THE UNIVERSITY OF CALGARY

# p53 Binds DNA as a Tetramer: How and Why

by

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A DISSERTATION

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR

THE DEGREE OF DOCTOR OF PHILOSOPHY

DEPARTMENT OF MICROBIOLOGY AND INFECTIOUS DISEASES

CALGARY, ALBERTA, CANADA

OCTOBER, 1997

## ABSTRACT

P53 tetramers bind to consensus DNA sequences as dimers of dimers. The two monomers within each dimer bind two consecutive quarter-sites (a half-site) and the two dimers within each tetramer bind pairs of half-sites. Although one dimer within a tetramer is sufficient for binding to DNA, both dimers bind cooperatively to a consensus sequence, resulting in a much longer p53-DNA half-life. In an environment of excess half-sites there is a marked decrease in the probability that free dimers, but not dimers of a tetramer, bind to adjacent half-sites in a consensus site. Consequently, in an environment of excess half-sites (such as occurs in the nucleus), p53 tetramers, but not dimers, bind to consensus DNA sites with high affinity.

In an environment of excess non-specific DNA, tetramers, but not dimers, bind consensus sequences with high fidelity. High fidelity DNA binding is due primarily to avidity, which results from tetramers dissociating from DNA only if both dimers simultaneously dissociate from the same molecule of DNA. Thus, the p53 tetramerization domain functions to increase the affinity and fidelity of DNA binding.

Another functional consequence of tetramerization is that p53 can bind to DNA via one conformationally wild type dimer ($PAb246^-/PAb240^-$) while the other dimer is conformationally mutant ($PAb246^-/PAb240^+$). This heterogeneous conformation exists in a population of p53 that is primarily mutant ($PAb246^-/PAb240^+$), suggesting that the mutant conformation is not necessarily dominant negative. Moreover, wild type p53 is

dominant in tetramers that are composed of one genotypically mutant dimer and one wild type dimer.

DNA-bound dimers are always conformationally wild type, and each dimer of a tetramer is in an independent conformational equilibrium. After one dimer binds to DNA, the second dimer of a tetramer can be 'trapped' in the mutant non DNA-bound conformation if the dimer binds to a protein such as PAb240. Alternatively, the second dimer can be 'trapped' in the wild type conformation if it binds to an adjacent half-site or to a protein such as PAb246. Excitingly, genotypically mutant p53 can be induced to bind DNA if it is 'trapped' in the wild type conformation by PAb246.

## PREFACE

Cancer is a world health problem, and as such has been the focus of an intensive international research effort for over 25 years. While tremendous progress has been made in understanding how the properties of cancer cells differ from those of normal cells, this knowledge has not yet been translated into a cure. During the past few years, it has become possible to begin looking at ways of curing cancer by targeting common differences between normal and cancer cells. Only a few common but specific molecular targets have been found, including p53, telomerase, ras, and Rb/cdk4/cyclin D/INK4. The most promising of these is p53, because activation of p53 in many cancer cells, but not in normal cells, causes cell death by apoptosis.

Initially my work on p53 was to focus on the oligomerization of the protein and to determine whether there were differences in this respect between mutant and wild type p53. Soon thereafter, p53 was reported to be a tetramer and a DNA-binding protein. Moreover, DNA binding proved to be the critical property required for p53 tumor suppressor activity. Thus, the focus of my research turned to investigating the specific conformation(s) of p53 that bind DNA, and how these conformations might be regulated.

Although many exciting leads were discovered, the few that were pursued were selected because they related directly to the property of wild type p53 that is inactivated in most tumors: DNA binding. The results that follow detail various important aspects of *how* p53 binds DNA at the molecular level and *why* p53 binds DNA as a tetramer.

## ACKNOWLEDGMENTS

I would like to thank the members of my supervisory committee, Drs. Karl Riabowol, Dylan Edwards, Susan Lees-Miller, and Patrick Lee. I would also like to thank the two additional members of my Ph.D. examining committee, Dr. David Bazett-Jones, and Canada's leading p53 expert, Dr. Sam Benchimol, for lending their expertise to the examination of my dissertation. Special thanks go to my supervisor, Dr. Lee, for being a mentor with good focus on molecular detail and for encouraging me to pursue whatever aspects of p53 research that I found most interesting.

I also acknowledge the various members of the lab, past and present, who have all contributed in their own way to the honest (for the most part) and very unique lab atmosphere. The people have made my six long years working in the basement of the Health Sciences Centre interesting and often quite entertaining.

Last and most importantly, I would like to thank my wife, Deanna, for her love, understanding, and support.

I dedicate this thesis to my son and to his generation, in the sincere hope that for them, the blight called cancer will be just another disease.

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| | |
|---|---|
| A344 | wild type human p53 with leucine 344 substituted by alanine |
| CON | p53 consensus DNA binding site |
| DMEM | Dulbecco's modification of minimal essential medium |
| DNA | deoxyribonucleic acid |
| DTT | dithiothreitol |
| EMSA | electrophoretic mobility shift assay |
| HEPES | N-[2-Hydroxyehtyl]piperazine-N'-[2-ethanesulfonic acid] |
| $k_D$ | dissociation rate constant |
| M24 | CON, but lacking specific binding to quarter-sites two and four |
| M34 | CON, but lacking specific binding to quarter-sites three and four |
| MUT | CON, but with only partial binding to quarter-sites two and four |
| NB | p53 DNA binding control lacking binding to all four quarter-sites |
| p53LZ332 | wild type murine p53 with a leucine zipper placed at position 332 |
| PAGE | polyacrylamide gel electrophoresis |
| PBS | phosphate-buffered saline |
| PMSF | phenylmethanesulfonyl fluoride |
| RRL | Rabbit reticulocyte lysate |
| SDS | Sodium dodecyl sulfate |
| $t_{1/2}$ | half-life |
| TBS | Tris-buffered saline |
| Tris | tris (hydroxymethyl) aminomethane |

What matters it to us what the many think?

Plato, Criton

# INTRODUCTION

## 1. Structure and Function of p53

### 1.1. *p53* is a tumor suppressor gene

The p53 tumor suppressor gene is the most frequently mutated cancer-associated gene yet identified, with p53 mutations occurring in approximately half of all human tumors (Nigro et al., 1989; Hollstein et al., 1991; Hollstein et al., 1996). Indeed, many characteristics that are selected for in cancer cells have been ascribed at least in part to either the loss of wild type p53 or the gain of mutant p53: immortalization (Jenkins et al., 1984; Rovinski and Benchimol, 1988; Harvey and Levine, 1991; Gollahon and Shay, 1996), co-operation with activated ras to transform cells (Parada et al., 1984; Eliyahu et al., 1984; Serrano et al., 1997), loss of DNA damage-induced apoptosis (Clarke et al., 1993; Lowe et al., 1993; Morgenbesser et al., 1994), angiogenesis (Dameron et al., 1994; reviewed by Bouck, 1996), resistance to chemotherapy (Lowe et al., 1993; Zastawny et al., 1993; Aas et al., 1996), increased metastatic potential (Baker et al., 1990; Crook and Vousden, 1992; Kemp et al., 1993), gene amplification (Livingstone et al., 1992; Yin et al., 1992), aneuploidy (Harvey et al., 1993), decreased DNA repair (Lee et al., 1994; Smith et al., 1995), and loss of the DNA damage cell cycle checkpoint (Kuerbitz et al., 1992). It is not known if tumor-derived mutant p53 proteins have lost (or gained) multiple molecular functions that contribute in different ways to tumorigenesis, or if only one or a few basic functions are lost that have pleiotropic effects. Several excellent recent reviews cover various aspects of p53 structure/function (Levine, 1997; Ko and

Prives, 1996; Soussi and May, 1996; Gottlieb and Oren, 1996).

In 1979 p53 was discovered as a cellular transformation antigen that could bind to SV40 large T antigen (Linzer and Levine, 1979; Lane and Crawford, 1979; DeLeo et al., 1979). Initially p53 was thought to be an oncogene due to immortalization of cells in culture (Jenkins et al., 1984) and co-operation with activated ras to transform primary rat embryo fibroblasts (Parada et al., 1984; Eliyahu et al., 1984). Wild type *p53* was subsequently discovered to be a tumor suppressor protein (Finlay et al., 1989; Baker et al., 1989). The initial report of an immortalization function of *p53* was a result of the inadvertent use of activated mutant *p53* cDNA and genomic clones, and wild type *p53* was shown not to immortalize primary cells (Eliyahu et al., 1989; Hinds et al., 1989).

Three initial lines of evidence indicated the tumor suppressor function of *p53* (reviewed in Levine, 1990): 1) both *p53* alleles are mutated in most tumors, suggesting a selection for loss of wild type function; 2) cotransfection of wild-type p53 drastically reduces the production of transformed foci in primary rat embryo fibroblasts transfected with activated *ras* plus either mutant *p53* or E1A; and 3) all three well-studied small DNA tumor viruses encode protein products which bind to and inactivate wt *p53*. The role of *p53* as a tumor suppressor was confirmed by studies which correlated germ line transmission of one mutated *p53* allele with individuals that developed early or multiple tumors in some cancer-prone Li-Fraumeni syndrome families (Malkin et al., 1990; Srivastava et al., 1990). Final proof came with the creation of *p53* null transgenic mice,

which developed spontaneous tumors within 6 months of birth (Donehower et al. 1992). Tumors arising in *p53* heterozygous mice invariably had lost the wild type *p53* allele, indicating that loss of wild type *p53* function was selected during tumorigenesis (ibid).

In *p53* heterozygous humans, the acceleration of tumor development is presumably due to the requirement of only one 'hit' at the wild type *p53* locus (or zero for *p53* knockout mice) to inactivate *p53*. This would accelerate the pattern of mutation in most tumors where one *p53* allele sustains a mutation, followed by loss of heterozygosity (Nigro et al., 1989; Baker et al., 1989; Hollstein et al., 1994). Loss of heterozygosity could happen after deletion of the wild type allele or by recombination of the alleles such that the wild type allele was replaced by the mutated allele. In either case, most tumors are not heterozygous for *p53*, indicating the tumorigenic selection for mutation of total loss of wild type *p53* function. It follows that a normal role of wild type *p53* is to suppress tumor formation.

An interesting feature of the *p53* mutation profile is that usually both alleles are not selected for deletion or nonsense mutation by tumor cells, but rather at least one sustains a missense (point) mutation (Nigro et al., 1989; Baker et al., 1989; Hollstein et al., 1994). This indicates that total loss of p53 is not as tumorigenic as is the presence of mutant p53 in the cell (reviewed by Zambetti and Levine, 1993). All tumor-derived point mutations of *p53* inactivate wild-type function, but others seem to also be activating mutations which lead to gain of function as assayed by augmentation of *ras* + *myc* or *ras*

+ E1A transformation (Parada et al., 1984; Eliyahu et al., 1984; Serrano et al., 1997). When mutant *p53* is introduced into p53 null cells, they display increased focus formation in soft agar and increased tumor formation in mice (Wolf et al., 1984; Dittmer et al., 1993). Moreover, the acquisition of mutant *p53* can confer metastatic potential (Hsiao et al., 1994) and might confer resistance to chemotherapeutic drugs via overexpression of P-glycoprotein, which indiscriminately transports large molecules out of the cell (Zastawny et al., 1993).

1.2. The p53 gene and conserved domains

The human *p53* gene has been localized to chromosome 17p13.1, and both a *p53* gene as well as a pseudogene has been identified in mice (reviewed by Montenarh, 1992). Clones from many species have been obtained, including human, monkey, mouse, rat, chicken, frog, squid, rainbow trout, and mollusks, but neither from *Drosophila* nor yeast (reviewed by Soussi and May, 1996). The genomic organization is similar between species, usually 11 exons with 10 introns. Interestingly, exon 1 is located 6-10kb upstream from exon 2, which in turn contains the p53 translational start site at its 5' end (reviewed in Soussi and May, 1996). At the other end of the gene, alternative splicing has been noted which gives rise to a C-terminally truncated p53 translation product in mice (Arai et al., 1986; Shaulsky et al., 1990b; Kulesz-Martin et al., 1994).

Sequence analysis of p53 predicts a 390 (murine) or 393 (human) amino acid protein consisting of three distinct domains (Figure 1). There is an N-terminal highly

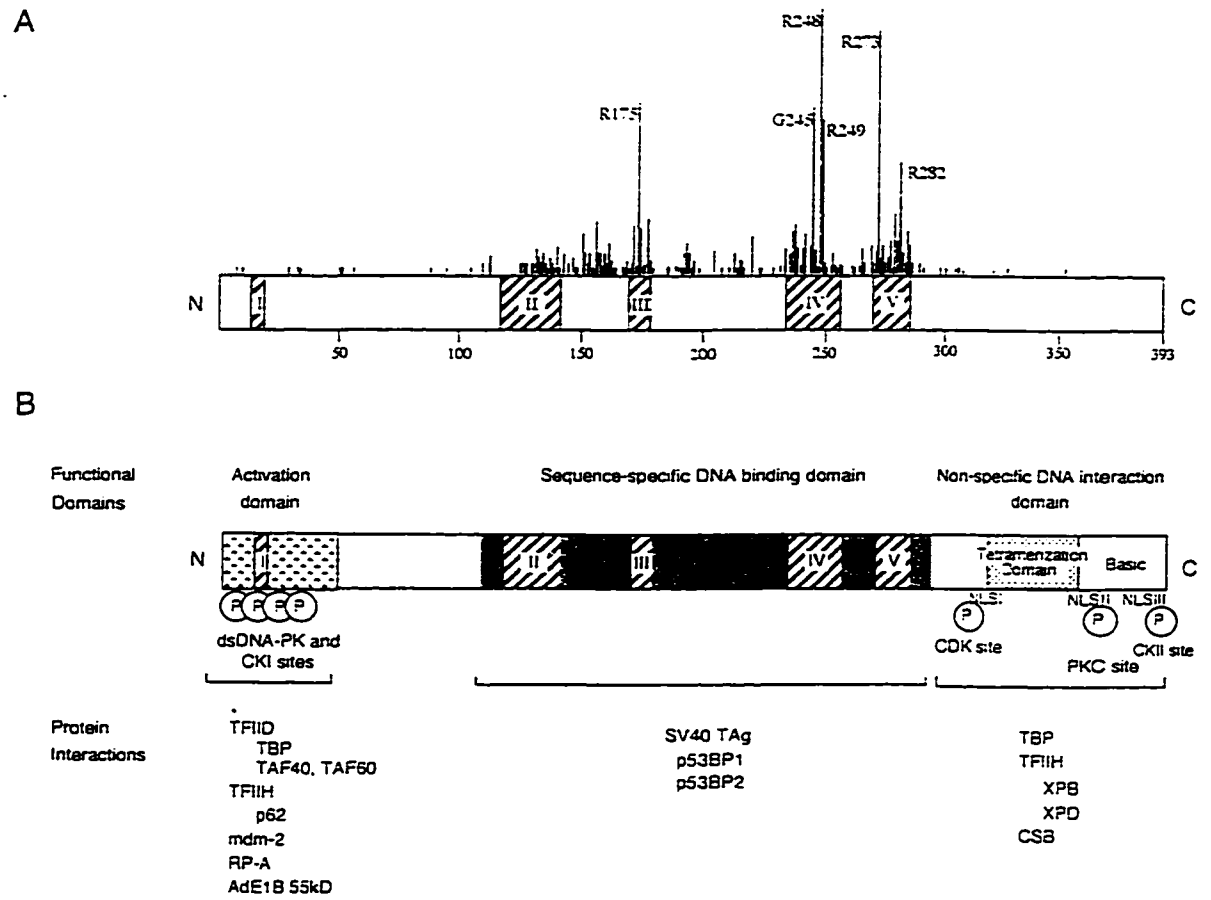



**Figure 1.** Landmarks of the human p53 protein. (*A*) p53 mutations found in human cancer. Hatched boxes represent evolutionarily conserved regions. Vertical lines above represent the frequency at which mutations are found at each particular residue and are clustered in conserved regions II–V. Several hot spots for mutations R175, G245, R248, R249, R273, and R282 are also indicated. (*B*) Structural organization of the p53 protein. Hatched boxes represent conserved regions. (Basic) The extreme carboxyl terminus, which contains several basic residues. Nuclear localization sequences (NLSs) and phosphorylation sites (circled Ps) are shown below. Additionally, analysis of murine p53 has also identified phosphorylation sites for INK kinase at amino acid 34 and MAP kinase at amino acids 73 and 83 (see text). However, corresponding residues in the human protein are not conserved acceptor sites for Ser/Thr phosphorylation. Of the many proteins demonstrated to interact with p53 (see Table 1), a subset of them is known to interact with particular regions of p53; these are shown at the *bottom*.

**Figure 1. Structural domains of p53.** This figure is reproduced without modification from Ko and Prives, 1996, pg. 1055, © 1996 by Cold Spring Harbor Laboratory Press.

charged acidic domain, followed by a hydrophobic, proline-rich central domain, and a C-terminal highly charged basic domain (Pennica et al., 1984). Five 6-20 amino acid domains have been evolutionarily conserved in p53, and all represent critical functionally active domains (Soussi et al., 1987; reviewed by Soussi and May, 1996). These domains span the following amino acid residues: I) 13-19, II) 111-136, III) 165-175, IV) 230-252, and V) 264-280 (Figure 1). Indeed, the last four of these conserved domains are hotspots for missense mutation (Hollstein et al., 1991, 1994). While domain I is involved in transcription and multiple protein-protein interactions, domains II to V are critical for p53 DNA binding ( Soussi and May, 1996).

1.3. Tumor suppressor activity of p53 requires sequence-specific DNA binding

Both wild type and mutant p53 can bind non-specifically to single-stranded DNA (Pavletich et al., 1993; Wang et al., 1993) and to a nuclear matrix/scaffold attachment DNA element (Weissker et al., 1992). Only wild type p53 binds to a double-stranded DNA consensus binding site (CON) containing two or more copies (consecutive or separated by one or two helical turns) of the ten base pair half-site 5'-PuPuPuC(A/T)(A/T)GPyPyPy-3', where Pu and Py represent purines and pyrimidines respectively (Kern et al., 1991; El-Deiry et al., 1992; Funk et al. 1992; Wang et al., 1995; Waterman et al., 1995). Thus, CON may be thought of as four inverted five base pair quarter-sites, where the first quarter-site is underlined. X-ray crystallography has revealed how one core domain monomer (the central half of p53, residues 102-292) binds to one such quarter-site (Cho et al., 1994).

The core domain has a very similar structure whether or not it is bound to DNA. Two β sheets form a sandwich, with one end being tightly packed and the other end being frayed (ibid). The frayed end of the sandwich positions the portions of p53 that contact DNA, consisting of a loop-β sheet-α helix motif and two large loops. Each element of the loop-sheet-helix contacts the major groove directly, one of the large loops (L3) contacts the minor groove, and the two large loops (L3 and L2) tetrahedrally coordinate a zinc atom.

It is very striking that these elements comprise the same regions of p53 that are evolutionarily conserved and harbour the greatest frequency of mutations (ibid; reviewed by Soussi and May, 1996; see Figure 1). The loop-sheet-helix motif consists of conserved domains II (the L1 loop and the S2 sheet) and V (part of the S10 sheet and the H2 helix). The L3 loop (conserved domain IV) that contacts the minor groove is partially positioned on DNA via a zinc atom that bridges the L3 loop to the L2 loop and H1 helix (conserved domain III) (ibid).

Six hotspots for p53 mutation account for about 40% of all tumorigenic p53 mutations (Figure 1). These are G245 (6%), R248 (10%), and R249 (6%) all from the L3 loop, R273 (9%) from the S10 sheet and R282 (4%) from the H2 helix, and R175 (6%) from the L2 loop (Cho et al., 1994; Hollstein et al., 1994). Only R248 and R273 directly contact DNA, but the other four hotspot mutants stabilize the tertiary structure of the L3 loop and helix-loop-helix (Cho et al., 1994). Thus, each hotspot mutation disrupts

sequence-specific DNA binding, although all amino acids that are critical for sequence-specific binding are not hotspots (Cho et al., 1994).

There is a satisfying correlation between the various mutations and the conformation of genotypically mutant p53. Mutation of any of the eight contact amino acids results in the loss of DNA binding, but the conformation of these genotypic p53 mutants is wild type (Cho et al., 1994; Bargonetti et al, 1993; Gannon et al., 1990). All other tumor-derived mutants that have been tested fail to bind to the p53 DNA consensus sequence, and also have a mutant conformation that is reactive with the mutant-specific antibody PAb240 (ibid). Thus, there are contact mutants and conformational mutants.

Remarkably, none of the residues that are hotspot mutants makes sequence-specific DNA contacts (Cho et al., 1994). This is somewhat surprising because sequence-specific DNA binding is a property of wild type p53 that is consistently lost in tumor-derived mutant p53. For example, the most crucial base in each p53 consensus quarter-site PuPuPuC(A/T) is the invariant C (El-Deiry et al., 1992). R280 contacts the G that is base-paired with the invariant C, but represents only 2% of the tumor-selected mutations (Cho et al., 1994). In fact, of the eight p53 residues that contact DNA, only two (R248 and R273) are mutated with high frequency (ibid). This is despite the fact that mutation of any of the eight contact residues abrogates DNA binding (reviewed by Friend, 1994). It may be concluded that the process of tumorigenesis strongly selects against p53 DNA binding, but another selection process also occurs during tumorigenesis that selects

against an additional, so far uncharacterised, property of wild type p53.

1.4. Subunit organization of DNA-bound p53

In transformed cells, p53 is present in high molecular weight forms (Kraiss et al., 1988). Wild-type p53 from cells as well as from *in vitro* translated mRNA was thought to exist as dimers to tetramers (McCormick et al., 1981). That p53 is a tetramer has been confirmed (Stenger et al. 1992; Sturzbecher et al., 1992; Friedman et al. 1993; Halazonetis and Kandil, 1993). Two domains that localize to the C-terminal 60 amino acids were initially thought to be involved in p53 tetramerization (Sturzbecher et al., 1992). A domain required for tetramerization resides in the correctly predicted amphipathic helix spanning residues 334-356, where mutation of hydrophobic amino acids abolishes oligomerization, but mutation of acidic residues reduces tetramers to dimers (ibid). However, the erroneous conclusion was reached that residues 334-356 formed the dimerization domain and 357-390 formed the tetramerization domain (ibid).

It was subsequently determined that C-terminal fragments of p53, spanning residues 311-367, formed tetramers in solution (Wang et al., 1993; Pavletich et al., 1993). Much more detailed information became available with the publication of the nuclear magnetic resonance and X-ray crystallography structure of the oligomerization domain (Lee et al., 1994; Clore et al., 1994, 1995a,b; Jeffrey et al., 1995). The initial NMR structure of the tetramerization domain (Clore et al., 1994) had correct secondary and tertiary structures for each of the four monomeric subunits, but the relative orientations of

the dimers were later shown to be off by about 55° due to incorrect assignment of critical inter-subunit NOEs (Lee et al., 1994; Clore et al., 1995a,b; Jeffrey et al., 1995). The isolated tetramerization domain forms a tetrahedrally symmetric dimer of dimers in which each of the four subunits are geometrically equivalent (Lee et al., 1994; Clore et al., 1994, 1995a,b; Jeffrey et al., 1995). However, a lower order of symmetry must be exhibited by DNA-bound p53 if all four subunits in the tetramer contact a consensus DNA binding site (Waterman et al., 1995).

A model was constructed in which four core domains can occupy the four quarter-sites in a full consensus sequence without steric clashes (Cho et al., 1994). This physical model of four p53 subunits bound to the consensus site is consistent with solution studies, where four p53 core domains can bind cooperatively to a consensus DNA sequence (Wang et al., 1995; Balagurumoorthy et al., 1995).

An important missing element in understanding p53 DNA binding is the global orientation of the oligomerization domains relative to the DNA binding domains. This is because the structures of the DNA binding and tetramerization domains, which consist of amino acids 102-292 and 324-355 respectively, have been determined separately but are joined by a flexible linker (reviewed by Arrowsmith and Morin, 1996). In an intact DNA-bound tetramer this linker would be sufficiently long that it could connect the carboxyl termini of any of the DNA binding domains to the amino termini of any of the oligomerization domains (Clore et al., 1994; Lee et al., 1994; Jeffrey et al., 1995).

Therefore, the relative orientations of the tetramerization and DNA binding domains cannot be deduced from available biophysical data.

Two different models have arisen that conceptually connect the DNA-binding domains to the oligomerization domains in an intact p53 tetramer. In one model, one dimer of a tetramer contacts the first and second quarter-sites and the other dimer binds to the third and fourth quarter-sites in the consensus sequence (Halazonetis and Kandil, 1993; Clore et al., 1994; Waterman et al., 1995). In the other model, one dimer contacts the first and third quarter-sites and the other dimer binds to the second and fourth quarter-sites (Cho et al., 1994; Lee et al., 1994; Jeffrey et al., 1995; Arrowsmith and Morin, 1996; Pennisi, 1996).

These models entail distinct predictions about the ability of isolated dimers to bind DNA, because core domains and tetramers both bind the first and second much better than the first and third quarter-sites (Wang et al., 1995). If stable binding occurs only when p53 subunits interact on adjacent first and second quarter-sites then of the two above-mentioned models, the former but not the latter predicts that dimers could bind to a half-site. The latter model proposes that a dimer bind to quarter-sites one and three, so the stabilizing inter-subunit interaction on quarter-sites one and two could only occur if two dimers both bound to a consensus sequence. Although isolated dimers do bind to a half-site (Halazonetis and Kandil, 1993; Waterman et al., 1995), the ability of dimers to bind to quarter-sites one and three has never previously been tested. It has not been

previously resolved which model of binding is correct or what the consequences are for understanding p53 DNA binding or tumor suppressor activity. Indeed, the reason why p53 is a tetramer is not known, because dimers can bind DNA (Halazonetis and Kandil, 1993; Waterman et al., 1995). Even high concentrations of p53 monomers can bind DNA, albeit with lower affinity than tetramers (Bargonetti et al., 1993; Pavletich et al, 1993; Wang et al., 1993, 1995; Balagurumoorthy et al., 1995).

1.5. The carboxyl terminal 30 amino acids of p53 negatively regulate DNA binding

Purified, bacterially expressed p53 exhibits weak sequence-specific DNA binding. However, an *in vitro* generated deletion mutant that lacked the C-terminal 30 amino acids was able to bind DNA strongly (Hupp et al., 1992). Wild type p53 was activated for DNA binding by several modifications of the C-terminus: removal by proteolysis, binding of PAb421, interaction with E. coli dnaK (homologous to hsp70), or phosphorylation by casein kinase II (ibid). At the time, Sturzbecker et al. (1992) had incorrectly concluded that the C-terminal 30 amino acids were required for tetramerization but not dimerization. This led Hupp et al. (1992) to propose that the conversion of p53 from tetramers to dimers activated DNA-binding. As discussed above, this conclusion was erroneous.

Nonetheless, the C-terminal 30 amino acids contain a domain that negatively regulates p53 DNA binding. Another modification of the C-terminus that activates DNA binding is phosphorylation of the PAb421 epitope by protein kinase C (Takenaka et al.,

1995). The result is loss of PAb421 binding, and has led to the suggestion that phosphorylation by CKII or PKC activates DNA binding, but might have different consequences for other protein-protein interactions (ibid). Addition of a peptide whose sequence spanned the C-terminal 30 amino acids was also able to activate DNA binding (Hupp et al. 1995; Jayaraman and Prives, 1995). This suggests that the negative regulation results from the negative regulatory domain binding to another region of p53, possibly the central core DNA binding domain (ibid). Excitingly, CBP/p300 activates p53 by inducing acetylation of the negative regulatory domain *in vitro* and *in vivo* (Gu and Roeder, 1997).

Another very interesting activity of the C-terminal 30 amino acids is the ability to bind DNA non-specifically (Pavletich et al., 1993; Wang et al., 1993). Single stranded DNA, 19-40 bases in length, activates p53 DNA binding dependent on the C-terminal domain (Jayaraman and Prives, 1995). Interestingly, this activation is unique in that binding is only detectable to a p53 consensus binding sequence in supercoiled plasmid DNA, but not to a short dsDNA oligonucleotide (ibid). This activation might be relevant to the 29 base single stranded DNA that is produced during mismatch repair (ibid), or to the DNA and RNA strand transfer/reannealing activity of p53 (Oberosler et al., 1993; Brain and Jenkins, 1994; Bakalkin et al., 1994, 1995; Wu et al., 1995).

## 2. Mechanisms That May Contribute To p53 Tumor Suppressor Activity

### 2.1. p53 is nuclear and is involved in DNA replication/repair

Upon serum-stimulation of growth arrested cells p53 localizes from a perinuclear distribution to the nucleus (Dippold et al., 1981). The nuclear localization of p53 occurs during early S phase, after cytoplasmic accumulation in G1, and p53 remains nuclear for about three hours (Shaulsky et al., 1990a; Takahashi and Suzuki, 1994). A temperature sensitive mutant p53 is cytoplasmic when in the mutant conformation, but when in the wild type conformation it becomes nuclear and inhibits DNA synthesis (Gannon and Lane, 1991). Interestingly, both wild-type and gain-of-function mutant p53 activities appear to rely on the ability of p53 to be transported into the nucleus via defined nuclear localization signals (Shaulsky et al., 1991; Shaulsky et al., 1990b; however see Slingerland et al., 1993). Additionally, p53 is transported into and out of the nucleus in an energy-dependent manner (Middeler et al., 1997).

Nuclear matrix association of p53 has been demonstrated (Deppert and Haug, 1986), and both mutant and wild type p53 bind specifically and with high affinity to a nuclear matrix/nuclear scaffold attachment DNA element, or MAR (Muller et al., 1996; Weissker et al., 1992). A role of p53 in the regulation of DNA synthesis is suggested because of competition between p53 and DNA polymerase-$\alpha$ for binding to the SV40 T antigen (Gannon and Lane, 1987). SV40 DNA synthesis is inhibited when p53 is overexpressed (Braithwaite et al., 1987), and in a DNA-binding-dependent manner (Miller et al., 1995), but not when normal levels of p53 are produced (von der Weth and

Deppert, 1993). Stronger support for a role in DNA replication is that the cellular Replication Protein A (RP-A) forms a complex with p53, which then stimulates DNA replication *in vitro* (Li and Botchan 1993). However, p53 also can block DNA replication *in vitro* (Cox et al., 1995; Miller et al., 1995).

RP-A is involved in both replication and repair, so the binding of p53 to RP-A could be relevant to one or both processes *in vivo* (Li and Botchan 1993; Dutta et al., 1993). In fact, p53 directly interacts with a number of proteins involved in DNA repair (Figure 1; reviewed by Ko and Prives, 1996; Gottlieb and Oren, 1996). CSB (ERCC6) is a strand-specific nucleotide excision repair factor that interacts with p53 (X. Wang et al., 1995). TFIIH is involved in both repair and transcription, and several TFIIH subunits bind p53, namely p62, XPB, and XPD (X. Wang et al., 1994; Xiao et al., 1994; X. Wang et al., 1995). These observations imply that p53 may be important for the regulation of DNA replication and/or DNA repair.

Indeed, cells that lack wild type p53 accumulate γ-radiation-induced dsDNA damage (X. Lee et al., 1994). As well, p53 is necessary for preventing gene amplification (Livingstone et al., 1992; Yin et al., 1992) and for maintaining a diploid genome (Harvey et al., 1993; Cross et al., 1995). Localization of p53 to the nucleus may therefore normally reflect the participation of p53 in a cell cycle control point that maintains integrity of the genome (Kastan et al. 1992; Levine, 1997).

2.2. DNA damage activates p53-dependent G1/S and G2/M cell cycle checkpoints

In order to avoid replicating damaged DNA and thereby propagating mutated genomes, normal cells ensure that any DNA damage has been repaired before entering the DNA synthesis (S) phase of the cell cycle (reviewed by Hartwell and Kastan, 1994). The enforced dependence of S phase on properly repaired DNA constitutes part of the G1/S cell cycle checkpoint (Kastan et al., 1992). This supports the proposed role of p53 in suppression of tumorigenesis (Lane 1992), where accumulation of p53 is induced by DNA-damaging agents (Maltzman and Czyzyk, 1984; Kastan et al. 1991) and is proposed to 'guard the genome' from being replicated before being repaired (Lane 1992).

Another aspect of p53 function that might prevent genomic instability is the participation of p53 in $G_2$/M arrest (Di et al., 1994; Agarwal et al., 1995; Stewart et al., 1995). When mitotic spindle inhibitors are added to dividing cells, p53 enforces a dependence of $G_1$ and S on previous completion of mitosis (Cross et al., 1995; Paules et al., 1995; Aloni-Grinstein et al., 1995; Harvey et al., 1993). Therefore, in addition to the $G_1$/S DNA damage checkpoint, p53 also participates in a $G_2$/M spindle checkpoint (ibid; reviewed by Ko and Prives, 1996).

The means by which p53 "senses" DNA damage is an extremely important aspect of p53 function. DNA damage could theoretically be indirectly detected by protein or lipid damage that is sustained at the same time as DNA damage, for example by radiation-induced free radical attack. However, p53 can be directly activated by

double-stranded DNA breaks, since the cleavage of genomic DNA by a restriction endonuclease *in vivo* is sufficient to activate p53 (Nelson and Kastan, 1994).

Moreover, p53 binds to DNA ends and mismatches (Lee et al., 1995; Reed et al., 1995; Bakalkin et al., 1994). Wild type p53 binds to the ends of single stranded DNA with much higher affinity than do mutant p53 proteins, and a role for p53 in repairing DNA damage has been suggested to be joining complementary ssDNA ends (Bakalkin et al., 1994). Alternatively, p53 may be regulated by a single-stranded 29 base product of excision repair (Jayaraman and Prives, 1995).

