Tumor Necrosis Factor α Induces the Adenovirus Early 3 Promoter by Activation of NF-κB*

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The early transcription unit 3 (E3) of human adenoviruses encodes proteins which appear to subvert host defense mechanisms. For example, the E3/19K protein inhibits the transport of major histocompatibility complex (MHC) class I molecules to the cell surface and thereby prevents cell lysis by cytotoxic T cells. Tumor necrosis factor α (TNF) stimulates expression of MHC molecules on the cell surface of normal cells but not of E3+ cells, rather, a further reduction of MHC expression is evident. This was attributed to the increased expression of E3/19K upon TNF treatment, an effect also observed for other E3 proteins. We investigated the mechanism of the TNF-mediated up-regulation of E3 products. We show that TNF stimulates expression of a luciferase reporter gene driven by the E3 promoter. Mutation of individual transcription factor binding sites within the E3 promoter reveals the importance of the NF-κB binding site κ2 for TNF inducibility. Electrophoretic mobility shift assays using antibodies directed against various members of the NF-κB family demonstrate that stimulation by TNF is mediated by the p50/p65 NF-κB complex. TNF inducibility does not depend on coexpression of E1A and can be observed during infection. Interestingly, the E3 promoter seems to be the only early promoter responsive to TNF and the only adenovirus promoter containing an NF-κB site. The implications of this regulatory mechanism for the adenovirus life cycle and its pathogenesis are discussed.

Human adenoviruses (Ads) cause acute infections of the respiratory and gastrointestinal tract as well as infections of the eye (1, 2). A proportion of patients develop persistent infections which can last for months or years (2, 3). A key role for regulating the interaction of the virus with its host and perhaps for viral persistence has been attributed to proteins encoded in the non-essential early transcription unit 3 (E3) of the virus (4–6). Several E3 proteins, 14.7, 14.5, and 10.4, can protect cells from TNF-mediated lysis (5), while the most abundant protein E3/19K down-regulates expression of host class I major histocompatibility complex (MHC) antigens on the cell surface (7, 8). This results in a profound reduction of cytotoxic T cell mediated lysis of virus-infected cells (6, 9–12). Both mechanisms may prolong survival of infected cells in vivo, allowing efficient virus reproduction in the face of an ongoing immune response. In support of this hypothesis, an Ad5 mutant with an E3/19K deletion shows a much stronger immunopathology in vivo than wild type viruses (13). Furthermore, E3 transgene products expressed in transplanted pancreas tissue seem to prevent tissue rejection, leading to prolonged survival of the allogeneic transplant (14).

Efficient transcription of the E3 region as well as of the other early regions, E1B, E2, and E4, is dependent on expression of the immediate early gene product E1A which has a variety of functions (15, 16). 130 base pairs immediately upstream of the E3 transcription start site are sufficient for both basal promoter activity and E1A induction of the E3 promoter in fibroblasts (17, 18). DNase I footprinting analysis and electrophoretic mobility shift assays (EMSA) revealed four protein binding sites within the E3 promoter, namely those for NF-1, p50, p52, and DNA. Various stimuli lead to the phosphorylation and degradation of IκB. This allows the nuclear translocation of an active heterodimer which can bind DNA and activate transcription (for reviews, see Refs. 25 and 26). The heterodimer is composed of a p50-like and a p65-like subunit. Two p50 homologues, p50 and p52, are distinguished whereas the second subunit can either be p65 (also called RelA), RelB, or c-Rel.

