kinase negative Jak1ΔB was generated using the inactivating lysine to glutamic acid substitution in motif II of the kinase domain (Hanks et al., 1988). This new mutant was designated Jak1ΔBK>E.

Stable clones of 2fTGH cells expressing levels of Jak1ΔBK>E comparable to that of Jak1ΔB were isolated. No apparent inhibitory effect of the Jak1ΔBK>E on the induction of CIITA or Class II mRNAs was seen (Figure 4.5), nor on the antiviral response (data not presented). FACS analysis indicated a residual partial inhibition of the expression of Class II proteins in response to IFNγ, but expression levels of Jak1ΔBK>E did not seem to correlate with the extent of this residual inhibition (data not shown).

The cellular localisation of Jak1ΔB is different to endogenous Jak1.

The fact that the phosphorylation of Jak1ΔB was not induced by IFNγ, but seen constitutively, suggested that the phosphorylation was not a result of interactions with the activated receptor complex. This possibility required verification because it would provide an insight into the site of the inhibitory effect. The subcellular distribution of Jak1ΔB was investigated by preparing membrane and cytosolic protein fractions from 2fJak1ΔB cells. The mutant protein is cytoplasmic whilst endogenous Jak1 is found at the membrane, presumably in association with cytokine-receptor complexes (Figure 4.6). A parallel Western blot for Class I HLA protein showed the absence of
contamination of the cytoplasmic extract with membrane protein (Figure 4.6), and lactate dehydrogenase assays (Figure 4.7) demonstrated negligible contamination of membrane-associated extracts with cytosolic protein.
Chapter 4. Jak1ΔB has no detectable effect on the Jak/STAT pathway.

Figure 4.1.

Analysis of the tyrosine phosphorylation of components of the Jak/STAT1 pathway in response to IFNγ in 2fTGH and 2fJak1ΔB.

2fTGH and 2fJak1ΔB#5 cells were plated out and the next day treated with IFNγ for 15 minutes. Cells were harvested along with untreated controls and lysed in Schindler buffer. The concentration of protein in the extracts was determined and equal amounts used to immunoprecipitate Jak1, STAT1, Jak2 and IFNGR1. The immunoprecipitated proteins were resolved on a 6.5% SDS-PAGE gel and transferred to PVDF. The membrane was probed with anti phosphotyrosine antibodies, stripped and reprobed for Jak1, Jak2, STAT1 and IFNGR1 protein, stripping after each Western blot.
Figure 4.1.

<table>
<thead>
<tr>
<th>Western blot</th>
<th>2fTGH</th>
<th>2fJak1ΔB</th>
</tr>
</thead>
<tbody>
<tr>
<td>con</td>
<td>IFNγ</td>
<td>con</td>
</tr>
</tbody>
</table>

STAT1 IP

- Anti P-tyr
- Anti STAT1

Jak1 IP

- Anti P-tyr
- Anti Jak1

Jak2 IP

- Anti P-tyr
- Anti Jak2

IFNGR1 IP

- Anti P-tyr
- Anti IFNGR1
Figure 4.2.

EMSA analysis of the effect of Jak1ΔB on DNA binding proteins activated in response to various ligands.

2fTGH and 2fJak1ΔB#5 cells were plated out and treated with IFNα, IFNγ and oncostatin M for 15 minutes. Following treatment the cells were lysed in Schindler buffer and DNA binding proteins analysed by EMSA analysis as described in Materials and Methods, using the hSIE oligonucleotide probe (193).
Figure 4.2.

Probe: hSIE

2fTGH

/ $\alpha$ $\gamma$ OSM

2fJak1$\Delta$B

/ $\alpha$ $\gamma$ OSM - treatment

Stat3/Stat3
Stat1/Stat3
Stat1/Stat1
Analysis of inducible serine phosphorylation of STAT1 in response to IFNγ.

2fTGH and 2fJak1ΔB cells were treated with IFNγ for the times indicated. In parallel cells were treated for 30 minutes with 1 μg/ml Anisomycin, a MAP kinase agonist (83). Following treatment cells were lysed in SDS sample buffer. Equal amounts of protein from each sample were resolved on a 10% SDS-PAGE gel. The proteins were transferred to PVDF and probed with antibodies specific for STAT1 phospho-serine 727, STAT1 phospho-tyrosine 701 and STAT1α protein, stripping the membrane between each Western blot.

I am indebted to Dr Ana Costa-Pereira for the data in Figure 4.3.
Figure 4.3.

Western blot

<table>
<thead>
<tr>
<th>Protein</th>
<th>0</th>
<th>2</th>
<th>4</th>
<th>6</th>
<th>8</th>
</tr>
</thead>
<tbody>
<tr>
<td>P-serine 727</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P-tyrosine 701</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>STAT1α protein</td>
<td></td>
<td></td>
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</tbody>
</table>

2fTGH

2fJak1ΔB
Figure 4.4a,b and c.

Assay of the specific autokinase activity of PKR in 2fTGH and 2fJak1ΔB#5.

2fTGH and 2fJak1ΔB#5 cells were treated with IFNα or γ for 18 hours. Cells were lysed, PKR immunoprecipitated from the extracts, and in vitro kinase reactions performed as described in the Materials and Methods. The PKR was then resolved on a 10% SDS-PAGE gel, which was dried, and phosphorylated PKR visualised by autoradiography (Figure 4.4a). In parallel, the same cell extracts were analysed for PKR protein by Western blot analysis (Figure 4.4b). Relative specific activity (Figure 4.4c) was determined by densitometric analysis of the data in (Figure 4.4a & b).
Figure 4.4a.

<table>
<thead>
<tr>
<th></th>
<th>2fTGH control</th>
<th>IFNα</th>
<th>IFNγ</th>
<th>2fJak1ΔB control</th>
<th>IFNα</th>
<th>IFNγ</th>
</tr>
</thead>
</table>

Autoradiograph showing PKR specific autokinase activity

Figure 4.4b.

Western blot with monoclonal antibody for human PKR

Figure 4.4c.

<table>
<thead>
<tr>
<th>Relative specific activity</th>
</tr>
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<td>1</td>
</tr>
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<td>0</td>
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</tbody>
</table>

Relative specific activity
Figure 4.5.

RNase protection analysis of the effect of a kinase-dead Jak1ΔB on IFNγ induced CIITA expression.

2fJak1ΔBK>E cells, which express the mutant Jak1ΔB containing the kinase-inactivating K>E substitution, were analysed for the induction of CIITA following 24 hours IFNγ treatment, in parallel with 2fTGH and 2fJak1ΔB#5. RNase protection analysis was performed using probes for Actin, 9-27, IRF1, 6-16, DRα and CIITA.
Figure 4.5.

2TGH  2JakΔB  2JakΔBK->E
-  γ  -  γ  -  γ  IFNγ treatment

-  γ  -  γ  -  γ  -

CIITA

DRα

6-16

IRF1

9-27

Actin
Chapter 4. Jak1ΔB has no detectable effect on the Jak/STAT pathway.

Figure 4.6.

Western blot analysis of Jak1 protein in membrane and cytoplasmic protein extracts from 2fJak1ΔB#5.

2fJak1ΔB cells were treated with IFNγ for 15 minutes, and together with untreated control cells, membrane and cytosolic protein extracts were isolated as described in Materials and Methods. The proteins were resolved on a 6.5% SDS-PAGE gel and transferred to PVDF. The membrane was probed for Jak1 protein, stripped and re-probed for the membrane-associated Class I HLA protein.

Figure 4.7.

Lactate dehydrogenase activity assay to monitor contamination of membrane protein extracts with cytosolic protein.

Membrane and cytosolic protein extracts from 2fJak1ΔB cells in Figure 4.6 were assayed for lactate dehydrogenase activity to monitor carry-over of cytosolic protein into membrane protein extracts.

I am indebted to Bjoern Lillimeier for the data in Figure 4.6 and 4.7.
Figure 4.6.

<table>
<thead>
<tr>
<th></th>
<th>unstimulated</th>
<th>+IFNγ</th>
</tr>
</thead>
<tbody>
<tr>
<td>total lysate</td>
<td></td>
<td></td>
</tr>
<tr>
<td>cytosol</td>
<td></td>
<td></td>
</tr>
<tr>
<td>membrane</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

- Jak1
- Jak1ΔB
- Class I HLA

Figure 4.7.

Graph showing Extinction@340nm over time (minutes) for:
- cytosol
- membrane
- cytosol IFNγ
- membrane IFNγ
Discussion.

Phosphotyrosine analysis showed no effect of Jak1ΔB on the phosphorylation of IFNGR1 and associated proteins (Figure 4.1) following IFNγ treatment, and EMSA analysis indicated no effect on the activation of STATs in response to several ligands (Figure 4.2) Serine phosphorylation of STAT1 (Figure 4.3) and the activity of PKR were also apparently unaffected (Figure 4.4). Importantly, the inhibitory effect of Jak1ΔB was disrupted by the kinase-inactivating K>E mutation (Figure 4.5), and it was shown that Jak1ΔB is cytosolic in contrast to endogenous Jak1 which localises with the membrane fraction (Figure 4.6).

The tyrosine phosphorylation of Jak1, Jak2, IFNAR1 and STAT1 are all seen in response to IFNγ in 2fJak1ΔB cells (Figure 4.1), consistent with Jak1ΔB not inhibiting receptor events and the subsequent activation of STAT1. This is confirmed by the EMSA analysis (Figure 4.2), which shows STAT activation in 2fJak1ΔB is comparable to wild type cells in response to IFNγ, IFNα and OSM. The absence of an observable effect on responses to these other ligands reinforces the distinction between Jak1ΔB and the kinase-negative mutants Jak1K>E and Jak1SFG, which do have a dominant inhibitory effect on these responses. The serine phosphorylation required for the full transactivating function of STAT1 is also apparently normal (Figure 4.3).

All this is consistent with the observed induction of Class I HLA which requires STAT1 activation, although slight inhibition of the IFNγ-induction of Class I HLA can be attributed to the inhibition of CIITA expression which also contributes to this response.

In addition to tyrosine phosphorylation of STAT1 there is increasing evidence for activation of other pathways by IFNγ. Here, the activation of PKR and p38 MAP
Chapter 4. Jak1ΔB has no detectable effect on the Jak/STAT pathway.

kinase were investigated and shown to be unaffected. A direct assay of PKR activity showed that both the level of PKR protein and its autokinase activity were comparable in wild type and Jak1ΔB expressing cells (Figure 4.4). However, the absolute change in activity seen was low (less than 10%) which suggests that PKR may play a limited role in the IFNγ response in these cells. Given its importance in antiviral responses, PKR would seem a likely candidate for inhibition by Jak1ΔB, and it is impossible to exclude that Jak1ΔB inhibits PKR below the threshold of detection in this assay.

Analysis of eIF2α, a substrate of PKR, was inconclusive (M. Clemens, pers. com), and other approaches such as the use of a dominant negative PKR or an inhibitor such as 2-aminopurine are complex, because of effects on protein synthesis. A potential target for PKR activity in the response to IFNγ is believed to be p38 MAPK, and through this STAT1 serine 727. Although there was no activation of p38 MAPK detected in these cells, the putative the target of the activated p38 MAPK - STAT1 - appears to be normally serine phosphorylated (Figure 4.3), which would be consistent with no inhibition of either p38 MAPK or PKR by Jak1ΔB. This is irrespective of whether either plays any role in STAT1 serine phosphorylation. Finally, the autokinase activation of p38 MAPK by Anisomycin indicates no effect of Jak1ΔB on p38 MAPK activity either (Figure 4.3).

Jak1ΔB is constitutively phosphorylated, which raises the obvious question as to whether the constitutive kinase activity is the cause of the dominant negative phenotype. The use of the kinase-inactivating K>E substitution in the active site of the kinase to generate Jak1ΔBK>E showed that the inhibitory effect is dependent on the kinase activity of Jak1ΔB. Accepting that Jak1ΔB has a constitutive kinase activity, it may aberrantly phosphorylate an unknown factor, a protein not normally a substrate of Jak1, resulting in the inhibitory phenotype. The identification of such a factor, which is required, for example, for the inducible expression of CIITA, could potentially be of comparable interest to the identification of a novel Jak1-substrate or interacting protein.
through a more classical dominant negative mode of action of Jak1ΔB. The possibility that Jak1ΔB interacts with other pathways known to inhibit expression of CIITA was mentioned in the previous chapter. The TNFα mediated mechanism affecting CIITA stability has already been addressed. The other ligand-dependent pathways known to inhibit IFNγ induction of CIITA, mediated by IL-1β (190) and TGFβ (163), cannot be discounted and are difficult to address since so little is known about them.

Accordingly, the two major alternative mechanisms (together with variations on them) have been born in mind throughout the remainder of this work, namely that tyrosine phosphorylated Jak1ΔB may (i) interact with or aberrantly phosphorylate a normal Jak1 substrate or adapter protein, thus interfering with signal transduction and leading to inhibitory effects, or (ii) constitutively phosphorylate/inactivate a factor “x” (a transcriptional co-activator, for example) required to sustain the inducible expression of CIITA and a subset of IFNγ inducible genes (see Chapter 6).

Whatever its effect, it is reasonable to conclude that the constitutive phosphorylation of Jak1ΔB is the result of autokinase activity. It is accepted that over-expressed Jaks can autophosphorylate, normally without a detectable effect on signal transduction. In Drosophila, however, two point mutations in the hopscotch gene product generated a protein hyperphosphorylated on tyrosine residues which resulted in overstimulation of a haematopoietic pathway and other abnormalities (143, 144). This is consistent with constitutive activation of the hopscotch protein, and introduction of one of these mutations into the corresponding site in mammalian Jak2 caused it to autophosphorylate and constitutively activate STAT5 (144). However, the hopscotch mutant was rescued by a Drosophila STAT loss-of-function mutant. In this respect, it would seem that Jak1ΔB is different from this type of mutant because there is no constitutive activation of STATs, and the responses which are inhibited by Jak1ΔB require STAT activation in the wild-type.
Jak1ΔB has no detectable effect on the Jak/STAT pathway.

Given that Jak1ΔB is constitutively active, constitutive activation of the receptor complex might be expected. However, neither IFNGR1, Jak2 or endogenous Jak1 were constitutively phosphorylated (Figure 4.1). Jak1ΔB might nevertheless interact with the receptor complex, and analysis of this would provide a possible insight into the target of the inhibitory mechanism.

For both uninduced and induced cells, Jak1ΔB localised to the cytosolic protein fraction, and endogenous Jak1 to the membrane fraction, presumably associated with the receptor complex (Figure 4.6). This is consistent with the view that endogenous Jak1 interacts with the receptor constitutively, and more importantly that the site of inhibition by Jak1ΔB lies away from the receptor. Jak1ΔB might still inhibit a receptor event, perhaps by sequestering a factor, preventing a functional interaction that is necessary as part of the response to IFNγ. It also suggests that the region deleted in Jak1ΔB may be essential for receptor interactions. It is interesting to note that the deleted region of Jak1ΔB overlaps with the newly identified FERM (4.1/ezrin-radixin-moesin) domain of Jak1, which could mediate interactions between Jak1 and the receptor complex (C. Hilkens, manuscript in preparation). This provides a reasonable basis for the failure of Jak1ΔB to localise to the receptor complex.

The constitutive activation of Jak1ΔB also raises issues about the effects of deleting this section of the protein. The deletion, encompassing JH3-5 is substantial - nearly 25% of the protein - and might be expected to have a severe effect on the structure of the protein. However, the rabbit polyclonal antibody to Jak1 from Santa Cruz recognises an epitope (residues 785-804) in the kinase-like domain JH2, and still recognises Jak1ΔB. At least this region must still be normally presented and folded for immunoprecipitations to work. The apparent kinase activity of the mutant is consistent with the kinase domain also being intact and functionally folded. On the other hand, the constitutive kinase activity of Jak1ΔB may indicate the loss of an autoregulatory
interaction. Various models for the activation of Jaks have been put forward, but recently a major regulatory role has been ascribed to the pseudokinase domain (265). Point mutations in the pseudo-kinase domain have produced constitutively active Jaks, such as those in the *Hopscotch* protein. It may be that the deleted region, being adjacent to JH2 interferes with this autoregulatory function allowing Jak1ΔB to autoactivate.

Alternatively the receptor or an associated factor may exert a regulatory effect and, as a result of the deletion, Jak1ΔB is free of this regulatory effect. A third explanation is that Jak1ΔB is similar to other Jaks which are over-expressed and autoactivate, but its inhibitory effect is a consequence of its failure to localise at the receptor. If somehow the same mutant could be associated with the receptor it might not have a dominant negative effect. Taking this argument a step further, perhaps any Jak1 over-expressed away from the receptor would have a dominant-negative effect.

*A priori,* it is possible that a constitutively active Jak1ΔB might lead to constitutive induction of the negative feedback mediated by SOCS proteins. If this was the case then the SOCS proteins would be expected to interfere with Jak/STAT activation in response to ligand and this is not apparent. Alternatively, Jak1ΔB might itself interact with SOCS proteins potentially leading to prolonged stimulation of the Jak/STAT pathway rather than its inhibition. Again there is no evidence of this either, and it seems unlikely that the SOCS proteins are involved in the inhibitory effect of Jak1ΔB.

Thus far it has been shown that Jak1ΔB selectively inhibits the expression of CIITA and Class II HLAs, and the antiviral response to IFNγ. But there is no detectable effect on the Jak/STAT pathway or serine phosphorylation of STAT1. The inhibition is dependent on both the level of expression and the kinase activity of Jak1ΔB, and the protein appears to be cytosolic, in contrast to the membrane association of wild-type
Jak1. No evidence was obtained for the inhibition of IFNγ-inducible PKR or for the involvement of p38 MAP kinase in the IFNγ response in these cells.

The inhibition of CIITA expression is more amenable to analysis than the antiviral response. The effect of Jak1ΔB on expression from CIITA promoter reporter constructs, and reporters responsive to specific transcription factors was now investigated.

Chapter 5

JAK1ΔB INHIBITS EXPRESSION FROM PROMOTER-REPORTER CONSTRUCTS REGULATED BY THE IFN GAMMA-INDUCIBLE CIITA PROMOTER-IV.

Introduction.

