BIOCHEMICAL AND IMMUNOLOGICAL STUDIES

OF THE CELLULAR TUMOUR ANTIGEN, p53

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Except where otherwise acknowledged, the results presented are the

work of the author.

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ABSTRACT

The cellular tumour antigen, p53, was first detected by virtue
of its association with Simian Virus 40 large T-antigen in SV40 -
transformed cells. Animals bearing tumours derived from cells
transformed by many agents, including SV40 and methylcholanthrene,
generate an immune response to p53. Such a response has not been
observed in healthy animals even though normal cells do contain p53,
albeit in small amounts. These and other data indicate that some
transformation-specific alteration to the structure or expression of
p53 occurs in transformed cells, suggesting that this protein may
play a role in the transformation process.

Monoclonal antibodies provide probes for p53 that are currently
essential for the study of this protein. The isolation and character-
isation of four new anti-p53 antibodies is described here. These define
at least two new antigenic determinants on human p53 and reveal
previously undefined levels of immunological complexity in both p53 and
the p53/T-antigen complex. One antibody defines a region of structural
similarity between p53 and an oligomeric form of T-antigen which may be
important in explaining how these two proteins associate.

The purification of p53 and of tryptic peptides derived from .
it and the subsequent determination of aminoacid sequences for some
of these peptides which are described here represent an important
advance in the study of p53, providing the first available sequence
data for this protein. These peptides have since been successfully
aligned with the predicted translation products of p53 cDNA clones
isolated by others, confirming the identity of these clones. Once the
complete sequence of p53 has been defined, it will be possible to map
the binding sites of the available anti-p53 antibodies on p53, so
associating the properties attributed to p53 via each determinant with
specific regions of the p53 protein.

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# Chapter 1

## THE CELLULAR TUMOUR ANTIGEN, p53.

### Introduction

It is an axiom of modern biology that all aspects of physiology and cell biology can ultimately be explained in molecular terms and one area of particular concern in recent years has been to discern the molecular events underlying a type of phenotypic change known as transformation, which can occur in many cell types during in vitro culture.

Generally, cells derived from healthy tissue will only survive a certain number of passages in vitro. However, it is possible to treat such cells with a variety of agents which have in common the ability to cause the outgrowth of clones with the potential for extended or indefinite growth. This enhanced growth potential is accompanied by changes in the degree of communication with neighbouring cells, cell shape and internal architecture, cell-surface and internal chemistry, the type and level of extra-cellular components secreted, cell motility and growth-factor requirements for division, which together constitute the transformed phenotype. Such transformed cells are frequently capable of forming progressively-growing, lethal tumours when introduced into a suitable host. Conversely, both naturally occurring tumours and tumours produced by specific agents in the laboratory will give rise to transformed cell lines, capable of growing and dividing for extended periods in culture.

Many agents can induce the transformed phenotype, including DNA and RNA viruses, chemicals and various types of radiation. Due both to the diversity of transformation inducers and to the

complexity of the transformed phenotype, it is generally assumed

that the molecular events which occur in a cell as it becomes

transformed must be extremely complex. Nevertheless, because the

transformation of cells in vitro appears to provide a good model

for the events which occur during spontaneous neoplastic transition

in vivo, in vitro transformation has been extensively studied

with a view to understanding transformation, and ultimately

tumourigenesis, at the molecular level.

Simian virus 40 (SV40), a DNA virus, is one agent capable

of causing transformation in vitro. Our detailed knowledge of

this virus has been recently reviewed (Acheson, 1980; Topp et al.,

1980; Tegtmeyer, 1980). However, since p53 was first detected in

SV40-transformed cells and much of the work on this protein

continues to be done in the context of SV40-mediated transformation,

I shall, before going on to discuss p53 in detail, briefly outline

some important aspects of SV40 biology.

SV40 was isolated from cultures of monkey kidney cells,

which it productively infects. Cells from most other species

cannot support the production of new SV40 particles. Instead,

such non-permissive cells can undergo a phenotypic transformation

after SV40 infection. These transformed cells generally revert

to normal phenotype after a period in culture with a concomitant

cessation of SV40 gene expression, but they can go on at low

frequency to generate stably transformed cell lines. The phenotype

of these transformants includes anchorage-independent growth,

overgrowth of monolayers on plastic and low serum-dependence for

growth. Introduction of such transformed cells into an

immunocompetent host induces an immune response to transformation-

specific antigens and can lead to formation of a tumour.

The major targets of anti-SV40 tumour sera are two proteins

with apparent molecular weights of around 94,000 and 17,000.
These are known as the SV40 large and small tumour (T) antigens
respectively and are the products of expression of one half of
the SV40 genome.  This is the region normally expressed early
in a lytic infection and is the only part of the viral genome
whose expression is necessary for the induction and maintenance
of transformation.  SV40 small t-antigen is a protein of 174
aminoacids whose function in lytic infection is not clear.
Genetic experiments suggest that small t-antigen is necessary for
initiating transformation in certain cell types.  Microinjection
experiments indicate that small t-antigen has the ability to
cause the dissociation of actin cables.  SV40 large T-antigen is
a protein of 708 aminoacids which is phosphorylated at multiple
serine and threonine sites.  In lytic infection, T-antigen
mediates both feedback inhibition of its own expression and the
initiation of viral DNA replication, apparently by binding to a
series of adjacent sites on the viral DNA in the vicinity of the
origin of replication and the promoter of early transcription.
Furthermore, purified T-antigen has intrinsic ATPase activity
when assayed in vitro.  In addition to its effects on viral
function, T-antigen also elevates the rate of cellular DNA
synthesis and can supply a 'helper' function that will allow
human adenoviruses to infect monkey cells.  In non-permissive
infections, T-antigen is essential for the induction and
maintenance of the transformed phenotype but it is not known
which, if any, of the known biochemical functions of large T are
involved in this process.

With viral gene expression in both lytic infection prior
to DNA synthesis and transformation apparently limited to the
large and small tumour antigens, it was assumed that, in order

to achieve in the first case the recruitment of the replication and other cellular machinery, and in the second the massive change in phenotype that constitutes transformation, one or both of these proteins had to be able to interact with at least one and probably several host cell components. The discovery, discussed in detail below, of the interaction of large T with a cellular protein, now known as p53, in transformed cells was therefore seen as the first example of these expected virus-host molecular interactions. p53 would be worthy of further study on this basis alone but, as I shall describe, subsequent work has also linked p53 with expression of the transformed phenotype in a much wider context and so considerable effort is being devoted to achieving an understanding of its functions.

## The Association of p53 with SV40 Large T-Antigen

p53 was probably first detected by several groups studying the specific tumour antigens of SV40-transformed cells by the technique of radioactive labelling of cell components in vivo, and subsequent immunoprecipitation and gel electrophoresis, using antiserum to SV40-induced tumours or -transformed cells (anti-T serum) (Tegtmeyer et al., 1977; Lichaa and Niesor, 1977; Griffin et al., 1978; Carroll et al., 1978; Gaudry et al., 1978; Edwards et al., 1979; Melero et al., 1979a; Chang et al., 1979; Kress et al., 1979). Each of these studies detected one or more proteins in the molecular weight range 48-56,000 that were precipitated specifically from SV40-transformed cells by anti-T serum, but, in general, there was no explanation offered for these results. Breakdown products of large T-antigen run in this size range and probably contributed to the material detected, but some of it was almost certainly what is now known as p53.

Two mechanisms by which a protein, such as these 48K-56K proteins, might be specifically precipitated by an antiserum can be envisaged. Antisera clearly contain many different antibodies with varying specificities. More than one protein may therefore be precipitated by an antiserum because of direct reactivity with antibodies in the polyclonal population. Alternatively, proteins might appear to be specifically precipitated by an antiserum if they can associate with a second protein against which the serum does have activity. Melero et al. (1979a), who detected 48K and 55K molecular weight polypeptides in extracts of SV40-transformed mouse cells, purported to show that the 48K protein shared antigenic determinants with large T-antigen thus explaining its specific precipitation by anti-T serum. However, they later found (Melero et al., 1979b) that their specific antiserum (Carroll et al., 1978) raised against the SDS-denatured large T-antigen polypeptide (anti-T polypeptide serum) also contained independent anti-48K antibodies as result of the protocol adopted to generate it, invalidating their original conclusion.

Ultimately, it was shown that an approximately 53,000 molecular weight protein (hereafter referred to as p53 although its estimated molecular weight is species-dependent) was precipitated by anti-T sera by either or both of the general mechanisms outlined above. Lane and Crawford (1979) described the precipitation of large T-antigen and p53 from a mouse SV40-transformed 3T3 cell line by a specific rabbit anti-T polypeptide serum (Lane and Robbins, 1978). They showed that these two antigens were precipitated with identical dose response curves and that the antiserum had intrinsic activity against large T-antigen but none against p53. From these data they concluded that p53 was precipitated by virtue of an association with large

T-antigen in these cells. Later McCormick and Harlow (1980), in
the converse experiment, prepared a specific antiserum to the mouse
p53 polypeptide and showed that it could immunoprecipitate T-antigen
from lysates of SV40-transformed mouse cells whilst having no
intrinsic anti-large T activity, providing the definitive proof
of the association of large T and p53 in SV40-transformed mouse
cells.

Some anti-tumour sera are essentially equivalent to anti-
large T polypeptide sera in lacking direct anti-p53 activity,
whilst others do contain both anti-T and anti-p53 antibodies
(Lane and Crawford, 1979; Linzer and Levine, 1979). Linzer and
Levine (1979), making use of an antiserum with dual reactivity to
large T and p53 and specific anti-large T-antigen monoclonal
antibodies (Martinis and Croce, 1978) made the important observation
that p53 could be precipitated, by some anti-T sera, from mouse
embryonal carcinoma (EC) cells (which do not contain T-antigen).
They proved that the EC cell p53 was similar    to that precipitated
from SV40-transformed cells by comparing their Staphylococcus aureus
V8 protease degradation products. Their results confirmed the
conclusions of Lane and Crawford and showed that p53 was encoded
by the host cell, not by SV40, a conclusion strongly supported by
both previous and subsequent comparisons of p53 and large T
methionine tryptic peptides (Melero et al., 1979a; Chang et al.,
1979; Kress et al., 1979; Smith et al., 1979; Linzer et al., 1979a;
McCormick et al., 1979; Stitt et al., 1981) which failed to detect
any significant similarity between the primary structures of the
two proteins. This conclusion is also supported by the
demonstration that mRNA encoding p53 did not hybridise to any
part of SV40 early region DNA (May et al., 1979).

Immunogenicity of p53

It is implicit in the foregoing discussion that p53 is somehow rendered immunogenic in mice and hamsters bearing SV40-induced tumours (Lane and Crawford, 1979; Linzer and Levine, 1979). Anti-p53 activity has been detected not only in the sera from such animals but also in sera from mice bearing methylcholanthrene-induced sarcomas (DeLeo et al., 1979), mice bearing Abelson murine leukaemia virus-induced tumours (Rotter et al., 1980) and some human breast and lung cancer patients (Crawford et al., 1982b; Crawford, 1983). In contrast, such activity has never been detected in normal serum. The immunogenicity of p53 in these situations has not only provided specific anti-p53 antisera, but has also facilitated the isolation of anti-p53 monoclonal antibodies (Gurney et al., 1980; Coffman and Weissman, 1981; Dippold et al., 1981; Harlow et al., 1981b) which have proved extremely useful in the study of p53.

At least two mechanisms can be envisaged by which the normal host tolerance for a cellular protein such as p53 could break down in a tumour-bearing animal. Firstly, the regulation of p53 expression in transformed cells could be altered so as to allow overproduction of p53 protein. Cell death and necrosis within a tumour containing high levels of p53 could be expected to expose the host immune system to unusually large amounts of the protein, which might be sufficient to swamp the normal mechanism of self-tolerance. The observation (Crawford, 1983) that breast cancer patients who were found initially to have high levels of anti-p53 activity in their serum, show a sharp decrease in the level of this activity in the period following surgical removal of their tumour would support such a suggestion. Secondly, p53 may be structurally altered in transformed cells, causing it to be

recognised as foreign by the immune system. Such an alteration

could take the form of a change in aminoacid sequence due to

mutation or some transformation-related modulation in the quality

or quantity of post-translational modification. As I shall describe,

the extent to which p53 expression and structure are altered in

transformed cells is not yet clear and it will obviously be

important to relate new data in these areas to the problem of

understanding how p53 becomes immunogenic.


## The Accurate Definition of p53

Before discussing the level of p53 expression in various

cells and tissues, it is necessary to consider how p53 can be

defined and hence accurately identified. Proteins are normally

defined by some assayable biological property, but the only

known biological property of p53 is its ability to bind to SV40

large T-antigen and to assay all proteins identified tentatively

as p53 for this would be very tedious and in some cases impossible.

Instead it is frequently assumed that immunoreactivity of a

putative p53 with one or more previously characterised anti-p53

antibodies or antisera is a sufficient test of identity but,

unfortunately, this is not necessarily the case. Two principal

sources of confusion that have arisen in the course of studies on

p53 are worth noting.

Firstly, since anti-tumour sera are polyclonal and

potentially multispecific, it is insufficient merely to show

precipitation of a polypeptide of the appropriate size on an

acrylamide gel by a serum with known anti-p53 activity in order to

establish that it is p53 being studied. Secondly, even monoclonal

antibodies, or monospecific anti-peptide antisera, have been

found to cross-react quite frequently with one or more otherwise

unrelated proteins (Lane and Hoeffler, 1980; Crawford et al., 1982a;

Lane and Koprowski, 1982). Since the probability of such a random

cross-reactivity occurring between two proteins with identical gel

mobilities is very low, there is usually no problem in interpreting

immunoprecipitation data obtained with such antibodies. However,

because of the possibility of cross-reaction, it cannot be assumed

that data obtained indirectly, for example, by immunofluorescence

analysis, relate to the same protein species as that identified

by immunoprecipitation with the same antibody.

Two related procedures do allow the comparison of

immunoprecipitated proteins at a detailed structural level.

Comparative sizing of the partial degradation products produced

from two proteins by Staphylococcus aureus V8 protease (Cleveland

et al., 1977) is a very rigorous test of similarity since any

variation in the distribution of either the labelled residues

(usually methionine) or the target residues (normally a restricted

number of the glutamic acid residues in a target protein) will

result in gross changes in the pattern of labelled degradation

products. Similarly, a comparison of labelled tryptic peptides

derived from two proteins will be a sensitive test of their

similarity. This technique is better suited to detecting limited

regions of sequence identity than the V8 technique but has the

disadvantage that, since the enzyme cuts more frequently than V8

protease (as usually employed), many more fragments will be

omitted from comparison because they are unlabelled. Both these

techniques have been used to confirm the identity of many putative

p53 proteins, although this has not been sufficient to prevent

p53 being erroneously attributed certain properties in the

published literature, as noted below.

## p53 in Transformed Cells and Tumours

By the use of suitable immunological and comparative
structural tests, p53 has now been shown to be a component of
many cell types in addition to SV40-transformants. Mouse cells
transformed by the closely related papovavirus, polyoma, contain
p53 (Hunter et al., 1978; Crawford et al., 1979), as do hamster
kidney cells or human embryo fibroblasts transformed by BK virus
(Simmons, 1980; Grossi et al., 1982) and hamster brain cells
transformed by JC virus (Frisque et al., 1980; Simmons, 1980).
A protein of the appropriate size has also been detected as a
tumour antigen in cells derived from bovine papilloma virus-
induced tumours (Breitburd et al., 1981). Several retrovirus-
transformed cell lines express p53. These include cells
transformed by Moloney and Kirsten murine sarcoma viruses and
Moloney murine leukaemia virus (DeLeo et al., 1979), Abelson
murine leukaemia virus (Rotter et al., 1980; Rotter et al., 1981)
and the Friend virus complex (Ruscetti and Scolnick, 1983).
There is, however, no evidence that the protein pp50 found
associated with the product of the Rous sarcoma virus oncogene in
transformed cells (Brugge et al., 1981) is in any way related
to p53. Cells transformed by adenoviruses (Sarnow et al., 1982a,b)
and by Epstein-Barr virus (Crawford et al., 1981) also express
p53.

It is not yet clear to what extent p53 is associated
with viral gene products in cells transformed by papovaviruses
other than SV40. Whilst it is likely that the T-antigens of
the human viruses BK and JC, which are closely related to SV40,
do form complexes with p53 in virus transformed cells (Simmons,
1980), efforts to show association between p53 and polyoma virus
large T-antigen have failed (Gurney et al., 1980). This is

somewhat surprising since the polyoma virus and SV40 large

T-antigens, whilst not completely homologous in structure, do

show considerable aminoacid sequence homology and do perform

very similar functions in lytic infection. Association with

p53 is not exclusively a property of papovavirus proteins. It

is now well documented that the human adenovirus type 5 early

region 1b product, a protein of 58,000 molecular weight, is

bound to p53 in adenovirus-transformed mouse cells (Sarnow et al.,

1982a,b).

It was originally reported that p53 was associated with

a 48,000 molecular weight form of the Epstein Barr Virus (EBV)

antigen, EBNA, in EBV-transformed cells (Luka et al., 1980;

Jörnvall et al., 1982; Luka and Jörnvall, 1982). Jörnvall et al.

(1982) claimed that this 53,000 molecular weight protein could

be bound by both the anti-p53 monoclonal antibody RA3-2C2 (Rotter

et al., 1980) and by an antiserum to the mouse methylcholanthrene-

induced sarcoma, CMS4, known to have anti-p53 activity (DeLeo

et al., 1979) implying that it was closely related to p53, but

they did not actually present these vital data. Using the N-

terminal sequence of the EBNA-associated protein determined by

Jörnvall et al. (1982), Luka et al. (1983) prepared 53K-specific

synthetic peptides to which they raised antisera. These reagents

immunoprecipitated a protein that was clearly of a higher molecular

weight than that precipitated by bona fide anti-p53 antibodies

from the same cells. It therefore appears that p53 and the EBNA-

associated protein are distinct entities. A possible explanation

of the earlier observation of an apparent immunological

relationship between p53 and the EBNA-associated 53K protein

is that the two proteins partially copurify. If so, the purified

53K of Luka et al. (1980) may have contained p53 as a minor

contaminant. Whilst such copurification might imply that the two proteins have some similar physical properties, there is at present no evidence that there is any significant structural or functional relationship between them.

Expression of p53 is not restricted to virally transformed cells. A highly significant early study by DeLeo et al.(1979), performed without reference to the SV40-transformation system, detected a p53 protein in a number of cell lines derived from murine methylcholanthrene (MC)-induced sarcomas by immunoprecipitation with antisera to MC-induced tumours. Similar results were obtained subsequently using a monoclonal antibody (Dippold et al., 1981). These sera also precipitated p53 from SV40-transformed 3T3 cells and this p53 was subsequently shown by peptide mapping analysis to be identical to the protein precipitated from these cells by anti-T serum (Maltzman et al., 1981). By the same criterion, p53 proteins precipitated by anti-MC serum from cells transformed by SV40, polyoma, Moloney murine sarcoma virus, Rous sarcoma virus and methylcholanthrene were also identical (Jay et al., 1979). p53 expression in these cells is not an artifact of prolonged passage in culture since methylcholanthrene-induced transformation of mouse embryo primary cells also led to a rapid induction of p53 expression (Rotter et al., 1983a), detected by metabolic labelling and precipitation with anti-p53 monoclonal antibodies.

As already discussed, p53 is a component of spontaneously transformed mouse embryonal carcinoma cell lines (Linzer and Levine, 1979). It has also been described in a transformed mouse lung fibroblast line (DeLeo et al., 1979; Jay et al., 1979; Dippold et al., 1981) and in primary cultures from a spontaneous murine B cell leukaemia and two mammary adenocarcinomas, as well as primary cultures derived from mouse T-cell lymphomas experimentally

induced with methylnitrosourea (Rotter, 1983). Crawford et al.

(1981) surveyed a wide range of cell lines isolated from human

tumours of varying type and origin and found the majority to express

p53. Some human tumours may also express p53 since a significant

number of antisera from human breast and lung cancer patients

contain anti-p53 activity whereas no such activity has been

found in control sera (Crawford et al., 1982b; Crawford, 1983).

However, one tumour cell line, HeLa, contained no detectable p53

as also shown by Gurney et al. (1980) and latterly two further cell

lines, human bladder carcinoma line EJ/T24 and mouse Abelson virus-

transformed lymphoid line L12, were also found to lack p53

(Crawford, 1982; Rotter et al., 1983c). To date, these are the

only transformed cells that have not been shown to express the p53

protein.