The presence of DNA damage may be relayed to p53 by means that are as yet unidentified, but which could involve genes that are defective in Bloom's Syndrome (Lu and Lane, 1993) or in ataxia-telangiectasia (Kastan et al., 1992; disputed by Lu and Lane, 1993). A very attractive candidate for detecting and relaying the presence of damaged DNA to p53 is the double-stranded DNA-dependent protein kinase (DNA-PK). This kinase consists of a large 470 kDa catalytic subunit and a regulatory Ku heterodimer that consists of a 70 and 80 kDa protein (Gottlieb and Jackson, 1993; Dvir et al., 1992). Ku80 is mutated in a radiosensitive cell line, and restoration of wild type Ku80 restores the normal response to DNA damage in these cells (Taccioli et al., 1994). The Ku protein binds to single-stranded regions of DNA, including ends, nicks, and transitions to secondary structure, which in turn activates the DNA-PK kinase activity (Morozov et al., 1994).

Since Ku80 is necessary for dsDNA break repair, and the binding of Ku80 to the ends of damaged DNA activates DNA-PK, DNA-PK could potentially activate p53 by phosphorylation. This possibility is very attractive given that DNA-PK is known to phosphorylate p53 *in vitro* on serines that are phosphorylated *in vivo* (Lees-Miller et al., 1992; Meek and Eckhart, 1988; Wang and Eckhart, 1992; Morgenbesser et al., 1994). The functional consequence(s) of phosphorylation of p53 by DNA-PK are unknown, but it might be expected that DNA-PK would activate p53 after DNA damage. Indeed, p53 is phosphorylated more efficiently by DNA-PK *in vitro* when both proteins are bound to nearby sites on the same stretch of DNA (Lees-Miller et al., 1992).

2.3. p53 can activate genes and initiate cell cycle arrest via transcriptional activation

A minimal transactivation domain was mapped to a 22 amino acid domain of p53, aa 20-42 (Unger et al., 1992; Miller et al., 1992), which is immediately C-terminal to conserved domain I. When fused to the DNA-binding domain of GAL4, the 22 residue acidic domain strongly activates transcription of a GAL4 reporter CAT construct (Unger et al., 1992; Miller et al., 1992), suggesting that p53 may activate transcription directly. P53-dependent transcription occurs *in vitro* (Farmer *et al.* 1992), and p53-mediated transactivation increases with increased binding of p53 to DNA *in vitro* (Kern et al., 1992). Finally, the amino terminal transactivation domain can be replaced with a heterologous transactivation domain, and the chimera retains transactivation function (Pietenpol et al., 1994).

The general transcription factor TBP interacts with the transactivation domain, as do the TBP-associated factors, *drosophila* $TAF_{II}40$ and $TAF_{II}60$ (Lin et al., 1994; Thut et al., 1995; Lu and Levine, 1995). Mutation of residues 22 and 23 abolishes both transactivation and TAF binding, but not TBP binding (ibid). Therefore, the transcriptional activation domain of p53 may act through binding TAFs, but not TBP.

Consensus binding sequences are found within DNA elements proximal to the ribosomal gene cluster (Kern et al., 1991), muscle creatine kinase (Weintraub et al., 1991; Zambetti et al., 1992), GADD45 (Kastan et al., 1992), CIP1/WAF1 (El-Deiry et al., 1993), MDM-2 (X. Wu et al., 1993; Juven et al., 1993), the anti-angiogenic factor TSP-1 (Dameron et al,. 1994), Rb (Osifchin et al., 1994), thrombospondin-1 (Dameron et al,. 1994), the EGF receptor (Deb et al., 1994), TGF-α (Shin et al., 1995), fas/APO-1 (Owen-Schaub et al., 1995), PCNA (Shivakumar et al., 1995), cyclin D (Chen et al., 1995), and IGF-BP3 (Bourdon et al., 1997). Interestingly, the locations of some of the p53 binding DNA elements are outside the immediate 5' promoter elements, either being far upstream (CIP1/WAF1) or within introns (GADD45, IGF-BP3, MDM-2, and TSP-1).

Another gene that is transactivated by p53 is bax, which promotes cell death through apoptosis (Zhan et al., 1994; Miyashita and Reed, 1995). In response to DNA damage, p53 directs cells to either cell cycle arrest or undergo apoptosis, so the activation of bax could be significant in some situations (reviewed by Ko and Prives, 1996; Gottlieb and Oren, 1996). However, it has been clear for some time that the transcriptional

activity of p53 is dispensable for inducing apoptosis in at least certain cell types (Caelles, 1994; Wagner et al., 1994; Crook et al., 1994).

Nonetheless, several genes are transactivated by p53 following DNA damage. The first gene that was identified to be transactivated by DNA damage-induced p53 was GADD45, which was transactivated in response to growth arrest and DNA damage only in cells that had wild type p53 (Kastan et al., 1992). The function of the GADD45 protein product is unknown, but GADD45 interacts with the proliferating cell nuclear antigen (PCNA) (Smith et al., 1994). PCNA in turn participates in the replication and repair of DNA (reviewed by Kelman, 1997).

The main transcriptional target of p53 is the p53-regulated inhibitor of cyclin-dependent kinase (cdk) activity, commonly known as p21, CIP1, or WAF1 (El-Deiry et al., 1993; reviewed by Hunter, 1993). After activation of p53, for example by DNA damage, levels of CIP1 rise (Dulic et al., 1994). Like GADD45, CIP1 can also bind to PCNA, and CIP1 was found to inhibit the activity of PCNA in replication but not in nucleotide excision repair (Flores-Rozas et al., 1994; Waga et al., 1994; Li et al., 1994). Thus, p53 might influence DNA replication/repair by activating transcription of at least two proteins, GADD45 and CIP1, that interact with PCNA.

Production of CIP1 protein also inactivates cdk activity in cyclin/cdk complexes via protein-protein interactions with the complex, thereby blocking phosphorylation of

the retinoblastoma protein pRb (Harper et al., 1993). This leaves pRb in a hypophosphorylated state, bound to E2F, which is thought to be responsible for blocking the cell cycle in G1 before DNA synthesis begins. CIP1 levels increase as does hypophosphorylated pRb after exposure of cells to ionizing radiation, thereby strongly supporting the model whereby p53 blocks the cell cycle after ionizing radiation in order to allow time for DNA repair (Dulic et al., 1994).

There is no doubt that in response to DNA damage p53 can induce a cell cycle arrest by transactivating downstream genes (Pietenpol et al., 1994; Crook et al., 1994). Such genes include CIP1, but CIP1 knockout mice develop normally and their cells retain some of the DNA damage-induced G1/S arrest (Deng et al., 1995). Cells from pRb knockout mice undergo G1 arrest after DNA damage, indicating that the G1/S DNA damage checkpoint might not necessarily require pRb either (reviewed by Levine, 1997). It is possible that other pRb family members (p107 or p130) can substitute for some aspects of pRb function in DNA damage-induced G1 arrest.

If a cell is to continue cycling after DNA damage-induced cell cycle arrest, p53 needs to be eventually turned off once the DNA is repaired. A good candidate for this is mdm-2, which is activated by p53 after γ-radiation (Barak et al., 1994; Chen et al., 1994; Price and Park, 1994). Mdm-2 then binds to and inhibits further activity of the transactivation domain of p53 (Momand et al., 1992; X. Wu et al., 1993; Oliner et. al., 1993; Kussie et al., 1996). It was recently found that upon DNA damage p53 is activated

until the subsequent accumulation of mdm-2 targets p53 for degradation (Haupt et al., 1997; Kubbutat et al., 1997). Mdm-2 is also required to inhibit p53 during embryogenesis *in vivo*, because the early lethality in mdm-2 knockout mouse embryos is alleviated by also knocking out p53 (Jones et al., 1995; Montes de Oca Luna et al., 1995).

2.4. p53 can repress transcription and direct apoptosis independent of transactivation

A property of p53 that appears to be closely related to transcriptional repression is apoptosis (reviewed by Gottlieb and Oren, 1996; Ko and Prives, 1996). In the presence of cycloheximide, wild type p53 reduces transcription of a CAT reporter gene under control of the serum-inducible promoter of c-fos (Ginsberg et al. 1991). Other "housekeeping" genes that are repressed by p53 include β-actin, p53, c-fos, c-jun, hsc 70 (Ginsberg et al. 1991), c-myc (Ragimov *et al.* 1993), Rb (Shiio *et al.* 1992), interleukin 6 (Santhanam *et al.* 1991), and P-glycoprotein (Zastawny et al. 1993). p53 binds Sp1 (Borellini and Glazer, 1993), and repression of hsp70 may occur through p53 binding and inhibiting CCAAT binding factor (Agoff et al., 1993). The interaction of p53 with E2F1 and DP1 also inhibits transactivation (O'Connor et al., 1995). Alternatively, adenovirus E1B 55K binds to DNA-bound p53 and in so doing represses the transcription of genes that are normally induced by p53 (Yew et al., 1994).

It has been suggested that the opposite effects of p53 in repressing or activating promoters might often depend on the presence of a TATA box (Lechner et al. 1992; Seto et al., 1992; Mack et al., 1993). The repression of TATA-driven promoters may be due to

squelching of TBP (ibid; Ragimov et al., 1993; Shaulian et al., 1995). Indeed, the C-terminus of p53 interacts with TBP and is necessary for repression (Horikoshi et al., 1995; Subler et al., 1994, Shaulian et al., 1995). Deletion of the C-terminal 75 amino acids abrogates transcriptional repression but not activation by overexpressed p53, which demonstrates that the two activities are separable (Sang et al., 1994; Subler et al., 1994). As well, overexpression of the C-terminus of p53 can repress p53-independent transcription (Shaulian et al., 1995).

Apoptosis is mediated by p53 even in the presence of the general transcription inhibitor actinomycin D or the translation inhibitor cycloheximide (Caelles et al., 1994; Wagner et al., 1994). Therefore, the transcriptional activation function of p53 is not required for apoptosis. Bcl2 inhibits p53-induced apoptosis and transcriptional repression without interfering with p53 transactivation, suggesting that repression and apoptosis may be linked (Shen and Shenk, 1994; Okan et al., 1995).

## 3. Conformations of p53

### 3.1. Antibody reactivity

Various monoclonal antibodies are available that recognize certain conformations of p53. These include murine-specific PAb242 (epitope at amino acids 9-25), PAb246 (aa 88-109), and PAb421 (aa 370-378) (Wade-Evans and Jenkins 1985). PAb240 recognizes p53 in the 'mutant' conformation, but not in the 'wild type' conformation (Gannon et al. 1990), whereas the converse is true for PAb246 and PAb1620 (Yewdell et

al. 1986, Milner et al. 1987). Upon denaturation of wild type p53, the epitope for 240 is exposed whereas the 246 epitope is destroyed. Under no circumstances are both epitopes displayed together on the same p53 subunit, although some p53, for example monomeric p53, displays neither epitope (Sturzbecher et al. 1992).

The relationship between the 248 and 421 epitopes has also been partially characterized in that they are sometimes displayed together, and at other times are not (Gamble and Milner, 1988). In p53 from SV40-transformed cells, there are distinct populations of $248^{+}/421^{-}$ and $248^{-}/421^{+}$. However, in RRL the p53 produced is either $248^{-}/421^{-}$ or just $421^{-}$. The reason for this difference may be that $248^{-}/421^{-}$ cellular p53 is stable, while the same p53 population produced in RRL is not stable, and is induced to display the 421 epitope during the conditions employed during immunoprecipitation, as found by Halazonetis et al. (1993).

The conformation of p53 also changes as p53 associates with other molecules. For example, after binding to DNA, murine p53 has been reported to adopt a 'mutant'-like conformation (Halazonetis et al. 1993). This is an erroneous description, for their data shows that the conformation of p53 actually became $246^{-}/240^{-}$ after binding to DNA. As well, the C-terminus only displays the 421 epitope after DNA binding, but not before (using low salt buffer, ibid). The loss of display of the 246 epitope (aa 88-109) and presentation of the 421 epitope (aa 370-378) together indicate that both N- and C-terminal conformational changes occur in p53 upon DNA binding in low salt.

3.2. Conformation of p53: wild-type versus mutant

Interestingly, wild type p53 can assume either the wild-type ($246^-/240^-$) or mutant ($246^-/240^+$) conformation, and in lymphocytes, a change from wild type to mutant is observed upon serum stimulation (Milner and Watson 1990). Exposure of the PAb240 epitope is believed to be at the expense of the PAb246 epitope, leading to the notion that wild type "suppressor" p53 is $PAb246^+$ and $PAb240^-$, and mutant p53 displaying the PAb240 epitope is always $PAb246^-$.

*In vitro*, the batch of rabbit reticulocyte lysate (RRL) used to translate p53 mRNA determines what phenotype of p53 is produced (Cook and Milner 1990). When two different batches are mixed, the RRL that produces the mutant conformation of p53 is dominant even when mixed 1:7 with wild type-producing RRL. This indicates that a factor, possibly a protein, is present in the mutant p53-producing RRL, and that this factor either prevents formation of the wild type form or converts wild type p53 to the mutant conformation. Intriguingly, it has recently been found that hsp90, which is present at variable levels in RRL, is required in order for p53 to attain the mutant conformation in RRL (Blagosklonny et al., 1996). Conversely, RRL that does translate wild type p53 fails to produce the $246^-$ conformation in a certain p53 mutant, as expected, but translation of the same mutant *in vivo* results in a $246^-$ conformation (Milner et al. 1993).

It is possible for p53 with a wild type primary sequence to be converted to the PAb246$^-$/PAb240$^+$ 'mutant' conformation. This has been demonstrated *in vitro* when wild type p53 is complexed with a dominant negative mutant p53 (Milner and Medcalf, 1991). Conversion to the mutant conformation also occurs when p53 is subjected to oxidation with physiological concentrations of Cu(II) (Hainaut et al., 1995; Hainaut and Milner, 1993). Intriguingly, p53 can be activated *in vivo* by the redox-regulatory protein Ref-1 (Jayaraman et al., 1997). Another event that converts wild type p53 to a mutant conformation is denaturation (Gannon et al., 1990). The latter observation suggests that the PAb240 epitope is linear, but is largely inaccessible in the wild type protein due to its localization in the hydrophobic core of the β-sandwich (Cho et al., 1994).

Two contrasting hypotheses regarding the biological significance of the ability of wild type p53 to adopt the 'mutant' PAb240$^+$ conformation have emerged. The first hypothesis is based on the crystal structure of the p53 DNA binding domain, which showed the PAb240 epitope to be buried in the hydrophobic core of the β-sandwich and to be removed from the DNA contact sites (Cho et al., 1994). It proposes that the display of the PAb240 epitope is accompanied by loss of DNA binding function and reflects a denatured state, rather than an alternate conformation of p53. In contrast, the 'conformation hypothesis' contends that the PAb246$^-$/PAb240$^+$ conformation of wild type p53 is not necessarily denatured, but reflects a cell cycle-regulated conformation of p53 which is growth promoting (reviewed by Milner, 1995). Whatever the case, the mutant conformation involves more than just the PAb240 epitope, as other regions of p53 also

become exposed in conformationally mutant p53 (Legros et al., 1994; Vojtesek et al., 1995).

The growth promoting conformation is the exclusive form of many tumor-derived mutant p53 proteins (Zambetti & Levine, 1993). The mutant conformation is thought to promote cell growth and transformation not just due to loss of wild type p53 growth suppressor function. Two additional properties of mutant p53 are an uncharacterized transforming function in the absence of endogenous wild type p53 and, when overexpressed, dominant negative inactivation of wild type p53 (ibid).

Both the mutant and wild type conformations of p53 can mediate conformation-specific protein binding. The gain-of-function exhibited by conformational mutants could be related to the two proteins, p38 and p42, that specifically bind to the mutant conformation of p53 (Y. Chen et al., 1994). Conversely, two proteins that bind to the wild type conformation of p53, 53BP1 and 53BP2, could mediate some aspect(s) of wild type p53 function (Iwabuchi et al., 1994). Intriguingly, a co-crystal structure of part of 53BP2 bound to the core domain of p53 revealed that the p53-DNA interface is very similar to the p53-53BP2 interface (Gorina and Pavletich, 1996). It is possible that 53BP2 is involved in the transcription-independent apoptosis induced by p53 following DNA damage (ibid). Indeed, Bcl2 competes with p53 for interaction with 53BP2, lending further support for a role of 53BP2 in apoptosis (Naumovski and Cleary, 1996).

3.3. Mutant p53 is Recessive to Wild-type p53 *In vivo*

When wild type and mutant p53 are overexpressed together in transfected cells, heteroligomers are formed which appear to possess a 'mutant' conformation (Eliyahu et al. 1988; Rovinski and Benchimol 1988; Finlay et al. 1989). In addition, *in vitro* translation demonstrates that mutant/wild type heteroligomers are formed cotranslationally, but not posttranslationally, and that the heteroligomers assume a mutant phenotype (Milner et al. 1991; Milner and Medcalf 1991). However, association of wild type p53 with conformationally wild type, pure loss-of-function mutants, demonstrates that the complexes retain similar or increased levels of transactivational ability compared to that of wild type p53 (Miller et al. 1993). One study found that various hotspot p53 mutants were not dominant negative when expressed *in vivo* (Williams et al., 1995).

The frequent selection for total loss of wild type p53 protein during tumorigenesis indicates that mutant p53 is not physiologically dominant negative, but that wild type p53 has one or more dominant functions that normally inhibit tumor progression (Lane and Benchimol, 1990). The tumor suppressor activity of p53 combined with the gain of function mutants explains the *in vivo* observation that most human tumors contain one mutant allele, which is frequently a gain of function mutant, in addition to a second deleted allele (Nigro et al., 1989; Hollstein et al., 1996).

Despite the clear evidence against p53 mutants being dominant negative *in vivo*, some confusion has arisen due to *in vitro* data that support a dominant negative effect of

p53 mutants. When wild type and mutant p53 are overexpressed together in transfected cells, heteroligomers are formed which possess a 'mutant' conformation (Eliyahu et al., 1988; Rovinski and Benchimol, 1988; Finlay et al., 1989). In addition, *in vitro* translation demonstrated that mutant/wild type heteroligomers formed cotranslationally, but not posttranslationally, and that the heteroligomers assumed a 'mutant' phenotype as determined by antibody analysis (Milner et al., 1991; Milner and Medcalf, 1991). Therefore, *in vitro* immunologic assays for p53 function do not correlate with the ability of wild type p53 to be dominant *in vivo*, so the dominant negative effect of mutant p53 may simply be a result of overexpression.

## 4. Summary

The p53 tumor suppressor gene is the most frequently mutated cancer-associated gene yet identified, with p53 mutations occurring in over half of all human tumors (Hollstein *et al.*, 1994). Most mutations occur in the DNA binding domain, where point mutations may disrupt either protein-DNA interactions directly or alter the overall conformation of the DNA binding domain (Cho *et al.*, 1994; Friend, 1994). Mutations in the p53 DNA binding domain likely contribute to tumorigenesis due to the requirement of DNA binding for p53 tumor suppressor activity (reviewed by Levine, 1997; Ko and Prives, 1996; Soussi and May, 1996). However, additional selective pressures occur during tumorigenesis that favour mutant p53 over lack of p53 (Zambetti and Levine, 1993).

P53 is required for the cellular response to DNA damage (Kastan et al., 1991; Kuerbitz et al., 1992). In fact, p53 can directly bind to irradiated DNA, to DNA which has a short mismatch, or to DNA ends (Lee et al., 1995; Reed et al., 1995; Bakalkin et al., 1994). The p53 protein can then halt the cell cycle to allow repair to occur, or it can induce apoptosis in the damaged cell (Clarke et al., 1993; Lowe et al., 1993), supporting the proposed role of p53 as "guardian of the genome" (Lane, 1992). Levels of p53 rise through transcriptional and post-translational stabilization, and p53 is activated for sequence-specific DNA binding, apoptosis, and transactivation of target genes (reviewed by Ko and Prives, 1996).

Growth arrest is one facet of p53 function, but an alternate and more important role of p53 is the induction of apoptosis in response to DNA damage (Miyashita & Reed, 1995; Caelles et al., 1994; Lowe et al., 1993; Diller et al., 1990; Kastan et al., 1991). Transcriptional activation and cell cycle arrest are linked to each other but not to suppression of transformation (Crook et al., 1994). This suggests that apoptosis might be more important for p53 tumor suppressor function. Indeed, efficacy of radiation and chemotherapy often correlates with the ability of p53 to induce apoptosis (reviewed by Levine, 1997).

Although p53-dependent transcriptional activation in response to DNA damage has been thoroughly investigated, this activity may be secondary for p53 tumor suppressor function. The more important role of p53 in DNA damage-induced apoptosis

has yet to be worked out mechanistically, but may involve one or more of the many proteins that interact with p53. A good candidate for such a protein is 53BP2 (Gorina and Pavletich, 1996; Naumovski and Cleary, 1996). Tumor-derived p53 mutants fail to bind DNA, so DNA binding may be required for apoptosis.

However, the basis for understanding a possible DNA binding-dependent, non-transcriptional activity of p53 that promotes apoptosis will likely require a greater understanding of how p53 binds DNA. In particular, it has not been previously established how each dimer of a p53 tetramer binds to DNA, or indeed why p53 is a tetramer. Additionally, the transcription-independent activity of p53 that promotes apoptosis may require p53 to bind DNA while interacting with other proteins. There has not previously been a theoretical basis for understanding what types of protein-p53 interactions are compatible with DNA binding. Clearly, the mutant and wild type conformations mediate conformation-specific protein-p53 interactions, and the conformation affects the ability of p53 to bind DNA. Thus, greater understanding of the relationship between the mutant and wild type conformations and DNA binding will also be important.

# MATERIALS & METHODS

## 1. Materials

Most routine chemicals were from Sigma or BDH. $^{32}$P-dNTP (6000 Ci/mmol) and $^{35}$S-methionine (cell labelling mix) were from Amersham. Electrophoresis reagents were from ICN, except SDS and TEMED were from BioRad.

## 2. Plasmid preparation

DNA constructs were grown in *E. coli* strains HB101 or XL1-Blue MRF' (Stratagene). Bacteria were grown overnight in Luria broth, and plasmid DNA was purified by the alkaline lysis method (Sambrook et al., 1989). Some plasmids were prepared from 1L cultures and then the supercoiled form was purified by centrifugation in CsCl, other plasmids were prepared from 5mL cultures and then purified using the glass filter-based Genie Prep Kit (Ambion).

The plasmids used were pSp53-Ala135, which encodes wild type murine p53, pSp53-Val135, which encodes temperature-sensitive mutant murine p53, and pSp65p53-H8, which encodes wild type human p53. These were sent by Dr. Jo Milner, but the pSp65p53-H8 contains the H-8 clone isolated in Dr. Varda Rotter's lab. The p53 dimer-encoding constructs used were p53LZ332, encoding wild type murine p53 with the GCN4 leucine zipper replacing p53 amino acids 332-393, and p53A344, encoding wild

type human p53 with a mutation from leucine to alanine at amino acid 344. These were constructed and sent by Dr. Thanos Halazonetis.

3. *In vitro* transcription and translation

Purified DNA plasmids were linearized downstream of the coding sequence with HindIII (Promega). The DNA was digested for 90 minutes at 37°C using 1-5 μg of plasmid DNA, 5U of HindIII per μg DNA, 5 μL 10X reaction buffer (Promega buffer 'B'), and $dH_20$ to 50 μL final volume. Linearized DNA was then purified using glass milk (Bio 101, Inc), and transcribed with Sp6 polymerase using the Megascript kit (Ambion). For translation of p53, aliquots of p53 RNA were added to micrococcal nuclease-treated rabbit reticulocyte lysate (Promega). Translation was carried out according to the manufacturer's instructions, except that twenty non-radiolabelled amino acids were added for all experments where binding to $^{32}$P-DNA was to be assayed, and translation was allowed to proceed at 37°C for 30-90 minutes.

4. Preparation of DNA sequences

The ability of p53 to bind DNA sequence-specifically was determined using various double-stranded sequences. Two unphosphorylated strands of each sequence (except RGC was 5' phosphorylated) were synthesized (U of C core DNA synthesis facility), then dissolved to an approximate concentration of 2mg/mL in TES buffer (10mM Tris, pH 8.0, 1mM EDTA, 25mM NaCl). The absorption at 260nm was determined spectrophotometrically (1 OD = 33 μg/mL ssDNA), then equal amounts of

each strand were mixed and incubated at 90°C for 10 minutes. The strands were allowed to hybridize by slowly cooling (60 minutes total) to 50°C, then 37°C, then 23°C, then 4°C.

The double-stranded sequences had 5' overhangs that were used for labelling with $^{32}P$. 4μL of [α-$^{32}P$]-dATP or [α-$^{32}P$]-dCTP (high specific activity, Amersham) was added to 1μl dsDNA (0.1 μg/μL), 1μl 10X low salt buffer (250 mM NaCl, 100mM $Mg(Cl)_2$, 100mM Tris pH 7.5), 0.75 μL DTT (20mM), 1.75 μL dNTP (0.2mM of each dNTP, with the $^{32}P$ deoxynucleotide omitted), 0.5 μL Klenow fragment (1U/μL, BRL or Boehringer Mannheim), and 1μL $dH_20$. The mixture was incubated at 23°C for 20 minutes then 4°C for 20 minutes. Labelled DNA sequences were then purified away from unincorporated nucleotide using the QIAquick Nucleotide Removal kit (QIAGEN) and isolated in 100μL TE buffer (1ng original DNA per 1μL final volume). For comparison of binding to different $^{32}P$ sequences, 2 μL of the labelled DNA was added to 3mL scintillation fluid (CytoScint, ICN) and counted in the $^{32}P$ channel of a scintillation counter (Beckman LS6500). The labelled DNA samples were then diluted to give equal counts per minute per μL of each DNA.

The top and bottom strands of CON are 5'-AGCTTAGACATGCCTAGACATG CCTA-3' and 5'-AGCTTAGGCATGTCTAGGCATGTCTA-3'. The top and bottom strands of M34 are 5'-AGCTTAGACATGCCTAGAGATCCCTAGCT-3' and 5'- AGCT TAGGGATCTCTAGGCATGTCTA-3'. The top and bottom strands of M24 are 5'-AGC TTAGACATCCCTAGACATCCCTAGCT-3' and 5'- AGCTTAGGGATGTCTAGGGA

TGTCTA-3'. The top and bottom strands of NB are 5'-AGCTTAGAGATCCCTAGAGA TCCCTAGCT-3' and 5'- AGCTTAGGGATCTCTAGGGATCTCTA-3'. The top and bottom strands of m1 (=m2) are 5'-AGCTTAGACATCCCTA-3' and 5'-AGCTTAGGG ATGTCTA-3'. The top and bottom strands of half are 5'-AGCTTAGACATGCCTA-3' and 5'-AGCTTAGGCATGTCTA-3'. The top and bottom strands of MUT are 5'-AGCT TAGACATGGCAAGACATGGCAA-3' and 5'-AGCTTTGCCATGTCTTGCCATGTC TA-3'. The top and bottom strands of RGC are 3'-pTTGCCTTGCCTGGACTTGCCTG GCCTGGCCTTGCCTT-3' and 5'-pTTAAGGCAAGGCCAGGCCAGGCAAGTCCAG GCAAGGC-3'. The top and bottom strands of S10 are 5'-AGCTTAGACATGCCT ATGAATTAATAGACATGCCTA-3' and 5'-AGCTTAGGCATGTCTATTAATTCAT AGGCATG TCTA-3'.

5. DNA binding analysis

Each DNA binding reaction contained: 2.5 μL of rabbit reticulocyte lysate containing *in vitro* translated p53, 0.25 μL dithiothreitol (0.1 M, Sigma), 1.0 μL $^{32}$P-radiolabelled DNA (1 ng/uL), 1.0 μL salmon testes DNA as nonspecific competitor (0.1 μg/μL, Sigma), 1.2 μL glycerol, 0.25-0.5 μL each monoclonal antibody (0.5-5 mg/mL purified antibody, Fab fragment, or ascites fluid), and tris-buffered saline (TBS: 25 mM Tris pH 7.5, 130mM NaCl, 3mM KCl) to 10 μL final volume. Reactions were incubated at 23°C for 30-60 minutes, then cooled to 4°C and electrophoresed in a high ionic strength, non-denaturing, electrophoretic mobility shift assay (EMSA) essentially as described (Chodosh, 1991).

The slab gel was prepared by mixing 19.3 mL $dH_20$, 4 mL acrylamide solution (24:1 acrylamide:bisacrylamide), 6.0 mL 5X gel running buffer (30.28 g Tris base, 142.7 g glycine, 0.98 g EDTA, and $dH_20$ to 1L), and 0.75 mL glycerol. Polymerization was induced by adding 25 uL TEMED (NNN'N'-tetramethylethylenediamine) and 225 uL 10% ammonium persulfate (APS), then the gel solution was poured into a vertical slab gel with 1.5 mm spacers. The gel was allowed to polymerize at 23°C for about 2 hours, then the lanes were rinsed with running buffer and the gel was pre-run for 30-60 minutes at 150V, 4 °C in 1X running buffer (prepared from the 5X stock used for the gel solution). The lanes were then rinsed again with running buffer and the DNA binding reactions were loaded and then run in the 4% polyacrylamide gel at 150V for between 1.5 and 3 hours at 4°C. The gel was then dried on Whatman 3mm filter paper and exposed to Reflection autoradiography film with a Reflection intensifying screen (Dupont) from between 30 minutes to overnight at -70°C.

When unlabelled dsDNA sequences were added for competition, 1 μL of an appropriate dilution was added per lane. The concentrations were checked by absorption at 260 nM (1 OD = 50 μg/mL dsDNA), then adjusted to give equal concentrations of quarter-sites (except NB, where the concentration was equal to the concentration of M34).

6. Densitometry

Autoradiography films were exposed to the $^{32}P$-gels such that the band intensities

were faint but readily visible. The film was scanned using a Hewlett Packard flatbed colour scanner (ScanJet 5d), but it was essential to convert the scanned image from colour to a greyscale image. No other computer adjustments were made to the image, which was then analysed using the very finicky, non user-friendly program SigmaGel (Jandel Scientific). Bands were quantified, and the only variable that was changed was the selection of a suitable threshold value that essentially represented background correction. Because half-lives were being measured, the threshold value was selected such that two separate pairs of time points in the same time course yielded nearly identical reduction in band intensity over a constant time interval (thanks go to Bill Panenka for this technique). This was found to be most reliable when faint bands (but more than barely visible) were analysed.

7. Determination of $t_{1/2}$ and $k_D$

The change in band intensity between two time points, obtained from densitometry of the scanned image, was converted to relative values. Data was only used if the percent reduction in band intensity was nearly identical between two different pairs of equally spaced time points in the same time course. An average percent dissociation was then taken from the two pairs of time points, and used to determine the half-life as follows (courtesy of Pauline Sabinin):

$$t_{1/2} = t(\ln 2)/\ln(N_o/N_t)$$

Where $t_{1/2}$ = half-life

$N_o$ = band intensity at time point 1

$N_t$ = band intensity at time point 2

t = time elapsed between time points 1 and 2

For 10-minute time intervals the half-life in minutes is therefore

$t_{1/2} = -6.93/[\ln(10xNt/No)-\ln(10)]$

If the dissociation follows first-order kinetics, then the overall dissociation constant, $k_D$, can be calculated using the value of the half-life (in seconds) as follows:

$k_D = -\ln(0.5)/ t_{1/2}$

As above, $t_{1/2}$ is the half-life of the p53-DNA complex.

8. Immunodepletion and immunoprecipitation

Aliquots of p53 were incubated with monoclonal antibody, or fetal bovine serum as a control, for 30 minutes at 23°C. The ratio of RRL containing p53 to ascites antibody solution (v:v) was 4:1. Then, 2.5 volumes of a 10% solution of inactivated *Staph. A* (IgSorb, The Enzyme Center) was pelleted at 12 000g in a 0.6 mL eppendorf tube for 12 seconds. The supernatant was aspirated, then the pellet was washed with PBS, then recentrifuged and the supernatant was aspirated again. To the pellet, the p53-antibody solution was added, then the pellet was resuspended by flicking the tube. Antibody complexes were allowed to bind to the *Staph. A* for 20 minutes at 23°C, with the tube being flicked (without introducing air bubbles) every 5 minutes to keep the *Staph. A* from settling. Bound p53 was precipitated by centrifugation for 12 seconds at 12 000g, and

supernatants were assayed for DNA binding as above. Immunodepletion using biotinylated PAb246 Fab fragments was as above, except streptavidin beads were substituted for *Staph. A*.

For immunoprecipitation analysis of total p53, p53 was bound to antibody and precipitated on *Staph. A* as above. The precipitate was washed three times with tris-buffered saline, then released by boiling in sodium dodecyl sulfate (SDS) for 5 minutes. Bound $^{35}$S-labelled p53 was then electrophoresed on an 8% polyacrylamide gel containing SDS (Laemmli et al., 1970). The gel was fixed in 10% acetic acid/15% methanol for at least one hour, dehydraded by two sequential 30 minute washes in dimethylsulfoxide (DMSO), then embedded with 20% diphenyloxazole (PPO, obtained from ICN) in DMSO (Laskey & Mills, 1975). The PPO was precipitated in the gel by washing with water for one hour, the gel was then dried and exposed to BioMax MR film (Kodak) at -70°C overnight.

