Evaluation of the role of cytokine activation in the multiplication of JC virus (JCV) in human fetal glial cells

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The human polyomavirus, JCV, is the etiologic agent of the fatal central nervous system demyelinating disease, progressive multifocal leukoencephalopathy. Progressive multifocal leukoencephalopathy occurs most frequently in patients with underlying immunosuppressive disorders and is the direct result of virus multiplication in oligodendrocytes, the myelin producing cell in the central nervous system. In this report we test the ability of cellular activation signals to modulate expression of the JCV genome in either transfected or infected human fetal glial cells. In addition, we analyze the binding of nuclear proteins isolated from untreated and cytokine treated human fetal glial cells to transcription factor binding sites in the JCV regulatory region. In contrast to the effects of cellular activation on the expression of the HIV-1 promoter in these cells, none of the cellular activators tested increased expression of JCV. The cytokine, TNF-α, increased binding of NFkB (p50/p65) to a JCV NFkB site but did not modulate the binding of nuclear proteins to the overlapping NF-1/AP1 region of the JCV enhancer. When taken together these results suggest that the response of JCV to cellular activation signals may be fundamentally different from the response of HIV-1 to these signals in human fetal glial cells and that the JCV NFkB site may not be required for JCV gene expression or multiplication in vivo.

Keywords: progressive multifocal leukoencephalopathy; transcription factors; NFkB

Introduction

JC virus belongs to the family papovaviridae and the genus polyomavirinae (Walker and Frisque, 1986; Major et al, 1992). Polyomaviruses are found in a wide variety of other species and are ubiquitous in their respective natural hosts. Seroepidemiological studies have indicated that greater than 70% of the human population worldwide have been exposed to JC virus (Walker and Padgett, 1983). The association of JC virus with a once rare human demyelinating disease known as progressive multifocal leukoencephalopathy (PML) was firmly established two decades ago with the cultivation of the virus from an afflicted individual whose initials were JC (Padgett et al, 1971). The incidence of PML has risen dramatically as a result of the AIDS pandemic. Approximately 2–4% of AIDS patients will develop PML (Berger et al, 1987; Kure et al, 1991).

In vivo, JCV has been detected in oligodendrocytes, astrocytes, in lymphoid tissues, and in peripheral blood lymphocytes of PML patients (Mazlo and Tariska, 1980; Richardson and Webster, 1983; Weiner et al, 1973; Tornatore et al, 1992; Houff et al, 1988; Schneider and Dorries, 1993; Dorries et al, 1994). In vitro, JCV infects glial cells derived from human fetal brain and, to a limited extent, several B lymphocyte cell lines (Atwood et al, 1992; Major and Vacante, 1989). This highly restricted cell type specificity is thought to be due to tissue specific transcription factors that interact with the regulatory region of JCV, cellular factors that interact with the JCV T antigen, or both (Feigenbaum et al, 1987; Ahmed et al, 1990a; Tada and Khalili, 1992; Ranganathan and Khalili, 1993; Chowdhury et al, 1990; Wegner et al, 1993; Renner et al, Kumar et al, 1994; Henson, 1994;
Major et al., 1990; Atwood et al., 1992; Tamura et al., 1990; Tamura et al., 1988). Studies of JCV regulation have mainly focused on the 98 base pair repeat region of the JCV genome that has been shown to contain binding sites for several transcription factors, including the nuclear factor-1 (NF-1) and the activator protein-1 (AP-1/jun) (Tamura et al., 1988; Amemiya et al., 1989, 1992). In addition to transcription factor binding sites in this region we and others have described the presence of a potential nuclear factor-κ (NFκB) binding site outside the JCV 98 base pair repeats (Major et al., 1990; Ranganathan and Khalili, 1993). Recently, transcription from a JCV promoter construct containing an NFκB site was found to be increased by treatment of human glioblastoma cells with the phorbol ester, PMA (Ranganathan and Khalili, 1993).

