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# Mouse p53 Represses the Rat Brain Creatine Kinase Gene but Activates the Rat Muscle Creatine Kinase Gene

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**The creatine kinases (CK) regenerate ATP for cellular reactions with a high energy expenditure. While muscle CK (CKM) is expressed almost exclusively in adult skeletal and cardiac muscle, brain CK (CKB) expression is more widespread and is highest in brain glial cells. CKB expression is also high in human lung tumor cells, many of which contain mutations in p53 alleles. We have recently detected high levels of CKB mRNA in HeLa cells and, in this study, have tested whether this may be due to the extremely low amounts of p53 protein present in HeLa cells. Transient transfection experiments showed that wild-type mouse p53 severely repressed the rat CKB promoter in HeLa but not CV-1 monkey kidney cells, suggesting that, in HeLa but not CV-1 cells, p53 either associates with a required corepressor or undergoes a posttranslational modification necessary for CKB repression. Conversely, mouse wild-type p53 strongly activated the rat CKM promoter in CV-1 cells but not in HeLa cells, suggesting that, in CV-1 cells, p53 may associate with a required coactivator or is modified in a manner necessary for CKM activation. The DNA sequences required for p53-mediated modulations were found to be within bp −195 to +5 of the CKB promoter and within bp −168 to −97 of the CKM promoter. Moreover, a 112-bp fragment from the proximal rat CKM promoter (bp −168 to −57), which contained five degenerate p53-binding elements, was capable of conferring p53-mediated activation on a heterologous promoter in CV-1 cells. Also, this novel p53 sequence, when situated in the native 168-bp rat CKM promoter, conferred p53-mediated activation equal to or greater than that of the originally characterized far-upstream (bp −3160) mouse CKM p53 element. Therefore, CKB and CKM may be among the few cellular genes which could be targets of p53 in vivo. In addition, we analyzed a series of missense mutants with alterations in conserved region II of p53. Mutations affected p53 transrepression and transactivation activities differently, indicating that these activities in p53 are separable. The ability of p53 mutants to transactivate correlated well with their ability to inhibit transformation of rat embryonic fibroblasts by adenovirus E1a and activated Ras.**

The creatine kinases (CKs) (EC 2.7.3.2) catalyze the reversible transfer of a high-energy phosphate group from creatine phosphate to ADP, thus regenerating ATP in cellular reactions which expend large amounts of ATP, e.g., muscle contraction and ion transport (55). Vertebrates express four distinct CK enzymes which are products of separate, single-copy genes: the brain isoform (CKB), the muscle isoform (CKM), and two mitochondrial isoforms (CKmi). The active form of the cytoplasmic CK is a dimer which exists in three, electrophoretically separable isoforms: MM (predominant form in adult skeletal and cardiac muscle), BB (predominant form in the brain and embryonic skeletal and cardiac muscle), and MB (present in embryonic and cardiac muscle in moderate amounts) (55). The CKmi self-associates to form dimers and octamers located on the outer surface of the inner mitochondrial membrane (55). It has been proposed that the ATP generated in the mitochondrion is used (by CKmi) to generate creatine phosphate, which is subsequently transported to the cytoplasm and used by the cytoplasmic CK (CKB or CKM) to regenerate ATP at sites of high ATP consumption (55).

Transcription of CKM is regulated in a highly tissue specific manner, being expressed almost exclusively in skeletal and cardiac muscle (23, 24, 35, 54). In undifferentiated dividing myoblasts, CKM is not expressed and CKB is barely detectable. Early after myoblast fusion, CKB mRNA is transiently expressed and then returns to basal levels, at which time expression of CKM mRNA begins and increases thereafter to allow CKM to become the predominant CK isoform (29, 40). Activation of CKM during myogenesis is regulated by an upstream (bp −1100) enhancer element containing several motifs, including an E-box sequence (CANNTG) (24) which is activated when bound by a heterodimeric *trans*-acting factor composed of a muscle-specific factor (either myoD, myogenin, or myf 5) and the ubiquitous factor $E_{12}$ (or $E_{47}$) (10). Also, the mouse CKM gene was shown to be transactivated in nonmuscle cells by the wild-type mouse p53 (p53wt) protein through a far-upstream element near bp −3160 which is independent of the MyoD protein (56).

Expression of rat CKB mRNA, however, is more widespread, being highest in astrocytes and oligodendrocytes in the brain (36), much lower in cardiac muscle and kidney, and barely detectable in some other tissues (e.g., liver) (23, 35, 54). The molecular basis of CKB regulation in different cell types is not completely understood. However, Hobson et al. (22) have identified four regulatory elements located within 85 bp of the transcription start site (bp +1) which increase basal transcription of the rat CKB gene transfected into HeLa cells: two CCAAT sequences (at bp −85 and −55), a consensus TATA AATA sequence (at bp −60), and a nonconsensus TTAA sequence (at bp −25). CKB promoter mutagenesis experiments coupled with DNA-protein binding assays indicate that both CCAATs and the −60 TATAAATA are binding sites for *trans*-acting (protein) factors which increase transcription exclusively from the downstream (−25) TTAA element (22, 23). Gasdar et al. have shown that CKB expression is also high in human lung tumors (17), many of which contain mutations in

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p53 alleles (51). In addition, we have observed high expression of the endogenous CKB mRNA in HeLa cells (28, 61). HeLa cells are cervical carcinoma cells transformed by human papillomavirus type 18 (HPV-18) (42), whose E6 protein binds to and destabilizes p53 (43).

In this study, we have tested whether high expression of CKB may be related to the extremely low amounts of p53 in HeLa cells (42). Indeed, we have found in transient-transfection experiments that mouse p53 severely repressed the rat CKB promoter in HeLa but not CV-1 cells. Conversely, p53 strongly activated the rat CKM promoter in CV-1 but not HeLa cells. We also identified a 72-bp promoter-proximal rat CKM fragment (from bp −168 to −97) necessary for the p53-mediated activation of CKM in CV-1 cells. In addition, missense mutations in conserved region II of p53 affected differentially its ability to repress CKB and activate CKM. Interestingly, the CKM transactivation activity of the p53 mutants correlated perfectly with their ability to inhibit transformation of rat embryonic fibroblasts induced by adenovirus (Ad) E1a and activated Ras.

