Expression of TNF-α by Herpes Simplex Virus-Infected Macrophages Is Regulated by a Dual Mechanism: Transcriptional Regulation by NF-κB and Activating Transcription Factor 2/Jun and Translational Regulation Through the AU-Rich Region of the 3′ Untranslated Region[1]

Søren R. Paludan,[2]* Svend Ellermann-Eriksen,* Veronique Kruys,† and Søren C. Mogensen*

Here we have investigated the regulation of TNF-α expression in macrophages during HSV-2 infection. Despite a low basal level of TNF-α mRNA present in resting macrophages, no TNF-α protein is detectable. HSV-2 infection marginally increases the level of TNF-α mRNA and protein in resting macrophages, whereas a strong increase is observed in IFN-γ-activated cells infected with the virus. By reporter gene assay it was found that HSV infection augments TNF-α promoter activity. Moreover, treatment of the cells with actinomycin D, which totally blocked mRNA synthesis, only partially prevented accumulation of TNF-α protein, indicating that the infection lifts a block on translation of TNF-α mRNA. EMSA analysis showed that specific binding to the κB#3 site of the murine TNF-α promoter was induced within 1 h after infection and persisted beyond 5 h where TNF-α expression is down-modulated. Binding to the cAMP responsive element site was also induced but more transiently with kinetics closely following activation of the TNF-α promoter. Inhibitors against either NF-κB activation or the activating transcription factor 2 kinase p38 abrogated TNF-α expression, showing a requirement for both signals for activation of the promoter. This observation was corroborated by reporter gene assays. As to the translational regulation of TNF-α, the AU-rich sequence in the 3′ untranslated region of the mRNA was found to be responsible for this control because deletion of this region renders mRNA constitutively translationable. These results show that TNF-α production is induced by HSV-2 in macrophages through both transcriptional and translational regulation. ***The Journal of Immunology*****, 2001, 167: 2202–2208.**

Macrophages are leukocytes of the mononuclear phagocytes lineage. They are endowed with a range of functions involved in clearance of infections. These include phagocytosis, Ag presentation, radical production, and cytokine secretion (1). One of the cytokines secreted by macrophages is TNF-α. This cytokine plays a beneficial role in clearance of a number of viral infections (2–5) as well as parasitic and bacterial infections (6–10). In contrast, TNF-α is also involved in the immunopathology of diseases such as diabetes, hepatitis, and rheumatoid arthritis (11–14). Moreover, a range of viruses elicit an immune response that contributes to the pathology associated with the infections, and TNF-α plays a role in this process (15–18). For instance, although TNF-α does contribute to control of HSV-1 replication (3), the cytokine also is an important factor in the pathogenesis of diseases triggered by primary and recurrent HSV infections (15, 19). Moreover, NO, which is induced by TNF-α in activated macrophages (20), has been reported to contribute to HSV-induced pneumonia and encephalitis (21, 22). Thus, to get the beneficial but not the self-destructive functions of this cytokine, it is essential for the organism to keep the expression of TNF-α under tight control.

The TNF-α promoter is very complex and contains *cis*-acting elements recognized by a number of transcription factors involved in induction of the proinflammatory response (see Fig. 1*A*). For instance LPS, a potent inducer of TNF-α expression in monocytes and macrophages, works through a mechanism highly dependent on NF-κB and the κB sites in the promoter (23, 24). Many other TNF-α-inducing stimuli rely mainly on the region encompassing the cAMP responsive element (CRE)[3] and κ3 sites, which bind to the heterodimer activating transcription factor (ATF) 2/Jun and NF-AT, respectively (25–28). Moreover, an Sp1 binding site in the proximal region is involved in regulation of the promoter (26, 29). The mechanism of TNF-α induction by virus infections has been investigated for some viruses. The HIV Tat protein as well as the EBV glycoprotein gp350 stimulate TNF-α expression in monocytes and macrophages through a mechanism dependent on NF-κB (30, 31). In contrast, hepatitis B virus X protein induces TNF-α expression in hepatocytes through NF-AT, independent of NF-κB activation (32).

Apart from the stimulus-specific regulation of the TNF-α promoter, it also is regulated in a cell type-specific manner. For instance, Goldfeld and associates (28) found that although induction of TNF-α expression in T cells was dependent on the κ3 site and

*Medical Microbiology and Immunology, University of Aarhus, Aarhus, Denmark; and †Department of Molecular Biology, Universite Libre de Bruxelles, Brussels, Belgium

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[2] Address correspondence and reprint requests to Dr. Søren R. Paludan, Department of Medical Microbiology and Immunology, Bartholin Building, University of Aarhus, DK-8000 Aarhus C, Denmark. E-mail address: srp@microbiology.au.dk

[3] Abbreviations used in this paper: CRE, cAMP responsive element; ATF, activating transcription factor; CsA, cyclosporin A; UTR, untranslated region; AUR, AU-rich; MOI, multiplicity of infection; CAT, chloramphenicol acetyltransferase; PDTC, pyrollidine dithiocarbamate.

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sensitive to the NF-AT activation inhibitor cyclosporin A (CsA), the same was not the case in B cells. A recent study from the same laboratory has investigated the stimulus-specific assembly of the TNF-α promoter and shown that in the absence of strong NF-AT activity, high output TNF-α expression is dependent upon Sp1 binding to the promoter (26).