In this report we examine the effects of cellular activation on the multiplication of JCV in infected and transfected human fetal glial cells. In addition we analyze the binding of nuclear proteins isolated from untreated and TNF-α-treated glial cells to oligonucleotides containing either the JCV NF1/AP1 site or the JC NFκB site. Using a panel of cellular activating agents we were unable to show activation of a reporter gene under the control of the JCV regulatory region. This correlated with gel shift assays which showed no differences in the binding of nuclear proteins to the NF-1/AP1 oligonucleotide from untreated or TNF-α treated glial cells. In contrast, treatment of the glial cells with TNF-α increased the binding of NFκB (p50/p65) to an oligonucleotide containing the putative JC NFκB site. Since our CAT construct did not include the JC NFκB site we infected glial cells with JCV virus to assess the contribution of this site to the multiplication of JCV in these cells. Treatment of the infected cells with a known NFκB inducer, TNF-α, did not result in an increase in the percentage of cells expressing T or V antigens or in the percentage of in situ hybridization positive cells. This is in contrast to the effects of TNF-α, IL-1β, and PMA on multiplication of HIV-1 in these cells (Atwood et al., 1994; Tornatore et al., 1994; Conant et al., 1994).

Our results suggest that the response of JCV to cellular activation signals may be fundamentally different from the response of HIV-1 to these signals in human fetal glial cells and that the JC NFκB site may not be required for JCV gene expression and multiplication in vivo.

Results

JC virus reporter gene expression in response to cellular activation

A JC virus construct (pM1CAT) containing the JCV regulatory region cloned upstream of a chloramphenicol acetyltransferase gene (CAT) was transfected into human fetal glial cells as described in materials and methods. The putative NFκB binding site is outside of the regulatory region of JCV and is therefore not included in this construct. Following transfection the cells were cultured for 48 h in media alone or with media containing either TNF-α, IL-1β, IL-2, IL-6, GM-CSF, or a mixture of PMA and the calcium ionophore A23187. Treatment of the transfected glial cells with these cytokines or PMA and calcium ionophore did not increase the expression of CAT from the JCV promoter (Figure 1). In replicate experiments, TNF-α inhibited JCV promoter activity by 52.9%, IL-1β inhibited by 72.2%, IL-6 inhibited by 53.0%, IL-2 inhibited by 48.1%, GM-CSF inhibited by 51.7%, and the combination of PMA and calcium ionophore (A23187) inhibited by 90.0%. The effect observed is specific to the JCV construct as under identical experimental conditions several of these reagents routinely increase the expression of CAT under the control of an HIV-1 promoter (Atwood et al., 1994).

JC virus reporter gene expression in response to cocultivation with B and T lymphocyte cell lines

It is possible that cell to cell signaling or soluble factors produced by cells other than the ones tested here may lead to increases in JCV gene expression. Since JCV infection is associated with lymphocytes in peripheral blood, in bone marrow, and in brain parenchyma, we examined JCV promoter activity in glial cells in the presence and absence of cocultivation with B and T lymphocyte cell lines (Atwood et al., 1992; Houff et al., 1988). Neither the Namalwa B cell line nor the A3.01 T cell line when cocultured in direct contact with transfected glial cells were capable of providing a signal that would activate expression of the JCV promoter (Figure 2). Cocultivation with either Namalwa cells or A3.01
cells resulted in a decrease in activity of the JCV promoter. This effect is specific to the JCV construct as identical experiments using a construct containing the HIV-1 promoter result in an increase in HIV-1 gene expression and multiplication (Tornatore et al., 1991).

