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# Tumor necrosis factor α activates human immunodeficiency virus type 1 through induction of nuclear factor binding to the NF-κB sites in the long terminal repeat

**(ACH2 T-cell line/provirus/retroviral transcription)**

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**ABSTRACT** **Expression of human immunodeficiency virus type 1 (HIV-1) can be activated in a chronically infected T-cell line (ACH2 cells) by a cytokine, human tumor necrosis factor α (TNF-α). TNF-α treatment of ACH2 cells resulted in an increase in steady-state levels of HIV RNA and HIV transcription. Gel mobility shift assays demonstrated that the transcriptional activation of the HIV long terminal repeat (LTR) by TNF-α was associated with the induction of a nuclear factor(s) binding to the NF-κB sites in the LTR. Deletion of the NF-κB sites from the LTR eliminated activation by TNF-α in T cells transfected with plasmids in which the HIV LTR directed the expression of the bacterial chloramphenicol acetyltransferase gene. Thus, TNF-α appears to activate HIV RNA and virus production by ACH2 cells through the induction of transcription-activating factors that bind to the NF-κB sequences in the HIV LTR.**

Infection by human immunodeficiency virus type 1 (HIV-1) is characterized clinically by a long period of latency preceding development of the acquired immunodeficiency syndrome (AIDS) (1, 2). Thus, elucidation of mechanisms that control the degree of viral replication during this period may have important clinical implications in understanding disease progression. Of particular interest are stimuli that might play a role in the activation of HIV expression in cells containing integrated proviruses.

The activation of the HIV promoter in the viral long terminal repeat (LTR) by mitogens (3–5), phorbol esters (3–8), retinoic acid (9), and antibodies to T-cell surface proteins (10) has been demonstrated by transfection experiments in which the LTR directed the expression of the bacterial enzyme chloramphenicol acetyltransferase (CAT) (11). Heterologous viruses including herpesviruses and papovaviruses have also been shown to activate CAT expression driven by the HIV LTR (12–17). These activating stimuli have been postulated to induce and/or augment expression of HIV from the latent provirus (2, 18, 19). Of particular importance is the possible role that physiological stimuli such as cytokines may play in activating HIV expression in infected cells of the immune system (2). In this regard, it has been demonstrated that granulocyte/macrophage colony-stimulating factor (GM-CSF) has an inductive effect on HIV expression in cells of promonocytic origin that contain integrated HIV proviruses (20).

To study the effects of various physiological stimuli on HIV expression in infected T cells, we recently developed a chronically infected T-cell line, ACH2, that contains a single integrated HIV provirus. ACH2 cells were derived following HIV-1 infection of A3.01 cells, a human $CD4^+$ T-cell line (21). ACH2 cells constitutively produce low levels of HIV particles (21); treatment with tumor necrosis factor α (TNF-α) markedly increased virus production (21, 22). In this study, we characterize the molecular mechanisms by which TNF-α activates HIV expression.

## MATERIALS AND METHODS

**Cell Culture.** ACH2 cells were cultured in RPMI-1640 containing penicillin/streptomycin, glutamine, 10 mM Hepes, and 10% fetal bovine serum at 37°C and 5% $CO_2$ in the presence or absence of TNF-α (0.5 mg/$10^7$ units; Genentech) at 100 units/ml.

**RNA Slot Blots.** Total RNA was extracted (23) from $5 \times 10^7$ cells 48 hr after addition of TNF-α. The RNA was denatured and transferred to nitrocellulose filters in an RNA slot blot apparatus (Schleicher & Schuell). Filters were hybridized overnight with a $^{32}$P-labeled 6.4-kilobase (kb) segment of HIV DNA (pBenn5; map position 1.7 kb to 8.1 kb; ref. 24) or with a molecular clone, X12, containing the fourth exon of the human class I HLA gene *A3* (a gift of Thomas Kindt, National Institutes of Health) and were washed as described (25). Filters were subjected to autoradiography for 6–24 hr at −70°C with an intensifying screen. Autoradiograms were analyzed with an LKB 2222-010 scanning densitometer.

