that an effector-signaling domain is present in the NH_2-terminus of GAP. Such a model is also supported by studies showing that the inhibition muscarinic atrial K^+ channel currents by Ras depends on interaction of Ras with GAP (*17*, *22*). The putative signaling domain on GAP seems to be located within the sequence containing Src-homology regions because experiments done with a truncated form of GAP lacking the NH_2-terminal hydrophobic region (nucleotides 392 to 3140) gave results in our experiments identical to those obtained with full-length GAP (*15*). However, expression of a fragment of GAP containing amino acids 1 to 701, which contains the SH2-SH3 domain but lacks the binding region to Ras, did not modify the transactivation of Py-TK-CAT in presence or absence of any of the plasmids described above (*15*). Thus it is not clear which domain on the NH_2-terminus of GAP is critical for signal transduction. Increased expression of GAP in CHO cells did not modify signaling by oncogenic Ras. It may be that GAP, as an effector, is already more abundant than Ras (*23*) or that the supply of GAP that acts as an effector is tightly regulated within the cell.

GAP may be a direct link between Ras-GTP and other downstream effectors. GAP enhances the action of transforming v-Ha-Ras and insulin in *Xenopus* oocytes (*24*), although GAP itself is unable to promote a full maturation response in oocytes (*25*). These data indicate that GAP may act to transmit signals to other effectors, although it is possible that GAP enhances the Ras effect by binding an inhibitor of maturation, such as a related *rap* gene product. One interpretation of our data is that GAP-C, lacking SH2-SH3 sequences and the regions necessary to bind lipids, recognizes and traps the oncogenic Ras-GTP complex and thereby prevents it from properly eliciting a downstream signal. This model also implies that GAP-C should induce phenotypic reversion. However, GAP-C does not have anti-oncogenic properties (*12*, *14*), perhaps because the affinity of GAP-C for Ras-GTP is not high enough. It is not known whether the relative or absolute amount of Ras-GTP is more important for signaling by Ras. However, a modified GAP-C with higher affinity for Ras, such as that displayed by NF1-GAP (an alternative effector for Ras-GTP in all cells) (*26*, *28*) might induce reversion of oncogenic Ras transformation. For example, Rap1a, which competes with GAP for binding to Ras in vitro (*29*, *30*), has anti-oncogenic properties in a cell line transformed by v-Ki-*ras* (*31*). Blocking the interaction between Ras-GTP and GAP is thus a potential mechanism of action for a therapeutic drug.

Note added in proof: The SH2-SH3 domain of GAP appears to participate in the inhibition of muscarinic atrial K^+ channel currents by Ras (*32*).

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Oncogenic Forms of p53 Inhibit p53-Regulated Gene Expression

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Mutant forms of the gene encoding the tumor suppressor p53 are found in numerous human malignancies, but the physiologic function of p53 and the effects of mutations on this function are unknown. The p53 protein binds DNA in a sequence-specific manner and thus may regulate gene transcription. Cotransfection experiments showed that wild-type p53 activated the expression of genes adjacent to a p53 DNA binding site. The level of activation correlated with DNA binding in vitro. Oncogenic forms of p53 lost this activity. Moreover, all mutants inhibited the activity of coexpressed wild-type p53, providing a basis for the selection of such mutants during tumorigenesis.

The gene for the nuclear phosphoprotein p53 is the most commonly mutated gene yet identified in human cancers (*1*). Missense mutations occur in tumors of the colon, lung, breast, ovary, bladder, and several other organs (*2*–*4*). The *p53* gene can suppress the growth of transformed murine (*5*) or human cells (*6*, *7*), but oncogenic forms lose this suppressor function.

Evidence suggests that p53 may regulate gene transcription. A nuclear protein, p53 can bind to DNA in vitro both sequence specifically (*8*, *9*) and nonspecifically (*10*). In addition, the NH_2-terminus of p53 behaves as an acidic transcriptional activation domain when fused to GAL4 (*11*, *12*).

