Distinct distal and proximal p53-binding sites in the MCK promoter govern the transcriptional response to p53

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Abstract We have investigated the functional importance of nucleotide sequence differences between proximal (P-) and distal (D-) p53-binding elements in the MCK promoter. P- and D-elements normally co-operate to permit synergistic promoter activation by p53. Interestingly, we find that P-elements cannot co-operate with each other. In contrast, co-operation between D-binding sites results in levels of p53-induced transcription far higher than those obtained by co-operation between P- and D-elements. These studies imply that distinct D- and P-p53-binding sites in the MCK promoter may dictate the promoter response to p53.

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Key words: p53; Muscle creatine kinase; Promoter; Transcription; DNA binding; Gene activation

1. Introduction

The protein encoded by the p53 tumour suppressor gene plays a major role in the cellular response to DNA damage (reviewed in [1,2]). There is now considerable evidence that at least part of the mechanism by which p53 functions, to either arrest cell growth or induce apoptosis, is through regulating the expression of other genes involved in these processes, such as the WAF1/CIP1 and GADD45 genes (reviewed in [3]). Transcriptional activation by p53 requires binding of p53 to specific sequences within the control regions of its target genes [4–7]. Several such target genes, including the muscle-specific creatine kinase (MCK) [8], WAF1 [9] and cyclin G [10] genes, contain multiple copies of this binding motif within their control sequences. Although the significance of this observation is not yet clear, in two recent studies which have addressed this issue using either a model promoter or the MCK promoter, evidence has been presented suggesting that if two p53-binding sites (a proximal element located adjacent to the TATA-box with a second, distal, element located several kilobases further upstream) are present, they co-operate to permit high-level, synergistic activation by p53 [11,12]. These data suggest that multiple p53-binding elements within a promoter may contribute to a mechanism of regulation by p53 more complex than previously envisaged.

A closer examination of the nucleotide sequences of p53-binding elements within the WAF1, cyclin G and MCK promoters indicates that though both distal (D-) and proximal (P-) elements contain sequences with homology to the p53 consensus binding sequence, each is distinctly different. In particular, within the MCK promoter, D- and P-sites differ at 9 out of 20 residues. The functional importance of these sequence differences is not understood. As one approach of investigation, we have constructed MCK-promoter reporter plasmids in which we have replaced either the D- or P-element with a copy of the remaining element. We report here that P- and D-elements show marked differences in their ability to co-operate, and that both D- and P-elements are required by the MCK promoter for a normal transcriptional response to p53.

2. Materials and methods

2.1. Cell culture, transfections and CAT assays

Monkey CV1 (monkey kidney epithelial) cells were maintained in Eagle's modified essential medium (EMEM; Gibco BRL, Grand Island, NY) supplemented with 5% heat-inactivated foetal bovine serum (FCS; Trace Biosciences, Sydney, Australia) and containing added L-glutamine (2 mM final concentration), penicillin/streptomycin (50 U/ml, Gibco BRL) and fungizone (2.5 μg/ml, Trace Biosciences).

In each experiment, 5×10^5 cells were seeded into 6 cm petri dishes and transfected using lipofectamine reagent (Gibco BRL, Life Technologies Inc., Gaithersburg, MD) exactly as described by the manufacturer. Optimal transfection was obtained with 9 μl of lipofectamine and 6 μg (2 μg CAT reporter plasmid and 4 μg of p53 expression plasmid) total DNA. The lipid/DNA complex was removed after 5 h, and cells harvested 18 h later for determination of CAT activity. Levels of CAT activity in transfected cell lysates were measured as described in detail previously [8]. Results presented are the means ± S.E. of at least three independent experiments carried out in duplicate.

2.2. Plasmids

Construction and characterisation of plasmids pMSV (control vector) and both pMSV wtp53 (wild-type mouse p53) and pMSV val135 (mouse mutant p53 with an Ala to Val change at residue 135) both expressing protein from the Moloney sarcoma virus promoter, have been described [13].