In addition to being regulated at the transcriptional level, TNF-α production also is subject to translational control. The 3′ untranslated region (UTR) of TNF-α mRNA contains an AU-rich (AUR) sequence (see Fig. 1*B*) that imposes a translational block on the mRNA in unstimulated cells. On treatment with, e.g., LPS and Sendai virus, the translational block is lifted and TNF-α protein is produced (33, 34). It has been shown that a protein complex constitutively binds the TNF-α mRNA AUR and that LPS stimulation and Sendai virus infection induces a second complex (35). Moreover, the second complex is induced only in cells able to produce TNF-α in response to the appropriate stimuli, suggesting that recruitment of specific proteins to the TNF-α mRNA AUR aids initiation of mRNA translation.

We have shown previously that HSV-2 infection of resting murine peritoneal cells and macrophage cell lines induces secretion of TNF-α to a modest extent and that simultaneous IFN-γ treatment dramatically enhances this production (36, 20). To further understand what governs the regulation of TNF-α expression during HSV infection, we have investigated the molecular mechanisms underlying this process.

Materials and Methods

Cell culture and virus infection

The mice used in our experiments were 7- to 8-wk-old female C57BL/6 mice. The mice were from Bomholtgaard Animal Breeding and Research Center (Ry, Denmark). Resting peritoneal cells were harvested by lavage of the peritoneal cavity with cold PBS supplemented with 2% FCS and 20 IU/ml heparin. The cells were washed once in RPMI 1640 medium supplemented with 5% FCS (HyClone, Logan, UT), 200 IU/ml penicillin, and 200 μg/ml streptomycin and seeded at a density of 1.5×10^6 cells/1.75 cm^2 (24-well tissue culture plates) and left overnight before stimulation and infection. RAW 264.7 cells were maintained in DMEM with 1% Glutamax I (Life Technologies, Rockville, MD) supplemented with antibiotics and 5% FCS. The RAW 264.7-derived cell lines CAT 3′TNF and CAT 3′TNF UA− have been described elsewhere (33) and were maintained under similar conditions plus 500 μg/ml Geneticin (Roche). For experiments, the cells were seeded in either 24- or 6-well tissue culture plates at a density of 6×10^5 and 2×10^5 cells/well, respectively, and left for 16–20 h before further treatment. The high-titer stock of the MS strain of HSV-2 used in this study was produced as described previously (36). Briefly, mycoplasma-free Vero cells in Eagle's MEM with 2% FCS, 200 IU/ml penicillin, and 200 μg/ml streptomycin were infected at a multiplicity of infection (MOI) of 0.01 (or left uninfected for mock preparations). When the cytopathic effect was nearly complete, the cells were freeze-thawed twice, and the supernatant was clarified by centrifugation at 3000 × *g* for 1 h. The virus was pelleted by ultracentrifugation at 45,000 × *g* for 1 h and resuspended in PBS supplemented with 0.1% BSA. After three 30-s periods of sonication at 40 W, the virus preparation was aliquoted and stored at −70°C until use. The virus stock had an infectivity titer of 8.9×10^7 PFU/ml as determined by plaque assay in Vero cells.

Chemical inhibitors

The chemical inhibitors of diverse cellular functions were: CsA (Sigma, St. Louis, MO), 1 μM; SB203580 (Calbiochem, La Jolla, CA), 10 μM; pyrollidine dithiocarbamate (PDTC; Sigma), 10 μM; *N*-tosyl-L-phenylalanine chloromethyl ketone (Sigma), 3 μM; H89 (Biomol, Plymouth Meeting, PA), 5 μM; GF109203X (Biomol), 1 μM; actinomycin D (Calbiochem), 1 μg/ml.

TNF-α bioassay

Measurement of TNF-α bioactivity was performed with the L929 cell-based bioassay. L929 cells were seeded (2×10^4 cells/well in 96-well culture plates) and left for 16–24 h at 37°C in a humidified atmosphere with 5% CO_2. The culture supernatants were removed and substituted with the samples to be assayed for TNF-α contents in successive 2-fold dilutions and incubated at 38.5°C with 1 μg/ml actinomycin D for 18 h. Cells were fixed in 10% formaldehyde and stained with crystal violet (1 mg/ml). Measurement of light absorbance at 580 nm and comparison with a TNF-α standard dilution series allowed assessment of TNF-α activity.

Isolation of RNA and RT-PCR

Total RNA was isolated with TRIzol (Life Technologies) according to the manufacturers recommendations. Two micrograms of RNA was subjected to reverse transcription with oligo$(dT)_{15}$ and the Expand Reverse Transcriptase (Roche, Gipf-Oberfrick, Switzerland). The cDNA was amplified by PCR with the following primers: TNF-α, 5′-TGT AGC CCA CGT CGT AGC AA-3′ (sense) and 5′-ATT GAC CTC AGC GCT GAG TT-3′ (antisense); β-actin, 5′-CCA ACC GTG AAA AGA TGA CC-3′ (sense) and 5′-GCA GTA ATC TCC TTC TGC ATC C-3′ (antisense); Chloramphenicol acetyltransferase (CAT), 5′-ATG TGT AGA AAC TGC CGG-3′ (sense) and 5′-GGC AAT GAA AGA CGG TGA-3′ (antisense). The products spanned 373 bp (TNF-α), 616 bp (β-actin), and 131 bp (CAT), respectively. The primers were obtained from DNA Technology (Aarhus, Denmark).