Dippold et al. (1981) described the detection of p53 in a

number of human mammary and renal carcinoma, melanoma and lymphoma

cell lines by indirect immunofluorescence with an anti-p53 monoclonal

antibody raised against mouse p53. However, this antibody has since

been shown to have little or no activity against human p53 (Crawford,

1983). It is therefore likely that the results of Dippold et al.

were due to cross-reaction of their antibody with other cell

proteins. Results obtained with the same antibody which suggested

that p53 was expressed in a passage-dependent manner in cultures of

normal human kidney epithelial cells and skin fibroblasts must

therefore be treated with considerable caution.

In summary, a wide variety of transformed cells and tumours,

both rodent and human, of epithelial, lymphoid or fibroblastic

origin and arising spontaneously, or by viral or chemical induction,

have now been shown to express p53, a protein identified originally

in the particular context of SV40-mediated transformation of

cultured mouse fibroblasts. Three transformed lines fail to synthesise detectable p53 and the explanation for this must clearly be sought. Nevertheless, expression of p53 does appear at this time to be a fairly general characteristic of the transformed cells that have been examined.


## p53 in Normal Cells and Tissues

Having shown that p53 was expressed in many transformed cells, it was necessary to determine whether p53 was also a component of normal cells, or whether its expression was truly transformation-specific. Many of the studies of transformed cells already discussed above were comparative studies of transformed and non-transformed cells and, almost invariably, the non-transformed cells were concluded to be negative for p53 expression, based on metabolic labelling (usually with $^{35}$S-methionine) and immunoprecipitation analysis. Amongst the normal cells and tissues in which p53 was not detected were BALB/c mouse 3T3 fibroblasts (Linzer and Levine, 1979; DeLeo et al., 1979; Carroll et al., 1980), lung fibroblasts, splenocytes, bone marrow cells and macrophages (DeLeo et al., 1979; Dippold et al., 1981), C57BL/6 mouse T-cell growth factor-dependent cytotoxic T-cells (Dippold et al., 1981) and precrisis cultures of human foreskin fibroblasts and mammary epithelial cells (Crawford et al., 1981).

Nevertheless, Linzer et al. (1979b) showed that p53 could after all be detected in immunoprecipitates of mouse 3T3 cell extracts, provided that autoradiography of the analytical gel used to separate components in the precipitate was carried out for a sufficient period, implying that p53 might be generally expressed at a low level in all non-transformed cells. Unfortunately, it is not certain how far one can generalise about normal cells on the

basis of data obtained from 3T3 cells. A recent study suggested
that the expression of p53 in precrisis cultures of mouse fibroblasts
passaged on a 3T3 regime depends on the strain of mouse from which
the cells were derived and their passage number. Cells from
"low-expression" strains showed rapidly increasing p53 expression
with time in culture. Furthermore, the frequency of crisis survival
in these cells was related to the level of p53 expression (Chen
et al., 1983). The implication of these results is that established
3T3 cell lines will have been selected for high-level expression of
p53 and so should not be regarded as typical of normal cells, at
least in this context.

In addition to its detection in mouse 3T3 cells, p53 has now
been detected in cultured monkey kidney cells (Simmons, 1980;
Harlow et al., 1981a) and in mouse thymus (DeLeo et al., 1979;
Dippold et al., 1981). Again, these cells may not be typical of
normal cells since one is capable of extended growth in culture and
the other is a highly differentiated tissue. It is therefore not yet
certain to what extent p53 is expressed in non-transformed cells
generally. The available data would be as consistent with the idea
that p53 is expressed selectively in normal tissues and generally
only in cells capable of continuous growth in culture, as with the
idea that p53 is expressed at very low levels in all normal cells
and tissues and at high levels in transformed cells. In
considering these different hypotheses, it must also be remembered
that, despite extensive efforts, p53 has not been detected in the
archetypal transformed cell line, HeLa. Its expression cannot
therefore be essential for viability and growth.

Many tumours and transformed cells derived from adult tissues
are known to re-express embryonic or foetal antigens (Edidin et al.,
1971; Coggin et al., 1971; Artzt et al., 1973; Gooding and Edidin, 1974)

that are not present in any normal adult tissues. The expression

of p53 in embryos has therefore been studied to determine whether

its presence in transformed cells is an example of such re-expression.

When analysed by metabolic labelling and immunoprecipitation, it

was found that primary cultures of mouse embryo cells expressed p53

in a stage-specific manner. Thus primary cultures derived from

day 10 to day 14 AL/N mouse embryos expressed readily detectable

amounts of p53 whereas after day 14 p53 expression declined rapidly

(Mora et al., 1980; Chandrasekaran et al., 1981a). In contrast,

DeLeo et al. (1979) found cultures derived from 10 - 15 day BALB/c

embryos were negative for p53, whilst Rotter et al. (1983a) detected

extremely low levels of p53 in primary cultures derived from

BALB/c X C57BL/6 F1 embryos at day 16. The discrepancies between

these sets of results may be due to strain and passage number

differences between the cells studied, given the findings of

Chen et al. (1983) discussed above. However, despite these

discrepancies, it is likely that p53 is expressed in embryos at

only certain stages of development. Expression of p53 in

transformed cells generally, and particularly in embryonal carcinoma

cell lines (Linzer and Levine, 1979) may therefore represent the

aberrant re-expression of a normal embryonic or foetal protein.


The Amounts of p53 in Normal and Transformed Cells

It is often stated, as part of a justification for its study,

that p53 is a protein present at higher levels in transformed cells

than normal cells. The experimental evidence for such a statement

was originally derived from metabolic radiolabelling of p53 in

various cell types and detection by immunoprecipitation and SDS gel

electrophoresis.

As already noted, p53 is almost undetectable in mouse 3T3

cells whereas, after infection with SV40, this protein is readily identified in immunoprecipitates (Linzer et al., 1979b). In the same study it was shown that the amount of detectable p53 increased rapidly after infection with SV40, reaching a plateau at around 22hr. post-infection whilst T-antigen levels increased slightly faster to peak at around 14hr. post-infection before falling again, due to feedback inhibition of its synthesis. Furthermore, studies of the effect on p53 expression of mutants defective in large T-antigen (tsA58) and small t-antigen (dl884) synthesis showed that expression of a functional large T protein was essential for the observation of elevated p53 levels in infected cells. Essentially similar results were obtained using an immunofluorescence assay (Carroll et al., 1980). SV40 also affects the synthesis of p53 during lytic infection of monkey cells, the rate of its synthesis, based on labelling with methionine, being elevated some 5-fold during a 72 hour time-course (Harlow et al., 1981a).

The same techniques have also been applied to determine the amounts of p53 in mouse embryo primary cultures and in embryonal carcinoma cell lines before and after differentiation. As already noted, p53 appears to be expressed in a stage-dependent fashion in mouse embryos, being expressed at high level between days 10 -14 (Mora et al., 1980; Chandrasekaran et al., 1981a). This same group extended their analysis to embryonal carcinoma cell lines and concluded that both cells undergoing spontaneous differentiation into non-tumourigenic cells and cells being induced to differentiate displayed a marked decrease in their level of p53 expression that was concomitant with the differentiation event (Chandrasekaran et al., 1982).

Finally, levels of p53 in cell lines derived from a wide

range of human tumours were compared with those in cultured precrisis

human cells on the basis of radioactive phosphate incorporation

(Crawford et al., 1981). On this basis virtually all the transformed

cells expressed p53 whereas the normal cells did not.

There are a number of difficulties in the interpretation of

these analyses, as was recognised in some cases by the investigators

involved. Firstly, metabolic labelling is a procedure that is very

sensitive to changes in the half-life of a protein. Thus it is not

possible to compare the levels of a protein in two cell types by

labelling unless the half-life in the two cases is similar.

Secondly, metabolic labelling as frequently practised, using high

specific-activity labelled metabolites for short periods of time,

can only reflect the rate of synthesis of a protein rather than

its steady state level. Rate of synthesis will, in general, not

be related to steady-state level in any simple or interpretable

way. Finally, the amount of label incorporated into a protein

by two cell types labelled in parallel can actually depend on the

choice of label. Cells will certainly vary in their rate of uptake

of metabolites from culture medium, but this will only affect the

overall incorporation of label into protein and can be allowed

for. More importantly, the amount of incorporation of radioactive

label into a specific protein will be sensitive to variations in

the chemistry of that protein in different cell types. $^{35}$S-

methionine is most frequently used to label proteins in vivo.

However, since it is a relatively infrequent component of proteins,

point mutations that alter the number of methionine residues in a

protein will cause a significant alteration in the observed

incorporation even if rate of synthesis and half-life are unaltered.

Even greater reservations attach to the interpretation of

data generated using $^{32}$P-phosphate as a metabolic label. Proteins

are labelled by this agent via post-translational phosphorylation of various aminoacid side chains and the level of labelling observed will clearly reflect the level of such modification. Since alterations in the extent and type of post-translational modification are a frequent means of modulating protein function, it is highly unlikely that the rate of phosphorylation and half-life of phosphorylation at each acceptor site on a protein will be sufficiently similar in two different cell types to allow any conclusions on relative protein levels based only on phosphate labelling.

Clearly, to make firm conclusions about the levels of p53 expression in different cells, one requires an assay for the actual amount of p53 protein in a cell. Such an assay was described by Benchimol et al. (1982). Anti-p53 monoclonal antibodies were used in non-competing pairs, the first to adsorb p53 from a cell lysate onto a plastic surface and the second, labelled with radioactive iodine, to tag the bound p53 with radioactivity. Having shown that the counts bound at this stage were proportional to the input of total cell protein (and thus to the input of p53) it was possible to compare the levels of p53 in a range of mouse cell lines, many of which had previously been studied by metabolic labelling. Surprisingly, it was found that whilst SV40-transformed 3T3 cells contained very high levels of p53 protein and cultures of normal cells contained virtually none, the expected result based on previous labelling studies, other transformed cell lines, notably mouse EC cells, contained as little as 1% of the p53 present in SV40-transformed cells. These cells routinely incorporate methionine into p53 with high efficiency and on this basis were estimated to contain 11% - 55% the amount of p53 in SV40 transformants (Linzer et al., 1979b; Chandrasekaran et al., 1981a).

Using a similar assay for human p53, the range of transformed cell lines found to contain p53 by $^{32}$P-phosphate labelling (Crawford et al., 1981) were also shown to contain p53 protein at levels that differed by up to 100-fold (Benchimol et al., 1982). Whilst p53 levels in all the non-transformed cells tested were uniformly low, the wide range in the levels of p53 protein in both mouse and human transformed cells, results which are in sharp contrast to the results of labelling studies, suggests that the level of p53 protein in a cell may not after all be linked in any simple way to expression of the transformed phenotype.

## The Control of p53 Expression in Different Cell Types.

It is possible that transformed cells can suffer various alterations to their mechanisms of control of p53 expression, perhaps explaining the differences in the steady-state levels of p53 in different transformants. Two pairings of transformed / non-transformed cells have been studied in detail to determine how the controls imposed on p53 expression in these cells compare.

As noted above, the SV40 T-antigen is required for the induction of p53 expression in SV40-infected mouse 3T3 cells and this induction results in the accumulation of high levels of p53. Measurements of the half-life of p53 in these cells suggest that this accumulation is due to a dramatic stabilisation of p53 in the SV40-transformed cell (Oren et al., 1981) and it is tempting to speculate, given that the effect requires a functional T-antigen, that stabilisation of p53 is directly due to its association with SV40 large T-antigen. Mouse embryonal carcinoma cells show a reduction in their apparent p53 levels on differentiation but in this case there was no alteration in the half-life of the protein. The difference in protein levels can however be explained by the

observation that the undifferentiated cells contained 6 to 10 fold
more translatable mRNA for p53 than the differentiated cells (Oren
et al., 1982). Both post-translational and transcriptional controls
have therefore been shown to regulate p53 levels in different
situations and it is possible, of course, that further mechanisms
may be found to operate in other cell types.

There are many events that could potentially underly an
observed change in the amount of p53 mRNA in a cell. Transcriptional
activity could be elevated or decreased by the differential
regulation of one or more p53 genes, by chromosome breakage or
translocation events that put p53 sequences under the control of
the wrong promoter, or by mutations in the p53 gene (or in the genes
for elements acting on the p53 gene) that alter directly or
indirectly its rate of transcription. Clearly in the particular
case of embryonal carcinoma cells, the elevated expression of p53
in the undifferentiated state is unlikely to be due to a mutational
event since differentiation is accompanied by a rapid alteration in
this aspect of cell phenotype. However, many other transformed cells,
in particular chemically transformed cells, might be expected to
have DNA damage as the cause of their phenotype and a detailed
characterisation of p53 gene organisation in these cells is
certainly needed.

As yet, our knowledge of the organisation of p53 genes is
very limited and no comparisons of p53 gene organisation in
different cell types have been made. There appear to be two loci
containing p53 sequences in BALB/c mice (Oren et al., 1983), one
of which is a pseudogene (Benchimol et al., in preparation), and
one inconclusive study has tentatively linked an expressed p53
gene with human chromosome 7 by immunoprecipitation analysis of
a bank of human/mouse hybrid clones (Stitt and Mangel, 1981).

These data are clearly insufficient to support any speculation as to the relevance of the above causes of altered transcription to the observed variability of p53 gene expression in normal and transformed cells.

## The Structure and Immunochemistry of p53

As noted earlier, the 53,000 molecular weight EBNA-associated protein (Luka et al., 1980) is now thought not to be p53 (Luka et al., 1983) and so the N-terminal sequence data derived from it (Jörnvall et al., 1982) is not relevant to the structure of p53. There is therefore no valid aminoacid sequence data for p53 available. Indeed, the complete aminoacid sequence is likely to be derived first by prediction from the sequence of cloned DNA complementary to the p53 message. Recently, the cloning of such cDNAs has been reported (Oren and Levine, 1983; Oren et al., 1983; Benchimol et al., in preparation) although as yet these clones do not cover the entire mRNA sequence. Rapid progress in this work is likely to provide a complete predicted aminoacid sequence for p53 in the near future, bringing nearer the prospect of directly comparing the structures of p53 proteins in normal and transformed cells.

In contrast to our current lack of knowledge of the p53 aminoacid sequence, there is already a considerable amount of data relating to post-translational modifications of p53. The protein is phosphorylated in many transformed cell types (DeLeo et al., 1979; Linzer and Levine, 1979; Crawford et al., 1981) but such phosphorylation is not readily detectable in non-transformed cells (Crawford et al., 1981). The nature of p53 phosphorylation has been studied in detail by van Roy et al.(1981). They found that monkey, human and rat p53 contained mostly phosphoserine, with smaller amounts of phosphothreonine. Mouse p53 reproducibly

contained only phosphoserine. None of the proteins tested

contained any detectable phosphotyrosine, a modified aminoacid

found in the products of several retroviral transforming genes

and also now in certain normal cellular components. Dissection

of the human p53 protein revealed at least three distinct sites

of phosphorylation, spread along the polypeptide.

p53 also displays heterogeneity in isoelectric point

(Crawford et al., 1979; Crawford et al., 1981; Fanning et al.,

1981a) and since all the species so identified appear to be

phosphorylated to some extent (Crawford et al., 1979) they may

reflect something other than differentially phosphorylated forms

of p53. Tryptic peptide analysis of p53 from SV40-transformed

mouse cells also suggests that it can carry some form of post-

translational modification distinct from phosphorylation. p53

from mouse SVT2 cells labelled in vivo contains several methionine-

containing tryptic peptides not present in p53 synthesised

in vitro from mRNA of the same cells (Oren et al., 1981) and

these extra peptides are not phosphorylated (Maltzman et al., 1981).

Potential alternative modifications of p53 that have been observed

in other proteins include glycosylation, fatty acid esterification

and ADP-ribosylation. The existence of such specific chemical

modifications on p53 has not been tested rigorously. However,

neither the enzyme endo-β-N-acetylglucosaminidase H, which cleaves

certain oligosaccharide linkages, nor tunicamycin, which inhibits

N-asparaginyl glycosylation had any effect on the apparent molecular

weight of p53 (Rotter et al., 1980; Crawford et al., 1981),

suggesting that the particular types of glycosylation affected

by these agents do not occur on p53.

Despite intensive efforts, p53 can still only be defined

antigenically. Considerable interest has therefore centred on the

immunochemistry of this protein. Six monoclonal anti-p53
antibodies have so far been described. The binding of these
antibodies to p53 was characterised in competition assays
(Benchimol et al., 1982; Crawford, 1982). It was shown that
three antibodies, PAb122 (Gurney et al., 1980), PAb410 and PAb421
(Harlow et al., 1981b) blocked each other's binding to p53 and so
appeared to recognise the same or closely related determinants.
Interestingly, all three of these antibodies arose from immunisation
of mice with syngeneic SV40-transformed cells. It is possible
that the use of SV40 large T-associated p53 as an immunogen biases
the immune response towards a particular antigenic region on p53
since, in the same test, antibodies RA3-2C2 (Coffman and Weissman,
1981) and 200-47 (Dippold et al., 1981), which were raised by
completely different protocols, were found to bind to two other
distinct determinants. A sixth antibody, PAb607, isolated by
L. Gooding and referenced by Benchimol et al.(1982), does not
compete with any of the other antibodies but has not otherwise
been characterised in any detail. None of the latter three
antibodies bind to human p53 (Crawford, 1983; Rotter et al.,
1983b), so that whilst probes exist for four determinants on
mouse p53, only one determinant on human p53 can so far be
recognised.

This situation is somewhat unsatisfactory since the
behaviour of a single antigenic determinant (distribution,
level of expression) may not be an accurate reflection of the
distribution and level of the protein, given that a protein
might well be antigenically heterogeneous. Ideally, studies of
a protein that employ monoclonal antibodies should be repeated
with antibodies specific for several different determinants, in
order to be certain of the results obtained. There is therefore

a need for more anti-p53 antibodies, particularly antibodies

with activity towards the human p53 protein.

Whilst p53s of different species appear virtually identical

by V8 protease partial digestion mapping (Fanning et al., 1981a),

the existence of anti-p53 antibodies capable of discriminating

between species suggest that a significant amount of sequence

divergence has occurred. Some attempt has been made to quantitate

the degree of homology between p53 proteins in several species

by calculating the percentage of methionine-tryptic peptide

shared between them (Simmons et al., 1980; Simmons, 1980).

Using these data, the hamster, rat, mouse, human and monkey p53

proteins were placed in an evolutionary tree. The rat and mouse

p53s shared more than 75% of their peptides, as did the human

and monkey proteins. The hamster p53 shared less than 50% of

its peptides with any of the other p53 proteins tested. It would

be expected from these data that p53 of any species would

potentially display one or more species-specific determinants

for which monoclonal antibodies could be isolated.


The Biochemistry and Biology of p53 and its Complex with SV40

T-Antigen

The best known feature of the biology of p53 is its

ability to associate with SV40 large T-antigen and a considerable

amount of time has been devoted to the study of this complex.

The association of p53 with SV40 large T-antigen was first

observed in SV40-transformed mouse cells but such complexes have

now also been observed in SV40-transformed rat, hamster and

human cells and are indeed not peculiar to transformed cells

since monkey cells lytically infected with SV40 contain a similar

T/p53 complex (Fanning et al., 1981a; Harlow et al., 1981a).

Mouse p53 has also been shown to bind to purified T-antigen in vitro (McCormick et al., 1981).

The strength of the interaction between large T and p53 appears to depend on the species origin of the p53. The mouse complex is very stable, being resistant to chelating agents, high salt, reducing agent and neutral detergent (McCormick and Harlow, 1980). In contrast, the monkey complex is very weak, dissociating completely within a few hours at $0^\circ$ in isotonic buffer (Harlow et al., 1981a) whilst the human complex displays a degree of stability intermediate between these two extremes. Probably as a result of this variable strength of association, the proportion of total p53 found in association with large T in cell extracts is also species-dependent. Little or no uncomplexed p53 can be found in SV40-transformed mouse cells (Crawford et al., 1979; Lane and Crawford, 1980) whilst in freshly prepared extracts of human SV40-transformants there is a considerable amount of free p53 (Gurney et al., 1980; Lane and Crawford, 1980; Fanning et al., 1981a) estimated in one case to be 20% of the total. In SV40-infected monkey cells, analysis is confused by the induction of p53 during the course of infection, but after 48 hours (Harlow et al., 1981a) and after 65 hours (Fanning et al., 1981a) there were significant amounts of both complexed and free p53 present.

