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Precise epitope mapping of the murine transformation-associated protein, p53

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Murine p53 cDNA sequences were cloned into an *in vitro* expression vector, Protem Hind. Four deletion libraries were generated using *Bal*31 double-stranded exonuclease; two being made from constructs encoding a fusion protein constructed from SV40 small t sequences and the p53 clone, p27.1a; and two from the full length p53 clone, pp53-5. Both 5′- and 3′-terminal deletions of the p53 gene were made. Transcription of these constructs using *Escherichia coli* RNA polymerase holoenzyme, followed by translation in mRNA-dependent rabbit reticulocyte lysate, gave *in vitro*, truncated protein products which were immunoprecipitated by a panel of anti-p53 monoclonal antibodies. This approach enabled us to map accurately the binding sites of seven different monoclonal antibodies, demonstrating four distinct antigenic sites on p53. A synthetic peptide was constructed corresponding to the predicted amino acid sequence of one of these epitopes. This peptide competes with the epitope on the full length p53 protein for the relevant monoclonal antibodies and dissociates the corresponding p53/antibody complexes.

*Key words: Bal*31 deletion mutants/epitope mapping/immunoprecipitation/*in vitro* expression/p53

Introduction

p53 is a cellular-encoded transformation associated protein that is present at elevated levels in a wide variety of transformed cell lines including spontaneous teratocarcinomas (Linzer and Levine 1979); cell lines derived from tumours induced by chemicals (DeLeo *et al.*, 1979), radiation (DeLeo *et al.*, 1979), DNA and RNA tumour virus infections (Lane and Crawford, 1979; Linzer and Levine, 1979; Rotter *et al.*, 1981; Ruscetti and Scolnik, 1983); and in several lines derived from human tumours (Crawford *et al.*, 1981). It is a phosphoprotein with an apparent mol. wt. of 53 kd on SDS-polyacrylamide gels (Lane and Crawford, 1979). This protein forms stable and specific complexes with two viral oncogene products – SV40 large T antigen (Lane and Crawford, 1979) and adenovirus E1b, a 57-kd protein (Sarnow *et al.*, 1982). The formation of such complexes stabilises p53, as the half-life of the protein increases from ~30 min to >22 h in 3T3 cells transformed by SV40 (Oren *et al.*, 1981). We have cloned cDNA sequences representing the entire murine p53 coding region (Jenkins *et al.*, 1984a) and have shown that transfection of p53 cDNA expression constructs into cells which have a finite lifespan in culture, results in cellular immortality and susceptibility to subsequent transformation by activated *ras* genes (Jenkins *et al.*, 1984b). In addition to elevated levels of p53 protein, there is also an alteration in its subcellular localisation in cells with a transformed phenotype. On transforming fibroblasts with SV40 the distribution of p53 changes from cytoplasmic to nuclear (Rotter *et al.*, 1983b). Also Concanavalin A treatment of mouse lymphocyte cultures results in epitope exclusion (Milner, 1984), suggesting another possible modification of the protein.

A number of monoclonal antibodies have been raised to p53, which show species specificities (Benchimol *et al.*, 1982; Rotter *et al.*, 1983a), indicating a variation in the primary structure of this protein, although certain characteristics, such as complex formation with SV40 large T, appear to be conserved. The purpose of this work was to map the epitopes recognised by the

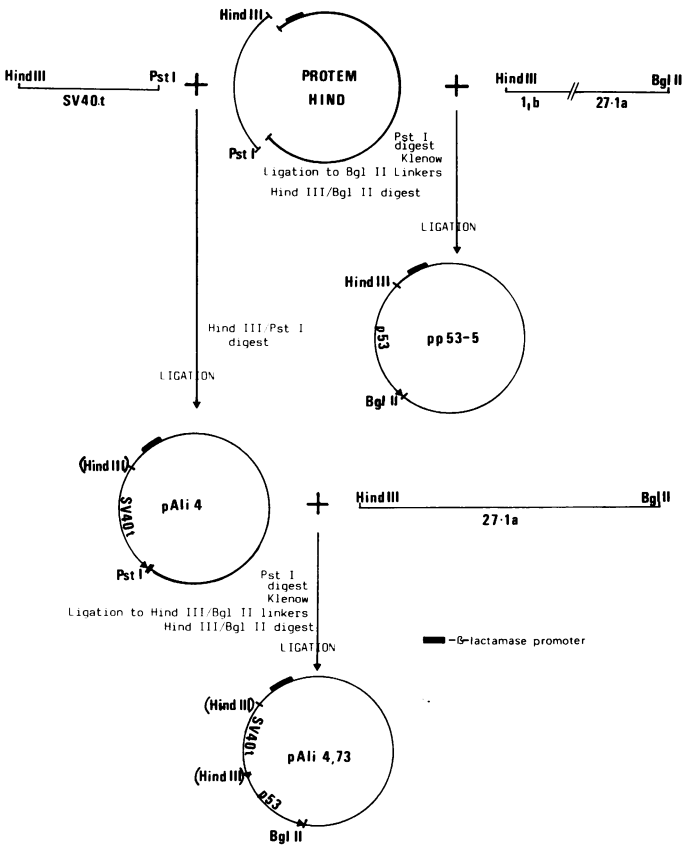

Fig. 1. Construction of expression vectors. The detailed construction of Protem Hind is given by Jenkins *et al.* (1984a). **pp53-5**. The *Hind*III/*Pst*I fragment of 1,b was ligated to the *Pst*I/*Bgl*II fragment of p27.1a to give a full length cDNA clone of p53. To be able to express this protein *in vitro*, the *Pst*I site of Protem Hind was altered to a *Bgl*II site by means of a synthetic linker and the p53 cDNA inserted by *Hind*III/*Bgl*II digest. Since the 5′non-coding region of the p53 gene was present the p53 was not placed in-frame with the β-lactamase AUG of Protem Hind. **pAli 4.** The *Hind*III/*Taq*I fragment of SV40 containing the majority of the coding region of small t was inserted into the *Hind*III/*Cla*I sites of pAT153 (Jenkins *et al.*, 1984a). The *Eco*RI site of pAT153 was converted to a *Pst*I site and the SV40t transferred to Protem Hind by a *Hind*III/*Pst*I insertion. The SV40t gene was placed in-frame with the β-lactamase AUG. **pAli4,73.** To generate a fusion protein between SV40t and p53, the *Hind*III/*Bgl*II fragment of 27.1a was ligated into the *Pst*I site of pAli4 (having ligated synthetic *Hind*III and *Bgl*II linkers onto the *Pst*I site already). The p53 gene was finally placed in the same reading frame as SV40t.

