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The Dual Effect of Adenovirus Type 5 E1A 13S Protein on NF-κB Activation Is Antagonized by E1B 19K

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The genomes of human adenoviruses encode several regulatory proteins, including the two differentially spliced gene products E1A and E1B. Here, we show that the 13S but not the 12S splice variant of E1A of adenovirus type 5 can activate the human transcription factor NF-κB in a bimodal fashion. One mode is the activation of NF-κB containing the p65 subunit from the cytoplasmic NF-κB–IκB complex. This activation required reactive oxygen intermediates and the phosphorylation of IκBα at serines 32 and 36, followed by IκBα degradation and the nuclear uptake of NF-κB. In addition, 13S E1A stimulated the transcriptional activity of the C-terminal 80 amino acids of p65 at a core promoter with either a TATA box or an initiator (INR) element. The C-terminal 80 amino acids of p65 were found to associate with E1A in vitro. The activation of NF-κB-dependent reporter gene transcription by E1A was potently suppressed upon coexpression of the E1B 19-kDa protein (19K). E1B 19K prevented both the activation of NF-κB and the E1A-mediated transcriptional enhancement of p65. These inhibitory effects were not found for the 55-kDa splice variant of the E1B protein. We suggest that the inductive effect of E1A 13S on the host factor NF-κB, whose activation is important for the transcription of various adenovirus genes, must be counteracted by the suppressive effect of E1B 19K so that the adenovirus-infected cell can escape the immune-stimulatory and apoptotic effects of NF-κB.

Infection with human adenovirus causes gastrointestinal, respiratory, urinary, and ocular infections, which may become persistent by the shedding of infectious virus from apparently healthy individuals several years postinfection (26, 28). The leftmost 11% of the adenovirus genome comprises the early region 1, which confers the ability to transform primary rodent cells (29). This region contains the transcription units for the E1A and E1B proteins. The E1B transcription unit encodes two major proteins, the E1B 19-kDa protein (19K) and E1B 55K. Both are necessary for the complete transformation of cells and suppression of apoptosis. E1B 55K interferes with p53-mediated apoptosis by directly masking the transactivation domain of p53, while p53 remains attached to its cognate DNA site (105). The cytoplasmic E1B 19-kDa protein is also able to prevent apoptosis mediated by different inducers, such as p53 (19), tumor necrosis factor alpha (TNF-α) (34, 101), and E1A (71). E1B 19K counteracts p53 activity by alleviating p53-mediated transcriptional repression (73). Furthermore, E1B 19K was found to increase the transcription from some viral and cellular promoters, such as the 70-kDa heat shock protein (hsp 70) promoter (1).

The E1A genes of adenovirus type 2 (Ad2) and adenovirus type 5 (Ad5) produce two major spliced mRNAs early in infection, and these mRNAs encode proteins of 243 (E1A 12S) and 289 (E1A 13S) amino acids, respectively. E1A proteins from a variety of adenovirus strains have three regions of homology, referred to as conserved regions (CRs) 1, 2, and 3 (45, 62). Two of the three CRs are found in both splice variants, whereas CR3 is unique to the 13S protein. CR1 and -2 are involved in transformation of cells (51, 104), suppression of enhancer activity (13, 92), and the induction of DNA synthesis (39, 51, 94). The 46-amino-acid CR3 domain is necessary for the transcriptional activation of six viral and some cellular promoters, such as the cellular proto-oncogenes c-*fos* and c-*jun*, the immunoglobulin κ chain, and proliferating cell nuclear antigen (PCNA) (1, 10, 32). These broad effects of E1A are thought to be exerted by interactions with numerous cellular proteins. These include the retinoblastoma gene product pRb (103), the pRb-like proteins p107 (104) and p130 (33, 50), retinoic acid receptor β (RARβ) (27), cyclin A (70), BS69 (35), p300 (23), the p300-related protein CBP (2, 58), some transcription factors such as ATF-2 (55), and a number of unidentified proteins (24).

The E1A protein contributes to the transforming ability of the virus, but complete transformation requires the expression of the E1B gene (38, 91). Only cells transformed by Ad12, but not by Ad5, are oncogenic when injected into immunocompetent syngeneic animals. This difference in oncogenicity has been ascribed to the lack of major histocompatibility complex (MHC) class I expression on Ad12-transformed cells, which can escape the action of cytotoxic T lymphocytes (12). Accordingly, it was shown that in Ad12-transformed cells the DNA-binding activity of transcription factor NF-κB, which plays an important role in the regulation of MHC class I genes, is downregulated (59, 64). In contrast, cells transformed by Ad5 were found to express MHC class I and to display constitutive NF-κB binding activity (59, 64). However, very little is known about how adenovirus proteins control the activation of NF-κB.

The DNA-binding form of NF-κB is composed of two subunits. Five different NF-κB DNA-binding subunits, p50, p65, p52, RelB, and c-Rel, have been characterized for vertebrates (85, 93). They all have a conserved domain of approximately 300 amino acids in their N termini, which is important for DNA binding, dimerization, and nuclear translocation. Three of the subunits, c-Rel, RelB, and p65, contain transactivation domains in their C termini (30). In most cell types, the DNA-

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binding subunits (which frequently are dimers of p50 and p65) are sequestered in the cytoplasm by an inhibitory subunit, called IκB (3). The IκB proteins also constitute a gene family, comprising IκBα, IκBβ, and Bcl-3; the precursor molecules for p50 and p52, called p100 and p105; and an alternatively spliced version of p105, called IκBγ (8, 40).

NF-κB becomes activated upon stimulation of cells with a variety of agents, including bacterial lipopolysaccharide, eukaryotic parasites, inflammatory cytokines, T-cell mitogens, and apoptotic and necrotic stimuli, as well as many viruses (85, 88, 93). Many of these stimuli cause inducible phosphorylation of IκBα on serines 32 and 36, which tags the protein for ubiquitinylation and subsequent degradation by the proteasome (15–17, 68, 89, 90). The released DNA-binding dimer can then enter the cell nucleus, bind to its cognate DNA, and induce transcription of its target genes, including those encoding immunoreceptors, cell adhesion molecules, cytokines, hematopoietic growth factors, acute-phase proteins, and transcription factors (93). The activation of NF-κB by many stimuli can be prevented by structurally diverse antioxidants, suggesting a requirement for reactive oxygen intermediates as messengers (42, 66). Recent experiments with cell lines stably overexpressing catalase or superoxide dismutase support this hypothesis (74). The induction of the active nuclear form of NF-κB is a prerequisite for the immune response, since it was demonstrated that targeted disruptions of the genes for the p50, p65, RelB, and c-Rel subunits all interfered with a proper immune response or the viability of the animal (9, 46, 84, 97).

In the present study, we found that the E1A 13S gene product of Ad5 is capable of activating the DNA-binding form of NF-κB by a mechanism which is sensitive toward an antioxidant and involves an inactivation of IκBα and the uptake of NF-κB into the cell nucleus. In a subsequent step, the E1A 13S protein can enhance NF-κB p65-dependent transactivation by physically associating with the transactivation domain of the p65 subunit. Both activating effects of E1A were counteracted by the E1B 19-kDa protein. We discuss a novel viral strategy by which virus-encoded proteins can either activate or repress a particular host transcription factor.

MATERIALS AND METHODS

Eukaryotic cell culture and transfections. Monkey COS7 cells and HeLa cells were grown at 37°C in Dulbecco's modified Eagle's Medium (DMEM) containing 10% fetal calf serum and 1% penicillin-streptomycin (all from GIBCO). COS7 cells (5×10^5 to 10×10^5) were transfected in solution as described elsewhere (57) and plated onto 10-cm-diameter dishes after transfection. Approximately 10^5 HeLa cells in 10-cm-diameter culture dishes were used for transfection with $CaPO_4$. Twelve hours prior to transfection, the culture medium was changed to DMEM supplemented with 5% fetal calf serum. After coprecipitation of the plasmids in ethanol, the dried pellet was resuspended in 90 μl of Tris-EDTA (pH 7.0) and 100 μl of 2× HBS buffer (274 mM NaCl, 40 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid], 1.4 mM Na_2HPO_4, pH 7.06) was added. The precipitate was formed by slowly pipetting, upon spinning the mixture in a vortex, 10 μl of 2 M $CaCl_2$ into the DNA solution. After a 30-min incubation at room temperature, the precipitate was added to the cells. Cells were subjected to a glycerol shock 12 h later. All plasmids used for transfections were purified on CsCl gradients. The amounts of reporter plasmids and expression vectors used are given in the figure legends.

