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p53-Dependent Activation of the Mouse MCK Gene Promoter: Identification of a Novel p53-Responsive Sequence and Evidence for Cooperation Between Distinct p53 Binding Sites

PAUL JACKSON,*[1] MARGARET SHIELD,† JEAN BUSKIN,† SHERYL HAWKES,*[2] MICHAEL REED,‡ KILIAN PERREM,* STEPHEN D. HAUSCHKA,† AND ANTONY BRAITHWAITE*

*Division of Cell Biology, John Curtin School of Medical Research, Australian National University, Canberra, ACT, Australia 0200
†Department of Biochemistry, University of Washington, Seattle, WA
‡Department of Microbiology, State University of New York, Stonybrook, NY

Transcriptional activation by p53 is dependent on the presence of a specific p53 binding site within control sequences of the target gene. One such target gene is the mouse muscle-specific creatine kinase (MCK) gene, which contains a p53 binding site between promoter residues −3182 and −3133 relative to the transcription start site. This DNA sequence is reported to be sufficient to confer p53-dependent activation on the MCK promoter. In contrast to this finding, evidence from promoter deletion studies suggests that sequences in the MCK promoter other than this p53 binding site also permit p53-dependent activation. To investigate this possibility, we have further examined sequences in the MCK promoter required for transcriptional activation by mouse p53. We report here identification of a second p53-responsive sequence within the MCK promoter. This novel sequence is situated between residues −177 and −81, and can confer p53-dependent, position- and orientation-independent activation on a heterologous promoter. Moreover, this sequence can specifically bind mouse and human p53. By promoter deletion studies, we provide evidence that these two elements cooperate to provide high-level, p53-dependent activation of the MCK promoter.

Tumor suppressor   p53   Muscle creatine kinase   Transcription factor

INACTIVATION of the p53 tumor suppressor gene by mutation or deletion is the most frequent genetic lesion observed in human cancer (Hollstein et al., 1991; Levine et al., 1991; reviewed in Donehower and Bradley, 1993). The loss of normal p53 function results in enhanced cellular proliferation and genomic instability, such as chromosomal rearrangements and gene amplification (Livingstone et al., 1992; Yin et al., 1992). The resulting accumulation of genetic mutations may well be important in the progression of cells from a normal to a tumorigenic phenotype (Weinberg, 1989).

There is now considerable evidence to support the notion that p53 plays a key role in the cellular response to DNA damage (Maltzman and Czyzyk, 1984; Fritsche et al., 1991; Kastan et al., 1991a; Kastan et al., 1992; Kuerbitz et al., 1992; Fritsche

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[1]Address correspondence to Dr. Paul Jackson.
[2]Present address: School of Biological Sciences, University of Auckland, Auckland, New Zealand.

et al., 1993; Hall et al., 1993; Zhan et al., 1993). Together these reports suggest that p53 functions as a G1 checkpoint control protein to monitor cellular DNA for damage and in particular for the appearance of DNA strand breaks (Nelson and Kastan, 1994). In the presence of such damage, the cell cycle is blocked, probably to allow time for repair of the DNA before the cells are committed to DNA replication. In the absence of p53, or in cells expressing mutant p53, there is no cell cycle arrest and cells continue to divide and accumulate DNA damage. Interestingly, the role of p53 in the cellular response to DNA damage may not be confined to pathways resulting in growth arrest. The growth arrest may be cell-type dependent, because thymocytes exposed to ionizing radiation or other agents associated with DNA damage do not G1 arrest but undergo p53-dependent programmed cell death or apoptosis (Lowe et al., 1993; Clarke et al., 1993).

A number of studies have shown that p53 regulates transcription from the promoters of diverse genes. Thus, p53 can downregulate transcription from the promoters of the c-fos gene (Ginsberg et al. 1991), the interleukin-6 gene (Santhanam et al., 1991), the Rb gene (Shiio et al., 1992), and the PCNA gene (Subler et al., 1992; Jackson et al., 1994). In addition, p53 represses transcription from promoters found in SV40, RSV, HIV, and HTLV-1 virus DNA (Subler et al., 1992; Jackson et al., 1993). On the other hand, when fused to the yeast transcription factor GAL4 DNA binding domain, p53 activates transcription from a test promoter containing GAL4 binding sites (Fields and Yang, 1990; Raycroft et al., 1990; Unger et al., 1992). Mammalian p53 proteins also activate transcription from the mouse muscle-specific creatine kinase (MCK) gene promoter (Weintraub et al., 1991; Zambetti et al., 1992; Jackson et al., 1993), the PCNA gene promoter in specific cell types (Jackson et al., 1994), and the mdm-2 gene (Barak et al., 1993; Wu et al., 1993). These data suggest that p53 may function in the control of cell cycling and DNA repair by regulating the expression of other genes involved in these processes. Consistent with this proposal, p53 activates transcription of genes involved in DNA repair (GADD45; Kastan, et al., 1992) and the control of cell growth (WAF1/CIP1; El-Deiry et al., 1993). GADD45 has recently been shown to bind PCNA, and to both stimulate DNA excision repair in vitro and prevent the entry of cells into S-phase (Smith et al., 1994), whereas WAF/CIP1 has been shown to inhibit growth of both normal and tumor cells by inhibiting the activity of cyclin-dependent kinases (Harper et al., 1993). Importantly, the cellular levels of both GADD45 and WAF1/CIP1 are elevated, in a p53-dependent manner, in response to DNA damaging agents (El-Deiry et al., 1994; Zhan et al., 1994).

One of the first genes found to be activated by p53 was the mouse MCK gene (Weintraub et al., 1991). Activation by mouse p53 was shown to be dependent on the presence of a 500-bp nucleotide sequence situated between promoter residues −3300 and −2800 upstream of the transcription start site. Subsequent studies identified a 50-bp fragment within this sequence (−3182 to −3133) to which p53 could bind (Zambetti et al., 1992). In support of the notion that the p53 binding site might act like an enhancer in the activation of transcription, this promoter fragment was capable of conferring p53-dependent, orientation-independent activation on a heterologous test promoter (Zambetti et al., 1992; Yuan et al., 1993). Such studies have implied that the presence of a single p53 response element is sufficient to explain activation of the MCK promoter by p53. There are two reports, however, that contain data inconsistent with this simple model. These studies clearly showed that p53 caused transcriptional activation of the MCK promoter in the absence of the known p53 binding site (Weintraub et al., 1991; Jackson et al., 1993), suggesting that overall activation of the MCK promoter by p53 might involve more than a single p53-responsive sequence.

In this study, we have further examined sequences within the MCK promoter that are required for transcriptional activation by mouse p53. We report here identification of a second p53-responsive element (NE) within this promoter. In contrast to the known p53 binding site, which is situated approximately 3 kb upstream of the transcription start site, the newly discovered p53 response element is located between promoter residues −177 and −81. This NE sequence specifically binds p53 and can confer position-independent, orientation-independent activation on a heterologous promoter. By using promoter deletion constructs containing one or both p53 binding sites, we show that these two p53 binding sites cooperate with each other to achieve high-level activation of the MCK promoter. The overall data are discussed in terms of a potential model for p53 activation of transcription.

MATERIALS AND METHODS

Cells and Transfection Procedure

Monkey CV1 cells were routinely maintained in minimal essential medium (MEM; Gibco BRL,

Grand Island, NY) containing 0.22% sodium bicarbonate and supplemented with 5% heat-inactivated fetal calf serum (FCS; ICN Biomedicals, Sydney, Australia). Human HeLa cells (ATCC CCL2) were grown in MEM plus 10% heat-inactivated FCS.