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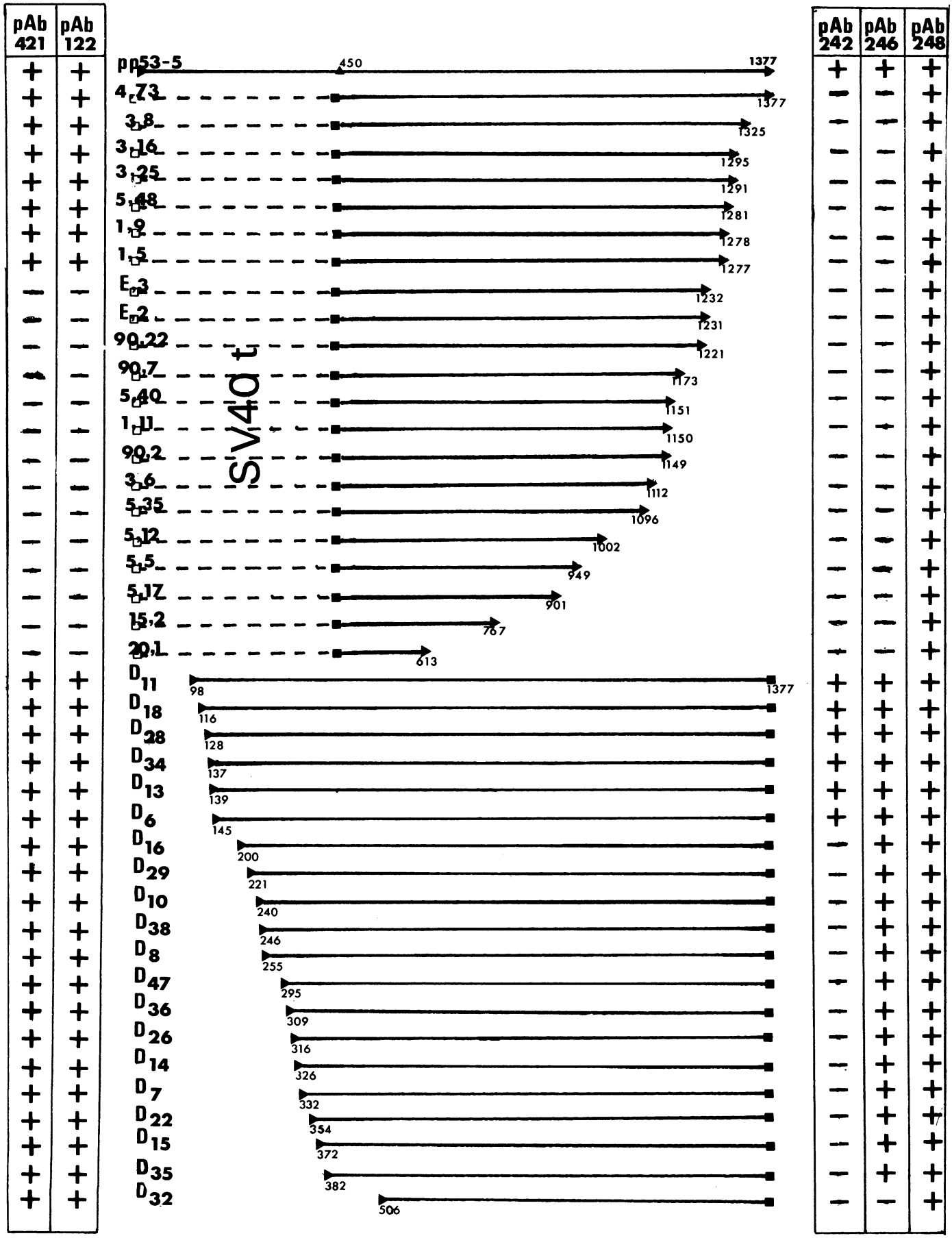
pAb 421
pAb 122
pAb 242
pAb 246
pAb 248
SV40 t
p53-5
450
1377
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3.25
1291
5.48
1281
1.9
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1096
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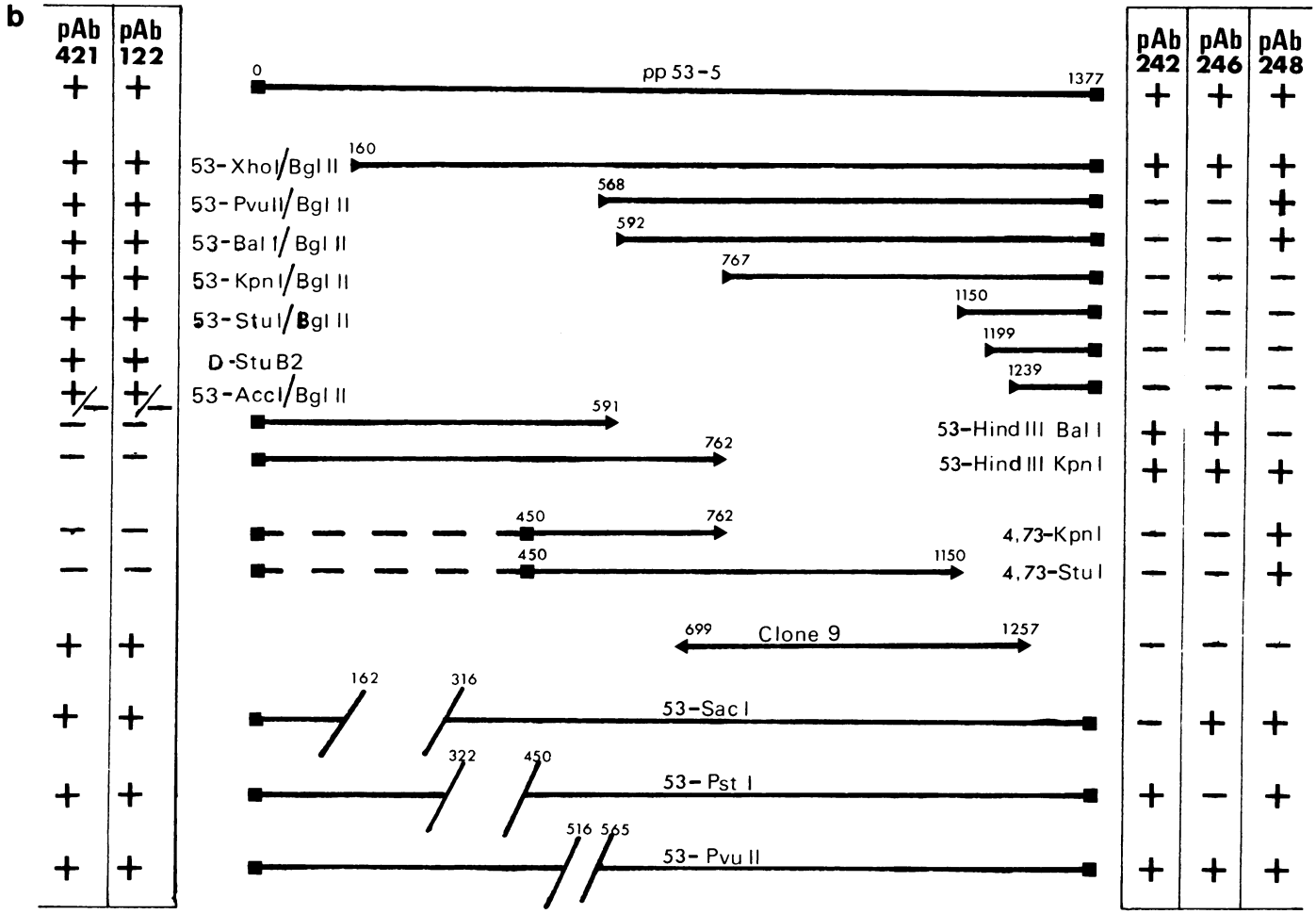

