The Control of Human Immunodeficiency Virus Type 1 Long Terminal Repeat Promoter Activity by Retinoic Acid.

by

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Abstract.

The control of human immunodeficiency virus type 1 (HIV-1) long terminal repeat (LTR) directed gene expression by the hormone all-trans-retinoic acid has been investigated. Cell type specific positive and negative modulation of basal and phorbol ester induced HIV-1 promoter activity is demonstrated. These effects of retinoic acid, shown to be dependent on retinoic acid nuclear receptors, are mapped to the NF-κB and Sp-1 binding region. Retinoic acid is shown to inhibit basal and phorbol ester stimulated HIV-1 promoter activity in the human promonocyte cell line U937 and in the human cervical carcinoma cell line HeLa. Phorbol ester stimulated gene expression from a heterologous promoter enhanced by multimers of NF-κB sites, from either the HIV-1 LTR or the human interleukin-2 gene, or the mouse major histocompatibility complex H2k gene, is also inhibited by retinoic acid. Furthermore, the activation of the HIV-1 promoter by the HIV-1 transactivator protein tat is shown to be inhibited by retinoic acid. Retinoic acid is also demonstrated to inhibit the stimulation of HIV-1 promoter activity by expression of NF-κB in the human T cell line Jurkat but, conversely, to synergise with the stimulation by phorbol ester treatment. Finally, retinoic acid receptor α is shown to be able to bind specifically to the HIV-1 NF-κB site in vitro, in the presence of the retinoic acid X receptor β. The mechanisms of the cell type specific positive and negative regulation of the HIV-1 promoter are discussed.
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1. Introduction.

1.1. The human immunodeficiency virus type 1. The aetiologic agent of acquired immune deficiency syndrome.

The human immunodeficiency virus type 1 (HIV-1) is the aetiologic agent for the vast majority of cases of acquired immune deficiency syndrome (AIDS). AIDS was first recognised in 1981 when unusual clusters of *Pneumocystis carinii* pneumonia (PCP) and Kaposi's sarcoma, a rare form of skin cancer, were reported in previously healthy homosexual men. Soon after these initial reports in the United States, AIDS was identified in Europe, the Caribbean and Africa and is now a global pandemic with the World Health Organisation (WHO) predicting between 40 and 100 million HIV infections by the year 2000. Many of the individuals infected with HIV-1 will progress to AIDS with the disease being ultimately responsible for their premature death.

HIV-1 and the related virus HIV-2 are members of the retroviral family, the lentiviruses. These viruses typically give rise to slowly progressive disease in their host. Other members of this family include the visna maedi, and progressive pneumonia viruses (PPV) that infect sheep, the caprine arthritis encephalitis virus that infects goats, the equine infectious anaemia virus which infects horses and the simian and feline immunodeficiency viruses which infect monkeys and cats respectively. Lentiviruses are also characterised by the complexity of their genomes as compared to other retroviruses. The human immunodeficiency viruses are the first members of the lentivirus family shown to infect human cells.

Advances in the treatment of HIV-1 disease will depend on an increased understanding of the molecular biology of the virus and the disease it causes. This thesis is concerned with the control of HIV-1 long terminal repeat (LTR) directed gene expression by retinoic acid. The effects of retinoic acid on HIV-1 directed gene expression are of particular interest as a binding site for retinoic acid receptor has recently been identified in the HIV-1 LTR sequences (1-3). Furthermore retinoic acid has been used to treat conditions associated with AIDS and thus an understanding of its direct effects on HIV-1 gene expression are of interest (4, 5).

1.1.1. The HIV-1 Life Cycle.

The initial events of the HIV-1 life cycle are similar to those of all other retroviruses. HIV-1 infects cells by virtue of the high affinity interaction between its outer envelope protein gp120 and the cellular receptor CD4 (6-8). The viral core is subsequently internalised by direct fusion of the viral envelope glycoprotein with the cell membrane in a pH independent manner involving a cleavage in gp120 at the V3
loop (9). The fusion is mediated by the viral protein gp41 which is non-covalently attached to gp120 (9). Upon internalisation the viral particle is converted to an active nucleoprotein complex. The viral RNA, still associated with core proteins, undergoes reverse transcription catalysed by the viral enzymes reverse transcriptase and RNase H to form double stranded viral cDNA, reviewed in (10).

The cDNA is then transported to the nucleus where integration into a host chromosome takes place, catalysed by the viral integrase protein. The enzymatic activities required for the synthesis of the HIV-1 DNA proviral intermediate and the integration into the host genome appear to be similar for HIV-1 and MLV (11). The viral DNA integrated into the host chromosome is longer than the viral RNA due to the duplication of a specialised sequence - the long terminal repeat (LTR) - of about 1000 bp at each end. The LTR is derived from a combination of sequences present at the 3' end (U₃), the 5' end (U₅), or both ends (R) of the RNA genome and has the structure U₃ - R - U₅. It contains the enhancer and promoter sequences responsible for the control of viral gene expression. The viral DNA is integrated so that the ends of the LTRs are directly joined to cellular sequences to form the integrated provirus.

Once integrated the provirus behaves in a similar manner to a cellular gene and is transmitted to any progeny cells that result from division of the host cell. Both cellular and viral factors are necessary to initiate high levels of viral gene expression. The cellular factors may be either constitutively produced or may be induced by a variety of activating signals. Once HIV-1 gene expression has been activated, viral genes are expressed, beginning with short, multiply spliced mRNAs which encode the regulatory proteins, particularly tat, rev and nef. The function of the viral transactivator protein tat will be discussed in a later section. The rev protein appears to play a critical role in the switch from production of the short mRNAs to the long singly spliced and unspliced mRNAs which encode the viral structural proteins. Viral protein synthesis leads to the production of viral particles which bud from the cell. The HIV-1 viral life cycle is summarised in Table 1.
Table 1. Summary of the HIV-1 viral life cycle. Adapted from (12).

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9
1.1.2. Immunopathogenesis of HIV-1 infection.

The typical course of HIV-1 associated disease and the activity of the major cell types involved in HIV-1 infection will be broadly summarised in this section (for reviews see (12, 13)). The period between initial HIV-1 infection and AIDS can be summarised as follows: primary infection; dissemination of the virus to the lymphoid organs; clinical latency; induction of viral expression and replication; cytopathicity; clinical disease characterised by immune dysfunction, opportunistic infection, wasting syndrome and neurodegenerative conditions. The mean time period between initial infection and clinical disease is approximately 10 years (14).

The course of HIV-1 infection is summarised in Figure 1.1. A few weeks after viral infection 50-70% of infected individuals suffer a burst of rapid viral replication and high levels of viremia (15). This causes a dramatic perturbation in the number of peripheral blood mononuclear cells (PBMC) usually with a substantial decline in CD4+ T lymphocytes. Within weeks to months a humoral (antibodies to HIV-1 p24 and env (Figure 1.1)) and cellular (HIV-1 specific cytotoxic T lymphocytes (CTL) (Figure 1.1)) immune responses to the virus are detected and the levels of culturable HIV-1 in peripheral blood decrease. The levels of most PBMC subsets return to normal, but the CD4+ T cells often equilibrate to moderately or even markedly depressed levels. The majority of patients then enter a long asymptomatic period, during which viremia is low, circulating HIV-1 p24 antigen is undetectable in plasma and viral replication is barely detectable (1 in 1000-10000 cells infected) in PBMC (16, 17). After a mean period of 8-10 years virus is again detected at high levels in the blood and the number of CD4+ T cells, which has been steadily decreasing, falls precipitously. Clinical disease is characterised by loss of immune function and opportunistic infections which eventually lead to death.

Over the past 2-3 years it has become increasingly apparent that the concept of a complete viral latency period is false and that at all stages of HIV-1 infection there are considerable levels of viral replication at some sites. HIV-1 infection is associated with peripheral generalised lymphadenopathy, hence the former name, lymphadenopathy virus (LAV), for the first HIV-1 isolate (7). Actively expressed virus is found in the lymphoid organs, especially the lymph nodes, and in tissue macrophages at all stages of infection at levels many times higher than those found in circulating T cells (18, 19). The next sections summarise the involvement of the major cell types implicated in the pathogenesis of HIV-1 disease and the control of the virus by and in these cells.
Figure 1.1. A schematic course of HIV-1 infection. See text for details. Adapted from (23).

Infection Seroconversion Death

Minor or no symptoms ARC/AIDS

CD4+ peripheral blood lymphocytes

Antibodies to HIV Env

HIV Specific CTL

Antibodies to HIV p24

Viremia

4-8 Weeks Up to 12 Years 2-3 Years

Time post-infection
1.1.21. The role of the lymph nodes in HIV-1 infection.

During an ordinary immune response the lymph nodes serve as filters to trap antigens in the thread-like processes of follicular dendritic cells (FDC) contained within germinal centres (reviewed in (20, 21)). T cells and B cells in the paracortical areas of the lymph nodes are consequently stimulated and an immune response is mounted. During the period after HIV-1 infection, large numbers of viral particles become trapped in the FDC network (22-24) leading to the infection of large numbers of permissive CD4+ T cells as they migrate to the lymph nodes in response to the antigentic challenge. In the microenvironment of the lymph node a general state of immune activation ensues.

The ability of HIV-1 to replicate in CD4+ T cells is strictly dependent on their state of activation. The mechanisms that maintain a constant pool of activated target cells in the lymph nodes are likely to include HIV-1 specific immune responses that stimulate and expand CD4+ T cells. This will generate a population of activated cells which can support a productive HIV-1 infection (25). In addition, HIV-1 infection leads to release of cytokines that may induce direct expression of HIV-1 or support viral replication indirectly by maintaining an increased level of cellular activation. These events do not occur in peripheral blood. The contact between large amounts of virus and activated CD4+ T cells in the lymph nodes leads to viral infection of large numbers of host cells and the propagation of infection. Sensitive techniques such as polymerase chain reaction (PCR) amplification of HIV-1 RNA and/or DNA in asymptomatic patients have shown that viral burden is 1-2 logs lower in peripheral blood than it is in lymphoid tissue taken from the same patient (19). Also RNA PCR analysis revealed that viral expression in peripheral blood is at least 1 log lower than in the lymph nodes (26). Electron microscopy (EM) analysis has demonstrated large amounts of opsonised virus (coated with antibody) in the lymph node at an early stage of infection. This virus may be responsible for some infection with the coating antibody allowing infection of CD4- cells (27).

It is possible that the low amount of virus in the peripheral blood is due to the efficient trapping of viral particles in the processes of the FDC (13). At later points in the disease when the CD4 count has fallen, plasma viremia is again detectable. EM studies of lymph nodes at this point reveal marked disruption of the germinal centres and necrosis of the FDC processes. The reasons for this FDC destruction remain unclear as FDC are rarely infected by HIV. It is likely that the loss of ability to trap virus contributes to the viremia seen at this stage. However CD4+ T cells are still being infected in the lymph nodes at this stage (13).
Bone marrow derived dendritic cells (DC) are also thought to play an important role in HIV-1 infection of CD4+ T cells. These cells carry virus into proximity with uninfected T cells and therefore amplify the cytopathic effect of a small amount of virus (28). After exposure to HIV-1 in vitro, DC continue to present antigen, forming clusters with T cells which are induced to divide making them particularly susceptible to HIV-1 infection. In this setting infection of DC cannot be detected but the clustered T cells form syncytia, release virus and die. This could lead to the loss of HIV-1 specific T cell clones in infected individuals.

11.2.2. The cellular targets of HIV-1 infection: the role of CD4+ T lymphocytes.

The cellular receptor for HIV-1 which allows the virus to enter cells is the CD4 differentiation antigen, expressed principally by T cells and cells of the monocyte/macrophage lineage (6, 7). Consequently these cells constitute the major target of HIV-1 infection. Other cell types such as epithelial and endothelial cells as well as neuronally associated cells such as astroglial cells, which are CD4 negative, have been shown to be infected by HIV-1 in vitro but their role in vivo remains controversial (12, 29). The control of HIV-1 gene expression by cytokines and related signalling molecules in T cells and cells of the monocyte/macrophage lineage is schematically summarised in Figure 1.2.

Resting T cells remain in G0 until stimulated by appropriate antigen although HIV-1 can bind CD4 and enter the cytoplasm of resting T cells, efficient viral DNA synthesis and integration require cellular activation (30-32). Investigation of the peripheral blood of asymptomatic individuals has shown that HIV-1 is found predominantly as full length unintegrated complementary DNA (33). This is probably due to the infection of quiescent T cells in G0, as activation of these cells in vitro can lead to integration of the viral DNA. Infected quiescent T cells may therefore represent a major inducible reservoir in infected individuals. Infection of activated, permissive T cells leads to replication and virion production within 24 hours (34).

The depletion of CD4+ T helper lymphocytes has been directly related to HIV-1 viral load by several groups (35-37). There are several postulated mechanisms whereby HIV-1 can kill CD4+ T cells both directly and indirectly. Direct mechanisms include the formation of pores in the cell membrane as a result of viral budding, or membrane insertion of env proteins which can disrupt membrane ion fluxes.

Infected cells expressing gp120 on their surface can also bind to CD4+ cells via gp120-CD4 interactions and fuse, to form large multi-nucleated syncytia. Syncytium formation probably leads to significant CD4+ cell death in vivo (6, 7). Syncytia die quickly in culture and would be expected to die even faster in the blood.
Figure 1.2. A simplified schematic summary of the control of HIV-1 gene expression by cytokines and related signalling molecules. Cytokines with ill-defined mechanisms of action are not shown e.g. IL-3, M-CSF and GM-CSF. For cytokines which exert opposite effects on HIV-1 replication only the best defined mechanism is shown. See (13). Adapted from (13). See text for details.
By incorporating non-infected cells into a syncytium, one gp120 expressing cell can eliminate many uninfected CD4+ cells. Another hypothesis which may explain CD4+ T cell killing is the induction of apoptosis. In HIV-1 infection CD4 may become crosslinked as a result of binding either gp120 or gp120-anti gp120 antibody complexes, which can cause the cell to apoptose following antigenic stimulation of the T cell receptor (TCR) (27). Intracellular CD4-gp120 complexes have been noted blocking nuclear pores. This and the accumulation of retroviral DNA in the cytoplasm may also lead to cell death by apoptosis. Several studies have supported the role of apoptosis in CD4+ cell depletion (38, 39).

Membrane associated viral proteins can also mark the cell for killing by cytotoxic T cells, the normal method of destroying virally infected cells by cell-mediated immunity. Furthermore CD4+ T cells may process HIV-1 proteins, especially gag derived antigens, and following antigen presentation, become targets for killing. Antibody dependent complement mediated cytotoxicity will also kill HIV-1 infected cells.

A puzzling aspect of HIV-1 infection is the lack of CD4+ T cell regeneration, either early in the disease or following antiretroviral therapy. This is probably partly due to the infection and killing of T cell precursors, recently demonstrated for both bone marrow and intrathymic T cell precursors (40, 41). However mechanisms of T cell homeostasis have also been suspected of playing a role, reviewed in (42).

Although HIV-1 infection is associated with a slow reduction in the amount of circulating CD4+ T cells, functional abnormalities have been noted at all stages of infection (43). Abnormal T cell responses to soluble antigen and defective T cell cloning are observed early in the course of HIV-1 infection. In addition it has been shown that in vitro exposure of CD4+ cells to specific HIV-1 proteins (gp120, gp41, gp24) in the absence of infection is sufficient to inhibit antigen specific responses. HIV-1 env proteins may block the interaction between CD4 and major histocompatibility complex (MHC) that occurs during antigen presentation and disrupt the normal post-receptor signalling pathways.

### 1.1.2.3. The control of HIV-1 expression in T cells.

The infection of resting CD4+ T lymphocytes has been shown to result in incomplete reverse transcription and abortive infection unless cell activation occurs within a few days of infection (31). However it is also likely that some T cells harbour genuinely latent HIV-1 proviruses which do not express mRNA. In vivo, latently infected PBL can be detected which carry complete integrated provirus (44, 45) which are probably memory cells which were infected while activated and became quiescent before virus replication could be completed (27). Consequently the
mechanisms whereby antigens and cytokines stimulate cellular activation and HIV-1 production by T cells are important factors in HIV-1 pathogenesis. Cytokines are peptide hormones which mediate inflammatory and immune reactions as well control the homeostasis of the immune system.

The first reports on the control of HIV-1 replication were produced by workers attempting to isolate the causative agent of AIDS. These workers utilised the mitogen phytohaemagglutinin (PHA) and the cytokine IL-2 to maximise the levels of cellular activation, seen to be conducive to the maximal virus production from infected lymphocytes (46, 47). In vivo the critical stimulus for T cell activation is provided by contact between antigen, presented by antigen presenting cell (APC), and the T cell antigen receptor on the surface of the T cell. Effective contact requires presentation of small fragments of antigen within a pocket of the major histocompatibility complex (MHC), on the antigen presenting cell surface (reviewed in (48) ). Cell surface adhesion molecules on the T cell also play an important role in this process. A second stimulation required to fully activate T cells is provided by the cytokines secreted by macrophages such as IL-1 and/or IL-6. These signals can be mimicked by phorbol esters, which activate protein kinase C, and anti-CD3 antibodies. The activation of T cells causes the induction of over 100 genes over a 2-3 week process. Among these are members of the family of inducible transcription factors, NF-κB. These proteins are able to directly activate the expression of HIV-1 gene expression. The activation of HIV-1 by NF-κB will be discussed in a later section.

It was also noted that the addition of anti-interferon α antibodies to cocultures of HIV-1 infected peripheral blood mononuclear cells (PBMC) and PHA stimulated allogeneic PBMC enhanced levels of virus production (46). TNF α was found to be largely responsible for the stimulation obtained using supernatants from PHA stimulated T cells or bacterial lipopolysaccharide (LPS) stimulated macrophages (49, 50). TNF α, also known as cachectin, and TNF β, also known as lymphotoxin, mediate their effects via interaction with cellular TNF receptors p55 and p75. TNF has been shown to upregulate HIV-1 expression in an autocrine/paracrine manner in infected primary T cells (51) as well as in an infected T cell line following stimulation with the phorbol ester PMA (52). Interferon γ (IFN γ), a lymphokine secreted by activated T lymphocytes, may also upregulate viral expression in an autocrine manner (51). Transforming Growth Factor β, a pleiotropic cytokine present in several isoforms has been reported to upregulate viral replication when added to infected primary T cell cultures (53).

In contrast to these stimulatory effects cytokines are also able to inhibit viral replication in infected T cells. Addition of IFN α to CEM T cells has been shown to
block an early event following viral entry, therefore preventing reverse transcription of the viral RNA and consequently productive HIV-1 infection (54). Interferon α has also been shown to inhibit the release of viral particles from the cell membrane of chronically infected T cell lines (55).

In summary the infection of a non dividing T cell will result in a form of latent infection that will persist for 2-3 days or until stimulation of the infected cell leads to permissive infection, virus production and cell death. The stimulation of T cells by antigen or cytokines such as TNF α/β, TGF β, or IFN γ is therefore an important control point of HIV-1 pathogenesis.

1.1.2.4 The role of B cells.

B cells are the immune cells responsible for the production of antibodies, and although B cells are not direct targets for HIV-1 infection, HIV-1 infected individuals exhibit severe abnormalities in B cell function. Some of these defects are secondary to T cell deficiency, whilst others are T cell independent. The majority of AIDS patients exhibit polyclonal B-cell activation with spontaneous B-cell proliferation, increased immunoglobulin secretion, and hypergammaglobulinaemia, suggesting chronic activation (56). A substantial proportion of these activated B cells are specific for HIV-1 antigens, particularly envelope antigens (57). Epstein Barr Virus (EBV) has been shown to play a major role in the high frequency of B cell lymphoma seen in AIDS patients (58).

In addition to spontaneous B cell hyperactivity, B cells from infected patients exhibit an intrinsic defect in antigen and mitogen induced responses at all stages of infection. B cells are regulated by cytokines such as IL-2, IL-4, IL-6, TGF β and TNF α and β, some of which operate in an autocrine manner (reviewed in (59)). Constitutive secretion of TNF α and IL-6, at levels capable of inducing virus replication in infected T cells and monocyctic cells, has been shown to occur in B cells from HIV-1 infected patients (60). This indicates that B cells may play a role in viral induction in vivo. Additionally B cells from infected patients can be stimulated to secrete cytokines by exposure to recombinant viral envelope gp120 (61). Recently this has been repeated in B cells from uninfected individuals with co-stimulation with IL-4 (62).

These findings support the hypothesis that, as discussed earlier, a fundamental factor in HIV-1 pathogenesis is the activation and accumulation of virus expression in the paracortical region of the germinal centres of lymph nodes where macrophages, CD4+ and CD8+ T cells, B cells and virus come into close cell to cell contact. In this environment secretion of stimulatory cytokines such as TNF α/β and IL-6 by B cells would contribute to the high levels of viral activity seen in lymphoid tissues.
1.1.2.5. The role of cells of the monocyte/macrophage lineage.

Cells of the monocyte/macrophage lineage also express the CD4 receptor on their surface (63, 64). However monocytes are not very infectable by HIV-1, with differentiation to macrophages being a requirement for efficient infection (65, 66). The reasons for this are unclear especially as the differentiation from monocytes to macrophages is associated with a reduction in levels of CD4 expression (67). Infection of macrophages appears to be persistent and poorly cytopathic with virus particles being found predominantly in cytoplasmic vacuoles which may shelter the virus from immune attack. Viral isolates have been discovered in the CNS with specific monocyte/macrophage tropism, found to be determined by the V3 loop region of the env glycoprotein. The role of circulating macrophages in HIV-1 pathogenesis is largely unknown although infected macrophages have been detected in specific tissues such as the lungs and the brain. This may mean that these cells play an instrumental role in the neuropathogenesis of HIV-1 infection by secretion of neurotoxic cytokines such as TNF α or by more direct cytopathic mechanisms (17, 68). At the very least sequestration of HIV-1 in tissue macrophages implies that these cells could function as a reservoir of virus infection as seen in other lentivirus infections in animals, such as visna-maedi virus and equine infectious anaemia virus. In HIV-1 infection infected tissue macrophages encountering activated T cells, particularly in the lymph nodes, could be responsible for the spread of HIV-1 in the host (12).

Important differences exist in the ability of HIV-1 to productively infect and replicate in T cells and macrophages. Cellular activation is thought to be required for the completion of reverse transcription and integration in T cells (31) whereas activation does not seem to be required in macrophages (69). Infection of macrophages, at least in vitro, is characterised by delayed and prolonged production of virus (70, 71). This may be due in part to the lower cytopathic effect in macrophages mentioned above. Furthermore, virion production occurs in intracellular golgi derived compartments, as well as at the plasma membrane, in macrophages infected in vitro (70, 71) and in vivo (72).

1.1.2.6. The modulation of HIV-1 replication in cells of the monocyte/macrophage lineage.

As in B cells, interaction of HIV-1 viral envelope gp120 with CD4 on the macrophage cell surface results in the activation of cellular genes, including TNF α and IL-1 as does activation of macrophages by HIV-1 antigens (73). Increased levels
of TNF α, IL-1 and IL-6 have been observed in macrophages obtained from HIV-1 infected individuals (74, 75). As HIV-1 gene expression is induced by TNF α, IL-1 and IL-6, this probably constitutes an important autocrine mechanism for upregulation of HIV-1 replication. Autocrine/paracrine upregulation of HIV-1 replication has been demonstrated in acutely infected U937 human promonocytic cells, where the effect was mediated by membrane bound and not secreted TNF α (76). It has also been recently demonstrated that TNF α itself can induce a TNF α mediated loop of HIV-1 expression in CD4+ chronically infected promyelocytic HL60 derived cells (77).

Neutralising-antibodies to IFN α have been shown to induce HIV-1 replication in acutely infected U937 cells, (78) indicating a negative regulatory role for this cytokine. Addition of TNF α/β or IFN α to primary macrophages or macrophage lines has confirmed the opposite roles of these cytokines in HIV-1 infection (66, 79, 80). IFN α can also potently suppress HIV-1 replication in primary monocyte derived macrophages (MDM) by affecting early steps in the HIV-1 life cycle (80). Interestingly the effects of cytokines can be dichotomous depending on the time of treatment. IFN γ has been shown to induce or inhibit HIV-1 replication when added prior to infection or after infection respectively (81). Similar results have been obtained in monocyte derived macrophages (MDM) treated with TGF β (82). HIV-1 replication has also been shown to be upregulated (66, 83, 84) or inhibited (66) by IL-4.

In addition to its dichotomous effects on virus replication IFN γ has been shown to directly stimulate HIV-1 gene expression in the chronically infected U1 cell line (85). It has also been shown to redirect the preferential site of maturation and release of virions from the plasma membrane to intra-cytoplasmic golgi derived compartments of PMA stimulated U1 cells (85). Under the same culture conditions IFN α blocked the release of mature virions from the plasma membrane of PMA stimulated U1 cells, as previously demonstrated in chronically infected T cell lines (55).

IL-6 has been demonstrated to upregulate HIV-1 expression in the chronically infected U937 derived U1 cell line by a transcription independent mechanism (79). Addition of IL-6 to U1 cells resulted in a stimulation of the synthesis of HIV-1 proteins without a concomitant increase in viral mRNA synthesis. Furthermore no increased levels of viral transcription were seen in U1 cells stimulated with IFN γ although this cytokine produced a significant increase in the production of HIV-1 mRNA. Both IL-6 and IFN γ were able to synergise with the increase in HIV-1 transcription induced by TNF α (79). These results demonstrate the ability of
different cytokines to stimulate HIV-1 replication in a synergistic manner via the ability to act at different stages of the viral life cycle.
1.2. The control of gene expression by the NF-κB and retinoic acid receptor families of transcription factors.

This thesis presents data concerned with the control of gene expression. The next section will therefore outline the mechanisms of the control of transcription. Transcription is the process by which the information contained in DNA is transcribed and processed into messenger RNA (mRNA). This enables the information to be translocated to the cytoplasm in the form of mRNA where it can be used to synthesise protein. The molecular biology of the NF-κB family of transcription factors will then be discussed followed by an outline of the transcriptional control of HIV-1 gene expression by cellular and viral factors. Finally the action of retinoic acid and its metabolites as regulators of transcription will be outlined.

1.2.1. Control of initiation and activation of transcription.

The various protein factors involved in the transcription of eukaryotic genes by RNA polymerase II (Pol II) can be classified into at least three groups. The first group, the general transcription factors, are those which are required for accurate transcription initiation and include, in addition to Pol II, TFIIA, TFIIB, TFIID, TFIIE, TFIIF, TFIIF, TFIIH and TFIJ. The various general transcription factors assemble in a stepwise fashion to form a pre-initiation complex on the promoter. A schematic model for the assembly of this complex has recently been described based on data obtained from kinetic assays, native gel electrophoresis and nuclease protection assays, reviewed in (86-88) (Figure 1.3.). This complex is capable of basal levels of transcription following an ATP dependent activation step.

The mechanism of action of RNA polymerase II is not well understood. The mammalian RNA Pol II consists of a number of subunits, the genes encoding some of which, have not yet been isolated. The genes encoding the two largest subunits have been cloned and include at least part of the catalytic site. The largest subunit contains a repetitive C-terminal domain (CTD) which may interact with the committed initiation complex via TFIIID (89). Hyperphosphorylation of the CTD blocks the incorporation of Pol II into the initiation complex (90, 91). TFIIF consists of two subunits, the smaller of which is also responsible for binding to Pol II, possibly via TFIIB. TFIIF probably complexes with Pol II prior to complexing with the initiation complex (92). TFIIF also affects transcript elongation by Pol II, requiring both subunits for this (93). The importance of this role is not clear.
Figure 1.3. A schematic model of transcription initiation complex assembly adapted from . An initial committed complex is formed by the binding of TFIID to the TATA box. This complex acts as a binding site for TFIIB which recruits RNA polymerase II and TFIIF into the complex. TFIIE and TFIIH are then able to bind the complex and transcription begins following an ATP dependent activation step.
To initiate transcription, the DNA near the initiation site must come apart to form a so-called open initiation complex. The minimal requirement for initiation of transcription is therefore the formation of such a complex. Which factors are minimally required for this is unclear at present, with some data suggesting that negative supercoiling of the template DNA can substitute for certain factors (94), whilst other data suggest that open complex formation requires a complete initiation complex and ATP hydrolysis (95).