9. Preparation of streptavidin beads and biotinylated PAb246 Fab fragments

Streptavidin beads were prepared by cross-linking streptavidin (Sigma) to cyanogen bromide-activated 4%-agarose beads as per the manufacturers instructions (Sigma). PAb246 Fab fragments were prepared with the ImmunoPure IgG1 Fab and F(ab')$_2$ Preparation Kit (Pierce). Briefly, purified PAb246 was digested with the immobilized protease ficin, then the Fc fragments were removed with protein A. The purified Fab fragments (1 mL of 0.15 mg/mL) were diluted with 1 mL PBS, then

centrifuged in a centricon 10 concentrator (Amicon) in a JA20 rotor (Beckman) at 4000g for 60 minutes. The retentate (0.5 mL) was diluted with 1.5 mL PBS and reconcentrated to 0.36 mL. 1 mL 0.1 M sodium bicarbonate, pH 8.5, was then added and the solution re-concentrated to 0.15 mL in the centricon 10 concentrator. This solution contained ~ 1 mg/mL protein, which was biotinylated by the addition of 11 uL NHS-LC-Biotin (Pierce) for 30 minutes at 23°C. The reaction was then diluted with 2mL TBS + 0.02% sodium azide, then centrifuged in a centricon 10 concentrator until 40 uL of retentate remained. The retentate was added to 60 uL TBS + 0.02% sodium azide and stored at 4 °C.

10. Antibodies

Ascites fluid was collected from mice in which monoclonal antibody-secreting cells were growing intra-peritoneally. Balb/c mice (four to six weeks old) were primed with 0.5 mL of Pristane (2,6,10,14-tetramethyl-pentadecane) injected intraperitoneally. Three to five days later the mice were injected intraperitoneally with 1mL of log phase hybridoma cells (at least $10^7$ cells/mL). The ascites fluid was collected after 5-14 days, incubated at 37°C for 1 hour, then at 4°C overnight. The ascites fluid was clarified by centrifugation at 1000g for 10 minutes. A 0.5 mL column of Protein A acrylic beads (Sigma) was prepared, and the ascites fluid was added to the column in 3.3M NaCl, 0.1 M sodium borate pH 8.9 because PAb240 and PAb246 are class IgG1 antibodies (Harlow and Lane, 1988). The column was washed and antibody eluted in 100mM glycine pH 3.0. The fractions were collected (0.5 mL each) into 50 uL 1M Tris pH 8.0 to minimize the time spent at low pH. Fractions were assayed for protein concentration by absorption at

280 nm (1 OD ~ 0.75 mg/mL antibody). Appropriate fractions were pooled and concentrated (as above) to 1-10 mg/mL in PBS in centricon 10 concentrators (Amicon). PAb246 Fab fragments were prepared using the IgG1 Fab purification kit (Pierce).

11. Cell culture and preparation of cell extract

NIH 3T3 cells were grown in Dulbecco's modification of minimal essential medium (DMEM) supplemented with 10% fetal bovine serum (Gibco). The cells were grown in 5% $CO_2$ at 37°C, then about 5 x $10^6$ cells were harvested at 80% confluency. Some cells were harvested after DNA damage was incurred by prior incubation for 16 hours in DMEM containing 300 ng/mL actinomycin D (Calbiochem). The medium was aspirated, the cells were rinsed with PBS, then the cells were scraped off the surface of the tissue culture flask in 1 mL ice-cold PBS using a rubber policeman. Removed cells were centrifuged at 1000g for 3 minutes, then washed in ice-cold PBS. The cell pellet was then resuspended and lysed in 100 µL of ice cold hypotonic buffer (10 mM HEPES, pH 7.6; 10 mM KCl; 1 mM MgAc; 1 mM DTT; 10% glycerol; 1 mM PMSF) for 10 minutes on ice, then passed through a 20-guage needle to further lyse the cells. After centrifugation at 12 000g for 5 minutes at 4°C, the supernatant was saved on ice and the pellet was extracted with continuous agitation in 40 µL hypertonic buffer (hypotonic buffer supplemented to 0.5 M KCl) for 30 minutes on ice. The nuclei (and other heavy membranes) were pelleted by centrifugation at 12 000g for 5 minutes at 4°C, and the supernatant was added to the cytoplasmic extract. The final concentration of KCl was therefore approximately 143 mM.

# RESULTS

The goal of the following experiments was to determine the subunit organization and conformation of wild type (tetrameric) p53 that was bound to DNA. Because a tetramer is a dimer-of-dimers, the binding of free dimers to DNA was examined in order to assess how dimers within a tetramer might bind. To find out how subunits within tetramers could bind to DNA, tetramer binding to DNA sequences that had various quarter-sites mutated was compared. Wild type p53 tetramers or dimers were translated *in vitro* in rabbit reticulocyte lysate (RRL). Binding to $^{32}$P-DNA sequences consisting of either a consensus p53 DNA binding sequence or variations thereof was assayed using the electrophoretic mobility shift assay.

## 1. Subunit Organization of DNA-Bound p53

As discussed above, one model of how tetrameric p53 binds to DNA has a half-site being bound by one dimer with the other half-site being bound by the other dimer. The other model has one dimer binding to quarter-sites one and three, with the other dimer binding to quarter-sites two and four. To determine which model is correct, the abilities of tetrameric or dimeric p53 to bind to consensus DNA sequences that had either the third and fourth (M34) or the second and fourth (MUT and M24) quarter-sites mutated were analyzed.

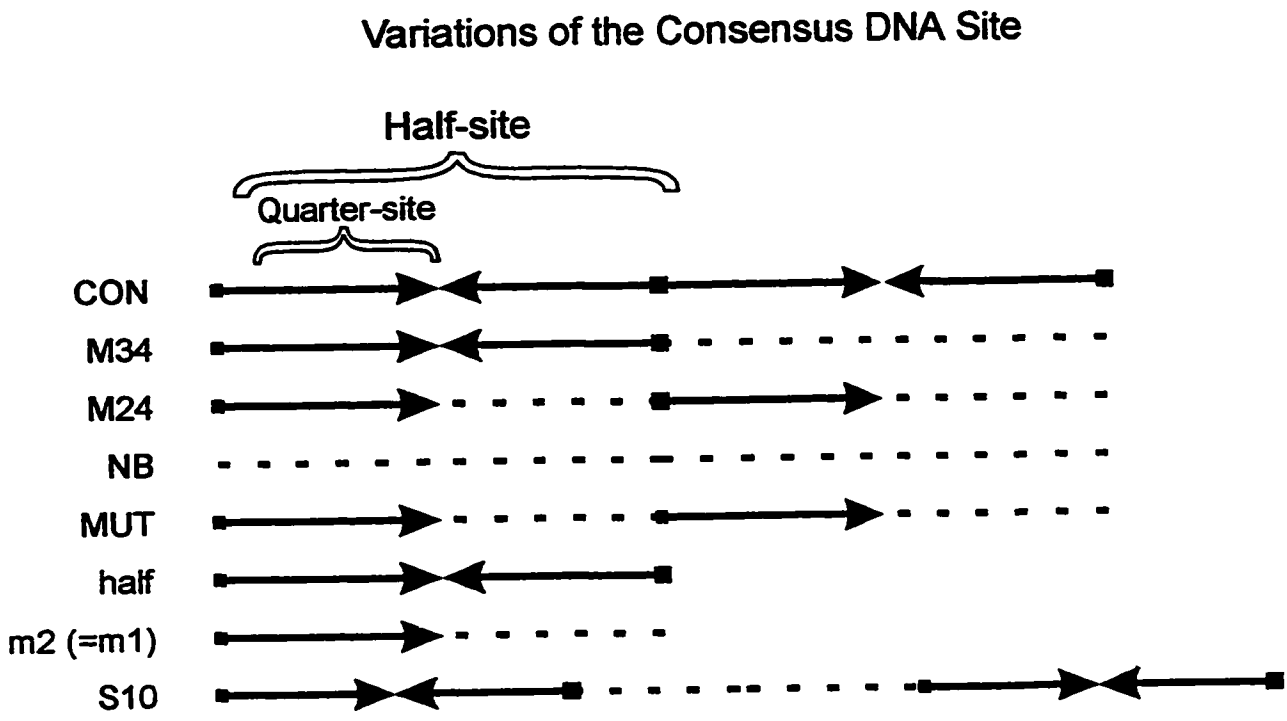
1.1. Wild type p53 binds to a half-site or to MUT

The first sequences synthesized in this project were CON and MUT. The definition of the consensus sequence was two copies of the 10 base pair sequence 5'-PuPuPuC(A/T)(A/T)GPyPyPy-3' (El-Deiry et al., 1992), and repeats of TGCCT (Kern et al., 1991). TGCCT corresponded to each half of the El-Deiry ten base-pair sequence, but oddly enough no perfect palindromes were selected by p53 for binding (El-Deiry et al., 1992). Therefore, CON was designed as two repeats of 5'-AGACATGCCT-3', which contains one perfect TGCCT following one inverted TGTCT (the underlined position was a T instead of C so as not to make a palindrome; see Figure 2).

As a control sequence to which p53 was predicted to not bind, MUT was synthesized as two repeats of 5'-AGACATGACA-3'. Since each monomeric subunit of p53 bound to one quarter-site of a consensus sequence (Cho et al., 1994), MUT was re-evaluated as a sequence that actually had the second and fourth quarter-sites mutated (Figure 2). It was also demonstrated by the p53-DNA co-crystal that the invariant **C** (or base-paired **G**) in each quarter-site were the most critical for DNA binding (ibid). It was at that point that NB and M34 were synthesized, but with base substitutions introduced at the invariant **C** (or base-paired **G**) (Figure 2).

When translated *in vitro* and assayed for DNA binding by EMSA, wild type p53 was able to bind to a $^{32}$P-labelled consensus DNA sequence (CON, Figure 2; Figure 3A, lane 3). Both a major slower migrating species and a minor faster migrating species of

**Figure 2**


Variations of the Consensus DNA Site
Half-site
Quarter-site
CON
M34
M24
NB
MUT
half
m2 (=m1)
S10

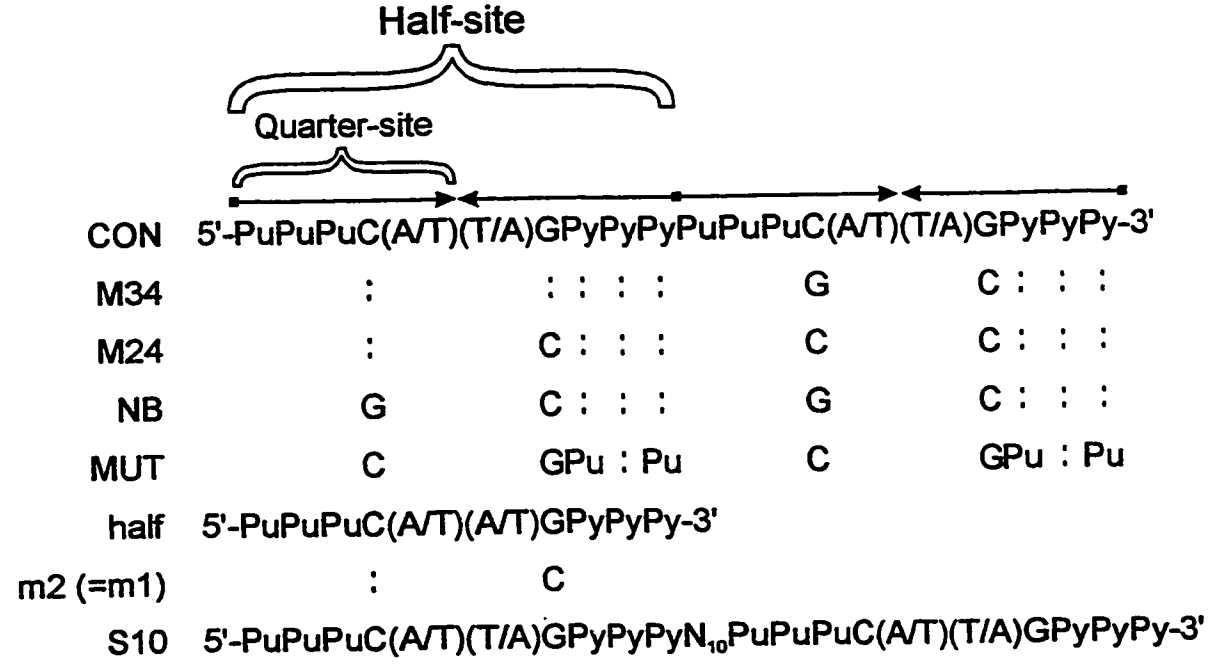
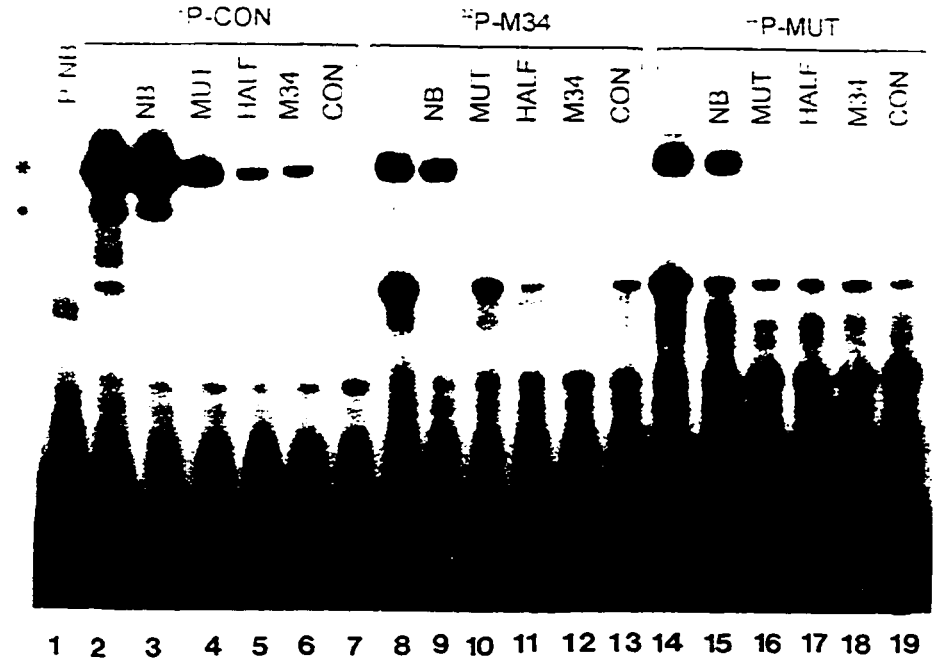

Half-site
Quarter-site
CON 5'-PuPuPuC(A/T)(T/A)GPyPyPyPuPuPuC(A/T)(T/A)GPyPyPy-3'
M34 : : : : : G C : : :
M24 : C : : : C C : : :
NB G C : : : G C : : :
MUT C GPu : Pu C GPu : Pu
half 5'-PuPuPuC(A/T)(A/T)GPyPyPy-3'
m2 (=m1) : C
S10 5'-PuPuPuC(A/T)(T/A)GPyPyPyN10PuPuPuC(A/T)(T/A)GPyPyPy-3'

**Figure 3. Binding of wild type p53 to a half-site or to MUT.** **A.** p53 was translated in RRL and then incubated in a DNA binding mixture containing 1ng of $^{32}$P-CON per lane, without (lane 2) or with (lane 3) PAb421. The migration rates of two forms of p53 are indicated by an asterisk and a dot. **B.** Wild type p53 was allowed to bind $^{32}$P-DNA sequences as indicated, all with PAb421. A 500-fold excess of unlabelled competitor DNA was added to each sample (as indicated) at the same time as the labelled DNA.

A


RRL + PAb421
p53
p53 + PAb421
1 2 3

B


P-CON
P-M34
P-MUT
NB
MUT
HALF
M34
CON
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19

full-length p53 were bound to DNA in the presence of PAb421 (indicated with an asterisk and a dot, respectively). These bands did not appear in rabbit reticulocyte lysate (RRL) that was mock translated (i.e. no p53 RNA was added) (lane 1), and were stabilized by the p53-specific antibody PAb421 (compare lanes 2 and 3). The requirement for PAb421 to inactivate a negative-regulatory C-terminal domain in p53, and thereby stabilize DNA binding, has been described by others (Hupp et al., 1992, 1995; Hainaut et al., 1994). Although 100-fold excess of non-specific competitor DNA was included in the DNA-binding reactions, two prominent background protein-DNA complexes were observed in RRL alone that migrated more quickly than did the p53-DNA complexes (lanes 1-3). At the bottom of each lane there was a relatively large amount of free $^{32}$P-CON.

The Non-Binding control sequence (NB, Figure 2) was not bound by murine p53 (Figure 3B, lane 1), nor did unlabelled NB compete with $^{32}$P-CON for p53 binding (lane 3). The sequence consisting of a single half-site competed for p53 binding similarly to the sequence that had one consensus half-site followed by mutated third and fourth quarter-sites (half and M34, Figure 2) (Figure 3B, lanes 5 and 6). The sequence that had the second and fourth quarter-sites mutated (MUT, Figure 2), also competed for p53 binding (Figure 3B, lane 4). If p53 could bind to MUT or M34, it was possible that two conformationally different populations of p53 bound to one or the other sequence.

Whether there were two different populations of p53 was assessed by cross-competition. Neither p53 binding to $^{32}$P-M34 nor $^{32}$P-MUT was competed off by NB

(Figure 3B, lanes 9 and 15). The population of p53 that bound to MUT and M34 was the same, as MUT competed for binding to M34 and vice versa (lanes 10 and 18). It should be noted that about twice as much $^{32}$P-MUT was added per binding reaction, because the intensity of the p53-MUT band is usually not strong (data not shown).

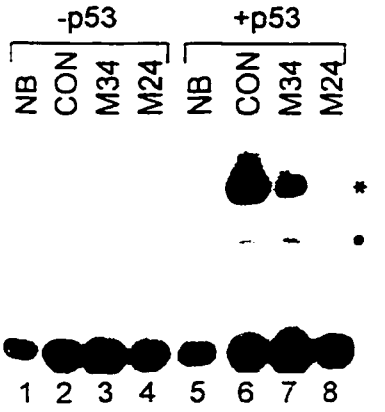
Because p53 was unable to bind NB (Figure 3B, lane 1), mutation of the invariant C (or G) in each quarter-site was sufficient to eliminate p53 sequence-specific DNA binding. It follows that the third and fourth quarter-sites in M34 probably also eliminated the binding of whichever subunits of tetrameric p53 normally bind to quarter-sites three and four. However, the second and fourth quarter-sites in MUT had base substitutions at different positions than the invariant C (or G), so MUT could have allowed some p53 subunit contact (Figure 2). At this point, M24 was synthesized such that the second and fourth quarter-sites had a base substitution at the invariant C (or G) (Figure 2).

1.2. Wild type p53 binds to a half-site, but not to M24

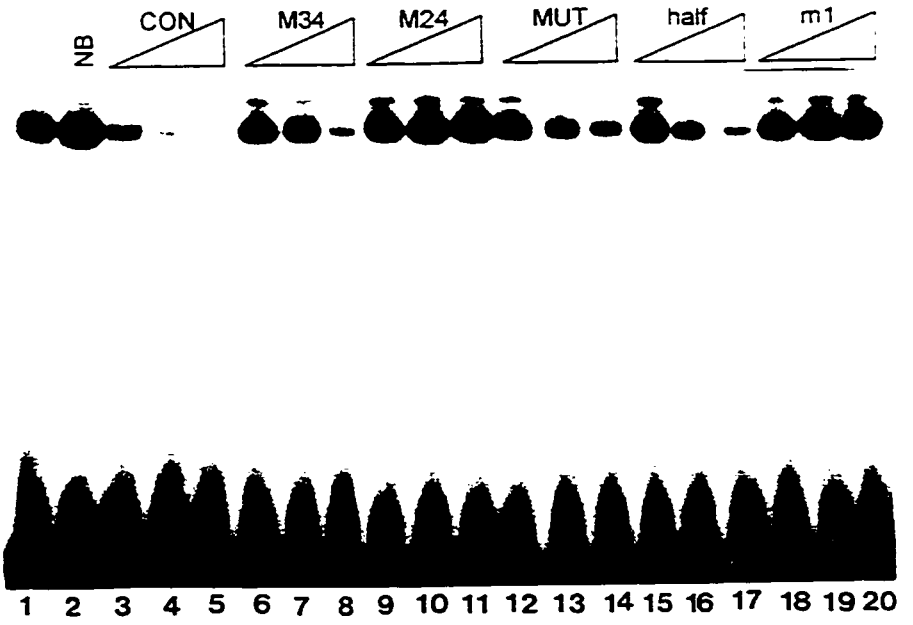
M34 was again bound by p53, but not as well as was CON (Figure 4A, compare the band intensities in lanes 7 and 6). In contrast, the sequence in which the second and fourth quarter-sites were mutated (M24, Figure 2) was not bound by p53 (Figure 4A, lane 8). Interestingly, the migration rates of both p53-CON and p53-M34 were the same, implying that the same molecular mass and therefore oligomeric form, of p53 was present in each complex (compare lanes 6 and 7). Thus, the same species of full-length p53 could bind either to the one half-site in M34 or to one or both of the two half-sites in CON, but not to alternating quarter-sites in M24. Identical results were seen with

**Figure 4. Binding of wild type p53 to a half-site or MUT, but not M24.** **A.** p53 or irrelevant RNA (luciferase) was translated in RRL and then bound to equal counts per minute of $^{32}$P-DNA as indicated. The migration rates of the two forms of p53 are indicated by an asterisk and a dot. **B.** Wild type p53 bound to $^{32}$P-CON was challenged with increasing amounts of unlabelled competitor DNA as indicated. 1μg of NB was added in lane 2, all other sequences were added at 0.01, 0.1, or 1μg, corresponding to the increasing height of the triangles.

A


-p53
+p53
NB
CON
M34
M24
NB
CON
M34
M24
*
•
1 2 3 4 5 6 7 8

B


NB
CON
M34
M24
MUT
half
m1
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20

murine and human p53 (data not shown).

Binding of p53 to MUT and M24 was directly compared by competition with $^{32}$P-CON. MUT but not M24 competed for p53 binding (Figure 4B, lanes 12-14 and 9-11, respectively). Competition by the half-site in 'half' or in M34 (lanes 15-17 and 6-8, respectively) was better than by MUT (lanes 12-14). Substitution of the invariant C (or G) in quarter-sites two and four eliminated binding, so the ability of p53 to bind MUT perhaps reflected subunits within p53 binding with low affinity to the altered second and fourth quarter-sites in MUT. Binding to M34 but not to M24 indicated that p53 tetramer binding was stabilized when two subunits of the tetramer interacted with the two quarter-sites in one half-site. However, these results do not differentiate between whether the dimers in a tetramer each bind to a half-site, or whether each half-site is bound by one subunit from each dimer that makes up a tetramer.

1.3. Free dimers bind to half-sites or pairs of half-sites, but not to MUT nor M24

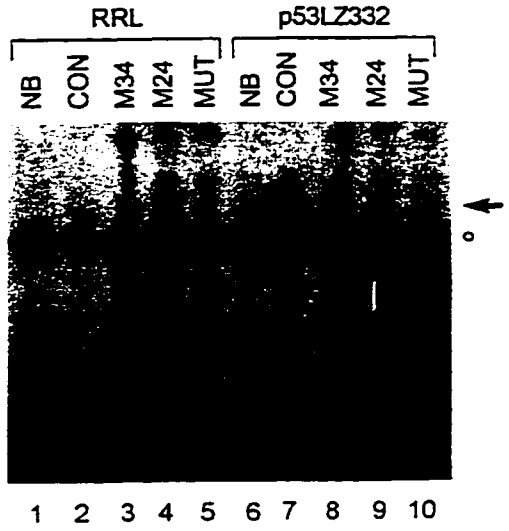
To assist in distinguishing between those two possibilities, the ability of free dimers to bind to the variations of CON was compared. A murine p53 construct was used that had the C-terminal 58 amino acids (oligomerization domain and negative regulatory domain) replaced with the leucine zipper of the yeast transcription factor GCN4. This fusion protein (p53LZ332) has been shown to bind DNA as a dimer (Halazonetis and Kandil, 1993).

In the RRL control lanes with no translation, a band was seen that binds similarly to all of the DNA sequences (Figure 5A, lanes 1-5, position marked by an open circle). This same position showed increased intensity only on CON and M34 when p53LZ332 was translated (compare lanes 7 and 8 with lanes 2 and 3). The likely explanation is that there was a p53LZ332-DNA complex that was specific for the half-sites in CON and M34. However, this complex comigrated with a non-sequence specific complex that was present in the absence of p53LZ332. In addition, a band unique to p53LZ332-CON migrated more slowly (lane 7, position marked by a closed tailed arrow). Of note is that p53LZ332 did not bind to MUT (compare lanes 5 and 10).

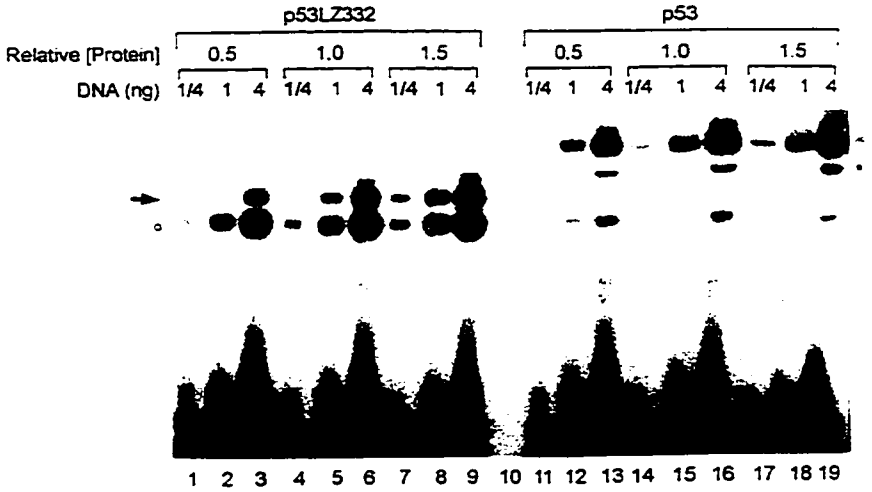
When compared to tetramer binding (Figure 4A), an interesting pattern is evident. There is a faster migrating complex of similar intensity on CON and M34 (Figure 4A, dot; Figure 5A, open circle). With either dimers or tetramers, a slower-migrating band is seen with CON, and on M34 this band is only evident with tetramer, but not with p53LZ332 (Figure 4A, asterisk; Figure 5A, arrow). This pattern of migration is consistent with the notion that one dimer was bound to one half-site in M34 (Figure 5A, lane 8), and that either one or two dimers could bind to the two half-sites in CON (lane 7, open circle and arrow respectively). Presumably, one tetramer could bind to one half-site in M34 and to one or both half-sites in CON (Figure 4A, asterisk). The more quickly migrating complex in the tetramer sample could represent a small population of dimers that did not form tetramers.

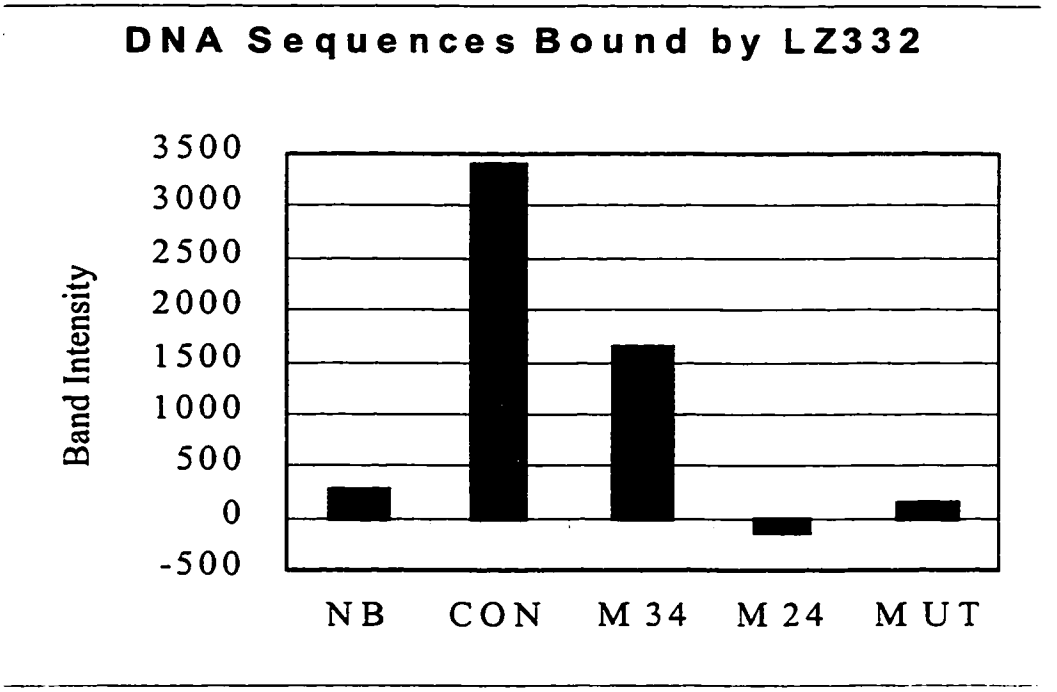
**Figure 5. DNA binding of p53LZ332.** **A.** RRL control (lanes 1-5) or p53LZ332 (lanes 6-10) was bound to $^{32}$P-DNA as indicated. The migration of two forms of p53LZ332 is indicated by an open circle and by an arrow. **B.** Increasing volumes of RRL containing p53LZ332 (lanes 1-9) or wild type p53 (lanes 11-19) was added to control RRL to the same final volume, containing PAb421. Each concentration of p53LZ332 or wild type p53 was added to increasing amounts of $^{32}$P-CON as indicated. Migration of a single p53LZ332 dimer is indicated by an open circle, pairs of p53LZ332 dimers by an arrow. The two forms of wild type p53 are indicated by an asterisk and a dot. **C.** Densitometry of p53LZ332 bound to each sequence in **A** after the RRL binding of each corresponding lane was subtracted. **D.** Densitometry of **B** showing the ratio of the lower band (circle) to the upper band (arrow).

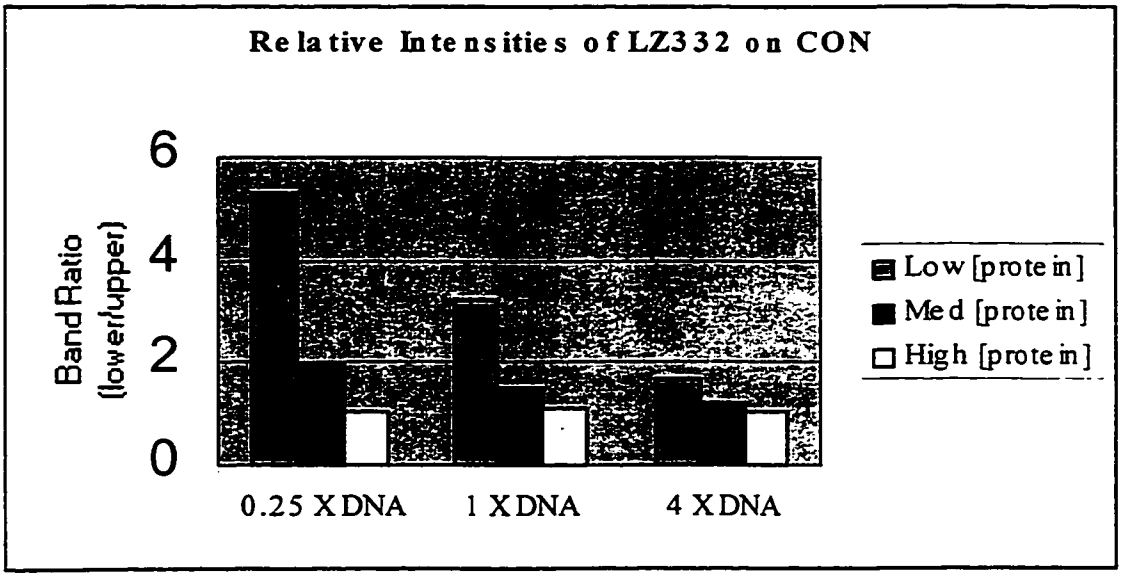
A


RRL
p53LZ332
NB
CON
M34
M24
MUT
NB
CON
M34
M24
MUT
1 2 3 4 5 6 7 8 9 10

B


p53LZ332
p53
Relative [Protein]
0.5
1.0
1.5
0.5
1.0
1.5
DNA (ng)
1/4 1 4 1/4 1 4 1/4 1 4 1/4 1 4 1/4 1 4 1/4 1 4
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19


DNA Sequences Bound by LZ332
Band Intensity
3500
3000
2500
2000
1500
1000
500
0
-500
NB
CON
M 34
M 24
MUT


Relative Intensities of LZ332 on CON
Band Ratio (lower/upper)
6
4
2
0
0.25 X DNA
1 X DNA
4 X DNA
Low [protein]
Med [protein]
High [protein]

If two p53LZ332 dimers were independently binding to the two half-sites in CON, then when the relative concentration of p53LZ332 is increased, the proportion of CON that has two versus one dimer bound should also increase. Translated p53LZ332 was diluted 1:2, 2:1, or not diluted with mock-translated RRL (i.e. no RNA was added), then allowed to bind to increasing amounts of CON (Figure 5B). With equal amounts of DNA, increasing the p53LZ332 concentration also increased the ratio of the upper band (arrow) to the lower band (open circle) (compare lanes 1 and 7, lanes 2 and 8, and lanes 3 and 9). Because the amount of the non-sequence specific co-migrating band was very low when tetrameric p53 was translated instead of p53LZ332, most of the lower band must have represented p53LZ332-CON (lanes 11-19, open circle). These results suggest that one dimer binds to M34 (open circle), and one (open circle) or two (arrow) dimers can bind to CON.

