 Nucleic Acids Research, 1997, Vol. 25, No. 7 **1319–1326**

DNA binding specificity of proteins derived from alternatively spliced mouse p53 mRNAs

Zoe Miner and Molly Kulesz-Martin*

Roswell Park Cancer Institute, Department of Experimental Therapeutics, GCDC Room 403, Elm and Carlton Streets, Buffalo, NY 14263, USA

Received January 3, 1997; Accepted February 18, 1997

ABSTRACT

The mouse p53 gene generates two alternative splice products encoding p53 protein and a naturally occurring protein (p53as) with changes at the C-terminus. In p53as the negative regulatory region for DNA binding and PAb421 antibody binding site are replaced, and p53as is constitutively active for sequence-specific DNA binding. Using the technique of randomized synthetic oligonucleotide in cyclic amplification and selection of targets, we have found that p53as and p53 proteins have the same DNA binding specificities but that these specificities frequently diverge from the consensus of two copies of PuPuPuCATGPyPyPy. The importance of tetranucleotide CATG was confirmed but there was a less rigorous requirement for patterns of flanking or intervening sequences. In particular, the three purines upstream and three pyrimidines downstream of CATG are not required for p53 or p53as binding, 29 or more intervening nucleotides are tolerated, and one CATG is sufficient where adjacent nucleotides contain a region of homology with certain previously reported non-consensus p53 binding sequences. These results suggested further definition of the non-consensus motifs, and database searches with these uncovered additional candidate genes for p53 protein binding. We conclude that p53as and perhaps other activated forms of p53 exert their effects on the same genes and that differential activities of p53 protein forms are not due to inherently different sequence selectivities of DNA binding.

INTRODUCTION

The tumor suppressor gene p53 encodes a protein with multiple functional domains, including an N-terminal region involved in transcriptional activation, a central hydrophobic sequence-specific DNA binding region and a C-terminal non-specific DNA binding region which can negatively regulate specific DNA binding. Most DNA binding studies of p53 require interaction with or modulation of a C-terminal inhibitory domain in p53. This is accomplished by addition of a C-terminal-specific antibody PAb421, which activates and shifts DNA–p53 complexes in electrophoretic mobility shift assays (EMSAs). The major form of p53 also can be activated by modifications of the C-terminus including phosphorylation, interaction with dnaK or truncation of the last 30 amino acids (1–3) or by non-specific binding of single-stranded DNA (4–8).

p53 functions as a transcription factor affecting the expression of genes involved in DNA repair, control of the cell cycle and apoptosis. DNA sequences which bind p53 and mediate its transcriptional regulation have been identified in the upstream promoter regions and introns of specific genes (9–17) and by *in vitro* studies using either genomic DNA fragments (18–20) or randomized oligonucleotides (21,22). These studies have delineated a consensus p53 binding sequence consisting of two copies of 5′-PuPuPuC(A/T)(T/A)GPyPyPy-3′ (19,22) which has been refined to two repeats of 5′-PuGPuCATGPyCPy-3′ (21).

Our laboratory has detected an alternatively spliced form of wild type p53 (designated p53as) in normal mouse cells and tissues (23,24) and shown that *in vitro* produced p53as constitutively binds to a p53 consensus sequence in gel shift experiments (6,25,26). p53as has 17 different amino acids at the C-terminus and is nine amino acids shorter than p53, resulting in losses of the PAb421 epitope, two of three nuclear localization signals, a 5.8S rRNA binding site and a casein kinase II phosphorylation site. Although the N-terminus and the central domain, which is responsible for sequence-specific interaction with DNA, are identical in the two proteins, other reports suggest that the C-terminus may be capable of modulating the specific DNA binding of p53 (1,2). p53as is preferentially associated with the G_2 phase of the cell cycle (24) while the p53 protein is associated with a G_1 arrest thought to be mediated by transcriptional activation of downstream genes (27,28). In addition, p53as responds to DNA damage with different kinetics compared to the major p53 form (Wu,Y. and Kulesz-Martin,M., unpublished).

These differences in properties and cell cycle expression of p53 and p53as suggest different functional specialization in cells. They could be explained by differences in the regulation of sequence-specific DNA binding due to the differences in their C-termini. Alternatively, p53as could have different DNA sequence specificity than p53 and exert its effects through differential transcriptional modulation of downstream genes. In order to test the hypothesis that the DNA sequence specificity of p53as and p53 are different, with implications for widening the search for specific p53as target genes, we sought p53as binding

*To whom correspondence should be addressed. Tel: +1 716 845 5918; Fax: +1 716 845 8857; Email: kulesz@S3101.med.buffalo.edu

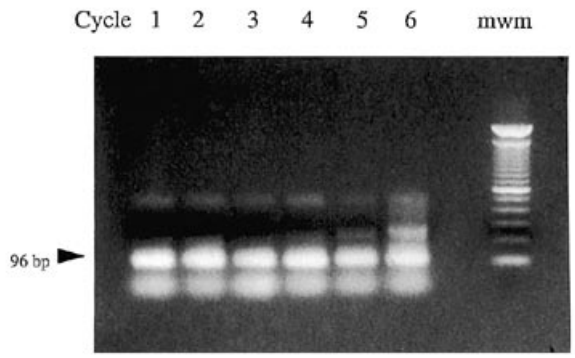

Figure 1. Agarose gel electrophoresis of the PCR products from CASTing cycles 1–6. Aliquots of PCR products were separated on a 1% agarose gel containing ethidium bromide. CASTing cycles 1–6 and the 100 bp ladder molecular weight marker (MWM) are indicated. The arrow indicates the expected 96 bp amplified DNA.

