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Transcriptional Activation of the Tumor Necrosis Factor α-Inducible Zinc Finger Protein, A20, Is Mediated by κB Elements*

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Alexandra Krikos‡, Carol D. Laherty‡§, and Vishva M. Dixit‡§¶

From the ‡Department of Pathology, §Cellular and Molecular Biology Program, the University of Michigan Medical School, Ann Arbor, Michigan 48109-0602

A20 was first identified as a tumor necrosis factor (TNF) primary response transcript encoding a 790-amino acid protein with a unique zinc finger motif. Recently, A20 was shown to protect cells from TNF-induced cytotoxicity in a variety of cell lines. Nuclear run-on studies previously established that TNF activates A20 at the transcriptional level. To further characterize the mechanism by which TNF activates the A20 gene, we have cloned the A20 5′-flanking sequences and identified TNF-responsive elements within the promoter. The transcription initiation site was mapped by both primer extension and S1 nuclease protection experiments to a position 4.2 kilobases (kb) upstream of the initiator methionine; the first and second exon were separated by a 3.9-kb intron. Sequences upstream of the transcription start site were 76% GC-rich and contained six Sp1 binding sites and a TATA-like sequence at −29 but lacked a consensus CCAAT site. Transfection of Jurkat T-cells with an array of A20 promoter CAT constructs showed that two κB elements residing at −54 and −66 were required for induction by TNF. Supporting this notion, DNA electrophoretic mobility shift assays using nuclear extracts from unstimulated and TNF-stimulated Jurkat cells demonstrated κB-specific binding of a TNF-activated factor to an end-labeled probe containing the two A20 κB sequences. Finally, evidence obtained from cotransfection experiments showed that A20 negatively regulated its own expression.

We have undertaken the cloning and characterization of primary response genes induced by tumor necrosis factor α (TNF)[1] in an effort to understand the molecular basis of its action (1–6). TNF has pleiotropic effects on cells and tissues which can be summarized as proinflammatory and catabolic in nature (reviewed in Ref. 7). In addition, TNF is capable of inducing hemorrhagic ischemic necrosis of fibrosarcoma tumors in mice and *in vitro* is cytostatic or cytotoxic to some tumor cell lines, whereas others are resistant. The molecular basis of resistance to TNF killing is only beginning to be understood. It is becoming evident that a subset of TNF-inducible genes confers resistance to TNF cytotoxicity. The first gene product found to partially protect cells from TNF-induced cytotoxicity was the mitochondrial enzyme manganous superoxide dismutase, an antioxidant that protects cells from reactive oxygen species thought to be generated by TNF action (8). Plasminogen-activator inhibitor type-2, a TNF-inducible protease inhibitor, also provides some protection from TNF cytotoxicity, indicating a potential role for proteases in TNF killing (9). More recently, we have identified a TNF-inducible primary response gene designated A20 which confers resistance to TNF cytotoxicity in stably transfected NIH 3T3 and WEHI 164 cells.[2] Further, human breast carcinoma cell lines resistant to TNF killing constitutively express the A20 gene product.[2]

The A20 gene product is composed of 790 amino acid residues (3). The C-terminal half contains 7 Cys_2/Cys_2 zinc finger repeats; 6 with the configuration Cys-X_4-Cys-X_{11}-Cys-X_2-Cys and 1 with the configuration Cys-X_2-Cys-X_{11}-Cys-X_2-Cys. A20 is distinct from other Cys_x finger proteins because it contains *multiple* repeated Cys_2/Cys_2 motifs and is the first example of a protein with a finger loop domain composed of 11 amino acid residues.

The A20 transcript is rapidly but transiently induced by TNF, reaching its highest level within 1 h following stimulation (1). In the concomitant presence of cyclohexamide (CHX), the transcript is greatly stabilized. This is consistent with the finding that the 3′-untranslated region of A20 mRNA contains four copies of the canonical sequence ATTTA (3) which confers CHX-inhibitable message instability to a number of short-lived transcripts including those of many oncogenes and cytokines (10, 11).

Nuclear run-on experiments have demonstrated that induction of the A20 gene by TNF is due to an increase in its rate of transcription (1). Given that this occurs in the absence of any intervening protein synthesis, the A20 promoter must be directly activated by transcription factors already present in the cell. To begin to delineate this pathway, we undertook the cloning and characterization of the A20 promoter. We show that induction of the A20 promoter by TNF is mediated through two κB elements. *In vitro*, the A20 κB elements formed a complex with a TNF-activated factor, presumably NF-κB. In addition, we provide evidence that A20 can negatively regulate its own promoter.

