Oncogene (1997) 15, 857–869

DNA-conformation is an important determinant of sequence-specific DNA binding by tumor suppressor p53

Ella Kim, Nils Albrechtsen and Wolfgang Deppert

Heinrich-Pette-Institut für Experimentelle Virologie und Immunologie an der Universität Hamburg, Martinistr. 52, D-20251 Hamburg, Germany

Sequence-specific transactivation of target genes is one of the most important molecular properties of the tumor suppressor p53. Binding of p53 to its target DNAs is tightly regulated, with modifications in the carboxy-terminal regulatory domain of the p53 protein playing an important role. In this study we examined the possible influence of DNA structure on sequence-specific DNA binding by p53, by analysing its binding to p53 consensus elements adopting different conformations. We found that p53 has the ability to bind to consensus elements which are present in a double-helical form, as well as to consensus elements which are located within alternative non-B-DNA structures. The ability of a consensus element to adopt either one of these conformations is dependent on its sequence symmetry, and is strongly influenced by its sequence environment. Our data suggest a model according to which the conformational status of the target DNA is an important determinant for sequence-specific DNA binding by p53. Modifications in the carboxy-terminal regulatory region of p53 possibly determine the preference of p53 for a given DNA conformation.

Keywords: p53-DNA binding; DNA conformation; p53 conformation; p53 consensus sequence

Introduction

Interaction with DNA in a sequence-specific manner is an important biochemical activity of the tumor suppressor p53, and is crucial for its ability to function as a transcription factor (reviewed in Donehower and Bradley, 1993; Deppert, 1994; Lane, 1994; Ko and Prives, 1996). The importance of DNA binding for the tumor suppressor function of p53 is underscored by the fact that most of the mutations in the p53 gene found in tumors affect the core domain of the p53 protein, which mediates sequence-specific DNA-binding (Kern *et al*., 1991; Bargonetti *et al*., 1993; Wang *et al*., 1993; Prives *et al*., 1994). The resolution of the three-dimensional co-crystal structure of the p53 core domain bound to a single half-site of a p53 consensus sequence (Cho *et al*., 1994) then revealed that amino acid residues most frequently mutated in human cancer occupy positions critical for sequence-specific DNA binding (Cho *et al*., 1994; Arrowsmith and Morin, 1996; Soussi and May, 1996), further strengthening the hypothesis that DNA binding and transactivation are required for tumor suppression by p53. The consensus for p53 binding sites consists of two copies (half-sites) of the sequence 5′-PuPuPuC(A/T)(T/A)GPyPyPy-3′, separated by 0 to 13 bp (El-Deiry *et al*., 1992). Binding of full-length p53 to its consensus DNA is complex, as the binding of such DNA via the p53 core domain is tightly regulated. An important role in this regulation is exerted by the carboxy-terminal regulatory domain of the p53 protein. According to our current understanding, modifications in the p53 carboxy-terminus which lead to a spatial separation of this regulatory domain from the p53 core domain result in the activation of the latent DNA binding properties of p53 (Hupp *et al*., 1992; Hupp and Lane, 1994; Wang and Prives, 1995; Waterman *et al*., 1995; Arrowsmith and Morin, 1996). Consequently, deletion of the carboxy-terminus abolishes negative regulation, and therefore leads to constitutive activation of p53 for sequence-specific DNA binding (Hupp *et al*., 1992). While the basic features of p53-DNA interactions are known, it is still poorly understood which parameters determine the efficacy and selectivity of these interactions. Sequence-specific DNA binding of p53 seems to be modulated by the phosphorylation state of its Cdk phosphorylation site (ser313 in mouse, or ser315 in human p53, respectively), supposedly influencing the conformation of the p53 core domain (Wang and Prives, 1995; Hecker *et al*., 1996). Interestingly, the binding to different p53 binding sites was differently affected by the same type of modification (Wang and Prives, 1995; Hecker *et al*., 1996). This suggested that, in addition to the conformational status of the p53 protein, the nature of the binding site itself might be another important parameter for determining the sequence-specific interactions of p53 with DNA. In agreement with the view that the p53 binding site itself, in addition to conforming to the consensus, provides features which determine its binding by p53, is the finding that some sequences are efficiently bound by full length p53 without prior activation of the protein (Kern *et al*., 1991; Halazonetis *et al*., 1993; Foord *et al*., 1993; Wu *et al*., 1993; Ludes-Meyers *et al*., 1996), while others strictly require activation of p53 for being bound (Hupp *et al*., 1992; Miyashita and Reed, 1995; Zhao *et al*., 1996). Furthermore, data obtained with synthetic oligonucleotides suggested that there is a hierarchy among p53 binding sequences, as not all sequences that perfectly match the consensus are bound by p53 with the same or even similar efficiency (Halazonetis *et al*., 1993). On the other hand, some sequences containing multiple mismatches, or even barely fitting the consensus are bound with high affinity (Bargonetti *et al*., 1991; Foord *et al*., 1993). Thus p53 binding sites operationally can be divided into 'low affinity' and 'high affinity' sites. However, so far it is poorly understood what distinguishes high

Correspondence: W Deppert
Received 30 May 1997; accepted 11 July 1997

affinity sites from low affinity sites. In this study we raised the question, whether structural features of the DNA substrate, in addition to conforming to the consensus, might determine sequence-specific binding by p53. Our finding that the ability of a given binding site to adopt a B- or a non-B-conformation greatly influences p53 DNA binding suggests a new model for understanding the regulation of p53-DNA interactions.

Results

Comparative analysis of natural p53 binding sites

The consensus for p53 binding sites has been established as two copies (half-sites) of the sequence 5′-PuPuPuC(A/T)(T/A)GPyPyPy-3′, separated by 0 to 13 bp (El-Deiry *et al*., 1992). A characteristic feature of this consensus is its rather low stringency, as only two out of 10 bases (C at position 4, and G at position 7, starting from the 5′-end) are conserved. This in turn suggests the existence of a large pool of potential p53 responsive elements with differing sequences, which indeed is the case for the sequences of the so far identified natural p53 responsive elements (Table 1a). The heterogeneity of these sites is even more pronounced, as the vast majority of natural binding sites contains bases that do not fit the consensus (Table 1a and b). Another factor adding to the heterogeneity of p53 binding elements is the variability in number and arrangement of half-sites in different p53 binding elements. Although p53 binding sites typically consist of two copies of the consensus half-site (Table 1a), many sites contain more than two half-sites (Table 1b). Such a variability in the sequence and in the arrangement of p53 binding sites is surprising, as it suggests a lack of stringency in sequence-specific recognition of DNA by p53 and, correspondingly, a somewhat 'relaxed' recognition of DNA by p53. This, however, is hard to imagine for such a protein as p53, whose crucial role in determining the destiny of cells after DNA damage strongly depends on its ability to specifically transactivate the appropriate target genes. Indeed, experimental data indicate that the interactions of p53 with its cognate target-sites are highly specific, as single base substitutions violating the homology of the consensus completely abolish binding (Halazonetis *et al*., 1993; El-Deiry *et al*., 1992). Furthermore, even some legitimate single-base substitutions that obey the consensus may lead to the failure of these DNAs to be bound by p53 (Halazonetis *et al*., 1993; and Table 1c). However, the effects caused by different single-base substitutions remain unclear, since there is no obvious correlation between the position of a certain base in a certain position of the cognate site, and its recognition by p53. For example, binding of p53 to the high affinity site BB.7, identified by Halazonetis *et al*. (1993), was completely abolished when G in position 2 (G^2) was substituted by A (A^2) in BB.13 DNA (see Table 2a). This can not simply be explained by the inability of p53 to bind sites that contain A^2, as in some natural binding elements this position is occupied by A (e.g. in the p53 binding elements of gadd45, p21, EGF, see Table 1a). These considerations suggest that fitting the consensus is necessary but not sufficient for a particular sequence to be recognized and bound by p53, and that some additional features other than fitting the consensus in the sequence of a binding site might have an impact on the sequence-specific interaction of p53 with DNA.

Comparison of the known p53 binding elements revealed that most of them, despite their sequence heterogeneity, share as a common feature an internal symmetry within the binding site (see Table 2b). A characteristic feature of sequences possessing an internal symmetry is their capacity to undergo structural transitions from B- to non-B DNA. Thus the presence of two or more symmetric half-sites in most p53 binding sites provides these DNAs with the ability to adopt different conformational isoforms. As

Table 1 **a** Heterogeneity of different natural binding elements in their sequences and in the extent of homology to the consensus

5′-PuPuPuC(A/T)(T/A)GPyPyPy-N_{0-13}-PuPuPuC(A/T)(T/A)GPyPyPy-3′

Site	*Sequence*	*Half-sites*	*Homology to the consensus [%]*	*Reference*
gadd 45	GAACATGTCT AAGCATGCTg	2	100/90	Kastan *et al*., 1992
Cyclin G	AcGCAAGCCC GGGCTAGTCT	2	90/100	Zauberman *et al*., 1995
p21	GAACATGTCC cAACATGTTg	2	100/80	El-Deiry *et al*., 1993
EGF	GAGCTAGacgTCC GGGCAgcCCC	2	100/80	Ludes-Meyers *et al*., 1996
CKM	GGGCAAGCagcacGcCTgGTTT	2	80/70	Zhao *et al*., 1996

b Many natural p53 responsive elements are composed of more than two halfsites

RGC	tGcCTTGCCT GGACTTGCCT GGcCTTGCCT	3	80/100/90	Kern *et al*., 1991
MCK	tGGCAAGCCTatGACATGgCCgGGGCcTGCCT	3	90/80/90	Zambetti *et al*., 1992
SV40	GGGCggGggCggGGGCggGggCggGGGCggGggC	3	60/60/60	Bargonetti *et al*., 1991
mdm2	GGtCAAGTTg GGACAcGTCC	4	80/90	Wu *et al*., 1993
	-17 bp-			
	GAGCTAaGTCCtGACATGTCT		100/90	
bax	GGGCTAtaTT	3	80	Miyashita and Reed, 1995
	tcACAAGTTagAGACAAGCCT GGGCgTGggCTAtaTT		70/100/70	

p53 binding sites were selected according to different homologies (expressed in % homology per each half-site) with regard to the p53 consensus sequence determined by El-Deiry *et al*. (1992) and shown above the Table 1a. Sequences within natural p53 responsive elements that conform to the consensus are typed in bold capital letters and underlined; small characters correspond to those bases within binding elements that do not match the consensus.

