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Sequence-specific DNA binding by p53: identification of target sites and lack of binding to p53–MDM2 complexes

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An immune selection procedure was employed in order to isolate p53 binding sites from mouse genomic DNA. Two DNA clones capable of tight specific interaction with wild type p53 were subjected to further characterization. In both cases, the p53 binding regions displayed a high degree of sequence homology with the consensus binding site defined for human genomic DNA. One of the clones was found to be derived from the LTR of a retrovirus-like element (a member of the GLN family). The region encompassing the GLN LTR p53 binding site could confer p53 responsiveness upon a heterologous promoter. Furthermore, the expression of the endogenous, chromosomally integrated GLN elements was significantly induced upon activation of wild type p53 in cells harboring a temperature sensitive p53 mutant. Finally, it was demonstrated that p53–MDM2 complexes fail to bind tightly to such a p53 binding site. This may contribute to the inhibition by MDM2 of p53-mediated transcriptional activation.

Key words: retrovirus-like elements/transcriptional activation/tumor suppressors

Introduction

Inactivation of the p53 tumor suppressor gene plays a central role in the development of many types of cancer (reviewed in Hollstein *et al.*, 1991; Levine *et al.*, 1991; Vogelstein and Kinzler, 1992; Oren, 1992). In many of the affected tumors the p53 genes carry point mutations. Consequently, such tumor cells fail to express the wild type form of p53 (wt p53), producing instead aberrant versions of the protein. The biochemical activities of p53 have been the focus of many recent studies (reviewed in Montenarh, 1992; Oren, 1992; Vogelstein and Kinzler, 1992). Such studies have revealed that wt p53 can interact specifically with defined DNA sequences, and that this property is usually abrogated by the mutations encountered in tumor cells (Bargonetti *et al.*, 1991; Kern *et al.*, 1991; El-Deiry *et al.*, 1992; Funk *et al.*, 1992; Hupp *et al.*, 1992). Combined with the presence of a potent transcriptional activation domain in p53 (Fields and Yang, 1990; O'Rourke *et al.*, 1990; Raycroft *et al.*, 1990), this suggested that p53 may act as a sequence-specific transcription factor. In agreement with this notion, it was indeed found that the introduction of one or more copies of a p53 binding site in front of a promoter could make it inducible by p53 (Farmer *et al.*, 1992; Funk *et al.*, 1992; Kern *et al.*, 1992; Scharer and Iggo, 1992; Zambetti *et al.*, 1992).

Recently, p53 was found to form specific complexes with a cellular polypeptide of 90–95 kDa apparent molecular weight, subsequently identified as the MDM2 protein (Barak and Oren, 1992; Momand *et al.*, 1992; Oliner *et al.*, 1992; Barak *et al.*, 1993). Overexpression of MDM2 can interfere with p53-dependent transcriptional activation (Momand *et al.*, 1992), suggesting that at least some p53 functions are impaired when it associates with MDM2. In line with this conjecture, amplification of the *mdm2* gene in certain tumors may eliminate the requirement for structural changes in their p53 genes (Oliner *et al.*, 1992).

In an attempt to identify additional p53 binding sequences and to explore their relevance to p53-mediated transcriptional activation, we applied an immune selection procedure to a mouse genomic DNA library. This led to the isolation of two novel high affinity p53 binding sites. One of these p53 binding sites resides within the LTR of an endogenous retrovirus-like element (GLN LTR; Itin and Keshet, 1986). The GLN LTR-derived site allows promoters to be positively regulated by wt but not mutant mouse p53. In support of the relevance of this observation, the expression of endogenous GLN elements was augmented in cells with high wt p53 activity. When one of the novel p53 binding sequences was used as a target, p53–MDM2 complexes failed to exhibit any detectable sequence-specific DNA binding. The latter finding may contribute to the ability of overexpressed MDM2 to block the biochemical and biological effects of wt p53.

Results

Isolation of p53 binding sequences from mouse genomic DNA

In order to isolate mouse DNA elements capable of sequence-specific interaction with wt p53, a library of small genomic DNA fragments was prepared in a plasmid vector (see Materials and methods). A mixture of such genomic DNA clones was subjected to three consecutive cycles of immune selection with a wt p53-rich cell extract and a combination of three p53-specific monoclonal antibodies. The extract was prepared from cells of Clone 6, overexpressing the temperature sensitive (ts) mouse p53 mutant p53val135 (Pinhasi-Kimhi *et al.*, 1986; Michalovitz *et al.*, 1990). The cells were maintained at 32°C for 24 h prior to the preparation of the extracts, to induce the accumulation of wt-like p53 protein. This extract was used for the selection of clones from the library. Control extracts containing comparable amounts of p53val135 in the mutant conformation were prepared from parallel cultures maintained at 37–38°C.

After the third selection cycle, the library contained ~4 × 10^5 clones. DNA was prepared from 30 individual clones, radiolabeled and subjected to a p53 binding assay essentially as done during the enrichment process. Of the 30

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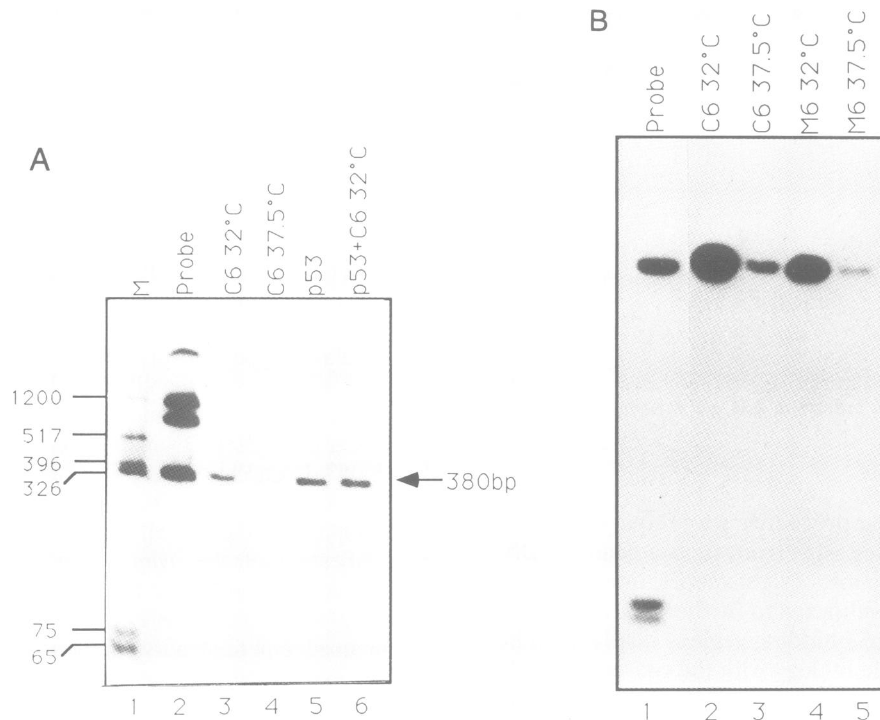

