JOURNAL OF VIROLOGY, Aug. 1986, p. 444–452
0022-538X/86/080444-09$02.00/0

Vol. 59, No. 2

Monoclonal Antibody Analysis of p53 Expression in Normal and Transformed Cells

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Received 27 January 1986/Accepted 24 April 1986

The cellular phosphoprotein p53 binds tightly and specifically to simian virus 40 T antigen and the 58,000-molecular-weight adenovirus E1b protein. Many human and murine tumor cell lines contain elevated levels of the p53 protein even in the absence of these associated viral proteins. Recently the cloned p53 gene, linked to strong viral promoters, has been shown to complement activated *ras* genes in transformation of primary rodent cell cultures. Overexpression of the p53 gene alone rescues some primary rodent cell cultures from senescence. We isolated three new monoclonal antibodies to the p53 protein, designated PAb242, PAb246, and PAb248, and mapped the epitopes they recognized on p53 in comparison with other previously isolated antibodies. At least five sterically separate epitopes were defined on murine p53. One of the antibodies, PAb246, recognizes an epitope on p53 that is unstable in the absence of bound simian virus 40 T antigen. This effect is demonstrable in vivo and in newly developed in vitro assays of T-p53 complex formation. Using the panel of anti-p53 antibodies and sensitive immunocytochemical methods, we found that p53 has a predominantly nuclear location in established but not transformed cells as well as in the vast majority of transformed cell lines. Several monoclonal antibodies to p53 showed cross-reactions with non-p53 components in immunocytochemical staining.

The cellular protein p53 is tightly complexed to simian virus 40 (SV40) T antigen in SV40-transformed (19, 24, 25) and productively infected (14) mammalian cells and is complexed with adenovirus 58,000-molecular-weight (58K) protein E1b in adenovirus-transformed mouse cells (39). The protein is very unstable in established but nontransformed cell lines, e.g., 3T3 cells, with a half-life of around 20 min, and is present in such cells in minute amounts. When p53 is complexed to T antigen, its half-life in the cell increases dramatically to around 24 h, and thus SV40-transformed 3T3 cells contain up to 1,000 times as much p53 as the parent 3T3 cells from which they were established (32). Many human and mouse transformed cell lines established either by in vitro transformation or from in vivo tumors contain elevated levels of p53 protein (1, 6, 7, 17, 35, 37, 42), and sera from tumor-bearing animals and human patients frequently contain anti-p53 antibodies (5, 7, 19, 24, 38). The normal cellular role of the p53 protein, the significance of its complexing to viral "oncogene" products in virus-transformed cells, and the basis of its elevated levels in many other transformed cells are unclear (3). Recent studies have strongly suggested that p53 may play a critical role in the control of cell growth and imply that alterations of p53 level may be causally associated with aspects of the neoplastic phenotype. Microinjection of monoclonal antibodies to p53 into the nucleus of quiescent fibroblasts can ablate the DNA synthesis response of the cells to serum stimulation (26, 27). Transfection of the cloned p53 gene linked to a strong viral promoter can rescue primary rodent cells from senescence (18); can complement activated *ras* genes in transformation of primary rodent cells (10, 18, 33); can convert a naturally regressing, p53-negative, Abelson virus-induced lymphoma cell line into a line that gives rise to progressive lethal tumors (44); and can make established cells tumorigenic (9). We have isolated three new monoclonal antibodies to p53, characterized them extensively in a variety of immunoassays, and compared them with six other existing anti-p53 monoclonal antibodies. We can define five sterically discrete epitopes on murine p53. One of the new antibodies recognizes an epitope on p53 that is stabilized in vivo and in vitro by the complexing of SV40 T antigen to p53. Immunoperoxidase staining of a range of transformed and normal cell lines with the panel of anti-p53 antibodies has shown that p53 is a nuclear protein in both transformed and nontransformed cells.

MATERIALS AND METHODS

Eucaryotic cells and virus stocks. Line 293 cells were obtained from P. Gallimore, University of Birmingham, Birmingham, U.K. SVA31 E7 cells were obtained from Y. Ito, Imperial Cancer Research Fund, London, U.K. 3T6 cells were from J. Milner, Cambridge University, Cambridge, U.K. T3T3 cells, a spontaneously transformed mouse cell line derived from 3T3 cells, were isolated at Imperial College, London. SV40-transformed human cells were obtained from B. Lane, Imperial Cancer Research Fund.

Adenovirus type 5 (Ad5) SVRIII virus was obtained from Y. Gluzman, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.; SV40 (strain 830) was from P. Rigby, Imperial College. All cells were grown at 37°C in a humidified incubator gassed at 10% (vol/vol) CO_2 in air. The cell culture medium was Dulbecco modified Eagle minimum essential medium supplemented with 10% (vol/vol) fetal calf serum plus 500 U of penicillin and 100 μg of streptomycin per ml.

Monoclonal antibodies. The antibodies used in this study and the original references describing their properties as anti-T or anti-p53 reagents are as follows: PAb421 and PAb419 (13); PAb122 (12); PAb607 (L. Gooding, unpublished data); PAb242, PAb246, and PAb248 (this paper); RA3-2C2 (37); 200.47 (8); PAb1005 (42); and PAb204 (20).

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Cells producing the following antibodies were gifts from the following: PAb419 and PAb421, E. Harlow; PAb607, L. Gooding; 200.47, W. Dippold; RA3-2C2, V. Rotter.

All the monoclonal cell lines were grown as described above, and the antibody they secreted was purified by protein A-Sepharose chromatography (11). The purified antibodies were iodinated using the iodogen-coated tube method to specific activities of 1 to 2 μCi/μg.

Cell extracts. Cell monolayers (SVA31 E7, T3T3, or 293 cells; 293 cells were infected 24 h earlier with Ad5 SVRIII virus at 5 PFU per cell) were rinsed in TD (25 mM Tris hydrochloride, pH 7.4, 136 mM NaCl, 5.7 mM KCl, and 0.7 mM Na_2HPO_4) and harvested by incubating in TD containing 10 mM EDTA until cells became detached from the dishes. The cells were collected, counted, and centrifuged (2,000 × *g* for 5 min), and the pellets were snap frozen in a bath of ethanol and solid CO_2. The pellets were stored at −70°C. Extracts were prepared by thawing the cell pellets rapidly and suspending them in extraction buffer (150 mM NaCl, 5 mM EDTA, 50 mM Tris hydrochloride, pH 8.0, 2 mM phenylmethylsulfonyl fluoride, 1% Nonidet P-40) at a ratio of 1 ml of buffer per 4×10^7 cells. The extract was kept on ice for 30 min and then clarified by centrifugation at 10,000 × *g* for 30 min. The supernatants were used immediately.

