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by

Ho-Sheng Lin

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ABSTRACT

INVESTIGATION OF THE MECHANISM RESPONSIBLE FOR THE DECREASED TRANSCRIPTION OF THE p53 TUMOR SUPPRESSOR GENE IN THE HUMAN SQUAMOUS CARCINOMA CELL LINE A253. **Ho-Sheng Lin, Teresita Munoz-Antonia, and Michael Reiss. Section of Medical Oncology, Department of Internal Medicine, Yale University, School of Medicine, New Haven, CT.**

The human head-and-neck squamous carcinoma cell line A253 was found previously to contain no mutations in the coding region of the p53 gene but to express an extremely low level of p53 mRNA. This greatly reduced level of p53 transcription may be the result of a mutation within the regulatory region of the gene or the result of an altered expression of cellular factors that regulate expression of the gene. In order to determine which of these two mechanisms is more likely to be responsible, transfection experiments were carried out to compare the transcriptional activity of a wild-type p53 promoter introduced into the A253 cell line to that in a non-neoplastic keratinocyte cell line, R12HKc/HPV16. The p53 promoter was linked to a luciferase reporter gene. The level of expression of this luciferase gene, determined by measuring the light reaction catalyzed by its protein product, reflects the transcriptional activity of the p53 promoter. Equal levels

of luciferase activity in both cell lines would indicate that the cellular environments of both cell lines were similar in their effect on the p53 promoter introduced, and therefore, a mutation in the regulatory region of the endogenous allele is a more likely mechanism responsible for the greatly reduced p53 transcription seen in A253 cells. On the other hand, lower luciferase activity in A253 cells than in R12HKc/HPV16 cells would indicate that alterations in cellular regulatory factor(s) in the A253 cells were responsible for this decreased p53 transcriptional activity.

Unfortunately, the findings from these experiments are inconclusive due to difficulties encountered with transfecting A253 cells. However, my experiments confirmed the existence of two promoter regions in the human p53 gene. The activity of the promoter within the 5' 1.2 kbp of intron 1 was found to be about 75% that of the promoter located upstream of the first exon.

INTRODUCTION

A. p53 Tumor Suppressor Gene

The proliferation of normal cells is regulated by the protein products of growth-promoting proto-oncogenes counterbalanced by the growth-constraining products of tumor suppressor genes. Thus, mutations that enhance the activities of proto-oncogenes would result in conditions that favor the growth of tumor cells. Similarly, genetic alterations that inactivate tumor suppressor genes would also favor the deregulated growth of cancer cells (1). Despite the wide biologic heterogeneity among human cancers, a long-standing hope is that the same biochemical pathways of growth control would be disrupted in many different kinds of cancer. The discovery of such a disrupted pathway would provide a central scheme for understanding, preventing, diagnosing, and treating these different types of cancer (2).

The pathway involving the p53 tumor suppressor gene appears to fulfill this promise, as alterations of this tumor suppressor gene appear to be involved, directly or indirectly, in the majority of almost all types of human cancers (2, 3). p53 is an important growth regulator and exerts its numerous cellular functions through the activation or repression of transcription of several different genes (3). The wild-type p53 protein forms a tetramer which binds to specific DNA sequences,

thereby stimulating or inhibiting the transcription of downstream genes that control growth (2). Dysfunction of the p53 tumor suppressor gene can occur in a variety of ways. Analyzing data from 1312 somatic mutations of p53, Harris and Hollstein found that 83% were missense mutations, 6% nonsense mutations, 10% deletions and insertions, while 1% were silent mutations (3). Furthermore, p53 dysfunction can occur via interactions with other cellular proteins, such as mdm-2, or viral oncoproteins which either neutralize or increase the rate of degradation of p53 proteins (3). Missense mutations, which are by far the most common mechanism responsible for p53 dysfunction, usually result in mutated p53 proteins which fail to bind DNA and which form oligomeric complexes with the wild-type p53 form, thereby disrupting its normal function (dominant-negative loss-of-function mutation). Alternatively, mutant p53 proteins can apparently gain new functions, above and beyond inactivating the wild-type protein, which promote the growth of cells (dominant gain-of-function mutation) (4). Furthermore, the effect of these mutant p53 proteins is exacerbated by their increased stability allowing them to accumulate to high concentration inside the cells. Thus, mutated p53 proteins can be doubly detrimental: they not only deprive cells of the wild-type p53's antiproliferative effect but can also stimulate abnormal cell growth. The wide range of amino acid substitutions resulting from various missense mutations

appears to result in p53 proteins which range from minimally dysfunctional to strongly dominant-negative proteins (1). Recent evidence suggests that wt p53 protects cells against the inherent mutability of the human genome in somatic cells (3). Thus, besides the lack of suppression and/or activation of unregulated cell growth, p53 dysfunction would render cells hypermutable, resulting in accumulation of mutations in cancer cells (3). Mild to moderate DNA damage in somatic cells, caused by either gamma-radiation or cytotoxic drugs, leads to a rapid increase in wild-type p53 proteins which serves to arrest cells in G1 phase so that repair of DNA damage can take place before proceeding to DNA synthesis and mitosis. In contrast, severe DNA damage results in p53-mediated cell death by apoptosis. Both mechanisms therefore reduce the probability of persistent somatic mutations (5, 6, 7). However, cells with mutant or absent p53 fail to arrest in G1 and do not undergo apoptosis in response to the DNA damage and will therefore tend to accumulate mutations and chromosomal rearrangements, resulting in rapid evolution of malignant clones and metastasis (8, 9, 10, 11). This may also be one of the reasons for the increased resistance of cancer cells to chemotherapeutic agents and ionizing radiation used in the treatment of cancer (12).

B. **Previous Work Done in This Laboratory**

Previous studies were performed in this laboratory to identify the mechanisms responsible for the inactivation of the p53 tumor suppressor genes in eight human squamous carcinoma cell lines of different origin (13). The mechanisms responsible for the decreased or absent expression of the wild-type gene product in these cell lines were highly diverse. They included deletion of the gene, gene rearrangement, accelerated rate of degradation of the p53 protein, and presumed mutations in the regulatory sequences of the gene (13). One cell line, A253, established from a squamous cell carcinoma of the submandibular region of a cancer patient (14, 15) failed to express any detectable p53 mRNA transcripts by Northern blot analysis (13) nor detectable p53 protein by Western blot (13). However, using a very sensitive technique called "reverse transcriptase polymerase chain reaction" (RT-PCR), a small amount of p53 mRNA was detected (Teresita Munoz-Antonia, unpublished data). When the genomic structure was evaluated by Southern blot analysis, it was found that A253 cells contained the expected 7 kb and 2.5 kb Hind III DNA fragments and 7.8 kb Bam HI fragment (13). Further study by chemical mismatch analysis of PCR-amplified cDNA revealed no mutations within exons 2 to 11 in the single p53 carried by A253 cells (13). Similarly, sequence analysis of a major portion of the first promotor region (210 bases 5' to the

first exon) (Figures 1 and 2) failed to reveal any mutation (unpublished data). These results indicate that the cell line A253 contains a single copy of p53 which does not carry any mutations in its coding region nor most of the first promoter region but which is transcribed at a subnormal level, detectable only by RT-PCR but not by Northern blot. Since the dysfunction of p53 in this cell line is apparently not due to a mutation nor a deletion in the coding sequence, it may be due to a novel mechanism not yet reported in the literature. Of interest, Stephen Friend reported recently that approximately 10% of human cancers carry a single wild-type p53 allele, which is not transcribed (Stephen Friend, personal communication). Therefore, I focused my attention on uncovering the mechanism responsible for this greatly reduced transcription of the p53 allele in A253 cells. One possible mechanism may be an alteration in an undefined cis- or trans-regulatory element upstream of the p53 promoter region or within the introns of the p53 gene such as from a point mutation or from a change in methylation pattern. The other possibility is an altered expression of cellular regulatory factors, such as, for example, an increase in a p53 suppressor protein or a decrease in a p53 transcriptional activator.

Figure 1. Map of the human p53 gene. The exons 1 through 11 are denoted by black boxes.