Fig. 1. (**A**) Binding of wt p53 to clone 15 DNA. Clone 15 DNA was digested with *Dra*I and *Eco*RI. The digested DNA was end-labeled with [^{32}P]dATP by the Klenow enzyme, and reacted with p53 of various sources, followed by immunoprecipitation with a mixture of the three p53-specific monoclonal antibodies, PAb248, PAb421 and RA3-2C2. Following elution from the immunoprecipitate, bound DNA was electrophoresed through a 5% non-denaturing polyacrylamide gel. The gel was dried and exposed for 4 h to X-ray film. Lane 1, radiolabeled marker (pBluescript SK DNA digested with *Hinf*I); lane 2, nonreacted radiolabeled DNA digest, equivalent to 5% of the amount taken for a binding reaction; lane 3, Clone 6 extract, 32°C (100 μg total protein); lane 4, Clone 6 extract, 37.5°C (100 μg total protein); lane 5, purified p53 generated in a baculovirus expression system (100 ng); lane 6, mixture of Clone 6 extract, 32°C (100 μg total protein) and purified baculovirus p53 (100 ng). (**B**) Binding of wt p53 to clone 255 DNA. Clone 255 DNA was digested with a combination of *Spe*I, *Nco*I and *Hin*dIII, and end-labeled with [^{32}P]dCTP and [^{32}P]dATP. Following incubation with various cell extracts, the DNA–protein complexes were precipitated essentially as described in (A), except that the eluted radiolabeled DNA was resolved on a 5% denaturing gel. Only the bottom part of the gel, containing a 62 bp fragment and a 30 bp fragment (both derived from the genomic DNA insert) is shown; the upper part, containing the plasmid vector DNA, has been cut out owing to the large size of the gel. The two strands of the 30 bp fragment were resolved on this denaturing gel into two distinct bands, reflecting the fact that the restriction enzyme cleavage left non-identical numbers of nucleotides on each strand. Lane 1, nonreacted radiolabeled DNA digest, equivalent to 2% of the amount taken for a binding reaction; lane 2, Clone 6 extract, 32°C (100 μg total protein); lane 3, Clone 6 extract, 37.5°C (100 μg total protein); lane 4, MCO1/cG9-6, 32°C (100 μg total protein); lane 5, MCO1/cG9-6, 37.5°C (100 μg total protein). EMBL database accession numbers for the clones are: Clone 15, X72083; Clone 255, X72084.

clones, five scored positive in this assay. Cross-hybridization experiments with DNA inserts from positive clones revealed that all five fell into a single cross-hybridizing group, which had apparently been strongly selected for in the library. As individual members of this group were found to carry inserts of various sizes (data not shown), it was concluded that they were probably derived from a multicopy gene family harboring a high affinity p53 binding site. A representative of this group, clone 15, which exhibited tight binding to p53, was taken for further analysis. Subsequently, we picked from the third cycle library 50 additional clones which did not cross-hybridize with the clone 15 insert. All 50 clones were subjected to a p53 binding assay, and three were found to be strongly positive. Of those, clone 255 was studied further.

Figure 1 depicts the behavior of these clones 15 and 255 in a p53 binding assay. DNA of clone 15 was digested with appropriate restriction enzymes, end-labeled and reacted with an extract of Clone 6 cells maintained at 32°C. Upon incubation with a mixture of p53-specific antibodies, a 380 bp fragment was specifically immunoprecipitated (Figure 1A, lane 3), indicating that this fragment contained a p53 binding site. Significantly less DNA was brought down by a similar amount of extract from Clone 6 cells grown at 37.5°C (lane 4), where the majority of the p53 protein is in the mutant conformation (Michalovitz *et al.*, 1990; Martinez *et al.*, 1991). The same fragment was also brought down specifically with highly purified wt mouse p53 generated in a baculovirus expression system (Figure 1A, lane 5).

When a similar reaction was carried out with plasmid clone 255 (Figure 1B), a 62 bp *Spe*I–*Nco*I fragment was specifically brought down (in this experiment the DNA was resolved on a long denaturing gel, of which only the bottom part is depicted). Again, the reaction was much stronger when the extract was prepared from Clone 6 cells maintained at 32°C rather than at 37.5°C (Figure 1B, lanes 2 and 3 respectively). A practically identical result was obtained with extracts of p53-deficient mouse cells stably transfected with the same ts p53 mutant (lanes 4 and 5). It is noteworthy that a 30 bp fragment derived from the same plasmid (bottom part of lane 1) was not brought down at all by wt p53, attesting to the high specificity of the reaction with the upper 62 bp fragment.

Next, the mouse genomic DNA inserts of clones 15 and 255 were subjected to DNA sequence determination (Figure 2). Clone 15 was found to represent a fusion of sequences derived from two distinct mouse repetitive DNA families (Figure 2A). The 5′ region of the insert corresponded to part of a long interspersed repeated DNA element (LINE

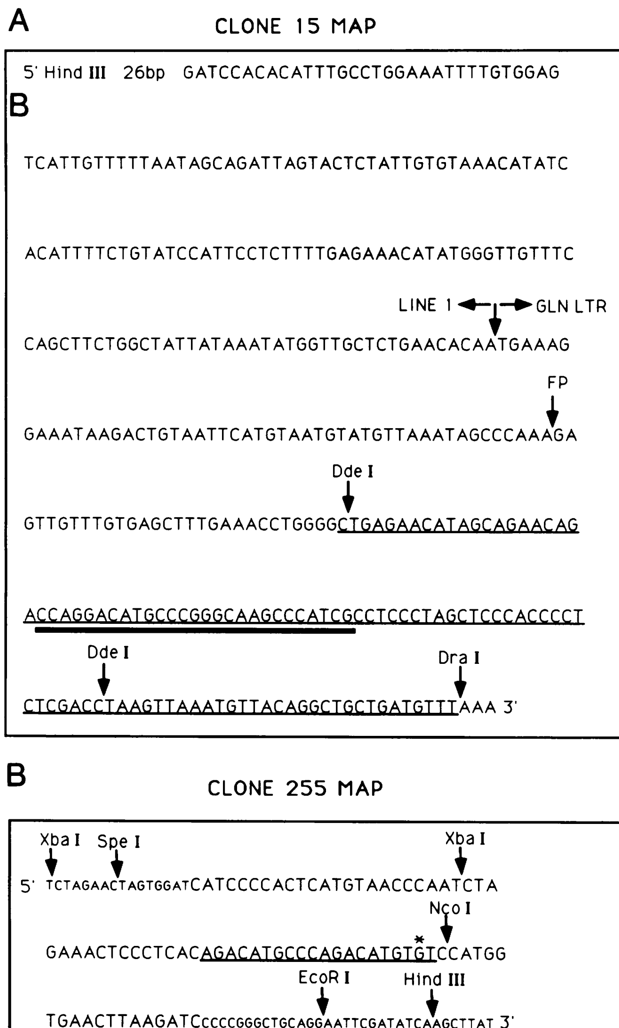

Fig. 2. Nucleotide sequences of DNA fragments containing p53 binding sites. (**A**) Clone 15. The *Hin*dIII site at the 5′ end, as well as the adjacent 26 bp stretch, are derived from the plasmid (pBluescript) polylinker. The underlined region (extending down to the *Dra*I site) corresponds to the 105 bp fragment employed for the construction of plasmid 100/T-CAT (see Figure 6A). The p53 binding site, as defined by DNase I footprinting (Figure 4), is doubly underlined. FP refers to the 5′ end of the fragment subjected to DNase I footprinting in Figure 4. This end was generated by Bal31 deletion of clone 15 DNA (data not shown). The 140 bp fragment used in Figure 4 was excised by cleavage at the *Dra*I site located at the 3′ of the sequence and at an *Hin*dIII site located in the polylinker adjacent to the site marked FP. The junction between LINE 1 and GLN LTR sequences is also indicated. (**B**) Clone 255. The region represented in smaller letters at the 5′ end, including the *Xba*I and *Spe*I sites, is from the polylinker. The 15 bp stretch and the 12 bp stretch indicated at the 3′ end are also from the polylinker, as are the pertinent *Eco*RI and *Hin*dIII sites. The underlined region indicates the 19/20 bp homology with the p53 binding consensus sequence of El-Deiry *et al.* (1992); the single mismatch with the consensus is indicated by an asterisk.