**Nuclear Run-On Transcription.** ACH2 cells were cultured in the presence or absence of TNF-α (100 units/ml). Nuclei were isolated (26) from $5 \times 10^7$ cells 12 hr after addition of TNF-α. The nuclei were incubated with [α-$^{32}$P]GTP and unlabeled nucleoside triphosphates for 30 min at 26°C as described by Pasco *et al.* (27). Total nuclear RNAs were purified (23) and hybridized (28) to nitrocellulose filters on which 5 μg of linearized and denatured plasmid DNA had been immobilized. The plasmid DNAs were pBenn5, containing HIV sequence, and HLA class I clone X12. The relative increase in nuclear transcription was determined by densitometric analysis of the autoradiogram obtained following a 3-week exposure.

**Preparation of Nuclear Extracts.** ACH2 cells were grown as suspension cultures in the presence or absence of TNF-α (100 units/ml). Twelve hours after treatment with TNF-α, the cells were harvested by centrifugation at 1000 × *g* and washed in phosphate-buffered saline. Nuclear extracts were prepared by the procedure of Dignam *et al.* (29) modified by Quinn *et al.* (30) such that buffer C contained 20% glycerol and 0.45 M NaCl and buffer D contained 20% glycerol and 80 mM KCl. Protein concentrations were determined by the method of Bradford (31).

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Abbreviations: TNF, tumor necrosis factor; HIV, human immunodeficiency virus; LTR, long terminal repeat; CAT, chloramphenicol acetyltransferase.

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**Gel Mobility Shift Assays.** Gel shift assays were performed as described (30, 32). Double-stranded oligonucleotides were made by annealing complementary oligonucleotides synthesized on a 380A DNA synthesizer (Applied Biosystems). They were then labeled with $^{32}P$ by incubation with the Klenow fragment of DNA polymerase in the presence of [$\alpha$-$^{32}P$]dGTP, [$\alpha$-$^{32}P$]dCTP, dATP, and dTTP. Radiolabeled oligonucleotides (5000–10,000 cpm) were incubated with nuclear protein (30 $\mu$g for the NF-$\kappa$B probe, 8 $\mu$g for the Sp1 probe) and 4.0 $\mu$g of poly(dI-dC) in a total volume of 6 $\mu$l. Nonradioactive DNAs (50 ng) were added for competition experiments. DNA–protein complexes were analyzed by electrophoresis in 5% polyacrylamide gels run in 22.3 mM Tris/22.3 mM boric acid/0.5 mM EDTA.

**Transfections and CAT Assays.** Plasmid DNAs were introduced into A3.01 cells ($6 \times 10^6$ per transfection) by a modification of the DEAE-dextran transfection procedure (33). The wild-type HIV LTR–CAT plasmid was a derivative of pBennCAT (12) in which a 719-base-pair (bp) *Xho* I–*Hin*dIII fragment (map position 8.9–9.6 kb on the full-length HIV provirus) was ligated to the CAT gene in the plasmid vector pIBI20 (IBI). In the mutant NF-$\kappa$B LTR construction, a 26-bp segment spanning the two NF-$\kappa$B sites was deleted and replaced with a 6-bp *Bcl* I oligonucleotide linker by oligonucleotide-directed, site-specific mutagenesis (J. Leonard, C. Parrott, A. Buckler-White, M. Martin, and A.B.R., unpublished work). The LTR–CAT plasmids (15 $\mu$g per transfection) were cotransfected with the HIV LTR–*tat* plasmid pAR (12) (30 $\mu$g per transfection) to amplify the amount of CAT protein produced. TNF-$\alpha$ (100 or 1000 units/ml) was added to the cells 4 hr after transfection. Cells were harvested and lysed 44 hr after addition of TNF-$\alpha$, and assays for CAT activity were performed as described (11). The relative increases in CAT activity were determined by liquid scintillation quantification of the acetylated and unacetylated [$^{14}C$]chloramphenicol from the chromatogram.