## MATERIALS AND METHODS

**Cell culture and transfection.** Cells were cultured as monolayers in Dulbecco's modified essential medium (DMEM) containing glucose (4.5 mg/ml), penicillin (50 U/ml), and streptomycin (50 μg/ml) in an atmosphere of 7% $CO_2$ at 37°C. CV-1 cells were supplemented with 10% fetal calf serum, and HeLa cells were supplemented with 7% heat-inactivated horse serum (JRH Biosciences). For transfection, approximately $5 \times 10^5$ cells were plated in 100-mm-diameter dishes (Costar) and cultured for 36 to 48 h to achieve approximately 40% confluence. About 4 h prior to addition of DNA, cells received 5 ml of fresh complete medium. Calcium phosphate-precipitated DNA was prepared by a modification of the method of Gorman (19) as described by Wilson et al. (57). Each set of DNA constructs was transfected onto duplicate or triplicate dishes. Unless stated otherwise, the cells in each dish received 2.2 pmol of either plasmid p2.9 CKB-CAT (10.2 μg) or p4.0 CKM-CAT (12 μg) plus 3 μg of plasmid $pSV_2 \cdot \beta$-gal (20) as a proposed internal control and pUC19 DNA to adjust to a total of 20 μg of DNA per dish. Where indicated in the figure legends, the cells received 1 μg (0.25 pmol) of plasmid p11-4 (52) expressing either mouse p53wt or mutant p53 (p53mt) under control of the simian virus 40 (SV40) promoter and enhancer. In control experiments designed to block p53 function, 1 μg (0.17 pmol) of plasmid expressing either HPV-16 E6 (p1436), HPV-16 E7 (p1434), or both HPV-16 E6 and E7 (p1321) proteins (under control of the human β-actin promoter) (38) was transfected, in the absence or presence of 1 μg of p11-4 · p53wt, into HeLa or CV-1 cells. The DNA remained in contact with CV-1 cells for 4 h or with HeLa cells for 8 h and was then withdrawn from the dish. The cells were rinsed twice with 5 ml of serum-free DMEM, cultured in 10 ml of fresh complete DMEM for an additional 34 to 38 h, and harvested prior to assay for chloramphenicol acetyltransferase (CAT) and β-galactosidase (β-Gal) enzyme activities.

**Assay for CAT and β-Gal enzyme activities.** Following transfection, cells were rinsed with phosphate-buffered saline (PBS), scraped from dishes in 40 mM Tris-HCl (pH 7.4)–1 mM EDTA–150 mM NaCl, centrifuged at 2,000 × *g*, and resuspended in 250 mM Tris-HCl (pH 7.8) (100 μl/100-mm-diameter dish). Cells were disrupted by three freeze-thaw cycles in dry ice-ethanol and centrifuged at 15,000 × *g* to remove particulate matter. Protein concentration of the supernatant (lysate) was measured as described by Bradford (7), using the Bio-Rad reagent. CAT activity was determined with 50 μg of HeLa lysate protein or 200 μg of CV-1 protein on the day of cell harvest as described by Gorman (19). Cell lysates were incubated at 37°C for 60 min with 0.25 μCi of [$^{14}$C]chloramphenicol (54 mCi/mmol; New England Nuclear), 0.4 mM acetyl coenzyme A (Sigma), and 0.1 M Tris-HCl (pH 7.8) in a volume of 200 μl. CAT activity was defined as the percentage of chloramphenicol converted to acetylated chloramphenicol product as determined by thin-layer chromatography (19) followed by scintillation counting the corresponding radioactive spots. In all cases, the relative CAT activities indicated in the figures are the averages of two to four experiments. We found that it was most accurate and reproducible not to normalize the CAT activity relative to the β-Gal activity, since the expression of $pSV_2 \cdot \beta$-Gal was inhibited by p53wt. β-Gal activity was measured as described by Wilson et al. (57).

**Construction of plasmids.** p2.9 CKB contained sequences from kb −2.9 to bp +5 of the rat CKB promoter. It was generated by a triple ligation between the *Hin*dIII-*Nco*I fragment (kb −2.9 to bp −161), the *Nco*I-*Bgl*II fragment (bp −161 to +5) from pLPwt (22, 23), and pUC$^{PL}$CAT (6) digested with *Hin*dIII-*Bgl*II. p0.2 CKB contained promoter sequences from bp −195 to +5. It was prepared by ligating the *Hin*dIII-*Bgl*II fragment from pCKBwt (22) into the *Hin*dIII and *Bgl*II sites of plasmid pUC$^{PL}$CAT. Plasmids p4.0 CKM, p1.5 CKM, and p0.47 CKM contained rat CKM promoter fragments with the 5′ end located at kb −4.0, −1.5, and −0.47, respectively, and the 3′ end terminating at bp +14 followed immediately by a synthetic *Bgl*II site. They were cloned into the *Nde*I-*Bgl*II sites of pUC$^{PL}$CAT (24). p0.17 CKM was prepared by obtaining the *Nco*I-*Bgl*II (bp −168 to +14) fragment from p0.47 CKM and cloning into the *Nde*I-*Bgl*II site of pUC$^{PL}$CAT. p0.1 CKM was generated by preparing the *Pvu*II-*Bgl*II (bp −96 to +14) fragment from p0.17 CKM and cloning into the *Nde*I-*Bgl*II site of pUC$^{PL}$CAT. p112N-1634 and p112R-1634 contained the 112-bp fragment from rat CKM (*Nco*I-*Msc*I, bp −168 to −57) cloned in the *Eco*RV site of p1634-CAT (60), in normal and reverse orientations, respectively. The *Nde*I ends of pUC$^{PL}$-CAT, the *Nco*I end (bp −168) of the *Nco*I-*Bgl*II fragment and *Pvu*II end (bp −99) of the *Pvu*II-*Bgl*II fragment, both ends of the 112-bp fragment, *Eco*RV ends of p1634-CAT, and *Hin*dIII ends of p0.2 CKB were filled in with T4 DNA polymerase before ligation with T4 DNA ligase. Plasmids p11-4 · p53wt and p11-4 · p53mt, respectively, code for p53wt and various missense p53mt proteins (45).

**Site-directed mutagenesis of p53.** Site-directed mutagenesis of the mouse p53wt cDNA was described previously (45, 48, 49).

**Preparation of plasmid DNA.** Plasmid DNA used in transfections was propagated in *Escherichia coli* DH5 and isolated by the alkaline lysis procedure of Birnboim (5), including the use of 2.5 M LiCl to remove bacterial protein and rRNA. Plasmid DNA was then purified by two successive CsCl gradient bandings and quantified by $A_{260}$ and agarose gel electrophoresis (57).

**Immunoprecipitation of p53wt and p53mt proteins.** Immunoprecipitation was performed as described by Schmieg and Simmons (45).