For each experiment, 10^6 cells in 10-cm petri dishes were transfected with the indicated amounts of plasmid DNA to a total of 20 μg DNA, using the calcium phosphate transfection system (Gibco BRL, Life Technologies Inc, Gaithersburg, MD) exactly as described by the manufacturers. After 24 h, transfected cells were washed with growth medium followed by phosphate-buffered saline (PBS), refed with growth medium, and incubated for a further 48 h at 37°C and 5% CO_2.

Chloramphenicol Acetyl Transferase (CAT) Assays

Unless otherwise indicated, the level of CAT activity in transfected cell lysates was determined essentially by the method of Gorman et al. (1992) as modified in Sambrook et al. (1989). Briefly, each dish of transfected cells was washed twice with ice-cold PBS. Cells were then harvested by scraping into 1 ml of ice-cold PBS, pelleted, and finally resuspended in 200 μl of 0.25 M Tris-Cl, pH 7.5. Extracts of cells were then prepared by three rounds of freezing (dry ice, 6 min) and thawing (37°C, 5 min). Cell lysates were vortexed thoroughly between each freezing step. Cell debris was removed by centrifugation for 15 min at 12,000 × g and 4°C. The supernatant was then heated to 65°C for 10 min to inactivate any CAT inhibitors in the lysates (Sleigh, 1986). To ensure that lysates from equal numbers of cells from different samples were used in each CAT assay, prior to heat inactivation the protein concentration in each supernatant was determined by measurement of the absorbance at 280 nm.

CAT activity in cell lysates was assayed by measuring the conversion of [^{14}C]chloramphenicol into its acetylated derivatives. Reaction mixtures contained lysate from transfected cells to a maximum of 35 μl and 5 μl of CAT reaction buffer. CAT reaction buffer consisted of 10 mg acetyl-CoA (Li salt; Sigma Chemical Co., St. Louis, MO), 58.3 μl [^{14}C]chloramphenicol (D-threo-[dichloroacetyl-1-^{14}C]chloramphenicol, 56 mCi/mmol, Amersham Australia Pty, Sydney, Australia), made up to 550 μl with water. In each reaction, the volumes of cell lysate and 0.25 M Tris-Cl, pH. 7.5, were adjusted to ensure CAT activities were determined from equal numbers of cells. After incubation for 6 h at 37°C, [^{14}C]chloramphenicol and its acetylated derivatives were extracted from the CAT reactions by addition of 1 ml ethyl acetate. Samples were vortexed for three periods of 10 s each, centrifuged for 5 min at 12,000 × g, and the upper phase then transferred to a fresh tube. The ethyl acetate was evaporated off, the remaining reaction products dissolved in 15 μl ethyl acetate, and then separated using thin-layer chromatography. Radioactive products were visualized by autoradiography. Percent conversion of [^{14}C]chloramphenicol was calculated by cutting out spots from the TLC plate and measuring the radioactivity in the spots by liquid scintillation counting.

When using the TLC CAT assay method in experiments with pBLCAT2 plasmids containing p53 response elements from the MCK promoter, we found that the basal activity in cell lysates transfected with these plasmids was very high. As a consequence, we were unable to clearly observe the effect of p53 on transcription from these constructs. To maintain equivalent transfection conditions compared with other experiments in this study and to accurately determine the effects of p53, we therefore measured the amount of CAT activity in such lysates using the method of Sleigh (1986). This method does not rely on TLC to separate radiolabeled reaction products and is useful for comparing extracts having high levels of CAT activity. In this method, CAT activity in cell extracts is determined by measuring the transfer of [^{14}C]acetyl groups from [^{14}C]acetyl-CoA into chloramphenicol. The amount of conversion is measured by scintillation counting as detailed in Jackson et al. (1993).

All transfection experiments were performed several times with similar qualitative results. Unless otherwise stated, data presented are representative of each experiment.

Plasmids

Plasmid pCMVNc9 (Eliyahu et al., 1989) expressing mouse wild-type (wt) p53 from the human cytomegalovirus (hCMV) promoter was obtained from M. Oren (Weizmann Institute, Rehovot, Israel).

Control plasmid pCMVneo (Southern and Berg, 1982) encodes the bacterial neo resistance gene from the hCMV promoter.

Construction and characterization of pMSV-wtp53 (encoding wt mouse p53) and pMSVval135 (encoding a mouse mutant p53 with an alanine to valine change at codon 135) both expressing p53 proteins from the Moloney sarcoma virus (MSV)

promoter, together with the control vector pMSV, have been described (Reed et al., 1993).

Construction and characterization of reporter plasmids containing various portions of the mouse muscle-specific creatine kinase fused to the bacterial CAT gene (p-3300MCKCAT, p-2800-MCKCAT, p-1674MCKCAT, p-1020 MCKCAT, p-776MCKCAT, and p-80MCKCAT) have already been described (Jaynes et al., 1988).

Promoter reporter plasmids containing sequences −588 to +7 (p-588MCKCAT), −300 to +7 (p-300MCKCAT), and −177 to +7 (p-177MCKCAT) from the MCK promoter fused to the CAT gene were generated by exonuclease III digestion followed by religation of Pst 1/Sal I cut, Sph 1/Acc 1 cut, and Pst 1/Acc 1 cut p-776MCKCAT, respectively. The nucleotide sequences of these constructs were verified by single-strand sequencing (M. Shield, Ph.D. Thesis, University of Washington).

Plasmid p-776Δ1MCKCAT was constructed by cloning the Hind III/Bgl I fragment from p-776MCKCAT (MCK promoter residues −776 to −190) into Hind III/Apa 1 cut p-177MCKCAT. The resulting construct contained MCK promoter sequences between −776 and +7 with a deletion between −189 and −81.

Plasmid p-3300Δ1MCKCAT was constructed by cloning the Sph 1 fragment from p-3300MCKCAT (MCK promoter residues −3300 to −723) into Sph 1 cut p-776D1MCKCAT. Correct orientation of the insert was verified by restriction digest analysis. The resulting plasmid contained MCK promoter sequences between −3300 and +7 with a deletion between −189 and −81.

pBLCAT2 (Luckow and Schutz, 1987) containing the Herpes simples virus thymidine kinase promoter was supplied by Dr. A. E. Reeve (University of Otago, Dunedin, New Zealand).

Recombinant reporter plasmids containing "putative" p53-responsive elements from the MCK promoter placed upstream of the HSV tk promoter in pBLCAT2 were constructed as follows.

MCK promoter sequence between −3195 and −3120 (BS: containing the known p53 binding site between −3133 and −3182) was amplified from p-3300MCKCAT by the polymerase chain reaction (PCR) using a 5′ primer, 5′-AACCGCATG CGGCGTGTGCTCCCTGGCAAGCCTAT-3′ together with a 3′ primer, 3′-CGGAGACTGG GAGTCACCGAGGGTACGTACGCCAA-5′. After purification on a QIAquick-spin column (QIAGEN, Chatsworth, CA), the amplified sequence was digested with Sph 1, repurified, and then subcloned into the Sph 1 site within the polylinker sequence of pBLCAT2. Correct (pBSCAT) or reverse (pSBCAT) orientation of the insert in pBLCAT2 was verified by restriction analysis. MCK promoter sequence between −177 and −81 (NE: containing the newly identified p53 response element) was amplified by PCR from p-300MCKCAT using a 5′ primer, 5′-AACC TCTAGACATACAAGGCCATGGGGCTGGGC AA-3′, with a 3′ primer, 3′-CTTTCGAGTAG ACGAGAGTCCCCGGAGATCTCCAA-5′. PCR products were purified as above, digested with Xba 1, repurified, and subcloned into the Xba 1 site within the polylinker sequence in pBLCAT2. Correct (pNECAT) or reverse (pENCAT) orientation of the insert in pBLCAT2 was verified by restriction analysis.