The size of the T-antigen/p53 complex has been determined by sucrose gradient analysis. Sedimentation coefficients for the mouse, human and monkey complexes of 22-25S have been obtained, which suggest an estimated molecular weight of 600-1000K (McCormick et al., 1979; McCormick and Harlow, 1980; Fanning et al., 1981a,b; Harlow et al., 1981a). Only one estimate of the ratio of large T to p53 in this complex has been made (Freed et al., 1983) and this figure, 0.87± 0.27 is subject to many errors and reservations

as a result of the method of determination, quite apart from the potential error in averaging six results ranging from 0.42 to 1.17. Based on molecular weights of the various T-antigen oligomers identified by Bradley et al.(1982) , the immediate precursor of the T/p53 complex may be a 16S T-antigen tetramer (see below). Within the wide limits of molecular weight estimated for the complex, one might therefore expect between 4 and 8 p53 monomers to be associated with each large T tetramer in a 23S complex.

The assembly of the high molecular weight T/p53 complex seems to follow a definite pathway. T-antigen, from either transformed or infected cells exists in discrete size classes from 5-7S up to approximately 23S (Prives et al., 1979; Bradley et al., 1982). These rapidly sedimenting forms seem, from pulse-chase experiments, to be formed from T-antigen first found in the 5-7S size-class (Greenspan and Carroll, 1981; Carroll and Gurney, 1982) and to be hyperphosphorylated, as compared with 5-7S T-antigen (McCormick and Harlow, 1980; Fanning et al., 1981b; Greenspan and Carroll, 1981). T-antigen produced by SV40 tsA58-transformed cells at the permissive temperature displays the same properties as wild-type T-antigen, but when produced at the non-permissive temperature it accumulates in an underphosphorylated 5-7S form that has no associated p53 (Prives et al., 1979; Greenspan and Carroll, 1981; Carroll and Gurney, 1982). It appears therefore that T-antigen oligomerisation and concomitant phosphorylation occur prior to the assembly of the ∿23S large T/p53 complex.

The oligomerisation of T-antigen (and therefore the assembly of the complex) may depend on the presence of divalent cations. 23S complex and 16S T-antigen are both dissociated into 5-7S T-antigen

by treatment with 20mM EDTA, an effect reversed by adding excess

calcium ions (Montenarh and Henning, 1983). This is in contrast

to the results of McCormick and Harlow (1980) who found 5mM EDTA

had no effect on the stability of the large T/mouse p53 complex.

However the discrepancy between these two results probably only

reflects the different concentrations of chelating agent used.

As yet, the p53 protein is not amenable to the type of

genetic analysis that would define the region(s) of the protein

involved in its interaction either with SV40 large T-antigen or

with the adenovirus 58K E1b gene product. Indeed, whilst it is

widely assumed that these associations are analogous and will

therefore involve the same parts of the p53 protein, there is no

proof of this at present. It might also be expected that the

binding sites on p53 for large T and 58K could be defined

antigenically, since they should contain determinants that would

be available for antibody binding only on uncomplexed p53.

However, none of the anti-p53 antibodies so far described appear

to define determinants of this type.

The region of SV40 large T-antigen involved in the binding

of p53 ought to be relatively easier to define than the site of

association on p53 since large numbers of mutants in the T-antigen

gene and antibodies to T-antigen are available. These have

allowed the division of the 708 aminoacid large T polypeptide

into a number of regions, on both functional and antigenic bases

(Harlow et al., 1981b; Pipas et al., 1983; Clark et al., 1983;

Soprano et al., 1983). It is therefore surprising that deletion

mutant analysis has not so far defined the p53 binding domain of

large T with any accuracy. The C-terminal portion of large T,

aa663-708, defined by deletion mutants dl1263, dl1265 (Cole et al.,

1977; van Heuverswyn et al., 1979), dl1066 and dl1140 (Pipas et al.,

1983) which are all viable and able to transform cells in vitro, is not required for p53 binding (Denhardt and Crawford, 1980). The N-terminal 82 aminoacids, deleted in the hybrid D2 antigen from virus Ad2$^+$D2 (Hassell et al., 1978), are also dispensable for this purpose (McCormick et al., 1981). However, truncated T-antigens that retain the normal N-terminal sequences but which lack 340 aminoacids or more from the C-terminal end fail to bind p53 (Reddy et al., 1982; Clayton et al., 1982; Colby and Shenk, 1982; Chaudry et al., 1982; Sompayrac et al., 1983). Thus deletion of the sequences in T-antigen between αα340 and αα662 destroys its ability to bind to p53.

This same region of T-antigen is also implicated in p53 binding by studies with antibodies. A number of anti-T antibodies, all of which require sequences in the region αα333-635 for binding (Harlow et al., 1981b), show a strong preference for binding to T-antigen free of p53 which suggests that they bind close to the site of p53 association on T-antigen. Interestingly, the tsA lesions that have been precisely mapped lie in this portion of T-antigen which also appears to provide elements essential for the T-antigen ATPase activity (Clark et al., 1981; Clark et al., 1983; E. Harlow, pers.comm.). Furthermore, variant T-antigens which emerge in populations of transformed cells and appear to confer on transformed cells a selective advantage over cells expressing wild-type T-antigen carry duplications of sequence that affect parts of this region. However, these variants retain the ability to bind to p53 (Lovett et al., 1982; Chaudry et al., 1983; May et al., 1983). The tentative mapping of the p53 binding site to a region of T-antigen which is so obviously important in other T-antigen functions tempts speculation as to a possible role for p53 in the expression or regulation of these

functions. However, a proper understanding of the importance of

p53 binding to T-antigen awaits more precise mapping data.

Despite our lack of knowledge about the purpose of large

T/p53 association, it seems reasonable to assume that this specific

virus-host interaction is not fortuitous. The weak association

between the two proteins must therefore be important to the

proper progress of lytic infection, in order to explain the

retention of the p53 binding site on large T. Indeed, it is

possible that it is the greater strength of this association in

rodent cells, rendering complex formation essentially irreversible,

that confers on these cells their non-permissive phenotype. On

this basis, several speculative explanations for the role of p53/

large T association in lytic infection can be advanced. Lane and

Hoeffler (1980) argued that, in order to subvert the cellular

replication machinery, T-antigen might have to replace a cellular

protein whose function in normal cells involved an interaction

with p53, but so far such a "T-equivalent" cellular protein has

not been unequivocally identified (Lane and Hoeffler, 1980;

Crawford et al., 1982a). Alternatively, p53 may be a protein

recruited by T-antigen to form a complex with activities distinct

from those of T-antigen alone. In this event, p53 could be needed

either to supply its normal cellular function, perhaps in some

unusual location to which it is directed by its association with

large T, or to create a multisubunit enzyme in which it has a

novel function, much as the bacteriophage Qβ replicase comprises

a phage-specified subunit and host-specified subunits that normally

function in protein rather than RNA synthesis. (Kamen, 1970; Blumenthal et al., 1972; Groner et al., 1972)

The importance of T-antigen/p53 association to SV40-mediated

transformation is also not certain, since there is some evidence

that the presence of the complex is not sufficient to maintain the

transformed state and may not even be needed for its induction.
Firstly, three revertants of SV40-transformed mouse fibroblasts
retain an apparently intact T/p53 complex in amounts similar to
those in transformed cells (Lane and Crawford, 1980). Secondly,
various fragments of SV40 DNA and SV40 deletion mutants capable
of expressing only severely truncated large T-antigens which
cannot bind p53 nevertheless transform cells in culture (Colby
and Shenk, 1982; Clayton et al., 1982; Sompayrac et al., 1983).
However, the transformation frequencies observed were very low
as compared with transformation by wild-type SV40 and it is
possible that these transformation events did not occur by the
normal mechanism. Whilst these data cast some doubt on the
importance of the large T/p53 complex in SV40-mediated transformation,
the observation that high level p53 expression in mouse primary
cells on passage correlates with their susceptibility to SV40-
mediated transformation (Chen et al., 1983) suggests that p53
plays at least an indirect role in this transformation process.

Immunofluorescence studies have shown that p53 has a
predominantly nuclear location in all cells expressing high levels
of the protein (McCormick and Harlow, 1980; Dippold et al., 1981;
Rotter et al., 1983a). Similar analysis of non-transformed cells,
which contain only small amounts of p53, suggests that p53 in
these cells is located in the cytoplasm but the immunofluorescent
signal on which this suggestion is based is only arguably above
background (Rotter et al., 1983a). Other groups have sought to
identify minor populations of p53 in SV40-transformed cells that
might, by virtue of association with T-antigen or otherwise, be
located on the cell surface (Luborsky and Chandrasekaran, 1980;
Schmidt-Ullrich et al., 1980; Chandrasekaran et al., 1981b; Santos
and Butel, 1982a,b). The available techniques are however

inadequate to answer the question unequivocally, since any
apparently plasma-membrane associated p53 detected by either
surface-labelling or subcellular fractionation could easily be
due in the first case to contamination of the cell sample with
a few permeable dead cells and in the second case to imperfect
separation of subcellular fractions during the analysis.

Very little is known about the biochemical functions of
either p53 or the T/p53 complex as distinct from T-antigen
alone. The only function so far analysed comparatively in free
T-antigen and the T/p53 complex is SV40 origin-binding activity.
T-antigen is known to bind tightly to several sites in the origin
region of SV40 DNA, based around concensus penta-nucleotides
(De Lucia et al., 1983). Both free T and T/p53 appeared able to
achieve this binding with equal efficiencies (Reich and Levine,
1982; Scheller et al., 1982). However, whilst it is assumed that
the patterns of T-antigen binding at these sites required for
feedback inhibition of early transcription and for the initiation
of replication to be observed are identical, there is no firm
evidence that this is the case. It is possible, therefore, that
there are some functionally significant differences between free
T and T/p53 binding at the origin despite the ability of both
forms to mediate the immunoprecipitation of SV40 origin-region
DNA. This and the other free T-antigen functions must be very
carefully studied in the T/p53 complex before it can be said how
tha activities of the two forms differ.

Independent studies by two groups have suggested a role
for p53 in normal cells in the process of commitment of a cell .
to DNA replication and ultimately division. In the first study,
performed initially with an anti-SV40 tumour serum containing
anti-p53 activity (Milner and McCormick, 1980) and subsequently

with anti-p53 monoclonal antibody (Milner and Milner, 1981) it

was shown that p53, which was undetectable in resting lymphocytes,

appeared in the same cells within 4 hrs. of stimulation with the

mitogen concanavalin A.  This apparent induction of p53 depended

on de novo mRNA synthesis (either of p53 mRNA or of mRNA for a

protein capable of altering p53 expression).  In the second study,

anti-p53 antibodies were microinjected into cell nuclei (Mercer

et al., 1982).  It was found that these antibodies, when injected,

specifically blocked the acceleration of cell division normally

observed in serum-starved, quiescent 3T3 cells when re-fed with

serum.  This blocking depended on the relative timing of injection

and refeeding since no blocking was observed when antibody was

introduced 4 hours or more after refeeding with serum.  Neither

could anti-p53 antibodies block DNA synthesis already in progress.

These studies, taken together, strongly suggest that p53 performs

its normal function as a cell is committed to move from $G_o$ into

$G_1$/S phase.

The function supplied by p53 during this process of

commitment to division is not known.  Indeed, so far there is only

one report which attributes any specific biochemical activity to

p53.  Jay et al.(1981) have described a serine / threonine

phosphotransferase (protein kinase) activity present specifically

in anti-p53 immunoprecipitates from methylcholanthrene or SV40-

transformed but not untransformed mouse 3T3 cells.  This is

clearly an interesting result, but by itself cannot be taken as

proof that p53 has an intrinsic kinase activity, since even

monoclonal antibody immune precipitates contain many proteins

apart from the known antigen and this result might be attributable

to a specifically or non-specifically coprecipitated protein.

Also, the in vitro kinase assay is extremely sensitive, being able

to detect very low levels of phosphotransferase activity. It is

therefore difficult to be certain that the amount of phosphate

transfer observed is sufficient to imply a truly catalytic

phosphotransferase activity in the major immune precipitate

component. Further studies, preferably employing purified

proteins, are needed to determine whether or not p53 is a

protein kinase and to determine the full extent and nature of

its biochemical activities.


The Correlation of Altered p53 Expression with Cell Phenotype

In considering how our observations on p53 expression may

correlate with the expression of a transformed phenotype, it is

important to bear in mind that transformed cells differ from

normal cells in a number of ways. In addition to having the

extended growth potential in vitro and tumourigenic potential

in vivo that are the essence of the transformed phenotype, they

also display a high rate of cell division and are usually

biochemically less differentiated than normal cells. Thus

biochemical events that appear initially to be markers of

transformation may instead be indicators of rapid cell division

or of the pluripotent, undifferentiated state.

The discovery that p53 was expressed in transformed cells

of many origins led to the suggestion that its expression was

transformation-specific. However, as already discussed, p53

is now known to be a component of normal cells and it is also

known that the level of p53 protein in transformed cells varies

over a range of at least 100-fold, being in many cases not

detectably above that in normal cells. There are also well-

characterised transformed cell lines that lack any detectable p53

expression, even at the level found in normal cells. Thus neither

p53 expression, nor its precise level, correlate with expression of the transformed phenotype. However, it remains true that p53 metabolism appears to be altered in some way in all transformed cell lines tested and both transcriptional and post-translational factors have been shown to be responsible for such alterations in different transformed cells.

The possibility that p53 expression might correlate with in vivo tumourigenicity has also been examined. Such a possibility is suggested by the detection of anti-p53 activity in the serum of some tumoured animals but not of healthy animals. A set of lineally descended rat cell lines comprising precrisis fibroblasts, a post-crisis non-transformed line, its SV40-transformed daughter, a revertant of this transformant and a line derived by methyl-cholanthrene treatment of the revertant, were tested for p53 expression by pulse labelling (Pollack et al., 1979). The expression of p53 in each member of this cell set was found to correlate closely with its tumourigenicity. However, this and another group have also concluded that p53 expression does not correlate with either tumourigenicity or any particular aspect of the transformed phenotype in similar series of mouse cell lines (Mora et al., 1980; Chen et al., 1981; Mora et al., 1982). It is not possible to make any conclusions from these apparently conflicting results at this stage. Rotter et al.(1983c), however, produced results that lend definite support to the idea of a correlation between p53 expression and tumourigenicity. They described an Abelson murine leukaemia virus transformant, L12, that was unusual in being successfully rejected by syngeneic mice and in lacking p53 expression. They showed that p53 expression could be induced in these cells by treatment with phorbol ester and that such treated cells were then able to form lethal tumours,

suggesting that the presence of p53 was linked with tumourigenicity.

A similar conclusion was reached in a study of Friend virus-infected

cells (Ruscetti and Scolnick, 1983).

There are conflicting data on whether p53 expression correlates

with either rate of cell division or state of differentiation. As

already noted, p53 expression decreases when mouse embryonal carcinoma

cells differentiate (Oren et al., 1982). However this differentiation

also represents a reversion of the transformed phenotype and is

frequently accompanied by a decrease in the rate of cell division.

The increase in p53 expression in dividing lymphocytes definitely

implies a correlation with rate of division (Milner and Milner, 1981)

but, in contrast, Ruscetti and Scolnick (1983) found no p53 in

rapidly dividing but pre-neoplastic Friend virus-infected mouse

cells. These authors also found no decrease in p53 expression

when Friend virus-transformed cells underwent erythroid differentiation,

a result in direct conflict with the recent analysis of Shen et al.

(1983). However, the latter authors used a $3\frac{1}{2}$ hour labelling

period instead of the 1 hour of Ruscetti and Scolnick. The

differences between the results of the two groups can be explained,

therefore, if there is a decrease in p53 stability with erythroid

differentiation to a half-life of about 1 hour. Shen et al. have

also linked reduced p53 expression with a prolongation of the $G_1$

phase of the cell cycle, i.e. with reduced rate of cell division.

The difficulty of dissociating the various phenotypic

features with which p53 expression might be linked makes it

difficult to draw firm conclusions. There is, however, fairly

compelling evidence that p53 expression is linked in some way with

the commitment of cells to divide and it is easy to envisage how

altering the structure or amounts of such a control protein could

lead to the expression of at least some elements of the transformed

phenotype. Postulating a controlling role for p53 can also accommodate the observation that certain rapidly dividing cells do not express detectable p53 protein. Further experiments will no doubt show whether or not this is the correct explanation of the vast array of largely qualitative observations on the expression of p53 that have been made, and will perhaps allow us to understand p53 function at the biochemical level.

Chapter 2

MATERIALS AND METHODS

In this chapter I shall describe in some detail the basic

methods used to generate the data to be presented subsequently.

However, procedures which I developed for the purification of p53

are presented in detail in the course of describing the results

obtained using them. Minor modifications to basic procedures which

can be quickly described are also presented in the text of Chapters

3 and 4.

Materials

All chemicals employed were analytical grade except as noted

below. Glycerol was Gold Label Grade from Aldrich Chemicals

(Gillingham, Dorset). Sodium dodecyl sulphate (SDS) was purchased

from Serva (Heidelberg, W. Germany) and, for the experiments

described in Chapter 4, was recrystallised twice from 80%$_\wedge^{(v/v)}$ethanol

after removal of contaminants with activated charcoal. The reagents

used in the analysis of aminoacid composition and determination of

peptide sequences were of the highest purity available and were

obtained from Rathburn (Walkerburn, Scotland) or BDH (Poole, Dorset).

TPCK-treated bovine trypsin was obtained from Worthington (Flow

Laboratories, Irvine, Scotland). Spectrapor membrane dialysis

tubing from Raven Instruments (Haverhill, Suffolk) was prepared for

use in the purification of p53 by heating to 80$^\circ$C for 5 min. in

10mM ethylenediaminetetraacetic acid (EDTA), pH 7.5, 5%$_\wedge^{(w/v)}$sodium

bicarbonate, then in ddH$_2$O and finally in 0.05%$_\wedge^{(w/v)}$SDS in which it was

subsequently stored at room temperature.

Table 2.1                    Cell Lines Employed

| Cell Line | Description | Provided by | Reference |
|---|---|---|---|
| SVA31E7 | Mouse SV40-transformed BALB/c 3T3 fibroblasts | Y.Ito | Unpublished |
| CQ15 | Mouse 3T12-like isolate of C3H-Q embryo fibroblasts | L.Gooding | Unpublished |
| P3-NS1-1-AG4-1 | Mouse 8-azaguanine$^R$ non-secreting BALB/c plasma-cytoma | R.Knowles via E.Harlow | Cowan et al. (1974) |
| SV80 | Human SV40-transformed skin fibroblasts | D.Livingston | Todaro et al. (1966) |
| VA13 | Human SV40-transformed WI38 lung fibroblasts | L.Gooding | Jensen et al. (1964a) |
| C33I | Human cervical carcinoma cell line | N.Auersperg | Auersperg (1964) |
| CV1 | African Green Monkey kidney cells | D.Livingston | Jensen et al. (1964b) |

Table 2.2                    Antibodies Employed

| Hybridoma | Specificity | Provided by | Reference |
|---|---|---|---|
| PAb122 | anti-p53 | E.Gurney via E.Harlow | Gurney et al. (1980) |
| PAb203/4/5 | anti-SV40 large T | D.Lane | Lane & Hoeffler (1980); Clark et al.(1981) |
| PAb400-series | anti-SV40 large T except PAb410/421 anti-p53 | E.Harlow | Harlow et al. (1981b) |
| PAb600-series | anti-SV40 large T except PAb607 anti-p53 | L.Gooding | unpublished |
| RA3-2C2 | anti-p53/anti-B lymphocyte differentiation antigen | R.Coffman | Coffman & Weissman (1981) |

## Cell Lines and Antibodies

Various cell lines used in the work described in Chapters

3 and 4 were isolated and provided by others.  Table 2.1 lists

these cell lines, their origins and from whom they were obtained.

SV40 virus, used to infect monkey CV1 cells, was the small plaque

strain, SV-S (Takemoto et al., 1966) derived from strain 776 of

Sweet and Hilleman (1960).  A large number of monoclonal antibodies

were also donated for use in the work I shall describe.  These are

listed in Table 2.2.  Lyophilised rabbit antisera with activity

against specific mouse immunoglobulin classes were obtained from

Miles Laboratories Inc. (Slough, England).

All cells except the mouse plasmacytoma line P3-NS1-1-AG4-1

were maintained in monolayer cultures on 90mm NUNC tissue culture

dishes.  Tissue culture media were Dulbecco's modified Eagles medium

(E4) supplemented with 10% foetal calf serum (FCS) for SV80 and CQ15,

E4 supplemented with 5% FCS for SVA31E7, E4 supplemented with 10% v/v

calf serum for CV1, or RPMI 1640 (Gibco Laboratories) supplemented

with 10% FCS for C33I and VA13.  SV80 and E7 cells were grown on a

preparative scale as monolayers on NUNC bioassay trays.  P3-NS1-1-AG4-1

(NS1) cells were grown in NUNC or Falcon tissue culture flasks in

RPMI 1640 medium supplemented with 10% FCS, 10% NCTC 135 (Gibco),

0.15mg/ml oxalacetate, 0.05mg/ml pyruvate and 0.2U/ml insulin

(hybridoma growth medium - see below).

