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Genetics

Use of genetic suppressor elements to dissect distinct biological effects of separate p53 domains

(tumor suppressor/dominant negative mutants/immortalization/drug resistance/retroviral library)

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ABSTRACT **p53 is a multifunctional tumor suppressor protein involved in the negative control of cell growth. Mutations in p53 cause alterations in cellular phenotype, including immortalization, neoplastic transformation, and resistance to DNA-damaging drugs. To help dissect distinct functions of p53, a set of genetic suppressor elements (GSEs) capable of inducing different p53-related phenotypes in rodent embryo fibroblasts was isolated from a retroviral library of random rat p53 cDNA fragments. All the GSEs were 100–300 nucleotides long and were in the sense orientation. They fell into four classes, corresponding to the transactivator (class I), DNA-binding (class II), and C-terminal (class III) domains of the protein and the 3′-untranslated region of the mRNA (class IV). GSEs in all four classes promoted immortalization of primary cells, but only members of classes I and III cooperated with activated *ras* to transform cells, and only members of class III conferred resistance to etoposide and strongly inhibited transcriptional transactivation by p53. These observations suggest that processes related to control of senescence, response to DNA damage, and transformation involve different functions of the p53 protein and furthermore indicate a regulatory role for the 3′-untranslated region of p53 mRNA.**

p53 is a growth regulatory gene that acts as an essential component of cell-cycle checkpoints (for review, see ref. 1). Stabilization of p53 occurs under conditions of stress, including DNA damage (2, 3), deregulation of microtubule assembly (4), detachment of cells from their normal substrate (5), hypoxia (6), and cell aging (7). Depending on the cell type, activation of p53 results either in growth-arrest or in apoptosis (8). p53 is a tetrameric nuclear transcription factor which activates several genes (1). In addition, it can suppress transcription from a number of promoters (9) and directly inhibit DNA replication (10). The activity of those p53-responsive genes that have been identified accounts in part for some p53-mediated checkpoint controls and for feedback regulation of p53 activity (1). However, many upstream and downstream components of p53 signaling pathways, as well as the factors determining cell-specific responses to p53 activation, have yet to be identified.

Inactivation of p53 by deletion, mutation or protein modification, which occurs in most human cancers (11), leads to immortalization (12), susceptibility to transformation by dominant oncogenes (13), loss of control over genomic stability (14), and resistance to radiation and chemotherapeutic drugs due to suppression of apoptosis (15). All of these effects may contribute to tumor progression. Consistently, knockout of the p53 gene in mice does not affect embryonic development, but does increases the probability of malignant disease (12), along with a dramatic loss of genome stability (16).

Structural analysis of the p53 protein has revealed its general organization and delineated the transactivator and oligomerization domains, as well as the regions that bind to DNA and to interacting proteins (1, 17). The properties of some p53 mutants indicate that different functions (for example, G_1 arrest or apoptosis) may be lost independently, suggesting that they are determined by different domains of the protein (18, 19).

A recently developed method employing genetic suppressor elements (GSEs) allows one to identify dominant negative peptides corresponding to different functional domains of a protein (20). GSEs, short fragments of cDNA encoding either inhibitory antisense RNA or dominant negative peptides, are isolated from expression libraries made from short random fragments of a target cDNA by selecting for inhibition of function. This strategy, first tested in a prokaryotic system (21), was then used in mammalian cells to select GSEs from a single gene (20) or from a total cellular cDNA pool (22).

In the present work, we have cloned and characterized short sense GSEs from p53. Considering that p53 acts through interaction of its functional domains with numerous cellular components, we hypothesized that p53-derived peptides, representing such domains, might be used to map, dissect, and analyze distinct functions of p53. This approach seemed feasible because biologically active truncated proteins and peptides derived from p53 have already been described (23–25). A retroviral GSE library was constructed from randomly fragmented p53 cDNA that was then delivered to mouse and rat embryo fibroblasts (MEFs and REFs), which have an intact p53 pathway. Comparison of the biological activities of the isolated GSEs revealed major differences among elements corresponding to different p53 regions, indicating that the processes of cellular senescence, response to DNA damage, and neoplastic transformation involve different functions of p53.