Solid-phase radioimmunoassay. The solid-phase radioimmunoassay used was a modification of that employed by Benchimol et al. (1). A 50-μl volume of purified antibody, (30 μg/ml in 10 mM phosphate buffer, pH 7.5) was pipetted into each well of a flexible microtitre dish (Falcon) and allowed to absorb overnight in a humidified chamber. The plate was rinsed in phosphate-buffered saline (PBS) and blocked in 3% bovine serum albumin for 3 h. Plates were finally rinsed in PBS before either immediate use or storage at −20°C. A 50-μl sample of clarified cell lysates (see above) was applied to each well of the antibody-coated plate, incubated overnight, and then rinsed in PBS. Then, 50 μl of ^{125}I-labeled antibody was pipetted into the wells, incubated for 3 h, and rinsed in PBS. Each well was counted in an LKB Wallac 80,000 gamma counter.

In vitro assay of p53 SV40 T-antigen association. The association between p53 and SV40 T antigen was assayed in vitro using methods based on the solid-phase radioimmunoassay. First, 50 μl of clarified cell lysate from T3T3 cells and 50 μl of Ad5 SVRIII-infected 293 cell lysate were incubated together for 3 h. The mixture was transferred to either an RA3-2C2 antibody-coated well or a PAb419 antibody-coated well in a plastic microtiter plate, allowed to incubate overnight, and then rinsed in PBS. A 50-μl volume of ^{125}I-labeled PAb419 (for RA3-2C2-coated plates) or PAb248 (for PAb419-coated plates) was added to each well, incubated for 3 h, and rinsed in PBS, and the individual wells were cut out and counted as described. For titration purposes the T3T3 or infected 293 cell extracts were diluted in extraction buffer.

Western blotting, immunoprecipitation, and gel electrophoresis. Western blotting, immunoprecipitation, and gel electrophoresis were carried out as described by Montano and Lane (30) and Mole and Lane (29).

Immunoperoxidase staining. Cells grown to 50% confluence were fixed on the plastic culture dishes or in the microtitre wells either with 50% acetone–50% methanol for 2 min or with 3% paraformaldehyde in PBS for 15 min followed by 50% acetone–50% methanol for 1 min. After fixation the cells were washed in PBS and incubated with monoclonal antibody, either as cell culture supernatants or as pure antibody diluted in 10% fetal calf serum in PBS, for up to 12 h. The plates were then rinsed in PBS and incubated with a rabbit anti-mouse immunoglobulin-horseradish peridoxase conjugate as described by Montano and Lane (30). The substrate *O*-dianisidine was prepared and used also as described earlier (30).

Hybridoma fusions, cloning, and screening. The method for fusion using polyethylene glycol and SP20/AG14 myeloma cells has been described (30). The fusions were plated out over 800 to 1,000 separate wells, and the hybrids were screened using immunocytochemical staining of SVA31 E7 cells and T3T3 cells which had been grown in microtitre dishes. Hybridoma cells from cells whose supernatants gave positive nuclear staining on both cell types were cloned three times through agarose (2), grown in bulk, and frozen down. The lines described have been completely stable in antibody production for the last 2 years.

RESULTS

Isolation of monoclonal antibodies. The new anti-p53 monoclonal antibodies described here were derived from two separate fusions and named PAb242, PAb246, and PAb248 (4). In the first fusion, from which PAb248 and PAb246 arose, a C57 Black mouse was the donor of the splenocytes. In the second fusion, from which PAb242 was isolated, the spleen donor was a BALB/c mouse. The immunization protocol used in both cases consisted of a series of interperitoneal injections of 5×10^6 cells of the SV40-transformed cell line BALB/c SVA31 E7 in Freund complete adjuvant, followed by a final boost without adjuvant 3 days before fusion. We devised a rapid and very reliable screening assay for these fusions by modifying the immunoperoxidase screen described by Lane and Lane (21). Supernatants from each hybridoma fusion were screened against a range of target cells which had been plated out over 96-well microtitre trays and then fixed with 50% acetone–50% methanol. The target cells used in the primary screen included SV40-transformed mouse fibroblasts, which showed strong nuclear staining with both existing anti-T and existing anti-p53 monoclonal antibodies; spontaneously transformed mouse fibroblasts that stained strongly with anti-p53 antibodies but not with anti-T antibodies; and untransformed 3T3 cells, which did not stain strongly with either type of antibody. The predominant phenotype of the hybridoma supernatants arising in these fusions was nuclear staining of the SV40-transformed fibroblasts only. These supernatants on subsequent, more detailed analysis proved to contain antibodies to SV40 large T. The three antibodies described here in detail were picked out originally because, in addition to staining the SV40-transformed cell nuclei, they also stained the nuclei of the spontaneously transformed 3T3 cells but failed to give a strong stain on the normal 3T3 cells. This method of screening is rapid, cheap, reliable, and very informative. The new hybridomas were cloned three times by the agarose technique (2). The three lines are stable and produce high levels of antibody when grown either in vitro or in vivo as ascites tumors (16). PAb248 and PAb246 were grown in BALB/c × C57F1 animals.

Characterization of the new antibodies by western blotting and immunoprecipitation. The three new putative anti-p53 antibodies were further characterized by Western blotting of extracts of SV40-transformed and spontaneously transformed 3T3 cells (Fig. 1) and by immunoprecipitation of ^{35}S-labeled extracts of these cells. PAb248 (Fig. 1, lane 1) and PAb242 (lane 3) both worked well in Western blots, binding to a single species of 53,000 molecular weight that

precisely comigrated with the species detected by PAb122 (lane 4) and PAb421 (lane 5). A band of identical mobility was also detected by RA3-2C2 (lane 6), but this antibody also reacted with other discrete bands in the extract. Antibody 200.47 (lane 7) gave a very weak reaction, whereas PAb607 (lane 8) and PAb1005 (lane 9) showed quite extensive nonspecific reactions. PAb204 (lane 10), used as a control, gave a strong reaction with a 68,000-molecular-weight protein (20).