Table 2.2    Antibodies are mouse x mouse hybrids except RA3-2C2 which is

a mouse myeloma x rat splenocyte hybrid.  Anti-p53 antibodies PAb421,

PAb410 and PAb122 were raised to mouse p53 and cross-react with p53

from other rodents, and primates.  PAb607 was raised to and is specific

for mouse p53 whilst RA3-2C2 was probably raised to a B-lymphocyte

differentiation antigen and cross reacts with mouse p53.

Buffers and Solutions

The following buffers and solutions were employed in the course of the work I shall describe and are referred to by the abbreviations indicated:

NET : 150mM NaCl

5mM EDTA

50mM tris/HCl pH 8.0

NET/gel : 150mM NaCl

5mM EDTA

50mM tris/HCl pH 7.4

0.05% v/v nonidet P40 (NP40)

0.02% w/v sodium azide

0.25% w/v gelatin

NET/BSA : NET/gel containing 2.0mg/ml of bovine serum albumin (BSA)

phosphate-buffered : 140mM NaCl
saline

2.7mM KCl

8.1mM $Na_2HPO_4$ )
) pH 7.2
1.5mM $KH_2PO_4$ )

0.9mM $CaCl_2$

0.5mM $MgCl_2$

tris-buffered : 140mM NaCl
saline

5.1mM KCl

0.7mM $Na_2HPO_4$

5.6mM dextrose

25mM tris/HCl pH 7.4

10 U/ml penicillin

0.1mg/ml streptomycin

.0015% w/v phenol red

NP40 lysis buffer : 1% v/v NP40

150mM NaCl

50mM tris/HCl pH 8.0

SDS gel sample   :     2% $^{w/v}$ SDS

       buffer           100mM dithiothreitol

                        10% $^{v/v}$ glycerol

                        25mM tris/HCl pH 6.8

   10% SAC   :     10% vol/vol suspension of formaldehyde-

                    fixed Staphylococcus aureus (strain Cowan 1)

                    in phosphate-buffered saline.

## Metabolic Labelling of Cultured Cells and Preparation of Extracts

Cells growing as monolayers were labelled just prior to confluence on 90mm NUNC dishes in 2.5ml E4 medium lacking phosphate with 1.0mCi $^{32}$P-phosphate (carrier-free, Amersham International) for 3 hrs or in 1.5ml medium lacking methionine with 1.0mCi $^{35}$S-methionine (>1000 Ci/mmol, Amersham International) for 3 hours. Alternatively, cells were labelled on 50mm NUNC dishes in 1.0ml E4 medium lacking leucine with 0.5mCi 4,5-$^3$H-leucine (130-190 Ci/mmol, Amersham International) for 2 hours. Hybridoma cells were cultured in suspension in E4 medium lacking methionine at a density of 5 X 10$^6$ cells/ml for 3 hrs in the presence of 0.5 Ci/ml $^{35}$S-methionine to label secreted antibody molecules.

Extracts were prepared from labelled monolayers after washing with tris-buffered saline at 0°. Cells were extracted on ice for 30 min with NP40 lysis buffer. Dishes were then scraped dry and the extracts clarified by centrifugation for 1 min in an Eppendorf microfuge. Generally, a monolayer containing around 10$^7$ cells was extracted with 0.5ml of NP40 lysis buffer and the extract distributed between ten immunoprecipitation reactions. However, when the extracted proteins were required in more concentrated form, e.g. for sucrose gradient analysis, this volume was reduced to 250µl.

Extracts of unlabelled cells for use in the purification of p53 were prepared either using NP40 lysis buffer as described above,

or by a homogenisation protocol that permitted the purification of

polysomes and T/p53 protein complex from the same cells. Preparation

of extracts by this latter procedure was kindly performed by

N. Williamson. Around $2 \times 10^9$ E7 cells were taken from monolayer

cultures on plastic and washed in phosphate-buffered saline. Washed

cells were broken in about 4ml per $10^8$ cells of 25mM tris/HCl pH 7.5 ,

25mM NaCl, 5mM MgCl$_2$ (homogenisation buffer) containing 2% Triton X-100,

1 mg/ml heparin, 1 µg/ml cycloheximide. The supernatant from a 5 min,

15,000 rpm centrifugation in a Sorvall SS34 rotor was made 0.1M in MgCl$_2$ and

left at $0^o$ for 1 hour before layering over an equal volume of

homogenisation buffer containing 0.5% Triton X-100, 0.5 mg/ml heparin,

1 µg/ml cycloheximide, 0.5M sucrose and centrifugation at 15,000 rpm for

15 min as above. The supernatant from this centrifugation was taken as the

large T/p53-containing cell extract.

In some experiments it was necessary to remove either T-antigen

or p53 from a labelled cell extract that initially contained both

these antigens. SV40-infected CV1 cell extracts and SV80 cell extracts

were cleared of all p53, including T-associated p53, by twice adding

anti-p53 antibody PAb421 at 37.5 µg/~$10^7$ cells to concentrated cell

extract (500 µl/~$10^7$ cells). Immune complexes were collected on each

occasion after 30 min at $0^o$ by adding the S. aureus cells from 500µl

of 10% SAC (Kessler, 1975). These were left for 15 min at $0^o$ and

removed by centrifugation (microfuge). A final addition of S. aureus

cells was then made to remove any residual immunoglobulins.

To prepare an SV80 extract lacking any T-antigen or large T/p53

complex the large T/p53 complex was first induced to dissociate.

Freshly prepared extract from $10^7$ cells (500µl) was diluted with 4.5ml

of NP40 lysis buffer containing 10% w/v sucrose, 1mM dithiothreitol,

further diluted with 5ml of NET/BSA buffer and incubated at +$10^o$

overnight. Large T-antigen was then removed by adding antibodies

PAb416 or PAb419 using the same protocol as described above for clearance of p53.

## Sucrose Gradient Analysis

Labelled proteins were in some cases separated by velocity sedimentation through sucrose before immunoprecipitation. 0.3ml of labelled cell lysate was layered over a 4.4ml linear 5-20% w/v sucrose gradient, formed in NP40 lysis buffer containing 1mM dithiothreitol on a 0.3ml cushion of 60% w/v sucrose. Gradients were then resolved by centrifugation in a Beckman SW55 rotor for 6 hrs at 55K rpm at +6°. Gradients were collected in approx. 0.2ml fractions which were then divided as required for parallel immunoprecipitation. Parallel gradients were calibrated with marker proteins having sedimentation coefficients 26S (glutamate dehydrogenase), 13.5S (phosphorylase a) and 7S (immunoglobulin G).

## Analytical Immunoprecipitations

Aliquots of labelled cell extract were diluted 5 to 10 fold with NET/BSA buffer and an appropriate amount of antibody added. Antibodies were either diluted from 10 mg/ml stocks in phosphate-buffered saline and amounts used quoted in μg protein or were used in the form of tissue culture supernatant from hybridoma cells and amounts quoted in μl. Tissue culture fluid generally contains antibody at around 30 μg/ml concentration. Reactions were allowed to proceed for 2 hours at room temperature. 0.25μl of rabbit anti-mouse Ig antiserum (except Figures 3.8 and 3.9, 0.5μl) was added to all small-scale reactions 30 min prior to the end of the reaction period. Immune complexes were collected on 20μl 10% SAC (Kessler, 1975) per 1.5μg purified antibody or 20μl tissue culture supernatant for 15 min at +4°, washed twice in NET/gel buffer and eluted into

1.25 volumes of SDS gel sample buffer per volume of 10% SAC employed.


## Gel Electrophoresis and Detection of Labelled Proteins

The components of analytical immunoprecipitates were separated

on SDS-polyacrylamide gels using the discontinuous tris/HCl - glycine

buffer system described by Laemmli (1970). A 10% $^{w/v}$ acrylamide

concentration was used, except where otherwise indicated. When

preparative gels were used to prepare p53 for the purpose of

immunising mice, this same buffer system was used. However, to avoid

contamination of purified proteins with glycine, a tris/borate/

sulphate buffer system, described by Neville (1971), was employed

when purifying p53 for sequence and composition analysis.

Analytical gels were fixed in 7.5% $^{v/v}$ acetic acid, 25% $^{v/v}$ methanol

for at least 30 minutes. At this stage, gels containing $^{3}$H-labelled

polypeptides were impregnated with diphenyloxazole as described by

Bonner and Lasky (1974). Gels were then dried and exposed either at

room temperature to Kodak SB-5 film ($^{35}$S-methionine and most $^{32}$P-

phosphate labelling experiments) or at $-70^{o}$ to preflashed Fuji RX

film with intensifying screens ($^{3}$H-leucine and a few $^{32}$P-phosphate

labelling experiments). Labelled species in preparative gels were

detected by exposure of the wet gel (unfixed), wrapped in Saranwrap,

to Kodak SB-5 film at room temperature.

Proteins of known molecular weight were used as markers during

gel electrophoresis. They were either used unlabelled and detected

by their binding of Coomassie-blue stain or used as $^{14}$C-methylated

or -acetylated derivatives and detected by autoradiography or

fluorography. $^{14}$C-methylated proteins (Amersham International) were

myosin (200K), phosphorylase b (92K), bovine serum albumin (68K),

ovalbumin (46K), carbonic anhydrase (30K), lysozyme (14.3K),

cytochrome c (12.5K), aprotinin (6.5K) and insulin A and B (2.35K

and 3.4K). $^{14}$C-acetylated proteins were phosphorylase b (92K) and

glutamate dehydrogenase (53K) and were prepared by L. Crawford using

1-$^{14}$C-acetic anhydride (60 -120 Ci/mmol, Amersham International).

Unlabelled proteins were phosphorylase b (92K), transferrin (80K),

catalase (60K), glutamate dehydrogenase (53K), alcohol dehydrogenase

(41K), carbonic anhydrase (30K), soybean trypsin inhibitor (21.5K)

and myoglobin (17.2K).

## Cell Fusion and Culture of Hybrid Cells

Methods for the production of hybridomas were taken from

Harlow et al. (1981b), adapted from the work of Köhler and Milstein

(1975) and Galfre et al. (1977). Spleens from immunised mice were

teased apart in RPMI1640 medium using sterile needles and the

resulting cell suspension decanted from any rapidly settling particles.

These splenocytes and $10^7$ NS1 plasmacytoma cells were washed twice

in RPMI 1640 medium and finally mixed and pelleted together. The

supernatant was removed and 0.5g of PEG 1000 in 0.5ml of RPMI 1640

gently stirred into the pellet over the course of one minute with

stirring continuing for a further 1 minute. 10ml of RPMI 1640 was

then stirred into the pellet over the next two minutes. The cells

were pelleted from this suspension and resuspended in around 200ml

of hybridoma growth medium (as described for the culture of NS1

plasmacytoma cells above). This suspension was split into around 400

0.5ml cultures in 24-well tissue culture plates. Cultures were

maintained at $37^\circ$ in a moist 10% $CO_2$ atmosphere.

After 24 hours (day 1), a further 0.5ml of growth medium was

added to each culture containing hypoxanthine, thymidine and

methotrexate to provide final concentrations of 0.1mM, 0.01mM and

0.01mM respectively. These agents were maintained in the growth

medium when the medium was changed on days 3 and 5. Methotrexate

was omitted from fresh medium from day 7 and from day 10 the cultures were maintained in unsupplemented growth medium.

NS1 cells have resistance to 8-azaguanine and therefore lack the enzyme hypoxanthine-guanine phosphoribosyl transferase (HGPRT) that would otherwise allow the lethal incorporation of this base analogue into cellular DNA. Methotrexate blocks de novo synthesis of both purines and thymidine by interfering with the transfer of single carbon atoms via the cofactor tetrahydrofolate. In this it is analagous to aminopterin which was used by Littlefield (1964) for the same purpose. Methotrexate was used here because of its greater stability and ease of handling. In the presence of this agent, cells depend on exogenous supplies of hypoxanthine and thymidine for survival and to utilise these they require the enzymes HGPRT and thymidine kinase. Thus NS1 cells will not survive in the selective medium whereas hybrids having the splenocyte parental HGPRT enzyme will grow. Unfused splenocytes will not divide in culture and will die within a few days. Hypoxanthine and thymidine were retained in the medium after withdrawal of methotrexate to prevent cell death due to the presence of residual methotrexate in the cultures.

Hybrid colonies were of a sufficient size for screening at around day 17. 100µl of tissue culture supernatant from each culture well that contained live cells was tested for the presence of antibody able to immunoprecipitate the large T/p53 complex from $^{32}$P-labelled SV80 cell extract. Procedures were as described above except that the S. aureus cell pellets were not washed before elution into SDS-gel sample buffer. Cultures giving a positive result were subcultured at various dilutions in 96-well Linbro microtitre trays, in equal volumes of growth medium and similar medium preconditioned by normal BALB/c splenocytes for 3 - 4 days at 37$^{\circ}$. Wells containing colonies were screened as above and a positive colony selected for further subculture.

Hybridomas were considered cloned when >99% of the wells containing

colonies gave positive results in the immunoprecipitation screen at

a dilution such that only 50% of the well contained colonies. These

clones were then transferred to mass culture and passaged at dilutions

of 1 in 5 every second or third day.


Purification of Antibodies

Antibodies of the PAb400-series and PAb122 were provided to me

in purified concentrated form by E. Harlow. They had been purified

from tissue culture supernatant as previously described (Harlow et al.

1981b), concentrated by ammonium sulphate precipitation and redissolved

at high concentration in phosphate-buffered saline. RA3-2C2 was

provided as a concentrated solution by R. Coffman. PAb1101, PAb1102,

PAb1103 and PAb1104 were partially purified on a small scale by

ammonium sulphate precipitation from tissue-culture supernatant.

PAb1103 and PAb1104 were obtained 90% pure and PAb1101 and PAb1102

about 10-20% pure as judged by Coomassie blue staining of samples after

gel electrophoresis. The only major contaminant had the molecular

weight expected of serum albumin. These purified antibodies were

iodinated for use as probes in radioimmunoassays either by a modification

of the chloramine-T method of Syvanen et al.(1973) described by

Benchimol et al.(1982) or by the method of Bolton and Hunter (1973).


Radioimmunoassays

Two types of radioimmunoassay were employed. The first, a

solution binding assay, was described by Lane and Robbins (1978) and

was used in a modified form described by Harlow et al.(1981b). This

assay studies the rebinding of SDS-denatured polyacrylamide gel-purified

antigens by antibodies in free solution. The bound antigen is

determined by collection on S. aureus protein A and counting in

scintillation fluid. Rebinding in this context has been taken as a measure of the stability of the antigenic determinant in question to protein denaturation.

The second type of assay utilises antibodies to immobilise antigens on a plastic surface. This assay was designed and developed by E. Harlow, L. Crawford and S. Benchimol and has been described previously in various forms (Harlow et al., 1981b; Crawford, 1982; Benchimol et al., 1982). It was applied here firstly to determine the class of antibody secreted by newly cloned hybridomas. Immuno-globulin fractions from class-specific rabbit anti-mouse Ig antisera were purified by affinity chromotography on protein A-sepharose (Pharmacia) using 0.1M phosphate buffer, pH 3 , to elute the bound material which was immediately restored to neutral pH by the addition of one tenth volume 1.0M phosphate buffer, pH 8 . These antibodies were diluted into 10mM sodium phosphate buffer, pH 7.0 , to a concentration of 20µg/ml and 1µg of protein in 50µl buffer allowed to bind to the surface of PVC microtitre wells overnight at room temperature in a moist atmosphere. $^{125}$I-labelled antibodies of known class were mixed with an excess of an unlabelled antibody, either of known class, the same or different from that of the labelled antibody, or an unknown antibody whose class was to be determined, and incubated on wells precoated with antibodies of the specificity appropriate to bind the labelled antibody. After 4 hours the wells were washed and the bound counts determined. Blocking of labelled antibody adsorption onto the well signified that the cold antibody included in excess was of the same Ig class as the labelled probe.

An assay that involved similar principles was used to determine the extent of interference between different pairs of determinants on SV40 large T-antigen and p53. An antibody that was able to bind the antigen required for examination was first bound to the surface of PVC

wells in 10mM phosphate buffer. Antigen was then bound to this

immobilised antibody from a concentrated (~2 X $10^7$ cells/ml) unlabelled

NP40 cell extract. After 2 hours, the wells were washed and mixtures

of various pairs of $^{125}$I-labelled and unlabelled antibodies added.

Each mixture comprised 10µl labelled antibody in NET/gel and 25µl

unlabelled antibody in the form of hybridoma tissue culture fluid

dialysed against 150mM NaCl, 50mM tris/HCl pH 7.4 , 1 mg/ml BSA.

After two hours the counts bound in each case were determined. A

low value indicated interference by the unlabelled antibody with the

binding of labelled antibody to the antigen.


## Performic Acid Oxidation of Proteins

Purified p53 was oxidised with performic acid to convert

cysteine residues to their stable cysteic acid derivatives (Hirs, 1967)

before preparation of tryptic peptides. Techniques were as described

by Smith et al. (1978). Briefly, approximately 50µg of protein was

dissolved in 100µl 98% v/v formic acid and mixed with 25µl methanol and

40µl performic acid (20:1 formic acid: 100 vol. $H_2O_2$ premixed at room

temperature for 2 hours). This was incubated at $-5^\circ$ in a salt/ice

bath for two hours and then lyophilised.


## Preparation of Tryptic Peptides

Approximately 1nmol of lyophilised, oxidised p53 was dissolved

in 100µl fresh 1% w/v ammonium bicarbonate and digested with 1 part in 50

(w/w) bovine trypsin for four hours at $37^\circ$. Peptides were lyophilised

three times from ddH$_2$O and redissolved in 50µl of 0.1% v/v trifluoracetic

acid (TFA), 6M urea for separation on a 75 X 4.5mm Synchropak RP-P

column (Synchron Inc., Linden, Indiana) by high pressure liquid

chromatography, HPLC (Bennett et al., 1980). The column was developed

with a linear 0 - 60% v/v gradient of acetonitrile in 0.1% v/v TFA over a

1 hour period. The eluate was monitored for absorbance at 206nm and collected in 0.5ml fractions.

## Aminoacid Composition Analysis

Lyophilised p53 (about 0.1nmol) was taken up in 50µl of 6N HCl and 0.5µl of 1,4-butanedithiol added. Samples were then deaereated, sealed under vacuum, and hydrolysed at $110^{\circ}$ for 22 hours. After removing the acid under vacuum, aminoacids in the hydrolysate were analysed by automated pre-column derivatisation with o-phthalaldehyde using a C-8 'Short One' reverse phase column (Rainni Insts., Woburn, Mass., USA) as described by Dabre and Waterfield (1983).

## Protein Sequence Analysis

Aminoacid sequences were determined using a gas phase sequencer built and operated as described by Hewick et al. (1981). High sensitivity detection methods for phenylthiohydantoin (PTH)-aminoacid analysis employed a C-8 Zorbax (Dupont) reverse phase column as described by Waterfield (1983).

Chapter 3


THE ISOLATION AND CHARACTERISATION OF MONOCLONAL

ANTIBODIES WITH ACTIVITY AGAINST HUMAN p53


Introduction

It will be evident from the foregoing discussion of our

knowledge of p53 (Chapter 1) that there exists a heavy dependence on

immunological reagents as probes for the presence and quantitation of

p53. This will continue to be the case until such time as functional

assays for p53 can be designed. The first immunological probes for

p53 were polyclonal antisera from animals bearing tumours induced by

injection of SV40- or methylcholanthrene-transformed cells. However,

such sera had the major disadvantage that they were not monospecific

and so the techniques of hybridoma isolation (Köhler and Milstein,

1975) were used by several groups to obtain monoclonal anti-p53

antibodies. As discussed in Chapter 1, six such antibodies have so

far been described. All of them bind to mouse p53 but only three, all

of them apparently recognising the same or similar determinants, also

bind to human p53. Only one antigenic area on human p53 can therefore

be probed with existing monoclonal antibodies.

This situation is unsatisfactory for two reasons. Firstly,

whilst monoclonal antibodies are in principle specific for an epitope

on a single antigen, it has been found that, when employed under

experimental conditions, these antibodies often display significant

affinity for other proteins (Crawford et al., 1982a; Lane and

Koprowski, 1982). This presumably reflects the presence on these

proteins of determinants that are related to some extent to the

antigen against which the antibody was raised. Depending on the

experimental approach employed, such cross-reactivities can obviously

confuse interpretation of the results obtained. Secondly, the set of anti-p53 antibodies currently available may not recognise typical p53 determinants. The splenocyte parents of five of these six hybridomas were taken from tumour-bearing animals that, for reasons which are not understood, mount an immune response to p53. It is clearly possible that the immunogenicity of p53 in this situation is the result of transformation-specific alterations in its structure. If so, anti-p53 antibodies arising out of immunisations with transformed cells might be expected to recognise determinants which are to some extent transformation-specific. Using such antibodies to determine, for example, the relative amounts of p53 in normal and transformed cells could easily produce an erroneous result.