In this report we test whether p53 can directly activate gene expression through its DNA binding site and whether oncogenic forms of p53 can interfere with this activation. Reporter plasmids (PG_n-CAT series) contained the polyomavirus early promoter and the chloramphenicol acetyl transferase (CAT) gene located downstream of a DNA sequence (PG) that binds p53 in vitro (Fig. 1). The reporter and an expression vector that encoded human wild-type p53 (*13*) were transfected together into the human colorectal cancer cell line HCT 116 (*14*). This cell line makes low amounts of p53 protein (*15*).

The intact wild-type p53 protein acti-

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vated CAT gene expression, and the activation correlated with the number of p53 DNA binding sites (PG repeats) upstream of the reporter (Fig. 2). The larger the number of PG repeats, the greater the binding to p53 in vitro (Fig. 2A) and the higher the CAT expression in vivo (Fig. 2B, lanes 2 to 7). Transactivation also increased with increasing amounts of p53 transfected (Table 1). When GC base pairs of the PG oligonucleotide were altered to form MG repeats (Fig. 1), the resulting sequence did not bind p53 in vitro (Fig. 3) or activate CAT expression in vivo (Table 1; Fig. 2B). The transactivation of CAT was independent of the orientation of the PG multimer (legend to Fig. 2B) and its distance from the promoter (*16*).

The correlation between p53 binding to the PG multimer in vitro (Fig. 2A) and the expression from PG_n-CAT reporters in vivo (Fig. 2B) suggested that p53 directly activated CAT gene expression in vivo by binding to PG multimers. If this activity were crucial to the tumor suppressor activity of wild-type p53, naturally occurring

Table 1. Activation of gene expression in human cells by p53. Transfection and CAT assays were performed as described (*14*) with 1.7 μg of reporter vector. Activities reflect the fraction of chloramphenicol converted to an acetylated form, expressed relative to the CAT activity observed with the p53-wt transfection in each experiment. In experiments 1, 2, and 3, 1.7, 0.85, and 2.55 μg of expression vector, respectively, were used. The CAT activity with the p53-wt expressor was 2.1-fold higher in experiment 3 than in experiment 2, which were done concurrently.

p53 expression vector	Reporter	Relative CAT activity		
		Exp. 1	Exp. 2	Exp. 3
p53-wt	PG_{13}-CAT	100	100	100
p53-wt	MG_{15}-CAT		<1	
p53-143	PG_{13}-CAT	3	1	2
p53-175	PG_{13}-CAT	2	3	3
p53-248	PG_{13}-CAT	2	4	3
p53-273	PG_{13}-CAT	2	2	1

Table 2. Activation of gene expression in yeast by p53. Wild-type or mutant p53 expression vectors and the β-gal reporter plasmids were transfected into *S. cerevisiae* and clones were obtained. The p53 expression was induced with galactose, and β-gal activity was measured in units of nanomoles per minute per milligram of protein. Two independent clones [experiment 1 and experiment 2] were tested. Strain construction and assays were performed [as described (*27*)]. Less than 1 unit was seen in the absence of galactose induction. The residual activity of the Yp53-143 mutant may have been due to a slight wild-type activity observable with valine to alanine substitution mutants at the relatively low temperature (30°C) used for yeast growth (*28*).

p53 expression vector	Reporter	β-gal activity	
		Exp. 1	Exp. 2
None	PG_{16}-*lacZ*	3	2
Yp53	PG_{16}-*lacZ*	13,000	8,000
Yp53-143	PG_{16}-*lacZ*	150	85
Yp53-273	PG_{16}-*lacZ*	5	2
Yp53	PG_4-*lacZ*	15,000	11,000
Yp53	PG_1-*lacZ*	2,600	3,000
Yp53	MG_{15}-*lacZ*	15	4