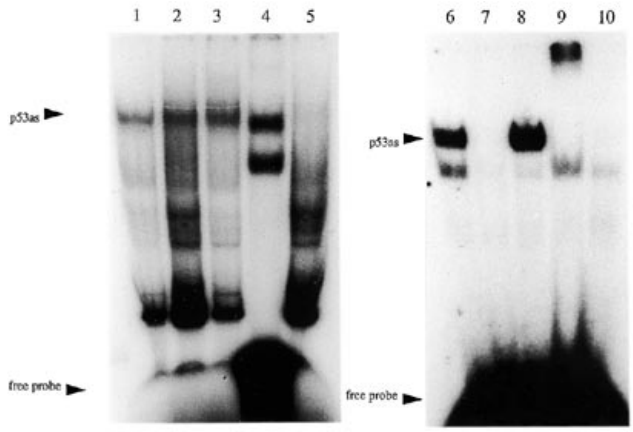

Figure 2. Autoradiogram from gel shift assays using CASTing cycles 3, 4 and 5. Aliquots of [α-^{32}P]PCR products as a binding probe for gel shift assays with *in vitro* translated p53as: lane 1, cycle 3; lane 2, cycle 4; lane 3, cycle 5. Lanes 4–10 contain [γ-^{32}P]p53 consensus probe (p53con). Lanes 4 and 6, p53as; lanes 5 and 10, reticulocyte lysate used in the *in vitro* translation; lane 7, p53as plus cold competitor p53 consensus DNA; lane 8, p53as plus cold competitor mutated p53 consensus DNA; lane 9, p53as plus ApAs antibody. Exposure time was 2 days at room temperature.

motifs using randomized synthetic oligonucleotides and the technique of cyclic amplification and selection of targets (CASTing) (29,30).

MATERIALS AND METHODS

Antibodies

Mouse monoclonal BC4-17 antibody and rabbit polyclonal ApAs antibody were generated against the unique 17 amino acids of p53as (24) and are available from Oncogene Research Products as Ab-9 and Ab-10 respectively. PAb421 (Oncogene Research Products) recognizes an epitope between amino acids 370 and 378 in the major form of p53 and absent in p53as.

In vitro transcription and translation

Plasmids and conditions used in these reactions were described previously (25).

CASTing

This procedure was essentially carried out as described (29,30) according to the following steps.

Polymerization of double-stranded DNA. A 96mer ssDNA was synthesized (Biopolymer Laboratory, Roswell Park Cancer Institute) beginning with an 18 nt primer site (5′-ATACCAGCT-TATTCAATT-3′) followed by 60 random bases and ending with an 18 nt primer site (5′-AGATAGTAAGTGCAATCT-3′) (31). Five µg of this oligonucleotide were made double-stranded by annealing a primer complementary to the 3′ flanking site (5′-AGATTGCACTTACTATCT-3′) in a 1:1 molar ratio followed by primer extension with 5 U *Taq* DNA polymerase in a standard reaction mixture (100 µl containing 10 mM Tris–HCl pH 8.3, 50 mM KCl, 1.5 mM MgCl, 0.001% gelatin and 200 µM of each deoxynucleoside triphosphate) at 94°C for 15 min, 60°C for 1 min and 72°C for 30 min.

Binding of p53as-specific antibody to magnetic beads. Twenty mg of sheep anti-mouse IgG coated magnetic beads (Dynabeads 280, Dynal Inc.) were washed in 5 ml PBS/0.1% BSA solution three times and resuspended at 30 mg/ml in the same solution. A 100 µl sample of the washed beads was added to 0.5 mg BC4-17 antibody and rocked at 4°C for 2.5 h. The beads were washed four times by adding 1 ml PBS/0.1% BSA, incubating at 4°C for 30 min, and recovering the beads with a magnet. Final resuspension was in PBS/0.1% BSA at a concentration of 30 mg beads/ml.

Association of dsDNA to p53as protein and antibody-coated magnetic beads. Double-stranded DNA (~5 µg) containing the randomized oligonucleotides was mixed with 4 µl of *in vitro* translated p53as protein and 2 µl buffer (20 mM HEPES pH 7.6, 100 mM KCl, 0.2 mM EDTA, 20% glycerol, 10 mg/ml BSA). After a 20 min incubation period at room temperature, 10 µl (300 mg) of the antibody-coated magnetic beads were added and the mixture gently agitated at room temperature for 60 min. Five hundred µl PBS containing 0.5% Nonidet P-40 and 0.1% BSA was added, the magnetic bead–p53as–DNA complexes were retrieved with a magnet and the supernatant removed. After three washes in the same solution, the bead–p53as–DNA complexes were washed once with PCR buffer (10 mM Tris–HCl pH 9.0, 50 mM KCl, 0.1% Triton X-100, 200 µM of each deoxynucleoside triphosphate, 1.9 mM $MgCl_2$). Final resuspension was in 40 µl PCR buffer.

Amplification of bound DNA sequences. Thirty µl of this resuspension were added to 1.2 mg of each primer and annealed in a thermal cycler (3 min at 80°C, 2 min at 94°C and 5 min at 46°C). During the period at 46°C, 0.5 µl *Taq* DNA polymerase (2.5 U, Boehringer Mannheim) and 0.5 µl Perfect Match (0.5 U, Stratagene) were added and 30 cycles of amplification (1 min at 94°C, 1 min at 46°C and 1 min at 72°C) followed by 10 min at 72°C carried out. Fourteen µl of the amplified DNA was used to initiate the next CASTing cycle. Six cycles were done. The last

