CYTOKINE GENE EXPRESSION IN HUMAN IMMUNODEFICIENCY VIRUS INFECTED MYELOID CELLS

by

MARIO G. D'ADDARIO JR.

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Department of Microbiology and Immunology
McGill University, Montreal
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ABSTRACT

Cytokines are polypeptide hormones secreted by immune and non-immune cells in response to infection. Cytokines mediate a number of actions including inflammatory responses, activation or suppression of ancillary cells, destruction of tumors, and elimination of pathogens. HIV selectively infects $CD4^+$ T lymphocytes and monocyte/macrophages resulting in the functional alterations of these cells. To further understand the effects of HIV infection on cytokine gene expression, we studied the production of cytokines from myeloid U937 and myelomonoblastic PLB-985 cells infected with HIV-IIIB. Results indicate that U9-IIIB and PLB-IIIB cells stimulated with PMA, LPS or recombinant cytokines transcribed and translated significantly higher levels of TNF-α, IL-1α, IFN-α, IL-1β, and IFN-β. Furthermore it appears that HIV infection of PLB-985 cells induces monocytic differentiation and alterations in the binding of NF-κB related proteins. PLB-IIIB cells demonstrated characteristics reflecting differentiation including morphological alterations, changes in *c-fms* and *c-myc* proto-oncogene expression, and increased expression of CD14 cell surface antigen. Examination of the IL-1β promoter revealed a DNA sequence capable of interacting with the NF-κB family of proteins; oligonucleotides containing 1-3 copies of this IL-1β-κB sequence were synthesized and shown to bind NF-κB proteins from LPS and PMA treated U937 and U9-IIIB cells. Recombinant NF-κB protein subunits specifically bound monomeric and dimeric copies of this IL-1β-κB sequence and binding was eliminated by competition with unlabeled wild type but not mutant oligonucleotides. The transcriptional activity of this IL-1β-κB site was examined by subcloning 1, 2, and 3 copies of this sequence upstream of an enhancerless SV_1CAT reporter plasmid. Monomeric, dimeric, and trimeric copies of the IL-1β-κB sequence were transcriptionally active and inducible by LPS and PMA in promonocytic U937, fibroblast 293, and Jurkat T cells. An IL-1β CAT reporter plasmid containing 4,400 bp of promoter appears to be more transcriptionally active in HIV infected cells than in uninfected myeloid cells. These results indicate that IL-1β gene expression may be significantly elevated in HIV infected cells and that transcriptional regulation by NF-κB/*rel* proteins may mediate its activation.

RÉSUMÉ

Les cytokines sont des hormones protéiques sécrétées par les cellules du système immunitaire et autres cellules en réponse à une infection virale. Les cytokines jouent un rôle de médiateur dans les réponses inflammatoires, l'activation ou suppression des cellules cibles, la destruction de cellules tumorales et l'élimination de substances pathogènes.

Le VIH infecte de façon sélective les lymphocytes T CD4+ et les monocytes/macrophages, rendant ces cellules anormalement fonctionnelles. Afin de comprendre les effets de l'infection du VIH sur l'expression génique, nous avons analysé la production des cytokines dans les cellules myéloides U937 et myélomonoblastiques PLB-985 infectées par la souche HIV-IIIB. Nos résultats montraient que dans les cellules U9-IIIB et PLB-IIIB induites au PMA, LPS ou par des cytokines recombinantes, la transcription et la traduction de TNF-α, IL-1α, IFN-α, IFN-β, et IL-1β se faisaient à des niveaux plus élevés. De plus, l'infection par le VIH semble induire la différentiation de ces cellules vers un profil monocytaire. Les PLB-IIIB présentaient plusieurs caractères de cellules différenciées; des changements dans la morphologie, dans l'expression des oncogènes cellulaires *c-fms* et *c-myc*, ainsi qu'une augmentation dans l'expression de l'antigène de surface CD14 étaient observés. L'analyse de la séquence du promoteur de l'IL-1β a révélé l'existence d'un site de fixation pour les protéines NF-κB; des oligonucléotides représentant 1 à 3 copies de la séquence IL-1β-κB étaient capables d'intéragir avec les protéines NF-κB des cellules U937 et U9-IIIB traitées au PMA et au LPS. Les sous-unités recombinantes de NF-κB se fixaient de façon spécifique aux copies monomériques et dimériques de la séquence IL-1β-κB, comme le démontraient des éxperiences de compétition. Les séquences IL-1β-κB étaient transcriptionellement actives et inductibles par LPS et PMA dans des cellules d'origine lympöide, promonocytaire, ou fibroblastique. Le plasmide IL-1β CAT 4.4 contenant un fragment de 4,400 bp du promoteur était transcrit plus éfficacement dans les cellules infectées que dans des cellules non infectées. Ces resultats indiquent que l'expression du gène IL-1β pourrait être augmentée de façon significative dans les cellules infectées par le VIH, et qu'un régulation par la famille de facteurs de transcription NF-κB/*rel* pourrait être responsable de cette activation.

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CONTRIBUTIONS TO ORIGINAL KNOWLEDGE

The following novel findings have been demonstrated in this thesis:

1. The production of cytokines TNF-α and IL-1β has been evaluated in HIV infected promonocytic U937 and myelomonoblastic PLB-985 cells. The results clearly demonstrate that stimulation of these cells with antigenic stimuli, LPS, and recombinant cytokines, such as TNF-α significantly upregulates transcription and translation of IL-1β and TNF-α. In HIV infected PLB-985, the rate of TNF-α RNA degradation was more rapid than in the parental PLB-985 population, indicating that elevated secretion of TNF-α protein from these cells was due to increased translational efficiency.

2. The interleukin-1β promoter was found to contain a DNA sequence binding to NF-κB proteins. This was demonstrated using proteins obtained from phorbol ester stimulated myeloid cells, using recombinant NF-κB p50, and IκB to inhibit binding of proteins from whole cell extracts. Competition using wild type, but not mutant unlabeled oligonucleotides confirmed this interaction, and also demonstrated that relative to the IFN-β P2 sequence, the protein-DNA adduct generated with the IL-1β-κB sequence was slightly less stable.

3. The insertion of monomeric, dimeric, and trimeric copies of the IL-1β-κB oligonucleotides upstream of an enhancerless CAT reporter plasmid demonstrated that these sites were transcriptionally active and inducible by both PMA and LPS in myeloid, lymphoid, and fibroblastic cells. These results demonstrate that this novel NF-κB sequence (GGGAAAATCC) is capable of interacting with NF-κB proteins, and is transcriptionally active in different cell lineages.

4. Activation of an interleukin-1β CAT reporter plasmid containing 4,400 bp of promoter sequence was observed in uninfected and HIV infected myeloid cells, and found to be more inducible by LPS and PMA in HIV infected cells. This result indicates that the transcriptional activity observed in HIV infected cells using PCR-mRNA phenotyping is reflected by the expression of the IL-1β CAT reporter plasmid.

PREFACE

The author was responsible for the all the data presented in this thesis, the data can be found in the following manuscripts:

D'Addario, M., G. Bensi, and J. Hiscott. The interleukin-1β promoter contains a functional NF-κB site (manuscript in preparation)

D'Addario, M., M. A. Wainberg, and J. Hiscott. 1992. Activation of cytokine genes in HIV-1 infected myelomonoblastic cells by phorbol ester and tumor necrosis factor. J. Immunol. 148: 1222-1229

Roulston, A., M. D'Addario, F. Boulerice, S. Caplan, M A. Wainberg, and J. Hiscott. 1992. Induction of monocytic differentiation and NF-κB like activities by human immunodeficiency virus-1 infection of myelomonoblastic cells. J. Exp. Med. 175: 751-763

D'Addario, M., A. Roulston, M. A. Wainberg, and J. Hiscott. 1990. Coordinate enhancement of cytokine gene expression in human immunodeficiency virus type-1 infected promonocytic cells. J. Virol. 64: 6080-6089

In addition, the candidate was also responsible for data found in the following manuscripts:

Figures 2A and 4A of,
Gosselin, J., L. Flamand, M. D'Addario, J. Hiscott, and J. Menezes. 1992. Infection of peripheral blood mononuclear cells by herpes simplex and Epstein Barr viruses: Differential induction of interleukin 6 and tumor necrosis factor alpha. J. Clin. Invest. 89: 1849-1856

Figures 5 and 6 of,
Gosselin, J., L. Flamand, M. D'Addario, J. Hiscott, J. Stefanescu, D. V. Ablashi, R. C. Gallo, and J. Menezes. 1992. Modulatory effects of Epstein Barr virus, herpes simplex and human herpes 6 viral infections and co-infections on cytokine synthesis: A comparative study. J. Immunol. 149: 181-187

Figure 3 of,

Boulerice, F., R. Geleziunas, S. Bour, H. Li, M. D'Addario, A. Roulston, J. Hiscott, and M. A. Wainberg. 1992. Differential susceptibilities of U937 cell clones to infection by HIV-1. J. Virol. 66: 1183-1187

Figures 1A, 2A, and 3A of,

Flamand, L., J. Gosselin, M. D'Addario, J. Hiscott, D. V. Ablashi, R. C. Gallo, and J. Menezes. 1991. Human herpes virus-6 induced interleukin-1β and tumor necrosis factor alpha but not interleukin 6 in peripheral blood mononuclear cell cultures. J. Virol. 65: 5105-5110

Figure 3 of,

Gosselin, J., L. Flamand, M. D'Addario, J. Hiscott, and J. Menezes. Epstein Barr virus infection and monokine synthesis, in: Epstein Barr Virus and Associated Diseases, Ablashi, D. (ed.), Humana Press, New York, 1991. pp 147-151

Figures 3A, B, C, and D of,

Gosselin, J., J. Menezes, M. D'Addario, J. Hiscott, L. Flamand, G. Lamoureux, and D. Oth. 1991. Inhibition of tumor necrosis factor-α transcription by Epstein Barr virus. Eur. J. Immunol. 21: 203-208

Figure 1 of,

Lacoste, J., M. D'Addario, A. Roulston, M. A. Wainberg, and J. Hiscott. 1990. Cell specific differences in activation of NF-κB regulatory elements of human immunodeficiency virus and beta interferon promoters by tumor necrosis factor. J. Virol. 64: 4726-4734

Conclusions from these studies will be reviewed in the Discussion of this thesis

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INTRODUCTION

A) Human Immunodeficiency Virus Infection, Pathogenesis and Monocyte/Macrophages.

The human immunodeficiency virus (HIV) infects a number of cell types through its interaction with cell surface CD4 molecules. Two of the most important target cells infected by HIV are the $CD4^+$ T lymphocytes and cells of the monocyte/macrophage lineage. Although HIV infected T lymphocytes are selectively depleted during HIV progression, the monocyte/macrophage lineage is capable of maintaining latent HIV DNA in its genome for extended periods of time. This cell lineage therefore serves as a reservoir of HIV, and stimulation of these cells by various agents can strongly activate HIV production (see Fauci, 1988, Levy, 1989, and Roy and Wainberg, 1988, for reviews).

Circulating monocytes have been shown to contain little HIV, but macrophages found in many tissues contain high levels of intracellular HIV. Recovery of HIV has been demonstrated for adherent mononuclear cells isolated from whole blood of seropositive patients, skin derived Langerhans cells, follicular dendritic cells of lymph nodes, bronchoalveolar macrophages, spinal and brain macrophages, and bone marrow stem cells (reviewed in Gendelman et al., 1989, Langhoff et al., 1991, Meltzer et al., 1990, and Rosenberg and Fauci, 1990). Monocyte/macrophages infected with HIV have been shown to have altered functions including decreased expression of cell surface markers (HLA-DR, and CD4), decreased receptor mediated phagocytic capabilities, decreased antigen presentation function, decreased intracellular killing of heterologous pathogens, decreased microbiocidal activity, decreased chemotaxis, and deregulated production of cytokines (Chantal-Petit et al., 1987, Ennen et al., 1990, and Gendelman et al., 1989). Also, as its name implies, HIV induced immunodeficiency results in the coinfection of a number of other

viruses including herpes simplex virus (HSV), human herpes-6 virus (HHV-6), Epstein Barr virus (EBV), cytomegalovirus (CMV), human T cell lymphotropic virus type 1 (HTLV-1), and hepatitis B virus (HBV); generally these viruses cause infections that would ordinarily be suppressed in non HIV-infected individuals. These coinfections may result in liver dysfunction induced by HBV, or CMV induced retinitis or blindness. Furthermore, late stage acquired immunodeficiency syndrome (AIDS) is associated with a number of other bacterial, fungal, or viral infections, and neurotropic disorders which contribute to the pathogenesis observed in these individuals (see Gendelman et al., 1989, for review).

Infection of T cells and monocyte/macrophages initially occurs through the interaction of the HIV gp120 protein with the cell surface CD4 molecule (reviewed in Zack et al., 1990). The CD4 surface antigen is present on cell types that play an important role in normal immune function, and functions to enhance the interaction between T cells and antigen presenting cells (reviewed in Parnes, 1989). Decreased cell surface CD4 expression caused by HIV infection contributes to the observed immunodeficiency (reviewed in Fauci, 1988). Monoclonal antibodies specific to the CD4 molecule are able to inhibit HIV entry into cells, while antibodies to other cell surface molecules do not prevent infection (Maddon et al., 1986, and Zack et al., 1990). Furthermore, expression of the CD4 molecule by transfecting the cloned CD4 gene in non-CD4 expressing cells renders these cells permissive to HIV, demonstrating the necessity of this surface antigen for infection (Maddon et al., 1986). Entry of HIV into cells after interaction with CD4 is thought to occur by fusion of the viral lipid bilayer with the cellular membrane resulting in a virus particle released into the cytoplasm as a free virion not enclosed in an endocytic vesicle. HIV virions containing the single stranded RNA genome then freely migrate to the nucleus

where HIV specific reverse transcriptase generates double stranded DNA that randomly integrates into the host cell genome.

Activation of latent virus is dependent on many factors (described later), but as demonstrated for other lentiviruses, replication of HIV is fully permissive in neural cells, and in lung and tissue macrophages. Persistant HIV infection is characterized by the ability of the virus to evade the host immune system by either remaining latent in the genome of host cells for extended periods of time, or by limiting the expression of viral proteins on the membranes of infected cells. Another mechanism by which HIV evades the immune response is the ability of the virus to produce mutated forms of its surface proteins, thereby evading a slower developing immune response (see Rosenberg and Fauci, 1990, for review).

One of the most interesting phenomenon associated with HIV infection, particularly with *in vitro* cell culture is that of cellular tropism (reviewed in Gendelman et al., 1989). It has been demonstrated that HIV isolates from patients transferred to *in vitro* cell culture and passaged in either monocyte or T cell cultures, acquired a strange characteristic. Viruses isolated from monocyte cultures grown in the presence of macrophage-colony stimulating factor (M-CSF) were capable of growth in T cell cultures, while those HIV isolates initially grown in T cells in the presence of phytohaemaglutinin (PHA) and interleukin-2 (IL-2) were unable to grow in monocyte cultures. Although the true nature of this tropism is not well understood, it is assumed that initial isolation of virus from HIV infected individuals produces a number of different tropic strains, and passage through either cell system preferentially allows for the survival of one strain.

Entry of HIV into cells and selective diminution of CD4 expression on membranes of infected cells along with other immunological changes places HIV infected individuals in a severely compromised position. The exact mechanism of T_4 lymphocyte cell death is not well known, but a few postulates have been made (reviewed in Rosenberg and Fauci, 1990). Firstly, release of HIV (virus budding) is thought to create microholes in the membrane, thus altering permeability of the membrane and disrupting the ionic balance present at the membrane. HIV infected cells may also accumulate large amounts of unintegrated viral DNA in the cytoplasm or nucleus that disrupt normal cellular activities. Another interesting phenomenon associated with HIV infected T cells is the formation of multinucleated giant cells (syncitia), which result from the fusion of HIV infected cells with non infected cells. The cells are thought to be so large that the membrane is thinned to a point whereupon it collapses. Interestingly, this phenomenon has not been observed as readily with infected monocyte/macrophage cultures as it has with T lymphocyte populations.

Pathogenesis in AIDS patients is due mainly to immune dysfunction resulting in AIDS related disorders such as autoimmune reactions against MHC class II, or haematological abnormalities including leukemias, anemias, and thrombocytopenias. These disorders may result not only from HIV infection of T cells and monocytes, but also of bone marrow-derived stem cells (described later). Cytokines released from HIV-infected cells are also capable of dysrupting normal physiology. Tumor necrosis factor alpha (TNF-α) which is known to cause wasting during chronic infections (Carswell et al., 1975, and reviewed in Vilcek and Lee, 1991) has been found to be produced at high levels in HIV infected persons. This cytokine, at excessive levels may contribute to the cachexia and wasting observed in late stage AIDS and AIDS related complexes (ARC). Furthermore, TNF-α has also been shown to be involved in the

activation of latent HIV in both T lymphocytes and monocyte/macrophages.

More than 60 % of AIDS patients have neurotropic disorders, commonly known as AIDS dementia complex or AIDS encephalopathy. HIV particles have been isolated from the cerebrospinal fluid (CSF) of both AIDS patients and asymptomatic seropositive individuals. In the CSF, HIV is thought to mediate its effects in part by inhibiting the growth of neurons. More specifically, HIV gp120, in the presence of the neurotropic growth factor neuroleukin, is able to suppress sensory neuron cell development. In conjunction with other heterologous pathogens, HIV may stimulate neural disorders such as CMV induced encephalitis, cryptococcal meningitis, and toxoplasmosis (reviewed in Rosenberg and Fauci, 1990). In these cases it appears that HIV stimulates other infectious agents to induce disorders normally suppressed in immuno-competent individuals.

Activation of latent HIV by a number of mitogens such as lipopoly-saccharide (LPS), phorbol ester (PMA), phytoheamagglutinin (PHA), or cytokines including interleukin-1 (IL-1), interleukin-6 (IL-6), and tumor necrosis factor-α (TNF-α), and virally encoded proteins from CMV, EBV, HSV, and HTLV-1 has been shown to occur both in T cells and monocyte/macrophages. These agents stimulate a number of constitutive and inducible host cellular transcription factors including promoter specific transcription factor Sp-1, activation protein-1 (Ap-1), nuclear factor κB (NF-κB), nuclear factor of activated T cells (NFAT-1), upstream sequence binding factor (USF), and general transcription factor IID (TFIID) (see Greene, 1990, and Vaishnav and Wong-Staal, 1991, for reviews). Activation of latent HIV usually involves these specific cellular factors interacting at discrete sites within the HIV long terminal repeat (HIV-LTR). The HIV genome consists of at least nine genes which are differentially activated and spliced to produce a number of different gene

products. Some genes -*gag*, *pol*, and *env*- code for structural proteins, while *tat*, *rev*, *nef*, *vpu*, *vpr*, and *vif* code for regulatory products. Initial activation of HIV gene expression by cellular factors induces the production of viral proteins Tat, Rev, and Nef from doubly spliced viral RNA transcripts (reviewed in Cullen and Greene, 1989, and Varmus, 1988). Tat can subsequently stimulate HIV expression at the RNA level through its interaction with the HIV trans-activation response element (TAR) present within the first 60 nucleotides of all HIV RNA transcripts (see Sharp and Marciniak, 1989, for review). This activity has also been demonstrated *in vitro* using a reconstituted transcription system containing Tat protein and DNA templates containing the TAR region (Marciniak et al., 1990). Conversely, expression of Nef protein may induce latency, and may act in opposition to tat to trans-repress HIV transcription.

The *rev* gene product is found preferentially in the nucleoli of infected cells and facilitates the conversion of HIV transcription to single spliced transcripts that include the gag, pol, and env structural proteins (reviewed in Varmus, 1988). The stimulation of gag, pol, and env transcripts by rev concomitantly suppresses the expression of doubly spliced messages encoding Tat, Rev, and Nef. Therefore rev allows for the production of viral structural proteins that ultimately form mature viruses, and simultaneously suppresses the production of HIV regulatory proteins. After the initial activation of latent HIV by host cell proteins, HIV is able to utilize viral regulatory proteins to modulate its own production.

B) Cytokines and HIV Infection.

Cytokines comprise a network of soluble mediators released predominantly by hematopoietic cells; in general, cytokines enhance or suppress ancillary cell functions that act to eliminate infectious agents (ie: bacteria, viruses, tumors etc.) or toxins. Production of different interleukins (ILs), interferons (IFNs), colony stimulating factors (CSFs), and tumor necrosis factors -α and -β (TNFs) have been shown to increase rapidly and to very high levels in response to infectious agents. These molecules have been shown to be secreted by a number of cells including T and B lymphocytes, monocyte/ macrophages, natural killer cells, neutrophils, fibroblasts, dermal dendritic, leukemic, and neural cells (astrocytes, and microglia) (reviewed in Arai et al., 1990). Cytokine release causes a number of events including inflammation, fever and other pyrogenic phenomenon, induction of an antiviral state and stimulation of cell proliferation. Cytokines are polypeptide hormones that upon secretion can act in distant tissues, and interact with specific receptors to alter the behavior of target cells. The precise biological effect of a cytokine will be determined by the milieu in which it acts, and the other effector cytokines present.

Bi) Interferons.

Interferons are proteins present in three distinct forms; interferon-α (IFN-α), interferon-β (IFN-β) and interferon-γ (IFN-γ). These proteins were first identified due to their antiviral properties, but have now been demonstrated to affect other cellular activities such as differentiation, cell proliferation and modulation of MHC class I surface antigen expression. IFN-α and IFN-β are secreted by most nucleated peripheral blood leucocytes, while IFN-γ is produced exclusively by activated T lymphocytes. The genes encoding IFN-α and IFN-β lack introns

while IFN-γ is coded by four exons. The proteins are also structurally different; IFN-α and -β are acid stable whereas IFN-γ is acid labile. IFN-α and -β interact with a common receptor while IFN-γ binds a distinct receptor. Activation of interferon genes occurs in response to a number of agents including double stranded RNA containing viruses, poly rI:rC. and cytokines (platelet derived growth factor, colony stimulating factor-1, and interleukin-1). A complete review of interferons can be found in Pestka et al. (1987); transcriptional regulation of these genes is discussed in Taniguchi (1988).

Bii) Interleukin-2.

The interleukins comprise a family of cytokines produced by a number of cells, one of the most important is interleukin-2 (IL-2). This factor is produced mainly by activated $CD4^+$ T lymphocytes and acts in an autocrine manner to stimulate T cell division and development. IL-2 is released in response to T cell mitogens phytohaemaglutinin (PHA) and concanavalin A (Con A), is ~15 kDa, and acts to stimulate T lymphocyte expansion, causes B lymphocyte growth, differentiation and secretion of immunoglobulins (Ig). This activation is due to the ability of IL-2 to enhance expression of the IL-2 receptor, and other membrane proteins such as MHC class II, transferrin and insulin receptors. IL-2 has potential anti-tumor activities due to its ability to activate the non-specific killing activity of lymphocyte activated killer cells and tumor infiltrating lymphocytes (reviewed in Arai et al., 1990, and Smith, 1988).

Biii) Interleukin-6.

One of the most intensively studied cytokines, due to the multitude of activities it encompasses is interleukin-6 (IL-6). This cytokine, produced by T and B lymphocytes, endothelial cells, monocyte/macrophages, and fibroblasts was formerly known as interferon-β_2, B cell stimulatory factor-2 (BSF-2), hepatocyte stimulatory factor (HSF), and hybridoma plasmacytoma growth factor (PCGF). IL-6 is produced by these cells in response to bacterial or viral infections, LPS treatment and cytokines such as IL-1 and TNF-α either alone or in combination with IFN-γ, platelet derived growth factor (PDGF) and granulocyte macrophage-colony stimulating factor (GM-CSF). The protein is 26 kDa, and interacts with receptors present mainly on T and B cells. Interaction with B cell receptors causes B cell growth and Ig secretion. IL-6 induces IL-2 secretion and thymic T cell differentiation in synergy with T cell mitogens, it also stimulates hematopoietic stem cell growth and differentiation. IL-6 is a potent mediator of inflammation, it activates the acute phase response proteins of hepatocytes and when released by astrocytes of the nervous system may function to induce the differentiation of neural cells (see Arai et al., 1990, and Van Snick, 1990 for reviews). One of its most detrimental characteristics is its ability to stimulate tumor cell growth. A number of myelomas, plasmacytomas and hybridomas have been shown to require IL-6 for growth. In multiple myeloma, neoplastic blast cells not only secrete elevated levels of IL-6, but use the cytokine to induce their own development and replication (Kawano et al., 1988). High levels of IL-6 have also been observed in the serum of AIDS patients, and are easily released from stimulated B lymphocytes of seropositive individuals (Honda et al., 1990)

C) Tumor Necrosis Factor-α and -β.

One of the major cytokines produced by immune cells is one that has also been found to be deleterious at high levels, tumor necrosis factor-α (TNF-α). Found over two decades ago to mediate nonspecific tumor cell killing, TNF-α was purified from endotoxin stimulated mice and determined to be the cause of wasting and anorexia in these animals (reviewed in Beutler and Cerami, 1989).