Polymerase Chain Reactions

PCR were performed in a reaction mixture containing the following: 100 pmol each primer, 2.5 mM of each dNTP, 10 ng DNA template, $MgCl_2$ to a final concentration of 2 mM (NE) or 6 mM (BS), 0.5 μl Vent DNA polymerase (New England Biolabs Inc., Beverly, MD), 10 μl of 10 × Vent reaction buffer, and water to a final volume of 100 μl.

PCR was performed in a Perkin Elmer 480 Thermal Cycler as follows: one cycle of 96°C for 5 min, 55°C for 1 min, and 72°C for 2 min, followed by 25 cycles of 96°C for 1.5 min and 72°C for 1 min. All reactions were then cooled to 4°C before purification of the amplified DNA sequence.

Gel Shift Assays

Nuclear extracts from HeLa cells were prepared essentially by the method of Schreiber et al. (1989). Once prepared, extracts were dialyzed overnight against 500 volumes of 20 mM Tris-HCl, pH. 7.2, 10% glycerol, 1% NP40, 5 mM EDTA, 100 mM NaCl (TEG buffer). Gel mobility shift assays were performed by incubating 40 μg of nuclear extract protein with TEG containing additional NaCl to a final concentration of 150 mM and 0.5 μg of double-stranded poly(dIdC) for 15 min at 15°C. During this time any competitors were also added. Competitors used were, 30- to 50-fold excess of unlabeled MCK BS or MCK NE sequence, or 50-fold excess of unlabeled double-stranded oligonucleotides corresponding to the consensus binding sites for Ap-2 (5′-GAT CGAACTGACCGCCCGCGGCCCGT-3′) or Sp1 (5′-ATTCGATCGGGGCGGGGCGAGC-3′)

transcription factors. To verify the presence or absence of p53 in any protein/DNA complexes, in certain experiments 500 ng of purified PAb1801 antibody (Banks et al., 1986) was included at this stage. Labeled oligonucleotide probe (NE sequence, 1.25 ng; 3000 cpm/ng and BS sequence, 0.6 ng; 12000 cpm/ng) was then added and the reaction continued for a further 15 min at 15°C. Protein/DNA complexes were resolved on a 6% polyacrylamide gel. Gels were prerun at 280 V for 10 min. After addition of samples, gels were run at 280 V for 5 min followed by 200 V for a further 2 h. All gels were run at 4°C. Gels were then fixed in 47% methanol/10% acetic acid, dried under vacuum for 20 min, and exposed to Kodak XAR-5 film at −70°C.

RESULTS

Activation of the MCK Promoter by p53 in the Absence of the p53 Binding Site

In a recent study (Jackson et al., 1993), we obtained preliminary evidence suggesting that activation of the MCK promoter by p53 might involve promoter sequences other than the p53 binding site identified by Weintraub et al. (1991) and Zambetti et al. (1992). To further investigate this possibility, we first confirmed that p53 activated transcription of the MCK promoter in the absence of the known p53 binding site. MCK gene promoter CAT constructs (Weintraub et al., 1991) (Fig. 1A) in which the p53 binding site was either present (p-3300MCKCAT) or absent (p-2800MCKCAT) and a truncated MCK promoter lacking all but the most proximal promoter sequence (p-80MCKCAT) were cotransfected into monkey CV1 cells with either a control plasmid (pCMV-neo; Southern and Berg, 1982) or a plasmid expressing wt mouse p53 (pCMVNc9; Eliyahu et al., 1989). CV1 cells were chosen because these cells were used in the original studies by both Weintraub et al. (1991) and Jackson et al. (1993). Results of a typical experiment presented in Fig. 1B show that in the absence of p53, basal levels of activity from the MCK promoter reporter plasmids were very low. In the presence of the p53 binding site, transfected p53 caused a marked activation of MCK promoter activity (p-3300MCKCAT; 61-fold). Importantly, in the absence of the p53 binding site, p53 was clearly still capable of activating the MCK promoter (p-2800MCKCAT; fourfold) though the level of activation was much less than that observed for p-3300MCKCAT. In contrast, there was no effect of p53 on activity from p-80MCKCAT.

The above studies made use of the pCMVNc9 vector, which expresses p53 from the human cytomegalovirus early enhancer. In a separate series of experiments, we also investigated the ability of p53 expressed from the Moloney sarcoma virus promoter (pMSVwtp53; Reed et al., 1993) to activate the MCK promoter. Typical results presented in Fig. 1C show that p53 expressed from this promoter was also able to activate transcription from both p-3300MCKCAT (44-fold) and p-2800MCKCAT (eightfold). Interestingly, under these conditions, p-80MCKCAT had the highest basal levels of activity in CV1 cells. However, p53 had no effect on this high level of basal activity.

Importantly, throughout these studies, we found that the absolute induction levels of p-3300MCKCAT and p-2800MCKCAT varied between experiments (compare for example, Fig. 1 and Fig. 5). However, we consistently observed that p-3300MCKCAT was activated to a much higher degree than p-2800MCKCAT, whereas p53 clearly had little effect on p-80MCKCAT.

Activation of the MCK promoter in the absence of the p53 binding site was further confirmed in dose-dependence studies, where we observed increasing activation of p-2800MCKCAT with increasing amounts of transfected pMSVwtp53 (Fig. 1D). Highest activation of this promoter was obtained with 5 μg of p-2800MCKCAT and 15 μg of pMSVwtp53. No activation of this promoter was observed with increasing amounts of a transfected p53 mutant (pMSVp53val135; Fig. 1D), even though such cells expressed higher levels of transfected p53 protein than did cells transfected with pMSVwtp53 (data not shown). Taken together, these data clearly show that wt p53 can activate transcription from the MCK promoter in the absence of the known p53 binding site.

Localization of a Novel p53-Responsive Sequence in the MCK Promoter

To locate possible promoter sequences that enabled p53 to activate the MCK promoter in the absence of the known p53 binding site, we made use of a series of reporter plasmids containing sequential deletions of the MCK promoter (Jaynes et al., 1988; Materials and Methods; illustrated in Fig. 2). These plasmids were cotransfected into CV1 cells along with a control plasmid (pMSV vector) or pMSVwtp53. Amounts of transfected DNA were chosen to obtain highest activation of the reporter plasmid (Fig. 1D). Expression vector