Isolation of nuclear extracts

To isolate nuclear proteins, the cell monolayer was washed twice with ice-cold PBS, scraped off the plate, and spun down (2000 μg for 1 min). The cells were resuspended in a hypotonic buffer (20 mM HEPES, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, 0.2 mM EDTA, 0.2 mM PMSF, 0.2 mM leupeptin, 0.2 mM pepstatin A, and 0.1 mM Na_3VO_4) and left on ice for 15 min. Nonidet P-40 was added to 0.6%, and the mixture was vortexed 15 s and centrifuged at 10,000 × g for 1 min. Extraction buffer (20 mM HEPES, pH 7.9, 20% glycerol, 1.5 mM $MgCl_2$, 420 mM NaCl, 0.5 mM DTT, 0.2 mM EDTA, 0.2 mM PMSF, 0.2 mM leupeptin, 0.2 mM pepstatin A, 0.1 mM Na_3VO_4, and 0.2% Nonidet P-40) was added to the nuclei and incubated 30 min at 4°C with rocking. The samples were centrifuged at 10,000 × *g* for 15 min at 4°C, and the supernatants were harvested as nuclear extracts.

EMSA

To assay for DNA-binding activity, 10 μg of protein in 3 μl of nuclear extraction buffer was mixed with 4 μg of poly[d(I-C)] and 20,000 cpm ^{32}P-labeled probe in 18 μl. The final concentrations were: 4 mM Tris-HCl, 23 mM HEPES, 66 mM NaCl, 5 mM $MgCl_2$, 0.7 mM EDTA, 1 mM DTT, and 14% glycerol. After 25 min of incubation at room temperature, the reaction mixture was subjected to electrophoresis on a nondenaturing 5% polyacrylamide gel in 0.5 × TBE buffer (45 mM Tris base, 45 mM boric acid, 1 mM EDTA). The gel was dried and analyzed by autoradiography. For competition assays, 100-fold excess of cold probe was added together with the labeled probe. For supershift analysis, 1 μg of Ab was added together with the nuclear proteins. The Abs used were: goat polyclonal anti p65, mouse monoclonal anti c-Rel, and rabbit polyclonal Abs against p50, STAT6, ATF2, and c-Jun (all obtained from Santa Cruz Biotechnology, Santa Cruz, CA). The probes corresponding to sites in the murine TNF-α promoter are shown in Fig. 1*A*. Other probes used were IFN-γ activation site (5′-CTG ATT TCC CCG AAA-3′) and IFN-stimulated response element (5′-AAA TGA AAA TGA AAA TGA AAA TGA-3′).

Plasmids

The wtTNF/CAT reporter construct was kindly provided by Dr. Dmitry Kuprash and contains the region from −1173 to +127 of the murine TNF-α promoter. The mutCRE/CAT mutant was generated using the GeneEditor Mutagenesis System (Promega, Madison, WI) and the following primer: 5′-CAC ATG AGA AGC TGG TTT TCT-3′ (mutated nucleotides underlined). The isolated plasmid was sequenced before use. To generate the dominant negative IκBα expression construct, mutant IκBα (a kind gift from Dr. J. Hiscott; Ref. 37) was amplified by PCR with the high-fidelity polymerase Pfu with the following primers: 5′-GAA TTC ATG TTC CAG GCG GCC GAG-3′ (sense), 5′-GTC GAC TTA GAA CTC TGA CTC TGT GTC-3′ (antisense). Restriction sites used for subcloning are underlined. The PCR fragment was cloned into the *Eco*RV site of pBluescript KS (Stratagene) and subcloned into the appropriate sites in pcDNA3.

Transient transfections

RAW 264.7 cells were seeded at a density of 5×10^5 per 10 cm^2 well and left overnight to settle. The cells were transfected with DNA by using lipofectAMINE (Life Technologies). Briefly, 3 μg of DNA (2.5 μg of wtTNF/CAT or mutCRE/CAT reporter gene construct and 0.5 μg of

mutIκBα or pcDNA3) was mixed with serum-free DMEM to 100 μl (A) and 8 μl lipofectAMINE was mixed with serum-free DMEM to 100 μl (B). A and B (C) were gently mixed and left for 20 min at RT. The cells were washed twice in PBS, and 800 μl of serum-free DMEM was added to C, which was gently spread over the cells. Four hours later, 4 ml of DMEM supplemented with 12.5% FCS was added to the cells to give a final FCS concentration of 10%. After an additional 20 h, the medium was exchanged with preheated DMEM + 5% FCS and left for 6 h before stimulation and infection.

CAT assay

For CAT assay, cells were lysed in 250 mM Tris-HCl, pH 7.8, by two cycles of freeze-thaw. Equal amounts of protein were incubated for 2 h at 37°C with 0.05 μCi of [^{14}C]chloramphenicol (Amersham, Arlington Heights, IL) and 20 μmol of acetyl-CoA (Roche) in a total volume of 150 μl. The chloramphenicol and acetylated derivatives were extracted with ethyl acetate and separated by thin-layer chromatography followed by exposure to x-ray films.