TNF-α is a non glycosylated ~17 kDa protein produced mainly by activated monocyte/macrophages, and in smaller quantities by lymphocytes, endothelial cells, astrocytes, and natural killer cells. In serum, TNF-α is not isolated as a monomer, but as a tightly packed immunoreactive trimer of ~50 kDa. This cytokine is capable of stimulating its own production IL-1, IL-6, IFN-β, CSFs and other growth factors, inflammatory mediators, and acute phase proteins. Significant accumulation of TNF-α RNA has been observed in stimulated cells indicating translation may also be tightly regulated (Sung et al., 1991). More generally, TNF-α stimulates glycerol release from adipose tissues, suppresses adipocyte differentiation, and activates neutrophil cytotoxicity, degranulation, and adherence. TNF-α is a potent endogenous pyrogen, it stimulates bone resorption, degradation of cartilage, and is directly cytolytic to a number of cells. Both the 55 and 75 kDa subunits of the TNF-α receptor are found on virtually all cells except erythrocytes; both receptors bind TNF-α with high affinity. The stimulation of either subunit with specific antibodies is sufficient to induce a wide range of TNF-α dependent phenomena (see Vilcek and Lee, 1991, for review). The cytolytic activities of TNF-α are due not only to the activation of particular signal transduction pathways inducing various intracellular functions, but also due to the capacity of TNF-α to physically enter the cell membrane, disrupt normal membrane permeability, and destroy ionic charge differences (Kagan et al., 1992).

Interaction of TNF-α with these high affinity receptors mediates a number of activities although the signal transduction pathway utilized by this cytokine receptor system is not well understood (described more completely in the discussion of this thesis). The receptors themselves have no intrinsic protein kinase activity, but a number of protein kinases have been shown to become activate within minutes of TNF-α interaction with receptor. Intracellular cAMP concentrations have also been demonstrated to increase with TNF-α addition to monocyte cultures, although less quickly than protein kinase activity. One activity which has been documented and will be described more completely in the discussion is the activation of transcription factor NF-κB by TNF-α. Many cytokines and viruses such as HIV have been shown to be transcriptionally regulated by TNF-α through NF-κB (Schütze et al., 1990). Therefore activation of HIV and cytokine genes in HIV infected cells may be mediated by NF-κB proteins in response to TNF-α. Furthermore, over expression of TNF-α in HIV infected persons may be stimulating viral and cellular gene activity, leading to increased viral burden in the host.

Tumor necrosis factor-β (TNF-β) or lymphotoxin is produced mainly by T lymphoid cells in response to phorbol esters, viruses, and cytokines such as IFN-γ, IL-2, and to T cell mitogens PHA, and Con A. The active peptide is glycosylated and is composed of a homotrimer of ~25 kDa. The protein is secreted from activated T cells and acts on epithelial cells to cause cytolysis. TNF-β stimulates the differentiation and expansion of other cells such as polymorphonuclear leukocytes and stimulates the expression of MHC class I antigens. TNF-β induces changes such as the redistribution of intracellular Ca^{++} stores, increased protein turnover, alterations in fatty acid metabolism, production of oxygen radicals, and induction of programmed cell death. A more detailed review of TNF-β activity can be obtained in Paul and Ruddle (1988).

D) Interleukin-1.

Produced by a number of cells including monocyte/macrophages, T and B lymphocytes, fibroblasts, neutrophils, and nervous system microglia and astrocytes, interleukin-1 (IL-1) affects almost all biological tissues and processes (reviewed in Dinarello, 1991, and Mizel, 1989). The steady state levels of interleukins *in vivo* is low, but production of interleukin 1, in response to inflammatory agents, bacterial, or viral infection, causes a rapid increase in serum levels, and a cascade of effects whose activities are evident in many tissues.

The effects of IL-1 are mediated by specific receptors which are responsive to both IL-1 subtypes (-α and -β); these receptors are found on many cell types including monocyte/macrophages, T and B lymphocytes, fibroblasts, hepatocytes, and endothelial cells. IL-1 induces a number of intracellular effects which are usually produced following the production of arachidonic acid metabolites including prostaglandins, and other lipoxygenase byproducts (see Dinarello, 1991, for review). The two IL-1 receptor types are of 68 and 80 kDa; they both contain extracellular domains found in the Ig superfamily, and although they can interact with both IL-1 -α and -β, demonstrate only ~30 % homology in the extracellular domain. The larger receptor type diverges from the smaller 68 kDa by possessing a larger intracellular carboxy terminal region. The intracellular domain does not have protein kinase activity per se, but has been shown to be phosphorylated at serine and threonine residues. It is not known if phosphorylation of these sites is required for biological activity. Although IL-1 and its receptors have been shown to activate the protein kinase A signal transduction pathway, evidence also indicates that once IL-1 binds to its receptor, the receptor-ligand complex can be directly translocated to the nucleus. Furthermore, this entry occurs prior to any IL-1 induced gene activation

event (Curtis et al., 1990).

Although both IL-1 forms (-α and -β) are ~17 kDa, and are initially generated as ~31 kDa precursors, they share only 25 % amino acid sequence homology. The immature 31 kDa peptide is processed in the cytoplasm, and has been shown to be immunologically active, while the 17 kDa peptide is secreted or membrane bound; release of membrane bound IL-1 may be due to apoptosis (Hogquist et al., 1991).

Formerly called endogenous pyrogen, IL-1 is a potent inducer of fever, sleep, inflammation, and release of acute phase response proteins of hepatocytes. It induces its own production, along with the production of IL-6, IL-2, IL-4, GM-CSF, and TNF-α. Although IL-1 is able to mediate a number of neurological functions including sleep induction, decrease in appetite, release of adreno-corticotropin hormone, and brain prostaglandins, one of the most interesting aspects of IL-1 biology is its inability to traverse the blood brain barrier. Therefore, its effects on the brain are thought to be due to its ability to activate the release of specific proteins from brain endothelial cells, a cell type that expressed both IL-1 receptor subtypes (reviewed in Pober and Cotran, 1990).

Transcriptional activation of the IL-1 gene has not been well characterized. Analysis of those agents capable of stimulating IL-1 production indicate that the gene is extremely responsive to bacterial endotoxin; treatment of cells with the protein synthesis inhibitor cycloheximide (CHX) causes superinduction of the gene. Furthermore, addition of actinomycin D (Act D) to cell cultures stimulated with LPS demonstrated that IL-1β RNA half life was very rapid, and may involve selective degradation (Fenton et al., 1988). Rapid degradation of cytokine RNA transcripts has also been observed for TNF-α, GM-CSF and IFN-β (reviewed in Arai et al., 1990). Many groups have analyzed the differential activation of both

IL-1α and IL-1β, and determined that LPS-induced monocytic cells produced mainly the -β form (Turner et al., 1989). One of the most interesting aspects of IL-1β gene activation is the finding that transcription and translation are under tight independent control (Schindler et al., 1990); significant amounts of IL-1β RNA can accumulate intracellularly without any appreciable secretion or membrane bound forms of the protein. Multiple levels of regulation of IL-1 release is not surprising when one considers the potentially harmful effects of IL-1β over-expression. Furthermore, a serum form of IL-1β called the IL-1 receptor antagonist protein exists. This protein is 26 % homologous to immunoreactive IL-1β protein, and is capable of interacting with both IL-1 receptor types without inducing cellular signal transduction pathways (Carter et al., 1990). This antagonist protein may serve as another level of IL-1β regulation.

Cytokines rarely function alone, but rather act in concert with other growth factors and cytokines. Experiments performed *in vivo* have shown for example that intravascular injection of LPS into mice causes the immediate production of serum TNF and IL-1, and the later production of IL-6 (Ulich et al., 1991). The antagonistic effects of different cytokines in response to various agents can also be demonstrated; when LPS is added to human monocyte cultures, significant transcription and secretion of TNF are observed. When IL-6 is added alone, similar results are observed. However when IL-6 is added prior to stimulation with LPS downregulation of TNF production and decreased monocyte target cell killing are observed. In addition, while GM-CSF is able to render human monocytes responsive to LPS and TNF-α, addition of GM-CSF with IL-6 causes cessation of growth and no responsiveness to LPS or TNF-α (Aderka et al., 1989). These types of examples indicate that such agonist-antagonist reactions occur in the immune system, and demonstrate how various cytokines are able

to either positively or negatively modulate other immune activities leading to either synergistic or antagonistic effects.

In response to invasive agents, cytokines and the immune system function in unison to remove the pathogen. In response to viral infection however, cytokines and other cellular processes may be differentially modulated. Infection of monocyte/macrophages with viruses such as vaccinia virus, herpes simplex virus (HSV), Epstein Barr virus (EBV), human herpes-6 virus (HHV-6), cytomegalovirus (CMV), influenza virus, Sendaï virus, and human immunodeficiency virus (HIV) results in a significantly altered production of cytokines (Aderka et al., 1986, Amadori et al., 1989, Breen et al., 1990, Clouse et al., 1991, D'Addario et al., 1990, 1992, Dudding et al., 1989, Flamand et al., 1991, Folks et al., 1987, Goldfeld et al., 1989, 1990, Gosselin et al., 1991, 1992a, 1992b, Honda et al., 1990, Merrill et al., 1989, 1992, Nain et al., 1990, Nakajima et al., 1989, Rieckmann et al., 1991a, Stellrecht et al., 1992, Voth et al., 1990, Vyakarnam et al., 1991, and Yamato et al., 1990). Together, these studies show that different viruses may differentially stimulate or repress certain cytokines in various cell systems.

With respect to HIV infection, many groups have demonstrated that antigenic stimulation of T cells and monocyte/macrophages infected with HIV results in significantly higher levels of cytokine gene transcription and translation. This evidence is derived not only from monocyte/macrophages and T cell lines infected with HIV, but also from monocytes isolated from blood of HIV infected individuals and from AIDS patients. The levels of cytokines such as IL-6 and TNF-α have been shown to be elevated in the serum of AIDS patients. Furthermore, purification of monocytes or macrophages from these patients followed by *in vitro* stimulation with antigenic agents indicates that these cells produce higher levels of cytokines, compared to equivalently stimulated

uninfected cell populations (Voth et al., 1990, and Vyakarnam et al., 1991).

Just as HIV may modulate cytokine gene expression, cytokines may differentially activate or suppress HIV production (Dubreuil et al., 1990, Gendelman et al., 1990a, Macé et al., 1989, Orholm et al., 1989, Poli et al., 1989, 1991). Many groups have demonstrated that IFN-α, TNF-α, IFN-γ, and GM-CSF added to cell cultures prior to HIV addition, strongly suppress the infection and spread of the virus. However, in the case of IL-1 and TNF-α, addition of these cytokines to cultures chronically infected with HIV stimulates the expression and expansion of the virus (Chester-Kalter et al., 1991, Clouse et al., 1989, Folks et al., 1987, 1989, Hazan et al., 1990, Israël et al., 1989, Kitano et al., 1991, Locardi et al., 1990, Poli et al., 1990, and Rieckmann et al., 1991b). Furthermore, B cells isolated from HIV infected individuals have been shown to spontaneously secrete a factor (IL-6), not produced from B cells of HIV negative donors, which also strongly activates HIV production from infected monocytic and T cells (Rieckman et al., 1991a).

E) Cytokines, HIV and the NF-κB Transcription Factor.

The nuclear factor κB (NF-κB) transcription factor interacts with the HIV LTR at two sites between -105 to -80 upstream of the transcription start site. NF-κB was originally described as a factor interacting with the immunoglobulin κ light chain gene enhancer (Lenardo et al., 1989a). Although originally identified in B lymphocytes, this family of proteins is now known to exist in a number of other cell types (reviewed in Baeuerle, 1991, and Lenardo and Baltimore, 1989b). NF-κB is a family of structurally and functionally related peptides that regulate transcription of viral genes (HIV, CMV, SV40, and Adenoviruses), genes coding for cell surface receptors (MHC class I (H-2K^b), IL-2 receptor α chain, T cell receptor β, and β_2 microglobulin), cytokine genes (TNF-α, IFN-β, GM-CSF, G-CSF, IL-6, IL-2, and TNF-β), and genes coding for transcription factors (IRF-1, c-myc, and NF-κB p50) (reviewed in Baeuerle, 1991).

NF-κB was originally characterized as two proteins of 50 and 65 kDa. Cloning of these factors by Ghosh et al. (1990b), and Kieran et al. (1990) revealed that a number of other proteins shared significant homology with these peptides in the amino terminal DNA binding region. The NF-κB protein family shares homology with the *rel* family of oncoproteins found in the avian reticuloendotheliosis virus (Rev-T), and the Drosophila developmental morphogen *dorsal* (reviewed in Gilmore, 1990). On the basis of homology in the amino terminal region, the NF-κB family now consists of a number of peptides ranging in molecular weight from ~100 kDa to 49 kDa. These proteins are found not only in B lymphocytes, but preexist in the cytoplasm of other cell types coupled to the inhibitor IκB. Activation of T and B lymphocytes, monocyte/macrophages, and fibroblasts by a number of agents including phorbol ester, ionizing radiation, oxygen radicals, LPS, viruses (HIV-1, CMV, HHV-6, HBV, HSV, and HTLV-1) or cytokines (IL-1, and TNF-α, -β) liberates the

DNA binding proteins from IκB, causing them to translocate to the nucleus where they can interact with DNA (Baeuerle and Baltimore, 1988, 1989, Brach et al., 1991, and Schreck et al., 1991) It has been determined that the 65 kDa subunit is the peptide interacting with IκB in the cytoplasm (Urban and Baeuerle, 1990). Once translocated to the nucleus, the p50 and p65 peptides or their rel homologs, interact in different combinations to a decameric DNA sequence containing the consensus motif 5'-GGGRNN(YYC)C-3' (where Y denotes pyrimidines, and N is any nucleotide). Oncoprotein cooperation to bind sequence specific DNA is not a novel phenomenon. The *Fos* and *Jun* proto-oncogene products interact with the AP-1 DNA sequence as a dimer complex (reviewed in Ransone and Verma, 1990), and more recent evidence has demonstrated that the Myc proto-oncogene interacts with another protein Max to bind DNA (Blackwood et al., 1992). Similar *rel*-related protein subunit interactions may be occuring. The activation of the DNA binding subunits and their translocation into the nucleus is mediated by the dissociation of the cytoplasmic complex. The latent cytoplasmic form is held together through interaction of p65 with IκB, and dissociation *in vitro* can occur by phosphorylation of the inhibitor IκB (Ghosh and Baltimore, 1990a).

c-Rel itself is an 85 kDa protein located in the cytoplasm of cells. It is capable of complexing with other NF-κB-like proteins to bind NF-κB DNA sequences, and is able to transcriptionally activate heterologous promoters containing κB sites (Hansen et al., 1992, and Inoue et al., 1991). Interestingly, while the virus-derived v-Rel protein is also able to complex with NF-κB peptides, it inhibits NF-κB transcription from κB dependent promoters (Ballard et al., 1992, and reviewed in Gilmore, 1990). These observations indicate that variations in DNA affinity, heterodimer formation, and induction kinetics may contribute to both positive and negative transcriptional control. A recent report

by Perkins et al. (1992) demonstrated that variants of the κB DNA binding sequence were transcriptionally activated by different combinations of κB proteins. This result indicates that various κB regulated genes containing slightly different κB sequences may be differentially regulated by different NF-κB subunit interactions. Several genes have been cloned recently that demonstrate significant homology to the rel proteins. These genes have been cloned from various cell systems, and have been found to be different from previously characterized members of the family through several criteria. Thus, genes containing κB sequences in their promoter, apart from possibly being transcriptionally regulated by other additional factors, can be differentially regulated by a number of different κB subunit combinations.

Induction of NF-κB activity occurs via a number of agents, and NF-κB can itself transcriptionally activate a number of genes. Although these proteins were originally thought to activate only immunoregulatory genes, NF-κB is now known to interact with widely different promoters. Data now indicates that NF-κB p50 may itself be transcriptionally regulated by two NF-κB DNA binding sequences located in its own promoter sequence (Meyer et al., 1991, and Ten et al., 1992). Cytokine genes such as TNF-α, IFN-β, GM-CSF, G-CSF, IL-6, IL-2, and TNF-β have all been shown to contain DNA sequences capable of interacting with NF-κB proteins (reviewed in Baeuerle, 1991). While the 65 kDa subunit of NF-κB was originally thought to be the only component mediating transcriptional activation (Schmitz and Baeuerle, 1991), *in vitro* evidence now indicates many other subunits may be involved. Using purified or recombinant NF-κB proteins, *in vitro* transcriptional activation has been demonstrated using NF-κB dependent sequences such as the IFN-β promoter (Cohen et al., 1991), and the HIV-LTR sequences (Kretzschmar et al., 1992).

The role of NF-κB in HIV infection has also been extensively analyzed. Many reports have demonstrated that HIV infection of monocyte/macrophages results in differentiation; one of the characteristics associated with this maturation is the increased expression of NF-κB proteins. HIV infected cells contain elevated levels of cytoplasmic NF-κB proteins (Bachelerie et al., 1991). Furthermore, HIV induced differentiation, apart from increasing NF-κB dependent HIV gene expression per se (Griffin et al., 1989), may alter preexisting cytoplasmic NF-κB complexes and generate novel protein associations containing different subunit combinations (Roulston et al., 1992). HIV specific proteins can also specifically stimulate one NF-κB subunit. The p50 NF-κB subunit is generated as a 105 kDa precursor which is processed into the smaller DNA binding 50 kDa form (Kieran et al., 1990). The processing event can also be performed by the HIV protease (Rivière et al., 1991). Therefore HIV infection may potentially liberate latent cytoplasmic NF-κB, thereby increasing NF-κB-dependent gene expression.

Cytokines IL-1 and TNF can also activate HIV through NF-κB (described in greater detail in the discussion). With elevated levels of circulating TNF and IL-1 in AIDS patients, potential activation of latent HIV is greatly enhanced. Taken together these observations indicate that HIV infection may disrupt NF-κB regulation. Initially described as a factor regulating immunogulatory genes, NF-κB may potentiate HIV expression through cytokine or antigenic induction.

RESEARCH OBJECTIVES

The objective of the work presented in this thesis was to analyze the mechanisms underlying the immune dysfunction occuring during HIV infection of monocytes. Our goal was to examine how the transcription of cytokine genes was modulated upon antigenic stimulation of HIV infected cells in culture. This analysis progressed to examine the transcriptional activation of the interleukin-1β gene. Specifically we sought to examine the role of transcription factor NF-κB in IL-1β gene expression in uninfected and HIV infected myeloid cells.

MATERIALS AND METHODS

A) Cell Culture.

U937, a human CD4+ promonocytic cell line (Sundstrom and Nilson, 1976), HIV-1 chronically infected U9-IIIB, CD4+ human myelomonoblastic PLB-985 cells (Tucker et al., 1987), HIV-1 chronically infected PLB-IIIB cells, Jurkat, a CD4+, CD8- T lymphoid cell line, and, 293, a human embryonic kidney cell line were grown in RPMI 1640 medium (GIBCO-BRL) supplemented with 5-10 % calf serum (HyClone) at 37°C, under 5.0 % CO_2, 1 % glutamine, 100 IU/ml penicillin, 20 µg/ml streptomycin, and 10 µg/ml gentamycin. U937 and PLB-985 cells were infected with HIV-1 strain IIIB (multiplicity of infection, 0.01 plaque forming units/cell), using virus from HIV-1 chronically infected U937 cells (obtained from Dr. M. A. Wainberg); after 21-28 days U9-IIIB and PLB-IIIB cultures were established that were >97 % positive for HIV p24 antigen. Induction of exponentially growing cells was performed as described for the individual experiments; in general cells were treated with phorbol 12-myristate 13-acetate (PMA; Sigma, 25 ng/ml), recombinant human tumor necrosis factor-α (TNF α; Genzyme, 10 ng/ml), lipopolysaccharide (LPS; Sigma, 100 µg/ml), or recombinant interferon-α2 (IFN-α2; Schering Biomedical, 1000 IU/ml) for 4 hr and then infected with Sendaï virus (500 heamaglutinating untis/ml; American Type Culture Collection) for 2 hr.

B) RNA Isolation and Polymerase Chain Reaction Analysis.

i) RNA Isolation Procedure.

RNA isolation was performed using the guanidinium thiocyanate procedure (Chomczynski and Sacchi, 1987). Briefly, uninduced, and PMA or LPS induced U937, U9-IIIB, PLB-985 or PLB-IIIB cells were collected by

centrifugation (1,200 rpm, 10 min), rinsed in phosphate buffered saline (PBS; 75 mM NaCl, 2 mM KCl, 8 mM NaH_2PO_4), and resuspended in 5 ml of solution D (4 M guanidinium thiocyanate, 25 mM Sodium Citrate, pH 7.0, 0.5 % Sarcosyl, and 100 mM β-mercaptoethanol). The cell pellet was vortexed, placed on ice for 15 min, then centrifuged in a Sorval Centrifuge for 20 min at 4°C and 10,000 rpm, after the addition of 5 ml phenol:H_20, 500 µl of 0.5 M Sodium Acetate (pH 4.0), and 2 ml of chloroform: isoamylalcohol (49:1). The upper aqueous phase was precipitated overnight at -20°C in corex tubes with an equal volume of isopropanol. RNA was pelleted at 10,000 rpm, for 20 min at 4°C, resuspended in solution D (600 µl), and isopropanol (600 µl), and again precipitated overnight at -20°C. RNA was pelleted in a microfuge (15,000 rpm, 4°C, 15 min), and total cellular RNA was treated with 1 U RNase free DNase (RQ_1 DNase; Promega) for 30 min at 37°C, phenol: chloroform: iso-amylalcohol extracted, ethanol precipitated and stored at -70°C.

ii) Polymerase Chain Reaction Analysis of RNA.

Reverse transcription (RT) was performed on total RNA (1 µg) using 40 U of Moloney murine leukemia virus reverse transcriptase (MMLV RT; GIBCO BRL) and 10 pmol of primer B (3' end) in buffer containing 50 mM Tris HCl (pH 8.3), 75 mM KCl, 10 mM dithiothreitol (DTT), and 3 mM $MgCl_2$, total volume was 50 µl. Polymerase chain reaction (PCR) assays were performed using 25 µl of RT product, in PCR buffer containing 100 mM Tris HCl (pH 8.4), 500 mM KCl, 15 mM $MgCl_2$, 100 µg/ml bovine serum albumin (BSA), 2 mM each; dATP, dTTP, dGTP, dCTP (Pharmacia), 10 pmole primer A (5' end), 10 pmole primer B (3' end) and 1.0 U *Thermas aquatis* DNA polymerase (Bio Can Scientific). After reverse transcription the products were held at 95°C for 5 min, annealed at 55°C for 1 min, extended at 72°C for 1 min, and denatured at 95°C for 1 min.

The annealing, extension, and denaturation procedure involved 28 cycles of amplification on a MJ Research Thermal Cycler. PCR products were analyzed on a 6.0 % denaturing Tris borate (TBE; 50 mM Tris HCl, pH 8.0, 50 mM Boric acid, and 1 mM EDTA [Ethylamine tetraacetic acid disodium salt]) polyacrylamide gel (PAGE, 19:1 crosslink-7 M Urea), and autoradiographed at -70°C using Cronex film (DuPont). Autoradiographs were obtained by using PCR primers which were 5' end labelled with [γ^{32}P] ATP (ICN Radiochemicals) and T_4 polynucleotide kinase (T_4 PNK; Pharmacia). Unincorporated radionucleotides were removed by gel filtration over a Sephadex G-25 (Pharmacia) minicolumn. Marker plasmid pAT153 x *Hae III*, was also 5' end labelled with [γ^{32}P] ATP and T_4 PNK. PCR amplified products were quantified relative to SV_2CAT DNA (9 x 10^6 copies/tube) by scintilation counting of counts per minute in the bands, thus normalizing for tube to tube differences in each PCR reaction. An equation to determine the number of cytokine RNA molecules per cell had been established (D'Addario et al., 1990):

$$x = \frac{W/(MxA)}{C} \times \frac{Y_1}{Y_0}$$

where x is the number of copies of cytokine RNA molecules per cell, W is the mass of SV_2CAT DNA in grams, M is the molecular mass of SV_2CAT (3.3 x 10^6 g/mol), A is Avogadro's number (6.023 X 10^{23} molecules/mol), C is the number of cell equivalents, Y_1 is the number of counts per minute in the cytokine cDNA PCR product and Y_0 is the number of counts in the SV_2CAT DNA PCR product. C is derived by dividing the total RNA yield by the number of cells. These values were further normalized to the amount of constitutively expressed GADPH RNA-PCR amplifed product. The sequence data and numbering of the oligonucleotides used in the RT-PCR reactions were derived from the following sources; Arcari et al. (1984) for GADPH, Bensi et al. (1987) for IL-1β, Devos et

al. (1982) for IFN-γ, Gorman et al. (1982) for SV_2CAT, Henco et al. (1985) for IFN-α1, IFN-α2, March et al. (1985) for IL-1α, Nedwin et al. (1985) for TNF-α, Shigeo and Taniguchi (1981) for IFN-β, and Yasukawa et al. (1987) for IL-6. The location of the primers within the cytokine genes are listed in table 1.

C) Assays for Cytokine Activities.

