Adenovirus E1B oncoprotein tethers a transcriptional repression domain to p53

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Many DNA tumor viruses express a protein that inhibits transcriptional activation by the tumor-suppressing transcription factor p53. We report that adenovirus E1B 55K represses p53-mediated activation by a mechanism not described previously. E1B 55K binds p53 without displacing it from its DNA-binding site. A fusion of E1B 55K to the GAL4 DNA-binding domain represses transcription from a variety of promoters with engineered upstream GAL4-binding sites. Mutations within E1B 55K that interfere with its transforming activity and its ability to inhibit p53-mediated *trans*-activation also interfere with transcriptional repression by the GAL4-55K fusion. These results demonstrate that E1B 55K functions as a direct transcriptional repressor that is targeted to p53-responsive genes by binding to p53.

[**Key Words:** Transcriptional repression; silencing; transformation; repressor]

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p53 protein functions as a tumor suppressor (Levine et al. 1991), and the gene encoding it is the most commonly mutated gene in human tumors (Vogelstein and Kinzler 1992). Recent studies demonstrate that p53 functions as a critical component of a G₁ checkpoint induced by DNA damage (Diller et al. 1990; Hartwell 1992; Lane 1992; Lin et al. 1992). Consequently, the absence of p53 function results in genomic instability (Livingstone et al. 1992; Yin et al. 1992). A model for a cell cycle checkpoint pathway postulates that DNA damage activates a protein or set of proteins encoded by AT genes that are defective in patients suffering from ataxia-telangiectasia (AT) (Kastan et al. 1992). The activated AT proteins then induce p53 expression, which in turn induces growth arrest and DNA damage-inducible (GADD) genes, including GADD 45 (Kastan et al. 1992). These events culminate in cell cycle arrest at the G₁/S transition where DNA repair may occur (Kastan et al. 1992; Lane 1992, 1993). In the absence of this G₁ checkpoint, the resulting genomic instability results in DNA rearrangements, gene amplification, and other types of mutations leading to activation of oncogenes, inactivation of tumor suppressor genes, and consequent oncogenic progression. Other studies also show that p53 expression induced by DNA damage results in apoptosis (programmed cell death), perhaps as an alternative to DNA repair (Shaw et al. 1992; Clarke et al. 1993; Lane 1993; Lowe et al. 1993). These results explain why the elimination of p53 activity is an important step in oncogenic progression (Hartwell 1992).

p53 is a sequence-specific DNA-binding transcrip-

tional activator. Considerable evidence suggests that this biochemical activity is necessary for p53 to function as a tumor suppressor (Vogelstein and Kinzler 1992). The model is that p53 transcriptionally activates genes that prevent entry into S phase and/or genes required for apoptosis. Recent studies show that p53 can bind to the TATA-binding protein (TBP) (Seto et al. 1992; Liu et al. 1993; Truant et al. 1993), a central component of the RNA polymerase II preinitiation complex, emphasizing the significance of p53's function as a transcriptional activator.

A number of different proteins have been discovered to bind p53 and inhibit its transcriptional activity. Some p53 mutants can oligomerize with wild-type p53, eliminating its DNA-binding ability and, consequently, its transcriptional activity (Bargonetti et al. 1992; Kern et al. 1992). The cellular oncoprotein MDM-2 binds p53 and abrogates its transcriptional activity (Momand et al. 1992). MDM-2 binds to the amino-terminal activation domain of p53, suggesting that it might function by sterically blocking interactions between the activation domain and general transcription factors (Oliner et al. 1993). However, a recent report indicates that the p53-MDM-2 complex cannot bind to a p53-binding site, providing an alternative explanation for MDM-2 repression of p53 activation (Zauberman et al. 1993). In addition, several proteins that are expressed by DNA tumor viruses inhibit p53 activity. The large tumor (T) antigen of SV40 forms a complex with p53, inhibiting its DNA-binding activity and, consequently, its transcriptional activity (Bargonetti et al. 1992; Farmer et al. 1992). The human papillomavirus type 16 (HPV-16) E6 protein complexes with p53 and, in cooperation with a cellular pro-

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tein, promotes the polyubiquitination of p53, resulting in its degradation (Scheffner et al. 1990).

The adenovirus (Ad) early 1B (E1B) 55K protein also forms a stable complex with p53 in adenovirus-transformed cells (Sarnow et al. 1982a). By studying a panel of E1B 55K in-frame insertion mutants, we found that 55K inhibited p53-mediated *trans*-activation in a manner that correlated strictly with the ability of E1B 55K to transform cells in cooperation with the early 1A (E1A) protein (Yew and Berk 1992). It is not known how E1B 55K negatively regulates p53's transcriptional activity, but there are several possible mechanisms. In adenovirus-transformed cells the E1B 55K protein has been shown to alter the localization of a large fraction of cellular p53 from its normal site in the nucleus to a discrete cytoplasmic body associated with the nuclear membrane (Zantema et al. 1985b). This sequestration of p53 in a presumably inactive complex has been postulated to be the manner in which E1B 55K may inactivate p53 function. However, two lines of evidence suggest that this is not the principal mechanism by which E1B 55K inhibits p53. First, studies with the Ad12 E1B 54K protein, the serotype 12 homolog of Ad2 E1B 55K, showed that the Ad12 54K protein could inhibit p53 *trans*-activation (Yew and Berk 1992) even though Ad12 54K does not sequester p53 in cytoplasmic bodies (Zantema et al. 1985a). Second, an E1B 55K insertion mutant at amino acid 443 (R443), which binds p53 like wild-type E1B 55K but is defective for both transforming activity and for inhibition of p53 *trans*-activation (Yew and Berk 1992), retains the ability to sequester p53 in cytoplasmic bodies (P.R. Yew and A.J. Berk, unpubl.). Sufficient p53 apparently enters the nucleus to permit activation of reporter genes. Consequently, there is no strict correlation between the ability of E1B 55K to sequester p53 in a cytoplasmic body and its ability to inhibit p53-mediated *trans*-activation.