We recently investigated the effect of TNF on cell surface expression of MHC molecules in E3+ fibroblasts. Unlike the stimulation of MHC expression in E3+ cells, TNF treatment further reduced MHC expression on the cell surface of E3+ cells. Concomitantly, an increased expression of E3/19K (27) as well as other E3 proteins was noted (28). This suggested that TNF stimulates E3/19K expression and thereby reduces MHC antigen levels on the cell surface. TNF is an inflammatory cytokine, produced primarily by macrophages but also by T cells (for reviews, see Refs. 29–31). It exhibits several antiviral properties. High doses of TNF can inhibit replication of RNA and DNA viruses and many virus-infected cells become sensi-

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† The abbreviations used are: Ad, adenovirus; AP-1, activator protein 1; ATF, activating transcription factor; CAT, chloramphenicol acetyltransferase; E3, early region 3; EMSA, electrophoretic mobility shift assays; IL-1, interleukin 1; CMV, cytomegalovirus; MHC, major histocompatibility complex; NF-1, nuclear factor 1; NF-κB, nuclear factor κB; TNF, tumor necrosis factor α.
tive to TNF-mediated lysis (32, 33). In addition, TNF activates neutrophils and stimulates the killing activity of macrophages and NK cells, both of which are involved in the early antiviral response of the innate immune system (29). Moreover, TNF also supports the adaptive immune system by up-regulating class I major histocompatibility (MHC) antigens, which in turn increases the efficiency of killing by cytotoxic T cells (34). Interestingly, Ad appears to induce TNF, IL-1, and IL-6 in the infected tissue in mice. Thus, the observed stimulation of E3 expression by TNF may be a feedback mechanism allowing efficient expression of immunosuppressive E3 proteins which might be beneficial for survival of the virus in its host. Consistent with this interpretation, there is good correlation between high levels of TNF in the blood and severity of Ad-induced disease in humans (35).

We have now investigated the mechanism by which TNF stimulates E3 expression. We found that TNF activates the E3 promoter by utilizing the cytosolic transcription factor NF-κB. Two NF-κB family members, p50/relA, strongly bind to the κ2 site within the E3 promoter and therefore seem to mediate this effect. Interestingly, E3 appears to be the only early promoter sensitive to the presence of TNF. The implications of this response mechanism for the natural infection are discussed.

MATERIALS AND METHODS

Plasmid Constructs—The EcoRV C fragment, subcloned in pBlue-script II KS+ (Stratagene, Heidelberg, Germany) encompasses the entire E3 region of Ad2 including 273 base pairs upstream of the E3 transcription start site (27). The E3 promoter, contained within a 360-bp pair long SacI fragment, was cut out of the subcloned EcoRV C fragment utilizing the SacI site of the vector and the E3 SacI site at nucleotide +29 and inserted into the SacI site in front of the luciferase reporter gene of the "pG52 basic" vector (Promega, Heidelberg, Germany). The orientation of the inserted fragment was determined by sequencing using the Sequenase kit (U. S. Biochemicals, Bad Homburg, Germany). Polymerase chain reaction-mediated site-directed mutagenesis (36) of the E3 promoter using vector and mutant primers listed in Table I was carried out as described previously (37). Mutagenized double-stranded fragments were cleaved with SacI and inserted into pG52 basic. Orientation and correct sequence of the resulting mutant E3 promoter constructs were confirmed by sequencing.

Cell Lines, Transient Transfections, and Enzymatic Assays—293 and HeLa cells were routinely grown in Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum, 2 mM glutamine and antibiotics. Transient transfections were performed by the calcium-phosphate precipitation method according to a standard protocol (38). Briefly, a 6-cm dish of cells was transfected with 5 μg of DNA of each luciferase construct using 200 μl of calcium-phosphate-DNA precipitate was added to a 6-cm dish of cells at 25% confluency. The luciferase activity measured was further increased by a factor of 3.3 (see top of Fig. 1). This level of induction is very similar to the increase of E3/19K protein synthesis that we reported previously (27). Therefore, it seems that the TNF-mediated up-regulation of E3 expression may be entirely due to activation of transcription driven by the E3 promoter.