Table 2 **a** Fitting the consensus is necessary but not sufficient for binding of DNA by p53 in vitro

5′-PuPuPuC(A/T)(T/A)GPyPyPy-N $_{0-13}$-PuPuPuC(A/T)(T/A)GPyPyPy-3′

DNA	*Sequence*	*Half-sites*	*Homology to the consensus [%]*	*Binding of p53*	*Reference*
BB.7	ccGGGCAAGTCC GGGCAAGTCCgggcatgt	2	100/100	+	Halazonetis *et al*., 1993
BB.13	ccGAGCAAGTCC GAGCAAGTCCgagcatgt	2	100/100	–	#
BB.18	tcGGGCAAGTTC GGGCAAGTTCgggcatgt	2	100/100	–	#

b Internal symmetry is a typical feature of many natural p53 binding elements

RGC	tGcCTTGCCT GGACTTGCCT GGcCTTGCCT	3	80/100/90	Kern *et al*., 1991
MCK	tGGCAAGCCTatGACATGgCCgGGGCcTGCCT	3	90/80/90	Zambetti *et al*., 1992
mdm2	GGtCAAGTTg GGACAcGTCC	4	80/90	Wu *et al*., 1993
	-17 bp-			
	GAGCTAaGTCCtGACATGTCT		100/90	
bax	tcACAAGTTagAGACAAGCCT GGGCgTGggCTAtaTT	3	70/100/70	Miyashita and Reed *et al*., 1995
Cyclin G	AcGCAAGCCC GGGCTAGTCT	2	90/100	Zauberman *et al*., 1995
p21	GAACATGTCC cAACATGTTg	2	100/80	El-Deiry *et al*., 1993

Vertical arrows in Table 2a point to the substituted bases differing sequences of BB.13 and BB.18 DNAs from BB.7 DNAs from BB.7 DNA. Horizontal arrows in Table 2b designate the elements of internal symmetry within binding sites. Axis of symmetry and interruptions of symmetry are indicated by squares and dots, respectively. All other symbols are as in Table 1

a consequence, transient alterations in DNA conformation could provide an additional level for determining the target-specificity in sequence-specific DNA binding of p53, if the affinity of p53 to its target DNA would be influenced by the local conformation of the consensus DNA. To test this hypothesis, we analysed DNA binding of murine p53 to a variety of DNA substrates, representing the same binding sites in different conformations.

The RGC binding site as a model substrate for sequence-specific DNA binding by p53

In our initial experiments we used DNA-oligonucleotides containing the RGC site, which is one of the first identified (Kern *et al*., 1991; see Table 1b), and best characterized natural p53 binding elements. It contains three half-sites, one of which (in the middle) perfectly matches the consensus. We first demonstrated the specific and reproducible binding of our p53 preparations to the double-stranded oligonucleotide RGC-3/ds (Table 3), containing three copies of the perfect RGC half-site (thereafter referred to as RGC half-site). As previously reported by others (Kern *et al*., 1991; Wang *et al*., 1995a), we also in our experiments observed strong binding of p53 without prior activation of the protein (Figure 1, lane 2). The specificity of this binding was confirmed by supershifting the DNA-protein complexes with PAb421 (lanes 3 and 4). In accordance with the finding that binding of p53 to the RGC-site does not require activation of a latent DNA binding activity of p53, addition of PAb421 to the DNA binding reaction induced a supershift, but did not increase the amount of RGC-3/ds DNA bound by p53. Non-specific antibodies had no effect on the DNA binding activity of p53 and did not induce a supershift (lanes 5 and 6). The specificity of binding was further confirmed by competition experiments, demonstrating that the labeled RGC-site could be effectively competed out of p53-DNA complexes by an excess of unlabeled RGC-3/ds DNA (lanes 7 and 8), but not by non-specific DNA (lanes 9 and 10).

The internal symmetry of the RGC-3 sequence (see Table 3) endows single-stranded RGC-3 DNA with the capacity to fold back and form hairpins. This can occur even after annealing with the complementary DNA strand, due to 'breathing' of the DNA, leading to the presence of a mixed population of duplexed and hairpin-containing molecules. This, however, will make it impossible to monitor the effects of different conformations of a single RGC half-site on DNA binding by p53. Therefore we in our following experiments used oligonucleotides that contained only one copy of the RGC half-site. Although the RGC-site usually is used in a multimeric form, a half-site alone is sufficient for binding and transactivation by p53 *in vitro* (Farmer *et al*., 1992; Kern *et al*., 1992). Due to the lack of a second symmetric half-site, a single RGC binding site does not have the ability to adopt an alternative conformation by itself. However, this potential can be endowed to this RGC-site by sequences flanking it (compare RGC-1b/ss and RGC-1a/ss in Table 3), rendering such a RGC-site a good model for studying the influence of different DNA conformations of a given p53 binding site on sequence-specific DNA binding by p53.

A characteristic feature of the natural RGC binding site is that its half-sites contain TGCCT repeats (see Table 1b). Since TGCCT repeats were found within or close to several 'natural' p53 binding elements (Kern *et al*., 1991; El-Deiry *et al*., 1992; Zambetti *et al*., 1992), it had been suggested that, in addition to the half-sites,

Table 3 Oligonucleotides used in binding assays

Name of oligonucleotide	*Sequence*	*Number of halfsites*	*Capacity to be in non-B DNA conformation*
RGC-3/ss	5′-cctgcctGGACTTGCCTggcctgcctGGACTTGCCTggcctgcctGGACTTGCCTgg-3′	3	+
RGC-1b/ss	5′-gacgccttaggtagggccctGGACTTGCCTcccgggatggtaccgcgg-3′	1	–
RGC-1a/ss	5′-gacgccttaggtacctggcctgcctGGACTTGCCTggcctgcctggatggtaccgcgg-3′	1	+
RGC-2/ss	5′-gacgggttacctacctGGACTTGCCTggcctgcctGGACTTGCCTggatggtaccgcgg-3′	2	+
RGC-du/ss	5′-gacgccttaggtacctgcctGGACTTGCCTggtcctccAGGCAAGTCCaggcaggatggtaccgcgg-3′	2	+
RGC-mut	5′-gacgccttaggtaGGACggtCCTggcaggatggtaccgcgg-3′	0	–
Lock	5′-ccgcggtaccattacctaaggcgtc-3′	0	–
Hu/La-1	5′-gccgccagcttAGACATGCCTAGACATGCCTatcgaccgccg-3′	2	+
Hu/La-2	5′-gccgccagcttAGACATGCCTatcgaccgccg-3′	1	+
Hu/La-du	5′-agcttAGACATGCCTAGACATGCCTa-3′	2	+
Lock-2	5′-cggcggtcgaagctggcggc-3′	0	–

Sequences of p53 binding sites are marked by capital characters, flanking and intervening sequences are given in small characters. Only the upper strand of the oligo is shown. Arrows designate symmetric elements with centers of mirror symmetry shown by V. Dots represent interuptions of symmetry. Unless indicated otherwise, double-stranded oligos were used in the binding assays (oligo/ds). Also shown is the capacity to adopt a non-B-DNA conformation due to the presence of internal symmetry. The sequence of the Hu-La-du DNA is identical to the sequence of the oligonucleotide originally used by Hupp *et al*. (1992)

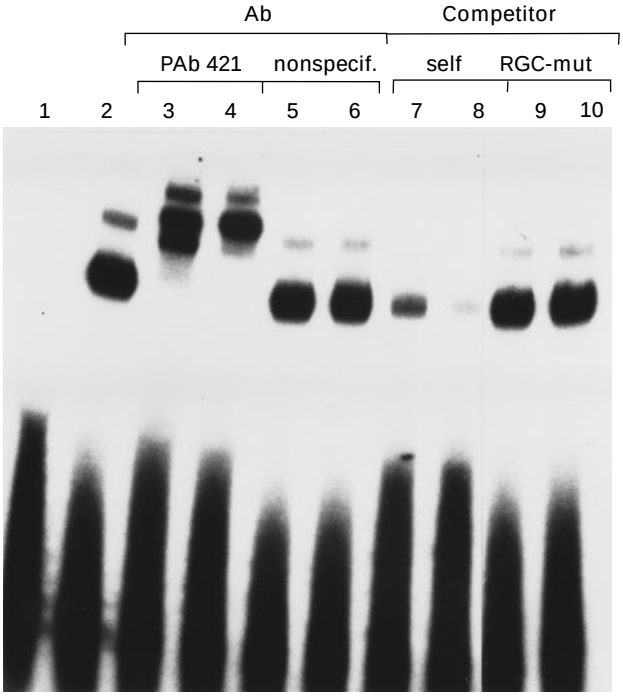


Figure 1 Binding characteristics of RGC-sequence composed of three p53 half-sites. wt p53 protein (5 ng) was present in all binding reactions containing radiolabeled double-stranded RGC-3/ds oligonucleotide, except in the control (lane 1). Binding was analysed by EMSA in a 4% native polyacrylamide gel. 0.1 or 0.5 μg of PAb421 (lanes 3 and 4, respectively), or the non-p53-specific antibody PAb108 (lanes 5 and 6), or 10-fold (lanes 7 and 9), or 20-fold (lanes 8 and 10) molar excesses of unlabeled competitor were added where indicated