Transactivation assays. After 36 to 48 h of expression, cells were washed once with ice-cold phosphate-buffered saline (PBS) and subsequently harvested by scraping with a rubber policeman. The protein concentration was determined by the method of Bradford (14), and equal amounts of proteins were assayed for chloramphenicol acetyltransferase (CAT) activity. Acetylated and nonacetylated forms of [^{14}C]chloramphenicol were separated by thin-layer chromatography, and the incubation conditions were chosen to result in conversion of [^{14}C]chloramphenicol not exceeding 60%. Transfections were performed at least in duplicate, and the results were quantified by liquid scintillation counting. Cells tested for luciferase activity were washed once in PBS and subsequently lysed for 5 min directly on the dish by addition of 300 μl of 1% (vol/vol) Triton X-100, 25 mM glycylglycine (adjusted to pH 7.8 with KOH), 15 mM $MgSO_4$, 4 mM EGTA [ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid] (adjusted to pH 8 with KOH), and 1 mM dithiothreitol (DTT). The lysates were transferred to Eppendorf tubes and centrifuged, and 100 μl of the supernatant was assayed for luciferase activity. This assay was performed by adding 50 μl of reaction buffer (25 mM glycylglycine [pH 7.8], 15 mM $MgSO_4$, 30 mM potassium phosphate [pH 7.6], 4 mM EGTA, 1 mM DTT, and 3 mM ATP) and measuring the light emission in a Microlumat LB96 P luminometer (Berthold). The luminometer was programmed to inject 100 μl of 0.3-mg/ml luciferin (Sigma) and to measure light emission for 30 s after injection.

Plasmids. The eukaryotic expression vectors pHβAPr-E1A 13S and pHβAPr-E1A 12S (96) and vectors encoding cytomegalovirus (CMV) 19K and CMV 55K (99), RcCMV-p65 (75), Rous sarcoma virus (RSV) luciferase (21), and RcCMV-IκB-α and RcCMV-IκB-α S32/36A (serines at positions 32 and 36 changed to alanines) (89) have been described previously. The NF-κB-dependent reporter plasmids used were the human immunodeficiency virus type 1 (HIV-1) luciferase plasmid (41) and the CAT reporter plasmids J16 and J32, which are controlled by wild-type and mutated NF-κB binding sites, respectively (69). The Gal4-dependent reporter plasmid (7) and the Gal-TATA-CAT and Gal-INR-CAT reporter genes were described previously (60). The construction of the plasmids pGal4-p65$^{471–551}$, pGal4-p65$^{286–521}$, pGal4-p65$^{521–551}$, pGal4-p65$^{286–470}$, pHis-Gal (1–147), and pHisGal-p65$^{471–551}$ is reported in reference 78.

Analysis of protein-protein interactions. The DNA-binding domain of the Gal4 protein (amino acids 1 to 147) and the Gal4-p65 fusion proteins were expressed in bacteria and purified to homogeneity as described elsewhere (78). These purified proteins, as well as bovine serum albumin (BSA) and lysozyme as additional control proteins, were coupled to CNBr-activated Sepharose 4B (Pharmacia Biotech Inc.) according to the instructions of the manufacturer. After the coupling procedure, the columns were equilibrated with binding buffer consisting of 12 mM HEPES-KOH (pH 8), 12% glycerol, 100 mM KCl, 5 mM $MgCl_2$, 1 mM β-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride (PMSF), 20 μM $ZnCl_2$, and 1 mM spermidine (BB). Proteins were labeled with [^{35}S]methionine (Amersham) by using the coupled TnT in vitro transcription-translation system from rabbit reticulocytes (Promega Inc.). Equal amounts of ^{35}S-labeled proteins were incubated with 50 μl of protein-coupled Sepharose beads in BB. Binding reaction mixtures were incubated on ice for 30 min. The beads were then washed four times in BB containing 200 mM KCl, and the bound proteins were eluted by incubation in BB containing 300 mM KCl. The eluates were subsequently loaded on sodium dodecyl sulfate (SDS)–12% polyacrylamide gels. Gels were dried and exposed to X-ray film at −80°C. Proteins tested in immunoprecipitation assays were labeled with [^{35}S]methionine by in vitro transcription-translation. Subsequently, proteins were dialyzed against 1× binding buffer (12 mM HEPES-KOH [pH 8], 12% glycerol, 100 mM KCl, 5 mM $MgCl_2$, 1 mM PMSF, and 20 μM $ZnCl_2$). Binding of proteins was allowed to proceed for 15 min at room temperature, and dithiobis succinimidyl propionate (Pierce) was added to a final concentration of 2 mM. The reaction was stopped after 20 min by the addition of 7 μl of 1 M ethanolamine. Subsequently, 100 μl of immunoprecipitation (IP) buffer (100 mM Tris-HCl [pH 8], 140 mM NaCl, 1% Triton X-100, and 0.1% SDS) was added, together with 30 μl of protein A-Sepharose beads (Pharmacia Biotech Inc.) preswollen for 30 min in IP buffer containing 20 μg of BSA (Sigma). The mixture was incubated for 1 h at 4°C on a spinning wheel. After centrifugation, the supernatant was incubated with 1 μl of an anti-p65 (α-p65) antibody (Santa Cruz) and 30 μl of protein A-Sepharose beads (Pharmacia Biotech Inc.) preswollen for 30 min in IP buffer with 20 μg of BSA (Sigma) and then incubated for 1 h at 4°C on a spinning wheel. The beads were washed six times in IP buffer. Subsequently, the beads were boiled for 5 min in 20 ml of 1× SDS sample buffer and proteins were separated on a reducing SDS–10% polyacrylamide gel. The gel was then dried and autoradiographed.

Western blotting (immunoblotting). COS cells were transfected with 2 pmol of expression plasmid by the DEAE-dextran method. After 36 h, cells were harvested and lysed in 1× SDS buffer and proteins were separated on a reducing SDS–10% polyacrylamide gel. Subsequently, the proteins were transferred from the SDS gel onto a polyvinylidene difluoride membrane (Bio-Rad) in a semidry-blot apparatus (Schleicher und Schüll) according to the instructions of the manufacturers. The detection of E1A proteins was performed by first washing the membrane twice in TBST (10 mM Tris-HCl [pH 8], 150 mM NaCl, 0.05% Tween 20) and then incubating it in TBST containing 5% nonfat dry milk powder for 1 h. The membrane was then incubated in a small volume of TBST, containing an 1:500 dilution of the α-E1A antibody (Santa Cruz). After a 4-h incubation at room temperature, the membrane was washed eight times in TBST and incubated for another hour in TBST containing a 1:3,000 dilution of the second anti-rabbit antibody coupled to horseradish peroxidase (Bio-Rad). After extensive washing, the bound antibodies were detected by using the ECL system (Amersham), according to the manufacturer's instructions.

EMSAs. HeLa cells (5×10^5) were transfected with the appropriate expression plasmids and harvested 12 h later. The amount of transfected DNA was kept constant by using equivalent amounts of empty expression vector (RcCMV; Invitrogen). The control stimulation was done by adding 50 ng of phorbol myristate acetate (PMA) per ml and incubating the mixture for 30 min. Nuclear extracts were prepared essentially as described elsewhere (82). Briefly, cells were washed twice with TBS buffer (25 mM Tris-HCl [pH 7.4], 137 mM NaCl, 5 mM KCl, 0.7 mM $CaCl_2$, 0.1 mM $MgCl_2$) and transferred to a precooled Eppendorf tube after being scraped off the plate with a rubber policeman. Cells were pelleted by centrifugation at 4°C for 2 min at 400 × *g*. The pellet was resus-

pended in 200 μl of cold buffer A (10 mM HEPES-KOH [pH 7.9], 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, and 0.5 mM PMSF) by gentle pipetting. After incubation for 10 min on ice, 5 μl of 10% Nonidet P-40 was added and cells were lysed by being spun in a vortex. The homogenate was centrifuged for 30 s in a microcentrifuge, and the pellet containing the cell nuclei was dissolved in 30 μl of buffer C (20 mM HEPES-KOH [pH 7.9], 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 0.5 mM PMSF, and 1% [vol/vol] aprotinin). The Eppendorf tubes were incubated for 15 min on ice and briefly spun in a vortex every 3 min. The extract was centrifuged for 5 min in a microcentrifuge at 4°C, and the supernatant was used for band shift assays. These were performed by incubation of 10 μg of nuclear extract, 2 μg of poly(dI-dC) (Sigma), and 10,000 cpm of a labeled oligonucleotide on ice in binding buffer (10 mM HEPES [pH 7.9], 25 mM KCl, 4% Ficoll 400 [wt/vol], 1 mM DTT, 0.1 mM PMSF, 5 mM $CaCl_2$, 0.5 mM spermidine). In the supershift experiments, 1 μl of a polyclonal rabbit α-p65 antiserum was added to the binding reaction mixture. The antiserum, which is specific for the p65 subunit of NF-κB, was obtained by immunization of rabbits with a bacterially produced p65 fragment. This fragment comprised the unique C-terminal 123 amino acids of p65 and was purified as described previously (76). The free and protein-bound oligonucleotides were separated on a 4% polyacrylamide gel. Gel and running buffers were identical and contained 25 mM Tris, 25 mM boric acid, and 0.5 mM EDTA. The gel was dried after electrophoresis and exposed to Kodak XAR5 film. The following oligonucleotide used for electrophoretic mobility shift assays (EMSAs) contains a single NF-κB binding site, which is shown underlined:

5′-AGTTGAGGGGACTTTCCCAGGC-3′
3′-TCAACTCCCCTGAAAGGGTCCG-5′

The oligonucleotide was labeled with [γ-^{32}P]ATP by using T4 polynucleotide kinase (Boehringer Mannheim).