**Western blot (immunoblot) analysis.** Transfected HeLa cells were washed once with PBS and once with PBS plus 3 mM $Mg^{2+}$ acetate and lysed in lysis buffer (100 mM Tris-HCl [pH 6.8], 25 mM $Mg^{2+}$ acetate, 10% glycerol, 0.5 mM dithiothreitol, 0.2 mM phenylmethylsulfonyl fluoride, 0.4% Nonidet P-40). Protein concentration was determined by the method of Bradford (7). One hundred micrograms of total protein was loaded on a sodium dodecyl sulfate (SDS)–12.5% polyacryl-

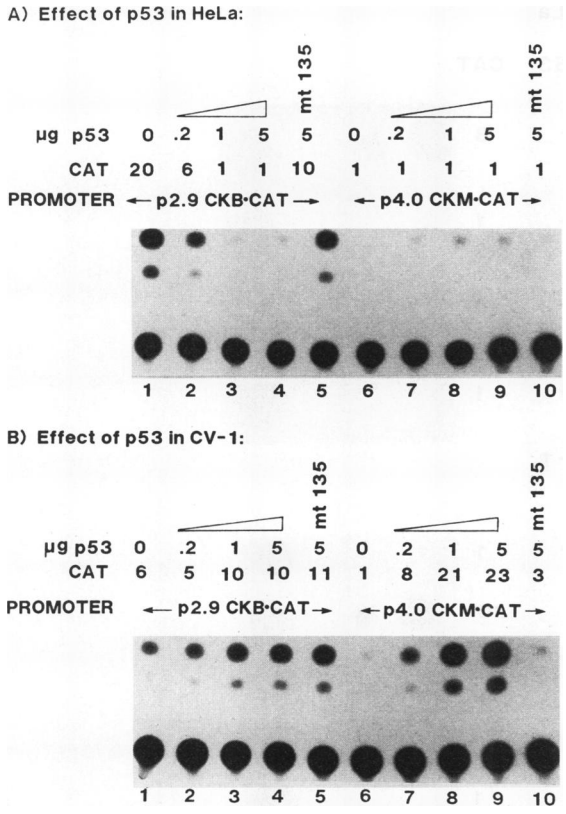


FIG. 1. p53-mediated repression of CKB in HeLa cells (A) and activation of CKM in CV-1 cells (B). Reporter plasmid p2.9 CKB-CAT (10.2 µg [2.2 pmol]) (lanes 1 to 5) or p4.0 CKM-CAT (12 µg [2.2 pmol]) (lanes 6 to 10) was cotransfected with 0, 0.2, 1, or 5 µg (lanes 2 to 4 and 7 to 9) of p53wt expression plasmid p11-4 · p53wt or 5 µg of p53mt 135AV (lanes 5 and 10) into HeLa (A) or CV-1 (B) cells at 37°C. CAT activity was determined with 50 µg of protein from HeLa cells and 200 µg of protein from CV-1 cells. CAT activities are the averages of two (A) and four (B) sets of independent experiments.

amide gel, blotted onto a nitrocellulose membrane, probed with primary antibody PAb421 followed by goat anti-mouse immunoglobulin G conjugated with peroxidase (Sigma), and visualized with an enhanced chemiluminescence kit (Amersham).

## RESULTS

**p53-mediated repression of CKB in HeLa cells and activation of CKM in CV-1 cells.** To determine if p53 regulates the rat CKB and CKM promoters, p53wt expression plasmid p11-4 · p53wt was cotransfected with either the p2.9 CKB or p4.0 CKM reporter plasmid (depicted in Fig. 3) into HeLa and CV-1 cells, respectively. Figure 1A shows that basal expression of p2.9 CKB in HeLa cells was readily detected (lane 1) and that cotransfection of p53wt severely repressed p2.9 CKB in a dose-dependent manner (lanes 2 to 4). While 1 µg of p53wt plasmid (0.25 pmol) maximally repressed p2.9 CKB by 20-fold (lane 3), as much as 5 µg of plasmid expressing mutant p53 135AV repressed CKB by only 2-fold (lane 5). p53mt 135AV has an Ala-to-Val change at residue 135 and is temperature sensitive, behaving like p53wt at 32°C and like a mutant at 37 to 39.5°C (2, 4, 13, 44); all of our experiments were performed at 37°C. In contrast to the results with p2.9 CKB, basal expression of p4.0 CKM in HeLa cells was extremely low (lane 6), as expected, as a result of the absence of muscle-specific *trans*-acting factors (10); cotransfection of either p53wt (lanes 7 to 9) or p53mt 135AV (lane 10) had no significant effect on p4.0 CKM.

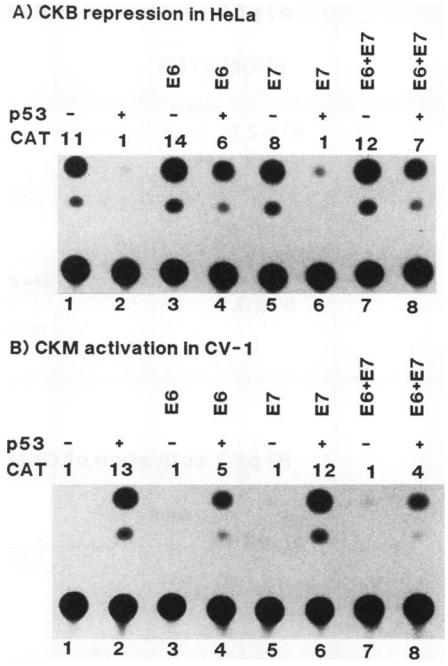


FIG. 2. The HPV-16 E6 protein blocks p53-mediated repression of CKB in HeLa cells (A) and activation of CKM in CV-1 cells (B). Plasmid p2.9 CKB-CAT (10.2 µg) (A) or p4.0 CKM-CAT (12 µg) (B) was cotransfected along with 1 µg (0.17 pmol) of plasmid expressing HPV-16 E6 (lanes 3 and 4), HPV-16 E7 (lanes 5 and 6), or HPV-16 E6 and E7 (lanes 7 and 8) in the absence (lanes 1, 3, 5, and 7) or presence (lanes 2, 4, 6, and 8) of 1 µg (0.25 pmol) of p11-4 · p53wt into HeLa (A) or CV-1 (B) cells. CAT activities are averages of three sets of independent experiments.

The rat CKB and CKM promoters displayed different patterns of modulation by p53 in CV-1 cells. Figure 1B shows that basal expression of p2.9 CKB was detectable in CV-1 cells (lane 1) and was not repressed when cotransfected with either p53wt (lanes 2 to 4) or p53mt 135AV (lane 5) but rather was consistently increased about 1.7-fold by p53wt. Conversely, basal expression of p4.0 CKM in CV-1 cells was very low (lane 6) but was greatly activated in a dose-dependent manner by p53 (lanes 7 to 9), consistent with the previously published transactivation of mouse CKM (56, 60). One microgram of p53wt activated p4.0 CKM by 21-fold to nearly maximal levels (lane 8); however, 5 µg of p53mt 135AV had no significant effect (lane 10).