## RESULTS

**TNF-$\alpha$ Activation of HIV Transcription.** We previously demonstrated (22) that activation of HIV-1 expression by TNF-$\alpha$ occurs at the level of the HIV LTR, as studied by transfection of LTR–CAT constructions. To determine whether the induction of HIV expression from the integrated provirus in ACH2 cells is due to an increase in HIV RNA levels, slot blot analysis of serially diluted RNA was performed (Fig. 1). Treatment of ACH2 cells with TNF-$\alpha$ resulted in a 4- to 5-fold increase in HIV RNA levels over untreated ACH2 cells. Class I HLA RNA levels were not increased by TNF-$\alpha$ treatment and, in fact, may have decreased slightly. Thus, the increased HIV RNA observed in TNF-$\alpha$-treated cells was not due to an artifact of RNA loading on the filter or to a generalized increase of cellular transcription in TNF-$\alpha$-treated cells.

Nuclear run-on assays were performed on ACH2 cells to determine whether the increase in steady-state levels of HIV RNA was a result of augmented transcription of the integrated provirus (Fig. 1*b*). Treatment of ACH2 cells with TNF-$\alpha$ resulted in a 3-fold increase in HIV transcriptional activity. Transcription of class I HLA RNA did not increase after TNF-$\alpha$ treatment. When nuclear run-on assays were conducted in the presence of $\alpha$-amanitin, an inhibitor of RNA polymerase II transcription, there was no difference in the levels of $^{32}P$-labeled transcripts synthesized by nuclei from treated and untreated cells (data not shown). Thus, TNF-$\alpha$ specifically activates HIV transcription by RNA polymerase II.

**TNF-$\alpha$ Induces Nuclear Factor Binding to the HIV LTR.** In an attempt to localize possible DNA sequences in the HIV LTR responsible for the TNF-$\alpha$ activation of HIV transcription, gel mobility shift assays were performed. Binding sites for two known human transcriptional factors, NF-$\kappa$B and Sp1, have been localized in the LTR (3, 34). Oligonucleotide probes spanning either the two tandem NF-$\kappa$B binding sites or the three Sp1 binding sites shown in Fig. 2 were used in these assays. When the NF-$\kappa$B probe was incubated with nuclear extracts from untreated ACH2 cells, no shifted bands were observed (Fig. 3*a*). Incubation with nuclear extracts from ACH2 cells treated with TNF-$\alpha$ resulted in the appearance of two shifted bands. These bands were eliminated by competition experiments in which a 50-fold molar excess of unlabeled NF-$\kappa$B oligonucleotide was included in the binding reaction mixture. Competition with nonspecific oligonucleotide ST (Figs. 2 and 3*a*) or with the Sp1 oligonucleotide (data not shown) did not reduce binding to the NF-$\kappa$B probe in the TNF-$\alpha$-treated extracts. Similar binding studies were performed using a mutated NF-$\kappa$B sequence (NF-$\kappa$B*) previ-