1.4. The major DNA-bound p53 species is a tetramer

There can be no direct comparison made between the migrations of p53 and p53LZ332 because p53 required PAb421 to inactivate the C-terminal negative-regulatory domain, whereas this domain was deleted in p53LZ332. Thus, PAb421 would have decreased the mobility of p53 but not p53LZ332. A344 is a full-length wild-type human p53 clone containing a point mutation at residue 344, from leucine to alanine, that disrupts the dimer-dimer interface and results in p53 forming dimers instead of tetramers (Waterman et al., 1995). This dimeric construct retains the PAb421 epitope, and its migration rate can therefore be directly compared to that of p53 tetramers.

Dimeric A344 bound neither to NB nor to M24 (Figure 6A, lanes 1 and 5). One A344-M34 complex was observed (lane 4, position marked by a dot). When bound to CON there were two molecular weight species, one migrating identically to that of A344-M34, and one migrating more slowly (lane 3, dot and asterisk respectively). Of particular interest was that PAb421 was required to activate (or stabilize) A344 dimer DNA binding (compare lanes 2 and 3). It should be noted that A344 did not bind MUT, and sometimes bound CON weakly in the absence of PAb421 (data not shown). The pattern of binding was very similar to p53LZ332, except the A344-DNA complexes had a slower mobility due to the presence of PAb421. Because the lower A344 band (dot) migrated distinctly from the non-specific band, the oligomeric identities of the two A344-DNA complexes could be unambiguously established.

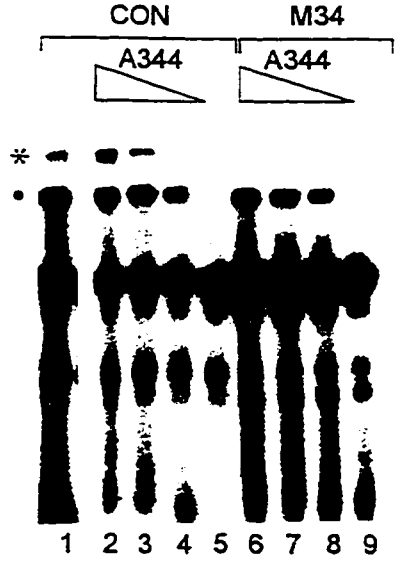
Whether the upper band represented two dimers bound to CON was assessed by adding decreasing amounts of A344 to a constant amount of $^{32}$P-CON (Figure 6B; lanes 2 to 4). The concentration of protein in lane 4 is one-third that of lane 2, resulting in a corresponding decrease in the intensity of the A344-DNA complex. In order to allow direct comparison of the ratio of two versus one dimer bound to CON, the autoradiography film exposure time of lane 4 was increased (lane 1) to give the dimer in lane 4 approximately equal intensity to the dimer in lane 2 (photographic processing was identical). The intensity of the more slowly migrating species on CON (asterisk) relative to the quickly migrating species (dot) was reduced when less versus more A344 was translated (Figure 6B, lanes 1 versus 2).

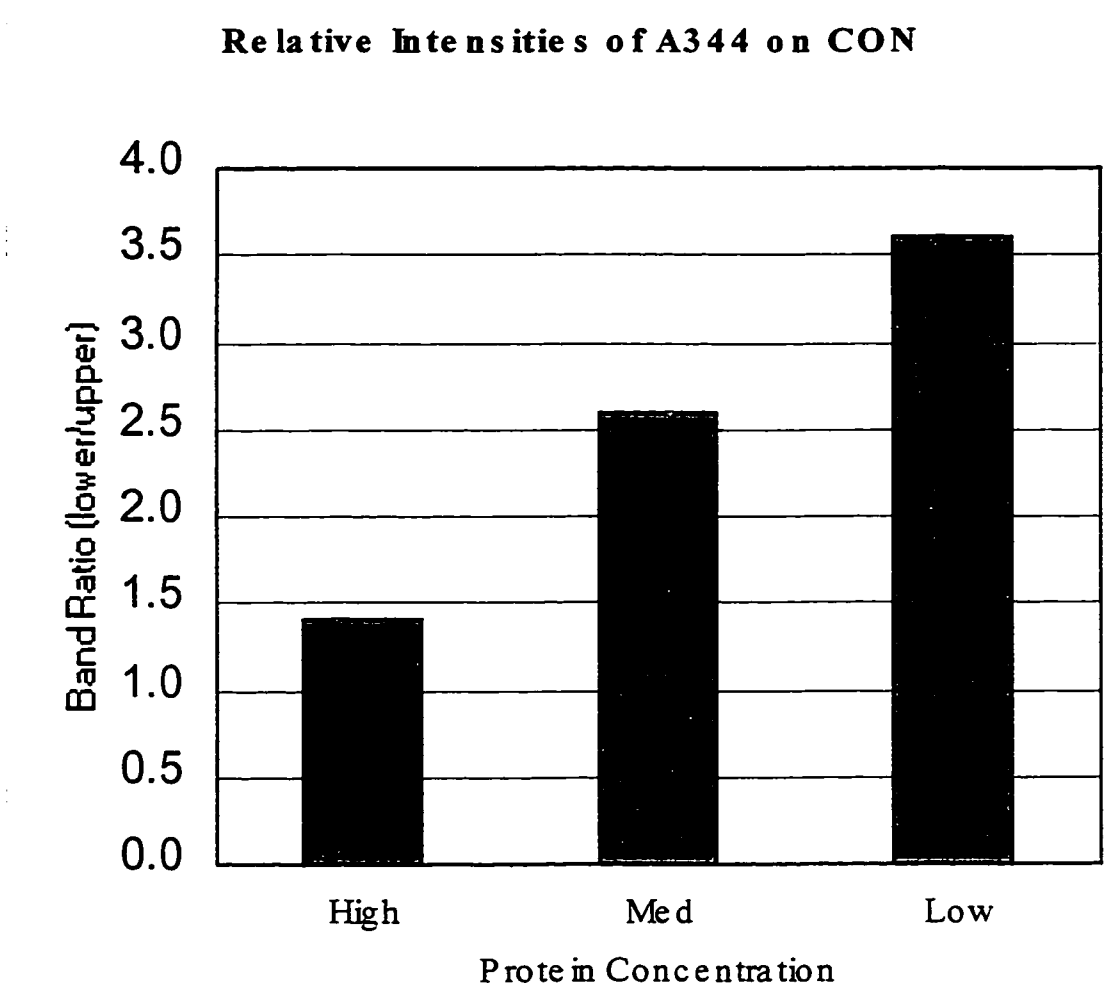
**Figure 6.** **DNA binding of A344 dimers.** **A.** A344 was bound to $^{32}$P-DNA, with or without PAb421, as indicated. The migration rates of two forms of A344 are indicated by an asterisk and a dot. **B.** Decreasing amounts of A344 were added to $^{32}$P-CON (lanes 2-4) or $^{32}$P-M34 (lanes 6-8). RRL alone served as a control (lanes 5 and 9). Lane 1 is an increased autoradiography film exposure time of lane 4. The migration of a single A344 dimer is indicated by a dot, pairs of A344 dimers by an asterisk. **C.** Densitometry of the ratio of lower (dot) to upper (asterisk) bands in B. The lanes in B that were scanned for C were lanes 2 (high [protein]), 3 (med [protein]), and 1 (low [protein]).

A


NB + PAb421
CON
CON + PAb421
M34 + PAb421
M24 + PAb421
*
1 2 3 4 5

B


CON
M34
A344
A344
*
1 2 3 4 5 6 7 8 9


Relative Intensities of A344 on CON
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
Band Ratio (lower/upper)
High
Med
Low
Protein Concentration

These results are expected for two A344 dimers binding independently to CON. Decreasing the concentration of dimer relative to DNA would give an unbound dimer a greater chance of binding one of two half-sites on an unoccupied CON molecule compared to one half-site on CON that already had one A344 dimer bound. Most likely, the more quickly migrating band of A344-CON (dot) represented one A344 dimer bound to one half-site in CON, whereas the more slowly migrating A344-CON band (asterisk) represented two dimers of A344 bound to the two half-sites in CON.

Does wild type p53 bind DNA similarly to A344 dimers? The less abundant, more quickly migrating species of p53-CON and p53-M34 migrated at a position identical to that of A344-M34, likely representing one dimer bound to DNA (Figure 7, lanes 2 and 3 compared to 7, dot). Unlike the p53 tetramer band (asterisk), the dimer band (dot) was present at equal intensities with both CON and M34 (Figure 4A, lanes 6 and 7; Figure 7, lanes 2 and 3). A344 dimers also bound CON and M34 to a similar extent (Figure 7, lanes 6 and 7). Also similar to p53 dimers, A344 did not bind M24 (Figure 7, lane 4). Thus, wild type p53 dimers could bind DNA similarly to A344 dimers.

Interestingly, both p53-CON and p53-M34 migrated at the same position as did the more slowly migrating species of A344-CON (Figure 7, lanes 2 and 3 compared to 6, position marked with an asterisk). Because the A344-CON slowly migrating band (lane 6) contained two A344 dimers, the comigrating p53-CON and p53-M34 complexes most likely contained one p53 tetramer. Therefore, the major p53 band represented a complex

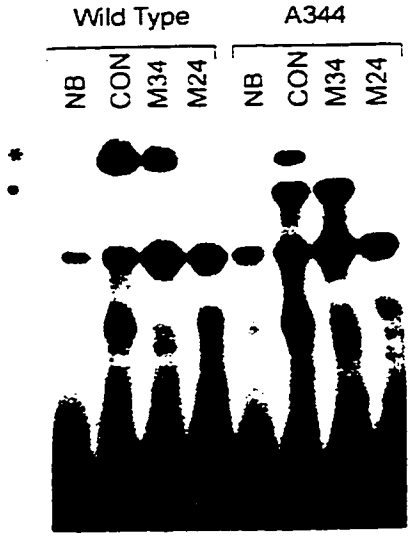



**Figure 7. Comparison of the migration of p53 tetramers and A344 dimers**. Wild type p53 or A344 was added to $^{32}$P-DNA sequences as indicated, in the presence of PAb421. The migration of a single dimer is indicated by a dot, pairs of A344 dimers by an asterisk.

consisting of one tetramer bound to DNA (lanes 2 and 3, asterisk). Furthermore, the predominance of tetrameric p53 that was bound to one half-site (M34) indicated that most wild-type p53 had already formed tetramers before binding to DNA (lane 3, asterisk compared to dot).

1.5. Cellular p53 binds DNA similarly to p53 translated *in vitro*

It was important to compare the DNA binding of *in vitro* translated p53 to cellular p53. To this end, subconfluent NIH 3T3 cells, which are known to contain wild type p53, were harvested and total cell extract was prepared. Because DNA damage is known to activate p53 and stabilize the p53 protein level in cells, extract was prepared from control cells or from DNA damaged cells, then assayed for DNA binding by EMSA. A low amount of endogenous cellular p53 in the absence of DNA damage was activated for DNA binding by PAb421 (Figure 8, lane 14), but DNA damage-induced stabilization of p53 yielded a much greater amount of PAb421-activated DNA binding complex (lane 6).

The most slowly migrating form of p53 on CON and M34 (Figure 8, lanes 6 and 7, asterisk) was the major DNA-bound species, and probably consisted of p53 tetramer-DNA complexes. The lower band (lane 6, dot) most likely represented a low concentration of free dimers, as was observed with *in vitro* translated p53. Interestingly, there was a low amount of DNA binding activity, unique to CON, which was activated by DNA damage and detected without PAb421 (lane 2). This presumably represented p53 DNA binding that was not inactivated by the C-terminal negative regulatory domain.

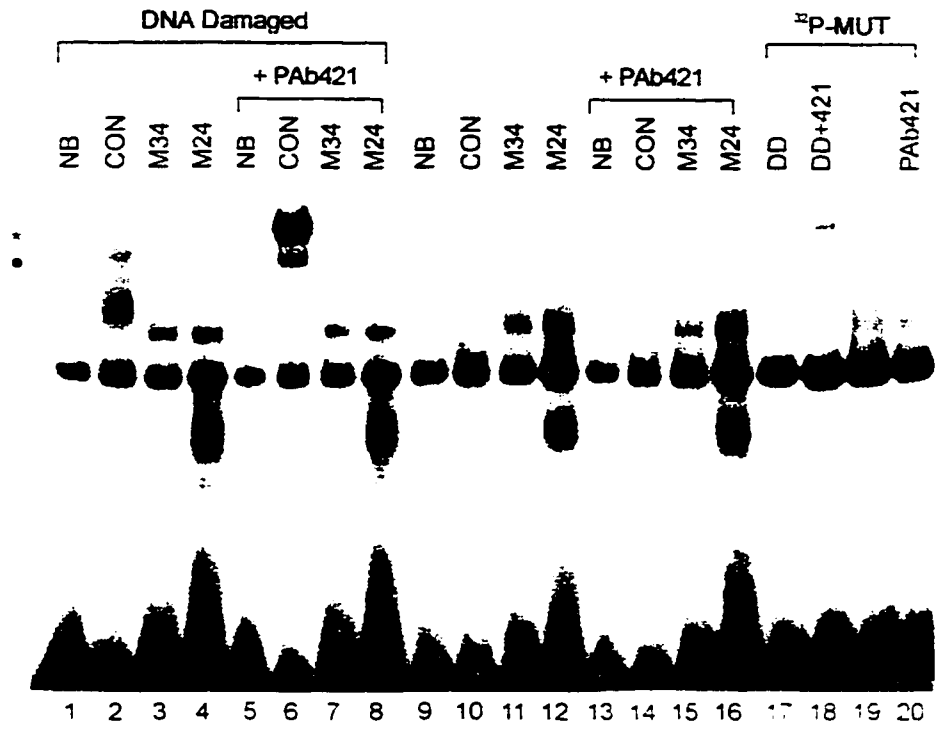



**Figure 8.** **Binding of endogenous p53 in cell extract.** Balb/c 3T3 total cell extract was prepared from cells with (lanes 1-8 and 17-18) or without (lanes 9-16 and 19-20) prior DNA damage (DD) by actinomycin D. Cell extract was added to $^{32}$P-DNA as indicated, either with or without PAb421 as indicated.

The pattern of DNA binding was very similar to *in vitro* translated p53, as CON was bound the best, with some M34 being bound but no M24 nor NB (Figure 8, lanes 6, 7, 8, and 5 respectively). MUT was also bound by cellular p53, in fact it was bound better than was M34 (lane 18 compared to lane 7). Another similarity to *in vitro* translated p53 was the non-specific band that migrated about 1/3 of the way down the gel, with a variable band just above (lanes 1-20). The pattern of DNA binding exhibited by cellular p53 was therefore similar to the pattern of *in vitro* translated p53 DNA binding.

## 2. Binding Affinity Results Entirely from Dimer-Dimer Cooperativity and Avidity

### 2.1. Tetramers bind CON, but not a single half-site, with high affinity

Interestingly, p53 tetramers (but not free dimers) appeared to bind better to CON than to M34 (Figure 4A, lanes 6 and 7). To assess whether this reflected a difference in affinity, p53-[$^{32}$P]-DNA complexes were allowed to form, then excess unlabelled CON was added to 'trap' any p53 that dissociated from the $^{32}$P-labelled sequence. A time course revealed that the half-life ($t_{1/2}$) of p53-CON was about 20-30 minutes, whereas p53-M34 had a $t_{1/2}$ much shorter than 5 minutes (Figure 9, lanes 2-8 and 9-14, respectively). The intensities of the bands on CON were quantified, and the half-life of p53-CON was calculated to be 25 minutes (Table 1). The dissociation of p53 from DNA probably follows first order kinetics, and therefore the overall dissociation constant of p53-CON is $4.62 \times 10^{-4}$ s$^{-1}$ (Table 1). It seemed likely that the greater stability of p53 on CON compared to M34 would arise from interaction of both dimers of a tetramer with the two half-sites in CON. The previous finding that two A344 dimers could bind side-by-side on CON was then exploited to determine whether dimers stabilized one another

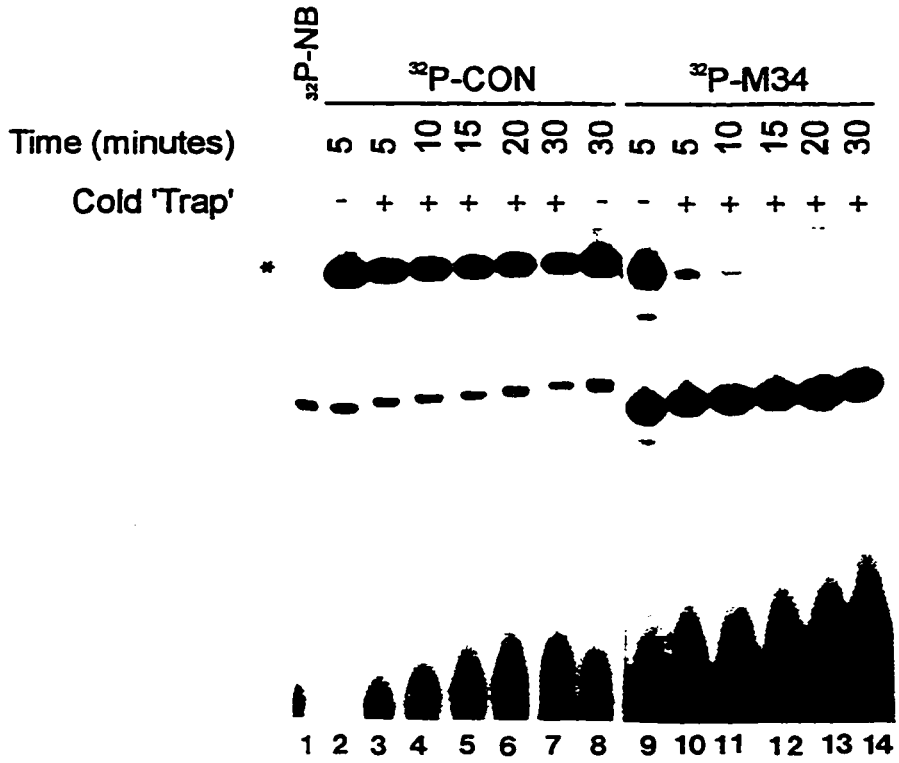



**Figure 9.** **Half-life of p53 on CON and M34.** P53 was allowed to bind to $^{32}$P-DNA for 45 minutes, at which point the time course started. 100-fold excess unlabelled CON (100 ng per lane) was then added to 'trap' any p53 that dissociated from $^{32}$P-DNA. Aliquots were loaded on a running gel at the time points indicated. For comparison, some aliquots did not have any competitor DNA added.

**TABLE I. HALF-LIVES OF p53-DNA COMPLEXES**

| Protein-DNA complex | % Remaining after 10 minutes | $t_{1/2}$ (minutes) | $k_D$ ($s^{-1}$) |
|---|---|---|---|
| p53 – CON | 75.4 +/- 0.1 | 25 | $4.6 \times 10^{-4}$ |
| p53 – S10 | 40.6 +/- 0.2 | 8 | $1.4 \times 10^{-3}$ |
| $(A344)_2$ - CON | 63.5 +/- 3.4 | 15 | $7.7 \times 10^{-4}$ |
| $A344_1$-CON | ~3 | ~1 second | $\sim 1.2 \times 10^{-2}$ |

by interacting after binding to DNA.

2.2. High affinity DNA binding results primarily from inter-dimer cooperativity

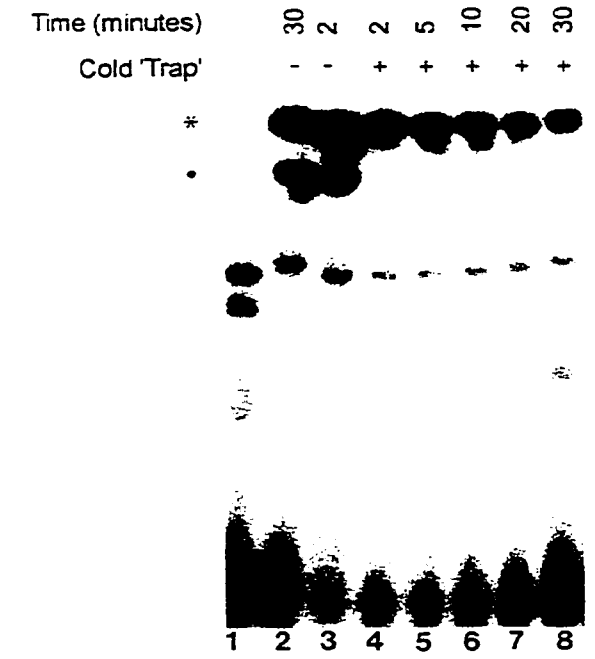
Two complexes of A344-CON were again observed: a faster migrating A344 dimer bound to one of the half-sites in CON and a slower migrating pair of A344 dimers bound to the two half-sites in CON (Figure 10A, lanes 2 and 3, positions marked by a dot and asterisk respectively). When excess unlabelled CON was added to preformed A344-$^{32}$P-CON complexes, the two A344 dimers bound side-by-side on CON had a $t_{1/2}$ of about fifteen minutes (lanes 1-8, asterisk; Table 1). This half-life corresponds to a dissociation constant of $7.7 \times 10^{-4}$, assuming first order kinetics (Table 1).

Strikingly, in contrast to the pair of A344 dimers, the single A344 dimer on DNA had a $t_{1/2}$ much less than two minutes (Figure 10A, compare lanes 3 and 4, dot). As expected, the single A344 dimer on M34 also had a short $t_{1/2}$ of less than two minutes (data not shown). Shorter time courses revealed that the single A344 dimer on CON had a $t_{1/2}$ much less than thirty seconds (Figure 10B). Consequently, the half-life of two dimers bound to CON (15 minutes) was much greater than double the half-life of a single dimer bound to one half-site (much less than 30 seconds).

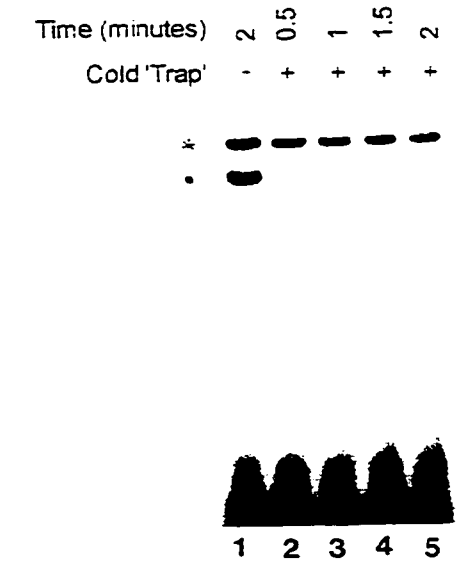
A very rough estimate of the half-life of one A344 dimer bound to one half-site can be obtained by estimating the percent decrease in the intensity of the A344 single dimer in thirty seconds (Figure 10B). If the intensity was 3% or less after thirty seconds,

**Figure 10. Half-life of A344 on CON.** **A** and **B**. A344 was allowed to bind to $^{32}$P-CON for 45 minutes, at which point the time course started. 100-fold excess unlabelled CON (100 ng per lane) was then added to 'trap' any A344 that dissociated from $^{32}$P-CON. Aliquots were loaded on a running gel at the time points indicated. For comparison, some aliquots did not have any competitor DNA added (cold 'trap'- ). The migration of one A344 dimer is marked by a dot, pairs of A344 dimers are marked by an asterisk.

A


Time (minutes)
30
2
2
5
10
20
30
Cold 'Trap'
- - + + + + +
*
•
1 2 3 4 5 6 7 8

B


Time (minutes)
2
0.5
1
1.5
2
Cold 'Trap'
- + + + +
*
•
1 2 3 4 5

then at least five half-lives must have passed ($2^{-5}$ = 3.125%). Thus, the half-life of one A344 dimer on one half-site is probably no greater than one second (3.125% of 30 seconds = 0.9375 seconds). This estimated value was included in Table 1, but the margin of error could be quite large. Interestingly, even though the half-life of one dimer on one half-site was probably about one second, the dimer-half-site complex was sufficiently stable to be visualized after about two hours of electrophoresis (Figures 6, 7, and 10). This is observed with other proteins, and is likely due to stabilization of protein-DNA complexes by the high ionic strength of electrophoresis buffer (Chodosh, 1991).

Therefore, one A344 dimer cooperatively stabilized the binding of the second dimer, which would also presumably occur in dimers of a tetramer bound to CON. Such inter-dimer cooperativity explained the increased $t_{1/2}$ from about one second to fifteen minutes. However, another property that would increase the half-life of tetramers from fifteen to twenty-five minutes must be lacking in side-by-side DNA-bound A344 dimers.

2.3. Avidity also increases the binding affinity of tetramers

Another possible contributing factor to the increased half-life of tetramer for CON was increased avidity. To separate the possible avidity effect from the cooperative inter-dimer interaction (above), a sequence consisting of two half-sites separated by one helical turn was employed (S10, Figure 2). If p53 was able to bind S10 with each dimer contacting one half-site, then each p53 tetramer would be bound to a single molecule of DNA, but the two dimers would be unlikely to interact at the DNA binding domains due to the spatial separation of one helical turn. Thus, any increase in the half-life of p53-S10

over p53-M34 would be due to the independent interaction of each dimer of a tetramer with the separated half-sites on the same molecule of S10. It logically follows that for a tetramer to dissociate from S10, the two non-interacting dimers of a tetramer must both simultaneously be dissociated from their respective half-sites if the p53 tetramer was to dissociate from S10.

The half-life of p53 on S10 was much greater than on M34 but less than on CON (Figure 11, lanes 8-13, 14-19, and 1-7 respectively). The half-life of p53 on S10 was eight minutes (Table 1), corresponding to a dissociation constant of $1.4 \times 10^{-3}$ (Table 2).

In contrast, two A344 dimers bound to S10 had a $t_{1/2}$ much shorter than two minutes (data not shown). The likely explanation for this finding is that the two dimers of a tetramer had a higher avidity for the two half-sites in S10. If, for example, the binding of one dimer to DNA bent or twisted the DNA in such a way as to allow the second dimer to bind better to DNA, then this stabilizing effect would appear with A344 dimers. Therefore, the dimers in a tetramer are probably binding with higher avidity to the two half-sites in S10, and therefore also in CON, because the tetramerization domain confers increased avidity to p53 DNA binding.

Interestingly, the sum of the half-lives of p53 (Table 1) on S10 (8 minutes) and of two A344 dimers on CON (15 minutes) is very close to the half-life of an intact tetramer on CON (25 minutes). This is expected if two independent, non-overlapping mechanisms both contribute separately to stabilizing the p53-DNA interaction. Thus, dimer-dimer

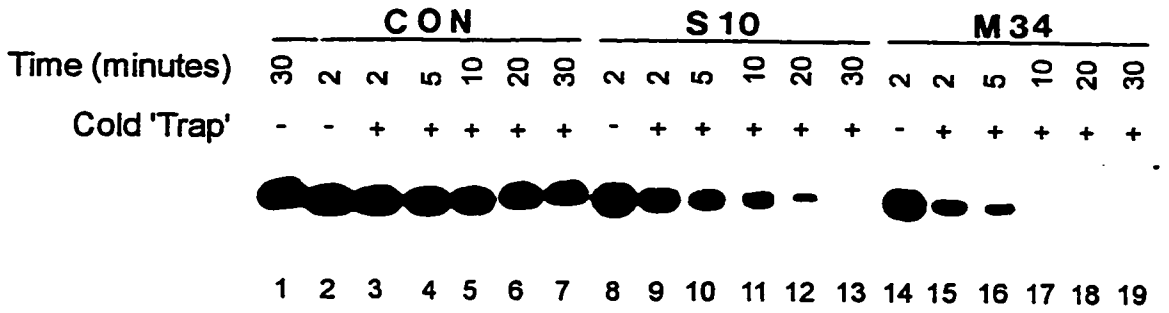



**Figure 11. Half-life of p53 on CON, S10, and M34.** P53 was allowed to bind to $^{32}$P-DNA for 45 minutes, at which point the time course started. 100-fold excess unlabelled CON (100 ng per lane) was then added to 'trap' any p53 that dissociated from $^{32}$P-DNA. Aliquots were loaded on a running gel at the time points indicated. For comparison, some aliquots did not have any competitor DNA added.

cooperativity and tetramer avidity are distinct and account for the entire (within experimental error) affinity of tetramer for consensus DNA.

## 3. High Fidelity Binding Results from Avidity

### 3.1. Tetramers bind CON with high fidelity

The rapid dissociation of tetramers from single half-sites, but not CON, could reflect the mechanism by which p53 selects consensus sites in a large pool of DNA. The following experiments address this possibility by adding human p53 to $^{32}$P-labelled sequences in the presence of excess unlabelled DNA. Whereas in the dissociation experiments the p53-$^{32}$P-DNA complex was allowed to form before unlabelled DNA was added to the reaction, the following experiments have labeled and excess unlabelled DNA present from time zero. As expected, neither NB nor M24 competed with $^{32}$P-CON for binding to a constant amount of p53 (Figure 12A, lanes 4-5 and 16-20, respectively). CON competed for p53 binding the best, followed by M34 (lanes 6-10 and 11-15, respectively). Therefore, wild-type p53 preferentially bound to CON, but selected M34 (but not M24) present in excess. Identical results were seen with murine p53 (data not shown).

Due to the nature of the selective mutations of two out of ten base pairs in the second M34 half-site, it was possible that the intact p53 oligomer was making other stabilizing contacts in the two mutated quarter-sites of M34. To test this possibility p53 binding to $^{32}$P-CON was challenged with a single half-site (HALF) not followed by a mutated half-site. There was virtually no difference between excess M34 and HALF

(Figure 12B, compare lanes 4-6 with lanes 7-9). Therefore, tetramers preferentially bind CON, but can bind excess single half-sites. Importantly, fidelity of binding to CON was maintained in the presence of excess M24 or NB.

3.2. A344 dimers bind CON with low fidelity

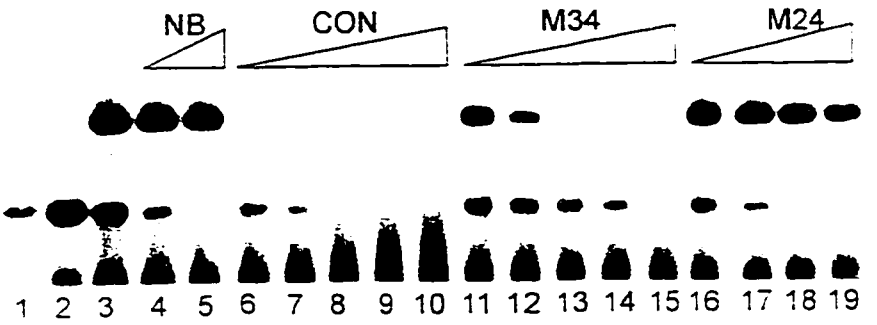
Because the half-life of dimers on CON was much greater than on M34 mainly due to cooperativity (Figure 10A), it might be expected that pairs of dimers would bind preferentially to CON. Notably, excess unlabelled M34 competed with $^{32}$P-CON for binding to single or pairs of A344 dimers (Figure 13A, dot and asterisk respectively). Not surprisingly, excess M34 also competed with $^{32}$P-M34 for binding to A344 dimers (Figure 13B). However, M34 competed better than an equal concentration of half-sites in CON.

Levels of A344 were then translated such that a greater portion of $^{32}$P-CON was bound by pairs of A344 dimers than by single dimers (Figure 14, lane 1). When excess unlabelled CON or M34 was added to A344 at the same time as $^{32}$P-CON, there was a preferential decrease in DNA-bound pairs of A344 dimers relative to single dimers (Figure 14, lanes 1-3, compare the ratio of pairs (asterisk) to single (dot) A344 dimers). Although an equimolar amount of half-sites was added for CON and M34, M34 competed for pairs of A344 even better than CON (compare lanes 4-6 with 1-3).

The ratio of pairs of A344 dimers to single A344 dimers was greater than one in lanes 1 and 4. When a moderate excess of CON or M34 was added at time zero, the ratio

**Figure 12. Competition of p53 binding to CON.** **A.** p53 was added to $^{32}$P-CON and increasing concentrations of unlabelled DNA as indicated. 100ng (lane 4) or 1000ng (lane 5) of NB was added, or increasing amounts of other sequences as indicated. M34 and M24 were added at 10ng (lanes 11 and 16), 33ng (lanes 12 and 17), 100ng (lanes 13 and 18), 333ng (lanes 14 and 19), and 1000ng (lanes 15 and 20). CON was added to give an equal concentration of quarter-sites as M34 and M24. As controls, lane 1 contains p53, PAb421, and $^{32}$P-NB. Lane 2 contains p53 and $^{32}$P-CON without PAb421. Lanes 3-19 contain p53, $^{32}$P-CON, and PAb421. **B.** p53 was added to cold competitors as indicated, 10ng (lanes 1, 4, and 7), 100ng (lanes 2, 5, 8, and 10), 1000ng (lanes 3, 6, 9, and 11).