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EMBL Data Bank with accession number(s) M96756.

¶ An Established Investigator of the American Heart Association. To whom correspondence and reprint requests should be addressed: Dept. of Pathology, the University of Michigan School of Medicine, 1301 Catherine St., P.O. Box 0602, Ann Arbor, MI 48109-0602.

[1] The abbreviations used are: TNF, tumor necrosis factor α; CHX, cyclohexamide; CAT, chloramphenicol acetyltransferase; RSV, Rous sarcoma virus; LTR, long terminal repeat; IL-2, interleukin 2; IL-6, interleukin 6; IL-8, interleukin 8; HIV, human immunodeficiency virus; kb, kilobase(s); bp, base pair(s).

[2] A. W. Opipari, H. M. Hu, R. Yabkowitz, and V. M. Dixit, manuscript submitted.

EXPERIMENTAL PROCEDURES

Screening of cDNA and Genomic Libraries—A random primed cDNA library in the plasmid vector, pcDNA1, was made using poly(A$^+$) RNA from human umbilical vein endothelial cells treated with human recombinant TNF (200 units/ml; a gift from Genentech, South San Francisco, CA) and CHX (10 μg/ml) for 4 h (Invitrogen, San Diego, CA). 10^6 colonies of the cDNA library were screened as described previously (12) using a random prime-labeled probe (13) with a specific activity of 1–8 × 10^8 cpm/μg and corresponding to the 5′ 435 bp of the previously characterized A20 cDNA (3). Plasmids from hybridizing colonies were isolated and subjected to restriction mapping and Southern analysis (14) to identify a cDNA (designated 36.1.1) with the most 5′-extended sequence.

4.5 × 10^5 colonies of a human placenta genomic library in pWE15 (Stratagene, La Jolla, CA) were screened as described previously using [^{32}P]dCTP random prime-labeled probes corresponding to the 5′ 1075 bp of the published A20 cDNA sequence (3). Hybridizing cosmids were purified, digested with a variety of restriction enzymes, and analyzed by Southern blot hybridization (14) to a [^{32}P]dCTP-labeled probe comprising the most 5′ 187 bp of the cDNA isolate 36.1.1. A hybridizing 6-kb *Hin*dIII fragment was subcloned into the plasmid vector pGEM-7zf (Promega).

DNA Sequencing and Analysis—The 6-kb genomic *Hin*dIII fragment was digested with *Pst*I and the resulting fragments were subcloned into pTZ18R (Pharmacia LKB Biotechnology Inc.) for sequence analysis. Cesium chloride-banded plasmid was sequenced on both strands by the dideoxy chain termination method using synthetic oligonucleotide primers and modified T7 DNA polymerase (Sequenase) according to the supplier's instructions (United States Biochemical Corp.). Sequence compressions were resolved using the deaza base analog of guanosine or by the use of terminal deoxynucleotidyl transferase as recommended by the supplier (United States Biochemical Corp.). DNA sequence was analyzed on an Apple Macintosh computer using the MacVector program, version 3.5.

Cell Culture—The Jurkat T-cell leukemia line was maintained in RPMI 1640 (Whittaker Bioproducts, Walkersville, MD) supplemented with 7.5% fetal bovine serum (Hyclone Laboratories, Logan, UT), 0.1 mM nonessential amino acids, penicillin (100 units/ml), streptomycin (100 units/ml), and 2.0 mM additional glutamine (GIBCO).

Primer Extension and S1 Nuclease Mapping—Total RNA was prepared from Thp1 cells, a myelomonocytic cell line, following treatment with TNF (200 units/ml) and CHX (10 μg/ml) for 8 h as previously described (1). Primer extension analysis was performed according to standard procedures (15) with 5 μg of RNA and 1–5 × 10^5 cpm of an antisense oligonucleotide complementary to nucleotides +54 to +30 and end-labeled with [γ-^{32}P]ATP to a specific activity of 1–5 × 10^7 cpm/pmol. For S1 nuclease mapping of the A20 transcription start site, a 62-base antisense oligonucleotide complementary to nucleotides +54 to −8 was end-labeled to a specific activity of 1–5 × 10^7 cpm/pmol using T4 kinase and annealed to 10 μg of RNA followed by S1 nuclease cleavage according to standard protocols (15).