**Effect of TNF-α on the binding of transcription factors to the overlapping NF-1/AP1 site in the JCV regulatory region**

Since the overlapping NF-1/AP-1 binding site in the JCV enhancer is critical to both transcription and replication of the JCV genome we examined nuclear protein binding to this region in the context of TNF-α stimulation. Several specific gel shifted complexes (Figure 3, A–D) were formed when nuclear proteins from untreated (Figure 3, lanes 2–4) and TNF-α treated (Figure 3, lanes 5–7) human fetal glial cells were interacted with the probe. These complexes are specific as they were completely competed for by an excess of unlabeled homologous probe (Figure 3, lanes 3 and 6) but not by an excess of unlabeled mutant probe (Figure 3, lanes 4 and 7). When the probe was reacted with nuclear extracts from glial cells that had been treated with TNF-α no discernible differences in the specific binding of any of the complexes were observed (Figure 3, compare lanes 2–4 with lanes 5–7). Since JCV is capable of infecting several B cell lines we also examined nuclear protein binding to our probe from untreated and TNF-α treated B cells (Namawla). Only the B complex was formed when nuclear extracts from these cells were interacted with our probe (Figure 3, lanes 8–10). Again, no differences were seen when the nuclear extracts were from Namawla cells treated with TNF-α (Figure 3, lanes 11–13).

**Effect of TNF-α stimulation on the binding of nuclear proteins to the JCV NFkB site**

To begin to assess the role of the putative NFκB site in the multiplication of JCV we tested whether or not nuclear proteins isolated from primary human fetal glial cells would bind to an oligonucleotide containing this site. Nuclear proteins isolated from human fetal glial cells formed two detectable gel shifted complexes when reacted with an oligonucleotide containing the JCVκB site (Figure 4, lanes 2–5, bold and light arrows). The complexes are specific as they were completely competed for an excess of unlabeled homologous probe (Figure 4, lane 3) but not by an excess of unlabeled mutant probe (Figure 4, lane 4). Three gel shifted complexes were evident when the probe was interacted with extracts from glial cells that had been treated with TNF-α (Figure 4, lanes 6–9, bold, light, and dashed arrows). The complexes are specific as they were completely competed for an excess of unlabeled mutant probe (Figure 4, lane 7) but not by an excess...
of unlabeled mutant probe (Figure 4, Lane 8). Binding to the JC NFkB probe was induced several fold by TNF-α treatment (Figure 4, compare lanes 2–5 with 6–8). An excess of unlabeled HIV-1 κB competitor competed for all the specific complexes from either uninduced or induced glial cells (Figure 4, lanes 5 and 9).

Identification of NFκB subunits present in each of the specific gel shifted complexes with subunit specific antisera

Nuclear proteins from untreated and TNF-α treated glial cells were reacted with the JC NFκB probe in the presence and absence of antisera to the p50 and p65 subunits of NFκB. Antiseras to an unrelated antigen, galactocerebroside, was used as a negative control in this experiment. Anti-p50 antiserum supershifted two of the specific bands (Figure 5, compare lanes 2 and 6 with lanes 3 and 7). The dashed-dotted arrow indicates the new position of the anti-p50 supershifted band. Anti-p65 antiserum abolished two of the specific bands (Figure 5, compare lanes 2 and 6 with lanes 4 and 8). The control anti-sera had no effect on the migration or the formation of any of the bands (Figure 5, lanes 5 and 9). The bold arrow indicates the position of a band that is affected by both the p50 and p65 specific antisera which indicates that both p50 and p65 subunits are present in the complex. The dashed arrow indicates the position of a band that is only affected by p50 antisera which indicates that p50 but not p65 is present in that complex. The solid-light arrow indicates the position of a band that is only affected by the p65 antisera which indicates that p65 but not p50 is present in that complex.