No apparent defect was detected in the activation of individual components of the Jak/STAT pathway, yet Jak1ΔB strongly inhibited IFNγ-induced expression of CIITA mRNA without a detectable effect on its stability (Chapter 3). Four promoters are known to regulate expression of the single copy CIITA gene using separate transcription start sites, but resulting in 3'co-terminal mRNAs (160). The promoters are designated on the basis of their relative positions 5’ to 3'; promoter I (pI) is primarily utilised in dendritic cells, pII appears to be little used and insignificant, pIII is active in B cells and is also weakly responsive to IFNγ in several cell types. But promoter IV (pIV) is the main IFNγ-responsive promoter in non-professional antigen presenting
cells such as fibroblasts (160). This promoter contains sequence elements which might bind a variety of transcription factors (illustrated in Figure 5.1) including an NFκB site, an NF-Gma site, a GAS element and an IRF1/2 site. Extensive analysis has demonstrated that STAT1, IRF1 and USF1 are all required for IFNγ induction (54, 159, 182). Importantly, STAT1 and USF1 - bound to the adjacent GAS and E Box sites - interact co-operatively (159). Analysis of this IFNγ-inducible promoter, and specific signals to it, might identify a promoter element and interacting factor sensitive to Jak1ΔB - potentially a new player in the regulation of CIITA promoter IV and also IFNγ signalling.

There is increasing evidence that additional nuclear partners may be required for STAT-mediated transcription of different genes. To date, transcription factors such as USF1 and IRF1, and co-activators such as CBP/p300, P/CAF, Nmi, BRCA1 and MCM5 have all been implicated as co-factors for STAT-mediated transcription (reviewed in (213)). Indeed inhibition of USF1 (274) and CBP/p300 (235) have been shown to interfere with IFNγ-induced expression of Class II HLAs. An effect of Jak1ΔB on a nuclear factor required for a specific subset of responses would also be an elegant explanation of the apparent specificity of inhibition achieved in the context of apparently normal Jak and STAT activation. Inhibition of the promoter-reporter constructs by Jak1ΔB would also provide strong evidence for an effect at the level of transcription rather than a post-transcriptional effect.

Here, reporter constructs regulated by both the full length and minimal IFNγ-responsive CIITA promoter (CIITA promoter IV) were initially analysed and found to be inhibited by Jak1ΔB. Reporters responsive to the individual cis-acting proteins IRF1 and STAT1, known to regulate this promoter, were then analysed. No evidence for an inhibition of activity was observed, and USF1 and STAT1 were seen to bind to the
promoter in a wild-type fashion. No function for the NFκB site found in CIITA
promoter IV - but not positively implicated in its regulation - could be demonstrated.
**Results.**

Jak1ΔB inhibits IFNγ-induction of a full length promoter IV-luciferase construct.

The response of promoter III which has been shown to be weakly IFNγ-inducible was briefly analysed, and appeared to be uninhibited by Jak1ΔB (data not shown), but initial and subsequent analysis focused on the major IFNγ-induced promoter IV. All the promoter reporter constructs relating to analysis of promoter IV are depicted for ease of reference in **Figure 5.2**.

Repeat experiments with pIV-luciferase reporter constructs demonstrated inhibition of reporter expression in response to IFNγ in cells expressing Jak1ΔB. The degree of inhibition was variable between experiments, but nonetheless significant (**Figure 5.3a**). In contrast, Jak1ΔB did not inhibit an IRF1 promoter-luciferase reporter analysed in parallel (**Figure 5.3b**), suggesting that the apparent inhibition of expression from the pIV-luciferase construct was authentic and consistent with the inhibition of the endogenous promoter.

A minimal promoter IV-globin reporter construct is also inhibited by Jak1ΔB.

The disparity between the degree of inhibition of the pIV-luciferase reporter and endogenous CIITA mRNA might be attributed to the differences in the nature of the assays, one measuring an endogenous RNA and the other measuring a protein. To address this, a promoter IV–β-globin reporter construct was used, expression from which was measured by RNase protection. The inhibition of the IFNγ-induction of this
reporter was more complete than for the luciferase construct (Figure 5.4), and closer to that seen for the endogenous CIITA mRNA itself (Figure 3.9).

**Analysis of promoter-reporter constructs responsive to individual components known to regulate IFNγ-induction of CIITA.**

There was no indication of an inhibition of the Jak/STAT pathway by Jak1ΔB, based on anti-phosphotyrosine, anti-phosphoserine and EMSA analysis of its components (Chapter 4). A further functional analysis of the *cis*-acting proteins known to regulate promoter IV was therefore undertaken. **Figure 5.5** is a schematic depicting the regulation of the IFNγ-inducible promoter IV. Promoter-reporter constructs responsive to STAT1 and IRF1 were transiently transfected into 2fTGH and 2fJak1ΔB cells and reporter expression in response to IFNγ was analysed.

Expression from the STAT1-responsive full length IRF1-promoter itself was unaffected by Jak1ΔB (Figure 5.3b). Two additional STAT1-responsive elements were analysed. The first was the GAS/E-Box element from CIITA promoter IV, which consists of STAT1-binding GAS site and an adjacent USF1-binding E-box site. The role of USF1 in regulation of promoter IV has been proven, and a functional interaction between USF1 and STAT1 has been postulated (159). Luciferase reporter constructs regulated by two and four juxtaposed copies of this CIITA GAS/E-box element were assayed in 2fTGH and 2fJak1ΔB cells. In parallel, reporter constructs regulated by two and six copies of the STAT1-responsive GAS element from the IRF1 promoter were also assayed. A possible slight inhibition of the induction of the CIITA GAS/E-box construct (**Figure 5.6a**) was seen, whilst the induction of the IRF1 GAS was not appreciably affected (**Figure 5.6b**).
Finally, the function of IRF1 was assayed. Expression of a luciferase reporter driven by four copies of the IRF1-responsive Hex element (62) was strongly induced by IFNγ and not inhibited by Jak1ΔB (Figure 5.7).

Note: the reporter data presented in Figures 5.3 - 5.7 is representative of at least three independent experiments with that particular reporter construct.

**EMSA analysis using the GAS/E-box element shows no inhibitory effect of Jak1ΔB.**

In view of the possible slight inhibition by Jak1ΔB of the reporter regulated by the CIITA GAS/E-box element (Figure 5.6a), DNA-binding complexes interacting with this element were analysed by EMSA. Nuclear extracts from control and IFNγ-treated cells were analysed with an oligonucleotide probe encompassing what is referred to as the NGE region of CIITA promoter IV (159), containing the GAS and E-Box sites, and also an NF-GMa site (210). Antibodies to USF1 and STAT1 were used to identify DNA-binding complexes containing these proteins. A constitutive complex containing USF1 is present and unaffected by Jak1ΔB (Figure 5.8a), and a complex containing STAT1 was induced by IFNγ and was also unaffected by Jak1ΔB (Figure 5.8b). This is consistent with previous EMSA analysis using an SIE-GAS probe indicating no effect on STAT1-GAS-binding (Figure 4.2), and furthermore with there being no inhibition of the DNA-binding function of USF1. An additional complex supershifted by antibody to both USF1 and STAT1 was also seen (V.Arulampulam, pers. com.), consistent with previous observations of co-operative interactions between the two factors (159). But reproducible resolution of this complex was frustrated, and the data is not presented here.
The NFκB site in CIITA promoter IV plays no role in inhibition by Jak1ΔB.

The NFκB element in CIITA promoter IV has not been attributed any role in IFNγ regulation. In view, however, of the inhibition by Jak1ΔB of NFκB activity in response to dsRNA (Chapter 6), and the possible activation of NFκB by IFNγ (A. Deb and B. R. G. Williams, unpublished data), the NFκB site in the pIV-Luciferase construct was mutagenised and induction of the mutant construct by IFNγ analysed in 2fTGH and 2fJak1ΔB cells. The mutant NFκB site had little or no effect on reporter expression either in terms of induction by IFNγ or inhibition by Jak1ΔB (Figure 5.9). If NFκB-activation is required for the response to IFNγ, but inhibited by Jak1ΔB, it would be expected that mutation of the NFκB site would inhibit the response in the wild-type towards that seen in 2fJak1ΔB. Alternatively, if the NFκB site is itself the target of an inhibitory signal causing the inhibition by Jak1ΔB, then its mutation should restore expression of the reporter to wild type levels. Neither effect was seen (Figure 5.9), and on the basis of this, it can be concluded that the NFκB site plays no role in the inhibitory effect of Jak1ΔB.
Figure 5.1.

The major regulatory elements in IFNγ-inducible promoter IV of CIITA.
Figure 5.1.

-350 -225 +1 +50

NFkB  GAS E-Box  IRF1

-154  -126  -66  -55

NF-GMa GAS E-Box  IRF1

TTGGGATGCCACTTCTGATAAGGACGTG GAAAGTGAAAGGTTCTGATAAACACGTGTIGGGATGCCAC
Figure 5.2.

Reporter constructs used to analyse the inhibitory effect of Jak1ΔB on CIITA promoter IV.
Figure 5.2.

**Promoter IV reporters**

- **pIV-Luciferase**
  
  -350
  NFkB  GAS E-Box  IRF1
  +50
  Luciferase

- **pIV-β-globin**
  
  -350
  NFkB  GAS E-Box  IRF1
  +50
  β-globin

- **pIVmkappaB-Luciferase**
  
  -350
  ΔNFkB  GAS E-Box  IRF1
  +50
  Luciferase

**STAT1-responsive reporters**

- **IRF1 promoter-Luciferase**
  
  -137  IRF1 promoter  +8
  Luciferase

- **2x (IRF1 GAS) Luciferase**
  
  (GAS from IRF1 promoter)
  TGATTTCCCCG

- **6x (IRF1 GAS) Luciferase**

**USF1/STAT1-responsive reporters**

- **2x (CIITA GAS E-Box) Luciferase**
  
  (GAS and E-Box from CIITA promoter IV)
  TTCTGATAAAGCAGTG

- **4x (CIITA GAS E-Box) Luciferase**

**IRF1-responsive reporter**

- **(Hex)4-Luciferase**
Figure 5.3a and b.

Activation of the minimal promoter IV-Luciferase construct in 2fTGH and 2fJak1ΔB cells.

Figure 5.3a.
2fTGH (2f) and 2fJak1ΔB (ΔB) cells were transiently transfected with the minimal IFNγ-promoter IV-luciferase construct (pIV-Luc) or the promoter-less pGL2Basic construct from which pIV-Luc is derived (pGL2). Cells were treated with IFNγ for 18 hours and harvested in parallel with untreated controls. Luciferase activity was assayed as described in the Materials and Methods.

Figure 5.3b.
2fTGH (2f) and 2fJak1ΔB (ΔB) cells were transiently transfected with either 1 or 2 μg of an IRF1 promoter-luciferase construct (IRF1-Luc). Cells were treated with IFNγ for 18 hours and harvested in parallel with untreated controls, and Luciferase activity was assayed.
Figure 5.4.

RNase protection analysis of 2fTGH and 2fJak1ΔB cells transiently transfected with the CIITA promoter IV-β-globin reporter construct.

Cells were transiently transfected with the promoter IV-β-globin reporter and treated for 6 hours with IFNγ as indicated. RNA was harvested from the cells and analysed by RNase protection using the β-globin and Actin probes to monitor the activity of the reporter construct.

I am indebted to Dr Gesan Arulampalam for the data in Figure 5.4.
Figure 5.4.

IFN treatment:  

2fTGH  

2fJak1ΔB  

β-globin

Actin

IFN treatment:  

-  γ  -  γ
The regulation of CIITA promoter IV by IFNγ.

IFNγ induces the tyrosine phosphorylation of STAT1 leading to expression of IRF1.

STAT1 and IRF1 are required for induction of CIITA promoter IV by IFNγ, and USF1 has been shown to co-operatively bind to the promoter in association with STAT1.
Figure 5.5.
Figure 5.6a and b.

Analysis of STAT1-responsive Luciferase reporters in 2fTGH and 2fJak1ΔB cells.

Figure 5.6a.
2fTGH (2f) and 2fJak1ΔB (ΔB) cells were transiently transfected with Luciferase reporters regulated by 2 or 4 copies of the CIITA GAS/E Box element. Cells were treated with IFNγ for 6 hours and harvested in parallel with untreated controls. Luciferase activity was assayed as described in the Materials and Methods.

Figure 5.6b.
2fTGH (2f) and 2fJak1ΔB (ΔB) cells were transiently transfected with Luciferase reporters regulated by 2 or 6 copies of the GAS element from the promoter of the IRF1 gene. Cells were treated with IFNγ for 6 hours and harvested in parallel with untreated controls, and Luciferase activity was assayed.
Figure 5.7.

Analysis of an IRF1-responsive Luciferase reporter in 2fTGH and 2fJak1ΔB cells.

The IRF1-responsive (Hex)$_4$-Luciferase reporter regulated by 4 copies of the Hex element was transiently transfected into 2fTGH (2f) and 2fJak1ΔB (ΔB) cells. Cells were treated with IFNγ for 6 hours and harvested in parallel with untreated controls. Luciferase activity was assayed as described in the Materials and Methods.
Figure 5.7.
Figure 5.8a and b.

EMSA analysis of DNA-binding complexes which bind to a composite NFGma-GAS-E Box (NGE) oligonucleotide probe in 2fTGH and 2fJak1ΔB cells.

Nuclear extracts were prepared as described in the Materials and Methods from 2fTGH and 2fJak1ΔB cells treated with IFNγ for 20 minutes and from untreated control cells. The protein concentration of the extracts was determined and equal amounts of protein from each sample were incubated with the NGE probe.

Figure 5.8a.
Anti-USF1 antibody identifies a complex containing USF1, which is disrupted by inclusion of the antibody in the EMSA.

Figure 5.8b.
A STAT1-containing complex is also identified using anti-STAT1 antibody in the EMSA.

I am indebted to Dr Gesan Arulampalam for the data in Figure 5.8.
Figure 5.8a.

Supershift antibody

2fTGH | 2fJak1ΔB
--- | ---
- | -
USF1 | USF1

20 min IFNγ treatment

- | - | - | - | - | γ

Figure 5.8b.

Supershift antibody

2fTGH | 2fJak1ΔB
--- | ---
- | -
Stat1 | Stat1

20 min IFNγ treatment

- | - | - | - | - | γ

Stat1

USF1
Figure 5.9a and b.

Analysis of the function of the NFκB site in CIITA promoter IV in relation to Jak1ΔB.

In order to investigate the possible function of the putative NFκB element in CIITA promoter IV, 2fTGH (2f) and 2fJak1ΔB (ΔB) cells were transfected with a promoter IV-Luciferase construct (pIV-Luc), and in parallel the same construct but with the putative NFκB element mutagenised (pIVmκB-Luc) as described in Materials and Methods.

Figure 5.9a.

Cells were transfected with 1 or 2 μg of the wild type pIV-Luc construct as indicated. Following 6 hours treatment with IFNγ cells were harvested along with untreated controls, and Luciferase activity was assayed.

Figure 5.9b.

In parallel, cells were transfected with 1 or 2 μg of the mutagenised type pIVmκB-Luc construct as indicated. Following 6 hours treatment with IFNγ cells were harvested along with untreated controls, and Luciferase activity was assayed.
Discussion.

Promoter-reporter studies showed that Jak1ΔB inhibits transcription regulated by the minimal IFNγ-inducible CIITA promoter IV (Figures 5.3 & 5.4). Analysis using reporters responsive to the individual proteins known to regulate transcription from promoter IV (illustrated in Figure 5.5) indicated no inhibition of their activity. Thus, transcription regulated by STAT1 (Figure 5.6) and IRF1 (Figure 5.7) was not inhibited in 2fJak1ΔB cells. No differences were seen in EMSA analysis of the binding of STAT1 and USF1 to their sites in promoter IV (Figure 5.8). Finally, a role for NFκB in either the regulation or Jak1ΔB-mediated inhibition of promoter IV was excluded (Figure 5.9).

The inhibition of the minimal promoter IV reporter constructs by Jak1ΔB indicates again that inhibition of CIITA induction does not reflect an effect on the stability of the mRNA. This, together with the absence of any obvious effect on the stability of mRNA from transfected CIITA, suggests that Jak1ΔB does not utilise the TNFα-activated pathway which inhibits IFNγ-induced expression of CIITA.

The results from the STAT1-responsive reporters (Figure 5.6) are consistent with the biochemical analysis of STAT1 and show that there is no inhibition of the activity of STAT1 per se. This is supported by data from the IRF1-promoter reporter (Figure 5.3b) which is primarily regulated by STAT1, and the observed induction of Class I HLA by IFNγ (Chapter 3, Figure 3.3), also dependent on STAT1.

The IRF1-responsive Hex-regulated reporter construct was not inhibited by Jak1ΔB, and this is consistent with the observed induction of Class I HLA by IFNγ (Chapter 3, Figure 3.3) which requires IRF1 (23, 86, 188).
The role of USF1 in IFNγ-induced expression of CIITA involves a co-operative interaction with STAT1 (54, 159), such that the binding of STAT1 to the GAS site is dependent on the binding of USF1 at the adjacent E Box site. The data here shows no inhibitory effect of Jak1ΔB on DNA binding by USF1 or STAT1 to this tandem GAS/E Box element (Figure 5.8), and no inhibition of transcription regulated by the same element (Figure 5.6a), consistent with Jak1ΔB not affecting this aspect of USF1 function.

The results for each of STAT1, IRF1 and USF1, the known factors involved in IFNγ induction of CIITA revealed no evidence for any inhibition of their individual activity by Jak1ΔB. Other naturally occurring pathways inhibiting CIITA also do not apparently affect STAT1, IRF1 or USF1, including the inhibition caused by TGFβ (163) and IL-1β (190). These inhibitory effects are also independent of an effect on mRNA stability. Once identified, their mechanisms may be revealing for Jak1ΔB.

The activation of NFκB by IFNγ has only recently been reported and is controversial. Nevertheless, given the apparent inhibition of the dsRNA activation of NFκB by Jak1ΔB (Chapter 6) and the presence of a putative NFκB site in promoter IV it suggested a possible alternative site for inhibition by Jak1ΔB. Mutagenesis of the NFκB site, however, did not affect IFNγ induction of pIV-luciferase in 2fTGH, or 2fJak1ΔB. Thus, NFκB plays no direct role through this site in either IFNγ-inducible transcription or its inhibition by Jak1ΔB.

It seems that the activity of the three factors known to regulate transcription at promoter IV is not inhibited by Jak1ΔB. STAT1 is normally serine and tyrosine phosphorylated (Chapter 4), STAT1 and IRF1 responsive reporters are not noticeably
inhibited, and USF1 binds to the E-box element. The role of USF1 in transcription remains unclear. The importance of USF1 to the expression of Class II HLA was recently demonstrated by the observation that *Chlamydia* induces the breakdown of USF1, thus avoiding host immune defence by abrogating Class II HLA (274). It should be noted that expression of ICAM1 and IRF1 are unaffected in the same context, which appears to be the case with Jak1ΔB. (Expression analysis using oligonucleotide microarrays - Appendix - gave conflicting assessments of ICAM1 expression, but no inhibition of ICAM1 induction by IFNγ was seen in FACS analysis Figure A.2.). It is plausible that Jak1ΔB inhibits a regulatory signal to USF1 which is required for interactions with co-factors, and this only becomes apparent when transcription regulated by the full promoter and depending on full integration of co-factor signals is assayed, as is the case for the promoter IV-luciferase construct.

