Definition of a consensus binding site for p53

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Recent experiments have suggested that p53 action may be mediated through its interaction with DNA. We have now identified 18 human genomic clones that bind to p53 *in vitro*. Precise mapping of the binding sequences within these clones revealed a consensus binding site with a striking internal symmetry, consisting of two copies of the 10 base pair motif 5'-PuPuPuC(A/T)(T/A)GPyPyPy-3' separated by 0-13 base pairs. One copy of the motif was insufficient for binding, and subtle alterations of the motif, even when present in multiple copies, resulted in loss of affinity for p53. Mutants of p53, representing each of the four "hot spots" frequently altered in human cancers, failed to bind to the consensus dimer. These results define the DNA sequence elements with which p53 interacts *in vitro* and which may be important for p53 action *in vivo*.

In human cancer p53 is the most commonly altered protein yet discovered[1]. Mutation or deletion of p53 results in the loss of tumour suppressor function, and this has been associated with tumour progression (reviewed in refs 2–5). Biochemically, the wild-type (wt) p53 has been identified as a sequence-specific DNA binding protein[6,7], with transcription activating properties[8-10]. Because mutant p53 proteins that lose tumour suppressor function fail to bind either nonspecifically[11,12] or specifically[6,7] to DNA, wt p53 action may be mediated through its interactions with DNA. To date however, only one genomic binding site has been identified[6] and this clone had little obvious similarity to a p53 binding sequence found near the SV40 origin of replication[7]. The human binding sequence had three copies of a TGCCT repeat. This repeat occurs frequently in the human genome, and taken alone, its presence could not predict whether any particular DNA sequence would bind to p53 or explain why sequences containing high TGCCT density do not necessarily bind to p53 (unpublished observations).

To understand further the nature of the interaction of p53 with human DNA, we attempted to identify, in an unbiased fashion, human genomic fragments that could bind to wt p53 protein *in vitro*. We have now identified 18 such fragments. Detailed analysis of the binding sites within these fragments revealed a remarkable similarity, allowing formulation of a consensus binding site with a striking internal symmetry. Mutant p53 proteins, derived from human tumours, consistently failed to bind to this consensus. These results have significant implications for understanding the *in vivo* targets of p53, and suggest strategies for discovering genes adjacent to these sequences which may mediate p53 action.

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Isolation of p53 binding sequences

Figure 1*a* outlines the experimental strategy used for the isolation and analysis of p53-binding sites. Total human genomic DNA fragments were ligated to specially designed 'catch' linkers[13,14] to allow for subsequent PCR amplification and cloning. The linked genomic DNA was incubated with wt p53 and precipitated with anti-p53 antibodies. The bound DNA was amplified by PCR using primers complementary to the catch linkers, and the process repeated. After four rounds of sequential immunoprecipitation and PCR, the amplified and selected (AS) DNA was cloned. Clones were picked at random and tested for p53 binding first by immunoprecipitation (IP), and then by methylation interference (MI) and DNase I protection (DP).

We tested the inserts of 530 clones for binding to p53 (outline in Fig. 1*a*). Restriction fragments of the clones were end-labelled and incubated with purified human wt p53 protein which had been produced in baculovirus-infected cells. The bound DNA fragments were precipitated using anti-p53 antibodies and protein A sepharose, and analysed by electrophoresis. Of the clones, 23 were found to contain fragments that bound to p53. Clone S61 (Fig. 1*b*, lanes 11B,C) contained a single genomic fragment which bound to p53. Clone N2 contained five fragments, only one of which (357 base pairs (bp)) bound to p53 (lanes 10B,C). Other examples of p53-binding fragments are shown in lanes labelled 2,3,5 and 9. In all, 23 of the 530 clones were found to contain p53-binding fragments, and each of these was subcloned for further analysis. In contrast, we found that none of over 1,000 clones containing unselected human DNA inserts of similar size bound to p53 using the IP assay. Thus the whole-genome PCR