PAb248 and PAb242 completely failed to react with the T antigen present in the SV40-transformed cell extracts in the blotting procedures (data not shown). PAb246 (Fig. 1, lane 2) gave no specific reaction with any of the extracts in the blotting procedure.

Immunoprecipitation analysis supported the identification of PAb248, PAb246, and PAb242 as anti-p53 antibodies since they each efficiently immunoprecipitated both p53 and complexed large T from ^{35}S-labeled extracts of SV40-transformed fibroblasts, but only p53 from extracts of ^{35}S-labeled spontaneously transformed 3T3 cells (data not shown).

The anti-p53 specificity of the three antibodies has recently been confirmed by their ability to immunoprecipitate in vitro translation products encoded by fragments of p53 mRNA produced from the cloned p53 gene in *Escherichia coli* (43). The three antibodies bind specifically to p53 β-galactosidase fusion proteins expressed in *E. coli* and to p53 chloramphenicol acetyltransferase fusion proteins expressed in eucaryotic cells (R. Bartsch, N. Williamson, J. Gannon, S. Mole, and D. P. Lane, manuscript in preparation).

Analysis of anti-p53 antibodies by radioimmunometric assay. (i) Competition studies using the T-p53 complex as antigen immobilized by anti-T antibody. PAb242, PAb246, and PAb248 were purified from cell culture supernatants by protein A-Sepharose chromatography (11) and then iodinated with ^{125}I to high specific activity by the iodogen method. The specificity of the antibodies and the steric relationship between the epitopes they recognized and the epitopes recognized by a range of existing anti-p53 antibodies were evaluated in a variety of radioimmunometric assays. The first assay design reflected that employed by Benchimol et al. (1), in which plastic microtitre trays are first coated with the anti-T antibody PAb419 and then incubated with cell extract containing T-mouse p53 complex. The binding of iodinated anti-p53 antibodies to the complex-coated wells is then evaluated in the presence and absence of unlabeled competitor anti-p53 antibody. We performed these experiments over a 1,000-fold range of unlabeled inhibitors for each antibody evaluated (Table 1). All three new antibodies bound the immobilized T-p53 complex effectively and specifically. There was a very low background level of binding to control wells that had been coated with antibody but not with antigen. A number of important conclusions can be drawn from the experiments, and these have been confirmed and extended in the subsequent studies described below. PAb242, PAb246, and PAb248 recognize three sterically discrete epitopes, and although effective homologous competition is seen, they do not compete for each other's binding. The binding of PAb242 to p53 is not inhibited by any other anti-p53 antibody in the panel, and this antibody therefore defines a new, sterically discrete epitope on p53. The binding of pAb248 is inhibited strongly by RA3-2C2, and a clear but less potent inhibition is also seen with PAb607. None of the other antibodies in the panel, apart from PAb248 itself, inhibits PAb248 binding. The binding of PAb246 is inhibited strongly only by PAb607. By combining the data obtained here with the earlier studies of Benchimol et al. (1) and the fragment mapping experiments of Wade-Evans and Jenkins (43), a rather comprehensive antigenic map of murine p53 can be drawn (Fig. 2). The data are in complete agreement at the points where the data obtained here duplicated those of Benchimol et al. (1). We also confirmed these results by reciprocating the inhibitor and the label. In particular, we verified that labeled RA3-2C2 binding to p53 is blocked by PAb248 but not by PAb607 or any other antibody.

In addition, we have shown that the primate-specific anti-p53 monoclonal antibody PAb1005 (42) competes for PAb421 but not PAb122 binding to human p53. PAb421 and PAb122 compete in this assay (Table 2). Thus all nine anti-p53 antibodies can be distinguished from each other by

TABLE 1. Steric competition between anti-murine p53 antibodies[a]

Unlabeled competing antibody	% Inhibition with ^{125}I-labeled antibody probe:		
	PAb242	PAb246	PAb248
PAb242	9	105	127
PAb246	102	2	94
PAb607	70	10	44
PAb248	146	102	16
RA3-2C2	92	73	20
PAb421	112	89	74
PAb122	79	82	80
200.47	91	90	97

[a] Results are expressed as percent inhibition of binding of the radiolabeled antibody in the presence of a large excess (100 μg/ml) of the indicated unlabeled competitor. Thus, in the absence of any inhibition the value would be 100. The test was repeated many times over a wide range of competing antibody concentrations with broadly similar results.

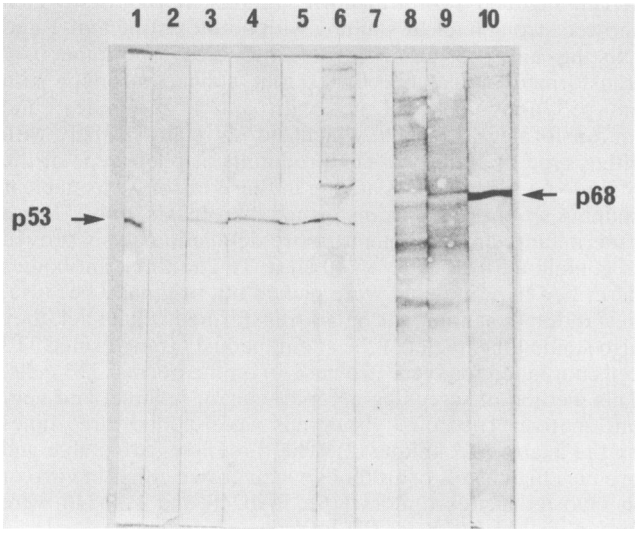

FIG. 1. Western blotting analysis of anti-p53 antibodies. A T3T3 cell extract was separated on a single-slot 15% polyacrylamide gel, and the separated proteins were transferred electrophoretically to nitrocellulose. The nitrocellulose sheet was cut into strips, and separate strips were incubated with the different antibodies tested. The blot was developed using a rabbit anti-mouse peroxidase second antibody and *O*-dianisidine substrate. Lanes: 1, PAb248; 2, PAb246; 3, PAb242; 4, PAb122; 5, PAb421; 6, RA3-2C2; 7, 200.47; 8, PAb607; 9, PAb1005; 10, PAb204.