STAT1 has been shown to interact with an increasing number of transcriptional "co-activators", including CBP/p300, P/CAF, MCM5 and Nmi. The varied role of these proteins remains to be fully defined, but it is likely that they may act as integrators between regulatory transcription factors and the transcription machinery. Again, it is possible that Jak1ΔB interferes with such an interaction with STAT1, and that this is only apparent in the context of the whole promoter. *Mycobacterium tuberculosis* was reported to inhibit the interaction of STAT1 and CBP/p300 without inhibiting phosphorylation of STAT1 (235), presumably resulting in suppression of an immune response to the intracellular parasite. This emphasises the potential importance of STAT1 co-activator interactions.

It remains possible that an unknown additional modification of one of STAT1, USF1 or IRF1 is inhibited. The importance of kinase activity of Jak1ΔB for its inhibitory effect emphasises the possibility of an inhibitory phosphorylation. The phosphorylation of IRF1 in general has not been rigorously monitored here. IRF1 is thought to be phosphorylated by casein kinase (136), but whether it is
inducible/regulatory or constitutive is unknown. Although no evidence for aberrant tyrosine phosphorylation of IRF1 by Jak1ΔB was obtained (data not shown) there may be a promoter-specific dependence on IRF1 phosphorylation. Similarly, USF1 may also be target of an inhibitory phosphorylation by Jak1ΔB. Recent reports describe a regulatory phosphorylation (27) which may regulate USF1 interactions with co-activators (184). USF1 should be monitored for both normal and aberrant phosphorylation. It would seem that STAT1 phosphorylation is not detectably affected by Jak1ΔB, but other modifications cannot be excluded. However 2D gel analysis failed to identify a difference in STAT1 between 2fTGH and 2fJak1ΔB cells, either constitutively or following IFNγ treatment (N. Rogers, unpublished data). But the possibility of an effect by Jak1ΔB on an unknown modification of STAT1 remains. Arginine methylation of STAT1 might be a novel regulatory pathway and an arginine methyltransferase was shown to be associated with the Type I IFN receptor (1). Acetylation of a number of transcriptional proteins including STAT2 has also been put forward as a regulatory modification which might influence transactivation (139, 176, 229) in addition to the role of histone acetyltransferases in regulating the accessibility of promoters. Any such modifications will probably regulate the interaction of the protein with a co-activator.

Further analysis of promoter IV regulation in the context of Jak1ΔB should centre around protein:protein interactions which may be required for full transactivation of the promoter. Although the behaviour of STAT1, USF1 and IRF1 in isolation was not affected by Jak1ΔB, their interaction with additional factors was not fully analysed, and should be investigated. Analysis of the phosphorylation of USF1 was attempted unsuccessfully, and should be re-addressed, since any such modification might affect additional interactions. Similarly, it is important to determine the phosphorylation status of IRF1. Finally, analysis of the interaction of STAT1 with known co-factors such as CBP, BRCA or Nmi might reveal an inhibitory effect of Jak1ΔB.
Here it has been shown that Jak1ΔB inhibits IFNγ-inducible transcription from minimal CIITA promoter reporter constructs. However, no defect in the known factor-promoter element interactions involved was detected, raising the possibility that the inhibition is at the level of higher transcription complexes linking STAT1, USF1 and IRF1 with transcriptional co-activators. This possibility is emphasised by the very recent data suggesting a requirement for Jak1 for the interaction of activated nuclear STAT1 with CBP/p300 (158). Interestingly, Jak1ΔB also inhibits the induction of ISGs by dsRNA, both through the induction of βIFN and directly (the dsRNA-direct response) (Chapter 6). Both responses are thought to involve complexes between transcription factors and the co-activator CBP/p300. Accepting the potential involvement of CBP/p300 in both the CIITA and dsRNA responses, the latter appeared appropriate for further analysis.
Chapter 6.

JAK1ΔB INHIBITS INDUCTION OF GENES BY DSrna.

Introduction.

Double-stranded RNA induces α and β IFNs with the consequent secondary induction of ISGs, but also induces a number of ISGs directly (the dsRNA direct response). Jak1ΔB inhibits both of these responses. The analysis of the activation of genes in response to dsRNA was initially undertaken as part of a wider analysis of Jak1ΔB. Responses to dsRNA are involved in the antiviral response and the priming of the dsRNA-response by pre-treatment with IFN is well recognised. The fact that Jak1ΔB inhibited the dsRNA response implied that the inhibitory effect was less specific than it had first appeared. This raised the possibility that the IFNγ-CIITA response and the dsRNA response required a common component, which would be an obvious candidate for the target of inhibition by Jak1ΔB.

There is no precedent for the involvement of Jaks in the response to dsRNA, although a mutant cell line called p2.1 was generated from Jak1-deficient U4C cells, and was characterised with a defective response to dsRNA (123). There is no defect in the dsRNA response in the U4C cells per se and, consistent with this, Jak1 failed to complement the defect in the 2.1 cells.

dsRNA both activates proteins and induces expression of genes. Major proteins activated include the dsRNA dependent protein kinase (PKR), and the family of 2′-5′ oligoadenylate synthetases which in turn activate RNaseL. Other proteins activated by
dsRNA include members of the MAP kinase superfamily, such as Jun N-Terminal kinases (JNK) and stress activated protein kinase (SAPK) (30, 93). Kinases activated by dsRNA (including PKR) perform at least two functions, firstly to regulate the activities of additional antiviral proteins, but also in activating transcription factors which upregulate dsRNA-responsive genes. For example, either dsRNA or virus can activate JNK leading to activation of AP-1 (30), and also cause PKR dependent NFκB activation through the IκB kinase (IKK)-dependent phosphorylation of the NFκB inhibitor IκBβ (53, 270).

Genes induced by dsRNA/virus include those of the IFNs themselves, and many recognised ISGs. A subset of these α/β IFN-responsive genes e.g. IFI56K, IRF1 and ISG15 are induced directly by dsRNA, and others are induced in a secondary response to the IFNs induced by dsRNA. The direct induction of ISGs by dsRNA is mediated through ISREs, but does not depend on the components of the IFN response pathway (11). The induction of the βIFN in response to virus or dsRNA has been well characterised. It is mediated through the IFN response element (IRE) or virus response element (VRE) (69), which is made up of several discrete positive and negative regulatory elements. A multi-protein complex called an enhanceosome assembles around the response element to induce expression of the gene (108, 155, 253, 266). The enhanceosome is known to include NFκB, ATF2/c-Jun heterodimers, IRFs and the enhanced mobility group protein HMG 1(Y). IRF3-dependent recruitment of the transcriptional co-activator CBP/p300 is essential for activation of the promoter (173).

ISREs which regulate dsRNA-responsive genes are of course also regulated by transcription factors activated by IFNs. It is possible a priori that a transcriptional regulatory protein which is inhibited by Jak1ΔB with respect to activation by IFNγ might also be inhibited with respect to activation by dsRNA. IFNγ does activate
transcription of genes through ISREs as well as the classical GAS element. If the target of inhibition by Jak1ΔB is not a sequence specific DNA-binding transcription factor but a co-factor then the nature of the activator (IFN or dsRNA) is irrelevant: the co-factor is required for either activator and its inhibition disruptions the response to both. On the basis of this reasoning, given that Jak1ΔB would appear to inhibit co-factors rather than the transcription factors themselves, it was reasonable to analyse the dsRNA response in the presence of Jak1ΔB.

Expression of βIFN mRNA and ISGs, and the activation of an NFκB responsive reporter by dsRNA were found to be inhibited by Jak1ΔB. Analysis of NFκB DNA-binding was consistent with inhibition by Jak1ΔB, but, as before, there was no evidence of inhibition of p38 MAPK or PKR. Apparent inhibition of IRF3 function was observed at a point downstream of IRF3 DNA-binding. Pre-treatment with IFNγ primes the transcriptional response to dsRNA, and such pre-treatment overcomes or circumvents the inhibition by Jak1ΔB.

**Results.**

**Expression of genes in response to dsRNA is inhibited by Jak1ΔB.**

Given the striking defect in the antiviral response to IFNγ caused by Jak1ΔB, and the potential for a common inhibition of elements of IFNγ and dsRNA-activated transcription, the response of cells to the dsRNA analogue polyinosinic-polycytidylic acid (poly(I)-poly(C)) was examined. Genes induced in response to poly(I)-poly(C) were analysed by RNase protection analysis (**Figure 6.1**). There is a clear inhibition of the primary induction of the mRNA of β-IFN, p48 and IRF1 in 2fJak1ΔB in
comparison to the wild-type 2fTGH. Therefore, Jak1ΔB inhibits both the induction of βIFN mRNA and the dsRNA-direct induction of ISGs.

**Activation of NFκB by dsRNA is inhibited by Jak1ΔB.**

NFκB is well characterised as a transcription factor activated by dsRNA. It is involved in the dsRNA activation of the βIFN gene (248), and therefore a candidate for inhibition by Jak1ΔB. The activation of NFκB in response to poly(I)-poly(C) was measured using an NFκB-responsive luciferase reporter construct (Figure 6.2). Moderate induction of the reporter gene by dsRNA was seen in 2fTGH and this was inhibited in 2fJak1ΔB. The activation of the same reporter by TNFα was measured in parallel, and repeatedly showed an enhancement in the presence of Jak1ΔB. The first observation is consistent with Jak1ΔB inhibiting the activation of NFκB by dsRNA, and analysis of DNA-binding by NFκB was carried out to confirm this. EMSA analysis of NFκB-binding to an oligo containing a consensus κB element demonstrated two effects of Jak1ΔB on NFκB activation by poly(I)-poly(C). There was a decrease in DNA-binding activity induced by dsRNA in the presence of Jak1ΔB (Figure 6.3) and an increase in the relative amount of the p50 component of the NFκB complex constitutively binding to the element (Figure 6.4), although there is clearly still poly(I)-poly(C)-induced binding of p65. The activation of NFκB by TNFα shown by EMSA analysis is not inhibited by Jak1ΔB (Figure 6.3), in agreement with the reporter data.
Repeat analysis suggested that the inhibition of NFκB-DNA binding activity was genuine. In view of this apparent effect on NFκB, the phosphorylation and breakdown of IκBβ, which regulates the activation of NFκB in response to dsRNA, was analysed, and showed no consistent effect attributable to the presence of Jak1ΔB (V. Arulampulam, unpublished data). It would appear, therefore, that the activation of NFκB by dsRNA but not TNFα is inhibited by Jak1ΔB. Accordingly, it was of interest to determine whether the activation by dsRNA of PKR or p38 MAPK was inhibited.

**Activation of PKR and p38 MAPK by dsRNA is unaffected by Jak1ΔB.**

Western blot analysis using a phospho-specific antibody that recognises activated p38 MAPK (Figure 6.5) indicates that the phosphorylation and activation of p38 MAPK in response to dsRNA was not detectably inhibited in the presence of Jak1ΔB. It is important to note that the activation of p38 MAPK is seen following overnight priming with IFNγ, whereas activation in unprimed cells is not detectable in this type of assay (data not shown). As seen previously (Figure 4.3), the stress kinase agonist Anisomycin leads to a strong phosphorylation of p38 MAPK in both cell lines.

The activation of PKR in cells following treatment with dsRNA was assayed (Figure 6.6). There was a moderate increase in PKR activity in 2fTGH cells and a similar increase in 2fJak1ΔB, with no indication of any inhibition of PKR by Jak1ΔB.

**Jak1ΔB inhibits IRF3 activity.**

IRF3 is another factor responsive to both virus infection and dsRNA (135, 253, 255, 267): the protein is serine phosphorylated in response to either activator, allowing it to dimerise and stabilising its nuclear localisation (119). Activated IRF3 regulates transcription as part of the βIFN enhancesome (253), and also regulates other dsRNA-
responsive promoters containing ISREs, such as those of RANTES and ISG15 (8, 38, 134, 164). Subsequent to its activation IRF3 is degraded by a ubiquitin-dependent pathway (135). IRF3(5D) is a recombinant IRF3 in which the serine residues phosphorylated in response to dsRNA have been replaced with phosphomimetic aspartic acid residues, resulting in a protein which behaves as a constitutively active IRF3 (134, 137). The stability of IRF3 and IRF3(5D) following dsRNA treatment were analysed (Figure 6.7), as an index of activation. In wild-type, but not Jak1ΔB-expressing cells, transiently transfected IRF3 is degraded following overnight treatment with poly(I)-poly(C). The degradation of IRF3(5D) in response to dsRNA is also inhibited by Jak1ΔB, suggesting that an event downstream of the activator-dependent phosphorylation of IRF3 is inhibited by Jak1ΔB.

In view of this it was important to ascertain whether Jak1ΔB inhibited the transcriptional regulatory function of IRF3 or just its degradation. RNase protection analysis was used to look at the induction of βIFN in response to poly(I)-poly(C) in cells transiently transfected with IRF3. Figure 6.8 shows a strong constitutive induction of βIFN mRNA in 2fTGH cells transfected with both the wild type IRF3 and the IRF3(5D), and no induction in cells expressing Jak1ΔB. Cells were also transfected with IRF3ΔN, an N-terminal deletion of the wild type which is not capable of binding to DNA, and which (as expected) did not induce βIFN expression at all in this assay, and inhibited induction in 2fTGH cells (Figure 6.8, lanes 7&8) relative to controls.

These results are consistent with Jak1ΔB inhibiting both the activation of NFκB and the activity of IRF3 with respect to expression of βIFN. Furthermore, overexpressing both IRF3 and IRF3(5D) is able to substantially restore the induction of p48 and IFI56K - which do not require NFκB - overcoming the inhibition by Jak1ΔB. It is reasonable to conclude from the data that Jak1ΔB inhibits transcriptional
activation through the IRF3-CBP/p300 complex which regulates the dsRNA-direct induction of ISGs.

The level at which this inhibition occurs is difficult to determine due to the apparent constitutive activation in wild-type cells by transfected IRF3 (Figure 6.8, lanes 3 & 4). This is most likely an effect of the transient transfection, having also been described by Kumar et al. (119), and is probably symptomatic of a wider activation of gene expression caused by the transfection reagents used in transient transfections which have been observed in the laboratory.

**DNA-binding by IRF3 is not affected by Jak1ΔB.**

Once activated by phosphorylation, IRF3 localises to the nucleus and is found as part of a DNA-binding complex known as a double-strand RNA activated factor, or DRAF which also contains a histone acetylase CBP or p300 (119, 255). The DRAFs can be identified by EMSA analysis of nuclear extracts, typically using the ISRE from the ISG15 promoter, and the effect of Jak1ΔB on these DNA-binding complexes was analysed (Figure 6.9). The antibody supershifts identify DNA-binding complexes containing IRF3, and also p48 which would be expected to bind the ISRE-containing probe. Both IRF3 and IRF3(5D) are clearly able to bind to the EMSA probe in extracts from transiently-transfected 2fTGH and 2fJak1ΔB cells. The activation of the wild type IRF3 as a result of the transfection is again apparent.

The DRAFs are relatively large and it is likely that the IRF3-containing complexes observed here are homodimeric IRF3 or IRF3(5D). Accordingly, the question of whether the formation of DRAF (IRF3-CBP/p300) complexes is inhibited by Jak1ΔB remains open until they can be detected and characterised in this type of assay. Nevertheless, it appears that Jak1ΔB does not directly inhibit the phosphorylation-dependent activation of IRF3, or its ability to directly bind DNA in isolation.
Figure 6.1.

Analysis of genes induced by dsRNA in 2fTGH and 2fJak1ΔB cells.

2fTGH and 2fJak1ΔB#5 cells were treated with poly(I)poly(C) for the times indicated and then harvested in parallel with untreated controls. RNA was extracted and RNase protection analysis was performed using probes for Actin, IRF1, p48/ISGF3γ and βIFN.
Figure 6.1.

The figure shows a gel analysis of Poly(I)·(C) treatment in two different cell lines: 2fTGH and 2fJak1ΔB. The treatment times are 0, 2, and 4.5 hours. The proteins of interest are β-IFN, p48, IRF1, and Actin. The gels indicate the expression levels of these proteins over the treatment time.
Figure 6.2.

Analysis of the activity of an NFκB-responsive Luciferase reporter in 2fTGH and 2fJak1ΔB cells.

2fTGH and 2fJak1ΔB cells were transiently transfected with a Luciferase reporter regulated by 3 copies of a consensus NFκB site (3xNFκB-Luc), or the equivalent promoter-less construct (ConA-Luc). Cells were treated with dsRNA (poly(I)poly(C)) or TNFα for 6 hours as indicated, then harvested along with untreated controls, and Luciferase activity was assayed.
Figure 6.2.

![Graph](image)
Chapter 6. Jak1ΔB inhibits induction of genes by dsRNA.

**Figure 6.3.**

Analysis of NFκB DNA-binding activity in 2fTGH and 2fJak1ΔB#5 cells treated with TNFα and dsRNA.

2fTGH and 2fJak1ΔB cells were treated with poly(I)poly(C) and TNFα for the times indicated. The cells were harvested and nuclear proteins extracted as described in the Materials and Methods. The concentration of protein in the extracts was determined and EMSA analysis of DNA-binding complexes in the extracts was performed using a consensus NFκB oligonucleotide probe.
Probe: κB consensus

<table>
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<th>Treatment (minutes)</th>
<th>TNFα</th>
<th>poly(I)•(C)</th>
<th>TNFα</th>
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<tr>
<td>120</td>
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2fTGH

2fJak1ΔB
Chapter 6. Jak1ΔB inhibits induction of genes by dsRNA.

Figure 6.4.

Analysis of the composition of NFκB DNA-binding complexes in 2fTGH and 2fJak1ΔB#5 cells treated with poly(I)poly(C) and IFNγ.

2fTGH and 2fJak1ΔB cells were treated with poly(I)poly(C) and IFNγ for the times indicated. The cells were harvested and lysed in Schindler lysis buffer, and the concentration of protein in the extracts determined. EMSA analysis of DNA-binding complexes in the extracts was performed using a consensus NFκB oligonucleotide probe. Antibodies added to the samples during the assay supershift complexes containing particular NFκB subunits. These are indicated by the arrows to the right of the gel: the lower arrow corresponds to p50, and the upper arrow to p65/Rel A complexes.
Figure 6.4.