Fig. 2. **(a)** Summary of immunoprecipitation studies on *Bal*31-deleted constructs. This figure summarises the results obtained from using the deletion libraries together with five different monoclonal antibodies. Deletions generated from pAli4,73 (encoding the fusion protein), using *Bal*31 digestion from the 3′ terminus of the p53 insert, are grouped in the top half of the figure – the dotted line indicating SV40t sequences and the full line p53 cDNA sequences. The numbers in small type refer to the base pair numbers designated to the full length cDNA clones (Jenkins *et al.*, 1984a). The numbers in bolder type refer to the name given to the plasmid. The lower half of the figure represents the deletion library obtained by *Bal*31 digestion of pp53-5 from the 5′ terminus of the p53 cDNA insert. All plasmids in this library have the prefix D. **(b)** Summary of immunoprecipitation studies on constructs deleted by using restriction enzyme digests. All constructs shown in this figure were deleted using restriction enzyme sites within the p53 cDNA clone. Any 3′-deleted constructs were sequenced across the deleted terminus and ligated to a synthetic *Hind*III linker, then placed in-frame with the β-lactamase AUG as described in the text. Any internal deletions were also kept in-frame by the insertion of an appropriate linker. A +/− results indicates difficulty in distinguishing whether there was an increase above background levels or not.

available monoclonal antibodies relative to the predicted amino acid sequence of p53 as part of a long term study of the structure-function relationships of this protein.

Results

Deletion plasmid construction

The plasmid used for the construction of the *in vitro* expression plasmids was Protem Hind, which is a derivative of pAT that has a unique *Hind*III site just downstream of the β-lactamase AUG (2 bp), the rest of the β-lactamase coding sequence has been deleted and replaced by a stuffer fragment from polyoma virus (Jenkins *et al.*, 1984). Initially two plasmids were constructed, one, pAli4,73 which has the *Hind*III/*Taq* fragment from SV40, containing the majority of the coding region of SV40t, inserted 5′ of the 27.1a fragment of p53 (terminal two-thirds of the coding sequence) (see Figure 1). This construct generates a fusion protein between SV40t and p53, since the two sequences have been placed in the same reading frame. The other plasmid, pp53-5, was constructed by the insertion of the full length p53 gene (27.1a+1,b) into Protem Hind by *Hind*III/*Bgl*II double digest, using *Bgl*II linkers at the unique *Pst*I site of Protem Hind (see Figure 1). Since the full length p53 gene also contains some 5′ non-coding sequence the p53 gene was not placed in-frame with the β-lactamase AUG in the pp53-5 construct. This resulted in a much weaker signal when the plasmid DNA was expressed *in vitro* (see Materials and methods) but it was still detectable. Deletion libraries of these two expression constructs, pAli4,73 and pp53-5, were generated by using *Bal*31 double-stranded exonuclease, after having linearised the plasmids with either *Hind*III (for 5′ deletions) or *Bgl*II (for 3′ deletions) – see Materials and methods.

Individual clones were transcribed and translated as described in Materials and methods. Resulting protein products were immunoprecipitated as described using the appropriate monoclonal antibodies. The results are summarised in Figure 2.

Immunoprecipitations

Only five of the seven monoclonal antibodies were able to immunoprecipitate protein products from pAli4,73, pAb421 and pAb410 (Harlow *et al.*, 1981), pAb122 (Gurney *et al.*, 1980),