Another possibility is that E1B 55K might interfere with p53's ability to bind to its DNA recognition sequence in a manner analogous to mutant p53, MDM-2, and SV40 T antigen. Because E1B 55K binds to the activation domain of p53 (Kao et al. 1990) it might function by sterically blocking or masking the p53 activation domain, preventing it from interacting with TBP or other possible activation targets. However, mutant R443 binds p53 as well as wild-type E1B 55K yet is defective in transformation and inhibition of p53-mediated transcriptional activation (Yew and Berk 1992). This result suggested to us that E1B 55K protein plays a more active role in inhibiting p53-mediated *trans*-activation than merely sterically masking the p53 activation domain.

To define how E1B 55K inhibits p53-mediated transcriptional activation, we tested whether p53 can bind to a p53 consensus DNA site while it is simultaneously bound to the E1B 55K protein. We also tested whether E1B 55K can itself act as a transcriptional repressor when it is brought directly to a promoter by a fused DNA-binding domain. Direct transcriptional repression (Levine and Manley 1989) or transcriptional silencing (Brand et al. 1985; Renkawitz 1990) is analogous to ac-

tivation by enhancer-binding proteins. Just as enhancer-binding proteins activate transcription from a variety of promoters by a mechanism largely independent of their position and orientation within a promoter region, direct transcriptional repressors inhibit transcription from a variety of promoters in a largely position- and orientation-independent manner. Our results demonstrate that E1B 55K binding to p53 does not interfere with p53's ability to bind to DNA. Consequently, E1B 55K can be brought to a promoter through its interaction with p53. We also show that a fusion of E1B 55K to the GAL4 DNA-binding domain can function as a potent transcriptional repressor when brought directly to heterologous promoters bearing GAL4-binding sites. This introduces a new function for E1B 55K as a direct transcriptional repressor and suggests a unique manner in which p53 function can be regulated by protein-protein interactions at a p53-responsive promoter.

Results

E1B 55K is brought to a p53-responsive promoter through its interaction with p53

We used two methods to determine whether the E1B 55K protein inhibits p53 binding to its specific DNA-binding site. First, we tested whether a 55K-p53-DNA complex can be specifically immunoprecipitated using a monoclonal antibody to 55K. Human p53 was purified from extracts of HeLa cells infected with a p53-expressing recombinant vaccinia virus (Fig. 1D). This p53 was competent to bind ³⁵S-labeled in vitro-translated E1B 55K protein in a coimmunoprecipitation assay (data not shown). A labeled DNA fragment containing five p53-binding sites from the ribosomal gene cluster (RGC) (Kern et al. 1991; Bargonetti et al. 1992) was incubated with purified p53 and immunoprecipitated with the anti-p53 monoclonal antibody pAb421. Under these conditions, ~2% of the probe was immunoprecipitated by the p53 antibody (Fig. 1A, lanes 3,4). A control DNA fragment was not immunoprecipitated (lanes 1,2). When in vitro-translated wild-type E1B 55K protein was added to the binding reaction, all of the DNA bound by p53 was immunoprecipitated by the anti-55K monoclonal antibody 2A6 (lane 7). No DNA was immunoprecipitated when using an equivalent concentration (Fig. 1B) of an E1B 55K mutant, H326, defective for p53 binding (Table 1), whereas DNA was efficiently precipitated with an E1B 55K mutant, R443, which binds p53 like wild-type 55K (Fig. 1A, lanes 8,9; Table 1). These results show that E1B 55K protein can bind to p53 while p53 is bound to DNA at a specific site.

An electrophoretic mobility-shift assay (EMSA) was used in a second assay for the effect of 55K on p53 DNA-binding activity. When a DNA fragment with five RGC p53-binding sites was incubated with purified p53 and increasing amounts of a control, unprogrammed reticulocyte lysate, three mobility-shift bands were observed (Fig. 1C, lanes 2-4). All three bands were completely competed away when the reaction was carried out with

Figure 1. E1B 55K forms a complex with p53 bound to DNA. (A) DNA immunoprecipitation assay. Purified p53 was incubated with a 32 P-labeled DNA fragment containing five RGC binding sites (lanes 3–10) or five GAL4 binding sites (lanes 1–2). Lanes 1 and 3 contain 2% of the total input counts used for each of the other samples. The anti-p53 monoclonal antibody pAb421 was used to immunoprecipitate p53, whereas the anti-55K monoclonal antibody 2A6 was used to immunoprecipitate E1B 55K. H326 is a 55K insertion mutant at amino acid 326 that is defective for p53 binding and R443 contains an insertion at amino acid 443 that does not affect p53 binding. (B) Immunoblot of in vitro-translated E1B 55K proteins. For each sample, 4 μ l of unlabeled in vitro-translated E1B 55K protein was analyzed by immunoblotting with 2A6 anti-55K antibody. (MW) Molecular weight markers. H326 and R443 are E1B 55K insertion mutants. (C) EMSA. The arrow indicates the mobility of unshifted free probe, and the brackets indicate the shifted p53 species and the 55K–p53 supershifted species. Lanes 2–13 contain 1 μ l of p53 and are noted (+). The volume of added reticulocyte lysate or lysate programmed with mRNA for the different E1B 55K proteins is indicated in microliters. DNA–protein complexes were analyzed by electrophoresis in a 1.4% agarose gel. (D) Purified p53. p53 (10 and 50 ng) purified from VV–p53-infected cell extracts was analyzed by SDS-PAGE followed by silver staining.

a 100-fold excess of cold RGC DNA fragment but not with a 500-fold excess of cold nonspecific DNA competitor (data not shown). When in vitro-translated E1B 55K protein was added to the reaction, the slowest mobility band decreased dramatically in intensity with a concom-

itant appearance of slower mobility complexes (lanes 5–7). The second slowest p53 mobility-shift band was also decreased in intensity, whereas the faster migrating band was unaffected. The supershifted bands were not observed with a mutant E1B 55K protein, H326, which

Table 1. Summary of E1B 55K mutant functions

	H17	N68	A143	H180	H224	A262	R309	H326	H354	S380	R443	F484
p53 Binding ^a	+	++	+++	—	+++	—	—	—	++	+	+++	+
Transformation ^b	++	++	+++	—	++	—	—	—	+++	++	—	+
Inhibition of p53 activation ^c	N.D.	N.D.	+++	N.D.	N.D.	—	—	—	+++	N.D.	—	N.D.
GAL4–55K repression	+++	+++	+++	—	+++	—	—	—	+++	+++	— ^d	—

(+ + +) Approximately same activity as wild-type E1B 55K; (—) approximately same activity as background; (N.D.) not done.

