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Wild-Type but Not Mutant p53 Immunopurified Proteins Bind to Sequences Adjacent to the SV40 Origin of Replication

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Summary

The DNA from a wide variety of human tumors has sustained mutations within the conserved p53 coding regions. We have purified wild-type and tumor-derived mutant p53 proteins expressed from baculovirus vectors and examined their interactions with SV40 DNA. Using DNAase I footprinting assays, we observed that both human and murine wild-type p53 proteins bind specifically to sequences adjacent to the late border of the viral replication origin. By contrast, mutant p53 proteins failed to bind specifically to these sequences. SV40 T antigen prevented wild-type p53 from interacting with this region. These data show that normal but not oncogenic forms of p53 are capable of sequence-specific interactions with viral DNA. Furthermore, they provide insights into the mechanisms by which viral proteins might regulate the control of viral growth and cell division.

Introduction

Mutation of the p53 gene appears to be a major determinant in many forms of human cancer (reviewed in Hollstein et al., 1991). In a high proportion of tumors from cancer patients, a loss of one p53 locus is coupled with missense mutations in the remaining p53 allele (Baker et al., 1989; Hollstein et al., 1991). The normal, noncancerous tissues of such patients usually do not contain mutations within the p53 gene, although in a few cases (the Li–Fraumeni syndrome), germline mutations of the p53 gene predispose patients to cancer development (Malkin et al., 1990; Srivastava et al., 1990). Mutant forms of p53 are not limited to human tumors, as they often occur in mouse erythroleukemias induced by Friend virus (Mowat et al., 1985; Munroe et al., 1987).

Several properties of p53 have been described. p53, a phosphoprotein, is found frequently, but not always, in the nucleus (reviewed in Lane and Benchimol, 1990; Levine, 1990). The p53 genes, which have been sequenced from a variety of vertebrate species, all encode proteins with acidic proline-rich amino-terminal portions, hydrophobic internal regions, and basic regions at the carboxyl terminus. Although regions of considerable divergence exist among p53 genes from evolutionarily distant species, all p53 genes contain four highly conserved regions within the hydrophobic inner region (reviewed in Soussi et al., 1990). Indeed, mutations within patients' tumors usually occur within these highly conserved regions of the protein (see Nigro et al., 1989), suggesting that they are important for the structure and/or normal function of p53. Wild-type p53 proteins display short half-lives, turning over within minutes, while mutant oncogenic p53 proteins are sometimes far more stable, with half-lives on the order of several hours (Finlay et al., 1988; Ginsberg et al., 1991).

Mutant p53 proteins can immortalize primary cells in culture and can cooperate with oncogenes such as *ras* to transform such primary cells (reviewed in Lane and Benchimol, 1990; Levine, 1990). By contrast, not only does wild-type p53 fail to immortalize cells or cooperate in their transformation, but it can suppress the ability of either mutant p53 or even other oncogenes to alter the growth properties of cells (Finlay et al., 1989; Michalovitz et al., 1990). Moreover, introduction of wild-type p53 into cancer cells can cause them to stop growing, often through arrest at the G1/S boundary of the cell cycle (Baker et al., 1990; Chen et al., 1990; Diller et al., 1990; Eliyahu et al., 1989; Martinez et al., 1991; Mercer et al., 1990). Viruses that require actively cycling cells for efficient replication might benefit from inactivation of gene products that suppress cell cycle progression. Consistent with this possibility, the products of at least three DNA tumor viruses—SV40 (Lane and Crawford, 1979; Linzer and Levine, 1979), adenovirus (Sarnow et al., 1982), and oncogenic forms of human papillomavirus (Werness et al., 1990)—bind to p53 in infected and transformed cells.

Further insights into possible functions of p53 within cells were derived from experiments demonstrating that p53 possesses a strong transactivation domain (Fields and Jang, 1990; Raycroft et al., 1990; O'Rourke et al., 1990). While these experiments did not address directly the possibility that p53 regulates transcription, the potency of its transactivation domain and the fact that this transactivation was not apparent in a mutant oncogenic p53 was highly suggestive. p53 was also shown to bind nonspecifically to DNA (Steinmeyer and Deppert, 1988; Kern et al., 1991a), and, importantly, several mutant p53 genes derived from human tumors were highly defective in their interactions with DNA–cellulose columns (Kern et al., 1991a). Taken together, these observations suggest that p53 might be a sequence-specific DNA-binding protein and that at least one mode by which it regulates cell growth would be through binding to and alteration of the function of unique regions of DNA. This idea has recently been supported by the demonstration that wild-type forms of p53 can bind to specific human DNA sequences (Kern et al., 1991b).

We and others previously showed that wild-type murine (Gannon and Lane, 1987; Braithwaite et al., 1987; Sturzbecher et al., 1988; Wang et al., 1989) and human (Friedman et al., 1990) p53 proteins strongly block SV40 large T antigen replication functions in vivo and in vitro. During

the course of experiments designed to understand mechanistically the basis for this inhibition, we examined, by DNAase I footprinting analysis, the influence of p53 and of T antigen on each other's interactions with viral DNA. Unexpectedly, we observed that wild-type p53 proteins alone bind to sequences adjacent to the viral core replication origin.