The elimination of determinant-specific effects from results of antibody-based p53 analyses could be achieved by performing all experiments with a panel of antibodies raised against p53 in different ways and comparing the results so obtained. To permit this type of comparative analysis, the isolation of further anti-p53 antibodies, in particular antibodies that react with human p53, was clearly necessary. To achieve this objective, I purified denatured p53 from an SV40-transformed human fibroblast cell line and used it to immunise BALB/c mice. The spleens from these animals provided splenocytes for fusion with immortal plasmacytoma cells. Hybrid clones were then selected for the ability to secrete antibody capable of binding to p53 in a detergent extract of the same human cells using an immunoprecipitation assay.

Denatured antigen was chosen as an immunogen for this work in the hope that monoclonal antibodies would be raised to previously uncharacterised p53 determinants. I anticipated that denaturation might remove any bias towards the recognition of only certain antigenic determinants on transformed cell p53 by the immunised

mouse. Ideally, the possibility of isolating antibodies to transformation-specific determinants could be eliminated by using p53 from normal cells as an immunogen but the p53 content of such cells is too low to allow purification of material sufficient for the several immunisations required. Having raised hybridomas to denatured p53, clones were selected for secretion of antibody that could recognise native p53 since this property was considered essential if the antibodies isolated were to be experimentally useful.

## Isolation of Hybridomas Secreting Anti-p53 Antibodies

p53 was prepared from the human SV40-transformed fibroblast line, SV80, by immunoprecipitation and gel electrophoresis. A typical batch purification procedure was performed as follows. An extract of between $1 \times 10^9$ and $2 \times 10^9$ SV80 cells, grown in monolayer culture, was prepared by the standard NP40 lysis procedure using 10ml of lysis buffer per $10^8$ cells. This was pooled with an extract prepared from around $3 \times 10^7$ SV80 cells, metabolically labelled with $^{32}$P-phosphate, to provide a radiolabelled tracer for p53 during subsequent steps. The pooled extract was cleared of material capable of binding non-specifically to S. aureus cells by two sequential 15 min incubations with 0.5ml of 10% SAC at $0^o$. At each stage, S. aureus cells were removed by centrifugation at 8K rpm for 10 min in a Sorvall SS34 rotor.

Large T-antigen and p53 were immunoprecipitated from the precleared extract with a monoclonal antibody cocktail comprising 7.5μg each of the anti-large T-antigen antibodies PAb405, PAb413, PAb416, PAb419, PAb423 and PAb440 with 22.5μg of the anti-p53 antibody PAb421, all isolated and generously provided in purified form by E. Harlow (Harlow et al., 1981b). After 30 min at room temperature, the immune complexes were collected with 500μl of

10% SAC. Following a 15 min incubation at $0^{\circ}$, the S. aureus cells

were pelleted, washed twice in NET/gel buffer and resuspended in SDS

gel sample buffer. They were then kept at $0^{\circ}$ for 1 hr to elute bound

proteins without dissociating immunoglobulin heavy chain dimers

(McCormick and Harlow, 1980). Proteins in the precipitate were

separated on SDS-polyacrylamide gels and labelled proteins detected

in the wet gel by autoradiography. Using these films, p53-containing

gel slices were located and excised. These were washed in ∿10 vols

$ddH_2O$ for 20 min with two changes to remove SDS and salts.

Mice were immunised initially by intraperitoneal injection of

a homogenate of p53-containing polyacrylamide gel in phosphate-

buffered saline. Four weeks later, a second immunisation was

performed, by tail vein injection of an eluate of similar gel slices

in phosphate-buffered saline. Each immunisation used about 5μg of

Coomassie-G stainable protein. Spleens were taken and fusions

performed four days after the second immunisation. The detailed

procedures used for the isolation of splenocytes from immunised

mice, the fusion of splenocytes with plasmacytoma cells and the

subsequent selection and cloning of hybridomas were broadly as

described by Harlow et al. (1981b), taken in part from Galfre et al.

(1977), and are detailed in Chapter 2.

Hybrid clones were screened for the production of antibody

able to immunoprecipitate the large T/p53 complex from lysates of

$^{32}$P-phosphate labelled SV80 cells. All immunoprecipitation screening

reactions were performed with the addition of rabbit anti-mouse

immunoglobulin antiserum to permit detection of anti-p53 antibodies

that did not have affinity for S.aureus protein A. Twelve cultures

gave positive results on an initial screen. Figure 3.1 shows an

autoradiogram displaying one of these positive reactions against a

background of results from negative cultures. Lanes C+ and C- show,

-64-

<u>Figure 3.1</u> (opposite) Hybrid clones were screened for the secretion

of antibody that could immunoprecipitate the SV80 large T/p53 complex.

100μl of growth medium was removed from each culture under sterile

conditions and used to immunoprecipitate antigens present in an

extract of about 2 x 10$^5$ $^{32}$P-phosphate-labelled SV80 cells, diluted

to 500μl with NET/BSA buffer. The reaction was allowed to proceed

overnight at +4$^o$, 0.25μl rabbit anti-mouse Ig antiserum was added and

this was followed after a further 30 minutes with 20μl of 10% SAC.

Pellets were collected after 30 min. at +4$^o$ and eluted into 25μl of

SDS gel sample buffer for electrophoresis on 10% polyacrylamide - SDS

gels. Proteins in the fixed and dried gels were detected by auto-

radiography.

# Hybridoma
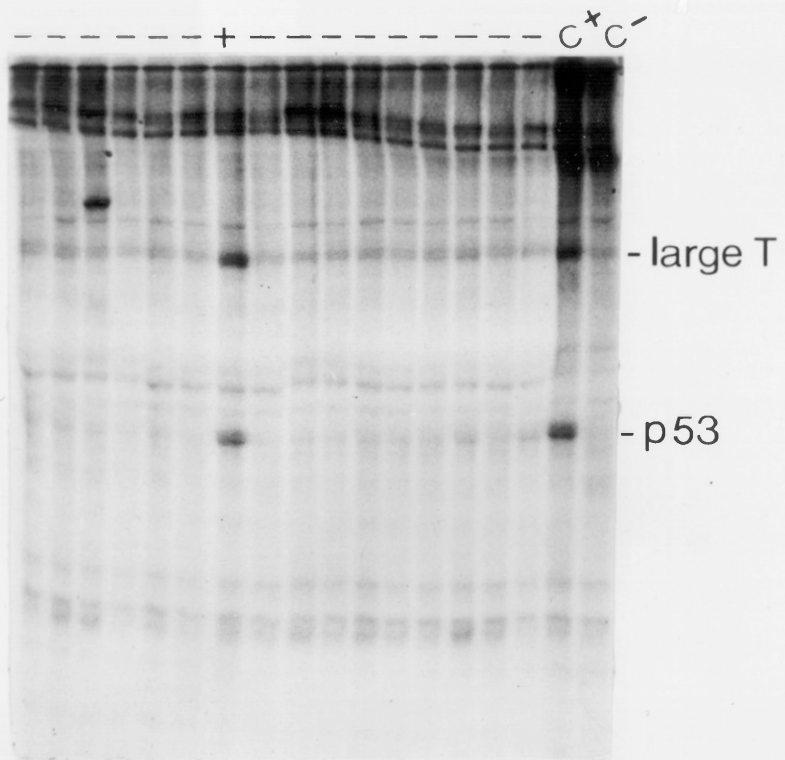## immunoprecipitation screen

### $^{32}$P - SV80 Extract



- large T

- p53

Figure 3.2 (opposite) Sepharose - protein A affinity - purified

antibodies from rabbit antisera directed against individual mouse IgM

and IgG classes and subclasses were allowed to adsorb to PVC microtitre

wells in 10mM phosphate buffer, pH 7.0 overnight. $^{125}$I-labelled

antibodies of the class appropriate to interact with each classing

antiserum (PAb405-IgG1; PAb421-IgG2a; PAb413-IgG2b; PAb430-IgG3;

RA3-2C2-IgM) were then added in 10μl NET/gel buffer, mixed with 40μl

of tissue culture supernatant containing either no antibody (column k),

test antibodies of the PAb1100-series (a to e) or control antibodies

of known class as indicated (f to j). Wells were washed in NET/gel

buffer after 4 hours at room temperature, dried and counted. All

results are expressed as counts per second on the ordinate.
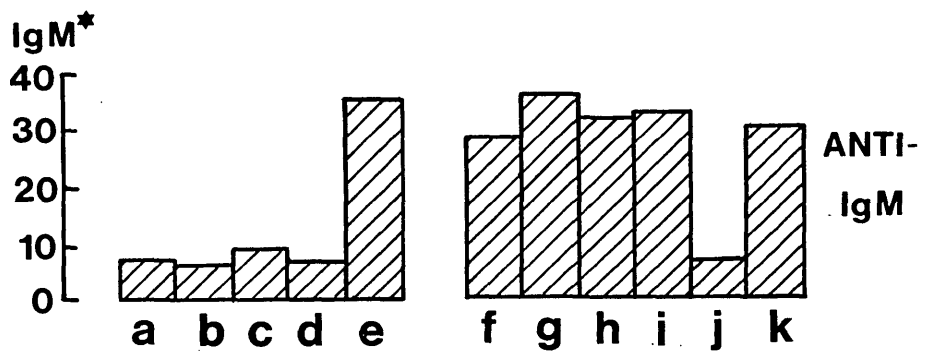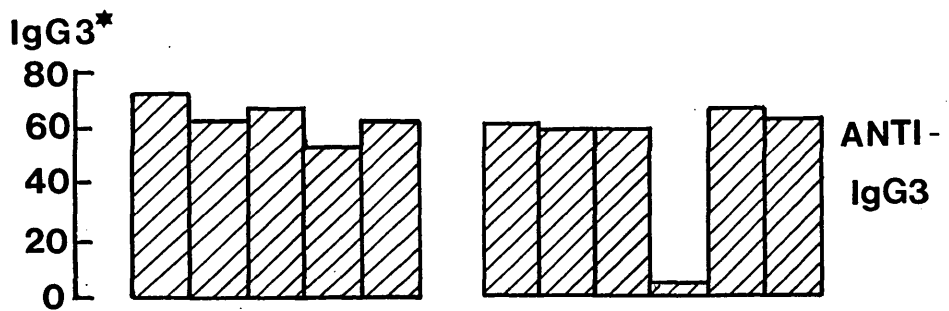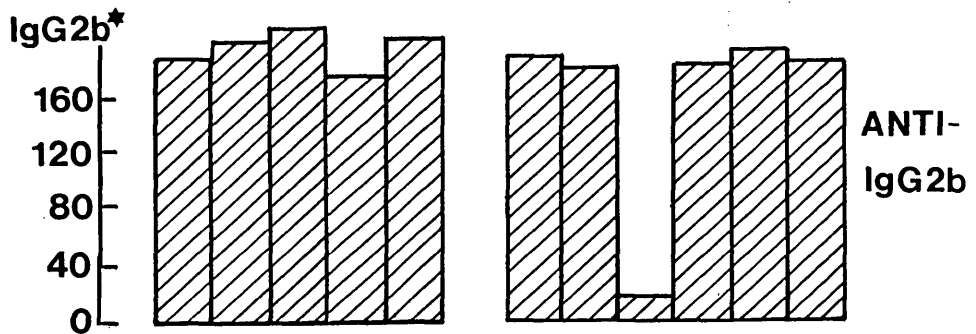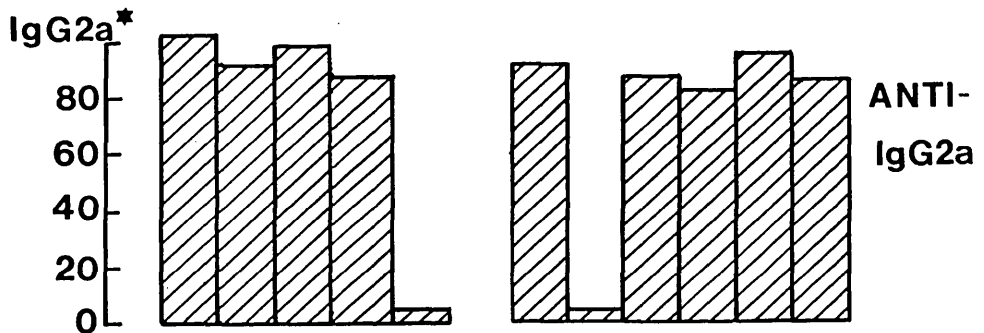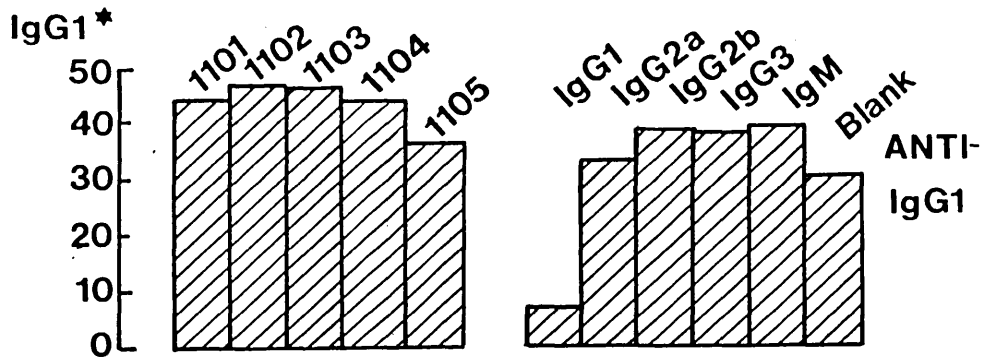
respectively, anti-p53-positive and -negative control reactions. Five hybridomas were successfully cloned from these initial positives and were designated PAb1101, PAb1102, PAb1103, PAb1104 and PAb1105 within the serial numbering system pr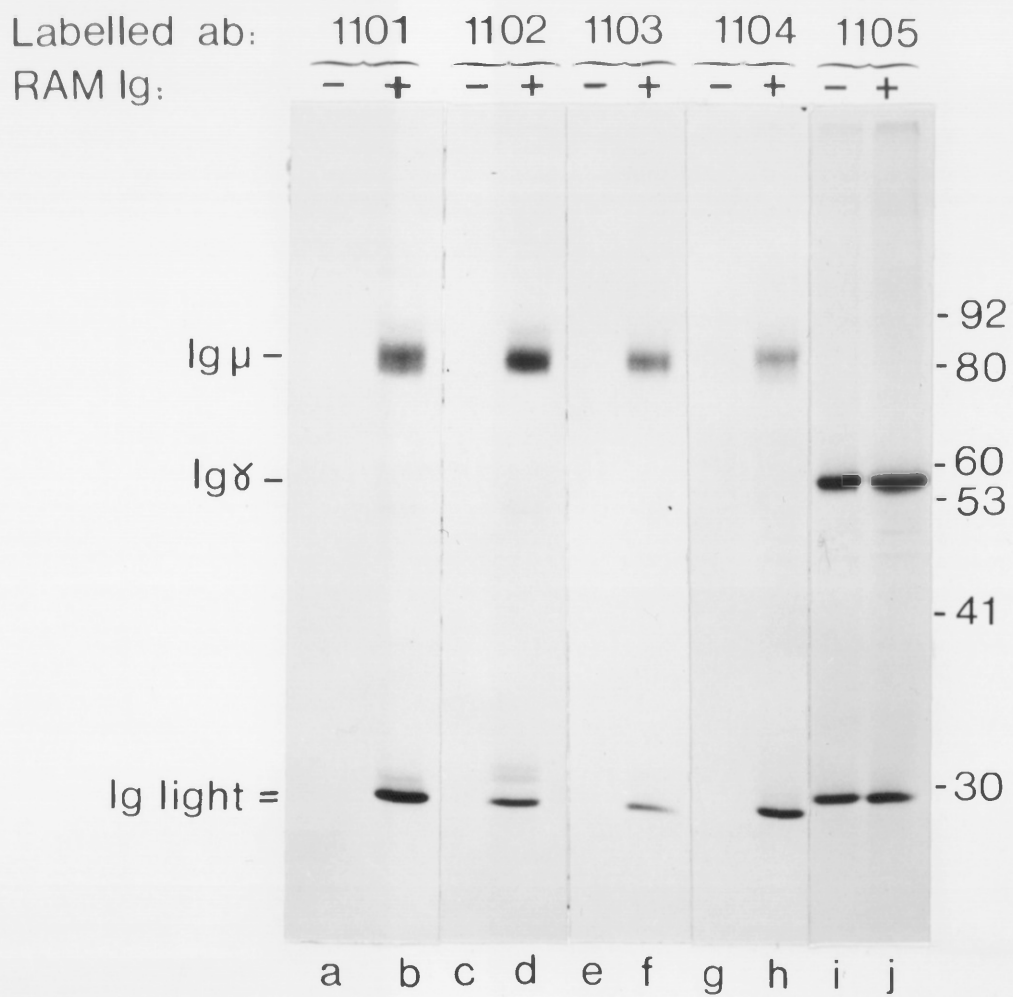oposed by Crawford and Harlow (1982). The culture that gave the positive reaction shown in Figure 3.1 gave rise ultimately to clone PAb1104.

The class of immunoglobulin secreted by each of the PAb1100-series hybridoma clones was determined in a radioimmune blocking assay (Harlow et al., 1981b). This procedure tests the ability of each test antibody to interfere with the binding of $^{125}$I-labelled antibodies of known class to antibodies with class-specific anti-mouse Ig activity. As shown in Figure 3.2, each of the antibodies PAb1101, PAb1102, PAb1103 and PAb1104 blocked the association of a known IgM antibody with anti-IgM antibodies but not the association of any class of IgG with the appropriate anti-Ig and all four were therefore classed as IgM. In contrast, PAb1105 blocked the binding only of known IgG2a to anti-IgG2a and was therefore classed as IgG2a. These conclusions were supported by an experiment in which antibodies secreted by each of the five clones were metabolically labelled with $^{35}$S-methionine and precipitated either directly on S. aureus protein A or via rabbit anti-mouse Ig antibodies. Figure 3.3 shows that PAb1101, PAb1102, PAb1103 and PAb1104 secrete Ig chains of ∿80K and 28K molecular weight (lanes b, d, f, h) which are not bound directly by protein A (lanes a, c, e, g), results which are typical of mouse IgM antibodies. PAb1105 secretes an antibody containing Ig chains of ∿50K and 28K which is efficiently bound by protein A (lanes i, j), properties which are typical of a mouse IgG2a antibody.

I have now studied the activities of antibodies PAb1101, PAb1102, PAb1103 and PAb1104 in some detail. In contrast, my knowledge of the properties of PAb1105 is limited and I will not

Figure 3.3 (opposite) Antibodies secreted by each of the PAb1100-

series clones were metabolically labelled by incubation of $10^7$

hybridoma cells in 2.0ml of culture medium containing 1.0mCi of

$^{35}$S-methionine for 3 hours. The labelled immunoglobulins in 50μl of

each tissue culture supernatant were then collected on S.aureus

protein A either directly (lanes a, c, e, g, i) or via rabbit

anti-mouse Ig antibodies (lanes b, d, f, h, j) and eluted into

SDS gel sample buffer. Their labelled polypeptide components were

then separated on a 10% polyacrylamide - SDS gel and detected by

autoradiography.

Labelled ab: 1101   1102   1103   1104   1105

RAM Ig:   −   +    −   +   −   +   −   +   −   +

Igμ −                                                    − 92
                                                         − 80

Igγ −                                                    − 60
                                                         − 53

                                                         − 41

Ig light =                                               − 30

          a   b    c   d   e   f   g   h   i   j

therefore discuss this antibody any further. Of the other four

antibodies, the specificity of PAb1104 is unusual and distinct from

that of the remainder of the series, making it convenient to discuss

its characterisation separately from that of PAb1101, PAb1102 and

PAb1103.


## Antibodies PAb1101, PAb1102 and PAb1103 Have Specific Anti-p53 Activity

PAb1101, PAb1102 and PAb1103 were selected for the ability to

immunoprecipitate SV80 T/p53 complex. An example of their interaction

with this complex is shown in Figure 3.4, lanes a-c. Each antibody

clearly precipitates the same $^{32}$P-labelled species as are precipitated

by the anti-p53 antibody PAb421 (lane d) and the anti-large T antibody

PAb419 (lane e). To determine whether or not each of these three new

antibodies had intrinsic activity towards the p53 component of the

T/p53 complex, their ability to immunoprecipitate p53 in the absence

of T-antigen was tested. C33I is a human cell line derived from a

cervical carcinoma (Auersperg, 1964). It expresses high levels of

p53 (Crawford et al., 1981; Benchimol et al., 1982). PAb1103 was

clearly able to precipitate p53 from $^{32}$P-labelled C33I cell extract

(Figure 3.4, lane h), as was PAb1101 (lane f), though with much lower

efficiency. PAb1102 (lane g) precipitated a very small amount of

labelled material having the same molecular weight as p53 which was

visible on the original autoradiogram.