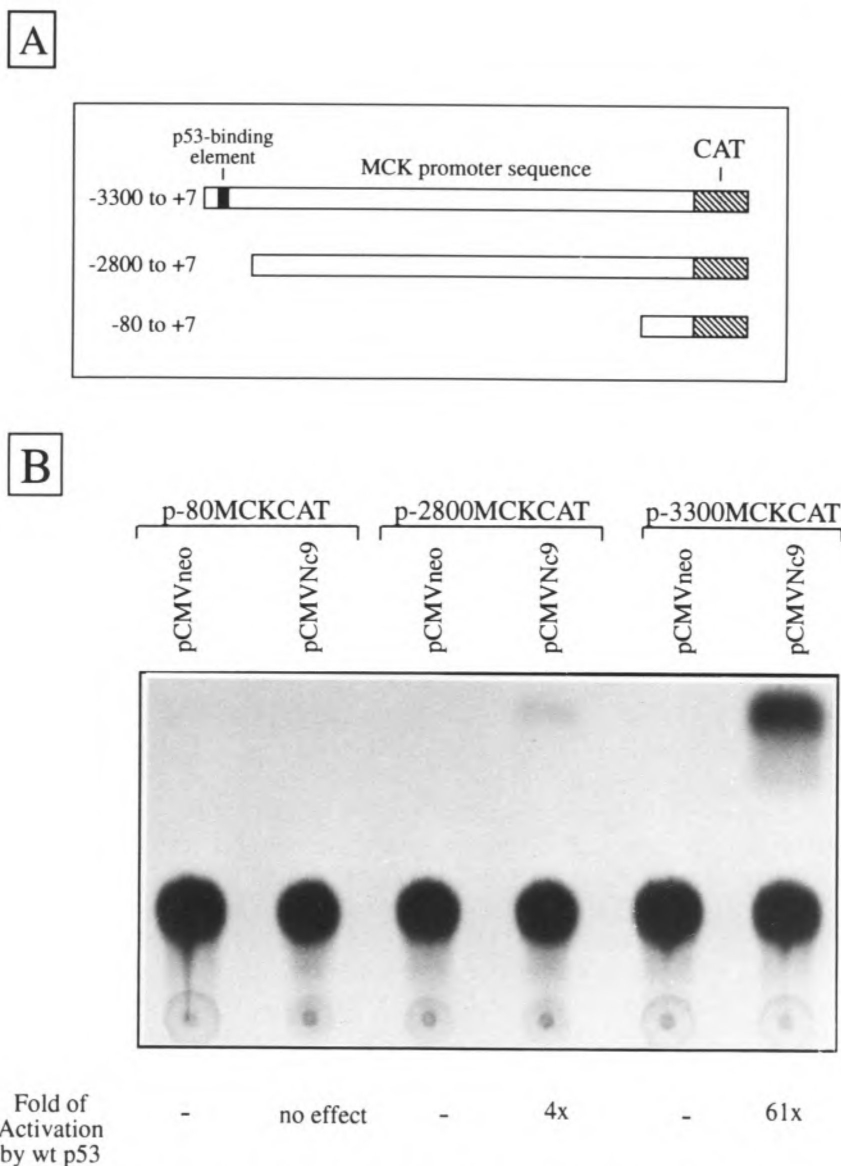
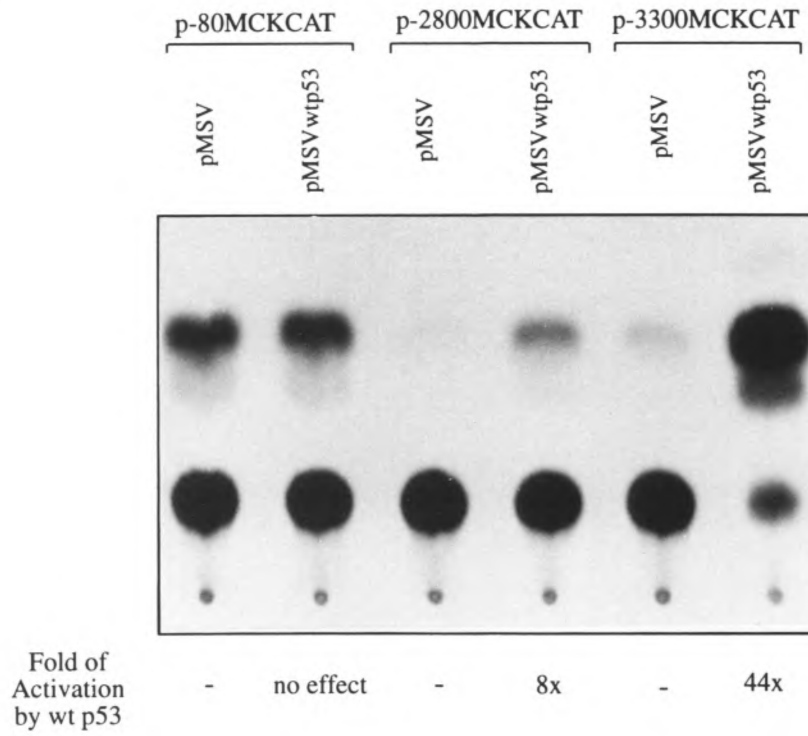


FIG. 1. Activation of the MCK promoter by p53 in the absence of the p53 binding site. (A) Schematic illustration of the MCK promoter/CAT reporter plasmids used. Numbers refer to the nucleotide sequence in the MCK promoter relative to the transcription start site. The approximate position of the distal p53 binding site is indicated. (B) CV1 cells were transfected with 10 μg each of the indicated MCK promoter/CAT reporter plasmid and 10 μg of either pCMVneo or pCMVNc9 as described in Materials and Methods. After 72 h, cell lysates were prepared, normalized for protein content, and used to determine CAT activity (also as described in Materials and Methods). Data presented are representative of six independent experiments. (C) Same as for (B) except that the MCK reporter plasmids were cotransfected with either pMSV vector only or pMSVwtp53 expression plasmids. (D) CV1 cells were cotransfected with 5 μg of p-2800MCKCAT and 5, 10, or 15 μg of either pMSVwtp53 or pMSVp53val135. Total DNA transfected was maintained at 20 μg by the inclusion of pMSV. Data are representative of two independent experiments.

pMSVwtp53 was used for this and all subsequent experiments because transfections with this plasmid gave consistently greater activation of p-2800MCKCAT than did pCMVNc9 (compare, for example, Figs. 1B and C; data not shown).

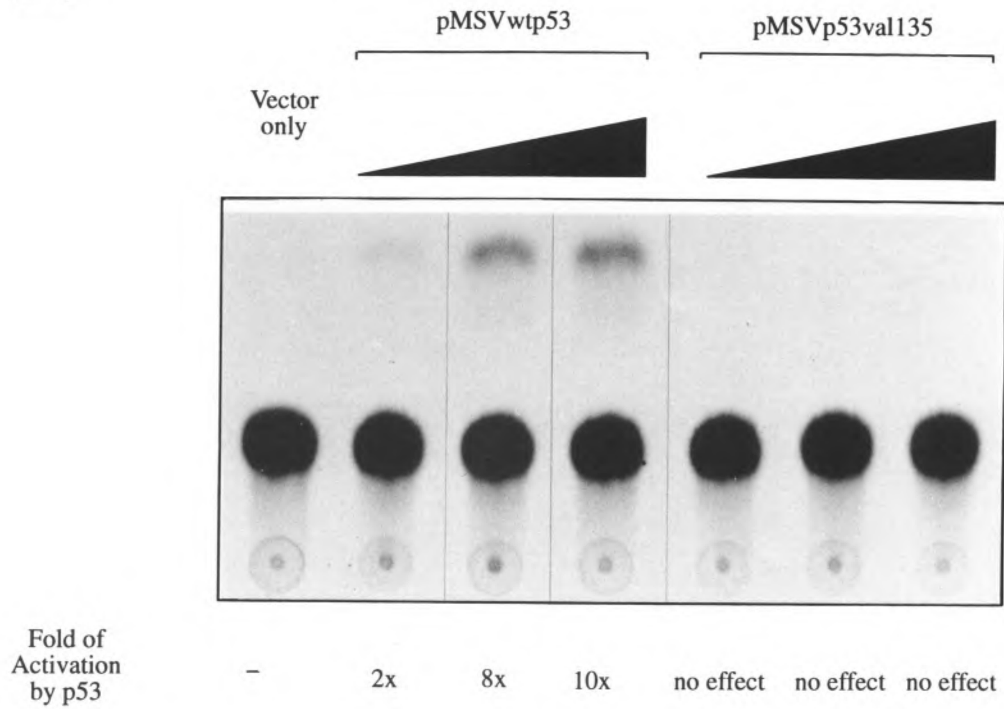
Results presented in Fig. 2 show that all MCK promoter constructs gave very low levels of basal CAT activity with p-80MCKCAT again having the highest activity. In agreement with our earlier experiments, p53 activated transcription from p-2800MCKCAT (13-fold). Furthermore, in spite of some variation between absolute levels of activa-

C



Fold of Activation by wt p53 - no effect - 8x - 44x

D



Fold of Activation by p53 – 2x 8x 10x no effect no effect no effect

tion, p53 activated transcription from all promoter reporter constructs except for the plasmid containing just 80 bp of MCK promoter sequence. These data suggested that a novel sequence responsive to p53 lies within MCK promoter residues −177 to −81.