Results

Wild-Type p53 Binds More Efficiently Than do Mutant p53 Proteins to SV40 DNA

Our studies were prompted by previous observations that murine and primate p53 proteins affect the interactions of SV40 T antigen with sequences at the SV40 replication origin (Wang et al., 1989; Sturtzbecher et al., 1988; Tack et al., 1989). To pursue these observations, recombinant baculoviruses were constructed expressing either murine, wild-type human, or mutant human p53 genes cloned from human carcinoma cells. The three mutant clones are representative of the mutational "hot spots" previously observed in human cancers (Nigro et al., 1989); each contains a single amino acid substitution at codon 143, 175, or 273. These viruses expressed abundant and comparable quantities of wild-type and mutant p53 proteins in infected insect cells. The murine and wild-type human p53 proteins described herein were capable of blocking the replication of SV40 *ori* DNA in vitro, as previously described (Wang et al., 1989; Friedman et al., 1990).

Figure 1 shows a silver-stained gel of the purified proteins used in these studies. Proteins were judged to be at least 80% pure as determined from silver-stained gels. T antigen or the complex of p53 with T antigen was purified by passing extracts over affinity columns containing the T antigen–specific monoclonal antibody PAb 419 (Harlow et al., 1981a). p53 proteins were purified similarly using an affinity matrix containing the p53-specific monoclonal antibody PAb 421 (Harlow et al., 1981a). Approximately equivalent quantities of either free or complexed human and murine p53 and T antigen proteins were obtained by these procedures.

Nitrocellulose filter binding assays were performed to compare the ability of wild-type and mutant human p53 proteins to bind to SV40 DNA (Figure 2). When the ability of purified p53 proteins to bind a ^{32}P end-labeled fragment spanning the SV40 regulatory region was tested, wild-type human p53 was markedly more effective at retaining the DNA fragment in this assay than were equivalent quantities of the mutant p53 proteins. We consistently observed that the ala(143) mutant was somewhat more effective than either the his(273) or his(175) mutant in binding to DNA. This is consistent with our observation that the ala(143) mutant binds weakly to T antigen and partially inhibits its ability to mediate SV40 *ori* DNA replication in vitro (P. N. F. and C. P., unpublished data). The relatively poor DNA binding by the mutant p53 proteins is consistent with observations that several mutant p53 proteins translated in reticulocyte lysates bound poorly to DNA–cellulose when compared with wild-type p53 (Kern et al., 1991a).

p53 Binds Specifically to Sequences Adjacent to the SV40 Replication Origin

To assess whether specific SV40 DNA sequences that are protected by p53 could be identified, purified p53 or T antigen was bound to a labeled fragment spanning the replication origin and adjacent regulatory sequences, and DNAase I footprinting experiments were performed (Figure 3). When we used a fragment labeled such that binding to the late template strand was detected, both wild-type human and murine p53 proteins displayed a unique protection pattern within the region of SV40 DNA that is directly adjacent to and on the late side of the replication origin (Figure 3A, lanes b and c). The region directly adjacent to the SV40 origin consists of one imperfect and two perfect copies of a 21 bp sequence (Tooze, 1981) that

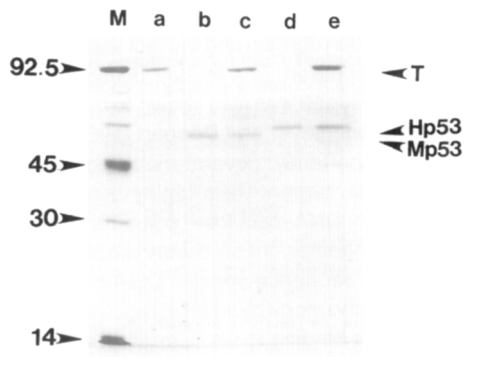

Figure 1. p53 and SV40 T Antigen Proteins

Sf27 cells were infected with recombinant baculoviruses encoding either SV40 T antigen (lane a), murine p53 (lane b), or human p53 (lane d) or were coinfected with viruses expressing SV40 T antigen and murine p53 (lane c) or viruses expressing SV40 T antigen and human p53 (lane e). p53, T antigen, and p53–T complexes were purified on immunoaffinity matrices as described in Experimental Procedures and analyzed on silver-stained 10% SDS–polyacrylamide gels. T = T antigen; Hp53 = human p53; Mp53 = murine p53.

