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The 5′ region of the p53 gene: evolutionary conservation and evidence for a negative regulatory element

Brigitta Bienz-Tadmor, Rina Zakut-Houri, Stephanie Libresco, David Givol and Moshe Oren

Department of Chemical Immunology, The Weismann Institute of Science, Rehovot 76100, Israel

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The 5′ regions of the mouse, rat and human functional p53 genes were isolated and analysed. All three genes possess a non-coding exon, comprising exclusively 5′ untranslated sequences. This exon contains extensive diad symmetry near the 5′ end of p53 mRNA, possibly allowing for the formation of a stable hairpin structure in this mRNA. The nucleotide sequence within this hairpin element is highly conserved among the species. A DNA stretch of 225 bp preceding the p53 mRNA cap site possesses distinct promoter activity when assayed in the CAT system. However, this activity is practically abolished when further upstream p53 sequences (~120 bp) are included in front of the CAT gene. This suggests that the control of p53 gene expression is complex and involves a negative regulatory element.

Key words: p53 tumor antigen/stem and loop structure/TATA box-less promoter/CAT assay/negative transcriptional control

Introduction

The p53 cellular tumor antigen has been implicated in the possible control of cell transition from a resting to an actively proliferating state (Milner and Milner, 1981; Mercer *et al.*, 1982, 1984; Reich and Levine, 1984). Furthermore, the p53 gene may be considered an oncogene, since it will transform primary normal cells in collaboration with activated Ha-*ras* (Eliyahu *et al.*, 1984; Parada *et al.*, 1984; Jenkins *et al.*, 1984). Hence deregulation of p53 expression may have significant consequences for cellular growth properties and promote neoplastic conversion. To understand further the regulation of 53 gene expression we attempted to determine the structural and functional properties of the p53 gene promoter.

Previous analysis of the 5′ region of the functional mouse p53 gene has revealed very extensive diad symmetry, predicting the formation of a physiologically stable stem-and-loop structure near the 5′ end of the corresponding mRNA (Bienz *et al.*, 1984). Attempts to determine precisely the p53 mRNA cap site were inconclusive, probably due to interference by the stem-and-loop structure (Bienz *et al.*, 1984; Zakut Houri *et al.*, 1984). Nevertheless, when S1 nuclease digestion conditions were modified, a distinct nuclease-protected fragment could be detected, suggesting that at least a fraction of mouse p53 mRNA is initiated 216 bp upstream to the 3′ end of exon 1 (Bienz *et al.*, 1984). These findings further indicated that the p53 gene promoter does not contain consensus TATA and CAAT boxes, which appear to constitute a common feature of most cellular promoters studied so far (Breathnach and Chambon, 1981).

To understand better the structure and function of the 5′-proximal p53-specific genomic DNA, we isolated and analysed the corresponding regions from several mammalian species. The results indicate that the stem-and-loop structure is remarkably conserved, suggesting that this element is functionally important. We also demonstrated that the region containing the putative p53 promoter is indeed capable of exhibiting promoter activity. Finally, evidence was obtained suggesting the presence of a negative transcriptional control element upstream of the mouse p53 promoter.

