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Two Distinct Regions of the Murine p53 Primary Amino Acid Sequence Are Implicated in Stable Complex Formation with Simian Virus 40 T Antigen

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We mapped regions of the mouse p53 primary amino acid sequence implicated in stable complex formation with simian virus 40 T antigen. A number of mutant p53 proteins failed to complex stably with T antigen in vivo but formed stable complexes with T antigen in in vitro association assays. In contrast to an earlier report (T.-H. Tan, H. Wallis, and A. J. Levine, J. Virol. 59:574–583, 1986), our study showed that two distinct regions of p53 primary amino acid sequence, highly conserved between mouse and *Xenopus laevis*, were implicated in stable complex formation. Our data support the proposal that, when in complex, T antigen may occupy a site on p53 that is implicated in the normal function of the protein.

p53 is a short-half-life cellular phosphoprotein that is present at elevated levels and in stable form in cells transformed by a variety of insults, including chemical mutagenesis (3), infection by DNA or RNA tumor viruses (11, 12), and irradiation (3), as well as in spontaneous transformants (12). p53 expression constructs immortalize primary rodent cells in vitro (9, 10, 14a) and render them sensitive to transformation by an activated *ras* oncogene (4, 10, 14). Specific in vitro mutations of the p53 coding sequence result in p53 proteins with enhanced activity in transformation assays and increased metabolic stability (9). Similar mutations can occur in vivo in association with malignant transformation by Friend leukemia virus (15), supporting the proposal (9) that mutational changes in the cellular p53 gene may constitute a mechanism by which p53 participates in multistage carcinogenesis.

p53 forms specific and stable complexes with simian virus 40 (SV40) T antigen (11, 12) and with the unrelated 57-kilodalton protein of the adenovirus 5 E1b region (16, 23). Expression of murine p53 in SV40 replication-permissive monkey COS cells results in the suppression of SV40 origin-dependent DNA synthesis (1). Murine p53 in in vitro assays can both displace polymerase α from T-antigen–polymerase α complexes and exist as a trimeric T-antigen–polymerase-α–p53 complex (5).

In the course of earlier work (19) and the present study, we found discrepancies between our own data and a report (20) which defined the binding domain for T antigen on p53. As we and others observed previously (8, 19), a variety of in-frame deletion and linker insertion p53 mutants fail to bind T antigen significantly in vivo but complex instead with hsp72/73 heat shock proteins. The location of these mutants (Fig. 1) suggests that a major part of p53, C terminal of amino acid 66, is implicated in T-antigen binding. To define the C-terminal boundary of p53 amino acid sequences required for T-antigen binding, we constructed a series of mouse p53 carboxyl-terminal deletion mutants and expressed them in the monkey COS cell transient-expression assay as we have described elsewhere (19). At 72 h after transfection, the cells were labeled for 2 h with [^{35}S]methionine, and T-antigen–p53 complexes were immunoprecipitated from cell lysates by using separately the mouse p53-specific monoclonal antibody PAb248 (22) and the T-antigen-specific monoclonal antibody PAb419 (6) (Fig. 2). Deletion of sequences at the carboxyl terminus up to amino acid 285 (mutant *Ctr*977) had no detectable effect on the ability of mouse p53 to bind T antigen, as evidenced by the coprecipitation of truncated mouse p53 anti-T-antigen immunoprecipitations. However, deletion of a further 12 amino acids (mutant *Ctr*942) totally abolished coprecipitation of the mouse p53 polypeptide. Overexposure of the autoradiograph (Fig. 2) confirmed that no mutant p53 was detectable in the anti-T-antigen immunoprecipitation of *Ctr*942-transfected cells. We conclude that the carboxyl-terminal junction between amino acid sequences essential and nonessential for complex formation lies within the region of amino acids 274 to 285.

As we described earlier (19) (Fig. 1), a number of linker insertion mutations abolish T-antigen binding in vivo. However, one p53 mutant carrying a linker insertion between amino acids 221 and 222 (*ins*785) did bind to T antigen (Fig. 1 and 3). Expression of the epitope recognized by monoclonal antibody PAb246 (22) has been shown to correlate well with T-antigen binding both in vivo (19, 22) and in in vitro association assays (22). Immunoprecipitation of *ins*785 containing COS cell lysates with PAb246 revealed that in addition to binding T antigen, the *ins*785 gene product expressed the 246 epitope, while linker insertions on either side (*ins*763 and *ins*816) and a neighboring deletion mutant (*dl*721) which mapped to within two amino acids amino-proximal of *ins*785 did not (Fig. 1 and 3). These results indicate that the primary amino acid sequences implicated in stable T-antigen–p53 binding subdivided into at least two separate stretches.