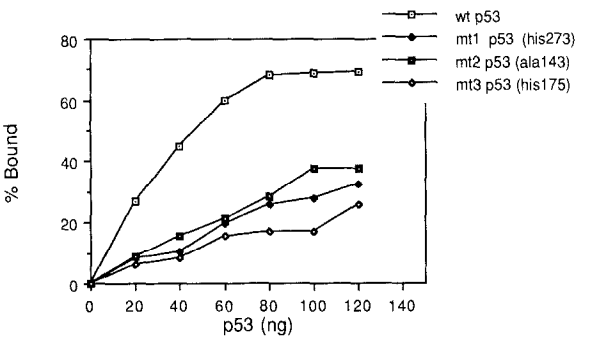

Figure 2. Wild-Type but Not Mutant p53 Proteins Bind Efficiently to SV40 DNA

Binding reactions containing increasing quantities of either wild-type or mutant p53 proteins, ^{32}P-labeled SVO DNA fragments (1 ng), and plasmid pAT153 DNA (10 ng) were incubated for 15 min at 35°C and filtered through 0.45 μm nitrocellulose filters. The filters were counted by liquid scintillation.

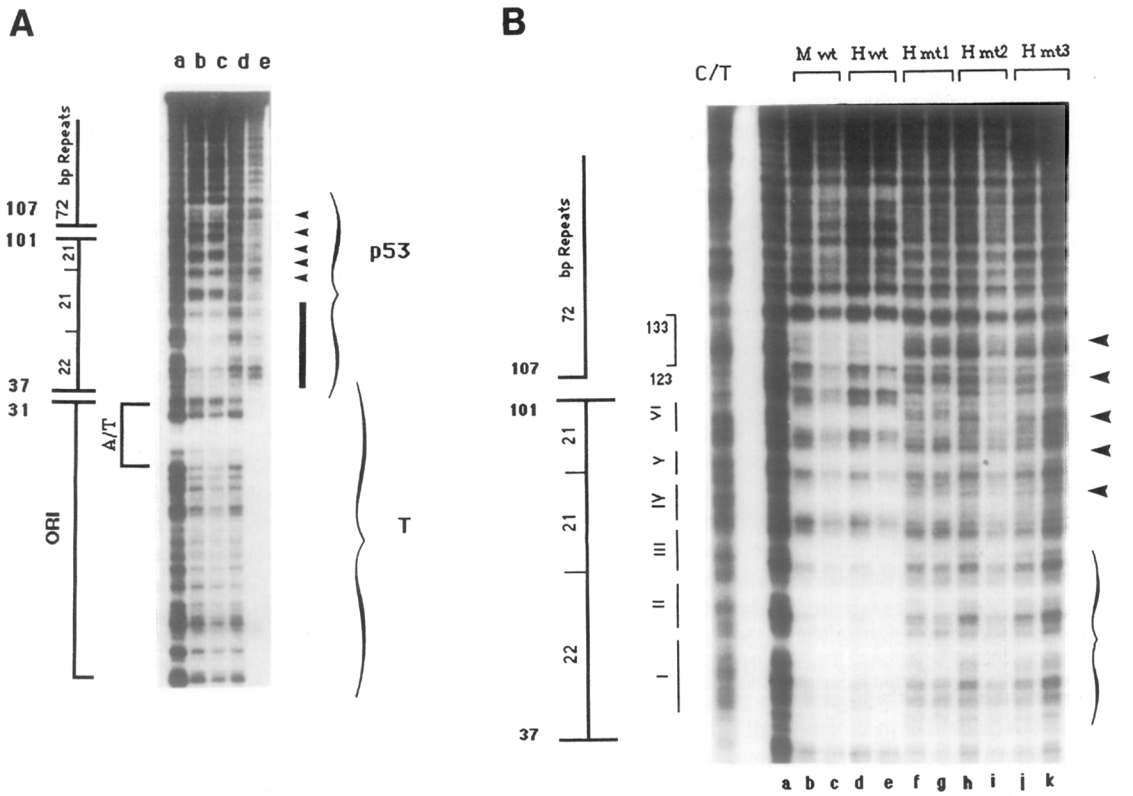

Figure 3. Wild-Type but Not Mutant p53 Proteins Bind Specifically to Sequences on the Late Side of the SV40 Replication Origin

DNAase I footprinting reactions were carried out as described in Experimental Procedures. The SVO fragment was labeled so that binding to the late template strand was detected.

(A) The SV40 origin and cis regulatory elements, along with nucleotide positions, are shown on the left. Binding regions identified for purified p53 and SV40 T antigen are indicated with braces. Discrete sites protected by p53 are indicated by the solid bar and arrowheads. Lane a contains no protein; lane b, 75 ng of murine p53; lane c, 75 ng of human p53; lane d, 75 ng of mutant human p53 his(273); lane e, 150 ng of SV40 T antigen.