The Nuclear Factor κB Elements of the E3 Promoter Play a Key Role in the TNF-mediated Induction of the E3 Promoter—Previous analysis of the E3 promoter led to the identification of transcription factor binding sites for AP-1, ATF, and NF-1. Two additional sites showing homology to the NF-κB consensus binding site were specifically occupied in lymphoid cells (18–23, 42) (see schematic representation on Fig. 1, left). To examine which cis-acting element of the E3 promoter is required for its TNF inducibility, each transcription factor binding site was separately mutated by substitution of 3–6 nucleotides (for details, see Table I). The inability of the mutated sites to bind the corresponding nuclear factor was confirmed by EMSA (see below, Fig. 2A). Mutated E3 promoters were inserted in front of the luciferase gene and the resulting constructs were transfected into 293 cells. The luciferase activity was determined in the presence and absence of TNF (Fig. 1). Luciferase activity obtained with the wild type promoter construct without TNF treatment was set to 1. For each construct, the ratio between

MgCl₂ containing the same concentration of protease inhibitors as above. Bovine caseins were extracted by adding 75 μl of NBE 0.7 (700 μM NaCl, 20 mM Hepes, pH 7.9, 25% glycerol, 1.5 mM MgCl₂ containing 0.5 M sucrose, 15 mM Tris, pH 7.5, 60 mM KCl, 0.25 mM dithiothreitol, and a mixture of protease inhibitors: 0.5 mM phenylmethylsulfonyl fluoride, 2 mM benzamidine, 5 μg/ml aprotinin, 5 μg/ml pepstatin, 5 μg/ml leupeptin), the nuclei were pelleted by centrifugation at 600 × g for 5 min at 4 °C. After resuspending the nuclei in 105 μl of NBE 0.14 (140 mM NaCl, 20 mM Hepes, pH 7.9, 25% glycerol, 1.5 mM
luciferase activities with and without TNF treatment is indicated on the right. As expected from previous studies (18–22, 42), both AP-1 and ATF mutations have a drastic effect on the basal level and/or E1A driven expression of the reporter gene, while TNF stimulation appears only weakly affected. Mutation of the NF-κB binding site alters neither the TNF induction potential of the promoter, nor its basal activity. A more pronounced reduction of the TNF-mediated induction is exhibited by mutating the potential binding sites for NF-κB, x1, and x2. In particular, the mutation of x2 reduces inducibility by 90%. A double mutation of x2 and x1 abolishes TNF induction completely. Thus, it seems that the NF-κB binding sites of the E3 promoter are primarily responsible for the induction of the E3 promoter by TNF. Apparently the x2 element plays the most critical role in this phenomenon. It is intriguing that the x2 site is strongly conserved among the known Ad E3 promoter sequences while x1 is not (Table I). Moreover, all x2 elements align well with the NF-κB consensus binding site (24). In contrast, the x1 sites generally do not fit the NF-κB consensus except for x1 of Ad2 and Ad5 which contains only one mismatch to the NF-κB consensus (Table II).

NF-κB Proteins Bind to the κ Elements of the E3 Promoter after TNF Treatment of Fibroblasts—To analyze which proteins bind to the E3 promoter in the absence and presence of TNF, EMSA were performed. Nuclear extracts of non-induced and TNF-induced 293 cells were prepared and tested with the probes indicated below the autoradiographs in Fig. 2A. The specificity of the binding reactions was confirmed by competition with a 200 μ excess of unlabeled wild type and mutant oligonucleotides. The mutant double-stranded oligonucleotides were identical to the primers used for generation of the E3 promoter constructs tested above (Fig. 1). While the wild type oligonucleotides efficiently competed with binding of the probes (Fig. 2A, lanes 4, 9, 14, and 19), the mutant oligonucleotides were unable to do so (Fig. 2A, lanes 5, 10, 15, and 20). This demonstrated that the introduced mutations disrupt the binding to the corresponding nuclear factors.