Plasmids—All CAT expression constructs were made in the pCAT-Basic vector (Promega). Plasmid −233/+12 A20CAT was made by inserting the 245-bp *Pst*I fragment containing the A20 transcription start site into the CAT vector. 5′ deletions were made by linearizing −233/+12 A20CAT at an upstream *Hin*dIII site in the vector polylinker. The linearized fragment was cleaved with either *Bss*HII (−144), *Nae*I (−111), *Bbr*PI (−74), or *Sma*I (−44) and recircularized with T4 ligase after filling-in 5′-protruding ends using the large fragment of DNA polymerase I. The resultant plasmids were designated −144/+12 A20CAT, −111/+12 A20CAT, −74/+12 A20CAT, and −44/+12 A20CAT. The deletion plasmid Δ-74/-45 A20CAT was constructed by cleavage of −233/+12 A20CAT with *Bbr*PI (−74) and *Sma*I (−44) followed by recircularization with T4 ligase. Plasmid dm A20CAT was constructed from the parental −233/+12 A20CAT plasmid using oligonucleotide site-directed mutagenesis as previously described (16). A 41-base oligonucleotide with the sequence 5′GT GACTTTGGAAAGATCTGTGGAAAAGATCGGGCCTACAAC3′ was used to replace the native A20 promoter sequence from −74 to −34 (mutations are *underlined*). RSV CAT contains the Rous sarcoma virus long terminal repeat (RSV LTR) inserted upstream of the CAT gene and has been described previously (17). The plasmid pSFFV-A20 contains an A20 cDNA encoding the entire open reading frame placed downstream of the Friend spleen focus-forming virus LTR in the expression vector pSFFV-neo[2] (18).

Transient Transfection and CAT Assay—Jurkat cell transfections were done as per published procedures (19) using 10 μg of plasmid/5 × 10^6 cells. Transfected cells were either untreated or treated with TNF which was added 30–36 h post-transfection at 200 units/ml for 12 h. Cells were harvested 42–48 h post-transfection, extracts were prepared, and CAT enzyme activity was assayed according to standard protocols (20, 21). CAT activity was quantitated using a Betascope 603 Blot Analyzer (Betagen, Waltham, MA). Induction of CAT activity by TNF is reported as the average from at least four separate transfections and represents the ratio of CAT activity from TNF-stimulated cells *versus* unstimulated cells.

Electrophoretic Mobility Shift Assays—Nuclear extracts used in electrophoretic mobility shift assays were prepared as described previously (22) from untreated Jurkat cells or cells treated with TNF (200 units/ml) for 1 h. Protein concentration was determined by the Bio-Rad assay according to manufacturer's instructions (Bio-Rad). A 43-bp A20 probe extending from −74 to −32 was made by annealing complementary synthetic oligonucleotides and labeling 5′-protruding ends with Klenow fragment. Binding reactions (23) containing 5 μg of nuclear protein extract, 1 μg of poly(dIdC·dIdC) (Pharmacia), 0.1 ng of A20 probe labeled to a specific activity of 2 × 10^8 cpm/μg, and KCl binding buffer (4% glycerol, 1 mM EDTA, 5 mM dithiothreitol, 0.01 M Tris-HCl, pH 7.5, and 5 mM KCl) in a volume of 15 μl were incubated for 15 min at room temperature and then resolved by electrophoresis through a 4% polyacrylamide gel in a Tris-glycine buffer (50 mM Tris, 0.4 M glycine, 2 mM EDTA, pH adjusted to 8.5). For competition experiments, 5 or 20 ng of unlabeled A20κB probe DNA, dmA20 (5′GTGACTTTGGAAAGATCTGTGGAAAAGATC GGGCCTACAACCC3′: *underlining* represents changes from native sequence), HIV-1 κB (5′GATCAGGGACTTTCCGCTGGGGACTT TCC3′), and IL-2 octamer (5′GATCCATATGTAAAACATTTTGC AATATGTAAAACATTTTA3′) double-stranded DNA fragments were added to binding reactions.

RESULTS

Isolation of an A20 cDNA Containing the 5′ End—An A20 cDNA was isolated and characterized previously (1, 3). This cDNA contained the entire A20 open reading frame but did not contain the extreme 5′ end of the transcript as demonstrated by preliminary primer extension analysis (data not shown). To obtain a cDNA clone containing the A20 extreme 5′ end, a random primed human umbilical vein endothelial cells cDNA library was screened using a probe containing the 5′ 435 bp of known A20 cDNA sequence (3). Six hybridizing clones were selected for sequencing based on the size of the inserts. A cDNA clone designated 36.1.1 provided an additional 148 bp of 5′ cDNA sequence.