(B) The same SV40 DNA fragment was used to test the specific binding ability of additional mutant p53 proteins. The regulatory elements are marked to indicate locations of the GC boxes (I–VI) and the protected areas in the enhancer. The C+T sequencing reaction of the probe is shown on the left. Lane a contains no protein; lanes b–k contain two levels (75 ng and 150 ng) of either wild-type or mutant p53 proteins. Lanes b and c, murine p53; lanes d and e, wild-type human p53; lanes f and g, mutant human p53 his(273) ("mt1"); lanes h and i, mutant human p53 ala(143) ("mt2"); lanes j and k, mutant human p53 his(175) ("mt3"). On the right, the arrowheads and brace indicate the sites of p53 protection.

comprises the six GC boxes (GGGCGG) that are bound strongly to the cellular transcription factor Sp1 (Dynan and Tjian, 1983). This region is contiguous with the duplicated 72 bp SV40 enhancer region, to which several cellular factors have been shown to bind (for review see Jones et al., 1988). By contrast, a mutant p53 protein, his(273), did not protect these sequences (Figure 3A, lane d). SV40 T antigen, at sufficiently high concentrations to bind completely to its well-defined sites within the replication origin (for review see Borowiec et al., 1990), only protected slightly some of the region adjacent to the late side of the core origin (lane e). The patterns of protection of SV40 DNA by T antigen and wild-type p53 were clearly different.

DNAase I footprinting experiments were repeated using wild-type human and murine p53 proteins and the additional human mutant p53 proteins listed in Figure 2. After the binding reactions, the DNAase I–cleaved DNA was subjected to electrophoresis for a longer time so that the region protected by p53 was more clearly delineated (Figure 3B). Again, striking patterns of protection by both wild-type human and murine p53 proteins were observed. The region that was the most strongly protected was that containing the first three of the six GC boxes. However, it appeared that sites within the remaining GC boxes and even extending well into the enhancer region, i.e., between nucleotides 101–108 and nucleotides 125–150, were also protected somewhat by p53. By contrast, neither the his(273) nor the his(175) mutant p53 protein displayed these specific interactions with the DNA. However, at the higher concentration of the mutant p53 ala(143), some protection of the origin-proximal region was noted. This is consistent with this mutant's increased ability to bind to the SV40 fragment using the nitrocellulose filter assay, and correlates with its increased ability to bind to and inhibit SV40 T antigen.

p53 Fails to Bind Specifically to a Deletion Mutant of SV40 DNA

The interaction of p53 proteins with the opposite (early template) strand of SV40 DNA within this region was exam-

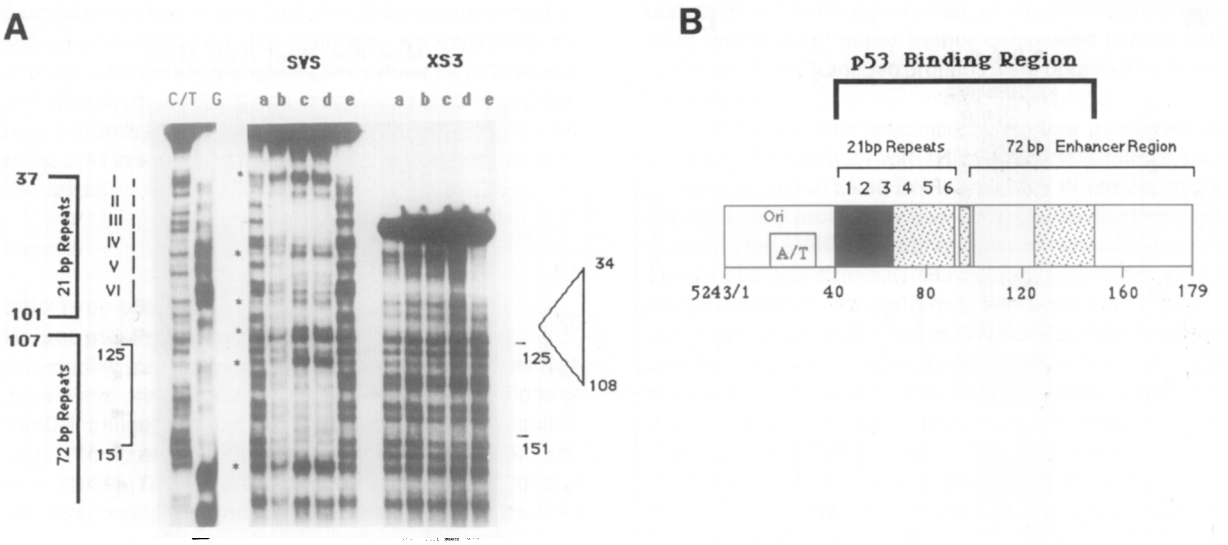

Figure 4. Wild-Type p53 Requires the GC Box Region for Specific Binding to SV40 DNA

(A) DNAase I protection protocols were as described for Figure 3. Early strand 5′ ^{32}P-labeled fragments containing SV40 nucleotides 1–294 containing or lacking nucleotides 34–108 were prepared from plasmids pSVS (panel SVS) and pXS3 (panel XS3), respectively. On the left, early region cis elements are marked to indicate locations of the GC boxes and the protected area in the enhancer. C+T and G sequencing ladders of the probe are also shown. On the right, the deleted region is marked, as is the enhancer region within SVS DNA to which p53 binds. Lanes a contain no protein; lanes b, 75 ng of murine p53; lanes c, 60 ng of wild-type human p53; lanes d, 90 ng of wild-type human p53; lanes e, 150 ng of SV40 T antigen.