The TFIIE complex is thought to consist of 4 subunits encoded by two genes with two of each subunit forming the complex. The function of TFIIE established thus far is the recruitment of TFIIH. TFIIH is also a multi-subunit complex possessing a kinase activity capable of phosphorylating the Pol II CTD as well as DNA-dependent ATPase and helicase activities. Transcription has been shown to require ATP hydrolysis (96) with the energy requirement being termed the activation of the basal initiation complex. As this energy is not required for TFIIE/TFIIH-independent transcription and TFIIH exhibits ATPase activity it is likely that this activation is mediated through this factor. Also ATP causes a conformational change in the initiation complex that correlates with dissociation of TFIIE or TFIIH or both from the initiation complex (97). The role of TFIIE and TFIIH may be the conversion of the initiation complex to an elongation complex able to transcribe long mRNA transcripts. This conversion has been termed promoter clearance.

For higher levels of gene expression further transcription factors are required to bind their upstream DNA binding sites and activate transcription, reviewed in (98). These proteins constitute the second group of transcription factors. Eukaryotic genes have upstream sequences called enhancers which contain binding sites for an array of transcription factors. These factors generally contain separate domains for DNA binding and transcriptional activation. A large body of evidence has accumulated to indicate that many activators stimulate transcription in vitro by increasing the proportion of DNA templates on which a functional pre-initiation complex is assembled. An activator could promote complex formation by increasing the equilibrium association constant for one or more of the assembly steps, thereby shifting the overall equilibrium in the forward direction. These effects could be mediated via the direct interactions which have been demonstrated between activating transcription factors and general transcription factors. The DNA between the sites has been proposed to loop out, allowing contact between proteins bound to sites on DNA large distances apart (reviewed in (99) ). In this way the binding of transcription factors to upstream sites leads to an increase in expression of the gene. Much work is concerned with the search for evidence of interactions between upstream transcription factors and the basal transcriptional machinery and much data has been produced.

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concerning interactions between upstream transcription factors and TFIID (reviewed by Greenblatt (100)).

The third group of factors comprises the coactivator proteins. These proteins are functionally described as factors that are required for activator function but do not affect activator-independent basal transcription. The coactivator proteins that have been best characterised are those present in the TFIID complex. It has been demonstrated that a single TFIID component, TATA binding protein (TBP), is sufficient for TATA element recognition, and subsequent basal transcription. However, sequence specific transcriptional activators can only activate transcription in vitro in the presence of partially purified TFIID, not with purified TBP (101). This is due to the fact that TFIID consists of TBP and at least eight TBP associated factors (TAFs) (reviewed in (102)). Many transcription factors interact with the pre-initiation complex via TAFs which interact directly with TBP. This may allow inducible activators to recruit basal transcription factors to the initiation complex, thus overcoming rate limiting steps of initiation of mRNA synthesis - the so-called coactivator hypothesis (101, 103). A three way interaction between an activator, herpes simplex VP16, a TAF, TAFII40 and general transcription factor TFIIB has recently been demonstrated (104). Furthermore, antibodies which disrupt this interaction inhibit transcriptional activation without affecting basal transcription (104). The use of a cell line expressing a temperature-sensitive TAF mutant has also confirmed the importance of TAF complexes in mediating activation (105).

Other components of the basal transcription complex such as TFIIA, TFIIB, TFIIE, TFIIF and TFIIH are also potential regulatory targets for activators. The VP16 activation domain shown to interact with TBP also interacts with TFIIB and recruits it to the initiation complex to activate transcription (106). Recently data have been produced demonstrating a conformational change in TFIIB induced by VP16 interaction such that binding sites for TFIIF and Pol II are exposed (107). An acidic activator can therefore affect initiation complex assembly both quantitatively, by recruiting TFIIB, and qualitatively by altering its conformation in such a way that initiation complex formation is driven forward (107).

It has been suggested that some transcriptional activation domains may assume the 3-dimensional conformation required for activation only upon interacting with a second factor or upon binding DNA (108). This would prevent non-productive interactions off DNA and provide further means for both specificity and flexibility in transcriptional control. The conformation assumed by the transcription factor NF-κB upon DNA binding has been shown to affect the level of transcriptional activation from different NF-κB binding DNA sequences. The various NF-κB sites cause different levels of conformational change. Consequently different levels of
transactivation are seen from these elements when they are linked to the same promoter (109).

The overall potency of a transcriptional activator is determined by several factors: the affinity of the factor for its site and for any other factors it must interact with to activate transcription, as well as the affinity of the interaction between the activation domain and its target in the initiation complex. Different types of activation domain have been classified including acidic and either glutamine or proline rich (reviewed in (98) ). The structural relationships and mechanisms of specificity of these different activation domains remain obscure. Mutational studies demonstrate that, for example, residues outside the glutamine rich sequence are most important for activation (110, 111). Furthermore, it has been demonstrated that different activation domains within a given class may not interact with the same target (111). This indicates different subtypes within the presently defined types of transcriptional activators.

The simple polymeric units commonly associated with activation domains are thought to make initial contact with multiple targets via weak interactions during different steps of the initiation complex formation. This will lead to a stable overall interaction (108). It should be noted that the expression of a gene is not generally controlled by the action of a single upstream transcription factor and that often the formation of 3-dimensional stereo-specific complexes are required. This has been demonstrated for the mouse T cell receptor α chain gene (112). The minimal enhancer was shown to contain the sites for at least three transcription factors, all of which are required for enhancer function.

In summary it seems that activators probably contact several distinct components within the basal initiation complex and that no single component is the specific target. These interactions between upstream activators and factors present in the basal initiation complex lead to the recruitment of the required factors and probably to conformational changes which drive forward assembly of the open initiation complex. Many of the studies carried out to date involve the use of in vitro systems, whose relevance in vivo remains to be seen. In addition, protein interactions are detected using assays which are much less effective at detecting the multiple weak interactions which are important in vivo (108).

1.2.2. Synergy in transcriptional activation.

Synergistic activation of gene expression by transcriptional mechanisms is common. The upstream regulatory regions of eukaryotic genes contain binding sites for several different activators and/or repressors. Synergism refers to a greater than additive increase in transcription when two or more activators are present.
simultaneously or when additional binding sites for an activator are introduced. For examples see (113-116). The fact that two factors can synergise demonstrates that they are acting in the same pathway or in convergent pathways. If they were acting independently, the maximum level of activation they could produce would be the sum of their two activations (117). For example, the fact that AP-1 and NFAT can synergise in the activation of interleukin-2 expression (118) means that they can both facilitate the stimulation of the same promoter element at the same time.

Several mechanisms have been proposed to explain the phenomenon of transcriptional synergy. The first and simplest explanation is that the synergy between two factors is due to their cooperative binding to DNA. This mechanism has been proposed to lead to transcriptional synergism in both yeast and mammalian cells (119-121). The second mechanism is distinguished from the first in that it can occur under conditions where the transcription factors are at saturating concentrations with respect to their binding sites. Cooperative binding cannot account for the synergy when the binding sites are saturated. This type of synergy has been described between multiple binding sites for the yeast transcription factor Gal4 (116) and between Gal4 and the mammalian activator ATF (115). It is postulated that this synergy is a consequence of interaction of each of the bound activators with some component of the transcriptional machinery rather than with each other. Gal4 and ATF have both been shown to interact with TFIID (122, 123) making this a potential target for this synergy. Furthermore, synergy has been demonstrated in a yeast in vitro transcription system between VP16 activation domains attached to a single Gal4 DNA binding domain (124).

Hershlag and Johnson have reviewed transcriptional synergy from a kinetic point of view (117). Essentially these authors discuss several hypothetical models which could provide mechanisms of synergy given that the process of transcription contains multiple steps, any of which could be regulated kinetically by transcriptional activators. These models thus provide hypothetical mechanisms by which transcription factors could synergise without contacting each other at any point. This means that not only could the transcription factors simultaneously contact different parts of the transcriptional machinery, but that they might be used at different times in the transcriptional initiation pathway. Distinguishing between physical synergism, e.g. cooperative binding to DNA, and kinetic synergism, e.g. lowering the kinetic energy of recruitment of two initiation factors to the initiation complex, is often not possible experimentally. The separation of these mechanisms will require careful dissection and analysis of the individual steps in transcriptional activation and initiation.
1.2.3. The NF-κB family of inducible transcription factors.

NF-κB is a pleiotropic inducible transcription factor with a central role in control of eukaryotic gene transcription including the control of the expression of human immunodeficiency virus type 1. NF-κB was first identified in 1986 as an apparently tissue specific factor which activated the Ig κ light chain intron enhancer during B lymphocyte development (125). Since then it has become increasingly apparent that NF-κB is widely expressed and is at the centre of a number of important cell signalling pathways. The versatility of the NF-κB system is evident in the large number of agents able to upregulate gene expression via NF-κB.

Signals from many parts of the cell are able to upregulate NF-κB. Cell surface receptors for cytokines such as interleukin-1 (IL-1) (126), IL-2 (127), tumour necrosis factors α and β (128, 129), lectins and bacterial lipopolysaccharide (LPS) activate NF-κB (130) as do factors activating second messengers at the cell membrane such as phorbol esters (125) or calcium ionophores (131). NF-κB is also activated by ds RNA (132), inhibition of protein synthesis (125) and by intranuclear proteins such as the Tax transactivator of the HTLV virus (133). NF-κB is also activated by oxidative stress (134). A list of the known NF-κB inducing agents is given in (135).

1.2.3.1. The role of NF-κB in the control of HIV-1 gene transcription.

The role of NF-κB in controlling HIV-1 transcription was first demonstrated in 1987 by Nabel and Baltimore (136). These workers linked the transcriptional activation of the HIV-1 promoter with the activation of T cells and the induction of NF-κB DNA binding activity. Mutational analysis demonstrated the NF-κB binding site to be a direct repeat sequence with homology to the Ig κ gene binding site. Since then the model whereby the intracellular environment of activated T cells leads to activation of the provirus, via NF-κB binding the LTR, has become central to the understanding of the control of HIV-1 transcription. The activation of HIV-1 gene expression then leads to viral replication, resulting in T cell death and eventual immune deficiency, characterising the disease AIDS. The central role of NF-κB in this process has ensured an unprecedented volume of research into its action and a much increased understanding of its role in immune function. NF-κB has also been shown to be responsible for the activity of the virus in cells of the monocyte/macrophage lineage (137, 138) which may act as reservoirs of productive infection (63, 64).
1.2.3.2. NF-\(\kappa\)B proteins contain homology to the v-rel oncogene.

NF-\(\kappa\)B constitutes a family of transcription factors able to homo and heterodimerise and modulate gene transcription. The various members of the NF-\(\kappa\)B family are shown in Figure 1.4. The similar DNA binding specificity and the ability of the NF-\(\kappa\)B proteins to homo and heterodimerise is due to a 300 amino acid long homology sequence. This homology region had previously been noted to be shared by the v-rel oncogene product from the avian retrovirus Rev-T and the \textit{Drosophila} maternal effect gene, dorsal. This region was called the NF-\(\kappa\)B/rel/dorsal or NRD domain and is recognised as the minimal requirement for DNA binding and dimerisation of the NF-\(\kappa\)B proteins, reviewed in (139, 140). The N terminal half of the NRD is involved in contacting the DNA (141) and the C terminal half is required for NRD/NRD domain interactions (142).

1.2.3.3. The NF-\(\kappa\)B p50 and p49 subunits are synthesised from large precursor proteins. The p65 subunit is not.

The cDNA for p50 encodes a 105 kD protein, p105, containing an N terminal NRD. The full length protein is unable to bind DNA and requires proteolytic processing to a mature form. The mature p50 comprises the N terminal half of p105 including the NRD. The C terminal portion of p105 contains yet another previously discovered sequence motif which had been noted in proteins involved in cell cycle control and cell architecture, reviewed in (139). This sequence motif with a length of 30-33 amino acids is called an ankyrin repeat, ANK. It is seen eight times in the p105 sequence. When p105 is proteolytically cleaved the ANK containing C terminal domain is removed to form a protein which can bind p50 and act as an inhibitor of p50 action. This will be discussed below. The protease responsible for this processing is thought to be a ubiquitin-dependent enzyme (143). The natural cleavage site of p105 is close to a glycine rich region which may serve as a hinge.

The mRNA for p105 is present in many tissues and can be upregulated by many of the same signals that activate NF-\(\kappa\)B activity (144, 145). This is largely due to the NF-\(\kappa\)B site in the p105 enhancer (146). Phorbol esters, which stimulate protein kinase C by virtue of their structural similarity with diacyl glycerol, or TNF \(\alpha\) strongly induce p105 mRNA in HL60 cells. In human peripheral blood lymphocytes stimuli such as the phorbol ester phorbol-12-myristate acetate (PMA) or the lectin phytohaemagglutinin (PHA) and calcium ionophore also elevate the p105 mRNA, (144). The immunosuppressive agent cyclosporin A strongly inhibits induction by calcium ionophore but only has a modest effect on induction by PMA or PMA/PHA.
Figure 1.4. A schematic summary of the structure of the primary members of the NF-κB family of transcription factors. The rel homology and ankyrin repeat regions are shown. See text for details. Adapted from (135).
Thus the prolonged activation of NF-κB, such as that seen in macrophage differentiation, may be due in part to increased p105 mRNA synthesis.

A third NF-κB subunit has recently been cloned which is very similar to p50 and hence has been called p49, p50B or NF-κB2 (147, 148). Like p50, p49 has a longer inactive precursor protein, p100, containing an N terminal NRD domain and a C terminal ankyrin repeat domain. The amino acid sequence homology between p100 and p105 is 60% in the NRD but then drops off to give a mean homology of 40% (147). As with p105, p100 is cleaved to give an active p49 which can heterodimerise with p65 and other rel proteins to bind DNA.

The p65 cDNA encodes a 549 amino acid protein and does not require proteolytic cleavage to an active form. The p65 mRNA is not significantly increased by mitogenic stimuli and is most closely related to the cellular version of the Rev-T oncogene v-rel (149, 150). Another subunit highly related to c-rel, rel-B has recently been identified (151).

1.2.3.4. Transcriptional activation by NF-κB.

The capability of the NF-κB proteins to activate transcription was tested by overexpression in established cell lines and assay of a reporter gene whose expression was controlled by NF-κB binding sites. In COS cells the separate expression of p65 and p50 led to detection of the appropriate homodimers binding their DNA elements in a band shift assay (152). In transactivation assays however, whilst p65 was able to activate reporter expression in several cell lines with several promoters (152-154), p50 was not (155), despite the fact that it could bind DNA. p49 also was not effective alone unless fused to the activation domain from the herpes virus protein, VP16 (156). Also when p50 or p65 sequences were linked to a Gal4 DNA binding domain only the p65 constructs could activate a Gal4-reporter construct (152, 153). These workers identified the p65 C terminal 30 amino acids as comprising a strong and independent activation domain making p50/p65 heterodimers strong transcriptional activators (157). The NF-κB c-rel and rel B subunits also contain putative activation domains (151, 158, 159). Coexpression of p50 and p65 subunits in Jurkat T cells led to a synergistic activation of NF-κB site-enhanced reporter genes (149, 154). p49 is also able to heterodimerise with p65, and p49/p65 heterodimers have been shown to be stronger activators than p50/p65 heterodimers with certain NF-κB elements (156).

The role of p50 in transcription remains in some dispute. p50 has not clearly been shown to activate transcription in eukaryotic cells but has been shown to be active in yeast (160) and in cell-free assays (109, 161). If p50 homodimers are unable to activate transcription, what is their function? Their main function may be to
suppress p50/p65 action, (152, 162) by binding DNA and blocking p50/p65 binding. Alternatively p50 could be seen as a helper subunit allowing the p50/p65 heterodimer to bind with high affinity to sites with low symmetry. As discussed below p50 activity is also closely involved with the activity of the NF-κB inhibitory proteins, I-κBαs.

1.2.3.5. The action of NF-κB is controlled by ankyrin repeat containing protein inhibitors - the I-κB family.

When p50 and p65 were overexpressed in cells both proteins were detectable in the nucleus (163-165). p50 was exclusively nuclear (163, 164) whilst p65 was at low nuclear concentrations and higher cytoplasmic concentrations. Higher expression of p65 however, led to its appearance in greater amounts in the nucleus (166), as if the p65 was sequestered in the cytoplasm until the sequestering factor was titrated out. Subsequently NF-κB was found to be bound to an inhibitor protein in the cytoplasm of non-activated cells by dissociation of the inhibitor NF-κB complex with detergents (167). This inhibitor protein was called I-κB. The activation of NF-κB by phorbol ester leads to a reduction of levels of NF-κB in the cytoplasm and a corresponding increase in the nuclear NF-κB binding activity (168). These data led to the current hypothesis that NF-κB remains complexed to the inhibitor, I-κB, in the cytoplasm until activated, when it is released and translocated to the nucleus. Biochemical analysis of I-κB has revealed that it exists in two isoforms, I-κBα, a 35-37 kD form (169) and a lesser form I-κBβ, 43 kD (170). I-κBα now appears to be equivalent to a previously cloned protein MAD3 (171). A further I-κB, pp40, has been cloned from chicken cells and found to be associated with the V-rel oncogene from the avian Rev-T virus mentioned above (172). This protein is immunologically related to I-κBβ (173). I-κBα and β bind and inhibit the action of p65 but not p50 (165, 171, 174, 175) and I-κBβ can bind c-rel and inhibit its binding to DNA (173).

As mentioned previously the p50 precursor protein is cleaved into two proteins, p50 and a C terminal ankyrin repeat containing protein, that can act as an inhibitor of p50 action. This protein is another I-κB protein, named I-κBγ. Consequently the synthesis of p50 is directly linked to the production of an inhibitor of p50 DNA binding activity. I-κBγ has also been identified as an independent entity, synthesised from a differentially spliced mRNA found mainly in lymphoid cells (176, 177). I-κBγ preferentially inhibits both DNA binding and nuclear localisation of p50. Another ankyrin repeat containing protein, the oncogene bcl 3, has similar activity, inhibiting p50 DNA binding (178, 179) and activating transcription as a consequence of inhibiting the negative effects of p50 homodimers (180). bcl 3 only inefficiently inhibits the activity of p65 and rel proteins (181) and forms a ternary complex on DNA with p49 (182).
1.2.3.6. The control of I-κB action.

The inhibition of NF-κB DNA binding by ankyrin repeat containing proteins, I-κBs, is now clear. The actual mechanisms of the release of NF-κB from I-κB however, are less well understood. As the p50/p65 purified from an activated nuclear preparation is indistinguishable from that purified from a cytoplasmic preparation it was assumed that the biochemical event activating NF-κB involved I-κB rather than p50/p65 (167), although transient modification of NF-κB could not be ruled out. Treatment of cytosolic extracts with a variety of protein kinases was shown to release NF-κB from I-κB (169, 183). Also I-κB which had been phosphorylated by PKC or HRI kinases was unable to inhibit NF-κB activity whereas untreated I-κB was (169). Thus it seems that NF-κB can be activated by phosphorylation of I-κB but further data illustrate the increasing complexity of this regulation. For example, although protein kinase A (PKA) treatment of cytoplasmic extracts will activate NF-κB, treatment of purified I-κB will neither phosphorylate it nor render it inactive. In addition to modification of I-κB, the DNA binding subunits can be modified such that they release I-κB. Human c-rel and p105 have been reported to be tyrosine phosphorylated on activation of Jurkat T cells with PMA/PHA (184). Also an IL-1 and LPS inducible NF-κB binding phosphoprotein has been described (185) which is associated with Ser/Thr kinase activity. Dephosphorylation of I-κB has been reported to activate NF-κB binding in cell free experiments with I-κB β (186). In summary there seem to be several mechanisms to activate NF-κB via I-κB and some of them at least involve phosphorylation and de-phosphorylation of I-κB or the DNA binding subunits themselves. I-κB proteins can also reduce the half life of NF-κB-DNA complexes (170), and so I-κB does not simply cover the DNA binding domain and must have some effect on NF-κB DNA interactions. Indeed I-κB is seen in the nucleus so newly formed I-κB may be able to terminate NF-κB dependent transcription in the nucleus.

I-κB controls NF-κB nuclear localisation. When I-κB α is overexpressed with p65 it can prevent the nuclear uptake of p65 (165, 166). Conversely p65 prevented nuclear localisation of I-κB α. Data supporting the hypothesis that I-κB covers the NF-κB nuclear localisation signal (NLS) has been derived with the use of antibodies to the NF-κB NLS. Recombinant I-κB α could prevent the precipitation of p65 by anti-p65-NLS (187). I-κB α could only prevent the precipitation by anti-p50NLS of p50/p65 heterodimers and not p50 alone. This suggests that with p50/65, I-κB α can bind the heterodimer and may mask both NLS sequences, thus preventing nuclear localisation of p50/p65. Data consistent with I-κB covering the NLS have come from Beg et al (165). These workers have shown that addition of an SV-40 NLS to the N-
terminus of p65 can override the inhibition, by I-κB, of nuclear uptake and that mutation of the p65 NLS abolishes I-κB binding. The situation is similar with p50 and I-κB γ. Experimentally the NLS of p50 is sensitive to protease unless bound to I-κB γ, and to a lesser extent I-κB α. Removal of the NLS by digestion generates a protein that can bind DNA and is not inhibited by I-κB γ. However it is unlikely that the NLS is the only point of contact between the I-κBs and NF-κB as a highly transforming p65 variant has been described which will not bind I-κB α but contains an intact NLS (165). Also bcl 3 can interact with p50 variants with no NLS, which are restricted to the cytoplasm (179), and transport them to the nucleus (188).

Once NF-κB has been activated the unbound I-κB α is degraded very rapidly. The half life of I-κB α in protein synthesis arrested B cells is approximately 2 hours (189). After PMA stimulation of these cells the half life is reduced to 1.5 minutes. I-κB α can be expressed in cells and have a longer half life so it is not simply that I-κB is destroyed when unbound (190). Inhibitors of proteolysis inhibit NF-κB activation so this I-κB destruction is a necessary step for activation (189). Phosphorylation may also play a role here, by modifying the substrate, the protease, and/or a regulator of the protease. An obvious advantage of a proteolytic reaction as opposed to a phosphorylation is the irreversibility of the elimination of the I-κB, so that the nuclear NF-κB can only be inactivated by its decay or by synthesis of new I-κB.

1.2.3.7. Signalling pathways involved in NF-κB activation.

The activation of T cells by antigen in the context of an appropriate major histocompatibility complex (MHC) molecule or by agents that mimic this interaction has been shown to lead to the induction of expression of over 100 different genes (191-193). NF-κB has been shown to be activated during this process and appears to be involved in the control of the expression of many of these genes especially during the early phases of the response to T cell receptor stimulation (194, 195). The regulation of cytokine gene expression appears to be one of the mechanisms whereby NF-κB is able to modulate T cell gene expression after activation. Although there has been some controversy regarding whether transcriptional or post transcriptional mechanisms are predominant in regulating cytokine expression (196) it has been shown that NF-κB plays a role in the regulation of the important T cell cytokine interleukin-2 (reviewed in (135)). Well defined NF-κB binding sites have also been found upstream of the IL-2 receptor α chain gene (197) the T cell receptor β chain gene (195) and the genes for the cytokines IL-8 (198), GM-CSF (199), G-CSF (200) and TNF α (201) and β (202) (reviewed in (135)).
Precisely how NF-κB is stimulated by signals initiating at the T cell antigen receptor remains unclear. Often a relatively simple signal will activate NF-κB such as phorbol ester or lectin treatment alone, as in murine thyoma EL4 cells (203) whereas in untransformed T cells PMA alone does not activate NF-κB (162). Stimulation of T cells by treatment with anti-CD3 or anti-CD28 also induces NF-κB activity demonstrating the ability of further T cell signalling pathways to activate NF-κB activity (204-206). The activation and/or differentiation of cells of the monocyte/macrophage lineage has also been demonstrated to involve the activation of NF-κB but the mechanisms by which this occurs are again not well understood (137, 138, 207).

The activation of NF-κB DNA binding activity by TNFα is better understood. In HL60 cells TNFα mediated NF-κB activation has been demonstrated to be mediated through the hydrolysis of sphingomyelin (208). This leads to the release of ceramide which can control the activity of specific protein kinases and phosphatases (209). Binding of TNFα to its receptor induces the production of ceramide without concomitant production of 1,2-diacylglycerol (DG) (210). These workers present data suggesting that the sphingomyelin pathway may mediate TNFα induced nuclear translocation of NF-κB directly. In Jurkat T cells however data have been obtained which suggest that a phosphatidylcholine specific phospholipase C (PLC) lies upstream of sphingomyelinase in the TNFα induced NF-κB activation pathway (211). In this pathway DG generated by an acid sphingomyelinase generates ceramide to activate NF-κB. These events were not detected in intact cells however and thus the data from (210) may be more relevant, at least for the activation of NF-κB by TNFα in HL60 cells. In the murine T cell line EL-4 IL-1β has been shown to activate the sphingomyelinase pathway (208) and mediate its effects through the induction of NF-κB (212). Further work has shown however that exogenous addition of phosphatidylcholine specific phospholipase C (PC-PLC) leads to production of DG and activation of NF-κB in human T cells and monocytic cell lines indicating that a cellular transduction pathway, dependent on PC breakdown, is functional in T cells and monocytes and may be used by transmembrane receptors to activate NF-κB.

Expression of v-src has been shown to activate NF-κB DNA binding in T cells (213) as has expression of raf-1, v-H-ras and v-mos (214). Cellular Raf-1 has been shown to be a common component utilised by multiple inducers of NF-κB activity (215).

1.2.3.8. NF-κB can bind a variety of DNA elements - implications for transcriptional control by NF-κB.
NF-κB is able to bind a wide variety of DNA elements differing significantly in their primary nucleotide sequence from the original immunoglobulin NF-κB binding sequence. Analysis of the binding of p50/p65 heterodimers has noted that p50 binds to the 5' more conserved half site and p65 binds to the less conserved 3' half site. Also the binding of rel homodimers is stronger to highly symmetric or palindromic motifs (216). The variation of NF-κB binding sites provides several mechanisms allowing differential specificity for NF-κB-mediated control of transcription. Binding to weak affinity sites is only possible at high concentrations of active NF-κB whereas binding to high affinity sites is possible at much lower concentrations, allowing different genes to respond to different levels of active NF-κB (217). Perkins et al (156) have shown that different NF-κB binding elements are activated preferentially by different combinations of NF-κB subunits which then activate transcription to varying degrees. Fujita et al (109) have elegantly demonstrated that the different NF-κB binding sites can cause varying degrees of conformational change in the bound NF-κB subunits. This was shown to affect the levels of activation seen with a specific NF-κB - DNA element combination. These workers found that p50 homodimers activate transcription preferentially from an MHC H2k gene NF-κB site but not from the β-interferon gene in vitro, despite the fact that the binding affinity for p50 homodimers is the same for both elements.
1.2.4. The transcriptional control of HIV-1.