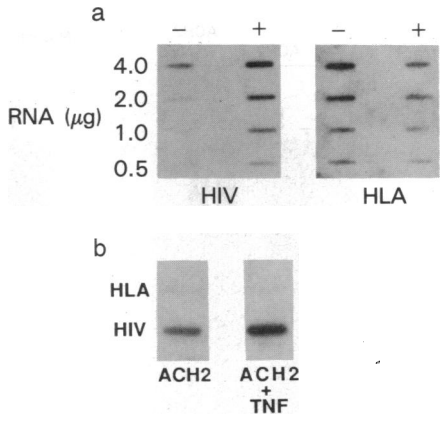


FIG. 1. (*a*) Slot blot analysis of HIV and class I HLA RNA from total RNA extracted from unstimulated ACH2 cells (−) or TNF-$\alpha$ (100 units/ml)-stimulated ACH2 cells (+). Serial dilutions of RNA (4.0, 2.0, 1.0, and 0.5 $\mu$g) were dotted on nitrocellulose, hybridized with $^{32}P$-labeled DNA probes, and subjected to autoradiography. (*b*) Nuclear run-on transcription of HIV RNAs in the presence or absence of TNF-$\alpha$. Nuclei were isolated from TNF-$\alpha$-treated and untreated ACH2 cells. Nuclear RNA was synthesized by run-on transcription in the presence of [$\alpha$-$^{32}P$]GTP and the labeled transcripts were hybridized to slot blots containing class I HLA or HIV DNA. Densitometry was performed to estimate the fold increase in transcriptional activity.

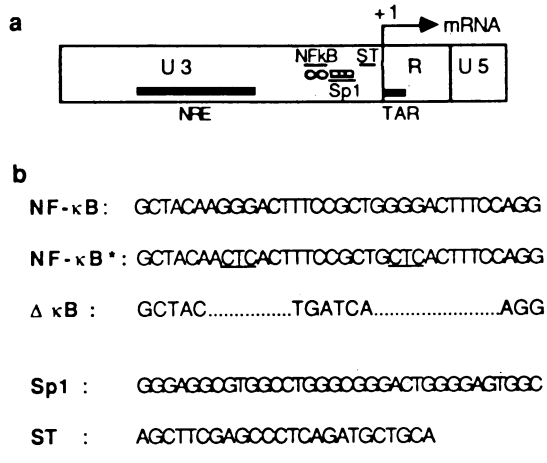


FIG. 2. (*a*) Structure of HIV LTR showing location of regulatory elements. The locations of oligonucleotide sequences (NF-$\kappa$B, Sp1, ST) used for gel mobility shift assays are indicated. U3, R, and U5 regions are indicated. NRE, negative regulatory element; TAR, transactivation response region. (*b*) DNA sequences of synthetic oligonucleotides used in gel mobility shift assays and of the $\Delta\kappa$B mutation made in the LTR–CAT construction for CAT assays.