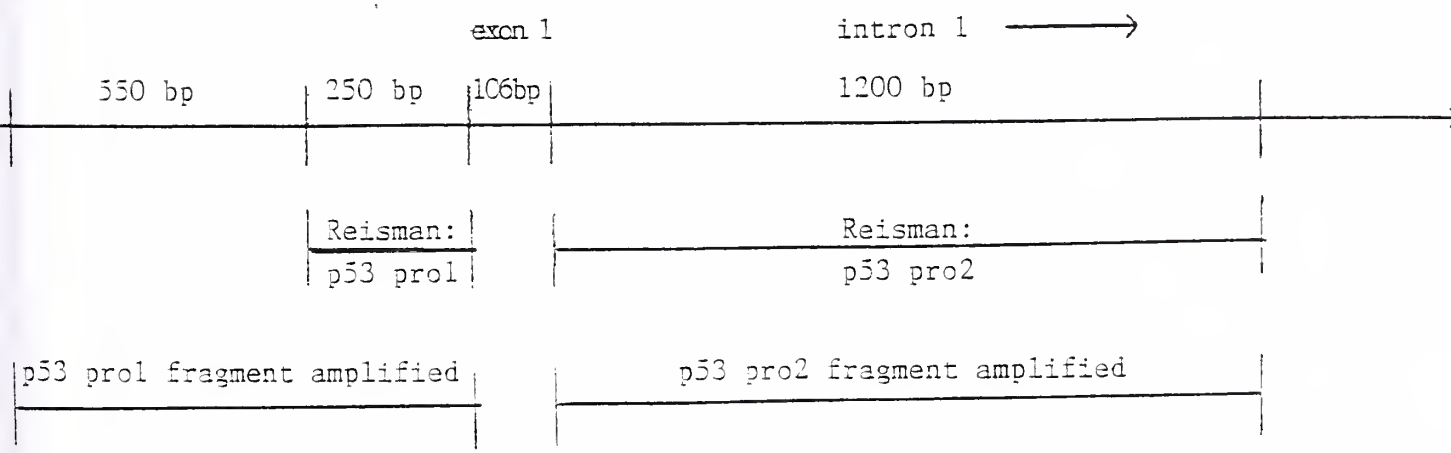


Figure 2. Schematic drawing of the 5' end of the p53 gene showing the two DNA sites identified by Reisman et al. (18) as having promoter activity. Also shown is the regions of DNA fragments, containing the p53 promoter 1 and 2, that were PCR amplified and cloned into the pGL2-Basic Vector in this study.

C. Regulation of Transcription of the p53 Gene

The human p53 gene is 20 kbp long and contains 11 exons. The first exon is noncoding and is followed by first intron of 10 kbp (16) (Figure 1). There are some discrepancies as to the exact localization of p53 regulatory regions. Lamb et al. identified a 350 bp region upstream of the 5' end of exon 1 as having full promoter activity (16). On the other hand, Tuck et al. analyzed 15 3' and 5' promoter deletion constructs and showed that an 85 bp fragment located 5' of the first exon retains at least 90% of the activity of the 350 bp fragment (17). Finally, Reisman et al. proposed the possibility that the p53 gene is regulated by two different promoters. They identified a first promoter (promoter 1) as being located 100-250 bp upstream of the 5' end of the first exon and a second promoter (promoter 2) being located within the 5' 1.2 kbp of intron 1 (18) (Figures 1 and 2). They also found the activity of the second promoter to be 3 to 9 times greater in the cell line K562, and 12 to 57 times greater in the cell line HL-60, compared to the first p53 promoter (18). In addition, when the human cell line HL-60 was induced to undergo terminal differentiation, the expression of the second promoter increased approximately 5 to 10 times while the expression of the first promoter remained constant (19). Based on these findings, the investigators concluded that

the expression of p53 is differentially controlled in normal, differentiating, and transformed cells (18, 19).

Although most authors (Tuck, Lamb, Reisman, and Bienz-Tadmor) agree that there is evidence for the existence of a negative regulatory element upstream of the p53 promoter region in murine cells, they disagree on the existence of a similar negative regulatory region in the human p53 gene (16-20). Reisman found evidence for an element upstream of the first promoter which negatively regulates the activity of the first and second promoter in a human cell line (18). However, Lamb and Tuck found no evidence for such an element (16, 17). Lamb suggested that either there may not be such a negative element or that there may be an additional positive regulatory element which counter-balances the negative effect of a repressor element (16). Further, some investigators have identified a number of potential regulatory sites within the human and murine p53 promoter region as well as a number of regulatory proteins that may bind to these sequences. At least four potential regulatory sites have been recognized in murine p53 promoter: These include an AP-1 like binding site, a potential SP-1 binding site, an NF-1 recognition sequence, and a conserved consensus recognition sequence for the basic helix-loop-helix (bHLH) family of DNA-binding proteins such as c-Myc, USF, and TFE3 (21, 22, 23). Reisman et al. have shown that changes in the level of one of these regulatory proteins, USF, can alter the level of activity of the murine p53

promoter in NIH-3T3, SVT2, and Cos cells. Furthermore, a deletion of the bHLH recognition motif leads to a reduction in the activitiy of the p53 promoter (21). Although these studies were carried out in murine cells, Ronen et al. found that a bHLH recognition site is also present in the human p53 gene (23).

D. Statement of Purpose and Design of Experiments

The published studies summarized above suggest the possible existence of repressor and, perhaps, enhancer elements upstream of the human p53 promoter (18, 19). Furthermore, a number of potential regulatory sites in the p53 gene as well as a number of potential regulatory proteins were found in murine cell lines (16-23). Thus, an alteration, either in a regulatory site of p53 or in any of the cellular regulatory proteins, might be responsible for the marked decrease in p53 gene transcription in A253 cells. Either of these would represent novel mechanisms of inactivation of p53. In order to investigate these two possibilities, I constructed two plasmids, one with the wild-type p53 promoter 1 and the other with the wild-type p53 promoter 2, linked to a reporter gene called luciferase. I then transfected a non-neoplastic keratinocyte cell line, R12HKc/HPV16 (which was found previously to contain normal amount of normal sized p53 mRNA and wild-type p53 protein) (13) and the A253 cell line with either of these plasmids and measured the luciferase activity as an indication of the

transfected p53 promoter's level of activity. This experiment might have one of two possible outcomes. In the first scenario, the luciferase activity in A253 cells might be equal to that in the non-neoplastic keratinocyte cell line. This would mean that the cellular environments in both cell lines were similar in their effect on the wild-type p53 promoter. We could then conclude that the greatly reduced expression of the endogenous p53 allele in the A253 cell line is most likely due to a mutation in some regulatory region(s) of the gene itself. On the other hand, if I found that the luciferase activity in A253 cells was lower than that in the non-neoplastic keratinocyte cell line, this would suggest that the altered cellular environment of the A253 cells was somehow responsible for the greatly reduced activity of the p53 promoter.

MATERIALS AND METHODS

A. Cell Culture: The squamous carcinoma cell line, A253, was obtained from the American Type Culture Collection (Rockville, MD) (13). Immortalized non-tumorigenic R12 HKc/HPV16 human keratinocytes were established by Dr. L. A. Pirisi, University of South Carolina, from a primary culture of human neonatal foreskin keratinocytes by transfection with recombinant human papillomavirus type 16 DNA, pMHPV16d (24). Both cell lines were adapted to and maintained in serum-free basal MCDB153-LB medium, supplemented with 70 ug/ml of bovine pituitary extract (Hammond Cell Technology, Alameda, CA), 10 ng/ml of epidermal growth factor, 5 ug/ml of insulin, 5 ug/ml of transferrin, 5 ng/ml of selenium, 1.4 x $10m^{-7}$ M hydrocortisone, 10^{-8} M triiodothyronine, 10^{-4} M ethanolamine, 10^{-4} M phosphoethanolamine, and 10 ug/ml of gentamycin (MCDB 153-LB++) as described by Pirisi et al. (24). For the culturing of A253, this complete medium was supplemented with 1% fetal bovine serum. The cells were grown in T-75 flasks and the media were changed every other day.

