Expression of the tumor suppressor protein p53 plays an important role in regulating the cellular response to DNA damage. During adenovirus infection, levels of p53 protein also increase. It has been shown that this increase is due not only to increased stability of the p53 protein but to the transcriptional activation of the p53 gene during infection. We demonstrate here that the E1a proteins of adenovirus are responsible for activating the mouse p53 gene and that both major E1a proteins, 243R and 289R, are required for complete activation. E1a brings about the binding of two cellular transcription factors to the mouse p53 promoter. One of these, ETF, binds to three upstream sites in the p53 promoter and one downstream site, whereas E2F binds to one upstream site in the presence of E1a. Our studies indicate that E2F binding is not essential for activation of the p53 promoter but that ETF is. Our data indicate the ETF site located downstream of the start site of transcription is the key site in conferring E1a responsiveness on the p53 promoter.

The tumor suppressor protein p53 plays an important role in maintaining the genomic integrity of a cell. Following exposure of a normal cell to genotoxic stress, with agents such as DNA-damaging drugs (1) and radiation (2), levels of p53 protein increase. As the p53 protein is a sequence-specific DNA binding transcription factor (reviewed in Ref. 3), this increase in p53 protein results in an increase in p53-dependent gene transcription, which in turn leads to cell cycle arrest or apoptosis (reviewed in Refs. 4–6). Cell cycle arrest is thought to be predominantly due to p53 transcriptionally activating the cyclin-dependent kinase inhibitor p21/WAF1 (7), which inhibits the protein kinase activities of G1 cyclin/cyclin-dependent kinase complexes, preventing phosphorylation of the retinoblastoma protein (8), thereby blocking cell cycle progression. It is less clear how p53 induces apoptosis, although several genes that play a role in regulating apoptotic pathways are transcriptionally regulated by p53 (9, 10). For example, p53 activates the bax gene (10), the product of which binds to and prevents the ability of Bcl-2 to block apoptosis (11). It appears that, in part, the level of p53 determines whether a cell enters a cell cycle arrest or apoptotic pathway (12), although transcription-independent apoptosis induced by p53 has been reported (13, 14).

The increase in p53 levels is due in part to increased stabilization of the protein (1). Although the mechanism by which the p53 protein is stabilized is still unclear (15), phosphorylation of p53 by the DNA-dependent protein kinase (16) or ATM kinase (17) may be responsible for activating the p53 protein. This phosphorylation may reduce the ability of p53 to interact with MDM2 (18) a negative regulator of p53 function (19), thereby preventing ubiquitin-mediated degradation of p53 (20). This in turn would enhance the ability of p53 to act as a transcriptional regulator (21) to bring about growth arrest or apoptosis.

However, two reports have raised the possibility that the p53 response to genotoxic stress may also be regulated at the transcriptional level (22, 23). These reports show an increase in transcription from the p53 gene in cells exposed to DNA-damaging drugs.

During adenovirus infection, the adenovirus early 1a (E1a) region expresses two major proteins, 243R and 289R, that differ by 46 amino acids that are present in 289R. Comparison of the primary amino acid sequences of 243R and 289R between serotypes suggests the presence of three conserved domains 1, 2, and 3 (CD1, CD2, and CD3) (reviewed in Ref. 24). These E1a proteins interact with numerous cellular proteins to drive cells through their cell cycle, thereby facilitating virus production (reviewed in Ref. 25). Expression of E1a has been shown to cause an increase in the level of p53 protein and induce p53-dependent apoptosis (26–28). Stabilization of p53 requires the amino terminus or CD1 of E1a and occurs through modification of a ubiquitin-protease pathway (29). Like DNA-damaging agents (22, 23), it has been shown that adenovirus E1a products not only stabilize p53 protein but also transcriptionally activate p53 expression. Early studies performed in normal rat kidney (NRK) cells using nuclear run-on assays demonstrated that stimulation of endogenous p53 expression by adenovirus was at the level of transcriptional initiation and that the E1a proteins were most likely responsible (30).

The adenovirus E1a proteins represent an extensively studied set of viral transactivators (31–33) that have been widely used in the study of regulatory systems that control cellular transcription. Neither of the major E1a proteins, 243R and 289R, is capable of binding to double stranded DNA in a sequence-specific manner (34); therefore, E1a must act through preexisting cellular transcription factors that interact with E1a-inducible promoters. Several sequence-specific DNA binding transcription factors, such as E2F (35), AP1 (36), ATF (37), Sp1, and USF (38), and components of the basal transcription initiation complex (39–41) have been shown to interact directly

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23777
with E1a and/or confer E1a responsiveness. In addition, E1a also interacts with the transcriptional cofactors CBP/p300 (42–44), P/CAB (45), and the retinoblastoma protein (46).