We were concerned that the loss of T-antigen binding exhibited in vivo by some of the mutant p53 proteins was

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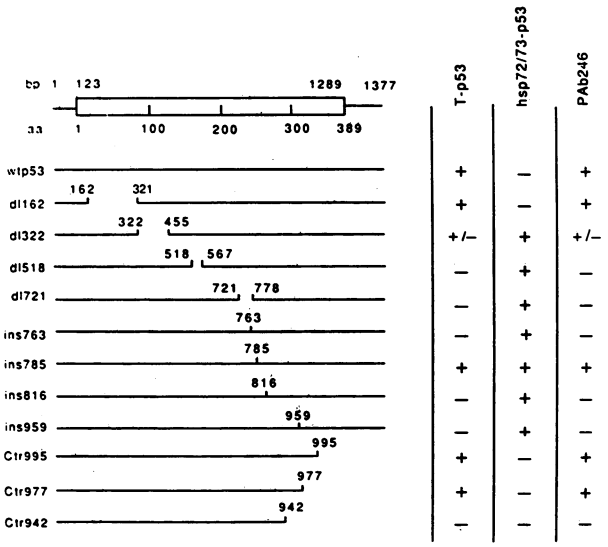

FIG. 1. T-antigen-binding and antigenic properties of mouse p53 mutants in transfected monkey COS cells. Nucleotide numbers of deletion boundaries and linker insertion positions are as shown. Deletion mutants have lost the following amino acid sequences: *dl*162, from 14 to 66; *dl*322, from 68 to 111; *dl*518, from 133 to 148; and *dl*721, from 200 to 219. Linker insertion mutants contained *Eco*RI 12-mer linkers that disrupted the protein sequence as follows: *ins*763, within the codon for amino acid 214; *ins*785, between amino acids 221 and 222; *ins*816, within the codon for amino acid 232; and *ins*959, between amino acids 279 and 280. Carboxyl-terminal truncations are missing amino acid sequences as follows: *Ctr*995, from amino acid 292 to the C terminus; *Ctr*977, from amino acid 286 to the C terminus; and *Ctr*942, from amino acid 244 to the C terminus. *dl*518 was originally called *dl*516 (9), *dl*721 was called *dl*717 (19), and *Ctr*995 was called *dl*1001 (9). These names have been changed as part of a rationalization of mutant nomenclature. Bp, Base pair; wt, wild type; aa, amino acid; +, T-antigen-p53 complex formation; −, no complex formation; +/−, poor complex formation.

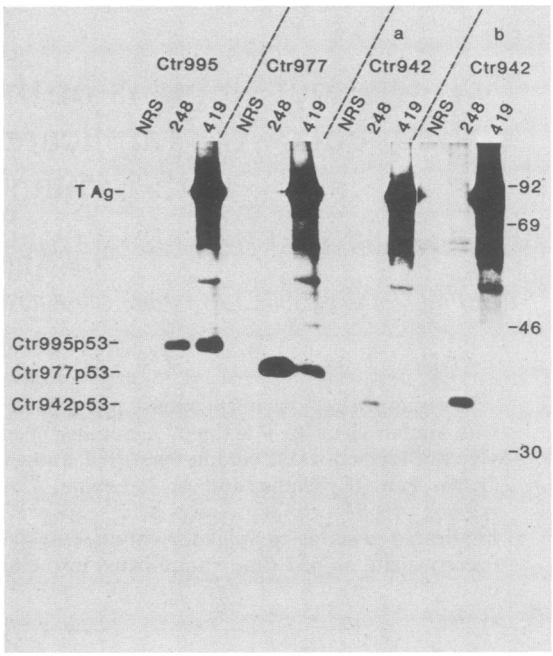

FIG. 2. Association of T antigen (T Ag) with mouse p53 carboxyl-terminal deletion mutants in monkey COS cells. p53 expression construct-transfected COS cells were labeled with [^{35}S]methionine for 2 h, and cell lysates were immunoprecipitated with anti-mouse p53 (PAb248) (22) or anti-T-antigen (PAb419) (6) monoclonal antibodies prior to being analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and autoradiography. Lane Ctr942 b is a longer exposure of lane Ctr942 a. Since newly synthesized p53 associates preferentially with mature rather than newly synthesized T antigen (2), the T-antigen/p53 signal ratio is correspondingly biased against T antigen in the anti-p53 immunoprecipitation lanes. Molecular sizes are given on the right in kilodaltons. NRS, Normal rabbit serum.