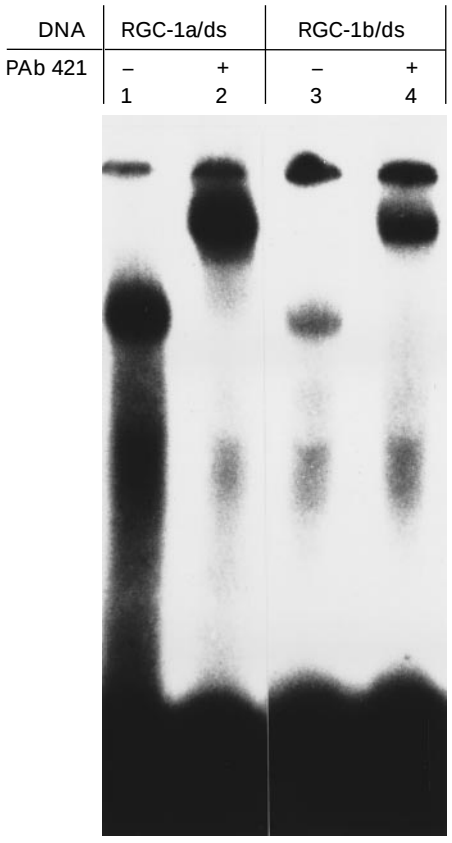


Figure 2 Binding of p53 to a single RGC half-site is stronger in the presence of TGCCT repeats. EMSA analysis of binding of wt p53 to radiolabeled RGC-1a/ds (lanes 1 and 2), or radiolabeled double-stranded RGC-1b/ds oligonucleotide (lanes 3 and 4) in the presence (lanes 2 and 4), or absence (lanes 1 and 3) of PAb421 as described in Figure 1

TGCCT repeats might also be important for the specific binding of p53 to DNA. To test this possibility, we analysed the binding of p53 to a single RGC half-site, surrounded by sequences that either contained (RGC-1a/ds), or lacked (RGC-1b/ds) TGCCT repeats (see Table 3). Both DNAs were bound by p53 (Figure 2), but binding was considerably stronger in the presence of TGCCT repeats (compare lanes 1 and 3), demonstrating that TGCCT repeats flanking the half-site facilitate binding of p53 to the RGC-site. Interestingly, binding of p53 to the half-site lacking TGCCT repeats dramatically increased in the presence of PAb421 (compare the intensity of retarded bands in lanes 3 and 4). This observation is in good agreement with the model of activation of a cryptic DNA binding activity of p53 (Hupp *et al*., 1992). However, no significant activation by PAb421 was observed when the same half-site was surrounded by TGCCT containing sequences (compare lanes 1 and 2).

Two possibilities might be considered to explain the 'helper effect' of TGCCT repeats for the binding of the RGC half-site by p53 in the absence of PAb421: First, TGCCT repeats themselves may be bound by p53 to some extent. However, data reported by Kern *et al*. (1991) strongly argue against this possibility, since these authors showed that mutations within the RGC half-site completely abolished binding of p53 despite the presence of two intact TGCCT repeats adjacent to the half-site. Alternatively, TGCCT repeats might facilitate binding due to the formation of a stem-loop structure in which GC-rich stems would be formed by GCC stretches from the TGCCT repeats (see Table 3). Due to the presence of TGCCT repeats endowing the RGC-1a/ds oligonucleotide with an internal symmetry (Table 3), this oligonucleotide could be present in two conformations – as a duplex or in a stem-loop structure (with the binding site located on the tip of the structure) – whereas RGC-1b/ds DNA that lacks internal symmetry (Table 3) can be present only as a duplex. If p53 indeed would be able to bind both duplex and stem-loop structures, then additive binding of these two isoforms of DNA would be a plausible explanation for the net-increase in binding seen with RGC-1a/ds DNA in comparison to RGC-1b/ds DNA (see Figure 2). To test this possibility we performed experiments in which the ability of p53 to bind stem-loop structures was examined using artificially formed conformational isoforms of the RGC consensus.

p53 preferentially binds to the RGC motif when it is presented in a stem-loop structure

Like most of the non-B-DNA structures, stem-loop structures are thermodynamically unfavorable compared to the canonical B-DNA duplex structure, and an input of additional energy is required for their extrusion within long stretches of duplexed DNA. *In vivo*, the energy derived from unconstrained supercoiling can be utilized to support structural transitions from B- to non-B-conformations in covalently closed chromosomal DNA (Liu and Wang, 1987; McClellan *et al*., 1990). *In vitro*, however, stem-loop structures can form even in the absence of supercoiling by short linear DNAs of oligonucleotides possessing an internal symmetry. Such oligonucleotides may readily fold back and form stem-loop structures which are stabilized by intrastrand base pairing. To address the problem of a possible interaction of p53 with a RGC-site present in a non-B-DNA conformation we made the following considerations: Under experimental conditions for analysing p53/DNA interactions by EMSA using short complementary oligonucleotides there is, due to DNA breathing, an equilibrium between spontaneously formed stem-loop structures resulting from intrastrand base pairing, and canonical B-duplex DNA. To separately analyse the binding of p53 to the chemically identical binding sites present in different conformational isoforms this equilibrium has to be shifted by means of artificial stabilization (locking) of the conformation of interest. To achieve this we used the strategy outlined in Figure 3a.

RGC half-sites were placed into different sequence contexts that would facilitate the formation of certain conformations (RGC-1a, RGC-1b and RGC-du oligonucleotides in Table 3). In addition to the RGC half-site(s) and varying flanking sequences, these three single-stranded oligonucleotides contained identical terminal sequences of 13 bp or 12 bp at their 5′- or 3′-ends, respectively. These sequences then were annealed with the 25 bp 'locking' oligonucleotide Lock (see Table 3), composed of sequences complementary solely to these terminal sequences, but lacking any sequence complementary to the RGC half-site itself, or to sequences immediately adjacent to it. Therefore, annealing of the different RGC-containing oligonucleotides to the Lock oligonucleotide will result in formation of three-way junction structures, where certain conformations of the RGC-site will be fixed by the Lock sequence. Since Lock itself lacks p53 binding sequences, binding of p53 to any of the three-way junction structures formed by the (labeled) Lock oligonucleotide and the (unlabeled) RGC-site containing oligonucleotide must represent a complex formed by p53 and its binding site carried by the unlabeled strand. Figure 3b shows the isolation of the various three-way junction structures, and their structural organization, with the RGC-site(s) being presented in a canonical B-duplex DNA in structure II, and in non-B-DNA conformations in structures I and III. Three-way junction structures were prepared and isolated as outlined and shown in Figure 3a and b and tested for their ability to bind p53 in EMSA. The results are shown in Figure 4 and demonstrate that p53 was able to bind all three structural isoforms of the RGC-site (lanes 3–8). No binding was observed with labeled single stranded Lock DNA (lanes 1 and 2) prior to its annealing with the various unlabeled RGC-oligonucleotides, thus providing evidence that p53 had bound specifically to the RGC half-site within a stem-loop structure on the unlabeled strands. However, the efficiency of RGC-site binding by p53 dramatically depended on the conformation within the RGC-site – the strongest binding to the RGC-site was observed when it was located at the tip of structure I, an intermediate binding was seen with structure II, imitating a regular DNA duplex, and the weakest binding occurred when two RGC half-sites were positioned within a 'bulb' in the middle of structure III. This indicates that not only the

presence of the binding motif in the non-B-DNA conformation as such, but also the shape of the structure resulting from such a structural transition is an important determinant for p53 DNA binding.