Characterization of A20 Genomic Clones—To identify genomic clones containing A20 5′-flanking sequences, a human placenta library in the cosmid pWE15 was screened using a probe containing the 5′ 1075 bp of the published A20 cDNA sequence (3). Twenty-two hybridizing clones were obtained from a primary screen of 4.5 × 10^5 colonies. Of these, two were further characterized by Southern analysis of restriction endonuclease digested cosmid DNA. A 6-kb *Hin*dIII genomic fragment was identified which hybridized to a probe containing the 5′ 187 bp of cDNA 36.1.1. Restriction enzyme mapping and partial DNA sequencing of the *Hin*dIII fragment showed that it contained about 1.4 kb of sequence upstream of the cDNA 5′ end and revealed a 3.9-kb intron 21 bp upstream of the ATG initiator methionine (Fig. 1*A*).

Mapping the A20 Transcription Initiation Site—A single transcription start site designated +1 (Fig. 1) was mapped 300 bp upstream of the initiator methionine in the cDNA sequence using a combination of S1 nuclease and primer extension analyses (Fig. 2). Two single-stranded antisense oligonucleotides having identical 5′ ends were used as probes for these reactions. For the primer extension reactions, an end-labeled 25-base oligonucleotide extending from +54 to +30 was annealed to RNA from Thp1 cells treated with TNF and CHX. Two primer extension products were observed; one 54 bases in length and another 33 bases. For S1 nuclease

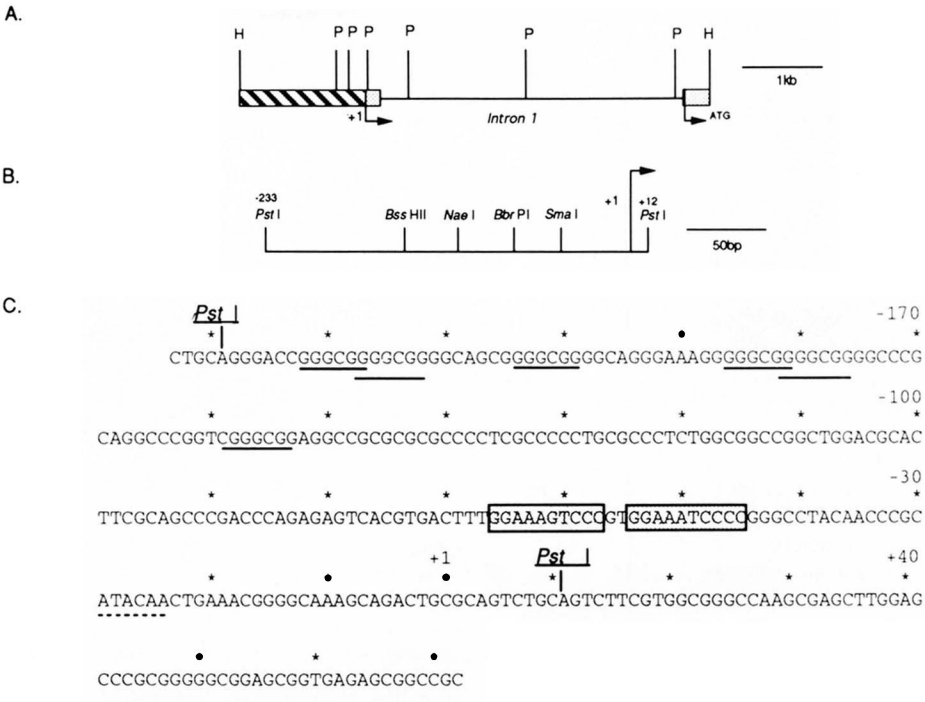

FIG. 1. **Restriction map and sequence of the A20 5′-flanking region.** *A*, a map of the 6-kb genomic *Hin*dIII fragment showing the position of the *Pst*I sites used for subcloning and subsequent DNA sequence analysis. The start of transcription is designated +1. *Hatched boxes* represent sequences upstream of +1, and *stippled boxes* represent exon sequences. A 3.9-kb intron is shown as a line just upstream from the ATG initiator methionine. *B*, a map of the *Pst*I fragment extending from −233 to +12 and containing the A20 transcription start site indicated by an *arrow* at +1. The restriction enzyme sites *Bss*HII (−144), *Nae*I (−111), *Bbr*PI (−76), and *Sma*I (−44) were used to generate a series of deletions used in Fig. 3. *C*, sequence of the A20 5′-flanking region extending from −233 to +72 and containing the 245-bp *Pst*I fragment used in the promoter studies. The six Sp1 binding sites are *underlined* with *solid lines*, a putative TATA element is *underlined* with *dashes*, and the two κB sites appear in *boxes*.