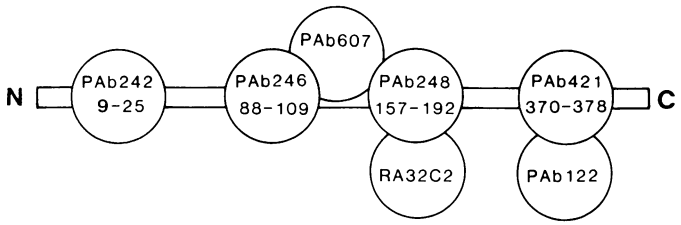

FIG. 2. Epitope map of murine p53. Data from Tables 1 through 3 have been combined with the linear mapping data of Wade-Evans and Jenkins (43) to provide an epitope map for p53. Steric competition is indicated by overlapping circles. The degree of overlap reflects the efficiency of competition (see text).

their unique behavior in these competition radioimmunoassays.

(ii) Competition studies using free p53 and the T-p53 complex as antigen immobilized by anti-p53 antibody. The steric competition studies were extended, using both T-p53 complex and free p53 (from extracts of spontaneously transformed 3T3 cell line T3T3) as antigen immobilized on the microtitre plate with a panel of different anti-p53 antibodies. In this experimental design the binding of iodinated anti-p53 antibodies to the solid-phase p53 was assessed directly in the absence of any competing antibody (Tables 3 and 4). It was clear that PAb242-coated plates were capable of immobilizing only small amounts of p53 and that ^{125}I-labeled PAb242 bound p53 relatively inefficiently. This result was confirmed over a range of concentrations of solid-phase pure PAb242 and with PAb242 iodinated under a variety of conditions. The simplest explanation is that the antibody is of relatively low affinity. PAb248 coats the plates very efficiently and is also very sensitive as a labeled antibody probe. The combinations of PAb248 and PAb421, or RA3-2C2 and PAb421, are probably the best for the radioimmunometric detection of murine p53. PAb248 is probably to be preferred to RA3-2C2 since it is far less cross-reactive, at least in the Western blot procedure (see Fig. 1).

Examination of the data obtained with PAb246 in these experiments revealed a curious anomaly. When T-p53 complex was used as the antigen, PAb246 acted as an effective solid-phase absorbant for the antigen and also as an effective iodinated probe; up to 56% of the input counts were retained at high antigen concentration immobilized on the noncompeting antibody RA3-2C2, for instance. When uncomplexed p53 was used as an antigen, PAb246 was able to act effectively as a solid-phase absorbant but failed completely to detect p53 when used as the iodinated probe. The extensive panel of data clearly indicates that the antigen is present in the extract and that both the iodinated PAb246 and the PAb246 antibody-coated plates are effective. The phenomenon was investigated over a wide range of antigen concentrations and is illustrated for the PAb248-PAb246 combinations in Fig. 3. This effect has been studied in some detail (see below) and appears to reflect a stabilization of the PAb246 epitope on p53 complexing to T.

In other respects, these assays using anti-p53 antibodies as solid phase confirmed the steric epitope maps obtained using anti-T-immobilized p53-T complex. It is notable, in comparing Tables 3 and 4, that the use of identical antibody as solid phase and iodinated probe detected p53 more efficiently in the SVA31 E7 extract than in the T3T3 extract, though always at a lower level than noncompeting pairs of antibodies. This phenomenon probably reflects the more multimeric nature of the T-p53 complex in the SVA31 E7 extracts rather than the free p53 in the T3T3 extracts. The homologous pairs presumably only measure multimeric p53.

In vitro assay of T-p53 complex formation. A radioimmunoassay was established to measure the formation of the complex between p53 and SV40 large T antigen in vitro. The assay exploits the specificity of anti-T and anti-p53 antibodies, and therefore neither the p53 nor T antigen need be pure. The assay does, however, function with completely pure T (40). The experiments here used an extract of 293 cells infected with the defective adenovirus-SV40 recombinant virus Ad5 SVRIII as a source of T antigen. None of this T antigen was detectably complexed to p53, probably because the p53 was already complexed to the adenovirus E1b protein present in the 293 cells. Extracts of the spontaneously transformed 3T3 cell line T3T3 were used as a source of p53. Complex assembly was assessed by preincubating the mixture of cell extracts at various ratios overnight and

TABLE 3. Sandwich assay of p53 in SVA31 E7 cells[a]

Solid-phase antibody	% ^{125}I-labeled antibody probe			
	PAb242	PAb246	PAb248	PAb421
PAb242	2.6	6	7	5
PAb246	3.5	12	34	22
PAb248	4.0	42	16	27
RA3-2C2	3.7	56	33	33
PAb421	3.9	41	42	10
PAb122	2.7	8.5	11	4
200.47	3.3	25	28	17
PAb419	4.2	51	48	37

[a] Two-site assay of p53 using a range of different anti-p53 antibodies as the solid phase and the labeled probe. The combinations shown were tested over a 1,000-fold range of SVA31 E7 cell extract concentrations, and the results are expressed as percent of the input antibody label bound using 50 μl of the undiluted extract.

TABLE 2. Steric competition between anti-human p53 antibodies[a]

Unlabeled competing antibody	% Binding of ^{125}I-labeled PAb1005 probe
PAb421	29
PAb122	68
PAb1005	35
PAb246	100

[a] Inhibition of binding of iodinated PAb1005 to the human p53-large T complex immobilized on PAb419 plates, expressed as percent of control binding in the absence of inhibitor. All the inhibiting antibodies were used over a range of concentration. Data shown were obtained using a concentration of 10 μg/ml.

TABLE 4. Sandwich assay of p53 in T3T3 cells[a]

Solid-phase antibody	% ^{125}I-labeled antibody probe			
	PAb242	PAb246	PAb248	PAb421
PAb242	0.4	0.4	2.8	2.6
PAb246	3.0	1.4	23.5	13.3
PAb248	2.3	1.6	4.8	18.4
RA3-2C2	2.5	2.0	11.2	26.2
PAb421	2.3	1.2	32.2	4.7
200.47	2.0	1.3	20.0	11.4
PAb419	0.4	0.2	0.4	0.4

[a] Two-site assay, using the same solid phase and labeled antibodies as in Table 4 to measure p53 in T3T3 cell extracts. The results are expressed as before, using the value of antibody bound using 50 μl of undiluted T3T3 cell extract.