<table>
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<th>poly(I)(C)</th>
<th>IFNγ</th>
<th>control</th>
<th>poly(I)(C)</th>
<th>IFNγ</th>
</tr>
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<tbody>
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<td>Supershift antibody:</td>
<td>p50 p65</td>
<td>p50 p65</td>
<td>p50 p65</td>
<td>p50 p65</td>
<td>p50 p65</td>
<td>p50 p65</td>
</tr>
</tbody>
</table>

2fTGH  2fJak1ΔB
Figure 6.5.

Analysis of the activation of p38 MAPK in response to dsRNA in 2fTGH and 2fJak1ΔB cells.

2fTGH and 2fJak1ΔB cells were primed overnight with IFNγ and then treated with poly(I)poly(C) for the times shown. In parallel, unprimed cells were treated with Anisomycin. Following treatments the cells were harvested along with untreated controls into SDS sample buffer. Equal amounts of protein were resolved on a 10% SDS-PAGE gel and transferred to PVDF. The membrane was probed with a phosphospecific antibody which recognises the phosphorylated, activated p38 MAPK. Following stripping, the membrane was Western blotted for p38 MAPK protein.

I am indebted to Dr Ana Costa-Pereira for the data in Figure 6.5.
Figure 6.5.

Western blot:

<table>
<thead>
<tr>
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<th>IFNγ o/n</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Aniso.</td>
</tr>
<tr>
<td>Phospho-p38</td>
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</tr>
<tr>
<td>p38 protein</td>
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<tr>
<td>Phospho-p38</td>
<td></td>
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<tr>
<td>p38 protein</td>
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</table>

- hrs poly(I)(C) treatment.

2fTGH

2fJak1ΔB
Analysis of the specific activity of PKR induced by dsRNA treatment of 2fTGH and 2fJak1ΔB cells.

2fTGH and Jak1ΔB cells were treated with poly(I)poly(C) for the times indicated. The cells were harvested, PKR was immunoprecipitated from the extracts, and *in vitro* kinase reactions performed as described in the Materials and Methods. The protein was resolved on a 10% SDS-PAGE gel, which was dried, and phosphorylated PKR visualised by autoradiography (Figure 6.6a). In parallel, the same cell extracts were analysed for PKR protein by Western blot analysis (Figure 6.6b). Relative specific activity (Figure 6.6c) was determined by densitometric analysis of the data in (Figure 6.6a & b).
Figure 6.6a.

Autoradiograph showing PKR-specific autokinase activity.

Figure 6.6b.

Western blot with anti-PKR mouse monoclonal antibody.

Figure 6.6c.

Relative specific activity.
Analysis of dsRNA-dependent degradation of IRF3.

2fTGH and 2fJak1ΔB cells were transiently transfected with IRF3 and IRF3(5D) expression constructs. Cells were treated with poly(I)poly(C) (IC) overnight and then harvested in Schindler lysis buffer along with untreated controls. The protein concentration of the extracts was determined and equal amounts of protein were resolved on a 10% SDS-PAGE gel. Proteins were transferred to PVDF and the membrane was probed by Western blot analysis for IRF3 protein. The positions of the IRF3 and the slower migrating IRF3(5D) are indicated by arrows on the left of the figure.
Figure 6.7.

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IRF3(5D) → IRF3

IRF3(5D) → IRF3
Figure 6.8.

Analysis of genes induced by IRF3.

2fTGH and 2fJak1ΔB cells were transfected with IRF3, IRF3(5D) and IRF3ΔN expression constructs, and control cells were mock transfected in parallel. The cells were treated with poly(I)poly(C) for 2.5 hours as indicated (IC), and then harvested in parallel with untreated control cells, and RNA was extracted. RNase protection analysis was performed using probes for Actin, IRF1, IFI56K, p48 and βIFN.
Figure 6.8.

<table>
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<td></td>
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2.5 hours treatment with poly(I)poly(C)

- βIFN
- p48
- IFI56K
- IRF1
- Actin
Figure 6.9.

EMSA analysis of IRF3 DNA-binding in 2fTGH and 2fJak1ΔB cells.

Cells were transiently transfected with IRF3 and IRF3(5D) expression constructs. Following treatment with poly(I)poly(C) (IC), cells were harvested in parallel with untreated controls in Schindler lysis buffer. EMSA analysis of the extracts was performed using an oligonucleotide containing the ISG15 ISRE as a probe. Antibodies to IRF3 and p48/ISGF3γ were used to identify DNA-binding complexes containing these proteins - indicated by the arrows on the right of the figure.
<table>
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**Figure 6.9.**

Transfection - IRF3 (5D)
Discussion.

The dsRNA-direct induction of ISGs and also βIFN was inhibited in cells expressing Jak1ΔB (Figure 6.1). Jak1ΔB inhibited NFκB-dependent reporter gene expression in response to dsRNA (Figure 6.2). There was also some evidence that DNA-binding by NFκB was inhibited (Figure 6.3-4) although there was no evidence for an inhibition of the dsRNA-induced degradation of IκB. No evidence was found for inhibition of the dsRNA activation of either p38 MAPK (Figure 6.5) or PKR (Figure 6.6). Transfection of wild-type IRF3 and IRF3(5D) was able to restore dsRNA-direct induction of ISGs but not the induction of βIFN (Figure 6.8), consistent with the inhibition of both IRF3 and NFκB activity. Jak1ΔB does not appear to inhibit DNA-binding by IRF3 (Figure 6.9).

The inhibition by Jak1ΔB of dsRNA-induced gene expression is a significant finding, potentially providing additional candidates for the inhibitory target of Jak1ΔB. A component involved in both the dsRNA induction of gene expression and the IFNγ induction of CIITA promoter IV would be a promising candidate, but the target of Jak1ΔB in each pathway is not necessarily a common factor.

The inhibition of the activation of NFκB by dsRNA establishes the dsRNA-direct activation of NFκB as a target for Jak1ΔB. PKR has been implicated in the activation of NFκB in the dsRNA response, but analysis of PKR activation in response
to dsRNA was inconclusive. As for IFNγ (Figure 4.4), the level of activation seen in the wild-type was low and an inhibitory effect was not discernible against this background (Figure 6.6). p38 MAPK has been assigned various roles in the dsRNA response (93). It was seen to be activated in cells primed with IFNγ (Figure 6.5) and this activation did not appear to be inhibited by Jak1ΔB. Therefore it is unlikely that the inhibitory effect of Jak1ΔB involves p38 MAPK.

Although the level of dsRNA-induced NFκB DNA-binding activity was reduced in the presence of Jak1ΔB, and there was evidence of an increase in the levels of p50 - which might have an inhibitory effect as a homodimer bound to DNA - there was no obvious inhibition of the breakdown of IκBβ, the major negative regulator of NFκB activity (data not shown). The high background of reporter activity in unstimulated cells also makes it difficult to determine the true extent of both activation of NFκB DNA-binding in the wild-type and its inhibition in the presence of Jak1ΔB. It is possible that the affect of Jak1ΔB on NFκB DNA-binding is negligible. This view is supported by the lack of a detectable inhibitory effect on IκBβ and that DNA-binding activity by NFκB is still substantial in 2fJak1ΔB cells.

The inhibition of the activity of IRF3 seems more clear cut. Overexpression of IRF3 in 2fTGH, but not 2fJak1ΔB, leads to constitutive expression of βIFN (Figure 6.8). However, overexpression of IRF3 does rescue expression of the dsRNA direct induction of IFI56K and p48 in cells expressing Jak1ΔB. It is thought that the dsRNA-direct response is dependent on IRF3-containing DRAFs, hence the restoration of this response, but the βIFN-response also requires NFκB whose activity (if not its DNA-binding) is still inhibited by Jak1ΔB.
Following activation, IRF3 is degraded by the proteasome system. This is seen in 2fTGH but not 2fJak1ΔB cells, even with the constitutively active IRF3(5D) (Figure 6.7). Together with the observation that IRF3(5D) is not constitutively degraded in 2fTGH this suggests that an additional post-activation modification is required to allow proteasome mediated degradation. This is supported by the fact that IRF3(5D) can bind DNA in 2fJak1ΔB (Figure 6.9). IRF3 is known to interact with co-factors as part of the βIFN enhancesome (155), and notably with the histone acetyltransferase proteins CBP/p300 as part of the DRAFs (255). These latter proteins are known to generally enhance transcription, possibly through acetylation of histones to open up promoter DNA, and also by interacting with additional transcription enhancers. There is evidence that their acetyltransferase activity is not restricted to histones (139, 229), and acetylation has been suggested as a possible regulator of ubiquitinylation and consequent degradation. A putative role for CBP/p300 or a co-factor in the ubiquitin-dependent pathway of IRF3 degradation, and the inhibition of IRF3-p300/CBP interactions by Jak1ΔB would explain the inhibition of both gene expression in response to IRF3 and also the inhibition of activator-dependent degradation of IRF3. This would support the accumulating evidence that Jak1ΔB may inhibit transcription co-activators.

Returning to the NFκB data, the apparent inconsistency between the level of DNA-binding and the inhibition of NFκB-dependent transcription could also be explained by a parallel with IRF3. In both cases it could be argued that DNA-binding is not affected, but another interaction required for transcription, such as with a common co-activator, is inhibited by Jak1ΔB, leading to their co-ordinated inhibition.

This brings up another issue, that of linking the inhibition of transcription dependent on NFκB or IRF3 with STAT1/USF1/IRF1 dependent transcription. The
most likely explanation is a shared dependency on co-factors which may be the target of inhibition of Jak1ΔB. The interaction of IRF3 with histone acetyltransferases is well characterised, and there are reports that IRF1 interacts with the histone acetylases P/CAF and GCN5 (152) albeit in response to IFNα. NFκB has also been shown to interact with CBP/p300 (240) but this was as part of a response to TNFα. There is no indication thatJak1ΔB inhibits IRF1 activation by IFNγ (Figure 5.7.) or the activation of NFκB by TNFα (Figure 6.2.). But, for example, should Jak1ΔB aberrantly phosphorylate or modify (either directly or indirectly) CBP/p300 or other co-activators, this might selectively affect interactions with only a subset of transcription factors. It is unknown whether the activation of NFκB by dsRNA requires CBP/p300, although the βIFN-enhanceosome involves both proteins. An alternative is that there is a co-factor specific for dsRNA-induced responses, in the same way that a co-factor such as BRCA1 can differentially regulate IFNγ induction of genes (171). In the same way, other co-factors might exist for receptor mediated responses going through NFκB and IRF1, and Jak1ΔB inhibits only a dsRNA specific co-factor.

Alternatively Jak1ΔB may affect post-translational regulatory modifications of IRF3 and NFκB. Both are known to be phosphorylated, regulating subsequent protein:protein interactions, but again the problem is that of how ISRE transactivation in response to dsRNA can be inhibited, whilst receptor mediated activation is apparently unaffected. There may be a specific dsRNA dependent modification pathway which is affected by Jak1ΔB. Direct monitoring of the phosphorylation of IRF3 and NFκB might identify differences between 2fTGH and 2fJak1ΔB. One attempt was made to determine whether Jak1ΔB influenced whether or not IRF3 was tyrosine
phosphorylated, but no phosphorylation was shown either in 2fTGH or 2fJak1ΔB cells (not shown).

As will be shown in Chapter 7, IFNγ-priming is able to overcome the inhibition by Jak1ΔB of gene expression in response to dsRNA. The mechanism of priming is not known. It could involve a novel pathway, or, as seems more likely, the induction by IFNγ of factors whose levels normally limit this aspect of the response to dsRNA. Given the dose dependency of inhibition by Jak1ΔB, overexpression of a required factor or downstream component could exceed the capacity of Jak1ΔB to inhibit responses. A possible extrapolation of this is that the factor inhibited by Jak1ΔB may be activated or induced by IFNγ. Inhibition of an activatable factor would be consistent with aberrant phosphorylation of a normal Jak1 substrate.

In summary, the data suggest again that Jak1ΔB inhibits transcriptional responses without inhibiting the activation of transcription factors themselves. Instead, it seems that Jak1ΔB inhibits the interactions of transcription factors with co-factors required for transcriptional activation.
Chapter 7. The effect of Jak1ΔB on responses to virus infection.

THE EFFECT OF JAK1ΔB ON RESPONSES TO VIRUS INFECTION.

Introduction.

The IFNγ induced antiviral state is complex. It is assumed that it involves the known IFNγ inducible antiviral proteins including PKR, 2′-5′OAS and GBP. In addition it may involve the following: (i) the induction of IFNα and β by IFNγ; (ii) the induction of IFNα/β and ISGs in response to viral dsRNA; (iii) the priming by IFNγ of these responses to dsRNA; (iv) apoptosis triggered by viral infection (101) (through or independently of, for example, PKR, the 2-5A system and p38 MAPK); (v) recruitment of elements of the IFNα/β response via IFNAR1 (231); (vi) additional, unrecognised mechanisms.

Given that Jak1ΔB inhibits both gene induction in response to dsRNA (Chapter 6) and the antiviral response to IFNγ (Chapter 3), it seemed possible a priori that dsRNA responses (through mechanisms (ii) and (iii) described above) might play a greater role in the antiviral response than had been generally appreciated. If so, then their inhibition might account for the inhibitory effect of Jak1ΔB on the antiviral response. In view of this the contribution to the IFNγ antiviral response of the induction of IFNβ (both directly and in response to dsRNA) and of ISGs (in direct response to
dsRNA, and as a secondary response via IFNβ) was assessed. There was no evidence for a predominant role for these pathways or, incidentally, none for one requiring the constitutive low level activation of the IFNα/β receptor (mechanism (v) above).

In parallel with the cytopathicity assays for antiviral activity (Chapter 3) it was noted that in the absence of IFN treatment, cells expressing Jak1ΔB appeared significantly more sensitive than wild-type cells to cell killing in response to EMC virus infection. This might reflect the inhibition by Jak1ΔB of a constitutive antiviral or cell survival response to viral dsRNA - normally augmented in response to IFNγ. Furthermore, unless inhibited by IFNγ treatment, this enhanced sensitivity to cell killing could be manifest as an apparent inhibition of the antiviral response during the routine cytopathic assay and might explain the Jak1ΔB-mediated inhibition of the IFNγ antiviral response. Preliminary analysis of the apparently enhanced sensitivity involved comparison of virus yield and apoptosis in wild-type and cells expressing Jak1ΔB. There was no evidence either for or against differences in either case.

**Results.**

**Jak1ΔB does not inhibit the IFNγ-primed induction of IFNβ or ISGs in response to dsRNA.**

The induction of mRNAs was analysed in response to dsRNA with or without IFNγ-priming in 2fTGH, U5A and 2f Jak1ΔB cells. U5A cells are defective in the response to Type I IFNs, and were used to exclude the induction of genes in response to βIFN induced by IFNγ.
\( \beta \text{IFN} \) mRNA is clearly induced by poly(I)-poly(C) treatment of 2fTGH cells, and the induction is enhanced by IFN\( \gamma \) priming. Importantly, IFN\( \gamma \) alone does not induce \( \beta \text{IFN} \) (Figure 7.1a). In the U5A cells \( \beta \text{IFN} \) is also induced by poly(I)-poly(C) and primed by IFN\( \gamma \) pre-treatment - allowing for under-loading of Figure 7.1a, lane 8 (IFN\( \gamma \)-primed U5A plus poly(I)-poly(C)). As expected, in 2fJak1\( \Delta \)B, \( \beta \text{IFN} \) is not induced by poly(I)-poly(C), but in IFN\( \gamma \)-primed cells the induction is restored.

IFI56K mRNA is also induced by poly(I)-poly(C) in 2fTGH and, as for \( \beta \text{IFN} \), IFN\( \gamma \) alone does not induce IFI56K, but IFN\( \gamma \) pre-treatment enhances induction by poly(I)-poly(C) (Figure 7.1b). However, in U5A cells there is no enhancement of IFI56K induction from IFN\( \gamma \) priming, suggesting that the dsRNA-direct response is not primed by IFN\( \gamma \). Finally, in 2fJak1\( \Delta \)B cells the induction of IFI56K in unprimed cells is inhibited relative to both 2fTGH and U5A cells, but as for the 2fTGH cells, IFN\( \gamma \) priming is able to enhance induction.

In U5A cells lacking the IFN\( \alpha/\beta \) receptor, in which secondary gene-induction in response to induced \( \beta \text{IFN} \) is not possible, the induction of IFI56K is an accurate measure of the direct induction of ISGs by dsRNA, even after treatment with IFN\( \gamma \). This dsRNA direct response of IFI56K was not enhanced by IFN\( \gamma \), suggesting no priming of the dsRNA-direct response by IFN\( \gamma \). There was no inhibition of the induction of either \( \beta \text{IFN} \) or IFI56K in U5A cells (Figure 7.1a & b). Together with the observation that there is no significant difference in the IFN\( \gamma \) antiviral response
Chapter 7. The effect of Jak1ΔB on responses to virus infection.

between 2fTGH and U5A cells (N.J. Rogers, unpublished data) this argues that there is no major role for dsRNA-induced βIFN in the IFNγ response.

The inhibition of the IFNγ antiviral effect in 2fJak1ΔB was greater than 100-fold (Figure 3.2), much greater than the inhibition of induction of βIFN and IFI56K after IFNγ pre-treatment in the same cells (Figure 7.1a & b). If the dsRNA pathways played a significant role in the antiviral response then greater inhibition of them by Jak1ΔB would be expected. However, it is still a possibility that the induction of IFI56K in 2fJak1ΔB cells primed with IFNγ is secondary, through the induction of βIFN, and the direct response to dsRNA is inhibited in these cells. It is even arguable that as IFNγ does not appear to prime the dsRNA-direct response, this is likely to be the case. It was not possible to generate stable transfectants of U5A cells expressing Jak1ΔB, preventing analysis of this possibility.

Cells expressing Jak1ΔB appear more sensitive to the cytopathic effects of virus infection.

Initially, confirmation was required of the original observation that there was enhanced killing by EMCV of cells which had not been pre-treated with IFN. To assess cell-death in the presence of virus without pre-treatment with IFN, a simple assay was used, in which cells were infected with a doubling-dilution series of virus concentrations to determine whether there was any difference in sensitivity to virus infection. Cells were infected and 24 hours later the plates were fixed, and stained with Giemsa to visualise surviving cells. It appears that (in this assay) wild-type cells can survive infections at much higher multiplicities of infection compared to those expressing Jak1ΔB (Figure 7.2). Alternatively, Jak1ΔB confers an acute sensitivity to virus infection even at very low levels of infection.
Virus yield is not enhanced in cells expressing Jak1ΔB.