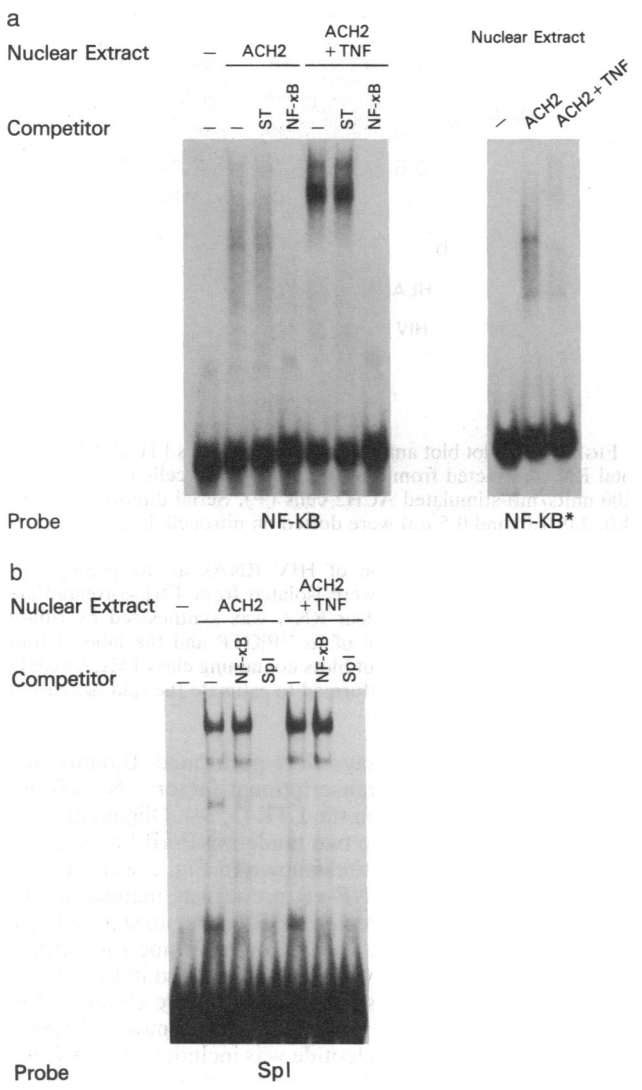


FIG. 3. Gel mobility shift analysis of the induction of protein binding to the HIV LTR by TNF-α. (*a*) Analysis of binding to the wild-type and mutant NF-κB binding sites. A $^{32}$P-labeled oligonucleotide spanning the wild-type or mutant NF-κB sites in the HIV LTR (Fig. 2) was incubated with nuclear extracts from untreated and TNF-α (100 units/ml)-treated ACH2 cells. For the NF-κB probe, competition experiments were performed by the addition of 50 ng (>50-fold molar excess) of unlabeled double-stranded oligonucleotides to the binding reaction mixture. Competitor oligonucleotides were the unlabeled NF-κB oligonucleotide and the ST oligonucleotide, containing sequences between the Sp1 binding sites and the TATA box in the LTR (Fig. 2). (*b*) Binding to the Sp1 sites. The $^{32}$P-labeled Sp1 oligonucleotide was used for gel mobility shift assays. Competitor DNAs were the unlabeled Sp1 or NF-κB oligonucleotides.

ously shown to be incapable of binding to factors present in activated Jurkat T cells (3). The NF-κB binding activity in TNF-α-treated ACH2 cells failed to bind to the mutant NF-κB sequence (Fig. 3*a*). Thus, treatment of ACH2 cells with TNF-α resulted in the induction of nuclear factor(s) that bind specifically to the NF-κB sites in the HIV LTR.

When the Sp1 oligonucleotide probe was used, the same pattern of shifting was observed upon incubation with nuclear extracts from both treated and untreated cells (Fig. 3*b*). Competition with nonspecific and specific unlabeled oligonucleotides revealed that the shifted bands were specific for the Sp1 oligonucleotide. Nuclear factor(s) binding to the Sp1 sites was therefore present at comparable levels in both treated and untreated cells.

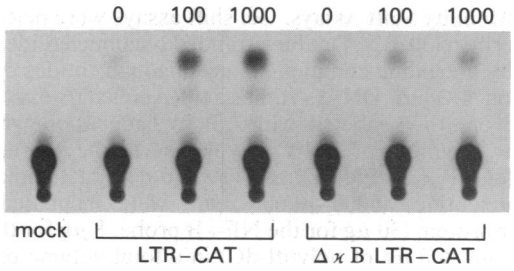


FIG. 4. Activation of the HIV LTR in A3.01 cells by TNF-α. A3.01 cells were transfected with either the wild-type or mutant (ΔκB) HIV LTR-CAT. The mutant lacked both NF-κB sites. HIV LTR–*tat* was cotransfected in all experiments to amplify the amount of CAT protein produced. The cells were treated with or without TNF-α (100 or 1000 units/ml) 4 hr after transfection. Cells were harvested for CAT protein 44 hr after treatment, and CAT activity was quantitated by liquid scintillation of the acetylated and unacetylated [$^{14}$C]chloramphenicol from the chromatogram.