Construction of reporter plasmids p-3300MCKCAT, p-2800MCKCAT and p-3300Δ1MCKCAT, all containing portions of the MCK promoter fused to the bacterial CAT gene, has been described previously [8,14]. For clarity, in the present report these plasmids have been retitled pD3300PMCKCAT, p2800PMCKCAT and pD3300MCKCAT respectively to indicate the presence of distal (D-) and/or proximal (P-)MCK p53-binding elements. The intervening number reflects the amount of MCK promoter sequence present in the plasmid. This system was used to name all derivative plasmids described below. Plasmid p2800MCKCAT was generated by *Sal*I digestion of pD3300MCKCAT and religation of the remaining vector. This plasmid contained MCK sequences between −2800 and +7, with a deletion between residues −189 and −81 and thus lacked both p53-binding elements. Plasmids pD2800PMCKCAT, pD1020PMCKCAT, pD776PMCKCAT and pD300PMCKCAT containing the distal MCK p53-binding elements separated by various intervals from the proximal MCK p53-binding element (Table 1) were generated as follows: MCK promoter sequences between −3195 and −3120 (D-fragment) were amplified from pD3300PMCKCAT by PCR using a 5′ primer 5′-AACCAAGCTTGGCGTGTGCTCCCTGGCAAGCC-TAT-3′ and a 3′ primer, 5′-AACCAAGCTTATGGGAGCCACT-GAGGGTCAGAGGC-3′. After purification, the amplified product was digested with *Hin*dIII, repurified and inserted into the *Hin*dIII sites within p2800PMCKCAT, p1020PMCKCAT, p776PMCKCAT

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Table 1
Co-operation between MCK P-elements is not restored by decreased promoter spacing between elements or reversal of relative promoter orientation

Plasmid used	Spacing between p53-response elements (kb)	p53-dependent synergy
pD3300PMCKCAT	3.00	+
pD2800PMCKCAT	2.65	+
pP2800PMCKCAT	2.65	−
prP2800PMCKCAT	2.65	−
pD1020PMCKCAT	0.87	+
pP1020PMCKCAT	0.87	−
prP1020PMCKCAT	0.87	−
pD776PMCKCAT	0.62	+
pP776PMCKCAT	0.62	−
pD300PMCKCAT	0.15	+
pP300PMCKCAT	0.15	−

CV1 cells were transfected with the indicated reporter plasmid and either pMSV, pMSVwtp53 or pMSV val135. To determine synergy, levels of wt p53-dependent activation of the above plasmids were compared with activation of the corresponding parent MCK reporter lacking the distally located p53-binding site. '+' and '−' indicate synergy and no synergy respectively.

and p300PMCKCAT [8]. After restriction enzyme analysis to confirm the presence of insert, correct orientation (5′-3′ with respect to the MCK promoter) was verified by PCR. Briefly, plasmid DNA from bacterial colonies obtained from the above procedure was analysed by PCR with the above 5′ primer and a novel 3′ primer, 5′-AACCTCTAGAGGCCCCTGAGAGCAGATGAGCTTTC-3′, which annealed to MCK promoter sequences between −105 and −81. Correct orientation of insert resulted in amplification of a fragment corresponding to the sequences between proximal and distal p53-binding elements.

Several MCK-reporter plasmids were generated in which a copy of the proximal p53-binding element replaced the normal distal p53-binding element. An MCK promoter fragment between −177 and −81 (P-fragment) with *Hin*dIII restriction enzyme sites at each end was amplified by PCR (for details of primers and conditions, see [8]). After digestion with *Hin*dIII and purification, this fragment was inserted into the *Hin*dIII site within p2800PMCKCAT, p1020PMCKCAT, p776PMCKCAT or p300PMCKCAT to form pP2800PMCKCAT, pP1020PMCKCAT, pP776PMCKCAT or pP300PMCKCAT respectively. Plasmids prP1020PMCKCAT and prP776PMCKCAT in which the orientation of the P-fragment was reversed relative to the MCK promoter were also made. All orientations of the P-fragment were confirmed by PCR.