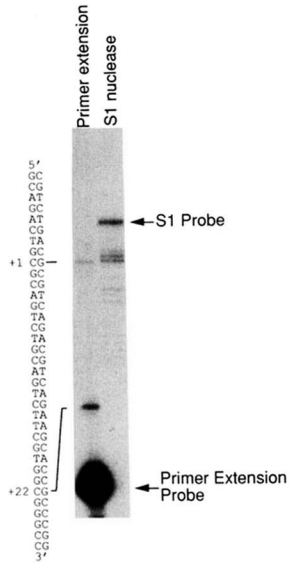

FIG. 2. **Primer extension and S1 nuclease mapping of the A20 transcription start site.** For primer extension analysis a synthetic oligonucleotide corresponding to bases +54 to +30 was end-labeled with [γ-^{32}P]ATP, annealed to total RNA from Thp 1 cells treated with TNF plus CHX, and extended with reverse transcriptase as described under "Experimental Procedures." Two products, 54 and 33 bases in length, were observed and designated +1 and +22, respectively. The transcription start site at +1 was confirmed by S1 nuclease mapping. An antisense 62-base oligonucleotide probe with the same 5′ end as the primer extension probe and extending to +8 was end-labeled and annealed to total RNA followed by digestion of unprotected probe with S1 nuclease. A 54-base protected product corresponding to the larger primer extension product at +1 was observed. The sequence corresponding to the primer extension and S1 nuclease protection products is shown on the *left*.

mapping, a 62-base end-labeled oligonucleotide extending from +54 to −8 was annealed to Thp1 RNA. Following S1 nuclease digestion of single-stranded DNA, three cleavage products of 54, 55, and 56 nucleotides were observed. Since the 54-base fragment was produced by both S1 nuclease and primer extension mapping, the start of transcription was assigned to a site 54 bases from the 5′ end of the probes used and designated +1 (Fig. 2). Repeated S1 cleavage reactions failed to confirm the presence of the smaller product observed in the primer extension reactions. The 56- and 55-base S1 products probably arose from incomplete S1 digestion near the hybrid junction since no corresponding primer extension products were observed. With these results, we mapped the start of transcription to a site 300 bp upstream of the initiator methionine in the cDNA sequence.

Sequence of the A20 5′-Flanking Region—The 305-nucleotide sequence shown in Fig. 1*C* encompasses 233 bp upstream of the transcription initiation site and 72 bp of the first exon. Inspection of the A20 upstream sequences showed an 88% GC-rich region that extends from −108 to −218 and contains six consensus Sp1 binding sites. Overall, the upstream sequences have a 76% GC content and probably are part of a methylation-free CpG island evident in most expressed genes (24–26). Consensus CCAAT and TATA box sequences are absent from the A20 upstream region, however, the sequence ATACAA at −29 resembles a TATA element, although it is not clear whether it can function as one. Two tandem κB elements, each in an inverted orientation, extend from −45 to −54 (5′GGAAATCCCC3′) and from −57 to −66 (5′GGAAAGTCCC3′). The 5′-most 10-bp κB element is identical to the canonical κB core sequence first identified in the immunoglobulin κ light chain enhancer as the recognition sequence for the transcription factor, NF-κB (27). The proximal 10-bp κB sequence lacks a G at position 6, however, this sequence falls within the variability observed in survey of functional κB elements (reviewed in Refs. 28 and 29).

TNF Induction of the A20 Promoter Is Mediated through κB Elements—TNF-mediated induction of A20 was originally described in human umbilical vein endothelial cells (1), a primary cell strain which is not transfectable. To identify a suitable cell line for promoter studies, we undertook a survey of transfectable cell lines. Jurkat T-cells, a transfectable leukemic line which displayed substantial induction of both A20 protein and transcript in response to TNF,[3] was chosen for subsequent promoter studies. Putative A20 promoter se-

[3] C. D. Laherty and V. M. Dixit, unpublished observations.