Results

HSV-2 infection of IFN-γ-activated macrophages stimulates secretion of TNF-α

We have reported previously that HSV-2 infection of murine macrophage cell lines and peritoneal cells triggers production of TNF-α and that concomitant IFN-γ treatment strongly amplifies this phenomenon (20, 36). In initial experiments, we confirmed these earlier findings (Fig. 2*A*) and further found that the same picture was seem at the level of mRNA (Fig. 2*B*). When examining the kinetics of TNF-α mRNA accumulation, we found that the mRNA started accumulating after between 1 and 2 h and that peak levels were detected after 3 h (Fig. 2*C*). After that time point, the levels rapidly declined to below basal levels. To validate the RAW 264.7 cell line as a model, we compared with murine peritoneal cells and observed that these cells also produced TNF-α in response to HSV-2 infection, synergized with IFN-γ, and that the two cell populations displayed the same kinetics of TNF-α induction (data not shown).

HSV-2-induced TNF-α expression is regulated at the level of transcription and translation

TNF-α expression in response to LPS has been investigated in great details and has been shown to be regulated at the level of transcription and translation (23, 34). In addition, Newcastle disease virus stimulates TNF-α secretion by activating transcription and stabilizing TNF-α mRNA (38). Thus, TNF-α can be regulated at multiple levels, and we wanted to investigate how HSV-2 infection of macrophages induces TNF-α production.

By reporter gene assay, we found that HSV-2 infection moderately enhanced TNF-α promoter activity in resting macrophages (Fig. 3*A*) and that concomitant IFN-γ treatment further augmented this. CAT accumulation was noticeable already 2 h after infection and reached maximal levels after around 8 h of infection.

In early experiments, we observed that TNF-α mRNA is detectable in resting macrophages when the number of PCR cycles were increased (Fig. 3*B*, *inset*). This allowed assessment of TNF-α mRNA stability in resting and HSV-2/IFN-γ-treated macrophages, which received actinomycin D before stimulation. As seen from Fig. 3*B*, the levels of TNF-α mRNA decreased slowly in untreated cells after de novo RNA synthesis was inhibited. IFN-γ treatment and HSV-2 infection did not stabilize TNF-α mRNA.

Because actinomycin D inhibits de novo RNA synthesis, measurement of TNF-α levels in supernatants from cells treated with this drug allowed us to evaluate whether HSV-2 infection, and IFN-γ stimulation led to translation of the constitutively present TNF-α mRNA. As seen in Fig. 3*C*, this treatment did indeed lead to TNF-α bioactivity despite the lack of de novo mRNA synthesis.

HSV-2 infection and IFN-γ stimulation induces binding to the κB#3 and CRE sites of the murine TNF-α promoter

Given the above finding that induction of TNF-α expression in macrophages by HSV-2 infection was at least partly attributable to enhanced transcription, we went on to explore the pattern of transcription factor recruitment to the TNF-α promoter after IFN-γ stimulation and HSV-2 infection. Nuclear extracts were harvested at different time points after treatment and subjected to EMSA with the probes shown in Fig. 1*A*. As seen from Fig. 4*A*, HSV infection and IFN-γ stimulation induced binding to the κB#3 site, with significantly elevated binding after 1 h and levels remaining elevated through 5 h. Three bands were observed with varying relative intensity between the experiments. By competition with excess of

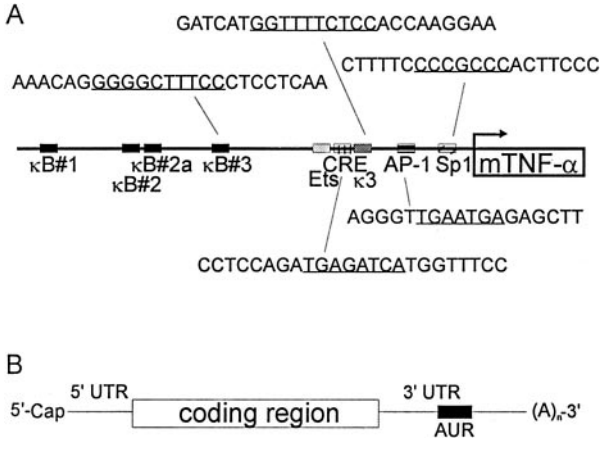

FIGURE 1. *A*, *cis*-acting elements in the murine TNF-α promoter. The highlighted sequences correspond to the probes used for EMSA. Core sequences in the probes are underlined. *B*, Structure of the TNF-α mRNA.