In comparison to the studies on the stabilization of the p53 gene, relatively little is known about the signal transduction pathways and transcription factors that regulate transcription from the p53 gene. The mouse p53 gene has a TATA-less promoter (47) that contains a region extending from −2 to +5 (all numbering is relative to the start site of transcription), which has homology to an initiator (Inr) element (48). Inr elements have been shown to position the start site of transcription in TATA-less promoters (49). Adjacent to the Inr (Fig. 1a), located between +5 and +17, is a NFI-1-like site (50, 51). Further downstream of the Inr is a helix-loop-helix (HLH) consensus binding motif (Fig. 1a). Several members of the HLH family, including USF, have been shown to bind to this site and enhance promoter activity (52, 53). Wu and Lozano (54) have demonstrated that NFκB also binds downstream of the Inr to a site located between +55 and +64 (Fig. 1a). Binding of NFκB in response to TNF-α, an inducer of NFκB activity, was shown to activate the mouse p53 promoter (54). The mouse p53 promoter also contains a consensus TRE-like AP1 binding site between −64 and −57 (Fig. 1a) that binds an unidentified factor designated p53 factor 1 (PF1) (50). Finally, the transcription factor ETF binds to a downstream region in the p53 promoter, and another unidentified factor PF2 binds to a site upstream (Fig. 1a) and appears to be essential for promoter activity (51).

From the literature, it appears that DNA-damaging drugs and expression of E1α during adenovirus infection result in both the transcriptional activation of the p53 gene and stabilization of the protein (1, 22, 23, 26, 30). Because the level of p53 may determine the fate of a cell (12), understanding the transcriptional mechanisms that regulate p53 expression is of importance. This study utilizes the ability of the viral transactivator E1α to activate p53 expression to identify several cellular factors that are involved in transcriptionally activating the p53 promoter, which may have relevance to the activation of p53 expression during the cellular response to genotoxic stress.

EXPERIMENTAL PROCEDURES

Plasmids—The plasmid pCAT3M contains the chloramphenicol acetyltransferase (CAT) gene but no eukaryotic promoter sequences upstream of this gene (55). pAACAT (47) contains −224 to +116 of the mouse p53 promoter blunt-end cloned in front of the CAT gene in pCAT3M (Fig. 1b). The plasmid pARCAT (47) contains −320 to +116 of the mouse p53 promoter also blunt-end cloned in front of the CAT gene in pCAT3M (Fig. 1b).

In the cytomegalovirus (CMV)-based plasmids, transcription is controlled by the immediate early enhancer-promoter of human CMV. pCMVE1a (56) contains a genomic fragment of adenovirus early region 1 that encodes all of the E1a proteins. pCMV125 and pCMV138 encode the adenovirus E1α proteins 243R and 289R, respectively (56).

Creation of ETF Mutants—In order to create the reporter plasmids with mutated ETF sites (see Fig. 6a) within pACAT, the technique of inverse polymerase chain reaction was used (57).

The following primer pairs were synthesized for pETF2CAT, 5'-GGTTC AAAGG ATTTT GCCCT CACCC TACAG C-3' and 5'-CGATG CCGG GGTC CTGGC TCTG-3'; for pETF4CAT, 5'-CTCAATA TTAGA ATCCT GACTC TGCAGA-3' and 5'-ATGTG GCCCT CACCA GGAGA G-3'; and for pETF7CAT, 5'-GTGTC CACCC TGGCT AAAGT TCTGT-3' and 5'-GTGTT ATGTT AAGAT CCAAG CACCA GC-3' (substitutions are underlined). For pETF2/4/7CAT, each mutated site was introduced by successive rounds of polymerase chain reaction/ligations using the above primers pairs. Polymerase chain reaction was performed with one cycle at 96 °C for 3 min, 67 °C for 15 s, and 72 °C for 5 min, followed by 25 cycles at 96 °C for 1 min, 67 °C for 15 s, and 72 °C for 5 min, and then 1 cycle of 72 °C for 9 min. This was done in 50 μl of reaction mixture containing 20 mM Tris-HCl, pH 8.8, 10 mM KCl, 10 mM (NH4)2SO4, 10 mM MgSO4, 0.1% Triton X-100, 100 mM each of dNTPs, 10 ng of the template pAACAT, 40 pmol each of the primers, and 0.5 unit of Vent™ DNA polymerase (New England Biolabs). The amplified linear DNA was agarose gel-purified and T4 polynucleotide kinase-tREATED, and then a portion was self-ligated in 15 μl Tris-HCl, pH 7.8, 5 mM MgCl2, 5 μM dithiothreitol, 0.25 mM ATP, 30 mM KCl, 1 mM hexamethylenamine, and 8 units of T4 DNA ligase at 21 °C for 1 h and then used to transform competent Escherichia coli DH5 cells. The required substitutions within each construct were confirmed by sequencing.

Cell Culture—NRK and HeLa cells were maintained at 37 °C, 10% CO2 in Dulbecco's modified Eagle's medium (Life Technologies, Inc.) supplemented with 10% fetal bovine serum. Rat embryo fibroblasts (REFs) were prepared as described previously (58) and routinely cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum at 37 °C and 10% CO2. REFs were used up to passage seven.

Mammalian Cell Transfection—Two transfection methods were used in this study: For REFs, 106 cells growing in a 10-cm dish were transfected using the calcium phosphate method (59) with the Transfinty kit from Life Technologies, Inc. Ten μg of plasmid DNA and 10 μg of carrier were used to transfect each 10-cm dish of cells. For HeLa cells, 2.5 × 105 cells were seeded into 35-mm dishes and transfected with FuGENE™ 6 (Roche Molecular Biochemicals) after 18 h. For each dish transfected, the total amount of DNA was kept at 4 μg. The amounts of reporter and expression plasmids used are indicated in the figure legends. Sonicated salmon sperm DNA was used as a carrier DNA to keep the total amount of DNA constant. The ratio of DNA (μg) to the volume of FuGENE™ 6 Reagent (μl) used was kept at 2:3 for each transfection.