Supernatants of PMA or rhTNF-α treated cells were collected 24 hr after treatment. Cells treated with rhTNF-α were washed in PBS and resuspended in fresh RPMI media after 2 hr of treatment to avoid interference in the TNF-α enzyme linked immunosorbant assays (ELISA). Supernatant samples were collected by centrifugation of cells (3 x 10^5 cells/ml) at 1,200 rpm for 10 min at 4°C and further clarified by centrifugation (45,000 rpm) for 30 min at 4°C in a Ti-45 rotor (Beckman Ultracentrifuge). Cytosolic IL-1β was obtained by washing and resuspending cell pellets in PBS; the cells were repelleted and swollen in 5 packed cell volumes of hypotonic buffer containing 10 mM Tris HCl (pH 8.0), 2 % dimethylsulfoxide (DMSO), 5 mM phenylmethylsulfonyl fluoride (PMSF), 10 mM N-2-hydroxyethylpiperazine-N'-2-ethane sulfonic acid (Hepes, pH 7.9), 0.75 mM Spermidine, 0.15 mM Spermine, 0.1 mM EDTA (pH 8.0), 0.1 mM EGTA (Ethylene bis (oxyethylene-nitrilo)-tetraacetic acid), 1 mM DTT, 10 mM KCl, and 1 μg/ml of each protease inhibitors pepstatin, leupeptin, and aprotonin for 20 min at 4°C. The cell suspension was lysed in a dounce homogenizer (20-25 strokes) and cytosolic extracts were collected by centrifugation in a Beckman Ti-45 rotor (45,000 rpm, 1 hr, 4°C) and frozen at -70°C. Samples (5 μg total cellular protein) were analyzed by ELISA for the presence of IL-1β, TNF-α, IL-1α and IL-6 according to the manufacturer's specifications (R & D Systems). All assays were performed at minimum in duplicate on three independent sets of induced cells. When the cytokine concentrations in the extracts exceeded the

manufacturer's standard curve, extracts were diluted appropriately and re-analyzed. The lowest limit of cytokine detection by the ELISA kits are; IL-6 31.3 pg/ml, IL-1β 31.3 pg/ml, IL-1α 31.3 pg/ml, and TNF-α 15.7 pg/ml.

D) S1 Mapping Analysis.

Total RNA was isolated from untreated, PMA treated (6 hr) or PMA treated, Actinomycin-D (Act-D; Boerhinger Mannheim, 1 μg/ml) treated PLB-985 or PLB-IIIB cells. Act-D was added to PMA stimulated cells begining at 6 hr after PMA addition; RNA was isolated 30 min, 1 hr, 2 hr and 4 hr thereafter and analyzed by S1 mapping. S1 mapping procedure was performed according to Xanthoudakis et al., (1989), briefly, 40 μg total RNA was lyophilized, resuspended in 80 % formamide hybridization buffer (80 % deionized formamide, 0.4 M NaCl, 40 mM PIPES (1, 4-Piperazinediethane sulfonic acid), and 1 mM EDTA) containing 20,000-40,000 cpm of end-labelled probe (1 X 10^6 cpm/pmol end), covered in paraffin oil and heat denatured at 75°C for 10 min. RNA samples and probe were incubated overnight at 50°C, then treated with S1 nuclease (Pharmacia; 330 U/ml) for 60 min at 30°C. The reaction was terminated by phenol-chloroform extraction and ethanol precipitation. RNA-DNA hybrids were resuspended in formamide dye loading buffer (80 % deionized formamide, 0.1 % xylene cyanol, 0.1 % bromophenol blue), heat denatured (95°C for 3 min), and analyzed on a 5 % denaturing PAGE. The probe was a 920 bp *Ban1-EcoR1* fragment of the TNF-α gene (TNF-α genomic clone was kindly provided by Genentech Inc.), end labelled with [γ^{32}P] ATP and T_4 PNK; the probe spanned the mRNA start site and generated a 307 bp DNA-RNA hybrid. The autoradiograph was scanned by laser densitometry (Pharmacia LKB Ultrascan XL), and the levels of DNA-RNA hybrids remaining after Act-D treatment were plotted relative to the level at 6 hr post PMA induction.

E) Northern Blot Analysis of RNA.

Total cellular RNA was isolated from untreated, or PMA treated PLB-985 and PLB-IIIB cells at specific times using a modified guanidinium thiocyanate procedure (previously described). Total RNA (20 μg) was electrophoresed in a 1.0 % denaturing formaldehyde gel (1 % agarose, and 1 X MOPS (3-(Morpholino) Propanesulfonic acid)), vacu-blot transferred (Vacu-Gene Blotting System, Pharmacia) for 2 hr onto a nylon membrane (Hybond-N; Amersham), and crosslinked through UV irradiation (312 nm transilluminator for 5 min; Fisher Biotech) hybridized with [α-^{32}P] ATP labelled probes (labelled using the Multiprime DNA Labelling System (Amersham) rapid protocol to ~1 x 10^9 cpm/μg). Hybridization occured overnight at 50°C in 5 X SSC (750 mM NaCl, 75 mM Sodium Citrate, pH 7.0), 50 % (v/v) deionized formamide, 0.5 mg/ml non-homologous herring sperm DNA (Boerhinger Mannheim), 5 X Denhardt's solution (0.5 % (w/v) Ficoll 400 (Sigma), 0.5 % (w/v) polyvinylpyrrolidine, and 0.5 % (w/v) BSA), and 10 % (w/v) Dextran sulfate (Pharmacia). The probes include a 1.23 kilobase (kb) *c-fms* fragment generated by *Eco R1* cleavage of clone pc-fms 104 (ATCC), a 1.1 kb β-actin fragment (kindly provided by Dr. M. A. Wainberg), a 4.5 kb *c-myc* fragment containing exons 2 and 3 produced by cleavage of plasmid pHSR-1 (ATCC) with *Xba1* and *Eco R1*, and pHB10, a 12.5 kb HIV-IIIB probe (a gift from Dr. F. Wong-Staal). The blots were washed sequentially in 50 ml solutions of, 5 X SSC, 15 min at 45°C, twice, then 1 X SSC and 0.1 % SDS (sodium dodecyl sulfate) at 50°C for 30 min, and then at 60°C for 15 min with 0.1 X SSC and 0.1 % SDS, air dried and exposed to X-OMAT film (Kodak) at -70°C. The relative amounts of each signal were quantified by laser densitometry (previously described).

F) Analysis of Cell Surface Markers.

PLB-985 and PLB-IIIB cells treated with PMA (25 ng/ml) were analyzed for expression of myeloid specific surface markers using monoclonal antibodies directed against CD13, CD14, CD33, and CD34 and analyzed in a flourescence activated cell sorter (Coulter Epics Profile II). Cells (4 x 10^5 cells/ml) were collected, washed and resuspended in PBS at a concentration of 1 x 10^6 cells/ml. Analysis was performed by the Hematology Department of the Jewish General Hospital.

G) Cell Transfections.

Exponentially growing U937, U9-IIIB, and Jurkat T cells were transfected by the DEAE-Dextran method according to Grosschedl and Baltimore (1985). Briefly, cells were washed in 1 X TS (25 mM Tris pH7.4, 137 mM NaCl, 5 mM KCl, 0.6 mM Na_2HPO_4, 0.7 mM $CaCl_2$, 0.5 mM $MgCl_2$) and resuspended at 2 X 10^7 cells/ml in TS, with the addition of DNA (5-10 µg) and DEAE-Dextran (0.5 µg/ml), total volume was 2 ml. This mixture was left at room temperature for 20 min, then incubated a further 60 min at 37°C with 20 ml fresh RPMI media and chloroquin diphosphate salt (Sigma, 100 µM). Cells were collected by centrifugation for 12 min and 1,200 rpm, reseeded in 15 ml fresh RPMI media and left for 24 hr before induction. Fibroblast 293 cells were transfected by calcium phosphate coprecipitation (Graham and Van der Eb, 1973). Briefly, subconfluent cells were washed in PBS and reseeded in Dulbecco's Modified Eagle Medium (DMEM) containing 5 % serum, and left for 4 hr. Precipitated DNA was resuspended in 250 mM $CaCl_2$, and bubbled through a solution of 2 X Hepes-buffered-saline (42 mM Hepes, and 274 mM NaCl, pH 7.1), and 2 X PO_4 (24.5 mM Na_2HPO_4, and 24.5 mM NaH_2PO_4). Precipitation was allowed to occur for 15 min and DNA was then aliquoted evenly over the cell monolayer.

Cells were incubated for 4 hr at 37°C, PBS washed, then reseeded in fresh RPMI medium and left overnight. All tranfections contained equivalent amounts of DNA, in those assays where less chloramphenicol acetyl transferase (CAT) reporter plasmid was used, inert pUC8 DNA was added. After 24 hr, cells were induced with PMA (25 ng/ml; Sigma), LPS (100 µg/ml; Sigma), and left for another 24 hr before lysis.

Protein extracts from suspension and adherent cells were obtained according to Ausebel et al., 1987. Briefly, suspension cells were obtained by centrifugation (1,200 rpm, 10 min), rinsing with 1 X PBS, and resuspending cell pellets in 100 µl of 250 mM Tris HCl (pH 7.8). Protein extracts from adherent 293 cells were obtained by aspirating the media 48 hr after transfection, washing the cell monolayer three times with 1 X PBS, and incubating the cells for 5 min at room temperature in 1 ml scrape buffer (40 mM Tris HCl (pH 7.8), 1 mM EDTA, and 150 mM NaCl). The cells were removed using a rubber policeman, collected into a microfuge tube, pelleted for 2 min, and resuspended in 100 µl 250 mM Tris HCl (pH 7.8). Soluble protein from both adherent and suspension cells was prepared by three successive 5 min freeze (-70°C) thaw (37°C) cycles followed by a 5 min centrifugation. Supernatant was collected and concentration was determined by the Bio Rad Protein Assay (according to the manufacturer's specifications). For each individual transfection, 50-100 µg of total protein extract was assayed for 1-4 hr at 37°C (described in each individual experiment). The reaction volume was brought to 60 µl with 250 mM Tris HCl (pH 7.8), and then up to 150 µl with 20 µl of 3.5 mg/ml acetyl co-enzyme A (Pharmacia), 0.5 µl D-threo-(dichloroacetyl-1-^{14}C) chloramphenicol (Amersham Radiochemicals, 54 mCi/mmol), and 70 µl 1M Tris HCl (pH 7.8). After the 37°C incubation, the protein was extracted with 1 ml of ice cold ethyl acetate and the organic phase was dried in a speed vac for 20 min. The protein

pellet was resuspended in 30 µl of ethyl acetate, spotted onto PE SIL G silica gel TLC plates (Whatman), and resolved by thin layer chromatography in buffer (95 % chloroform - 5 % methanol). TLC plates were sprayed three times with enhance spray (DuPont), and autoradiographed at -70°C.

The percentage of acetylated chloramphenicol was determined by cutting out the non acetylated and acetylated forms of chloramphenicol on the TLC plates and measuring the amount of C^{14} radioactivity by liquid scintillation counting. All transfections were performed at least twice in each cell type.

H) Plasmid Construction and Oligonucleotide Synthesis.

Plasmids SV_2CAT, SV_1CAT, P2(1)CAT, and P2(2)CAT have all been previously described (Leblanc et al., 1990, Lacoste et al., 1990), and are derivatives of pSV_2CAT (Gorman et al., 1982). pIL-1βx1-κBCAT, pIL-1βx2-κBCAT, and pIL-1βx3-κBCAT plasmids were obtained by subcloning synthetic oligonucleotides containing monomeric, dimeric and trimeric IL-1β NF-κB sequences (described below) with *Acc1-Sph1* ends into the *Acc1-Sph1* site of SV_1CAT using T_4 DNA ligase (GIBCO BRL). Oligonucleotides were synthesized on a Pharmacia Gene Assembler, single strand purified on a 12 % denaturing PAGE, and equivalent amounts of both oligonucleotides were anealled overnight in 1 X TNE (10 mM Tris HCl, pH 7.5, 100 mM NaCl, and 1 mM EDTA) to generated double stranded forms which were further purified on a 20 % non-denaturing PAGE. The sequences of the oligonucleotides including the *Acc1-Sph1* ends are as follows:

pIL-1βx1κBCAT is 5'-AGACGGGAAAATCCGTACG-3',

pIL-1βx2κBCAT is 5'-AGACGGGAAAATCCGGGAAAATCCGTACG-3', and

pIL-1βx3κBCAT is 5'-AGACGGGAAAATCCGGGAAAATCCGGGAAAATCC

GTACG-3'.

Clones containing the corret insert were analyzed using the rapid boiling lysis method (Holmes and Quigley, 1981), and sequenced (Sequenase Kit, United States Biochemical Corp.) for correct insertion of the oligonucleotide into the parental plasmid. The IL-1βCAT4.4 kb plasmid has been previously described (Bensi et al., 1990), and is shown diagramatically in figure 10.

I) Whole Cell Extract Preparation.

Whole cell extracts (WCE) were prepared from exponentially growing U937 and U9-IIIB cells after induction with PMA (25 ng/ml) for 6 hr. Extracts were prepared according to Cohen et al. (1991), briefly, cells were collected by centrifugation (1,200 rpm, 10 min), washed once in 1 volume of 1 X PBS and resuspended in 3 volumes of lysis buffer (20 mM Hepes pH 7.9, 0.5 mM EDTA, 0.5 mM EGTA, 0.5 mM Spermidine, 10 % glycerol, 10 mM Sodium Molybdate, 0.5 mM PMSF, and 1 mM DTT) containing 1 µg/ml each pepstatin, leupeptin and aprotinin protease inhibitors. Cells were lysed by adding 2 M KCl dropwise, to a final concentration of 0.5 M KCl, gently mixed by rotation at 4°C for 30 min, and then centrifuged at 35,000 rpm for 60 min at 4°C in a Ti60 rotor (Beckman Ultrancentrifuge). Supernatants were diluted to 0.1 M KCl with lysis buffer and protein concentrations were determined using the Bio Rad Protein Assay.

J) Electrophoretic Mobility Shift Assay.

A 10-15 µg sample of crude WCE from U937 or Jurkat cells was preincubated with 5 µg poly (dI:dC) in nuclear dialysis buffer (NDB; 20 mM Hepes, pH 7.9, 0.1 mM EDTA, 0.1 mM EGTA, 0.1 mM KCl, 2 mM DTT, and 5 % glycerol) for 10 min at 4°C. [γ^{32}P] ATP labelled (5'-GGGAAATTCCC GGGAAATTCC-3') from the PRDII domain of the IFN-β promoter or pIL-1βx1-κBCAT, pIL-1βx2-κBCAT oligonucleotides (sequences described earlier) were

incubated at room temperature for 30 min. The sequence of the G mutant is 5'-**ACT**AAATTCC**ACT**AAATTCC-3', and A mutant is 5'-GGGA**CGT**TCC GGGA**CGT**TCC-3'. The mutations are typed in bold (relative to the IFN-β P2 sequence). In competition assays using various amounts of unlabelled oligonucleotide, cold competitors were added during preincubation. Pre-incubation of protein extracts occured for 10 min at 4°C, followed by the addition of 20 ng of [γ^{32}P] ATP labelled oligonucleotide and incubation at room temperature for 30 min. Samples were analyzed on a 6 % native Tris glycine PAGE, run at 150 V for 5 hr, dried and exposed to Cronex film (DuPont). All oligonucleotides were 5' end labelled with [γ^{32}P] ATP (5000 Ci/mmol; ICN Radiochemicals) and 20 U of T_4 PNK (Pharmacia). The relative intensities of the protein-DNA complexes were measured using the Pharmacia LKB Ultrascan XL scanning laser densitometer.

K) Generation of Recombinant p50 and IκB.

Recombinant p50 was obtained initially as a 105 kDa (p105) construct from Alain Israël (Kieran et al., 1990). The p105 coding sequence was excised from Bluescript (Stratagene) by *Stu1-EcoR1* cleavage, blunt ended with S1 nuclease (3 U/ml; Pharmacia) for 10 min, and subcloned into the *Sma1* site of pGEX-3X (Pharmacia). Cleavage of pGEX-3X with *Xba1* and *EcoRI* liberated a DNA fragment which left the the p50 coding sequence intact, in frame, and under the control of the lac Z promoter. This construct was inducible by 1 mM IPTG (Isopropyl-β-D-thiogalactopyranosoid; GIBCO-BRL), and produced a GST-p50 fusion protein (glutathione-S-transferase). Bacterial cells (DH5') were transformed with pGEX-3X-p50, grown in 750 ml LB broth until $O.D._{650}$ of 0.5, induced with IPTG for 2-4 hr, pelleted by centrifugation (3,000 rpm for 20 min), and resuspended in 10 ml PBS. Cell suspension was sonicated at 4 μm, 5

34times for 30 seconds, and once for 1 min. Triton X-100 was added to a final concentration of 1 %, cell supernatant was obtained by centrifuging cell debris in a SS-34 rotor (Sorval, 5 min, 4°C, 10,000 rpm), and clarified GST-p50 fusion protein was mixed with glutathione sepharose beads (Pharmacia). The cell extract-sepharose bead mixture was mixed gently for 10 min at room temperature, spun down (3,000 rpm, 5 min) and the supernatant discarded. Beads were washed once with wash buffer (50 mM Tris HCl (pH 7.5), and 150 mM NaCl) and then incubated with Factor X (Pharmacia) cleavage buffer (1.0 mM $CaCl_2$, 50 mM Tris HCl (pH 7.5), and 150 mM NaCl) for 2-4 hr.

Recombinant IκB was generated by PCR amplification of the cDNA obtained from unstimulated Jurkat cellular total RNA using specific primers to positions 81 to 99 (5'-ACGTGAATTCAGCTCGTCCGCGCC-3') and 1151 to 1171 (5'-ATATAGGTGTGACGTGTGACCTTAAG-3'; sequences obtained from Haskill et al., 1991). The 5' end of each oligonucleotide primer contained an *EcoR1* cleavage site used for ligation of the amplified product into pGEX-2T plasmid (Pharmacia). Recombinant IκB is liberated from bacterial lysogens (as described above for p50), but IκB was removed from GST-IκB fusion protein by thrombin cleavage (Pharmacia).

RESULTS

The activation of cytokine genes was determined using the newly developed polymerase chain reaction (PCR, Mullis and Faloona, 1987, and Saiki et al., 1988). We extended this protocol to include the reverse transcription reaction (RT). A number of experiments, described in D'Addario et al. (1990), were performed to demonstrate that the conditions used for the coupled RT-PCR reaction is quantitative. To quantify these observations, conditions for mRNA phenotyping were standardized with two sets of reference primers: GADPH (glyceraldehyde 3-phosphate dehydrogenase) gene as an endogenous RNA control, and SV_2CAT DNA as an exogenously added standard to control for tube to tube variations. Controls demonstrating quantitation of the RT-PCR reaction and its standardization were performed and are shown in D'Addario et al. (1990). The work presented in this thesis is a portion of the research published by D'Addario et al. (1990), D'Addario et al. (1992), and D'Addario et al. (in preparation). Some data will not be described fully, but will only be referred to from these articles.

A) Induction of Cytokine RNA by Paramyxovirus Coinfection and PCR mRNA Phenotyping.

To examine the inducibility of interferons and other monocyte-derived cytokines, a series of oligonucleotide primers was produced for reverse transcription - polymerase chain reaction (RT-PCR) amplification of cytokine RNAs. The cytokine primers, their location within the nucleotide sequence of the gene, the sizes of the expected PCR products, and their restriction enzyme cleavage products are listed in Table 1. The expression of cytokine gene mRNA was examined in U937 and U9-IIIB cells 8 hr after Sendaï paramyxovirus

TABLE 1: Sequence Data and Position of the Oligonucleotides used in the Reverse Transcription - Polymerase Chain Reaction Analysis.

Primer A is the 5' primer (sense), and primer B is the 3' primer (antisense). Sequences of each of the genes was obtained from the following sources; Arcari et al. (1984) for GADPH, Bensi et al. (1987) for IL-1β, Gorman et al. (1983) for SV_2CAT, Henco et al. (1985) for IFN-α1, IFN-α2, Kaushansky et al. (1986) for IL-1α, Nedwin et al. (1985) for TNF-α, Shigeo and Taniguchi (1981) for IFN-β, and Yasukawa et al. (1987) for IL-6.

Transcripts	Location Of Primers In Oligonucleotide Sequences		PCR Fragment Sizes	
	5' primer	3' primer	Full Length	Cleavage Products
IFN-α1	911-930	1573-1554	662	*Bgl II*- 280, 382
IFN-α2	1181-1200	1460-1440	280	*Pvu II*- 96, 184
IFN-β	-61-42	577-558	638	*Pst 1*- 265, 373
IFN-γ	402-421	5001-4982	860	*Taq 1*- 140, 283, 437
IL-1α	1-21	480-460	480	*Hind III*- 192, 278
IL-1β	1-21	540-520	540	*Hind III*- 140, 400
IL-6	4121-4144	4320-4299	201	*Pst 1*- 68, 64, 48, 20
TNFα	2175-2199	2820-2801	412	*Pvu II*- 233, 94, 85
GADPH	371-389	546-567	196	*Mbo 1*- 125, 71
SV2CAT	40-17	4780-4757	303	*Rsa 1*- 168, 135

coinfection. Sendaï virus was chosen as an inducing agent, since its cytokine-activating potential in U937 cells has been previously characterized (Hiscott et al., 1990). TNF-α, IL-1β, and IFN-β transcripts were not detected in unstimulated U937 cells; Sendaï virus infection of U937 cells induced IFN-β transcription but only weakly stimulated TNF-α and IL-1β genes. In contrast, a distinct pattern of cytokine gene activation was observed in U9-IIIB cells; IL-1α RNA was constitutively expressed, and after virus infection, TNF-α, IL-1α, IL-1β, and IFN-β RNA levels were increased in Sendaï virus-induced U937 cells (data not shown, D'Addario et al., 1990). The differential activation of IFN-β RNA in U937 and U9-IIIB cells was also confirmed independently by S1 mapping analysis (D'Addario et al., 1990). This experiment suggested that the IFN-β gene, while not constitutively activated in HIV-1 infected cells may be more easily induced following antigenic stimulation of HIV-infected cell population. Similarly, TNF-α gene transcription was strongly induced by Sendaï virus infection of U9-IIIB but not U937 cells; a low constitutive level of TNF-α RNA was detected in U9-IIIB cells. The IL-1β gene appeared to be expressed constitutively as a consequence of HIV-1 infection of promonocytic cells and was also stimulated by coinfection with a heterologous virus.

B) Induction of Cytokine RNA by Lipopolysaccharide Treatment.

When lipopolysaccharide (LPS) was used as inducer, a different kinetic pattern of cytokine response was observed. TNF-α and IL-1β RNA levels were increased within 2 hr of induction in U9-IIIB cells, with RNA levels remaining elevated for more than 10 hr (Figure 1A, and 1B, lanes 8 to 13); by 24 hr, the steady state levels of TNF-α and IL-1β RNA had returned to basal levels (Fig. 1A, and 1B, lane 14). In U937 cells, transcription of the TNF-α or IL-1β gene was not strongly stimulated by LPS (Fig. 1A, and 1B, lanes 1-6). Interestingly,

FIGURE 1: Induction of IL-1β and TNF-α Transcription by LPS.

Total RNA (1 μg), isolated from U937 (lanes 1-7) or U9-IIIB (lanes 8-14) cells 0, 2, 4, 6, 8, 10, and 24 hr (indicated below the autoradiograph) after LPS treatment (1 μg/ml), was analyzed for IL-1β (**A**) and TNF-α (**B**) specific RNA by PCR-mRNA phenotyping. A constant amount of SV_2CAT (9×10^6 copies/tube) was added to each reaction after reverse transcription and coamplified. Lane M indicates marker pAT153 x *Hae III*, and the sizes are indicated on the left side of figure B. The correctly sized PCR products are indicated by the arrows on the right side of both figures.

A.