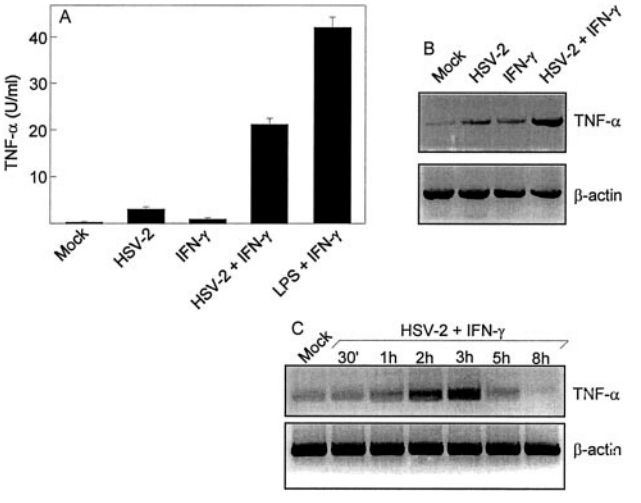

FIGURE 2. Production of TNF-α in macrophages. *A*, RAW 264.7 cells were treated with 100 IU/ml IFN-γ and infected with 3×10^5 PFU/ml HSV-2 (MOI 0.6) for 8 h. Supernatants were harvested and assayed for TNF-α bioactivity. As a positive control one set of cells were treated with 100 IU/ml IFN-γ and 100 ng/ml LPS. Results are shown as means of duplicate cultures ± SEM. *B* and *C*, RAW 264.7 cells were stimulated and infected as above. For the experiments presented in *B*, cells were left for 3 h, while the indicated incubation periods were used for the experiment presented in *C*. The cells were lysed, and RNA was extracted and analyzed by RT-PCT using specific primers for TNF-α and β-actin. The cDNA was amplified by 25 cycles of PCR.

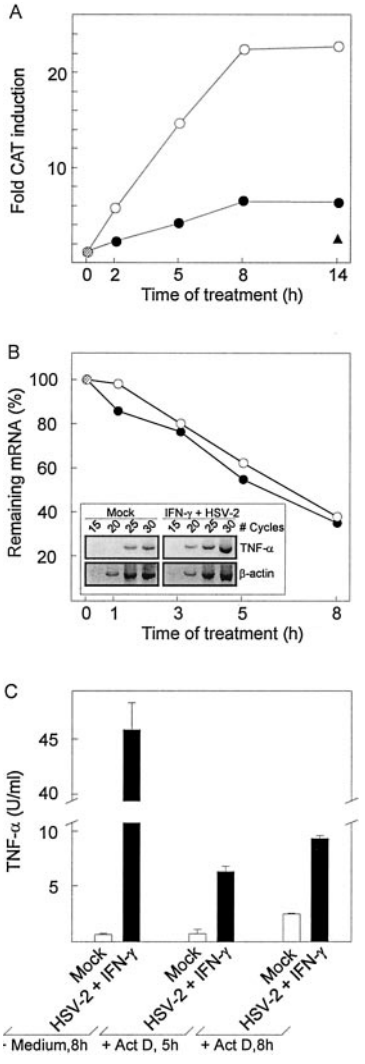

FIGURE 3. Regulation of TNF-α production in macrophages. *A*, RAW 264.7 cells were transfected with a reporter gene construct driven by the TNF-α promoter and treated with 100 IU/ml IFN-γ and infected with 3×10^5 PFU/ml HSV-2 (MOI 0.6). At the indicated time points postinfection cells were lysed and assayed for CAT activity. ●, HSV-2; ○, IFN-γ + HSV-2; ▲, IFN-γ. *B*, RAW 264.7 cells were treated with 1 μM of actinomycin D 15 min before stimulation and infection. At the indicated time points, RNA was isolated and analyzed as above. Thirty cycles of PCR were performed. The intensity of bands was quantified by densitometry, and TNF-α levels were normalized against β-actin. The results are shown as a percentage of remaining TNF-α mRNA. ●, Untreated; ○, IFN-γ + HSV-2. *B, Inset,* Amplification of TNF-α and β-actin mRNA by RT-PCR. mRNA from mock-infected or HSV-2/IFN-γ-treated RAW 264.7 cells was reverse transcribed and subjected to an increasing number of PCR cycles. *C*, The cells were treated as in *B* for the indicated time intervals, at which point supernatants were harvested and assayed for TNF-α bioactivity. Results are shown as means of duplicate cultures ± SEM.

cold probe, the specificity of the binding was examined (data not shown). It was found that although the probes for CRE-, AP-1-, and IFN-stimulated response element were unable to compete with any of the bands, cold κB#3 totally abolished binding to the hot κB#3 probe. Interestingly, cold probes for κ3 and IFN-γ activation site moderately decreased binding to the κB#3 probe, with the middle band being most affected. This is consistent with previous findings reported in the literature that these DNA sequences under certain conditions can interfere with NF-κB binding (39, 40). By supershift analysis, we found that p50 and p65 were present in the induced κB#3-binding complexes, whereas the contribution of c-*Rel* seems to be minor.

When examining binding to the CRE in the TNF-α promoter, we found that CRE-binding was indeed induced, but only transiently, with peak levels observed after 1 h (Fig. 4*B*). The binding was specific, and supershift analysis revealed that both ATF-2 and c-Jun were present in the DNA-binding complexes. Finally, we examined binding to the AP-1, κ3, and Sp1 sites. We were unable to detect specific constitutive or inducible binding to the two former sites but did observe a strong specific constitutive binding to the Sp1 site (data not shown).

To explore whether the dual treatment was necessary to bring about enhanced DNA-binding activity to the κB#3 and CRE sites, we generated nuclear extracts from cells treated with either IFN-γ and HSV-2 alone or both. By EMSA, we found that HSV-2 infection alone was sufficient to trigger binding to the κB#3 site and that concomitant IFN-γ stimulation further enhanced this binding (Fig. 4*A*). By contrast, the enhanced CRE binding was not observed in extracts from cells receiving either stimuli alone, but did require the dual treatment (Fig. 4*B*).