1; Berg and Howe, 1989), whereas the rest of the insert was highly homologous (98%) to a previously described member of the GLN family of retrovirus-like elements (Itin and Keshet, 1986). Most notably, the region contained within this fragment corresponded to the LTR of the GLN element (GLN LTR). The fusion between the two repetitive elements may have been achieved through the integration of the GLN element cDNA into the genomic LINE 1 DNA. Further analysis (see below) demonstrated that the p53 binding site resided within the GLN LTR portion of clone 15. No meaningful homology was found between the insert of clone 255 (Figure 2B) and any previously described DNA sequence.

Interactions between p53 and its DNA binding sites

The short size of the p53 binding DNA fragment of clone 255 made it a convenient target for electrophoretic mobility shift assays. An 82 bp *Xba*I–*Hin*dIII DNA band was radiolabeled and reacted with purified wt p53 generated in a baculovirus expression system (Figure 3A). This manipulation resulted in a prominent mobility shift (lane 1, band B); in addition, a much fainter, more slowly migrating complex could also be observed (band C). To establish that p53 was indeed a component of the shifted complexes, a similar analysis was performed in the presence of p53-specific antibodies. When a mixture of three monoclonal antibodies was added to the reaction after the DNA–protein complexes had been allowed to assemble, all the complexed DNA was supershifted into a more slowly migrating form (lane 3, band D). Hence, the complex formed with clone 255 DNA contains p53. Given the high degree of purity of the p53 protein in these preparations (Ragimov *et al.*, 1993), it appears most likely that p53 itself is directly responsible for the binding to this DNA target. When the antibody mixture was added to the p53 protein before the radiolabeled DNA probe (lane 2), the intensity of the supershifted band was reduced (a more pronounced effect is evident in Figure 3B, lanes 2 and 3). This particular combination of antibodies thus appears to interfere partially with the formation of DNA–p53 complexes, though not with the retention of pre-formed complexes.

In lanes 2 and 3, a mixture of three p53-specific monoclonal antibodies was employed. We next assessed the relative contribution of the individual antibodies to the observed supershift. PAb248 (lane 5) and RA3-2C2 (lane 6) each induced an efficient supershift. With RA3-2C2, all the supershifted complex was trapped in the gel origin, suggestive of a high molecular weight complex. This observation is consistent with the fact that RA3-2C2 is an IgM antibody (Coffman and Weissman, 1981), whereas the other monoclonal antibodies used in this study are IgGs. PAb421 failed to elicit any supershift (lane 4). The latter result was not surprising, as the baculovirus p53 used in this experiment contained a high concentration of the PAb421 epitope peptide, employed for the elution of p53 from the immunoaffinity column (Ragimov *et al.*, 1993).

A similar mobility shift experiment was also performed with extracts from Clone 6 cells maintained at 32°C (Figure 3B). In the absence of any added antibodies, a shifted band was observed whose position was indistinguishable from that of band B (see Figure 3A) obtained with purified p53 (data not shown). In addition, we observed a set of more rapidly migrating bands (N), whose identity has not been further investigated. The mixture of p53-specific antibodies again supershifted the complex very effectively (Figure 3B, lane 3), while strongly interfering with its formation when added to the extract before the DNA (lane 2). When each of the monoclonal antibodies was applied separately, the effects of PAb248 and RA3-2C2 (lanes 5 and 6) were similar to those observed in Figure 3A. Unlike the baculovirus-derived p53 preparation, Clone 6 extracts do not contain any cognate PAb421 epitope peptide that can interfere with the binding of this antibody. Under those conditions, incubation with PAb421 led to a marked increase in the total amount of

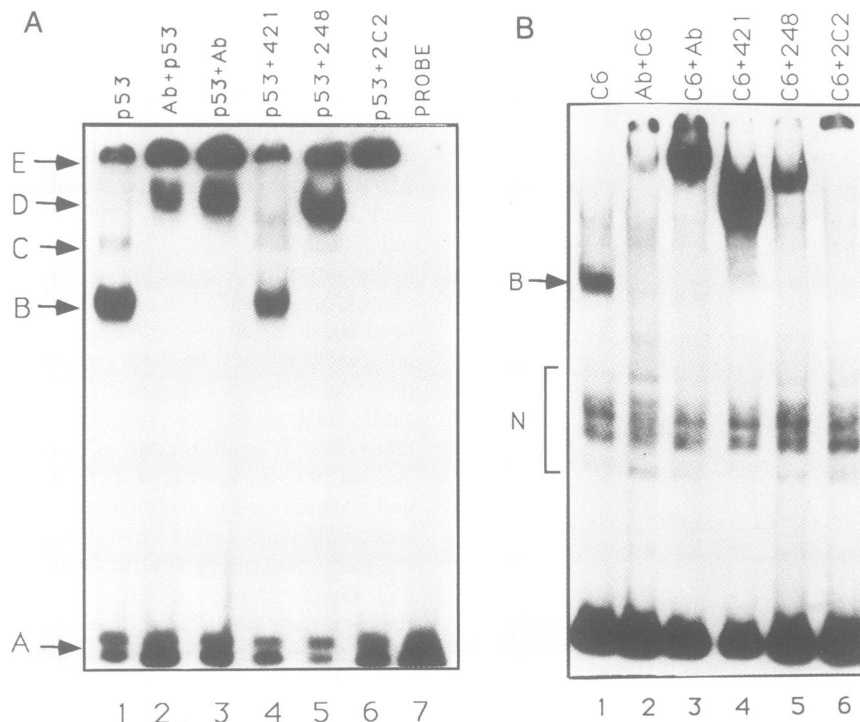

Fig. 3. (**A**) Electrophoretic mobility shift analysis of clone 255 DNA in the presence of purified wt p53. Clone 255 DNA was digested with *Xba*I and *Hin*dIII. An 82 bp fragment, extending from the internal *Xba*I site down to the *Hin*dIII site of the polylinker (see Figure 2B) was isolated from a non-denaturing polyacrylamide gel. The fragment was end-labeled with [^{32}P]dATP + [^{32}P]dCTP. Equal aliquots (50 000 c.p.m.) were reacted with purified baculovirus-expressed wt mouse p53 (100 ng per reaction), without or with various p53-specific monoclonal antibodies. Reactions in lanes 2 and 3 contained a combination of the three p53-specific antibodies, PAb248, PAb421 and RA3-2C2. In lane 2, the antibodies were incubated with the purified p53 before addition of the radiolabeled probe, whereas in lane 3 the antibodies were added only after the protein and probe had been incubated together for 20 min at 0°C. In lanes 4–6, details were as in lane 3 except that a single monoclonal antibody (indicated above the lane) was used in each reaction. Subsequently, the DNA–protein mixtures were resolved on a 5% non-denaturing polyacrylamide gel. Lane 7 contained probe only. The unshifted probe (A), as well as shifted bands of various electrophoretic mobilities (B–E) are indicated. (**B**) Gel mobility shift analysis of clone 255 DNA in the presence of wt p53-rich cell extract. The radiolabeled probe was prepared as in panel A, and reacted with Clone 6 extract, 32°C (100 μg total protein) with or without added antibodies. In lane 1, the probe was incubated with extract only (20 min at 0°C). In lane 2, the extract was incubated with a mixture of p53-specific monoclonal antibodies (PAb248, PAb421 and RA3-2C2) for 20 min at room temperature plus 15 min at 0°C; the probe was then added, followed by incubation for 20 min at 0°C. In lane 3, the probe was first incubated with extract for 20 min at 0°C; subsequently, the antibody mixture was added and incubation continued for 35 min at room temperature. In lanes 4–6 the details were as for lane 3, except that a single antibody (PAb421, PAb248 or RA3-2C2, respectively) was used rather than a mixture.

radiolabeled probe retained in specific DNA–protein complexes (compare lanes 1 and 4, Figure 3B; see also Figure 5). Thus, PAb421 has a pronounced positive effect on the efficiency of complex formation between clone 255 DNA and p53. The effect of PAb421 could reflect either a stabilization of pre-formed complexes or an enhancement of p53–DNA interactions, presumably through some conformational alteration of the p53 protein. Similar findings have been reported by Funk *et al.* (1992) and Hupp *et al.* (1992), using different DNA targets.