Plasmid pD3300DMCKCAT (in which a D-p53-binding element replaced the normal proximal p53-binding element) was constructed by amplifying an MCK promoter fragment (−3195 to −3120) with *Sph*I restriction enzyme sites at each end. After purification and digestion with *Sph*I, this fragment was inserted into *Sph*I-cut p80MCKCAT [8] which placed the D-fragment adjacent to residue −80 in the MCK promoter sequence in plasmid p80DMCKCAT. MCK sequences between −3182 and −178 were then amplified by PCR using the 5′ primer described above (with *Hin*dIII ends) and a 3′ primer, 5′-AACCAAGCTTGGCTGCCCCAAGGGCTGACTTGCTC-3′. After cutting with *Hin*dIII and subsequent purification, this fragment was inserted into the *Hin*dIII site adjacent to the 5′ end of the D sequence in p80DMCKCAT. Correct orientation of the introduced fragment was verified by restriction enzyme analysis.

2.3. Polymerase chain reaction (PCR) conditions

Amplification of P- (previously called NE) and D- (previously called BS) MCK fragments by PCR in a Hybaid Thermocycler was performed as previously described in detail [8]. MCK sequences between −3182 and −178 were amplified in a reaction mixture containing 100 pmol of each primer, 50 ng DNA template, $MgCl_2$ at a final concentration of 2 mM, 1.0 μl of Pfu enzyme (Stratagene, La Jolla, CA), 5.0 μl of 10× Pfu reaction buffer and water to a total volume of 50 μl. Reaction conditions were, an initial hot start using 94°C, 3 min; addition of enzyme; 72°C for 7 min. Then one cycle of 94°C, 5 min; 65°C, 1 min; 72°C for 7 min, followed by 94°C, 2 min; 72°C, 7.5 min for 25 cycles and a final cycle of 72°C, 10 min. After cooling to room temperature, reaction products were purified.

3. Results

3.1. Co-operation between P- and D- but not P- and P-MCK p53-binding elements

The mouse MCK promoter contains D- and P-p53-binding elements [8] which co-operate to allow high-level, synergistic activation by p53 [8,12] (Fig. 2B). Within these elements are nucleotide sequences with homology to the p53 consensus binding site, however, D- and P-elements differ from each other at 9 out of 20 residues (Fig. 1). We first investigated the functional importance of these differences by constructing an MCK-reporter plasmid containing two copies of the P-element (Fig. 2A). MCK promoter sequences between −177 and −81 were amplified and cloned into the *Hin*dIII site in p2800PMCKCAT forming pP2800PMCKCAT in which the two P-binding elements are separated by ~2.6 kb. A control reporter was made by inserting the D-element (MCK promoter residues −3182 to −3133) into a similar position in p2800PMCKCAT to form pD2800PMCKCAT. In co-transfection experiments, transcriptional activation of these reporters by wtp53 (expressed from pMSVwtp53) or mutant p53 (expressed from pMSV val135) was compared with activation of pD3300PMCKCAT (full promoter with both P- and D-elements), p2800PMCKCAT (P-element only) and p2800MCKCAT (no p53-binding sites). Consistent with previous findings [8,12] wt but not mutant p53 caused modest activation of p2800PMCKCAT (2.5-fold, Fig. 2B). High-level, synergistic activation was obtained with both pD3300PMCKCAT (14-fold) and pD2800PMCKCAT (17-fold). In contrast, we were unable to obtain any evidence for co-operation using pP2800PMCKCAT, rather activation dropped to levels observed for a single P-binding element (3-fold). In the absence of p53-binding elements neither wt or mutant p53 had any effect on the MCK promoter. Similar results were obtained in a heterologous promoter system in which P- and D-elements were cloned upstream of the thymidine kinase promoter in plasmid pBLCAT2 (data not shown). We conclude that MCK P-elements cannot co-operate to permit synergistic promoter activation by p53.

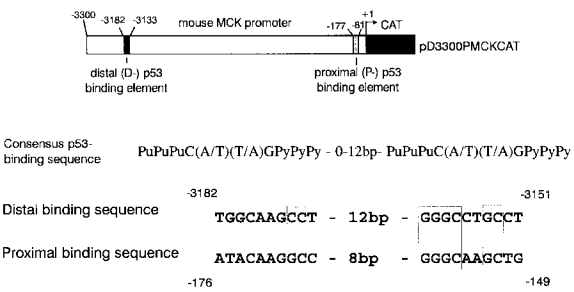

Fig. 1. Schematic illustration of the MCK promoter and MCK p53-binding elements. Numbers indicate nucleotide position relative to the transcription start site in the MCK promoter. Pu represents purine and Py, pyrimidine.