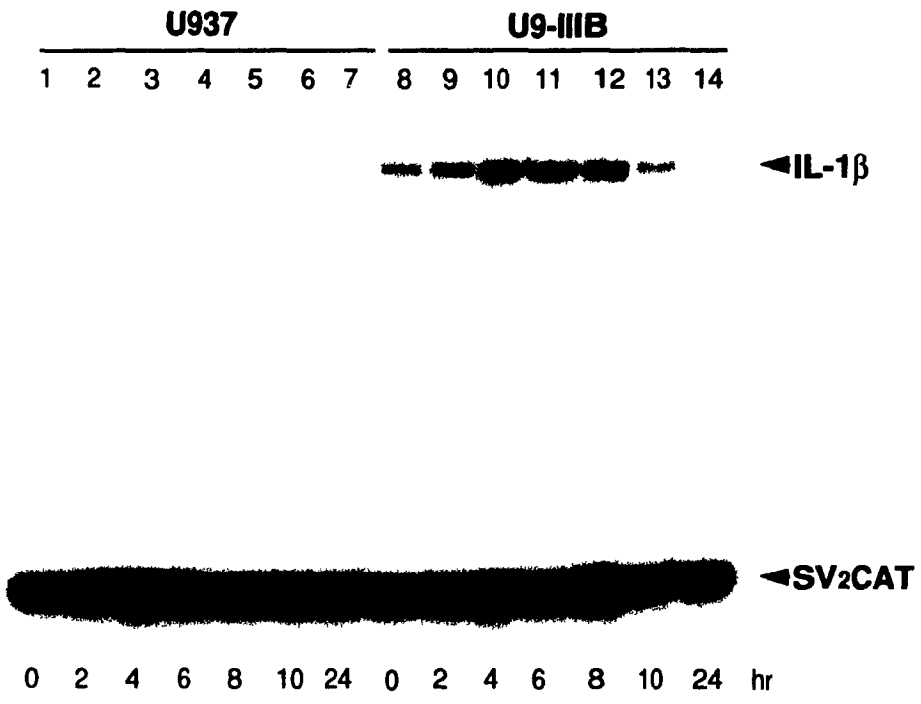
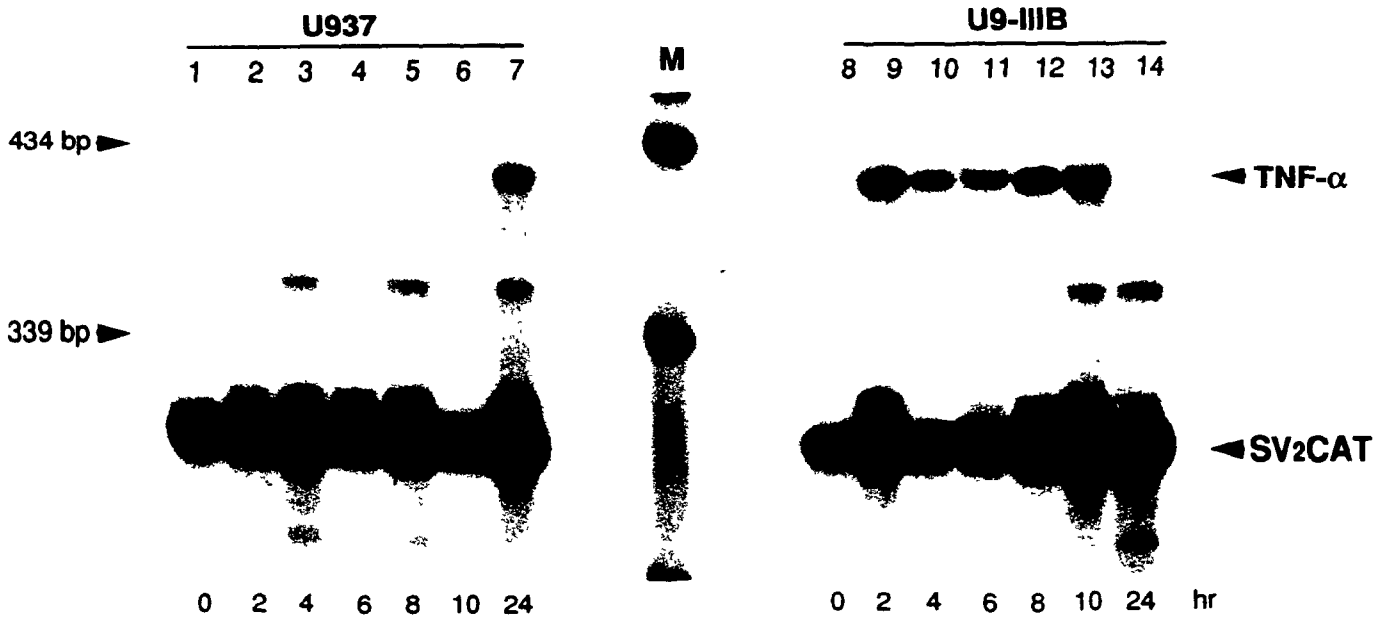

U937
U9-IIIB
1 2 3 4 5 6 7 8 9 10 11 12 13 14
◄IL-1β
◄SV2CAT
0 2 4 6 8 10 24 0 2 4 6 8 10 24 hr

FIGURE 1: (Cont'd...)

B.


U937
1 2 3 4 5 6 7
M
U9-IIIB
8 9 10 11 12 13 14
434 bp
339 bp
TNF-α
SV2CAT
0 2 4 6 8 10 24
0 2 4 6 8 10 24 hr

TNF-α RNA was present in U937 cells at 24 hr after LPS treatment (Fig. 1B, lane 7); it is possible that the TNF-α gene was secondarily activated as consequence of LPS, via activation of another distinct cytokine. This effect was shown by Clouse et al. (1989) to be the mechanism of LPS induction of HIV-1. More recently Gong et al. (1991) found that LPS-induced activation of influenza A virus was due to LPS-induced TNF-α secretion. Transcription of the IFN-α and IFN-β genes was not induced in either cell type by LPS treatment (data not shown). Similarly, a low basal level of IL-1α transcription was observed in U937 cells and was not elevated in U9-IIIB cells upon LPS stimulation (data not shown). Taken together these experiments demonstrate that expression of specific cytokine transcripts such as TNF-α, IFN-β, and IL-1β was potentiated in HIV-infected cells by paramyxovirus coinfection. However, while LPS treatment stimulated IL-1β and TNF-α expression in U9-IIIB cells, IFN genes were not activated.

C) HIV Infection of Myelomonoblastic PLB-985 Cells.

PLB-985 cells were originally characterized as a diploid myelomonoblastic cell line capable of inducer-mediated differentiation along either the monocytic or granulocytic lineage (Tucker et al., 1987). By virtue of the expression of CD4 on their cell surface, PLB-985 cells were infected with HIV-IIIB using culture fluids from U9-IIIB cells, following a protocol similar to the one described in Dubreuil et al. (1990). Two months after initial infection, a chronically infected cell population emerged (>95 % positive for HIV p24 antigen, Roulston et al., 1992).

D) Expression of Myeloid Specific Surface Markers.

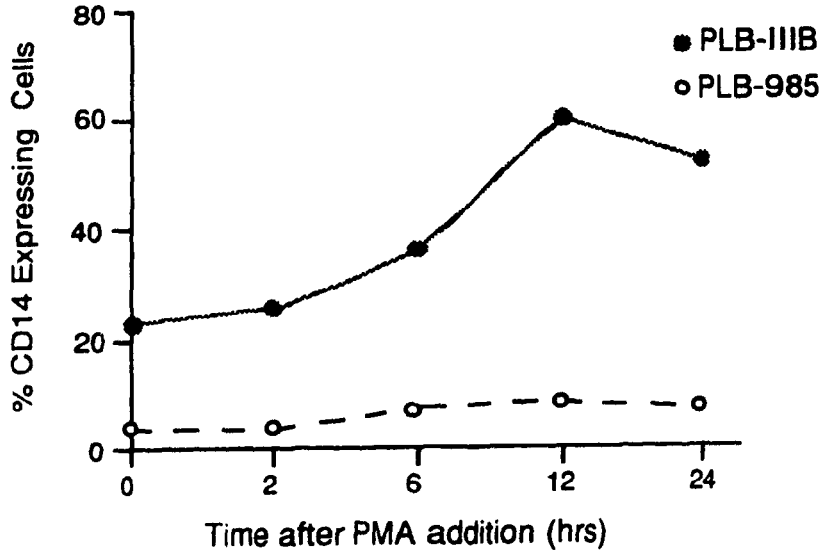
After having established a population of PLB-985 cells that was chronically infected with HIV (PLB-IIIB), the cells were induced with phorbol ester for various times and stained with Giemsa. PLB-985 cells stimulated with PMA became adherent within 12 hr and monocyte-like. Without induction, PLB-IIIB cells displayed several characteristics of monocyte maturation, including less basophilic cytoplasm, decreased nucleus/cytoplasm ratio, production of vaccuoles, less visible nucleoli, ruffling of the cytoplasmic membrane, and lobularization of the nucleus (data not shown, Roulston et al., 1992). These characteristics were all potentiated by phorbol ester treatment. In contrast, PLB-985 blast cells required PMA stimulation for up to 12 hr before demonstrating such characteristics. Similar phorbol ester induced cytological alterations were also observed by Tucker et al. (1987) and have been described by Lübbert et al. (1991).

To determine whether the morphological alterations observed after HIV infection were accompanied by other changes in the PLB-IIIB phenotype, expression of myeloid specific markers was determined by flow cytometery. Unstimulated PLB-985 were <5 % positive for the monocyte specific surface marker CD14, whereas PLB-IIIB cells were ~20 % positive for the monocyte-specific surface antigen. After 12 hr of PMA treatment, while ~12 % of PLB-985 cells became CD14 positive, >60 % of the HIV-infected cells expressed CD14 (Figure 2A). The levels of other myeloid markers CD13 (monoblast specific), and CD33 (leukemia specific), remained constant before and after PMA addition. Similarly 5-15 % of both cell types were CD34 (anti-myeloblast) positive both before and after infection and stimulation (Fig. 2B). Although our analysis did not extend beyond the myelomonoblastic PLB-985 and PLB-IIIB cell models, experiments carried out by Pautrat et al. (1990) demonstrated

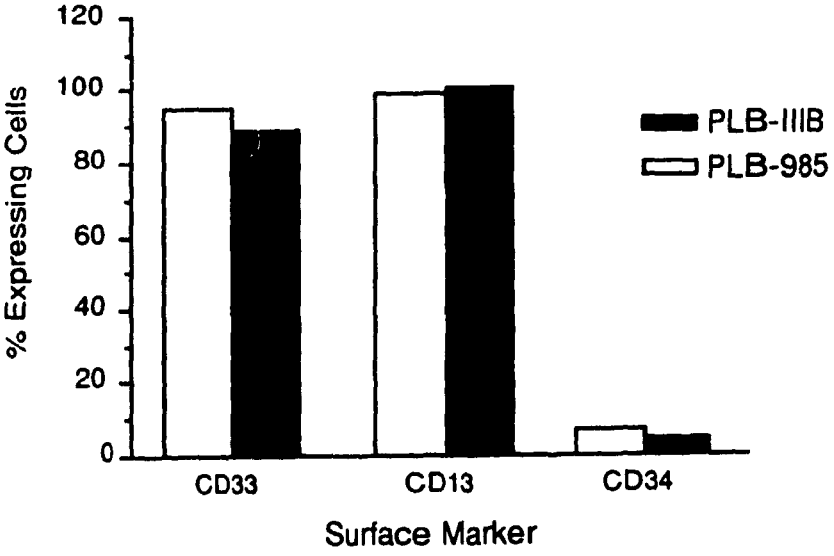
FIGURE 2: Myeloid Specific Marker Expression.

PLB-985 and PLB-IIIB cells were either untreated or PMA treated for various times before surface marker analysis using the EPICS cytofluorograph (Coulter Electronics Inc.). In **A** the percentage of CD14 positive cells is plotted as a function of time after PMA treatment. The levels of CD13, CD33, and CD34 positive PLB-985 and PLB-IIIB cells are indicated in **B**.

A.


80
60
40
20
0
% CD14 Expressing Cells
PLB-IIIB
PLB-985
0
2
6
12
24
Time after PMA addition (hrs)

B.


120
100
80
60
40
20
0
% Expressing Cells
PLB-IIIB
PLB-985
CD33
CD13
CD34
Surface Marker

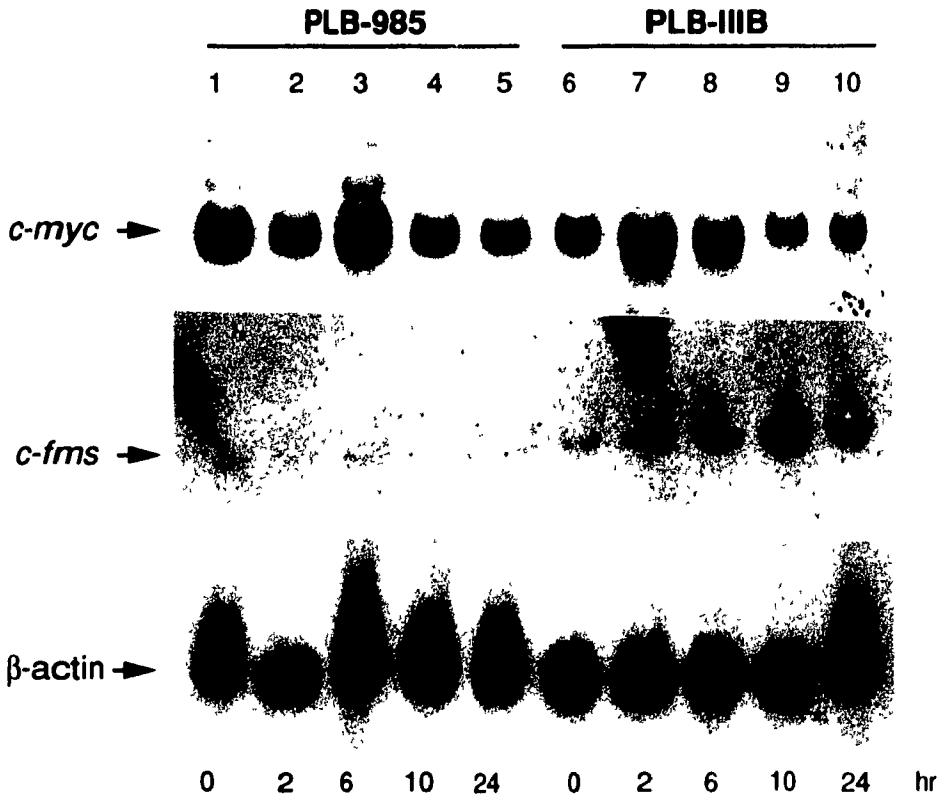
similar results in promonocytic U937 cells. Unstimulated HIV infected U9-IIIB cells expressed ~10 % CD14; this level was enhanced to >60 % by 48 hr of PMA stimulation. This data indicates that expression of the CD14 surface marker is a characteristic alteration associated with HIV infection and differentiation.

E) Expression of *c-fms* and *c-myc* Proto-Oncogenes.

Alteration of proto-oncogene expression is a recognized consequence of differentiation in a variety of hematopoietic cell types (Koeffler, 1983, and Lübbert et al., 1991). To examine the effects of HIV-1 infection on PMA-induced proto-oncogene expression, the steady state mRNA levels of *c-myc* and *c-fms* were measured at different times after PMA treatment of PLB-985 and PLB-IIIB cells (Figure 3). In both cell types, *c-myc* RNA levels decreased 2-3 fold by 2 hr after PMA treatment (Fig. 3, top panel). In contrast, *c-fms* RNA was only weakly detectable by northern blot analysis in PLB-985 cells before or after PMA induction. In untreated PLB-IIIB cells, *c-fms* RNA was present in low quantities. This level of *c-fms* was induced more than 20 fold by PMA treatment (Fig. 3, middle panel). The level of β-actin RNA did not fluctuate significantly with induction of differentiation (Fig. 3, bottom panel). Analogous phorbol ester-induced changes in *c-myc* and *c-fms* proto-oncogene expression in the human promyelocytic HL-60 cell line were shown by Sariban et al. (1985) to be characteristic of differentiation. After granulocytic induction of PLB-985 and PLB-IIIB cells with dibutyryl cAMP, no increase in *c-fms* was observed (data not shown). Finally, mophological and histochemical studies demonstrated that PLB-IIIB cells were unresponsive to inducers of granulocytic differentiation, indicating that the cell population had irreversibly differentiated along the monocytic lineage (data not shown).

FIGURE 3: Modulation of *c-myc* and *c-fms* RNA Levels in PMA Treated PLB Cells.

Total RNA (20 μg) was electrophoresed in a 1 % formaldehyde gel, transferred to a nylon membrane, and hybridized sequentially with probes for *c-fms*, *c-myc*, and β-actin, as described in the Materials and Methods. RNA was isolated at 0, 2, 6, 10, and 24 hr after treatment (shown below the autoradiograph). The arrows indicate the position of the correct RNA signal.


PLB-985
PLB-IIIB
1 2 3 4 5 6 7 8 9 10
c-myc
c-fms
β-actin
0 2 6 10 24 0 2 6 10 24 hr

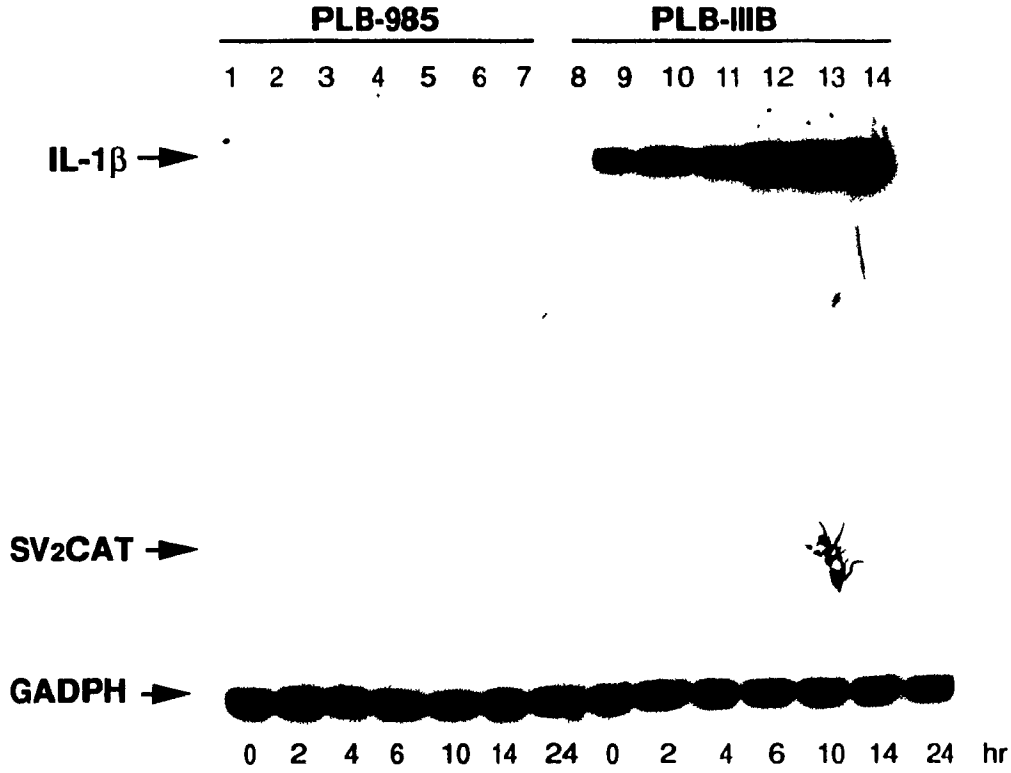
F) Enhanced IL-1β Gene Expression in HIV-1 Infected Myeloid Cells.

To further characterize cytokine gene expression, we investigated the induction of IL-1β, IL-1α, TNF-α, and IL-6 in both HIV infected myelomonoblastic PLB-985 (PLB-IIIB) cells, and promonocytic U937 cells (U9-IIIB) following treatment with phorbol esters or recombinant human tumor necrosis factor-α (rhTNF-α). Steady state IL-1β transcript levels were strongly stimulated in PLB-IIIB cells by PMA (Figure 4A, lanes 8-14, relative levels of IL-1β gene transcripts are shown below the autoradiograph); at 10-14 hr after treatment, 2,000-3,000 copies of RNA were detected by PCR mediated RNA amplification. rhTNF-α treatment (Fig. 4B, lanes 7-12) of PLB-IIIB cells also increased the levels of IL-1β RNA to about 100 copies/cell; in both cases, the magnitude of the increase was 20-30 fold compared to the induction observed in the parental PLB-985 cells (Fig. 4A, lanes 1-7; Fig. 4B, lanes 1-6). In both cell types, no IL-1β transcription was detected in uninduced HIV infected cells (Fig. 4A, lane 8; Fig. 4B, lane 7). In each case, the cytokine RNA levels were quantified relative to a constant amount of heterologous DNA (9 X 10^6 molecules of SV_2CAT per tube) added to each reaction and co-amplified with specific primers as previously described (D'Addario et al., 1990). A 40-fold induction of IL-1β transcripts was also observed at 10 and 14 hr after PMA treatment in U9-IIIB cells (Fig. 4C, lanes 8-12), while uninduced cells contained no detectable IL-1β RNA (Fig. 4C, lanes 1 and 7). Using less quantitative northern blot analysis, Yamato et al. (1990) observed similar results, and also noted that IL-1β RNA half life was not significantly different in HIV infected cells, indicating that IL-1β RNA accumulation is due to transcriptional activation, and not to increased RNA stability.

FIGURE 4: IL-1β Transcription in PLB and U937 Cells.

Total RNA (1 μg) was isolated from PLB-985 (lanes 1-7 in **A**, and 1-6 in **B**), and PLB-IIIB (lanes 8-14 in A, and 7-12 in B) or U937 (lanes 1-6 in **C**), and U9-IIIB (lanes 7-12 in C) cells after PMA (**A** and **C**) or TNF-α (**B**) treatment for various times (shown below the GADPH autoradiograph), and analyzed by PCR-mRNA phenotyping. Reverse transcription and PCR mediated amplification was carried out together with SV_2CAT DNA (9×10^6 copies/tube). The relative RNA levels are shown in the graphs below the autoradiographs; quantitation is described in the Materials and Methods. In **4C**, M indicates the marker lane (pAT153 X *Hae III*), and the sizes are shown on the right.

A.


PLB-985
PLB-IIIB
1 2 3 4 5 6 7 8 9 10 11 12 13 14
IL-1β
SV2CAT
GADPH
0 2 4 6 10 14 24 0 2 4 6 10 14 24 hr

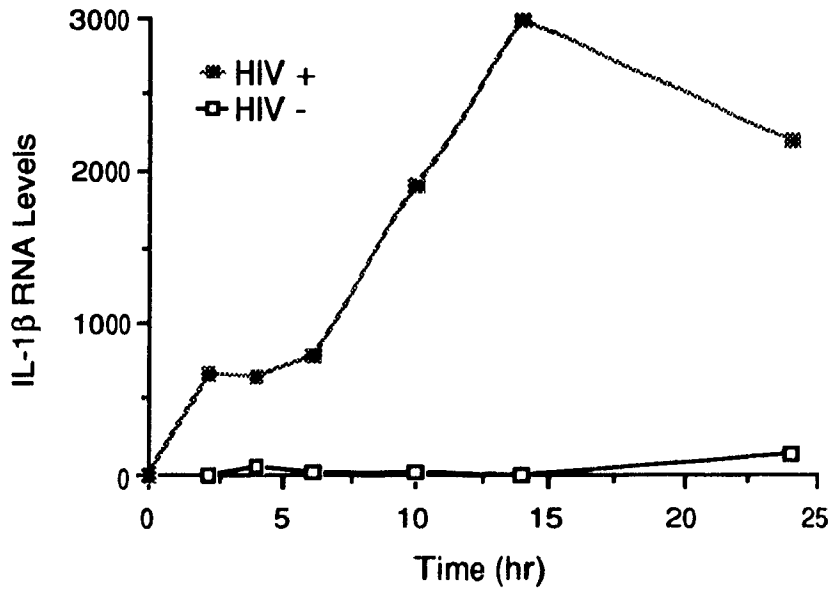
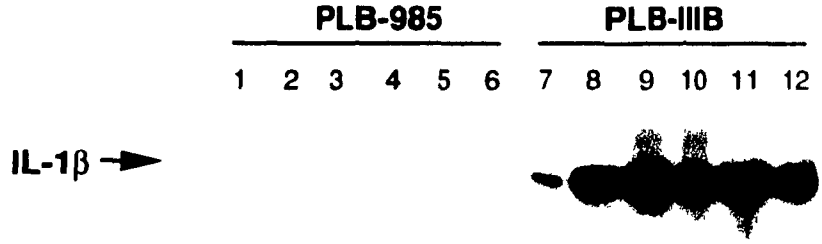
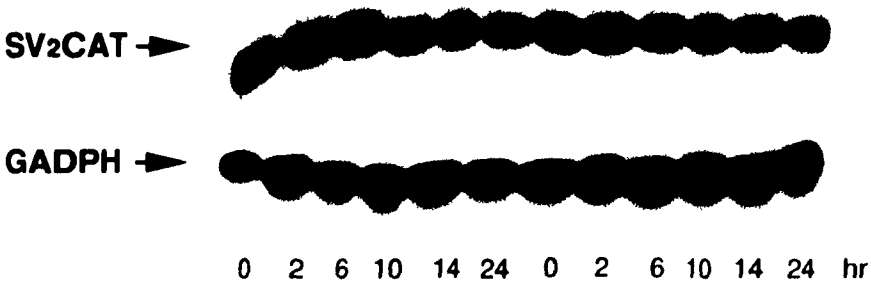

3000
2000
1000
0
HIV +
HIV -
IL-1β RNA Levels
0 5 10 15 20 25
Time (hr)

FIGURE 4: (Cont'd...)

B.


