is also possible that an enzyme could be activated when phosphorylated at an active site, if the substrate contains a positively charged group. Thus, covalent modification at the active site provides an alternative mechanism to the allosteric mechanism demonstrated for phosphorylase (*10*).

Site-directed mutagenesis provides a means to analyze the mechanisms by which the phosphorylation of serine residues regulates protein function. Substitution with aspartate or glutamate should reveal the magnitude of the electrostatic effects, while substitution with bulky residues, such as tyrosine, should analyze the steric effects. The approach becomes particularly powerful if a combination of substrate analogs and mutagenized protein is used to evaluate the quantitative contribution of each factor.

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Presence of a Potent Transcription Activating Sequence in the p53 Protein

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The p53 gene is frequently mutated in a wide variety of human cancers. However, the role of the wild-type p53 gene in growth control is not known. Hybrid proteins that contain the DNA binding domain of yeast GAL4 and portions of p53 have been used to show that the p53 protein contains a transcription-activating sequence that functions in both yeast and mammalian cells. The NH_2-terminal 73 residues of p53 activated transcription in mammalian cells as efficiently as the herpes virus protein VP16, which contains one of the strongest known activation domains. Combined with previous data that showed p53 is localized to the nucleus and can bind to DNA, these results support the idea that one function of p53 is to activate the transcription of genes that suppress cell proliferation.

THE CELLULAR PROTEIN P53 IS INvolved in normal cell growth control and in transformation. It was originally identified as a protein that forms a stable complex with the SV40 large tumor antigen and the adenovirus E1b protein (*1*). In many transformed cells, p53 is found with a much increased half-life and in correspondingly elevated concentrations (*2*). Although introduction of p53 into primary cells was shown to result in immortalization and (in cooperation with an activated *ras*) to result in transformation (*3*), experiments have shown that these p53 genes contained mutations (*4*). The wild-type p53 gene is incapable of transforming cells and can inhibit transformation by mutant p53 genes and other oncogenes (*5*). In addition, mutant versions of the p53 gene are found in a large variety of human tumors, and the encoded proteins may inactivate the wild-type p53 protein by forming inactive oligomeric complexes (*6*). Thus wild-type p53 is classified as a tumor suppressor gene or recessive oncogene, whose inactivation can lead to the transformation process (*5, 6*).

The biochemical mechanisms by which p53 acts in cell proliferation are as yet unknown. The protein is mainly localized to the nucleus and at least some fraction of the protein appears to be associated with chromatin or the nuclear matrix (*7*). The p53 protein is multiply phosphorylated (*8*) and is capable of binding to double-stranded and single-stranded DNA (*9*). In cotransfection assays with a reporter gene containing the long terminal repeat (LTR) promoter of an intracisternal A particle, p53 was able to increase the activity of the reporter gene (*10*). However, these experiments did not provide evidence that p53 specifically affected transcription, nor did they show whether the observed enhancement was a direct or indirect effect of the transfected p53. Although p53 may function as an activator of transcription, a direct test of this function cannot be performed in the absence of knowledge of its target genes. We now show that p53 has an activating domain by constructing hybrid proteins between portions of p53 and a heterologous DNA binding domain, in this case the DNA binding domain from the yeast protein GAL4.

For assaying transcriptional activation in yeast, we used strain GGY1::171 (*11*), which is deleted for *GAL4* and contains an integrated *GAL1-lacZ* under the regulation of the GAL4 protein (Fig. 1A). The source of p53 sequences was the plasmid pR4-2 (*12*), which contains a cDNA copy of a mutant p53 mRNA from the human A431 cell line. The mutation is His instead of Arg at residue 273, which has also been observed in breast and colon tumors (*6*). We constructed five plasmids (*13*) encoding GAL4-p53 hybrids that could be expressed in yeast (Fig. 1B), using as a parental vector the plasmid pMA424 (*14*). Vector pMA424 contains the yeast *ADH1* promoter fused to a fragment of the *GAL4* gene encoding the NH_2-terminal domain (amino acids 1 to 147) of GAL4. This region of GAL4 (designated $GAL4_D$) is capable of localizing to the nucleus and binding to specific sequences upstream of the *GAL1-lacZ* gene, but will not itself activate transcription (*15*). Thus β-galactosidase activity is a measure of the activating function of the sequences fused to the DNA binding domain. In addition, pMA424 carries sequences for selection in yeast and for replication in high copy. As controls, we used the GAL4 DNA binding domain alone and the entire GAL4 protein.