Inhibitors against NF-κB and p38 reduce induction of TNF-α by HSV-2 and IFN-γ

From the results shown above, it seemed possible that transcription factors of the NF-κB and ATF/Jun families were involved in activation of TNF-α gene transcription. To examine this, and to explore the potential participation of other signaling pathways, we incubated cells with a range of chemical inhibitors before stimulation and infection. After 3 h, total RNA was harvested and analyzed for the presence of TNF-α mRNA. As seen from Fig. 5*A*, the ability of HSV-2 and IFN-γ to induce TNF-α expression in RAW 264.7 cells was not affected by CsA, indicating that NF-AT is not involved in the process. This is consistent with the lack of inducible DNA binding to the NF-AT-responsive κ3 site in the TNF-α promoter following infection. By contrast, SB203580, an inhibitor of the ATF2 kinase p38, and the inhibitors of NF-κB activation PDTC and *N*-tosyl-L-phenylalanine chloromethyl ketone were able to strongly reduce TNF-α mRNA accumulation. Inhibitors against PKA (H89) and PKC (GF109203X) showed a modest effect on the TNF-α levels. Essentially similar results were obtained when TNF-α bioactivity was measured in culture supernatants from RAW 264.7 and murine peritoneal cells treated with the inhibitors (Fig. 5, *B* and *C*).

Overexpression of nondegradable IκBα and mutation of the CRE site abolish TNF-α promoter activation following HSV-2 and IFN-γ treatment

Based on the above results, NF-κB and ATF2/Jun seemed to be important for TNF-α expression in HSV-2-infected IFN-γ-activated macrophages. To test this more thoroughly, we performed reporter gene experiments where RAW 264.7 cells were either 1) cotransfected with the wild-type TNF-α promoter and the nondegradable IκBα mutant S32A/S36A or empty vector; or 2) were transfected with wild-type or CRE mutant TNF-α promoter. Subsequently, the transfected cells were stimulated with IFN-γ and infected with HSV-2. Twenty hours later, cells were lysed and CAT activity was assayed.

Cells transfected with wild-type TNF-α promoter and empty vector control displayed a basal reporter activity after mock infection (Fig. 6*A*) and a 14.7-fold induction after treatment with LPS (data not shown). When the cells were infected with HSV-2 and stimulated with IFN-γ, the promoter activity was enhanced >4.5-fold. Interestingly, this activation was abrogated if the cells had been cotransfected with the nondegradable IκBα mutant, thus

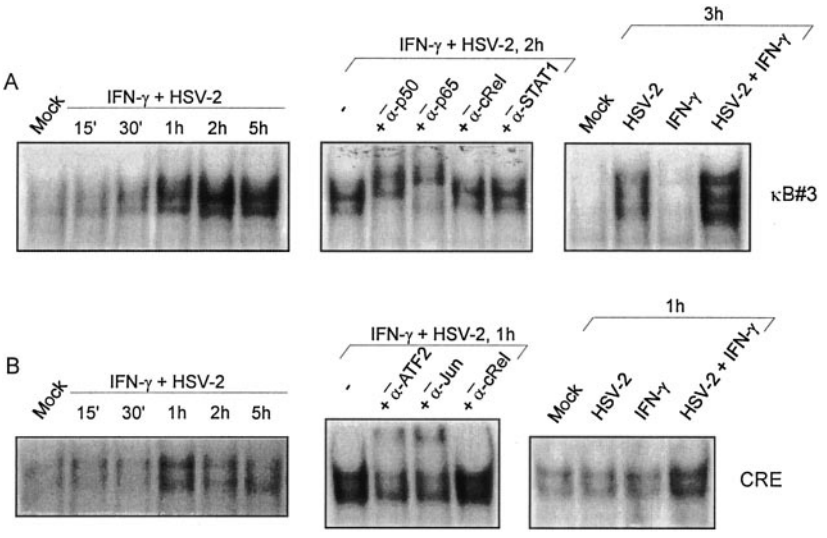

FIGURE 4. Binding activity to the κB#3 and CRE sites of the murine TNF-α promoter. RAW 264.7 cells were treated with 100 IU/ml IFN-γ and infected with 3×10^5 PFU/ml HSV-2 (MOI 0.6) for the indicated time periods, and nuclear extracts were prepared. Inducibility, complex composition, and requirement for dual stimulation was analyzed using the κB#3 (*A*) and CRE (*B*) sites from the murine TNF-α promoter as probe. Ten micrograms of nuclear proteins and 20,000 cpm of ^{32}P-labeled probe were used per reaction. One microgram of Ab per sample was used for supershift analysis.

strongly suggesting a role for NF-κB in TNF-α induction by HSV-2 in IFN-γ-activated macrophages.

To evaluate the role of CRE-binding proteins in TNF-α induction, we used the TNF-α promoter mutant mentioned above. As in Fig. 6*A*, we found that HSV-2 infection and IFN-γ stimulation raised the wild-type promoter activity severalfold over the CAT levels in extracts from mock-infected cells (Fig. 6*B*). Mutation of the CRE site rendered the reporter construct unresponsive to IFN-γ stimulation and HSV-2 infection. Thus, the CRE site of the murine TNF-α promoter is essential for its activation by HSV-2 and IFN-γ in RAW 264.7 cells.