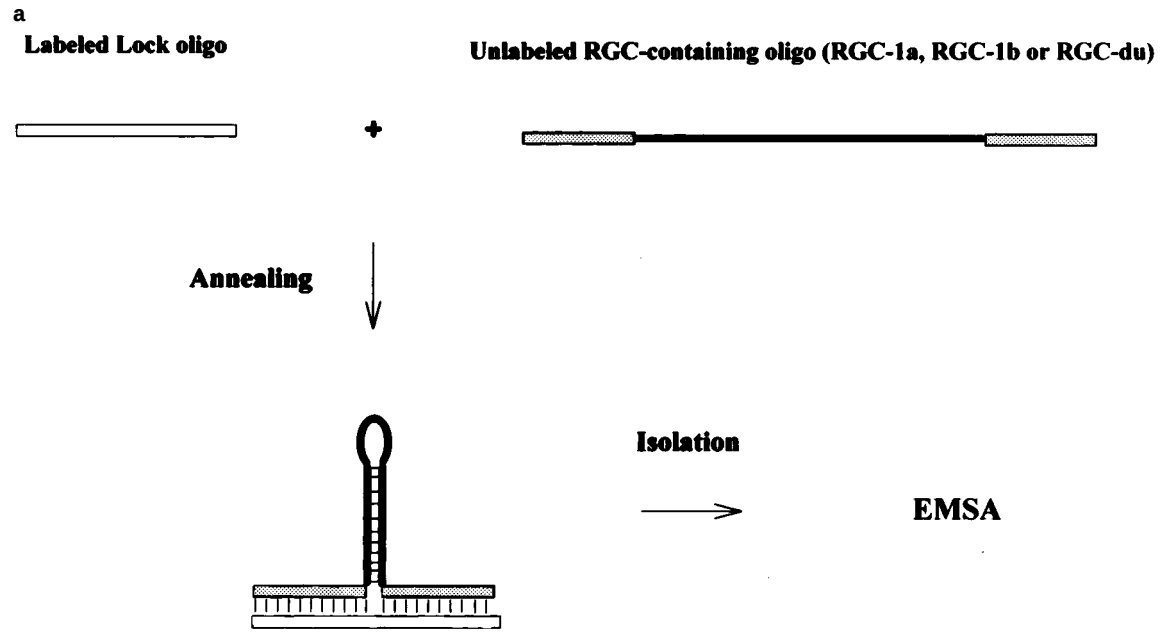
The results in Figure 4 are also compatible with the idea that the overall enhancement in p53 DNA binding, observed when the RGC-motif contained adjacent TGCCT repeats (Figure 2, lanes 1 and 2), was due to the formation of non-duplex DNA structures. These structural isoforms then were bound with higher affinity compared to duplex DNA, the only structure formed in the absence of TGCCT repeats (Figure 2, lanes 3 and 4).

p53 binds to RGC-sites present in stem-loops formed by single-stranded DNA

It recently has been reported that p53 can bind to Holliday junctions with high affinity (Lee *et al*., 1997). The results presented above therefore raised the question whether the recognition of three way junctions containing RGC sites by p53 reflected its binding to RGC sites within the stem-loop structures, as we propose. To answer this question is important for judging the relevance of the observed binding of p53 to non-duplex DNA as shown in Figure 4. In contrast to three-way junction structures that are very unlikely to occur *in vivo*, stem-loop structures formed by complementary strands would result in the extrusion of cruciforms, whose existence and roles in the regulation of gene expression have been well documented *in vivo* and *in vitro* (Ward *et al*., 1990, 1991; Dayn *et al*., 1992; Hanke *et al*., 1995; Spiro *et al*., 1995). To distinguish between three-way junction specific and stem-loop specific binding of p53 we performed experiments with the single stranded RGC-oligonucleotides that were used in our previous experiments (shown in Figure 3) to build up the various conformational forms of the RGC-site. These oligonucleotides exhibit a propensity for intrastrand base pairing due to the internal symmetry. Despite the presence of multiple non-matching bases within their regions of symmetry, these DNAs still have the capacity to form stem-loop structures with a stable stem formed by triads of G : C pairs (see Table 3). This raised the possibility that even in the absence of the Lock oligonucleotide, these RGC-oligonucleotides will still be able to spontaneously form stem-loop structures, although these structures will be less stable than in the presence of the Lock oligonucleotide. We therefore postulated that we should be able to detect binding of p53 to the stem-loops formed spontaneously by single-stranded RGC-DNA, if p53 indeed recognizes a RGC site in a stem-loop structure.

The same single-stranded and RGC-site containing oligonucleotides that were used for the preparation of artificial stem-loop structures in the experiments shown above (upper strands in Figure 3b) were labeled and tested in EMSA. The results are presented in Figure 5a and show that there is a good correlation between the capacity of a single-stranded oligonucleotide to form a stem-loop structure and its ability of being bound by p53: Binding was observed only with those single stranded DNAs (RGC-1a, RGC-2 and RGC-du) that were capable of intrastrand base pairing and formation of a stem-loop structure (Figure 5a, lanes 1–6). The observed binding was not due to the ability of p53 to nonspecifically bind single stranded DNA through its C-terminus (Bakalkin *et al*., 1994, 1995), as p53 failed to bind single stranded RGC-1b DNA that lacks any internal symmetry, and therefore does not have the potential for intrastrand base pairing (lanes 7 and 8). The failure of p53 to bind single-stranded RGC-1b DNA was not due to an improper sequence arrangement, because this DNA was efficiently and specifically bound by p53 after annealing with its complementary strand (Figure 2, lanes 3 and 4, RGC-1b/ds). These results therefore demonstrate that p53 is able to bind RGC half-sites located within stem-loop structures that would be formed by cruciform extrusions.

As expected, the order of preference in binding to the different structures was the same as in the experiments shown in Figure 4, with p53 exhibiting the greatest affinity for stem-loop structure I (RGC-



1a), a moderate one for the B-duplex of structure II (RGC-du), and the weakest one for structure III (RGC-2). In accordance with this interpretation are results of competition experiments, where single-stranded RGC DNAs were used as competitors in binding of p53 to the double-stranded RGC-3/ds oligonucleotide. These results reveal a direct correlation between the capacity of a single stranded competitor DNA to form stem-loop structures, and its ability to compete for p53 specific binding (Figure 5b). Single-stranded RGC-1a DNA with a high capacity to form structure I (lanes 6 and 7) competed for binding with a similar efficiency as the unlabeled double-stranded probe itself (lanes 2 and 3). Competition by RGC-2 was significantly weaker, reflecting the lower affinity of p53 for structure III DNA (lanes 4 and 5). No displacement was observed with either RGC-1b/ss DNA which is unable to form any

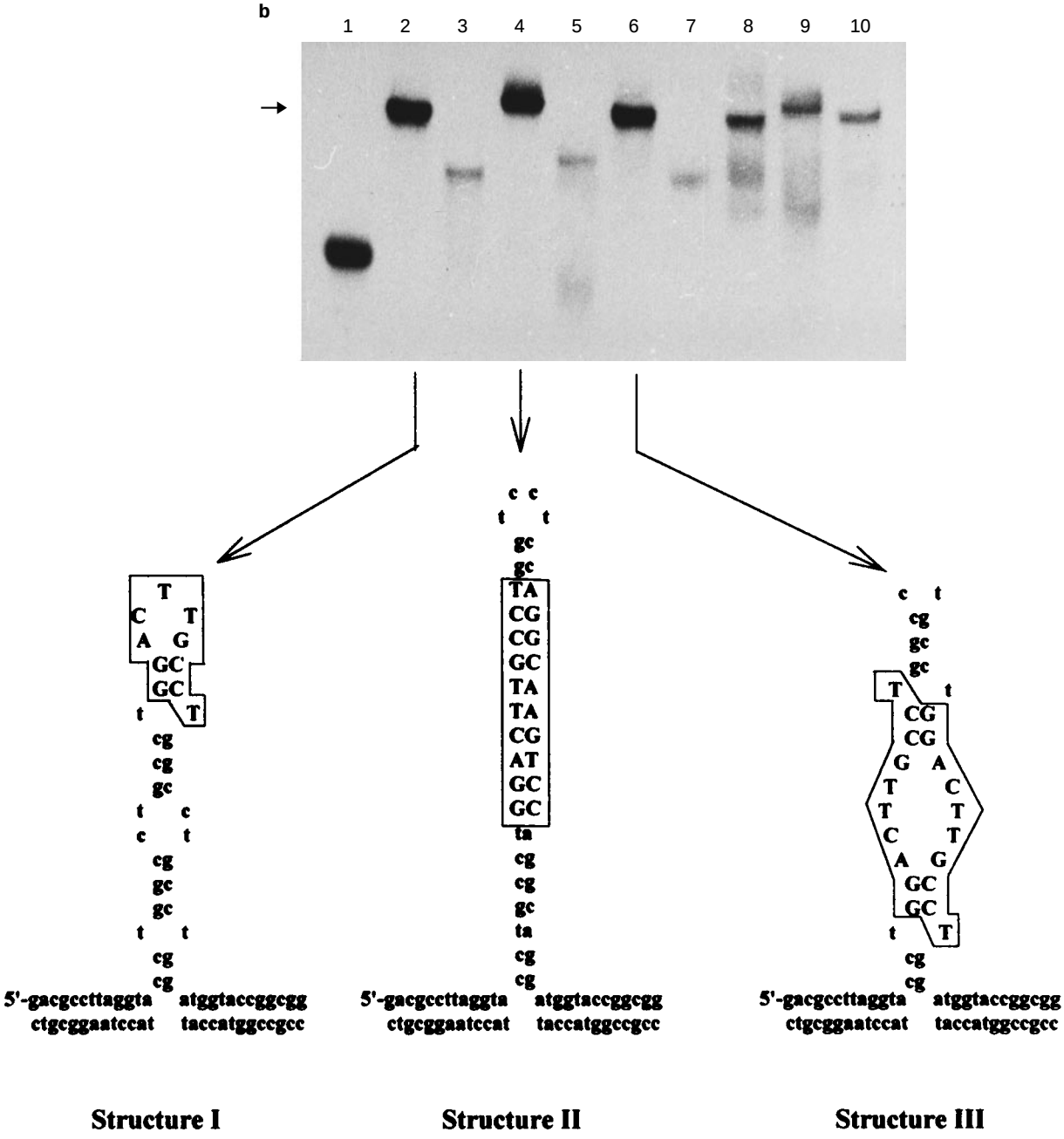


Figure 3 Strategy for generation and isolation of conformational isoforms of the RGC-binding site. Schematic presentation of the formation (**a**), and isolation and graphic representation (**b**) of three-way junction structures representing conformational isoforms of the RGC binding site. Unlabeled oligonucleotides RGC-1a, RGC-du or RGC-2, respectively, were annealed with radiolabeled oligonucleotide Lock. Annealing of Lock with RGC-1a generates structure I, annealing with RGC-du generates structure II, and annealing with RGC-2 generates structure III (as depicted in the bottom part). A 12% PAGE (top) of the resulting DNA isoforms shows that structures resulting from the annealing of the labeled Lock to the unlabeled RGC-containing oligonucleotides (lanes 2, 4 and 6) migrated with the same electrophoretic mobility (indicated by an arrow) as structures formed after annealing of labeled RGC-containing oligonucleotides to the unlabeled Lock DNA (lanes 8, 9 and 10, respectively). This confirms that the expected stem-loop structures (depicted at the bottom) were formed by the annealing of RGC-containing oligonucleotides with the Lock oligonucleotide. To distinguish between three-way junction structures and non-annealed DNAs, single stranded labeled DNAs of Lock, RGC-1, RGC-du and RGC-2 were heated at +85°C for 5 min, quickly cooled on ice and immediately loaded on gel (lanes 1, 3, 5 and 7, respectively)