The wild-type GAL4 protein activated significant transcription of the *GAL1-lacZ* gene, and hybrids containing the first 137 amino acids, 346 amino acids, or all (393 amino acids) of the p53 protein activated to varying degrees, ranging from approximately

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10% to 50% of the wild-type GAL4 (Fig. 1B). A hybrid containing the COOH-terminal 234 amino acids of p53 did not activate. We could not obtain stable yeast transformants of the hybrid containing only the first 73 amino acids of p53, although very small colonies carrying this hybrid did produce a blue color on plates containing X-gal, indicating that this NH_2-terminal fragment also could activate transcription (*16*). RNA analysis, by primer extension, indicated that transcription activated by p53 used the same start site as that activated by the native GAL4 protein (Fig. 1C).

Unlike the parental vector or any of the three plasmids that could transform yeast and carry only portions of the p53 gene, the plasmid carrying $yGAL4_D$-p53(1–393) caused slow growth in yeast, seen as small colonies on a plate or a much increased doubling time (Fig. 1D). Continued incubations of plates containing yeast expressing this hybrid resulted in the appearance of faster growing colonies, which had much reduced or no β-galactosidase activity (*16*). In addition, the activity of individual transformants with $yGAL4_D$-p53(1–393) showed considerable variation. Thus the presence of the entire mutant p53 protein, expressed as part of a hybrid with the GAL4 DNA binding domain under a strong promoter, is detrimental to yeast, and may lead to mutations in the plasmid that eliminate both the unhealthy phenotype as well as the transactivation function.

To determine whether p53 could activate in mammalian cells, we used reporter plasmids (*17*) (Fig. 2A) that carried the adenovirus E1b TATA box upstream of the chloramphenicol acetyltransferase (CAT) gene, either with or without five GAL4 binding sites inserted upstream of the TATA box. Sequences containing two of the p53 fragments were placed into the vector pSG424 (*18*), which encodes the same GAL4 DNA binding domain under the transcriptional regulation of the SV40 early promoter (Fig. 2B). We transfected these plasmids into Chinese hamster ovary (CHO) cells, as well as transfecting plasmids encoding the DNA binding domain alone, the entire GAL4 protein, and the GAL4 DNA binding domain fused to part of the herpes simplex virus protein VP16 (a strong activator of transcription) (*19*). The $GAL4_D$-p53 hybrids were capable of inducing high amounts of transcription of the CAT gene from the promoter carrying GAL4 binding sites (Fig. 2, B and C). The GAL4 DNA binding domain alone was inactive (Fig. 2C,