In the previous experiment, cells were infected with m.o.i. of less than one. Effectively this means that there are fewer virus particles than cells, and that two or more rounds of virus replication must occur for all cells to be killed. It is possible that the yield of virus from an individual infected cell expressing Jak1ΔB might be enhanced, or much more rapid, relative to that from a wild type cell, as a result of an inhibition by Jak1ΔB of the dsRNA response to virus. This would result in earlier, more extensive cell killing in the population of cells expressing Jak1ΔB in comparison to the wild-type.

Accordingly, virus replication was monitored for a single round in wild-type, U5A and 2fJak1ΔB cells, by measuring the incorporation of $^3$H-uridine into viral RNA in the presence of Actinomycin D (to inhibit endogenous DNA-dependent RNA polymerase). There was no significant difference in the rate or magnitude of RNA synthesis in the three cell types (Figure 7.3).

In a second assay of virus yields, 2fTGH, U5A and 2fJak1ΔB cells were infected at the same m.o.i. and following a single round of virus replication the cells and the tissue culture supernatant containing progeny virus were harvested and freeze-thawed to ensure maximum release of virus. The resulting “supernatant” was used to infect murine L929 cells, using a series of dilutions. The more virus produced by the initial infection the lower the dilution required at which the L929 cells would die. U5A cells were used to exclude any inhibition of virus growth through the rapid induction of Type I IFNs by virus dsRNA. No apparent difference was observed between these cell types in the assay (Figure 7.4).

Thus, there was no evidence for more rapid or extensive virus replication in cells expressing Jak1ΔB. In view of this, evidence for an effect on cell death through
apoptosis was sought. It is recognised that cell killing by virus is not just through lysis but also through apoptosis, which may or may not be dependent on virus yield.

Two markers of apoptosis are cell-surface phosphatidyl serine (150), which can be analysed by Annexin V staining (245), and the cleavage of poly(ADP-ribose) polymerase (PARP) by caspases (233). Western blot analysis of protein from a time course in virus infected cells did not identify significant changes in PARP following virus infection of 2fTGH or 2fJak1ΔB cells (data not shown). Similarly, treatment of cells with Actinomycin D and poly(I)-poly(C) - which can induce apoptosis in a similar fashion to virus infection - and subsequent FACS analysis of Annexin V staining was also uninformative (data not shown). On the basis of this it is not possible to make any assessment of apoptotic processes in relation to Jak1ΔB.
Analysis of gene expression in cells primed with IFNγ and induced by dsRNA.

2fTGH, U5A and 2fJak1ΔB cells were primed overnight with IFNγ and treated with poly(I)poly(C) for 4 hours (IC) in parallel with unprimed cells as indicated. The cells were harvested along with untreated controls and RNA was extracted. Gene expression was assayed by RNase protection analysis.

Figure 7.1a.
RNase protection analysis was performed using probes for Actin, IRF1 and βIFN with the RNA from cells treated as described above.

Note: the lane corresponding to the U5A sample primed with IFNγ and treated with poly(I)poly(C) appears to be underloaded, according to the signal from the Actin probe.

Figure 7.1b.
RNase protection analysis was performed using probes for Actin and IFI56K with the RNA prepared as described above.
Figure 7.1a.

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β-IFN

IRF1

Actin

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Figure 7.2.

Assay of the sensitivity of cells to killing by Encephalomyocarditis virus.

2fTGH and 2fJak1ΔB cells were seeded into a 96well plate and infected with a doubling dilution series of virus. 24 hours after infection the plates were fixed with formol saline and stained with Giemsa.
Effective multiplicity of infection (m.o.i.).

5x10^-4
4x10^-3
3x10^-2
0.06
0.125
0.25
0.5

Decreasing m.o.i.

Uninfected

Figure 7.2
**Figure 7.3.**

**Assay of the rate of virus replication.**

The rate of virus replication in 2fTGH, U5A and 2fJak1ΔB cells was measured through the incorporation of $^3$H-uridine into viral RNA in the presence of Actinomycin D, as described in the Materials and Methods. The total incorporation (measured in counts per minute) is shown for the time-points assayed.
Figure 7.3.
Figure 7.4.

Assay of the yield of virus from a single round of infection in 2fTGH, U5A and 2fJak1ΔB cells.

2fTGH, U5A and 2fJak1ΔB cells were infected with 0.5 pfu per cell, and virus harvested from the infected cells 14 hours later as described in Materials and Methods. Murine L929 cells were seeded into 96 well plates and infected with various dilutions of the extracts containing virus from the infected 2fTGH-type cells. 24 hours later the plates were fixed with formol saline and stained with Giemsa.
Figure 7.4.

Uninfected control

Initial dilution of cell extract.

1/10

Extract from infected 2fTGH

1/100
1/1000
1/10000

Extract from infected U5A

1/10
1/100
1/1000
1/10000

Extract from infected 2fJak1AB

1/10
1/100
1/1000
1/10000

Doubling dilution series
(1/2, 1/4, 1/8, 1/16, etc)
Discussion.

IFNγ alone did not induce expression of βIFN (Figure 7.1a). The poly(I)-poly(C) induction of βIFN is inhibited in 2fJak1ΔB, but IFNγ priming is able to restore inducibility (Figure 7.1a). Similarly, IFNγ alone does not induce expression of IFI56K (Figure 7.1b), and induction of IFI56K is inhibited by Jak1ΔB, but priming is able to compensate for this inhibition (Figure 7.1b). The priming of IFI56K induction seen in 2fTGH cells is not seen in the U5A cells unresponsive to βIFN (Figure 7.1b).

Initial observations that cells expressing Jak1ΔB are more sensitive to cell killing by virus were confirmed (Figure 7.2). But there was no evidence for an enhanced rate of replication or greater yield of infectious particles (Figure 7.3 & 7.4).

The role of genes induced by dsRNA in the IFNγ antiviral response can be appraised on the basis of data from experiments described in this section. The evidence indicates that there is no direct induction of βIFN by IFNγ in these cells, and IFNγ priming of the dsRNA-direct induction of genes was not observed in the U5A cells. But there is clearly priming of βIFN induction itself, and induction of ISGs through βIFN induction as seen in 2fTGH. Accepting that there is no inhibition of the IFNγ antiviral response in U5A cells compared to 2fTGH (N. J. Rogers, unpublished data), these secondary responses do not play a major role in the antiviral effect as assayed here. Nevertheless, it is likely that in vivo the dsRNA induction of ISGs through induction of βIFN will play an important role.
Very recently, Takaoka et al. (231) have presented data consistent with a requirement for constitutive activation of the Type I receptor in the response to IFNγ in mouse embryo fibroblasts. It is thought that this involves recruitment of IFNAR1 and elements of the IFNα response to the IFNγ receptor. Again, the absence of any major difference in the antiviral response to IFNγ in U5A and wild-type (2fTGH) cells argues against a major role for such a mechanism operating here.

On the basis of the limited data presented here, Jak1ΔB does not significantly inhibit induction of βIFN and IFI56K mRNAs in cells pre-treated with IFNγ (Figure 7.1a &b), in contrast to the response in unprimed cells. The mechanisms governing this priming effect of IFNγ and the resulting complementation or circumvention of the inhibitory effect of Jak1ΔB remain to be established. An obvious candidate for the complementing factor is IRF1, which can regulate induction of βIFN (188), is strongly induced by IFNγ, and can regulate transcription through ISRE elements (174, 181).

The data from U5A cells, which are unresponsive to βIFN, indicate that it is unlikely that the dsRNA-dependent induction of βIFN plays a major role in the IFNγ antiviral responses assayed here (Chapter 3). Consistent with this, there is little inhibition of induction of βIFN and IFI56K mRNAs in response to dsRNA in IFNγ primed 2fJak1ΔB (Figure 7.1a &b) even though there is a major inhibition of the antiviral response (Figure 3.2). This argues that these responses do not play a major role in the antiviral response and, additionally, that their inhibition is not the basis for the inhibition of this response by Jak1ΔB. In spite of the primed induction of βIFN and secondary induction of ISGs, a role for a residual inhibition of the dsRNA direct response in the IFNγ treated cells expressing Jak1ΔB cannot be excluded.
The observation that cells expressing Jak1ΔB are more sensitive to cell killing by EMCV may help explain the failure of the antiviral response to IFNγ. However, if this is the case, then pre-treatment with IFNα must be able to offset the effects of Jak1ΔB in some way.

One possible mechanism for enhanced killing would be more efficient virus replication in the presence of Jak1ΔB. Two simple assays presented above suggest that this is not the case. Firstly, there is no evidence that the rate of viral RNA synthesis - an index of the replication of the genome of RNA viruses - is greater in 2fJak1ΔB, and nor is the amount synthesised apparently any greater (Figure 7.3). Secondly, there was no evidence that the numbers of infectious progeny produced from an infection is greater in 2fJak1ΔB (Figure 7.4).

The ³H-uridine incorporation assay has been used effectively before, in the analysis of cells expressing the kinase negative Jak1K>E. On that occasion, virus yield was greater in Jak1K>E cells (18), correlating with the inhibition of the IFNγ antiviral effect. Unfortunately, the assay requires that every cell is infected, otherwise the level of ³H-uridine incorporation in the presence of Actinomycin D is too low to register above background levels. It may be that the effect of Jak1ΔB on virus replication only becomes apparent at very low levels of virus infectivity, outside the range of this assay.

The other possibility investigated is that Jak1ΔB enhances an apoptotic response, and that this leads to the enhanced killing by virus. Analysis of two markers of apoptosis - PARP and Annexin V staining of cell-surface phosphatidyl serine - failed to identify differences between 2fTGH and 2fJak1ΔB. Confirmation of this, together with more extensive analysis such as monitoring of caspases, would be necessary before an effect on apoptosis could be eliminated. Repeating the cell killing assay of the type in Figure 7.2 in the presence of a caspase inhibitor such as z-VAD might be also be informative.
It would be interesting to apply the same simple virus yield and apoptosis assays described here to analyse cells pre-treated with IFNγ prior to infection, and also to again show the dose-dependent effect of Jak1ΔB (Chapter 4) by using other clones expressing the mutant.

However unenlightening the results presented are, the initial observation of enhanced cell death remains, although it is difficult to follow up. Cell-killing by any virus would seem to be complex enough without the added possibility of apoptotic death, but it is likely that the presence of Jak1ΔB enhances some apoptotic or cytopathic effect. More extensive analysis of apoptotic pathways is possible, and other assays of virus replication or yield are an option, but an approach which will identify an unknown may ultimately be more successful in finding an explanation for this aspect of the Jak1ΔB phenotype.
Chapter 8.

DISCUSSION.

As part of on-going efforts to characterise novel Jak1-dependent signalling in response to IFNγ, a deletion mutant of Jak1 was identified. Jak1ΔB has no apparent effects on the Jak/STAT pathway in response to IFNs or other ligands, but grossly inhibited the IFNγ induced expression of CIITA mRNA, and also the IFNγ antiviral response. Expression of βIFN and the direct induction of ISGs in response to dsRNA was also inhibited. Consistent with this, inhibition of NFκB and IRF3-dependent gene expression was observed but there was no clear inhibition of DNA-binding by the two transcription factors. The inhibition of dsRNA-dependent gene expression was largely circumvented by IFNγ priming arguing against a role for this inhibition in the inhibition of the IFNγ antiviral effect. There is evidence that cells expressing Jak1ΔB are more sensitive to virus killing than wild-type cells, and microarray analysis suggested that the inhibitory effects might be more widespread than was initially apparent.

Jak1ΔB, IFNγ-signalling and the antiviral response.

The minimum requirements for the antiviral response are likely to be a functional receptor complex and an uncompromised signal transduction pathway leading to upregulation of genes. The IFNγ-induced antiviral response to EMCV infection is substantially inhibited in cells expressing Jak1ΔB (Figures 3.2 & 3.7). In contrast, there was a minor effect on the antiviral response to IFNα. However,
activation of neither the receptor nor the Jak/STAT pathway leading to gene induction are detectably affected in the presence of Jak1ΔB. Antiphosphotyrosine analysis of the receptor components showed no detectable effect of Jak1ΔB (Figure 4.1), whilst STAT1-responsive reporters (Figure 5.6) and IFNγ induction of mRNAs for IRF1 and p48 were also unaffected (Figure 3.4), and neither was the induction of Class I HLA s (Figure 3.3). However, the upregulation of CIITA expression is inhibited (Figure 3.9 & 3.10), indicating that there is inhibition of a subset of IFNγ-induced genes. One obvious explanation is that a subset of antiviral genes and CIITA are regulated by a common transcriptional signal not required by other genes assayed, and that this is somehow targeted by Jak1ΔB.

Soh et al. have shown that mouse macrophages expressing the human IFNGR1 and 2 chains support induction of MHC Class I and II in response to human IFNγ, but without sustaining an antiviral response to EMCV (125, 218). This suggests that an additional species-specific signalling component is required to provide the antiviral response. This situation is only weakly analogous to the Jak1ΔB phenotype: the IFNγ-induced expression of MHC Class II seen in the macrophages is obviously inhibited in cells expressing Jak1ΔB. On the other hand, there are differences between the regulation of the murine and human IFNγ-inducible CIITA promoter IV (160), and macrophages are also reported to upregulate promoter III in response to IFNγ (165). This would leave open the possibility that the two situations are related.

Experiments with the kinase-dead Jak1K→E in Jak1-negative U4 cells showed that it would support both Jak/STAT activation and inducible gene expression in response to IFNγ, but did not support the antiviral response (18). Here again is evidence that activation of STATs leading to gene expression is not sufficient to support an antiviral response to IFNγ. Moreover, the kinase activity of Jak1 is the critical
function, and it was suggested that reduced tyrosine phosphorylation of a site on Jak1K>E inhibited the recruitment of proteins required for the antiviral response. There are significant differences between the two mutants. Jak1ΔB is apparently constitutively active as a kinase and Jak1K>E is of course specifically kinase-dead. Jak1K>E interacts with the IFNγ-receptor complex whilst Jak1ΔB appears to be cytoplasmic. However, the two mutants appear to disrupt the antiviral response to similar levels. Moreover, in U4A cells, in spite of inducing other responses, Jak1K>E does not sustain substantial induction of Class II HLA's.

It is possible that cytoplasmic Jak1ΔB, which is constitutively-phosphorylated (Figure 4.1), interferes with a signal between the receptor and the nucleus by sequestering a factor normally required for signalling through endogenous Jak1 at the receptor. In a similar way Jak1K>E is unable to induce the same signal as endogenous Jak1 because it lacks the kinase activity to trigger a signal, or the requisite phosphorylation to recruit a signalling factor.

Irrespective of the mechanism, the phenotypes of both Jak1ΔB and Jak1K>E suggest that the expression of a subset of IFNγ responsive genes is regulated in some way by Jak1, in addition to requiring STAT activation.

**Priming and the IFNγ antiviral state.**

It is recognised that the antiviral response to α/β IFNs is much more effective than that to IFNγ. One possibility is that a substantial component of the IFNγ antiviral effect is through priming the production of IFNα/β. Our observations here suggest that this is not the case and that priming plays a minor role, at least in the primary response in tissue culture. In U5A cells, which do not respond to α/βIFNs, no difference was detected in the antiviral effects of IFNγ compared to the wild type cells. Similarly,
IFNγ-priming circumvents or complements the Jak1ΔB-mediated defect in the dsRNA-induction of IFNβ and ISGs (Figure 7.1), even though the IFNγ antiviral effect remains massively inhibited (Figure 3.2). The observation that IFNγ priming rescues dsRNA-dependent expression of βIFN in 2fJak1ΔB is not surprising given that IRF1 protein is induced by IFNγ. However, this requires that IRF1 function is not impaired, an issue discussed below in relation to CIITA induction. IRF1 and IRF3 are thought to be redundant with respect to induction of β-IFN (253), thus IRF1 may compensate for the inhibition of IRF3 by Jak1ΔB. IRF1 and IRF3 share the conserved IRF-family DNA-binding domain, but have divergent transactivation domains implying different mechanisms or requirements for transactivation. Alternatively, given the dose dependency of the inhibition by Jak1ΔB (Figures 3.5-3.8), overexpression of any factor which is a downstream target may swamp the capacity of Jak1ΔB to inhibit transcription, rescuing the response. This also raises the possibility that the factor(s) inhibited by Jak1ΔB are IFNγ induced, hence priming can offset inhibition. If this is the case, those factors inhibited by Jak1ΔB in dsRNA responses must be different to those inhibited in the CIITA response. This would argue against a role for IRF1, either as a priming factor, or as the target of Jak1ΔB inhibition with respect to CIITA expression.

Very recently Takaoka et al (231) have presented data from mouse embryo fibroblasts, consistent with a requirement for constitutive activation of the Type I receptor to facilitate recruitment of IFNAR1 and elements of the IFNα response in the response to IFNγ. The absence of any major difference in the antiviral response to IFNγ in U5A and wild-type (2fTGH) cells (N.J Rogers, unpublished data) argues against a major role for such a mechanism operating here. Nevertheless, very low level activation of STAT2 has been observed in response to IFNγ in the HT1080-based cell lines used
here (H.Is'harc, unpublished data). A more minor role for IFNα/β responses cannot, therefore, be excluded, and the apparent contrast between the results for the HT1080 cells and those obtained with MEFs emphasises the potential for variation between cell types. In addition, the primed induction of βIFN in response to viral dsRNA can be assumed to play a much more significant role in mediating the antiviral responses to IFNγ in vivo.

Overall, while more extensive studies will be required to define fully the roles of dsRNA and IFNα/β type responses in the IFNγ response, their inhibition as a basis for the inhibitory effect of Jak1ΔB on the IFNγ antiviral response (as assayed here) appears unlikely, although it remains to be conclusively established.

**IFNγ induction of CIITA promoter IV.**

Jak1ΔB inhibits IFNγ-induced expression of CIITA in the absence of a detectable effect on Jak/STAT activation or induction of the majority of IFNγ-induced genes. Jak1ΔB also inhibits expression of reporter constructs regulated by a minimal CIITA IFNγ-responsive promoter (Figure 5.3 & 5.4). Individual proteins known to regulate the promoter are STAT1, IRF1 and USF1 (159), and there was no detectable inhibition of the function of these factors individually (Figure 5.6-8). In this respect, Jak1ΔB appears selectively to inhibit the promoter IV which is regulated by all three.