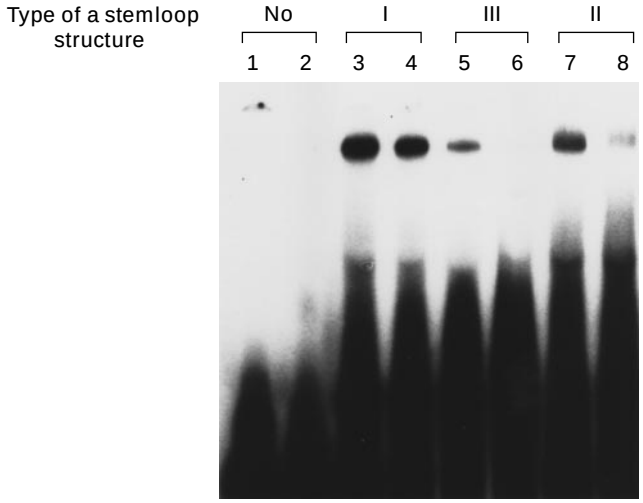


Figure 4 Binding of wt p53 to the RGC-site present in different DNA conformations. The binding of p53 to single-stranded radiolabeled Lock oligonucleotide alone (lanes 1 and 2), and to radiolabeled Lock oligonucleotide after its annealing with unlabeled oligonucleotides RGC-1a (lane 3 and 4), RGC-2 (lanes 5 and 6), or RGC-du (lanes 7 and 8), respectively, was analysed by EMSA in a 4% polyacrylamide gel. The binding reaction contained 10 ng (lanes 1, 3, 5 and 7) or 5 ng (lanes 2, 4, 6 and 8) or purified wt p53 protein

secondary structure (lanes 8 and 9), or with a single stranded DNA containing a mutated RGC sequence (lanes 10 and 11). These results cannot be explained by size-dependent effects of single-stranded DNAs on sequence-specific binding by p53 as shown by Jayaraman and Prives (1995). In their experiments nonspecific oligomers up to 40 bp stimulated binding of p53 to the GADD45 promoter, whereas longer ones (66 bp and 92 bp) inhibited sequence-specific binding (Jayaraman and Prives, 1995). Accordingly, one would have expected a stimulatory effect of RGC-mut (42 bp) and RGC-1b (48 bp) oligomers on p53 binding to the double stranded RGC-site. However, no such effect was observed (compare lanes 8 and 9; 10 and 11 in Figure 5b). Even more difficult to explain by this interpretation would be the strikingly different effects of the RGC-2 and RGC-1a DNAs (compare lanes 5 and 7, Figure 5b), as their DNAs differ in length by only one base (58 and 59 bp, respectively). These considerations strongly suggest that neither the presence of a RGC site in a three-way junction structure, nor single-strandedness as such, but the ability to form a proper stem-loop structure determines whether a RGC half-site will be recognized by p53 as a binding site.

DNA-binding of p53 to a consensus element in non-B-DNA conformation does not require activation of a latent p53 DNA-binding activity

Our data so far strongly suggest that wt p53 is capable of binding to different conformations of its cognate DNA elements, and that there is a hierarchy for binding among different structures. Our results also suggest that the efficiency of binding of p53 to the RGC-site in B-DNA conformation (structure II) could be modulated by shifting to a non-B-DNA conformation (structures I or III). Depending on the type of structure, binding could be enhanced (structure I), or

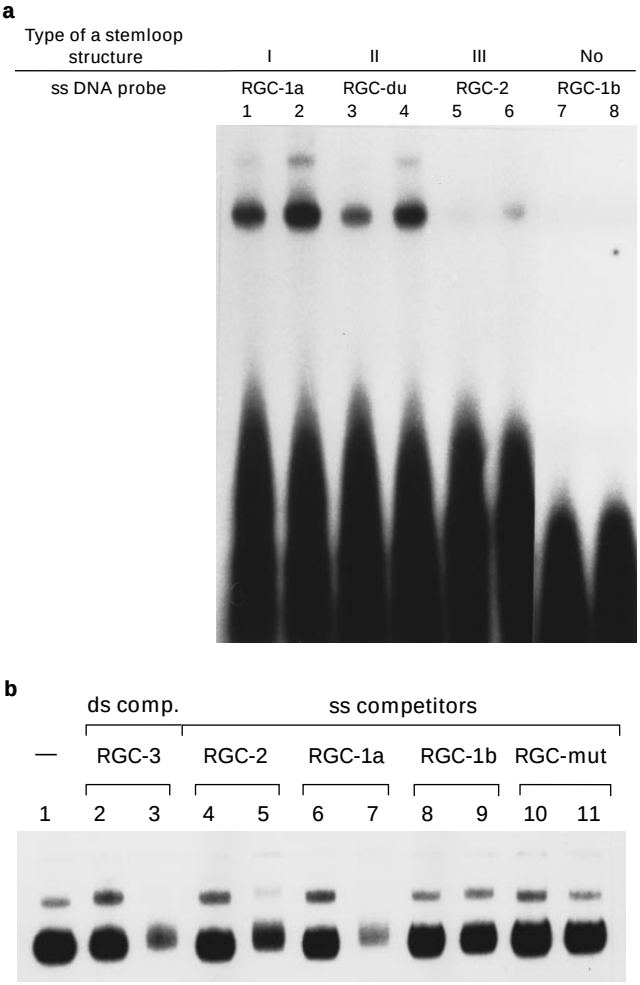


Figure 5 Binding of p53 to single-stranded oligonucleotides capable of forming stem-loop structures. (**a**) Binding of p53 to the various labeled single stranded RGC oligonucleotides was analysed by EMSA in the presence of 5 ng (lanes 1, 3, 5 and 7) or 10 ng (lanes 2, 4, 6 and 8) of wt p53. (**b**) Binding reactions were performed with labeled double-stranded oligonucleotide RGC-3/ds in the absence (lane 1) or in the presence of fivefold (lanes 4, 6, 8 and 10), or 10-fold (lanes 5, 7, 9 and 11) molar excesses of different unlabeled single stranded competitor oligonucleotides. Self-competition in the presence of fivefold and 10-fold molar excess of unlabeled double stranded RGC-3/ds is shown in lanes 2 and 3, respectively

significantly reduced (structure III). Our data also explain the different binding of identical RGC half-sites depending on the context in which they were placed (Figure 2). However, these data do not explain the dramatic difference in the enhancing effect of PAb421 on the binding of the RGC-1a *versus* the RGC-1b substrate (Figure 2). As an exciting possibility we considered that this difference might reflect the binding of p53 to the RGC-site present in a different conformation. To test whether the effects of PAb421 on DNA binding of p53 would differ for different DNA conformations, we analysed the binding of p53 to the binding sequence used by Hupp *et al*. (1992). This sequence is particularly interesting insofar, as p53 binds this DNA, in contrast to RGC-DNA, only after activation, e.g. by addition of PAb421 (Hupp *et al*., 1992, 1993, 1994). Using the approach outlined in Figure 4, three different isoforms were prepared in which this site was present in different conformations, as shown in Figure 6a (referred to as Hu/La-du/ds, Hu/