MATERIALS AND METHODS

Plasmids. The retroviral vector pLXSN and its derivative pLAPSN carrying human placental alkaline phosphatase were kindly provided by A. Dusty Miller (26). The retroviral vectors pPS-neo and pPS-hygro and their derivatives carrying the mutant human p53 cDNAs C141Y and R175H (pPSneo-p53^{141} and pPSneo-p53^{175}), or *N-ras* cDNA (pPS-N-ras-hygro) have been described (27, 28). The reporter chloramphenicol acetyl-

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Abbreviations: MEFs and REFs, mouse and rat embryo fibroblasts; GSE, genetic suppressor element; CAT, chloramphenicol acetyltransferase.

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transferase (CAT) plasmid (pAd-CAT), containing the p53-responsive adenosine deaminase promoter, has been described (29).

Cell Cultures. Cultures of MEFs and REFs were derived from 11- to 16-day-old embryos of Swiss mice. Cells passaged according to 3T3 protocol (30) were frozen at every second passage. Line 10(1) of $p53^{-/-}$ MEFs, lacking both alleles of p53, was kindly provided by Arnold Levine (31). The ecotropic retroviral packaging cell line BOSC23 was kindly provided by Warren Pear and David Baltimore (32). Ecotropic and amphotropic packaging lines derived from NIH 3T3 cells (GP+E86 and GP+envAm12) were kindly provided by Arthur Bank (33). All cells were maintained in Dulbecco's modified Eagle's medium containing 10% fetal (newborn for MEFs and REFs) bovine serum supplemented with penicillin and streptomycin.

p53 GSEs. The library was constructed as described (20). Briefly, rat p53 cDNA (34) was digested by DNase I and 100- to 300-bp fragments were blunt-end ligated to a mixture of two short double-stranded DNA adaptors obtained by annealing two complementary synthetic oligonucleotides. One adaptor carried three initiator ATG codons and the other three stop codons in all three reading frames, as well as *Eco*RI or *Bam*HI restriction sites (see Fig. 1). After *Eco*RI and *Bam*HI digestion, those fragments ligated to two different adaptors were amplified by PCR and cloned into pLXSN. The resulting library of 30,000 independent recombinant clones was amplified once on solid L-agar.

GSE Selection Procedures. The library was transfected into a mixture of ecotropic and amphotropic packaging cells and delivered to MEF recipient cells after "ping-pong" amplification (19, 21), MEFs, $1–3 \times 10^5$ per 100-mm dish, were plated and infected twice at 24-hr intervals. The efficiencies of transfection and infection were monitored by either G418 selection or by adding a plasmid expressing human placental alkaline phosphatase to the transfecting DNA, followed by staining for alkaline phosphatase activity. To assay focus formation, MEFs incubated after infection in 5% bovine serum for 2–4 weeks without passaging were stained with methylene blue. For etoposide selection, 2×10^5 infected MEFs per 100-mm dish were plated and treated for 24 hr with etoposide (100–400 ng/ml), followed by incubation in drug-free medium for 10 days. Etoposide-resistant cells formed dense colonies on the sparse monolayer of arrested cells. p53 cDNA inserts from the selected cells were isolated by PCR, using DNA from foci or drug-resistant colonies as template and sense strands of the adaptors as primers. The conditions necessary include 1 min at 94°C, 1 min at 60°C, and 1 min at 72°C, followed by a 10-min incubation at 72°C. The PCR products were recloned into pLXSN after *Eco*RI and *Bam*HI digestion. Alternatively, integrated proviruses were rescued by fusion with packaging cells. The selected cells were mixed with ecotropic packaging cells (1:2), plated at 6×10^6 per 100-mm dish, and fused by exposure to 50% polyethylene glycol, MW1300 (Sigma) for 1 min. Virus collected 24 and 48 hr after fusion was used to infect amphotropic packaging cells, which were selected with G418, propagated, and used directly to produce virus or mixed with ecotropic packaging cell lines to produce virus at a higher titer. The resulting retrovirus mixture, probably enriched for the desired GSEs, was subjected to a second round of similar selection, using lower virus titers to reduce the number of inserts per cell. Most cell clones from the second screening did contain single inserts, which were isolated by PCR, then recloned and sequenced as described above.