RESULTS

Ad5 E1A 13S is a potent activator of transcription factor NF-κB. In an attempt to define the viral gene product mediating NF-κB activation by Ad5, we cotransfected expression vectors encoding proteins of the early region 1 together with a κB-dependent CAT reporter gene construct into COS7 or HeLa cells. When the two splice variants of the Ad5 E1A gene were tested in such an assay, only the E1A 13S protein was able to stimulate transcription from the reporter gene (Fig. 1A, column 3). The 12S splice variant, which lacks CR3, failed to display any transcriptional activity in COS and HeLa cells. The stimulatory effect of E1A was dependent on the NF-κB binding sites in the promoter region of the reporter plasmid, since a reporter gene with mutated κB sites was inactive (Fig. 1A, column 9). The relative expression levels of the two E1A splice variants were found to be roughly comparable, as judged from Western blots (Fig. 1B). This experiment shows that the ability of the Ad5 virus to activate NF-κB can be attributed to the 13S splice variant of the E1A gene. The transcriptional activity of E1A 13S alone in combination with an NF-κB p65-overexpressing plasmid (Fig. 1A, column 4) was significantly higher than the activity of the p65 or E1A 13S vector alone (columns 2 and 3).

Ad5 E1A 13S enhances the transactivating potential of p65 NF-κB. To test the possibility that E1A proteins also influence the activated nuclear form of NF-κB, fusion proteins containing the transactivating C terminus of p65 and the Gal4 DNA-binding domain were tested for their activity in the presence of E1A proteins. A Gal4-dependent CAT reporter gene was cotransfected with different combinations of plasmids encoding E1A proteins and a fusion protein containing the DNA-binding domain of Gal4 and the transactivating C terminus of p65. As shown earlier (75), the C-terminal portion of p65 fused to Gal4 strongly activated the Gal4-controlled CAT reporter gene (Fig. 2). The expression of E1A 13S markedly stimulated the p65-dependent transactivation. The 12S splice variant of E1A failed to support p65-mediated transactivation. The cotransfection of both E1A variants alone had no effect on the Gal4-dependent reporter plasmid. To investigate the possible influence of E1A 13S on the transcriptionally inactive p50

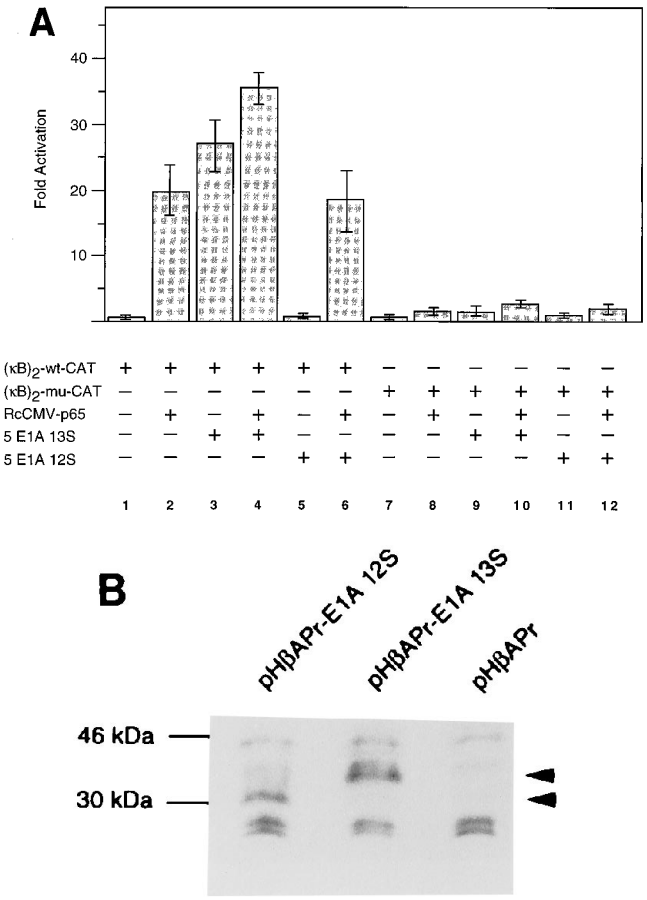


FIG. 1. Transcriptional analysis of E1A proteins. (A) Ad5 E1A 13S but not Ad5 E1A 12S stimulates NF-κB-dependent transcription. COS7 cells were cotransfected with 1.5 μg of expression vectors for p65, E1A 12S, and E1A 13S and 4 μg of the CAT reporter plasmids J16 [$(κB)_2$-wt-CAT] and J32 [$(κB)_2$-mu-CAT] as indicated. The J16 reporter plasmid contains two NF-κB binding sites in a truncated c-*fos* promoter region, which are mutated and nonfunctional in the control plasmid, J32 (69). Two days after transfection, cells were harvested and gene activation was assayed as percent acetylation of [^{14}C]chloramphenicol. The activity of the reporter plasmid alone was set at 1. The standard deviations, which were obtained from four independent experiments, are indicated by error bars. (B) Protein expression analysis of both E1A splice variants. COS cells were transfected with the indicated expression vectors for E1A 12S and E1A 13S and the empty vector alone (pHβAPr) as a control. Cells were harvested, and the cellular proteins were separated on a reducing SDS gel. The relative expression of the E1A proteins was monitored by Western blotting with α-E1A antibodies. The positions of prestained molecular mass markers are indicated; the arrowheads point to the E1A proteins.

subunit of NF-κB, a fusion protein containing Gal4 and the p50 subunit was tested for modulation by E1A. The background activity of Gal4-p50 remained unaltered upon coexpression of E1A 13S (data not shown). These experiments show that E1A 13S can further increase the activity of nuclear NF-κB by stimulating the transactivation potential of the p65 subunit.

Expression of Ad5 E1A activates the nuclear DNA-binding form of NF-κB. In order to assess whether the E1A-dependent stimulation of NF-κB-dependent transcription is accompanied by an induction of its DNA-binding form, EMSAs were performed. HeLa cells were transfected with an expression plasmid encoding E1A 13S, and nuclear extracts were assayed for NF-κB DNA-binding activity. The expression of E1A 13S in HeLa cells led to the appearance of a nuclear DNA-protein complex, which comigrated with the DNA–NF-κB complex

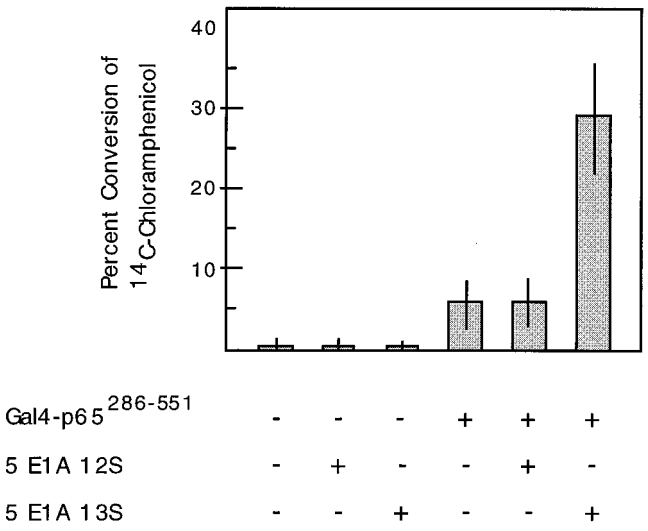


FIG. 2. Ad5 E1A 13S but not Ad5 E1A 12S enhances the transactivating potential of p65 NF-κB. COS7 cells were cotransfected with 4 μg of a Gal4-dependent CAT reporter gene together with expression vectors for Gal4-p65$^{286–551}$ (0.5 μg) and both E1A variants (3 μg) as indicated. Two days after transfection, the cells were harvested and the activity of the CAT reporter gene was determined. Transcriptional activity was determined as percent acetylation of [^{14}C]chloramphenicol. The error bars indicate the standard deviations, which were obtained from four independent experiments.

induced by treatment of cells with PMA (Fig. 3, compare lanes 2 and 4). In contrast, expression of E1A 12S did not induce the DNA-binding form of NF-κB (data not shown). The E1A 13S-induced complex was further characterized by supershift experiments using a polyclonal antiserum specific for the transactivating C terminus of the p65 subunit. The addition of the α-p65 antiserum partly prevented the formation of the DNA–NF-κB complex and supershifted the remaining DNA-binding activity (Fig. 3, lane 3). Incubation with a preimmune serum did not alter the binding of E1A-induced NF-κB to its cognate DNA (data not shown). The data show that E1A 13S induces a prototypic NF-κB complex containing the NF-κB p65 subunit.