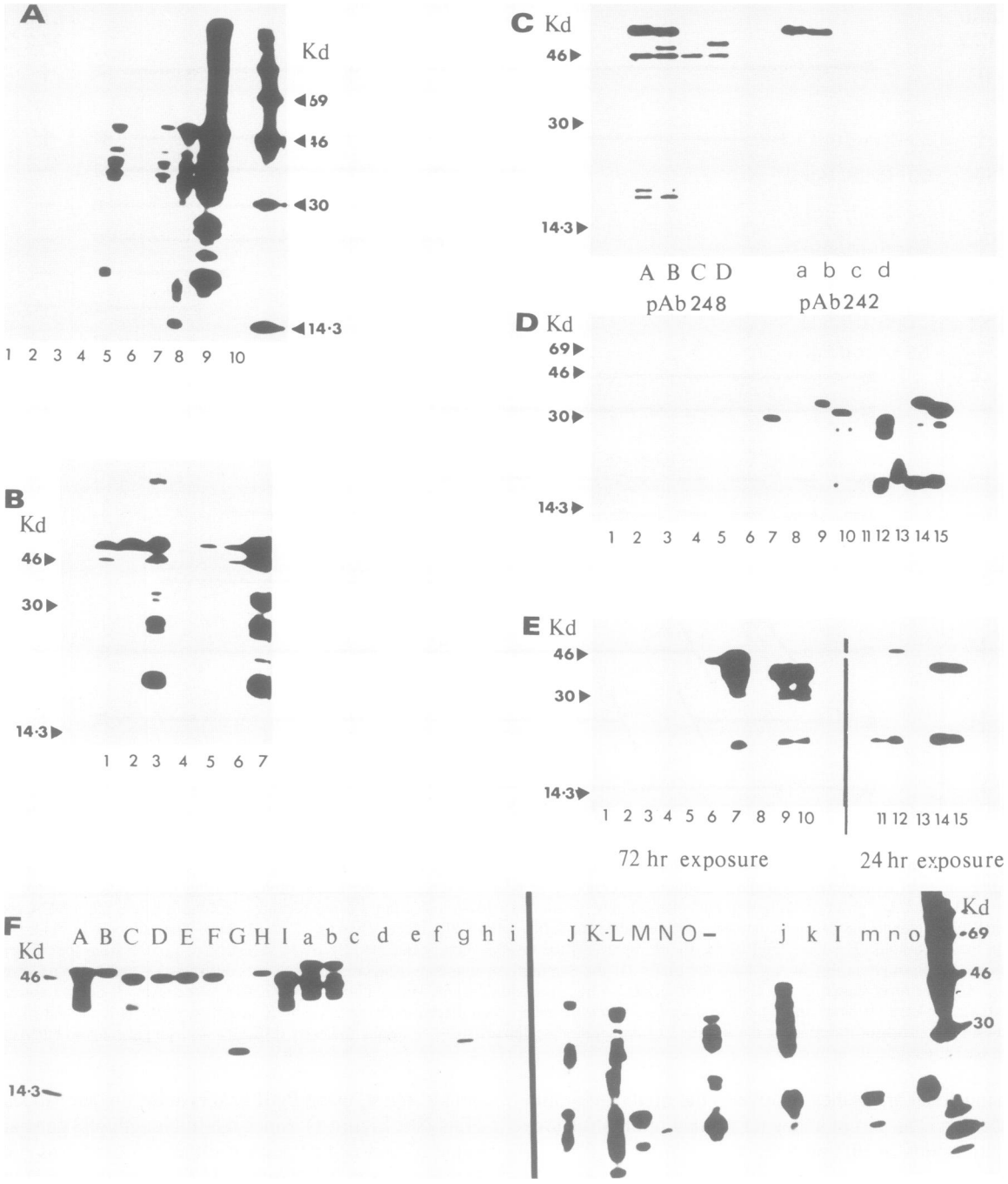

Fig. 3. **(A)** Immunoprecipitation studies on the fusion protein encoded by pAli4,73. The fusion protein from pAli4,73 was immunoprecipitated as described in the text **(1)** pAb607 – anti-p53, **(2)** blank, **(3)** pAb246 – anti-p53, **(4)** pAb242 – anti-p53, **(5)** pAb248 – anti-p53, **(6)** RA3 2C2 – anti-p53, **(7)** pAb200-47 – anti-p53, **(8)** pAb419 – anti-SV40t/T, **(9)** pAb421 – anti-p53, **(10)** pAb416 – anti-SV40T only. **(B)** Immunoprecipitation studies on protein encoded by pp53-5. The full length p53 protein from pp53-5 was immunoprecipitated as described in the text by **(1)** pAb248, **(2)** pAb242, **(3)** pAb421, **(4)** pAb419, **(5)** pAb200-47, **(6)** RA3 2C2, **(7)** pAb122. **(C)** Mapping of the epitope recognised by pAb242. The following protein products from deletions of pp53-5 were precipitated as described in the text by either pAb248 (lanes marked in capitals) or pAB242 (lanes with small letters). **Lanes (A)** and **(a)** pD,13 (139 – 1377 bp), **lanes (B)** and **(b)** pD,6 (145 – 1377 bp), **lanes (C)** and **(c)** 53-*Xho*I/*Bgl*II (159 – 1377 bp), and **lanes (D)** and **(d)** pD,16 (200 – 1377 bp). **(D)** Mapping of the epitope recognised by pAb248. The following protein products were immunoprecipitated as described in the text using pAb419 **(lanes 1 – 5)**, pAb248 **(lanes 6 – 10)**, or pAb122 **(lanes 11 – 15)**, **Lanes 1,6** and **11** are the immunoprecipitation products from 53-*Hind*III/*Bal*I (0 – 591 bp), **lanes 2,7** and **12** from 53-*Bal*I/*Bgl*II (592 – 1377 bp), **lanes 3,8** and **13** from Clone 9 (699 – 1257 bp), **lanes 4,9** and **14** from pD,35 (382 – 1377 bp) and **lanes 5,10** and **15** from pD,32 (506 – 1377). **(D)** Mapping of the epitope recognised by pAb246. The following protein products were immunoprecipitated as described in the text using pAb419 **(lanes 1 – 5)**, pAb246 **(lanes 6 – 10)** or pAb421 **(lanes 11 – 15)**. **Lanes 1,6** and **11** are the immunoprecipitation products from 53-*Xho*I/*Bgl*II (159 – 1377 bp), **lanes 2,7** and **12** from pD,29 (221 – 1377 bp), **lanes 3,8** and **13** from pD,22 (354 – 1377 bp), **lanes 4,9** and **14** from pD,15 (372 – 1377 bp), and **lanes 5,10** and **15** from pD,35 (382 – 1377 bp). **(F)** Mapping of the epitope recognised by pAb421, pAb122 and pAb410. The following truncated protein products of pAli4,73 were immunoprecipitated as described in Materials and methods, by pAb419 (lanes indicated by capitals in **A** and **B**) or by pAb421 (lanes marked with small letters in **A**) or by pAb122 (lanes marked with small letters in **B**). **Lanes (A)** and **(a)** pI,5 (450 – 1277 bp), **lanes (B), (b), (J)** and **(j)** pI,9 (450 – 1278), **lanes (C)** and **(c)** pE,2 (450 – 1231 bp), **lanes (D)** and **(d)** pE, 21 (450 – 1231 bp), **lanes (E)** and **(e), (K)** and **(k)** pE,3 (450 – 1232 bp), **lanes (F)** and **(f)** pE,9 (450 – 1232 bp), **lanes (G), (g), (M)** and **(m)** 53-*Stu*I/*Bgl*II (1199 – 1377 bp), **lanes (H)** and **(h)** pI,30 (450 – 1260 bp), **lanes (I)** and **(i)** pI,11 (450 – 1150 bp), **lanes (L)** and **(l)** p90-22 (450 – 1221 bp), **lanes (N)** and **(n)** 53-*Acc*I/*Bgl*II, and **lanes (O)** and **(o)** Clone 9.