A


NB
CON
M34
M24
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19

B

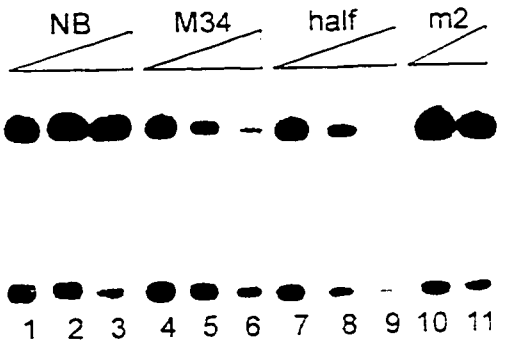
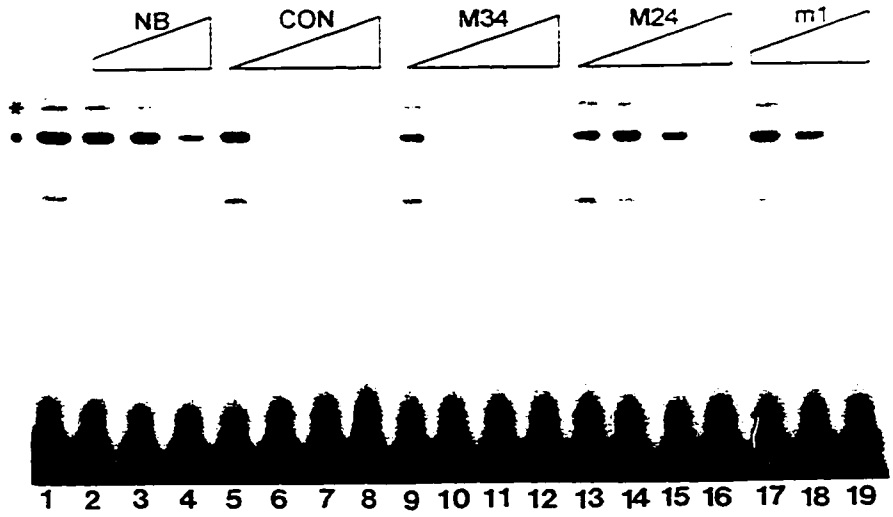
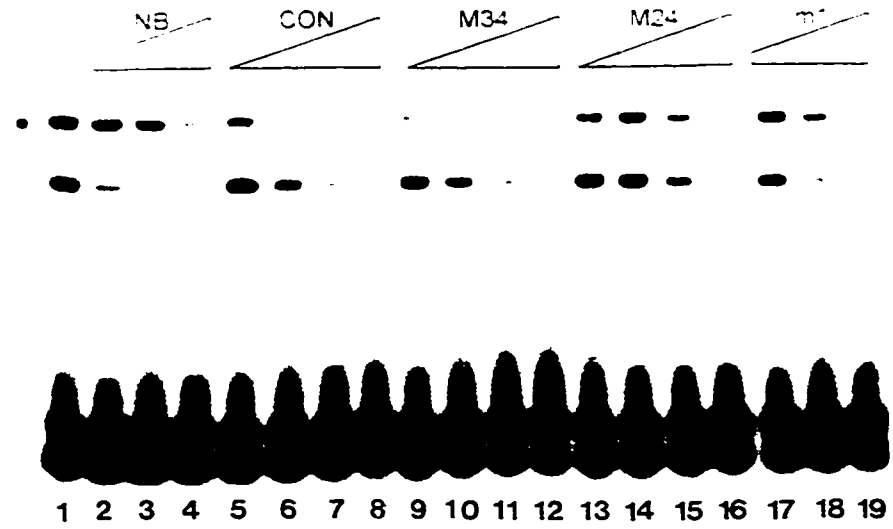

NB
M34
half
m2
1 2 3 4 5 6 7 8 9 10 11

**Figure 13. Competition of A344 binding to DNA.** **A**. A344 was added to $^{32}$P-CON and increasing concentrations of unlabelled DNA as indicated. The amounts of NB and m1 added were 10ng, 100ng, and 1000ng. M34 and M24 were added at 1ng, 10ng, 100ng, and 1000ng. CON was added to give an equal concentration of quarter-sites as M34 and M24. **B**. A344 was added to $^{32}$P-M34, otherwise lanes are as in A. Single dimers are indicated by a dot, pairs of dimers by an asterisk.

A


NB
CON
M34
M24
m1
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19

B


NB
CON
M34
M24
m1
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19

of pairs to single A344 dimers on DNA clearly decreased below 1 (Figure 14). When presented with a choice of binding to excess half-sites, A344 dimers did not preferentially bind side-by-side on CON. These data suggest that it is easier for A344 dimers to bind to a free half-site than to a half-site on CON that is adjacent to a half-site that is already occupied by another A344 dimer.

Surprisingly, at the highest concentration used, NB and M24 competed with $^{32}$P-CON or with $^{32}$P-M34 for binding to A344 (Figures 13A and 13B respectively). A single quarter-site (m1; Figure 2) competed for A344 binding the same as did M24, indicating that consensus pentamers could weakly bind to A344 dimers (Figures 13A and 13B). Thus, an A344 dimer did not bind to DNA containing consensus first and third quarter-sites any better than to DNA containing only one quarter-site. Importantly, and in contrast to tetramers (Figure 12), excess NB or single quarter-sites competed with $^{32}$P-CON for binding to A344 dimers (Figures 13A and 13B). In the presence of excess NB or quarter-sites, dimers did not maintain their fidelity of binding to CON.

3.3. Avidity is the primary contributing factor to tetramer fidelity

The ability of NB and m2 to compete with $^{32}$P-CON for binding to A344 dimers but not to wild-type tetramers was noteworthy in that it reflected a difference in the fidelities of dimer versus tetramer DNA binding. In other words, in an environment of excess non-consensus DNA, tetramers, but not dimers, faithfully bound to the consensus sequence. It was important to determine whether tetrameric p53 had increased fidelity due to the cooperative side-by-side interaction of both dimers of a tetramer with DNA, or

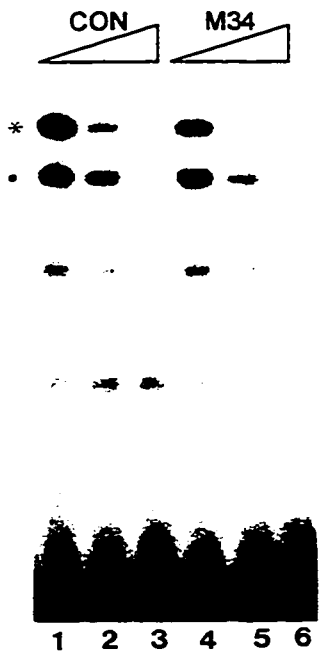



**Figure 14. Competition of pairs of A344 dimers by CON versus M34.** A344 was added to $^{32}$P-CON with increasing amounts of unlabelled CON (3.3, 10, or 33 ng) in lanes 1-3. M34 was added to give an equivalent mass of half-sites. The migration of a single dimer is indicated by a dot, pairs of A344 dimers by an asterisk.

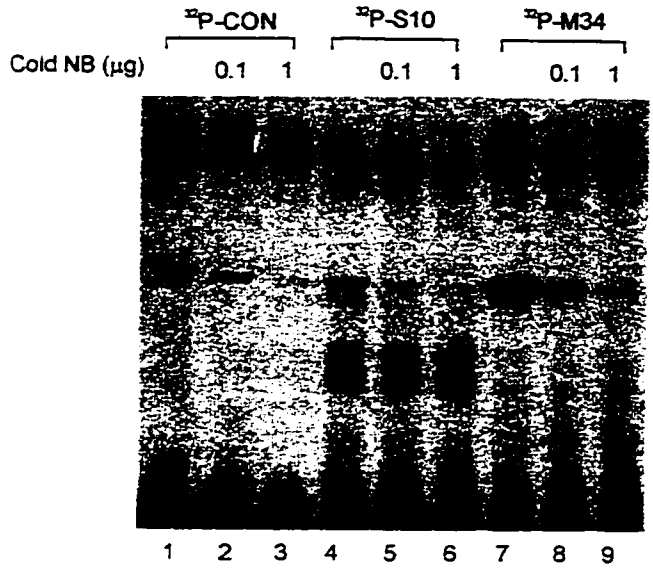
due to the increased avidity of tetramer binding.

High levels of NB did not compete out tetramer binding to $^{32}$P-CON, whereas binding to $^{32}$P-S10 was competed minimally and to $^{32}$P-M34 was competed out better (Figure 15A, compare lanes 4 and 6, 7 and 9 respectively). The ability of NB to compete for tetramer binding to M34 would be expected to be similar to A344 dimer binding, but NB did not compete tetramer binding to M34 nearly as well as it did A344 binding (compare Figure 15A to Figures 13A and 13B). One explanation might be that the second dimer of a tetramer was interacting non-specifically to adjacent DNA, so weakly stabilized the p53-M34 complex. Nonetheless, excess NB competed for tetramer binding to M34 but not to S10 or to CON.

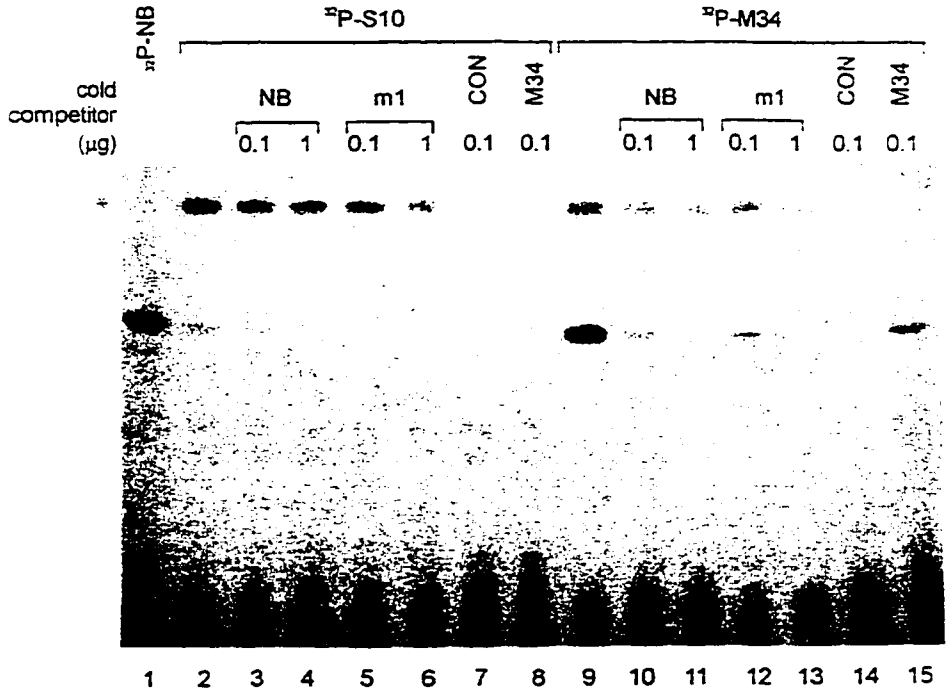
An excess of single quarter-sites competed out tetramer binding to $^{32}$P-S10 better than did excess NB (Figure 15B, compare lanes 5 and 6 to lanes 3 and 4). However, tetramer binding to $^{32}$P-M34 was substantially lower in the presence of high levels of quarter-site (Figure 15B, compare lane 13 to lane 9). As with NB, the large difference between quarter-site competition of tetramer binding to $^{32}$P-S10 (lane 6 compared to 2) and $^{32}$P-M34 (lane 13 compared to 9) can be attributed to avidity. The difference between tetramer binding to M34 and S10 was avidity, and the high fidelity of tetramer binding to $^{32}$P-CON in the presence of excess non-consensus DNA was also due to avidity. Therefore, avidity was the main factor contributing to high fidelity tetramer binding to CON.

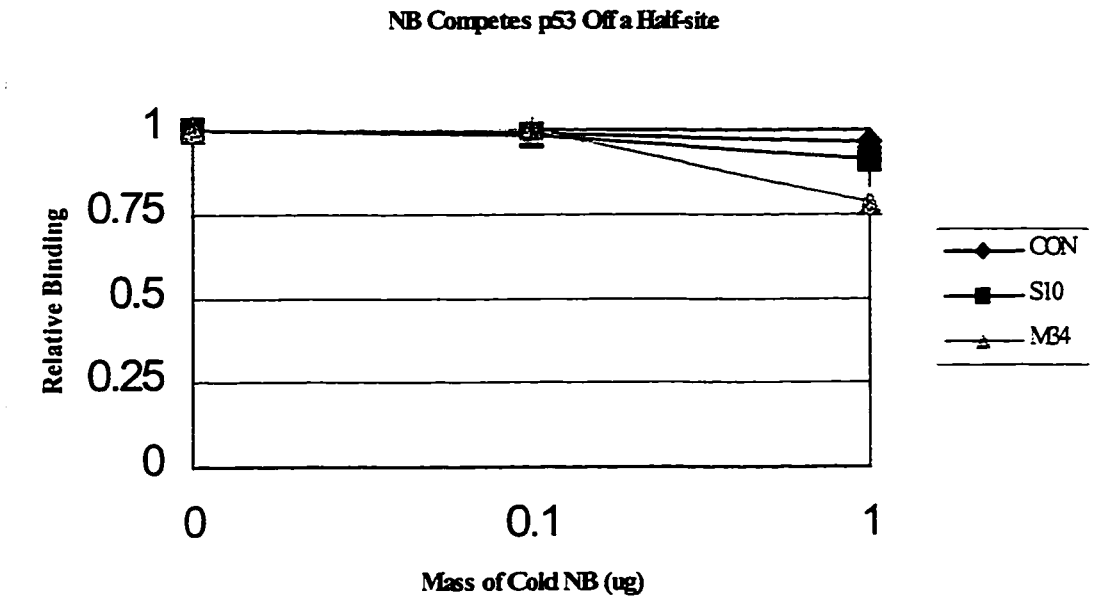
**Figure 15. Fidelity of tetramer DNA binding.** A. Competition of p53 binding to $^{32}$P-DNA by unlabelled NB was as indicated. B. p53 was added to $^{32}$P-DNA and unlabelled DNA as indicated. C. and D. Densitometry of A and B, respectively, showing the percent reduction in each p53-DNA complex.

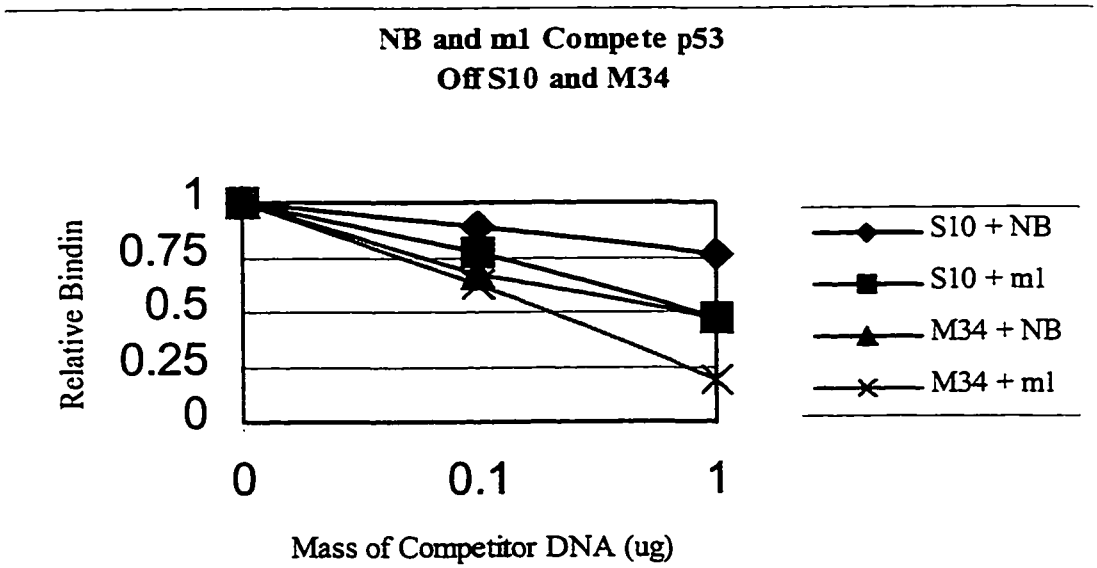
A


$^{32}$P-CON
$^{32}$P-S10
$^{32}$P-M34
Cold NB (μg)
0.1 1 0.1 1 0.1 1
1 2 3 4 5 6 7 8 9

B


$^{32}$P-NB
$^{32}$P-S10
$^{32}$P-M34
cold competitor (μg)
NB
m1
CON
M34
0.1 1 0.1 1 0.1 0.1 0.1 1 0.1 1 0.1 0.1
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15


NB Competes p53 Off a Half-site
Relative Binding
1
0.75
0.5
0.25
0
0
0.1
1
Mass of Cold NB (ug)
CON
S10
M34


NB and m1 Compete p53
Off S10 and M34
Relative Bindin
1
0.75
0.5
0.25
0
0
0.1
1
Mass of Competitor DNA (ug)
S10 + NB
S10 + m1
M34 + NB
M34 + m1

3.4. Tetramerization confers higher fidelity to cellular p53 DNA binding

It was important to compare whether cellular p53 bound with similar fidelity as *in vitro* translated p53. 'Normal' NIH 3T3 cells were exposed to the DNA damaging agent actinomycin D and cell extract was then prepared and assayed for DNA binding in the presence of PAb421. Cellular p53 binding to $^{32}$P-CON was competed best by CON, then by M34 (Figure 16). M24 and m1 competed marginally, and NB not at all.

In contrast, excess NB competed well with $^{32}$P-M34 for cellular p53 binding (Figure 16). Excess single consensus quarter-sites competed with $^{32}$P-M34 even better (lanes 19-20). Cellular p53 high fidelity binding was therefore not maintained when only one dimer interacted with the one half-site in M34. Presumably, if cellular 53 were dimeric then it would bind with the low fidelity of tetramers binding to M34. Thus, cellular p53 could bind DNA with high fidelity in the presence of excess NB only when both dimers interacted with CON.

## 4. All DNA-Bound p53 is Conformationally Wild Type

DNA binding is one property of wild type p53 that is lost in tumor-derived mutant p53. Another property that is frequently altered is the conformation, which can be wild type (i.e. p53 that displays the PAb246 epitope) or mutant (PAb240$^+$). To avoid confusion over terminology, the p53 that is able to bind to PAb246 shall be referred to hereinafter as PAb246$^+$ p53. The rabbit reticulocyte lysate (RRL) *in vitro* translation system was again exploited since it contains many cellular proteins that could potentially regulate p53 activity and conformation, for example hsp90 (Blagosklonny et al., 1996).

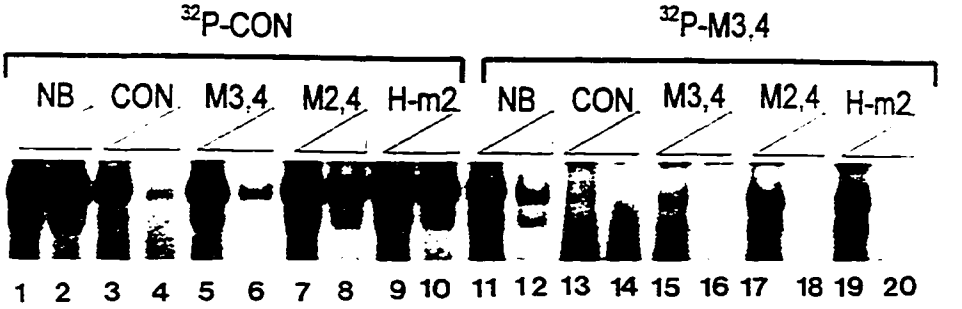



**Figure 16. Fidelity of endogenous cellular p53 DNA binding**. Balb/c 3T3 total cell extract was prepared from cells with prior DNA damage by actinomycin D. Cell extract was added to 1ng $^{32}$P-DNA as indicated, all with PAb421. Either 0.01 or 1 μg of unlabelled competitor DNA was added.

RRL has been demonstrated to variably contain an unidentified factor that regulates the PAb246/PAb240 conformation of p53 (Cook & Milner, 1990).

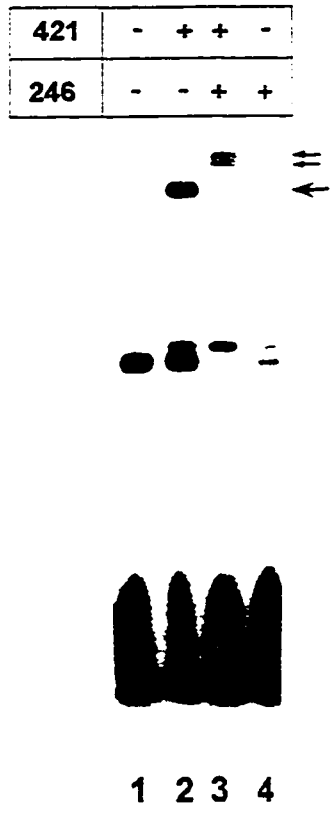
4.1. All DNA-bound p53 is conformationally wild type, and PAb246 can dissociate p53 from DNA

As usual, PAb421 was required to inactivate the negative-regulatory C-terminal domain (Figure 17A, lane 2). When PAb246 was also included in the DNA binding reaction mixture, the migration of the entire p53-DNA complex was altered (lane 3). The migration of the complex was retarded by PAb246, and two bands were visible compared to only one in the absence of PAb246 (lane 2). PAb246 in the absence of PAb421 did not give rise to any new DNA binding activity, indicating that both bands in the doublet were dependent on the inclusion of PAb421 (lane 4). Since PAb246 was present in vast molar excess over the estimated amount of p53, all available PAb246 epitopes should have been occupied. Thus, the presence of two bands only after PAb246 addition most likely reflected the presence of two intrinsically different forms of DNA-bound p53.

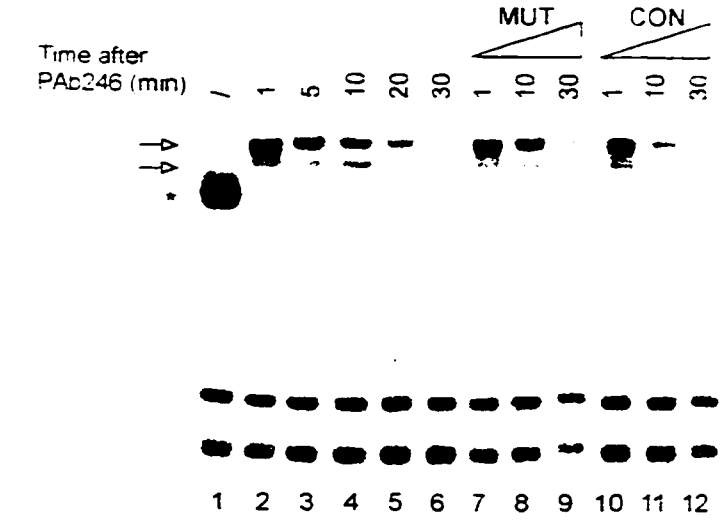
The difference in migration of these two forms may be due to differences in charge, conformation, and/or molecular weight of the p53-DNA complex. The migration of the entire p53-DNA complex was affected by PAb246, since there was no band evident at the position of the initial p53-CON complex after PAb246 is added (Figure 17A, lane 3 vs. 2). However, the combined intensities of the two PAb246 supershifted bands did not add up to the intensity of the one band in the absence of PAb246 (lane 3 versus 2). Some DNA binding was most likely abrogated by PAb246.

**Figure 17. Reactivity of p53 with PAb246.** **A.** p53 was incubated with $^{32}$P-CON and anitbodies as indicated. P53 is indicated by a large arrow, PAb246-supershifted p53 is indicated by two smaller arrows. **B.** p53 was allowed to bind $^{32}$P-CON, then PAb246 was added for the times indicated, at which point the reaction was loaded into ice cold, high ionic strength gel running buffer. Unlabelled MUT was added at 100ng per lane (lanes 7-9), or unlabelled CON was added at 10 ng per lane (lanes 10-12). The migration rate of p53-DNA is marked by an asterisk, PAb246-supershifted p53 by two open arrows.

A


421 - + + -
246 - - + +
1 2 3 4

B


MUT
CON
Time after
PAb246 (mn)
/ 1 5 10 20 30 1 10 30 1 10 30
*
1 2 3 4 5 6 7 8 9 10 11 12

The PAb246-induced dissociation of p53 from CON was followed over time (Figure 17B). Upon addition of PAb246 to preformed p53-CON there was a very rapid dissociation of most of the p53 from DNA (compare the intensity of p53 in lane 2, open arrows, to lane 1, asterisk). The supershifted p53 that remained bound to DNA was again shifted into a doublet (open arrows). The lower PAb246-supershifted band was relatively stable over 30 minutes, whereas the upper band dissociated more readily (lanes 2-6).

When excess unlabelled CON was added to trap p53 that dissociated from $^{32}$P-CON, the half-life of the upper band was clearly less than 10 minutes (Figure 17B, lanes 10-12). However, the half-life of the lower PAb246-supershifted band was greater than 10 minutes. Most of the p53-DNA complex was rapidly dissociated by PAb246, so perhaps the relatively unstable upper band represented the bulk of p53, most of which had already dissociated from DNA. It should be noted that both bands contain p53 and PAb246, because neither appeared when murine-specific PAb246 was added to human p53-DNA complexes (Figure 25B). Clearly, the two PAb246-supershifted bands represented different forms of p53, which could have differed in any of a number of properties, including conformation, phosphorylation, or complex formation with other proteins in the RRL.

4.2. All DNA binding-competent p53 is conformationally wild type

To determine whether all p53 that is capable of binding to DNA was already in the wild type conformation, aliquots of p53 were immunodepleted with either PAb246 or control antibody, and the supernatant was assayed for any remaining DNA binding

activity (Figure 18A, lanes 5 & 6). When PAb246 was used to immunodeplete the reaction of p53 that was in the wild type conformation ($PAb246^{+}$) before DNA was added, all of the p53 that was capable of binding to DNA (binding-competent p53) was removed (lane 5). In contrast, p53 remained in the precleared control lane that was treated identically except that no PAb246 was added to immunodeplete p53 (lane 6). As well, some p53 was bound to MUT (lane 8), the sequence to which tetramers but not dimers could bind (Section 1).

4.3. PAb246 dissociates all of the p53 that binds to MUT

Some of the p53 that was bound to the consensus DNA binding site (CON) was supershifted and some was displaced from DNA by PAb246 (Figure 17A and 17B). However, when p53 was bound to the altered binding site MUT (Figure 2), PAb246 displaced all of the bound p53 (Figure 18B, compare lanes 5 and 6). When conformationally wild type ($PAb246^{+}$) p53 was precleared with PAb246 from the binding reaction no p53-consensus DNA complex was apparent (Figure 18B, lanes 3 and 4). In addition, no DNA binding-competent p53 remained to bind to MUT when p53 was precleared with PAb246 (lanes 7 and 8). Therefore, p53 that could bind to MUT and p53 that was bound to MUT was in the wild type conformation.

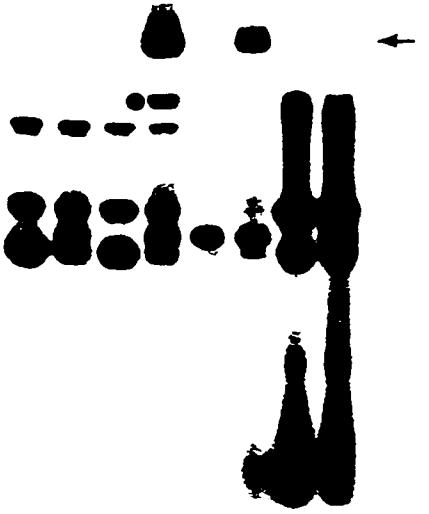
**5. Some DNA-Bound p53 is Concurrently Wild Type and Mutant, or Dual Positive**

The results presented in Section 5, except for Figures 19C, 20, and 24, and including Figure 29, were previously published (McLure and Lee, 1996). These figures and text are reproduced here with the express written permission of the copyright holder.

**Figure 18. Immunodepletion of p53 with PAb246.** **A.** p53 was incubated with $^{32}$P-CON or MUT and PAb421 as indicated. Aliquots of p53 were added to control antibody (lane6) or PAb246 (lane 5), then any reactive p53 was precipitated by insoluble protein A and the supernatant assayed for DNA binding. **B.** p53 was immunodepleted as in A, then the supernatants were allowed to bind $^{32}$P-CON or $^{32}$P-MUT, with or without PAb246 as indicated. The migration rate of p53-DNA is marked by a large arrow, PAb246-supershifted p53 by two smaller arrows.

A

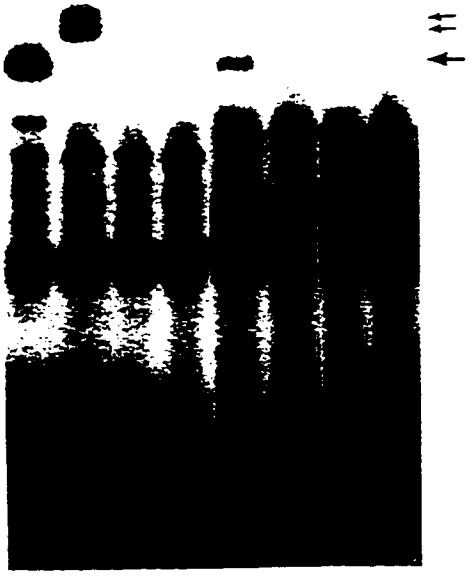
| | CON | | | | | | MUT | |
|---|---|---|---|---|---|---|---|---|
| Immunodepletion | None | | | | 246 | Ctrl Ab | None | |
| p53 | - | | + | | + | + | + | |
| 421 | - | + | - | + | + | + | - | + |



1 2 3 4 5 6 7 8

B

| | CON | | | | MUT | | | |
|---|---|---|---|---|---|---|---|---|
| Immunodepletion | Ctrl Ab | | 246 | | Ctrl Ab | | 246 | |
| 246 | - | + | - | + | - | + | - | + |



1 2 3 4 5 6 7 8

5.1. Some DNA-bound p53 is conformationally mutant

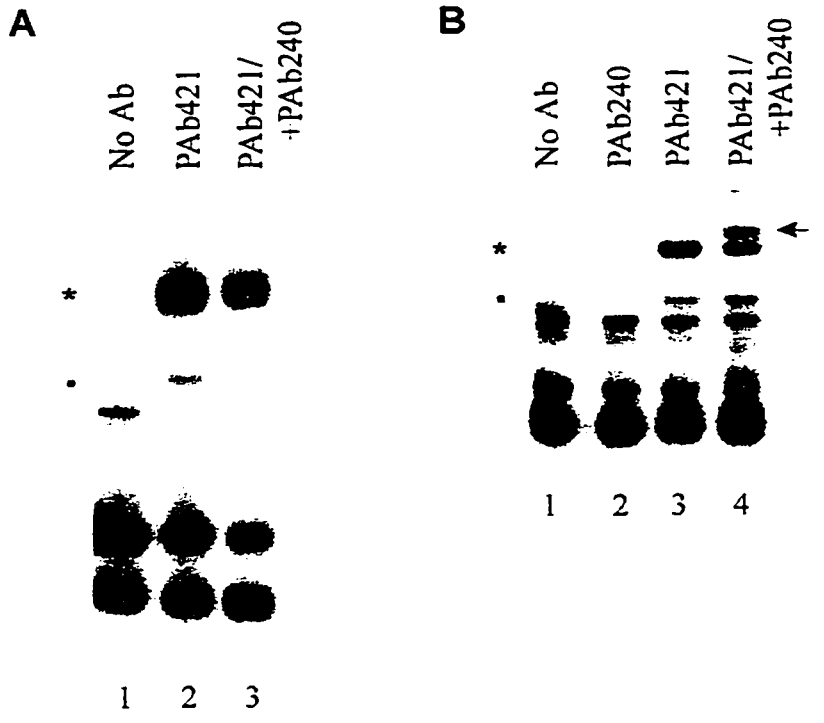
Conformationally wild-type murine p53 generated *in vitro* from different batches of rabbit reticulocyte lysate (RRL) was assayed for DNA binding and for recognition by PAb240 after this binding. Depending on the batch of RRL used, two different sets of results were typically obtained (shown in Figure 19 and Figure 20). For the batch of RRL represented in Figure 19A, the addition of PAb240 to the DNA binding mixture had no effect on the DNA-bound p53 (lane 3), suggesting that this p53 species was unreactive with PAb240.

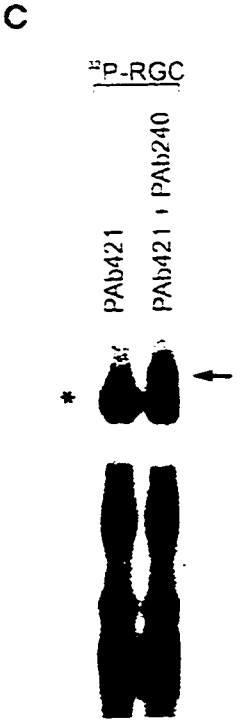
For the batch of RRL represented in Figure 19B, approximately 50% of the PAb421-p53-DNA complex was supershifted by PAb240 (Figure 19B, lane 4, indicated by an arrow). PAb240 by itself (in the absence of PAb421) had no effect (lane 2). It should be noted that the lower dimeric p53 band was unchanged in the presence of PAb240 (lane 4, dot). When bound to another consensus DNA sequence, RGC (a p53-responsive element from the ribosomal gene cluster), p53 was again supershifted by PAb240 (Figure 19C). These experiments therefore illustrate that some DNA-bound p53 can display the PAb240 epitope previously believed to be inaccessible in wild-type p53. However, the remote possibility that the supershifted band arose from an indirect effect of PAb240 (and therefore contained no PAb240) could not be ruled out from this experiment.