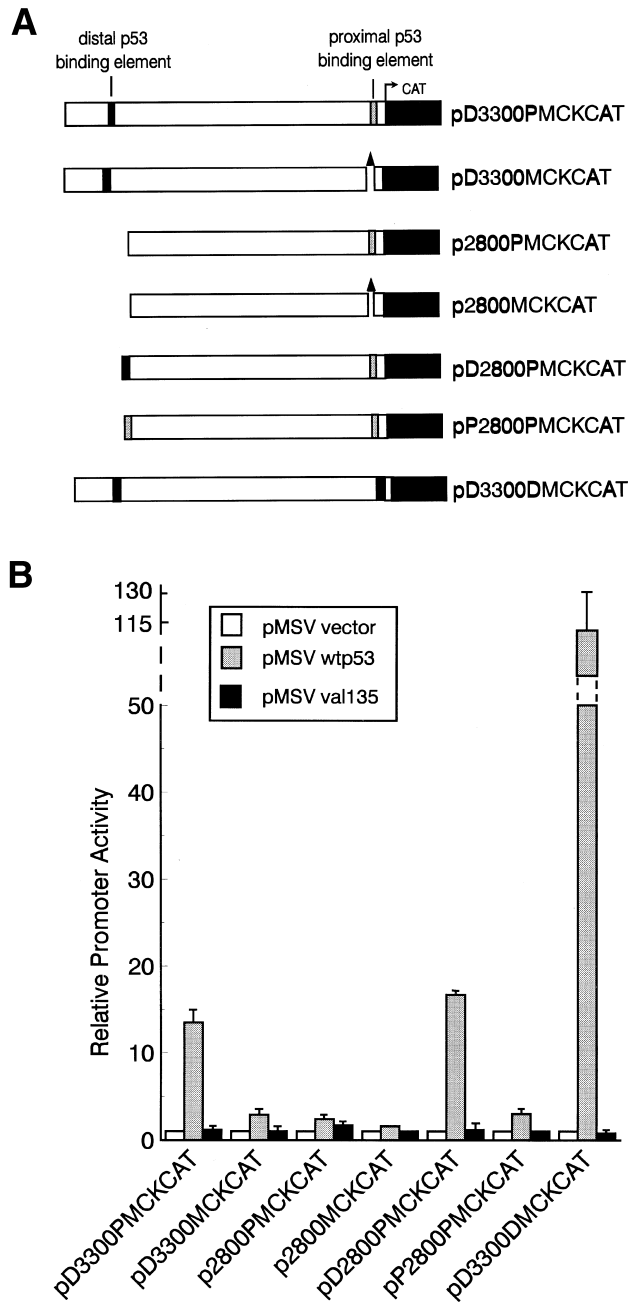

Fig. 2. Distinct proximal and distal MCK p53-binding elements modulate synergistic promoter activation by p53. A: Schematic illustration of MCK promoter reporter plasmids used. B: CV1 cells were transfected with 2 μg of reporter plasmid and 4 μg of either pMSV, pMSVwtp53 or pMSV val135. Results presented are the means+S.E. of at least three independent experiments.

3.2. *Failure of P-elements to co-operate is not restored by reducing promoter spacing between elements or inverting relative orientation*

In a previous study, we used a series of MCK reporter plasmids in which the intervening DNA sequence between D- and P-elements was gradually deleted to demonstrate that reducing this sequence from 3 kb (as in the full MCK promoter) to only 150 bp had no effect on synergy [12]. We used these plasmids as the basis to examine if a reduction in spacing between P-elements might be sufficient to restore synergistic activation. Thus reporter plasmids were constructed in which the spacing between P-elements varied between 2.65 kb and 150 bp (Table 1). As controls corresponding plasmids were used in which the D-element was present. Results summarised in Table 1 indicate that reducing spacing to 150 bp had no effect on co-operation between D- and P-elements. In contrast, co-operation between P-elements was not detected with any of the NE-reporter plasmids.