pAb200-47 (Dippold *et al.*, 1981) and pAb248 (Yewdell *et al.*, in preparation) (Figure 3A; results from pAb410 not shown), whereas all seven were able to immunoprecipitate the proteins encoded by pp53-5 (Figure 3B). Finer mapping of the individual epitopes recognised by each monoclonal was achieved by immunoprecipitation of each available set of deletions and is summarised in Table I and Figure 4.

Mapping of the epitope recognised by pAb242 (Yedwell *et al.*, in preparation) is shown in Figure 3C. The truncated protein encoded by the deletion mutant of pp53-5, pD,6, which has the first 145 bp of the p53 deleted, is still immunoprecipitated by pAb242. However the product encoded by the mutant pD,16 (missing the first 200 bp) is not precipitated by this antibody, indicating that the region of the p53 gene encoding the epitope lies between base pairs 145 and 200, or that base pair 200 is involved in the formation of the primary amino acid sequence of the epitope.

Mapping of the epitope recognised by pAb248 (Yewdell *et al.*, in preparation) is shown in Figure 3D. From Figure 3A, it is clear that the epitope lies within the p53 protein domain encoded by pAli4,73 (base pairs 450 – 1377). Immunoprecipitation of the proteins encoded by p53-*Pvu*II/*Bgl*II and Clone 9 (Benchimol *et al.*, 1983) by pAb248, indicated that the former was positive (data not shown) and the latter negative (Figure 3D). These two constructs thus indicate a stretch of amino acid residues that contain the epitope recognised by pAb248 (568 – 699 bp). Figure 3D also shows that deletion pD,32 encodes a protein that was precipitated by pAb248 (as expected since p53-*Pvu*II/*Bgl*II was positive) and the results obtained from immunoprecipitation of the p53-*Hind*III/*Bal*I and p53-*Bal*I/*Bgl*II products. The latter of these products gave a positive result indicating that the DNA sequence that encodes the antigenic site recognised by pAb248 lies downstream of the *Bal*I site and before the start of clone 9.

Mapping of the epitope recognised by pAb246 (Yewdell *et al.*, in preparation) is shown in Figure 3E. All deletion mutants up to and including pD,35 (missing the first 382 bp of p53) encode protein products that can be immunoprecipitated by pAb246. The fact that pAb246 cannot preciptate any protein products from pAli4,73, which contains the terminal two-thirds of p53, localises the epitope to being encoded by the first 450 bp of p53. Therefore the epitope lies on the stretch of amino acids encoded by base pairs 382 – 450.

The epitopes recognised by pAb421 (Harlow *et al.*, 1981) and pAb122 (Gurney *et al.*, 1980) are mapped in Figure 3F. The deletion mutants which encode protein products that indicate the boundaries of the epitope(s) are pI,9 and pE,3. pI,9 (missing base pairs 1278 – 1377 of p53) encodes a fusion protein that can be immunoprecipitated by pAb421 and pAb122 but those encoded by pE,3 (missing base pairs 1232 – 1377) cannot be precipitated by these antibodies. This indicates that the region encoding the epitope(s) recognised by pAb421 and pAb122 lies between base pairs 1232 and 1278. A finer mapping is obtained by immunoprecipitation of products encoded by Clone 9 (Benchimol *et al.*, 1983), base pairs 699 – 1257, which narrows the region down to 1232 – 1257. Although not shown, pAb410 gave identical immunoprecipitation patterns to pAb421 and pAb122. We were unable to distinguish between the epitope recognised by pAb421 and pAb122 at this resolution, although there is evidence that these epitopes are not identical (Crawford, 1982).

Table I. Summary of epitope mapping

Monoclonal antibody	Region containing the epitope	
	Base pair no.	Amino acid residue
pAb242	145(+) – 200(–)	9 – 25
pAb246	382(+) – 450(–)	88 – 109
pAb248	592(+) – 699(–)	157 – 192
pAb421	1232(–) – 1257(+)	370 – 378

The p53 cDNA (Jenkins *et al.*, 1984a) has 1377 bp, including the 3′ and 5′ non-coding regions and consists of a stretch of 389 amino acid residues.