**HPV-16 E6 protein blocks p53-mediated modulation of CK genes.** To verify that modulation of the CKB and CKM promoters was mediated by p53, we attempted to block these effects by coexpression of the HPV-16 E6 protein. E6 has been shown to bind and destabilize p53 in vitro (43). In HeLa cells, E6 interfered with p53-mediated repression of CKB (Fig. 2A). Cotransfection of as little as 1 µg (0.17 pmol) of plasmid expressing HPV-16 E6 protein reduced p53-mediated repression of p2.9 CKB from 11-fold (lanes 1 and 2) to only 2-fold (lane 4). E6 had no significant effect on basal expression (in the absence of p53) of CKB (lane 3). The E7 protein is another HPV-16 early protein which does not bind to p53 but rather interacts with the retinoblastoma protein (30). Lane 6 shows

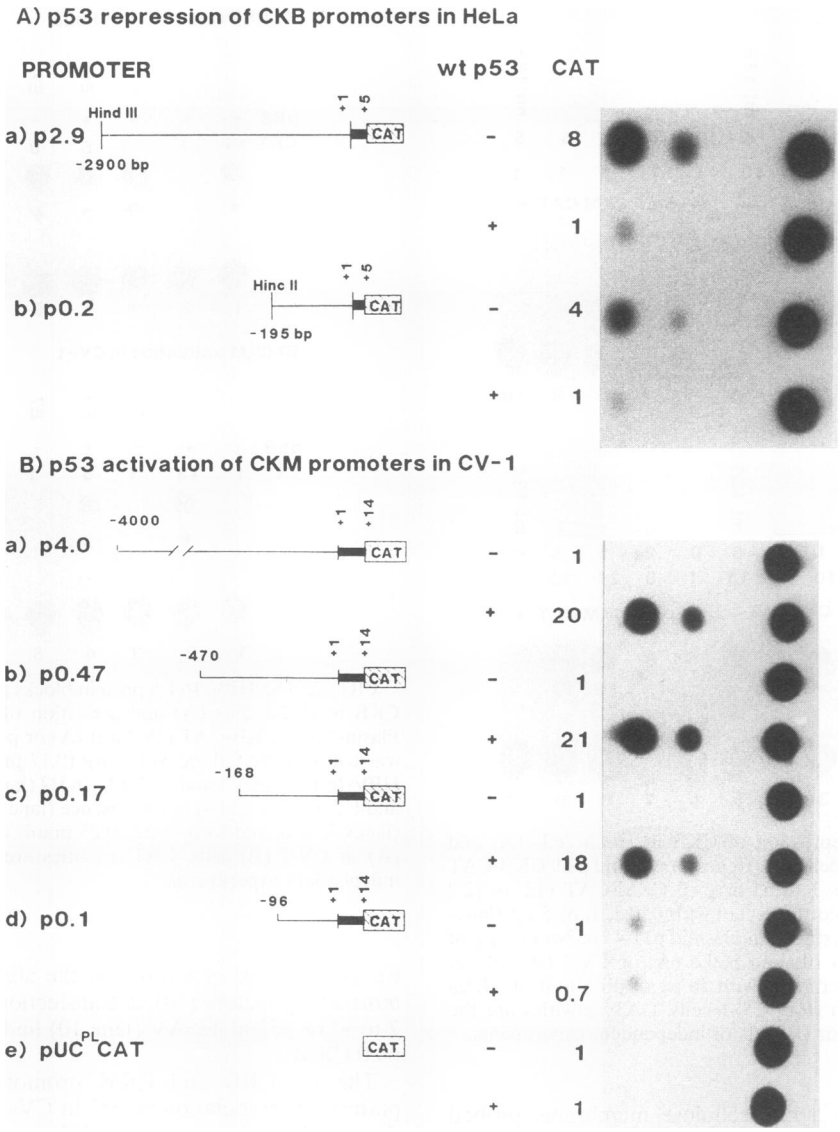


FIG. 3. Localization of sequences responsible for p53-mediated modulation of CK promoters. A 2.2-pmol amount of the indicated CKB-CAT reporter plasmid (10.2 μg of p2.9 CKB or 6.4 μg of p0.2 CKB) (A) or CKM-CAT plasmid (12 μg of p4.0 CKM, 6.8 μg of p0.47 CKM, or 6.4 μg of p0.17 CKM, p0.1 CKM, or pUC$^{PL}$CAT) (B) was transfected into HeLa (A) and CV-1 (B) cells in the absence or presence of 1 μg (0.25 pmol) of p11-4 · p53wt. CAT activities are averages of three sets of independent experiments.

that the E7 protein did not interfere with p53-mediated repression of CKB and thus served as a negative control. Similar to expression of E6, coexpression of both E6 and E7 proteins reduced p53-mediated repression of CKB from 11-fold to only 2-fold (lanes 1, 2, and 8), without affecting basal expression of CKB (lanes 1 and 7).

In CV-1 cells, the E6 protein also interfered with p53-mediated activation of CKM (Fig. 2B); E6 reduced p53-mediated activation of CKM from 13-fold to 5-fold (lanes 1, 2, and 4). A similar reduction was observed when both E6 and E7 proteins were expressed (lane 8). Expression of E7 protein alone did not affect the p53-mediated activation of CKM (lane 6). The low basal expression of p4.0 CKM was not affected by either E6 or E7, alone or in combination (lanes 3, 5, and 7).

**Localization of sequences responsible for p53-mediated modulation of CK genes.** To localize the DNA sequences responsible for p53-mediated modulation of CKB and CKM, deletion analyses were performed on the CKB and CKM promoters. Figure 3A shows that both long p2.9 CKB and short p0.2 CKB promoters were completely repressed by p53wt in HeLa cells. This result indicated that if there is a specific sequence responsible for CKB repression, it is located between bp −195 and +5 of the CKB promoter. Similarly, Fig. 3B shows that p4.0 CKM, p0.47 CKM, and p0.17 CKM promoters were all strongly activated by p53wt in CV-1 cells (data sets a to c); p1.5 CKM was also activated to the same extent by p53 (data not shown). Conversely, p0.1 CKM was not activated by p53 but rather was reproducibly repressed by 30% (data set d). The lack of p53-mediated activation of p0.1 CKM is not likely due to the absence of the sequences required for basal transcription, since it still contained a consensus TATA box at bp −30, and more importantly, basal expression of p0.1 CKM was reproducibly two- to threefold higher than for all of the other CKM-CAT constructs. This finding indicated that se-