Other Methods. Western analysis of soluble proteins was performed as described (35), using the anti-p53 monoclonal antibodies PAb421 and PAb246 (kind gifts of Arnold Levine). Horseradish peroxidase conjugated to goat anti-mouse antibodies (Bio-Rad) was used for visualization by enhanced chemiluminescence (Renaissance reagent; DuPont/NEN). For immortalization assays, REFs, frozen four or six passages before crisis, were plated at $3–10 \times 10^5$ cells per 100-mm dish, infected or transfected with individual GSEs, and selected with G418. For p53 transactivation assays, cells (2×10^5 per 60-mm dish) were transfected with 12 μg of plasmid DNA containing 5 μg of pAd-CAT using the standard calcium phosphate procedure as described (29). Extracts, prepared by freezing and thawing the cells three times, were normalized for their protein content. The efficiency of transfection was determined by using a quantitative β-galactosidase assay in which the enzyme is expressed from a pCMV-lacZ plasmid (2 μg), then added to each transfection mixture.

RESULTS

Isolation of Focus-Forming p53 GSEs. Biologically active GSEs from the p53 GSE library (Fig. 1) were isolated by using

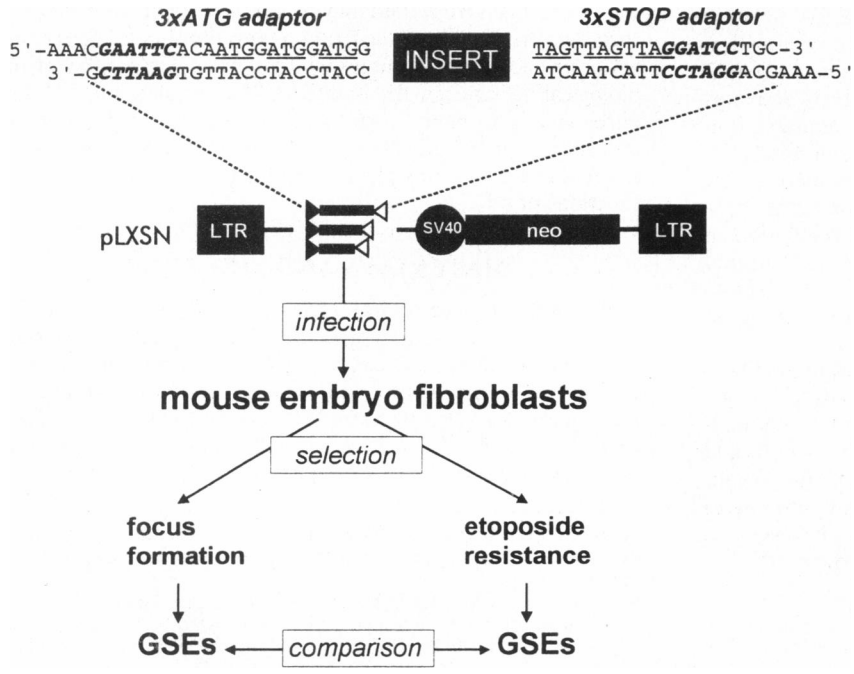

FIG. 1. Construction of the p53 GSE library and general strategy of selection. p53 cDNA fragments generated by DNase I digestion (INSERT) were blunt end-ligated to synthetic 3xATG and 3xSTOP adaptors. Initiator ATG codons and termination TAG codons in the adaptors are underlined. *Eco*RI and *Bam*HI sites in the adaptors are shown in an italicized boldface font. The directionality of ligation was provided by the presence of a single blunt end in each adaptor. PCR-amplified adaptor-ligated inserts were cloned under the control of the long terminal repeat (LTR) promoter in the pLXSN vector. The resulting GSE library was delivered to MEFs, which were then selected for two p53-related phenotypes. The structures and biological effects of GSEs isolated in the alternative selections were then compared.

early-passage MEFs, chosen because they have an intact p53 pathway and alter their phenotype in response to suppression of p53 function. In these cells, p53 controls senescence, sensitivity to transformation by dominant oncogenes (e.g., *ras*), as well as sensitivity to DNA-damaging drugs and radiation, allowing several alternative ways of selecting and testing GSEs. MEFs express relatively low levels of p53 protein, favoring inhibition by GSEs, and they are very sensitive to infection with ecotropic retroviruses.