(B) A diagram of the p53-binding region in SV40 DNA based on DNAase I footprint data. Shaded regions represent discrete p53-binding sites, with black areas representing stronger interactions and stippled areas representing sites less well protected by p53.

ined (Figure 4A, panel SVS). Results similar to those obtained with the late template strand were obtained when the wild-type human (lanes c and d) and murine (lane b) proteins were compared to each other and to T antigen (lane e). Again, the region that was most strongly protected by p53 was that containing the three GC boxes adjacent to the replication origin. On this strand, however, we also noted consistently not only protection by p53 but also strong DNAase I hypercutting induced by p53 at several specific sites (asterisks in Figure 4A). DNA directly adjacent to the borders of the three GC boxes, especially at the origin-proximal site, was hypercleaved by DNAase I in the presence of p53, as was DNA bordering the enhancer region protected site between nucleotides 125 and 151. We also observed somewhat different patterns of protection on this strand by murine and human p53 proteins in that the overall protection of this region was greater by the murine p53, while the human p53 induced hypercutting by DNAase I more effectively. T antigen exhibited no protection on this DNA strand other than within the core replication origin (top of lane e).

To examine whether p53 protects the DNA that is adjacent to the replication origin in a non-sequence-specific manner, we utilized a deletion mutant, pXS3 (Fromm and Berg, 1982), that lacks nucleotides 34–108 containing the region with the six GC boxes but retains the SV40 enhancer region now positioned directly adjacent to the replication origin (Figure 4A, panel XS3). When the murine (lane b) or the human (lanes c and d) p53 protein was bound to the same fragment generated from mutant pXS3 DNA, the DNAase I footprints showed that essentially no specific strong interactions of these proteins with DNA had occurred (compare lanes a–d), although slight hypercutting was occasionally observed. As expected, T antigen protected those sequences within the origin that could be discerned using the XS3 fragment (see top of lane e). The pattern of DNAase I cutting of the XS3 fragment in the presence of p53 was essentially identical to the pattern seen in the absence of protein (lane a). It is important to note also that with the XS3 fragment no sequences were protected within the enhancer region. This shows that the sites protected by p53 within the enhancer may be the result of secondary interactions of the protein with DNA resulting from its specific interactions within the region containing the GC boxes. The regions on SV40 DNA that are protected are shown in Figure 4B.

SV40 T Antigen Inhibits the Sequence-Specific Binding of p53 to DNA

Previous studies have examined the consequences of p53 binding to T antigen on the ability of the viral protein to replicate viral DNA. These studies did not address the perhaps more generally significant question of the effect on p53 of its binding to T antigen. It was therefore of considerable interest to determine whether T antigen is capable of altering the interaction of p53 with SV40 DNA. Two alternative approaches were taken. In the first case, p53–T antigen complexes were purified from insect cells that had been coinfected with both recombinant p53 and T antigen baculoviruses using a T antigen–specific mono-

clonal antibody column (see Figure 1). We determined that the two proteins, when purified in this manner, remained bound to each other (data not shown).

The ability of similar quantities of free p53 protein or p53 protein complexed to T antigen to bind to the previously identified sites on SV40 DNA using the labeled late template strand was examined (Figure 5A). Neither human nor murine p53 protein when bound to T antigen displayed the p53 protection patterns seen with free p53 proteins (compare lanes c and e in Figure 5A, containing murine and human p53 proteins alone, to lanes d and f, containing these proteins complexed to T antigen). Some slight protection of the origin-proximal region was apparent with the human p53–T antigen complex, which is most likely related to the fact that primate-derived p53 is known to bind less tightly to T antigen than is p53 from rodent cells (Harlow et al., 1981b; P. N. F. and C. P., unpublished data). Consistent with our previous studies (Wang et al., 1989), the origin region sequences normally protected by T antigen alone (see Figure 5A, lane b) were protected somewhat less well by the murine p53–T antigen complex (see lane d), confirming that T antigen that is bound by murine p53 is itself inhibited from interacting with the viral origin. These data thus demonstrated that preformed p53–T antigen complexes were greatly compromised in their ability to protect sequences adjacent to the viral replication origin.

To address further the alteration of p53-specific DNA binding by T antigen, we also added separately purified T antigen and human p53 and examined their binding to these sequences (Figure 5B). Using SV40 DNA labeled on the late template strand, we again observed that p53-specific interactions with SV40 DNA (lane c) were reduced in the presence of added T antigen (lane d). In these experiments the ratio of p53 to T antigen was not sufficiently high for p53 to affect binding by T antigen to the origin region. When DNA labeled on the early template strand was used, we observed similarly that the DNAase I protection and hypercutting pattern that was characteristic of p53 was abolished in the presence of added T antigen (data not shown). Taken together, our experiments show that T antigen strongly inhibits the binding of p53 to viral DNA.