To test this possibility, we made use of two further MCK promoter reporter plasmids. One reporter contained 776 bp of MCK promoter sequence (p-776MCKCAT; Fig. 2) whereas the second contained the same 776 bp of promoter sequence from which residues −190 to −81 had been deleted (p-776Δ1MCKCAT; Fig. 2). Results shown in Fig. 2 indicated that wt p53 activated p-776MCK- CAT (20-fold) to levels similar to that previously observed for p-2800MCKCAT. In contrast, the activation of p-776Δ1MCKCAT cotransfected with wt p53 was severely impaired, being only threefold above the activity observed when transfected with the control vector. These data again are consistent with the presence of a novel p53-responsive element situated between residues −177 and −81 of the MCK promoter.

Position- and Orientation-Independent Activation of Transcription by a Novel p53 Response Element in the MCK Promoter

Although we had identified a possible novel p53-responsive sequence within the proximal MCK promoter, it was important to demonstrate that this sequence in isolation was capable of permitting p53-dependent activation of transcription. Therefore, MCK promoter sequence between −177 and −81 (NE) was amplified by PCR as described in Materials and Methods, and then cloned into the polylinker sequence within the reporter plasmid pBLCAT2 (Luckow and Schutz, 1987) such that the MCK promoter sequence was upstream of the HSV tk promoter within pBLCAT2 (pNECAT; Fig. 3). The NE sequence was inserted in both normal (5′-3′, as found in the MCK promoter) and reverse (EN; pENCAT) orientations. As controls for our study, we also amplified MCK promoter sequences between −3195 and −3120 (BS), which contains the known distal p53 binding site between residues −3182 and −3133, and cloned these in both normal (pBSCAT) and reverse (pSBCAT) orientations into the polylinker sequence upstream of the HSV tk promoter in pBLCAT2 (Fig. 3).

Any effect of p53 on the HSV tk promoter in pBLCAT2 would make the effect of p53 on recombinant pBLCAT2 plasmids difficult to interpret. Therefore, in a preliminary series of transfection experiments with this plasmid, we investigated the effect of p53 on transcription from the tk promoter. We found that pBLCAT2 had a very low level of basal activity within CV1 cells, and this activity was not significantly affected by cotransfection with pMSVwtp53 (see, for example, Fig. 3).

Recombinant pBLCAT2 reporter plasmids were cotransfected into CV1 cells with pMSV vector and either pMSVwtp53 or pMSVp53val135. Results presented in Fig. 3 showed that wt, but not mutant p53, caused consistent activation of transcription from pBSCAT (average of 19-fold). In addition, wt p53, but not mutant p53, activated transcription from pSBCAT (average 9.5-fold), though to a level somewhat less than that observed for pBSCAT. These data are consistent with previous reports that the p53 binding site from the MCK promoter can confer position- and orientation-independent transcription on a heterologous promoter (Zambetti et al., 1992).

Similar effects on transcription by wt, but not mutant p53, were found for reporter plasmids containing the MCK NE promoter sequence (Fig. 3). Wild-type p53 clearly activated both pNECAT (average 9.0-fold) and to a lesser extent pENCAT (average 4.5-fold). These data indicate that sequences between −177 and −81 within the MCK promoter can confer wt p53-dependent and orientation-independent activation on a heterologous promoter.

Binding of p53 to the Novel p53 Response Element in the MCK Promoter

Analysis of genes activated by p53 has identified a consensus p53 binding sequence consisting of two copies of 5′-PuPuPuC(A/T)(T/A)GPyPyPy-3′ separated by up to 13 bp. Within the nucleotide sequence of the newly identified p53 response element in the MCK promoter are two regions with similarity to this consensus binding sequence (underlined residues in Fig. 4A). The first motif (ATACAAGGCC; Motif 1) matches the consensus in 8 of 10 residues, whereas the second motif (GGGCAAGCTG; Motif 2) matches the consensus at 9 of 10 residues. The two motifs are separated by 8 bp of intervening sequence.

The similarity between these motifs and the consensus binding sequence suggested that p53 might bind to the newly identified p53-responsive sequence in the MCK promoter sequence. To investigate this possibility, we used the amplified MCK proximal promoter NE sequence (−177 to −81) in gel mobility shift assays. As a control for