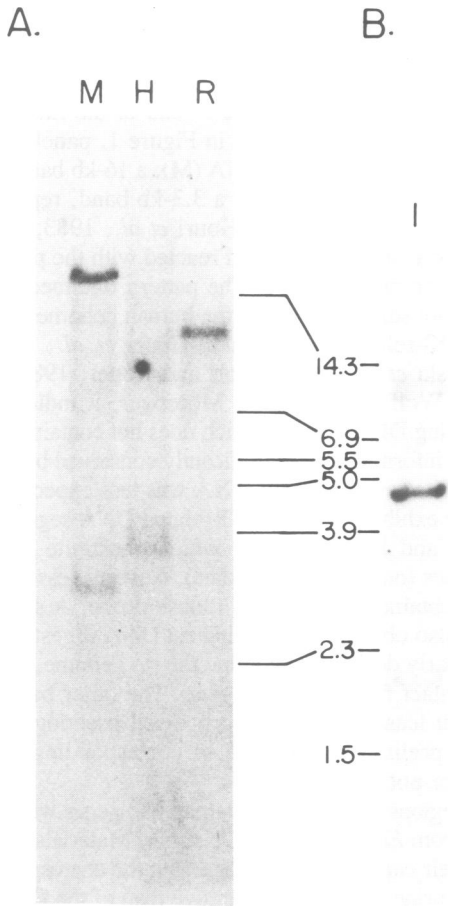

Fig. 1. Detection of p53-specific sequences in genomic DNA from various species. **A.** Hybridization with the 5′ region of the mouse p53 gene. BALB/c mouse liver (M), human placenta (H) or rat fibroblast (R) genomic DNA (10 μg/lane) was digested with *Eco*RI, separated on a 0.6% agarose gel, transferred to nitrocellulose and hybridized to the nick-translated 0.7-kb *Eco*RI-*Hind*III fragment of clone Ch53-7 (Bienz *et al.*, 1984) as detailed in Materials and methods. Numbers on the left denote the positions and sizes (in kb) of co-electrophoresed mol. wt. markers. **B.** Rat p53-specific sequences. Rat genomic DNA was digested with *Eco*RI and analyzed as in **A**, except that the probe was the *Xho*I-*Sac*II 950 bp fragment of the mouse p53 cDNA clone pp53-176 (Zakut-Houri *et al.*, 1983). This probe is derived exclusively from within the protein-coding region.

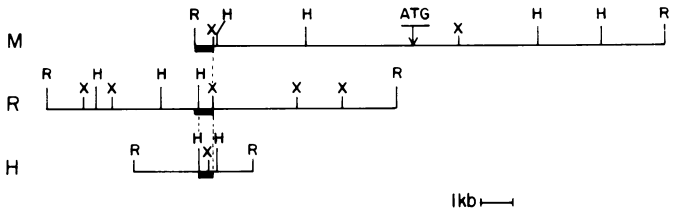

Fig. 2. Physical map of the *Eco*RI fragments containing the 5′ regions of the functional p53 genes from mouse (M), rat (R) and man (H). The three DNA segments are aligned at the first (non-coding) p53 exon. The black boxes indicate the region whose sequence is presented in Figure 3. The position of the initiator codon of mouse p53 is also shown (ATG). H, *Hind*III; R, *Eco*RI; X, *Xba*I.

Results

Structural analysis of 5′ regions from rat and human functional p53 genes

Evolutionary conservation of DNA sequences is usually taken as an indication of their functional importance. We therefore attempted to isolate the 5′ regions of p53 genes from different mammalian species and compare their respective sequences. Mouse, rat and human genomic DNA were digested with *Eco*RI, fractionated on an agarose gel and hybridized with the 0.7-kb *Eco*RI-*Hind*III fragment containing the 5′ end of the mouse p53 gene (Bienz *et al.*, 1984). As shown in Figure 1, panel A, the probe detected two bands in mouse DNA (M): a 16-kb band, corresponding to the functional gene and a 3.3-kb band, representing the processed pseudogene (Zakut-Houri *et al.*, 1983; Bienz *et al.*, 1984). Only a single DNA band reacted with the probe in either human (H) or rat (R) DNA. The pattern obtained with human DNA was not surprising, since the human genome contains only a single p53-related gene (Zakut-Houri *et al.*, 1984, 1985); Matlashewski *et al.*, 1984; Wolf and Rotter, 1985; Harlow *et al.*, 1985; Wolf *et al.*, 1985). Moreover, it indicated that the corresponding DNA region, which does not contain any p53 protein coding information, is significantly conserved between mouse and man. The result with rat DNA was less expected, since the rat genome exhibits multiple p53-related DNA segments (Figure 1B) (Wolf and Rotter, 1985), which constitute at least three distinct genes (our unpublished data). Nevertheless, only a single band was obtained using the 5′ mouse probe. A single reactive band was also obtained employing rat DNA digested with *Pvu*II or *Pst*I, clearly demonstrating that the rat genome, too, contains only one intact functional p53 gene. The other bands probably represent at least two distinct processed pseudogenes, as confirmed by preliminary analysis of corresponding rat genomic clones (data not shown).