Discussion

We have shown that p53 protein preparations immunopurified from insect cells are capable of protecting a region within SV40 DNA, while similar preparations containing oncogenic mutant p53 proteins exhibit greatly reduced binding to and very little specific interaction with this DNA. We have further shown that SV40 T antigen prevents this sequence-specific interaction by the wild-type p53 preparations. The DNAase I footprints of p53 on SV40 DNA suggest strongly that the region that is directly adjacent to the late side of the replication origin contains the primary site of protection by p53. This region, comprising sequences that are well known to regulate expression of both early and late promoters, contains several GC-rich elements that are known to bind specifically to additional factors such as Sp1 (Dynan and Tjian, 1983), AP-2 (Imagawa et al., 1987; Mitchell et al., 1987), and LSF (Huang et al., 1990). The most well characterized of these is the zinc finger–containing protein Sp1 (Kadonaga et al., 1983). It is unlikely that p53 is closely related to the Sp1 protein, as it contains virtually no strong homology to Sp1 and has, at best, one very poor zinc finger motif and no other commonly recognized DNA-binding motifs (Soussi

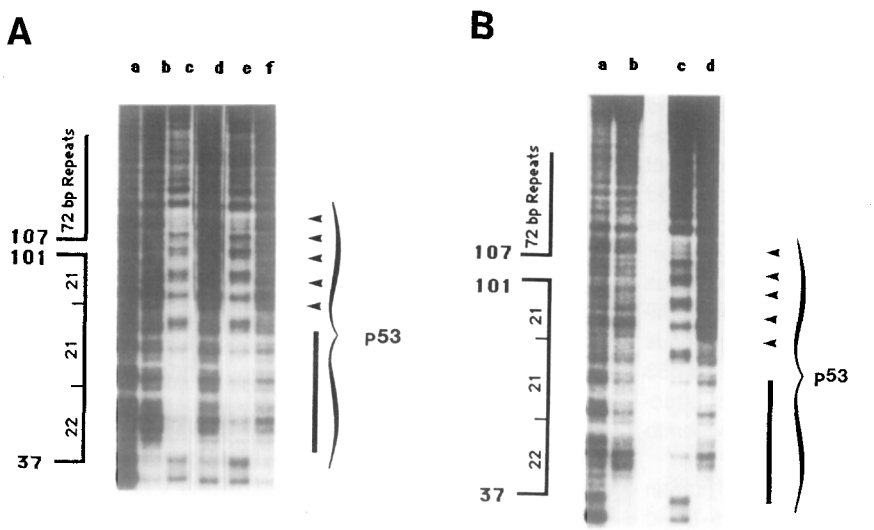

Figure 5. SV40 T Antigen Inhibits Specific Binding by p53 to SV40 DNA

(A) DNAase I protection analysis of the late template strand of the ^{32}P-labeled SVO fragment (as shown in Figure 3) bound to no protein (lane a), 300 ng of SV40 T antigen (lane b), 150 ng of murine p53 (lane c), murine p53–T antigen complex (300 ng total protein) (lane d), 150 ng of human p53 (lane e), and human p53–T antigen complex (300 ng total protein) (lane f).

(B) DNAase I protection analysis of the late template strand bound to no protein (lane a), 300 ng of T antigen (lane b), 150 ng of human p53 (lane c), and 150 ng of human p53 and 300 ng of T antigen (lane d). p53-binding sites are indicated by bar and arrowheads as in Figure 3.

et al., 1990). Furthermore, examination of the p53 protection pattern within the GC boxes shows that it displays a near mirror image of the pattern seen with Sp1. Thus, while p53 bound preferentially to the origin-proximal GC boxes 1, 2, and 3 and protected the origin-distal boxes less well, Sp1 displays increased protection of GC boxes 4, 5, and 6 relative to 1, 2, and 3 (Dynan and Tjian, 1983). Moreover, examination of the protection pattern within each box indicated that p53 protected well the origin-distal part (CGG) of each consensus GGGCGG region but protected poorly the more proximal part of this sequence (GGG). Similarly, although the transcription factor AP-2 was shown to protect the SV40 GC box–containing region as well as G-rich sequences within the enhancer, it displays a different pattern of interaction with the former and indeed protects an entirely different region within the enhancer from what we have observed with p53 (Mitchell et al., 1987; Imagawa et al., 1987). It will be of interest to determine whether p53 and these other factors influence each other's interactions with this region.

In a related study, wild-type but not mutant p53 proteins were shown to bind to unique fragments isolated from cellular DNA that contain repeats of the sequence (TGCCT) (Kern et al., 1991b). However, the SV40 GC box region does not contain repeats of this sequence motif. Within the SV40 regulatory region, in addition to the GC boxes, p53 also protected two discrete regions, between nucleotides 101 and 108 and between nucleotides 125 and 150, the latter of which contains one copy of this sequence motif. However, it is likely that these regions represent secondary binding sites because deletion of the GC box–containing region virtually abolished protection by p53 of these additional regions.