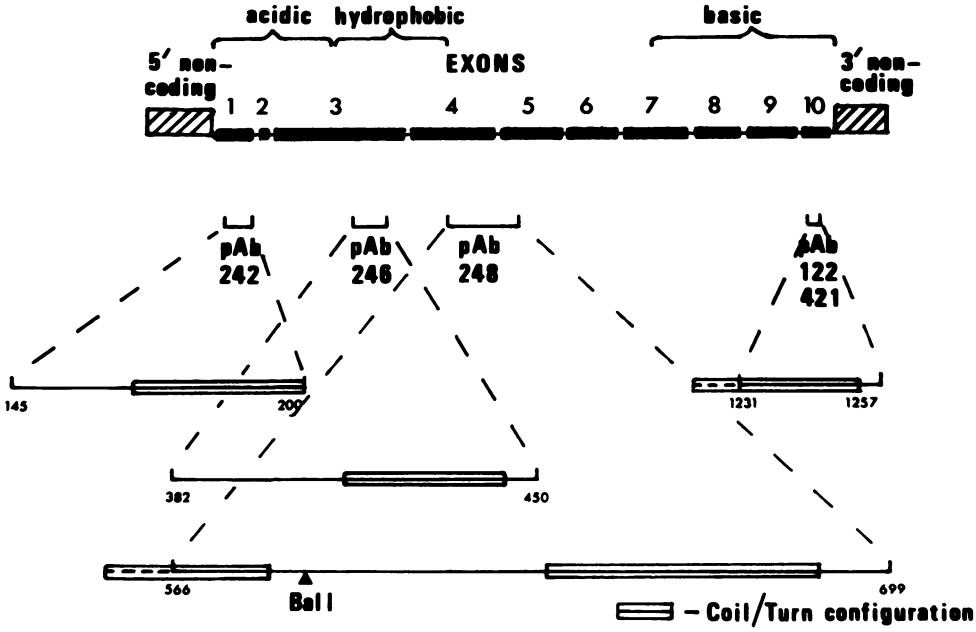

Fig. 4. Schematic representation of epitope mapping data. The solid boxes represent exons mapped within the p53 gene by Bienz *et al.* (1984). The cross-hatched areas indicate non-coding regions of the gene. The open boxes are regions of the gene which encode protein sequences capable of adopting coil/turn configurations (see text). The DNA sequences which encode the amino acid residues constituting the epitopes are indicated by a solid line below the diagrammatic representation of the gene. The numbers in small type at each end of these sequences are the nucleotide numbers given to the p53 cDNA clone (Jenkins *et al.*, 1984a).

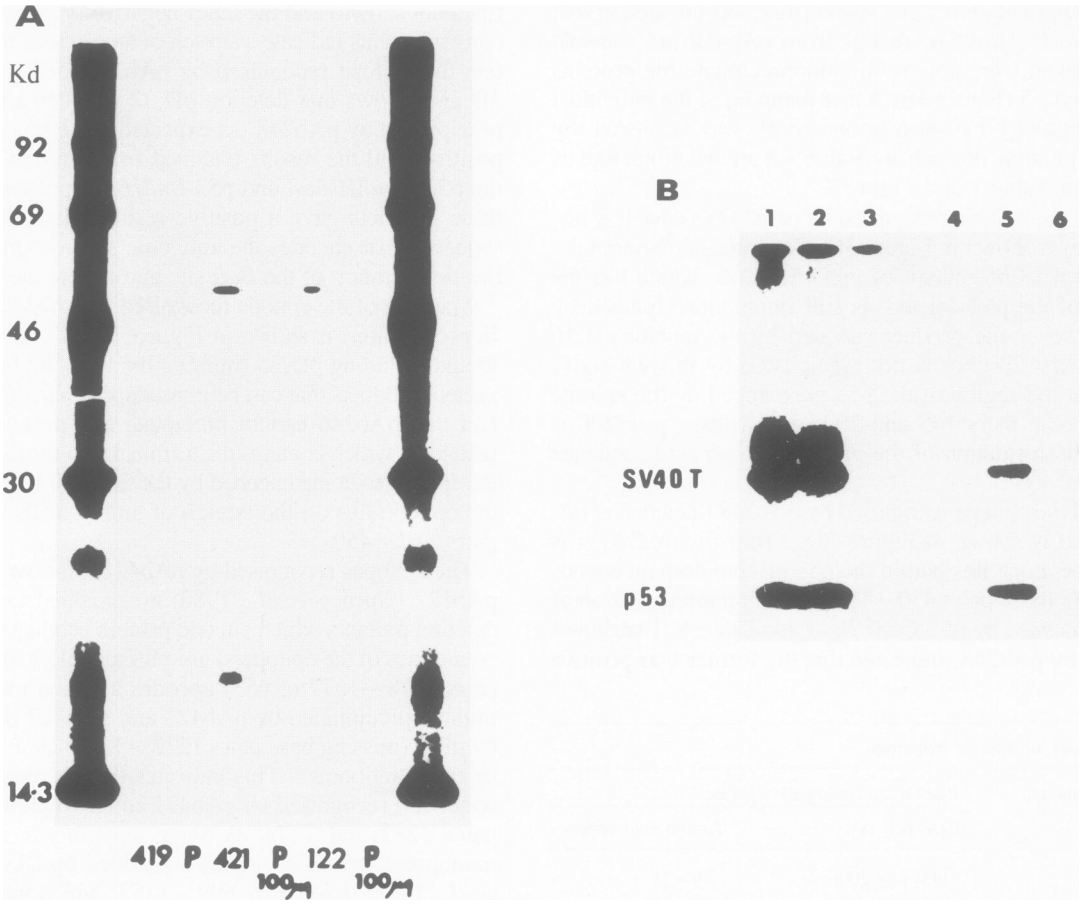

Fig. 5. (A) Competition studies between *in vitro* synthesised p53 and the synthetic peptide. The monoclonal/p53 complex (either pAb421, pAb122 or pAb419) was immobilised on Protein A-Sepharose beads as described in the text. The protein was eluted from the monoclonal antibody using 100 μg peptide, which was a vast molar excess over the antibody concentration, as shown in tracks marked P adjacent to the corresponding monoclonal. **(B)** Competition studies between *in vivo* synthesised p53 and the synthetic peptide. Either pAb421 or pAb419 was immobilised on Protein A-Sepharose beads as described in the text. Bound p53 from *in vivo* labelling of SV3T3 cells was eluted from the antibodies by adding 100 μg of peptide. **Lanes 1–3** are without peptide and **lanes 4–6** are samples of the eluant after peptide competition. **Lanes 1** and **4** are using pAb419, **lanes 2** and **5** are using pAb421 and **lanes 3** and **6** are using normal mouse serum.

The regions summarised in Table I, containing the sequences in which the epitopes lie, are defined by the closest pair of positive and negative deletions. It is possible that a negative deletion (indicated by − in Table I) has in fact interrupted the sequence that encodes the epitope. This appears to happen in the case of the p53-*AccI*/*BglII* deletion, where there is an interaction between pAb122 (and pAb421, not shown) and the truncated protein, but it is greatly reduced in intensity (Figure 3F, tracks N and n). So the boundaries to these epitopes, defined in Table I, are conservative.