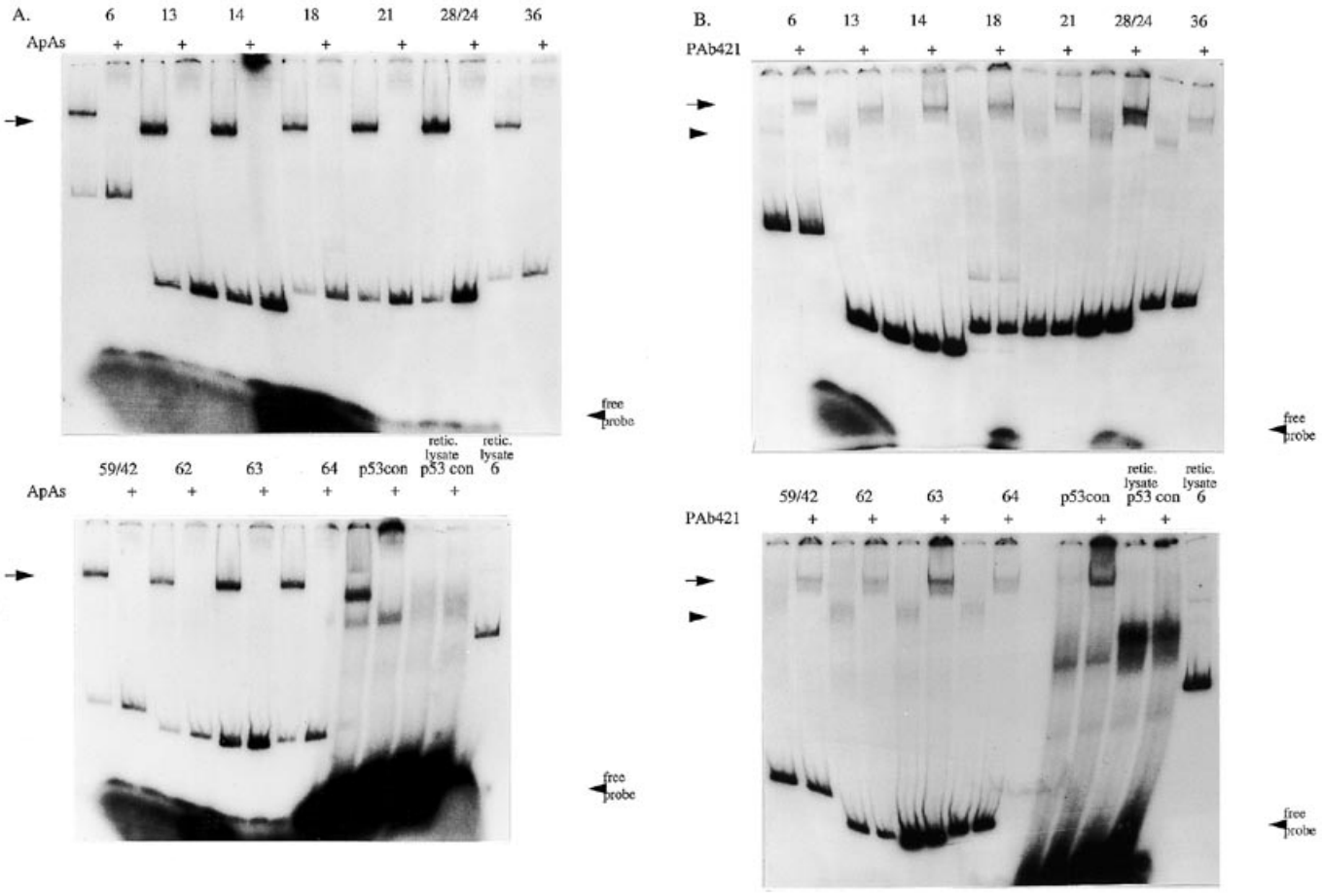

Figure 3. Autoradiogram from gel shift assays of *in vitro* translated p53as or p53 binding to end-labeled CASTing sequences. The number of each labeled CASTing sequence or the p53 consensus sequence (p53con) and the antibodies present in the DNA binding reactions are indicated. Fragments containing the CASTing sequences were excised from the plasmid, agarose gel purified and end-labeled. Lanes labeled 'retic. lysate' contain unprogrammed reticulocyte lysate used for *in vitro* translation. (**A**) Assays of p53as protein in the presence or absence of ApAs. Arrow indicates the specific p53as–DNA complex. Exposure time was overnight at room temperature. (**B**) Assays of p53 protein in the presence or absence of PAb421. The arrowhead indicates the expected position of an active p53–DNA complex; the arrow indicates the PAb421-activated and supershifted protein–DNA complex. Exposure time was 2 days with intensifying screens at –80°C.

four cycles also contained 10 µCi [α-^{32}P]dCTP during the amplifications.

Molecular cloning of PCR products

Cycle 5 was used for cloning to decrease the potential of ligating the higher molecular weight molecules observed in the greatest amounts in cycle 6. One µl of the cycle 5 PCR reaction was used with the TA Cloning Kit following the instructions of the manufacturer (Invitrogen) and transformants were plated in the presence of X-gal. Plasmid DNA isolated from white colonies was used in PCR reactions containing [α-^{32}P]dCTP followed by DNA binding assays to identify clones which contained a p53as binding sequence.

DNA binding assay

Fifteen µl reactions were carried out on ice for 30 min and contained 3 µl of an *in vitro* protein reaction, 10 µg poly[d(I–C)], 0.1 mg/ml BSA and 0.1 µl of a labeled PCR reaction (~100 000 c.p.m.) or 20 000–70 000 c.p.m. of an end-labeled DNA fragment in a buffer composed of 20 mM Tris–HCl pH 7.2, 80 mM NaCl, 1 mM EDTA, 5 mM DTT, 4% glycerol and 0.1% Triton X-100. Supershifting of protein–DNA complexes was carried out using 100 ng PAb421 or 200 ng ApAs. Reactions products were run on a 4% non-denaturing polyacrylamide gel in 0.5× TBE at 4°C, the gel dried and products visualized by autoradiography. A p53 consensus DNA binding sequence is used as a positive control (p53con) (19,25). A mutated p53 consensus DNA binding sequence was used for competition as has been previously described (19,25).