The 5′ regions of the rat and human p53 genes were molecularly cloned from *Eco*RI-digested DNA (see Materials and methods) utilizing their capacity to hybridize with the corresponding probe from the murine gene. A comparative map of the DNA segments studied is shown in Figure 2. As demonstrated (Bienz *et al.*, 1984), the first codon of the p53 protein coding region is separated from the first (non-coding) exon of the mouse gene by a 6.1-kb intron, which is entirely contained within the 16-kb *Eco*RI fragment (Figure 2). In the human gene, the first exon resides on a 3.8-kb *Eco*RI fragment (Figure 2), which is followed by a ~9-kb intron. The initiator ATG is located on the next *Eco*RI fragment, which is ~15 kb long (data not shown).

The regions comprising the first exon and the immediate 5′ flanking DNA were sequenced, and the results are presented in Figure 3. The mouse and human genes contain, upstream to the

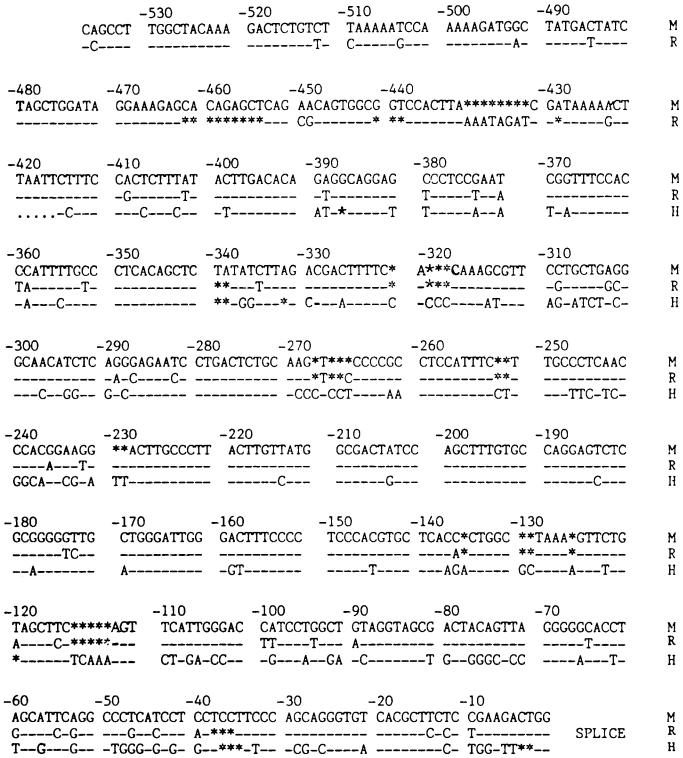

Fig. 3. DNA sequence of the 5′ regions of the mouse, rat and human functional p53 genes. The mouse (M) sequence is presented in full, whereas the rat (R) and human (H) sequences detail only those nucleotides which differ from the mouse. Identities to the mouse sequence are indicated by horizontal bars, while asteriks denote gaps introduced in the sequence for better alignment. Numbering refers to the mouse sequence, with position −1 being the most 3′ nucleotides of the first (non-coding) exon. In the mouse mRNA, this is immediately followed by the initiator AUG codon (Bienz *et al.*, 1984). The putative major cap site is at position −216 (Bienz *et al.*, 1984).

splice site, 157 and 107 nucleotides, respectively, which are identical to those found in the corresponding p53 cDNA clones described (Zakut-Houri *et al.*, 1983, 1985). These nucleotides constitute part of the first (non-coding) exon. The sequence comparison reveals that some 5′ p53 elements are remarkably conserved among species. Most notably, the stretch extending from nucleotide −211 to −114 in the mouse gene, which is involved in the formation of the putative stem-and-loop (Bienz *et al.*, 1984) exhibits a very high degree of conservation (95% between mouse and rat, 85% between mouse and man). Moreover, the alterations that do occur do not significantly affect the stability of this structure. Thus, the calculated ΔG° values (Tinoco *et al.*, 1973) are −56, −57 and −49 kcal/mol for the mouse, rat and human elements, respectively.