One possible explanation is that the assembly of a higher order transcription complex required to activate promoter IV is inhibited by Jak1ΔB. STAT1 and IRF1 are known to interact with a variety of transcriptional co-activators, and these interactions are probably essential for upregulation of promoter IV. Jak1ΔB may interfere with the
specific assembly of such a complex, leading to the selective inhibition of promoter IV. As discussed in Chapter 5, it remains a possibility that Jak1ΔB interferes with a post-translational modification of IRF1 or USF1 leading to the failure to upregulate promoter IV. Although the function of IRF1 appears unaffected by Jak1ΔB (as measured by the Class I HLA response and the IRF1-responsive reporter), an additional modification may be required for the regulation of promoter IV. IRF1 is known to be post-translationally modified by a serine-threonine kinase, thought to be casein kinase II, and this phosphorylation is important for the activity of IRF1 (136, 249). An effect of Jak1ΔB on IRF1 function would not be unexpected given that IRF1 plays a major role in the antiviral response to EMCV (107).

Analogous to Jak1ΔB, inhibition of IFNγ-induced CIITA expression is seen in several situations where Jak/STAT function is not inhibited. In a cell line supporting a permissive infection by HCMV, expression of CIITA is inhibited without inhibition of STAT1 activation or IFNγ-induced Class I HLA (121). Interestingly, HCMV proteins are known to interact with CBP and P/CAF (205), two proteins known to interact with STAT1, and which could be required for CIITA expression. *Chlamydia* interferes with CIITA expression by targeting USF1 for degradation (274), emphasising both the importance of USF1 and the requirement for all three known DNA-binding proteins at promoter IV for transcription.

**Induction of gene expression by dsRNA.**

Induction of ISGs in response to dsRNA is thought to be mediated by complexes containing IRF3 and CBP/p300 (98, 255). There is also a requirement for the activation of NFκB for the induction of βIFN (126, 248). Jak1ΔB inhibits the induction of ISGs and βIFN by dsRNA (Figure 6.1), apparently through inhibition of the activity of IRF3 (ISGs and βIFN) and NFκB (βIFN). Inhibition of NFκB was
thought initially to be a consequence of reduced DNA-binding, but there was no evidence of inhibition by Jak1ΔB of dsRNA-dependent IκB degradation. Accepting that the inhibition of IRF3 appears to be at a step subsequent to DNA-binding, it seems possible that in addition to any putative inhibition of its activation, the activity of NFκB might also be inhibited downstream of DNA-binding.

The inhibition of βIFN induction by dsRNA is reminiscent of the inhibition of CIITA promoter IV, in that the activation of individual DNA-binding proteins NFκB and IRF3, or STAT1, USF1 and IRF1 - was not inhibited by Jak1ΔB, but their activity in the context of the specific promoters was. The requirement for transcriptional co-activators at the βIFN promoter is well characterised, with CBP/p300 being recruited to the βIFN enhanceosome. Inhibition of this type of interaction by Jak1ΔB would disrupt promoter upregulation, independently of DNA-binding by transcription factors, and suggests a link between the inhibition of dsRNA-induced gene expression, and the inhibition of IFNγ-induced CIITA expression.

A common mechanism by which many viruses attempt to inhibit the host cell response is through proteins which sequester essential factors. Viral IRF homologues (vIRFs) are common-place (146) and have been shown to inhibit cellular IRFs directly, and also to bind to transcription co-activators such as CBP/p300. In this way vIRFs inhibit the cellular response to dsRNA, and many other viral proteins also interact with CBP/p300 including Adenovirus E1A, HPV E6 and HCMV IE86 (24, 175, 205), potentially resulting in a similar inhibition of responses to dsRNA. As discussed above, HCMV also inhibits IFNγ-induced expression of CIITA, implying that there might be a common transcriptional requirement between CIITA expression and dsRNA induction of genes.
The relationship between the inhibition of both IRF3 activation and the IFNγ response is interesting because nuclear localisation of both IRF3 and STAT1 is regulated by Crm1. IRF3 has been shown to interact with Crm1 in a Ran-dependent manner, and treatment of cells with Leptomycin B - a specific inhibitor of Crm1-mediated nuclear export - leads to the nuclear accumulation of IRF3 (119) and tyrosine-phosphorylated STAT1 (158). Potentially the same could be true for other Crm1 regulated proteins, of which IκBα is thought to be one (96). This is interesting in view of the apparent inhibition by Jak1ΔB of NFκB-dependent transcription in response to dsRNA, but there is an obvious conflict here given the apparent normal activity of NFκB in response to TNFα in 2fJak1ΔB (Figure 6.2 & 6.3). On the other hand, TNFα might activate a different signal for nuclear retention. The work of Mowen and David also suggests that Jak1 plays a role in regulating the nuclear retention of STAT1, which is defective in Jak1-deficient cells (both U4A and HeLa Jak1-/-) and also in U4A cells expressing the kinase-negative Jak1K>E. This emphasises the more attractive possibility of nuclear retention of the transcription factors actively involved in transcription perhaps through interactions with nuclear binding partners, or further modification. Acetylation of CIITA by P/CAF has been shown to regulate its nuclear localisation (220), and IFN-dependent acetylation of STAT2 was recently reported (176), setting a precedent for other STATs. Hypothetically, enhanced nuclear retention of transcription factors required for induced gene expression could be dependent on acetylation by co-activators such as P/CAF, CBP or p300. A ligand could both activate a transcription factor and also enhance its nuclear retention by signalling to nuclear co-factors.
Does Jak1ΔB interfere with histone acetyltransferase activity required for activation of specific promoters?

Acetylation is increasingly recognised as an important regulator of transcription (reviewed in (64, 114)). A steadily increasing number of proteins are being isolated with acetyltransferase activity, and the numbers and types of proteins which are acetylated is also increasing. Briefly, acetylation of histones relaxes chromatin structure, increasing the accessibility of promoter DNA to the transcriptional apparatus. Acetylation of several transcription factors has been demonstrated to affect their DNA-binding ability, and is also reported to affect nuclear localisation. Finally, acetylation of proteins can regulate their stability.

It is believed that the co-ordinated activities of multiple histone acetyltransferases play a central role in specific transcriptional regulation by regulating chromatin structure of promoters, acting as physical bridges to recruit additional co-factors, and possibly acetylating non-histone proteins such as transcription factors. Acetylation of c-jun-associated histone H3 has been reported, dependent on phosphorylation of the histone itself (33). It is possible that the phosphorylation acts as a cue for acetylation, and the histone is targeted by its association with c-jun at the promoter. Another report describes p38 MAPK-dependent phosphorylation of histone H3 in response to IFN treatment (236). It is plausible that a co-ordinated pattern of transcription factor activation and modifications of histones or other nucleosomal proteins at specific promoters is required for transcription. Thus, for CIITA promoter IV, it is not sufficient to activate the DNA-binding STAT1 and IRF1, but an additional signal regulating recruitment of co-activators is also required, and is inhibited by Jak1ΔB.

The involvement of histone acetyltransferases in IFN-signalling is emerging with the characterisation of the roles of CBP/p300, P/CAF, MCM5 and BRCA1. Importantly, BRCA1 has a proven role in mediating upregulation of specific promoters
by IFNγ (171). It is possible that the inhibitory effect of Jak1ΔB affects a specific acetyltransferase activity regulating a subset of promoters.

The mutant does not appear to inhibit the activation of individual transcription factors, but does affect expression from promoters collectively regulated by them, possibly by influencing the recruitment or activity of acetyltransferases specifically required for subsets of promoters. It is recognised that acetyltransferases are present in cells in limiting quantities, and transfection of them can enhance reporter expression. Jak1ΔB’s inhibitory effects are related to its level of expression, suggesting that it interacts with a limiting factor or signal.

The signal-dependent activation of different co-factors would also explain why responses to IFNα and TNFα are not inhibited by Jak1ΔB. Finally, the activator-dependent degradation of IRF3 is inhibited by Jak1ΔB although activation of IRF3 and subsequent DNA-binding α not inhibited. This suggests that an additional process is required to initiate degradation, which could be an acetylation event. The induction of βIFN mRNA by dsRNA is rapid but transient, and degradation of critical transcription factors would be consistent with this. Integrating the degradation signal with the process of promoter upregulation would be a sensible way to ensure tight regulation of the βIFN promoter.

The importance of CBP/p300 as common transcriptional co-activators involved in a wide range of transcriptional responses is now established (64, 212). The levels of these proteins are believed to be limiting, with the result that transcription is enhanced by transfection/overexpression, and competition for these proteins has been suggested as a mechanism for interference between signalling pathways e.g. Ras/AP1 and Jak/STAT (87). Given the role of CBP/p300 in STAT, IRF and NFκB-mediated transcription, these two factors and also P/CAF are certainly putative targets for Jak1ΔB.
Mowen and David (158) showed that the product of a CBP-GST fusion construct could be used to purify STAT1 from extracts from cells expressing Jak1, but not from Jak1-deficient cells. The implication of this is that a Jak1-dependent signal is necessary for the interaction of STAT1 with CBP. Either this signal is targeted at STAT1 itself rather than CBP, or a co-factor required by CBP for the interaction may be the target. It is a possibility that Jak1ΔB inhibits the STAT1-CBP interaction or the subsequent activity of CBP, inhibiting promoter IV. Jak1 has been observed in the nucleus (140), the significance of which has been unclear, although the localisation of Jak1 to the nucleus may be important for regulating the interaction of STAT with other nuclear factors.

The evidence is highly circumstantial, but an effect of Jak1ΔB on interactions between DNA-bound transcription factors and co-activators provides a possible common site for the dsRNA response and IFNγ induction of CIITA. The mechanism may involve aberrant modification of STAT1, NFκB and IRF, inhibiting recruitment of specific co-activators to particular promoters. Alternatively, Jak1ΔB may affect a specific co-factor or subset of co-factors directly, preventing their interaction with the DNA-bound transcription factors at complex promoters.

Several approaches could help to identify the possible mechanism. Cross-linking of proteins interacting with promoter DNA has been used to analyse transcriptional complexes such as the βIFN enhanceosome (253). Analysis of IRF3-CBP (i.e. the DRAFs) and STAT1-CBP interactions or additional proteins interacting might be informative. Overexpression of candidate critical factors might overcome the inhibition by Jak1ΔB. Perhaps the most obvious approach to identify a candidate would be to show a direct interaction with, and/or phosphorylation by, Jak1ΔB, or the absence of an interaction between endogenous Jak1 and a factor in the presence of Jak1ΔB. Efforts aimed at purifying Jak1-interacting proteins in the lab have lead to an optimised
approach using a tagged Jak1, and this approach could easily be utilised for Jak1ΔB too.

The effect of Jak1ΔB is an intriguing problem, the solution to which may help to explain how specificity at the promoter level is controlled. Analysis of the inhibition of the antiviral response has also raised interesting questions concerning the mechanism of this response. The data have highlighted IFNγ priming of the dsRNA induction of IFNβ. Although not a major factor in the primary antiviral response, it is likely to be important in vivo. In addition, the data suggest that a major effect of IFNγ may be upon the inhibition of virus-dependent cell killing (whether necrotic or apoptotic). In this respect there is also much to be learned.


mutant cell line: central role of the 91 kDa polypeptide of ISGF3 in the interferon-α and -γ signal transduction pathways. EMBO Journal 12:4221-4228.


is mediated by cooperation between signal transducer and activator of transcription 1 and nuclear factor kappaB. J Biol Chem 272:14899-907.


Bibliography.


Appendix 1.

EXPRESSION PROFILING BY OLIGONUCLEOTIDE MICROARRAY ANALYSIS: JAK1ΔB INHIBITS THE INDUCTION OF A SUBSET OF GENES IN RESPONSE TO IFN-GAMMA.

Introduction.

Up to now, analysis of the effect of Jak1ΔB on signal transduction provided little clue as to the nature of its inhibitory mechanism, other than that the effect was probably mediated at the level of the promoter rather than being an inhibition of the Jak/STAT pathway. Analysis of the Jak/STAT signalling pathway and associated proteins thought to modulate its activity, such as PKR and p38 MAPK, failed to identify differences between 2fTGH and 2fJak1ΔB. Limited promoter analysis using reporter gene constructs demonstrated that reporters driven by the IFNγ-inducible CIITA promoter IV were indeed inhibited, consistent with the inhibition of transcription from the endogenous promoter. However, reporters regulated by individual elements that contribute to overall control of promoter IV were not appreciably affected, correlating with physical analysis suggesting no discernible effect on the individual proteins.

Recent advances in the field of expression profiling and microarray analysis provide the technology to simultaneously analyse the expression of thousands of genes in parallel - an undertaking which would be impossible using traditional methods such as Northern blotting, RNase protection analysis or RT-PCR. During 1997/1998 the Lerner Research Institute at the Cleveland Clinic Foundation set up an Affymetrix
Appendix 1. Microarray analysis of the effect of Jak1ΔB on gene-expression.

oligonucleotide microarray-based system. The Stark, Williams and Silverman laboratories carried out analysis of IFNα, β and γ-induced gene-expression in HT1080 cells (52) - the parental line for 2fTGH and 2fJak1ΔB. These labs, with whom there has been extensive collaboration in the past, kindly agreed to make the Cleveland facility available to us for a limited series of experiments. The objectives were: (i) generally to assess the Affymetrix oligonucleotide microarray-based system for consideration by ICRF in comparison to cDNA-based systems of expression profiling; (ii) to obtain expression profiles comparing the IFNγ responses of wild type 2fJak1ΔB and U4A (Jak1-negative) cell lines.

The identification of additional IFNγ-induced genes inhibited by Jak1ΔB in a similar way to CIITA would provide useful data for several reasons:

- Identifying such genes would potentially allow the identification of common factors regulating the promoters of genes inhibited and, therefore, likely candidates for the target of Jak1ΔB’s inhibitory effect. Furthermore, a simpler promoter to that of CIITA might be identified, which would be easier to analyse.
- If the inhibitory effect of Jak1ΔB was limited to expression of only a small number of genes, it might identify crucial known or novel antiviral proteins upregulated during the IFNγ antiviral response, and whose expression was inhibited by Jak1ΔB.
- Analysis of the promoters of such genes might lead to the identification of novel sequence motifs associated with antiviral responses.

It was uncertain whether genes other than those governed by CIITA were inhibited by Jak1ΔB. The fact that, of the other genes analysed so far, only the induction of Class I HLA was slightly inhibited, and that this could be attributed to the inhibition of CIITA, was evidence that a degree of specificity was involved in the inhibition. This specificity may be evidence that ligand-dependent signal transduction
does not just depend on the STATs activated at the receptor, and other signals are integrated through higher transcription complexes at the promoter level to introduce specificity. For example, BRCA1 has been shown to co-operate with STAT1 in the differential regulation of IFNγ-induced genes (171). It is possible that other co-factors mediate additional specificity, and ligand dependent signals regulate these co-factors. Obtaining information about the effect of Jak1ΔB on a broader range of IFNγ-induced genes could give an insight into this specificity.

The experiments described in this section were carried out during visits to Cleveland in the autumn of 1998 and spring of 1999. mRNA samples were prepared both at ICRF and in Cleveland, and checked by RNase protection analysis. The cDNAs and biotinylated cRNAs were prepared during the visits to Cleveland. Microarrays were hybridised and scanned as a service by the Lerner Institute Gene Core facility. I am indebted to Dr Sandy Der for help with analysis of the data and sample preparation, and to Dr Leslie Ann Hawthorn for hybridisation and scanning of the microarrays.

There were initial problems with the quality of the microarrays (acknowledged by Affymetrix), and irregularities in the hybridisation process or sample preparation limited the integrity of the analysis and the conclusions which can be reached. Nevertheless, the results indicated that the inhibition by Jak1ΔB is not specific for CIITA (and CIITA-dependent genes) but almost certainly affects a substantial subset of IFNγ inducible genes.

**Results.**

Messenger RNA samples were prepared in parallel from untreated and IFNγ-treated 2fTGH and 2fJak1ΔB cells, and the cDNA and biotinylated copy RNA (cRNA) for each sample synthesised as described in the Materials and Methods. The cRNA was
Appendix 1. Microarray analysis of the effect of Jak1ΔB on gene-expression.

hybridised to Hu6800 arrays and the arrays were scanned to generate the raw data to be analysed.

The analysis of the Affymetrix microarray data is a two step process. Initially, a baseline expression profile is generated from the four chips that constitute a chip set hybridised with a particular sample. This profile is based on the absolute intensities of fluorescence generated by sample hybridisation with the probe sets and gives absolute values for expression levels of the ~6800 genes analysed. This baseline is then used to determine the relative change in expression levels in other samples, which are called the experimental samples. In doing this the GeneChip analysis software takes into account differences between profiles caused by experimental variation (e.g. the hybridisation process) or the quality of the samples.

Each microarray consists of a set of four chips A-D with oligonucleotide probes for the mRNA from ~1700 genes on each chip. The probe set for a gene consists of 20 specific 25-mer oligonucleotide probes which hybridise to sequences in cRNA generated from the mRNA sample of interest. These are called the perfect match (PM) oligonucleotides. For each PM oligonucleotide there is a corresponding mismatch (MM) oligonucleotide with a single base substitution which provides a measure of non-specific hybridisation for the PM oligonucleotide. The expression level of a mRNA is calculated by subtracting the intensities of the MM probes from the PM probes, giving a net intensity value.

The abundance of house-keeping genes whose expression levels should not vary greatly is used as a standard for the expression levels of other genes, and the values for these in the experimental file are adjusted so that they match those of the baseline file, a process called scaling, with the values for all other genes adjusted accordingly. Then the total intensity of all the genes is adjusted in the experimental file and baseline file, the values being scaled up or down to give the same total value, a process called normalising to all genes. The output from the software is in a spreadsheet
format which can be manipulated within certain limits using Excel. The major parameters used in this section are listed in the table below.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abs Call</td>
<td>Absolute call: can be present (P), absent(A) or marginal(M) - whether a mRNA is present in the experimental sample.</td>
</tr>
<tr>
<td>Average Difference (Intensity)</td>
<td>Average difference - the average of the perfect match minus the mismatch probe intensity for all the probe pairs used from the probe set for the gene.</td>
</tr>
<tr>
<td>Avg. Diff. Change (Change)</td>
<td>The difference between the experimental and baseline average differences.</td>
</tr>
<tr>
<td>Diff.</td>
<td>Difference Call: change in expression between baseline and experimental sample; can be increase(I), decrease (D), marginal increase or decrease (MI or MD), or no detectable change (NC).</td>
</tr>
<tr>
<td>B=A</td>
<td>A * in this column signifies that the transcript is absent from the baseline sample.</td>
</tr>
<tr>
<td>Fold change</td>
<td>The ratio of the average differences between the two samples.</td>
</tr>
<tr>
<td>Sig (Significance)</td>
<td>Significance - assigned by the software: the larger the value the more reliable the measured difference between baseline and experimental. A negative value signifies a decrease between baseline and experimental files, and a positive corresponds to an increase.</td>
</tr>
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</table>

Previous experiments using the Affymetrix system with HT1080 cells provided a control set of data against which to assess the quality of data from the expression analysis in 2fTGH and 2fJak1ΔB cells. Comparison with the data from HT1080 cells - from which 2fTGH cells are derived - would provide a benchmark both for the numbers of genes seen to be expressed and induced in 2fTGH, and also for differences between the 2fTGH and 2fJak1ΔB expression patterns.
Appendix 1. Microarray analysis of the effect of Jak1ΔB on gene-expression.