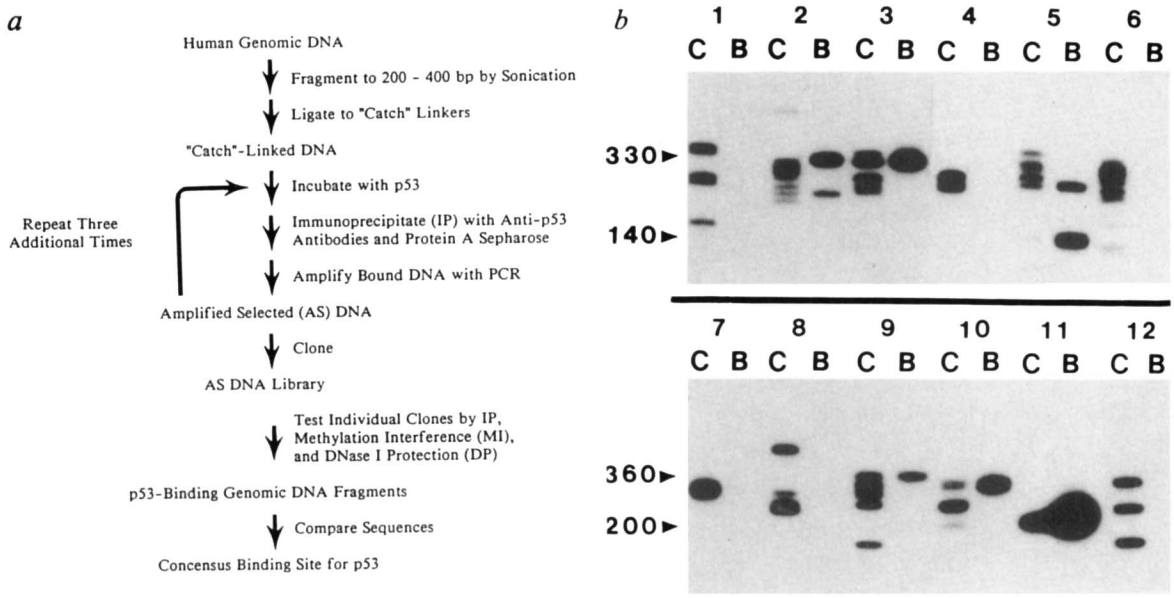


Fig. 1 Isolation of human genomic sequences that bound to p53. *a*, Experimental strategy used for isolation and analysis of human genomic DNA fragments which bound to p53. *b*, Immunoprecipitation (IP) assays of cloned fragments. Clones of AS DNA were tested for the presence of p53-binding fragments by IP. For each clone, the bound DNA is shown in the B lane, adjacent to a control (C) lane containing 2% of the total end-labelled DNA used in the binding assay. In this representative experiment, eight binding fragments were identified, representing six unique genomic fragments. The inserts from the clones in lanes labelled 2, 3, 5, 9, 10 and 11 contained p53-binding fragments, while the other lanes contained none. The clones in lanes 2 and 5 each contained two binding fragments.

procedure significantly enriched for p53-binding sequences.

Mapping of p53 binding sites

Localization of the regions bound by p53 was obtained by DP or MI assays using the subcloned DNA fragments as probes. In control experiments, we found that the fragments were not immunoprecipitated by antibodies in the absence of p53, and that they bound to p53 in the absence of antibodies. The stability of the p53-DNA complexes however, was apparently enhanced by the presence of antibodies, so we generally used IP to isolate and subsequently analyse bound DNA. For MI, the fragments were methylated at G residues and bound to p53 (Fig. 2). Methylation of G residues critical for p53 binding resulted in interference with IP. For example, methylation at nucleotides 217, 222, 227–229 and 233 of the 248 bp insert from clone 11B3 completely interfered with the binding of this fragment to p53 (Fig. 2, footprint 2). When the opposite strand was analysed, interference was observed at the G residues corresponding to nucleotides 219, 223, 224, 230, 235 and 236 (Fig. 2, footprint 1). For DP, labelled DNA fragments were first subjected to IP, then incubated with various amounts of DNase I. For clone N22, p53 binding provided protection against DNase I cleavage at residues 187–211 (Fig. 2, footprint 9). MI showed interference by G residues only within the region protected by DNase I (Fig. 2, footprint 10).