Once the provirus has successfully integrated into a host cell chromosome the initial transcriptional control of the HIV-1 promoter is by cellular transcription factors. The binding sites for the principal transcription factors shown to bind the HIV-1 long terminal repeat sequences are shown in Figure 1.5. The constitutively expressed transcription factors Sp-1 and the TATA binding complex TFIID are responsible for only a very low constitutive promoter activity in the cell types tested. These sites are essential for promoter activity however, as their mutation results in a defective provirus (218-220).

1.2.4.1. The regulation of HIV-1 gene expression by NF-κB.

Higher levels of transcription are achieved by activation of the inducible transcription factor, NF-κB. The regulation of HIV-1 gene expression by NF-κB has been investigated by transfecting a Jurkat T cell line with a construct in which the expression of the chloramphenicol acetyl transferase (CAT) gene is controlled by HIV-1 LTR promoter sequences. The activity of the reporter gene in transfected cell extracts is then assumed to be a measure of the activity of the HIV-1 promoter. Cotransfection of expression vectors for NF-κB p50 and p65 led only to low level stimulation of CAT activity, whereas expression of NF-κB p49 and p65 led to 8 fold stimulation (147). Expression of p50 and p65 was able however, to activate a CAT reporter construct containing 4 copies of the HIV-1 NF-κB element enhancing an SV40 minimal promoter in Jurkat cells (156). NF-κB c-rel is unable to activate HIV-1 promoter activity (221).

It seems likely therefore that p49/p65 heterodimers are the NF-κB proteins in T cells responsible for activation of HIV-1 gene expression. The modest stimulation of HIV-CAT activity by p49/65 heterodimers is much increased by the presence of the HIV-1 transactivator protein tat (221). These workers showed a 16 fold stimulation of HIV-CAT with p49 and p65 or an 80 fold stimulation with expression of tat. Expression of p49, p65, and tat however gave a 400 fold stimulation. Expression of p50 and p65 had little effect on the stimulation mediated by tat. Tat was also able to synergise with fusion proteins containing the DNA binding domain of either p105 or c-rel and the acidic transcriptional activation domain of the herpes simplex protein VP16 (156). This result suggests that the synergy with p49/p65 and tat correlates with transcriptional activation rather than a direct interaction between tat and NF-κB (156).

The activation of HIV-1 promoter activity by NF-κB has also been shown to be dependent on the 3 Sp1 sites directly 3' to the NF-κB binding repeat in the HIV-1
Figure 1.5. Schematic diagram of the HIV-1 long terminal repeat sequences. Known and putative transcription factors binding sites are shown. LTR positions shown are relative to the transcription start site. See text for details.
LTR (222). These workers demonstrate that a protein-protein interaction must occur between NF-κB and Sp1 bound to an adjacent site for NF-κB to activate transcription. The interaction between NF-κB and Sp1 is dependent on orientation and position and is not observed with other elements including GATA, CCAAT or octamer. Moreover an increase in the spacing prevents interaction and thus activation. The Sp1 sites are therefore an integral part of the HIV-1 enhancer and essential for activation of promoter activity by NF-κB.

Chronic infection of macrophages has been shown to induce NF-κB DNA binding activity (223). This increase in NF-κB binding activity may enable the virus to replicate in macrophages. The activation of NF-κB may be through proteolysis of the NF-κB precursors p105 and p100 by the HIV-1 protease (224).

1.2.4.2. The regulation of HIV-1 gene expression by non NF-κB transcription factors.

A binding site for the AP-1 transcription factor complex has been described (225) but this factor has not been shown to activate the HIV-1 promoter, nor has AP-1 been formerly demonstrated to bind the site. A transcription factor found to regulate T cell specific genes on T cell activation (Nuclear Factor Activated T Cells, NF-AT) has been described to bind the LTR and activate transcription (226) but more recent studies have not confirmed the role of this factor in the control of the HIV-1 promoter (227).

Binding sites for 2 constitutive transcription factors have also been described, leader binding protein (LBP) and upstream stimulatory factor (USF). Binding of LBP has been demonstrated in vivo and in vitro to a site 3' to the transcription start site (228-230). Mutation of the LBP binding site was shown to affect in vitro transcription initiation and efficiency (231) but transient transfection of LBP-site mutant HIV-CAT constructs failed to demonstrate any effect on basal or tat stimulated promoter activity (230). USF was originally described as a HeLa cell protein able to increase the activity of the late adenovirus promoter (232) and has been shown to bind the HIV-1 LTR (228). This protein has been shown to have a modest negative effect on HIV-1 transcription (228, 233).

Rosen et al (234) have shown that a region of the LTR (positions -340 to -185 called the negative regulatory region, NRE) is able to act as a negative regulator of promoter activity. It is able to confer this activity to a heterologous promoter (SV-40) in an orientation independent manner in feline epithelial CCCS+L- cells.

In addition to cellular transcription factors it has been shown that HIV-1 gene expression can be regulated by transcription factors encoded by super-infecting viruses. The human T cell leukaemia virus type I (HTLV I), the human
Spumaretrovirus (HSRV), and members of the adenovirus, the herpes virus and the hepatitis B virus families all encode gene products able to enhance HIV-1 promoter activity (reviewed in (235)). Which of these factors are relevant to pathogenesis and which of these viruses are able to act as cofactors in HIV-1 pathogenesis remains unclear. Two strong candidates however, are HTLV 1 and human herpes virus type 6 (HHV-6) due to their ability to infect T cells. Co-infection of HIV-1 and either of these viruses has been shown to result in enhanced replication of HIV-1 and in accelerated cell death in cultured CD4+ T cells (236).

The initial mRNA transcripts produced after activation of HTV-1 infection are short and multiply spliced. These transcripts encode the viral regulatory proteins tat, rev and nef. The viral transactivator protein tat is a very powerful activator of HIV-1 LTR directed gene expression.

1.2.4.3. The control of viral gene expression by the virally encoded transactivator protein, tat.

The human immunodeficiency viruses 1 and 2, in common with other members of the lentivirus family, encode related transactivator proteins. The HIV-1 transactivator protein is called tat and its function is essentially to increase the synthesis of viral mRNAs when viral gene expression is activated. One of the novel features of HIV-1 tat action is that it acts via an interaction with a conserved sequence in the nascent viral mRNA called the TAR region. HIV-1 TAR encompasses nucleotides +1 to +44 relative to the transcription start site. It forms a stem loop structure with nucleotides +30 to +33 unpaired at the top of the loop. The determinants in TAR upon which tat action depends have been extensively characterised (237) (reviewed in (238)). The TAR stem contains a critical U rich trinucleotide bulge. It has been proposed that the bulge distorts the major groove of the RNA stem allowing interaction with the tat basic region. Amino acids flanking the basic region are also involved. Genetic experiments further suggest that interaction between a cellular factor(s) facilitates tat binding in vivo (239) and references therein). As TAR may be substituted with target sequences for other RNA binding proteins when the appropriate binding domain is fused to tat, it appears that the sole function of TAR is to direct tat to the LTR (240, 241).

The mechanism by which tat activates HIV-1 transcription is not well understood but is known to include elements of stabilisation of elongation as well as a function similar to that of classical transcriptional activators. The HIV-1 promoter directs the synthesis of high levels of short RNA transcripts (242, 243) and it appears that tat can function by increasing the expression of the full length viral mRNAs. Evidence from nuclear run-on assays (242-244) and from in vitro transcription assays
(245-247) has supported the idea that tat acts to increase the efficiency of RNA chain elongation from the HIV-1 promoter. This model suggests that the HIV-1 promoter is unable to assemble a transcription complex that efficiently elongates RNA in the absence of tat. When tat is bound to TAR, however, elongation is efficient, and full length transcripts are produced with tat acting as a RNA sequence specific processivity factor (245, 248).

In addition to this, tat has been shown to be able to activate transcription in a similar way to classical transcriptional activators. Domain substitution experiments indicate that tat has a modular structure similar to other eukaryotic transcriptional activators. The N-terminal 48 amino acids of tat have been shown to activate transcription autonomously when targeted to the HIV-1 LTR or certain heterologous promoters, either through DNA binding sites located upstream of the transcription start site, or via downstream RNA binding sites (249).

Direct interaction of the tat protein with the general transcription factor TATA binding protein (TBP) has been demonstrated (250). Tat may therefore function by recruiting or inducing conformational change in basal transcription factors, such as TFIID, in a manner similar to classical acidic activators. When tat action was compared with the mechanism of action of the acidic transcriptional activator herpes simplex virus VP16, tat was shown to act via a mechanism distinct from that of VP16 at different steps in the transcription process (251). A similar conclusion was drawn by Kamene et al who also demonstrated a dependence of tat on Sp1 (252). Furthermore interaction between Sp1 and tat has been demonstrated in vitro (253) and a ternary interaction between Sp1, tat and TATA binding factors has been suggested to be important for tat function (254). It appears that other transcription factors such as AP-1, ATF and USF can partially substitute for Sp1 (255-257). Tat has also been shown to have a preference for TATA motifs similar to those present in the HIV-1 LTR (255, 258).

An interesting additional property of tat is its ability to activate transcription of genes encoding cytokines able to stimulate HIV-1 gene expression. The best characterised example of this is the stimulation of TNF α and β. Activation of transcription has been shown to depend on a TAR-like structure and binding sites for Sp1 and NF-κB in the TNF β promoter (259). Tat is also able to induce the expression of IL-6 (260) and TGF β (261, 262).

Retroviruses must ensure that poly(A) signals in the 3' LTR are highly active while identical signals in the 5' LTR are inactive. Tat has been implicated in the control of this process in that elongating RNA polymerase II, which has been activated by tat, appears to specifically occlude the 5' HIV-1 poly(A) site (263).
12.4.4. The activation of HIV-1 gene expression by some extracellular signals is mediated via activation of NF-κB.

As mentioned earlier the activation of T cells by antigen has been shown to result in the activation of NF-κB. Consequently T cell activation will lead to the activation of the HIV-1 promoter in an infected cell. However the successful infection of T cells and integration of the HIV-1 viral DNA into the host cell chromosome requires an activated cellular environment. NF-κB will therefore be found in an active form when the cellular environment is conducive to viral integration. Macrophages however can be infected before activation and the expression of viral proteins has been shown to depend, to some extent, on the levels of active NF-κB in the infected cells (138). This will be discussed further in Chapters 3-6.

Cytokines such as TNF-α and β have been shown to induce HIV-1 expression by virtue of their ability to activate NF-κB in T cells as well as in cells of the monocyte/macrophage lineage (264, 265). NF-κB has also been assumed to mediate stimulation of HIV-1 expression by stimulation of cell surface signalling molecules such as CD3 (266) CD2 (204, 267) and CD28 in T cells (204, 266) (see Figure 1.2.). NF-κB has also been implicated in the induction of HIV-1 promoter activity by IL-1 in a variety of T cell lines (264). In a chronically infected human promonocytic line (U937-derived U1 cells) it has been suggested that IL-1 can induce viral replication by a partially NF-κB independent mechanism (268).

Recent data have demonstrated that the HIV-1 promoter is highly active in several brain regions of transgenic mice (269). This has been correlated with active NF-κB p50 and p65 in rat neurones in vivo and in vitro (270, 271). It has also been shown that a TAR-independent tat activity exists in central nervous system derived cells which is dependent on the NF-κB binding direct repeat (272). The novel astrocyte derived NF-κB DNA binding activity is retained on a tat affinity column whereas prototypical NF-κB is not.

12.4.5. Activation of NF-κB is not necessarily sufficient for activation of HIV-1 gene expression.

It should be noted that despite a large body of evidence demonstrating that the activation of the HIV-1 LTR by a wide variety of signals is mediated via NF-κB, there are some important examples where induction of NF-κB binding activity is not sufficient for LTR activation. Stimulation of T cells with anti-CD3 antibody has been demonstrated to activate NF-κB but not HIV-1 LTR activity in some cells (127). This study also demonstrated an induction of NF-κB by TNF which was unable to activate the HIV-1 LTR. Interestingly a viral clone deleted in its NF-κB binding region was
found to be infective and inducible by PMA but not by TNF α in the T cell line Jurkat. This result indicates that, at least in T cells, NF-κB binding is required for a response to TNF α but not to PMA. The PMA response was shown to be dependent on both Sp1 and TATA binding factors (273). Further studies have shown that deletion of the Sp1 binding sites does not prevent viral replication in several T cell lines or in primary PHA-stimulated PBMC. Addition of TNF α to a T cell infected with Sp1 deleted virus led to a significant increase in viral production (274). Deletion of both sites however, prevented replication in all cell types tested (220, 274, 275).
1.2.5. Retinoic acid. Its activity as a regulator of transcription.

The action of retinoic acid, a metabolite of vitamin A, on the control of HIV-1 LTR directed gene expression will be discussed in this thesis. Therefore a discussion of the mechanism of action of retinoic acid and its metabolites will be presented in this section. Vitamin A is metabolised to a wide range of biologically active retinoids which have pleiotropic effects on growth, differentiation, proliferation and development (reviewed in (276-280)). The complexity of the retinoid signalling system arises from the combination of several active retinoid metabolites, multiple cytoplasmic binding proteins and distinct ligand specificities and functional properties of retinoid receptors. Retinoid action is mediated through these receptors which are members of the steroid/thyroid nuclear receptor superfamily of transcription factors (281, 282). Additional diversity is generated by heterodimeric interaction between the retinoic acid receptors, (RARs), RAR α, RAR β, and RAR γ and the retinoic acid X receptors, (RXRs), RXR α, RXR β, and RXR γ. The RXRs are also able to heterodimerise with other members of the nuclear receptor superfamily such as the thyroid receptor (283, 284) and the vitamin D3 receptor (284).

1.2.5.1. Retinoids: the metabolites of vitamin A.

RARs and RXRs are ligand-inducible transcription factors which modulate expression of target genes via cis-acting DNA response elements. The action of these receptors is controlled in part by the binding of the metabolites of vitamin A. The three major species of vitamin A metabolites are all-trans-retinoic acid, 3,4-didehydroretinoic acid (ddRA) and 9-cis-retinoic acid (9C-RA) (285-288). All-trans-retinoic acid is the major species of retinoic acid and is the isomer used in this study. It will be referred to henceforth simply as retinoic acid. ddRA is thought to play an important role in retinoid signalling in development as its concentration is six-fold greater than that of retinoic acid in developing chick limb buds (286). 9C-RA is a stereo-isomer of retinoic acid and has been demonstrated to be the ligand for the RXRs (287, 288). Retinoic acid is converted in cultured animal cells to 9C-RA so RXRs can activate transcription in cells treated with high concentrations of retinoic acid (289).

1.2.5.2. The therapeutic role of retinoids.

The differentiating and anti-inflammatory properties of retinoid have led to their use as anti-neoplastic agents useful in the therapy of a variety of cancers (290-293). The t(15;17)(q21-q11-22) translocation associated with acute promyelocytic
leukemia (APL) has been shown to involve the retinoic acid receptor α gene. The resulting gene product leads to the leukemic phenotype in promyelocytes. Differentiation therapy with all-trans-retinoic acid is able to terminally differentiate the leukemic promyelocytes and lead to complete remission in the majority of cases. Retinoids have also been used successfully to treat squamous cell carcinoma of the head and neck (292). Retinoids or retinoid analogues have been used successfully to treat skin conditions such as acne vulgaris (reviewed in (294)), as well as psoriasis (295) and eczema (296).

1.2.5.3. The retinoic acid receptors are members of the steroid/thyroid binding superfamily of nuclear receptors.

The amino acid sequence of members of the nuclear receptor superfamily can be divided into six regions, designated A-F. These are based on regions of homology between the different types of receptors (297) (Figure 1.6.). The amino terminal region A of RARs is type and isoform specific, whereas region B is moderately conserved between the RARs of a given species but highly conserved between RARs of the same type across species. For example, the human region B and the mouse region B of retinoic acid receptor α are more homologous than the B regions of the human α and β retinoic acid receptors.

Regions A and B contain ligand independent transactivator function. These are regions of the receptor which have been defined as being responsible for the activation of gene expression in a ligand independent manner. Region C is highly conserved and contains two zinc fingers. This region corresponds to the core of the DNA binding domain and is therefore responsible for the recognition of retinoic acid response elements in DNA (297, 298). The ligand binding domain, region E, is also well conserved between receptor types. Region E contains a ligand-dependent transactivation function and a dimerisation interface. The functions of regions D and F are unknown although these regions are also well conserved for a given RAR type across species. The central part of region D is markedly RAR type specific, and thus may be required for an undefined type specific function. The amino terminal of region D is highly basic and may function as a nuclear localisation signal.

mRNA isoforms display tissue-specific and stage-specific expression during development and have been characterised, in mouse, for all RAR types (299-301). The transcripts of all RARs display specific spatio-temporal patterns of expression during development and in the adult (299). These data suggest that RARs may perform specific and important functions in specific places from the earliest stages of morphogenesis, although certain functions seem to be able to be mediated by any of
Figure 1.6. Comparison of the amino acid sequence of human (h) RAR α1, β2 and γ1 isoforms, and of these receptors with their mouse (m) counterparts. The amino acid positions corresponding to the boundaries between different regions (A-F) and the percentage homology between receptor types are given.
the RARs e.g. retinoic acid induced differentiation of HL60 cells (302). In agreement with the housekeeping nature of its promoter, RAR α expression appears almost ubiquitous (299). The isoforms of a given RAR type arise from the use of different promoters and alternative splicing. The mRNAs diverge at a point corresponding to the splice junction between exons encoding regions A and B, differing in their 5' untranslated region and in their A regions, which are fused to a common B-F sequence.

The three retinoic acid X receptors are most closely related to the vertebrate orphan receptor COUP-TF (303) and the *Drosophila* ultraspiracle gene product (304). An orphan receptor is a protein defined as a member of the steroid/thyroid receptor family of transcription factors by its homology with other members of the family. The term orphan is used to denote the fact that no ligand has been discovered for the receptor. COUP-TF was originally described via its interaction with a response element in the chicken ovalbumin upstream promoter and has been defined as an orphan member of the steroid/thyroid class of transcription factors (303). Interestingly there is no *Drosophila* RAR homologue (304). This indicates the ancient evolutionary origin of the RXRs and raises the question as to whether RXRs may represent the original retinoid signalling system.

The primary sequence of the RXRs can also be divided into regions A-F and, in general, the RXRs follow a similar pattern of inter-type and inter-species conservation. In the mouse RXRs α and β tend to be diffusely expressed at early stages of development when RARs are showing distinct patterns of expression (279). By contrast RXR γ is much more restricted early on in development (305). In late mouse organogenesis and in the adult, expression of RXR α is more restricted and is predominantly seen in the skin, liver and digestive tract epithelia (305). RXR β however remains at ubiquitous low levels in the adult mouse (305).

1.2.5.4. Retinoic acid receptors have two autonomous activating functions.

Two autonomous transcriptional activation functions (AFs) have been characterised in the estrogen receptor (ER), progesterone receptor (PR) and the glucocorticoid receptor (GR). A constitutive AF and a hormone-dependent AF are located within the N-terminal A/B region (AF-1 formerly called TAF-1) and the ligand binding domain (region E, formerly called TAF-2), respectively. As mentioned earlier, activator functions are regions of the receptor which have been defined as being responsible for the activation of gene expression in either a ligand-dependent or independent manner. Both AFs of a given receptor exhibit distinct cell type and promoter context specificity, and specific transactivation characteristics. Recently, experimental use of chimeric and deleted RARs has revealed the existence of a
similar ligand-dependent activator function, AF-2, mapping to a region overlapping
the ligand binding domain in RARs (289). RAR AF-2s are activated to a similar
degree by all-trans-retinoic acid, ddRA and 9C-RA. The RXR AF-2s however are
only efficiently activated by ddRA and 9C-RA (289). The existence of AF-2s is also
demonstrated by the ability of all RARs and RXRs, deleted for regions A and B, to
activate transcription from some RAREs (306). The synergising ligand-independent
AF-1 functions have been located by the use of deletion mutants and mapped to the A
and B regions of the receptors (306).

The activator functions from a given receptor type are specific to that type and
the AF-1 of one receptor type will not synergise with the AF-2 of another type (306). Differential ligand binding of the various retinoid metabolites between receptor types
suggests that the ligand binding domains, and/or the ligand-dependent activator
functions (AF-2s) may be somewhat distinct between receptor types, i.e. α β γ. The
ligand binding domains are better conserved for a given RAR type across species than
for the three RAR types within a species. Thus the overall transactivation potential of
a given receptor will probably be determined by a common ligand-dependent AF-2,
located in the type-specific ligand binding domain, and an isoform-specific, cell type
and developmental stage-specific AF-1 located in the promoter determined region A.

1.2.5.5. Retinoid action is modulated by cellular retinol binding and cellular
retinoic acid binding proteins CRBPs and CRABPs.

Many specific intracellular retinol and retinoic acid binding proteins, CRBPs
and CRABPs respectively have been identified which show a high degree of
homology (reviewed in (307) ). Expression of CRBPs and CRABPs during
embryogenesis and in the adult is spatially and temporally restricted, suggesting that
these proteins play specific roles. Tissue distribution studies have found CRBP I in
the liver, lung, kidneys, epididymis and testes, whereas CRABP I is most
concentrated in the testes, skin and eyes (308-310). The two proteins are often
concentrated in different cell types within a given organ. CRBP II expression is more
restricted and the protein is found only in the intestine (311).

The CRABP II proteins have been found to bind all-trans-retinoic acid with
much higher affinity than 13-cis-retinoic acid, whereas CRABP I binds both ligands
with similar affinity. CRABP I is widely expressed in the chick embryo whereas
CRABP II expression is restricted to skin, muscle and bone in the embryo and is not
expressed in the adult (312, 313). The importance of cellular retinoid-binding proteins
for retinoid function is indicated by mutant cell lines unable to express CRABPs
which are unresponsive to retinoic acid (307).
It is likely that the intracellular concentration of retinoic acid is a factor determining the extent of retinoic acid receptor activation. It is therefore interesting to note that CRBP I (314) and CRABP II (315, 316) gene expression is induced by retinoic acid, and that these proteins have been proposed to fine tune the intracellular concentration of free retinoic acid, either by its sequestration or by facilitating its metabolism (285, 317, 318). In the limb of the chick embryo the distribution of CRABP II is inversely related to that of retinoic acid. CRABP II may therefore function to steepen the retinoic acid concentration gradient thereby facilitating chick wing development. Many retinoic acid responsive cells do not express the defined binding proteins (311, 319) so although some cells require them, other cells, or processes within the same cells, seem to function without the binding proteins.

1.2.5.6. Retinoic acid receptors modulate gene expression via DNA binding sites - retinoic acid response elements, RAREs.

The various RAR/RXR heterodimers transcriptionally activate gene expression from naturally occurring and synthetic retinoic acid responsive elements (RAREs) which generally consist of the direct repetition of two core motifs in the form of PuGGTTCA(X)nPuGGTTCA (316). These sequences have been recognised in the enhancer region of several genes whose expression is regulated by retinoic acid in cultured cells. These include the enhancers for cellular retinol binding protein I (CRBP I) (320) and cellular retinoic acid binding protein 2 (CRABP II) (316). The promoter regions of a number of additional genes have been shown to contain sequences which will bind retinoic acid receptors in gel mobility shift assay (GMSA) experiments and can mediate retinoic acid induced gene expression when 5' to a heterologous promoter, but cannot experimentally induce the expression of the cognate gene. Such is the case for the putative RAREs located in the upstream regions of the phosphoenolpyruvate carboxykinase gene, the CRBP II gene, and the major histocompatibility complex class 1 genes (276, 321-323). These genes and others demonstrate that the presence of an RARE in an enhancer does not guarantee the retinoic acid inducibility of the gene.

Much experimentation has been carried out to determine whether the various nuclear receptors preferentially bind to and transactivate from response elements of different core sequences, or response elements containing different spacing between the half sites (reviewed in (279) ). From these data some generalities have been deduced. Directly repeated motifs (DRs) separated by a 5 (DR5), 4, 3 or 1 bp space correspond to specific binding sites for RARs, thyroid hormone receptors (TRs), vitamin D receptors (VDRs), and RXRs respectively. However additional systematic binding studies with various synthetic direct repeat elements have indicated that
bacterially expressed RARs bind to DR 2, 3, 4 and 5 elements whereas bacterially expressed RXRs bind preferentially to DR1 elements and with less efficiency to DR2 and DR5 elements (324). DR1 elements are rather promiscuous in these experiments and are recognised by a number of members of the nuclear receptor superfamily including the PPAR (325), COUP-TF (326) and the related ARP-1 (327) and EAR-2 (326). ARP-1 and EAR-2 are orphan receptors. ARP-1 was characterised via its ability to regulate the apolipoprotein A1 gene (328), and EAR-2 was cloned by virtue of its homology to members of the steroid/thyroid receptor superfamily (329).

RAR/RXR heterodimers form in solution and their stability is increased by binding to DNA. In *in vitro* experiments RAR/RXR heterodimers have been shown to bind with much greater efficiency to many RAREs. The specificity of the heterodimers is much altered, with DR5 and DR2 of the PuGGTTCA motif being most efficiently bound. The receptor domains required for dimerisation overlap the LBD and include multiple heptad repeats of hydrophobic residues which have been suggested to form coiled coil dimerisation interfaces (330).

Much of these data were determined *in vitro* using receptors expressed in bacteria, so to what degree the data reflect the binding characteristics of the activated receptors in a nucleus, remains to be seen. Indeed the two RAREs in the promoter of the retinoic acid responsive CRABP II gene correspond to a DR1 and a DR2, so if there is a recognition code based on spacer length it must be highly degenerate. TR has been shown to bind ER binding sites with high affinity and inhibit hormonal activation (331), and TR and RARs can activate transcription from the same elements (332). The factors determining which receptors bind and activate transcription from a particular response element will undoubtedly depend on the relative abundance and activity of the various steroid receptors, including the RXRs, as well as further cell specific parameters.

The formation of heterodimers between RAR and RXR is responsible for generating enormous diversity in the retinoid signalling pathway (reviewed in (279) ). The formation of a wide variety of dimers between RARs and RXRs is likely to facilitate the ability to bind a wide variety of RAREs including palindromic, direct repeat, and imperfect repeat sequences. Asymmetrical elements probably bind RAR/RXR heterodimers in a specific orientation, for example RAR to the 5' half site and RXR to the 3' half site, or vice versa. Heterodimer binding orientation may depend on spacer length and involve a different dimerisation interface for different spacer lengths. It is also likely that various RAR/RXR heterodimers display some RARE specificity *in vivo* although this is not obvious *in vitro* (333).

Heterodimer formation will also generate much diversity at the level of transcriptional activation. The combination of the various distinct AF-1 and AF-2 activator functions of the members of a heterodimer is likely to result in the
generation of subtly novel activating potentials. Furthermore, heterodimerisation may cause conformational change in the receptors which could modulate DNA binding, ligand binding, or activator function. Conformational changes induced on DNA binding may also affect heterodimers differently to homodimers. In summary heterodimerisation has the potential to subtly change any or all of the receptor functions from transcriptional activation to DNA element recognition, and is the single most important factor generating the level of diversity seen in the retinoid signalling system.

1.2.5.7. Activation of steroid hormone receptor by hormone binding.

Although it is now clear that the binding of retinoic acid to its receptors leads to activation of gene expression, the mechanism by which this occurs remains unclear. According to the classical two step model of hormone action, ligand binding induces a conformational change in the receptor that enables the complex to translocate to the nucleus and bind its cognate DNA binding site. However, apart from the glucocorticoid receptor (GR), steroid receptors are predominantly nuclear (334) due to a strong constitutively active nuclear localisation signal (NLS) (335). The heat shock protein Hsp 90 has been found complexed with steroid receptors and it has been proposed that this protein masks the NLS in the GR when no ligand is bound. Hsp 90 also appears to be important for stabilising the unliganded GR and facilitating hormone binding (335). The physiological significance of heat shock proteins complexing with receptors other than GR is less clear and somewhat controversial. TR does not complex with heat shock proteins and is able to bind DNA in the absence of ligand as are RARs (336).