Competition studies

A peptide was synthesised which covered the epitope predicted by pAb421, pAb410 and pAb122, stretching from nucleotides 1224 to 1253 inclusive (Jenkins *et al.*, 1984a); Lys-Lys-Gly-Gln-Ser-Thr-Ser-Arg-His-Lys. The pE,3 deletion terminates at the Gly position and the p53-*AccI*/*BglII* deletion commences within the first serine residue. Clone 9 terminates one lysine residue away from the right-hand end of the peptide. This decapeptide should thus contain within it the amino acid sequence required to form the epitope recognised by pAb421. It is also a denaturation-resistant epitope, suggesting that the tertiary structure of the protein is not required for its expression.

We then studied the ability of the peptide to elute p53 from pAb421 and pAb122 by using both *in vitro* and *in vivo* synthesised p53. Antibody/antigen complexes were immobilised on Protein A-Sepharose beads followed by incubation with peptide in vast molar excess (100 μg) with respect to the antibody concentration as described in Materials and methods. From Figure 5a and b it is clear that the peptide is capable of competing with both *in vitro* (Figure 5a) and *in vivo* (Figure 5b) synthesised p53 for the relevant monoclonal antibody. However the peptide cannot displace the full length protein from antibody/antigen complexes when an irrelevant antibody, such as pAb419, is used (Figure 5b, lanes 1 and 4). These data confirm the prediction that the peptide contains the antigenic site(s) recognised by pAb421 and pAb122.

Discussion

Using the techniques described above, we have demonstrated the presence of four distinct antigenic sites on p53, three of which, pAb242, pAb246 and pAb248, are mouse specific (D.P.Lane, personal communication), and one which is common to mouse and human p53 (Harlow *et al.*, 1981). The epitopes recognised by the monoclonal antibodies specific for mouse p53 lie towards the amino terminus of the molecule, either within the acidic region (residues 1–75, base pairs 123–347), pAb242; or close to the hydrophobic region (residues 76–149, base pairs 348–570), pAb246 and pAb248 (Figure 4), suggesting that the amino-

terminal region (30%) of this protein differs significantly between species. The epitopes present on human, monkey, rat and mouse p53, recognised by pAb421, pAb122 and pAb410, lie very close to the carboxy terminus of the p53 protein, within the basic region (residues 275–389) (Figure 4.) Since p53 from both human and mouse cells complexes with SV40 large T antigen, it is highly probable that the region of the protein that is involved in T-binding is that which is conserved between the two species, i.e., the carboxy-terminal basic region of the molecule. It is interesting that serum from a breast cancer patient also recognises the pAb421/pAb122 antigenic site, supporting the suggestion that this site can be immunogenic in a syngeneic host (Milner, 1984). Data from Milner (1984) indicating that the pAb241 epitope is only expressed in Concanavalin A-treated lymphocytes, but not in resting cultures and *vice versa* for pAb428, suggested the possibility of two p53 genes which can be switched on and off. Our data, however, indicate that both epitopes can be present on protein molecules encoded by a single cDNA, and therefore the treatment of lymphocytes with Concanavalin A must either induce a modification of the gene or a post-translational modification of the protein. Our mapping data locate the epitope recognised by pAb248 as being in the same region as a putative tyrosine phosphorylation site at amino acid residue 159, as described earlier (Jenkins *et al.*, 1984a).

Comparison of the epitope mapping data with a published structure for the mouse p53 genomic gene (Bienz *et al.*, 1984) reveals that each epitope lies within a separate exon (Figure 4). Secondary structure predictions using the algorithm of Chou and Fasman (1974) suggest that the mapped epitopes lie in regions of the protein capable of adopting a turn/coil configuration (Figure 4). It is interesting that recent studies (Tainer *et al.*, 1984) suggest that antigenicity correlates better with the mobility of exposed surface areas of the protein, rather than their ability to form coil or loop structures. It may be that the antigenic sites we have mapped represent plastic elements of the p53 protein surface. The epitope recognised by pAb421, pAb122 and pAb410 and the serum from a human cancer patient, has a high hydrophilicity index, whereas the other epitopes map in regions that do not correlate so precisely with the predicted hydrophilicity profile.

Since none of these monoclonal antibodies interfere with SV40 large T/p53 protein, it seems likely that the regions of primary amino acid sequence that flank these epitopes cannot be located at the interface of the p53/SV40 T complex.

The data we present show that epitope mapping can be applied as a novel route in the prediction of useful synthetic peptides, which can be used in conjuction with well characterised monoclonals in the immunoaffinity purification of non-abundant proteins.

Materials and methods

Bacterial strains

All plamids were transfected into *Escherichia coli* HB101 strain (Mandel and Higa, 1970). Transfections were plated out on LB agar containing 10 μg/ml tetracycline. Colonies were grown up as midi-preps in 10 ml of LB broth + 10 μg/ml tetracycline or as 400 ml maxi-preps (Birnboim and Doly, 1979).

Preparation of plasmid DNA

Plasmid DNA was isolated from cultures grown to stationary phase using the alkaline method of Birnboim and Doly (1979), followed by purification by banding in caesium chloride.

Reagents

Restriction enzymes, T4 DNA ligase, *E. coli* RNA polymerase and synthetic linkers were purchased from New England Biolabs. *Bal*31 exonuclease was purchased from Boehringer Mannheim. Klenow fragment of DNA polymerase was obtained from P-L Laboratories. The decapeptide, Lys-Lys-Gly-Gln-Ser-Thr-Ser-Arg-His-Lys, was purchased from Merseyside Laboratories.

Construction of expression vector and deletion libraries

The construction of Protem Hind, the expression vector used in this study, was described by Jenkins *et al.* (1984a), and is summarised in Figure 1. It contains the β-lactamase promotor and AUG initiation codon, 2 bp of the 5′ terminus of the β-lactamase coding region and 118 bp of the 3′ terminus. The p53 gene, with SV40 small t sequences upstream (generating the fusion protein) was inserted in-frame with the β-lactamase initiation codon, using *Hind*III/*Bgl*II restriction enzyme sites, pAli4,73. The full length p53 gene, which has some 5′ non-coding region present, was not placed in-frame, pp53-5.