perhaps due to some competition between T antigen and hsp72/73 for p53. We had previously shown that [^{35}S] methionine-labeled mouse p53 proteins synthesized in an mRNA-dependent rabbit reticulocyte lysate express denaturation-sensitive epitopes (21), and p53 readily complexes with T antigen in in vitro association assays to give detergent- and urea-resistant complexes indistiguishable from complexes formed in vivo (13). However, we were unable to form in vitro complexes between wild-type or mutant p53 and hsp72/73 (18). We therefore tested the ability of p53 mutants to bind T antigen in in vitro association assays. After association, immunoprecipitated T-antigen–p53 complexes were washed sequentially in RIPA buffer containing 0.1% sodium dodecyl sulfate and 2 M urea (13) (Fig. 4). Wild-type p53 (Fig. 4B) and the protein product of *dl*162 (Fig. 4A) coprecipitated readily with T antigen in PAb419 immunoprecipitates in vitro as they did in vivo and served as positive controls in this assay. The *dl*322 protein, which bound poorly in vivo, behaved the same in vitro, with much reduced but still detectable coprecipitation of p53 protein in anti-T-antigen immunoprecipitations. The *ins*785 gene product coprecipitated in T-antigen immunoprecipitates indistinguishably from wild-type p53; *dl*518 and *ins*959 behaved in vitro as they did in vivo, with no significant coprecipitation of the encoded mutant p53 T-antigen immunoprecipitations. Strikingly, the mutant protein encoded by *dl*721 readily coprecipitated with T antigen (Fig. 4A) and the *ins*816 gene

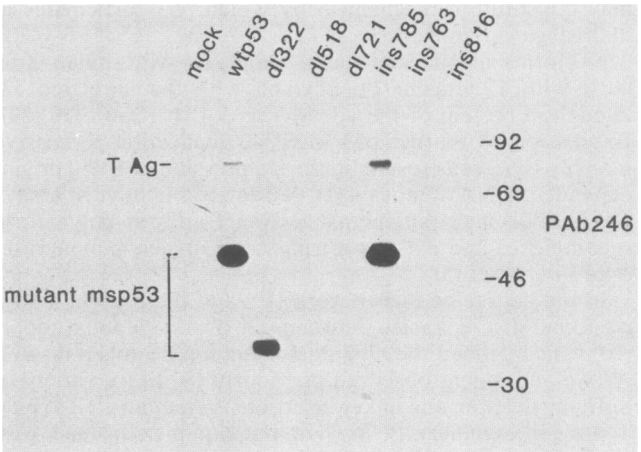

FIG. 3. Immunoprecipitation of p53 proteins from COS cells transfected with different internal deletion and linker insertion mutants. Labeled cell lysates were immunoprecipitated with monoclonal antibody PAb246 (22), which recognizes the conformational form of mouse p53 (msp53) associated with T-antigen (T Ag) binding. Immunoprecipitations were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis as for Fig. 2. Gel loading was normalized to give equivalence of PAb246-precipitable signal, since only a minor subset of *dl*322 protein is bound to T antigen. Molecular sizes are given on the right in kildaltons. Wt, wild type.