IκBα-dependent and -independent pathways of E1A 13S activity. The two modes by which E1A 13S activates NF-κB were characterized in more detail. COS7 cells were transfected with a κB-dependent CAT reporter gene, the activator molecule E1A 13S, and increasing amounts of an expression vector encoding IκBα. The activation of the κB-dependent CAT reporter gene by E1A 13S was almost completely inhibited by IκBα in a dose-dependent manner (Fig. 4A). This suggests that the activation of NF-κB by E1A involved the loss of IκB proteins. In contrast, the cotransfection of increasing amounts of IκBα did not significantly change the inducing effect of E1A 13S on the activity of a Gal4-dependent CAT reporter gene activated by Gal4-p65$^{286–551}$ (Fig. 4B). The finding that IκBα almost completely blocked the κB-dependent transactivation without influencing the effects of E1A 13S on nuclear p65 suggests that the cytoplasmic NF-κB activation by E1A 13S precedes its superactivating effect in the nucleus.

Activation of NF-κB by E1A 13S is prevented by an antioxidant and appears to rely on phosphorylation of IκBα on serines 32 and 36. Many of the diverse NF-κB activators have in common the property of increasing the intracellular level of reactive oxygen intermediates (ROIs) (66). The potential involvement of ROIs in the activation of NF-κB by E1A 13S was investigated by monitoring the effects of the antioxidant pyrrolidinedithiocarbamate (PDTC) on κB-dependent transcription. HeLa cells were cotransfected with a luciferase reporter gene under the control of the HIV-1 long terminal repeat (LTR), which contains two binding sites for NF-κB, and the E1A 13S expression plasmid. The addition of increasing amounts of PDTC strongly impaired the stimulatory effect of E1A 13S on κB-dependent transactivation (Fig. 5A), suggesting that ROI production was involved in the activation of NF-κB by this adenovirus protein. In contrast, the superactivation of Gal4-p65$^{286–551}$ by E1A 13S was not significantly altered by the antioxidant (Fig. 5B). ROIs appear to be important only during the cytoplasmic events leading to the dissociation of IκBα.

IκBα phosphorylation at serines 32 and 36 occurs in response to many distinct stimuli and was shown to target IκBα for degradation (15–17, 68, 89, 90). Using a transdominant negative mutant (S32/36A) of IκB-α (15, 16, 89), we tested whether the mutant protein is superior to the wild-type IκBα in suppressing E1A 13S-induced activation of κB-dependent transcription. This strategy was chosen because limited transfection efficiencies do not allow monitoring of E1A-induced degradation of IκB directly. HeLa cells were transiently transfected with an HIV-1 LTR-controlled luciferase reporter gene, the E1A 13S expression plasmid, and small amounts of either the wild type or the doubly mutated S32/36A mutant of IκBα. The S32/36A double mutant showed a significantly stronger inhibition of E1A-induced NF-κB activity than did the wild-type form of IκBα (Fig. 5C). This difference in inhibitory activity corresponded to that observed with okadaic acid, PMA, and TNF-α (89). Taken together, these results suggest

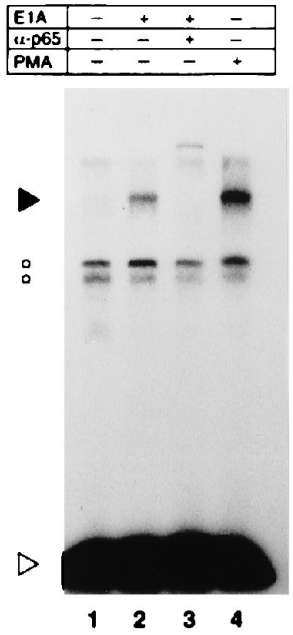


FIG. 3. Expression of Ad5 E1A 13S leads to the appearance of nuclear NF-κB containing the p65 subunit. HeLa cells were transfected with 10 μg of an expression plasmid encoding the E1A 13S protein (lanes 2 and 3) or the same amount of an empty RcCMV expression vector (lane 1) or treated with 50 ng of PMA per ml as indicated. EMSAs were performed by incubating equal amounts of nuclear protein extracts with a ^{32}P-labeled oligonucleotide containing an NF-κB binding site. The binding reaction mixture shown in lane 3 was incubated with an antiserum directed against the unique C terminus of p65. The open arrowhead points to the unbound DNA oligonucleotide, the two small circles indicate the positions of nonspecific DNA-protein complexes, and the filled arrowhead indicates the position of the NF-κB–DNA complex.