**NF-κB Sequences Are Required for TNF-α Activation of the HIV LTR.** The transcriptional activation of the HIV LTR by TNF-α is, therefore, associated with the induction of a nuclear factor(s) binding to the NF-κB sites in the LTR. The possible role of these factors in mediating activation by TNF-α was tested with LTR–CAT constructions. The parent cell line A3.01 (24) was transfected with wild-type or mutant LTR–CAT constructions and maintained in the presence or absence of TNF-α. The *tat* expression plasmid pAR (12) was cotransfected in all cases; transfection of the pIBI-based LTR–CAT plasmids in A3.01 cells without *tat* resulted in undetectable levels of CAT activity (E.J.D., unpublished observations). As shown in Fig. 4, TNF-α activated the wild-type LTR–CAT construct in a dose-dependent manner, resulting in a 10-fold increase in CAT activity in the presence of TNF-α at 100 units/ml. Activation of the LTR, however, was virtually abolished (1.3-fold) after deletion of the NF-κB sites in the LTR. Similar results were observed when the Tat protein was supplied by cotransfection of the simian virus 40-based expression plasmid pSVtat12 (data not shown; ref. 35). These experiments therefore suggest a functional role for the factors binding to the NF-κB sites in the transactivation of the HIV LTR by TNF-α.

## DISCUSSION

An important unresolved question in the study of the pathogenesis of HIV infection is the mechanisms that contribute to disease progression and the development of AIDS. Chronically or latently infected cells expressing little or no HIV that can be induced to produce high levels of virus have been hypothesized to contribute to the relentlessly progressive nature of HIV infection (2, 36). Recently, clonal cell lines have been developed that provide models of such chronically or latently infected cells (20, 21). ACH2, a T-cell clone containing a single HIV provirus, has been particularly useful in identifying physiological stimuli that can activate the expression of integrated proviral DNA (21, 22). The supernatant of lipopolysaccharide-stimulated human monocytes (21) and the recombinant human cytokines TNF-α and TNF-β (22) are particularly potent activators of HIV production in this line. TNF-β also activated HIV expression in chronically infected MOLT-4 cells (37). These studies suggest that TNFs may stimulate expression of HIV proviruses *in vivo* and thus play a role in disease progression in AIDS patients. Stimuli that lead to secretion of TNF-α from monocytes, such as the products of bacterial and mycobacterial infection, may result in elevated plasma TNF levels (38, 39). In fact, elevated TNF levels have been detected in AIDS patients as compared to asymptomatic HIV-seropositive individuals (40). In such

patients, TNF may result in continued stimulation of viral replication that could potentiate destruction of $CD4^+$ lymphocytes and subsequent deterioration of immune function.

In the present study, we have examined the molecular mechanisms by which TNF-$\alpha$ activates HIV expression in ACH2 cells. TNF-$\alpha$ results in transcriptional activation of the integrated HIV provirus. A 4- to 5-fold increase in steady-state HIV RNA and a 3-fold increase in HIV transcription were observed. These increases were comparable to the 4- to 5-fold TNF-induced augmentation in virion production from ACH2 cells previously reported (22). This activation appears to be mediated by the induction of cellular transcriptional factors that bind to the NF-$\kappa$B sequences in the LTR. Even small increases in HIV transcription could then result in the production of sufficient amounts of the viral regulatory proteins Tat and Rev to allow production of high levels of HIV. As TNF-$\beta$ appears to bind to the same receptor as TNF-$\alpha$ (41), it is likely that TNF-$\beta$ activation of HIV expression follows the same pathway.

The induction of nuclear factors that bind to the NF-$\kappa$B sites of the HIV LTR has been observed to be responsible for activation of the LTR by a number of stimuli in transient-transfection assays. Treatment of Jurkat T cells by phorbol esters and phytohemagglutinin resulted in increased activity of LTR–CAT plasmids mediated through the NF-$\kappa$B sites (3, 5, 8). Herpes simplex virus infection of cells also activates HIV LTR expression, at least in part through induction of NF-$\kappa$B binding activity (32). A similar mechanism may explain the reported activation of the LTR by the Tax protein of human T-cell leukemia virus type I (42–44). Thus, induction of nuclear factor binding to the NF-$\kappa$B sequences appears to be a common pathway for activation of the HIV LTR and may be important *in vivo* for physiologically relevant stimuli such as TNF-$\alpha$ that interact with infected T cells. Proteins of different molecular masses have been identified that bind to the HIV NF-$\kappa$B/core enhancer sequences. NF-$\kappa$B proteins of 42–50 kDa have been purified by oligonucleotide affinity chromatography from B cells (45) and bovine spleen (46). Proteins of 55–60 kDa, called EBP-1, have been purified from HeLa cells (47). In addition, a microscale affinity assay has been used to demonstrate the induction of an 86-kDa protein in stimulated T cells that has been called HIVEN86A (48). A mechanism of phorbol ester-induced activation of the NF-$\kappa$B protein involving the dissociation of a cytoplasmic inhibitor molecule, I$\kappa$B, has been demonstrated recently (49).