1.2.5.8. The transcriptional control of HIV-1 LTR directed gene expression by retinoic acid.

The transcriptional control of HIV-1 LTR directed gene expression by retinoids has been investigated by several groups. A sequence in the LTR which resembles a retinoic acid response element has been previously described (1) (Figure 1.7). The site was originally described as a 5 base palindrome with a nine base spacer. This sequence has been shown to be able to bind retinoic acid receptors in vitro and to confer retinoic acid responsiveness to a heterologous promoter (2). This study also demonstrated that the HIV-1 LTR is unresponsive to retinoic acid treatment in F9 murine embryonal carcinoma cells. These cells differentiate in response to retinoic acid and have been shown to express retinoic acid receptors. However a recent study
Figure 1.7. The structure of the nuclear receptor responsive region (NRRE). See text for references and further details. Positions shown are relative to the transcription start site. The sequence shown is from the HIV-1 variant HXB2.
has shown that again site B is able to confer a retinoic acid response to a heterologous promoter in CV1 cells, a monkey kidney cell line. These workers also showed that either all-trans-retinoic acid or 9-cis-retinoic acid was able to stimulate HIV-1 LTR directed gene expression in these cells. These data have been confirmed by Ladias (3) who has extended the recognised steroid receptor binding region to contain a further 3' half site and 5 heptanucleotide repeats (Figure 1.7) This study demonstrated that RXR homodimers were able to bind the region in the presence of the RXR ligand, 9-cis-retinoic acid. Furthermore 5 nuclear orphan receptors with unknown ligands, apolipoprotein A1 regulatory protein (ARP-1), v-erbA-related proteins -2 and -3 (EAR-2 and EAR-3), hepatocyte nuclear factor-4 (HNF-4), and nerve growth factor-inducible protein-B (NGFI-B), as well as heterodimers between RXR α and either ARP-1, EAR-2, EAR-3, RAR α or the peroxisome proliferator-activated receptor were able to bind this region in vitro. Ladias confirmed the activation of the HIV-1 promoter by all-trans-retinoic acid and 9-cis-retinoic acid and demonstrated a small increase in promoter activity when these orphan receptors were overexpressed in the presence of RXR and 9-cis-retinoic acid in human choriocarcinoma JEG-3 cells. The orphan receptor COUP-TF has also been demonstrated to bind to Site B (338) and overexpression of this protein has been demonstrated to have a mild negative regulatory effect on HIV-1 promoter activity (337). No mutation of the LTR was carried out in either of these studies (3, 337) to clarify the role of the nuclear receptor responsive element (NRRE), (Site B) in these responses. Alignment and comparison of the NRRE as defined by Ladias (-358 to -320) has revealed some sequence conservation in various viral isolates but the role of this region in controlling viral replication rates remains unconfirmed. The regulation of HIV-1 gene expression by retinoic acid and in cells differentiated by retinoic acid will be discussed further in Chapters 3-6.

1.2.6. Modulation of gene expression by steroid hormones.

1.2.6.1. Activation of gene expression. Evidence for interaction between steroid hormone receptors and basal transcription factors.

The precise mechanism by which steroid receptors activate transcription remains unclear but some, including the progesterone receptor (PR), the estrogen receptor (ER), and the GR have been shown to stabilise the formation of pre-initiation complexes on DNA. Recent data has also demonstrated an interaction between TFIIB and the orphan receptor COUP-TF (339). These authors demonstrate that the interaction is not a passive one and that TFIIB is required for induction of transcription by the PR and the ER. Association of TFIIB with the transcription
initiation complex has been reported to be rate limiting. Thus the stabilisation of DNA-COUP-TF complex by TFIIB implies a role in stabilisation of the initiation complex by COUP-TF and hence a mechanism of transcriptional activation. As the interaction between the receptor and TFIIB is direct, this factor may be a general target for the steroid receptor superfamily (339).

1.2.6.2. Negative modulation of gene expression by steroid hormones. Mutually exclusive binding to overlapping binding sites.

Steroid hormones are able to modulate the action of other transcription factors by mutually exclusive binding to overlapping cis-elements. Some of these sites may allow hormone-activated receptor to bind the site and activate transcription, whereas others are sites from which the receptor is unable to activate transcription. The latter sites have been called negative response elements, NREs or nHREs. This inability to activate transcription can be due to the sequence of the site; the nGRE in the prolactin gene can be converted to a classic, fully responsive GRE with two base changes (340), although surrounding sequences may be involved as there is no clear consensus sequence (341). The binding of the receptor to an nGRE may result in a conformation in which the GRE is unable to activate transcription.

Diamond et al (342) have characterised the response of the mouse proliferin gene in which a GRE overlaps an AP-1 binding site. The element alone is a positive modulator of transcription by virtue of either jun homodimer or fos/jun heterodimer binding. Activated GR will further activate transcription in the presence of the jun homodimer, which is a weak activator, or inhibit transcription in the presence of fos/jun heterodimers, which are strong transactivators. The response to GH therefore, depends on the ratio of fos to jun present and is consequently cell type specific. The opposite is true for the steroid receptor binding element in the osteocalcin gene. This element mediates upregulation of expression in the presence of retinoic acid or vitamin D and inhibition in the presence of jun/fos heterodimers (343). In the chorionic gonadotropin α subunit gene a cAMP response element co-localises with a GR binding site (344, 345). In this case DNA binding is all that is required for repression and the amino and carboxy terminal halves of the receptor can be removed without impairing repression (345).

1.2.6.3. Negative modulation of gene expression by steroid hormones. Interaction between glucocorticoid receptor and AP-1 c-fos/c-jun complexes.

The regulation of the collagenase gene by glucocorticoid hormone (GH) and AP-1 has been studied extensively and has demonstrated a novel mechanism of
repression of gene expression (346-348). The collagenase promoter contains an AP-1 element that serves as the major mediator of gene induction by phorbol ester, inflammatory mediators and growth factors (349-351). Treatment of phorbol ester stimulated cells with GH leads to a significant reduction in the induction of collagenase promoter activity by phorbol ester (346, 348). The repression is dependent on an interaction between the GR and AP-1 complex as demonstrated by co-immune precipitation of GR and AP-1 complexes with anti c-jun antibodies (346). As ternary DNA-AP-1-GR complexes were not detected, AP-1 DNA binding may not be required, suggesting that the two proteins, c-jun and GR, form a complex unable to bind DNA. However the GR DNA binding domain is required, suggesting that this domain may have further functions, although the question of whether GR can inhibit AP-1 DNA binding and not merely interfere with activation, remains controversial (352). The reverse situation also appears to be true and GH stimulation of the mouse mammary tumour virus (MMTV) LTR can be inhibited by expression of either c-fos or c-jun, by a similar mechanism (346).

1.2.6.4. Negative modulation of gene expression by steroid hormones. Inhibition of AP-1 DNA binding by activated retinoic acid receptors.

Retinoic acid has also been shown to be able to inhibit the collagenase promoter in an AP-1 site dependent manner (353). Again the DNA binding domain of the retinoic acid receptor was required for the repression which was mediated by inhibition of AP-1 DNA binding. Interestingly, the ligand binding domain was required, as was receptor activation by retinoic acid, distinguishing the mechanism of repression from that described above for the GR. Retinoid X receptors were unable to repress collagenase expression. These data parallel those obtained by investigation of the effects of retinoic acid on the stromelysin promoter (354). In this case retinoic acid was shown to inhibit both AP-1 dependent stromelysin promoter activity and activity of an AP-1 reporter construct. This was shown to be due to inhibition of AP-1 DNA binding. Despite efforts to co-immune precipitate AP-1 and retinoic acid in a manner similar to that used to demonstrate interaction of GR and AP-1, no retinoic acid receptor/AP-1 complexes were detected. Furthermore no retinoic acid receptor/AP-1/DNA ternary complexes were detected. The investigators did not rule out the possibilities that either the antibodies interfered with the interactions or that the interactions were weak and undetectable by immune precipitation.
Chapter 2: Materials and Methods

2.1. Plasmid DNA.

The HIV-CAT construct used is essentially an HXB2 version of pU3R-III. pU3R-III contains the XhoI-Hind III long terminal repeat (LTR) fragment isolated from clone C15 of HTLV III cloned into pSVIXCAT. See (234) and references therein. HIV-CAT has the Kpn I to Sac I C15 LTR fragment replaced with a similar LTR fragment from strain HXB2 (355) Genbank accession number KO3455.

The GVL3-CATs (356) construct contains the HIV-1 LTR cloned into an MSV based retroviral construct containing an MSV LTR and an SV40 promoter driving expression of the neomycin resistance gene, both in a sense orientation with respect to the HIV-1 LTR.

HIV-1 deletion mutants were prepared by treating pUC 18 containing the Kpn 1-Hind III HIV-1 LTR fragment from HXB2, with Kpn 1 and Hind III to remove the LTR fragment. This fragment was gel purified, gene cleaned and partially digested with Rsa I generating fragments of lengths 215 and 380 base pairs, each with a blunt Rsa I 5' end and a Hind III cut 3' end. These fragments were then ligated into Sma I, Hind III cut pUC 18. These 2 plasmids were then treated with Kpn I and Sac I to remove the shortened LTR fragments which were used to replace the full length fragments from a Kpn I, Sac I cut HIV-CAT. Sequence was confirmed by chain termination DNA sequencing.

The NF-κB HIV-CAT mutant was created by site directed mutagenesis (SDM) on wild type HIV-CAT using the Amersham SDM kit and protocols. The mutant oligo sequence 5' GAGCTTGCTACAATCTACTTTCCGCTGTCTACTTTCCAGGGAGG- 3' has GGG replaced by TCT, underlined. Sequence was confirmed by chain termination DNA sequencing.

The HIV-CAT site B mutant plasmid was also created using the Amersham SDM kit and protocols. The mutant oligo sequence 5' TCGACACACTAGCGATATCCACGCTAGTGC-3', has 5' GGGGTCA and 3' TGACCTT replaced with 5' CACTAGC and 3' GCTAGTG, underlined. Sequence was confirmed by chain termination DNA sequencing.

CAT reporter plasmids containing 4 copies of the wild type HIV-1 NF-κB site, or 4 copies of the mouse class 1 major histocompatibility complex (MHC) H2k gene NF-κB site or 3 copies of the human interleukin 2 (IL-2) gene NF-κB site or 4 copies of a mutant NF-κB binding site which does not bind NF-κB were a kind gift from Dr. N. Perkins and have previously been described, (133, 156).
CAT reporter plasmid TRE3-CAT contains a single copy of the synthetic palindromic retinoic acid responsive element sequence TRE3, described by (357), ligated into Hind III, Xba 1 cut pBL2-CAT (358). Sequence was confirmed by chain termination DNA sequencing.

RSV-CAT contains the Rous sarcoma virus 5' long terminal repeat promoter sequences driving CAT expression and has previously been described (359).

SV2CAT contains the SV-40 immediate early enhancer and promoter sequences driving expression of CAT and has previously been described (360).

Retinoic acid expression vectors are the human retinoic acid receptor alpha, beta and gamma cDNAs cloned into the SV40 based expression vector pSG5 (361). These plasmids were a kind gift from Dr. F. Farzaneh.

NF-κB expression vectors are the human NF-κB cDNAs subcloned into the Hind III - Bgl II sites of RSV-Beta globin (359) with the Beta globin cDNA removed. These plasmids were a kind gift from Dr. N. Perkins and have previously been described (156). An empty RSV expression vector was made by excising the NF-κB p50 cDNA with Hind III and Bgl II and filling in the 5' overhangs with klenow as described in section 2.4 and religating.

pSV2tat72 (362) is the SV-40 immediate early promoter and enhancer sequences driving expression of a 72 amino acid HIV transactivator tat protein. This plasmid was a kind gift of Dr. J. Karn.

2.2. Digestion of plasmid DNA by Restriction enzymes.

Plasmid DNA was cut with restriction enzymes (Northumbria Biologicals and Boehringer Mannheim) as specified by the manufacturers. The reaction was stopped by the addition of gel loading buffer (100 mM EDTA, 50 % glycerol, 0.25 % bromophenol blue) followed by agarose gel electrophoresis.

2.3. Removal of 5' Phosphate to prevent self ligation.

5' phosphate was removed by the addition of 1 µl calf intestinal phosphatase (Boehringer Mannheim) and 1 µl 10 x buffer to 8 µl DNA and incubation at 37 °C for 10-30 minutes. The reaction is stopped by either incubation at 56 °C for 30 min. or gene cleaning, see below.

2.4. Creating blunt ended DNA fragments from sticky ended fragments.

Where DNA fragments are created with 5' overhangs and a blunt end is required the end can be filled in using DNA polymerase klenow fragment (Boehringer
Mannheim). Klenow buffer is added to a final concentration of 10 mM Tris-HCl pH 7.5, MgCl₂ 0.5 mM, DTT 0.75 mM, dNTPs 0.05 mM and enzyme is added at 1 µl in 20 for 15 min. at room temperature. The DNA is then cleaned by phenol/chloroform extraction and ethanol precipitated with 0.1 M NaCl and 2.5 volumes of ethanol at -20 °C.

2.5. Agarose gel electrophoresis.

The products of plasmid DNA digestion were separated on the basis of size by electrophoresis in 1-2 % agarose gels containing 0.5 µg/ml ethidium bromide and 1 X TAE (40 mM Tris-acetate, 10 mM EDTA pH 8.0). The size of the DNA fragments was determined by electrophoresis of standard DNA ladder size markers (Gibco BRL) in parallel. DNA was visualised with a standard transilluminator and quantified by comparison with known amounts of size marker DNA.

2.6. Purification of DNA fragments from agarose gels.

DNA was purified using a 'Gene Clean' kit (BIO 101). DNA fragments were cut from the agarose gel and dissolved in 2 volumes of potassium iodate at 55 °C. 10 µl of glass milk (BIO 101) was added and the mixture rotated at room temperature for 10 minutes. Glass beads and DNA was recovered by centrifugation in a microcentaur. The pellet was washed three times in 700 µl NEW wash (BIO 101). DNA was separated from the glass beads by resuspension in 10 µl water, incubation at 55 °C for 10 minutes and pelleting of the glass beads by centrifugation. Supernatant containing the clean DNA was removed and quantified by electrophoresis with ethidium bromide as above.

2.7. Ligation of DNA fragments.

Generally 100 ng of plasmid backbone was ligated to a 3-5 fold molar excess of DNA insert with 1 µl T4 DNA ligase enzyme (Boehringer Mannheim) and 1 µl 10 X reaction buffer (Boehringer Mannheim) in a 10 µl reaction volume for 2-3 hours at room temperature.

2.8. Making competent E. Coli.

200 µl of a fresh overnight culture were inoculated into 200 ml of L-broth and grown until the O.D.600 was ~ 0.6. Cells were then harvested by centrifugation (3000 rpm, 10 min.) in a 50 ml falcon tube and resuspended in 40 ml buffer 1 (30 mM...
KOAc, 100 mM RbCl, 10 mM CaCl₂, 50 mM MnCl₂ pH 5.8) and incubated on ice for 10 min. Cells are centrifuged as before and resuspended in 3 ml ice cold buffer 2 (10 mM MOPS, 75 mM CaCl₂, 10 mM RbCl, 15 % v/v glycerol pH 6.5 with KOH), aliquoted into 100 µl aliquots, frozen in liquid nitrogen and stored at -70 °C. Cells made in this way are good for ~1 year.

2.9. Transformation of competent E. Coli.

50 µl of competent bacteria were added to the ligation reaction or 1 ng of plasmid DNA and incubated on ice for 20 minutes. Transformations were then heat shocked at 42 °C for 90 seconds before addition of 200 µl of L-Broth and incubation at 37 °C for 45 minutes. Finally, bacteria were plated onto L-agar plates containing 50 µg/ml ampicillin and incubated overnight at 37 °C.

2.10. Preparation of plasmid DNA ' maxi-prep method '.

Large quantities (2-5 mg) of clean plasmid DNA were obtained by the alkaline lysis method and purified by ultracentrifugation through caesium chloride as described in (363).

2.11. Preparation of plasmid DNA ' mini-prep method '.

Small quantities of DNA (5-20 µg) were obtained by the alkaline lysis method as described in (363).

2.12. DNA sequencing.

Plasmid DNA was sequenced by chain termination sequencing using a Sequenase kit (United States Biochemical Corporation) according to the manufacturers instructions. Samples were electrophoresed in wedge type denaturing gels (8.3 M urea, 6 % acrylamide (19:1 monomer:bis )) in 1 X TBE (45 mM Tris-borate, 10 mM EDTA, pH 8.0). Following electrophoresis gels were fixed in 10 % acetic acid 12 % methanol for 1 hour, dried at 80 °C for 2 hours and autoradiographed.

HeLa (human cervical carcinoma) cells (364) and 293 (human kidney) cells were maintained in Dulbecco's modified eagle medium (Gibco) with 10 % heat inactivated (56 °C 30 min) foetal calf serum (FCS) (Gibco) in a 10 % CO₂ incubator.

U937 (human promonocytic) cells (365) and HL60 (human myelocytic cells) (366) were maintained in RPMI 1640 (Gibco) with 10 % heat inactivated FCS (Gibco) in a 5 % CO₂ incubator.

Jurkat (human T cell leukaemia) cells were maintained in RPMI 1640 (Gibco) with 5 % heat inactivated FCS (Gibco) in a 5 % CO₂ incubator.

U38 Cells (356) were derived from the U937 promonocytic cell line and have been stably transfected with the construct GVL3-CATs, a retroviral vector containing the HIV-1 LTR sequences linked to a CAT reporter gene. These cells were maintained as U937 cells.

HL60 (human promyelocyte) stable GVL3-CATs transfectants (HL60 B10) were maintained as for U937 cells.


FCS was heat treated (56 °C 30 min) and rolled with 5 g activated charcoal (Sigma) and 12.5 g AG1-X8 (D) ion exchange resin (Biorad) for 4 hours at RT. The FCS was then centrifuged (4000 rpm, 20 min, Beckman J6) and the pellet discarded. This was repeated 4 times. Finally supernatant was centrifuged (10,000 rpm 30 min Beckman J2-21) and filtered through 0.45 μm filters and then 0.2 μm filters. Serum was stored at -20 °C.

2.15. Transient Transfection of adherent cells.

These cells were transfected by calcium phosphate-DNA co-precipitation as follows. Cells were plated at 5 X 10⁵ / 10 cm plate 24 hrs prior to transfection. Plasmid DNA (15 μg total made up with pUC DNA) was added to 500 μl of 0.25 M CaCl₂. To this mixture was added 500 μl of 2 X HBS DNA precipitation buffer (50mM Hepes, 1.5 mM Na₂HPO₄, 10mM KCl, 280 mM NaCl, 12 mM glucose, pH 7.05) and a precipitate allowed to form for 15 minutes. The mixture was then added to a plate containing 10 ml medium and cells and incubated overnight. The cells were then washed twice in 6 mls phosphate buffered saline (PBS), followed by addition of 10 mls DMEM 10 % FCS containing the appropriate pharmacological agents. Cells were incubated for a further 24-36 hours before harvesting, standardisation and assay for chloramphenicol acetyl transferase (CAT) activity.
2.16. Transfection of HL60 and U937 cells.

These cells were transfected by electroporation as follows. Cells were grown to approximately $3 \times 10^5$/ml, centrifuged, and resuspended at $4 \times 10^7$/ml. The medium was kept as conditioned medium. 500 µl aliquots of cells ($2 \times 10^7$ cells) were electroporated in duplicate with 15 µg of HIV-CAT at 250V, 960µF. Following electroporation cells were diluted into 5 mls conditioned medium. Cells from duplicate electroporations were pooled and incubated for 3-4 hours. If transfections were part of the preparation of a stable transfected cell line the cells were incubated in 10 mls conditioned medium for 2-3 days before selection for transfected cells in 1-2 mg/ml G418. Where the transfections were transient, each 10 ml of cells was made up to 40 ml in RPMI + 10 % stripped FCS and divided between 4 10 cm plates at 10 ml per plate. Each plate was then dosed with the appropriate agents. The cells were incubated for 48 hours before harvesting, standardisation and assay for CAT activity.

2.17. Transient transfection of Jurkat cells.

Cells were grown to a maximum concentration of $5 \times 10^5$/ml and harvested by centrifugation at 1000 rpm for 10 minutes. The cells were washed twice in TS buffer. TS buffer is made by adding 1 ml TS salts (MgCl$_2$ 10mg/ml, CaCl$_2$ 10 mg/ml) to 100 ml TS solution (0.14M NaCl, 5 mM KCl, 0.85 mM Na$_2$HPO$_4$, 25 mM Tris base, pH 7.4). Cells were then resuspended at $8 \times 10^6$/ point in 1 ml TS buffer containing 0.5 mg DEAE dextran and 2 µg of the appropriate reporter gene made up to 10 µg/point with pUC DNA. The cells were incubated at room temperature for 15 minutes, followed by addition of 9 ml of media containing 1 mM chloroquine (Sigma) and incubation at 37 °C, 5 % CO$_2$ for 30 minutes. Cells were then pelleted 1000 rpm 10 minutes, washed twice in 5 ml media, and finally resuspended in 20 ml media and split between two 10 cm plates. Following incubation at 37 °C 5 % CO$_2$ overnight cells were dosed with the appropriate pharmacological agents and incubated for a further 24 hours before harvesting.

2.18. Cell harvesting and standardisation for DNA uptake.

For adherent cells medium was removed and cells scraped off the plates into phosphate buffered saline (PBS) and washed twice in 10 mls PBS. Medium containing U937 cells was centrifuged and adherent cells scraped into PBS. The cells were washed twice in 12 mls PBS. Cells were then resuspended in 120 µl extract buffer (0.25 M Tris.HCl pH 7.5, 17 % glycerol, 5 mM EDTA). The cells were freeze
thawed (liquid nitrogen $\leftrightarrow$ 37 °C) 4 times and heat inactivated (65 °C 10 min) centrifuged and the pellet discarded. 20 µl (HeLa or Jurkat) or 10 µl (U937) of this extract was treated with RNase (100 µg/ml, 30 min) and proteinase K (100 µg/ml, 30 min) in a total volume of 50 µl. 25 µl of these samples were then denatured (0.25M NaOH, 0.5M NaCl, 10 min RT.) and diluted to 210 µl in 0.1 X SSC, 0.125M NaOH, on ice. Duplicate 100 µl aliquots were then loaded on to "GeneScreen Plus" hybridisation membrane (Dupont) using a Biodot apparatus, slot format, (Biorad). Standard amounts of HIV-CAT from 10 ng to 5 pg were added to untransfected cell extracts and loaded to produce a standard curve. Slot blots were pre-hybridised and hybridised at high stringency (1M NaCl, 1 % SDS, 10 % dextran sulphate, 50 % deionised formamide and 0.3 mg/ml denatured salmon sperm DNA at 42 °C overnight) with a $^{32}$P oligo-labelled CAT fragment (excised from pBL2-CAT with Sma I and Bgl II). Blots were washed (2 X SSC, 5 min RT twice; 2 X SSC, 1 % SDS 30 min 65 °C twice; 0.1 X SSC 30 min RT once) and exposed to a Phosphorimager (Molecular Dynamics). Samples were quantitated for transfected HIV-CAT with reference to the standard curve. See chapter 3 for an example calculation.

2.19. Activation of U38 cells and HL60 stable GVL3-CAT transfectants.

U38 (356) and HL60 B10 cells were plated at 2.5 X 10$^5$/ml (10 mls) and activated with retinoic acid (1 µM) and/or PMA (10ng/ml) or left untreated as a control and incubated for 48 hr. Cell extracts were made as above.

2.20. Assay of Chloramphenicol Acetyl Transferase (CAT) activity.

Samples were assayed for CAT activity according to Gorman et al (367) on the basis of similar amounts of transfected DNA for transient transfection and for similar amounts of protein for stable transfection. In all CAT assays the % CAT conversion was kept in the linear detectable range for the assay (0.1 % - 40 %). Protein content of cellular extracts was measured using a Biorad protein assay kit according to the manufacturers instructions.

2.21. S1 nuclease mapping.

A 465 bp fragment containing 138 bp of sequence upstream and 77 bp downstream of the HIV-1 initiation site as well as 250 bp of CAT sequence was restricted from HIV-CAT with Sca 1 and Eco R1. This fragment was ligated into the M13 vector M13mp19 cut with Sma 1 and Eco R1. A 520 bp fragment containing the HIV-1 LTR sequences was then excised from the M13 mp19 vector with Xba 1 and
used to make the single stranded DNA probe. S1 nuclease mapping was carried out by the method of Berk and Sharp, (368).

2.22. Preparation of nuclear extracts.

293 cells were transfected transiently as described above. Cells (1-2 x 10^7) were harvested by trypsinisation and centrifugation at 1000 rpm, washed once in 5 ml ice cold PBS and 3 times in 1 ml ice cold buffer A (10 mM Hepes pH 7.9, 1.5 mM MgCl₂, 10 mM KCl, 0.5 mM DTT, 0.5 mM PMSF). Cells were resuspended in 2 x pellet volume (PV) buffer A containing 0.1 % NP-40 and incubated at 4 °C for 5 minutes. Cells were spun for 10 minutes at 4 °C in a microfuge and the supernatant discarded. The nuclear pellet was resuspended in 2 x PV buffer C (20 mM Hepes pH 7.9, 0.42 M NaCl, 1.5 mM MgCl₂, 0.5 mM DTT, 0.5 mM PMSF 25 % v/v glycerol) and incubated for 15 minutes at 4 °C on a tumbler, followed by a gentle vortex. The lysed nuclei were then spun at 4 °C for 12 minutes in a microfuge. The supernatant was diluted with modified buffer D (20 mM Hepes pH 7.9, 0.5 M KCl, 0.5 mM DTT, 0.5 mM PMSF 20 % v/v glycerol) keeping the ratio of modified buffer D to buffer C at 6:1. The extracts were then frozen quickly on dry ice and stored at - 70 °C.

2.23. Gel Mobility Shift Assays (GMSA).

The oligonucleotide used as a positive control for retinoic acid receptor binding was a previously described retinoic acid response element from Mus Musculus and had the sequence 5'-GGGTAGGGTTCACCGAAAGTTCACTCG-3' (369). The NF-κB binding probe derived from the HIV-1 LTR had the sequence 5'-GAGCTTGCTACAAGGGACTTTCCCGCTGGGGACTTTCCAGGGAGG-3'.

GMSA binding reactions were carried out in a 30 µl reaction volume at room temperature for 30 minutes. The 30 µl binding reaction contained; 10 fmols reverse transcriptase 32P labelled oligonucleotide probe, 25 mM Tris-Cl pH 7.8, 100 ng poly(dI-dC), 0.5 mM EDTA, 80 mM KCl, 100 ng of aprotinin, 3 µg albumin, 10 mM β-mercaptoethanol, 0.05% Triton X-100, 10% glycerol. After incubation the samples were incubated on ice for 15 minutes and then loaded onto a 6 % non-denaturing polyacrylamide gel (acrylamide:bis-acrylamide ratio, 37.5:1). Gels were pre-run for 1 hour prior to loading samples and were then run at 10 V/cm for 3 hours at 4 °C in 0.25 X TBE (23 mM Tris-Borate, 0.5 mM EDTA pH 8.0). Radioactivity was visualised by autoradiography with Kodak X-Omat film.
3.1. Introduction.

Human cells from a variety of tissues are susceptible to HIV infection and amongst these, cells of the blood represent the major target. CD4+ T lymphocytes are severely depleted during the course of HIV-1 infection and their loss is thought to be a major contribution to the loss of immune function suffered in AIDS.