Subsequently, I identified conditions under which the large T/p53

complex of SV80 cells will dissociate in vitro (described in Chapter 2).

Under these conditions SV80 p53 can be prepared free of large T-

antigen by clearance of the SV80 cell extract with an anti-large T-

antigen antibody. As shown in Figure 3.4, lanes k-m, PAb1101 and

PAb1103 and, with low efficiency, PAb1102, precipitated the

characteristic p53 doublet from such a pretreated SV80 cell extract.

Figure 3.4 (opposite)  Aliquots of $^{32}$P-phosphate - labelled SV80 cell

extract (lanes a to e), C33I cell extract (lanes f to j) or SV80

extract treated to dissociate the T/p53 complex and cleared of large

T-antigen (lanes k to o) were immunoprecipitated with 1.0μl (a,f) or

10μl (k) of PAb1101, 5.0μl (b,g) or 10μl (l) of PAb1102, 5.0μl (c,h)

or 10μl (m) of PAb1103, 1.5μg of PAb421 (d,i,n) or 1.5μg of PAb419·

(e,j,o) for a total of 2 hours with 0.25μl/reaction rabbit anti -

mouse Ig antiserum added for the final 30 minutes. Precipitated

proteins were separated by electrophoresis through 10% polyacrylamide

- SDS gels and detected by autoradiography.

$^{32}$P - SV80

$^{32}$P - C33I

$^{32}$P - SV80
- cleared of T

1101  1102  1103  421  419

1101  1102  1103  421  419

1101  1102  1103  421  419

large T -

p53 =

p53 -

p53 =

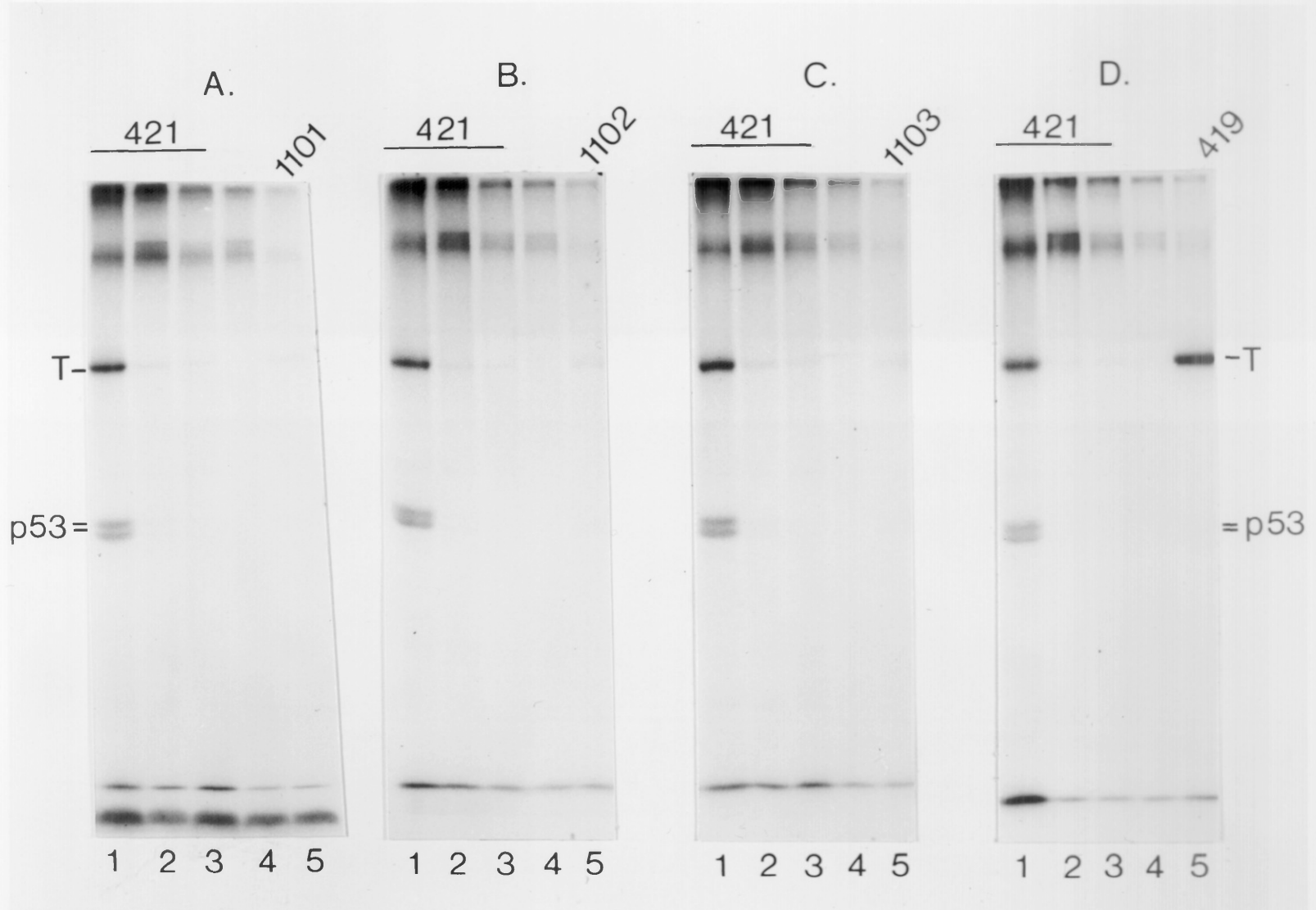a  b  c  d  e

f  g  h  i  j

k  l  m  n  o
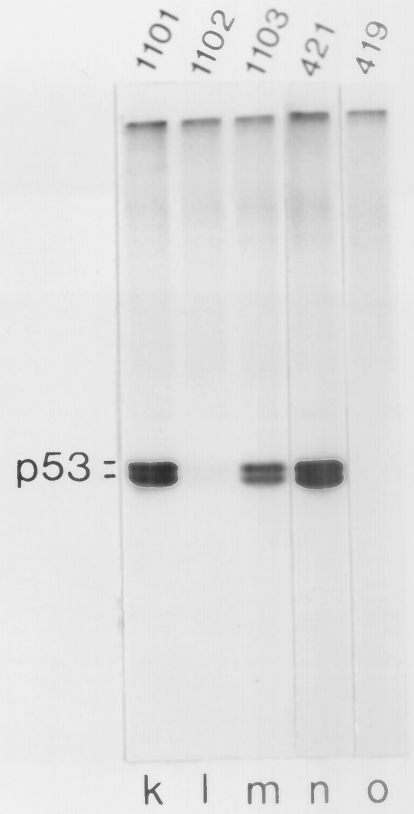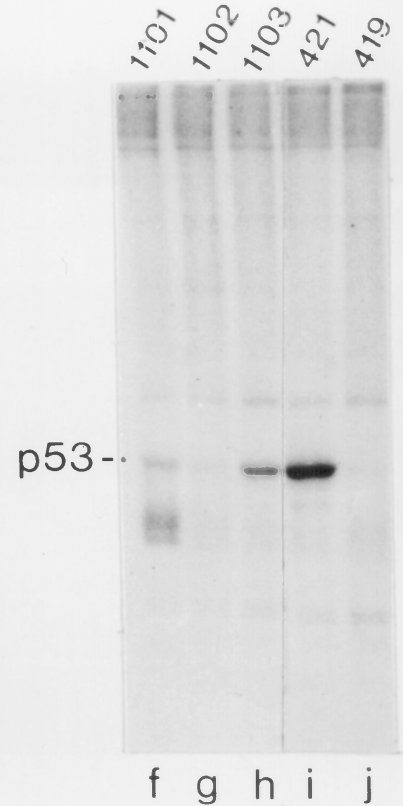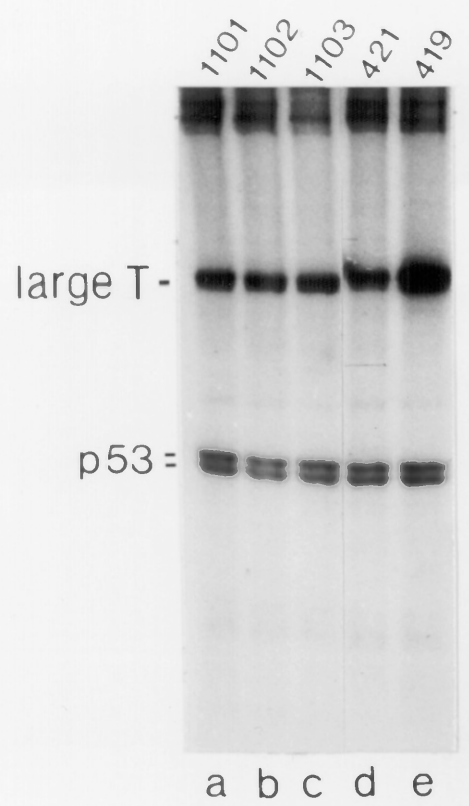
In parallel experiments the anti-p53 antibody PAb421 (lane n) but not the anti-large T antibody PAb419 (lane o) precipitated this same doublet of p53. I therefore concluded that each of the three anti-bodies PAb1101, PAb1102 and PAb1103 had an intrinsic anti-p53 activity.

To confirm that these antibodies had no activity towards SV40 large T-antigen, the other known component of the complex against which activity was originally selected during isolation of hybridomas, I performed cascade immunoprecipitations on labelled SV80 extract. This procedure involves successive treatments of a cell extract with antibodies of one type, to remove all antigen against which those antibodies have activity, followed by examination of the antigens remaining in the extract using one or more different antibodies. SV80 cell extract contains a considerable amount of free T-antigen and so it is possible to remove from it all p53 and large T/p53 complex with anti-p53 antibodies and to use this cleared extract to test antibodies for anti-T activity. As shown in Figure 3.5, (panels A, B and C, lane 5) neither PAb1101, PAb1102 nor PAb1103 had any activity towards the free T-antigen remaining after clearance of p53 from labelled SV80 extract (each panel, lanes 1-4) whereas this T-antigen was readily detected in the same extract using anti-T antibody PAb419 (panel D, lane 5). These data, together with those presented in Figure 3.4, show that antibodies PAb1101, PAb1102 and PAb1103 have genuine anti-p53 activity but no anti-large T activity. This anti-p53 activity must therefore entirely account for the observed binding of these antibodies to the SV80 large T/p53 complex.

The anti-p53 antibodies previously described vary in the extent of their cross-reaction between the p53 proteins of different species. I therefore examined the possibility that PAb1101, PAb1102 and PAb1103 might react with p53 from cells of non-human origin. The

Figure 3.5 (opposite) Aliquots of $^{32}$P-phosphate - labelled SV80 cell

extract were subjected to three sequential immunoprecipitations with

PAb421 (each panel, lanes 1 to 3) followed by an additional clearance

with S.aureus cells (lanes 4). Finally, the extracts were immuno-

precipitated (lanes 5) with PAb1101 (panel A), PAb1102 (panel B),

PAb1103 (panel C) or PAb419 (panel D). Antibody inputs were 1.5μg of

PAb421 or PAb419 , or 20μl of tissue culture fluid from PAb1101,

PAb1102 or PAb1103. All reactions were for a total of 1 hour with

0.25μl rabbit anti-mouse Ig antiserum added after 30 min. Immune

complexes were collected on 20μl 10% SAC for 15 min. at +4$^{\circ}$ and

supernatants immediately transferred to the next reaction.

Precipitated proteins were separated on 10% polyacrylamide - SDS gels

and detected by autoradiography.

A.　B.　C.　D.

421　1101　421　1102　421　1103　421　419

T-　　　　　　-T

p53=　　　　　　=p53

1　2　3　4　5　　1　2　3　4　5　　1　2　3　4　5　　1　2　3　4　5

results of an immunoprecipitation analysis of two mouse cell extracts that employed these antibodies is shown in Figure 3.6. PAb1102 and PAb1103 showed no interaction with either SVA31E7 large T/p53 complex (lanes b, c) or CQ15 p53 (lanes g, h) at antibody inputs sufficient to precipitate all the complex from an aliquot of a human cell extract (Figure 3.4). In the same experiment, PAb1101 showed weak activity towards mouse T/p53 complex (lane a) but none against free p53 (lane f). Subsequently, titration of antibody PAb1101 with SVA31E7 extract clearly showed precipitation of T/p53 complex at higher antibody inputs (Figure 3.6, lanes k-o).

Similar experiments were also performed with CV1 monkey kidney cells, with and without SV40 infection, the results of which are shown in Figure 3.7. The inputs of antibody used were, for each of the PAb1100-series, 5-fold higher than those employed with SV80 and C33I extracts in Figure 3.4 and SVA31E7 and CQ15 extracts in Figure 3.6. In the case of PAb1101 and PAb1102, this was sufficient for precipitation of the large T/p53 complex from the infected cell extract to be observed (lanes a, b) but no activity towards monkey p53 could be detected in PAb1103 antibody (lane c).

I concluded from these data that each of the antibodies tested had a distinct species-specificity. PAb1101 was the most widely reactive, binding to human, monkey and, with lower efficiency, to mouse p53 proteins. PAb1102 reacted with human and monkey proteins and PAb1103 only with human p53.

The Recognition of Large T-Associated and Free p53 by PAb1101, PAb1102 and PAb1103

The initial characterisation of the activities of PAb1101, pAb1102 and PAb1103 towards SV80 and C33I antigens (Figure 3.4, lanes a-j) was performed using the same amounts of each antibody in

Figure 3.6 (opposite) $^{32}$P-phosphate - labelled extracts of mouse

SVA31E7 (lanes a to e, k to o) and CQ15 (lanes f to j) cells were

immunoprecipitated with 1.0μl PAb1101 (a,f), 5.0μl PAb1102 (b,g),

5.0μl PAb1103 (c,h), 1.5μg PAb421 (d,i), 1.5μg PAb419 (e,j) or

PAb1101 at inputs decreasing from 50μl to .08μl in five-fold dilution

steps (k to o) for 2 hours at room temperature with 0.25μl per

reaction of rabbit anti-mouse Ig antiserum added for the final 30

minutes. Precipitated proteins were separated by electrophoresis

through 10% polyacrylamide - SDS gels and detected by autoradiography.

Figure 3.7 (opposite) $^{32}$P-phosphate - labelled extracts of CV1 monkey

cells labelled 48 hours after infection with 7.5 pfu/cell SV40 virus

(lanes a to e) or mock infected CV1 cells (lanes f to j) were

immunoprecipitated with 5.0μl PAb1101 (a,f), 20μl PAb1102 (b,g), 20μl

PAb1103 (c,h), 1.5μg PAb421 (d,i) or 1.5μg PAb419 (e,j) for 2 hours

at room temperature with 0.25μl per reaction of rabbit anti-mouse Ig

antiserum added for the final 30 min. Precipitated proteins were

separated by electrophoresis through 10% polyacrylamide - SDS gels

and detected by autoradiography.