The ability of p53 to contact several distinct regions on SV40 DNA is not surprising, nor is it unprecedented (Imagawa et al., 1987; Mitchell et al., 1987; Sturm et al., 1987; Davidson et al., 1988). For example, AP-2 (Mitchell et al., 1987; Imagawa et al., 1987) and LSF (Huang et al., 1990) each bind to distinct regions within both the enhancer and the 21 bp repeat region. Using several approaches to analyze the size and shape of the purified p53 proteins that we have utilized in these experiments, it was determined that they exist in solution as large oligomeric structures (P. N. F. and C. P., unpublished data; P. Tegtmeyer, personal communication). This is consistent with previous studies indicating that extracts of cells contain predominant forms of p53 that are very large and heterogeneous in size (Kraiss et al., 1988, and references therein). As p53 consists almost exclusively of large, higher order structures, it is conceivable that it can extend its protection to sequences distal to its primary binding site.

It is of considerable interest that both murine and human wild-type p53 proteins bound specifically to the SV40 origin-proximal region. That both murine and human wild-type p53 proteins displayed similar, although not identical, protection patterns is also significant. This supports the notion that p53 function may be fairly conserved among vertebrate species. By contrast, three different human mutant p53 proteins failed to protect this region. The mutant proteins are encoded by sequences cloned from human tumor cells. The mutations map within conserved regions of p53, and two of them, at codons 175 and 273, occur frequently in several types of tumor (Nigro et al., 1989). The inability of the mutants to bind to this region is most likely related to the fact that the several mutant p53 proteins that were used in this study all displayed generally reduced nonspecific and specific interactions with DNA (Kern et al., 1991a, 1991b; J. B. and C. P., unpublished data). This, in turn, may reflect a general alteration in the structure of mutant p53 forms.

Nearly all mutant p53 proteins that we have tested display reduced binding to T antigen and commensurate inability to inhibit its replication functions (P. N. F., S. E. K., B. V., and C. P., unpublished data). While this may suggest either that the DNA-binding domain of p53 is in close proximity to or overlaps its T antigen–binding region, it may also reflect more profound global changes in the structure of the protein. This second alternative is supported by the fact that many oncogenic forms of p53, but not wild-type p53, are bound by the hsp/hsc heat shock proteins (Ehrhart et al., 1988; Hinds et al., 1987; Pinhasi-Kimhi et al., 1986; Sturzbecher et al., 1987), an interaction that may suggest the availability of an unfolded region(s) in the mutant proteins. Furthermore, at least two monoclonal antibodies have been identified that display preferential association with wild-type (Yewdell et al., 1986; Finlay et al., 1988) or mutant (Gannon et al., 1990) forms of p53. Note, however, that only a subset of mutant p53 proteins bind to heat shock proteins or specific monoclonal antibodies, while all mutant forms of p53 so far tested bind poorly to DNA. This includes the his(273) mutant, which does not bind to hsp or "mutant-specific" antibodies but has lost the ability to bind to DNA (e.g., Figure 3B). Thus, abnormal DNA binding appears to reflect a more consistent defect associated with in vivo derived p53 mutations than does abnormal protein binding.

The ability of T antigen to inhibit specific DNA binding by p53 adds new significance to the p53–T antigen complex, which is found in virally infected and transformed cells. There are at least three separate transforming DNA viruses that encode p53-binding proteins. It has been suggested that viral oncoprotein binding to p53 (or to the retinoblastoma protein RB) might facilitate transformation by functionally inactivating p53 (or RB) tumor suppressor functions (Green, 1989; Levine and Momand, 1990). However, it is more likely that this binding has evolved to facilitate some aspect of the life cycle necessary for viral propagation rather than viral transformation; from the viral point of view, the latter is a "dead end."

One can envisage at least two ways in which the blocking of p53 DNA binding by T antigen might influence viral propagation. First, p53 binding to viral DNA might strongly interfere with the viral cycle. This might be reflected in either deregulated early and/or late viral transcription or repressed viral DNA synthesis. By this mode the inactivation of p53 by T antigen would be beneficial and possibly even obligatory for the viral cycle. Second, T antigen might well be capable similarly of repressing or altering the interactions of p53 with sequences within cellular DNA. As a growth suppressor, it can be envisioned

that p53 helps to maintain cells in a resting state, one that is not favorable for viral replication. That p53 possesses both a strong transactivation domain and the capacity to bind specifically to DNA suggests that it may be a factor that regulates the expression of select genes. However, it is interesting that the only sites of cellular DNA so far shown to bind to p53 are near potential origins of replication (Kern et al., 1991b). Identification of cellular p53 binding sites that are blocked or changed by T antigen might provide a unique window into sequences that regulate cell growth. Further analysis of p53 binding sites in viral and cellular DNA may provide crucial insights into both viral and cellular growth control.

The p53 used in these studies was purified from insect cells infected with p53-expressing recombinant baculoviruses by a simple immunoaffinity procedure and was judged to be ~80% pure. Accordingly, it as as yet unproven that p53 itself is directly contacting the SV40 DNA. Therefore, although we refer in this study to the entity that is interacting with the SV40 DNA as p53, this assertion awaits more rigorous proof. Another scenario exists whereby wild-type p53 forms a tight complex with one or more minor but potent sequence-specific DNA-binding proteins from insect cells. Such a protein or proteins would interact with the wild-type but not the mutant p53 proteins and would also be blocked by T antigen. However, although we consider this to be unlikely, this observation would itself be of considerable interest.