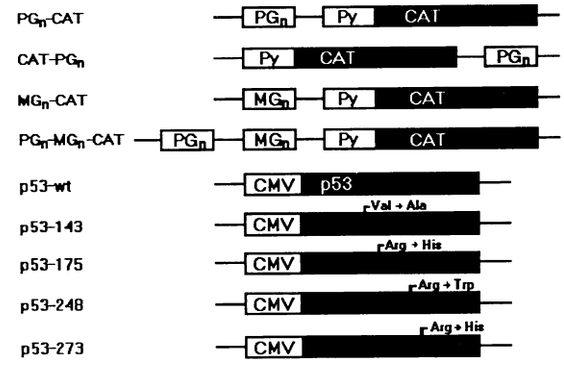

Fig. 1. Reporter and expresser constructs used in transfections. PG_n, n copies of the p53 binding sequence PG; MG_n, n copies of a mutated sequence, which does not bind p53; CAT, the chloramphenicol acetyltransferase coding sequence; Py, the early gene promoter from polyomavirus; CMV, the cytomegalovirus promoter from the parent vector pCMVNeoBam. Constructions are detailed in (*13*).

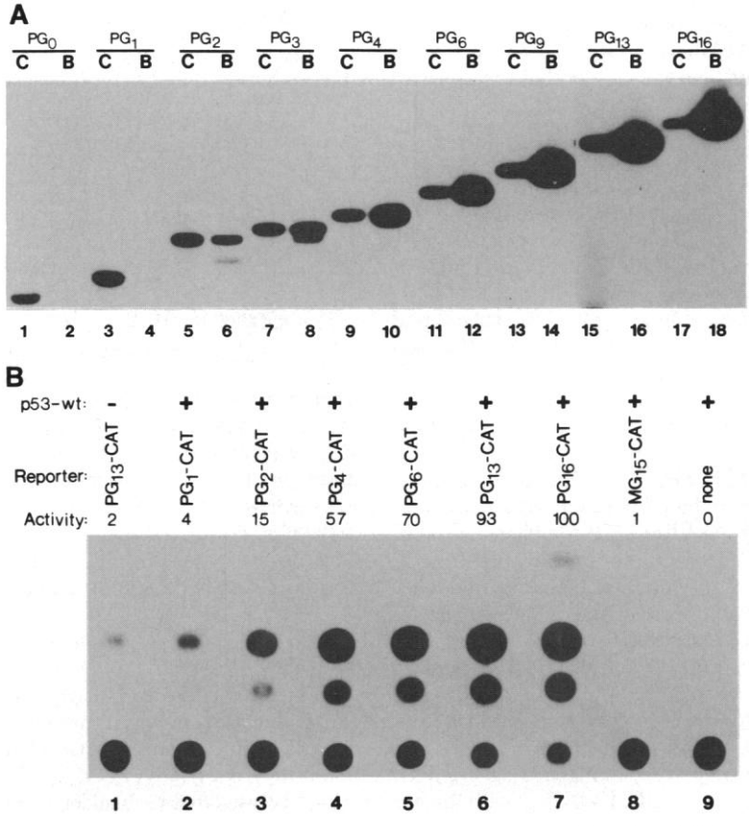

Fig. 2. Correlation of DNA binding and transactivation. (**A**) Relative DNA binding abilities of clones containing repeats of a p53-binding sequence (PG_n series), in an immunoprecipitation assay. Clones were cleaved by restriction endonucleases to extricate the PG_n repeat, end-labeled, incubated with purified baculovirus-produced wild-type human p53, and immunoprecipitated with anti-p53 and protein A–Sepharose; bound fragments were recovered and separated on a nondenaturing polyacrylamide gel [as described in (*8*)]. In the absence of antibodies, less than 0.5% of the labeled DNA was found in the precipitate. C, control lane, containing 2% of the labeled DNA used in the binding reactions; B, bound DNA recovered from the immunoprecipitates. (**B**) Transactivation efficiencies of reporters containing the various PG_n concatemers compared by CAT assay. Equal amounts (1.7 μg) of the given reporter and the expression vector p53-wt were transfected into HCT 116 cells. Reporters had one orientation of the PG_n sequence (. . . TGCCT . . . Py . . . CAT . . .), except for PG_2-CAT and PG_{13}-CAT, which had the opposite orientation (. . . AGGCA . . . Py . . . CAT . . .). CAT assays were performed [as described (*14*)]. Results are expressed relative to the CAT activity in lane 7, which was arbitrarily set at 100.