RESULTS

p53as-binding sequences obtained by CASTing

Aliquots of PCR reactions from six successive PCR cycles were observed by agarose gel electrophoresis as shown in Figure 1. The expected length of the bound DNA fragment was 96 nt and is indicated by the arrow. Increasing amounts of higher molecular weight products visible immediately above the putative specific product are commonly observed in successive cycles using this

Table 1. Sequences retrieved by CASTing

		R/Y	F	I	#I	*
6	ATGGGGG**CATG**AGATTAAATAACCCAAATGAGGG**CATG**CGC**CATG**TCTGGCCC	+/-	-	-	23,3	-
13	AGCGTCAACAAGCGATTAGACACACG<u>GAA</u>**CATG**<u>TCCGGA</u>**CATG**<u>TTC</u>TAGCAAGAGCGTCT	+	+	+	6	-
14	ATGGGGG**CATG**AGATTAATAACCCAAATGAGGGCATCCG**CATGCATG**<u>CCT</u>CGGCCC	+/-	-	-	28,0	-
18	CCACCGGGA**CATG**TCGTT<u>CTTG</u>TTTTTCTGAATGGA<u>CTTG</u>TTCGCATAAAGAGCCTGG	-	NA	NA	NA	+
21	GCCAACGAGTGAACGGGCATCCG**CATG**TTAA<u>CGGTTTTGGC</u>TTATCGCTGTGAT	-	NA	NA	NA	+
28/24	TATCACAGCGATAAGCCAAACCGATTAA**CATG**CG**CATG**<u>CCGGTTC</u>ACTC<u>TTGGCG</u>G (54N PRIMER) CAGAAGAC<u>TGCCCGGG</u>**CATG**TTCTACCAAACTGTTCCCCTTCCTCCTGTG	+/-	-	-	2,88	+
36	GCCAACGAGTGAACGGG**CATG**CG**CATG**TTAACGGA (18N PRIMER) GCCACGGACCTGATCACGGGTCTGTCTAAAG<u>CGGTTT</u>GAATCGT**CATG**GGTGAATGGTAA	-	-	-	2,70	+
59/42	GCCAACGAGTGAACGGG**CATG**GCG**CATG**TTAA<u>CGGTTTTGGC</u>TTATCGCTGTGGT	-	-	-	3	+
62	ACGTGTAA**CATG**<u>CCCGGG</u>ATGTTGTTCGAGCCCGAAGAGTCAAAGGGTTGAGACTAGGC	-	NA	NA	NA	-
63	A**CATG**AACAGGTTTAAGGTTAATGA**CATG**<u>CCCGGG</u>**CATG**CCGCGGTCTTTCAGTAGGGT	-	-	+	20,6	-
64/38	AGCCGCT<u>CTTG</u>CTAG<u>AA</u>**CATG**<u>TCCGGA</u>**CATG**<u>TTC</u>GTGTGTCTATCG<u>CTTG</u>TTGACGCT	+	+	+	6	+

Underlined sequences are motifs shared with known p53 binding sequences shown in Table 3. CATG sequences are in bold.
R/Y, + indicates three purines upstream and three pyrimidine downstream of CATGs; +/– indicates some, but not all, of the upstream or downstream sequences conform to three purines upstream and three pyrimidines downstream of CATGs; – indicates none of the upstream or downstream nucleotides conform to three purines upstream and three pyrimidines downstream of CATGs.
F, 3 nt flanking CATGs hybridize.
I, intervening sequences (between CATGs) hybridize.
#I, number of intervening nucleotides between CATG sequences.
*, homology with MgBH6 (ref. 20); see Tables 3 and 4.

technique, and have been attributed to futile cycling due to limiting reagents (29,30). The lowest molecular weight products are primer dimers.

Figure 2 depicts gel shift assays carried out with aliquots of labeled PCR reactions from cycles 3, 4 and 5 (as in Fig. 1) and *in vitro* translated p53as protein. The arrow indicates the shifted band containing p53as present in each cycle, which migrates similarly to the p53as bound to a consensus p53 binding sequence shown in lane 4. The specificity of this band was determined by competition with cold probe DNA (abrogation of band by wild type p53 binding consensus sequence, lane 7, but not with mutated sequence, lane 8) and by supershifting with ApAs antibody (lane 9). The faster migrating band visible in lanes 4 and 6 was non-specific as it was not supershifted in the presence of ApAs.

An aliquot of cycle 5 was used to generate clones of these putative p53as binding sequences for verification of p53as binding by gel shift assays, comparison of binding of p53as and p53, and sequence analysis of binding motifs. Plasmid DNA was isolated from 64 transformants and used in PCR reactions containing [α-^{32}P]dCTP. The labeled PCR products were used in gel shift assays with *in vitro* translated p53as. Fourteen of the 64 clones were verified positive for p53as binding. Their randomized 60 bp inserts were sequenced on both strands and results are presented in Table 1. Flanking primer sequences were present in all clones and are not shown. Three clones were represented twice (28/24, 59/42 and 64/38). Common artifacts when cloning PCR products were observed in several clones but are unlikely to affect the analysis: several clones had two or more copies of the primers at either or both ends, and clones 28/24 and 36 had two randomized 60 bp segments separated by multiple primers, as indicated in Table 1. The 11 unique cloned sequences were verified for binding to p53as and also were found to bind p53. Figure 3A depicts an autoradiogram of gel binding assays containing the 11 unique CASTing sequences and p53as protein in the presence or absence of ApAs. An arrow indicates the specific protein–DNA complex which is abrogated or supershifted upon the addition of ApAs (as previously reported ref. 25). Slight differences in mobility of bands reflect differences in the labeled probes. The mobilities of non-specific bands are not affected by the addition of ApAs. Figure 3B shows the same labeled CASTing sequences in gel shift assays containing the regular form of p53 in the presence or absence of PAb421. As expected, little of the *in vitro* translated p53 is in the active form and able to bind DNA (arrowhead) unless PAb421 is present for activation and supershifting (arrow). The presence of doublets in the supershifted complexes has been occasionally observed and is reticulocyte lysate dependent. It also may reflect the ability of these relatively long DNA probes to bind more than one p53 tetramer.