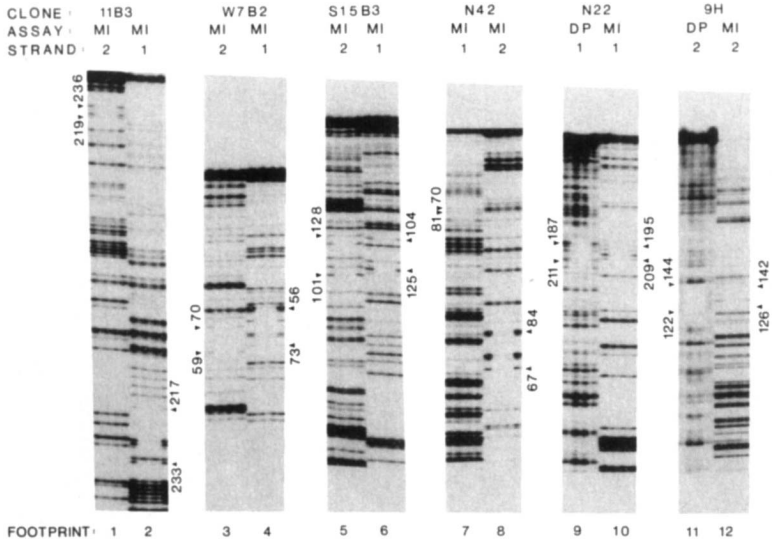
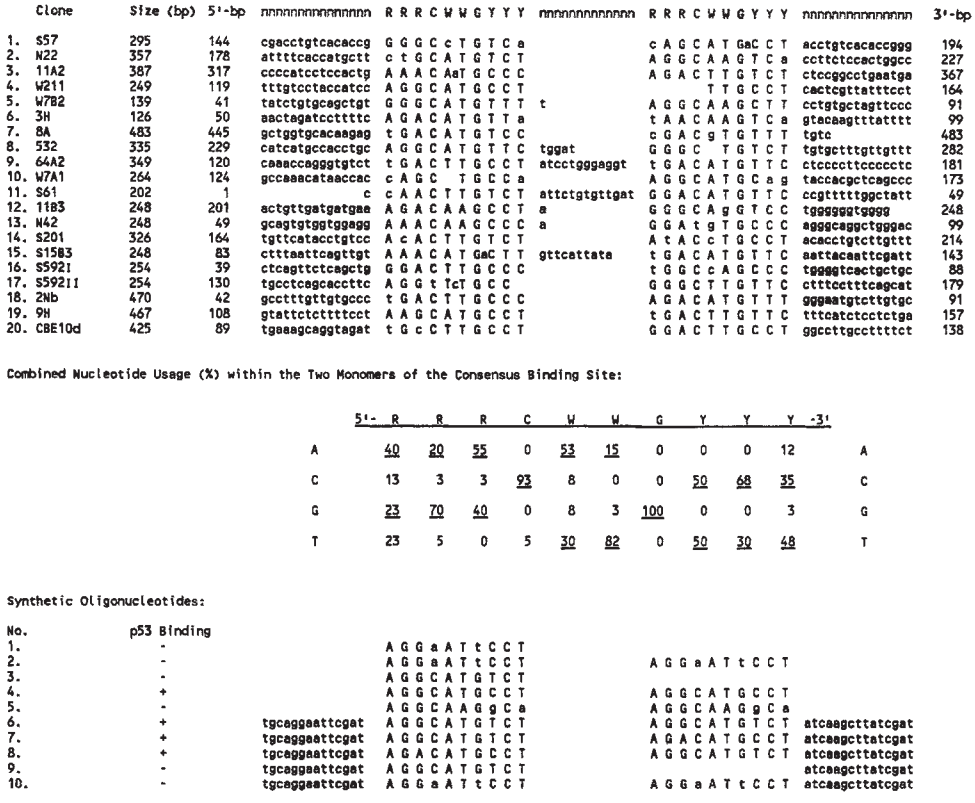


Fig. 2 Mapping of p53-binding sites by DNase I protection (DP) and methylation interference (MI). For each footprint, the first and fourth lanes contain control samples of the total labelled DNA, whereas the middle two lanes contain an equivalent amount of p53-bound DNA. DNA sequences corresponding to the p53-binding regions on strand 1 are shown in Fig. 3.