We next attempted to determine more precisely the sequence of the new p53 binding site residing in clone 15. As a first step, Bal31 deletion analysis was employed in order to narrow down the borders of the p53 binding site to within a 140 bp fragment (data not shown). This fragment was then subjected to DNase I footprinting analysis (Figure 4). In the presence of purified baculovirus-derived wt p53, a 27 bp stretch was clearly protected from digestion (lane 3). The sequence of this region, shown on the right, matched at 20 out of 20 positions the p53 consensus binding site proposed by El-Deiry *et al.* (1992). Thus, the p53 binding site of clone 15, originating in mouse DNA, is practically indistinguishable from analogous sites on human DNA. Similarly, clone 255 contains a stretch of nucleotides that matches the El-Deiry consensus in 19 out of 20 positions (see Figure 2B). This suggests that the structural motifs conferring DNA binding specificity are probably highly conserved between mouse and human p53.

To determine whether different putative p53 binding DNA elements vary with respect to their ability to form tight DNA–protein complexes, we compared the performance of sequences from clone 15, clone 255 and the ribosomal gene cluster (RGC) site described by Kern *et al.* (1991). The three target sites were subjected to a gel mobility shift assay with extracts of Clone 6, prepared from cells maintained at either 32 or 37.5°C. As seen in Figure 5, the various DNA targets differed considerably with regard to their ability to form stable interactions with wt p53. Whereas both clone 255 and clone 15 could be easily shifted by p53, no mobility shift of the RGC site oligonucleotide was observed under these experimental conditions (compare lanes 4 and 12 with lane 8). The clone 15 site appeared to possess the tightest interaction with p53, as reflected by its ability to be shifted very efficiently even in the absence of added antibodies (lane 12). Addition of the monoclonal antibody mixture enhanced the interaction between p53 and the clone 255 probe (lane 5), presumably reflecting the effect of PAb421; yet, in our hands it was insufficient to elicit a detectable supershift with the RGC probe

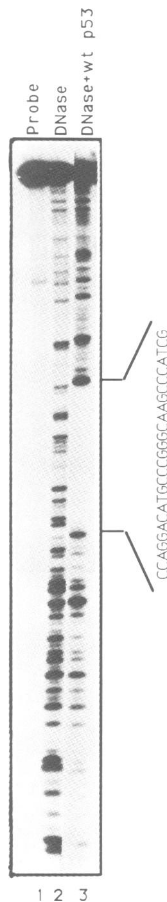

Fig. 4. DNase I footprinting analysis of p53–DNA interaction. A 140 bp *Dra*I–*Hin*dIII fragment, derived from the GLN LTR region of clone 15, was end-labeled with [^{32}P]dATP. Equal amounts of the probe (30 000 c.p.m.) were incubated without (lanes 1 and 2) or with (lane 3) purified baculovirus-expressed wt mouse p53 (1 μg), as detailed under Materials and methods. At the end of the incubation period, the samples in lanes 2 and 3 were subjected to digestion with pancreatic DNase I. The digested DNA was resolved on a 5% denaturing polyacrylamide gel. The sequence of the protected region in lane 3 is indicated to the right of the autoradiogram.

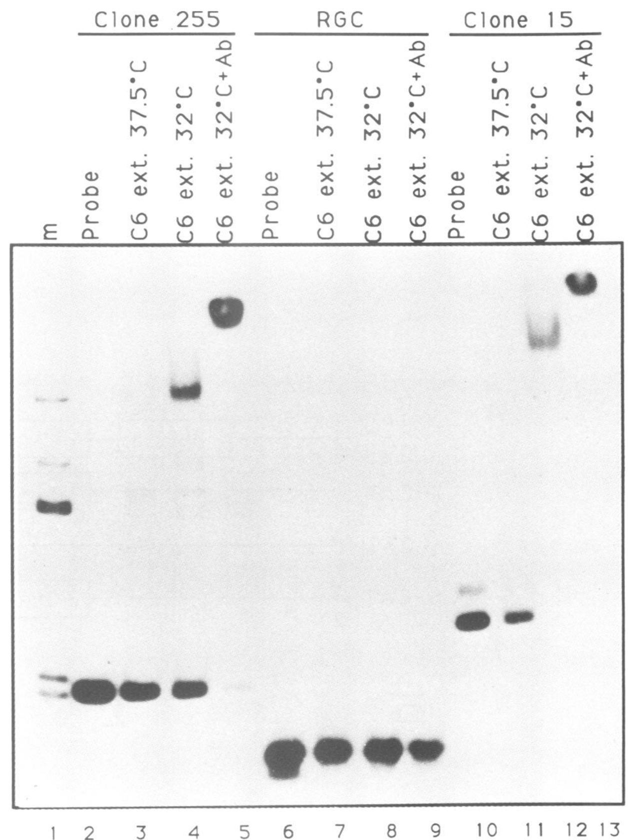

Fig. 5. Comparative gel mobility shift analysis of various p53 binding sites. Three different DNA fragments containing p53 binding sites (indicated at the top of the figure) were end-labeled with [^{32}P]dATP and subjected to a gel mobility shift assay essentially as described in Figure 3. The labeled fragments were as follows. Clone 255, a 70 bp fragment, extending from the internal *Xba*I site to the *Eco*RI site of the polylinker (see Figure 2B). Clone 15, a 105 bp *Dra*I–*Hin*dIII fragment of the GLN LTR; this fragment is identical to the region underlined in Figure 2A, and the *Hin*dIII site is from the plasmid polylinker into which the DNA was ligated following Bal31 deletion. RGC, a ribosomal gene cluster (Kern *et al.*, 1991) synthetic double-stranded oligonucleotide. Cell extracts were from Clone 6 cells maintained at either 32 or 37.5°C (100 μg per reaction). In lanes 3, 4, 7, 8, 11 and 12 the probe was incubated with the indicated cell extract for 20 min at 0°C prior to loading onto the gel. In lanes 5, 9 and 13, the probe was first incubated with extract for 20 min at 0°C; subsequently, a mixture of PAb248, PAb421 and RA3-2C2 was added and incubation continued for 20 min at room temperature. Lanes denoted 'Probe' represent 2% of the amount of probe taken for each pertinent binding assay. m, marker (pBluescript SK DNA digested with *Hin*fI).

(lane 9). It is noteworthy that the same RGC probe could still form detectable complexes with wt p53 when assayed by a different procedure, involving immune selection (Shaulian *et al.*, 1992). This is probably due to the different stringencies of the two procedures.

A p53 binding site can confer p53-dependent transcriptional activation

Insertion of p53 binding sites upstream of various promoters was previously shown to confer upon them the ability to be transactivated by wt p53 (Farmer *et al.*, 1992; Funk *et al.*, 1992; Kern *et al.*, 1992; Scharer and Iggo, 1992; Zambetti *et al.*, 1992). The GLN LTR is a naturally occurring transcriptional control element containing a p53 binding site. Moreover, the p53 binding site is located 116 bp upstream of the TATAA box (data not shown), in a region which by analogy with other retroviral LTRs is probably part of the GLN LTR enhancer. It was therefore of interest to determine whether this site, too, could confer p53-responsiveness. As shown in Figure 6, this was indeed found to be the case. Three copies of a 105 bp fragment of the GLN LTR, encompassing the p53 binding site (underlined in Figure 2A) were placed in front of an enhancerless SV40 early promoter (plasmid 100/T-CAT, Figure 6A). When transfected into p53-deficient mouse fibroblasts, this chimeric transcriptional control element was strongly stimulated by wt p53 (Figure 6B, lane 1); two different tumor-derived p53 mutants, p53phe132 (Figure 6B, lane 2) and p53cys270 (lane 3), failed to exhibit a similar stimulation. The positive effect of wt p53 was specific; when the c-*fos* promoter was assayed under the same conditions, it was actually repressed by wt p53; here, too, p53phe132 had no effect (Figure 6B, lanes 5–7). This is in agreement with earlier reports (Ginsberg *et al.*, 1991; Santhanam *et al.*, 1991; Lechner *et al.*, 1992).