All 11 of the non-duplicated p53as binding sequences analyzed contained tetranucleotide CATG and eight of the 11 contained two or more CATG motifs (shown in bold print in Table 1), as found in the p53 refined consensus sequence (′5′-PuGPuCATG-PyCPy-3′) (21). Only two of these, designated 13 and 64/38, fit the consensus sequence with respect to three purines followed by a CATG followed by three pyrimidines. In both these cases complementarities also were created by the flanking sequences and within the intervening 6 nt (those between the two CATGs), a property shared by two published consensus sequences(19,22). Neither 13 nor 64/38 fit the refined consensus requirement for ‘GPu’ immediately upstream and ‘PyC’ immediately downstream of the CATG (21). Two sequences, 6 and 14, did adhere to this requirement if only the 3 nt upstream of the first CATG and the

Table 2. Synthetic DNA sequence tested in gel shift assays with p53 and pS3as proteins translated *in vitro*

				p53/p53as binding
1CATG	CAAACGCGTAGC	GGA**CATG**TCC	TGCGCGACAGGTCC	-
2CATG0	CAAACGCGTAGC	**CATGCATG**	TGCGCGACAGGTCC	-
2CATG1	CAAACGCGTAGC	**CATG**C**CATG**	TGCGCGACAGGTCC	-
2CATG3	CAAACGCGTAGC	**CATG**CCA**CATG**	TGCGCGACAGGTCC	-
2CATG6	CAAACGCGTAGC	GGA**CATG**TCCGGA**CATG**TCC	TGCGCGACAGGTCC	+
2CATG29	GGA**CATG**TCC	CAAACGCGTAGCTGCGCGACAGGTCC	GGA**CATG**TCC	+
WAF-1	CAAACGCGTAGC	**CATG**TCCCAA**CATG**	TGCGCGACAGGTCC	+
mWAF-1		GAA**CATG**CCCAA**CATG**AA		+
CCCGGG	CAAACGCGTAGC	**CATG**CCCGGG	TGCGCGACAGGTCC	-

The flanking sequences used in all oligonucleotides were described previously (21). p53 was tested in the presence or absence of PAb421 and bound only in its presence. WAF-1 has the wild type intervening sequence but not the flanking sequences. mWAF-1 has changes in intervening and flanking sequences compared with WAF-1 (see Table 3). CCCGGG has only one CATG sequence upstream of a hepta-nucleotide found in other published p53 binding sequences (see Table 3).

Table 3. Known p53 binding sequences

		p53as binding	R/Y	F	I	#I	*
WAF-1	GAA**CATG**TCCCAA**CATG**TTG	+	+/-	-	-	6	-
P53CON	GAA**CATG**CCCGGG**CATG**TCC	+	+	+	+	6	-
MgBH6	GACACTGGTCACACTTGGCTGCTTAGGAAT	ND	NA	NA	NA	NA	+
CYCLIN G	AGGCCAGACCTGCCCGGGCAAGCCTTGGCA	+	NA	NA	NA	NA	+
RGC	GTTTGCCTTGCCTGGACTTGCCTGGCCTTGCCTT	+/-	NA	NA	NA	NA	+/-
50-2	TGGCAAGCCTATGA**CATG**GCCGGGGCCTGCCTCTCTCTGCCTCTGACCCT	ND	NA	NA	NA	NA	-

CATGs are shown in bold; underlined sequences are motifs shared with CASTing sequences.
R/Y, + indicates three purines upstream and three pyrimidine downstream of CATGs; +/– indicates some, but not all, of the upstream or downstream sequences conform to three purines upstream and three pyrimidines downstream of CATGs.
F, 3 nt flanking CATGs hybridize.
I, intervening sequences (between CATGs) hybridize.
#I, number of intervening nucleotides between CATG sequences.
*, homology with MgBH6 (ref. 20).

3 nt downstream of the last CATG are considered (indicated +/– in column R/Y, Table 1). Three sequences, 18, 21 and 62, contained only one CATG although two of these, 18 and 62, also had CTTG or CTAG respectively. This is consistent with the less stringent consensus sequence (5′-PuPuPuC(A/T) (T/A)GPy-PyPy-3′) (19,22) but, unlike CATG itself, is not a perfect hybrid with the upstream CATG. Sequence 21 had only one CATG and did not have an additional C(A/T)(T/A)G. Furthermore, eight of the 11 sequences deviated from the consensus in that the CATG was either not repeated, or the intervening nucleotides numbered <6 or >19. Therefore we designed synthetic oligonucleotides to test these points further.

DNA binding assays using synthesized DNA

Several oligonucleotides and their complement sequences were synthesized, annealed and used in DNA binding assays with *in vitro* translated p53as or p53. The sequences used and the results of these assays are shown in Table 2. Each oligonucleotide contains a central test sequence flanked by a common unrelated sequence (21). p53as binding corresponded to p53 binding (in the presence of PAb421), so both are shown together.