quences linked upstream of the CAT gene were transfected into Jurkat cells. Thirty h post-transfection the cells were split into two groups; one was treated with TNF (200 units/ml) for 12 h, and the other was left untreated. Transfection of a 245-bp *Pst*I fragment extending from −233 to +12 cloned upstream of the CAT reporter gene (−233/+12) resulted in a 24-fold induction of CAT activity by TNF (Fig. 3). No significant difference in TNF-induced CAT activity was observed with an A20 insert extending 5′ to the *Hin*dIII site at −1.4 kb and 3′ to +70 (data not shown), therefore, subsequent constructions were derived from −233/+12 A20CAT. A series of 5′-nested deletions linked upstream of the CAT gene was constructed, and the results of transfections using these constructs are shown in Fig. 3. Deletion of the six Sp1 consensus binding sites clustered between −220 and −150 (plasmid −144/+12) did not affect the extent of induction by TNF. Further deletions that removed 5′ sequences up to −74 (plasmids −111/+12 and −74/+12) also did not alter inducibility by TNF. However, induction of CAT activity was eliminated with the deletion plasmid −44/+12 A20CAT suggesting that critical elements responsible for induction by TNF must reside between −74 and −44. In addition, a deletion from −74 to −45 (Δ-74/-45) prevented induction of CAT activity by TNF (Fig. 3). It appeared likely that the κB sites which were deleted in both promoter constructs not displaying TNF inducibility were critical for induction. To confirm this, point mutations were introduced to alter both κB sites in the parental −233/+12 A20CAT plasmid (Fig. 4). Changes were chosen that abolish binding by NF-κB (16, 28). As expected, the mutant plasmid, dm A20CAT, was not inducible by TNF (Fig. 4), supporting the hypothesis that induction of the A20 promoter by TNF was mediated through the κB sequences.

TNF Activates a Factor That Binds to the A20-kb Sites—DNA electrophoretic mobility shift assays demonstrated binding of a factor from TNF-treated cells to the A20 κB sites. An end-labeled 43-bp double-stranded oligonucleotide probe containing the two A20 κB sequences was incubated with nuclear extract from either TNF-treated or untreated Jurkat T-cells. Both extracts produced a protein-DNA complex that appeared as an electrophoretically retarded band in Fig. 5 (*lanes 1* and *2*). However, only extract from TNF-treated cells produced, in addition, a TNF-inducible protein-DNA complex indicated by the *arrow* (*lane 2*). This TNF-inducible complex was

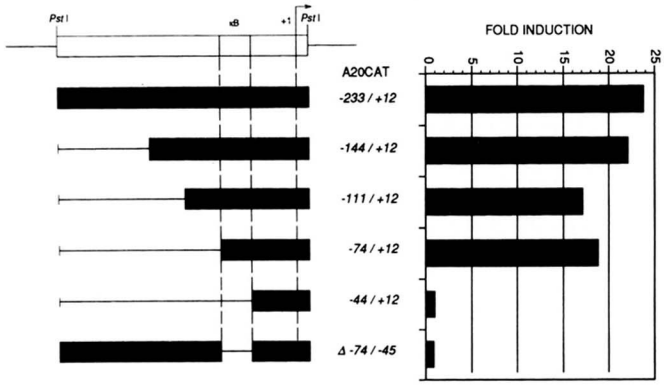

FIG. 3. **A20 promoter-CAT constructs and induction by TNF.** Six A20 promoter-CAT constructs comprising the parental −233/+12 A20CAT plasmid and five deletion derivatives are shown diagrammatically in the *left panel*. Also shown is a diagram aligning the start of transcription (+1) and the κB sites with the *outlined constructs, below*. -Fold induction by TNF is shown as a *bar graph* to the *right* of each construct and was calculated as a ratio of CAT activity from TNF-stimulated cells to that from unstimulated cells and is the average from at least four separate transfections, done in duplicate.

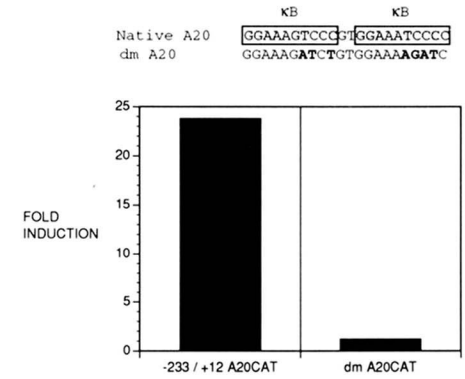

FIG. 4. **Construction of the A20 κB double mutant plasmid, dm A20CAT, and induction by TNF.** The native A20 κB sequences in the −233/+12 A20CAT plasmid shown at the *top* of the figure (*boxed* sequences) were altered by site-directed mutagenesis to yield the plasmid dm A20CAT. Altered bases are indicated in *bold print*. The *bar graph* compares the -fold induction from native A20CAT and dm A20CAT by TNF and is expressed as the ratio of CAT activity from cells stimulated with TNF to that from unstimulated cells. All experiments were done at least seven times, in duplicate.