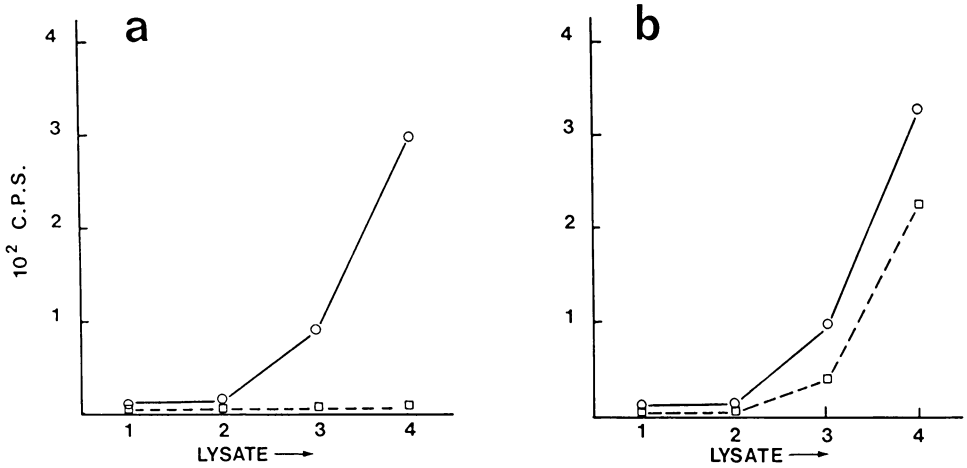

FIG. 3. Solid-phase radioimmunoassay of p53 in SV40-transformed and spontaneously transformed 3T3 cells. (a) Solid-phase antibody, PAb248; radiolabeled antibody, PAb246. (b) Solid phase, PAb246; radiolabeled antibody, PAb248. Titers of cell lysates from the SV40-transformed (○) and the spontaneously transformed (□) cells were determined over a 10,000-fold range of dilutions. Point 1 is equivalent to 2×10^3 cells, and point 4 is therefore equivalent to 2×10^6 cells.

then adding the extracts to plates coated with anti-T (PAb419) or anti-p53 (RA3-2C2) and probing for the presence of complex with iodinated anti-T or anti-p53 antibodies, as appropriate. The assay yields reproducible data and permits titration of both the T and p53 components into the complex. A titration of T is illustrated in Fig. 4. Background in the absence of complex but in the presence of either individual cell extract was exceptionally low. Using noncompetitive iodinated anti-p53 antibodies (PAb421) or noncompetitive solid-phase anti-T antibodies (PAb205), it was possible to measure both components of the complex individually. From these results it appears that whereas all p53 molecules are able to complex to T, only a fraction of T molecules are able to complex to p53. This does not appear to be a unique property of Ad5 SVRIII T antigen, since similar results have also been obtained using T antigen derived from lytic infections of CV1 cells (D. P. Lane and J. V. Gannon, manuscript in preparation).

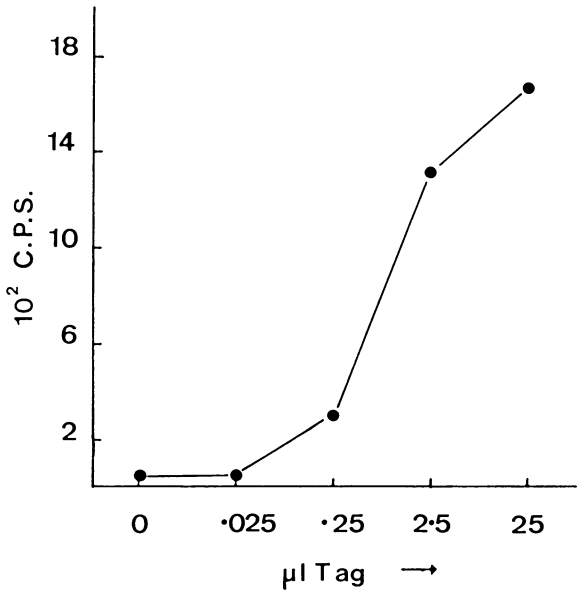

FIG. 4. In vitro formation of the T-p53 complex. A 50-μl volume of T3T3 cell extract (p53 extract) was incubated with the indicated quantities of extract from Ad5 SVRIII-infected 293 cells (large T; T ag). The mixtures were transferred to RA3-2C2-coated plates (anti-p53) and probed with iodinated anti-T PAb419. In the absence of the p53 extract, binding of PAb419 to the plate was at background levels even at the highest concentration of T extract used.

The effect of complexing to T antigen on the epitope structure of p53 was investigated by measuring p53 concentrations with various combinations of anti-p53 antibodies in the presence of increasing amounts of T antigen. This assay has the potential to reveal masking of epitopes by the site of complex formation. No such result was obtained, and further studies have indicated that none of the anti-p53 antibodies inhibits complex formation, whereas certain anti-T antibodies clearly do so (Lane and Gannon, in preparation). A striking effect was observed on the PAb246 epitope, however. When PAb246 was used as the iodinated probe and PAb248 was the solid phase, then the PAb246 epitope could only be detected on p53 in the presence of T antigen (Fig. 5). The titration of the PAb246-positive p53 molecules closely reflected the titration of the formation of the T-p53 complex. These results fit in well with the results described above, obtained with PAb246 as an iodinated probe in assays of p53 from extracts of cells containing T-p53 or free p53.