5.2. p53 can be conformationally mutant before binding to DNA

To demonstrate conclusively that the PAb240 supershift actually represented

**Figure 19. Display of the PAb240 epitope by DNA-bound p53.** **A** and **B**. p53 was translated in two different batches of RRL and assayed for binding to $^{32}$P-CON, in the presence of antibodies as indicated. **C**. p53 was translated in a B-type batch of RRL, and bound to a consensus sequence that differs from CON (RGC) with or without PAb240.


A
No Ab
PAb421
PAb421/
+PAb240
*
•
1 2 3
B
No Ab
PAb240
PAb421
PAb421/
+PAb240
*
•
1 2 3 4


C
$^{32}$P-RGC
PAb421
PAb421 + PAb240
*

antibody bound to p53, p53 was immunodepleted before DNA binding, and the supernatants assayed for DNA binding. Initially, p53 was incubated with *immobilized* PAb240, PAb246, or an identical control with no antibody (Figure 20). Only immobilized PAb246 but not immobilized PAb240 was able to clear p53 before DNA binding (lanes 7-9 and 4-6, respectively). This was initially exciting because one explanation for some p53 being $PAb240^+$ after binding to DNA was that upon DNA binding, p53 underwent conformational change to a 'mutant' conformation (lane 2).

*Immobilized* PAb240 might not be able to access the PAb240 epitope on p53 as well as PAb240 in solution, so the above experiment was repeated using *soluble* PAb240 to immunodeplete p53 before DNA binding. In a batch of RRL that produced $PAb240^-$ DNA-bound p53 (Figure 21A, lanes 1 and 2), preadsorption with soluble PAb240 did not reduce the amount of DNA-bound p53 (lanes 3 and 4). In contrast, in a batch of RRL that produced $PAb240^+$ p53 (Figure 21B, lanes 1 and 2), most of the DNA binding-competent p53 was precleared by soluble PAb240 but not by control antibody (lanes 3 and 4 compared to lanes 1 and 2, respectively). Interestingly, the amount of p53 removed by preadsorption with PAb240 (lanes 3 and 4) was larger than that present in the PAb240-supershifted p53 complex (lane 2).

This experiment therefore confirmed that the PAb240-supershifted band indeed contained PAb240. Moreover, it also demonstrated that populations of p53 that were $PAb240^+$ after DNA binding were also $PAb240^+$ before binding to DNA. Thus, there was no conformational change from wild type to mutant after binding to DNA.

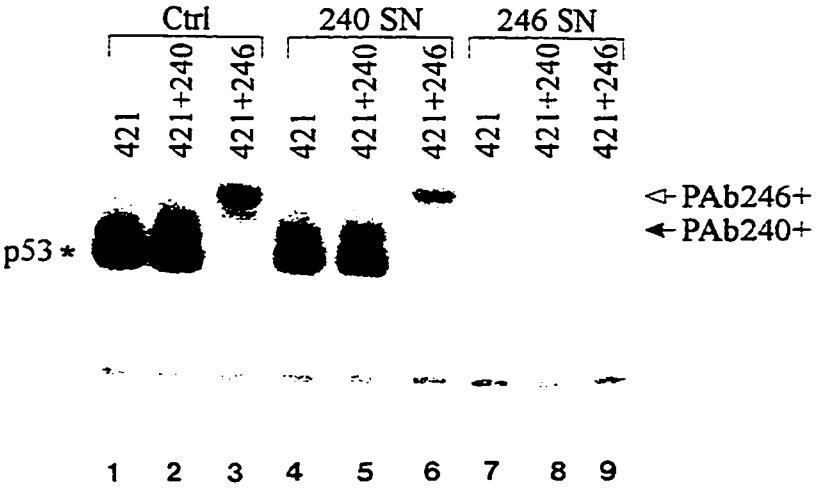
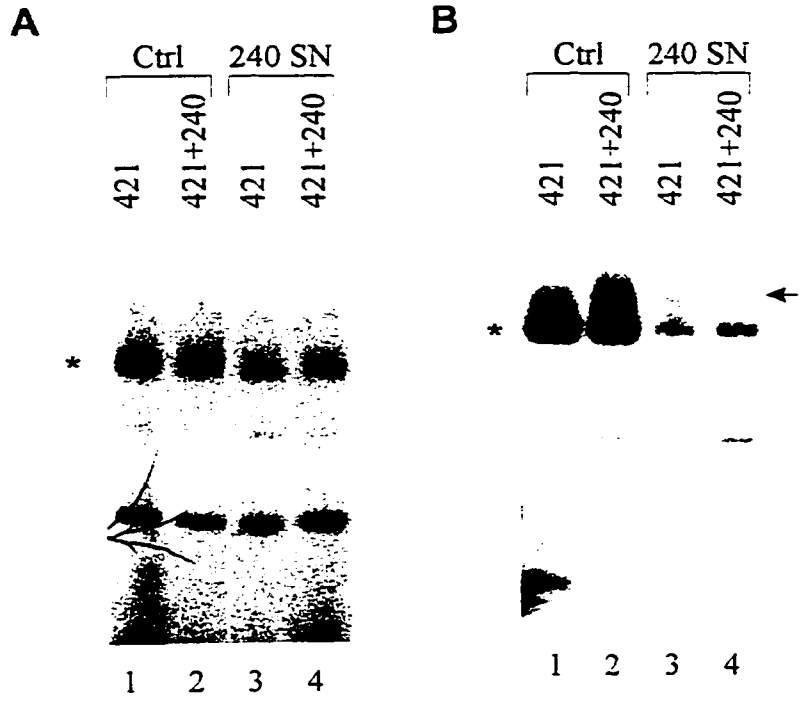



**Figure 20. Reactivity of p53 with immobilized PAb240 and PAb246.** p53 was incubated with immobilized PAb240, PAb246, or control antibody. Supernatants were assayed for binding to $^{32}$P-CON with antibodies as indicated.

**Figure 21. Reactivity of p53 with soluble PAb240 before DNA binding. A** and **B.** p53 synthesized in two different batches of RRL (A and B as in Figure 19) was precleared with control antibody (lanes 1 and 2) or PAb240 (lanes 3 and 4) and then assayed for binding to $^{32}$P-CON. The position of p53 is indicated by an asterisk, and the PAb240-supershifted band is indicated by an arrow.


A
Ctrl
240 SN
421
421+240
421
421+240
*
1 2 3 4
B
Ctrl
240 SN
421
421+240
421
421+240
*
1 2 3 4

## 5.3. DNA-bound p53 can be simultaneously conformationally mutant and wild type, or 'dual positive'

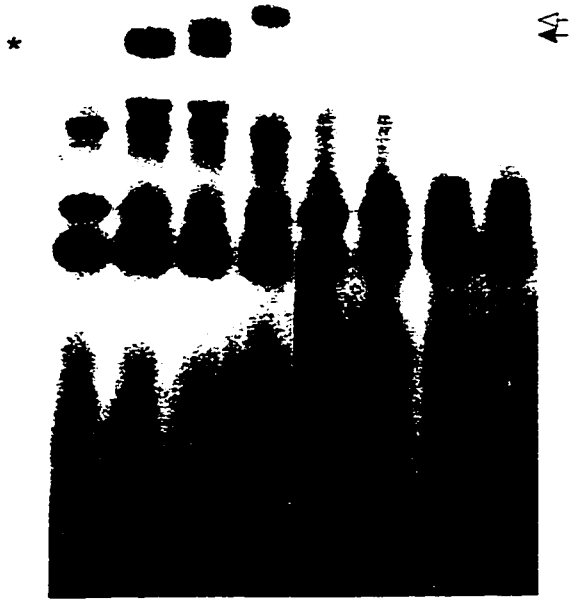
Approximately one half of the DNA-bound p53 was supershifted by PAb240, so it might be expected that the other half would be recognized by PAb246, since these two antibodies are thought to recognize mutually exclusive conformations of p53 (reviewed by Zambetti and Levine, 1993). However, instead of only some of the p53-DNA complex being PAb246$^+$ the entire complex was recognized by PAb246 (Figure 22A, lane 4 compared to lane 2). Some of the p53-DNA complex was supershifted by PAb246 (lane 4, open arrow), while the rest was dissociated by PAb246 as judged by the lack of any residual PAb421-p53-DNA complex (lane 4 compared to lane 2). Both the PAb240$^+$ and PAb246$^+$ p53 populations were sequence-specifically bound to DNA, as shown by the lack of binding to NB (lanes 5-8).

The observation that the entire p53-DNA complex was in a PAb246$^+$ conformation and ~50% of the p53-DNA complex was in a PAb240$^+$ conformation, has led to the interesting question as to whether the DNA-bound PAb240$^+$ p53 was concurrently PAb246$^+$. Since the PAb246-supershifted p53-DNA complex sometimes migrated as a doublet (shown in Section 4) which would in turn make the interpretation of PAb240 supershift difficult, purified PAb246 Fab fragments (PAb246') were used for the next set of experiments. Figure 22B shows that like the intact PAb246, a good portion of the p53-DNA complex was supershifted into a single band by PAb246' (lane 5, lower open arrow). PAb246' also caused some p53 to dissociate from DNA (compare the p53-DNA intensities in lanes 2 and 5). In the presence of PAb240, a population of the

**Figure 22. Relationships of the PAb240$^+$ and PAb246$^+$ conformations of DNA-bound p53.** **A.** p53 was bound to CON or NB in the presence of antibodies as indicated. The position of the p53-DNA complex is indicated by an asterisk, PAb240 and PAb246-supershifted complexes are indicated by closed and open arrows respectively. **B.** Binding conditions were as in A, but PAb246 Fab fragments (PAb246') were used instead of PAb246. The PAb246' and PAb240 supershifted complex is indicated by a double arrow.

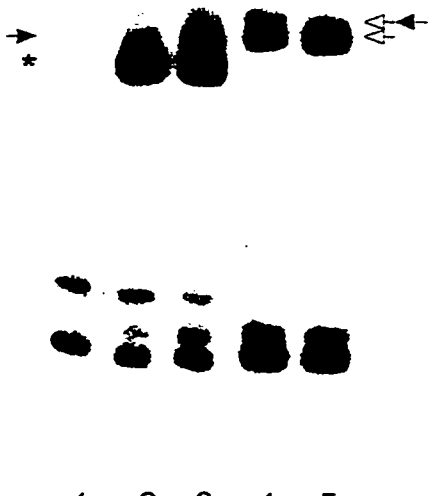
A

| | CON | | | | NB | | | |
|---|---|---|---|---|---|---|---|---|
| PAb421 | - | + | + | + | - | + | + | + |
| PAb240 | - | - | + | - | - | - | + | - |
| PAb246 | - | - | - | + | - | - | - | + |




B

| | | | | | |
|---|---|---|---|---|---|
| PAb421 | - | + | + | + | + |
| PAb240 | - | - | + | + | - |
| PAb246' | - | - | - | + | + |



PAb246' supershifted p53-DNA complex was further shifted (lane 4, double arrow), suggesting that some DNA-bound p53 displayed both PAb246 and PAb240 epitopes.

To demonstrate that PAb246' was indeed present in the p53-DNA complex, biotinylated PAb246' (bio-PAb246') was used instead of regular (non-biotinylated) PAb246' in the band shift experiment. Figure 23 shows that as was observed with PAb246', the DNA-bound p53 was shifted by bio-PAb246' (lane 3), and some of this new complex was further shifted by PAb240 (lane 5). Streptavidin-agarose beads were then added to the latter reaction mixture to precipitate all free bio-PAb246' and bio-PAb246'-associated complexes. After removal of the beads by low-speed centrifugation, the supernatant was then assayed for DNA binding activity. None was found (lane 6). That the streptavidin preclearing was specific for bio-PAb246' complexes was illustrated by the demonstration that both $PAb240^-$ (lower open arrow) and $PAb240^+$ p53-DNA complexes (double arrow) remained in the supernatant when PAb246' was substituted for bio-PAb246' (lane 7). Thus, for some DNA-bound p53, not only are the PAb246 and PAb240 epitopes simultaneously displayed, they can both be occupied with no loss of DNA-binding activity.

It is important to note that of many different batches of RRL (dozens) that have been examined, all produced p53 capable of binding to DNA and the p53-DNA complex was always 100% recognized by PAb246 (data not shown). However, depending on the batch of RRL used, 0-50% of the p53-CON complex could react with PAb240 (data not shown). Therefore, all DNA-bound p53 is $PAb246^+$, but only a subpopulation of this is

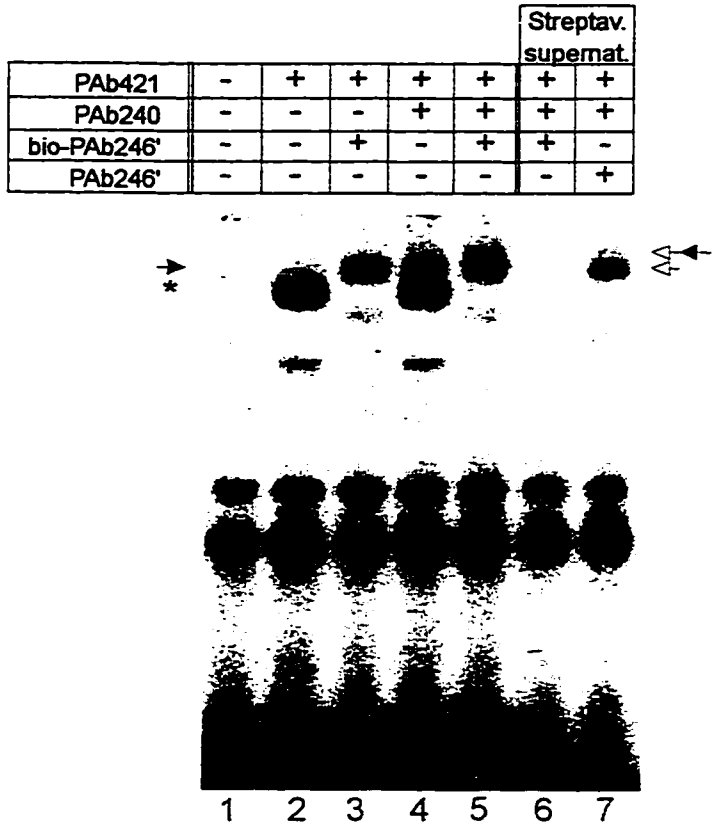



**Figure 23. PAb240$^{+}$ p53 is precleared by PAb246.** p53 was bound to $^{32}$P-CON in the presence of antibodies as indicated. After incubation with PAb246 Fab fragments (PAb246', lane 7) or biotinylated PAb246' (bio-PAb246', lane 6), p53-bio-PAb246' complexes were precipitated with immobilized streptavidin and the supernatant assayed for DNA binding.

PAb240$^{+}$, or 'dual positive'. The 'dual positive' form of p53 can bind to DNA both before and after interacting with either or both PAb240 and PAb246.

5.4. PAb240 can protect DNA-bound p53 from PAb246-induced dissociation

The relationship between PAb240 binding and the two PAb246-supershifted forms of p53 was further investigated. As expected from Figure 17B, when PAb246 was added to p53 for 40 minutes most of the p53 had dissociated from DNA, leaving mainly the lower band of the PAb246 doublet (Figure 24, lane 7, open arrow). However, when PAb246 and PAb240 were added sequentially, an interesting difference was apparent. After 10 minutes of incubation with PAb246, PAb240 was added to the binding reaction for a further 30 minutes (lane 6). Although PAb246 was present for 40 minutes, enough time to dissociate most of the DNA-bound p53 (lane 7, open arrow, compared to lane 3, asterisk), PAb240 stabilized the upper band (lane 6, closed arrow-open arrow). Unfortunately, it cannot be said whether the PAb246-supershifted upper band of the doublet was stabilized by PAb240. The lower band of the doublet could have been stabilized and supershifted by PAb240.

When PAb240 was allowed to bind to p53 for 30 minutes, followed by PAb246 for 10 minutes, the upper band was even stronger (Figure 24, lane 5). Interestingly, the intensity of the dual positive band was very close to the intensity of the PAb240$^{+}$ band in the absence of PAb246 (lane 5, closed arrow-open arrow and lane 4, closed arrow respectively). Although both PAb240 and PAb246 can simultaneously bind to DNA-

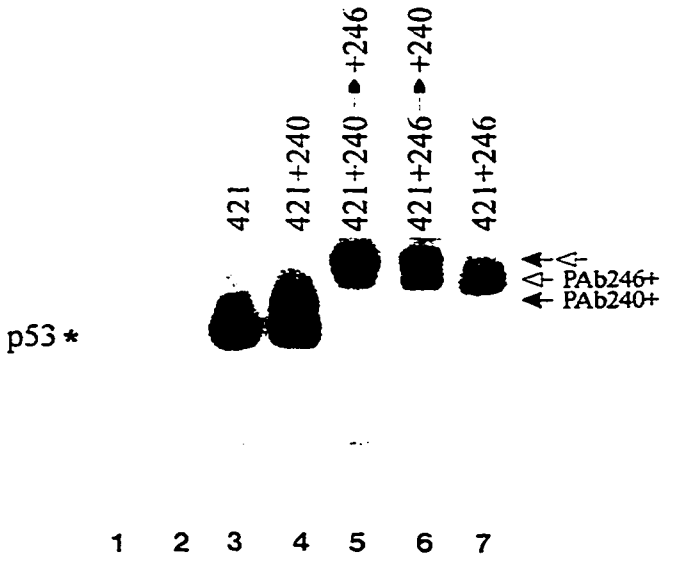



**Figure 24. Effect of PAb240 on PAb246-induced dissociation.** p53 was incubated with $^{32}$P-CON and PAb240 (lane 4) or PAb246 (lane 7) for 40 minutes. After 30 minutes of incubation with PAb240, PAb246 was added for a further 10 minutes (lane 5). After 10 minutes of incubation with PAb246, PAb240 was added for a further 30 minutes (lane 6). The two control lanes are p53 with either no antibody added or with PAb240 and PAb246 (lanes 1 and 2, respectively).

bound p53, the above experiments do not reveal which subunits of p53 tetramers are wild type, and which are mutant.

## 6. Each Dimer of a Tetramer is in an Independent Conformational Equilibrium

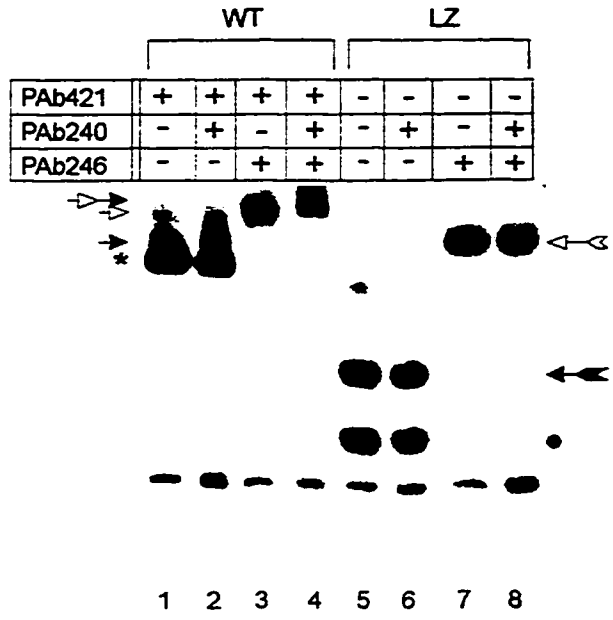
### 6.1. Tetramerization is required for the mutant phenotype of DNA-bound p53

Since wild type p53 binds DNA as tetramers (dimer-of-dimers), it was of interest to determine whether the PAb246 and PAb240 epitopes could coexist on the same dimer of p53. The murine p53LZ332 protein bound the consensus DNA as two different species, whose migration is marked by a dot and solid tailed arrow (Figure 25A, lane 5). While some full-length (WT) p53 was supershifted by PAb240 (lane 2, solid arrow), p53LZ332 was unreactive with PAb240 (lane 6). However, all p53 species were reactive with PAb246 (lanes 3 and 7, open and open tailed arrows respectively). Importantly, the full-length, but not leucine zipper, PAb246-reactive p53 was further supershifted by PAb240 (lanes 4 and 8 respectively). Therefore, the p53 leucine zipper dimer was $PAb246^{+}/PAb240^{-}$ under conditions where full-length wild type p53 was $PAb246^{+}/PAb240^{+}$.

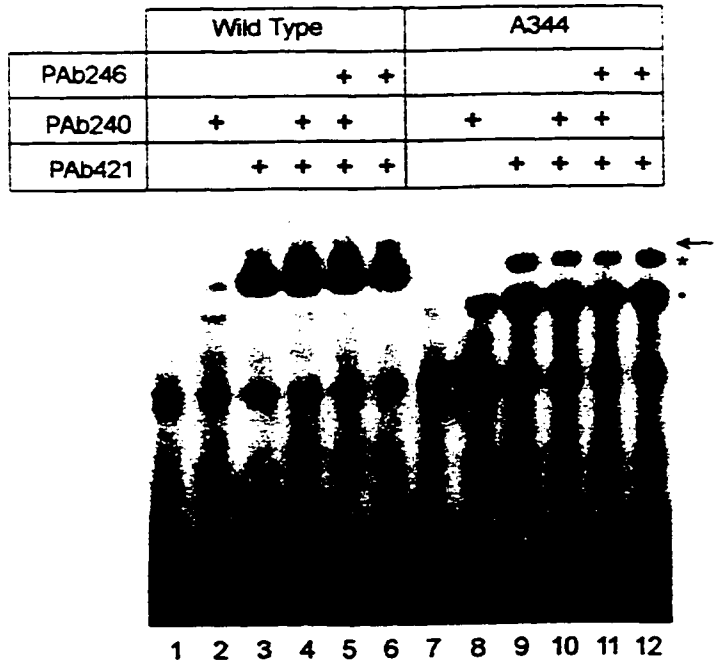
It was shown above that the two p53LZ332 bands on CON represent one or two dimers (Figure 5A). The finding that the single p53LZ332 dimer on CON is $PAb246^{+}$ demonstrated conclusively that the wild type conformation is defined at the level of a dimer (Figure 25A, lane 7, open tailed arrow). Even two dimers bound side-by-side on CON failed to display the mutant conformation (compare lanes 5 and 6, solid tailed arrow).

**Figure 25. Reactivity of dimeric p53 with PAb240 and PAb246.** **A.** Wild type murine p53 (lanes 1-4) or p53LZ332 (lanes 5-8) was bound to $^{32}$P-CON in the presence of antibodies as indicated. The migrations are indicated as follows: p53, asterisk; PAb240-supershifted p53, solid arrow; PAb246-supershifted p53, open arrow; p53 supershifted by both PAb240 and PAb246, open arrow followed by a solid arrow; single p53LZ332 dimers, dot; pairs of p53LZ332, solid tailed arrow; PAb246-supershifted p53LZ332, open tailed arrow. **B.** Wild type human p53 (lanes 1-6) or A344 (lanes 7-12) was bound to $^{32}$P-CON in the presence of antibodies as indicated. The migration of single dimers is indicated by a dot, pairs of dimers or tetramers by an asterisk, and PAb240-supershifted p53 by an arrow.

A


WT
LZ
PAb421 + + + + - - - -
PAb240 - + - + - + - +
PAb246 - - + + - - + +
1 2 3 4 5 6 7 8

B


Wild Type
A344
PAb246 + + + +
PAb240 + + + + + + +
PAb421 + + + + + + + +
1 2 3 4 5 6 7 8 9 10 11 12

The C-terminal 58 amino acids of p53 were removed in p53LZ332, leaving wild type p53 residues 1 to 332 (Halazonetis and Kandil, 1993). Thus, the dimerization domain (residues 326-334), tetramerization domain (residues 337-355), and residues 360-390 were all missing in p53LZ332. However, the dimerization domain was functionally replaced with the leucine zipper from GCN4 (Halazonetis and Kandil, 1993). The two remaining possibilities that could explain why p53LZ332 could not bind DNA while displaying the mutant phenotype were that either tetramerization or residues 360-390 were required.

A344 and p53LZ332 were similar in that both bound CON as pairs of dimers (Figures 5 and 6). However, A344 is dimeric due to a single point mutation at residue 344 and thus retains the C-terminal 30 residues (Waterman et al., 1995). Therefore, A344 could be used to discriminate between whether tetramerization was required for p53 to display the mutant phenotype while bound to DNA.

Neither one nor two A344 dimers were PAb240$^+$ when bound to CON (Figure 25B, lane 10, dot and asterisk). However, wild type human p53 translated in parallel to A344 was PAb240$^+$ on CON (lane 4, arrow). Not surprisingly, PAb246 had no effect on human wild type or human A344 p53, because PAb246 is murine-specific (lanes 5, 6, 11, and 12). Nonetheless, even in the presence of the C-terminal 30 amino acids, dimeric p53 was not able to be in the mutant conformation when bound to DNA. By elimination, the most probable requirement for DNA-bound p53 to be able to display the PAb240 epitope was tetramerization.

## 6.2. One wild type dimer of a tetramer can bind to one half-site while the other dimer is in an unbound mutant conformation

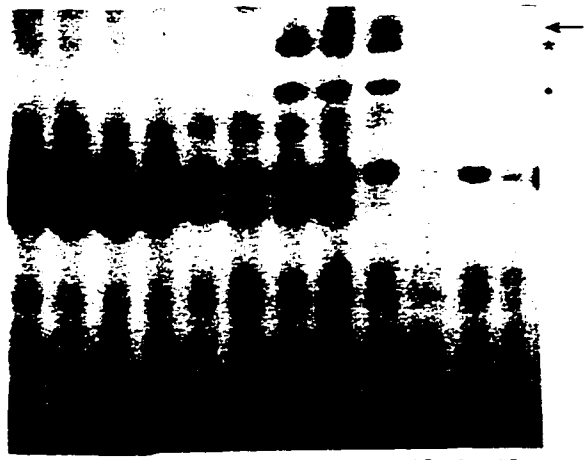
It was of interest to determine the conformation of tetramers that were bound to DNA via one dimer. Binding of PAb240$^+$ p53 to CON was competed by half and M34, but not NB (Figure 26A, lanes 10, 11, and 9 respectively, arrow). Additionally, tetramers could bind to M34 in a PAb240$^+$ conformation (Figure 26B, lane 6, arrow). In contrast, A344 dimers were PAb240$^-$ when bound to CON or M34 (lanes 10 and 12). DNA-bound dimers are conformationally wild type but not mutant (Figure 25A), so the dimer in a tetramer that binds DNA is likely also wild type but not mutant. Thus, the single half-site in M34 was bound by a wild type dimer in a tetramer, leaving the other dimer to bind PAb240.

One possibility was that one wild type dimer could bind to DNA, while the other mutant dimer did not interact with DNA. If this were the case, tetramers that were bound to DNA via both dimers simultaneously would be PAb240$^-$. Serendipitously, MUT was bound by tetramers but not dimers, and like p53-CON, all p53-MUT was conformationally wild type (Sections 1 and 4). Although p53 was PAb240$^+$ on CON (Figure 27, lane 2), p53 bound to MUT was PAb240$^-$ (lane 4). Since only tetramers could bind to MUT (Figures 4 and 7), it may be concluded that the dual positive p53 conformation is incompatible with both dimers of a tetramer binding to DNA. Thus, PAb240$^+$ p53 bound to DNA via one wild type dimer, and the other dimer could bind PAb240 but not DNA.

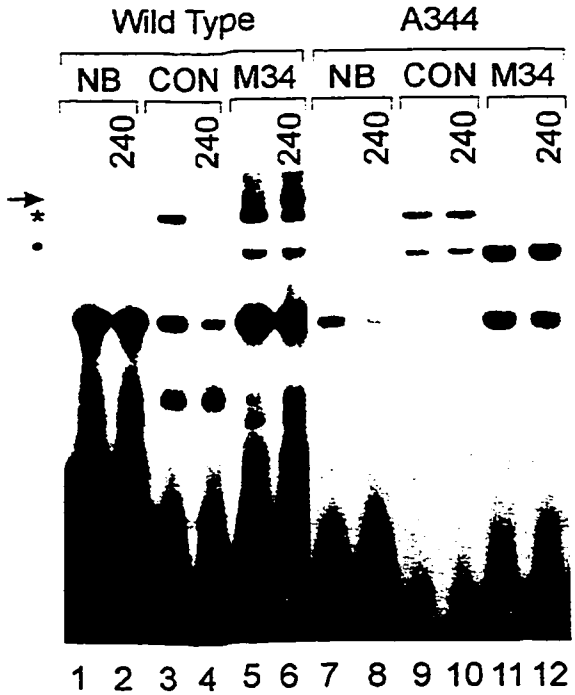
**Figure 26. Binding of PAb240$^+$ p53 to M34.** **A.** p53 was bound to $^{32}$P-NB (lanes 1-5) or $^{32}$P-CON (lanes 6-12) in the presence of PAb240 as indicated. 500ng unlabelled competitor DNA was included as indicated. **B.** Wild type p53 or A344 was bound to $^{32}$P-NB (lanes 1-2 and 7-8), $^{32}$P-CON (lanes 3-4 and 9-10), or $^{32}$P-M34 (lanes 5-6 and 11-12) in the presence of PAb240 as indicated.

A

| P-DNA | NB | | | CON | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Competitor | | | | | NB | half | M34 | CON | |
| PAb240 | + | | + | + | | + | + | + | + |
| PAb421 | | + | + | + | + | + | + | + | + |




B



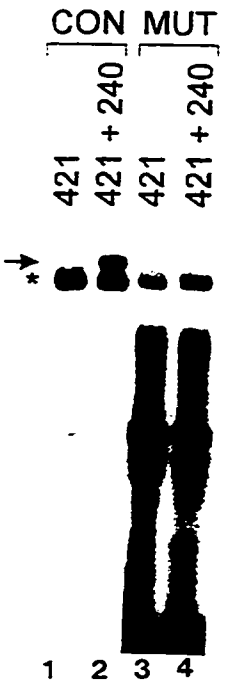



**Figure 27. Comparison of PAb240$^{+}$ p53 binding to CON and MUT.** p53 was bound to $^{32}$P-CON (lanes 1-2) or $^{32}$P-MUT (lanes 3-4) in the presence or absence of PAb240. The migration of p53 is indicated by an asterisk, PAb240-supershifted p53 by an arrow.

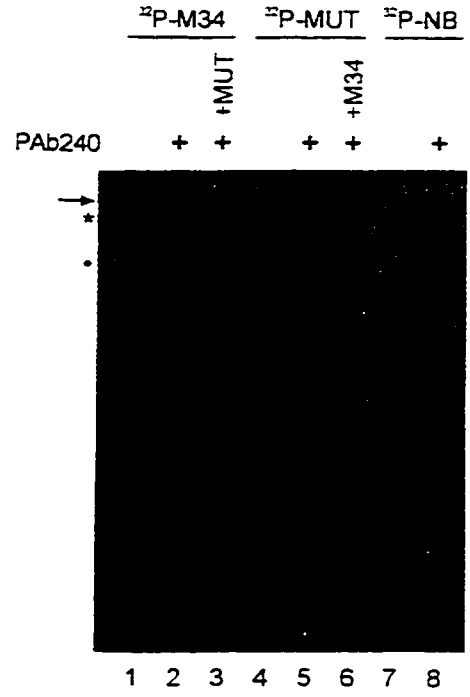
6.3. Each dimer within a tetramer is in conformational equilibrium

As PAb240$^+$ genotypically wild type p53 always coexisted with PAb240$^-$ p53, an important concept to clarify was whether the conformation of each dimer within a tetramer was fixed or whether it was changing between mutant and wild type. If the conformation of dimers was in equilibrium, the second half-site in CON might compete with PAb240 to 'trap' both dimers of a tetramer in the wild type conformation, thus explaining why only 30-50% of the p53-DNA complexes were usually PAb240$^+$. Therefore, the same starting population of p53 was allowed to bind to M34 via one wild type dimer (Figure 28A, lane 1). Conditions were found where over 50% of the DNA-bound p53 was PAb240$^+$ (lane 2). As in Figure 26B, the ability of only one dimer of a tetramer to bind to the one half-site in M34 did not force the other dimer to be PAb240$^+$ (lane 2, asterisk). The PAb240$^+$ p53 was bound to M34 via one dimer, and MUT could only be bound by both wild type dimers of a tetramer (Figures 25-27).

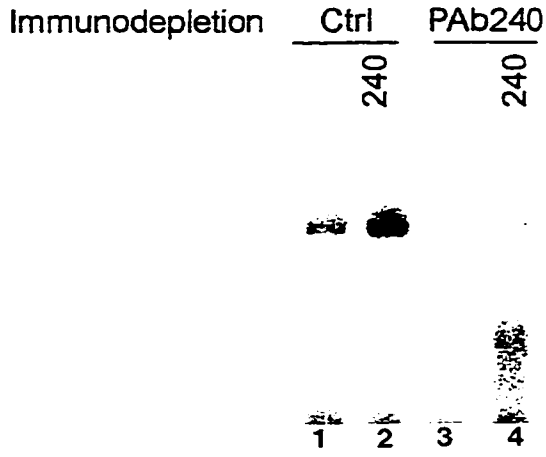
Therefore, if the conformations were fixed, MUT should compete out PAb240$^-$ but not PAb240$^+$ p53 from binding to M34. However, MUT competed out both forms of p53 (Figure 28A, lane 3). As confirmation that the same population of p53 was binding to MUT and M34, M34 also competed off p53 binding to MUT (lane 6). Thus, one wild type dimer of a tetramer could bind M34 (lane 1), and the other dimer could either be in the wild type conformation and bind MUT (lane 3), or be conformationally mutant and bind PAb240 (lane 2).