A consensus binding site for p53

We compared the sequences of the 23 clones. The average insert was 307 bp (range 139 – 470). Ten of the 23 clones were not unique, showing at least 100 contiguous nucleotides identical to one other clone. Thus the 23 clones represented only 18 independent genomic DNA fragments. We found no significant homologies among these 18 fragments by computer methods, but when we aligned the regions involved in p53 binding (as assessed by MI and DP), a striking and consistent feature of the clones became apparent (Fig. 3). Each of the binding sites



	Clone	Size (bp)	5'-bp	nnnnnnnnnnnnnn	R R R C W W G Y Y Y	nnnnnnnnnnnn	R R R C W W G Y Y Y	nnnnnnnnnnnnnn	3'-bp
1.	S57	295	144	cgacctgtcacaccg	G G G C c T G T C a		c A G C A T GaC C T	acctgtcacaccggg	194
2.	N22	357	178	attttcaccatgctt	c t G C A T G T C T		A G G C A A G T C a	ccttctccactggcc	227
3.	11A2	387	317	ccccatcctccactg	A A A C AaT G C C C		A G A C T T G T C T	ctccggcctgaatga	367
4.	W211	249	119	tttgtcctaccatcc	A G G C A T G C C T		T T G C C T	cactcgttatttcct	164
5.	W7B2	139	41	tatctgtgcagctgt	G G G C A T G T T T t		A G G C A A G C T T	cctgtgctagttccc	91
6.	3H	126	50	aactagatccttttc	A G A C A T G T T a		t A A C A A G T C a	gtacaagtttatttt	99
7.	8A	483	445	gctggtgcacaagag	t G A C A T G T C C		c G A C g T G T T T	tgtc	483
8.	532	335	229	catcatgccacctgc	A G G C A T G T T C tggat		G G G C T G T C T	tgtgctttgttgttt	282
9.	64A2	349	120	caaaccagggtgtct	t G A C T T G C C T atcctgggaggt		t G A C A T G T T C	ctcccctttcccctc	181
10.	W7A1	264	124	gccaaacataaccac	c A G C T G C C a		A G G C A T G C a g	taccacgctcagccc	173
11.	S61	202	1	c	c A A C T T G T C T attctgtgttgat		G G A C A T G T T C	ccgtttttggctatt	49
12.	11B3	248	201	actgttgatgatgaa	A G A C A A G C C T a		G G G C A g G T C C	tgggggtgggg	248
13.	N42	248	49	gcagtgtggtggagg	A A A C A A G C C C a		G G A t g T G C C C	agggcaggctgggac	99
14.	S201	326	164	tgttcatacctgtcc	A c A C T T G T C T		A t A C c T G C C T	acacctgtcttgttt	214
15.	S1583	248	83	ctttaatttcagttgt	A A A C A T GaC T T gttcattata		t G A C A T G T T C	aattacaatttcgatt	143
16.	S592I	254	39	ctcagttctcagctg	G G A C T T G C C C		t G G C c A G C C C	tgggggtcactgctgc	88
17.	S592II	254	130	tgcctcagcaccttc	A G G t TcT G C C		G G G C T T G T T C	ctttcctttcagcat	179
18.	2Nb	470	42	gcctttgttgtgccc	t G A C T T G C C C		A G A C A T G T T T	gggaatgtcttgtgc	91
19.	9H	467	108	gtattctctttttcct	A A G C A T G C C T		t G A C T T G T T C	tttcatctcctctga	157
20.	CBE10d	425	89	tgaaagcaggtagat	t G c C T T G C C T		G G A C T T G C C T	ggccttgccttttct	138

Combined Nucleotide Usage (%) within the Two Monomers of the Consensus Binding Site:

	5'- R	R	R	C	W	W	G	Y	Y	Y -3'	
A	40	20	55	0	53	15	0	0	0	12	A
C	13	3	3	93	8	0	0	50	68	35	C
G	23	70	40	0	8	3	100	0	0	3	G
T	23	5	0	5	30	82	0	50	30	48	T

Synthetic Oligonucleotides:

No.	p53 Binding			
1.	-	A G G a A T t C C T		
2.	-	A G G a A T t C C T	A G G a A T t C C T	
3.	-	A G G C A T G T C T		
4.	+	A G G C A T G C C T	A G G C A T G C C T	
5.	-	A G G C A A G g C a	A G G C A A G g C a	
6.	+	tgcaggaattcgat A G G C A T G T C T	A G G C A T G T C T	atcaagcttatcgat
7.	+	tgcaggaattcgat A G G C A T G T C T	A G A C A T G C C T	atcaagcttatcgat
8.	+	tgcaggaattcgat A G A C A T G C C T	A G G C A T G T C T	atcaagcttatcgat
9.	-	tgcaggaattcgat A G G C A T G T C T		atcaagcttatcgat
10.	-	tgcaggaattcgat A G G a A T t C C T	A G G a A T t C C T	atcaagcttatcgat

Fig. 3 Definition of a consensus binding site for p53. The p53 binding sites of 18 cloned human genomic DNA fragments, determined by footprinting methods (Fig. 2), are displayed along the central axis of symmetry which separates the two 10 bp consensus monomers. Nucleotides in capital letters represent identity of a genomic sequence to the consensus, whereas lower case letters identify disparity with the consensus. Sequences surrounding the consensus or separating the two 10 bp monomers are also shown in lower case. Combined nucleotide usage within the two monomers of the consensus binding site is shown in the middle. Some of the synthetic oligonucleotides investigated for the ability to be bound by p53 are shown at the bottom. Oligonucleotides 6–10 were tested after cloning into plasmid vectors.

contained two copies of the 10 bp motif 5'–PuPuPuC(A/T)(T/A)GPyPyPy–3', separated by 0–13 bp. One clone (S592) contained two separate areas of footprinting, and both regions contained a dimer of the 10 bp motif (Fig. 3). In all clones, the regions displaying DP and MI were always centred within the dimers, and G residues within the 10 bp motif strongly interfered with binding to p53 (see Fig. 2). The 10 bp consensus monomer contained an internal symmetry, with two oppositely oriented half-sites of the form 5'–PuPuPuC(A/T)–3'. This symmetry was extended in the dimers, which contained four half-sites oriented in alternating directions, forming a pseudopalindromic structure, sometimes with an intervening loop. This consensus dimer was also recognized in the previously identified p53 binding sequence mapped within plasmid CBE10d (Fig. 3). Despite the remarkable symmetry noted for all p53 binding sequences, none of the genomic sites were palindromic.

Alterations of the consensus

To determine if the 10 bp consensus monomer could bind to p53, a synthetic oligonucleotide (5'–AGGCATGTCT–3') containing the consensus sequence was studied. Oligonucleotide duplexes were tested either directly or after cloning into plasmid vectors. The monomer did not bind to p53, either alone (not shown) or flanked by 43 nucleotides of plasmid sequences (Fig. 4*a*, lane 5). In contrast, the dimers (composed of two copies of the monomer arranged in head-to-head, tail-to-tail, or head-to-tail orientation), each bound strongly to p53 protein (Fig. 4*a*, lanes 1–4, 6). Higher-order oligomers of the monomer did not bind any better than the dimer in the IP assay (Fig. 4*a*, lanes 6–8). A different monomer, still fitting the consensus sequence, but perfectly palindromic, also bound as a dimer and tetramer, but not as a monomer (Fig. 4*b*, lanes 4–6). Two variants of the consensus motif were also tested for binding. In the first, the two critical G:C bps at positions 4 and 7 of the monomer were substituted with A:T bps (Fig. 3, synthetic oligonucleotide No. 1). Although this sequence was perfectly symmetrical, it did not bind to p53 either as a monomer or as a multimer (Fig. 5*b*, lanes 1–3). We also tested direct repeats of the 5'–PuPuPuC(A/T)–3' half-site, and found that these did not bind to p53 (Fig. 3, synthetic oligonucleotide No. 5). Thus the mirror-image symmetry of the half-sites within the 10 bp consensus monomer was critical for its activity. Finally, we tested p53 mutants representing each of the four 'hot-spots' frequently altered in human cancers[15,16] for ability to bind to the consensus dimer. None of the p53 mutants bound appreciably to this sequence under conditions where the wt protein bound strongly (Fig. 5). These experiments also showed that *in vitro* translated p53, as well as that purified from baculovirus-infected insect cells, had the capacity to bind DNA specifically.