As can be seen, NF-1, AP-1, and ATF bind to the E3 promoter in the absence of TNF (Fig. 2A, lanes 2, 12, and 17). In agreement with the results of the mutational analysis shown in Fig. 1, induction with TNF has no effect on proteins binding to the NF-1 and ATF probes (Fig. 2A, lanes 3 and 18), whereas a slight increase is observed with the AP-1 probe (1.6-fold; Fig. 2A, lane 13). In contrast, a protein complex with x2 is only formed when extracts from TNF-treated 293 cells are used (Fig. 2A, compare lanes 7 and 8). A complex of similar size but in much lower amounts can be detected using the κ1 probe after TNF induction (data not shown). We therefore compared the affinities of κ1 and κ2 for the TNF-induced complex in competition assays using radiolabeled x2 (Fig. 2B). A 10 times higher concentration of cold κ1 is necessary to give an equivalent competition to κ2 (Fig. 2B, compare lanes 6 and 7). This showed that the affinity of the bound protein(s) for x2 is much greater than for κ1.

The question arises which members of the NF-κB family of transcription factors bind to the NF-κB elements within the E3 promoter. Therefore, supershift experiments were performed using antibodies directed against individual NF-κB subunits (Fig. 3). Antibodies against p50 and p65 decrease the migration of the complex whereas antibodies against RelB, c-Rel, and p52 (data not shown) seem to have no effect. Thus, we conclude that the TNF-activated transcription factor binding to the x2 and κ1 elements of the E3 promoter in 293 cells is composed of the

**TABLE I**

| Oligonucleotide | Oligonucleotide sequence |
|-----------------|-------------------------|
| E3 x2 WT sense  | TGTACCAGGAAAGTCGCCGCTCCC |
| E3 x2 antisense | TGGTGGGAGCCGGGATTCCCTGGT |
| E3 x1 WT sense  | ACCACTGTGGTACTTCCAAGGG |
| E3 x1 antisense | CCGAAGGTTCGAGGAGCTC |
| E3 AP-1 WT sense| CCGAAGTTCCAGATGATCC |
| E3 AP-1 antisense| CTTGAGTTAGTCTGAGG |
| E3 AP-1 WT antisense| CCTGAGTTAGTCTGAGG |
| E3 AP-1 antisense| CCGAAGGTTCGAGGAGCTC |
| E3 AP-1 WT antisense| CCGAAGTTCCAGATGATCC |
| E3 AP-1 WT antisense| CCGAAGGTTCGAGGAGCTC |
| E3 AP-1 WT antisense| CCGAAGTTCCAGATGATCC |
| E3 AP-1 WT antisense| CCGAAGGTTCGAGGAGCTC |
| E3 AP-1 WT antisense| CCGAAGTTCCAGATGATCC |
| E3 AP-1 WT antisense| CCGAAGGTTCGAGGAGCTC |
| E3 AP-1 WT antisense| CCGAAGTTCCAGATGATCC |
| E3 AP-1 WT antisense| CCGAAGGTTCGAGGAGCTC |
| E3 AP-1 WT antisense| CCGAAGTTCCAGATGATCC |
| E3 AP-1 WT antisense| CCGAAGGTTCGAGGAGCTC |
| E3 AP-1 WT antisense| CCGAAGTTCCAGATGATCC |
| E3 AP-1 WT antisense| CCGAAGGTTCGAGGAGCTC |
| E3 AP-1 WT antisense| CCGAAGTTCCAGATGATCC |
| E3 AP-1 WT antisense| CCGAAGGTTCGAGGAGCTC |
| E3 AP-1 WT antisense| CCGAAGTTCCAGATGATCC |
| E3 AP-1 WT antisense| CCGAAGGTTCGAGGAGCTC |
| E3 AP-1 WT antisense| CCGAAGTTCCAGATGATCC |
| E3 AP-1 WT antisense| CCGAAGGTTCGAGGAGCTC |
| E3 AP-1 WT antisense| CCGAAGTTCCAGATGATCC |
| E3 AP-1 WT antisense| CCGAAGGTTCGAGGAGCTC |
| E3 AP-1 WT antisense| CCGAAGTTCCAGATGATCC |
| E3 AP-1 WT antisense| CCGAAGGTTCGAGGAGCTC |
| E3 AP-1 WT antisense| CCGAAGTTCCAGATGATCC |
| E3 AP-1 WT antisense| CCGAAGGTTCGAGGAGCTC |

a Substitutions in the mutant oligonucleotides are underlined.
NF-κB proteins p50 and p65 (RelA).