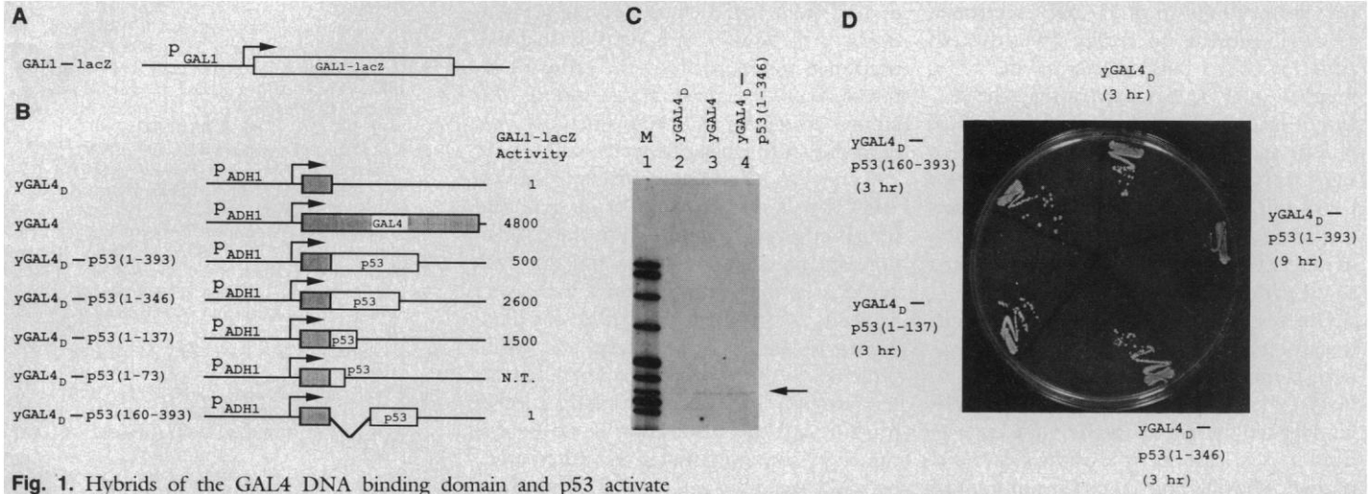

Fig. 1. Hybrids of the GAL4 DNA binding domain and p53 activate transcription in yeast. (**A**) The reporter gene is an integrated *GAL1-lacZ* fusion (*11*), which is regulated by the upstream activation sequence (UAS) for the *GAL1,10* genes. (**B**) Hybrids for expression in yeast are prefaced by the letter y; the GAL4 DNA binding domain (amino acids 1 to 147) is designated $GAL4_D$; the entire GAL4 protein (amino acids 1 to 881) is designated GAL4; and the amino acids from p53 are as indicated. Transformants were grown and assayed for GAL1-lacZ activity as described (*25*). N.T., not tested; this plasmid did not produce stable transformants. (**C**) Primer extension assays for the start site of the *GAL1-lacZ* mRNA were performed as described (*14*); lane 1, labeled pBR322 Msp I fragments; lanes 2 to 4, assays containing RNA from cells transformed with the indicated activator. (**D**) Yeasts transformed with the hybrid containing all of the p53 protein grow more slowly than those carrying the other indicated hybrids. The plate contains synthetic minimal media minus His, to maintain selective pressure for the plasmids that carry the *HIS3* gene. Doubling times for these transformants in liquid media minus His are indicated in parentheses.

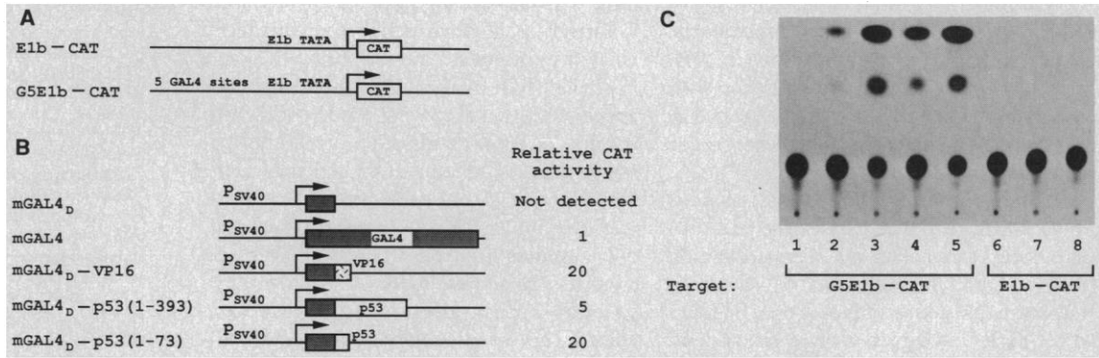

Fig. 2. $GAL4_D$-p53 hybrids activate transcription in mammalian cells. (**A**) The reporter plasmids have the CAT gene containing the E1b TATA either without or with five upstream GAL4 binding sites. (**B**) Hybrids for expression in mammalian cells are prefaced by the letter m; $mGAL4_D$ is carried on plasmid pSG147 (*19*), mGAL4 on pSG4 (*19*) and $mGAL4_D$-VP16 on pSGVP (*19*). CAT activity was normalized to mGAL4. (**C**) The reporter and activator plasmids (5 μg each) were transfected by electroporation, as described by the supplier (BRL), into CHO cells. Cells were collected 48 hours after transfection and assayed for CAT activity (*26*), which was quantified by counting the acetylated choramphenicol forms in an AMBIS Radioanalytic Imaging System. The activators were as follows: lane 1, $mGAL4_D$; lane 2, mGAL4; lane 3, $mGAL4_D$-VP16; lane 4, $mGAL4_D$-p53(1–393); lane 5, $mGAL4_D$-p53(1–73); lane 6, mGAL4; lane 7, $mGAL4_D$-p53(1–393); and lane 8, $mGAL4_D$-p53(1–73).