Effect of TNF-α stimulation on the multiplication of JC virus in infected human fetal glial cells

Since our molecular construct did not contain the putative JC NFκB binding site we assessed the effects of TNF-α stimulation on JC virus infected glial cells. The MAD-4 strain of virus used in this experiment has been sequenced and found to contain the putative κB site (our unpublished results). Following infection of cell monolayers with virus the cells were maintained in media alone or in media containing TNF-α (1000 U ml⁻¹). At 5 days post infection the percentages of T and V antigen positive cells as well as the percentage of in situ hybridization positive cells were determined. In cultures maintained without TNF-α, 46.9% of the
Table 1  Effect of TNF-α on JCV gene expression and multiplication

<table>
<thead>
<tr>
<th>Treatment</th>
<th>T antigen positive cells (%)</th>
<th>V antigen positive cells (%)</th>
<th>Cells replicating viral DNA (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>- TNF-α</td>
<td>46.9 (18.5)</td>
<td>26.1 (5.8)</td>
<td>15.9 (3.5)</td>
</tr>
<tr>
<td>+ TNF-α</td>
<td>47.1 (15.1)</td>
<td>21.7 (4.2)</td>
<td>13.4 (5.4)</td>
</tr>
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cells were T antigen positive, and 26.1% of the cells were V antigen positive (Table 1). In cultures maintained in the presence of TNF-α, 47.1% of the cells were T antigen positive, and 21.7% of the cells were V antigen positive (Table 1). In situ hybridization with a biotinylated JCV DNA probe detected 15.9% positive cells in the untreated cultures and 13.4% positive cells in the TNF-α treated cultures (Table 1). Note that we have previously determined that the sensitivity of in situ hybridization with biotinylated JCV DNA probes is such that several hundred copies of the JCV genome need to be present in order to generate a positive signal. Representative T antigen, V antigen, and in situ hybridization positive cells are shown in Figure 6, panels A, B, and C respectively. Standard error of the mean is indicated by the numbers in parentheses.

Discussion

JC virus is an extremely successful human pathogen, infecting greater than 70% of the population worldwide. Part of this success no doubt comes from the ability of JCV to establish life long persistent or latent infections in its host. Periodic reactivation of the virus and its excretion in urine probably contribute to the high carrier rate of virus in the population. Intensive research on JCV began nearly two decades ago when the virus was associated with the rare but fatal demyelinating disease, progressive multifocal leukoencephalopathy (PML). Despite this, little is known about the mechanisms of virus transmission, virus latency, or the signals that lead to its reactivation.

Since a number of inflammatory cytokines are increased in acquired immune deficiency syndrome (AIDS) patients, in particular in CNS tissue, we sought to determine what role, if any, cytokines play in the life of JCV in vivo (Wesselingh et al., 1993). Using a panel of cellular activating agents that included TNF-α, IL-1β, IL-2, IL-6, GM-CSF, PMA in combination with the calcium ionophore A23187, and cocultivation with B cells and T cells, we were unable to increase expression of a reporter gene under the control of the JCV regulatory region. In fact, these treatments all led to lower expression of the reporter gene in these cells. Consistent with this lack of induction is the fact that we could not detect any modulation of binding of nuclear proteins to the critical NF1/AP1 site in the JCV regulatory region. The overlapping NF1/AP1 binding site is highly conserved in all strains of JCV and functions in both transcription and replication of the virus (Tamura et al., 1988, 1990; Sock et al., 1991). This region has also been shown to bind purified nuclear factor-1 and c-jun (Amemiya et al., 1989).

We next investigated binding of nuclear proteins to an oligonucleotide containing the putative JC NFkB site both in the presence and absence of TNF-α, a known inducer of NFkB.

NFkB is constitutively expressed in B lymphocytes where it functions to increase transcription of the gene that codes for the κ light chain of immunoglobulins (Lenardo et al., 1987). In most other cells NFkB is retained in the cytoplasm as an inhibitor protein referred to as inhibitor κB (IκB) (Bours et al., 1992b; Brown et al., 1993). Activation of cells by a variety of signals, including TNF-α and IL-1β, result in the dissociation of NFkB from IκB and translocation of NFkB to the nucleus. In the nucleus NFkB activates the expression of several viral and cellular genes (Kawakami et al., 1988; Lenardo and Baltimore, 1989; Lenardo et al., 1989). The prototypical and most abundant form of NFkB is a heterodimer composed of p50 and p65 subunits. Recently, however, several other homodimeric and heterodimeric forms of NFkB have been described (Molitor et al., 1990; Bours et al., 1992b; Baueerle, 1991; Mercurio et al., 1993; Bours et al., 1992a). These include p50/p50 homodimers and p65/p65 homodimers (Ganchi et al., 1993).