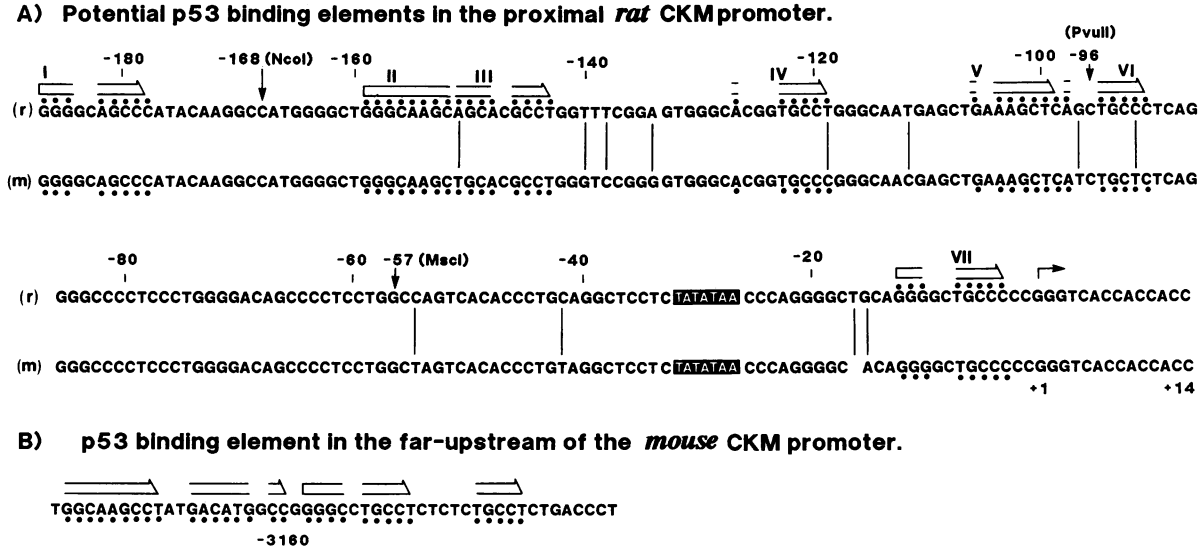


FIG. 4. Sequence comparison of 187 bp in the proximal rat (r) and mouse (m) CKM promoters. (A) Positions of the seven potential p53 motifs in the rat CKM promoter are indicated by roman numerals and large arrows; dots indicate the nucleotides which match the consensus p53-binding motif; vertical lines indicate the nucleotide differences between the rat and mouse CKM promoters. The TATA box is highlighted, and the bent arrow at +1 marks the site of transcription initiation. (B) The p53-binding motifs in the far-upstream mouse CKM 5′ flanking region near bp −3160.

quences located between bp −168 to −97 of rat CKM are necessary for p53 transactivation. These results, however, do not eliminate the possibility that an additional (or redundant) p53 element(s) exists further upstream of −168 bp of the CKM promoter.

**A CKM promoter-proximal fragment from bp −168 to −57 confers p53-mediated activation on a heterologous promoter.** Weintraub et al. (56) showed that the DNA element responsible for the greatest p53-mediated activation (21- to 90-fold) of the mouse CKM gene was located far upstream near bp −3160. Shorter mouse CKM promoters ranging down to bp −776 were also activated by p53 but only 1/10 as much as the 3.3-kb CKM promoter (56). For comparison, Fig. 4B shows that the far-upstream p53 elements located near bp −3160 of the mouse CKM gene adhere more closely to the consensus p53 motif 5′-PuPuPuC(A/T)(T/A)GPyPyPy-3′ (11, 15, 60) than the motifs in the proximal rat CKM promoter (Fig. 4A).

We wished to know if the bp −168 to −57 fragment of the rat CKM proximal promoter was able to confer p53-mediated activation when cloned into a heterologous promoter (i.e., p1634) and how it compared with the activation conferred by the far-upstream (bp −3160) mouse p53 element originally characterized by Zambetti et al. (60), who used plasmid pMCK-1634 (Fig. 5A). Figure 5A shows diagrams of two rat CKM constructs, p112N-1634 and p112R-1634, which contained the 112-bp fragment (*Nco*I-*Msc*I; bp −168 to −57) of rat CKM cloned in the normal and reverse orientations, respectively, into the *Eco*RV site of p1634, which has the Ad major late promoter TATA box fused to the terminal deoxynucleotidyl transferase gene initiation region (60). Both p112N-1634 and p112R-1634 were strongly activated by p53 in CV-1 cells (Fig. 5B, lanes 5 to 8) such that, in the presence of p53, their promoter activity was comparable to that of p53-activated pMCK-1634 (lane 10), which has a 500-bp fragment containing the far-upstream mouse CKM p53 element cloned into p1634 (60). However, since the basal expression of p112N-1634 and p112R-1634 was reproducibly 4-fold higher than that of pMCK-1634, the extent of activation of p112N-1634 and p112R-1634 was 8-fold, compared with 37-fold for pCKM-1634. Also, the average p53-mediated activation of rat p0.17 CKM was 37-fold (lanes 1 and 2), and its promoter activity in the presence of p53 was more than 4-fold higher than that of any other construct. This finding indicates that the natural 168-bp rat CKM promoter context is important for maximal activity but that a smaller region (bp −168 to −57) also has significant activity.

**Missense mutations in region II of p53 affect its transrepression and transactivation activities differentially.** Since this report and previous studies have shown that p53 exhibits both transrepression (18, 33, 41) and transactivation (56, 60) activities, we wished to determine how different point mutations would affect these two activities. Therefore, missense mutations in conserved region II of the p53 protein (45) were analyzed for the effects on CKB repression in HeLa cells and CKM activation in CV-1 cells. Conservative substitutions were chosen to minimize changes in amino acid charge, polarity, and hydrophobicity. Region II is one of the five evolutionarily conserved regions (50), and it is the site of two naturally occurring mutations which have activated p53 for transformation in the mouse (i.e., 132CF and 135AV) (2, 4, 13, 44). In addition, region II appears to influence the overall structure of p53 (13, 32, 39) as well as the binding of p53 to SV40 large T antigen (T Ag) (45, 52).

We previously generated three classes of p53 mutants that differed in the binding to SV40 T Ag and monoclonal antibodies PAb246 and PAb240 (45). PAb246 recognizes an epitope on p53wt that is lost upon denaturation (59), while PAb240 does not bind well to p53wt but rather binds to denatured p53 (16). The CKB transrepression and CKM transactivation activities of six of these mutants were analyzed (Table 1 and Fig. 6). Table 1 shows that the class I mutant (136KR) is most similar to p53wt since it binds to T Ag (T Ag$^+$) as well as to PAb246 (PAb246$^+$) but does not bind to PAb240 (PAb240$^-$). 136KR retained 100% of the p53wt transrepression activity (Fig. 6A; compare samples 1, 2, and 7) and 57% of p53wt transactivation (Fig. 6B, samples 1, 2, and 7). The class II