One copy of 5′-PuGPuCATGPyCPy-3′ was insufficient to confer p53as binding, but two copies of CATG with five (mWAF-1) or six (WAF-1) intervening nucleotides was sufficient for binding even without internal symmetry or consensus flanking sequences. Binding was not observed when either 0, 1 or 3 intervening nt were present, indicating that the CATGs separated by 0–3 nt in CASTing sequences 6, 14, 28/24, 36 and 59/42 were not sufficient for the observed p53as–p53 binding and that regions within the longer stretches between CATGs (ranging from 23 to 88 nt) or sequences other than CATG (sequence 59/42) must be involved. DNA sequences containing two copies of

Table 4. Summary of motifs and sequences in which each is found

Motif	Casting Sequences	Published Sequences	References
PuPuPuCA/TT/AGPyPyPy	13, 64/38		(19,22)
GAACATGTCCGGACATGTTC	13, 64/38		
TTGGC*	21, 28/24, 59/42 (inverted repeats) and 36	MgBH6	(20)
CTTG	18, 28/24, 64/38)	cyclin G, RGC, MgBH5	(16,18,20)
ACTTG	18, 28/24 (inverted repeats)	RGC, MgBH6	(18,20)
TGCCT	14	RGC, 50-2	(18,32)
TGCCCGGG	28/24, 62, 63	cyclin G, p53con	(16,22)
TGTCC	13, 64/38	WAF-1, p53con	(13,22)
GGCAAGCCT	none	cyclin G, 50-2	(16,32)

*, extended to TTGGCT in 21, 59/42 and MgBH6.

5′-PuGPuCATGPyCPy-3′ in which the CATG sequences are separated by 6 or 29 nt (2CATG6 and 2CATG29) were bound by both proteins, supporting the results with CASTing sequences 6, 14, 28/24 and 36 where the number of intervening nucleotides was either <5 or >23.

The results with synthetic probes did not answer the question of whether two CATG motifs separated by >29 nt are sufficient for p53as–p53 binding. In addition, the probe designated CCCGGG for the sequence adjacent to the only CATG in sequence 62 [and also found in the p53 binding sites of p53con (22) and the cyclin G promoter (16)] was not bound by either protein, indicating that other sequences are necessary for p53as–p53 binding. In the case of sequence 62 the other motif may be the CTAG present 41 bp downstream of the CATG. The need for additional unidentified sequence(s) for binding must be invoked for the sequences 21, which has only one CATG, and 59/42, which has two CATG sequences separated by 3 nt and no additional C(A/T)(T/A)G motifs. Therefore, we examined the sequences retrieved by CASTing for homology with other reported p53 binding sequences presented for reference in Table 3.

Sequence comparison with other known p53 binding sequences

All of the sequences except CASTing sequence 6 (which had three CATGs separated by 23 and 3 nt) contained one or more copies of regions found in reported p53 binding sequences. Of the sequences which were tested, all bound to p53as as indicated in Table 3. The homologous regions from these reported sequences and the sequences retrieved by CASTing with p53as protein are underlined in Table 3 and summarized in Table 4. As noted above, we were particularly interested in whether other non-consensus p53 binding motifs were present in the six out of 11 sequences in which CATG was unique (sequences 18, 21 and 62) or in which two CATGs were separated by <5 (59/42) or >29 (28/24, 36) nt. It was striking that five of these six sequences (all but sequence 62) contained a region of homology to the non-consensus sequence, MgBH6 (20) (see the column headed * in Table 1). Cyclin G and RGC also contain regions of homology to MgBH6 (see Table 3). As detailed previously, the p53 binding sequence MgBH6 was obtained from mouse fibroblast DNA and significant sequence homology to known genes was not found at the time. Furthermore, the entire region shown in that report was protected by p53 protein produced in *Escherichia coli* in DNA footprinting assays and conferred activation by p53 of a reporter gene in transcription assays in cells (20).

Affinity of p53 and p53as for DNA binding

Two of the synthesized DNAs that were positive for binding, 2CATG6 and 2CATG29, were examined for differences in affinity between *in vitro* translated p53 and p53as. Gel shift assays that used 2 ng ^{32}P-labeled consensus sequence (see Materials and Methods) probe were subjected to increasing amounts of 2CATG6 or 2CATG29 unlabeled probe. Figure 4 shows the autoradiogram from one of several such experiments. The affinity of both proteins for 2CATG6 was greater than for 2CATG29 since 2 ng cold 2CATG6 probe was able to compete all binding of the labeled consensus sequence probe while 10 ng cold 2CATG29 did not completely compete binding to the labeled probe for either protein.

DISCUSSION

Previous studies have shown that p53 binds two repeats of 5′-PuPuPuC(A/T)(T/A)GPyPyPy-3′ and that the spacing between these repeats ranged from 0 to 13 nt (19), which is equivalent to 6–19 nt between CATGs. The independent retrieval of p53as binding sequences confirms certain features of published p53 DNA binding and indicates a lack of strict requirement for others as follows: (i) confirms one or more CATG motifs; (ii) no strict requirement either for specific sequence or for secondary structure of the internal nucleotides between two copies of CATG; (iii) lack of requirement for three purines preceding the CATG or three pyrimidines following the CATG; (iv) no requirement for flanking nucleotide triplets to contain particular nucleotides or secondary structure; and (v) intervening space between two CATGs can contain 29 nt or more. p53as and p53