^aIn vivo p53 binding.

^bTransformation of primary baby rat kidney cells.

^cInhibition of transcriptional activation by GAL4–p53 and murine p53.

^dNot completely defective.

does not bind p53, and were again generated by mutant R443, which binds p53 like wild-type E1B 55K (lanes 11–13).

The different mobility-shift bands generated by p53 may represent binding of p53 to increasing numbers of binding sites or the binding of alternative p53 oligomers. The E1B 55K protein appears to preferentially supershift the slowest mobility-shift band, which may indicate that 55K binds preferentially to p53 concentrated at several sites. When the DNA immunoprecipitation assay was used, large amounts of in vitro-translated E1B 55K protein could be added, resulting in very efficient immunoprecipitation of p53–DNA complexes. In contrast, the EMSA had strict volume limitations. Consequently, much less E1B 55K protein could be added, which resulted in reduced binding of the p53–DNA complex by E1B 55K compared with the immunoprecipitation assay. Also, the intensity of the supershifted complexes did not equal the intensity of the slowest p53 mobility-shift band. This appears to result from the fact that in the presence of E1B 55K or R443 protein, many counts were found in the wells of the gel, indicating that some very large complexes had formed. These results confirm that E1B 55K protein can bind to DNA-bound p53 stably enough to supershift a p53–DNA complex. The fact that the supershifted probe in Figure 1C formed a smear rather than a distinct band raises the possibility that 55K destabilizes the p53–DNA complex, leading to its dissociation during electrophoresis. However, in the experiment of Figure 1A, the same amount of specific DNA was immunoprecipitated in the 55K–p53–DNA complex as in the p53–DNA complex (cf. lanes 7 and 9 with lane 4). Because the immunoprecipitation procedure involved extensive washing of the immunoprecipitates, it is clear that 55K binding to p53 does not greatly decrease the stability of the p53–DNA complex.

Taken together, these results demonstrate that the E1B 55K protein does not prevent p53 from binding to its specific DNA-binding sequence. Moreover, the E1B 55K protein can bind to p53 that is bound to p53 DNA-binding sites and thus can be brought to a p53-responsive promoter through its specific interaction with p53.

E1B 55K acts as a direct transcriptional repressor when directly bound to a promoter region

In earlier work we found that E1B 55K binds to the amino-terminal activation domain of p53 (Kao et al. 1990). This raised the possibility that E1B 55K might inhibit p53 activation simply by sterically blocking the interaction of the p53 activation domain with target molecules in the preinitiation complex. However, a mutant of E1B 55K, R443, was constructed (Yew et al. 1990) that binds to p53 equivalently to wild-type E1B 55K yet fails to repress p53-activated transcription (Yew and Berk 1992; Table 1). This result suggested to us that E1B 55K might have an activity in addition to p53 binding required for inhibition of p53-activated transcription. This second activity would be defective in the R443 mutant. We questioned whether this second activity might be

similar to that of transcriptional repressors such as the yeast $\alpha 2$ -Mcm1 (Johnson and Herskowitz 1985), mammalian thyroid receptor in the absence of hormone (Damm et al. 1989), and *Drosophila* Krüppel (Licht et al. 1990). These transcriptional repressors inhibit a variety of promoters largely independently of the position at which they bind.

To test whether E1B 55K has the activity of a transcriptional repressor, we constructed a fusion between the DNA-binding domain of GAL4(1–147) and the E1B 55K protein and tested whether the fusion protein could repress expression of reporter genes with upstream GAL4-binding sites. Reporter plasmids contained zero, one, or five GAL4-binding sites upstream of the herpes simplex virus thymidine kinase (HSV TK) promoter (–105 to +51) controlling expression of chloramphenicol acetyltransferase (CAT) (Fig. 2A). CAT expression was unchanged when the G0TKCAT reporter was cotransfected with increasing amounts of a plasmid expressing GAL4–55K. However, when the G1TKCAT or G5TKCAT reporter was cotransfected with the GAL4–55K-expressing plasmid, a marked repression in CAT activity was observed, whereas no repression was observed with the GAL4 DNA-binding domain alone (Fig. 2B). CAT activity was reduced to 13% of the activity observed in the absence of a GAL4 fusion protein for G1TKCAT and 8% for G5TKCAT. Cotransfection of vectors expressing wild-type or mutant 55K proteins that are not GAL4 fusions had no effect on CAT expression from G5TKCAT (Fig. 2C), demonstrating that the 55K protein must be bound to the TK promoter region by the GAL4 DNA-binding domain to repress expression. The effects of other GAL4 fusion proteins were analyzed to test the specificity of repression by GAL4–55K (Fig. 2C). As expected, GAL4–VP16 markedly increased the level of CAT activity observed, whereas a fusion to a transcriptionally defective mutant of the adenovirus E1A activation domain (Martin et al. 1990) had no effect. Significantly, a GAL4 fusion to the homologous Ad12 54K E1B protein also repressed CAT expression from G5TKCAT. GAL4–55K repression was also observed using CV-1, NIH-3T3, and COS cells, indicating that the repression was not cell line specific (data not shown).

These results demonstrate that the E1B 55K protein can function as a potent transcriptional repressor when directly bound to the TK promoter region. This is the first biochemical activity to be attributed to the adenovirus E1B 55K protein, aside from specific p53-binding activity. These results indicate that the E1B 55K protein does not merely block the transcriptional activity of p53 by masking its activation domain but rather plays an active role in repressing transcription from p53-responsive promoters.