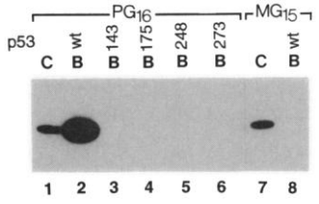

Fig. 3. Comparison of the ability of wild-type and mutant p53 to bind to PG_{16} and lack of DNA binding ability of MG_{15}. Each p53 form was produced in a rabbit lysate (*10, 23*) and equalized for p53 quantity by Western blot analysis (*18*). C, control lanes, containing 2% of the labeled DNA used in the binding reaction. B, bound DNA recovered from immunoprecipitations, performed as described (*23*).

p53 mutants might be defective in this function. To test this, we transiently cotransfected mutant p53 genes into cells together with PG_{13}-CAT (*17, 18*). All tumor-derived mutations examined lost the ability to transactivate PG_{13}-driven CAT expression (Table 1). The inability of p53 proteins having tumor-derived mutations to bind specifically to the p53 recognition sequences in this construct (Fig. 3, lanes 3 to 6) was thus consistently reflected as defects in transactivation (*19*).

If the ability of p53 to bind to DNA sequences in vivo and activate the transcription of adjacent genes were an intrinsic feature of the protein, this activity should be transferable to simpler eukaryotes. Indeed, the NH_2-terminal acidic activation domain of p53 has been reported to function in *Saccharomyces cerevisiae* when fused to the DNA binding domain of GAL4 (*11*). We thus stably transformed yeast with the *lacZ* reporter gene placed downstream of PG multimers (PG_n-*lacZ*) and a galactose-inducible p53 expression vector (Yp53). The addition of galactose to the medium resulted in p53 expression accompanied by a striking elevation in β-galactosidase (β-gal) activity (Table 2). When the MG multimer (non–p53 binding) was substituted for the PG multimer, little activation of β-gal was observed (Table 2). Moreover, the ability of p53 mutants to transactivate β-gal in yeast cells was reduced by more than 80-fold compared to the ability of the wild-type p53. Thus, the results in *S. cerevisiae* were similar to those observed in human cells.

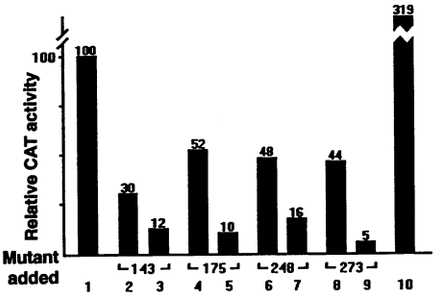

Fig. 4. Dominant-negative effects of various mutant p53 proteins. The p53-wt (0.85 μg) was used in all transfections, without (lane 1) or with the addition of 0.85 μg (lanes 2, 4, 6, 8) or 2.55 μg (lanes 3, 5, 7, 9) of mutant p53 construct, or with an additional 2.55 μg of p53-wt (lane 10). The PG_{13}-CAT reporter (1.7 μg) was used in each transfection. The results shown are representative of at least two transfections done on separate days.

It is thought that mutant p53 can inhibit the growth suppressor effects of wild-type p53 through a "dominant negative" action (*5–7, 20, 21*). To determine whether an analogous dominant negative effect could be observed on transcriptional activation, we cotransfected wild-type and mutant p53 with the PG_{13}-CAT reporter into HCT 116 cells. When equal amounts of wild-type and mutant p53 expression vectors were used, the expression of CAT decreased by approximately 50% compared to that achieved by wild-type p53 alone (Fig. 4, lanes 2, 4, 6, and 8). Increasing the ratio of mutant p53 to wild-type p53 (*22*) produced an 84 to 95% reduction (Fig. 4, lanes 3, 5, 7, and 9). When additional wild-type p53 expression vector was substituted for mutant p53 expression vector in the cotransfection experiment, the CAT expression increased rather than decreased, as expected (Fig. 4, lane 10).