Essentially identical results were obtained when plasmid 100/T-CAT was assayed in p53-deficient human Saos-2 osteosarcoma cells (Figure 6C). In this case, p53 expression was driven by the CMV immediate early enhancer/promoter.

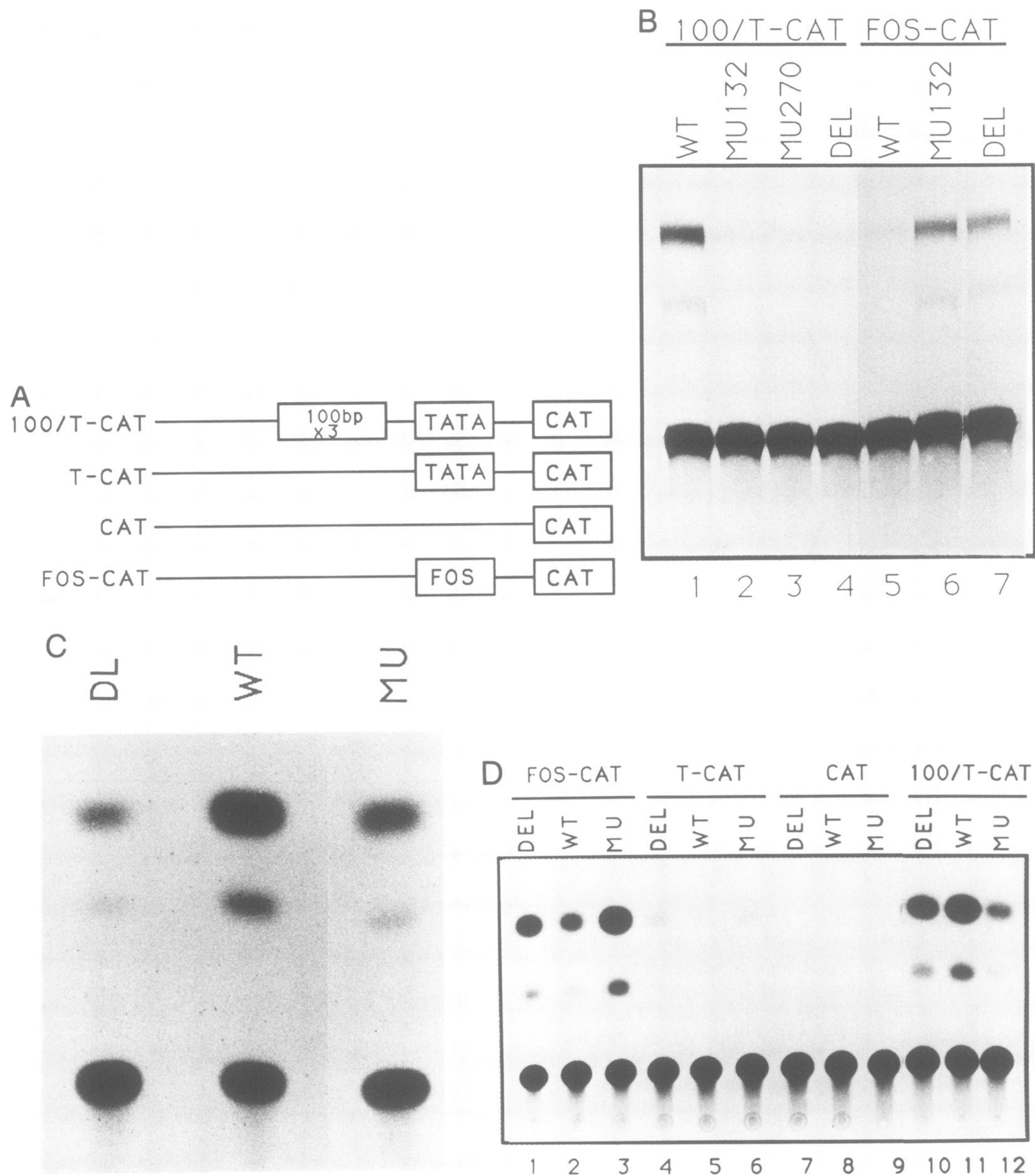

Fig. 6. Transcriptional modulation by the GLN LTR p53 binding region in the presence of wt and mutant p53. (**A**) Schematic structures of reporter plasmids. In 100/T-CAT, three tandem copies of the 105 bp region indicated in Figure 2A (underlined) were placed in front of an enhancerless SV40 early region promoter. T-CAT contains the SV40 early region promoter inserted upstream of the CAT gene. CAT contains the CAT gene without any transcriptional control elements. FOS-CAT contains the human c-*fos* gene promoter (Ginsberg *et al.*, 1991). (**B**) Transcriptional effects of p53 in p53-deficient mouse fibroblasts. Clone 314 fibroblasts, established from a p53 double knock-out mouse embryo (Donehower *et al.*, 1992), were transfected with a combination of the indicated CAT reporter plasmid (4 μg) and various p53 expression vectors (12 μg). Cell extracts were prepared 48 h later, and subjected to CAT analysis. The autoradiogram of the TLC plate is shown. WT, pLTRp53cGwt (Michalovitz *et al.*, 1990); MU132, pLTRp53cGphe132 (Michalovitz *et al.*, 1990); MU270, pLTRp53cGcys270 (Halevy *et al.*, 1990); DEL, pLTRp53dl, a derivative of pLTRcGwt from which the bulk of the p53 coding region has been deleted (Michalovitz *et al.*, 1990). (**C**) Transcriptional effects of p53 in p53-deficient human osteosarcoma cells. Saos-2 cells were transfected with combinations of various p53 expression vectors (0.1 μg per dish) and plasmid 100/T-CAT (2.5 μg per 60 mm dish). Cell extracts were prepared 46 h later and subjected to CAT analysis. WT, pCMVp53wt; DL, pCMVp53dl, a derivative of pCMVp53wt from which the bulk of the p53 coding region has been deleted; MU, pCMVp53m, expressing a double mutant p53 protein. All three p53 plasmids were described in Eliyahu *et al.* (1989). (**D**) Transcriptional effects of p53 in cells expressing endogenous wt p53. Low passage non-established rat embryo fibroblasts were transfected with the indicated reporter plasmids (see A) together with various p53 expression vectors essentially as in Figure 6B, extracted 48 h later and subjected to CAT analysis. The autoradiogram is shown.

Once again, wt p53 could strongly activate expression of a reporter gene driven by three repeats of the GLN LTR p53 binding region.

A similar experiment was carried out in primary rat embryo fibroblasts (REF), which express physiological levels of endogenous wt p53. Again, the coexpression of transfected wt p53 stimulated the activity of 100/T-CAT (Figure 6D, compare lanes 10 and 11), but the fold induction was less than in p53-deficient cells. Unlike in p53-deficient cells, p53phe132 actually reduced expression from 100/T-CAT in REF (lane 12). The differences between the two cell types most probably stem from the ability of the endogenous REF wt p53 to induce a significant basal level of transcription from this promoter, and of the cotransfected mutant p53 to compromise this basal transcription, presumably through a negative dominant mechanism (Farmer *et al.*, 1992; Kern

et al., 1992; Shaulian *et al.*, 1992). The c-*fos* promoter was repressed by cotransfected wt p53 in REF, but not as strongly as in p53-deficient cells (Figure 6D, lanes 1 and 2); in this experiment, mutant p53 actually had a stimulatory effect on the c-*fos* promoter in REF (lane 3). Finally, no significant activity was exhibited by either the promoterless CAT gene (CAT, lanes 7–9) or by the enhancerless SV40 early promoter (T-CAT, lanes 4–6) under the same experimental conditions. Hence, GLN LTR-derived p53 binding sites can act as efficient targets for p53-mediated transcriptional activation.