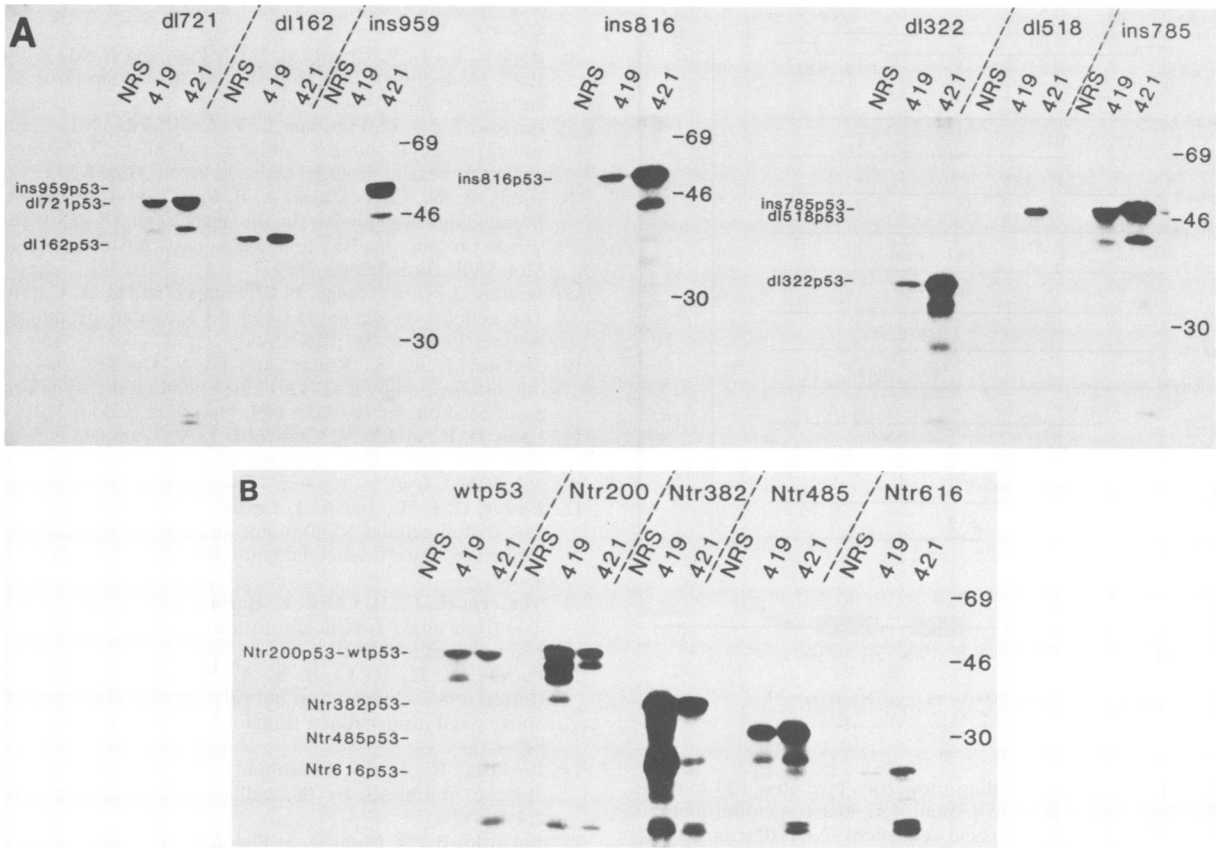

FIG. 4. In vitro association between T antigen and reticulocyte lysate-derived p53 linker insertion and deletion mutants (A) and amino-terminal truncation mutants (B). After in vitro association, lysates were immunoprecipitated with monoclonal antibody PAb419, which recognizes SV40 T-antigen (6), or monoclonal antibody PAb421 (6), which recognizes an epitope located at the extreme carboxyl terminus of p53 (21). The lower-molecular-weight-labeled peptides coprecipitating in these assays are partly products of internal translation initiation within the cRNA coding sequence (21) and partly degradation products reflecting the relative instability of some p53 mutants in mixed-lysate in vitro association assays. NRS, Normal rabbit serum. Molecular sizes are given on the right in kilodaltons.

product at reduced but clearly detectable levels (Fig. 4A). A series of amino-terminal deletions removing up to 164 amino acids did not affect T-antigen binding (Fig. 4B), although we could not usefully perform in vitro mapping beyond this point because of the increasing instability of the truncated products. Combining the in vitro (Fig. 5A) and in vivo (Fig. 1) data, we are able to define the T-antigen-binding sequence requirements in more detail. Fig. 5B is a map of the regions of the p53 amino acid sequence in which we propose that these sequences must lie. In each case, junctions between essential and nonessential sequences have been assigned on a worst-case basis. For example, the boundary at amino acid 199 corresponds to the amino-terminal limit of the deletion in mutant *dl*1721, the boundary at amino acid 232 corresponds to the site of the linker insertion in *ins*816, and the amino-terminal boundary at amino acid 165 is dictated by the instability in vitro of mutant proteins deleted beyond this point.