Immunocytochemistry of p53. The patterns of reactivity of the anti-p53 antibodies were assessed by using the sensitive immunoperoxidase test to stain fixed cells of a range of species. In examining SV40-transformed cells of human, rat, and mouse origin, clear differences in reactivity were observed. All the antibodies gave a clear positive nuclear stain on SV40-transformed mouse fibroblasts and on the spontaneously transformed mouse T3T3 fibroblast line, with the sole exception of PAb1005. Clear nuclear staining of the SV40-transformed rat cell line was seen with PAb122 and PAb421. PAb242, PAb246, and PAb1005 were clearly negative on these cells. PAb607 and RA3-2C2 showed quite a strong diffuse stain on these cells, which is probably cross-reactive, reflecting their behavior on Western blots. PAb248 stained a discrete extranuclear structure, possibly the Golgi apparatus, in the SV40-transformed rat cells, but failed to stain the nucleus. PAb122, PAb421, and PAb1005 all clearly stained the nuclei of the SV40-transformed human cells. No staining was seen in human cells with PAb242, PAb246, or

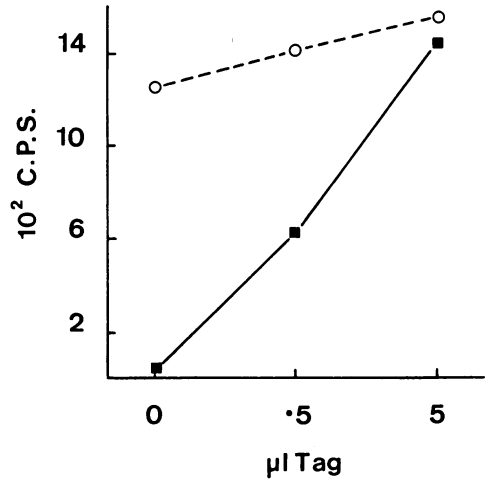

FIG. 5. In vitro stabilization of the PAb246 epitope by large T. A 50-µl sample of p53 extract was incubated with the indicated amount of a large-T extract as described for Fig. 4. The effect of complex formation on the PAb246 epitope was evaluated by transferring the reaction mixture to a PAb248 solid-phase plate and then probing with either radiolabeled PAb246 (□) or, as control, radiolabeled PAb421 (○).

200.47. RA3-2C2 and PAb607 again gave a weak diffuse staining pattern, although staining with RA3-2C2 was predominantly nuclear; PAb248 again stained a discrete extranuclear structure but did not stain the nucleus.

These results on the species specificity of the anti-p53 antibodies are in good agreement with earlier studies for those antibodies used in both sets of experiments, e.g., RA3-2C2, PAb122, and PAb421. The species specificity of PAb242, PAb246, and PAb248 was also confirmed in radioimmunometric assays in which all three antibodies failed to bind rat p53-T complex immobilized by PAb419; PAb421 was able to bind efficiently to this complex. The antibody 200.47, earlier reported to react with human p53 (8), did not stain the nucleus of the SV40-transformed human cells, though some diffuse stain was seen. Iodinated 200.47 bound anti-T-immobilized T-mouse p53 complex but did not bind similarly immobilized T-human p53 complex, even though the latter reacted strongly with iodinated PAb421. Staining of non-SV40-transformed mouse cells with anti-p53 antibodies revealed a further degree of complexity. A line of 3T6 cells was found to give intense nuclear staining with all murine-reactive antibodies except PAb246 and PAb607. In the case of PAb246, no staining at all was seen on acetone–methanol-fixed cells, whereas PAb607 gave a diffuse stain similar to its behavior on human and rat SV40-transformed cells.

The absence of the PAb246 staining was investigated further. When 3T6 cells were abortively infected with SV40, those cells that expressed T antigen also stained strongly with PAb246. The surrounding uninfected cells remained negative (Fig. 6). A time course of infection was analyzed. PAb246-positive cells were detected as early as 9 h postinfection, as soon as the first anti-T-positive cells could be seen. This time course is in striking contrast to that seen with p53 staining of abortively infected 3T3 cells. In this case, whereas T-antigen-positive cells were detected as early as 10 h postinfection, p53-positive cells did not appear until 24 h postinfection, and PAb246-positive cells appeared at the same time as those staining with other anti-p53 antibodies.

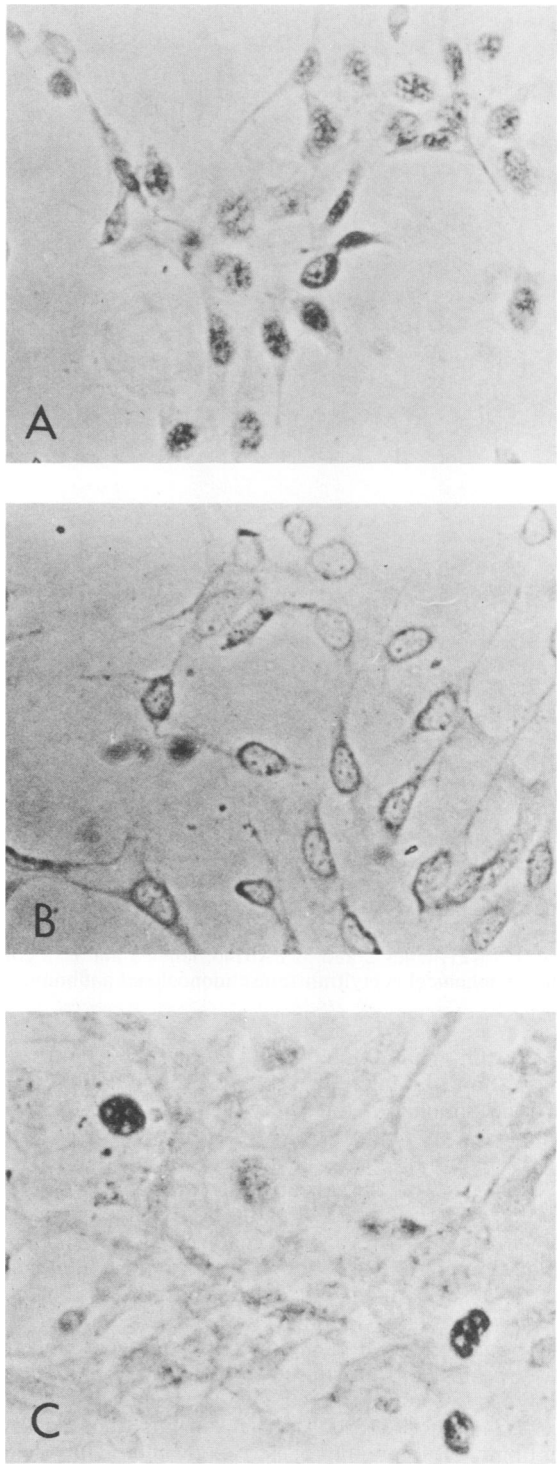

FIG. 6. Induction of the PAb246 epitope by SV40 infection of 3T6 cells. Subconfluent cultures of 3T6 cells were fixed in acetone-methanol and stained with anti-p53 antibodies by the immunoperoxidase technique. (A) Uninfected 3T6 cells stained with PAb421. (B) Uninfected 3T6 cells stained with PAb246. (C) 3T6 cells, infected 20 h earlier with 5 PFU of SV40 per cell, stained with PAb246.