Cells of the monocyte/macrophage lineage are also critically involved in the pathology of the disease caused by HIV-1. Virus has been detected at high frequency in macrophages in the brain (370, 371), lymphatic (372), and lung (373) tissues of AIDS patients. HIV infected macrophages may then constitute an important reservoir of infection in the blood of infected individuals (63, 64) as has been noted for other lentivirus infections (374, 375).

Retinoids have long been known to have immunomodulatory and anti-inflammatory functions (376-378) but the mechanisms of these functions are not well understood. Macrophages have been demonstrated to play diverse roles immunological processes and the effects of retinoids on cells of the monocyte/macrophage lineage are therefore of great interest. Cells of the monocyte/macrophage lineage are able to differentiate to perform a specific set of functions depending on signals received by the cells. An appropriate balance between activation or stimulation, and suppression of macrophage function is essential, as inappropriate activation or insufficient suppression can lead to extensive tissue damage. Retinoids have been shown to regulate both stimulatory and inhibitory functions in these cells and are therefore thought to play an essential role in the homeostasis of the immune system. Retinoids have been shown to stimulate the production of cytokines interleukins 1 and 3 (IL-1 and IL-3) (379, 380) as well as stimulate tumouricidal activity (381-383), phagocytosis (384), Fc receptor expression (385), and expression of the macrophage enzyme transglutaminase (386-388). Transglutaminase is responsible for catalysis of covalent cross linking of proteins and is thought to have an important role in the activation and differentiation of macrophages (386). Retinoids have additionally been demonstrated to inhibit certain monocyte/macrophage functions including procoagulant activity (389) synthesis of certain enzymes such as collagenase (390) and release of cytokines such as TNF α (391). It is therefore of interest to investigate the effects of retinoid treatment on HIV-1 in cells of the monocyte/macrophage lineage. Furthermore, given the intimate association between viral and host regulatory factors that has been established to exist in other cell systems, it is likely that the intracellular environment will play a central role in the control of HIV-1 replication in cells of the monocyte/macrophage lineage.
Consequently investigation of the effects of differentiation and cellular activation of these cells on viral replication, or the activity of the HIV-1 LTR promoter would be useful in understanding the pathology of the disease.

There are essentially two approaches to the investigation of the ability of HIV-1 to replicate in a particular cell type. One can infect cells with virus and measure the level of viral load and hence rates of replication with techniques such as assay of particle associated reverse transcriptase (RT) or assay of viral mRNA. Alternatively a simpler method is the transfection and assay of an HIV-1 LTR controlled reporter gene. To do this, a construct is prepared whereby the HIV-1 long terminal repeat (LTR) promoter sequences control the activity of a reporter gene such as the bacterial enzyme, chloramphenicol acetyl transferase (CAT). This construct is then transiently or stably transfected into the cells of interest and the activity of the reporter gene product is used as a measure of the activity of the HIV-1 LTR. The activity of the HIV-1 LTR can provide insights into the control of viral replication, at the level of gene expression, under the experimental conditions employed.

When investigating the effect of differentiation or cellular activation on transcriptional control of a gene or virus one must carefully consider experimental design. An attempt should be made to separate the direct effect of differentiation or activation, from the secondary effects of switching on or off cellular genes which can modulate gene expression. For example when considering the action of retinoic acid on HIV-1 directed transcription in cells of the monocyte/macrophage lineage there are two mechanisms of action to be considered. Retinoic acid acts through DNA binding nuclear receptors of the steroid/thyroid class. These receptors bind retinoic acid and activate transcription of cellular genes, causing differentiation of responsive cells. Among these genes may be cytokines such as TNF or interleukins, and transcription factors such as NF-κB, any of which may then activate HIV-1 transcription. Alternatively the retinoic acid receptors may bind HIV-1 LTR sequences and directly modulate HIV-1 gene expression. The key to experimental design is to be able to differentiate, where possible, between these two mechanisms.

The consequences of the state of differentiation of myeloid cells on HIV-1 infectivity and viral production have been previously investigated (53, 392-394). These studies have demonstrated that virus production by infected cells of the monocyte/macrophage lineage can be altered by differentiation of the cells by cocaine (53), PMA or retinoic acid (392, 393) or by transforming growth factor β (394). Regulation of virus expression appears to be dependent on the state of activation and/or differentiation of the cells at the time of infection. Often the effect of an agent is dichotomous, its effect depending on at what stage of infection the cells are treated. Opposite effects on virus production have been seen in monocyte derived macrophages (MDM) stimulated with γ interferon before or after HIV-1 infection.
Also IL-4 stimulation enhanced HIV-1 replication in freshly isolated monocytes but suppressed HIV infection in 5 day old MDMs (82).

The effects of treating monocyte/macrophages with retinoic acid on the rate of HIV-1 replication have been investigated by Turpin et al (395). These workers demonstrated that treatment of monocytes, recovered from HIV-1 negative blood, with retinoic acid prior to HIV-1 infection led to an increase in viral replication as measured by assay of reverse transcriptase activity. The data showed a 10 fold increase in levels of RT activity in culture fluids of infected monocytes treated with retinoic acid 1 week before infection, in comparison to untreated, infected monocytes. These effects persisted without further treatment for at least 4 weeks. HIV associated cytopathic changes in the infected monocyte cultures were also increased in both frequency and extent with retinoic acid treatment. Detailed analysis of HIV infectivity and the time course of infection showed no differences in susceptibility to infection between retinoid treated and control cells. The effects seen were due to increased levels of virus replication in infected cells rather than an increase in the number of HIV infected cells per culture. Similar data were obtained using the human myeloid cell line THP-1 (395). These cells were noted to differentiate in response to retinoic acid, become more macrophage like morphologically and showed an increase in attachment to substrate.

Turpin et al (395) suggested that the observed increase in HIV-1 replication was due to an increase in HIV-1 LTR driven gene expression. This conclusion was drawn from data derived using the U937 derived cell line U38. U937 cells are a human monocytic cell line which differentiate into cells with macrophage-like characteristics with retinoic acid treatment. U38 cells have a stably integrated HIV-CAT construct (GVL3-CATs) (356) (Figure 3.1). GVL3-CATs is a murine sarcoma virus (MSV) based retroviral construct containing the HIV-1 5' long terminal repeat sequences driving expression of the CAT reporter gene. Treatment of these cells with 1 μM retinoic acid stimulated reporter activity 3 fold.

Conversely retinoic acid has also been reported to have inhibitory effects on HIV-1 replication in cells of the monocyte/macrophage lineage. Using the chronically HIV-1 infected U937 cell line U1, Poli et al (82) have demonstrated that co-treatment with retinoic acid was able to inhibit phorbol ester induced viral replication by 50-90%. U1 cells contain 2 integrated copies of the HIV-1 provirus and no detectable viral expression was seen unless the cells were treated with a stimulating agent. Retinoic acid was also able to completely suppress the stimulation of HIV-1 expression by IL-6 or GM-CSF when it was added with the cytokine to the cells. These data were produced by assay of particle associated reverse transcriptase activity, as was the case...
Figure 3.1. The constructs used to investigate the retinoic acid response of the HIV-1 LTR. The construction of HIV-CAT is described in Materials and Methods and the construction of GVL3-CATs has previously been described, see text for details. The position of the probe used in the S1 nuclease assay is shown.
in Turpin et al (395). The same inhibition was seen in levels of steady state HIV-1 mRNA and in reduced levels of nuclear run on viral transcription. No significantly enhanced transcription of viral mRNA was seen with retinoic acid treatment alone unless, as in Turpin et al (395), the cells were pre-treated for several days.

Poli et al also demonstrated an increase in HIV-1 production from U1 cells stimulated with TNF α (82). This stimulation was not inhibited by retinoic acid treatment. TNF α has been shown by several groups to act via activation of NF-κB binding (128, 265, 396). As PMA has also been shown to act by upregulating NF-κB binding activity in these cells (137) it would be expected that retinoic acid would be able to inhibit stimulation by TNF α. These data will be discussed further in Chapter 6.

These two studies (82, 395) have demonstrated opposing effects of retinoic acid on control of viral replication in cells of the monocyte/macrophage lineage. Turpin et al (395) have differentiated monocytic cells with retinoic acid, infected them and then measured the effect of the differentiated phenotype on viral replication. Poli et al (82) have measured the direct action of retinoic acid on viral replication without the time lag required for these cells to attain the differentiated phenotype. These workers noted that pretreatment of U1 cells with retinoic acid was able to stimulate viral replication, demonstrating both aspects of retinoic acid treatment in a single system as has been done for γ interferon and IL-4 (81, 82). This chapter presents data demonstrating the direct action of retinoic acid treatment on basal and phorbol ester stimulated HIV-1 promoter activity in cells of the monocyte/macrophage lineage.

3.2. Results.
3.2.1. HIV-1 promoter activity is stimulated in retinoic acid treated U937 and HL60 cell lines carrying the HIV-CAT reporter plasmid GVL3-CATs. Retinoic acid treatment can synergise with phorbol ester treatment.

In order to investigate the response of the HIV-1 LTR promoter to retinoic acid in human monocytic cell lines, the U38 cell line was obtained and the construct GVL3-CATs was used to prepare an additional HL60 cell line derivative, named HL60 B10. HL60 cells are human promyelocytic cells and differentiate into granulocytic cells following retinoic acid treatment (319). These lines were tested for a response to retinoic acid and phorbol ester both singularly and in combination (Figure 3.2). CAT activity was stimulated 5 fold by retinoic acid in agreement with previous data obtained using the U38 line (395). In addition this experiment demonstrated a synergistic response of 130 fold to retinoic acid and PMA when both agents were added together. In HL60 B10 cells retinoic acid stimulated reporter activity by 15 fold and again retinoic acid and PMA treatment gave a synergistic
Figure 3.2. The effect of phorbol ester and retinoic acid treatment on CAT activity in U937 and HL60 cell lines stably transfected with GVL3-CATs. Cells were plated at 10 ml of 2.5 X 10^5/ml and treated with the following agents, 1 untreated control, 2 retinoic acid (1μM), 3 PMA (10ng/ml), 4 retinoic acid (1 μM) and PMA (10 ng/ml). Cells were incubated for 48 hours before cell extracts were made and assayed for CAT activity. Cell extract volumes were standardised on the basis of similar amounts of protein. See Materials and Methods for further details. Fold stimulations are based on the value of the untreated control which is arbitrarily set at 1. Data is representative of at least two separate experiments.
response of 90 fold above the basal activity. These data demonstrate a synergy between retinoic acid and PMA on reporter activity and extend the response to retinoic acid to another human haematopoietic cell line, HL60. Transient transfection of GVL3-CATs into U937 cells and activation with retinoic acid and/or PMA gave similar results to the stably transfected U937 cells U38 (data not shown). These data are clearly contradictory to those obtained by assay of viral activity after retinoic acid treatment of the U1 cell line (82).

3.2.2. Analysis of the transcription start site in GVL3-CATs derived stable cell lines by S1 nuclease protection assay.

In addition to the HIV-1 LTR the GVL3-CATs construct contains regulatory elements from an SV-40 promoter enhancer cassette and a murine sarcoma virus (MSV) LTR, both in a sense orientation (Figure 3.1). In order to determine whether the CAT induction seen in response to retinoic acid and PMA was due to the HIV-1 LTR, an S1 nuclease protection assay was carried out on the HL60 B10 line and the U38 line after stimulation with retinoic acid and/or PMA. The position of the single stranded probe used is shown in Figure 3.1.

This experiment tests whether the transcriptional start site in the HIV-1 LTR is utilised in response to retinoic acid and/or PMA treatment, or whether the mRNA coding for CAT is initiated upstream of the HIV-1 start site. The mRNA may initiate at an MSV or SV-40 start site. If the predominant mRNA initiates at the correct start site within the HIV-1 LTR a 327 base pair section of the probe will be protected from digestion by pairing with the mRNA. This protected sequence will be between the 5' start site and the 3' Eco R1 site where the probe homology to the CAT gene ends, see Materials and Methods and Figure 3.1 for preparation of probe. If the mRNA initiates upstream from the HIV-1 LTR start site more probe will be protected in a 5' direction. The maximum probe length that can be protected is 465 base pairs (bp) which will indicate a start site 5' to the HIV-1 sequences.

The results for the S1 mapping are presented in Figure 3.3. Lanes 1 and 2 show the data for the HL60 stable line HL60 B10. In both untreated control (lane 2) and retinoic acid stimulated (lane 1) samples, protected fragments are present for both the 327 and the 465 bp sizes. This indicates that the mRNA initiates both at the correct site and at a site 5' to the HIV-1 LTR for both basal and retinoic acid stimulated CAT mRNA. The fold stimulation is greater for the upstream initiated mRNA but a stimulation on addition of retinoic acid is evident for both mRNAs. The samples from the U38 cell line (lanes 3-6) show only protected probe sizes of 465 bp corresponding to an upstream initiation site. This indicates that none of the CAT mRNA is initiated in the HIV-1 sequences. This mRNA is stimulated by retinoic acid
Figure 3.3. S1 nuclease protection assay. Lanes contain samples prepared from cells as follows; Lane 1, HL60 B10 cells treated with retinoic acid (1μM). Lane 2, HL60 B10 cells untreated control. Lane 3, U38 cells untreated control. Lane 4, U38 cells treated with retinoic acid (1μM). Lane 5, U38 cells treated with PMA (10 ng/ml). Lane 6, U38 cells treated with retinoic acid (1μM) and PMA (10 ng/ml). Size markers, (S) and undigested probe (P) are also shown. See text and Materials Methods for further details.
and phorbol ester and the mRNA is stimulated synergistically by both agents added together.

These mRNA levels parallel the stimulated CAT activities seen in Figure 3.2, showing that, as expected, CAT activity is controlled at the level of mRNA in these cells. However the CAT activity in these lines following PMA/retinoic acid treatment does not represent the activity of the HIV-1 promoter. CAT mRNA is initiated upstream of the HIV-1 sequences and therefore CAT expression will be influenced by upstream sequences other than the HIV-1 sequences. This may explain the differences in the retinoic acid response of the U38 cell line, (395) and Figure 3.2, and the effects of retinoic acid seen with the U1 cell line (82).

3.2.3. Retinoic acid is able to inhibit the phorbol ester induced HIV-1 promoter activity in U937 cells transiently transfected with HIV-CAT.

In order to investigate further the response of HIV-1 activity to retinoic acid transient transfection techniques were established for U937 cells using electroporation. The HIV-CAT plasmid used contained the 5' HIV-1 LTR from HIV-1 strain HXB2 linked to the CAT gene (see Figure 3.1. and Materials and Methods). No further regulatory sequences were present. This construct will be referred to subsequently as HIV-CAT.

3.2.3.1. Standardisation of transient transfection efficiency.

In all transient transfection experiments there must be standardisation for transfection efficiency between separate points in the same experiment. The simplest way to do this is to split the cells from a single transfection point to separate plates or flasks and then add the test agent to one of the plates and not the other. This should ensure that the cells in each plate have been transfected with similar efficiency. Additionally a protein assay can be used to determine that the drug does not cause an alteration in cell number, i.e. cell death or proliferation. This method however cannot be used where the transfections contain different plasmids or different amounts of plasmid. A favoured method to standardise transfections in this situation is cotransfection, into each point, a plasmid containing a different promoter driving expression of an alternative reporter gene. For example Rous sarcoma virus (RSV) LTR or SV-40 immediate early promoter driving beta galactosidase expression plasmids are popular for this purpose. However this approach must be used with caution as it is very prone to giving artefactual data. By using this method one is comparing the activity of one promoter to another with no method of measuring the base line. For example if the test promoter responded to a drug or was activated by a
transcription factor by the same fold as the control promoter the resulting perceived stimulation would be zero. This is an extreme case and more common is a two or three fold induction or inhibition of the control promoter activity which silently alters the observed activity of the test promoter. Many workers attempt to avoid this by initially testing the control promoter for a response before employing it as the control. However this experiment is often uncontrolled and should be treated with caution. Essentially this type of control should only be used if it is ascertained that the activity of the control promoter is not modulated by any of the test situations employed.

In many of the transfection experiments here the phorbol ester PMA is used. This drug activates protein kinase C as discussed in the Introduction and causes activation of both NF-κB and AP-1 as well as further less well defined factors. Consequently PMA will activate any promoters enhanced by these sites and is able to activate all the promoters tested. These include herpes simplex immediate early, SV-40, RSV, and herpes simplex thymidine kinase promoters, data not shown.

In order to overcome this problem I have employed a method of measuring the amount of DNA released from the cells after they have been thoroughly washed and lysed (397) (see Materials and Methods). This method allows a direct measurement of the transfected DNA and therefore provides an accurate control for transfection efficiency. Essentially the extract samples containing the transfected DNA were blotted on to a hybridisation membrane which was then hybridised to a radioactive CAT probe. The radioactivity associated with each sample was measured and correlated with the amount of DNA in the extract. Standard curves were derived by adding known amounts of plasmid DNA to mock transfected cell extract and plotting radioactive volume against DNA in pg. Radioactivity was measured using a phosphorimager. The data concerning measures of radioactivity are in units of radioactive volume as as measured by the phosphorimager. The method of controlling for transfection efficiency and calculating promoter activity is detailed below with a typical example.

3.2.3.2. Calculation of data for U937 transfections.

HIV-CAT was transiently transfected into U937 cells as described in Materials and Methods. The transfections were controlled for transfection efficiency by measuring the DNA in the cell extracts as in Materials and Methods. The data for the standard plots were obtained by slot blotting mock transfected extracts containing known amounts of HIV-CAT plasmid DNA onto hybridisation membrane. The radioactive volume values for each sample were obtained by scanning slot blotted membranes following hybridisation to $^{32}\text{P}$ labelled CAT DNA probe on a phosphorimager. These values were then used to plot a standard graph of radioactive
volume vs DNA in extracts. A typical example is shown in Figure 3.4. The equation of the standard curve obtained from this plot was then used to calculate the amount of DNA per slot in the test samples using the formula \( y = mx + c \) where \( y \) = the radioactive volume as measured by the phosphorimager and \( x \) = the amount of DNA present on the membrane for each point. As the test samples were slot blotted in duplicate two volume values were obtained for each transfection point. The mean of these values was calculated. The value for \( C \) as derived from the standard curve was subtracted from the mean. The amount of DNA/slot was then calculated by dividing the mean - \( C \) value by the value for \( m \) as derived from the standard curve. The DNA/slot was used to calculate extract volumes that contained equal amounts of transfected DNA for each sample. These values were calculated by dividing the constant value \( K \) by the pg DNA/slot. The value of \( K \) for U937 transfections was derived by multiplying the lowest amount of DNA measured in each experiment by 80. In this example \( K = 307 \times 80 = 24560 \). The number 80 was derived empirically and ensures that the volumes CAT assayed give % conversion values in the detectable linear range. The extracts were then assayed for CAT activity as in Materials and Methods. The TLC plates obtained following separation of acetylated products were scanned on a phosphorimager and radioactive volumes were measured for the upper and lower spots by the phosphorimager. % conversions were calculated (Table 3.2) by dividing the value for the upper acetylated product spot by the sum of the upper and lower spots. Fold stimulations are calculated assigning the untreated control the value of one. An example CAT assay plate for the transfection of HIV-CAT into U937 cells is shown in Figure 3.5.
Figure 3.4. Example standard plot for U937 transfection. Radioactive volume values associated with each standard sample are as measured by a phosphorimager (Molecular Dynamics). Amounts of DNA standard (HIV-CAT) are in picograms. The plot is representative of standard plots for all U937 cell transfections. The equation representing the straight line drawn through the points is shown. This equation is used to calculate the volumes of cell extract to assay for CAT activity. See text for details. 

$P = 0.986$
Table 3.1. Values derived from the standardisation of transfection efficiency for U937 cells transfected with HIV-CAT. See text and Materials and Methods for details of the transfection standardisation procedure.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Volume 1</th>
<th>Volume 2</th>
<th>Mean Volume</th>
<th>Mean Volume - C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>44760</td>
<td>38250</td>
<td>41505</td>
<td>41437</td>
</tr>
<tr>
<td>Retinoic acid (1\muM)</td>
<td>39990</td>
<td>45860</td>
<td>42925</td>
<td>42857</td>
</tr>
<tr>
<td>PMA (10ng/ml)</td>
<td>21780</td>
<td>20540</td>
<td>21160</td>
<td>21092</td>
</tr>
<tr>
<td>Retinoic acid + PMA</td>
<td>33520</td>
<td>32980</td>
<td>33250</td>
<td>33182</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Treatment</th>
<th>pg DNA/Slot</th>
<th>Vol CAT (\mu l)</th>
<th>Extract Assayed</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>604</td>
<td>40.7</td>
<td></td>
</tr>
<tr>
<td>Retinoic acid (1\muM)</td>
<td>625</td>
<td>39.3</td>
<td></td>
</tr>
<tr>
<td>PMA (10ng/ml)</td>
<td>307</td>
<td>79.9</td>
<td></td>
</tr>
<tr>
<td>Retinoic acid + PMA</td>
<td>484</td>
<td>50.8</td>
<td></td>
</tr>
</tbody>
</table>
Figure 3.5. Representative CAT assay for the transfection of HIV-CAT into U937 cells. Cells were treated as follows: 1. Control, 2. Retinoic acid (1μM), 3. PMA (10 ng/ml), 4. Retinoic acid (1μM) and PMA (10 ng/ml). See text for further details.

Table 3.2. Values derived from the CAT assay TLC plate by the phosphorimager. Upper volume refers to radioactive volume associated with upper acetylated product and lower volume refers to radioactive volume associated with unreacted substrate. % conversion values were derived by dividing the upper volume value by the sum of the upper and lower volume values. Fold stimulation values are based on the % conversion value for the untreated control which is arbitrarily set at 1.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Upper Volume</th>
<th>Lower Volume</th>
<th>% Conversion</th>
<th>Fold Stimulation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>4114</td>
<td>237974</td>
<td>1.7</td>
<td>1.0</td>
</tr>
<tr>
<td>Retinoic acid (1μM)</td>
<td>2404</td>
<td>272938</td>
<td>0.9</td>
<td>0.5</td>
</tr>
<tr>
<td>PMA (10ng/ml)</td>
<td>15878</td>
<td>235427</td>
<td>6.3</td>
<td>3.7</td>
</tr>
<tr>
<td>Retinoic acid + PMA</td>
<td>6804</td>
<td>261654</td>
<td>2.5</td>
<td>1.5</td>
</tr>
</tbody>
</table>
HIV-CAT was transfected transiently into U937 cells and the cells were treated with retinoic acid or PMA or both agents together. The result is shown in Figure 3.6 and a representative CAT assay is shown in Figure 3.5. Treatment with PMA stimulated HIV-1 promoter activity by 3-4 fold. Retinoic acid treatment however inhibited the basal and the PMA induced level of promoter activity. These data are in agreement with those produced previously investigating the direct action of retinoic acid on viral replication in these cells (82).

3.2.4. The retinoic acid receptor binding site, site B, is not responsible for the retinoic acid mediated inhibition of HIV-1 promoter activity.

It has been shown that a site within the HIV-1 LTR (site B) is able to bind retinoic acid receptor and confer retinoic acid responsiveness to a heterologous promoter (2). In order to determine whether this site was responsible for the inhibition of HIV-1 promoter activity the site was mutated to a sequence reported to be unable to bind retinoic acid (2) by site directed mutagenesis (see Materials and Methods). This site B mutant HIV-CAT plasmid was then transiently transfected into U937 cells and tested for a response to retinoic acid and PMA (Figure 3.7). The mutant construct was induced to a similar degree (3-4 fold) by PMA and this induced activity was reduced to almost basal levels with retinoic acid treatment.
Figure 3.6. Transient transfection of HIV-CAT into U937 cells. $2 \times 10^7$ cells were electroporated with 15 µg of HIV-CAT DNA in duplicate and samples were mixed and divided into 4 10cm plates containing 10 ml media. See Materials and Methods for details. Cells were left overnight and then treated as follows, 1 untreated control, 2 retinoic acid (1µM), 3 PMA (10 ng/ml), 4 retinoic acid (1µM) and PMA (10 ng/ml). After 24 hours incubation cell extracts were made and standardised for transfection efficiency. Finally equalised amounts of extracts were assayed for CAT activity. The fold stimulations are based on the value for the untreated control which is arbitrarily set at 1. The data is the mean of at least three separate transfections +/- standard error of the mean.
Figure 3.7. Transient transfection of Δ site B HIV-CAT into U937 cells. 2 × 10^7 cells were electroporated with 15 μg of Δ site B HIV-CAT DNA in duplicate and samples were mixed and divided into 4 10cm plates containing 10 ml media. See Materials and Methods for details. Cells were left overnight and then treated as follows, 1 untreated control, 2 retinoic acid (1μM), 3 PMA (10 ng/ml), 4 retinoic acid (1μM) and PMA (10 ng/ml). After 24 hours incubation cell extracts were made and standardised for transfection efficiency. Finally equalised amounts of extracts were assayed for CAT activity. The fold stimulations are based on the value for the untreated control which is arbitrarily set at one. The data is the mean of at least three separate transfections +/- standard error of the mean.
3.3. Conclusions.

The data produced by treatment of the GVL3-CATs stable lines U38 and HL60 B10 are in agreement with those previously obtained using the U38 line (395), in that retinoic acid and phorbol ester are able to stimulate CAT activity in these cells. However the data produced by the S1 nuclease protection assay demonstrate that the CAT activity in these cells produced in response to retinoic acid or PMA treatment is not dependent on HIV-1 elements, as the dominant mRNA species is not initiated in the HIV-1 LTR. Enhancer elements have been reported to be active at a distance of several kilobases (99). Therefore the transcriptional control of any transcripts initiating at the correct site in the HIV-1 LTR, (Figure 3.3 lanes 1-2) are likely to be influenced by the strong control elements in the upstream MSV LTR and SV-40 enhancer sequences. It is therefore unlikely that the synergy between retinoic acid and PMA seen in both the U38 and HL60 B10 lines is dependent on HIV-1 LTR sequences alone.

It is clear however, from the data obtained by transient transfection of U937 cells with HIV-CAT that retinoic acid is able to inhibit the basal and PMA induced HIV-1 promoter activity. These data are in agreement with that obtained by treatment of the HIV-1 infected U937 cell line U1 (82). Mutation of the retinoic acid receptor binding site, site B had no significant effect on this retinoic acid mediated inhibition in U937 cells. This site is therefore unlikely to be involved in this effect.
Chapter 4: The response of the HIV-1 promoter to retinoic acid in HeLa cells.

4.1. Introduction.

The negative response of the HIV-1 promoter to retinoic acid has been demonstrated in the haematopoietic line U937 (Chapter 3). These cells are difficult to transfect and variations in their differentiation state can lead to differing responses to the agents used in these experiments. HeLa, a human cervical carcinoma cell line, has been widely used to investigate the control of HIV-1 LTR promoter activity (234, 256, 398, 399). These cells were therefore employed to further investigate the response of the HIV-1 promoter to retinoic acid.

In order to control for retinoic acid activity a thyroid response element TRE3-CAT construct which responds to retinoic acid treatment was obtained. The activity of the herpes simplex thymidine kinase promoter is stimulated by retinoic acid treatment by virtue of an upstream single copy synthetic response element, called TRE3, which has been previously described (357). In addition, two further constructs were obtained to control for the response to PMA. These were RSV-CAT (359) and SV2-CAT, constructs in which the 5' LTR from rous sarcoma virus, or the immediate early enhancer/promoter sequences from SV-40 virus respectively, control CAT expression. Although HeLa cells have been shown to express retinoic acid receptors (333), it has been found that when transiently transfecting reporter constructs into cells, cotransfection of receptor expression vectors ensures maximal effects (306). Consequently in all of these HeLa transfections an RAR α expression vector was cotransfected, unless otherwise stated.