The first selection protocol was based on the observation that infection of MEFs with a retroviral vector carrying the p53 mutant C141Y leads to the formation of foci (Fig. 2). MEFs infected with the p53 GSE library also yielded foci under similar conditions (Fig. 2). These were picked and PCR was used to isolate the inserts, which were then recloned in pLXSN. These secondary sublibraries were used for a new round of selection, which yielded a significant enrichment of focus-inducing clones (Fig. 2).

The distribution of the recovered fragments was not random with respect to orientation or position. Most of the GSEs (14 of 15) were sense-oriented. As shown in Fig. 2, they are derived from three separate regions of p53 mRNA, each represented by a series of overlapping fragments. Group I consists of four elements, representing the very beginning of the protein-coding region, which corresponds to the transactivator domain (36). Group II includes seven overlapping elements in the region downstream of the transactivator domain and proximal to part of the DNA-binding domain, from amino acids 58 to 171. Finally, three elements of group III map to a specific area (nucleotides 1344–1484) of the 3′-untranslated region of the mRNA (34).

We chose four GSEs to represent the three groups (marked with asterisks in Fig. 2) for functional testing (Table 1). All were active in infected MEFs, inducing formation of a dense culture of morphologically altered cells (data not shown). Although the rest of the elements shown in Fig. 2 were not tested individually, they are very similar in sequence to the confirmed GSEs and therefore are likely to possess similar biological activity.

Sense-oriented GSEs are expected to act as dominant negative polypeptides. To verify that biological activity de-