E1B 55K can function as a general transcriptional repressor on both weak and strong promoters

To determine whether the E1B 55K protein can function as a general repressor of transcription, we tested the effect of GAL4–55K on expression from the strong SV40

Figure 2. GAL4-55K represses CAT activity from a TK promoter with upstream GAL4-binding sites. (A) TK reporter templates. G0TKCAT contains no GAL4-binding sites, whereas G1TKCAT contains one GAL4-binding site 111 bp from the transcription initiation site. G5TKCAT contains five GAL4-binding sites 120 bp from the transcription initiation site. (B) CAT assay on TK reporters. The reporters were transfected at 7 μ g per sample. Each reporter was tested with the reporter alone (NONE), with increasing amounts of the GAL4 plasmid expressing the DNA-binding domain of GAL4(1–147) at 0.2 μ g and 2.5 μ g, and with increasing amounts of the GAL4-55K-expressing plasmid at 0.2, 0.7, and 2.5 μ g. The CAT activities for each sample were quantitated using a Molecular Dynamics PhosphorImager. The values were all normalized to the CAT activity of the reporter alone (NONE), which was set at 100%. These values are representative of at least three experiments for each sample tested. The values are listed in order from left to right for each panel. G0TKCAT: 100, 110, 96, 85, 90, 82. G1TKCAT: 100, 114, 98, 16, 14, 13. G5TKCAT: 100, 200, 243, 24, 12, 8. (C) CAT assay with other GAL4 fusions and E1B 55K proteins. GAL4-Ad2 55K expresses a fusion protein between GAL4 and E1B 55K of adenovirus serotype 2, and GAL4-Ad12 54K expresses a fusion between GAL4 and E1B 54K of adenovirus serotype 12. GAL4-VP16 expresses GAL4 fused to the activation domain of the HSV transcriptional activator VP16. GAL4-E1A 171/174 expresses GAL4 fused to a mutant of the E1A transcriptional activator that is defective for transcriptional activity (Martin et al. 1990). The plasmids expressing Ad2 E1B 55K wild type and 55K insertion mutants A143, A262, H354, and R443 were all expressed from the strong pcDL-SR α -296 expression vector (Yew and Berk 1992). The G5TKCAT reporter was transfected at 6.5 μ g per sample, along with 2.5 μ g of the GAL4 expressing plasmids except GAL4-VP16, which was transfected at 0.1 μ g. The E1B 55K-expressing plasmids were transfected at 1 μ g per sample. The levels of CAT activity were quantitated and are listed in order from left to right where the activity of the reporter alone (NONE) was set at 100%. G5TKCAT: 100, 173, 14, 17, 435, 187, 80, 136, 62, 98, 91. These values are representative of at least three experiments for each sample tested.

early control region (complete enhancer plus promoter) and the relatively weak (in uninfected cells) adenovirus 2 major late promoter (MLP), both placed downstream of five GAL4-binding sites (Fig. 3A). Both promoters were strongly repressed by GAL4-55K (Fig. 3B,C) but not by a GAL4 fusion to a mutant of 55K defective for p53 repression (Table 1). These results demonstrate that GAL4-55K can function as a transcriptional repressor when bound to three different promoters that have no single activator binding element in common. In the case of the strong SV40 promoter, GAL4-55K represses transcription from a distance of $\sim$ 530 bp from the transcription initiation site. This ability to repress transcription of multiple promoters from a distance is referred to as di-

rect transcriptional repression (Levine and Manley 1989) or transcriptional silencing (Renkawitz 1990).

E1B 55K direct repression function maps to three regions of the protein

An E1B 55K mutant with a 4-residue insertion at amino acid 443 (mutant R443) is defective for inhibition of p53-mediated activation even though it retains a wild-type ability to bind p53 (Yew and Berk 1992; Table 1) and to bind to a p53-DNA complex (Fig. 1A). We tested whether this defect in p53 repression is the result of a defect in function as a direct repressor by constructing a GAL4 fusion to the R443 mutant and assaying its ability

Figure 3. E1B 55K represses CAT activity from the SV40 control region and the Ad2 major late promoter. (A) SV40 and MLP reporters. G5SV40CAT (*top*) bears five GAL4-binding sites 180 bp upstream from the SV40 enhancer and promoter or ~530 bp from the transcription initiation site. G5MLPCAT (*bottom*) contains five GAL4-binding sites 15 bp upstream of the MLP (including the upstream stimulating factor, CCAAT, TATA, and initiator elements) or ~264 bp from the transcription initiation site. (B) SV40 control region. CAT assay showing increasing amounts of GAL4(1–147), GAL4–55K, and GAL4–A262 expression plasmids cotransfected with either 0.2 μ g of G5SV40CAT or 0.2 μ g of G0SV40CAT reporter plasmids. The amount of plasmid DNA transfected in each lane is shown below each wedge. (Left) Results with the SV40 reporter bearing five GAL4-binding sites; (right) results obtained with the SV40 reporter bearing no GAL4-binding sites. GAL4–A262 expresses a GAL4 fusion protein with an E1B 55K mutant containing a 4-amino acid in-frame insertion at amino acid 262. GAL4–VP16 expresses a GAL4 fusion with the activation domain of the HSV transcriptional activator VP16. CAT activities were quantitated and are listed in order from *left to right*. The activity of the reporter alone (NONE) was set at 100%. G5SV40CAT: 100, 75, 87, 17, 14, 28, 71, 68, 82, 327. G0SV40CAT: 100, 266, 180, 154, 260. These values are representative of at least two experiments for each sample tested. (C) MLP. CAT assay with the MLP reporter bearing five GAL4-binding sites (*left*) and no GAL4-binding sites (*right*) with increasing amounts of plasmid DNA expressing GAL4 fusion proteins with the amount of DNA transfected in micrograms shown below each wedge. Five micrograms of each reporter was transfected. CAT activities were quantitated and are listed in order from *left to right*. The activity of the reporter alone (NONE) was set at 100%. G5MLPCAT: 100, 87, 77, 12, 8.5, 6.4, 119, 72, 54, 204, 100. (The two values of the reporter alone were averaged and set at 100%.) G0MLPCAT: 100, 99, 90, 89, 79. These values are representative of at least two experiments for each sample tested.

to repress transcription from the TK, MLP, and SV40 control regions. The GAL4–R443 mutant exhibited a clear defect in repression compared with wild-type GAL4–55K (Fig. 4, data for the SV40 control region not shown). This indicates that the R443 mutant is defective for the activity required to make it an effective direct repressor. However, when high concentrations of GAL4–R443 plasmid DNA (>2 μ g) were cotransfected with G5TKCAT, the R443 defect was diminished (Fig. 5C), indicating that the mutation does not completely eliminate 55K repression function.