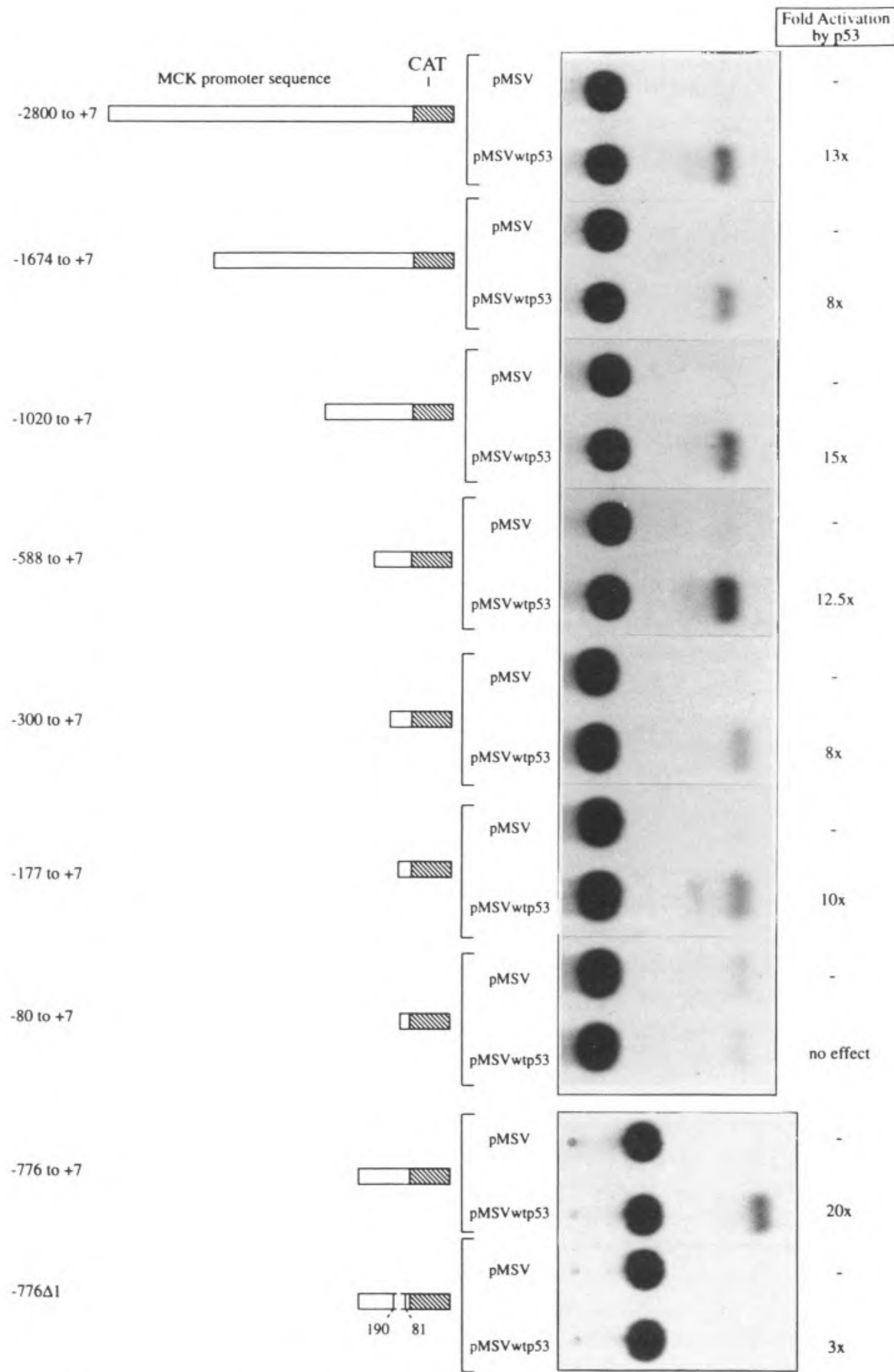


FIG. 2. Localization of a novel p53-responsive sequence within the MCK promoter. Schematic illustration of the MCK promoter/CAT reporter plasmids used is shown. Numbers refer to the nucleotide sequence in the MCK promoter relative to the transcription start site. Dashed lines indicate the boundaries of deletions. To identify MCK promoter sequences responsve to p53, CV1 cells were cotransfected with 5 μg of the indicated promoter reporter plasmids and 15 μg of either pMSV vector or pMSVwtp53 as described in Materials and Methods. After 72 h, cell lysates were prepared and assayed for CAT activity (also as described in Materials and Methods). Data presented are representative of three independent experiments.

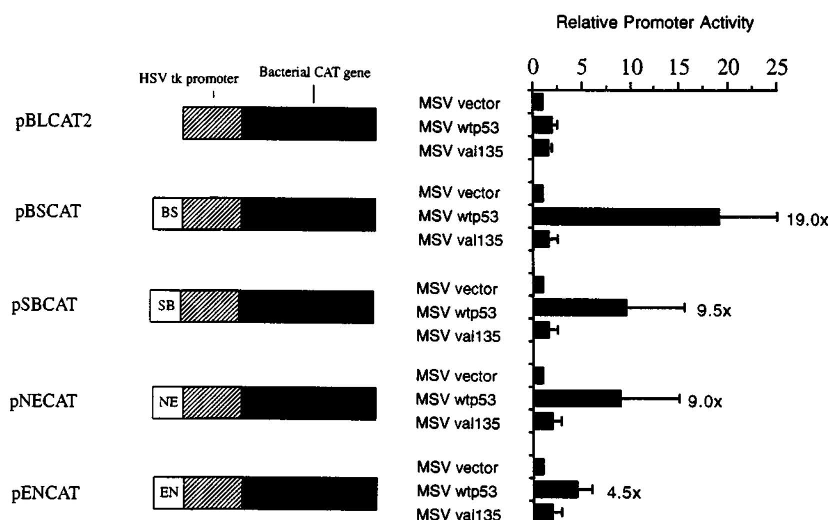


FIG. 3. MCK promoter residues between − 177 and − 81 confer p53-dependent activation on a heterologous promoter. Illustration of recombinant plasmids used. Nucleotide sequences NE (MCK promoter residues − 177 to − 81) and BS (MCK promoter residues − 3195 to − 3120) were amplified by PCR, purified, and cloned into the polylinker sequence of pBLCAT2 to form plasmids pNECAT and pBSCAT, respectively, as described in Materials and Methods. Similar constructs were made with the NE (pENCAT) and BS (pSBCAT) cloned in the opposite orientation. CV1 cells were cotransfected with 5 μg of the indicated reporter plasmid and 10 μg of either pMSVwtp53 or pMSVp53val135 as described in Materials and Methods. After 72 h, cell lysates were prepared and CAT assay determined by the method of Sleigh (1987) as described in Materials and Methods. Data presented are the mean with standard deviation of four independent experiments.

these studies we also used the MCK distal promoter BS sequence (− 3195 to − 3120), which has already been shown to bind p53 (Zambetti et al., 1992). CV1 cells contain endogenous monkey p53, which might make results from gel shift assays difficult to compare with the results of cotransfections using the mouse p53; therefore, we decided to make nuclear extracts from human HeLa cells (that express low levels of endogenous human wt p53, which can be detected by gel shift assay; Hoppe-Seyler and Butz, 1993). Results of these gel shift experiments are presented in Fig. 4B. We observed a single major DNA–protein complex involving the MCK BS sequence and this complex was specifically competed by inclusion of a 50-fold excess of cold BS sequence in the binding reaction. The presence of p53 in this protein–DNA complex was confirmed by supershifting in the presence of the p53 specific monoclonal antibody, PAb1801 (Banks et al., 1986). Two major and two minor protein–DNA complexes were observed using nuclear extracts with the MCK NE element. All bands, however, were specifically competed with a 30-fold excess of a specific competitor DNA (cold MCK NE sequence). Protein–DNA complexes were also competed with a 50-fold excess of cold BS sequence, suggesting that p53 was involved in the formation of protein–DNA complexes involving the NE sequence. The presence of p53 was confirmed by supershifting of all bands into a single slower mobility complex by PAb1801. We have obtained essentially similar results using nuclear extracts from NIH3T3 cells, which express endogenous mouse p53 with mutations at residues 236 and 243, but which retain wild-type function (Chen and Defendi, 1992; Denko et al., 1994; data not shown). Interestingly, we observed an increase in protein–DNA complex formation when binding reactions included PAb1801. Similar enhancement of p53 binding to DNA in the presence of antibodies has been reported previously (Hupp et al., 1992).

Further examination of the MCK NE sequence revealed the presence of a putative Spl factor binding site and several putative Ap-2 factor binding sites (Fig. 4A). To determine whether or not these transcription factors could bind the NE sequence in our system, we performed competition gel shift experiments as above and included a 50-fold excess of oligonucleotides containing consensus binding sites for either Spl or Ap-2. Results illustrated in Fig. 4B clearly show that neither Sp-1

nor Ap-2 oligonucleotides were able to compete for factors in the NE DNA–protein complexes observed in the HeLa cell nuclear extracts. In contrast, the presence of these oligonucleotides somewhat enhanced the formation of protein–DNA complexes. We conclude from these gel shift experiments that p53, but not Sp1 or Ap-2, is present in protein–DNA complexes formed with the MCK NE sequence.

Cooperation Between p53 Response Elements in the MCK Promoter

Results presented in previous sections of this report have indicated that the MCK promoter contains two p53-responsive sequences. We were interested to determine whether or not these two elements might function together to permit the high level of p53-dependent activation observed for the full-length MCK promoter (see, for example, Fig. 1C). If this notion is correct, then one might reasonably expect that deletion of either one of the two p53 response elements from this promoter should result in reduction, but not abolition, of the ability of p53 to activate transcription. Consistent with this proposal, activation of p-2800MCKCAT by p53 is much reduced compared with p-3300MCKCAT (see, for example, Fig. 1C). To further test the hypothesis, we constructed an MCK promoter reporter construct containing 3300 bp of promoter sequence from which residues between −190 and −81 were deleted (p-3300Δ1MCKCAT). We prepared this construct (using the MCK promoter) rather than use the model recombinant pBLCAT2 system, because we reasoned that intervening MCK promoter sequences might be important for any cooperative interaction between the two p53 binding sites. This construct was cotransfected into CV1 cells along with pMSV vector and either pMSVwtp53 or pMSVp53val135. As controls, in these experiments, we again used p-3300MCKCAT, p-2800MCKCAT, and p-80MCKCAT. Results presented in Fig. 5 showed that wt p53 strongly activated the full-length MCK promoter (163-fold; both p53 binding sites). On the other hand, activation of p-3300Δ1MCKCAT was much less (15-fold; distal p53 binding site only) and similar to the level of activation obtained with p-2800MCKCAT (14-fold; proximal p53 binding site only). Wild-type p53 had little effect on activity from the p-80MCKCAT reporter (neither p53 binding site). For each promoter construct, mutant p53val135 was severely defective for activation.