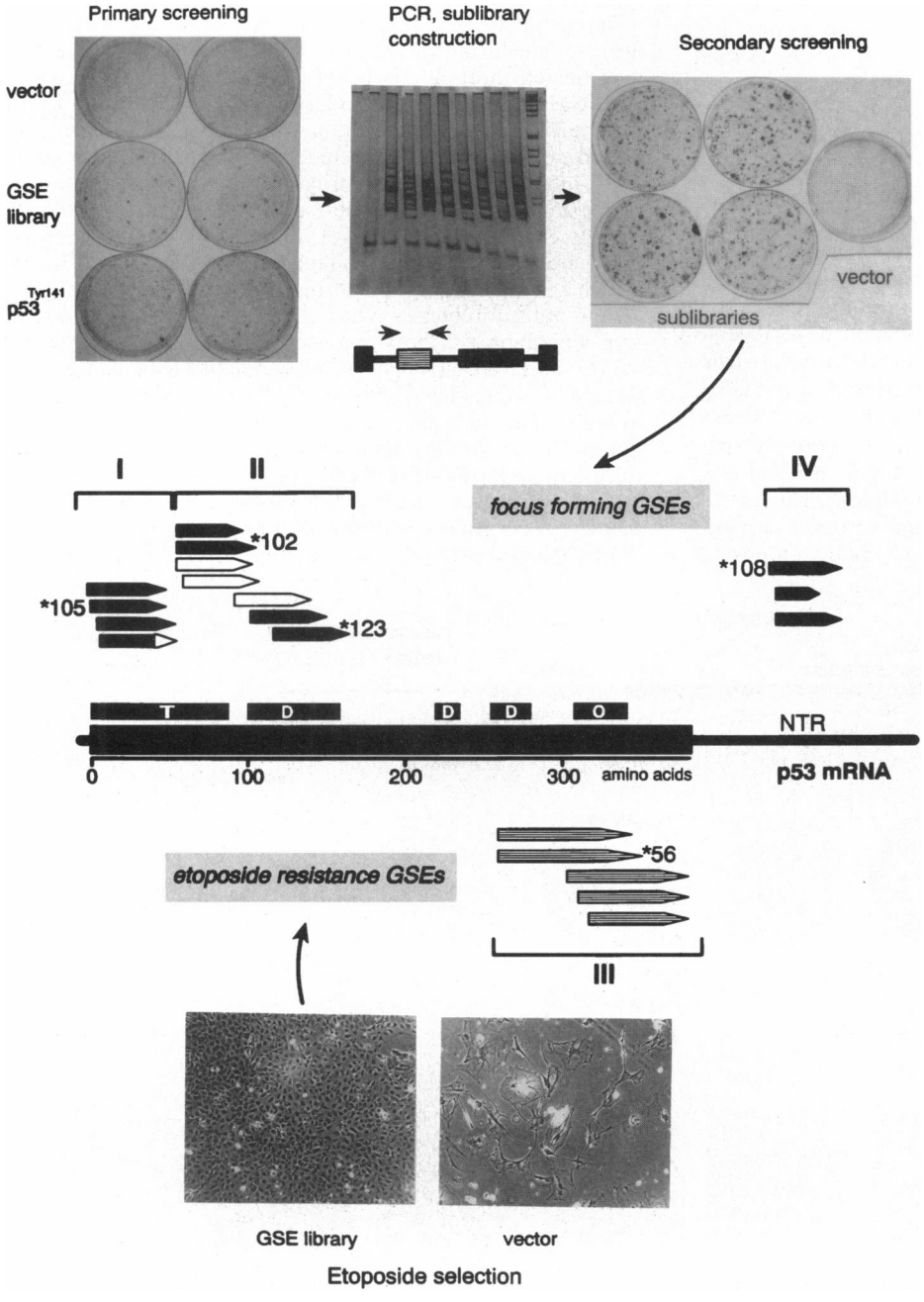

FIG. 2. Isolation of p53 GSEs using alternative selections in MEFs. The p53 GSE library was transduced into MEFs, which developed foci (second row of plates, *Top Left*) similar to those induced by the p53 mutant C141Y (bottom row). p53 fragments were isolated from the DNA of individual foci by PCR (top of the *Middle* panel; the left lane contains PCR products derived from DNA of library-transduced MEFs before selection, the other lanes represent PCR products obtained from individual foci) and recloned back into pLXSN. The resulting sublibraries, delivered to fresh MEFs, caused a significant increase in focus formation (*Top Right*). The difference in growth of MEFs containing the control vector or the GSE library after etoposide treatment is shown at the *Bottom*. The positions of isolated GSEs are shown above (focus-forming GSEs) and below (etoposide-resistance GSEs) the diagram of p53 mRNA. The locations of the transactivator (T), DNA-binding (D), and oligomerization (O) domains of the p53 protein and the untranslated region (NTR) of the p53 mRNA are indicated. Those GSEs used for functional testing are marked by asterisks. White arrows correspond to elements that were mapped but not completely sequenced. The white head on one of the GSE arrows indicates loss of a nucleotide, causing a frameshift mutation near the 3′ end of the element.

Table 1. Position, orientation, and activity of p53 GSEs and the encoded peptides

Identification number of GSE	Position*		Activity
	Nucleotides	Amino acids	imm/eto/*ras*†
105	−4–147	1–50	+/−/+
102‡	172–329	58–109	+/ND/ND
123	365–516	123–171	+/−/−
19	823–1079	275–359	+/+/ND
56	823–1105	275–368	+/+/+
13	901–1182	301–391	+/+/ND
22	937–1199	312–391	+/+/+
50	964–1205	322–391	+/+/ND
108	1322–1458	None	+/−/−

ND, not determined.
*Number 1 is the first nucleotide of the initiator codon in rat p53 cDNA (34).
†Immortalization/etoposide resistance/cooperation with *ras*.
‡Contains a mutation changing the 72nd residue form E to G.

pends on protein-coding capacity, we constructed a nonsense mutant of a class II p53 GSE (123, see Fig. 2). A stop codon was created immediately downstream and in-frame with the initiator codon by a single nucleotide substitution. The nonsense mutation completely inactivated the ability of the GSE to induce immortalization of REFs, indicating that the biological activity of this element depended on its translation.

Isolation of p53 GSEs Conferring Etoposide Resistance. Etoposide induces DNA damage by stabilizing cleavable complexes involving topoisomerase II (37). Primary MEFs infected with the GSE library were treated with etoposide under the minimal drug concentration, leading to complete and irreversible growth arrest of control cells infected with the insert-free vector. In contrast, numerous colonies appeared in the GSE library cell population, forming a dense monolayer 2 weeks after the application of etoposide (Fig. 2). Integrated proviruses carrying p53 fragments were rescued and enriched in a second round of selection under similar conditions. The inserts were isolated by PCR, cloned into the same retroviral vector, and tested individually for their ability to confer etoposide resistance. The positions of five GSEs with confirmed activity are shown in Fig. 2 and Table 1.