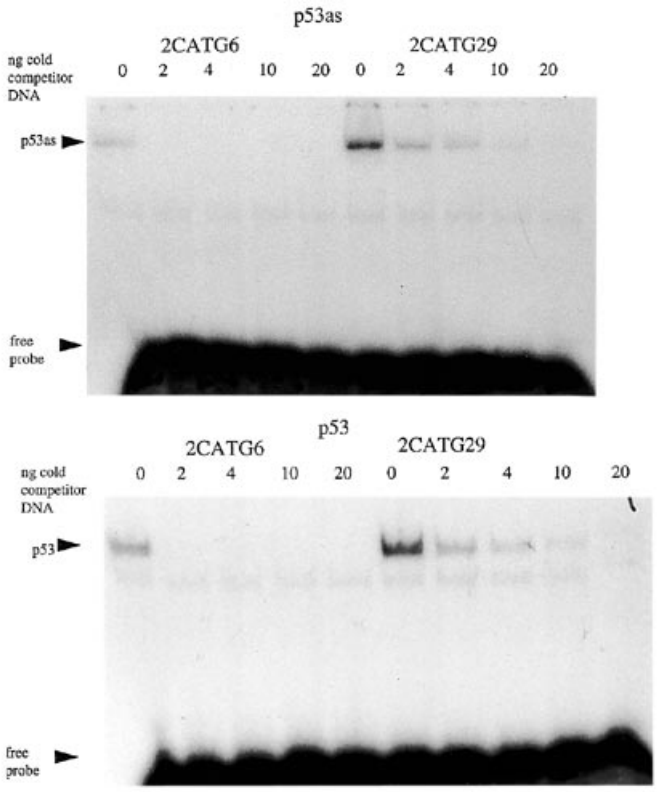

Figure 4. Autoradiogram of gel shift assays containing *in vitro* translated p53as or p53, [γ-^{32}P]p53 consensus probe, and increasing amounts of cold competitor 2CATG6 or 2CATG23 DNA as indicated. The positions of p53as or PAb421-shifted p53 DNA are indicated. Exposure time was overnight at room temperature.

proteins showed the same sequence specificities and the affinities of binding to sequences with CATGs separated by 6 or 29 intervening nt was the same for both proteins.

All of the CASTing sequences contained one or more copies of CATG. Two of the retrieved sequences, 13 and 64/38, fit the consensus sequence (19,22), and three additional sequences, 6, 14 and 63, fit a refined consensus (21) with respect to the placement of G 2 nt upstream and C 2 nt downstream of a CATG. However two (6,14) of these five sequences with similarities to the consensus had more than the reported 19 nt between the CATG motifs and no additional reported p53 binding motifs, supporting the results with synthetic oligos and the conclusion that ≤29 bp between CATGs are tolerated. Of the six additional sequences (18, 21, 28/24, 36, 59/42, 62) which did not fit the consensus, having only one CATG or <5 or >19 nt between two CATG motifs, five contained a region of homology with the p53 binding sequence MgBH6 (20). The remaining sequence, 62, contained a CATGCCCGGG sequence and a downstream CTAG with additional intervening potential for secondary structure. Both p53as and p53 bound a sequence from the cyclin G promoter (Wu,Y., unpublished) which contains one copy each of TGCCCGGG (found in p53con) and TTGGC (found in MgBH6) but does not contain CATG (16). The sequences TGCCT, TGTCC and TGCCC either are adjacent to or overlap a C(A/T)(T/A)G consensus in retrieved sequences and in all but one of the reported consensus sequences noted, making their significance ambiguous. The exception, 50-2, contains no overlaps with a C(A/T)(T/A)G consensus, but does contain one CATG upstream of two copies of TGCCT. While not tested by gel binding assays, the p53as protein transcriptionally activated a reporter via the 50-2 sequence (32) shown in Table 3 (Huang,H. and Kulesz-Martin,M., unpublished).

The affinity of p53as and p53 for CATG separated by 29 nt was within the range observed for other published p53 binding sites such as RGC (18) and WAF-1 (13). However, one study reports that DNA binding sequences that contained >10 nt between the CATG motif were transcriptionally inactive when tested by *in vivo* assays in yeast (33) suggesting that *in vitro* DNA binding does not necessarily convey *in vivo* function.

The sequences retrieved by CASTing for p53as binding further defined p53 binding motifs and suggest additional combinations for database searches of potential p53 target sites. We performed FASTA searches of the GenBank and EMBL databases using several combinations of the motifs found in the p53as retrieved sequences or shared with known p53 binding sequences. The identical consensus-like sequence (GAA**CATG**TCCGGA-**CATG**TTC) independently retrieved in sequences 13 and 64/38 was not found as a perfect match to any known sequence; however, homologous sequences were found in many genes including the 5′ promoter region of AP-1 (accession no. L16546, a rat homologue of human multidrug resistance gene), and the 3′ region of mRNA from the human ICAM gene (accession J03132). An inverted repeat, CGGTT-TTGGC, of a segment (TTGGC) of the published sequence MgBH6 (20) which is also present in 21, 28/24 and 59/42 was found in intron 6 of the thrombospondin gene (accession no. J05605), the human CMV enhancer region (accession no. M64944) and in the coding region of a human homologue of dnaJ (accession no. D13388). Certain of these genes are transcriptionally modulated by p53 or their family member proteins are known to bind p53. We speculate that searches of combinations of motifs more specifically defined by p53as binding in the current studies is warranted, followed by testing of any naturally occurring regions for p53 or p53as binding, transcriptional activity of reporter genes in mammalian cells, and p53as- or p53-dependent modulation of endogenous gene expression.

We conclude from these results that the DNA binding sequences of p53as and p53 are the same. However, since p53as is constitutively active for DNA binding, and p53 oligomerization with p53as blocks that binding (25), the p53:p53as ratio may be important in regulating expression of transcriptional target genes. We conclude further that any differences in the function of p53 and p53as proteins must result not from inherent differences in DNA sequence specificity of binding, but from differential expression of the proteins or from differential regulation of their function by modifications at the C-terminus, either by post-translational modifications of amino acids present in p53 but not in p53as and/or by differential binding of p53-associated proteins.