The inhibitory effect could be caused by a failure of the mutant–wild-type complexes to bind to DNA or by a failure to activate transcription once bound. To distinguish between these two possibilities, we cotranslated wild-type p53 and the His^{175} mutant of p53 in vitro. Under these conditions, it has been shown that wild-type p53 forms hetero-oligomers with mutant p53 (*23*). The mutant p53 protein significantly inhibited the ability of wild-type protein to bind DNA in these experiments (Fig. 5), providing an explanation for the inhibitory effects seen in vivo (Fig. 4).

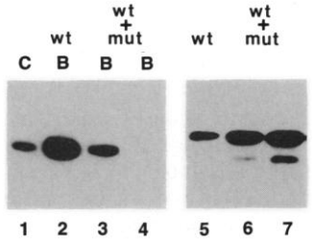

Fig. 5. Inactivation of in vitro DNA binding of wild-type p53 by mutant forms. An end-labeled fragment containing the PG_{13} repeat was immunoprecipitated with 10 μl of an in vitro translation reaction made with 0.1 μg of wild-type p53 RNA (lane 2). In lanes 3 and 4, 0.1 and 0.3 μg, respectively, of His^{175} RNA was used in addition to 0.1 μg of wild-type p53 template. In vitro translations were performed as described (*23*). B, bound DNA recovered from immunoprecipitations; C, control lane containing 2% of the labeled DNA used in the binding reactions. In lanes 5 to 7, 10 μl of the in vitro translation reactions used for lanes 2 to 4, respectively, were separated by SDS–polyacrylamide gel electrophoresis and assessed by Western blot for p53 protein concentration (*18*).

Although several properties of p53 have been previously described (*21*), the ability to bind to specific DNA sequences and control adjacent gene transcription is the only functional property consistently lost in mutants from all four evolutionarily conserved domains of the p53 protein (Fig. 3 and Table 1). This suggests that transcriptional regulation is fundamental to wild-type p53 function.

The *p53* gene can inhibit the expression of some reporter gene constructs (*24*); however, it is not known whether such effects are mediated by p53 binding to adjacent sequence elements. Our results do not exclude the possibility that p53 can directly inhibit, as well as activate, transcription of genes. Precedents exist for the ability of a single transcription factor to either activate or inhibit expression, depending on the sequence used to drive expression (*25*).

The simplest interpretation of the dominant-negative effects of the p53 mutants is that mutant and wild-type p53 form a heteromeric complex (*23, 26*) that is debilitated in its ability to bind DNA, and therefore cannot control transcription of genes adjacent to these binding sites. Such adjacent genes may include those required for inhibition of cell growth or invasion; this would explain how p53 mutations lead to tumor progression.

