Proc. Natl. Acad. Sci. USA
Vol. 88, pp. 4128–4132, May 1991
Genetics

Expression from the murine p53 promoter is mediated by factor binding to a downstream helix-loop-helix recognition motif

(oncogene expression/DNA-binding proteins/trans-activation)

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Communicated by Christian B. Anfinsen, January 31, 1991

ABSTRACT **Expression of the p53 gene plays an important role in the regulation of cellular proliferation and malignant transformation. Overexpression of mutant forms of p53 is in fact a common feature of many transformed cells. Studies dealing with the transcriptional regulatory regions of the p53 gene indicate that, unlike most promoters transcribed by RNA polymerase II, the p53 promoter contains no TATA-like sequence upstream of the transcription start site. Here we demonstrate that the murine p53 promoter contains a cis-acting element that maps downstream to the transcription initiation site. The integrity of this element is required for high-level expression from the promoter in transformed cells. By DNase I protection and mobility-shift analysis, we show that a nuclear factor binds to this downstream element through the consensus recognition sequence for the helix-loop-helix (HLH)-containing proteins of the myc/MyoD family of transcriptional regulators. We propose that the activity of one or more members of this family of transcription factors is an important determinant in the expression of p53 and that at least one level of p53 overexpression in transformed cells may thus be due to aberrant expression of the relevant factor(s). Furthermore, the possibility that the regulation of expression of p53 occurs, in part, by means of a potential HLH-containing factor provides a possible mechanism for the suppression of proliferation by the MyoD family of transcriptional regulators.**

Recent evidence has indicated that p53 is a growth modulator and that aberrant expression or inactivation of p53 is a frequent event in the genesis of many malignancies. The role of p53 in growth regulation is supported by the observations that overexpression of wild-type p53 inhibits cellular proliferation (1–4), that expression of the gene is induced prior to S phase (5), and that p53 is phosphorylated by the cell cycle regulator $p34^{cdc2}$ (6, 7). In addition, the wild-type p53 gene has been shown to inhibit transformation *in vitro* (4, 8) and a frequent property of many malignantly transformed cells is either the loss of wild-type p53 expression (9–13) or overexpression of mutant forms of p53 (14, 15). Furthermore, in cooperation with the *ras* oncogene, overexpression of mutant p53 leads to the transformation of normal embryonic cells (16–18). Mutant forms of p53 have been proposed to function in transformation by a dominant negative mechanism (19), possibly by interacting with endogenous wild-type p53 (8) or its cellular targets.

Although the molecular basis of p53 involvement in cell proliferation and malignant transformation has not yet been defined, a role in the regulation of DNA replication and mRNA transcription is suggested by recent findings. p53 was shown to compete with DNA polymerase α for binding to the simian virus 40 (SV40) large tumor antigen and to inhibit the initiation of SV40 replication *in vitro* (20–22). In addition, it was recently demonstrated that wild-type p53, but not mutant p53, can function as a transcriptional activator when present in cells as a fusion with the DNA-binding domain of the yeast transcription factor GAL4 (23, 24). Both of these functions are consistent with the active nuclear localization of p53 (25–27).

To define elements involved in regulating transcription of the p53 gene, we and others have been analyzing regulatory regions of the gene (28–32). Unlike many genes transcribed by RNA polymerase II, the p53 promoter lacks a TATA box (28, 33). Other promoters that lack the TATA box have recently been shown to contain sequences downstream of the transcription start site that bind factors that influence the activity of the promoter (34–37). By analogy, we predicted that the p53 promoter may contain similar regulatory domains. Here we report the identification of an essential downstream element that binds to a cellular factor through a consensus recognition sequence for the helix-loop-helix (HLH)-containing myc/MyoD family of transcriptional activators (38–40). Binding to this downstream element correlates with elevated expression from the p53 promoter and thus may be necessary for maximal trans-activation of the promoter in transformed cells. The finding that positive regulation of expression of p53 may occur by means of a HLH-containing factor provides one possible mechanism for the suppression of proliferation by the MyoD family of transcriptional regulators.