To further characterize regions of 55K required for its function as a direct transcriptional repressor, a number of deletion and insertion mutants (Fig. 5A) were analyzed as fusions to the GAL4 DNA-binding domain. Deletion mutants removing amino acids in the central portion of E1B 55K between amino acids 215 and 354 eliminated transcriptional repressing function (Fig. 5B). Deletion of

amino acids 17 to 68, removing an alanine-rich region, had a minimal effect on repression. Surprisingly, deletion of amino acids 354–443 had no effect on repression, even though an insertion of 4 amino acids at residue 443 was defective for repressing activity.

The insertion mutants defined three regions of the 55K protein necessary for repression. Insertions at amino acid 180, between amino acids 262 and 326, and at amino acids 443 and 484 were all reduced for repressing activity (Fig. 5C). It was not possible to detect these GAL4–55K fusion proteins in the transfected HeLa cells. Therefore, to test whether these defects in repressing activity might be the result of instability of the GAL4 fusion proteins in mammalian cells, expression of the proteins was analyzed directly in COS cells where replication of the transfected expression vectors led to higher expression of the encoded proteins. With the exception of GAL4–F484, all of the insertion mutants accumulated to levels near

Figure 4. R443 is defective for transcriptional repression. (A) Effect of GAL4-R443 on G1TKCAT. G1TKCAT was transfected at 7 μ g per lane along with increasing amounts of plasmid DNA (between 0.01 and 1 μ g) expressing GAL4(1–147), GAL4-55K, GAL4-A262, and GAL4-R443. A262 and R443 are insertion mutants of E1B 55K. Amounts of DNA transfected for each GAL4 fusion were indicated in micrograms. CAT activities were quantitated and are listed in order from left to right. The activity of the reporter alone (NONE) was set at 100%. G1TKCAT: 100, 86, 167, 86, 32, 27, 17, 125, 118, 123, 170, 86, 85, 81, 42. These values are representative of at least three experiments for each sample tested. (B) GAL4-R443 in CV-1 cells. CAT assay showing the effect of GAL4-R443 on the G5TKCAT reporter in CV-1 cells. GAL4 fusion plasmids (2 and 5 μ g) were transfected with 7 μ g of G5TKCAT. GAL4 expresses amino acids 1–147, and A262 and R443 are E1B 55K insertion mutants. CAT activities were quantitated and are listed in order from left to right. The activity of the reporter alone (NONE) was set at 100%. G5TKCAT: 100, 276, 293, 17, 18, 188, 404, 85, 89. These values are representative of at least three experiments for each sample tested. (C) GAL4-R443 on G5MLPCAT. CAT assay with increasing amounts of GAL4-R443 on the G5MLPCAT reporter in HeLa cells. Amounts of transfected DNA are indicated in micrograms. GAL4 expresses the DNA-binding domain of GAL4(1–147) and R443 is an insertion mutant of E1B 55K. CAT activities were quantitated and are listed in order from left to right. The activity of the reporter alone (NONE) was set at 100%. G5MLPCAT: 100, 77, 74, 11, 8.8, 6.7, 36, 24, 24. These values are representative of at least two experiments for each sample tested.

or above the wild-type E1B 55K fusion. Similar results were observed for the original 55K mutants (as opposed to GAL4 fusions) in Western blots of extracts from HeLa cells (Yew et al. 1990) and primary baby rat kidney cells (Yew and Berk 1992) infected with adenovirus mutants. Consequently, with the exception of GAL4-F484, the failure of GAL4-55K mutants to repress CAT expression was probably not the result of reduced protein concentrations. The 12 insertion mutants have also been tested for their ability to transform primary rodent cells in association with the adenovirus E1A proteins (Yew and

Berk 1992; Table 1). It is striking that transcriptional repression by the GAL4-55K mutants correlates precisely with the transforming activities of the equivalent 55K mutants.

p53 is not required for GAL4-55K transcriptional repression

The region between residues 262 and 326 required for 55K repression overlaps the region of 55K required to bind p53 (Kao et al. 1990; Yew and Berk 1992; Table 1).

Direct repression by E1B 55K

Figure 5. Regions of E1B 55K required for transcriptional repression. (A) E1B 55K insertion and deletion mutants. Schematic drawing of the E1B 55K protein from the amino terminus to the carboxyl terminus with deletion mutants diagrammed *above* and insertion mutants indicated *below*. The letters represent the restriction site used to make the insertion, and the number represents the amino acid at or preceding the insertion site (Yew et al. 1990). The E1B 55K protein is 495 amino acids in length. An alanine-rich region within E1B 55K is shown by the hatched rectangle between amino acids 17 and 68. Inhibition domains are indicated: solid rectangles indicate regions of E1B 55K defective when mutated; the hatched rectangle represents a region of 55K defective in a concentration-dependent manner when mutated. (B) E1B 55K deletion mutations. CAT assay showing the effect of different GAL4-55K deletion mutants on the G5TKCAT reporter. GAL4 fusion expression vectors were transfected at 2.5 μ g per lane, and G5TKCAT reporter was used at 6.5 μ g per lane. Deletions are named by the regions removed between two specified insertion mutants. CAT activities were quantitated and are listed in order from *left* to *right*. The two values of the reporter alone (NONE) were averaged and set at 100%. G5TKCAT: 100, 135, 9.2, 17, 132, 7.9, 129, 100. These values are representative of at least three experiments for each sample tested. (C) E1B 55K insertion mutations. CAT assay with the G5TKCAT reporter and GAL4-55K insertion mutants. GAL4-55K insertion mutants are indicated by their names as shown in Fig. 5A. GAL4 fusion plasmids were transfected at 2.5 μ g per lane, and G5TKCAT was used at 6.5 μ g per lane. CAT activities were quantitated and are listed in order from *left* to *right*. The activity of the reporter alone (NONE) was set at 100%. G5TKCAT: 100, 157, 8.4, 7.3, 3.7, 8.5, 72, 8.7, 90, 56, 56, 6.9, 4.6, 17, 182. These values are representative of at least two experiments for each sample tested. (D) Immunoprecipitation of GAL4 fusion proteins. COS cells were transfected with 5 μ g of GAL4 fusion constructs per sample, and cells were metabolically labeled. Double immunoprecipitations were carried out with either a rabbit antiserum against GAL4 shown at *left* or with the anti-55K monoclonal antibody 2A6 at *right*. 14 C-labeled molecular weight markers are indicated at *right*.