B. Construction of Recombinant Plasmids:

a) Preparation of wild-type p53 promoter 1 and 2 DNA fragments: Wild-type p53 promoter 1 and 2 fragments were isolated by PCR. For the first p53 promoter, the plasmid phuRxCAT (courtesy of Moshe Oren, Department of Chemical

Immunology, Weizmann Institute of Science, Israel) containing the wild-type p53 promoter linked to a chloramphenicol acetyltransferase (CAT) reporter gene was used as the DNA template. The 5' primer used was 5' GCGCGGTACCTTCCCATCAAGCCCTAGGGC 3' with a KpnI restriction nuclease recognition sequence at the 5'end. The 3' primer used was 5' GCGCAGATCTTTTTGAGAAGCTCAAAAC 3' with a BglII restriction nuclease recognition sequence attached to its 5' end. A DNA fragment extending from the 5' end of the first exon to 842 bp upstream was PCR amplified (26) in buffer containing 500 mM Tris, pH 9, 500 mM KCl, and 15 mM $MgCl_2$. For the second p53 promoter, genomic DNA from keratinocytes was isolated as described by Blin and Stafford (27) and used as template. The 5' primer used was 5' GCGCGGTACCATTGGGTAAGCTCCTGAC 3' with a KpnI site at the 5' end. The 3' primer used was 5'GCGCAGATCTTTCAGCCTGCGTCTGGAAC 3' with a BglII site at the 5' end (thereby generating a BglII site at the 3' end of the PCR amplified DNA fragment). The DNA fragment amplified by PCR extends from the 3' end of exon 1 to about 1.2 kbp downstream. It was amplified in buffer containing 840 mMTris pH 9, 430 mM KCl, and 13 mM $MgCl_2$. The parameters for PCR amplification were one initial denaturation at 94^oC for 5 minutes followed by 31 cycles of denaturing at 94^oC for 30 seconds, annealing at 55^oC for 1 minute, and elongating at 72^oC for 30 seconds for the first 30 cycles and 10 minutes for the last cycle. The

two DNA fragments (containing the first and second p53 promoters) amplified are shown in Figure 2.

b) Ligation of p53 promoter DNA fragment to pGL2-Basic Vector: The PCR amplified wild-type p53 promoter 1 and 2 DNA fragments were phenol-chloroform extracted and precipitated in ethanol. They were then digested with the restriction nuclease enzymes KpnI and BglII to create a staggered cleavage at the 5' KpnI restriction nuclease site and the 3' BglII site. These fragments were then separated by electrophoresis in a low melting point agarose gel. After the gel slices were melted by heating at 65^{o}C for 5 to 10 minutes, the DNA fragments were purified by phenol:chloroform extraction and ethanol precipitation. The pGL2-Basic Vector was obtained from Promega (Fig 3).

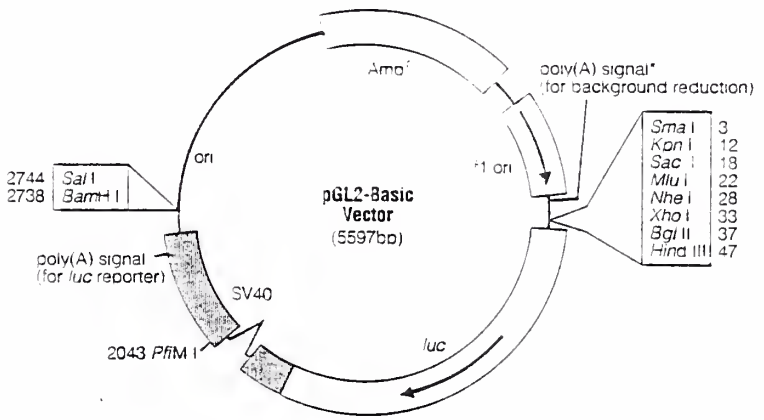


Figure 3. pGL2-Basic Vector.

This vector lacks any promoter or enhancer sequences but contains a luciferase reporter gene downstream from multiple restriction nuclease recognition sites where exogenous promoter sequences may be introduced. The pGL2-Basic Vector was digested with the restriction endonucleases KpnI and BglII and ligated with the 842 bp DNA fragment containing the first p53 promoter to form the recombinant plasmid pGL2-p53pro1. Similarly, the vector was ligated to the 1.2 kbp DNA fragment containing the second p53 promoter to form pGL2-p53pro2. The ligation was carried out by incubating 100 ng of pGL2-Basic Vector and 45 ng of p53 promoter fragment with T_4DNA Ligase (Promega, Madison, WI) at 16^{o}C for 16 hours in a buffer contining 3 mM Tris-HCl, pH 7.8, 10 mM $MgCl_2$, 10 mM DTT, and 5 mM ATP. At the end of the incubation, the mixtures were heated at 65^{o}C for 10 minutes to inactivate the T_4DNA Ligase.

c) Selection of the ligated recombinant plasmids using miniprep: The mixtures containing the ligated recombinant plasmids were used to transform competent *E. coli*. cells, DH5-alpha. The resulting clones were analyzed by restriction enzyme digestion. Based on the size of the inserts after KpnI and BglII digestion, two clones were selected, one containing a 842 bp insert (pGL2-p53pro1) and the other containing a 1.2 kbp insert (pGL2-p53pro2). Both recombinant plasmids were isolated using the Triton-lysozyme method and purified by banding on a CsCl gradient.

C. Verification of Recombinant Plasmids:

a) Digestion with various restriction nuclease enzymes to verify the recombinant plasmids: The identity of the pGL2-p53pro1 plasmid was verified by digestion with restriction nucleases KpnI, BglII, SalI, SacII, HindIII, and SmaI individually and in various combinations to determine whether they yield DNA fragments of the expected sizes. The digestions were carried out by incubating the pGL2-p53pro1 plasmid and the restriction endonuclease(s) at 37^{o}C (except for SmaI which is incubated at 25^{o}C) for 2 to 3 hours. At the completion of digestion, the sizes of the digested fragments were analyzed by electrophoresis on a 1% (w/v) agarose gel.

b) Direct DNA sequencing of the p53 promoter 2 fragment from pGL2-p53pro2 for further verification: Sequencing was performed by annealing an unlabeled primer to the denatured p53 promoter 2 DNA fragments for 3 minutes at 95^{o}C, extending the primer one base with the 32-P labelled dTTP and Sequenase (U. S. Biochemical,Cleveland, OH) for 2 min. at 25^{o}C, adding Sequenase termination mixes , and sequencing for 5 min. at 37^{o}C (13).

D. Other Plasmids:

a) pRSV-luc plasmid: The plamid pRSV-luc was used in experiments that were conducted to determine the optimal conditions for transfection. The strong transcriptional activity of the long terminal repeat of Rous sarcoma virus (29) makes the luciferase activity much easier to measure during this optimization process.

b) pSV-2CAT plasmid: pSV-2CAT plasmid was co-transfected with the test plasmid (pGL2-p53pro1 or pGL2-p53pro2) in order to correct for any differences in the luciferase activity that was due to the difference in transfection efficiency between the two cell lines, A253 and R12HKc/HPV16.

E. Determination of the Level of p53 Transcriptional Activity:

a) Transient transfection assay:

Three different methods of transfections were tried.

1) Transient transfections using Lipofectin (GIBCO-BRL, Bethesda, MD) were carried out according to the manufacturer's specifications. The cells to be transfected were grown to 50-90% confluency in 10 cm dishes at 32^{o}C in the presence of 5% CO_2. 10 ug of recombinant plasmid DNA containing the wild-type p53 promoter were mixed with 5 ug of pSV-2CAT plasmid and 50 ul of lipofectin solution. The

cells were washed twice with the basal MCDB153-LB medium and the plasmid and lipofectin mixture was added dropwise to the cells on the plates. The plates were then incubated at 32°C for 5-6 hours before one ml of complete medium (MCDB153-LB++) was added. The plates were then incubated overnight at 32°C. Twenty-four hours after transfection, the transfection medium was completely replaced with 10 ml MCDB153-LB++ complete medium. The cells were incubated for an additional 24 hours before proteins were extracted.