4.2. Results.

4.2.1. Transfection of HIV-CAT and retinoic acid receptor expression vectors into HeLa cells.

HeLa cells were cotransfected with HIV-CAT and expression vectors containing either no cDNA or cDNAs encoding the retinoic acid receptors (RARs) α, β or γ, using a calcium phosphate co-precipitation protocol (see Materials and Methods). After 24 hours the cells were treated with retinoic acid (1μM), and/or PMA (10ng/ml) or left untreated as a control. After a further 24 hour incubation the cells were harvested and reporter activity was assayed.

The transfection standardisation for HeLa cells was essentially the same as that for U937 cells detailed in Chapter 3. The standard plots for HeLa transfections were calculated as those for U937 transfections (Chapter 3). The standard plot for the standardisation of the transfection described above, which is typical of all HeLa standard plots, is presented in Figure 4.1. The radioactive volumes for the test
transfections are presented in Table 4.1. The results for the transfections containing cotransfected α receptor are presented in the 1st to the 4th rows, the β receptor 5th-8th, and the γ receptor 9th-12th. Cell extract samples were blotted in duplicate giving rise to two volume values (Columns 1 and 2) which were meaned (Column 3). The value for the pg DNA associated with each slot was determined by use of the equation derived from the standard plot in Figure 4.1 as detailed in Chapter 3 (Column 4). The value for K used in this experiment was derived by multiplying the lowest value for pg DNA/μl extract by 40. In this example K = 2.1 × 40 = 84. As for U937 transfections the value 40 was empirically derived to ensure that the extract volumes used in the CAT assay gave % conversion values in the detectable linear range. K was used to determine the volume of each cell extract (so that it contained a similar amount of transfected DNA) to be assayed for CAT activity. The resultant CAT assay for the example HeLa transfection is presented in Figure 4.2. Table 4.2 contains the conversion values and calculated fold stimulations for this experiment. The fold stimulations are based on the untreated control conversion value for each receptor type which is arbitrarily set at a fold stimulation of 1. Table 4.2 demonstrates that the conversion values of the control points for the three retinoic acid receptors did not vary significantly. This experiment is representative of the experiments used to calculate Figure 4.3.
Figure 4.1. A standard plot for standardisation of transfection efficiency in HeLa cells cotransfected with HIV-CAT and retinoic acid receptor expression vectors. This plot is representative of standard plots for all HeLa transfections. The equation representing the straight line drawn through the points is shown. This equation is used to calculate the volumes of cell extract to assay for CAT activity, see text for details. P = the coefficient of correlation for a straight line which = 1 when the line has perfect correlation with the data.
Table 4.1. Values derived from the standardisation of transfection efficiency for HeLa cells cotransfected with HIV-CAT and retinoic acid receptor expression vectors. See Chapter 3 and Materials and Methods for details of the transfection standardisation procedure.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Volume 1</th>
<th>Volume 2</th>
<th>Mean Volume</th>
<th>Mean Volume - C</th>
</tr>
</thead>
<tbody>
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<td>5202</td>
<td>4306</td>
<td>4754</td>
<td>4482</td>
</tr>
<tr>
<td>Retinoic acid (1µM)</td>
<td>2917</td>
<td>2806</td>
<td>2861</td>
<td>2589</td>
</tr>
<tr>
<td>PMA (10ng/ml)</td>
<td>836</td>
<td>866</td>
<td>851</td>
<td>579</td>
</tr>
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<td>502</td>
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<tr>
<td>Control</td>
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<td>3014</td>
<td>2770</td>
<td>2498</td>
</tr>
<tr>
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<td>1825</td>
<td>1553</td>
</tr>
<tr>
<td>PMA (10ng/ml)</td>
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<td>746</td>
<td>818</td>
<td>546</td>
</tr>
<tr>
<td>Retinoic acid + PMA</td>
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<td>769</td>
<td>733</td>
<td>461</td>
</tr>
<tr>
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<td>2993</td>
<td>2723</td>
<td>2450</td>
</tr>
<tr>
<td>Retinoic acid (1µM)</td>
<td>1954</td>
<td>2642</td>
<td>2298</td>
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<td>724</td>
<td>765</td>
<td>493</td>
</tr>
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<td>1343</td>
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84
<table>
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<tr>
<th>Treatment</th>
<th>pg DNA/slot</th>
<th>pg DNA/µl extract</th>
<th>Volume assayed (µl)</th>
<th>CAT</th>
</tr>
</thead>
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<tr>
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<td>4</td>
<td></td>
</tr>
<tr>
<td>Retinoic acid (1µM)</td>
<td>60</td>
<td>12</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td>PMA (10ng/ml)</td>
<td>14</td>
<td>3</td>
<td>31</td>
<td></td>
</tr>
<tr>
<td>Retinoic acid + PMA</td>
<td>12</td>
<td>2</td>
<td>36</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>58</td>
<td>12</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td>Retinoic acid (1µM)</td>
<td>36</td>
<td>7</td>
<td>12</td>
<td></td>
</tr>
<tr>
<td>PMA (10ng/ml)</td>
<td>13</td>
<td>3</td>
<td>33</td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Control</td>
<td>57</td>
<td>11</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td>Retinoic acid (1µM)</td>
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<td>9</td>
<td></td>
</tr>
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<td>PMA (10ng/ml)</td>
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<td>2</td>
<td>37</td>
<td></td>
</tr>
<tr>
<td>Retinoic acid + PMA</td>
<td>21</td>
<td>4</td>
<td>20</td>
<td></td>
</tr>
</tbody>
</table>
Figure 4.2. Representative CAT assay for the cotransfection of HIV-CAT and retinoic acid receptor expression vectors into HeLa cells. Cells were treated as follows: 1. Control. 2. Retinoic acid (1μM). 3. PMA (10 ng/ml). 4. Retinoic acid (1μM) and PMA (10 ng/ml). Left hand 4 lanes contain samples from cotransfected RAR α, the middle 4 lanes, cotransfected RAR β and the right hand 4 lanes, RAR γ. See legend for Figure 4.3. and text for further details.
Table 4.2. Values derived from the CAT assay TLC plate from the example CAT assay in Figure 4.2 by the phosphorimager. Upper volume refers to radioactive volume associated with upper acetylated product and lower volume refers to radioactive volume associated with unreacted substrate. % conversion values were derived by dividing the upper volume value by the sum of the upper and lower volume values. Fold stimulation values are based on the conversion value of the untreated control point for each receptor which is arbitrarily set at 1.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Upper Volume</th>
<th>Lower Volume</th>
<th>% Conversion</th>
<th>Fold Stimulation</th>
</tr>
</thead>
<tbody>
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<td>Control</td>
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<td>1515285</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>Retinoic acid (1μM)</td>
<td>13280</td>
<td>1472595</td>
<td>0.9</td>
<td>0.9</td>
</tr>
<tr>
<td>PMA (10ng/ml)</td>
<td>202931</td>
<td>1195180</td>
<td>14.5</td>
<td>14.5</td>
</tr>
<tr>
<td>Retinoic acid + PMA</td>
<td>103465</td>
<td>1281928</td>
<td>7.5</td>
<td>7.5</td>
</tr>
<tr>
<td>Control</td>
<td>12603</td>
<td>1410421</td>
<td>0.9</td>
<td>1.0</td>
</tr>
<tr>
<td>Retinoic acid (1μM)</td>
<td>6919</td>
<td>1746070</td>
<td>0.4</td>
<td>0.4</td>
</tr>
<tr>
<td>PMA (10ng/ml)</td>
<td>72524</td>
<td>1323892</td>
<td>5.2</td>
<td>5.9</td>
</tr>
<tr>
<td>Retinoic acid + PMA</td>
<td>38433</td>
<td>1407734</td>
<td>2.7</td>
<td>3.0</td>
</tr>
<tr>
<td>Control</td>
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<td>1378264</td>
<td>1.2</td>
<td>1.0</td>
</tr>
<tr>
<td>Retinoic acid (1μM)</td>
<td>2364</td>
<td>1331465</td>
<td>0.2</td>
<td>0.1</td>
</tr>
<tr>
<td>PMA (10ng/ml)</td>
<td>90213</td>
<td>1287838</td>
<td>6.5</td>
<td>5.3</td>
</tr>
<tr>
<td>Retinoic acid + PMA</td>
<td>50314</td>
<td>1307251</td>
<td>3.7</td>
<td>3.0</td>
</tr>
</tbody>
</table>
A representative CAT assay is shown in Figure 4.2 and the results are presented in Figure 4.3. The addition of retinoic acid inhibited the basal rate of promoter activity in the presence and absence of cotransfected retinoic acid receptors. This inhibition was much greater with RAR γ (80 %) than with either RAR α (42 %) or RAR ß (29 %) or no receptor (51 %). Stimulation of HIV-CAT, cotransfected with empty expression vector, with PMA resulted in a mean stimulation of 12 fold basal promoter activity. This stimulation was decreased to 6 fold by overexpression of RARs α, ß or γ, and was further reduced, by treatment with retinoic acid, to a 3 fold stimulation. All three RARs tested mediated similar inhibitions of the PMA stimulation.

The degree of stimulation of promoter activity with PMA varied between approximately 5 and 15 fold basal activity in repeat experiments, although PMA was able to stimulate promoter activity in all experiments. This variation is probably due to the day to day condition of the cells, their density, growth rate etc. The reduction of this stimulation by retinoic acid as a % inhibition of the given stimulation in each experiment was reproducible. The results are therefore expressed as mean % inhibitions +/- standard error of % inhibition, as these figures demonstrate the level of inhibition, the significant parameter in this experiment.

The response of the TRE3-CAT construct to retinoic acid was a 5 fold stimulation (Figure 4.4). The basal rate of SV-40 promoter activity was unaffected by retinoic acid treatment but was stimulated by 2-3 fold by PMA treatment (Figure 4.4). RSV-CAT activity was stimulated 5 fold by PMA and this stimulation was not inhibited by retinoic acid. Retinoic acid treatment alone did not significantly affect RSV promoter activity (Figure 4.4). The inhibition by retinoic acid is therefore specific to the HIV-1 LTR.

These data demonstrate that the response of the HIV-1 LTR promoter is similar in the human monocytic cells U937 and the human epithelial cell line HeLa.

4.2.2. Mapping the retinoic acid responsive site in HeLa cells.

In order to map the site in the LTR responsible for the retinoic acid response two deletion mutants were constructed (Figure 4.5. and Materials and Methods). The wild type construct contains the entire R and U3 regions of the HIV-1 LTR. The Δ-308 HIV-CAT construct contains all the LTR sequences 3' to position -308 relative to the transcription start site. The sequence deleted in the Δ-308 mutant contains the retinoic acid receptor binding site, site B, (2) and a putative AP-1 binding site (225, 234). The use of this mutant can establish whether or not the retinoic acid response is due to a negative transcriptional effect of the RARs binding to this region. RAR may
Figure 4.3. CAT assay of HeLa cells transiently cotransfected with HIV-CAT and either empty vector or expression vectors for the human retinoic acid receptors. 5 X 10^5 cells/10 cm plate were plated out in 10 ml media and incubated overnight. The next day cells were transfected with 5 µg of HIV-CAT and 2 µg expression vector/plate. After a further 24 hours cells were left untreated, 1, or treated with 2. retinoic acid (1µM), 3. PMA (10 ng/ml), 4. retinoic acid (1µM) and PMA (10 ng/ml). In at least three replicate experiments the basal activity of the LTR was inhibited by retinoic acid by 51 % +/- 11 % (empty expression vector) 42 % +/- 17 % (alpha receptor) 29 % +/- 13 % (beta receptor) and 80 % +/- 5 % (gamma receptor). The stimulation of the LTR by PMA was inhibited by retinoic acid by 51 % +/- 14 % (alpha receptor), 29 % +/- 10 % (beta receptor) and 51 % +/- 10 % (gamma receptor). No significant reduction (3 %) was observed in the presence of empty expression vector. See Materials and Methods for further details of transfection procedure.
Figure 4.4. CAT assay of HeLa cell extracts transiently cotransfected with either TRE-CAT, SV2CAT or RSV-CAT and RAR alpha expression vector. Cells were transfected with 5 μg reporter and 2 μg receptor expression vector/plate. 24 hours after transfection cells were treated as follows 1. untreated control, 2. retinoic acid (1μM), 3. PMA (10 ng/ml), 4. retinoic acid (1μM) and PMA (10 ng/ml). Fold stimulations are based on the value for the untreated control for each reporter, which is arbitrarily set at 1. The data are the mean of at least three transfections and are shown +/- standard error of the mean.
Figure 4.5. Schematic diagram of the HIV-1 LTR showing binding sites for transcription factors important in HIV-1 gene expression. The deletion mutants used to map the retinoic acid responsive region of the LTR are shown. See Materials and Methods section for details of mutant construction.
have a direct negative transcriptional effect or it may bind and block the binding of an activating transcription factor, such as AP-1. The deleted region also contains the 5' end of the negative regulatory region (NRE) which spans nucleotides -340 to -185 as defined by Rosen et al (234). These authors demonstrated that this region of the LTR was able to confer an orientation independent negative effect on the HIV-1 promoter or on an SV-40 promoter in feline epithelial CCCC+L cells.

The second deletion mutant Δ-139 HIV-CAT contains the LTR sequence 3' to position -139 relative to the transcription start site. The deleted region contains the entire NRE as defined above. It also contains a putative NFAT-1 binding site (226) although the relevance of this site in HIV-1 transcription is controversial (227). The deleted region also contains a binding sites for the transcription factor upstream stimulatory factor (USF) (228). USF was originally described as a protein present in HeLa cells which increased the activity of a late adenovirus promoter (232) and has been shown to have a small negative effect on HIV-1 promoter expression (228, 233). The deleted region also contains sequence homologous to the interleukin-2 receptor upstream regulatory region (400). The Δ-139 mutant essentially contains the NF-κB binding direct repeat and the 3 Sp1 sites upstream of the transcription start site.

The deletion mutant HIV-CAT constructs were transiently cotransfected with RAR α expression vector into HeLa cells and treated with retinoic acid and/or PMA or left untreated as a control. The results are shown in Figure 4.6. The fold stimulations are based on transfection of HIV-CAT wild type. The basal promoter activity in both constructs is increased relative to wild type HIV-CAT and the deletion mutants are still stimulated by PMA due to the presence of the NF-κB sites in both constructs. The inhibition of basal and PMA stimulated promoter activity by retinoic acid is however, similar in magnitude to wild type HIV-CAT for both deletion mutants.

4.2.3. The effect of retinoic acid on the activity of NF-κB responsive heterologous promoters.

In order to investigate the role of the NF-κB sites in the retinoic acid mediated inhibition, a series of NF-κB reporter constructs were obtained. These constructs contain a minimal SV-40 promoter from which the two 72 bp enhancers have been deleted. The activity of this promoter is controlled by multiple copies of the NF-κB binding sites from either the HIV-1 enhancer, the human interleukin-2 (IL-2) gene enhancer or the mouse major histocompatibility complex (MHC) H2k gene enhancer. In addition to these constructs a similar construct was obtained containing the same minimal promoter enhanced by 4 copies of a mutant HIV-1 NF-κB site which has
Figure 4.6. CAT assay of HeLa cell extracts transiently cotransfected with HIV-CAT deletion mutants and RAR alpha expression vector. These mutants have 5' deletions to positions -308 and -139 relative to the transcription start site. See text and Materials and Methods section for further details of constructs. Cells were transfected with 5 μg reporter and 2 μg receptor expression vector/plate. 24 hours after transfection cells were treated as follows: 1. control, 2. retinoic acid (1μM), 3. PMA (10 ng/ml), 4. retinoic acid (1μM) and PMA (10 ng/ml). Fold stimulations are based on the value for the untreated wild type HIV-CAT control which is arbitrarily set at 1. In at least three replicate experiments the basal activity of the Δ-308 or the Δ-139 mutant was inhibited by retinoic acid by 46% ± 6% and 42% ± 3% respectively and the PMA stimulated activity by 50% ± 4% and 62% ± 3% respectively.
been shown to be unable to bind NF-κB (136) (See Materials and Methods and (133)).

These constructs were transiently transfected into HeLa cells and tested for responses to retinoic acid and PMA. The response of the 4 X HIV-1 NF-κB CAT construct to retinoic acid and/or PMA is presented in Figure 4.7. The responses of the IL-2 and MHC H2k based constructs are presented in Figures 4.8 and 4.9 respectively. The promoters of all three constructs are stimulated by PMA treatment. Furthermore the PMA induced activity of these constructs is reduced by treatment with retinoic acid. Interestingly the inhibition of the 4 X HIV-1 NF-κB CAT construct (12 % +/- 6 %)(Figure 4.7) is less than that observed for the HIV-1 LTR-CAT construct (42 % +/- 17 %)(Figure 4.3). The inhibition of PMA stimulation of the IL-2 and MHC H2k enhanced promoters is greater at (62 % +/- 3 %)(Figure 4.8.) and (60 % +/- 8 %)(Figure 4.9.) respectively.

Transient transfection of HeLa cells with the mutant 4 X HIV-1 NF-κB CAT construct and assay of CAT activity demonstrated that this construct is inactive both in the presence and absence of PMA and/or retinoic acid in these cells, data not shown. This demonstrates the complete dependence of these minimal promoter constructs on the presence of competent NF-κB binding sites and as well as a role for the wild type NF-κB sites in the retinoic acid mediated inhibition.

4.2.4. The effect of retinoic acid and phorbol ester on the activity of a mutant ΔNF-κB HIV-CAT construct.

Additionally a mutant HIV-CAT ΔNF-κB construct was prepared, by site-directed mutagenesis, in which the NF-κB sites were mutated to a sequence shown to be unable to bind NF-κB (136) (See Materials and Methods). This construct allows the investigation of the effect of retinoic acid on HIV-1 promoter activity to be determined in the absence of NF-κB binding. Transient transfection of this construct into HeLa cells and assay for CAT activity demonstrated that the basal activity of this construct was greatly reduced (5 fold) by the mutation of the NF-κB sites (Figure 4.10). Furthermore the HIV-CAT ΔNF-κB construct was only slightly stimulated by PMA treatment (2 fold), and retinoic acid treatment inhibited promoter activity to undetectable levels.
Figure 4.7. CAT assay of HeLa cell extracts transiently cotransfected with a construct in which 4 copies of the HIV-1 NF-kB site are enhancing a minimal SV-40 promoter driving CAT expression and an expression vector for RAR alpha. Cells were transfected with 3µg reporter and 2µg expression vector/plate. See Materials and Methods section for further details of the transfection procedure. Cells were treated as follows, 1. untreated control, 2. retinoic acid (1 µM), 3. PMA (10 ng/ml), 4. retinoic acid (1 µM) and PMA (10 ng/ml). Fold stimulations are based on the value for the untreated control, which is arbitrarily set at 1. In at least three replicate experiments treatment with retinoic acid inhibited the PMA stimulated promoter activity by 23 % +/- 6 %. Error is +/- standard error of the mean.
Figure 4.8. CAT assay of HeLa cell extracts transiently cotransfected with a construct in which 3 copies of the human interleukin-2 NF-kB site are enhancing a minimal SV-40 promoter driving CAT expression and an expression vector for RAR alpha. Cells were transfected with 3μg reporter and 2μg expression vector/plate. See Materials and Methods for further details of the transfection procedure. Cells were treated as follows, 1. untreated control, 2. retinoic acid (1 μM), 3. PMA (10 ng/ml), 4. retinoic acid (1 μM) and PMA (10 ng/ml). Fold stimulations are based on the value for the untreated control, which is arbitrarily set at 1. In at least three replicate experiments treatment with retinoic acid inhibited the basal promoter activity by 66 % +/- 4 % and the PMA stimulated promoter activity by 62 % +/- 3 %. Inhibitions are +/- standard error of the mean.
Figure 4.9. CAT assay of HeLa cell extracts transiently cotransfected with a construct in which 4 copies of the mouse major histocompatibility H2k gene NF-kB site are enhancing a minimal SV-40 promoter driving CAT expression and an expression vector for RAR alpha. Cells were transfected with 5μg reporter and 2μg expression vector/plate. See Materials and Methods for further details of the transfection procedure. Cells were treated as follows, 1. untreated control, 2. retinoic acid (1 μM), 3. PMA (10 ng/ml), 4. retinoic acid (1 μM) and PMA (10 ng/ml). Fold stimulations are based on the value for the untreated control, which is arbitrarily set at 1. In at least three replicate experiments treatment with retinoic acid inhibited the PMA stimulated promoter activity by 60 % +/- 8 %. Inhibition is +/- standard error of the mean.
Figure 4.10. CAT assay of HeLa cell extracts transiently cotransfected with an HIV-CAT construct in which the NF-kB sites have been mutated and an expression vector for RAR alpha. The data from Figure 4.3 obtained with the wild type HIV-CAT are shown for comparison. Cells were transfected with 5μg reporter and 2μg expression vector/plate. See Materials and Methods for further details of the NF-kB mutation transfection procedure. Cells were treated as follows, 1. untreated control, 2. retinoic acid (1 μM), 3. PMA (10 ng/ml), 4. retinoic acid (1 μM) and PMA (10 ng/ml). Fold stimulations are based on the value for an untreated wild type HIV-CAT control, which is arbitrarily set at 1. In at least three replicate experiments treatment with retinoic acid inhibited the basal and PMA stimulated promoter activity to undetectable levels.
4.2.5. Retinoic acid receptor is able to bind the HIV-1 NF-κB site in vitro in the presence of RXR.

As previously discussed in the Introduction, steroid hormone receptors have been demonstrated to negatively regulate gene expression by binding sites for activating transcription factors thereby preventing their action (342, 343). The inhibition of the activity of the minimal promoter enhanced by 4 copies of the HIV-1 NF-κB site (Figure 4.7) suggests an involvement of the NF-κB site in the retinoic acid mediated effect. Retinoic acid may exert its action via binding of retinoic acid receptor to the NF-κB site and thus occlusion of NF-κB binding, thereby preventing NF-κB activity. A gel mobility shift assay (GMSA) was therefore carried out to test whether retinoic acid receptor could bind the HIV-1 NF-κB site. A nuclear extract from 293 cells (a human kidney derived cell line) transfected with RAR α expression vector was used as a source of RAR α. Recombinant RXR was a gift from Dr. H. Stunnenberg. The probes used were radioactively labelled oligonucleotides encoding a defined retinoic acid receptor binding site or the HIV-1 NF-κB binding site (see Materials and Methods). The result is shown in Figure 4.11. Lane 1 contains the control RARE probe and RAR α extract. A retarded band representing RAR bound to the probe is clear. A weak upper band is also present and this probably represents the probe bound to an RAR/RXR heterodimer between the transfected RAR and the endogenous RXR. Lanes 2 and 3 contain the HIV-1 NF-κB probe and demonstrate that both the RAR α extract (Lane 2) and the recombinant RXR (Lane 3) are unable to bind the NF-κB probe alone. Lane 4 clearly shows a retarded band co-migrating with the upper band in lane 1. This represents an RAR/RXR heterodimer bound to the NF-κB probe. These data provide evidence that RAR α is able to bind the HIV-1 NF-κB site when heterodimerised with RXR β.

4.2.6. The inhibition of tat activated HIV-1 promoter activity in HeLa cells.

The previous sections have presented data concerning the inhibition of basal and phorbol ester stimulated HIV-1 LTR promoter activity by retinoic acid in HeLa cells. It is also of interest to investigate whether retinoic acid is able to modulate the stimulation of the HIV-1 promoter by the viral transactivator protein tat. Once gene expression is stimulated in the integrated provirus, the HIV-1 tat protein is synthesised and further gene expression is stimulated by its action. The mechanisms of activation of HIV-1 gene expression by tat are complex and are discussed in the Introduction. Essentially if tat acts by stimulating the elongation of RNA polymerase II transcripts, that have already been initiated, then retinoic acid would be expected to
Figure 4.11. Gel mobility shift assay. Lanes contain as follows; Lane 1, 10 fmols of RARE probe, 1 μl RAR α extract. Lane 2, 10 fmols NF-κB probe, 1 μl RAR α extract. Lane 3, 10 fmols NF-κB probe, 1 μl RXR β extract. Lane 4, 10 fmols NF-κB probe, 1 μl RAR α extract, 1 μl RXR β extract. See text and Materials and Methods for further details.
inhibit their initiation by interference with NF-κB binding and/or NF-κB mediated transcriptional activation. Also if tat acts as a transcriptional activator then retinoic acid receptor may bind the LTR and act as a non-specific transcriptional inhibitor.

HeLa cells were cotransfected with HIV-CAT, expression vector for RAR α, and an increasing amount of expression vector for the transactivator protein tat. After 24 hrs the cells were treated with retinoic acid (1μM) or left untreated as control. After a further 24 hour incubation the cells were harvested and reporter activity was assayed. The fold stimulations are calculated on the basis that the basal fold stimulation without tat expression is 1.

The results are shown in Figure 4.12. Increasing expression of tat progressively stimulated HIV-1 promoter activity, as expected, and retinoic acid treatment was able to inhibit this stimulation by a similar degree to that seen with the inhibition of PMA stimulated promoter activity in these cells.

The action of retinoic acid on the stimulation of the ΔNF-κB HIV-CAT mutant by tat expression, was also investigated. Due to the very low basal CAT conversion levels of this mutant the data are expressed as mean % CAT conversions (Figure 4.13.). The degree of inhibition of the ΔNF-κB HIV-CAT mutant was greater than that of the wild type construct. These data (Figures 4.12 - 4.13.) clearly demonstrate that retinoic acid is able to inhibit the tat mediated activation of both the wild type and the ΔNF-κB HIV-CAT mutant.
Figure 4.12. CAT assay of HeLa cells cotransfected with HIV-CAT and expression vectors for the HIV-1 transactivator protein tat and retinoic acid receptor alpha. Cells were transfected with 5μg reporter, 2μg RAR expression vector/plate and amounts of tat expression vector as indicated. See Materials and Methods for further details of transfection procedure. Cells were treated with retinoic acid (1μM) where indicated. Fold stimulations are based on the value for the untreated HIV-CAT point with no cotransfected tat. Results show the mean of two independent transfections +/- standard error of the mean.
Figure 4.13. CAT assay of HeLa cells cotransfected with ΔNF-κB HIV-CAT and expression vectors for the HIV-1 transactivator protein tat and retinoic acid receptor alpha. Cells were transfected with 5μg reporter, 2μg RAR expression vector/plate and amounts of tat expression vector as indicated. See Materials and Methods for further details of transfection procedure. Cells were treated with retinoic acid (1μM) where indicated. % conversions are used as basal activity was undetectable in unstimulated points. Results show the mean of two independent transfections +/- standard error of the mean.
4.3. Conclusions.

The data presented in Figure 4.3 and Figure 3.5 demonstrate that the response of the HIV-1 promoter to retinoic acid is similar in the human monocytic cells U937 and the human epithelial cell line HeLa. Overexpression of the retinoic acid receptors without retinoic acid addition was able to inhibit the phorbol ester stimulated response by 50%. This may be due to the presence of retinoids in the 10% foetal calf serum added to the growth medium or to an effect of the receptor in the absence of ligand. A further ligand-inducible inhibition of promoter activity of approximately 25% is also demonstrated. There appears to be no significant difference in the control of HIV-1 promoter activity between the 3 receptor types other than the observation that the γ receptor can reduce the basal level of promoter activity in the presence of retinoic acid to 20% of the untreated level, compared to 60-70% for the α and β receptors.