PLB-985
PLB-IIIB
1 2 3 4 5 6 7 8 9 10 11 12
IL-1β


SV2CAT
GADPH
0 2 6 10 14 24 0 2 6 10 14 24 hr

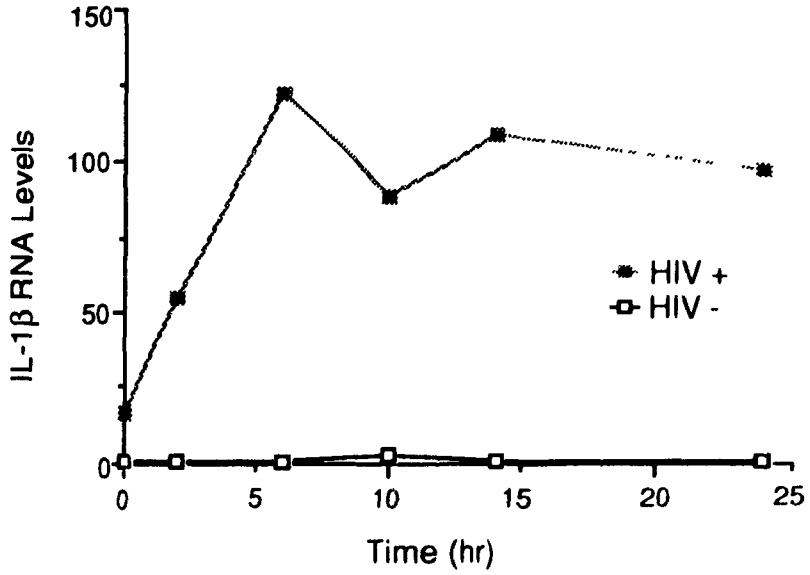
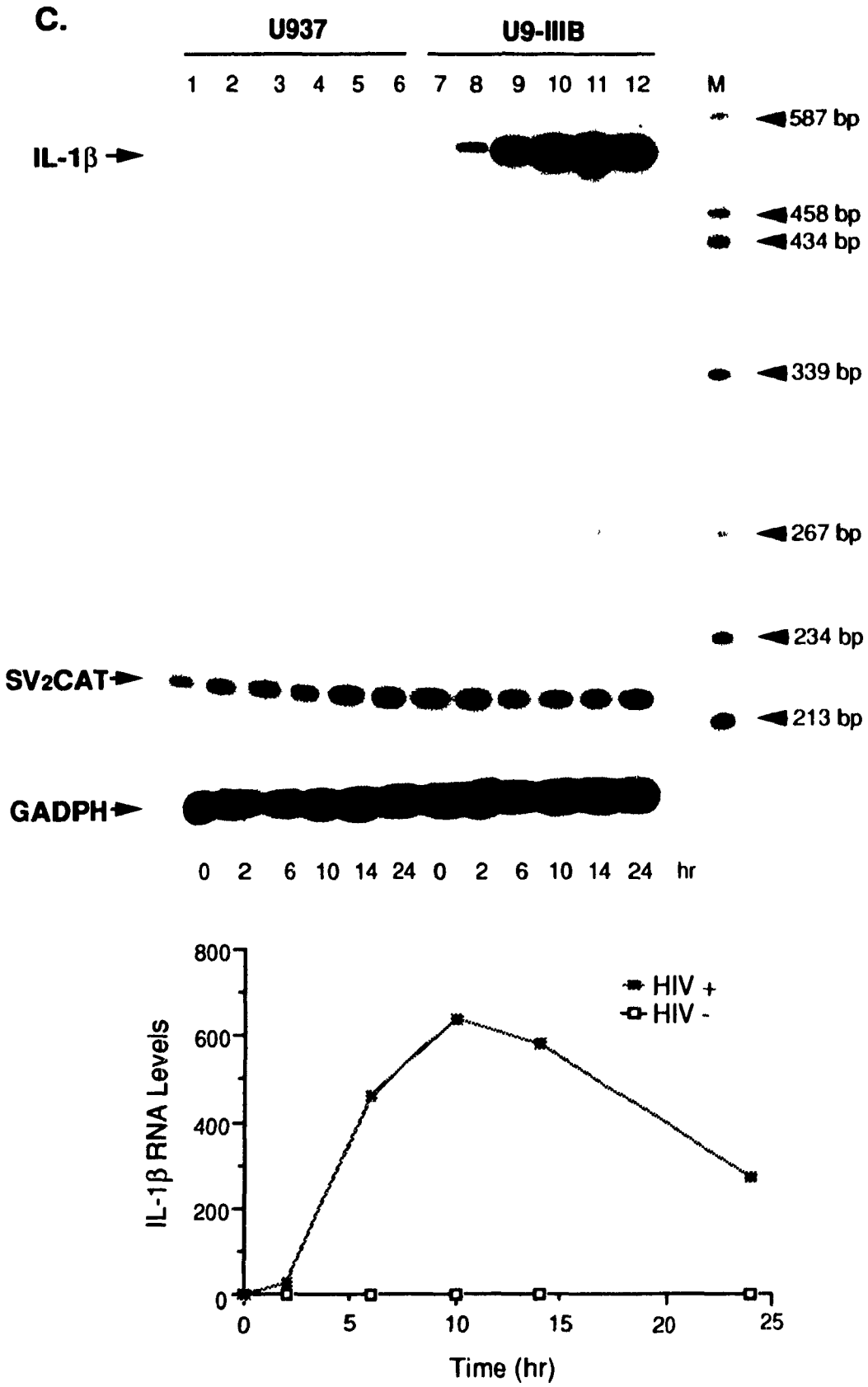

150
100
50
0
IL-1β RNA Levels
HIV +
HIV -
0 5 10 15 20 25
Time (hr)

FIGURE 4: (Cont'd...)

C.


U937
U9-IIIB
1 2 3 4 5 6 7 8 9 10 11 12
M
IL-1β
587 bp
458 bp
434 bp
339 bp
267 bp
234 bp
213 bp
SV2CAT
GADPH
0 2 6 10 14 24 0 2 6 10 14 24 hr
800
600
400
200
0
IL-1β RNA Levels
HIV +
HIV -
0 5 10 15 20 25
Time (hr)

G) Expression of IL-1β Protein in HIV-1 Infected Cells.

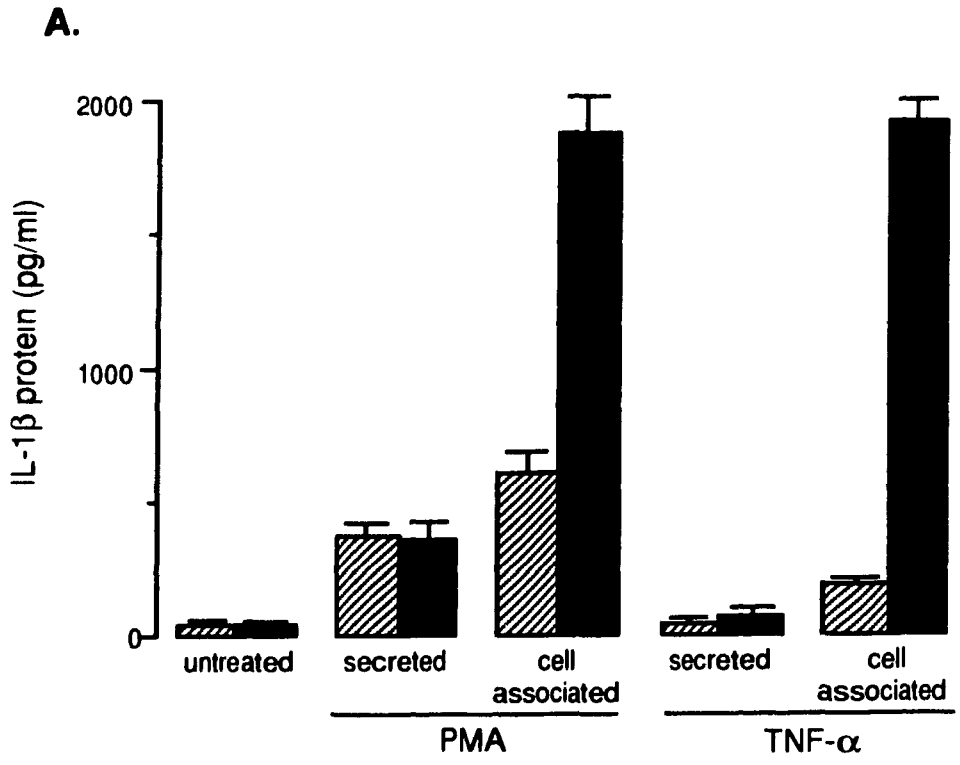
When secretion of IL-1β was examined in unstimulated PLB-IIIB cells, basal levels of immunoreactive IL-1β (38 ± 3.3 pg/ml) were detected in culture supernatants by ELISA (Figure 5). Since previous results had demonstrated that significant amounts of IL-1β may remain membrane bound (Lonnemann et al., 1989), both secreted and cell associated levels of IL-1β were examined. At 24 hr after PMA treatment, most of the IL-1β activity in the PLB-IIIB cells was cell-associated; a three fold increase in cell-associated IL-1β was observed in PLB-IIIB cells compared to PLB-985 cells (1,823 ± 249 pg/ml versus 596 ± 89.6 pg/ml) (Fig. 5A); rhTNF-α treatment produced a ten-fold difference (1,907 ± 139 pg/ml in PLB-IIIB, and 188 ± 39 pg/ml in PLB-985 cells) (Fig. 5A). Similarly, PMA treatment of U9-IIIB cells resulted in a nine-fold increase in the amount of cell-associated IL-1β compared to U937 (1,493 ± 116 pg/ml and 160 ± 34 pg/ml) (Fig. 5B). Thus, increased IL-1β mRNA and protein levels were associated with PMA and TNF-α induction of HIV-1 infected myeloid cells. These results were not unexpected, but confirmed Western blot data from Folks et al. (1987). The authors demonstrated that membrane fractions from rhTNF-α-stimulated HIV infected promonocytic cells contained higher levels of IL-1β protein than uninfected cells.

H) Expression of TNF-α in HIV Infected Myelomonoblastic Cells.

In contrast to the results obtained with IL-1β, TNF-α gene transcription was rapidly induced in both PLB-985 and PLB-IIIB cells following treatment with either PMA or TNF-α (Figure 6). Exposure to PMA led to a rapid induction of TNF mRNA in both uninfected and HIV-1 infected cells, reaching peak levels of 800-1,000 copies/cell between 2-10 hr (Fig. 6A, lanes 10-15, quantitation

FIGURE 5: IL-1β Protein Activity in PMA or TNF-α Treated Cells.

PLB-985, PLB-IIIB (**A**), and U937, U9-IIIB cells (**B**) were treated with PMA or TNF-α for 24 hr; cells were centrifuged for 10 min at 1,200 rpm and the supernatants were assayed for IL-1β activity. Preparation of cell lysates for analysis of cell associated IL-1β is described in the Materials and Methods. In both A and B, solid bars represent HIV infected cells, and hatched bars represent non infected cells.


A.
2000
1000
0
IL-1β protein (pg/ml)
untreated
secreted
cell associated
secreted
cell associated
PMA
TNF-α

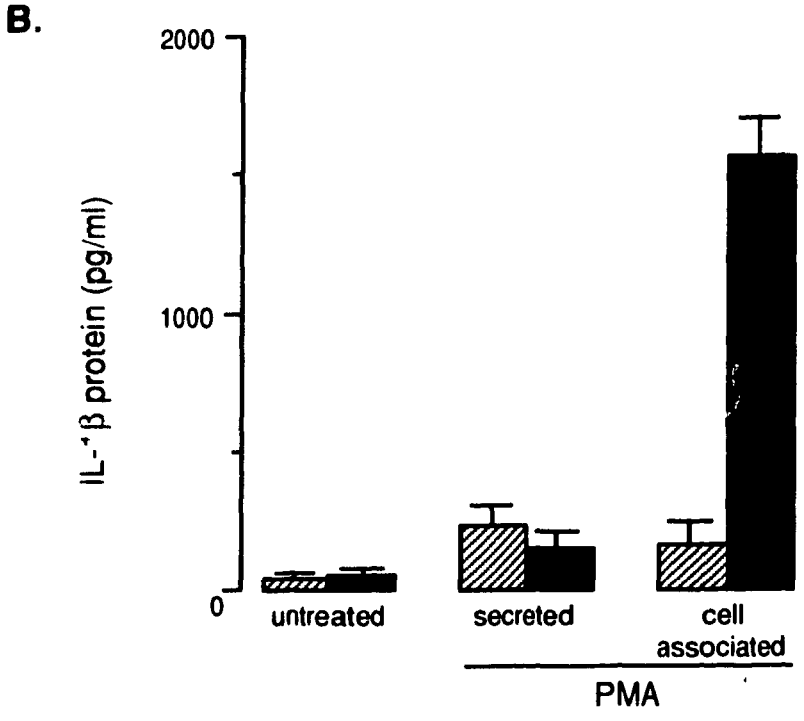
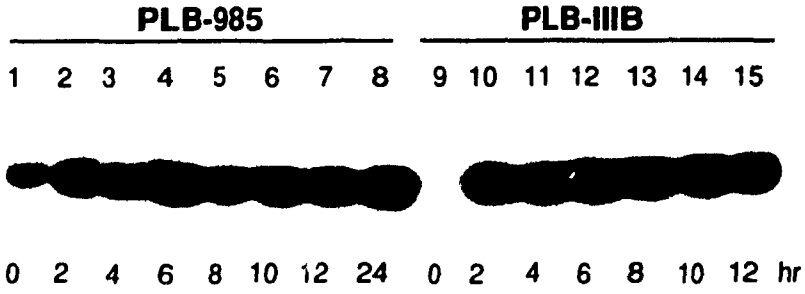

B.
2000
1000
0
IL-1β protein (pg/ml)
untreated
secreted
cell associated
PMA

FIGURE 6: TNF-α Transcription in PLB-985 and PLB-IIIB Cells.

Total RNA (1 μg) was isolated from PLB-985 (lanes 1-8 in A, lanes 1-6 in B), and PLB-IIIB cells (lanes 9-15 in A, lanes 7-12 in B) following different times of PMA (**A**) or rhTNF-α (**B**) treatment (indicated below the autoradiograph). TNF-α RNA levels are expressed relative to SV_2CAT DNA (9×10^6 copies/tube), and normalized to the levels of GADPH RNA, as described in the Materials and Methods.

A.


PLB-985
PLB-IIIB
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
0 2 4 6 8 10 12 24 0 2 4 6 8 10 12 hr

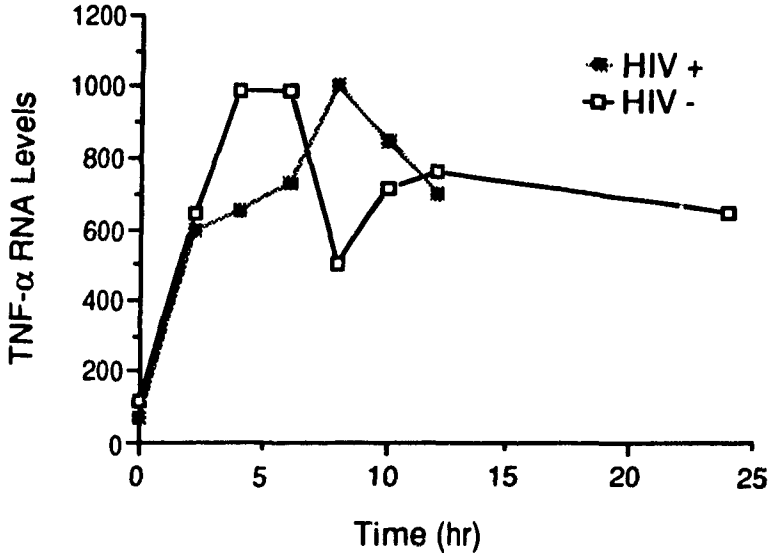
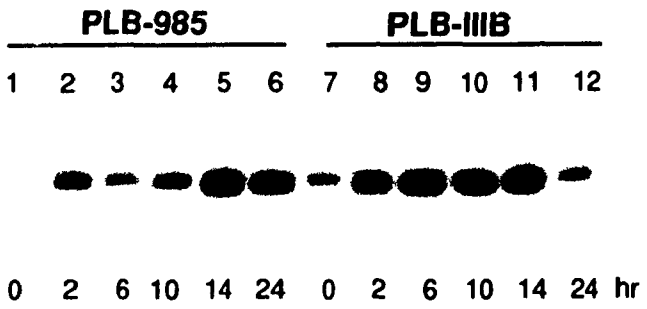
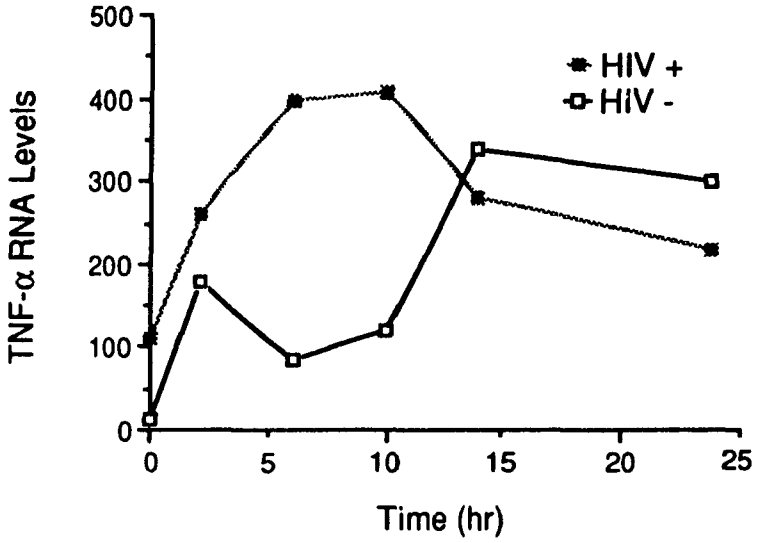

TNF-α RNA Levels
1200
1000
800
600
400
200
0
0 5 10 15 20 25
Time (hr)
HIV +
HIV -

FIGURE 6: (Cont'd...)

B.


PLB-985
PLB-IIIB
1 2 3 4 5 6 7 8 9 10 11 12
0 2 6 10 14 24 0 2 6 10 14 24 hr


TNF-α RNA Levels
500
400
300
200
100
0
0 5 10 15 20 25
Time (hr)
HIV +
HIV -

demonstrated below the autoradiograph). TNF-α transcript levels were increased to 100-400 copies/cell by TNF treatment; a difference in the kinetics of induction was noted between uninfected and HIV infected cells (note the difference in Fig. 6B, lanes 1 and 7). The same inducers produced different results in U937 and U9-IIIB cells; a constitutive level of TNF-α RNA was detected in the latter (data not shown). In response to rhTNF-α treatment, TNF-α transcription was only weakly inducible in U937 and U9-IIIB cells (data not shown). Surprisingly, supernatants from PMA-stimulated PLB-IIIB cells contained about 14-fold more TNF-α protein than uninfected PLB-985 cells (2,300 ± 141 pg/ml versus 168 ± 13.3 pg/ml) (Figure 7), even though both cell types produced equivalent final amounts of TNF-α RNA transcripts (Fig. 6A, lanes 8 and 15). Supernatants from TNF-treated PLB-IIIB cells contained 5 times more TNF protein than uninfected controls (1,015 ± 35 pg/ml and 203 ± 31.3 pg/ml, respectively), although the relative TNF-α induced levels at 24 hr were lower.

To examine the possibility that TNF RNA was stabilized in HIV infected cells, an actinomycin D (Act D) chase experiment was performed at 6 hr after PMA induction of PLB-985 and PLB-IIIB cells as described in the Materials and Methods (Figure 8). PMA-treatment led to a 15-40 fold increase in TNF-α specific mRNA by 6 hr after treatment as detected by S1 mapping (data not shown), thus independently confirming the results obtained by RT-PCR. Total RNA was isolated at 30 min, 1 hr, 2 hr, and 4 hr after addition of Act D (1 μg/ml) and the amount of RNA remaining at each time point was determined by S1 mapping. In PLB-985 cells, TNF RNA decayed with a $T_{1/2}$ of 90 min while in PLB-IIIB cells the half life of TNF message was about 30 min. Clearly, TNF RNA is less stable in HIV infected cells than in the uninfected cells. Therefore these data indicate that 1) HIV infection may lead to increased translational efficiency

FIGURE 7: TNF-α Protein Activity in PMA and rhTNF-α Treated PLB Cells.

Supernatants from 3 X 10^5 cells treated with PMA (left) or rhTNF-α (right) were assayed for TNF-α activity by ELISA (R & D Systems). The solid bars represent HIV infected PLB-IIIB cells, and the hatched bars represent PLB-985 cells.

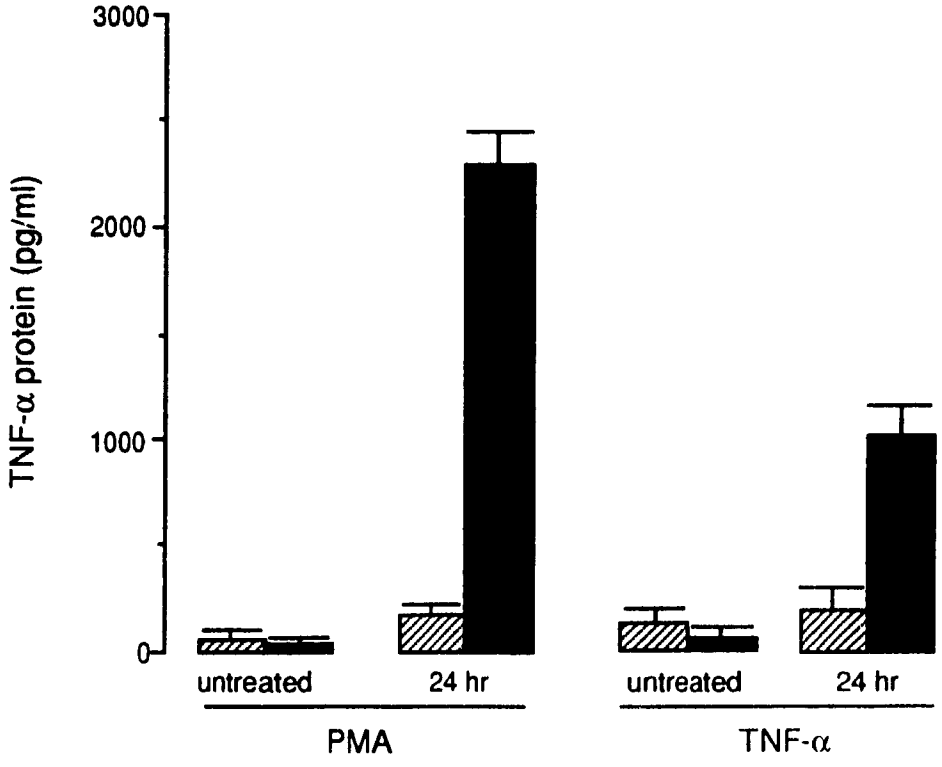
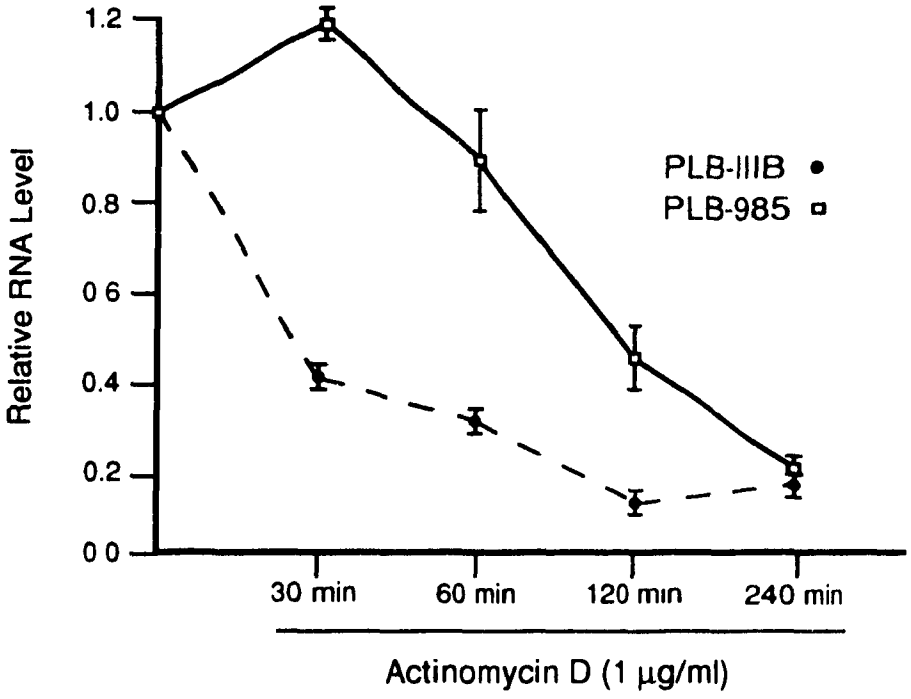

3000
2000
1000
0
TNF-α protein (pg/ml)
untreated
24 hr
untreated
24 hr
PMA
TNF-α

FIGURE 8: TNF-α RNA Stability in PMA Induced PLB Cells.

Total RNA was isolated from PLB-985 or PLB-IIIB cells at 6 hr after PMA treatment (25 ng/ml). Actinomycin D (1 μg/ml) was added to cells and RNA was isolated 30 min, 1 hr, 2 hr, and 4 hr thereafter; TNF-α specific RNA:DNA hybrids were analyzed by S1 mapping, as described in the Materials and Methods. The autoradiographs were scanned by laser densitometery, and the amount of TNF RNA remaining at each time was plotted as a function of the amount of RNA present at 6 hr after PMA addition.


1.2
1.0
0.8
0 6
0.4
0.2
0 0
Relative RNA Level
PLB-IIIB •
PLB-985 ▫
30 min
60 min
120 min
240 min
Actinomycin D (1 μg/ml)

of TNF mRNA in PLB-985 cells, and 2) PMA is a more potent stimulator of translation than recombinant TNF.

I) Transcription of Interleukin-1α and Interleukin-6.

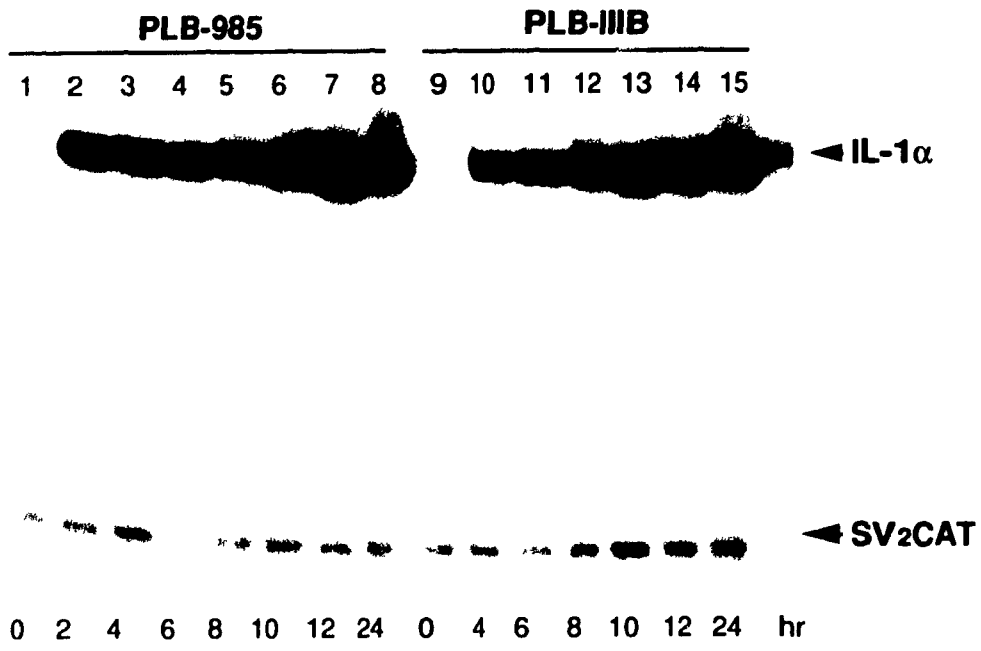
Transcription of IL-1α was induced by PMA treatment both in PLB-985 and PLB-IIIB cells; IL-1α transcript levels increased to 50-150 copies/cell by 10 hr after induction (Figure 9A). The results of TNF stimulation of IL-1α were similar (data not shown). In PMA-treated PLB-985 cells, a low level of IL-1α protein was secreted into the culture supernatant, whereas in PLB-IIIB cells (Fig. 9B) detectable PMA-induced IL-1α was predominantly cell-associated. Similar to IL-1β, significant amounts of IL-1α also remain membrane bound after stimulation (Lonnemann et al., 1989).