Analysis of promoter activity by the CAT-assay

Due to the unusual structural features exhibited by the putative p53 promoter, it was necessary to demonstrate directly that the implicated DNA segment does possess the ability to act as a promoter. To that end, the *Ava*II-*Ava*II 337 bp fragment, extending between nucleotides −441 and −104, was introduced in front of the chloramphenicol acetyl-transferase (CAT) gene (Gorman *et al.*, 1982). This piece of DNA should contain the p53 mRNA cap site (nucleotide −216, Bienz *et al.*, 1984), as well as the proximal promoter sequences. As seen in Figure 4, this *Ava*II-*Ava*II fragment indeed exhibited easily detectable promoter function, manifest as CAT activity upon transfection into either-

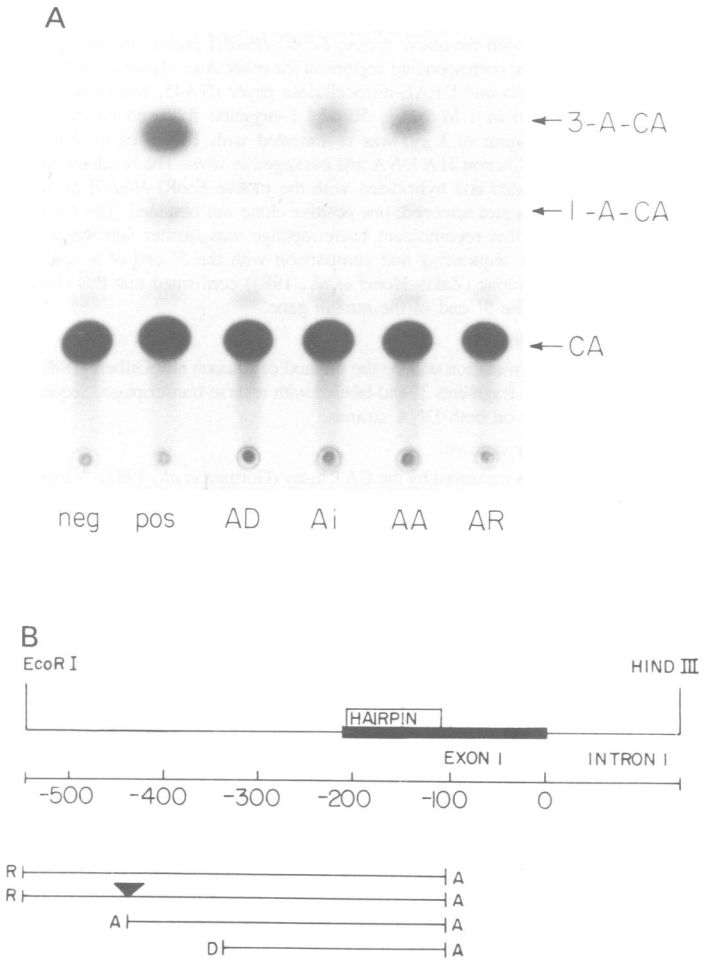

Fig. 4. CAT activity of 5′ p53 plasmids in rat embyro fibroblasts. The various plasmids were constructed and analyzed as described under Materials and methods. **A**. Autoradiogram of chromatographed [^{14}C]chloramphenicol conversion products. CA, unconverted chloramphenicol; 1-A-CA, 1-acetate chloramphenicol; 3-A-CA, 3-acetate chloramphenicol. The following plasmids were used as controls: neg, pCAT3M, serving as a negative control; pos-PSV2CAT (Gorman *et al.*, 1982) serving as a positive control. All other plasmids contained a 5′ genomic fragment in front of the CAT gene, as follows: AD, *Dde*I-*Ava*II; Ai, *Eco*RI-*AvaII*, with an 12 bp insertion in the 5′ *Ava*II site; AA, *Ava*II-*Ava*II; AR, *Eco*RI-*Ava*II. **B**. Schematic map of the 5′ *Eco*HI-*Hind*III mouse p53 genomic fragment and of the various subfragments employed for CAT analysis. Indicated on top are the positions of the first p53 exon (thick bar) and of the predicted hairpin loop structure. Numbering is as in Figure 3. The bottom lines describe the relative positions of the DNA subfragments assayed for promoter activity in **A**. R, *Eco*RI; A, *Ava*II; D, *Dde*I. The inverted triangle indicates the position of the 12 bp insertion.

primary rat embryo fibroblasts (Figure 4, lane AA) or NIH/3T3 cells (data not shown). The level of activity displayed by this plasmid in the CAT assay was ~2- to 3-fold lower than that obtained by transfection with a plasmid in which CAT expression was driven by the simian virus 40 (SV40) early promoter (lane pos). These results further confirm the previous assignment of the p53 promoter to the region located upstream to nucleotide −216. In an attempt to define more precisely the location of the promoter, a shorter DNA piece extending only between the *Ava*II site at nucleotide −104 and the *Dde*I site at position −293 (Figure 4, AD), was similarly tested for promoter activity. It is clear from Figure 4 that this plasmid induces no detectable CAT activity, thus positioning the 5′ end of the minimal p53 promoter upstream to nucleotide −293.