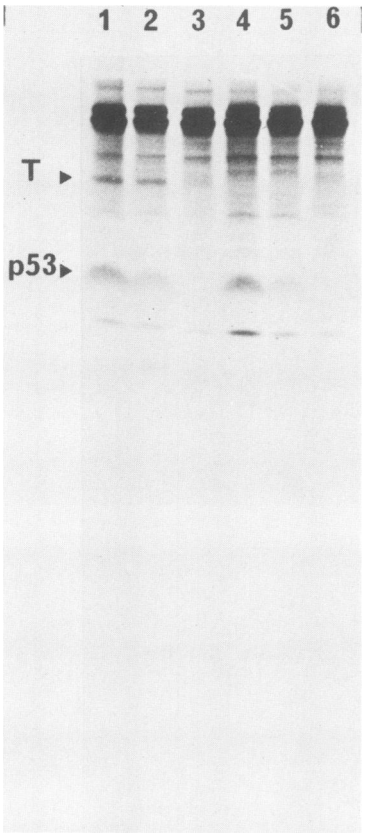

FIG. 7. Immunoprecipitation analysis of p53 in 3T6 cells. 3T6 cells, either uninfected or infected 24 h earlier with SV40, were labeled with [^{35}S]methionine, and the labeled extracts were immunoprecipitated with anti-p53 antibodies. Lanes 1, 2, and 3 are immunoprecipitates of infected 3T6 cells; lanes 4, 5, and 6 are immunoprecipitates of uninfected 3T6 cells. Antibodies used: lanes 1 and 4, PAb421; lanes 2 and 5, PAb246; lanes 3 and 6, a control anti-chloramphenicol acetyltransferase monoclonal antibody.

Similar results were obtained with PAb607, except that this antibody showed a high level of background staining.

PAb246 staining does not always require the p53 protein to be complexed to T. In the T3T3 cell line, PAb246 gives a strong nuclear stain in the absence of large T. Indeed, this assay was part of the original screen that selected the hybridoma. When the p53 staining of T3T3 cells was compared with that of the SV40-transformed SVA31 E7 cells after the differential extraction procedure of Staufenbiel and Deppert (41), we found that the nuclease-treated nuclei of both cell lines stained strongly with PAb246, PAb248, and PAb421. Salt treatment of the nuclease-treated cells left the nuclei of both cells still staining with PAb248 and PAb421, but whereas the salt-extracted SVA31 E7 nuclei still stained with PAb246, the T3T3 cell nuclei did not stain at all with this antibody. Radioimmunoassay experiments have shown that the PAb246 epitope of T3T3 cells is not destroyed by 2 M NaCl treatment.

To further study the immunochemistry of the PAb246 epitope in infected 3T6 cells, we employed immunoprecipitation of [^{35}S]methionine-labeled extracts and radioimmunometric assays. PAb246 and PAb421 both immunoprecipitated the T-p53 complex from extract of [^{35}S]methionine-labeled 3T6 cells labeled for 3 h at 24 h postinfection. Surprisingly, both PAb246 and PAb421 were able to immunoprecipitate p53 from uninfected 3T6 cells (Fig. 7). Similarly, p53 from 3T6 cells could be immobilized on PAb246-coated plates and detected with a wide range of ^{125}I-labeled antibodies. When freshly prepared cell extract was used, some ^{125}I PAb246 label was able to bind to p53 from T3T3 cells immobilized on plates coated with PAb248, PAb421, and RA3-2C2. No binding at all was seen between this probe and the immobilized p53 from 3T6 cells. This gives further support to the idea suggested by the immunocytochemical studies that the PAb246 epitope is even more labile on 3T6 cell p53 than it is on T3T3 p53. ^{125}I-labeled PAb607 showed binding characteristics similar to those of ^{125}I-PAb246 in this study (data not shown).

To localize p53 in nontransformed 3T3 cells, we first removed those antibodies from the panel that showed background in the cross-species survey, as these could be expected to give misleading information when trying to detect very low levels of p53. Only two monoclonal antibodies that reacted with mouse p53 gave really clean backgrounds. These were PAb242 and PAb246; RA3-2C2, 200.47, PAb607, and PAb248 all showed some nonspecific background staining. PAb421 and PAb122 reacted with p53 of all species assessed, so their background level of nonspecific staining could not be evaluated. We therefore stained the 3T3 cells with PAb242, PAb246, and PAb421, with a mixture of PAb242 and PAb246, and also with a mixture of all three antibodies. PAb421 alone stained all the 3T3 cells quite strongly. The staining pattern was quite different from that seen when PAb421 stained SV40-transformed cells since it was not confined to the nucleus; the whole cytoplasm of the cell was also stained in a homogeneous manner. The results with PAb242 and PAb246 were quite distinct, since neither antibody gave any staining on the 3T3 cells when used alone. The mixture of PAb242 and PAb246 demonstrated clear nuclear staining in about 5% of the cells. The stain was not particularly intense but clearly did not extend to the cytoplasm. When 3T3 cells were stained with all three antibodies, the PAb421 pattern was dominant (data not shown).

The rather strong cytoplasmic staining of 3T3 cells with PAb122 observed by others (36) is not readily reconciled with our data and may represent some cross-reaction with the antibody and a cytoplasmic non-p53 species (15).