The stimulation of TRE3-CAT with retinoic acid (Figure 4.4) was in agreement with previously published data using this element (357). This result confirms the ability of retinoic acid to stimulate transcription mediated by a retinoic acid response element in HeLa cells. The activities of the SV-40 enhancer promoter and the RSV LTR promoter are not inhibited by retinoic acid treatment. This provides evidence that the effect is specific for the HIV-1 promoter. Furthermore the stimulation of both the SV40 and the RSV promoters (Figure 4.4) demonstrates that PMA is stimulating these cells and that the retinoic acid effect is not simply due to upstream inhibition of the action of PMA by affecting, for example, protein kinase C activation or expression.

The increase in basal promoter activity of both deletion mutants (Figure 4.6) is due to the deletion of the 5' end of the NRE in the Δ-308 mutant and the entire NRE in the Δ-139 mutant. As both mutants are inhibited to the same degree by retinoic acid as the wild type HIV-CAT, the site responsible for this effect must lie 3' to position -139 relative to the transcription start site. This region contains the NF-κB binding direct repeats and the 3 Sp1 binding sites.

The inhibition of the 4 X HIV-1 NF-κB CAT construct by retinoic acid (Figure 4.7) implies that the inhibition of HIV-1 promoter activity is at least partly mediated through the NF-κB sites and shows that this inhibition can be conferred to a heterologous promoter. The inhibition of this construct however is less than that seen with the full length LTR. This construct depends entirely on NF-κB for its promoter activity, a similar construct containing mutant NF-κB sites is inactive in these cells. Interestingly 2 further constructs containing NF-κB sites from the human IL-2 gene
and the mouse MHC H2k gene are also stimulated by PMA and this stimulation is inhibited by retinoic acid.

The NF-κB mutant HIV-CAT is also inhibited by retinoic acid (Figure 4.10). The small stimulation of the NF-κB mutant by PMA has been previously noted (401) and has been ascribed to the putative NFAT-1 site (226) as well as TATA binding factors (273). The involvement of the NFAT-1 site in the response to PMA however, remains controversial (227). The inhibition of this construct demonstrates that the effect can be mediated without NF-κB binding activity.

The GMSA data presented provides evidence that retinoic acid may inhibit HIV-1 gene expression, at least in part, by binding the NF-κB site, preventing NF-κB binding, and consequently inhibiting NF-κB mediated activation of the HIV-1 promoter. Inhibition of basal levels of NF-κB binding could lower the rate of basal promoter activity, as is seen with retinoic acid treatment. This may therefore represent a mechanism through which retinoic acid treatment is able to inhibit the HIV-1 promoter directly.

Figures 4.12 and 4.13. demonstrate that retinoic acid is able to inhibit the activation of the HIV-1 promoter by tat. This is shown to be true for both the wild type HIV-CAT construct and the ΔNF-κB HIV-CAT mutant. This result demonstrates that retinoic acid is able to inhibit the tat mediated activation of the HIV-1 promoter in an NF-κB independent manner. The inhibition of tat mediated stimulation is discussed further in Chapter 6.
Chapter 5: The role of NF-κB in the modulation of HIV-1 promoter activity by retinoic acid in Jurkat cells.

5.1. Introduction.

In order to investigate further the role of NF-κB in the retinoic acid mediated inhibition of HIV-1 promoter activity HeLa cells were cotransfected with NF-κB expression vectors and HIV-CAT. Expression of active NF-κB p50 and p65 or p49 and p65 was unable to significantly activate the HIV-1 promoter in HeLa cells (data not shown). These experiments were therefore repeated in the human CD4+ T cell line Jurkat. The CD4+ T cell is thought to be the principal reservoir of HIV-1 in the peripheral blood of infected individuals (44). Infection of quiescent T cells leads to incomplete infection, with the viral DNA existing as circular forms in the cytoplasm. Unless the cell is activated within 2-3 days, the infection is abortive. Once integrated the provirus is stimulated to productive infection, initially by host cell factors such as NF-κB. Consequently the processes of T cell activation and induction of NF-κB are important factors in the viral life cycle in T cells.

It is of interest to establish whether the inhibitory action of retinoic acid on HIV-1 promoter activity seen in cells of the monocyte/macrophage lineage is functional in T cells. Retinoic acid has been shown to be able to influence T cell differentiation via modulation of the major T cell growth factor interleukin-2 (IL-2) (402, 403) which is involved in the control of T cell development (404, 405). This modulation is mediated via RAR inhibiting the DNA binding of a transcription complex containing octamer-binding transcription factors and AP-1 (402, 406). Retinoids have also been shown to be important co-factors in T cell activation (407). The activation of resting T cells is initiated by the interaction of the T cell receptor (TCR) with antigen peptide bound to major histocompatibility complex (MHC) on antigen presenting cells (APC), reviewed in (48). This activation has been shown to be dependent in vitro on the presence of retinol and, although retinol is not sufficient to cause activation, it has been shown to be a crucial co-factor for the activation of CD4+ and CD8+ thymic T cells and CD4+ peripheral T cells (407). Retinoic acid is also able to increase stimulation of T cell HOXB cluster expression by T cell activation (408).

In view of the importance of CD4+ T cells in the aetiology of HIV-1 disease and the importance of retinoic acid as a co-factor in T cell differentiation and activation, the effects of retinoic acid on HIV-1 promoter activity in T cells were investigated. Jurkat cells are a CD4+ human T cell line readily infectable by HIV-1. They have been used as a human T cell model by many groups to investigate the
activation of latent HIV-1 provirus and control of HIV-1 LTR directed gene expression (222)

5.2. Results.

Jurkat cells were transiently cotransfected with expression vectors for the NF-κB subunits p50 and p65 and with expression vector for RAR α, using a DEAE dextran protocol (see Materials and Methods). The cells were left overnight and then each transfected flask was split into two. Half of the cells were treated with retinoic acid (1μM) and the other half left untreated as a control. The transfections were standardised for transfection efficiency by the method detailed in Chapter 3 and Materials and Methods. The standard plots for standardisation of transfection efficiency for Jurkat transfections were calculated as in Chapter 3. A representative example for the stimulation of HIV-1 promoter activity by NF-κB subunit expression is presented in Figure 5.1. The radioactive volumes from the test transfections are presented in Table 5.1. Cell extract samples were slot-blotted in duplicate (Columns 1 and 2). These values were meaned (Column 3). The value for the pg DNA associated with each slot was determined by use of the equation derived from the standard plot in Figure 5.1 as detailed in Chapter 3 (Column 4). The value for K used in the Jurkat transfections was derived by multiplying the minimum pg DNA/slot by 40. As with the HeLa transfection standardisations, the empirically derived value of 40 was found to give a % CAT conversion within the linear range of the assay. The value for K used in this example experiment was 40 x 9 = 360. This value was used to determine the volume of each cell extract (which contained a similar amount of transfected DNA) to be assayed for CAT activity.

The resultant CAT assay is shown in Figure 5.2 and the % conversion and fold stimulation values for the CAT activity derived from this assay are presented in Table 5.2.
**Figure 5.1.** The standard plot for standardisation of transfection efficiency in Jurkat cells cotransfected with HIV-CAT and expression vectors for NF-κB subunits p50 and p65 and RAR α. This plot is representative of standard plots for all Jurkat cell transfections. The equation representing the straight line drawn through the points is shown. This equation was used to calculate the volumes of cell extract to assay for CAT activity. See text for details. P = the coefficient of correlation for a straight line which = 1 when the line has perfect correlation with the data.
Table 5.1. Values derived for the standardisation of transfection efficiency from cotransfection of Jurkat cells with HIV-CAT and NF-κB expression vectors.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Volume 1</th>
<th>Volume 2</th>
<th>Mean Volume</th>
<th>Mean Volume - C</th>
</tr>
</thead>
<tbody>
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<td>Untreated</td>
<td>119501</td>
<td>123776</td>
<td>121638</td>
<td>115614</td>
</tr>
<tr>
<td>Retinoic acid (1μM)</td>
<td>28476</td>
<td>27602</td>
<td>28039</td>
<td>22014</td>
</tr>
<tr>
<td>Untreated</td>
<td>52890</td>
<td>55471</td>
<td>54180</td>
<td>48156</td>
</tr>
<tr>
<td>Retinoic acid (1μM)</td>
<td>44995</td>
<td>42953</td>
<td>43974</td>
<td>37949</td>
</tr>
<tr>
<td>Untreated</td>
<td>26953</td>
<td>29903</td>
<td>28428</td>
<td>22403</td>
</tr>
<tr>
<td>Retinoic acid (1μM)</td>
<td>25070</td>
<td>24262</td>
<td>24666</td>
<td>18641</td>
</tr>
<tr>
<td>Untreated</td>
<td>30801</td>
<td>30486</td>
<td>30644</td>
<td>24618</td>
</tr>
<tr>
<td>Retinoic acid (1μM)</td>
<td>34794</td>
<td>33316</td>
<td>34055</td>
<td>28030</td>
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</table>

<table>
<thead>
<tr>
<th>Treatment</th>
<th>pg DNA/slot</th>
<th>pg DNA/μl extract</th>
<th>Volume CAT assayed (μl)</th>
</tr>
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<tr>
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<td>11.9</td>
<td>6.1</td>
</tr>
<tr>
<td>Retinoic acid (1μM)</td>
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<td>2.3</td>
<td>31.9</td>
</tr>
<tr>
<td>Untreated</td>
<td>25</td>
<td>4.9</td>
<td>14.6</td>
</tr>
<tr>
<td>Retinoic acid (1μM)</td>
<td>19</td>
<td>3.9</td>
<td>18.5</td>
</tr>
<tr>
<td>Untreated</td>
<td>11</td>
<td>2.3</td>
<td>31.3</td>
</tr>
<tr>
<td>Retinoic acid (1μM)</td>
<td>10</td>
<td>1.9</td>
<td>37.6</td>
</tr>
<tr>
<td>Untreated</td>
<td>13</td>
<td>2.5</td>
<td>28.5</td>
</tr>
<tr>
<td>Retinoic acid (1μM)</td>
<td>14</td>
<td>2.9</td>
<td>25.0</td>
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</table>
Figure 5.2. Representative CAT assay for the cotransfection of HIV-CAT, expression vectors for NF-κB p50, NF-κB p65 and retinoic acid receptor α into Jurkat cells. Amounts of NF-κB p50 and p65 expression vectors are as in Figure 5.3 as follows; 1st pair none, 2nd pair 1 μg, 3rd pair 2 μg, 4th pair 5 μg. Cells were treated as follows: 1. Untreated control. 2. Retinoic acid (1μM). See text for further details.
Table 5.2. Values derived from the CAT assay TLC plate from the example CAT assay in Figure 5.2 by the phosphorimager. Upper volume refers to radioactive volume associated with upper acetylated product and lower volume refers to radioactive volume associated with unreacted substrate. % conversion values were derived by dividing the upper volume value by the sum of the upper and lower volume values. Fold stimulation values are based on the conversion value of the untreated control point with no cotransfected NF-κB which is arbitrarily set at 1.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Upper Volume</th>
<th>Lower Volume</th>
<th>% Conversion</th>
<th>Fold Stimulation</th>
</tr>
</thead>
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<td>6978591</td>
<td>1.6</td>
<td>1.0</td>
</tr>
<tr>
<td>Retinoic acid</td>
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<td>4444473</td>
<td>5.0</td>
<td>3.1</td>
</tr>
<tr>
<td>(1μM)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Untreated</td>
<td>368110</td>
<td>6221573</td>
<td>5.6</td>
<td>3.5</td>
</tr>
<tr>
<td>Retinoic acid</td>
<td>203820</td>
<td>5466897</td>
<td>3.6</td>
<td>2.3</td>
</tr>
<tr>
<td>(1μM)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Untreated</td>
<td>712727</td>
<td>4716020</td>
<td>13.1</td>
<td>8.3</td>
</tr>
<tr>
<td>Retinoic acid</td>
<td>379593</td>
<td>4550875</td>
<td>7.7</td>
<td>4.9</td>
</tr>
<tr>
<td>(1μM)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Untreated</td>
<td>963958</td>
<td>4236497</td>
<td>18.5</td>
<td>11.7</td>
</tr>
<tr>
<td>Retinoic acid</td>
<td>346166</td>
<td>3911462</td>
<td>8.1</td>
<td>5.1</td>
</tr>
<tr>
<td>(1μM)</td>
<td></td>
<td></td>
<td></td>
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</tbody>
</table>
Expression of increasing amounts of NF-κB p50 and p65 stimulated HIV-1 promoter activity to a maximum of 8 fold above basal level in Jurkat cells (Figure 5.3). This stimulation by NF-κB expression was inhibited by approximately 50 % with each amount of NF-κB by treatment with retinoic acid. Although these cells have been shown to express retinoic acid receptor (402), further receptor was expressed by cotransfection of retinoic acid receptor α expression vector to ensure maximal effects.

The response to retinoic acid was controlled for by transfecting the retinoic acid responsive TRE3-CAT construct into Jurkat cells. This construct is stimulated by retinoic acid by virtue of a single copy RARE, upstream of the herpes simplex thymidine kinase promoter (See Materials and Methods). The cells were treated with retinoic acid (1μM) or left untreated as a control. The result is shown in Figure 5.4. Retinoic acid stimulated promoter activity by 70 fold demonstrating that retinoic acid is active in these cells and that functional retinoic acid receptors are synthesised when RAR expression vectors are transfected into these cells.

To examine whether the inhibition of NF-κB stimulated promoter activity could be increased above 50 % by increased expression of retinoic acid receptor the following transfection was carried out. Jurkat cells were cotransfected with HIV-CAT and 2 μg of NF-κB p50 and 2 μg of NF-κB p65 expression vector to give a stimulation of approximately 5 fold above unstimulated promoter activity. These cells were also cotransfected with an increasing amount of RAR α expression vector. Each point was split into 2 and half was treated with retinoic acid (1μM) and half was left untreated as a control. The results are presented in (Figure 5.5). The inhibition of HIV-CAT activity increased from 32 % inhibition (no cotransfected RAR) to a maximum of 68 % (1μg cotransfected RAR), with no further increase in inhibition at 2 μg cotransfected RAR. This result demonstrates that further expression (> 1 μg) of retinoic acid receptor is unable to inhibit NF-κB mediated stimulation of HIV-1 promoter activity by more than 70 %. This result shows that retinoic acid receptor is not limiting in these experiments.

Phorbol ester has previously been demonstrated to induce NF-κB (136, 194) and activate HIV-1 promoter activity in Jurkat cells. It is therefore of interest to test whether PMA stimulated HIV-1 promoter activity is inhibited by retinoic acid treatment as it is in U937 (Chapter 3) and HeLa (Chapter 4) cell lines. HIV-CAT was transiently cotransfected into Jurkat cells with RAR α expression vector and treated with retinoic acid and/or PMA. The results are shown in Figure 5.6. HIV-1 promoter activity was stimulated 15 fold above the level of the untreated control by PMA treatment. In contrast to the inhibition seen in U937 and HeLa cells, retinoic acid was stimulatory in Jurkat cells, giving a small stimulation above control which was
synergistic with the 15 fold PMA stimulation, producing a co-stimulation of approximately 30 fold above control levels.

As mentioned previously retinoic acid receptor has been shown to bind a site, site B, in the HIV-1 LTR (2). To test whether the retinoic acid response was mediated through site B, a site B mutant HIV-CAT construct was prepared (see Materials and Methods). The site B mutant HIV-CAT was mutated to a sequence which no longer bound retinoic acid receptor at site B. This construct was transiently cotransfected into Jurkat cells with RAR α expression vector. The transfected cells were stimulated with retinoic acid and/or PMA and the results are shown in Figure 5.7. Mutation of site B leads to an approximately 2 fold increase in the level of stimulation by PMA but the stimulation by retinoic acid is unaffected.

The Δ-139 HIV-CAT deletion mutant (see Chapter 4 and Materials and Methods) is also able to mediate the stimulation by retinoic acid as well as the synergy with PMA (Figure 5.8). The site(s) responsible for this effect of retinoic acid must therefore be situated 3' to position -139 relative to the transcription start site. The effect of retinoic acid alone is increased in this construct, compared to wild type. In addition the PMA stimulation is reduced to 8 fold. Retinoic acid/PMA cotreatment increases the stimulation to 16 fold above basal levels.
Figure 5.3. CAT assay of Jurkat cell extracts cotransfected with HIV-CAT and expression vectors for NF-kB subunits p50 and p65 and RAR alpha. 8 x 10^6 cells were transfected with 2 μg of HIV-CAT, 1 μg of RAR alpha expression vector and the indicated amount of NF-kB expression vector/point. 24 hours after transfection each point was split into 2 and half was treated with retinoic acid (1 μM) and the other half was left untreated as a control. Cells were transfected using a DEAE dextran protocol. See Materials and Methods section for further details of transfection protocol. Fold stimulations are based on the value for the untreated HIV-CAT point with no cotransfected NF-kB. The value for this point is arbitrarily set at 1. The data represent the mean of at least three separate transfections and errors shown are standard errors of the mean.
Figure 5.4. CAT assay of Jurkat cell extracts cotransfected with TRE3-CAT and expression vector for RAR alpha. 8 x 10^6 cells were transfected with 2μg of TRE3-CAT and 1μg of RAR alpha expression vector. 24 hours after transfection each point was split into 2 and half was treated with retinoic acid (1μM) (Column 2) and the other half was left untreated as a control (Column 1). Cells were transfected using a DEAE dextran protocol. See Materials and Methods section for further details of transfection protocol. Fold stimulations are based on the value for the untreated TRE3-CAT point which is arbitrarily set at 1. The data represent the mean of at least three separate transfections and errors shown are standard errors of the mean.
Figure 5.5. CAT assay of Jurkat cell extracts cotransfected with HIV-CAT, 2μg of NF-kB p50, 2μg of NF-kB p65 expression vectors and increasing amounts of RAR alpha expression vector as indicated. 24 hours after transfection all points shown were treated with retinoic acid (1μM). Results are expressed as % inhibition, by retinoic acid, of the stimulation of CAT activity as compared to an untreated control for each amount of RAR. Cells were transfected using a DEAE dextran protocol. See Materials and Methods section for further details of transfection protocol.
Figure 5.6. CAT assay of Jurkat cell extracts cotransfected with HIV-CAT and RAR alpha expression vector. 8 x 10^6 cells were transfected with 2μg of HIV-CAT and 1μg of RAR alpha expression vector/transfection point. 24 hours after transfection cells were treated as follows, 1. untreated control, 2. retinoic acid (1μM), 3. PMA (10 ng/ml), 4. retinoic acid (1μM) and PMA (10 ng/ml). Cells were transfected using a DEAE dextran protocol. See Materials and Methods section for further details of transfection protocol. Fold stimulations are based on the value for the untreated control which is arbitrarily set at 1. The data represent the mean of at least three separate transfections and errors shown are standard errors of the mean.
Figure 5.7. CAT assay of Jurkat cell extracts cotransfected with Δ site B HIV-CAT mutant and RAR alpha expression vector. The Δ site B HIV-CAT mutant has been mutated at site B so that this site no longer binds retinoic acid receptor. See Materials and Methods for details. 8 x 10^6 cells were transfected with 2μg of mutant HIV-CAT and 1μg of RAR alpha expression vector/transfection point. 24 hours after transfection cells were treated as follows, 1. untreated control, 2. retinoic acid (1μM), 3. PMA (10 ng/ml), 4. retinoic acid (1μM) and PMA (10 ng/ml). Cells were transfected using a DEAE dextran protocol. See Materials and Methods section for further details of transfection protocol. Fold stimulations are based on the value for the untreated control which is arbitrarily set at 1. The data represent the mean of at least three separate transfections and errors shown are standard errors of the mean.
Figure 5.8. CAT assay of Jurkat cell extracts cotransfected with HIV-CAT deletion mutant Δ-139 and RAR alpha expression vector. 8 x 10^6 cells were transfected with 2µg of mutant HIV-CAT and 1µg of RAR alpha expression vector/transfection point. See Materials and Methods and Chapter 4 for details of HIV-CAT deletion mutant. 24 hours after transfection cells were treated as follows, 1. untreated control, 2. retinoic acid (1µM), 3. PMA (10 ng/ml), 4. retinoic acid (1µM) and PMA (10 ng/ml). Cells were transfected using a DEAE dextran protocol. See Materials and Methods section for further details of transfection protocol. Fold stimulations are based on the value for the untreated control which is arbitrarily set at 1. The data represent the mean of at least three separate transfections and errors shown are standard errors of the mean.
5.3. Conclusions.

In accordance with previously published data HIV-1 promoter activity is shown to be induced by expression of active NF-κB subunits in the Jurkat T cell line. Also in agreement with the data presented in Chapter 4 this stimulation is inhibited by expression of retinoic acid receptor and treatment with retinoic acid. The inhibition is shown to be maximal at 1μg cotransfected receptor with an inhibition of 68%. This demonstrates that the amount of retinoic acid receptor α is not limiting in these experiments.

Also in accordance with previously published data (136) the phorbol ester PMA is able to stimulate HIV-1 promoter activity. However, despite the ability of retinoic acid to inhibit HIV-1 promoter activation by cotransfected NF-κB subunits, co-stimulation with PMA and retinoic acid led to a synergistic increase in promoter activity (Figure 5.6). This activity is not dependent on the retinoic acid receptor binding site, site B (Figure 5.7), nor is it dependent on any LTR sequences 5' to position -139 relative to the transcription start site (Figure 5.8). This synergy is a surprising result as PMA has been shown to activate the HIV-1 promoter predominantly by inducing NF-κB (136, 194), and a synergy between active NF-κB and retinoic acid is contradictory to the retinoic acid mediated inhibition shown in Figure 5.3 and 5.4. There is evidence, however, to suggest that PMA also activates HIV-1 via NF-κB independent mechanisms (273). It is possible that this synergy is the sum effect of an NF-κB independent activation of the promoter and the small (2 fold) stimulation of HIV-1 promoter activity by retinoic acid. This will be discussed further in Chapter 6.
6.1. The effects on HIV-1 replication of differentiation of cells of the monocyte/macrophage lineage.

A stimulatory effect of retinoic acid on HIV-1 replication in cells of the monocyte/macrophage lineage has been clearly demonstrated (82, 395). This stimulation has been shown to be dependent on pre-treatment of the cells with retinoic acid (82). This will lead to a differentiated cell phenotype which Turpin et al (395) and Poli et al (82) have demonstrated will support a higher level of viral replication. This effect is apparent for several weeks following retinoic acid treatment and is not due to increased expression of the viral receptor CD4, or to increased levels of HIV-1 uptake (395). Turpin et al assume that the positive effect is due to a direct stimulation of HIV-1 transcription by retinoic acid, mediated through retinoic acid nuclear receptors. This is based on the data obtained with the U38 cell line, demonstrated to be artefactual (Chapter 3). S1 nuclease protection assay of both the U38 cell line and a promyelocytic HL60 derived line, stably expressing the same HIV-1 LTR-CAT construct, demonstrated that both lines are unsuitable for analysis of HIV-1 promoter activity. This is due to reporter mRNA initiating at a site 5' to the HIV-1 sequences (Figure 3.3.).

Turpin et al explain their results by citing data claiming an inhibition of HIV-1 LTR promoter activity by AP-1 and data demonstrating an inhibition of AP-1 binding by retinoic acid. Thus retinoic acid is assumed to act by preventing an inhibitory effect of AP-1. However the evidence concerning the inhibition of HIV-1 promoter activity by AP-1 (409) is simply a mutational analysis of the HIV-1 LTR in which deletion of a putative AP-1 site, leads to a virus with higher replication rates in U937 and Jurkat T cells. No activation or expression of AP-1 is demonstrated nor is AP-1 shown to bind the LTR. Taking such points into consideration, together with the S1 nuclease protection assay result, this mechanism of retinoic acid mediated stimulation is unlikely.

The differentiation of monocytic type cells to macrophage type cells has been reported to be associated with constitutive activation of NF-κB (137, 207). An increased level of activation of NF-κB in mature macrophages, differentiated with retinoic acid, could explain the increase in viral replication rates seen in (82, 395). Infection of THP-1 cells with HIV-1, and characterisation of resultant clones has allowed the comparison of NF-κB DNA binding activity in clones with varying levels of HIV-1 replication (138). These workers have shown that the replication of HIV-1 in these mature cells of the monocyte/macrophage lineage is dependent on the level of DNA binding activity of an NF-κB p50/p65 heterodimer. Clones supporting low
levels of viral replication were found to have low levels of this activity. The activity of NF-κB p50/p65 in these cells was shown to be controlled by the presence of an inhibitor of p50/p65, probably an IκB.

Turpin et al (395) note cell morphology changes associated with differentiation when HIV-1 infected THP-1 cells are treated with retinoic acid. These cells also supported increased levels of viral replication after retinoic acid treatment. Furthermore it has also been shown that primary human macrophages upregulate HIV-1 promoter activity via the NF-κB site when induced to differentiate through contact with autologous activated nonadherent cells (410). Retinoic acid induced differentiation however, does not necessarily promote NF-κB activation in these cells. Suzan et al found no induction of NF-κB DNA binding activity in retinoic acid treated U937 cells or in purified human monocytes (411). Furthermore, it has been recently shown that retinoic acid differentiated U937 cells have an increased ability to stimulate HIV-1 promoter activity on PMA treatment (412). This affect was shown to be due to the induction of a TATA binding factor which is likely to account for at least part of the increased activity of the HIV-1 promoter in cells of the monocyte/macrophage lineage, differentiated by retinoic acid treatment.

In summary, although differentiation of cells of the monocyte/macrophage lineage can lead to induction of NF-κB (137, 138, 207, 410), this is not always the case (138, 411). IκB α has also been reported to be induced on macrophage adherence (171). It is clear that the cellular differentiation state is an important determinant of the level of HIV-1 replication supported in cells of the monocyte/macrophage lineage (82, 395, 410). Retinoic acid treatment of these cells prior to HIV-1 infection can lead to a differentiated phenotype able to support a higher rate of viral replication (82, 395). This has been shown to be due to increased levels of active NF-κB (137, 138, 207, 410), and/or induction of TATA binding factors (412).

6.2. The inhibition of HIV-1 replication by retinoic acid.

The treatment of HIV-1 infected U937 cells with retinoic acid has been shown conclusively to inhibit viral replication stimulated by cotreatment with either phorbol ester, or GM-CSF, or IL-6 (82). These workers conclude that despite the similarity in the effects of retinoic acid and transforming growth factor β (TGF β) on HIV-1 expression, the retinoic acid mediated effects are not due to the production of this growth factor. This conclusion is drawn from an absence of detectable TGF β in culture supernatants and from a lack of effect of antibodies to TGF β. Figure 3.6 demonstrates an inhibition of the basal and phorbol ester stimulated HIV-1 promoter activity in U937 HIV-CAT transient transfectants. These data are in agreement with
those presented by Poli et al (82) who demonstrate retinoic acid mediated inhibition of PMA stimulated HIV-1 replication in HIV-1 infected U937 cells. As a positive retinoic acid response requires that the cells be pre-treated with retinoic acid before viral infection (82, 395), it seems likely that the direct transcripational effects of retinoic acid, mediated through RARs binding to the HIV-1 LTR, are inhibitory in these cells. Figure 3.7 demonstrates that the retinoic acid receptor binding site, site B (2), is not required for the observed inhibition.

6.3. The mechanism of down regulation of HIV-1 promoter activity by retinoic acid in HeLa cells.

The regulation of HIV-1 promoter activity was further studied in HeLa cells. The inhibition of phorbol ester stimulated and basal HIV-1 promoter activity by retinoic acid treatment in HeLa cells (Figure 4.3) parallels the effects seen in the monocytic line U937 (Figure 3.6). This inhibition by retinoic acid requires expression of retinoic acid receptors, providing evidence for their involvement in the effect (Figure 4.3.). The similar response in U937 and HeLa cells enables the site(s) responsible for the effect of retinoic acid to be mapped in HeLa cells. These cells have been widely used by many groups to investigate the control of HIV-1 promoter activity (234, 256, 398, 399).