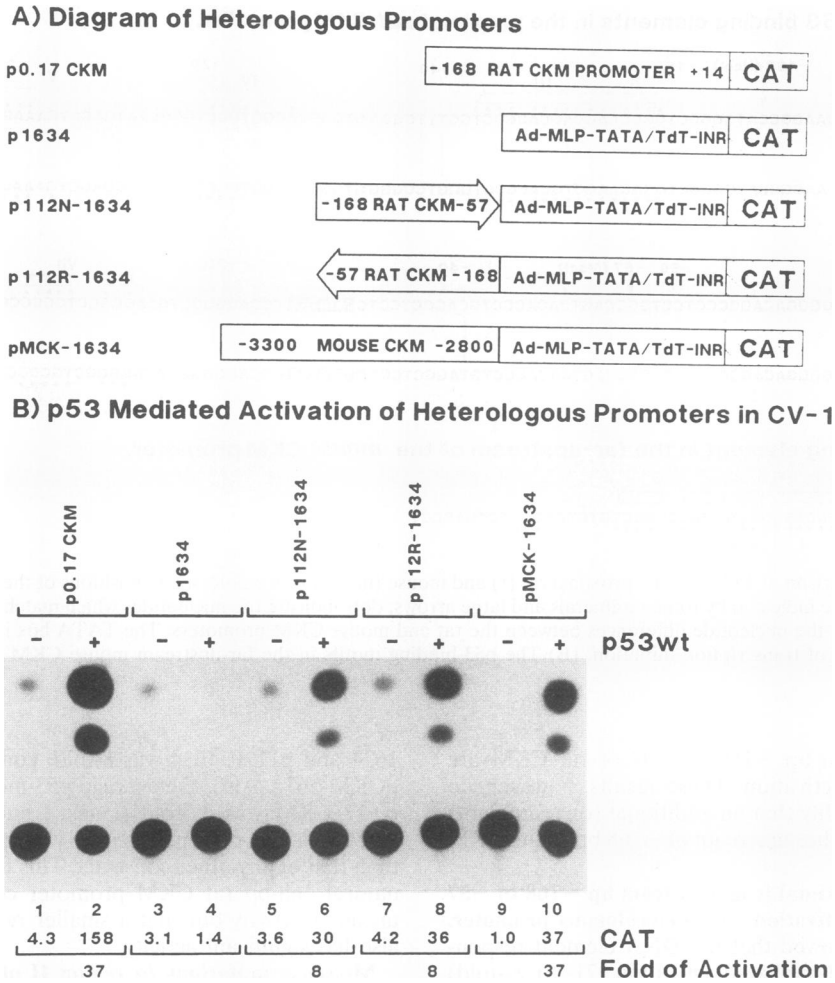


FIG. 5. p53-mediated activation of heterologous promoters containing the bp −168 to −57 rat CKM fragment. (A) Schematic diagram of the heterologous promoters. Ad-MLP, Ad major late promoter; TdT-INR, terminal deoxynucleotidyltransferase initiation region. (B) Reporter plasmids indicated in panel A (2.2 pmol) were transfected in the absence (−) or presence (+) of 1 μg of p11-4 · p53wt into CV-1 cells. One experiment is shown; the other two gave similar results.

mutants (129KR, 131FL, and 135AV) are T Ag$^-$ PAb246$^-$ PAb240$^+$. All three completely lost the ability to activate CKM (Fig. 6B, samples 3, 4, and 6) but retained partial ability (12, 20, and 14%, respectively, of p53wt activity) to repress CKB (Fig. 6A, samples 3, 4, and 6). The class III mutants (134LV and 137TS) are T Ag$^-$ PAb246$^+$ PAb240$^-$ and differ from each other in transrepression and transactivation activities. Mutant 134LV retained essentially total ability to repress CKB (Fig. 6A, sample 5) and to activate CKM (Fig. 6B, sample 5) and thus resembled the class I mutant 136KR. Mutant 137TS

TABLE 1. Properties and activities of wild-type and mutant p53

| Class | T Ag binding[a] | PAb246 binding | PAb240 binding | Mutant | % of p53wt CKB transrepression[b] | % of p53wt CKM transactivation | Transformation inhibition[c] |
|---|---|---|---|---|---|---|---|
| Wild type | + | + | − | | 100 | 100 | + |
| I | + | + | − | 136KR | 100 | 57 | + |
| II | − | − | + | 129KR | 12 | 0 | − |
| | − | − | + | 131FL | 20 | 0 | − |
| | − | − | + | 135AV | 14 | 0 | − |
| III | − | + | − | 134LV | 100 | 86 | + |
| | − | + | − | 137TS | 0 | 25 | + |

[a] The extent of binding of wild-type and mutant p53 proteins to either PAb246, PAb240, or SV40 T Ag, which serves as the basis for the assignment of the + or − values, can be found in reference 45. The percentages of p53wt repression and activation activities are averages of at least three sets of independent experiments.

[b] Defined as the extent of CKB repression mediated by p53mt divided by the CKB repression mediated by p53wt; similarly, the percent transactivation activity of p53mt is the extent of CKM activation mediated by p53mt divided by the CKM activation mediated by p53wt.

[c] The cell transformation inhibition assay was done at least three times; see reference 45 for details.