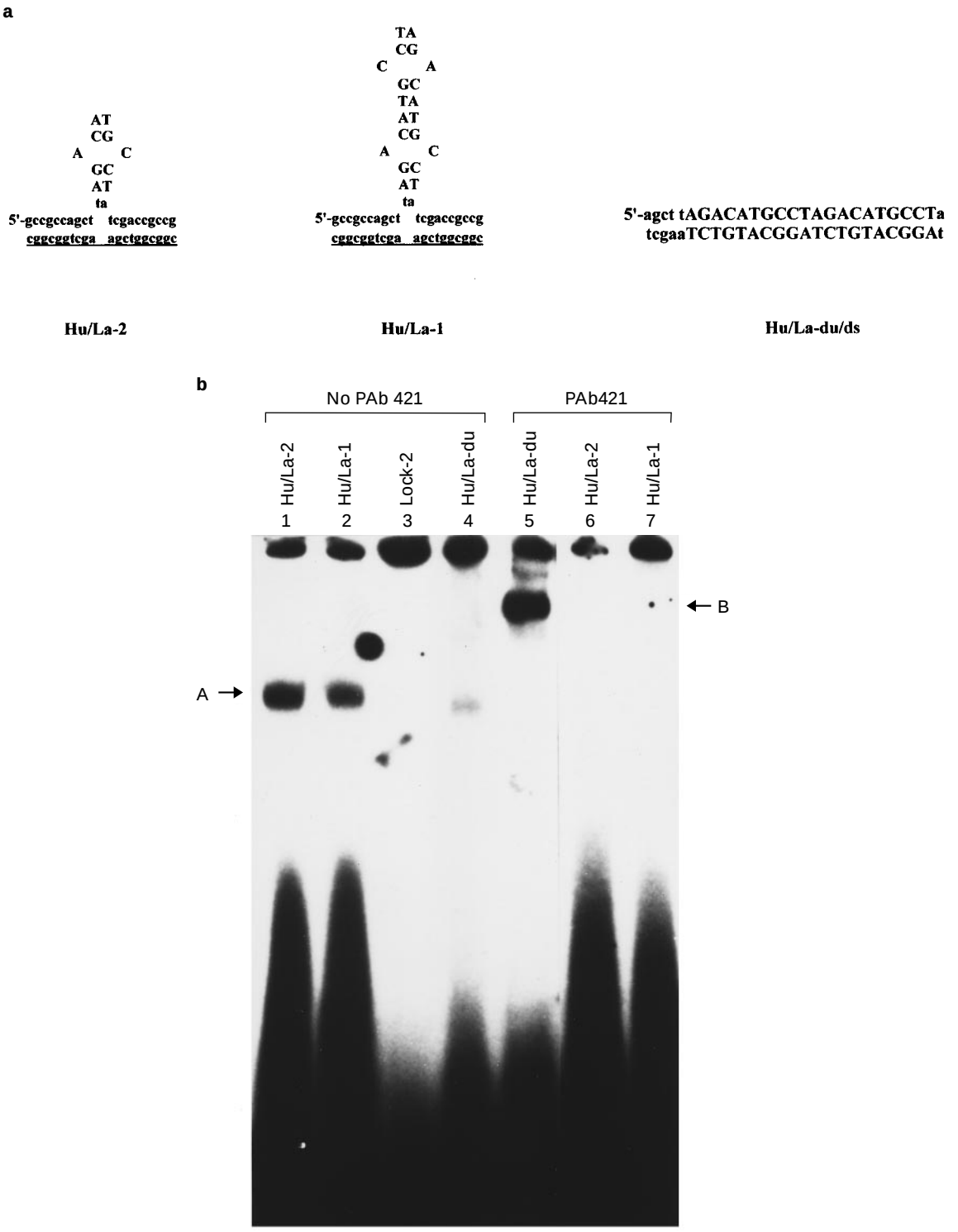


Figure 6 The requirement for activation of p53 for DNA binding depends on the conformational status of the target DNA. (**a**) Schematic representation of different conformational isoforms of the Hu/La site prepared as outlined in Figure 4. The Hu/La-du/ds is a duplex prepared by reannealing the Hu/La-du oligonucleotide (Hupp *et al*., 1992) with its complementary sequence; non-B-DNA isoforms Hu/La-2 and Hu/La-1 were prepared by annealing of the corresponding single-stranded oligonucleotides Hu/La-2 and Hu/La-1 with the Lock-2 oligonucleotide (underlined). (**b**) Binding of purified wt p53 to the Hu/La site in different conformations (lanes 1, 2 and 4–7). All reactions were performed in presence of wt p53. PAb421 was added to the reaction mixture in lanes 5–7. Arrows indicate p53-DNA complexes formed in the absence (**a**) and presence (**b**) of PAb 421

La-1, and Hu/La-2). Analysis of p53 binding to these DNAs by EMSA (Figure 6b) revealed striking differences in the binding capacity of p53, depending on whether the Hu/La site was present in the B-conformation, or in non-B-conformations. In accordance with the findings of Hupp *et al*. (1992), binding of p53 strictly depended on the presence of PAb421, but only when the DNA was in duplex conformation (compare lanes 4 and 5, complexes A and B; the slower mobility of complex B compared to complex A is due to supershifting of this complex in the presence of PAb421). However, when the Hu/La site was present in a non-B-DNA conformation, strong binding of p53 was observed in the absence of PAb421 (lanes 1 and 2). No

shifted band was detected with the bottom locking strand Lock-2, lacking the p53 consensus sequence (lane 3). These data demonstrate that activation of p53 for recognition and binding to this particular site is not necessary when it is present in a stem-loop conformation, while binding of p53 to the same site requires prior activation with PAb421 when it is present in the duplex B-conformation. Of particular interest is that PAb421 strongly inhibited the binding of p53 to this site when it was presented in stem-loop structures. This is in marked contrast to the enhancing effect of PAb421 on p53 binding to the Hu/La site when it was presented as duplex DNA (compare lanes 1 and 2 with lanes 6 and 7). This observation suggests that binding of PAb421 to p53 might alter the conformation of the p53 DNA binding domain in a way that favors the binding of this consensus DNA when it is present in a duplex B-DNA conformation, but reduces the affinity for this DNA when it is present in a non-B-DNA conformation.

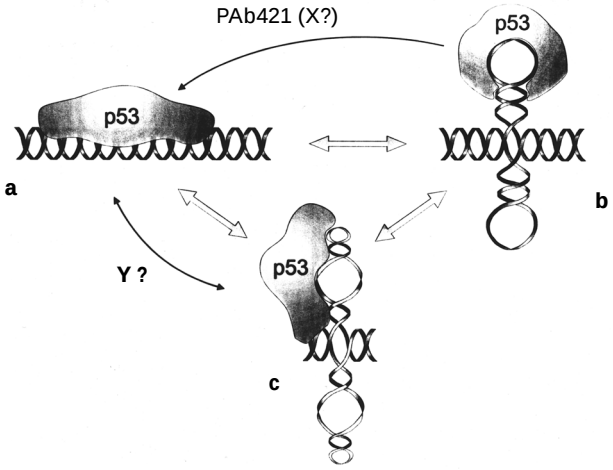


Figure 7 p53 discriminates between different conformations of DNA within its binding sites. Depending on the conformational status of the target DNA, the p53 binding site could be exposed to p53 in different DNA conformations – as B-, (**a**) or non-B-DNA [(**b**) and (**c**)]. Structural flexibility of p53 itself would allow the protein to switch between different conformations that fit particular isoforms of its target, DNA. In the case of the Hu/La p53 binding sequence, PAb421 is able to adjust the conformation of p53, allowing its binding to a duplex *in vitro*. This may imitate the effect of other factors (X and Y) that participate in such adjustments *in vivo*

Discussion

The marked sequence-heterogeneity of the known p53 binding elements asks for additional features other than sequence-specificity of the cognate DNA sites alone that will contribute to the specific recognition and binding of p53 to its DNA-targets. Sequence-heterogeneity could be due to a relaxed or promiscuous binding of p53 to its target DNAs. However, the different and unpredictable effects (enhancement, reduction or complete abrogation of p53 binding) caused by different consensus – matching single base substitutions (Halazonetis *et al*., 1993) are not compatible with this simple interpretation. As sequence comparison of the p53 consensus elements suggested that these DNAs might be endowed with the capacity of forming non-B-DNA structures, we asked whether the conformation in which these elements are presented to p53 might be an important determinant for the specificity and affinity of binding. Our results show that this is indeed the case. Using two different consensus elements – the RGC-site (Kern *et al*., 1991), and the consensus oligonucleotide Hu/La used by Hupp *et al*. (1992) which were presented either as a canonical B-DNA duplex, or locked in different non-B-DNA structures (see Figures 3b and 6a), we found that p53 is able to bind to different conformations of the same consensus sequence, albeit with different efficiencies (see Figures 4 and 6b).

Our results demonstrate that the ability of p53 to bind a given half-site greatly depends on the conformation of the target DNA – p53 bound a single RGC half-site within stem-loop structure I more efficiently than the same half-site in the canonical B-DNA duplex presented by structure II (Figures 2 and 4). Interestingly, however, not each stem-loop structure will necessarily promote binding – stem-loop structure III, formed by two copies of the RGC half-site, bound even weaker than a single half-site in B-duplex conformation (Figure 4). In the case of the Hu/La site, the effect of conformational changes was even more dramatic: As described previously (Hupp *et al*., 1992), binding of p53 to this site in the B-duplex DNA conformation required activation of p53 with PAb421 (Figure 6b). In contrast, the presence of this site in the stem-loop structures Hu/La-1 and Hu/La-2 resulted in strong binding by p53 without prior activation (Figure 6b). These results led us to conclude that a stem-loop structure with a single binding half-site located at its tip (structures I in Figure 3b and Hu/La-2 in Figure 6a) is a very favorable binding substrate for p53 which does not require the activation of a latent DNA-binding activity of p53. In contrast, binding of duplex B-DNA seems to be much less preferred by carboxy-terminally unmodified p53, and, in the case of the Hu/La site, binding to duplex DNA even will not occur at all without prior activation.