DISCUSSION

The epitope map of p53 derived from the experiments described here and those of Benchimol et al. (1) and Wade-Evans and Jenkins (43) gives a useful base line for the study of p53 using monoclonal antibodies. This is particularly relevant since recent studies have indicated rearrangements and deletions of the p53 gene in certain tumors (22, 31). The correlations between the linear mapping of epitopes using gene fragment protocols and the steric mapping are interesting. None of the antibodies that have been mapped to distinct sites on the linear structure interferes sterically with the binding of the others to p53 (Fig. 2). Thus PAb242, 246, 248, and 421 are all noncompetitive. Encouragingly, some antibodies bridge the gap between adjacent epitopes on the linear structure. Thus PAb607 competes for both PAb246 (epitope mapped to between amino acids 88 and 109) and PAb248 (epitope mapped to between amino acids 157 and 192).

The species specificity of the new anti-p53 antibodies allows a closer analysis of their likely binding sites. In Table 5 the sequences of mouse and human p53 are compared within the epitopes of these three mouse-specific antibodies.

TABLE 5. p53 epitopes[a]

Antibody (binding site)	Species	Sequence of epitope
PAb242 (amino acids 9–25)	Mouse	S D I S L E L P L S Q E T F S G L
	Human	P V P D
PAb246 (amino acids 88–109)	Mouse	P L S S F V P S Q K T Y Q G N Y G F H L G F
	Human	S S R
PAb248 (amino acids 157–192)	Mouse	A M A I Y K K S Q H M T E V V R R C P H H E R C S D G D G L A P P Q H L
	Human	Q S

[a] The three antibodies PAb242, PAb246, and PAb248 react with mouse but not human p53. The sequences of mouse and human p53 are compared here within the regions which encompass the binding sites of the antibodies determined earlier (43). The sequences are taken from the data listed by Harlow et al. (15) and are identical except for the indicated substitutions.

There are four substitutions within the PAb242 site, three within the PAb246 site, and two within the PAb248 site. While it is possible for PAb242 and PAb246 that the species specificity of the antibody is dictated by more than one of these substitutions, this seems less likely in the case of PAb248, as the two substitutions are separated by 19 amino acids. It is interesting that of eight anti-p53 antibodies raised by autoimmunization, six are species specific and the two that are not recognize nearly the same epitope. Comparison of the human and mouse p53 sequences shows them to be quite highly conserved, so the concentration of the immune response on the few points of difference is intriguing.

The stability of the epitope on p53 recognized by PAb246 shows a striking variation between different cell lines. This variation is seen in both radioimmunometric assays and immunocytochemical experiments. Whereas T3T3 cells and SVA31 E7 cells stain intensely with PAb246, 3T6 cells show no stain, though p53 is readily detected in these cells by using six other anti-p53 antibodies. 3T6 cells expressing T-antigen after abortive SV40 infection do, however, stain strongly with PAb246. In radioimmunometric assays the PAb246 epitope is more stable at room temperature in the presence of T antigen, either when complexed to p53 in vivo (as in the cell extract experiment) or in an in vitro assembly system. The basis of this stabilization is not yet clear. A number of possibilities can be considered for the loss of the epitope on fixation and on incubation at room temperature in in vitro extracts. It is clear that the two processes could be distinct. The fixation experiment suggests a conformational shift in the molecule which is prevented in the presence of large T. This explanation could also explain the radioimmunometric assay results, though here the possibility of loss of a posttranslational modification on a selective proteolysis must also be considered. The PAb246 epitope is conformational in that PAb246 fails to interact with sodium dodecyl sulfate-denatured p53; however, the creation of the epitope does not require the entire p53 molecule since it can be detected on in vitro translation products of an element of the p53 gene (43). If the epitope relies on posttranslational modification, this must occur in in vitro translation systems and also in *E. coli* (Bartsch et al., in preparation). If proteolysis is involved in the in vitro loss of the epitope, it must be highly selective for the epitope, as the radioimmunoassays demonstrate that the PAb246-negative molecules are still intact, i.e., the N-terminal and C-terminal epitopes are still connected.

The possibility exists that the vast majority of p53 molecules in 3T6 cells do not express the PAb246 epitope. Incubation in solution with PAb246 or with large T in the in vitro assays may actually shift the equilibrium in favor of the PAb246 state. This idea is consistent with the complete absence of PAb246 staining of uninfected 3T6 cells fixed with either paraformaldehyde or acetone-methanol. If p53 can exist in two conformations, a PAb246-positive T-bound state and a free PAb246-negative state, then one can speculate that, in those non-SV40-transformed cells in which p53 displays the pAb246 epitope, this conformation of p53 has been stabilized by some other cellular or viral protein. Recently Pinhasi and Oren (34) have presented data that suggest complexing of p53 to a 68,000-molecular-weight protein in some tumor cell lines. Although we have not identified this species in our experiments, it nevertheless represents a provocative candidate for a host-coded p53-stabilizing protein. It will be important to measure the half-life of p53 in the different cell types described here to see whether PAb246-positive p53 molecules are more stable than PAb246-negative p53 molecules.

There may be a counterpart of the PAb246 antibody in the human system, as Leppard and Crawford (23) have isolated anti-human p53-specific monoclonal antibodies that seem to bind more efficiently to T-complexed p53 than to free p53 in immunoprecipitation analysis. Recently Milner (28) has presented evidence suggesting the existence of two discrete antigenic forms of murine p53: one form, present in resting lymphocytes, expresses the PAb248 and RA3-2C2 epitopes but not the PAb421 and PAb122 epitopes, and the other, present in activated lymphocytes, shows the opposite behavior. In our experiment in the fibroblast systems we can find no evidence for the existence of these discrete forms. The radioimmunoassays of free p53 from T3T3 cells imply that it is not highly polymerized but nevertheless bears both the RA3-2C2 and PAb248 epitope(s) and the PAb122 and PAb421 epitopes. It is clear that a single p53 cDNA species can on transfection into a wide range of mammalian cells direct the synthesis of both PAb248 and PAb421 epitopes (Bartsch et al., in preparation). It is possible that in the lymphocytes p53 becomes associated with host proteins that obscure certain epitopes and that these interactions alter during lymphocyte activation, but it seems unlikely that there is more than one primary sequence product of the p53 gene.

ACKNOWLEDGMENTS

We thank those mentioned in the text for their generous gifts of antibodies, cells, and viruses. We also thank Steve Brennan and Elizabeth Woodruff for their excellent technical assistance.

This research was supported by a grant from the Cancer Research Campaign.

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