Co-transfection of the NF-κB Subunits p65 or IκB Alters TNF Induction of the E3 Promoter—To corroborate our conclusion that TNF induction of the E3 promoter is mediated by NF-κB, we sought to overexpress the activating subunit of NF-κB, p65. Overexpression of p65 would be expected to activate gene expression driven by the E3 promoter, if this were NF-κB dependent. Therefore, pE3Luc was transfected with a vector expressing p65 under control of the cytomegalovirus promoter (pCMVp65, a kind gift from P. Baeuerle). As expected, co-transfection of pCMVp65 strongly activates expression of the pE3Luc construct. Furthermore, TNF treatment does not lead to a further increase of the measured luciferase activity (Fig. 4). The opposite effect is observed when the inhibitory subunit IκB is overexpressed by co-transfection of pCMVIκB (a kind gift from P. Baeuerle). Overexpressed IκB abolishes TNF inducibility of the E3 promoter (Fig. 4). These two findings strongly confirm the involvement of NF-κB in the induction of the E3 promoter by TNF.

TNF Does Not Activate the E2 and E4 Promoter—Considering the function of E3 proteins, which seem to counteract functions of the host immune system, it is interesting to know whether TNF inducibility is a specific feature of the E3 promoter. To answer this question, we first tried to find other NF-κB binding sites in the Ad2 or Ad5 genome by searching with the NF-κB consensus sequence. Apart from those in the E3 promoter, no other Ad promoter contained an NF-κB site. Second, we tested the effect of TNF on two other early promoters, namely E2 and E4. E2CAT, E3CAT, and E4CAT plasmids (43; kindly provided by M. B. Mathews) were transfected into 293 cells and the CAT activity was measured in TNF-treated and untreated cells. As shown in Fig. 5, only the E3 promoter is up-regulated upon TNF treatment. These results, together with our previous data indicating that E1A is also not up-regulated by TNF (27), provide strong evidence that within the Ad genome TNF-inducibility is a special feature of the E3 promoter.

TNF Up-regulation of E3 in the Absence of E1A—E1A proteins play a key role for the efficient expression of the Ad early genes (for reviews, see Refs. 15, 16, and 44). All the above described experiments were performed with 293 cells, a human embryonic kidney cell line transformed with the E1 region of Ad5 (41). These cells constitutively express E1A proteins which activate the E3 promoter. To test whether TNF induction is dependent on the presence of E1A, we performed transient transfection assays with pE3Luc in HeLa cells with and without co-transfection of a plasmid carrying the E1A region (Fig. 6). As expected, the activity of the E3 promoter in the absence of E1A is much lower than in 293 cells (30-fold lower, data not shown). Coexpression of E1A increases its activity by a factor of 7. In the presence of TNF, the activity of the E3 promoter is up-regulated by a factor of ~3, irrespective of the presence of E1A. Furthermore, the double mutant construct κ2mut1 and κ2 double stranded oligonucleotides as indicated. The arrowhead marks the position of the NF-κB complex.