2) Transient transfections using electroporation were carried out essentially according to the protocol described by Reiss et al. (28). Cells grown to about 80% confluency were detached by trypsinization and resuspended in 0.5 ml of ice-cold phosphate buffered saline. 50 ug of pRSV-luc and 50 ug of pSV-2CAT plasmids were added to the cell suspension and incubated for 10 minutes in ice. The mixture was then transferred to sterile 0.4 cm polysterene cuvettes provided with two aluminium electrodes as described by Potter et al. (30). An electric pulse of 100 to 200 V was then applied to the electroporation chamber. After the shock, the cell suspension was kept in ice for 10 minutes, diluted with warm culture medium, and transferred to a new dish. The cells were incubate at 37°C for 30 hours before they were harvested for protein extraction.

3) Transient transfections by calcium phosphate precipitation were also carried out. 12-16 hours prior to transfection, 1.5 x 10^6 cells were plated onto 10 cm dishes. The medium was replaced with fresh MCDB153-LB++ two hours before transfection. 22.5 ug of pRSV-luc and 11.25 ug of pSV-2CAT were mixed with 962 ul of TE (1mM Tris and 0.1mM EDTA, pH 7.9) and 141 ul of 2M $CaCl_2$ to a final volume of 1125 ul. This mixture was added slowly to the 2xHBS solution (280mM NaCl, 50mM hepes, 1.5mM Na_2HPO_4, pH 7.1) while simultaneously bubbling the 2xHBS with a 1 ml pipette attached to a mechanical pipettor. After incubation for 40 minutes at room temperature, the mixture was added dropwise to each plate. The cells were then incubated at 37^{o}C for 4 hours before they underwent glycerol shock using 11.25 ml of 15% glycerol for 1-2 minutes to increase transfection efficiency. After washing the glycerol off with MCDB153-LB++, the cells were incubated for 48 hours at 37^{o}C incubator before protein extraction.

b) Preparation of protein extract: Thirty hours (for electroporation) and forty-eight hours (for lipofectin and calcium phosphate precipitation) after the start of transfection, the plates were rinsed with ice-cold PBS and the cells were gently scrapped with a rubber policeman using 1.5 ml of scrapping buffer (0.04 M Tris, pH 7.4, 0.15 M NaCl, and 0.001 M EDTA). The cells were then transferred to a microcentrifuge tube and centrifuged at 14,000 rpm for 4

minutes at 4°C. The supernatant was discarded and the cell pellet was resuspended in 50 ul of extraction buffer (100 mM KH_2PO_4 and 1 mM DTT). The cells were lysed by 3 cycles of freezing in a dry ice/ethanol bath for two minutes and thawing in a 37°C water bath for 2 minutes. The cell debris was then collected by spinning it down in a microcentrifuge The supernatant containing the protein extract was transferred to a clean tube and stored at -70°C.

c) Determination of protein concentration: The concentration of protein extract was determined using the Bio-Rad Protein Assay (BIO-RAD Laboratories, Richmond, VA) based on the method of Bradford (31).

d) Luciferase assay: The reporter gene luciferase codes for an enzyme which oxidizes luciferin to produce a photon. The measurement of the light intensity can then give an accurate indication of the level of expression of the gene and, indirectly, of the transcriptional activity of the promoter which drives the reporter gene. This measurement is done using the Luciferase Assay System (Promega, Madison, WI) according to the manufacturer's specifications. A measured amount of protein extract was diluted to a volume of 20 or 40 ul and mixed with 100 or 200 ul of luciferase assay reagent, respectively. These mixtures were immediately placed in a Series 20 Barthda Luminometer (Turner Designs, Sunnyvale, CA) for measurement of light production.

e) Quantitative determination of chloramphenicol acetyl transferase (CAT) in transfected cells: The amount of CAT enzyme was determined using the CAT ELISA Assay from Boehringer Mannheim Corporation (Indianapolis, IN) according to the manufacturer's protocol. Briefly, 50 ug of protein extract was diluted to a volume of 200 ul using sample buffer (Solution III from the CAT ELISA Assay kit). Different dilutions of CAT protein were used to create a standard curve. The protein extract to be measured and the standards were incubated in a 96 well plate containing anti-CAT antibodies for 2 hours at 37^{o}C while rocking slowly. The solution was then discarded, the wells washed four times with washing buffer containing 0.5% (v/v) Tween-20 in PBS buffer, and a digoxigenin-labeled antibody to CAT was added. After one hour incubation, the solution was discarded and wells washed as before. An antibody to digoxigenin conjugated to peroxidase was then added for another one hour incubation. Lastly, the peroxidase substrate was added. Since the peroxidase enzyme cleaves the substrate to produce a colored precipitate, the absorbance of the sample precipitate can be determined and is an indication of the level of CAT protein present in the cell extracts.

RESULTS

Verification of Recombinant Plasmids:

After ligation of the p53 promoter 1 and p53 promoter 2 DNA fragments into the pGL2-Basic Vector and transformation into competent E. coli. cells, the recombinant plasmids were isolated and digested with the restriction nucleases KpnI and BglII. Figure 4 shows that 4 out of 6 clones yielded the expected 5.6 kbp fragment for the linear pGL2-Basic Vector and the 0.84 kbp fragment for p53 promoter 1 and 9 out of 13 clones had the expected 5.6 kbp pGL2-Basic Vector fragment and the 1.2 kbp fragment for the p53 promoter 2. Clone 1A was grown for isolation of the plasmid pGL2-p53pro1 by Triton-lysozyme method and clone 20 was selected for the plamid pGL2-p53pro2. The recombinant plasmid pGL2-p53pro1 was digested using SalI+SacII, KnpI+BglII, and SacII alone. The sites for these restriction nucleases are shown on Figure 5, and Figure 6 shows that the digestions yielded fragments of the correct size on gel electrophoresis.