To determine whether the *Ava*II-*Ava*II fragment contained the entire p53 promoter, we substituted it by a larger segment extending further up to the *Eco*RI site constituting the 5′ end of the cloned mouse p53 genomic DNA (Figure 4, AR). Unexpectedly, the inclusion of these additional 5′ *Eco*RI-*Ava*II sequences markedly reduced the CAT activity of the resulting plasmid. These results suggested that a negative control element is located in front of the active portion of the p53 promoter. This idea was further explored by introducing a 12 bp linker into the *Ava*II site at position −441. The resultant plasmid (Figure 4, Ai) clearly regained much of the basal promoter activity. The most likely interpretation is that the insertion had occurred within or very close to the negative control element, thereby preventing it from efficiently interfering with promoter activity.

Discussion

The work presented here deals with the characterization of the 5′ ends of the p53 genes from three mammalian species. All three genes possess a non-coding exon, comprising exclusively 5′ non-translated sequences. This exon contains a region of extensive dyad symmetry which is highly conserved among species and most likely leads to the formation of a stable stem-and-loop structure in the resultant mRNA. It is tempting to suggest that this structure is somehow essential for the proper expression of p53, probably by affecting mRNA metabolism or translation efficiency. In this respect it is noteworthy that extensive dyad symmetry is also found in the 5′ half of c-*myc* mRNA, although in this case it extends to the second, coding exon (Saito *et al.*, 1983). Removal of the putative stem-and-loop structure from c-*myc* mRNA does indeed increase substantially the translatability of this mRNA (Darveau *et al.*, 1985) lending further support to its possible involvement in control of gene expression. It still remains to be determined whether the corresponding element in p53 mRNA, which is limited to the first non-coding exon, can play a similar role.

The analysis of the p53 promoter is of particular interest. The precise localization of the p53 mRNA cap site is complicated due to interference of the stem-and-loop structure with the analysis. Nevertheless, the data are highly consistent with the p53 promoter being located as suggested in the text. Thus, the p53 promoter is devoid of any recognizable TATA and CAAT boxes. Since the absence of a TATA box is frequently associated with multiple transcripition initiation sites (Benoist and Chambon, 1981; Allan *et al.*, 1983; Reynolds *et al.*, 1984; Calabi and Neuberger, 1985), this may also account for the rather heterogeneous migration of p53 mRNA on agarose gels (Oren *et al.*, 1983). Although such cap site heterogeneity appears to be in variance with the S1 analysis data (Bienz *et al.*, 1984) the relatively weak signal corresponding to the putative major cap site may suggest that alternative minor mRNA start sites may have simply escaped detection.