MATERIALS AND METHODS

Recombinant Plasmids. A series of 3′ deletions were generated within a 0.7-kilobase-pair (kbp) *Eco*RI–*Hin*dIII fragment (see Fig. 1) containing the 5′ end of the murine p53 gene (28). This DNA fragment contains a functional promoter as well as sequences that extend 348 bp downstream of the initiation site comprising the 216-bp noncoding first exon and 132 bp from the first intron. Deletions were generated either by digestion of the DNA with appropriate restriction enzymes (e.g., *Hae* III for p0.49CAT and *Ava* II for pAACAT) or by digestion with BAL-31. The deletion endpoints were approximated (within 5 bp) by electrophoresis of the fragments through 2% agarose relative to appropriate markers. This intact fragment and the deletion derivatives were cloned directly upstream to the *Escherichia coli* chloramphenicol acetyltransferase (CAT) gene on the vector pUC18CAT.

Cell Culture, DNA Transfections, and CAT Assays. The murine cell lines BALB/c-3T3, NIH 3T3, SVT2, and MethA

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Abbreviations: HLH, helix-loop-helix; CAT, chloramphenicol acetyltransferase; SV40, simian virus 40; LTR, long terminal repeat; Mo-MuLV, Moloney murine leukemia virus.

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were cultured in Dulbecco's modified Eagle's minimal essential medium containing 10% fetal bovine serum. Fifteen micrograms of each plasmid was introduced into $\approx 1 \times 10^6$ cells from each cell line by calcium phosphate coprecipitation (41). After 5 hr in the presence of the precipitate, the cells were treated with 20% glycerol in complete medium for 2 min. The cells were washed and maintained in complete medium. After 48 hr, extracts were prepared and assayed for CAT activity (42) using equivalent amounts of protein. The percentage of [^{14}C]chloramphenicol acetylated was quantified by eluting the acetylated and nonacetylated species and measuring the amount of radioactivity by scintillation counting. Control experiments showed that all assays were in the linear range. The values shown are the average of at least three independent transfections. The CAT vector (pUC18-CAT) yielded $\approx$0.3% acetylation.

Preparation of DNA Probes and DNA–Protein-Binding Assays. An 80-bp *Alu* I restriction fragment extending from nucleotides +18 to +98 was subcloned into the *Eco*RV site of the polylinker of pBluescript (Stratagene). The orientation of the insert was determined by the nucleotide sequence of the junctions. The insert was released from the vector by digestion at the polylinker with *Xba* I plus *Sal* I and purified by electrophoresis through 5% polyacrylamide. The ends of the fragment were radiolabeled by reaction with [α-^{32}P]dATP (>3000 Ci/mmol; 1 Ci = 37 GBq) and *E. coli* DNA polymerase I (Klenow fragment). To generate singly end-labeled fragments, the ^{32}P-labeled DNA was digested with either *Hin*dIII or *Bam*HI to remove the radioactivity from the noncoding or coding strand, respectively. Nuclear extracts were prepared from SVT2 cells as described (43). For DNase I protection analysis, binding reaction mixtures contained nuclear extract, 20 fmol of end-labeled DNA, 1 μg of poly(dI-dC), 25 mM Tris·HCl (pH 7.9), 50 mM KCl, 6.25 mM $MgCl_2$, 0.5 mM EDTA, 1 mM dithiothreitol, and 10% glycerol in a volume of 50 μl. Samples were treated with DNase I, purified, and separated by electrophoresis through 7.0% acrylamide/8 M urea gels as described (44). For electrophoretic mobility-shift assays, binding reaction mixtures contained 10 μg of nuclear extract, 20 fmol of end-labeled DNA, 2 μg of poly(dI-dC), 2 μg of calf thymus DNA, 80 mM NaCl, 10 mM Tris·HCl (pH 7.5), 1 mM EDTA, 0.1% Triton X-100, 10 mM 2-mercaptoethanol, and 4% glycerol in a volume of 25 μl. The mixture was incubated on ice for 15 min followed by incubation at 20°C for 15 min and electrophoresed through 4% polyacrylamide gels in the presence of 0.045 M Tris-borate/1 mM EDTA (0.5× TBE). For competition experiments, oligonucleotides were added to the binding reaction mixtures prior to addition of nuclear extract. An oligonucleotide containing an NF-1-binding site contains the sequence 5′-GATCCCCCGGCTCAGGGCCAAGAAC-3′ and was taken from position 8056 to 8076 from the long terminal repeat (LTR) of Moloney murine leukemia virus (Mo-MuLV) (45, 46).