**Figure 28. Comparison of p53 binding to MUT and to PAb240.** **A.** p53 was bound to $^{32}$P-M34 (lanes 1-3), $^{32}$P-MUT (lanes 4-6), or $^{32}$P-NB (lanes 7-8). 1 μg of unlabelled MUT was added to lane 3, or M34 to lane 6. PAb240 was added at the same time to lanes 2, 3, 5, 6, and 8. **B.** p53 was cleared with control antibody (lanes 1 and 2) or PAb240 (lanes 3 and 4), then the supernatants were assayed for binding to $^{32}$P-MUT.

A


$^{32}$P-M34
$^{32}$P-MUT
$^{32}$P-NB
+MUT
+M34
PAb240
+ + + + +
1 2 3 4 5 6 7 8

B


Immunodepletion
Ctrl
PAb240
240
240
1 2 3 4

If that were the case, then the p53 that could bind to MUT in a PAb240$^-$ conformation could have been PAb240$^+$ before binding to MUT. Indeed, PAb240 immunodepleted all of the p53 that was able to bind MUT (Figure 28B, lanes 3 and 4). These findings suggest that one initial population of p53 contained tetramers in which the dimers were in conformational equilibrium. It might be recalled at this juncture that more p53 was precleared by PAb240 than was subsequently supershifted on DNA by PAb240 (Figure 21A).

## 7. The Mutant Conformation can be Recessive

### 7.1. Dual positive p53 is a small component of a largely mutant (PAb246$^-$/PAb240$^+$) p53 population generated in RRL

The very fact that some wild type p53 could bind DNA with one dimer being PAb240$^+$ demonstrated that the mutant conformation is not necessarily dominant negative. In RRL that produced PAb240$^+$ p53, most of the p53 that could bind DNA was precleared by PAb240 or by PAb246, indicating that most of the DNA binding-competent p53 could be dual positive. The size of the PAb246$^+$ p53 population relative to total p53 generated in a typical RRL reaction was determined by immunoprecipitating $^{35}$S-methionine labeled, *in vitro*-translated wild type p53 with PAb246 and PAb240. The majority of the p53 synthesized was PAb240$^+$, and only a small population was PAb246$^+$ (Figure 29). This is despite the fact that this same batch of RRL produced DNA-bound, PAb240$^+$ p53 that was all recognized by PAb246 in a bandshift assay (similar to Figures 19 and 21). Therefore DNA-bound 'dual positive' p53 existed under conditions where the majority of p53 was in the PAb240$^+$/PAb246$^-$ conformation.

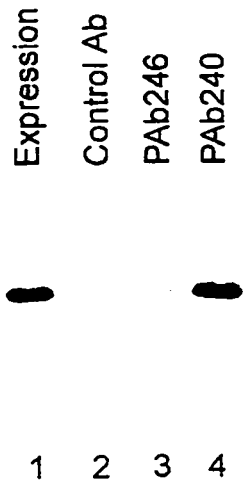



**Figure 29. Comparison of relative amounts of PAb240$^+$ and PAb246$^+$ p53 synthesized in RRL.** p53 translated in the presence of $^{35}$S-methionine was precipitated with antibodies as indicated and analysed by SDS-PAGE.

It is particularly significant that even in a population of p53 that was predominantly in a mutant conformation, the dimers within a tetramer were still in conformational equilibrium. Interestingly, genotypically wild type p53 could always bind to CON, and therefore contained at least some wild type dimers within the tetramers (data not shown). The proportion of this p53 that also contained PAb240$^{+}$ dimers was variable, between 0 and 50%, suggesting that the equilibrium was shifted towards the wild type conformation in the presence of DNA.

7.2. Genotypically mutant p53 is in a conformational equilibrium

If dimers of wild type p53 are in conformational equilibrium even when most of the p53 is PAb240$^{-}$, then the mutant conformation is not dominant negative in and of itself. Perhaps genotypic mutant p53 could also be in equilibrium. To test this idea, a temperature-sensitive murine mutant ($Val_{135}$) was used because it can undergo a rapid conformational shift from wild-type to mutant when the temperature is increased from 32°C to 37°C (reviewed by Milner, 1995).

$Val_{135}$ was translated at 37°C, then allowed to bind to CON for 20 minutes at 37°C. No $Val_{135}$ was able to bind DNA (Figure 30, lane 1). Addition of PAb246 actually did trap a small amount of $Val_{135}$ in a wild type conformation that could bind DNA (lane 5). Surprisingly, the murine-specific PAb242 cooperated with PAb246 in stabilizing DNA binding of mutant p53 (lane 4). The murine constitutive mutant $Phe_{132}$ was also stabilized in a DNA-bound conformation by PAb242 (data not shown). Most

importantly, despite PAb246 dissociating most p53 from DNA (Figure 17B), some $Val_{135}$ was trapped in a wild type, DNA-bound conformation by PAb246 (Figure 30, lane 5).

7.3. Wild type p53 can stabilize mutant p53 in a wild type conformation

When $Val_{135}$ was co-translated (Co-TL) with wild type human p53, nearly all of the human p53 DNA binding was eliminated (Figure 30). However, when $Val_{135}$ and human p53 were mixed post-translationally (Post-TL Mix), most of the human p53 DNA binding remained. Therefore, $Val_{135}$ was dominant negative at 37°C when cotranslated with wild type p53.

For $Val_{135}$ to be dominant negative, it must have formed heteroligomers with wild type human p53. By chance, some of these heteroligomers would have been composed of one wild type dimer and one $Val_{135}$ dimer, so it was of interest to determine whether such a tetramer would be 'dual positive'. Surprisingly, none of the DNA-bound co-translated p53 was supershifted by PAb240 (lane 8). However, about one half of the DNA-bound co-translated p53 was supershifted by PAb246 (lane 10).

When $Val_{135}$ and human wild type p53 were mixed post-translationally, $Val_{135}$ was not dominant negative. Half the amount of human p53 in lanes 16-20 was mixed with $Val_{135}$, and the intensity of DNA-bound p53 was about half (lanes 11-15). Compared to the co-translated sample (lane 10), the amount of PAb246-supershifted p53 was greater with post-translational mixing, although the ratio to $PAb246^-$ p53 was smaller (lane 15). Human p53 was not recognized by PAb246 (lane 20), only heteroligomers

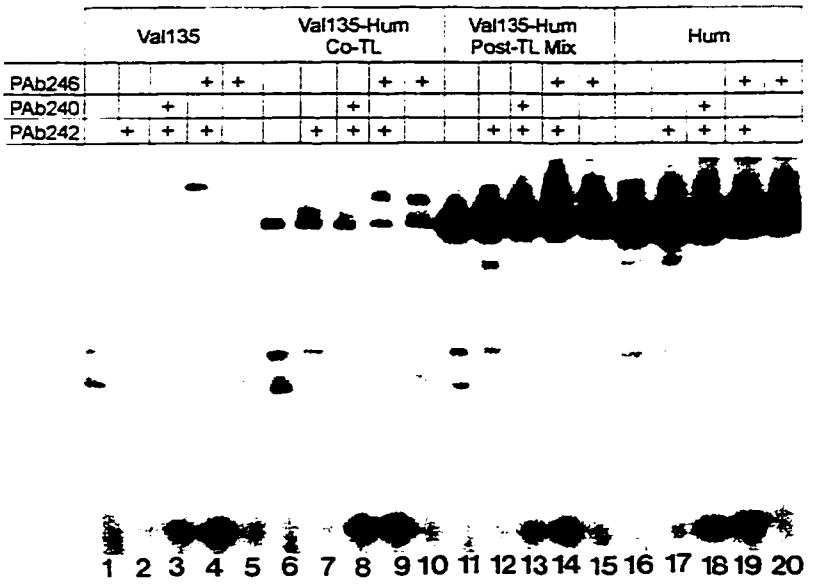



**Figure 30. Effects of wild type p53 or antibodies on mutant p53 DNA binding.** The temperature-sensitive mutant murine p53 Val135 and/or wild type human p53 were translated and bound to $^{32}$P-CON at 37°C. Val135 was co-translated with human p53 or mixed with human p53 post-translationally as indicated. Murine-specific antibodies PAb242 and PAb246 were included in the indicated lanes. Mutant conformation-specific PAb240 or wild type conformation-specific PAb246 were included as indicated.

were PAb246$^{+}$ (lanes 10 and 15).

The wild type conformation was defined at the level of dimers (Figure 25A), so $Val_{135}$ that was PAb246$^{-}$ probably also contained at least one conformationally wild type dimer in a tetramer. The other dimer of these tetramers was human, because $Val_{135}$ alone bound DNA to a much smaller extent (Figure 30, compare lanes 5, 10, and 15). This finding is very significant because $Val_{135}$ dimers did not convert wild type human dimers to a mutant conformation. In fact, the wild type human dimers converted at least some mutant $Val_{135}$ dimers to a wild type conformation (lanes 10 and 15). The fact that $Val_{135}$ inactivated wild type human DNA binding when they were co-translated suggests that co-translation might result in the majority of tetramers being formed by two dimers, each of which contained at least one mutant subunit. However, when one wild type human dimer was present in the complex (revealed by p53 that could bind DNA), the second genotypically mutant dimer was induced to assume the wild type conformation that could be trapped by PAb246. Therefore, mutant p53 was not dominant negative when at least one dimer consisted of wild type p53. In such heteroligomers, the mutant conformation was recessive.

# DISCUSSION

## 1. Significance of Understanding the Mechanism of DNA Binding by p53

### 1.1. A tetramer binds DNA with each dimer of the tetramer contacting its own half-site

Although the individual structures of the major functional domains of p53 have been determined (reviewed by Pennisi, 1996), a major unresolved issue has been how the four DNA binding domains are related to the oligomerization domains in a DNA-bound p53 tetramer (reviewed by Arrowsmith and Morin, 1996). Since the isolated oligomerization domain forms a very stable tetramer in solution at neutral pH (Johnson et al., 1995), it is likely that the relative orientation of each subunit remains fixed in the tetramerization domain. Similarly, four core domains bind to the four quarter-sites in a consensus sequence, and the four subunits of a tetramer all engage the consensus sequence (Wang et al., 1995; Balagurumoorthy et al., 1995).

Consequently, the relative order of each DNA binding domain on DNA would remain fixed once a tetramer became bound to a consensus sequence. Thus, the full-length tetramer may be viewed as a dimer-of-dimers as defined at the tetramerization domain, with the DNA binding domains extending to contact DNA. In order to demonstrate how full length p53 binds DNA it was necessary to determine the linkage between these two fixed structures.

It is demonstrated here for the first time how the DNA binding domains are connected to the oligomerization domains in DNA-bound p53 tetramers. Tetramers could bind to a DNA sequence that contained quarter-sites one and two (M34), but not to a sequence that contained only quarter-sites one and three (M24). This indicated that the critical cooperative interaction occurred between subunits of the tetramer that bound adjacently to the two quarter-sites in one half-site. However, this finding did not discriminate between which subunits of a tetramer were interacting on a half-site, because either both subunits of one dimer or one subunit of each dimer of a tetramer could have been binding to one half-site.

Dimeric p53 (A344 and p53LZ332) could bind to M34 but not to M24, indicating that the two subunits of one dimer could bind to one half-site. It is likely that the A344 mutation did not significantly alter the structure of p53, but merely resulted in the formation of dimers that bound DNA similarly to wild type dimers. Because A344 dimers and wild type dimers both bound M34 but not M24, the cooperativity of binding of two subunits in a dimer must be due to interaction with the adjacent quarter-sites in one half-site. Therefore, the binding of tetramers to M34 is likely achieved via the interaction of one dimer in a tetramer to the one half-site in M34, leaving the other dimer either 'hanging off' the DNA or perhaps interacting non-specifically with DNA. One of the two dimers in a tetramer would contact one of the two half-sites in CON, while the other dimer in the tetramer could contact the other half-site in CON.

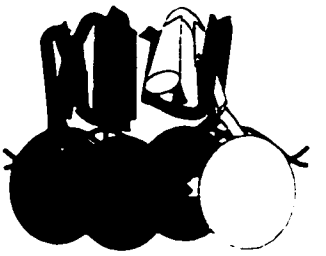
It should be noted that the assignment of oligomerization status to p53 is based primarily on the results of others, who have shown that wild type p53 binds consensus DNA as a single tetramer (Halazonetis and Kandil, 1993; Waterman et al., 1995; Wang et al., 1995; Balagurumoorthy et al., 1995). Multiple tetramers have also been demonstrated to bind DNA, although the migration of the p53-DNA complex was significantly decreased (Waterman et al., 1995). The observation that pairs of A344 dimers migrate more slowly than single dimers on CON, and tetramers comigrate with pairs of A344 dimers, is consistent with p53 binding to CON as a single tetramer.

It can be concluded that the two subunits of p53 that form one of the two dimers in a tetramer bind to one half-site in the consensus DNA sequence (Figure 31A). The other two subunits of p53 that form the second dimer in the tetramer bind to the second half-site. Thus, p53 can be visualized as a pair of clamps, with each dimer being one clamp. This model is schematically depicted in Figure 31A, which incorporates the present findings as well as other previous suggestions for various aspects of p53 DNA binding.

This model of p53 DNA binding is consistent with other results that led to the suggestion that tetramers might bind DNA with each dimer contacting one half-site (Halazonetis and Kandil, 1993; Clore et al., 1994; Waterman et al., 1995). The DNA binding core domains are modeled as ellipses for simplicity, with each core domain binding to one quarter-site such that the bulk of the four core domains lies on one face of

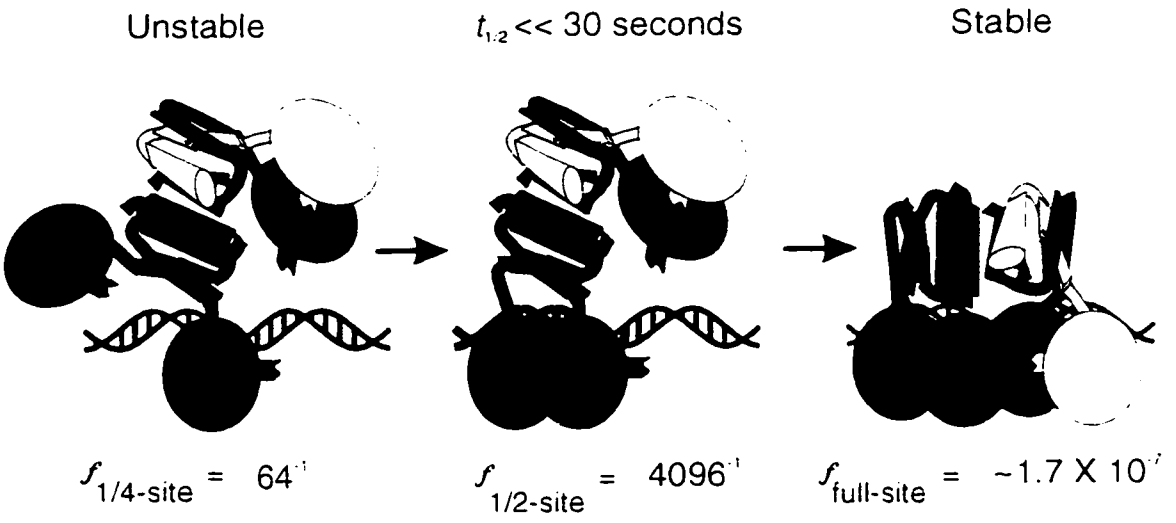
## Figure 31

A



Model of a tetramer binding to a consensus sequence. One dimer (green and purple) binds to one half-site on the DNA. The second dimer of the tetramer binds to the adjacent half-site. After binding, the dimers interact to stabilize binding.

B




Predicted change with time in the major population of p53-DNA complexes in an environment of excess, random DNA. Initially, the average p53 tetramer interacts transiently with non-specific DNA (not shown), but one subunit rapidly encounters a frequently occuring quarter-site (blue DNA, left). Better binding occurs when a less frequently occuring half-site is encountered (middle), but the tetramer becomes stably bound to DNA only once it encounters an infrequent full consensus sequence (right).

the DNA helix (Cho et al., 1994). Flexible linkers extend to the other face of the helix and may have no fixed structure, thereby allowing the tetrahedrally symmetric tetramerization domain to retain its symmetry while the attached core domains are bound to DNA with a lower order of two-fold cyclic symmetry (Waterman et al., 1995). The oligomerization domain is a tetrahedrally symmetric dimer-of-dimers, consisting of two pairs of interlocking subunits (Lee et al., 1994; Clore et al., 1994, 1995a,b; Jeffrey et al., 1995). Each dimer is formed by two monomers that dimerize via an anti-parallel B-sheet followed by a turn and an alpha helix, the latter of which forms the dimer-dimer interface (ibid). The tetramerization domain likely resides on the opposite side of the helix relative to the core domains (Lee et al., 1994; Jeffrey et al., 1995).

A model that has the DNA binding domains of one dimer in a tetramer binding to one half-site of a consensus DNA sequence has been previously postulated, but not demonstrated. One group demonstrated that free dimers could bind to half-sites, but it was not ascertained whether dimers in a tetramer could bind to quarter-sites one and two or one and three in the consensus DNA (Halazonetis and Kandil, 1993; Waterman et al., 1995). Another group showed that tetramers (lacking the C-terminal negative regulatory domain) could bind to half-sites much better than to DNA sequences that had only the first and third quarter-sites (Wang et al., 1995). However, there was no discrimination between whether each dimer in the tetramer bound to one half-site or whether one subunit from each dimer bound to a half-site. This distinction is critical because these same authors demonstrated that four free monomers bound cooperatively to the four quarter-

sites in consensus DNA. Tetramers also occupied all four quarter-sites, and presumably bound DNA cooperatively (Wang et al., 1995). Therefore, the cooperativity between two subunits that contacted one half-site could have been due to an interaction between either two subunits from one dimer or one subunit from each dimer within the tetramer.

Yet another group has found that monomeric core p53 DNA binding domains, co-crystallized with DNA, bind with one monomer contacting the first quarter-site and a second monomer contacting a third (imperfect) quarter-site (Cho et al., 1994). Although a consensus half-site was present in the DNA used for crystallization, only one quarter-site was bound by a core domain. A second core domain bound not to the adjacent quarter-site, but rather to the quarter-site that would be the third in a consensus sequence. This site had homology to a consensus quarter-site, but lacked the essential, invariant C in 5'-PuPuPuC(A/T). In addition, one of three protein subunits present in the crystal structure did not contact DNA at all, but was positioned according to crystal packing forces (Cho et al., 1994). Therefore, the crystallized arrangement of monomeric subunits on DNA was influenced more by crystal packing forces than by the protein-protein interactions that normally stabilize DNA binding in solution.

1.2. Cooperative inter-dimer interactions and avidity stabilize tetramer binding

Tetramers could bind via one dimer interacting with one half-site, but binding was stabilized, from much less than 30 seconds to about 25 minutes (Figure 10; Table 1), if

the second dimer of a tetramer simultaneously bound DNA beside the first dimer. This is consistent with previous observations that cooperative interactions occur between p53 monomers that bind to one or two half-sites (Pavletich et al., 1993; Wang et al., 1995; Balagurumoorthy et al., 1995). Of note is that p53 dimer-dimer DNA binding cooperativity has not previously been reported, as cooperativity has previously been limited to the binding of subunits to the two quarter-sites of a half-site (Pavletich et al., 1993; Wang et al., 1995; Balagurumoorthy et al., 1995).

Although the specificity of DNA binding is dictated by the core domains (Bargonetti et al., 1993; Pavletich et al., 1993; Wang et al., 1993), the equilibrium constant of a tetramer for the consensus site is about 10-100 fold better than that of four individual core domains (Bargonetti et al., 1992; Hall and Milner, 1995; Balagurumoorthy et al., 1995; Pavletich et al., 1993). Although the overall equilibrium constant ($K = 8.3 \times 10^{-8}$ M) was measured for monomers on consensus DNA, the authors refer to this constant as the dissociation rate constant $K_d$ (Balagurumoorthy et al., 1995). This should not be confused with the (non-equilibrium) dissociation constant ($k_D$) determined here as $4.6 \times 10^{-4}$ $s^{-1}$. What this number means is that of $4.6 \times 10^4$ molecules of tetramers bound to CON, one random tetramer dissociates from DNA per second. The ability of that or other unbound molecules to reassociate with DNA is not reflected by the $k_D$.

There were three explanations for two dimers of a tetramer binding better to two

half-sites (CON) than to one (M34). One possibility was that one dimer could propagate a conformational shift through the oligomerization domain to the second dimer in a tetramer in such a way as to increase the binding of the second dimer. Another possibility was simply that because two dimers are physically linked in a tetramer, the avidity of both dimers binding to DNA was greater than that of one dimer binding. The third possibility was that when bound side-by-side on CON, the two dimers interacted cooperatively, perhaps at the DNA binding domains' dimer-dimer interface.

The first of these possibilities would be consistent with a conformational shift that has been proposed to propagate through the oligomerization domain (Halazonetis and Kandil, 1993). Indeed, the half-life of tetramers on one half-site was greater than the half-life of dimers on one half-site (Figures 4 and 10). However, further experiments are required to determine whether conformational change was actually responsible.

One trivial, but likely, explanation for the slightly longer half-life of tetramers compared to dimers on a half-site is that the dimer of the tetramer that was not bound to a half-site was binding 'loosely' to an adjacent non-consensus half-site. In support of this conjecture is the observation that the half-life of tetramers on M34 was greater than the half-life of dimers bound to a single half-site (Results section 2). Indeed, this possibility makes accurate determination of the half-life of tetramers on M34 misleading, because the number might not reflect the affinity of one dimer of a tetramer for one half-site.

The second possibility for dimers binding better to CON than to M34 was increased avidity, which is a concept first applied to antibodies. Avidity simply means that if two domains of an oligomer are independently binding to and dissociating from two different binding sites in the same molecular substrate, the affinity of the complex is increased because the substrate (DNA) is only released if both binding domains (dimers) simultaneously release the substrate (DNA). The concept of avidity does not imply that the binding of one domain affects the affinity of the second domain for substrate. Strong support for this mechanism is offered by the finding that when one tetramer bound to two separated half-sites on one molecule of DNA (S10), binding was stabilized relative to the binding to a single half-site (Table 1).

The binding to spatially separate half-sites in S10 likely required that the two half-sites be positioned on the same face of the DNA helix (Wang et al., 1995). Each dimer in a tetramer could therefore occupy the half-sites in S10, but most likely there was no inter-dimer interaction via the DNA binding domains, although the DNA could be bent (Balagurumoorthy et al., 1995). If there was interaction between dimers outside of the oligomerization domain even on S10, such an interaction should also be able to occur with pairs of A344 dimers on S10. However, no stabilization was seen for pairs of A344 dimers on S10 (Figure 11). The dimers of a tetramer could still interact via the oligomerization domain, resulting in increased avidity on S10 because each dimer in a tetramer is in an independent equilibrium (discussed below). Despite this increased avidity, the half-life of p53-CON (25 minutes, Table 1) was still greater than p53-S10 (8

minutes), pointing to additional factors to stabilize p53 on a consensus sequence.

The third possibility was that dimer-dimer interaction contributed to tetramers binding better to a consensus sequence than to one half-site. This possibility is strongly supported by the finding that pairs of A344 dimers bound to CON with a much greater half-life (15 minutes; Table 1) than did single A344 dimers (< 1 second; Table 1). It is possible that the dimer-dimer stabilization of DNA binding was mediated by the interaction that occurs between monomers in the crystal structure, namely Ser96, Ser99, and Thr140 of one subunit interacting with Thr140, Glu198, Gly199, and Glu224 of another subunit (Cho et al., 1994). This interaction has been interpreted as the primary intra-dimer, inter-monomer interaction in the model of tetramers binding DNA as one dimer binding to quarter-sites one and three, the other dimer binding sites two and four (Cho et al., 1994; Lee et al., 1994; Jeffrey et al., 1995; Arrowsmith and Morin, 1996; Pennisi, 1996). The same interaction can be reinterpreted as possibly stabilizing a DNA-bound tetramer at the dimer-dimer interface.

It is satisfying that the contributions of avidity and dimer-dimer cooperativity to the p53-consensus DNA half-life, 8 and 15 minutes respectively, account for virtually all of the 25 minute half-life of p53-DNA within experimental error (Table 1). This suggests that no other important mechanisms contribute to p53-DNA stabilization. That being said, some mechanistic details could be subsumed by the dimer-dimer cooperativity. For example, after binding to DNA one dimer could undergo a conformational change that

might increase the affinity constant of the second unbound dimer. Such an effect would likely also occur in A344 dimers that bound side-by-side, but there is no evidence to support such an effect at physiological salt concentration (see Halazonetis et al., 1993, for conformational effects of DNA binding in low salt).

1.3. Tetramerization confers fidelity and affinity

Consideration of why p53 forms a tetramer before binding DNA is very illuminating in understanding p53 structure/function. It is particularly noteworthy that the role of p53 tetramerization is revealed by careful consideration of the relative binding affinities and fidelities of tetramers and dimers. Most DNA binding proteins are dimers, and dimers of p53 bind DNA well *in vitro*, so one might pose the previously unanswered teleological question "Why is p53 a tetramer instead of a dimer?"

It was very important that in the presence of excess NB or quarter-sites, tetramers but not A344 dimers bound CON to the same extent as when no competitor sequences were present. Moreover, like an A344 dimer, one dimer of a tetramer bound to one half-site with lower fidelity. Concurrent interaction of both dimers of a tetramer with distal half-sites (in S10) preserved the fidelity of binding in an environment of excess NB, but not quarter-sites. This effect was stronger than the fidelity of pairs of A344 dimers binding, so avidity conferred fidelity to DNA binding.

Nonetheless, it is likely that the increased half-life of binding CON that was conferred by dimer-dimer interaction also accounted for some of the high fidelity of binding CON in the presence of excess quarter-sites. Although pairs of A344 dimers bound CON with an increased half-life, this dimer-dimer interaction was not sufficient to confer high fidelity binding in the presence of excess NB. Most likely, this reflected the fact that A344 dimers could bind weakly to NB, compounded by the fact that one bound dimer introduced a barrier to a second dimer binding adjacently. Because of the short half-life of one dimer bound to DNA, another dimer that could have interacted with DNA to form a pair of stable dimers would instead bind weakly to many different non-consensus sequences. Thus, the fidelity of tetramer binding required both a cooperative dimer-dimer interaction that could occur independently of tetramerization, and an increased avidity of binding that resulted from tetramerization.

Based on the relative capacity of p53 tetramers to bind various DNA sequences, the journey that an average tetramer might take when it encounters a large excess of DNA (e.g. in the nucleus) can be envisioned (Figure 31B). Weak affinity of the dimers within a tetramer for non-specific DNA would position p53 on DNA, possibly mediated by the non-specific DNA binding activity resident in the C-terminus (Pavletich et al., 1993; Wang et al., 1993). In a random sequence of DNA, single quarter-sites would frequently be encountered (1/64 five base pair sequences is a quarter-site), but relatively stable binding would only occur if a half-site was encountered. Tetramers bound to a half-site still have a short half-life, so they would be able to rapidly sample many more sequences

of DNA. Finally, when a rare consensus site was encountered, a p53 tetramer could bind with a longer half-life of 25 minutes. DNA-bound p53-directed tumor suppressor activities would then presumably ensue.

Pairs of free dimers were able to bind DNA with a relatively long half-life of 15 minutes compared to the tetramer-DNA half-life of 25 minutes, so dimers appear at first glance to bind DNA 60% as well as tetramers. Considering that p53 protein levels are stabilized to varying degrees after different types of DNA damage (Richard Woo, personal communication), A344 dimers might reasonably be expected to function similarly to tetramers in a physiological setting.

If this were the case, p53 DNA binding would be similar to that of activated Stat1, where two free dimers bind to proximal sites on DNA, resulting in a greatly increased half-life of the bound dimers (Vinkemeier et al., 1996). However, in the nucleus there would be competition between weak binding to plentiful non-specific DNA and stronger binding to infrequent half-sites or rare consensus sites. Because free dimers were easily 'distracted' by non-specific DNA, the probability of a second dimer binding independently and simultaneously to the second half-site in a consensus sequence was low when an unbound dimer had the choice to bind weakly to other non-specific DNA.

Therefore, although free dimers can bind cooperatively side-by-side *in vitro*, this cooperativity is a reflection of the dissociation constant. Conditions *in vivo* would make

the formation of such cooperatively bound dimers unlikely, because the association of a second dimer for the second half-site of a partially occupied consensus site was preferentially competed by excess free half-sites (a reflection of the association constant). If p53 was dimeric, the cell could theoretically overcome this obstacle by producing very high concentrations of dimers. However, tetramerization also overcomes this obstacle by increasing the probability that once one dimer of the tetramer binds DNA, the other dimer will encounter the adjacent half-site in a consensus sequence rather than other non-specific DNA.

Thus, pairs of A344 dimers only formed *in vitro* when the ratio of dimers to DNA consensus sequences was very high. In essence, this was an *in vitro* artifact because *in vivo* the dimers would be expected to encounter a 4096-fold excess of non-specific (random) DNA over single half-sites, or a $1.7 \times 10^7$-fold excess of non-specific DNA over consensus sites. Even when non-specific DNA (NB) was added at a 1000-fold excess, A344 dimers were no longer able to bind to consensus DNA with high fidelity (i.e. A344 dimers were competed off). Moreover, the ratio of half-sites to consensus sites would be 4096 (in a genome of random sequence), and even moderate excess (10 to 100-fold) of half-sites competed for A344 binding *in vitro*.

Consequently, pairs of dimers might bind to DNA with high affinity *in vivo* except for pairs of dimers would form only very rarely, because of the low probability of two dimers binding adjacently. In fact, *in vitro*, single A344 dimers preferred to bind to

free half-sites rather than to a half-site beside a half-site that was already occupied by another A344 dimer (Figure 14). This is a reflection of the affinity constant, as opposed to the dissociation constant, and could be explained if there was an energy barrier to the formation of DNA-bound pairs of dimers. Mechanistically it might be envisioned that one A344 dimer would partially block the access to an adjacent half-site of a consensus sequence.

Therefore, one function of the tetramerization domain may be to position the dimers of a tetramer such that once one dimer bound to a half-site, the probability of the second dimer binding to an adjacent half-site in a consensus sequence would be considerably elevated. One result was high affinity binding, another was high fidelity of binding. In other words, the tetramerization domain acts as a catalyst to overcome the energy barrier of the energetically favourable second dimer binding adjacently to another dimer on DNA.

## 2. Relationship of the Wild Type and Mutant Conformations

### 2.1. DNA binding requires the wild type conformation

Although not all of the *in vitro* translated p53 was PAb246$^-$, all translated p53 was required to be in the wild type, PAb246$^+$ conformation in order to bind DNA sequence-specifically. While it has been found previously that the wild type PAb246$^-$ conformation is tightly correlated with DNA binding (Hainaut et al., 1995; Hainaut &

Milner, 1993), these results directly and unambiguously demonstrate that the PAb246$^-$ conformation is prerequisite for DNA binding. The ability of the cell to regulate the wild type conformation of p53 via a redox mechanism (Hainaut & Milner, 1993; Hainaut et al., 1995; Jayaraman et al., 1997) would thereby regulate the ability of p53 to bind DNA sequence-specifically.

Once p53 has bound to DNA, it has been proposed that p53 must undergo a conformational change if all four subunits of tetrameric p53 are bound to a symmetric DNA sequence (Waterman et al., 1995). Although it has been claimed that such a change is detectable at the PAb246 epitope, such a change was not detected using PAb246, since all DNA binding-competent p53 was PAb246$^-$ and remained so after binding to DNA.

PAb246 altered the migration of the entire p53-DNA complex, and this was interpreted here as being indicative that all DNA-bound p53 is conformationally wild type. While bound to DNA, p53 was not only PAb246$^-$ but was able to bind both PAb246 and DNA simultaneously. All of the p53 that was bound to DNA remained PAb246$^-$ after binding to DNA, but the consequence of PAb246 binding to the p53-DNA complex was that some of the complex was supershifted and some was dissociated.

The conclusions regarding the PAb246 conformation contradict other conclusions that p53 is not conformationally wild type after binding to DNA (Halazonetis et al., 1993) and that PAb246 blocks DNA binding (Wolkowicz et al., 1995). When PAb246 was

added to the PAb421 activated p53-DNA complex, the p53-DNA complex was partly supershifted and partly dissociated (Fig. 3a in Halazonetis et al., 1993). It could therefore be concluded that all of their DNA-bound p53 likewise remained PAb246$^-$, and the result of PAb246 binding to this complex was partly a supershift and partly dissociation of the p53-DNA complex. In support of the idea that all DNA-bound murine p53 is PAb246$^-$, another group also found that DNA-bound p53 retains the wild type PAb246$^-$ conformation, although their focus was mainly on the similar PAb1620 (Hall & Milner, 1995).

If two different populations of conformationally wild type p53 bind to DNA, and their DNA binding is regulated differently by PAb246, it is possible that the two DNA-bound populations are functionally different. Of course, there is no PAb246 in the cell, and therefore the regulation of p53 DNA binding activity via PAb246 binding to the PAb246 epitope would not occur. However, another cellular protein could potentially bind to the exposed region of the PAb246 epitope, and in the absence of other activities of the putative cellular PAb246 counterpart the two different populations of DNA-bound p53 might be regulated as they are with PAb246 (although the putative cellular PAb246 counterpart might have additional activities).

The consequence of a PAb246 cellular counterpart protein binding to p53 would be predicted to be dissociation of p53 from DNA. It is interesting in this respect that such a protein might be an oncogene, because overexpression or constitutive activation would

inactivate p53 DNA binding. Since PAb240 could bind to DNA-bound p53 and protect it from PAb246-induced dissociation, a PAb240 cellular counterpart might paradoxically be expected to activate p53 DNA binding in some circumstances. Candidates for such a protein include the mutant-specific p38 and p42 (Chen et al., 1994).