A frequently observed feature of cellular TATA-box-less promoters is the presence of multiple repeats of the motif CCGCCC (Reynolds *et al.*, 1984; Calabi and Neuberger, 1985; Ishii *et al.*, 1985). Such motifs are not found in the p53 promoter, but there are in fact two repeats of the sequence CTTGCCC (positions −252 and −229) as well as a CCCGCC (position −265), all of which are strictly maintained in both mouse and rat. It is, therefore, possible that these elements also play a role in determining the activity of the p53 promoter. The absence of a TATA box has been suggested to be characteristic of so-called 'housekeeping genes' (Reynolds *et al.*, 1984). Recent studies have

indicated that this feature is shared by several cellular oncogene homologues and growth-related genes, such as the ones encoding the epidermal growth factor (EGF) receptor (Ishii *et al.*, 1985), N-*ras* (Hall and Brown, 1985) and transforming growth-factor (TGF-β) (R.Derynck, personal communication). Thus, control of transcriptional initiation in many of those genes may involve different factors and signals than those employed by most of the genes studied to date.

Another remarkable feature of the p53 promoter is the presence of an upstream DNA segment which seems to act as a negative transcriptional control element. A small insertion at position −441 abolishes much of this negative effect, suggesting that the responsible element is located in close proximity to this position. Comparison of mouse and rat DNA reveals that the nucleotide sequence around this point (the *Ava*II site) is not well conserved between those closely related species. However, since the negative effect of the mouse element is clearly demonstrable in rat fibroblasts (Figure 4), it is likely that the same or very similar signals are recognized in both species. Therefore, we would like to suggest that the crucial part of the negative element is located slightly upstream to the *Ava*II site within a stretch of nucleotides that is highly homologous in the two species.

We have no evidence that this negative element is functional *in vivo*. Nevertheless, it is noteworthy that very similar observations were made for several other genes, such as c-*myc* (N.Wilkie, personal communication), N-*ras* (A.Hall, personal communication) and the EGF receptor gene (G.Merlino, personal communication). In all these cancer-related genes it was observed that removal of a DNA region upstream to the essential promoter sequences resulted in a remarkable increase in promoter activity. Therefore, it is plausible that all these genes, including the one for p53, interact at the 5′ region with a repressor-like protein, which has to be displaced in order for transcription to initiate efficiently. The nature of these 'repressor' molecules as well as the factors affecting their interaction with the target upstream elements remain to be elucidated.

Materials and methods

Analysis of genomic DNA

High mol. wt. genomic DNA was prepared by the method of Blin and Stafford (1976). Aliquots (10 μg) were digested to completion with *Eco*RI and subjected to agarose gel electrophoresis and Southern blotting (Southern, 1975). Immobilized DNA was hybridized with a nick-translated probe (sp. act. 1×10^8 c.p.m./μg) in 50% formamide, 0.75 M NaCl, 75 mM sodium citrate, 50 mM sodium phosphate, pH 6.5, 5% Dextran-sulfate and 5 × Denhardt's solution in the presence of 50 μg/ml heat-denatured salmon DNA. Hybridization was at 34°C for 20 h, followed by washes in 3 × SSC at 55°C (2 × 30 min) and 65°C (2 × 30 min).