This raised the possibility that p53 might be involved in GAL4-55K repression. To test this possibility, we assayed the ability of GAL4-55K to repress CAT activity from the TK promoter in two p53-minus cell lines. The Saos-2 and (10)1 cell lines have large deletions in their p53-coding regions and express no p53 mRNA or protein (Masuda et al. 1987; Harvey and Levine 1991). GAL4-55K protein retained the ability to efficiently repress

CAT expression from the TK promoter in these cell lines (Fig. 6), demonstrating that p53 is not required for transcriptional repression by GAL4-55K.

Discussion

A model for E1B 55K repression of p53-activated genes

E1B 55K inhibits transcriptional activation by p53, a

Figure 6. p53 is not required for GAL4–55K transcriptional repression. (A) Saos-2 cells. CAT assay using the G5TKCAT reporter at 7 μ g per sample with increasing amounts of GAL4–55K and GAL4–A262 (0.3 to 3 μ g). GAL4 represents the DNA binding domain of GAL4(1–147), and A262 is an E1B 55K insertion mutant. Saos-2 cells are defective for expression of p53 protein. CAT activities were quantitated and are listed in order from left to right. The two values of the reporter alone (NONE) were averaged and set at 100%. G5TKCAT: 100, 158, 22, 17, 18, 78, 98, 100, 100. These values are representative of at least three experiments for each sample tested. (B) (10)1 cells. CAT assay showing the effect of increasing amounts of GAL4, GAL4–55K, and GAL4–A262 (0.5 to 4 μ g) on the G5TKCAT reporter (7 μ g) bearing five GAL4-binding sites. GAL4 expresses only the DNA binding domain (1–147), and A262 is an E1B 55K insertion mutant. (10)1 cells do not express any p53 protein. CAT activities were quantitated and are listed in order from left to right. The two values of the reporter alone (NONE) were averaged and set at 100%. G5TKCAT: 100, 167, 288, 29, 33, 19, 90, 86, 80, 100. These values are representative of at least two experiments for each sample tested.

function required for E1B 55K transforming activity (Yew and Berk 1992). The results reported here demonstrate that E1B 55K forms a complex with p53 without displacing p53 from its specific DNA-binding site (Fig.

1). Furthermore, we found that 55K contains a potent transcriptional repression domain because a GAL4–E1B 55K fusion protein represses expression of reporter genes when bound within a few hundred bases of their transcription initiation site (Figs. 2 and 3). These results suggest the model shown in Figure 7. In this model the repression domain of E1B 55K inhibits the activity of a general transcription factor required for initiation by RNA polymerase II, or the polymerase itself. This repression domain is targeted to promoters activated by p53 through the specific p53-binding activity of E1B 55K first observed by Sarnow et al. (1982a). Strong support for this model comes from the perfect correlation between the effect of E1B 55K mutations on 55K transforming activity and on the transcriptional repression activity of the GAL4–55K fusion (Table 1). These results indicate that the E1B 55K protein contributes to adenovirus transformation by functioning as a direct transcriptional repressor targeted to p53-activated genes. It is also possible that the E1B 55K repression domain is targeted to additional DNA sites by binding to transcription factors other than p53.

Comparison of E1B 55K to other eukaryotic repressors

The E1B 55K protein appears to function as a direct transcriptional repressor (Levine and Manley 1989) in a manner similar to several other proteins with transcriptional repressing activity from different eukaryotes: yeast α 2–Mcm1 (Johnson and Herskowitz 1985), yeast proteins interacting with the silent mating-type loci silencers (Brand et al. 1985), the human thyroid receptor in the absence of hormone (Damm et al. 1989), the *Drosophila*

Figure 7. Model for E1B 55K inhibition of p53-mediated transcriptional activation. (A) When E1B 55K is directly brought to a promoter bearing GAL4 sites by the DNA-binding domain of GAL4, 55K mediates transcriptional repression on heterologous promoters. (B) E1B 55K is directed to a promoter by specific binding to p53. E1B 55K then mediates transcriptional repression by modifying the general transcriptional machinery, repressing p53 target genes. In this way, E1B 55K would specifically inhibit transcription of p53-responsive genes that may be required for the correct implementation of a G₁ checkpoint and/or apoptosis.

Krüppel protein (Licht et al. 1990), and other eukaryotic "direct repressors" that have been described (see Han and Manley 1993). Each of these proteins can repress promoters they do not normally regulate when their binding sites are placed within a few hundred base pairs of the transcription initiation site. E1B 55K is distinct from these in that it does not bind DNA directly. It may be similar in this regard to the complex of yeast Ssn6 and Tup1 required for repression by the DNA-binding proteins $\alpha 2$ -Mcm1, $\alpha 1$ - $\alpha 2$, and Mig1 (Keleher et al. 1992). In the case of E1B 55K, the specific binding of E1B 55K to p53 tethers the potent transcriptional repression domain of E1B 55K to promoters with p53-binding sites.