All the etoposide-selected GSEs fell into one area of p53 mRNA, near the C terminus of the coding region. This part of the p53 protein was shown previously to act as a dominant negative suppressor of p53 function (23, 25). Interestingly, none of the GSEs isolated by focus selection were in the C-terminal domain and none of the etoposide-selected GSEs fell into to any region identified by focus selection. The striking difference in the sets of GSEs isolated by the two types of selection reveals that GSEs of different classes have different biological activities, reflecting different functional mechanisms. To explore this aspect further, we compared the properties of representative GSEs from of each of the four groups.

Biological Effects of Different Classes of p53 GSEs. All four classes were tested in an immortalization assay in which the GSEs and control insert-free vector were delivered to middle passage REFs, either by direct transfection or by virus infection. Small colonies consisting of senescent cells developed in the control population while many, large, fast-growing colonies arose in all four populations infected with the p53 GSEs (Fig. 3, top row). These cells did not show any decrease in growth rate during the next seven passages, indicating that they had passed through crisis. We conclude that GSEs from all four classes are capable of inducing immortalization of REFs.

A different result was obtained when the GSEs were compared for their ability to induce resistance to etoposide. GSE-infected REFs were treated with a concentration of etoposide inducing irreversible growth arrest in control, vector-infected cells. Colonies of etoposide-resistant REFs appeared only in the population infected with the class III GSE, which was isolated initially through etoposide selection. None of the other three classes had any effect on drug resistance.

Cooperation with the *N-ras* oncogene was also used to assay the p53 GSEs. Expression of activated *ras* is lethal for rodent embryo fibroblasts expressing wild-type p53. However, some p53 mutant proteins facilitate the survival and transformation of *ras*-transduced REFs (38). To test whether any of the GSEs possess such activity, REFs infected with GSE viruses were superinfected with the retroviral vector pPS-N-ras-hygro, carrying N-ras^{asp12} cDNA and a gene conferring hygromycin resistance. Control REFs infected with the insert-free virus and REFs transduced

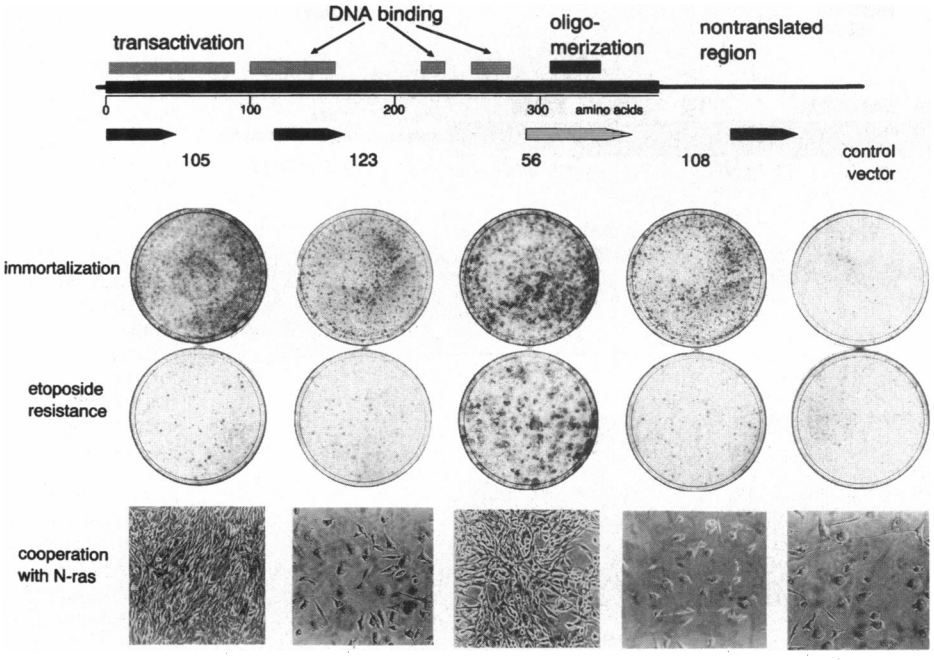

FIG. 3. Biological effects on REFs of GSEs representing different portions of p53 mRNA. See the text for details.

with class II and class III GSEs yielded no hygromycin-resistant colonies. Class III and, to a lesser extent, class I GSEs generated numerous hygromycin-resistant colonies (Fig. 3), indicating that they inhibit *ras*-induced cell death.