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13. Direct repeats of the oligonucleotide PG (5′-CCTGCCTGGACTTGCCTGG-3′) were used for the p53 binding sequences. Each copy of the repeat contained the binding region of plasmid C_{BE}, previously shown to bind p53 in vitro (*8*). For the nonbinding control sequence, repeats of the oligonucleotide MG (5′-CCTTAATGGACTTTAATGG-3′) were used. For the CAT reporters, repeats of PG were ligated into the Eco RV site of pBluescript II SK+ (Stratagene, La Jolla, CA) to form the PG_n and MG_n series. The Bgl II–Bam HI fragment of pPyOlCAT [Y. Murakami, M. Asano, M. Satake, Y. Ito, *Oncogene* **5**, 5 (1990)], containing the polyomavirus early promoter and the CAT gene coding region, was then ligated into the Bam HI site of the PG_n and MG_n series clones to form the PG_n-CAT and MG_n-CAT series. The PG_n-MG_n-CAT series was formed by excising the Hind III–Sal I fragments of PG_n-CAT, blunt-ending, attaching Xba I linkers, and ligating into the Xba I site of the MG_n-CAT series plasmids. The construction of the p53–wild-type (p53-wt) expression construct has been described [identical to pC53-SN3 in (*6*)]; the mutant expression plasmids (Fig. 1) were constructed similarly from previously described clones (*10*). The mutants studied included one which was typical of those found in the germ line of Li-Fraumeni patients (Trp^{248}) [D. Malkin *et al.*, *Science* **250**, 1233 (1990); S. Srivastava, Z. Zou, K. Pirollo, W. Blattner, E. H. Chang, *Nature* **348**, 747 (1990)] and three found commonly in a variety of human tumors (Val^{143}, His^{175}, His^{273}) (*4*).
14. Cultures of HCT 116 cells at 50 to 80% confluence in 25-m^2 flasks were transfected with Lipofectin [Bethesda Research Laboratories (BRL), Gaithersburg, MD] according to the manufacturer's instructions. All flasks within an experiment were transfected with the same total amount of plasmid, with additional pCMVneoBam DNA (*6*) as necessary. Cells were harvested at 20 to 24 hours, and the CAT activity of the lysates was measured by acetylation of ^{14}C-labeled chloramphenicol (ICN, Costa Mesa, CA) as previously described [C. M. Gorman *et al.*, *Mol. Cell. Biol.* **2**, 1044 (1982)]. The Bio-Rad protein assay was used to assure equivalence of lysate protein. Percent conversion to the acetylated form of chloramphenicol was calculated after quantitation of excised chromatographic spots by scintillation counting. Results reported are representative of at least two transfections done on separate days.
15. Exons 5 to 8 of the p53 gene from HCT 116 cells were amplified by polymerase chain reaction (PCR) and sequenced as described in [D. Sidransky *et al.*, *Science* **252**, 706 (1991)]. Previously, these exons had been shown to contain more than 90% of the mutations observed in human tumors (*2–4*). No mutations were observed in HCT 116 cells. Small amounts of apparently wild-type protein could be detected in Western blots of HCT 116 protein, performed as described (*18*). In cells transfected with p53 expression vectors, p53 protein concentrations increased by more than 20-fold.
16. Placing an additional 59 to 333 base pairs between the PG multimer and the promoter (PG_n-MG_n-CAT reporters, Fig. 1) had little effect on transactivation. However, placement of the PG binding sequences downstream of the CAT gene (CAT-PG_n, Fig. 1) did not allow transactivation (S. E. Kern *et al.*, unpublished data).
17. The mutant p53 gene expression construct used in these experiments were not toxic to transfected cells, as shown by (i) continued cell cycle progression after transient expression (*6*); (ii) establishment of stable clones from these transfectants (*6*); and (iii) noninhibition of CMV-driven gene expression, as assessed by measurements of the exogenous p53 protein produced in these cells (*18*).
18. The transfection and expression efficiency of mutant p53 expression vectors was assessed by Western blot analysis. Protein (100 μg) from each cell lysate was separated on a 10% SDS–polyacrylamide gel, transferred onto a polyvinyl membrane, blocked with 5% nonfat milk, incubated with PAb1801 (1 μg/ml) (Oncogene Sciences, Manhasset, NY) then with ^{125}I-labeled goat antibody to mouse immunoglobulin (IgG) (NEN, Boston, MA), and autoradiographed. Expression of mutant p53 was approximately 0.5-, 1.5-, 2.5-, and 1.5-fold that achieved with the p53-wt vector when p53-143, p53-175, p53-248, and p53-273 were used, respectively. For each expression vector, the concentration of p53 protein increased by two- to fourfold when 2.55 μg instead of 0.85 μg of expression vector was used for transfection.