$^{32}$P - E7  $^{32}$P - CQ15  $^{32}$P - E7

50  µl  ·08

1101

1101 1102 1103 421 419    1101 1102 1103 421 419

large T-                                            -large T

p53-                              -p53              -p53

a b c d e        f g h i j        k l m n o

SVS - inf CV1        CV1

1101 1102 11Q3 421 419    1101 1102 1103 421 419

T-

p53-                                        -p53
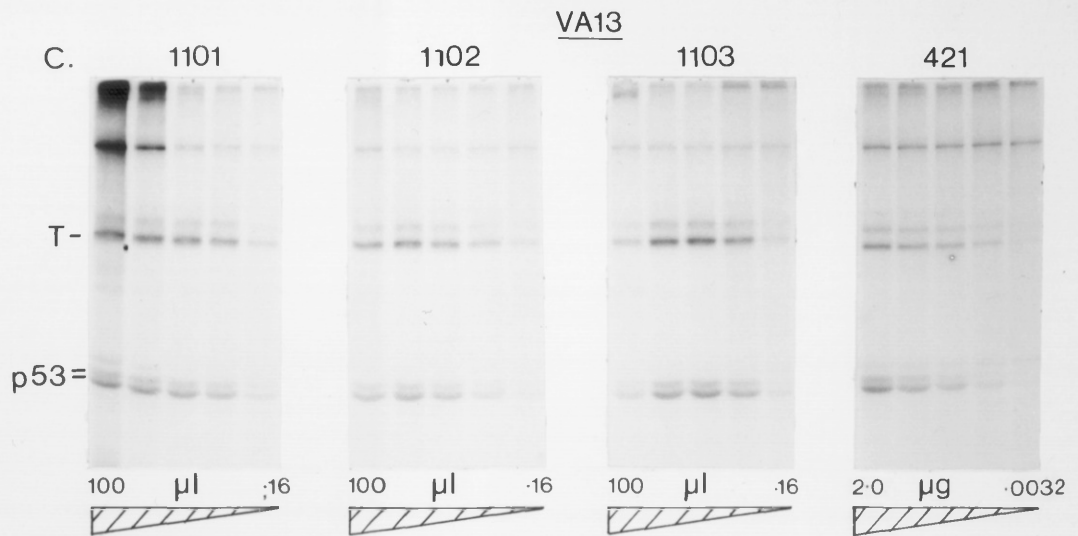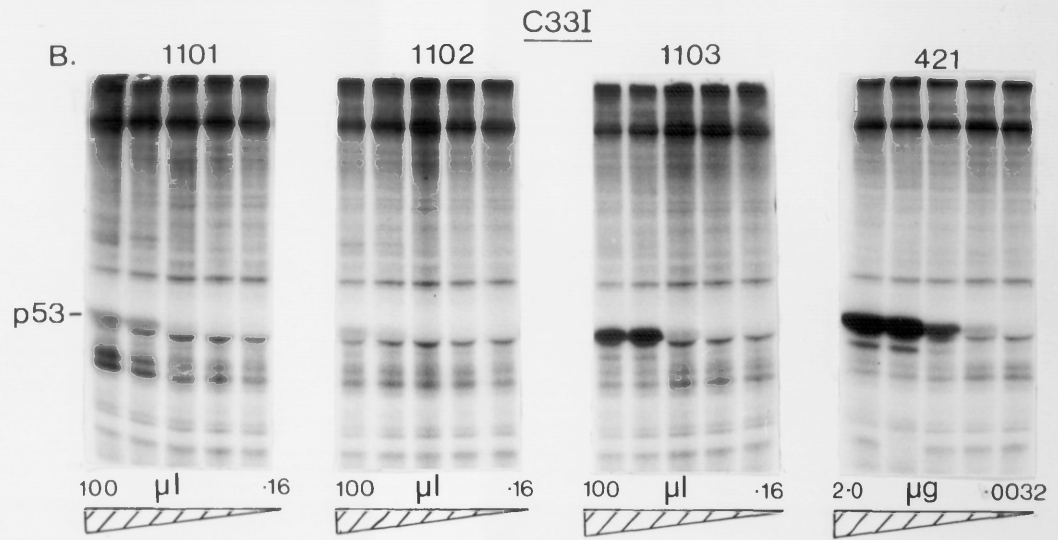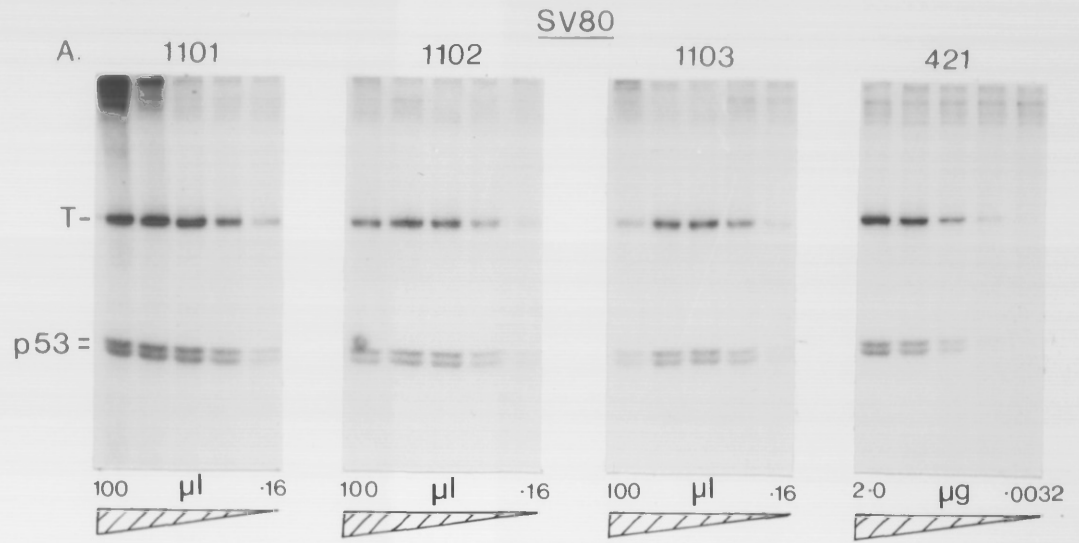
a b c d e        f g h i j
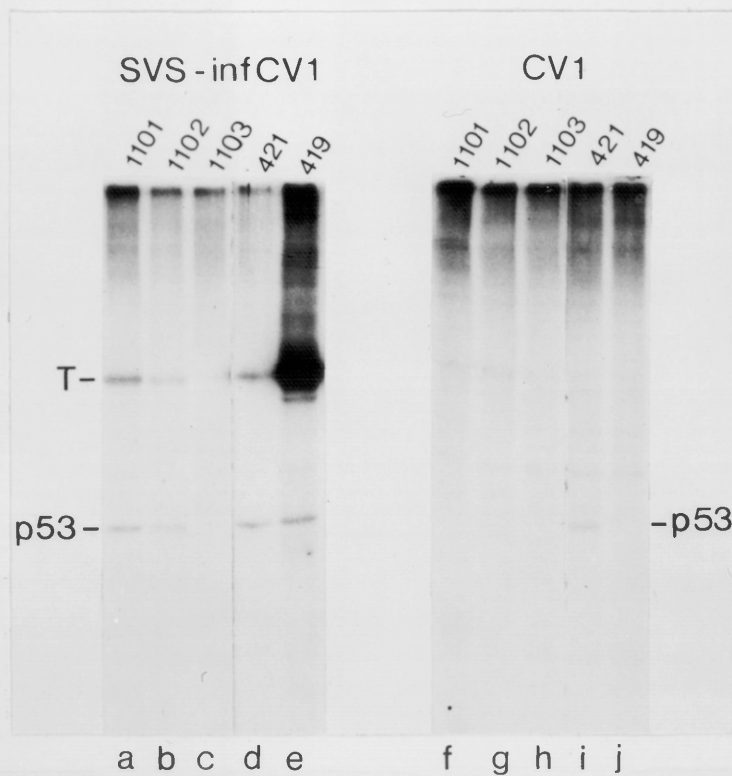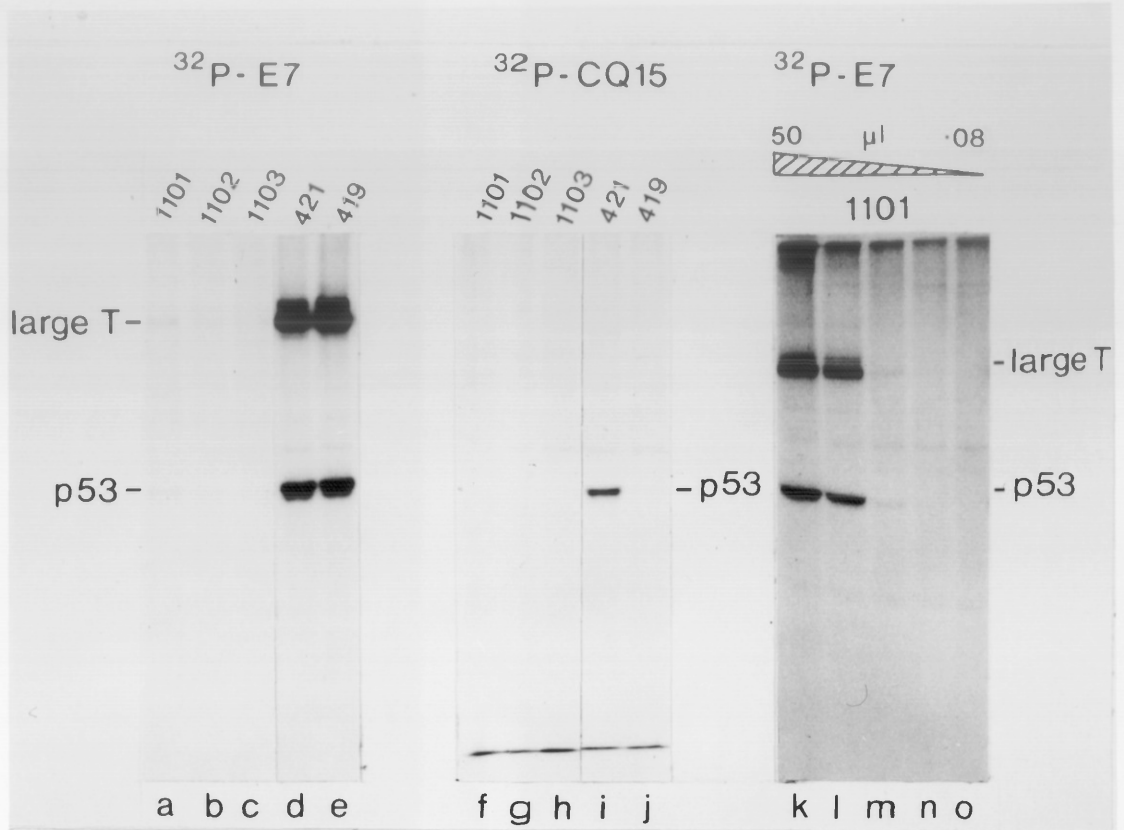
the two cases. However, whereas these amounts of the antibodies were

sufficient to precipitate the same quantity of T/p53 complex from

SV80 extract as an excess of the anti-p53 antibody, PAb421, these

same inputs were not adequate to produce the same response as an

excess of PAb421 from C33I extract. I had also noted that antibodies

PAb1101 and PAb1102 could precipitate CV1 p53 when it was complexed

with T-antigen but not when it derived from uninfected cells (Figure
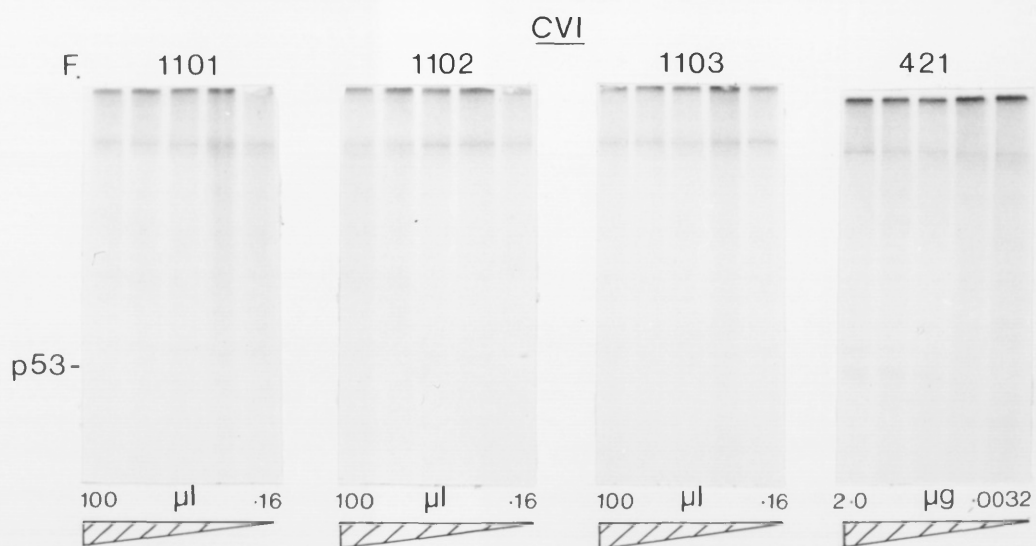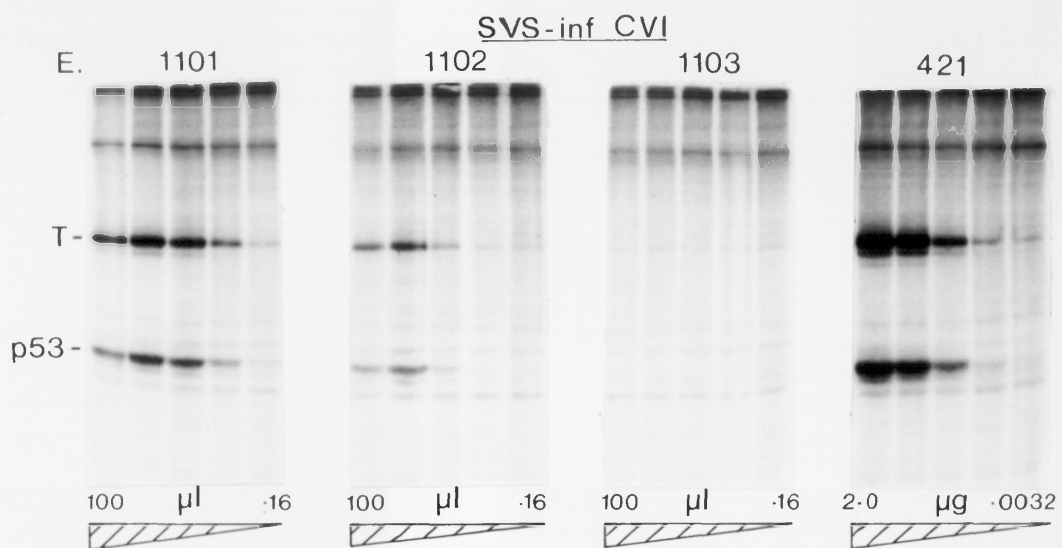
3.7, lanes a, b, f, g).

To investigate these apparent differences in the efficiency

of precipitation of different p53 forms by PAb1101, PAb1102 and

PAb1103, I carried out antibody titration experiments using various

labelled cell extracts as sources of antigen. The results of these

analyses are shown in Figure 3.8. At the right of each of panels A-F

is the result of titrating PAb421 against one of 6 different $^{32}$P-

labelled antigen sources. In each case, the precipitation of p53

or large T/p53 shows approximately the same dose-response curve,

reaching a plateau at inputs of PAb421 between 0.4µg and 2.0µg per

reaction. In contrast, antibodies PAb1101, PAb1102 and PAb1103

reacted well with large T-associated human p53 (SV80-panel A; VA13-

panel C) and relatively poorly with free human p53 (C33I-panel B;

SV80 p53-panel D). Where activity could be observed, there was a

similar discrepancy between the activity displayed by these antibodies

towards T-associated and free monkey p53 (panels E, F).

Some quantitative measure of the efficiency of recognition of

p53 from each source by PAb1101, PAb1102 and PAb1103 was obtained by

comparing the amounts of these antibodies that were needed to achieve

the same immunoprecipitation response as a subsaturating amount of

PAb421 in each extract. These data, together with the method by which

they were derived from the immunoprecipitation analysis shown in

Figure 3.8, are displayed in Table 3.1. They show that PAb1103 is

Figure 3.8 (A to C, opposite; D to F, overleaf)  Aliquots of extracts

of $^{32}$P-phosphate labelled cells (SV80 - panel A; C33I - panel B;

VA13 - panel C; SV80, precleared of T-antigen - panel D; SVS-infected

CV1 - panel E; CV1 - panel F) were immunoprecipitated with the

amounts of antibody indicated for 2 hours at room temperature with

0.5μl rabbit anti-mouse Ig antiserum added for the final 30 minutes.

Proteins were separated on 10% polyacrylamide - SDS gels and detected

by autoradiography. Each antibody was used at five inputs in five-

fold dilutions from left to right with the maximum and minimum inputs

indicated in each case.

A. SV80

1101    1102    1103    421

T-

p53=

100    µl    ·16    100    µl    ·16    100    µl    ·16    2·0    µg    ·0032

B. C33I

1101    1102    1103    421

p53-

100    µl    ·16    100    µl    ·16    100    µl    ·16    2·0    µg    ·0032

C. VA13

1101    1102    1103    421

T-

p53=

100    µl    ·16    100    µl    ·16    100    µl    ·16    2·0    µg    ·0032

Decreasing antibody input 5-fold steps, left to right.

SV80 p53

D.  1101          1102          1103          421

T-

p53=

100    µl    ·16    100    µl    ·16    100    µl    ·16    2·0    µg    ·0032

SVS-inf CVI

E.  1101          1102          1103          421

T-

p53-

100    µl    ·16    100    µl    ·16    100    µl    ·16    2·0    µg    ·0032

CVI

F.  1101          1102          1103          421

p53-

100    µl    ·16    100    µl    ·16    100    µl    ·16    2·0    µg    ·0032

Decreasing antibody input  5-fold steps, left to right.

-84-
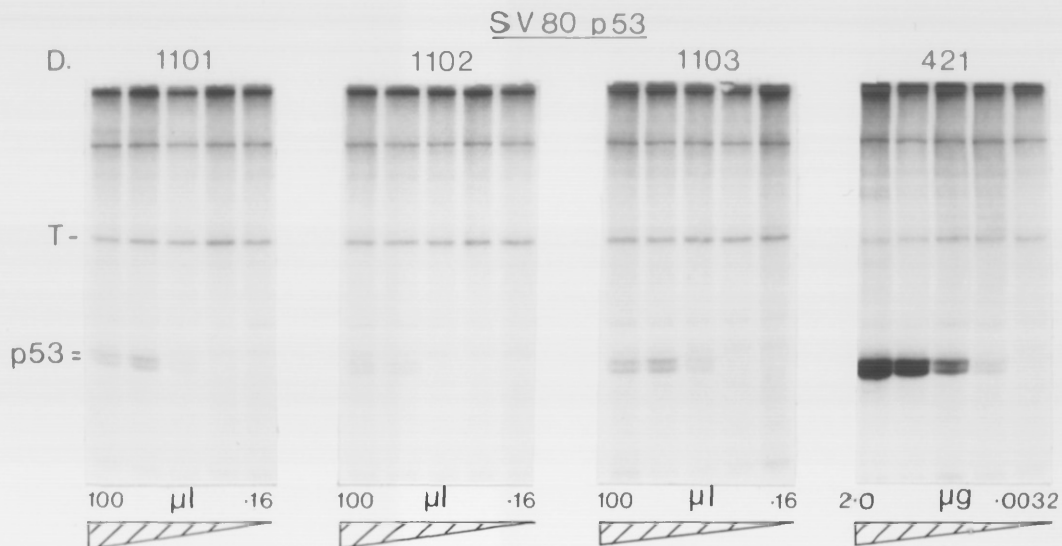
Table 3.1

Relative Efficiencies of p53 Binding by Anti-p53 Antibodies

| Cell Extract | Antigen | PAb421 /units | PAb1101 /units | PAb1102 /units | PAb1103 /units |
|---|---|---|---|---|---|
| SV80 | T/p53 | 1 | 1 | 1 | 1 |
| C33I | p53 | 1 | 125 | 125-625 | 25 |
| VA13 | T/p53 | 1 | 1 | 1 | 1 |
| SV80p53 | p53 | 1 | 125 | 125 | 25 |
| SV-infCV1 | T/p53 | 1 | 1-5 | 25 | >125 |
| CV1 | p53 | 1 | >125-625 | >125 | >125 |

These data have been generated by comparing by eye the antibody titration results with different cell extracts presented in Figure 3.8. The amounts of PAb1101, PAb1102 and PAb1103 necessary to immunoprecipitate from SV80 extract the same amount of p53 as 0.08µg PAb421 were defined in each case as 1 unit of antibody. The number of units of each antibody required to mimic the response to either 0.08µg (C,E,F) or 0.016µg (B,D) of PAb421 in each of the other extracts was then determined. Where it was convenient to use the 0.016µg PAb421 result for this comparison, the figures quoted have been adjusted pro rata to be equivalent to 0.08µg of PAb421.

25-fold less efficient at binding free human p53 than large T-associated p53, whilst for PAb1101 and PAb1102 the difference is 125-fold. There is also at least a 5 to 25-fold difference between the efficiencies of precipitation of monkey p53 in the presence or absence of T-antigen by both PAb1101 and PAb1102. This figure is only a minimum estimate as, even at the highest inputs employed, precipitation of uncomplexed CV1 p53 was not detected with any of the PAb1100-series antibodies whilst PAb421-reactive material was clearly present.

In some of the titrations shown in Figure 3.8 the precipitation of p53 by PAb1101, PAb1102 and PAb1103 appears to plateau before reaching the levels achieved by high inputs of PAb421. Whilst this might be taken as evidence for the recognition of immunological subsets of p53 by these antibodies in certain cases, it is more likely that this phenomenon is due to the amounts of available rabbit anti-mouse Ig antibodies becoming limiting at high inputs of anti-p53 antibodies. Immunoprecipitation by PAb1101, PAb1102 and PAb1103 depends on the presence of this second antibody whereas PAb421 can bind directly to S. aureus protein A. Unfortunately, it was not practicable to increase substantially the amounts of second antibody employed in these reactions because of the distortion of the electrophoretic pattern that would have been caused in the region of interest by an overloading of immunoglobulin γ chains. However, any effects of second antibody levels on the data shown in figure 3.8 are restricted to the results of the highest antibody inputs and so do not affect the conclusions that have been drawn from them.

## PAb1104 Recognises Both p53 and SV40 Large T-Antigen

To determine which of the two known components of the SV80 T/p53 complex was recognised by PAb1104, immunoprecipitation reactions were performed using SV80 lysate that had been precleared of either

T-antigen or p53 by the procedures outlined in Chapter 2. As shown

in Figure 3.9, PAb1104 (lane c) immunoprecipitated the same doublet

of p53 as was precipitated by PAb421 (lane b) from SV80 lysate cleared

of large T under conditions where no indirect precipitation of p53

via large T and an anti-large T antibody, PAb419, was observed (lane

d). Equally, PAb1104 (lane f) also precipitated the same large T-

antigen species as was precipitated by PAb419 (lane g) from SV80

lysate cleared of p53, when no precipitation of T-antigen via p53

was seen (lane e). It appeared therefore that the antibody secreted

by hybridoma PAb1104 had distinct anti-large T and anti-p53 activities,

a characteristic not observed either in previously described anti-p53

antibodies or in the other antibodies of the PAb1100-series.

An apparent dual reactivity of a monoclonal antibody could be

easily explained if the hybridoma producing it had not been properly

cloned or the clone secreted a mixed antibody population. During the

isolation of hybridoma PAb1104 cells were cloned three times, until

they were monoclonal. However, to exclude the remote possibility that

the dual activity of PAb1104 resulted from the secretion by the

clone of a mixed population of antibodies, I metabolically labelled

PAb1104 cells with $^{35}$S-methionine and determined the size of the

secreted, labelled polypeptides that comprised the antibodies capable

of binding to large T and to p53, Figure 3.10. Since PAb1104 does

not bind to protein A, it can only be precipitated on S. aureus cells

indirectly. In this experiment it was bound either via large T and

a protein A-binding anti-T antibody, PAb416, or via p53 and a protein

A-binding anti-p53 antibody, PAb421. As shown in lanes a and g, the

labelled PAb1104 immunoglobulin subunits precipitated in these two

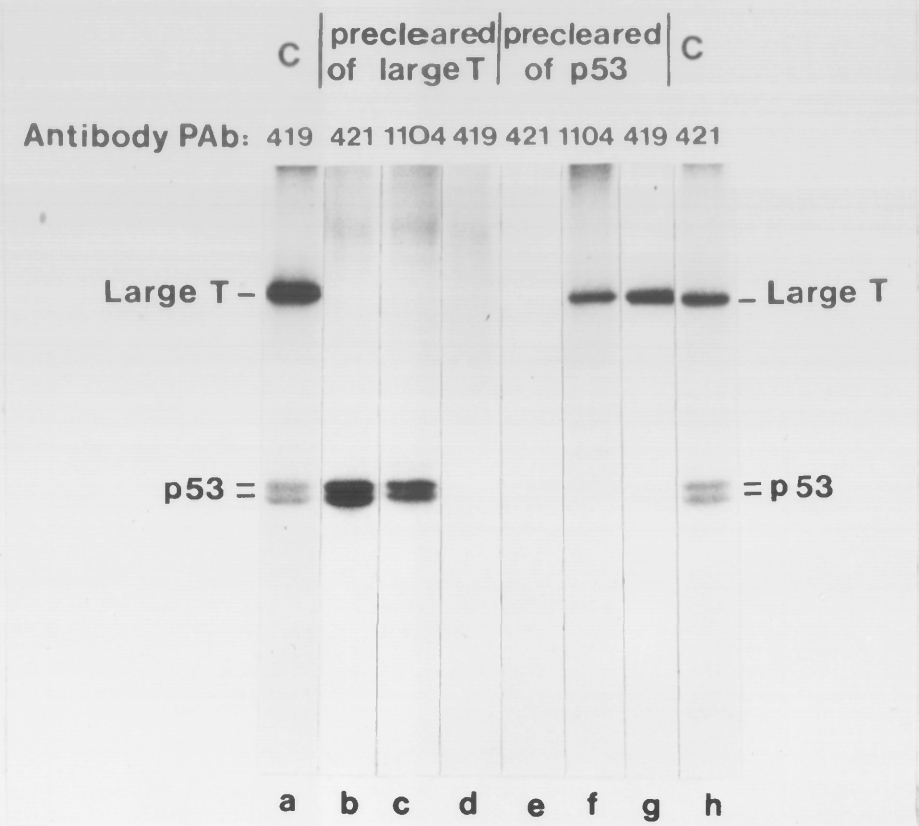reactions behaved identically on SDS-polyacrylamide gels.