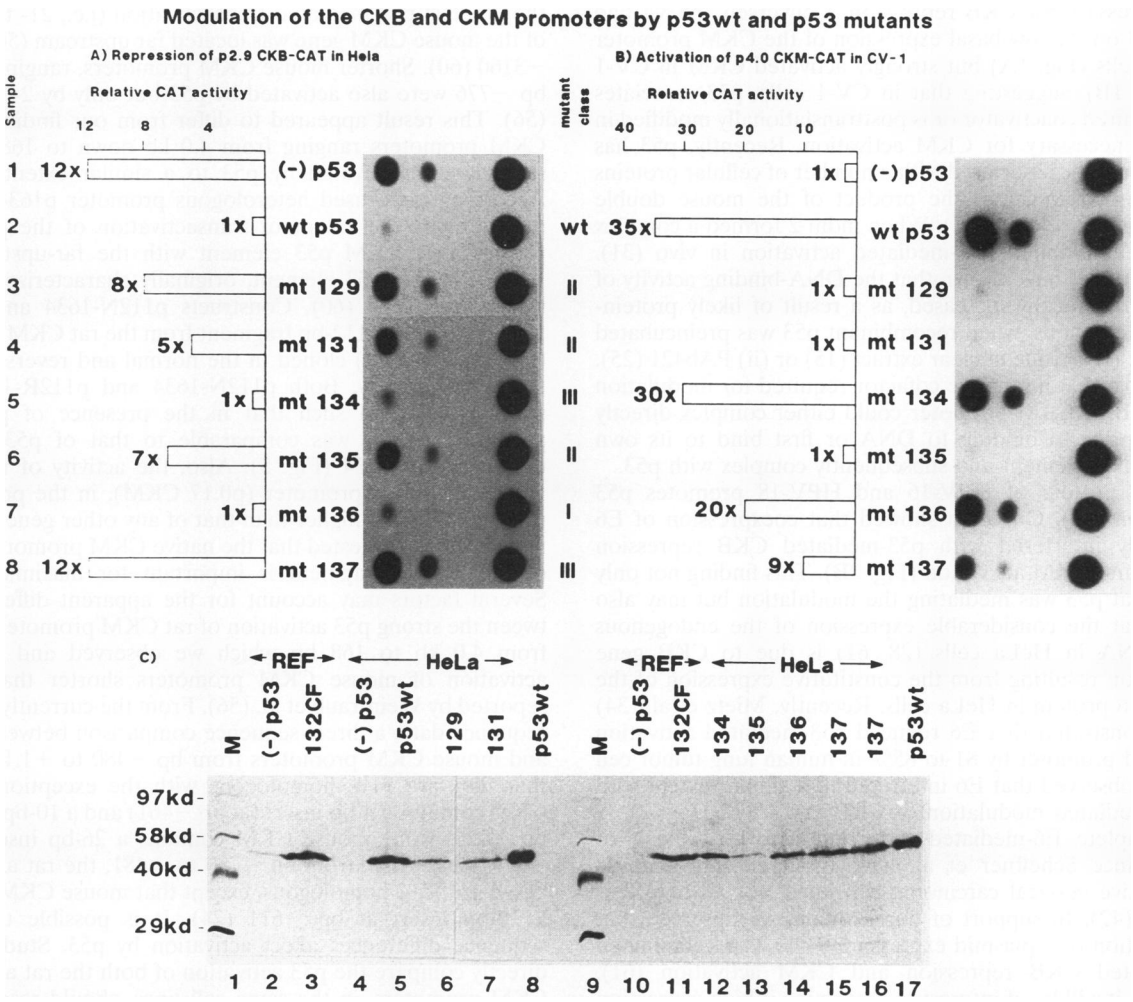


FIG. 6. Missense mutations in conserved region II of p53 differentially affect its transrepression and transactivation activities. One microgram (0.25 pmol) of plasmid encoding either p53wt or the indicated missense mutants was cotransfected with p2.9 CKB-CAT (2.2 pmol) into HeLa cells (A) or with p4.0 CKM-CAT (2.2 pmol) into CV-1 cells (B). CAT activities are averages of three sets of independent experiments. (C) Western blot analysis of p53 protein levels in transiently transfected HeLa extracts. One microgram of plasmid expressing p53wt (lane 5) or indicated p53 mutants (lanes 6, 7, and 12 to 16) was cotransfected with 2.2 pmol of p2.9 CKB into HeLa cells; 100 μg of total protein was subjected to SDS-polyacrylamide gel electrophoresis, transferred to a nitrocellulose membrane, probed with PAb421 followed by goat anti mouse immunoglobulin G, and visualized by enhanced chemiluminescence. M, molecular weight markers (lanes 1 and 9); REF, rat embryonic fibroblasts stably transformed with either E1a and Ras (lane 2 and 10, negative control) or E1a, Ras, and mouse p11-4 · p53mt/132CF (lanes 3 and 11, positive control); lanes 8 and 17, positive control mouse p53wt expressed in baculovirus and affinity purified with PAb421. Two separate transfections with different DNA preparations of mutant 137TS are shown (lanes 15 and 16).

displayed a distinct phenotype, since it completely lost ability to repress CKB (Fig. 6A, sample 8) yet maintained the ability to significantly activate CKM (i.e., 137TS activated CKM by ninefold, which was 25% of p53wt activity; Fig. 6B, sample 8). It should be noted that the ability of mutants 129KR, 131FL, and 135AV to repress CKB shown in Table 1 is a minimum estimate, since Western blot analysis (Fig. 6C) showed that the steady-state level of 129KR and 131FL in HeLa cells was about 50% of the p53wt level (lanes 5 to 7), while the level of 135AV (lane 13) was less than 25% of the p53wt level. On the other hand, the protein levels of mutants 134LV, 136KR, and 137TS were similar (and comparable to the level of p53wt) (Fig. 6C, lanes 4, 12, 14, 15, and 16), and therefore their different CKB transrepression activities would appear to be due to their intrinsic characteristics rather than their level of expression. Currently, we have no explanation for the low expression level of 135AV in HeLa cells. We also attempted numerous times to demonstrate the protein levels of various p53 mutants in CV-1 cells without success, probably because of the low transfection efficiency of CV-1 cells. In summary, different region II mutations affected the transrepression and transactivation activities of p53 differentially.

## DISCUSSION

These studies employed transient-transfection assays to examine the effects of wild-type or mutant p53 proteins on the expression of the rat CKB and CKM promoters. Expression of p53wt severely repressed the CKB promoter in HeLa cells (Fig. 1A) but not in CV-1 cells (Fig. 1B), possibly suggesting that in HeLa but not CV-1 cells, p53 either associates with a required corepressor or undergoes a posttranslational modifi-

cation necessary for CKB repression. Conversely, p53wt had little effect on the low basal expression of the CKM promoter in HeLa cells (Fig. 1A) but strongly activated CKM in CV-1 cells (Fig. 1B), suggesting that in CV-1 cells, p53 associates with a required coactivator or is posttranslationally modified in a manner necessary for CKM activation. Recently, p53 has been shown to be associated with a number of cellular proteins in vivo, one of which is the product of the mouse double minute 2 gene (*mdm-2*) (31). When mdm-2 formed a complex with p53, it inhibited p53-mediated activation in vivo (31). Separate studies have shown that the DNA-binding activity of p53 was dramatically increased, as a result of likely protein-protein interactions, when recombinant p53 was preincubated with either (i) a crude nuclear extract (15) or (ii) PAb421 (25). It is possible that a putative cofactor required for modulation of the CKB or CKM promoter could either complex directly with p53 prior to binding to DNA or first bind to its own cognate DNA element and subsequently complex with p53.

The E6 protein of HPV-16 and HPV-18 promotes p53 degradation (43). Our data showed that coexpression of E6 significantly interfered with p53-mediated CKB repression (Fig. 2A) and CKM activation (Fig. 2B). This finding not only verified that p53 was mediating the modulation but may also suggest that the considerable expression of the endogenous CKB mRNA in HeLa cells (28, 61) is due to CKB gene derepression resulting from the constitutive expression of the HPV-18 E6 protein in HeLa cells. Recently, Mietz et al. (34) have demonstrated that E6 reduced p53-mediated activation of a hybrid promoter by 81 to 85% in human lung tumor cell lines. We observed that E6 interfered to a similar extent with the p53-mediated modulation of CKB and CKM (Fig. 2). A more complete E6-mediated reduction may be difficult to achieve, since Scheffner et al. have observed that even in HPV-positive cervical carcinoma cell lines, not all of p53 is degraded (42). In support of our E6 data, we observed that cotransfection of a plasmid expressing SV40 T Ag eliminated p53-mediated CKB repression and CKM activation (61). Therefore, it will be of interest to determine if high expression of the endogenous CKB gene is generally observed in tumor cells which are transformed by viruses that express viral proteins that interfere with p53 function (e.g., SV40, HPV, and Ad [30]).