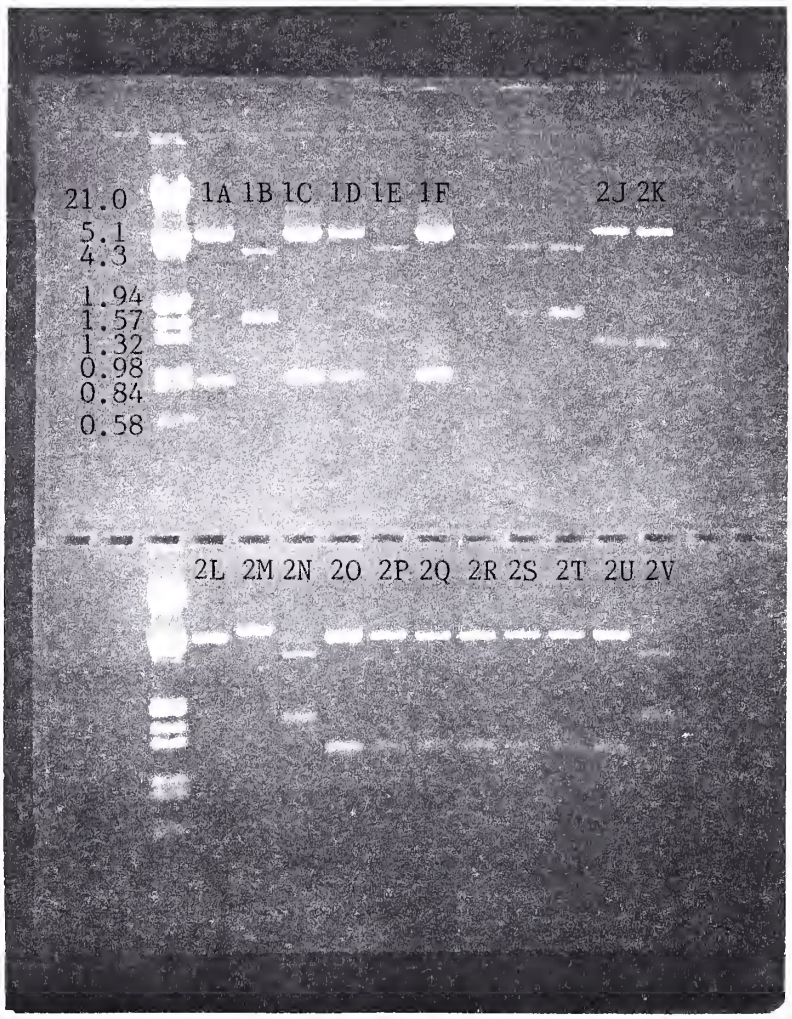
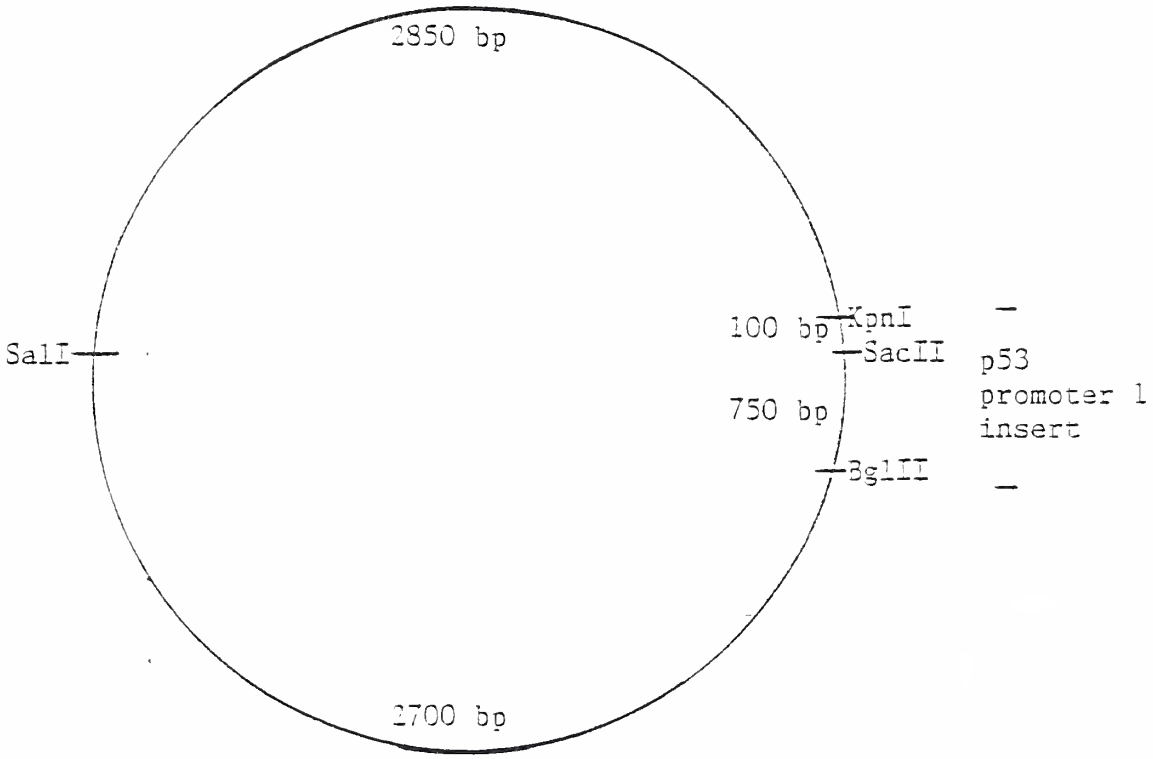


Figure 4. Digestion of plasmid DNA prepared from transformed E. coli. (DH5-alpha) colonies. The restriction nucleases used were KpnI and BglII which released the inserted p53 promoter fragment from the recombinant plasmid. Lane one shows the sizes of DNA standard lambda DNA EcoR1/HindIII prepared by V. F. Vellucci. Lanes 1A to 1F show the digestion of plasmid DNA from colonies that were transformed by the ligated product of pGL2-basic vector and p53 promoter 1 fragment. Lanes 1A, 1C, 1D, and 1F all showed the expected fragment sizes of 0.84 kbp for p53 promoter 1 fragment and 5.6 kbp for the pGL2-basic vector. Lanes 2J to 2V represent the digestion of plasmid DNA from colonies that were transformed by the ligated product of pGL2-basic vector and p53 promoter 2 fragment. Lanes 2J, 2K, 2O, 2P, 2Q, 2R, 2S, 2T, and 2U all showed the expected fragment sizes of 1.2 kbp for the p53 promoter 2 fragment and 5.6 kbp for the pGL2-basic vector.



expected sizes of fragments from digestion by:

SalI and SacII --> 2.85 kbp and 3.45 kbp

KpnI and BglII --> 0.84 kbp and 5.60 kbp

SacII --> linear 6.4 kbp

Figure 5. pGL2-p53pro1 recombinant plasmid restriction nuclease sites and expected sizes of fragments from nuclease digestions.

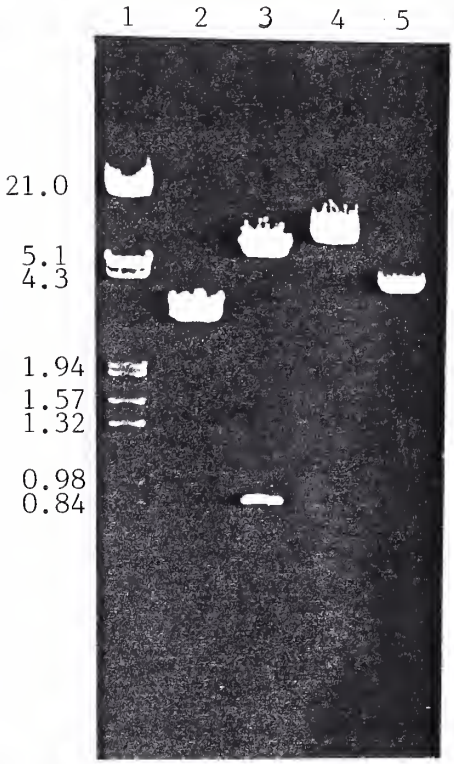
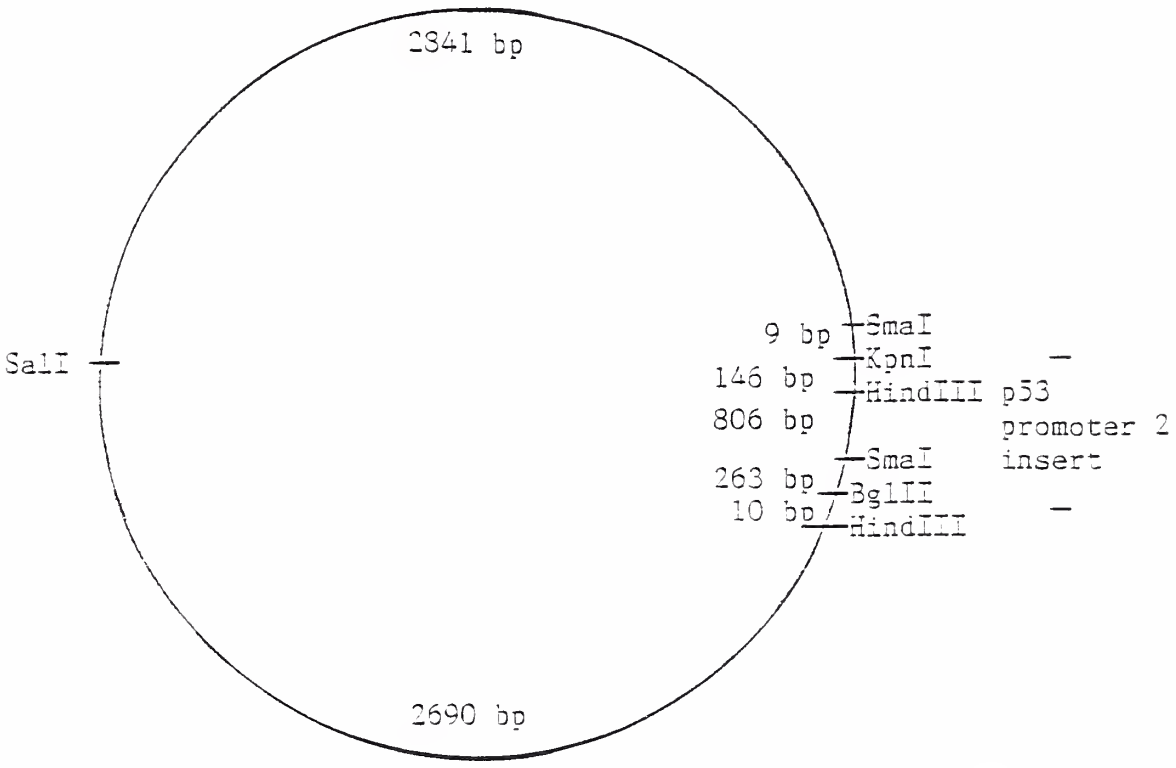