Although induction of binding to the NF-$\kappa$B sites represents a common feature of stimuli that activate the HIV LTR, a second sequence referred to as NFAT-1 has been suggested to play a role in the activation of the LTR by antibodies directed against T-cell surface proteins (10). Activation of Jurkat cells by treatment with antibodies directed against the T-cell receptor complex induces NFAT-1 binding activity (50). We have observed no evidence of induction of nuclear factor binding to the NFAT-1 site by TNF-$\alpha$ treatment of ACH2 cells as monitored by gel shift assays (data not shown). Furthermore, TNF-$\alpha$ treatment of HIV LTR–CAT plasmids lacking the NF-$\kappa$B sites resulted in only a 1.3- to 1.4-fold increase in CAT activity, as compared to the 10- to 13-fold increase observed with wild type. Thus, the potent activation of the LTR by TNF-$\alpha$ appears to proceed primarily through NF-$\kappa$B activation, although contributions of other LTR sequences cannot be excluded.

TNF-$\alpha$ exerts pleiotropic effects *in vivo* (38, 39) including effects on adipocyte metabolism (51) and fibroblast growth (52), mediation of the symptoms of endotoxic shock (38, 39), and immunomodulatory effects (52–55). Some of these diverse activities may be mediated by TNF-$\alpha$-induced alterations in gene expression; TNF-$\alpha$ has been shown to directly and specifically affect the transcriptional activity of a number of genes. The transcription of some genes, such as those encoding lipoprotein lipase (56, 57) and $\alpha$1 collagen (58), may be decreased by TNF treatment. The transcription of a variety of other genes, including ferritin heavy chain (59), monocyte-derived neutrophil chemotactic factor (60), c-*fos* and c-*myc* (61), class I HLA (53, 62), and interferon $\beta_2$ (63) genes, may all be activated by TNF-$\alpha$. Interestingly, the promoters of the class I HLA and interferon $\beta_2$ genes both contain sequence elements related to the NF-$\kappa$B binding site, and activation of NF-$\kappa$B-like proteins has been hypothesized to explain lymphokine induction of HLA synthesis (64). In ACH2 cells, no induction of HLA RNA was seen following TNF treatment (Fig. 1). This is in contrast to the induction of HLA mRNA seen in endothelial cells (53) and may be due to cell-type-specific differences in transcriptional regulatory proteins. The role of the NF-$\kappa$B-like sequence in the interferon $\beta_2$ promoter has not been clearly established. A deletion analysis of the interferon $\beta_2$ promoter suggested that sequences 5′ to the NF-$\kappa$B-like element were required for TNF inducibility (63). Furthermore, many of the TNF-inducible genes do not contain recognized NF-$\kappa$B sites. Therefore, TNF-$\alpha$ induction of cellular gene transcription may require the activation of other DNA-binding proteins in addition to NF-$\kappa$B. Further analysis of the promoter elements required for TNF inducibility may provide a greater understanding of the mechanisms by which cytokines alter gene expression and induce their pleiotropic effects.

**Note Added in Proof.** After submission of the manuscript for review, two reports were published demonstrating the induction of NF-$\kappa$B binding activity in human T cells by TNF-$\alpha$ (65, 66).

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