Our observation that PAb421 had opposite effects on the binding of p53 to the Hu/La site in different conformations might provide an explanation for the different binding behavior of p53 towards binding sites present either in B-DNA duplex or stem-loop conformations. As PAb421 enhanced the binding of p53 to duplex B-DNA, while it decreased p53 binding to this site when it was present in stem-loop structures (Figure 6b), we suggest that p53 might be able to adopt two different conformations when interacting with its target DNAs; one, in which it preferentially binds to its cognate sites when they are presented in a stem-loop structure, and another one, which preferentially binds to duplex B-DNA. Then binding of PAb421, or possibly any other activating modification of the carboxy-terminal regulatory domain of p53, would shift the conformation of p53 in such a way that p53 preferentially binds to duplex B-DNA. As truncation of the carboxy-terminus seems to have a similar effect on p53 DNA binding as its modification (Hupp *et al*., 1992), this view also is in accordance with the crystal structure of the p53 core domain bound to duplex B-DNA (Cho *et al*., 1994). The DNA used for co-crystallization is formed by the synthetic consensus pentamer sequence GGGCA-AGTCT within a sequence environment that has a

low capacity to form non-B-DNA structures. It is important to recall that the isolated core domain possibly will be able to assume only one conformation, especially in the presence of B-duplex DNA. Therefore a complete understanding of the different interactions of p53 with p53 consensus DNA will await co-crystallization of full length p53 with various DNA substrates. Regarding the importance of structural features of DNA for p53/DNA interactions, it is interesting that Lee *et al.* (1997) showed that wt p53 is able to bind to Holliday junctions, and that our laboratory provided evidence for the specific binding of mutant p53 to MAR/SAR DNA elements, which contain a variety of sequence elements promoting the formation of non-B-DNA structures (Weissker *et al*., 1992; Müller *et al*., 1996; Deppert, 1996).

Based on this ability of wt p53 to discriminate between different DNA conformations we propose that the specific binding of p53 to its target sites is regulated via conformational changes of the DNA within the p53 binding site, as well as via regulatory modifications of the p53 protein itself (Figure 7). This model can explain, and bring together, several seemingly contradictory observations, e.g. that in some cases at least two half-sites were shown to be required for binding (El-Deiry *et al*., 1992; Halazonetis *et al*., 1993), while in others the presence of a single half-site was sufficient for binding (Kern *et al*., 1992; Wang *et al*., 1995b), or the requirement for carboxy-terminal activation of the p53 protein for binding to some, but not to all target sites.

In accordance with this model is the fact that many p53 binding sequences are composed by several repeats. The presence of at least two or more half-sites with an internal symmetry in most of the natural binding elements (see Table 1) endows these DNAs with the flexibility that allows reversible shifting between different conformations. This might be important for regulating the binding of p53 to these sequences, and thus for the transactivation of p53 target genes. In contrast, the presence of just a single half-site would not allow such a flexibility in DNA conformation, unless the binding site is located within an appropriate sequence environment, like the RGC-site flanked by TGCCT repeats. Furthermore, the postulated interaction of p53 with non-B-conformations of its target DNAs also could explain the relative low stringency of the p53 consensus sequence; due to the imperfect symmetry of these DNAs, secondary structures extruded by p53 binding sites would contain unpaired bases that would facilitate further structural transitions upon changes in environmental conditions. In contrast, a perfect internal symmetry of the half-sites would significantly decrease the structural flexibility of the target DNA, rendering conformational shifting energetically difficult, if not impossible. Formation of non-B-DNA *in vivo* has been documented (Lee *et al*., 1987; Sen and Gilbert, 1988; Wittig *et al*., 1989, 1992; Ward *et al*., 1991; Michelotti *et al*., 1996), and topologically constrained chromosomal DNA undergoes conformational changes that are regulated and coordinated with cellular processes (reviewed in: Dröge, 1994; van Holde and Zlatanova, 1994; Leach, 1994). Structural transitions may be supported by other proteins that could stabilize or even promote the extrusion of the alternative conformations. The existence of proteins whose interaction with DNA is based on conformation – but not sequence-specific – recognition has been documented (Bianchi *et al*., 1988; Herbert *et al*., 1993; Leith and Russel, 1993; Pearson *et al*., 1995; Solaro *et al*., 1995). Transcriptional regulation of many genes involves conformational changes in DNA (Delic *et al*., 1991; Hanke *et al*., 1995; Iyer and Struhl, 1995; Spiro *et al*., 1995; Michelotti *et al*., 1996), and binding of some transcription factors depends on the conformation of DNA within or adjacent to their binding sites (Grigoriev *et al*., 1992; Spiro *et al*., 1993; Hu *et al*., 1994; Mayfield and Miller, 1994). At the level of the p53 protein, modifications of the p53 carboxy-terminal regulatory domain either by posttranslational modifications, alternative splicing (Kulesz-Martin *et al*., 1994; Flaman *et al*., 1996), or by binding of proteins mimicking the effect of PAb421 binding would provide another level for determining the specificity for transactivation of the ever growing list of potential p53 target genes. Discrimination between different DNA conformations by p53 molecules of different conformational status then would provide the molecular basis for an additional and higher level of specificity than just the presence of a p53 consensus binding site in a promoter of a putative p53 target gene.

Materials and methods

Overexpression and isolation of the recombinant murine wt p53

Sf9 insect cells at 80% confluency were infected with recombinant baculovirus expressing wild-type p53 and harvested 48 h days after after infection. After being washed four times with phosphate-buffered saline (PBS) at +4°C, cells were resuspended in 10 mM HEPES, pH 7.4, 1.5 mM $MgCl_2$ and 5 mM KCl (buffer A) and incubated on ice for 60 min. From this step on all solutions used contained 2 mM phosphomethylsulfofluoride (PMSF), 1 mM dithiotreitol (DTT), 50 μg ml^{-1} of leupeptin and 30 μg ml^{-1} aprotinin (Trasylol, Bayer) as inhibitors of proteases. This cell suspension was transferred to a dounce-homogenizer, and cellular membranes were disrupted by stroking cells on ice 30 times. Cell lysis was controlled by staining nuclei with trypan blue. The lysate next was extracted by a 45 min incubation on ice, followed by centrifugation at 5000 *g* at 4°C for 10 min. Supernatant A (SA) was collected, and the pellet (PA), after being resuspended in 10 mM HEPES, pH 9.0, 1.5 mM $MgCl_2$, 5 mM KCl (buffer B), was subjected to a second extraction yielding supernatant SB and pellet PB. Two more extraction steps were consequently performed using buffer B containing 0.2 or 0.5 M KCl, and resulting in pellets PC and PD and supernatants SC and SD, correspondingly. Supernatants SA, SB, SD and SC were cleared by centrifugation at 10^5 *g* for 30 min at 4°C and analysed for the presence of p53. The majority of wt p53 was present in supernatant SC. p53 was greater than 80% pure when stained with Coomasie Blue in an SDS polyacrylamide gel. The identity of a single band migrating with a mobility corresponding to that of wt p53 as representing p53 was confirmed in Western blot analysis. p53-containing fraction SC was aliquoted and stored frozen at −70°C. Although most of the experiments reported here were performed with such a p53 preparation, p53 protein purified to homogeneity by immunoaffinity procedures was used to verify the major conclusions made in this manuscript. Protein A-Sepharose columns cross-linked with p53-specific monoclonal antibody PAb421 were used to purify p53 protein from the extracts of infected cell as described (Mummenbrauer *et al*., 1996; Zhao *et al*., 1996).

Gel mobility shift assay

Synthetic oligonucleotides (sequences shown in Table 2) were end-labeled using T4 polynucleotide kinase and γ-^{32}P-ATP, gel purified and used as probes in binding reactions without or after annealing with unlabeled complementary strands. Binding reaction mixtures with or without p53 (5–10 ng) were preincubated for 20 min with 2 μg of poly dI : dC (Pharmacia Biotech) in 10 mM HEPES (pH 7.8) with 50 mM KCl, 1 mM EDTA, 5 mM $MgCl_2$ and 10% glycerol at room temperature (RT). Antibodies or unlabeled competitor DNA were also added to the preincubation mixtures where indicated. 2×10^{-7} M (2×10^4 c.p.m.) of radiolabeled probe was added after preincubation and binding was allowed for another 30 min at RT. Reaction products were analysed by electrophoresis in 4% native polyacrylamide gels using 40 mM EDTA, 37 mM sodium acetate, pH 7.8, as the gel electrophoresis buffer.

Isolation of artificial three way junction structures

1 μg of Lock or Lock-2 oligonucleotides were end labeled with T4 polynucleotide kinase and γ-^{32}P-ATP and annealed with unlabeled DNAs containing complementary sequences, followed by separation of the annealed DNAs in 12% native polyacrylamide gel as depicted in Figure 4. After electrophoresis, the gels were shortly exposed to Kodak film and bands corresponding to the three way junction structures were cut out from the gel. Gel stripes were crashed and soaked in STE, pH 7.8 for 2 h at +4°C with agitation followed by ethanol precipitation of DNA from the eluates. After thorough washing with 70% ethanol, the eluted DNA was counted for radioactivity, redissolved in TE, pH 7.8 and used in EMSA as described above.

Acknowledgements

We wish to thank Drs P Vogt, G Wahl and G Walter for helpful and inspiring discussions. We also thank Martina Hintz-Malchow for the assistance with editing this manuscript and Doris Weidemann for help with photographic documentation of the data. This study was supported by grant De 212/17-1 from the Deutsche Forsschungsgemeinschaft, grant W92/94/De2 from the Deutsche Krebshilfe (Dr Mildred Scheel Stiftung), and the Fonds der Chemischen Industrie. The Heinrich-Pette-Institut is financially supported by Freie und Hansenstadt Hamburg and Bundesministerium für Gesundheit. EK is supported by a fellowship from the EMBO.

References

Arrowsmith CH and Morin P. (1996). *Oncogene*, **12,** 1379–1385.