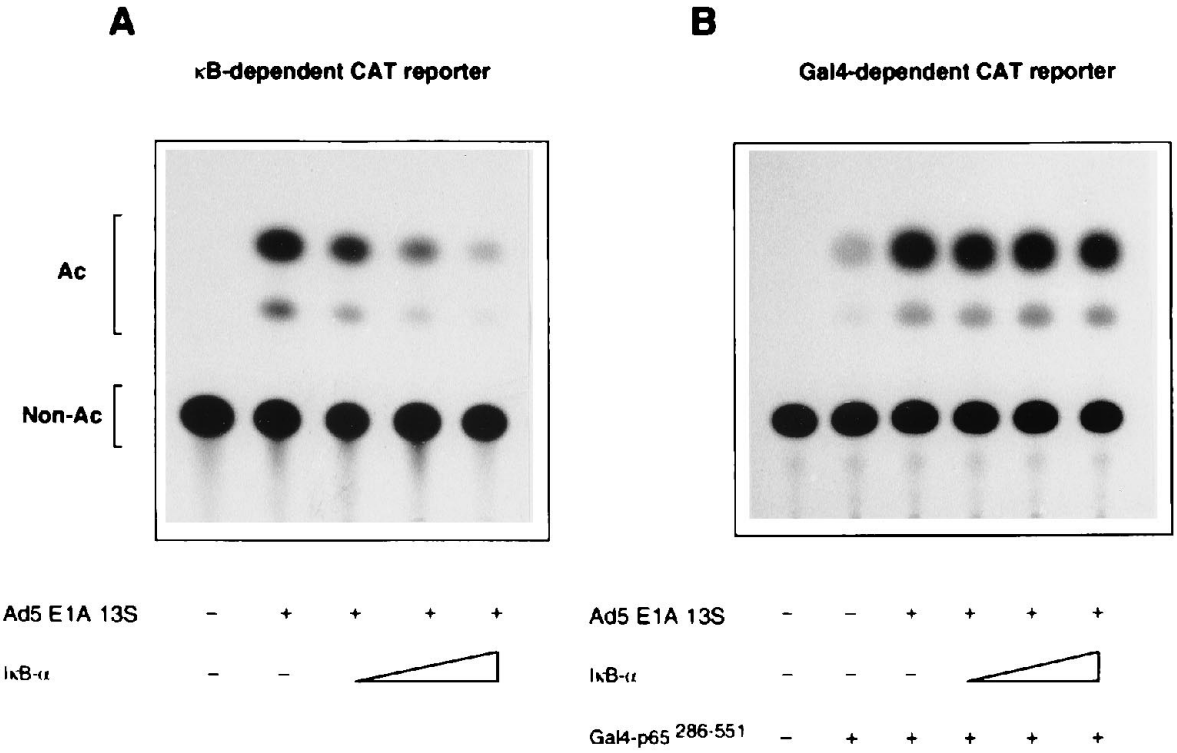


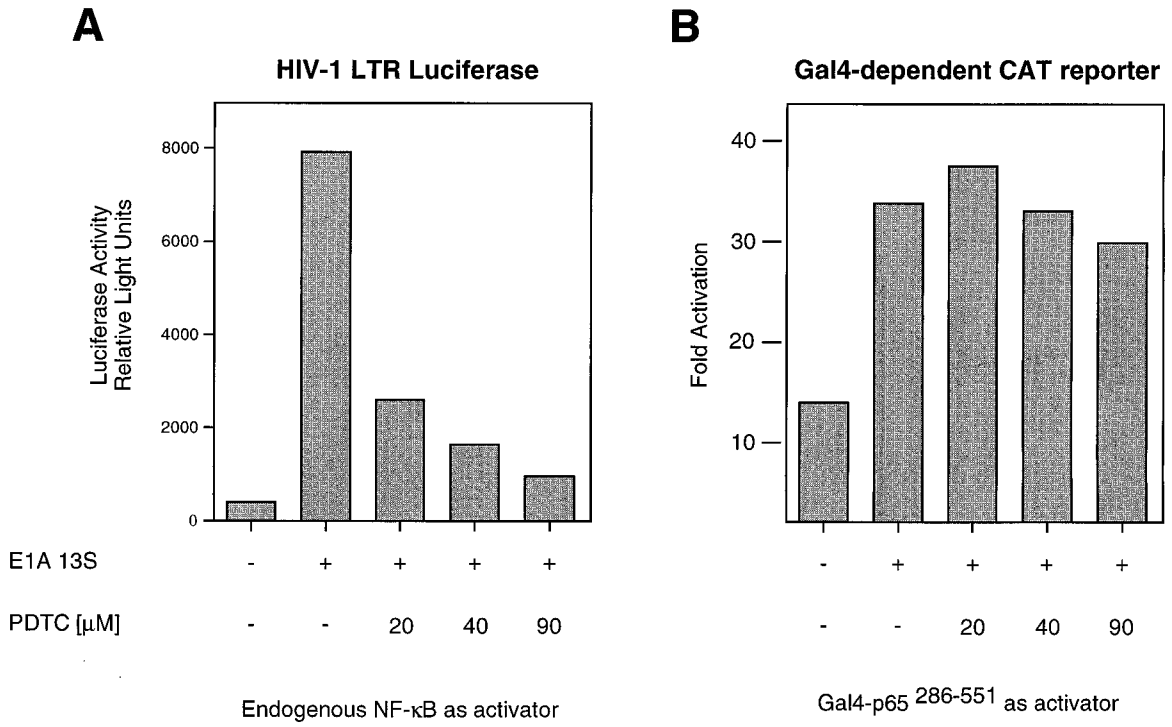
FIG. 4. Ad5 E1A 13S activates NF-κB by an IκB-dependent and an IκB-independent pathway. (A) IκBα cotransfection abrogates the NF-κB-dependent transcription induced by E1A 13S. COS7 cells were cotransfected with 4 μg of the κB-dependent reporter plasmid J16 and 0.5 μg of an E1A 13S expression vector, together with increasing amounts (10, 40, and 160 ng) of an expression vector encoding IκBα, as indicated. Two days after transfection, cells were harvested and assayed for CAT activity. (B) IκBα cotransfection does not influence E1A 13S-dependent superactivation of p65. Plasmids encoding Gal4-p65$^{286–551}$ (0.5 μg) and E1A 13S (3 μg) and 4 μg of a Gal4-dependent CAT reporter gene were cotransfected into COS7 cells together with increasing amounts (10, 40, and 160 ng) of IκBα, as indicated. Two days after transfection, cells were harvested and the CAT activity was determined. Results from a typical CAT assay are shown; the positions of acetylated (Ac) and non-acetylated (Non-Ac) [^{14}C]chloramphenicol are indicated.

that the first steps of cytoplasmic NF-κB activation by E1A 13S are identical to those of other stimuli involving the generation of ROIs and the inducible phosphorylation of IκBα at serines 32 and 36.

E1A 13S stimulates the activity of the C-terminal 80 amino acids of NF-κB p65 on core promoters with TATA or initiator (INR) elements. The E1A-responsive region in the C-terminal half of p65 was further defined. The coexpression of E1A 13S in COS7 cells stimulated transactivation from a Gal4-dependent CAT reporter gene not only by Gal4-p65$^{471–551}$ (Fig. 6A) but also by the short, most C-terminal transactivation domain, transactivation domain 1 (TA_1), present in the plasmid Gal4-p65$^{521–551}$. The fusion protein Gal4-p65$^{286–521}$, which encompasses transactivation domain 2 (TA_2) of NF-κB p65, was equally responsive to stimulation of transcription by E1A 13S. However, Gal4-p65$^{286–470}$, which contains only a part of the TA_2 domain, was no longer responsive to E1A stimulation. This maps the E1A-responsive region to the C-terminal 80 amino acids of p65.

Since it is known that basal promoter elements are selective determinants of some transcriptional activators (18, 25), the influence of two different core promoters on the p65-dependent superactivation by E1A 13S was investigated in further detail. Two distinct reporter plasmids with different core promoters were tested in transient transfection assays for the ability of E1A 13S to superactivate transactivation by Gal4-p65$^{521–551}$. One CAT reporter plasmid contained Gal4 binding sites in the context of a TATA box in its core promoter region, and the other one contained Gal4 binding sites in the context of an INR element in its core promoter region. Figure 6B shows that the coexpression of E1A 13S stimulated transactivation by Gal4-p65$^{521–551}$ with both TATA- and INR-controlled promoters.

E1A 13S can bind to the C terminus of p65 NF-κB. A possible direct association of the E1A 13S protein with p65 was investigated. Interaction between in vitro-translated [^{35}S]methionine-labeled E1A 13S and NF-κB p65 protein was tested by immunoprecipitation experiments. E1A 13S was precipitated by an α-p65 antiserum only in the presence of the full-length p65 protein (Fig. 6C). No significant precipitation of E1A 13S is seen in the absence of NF-κB p65, suggesting a direct interaction between these two proteins. Since the C-terminal 80 amino acids of p65 are E1A responsive (Fig. 6A), we tested the ability of a His-tagged fusion protein consisting of Gal4 (positions 1 to 147) and the 80 C-terminal amino acids of p65 (positions 471 to 551) to directly bind E1A. Gal4-p65$^{471–551}$ and, as a control, the Gal4(1–147) protein were expressed in *E. coli* and purified to homogeneity (78). These purified proteins, as well as BSA and lysozyme as additional controls, were coupled to CNBr-activated Sepharose 4B. Subsequently, equal amounts of in vitro-translated [^{35}S]methionine-labeled E1A 13S protein were passed over the Sepharose beads coupled with either BSA, lysozyme, Gal4, or Gal4-p65$^{471–551}$. After four washes of the columns with a binding buffer containing 200 mM KCl, the columns were eluted with binding buffer containing 300 mM KCl. The eluted proteins were analyzed by SDS-polyacrylamide gel electrophoresis and visualized by autoradiography. In accordance with results ob-