Fig. 2. Nuclear proteins binding to the E3 promoter in untreated or TNF-treated 293 cells. A, electrophoretic mobility shift assays with the different cis-acting elements of the E3 promoter. The different probes indicated below the autoradiograph were run without or with prior incubation with nuclear extracts from non-induced and TNF-induced 293 cells. The specificity of binding was tested with the cold wild type (WT) and mutant probe (mut) as indicated on top of the figure. B, competition of κ2 binding with increasing amounts of κ1 and κ2 double stranded oligonucleotides as indicated. The arrowhead marks the position of the NF-κB complex.
TNF-responsive Element in the Adenovirus E3 Promoter

Potential binding sites for NF-κB within the E3 promoters of different adenovirus subtypes

| Subgroup | Ad subtype | κ1 (antisense) | κ2 (antisense) |
|----------|------------|----------------|----------------|
| A        | Ad12       | aGGcA Aaaagt   | GGAT TTTCC     |
| B        | Ad35       | aGGAA GTaat    | GGAC TTCC      |
| C        | Ad2 and 5  | GGGAT GtaCC    | GGGG TTCC      |
| D        | Ad19       | cGGGA GTagt    | GGGGA TTCC     |
| E        | Ad40       | tGGcA Gagg     | GGGT TTCC      |
| F        | Ad41       | tGGcG Agaga    | GGGT TTCC      |

* Nucleotides not aligning with the consensus binding site for NF-κB (GGGRNYYCC (24)) are written in lower case. The sequences for Ad12, Ad35, Ad2, Ad5, Ad19, Ad40, and Ad41 have been published (52–58).

FIG. 3. Composition of the NF-κB complex binding to the E3 promoter in TNF-stimulated 293 cells. Antibodies specific for NF-κB subunits were preincubated with nuclear extracts of TNF-treated 293 cells for 1 h before adding the κ2 radioactive probe. Controls include incubation with preimmune serum (PL lane 4), no serum added (lanes 1–3), no TNF treatment (lanes 1 and 2), and no protein added (lane 1).

~3 for 14 h and incubated with or without TNF. Then, the most abundant E3 protein, E3/19K, was immunoprecipitated and analyzed by SDS-polyacrylamide gel electrophoresis (Fig. 7). In accordance with the observed 3-fold higher E3 promoter activity, TNF treatment increases the expression of E3/19K in Ad2 and Ad5 infected cells by a factor of 3 (average value from five independent experiments, see also Ref. 27). The induction is much higher (6–20-fold in three different experiments) in cells infected with the E1A deletion mutant dl312. The level of E3/19K expression can reach that of Ad2 and Ad5 (+E1A; Fig. 7, compare lane 6 with lanes 1 and 3). Therefore, during infection, TNF can substitute for the E1A-mediated induction of the E3 promoter.

DISCUSSION

We previously reported that TNF amplifies the effects of the adenovirus E3/19K protein by increasing its expression at the protein and mRNA level (27). The subsequent observation that all E3 proteins tested seem to be up-regulated, suggested that TNF might activate the E3 promoter (28). Using E3 luciferase reporter constructs, we demonstrate that this is indeed the case (Fig. 1). By mutating individual transcription factor binding sites within the E3 promoter, it was shown that neither the NF-1 nor the ATF site contributed to TNF-mediated induction of the E3 promoter. Most critical for this effect was one of the two potential NF-κB binding sites, termed κ2. Mutating this site resulted in a 90% reduction of TNF inducibility, whereas mutations in κ1 reduced the TNF induction only by 36% (Fig. 1). The reason for this differential influence appears to be a 10-fold lower affinity of the κ1 site for the NF-κB complex (Fig. 2B). Nevertheless, κ1 contributes to the TNF-mediated induction of the E3 promoter since mutation of both κ1 and κ2 completely abrogated the ability of the promoter to respond to TNF. The same construct also showed a drastic reduction of constitutive promoter activity indicating that this portion of the promoter influences basal and/or E1A mediated activity. At present, we do not know whether additional factors bind to the NF-κB site in the uninduced state or the low amount of NF-κB detected after long exposure contributes to basal transcription. Confirming the importance of NF-κB, overexpression of an inhibitor of NF-κB, IκB, essentially eliminated the stimulatory capacity of the E3 promoter for TNF (Fig. 4).