lane 1), the full-length GAL4 protein activated weakly (lane 2), and the $GAL4_D$-VP16 hybrid was a potent activator (lane 3), as shown previously (*19*). The hybrid carrying all of p53 was a strong activator (lane 4), and the hybrid containing the first 73 amino acids of p53 (lane 5) was as active as $GAL4_D$-VP16, although we have not determined the relative protein concentrations of the p53 and VP16 hybrids. The activation observed was strictly dependent on the presence of GAL4 binding sites, as no CAT activity was observed in their absence (lanes 6 to 8), and the transcriptional start site for RNA induced by the p53(1–73) hybrid was the same as that induced by the VP16 hybrid (*16*). The NH_2-terminal p53 fragment may be a stronger activator than the full-length protein for several reasons. The acidic residues may be more accessible to the transcriptional machinery, the full-length protein may bind to other DNA sequences or other proteins to reduce its activation from the GAL4 binding sites, or the acidic fragment may be more stable than the full-length protein. That the first 73 residues of p53 are such a potent activator in CHO cells suggests that in yeast this fragment may not be tolerated because it titrates out an essential factor and thus reduces the transcription of other yeast genes, an inhibition described as squelching (*20*).

Our results, that hybrids containing portions of p53 are able to activate transcription, combined with previous evidence (*7, 9*) for the nuclear localization and DNA binding activity of p53, suggest that transactivation is at least one role of the native p53 protein. The p53 domain capable of transactivation is at the NH_2-terminus, and this domain is highly acidic, with a net negative charge of −15 in the first 73 amino acids. Similarly charged regions of other proteins have also been shown to be effective transcriptional activators (*21*). In human and mouse p53 amino acid sequences, 29 of the NH_2-terminal 73 residues differ (after introduction of gaps for alignment), although the acidic nature is strictly maintained (*22*). Such divergence in primary sequence with conservation of charge might be expected of an activation domain, for which no precise sequence is required (*14*).

A transactivation function has obvious implications for the activity of the p53 protein in the mammalian cell. Transformation by loss of p53 function may be related to transcriptional activation, as it may be related for such oncogenes as *myb*, *jun*, and *fos*. Given that the wild-type p53 gene is a tumor suppressor gene, normally limiting uncontrolled growth, it might be necessary for the transcription of negative regulators of cell proliferation (Fig. 3). Although the

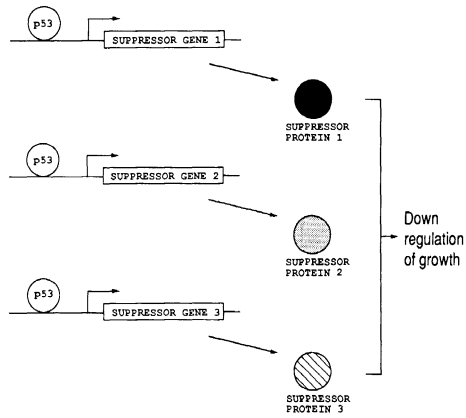

Fig. 3. A possible model for the transactivation function of p53 in cell proliferation. The wild-type p53 protein could coordinately activate the transcription of a number of genes whose products negatively regulate events in cell proliferation.

$GAL4_D$-p53 hybrids we have tested were derived from a p53 clone carrying a mutation at residue 273, it is unlikely that this particular mutation affects the protein's intrinsic ability to activate transcription. Mutations activating for transformation have been shown to cluster between amino acids 132 and 281 (*6*) and only the first 73 amino acids of p53 are necessary for its transcriptional activation function. Thus these mutations are more likely to affect a function such as DNA binding or interactions with other proteins, as already demonstrated for binding to SV40 large T antigen and hsc70 protein (*23*). For example, p53 may activate transcription in association with another protein, and this association could be disrupted by activating mutations. Alternatively, p53, like the adenovirus E1A products (*24*), may have separate functions for transcription and tumor suppression, and it is this latter function that is altered by mutation. In any event, elucidation of potential cellular targets for transactivation by p53 may clarify its normal roles in proliferation. In addition, it will be of interest to determine whether binding of p53 to SV40 large T antigen or adenovirus E1b protein affects its ability to activate transcription.

Finally, two of the $GAL4_D$-p53 hybrids appear detrimental to yeast. The hybrid with the first 73 amino acids of p53 could not be stably introduced into yeast and may activate too strongly to be tolerated. The hybrid with the full-length p53 causes considerable unhealthiness in yeast, whereas shorter derivatives that activate more *GAL1-lacZ* transcription appear to have little deleterious effect. This result suggests that the full-length hybrid is affecting growth control in yeast, perhaps through its direct effect on the transcription of other genes, in addition to those containing GAL4 binding sites. Another possibility is that the full-length p53 might bind to a critical yeast protein, for example, a homolog of p53, and thereby prevent it from exerting its activity. In either case, the ability to obtain faster growing mutants of yeast expressing the full-length hybrid may allow a genetic dissection in yeast of the role of p53.