It is important to emphasise that the data shown here from the HT1080 experiment is different to that published in PNAS (52). This reflects an upgrade in the software used to analyse the data, together with the discovery that many of the oligonucleotides in the probe sets were incorrectly derived and corresponded to intronic sequences. The GeneChip 3.1 software used here has been designed to ignore these faulty probe sets. This can lead to differences in output from the same original data analysed with the old software. An extreme example is that of RING4 which, using GeneChip 3.0, in HT1080 cells is induced 49.2 fold by IFNγ after six hours. Using GeneChip 3.1 however, the software ignores faulty oligonucleotides in the probe set, and the gene is called as absent in both control and IFNγ-treated samples.

It seemed that the most appropriate analysis would be to compare expression changes following IFNγ treatment relative to the corresponding untreated control; the alternative analysis of comparing differences in expression between the IFNγ-treated samples might be profoundly influenced by clonal variation. After the initial analysis it would then be possible to compare changes in genes between the cell lines e.g. identify those genes induced in 2fTGH and for these compare the level of induction in 2fJak1ΔB.

It should be emphasised that where data for individual genes from the GeneChip comparative analysis is shown, it is only selected data i.e. the 30 or so genes most highly induced or showing the greatest differential between samples from that analysis.

Comparison of 2fTGH data with previous HT1080 data.

Initial analysis through a straightforward visual examination of the raw fluorescence data from the chip scanner indicated problems with some aspects of the hybridisation in the 2fTGH experiment. Figure A.1 shows typical false colour images
Appendix 1. Microarray analysis of the effect of Jak1ΔB on gene-expression.

generated by the confocal laser scanner, depicting a chip from the original experiment in
HT1080 cells, and an example of the corresponding chip hybridised with RNA from
the 2fTGH experiment. There is a clear difference in the intensity of fluorescence and
also the pattern, with the HT1080 chip having a more homogeneouss pattern and
generally lower intensity. The edge of the array is clearly identified by white (highly
fluorescent) border features exemplified by the chip from the HT1080 experiment.
There are no oligonucleotides present on the area outside the border features which
should be totally dark as is the case for the HT1080 chip. It is apparent that there is a
significant amount of fluorescence in this blank area in the chip for the 2fTGH
experiment, indicative of either non-specific staining by streptavidin-PE or non-specific
hybridisation of cRNA target to the array surface. Either scenario reflects a problem
with the hybridisation itself or the glass substrate of the chip onto which the
oligonucleotide probes are arrayed.

Examples of intensity values from the control probe sets present on each array
are listed in Table A.1. External control RNAs from \textit{in vitro} transcribed bacterial and
phage genes are spiked into experimental samples at known concentrations prior to
hybridisation. The internal control mRNAs such as GAPDH (M33197) and Actin
(X00351) are represented by multiple probe sets. These control probe sets facilitate
comparisons between arrays and experiments; analysis of the data tended to confirm
that there was variability between chips. The data are from the same cRNA preparation
hybridised in parallel to the four sub-arrays A, B, C and D - which together constitute
one chip set.

Even after normalising to take into account variation in the general level of
intensity between the chips, there are two points of interest. Firstly, looking at the
GAPDH and Actin probe sets, there are clear differences in signal intensities between
chips, with the B-chip consistently most intense and D-chip the least intense. Bearing in
mind that the same sample is hybridised to each chip in parallel, this suggests a
significant variation between chip performance.
Appendix 1. Microarray analysis of the effect of Jak1ΔB on gene-expression.

The second observation is that the external control RNA's - BioC, BioDn, and CreX - spiked into the hybridisation sample at known concentrations show consistent variations between chips as well. However, for these probes it is the A chip giving consistently higher intensities, the other three giving relatively similar intensities. It would be expected that if an individual chip was not performing this would be consistently seen with all probe sets. The fact that a theoretically homogenous sample gives quantitatively variable readings on different chips, and also that the variation itself is inconsistent, reinforces that there is a significant problem with the chips.

Given the apparent problems with the arrays themselves, new chips were obtained, and hybridised with the same samples as the original chips, giving more acceptable data from the control probe sets. Subsequent data presented here is from this second series of hybridisations.

A comparison of gene expression in HT1080, 2fTGH and 2fJak1ΔB cells.

The data overleaf allows a general comparison between the cell lines and also highlights differences between the quality of the HT1080 experiments and the comparative analysis of 2fTGH and 2fJak1ΔB.

By way of explanation, a gene is called present if the average intensity values for the perfect match (PM) probes are greater than those of the mismatch (MM) probes. The decision to “call” a probe set as an increase, decrease, or a marginal call is based on the numbers of probe pairs where a change is seen - the higher the proportion of probes which show change relative to those which do not, the more likely the call will be a definite increase or decrease.
Appendix L Microarray analysis of the effect of Jak1ΔB on gene-expression.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>HT1080 6hrs IFNγ</th>
<th>2fTGH con Vs 2fTGH 6hrs IFNγ</th>
<th>2fJak1ΔB con Vs 2fJak1ΔB 6hrs IFNγ</th>
</tr>
</thead>
<tbody>
<tr>
<td>Genes present</td>
<td>2200</td>
<td>1300</td>
<td>1300</td>
</tr>
<tr>
<td>Genes changed*</td>
<td>2100</td>
<td>1400</td>
<td>1200</td>
</tr>
<tr>
<td>Increase</td>
<td>690</td>
<td>440</td>
<td>480</td>
</tr>
<tr>
<td>Decrease</td>
<td>310</td>
<td>210</td>
<td>170</td>
</tr>
<tr>
<td></td>
<td>160</td>
<td>130</td>
<td>150</td>
</tr>
</tbody>
</table>

* Genes changed = sum of those called as Increase, Decrease, and also Marginal Increase & Marginal Decrease, the numbers for which are not displayed above.

The data sets for the 2fTGH and 2fJak1ΔB cells are substantially different from that of the HT1080 cells, with only half as many genes called present by the software. This reflects the higher global intensity pattern, which, when intensities are “normalised”, gives a higher background. This effectively reduces the relative intensity of every probe set, and low-abundance genes are swamped by the background. This higher global intensity was seen in spite of “better” chips being used in the second series of hybridisations, and was attributed to non-specific hybridisations, possibly an artefact of the hybridisation process. In spite of this, the data sets for 2fTGH and 2fJak1ΔB seem comparable and allow some meaningful analysis.

A comparison of genes induced in 2fTGH and HT1080.

Comparing the responses of genes highly induced in the 2fTGH experiment with their response as measured in the prior HT1080 experiment, there are some large
Appendix 1. Microarray analysis of the effect of Jak1ΔB on gene-expression.

discrepancies apparent (Table A.2). Of the thirty genes most highly induced in 2fTGH, seventeen are categorised in HT1080s as No Change by the software, given a low Fold Change value, and low Difference Change. It is interesting to note that for these genes, eleven of the probe sets are on the D chip and five are on the C chip. One interpretation might be that the quality of the chips used for the 2fTGH samples was again suboptimal, otherwise a more even distribution might be expected.

Conversely, starting with the genes highly induced in HT1080 and looking at their response in 2fTGH (Table A.3), the correlation is better, but by no means perfect. The Difference Call given by the software varies widely, with eighteen genes called as No Change, but the probe sets for these genes give higher (Difference) Change values and Fold (Change) values in comparison to those which were induced in 2fTGH and not in HT1080. The apparent contradiction between Difference Call and Change reflects the high background intensity of the chips in the 2fTGH experiment; the software is reluctant to make calls against high background intensities using low absolute changes in intensity.

On the basis of this comparison it seemed reasonable, when looking at differences between 2fTGH and 2fJak1ΔB cells, to concentrate on those genes induced in 2fTGH also induced in HT1080. This would eliminate probe sets affected by chip quality in the 2fTGH experiment where apparent changes might be artefacts of the variability between individual chips.

A comparison of gene expression in 2fTGH and 2fJak1ΔB cells after 6 hours treatment with IFNγ.

Table A.4 compares expression data for genes from the 2fTGH and 2fJak1ΔB experiments, looking only at genes identified as IFNγ-induced in the HT1080 experiment. The majority of the genes induced in 2fTGH appear to be authentic IFN-responsive genes e.g. GBP, 9-27, IRF1, STAT1, and several from the MHC loci. Others are less obviously candidates that might be induced by IFNγ e.g. an H+-
ATPase, a putative N-acetyl transferase, and nicotinamide N-methyltransferase. There are a large number of genes apparently induced in 2fTGH but not induced in 2fJak1ΔB - these being given a No Change for the Difference Call, and having zero fold change - which might therefore be inhibited by Jak1ΔB. These include LMP2, GBP, IP-30, p48, ICAM-1 and IFP35, which are all well characterised as IFNγ-inducible.

A comparison of the response of genes determined by the GeneChip analysis with that seen in RNase protection analysis is useful (Table A.5). Qualitatively the GeneChip and RNase protection data are comparable for IRF1, STAT1α and 9-27, although for 9-27 the fold change value is five-fold greater in 2fTGH, which does not appear to be the case in the RNase protection. Generally, the intensity values are lower in 2fJak1ΔB, which may be a reflection on the quality of sample hybridised to the arrays for 2fJak1ΔB. Interestingly, the analysis for 6-16 would suggest it is inhibited in 2fJak1ΔB, even though the change in intensity between untreated and treated is very similar to that in 2fTGH. The “No Change” call reflects the lower absolute intensity being too close to background levels in 2fJak1ΔB, preventing the software from acknowledging the change in intensity between probe sets. RNase protection analysis shows comparable induction of 6-16 between the two cell lines.

The inhibition of ICAM-1 expression which is suggested by the GeneChip experiment seems to contradict the results of FACS analysis in which ICAM1 was shown to be induced to wild-type levels in 2fJak1ΔB albeit after 48 hours induction with IFNγ (Figure A.2). A closer look at the GeneChip data reveals that the intensity values for the probe sets are low for samples from HT1080 and 2fTGH - which do show an induction - and even lower in 2fJak1ΔB.

Unfortunately, for those genes which are demonstrably inhibited by Jak1ΔB using RNase protection analysis, the GeneChip expression analysis is inconclusive, since neither CIITA nor Invariant chain are either detectable or induced in 2fTGH or 2fJak1ΔB. Even in the HT1080 experiment the intensities for both probe sets are low.
Appendix 1. Microarray analysis of the effect of Jak1ΔB on gene-expression.

One reason for this is that they may be low abundance mRNAs, or alternatively, the oligonucleotide probes selected for these genes might be inadequate.

Jak1ΔB appears to have a broad inhibitory effect on gene expression after 18 hours treatment with IFNγ.

Table A.6 shows expression analysis of 2fJak1ΔB after 18 hours IFNγ in comparison to a baseline of 2fTGH after 18 hours IFNγ, looking at genes inhibited in 2fJak1ΔB relative to 2fTGH. Juxtaposed with this first data is the analysis of 2fJak1ΔB after 18 hours with 2fJak1ΔB untreated as a baseline. Data for Invariant chain suggests a strong inhibition in 2fJak1ΔB at 18 hours relative to 2fTGH, and no induction in 2fJak1ΔB relative to the untreated control. Also 9-27 appears to be inhibited, again comparing 2fJak1ΔB to 2fTGH, but the comparison with the untreated control suggests a substantial induction still occurs in 2fJak1ΔB, consistent with RNase protection data. There are many other genes which are called as a decrease between 2fTGH and 2fJak1ΔB and as “No Change” after 18 hours IFNγ induction in Jak1ΔB. Some are obscure in terms of their association with IFNs (e.g. Collagen, MIC2, Mac25) but others are authentic ISGs such as complement proteins and HLA’s.

A summary of the data from the expression analysis at 18 hours is shown here:

<table>
<thead>
<tr>
<th></th>
<th>Experiment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2fTGH 18 hrs IFNγ Vs 2fJak1ΔB 18 hrs</td>
</tr>
<tr>
<td>Genes present</td>
<td>1300</td>
</tr>
<tr>
<td>Genes changed*</td>
<td>420</td>
</tr>
<tr>
<td>Increase</td>
<td>70</td>
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<tr>
<td>Decrease</td>
<td>230</td>
</tr>
</tbody>
</table>
Appendix 1. Microarray analysis of the effect of Jak1ΔB on gene-expression.

It seems that on the basis of expression analysis at 18 hours using GeneChip there is widespread inhibition by Jak1ΔB compared to the wild type, but at the same time there are still genes being induced in 2fJak1ΔB. Also, for the 2fJak1ΔB 18 hour data set the intensity values are low (Table A.6) which might inflate the numbers of genes apparently inhibited in comparison to the 2fTGH cells. Given the problems with chip quality, the analysis of genes whose probe sets give low intensity values should be treated with caution, especially when additional data from a more robust, reliable assay gives contradictory results. It was anticipated that this analysis would identify later genes perhaps dependent on those induced at earlier times. Expression at late times was not analysed in HT1080 cells, so no “known, good” bench mark was available with which to compare expression in 2fTGH and 2fJak1ΔB.

Figure A.3 shows the individual probe sets for three genes - Invariant chain, Mac-25 and CIITA - from the chips hybridised to the 18 hour IFNγ treated RNAs. The grid superimposed on the images to identify each probe set is generated by the GeneChip software, and each square corresponds to the area of the array surface where a particular oligonucleotide is present. The top row of each grid corresponds to the 20 perfect match (PM) oligonucleotides, and below each perfect match is the corresponding mismatch (MM) oligonucleotide.

Looking at the probes for Invariant chain, in the 2fTGH 18 hours IFNγ sample, there are a couple of PM oligonucleotides recognised by the RNA sample, one in particular producing a very high intensity signal, and the corresponding MM oligos are much less intense. Invariant chain was described as a decrease between the 2fTGH and 2fJak1ΔB samples, and there is clearly a lower overall intensity of hybridisation in the 2fJak1ΔB sample, whilst the pattern of hybridisation in terms of the relative intensity of the oligos is similar, which is reassuring. The probe sets for Mac-25 also reflect the software’s analysis - a decrease from 2fTGH to 2fJak1ΔB - with two PM oligos
Appendix 1. Microarray analysis of the effect of Jak1ΔB on gene-expression. 266

producing a high signal in the 2fTGH sample and not for 2fJak1ΔB. Finally, the probe sets for CIITA show virtually no signal, reflected by the low intensity values in the analysis. It should be noted that the two diagonally aligned high intensity features in the grid are not oligo probes for CIITA, but features distributed across the surface of each chip to allow the software to align the position of probe sets during analysis. These are similar to the edge features clearly visible in Figure A.1 on the image of the chip from the HT1080 experiment.

Additional analysis looking for genes highly induced in 2fJak1ΔB relative to 2fTGH was uninformative. Any such genes would have been candidates for inhibitors of the antiviral response, or of responses to IFNγ more generally, whether as a direct result of their action, or indirectly through competition for common limiting factors. In view of the apparent constitutive phosphorylation of Jak1ΔB, aberrant constitutive gene expression might be expected, but no clear evidence was found.

In summary, the expression analysis provides somewhat contradictory findings. This is demonstrated by Table A.7 showing the output from two analyses, the first comparing expression in 2fJak1ΔB after 6 hours IFNγ to a baseline of 2fTGH at 6 hours, and the second 2fJak1ΔB after 18 hours IFNγ to 2fTGH after 18 hours IFNγ. The analysis at 6 hours, as previously described, is consistent with inhibition of several genes by Jak1ΔB, but at 18 hours these same genes do not appear to be fundamentally inhibited, even though Invariant chain is strongly inhibited, in agreement with previous data. One explanation of this apparent disparity is that expression of the genes induced in the 2fTGH at 6 hours had diminished again by 18 hours back to levels comparable to those in an uninduced situation and also to a situation where the inhibition by Jak1ΔB was limiting gene expression. It is reasonable to conclude that, in spite of problems with the quality of arrays supplied and other issues relating to the processing of samples, the inhibition by Jak1ΔB is probably not restricted to CIITA or CIITA-
Appendix 1. Microarray analysis of the effect of Jak1ΔB on gene-expression. 267

dependent genes, but the results provided by microarray analysis must be confirmed by
more robust assays such as RT-PCR, Northern blotting or RNase protection analysis.
Appendix 1. Microarray analysis of the effect of Jak1ΔB on gene-expression.

Figure A.1.

Examples of oligonucleotide arrays used in the analysis of the effect of Jak1ΔB on gene-expression.

False colour images produced by confoccal laser scanner representing fluorescence intensities of samples following hybridisation to Affymetrix microarrays.
Figure A.1.

HT1080 experiment

2fTGH experiment
Table A.1. Control probe intensities for 2fJakΔB (6 hours IFNγ).

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Gene Name</th>
<th>Intensity</th>
<th>Change</th>
</tr>
</thead>
<tbody>
<tr>
<td>5v4N all CHIP A</td>
<td>AFFX-BioC-3_at</td>
<td>132</td>
<td>-34</td>
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<tr>
<td>5v4N all CHIP B</td>
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</tr>
<tr>
<td>5v4N all CHIP D</td>
<td>AFFX-BioC-3_at</td>
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<td>33</td>
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<tr>
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<td>5v4N all CHIP B</td>
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Table A.2. A comparison of genes induced in 2fTGH and HT1080 by 6 hours treatment with IFNγ.

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<th>Probe Set</th>
<th>2fTGH Intensity</th>
<th>2fTGH vs 2fTGH 6hrs Change</th>
<th>Fold</th>
<th>Sig</th>
<th>2fTGH Intensity</th>
<th>2fTGH vs 2fTGH 6hrs Change</th>
<th>Fold</th>
<th>Sig</th>
<th>HT1080 Intensity</th>
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<th>Sig</th>
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Table A.3. A comparison of genes induced in HT1080 and their induction in 2fTGH after 6 hours treatment with IFNγ.