Figure 6. Digestion of the recombinant plasmid pGL2-p53pro1 by SalI and SacII (lane 2), KpnI and BglII (lane 3), and SacII alone (lane 4). Lane 1 shows the sizes of the standard lambda DNA EcoRI/HindIII stardard. Lane 2 shows the 2.95 kbp and 3.45 kbp digest products from SalI and SacII; lane 3 shows the 5.6 kbp and .84 kbp products from KpnI and BglII digestion; lane 4 shows the linear 6.4 kbp fragment from single cut made by SacII; and lane 5 shows the undigested pGL2-p53pro1 cirular plasmid of 6.4 kbp. The result of these digestions confirmed that the correct recombinant plasmid was cloned since they produced fragments of expected sizes.

The restriction nuclease digestion sites for pGL2-p53pro2 together with the expected sizes of digestion fragments are shown in Figure 7. The result of the digestion is shown in Figure 8. Surprisingly, lanes 8 and 9 revealed fragments of unexpected sizes. On lane 8, the digestion by SalI and SmaI should give fragments of 2.97 kbp, 2.85 kbp, and 0.96 kbp; instead, fragments of about 3.9 kbp and 2.85 kbp are seen. On lane 9, digestion by SmaI alone should give rise to fragments of 5.8 kbp and 0.96 kbp; instead, a fragment of similar size to lane 10 is seen suggesting only a single cut was made by SmaI. The observed sizes of digestion products in lanes 8 and 9 would be expected if there was no SmaI restriction site within the 1.2 kbp p53pro2 fragment, leaving only one SmaI site on the pGL2-vector close to the KpnI site. Thus, although there is a HindIII site at the expected region within the p53pro2 fragment as shown in lane 6, the results from lane 8 and 9 appear to indicate that the expected SmaI site within the p53pro2 fragment is missing. This raises the possibility that there is a SmaI polymorphism within the p53pro2 fragment or that the fragment cloned is not really the p53pro2 fragment. In order to resolve this issue, direct DNA sequencing of the 5' and 3' region of the 1.2 kbp fragment was done. 150 base pairs were read from the 5' end and also from the 3' end which matched the wild-type sequence of p53 promoter 2 region. This verifies that the right fragment had been cloned into the pGL2-Basic Vector. Unfortunately, the

putative SmaI site is 260 base pairs from the 3' end and I was therefore unable to confirm the existence of a polymorphism at this site. Since the p53pro2 fragment is derived from the intron 1 region, which may not be an important region to be conserved during evolution, it is possible that a SmaI polymorphism exists among individuals. Polymorphisms within intron 1 of p53 have also been reported by others (32, 33).



expected sizes of fragments from restriction nuclease digestion:

HindIII --> 5.7 kbp and 1.08 kbp (lane 6)

KpnI and BglII --> 5.55 kbp and 1.2 kbp (lane 7)

SalI and SmaI --> 2.97 kbp, 2.85 kbp, and 0.96 kbp (lane 8)

SmaI --> 5.8 kbp and 0.96 kbp (lane 9)

SalI --> linear 6.75 kbp (lane 10)

if SmaI site is absent in the p53 promoter 2 fragment:

SalI and SmaI --> 3.9 kbp and 2.85 kbp (lane 8)

SmaI --> linear 6.75 kbp (lane 9)

Figure 7. pGL2-p53pro2 recombinant plasmid restriction nuclease sites and the expected sizes of DNA fragments from nuclease digestions. If the SmaI restriction site is missing in the p53 promoter 2 fragment, then fragment sizes different from that expected would appear on lanes 8 and 9.

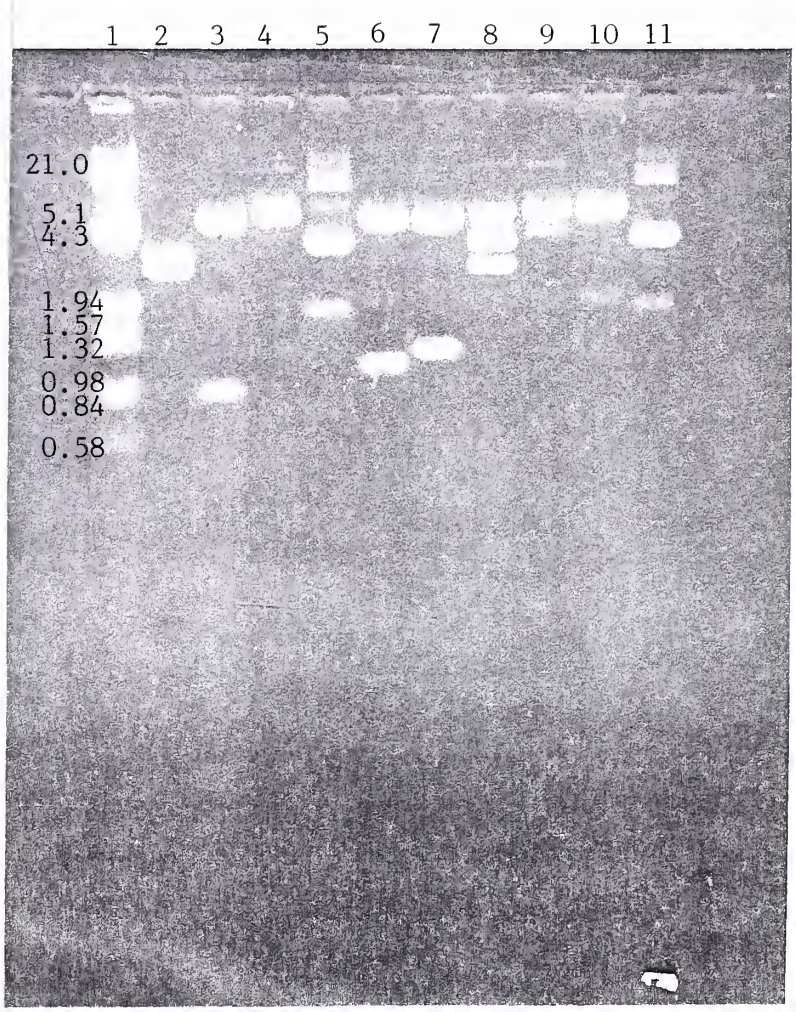


Figure 8. Restriction nuclease digestion of the recombinant plasmid pGL2-p53pro2. Lane 1 shows the sizes of the standard lambda DNA EcoRI/HindIII and lanes 2 through 5 are the digestion products of pGL2-p53pro1 as shown earlier on Figure 6. Lanes 6 to 11 show the digestion products of pGL2-p53pro2. In lane 6 the expected 5.7 kbp and a 1.08 kbp fragments from a HindIII digestion are seen. Lane 7 also reveals the expected sizes of fragments, 5.55 kbp and 1.2 kbp, from digestion by KpnI and BglII. A linear fragment of 6.75 kbp from the single cut of SalI is shown on lane 10, and lane 11 shows the circular 6.75 kbp fragment from an undigested pGL2-p53pro2 plasmid. Lanes 8 and 9 revealed fragments of unexpected sizes. On lane 8, the digestion by SalI and SmaI should give fragments of 2.97 kbp, 2.85 kbp, and 0.96 kbp; instead, fragments of 3.9 kbp and 2.85 kbp are seen. On lane 9, digestion by SmaI alone should give rise to fragments of 5.8 kbp and 0.96 kbp; instead, a fragment of 6.75 kbp is seen.