Bakalkin G, Yakovlela T, Selivanova G, Magnusson KP, Szekely L, Klein G, Terenius L and Wiman KG. (1994). *Proc. Natl. Acad. Sci. USA*, **91,** 413–417.

Bakalkin G, Selivanova G, Yakovleva T, Kiseleva E, Kashuba E, Magnusson KP, Szekely L, Klein G, Terenius L and Wiman KG. (1995). *Nucl. Acids Res.*, **23,** 362–369.

Bargonetti J, Friedman PN, Kern SE, Vogelstein B and Prives C. (1991). *Cell*, **65,** 1083–1091.

Bargonetti J, Manfredi JJ, Chen X, Marshak DR and Prives C. (1993). *Genes Dev.*, **7,** 2565–2574.

Bianchi ME, Beltrame M and Paonessa G. (1988). *Science*, **243,** 1056–1059.

Cho Y, Gorina S, Jeffrey P and Pavletich NP. (1994). *Science*, **265,** 346–355.

Dayn A, Malkosyan S and Mirkin SM. (1992). *Nucl. Acids Res.*, **20,** 5991–5997.

Delic J, Onclerco R and Moisan-Coppey M. (1991). *Biochem. Biophys. Res. Comm.*, **181,** 818–826.

Deppert W. (1994). *Seminars in Cancer Biol.*, **5,** 187–203.

Deppert W. (1996). *J. Cell. Biochem.*, **62,** 172–180.

Donehower LA and Bradley A. (1993). *Biochem. Biophys. Acta*, **1155,** 185–205.

Dröge P. (1994). *BioEssays*, **16,** 91–99.

El-Deiry WS, Kern S, Pietenpol JA, Kinzler KW and Vogelstein B. (1992). *Nature Genet.*, **1,** 45–49.

El-Deiry WS, Tokino T, Velculescu VE, Levy DB, Parsons R, Trent JM, Lin D, Mercer WM and Vogelstein B. (1993). *Cell*, **75,** 817–825.

Farmer G, Bargonetti J, Zhu H, Friedman P, Prywes R and Prives C. (1992). *Nature*, **358,** 83–86.

Flaman JM, Waridel F, Estreicher A, Vannier A, Limacher JM, Gilbert D, Iggo R and Frebourg T. (1996). *Oncogene*, **12,** 813–818.

Foord O, Navot N and Rotter V. (1993). *Mol. Cell. Biol.*, **13,** 1378–1384.

Grigoriev M, Peuth D, Robin P, Hemar A, Saison-Behmoa T, Dautry-Varsat A, Thuong NT, Helene C and Harel-Bellan A. (1992). *J. Biol. Chem.*, **267,** 3389–3395.

Halazonetis TD and Kandil AN. (1993). *EMBO J.*, **12,** 5057–5064.

Halazonetis TD, Davis LJ and Kandil AN. (1993). *EMBO J.*, **12,** 1021–1028.

Hanke JH, Hambor JE and Kavanthas P. (1995). *J. Mol. Biol.*, **246,** 63–73.

Hecker D, Page G, Lohrum M, Weiland S and Scheidtmann KH. (1996). *Oncogene*, **12,** 953–961.

Herbert AG, Spitzner JR, Lowenhaupt K and Rich A. (1993). *Proc. Natl. Acad. Sci. USA*, **90,** 3339–3342.

Hu CH, McStay B, Jeong SW and Reeder RH. (1994). *Mol. Cell. Biol.*, **14,** 2871–2882.

Hupp TR, Meek DW, Midgley CA and Lane DP. (1992). *Cell*, **71,** 875–886.

Hupp TR, Meek DW, Midgley CA and Lane DP. (1993). *Nucl. Acids Res.*, **21,** 3167–3174.

Hupp TR and Lane DP. (1994). *Cold Spring Harbor Symp. Quant. Biol.*, **59,** 195–206.

Iyer V and Struhl K. (1995). *EMBO J.*, **14,** 2570–2579.

Jayaraman L and Prives C. (1995). *Cell*, **81,** 1021–1029.

Kern SA, Kinzler KW, Bruskin A, Jarosz D, Friedman P, Prives C and Vogelstein B. (1991). *Science*, **252,** 1708–1711.

Kern SE, Pietenpol JA, Thiagalingam S, Seymour A, Kinzler K and Vogelstein B. (1992). *Science*, **256,** 827–830.

Ko LJ and Prives C. (1996). *Genes Dev.*, **10,** 1054–1072.

Kulesz-Martin MF, Lisafeld B, Huang H, Kisiel ND and Lee L. (1994). *Mol. Cell. Biol.*, **14,** 1698–1708.

Lane DP. (1994). *Br. Med. Bull.*, **50,** 582–599.

Leach D. (1994). *BioEssays*, **16,** 893–900.

Lee JS, Burkholder GD, Latimer LJP, Haug B and Braun RP. (1987). *Nucl. Acids Res.*, **15,** 1047–1061.

Lee S, Cavallo L and Griffith J. (1997). *J. Biol. Chem.*, **272,** 7532–7539.

Leith IR and Russel WC. (1993). *Biochem. Biophys. Res. Comm.*, **196,** 601–610.

Liu LF and Wang JC. (1987). *Proc. Natl. Acad. Sci. USA*, **84,** 7024–7027.

Ludes-Meyers JH, Subler MA, Shivakumar CV, Munoz RM, Jiang P, Bigger JE, Brown D, Deb SP and Deb S. (1996). *Mol. Cell. Biol.*, **16,** 6009–6019.

Mayfield C and Miller D. (1994). *Nucleic Acids Res*., **22,** 1909 – 1916.
McClellan JA, Boublikova P, Palecek E and Lilley DMJ. (1990). *Proc. Natl. Acad. Sci. USA*, **87,** 8373 – 8377.
Michelotti GA, Michelotti EF, Pulinar A, Duncan RC, Eick D and Levens D. (1996). *Mol. Cell. Biol*., **16,** 2656 – 2669.
Miyashita T and Reed JC. (1995). *Cell*, **80,** 293 – 299.
Müller BF, Paulsen D and Deppert W. (1996). *Oncogene*, **12,** 1941 – 1952.
Mummenbrauer T, Janus F, Müller B, Weissmüller L, Deppert W and Grosse F. (1996). *Cell*, **85,** 1089 – 1099.
Pearson CE, Zannis-Handjopoulos M, Price G and Zorbas H. (1995). *EMBO J*., **14,** 1571 – 1580.
Prives C, Bargonetti J, Farmer G, Ferrari E, Friedlander P, Wang Y, Jayaraman L, Pavletich N and Hubscher U. (1994). *Cold Spring Harbor Symp. Quant. Biol*., **59,** 207 – 213.
Sen D and Gilbert W. (1988). *Nature*, **334,** 364 – 366.
Solaro P, Greger B and Kemper B. (1995). *Eur. J. Biochem*., **230,** 926 – 933.
Soussi T and May P. (1996). *J. Mol. Biol*., **260,** 623 – 637.
Spiro C, Bazett-Jones DP, Wu X and McMurray CT. (1995). *J. Biol. Chem*., **270,** 27702 – 27710.
Spiro C, Richards JP, Chandraserakan S, Brennan RJ and McMurray CT. (1993). *Proc. Natl. Acad. Sci. USA*, **90,** 4606 – 4610.
van Holde K and Zlatanova J. (1994). *BioEssays*, **16,** 59 – 68.
Wang Y and Prives C. (1995). *Nature*, **376,** 88 – 91.
Wang Y, Farmer G, Soussi T and Prives C. (1995a). *Oncogene*, **10,** 779 – 784.
Wang Y, Reed M, Wang P, Stenger JE, Mayr G, Anderson ME, Schwedes JF and Tegtmeyer P. (1993). *Genes Dev*., **7,** 2575 – 2586.
Wang Y, Schwedes JF, Parks D, Mann K and Tegtmeyer P. (1995b). *Mol. Cell. Biol*., **15,** 2157 – 2165.
Ward GK, McKenzie R, Zannis-Hadjopoulos M and Prives GB. (1990). *Exper. Cell Res*., **188,** 235 – 246.
Ward GK, Shihab-el-Deen A, Zannis-Handjopoulos M and Price GB. (1991). *Exper. Cell Res*., **195,** 92 – 98.
Waterman JLF, Shenk JL and Halazonetis TD. (1995). *EMBO J*., **14,** 512 – 519.
Waterman MLJ, Waterman JLF and Halazonetis TD. (1996). *Cancer Res*., **56,** 158 – 163.
Weissker S, Müller B, Homfeld A and Deppert W. (1992). *Oncogene*, **7,** 155 – 167.
Wittig B, Dorbic T and Rich A. (1989). *J. Cell. Biol*., **108,** 755 – 764.
Wittig B, Wolfl S, Dorbic T, Vahrson W and Rich A. (1992). *EMBO J*., **11,** 4653 – 4663.
Wu X, Bayle JH, Olson D and Levine AJ. (1993). *Genes Dev*., **7,** 1126 – 2232.
Zambetti GP, Bargonetti J, Walker K, Prives C and Levine AJ. (1992). *Genes Dev*., **6,** 1143 – 1152.
Zauberman A, Lupo A and Oren M. (1995). *Oncogene*, **10,** 2361 – 2366.
Zhao J, Schmieg FI, Logsdon N, Freedman D, Simmons DT and Molloy GR. (1996). *Oncogene*, **13,** 293 – 302.