We suggested that E1B 55K inhibits the activity of a general transcription factor because the GAL4-55K fusion repressed transcription from three different promoters with no single activator in common. Transcriptional repressing domains from *Drosophila* even-skipped protein (Han and Manley 1993) and mammalian thyroid and retinoic acid receptors (Baniahmad et al. 1992) also probably target a general transcription factor because they repress transcription from minimal promoters consisting of only a TATA box or initiator element. The target of repression could equally well be the polymerase itself. This possibility has been raised for the yeast repressor Sin1 (Spt2) because a genetic interaction has been reported between Sin1 and the carboxy-terminal repeat of the large subunit of RNA polymerase II (Peterson et al. 1991).

We cannot tell from these *in vivo* experiments whether E1B 55K represses transcription directly or whether additional factors interacting with 55K are required. As mentioned above, in yeast it appears that repression by the DNA-binding proteins $\alpha 2$ -Mcm1, $\alpha 1$ - $\alpha 2$, and Mig1 require that these factors bind an additional complex of the Ssn6 and Tup1 proteins (Keleher et al. 1992). The finding that high levels of GAL4-55K reproducibly diminished repression of the SV40 early promoter and enhancer compared with lower GAL4-55K concentrations (Fig. 3B) might be explained by the titration of similar "corepressors" required for 55K repression. This "squenching" of repression might be analogous to the squenching of activation by high concentrations of activators (Gill and Ptashne 1988).

Other transcriptional repression domains have been shown to be alanine rich (Licht et al. 1990), highly basic (Baniahmad et al. 1992; Saha et al. 1993), and rich in proline and hydrophobic residues (Madden et al. 1991; Han and Manley 1993). E1B 55K contains an alanine-rich region between amino acids 47 and 60 (8 of 14 residues are alanine). However, deletion of this region from GAL4-55K had only a small effect on repressing activity (Fig. 5). The regions of E1B 55K where deletions and insertions did interfere with repression are neither highly basic nor proline rich. The central region of 55K spanning amino acids ~250-350, where three insertions inactivated repression by GAL4-55K, does contain a high proportion of hydrophobic amino acids. However, these insertions may disrupt the tertiary structure of the protein because they also inhibit p53 binding by 55K and an

apparently distinct function of the E1B 55K protein involved in the preferential nuclear-cytoplasmic transport of viral mRNAs during the late phase of infection (Yew et al. 1990; see Ornelles and Shenk 1991).

p53 is not required for GAL4-55K repression

Not surprisingly, the regions of E1B 55K required to inhibit p53-mediated transcriptional activity included a region required for p53 binding (Yew and Berk 1992). In analyzing regions of 55K required for repression by the GAL4-55K fusion, we did not expect to observe a requirement for the p53-binding domain. We anticipated that this function would be replaced by the GAL4 DNA-binding domain. Surprisingly, all the insertion mutations analyzed that disrupt E1B 55K-p53 binding also inactivated repression by GAL4-55K. This raised the question of whether p53 was a cofactor required for GAL4-55K repression. p53 itself may mediate transcriptional repression of promoters containing CCAAT boxes by interacting with the CCAAT-binding factor (Agoff et al. 1993). It has also been suggested that p53 may repress transcription by its interaction with TBP (Seto et al. 1992; Mack et al. 1993). Overexpressed p53 has also been shown to reduce the transcriptional activity of a number of promoters that do not contain consensus p53-binding sites (Ginsberg et al. 1991). Despite the many reasons for considering that p53 might be required for transcriptional repression by GAL4-55K, we found that GAL4-55K continued to repress expression from target promoters in cells completely lacking p53. Further studies of E1B 55K as a transcriptional repressor should add to the limited but growing knowledge concerning direct transcriptional repressors or silencers and the mechanism by which they repress transcription.

Materials and methods

Cell culture, transfection, and CAT assay

HeLa and CV-1 cells were grown in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% newborn calf serum and 5% fetal calf serum (FCS), respectively and were transfected as described previously (Yew and Berk 1992). Saos-2 (Masuda et al. 1987), and (10)1 (Harvey and Levine, 1991) cells were grown in DMEM supplemented with 10% FCS and transfected as described (Zambetti et al. 1992). Cells were harvested 48 hr post-transfection. The amount of reporter plasmid transfected was as follows: 6.5–7 μ g of TKCAT reporters, 0.2 μ g of SV40CAT reporters, and 5 μ g of MLPCAT reporters. All samples for CAT assays were normalized for β -galactosidase activity expressed from a cotransfected control expression vector and used in CAT assays as described (Yew and Berk 1992).

Vaccinia virus construction and p53 purification

pTM.1-ep53, expressing epitope-tagged p53, was constructed by PCR amplification of human p53 from the pcDNA-p53 plasmid (Liu et al. 1993), using primers that introduce an *Nco*I site and the amino acid sequence MYPYDVPDYA at the 5' end, and a *Bam*HI site at the 3' end. The amplified DNA fragments were cloned between the *Nco*I and *Bam*HI sites of the pTM.1 vector

(Elroy-Stein et al. 1989). Recombinant vaccinia virus expressing epitope-tagged human p53 was constructed by the procedure of Elroy-Stein et al. (1989). Correct recombinant vaccinia virus was confirmed by Western blot analysis of cell extracts prepared from infected cells. Recombinant virus (VV-ep53) was plaque purified, viral stocks were grown, and viral titers were determined by plaque assay on HeLa cells.

Epitope-tagged p53 was affinity-purified from vaccinia virus-infected HeLa cells. Two liters of HeLa cells were infected with recombinant viruses VV-ep53 and v-TF7-3 (which expresses T7 RNA polymerase), each at an m.o.i. of five. Cells were harvested at 24 hr postinfection and nuclear extract was prepared (Dignam et al. 1983). One milliliter of the nuclear extract was incubated with 150 μ l of packed beads of protein A-Sepharose covalently coupled to monoclonal antibody 12CA5 at 4°C for 6 hr (Liu et al. 1993). The 12CA5 affinity beads were washed three times with 0.4 M KCl D buffer (20 mM HEPES, 20% glycerol, 0.2 mM EDTA, 1 mM dithiothreitol, 1 mM PMSF) and two times with 0.1 M KCl D buffer. ep53 was eluted with 60 μ l of the influenza virus epitope oligopeptide (YPYDVPDYA) at 1 mg/ml in 0.1 M KCl D buffer.