These findings are consistent with the possibility that two sequences within the MCK promoter may cooperate for activation of the MCK promoter by p53.

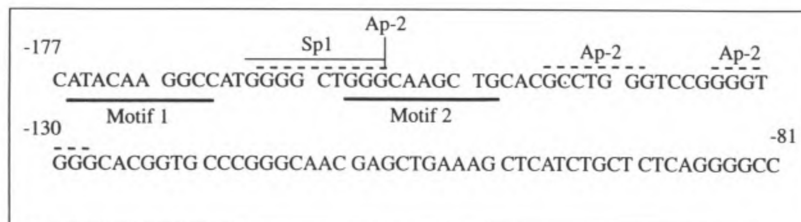
DISCUSSION

Previous studies into the molecular basis by which mouse p53 activates transcription of the mouse MCK promoter have suggested that the presence of a single p53 binding site is sufficient to fully explain p53-dependent activation of this promoter (Weintraub et al., 1991; Zambetti et al., 1992). Contrary to this model, data from promoter deletion studies have provided preliminary evidence to suggest that other sequences within the MCK promoter might contribute to the ability of p53 to activate transcription (Weintraub et al., 1991; Jackson et al., 1993). Given this consideration, we have further examined sequences within the MCK promoter required for p53-dependent activation of transcription. In this report, we provide evidence for a second p53-responsive element in the MCK promoter. This element lies within promoter residues −177 to −81 upstream of the transcription start site and can also confer p53-dependent, orientation-independent activation on a heterologous promoter.

The presence of two p53 response motifs within the MCK promoter raises the possibility that both response elements might be important for activation of this promoter by p53. We have obtained some evidence to support this model by analyzing the ability of p53 to activate MCK promoter reporter plasmids lacking one or the other of the p53 response motifs. Results from these experiments clearly showed that each individual p53 response element could contribute to activation of the MCK promoter by p53. Importantly, however, the presence of both response elements in the same promoter resulted in a synergistic activation of transcription. During the preparation of this manuscript, Stenger et al. (1994) reported that in a model promoter, when separated by an appropriate distance, two p53-responsive elements could synergize to activate transcription. Our findings, therefore, provide the first evidence that cooperation between two p53 binding sites can occur in a complex, natural promoter.

A consensus p53 binding motif has been defined that consists of two copies of 5′-PuPuPuC(A/T)(T/A)GPyPyPy-3′ separated by

A



B

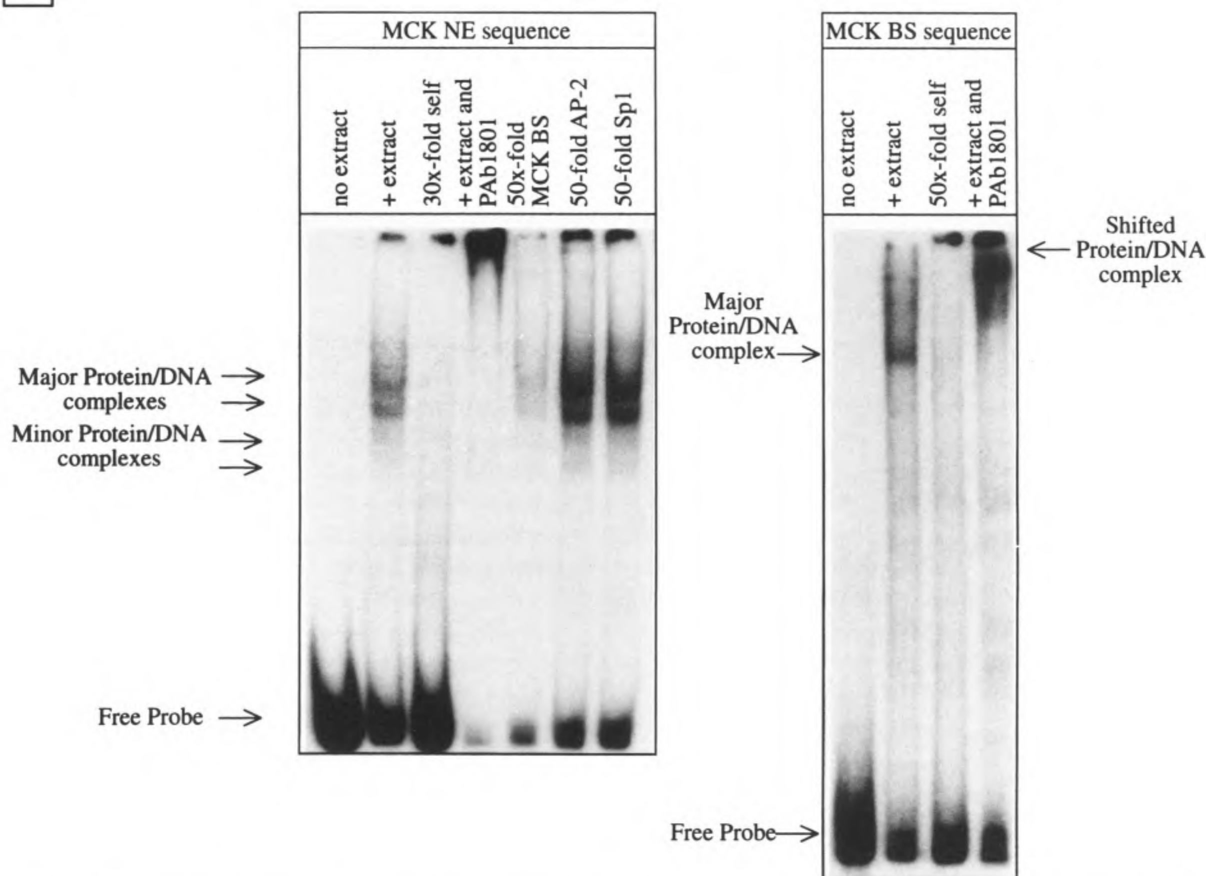


FIG. 4. p53 is present in protein–DNA complexes formed using the proximal p53 response element in the MCK promoter. (A) Nucleotide sequence of the proximal p53 response (NE) element in the MCK promoter. Nucleotide numbers refer to the residues in the MCK promoter sequence relative to the transcription start site. Sequences with similarity to the consensus p53-binding site (-); labeled Motif 1 and Motif 2, respectively. The positions of potential binding sites for Sp1 and Ap-2 transcription factors are indicated. (B) Gel shift assays using oligonucleotides corresponding to the proximal and distal MCK promoter NE and BS p53-responsive sequences with nuclear extracts derived from HeLa cells. Assays were performed in the absence or presence of competitor DNA, or the p53-specific monoclonal antibody PAb1801, as described in Materials and Methods. The positions of free probe and specific protein–DNA complexes are indicated.

up to 13 bp (Kern et al., 1991; El-Deiry et al., 1992; Funk et al., 1992; Halazonetis et al., 1993). The distal p53 binding site in the MCK promoter identified by Zambetti et al. (1992) conforms to this consensus having two copies of the binding motif separated by 12 bp. The 5′ copy is situated within promoter residues −3182 to −3173 (TGGCAAGCCT; matches consensus at 9 of 10 residues), whereas the 3′ copy is located within residues −3160 to −3151 (GGGCCTGCCT; 9 of 10 matches with the consensus). The novel, proximal p53 response element we have identified in the MCK promoter (NE sequence) also contains two sequences [promoter residues −176 to −167 (Motif 1) and −158 to −149 (Motif 2)] with similarity to the consensus sequence and separated by 8 bp.