All deletion libraries were generated using *Bal*31 double-stranded exonuclease (Boehringer-Mannheim) at a concentration of 0.5 Units/μg linearised plasmid DNA. The digest reactions contained 0.5 M NaCl, 40 μg/ml BSA, 4 mM Tris-HCl pH 8.0, plus 4 mM CaCl and were incubated at 30°C. Samples were removed at various time intervals and immediately transferred to two-thirds volume of 5 M ammonium acetate + 1 mM EDTA plus an equal volume of phenol, vortexed and stored on ice. After transferring the upper aqueous layer, the samples were precipitated by adding three volumes of ethanol, placing on dry ice for 5 min, warming to room temperature, then centrifuging on a bench micro-centrifuge for 5 min. The samples were precipitated a second time using 0.3 M sodium acetate and 75% ethanol, before ligating the deleted plasmid to synthetic *Bgl*II linkers (3′ deletions) or *Hind*III linkers (5′ deletions). The deleted gene was then removed from the plasmid by either *Hind*III/*Bgl*II digestion, or *Eco*RI/*Bgl*II digestion in the case of pAli4,73; and separated on a 0.8% LGT agarose gel containing 1 μg/ml ethidium bromide. The inserts were religated to an undeleted expression vector (Protem Hind digested with *Hind*III/*Bgl*II).

The plasmid containing the full length p53 gene, pp53-5, was used to generate two series of deletions. By linearising pp53-5 with *Hind*III and treating with *Bal*31 exonuclease, as described above, a set of 5′ deletions of p53 were obtained; by linearising the same plasmid with *Bgl*II and treating with *Bal*31, a set of 3′ deletions were obtained (Figure 2). In order to keep the 5′ deletions in-frame with the β-lactamase AUG, the *Hind*III site was filled in by T4 DNA polymerase (Klenow fragment) repair, followed by religation; or by Klenow repair and ligation to a synthetic *Bam*HI linker.

The plasmid, pAli4,73 containing the SV40/p53 fusion protein gene, was used to generate two sets of deletions; by linearising using *Bgl*II, followed by *Bal*31 digestion, 3′ deletions were constructed as with pp53-5 (Figure 2). However 5′ deletions were made using unique restriction enzyme sites within the p53 gene, due to the loss of the 5′-terminal *Hind*III site (Figure 2).

All deletions were sequenced using the method of Maxam and Gilbert (1977) to determine their exact positions within the gene.

Monoclonal antibodies

The monoclonal antibodies, pAb419 (Harlow *et al.*, 1981), pAb421 (Harlow *et al.*, 1981), pAb410 (Harlow *et al.*, 1981), pAb607 (Gooding, unpublished), RA3 2C2 (Coffman and Weissman, 1981) and pAb200-47 (Dippold *et al.*, 1981) were a gift from D. P. Lane. The hybridoma pAb122 was generously provided by E. Gurney (Gurney *et al.*, 1980). pAb242, pAb246 and pAb248 were also generously provided by D. P. Lane, having been produced and characterised in his laboratory (Yewdell *et al.*, in preparation).

In vitro transcription/translation and immunoprecipitations

All methods used were as described by Jenkins *et al.* (1984a).

In vivo labelling of p53

A 150 cm^3 flask of subconfluent SVA3 1E7 was incubated for 4 h in low phosphate Dulbecco's modified Eagle's medium (DMEM), then labelled for 4 h at 37°C in 5 ml of phosphate free DMEM + 2 mCi of [^{32}P]orthophosphate (Amersham). The cell monolayer was washed twice with PBS and then lysed in 5 ml of RIPA buffer, as described by Van Roy *et al.* (1981), but omitting the SDS. The supernatant was harvested by decanting from the flask and clarified by centrifugation at 30 K for 30 min in a Sorvall angle-head rotor. 400 μl aliquots were stored at −70°C. Before use the aliquots were thawed and pre-cleared with Protein A-Sepharose (Pharmacia).

Competition assay between synthetic peptide and in vitro synthesised p53

The p53 cDNA constructs pAli4,73 and pp53-5 were transcribed and translated as above. The translation mix was taken up in 1 ml NET/BSA + 0.5 M NaCl and pre-cleared with 40 μl Protein A-Sepharose (Pharmacia) for 15 min at room temperature. The sample was spun in a bench microcentrifuge for 3 min to remove any Sepharose beads and the supernatant incubated overnight with the appropriate monoclonal antibody – using pAb421 (Harlow *et al.*, 1981) or pAb122 (Gurney *et al.*, 1980) as test monoclonals and pAb419 as a control. 40 μl of Protein A-Sepharose was added to each sample and incubated for 15 min at room temperature. The Protein A-Sepharose was pelleted as before and washed with 1 ml NET/GEL + 0.5 M NaCl. The pellet was resuspended in 100 μl NET/GEL containing 100 μg of peptide and incubated at room temperature for 1 h. The sample was respun

and the pellet washed three times as in a normal immunoprecipitation (Jenkins *et al.*, 1984a). The resulting pellet was resuspended in 20 μl loading buffer, containing β-mercaptoethanol, and boiled for 5 min prior to loading onto a 10% SDS-polyacrylamide gel as described by Laemmli (1970), with the same modifications as previously (Jenkins *et al.*, 1984a).

Competition assay between synthetic peptide and in vivo labelled p53

SVA3 1E7 cell monolayers were labelled and the cell lysates harvested as described above. 100 μl of cell lysate was diluted with 0.5 ml NET/BSA + 0.5 M NaCl, pre-cleared with Protein A-Sepharose (Pharmacia), then incubated for 30 min at 0°C with either pAb421 or pAb419 (Harlow *et al.*, 1981). 40 μl of 1 mg/ml Protein A-Sepharose was added to each sample and incubated for 15 min at room temperature. The Protein A-Sepharose was pelleted by centrifugation. The pellet was washed with NET/GEL and then incubated with 100 μg peptide in 40 μl NET/GEL for 30 min at room temperature. The Protein A-Sepharose was pelleted again and 20 μl of the supernatant removed and added to 30 μl load buffer. A control experiment in which the cell lysate was diluted in NET/BSA + 0.5 M NaCl, pre-cleared as before, but not incubated with peptide was also done. The pellet from this experiment was washed with NET/GEL as before, then taken up in 20 μl load buffer. All samples were boiled prior to loading onto 10% polyacrylamide gels as before.

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