Transient Transfection Studies:

The difference in the measured activity of a reporter protein (CAT or luciferase) by a particular promoter, in the two different cell lines, A253 and R12HKc/HPV16, may be due to many factors:

1. Different number of cells present in different cell culture plates. This is corrected by expressing the level of reporter protein activity per unit of protein extract.

2. Difference in the transfection efficiency between the two cell lines. In order to correct for this factor, the control plamid pSV-2CAT was transfected at the same time with the test plamid (pRSV-luc, pGL2-p53pro1, or pGL2-p53pro2). Assuming that the control plamid and test plasmid are transfected with equal efficiency into the same cell line and assuming that the two cell lines affect the SV-2 promoter similarly, then the expression of the luciferase activity per ug of protein over CAT enzyme concentration would correct for any difference due to different transfection efficiency between the two cell lines.

3. Differences in the cellular environment (e.g. presence or absence of certain gene regulatory proteins) of the two cell lines which alter the activity of a particular promoter causing a difference in the expression of reporter protein. This is the difference which we expect to measure in these experiments.

Optimization of Transfection Conditions For A253 and R12HKc/HPV16 Cell Lines Using the Plasmids pRSV-luc:

Three different methods of transfections (electroporation, calcium phosphate precipitation, and lipofection) were tested in order to determine which technique would yield the highest transfection efficiency in the two cell lines. The plasmid pRSV-luc was used in these optimizing experiments because of the very strong transcriptional activity of the long terminal repeat of Rous sarcoma virus which makes the luciferase activity much easier to measure (29).

1. Transfection of A253 and R12HKc/HPV16 Cells Using Electroporation Technique:

Each cell line was shocked using Gene Pulser apparatus & capacitance extender (Bio-Rad Laboratories, Hercules, CA) with an electric pulse of 100 V or 200 V in the presence of 50 ug of the test plasmid, pRSV-luc. Control cells were shocked at 200 V without the pRSV-luc plasmid. Table 1 shows that the transfection efficiency under this experimental condition was low for both cell lines. The measured luciferase activity per ug protein from cells shocked in the presence of pRSV-luc is close to the background value obtained with mock-transfected cells.

		luciferase activity/ug protein
A253 Cells:	100 V with pRSV-luc	2.27/ug protein
	200 V with pRSV-luc	2.90/ug protein
	200 V (control)	0.72/ug protein
R12HKc/HPV16:	100 V with pRSV-luc	0.81/ug protein
	200 V with pRSV-luc	2.16/ug protein
	200 V (control)	1.14/ug protein

Table 1. Measured luciferase activity per ug protein from A253 and R12HKc/HPV16 cells transfected with pRSV-luc using electroporation at 100 V and 200 V.

2. Transfection of A253 and R12HKc/HPV16 Cells Using Calcium Phosphate-DNA Precipitate:

Calcium phosphate transfection using pRSV-luc and pSV-2CAT was also tried. The luciferase activity measured was also low. For the A253 cells, the luciferase activity is shown in Table 2. The R12HKc/HPV16 cells appeared to be killed under the conditions used in these experiments.

		luciferase activity/ug protein
A253 Cells:	with glycerol shock	1.50/ug protein
	without glycerol shock	2.10/ug protein

Table 2. Luciferase activity of the A253 cells transfected with pRSV-luc using the calcium phosphate precipitate method with or without glycerol shock.

3. Transfection of A253 and R12HKc/HPV16 Cells Using Lipofectin:

Experiments varying the incubation time and the confluency of the cells were carried out to determine the optimal conditions for transfection by lipofection. Table 3 shows the results from the experiment in which the length of incubation time (from the start of transfection to the extraction of proteins from the cells) was varied. Both the A253 and R12HKc/HPV16 cell lines yielded the greatest luciferase activity at 48 hour post-transfection.

	time	luciferase activity/ug protein
A253 Cells:	24 hr.	2.1/ug protein
	48 hr.	153.6/ug protein
	72 hr.	7.6/ug protein
R12HKc/HPV16:	24 hr.	57.9/ug protein
	48 hr.	1798.5/ug protein
	72 hr.	89.6/ug protein

Table 3. Luciferase activities from A253 and R12HKc/HPV16 cell harvested at different time after transfection with pRSV-luc.

Another variable tested was the confluency of the cells in the plate at the time of transfection. Cells were grown to between 50-90% confluency at the time of transfection with pRSV-luc to determine the optimal cell density. The

incubation time was kept at 48 hours. The results are shown in Table 4 and appear to indicate that for both A253 and R12HKc/HPV16 cells, 80-90% confluency at the time of transfection yielded the highest rate of transfection efficiency and therefore the highest luciferase activity.

	confluency	luciferase activity/ug protein
A253 Cells:	60-70%	2.57/ug protein
	70-80%	23.61/ug protein
	85-95%	40.21/ug protein
R12HKc/HPV16:	50-60%	38.78/ug protein
	60-70%	610.61/ug protein
	70-80%	1521.93/ug protein

Table 4. Luciferase activity of A253 and R12HKc/HPV16 cells from transfection with pRSV-luc at different confluency levels.

From these experiments, transfection using lipofectin appear to yield the highest transfection efficiency in both the A253 and the R12HKc/HPV16 cells. The optimal incubation time was determined to be 48 hours and the optimal cell density at the time of transfection was at 80-90% confluency. However, it is also clear from these experiments that the measured luciferase activity per ug protein in the A253 cells was much lower than that in the

R12HKc/HPV16 cells. Since both of these cell lines were transfected with the test plasmid, pRSV-luc, the lower luciferase activity in A253 cells appears to be due to an intrinsically low transfection efficiency of these cells.

Determination of the Level of p53 Promoter Activities in the Cell Line A253 and a Non-neoplastic Keratinocyte Cell Line, R12HKc/HPV16:

After the transfection condition using lipofectin was optimized in the previous experiments, the A253 and R12HKc/HPV16 cells were transfected using the recombinant plasmids, pGL2-p53pro1 and pGL2-p53pro2, in order to determine the level of p53 promoter 1 and promoter 2 activity in these two cell lines. The incubation time was kept at 48 hours. Table 5 shows the luciferase activity per ug protein, amount of CAT enzyme per ug protein, and the luciferase activity over CAT protein concentration to correct for difference due to transfection efficiency. Even though the values for the luciferase activity per CAT protein appear to suggest that the transcriptional activity of the p53 promoter 1 and 2 was much lower in the A253 cells compared to that in the R12HKc/HPV16 cells, however, the luciferase activities from the A253 cells were so low that interpretation of these data may be unreliable.

	luc/ug protein	CAT/ug protein	luc/CAT
A253 Cells:			
p53 promoter 1	1.7/ug protein	0.47/ug protein	3.56
p53 promoter 2	2.2/ug protein	0.82/ug protein	2.73
R12HKc/HPV16 Cells:			
p53 promoter 1	392.7/ug protein	6.91/ug protein	56.82
p53 promoter 2	357.6/ug protein	6.58/ug protein	54.30

Table 5. Results of the transfection experiment in A253 and R12HKc/HPV16 cells using the recombinant plasmids, pGL2-p53pro1 and pGL2-p53pro2.

Comparison of the Level of Transcriptional Activities of p53 Promoter 1 and Promoter 2 in the R12HKc/HPV16 Cells:

The transcriptional activity of promoter 2 had previously been found to be 3 to 9 times greater in the human leukemic cell line K562 and 12 to 57 times greater in the human leukemic cell line HL-60, compare to that of the promoter 1 (18). Our previous experiments have shown that transfection of plasmids into the R12HKc/HPV16 cells was largely successful with good measured luciferase activity. Thus, we decided to use R12HKc/HPV16 cell line to compare the strength of transcriptional activity between the p53 promoter 1 and promoter 2. As shown in Table 6 under the luc/CAT section, the transcriptional activity of the first promoter construct was found to be twice that of the second

promoter in one case and about the same as the second promoter in another experiment.

	luc/ug protein	CAT/ug protein	luc/CAT
exp. 1:			
p53 promoter 1	2141.9/ug protein	2.32/ug protein	922.0
p53 promoter 2	978.5/ug protein	2.06/ug protein	473.01
exp. 2:			
p53 promoter 1	392.7/ug protein	6.91/ug protein	56.82
p53 promoter 2	357.6/ug protein	6.59/ug protein	54.30

Table 6. Luciferase activities of p53 promoter 1 and promoter 2 in the cell line, R12HKc/HPV16, expressed per ug protein and per unit CAT protein concentration.