Transcription of the IL-6 gene was only weakly inducible by PMA and TNF-α in normal or HIV infected PLB cells, although more RNA was detected in HIV infected cells than parental cells (data not shown). Measurement of IL-6 protein in culture supernatants also reflected the poor inducibility of IL-6, ranging between <20-70 pg/ml (data not shown). This poor level of IL-6 induction was surprising since *in vivo* evidence indicates that IL-6 is found at very high levels in the serum of seropositive patients, and is inducible by LPS in *ex vivo* stimulated monocytes/macrophages (Honda et al., 1990). Although both IL-1α and IL-6 were stimulated by PMA and rhTNF-α in both cell types, this data indicates that transcriptional activation of these cytokines genes are not dramatically different in uninfected and HIV infected myelomonoblastic cells.

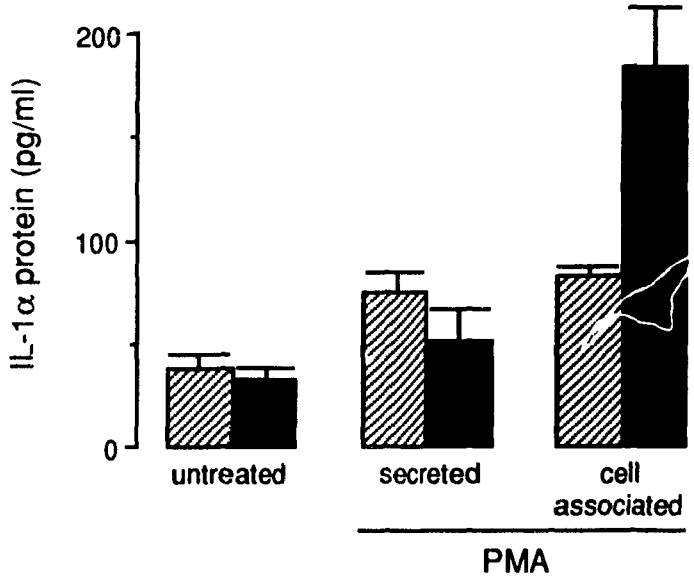
FIGURE 9: Transcription of the IL-1α Gene in PLB Cells.

A) Total RNA (1 μg) was isolated from uninduced PLB-985 (lane 1) and PLB-IIIB (lane 9) cells or PMA stimulated cells (lanes 2-8 for PLB-985, and lanes 10-15 for PLB-IIIB cells, indicated above the autoradiograph) for various amounts of time (indicated below) and analyzed by PCR-mRNA phenotyping. An SV_2CAT control (9×10^6 copies/tube) was also performed. **B)** Relative amounts of IL-1α protein produced by PMA stimulated cells were analyzed by ELISA. Cells associated forms were obtained according to the Materials and Methods. Solid bars represent HIV infected cells, and non-infected cells are shown by the hatched bars.

A.


PLB-985
PLB-IIIB
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
IL-1α
SV2CAT
0 2 4 6 8 10 12 24 0 4 6 8 10 12 24 hr

B.


IL-1α protein (pg/ml)
200
100
0
untreated
secreted
cell associated
PMA

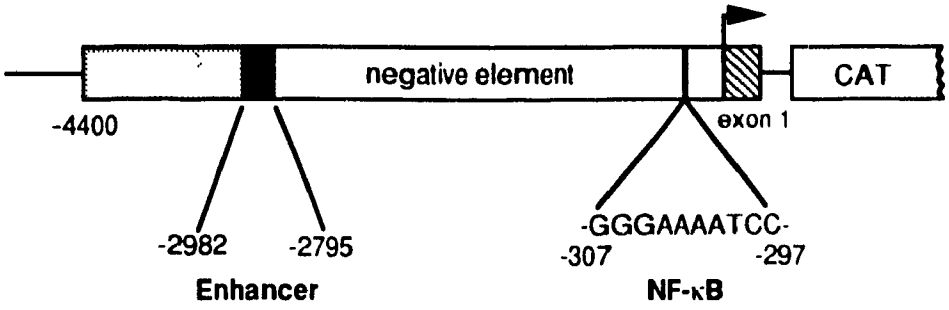
J) Proteins Interacting with the IFN-β PRD II κB Sequence Bind an Interleukin-1β NF-κB Site.

Examination of the interleukin-1β (IL-1β) promoter sequence revealed a sequence located at position -307 to -297 (5'-GGGAAAATCC-3') with 90 % similarity to the positive regulatory domain (PRD)II/NF-κB sequence in the IFN-β promoter (5'-GGGAAATTCC-3'). Figure 10 illustrates the location of the putative NF-κB site within the IL-1β promoter, and compares the sequence to the PRD II site (IFN-β P2, shown below). Other features of the IL-1β promoter previously described by Bensi et al. (1990) are also shown. The only difference between the two κB sites is a T to A transition at position 7 of the IL-1β sequence. The sequences of the G and A mutant oligonucleotides are also shown below. To test the DNA binding properties of the IL-1β site, oligonucleotides containing 1 to 3 copies of the IL-1β sequence were analyzed for their ability to interact with NF-κB proteins.

Protein extracts were prepared from U937 cells stimulated with PMA for 6 hr and analyzed for binding to the IL-1β or the IFN-β NF-κB site by electrophoretic mobility shift assay (EMSA, Figure 11). PMA-induced extracts contained about 20-fold higher levels of specific DNA binding proteins capable of binding to either the IFN-β or the IL-1β-κB sites (Fig. 11, lanes 1 and 7) than uninduced extracts (data not shown). Binding to the IL-1β·κB site was completely abolished by the addition of 200-fold molar excess of unlabeled IL-1β or P2 competitor DNA (Fig. 11, lanes 2, 4, and 5), while mutation of the first three GGG residues of the P2 site or mutation of the AA residues at postitions 5 and 6 eliminated the ability of the P2 site to compete for IL-1β complex formation (Fig. 11, lanes 3 and 6). In contrast, binding to the IFN-β P2 site was about 80 % competed by the addition of a 200-fold molar excess of oligonucleotides containing either 1, 2, or 3 copies of the IL-1β-κB site (Fig. 11,

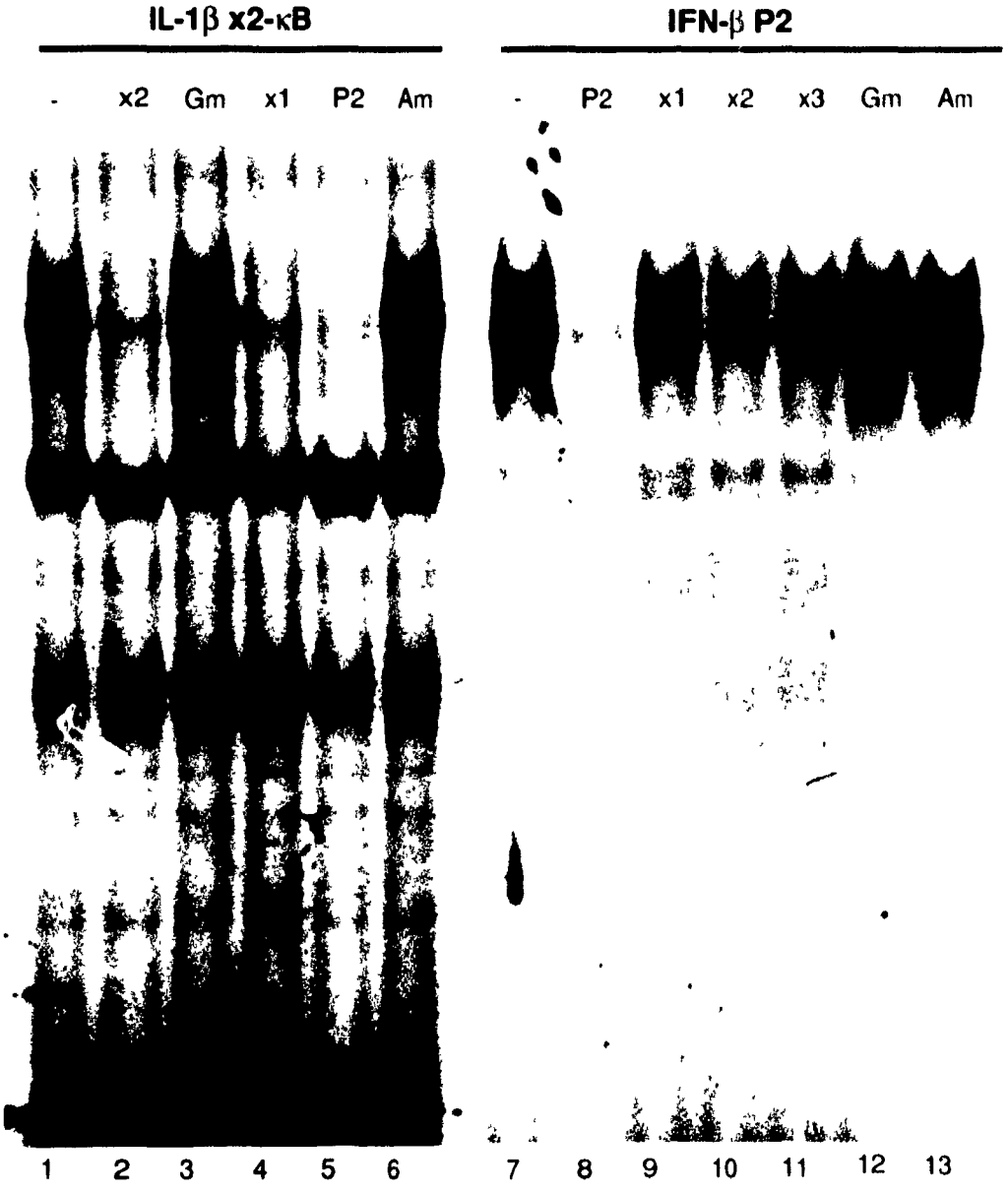
FIGURE 10: Schematic Representation of the Interleukin-1β Gene Promoter.

The schematic, reproduced from Bensi et al. (1990) indicates the putative NF-κB binding site located at postion -307 to -297, and its sequence. Included in this diagram are the enhancer located between -2982 and -2795, the putative negative element, intron 1 and exon 1. The upper diagram indicates the location of the IL-1β gene-CAT fusion. The lower panel describes the oligonucleotides used in the electrophoretic mobility shift assays, the abreviations are explained in the Materials and Methods, and the mutations are written in lower case.



IFN-β P2-	GGGAAATTCCGGGAAATTCC
IL-1β-1κB-	GGGAAAATCC
IL-1β-2κB-	GGGAAAATCCGGGAAAATCC
IL-1β-3κB-	GGGAAAATCCGGGAAAATCCGGGAAAATCC
A mut-	GGGAc g TTCCGGGA c gTTCC
G mut-	a c t AAA TTCCa c t A AATTCC

FIGURE 11: Binding of NF-κB Proteins to the IL-1βx2-κB Sequence. WCE were prepared from PMA induced U937 cells; 10 μg were assayed for binding to the IL-1βx2-κB and IFN-β P2 sequences. Extracts were preincubated without unlabeled competitor (lanes 1 and 7, shown below the autoradiograph), or 200-fold molar excess of various oligonucleotides (lanes 2-6, and 8-13), and analyzed on a 6 % native tris-glycine PAGE; x2 indicates IL-1βx2-κB, Gm indicates G mutant, Am indicates A mutant, x1 indicates IL-1βx1-κB, x3 indicates IL-1βx3-κB, and P2 indicates the IFN-β P2 sequence.


IL-1β x2-κB
IFN-β P2
- x2 Gm x1 P2 Am
- P2 x1 x2 x3 Gm Am
1 2 3 4 5 6 7 8 9 10 11 12 13

lanes 9-11).

Using extracts from PMA-stimulated U937 cells, competition profiles were generated using the IL-1β (Figure 12A) or the IFN-β NF-κB probes (Fig. 12B); however, oligonucleotides containing 1 or 2 copies of the IL-1β site were less effective in competing for the complexes formed on the P2 probe, as suggested by the results of Fig. 11. This result, together with a decreased amount of protein-DNA complex formed using the IL-1β probe in multiple experiments (compare Fig. 11, lanes 1 and 7), indicated that the single T to A transition at position 7 of the IL-1β-κB sequence resulted in a 4-fold weaker binding site than the IFN-β site. Nonetheless, co-migration of the protein-DNA complexes indicated that both sites bound NF-κB-related proteins.

K) NF-κB p50 and p65 Subunits Interact with the IL-1β NF-κB Site.

Recombinant NF-κB p50 and IκB/MAD3 proteins, generated as GST fusion polypeptides and then cleaved to release functional proteins, were used to assess interaction of the subunits with the IL-1β κB site. Recombinant p50 (50 ng) specifically interacted with the P2 and the IL-1β NF-κB sites (Figure 13, lanes 1 and 6); complex formation was eliminated by the addition of a 200-fold molar excess of unlabeled competitor P2 or IL-1β oligonucleotides (Fig. 13, lanes 2-5, and 7-10).

Recombinant IκB (rIκB) was used together with PMA-induced U937 protein extracts to examine the involvement of p65 in IL-1β-κB complex formation. Previous studies have demonstrated that IκB interacts with the p65 subunit of the NF-κB complex to cause dissociation of DNA bound p50-p65 complexes (Zabel and Baeuerle, 1990), and forms the basis of a functional assay for IκB and p65 DNA binding activity. Addition of rIκB to protein-DNA complexes generated with either IFN-β (Figure 14, lanes 1 and 2) or IL-1β sites

FIGURE 12: Relative Binding Affinities of NF-κB to the IL-1βx2-κB and IFN-β P2 Sequences.

WCE from PMA stimulated U937 cells (10 μg) were assayed for binding to the IL-1βx2-κB (**A**) or the IFN-β P2 sequence (**B**) with increasing concentrations of unlabeled competitor ranging from 50 to 1000-fold molar excess added during preincubation. Gels were autoradiographed, scanned by laser densitometery, and the levels of bound material were quantitated relative to the κB protein-DNA complexes formed without addition of competitors (C/C_o).

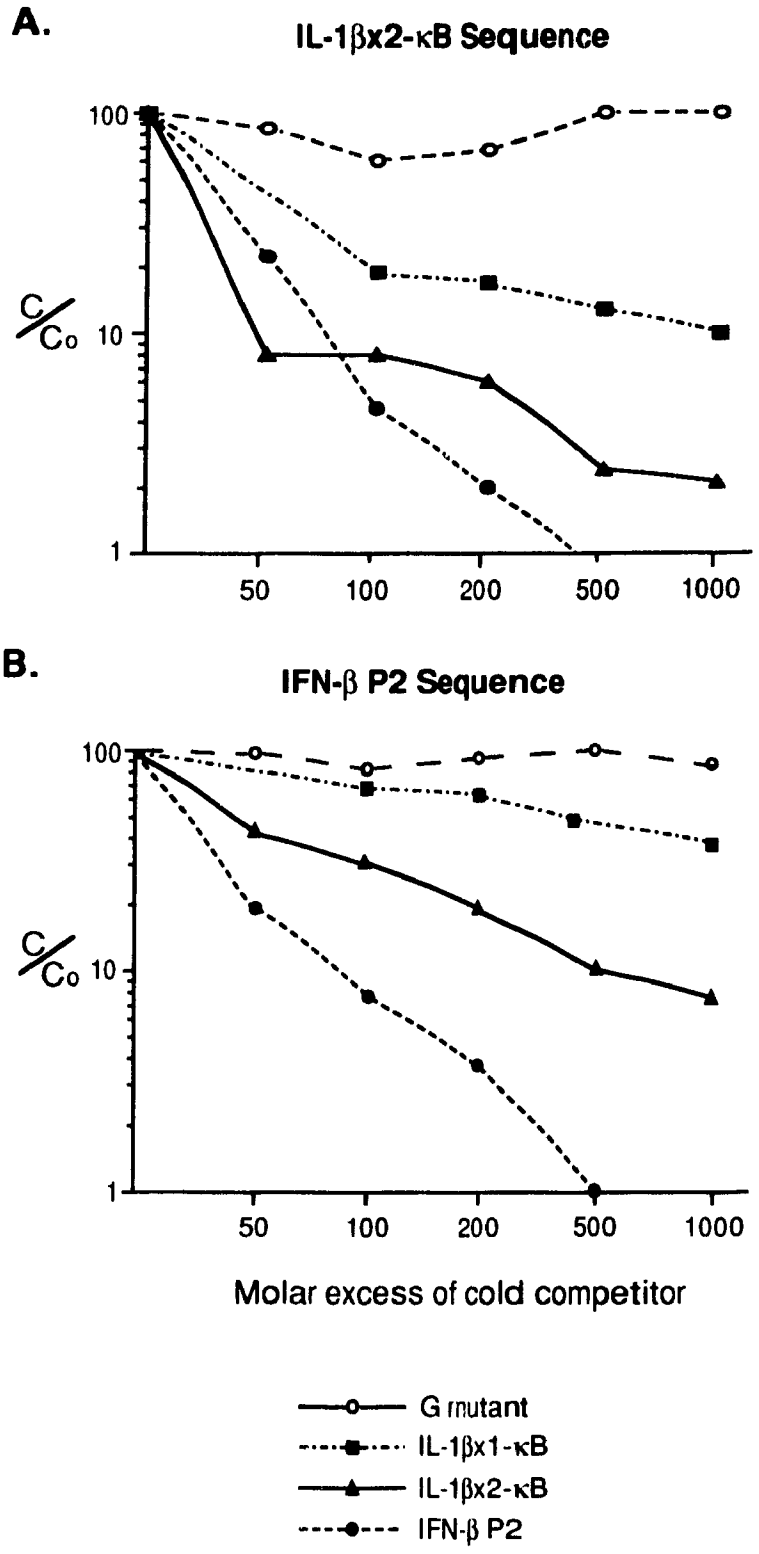
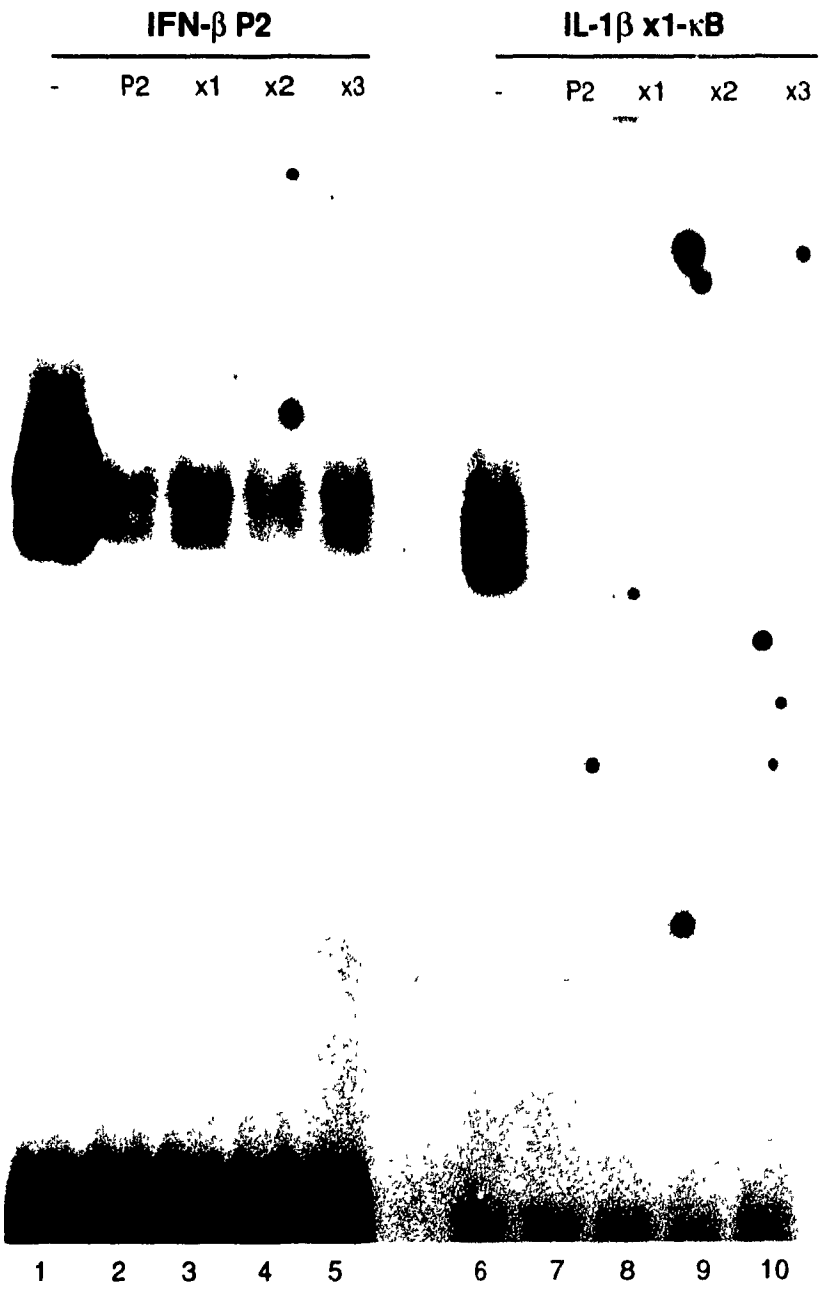

A.
IL-1βx2-κB Sequence
100
10
1
C/Co
50
100
200
500
1000
B.
IFN-β P2 Sequence
100
10
1
C/Co
50
100
200
500
1000
Molar excess of cold competitor
G mutant
IL-1βx1-κB
IL-1βx2-κB
IFN-β P2

FIGURE 13: Specific Interaction of NF-κB p50 with IL-1βx1-κB.

Gel shift analysis using ^{32}P-labelled IL-1βx1-κB and IFN-β P2 oligonucleotides as probes and 50 ng recombinant NF-κB p50 subunit. Extracts from bacteria transformed with plasmid pGEX-3X containing GST-NF-κB p50 fusion were induced with 1 mM IPTG, purified through a glutathione column, and assayed for gel retardation (described in the Materials and Methods). Lanes 1 and 6 contained no competitor while lanes 2-5 and 7-10 contained 200 fold molar excess of various cold oligonucleotides (listed above each lane).


IFN-β P2
IL-1β x1-κB
- P2 x1 x2 x3
- P2 x1 x2 x3
1 2 3 4 5 6 7 8 9 10

(Fig. 14, lanes 3-6) dissociated the κB complex (Fig 14, lanes 2, 4, and 6); addition of rIκB to protein-DNA complexes using recombinant p50 did not decrease complex formation, indicating the specificity of the rIκB for p65 containing complexes (data not shown). Furthermore, rIκB did not affect the faster migrating IL-1β specific complex (Fig. 14, lanes 3-6); the nature of this complex termed NF-βB, which does not form to a significant extent with the P2 probe (Fig. 14, lanes 1 and 2) is currently under investigation (M. Fenton, personal communication).

L) Functional Activity of IL-1β NF-κB Dependent Promoters in U937, Jurkat, and 293 Cells.

To examine the transcriptional activity of the IL-1β NF-κB site, 1 to 3 copies of the IL-1β–κB site were subcloned into the *Acc1-Sph1* cleaved SV_1CAT and compared to the activity of reporter plasmids containing 2 and 4 copies of the IFN-β NF-κB sequences, P2(1)CAT and P2(2)CAT respectively, following transient transfection into either promonocytic U937, fibroblastic 293, or Jurkat T cells (Figure 15). CAT reporter activity was assessed at 24 hr after addition of phorbol ester (PMA) or lipopolysaccharide (LPS). The upper panel of Fig. 15 illustrates a representative analysis of the inducibility of the IL-1β-κB-dependent constructs in U937 cells in response to PMA or LPS, while the lower bar graphs summarizes the average of duplicate experiments. With all the NF-κB constructs, low basal level activity was observed in U937 cells (Fig. 15, open bars). Induction with PMA led to increases in IL-1β-κB-dependent CAT activity ranging from 5 to 50 fold (Fig. 15, solid bars); the level of PMA inducibility of the constructs was directly dependent on the number of IL-1β-κB enhanson sites present. As demonstrated previously, multimerization of the PRD II elements in CAT-based reporter plasmids also led to a synergistic stimulation of

FIGURE 14: Recombinant IκB Induced Inhibition of NF-κB Binding to the IL-1β-κB Sequence.