The expression of endogenous GLN elements is stimulated by wt p53 activity

In the transfection experiments discussed in the previous section, as well as in many earlier reports, the p53 binding sites were placed in a very artificial context. One could therefore question whether their response truly reflected the behavior of authentic endogenous chromosomal p53 targets. Having a p53 binding site within a well defined, natural transcriptional control element, offered the advantage of being able to test directly whether the corresponding unmanipulated genes were also p53-responsive. To that end, RNA was extracted from LTR-6 cells, which are M1 mouse myeloid leukemic cells stably transfected with the ts p53 mutant p53val135 (Yonish-Rouach *et al.*, 1991) and subjected to hybridization with a probe specific for GLN element transcripts. The full length transcripts of intact GLN elements are expected to be ~8 kb long (Keshet *et al.*, 1990). As can be seen in Figure 7, such transcripts were indeed present in M1-derived cells. In cells maintained at 37.5°C, where the ts p53 is in its mutant conformation (Michalovitz *et al.*, 1990), the levels of these transcripts were relatively low (first lane). On the other hand, the induction of wt p53 activity upon shift down to 32°C resulted in a several-fold increase in the amounts of GLN element RNA. The increase was clearly evident within 5 h after the temperature shift and continued at later times. No increase in GLN RNA was seen when similar cells expressing the non-ts p53 mutant p53phe132 (Yonish-Rouach *et al.*, 1991) were shifted to 32°C (data not shown). Hence, the induction of GLN element gene expression correlated with the activation of wt p53. These findings demonstrate that wt p53 can transactivate potential target genes also within their natural chromosomal context, and further support the contention that wt p53 is indeed an effective transcriptional activator.

p53–MDM2 complexes fail to bind tightly to p53 target DNA

The p53 protein can form specific complexes with the product of the *mdm2* gene (Momand *et al.*, 1992; Oliner *et al.*, 1992; Barak *et al.*, 1993). This interaction can abrogate the ability of wt p53 to transactivate promoters carrying a p53 binding site (Momand *et al.*, 1992). To explore the basis for the inhibitory effect of MDM2 on the activity of wt p53, we asked whether p53—MDM2 complexes retained efficient binding to a cognate DNA target. To that end, advantage was taken of the fact that in Clone 6, as well as in other lines of similar background, the induction of wt p53 activity at 32°C leads to a rapid increase in the cellular levels of MDM2 mRNA and protein (Barak *et al.*, 1993). Consequently, these cells contain substantial amounts of p53—MDM2 complexes at the permissive temperature (Barak and Oren, 1992; Barak *et al.*, 1993).

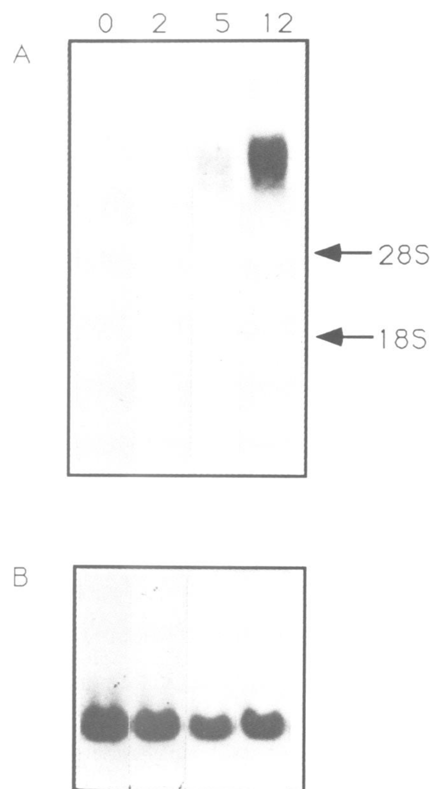

Fig. 7. Induction of GLN element transcripts by wt p53. Cells of line LTR-6, derived by transfection of p53-negative M1 myeloid leukemic cells with the ts p53 mutant p53val135 (Yonish-Rouach *et al.*, 1991; data not shown) were shifted down to 32°C for the time periods (in hours) specified above each lane. RNA was extracted and 10 μg of each sample was electrophoresed on a formaldehyde–agarose gel. Following transfer on to a nitrocellulose membrane, the blot was hybridized sequentially with a probe containing the entire GLN LTR (480 bp *Spe*I–*Hin*dIII fragment derived from clone 15; panel A) and with a rat β-actin cDNA probe (panel B).

A radiolabeled DNA fragment derived from clone 255 was incubated with portions of an extract from Clone 6 cells maintained at 32°C. DNA–protein complexes were brought down with either a mixture of the p53-specific monoclonal antibodies PAb248 and RA3-2C2, or an MDM2-specific rabbit polyclonal serum. The use of PAb421 as a p53-specific monoclonal antibody was deliberately avoided, owing to the potential bias that its positive effect on DNA binding (see Figure 3B) might have introduced into the assay. Half of each immunoprecipitate was subjected to DNA gel analysis as in Figure 1 (Figure 8A, top panel), while the other half of the same sample was processed for the quantitation of p53 protein by Western blotting (Figure 8A, bottom panel). When protein–DNA complexes were collected with p53-specific antibodies, the amount of specifically precipitated target DNA correlated well with the amount of p53 in the extract (Figure 8A, lanes 1–5). On the other hand, while substantial amounts of p53 were also precipitated by the anti-MDM2 antibodies (Figure 8A, bottom panel, lanes 6 and 7), this did not result in any detectable coprecipitation of clone 255 target DNA (Figure 8A, top panel, lanes 6 and 7). This was not due to an adverse effect of the MDM2 antiserum on sequence-specific DNA binding, as addition of this antiserum to a mixture of p53-specific monoclonal antibodies did not interfere with the ability of the latter to precipitate the expected amount of target DNA (compare lanes 2 and 9, Figure 8A, top panel).