Plasmid construction

The in vitro translation vectors expressing wild-type E1B 55K and H326 were constructed by cloning the 1865-bp *Sst*I fragment from pUC19.R-/Ad1767-3632 (Yew et al. 1990) into the *Sst*I site of pSP65. The in vitro translation vector for R443 was constructed by cloning the 1720-bp *Bst*EII-*Bam*HI fragment from pSR α -R443 (Yew and Berk 1992) with the 3150-bp *Bst*EII-*Bam*HI fragment from pSP65/55K. The GAL4-55K-expressing plasmid was constructed by cloning a *Bam*HI-*Sst*I E1B 55K PCR fragment into the *Bam*HI-*Sst*I sites of the plasmid, pSG424 (Sadowski and Ptashne 1989) to make pSG424/55K PCR. This construct was sequenced through the GAL4 fusion region and beyond the *Asp*718 site in the adenovirus E1B 55K sequence (Ad2 sequence 2045). GAL4 fusion constructs were then made for wild-type E1B 55K, and 55K mutants H17, N68, A143, H180, H224, A262, R309, H326, H354, S380, R443, F484, dlH354-R443, dlA262-F484, dlH215-H326, dlH17-N68 by cloning the *Asp*718-*Sst*I 1600-bp fragment from pAd2.17.R/CA-/19K-55K mutants and wild type (Yew and Berk 1992) between the *Asp*718 and *Sst*I sites of pSG424/55K PCR.

The CAT reporter plasmids were constructed as follows: The G5TKCAT and G1TKCAT plasmids were constructed by cloning the GAL4-containing *Xba*I-*Sst*I fragments from G5E4TATA/pGEM-3 or G1E4TATA/pGEM-3, respectively, between the *Xba*I and *Sst*I sites of G5TKCAT (Shi et al. 1991). G5SV40CAT was constructed by cloning an end-filled *Xba*I-*Hind*III 100-bp fragment containing five GAL4-binding sites from G5E4TATA/pGEM-3 into the filled-in *Acc*I site (SV40 520 bp) of pSV2CAT (Gorman et al. 1982) that contains the entire SV40 enhancer and early promoter from SV40 340 bp to SV40 5018 bp. The G5MLPCAT plasmid was constructed by cloning a 250-bp *Xba*I-*Xho*I PCR fragment containing the MLP from Ad2 5790 bp to Ad2 6062 bp between the *Xba*I and *Xho*I sites of G5TKCAT, substituting the Ad2 MLP for the 150-bp TK promoter region.

Oligonucleotides containing one p53-binding site were made as complementary 24-mers containing one RGC site (5'-CTTGCCTGGACTTGCTGG) with a *Xho*I site at the 5' end and a *Sal*I site at the 3' end. The plasmid pB5E4T was constructed by ligating five tandem p53-binding oligonucleotides into the *Sal*I site of p Δ -38, 23 bp upstream from the E4 TATA box (Carey et al. 1990).

Immunoblotting and immunoprecipitation

Immunoblotting was performed on in vitro-translated proteins by the method described previously (Yew and Berk 1992) using anti-55K 2A6 (Sarnow et al. 1982b) hybridoma supernatant diluted 1:10. For the immunoprecipitation, COS cells were labeled with Trans³⁵S-label 60 hr post-transfection for 2.5 hr and were then harvested in 10S buffer [250 mM NaCl, 50 mM HEPES (pH 7.2), 0.3% NP-40, 0.1% Triton X-100, and 0.005% SDS]. The extracts were precipitated with trichloroacetic acid (TCA), and 5×10^7 TCA-precipitable counts were used for each sample with 1 μ l of anti-GAL4 antibody using a double immunoprecipitation protocol (Yew and Berk 1992).

DNA immunoprecipitation

The DNA immunoprecipitation assay was performed essentially as described with modifications (Kern et al. 1991). A 160-bp *Hind*III-*Xba*I DNA fragment containing five RGC sites from the pB5E4T plasmid was used. The RGC fragment was ³²P-labeled by end-filling, and 5×10^5 cpm of probe was used in each reaction. DNA probe, 20 μ l of purified p53 from vaccinia virus, and 125 μ l of in vitro-translated E1B 55K protein were incubated for 1 hr at 30°C in 200 μ l with 50 ng/ μ l of poly[d(I-C)] in binding buffer (Kern et al. 1991). Protein A-Sepharose was incubated with 600 μ l of hybridoma supernatants pAb421 (Harlow et al. 1981) or 2A6 for 1.5 hr at room temperature and was washed twice with binding buffer. The antibody bound to protein A-Sepharose was mutated with the DNA, p53, and E1B 55K mixture for 1 hr at room temperature. The reaction was washed twice with binding buffer, processed as described (Kern et al. 1991), and analyzed by electrophoresis in a 5% polyacrylamide TBE gel, followed by autoradiography.

EMSA

The 160-bp DNA fragment used in the mobility-retardation assay was cut from pB5E4T using *Bam*HI and *Hind*III and was ³²-end-labeled with T4 polynucleotide kinase. Purified ep53 protein (1 μ l) was added to unprogrammed reticulocyte lysate or in vitro-translated E1B 55K protein (1, 2, or 4 μ l) and incubated with 1000 cpm of ³²P-labeled DNA probe in mobility-shift buffer (60 mM KCl, 12% glycerol, 5 mM MgCl₂, 1 mM EDTA, 10 mg/ml of poly[d(GC)] at 30°C for 40 min. Purified ep53 alone did not produce as intense a mobility-shift signal as when either 50 μ g/ml of BSA or reticulocyte lysate was added with the purified p53 to provide carrier proteins. Samples were analyzed on a 1.4% agarose gel containing 0.5 $\times$ TBE (0.045 mM Tris-borate, 0.045 mM sodium borate, 0.001 mM EDTA).

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