An even greater reduction of basal promoter activity was observed with the promoter mutated at the AP-1 site. This can be accounted for by the loss of E1A-mediated stimulation of E3 promoter activity (18, 21, 46). However, inducibility by TNF was also reduced by 30%. Consistent with these data, induction with TNF increases the signal seen with an AP-1 oligonucleotide (Fig. 2A). This suggests that TNF treatment also influences the occupancy of the AP-1 site, presumably by induction of c-Fos and c-Jun, the constituents of AP-1 (47).

Having identified NF-κB as a predominant regulator of TNF-mediated induction of the E3 promoter, we next examined which members of the NF-κB family of transcription factors p50, p52, RelA, RelB, or c-Rel are mediating the response. Several criteria suggest that p50/RelA activate the E3 promoter in response to TNF. First, overexpression of p65 (RelA) increases promoter activity 7-fold in the absence of TNF and TNF treatment does not further increase this factor (Fig. 4). Second, EMSA experiments in the presence of antibodies directed to p50 and p65 induced a supershift which was not observed with RelB or c-Rel specific antibodies (Fig. 3) nor with antibodies directed to p52 (data not shown).

We further investigated whether or not induction of the E3 promoter by TNF is independent of the E1A protein. Two types of experiments were performed: HeLa cells lacking E1A were transiently transfected with the E3 reporter construct in the presence and absence of TNF. Fig. 6 shows that the induction by TNF was basically identical to that observed in 293 cells which constitutively express E1A. Co-transfection of an E1A expression vector did not alter the inducibility of the E3 promoter (Fig. 4).

Again, no induction was seen when both NF-κB sites were mutated, while mutating AP-1 did not affect TNF induction in HeLa cells. Moreover, TNF induction of E3/19K protein synthesis was observed upon infection with an Ad virus containing a deletion of the E1A genes (dl312). While basal expression of E3/19K was poor in the uninduced state, TNF treatment raised the level of E3/19K to that of Ad2, which expresses E1A. Thus, TNF can functionally replace E1A for efficient expression of E3 proteins. No evidence is available that TNF substitutes for E1A...
The value obtained after transfection of pE3Luc into non-treated cells was arbitrarily set to 1. Luciferase activity was normalized with the β-galactosidase assay. The values represented by the bars correspond to the average of three experiments and the error bars are standard deviations.

FIG. 4. Co-transfection into 293 cells of pE3Luc with expression vectors coding for the NF-κB subunits P65 and 1κB in the presence or absence of TNF. The value obtained after transfection of pE3Luc into non-treated cells was arbitrarily set to 1. Luciferase activity was normalized with the β-galactosidase assay. The values represented by the bars correspond to the average of three experiments and the error bars are standard deviations.

FIG. 5. TNF activates the E3 promoter but not the E2 or E4 promoters. The E2CAT, E3CAT, and E4CAT plasmids (43) were transfected into 293 cells and TNF treatment was carried out as described under “Materials and Methods.” For each construct, CAT activity of non-treated cells was set to 1. CAT activity was normalized with the respective β-galactosidase activity. The values represented by the bars correspond to the average of four independent experiments and the error bars are standard deviations.

FIG. 6. E3 promoter activity in HeLa cells. The E3 promoter elements are schematically outlined in the left of the figure. Mutations are indicated by a cross. Luciferase activity of the wild type E3 promoter in the absence of TNF and E1A was set arbitrarily to 1. The numbers on the right represent the stimulatory factors as determined by the ratio of luciferase activity in TNF-treated versus untreated cells. Luciferase activity was normalized with the β-galactosidase activity measured in the same extracts. The values represented by the bars correspond to the average of three experiments and the error bars are standard deviations.