RNA Analysis. Total RNA was isolated by extraction with guanidium isothiocynate and purified by centrifugation through 5.7 M CsCl (47); 50 μg of RNA from each cell line was resuspended in 50% formamide/6% formaldehyde/1× SSC (SSC = 0.15 M NaCl/15 mM sodium citrate) and heated at 60°C for 10 min. Two-fold serial dilutions were prepared and applied to duplicate nitrocellulose filters. The filters were baked at 80°C for 2 hr and hybridized to ^{32}P-labeled CAT coding sequences or to ^{32}P-labeled actin-specific sequences.

RESULTS

Identification of a Downstream Promoter Element Within the Murine p53 Gene. To determine whether sequences downstream of the transcription initiation site influence the activity of the p53 promoter, we generated a series of 3′ deletions within an $\approx$0.7-kbp *Eco*RI–*Hin*dIII fragment containing the 5′ end of the murine p53 gene (27). This DNA fragment contains a functional promoter as well as sequences that extend 348 bp downstream of the initiation site comprising the 216-bp noncoding first exon and 132 bp from the first intron (Fig. 1). This intact fragment and the 3′ deletion derivatives, generated by digestion with BAL-31 exonuclease, were cloned directly upstream to the *E. coli* CAT gene and tested for their ability to transiently direct expression of the reporter gene in the SV40-transformed murine cell line SVT2. These cells were chosen because they express high levels of endogenous p53 (data not shown) and are therefore well suited for characterizing the molecular basis of p53 overexpression. As shown in Fig. 1, deletion of exon 1 sequences between positions +66 and +112 resulted in a 5-fold reduction in expression of the CAT gene (pAACAT vs. p0.39CAT). Deletion of an additional 25 bp from the 3′ end of exon 1 (p0.36CAT) did not lead to a further significant reduction (<2-fold) in expression of the CAT gene. These results indicate that sequences downstream to the p53 transcription initiation site, contained between nucleotides +66 and +112, are required for high-level expression from the p53 promoter in SVT2 cells. It should be noted, however, that deletion of this element did not result in a complete loss of promoter activity. A basal level of activity, $\approx$6-fold above that obtained with the promoterless CAT vector (pUC18-CAT), indicates that additional cis-acting elements that lie upstream are required for a basal level of promoter activity in these cells.

To determine whether this downstream element contains specific protein-binding sites, DNase I protection analysis was carried out with nuclear extracts prepared from SVT2 cells on an 80-bp *Alu* I restriction fragment extending from nucleotides +18 to +98 (Fig. 2*c*). One protein-binding site was identified within this downstream element, as indicated by a region of protection that spans nucleotides +65 to +79

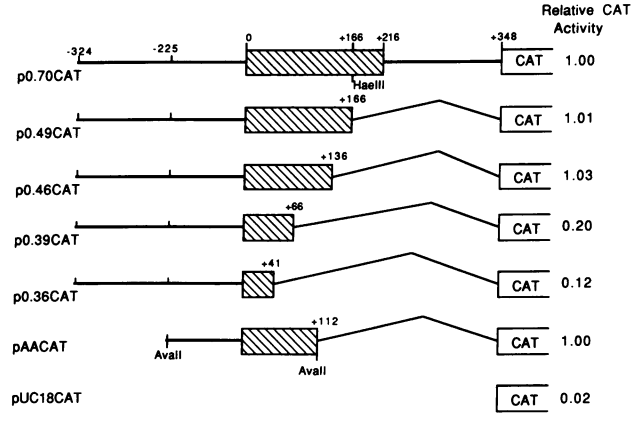

FIG. 1. Deletion analysis and identification of a downstream promoter element in the p53 gene. The construct p0.70CAT contains a 672-bp *Eco*RI–*Hin*dIII fragment from the 5′ end of the murine p53 gene cloned into the *Hin*dIII site of pUC18CAT. Nucleotides −324 to −1 contain upstream sequences shown previously to have promoter activity, nucleotides 0 to +216 (hatched box) contain the noncoding first exon, and nucleotides +217 to +348 are derived from the 5′ end at the first intron (28). Deletions were generated either by digestion of the DNA with appropriate restriction enzymes (e.g., *Hae* III for p0.49CAT and *Ava* II for pAACAT) or by digestion with BAL-31 exonuclease from a unique restriction site within the polylinker. The nucleotide positions of the deletion endpoints are indicated. All deletion derivatives were subcloned immediately upstream to the *E. coli* CAT gene into *Hin*dIII–*Bgl* II sites of pUC18CAT (open box). The resulting constructs were transfected onto SVT2 cells and after 48 hr cell lysates were prepared for CAT assays. Relative CAT activity represents the fraction of measured activity relative to the parental plasmid p0.70CAT ($\bar{x}$ = 15.6% chloramphenicol acetylated).