HSV-2 and IFN-γ lift an AUR-dependent translational block on TNF-α mRNA

In Fig. 3*C* we showed that HSV-2 infection and IFN-γ stimulation induced secretion of TNF-α protein even under conditions where de novo mRNA synthesis was totally inhibited. This suggested that HSV/IFN-γ treatment renders the constitutively present TNF-α mRNA translationable. For a range of other stimuli, this phenomenon previously has been demonstrated to occur through an AUR-dependent mechanism, and we wanted to test whether this was the case in our system. For this purpose, we used the previously reported system (33, 34) where the wild-type or AUR mutant TNF-α mRNA 3′ UTR was placed downstream of a CAT reporter expressed from a SV40 promoter. The cells stably expressing the AUR mutant (CAT 3′TNF UA$^-$) contained much more CAT mRNA than the cells expressing the wild-type 3′ UTR of the TNF-α mRNA (CAT 3′TNF), and also exhibited a 20-fold higher CAT activity (Fig. 7). On HSV-2 infection, an ~2-fold increase in CAT activity was observed for CAT 3′TNF UA$^-$, and the CAT levels were increased >10 times in the CAT 3′TNF-transfected RAW 264.7 cells. Given the presence of κB and CRE sites in the SV40 promoter, the effect of infection on CAT 3′TNF UA$^-$ is likely to be transcriptional. Treatment with IFN-γ alone or along with HSV-2 infection did not affect CAT activity. These results show that instability of TNF-α mRNA is conferred by the AUR and that this region also participates in regulation of TNF-α mRNA translation in HSV-infected macrophages.

Discussion

The early immune response to a viral infection is important for the ability of the organism to eventually control the infection. The cytokine TNF-α is a central factor in this nonspecific immune response. For instance, inactivation of TNF-α by Ab-mediated neutralization or deletion of the genes encoding the cytokine or the TNF receptors renders mice more susceptible to infection with the poxviruses ectromelia virus and vaccinia virus (41) and the herpes viruses CMV and HSV (3, 4). In addition to the beneficial role of TNF-α in host defense, an increasing number of studies have shown that this cytokine may also participate in the immunopathology induced by viruses. For HSV, it has been reported that TNF-α and TNF-α-induced products contribute to the pathogenesis of virus-induced keratitis, encephalitis, and pneumonia (13, 15, 21, 22). Hence, it is important to keep TNF-α expression under rigid control. In the present study, we have found that HSV-2 induces expression of TNF-α in IFN-γ-activated murine macrophages and that this is brought about by a dual mechanism: activation of the TNF-α promoter and release of a block on TNF-α mRNA translation.

The mechanism of transcriptional activation shares some similarities with what has been found for other TNF-α-inducing viruses and shows some unique features. The requirement for NF-κB in the process has previously been reported for EBV glycoprotein gp350 and the HIV Tat protein (30, 31). Moreover, the nonviral products LPS and staphylococcal enterotoxin A are also known to induce TNF-α expression through an NF-κB-dependent mechanism (23, 24, 42). However, the requirement for both NF-κB and ATF2/Jun in TNF-α expression has not been reported previously. This observation illustrates an interesting phenomenon, namely that although NF-κB is involved in expression of many HSV-induced products, including TNF-α, IL-6, and NO synthase type 2 (S. R. Paludan, unpublished results and Ref. 20), these are expressed with very different kinetics because NF-κB works in concert with other transcription factors in activation of specific promoters. This allows the infected cell to use NF-κB in regulation of many inducible genes, yet fine-tune the expression by cooperation with different transcription factors.

The hepatitis B virus X protein has previously been shown to induce TNF-α in hepatocytes via NF-AT (32), and the ability of Sendai virus to bring about TNF-α expression in T cells, B cells, and fibroblasts has also been reported to involve NF-AT (26). In our experiments, NF-AT was not found to play any major role in HSV-2-induced TNF-α expression in macrophages. Moreover, no inducible or sequence-specific NF-AT binding was observed. These results demonstrate that different viruses use diverse mechanisms to stimulate TNF-α expression.