We definitively identified a promoter-proximal sequence (bp −168 to −97) that was responsible for p53-mediated activation of rat CKM in CV-1 cells. Moreover, a 112-bp fragment encompassing this 70-bp sequence conferred p53-mediated activation on a heterologous promoter. Previous reports have established that the most prevalent p53 core response element is composed of two copies of the 10-bp motif 5′-PuPuPuC(A/T)(T/A)GPyPyPy-3′ separated by 0 to 13 bp (11, 15, 26, 27, 60). The 168-bp rat CKM promoter contains five copies of a potential p53 motif between bp −168 and −90, of which four are homologous to the consensus in the (T/A)GPyPyPy-3′ region but less homologous in the 5′-PuPuPuC(A/T) region (Fig. 4A). However, recent reports indicate that somewhat different sequence elements can respond to p53. The human T-cell leukemia virus type I (HTLV-I) enhancer contains a p53 element, 5′-GCCCTGACGTGTCCCC-3′ (1), the SV40 origin of replication has a weak p53-binding element with two copies of 5′-GGGCGGAGTTA-3′ (3), and most recently, mouse genomic DNA clones with a p53-binding element of 5′-GACACTGGTCACACTTGGCTGCTTAGGAAT-3′ have been found (14). The degeneracy in sequence has led to the supposition that in some instances, p53 binds to DNA in a complex with another protein (1, 15).

It has been reported that the DNA element responsible for the greatest p53-mediated transactivation (i.e., 21- to 90-fold) of the mouse CKM gene was located far upstream (56) near bp −3160 (60). Shorter mouse CKM promoters, ranging down to bp −776 were also activated by p53 but only by 2- to 12-fold (56). This result appeared to differ from our finding that rat CKM promoters ranging from 4.0 kb down to 168 bp were strongly transactivated by p53 to a similar extent (Fig. 3). Accordingly, we used heterologous promoter p1634 to compare directly the extent of transactivation of the promoter-proximal rat CKM p53 element with the far-upstream (bp −3160) mouse p53 element, originally characterized in plasmid pMCK-1634 (60). Constructs p112N-1634 and p112R-1634 contained a 112-bp fragment from the rat CKM promoter (bp −168 to −57) cloned in the normal and reverse orientations, respectively. Both p112N-1634 and p112R-1634 were strongly activated such that in the presence of p53, their promoter activity was comparable to that of p53-activated mouse pMCK-1634 (Fig. 5). Also, the activity of the native 168-bp rat CKM promoter (p0.17 CKM), in the presence of p53, was fourfold higher than that of any other gene construct tested, which suggested that the native CKM promoter context of the 112-bp fragment is important for maximal activity. Several factors may account for the apparent difference between the strong p53 activation of rat CKM promoters ranging from 4.0 kb to 168 bp which we observed and the weak activation of mouse CKM promoters shorter than 3.3 kb reported by Weintraub et al. (56). From the currently available sequence data, a direct sequence comparison between the rat and mouse CKM promoters from bp −480 to +1 has shown that they are 91% homologous with the exception that rat CKM contains a 4-bp insert (at bp −461) and a 10-bp insert (at bp −424) while mouse CKM contains a 26-bp insert (at bp −379) (24). Also, from bp −780 to −481, the rat and mouse CKM are 85% homologous, except that mouse CKM contains a 10-bp insert at bp −611 (24). It is possible that these sequence differences affect activation by p53. Studies which directly compare the p53 activation of both the rat and mouse CKM promoters, in the same cell lines, should resolve these differences. The results of our studies do not eliminate the possibility that an additional (or redundant) p53 element exists upstream of bp −168 in the rat CKM (Fig. 3 and 4). Since the published sequence data of the rat CKM promoter extend only up to bp −1512 (24), it is not known if a consensus p53 element is located far upstream (near bp −3160), as shown in mouse CKM (60).

We identified the sequence in the CKB promoter from bp −195 to +5 as being involved in p53-mediated repression of CKB. However, the mechanism by which p53 represses CKB remains elusive. CKB repression does not appear to be mediated by the typical 5′-PuPuPuC(A/T)(T/A)GPyPyPy-3′ motif, since not even a degenerate one is present in the 195-bp rat CKB promoter. A 5′-GGAAGTGA-3′ element has been proposed to be responsible for p53-mediated repression of the human retinoblastoma protein (47). Interestingly, a similar sequence (5′-GGAATGA-3′) exists in the rat CKB promoter (at bp −79), but its role in repression is not established. However, repression by p53 does not appear to solely involve a GGAAGTGA sequence, since p53 has been shown to repress a number of other promoters (18, 41), at least some of which do not contain a GGAAGTGA (e.g., human interleukin-6 [41] and rat p53 [8]). Recent reports demonstrating that p53 binds to the TATA-binding protein and inhibits transcription initiation offer a possible explanation for the repression of numerous promoters (46, 53).

It has been suggested that p53 regulates normal cell growth by activating transcription of genes whose products suppress

growth and tumor formation (3, 12, 21, 37). Conceivably, p53 may also repress genes whose products function to initiate and/or sustain accelerated growth. The latter class of genes may include CKB, since this enzyme functions to regenerate ATP in cell types with high energy expenditure, e.g., transformed cells (17). Recent reports have indicated that the ability of p53 to activate genes is critical for normal growth control (12, 21). First, while p53wt strongly activated an HTLV-I promoter containing p53 elements, all p53 point mutants isolated from human tumors lacked transactivation (1). Second, transformation of primary cells by Ad2 requires binding of the Ad2 E1b protein to p53. Analysis of wild-type and mutant Ad2 E1b proteins showed a strong correlation between the ability of E1b to inhibit p53-mediated gene activation and its ability to transform cells, in cooperation with Ad E1a (58). Accordingly, we generated three classes of p53 mutants which differed in the ability to bind SV40 T Ag, PAb246, or PAb240 and tested not only their transactivation and transrepression activities but also if there was any correlation between the latter two activities and the ability of p53 to inhibit cell transformation (45). We found that there was no direct correlation between the binding of T Ag by p53 and the CKB transrepression and CKM transactivation activities of p53; e.g., the class III mutant 134LV was T $Ag^-$ and yet retained essentially complete ability to activate CKM and repress CKB. On the other hand, there was a correlation between the binding of PAb246 by p53 and the ability of p53 to transactivate CKM. For example, class I mutant 136KR and class III mutants 134LV and 137TS are $PAb246^+$, and they all significantly transactivate CKM. Interestingly, separate studies showed that all the mutants which retained at least some transactivation activity (136KR, 134LV, and 137TS) were capable of inhibiting transformation of rat embryonic fibroblasts induced by Ad E1a and activated Ras (Table 1, reference 45, and unpublished data).

It is not known whether p53 plays a role in myogenesis or in the normal activation of CKM in vivo. It is possible that p53 is involved in the obligatory cessation of myoblast cell division prior to myotube formation (10). p53 null mice apparently develop normally up to birth but display early tumor formation (9). It would be interesting to determine whether the expression patterns of CKB and CKM in brain and skeletal muscle tissues from p53 null mice are altered.

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