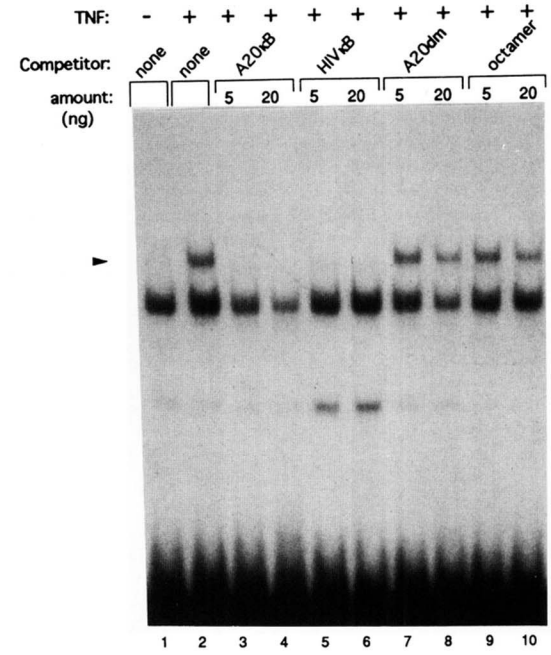

FIG. 5. **Electrophoretic mobility shift assays.** A ^{32}P end-labeled A20 κB double-stranded probe extending from −76 to −45 was incubated with nuclear extract from TNF treated (+) or untreated (−) Jurkat T-cells in the absence or presence of the indicated competitors. The specific TNF-inducible DNA-protein complex is indicated by the *arrow*. 0.1 ng of labeled probe was used, while the competitors were added at 5 or 20 ng corresponding to a 50- or 200-fold molar excess for the A20 κB, dmA20, and octamer sequences and 70- and 280-fold molar excess for the HIV-1 κB sequence.

sequence-specific as demonstrated first, by the ability of a 50-fold molar excess of unlabeled A20 probe to compete for binding of the TNF-activated factor to the labeled A20 probe (*lanes 3* and *4*), and second, by the inability of the unrelated IL-2 octamer to compete (*lanes 9* and *10*). To confirm that the A20 κB elements were involved in the formation of the TNF-inducible complex, unlabeled oligonucleotide containing mutations in the κB sequence was used as competitor. The mutated oligonucleotide was unable to compete even at 200-fold molar excess, and the gel-shift pattern was identical to that seen for reactions with no competitor (*lanes 7* and *8*). To

examine whether the sequence similarities between the HIV-1 κB motif and the A20 elements were functionally significant, cross-competition experiments were performed using a 29-bp HIV-1 LTR oligonucleotide that contained two κB sites which are identical to the core NF-κB binding site in the immunoglobulin κ light chain enhancer (16, 27) and have been shown to interact with NF-κB (16). As shown in Fig. 5, an excess of HIV-1 κB sequences was as effective a competitor as the native A20 sequence. Taken together, these results indicate that proteins interacting with A20 κB sequences are also capable of binding the HIV-1 κB motif.

A20 Negatively Regulates Its Own Expression—Previous work has shown that the A20 transcript is rapidly but transiently expressed following TNF treatment of human umbilical vein endothelial cells (1). This transient induction may, at least in part, be due to feedback inhibition with newly synthesized A20 inhibiting its own promoter. Since such feedback inhibition has been reported for c-*fos*, (30, 31) a primary response gene, we decided to directly test this possibility in cotransfection experiments. The A20 expression plasmid, pSFFV-A20, which directs the constitutive expression of A20 or the vector control (pSFFV-neo), was cotransfected with an A20 promoter CAT construct (−233/+12) followed by treatment with TNF (200 units/ml) for 12 h. TNF activation of the A20 promoter was reduced 11-fold in cotransfections with pSFFV-A20 compared to vector alone (Fig. 6) consistent with A20 exerting an inhibitory influence on its own promoter. To ask whether A20 was a general inhibitor of transcription the promoter from the RSV LTR which is not NF-κB-responsive was cotransfected with either the A20 expression plasmid or the vector control. CAT activity from the RSV promoter was neither induced by TNF nor altered in cotransfections with pSFFV-A20 (data not shown). In aggregate, these results suggest that A20 either directly or indirectly acts as a negative regulator of its own expression.

DISCUSSION

A20 was first identified as a TNF primary response transcript whose induction occurs in the absence of intervening protein synthesis (1). To understand the mechanism for its immediate induction, the promoter region of the A20 gene was cloned and analyzed in transfection studies. Two κB elements that reside between −66 and −45 were shown to be essential for TNF-regulated expression of A20 since deletion and site-directed mutagenesis of the κB elements in constructs using the CAT gene as a reporter resulted in elimination of induction by TNF (Figs. 3 and 4).