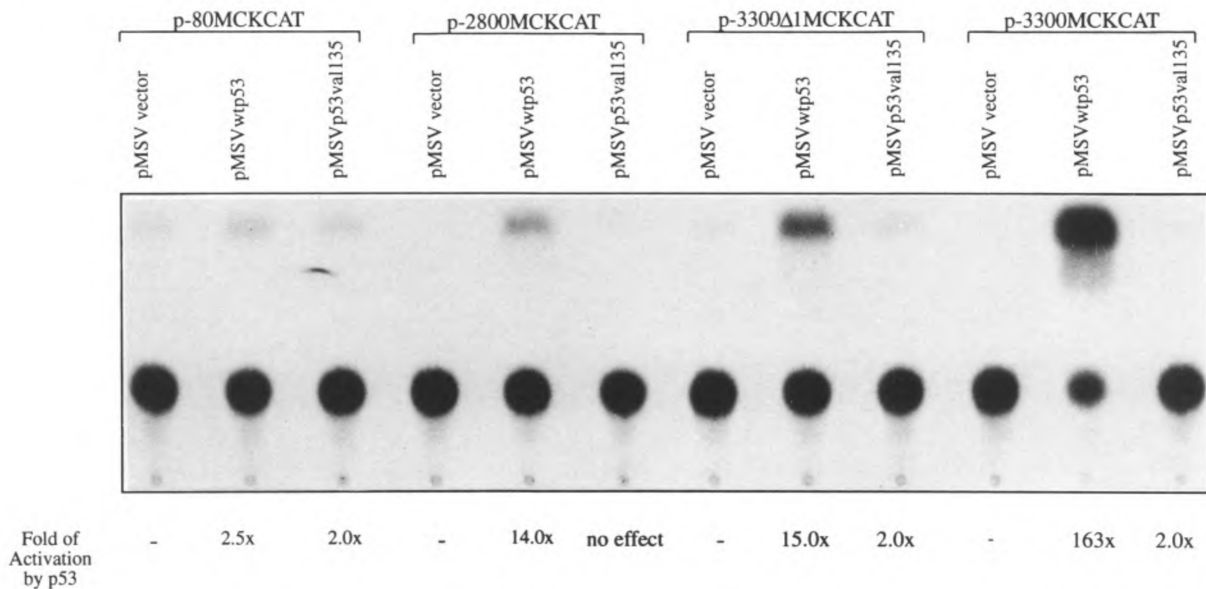


FIG. 5. Two independent response elements contribute to the activation of the MCK promoter by p53. CV1 cells were transfected with 5 μg of the indicated MCK promoter/CAT reporter plasmids and 15 μg of either pMSV, pMSVwtp53, or pMSVp53val135. Transfection and determination of CAT activity in cell lysates were perfomed as described in the legend to Fig. 2. Results illustrated are representative of two independent experiments.

Motif 1 (ATACAAGGCC) matches the consensus in 8 of 10 residues, whereas Motif 2 (GGGCAAG-CTG) matches the consensus at 9 of 10 residues. The similarity between the NE p53 response element and the consensus p53 binding sequence strongly suggested that p53 should bind this DNA sequence. With the use of gel mobility shift assays we found that both human and mouse p53 were present in protein–DNA complexes formed with the NE sequence. In addition, though the nucleotide sequence for the NE sequence predicted the presence of both Sp1 and Ap-2 transcription factor binding sites, by competition experiments we were unable to demonstrate binding of either of these protein factors to the NE sequence. Furthermore, competition for protein factor binding to the MCK NE sequence was only observed using the MCK BS sequence. Based on these data and in the absence of purified factor binding studies, we would suggest that p53 is the only protein factor binding to the MCK NE sequence.

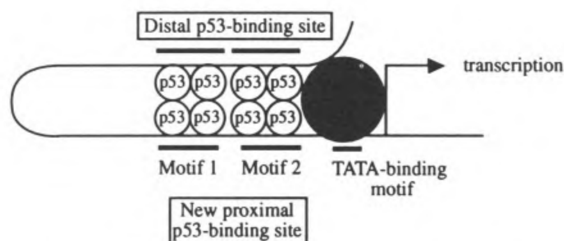


FIG. 6. Schematic illustration of the possible mechanism by which the two p53 binding sites in the MCK promoter may contribute to transcriptional activation. Distal binding site refers to the p53 binding site identified by Zambetti et al. (1992). ● represents the complex formed by TFIID and its associated proteins to initiate transcription. Each p53 symbol represents a p53 protein monomer.

The precise mechanism by which p53 is able to activate transcription is not yet understood. The fact that each p53 response element in the MCK promoter can activate transcription in both a position- and orientation-independent manner supports the notion that p53 binding sites may act as enhancer elements. Recent reports have indicated that p53 can bind to other protein components of the transcription machinery including Sp1 (Borellini and Glazer, 1993), CBP (Agoff et al., 1993), and both TBP and TFIID (Seto et al., 1992; Truant et al., 1993). Importantly, p53 and TFIID can interact to promote cooperative binding of p53 to DNA with a concomitant cooperative enhancement of the ability of p53 to activate transcription (Chen et al., 1993). Recent reports have also shown that p53 binds DNA as a tetramer (Friedman et al., 1993; Cho et al., 1994) with each half p53 binding site (single copy of the consensus sequence) interacting with two molecules of p53. Taking all these observations into consideration, we would suggest the basis for a model to explain the mechanism by which p53 activates transcription of the MCK promoter, as illustrated in Fig. 6. In this model, the cooperative interaction between

two independent p53 binding sites in the MCK promoter is achieved by binding of p53 dimers to each individual half-site and looping-out of the intervening DNA. In support of this model, looping-out of DNA and oligomerization of p53 has been shown to be associated with transcriptional activation by p53 (Stenger et al., 1994) and is also consistent with some preliminary observations we have obtained using recombinant reporter plasmids suggesting that sufficient space between the two p53 response elements is important for cooperative activation of transcription (P. Jackson, unpublished data). The p53 protein may then promote activation of transcription by interacting with TBP or TFIID, which is bound to the TATA sequence in the MCK promoter (residues −41 to −35). To further explore the feasibility of this model, we are presently investigating a possible interaction between p53 and TBP in the activation of the MCK promoter and the stoichiometry of binding of p53 to each of the specific response elements.

Given the notion that p53 functions by regulating transcription of genes involved in controlling cell growth, DNA repair, and apoptosis, there is currently considerable effort directed towards identification of potential in vivo targets for p53 function. In this respect, GADD45, WAF1/CIP1, and Mdm-2 appear to be clear candidates. The in vivo significance for the activation of the MCK promoter by p53 is far less understood. Transfection studies using wt human p53 (Weintraub et al. 1991) and Northern blot analysis of fibroblasts derived from p53-null mice (Harvey et al., 1993) have provided no evidence to show that endogenous MCK gene levels are regulated by p53. Moreover, p53-null mice appear to develop normally with no apparent muscle defects (Donehower et al., 1992). On the other hand, there is evidence from studies of skeletal muscle cells in vitro that p53 levels are increased as such cells cease proliferation and undergo differentiation (Halevy, 1993). Conceivably, the elevated levels of p53 may contribute both to cessation of cell growth and induction of genes that are activated during muscle differentiation. Consistent with this possibility, high levels of MCK are found only in postmitotic myocyte cultures (Eppenberger et al., 1964; Pearson and Epstein, 1982) and accumulation of MCK mRNA in the MM14 mouse myoblast cell line begins almost immediately after cells have committed to terminal differentiation (Chamberlain et al., 1985). Although there is evidence to support the notion that p53 may be involved in differentiation (Kastan et al., 1991b; Shalusky et al., 1991; Kosaka et al., 1992), a clear role for p53 in activation of the MCK promoter and/or muscle differentiation remains to be determined.

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