In contrast to our results using the NF1/AP1 probe, TNF-α treatment of glial cells led to a several fold induction of binding of nuclear proteins to an oligonucleotide probe containing the JC NFkB site. Supershift analysis with NFkB specific antisera was used to confirm that the gel shifted bands were indeed due to the binding of NFkB p50 and p65 subunits. The predominant band is due to the binding of p50/p65 heterodimers, and the other minor bands are most likely due to the binding of p50/p50 and p65/p65 homodimers. The HIV-1 NFkB site effectively competed for all three bands in both induced and uninduced cells. These results are consistent with our previous report of TNF-α induction of NFkB binding to an oligonucleotide containing an HIV-1 κB binding site (Atwood et al., 1994).

Since our molecular construct did not contain a κB site we sought to test the contribution of this site by infecting glial cells with virus. We measured early and late gene expression and virus multiplication at 5 days post infection. This time was chosen as it would allow us simultaneously to measure the effects of TNF-α at all stages of the JC virus life cycle. Treatment of the cultures with TNF-α did not increase either the percentage of cells expressing the early viral protein, T antigen, or the percentage of cells expressing the late viral virion protein, VP1. Similarly, the percentage of in situ hybridization
positive cells was not increased by TNF-α treatment of the infected cells. The sensitivity of our DNA:DNA in situ hybridization with biotinylated probes is such that a minimum of 200 copies of viral DNA must be present in a cell in order to be detected (Aksamit et al., 1985). This method therefore only detects a high copy number of viral DNA which can only occur if the DNA is being replicated in the cells.

These data are consistent with our transient transfection assays in that TNF-α actually reduced virus gene expression. It is possible that the negative effects of these reagents is due to toxicity of the reagents for the cells. We feel, however, that this is unlikely as these same reagents increase the expression and multiplication of HIV-1 in these same cells (Atwood et al., 1994; Tornatore et al., 1991). Also, several of these cytokines, including TNF-α and IL-6, are actually produced by our glial cell cultures (Vitkovic et al., 1991).

There are several possibilities that could account for the lack of increased JCV gene expression by TNF-α despite the induction of binding of NFκB to the JC NFκB oligonucleotide. One possibility is that binding of a transcription factor to an isolated binding site on an oligonucleotide may not reflect what actually occurs in vivo. Gel shift assays with oligonucleotides that include surrounding JCV DNA sequences as well as in vivo footprinting experiments will need to be done to determine whether NFκB binds to this region in vivo. Another possibility is that the binding site for NFκB is located in the early coding region for the large T protein and binding here could be inhibitory to T protein synthesis. We are currently examining the effects of TNF-α on both early and late mRNA synthesis in JCV infected cells to assess this possibility. Also, we are investigating other types of signalling pathways that may lead to increased JCV expression in cells derived from both brain and lymphoid tissue.

Materials and methods

Cells and plasmids
Preparation of glial cell cultures from human fetal brain has been described in detail (Major and Vacante, 1989). Briefly, primary (8–16 weeks gestation) human fetal glial cells (HFG) were grown in Eagle’s minimum essential medium with 10% fetal bovine serum, L-glutamine (3.0 mg/ml), and antibiotics. Cells were grown at 37°C with 5% CO₂ in poly-D-lysine coated plasticware. Cultures in which greater than 98% of the cells stained with the astrocyte specific marker GFAP were used in these experiments. The Namalwa EBV positive human B cell line was obtained from the American Type Culture Collection (ATCC, Rockville, MD). A3.01 human T cells were kindly provided by Malcolm Martin (NIH, Bethesda, MD). Both the Namalwa and