Viruses—Wild-type human adenovirus serotype 5 (Ad5) was obtained from the American Type Culture Collection and was free from adenovirus-associated virus. The mutant adenovirus dl312 (60) expresses no E1a proteins, due to a deletion of base pairs 448–1349. dl347 (61), which expresses the 243R protein but not 289R, was created when the E1α gene in dl309 (60) was replaced with the 2IS cDNA. The mutant adenovirus dl348 (61), which expresses the 289R protein but not 243R, was created when the E1α gene in dl309 (60) was replaced with the 13S cDNA. dl337 was created when base pairs 1770–1915 were deleted from dl309, so it expresses a truncated E1b 19-kDa protein (62). dl338 was created when base pairs 2050–3298 were deleted from dl309 and does not express any E1b 55-kDa protein (63). dl292 (referred to as dl324 in Ref. 64) has a deletion in the protein coding region of E3, so it expresses only the minor E3 proteins of 12.5 and 3.6 kDa. The mutant dl808 has the open reading frames 2–7 of E4 in adenovirus 2 deleted, so it expresses no E4 proteins (65).

Viruses Infection—Monolayers of cells in 10-cm dishes were infected with Dulbecco's modified Eagle's medium containing virus, 40 h postseeding or 18 h posttransfection. The multiplicity of infection (MOI) was dependent on the experiment and is indicated in the figure legends. Cells were incubated for 1 h at 37 °C and 10% CO2, after which, the medium was replaced with 10 ml of Dulbecco's modified Eagle's medium containing 2% fetal bovine serum.

CAT Assay—Sixty hours posttransfection, the supernatant of the treated cells was harvested twice in ice-cold PBS and then resuspended in 100 μl of 0.25 M Tris-HCl, pH 7.5. Extracts of cells were then prepared by three rounds of freezing and thawing followed by centrifugation for 15 min at 12,000 rpm and 4 °C to remove cellular debris. The supernatant was then heated to 65 °C for 10 min to inactivate a CAT inhibitor previously reported (66). CAT activities from a standard amount of lysate were determined as described by Sleigh (66).

Details of this procedure have been described previously (67).

Preparation of Nuclear Extracts—Forty-eight hours postinfection, NRK cells in 10-cm dishes were washed twice with cold PBS, harvested by scraping, and transferred to microcentrifuge tubes. Nuclear proteins were isolated using a modified small-scale preparation method (68), after which, the nuclear proteins were centrifuged for 5 min at 4 °C, the supernatant was then dialyzed against 100 volumes of Buffer D (20 mM HEPES, pH 7.9, 20% glycerol, 0.1 mM KCl, 0.2 mM EDTA, 0.5 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride) for 18 h at 4 °C.

Competitor DNA—Double stranded oligonucleotides containing the consensus binding sites for AP2, ATF and Sp1 were purchased from TClonase. The cDNA. The details of this procedure have been described previously (69).

Electrophoretic Mobility Shift Assays (EMSAs)—Binding reactions were performed in a volume of 15 μl containing 15–35 μg of nuclear extract, 0.5–2 μg of poly(dI:dC)poly(dI:dC), 10 μM Tris-HCl, pH 7.5, 30 mM NaCl, 1 mM MgCl2, 0.5 mM dithiothreitol, 0.5 mM EDTA, 4% glyco-
erol. Reactions were allowed to proceed for 15 min at room temperature. 1 × 10^6 cpm of target oligonucleotide (oligomers 1–7), end-labeled with 32PdCTP, was added to the binding reaction and incubated at room temperature for a further 15 min. Following this, 1.5 μl of 10× DNA loading dye was added, and the binding reaction was immediately loaded on to a polyacrylamide gel. After electrophoresis, gels were fixed in 10% acetic acid for 10 min, dried for 30 min at 80 °C, and exposed to Kodak X-OMAT AR film at −70 °C, usually for 7–14 h. For competition EMSAs, unlabeled oligonucleotides were added to the binding reactions prior to addition of the radiolabeled oligonucleotide.