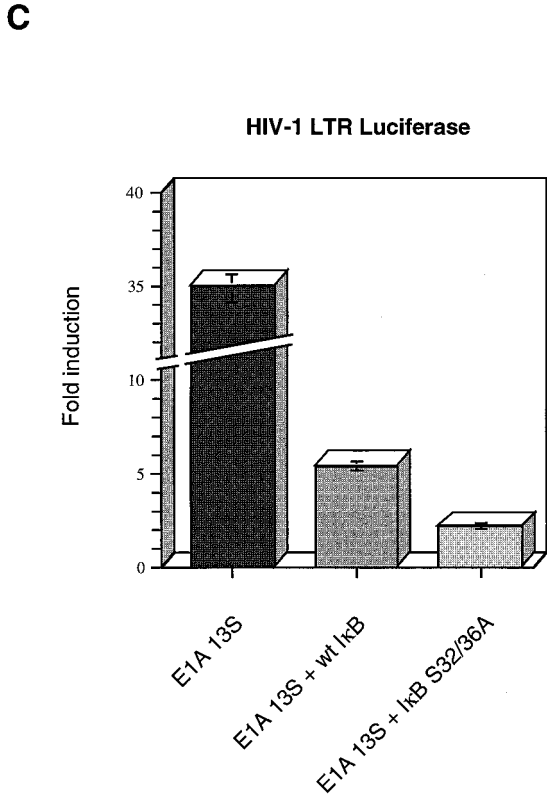


FIG. 5. Inhibition of the cytoplasmic E1A-mediated NF-κB activation by antioxidants and the transdominant IκBα mutant S32/36A. (A) Inhibiting effect of the antioxidant PDTC on E1A-induced NF-κB transcription. HeLa cells were transfected with 4 μg of a luciferase reporter gene controlled by the HIV-1 LTR together with 1 μg of an expression plasmid for E1A 13S as indicated. Twenty hours after transfection, cells were treated for 14 h with the indicated amounts of PDTC. After this time, cells were harvested and the luciferase activity was determined. (B) Effect of PDTC on E1A-induced transcriptional activity of nuclear p65. A Gal4-dependent CAT reporter gene was cotransfected with expression plasmids for Gal4-p65$^{286–551}$ (0.5 μg) and E1A 13S (3 μg) as indicated. Cells were treated as described for panel A prior to the determination of CAT activity. Transcriptional activity is given as fold induction, which was calculated by comparison with the basal level of transcription of the Gal4-dependent CAT reporter gene alone. (C) Effect of S32/36A mutants on the expression of a κB-dependent reporter gene. HeLa cells were transfected with 2 μg of an HIV-1 LTR-controlled luciferase reporter gene and 250 ng of an E1A 13S expression vector in order to induce transcription from the HIV-1 promoter. HeLa cells were additionally transfected with 100 ng of either wild-type (wt) IκBα or the S32/36A IκBα mutant. The transcriptional activities are given as fold induction, which was calculated by comparison with the basal level of transcription of the luciferase reporter gene alone. The standard deviations obtained from four experiments are indicated by error bars.

tained in a previous study (56), no significant binding of E1A 13S to the DNA-binding domain of Gal4 was detected (Fig. 6D, lane 2). Most E1A protein was recovered from the Gal4-p65$^{471–551}$ column, while almost no E1A was retained by the lysozyme- and BSA-coupled Sepharose (Fig. 6D, compare lanes 3 and 4). Subsequent washing with a buffer containing 0.4% SDS revealed that most of the E1A protein had eluted from the columns after incubation in the binding buffer containing 300 mM KCl (data not shown). In order to exclude the possibility that this binding is mediated by a bridging factor(s) present in the reticulocyte lysate and to characterize this interaction in more detail, the Gal4-p65$^{471–551}$ and the E1A 13S proteins were bacterially expressed and purified to apparent homogeneity. The binding affinity and the kinetics of this protein-protein interaction were further analyzed by surface plasmon resonance analysis. By using a BIAcore device, the association rate constant between the Gal4-p65$^{471–551}$ and the E1A 13S proteins was determined to be 4.9×10^4 M^{-1} s^{-1} and the dissociation rate constant was determined to be 1.9×10^{-3} s^{-1}. The standard deviation in these experiments was below 10%, and no significant binding to the control protein BSA was measured (65). In conclusion, E1A 13S appears to superacti-

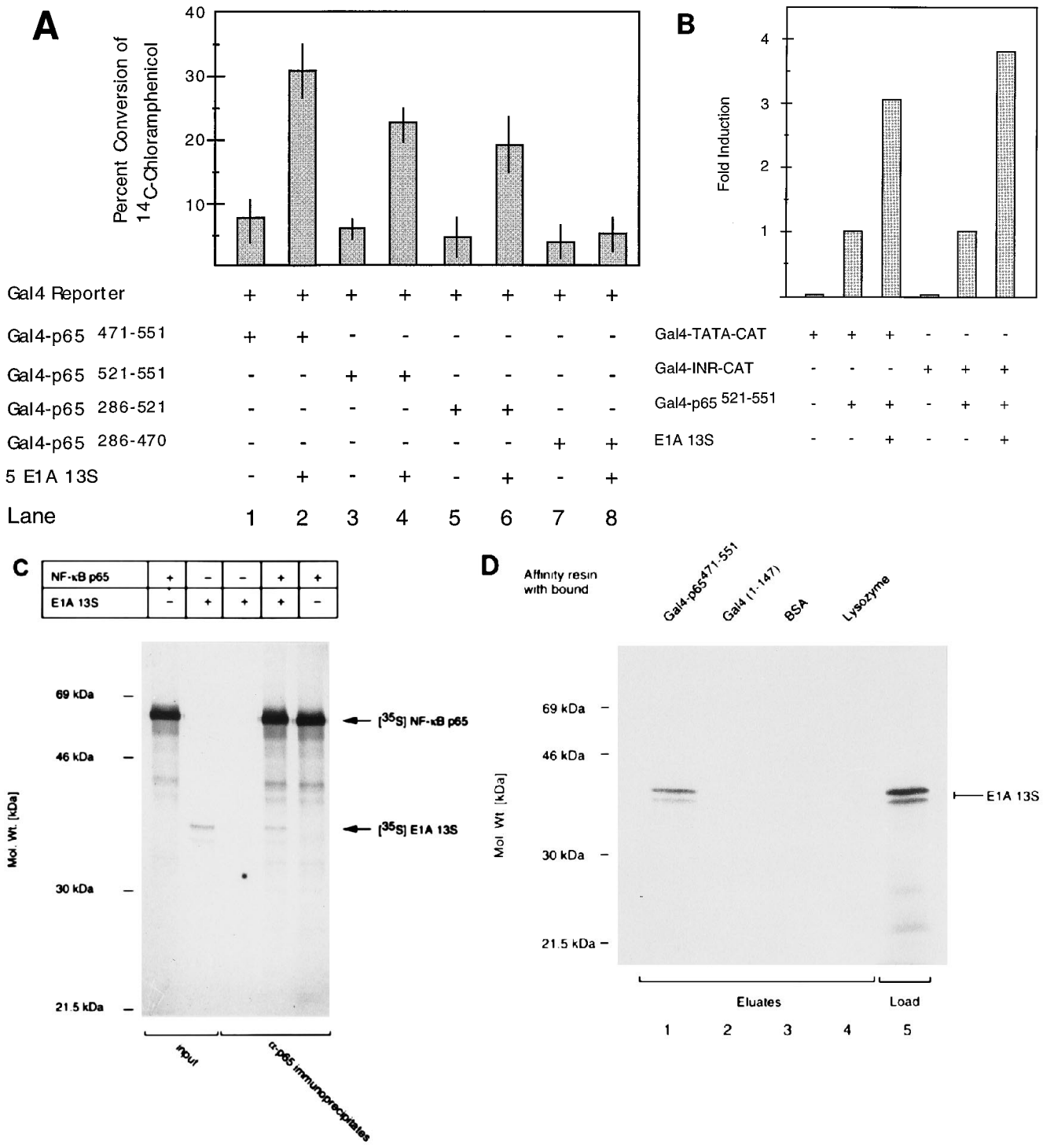


FIG. 6. Effects of E1A 13S in the nucleus. (A) Mapping of the E1A-responsive region in p65. COS7 cells were cotransfected with a Gal4-dependent CAT reporter gene, expression vectors encoding various fusion proteins composed of p65 and the DNA-binding domain (amino acids 1 to 147) of Gal4, and E1A 13S, as indicated. Two days after transfection, the cells were harvested and equal amounts of protein were assayed for CAT activity. The incubation times for the CAT assays were chosen individually for every Gal4-p65 fusion protein in order to result in a [^{14}C]chloramphenicol conversion rate within the linear range. The standard deviations obtained from five individual experiments are indicated by error bars. (B) E1A superactivates p65 on both TATA- and INR-controlled core promoters. COS7 cells were cotransfected with expression plasmids encoding Gal4-p65$^{521–551}$, E1A 13S, and two different reporter plasmids as indicated. Two days after transfection, cells were harvested and assayed for CAT activity. The relative transactivation obtained by cotransfection of Gal4-p65$^{521–551}$ and the respective reporter gene was set at 1 in order to allow a direct comparison of the relative induction by E1A 13S. Results of a representative experiment are shown. (C) E1A 13S directly associates with NF-κB p65 in vitro. Rabbit reticulocyte lysates were programmed in the presence of [^{35}S]methionine with cDNAs for p65 and E1A 13S. The labeled proteins were incubated in the indicated combinations and subsequently precipitated with an anti-p65 antibody. An autoradiogram of an SDS–12% polyacrylamide gel is shown, and positions of the prestained marker proteins are indicated on the left. The arrows mark the positions of the respective proteins. (D) The E1A 13S protein associates with the 80 C-terminal amino acids of p65. Equal amounts of in vitro-translated [^{35}S]methionine-labeled E1A 13S protein were incubated with Gal4(1–147), Gal4-p65$^{471–551}$, BSA, and lysozyme, which were covalently coupled to Sepharose 4B beads as indicated. The beads were washed with binding buffer and eluted with a binding buffer containing 300 mM KCl. The eluates were analyzed by SDS–12% PAGE. The autoradiogram of a representative experiment is shown. The positions of the marker proteins are indicated on the left; the bar on the right indicates the E1A 13S protein.