In a recent report, Soussi et al. (17) described the isolation and sequence of a cDNA gene encoding a *Xenopus* p53. Comparison of toad, human, and mouse p53 genes reveals that conservation of protein sequence is restricted to limited regions of the polypeptide, while elsewhere homology is patchy (17). A comparison of the regions we define here as being implicated in T-antigen binding with the sequence blocks defined by Soussi as being evolutionarily conserved reveals a significant correspondence (Fig. 5B). Thus, our sequence block A includes their conserved block III, and, strikingly, our block B coincides almost exactly with their blocks IV and V. It may be significant that block B encompasses the stretch of amino acid sequence most highly conserved between *Xenopus* sp., human, and mouse p53. Thus it appears that the protein domains implicated in our study in complex formation with the papovavirus oncoprotein T antigen are conserved during evolution. Indeed, recent collaborative work by Soussi et al. and us showed that *Xenopus* p53 forms stable complexes with SV40 T antigen in vivo and in vitro. These data strongly suggest that T antigen interacts with regions of p53 that are directly involved in the normal function of the protein and support the notion that, when complexing with p53, T antigen may be usurping the role of some cellular T-antigen-equivalent protein which is a normal target for p53.

The biological relevance of the T-antigen–p53 and hsp72/73-p53 complexes remains obscure, and a detailed understanding of the molecular architecture underlying this association will be useful in determining any functional role of p53 in SV40 replication or T-antigen-mediated cell transformation.

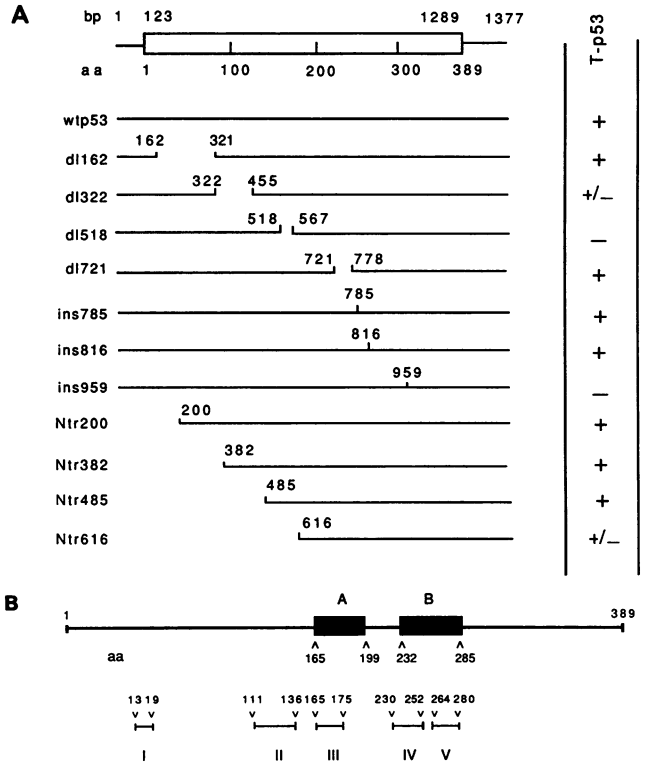

FIG. 5. (A) T-antigen-binding properties of mouse p53 mutants in association assays in vitro. Amino-terminal truncation mutants have lost the following amino acid sequences: *Ntr*200, from 1 to 25; *Ntr*382, from 1 to 87; *Ntr*485, from 1 to 121; and *Ntr*616, from 1 to 165. Deletion and insertion mutants are described in the legend to Fig. 1. Bp, Base pair; wt, wild type; aa, amino acid; +, T-antigen–p53 complex formation; −, no complex formation; +/−, poor complex formation. (B) Regions of the mouse p53 amino acid sequence implicated in stable association with T antigen. Boxes represent regions of primary amino acid sequence whose disruption resulted in loss of stable T-antigen binding. Bars indicated by roman numerals represent amino acid sequence blocks highly conserved between mouse, human, and *Xenopus* p53 (17). aa, Amino acid.

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