Historically, the κB element was first described as one of

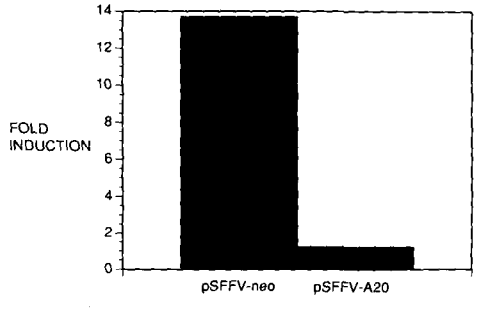

FIG. 6. **A20 negatively regulates its own promoter.** The *bar graph* compares the -fold induction by TNF of the −233/+12 A20CAT plasmid when it is cotransfected with either the vector (pSFFV-neo) or with an A20 expressing plasmid (pSFFV-A20). Plasmids were cotransfected in approximately equimolar amounts of A20CAT and pSFFV-A20. Values represent the mean of seven separate transfections, done in duplicate.

at least four protein binding sites in the κ light chain enhancer and is the recognition sequence for the transcription factor, NF-κB (27). Activation of NF-κB involves its dissociation from a cytosolic inhibitor (I-κB) and its subsequent translocation to the nucleus (reviewed in Ref. 29). Since NF-κB is activated *post-translationally* by a variety of cellular signals including TNF (22, 32, 33), it serves as a rapid conduit for TNF signals to the transcriptional apparatus.

A survey of NF-κB-responsive genes shows a preponderance of genes whose protein products are central to the immune and inflammatory responses; many of these are also TNF-responsive including the genes of the class I major histocompatibility complex (34), the cytokines IL-6 (35, 36), IL-8 (37), and *gro* (38), and the IL-2 α chain receptor (32, 39).

In addition to NF-κB, TNF induces the expression of three known transcription factors: interferon regulatory factor 1 which binds to a hexameric sequence present in the α and β interferon enhancers (40), NF-GMa which binds to the cytokine 1 element present in the regulatory region of many hemopoietic growth factors (41), and AP-1 (2, 42) shown to bind the 12-*O*-tetradecanoylphorbol-13-acetate-responsive element of several genes (43). However, it is important to note that these factors need to be transcriptionally activated. This is in distinct contrast to NF-κB, which is activated post-translationally.

Evidence for the involvement of NF-κB in TNF-mediated induction of A20 came not only from deletional and mutagenic analyses of A20 promoter-CAT constructs (Figs. 3 and 4) but also from electrophoretic mobility shift assays which showed that TNF activated a factor that bound to the A20 κB elements (Fig. 5). Identification of this factor as NF-κB was supported by the finding that oligonucleotides containing the HIV-1 κB sequences effectively competed the binding of A20 κB sequences to nuclear extracts prepared from TNF-treated cells. In contrast, both the unrelated IL-2 octamer sequence and the dm A20 κB sequence, in which invariant nucleotides within the consensus NF-κB binding site were altered, failed to compete.

These findings strongly suggest that one of the outcomes of TNF mediated NF-κB activation is induction of A20, a zinc finger protein that has been shown recently to inhibit TNF-induced cytotoxicity.[2] Transfection of TNF-sensitive cell lines with an A20-expressing plasmid resulted in the development of a remarkable resistance to TNF killing. Additionally, the constitutive expression of A20 in TNF-resistant but not in TNF-sensitive breast carcinoma cell lines further underscores the important role that A20 plays in mediating resistance to TNF cytotoxicity. It is known that pretreatment of TNF-sensitive cell lines with a sublethal dose of TNF renders such lines resistant to subsequent TNF challenge (44, 45). It is conceivable that pretreatment results in the rapid activation of NF-κB and subsequent induction of A20 leading to a state of resistance. Such a state would be beneficial for bystander host cells during inflammation to provide protection from TNF and the related molecule, lymphotoxin, released by inflammatory cells.

Given that A20 contains zinc fingers which are a distinctive feature of several known site-specific DNA binding proteins that function as transcription factors (reviewed in Ref. 46), it is possible that A20 controls expression of genes further downstream in the TNF signalling cascade. One potential target for A20 control is its own promoter, which would be consistent with A20 transcript being *transiently* induced following TNF stimulation (1). Feedback inhibition has been demonstrated for other transcription factors including the c-Fos component of the dimeric transcription factor AP1 (30,

31). In transfection experiments (Fig. 6), an A20-expressing plasmid markedly inhibited TNF inducibility of a cotransfected A20 promoter-CAT construct but did not alter activity from a RSV promoter-CAT construct, suggesting that inhibition was specific and not simply due to a general inhibition of transcription.

At present it is unclear whether this feedback inhibition is occurring directly, with A20 binding to its own promoter, or indirectly, with A20 modulating the activity of intermediary proteins which in turn shut off the promoter. This question is the present focus of our investigations.

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