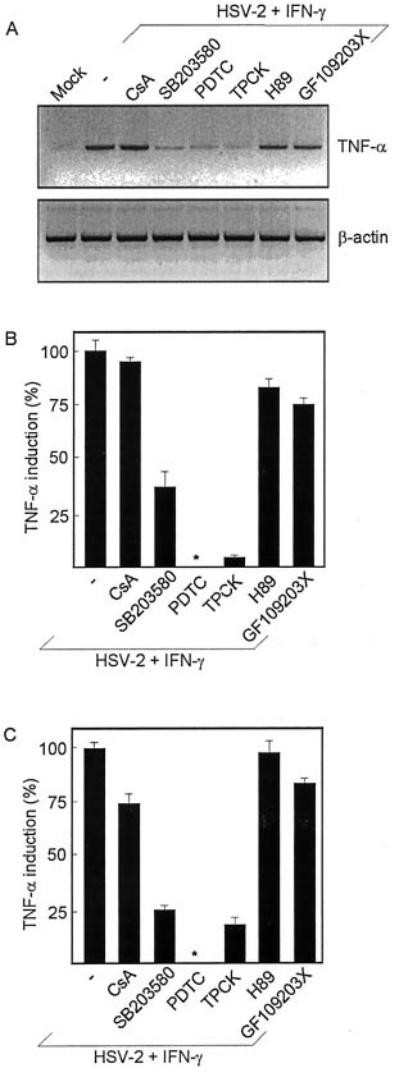

FIGURE 5. Effect of chemical inhibitors on TNF-α induction. RAW 264.7 cells and murine peritoneal cells were pretreated with the indicated inhibitors (for concentrations see *Materials and Methods*) 15 min before stimulation. RAW 264.7 cells received 100 IU/ml IFN-γ and 3×10^5 PFU/ml HSV-2 (MOI 0.6), while the peritoneal cells were treated with the same amount of IFN-γ and 7.5×10^5 PFU/ml HSV-2 (MOI 0.6). After 3 h of infection, total RNA was isolated while supernatants were collected after 8 h. *A,* The RNA from RAW 264.7 cells was reverse transcribed and subjected to 25 cycles of PCR with primers specific for TNF-α and β-actin. *B* and *C,* The supernatants from RAW 264.7 (*B*) and PCs (*C*) were assayed for TNF-α κB bioactivity. $*$, PDTC was toxic to the L929 cells used in the TNF-α bioassay. The results are shown as fold induction of TNF-α secretion $\pm$ SEM. Supernatants from cells receiving IFN-γ and HSV only is set to 100%.

Our finding that HSV-2 activates TNF-α mRNA translation in macrophages parallels results from previous studies on TNF-α induction by LPS and Sendai virus (34). As for the latter two, HSV-2 infection was able to lift a block on TNF-α mRNA translation, and this function was dependent on the AUR in the 3′ UTR. As to how the AUR is involved in the translational control, it has been shown that constitutive and inducible protein complexes bind to this region and that inhibition of recruitment of the inducible complex by tyrosine kinase inhibitors impairs TNF-α mRNA translation (34). These results suggest that protein complexes bound to the AUR facilitate mRNA translation possibly by interacting with the translational machinery.

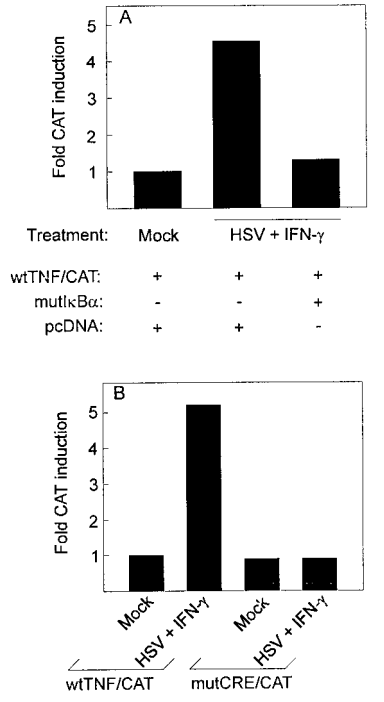

FIGURE 6. Role of NF-κB and CRE-binding factors in activation of the TNF-α promoter by HSV-2 and IFN-γ. RAW 264.7 cells were seeded and transfected with the indicated DNA plasmids and treated with 100 IU/ml IFN-γ and 3×10^5 PFU/ml HSV-2 (MOI 0.6) 30 h later. After 20 h of incubation, the cells were lysed and assayed for CAT activity. *A,* Activation of the wild-type TNF-α promoter (wtTNF/CAT) in the presence and absence of nondegradable IκBα (mutIκBα). *B,* Activation of wtTNF/CAT and a CRE mutant of the TNF-α promoter (mutCRE/CAT) by IFN-γ and HSV-2.

Collectively, the results of the present study clearly demonstrate that TNF-α expression during HSV-2 infections is tightly regulated. First, the ability of HSV-2 to induce high out-put TNF-α is dependent on the presence of IFN-γ. Second, at the transcriptional level, both NF-κB and ATF2/Jun are required for promoter activation. Third, TNF-α mRNA is not translated in resting uninfected cells, but only after infection of the macrophages. Such a tight regulation is desirable for the host because it limits TNF-α expression in magnitude and duration. The rapid and transient expression

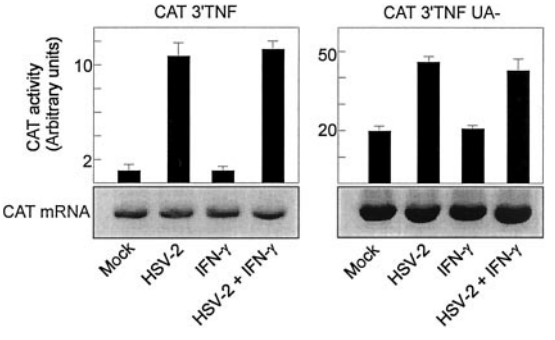

FIGURE 7. Involvement of the TNF-α mRNA AUR in regulation of expression. RAW 264.7 cells stably transfected with the CAT 3′TNF or CAT 3′TNF UA$^-$ constructs (described in the text) were seeded and treated with 100 IU/ml IFN-γ and 3×10^5 PFU/ml HSV-2 (MOI 0.6). Sixteen hours later, the cells were lysed and assayed for CAT activity (*top*) or analyzed for the presence of CAT mRNA (*bottom*). The results of the CAT assay are shown in arbitrary units $\pm$ SEM.

of TNF-α probably allows initiation of a beneficial immune response without causing significant damage to the host.

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