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13. Yeast GAL4-p53 constructions derive from pMA424 (*14*) and pR4-2 (*12*). The Nco I site at amino acid 1 of the p53 gene was converted with an oligodeoxynucleotide adaptor to an Eco RI site (plasmid pTU6) and the resulting Eco RI fragment carrying the entire coding sequence of p53 was ligated to Eco RI–cut pMA424 to generate $yGAL4_D$-p53(1–393). $yGAL4_D$-p53(1–346) and $yGAL4_D$-p53(1–137) were similarly derived after Stu I–Sma I and Bal I–Sma I truncations, respectively, of pTU6, and carry 28 non-p53 amino acids before terminating. $yGAL4_D$-p53(1–73) was constructed by first ligating an Eco RI–Hae III fragment from pTU6 into Eco RI–Sma I–cut pUC18 [C. Yanisch-Perron, J. Vieira, J. Messing, *Gene* **33**, 103 (1985)] to yield pUB34, with the resulting Eco RI–Bam HI fragment of the p53 gene ligated into pMA424. This hybrid carries an additional nine non-p53 amino acids before terminating. $yGAL4_D$-p53(160–393) was constructed by changing the Nco I site at amino acid 160 of the p53 gene with an oligodeoxynucleotide adaptor into an Eco RI site, followed by ligation of the resulting Eco RI fragment into pMA424. yGAL4 is the entire GAL4 coding sequence under the control of the *ADH1* promoter, carried on a centromere plasmid.
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18. pSG424 is similar to pSG147 (*19*) with additional polylinker restriction sites, and was provided by the Ptashne laboratory. $mGAL4_D$-p53(1–393) was constructed by ligating the Eco RI fragment containing

the entire p53 coding sequence (*13*) into the Eco RI site of pSG424. mGAL4$_D$-p53(1–73) was constructed by ligating an Eco RI–Xba I fragment from pUB34 (*13*) into the Eco RI–Xba I sites of pSG424.
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Transcriptional Activation by Wild-Type But Not Transforming Mutants of the p53 Anti-Oncogene

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The protein encoded by the wild-type p53 proto-oncogene has been shown to suppress transformation, whereas certain mutations that alter p53 become transformation competent. Fusion proteins between p53 and the GAL4 DNA binding domain were made to anchor p53 to a DNA target sequence and to allow measurement of transcriptional activation of a reporter plasmid. The wild-type p53 stimulated transcription in this assay, but two transforming mutations in p53 were unable to act as transcriptional activators. Therefore, p53 can activate transcription, and transformation-activating mutations result in a loss of function of the p53 protein. The inability of the p53 mutant proteins to activate transcription may enable them to be transformation competent.

THE WILD-TYPE P53 PROTEIN FUNCTIONS to suppress transformation, and mutations have been reported that result not only in the loss of this function, but the gain of another function, the ability to actively transform a cell (*1–6*). In these tissue culture experiments, the expression of the normal p53 gene appears to inactivate the tumorigenic potential of the mutant p53 gene. Other data also suggest that the function of the wild-type p53 gene suppresses transformation. One of the most common genetic alterations that occurs during the development of colorectal tumors is a deletion of the short arm of human chromosome 17, the location of the p53 gene. In two colorectal tumors, both p53 alleles were shown to be altered (*7*), and the tumors did not make a normal p53 gene product. Additional studies with small cell lung cancer tumors and osteosarcoma samples have shown that these types of tumors also contain genomic rearrangements, deletions, or small mutations in the p53 gene in approximately half of the samples studied (*8, 9*).