ACKNOWLEDGEMENTS

We are grateful to Jean Seo for her help in beginning this project and Dr Bruce Dolnick for helpful suggestions. This work was supported by a US Army Department of Defense Medical Research Grant DAMD17-94-J-4341 and by Roswell Park Cancer Institute NIH Core Grant CA16056.

REFERENCES

1 Hupp,T.R., Meek,D.W., Midgley,C.A. and Lane,D.P. (1992) *Cell*, **71**, 875–886.
2 Hupp,T.R., Meek,D.W., Midgley,C.A. and Lane,D.P. (1993) *Nucleic Acids Res.*, **21**, 3167–3174.
3 Hupp,T.R. and Lane,D.P. (1994) *Cold Spring Harbor Symp. Quant. Biol.*, **59**, 195–206.
4 Wu,L., Bayle,J.H., Elenbaas,B., Pavletich,N.P. and Levine,A.J. (1995) *Mol. Cell. Biol.*, **15**, 497–504.
5 Bakalkin,G., Selivanova,G., Yakovleva,T., Kiseleva,E., Kashuba,E., Magnusson,K.P., Szekely,L., Klein,G., Terenius,L. and Wiman,K.G. (1995) *Nucleic Acids Res.*, **23**, 362–369.
6 Bayle,J.H., Elenbaas,B. and Levine,A.J. (1995) *Proc. Natl. Acad. Sci. USA*, **92**, 5729–5733.
7 Jayaraman,L. and Prives,C. (1995) *Cell*, **81**, 1021–1029.
8 Hupp,T.R., Sparks,A. and Lane,D.P. (1995) *Cell*, **83**, 237–245.
9 Weintraub,H., Hauschka,S. and Tapscott,S.J. (1991) *Proc. Natl. Acad. Sci. USA*, **88**, 4570–4571.
10 Miyashita,T. and Reed,J.C. (1995) *Cell*, **80**, 293–299.
11 Shivakumar,C.V., Brown,D.R., Deb,S. and Deb,S.P. (1995) *Mol. Cell. Biol.*, **15**, 6785–6793.
12 Dameron,K.M., Volpert,O.V., Tainsky,M.A. and Bouck,N. (1994) *Cold Spring Harbor Symp. Quant. Biol.*, **59**, 483–490.
13 El-Diery,W.S., Tokino,T., Velculescu,V.E., Levy,D.B., Parsons,R., Trent,J.M., Lin,D., Mercer,W.E., Kinzler,K.W. and Vogelstein,B. (1993) *Cell*, **75**, 817–825.
14 Kastan,M.B., Zhan,Q., El-Diery,W.S., Carrier,F., Jacks,T., Walsh,W.V., Plunkett,B.S., Vogelstein,B. and Fornace,A.J.,Jr. (1992) *Cell*, **71**, 587–597.
15 Miyashita,T., Harigai,M., Hanada,M. and Reed,J.C. (1994) *Cancer Res.*, **54**, 3131–3135.
16 Okamoto,K. and Beach,D. (1994) *EMBO J.*, **13**, 4816–4822.
17 Wu,X., Bayle,J.H., Olson,D. and Levine,A.J. (1993) *Genes Dev.*, **7**, 1126–1132.
18 Kern,S.E., Kinzler,K.W., Bruskin,A., Jarosz,D., Friedman,P., Prives,C. and Vogelstein,B. (1991) *Science*, **252**, 1708–1711.
19 El-Diery,W.S., Kern,S.E., Pietenpol,J.A., Kinzler,K.W. and Vogelstein,B. (1992) *Nature Genet.*, **1**, 45–49.
20 Foord,O., Navot,N. and Rotter,V. (1993) *Mol. Cell. Biol.*, **13**, 1378–1384.
21 Thukral,S.K., Lu,Y., Blain,G.C., Harvey,T.S. and Jacobsen,V.L. (1995) *Mol. Cell. Biol.*, **15**, 5196–5202.
22 Funk,W.D., Pak,D.T., Karas,R.H., Wright,W.E. and Shay,J.W. (1993) *Mol. Cell. Biol.*, **12**, 2866–2871.
23 Han,K. and Kulesz-Martin,M.F. (1992) *Nucleic Acids Res.*, **20**, 1979–1981.
24 Kulesz-Martin,M.F., Lisafeld,B., Huang,H., Kisiel,N.D. and Lee,L. (1994) *Mol. Cell. Biol.*, **14**, 1698–1708.
25 Wu,Y., Liu,Y., Lee,L., Miner,Z. and Kulesz-Martin,M. (1994) *EMBO J.*, **13**, 4823–4830.
26 Wolkowicz,R., Peled,A., Elkind,N.B. and Rotter,V. (1995) *Proc. Natl. Acad. Sci. USA*, **92**, 6842–6846.
27 Lane,D.P. (1992) *Nature*, **358**, 15–16.
28 Donehower,L.A. and Bradley,A. (1993) *Biochim. Biophys. Acta*, **1155**, 181–205.
29 Wright,W.E., Binder,M. and Funk,W. (1991) *Mol. Cell. Biol.*, **11**, 4101–4110.
30 Brown,L. and Baer,R. (1994) *Mol. Cell. Biol.*, **14**, 1245–1255.
31 Crameri,A. and Stemmer,W.P.C. (1993) *Nucleic Acids Res.*, **21**, 4410.
32 Zambetti,G.P., Bargonetti,J., Walker,K., Prives,C. and Levine,A.J. (1992) *Genes Dev.*, **6**, 1143–1152.
33 Tokino,T., Thiagalingam,S., El-Diery,W.S., Waldman,T., Kinzler,K.W. and Vogelstein,B. (1994) *Hum. Mol. Genet.*, **3**, 1537–1542.