Isolation of 5′ p53-specific genomic clones

The 0.7-kb *Eco*RI-*Hind*III fragment carrying the 5′ end of the functional mouse p53 gene was subcloned from the genomic bacteriophage clone Ch 53-7 (Bienz *et al.*, 1984). For the isolation of the corresponding rat DNA segment, we used the above 0.7 kb *Eco*RI-*Hind*III fragment to probe a partial *Eco*RI rat genomic library (provided by I.Schechter). Several positive clones were obtained and further subjected to detailed restriction enzyme analysis followed by hybridization with various mouse p53-specific probes. Two clones had the DNA reactive with the 0.7 kb 5′ probe positioned immediately next to a stretch reacting with a probe derived from the central coding portion of the mouse gene; these were discarded as putative rat p53 processed pseudogenes (see Zakut-Houri *et al.*, 1983). A third clone, containing an 11 kb rat DNA insert, had no other p53-specific sequences at least 4.5 kb to each side of the region reactive with the mouse 5′ probe. The corresponding 11-kb *Eco*RI fragment was subcloned in pBR322. Nucleotide sequence analysis confirmed that this clone indeed contained the first (non-coding) rat p53 exon as well as the upstream sequences and part of the first intron of the functional rat p53 gene (see Figures 2 and 3).

To clone the 5′ end of the human p53 gene, human placenta DNA was digested to completion with *Eco*RI and fractionated on a 0.7% agarose gel. The region containing the desired 3.8 kb fragment (see Figure 1A) was localized by hybridization of one gel lane with the above mouse *Eco*RI-*Hind*III probe, and the DNA was retrieved from the corresponding regions of the other lanes. Isolation of DNA was by electrophoresis into DEAE-nitrocellulose paper (NA45, Schleicher and Schuell), and elution in 1 M NaCl, 50 mM L-arginine followed by ethanol precipitation. An aliquot (0.3 μg) was re-annealed with 1.5 μg of *Eco*RI-cut phosphatase-treated Charon 21A DNA and packaged *in vitro*. The resultant bacteriophages were plated and hybridized with the mouse *Eco*RI-*Hind*III probe. Out of ~300 000 plaques screened, one positive clone was obtained. The 3.8-kb *Eco*RI fragment of this recombinant bacteriophage was further subcloned in pBR322. Nucleotide sequencing and comparison with the 5′ end of a human p53-specific cDNA clone (Zakut-Houri *et al.*, 1985) confirmed that this clone indeed represented the 5′ end of the human gene.

Nucleotide sequencing

DNA sequence determination was by the method of Maxam and Gilbert (1980), employing restriction fragments 3′ end-labeled with reverse-transcriptase. Sequencing was performed on both DNA strands.

Analysis of promoter activity

Promoter activity was measured by the CAT assay (Gorman *et al.*, 1982). Various DNA segments derived from the 5′ *Eco*RI-*Hind*III fragment of Ch 53-7 were blunt-ended with the aid of the large fragment (Klenow fragment) of *Escherichia coli* DNA polymerase I. Each of these DNA segments was introduced into the *Bgl*II site of plasmid pCAT3M (gift of P.Gruss; Laimins *et al.*, 1984) which had also been similarly blunt-ended. To construct plasmid Ai, the DNA of plasmid AA was further cleaved at the unique *Xba*I site located in front of the above *Bgl*II site (Laimins *et al.*, 1984) and treated with the Klenow fragment to create blunt ends. The fragment extending from the 5′ *Eco*RI site to the *Ava*II site at position −441 was similarly treated and ligated in the proper orientation into the converted *Xba*I site. This procedure resulted in the introduction of a 12 bp insertion at position −441 of the mouse DNA. Primary rat embryo fibroblasts or NIH 3T3 cells were grown to 60% confluence in Dulbecco Modified Eagle's Medium supplemented with 10% fetal calf serum. 1 h prior to transfection, cells were re-fed with fresh medium. Transfection was performed by the calcium-phosphate method (Graham and van der Eb, 1973) as described (Eliyahu *et al.*, 1984), employing 10 μg of plasmid DNA without added carrier. 48 h post-transfection, cells were harvested and assayed for CAT activity essentially as described by Gorman *et al.* (1982). Protein concentration in the extracts was determined prior to analysis according to Bradford (1976), and equal amounts of total cell protein were taken for the CAT assay.

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