Discussion

Human genomic DNA fragments which could bind p53 were isolated and used to define a consensus binding sequence for p53. The consensus contained two copies of the double-stranded 10 bp motif 5'–PuPuPuC(A/T)(T/A)GPyPyPy–3' separated by 0–13 bp. The symmetry of the four half-sites within the consensus suggests that p53 interacts with DNA as a tetrameric protein. This is consistent with studies suggesting that p53 assembles into homotetramers *in vivo* and *in vitro*[17,18].

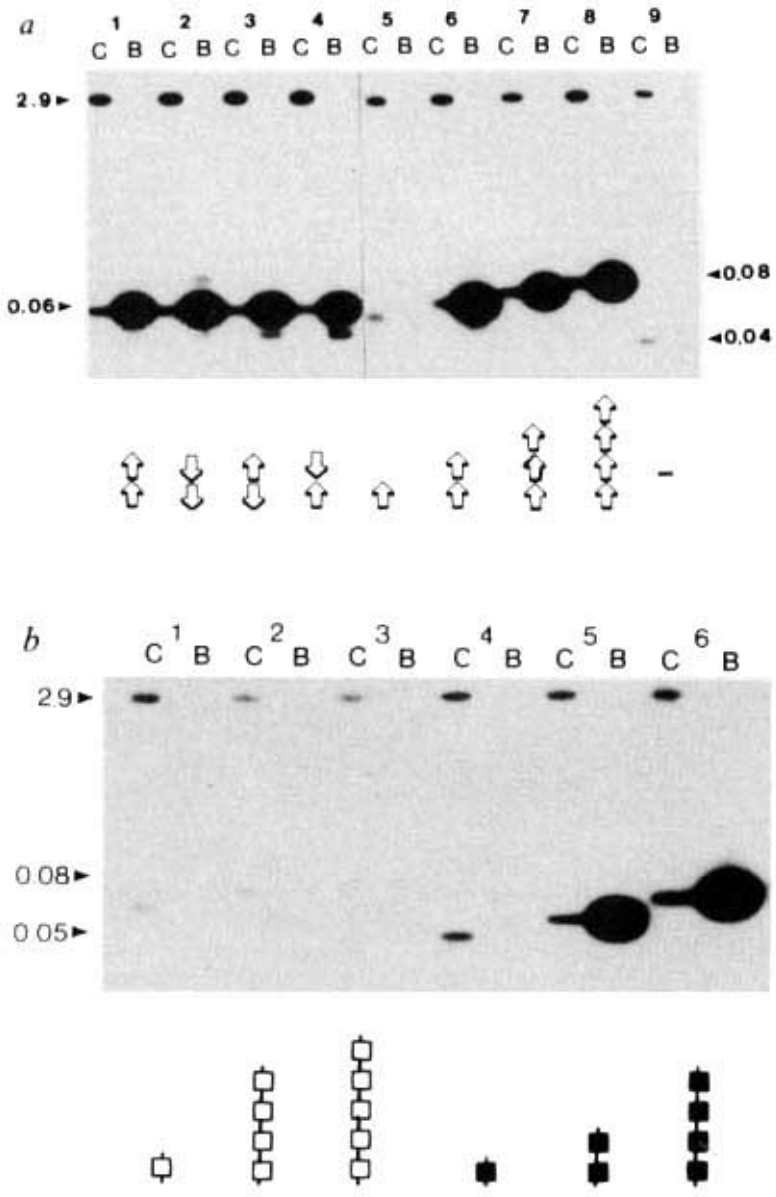


Fig. 4 Binding of cloned synthetic oligonucleotides to wt p53. The 10 bp consensus monomer was insufficient for binding, whereas dimers in various orientations or multimers of the 10 bp consensus bound strongly to p53. For each sample, the control lane (C) contained 2% of the total DNA used in the binding reaction, and was composed of two fragments: a 2.9 kilobase vector DNA fragment, and a fragment of 40 to 80 bp containing no insert (*a*, lane 9) or inserted oligonucleotides surrounded by plasmid sequences. Bound DNA from the IP is shown in the B lanes. In *a*, the inserts contained the 10 bp consensus monomer sequence 5'–AGGCATGTCT–3' (lane 5), or multimers of this sequence arranged as indicated (lanes 1–4, 6–8). In *b*, the inserts contained the 10 bp palindromic consensus monomer 5'–AGGCATGCCT–3' (lane 4) or multimers of this sequence (lanes 5, 6); or the palindromic nonconsensus monomer 5'–AGGAATTCCT–3' (lane 1), or multimers of this sequence (lanes 2, 3).