RESULTS

Adenovirus E1a Transcriptionally Activates p53 Promoter Constructs—Adenovirus infection has been shown to increase levels of p53 mRNA and protein in mouse Swiss 3T3, hamster tsAF8, and NRK cells (30, 71). Braithwaite et al. (30) demonstrated that Ad5 can transcriptionally activate the endogenous p53 gene in NRK cells. To determine the region of the p53 promoter required for activation by Ad5, two p53 promoter fragments were tested for their ability to respond to Ad5. The construct pAACAT contains the region of the p53 promoter from −224 to +116, whereas pARCAT contains the region from −320 to +116 cloned in front of the reporter CAT gene (Fig. 1b). These constructs along with the promoterless CAT construct pCAT3M were transfected into REFs. Eighteen hours post-transfection, the REFs were infected with wild-type Ad5 at varying MOIs of 10, 20, or 30 infectious units (iu) per cell. Forty-eight hours after infection, cell lysates were prepared and assayed for CAT activity. As shown in Fig. 1c, both pAACAT and pARCAT were activated by Ad5 in a dose-dependent manner. However, whereas at an MOI of 30 iu per cell, activity from pAACAT was enhanced 8-fold, pARCAT activity was enhanced only 3-fold. There was no enhancement of pCAT3M activity at the same MOI (Fig. 1c). The reduced level of activity from the longer construct (pARCAT) in response to Ad5 is reproducible and may be due to the presence of elements that “dampen” the activation of p53 in this construct. The region between −320 and −224, located in pARCAT, contains a putative negative transcriptional regulatory element (47), and although it is not involved in basal expression (51), it may interfere with activation of the p53 promoter by Ad5. Nonetheless, these results in REFs show that the elements required for transcriptional activation of p53 by Ad5 are located between −224 and +116 of the mouse p53 promoter. Similar results were obtained in L929 cells (data not shown).

Although the E1a proteins of adenovirus are extensively studied transcriptional regulators (31–33), other early region genes have also been shown to be involved in regulating transcription. For example, the E4 19-kDa protein stabilizes the E2F complex on the adenovirus E2 promoter (72). To identify involvement of any other early region proteins in transcriptionally activating the p53 promoter, REFs were transfected with pAACAT and then infected with a panel of viruses that are each deficient in different early region gene products, the weight of the evidence here, as well as from the literature (30, 73), argues that it is the E1a proteins that are solely responsible for activating transcription from the p53 promoter.

Both 243R and 289R Can Activate p53 Expression—The above results show that the E1a proteins of Ad5 are responsible for activating p53 expression. There are two major E1a proteins expressed during adenovirus infection, 243R and 289R, both of which have been shown to be transcriptional regulators (31, 32). To determine whether one or both of these E1a proteins are required to transcriptionally activate the p53 promoter, the adenovirus mutants dl347 and dl348, which express 243R and 289R, respectively (61), were tested for their ability to enhance activity of pAACAT. REFs were transfected with pAACAT and then infected with either dl312, dl347, dl348, or wild-type Ad5. Fig. 2a shows that infection of dl312 had no effect on expression from pAACAT as shown above (Fig. 1d). The two mutant adenoviruses dl347 and dl348 activated the pAACAT construct to a similar level, which was approximately 4-fold above basal expression. However, in general, this activation is not as effective as that of wild-type Ad5 (5–6-fold activation in this experiment).

To confirm that it is only these E1a products that are required for activation of the p53 promoter, the expression constructs pCMV12S and pCMV13S, which express the 243R protein and the 289R protein, respectively (56), and pCMVE1a, which expresses all the E1a proteins (56), were assayed for their ability to activate p53 expression using pAACAT. HeLa cells were cotransfected with pAACAT and either 0.5 or 1 μg of pCMV12S, pCMV13S, or pCMVE1a. The reason for using HeLa cells and not REFs in this experiment is discussed below. Fig. 2b shows that all expression constructs are capable of stimulating expression from pAACAT; however, their profile of activation varies. In this experiment, a marked activation (15–17-fold) of pAACAT activity occurs when pCMVE1a is cotransfected. Similarly, cotransfection of pCMV12S and pCMV13S also activated pAACAT, although neither activates as well as pCMVE1a, consistent with the virus infection studies in REFs (Fig. 2a).

Interestingly, although it has little effect on either pCMVE1a or pCMV12S, increasing the dose of pCMV13S caused a reproducible reduction (approximately 50%) in activation of p53 expression. This suggests that increasing the amount of the 289R protein has transcriptional repression properties. This repression may result from transcription factor squelching (74), as the region unique to 289R (contained within CD3) is thought to act as a transcriptional cofactor interacting with promoter bound factors (38) and the general transcriptional machinery (75). Therefore, if 289R is in excess, it may be interacting with its target factor off the promoter, and if this target factor is limiting, transcription will be reduced. This has not been explored further.

Transfection studies similar to these have also been done in L929 cells and REFs. Although similar results were obtained in L929 cells (data not shown), in REFs, we observed a dose-dependent inhibition of pAACAT expression with pCMVE1a (data not shown). This appears to be due to the E1a products causing apoptosis, as has been reported previously (76), thereby resulting in a loss of transfected cells from the transient expression assay.

Nonetheless, the data in Fig. 2 demonstrate that the E1a proteins 243R and 289R can activate p53 expression independently, although neither protein is as effective in activating p53 expression as when all E1a products are present. Although the level of activation may vary, the ability of E1a to activate p53 expression does not appear to be limited to a particular cell
type, consistent with earlier reports from Liu et al. (71) and Braithwaite et al. (30).