(Fig. 2*a*). An examination of the nucleotide sequence of the protected region revealed the presence of the sequence CACGTG (nucleotides +70 to +75). This nucleotide sequence is a match to the consensus recognition site (CANNTG) for the myc/MyoD family of HLH-containing transcriptional activators (39, 48). To test whether this specific site is required for protein binding we performed electrophoretic mobility-shift assays on the 80-bp *Alu* I fragment with nuclear extracts prepared from SVT2 cells. Double-stranded oligonucleotides containing either the putative wild-type HLH recognition sequence (CACGTG) or mutations in this sequence (GGTGTG) were used as competitors in the binding reactions. As shown in Fig. 2*b*, the wild-type sequence, which contains the HLH-binding motif, competed for binding to the 80-bp *Alu* I fragment, whereas mutations introduced into this sequence abolished the competition for binding (Fig. 2*b*). These results, as well as the requirement of this downstream element for activity of the promoter, indicate that protein binding to the HLH recognition motif is likely to be required for high levels of expression of the p53 promoter.

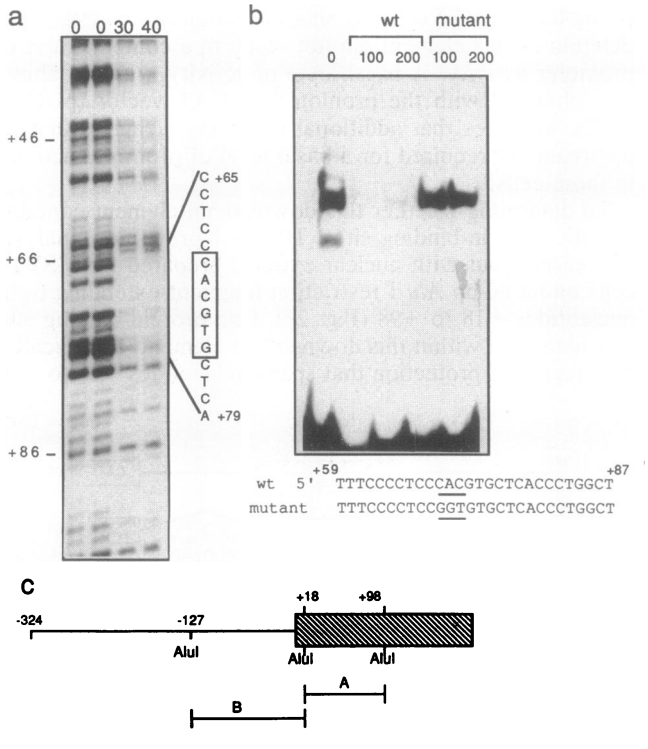

FIG. 2. Factor-binding site containing a HLH recognition motif maps downstream to the p53 initiation site. (*a*) The ^{32}P-labeled coding strand of an 80-bp *Alu* I restriction fragment, containing sequences between nucleotides +18 and +98, was assayed for digestion by DNase I in the absence (0) or presence of increasing amounts (30 and 40 μg) of nuclear extract prepared from SVT2 cells. The numbers identify the nucleotide positions as described in the legend to Fig. 1. The region of protection is indicated along with the nucleotide sequence. The boxed sequence is the HLH consensus recognition site (38, 48). Positions of the nucleotides were determined from Maxam–Gilbert sequencing reactions (A+G). (*b*) Effect of mutations in the HLH recognition motif on competition for binding to p53 downstream sequences. Nuclear extracts from SVT2 cells were incubated with the end-labeled *Alu* I restriction fragment (+18 to +98) in the absence (0) or presence of a 100- or 200-fold molar excess of either a wild-type (wt) or mutant oligonucleotide. Products were assayed by electrophoretic mobility shift. The sequences of the oligonucleotides are shown. (*c*) Illustration of 5′ end of the murine p53 gene showing first exon (hatched box) and upstream sequences. The probe for DNase I protection and mobility shift was the *Alu* I fragment from the first exon (+18 to +98) and is labeled as fragment A. Fragment B is referred to in Fig. 4.