10 μg of WCE from PMA induced U937 cells was preincubated with or without 50 ng of rIκB obtained from bacterial extracts, and analyzed for binding to the IFN-β P2 (lanes 1 and 2), IL-1βx1-κB (lanes 3 and 4), and IL-1βx2-κB (lanes 5 and 6).


IL-1β NF-κB
IFN-β P2
1 copy
2 copies
I-κB
- + - + - +
1 2 3 4 5 6

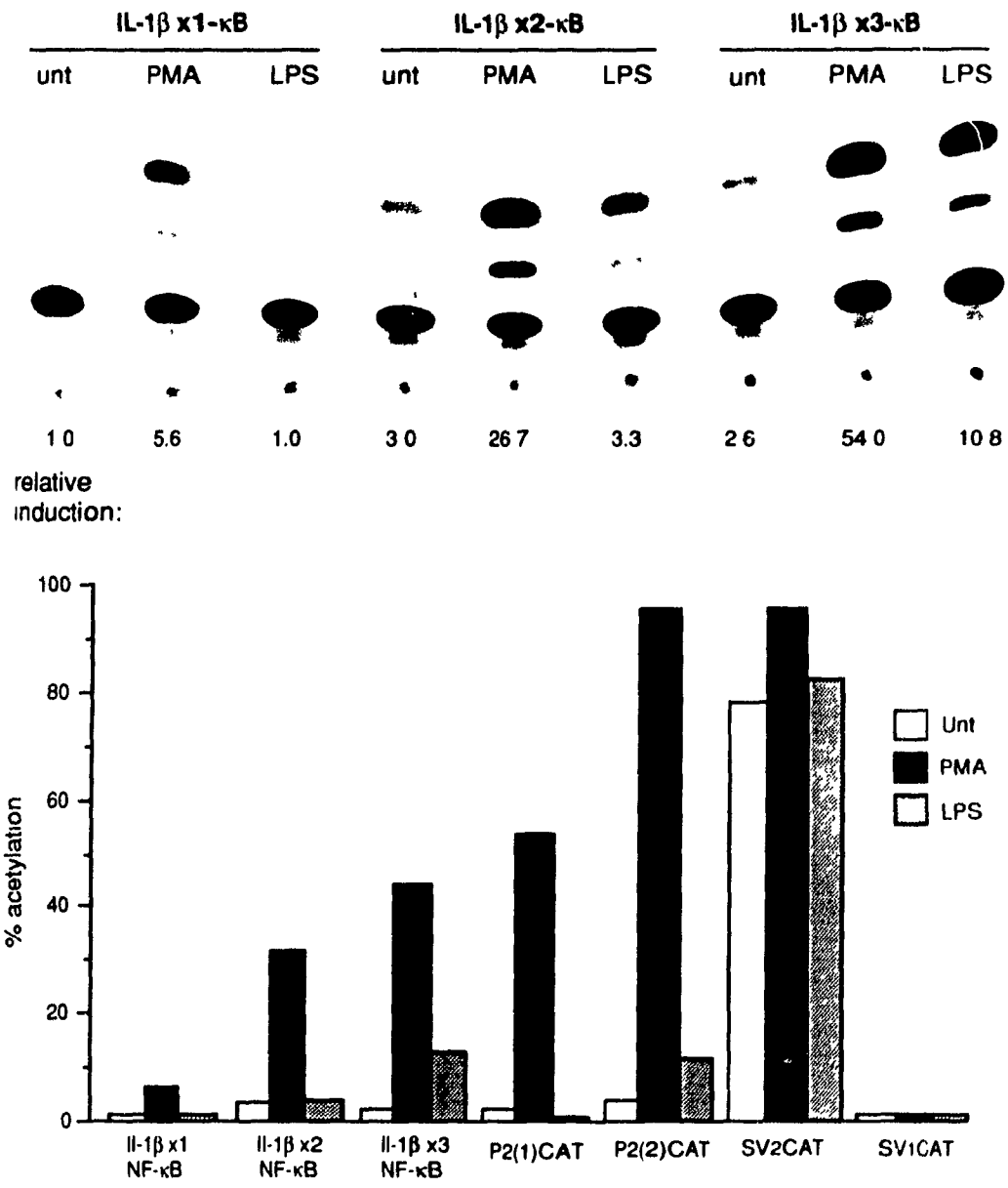
transcriptional activity (Fig. 15, lower panel). The synergistic activity of both the IL-1β and IFN- κB elements is consistent with the definition of the κB element as a functional proto-enhancer (Leblanc et al., 1990). In all experiments, the PRD II sequences were stronger transcriptional elements than the IL-1β sequences, indicating that differences in the binding affinities of the two sites was also reflected in distinct levels of functional transcriptional activity.

In contrast to the results of PMA induction, LPS treatment of transfected U937 cells did not significantly stimulate κB-dependent transcription with reporter constructs containing 1 or 2 copies of either the IL-1β or the PRD II elements. Multimerization of IL-1β or the PRD II sites - to 3 and 4 copies, respectively - led to a 5-10 fold increase in CAT activity following LPS stimulation (Fig. 15, hatched bars). These results demonstrate that LPS stimulation is sufficient to stimulate NF-κB-dependent reporter gene activity in U937 cells as well as NF-κB DNA binding activities (data not shown); the fact that multimerization of the κB elements was necessary to detect LPS responsiveness however suggested that LPS was a weaker inducer of these activities *in vivo*.

The results differed slightly in fibroblast 293 cells, where the 1 and 3 copy IL-1β-κB sites were relatively strong transcriptional activators without the addition of inducers (data not shown). In general the basal transcriptional activity in these cell types was high, and induction by LPS or PMA was not as significant as in the U937 cell transfectants. Within the context of the 293 cellular background, constructs containing 1 or 2 IL-1β-κB sites had a 3-4-fold higher basal level of CAT activity than the same constructs in U937 cells. PMA induction of U937 cells transfected with these constructs resulted in a further 3-5 fold stimulation of activity. Multimerization of IL-1β-κB sites generated a constitutively active enhancer element that was slightly induced (< 2 fold) by

FIGURE 15: Induction of IL-1β and IFN-β NF-κB Hybrid Promoters.

U937 cells were transfected by the DEAE-Dextran method, and induced 24 hr later with PMA (25 ng/ml) or LPS (100 μg/ml). Cells were harvested 48 hr after transfection, and 50 μg of total cell lysate was assayed for CAT activity for 1 hr. Transfections were performed twice; one representative TLC plate is shown above, and the mean values obtained for two transfections are shown below. Average induction ratios are shown below the TLC, and are calculated relative to the % conversion obtained for the untreated IL-1βx1-κB cell transfections. Each transfection contained SV_1CAT and SV_2CAT controls.


IL-1β x1-κB
IL-1β x2-κB
IL-1β x3-κB
unt
PMA
LPS
unt
PMA
LPS
unt
PMA
LPS
1 0
5.6
1.0
3 0
26 7
3.3
2 6
54 0
10 8
relative induction:
100
80
60
40
20
0
% acetylation
Unt
PMA
LPS
Il-1β x1 NF-κB
Il-1β x2 NF-κB
Il-1β x3 NF-κB
P2(1)CAT
P2(2)CAT
SV2CAT
SV1CAT

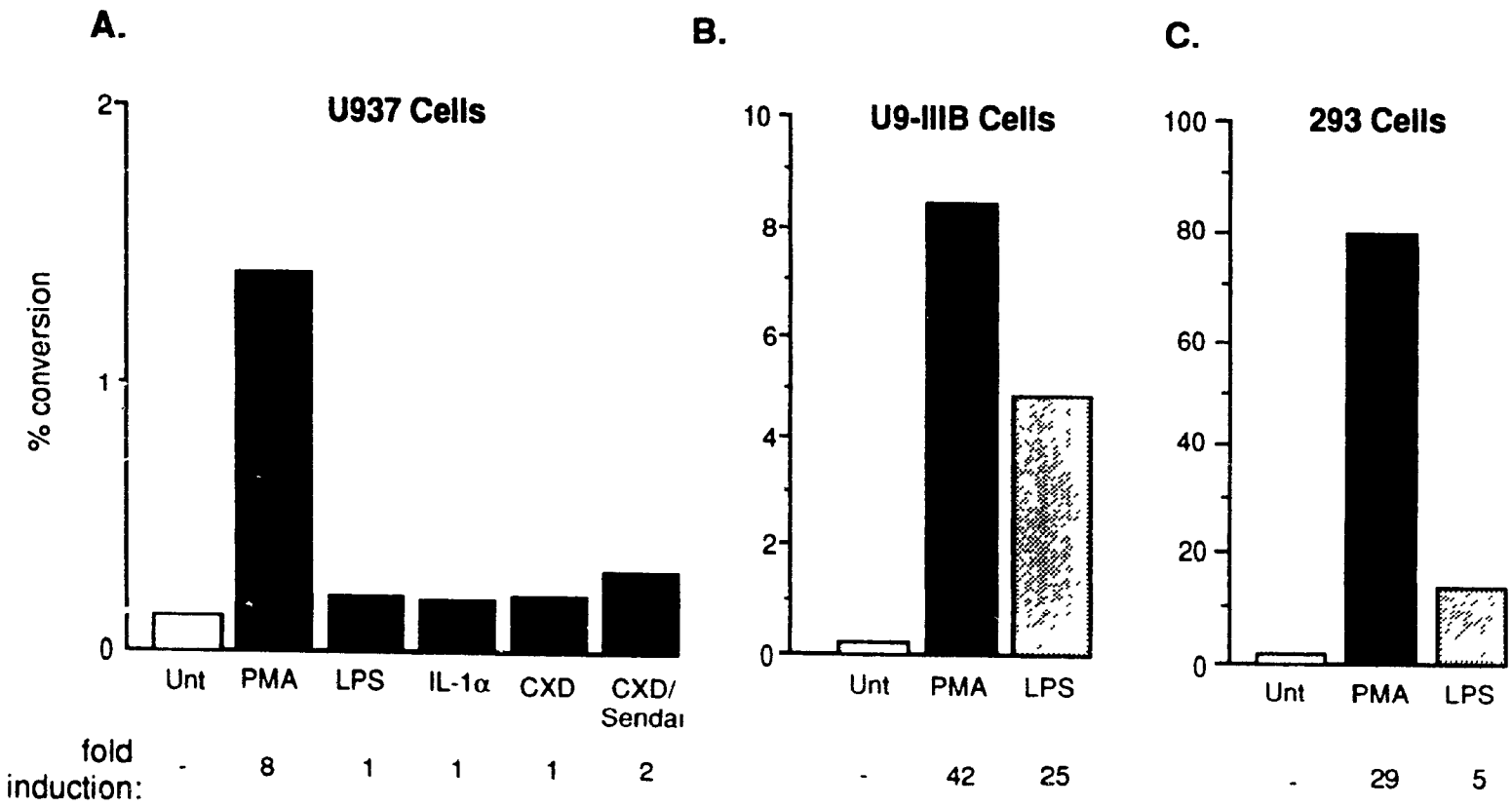
PMA treatment. Similarly, the constructs containing 2 or 4 copies of the PRD II element had higher basal levels of activity in 293 cells compared to U937 cells. Transient transfection of mouse fibroblast L929 cells by Goldfeld et al. (1990) using a 2 X PRD II CAT construct (similar to P2(2)CAT) interestingly produced similar results. The authors found that multimerization of this IFN-β κB sequence and transfection into fibroblast cells produced high basal levels of transcription which were only mildly inducible by Sendaï paramyxovirus. The same report demonstrated that multimerization of the putative TNF-α κB sites also produced high basal levels of activity which were not significantly inducible by either LPS or virus. Therefore, high levels of basal NF-κB dependent transcription in fibroblast cells followed by weak induction is not unique to our system.

Similar but not identical patterns of transcriptional activation using the same IL-1β and IFN-β-based reporter plasmids were observed when Jurkat T cells were transfected (data not shown). In order to establish reliable CAT activation, all transfections were repeated at minimum twice, and also with the use of a CMV-gal-based plasmid, we were able to determine that transfection efficiencies did not vary significantly (<= 10 %) between experiments.

To determine whether activation of an IL-1β CAT reporter plasmid (IL-1β 4.4 CAT) containing 4,400 bp of promoter was also inducible in these same cell types, transfections and induction by LPS or PMA was performed (Figure 16). DEAE-Dextran transfection into U937 cells indicated that constitutive levels of IL-1β 4.4 CAT was low, and induction by a variety of agents did not stimulate plasmid activity significantly. Only PMA treatment resulted in a 7-fold induction (Fig. 16A, inducers are indicated below the graphs, and fold induction is indicated below the inducing agents). However, when the plasmid was expressed in HIV infected U937 cells, both LPS and PMA strongly stimulated activity to 42 and 25 fold respectively (Fig. 16B).

FIGURE 16: Induction of the IL-1β CAT 4.4 Plasmid by PMA.

The IL-1β CAT 4.4 expression plasmid (shown in figure 10) was transfected (5 μg) into U937 (**A**), and U9-IIIB (**B**) cells by the DEAE-Dextran procedure, and into 293 cells (**C**) by calcium phosphate co-precipitation (described in the Materials and Methods). Cells were untreated (unt) or induced with phobol ester (PMA), lipopolysaccharide (LPS), recombinant interleukin·1α (IL-1α), cyclo-heximide (CXD), or CXD and Sendaï virus (CXD/Sendaï) for 24 hr. 100 μg of total U937 and U9-IIIB cellular proteins were analyzed for CAT activity for 4 hr at 37°C, while 50 μg of 293 cell extracts were analyzed for 2 hr. Calculation of fold induction is described in the Materials and Methods and the numbers are indicated below the graphs.


A.
U937 Cells
% conversion
2
1
0
Unt
PMA
LPS
IL-1α
CXD
CXD/
Sendai
fold
induction:
-
8
1
1
1
2
B.
U9-IIIB Cells
10
8
6
4
2
0
Unt
PMA
LPS
-
42
25
C.
293 Cells
100
80
60
40
20
0
Unt
PMA
LPS
-
29
5

Surprisingly, expression of the IL-1β CAT 4.4 plasmid in fibroblast 293 cells (Fig 16C) resulted in significantly elevated production of CAT activity compared to U937 and U9-IIIB cells (note the difference in the % conversion scale). The relative fold induction by LPS and PMA however were not dramatically higher, fold induction was calculated at 5 and 29 fold for LPS and PMA respectively. This data indicates that an IL-1β reporter plasmid containing 4,400 bp of promoter was more transcriptionally active in HIV-infected cells reflecting the transcriptional activation of the endogenous gene as determined previously by RT-PCR analysis.

DISCUSSION

A) Regulation of Cytokine Gene Expression During Viral Infection.

The results presented in this thesis demonstrate that HIV-1 infected promonocytic cells (U9-IIIB) express increased levels of cytokines IL-1β, IFN-β, and TNF-α mRNA following coinfection with Sendaï paramyxovirus. Stimulation of HIV-1-infected myelomonoblastic cells (PLB-985) with lipopolysaccharide (LPS) or phorbol ester (PMA) resulted in higher TNF-α, and IL-1β transcription, but not IFN-β or IL-6 levels. Furthermore, the interleukin-1β promoter was found to contain a DNA sequence capable of interacting with NF-κB proteins. Insertion of 1 to 3 copies of this sequence upstream of an enhancerless reporter plasmid indicates that this sequence is transcriptionally active and inducible by LPS and PMA in myeloid, lymphoid, and fibroblastic cells.

Analysis using other viral models demonstrates that the results obtained using the HIV-monocyte cell model are not unique. Stellrecht et al (1992) recently demonstrated that Jurkat T cells infected with vaccinia virus could preferentially activate interleukin-2 (IL-2) production while not significantly modulating interleukin-1β (IL-1β) or interferon-γ (IFN-γ). However. when these cells were stimulated with PHA-PMA, IFN-γ secretion was upregulated while IL-2, IL-1β, and IL-6 expression were not stimulated. Similar virus-induced cytokine gene expression has also been demonstrated using cytomegalovirus (Dudding et al., 1989), Sendaï virus, (Aderka et al., 1986), and influenza A virus (Nain et al., 1990, and Gong et al., 1991). Using the herpes virus model and purified monocytes from whole blood, Gosselin et al (1991, 1992, 1992) found that HHV-6 could stimulate IL-1β, while HSV, and EBV did not, and conversely that IL-6 and TNF-α were significantly repressed by HHV-6, and EBV, while HSV addition was refractive. Modulation of cytokine gene expression by herpes

viruses is supported by the recent evidence indicating an Epstein Barr virus protein, BCRFI, is analogous to human IL-10 (Vieira et al., 1991). In contrast, dysregulated cytokine production by HIV-infected cells is not easily explained.

Many groups have demonstrated that antigen-stimulated HIV-infected monocyte/macrophages or T lymphocytes transcribe and translate significantly higher levels of cytokines. Addition of LPS, PMA, or Sendaï virus to HIV-infected cell cultures produces elevated levels of IL-1, TNF, IFN-α, IFN-β, and IL-6 at the transcriptional and translational level (Breen et al.,1990, D'Addario et al., 1990, 1992, Folks et al., 1987, Roulston et al., 1992, and Yamato et al., 1990). Vyakarnam et al. (1991) found that *in vitro* LPS-stimulated peripheral blood mononuclear cells (PBMC) isolated from six HIV infected individuals resulted in significantly higher secretion of TNF-α,TNF-β, and IFN-γ. Similar results were obtained by Allen et al. (1990), Honda et al. (1990), and Voth et al. (1990) using mononuclear cells isolated from HIV-infected persons. These reports also demonstrate that IL-2 cytokine receptor expression on monocytes and T lymphoid cells may also be modulated by HIV infection. Enhanced production of cytokine RNA was not restricted to monocytic cells since IFN-γ transcription was also increased in a chronically infected T lymphoid cell line following PMA stimulation (M. D'Addario, unpublished observations). These results indicated that antigenic stimulation of HIV-infected cells may lead to the production of significantly higher levels of cytokines which may further disrupt normal immune function

The relative contributions of increased transcriptional initiation, and increased mRNA stability to the coordinate enhancement of cytokine gene expression have been examined. Many transiently expressed genes encoding cytokines, growth factors, and certain proto-oncogenes, possess AU rich sequences in the 3' untranslated region of mRNA which decrease the half life of

these specific mRNAs by a poorly defined mechanism (Caput et al., 1986, Shaw and Kamen, 1986, and Peppel et al., 1991). The AU rich instability sequences of multiple cytokine RNA species represent a distinct stage of RNA metabolism at which these genes could be coordinately regulated as a consequence of HIV infection. Our results (D'Addario et al., 1992), and data from Yamato et al. (1990) indicate that no significant difference in the stability of IL-1β and TNF-α RNAs in HIV infected monocytic and myelomonoblastic cells.

Several HIV regulatory proteins have been characterized, including Tat, Rev and Nef, that could potentially modulate cellular gene transcription. Of these, the 15 kDa Tat protein is known to interact with the stem-loop structures at the 5' end of HIV RNA and *trans*-activate expression of all sequences linked to the HIV LTR (reviewed in Sharp and Marciniak, 1989). As yet however, no cellular genes have been identified which are activated by the unique *trans*-activation mechanism of the *tat* gene. However, recent data from Jagannadha-Sastry et al. (1990), and Farrell et al. (manuscript submitted) demonstrate that Raji B cells stably transfected and expressing Tat protein transcribe higher levels of TNF-β. When Hela-Tat cells were examined for the production of IL-1β however, the levels produced by these cells was found to be depressed. In addition, while our HIV infected cells transfected with IL-1β CAT produced very high levels of activity, transfection of the IL-1β CAT vector into Tat expressing cells did not produce similar results. The involvement of Tat and elevated levels of cytokines is reinforced by the evidence demonstrating that tat protein not only stimulates Kaposi's sarcoma-derived cells *in vitro* (Ensoli et al., 1990), but that growth of these Kaposi's sarcoma-derived cells involves the secretion of high levels of IL-1β, basic fibroblast growth factor, and other angiogenic agents (Ensoli et al., 1989). Cotransfection of a tat expressing plasmid and an IL-1β CAT vector into U937 cells also did not demonstrate increased transcriptional

activity, indicating that Tat protein may not be completely responsible for the effects observed.

Recently, a novel HIV-1 regulatory gene, *vpr*, was characterized which increased the rate of replication, and accelerated the cytopathic effect of the virus in T cells (reviewed in Greene, 1990). *vpr* was also capable of *trans*-activating the HIV LTR as well as other heterologous viral promoters (Cohen, E., et al., 1990). This initial characterization of *vpr* suggests that HIV-chronically infected cells may constitutively express a non-specific *trans*-activator protein capable of enhancing transcription of specific cellular genes. Coexpression of a *vpr* expressing plasmid and a cytokine reporter plasmid should help determine if Vpr protein plays a role in increasing cytokine gene transcription.

One other interesting aspect of cytokine gene activation by HIV explores more specifically the activities of specific viral proteins. The use of recombinant HIV proteins has demonstrated that activation of cytokine genes may be partially mediated by the interaction of HIV gp120 with cell surface CD4 (Clouse et al., 1991, Merrill et al., 1989, 1992, Rieckmann et al., 1991a, and Wahl et al., 1989). Interaction of recombinant HIV gp120 with CD4 causes specific intracellular signals that activate the transcription of IL-1β, TNF-α, IL-6, and GM-CSF from monocytes, T cell lines and primary cultures. Recent evidence from Merrill et al. (1992) indicates that brain cultures can also be stimulated to produce IL-1 and TNF when induced *in vitro* with recombinant HIV gp160 or gp41. Therefore, the interactions of gp120 on HIV infected cells with CD4 may be a mechanism partially responsible for elevated levels of cytokines in the serum of HIV infected persons.

Although the above results demonstrate that HIV may alter cytokine gene expression, the reverse may also be true. Cytokines such as TNF-α, IL-1, M-CSF, and GM-CSF have been shown to stimulate HIV replication and

propagation in monocytes, T lymphocytes, and bone marrow stem cells (Chester Kalter et al., 1991, Clouse et al., 1989, Folks et al., 1989, Hazan et al., 1990, Israël et al., 1989, Kitano et al., 1991, Koyanagi et al., 1989, Locardi et al., 1990, Poli et al., 1990, and Rieckmann et al., 1991b). This activation was found to occur at the transcriptional level by the activation of the HIV LTR in CAT assays, and by secretion of infectious HIV particles from infected cells. Furthermore Amadori et al. (1989), and Rieckmann et al. (1991a) recently found that B lymphocytes isolated from HIV-seropositive patients constitutively produce IL-6, which can activate HIV from autologous monocytes and T cells. Another interesting result which closely examined the role of HIV in hematopoietic development showed that GM-CSF added to HIV infected bone marrow stem cells not only stimulated cytokine production and cell differentiation, but also induced HIV replication in the bone marrow (Kitano et al., 1991). Therefore, elevated levels of circulating cytokines due to antigenic stimulation of HIV-infected cells, may not only result in immune dysregulation, but in selective activation of latent HIV, thus increasing viral burden in the host.

Although cytokines may be upregulated upon HIV infection, one interesting set of results demonstrates that cytokines may also suppress HIV infection and spread. Many groups, including our own, have shown that addition of interferon-α (IFN-α) to cell cultures infected with HIV, or addition of this cytokine prior to HIV infection strongly suppresses HIV infection and/or spread (Dubreuil et al., 1990, Gendelman et al., 1990a, Orholm et al., 1989, and Poli et al., 1989, 1991). Macé et al. (1989) demonstrated that IFN-α-induced HIV inhibition could be abrogated by the addition of antibodies to IFN-α resulting in the continued spread of the virus. Poli et al. (1989) showed that IFN-α could suppress HIV propagation in chronically infected cells more efficiently than the antiviral base analogue 3'-azido-3'-deoxythimidine (AZT). The intriguing aspect

of the anti-HIV potential of IFN is its mechanism. While AZT is known to act by chain terminating HIV reverse transcription, the mechanism utilized by IFN is not completely understood. Poli and collegues suggest that post-translational inhibition of virus maturation is involved, due to the accumulation of HIV particles in the infected cells. Although TNF-α may activate HIV gene expression, it has been shown that addition of TNF-α and IFN-γ prior to HIV infection resulted in diminution of HIV entry and spread in T cell cultures (Faltynek et al., 1989, and Wong et al., 1988). More elaborate molecular analyses have also shown that IFN genes (IFN-α2, and IFN-β) constitutively expressed or induced in HIV-infected cells suppress HIV proliferation (Bednarik et al., 1989, and Macé et al., 1991).

B) HIV Infection and Monocyte Differentiation.

One of the alterations of monocyte physiology induced by HIV infection is the induction of differentiation. Analysis using another lentivirus model demonstrated that infection of monocytes by sheep lentiviruses was dependent on monocyte maturation (Gendelman et al., 1985). Monocytes isolated from sheep peripheral blood supported virus entry and replication poorly, while *in vitro* differentiated monocytes did not restrict viral infection or expansion. However, the mechanisms associated with this requirement for differentiated cells are not well understood. Activation of T cells has also been shown to be necessary for *in vitro* infection by HIV (Gowda et al., 1989). In this case, activation of T cells by PHA or anti-CD3 stimulated HIV envelope fusion with the cell membrane, while unstimulated T cells did not initiate nucleocapsid entry.