**E1a Stimulates Transcription Factor Binding to the p53 Promoter**—To identify regions of the p53 promoter that are bound by transcription factors in the presence of E1a, a set of seven partially overlapping double-stranded oligonucleotides (referred to as oligomers 1–7) were synthesized that span the region −224 to +101 of the mouse p53 promoter (Fig. 3a). This essentially contains the region of the p53 promoter within pAACAT that is responsive to activation by E1a (Figs. 1 and 2). The seven oligomers were radiolabeled in vitro and incubated with nuclear extracts prepared from dl312 or wild-type Ad5-

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**Fig. 1. Adenovirus activates expression of the p53 promoter. a, regulatory motifs present in the mouse p53 promoter. A diagrammatic representation of the p53 promoter showing the location of transcription factor binding sites. The PF1 (−64 to −57) and NF1-like (+5 to +17) sites were shown to bind nuclear factors by Ginsberg et al. (50). Both USF (53) and Myc (52) can bind to the HLH recognition motif (+70 to +75) located within exon 1, whereas NF-xB also binds to a site (+55 to +64) that maps within exon 1 (54). The region from −195 to −165 was shown to bind an activity termed PF2 (51). The Inr motif was identified by nucleic acid sequence analysis (−2 to +5). b, schematic diagram of the mouse p53 promoter constructs, pAACAT and pARCAT (47). c, REFs were transfected with 10 µg of pAACAT or pARCAT and then mock-infected or infected with Ad5 at varying MOIs of 10, 20, or 30 iu/cell. Forty-eight hours postinfection, the cells were assayed for CAT activity. REFs were also transfected with 10 µg of the promoterless CAT construct pCAT3M and infected with Ad5 at an MOI of 30 iu/cell. d, REFs were transfected with 10 µg of pAACAT and mock-infected or infected with dl312, dl337, dl338, dl327, dl808, or Ad5 at an MOI of 30 iu/cell. Forty-eight hours postinfection, the cells were assayed for CAT activity. REFs were also transfected with 10 µg of pCAT3M and infected with Ad5 at an MOI of 30 iu/cell.
infected NRK cells. NRK cells were used to prepare nuclear extracts, as the study by Braithwaite et al. (30) showed the endogenous p53 gene to be transcriptionally activated by E1a in NRK cells. However, REFs were used for the above expression studies (Figs. 1 and 2a), because in general we found NRK cells difficult to transfect without toxic side effects, no matter what technique was employed.

Protein-DNA complexes were observed on all oligomers when the nuclear extracts contained E1a (Fig. 3b). Complexes formed on oligomers 1 and 6 were the same as those seen with dl312-infected NRK extracts (Fig. 3b) and also with mock-infected extracts (data not shown). There is a minor upper complex formed on oligomer 1 in Ad5-infected NRK nuclear extracts; however, this complex was shown to be due to non-specific binding (data not shown). The complexes formed on oligomers 1 and 6 have been shown to contain PF2 and NF1, respectively (51). For oligomer 7, there is a change in complex intensity in the presence of E1a, with an increase in binding of the upper complex previously shown to be ETF (51), whereas the lower complex, previously shown to be USF (51), is decreased (Fig. 3b). Protein-DNA complexes were formed on oligomers 2, 3, 4 and 5 with Ad5-infected extracts that were not seen with dl312-infected (Fig. 3b) or mock-infected extracts (data not shown).

These data suggest that transactivation of the p53 promoter by E1a involves activation of cellular transcription factor binding to discrete regions of the p53 promoter, within oligomers 2, 3, 4, and 5 with Ad5-infected extracts that were not seen with dl312-infected (Fig. 3b) or mock-infected extracts (data not shown).

ETF Binds to Several GC-rich Regions in the Mouse p53 Promoter—To identify the transcription factors involved in the protein-DNA complexes formed on oligomers 2, 3, 4, and 5 (Fig. 3b), competition EMSAs were performed with these oligomers and double-stranded oligonucleotides that contain the recognition sites of known transcription factors (Fig. 4).

Fig. 2. Both E1a proteins are required for full activation of p53 expression. a, REFs were transfected with 10 μg of pAACAT. Eighteen hours posttransfection, cells were mock-infected or infected with dl312, dl347, dl348, or wild-type Ad5 at an MOI of 30 iu/cell. Forty-eight hours postinfection, cells were harvested and assayed for CAT activity. REFs were also transfected with 10 μg of the promoterless CAT construct pCAT3M and infected with Ad5 at an MOI of 30 iu. b, HeLa cells were cotransfected with the mouse p53 promoter construct pAACAT (1 μg) and either 3 μg of carrier DNA (mock) or 0.5 or 1 μg of pCMVE1a (expresses all E1a products), pCMV12S (expresses 243R only), and pCMV13S (expresses 289R only). Sixty hours posttransfection, cells were harvested and assayed for CAT activity. HeLa cells were also cotransfected with pCAT3M (1 μg) and either 3 μg of carrier DNA (mock) or 0.5 μg of pCMVE1a.

FIG. 3. Comparison of factors binding to the p53 promoter in dl312- and Ad5-infected NRK cells. a, schematic diagram indicating the location of the seven oligomers (oligomers 1–7) that span the mouse p53 promoter. b, the seven end-labeled oligomers were incubated with 35 μg (for oligomers 1–5) or 15 μg (for oligomers 6 and 7) of nuclear extracts prepared from dl312 or wild-type Ad5-infected NRK cells, and the mixtures were separated on a polyacrylamide gel.

ETF binds to several GC-rich regions in the mouse p53 promoter—To identify the transcription factors involved in the protein-DNA complexes formed on oligomers 2, 3, 4, and 5 (Fig. 3b), competition EMSAs were performed with these oligomers and double-stranded oligonucleotides that contain the recognition sites of known transcription factors (Fig. 4).