It is interesting to compare the consensus binding site defined in Fig. 3 with that of the two previously identified p53 binding sequences. The sequence of one of the latter (CBE from the ribosomal gene cluster) is included in Fig. 3 for comparison. The binding sequence of clone CBE matched the consensus reasonably well, with only two mismatches out of 20 bps. However, one of the two mismatches was apparently critical, as all of the other binding sites contained a purine at the third position, whereas in CBE this position was occupied by a C. A sequence from SV40 has also been shown to bind to p53 (ref. 7). Although the SV40 residues responsible for binding have not been precisely identified, the binding region contains two copies of the sequence 5'–GGGCGGAGTTA–3', separated by 10 bp. This sequence matches the consensus binding element at 8 of the 10 residues, with a G insertion in the middle. p53 binds to CBE and SV40 sequences much less efficiently (by 5- and 15-fold, respectively) than to the consensus sequence, as assessed by quantitative immunoprecipitation of labelled DNA fragments (unpublished observations).

A critical issue for future research is the identification of genes whose expression is controlled by p53. Although the sequences required for p53 binding are now known, this is likely to prove a difficult task. The consensus binding sequences for other oncogenic proteins (for example myc, myb, jun, fos) have been known for some time, but the genes controlled by these DNA binding proteins, which presumably mediate their effects, have not yet been identified. For p53, such identification could be facilitated in two ways by the data presented here. First, genes of interest (for example, those controlling growth or invasion) could be examined for the presence of the consensus binding site. Those genes which contain this binding site in their regulatory regions would be good candidates for p53-mediators. Second, the 18 human DNA clones which defined the consensus may prove to be biologically relevant to p53 action. A search of the current DNA databases (Genbank version 70, EMBL 29) revealed

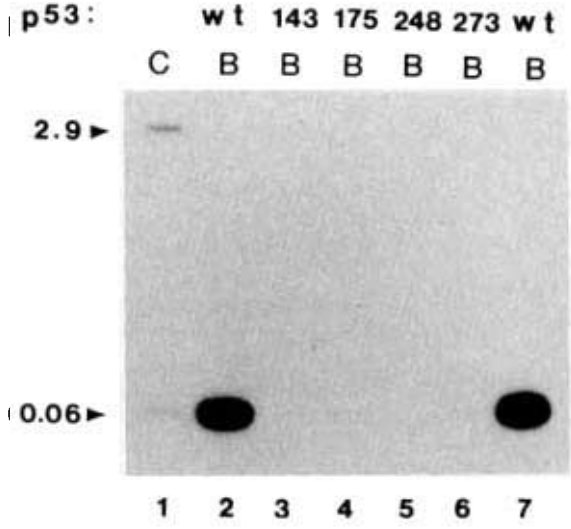


Fig. 5 Comparison of wt and mutant p53 protein binding to the consensus dimer. *In vitro* translated p53 proteins were tested for the ability to bind the consensus dimer by IP. 2% of the total DNA used for binding is shown in lane 1. Lane 7 shows binding to baculovirus-produced human wt p53 protein. Lanes 2–6 show binding of *in vitro* translated wild-type and mutant p53 proteins. The mutant p53 proteins contained changes at codon 143 (val to ala), 175 (arg to his), 248 (arg to trp) and 273 (arg to his).

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that none of the sequences from these clones has been recorded previously. Through a combination of genomic and cDNA library screening, these clones can now be used as probes to find adjacent genes whose expression may be controlled by p53.