Both etoposide-resistance and *ras*-cooperation assays were also done with p53-null 10(1) cells, to determine whether the GSEs act against p53 itself or in a p53-independent manner. In contrast to cells with wild-type p53, no differences were found between the control and GSE-transduced populations, demonstrating that the effect of the p53 GSEs requires the presence of p53 (data not shown).

Effects of GSEs on p53 Expression and Function. GSEs in classes I, II, and IV did not alter p53 protein levels, analyzed by immunoblotting, indicating that they have no direct effect on the synthesis or stability of p53. In contrast, a dramatic increase in p53 content was observed in REFs transduced with a class III GSE (Fig. 4*A*). Thus, the C-terminal GSEs seem to convert p53 into a stable but functionally inactive form.

The effects of p53 GSEs on transactivation by p53 were studied by using a reporter construct expressing the CAT gene under the control of a minimal thymidine kinase promoter containing the p53-responsive element from the human adenosine deaminase gene. The construct was cotransfected into REFs or into p53-null 10(1) cells, together with plasmids expressing individual GSEs and, in the case of the p53-null cells, wild-type p53. As controls, we used either insert-free vector or a vector expressing the p53 mutant R175H, a strong dominant inhibitor of wild-type p53 function (39). A representative result of one of the experiments

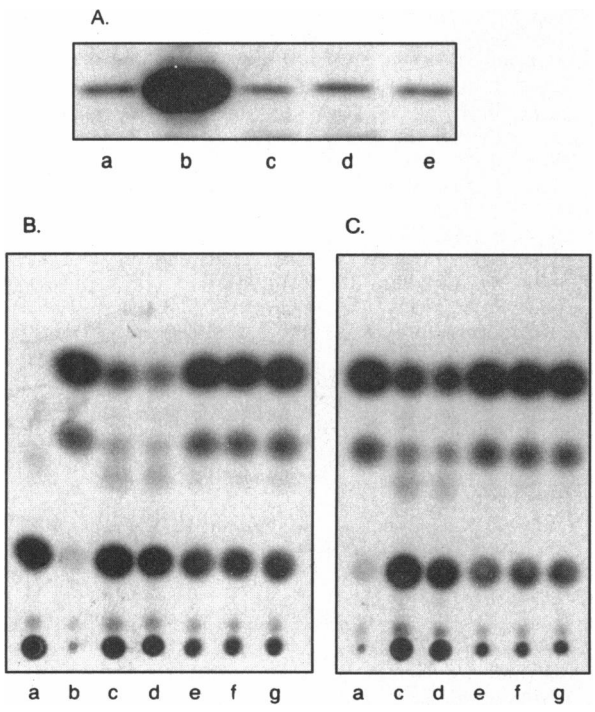

FIG. 4. (*A*) Levels of p53 protein in REFs transduced with different p53 GSEs. Lanes: a, insert-free vector (negative control); b, GSE 56 (oligomerization domain); c, GSE 105 (transactivator domain); d, GSE 108 (3′-untranslated region); e, GSE 123 (DNA-binding domain). (*B* and *C*) Effect of p53 GSEs on transactivation of a CAT gene controlled by a p53-responsive promoter (pAd-CAT). (*B*) Transient transfection of p53-null 10(1) cells with pAd-CAT in combination with insert-free vector (negative control) (lane a), wild-type p53 plus insert-free vector (positive control) (lane b), wild-type p53 plus R175H (lane c), wild-type p53 plus GSE56 (oligomerization domain) (lane d), wild-type p53 plus GSE105 (transactivator domain) (lane e), wild-type p53 plus GSE123 (DNA-binding domain) (lane f), wild-type p53 plus GSE108 (3′-untranslated region) (lane g). (*C*) Transfection of REFs with insert-free vector (positive control) (lane a) or p53 GSEs (same order as in *B*) (lanes c–g).

(Fig. 4 *B* and *C*) shows that GSEs from classes I, II, or IV produced a marginal but reproducible decrease in p53-driven CAT expression. In contrast, the class III GSE, which maps to the oligomerization domain, reduced CAT expression strongly, even more than the mutant p53 protein.