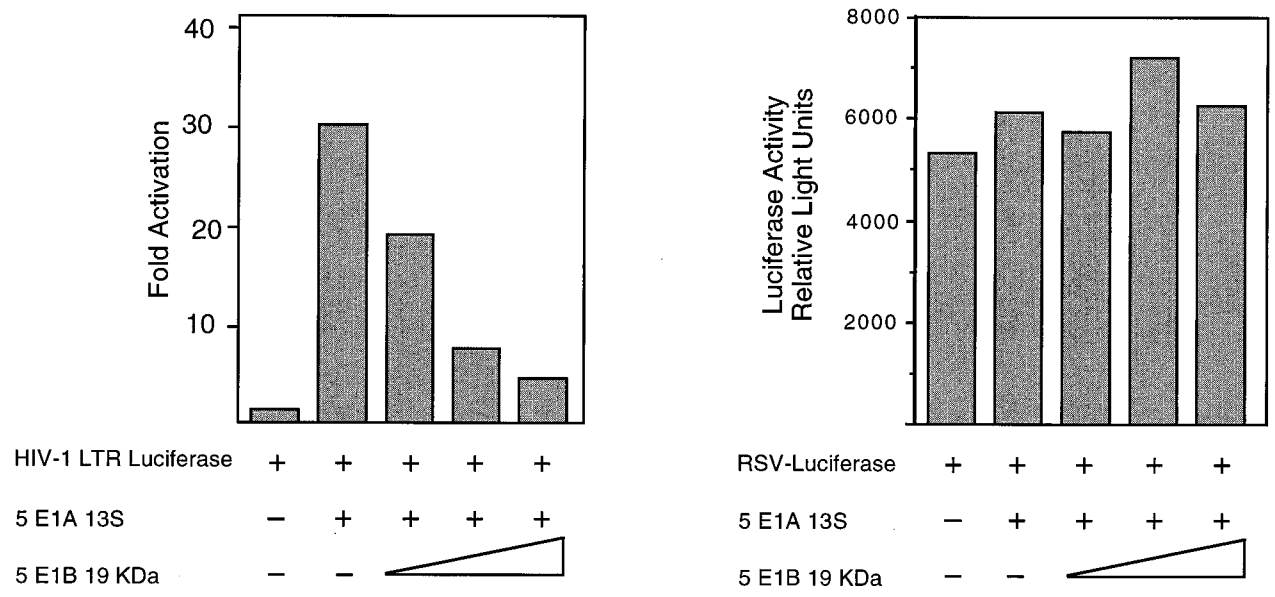


FIG. 7. Ad5 E1B 19K expression inhibits NF-κB activation. (Left) Increasing amounts of an expression vector encoding E1B 19K were cotransfected into COS7 cells together with 4 μg of the HIV-1 luciferase reporter plasmid and 500 ng of an E1A 13S expression vector, as indicated. One day after transfection, cells were harvested and assayed for luciferase activity. The results of a typical set of experiments are shown. (Right) In the control experiment, the same amounts of E1B 19K- and E1A 13S-encoding plasmids as used in the leftmost panel were used, and plasmids were tested for their influence on the activity of a constitutively active RSV luciferase construct.

vate p65-dependent transcription by binding to the C-terminal 80 amino acids of p65 with a dissociation constant of approximately 3.8×10^{-8} M.

Ad5 E1B 19K represses NF-κB activity. E1B, another protein encoded by the adenovirus early region 1, counteracts the detrimental effects of E1A on cell viability (100, 102). A potential opposing effect of E1B expression on the activation of NF-κB by E1A was tested for in transient transfection studies using a κB-dependent HIV-1 luciferase reporter gene. The inductive effect of E1A 13S on κB-dependent transactivation in COS7 cells was attenuated in a dose-dependent manner by cotransfection of increasing amounts of E1B 19K (Fig. 7, left). This suppressing effect was specific, since in a control experiment the expression of a cotransfected luciferase gene under the control of a constitutive RSV enhancer-promoter was unaltered by increasing amounts of E1B 19K (Fig. 7, right). The larger, 55-kDa splice variant of E1B displayed no suppressing effect on NF-κB activation when coexpressed in COS7 cells (data not shown).

The mechanism by which E1B 19K inhibited NF-κB activation was further investigated. HeLa cells were cotransfected with expression plasmids for E1A 13S and increasing amounts of E1B 19K and subsequently tested for the occurrence of nuclear DNA-binding activity. The E1A 13S-induced NF-κB DNA-binding activity was dose-dependently inhibited by the coexpression of increasing amounts of E1B 19K (Fig. 8A, lanes 1 to 3). This suggests that E1B acted in the cytoplasm by preventing the release of IκB from NF-κB. The potential influence of E1B 19K on E1A-dependent superactivation of p65 in the nucleus was also investigated by cotransfection experiments with COS7 cells. The coexpression of E1B 19K did not influence the potential of Gal4-$p65^{521-551}$ to transactivate a Gal4-dependent reporter gene (Fig. 8B, compare second to fourth columns). However, the stimulatory effect of E1A 13S on transactivation by $p65^{521-551}$ was dose-dependently reduced upon coexpression of E1B 19K (Fig. 8A). These data show that E1B 19K can counteract both activities of E1A: its activation of NF-κB in the cytoplasm and its superactivation of p65 in the nucleus.

DISCUSSION

Dual activation of NF-κB by E1A 13S. In this work, we show that the E1A 13S gene product of Ad5 is sufficient to strongly activate the transcription factor NF-κB. The cysteine-rich CR3 region of E1A 13S is necessary for this effect, because the 12S splice variant of E1A failed to activate NF-κB. E1A 13S has been shown to exert its transcriptional effects by a variety of mechanisms. First, E1A can induce the phosphorylation of some transcription factors, such as E2F, E4F, and TFIIIC (5, 37, 72). This activity apparently requires the presence of CR3. Secondly, E1A can be recruited to promoters through direct binding to transcription factors, such as ATF-2, TATA-binding protein (TBP), Oct-4, c-Jun, USF, and Sp1 (47, 55, 56, 79). Thirdly, E1A can activate adenovirus early region 2 (E2) by dissociating the E2F transcription factor from pRb (5, 6). This study adds a novel clue to the pleiotropic effects of E1A 13S.

As suggested by the inhibitory effects of the antioxidative compound PDTC, the expression of the viral protein induces the generation of ROIs, which subsequently induce the phosphorylation and degradation of IκB. Because IκB phosphorylation at serines 32 and 36 is also observed after stimulation of cells with a large variety of NF-κB inducers, such as TNF-α, phorbol ester, and okadaic acid, we consider it unlikely that IκBα becomes directly phosphorylated by E1A. Rather, the adenovirus protein exerts its stimulatory effects on NF-κB by a more general signalling cascade involving oxidative stress. Proteins encoded by other viruses, such as Tat, Tax, and hemagglutinin, can also activate NF-κB and are suppressed in this activity by antioxidants (67, 81, 98). Except in the case of the HIV-1 Tat protein, which induces oxidative stress by down-regulation of the mRNA for the radical-scavenging enzyme Mn-dependent superoxide dismutase (MnSOD) (98), it is currently not clear how expression of viral proteins increases the