Binding to the Downstream HLH Recognition Motif Correlates with Expression of the p53 Promoter in Transformed Cells. Since the mutant p53 gene is overexpressed in many transformed, but not immortalized, cells, we sought to determine whether binding to the downstream promoter element correlates with high-level expression from the p53 promoter. We first assayed the expression of the CAT gene under control of the intact p53 promoter (p0.7CAT) in two transformed murine cell lines, SVT2 and MethA, and in two immortalized cell lines, NIH 3T3 and BALB/c-3T3. Measurement of the resulting CAT activities revealed that expression from the intact p53 promoter in SVT2 and MethA was ≈10-fold higher than in NIH 3T3 or BALB/c-3T3 (Fig. 3*A*), consistent with the overexpression of the gene in these cells. As controls, the SV40 early promoter and the Moloney murine sarcoma virus (Mo-MuSV) LTR were also tested in this assay. These control promoters expressed the CAT gene at similar levels in all four cell types, indicating that the transfection efficiencies were similar and that the elevated expression of the p53 promoter in the transformed cells was specific. To confirm that the elevated CAT activity was due to increased levels of CAT mRNA, we measured the steady-

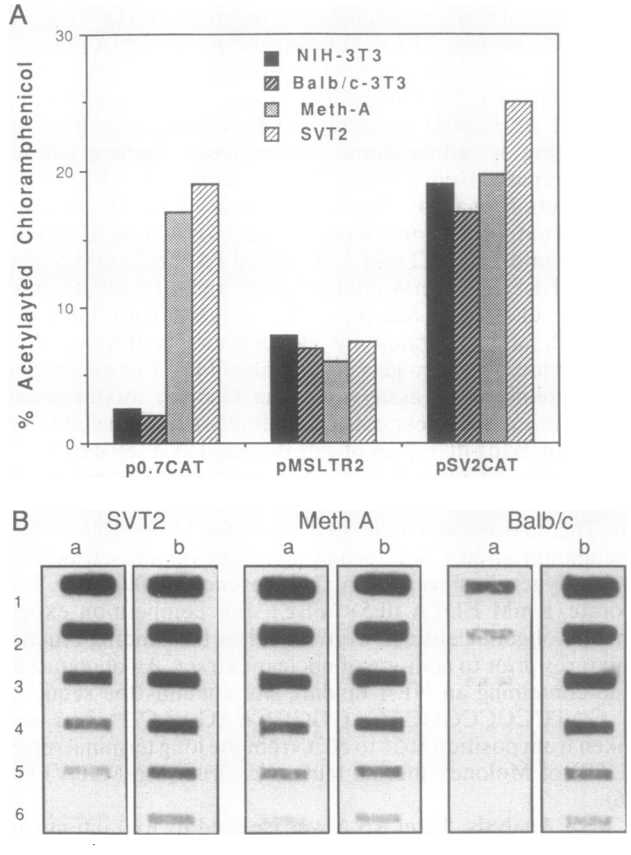

FIG. 3. Selective activation of the p53 promoter in transformed MethA and SVT2 cells. (*A*) Plasmids (15 μg) containing the intact murine p53 promoter (p0.7CAT, Fig. 1), the Moloney murine sarcoma virus (Mo-MuSV) LTR (pMSLTR2), and the SV40 early promoter (pSV2CAT) upstream of the CAT gene were transfected onto immortalized NIH 3T3 and BALB/c-3T3 or onto transformed MethA or SVT2 cells by calcium phosphate coprecipitation. After 48 hr, cells were harvested and equal amounts of cell extract were assayed for CAT activity. pMSLTR2 and pSV2CAT serve as controls for specificity of the response and for transfection efficiency. The results represent the average of three independent transfections. (*B*) Relative levels of CAT-specific RNA after transient expression of p0.7CAT were measured by slot-blot hybridization. The filters were hybridized to ^{32}P-labeled CAT coding sequences (lanes a) or to ^{32}P-labeled actin-specific sequences (lanes b). Rows 1–6 contain 50, 25, 12.5, 6.25, 3.15, and 1.56 μg of RNA.