## 2.2. Conformationally mutant dimers of p53 can be tethered to DNA via the other dimer in a tetramer

It has long been held that the PAb246 epitope is displayed only on functional, wild type p53, and the PAb240 epitope is specific for mutant or denatured p53 forms. Using RRL that produced mainly $PAb246^-/PAb240^-$ p53, the total p53 population was found to harbour a minor subpopulation that displayed both the wild type and mutant epitopes (dual positive, i.e., $PAb246^-/PAb240^+$). The two epitopes were displayed both before and after DNA binding, and both epitopes could be occupied by the antibodies while p53 was bound to DNA. Furthermore, although the size of this subpopulation relative to total p53 generated was quite small, the observation that it accounted for up to 50% of all DNA-binding p53 suggests that at least some of the known functions of p53 may be attributable to this particular species.

Dual positive p53 has indeed been previously detected using an ELISA assay, but was thought to be functionally inactive due to its inability to bind to the SV40 large T antigen (Gannon et al., 1990). Thus, the preferential reactivity of PAb240 with p53 that

was inactive for T antigen binding, coupled with the finding that structural mutants of p53 are PAb240$^+$/PAb246$^-$, has led to the conclusion that PAb240 reactivity is indicative of a nonfunctional 'mutant' conformation (reviewed by Zambetti and Levine, 1993). More recently, based on X-ray crystallography of the central core domain bound to DNA, Cho et al (1994) deduced that the "mutant conformation" most likely represents denatured states of p53, rather than a well-defined, alternative conformational state of the protein.

Their reasoning was as follows. First, the peptide epitope for PAb240 [residues 212-217 (Stephan and Lane, 1992)] is located on the S7 β strand of wild-type p53, part of which is packed within the hydrophobic core of the β sandwich, thereby rendering the PAb240 epitope mostly inaccessible to the antibody. Second, the majority of mutations that result in the manifestation of the PAb240$^+$ phenotype most likely cause global denaturation since they mainly occur at the L3 loop and the loop-sheet-helix motif that are quite distant from the PAb240 epitope. Third, the PAb240 antibody was raised against denatured p53 (Gannon et al., 1990) and therefore recognizes all p53 forms (including wild-type p53) that are denatured. Collectively, these considerations have led Cho et al. to suggest, in essence, that PAb240$^+$ p53 is an inactive, nonfunctional, and therefore relatively passive entity.

An alternative view challenges the above notion and portrays the PAb240$^+$ phenotype as being an altered conformational, rather than a denatured, state of p53 (Milner, 1995). It was noted that most PAb240$^+$ mutants exhibit the same

oligomerization profile as the wild-type protein (Milner et al., 1991; Friedman et al., 1993), and that some PAb240$^-$ mutants share functional similarity with wild-type p53 in terms of specific DNA binding and transcriptional activity (Chen et al., 1993). Furthermore, exposure of the PAb240 epitope has been found to be reversible (Milner and Medcalf, 1990., Hainaut and Milner, 1992). The finding that hsp90 is required for p53 to assume the mutant conformation strongly supports the conformation hypothesis (Blagosklonny et al., 1996). Taken together, these observations suggest that p53 is conformationally flexible and that the PAb240$^-$ conformation is not necessarily a static one with no functional significance. The present finding that a sizable population of DNA-binding p53 generated *in vitro* displays the PAb240$^+$ conformation is in congruence with this view.

According to the conformation hypothesis (Milner, 1995), the PAb246$^-$ and PAb240$^+$ conformations represent alternative (i.e., mutually exclusive) p53 forms. Thus PAb246$^+$ p53 is proposed to be always PAb240$^-$ and vice versa, and structural changes that cause loss of PAb246 activity are presumed to be invariably accompanied by exposure of the normally cryptic PAb240 epitope. It is also believed that some of these PAb240$^+$ p53 mutants can promote cell proliferation. While the conformation hypothesis is sound in principle, it is proposed here that a further level of control could be exercised by the cell by toggling the PAb240 epitope independently of the PAb246 conformation and DNA binding, as evidenced by the existence of 'dual positive' p53.

In view of the present observation that dual positive p53 can interact with both PAb246 and PAb240 while it is bound to DNA, it may be speculated that there could be cellular counterparts to these antibodies that interact with p53 in a similar manner. It is interesting to note in this regard that two cellular proteins, 53BP1 and 53BP2, have been found that bind to wild type, but not mutant p53 (Iwabuchi et al., 1994). However, 53BP2 probably does not interact with the PAb246 epitope because it binds to the DNA binding surface of p53 (Gorina and Pavletich, 1995). Nonetheless, the effect of these two p53 binding proteins on p53 DNA binding is similar to that observed with PAb246 and PAb1620 (Halazonetis et al., 1993; Wolkowicz et al., 1995; Hall and Milner, 1995). Additionally, two other cellular proteins have been found to interact with mutant, but not wild type p53, a property that is reminiscent of PAb240 (Chen et al., 1994). These observations are again in favor of the argument that p53 with the mutant conformation may also play a functional role by regulating cell growth and/or apoptosis via interaction with cellular factors.

In light of *in vitro*-translated p53 binding DNA as a tetramer (dimer of dimers), it was of interest to determine whether the PAb246 and the PAb240 epitopes were expressed on the same or different dimers of the tetramer. Two DNA-binding dimeric p53 constructs were used, one which had the C-terminal 58 amino acids of wild-type p53 replaced with a leucine zipper, the other which had a mutation at residue 344 from alanine to leucine (Halazonetis and Kandil, 1993; Waterman et al., 1995). The murine p53LZ332 was PAb246$^+$/PAb240$^-$, and both dimers were PAb240$^-$ even when two dimers

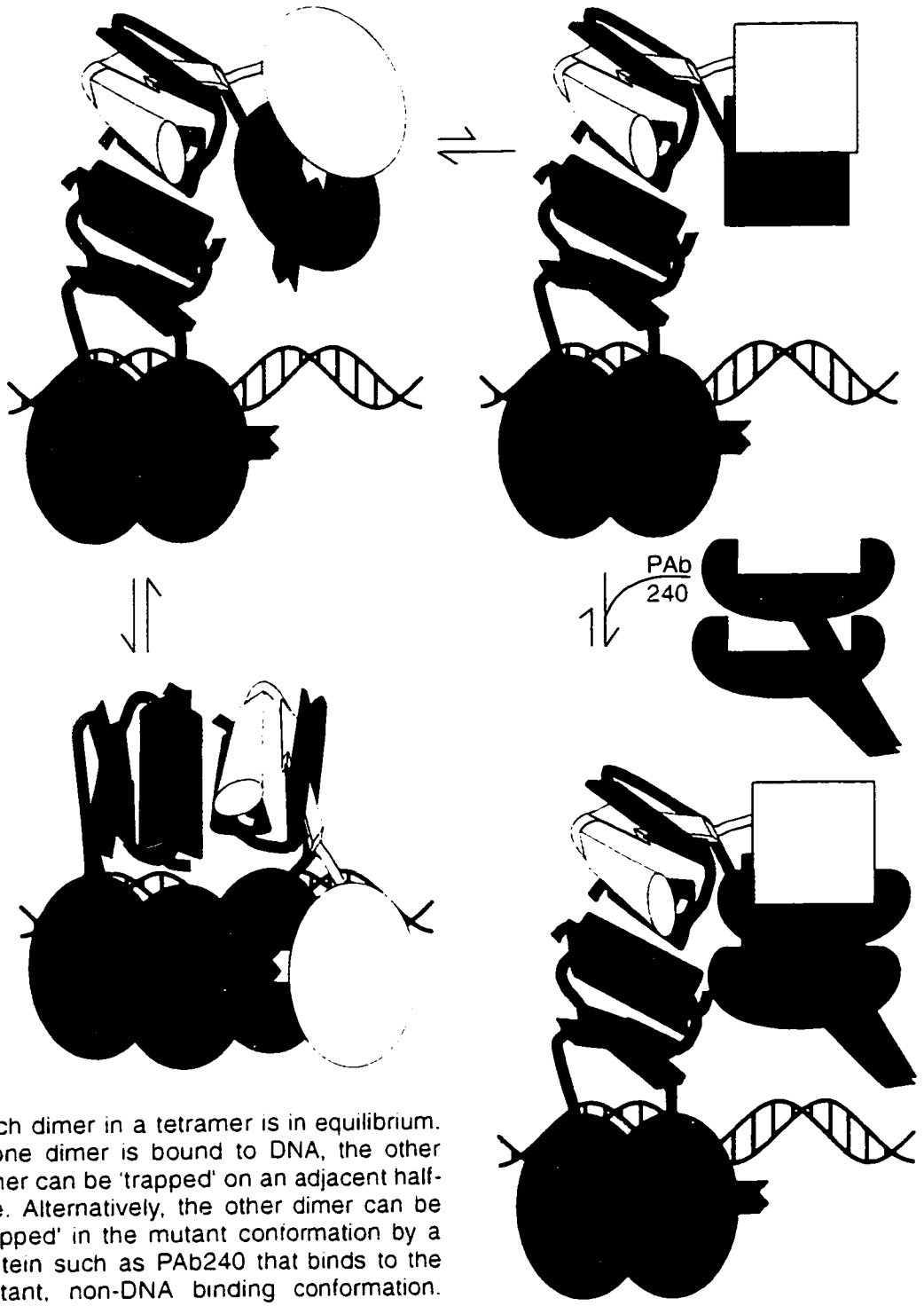
were bound adjacently on a consensus sequence. This demonstrated that tetramerization was necessary for the PAb246$^-$/PAb240$^-$ phenotype.

Using the altered consensus sequence MUT it was demonstrated that one starting population of p53 tetramers could bind DNA in either of two conformations. If both dimers of the tetramer contacted DNA, they would both be conformationally wild type. Some p53, however, could bind DNA via one dimer while the other dimer of the tetramer was bound to PAb240 instead of binding to DNA. It follows that the dimers of a tetramer in the initial population of p53 were in equilibrium, because they could be conformationally either mutant or wild type.

In line with this reasoning, PAb240 depleted nearly all of the DNA binding-competent p53, but when bound to DNA only about 25-50% of the p53 was PAb240$^-$. Therefore, in the absence of DNA there was a greater chance that a dimer in a tetramer could react with PAb240. One logical explanation is that in the presence of DNA, when one dimer in a tetramer was bound to a half-site, the second dimer was still in conformational equilibrium. While the second dimer was in the wild type conformation it could bind DNA, or while in the mutant conformation the second dimer could bind PAb240 (schematically presented in Figure 32).

The binding of antibody to antigen is usually very tight, so once PAb240 bound to the unbound dimer of DNA-bound dual positive p53, the conformation of the second

**Figure 32**




Each dimer in a tetramer is in equilibrium. If one dimer is bound to DNA, the other dimer can be 'trapped' on an adjacent half-site. Alternatively, the other dimer can be 'trapped' in the mutant conformation by a protein such as PAb240 that binds to the mutant, non-DNA binding conformation.

dimer would likely be 'locked' in the mutant conformation (Figure 32). This dimer of p53 is presented as not binding to DNA for clarity, because sequence-specific DNA binding would be precluded. However, mutant p53 still possesses the ability to bind DNA non-specifically, including interacting via the extreme C-terminus (Wang et al., 1993; Pavletich et al., 1993; Bargonetti et al., 1993). Support for the possibility that an unbound dimer of a DNA-bound tetramer can interact nonspecifically with DNA is found in the observation that tetramers bound to a single half-site in M34 have a longer half-life than single A344 dimers bound to a half-site (Results section 2).

Alternatively, an unbound dimer of a DNA-bound tetramer could be in the wild type conformation and bind to an adjacent half-site in a consensus site (Figure 32). Pairs of A344 dimers could bind to a consensus site with a half-life of 15 minutes (Table 1), so when both dimers of a tetramer bound to a consensus site, the half-life attributed to dimer-dimer cooperativity would also be 15 minutes. Tetramers bound with a half-life of 25 minutes, so 15 of those 25 minutes (60%) was attributable to dimer-dimer cooperativity.

It is interesting to consider what is happening for the 8 minutes (out of 25 minutes, Table 1) that the average DNA-bound tetramer remained bound due to avidity. The dimers in a tetramer were in equilibrium between bound/unbound conformations, and avidity applies to two independently binding subunits that bind the same molecule of substrate. Thus, for 8 of 25 minutes (32%), one dimer was bound to DNA while the other

dimer was unbound. The unbound dimer was not necessarily in the mutant conformation, but it was in conformational equilibrium while it remained unbound. Regardless of whether the PAb240$^{-}$ conformation actually contributes to p53 activity, this data shows that DNA-bound p53 tetramers are not bound to DNA in a static manner. Rather, DNA-bound tetramers are in a dynamic, conformational equilibrium (Figure 32).

One consequence of the binding mechanism is that the concept of p53 binding to DNA in a tight (T state) or relaxed (R state) conformation needs clarification (Halazonetis and Kandil, 1993; Waterman et al., 1995). Each dimer has been depicted as following the concerted model of Monod, Wyman, and Changeux (ibid; Monod et al., 1965). Because each dimer can be only wild type or only mutant, the DNA binding by subunits of a dimer can indeed be modeled as concerted. However, because the two dimers of a tetramer are in conformational equilibrium even in DNA-bound p53, the binding of p53 tetramers to DNA must be modeled by the Koshland sequential model of substrate binding (Koshland et al, 1966).

2.3. Re-examination of the dominant negativity of mutant p53

The finding that only p53 that contains the C-terminal tetramerization domain could be dual positive was consistent with the idea that the dual positive phenotype may occur as a result of interaction between two dimers that form a tetramer. In such a case, one PAb246$^{+}$/PAb240$^{-}$ dimer may be complexed with a PAb246$^{-}$/PAb240$^{-}$ dimer,

implying that the PAb246$^-$/PAb240$^-$ conformation may not be dominant negative in and of itself. This conclusion is supported by the observation that dual positive PAb246$^-$/PAb240$^+$ p53 bound DNA even when PAb246$^-$/PAb240$^-$ p53 was present in vast excess.

One would expect that in a tumor which has progressed via p53 mutation, if the first p53 mutation was a deletion, then wild type p53 would still be produced and the wild type allele would be selected for future mutation. Alternatively, if the first p53 mutation were a point mutation, then both wild type and mutant p53 would be expressed equally in the cell (although the mutant protein has a longer half-life). In this latter case, mutation or deletion of the second p53 allele would be selected for only if wild type p53 was dominant, but not if mutants were dominant negative.

The case of wild type p53 being dominant, but not recessive, to mutant p53, is supported by two *in vivo* observations. One mutated/one deleted p53 allele is common in tumors (Nigro et al., 1989; Hollstein et al., 1994), and deletion of the wild type allele occurs in tumors of Li-Fraumeni patients with a germline p53 mutation (Malkin et al., 1990). Moreover, mutation of one allele only is detected infrequently in carcinomas (Baker et al, 1990). Therefore, mutant p53 is recessive to wild-type p53, and inactivation of both p53 alleles is strongly selected for during tumorigenesis.

Experimental evidence supporting the recessive negative nature of p53 mutations

is ample. When wt p53 is cotransfected with adenovirus E1A plus activated ras or mutant p53 plus activated ras, the wild type p53 dominantly inhibits focus formation by up to 80% (Eliyahu et al., 1989; Finlay et al., 1989). More exhaustive analysis in a model tissue culture system leaves little doubt as to the recessive mutant nature of p53 mutations.

Saos-2 cells, human osteosarcoma cells which express no endogenous p53 and are highly tumorigenic in nude mice, were transfected with wild type, mutant, or a combination of both p53 constructs (Chen et al., 1991). Mutant p53 enhanced focus formation, but transfection of wild type p53 abolished both endogenous focus formation/tumorigenicity of Saos-2 cells as well as that augmented by mutant p53. Another study found that various hotspot mutants failed to be dominant negative over endogenous wild type p53 (Williams et al., 1995). In addition, when one mutant and one wild type allele were coexpressed at 'normal' levels *in vivo*, p53-dependent transcription and inhibition of colony growth were at near wild type levels (Frebourg et al., 1994).

These results raise the question of what properties in addition to the mutant $PAb246^{-}/PAb240^{-}$ conformation allow mutant p53 to be dominant negative. The early report showing that mutant p53 is dominant negative *in vitro* has been very influential in the p53 field (Milner and Medcalf, 1991). The dominant negative nature of mutant p53 was assayed purely on the basis of $Val_{135}$ reactivity with PAb240 and PAb246 (ibid). Because dual positive p53 can exist in a population of p53 that is overall

PAb240$^{+}$/PAb246$^{-}$, and $Val_{135}$ can be induced by wild type human p53 to bind DNA in a wild type conformation, the significance of the immunoprecipitation analysis of Milner and Medcalf, 1991, must be reevaluated. Milner and Medcalf (1991) also found that the dominant negative property of mutant p53 was dependent on co-translation. Because dimers were shown here to define the level of the mutant/wild type conformation, and mutant and wild type dimers can coexist in a tetramer, it seems possible, if not likely, that the dominant negative effect applies only to tetramers that contain heterodimers with at least one mutant subunit per dimer.

That being so, it may be further deduced that dimers form co-translationally, but not post-translationally. Because many ribosomes are translating nascent protein from any given mRNA strand, it is apparent that the local concentration of nascent proteins from the same genetic sequence would be high relative to heterologous nascent strands in solution. Therefore, if mutant p53 cotranslationally inactivates wild type p53 by forming heterodimers, the concentration of mutant mRNA would have to be very high. This may explain why mutant p53 can be dominant negative *in vitro*, where high concentrations of RNA are typically translated, and in transfection studies, where proteins are typically massively overexpressed. No mechanistic explanation has been previously published explaining the dominant negative *in vitro* artifact.

## 2.4. Significance of understanding p53 DNA binding to designing therapeutic cancer strategies

It is interesting to consider the potential role of cancer therapy that is centered on p53. Firstly, in cancer cells that harbor wild type p53, there is a good response to chemotherapy. This is presumably due to the activation of p53 by chemotherapeutic agents, which results in cell cycle arrest in normal cycling cells, but can result in apoptosis of tumor cells (reviewed by Ko and Prives, 1996; Levine, 1997).

Restoring wild type activity to mutant p53 depends on whether the mutant is a contact or a conformational mutant. The former lacks one of the amino acid side chain-DNA interactions (Cho et al., 1994), so would require the design of a drug that compensated for this lack of DNA binding without interfering with p53 activity. The latter would require the design of a drug that converted the tertiary structure of the endogenous mutant p53 from the mutant into a wild type conformation, a task previously described as "Herculean" (Friend, 1994). While these approaches at first might seem daunting, understanding the precise manner in which p53 binds DNA provides the basis for a molecular approach to designing small molecules that might restore binding to mutant p53 proteins. Such an understanding includes the p53 core domain-DNA co-crystal, tetramer binding to a consensus site as a dimer-of-dimers with each dimer contacting one half-site, and mutant p53 dimers being in conformational equilibrium with the DNA binding wild type conformation. The contents of this dissertation strengthen the understanding of p53 DNA binding by demonstrating the tertiary relationships of dimers

within a tetramer.

The exercise of designing drugs to restore wild type p53 activity to mutant p53 proteins might seem futile if the mutant conformation of p53 was the result of an alternate folding pathway that precluded the wild type conformation, as suggested by Cho et al. (1994). However, this dissertation presents a clear indication that the mutant and wild type conformations are in fact in equilibrium. Whereas p53 that is genotypically wild type is shifted towards the wild type phenotype (defined by antibody reactivity and DNA binding), mutant p53 is strongly shifted towards the mutant conformation. It is particularly noteworthy that even mutant p53 can be in equilibrium with the wild type phenotype, and this conformation can be trapped and thereby induced to bind to the p53 DNA consensus sequence. Indeed, two hotspot mutants were found to bind consensus DNA, perhaps indicating that some mutants are in conformational equilibrium (Zhang et al., 1993).

Importantly, the ability of PAb246 to stabilize $Val_{135}$ DNA binding could be a general consequence of 'trapping' mutant p53 in the wild type, DNA binding conformation. This would be true if conformational mutants are generally in equilibrium with the wild type conformation. However, because $Val_{135}$ is a temperature sensitive mutant, it might be expected to be more conformationally flexible than other mutants. Nonetheless, many human tumor-derived p53 mutants are also temperature sensitive (Rolley et al., 1995).

Interestingly, of ten structural mutants that are PAb240$^{-}$/PAb1620$^{-}$ at 37°C but PAb240$^{+}$/PAb1620$^{-}$ at 30°C, nine gain DNA binding activity at 30°C (ibid). PAb1620 recognizes an epitope which is conformationally related to the PAb246 epitope, and the effects of PAb1620 and PAb246 on p53 DNA binding are also similar (Milner *et al.*, 1987; Halazonetis *et al.*, 1993; Wolkowicz *et al.*, 1995; Hall & Milner, 1995). Based on the 'dual positive' data, these activated mutants might also bind DNA in the 'dual positive' conformation, which would be PAb240$^{+}$/PAb1620$^{-}$ for human p53 since PAb246 is murine specific.

It is interesting in this respect that wild type p53 was inactivated by PAb246, as judged by the marked decrease in DNA-bound p53 over time. Therefore, the low level of mutant p53 that was induced to bind DNA by PAb246 could result from either of two mechanisms. One possibility is that only a small percentage of the mutant p53 was able to adopt the wild type conformation for long enough to be 'trapped' by PAb246. Another possibility is that all of the mutant p53 could adopt the wild type conformation and be bound by PAb246, but once PAb246 reacted with the mutant p53 DNA binding was dissociated as with wild type p53. This latter possibility would be exciting in that PAb240 was able to protect wild type p53 from such dissociation, so might function similarly with mutant, PAb246-activated p53.

### 3. Future Directions

Many avenues of research could be pursued based on the findings of this

dissertation. All of the results were obtained *in vitro*, so an *in vivo* confirmation of the principal conclusions would be valuable. Regarding the mechanisms contributing to tetramer DNA binding, p53 responsiveness could be tested of plasmid constructs containing a reporter gene downstream of CON, M34, or S10. Obviously, the nucleus contains a vast excess of non-specific DNA, so p53 transcriptional activity would require that p53 tetramers bind with high enough fidelity to activate transcription. Endogenous p53 could be activated by DNA damage and the difference between transactivation of M34 and CON would represent the *in vivo* contributions of tetramer cooperativity and avidity. The contribution of dimer-dimer cooperativity to p53-activated transcription could be assayed by comparing the difference between transactivation of S10 and CON. Similarly, the contribution of avidity to p53-activated transcription could be assessed by comparing the difference between transactivation of S10 and M34.

Another interesting experiment would be to identify the dimer-dimer cooperativity interaction. In the model of p53 tetramer DNA binding, the cooperativity was depicted as occurring at the DNA binding domains (Figures 31 and 32). This was based on the interactions identified in the crystal structure, where Ser96, Ser99, and Thr140 of one subunit interacted with Thr140, Glu198, Gly199, and Glu224 of another subunit (Cho et al., 1994).

Confirmation of the dimer-dimer interaction implied by Figure 31 could come from studies of p53 in which one, or combinations of multiple, of the above amino acids

were mutated by site-directed mutagenesis. The appropriate assay would require detection of mutated p53-DNA complexes as confirmation that the mutation did not structurally interfere with DNA binding. Following that, it can be predicted that disruption of the putative dimer-dimer interaction would decrease the half-life of p53-CON from 25 to 8 minutes (Table 1). Of course, a negative result would indicate that the site-directed mutation did not interfere with dimer-dimer cooperativity because either the mutation still allowed interaction at the dimer-dimer interface, or another region of p53 could confer dimer-dimer stabilization.

A conceptually broad area that could be pursued would be to assay the multitude of p53-binding proteins (reviewed in Ko and Prives, 1996) for the ability to bind to DNA-bound p53. The p53 domains to which many proteins bind are located outside the DNA binding domain, so most proteins should be able to bind to DNA-bound p53. However, even proteins that do bind to the DNA binding domain, such as 53BP2 (Gorina and Pavletich, 1996), could interact with p53 that was bound to DNA via only one dimer in a tetramer. Detection of any such interactions would rely heavily on the relative concentrations of p53, DNA, and p53 binding protein, and the relevance to p53 activity *in vivo* would require extensive work.

It would also be interesting to determine whether the dual positive conformation contributes to wild type p53 activity *in vivo*. Clearly, there is the potential that the two mutant proteins p38 and p42 (Chen et al., 1994) could bind to DNA-bound wild type p53

*in vivo*. Unfortunately, those two proteins were only characterized by antibodies, which are not available (even from the authors), and no gene sequences were published (ibid). Given that the PAb240$^{-}$ conformation can represent a large portion of the total DNA-bound p53, it may be valuable to clone any mutant-specific p53 binding proteins. This could be most easily accomplished by using the yeast two-hybrid system, using a peptide containing the PAb240 linear epitope as bait. Any positives could be further confirmed by assaying for binding to a labeled peptide containing the PAb240 epitope. Of course, confirmation of a protein being a p53 binding protein would require co-immuno-precipitation to demonstrate a physical association between the two proteins.

Another general approach to probe whether the mutant conformation contributes to p53 activity *in vivo* would be to introduce PAb240 into cells and assay for any perturbations to p53 function. The antibody could be introduced either by electroporation or by microinjection, and functional assays would include p53-dependent responses such as radiation-induced apoptosis and cell cycle arrest. It is interesting in this respect that other antibodies have the same effect *in vivo* that would be predicted from *in vitro* studies. For example, PAb246 dissociates p53 from DNA *in vitro*, and inhibits p53 function *in vivo* (Hupp et al., 1995). PAb421 activates p53 DNA binding *in vitro*, as well as *in vivo* (ibid; Mercer et al., 1982; 1984; Shohat et al., 1987). Thus, PAb240 might bind to DNA-bound, genotypically wild type p53 *in vivo* and thereby regulate p53 function.

A short set of useful experiments would probe whether the PAb240$^-$ conformation was indicative of a general or a local conformational change in p53. For example, the conformational equilibrium detected in a DNA-bound tetramer could represent transitions between two completely altered conformations, or the local conformation about the PAb240 epitope could be in equilibrium. A series of mutant-specific antibodies has been developed, which react with epitopes spanning the central DNA-binding core domain of p53 (Legros et al., 1994; Vojtesek et al., 1995). These antibodies would therefore be ideal for determining the extent of conformational flux in wild type p53.

The generality of the recessive nature of $Val_{135}$ and the stabilization of $Val_{135}$ DNA binding by PAb246 should be explored using other tumor-derived mutants. It might be expected that constitutive mutants cannot assume the wild type conformation, but it would certainly be interesting to screen a panel of mutants under conditions that might allow heterotetramers to form with wild type p53. Perhaps, like $Val_{135}$, other mutants are not totally dominant negative when expressed at more physiologically relevant levels.

Constitutive mutants might not be dominant negative, but they would be unlikely to convert to the wild type conformation when complexed with wild type p53 (although that would be important if such an effect occurred). More likely, a wild type dimer of a tetramer might bind to DNA while tethering a constitutively mutant dimer via the tetramerization domain (i.e. constitutively 'dual positive' p53). If this were found *in vitro*, it would pave the way for experiments *in vivo* expressing 'normal' levels of wild

type and constitutively mutant p53. Cell extract could be assayed for 'dual positive' p53, and if found would be worthy of pursuit regarding p53 function *in vivo*.

Of particular relevance is what normally happens when a tumor cell sustains a mutation in one allele only. A possible function of dual positive p53 might be unveiled in such circumstances, where it would be advantageous for the cell to undergo apoptosis rather than allow the second allele to become mutated. Along with other considerations, the 'dual positive' conformation of p53 might be relevant not to transcriptional activation, but rather to p53-dependent, transcription-independent apoptosis.

It is significant that PAb246 is murine-specific, and is therefore not directly relevant to human p53. Nonetheless, PAb1620 is similar to PAb246 in that they are both wild type-specific, but PAb1620 supershifts murine but not human p53-DNA complexes (e.g. see Hall and Milner, 1995). Thus, the $Val_{135}$ experiment could be repeated using murine (and human) mutants and PAb1620. If the antibody could stabilize mutant murine p53 DNA binding, other smaller molecules could be designed or screened for similar binding and stabilization of mutant human p53 DNA binding. Of course, the property of PAb246 dissociating p53 from DNA would have to be circumvented if a wild type conformation-stabilizing drug was designed to bind similarly to the wild type conformation-specific antibodies. It would be interesting to test the wild-type binding peptides isolated by a phage display library, to find out whether peptides could stabilize conformational mutant human p53 proteins (Daniels and Lane, 1994).

Any or all of the above directions could be extremely rewarding not only in terms of unravelling details of the p53 DNA binding mechanism, but also in terms of understanding what molecular interactions (inter-dimer or intra-dimer) are necessary for p53 to bind DNA *in vivo*. Such an understanding could lead to rational design of small molecules to reconstitute the critical structural aspects of DNA binding-competent p53 that are lost in tumor-derived conformationally mutant p53 proteins.

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# IMAGE EVALUATION
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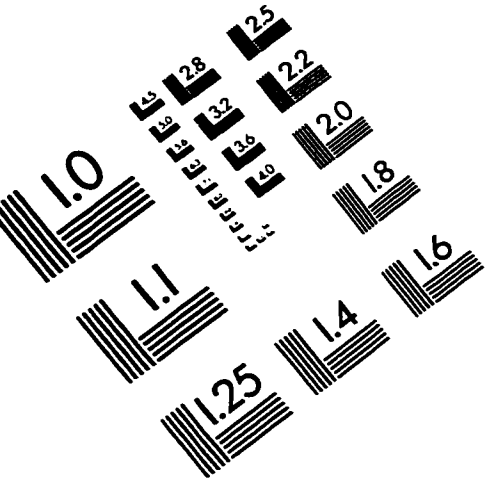


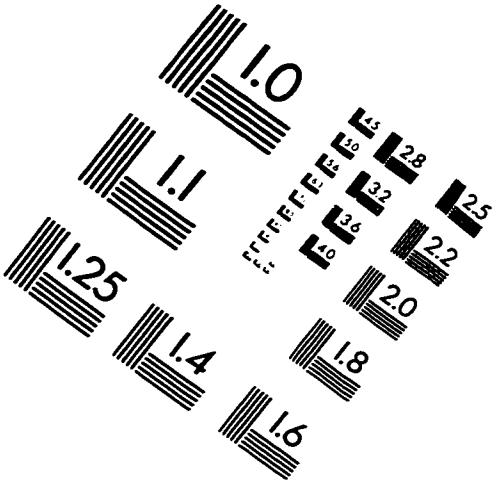


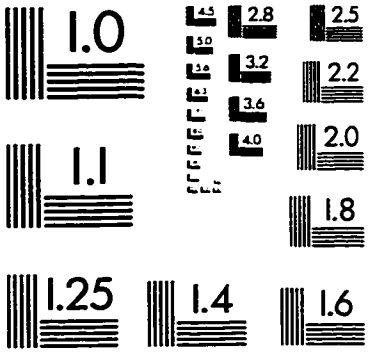


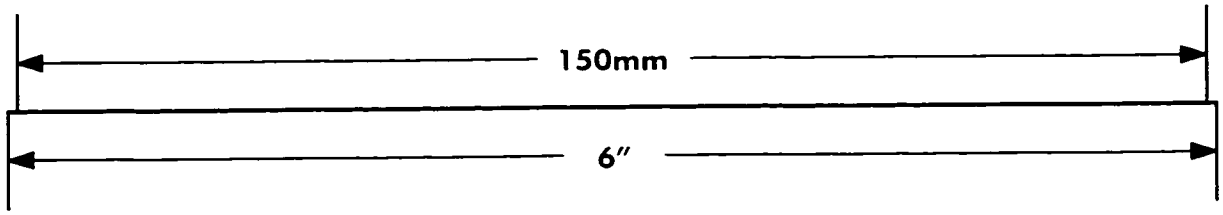


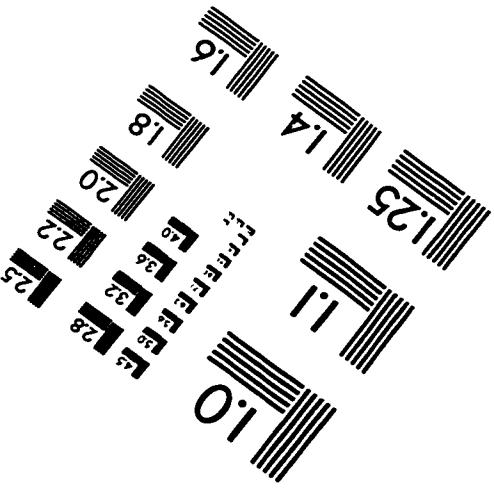


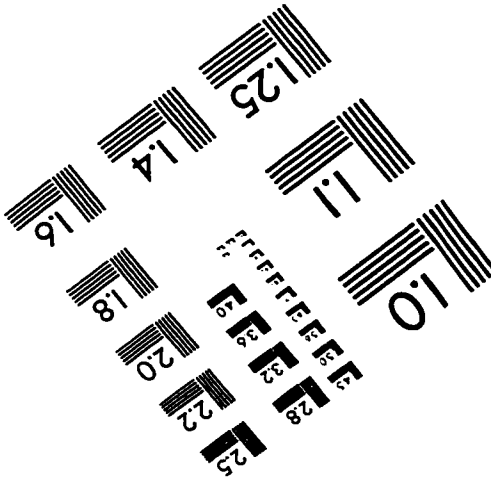


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