FIG. 7. TNF increases E3/19K synthesis in infected cells even in the absence of E1A. HeLa cells were infected and TNF-treated for 14 h with a low multiplicity of infection with Ad2, Ad5, and dl312 (an E1A deletion mutant of Ad5). After metabolic labeling E3/19K was immunoprecipitated with the monoclonal antibody Tw1.3 (51). The amount of E3/19K precipitated was determined by PhosphorImager analysis. In this particular experiment the ratio of immunoprecipitated E3/19K from TNF-treated versus untreated cells was 2.5 for Ad2, 3.2 for Ad5, and 9.0 for dl312. Average numbers are given in the text.

We believe that the regulation of the E3 promoter by TNF/NF-κB is biologically important for the Ad replication cycle in vivo and for Ad pathogenesis. Considering the known activities of E3 proteins, inhibition of antigen presentation by E3/19K and interference with TNF lysis by 14.5K, 10.4K, and 14.7K, it is intriguing that the sensitivity to TNF/NF-κB appears to be restricted to the E3 promoter. Searching the Ad2 and Ad5 genome sequences did not reveal NF-κB consensus sequences in other promoters than the E3 promoter (data not shown). Furthermore, examination of CAT reporter constructs driven by the early promoters E2, E3, and E4 indicated that only the E3 promoter is activated by TNF (Fig. 5). Strikingly, the high affinity binding site for NF-κB, κ2, is particularly well conserved among Ad subtypes and in all cases conforms to the consensus sequence for NF-κB binding sites (Table II). This strongly suggests an important role for this element in the virus life cycle.

On the basis of occupancy of the E3 NF-κB sites in lymphoid cells and the activity of CAT reporter constructs, Williams and co-workers (23) previously suggested that NF-κB might drive E3 expression in lymphoid cells and thereby might support persistence of Ad in lymphoid tissue. Our data extend these studies by showing that in other cell types, such as fibroblasts and probably epithelial cells, TNF can activate NF-κB, thereby promoting E3 expression and thus enhancing the functional activity of these viral proteins. When infection conditions were chosen which mimic the in vivo situation, that is, when cells were infected with a low multiplicity of infection, induction of E3/19K by TNF was about 10-fold (27). In light of earlier in vivo data showing that adenovirus infection induces TNF in infected lung tissue in mice (48), we suggest the existence of an autocrine feedback loop to insure efficient E3 expression. The following scenario can be considered: macrophages/monocytes first infiltrate the site of infection and produce the inflammatory cytokines TNF, IL-1, and IL-6. If E3 expression is not sufficiently high, TNF may be able to lyse infected cells. However, binding of TNF to the TNF receptor activates NF-κB leading to increased transcription of MHC genes. The simultaneous activation of the E3 promoter allows efficient expression of E3 proteins and therefore protect infected cells from the lytic activity of TNF and the subsequently infiltrating cytotoxic T cells (6, 48). Several lines of evidence support this model. First, TNF treatment of E3+ target cells did not increase their lysis.
by cytotoxic T cells. Second, lipopolysaccharide treatment of mice transgenic for the E3 region of Ad2 induces TNF-activated E3 transcription in several organs which were E3-negative in the absence of TNF. The induction in organs showing a basal activity of the E3 promoter ranged from 2-fold in spleen to 62-fold in the lung (49). These data indicate that the E3 promoter can be regulated by TNF in vivo. Moreover, there is evidence that the severity of an adenovirus infection (those with fatal outcome) correlates with high levels of TNF (and IL-6 and IL-8) in the sera of patients (35). Further work is necessary to clarify whether TNF causatively determines the outcome of Ad infections or whether the above correlation reflects an increased tissue damage triggered by adenovirus.

There is also evidence that viral membrane proteins such as E3/19K which accumulate in the endoplasmic reticulum may cause endoplasmic reticulum stress resulting in NF-κB activation (50). This can be interpreted as a primitive response mechanism of cells to virus infection with the consequence of activating immunologically important genes. However, the significance of this phenomenon during infection remains to be investigated.

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