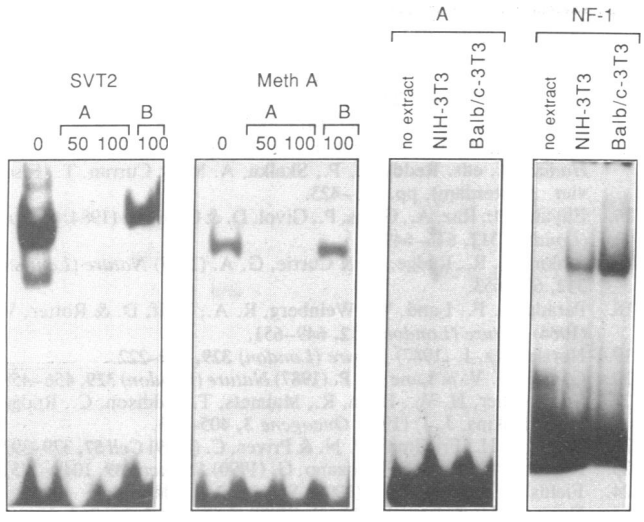

FIG. 4. Binding of nuclear proteins from transformed MethA and SVT2 cells to p53 downstream sequences. Nuclear extracts were prepared from the cells indicated and assayed for binding to the downstream *Alu* I DNA fragment (+18 to +98) by electrophoretic mobility shift. Competitor DNA (50- and 100-fold molar excess) with either the homologous +18 to +98 fragment (A) or a nonspecific DNA (B) that maps between nucleotides −127 to +18 (fragment B; see Fig. 2) was used. The oligonucleotide containing the NF-1-binding site contains the sequence 5′-GATCCCCCGGCTCAGGGC-CAAGAAC-3′. The DNA sequence of the NF-1 site was taken from positions 8056–8076 from the LTR of Mo-MuLV (45, 46).

state level of CAT-specific RNA transcribed from the p53 promoter by hybridization analysis. Two-fold serial dilutions of total cellular RNA were applied to nitrocellulose filters and hybridized to CAT- and actin-specific probes. Although the level of actin RNA was similar in BALB/c, MethA, and SVT2, the level of CAT RNA transcribed from the p53 promoter was 8- to 10-fold higher in MethA and SVT2 than in BALB/c (Fig. 3*B*). These data demonstrate that the p53 promoter is trans-activated in MethA and SVT2 cells.

To determine whether the trans-activation of the p53 promoter in MethA and SVT2 cells correlates with the presence of the downstream binding activity, we performed electrophoretic mobility-shift assays using nuclear extracts obtained from the cell lines described above. The DNA fragment used to detect binding was the 80-bp *Alu* I fragment described in the legend to Fig. 2 that extends from +18 to +98, spanning the protected HLH-binding motif. A specific protein–DNA complex was observed only when using extracts from SVT2 or MethA cells (Fig. 4). Formation of the complex was inhibited by a 50-fold molar excess of the homologous DNA (fragment A) but not by a 100-fold molar excess of a nonspecific competitor (fragment B). No such protein–DNA complex was observed using extracts from either NIH 3T3 or BALB/c-3T3. These same NIH 3T3 and BALB/c-3T3 extracts, however, formed complexes with a control double-stranded oligonucleotide containing an NF-1-binding site (Fig. 4). This finding indicates that the lack of complex formation with the p53 sequences using extracts derived from NIH 3T3 or BALB/c-3T3 was due to the absence of this downstream binding activity rather than a general lack of DNA-binding activity. These results demonstrate that factor binding to the downstream HLH motif within the p53 gene does correlate with expression from the p53 promoter and thus may be required for maximal trans-activation of the promoter in transformed cells.

DISCUSSION

Previous analysis of the murine p53 promoter has demonstrated that it lacks an upstream TATA box (28). Recent findings with other promoters that lack a TATA box, such as the terminal deoxynucleotidyltransferase (49), dihydrofolate reductase (DHFR) (35, 50), SV40 major late (34, 36), and adenovirus IVa2 (37) indicate that features of this class of promoters include upstream (G+C)-rich Sp1-binding sites (51), protein binding at the transcription initiation site, and downstream regulatory elements. Binding at the initiation site appears to be required for correct positioning of transcription initiation (50), whereas the downstream elements positively modulate the level of transcription (34–37). The murine p53 promoter contains structures analogous to those found in these other non-TATA box promoters. Sequence analysis indicates the presence of one short (G+C)-rich region between positions −41 and −51 containing a consensus Sp1-binding site. In addition, Ginsberg *et al.* (32) identified an AP-1-like binding site between positions −52 and −68 as well as an NF-1-like binding site that overlaps the transcription initiation site (nucleotides −1 to +24). It remains to be determined whether, as in the case of the DHFR promoter, binding at the initiation site of the p53 promoter is required for correct positioning of transcription initiation. Here we have shown that the p53 promoter shares an additional feature of this class of promoters, a downstream regulatory element.