To determine if P-element orientation was important in co-operation, in a further series of experiments, we generated reporter plasmids in which we reversed the orientation of a distally located P-element relative to the normal P-element (prP2800PMCKCAT and prP1020PMCKCAT; Table 1). These plasmids also failed to demonstrate synergistic activation by wtp53 (Table 1). We conclude that the inability of two P-elements to co-operate cannot be restored by reducing promoter spacing or inverting relative element orientation and is due to the nature of the element.

3.3. *Enhanced co-operation between D-p53-binding elements*

To determine if MCK D-elements co-operate with each other, we constructed pD3300DMCKCAT in which the normal MCK P-element was replaced by a second D-element (Fig. 2A). Plasmid pD3300MCKCAT (D-element only) was used as a control. In agreement with previous findings [8,12], this reporter showed modest activation (4-fold) by wt but not mutant p53 (Fig. 2B). Somewhat surprisingly, pD3300-DMCKCAT was activated >100-fold, $\sim 8\times$ the synergistic level of activation of a normal MCK promoter in plasmid pD3300PMCKCAT (Fig. 2B). These data indicate that D-elements co-operate far more effectively than do P- and D-elements, and furthermore suggest that a specific combination of D- and P-elements in the MCK promoter is required for normal activation of this promoter by p53.

4. Discussion

Several of the genes which are cellular targets for transcriptional activation by p53, including the WAF1, cyclin G and MCK genes, contain multiple copies of the p53 consensus binding sequence in their control regions [8–10]. Although the significance of this feature is not yet clear, two recent reports using model promoters and the MCK promoter have suggested that multiple p53-binding sites co-operate to cause high level, synergistic activation of a target promoter by p53 [11,12].

We have extended these studies by examining the significance of distinct D- and P-p53-binding elements using the MCK promoter system. This promoter contains D- and P-p53-binding elements [8] (Fig. 1A). Within these elements are sequences with homology to the p53 consensus binding sequence. However, D- and P-sequences differ from each other at 9 out of 20 (45%) residues (Fig. 1). Using a series of MCK-reporter plasmids, we have confirmed that P- and D-elements co-operate even when the intervening promoter is reduced to as little as 150 bp (Table 1) [12]. In contrast, with plasmids containing only P-elements we were unable to detect co-operation (i) using a full-length promoter with two P-elements, (ii) using promoters in which the spacing between two P-elements was progressively deleted, (iii) using promoters where the relative orientations of two P-elements was reversed (Fig. 2B, Table 1). At present, we cannot for-

mally exclude the possibility that flanking sequences around the specific p53-binding sites within the P- (and D-) elements may be influencing their ability to co-operate or that placing P-element sequences out of context played a role in the failure to synergise. However, the fact that D- and P-elements co-operate in spite of large deletions in the intervening promoter sequence [12] and can also co-operate to synergistically activate a heterologous promoter [12], suggest that the simplest explanation for our data is that the P-element sequence does not permit co-operation between identical elements. In contrast to these findings, the MCK D-elements co-operate very effectively resulting in levels of activation by p53 which were more than 10-fold greater than those normally obtained between D- and P-elements (Fig. 2B). Thus the two MCK p53-binding elements vary markedly in their ability to co-operate and activate transcription. Together, these data suggest that having distinct D- and P-elements within the MCK promoter is functionally important for the promoter response to p53. In particular, we suggest that the MCK P-element limits the degree of activation by p53. We are presently trying to obtain further evidence in support of this proposal by examining the importance of sequence differences between p53-binding sites in the WAF1 and Cyclin G promoters.

Although the biochemical consequences of the sequence differences between D- and P-elements are not clear, they may well affect the binding of p53. Indeed there is accumulating evidence to suggest that wt, and indeed mutant, p53 proteins possess different affinities for different versions of the p53-binding sequence, which in turn can affect activation of promoters containing the different binding sequences (for example [15,16]). Moreover, gel shift assays using MCK D- and P-elements have indicated that p53 binds with somewhat higher affinity to the D-element compared with the P-element [8]. The importance of different p53-binding sites for synergy is not yet clear, however, binding of p53 to DNA is reported to cause profound DNA bending [17]. Conceivably, in conjunction with DNA looping [11,12], differential bending by p53 may permit p53 molecules bound to distant elements to correctly orientate for co-operation.

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