Morphological and ultrastructural changes have also been demonstrated following HIV infection of promonocytic cells. Adherence to plastic, accumulation of larger more abundant cytoplasmic vacuoles, lobularized nuclei,

ruffling cytoplasmic membrane, larger cytoplasm to nucleus ratio, less basophilically staining cytoplasm (determined with Giemsa), and enhanced expression of cell surface CD14 have all been demonstrated to be characteristic changes associated with HIV infection or phorbol ester induced differentiation (Koeffler, 1983, Lübbert et al., 1991, Minta and Pambrum, 1985, Pautrat et al., 1990, and Roulston et al., 1992). Molecular analysis of *c-fms* and *c-myc* proto-oncogene expression indicated that HIV-infected myelo-monoblastic cells have differentiated into monocyte like cells (Roulston et al., 1992). Interestingly the CD14 surface marker has recently been demonstrated to be responsible for the interaction with LPS (Wright et al., 1990), and increased expression of this receptor on HIV-infected cells may lead to increased responsiveness to bacterial antigen, and hence increased immunological activation.

Another important feature of HIV-induced monocyte differentiation is the transcriptional activation of genes mediated by specific cellular factors. One of the most important transcription factors regulating HIV activation is NF-κB (Nabel and Baltimore, 1987). Several pieces of evidence indicate that HIV-infected monocytes express elevated levels of transcriptionally active NF-κB, and that antigenic stimulation of these cells results in significantly higher levels of NF-κB-mediated gene expression (Bachelerie et al., 1991, Griffin et al., 1989, Pautrat et al., 1990, Roulston et al., 1992, and Suzan et al., 1991). These results appear to indicate that undifferentiated cells express levels of NF-κB that can be significantly elevated as HIV infection progresses (Bachelerie et al., 1991).

Our results are in agreement with previous experiments that used several different myeloid cell lines to show that NF-κB was constitutively expressed in mature monocytic cell lines and primary macrophages; in those studies, NF-κB binding activity appeared to be induced during the promyelocyte to monocyte

transition. In HL-60 and U937 cells, NF-κB binding activity, expression of a HIV-LTR reporter CAT construct, and monocytic differentiation were coordinately induced by PMA treatment, whereas in P388, THP-1 and PU5-1.8 cells, NF-κB binding activity and basal level expression of the LTR-CAT plasmid were constitutive and not further induced by PMA (Griffin et al., 1989). Similarly, we found that in PLB-985 cells, PMA treatment resulted in the induction of NF-κB binding activity and monocytic differentiation (Roulston et al., 1992).

It is now clear that NF-κB represents a family of related DNA binding polypeptides that include p45, p50, p55, p65, p75 and p85 (*c-rel*), each of which is capable of binding to a single DNA sequence motif (reviewed in Baeuerle, 1991). The relationship of all these proteins to one another remains to be established as does the functional consequences of the protein-DNA interactions with regard to gene expression. At least some of the individual subunits possess unique affinities for the recognition site that can be altered significantly by point mutations. Furthermore, the degree of heterodimer formation between distinct subunits also differs. Temporal variations in the induction of NF-κB proteins have been described; in phorbol ester-induced Jurkat T cells, a rapid induction of p55 and p75 forms of NF-κB - occurring within minutes of induction - was detected, while the p85 and p50 DNA binding activities were induced after several hours of PMA treatment (Molitor et al., 1991). These observations indicate that variations in DNA affinity, heterodimer formation, and induction kinetics may contribute to both positive and negative transcriptional control.

The impact of HIV-1 infection on transcriptional regulation by NF-κB is complicated by the recent observation that the p105 precursor of the p50 NF-κB subunit is cleaved by the HIV-1 protease during acute infection (Rivière et al., 1990). Proteolytic cleavage of the NF-κB precursor may contribute to increased

NF-κB activity in the nucleus of infected cells, and augment viral transcription; alternatively, cytokines such as TNF-α or IL-1, secreted in response to HIV infection, may increase NF-κB translocation to the nucleus.

Perhaps the most striking consequence of HIV-1 infection appears to be the identification of a new higher molecular weight constitutive NF-κB protein-DNA complex in PLB-IIIB cells (Roulston et al., 1992). In addition, almost no "characteristic" NF-κB complexes were detected in these PLB-IIIB cells. These proteins possessed a higher affinity for the more symmetric IFN-β-PRDII binding site than for the HIV enhancer sites, indicating a different pattern of NF-κB proteins may be present in HIV-infected cells which preferentially bind the IFN-β sequence.

C) Monocyte Differentiation and Cytokine Gene Expression.

One of the consequences of differentiation is the activation of cellular pathways not yet fully developed in the undifferentiated cell. Monocytes are active producers of cytokines, and many pieces of evidence indicate that monocyte differentiation causes increased sensitization to antigenic agents which is manifested by an increase in transcription, and translation of cytokine genes.

An excellent set of experiments describing the maturation of murine embryonic stem cells and the expression of cytokines and their receptors was recently published (Schmitt et al., 1991). The authors show that *in vitro* development of murine stem cells into macrophages, mast cells, erythrocytes, neutrophils, and basophils by colony stimulating factors caused characteristic alterations in the expression of cell surface markers, and cytokine genes. The results indicate specific temporal patterns of cytokine genes (IL-6, IL-3, IL-1, IL-4, and various CSFs), cytokine receptor genes (IL-3, G-CSF, IL-1, IL-4, and

CSF-1), and cell surface marker (erythropoietin, and CD45) expression occurs in the developing cell lineages. In general however, most of the genes analyzed become much more transcriptionally active as development progresses indicating that the mature cells were transcribing and translating higher levels of cytokine genes.

Dudding et al. (1989) recently analyzed the production of cytokines from cytomegalovirus (CMV)-infected promyelocytic ML-3 cells. The authors found that IL-1β production from ML-3 cells infected with CMV was not significantly higher than background. However, when these cells were induced to differentiate with PMA prior to CMV infection, transcription, translation, and stability of IL-1β and TNF-α mRNA were dramatically higher, indicating that differentiation caused sensitization to viral infection. In an analogous series of experiments, Aderka et al. (1986) found that Sendaï paramyxovirus produced similar results. Fractionation of peripheral blood mononuclear cells into non-adherent (monocytes) or adherent (macrophages) cells followed by Sendaï virus infection resulted in TNF-α protein levels ~200 times higher in the adherent population. When these two cell populations were stimulated with either LPS or PMA similar differences were observed indicating the mature cell group was much more responsive to antigenic stimulation.

D) Transcriptional Regulation of Cytokine Genes by NF-κB.

Cytokine genes are rapidly and transiently induced by a number of agents. The promoter regions of many cytokine genes have been found to contain sequences capable of interacting with the NF-κB family of transcription factors. Although the extent of characterization of the DNA sequences among genes varies tremendously, NF-κB interactions in part regulate the transcription of IFN-β, IL-6, TNF-α, TNF-β, GM-CSF, and IL-2 genes (reviewed in Baeuerle, 1991).

Although activation of NF-κB by various inducers can occur in a number of cell types, differential activation of cytokine genes presents an interesting question. The NF-κB DNA binding sequences found in these genes have a certain degree of variability which may contribute to differential activation, but all share the consensus sequence 5'-GGGRNNYYCC-3' Therefore the ability to preferentially activate certain immunoregulatory genes upon inflammation and not other genes suggests a complex pattern of gene regulation which may involve a number of transcription factors.

The transcriptional regulation of certain cytokine genes such as IL-6, TNF-α, and IFN-β have been well characterized and have been shown to involve a number of transcription factors interacting with their respective DNA sequences (see Taniguchi, 1988, for review). Arguably, one of the best transcriptionally characterized cytokine genes is interferon-β. In the case of IFN-β, evidence indicates that the gene is ordinarily under negative control (Goodbourn et al., 1986), and that this repression is probably mediated by a repressor protein, PRDI-BF1, interacting with a specific DNA sequence in the promoter element (Keller and Maniatis, 1991). Activation of a number of proteins including the NF-κB family, and interferon regulatory factor-1 proteins, and their interaction with discrete sequences in the promoter (Goodbourn et al., 1985, Fujita et al., 1987, Miyamoto et al. 1988, Xanthoudakis et al. 1987a, 1987b, 1989, and Xanthoudakis and Hiscott, 1987) leads to activation of this cytokine gene to high, but transient levels (Whittemore and Maniatis, 1990). More detailed analysis using various subfragments of the IFN-β promoter demonstrated the activity of these sequences, and their responsiveness to various stimuli (Goodbourn and Maniatis, 1988, and Leblanc et al., 1990). Analysis of other cytokine gene promoters has not been as extensive, and in many cases only a few regulatory sequences have been analyzed. In the cases of TNF-α, IL-6, IL-

2, GM-CSF, and TNF-β, evidence indicates that NF-κB may in part play a role in gene regulation (Cohen, L, et al., 1991, Collart et al., 1990, Freimuth et al., 1989, Goldfeld et al., 1990, 1991, Lenardo et al., 1989b, Libermann and Baltimore, 1990, Lowenthal et al., 1989, Mackman et al., 1991, Nishizawa and Nagata, 1990, Nonaka and Huang, 1990, Schreck and Baeuerle, 1990, Shakhov et al , 1990, Shimizu et al., 1990, Xanthoudakis et al., 1987a, 1987b, and Zhang et al., 1990).

More recently transcriptional activation of interleukin-6 has been shown to be modulated by NF-κB. In this case, rapid induction by LPS, PHA, TNF-α, or polyI:C in promonocytic U937 cells is mediated by a NF-κB site (5'-GGGATTTTCCC-3') located at -63 to -73 relative to the CAP site (Libermann and Baltimore, 1990). In addition, nuclear extracts from PMA-stimulated Jurkat cells produce NF-κB complexes in gel shift assays which were indistinguishable from those produced by immunoglobin κ light chain gene κB sequence. Transcriptional activation of IL-6 by recombinant cytokines IL-1 and TNF was also shown to be dependent on this κB site. Although the IL-6 promoter contains DNA sequences capable of binding AP-1, and CRE proteins, transfection of an IL-6 CAT reporter plasmid containing a mutated κB sequence drastically reduced responsiveness to both IL-1 and TNF (Shimizu et al., 1990).

The involvement of distinct κB protein subunits was analyzed by Nakayama et al. (1992). The authors found that the IL-6 κB site is capable of binding p85 (c-rel) homodimers, and p50-p85 heterodimers. In addition, a lymphoid specific TNF-α-inducible factor bound multimerized copies of the IL-6κB sequence, and preferentially interacted with c-rel homodimers and not p50-p85 heterodimers. This high molecular weight lymphoid specific factor, IL-6κB BFII, was not found in monocytic, or nonhematopoietic cells, but only in T and B lymphoid cells, and bound more tightly to IL-6 and IFN-β κB motifs than the

SV40 and MHC class I κB sites. More recently IL-6 gene activation was also shown to involve NF-IL6, a nuclear protein present in low amounts in various cells but extremely inducible by LPS, IL-6, TNF-α and IL-1, which binds a 14 bp pallindromic sequence responsive to cAMP (Isshiki et al., 1990, and Kinoshita et al., 1992). The analysis of the IFN-β and IL-6 promoter sequences demonstrate that although our analysis concentrated on κB-mediated transcriptional activation, other specific factors interacting with the upstream sequence contribute to gene regulation.

Cell and tissue-specific factors have previously been shown to bind and differentially activate transcription from various κB sequences. Stimulation of Jurkat T cells by TNF-α activates the HIV-LTR, but not the IL-2 receptor α chain (IL-2_Rα) (Freimuth et al., 1989). However, stimulation of more immature T cells (YT-1) with TNF-α or IL-1 causes activation of an IL-2_Rα κB CAT construct, indicating that cell specific factors may mediate transcriptional activation. Data from Lacoste et al. (1990) shows that activation of IFN-β-based P2(2)CAT in Jurkat T cells is sensitive to TNF-α, but that stimulation of monocytic U937 cells with this cytokine does not produce similar activation. This difference in transcriptional activation is also reflected in gel shift assays showing that TNF-α –induced κB binding to P2 sequences is higher in Jurkat T, but not monocytic cells. Differential activation of cytokine genes by different NF-κB subunits has been demonstrated for the interferon-γ (IFN-γ) intronic κB sequence. The κB sequence (5'-TGATTTTCC-3') in this promoter is similar to the IL-2_Rα sequence (5'-GGGAATCTCC-3'), and is shown to preferentially interact with bacterially expressed p85, and not purified p50 (Sica et al., 1992). Similar differences were found by Cross et al. (1989) to be the cause of different trans-activation efficiencies when they compared the Ig κ light chain and IL-2_Rα κB sequences. This demonstrates that cell specific factors are probably involved in assisting κB

mediated transcriptional activation, and that various subunit interactions may differentially activate κB dependent gene expression.

Another gene which has been shown to be transcriptionally regulated by NF-κB is tumor necrosis factor-α (TNF-α). Evidence from Collart et al. (1990) indicates that the promoter of this gene contain four NF-κB like sequences regulating its transcriptional activation by various inducers. The most interesting aspect of this work is that the four κB sites contain slight sequence variations, and the authors demonstrate that these differences are reflected in their ability to interact with purified NF-κB proteins in gel shift assays. However, while transcriptional activation of multimerized κB sequences is inducible by Sendaï virus, PMA, or LPS, mutation of these sites in the context of the promoter does not inhibit activation of the TNF-α gene *in vivo* (Goldfeld et al., 1989, 1990, 1991, and Shakhov et al., 1991). Moreover, Goldfeld et al. (1990, and 1991) found significant differences in the virus inducibility of the κB multimers in fibroblastic and monocytic cell lines; while Sendaï paramyxovirus strongly induced κB sequences in monocytes, fibroblast cells were not significantly activated Similarly, PMA was found to activate κB sequences in B cells, but not T cells, indicating cell specific factors may be involved in assisting κB-dependent transcriptional activation.

E) Interleukin-1 Gene Activation.

Detailed analysis of the IL-1β promoter has not been undertaken, only recently has evidence emerged about its transcriptional regulation (Bensi et al. 1990). As mentioned previously and reviewed in Dinarello (1991) activation of IL-1 gene transcription can occur in response to LPS, IL-1, TNF-α, IL-6, and PMA. IL-1 gene expression can be superinduced in monocytic cells by the addition of cycloheximide (CHX) indicating that the RNA is transiently produced

and post-transcriptionaly degraded or repressed by a newly synthesized protein (Fenton et al., 1987). While LPS induced IL-1β activation produces a transient pattern of gene transcription, PMA induction produces a more stable RNA species with a longer half life which is not significantly degraded even after 12 hr (Fenton et al., 1988). Addition of actinomycin D (Act D) to PMA-stimulated cells did not result in rapidly degrading RNA, but rather allowed low continuous expression of IL-1β transcripts. Thus LPS and PMA may induce IL-1β gene transcription through different mechanisms. Schindler et al. (1990) recently found that transcriptional activation of the IL-1β gene by LPS or *Staphlococcus epidermidis* in peripheral blood mononuclear cells resulted in different patterns of IL-1β protein synthesis. The authors found that in the absence of LPS stimulation, IL-1β mRNA accumulated intracellularly, and was not translated, indicating post-transcriptional or translational modifications may play a role in IL-1β protein synthesis.

Cyclic AMP-dependent protein kinases have recently been shown to be involved in the transcriptional activation of the IL-1β gene. Agents known to upregulate intracellular cAMP were not only shown to activate IL-1β, but not IL-1α gene transcription levels, but also potentiate LPS induced transcription (Ohmori et al., 1990). The data also indicated that the increased levels of IL-1β RNA were not due to increased stability, as dB2cAMP treated cells did not have significantly different rates of decay. Conclusive evidence for the involvement of cAMP in the activation of IL-1β was obtained by Cheadle and Mizel (1990) who found that addition of cAMP-inhibitors abrogated the cAMP-induced activation of an IL-1β-CAT vector in B cells. They found that IL-1 stimulated cAMP dependent protein kinases (PKA) could be blocked by a protein inhibitor that interacted with only the catalytic subunit of PKA, and not PKC. PMA-induced IL-1 activation was unaffected by cotransfection with the inhibitor, demonstrating

the specificity of the inhibitor for the PKA and not PKC pathway.

The DNA sequences conferring PMA inducible expression of IL-1β in monocytes and B cells were found by Bensi et al. (1990) to be present in an enhancer element present at -3,000 bp from the transcription start site. This 180 bp enhancer, located between -2987 and -2787, contains motifs present in the interferon-β PRDI, and collagenase AP-1 sequences. Insertion of this enhancer sequence upstream of an enhancerless SV40 reporter plasmid conferred TPA inducibility, and multiple copies increased the level of transcriptional activation. To complement this data, we found that the IL-1β promoter contains a sequence which confers NF-κB-dependent transcriptional regulation (D'Addario et al., manuscript in preparation). This site (5'-GGGAAAATCC-3') was found to interact with NF-κB proteins from U937 whole cell extracts. Using recombinant NF-κB proteins, we found that this sequence specifically interacted with both the p50 and p65 subunits of NF-κB. Multimers of the IL-1β-κB sequences were also found to be transcriptionally active and inducible by PMA and LPS in myeloid, lymphoid, and fibroblastic cells. In addition, transfection of an IL-1β CAT reporter plasmid containing 4,400 bp of promoter was more active in HIV infected cells than uninfected myeloid cells. To determine if the IL-1β-κB sequence is responsible for elevated levels of IL-1β expression in HIV infected cells, mutation of this site and reintroduction into these cells would have to be performed. Thus far, we have found that HIV-infected persons have elevated levels of circulating cytokines in their serum. Antigenic stimulation of HIV infected cells may also lead to increased secretion of cytokines from these individuals which may potentially activate latent HIV. Many groups have also shown that transcriptional activation of many cytokine genes and the HIV-LTR is dependent on κB DNA sequences; the question that must be addressed is how do the elevated levels of circulating cytokines activate κB-dependent HIV

expression.

F) Activation of NF-κB by Cytokines IL-1 and TNF.

The activation of NF-κB translocation into the nucleus and binding to sequence specific DNA was shown to require the phosphorylation of IκB. The activation of DNA binding activity *in vitro* was found to occur with protein kinase C, cAMP dependent protein kinase A, and haem regulated eIF-2 kinase (Ghosh and Baltimore, 1989a). The activation of latent HIV was found to be dependent on two NF-κB sites located in the long terminal repeat (Nabel and Baltimore, 1987). DNA binding to these κB sites was inducible by PMA and PHA in Jurkat T cells, and trans-activation of κB CAT constructs, but not mutant κB sequences indicated that expression of HIV is dependent on NF-κB. Kinter et al. (1990) recently found that activation of latent HIV could occur using synthetic PKC activators, while synthetic molecules which activate cAMP-dependent PKA did not produce elevated HIV expression. Using a chronically infected promonocytic cell line which produces undetectable levels of HIV, the authors found that 70-90 % of PKC- or TNF-α-mediated HIV activation could be eliminated by the use of PKC specific inhibitors. No such inhibition was observed when cAMP-dependent PKA inhibitors were used, demonstrating that protein kinase C transcriptionally activates HIV gene expression.

Although activation of NF-κB has been demonstrated using PMA, a molecule which directly activates protein kinase C, activation of HIV gene expression by TNF or IL-1 is less well understood. Many groups have shown that both these cytokines can directly activate κB-mediated trans-activation of HIV (Folks et al., 1989, Osborn et al., 1989, and Poli et al., 1990), and this induction can be correlated with increased levels of NF-κB DNA binding to the HIV LTR κB sequences (Israël et al., 1989, Lowenthal et al., 1989, and Osborn

et al., 1989). All these groups demonstrate that basal κB DNA binding, HIV LTR CAT, and HIV specific p24 antigen expression can all be significantly enhanced by TNF-α or IL-1 treatment of HIV-infected cells.

Two TNF-α receptors have been identified and their differential expression on various cells suggests that both receptors may be independently regulated. Although both share homology in the extracellular area, the intracellular portions do not show significant homology and suggest both may activate different intracellular signalling pathways (see Vilcek and Lee, 1991 for review). Thoma et al. (1990) recently found that the lower molecular weight receptor (TR60), when bound by a specific antibody could inhibit activities mediated by TNF-receptor interaction. In those cells where both receptors were expressed at comparable levels, the authors found that blocking TR60 with antibody, and binding the higher molecular weight with TNF-α did not produce TNF-α-dependent activities such as growth inhibition, and HLA-DR induction. Schütze et al. (1990) found that interaction of TNF-α with receptor produced PKC alterations which were similar to those induced by phorbol ester treatment. They found that some cell lines treated with TNF-α translocated PKC from the cytosol to the membrane and concomitantly increased the expression of TNF receptors (Scheurich et al, 1987). PMA also induced translocation of PKC from the cytosol to the membrane, but synergistically downregulated expression of TNF receptors. The authors conclude that PKC may play a role in TNF-α mediated signal transduction in some, but not all cells. However a short time later, the same group (Meichle et al., 1990) found that TNF-α activation of κB DNA binding activity did not require PKC in K562 and Jurkat T cells. While pretreatment of cells with PKC inhibitors H7 or staurosporine completely blocked PMA-induced κB DNA binding, TNF-α-induced κB activity was slightly, but not completely eliminated, indicating that TNF-α may activate κB DNA

binding activity through another mechanism. Although Zhang et al. (1988) had previously demonstrated that TNF-α stimulated cAMP-dependent PKA activity in fibroblasts, similar analysis in promyelocytic HL60 cells did produce equivalent results. Hohmann et al. (1991) found that both TNF species did not increase intracellular cAMP concentrations, and while forskolin enhanced cAMP PKA, it was not able to activate NF-κB binding activity per se. This data is surprising since Ghosh and Baltimore (1988) found that *in vitro,* PKA could enhance κB DNA binding, and that cAMP-dependent PKA was found in fibroblasts to mediated IL-6 induction by TNF-α (Zhang et al., 1988). However, Zhang et al. (1990) did observe that forskolin-induced IL-6 κB CAT transcriptional activation was weak when compared to TNF-α or IL-1 induction.

Both TNF subtypes bind to the same two types of receptors (Hohmann et al., 1990), and have been shown to activate NF-κB DNA binding activity to the Ig κ light chain sequence within 2 min of addition. Although slight differences in the amount of binding was observed using different cell systems, in both cases concentrations of TNF-α used to initiate DNA binding were roughly 20 times lower than the amounts of TNF-β. In the case of TNF-α, this κB DNA binding activity was observed when only 5-20 % of receptors were bound to the ligand. More recently Kruppa et al. (1992) analyzed TNF-α-induced κB DNA binding to the HIV κB sequences. Activation of κB DNA binding and HIV-LTR CAT induction by TNF-α and LPS was found to be completely eliminated by the blockage of TR60 with a specific monoclonal antibody. To further demonstrate the involvement of the receptor subunit in TNF-α induced κB activity, the authors expressed this receptor in 70Z/3 cells, a population which does not contain endogenous TNF receptor. Whereas stimulation of 70Z/3 cells with TNF-α did not produce any κB DNA binding activity, addition of TNF-α to 70Z/3 cells expressing TR60 responded by inducing κB binding to the HIV sequences.

They also demonstrate that LPS and TNF-α, but not PMA-induced transcriptional activation of an HIV-LTR κB CAT construct can be inhibited by this monoclonal antibody. In addition to showing that TNF-α signal transduction occurs through this receptor subtype, this data also indicates that LPS-induced TNF-α secretion is responsible for κB DNA binding activity. It must be remembered that TNF-α-induced transcriptional activation is not completely dependent on κB sequences. TNF has also been shown to activate PKC-dependent AP-1; therefore although this section dealt only with NF-κB and its induction by TNF, other transcription factors will certainly be induced by this pluripotential cytokine.

As mentioned above, IL-1 can induce HIV gene expression, and the activation of a number of cytokine genes (see Dinarello, 1991, for review). However, the signal transduction pathway used by this cytokine-receptor system has not been very well characterized. In 1989, Elias et al. found that IL-1 could act in synergy with TNF to stimulate transcription and translation of IL-1 in fibroblasts. It now appears that this induction may be mediated by cAMP-dependent PKA. Three reports by Mizel's group (Shirakawa and Mizel, 1989a, Shirakawa et al., 1989b, and Chedid and Mizel, 1990) demonstrated that IL-1-induced activation of NF-κB binding to immunoglobin κ light chain-κB sequence and κB-dependent CAT constructs was dependent on protein kinase A. The use of specific inhibitors to PKA proved this intracellular pathway and not PKC is involved in IL-1 mediated gene activation.

G) Conclusions.

Taken together, our results indicate that HIV-infected cells may transcribe and translate higher levels of cytokines upon antigenic stimulation, or activation with recombinant cytokines. These higher levels of cytokines may lead to the direct activation of NF-κB-dependent gene expression, including activation of other cytokines and latent HIV. HIV-infected cells have already been shown to possess higher levels of cytoplasmic NF-κB which may be activated by cytokines, or HIV-specific proteases; this enhanced level of NF-κB may potentiate cytokine gene activation and immune dysregulation. Apart from the involvement of IL-1 and TNF in the activation of NF-κB-dependent gene expression, IL-1 is also involved in Kaposi's sarcoma of AIDS patients. Our observation that IL-1β is produced at extremely high levels by antigenically stimulated monocytes indicates that this factor may be critically involved in AIDS pathogenesis.

Although data from our lab indicates HIV Tat protein may not be the only factor activating IL-1β gene transcription, co-expression of HIV Vpr-regulatory protein with the IL-1β reporter plasmid would determine if this factor aids hyper expression of the IL-1β gene in HIV-infected cells. The involvement of NF-κB in IL-1β gene transcription can now be studied by cotransfection with distinct NF-κB subunits with the IL-1β reporter plasmid. This would determine if this family of transcription factors is involved in the activation of this cytokine gene, and mutation of the IL-1β-κB site within the context of the promoter would determine if NF-κB is critical or whether other factors are involved.

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