Figure 6  Immunofluorescence and in situ hybridization analyses of T, V, and DNA positive cells. (a) Representative field of T antigen positive cells. (b) Representative field of V antigen positive cells. (c) Representative field of in situ hybridization positive cells.
A3.01 cell lines were maintained in RPMI 1640 supplemented with 10% fetal bovine serum, L-glutamine (3.0 mg ml\(^{-1}\)), and antibiotics. The plasmid pM1CAT contains the regulatory region of the Mad-1 strain of JCV (Hind III to Pvu II) cloned into pA10CAT upstream of the reporter gene chloramphenicol acetyl transferase. Transcription from the JCV promoter is in the early orientation. pA10CAT has been described elsewhere (Gendelman et al., 1986).

Transfection and CAT assays
Cells were transfected in triplicate by calcium phosphate precipitation with 10 μg of pM1CAT DNA, 10 μg of a negative control construct (pA10CAT), or with 10 μg of calf thymus DNA. Following transfection, the cells were grown for 48 h in media alone or in media containing TNF-α (1000 U ml\(^{-1}\)), IL-β (10 U/ml), IL-2 (50 u ml\(^{-1}\)), GM-CSF (400 U ml\(^{-1}\)), IL-6 (500 u ml\(^{-1}\)), or PMA and the calcium ionophore A23187 at 10^4 M each. In the cocultivation experiments Namalwa or A3.01 cells were added in direct contact with the transfected glial cells at a ratio of 1:1 (lymphocytes:glial cells). The transfected cells were then washed 2x in phosphate buffered saline, pH 7.2 (PBS), 1x in mM Tris-HCL (pH 7.1), and lysed by successive freeze thaw cycles in 100 μl of 250 mM Tris-HCL (pH 7.1). After removal of cell debris by centrifugation, the protein concentration in the cell extract was determined by the method of Bradford (Bradford, 1976). Equivalent amounts of protein were assayed for CAT enzyme activity using the fluor diffusion method of Neuman (Neuman et al., 1987). Briefly, 80 μg of protein, diluted in 250 mM Tris-HCL (pH 7.1), was added to a reaction mixture containing 0.25 mM chloramphenicol, and 0.5 μCi (2.5 nM) of 3H-acetyl coenzyme A. The mix was then overlaid with 5.0 ml liquid scintillation cocktail (Econofluor-2 Dupont) and counted in a β counter (Beckman) for 3 h. Efficiency of transfection within each experiment was controlled for by slot blot hybridization of DNA isolated from the transfected cells.

Infection
1 x 10^4 cells growing on poly-D lysine coated coverslips were washed 2x in media containing 2% FCS and then incubated with 400 HAU of JCV (MAD-4 strain) in 1.0 ml media containing 2% FCS for 1.5 h at 37°C. The inoculum was removed and the cells were maintained in media with and without TNF-α at 1000 U ml\(^{-1}\).

Immunofluorescent analysis of T and V antigens
At 5 days post infection cells were washed 3x with PBS and fixed for 10 min in ice cold acetone. Duplicate coverslips were then incubated with anti-SV40 T antigen antibody (PAB 416, Oncogene Sciences, 1:10), anti-SV antigen antibody (PAB 597, a generous gift of L Norkin, 1:100), or with negative control antibodies for 45 min at 37°C. The cross reactivity of these anti-SV40 antibodies with JCV proteins has been previously published (Major et al., 1987). The coverslips were then washed 3x in PBS and incubated with secondary goat anti-mouse antibody conjugated to FITC (Jackson Immunoresearch, West Grove, Pennsylvania, 1:15) for an additional 0.5 h. The coverslips were washed 3x in PBS and mounted on glass slides with 90% glycerol. T and V antigen positive cells were visualized on a Zeiss epifluorescent microscope.