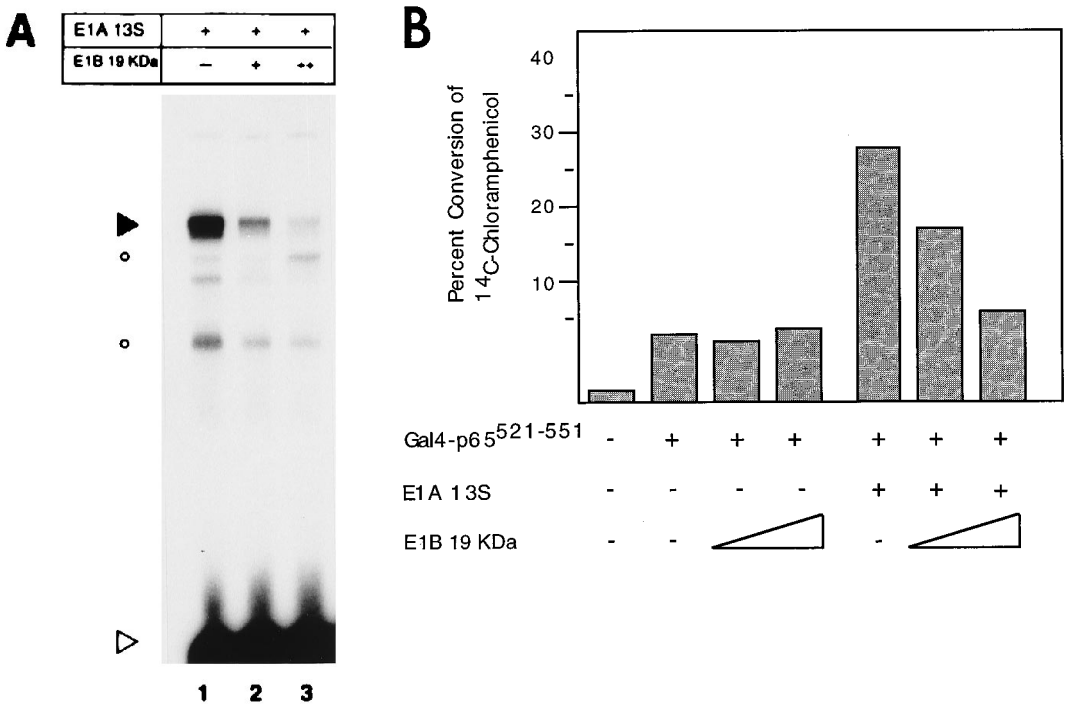


FIG. 8. Ad5 E1B 19K represses the cytoplasmic and nuclear effects of E1A 13S. (A) Expression of E1B 19K prevents the generation of the nuclear, DNA-binding form of NF-κB. HeLa cells were transiently transfected with 5 μg of expression vectors for E1A 13S or with E1A together with 6 (+) and 12 (++) μg of E1B 19K as indicated. After 12 h of expression, cells were harvested and nuclear extracts were tested for NF-κB-binding activity by EMSA. The filled arrowhead indicates the location of the NF-κB–DNA complex, the two small circles indicate the positions of nonspecific DNA-protein complexes, and the open arrowhead points to the position of the unbound DNA oligonucleotide. (B) COS7 cells were cotransfected with 4 μg of a Gal4-dependent CAT reporter gene and plasmids encoding Gal4-p65$^{521–551}$ (100 ng), E1A 13S (3 μg), and various amounts of E1B 19K, as indicated. Two days after transfection, cells were harvested and the CAT activity was determined. The transcriptional activity is given as fold induction, which was calculated by comparison with the basal level of transcription of the Gal4-dependent CAT reporter gene alone.

production of ROIs. It is possible that the ROIs generated by the E1A 13S protein originate from the mitochondria, as seen after stimulation of cells with TNF-α (83). An alternative possibility would be the activation of radical-producing systems such as plasma membrane-associated NADPH oxidases, xanthine oxidase, peroxisomal fatty acyl-coenzyme A oxidase, cytochrome P-450, glucose oxidase, or enzymes involved in arachidonic acid metabolism. Supershift experiments showed that the majority of NF-κB complexes activated upon expression of E1A 13S contained the strongly transactivating p65 subunit. This NF-κB subunit is frequently detected in NF-κB complexes induced by numerous stimuli, but it does not usually contribute to the constitutive NF-κB activity detected in B and T cells (48, 61).

In contrast to the human T-cell leukemia virus-encoded Tax protein, which activates NF-κB by alternative mechanisms including its association with IκB, p100, p105, and p50 (36, 43, 63, 95), the E1A 13S protein induces NF-κB DNA-binding activity by a pathway shared with many other NF-κB-inducing stimuli. This involves sensitivity toward antioxidants and the effect of a transdominant negative IκBα mutant in which S-32 and S-36 are substituted by alanines.

The coexpression of the E1A 13S protein did not significantly influence the activity of p50, suggesting that this subunit is not important for the effects of E1A on the transcriptional activity of nuclear NF-κB. The potentiation of p65-mediated transactivation is most likely due to the direct association of E1A 13S with the unique C-terminal 80 amino acids of p65. Since the CR3 domain itself displays strong transactivation properties (52), it is possible that the enhanced transcription is due to the activity of this additional transactivation domain to p65 by bound E1A. Alternatively, or in addition, it is possible that E1A 13S alters the phosphorylation status of the p65 C terminus, which was shown to correlate with its transcriptional activity in intact cells (77). As a further potential mechanism, E1A could work as a bridging factor by bringing p65 into the vicinity of other transcription factors which are also able to interact with E1A 13S. It might also be possible that E1A associates with multimeric transcription factor complexes containing NF-κB. Such a mechanism possibly accounts for the E1A-dependent cooperativity between the retinoic acid receptor and TFIID in complex (11). Further potentially interesting candidates are complexes between p65 and ATF-2, which are important in the regulation of human beta-interferon and the E-selectin genes (22, 44). These direct protein-protein interactions can greatly enlarge the regulatory potential, so that a limited number of transcription factors can specifically regulate a large number of genes. Since it is hard to image that all of the described numerous protein partners for E1A and p65 are simultaneously binding to these molecules, it is tempting to speculate that at least some of these interactions occur rather rarely and are transient. At present, we cannot rule out the possibility that the dual effect of E1A 13S on NF-κB activation requires additional E1A-associated proteins. Future studies employing E1A 13S mutants which cannot bind to their cellular ligands will help to address this question.

The antagonistic effects of E1A 13S and E1B 19K may ensure adenovirus survival. Besides other host transcription factors, the expression of Ad5 requires the activity of transcription factor NF-κB, which, for instance, participates in the expres-

sion of the E3/19K protein (20). Accordingly, the E1A gene product, which is encoded in early region 1, triggers NF-κB activation as an early event in the viral life cycle in order to stimulate the expression of late adenovirus genes. As shown earlier, an elevated NF-κB activity coincides with enhanced transcription of some viral as well as cellular NF-κB target genes, such as MHC class I (80). Of note, the transcription of early region 1 itself is apparently not controlled by NF-κB binding sites, rendering the master gene product E1A independent from the activity of the subordinate host factor NF-κB. E1A-induced NF-κB not only contributes to the expression of some late adenovirus genes but also is a master regulator of the cellular immune response. This is achieved by its potential to coordinately induce the activation of inflammatory cytokine genes, such as TNF-α and -β, interleukin-2 (IL-2), IL-6, IL-8, granulocyte-macrophage colony-stimulating factor, and granulocyte colony-stimulating factor (93). These and many more gene products stimulate the immune system and contribute to the activation of monocytes, macrophages, and the T-cell response. Since NF-κB induction is also seen in some apoptotic pathways (54, 86), we assume that at least one of the mechanisms by which E1B 19K prevents programmed cell death is the inhibition of NF-κB activity. These apoptosis-inducing and immune-stimulatory effects of NF-κB are potentially detrimental for the virus. We assume that this is the reason why the E1B 19-kDa protein has evolved to counteract E1A-induced NF-κB activity.

The relative amounts of E1A 13S and E1B 19K in the host cell allow the virus to finely tune the cellular NF-κB activity, so that the balance between these two proteins finally determines NF-κB activity. The E1B 19-kDa protein was found to inhibit both effects of E1A on NF-κB. We consider a direct effect of E1B 19K on the E1A protein to be unlikely, because E1B 19K also inhibited NF-κB activated by unrelated stimuli, such as TNF-α and PMA (53). We have recently discovered that the Bcl-2 protein, like E1B, can reduce the activity of nuclear NF-κB (31). Both proteins, Bcl-2 and E1B 19K, have antiapoptotic activity, and Bcl-2 was found to be able to functionally replace E1B 19K (87). Bcl-2 expression was found to allow conversion of lytic to persistent alphavirus infection in several cell lines (49). By analogy, we speculate that the NF-κB-repressing activity of E1B 19K is necessary for the persistence of Ad5. Deletion of a short hydrophobic region within E1B 19K, which contains a region with homology to Bcl-2, abrogated this inhibiting activity (53). The observation that E1B 19K expression confers cellular resistance to cytotoxicity of TNF-α (34, 101) could be mechanistically explained by the inhibition of NF-κB activity by this viral protein. A possible mechanism for this NF-κB-inhibiting activity of E1B 19K is potentially provided by a direct comparison of the two cell lines KB and KB 18. KB 18 cells are infected with an adenovirus expressing an intact E1B 19K gene, whereas the adenovirus in KB cells carries a deletion mutant of E1B 19K. The KB 18 cells, which are much less sensitive to TNF killing, were found to have MnSOD mRNA levels 20-fold increased relative to levels in KB cells (34). It is tempting to speculate that this E1B 19K-induced upregulation of MnSOD expression may account for its NF-κB-inhibiting activity.

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