Fig. 4a shows that a 200-fold molar excess of unlabeled self or ETF abolished formation of the complex (2b) on oligomer 2, whereas the complex was not abolished with a 200-fold molar excess of Sp1. Thus, the factor present in complex 2b is likely to be ETF.

As shown in Fig. 4b, the faster migrating, more abundant complex (4b) formed on oligomer 4 was lost in the presence of a 200-fold molar excess of unlabeled self and ETF, but was not competed by a 200-fold molar excess of AP2 or PIF1. The slower migrating, less abundant complex (4a) was abolished with a 200-fold molar excess of self and AP2. This result also shows...
that the binding activity termed PF1 (50), does not bind to its reported site between −64 and −57 in the presence of E1a.

Fig. 4c shows that the faster migrating, more abundant complex b formed on oligomer 5 was reduced in the presence of a 200-fold molar excess of unlabeled self and completely abolished in the absence of a 200-fold molar excess of ETF but was not lost in the presence of a 200-fold molar excess of Sp1 or AP2. The slower migrating complex (5a) appears to contain AP2 because it was lost in the presence of a 200-fold molar excess of AP2, but not Sp1, although it was also reduced in the presence of the ETF competitor.

The results of these competition EMSAs suggest that the transcription factor ETF binds to three upstream sites in the mouse p53 promoter in the presence of E1a. ETF has been shown to bind to a variety of GC-rich sequences, the core sequence of which is 5′-CCCCC-3′ (69). Examination of the sequence in oligomers 2, 4, and 5 reveals the presence of putative ETF motifs within these oligomers.

With the oligomers that bind ETF, a slower migrating complex was often observed. In Fig. 4, it was seen with oligomers 2, 4, and 5 (complexes labeled a); however, it was also often seen with oligomer 7 (this variation is probably due to extract preparation). From the competition EMSAs performed with oligomers 4 and 5, it appears that this complex contains AP2, as it was absent in the presence of a 200-fold molar excess of AP2 (Fig. 4, b and c). However, oligomers 4 and 5 do not contain the consensus sequence for AP2, CCCCAGGC (78). Because AP2 is capable of binding to other GC-rich sequences (79), it is likely that AP2 is binding to the same GC-rich motifs present in the p53 promoter as ETF. Consistent with this interpretation, the oligomer containing an ETF site from the epidermal growth factor receptor promoter (69) causes partial competition of AP2 (Fig. 4, b and c). We conclude from these data that two transcription factors, ETF and AP2, which are both capable of binding to GC-rich DNA motifs, bind to the p53 promoter; however, it appears that AP2 represents a minor activity compared with ETF present in the NRK/Ad5-infected nuclear extracts.

To confirm that ETF binds to the p53 promoter in response to E1a in other cellular backgrounds, oligomers 4 and 7 were used in EMSAs with nuclear extracts prepared from mock- and Ad5-infected NRK cells, which were lost in the presence of a 200-fold molar excess of unlabeled self or E2F. However, the complex was still present in the presence of a 200-fold excess of E2F. This result shows that E2F binds to oligomer 3 in the presence of E1a. Adding support to this conclusion is the presence of a putative E2F site between −96 and −90 within oligomer 3. Therefore E2F, as well as ETF and AP2, binds to the p53 promoter in the presence of E1a and may contribute to the E1a-dependent transactivation of the mouse p53 promoter.

243R Is Required for the Binding of Both ETF and E2F to the p53 Promoter—Both E1a proteins, 243R and 289R, are transcriptional activators; however, they are thought to bring about activation by different mechanisms (38, 77, 80). Expression of E1a was shown to stimulate the binding of three transcription factors, ETF, E2F, and AP2, to the p53 promoter (Figs. 3b and 4). To determine the requirements for 243R and 289R in stimulating binding of these factors to the p53 promoter during activation by adenovirus, the five oligomers (oligomers 2, 3, 4, 5, and 7) that exhibited a different pattern of factor binding in the presence of E1a (wild-type Ad5 infection), than in its absence (dl312 infection), as shown in Fig. 3b, were radiolabeled in vitro and incubated with nuclear extracts prepared from dl312-, dl347-, dl348-, or Ad5-infected NRK cells. Fig. 5 shows that the pattern of binding with dl312- and Ad5-infected nuclear extracts was the same as that seen in Fig. 3b. That is, no complexes were formed on oligomers 2, 3, 4, and 5 with dl312-infected nuclear extracts. With Ad5-infected NRK nuclear extracts E2F bound to oligomer 3, and ETF (Fig. 5, complexes labeled b) and AP2 (complexes labeled a) formed complexes on oligomers 2, 4, and 5, whereas oligomer 7 shows increased binding of ETF (complex b) with Ad5-infected NRK nuclear extracts compared with the dl312-infected extracts.

Fig. 5 shows a difference in the binding pattern between dl348-, dl347-, and Ad5-infected NRK nuclear extracts. In both dl347- and dl348-infected NRK nuclear extracts, ETF complexes were formed on oligomers 2, 4, 5, and 7 (Fig. 5), indicating that both the 243R and 289R proteins are capable of stimulating ETF binding to the p53 promoter. This suggests that
the region unique to 289R (CD3) is not required for ETF to bind to the p53 promoter, although there appeared to be more ETF bound in Ad5-infected extracts.