Methodology

Whole genome PCR. Whole-genome PCR was performed as described previously, except that only one oligonucleotide (5'–GAGTAGAATTCTAATATCTC–3') was used for amplification[13,14]. 200 ng of 'catch'-linked human genomic DNA was incubated with 100 ng of baculovirus-produced human wt p53 (ref. 19), and immunoprecipitated as described below. After four rounds of IP and PCR, the AS DNA was cleaved with *Eco*R1 and cloned into either the vector λZap II or pBluescript II SK+ (Stratagene). Individual clones were picked at random and tested for p53 binding after cleavage with *Eco*R1 and end-labelling by Klenow fill-in. For IP (ref. 20), 10 ng of DNA were incubated with 100 ng of baculovirus-produced human wt p53 and 100 ng of poly dI-dC at 4 °C for 30 min in 100 µl of 'DNA-binding buffer' containing 100 mM NaCl, 20 mM Tris, pH 7.0, 10% glycerol, 1% NP40 and 5 mM DTT. DNA fragments bound to p53 were complexed to antibodies by the addition of 8 µl containing 400 ng each of anti-p53 antibodies PAb421 and PAb1801 (Oncogene Science), and incubated for 30 min at 4 °C. The DNA-p53-anti-p53 antibody complexes were precipitated following the addition of 26 µl of DNA-binding buffer containing 1.5 mg protein A Sepharose and 10 µg of poly dI-dC and mixing at 4°C for 30 min. After removal of the supernatant, the immunoprecipitate was washed twice with 1 ml of DNA-binding buffer. Bound DNA was purified by treatment with SDS and proteinase K at 48 °C for 30 min, extracted with phenol and chloroform, precipitated with ethanol, separated by electrophoresis on a 10% nondenaturing polyacrylamide gel, and autoradiographed.

DNase protection and methylation interference. p53-binding DNA fragments were subcloned and labelled on one end, gel-purified and subjected to DP or MI mapping. For MI, 10 ng of DNA were incubated in 200 µl of 50 mM Na cacodylate, 1 mM EDTA, pH 8.0 and 5 µl of 10% dimethylsulphate/90% ethanol for 5 min at 20 °C to methylate G residues. 50 µl containing 1.5 M Na acetate, 1 M beta-mercaptoethanol and 60 µg of glycogen were added. The mixture was ethanol-precipitated, washed, and resuspended in 5 µl of 3 mM Tris, 0.2 mM EDTA, pH 7.5, and allowed to bind to wt p53 as described above. IP and DNA purification were performed as described above, and the samples were incubated with 100 µl of 1 M piperidine at 90 °C for 30 min. The samples were then dried under vacuum and separated electrophoretically on a 6 % polyacrylamide sequencing gel. The control DNA samples were carried through all incubations except no p53 was added. For the control samples, the protein A Sepharose pellets were treated with SDS and proteinase K without removal of the supernatants (which contained the labelled DNA in the absence of p53).

For DP assays, end-labelled DNA fragments were immunoprecipitated and the precipitates incubated for 2 min at 25 °C with 200 ng DNase I in DNA binding buffer supplemented with 5 mM $MgCl_2$. After purification of the DNA, samples were separated by electrophoresis on sequencing gels and loaded as described above for MI. MI was performed on all 18 genomic DNA fragments that bound to p53. DP assays were performed on 13 fragments and the regions of protection uniformly coincided with those indicated by the MI assays.

Oligonucleotide experiments. Double-stranded oligonucleotides corresponding to the consensus monomer sequence 5'–AGGCATGTCT–3' or variants thereof were ligated and cloned into the *Eco*RV site of pBluescript II SK+ (Stratagene). Individual clones containing no insert, monomer insert, dimers, trimers and tetramers were isolated and sequenced to confirm their identity. End-labelling and p53 binding were performed on *Xho*I plus *Pst*I-generated fragments of plasmid DNA. These fragments contained the inserted oligonucleotides surrounded by 43 nucleotides of plasmid sequences. One hundred ng of baculovirus produced or *in vitro* translated p53 was precipitated with PAb 421 and PAb 1801 as described above. The precipitates were washed three times with DNA binding buffer and resuspended in 100 µl DNA binding buffer containing 100 ng poly dI-dC and 10 ng end-labelled DNA. After 30 min at 4 °C, 10 µg of poly dI-dC was added and incubated an additional 30 min. The bound DNA was purified and analysed as described above. *In vitro* translations were performed as described previously[12].

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