The p53 downstream element maps within the noncoding first exon of the gene between nucleotides +66 and +112. DNase I protection analysis of this region indicates that a protein binds within this sequence, protecting nucleotides +65 to +79. Interestingly, the protected region contains a consensus HLH protein-binding motif (CANNTG, refs. 38–40, 48), which we demonstrate is required for binding to this element. We conclude from our findings that binding to this downstream regulatory element is most likely necessary for high-level expression of the p53 promoter. This conclusion is based on the demonstration that deletion of this element reduces promoter activity ≈5-fold in SVT2 cells as well as on the demonstration that factor binding to this element is not detected in cells that do not efficiently express the promoter.

A comparison of the murine and human p53 nucleic acid sequences revealed the presence of a similar HLH recognition motif in the promoter of the human gene (CATGTG). Interestingly, as shown below, the sequences surrounding this element are also highly conserved (82%) (H, human; M, murine; C, consensus).

```
                                                          -12
H   GGGATTGGGGtTTTCCCCTCCCAtGTGCTCAagaCTGGCgcTAA
M   GGGATTGGGacTTTCCCCTCCCAcGTGCTCA-ccCTGGC--TAA
C                        CANNTG                    +89
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Although the putative HLH recognition site maps downstream to the transcription initiation site in the murine gene (+70 to +75), it maps upstream of the transcription initiation site in the human gene (−29 to −34). The fact that the sequences surrounding this site are so highly conserved indicates that they probably represent the same functional element in both genes. This suggests that the position of the element relative to the transcription start site may be less important than the presence of the element within the promoter. In this regard, sequences downstream of the transcription start site, within the noncoding first exon of the murine gene, thus constitute part of the functional promoter. Since in the murine gene this element is transcribed into mRNA, we cannot exclude the formal possibility that trans-activation may additionally involve protein–mRNA interactions as in case of the human immunodeficiency virus trans-activator Tat (52).

Alterations in the function or the expression of p53 by either point mutations, rearrangements, or deletions of the gene have been implicated as being an important and possibly necessary event in the malignant transformation of many cell types. In transformed cells where the p53 gene has undergone

point mutations, the gene is often found to be overexpressed. One mechanism for the accumulation of elevated levels of p53 in these cells was shown to be due to increased stabilization of the protein, possibly due to formation of a complex with heat shock protein hsc70 (53, 54). Overexpression of the mutant gene in these cells may be required to generate a sufficient level of protein to facilitate its transforming or dominant negative functions (55). The data presented here implicate the binding of a potential HLH-like transcription factor to a downstream regulatory element in the activation of the p53 promoter in transformed cells, thus providing another potential level of overexpression of the gene. Furthermore, we propose that the activity of a member of this family of transcription factors may also be an important determinant in the regulated expression of p53 in normal cells. The HLH domain has been shown to occur in a growing family of transcriptional regulatory proteins involved in the control of gene expression and cellular differentiation (38, 56) and functions in DNA binding and dimerization among different members of the family (38). The activity of the HLH DNA-binding transcription factors in cellular differentiation and the role of p53 in growth suppression raise the intriguing possibility that the growth arrest associated with terminal differentiation may in some cases be a result of HLH-induced expression of p53. At least one level of p53 overexpression in transformed cells may thus be due to aberrant expression of relevant transcription factors. One possibility is that mutations or genomic rearrangements affecting the activity of transcription factors, as in the case of the recently described HLH-containing protein, lyl-1, in some T-cell leukemias (57), may generate altered factors that aberrantly activate expression of p53.

This work was supported in part by a grant from the Leo and Julia Forcheimer Center for Molecular Genetics at the Weizmann Institute of Science. V.R. is an incumbent of the Norman and Helen Asher Professional Chair in Cancer Research and holds a Career Development Award from the Israel Cancer Research Fund. D. Ronen and D. Reisman have been supported by awards from the Israel Cancer Research Fund.

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