Fig. 5 shows that E2F only binds to oligomer 3 when 243R is present (in $d_{347}$- and Ad5-infected nuclear extracts), as the E2F complex is absent from $d_{348}$-infected extracts. This indicates that 243R is necessary for E2F binding to the p53 promoter; however, it also implies that binding of E2F is not essential for activation, as Fig. 2 shows that 289R alone is able to activate p53 expression.

The minor binding activity AP2, which in Fig. 5 binds to oligomers 2, 4, 5, and 7 (complexes labeled a), also requires the presence of 243R as it is only seen in $d_{347}$- and Ad5-infected nuclear extracts.

Therefore these results suggest that, with regard to activation of p53 expression by adenovirus E1a, the key cellular transcription factor is ETF.

**The Downstream ETF Site Is Important for Activation by E1a**—To test the possibility that ETF alone can confer E1a responsiveness on the p53 promoter, three of the four ETF sites in the p53 promoter were mutated and tested for their ability to mediate transactivation by E1a. Examination of the sequences within oligomers 2, 4, 5, and 7 revealed the presence of four putative ETF DNA binding motifs. Three of these sites (sites 2, 4, and 7; their sequences are shown in Fig. 6a) were confirmed as ETF binding sites when they were mutated and tested for their ability to bind ETF in EMSAs (data not shown). Only analysis of the ETF binding site within oligomer 5 was inconclusive, and this is likely to be due to the extremely GC-rich nature of this region (−55 to −1) of the p53 promoter. Four p53 promoter/CAT constructs were created when these mutant ETF binding sites were introduced into pAACAT (Fig. 6a); these constructs were then tested for their ability to respond to wild-type Ad5 or E1a alone.

First, REFs were transfected with either pAACAT, the ETF mutant p53 promoter/CAT constructs (Fig. 6a), or pCAT3M and then infected with wild-type Ad5. Fig. 6b shows that although expression from pAACAT is activated 5-fold by infection of Ad5, mutation of either of the two upstream sites between −148 and −143 (site 2 in pETF2CAT) and between −73 and −69 (site 4 in pETF4CAT), causes a slight reduction in the level of activation upon Ad5 infection, as these constructs were activated 3- and 4-fold, respectively. However, expression from the constructs pETF7CAT, which contains a mutation in the downstream ETF site located between +63 and +70 (site 7), and the triple ETF mutant (pETF2/4/7CAT), were completely unresponsive to infection by Ad5. This result demonstrates that although disruption of the two individual upstream ETF sites has little effect on the ability of Ad5 to activate p53 expression, it is the downstream ETF site that appears to be essential for activation.

To determine whether a similar result would be observed when E1a is expressed alone, HeLa cells were cotransfected with either pAACAT, the ETF mutant p53 promoter/CAT constructs or pCAT3M, and the E1a expression construct pCMVE1a. As shown in Fig. 6c, pAACAT was activated approximately 10-fold by expression of E1a. Mutation of either ETF site 2 or site 4, located between −148 and −143 (pETF2CAT) and between −73 and −69 (pETF4CAT), respectively, did not affect the ability of E1a to activate p53 expression, although pETF7CAT, which carries the mutant downstream ETF site 7, is activated only 3-fold by E1a. The triple ETF mutant (pETF2/4/7CAT) is only activated 2-fold by E1a. These data demonstrate that loss of the downstream ETF site markedly reduces the ability of E1a to activate p53 expression, but that mutation of all sites has a further influence.

We conclude that ETF confers E1a responsiveness on the p53 promoter in both these cell types and that the ETF site located between +63 and +70 (in oligomer 7) is most important.

**DISCUSSION**

Several reports have shown that the p53 gene is transcriptionally activated in response to a number of different agents. These include the DNA tumor viruses SV40 (81) and adenovirus (30, 71), serum and phorbol esters (50), and, more recently, DNA-damaging drugs (22, 23). Despite this, little is known about the transcriptional regulation of the p53 gene by any of these agents.

In this paper, we have studied the transcriptional control of the mouse p53 promoter after infection with human Ad5. As the adenovirus E1a proteins induce a p53-dependent apoptosis (26), transcriptional activation by E1a may contribute to the process of virus-induced cell death.

Initial experiments showed that Ad5 stimulated activity of the p53 promoter/CAT construct pAACAT 8-fold in REFs (Fig. 1) and in mouse L929 cells (data not shown). This level of induction with the p53 promoter/CAT construct is similar to that observed for induction of the endogenous p53 gene by Ad5 (30). Further studies in which REFs were infected with Ad5 mutants confirmed a requirement for the E1a proteins (Figs.
1d and 2a), and cotransfection experiments with E1a expression plasmids in HeLa cells demonstrated that the E1a products alone are responsible for transactivation of the p53 promoter (Fig. 2b).