DISCUSSION

To dissect the complex functions of p53, we isolated several classes of p53 GSEs with different properties. While all GSE classes induced cell immortalization, only two rescued REFs from the cytotoxic effect of mutant *ras* expression and only one of these two conferred resistance to the DNA-damaging drug etoposide (Table 1). These results suggest that activities of p53 involved in cell mortality, the responses to an activated *ras* oncogene, or DNA damage involve distinct functions of the protein.

The vast majority of the p53 GSEs isolated are sense-oriented, indicating that, under our experimental conditions, inhibition of p53 function by an antisense mechanism was inefficient. It is likely that selection of the rat GSE library in mouse cells contributes to the bias against antisense GSEs, since the differences between mouse and rat RNA sequences would suppress hybridization.

Three classes of GSEs correspond to different portions of the coding region of p53 cDNA and are likely to act by encoding inhibitory peptides. The dependence of the activity of class II GSEs on translation was directly demonstrated by introduction of a nonsense mutation. The differential effect of class I and class III GSEs on different p53-associated phenotypes is most consistent with a functional peptide. In contrast, the activity of class IV GSEs, derived from the 3′-untranslated region of p53 mRNA, hints at a novel mechanism of p53 regulation that may involve interaction of this part of the mRNA with factors that regulate p53 synthesis through changes in mRNA metabolism or translation efficiency. The biological activity of a separately expressed 3′-untranslated region of an mRNA was previously demonstrated for α-tropomyosin, which displayed tumor suppressor activity by an unknown mechanism (40).

The well-studied domain structure of p53 allows us to link the selected GSEs to known functional domains of the protein. GSEs of class I and the 3′ subset of class II correspond to the transactivator and DNA-binding domains, respectively, and therefore could be expected to inhibit transactivator function. However, the weak effect of these GSEs on p53-dependent promoter function and the clear differences in the ability of class I and class II GSEs to cooperate with *ras* argue that GSEs in these classes may rather act by interfering with other protein–protein interactions. In fact, these elements contain the sites of p53 binding to mdm-2, TAF31, large simian virus 40 T-antigen, TATA-binding protein, 53BP1, and 53BP2 (41–45). It should be noted that the class II GSEs cover a rather long region of the protein and therefore may include elements with different mechanisms of action. We are now analyzing the most N-terminal GSE from group II, which carries a sequence conserved between the transactivator and oligomerization domains of p53.

The class III GSEs correspond to the C-terminal portion of the protein, which contains the oligomerization domain (23), regulating sequence-specific DNA binding (24), and interaction with broken DNA (46). An anti-p53 activity of a separately expressed C-terminal portion of the protein has been characterized earlier (19, 23, 25, 47). Consistently, GSEs derived from this region show strong biological activity in several different assays: they promote immortalization, cooperate with *ras*, induce resistance to DNA-damaging treatments, and strongly inhibit transactivation by p53. Expression of these elements is accompanied by a dramatic accumulation of the p53 protein which, however, does not lead to growth inhibition, indicating that the accumulated protein is inactive. The C-terminal GSEs may interfere with the oligomerization of p53

and may induce conformational changes that alter the activity or metabolism of the protein.

Class III elements were selected for resistance to etoposide but were not isolated in the focus-formation assay, even though they were as efficient in the latter assay in some population of MEFs as the other classes of GSEs. The failure to isolate class III GSEs through focus formation could be related to quantitative differences in p53 activity among different preparations of MEFs. This parameter remains poorly controlled and may depend on the stage of the embryos used for the preparation of MEF cultures.

The peptide-encoding GSEs should provide valuable tools to identify proteins that interact with p53 in different regions. Their use as bait in the two-hybrid system (48) may yield specific p53 binding partners and is a major direction of our further studies.

The strategies used in the present work limit the range of GSEs isolated to those that inhibit function in normal rodent fibroblasts. Some of the p53 GSEs that may have been missed may include those interfering with p53-mediated apoptosis, to which normal MEFs are relatively resistant. GSEs that inhibit apoptosis in several apoptosis-sensitive cell systems are being isolated and compared with the elements cloned in the course of the present study.

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