Experimental Procedures

Cells and Viruses

Spodoptera frugiperda insect cells (Sf27 cells) and recombinant baculoviruses vEV55SVT (SV40 T antigen) and vEV55p53 (murine p53) were kindly provided by D. O'Reilly and L. Miller (O'Reilly and Miller, 1988). The murine p53 used in these studies was derived from a clone isolated by Jenkins et al. (1984). Although this p53 contains changes at amino acid residues 48, 79, and 81 when compared with the wild-type p53 as designated by Finlay et al. (1988), these changes are not within the p53 conserved regions, and we have observed that this protein functions equivalently to authentic wild-type murine p53 in all assays that we have used, including in its ability to block SV40 *ori* DNA synthesis (P. N. F., M. Oren, and C. P., unpublished data). Recombinant baculoviruses expressing mutant p53 his(273) or wild-type p53 were as described (Friedman et al., 1990). Recombinant baculoviruses expressing mutant p53 ala(143) and his(175) were generated in the same manner (P. N. F. et al., unpublished data). Sf27 cells were grown at 27°C in TC-100 medium (GIBCO) containing 10% heat-inactivated fetal calf serum and 0.25% tryptose broth.

Purification of p53 Proteins and SV40 T Antigen

Sf27 cells (2.5×10^7 per 150 mm dish) were infected with recombinant viruses and harvested 48 hr postinfection. Extracts of infected cells were prepared as described (Wang et al., 1989). p53 proteins and T antigen were purified from cell lysates by immunoaffinity procedures (Wang et al., 1989). A monoclonal antibody column with p53-specific PAb 421 (Harlow et al., 1981a) cross-linked to protein A–Sepharose was used to purify p53 proteins. p53–T antigen complexes and SV40 T antigen were purified using a column with T antigen–specific PAb 419 (Harlow et al., 1981a) cross-linked to protein A–Sepharose.

DNA Filter Binding Assay

Reaction mixtures (50 μl) containing 40 mM creatine phosphate, 7 mM $MgCl_2$, 0.5 mM DTT, 0.2 mg/ml bovine serum albumin, 4 mM ATP, 10 ng of pAT153 nonspecific plasmid DNA (Twigg and Sheratt, 1980), and 1 ng of ^{32}P-labeled SVO DNA fragment (SV40 nucleotides 5171–294) and the indicated p53 protein were incubated for 15 min at 35°C. Reaction mixtures were filtered through 0.45 μm nitrocellulose filters presoaked in wash buffer (25 mM HEPES), washed twice with the same buffer, dried, and counted.

DNAase I Footprinting

Reaction mixtures (50 μl) containing 40 mM creatine phosphate (di-Tris salt; pH 7.7), 4 mM ATP, 7 mM $MgCl_2$, 0.2 mg/ml bovine serum albumin, 0.5 mM DTT, 10 ng of the plasmid pAT153 (Twigg and Sheratt, 1980), and 1 ng of 5′ ^{32}P-labeled DNA fragment were preincubated for 5 min at 37°C. Protein was then added as indicated in the figure legends. Binding was allowed to proceed for 15 min at 37°C. The amount of DNAase I required to produce an even pattern of partial cleavage products was pretested empirically; in general, 4–10 μl of a freshly diluted 0.5 μg/ml solution in 20 mM $CaCl_2$ was used. Following digestion, 50 μl of DNAase stop solution (2 M ammonium acetate, 100 mM EDTA, 0.2% SDS, 100 μg/ml sheared salmon sperm DNA) was added and the DNA was extracted with phenol and ethanol precipitated. The DNA was then subjected to electrophoresis on either 6% or 8% denaturing polyacrylamide gels.

End-labeled DNA fragments for binding studies were prepared using standard techniques (Maxam and Gilbert, 1980). A fragment labeled downstream of the 21 bp repeats was prepared from pATSVO, a plasmid containing SV40 nucleotides 5171 to 294 inserted into pAT153. The plasmid pATSVO was digested with HindIII, and the fragments were labeled by filling in with the large fragment of DNA polymerase I and [^{32}P]dNTPs. The mixture was then digested with EcoRI, and the desired single end-labeled fragment was gel purified. This HindII–EcoRI fragment corresponds to the SV40 DNA strand from which transcription of the late viral genes takes place and is referred to as the late template strand. Fragments labeled on the opposite strand, the early template strand, were prepared from plasmids pSVS and pXS3 (Fromm and Berg, 1982). Plasmid pSVS contains the wild-type SV40 genome combined at its unique EcoRI site with the PvuII–EcoRI fragment of pBR322 (Fromm and Berg, 1982). Plasmid pXS3 was derived from pSVS and is deleted for SV40 nucleotides 34 to 108 (Fromm and Berg, 1982). These plasmids were digested with BanI, end labeled with the large fragment of DNA polymerase I, cut again with BglI, and the appropriate fragments gel purified.

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