19. The data on transcriptional activation by wild-type p53, and loss of this activity in p53 mutants, were also observed in transiently transfected HeLa cells (S. E. Kern *et al.*, unpublished data).
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22. When 0.85 μg of p53-wt and 2.55 μg of p53 mutant expression vectors were used for cotransfection, the amount of total p53 protein in the transfected cells was two- to fourfold higher than that achieved with 0.85 μg of p53-wt alone, as assessed by Western blot analysis (*18*).
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27. For the yeast β-gal reporter plasmids, PG and MG sequences in Sal I–Sma I fragments were ligated to the Sal I and filled in Xho I sites of pCZ [A. R. Buchman *et al.*, *Mol. Cell. Biol.* **8**, 5086 (1988)] to create PG_n-*lacZ* and MG_n-*lacZ*. The construction of the yeast p53 expression vectors has been described [J. M. Nigro, R. Sikorski, S. I. Reed, B. Vogelstein, *ibid.* **12**, 1357 (1992)]. The Yp53 expressed wild-type p53, whereas Yp53-143 and Yp53-273 have the same p53 mutation as p53-143 and p53-273, respectively. The reporter and expressor vectors were introduced into *S. cerevisiae*, strain YPH420, and clones were obtained. Overnight cultures grown at 30°C in synthetic liquid medium containing raffinose as the carbon source were diluted to an absorbance at 600 mm of 0.1 in medium containing raffinose and galactose (1:1) and growth continued for another 18 to 24 hours. Cells were harvested and resuspended in breaking buffer [100 mM tris-HCl (pH 8.0), 1 mM dithiothreitol, 0.1 mg/ml of bovine serum albumin, and 20% glycerol]. Cells were disrupted in the presence of 2 mM phenylmethylsulfonyl fluoride by vortexing with glass beads (0.4 mm), clarified by centrifugation; protein concentration was determined by the Bio-Rad assay. The β-gal assays were performed with *o*-nitrophenyl-β-D-galactoside as described [J. H. Miller, *Experiments in Molecular Genetics* (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1972)]. After galactose induction, p53 expression was similar in transformants containing wild-type and mutant expression vectors as assessed by Western blot analysis (*18*).
28. Conversion of an alanine to a valine substitution mutant to a wild-type p53 form at low temperatures has been observed in mammalian cells [D. Michalovitz, O. Halevy, M. Oren, *Cell* **62**, 671 (1990); J. Martinez, I. Georgoff, J. Martinez, A. J. Levine, *Genes Devel.* **5**, 151 (1991)].
29. We thank D. Sidransky for performing the sequence analyses of HCT 116 p53 genes, C. Prives for the recombinant baculoviruses encoding p53, P. Hieter for advice on the yeast experiments, A. Buchman for providing pCZ, M. Brattain for HCT 116 cells, and Pharmagenics, Inc. Allendale, NJ, for synthesis of oligonucleotides. Supported by the Clayton Fund, McAshan Fund, Preuss Foundation, and grants CA06973, CA09243, and CA35494.

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Cholecystokinin Antianalgesia: Safety Cues Abolish Morphine Analgesia

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Environmental stimuli that signal the occurrence of aversive or dangerous events activate endogenous opiate analgesia systems. Signals for safety (the nonoccurrence of aversive events) produce the opposite and inhibit environmentally produced analgesia. Stimuli that signal safety are now shown to abolish the analgesic effect of morphine, even when morphine is applied directly to spinal cord. Further, this antiopiate effect occurs because the environmental stimulus leads to release of the neuropeptide cholecystokinin in the spinal cord. This process may contribute to the regulation of pain and the development of opiate tolerance.

The discovery of endogenous pain inhibitory circuitries in the central nervous system (CNS) led to investigation of environmental events that trigger their activation (*1*). In general, these neural circuits are activated by contact either with noxious stimuli or with cues that signal such noxious events (that is, innate or learned danger signals) (*1*). The CNS may also contain circuitry that can inhibit pain inhibition systems (*2–4*). This evidence rests, to date, on the effects of administering exogenous agonists and antagonists of possible antianalgesia neurotransmitters. In agreement with the concept of antianalgesia systems, agonists block whereas antagonists or anti-

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