DISCUSSION

p53 tumor suppressor gene is the most commonly identified mutated gene in human tumors to date. About 70% of the 6.5 million people diagnosed with cancer each year carry p53 mutations in their tumors (34). In addition, approximately 30% of the remaining cases carry a single wild-type p53 allele, which is not transcribed (Stephen Friend, personal communication). This pervasive involvement of p53 dysfunction in almost all types of human cancers raises the hope that understanding the mechanism of p53 inactivation will provide new leads for the prevention, diagnosis, and treatment of cancer. The finding that a transfected wild type p53 gene is able to suppress growth and/or tumorigenicity of a variety of human cancer cell lines (35-44) has raised numerous new therapeutic opportunities. One such therapy would involve the introduction of a wild-type copy of the p53 gene into human tumors via gene therapy. Such therapy would have a high therapeutic index since the overexpression of wild-type p53 transfected into nonmalignant cells has minimal deleterious effects (3). In fact, one such trial has already been approved for 14 patients with advanced lung cancer (34).

Perhaps, one of the most widely investigated area involves the evaluation on the use of p53 status as a potential biologic marker to aid in treatment decisions, in

assessing prognosis, and in detecting early tumors (45-59). By far, the most common mechanism responsible for p53 dysfunction is a missense mutation, accounting for about 83% of cases (3). Such mutations usually result in a mutated p53 protein with increased stability, increasing their half-life from minutes to hours and allowing them to accumulate to high concentration inside the malignant cells (60, 61). This increased level of p53 protein in these malignant cells can be detected immunohistochemically using antibodies against the p53 protein (3). On the other hand, the relatively low levels of wild-type p53 protein in normal cells will not be detected using this immunohistochemical analysis (3). This finding is potentially very useful clinically because immunocytochemistry is a simple technique which can be used to detect p53 mutations in tissue biopsy materials, sputum cytologic specimens, or cells from aspirates (3). This could lead to early detection of cancer as well as aid in assessing the prognosis and determining the response to treatment. Although promising, the use of immunohistochemical techniques to detect p53 dysfunction is not perfect. As stated before, the majority (83%) of cases of p53 dysfunction are due to missense mutations that lead to mutant p53 proteins. These mutant proteins usually, but not always, accumulate to high levels in the cells. Harris and Hollstein reported that of the 100 tumors with p53 missense mutations they examined, greater than 90% were shown to have abnormal accumulation of p53 proteins, not

100% (3). Furthermore, when the other mechanisms responsible for 17% of the p53 dysfunction result in truncated protein or a normal, decreased, or even absent level of p53 protein, the immunohistochemical technique will fail to detect them. Cells carrying these types of inactivation would show up as false negatives. The sensitivity for detecting cells carrying p53 defects can be increased by methods such as restriction fragment length polymorphism analysis to detect deletion or single-stranded conformation polymorphism analysis to detect p53 gene mutation. However, neoplastic cells such as the A253 cell line being investigated in this study in which the p53 gene contains no mutation and the p53 protein is present at a very low level will not be detected by any of these techniques. The elucidation of other mechanisms which may be responsible for p53 dysfunction is therefore important clinically. If, for example, a cellular factor is identified as responsible for the suppression of p53 gene expression, this factor may represent a new oncogene and may serve as a biologic marker for detection of these neoplastic cells.

In this study, we have successfully constructed two recombinant plasmids, one containing the first promoter region and the other containing the second promoter region of p53 driving the reporter gene, luciferase. Our transfection experiments indicate that there is indeed

promoter activity in the 5' 1.2 kbp of the intron region which is in agreement with the observation made by Reisman et al. In their study, they found the activity of the second p53 promoter to be 3 to 9 times greater than the first promoter in the cell line K562 and 12 to 57 times greater in the cell line HL-60 (18). However, in our transfection experiments using R12HKc/HPV16 cells, we found the transcriptional activity of the second promoter to be half that of promoter 1 in one case and equal to that of promoter 1 in another instance (Figure 7). This discrepancy between our studies and that of Reisman's group may be due to at least two factors. First, the second p53 promoter region we used in our experiments was cloned from a non-tumorigenic cell line immortalized by transfection with recombinant human papillomavirus type 16 DNA, and therefore, it can be assumed to be wild-type. On the other hand, Reisman et al. isolated their second promoter region from a transformed human cell line SV80 which overexpresses p53 proteins. In their paper, they raised the possibility that there may be mutations which activate the second promoter region in the cell line SV80 which may therefore be responsible for the overexpression of p53 proteins (18). This possibility could account for the difference between their data and our own observation. Secondly, we performed the transfections of the recombinant plasmids containing the wild-type p53 promoters into a non-neoplastic keratinocyte cell line, R12HKc/HPV16. Reisman's group, however,

conducted their experiments using the human leukemic cell lines HL-60 and K562, both derived from leukemic patients (18). In their study, they found that the level of transcriptional activity of the first p53 promoter was about the same in both of these cell lines. The activity of the second promoter, however, differs greatly between the HL-60 and K562 cell lines. This difference in the second promoter's activity account for their observation that the activity of the second p53 promoter is greater than the first promoter by a factor of about 3 to 9 times in the cell line K562 and by a factor of 12 to 57 times in the cell line HL-60. This difference of transcriptional activity of p53 promoter 2 may be due to differences in the cellular factor(s) present in these two cell lines. The high level of p53 promoter 2 activity in these two cell lines may possibly be due to certain abnormally expressed activator proteins that act on the second promoter, and that the HL-60 cell line has a higher level of such putative activators than the K562 cell line. It may also be possible that the R12HKc/HPV16 cell line we used in our experiment lacks this abnormal activator protein and therefore expresses a lower level of p53 promoter 2 activity. The possibility of an activator protein can also explain the finding by Reisman et al. that during the terminal differentiation of the HL-60 cells, the expression from p53 promoter 2 was increased 5- to 10-fold, while expression from the first promoter remained constant (19). Clearly, it is possible that when

this transformed cell line was induced to undergo differentiation, it may increase the level of certain cellular factor responsible for the activation of the second p53 promoter.

Multiple transfection experiments were conducted in order to compare the level of transcriptional activity of the wild-type p53 promoters in the squamous carcinoma cell line A253 and a non-neoplastic keratinocyte cell line R12HKc/HPV16. The two possible results which we would expect from these experiments are that either the level of transcriptional activities of the p53 promoter in the two cell lines are the same or that the level of activity in the A253 cells are much lower than that in the R12HKc/HPV16 cells. The result would allow us to determine if the mechanism responsible for the greatly reduced level of p53 expression in the squamous carcinoma cell line A253 is more likely due to alteration in the regulatory region of the gene (if the activities are the same in the two cells) or due to alteration in the gene regulatory proteins (if the activities of the A253 is much lower than that in the R12HKc/HPV16). However, these transfection experiments were unsuccessful mainly due to the difficulty encountered in transfecting the A253 cells. Although we were able to obtain a satisfactory level of luciferase activity from the transfection experiments done in R12HKc/HPV16 cells, the luciferase activity measured in the transfected A253 cells

was consistently very poor, close to the baseline and therefore uninterpretable. Numerous attempts were made to optimize the transfection conditions but the improvement in transfection efficiency seen in A253 cells was not significant. Further investigations in the search for a possible novel mechanism of p53 inactivation will require the finding of an effective way of introducing the DNA plasmids into the A253 cell line. A second possibility is to conduct these experiments on another cell line, if one such exists, that has the same attributes as the A253 cells, such as the existence of an intact, nonmutated p53 gene but a very low level of p53 mRNA production.

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