In situ hybridization
In situ hybridization was performed as previously described (Aksamit et al., 1985). Briefly, cells grown on coverslips were washed in PBS and fixed for 0.5 h in 4% paraformaldehyde at room temperature. The cells were then dehydrated in serial ethanol washes. Cells were acid hydrolyzed in HCL, washed in Triton-X, subjected to limited protein digestion with pronase, and washed in glycine buffer. The cells were hybridized with 25-40 μl of a probe mixture that contained 50% formamide, 10% dextran sulfate, 0.4 mg ml\(^{-1}\) calf thymus DNA, 2 μg ml\(^{-1}\) biotinylated JCV DNA probe (ENZO Biochem), and 2x SSC (300mM sodium chloride and 30 mM sodium citrate). Probe DNA and cellular DNA were denatured by incubation at 85°C for 10 min. Hybridization was performed at 37°C overnight. Cells were then washed with 2x SSC for 2 min, with 0.1% Triton-X in PBS for 2 min, and in PBS for 3 min. Detection of the biotinylated probe was carried out immediately by direct affinity cytochemistry using the streptavidin-biotin-horseradish peroxidase kit (Detek I-hrp, ENZO, Biochem.). A fresh solution of diaminobenzidine tetrahydrochloride (DAB) was used as the chromogen. Cells were then washed in PBS, counterstained with hematoxylin, dehydrated, and mounted.

Preparation of nuclear extracts
Cultures of human fetal glial cells (1-10 x 10^6 cells) were grown for 48 h in the presence or absence of TNF-α (1000 U ml\(^{-1}\)). Nuclear extracts were prepared by a modification of the procedure of Dignam and has been previously described (Amemiya et al., 1989; Atwood et al., 1992; Dignam et al., 1983). The protein concentration of the samples was determined by the method of Bradford (Bradford, 1976).

Oligonucleotide probes and gel shift assays
The following oligonucleotide probes were synthesized on an Applied Biosystems (Foster City, California) 380A DNA synthesizer:

JCV NFκB:
(5’-GATCTGGAGGCGCCGGAAAATTCCTTTGTTT-TA-3’)
mutant Jκ B:
(5’-GATCTGGAGGCGGACTAATTTAAAA-3’).
Double stranded oligonucleotides were labeled with $\gamma^{32}P$-ATP and gel purified. The labeled JC NFkB probe (40,000 CPM) was incubated with 20 $\mu$g of nuclear extract from untreated or TNF-α treated cells in the presence or absence of a 10-fold molar excess of unlabeled homologous probe, unlabeled mutant probe, or unlabeled HIV-1 NFkB probe. The DNA binding reactions also contained 10 mM Tris-HCL (pH 7.9), 50 mM NaCl, 5 mM MgCl₂, 0.5 mM EDTA, 1.0 mM DTT, 10% glycerol (v/v), and 4 µg of the non-specific competitor, poly (dl-dC) (Pharmacia). The reactions were carried out at room temperature for 15 min and electrophoresed on a 6% polyacrylamide Tris-glycine gel. The gel was dried and samples visualized by autoradiography with Kodak xAR-5 film with an intensifying screen.

The NF1/AP1 probe was used in a similar manner except that only 10 µg nuclear extracts were used in the reactions.

**Supershift assays**

The radiolabeled JC NFkB probe was incubated for 15 min at room temperature with 20 µg of nuclear extract from untreated or TNF-α treated HFG cells under the conditions described above. After the complexes were formed, 1.0 µl of either rabbit anti-p50 serum (a generous gift from Ulrich Siebenlist), rabbit anti-p65 serum (Santa Cruz Biochem Inc, Santa Cruz, California), or rabbit hyperimmune anti-galactocerebrosides serum, as a negative control, were added to the reactions and incubated on ice for an additional 30 min. The complexes were resolved on 6% Tris-glycine gels as described above.

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