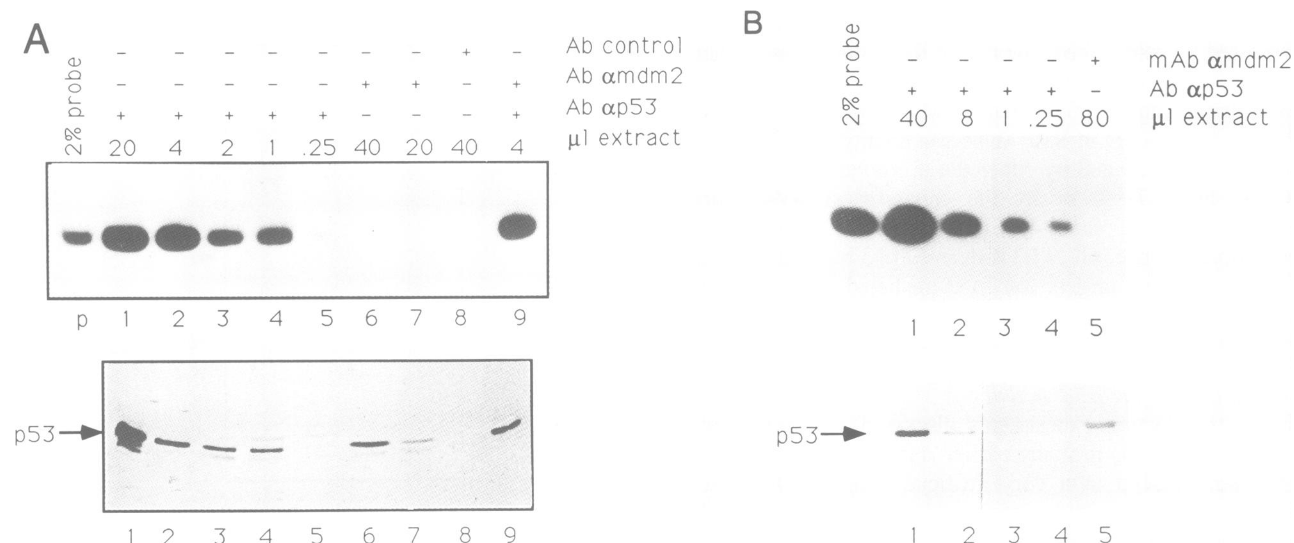

Fig. 8. Complexes between p53 and MDM2 fail to bind detectably to a p53 DNA target. (**A**) Analysis with polyclonal anti-MDM2 serum. Clone 255 DNA was digested with *Xba*I and *Eco*RI. A 70 bp fragment, extending from the internal *Xba*I site down to the *Eco*RI site of the polylinker (see Figure 2B) was isolated from a non-denaturing polyacrylamide gel. The fragment was end-labeled with [^{32}P]dATP and equal amounts of the probe (2×10^5 c.p.m. per reaction) were incubated with various amounts of a Clone 6, 32°C extract, as indicated (in μl) above each lane. The protein concentration in the extract was 12 mg/ml. Incubation was under the same conditions as in Figure 1, except that $ZnSO_4$ was included at a final concentration of 2 μM. Following incubation on ice for 60 min, the indicated antibodies (a mixture of PAb248 and RA3-2C2 or a polyclonal anti-MDM2 serum, each at a total amount of 4 μg) were added, and incubation was continued for an additional 30 min on ice. In lane 8, preimmune rather than immune rabbit serum was used. Immune complexes were collected on protein A–Sepharose beads. Half of each bead suspension was treated with SDS to release bound DNA, which was then electrophoresed through a 5% non-denaturing polyacrylamide gel (top panel). The other half of the suspension was mixed with protein sample buffer and boiled. The released polypeptides were subjected to electrophoresis on a 10% SDS–polyacrylamide gel, followed by Western blotting. The p53 proteins in each lane were detected by hybridizing the blot with a mixture of PAb421 and PAb248. The reaction was developed with the ECL reagents (Amersham), using goat anti-mouse IgG (bottom panel). (**B**) Analysis with MDM2-specific monoclonal antibodies. The experiment was performed essentially as in panel A, except that the MDM2-specific monoclonal antibody IF-2 (generous gift of Dr B.Vogelstein) was substituted for the polyclonal serum. The autoradiogram of the DNA binding assay is shown in the top panel, while the bottom panel depicts the p53 protein analysis data. The lower band of the doublet shown in lane 5 represents the p53 polypeptide, whereas the upper band represents the heavy chain of the anti-MDM2 antibody, which was used in large excess in order to ensure quantitative precipitation of p53–MDM2 complexes. The heavy chains of the p53-specific monoclonal antibodies which were used at lower concentrations could only be observed after longer exposure times (data not shown; see also panel A).

It is rather unlikely that the anti-MDM2 serum itself interfered directly with DNA binding by p53, given that the antiserum was added only after DNA–protein complexes had already been allowed to form. In fact, Figure 3 indicates that even in the case of antibodies against the actual DNA binding polypeptide, pre-formed DNA–p53 complexes cannot be disrupted by antibodies that otherwise interfere with the binding. Yet, one could argue that the use of a polyclonal serum might have resulted in the binding of many antibody molecules to the MDM2 polypeptide and the generation of a bulky complex which imposed a steric barrier on DNA binding by p53. Therefore, an essentially similar experiment was performed with an MDM2-specific monoclonal antibody. As seen in Figure 8B, the results were essentially identical to those obtained with the polyclonal serum. Hence, the sub-population of wt p53 that is associated with MDM2 appears incapable of binding tightly to its cognate target in clone 255 DNA. At present, it is unclear whether the mere physical association between the two proteins is sufficient to block binding, or whether the p53 molecule undergoes a subsequent alteration which renders it intrinsically binding-incompetent. In either case, the loss of sequence-specific binding can provide a molecular basis for the ability of MDM2 to abrogate transcriptional activation by wt p53.

Discussion

Using an immune selection procedure, two high affinity p53 binding sites were isolated from mouse genomic DNA. While one is apparently unique and does not correspond to any known gene, the other is derived from the LTR of a repetitive murine retrovirus-like element, belonging to the GLN family. The high frequency at which such GLN LTR regions emerged from the immune selection suggests that they constitute a major component of the high affinity p53 binding sites in the mouse genome. The mouse genome contains ~20–50 copies of full length GLN elements, as well as 1000–1500 additional GLN family LTRs, some of which may be present as solitary LTRs (Itin and Keshet, 1986). When LTR-6 cells were induced to overexpress wt p53 activity, the levels of the major GLN element transcript became markedly elevated. Overexpressed wt p53 can often cause a G_1 growth arrest (Levine *et al.*, 1991; Vogelstein and Kinzler, 1992; Oren, 1992). It could be argued that the induction of GLN element gene expression is a consequence of the cell cycle arrest. However, we have recently found that the activation of wt p53 in LTR-6 does not lead to any growth arrest, and the cells continue to move through the cell cycle at 32°C (Yonish-Rouach *et al.*, 1993). Hence, the stimulation of GLN element gene expression most probably stems from a direct effect of wt p53, presumably involving the interaction of p53 with the GLN LTR. At present, very little is known about the proteins whose expression is normally driven by the GLN LTRs (Keshet *et al.*, 1990). It is thus hard to assess whether the fact that they can serve as potential targets for activation by wt p53 has any biological significance. One provocative possibility is that the putative retrovirus giving rise to the GLN element has evolved high affinity p53 binding sites (two per

proviral DNA molecule) as a strategy for tying up and presumably titrating out p53, while upregulating its own expression. So far, however, we have no evidence to support this notion. It is also noteworthy that p53 can act as an RNA binding protein, covalently (Samad and Carroll, 1991) and perhaps also non-covalently (Steinmeyer and Deppert, 1988). Given that the p53 consensus site sequence is expected to be contained also within the GLN element transcript, it will be of interest to find out whether it exhibits any preferential binding to p53 when present within single stranded RNA.

Under the experimental conditions used in this study, different p53 binding sites exhibited pronounced differences with regard to their respective ability to associate tightly with wt p53. This could imply that the repertoire of DNA elements actually engaged in interaction with p53 may vary under various conditions, with additional low affinity sites being recruited only when cellular p53 concentrations exceed a certain threshold. Situations of the latter type may occur, for instance, upon exposure of cells to DNA damaging agents (Maltzman and Czyzyc, 1984; Kastan *et al.*, 1991; Kuerbitz *et al.*, 1992; Lane, 1992). Alternatively, the affinity of p53 for such 'weak' sites may be increased through covalent modifications of the protein, as well as through the formation of complexes between p53 and other cellular proteins. Thus, the relative performance of a given site in an *in vitro* binding assay does not necessarily reflect its likelihood to engage in p53 binding *in vivo*.

Numerous studies have shown that promoters containing p53 binding sites, either as part of their normal sequence or added deliberately, can be activated by wt p53. Yet, these studies typically employed recombinant plasmids, in which these promoters were placed out of their natural chromosomal context. In the present study, we show that wt p53 can also enhance the expression of unmanipulated genes containing p53 binding sites within their transcriptional control elements. This finding lends further credibility to the proposal that at least some biological effects of wt p53 are mediated through its ability to act as a transcriptional regulator, and that the role of p53 binding sites is to make the adjacent genes p53-responsive. Yet, while this is a reasonable proposal, it is far from proven. At present, it is still equally plausible that high affinity p53 binding sites actually serve other purposes, for instance in the context of imposing restrictions on DNA replication in cells harboring genetic damage (Kastan *et al.*, 1991; Lane, 1992; Livingstone *et al.*, 1992; Yin *et al.*, 1992).