Experiments with cells in culture suggest that p53 has a regulatory role, although the actual function has remained elusive. Both the p53 mRNA and protein have extremely short half lives, and a nuclear targeting signal directs p53 to the nucleus (*10–13*). In addition, p53 also appears to be involved in regulation of the cell cycle (*10*). The concentrations of p53 mRNA are decreased in primary cells in culture on contact inhibition

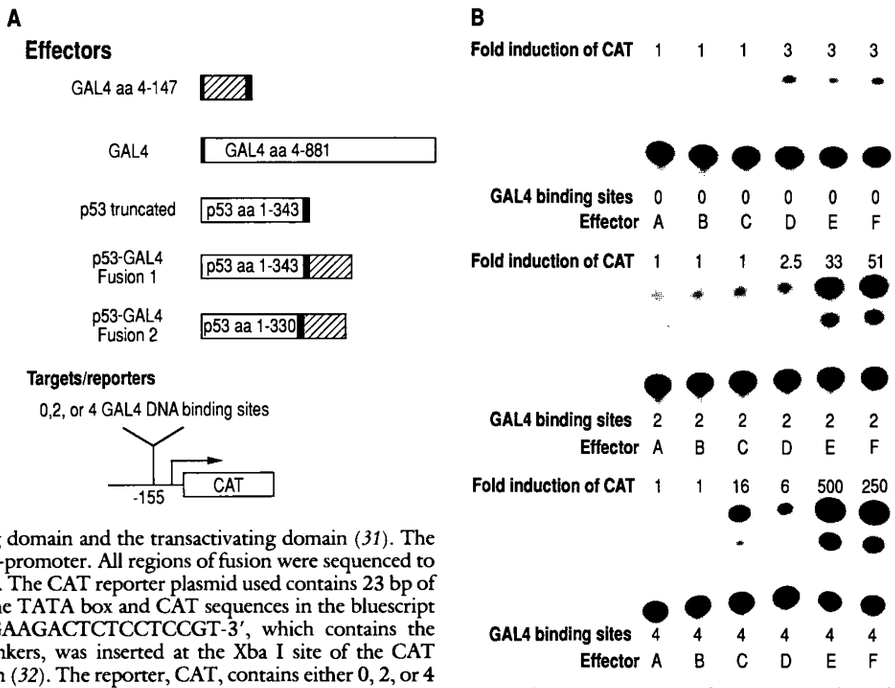

Fig. 1. The p53-GAL4 fusion proteins specifically activate transcription of CAT. (**A**) A diagrammatic representation of the different plasmids used. The effector plasmids contain the NH_2-terminal amino acids of p53 and the sequence coding for the GAL4 DNA binding domain; the reporter plasmid contains CAT coding sequences driven by the SV40 promoter. Two individual in-frame p53-GAL4 fusion proteins were made that contain the p53 transactivating domain and the GAL4 DNA binding domain. The p53-GAL4 plasmids consist of the Harvey murine sarcoma virus (H-MSV)–long terminal repeat, wild-type p53 coding sequences from amino acid 1–343 (fusion 1) or amino acids 1–330 (fusion 2) dispersed by p53 introns 2 and 3 and fused to GAL4 sequences (amino acids 4–147). The DNA construct labeled "p53 truncated" contains the H-MSV LTR and encodes only p53 amino acids 1–343. All p53 clones retain the nuclear localization signal of p53. GAL4 (amino acids 4–147) encodes only the GAL4 DNA binding domain. GAL4 (amino acids 4–881) encodes the entire GAL4 protein consisting of both the DNA binding domain and the transactivating domain (*31*). The GAL4 plasmids also use the H-MSV LTR enhancer-promoter. All regions of fusion were sequenced to ensure that the protein sequences remained in-frame. The CAT reporter plasmid used contains 23 bp of the SV40 enhancer, the SV40 21-bp repeats, and the TATA box and CAT sequences in the bluescript vector (*27*). The oligonucleotide 5′-CTAGACGGAAGACTCTCCTCCGT-3′, which contains the GAL4 recognition sequence bounded by Xba I linkers, was inserted at the Xba I site of the CAT plasmid, 155 bp upstream of the start of transcription (*32*). The reporter, CAT, contains either 0, 2, or 4 DNA recognition sequences for the GAL4 binding domain. (**B**) The activator and reporter plasmids (10 μg of each) were cotransfected with a plasmid containing the β-galactosidase gene (5 μg) with calcium phosphate precipitation into HeLa cells essentially as described (*33*). A β-galactosidase plasmid was used to monitor and normalize for transfection efficiency. The activity of CAT is measured by the conversion of [^{14}C]chloramphenicol (lower dot) to acetyl and diacetyl chloramphenicol, respectively (*34*). Effector A, none; effector B, GAL4 (amino acids 4–147); effector C, GAL4; effector D, truncated p53; effector E, p53-GAL4 (fusion 1); and effector F, p53-GAL4 (fusion 2).