The stimulation of the TRE3-CAT construct (Figure 4.4.) by retinoic acid treatment demonstrates that retinoic acid is able to activate gene expression from a typical retinoic acid responsive element (RARE) as previously described (357), and that cotransfection of retinoic acid receptor expression vectors leads to synthesis of functional retinoic acid receptors in these cells. The promoter activity of the RSV LTR stimulated by PMA is not inhibited by retinoic acid treatment (Figure 4.4). This shows that the inhibition by retinoic acid is not simply due to retinoic acid inhibiting the action of PMA by affecting an activation step upstream of the induction of transcription. This provides evidence that the retinoic acid mediated effect is specific to the HIV-1 LTR.

Transfection of SV2-CAT, a construct in which the SV-40 immediate early promoter controls CAT expression, into HeLa cells, and treatment with retinoic acid and/or PMA, demonstrates that neither the basal nor the stimulated promoter activity are inhibited by retinoic acid. The SV-40 enhancer region does contain a single element (GGGACCTTCC) (413) identical to half of the NF-κB binding direct repeat sequence in the HIV-1 enhancer. The lack of effect of retinoic acid on SV2-CAT demonstrates that the effect is specific to the HIV-1 LTR.

The data obtained with the HIV-CAT deletion mutants have enabled the site responsible for the retinoic acid response to be mapped to the region of the LTR 3' to
position -139 relative to the transcription start site of (Figure 4.6). This mapping rules out any involvement of the retinoic acid binding site, site B, or the putative AP-1 binding site (225, 409). Consequently, retinoic acid cannot be acting via a mechanism whereby it inhibits AP-1 binding to the putative AP-1 site. Inhibition of AP-1 DNA binding has been shown previously to be responsible for the inhibition of phorbol ester stimulation of collagenase (353) and stromelysin gene expression (354). The transcription factor binding sites in the region 3' to position -139 which have been characterised are the NF-κB binding direct repeat and the 3 Sp1 binding sites.

The inhibition of the PMA stimulation of the SV-40 based 4 x HIV-1 NF-κB CAT construct (Figure 4.7.) suggests that at least part of the effect of retinoic acid is to inhibit the action of NF-κB. This is in contrast to the lack of effect of retinoic acid on the complete SV-40 promoter enhancer construct (Figure 4.4.) which, as mentioned above, contains an identical single NF-κB site. This may be because a single copy of this repeat sequence is not sufficient for the inhibitory effect of retinoic acid, however interference from confounding effects of the SV-40 enhancer cannot be ruled out. It should be noted that the degree of inhibition of the 4 x HIV-1 NF-κB CAT construct is only half that seen with the complete LTR and HIV-1 promoter (23% +/- 6% rather than 51% +/- 14%). The data presented in Figures 4.8 and 4.9 demonstrate a retinoic acid mediated inhibition of the promoter activity of two further constructs in which the same SV-40 minimal promoter is enhanced by three copies of the NF-κB binding sites from the human interleukin-2 (IL-2) gene or from the mouse MHC H2k gene respectively. These promoters are stimulated by PMA treatment and inhibited by retinoic acid to a similar degree to the HIV-1 LTR promoter.

The inhibition of the PMA stimulated activity of the HIV-1, and the minimal promoter constructs containing NF-κB sites, could be explained by binding of RAR to the NF-κB sites in a conformation unable to activate transcription. This would prevent the activation of transcription by NF-κB. Although the HIV-1 NF-κB binding direct repeat sequence would not be expected to bind steroid/thyroid receptors, Figure 4.11 demonstrates that RAR α, in combination with RXR β, is able to bind the HIV-1 NF-κB binding direct repeat sequence in vitro. Close examination of the NF-κB binding region reveals that this sequence contains two half sites previously described to be responsive to thyroid hormone in the human thyroid stimulating hormone alpha (TSH α) gene (414) (Figure 6.1). The 5' NF-κB site contains the sequence GGACCTT at position -102 to -97 and the 3' site contains this sequence at position -88 to -83. There is an 8 bp spacing between the half sites. There is a further putative half site (AGGGGAC) at position -104 to -99 overlapping the 5' half site detailed above. This half site has been previously described in the rat growth hormone gene (415, 416) and would give a spacing between the half sites of 10 bp. Both of these half sites are
Figure 6.1. Sequences of NF-κB binding sites from the HIV-1 long terminal repeat sequences or from 5' enhancer regions of respective genes. The sequences in the HIV-1 site homologous to thyroid responsive elements are shown. See text for further details.
destroyed by the mutation employed to prevent NF-κB binding (GGG → TCT) (Figure 6.1).

These data are in agreement with recently published data (417) which also demonstrate retinoic acid receptor binding to the HIV-1 NF-κB site as a heterodimer with recombinant RXR β in vitro. These authors found no effect of retinoic acid on HIV-1 promoter activity in their system but showed a stimulatory effect of the thyroid hormone T3, mediated through its nuclear receptors. Thyroid receptor, which belongs to the same nuclear receptor family as retinoic acid receptors, was also shown to bind the HIV-1 NF-κB site. The binding of thyroid receptor, or retinoic acid receptor, and NF-κB to the NF-κB site was shown to be mutually exclusive (417). These data are in accordance with previously published data demonstrating that retinoic acid receptors and thyroid hormone receptors are able to bind and act through the same DNA binding sites (332). Desai-Yajnik also show that thyroid hormone is unable to bind the GGG → TCT mutant sequence (417).

These authors have investigated the activation of the HIV-1 promoter by ligand activated thyroid receptor and NF-κB in HeLa cells transiently cotransfected with an HIV-CAT 5′ deletion mutant. The data produced by these workers are summarised in Figure 6.2. Expression of thyroid receptor and treatment with thyroid hormone led to a 7-fold increase in HIV-1 promoter activity. Expression of either NF-κB p49 or NF-κB p50 was able to inhibit the activation of the HIV-1 promoter by thyroid hormone by approximately 50%. Furthermore a 2 to 3-fold stimulation by expression of NF-κB p49 or p105 was inhibited by expression of thyroid receptor. When NF-κB p50 and p65 were co-expressed with thyroid receptor, treatment with thyroid hormone resulted in a promoter activity similar to that measured without NF-κB expression. However as NF-κB p50/p65 expression stimulates the promoter, the fold stimulation with thyroid hormone treatment is decreased.

In summary the data produced by Desai-Yajnik et al (417) demonstrate an ability of thyroid hormone to stimulate HIV-1 gene expression. The HIV-1 NF-κB binding direct repeat sequence is shown to be responsible for this effect. This sequence is also shown to be able to confer the response to thyroid hormone to a heterologous promoter. Furthermore, thyroid hormone receptor is able to inhibit the action of NF-κB and NF-κB is able to inhibit the action of ligand activated thyroid receptor. The activation of promoter activity by thyroid receptor and by NF-κB is completely non-cooperative and it is assumed that these antagonistic effects are mediated through the mutually exclusive binding of thyroid receptor and NF-κB to the HIV-1 NF-κB site. It is reasonable to presume that retinoic acid is able to act in a similar way to inhibit NF-κB mediated stimulation. Retinoic acid is not however, able
Figure 6.2. Summary of the data produced by Desai Yajnik et al (417). HeLa cells were co-transfected with an HIV-CAT deletion mutant and expression vectors for various NF-kB subunits and/or the thyroid receptor (TR) as indicated. Cells were treated with thyroid hormone (T3, 100 nM) as indicated. See text for further details.
to stimulate promoter activity, perhaps due to the induction of an inactive receptor conformation when binding the NF-κB site in these cells.

Desai-Yajnik et al (417) also demonstrated that both retinoic acid receptors and thyroid receptors were able to bind the HIV-1 Sp-1 binding sequence. Furthermore the binding of thyroid hormone receptor or retinoic acid receptor and Sp-1 to the Sp-1 sites was shown, in vitro, to be mutually exclusive. These data provide a model whereby retinoic acid is able to inhibit the unstimulated, basal rate of HIV-1 promoter activity as well as a model for the inhibition of the mutant NF-κB HIV-CAT promoter (Figure 4.10.). It should be noted however, that NF-κB is responsible for a considerable level of basal HIV-1 promoter activity in HeLa cells, as shown by the 5-fold decrease in promoter activity on mutation of the NF-κB binding sites (Figure 4.10). The inhibition of basal activity could therefore be due to inhibition of NF-κB binding as well as inhibition of Sp-1 binding.

Desai-Yajnik et al (417) also showed that the activity of an HIV-1 LTR, with the NF-κB sites deleted, was inhibited by thyroid hormone. This is explained by the binding of the receptor to the Sp-1 sites and occlusion of Sp-1 binding. It is interesting that thyroid receptor is unable to activate transcription from the Sp-1 binding region in the same way that it can from the NF-κB sites. Presumably this is due to the induction of an inactive conformation when bound to DNA. However thyroid hormone is able to activate transcription from the HIV-1 Sp-1 sites in the presence of the HIV-1 transactivator protein tat. Tat may provide a bridge between Sp-1 site-bound thyroid hormone receptor and the initiation complex allowing transcriptional activation. The possible mutually exclusive binding of Sp-1 and RAR to the HIV-1 Sp-1 site would provide a mechanism for the inhibition, by retinoic acid, of NF-κB mediated HIV-1 promoter stimulation. It has previously been shown that the activation of the HIV-1 promoter by NF-κB is dependent on the 3 Sp-1 sites 3' to the NF-κB sites (222). The cooperation between Sp-1 and NF-κB, both in DNA binding and in transcriptional activation, is mediated by a direct protein-protein interaction between the two transcription factors. Interference of Sp-1 binding by RAR would therefore decrease both DNA binding and transcriptional activation by NF-κB. This may also explain why the 4 X HIV-1 NF-κB site-CAT construct is only inhibited by 23 % as opposed to 51 % for the complete enhancer in HIV-CAT. The absence of Sp-1 sites in the 4 X HIV-1 NF-κB-CAT construct confines the inhibition to the component directly affecting NF-κB binding as any affect on NF-κB binding or transcriptional activation via occlusion of Sp-1 binding would be absent.

Figure 6.1. demonstrates that the sequences of the HIV-1, the mouse MHC H2k and the human IL-2 NF-κB binding sites vary considerably. These sites would not be expected to contain a common binding site for the retinoic acid receptors. Desai-Yajnik et al defined the site responsible for the effects of thyroid hormone as in
Figure 6.1. These authors demonstrated that the HIV-1 NF-κB site could confer a response to thyroid hormone to a heterologous promoter but that the NF-κB responsive APRE from the rat angiotensinogen gene which contains a C between the A and the T (Figure 6.1.) could not bind thyroid hormone receptor or confer responsiveness to thyroid hormone. It therefore seems possible that the inhibition of the 4 X MHC and the 4 X IL-2 NF-κB CAT constructs might be mediated by another mechanism in addition to direct RAR binding of the NF-κB sites. Mutational analysis of NF-κB sites to define the requirements for RAR binding would be of future interest. It is possible that retinoic acid receptors interact with NF-κB subunits via protein-protein interactions, independently of DNA binding, and alter either NF-κB DNA binding or ability to activate transcription. Recently glucocorticoid hormone receptor, a member of the steroid/thyroid hormone nuclear receptor superfamily, has been shown to associate physically with NF-κB p65 and inhibit transcriptional activation (418). Previously both glucocorticoid hormone (346-348) and retinoic acid receptors (353, 354) had been shown to inhibit AP-1 mediated promoter activation by interaction with the AP-1 fos/jun complex. It is possible that retinoic acid is again able to act in a similar way to glucocorticoids in inhibition of NF-κB activity through RAR/NF-κB p65 interactions.

Either of the 2 models discussed above could explain the observed inhibition of tat mediated activation of HIV-1 promoter activity by retinoic acid (Figure 4.12.). Tat has previously been shown to synergise with NF-κB in stimulating HIV-1 promoter activity (221). In a situation where levels of NF-κB were basal and tat was activating transcription, inhibition of 50% of the NF-κB contribution to this synergy could reduce the total rate of transcription by 50%. The inhibition of promoter stimulation by tat could therefore be due to the inhibition of basal NF-κB binding and loss of the NF-κB component of the synergy. Tat has been shown to promote elongation of initiated mRNA transcripts (243, 244, 246) and so inhibition of transcriptional initiation via inhibition of NF-κB activity would also explain the inhibition of tat stimulated promoter activity. These data do not rule out a direct inhibition of tat action by retinoic acid via an NF-κB independent mechanism. The function of tat has also been demonstrated to be dependent on Sp-1 (252-254). A further mechanism of inhibition of tat action is therefore the inhibition of Sp-1 binding to the LTR, as described above, thereby preventing Sp-1 tat interactions. This mechanism would also mediate the inhibition of the action of tat on the NF-κB mutant HIV-CAT (Figure 4.13).

The data presented in this thesis and by Desai-Yajnik et al (417) do not rule out the possibility that RAR is able to bind and mediate the HIV-1 promoter inhibition from site(s) other than the NF-κB and Sp-1 sites. To determine whether this is the case, an HIV-CAT mutant containing mutations in the NF-κB and Sp-1 sites
would be required. This construct would be expected to have a very low basal activity and be unresponsive to phorbol ester treatment which would make it very difficult to determine whether the mutant promoter activity was inhibited by retinoic acid.

In summary the data fit two models which explain retinoic acid mediated inhibition of HIV-1 promoter activity; 1, retinoic acid receptor binds to the NF-κB binding repeat and/or the Sp-1 binding sites in the HIV-1 LTR in an inactive conformation, thereby occluding NF-κB and/or Sp-1 binding and consequently transcriptional activation; 2, retinoic acid receptor interacts with NF-κB and interferes with either DNA binding or transcriptional activation. These models are not mutually exclusive and both may contribute to the observed effects of retinoic acid on HIV-1 promoter activity in U937 and HeLa cells.

Retinoic acid has been shown to inhibit the induction of HIV-1 replication in the HIV-1 chronically infected U1 cell line stimulated by either PMA, GM-CSF or IL-6. It is interesting to note that the regulation of HIV-1 replication by GM-CSF and IL-6 in the U1 cell line has been reported to be post-transcriptionally mediated (419). Therefore any factor reducing the level of HIV-1 promoter activity would cause a reduction in the amount of nascent mRNA available for the post transcriptional stimulation. Consequently any mechanism whereby retinoic acid inhibited HIV-1 promoter activity would be expected to reduce the level of stimulation by these cytokines. This is in contrast to the lack of effect of retinoic acid on the stimulation of HIV-1 by TNF α. The data presented in this thesis and by Desai-Yajnik et al (417) support the inhibition of NF-κB or Sp-1 binding to the HIV-1 LTR as a mechanism of inhibition of HIV-1 promoter activity. It is therefore surprising that the stimulation by TNF α is not inhibited by retinoic acid treatment (82) as TNF α has been clearly shown to upregulate HIV-1 expression by induction of NF-κB (265). Retinoic acid has also been shown to counteract the effects of TNF α treatment in endothelial cells (420).

A possible explanation for the disparate effects of retinoic acid in U1 cells may lie in the ability of TNF α and PMA to stimulate NF-κB by distinct pathways. This has been shown to be the case in T cells. In EL-4 cells, a T cell line, PMA is less selective than TNF α in that it induces lymphokine production as well as NF-κB activation (126). Furthermore PMA has been shown to be able to stimulate either an NF-κB mutant promoter (273) or the expression of an NF-κB mutant virus in T cells (421), whereas TNF α cannot. It is therefore possible that the reason for the disparate effects of retinoic acid on PMA and TNF α stimulation of HIV-1 replication in U1 cells (82) is due to of a similar difference in the action of PMA and TNF α in cells of the monocyte/macrophage lineage.

6.4. The control of HIV-1 promoter activity in Jurkat T cells.
Further evidence for the involvement of NF-κB in the inhibition of HIV-1 promoter activity by retinoic acid is provided by transient transfection experiments in the human T cell line Jurkat. T cells have been shown to express retinoic acid receptor (402) and retinoids have been shown to modulate T cell function as discussed in Chapter 5. The data presented in Chapter 5 demonstrate that retinoic acid is able to inhibit the NF-κB mediated stimulation of the HIV-1 promoter in a further cell type, which is also critically involved in the pathogenesis of HIV-1 infection. Overexpression of NF-κB, by transfection of expression vectors for active NF-κB subunits, stimulates cotransfected HIV-1 promoter activity as previously described (156, 222). Treatment of these cells with retinoic acid inhibits the activation of the HIV-1 promoter (Figure 5.3.) as was shown for the PMA stimulation in U937 cells (Figure 3.6.) and HeLa cells (Figure 4.3.). This inhibition, in Jurkat cells is increased by the overexpression of RAR α (Figure 5.5.) as was shown in HeLa cells (Figure 4.3.), further confirming the role of the retinoic acid receptors in the observed effects. Over-expression of RAR cannot inhibit the activation of NF-κB mediated stimulation by more than 60 %. This is also true in HeLa cells and presumably reflects the equilibrium between RAR and NF-κB or Sp-1 binding.

The basal rate of HIV-1 promoter activity in these cells is slightly stimulated by retinoic acid treatment. However the inhibition of NF-κB stimulated HIV-1 promoter activity by retinoic acid is presumably mediated by a mechanism similar to that in U937 and HeLa cells. Retinoic acid receptor could compete with NF-κB and Sp-1 for their binding sites in the LTR. This competition, between the strongly activating NF-κB-Sp-1 synergy and the weakly activating retinoic acid receptor, would lead to an intermediate level of activation. This model requires that the basal stimulation by NF-κB in these cells is weak, and that NF-κB or Sp-1, and retinoic acid receptor binding are mutually exclusive. Basal levels of NF-κB in Jurkat cells have been shown to be low: mutation of the NF-κB sites in the LTR reduces basal promoter activity by approximately two fold (222). Furthermore, retinoic acid receptor has been shown to bind the Sp-1 and NF-κB sites in a mutually exclusive manner in the presence of RXR β (417). A similar mutually exclusive binding model has been described for the regulation of the mouse proliferin promoter by glucocorticoid hormone and AP-1, (342) (see section 1.2.6.2.).

The small stimulation of HIV-1 promoter activity by retinoic acid is able to synergise with the stimulation by PMA treatment (Figure 5.6). This effect is not mediated by the retinoic acid receptor binding site, Site B, (Figure 5.7) or any site 5' to position -139 relative to the transcription start site (Figure 5.8), as demonstrated by the use of the site B mutant HIV-CAT construct and the -139 HIV-CAT deletion mutant respectively. The synergy is mediated by an activity induced by PMA which
is distinct from the activation by NF-κB p50 or p65, as shown by the fact that activation of the HIV-1 promoter by expression of these proteins is not able to synergise with the activation by retinoic acid. This result is in contrast to that produced by retinoic acid and PMA treatment of U937 cells or HeLa cells transfected with HIV-CAT. PMA treatment has been clearly shown to stimulate NF-κB in these cells (136, 194) so it appears that PMA is stimulating an activity in addition to NF-κB which is able to cooperate with retinoic acid. It is therefore of interest to note that T cells infected with an NF-κB mutant virus can be induced to produce virus by PMA treatment (421). Furthermore, a TATA binding protein has been shown to be induced by PMA treatment of T cells (273). The induction of this protein, which is able to activate an NF-κB mutant HIV-1 promoter, could alter the nature of the initiation complex, thereby allowing both NF-κB and ligand activated RAR to activate transcription of the same promoter. The ability of both proteins to activate transcription of the same promoter would result in the synergistic promoter activation seen in Figures 5.6-5.8. Hershlag and Johnson (117) have argued that this type of synergy does not require that both activators be present on the DNA at once. The synergy could therefore be mediated despite mutually exclusive binding of NF-κB or Sp-1 and RAR. However the PMA induced factor could allow RAR to cooperate with NF-κB when bound to the Sp-1 site. A similar phenomenon was described for thyroid hormone receptor, which is only able to activate transcription from the Sp-1 site in the presence of the viral transactivator protein tat (417). In this case the tat protein may provide an intermediate for, or allow an interaction between, ligand activated thyroid receptor and a member of the basal transcription machinery. A further possibility to explain the cooperation between PMA and retinoic acid is that retinoic acid is able to induce expression of NF-κB which PMA is then able to activate.

As discussed in Chapter 5, retinoic acid has been shown to have wide ranging effects on T cells. One of the best characterised effects is the retinoic acid mediated inhibition of IL-2 synthesis in T cells (402, 406). Expression of the IL-2 gene is controlled by an upstream enhancer containing binding sites for AP-1, NF-κB, Octamer and NF-AT (nuclear factor activated T cells). These binding sites are non-consensus and stimulation of IL-2 requires the cooperation between factors activated by protein kinase C activation as well as calcium mediated signals, reviewed in (48, 422). It has been shown that one target for the retinoic acid mediated inhibition of IL-2 is an octamer motif which binds the inducible nuclear factor complex OAP-40. The constituents of this complex include oct 1 and oct 2, as well as the AP-1 subunits fos and jun (422-425). Fos and jun are required for transcriptional activation by this complex (406) and RAR α has been shown to be able to inhibit the formation of the AP-1-DNA complex in vitro. This results in an interference with the oct-2 dependent cis-regulatory function of this octamer motif and consequently an inhibition of IL-2
expression (406). This octamer element was also shown to be able to confer negative modulation by retinoic acid to a heterologous promoter (402).

The IL-2 enhancer also contains a binding site for NF-κB. Mutation of this site has been shown to affect IL-2 promoter activity in transient transfection experiments (426). Figure 4.8 demonstrates that the NF-κB site from the IL-2 promoter is also able to confer negative regulation by retinoic acid to a heterologous promoter. It is therefore likely that the inhibition of IL-2 gene expression by retinoic acid is mediated in part through the inhibition of NF-κB activity. Furthermore the inhibition of the activity of a promoter enhanced by 4 copies of the NF-κB site from the mouse MHC H2k gene provides further evidence that retinoic acid is able to modulate the action of NF-κB in transcriptional activation (Figure 4.9.). The transcriptional control of HIV-1 promoter activity by retinoic acid, mediated through its nuclear receptors, has been shown by the data presented here and in references cited to depend on cell type specific factors. Retinoic acid can upregulate promoter activity through the NRRE (3, 337) and can inhibit, and perhaps stimulate, promoter activity through the NF-κB and/or Sp-1 sites. Differentiation of cells, infected by HIV-1, by retinoic acid is also able to modulate HIV-1 promoter activity through NF-κB (137, 138, 207) and TATA binding factors (412). Retinoids have been used with some success in the treatment of AIDS related conditions such as Kaposi's sarcoma (427), pseudo hairy leukoplakia (428), Reiters syndrome (4, 5) and rectal ulcer (429) but due to the complexity of the control of HIV-1 promoter activity by retinoic acid it is unlikely that any therapeutic effects could be predicted from these in vitro data.

6.5. Implications from the inhibition of NF-κB function by retinoic acid.

The inhibition, by retinoic acid, of NF-κB mediated activation demonstrated for both IL-2 and HIV-1 promoters, as well as the MHC H2k NF-κB site enhanced minimal promoter may represent a further mechanism whereby retinoic acid is able to regulate immune function. As discussed in the Introduction, NF-κB has been implicated in the control of the expression of a wide variety of genes involved in immune function. Cellular activating signals induce NF-κB activity which then mediates further expression of genes necessary for the active phenotype. For example, activation of T cells by a wide variety of activating signals, including antigen, treatment with anti CD3, anti-CD2, anti-CD28, Ca-ionophores, phorbol esters, lectins, or TNF α leads to induction of NF-κB. Thus NF-κB is regulated by a wide variety of stimuli which in turn regulate a wide variety of genes, mediated in part through the activation of NF-κB.

The diversity of signalling pathways involving NF-κB is mediated, in part, by the combination of several different NF-κB subunits which heterodimerise to form
transcription factors with different specificities and activation potentials. The diversity achieved by NF-κB is further increased by its ability to activate transcription synergistically with a number of transcription factors. These include Sp-1 in the HIV-1 enhancer (222) and possibly in the enhancers for IL-2 receptor α chain (430, 431), the ICAM-1 enhancer (432) and the GM-CSF enhancer (199). NF-κB appears to cooperate with NF-IL6 in the IL-6 enhancer (433), NF-ELAM1 on the NF-ELAM1 enhancer (434), helix-loop-helix proteins in the κ light chain enhancer (435), and with several factors in the IL-2 enhancer (436). It is therefore possible that a further factor able to regulate the expression of genes controlled by NF-κB is retinoic acid. These effects will be dependent on the presence of the retinoic acid receptors, as demonstrated in Chapters 4 and 5, and are likely to be further affected by the receptor subtype expressed, see Figure 4.3, where the γ receptor is able to inhibit basal HIV-1 promoter activity to a greater degree than the α and β receptors.

Retinoic acid has been shown to have wide ranging effects on immune function, the mechanisms of which are not well understood. Derivatives of vitamin A have anti-inflammatory effects in chronic dermatological diseases in humans as well as in experimental models of inflammation such as adjuvant arthritis (376) or ultraviolet-induced erythema (437). The mechanisms of action of retinoids in anti-inflammation are generally unclear although the beneficial effects of retinoic acid in adjuvant arthritis have been assumed to be explained, at least in part, by the inhibition of the synthesis of collagenase (376, 438). It has been shown that retinoic acid is able to antagonise the effects of the transcription factor AP-1 in a DNA independent manner. This effect has been demonstrated to be directly responsible for the inhibition of the synthesis of the enzymes collagenase and stromelysin. Both of these proteins are proteases secreted by synoviocytes and are implicated as causative factors in inflammatory conditions, such as rheumatoid arthritis, where they play an important role in joint destruction (439-441). This may explain the anti-inflammatory mechanism of retinoic acid in this case. Additionally retinoic acid has been shown to inhibit collagenase synthesis in monocytes in a dose dependent manner (390), and to modulate the actions of the inflammatory cytokine IL-1 on endothelial cells (442).

The antagonistic effects of retinoic acid on the action of the tumour promoter PMA in the mouse skin carcinogenesis model have long been known (443, 444). Retinoids have also been shown to be effective in the treatment of human skin cancer (290). These effects have recently been suggested to be due to the ability of retinoic acid to antagonise AP-1 (445). However the ability of PMA to activate NF-κB and the ability of retinoic acid to inhibit the action of NF-κB, as shown here, suggest that the antagonism of NF-κB action may also play a role in anti-tumour-promotion by retinoic acid. Additionally the retinoic acid analogue RO-109359 has been shown to
inhibit the PMA induced GM-CSF mRNA levels in an epidermal model of tumour-promotion (446).

The interactions between members of the AP-1 complex and retinoic acid receptors have now been well established (353, 354, 445) and are likely to contribute to the ability of retinoic acid to regulate such wide ranging processes. However, the wide role of NF-κB in inflammatory processes detailed in the Introduction and the ability of retinoic acid to antagonise transcriptional activation by NF-κB, as demonstrated in this thesis, may indicate a further mechanism by which retinoic acid may mediate its anti-inflammatory and anti-tumour-promoter properties. Interestingly a new class of synthetic retinoid has recently been described which are able to selectively inhibit the action of AP-1 but are unable to activate receptor mediated transcriptional activation. These retinoids are able to inhibit the proliferation of several tumour cell lines but have no effect on F9 differentiation which is mediated through the retinoid nuclear receptors (447). These retinoids may be of great clinical use as their side effects, known to be a problem in retinoid therapy (290, 292, 448, 449), will be minimised by their specificity. It will be interesting to determine whether these retinoids are able to modulate NF-κB activity.
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