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<th>Change</th>
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<td>9.02</td>
<td>MHC-encoded proteasome subunit gene LAM</td>
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| HG2915- | 347 I         | 369    | -9.7 | 5.28 | 1055 I        | 762    | 6.5  | 5.59 | Major Histocompatibility "Complex," Class "I,"
| M87503  | 430 I         | 323    | 4    | 2.02 | 399 I         | 470    | 7.6  | 5.17 | Human IFN-responsive transcription factor su |
| J03909  | 401 NC        | 364    | -7.0 | 3.51 | 236 I         | 209    | -9.0 | 3.79 | Human gamma-interferon-inducible protein (II) |
| X66401  | 739 I         | 483    | 3.5  | 1.96 | 737 I         | 643    | 4.7  | 3.54 | LMP2 gene extracted from H.sapiens genes |
| HG2917- | 480 I         | 421    | 4.2  | 2.46 | 958 I         | 718    | 4    | 2.97 | Major Histocompatibility "Complex," Class "I,"
| M62800  | 100 NC        | 167    | -3.4 | 0.91 | 169 I         | 186    | -7.6 | 2.85 | Human 52-kD SS-A/Ro autoantigen "mRNA,"
| U43142  | 45 I          | 307    | -5.5 | 0.6  | 249 I         | 163    | -6.8 | 2.69 | Human vascular endothelial growth factor regulator |
| Z50194  | 148 NC        | -91    | -1.6 | -0.16| 221 I         | 182    | 5.6  | 2.31 | Homo sapiens mRNA for PQ-rich protein |
| M24283  | 214 NC        | 142    | 3.3  | 0.98 | 101 I         | 112    | -7.9 | 2.29 | Human major group rhinovirus receptor (HRV) |
| U72882  | 415 I         | 259    | 2.7  | 0.9  | 269 I         | 216    | 5.1  | 2.27 | Human interferon-induced leucine zipper protein |
| L22342  | 354 NC        | 210    | 2.5  | 0.7  | 132 I         | 136    | -6.2 | 2.14 | Human nuclear phosphoprotein "mRNA," complete CDS |
| M97936  | 295 I         | 441    | -7.4 | 3.55 | 69 I          | 141    | -6.0 | 2.12 | Human transcription factor ISGF-3 mRNA sequence |
| U51010  | 1668 NC       | 254    | 1.2  | 0.06 | 1184 I        | 773    | 2.9  | 1.8 | Human nicotinamide N-methyltransferase "gene" |
| M55452  | 495 I         | 542    | -8.9 | 1.43 | 143 I         | 126    | -5.4 | 1.71 | Human guanylate binding protein isoform I (G) |
| X71874  | 774 I         | 673    | 8.5  | 6.85 | 575 I         | 371    | 3.4  | 1.67 | Proteasome-like subunit MECL-1 gene extracted from H.sapiens |
| U08021  | 1880 I        | 1032   | 2.2  | 1.23 | 1769 I        | 1048   | 2.5  | 1.54 | Human nicotinamide N-methyltransferase (NMNMT) |
| U63824  | 73 NC         | -2     | -1   | 0    | 152 I         | 113    | -5.0 | 1.51 | Human transcription factor RTF1 (RTF1) "gene" |
| L07633  | 459 I         | 67     | 1.1  | 0.03 | 736 I         | 453    | 2.9  | 1.4 | Homo sapiens (clone 1950.2) interferon-gamma |
| J04080  | 178 NC        | 150    | -3.5 | 0.95 | 112 I         | 98     | -4.7 | 1.28 | Human complement component C1r "mRNA," complete CDS |
| U82897  | -151 NC       | -57    | -2.7 | -0.15| 125 I         | 106    | -4.4 | 1.23 | Human Bcl-2 binding component 3 (bbcl3) "mRNA, complete CDS |
| M83667  | 110 NC        | 52     | 1.9  | 0.19 | 119 I         | 95     | 4.2  | 1.16 | Human NF-IL6-beta protein "mRNA," complete CDS |
| M37435  | 471 NC        | 210    | 1.8  | 0.33 | 293 I         | 202    | 3.2  | 1.12 | Human macrophage-specific colony-stimulating factor |
| M79462  | 66 NC         | 65     | -1.9 | 0.11 | 115 I         | 90     | -4.2 | 1.09 | Human PML-1 "mRNA," complete CDS |
| D89052  | 819 NC        | 189    | 1.3  | 0.1  | 1082 I        | 563    | 2.3  | 1.03 | Human mRNA for proton-ATPase-like "protein" |
| M94880  | 469 NC        | 31     | 1.1  | 0.01 | 419 I         | 236    | 2.8  | 0.96 | Human MHC class I (HLA-A*8001) mRNA - comp |
| AF00844 | 500 NC        | 313    | 2.7  | 0.99 | 213 I         | 145    | 3.1  | 0.9 | Homo sapiens phospholipid scramblase "mRNA, comp |
| L13210  | 217 NC        | -54    | -1.2 | -0.04| 963 I         | 527    | 2.2  | 0.87 | Human Mac-2 binding protein "mRNA," comp |
Table A.4. Comparison of genes induced in 2fTGH and 2fJak1ΔB after 6 hours treatment with IFNγ.

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<td>Vacuolar H+-ATPase Mr &quot;56,000&quot; subunit (Hsp60)</td>
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Table A.5. Analysis of the induction of selected genes after 6 hours IFNγ.

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<th>HT1080 con vs 6hrs IFNγ</th>
<th>2fTGH con vs 6hrs IFNγ</th>
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Proteasome-like subunit MECL

MHC Class I

IFR1

ISGF3A/STAT1alpha

IP-30

ISGF3γ/p48

LMP2

ICAM-1

IFP35

hPA28

IF16

Human ISG-54K gene

Human ubiquitin carrier protein 1

Human HLA-A8

Human MHC class I (HLA-A*3101)

Human HLA-A26

Human Fas ligand

Human Bcl-2 binding component

Human Factor NF-IL6
Appendix 1. Microarray analysis of the effect of Jak1ΔB on gene-expression.

Figure A.2.

FACS analysis of the effect of Jak1ΔB on the induction of ICAM1 expression in response to IFNγ treatment.

2fTGH and 2fJak1ΔB cells were treated with IFNγ for 48 hours. Following treatment the cells were harvested and stained alongside untreated controls with antibodies specific for ICAM1. Cell surface expression of ICAM1 was monitored by FACS analysis.

I am indebted to Diane Watling for the data shown in this figure.
Table A.6. A comparison of differences in gene expression between 2fTGH and 2fJak1AB after 18 hours IFNγ.

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<th>Fold</th>
<th>Sig</th>
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<th>Diff</th>
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<td>119</td>
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<td>-3.89</td>
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<td>RbP gene (renin-binding protein) extracted from l</td>
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<td>-3.71</td>
<td></td>
<td>Alpha-tubulin (b alpha 1)</td>
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<td>-1.9</td>
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<td>mRNA for NADPH-flavin reductase, complete cc</td>
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<td>-3.65</td>
<td></td>
<td>mRNA for TGF-beta superfamily protein, compl</td>
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<td>-1927</td>
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<td>-0.99</td>
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Figure A.3.

Comparison of probe set fluorescence intensities for selected genes, demonstrating relative expression in 2fTGH and 2fJak1ΔB cells.

False colour images of the individual probe sets for three genes following hybridisation of microarrays with the samples prepared from 2fTGH cells treated with IFNγ for 18 hours and the corresponding 2fJak1ΔB cells.
Figure A.3.

M13560 - Invariant chain

HG987-HT987 - Mac25

U18259 - CIITA
Table A.7. Differences in gene expression between 2fTGH and 2fJak1ΔB following 6 and 18 hours IFNγ.

<table>
<thead>
<tr>
<th>Probe Set</th>
<th>Intensity 6hrs IFNγ vs 2fTGH 6hrs IFNγ</th>
<th>Intensity 18hrs IFNγ vs 2fTGH 18hrs IFNγ</th>
<th>Entrez Definition - AffxDefinition</th>
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<tr>
<td></td>
<td>Diff</td>
<td>Change</td>
<td>Fold</td>
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<td>-467</td>
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Discussion.

Although it is apparent that the experiments are flawed through both variation in the quality of the chips and issues surrounding hybridisation of samples, the data still provides some points of interest.

The quality of the chips was variable throughout the experiment, and this was initially evident through analysis of the control RNA probe sets which gave a variable signal from what should have been an identical sample during the first set of hybridisations. Subsequent hybridisations gave more acceptable comparisons between chips hybridised with the same sample, but the number of genes called present was still lower than would have been expected, and the general feeling was that the quality of the arrays was still poor (S. Der, pers. com.). There was a bias in the distribution of the probe sets which showed changes in expression, with many of the probe sets being located on a particular sub-array (Table A.2). This reinforces the impression that there was variation in the performance of individual chips, often giving uniformly low intensity values for those probe sets present on it.

If the inhibition of genes highlighted by the comparisons of 6 hour time-points is real, it points to much wider inhibition by Jak1ΔB than previously suspected. This in turn supports the view that the target of Jak1ΔB is a factor more widely involved in the regulation of transcription. If the same general trend of inhibition is confirmed by another method, such as an alternative array format (e.g. a cDNA array), it would raise additional questions.

If the inhibition by Jak1ΔB is more widespread than previously indicated, but there is no indication of inhibition of the Jak/STAT pathway, how is Jak1ΔB able to inhibit a relatively broad range of gene-expression? The role of transcriptional co-factors in induction of gene expression is emerging, and with it the importance of
Appendix 1. Microarray analysis of the effect of Jak1ΔB on gene-expression.

Factors such as histone acetyltransferases. The dependence of STATs on such cofactors is becoming apparent, and it may be that, as previously suggested, Jak1ΔB inhibits co-factor interactions at the promoters of IFNγ-induced genes, leading to a broad range of inhibition. This would be consistent with the whole promoter analysis using CIITA promoter IV.

As yet, no one particular co-factor has been identified as a universal co-factor for STAT1-dependent IFNγ-induced transcription, but if such a factor did exist then it would be a realistic candidate for the target of Jak1ΔB. Modulation of such a factor by Jak1ΔB might differentially affect its function depending on which factors, in addition to STAT1, were required for promoter function. CBP is a potential candidate since overexpression of CBP leads to potentiation of STAT1-dependent IFNγ-induced transcription (87), as does Nmi overexpression (277), which is believed to enhance STAT1-CBP interactions. Analysis of the effect of Jak1ΔB on such interactions would be informative.

Does Jak1ΔB really not have an inhibitory effect on responses to IFNα? There is a slight inhibition of the antiviral response to IFNα in 2fJak1ΔB, but nothing approaching that seen for the IFNγ response. An antiviral response is a complex phenotype, the product of the co-ordinated action of many gene products, and the inhibition of the IFNγ antiviral effect is unsurprising if the broad inhibition of IFNγ-induced genes by Jak1ΔB suggested by the microarray data is to be believed. In view of this, the limited inhibition of the IFNα antiviral effect suggests that expression analysis of genes induced by IFNα in 2fJak1ΔB would show much less inhibition in comparison to analysis of IFNγ-induced genes.
Appendix 1. Microarray analysis of the effect of Jak1ΔB on gene-expression.

It is recognised that IFNα can regulate GAS sites in IFNγ responsive genes, given the formation of STAT1 homodimers (GAFs) following IFNα stimulation (76, 132). These may play a role in the antiviral response to IFNα and Jak1ΔB may inhibit their activity as it might do in the IFNγ response, hence the slight inhibition of the IFNα antiviral response.

Alternatively, IFNα responsive genes may be less dependent on co-factors. ISGF3 is the major transcription regulator activated by IFNα and the presence of p48 or STAT2 may reduce a co-factor requirement. If co-factors are inhibited by Jak1ΔB this effect might be less pronounced on ISGF3 dependent transcription compared to GAF-dependent transcription.

Does Jak1ΔB inhibit expression of genes regulated independently of the Jak/STAT pathway e.g. constitutively expressed genes, or genes regulated by the TGFβ/SMAD pathway? If Jak1ΔB inhibits a co-factor which is also required for responses mediated by other transcription factors (e.g. the SMADs, Ets, MyoD) then this is a possibility. Alternatively, all gene expression is dependent on the general transcriptional apparatus of the cell (RNA polymerase etc.) which is recruited to promoters by transcription factors like the STATs; but it seems unlikely that Jak1ΔB targets this, otherwise the inhibition would be more widespread, substantially affecting the IFNα Class I HLA and antiviral responses.

There may be inhibition of constitutive gene expression, and the evidence may be hidden in the data from the microarray analysis, but the lack of confidence in this data makes the investigation of this possibility unrealistic.
Appendix 1. Microarray analysis of the effect of Jak1ΔB on gene-expression.

Returning to the GeneChip analysis, results which contradict the response demonstrated by RNase protection are difficult to explain. It could be argued that the microarray technique with its reverse-transcription and poly-A selection might not provide populations of cRNA comparable to the original mRNA used in the RNase protection assay. However, any selective enrichment or impoverishment would affect the same particular mRNA in any samples compared, and the relative difference should be maintained. For example, RNase protection analysis of mRNA for p48 and 9-27 indicates no gross difference between 2fTGH and 2fJak1ΔB, but the same mRNA, following sample preparation for GeneChip analysis, indicates substantial inhibition of both genes in 2fJak1ΔB relative to 2fTGH. This sort of effect could be attributed to an artefact of chip variation, in which case almost every gene which shows a differential response must be treated with caution.

Some of the discrepancies can be attributed to the choice of oligonucleotide probe for particular genes, such as those for CIITA, whose expression was not detected with the microarrays (Figure A.3). In the case of IRF1 mRNA which was repeatedly assayed in RNase protections, together with the IRF1 promoter-luciferase data, both showing no apparent difference between 2fTGH and 2fJak1ΔB, it is likely that the difference seen using the microarray may be an experimental artefact. These examples serve to emphasise the need for confirmation of any observations by alternative techniques.

The uncertainty surrounding the quality of the microarray analysis and the apparently broad inhibition demonstrated by Jak1ΔB - if the results are to be believed - makes further analysis difficult to address. Until the authenticity of the results can be more generally confirmed, deeper analysis of individual genes and their promoters would be foolhardy. But a brief assessment is interesting.

Looking at the genes which may be inhibited, and their putative functions, several genes which are induced in 2fTGH at 6 hours but inhibited in 2fJak1ΔB may play a role in the antiviral response of cells, indicating that the inhibition suggested by
Appendix 1. Microarray analysis of the effect of Jak1ΔB on gene-expression. 285

the GeneChip analysis may be authentic. The role of GBP as an antiviral protein has been proven by antisense and overexpression approaches (6), and its inhibition would be consistent with a reduced antiviral response.

The apparent inhibition of several proteasome components and associated factors is pertinent to the inhibition of the antiviral response. It is recognised that the activity of proteasomes alters following IFN treatment. Changes in the expression of proteasome components after IFN treatment lead to altered specificity and changes in the nature of peptides expressed on MHC Class I (206). This may feed into cell-mediated antiviral responses, but would not account for the inhibited response seen in tissue-culture.

Other genes whose expression is inhibited by IFNγ in 2fTGH, but not in 2fJak1ΔB, may be essential for virus replication, and continued expression of these in 2fJak1ΔB would enhance virus replication. Unfortunately, this aspect of the analysis failed to produce any meaningful comparisons.

There are almost certainly unidentified antiviral proteins and the Affymetrix arrays represent only a proportion of the possible numbers of genes which might be induced by IFNs. It would not be surprising if comparison of 2fTGH and 2fJak1ΔB failed to identify novel antiviral proteins. Also, antiviral mechanisms do not depend solely on induction of genes, but also on the modification or activation of pre-existing proteins such as eIF2α and RNase L, which would not be identified by the GeneChip.

It is ironic that the power of the Gene Chip is its ability to gather a large amount of data rapidly, which can provide answers to many different questions, but in this case the uncertainties over the integrity of the experiment and the apparent complexity of the differences in responses makes further analysis difficult to address.

At best the Gene Chip analysis might have identified novel candidate antiviral genes inhibited by Jak1ΔB, cellular genes which an infecting virus might depend on for replication and which are inhibited during the antiviral response in 2fTGH but not in
2fJak1ΔB, co-regulated promoters inhibited by Jak1ΔB, or genes constitutively induced in 2fJak1ΔB compared to 2fTGH. Realistically, technical problems allow only the very limited conclusion that it is likely that the inhibition by Jak1ΔB affects a significant proportion (perhaps 10%) of IFNγ inducible genes. But even this will require further confirmation.
Appendix 2.

ABSTRACT OF MANUSCRIPT IN PREPARATION.

The Interferon-gamma antiviral response in human cells.

Aspects of the IFN-γ antiviral response versus EMC virus have been analysed in wild type human HT1080 cells and in mutants thereof which are defective in different elements of the IFN-α/β response pathway. IFN-γ has only a partial inhibitory effect (approximately 50%) on viral RNA production in a single cycle replication assay. It is nevertheless significantly inhibitory in typical virus yield or cell death assays, the effect in the latter (10,000 fold) substantially exceeding that based on virus yield (100 fold). Very low level STAT2 phosphorylation and induction of the IFN-α/β ‘specific’ IFI56K mRNA are observed in response to high levels (>5000 IU/ml) of IFN-γ in these cells. IFN-γ per se does not, however, directly induce detectable IFN-β mRNA. On the other hand, the production of IFN-β mRNA in response to dsRNA or virus infection is greatly enhanced by priming (pretreatment) with IFN-γ, as is the activation of the p38 MAP kinase but not the production of dsRNA-inducible ISGs. Priming requires prolonged (18 hr) pretreatment with IFN-γ and likely reflects enhanced responses through IFN-γ-induced proteins. Inhibition of the p38 MAP kinase was without effect on IFN-β mRNA production. Despite the IFN-γ priming of dsRNA responses, in typical in vitro assays the antiviral response to IFN-γ was effectively identical in wild-type cells and cells lacking an IFN-α/β response. Accordingly, in apparent contrast to increasing evidence for a role for the IFN-α/β response in the primary IFN-gamma response in mouse embryo fibroblasts (e.g. Takaoki et al. Science (2000) 288, 2357-60), no evidence was obtained for a significant role for α/β IFN in the primary IFN-γ response in this human cell system. IFN-γ priming of the dsRNA response and of IFN-β production can reasonably be assumed, however, to play an important role(s) in enhancing the IFN-γ antiviral response(s) in vivo.