Finally, we demonstrate that when p53–MDM2 complexes are brought down by MDM2-specific antibodies, they fail to exhibit a detectable association with a p53 DNA target. Even though we cannot rule out completely an effect of the antibodies on DNA binding, the most likely interpretation of our data is that the complexes formed *in vivo* between p53 and the MDM2 protein are defective in their ability to interact with that target. It is unclear how general this observation is. Thus, while failing to associate stably with the p53 binding element of clone 255, p53–MDM2 complexes may nevertheless bind efficiently to other types of p53 target sites. Moreover, these complexes may in fact endow upon p53 a different DNA target specificity. This suggestion is particularly provocative in view of the fact that the MDM2 polypeptide possesses properties consistent with its being a putative DNA binding protein (Fakarzadeh *et al.*, 1991). Such a possibility may gain further significance in light of the finding that activation of wt p53 leads to enhanced expression of the *mdm2* gene and to a marked increase in the levels of p53–MDM2 complexes (Barak *et al.*, 1993). Hence, while our findings offer a very logical explanation for the ability of MDM2 to abort the activation of promoters containing high affinity p53 binding sites, they still do not rule out the option that p53–MDM2 complexes actually lead to the induction of a new activity. Further investigation into the possible interaction of such complexes with different DNA elements should help clarify this issue.

Materials and methods

Cell lines and antibodies

Clone 6 cells are derived from rat embryo fibroblasts transformed with a combination of activated human Ha-ras and the ts p53 mutant p53val135 (Pinhasi-Kimhi *et al.*, 1986; Michalovitz *et al.*, 1990). The MCO1/cG9-6 line was generated by transfecting the p53-negative MCO1 cells (Halevy *et al.*, 1991) with plasmids encoding p53val135 and resistance to hygromycin B, followed by selection in the presence of the antibiotic (data not shown). Immortalized 314 fibroblasts were derived from a p53-deficient mouse embryo (Donehower *et al.*, 1992) at 14 days of pregnancy, and were kindly provided by Dr L.Donehower. The cells were passaged every 3 days at a density of 3×10^6 cells per 100 mm dish, for over 30 passages.

Hybridoma cell lines producing p53-specific monoclonal antibodies were injected into the intraperitoneal cavity of BALB/c mice. Ascites fluid was collected, and antibodies were purified by chromatography on protein A–Sepharose (Sigma). The rabbit polyclonal anti MDM2 serum has been described elsewhere (Barak *et al.*, 1993).

Construction of genomic library and screening for p53 binding clones

Mouse (strain C57/Bl) genomic DNA was digested to completion with *Mbo*I. The resultant mixture of digestion products was ligated into the *Sma*I site of plasmid pBlueScript SK– (Stratagene), followed by electroporation into competent HB101 bacteria. The electroporated bacteria were grown in a mass liquid culture. Plasmid DNA was then extracted from the cultured bacteria, and used for immune selection as outlined below. The primary library, taken for the first round of immune selection, contained $\sim 2 \times 10^6$ independent clones, ~90% of which contained genomic DNA inserts (data not shown).

For immune selection, 20 μg plasmid DNA from the library was mixed with 300 μg of extract from Clone 6 cells maintained at 32°C (see below), in a binding buffer containing a final concentration of 10% glycerol, 5 mM EDTA, 20 mM Tris–Cl pH 7.2, 100 mM NaCl and 0.1% NP40 (total volume = 100 μl). Following incubation for 1 h on ice, a mixture of the p53-specific monoclonal antibodies PAb421, PAb248 and RA3-2C2 (1 μg each) was added, and incubation on ice resumed for another 30 min. Finally, 2 mg protein A–Sepharose beads (Sigma) were added, followed by incubation on ice for 30 min. The beads were then washed three times with washing buffer (identical to the above binding buffer, but containing only 2% glycerol). The DNA was released from the beads by incubation for 10 min at 45°C in a solution containing 1% SDS. The DNA was extracted with phenol–chloroform, ethanol precipitated in the presence of glycogen (10 μg) as a carrier, and used to transform electrocompetent HB101. The immune selection procedure was then repeated twice more to generate the final enriched library. After the third selection cycle, individual transformed HB101 colonies were grown up. Plasmid DNA was extracted from each colony, and representative clones were subjected to digestion with *Xba*I plus *Hind*III. Individual inserts were isolated from a preparative agarose gel, end-labeled and tested for hybridization with other clones from the enriched library. Following the elimination of cross-hybridizing clones, DNA from the remaining plasmids was digested as above, end-labeled with ^{32}P as indicated in the corresponding figures, and subjected to a p53 binding assay essentially under the conditions used for the processing of the library, but with reduced amounts of extract and antibodies (see figure legends for specific details). The radiolabeled DNA was then extracted, ethanol precipitated and resolved on a 5% polyacrylamide gel.

Preparation of cell extracts and proteins

Clone 6 and MCO1/cG9-6 cells (80% confluent) were maintained at either 37.5 or 32°C. For the preparation of extracts, cells were washed twice with ice-cold PBS, and extracted as described before (Maltzman *et al.*, 1981). The protein extract was frozen in small aliquots and stored at −70°C for subsequent use. Extracts prepared by this method were used for immune selection assays, as well as for isolating p53 binding DNA clones. For electrophoretic mobility shift assays and DNase I footprinting, the extraction procedure of Bagchi *et al.* (1990) was found to yield better results (data not shown), and was therefore employed routinely for these assays.

Recombinant wt p53 was prepared from insect cells infected with the corresponding baculovirus. The protein was purified by a two step procedure, involving chromatography over phosphocellulose followed by immunoaffinity chromatography over a PAb421 column. The construction of the recombinant baculovirus and the purification procedure are detailed elsewhere (Ragimov *et al.*, 1993).

Electrophoretic mobility shift assays

Cell extracts or purified protein were mixed with 1−2 μg of poly[d(I-C)] (Boehringer Mannheim) in 20 μl of binding buffer containing 110 mM KCl, 4 mM $MgCl_2$, 4 mM Tris−Cl pH 7.6, 4% glycerol, 0.05 mM $ZnCl_2$ and 0.25% bromophenol blue. The mixture was kept on ice for 10 min and then the end-labeled probe was added and incubation was continued for another 20 min on ice. The reaction was loaded on a 5% neutral polyacrylamide gel and run in a Tris−glycine running buffer (25 mM Tris−Cl pH 8.3, 190 mM glycine and 10 mM EDTA). In some reactions, various antibodies were included as indicated in the corresponding figure legends.

DNase I footprinting

Purified p53 was mixed with 1 μg poly[d(I-C)] in 50 μl of a solution identical to the binding buffer used for electrophoretic mobility shift assays, excepting bromophenol blue. After 10 min at 0°C, end-labeled probe was added for an additional 20 min on ice. 50 μl of 2 × DNase I buffer (5 mM $CaCl_2$, 10 mM $MgCl_2$) containing 20 pg DNase I were then added for 60 s at room temperature. The reaction was stopped by adding EDTA to 25 mM and SDS to 0.05%. Proteinase K was added to a concentration of 200 μg/ml and incubated for 30 min on ice. The mixture was phenol−chloroform extracted, ethanol precipitated and loaded on a 5% denaturing polyacrylamide−urea gel.

Expression studies

CAT analysis was performed as described before (Ginsberg *et al.*, 1991). RNA was prepared by the LiCl method and analyzed as described before (Einat *et al.*, 1985).

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