To identify the regions of the p53 promoter that are responsive to E1a, a series of overlapping double-stranded oligonucleotides (oligomers 1–7) were synthesized that span the minimum region of the mouse p53 promoter (224 to 1101) required for transactivation by E1a (Fig. 1). EMSAs carried out with these oligomers using nuclear extracts of NRK cells either infected with wild-type Ad5 or the E1a-deficient virus, dl312, showed that whereas some oligomers bound proteins irrespective of the presence of E1a, others showed binding only in the presence of E1a (Fig. 3). These results suggested that transactivation of the p53 promoter occurs by E1a facilitating binding of (new) transcription factors to the promoter.

To determine whether the above hypothesis is correct, competition EMSAs were first carried out to determine which factors bound to the various oligomers (summarized in Fig. 7). This was then followed by site-directed mutagenesis of certain key factor binding sites within the promoter. The binding studies demonstrated that transcription factors E2F and ETF bound to the oligomers (or showed increased binding as for oligomer 7) in the presence of E1a proteins (Figs. 3 and 4).

Further studies using an adenovirus mutant (dl348), which expresses only the largest E1a protein, 289R, indicated that although E2F did not bind after infection with this mutant adenovirus (Fig. 5), dl348 could nonetheless transactivate the p53 promoter (Fig. 2a). Therefore, binding of E2F is not likely to be essential for transactivation of p53 expression by E1a. Thus, given the other binding data, the transcription factor ETF seems to be the most likely candidate to transactivate the p53 promoter in response to E1a expression.

ETF is a GC-rich DNA-binding protein known to activate TATA-less promoters (69) that has not previously been implicated in E1a-mediated activation. To determine whether ETF binding is critical to E1a transactivation of the mouse p53 promoter, mutagenesis of three of the four ETF sites was carried out. The mutated sites are shown in Fig. 6a. The fourth site within oligomer 5 (from 255 to 21) could not be mapped, presumably due to a very high GC content in this region.

Mutagenesis of the individual ETF sites had relatively little effect on promoter activity in the absence of E1a. Mutation of site 2 (from 2148 to 2143) and site 4 (from 2473 to 2469) also had only a small effect on activity upon adenovirus infection (Fig. 6b). However, mutation of the ETF site in oligomer 7 located between 163 and +70 (site 7) also had only a small effect on activity upon adenovirus infection (Fig. 6b) or in the presence of E1a (Fig. 6c). However, mutation of the ETF site in oligomer 7 located between +63 and +70 (site 7) had a marked effect on the ability of E1a to transactivate the p53 promoter. For example, pAACAT was transactivated 5-fold by Ad5, and the ETF site 2 mutant (pETF2CAT) was transactivated 4-fold; however, the site 7 mutant (pETF7CAT) was not transactivated at all (Fig. 6b). Likewise, in transfected HeLa...
Transactivation of p53 by Adenovirus E1a

10-fold to around 3-fold (Fig. 6). Thus, for both adenovirus and E1a alone (expressed from a plasmid), transactivation of the p53 promoter would appear to be dependent primarily on increased binding of ETF to its downstream site located between +63 and +70.

The studies herein have shown that Ad5 E1a can transactivate the p53 tumor suppressor gene promoter in different cell types (Figs. 1, 2, and 6), consistent with previous reports of stimulation of endogenous p53 gene expression after adenovirus infection in several cell lines (30, 71). DNA binding studies also showed that upon adenovirus infection, ETF and E2F were bound to the p53 promoter in the presence of E1a (Figs. 3 and 4). However, the nuclear extracts used for these binding studies came from cells (NRK cells) that are different than those used in the transactivation studies that were performed in REFs and HeLa cells. In the transactivation studies, site-directed mutagenesis of the ETF site (resulting in a loss of ETF binding to the p53 promoter) showed that the ETF site downstream of the transcription start site was critical to transactivation of the p53 promoter by E1a (Fig. 6). These results, taken together with the binding studies, indicate that the ability of ETF to bind to its downstream site in the p53 promoter is required for transactivation of p53 expression by E1a, in different cell types. One might reasonably conclude then that E1a transactivates the p53 promoter by activating ETF in some way to bind to its downstream site within the promoter. At present, this activation process is unknown; however, it does appear to require the presence of the E1a proteins 243R and 289R for full activation of p53 expression (Fig. 2).

Of particular interest here is the fact that the downstream ETF site between +63 and +70 is the most important region for mediating transactivation by E1a. In the mouse p53 promoter, two other transcription factor binding motifs overlap this ETF site (Fig. 1a). An NF-κB site is located between +55 and +64 (54), whereas an HLH motif is located between +70 and +75 (52, 53). These data suggest this downstream region of the p53 promoter, which is bound by several transcription factors, is a critical control region in the p53 promoter in response to different transactivating agents. Binding of NF-κB to its site appears to play a role in transcriptionally activating the p53 promoter in response to DNA damage induced by the drug daunomycin (23), whereas USF and the immediate early protein Myc have been shown to regulate p53 expression through the HLH motif (52, 53). Members of the HLH family of DNA-binding proteins are often involved in regulating cell growth and differentiation (82). In addition, Myc, like E1a, can induce apoptosis (83). Therefore, this downstream region in the p53 promoter appears to play an important role in controlling the levels of p53 protein and hence in determining the cellular response to several environmental cues, whether it be growth arrest or apoptosis.

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Transactivation of p53 by Adenovirus E1a

3547–3552

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