These same aliquots of labelled PAb1104 were then further

precipitated with either large T, PAb416 and S. aureus cells

Figure 3.9 (opposite)  SV80 cells were metabolically labelled with
$^{32}$P-phosphate and lysate immunoprecipitated either directly (lanes a,h)

or after preclearance of all large T-antigen (lanes b,c,d) or p53

(lanes e,f,g) as described in Chapter 2. Aliquots of lysate derived

from ∿$10^6$ cells were precipitated with 1.5µg PAb419 (lanes a,d,g),

1.5µg PAb421 (lanes b,e,h) or 5µl PAb1104 (lanes c,f) and precipitated

proteins separated by electrophoresis through a 10% polyacrylamide -

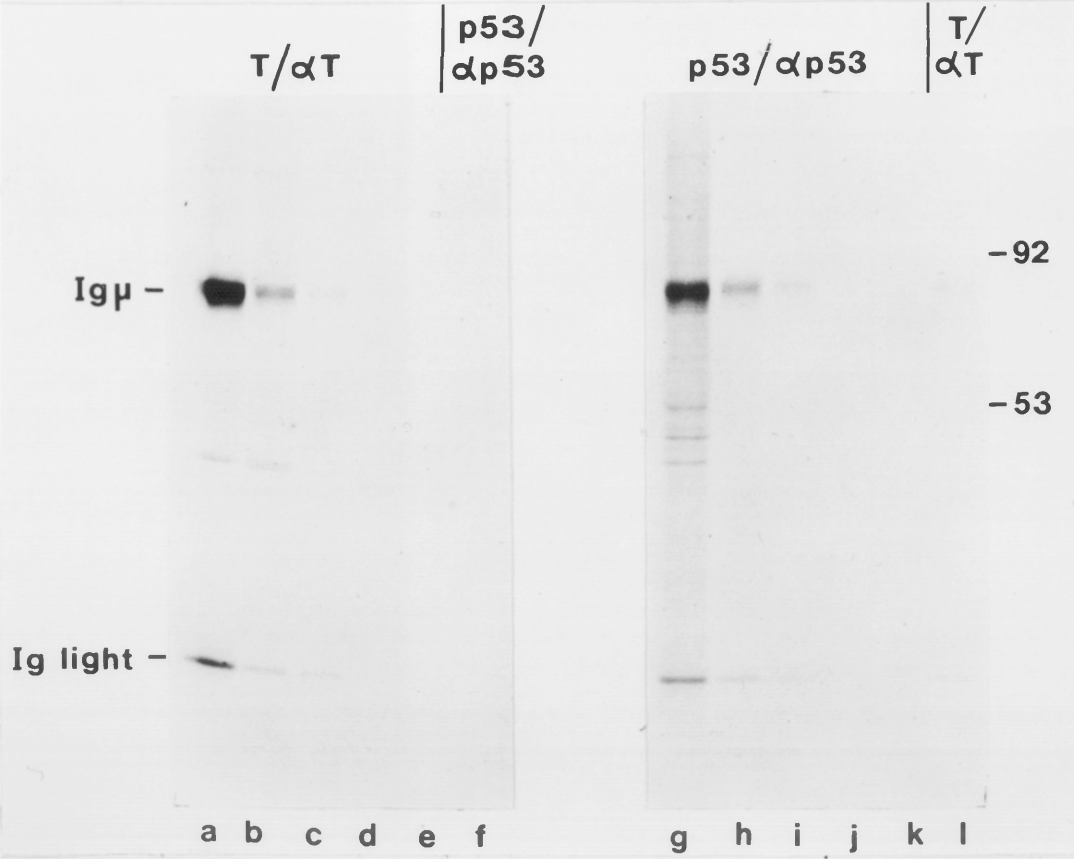SDS gel and detected by autoradiography.


Figure 3.10 (opposite)  PAb1104 does not bind to S.aureus protein A and
so can only be precipitated indirectly on S.aureus cells.  In this
experiment T-antigen and a protein A - binding anti-T antibody, or p53
and a protein A - binding anti-p53 antibody were employed to provide
bridges which would allow association of PAb1104 antibodies of the
appropriate specificity with protein A.  PAb1104 hybridoma cells were
cultured in medium containing $^{35}$S-methionine to label secreted anti-
bodies.  Two 50µl aliquots of culture medium containing labelled PAb1104
antibody were taken and subjected to a series of sequential indirect
immunoprecipitation reactions to remove either all labelled anti-T
antigen antibodies (a to e) or all labelled anti-p53 antibodies (g to k).
Each of these antibody-depleted aliquots of PAb1104 tissue culture
supernatant was then tested for the presence of any residual labelled
antibody having the opposite specificity to that already removed, ie.
anti-p53 antibodies (lane f) and anti-T antibodies (lane l).

Each of steps a to c, f to i, comprised the addition at 30 min.
intervals to PAb1104 supernatant, diluted to 0.5ml with NET/BSA buffer,
of 100µl cell extract, 1.5µg antibody and 20µl 10% SAC.  Steps d,j
omitted the addition of cell extract and steps e,k the addition of both
cell extract and antibody.  Steps a to d, and l used large T extract
and PAb416;  steps f to j used p53 extract and PAb421.  Large T-
containing extract was prepared from CV1 monkey cells at 2 x $10^7$ cells/ml
48 hours after infection with 7.5pfu/cell SV40 small plaque strain, SV-S
and cleared of p53 as described in Chapter 2.  p53-containing extract
was prepared from C33I cells at 2 x $10^7$ cells/ml.  Immunoprecipitated
proteins were separated on a 10% polyacrylamide - SDS gel and
detected by autoradiography.

$^{32}$P-SV80

| | C | precleared of large T | | | precleared of p53 | | | C |
|---|---|---|---|---|---|---|---|---|
| Antibody PAb: | 419 | 421 | 1104 | 419 | 421 | 1104 | 419 | 421 |

Large T –

– Large T

p53 =

= p53

a  b  c  d  e  f  g  h

$^{35}$S-PAb 1104

| T/αT | | p53/αp53 | | p53/αp53 | | T/αT | |
|---|---|---|---|---|---|---|---|

Igμ –

–92

–53

Ig light –

a  b  c  d  e  f      g  h  i  j  k  l

(Figure 3.10, lanes b-e) or p53, PAb421 and S. aureus cells (lanes h-k) to remove all antibody molecules capable of binding to large T and p53 respectively. Finally, any labelled PAb1104 left in the reaction after this clearance was precipitated with the alternative combination of antibody and antigen (p53, PAb421, S. aureus - lane f; large T, PAb416, S. aureus - lane 1). It was found that no PAb1104 anti-p53 activity remained after depletion of the anti-T activity in the labelled supernatant and that only a very small amount of anti-T activity remained after anti-p53 depletion, probably reflecting incomplete depletion in the first phase. The distinct and independent anti-T and anti-p53 activities in PAb1104 supernatant therefore reside in the same population of antibody molecules. This unique feature of antibody PAb1104 makes it difficult to study the species-specificity of its anti-p53 activity in the manner already described for PAb1101, PAb1102 and PAb1103 since it can bind to both components of the T/p53 complex. It binds neither to monkey nor mouse p53 in the absence of large T-antigen but this could reflect a strong preference for binding to T-associated p53 of the type shown by the other PAb1100-series antibodies rather than a true species-specificity.

Whilst there have been reports of monoclonal antibodies cross-reacting with one or more otherwise uncharacterised antigens in a cell lysate in addition to their known target (Lane and Hoeffler, 1980; Crawford et al., 1982a; Lane and Koprowski, 1982), it is extremely surprising to find a single antibody species with activity to two already well defined antigens which, furthermore, are known to associate in vivo. The improbability of such a specific cross-reaction occurring by chance led me to suppose that the observed dual-reactivity of PAb1104 might reflect the existence of some significant structural similarity between the two antigens involved. Nevertheless, it was still possible that PAb1104 was a widely cross-reactive antibody which
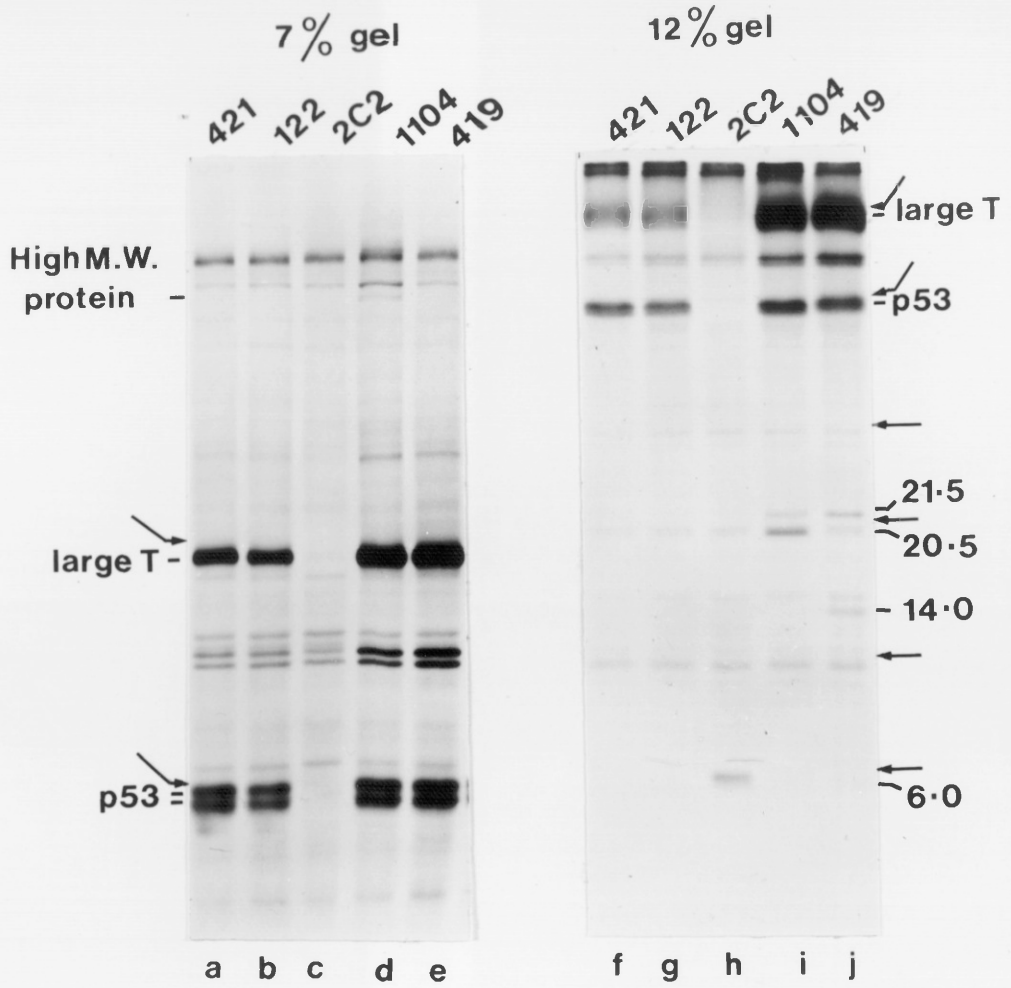
recognised a very simple determinant and that the observed specificity

of its immunoprecipitation of large T and p53 was only apparent, as a

consequence of labelling only a subset of the proteins in a cell extract

with $^{32}$P-phosphate.

To test this possibility, SV80 cells were labelled with $^3$H-

leucine, which should be incorporated into almost all proteins, and

extracts immunoprecipitated with either PAb1104 or control antibodies.

The results of this analysis are shown in Figure 3.11. RA3-2C2

(lanes c, h) is a negative control in this system since it does not

bind to human p53. The only proteins precipitated by PAb1104 (lanes

d, i) and not by RA3-2C2 apart from large T and p53 are, as indicated,

a very high molecular weight protein, and proteins of 21.5K and 20K.

The 21.5K protein is also precipitated by PAb419 (lane j), which

additionally precipitates a 14K protein. RA3-2C2 precipitates a

protein of around 6K. There were no qualitative differences in the

immunoprecipitation pattern observed when, as an alternative procedure,

labelling was carried out for 30 min. with or without prior depletion

of leucine pools for 30 min. Although I cannot exclude the possibility

that other specifically precipitated proteins might have been detected

under different labelling conditions, I believe that these data show

that PAb1104 is not a widely cross-reactive antibody. Rather, these

data support the idea that the antigenic relationship between SV40

large T-antigen and p53 detected by PAb1104 is a reflection of a

genuine structural similarity between these two proteins.

A characteristic of the anti-T monoclonal antibodies that have

been shown to cross-react with cellular proteins (Crawford et al.,

1982a) is that they recognise determinants that survive SDS denaturation,

as judged by their ability to rebind $^{35}$S-methionine-labelled antigen

after elution from an SDS-polyacrylamide gel. To test the ability of

PAb1104 to bind denatured antigen, I prepared $^{35}$S-labelled large T and

Figure 3.11 (opposite)  SV80 cells were labelled with $^{3}$H-leucine for

2 hours and extract prepared. Aliquots were immunoprecipitated with

20μl PAb421 (lanes a,f), PAb122 (lanes b,g), RA3-2C2 (lanes c,h) or

PAb419 (lanes e,j) or with 10μl PAb1104 (lanes d,i). After resuspension

in sample buffer, each precipitate was divided into two parts and

one part separated on a 7% polyacrylamide - SDS gel (lanes a to e)

and the other on a 12% polyacrylamide - SDS gel (lanes f to j).

Precipitated proteins were detected by fluorography. The positions in

the gels to which marker proteins of molecular weights 92K, 53K, 30K,

21K, 12.5K, and 6.5K, described in Chapter 2, migrated are indicated
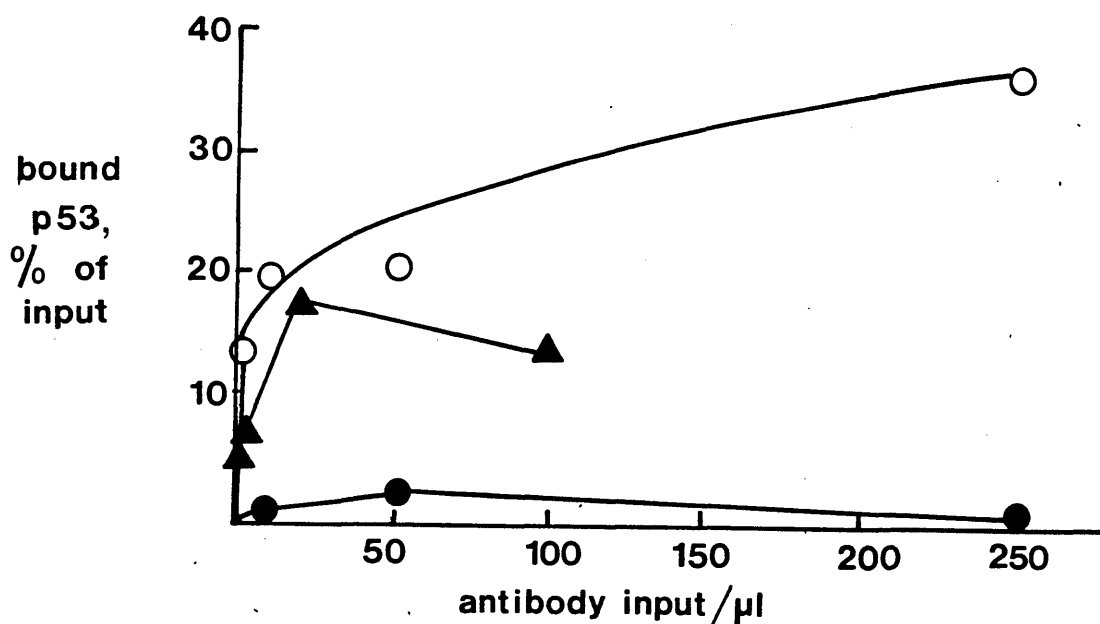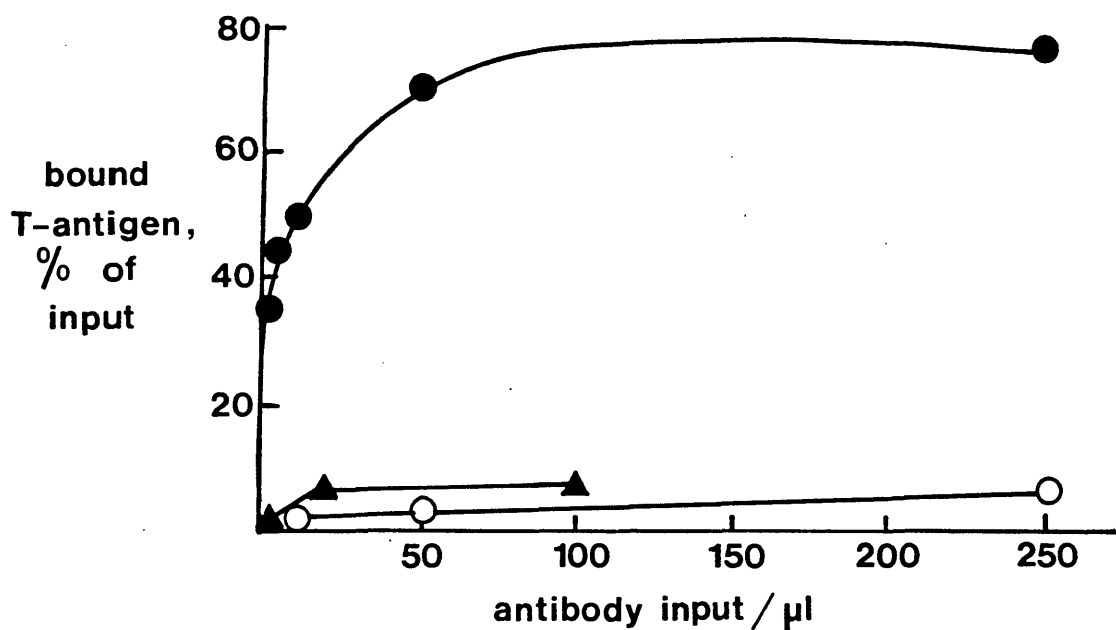
by arrows.

$[^3\text{H}]$ leu-SV80

7 % gel                    12 % gel

421  122  2C2  1104  419        421  122  2C2  1104  419

large T

p53

High M.W. protein —

large T —

p53 =

21·5
20·5

14·0

6·0

a  b  c  d  e        f  g  h  i  j

**Figure 3.12** Lysate from $5 \times 10^7$ SV80 cells was immunoprecipitated with 200µl PAb421 and 200µl PAb419, and proteins separated by SDS - gel electrophoresis. Gel slices containing large T and p53 were excised and eluted in 0.1M ammonium bicarbonate, $0.05\%^{w/v}$ SDS at $25^\circ$ for 48 hours. 5µl aliquots of filtered eluate, containing 2200cpm large T or 1500cpm p53, were allowed to rebind to varying inputs of antibodies PAb421, PAb1104 or PAb416 in 100µl NET/gel buffer overnight at $+4^\circ$. 0.5µl of rabbit anti-mouse Ig antiserum was added to each reaction 30 min. prior to collecting immune complexes with 20µl 10% SAC. Washed pellets were resuspended in 10µl $ddH_2O$ and counted in 1.0ml Aquasol.
● - PAb416 ; ▲ - PAb1104 ; O - PAb421.

p53 from metabolically labelled SV80 cells by immunoprecipitation,

gel electrophoresis and passive elution and used these as antigens in

a direct solution binding assay (Lane and Robbins, 1978; Harlow et al.,

1981b). As shown in Figure 3.12, the positive control antibodies

PAb416 and PAb421 rebound 75% and 36% of the large T and p53 probes

respectively. In parallel experiments, PAb1104 bound only 5% of

large T and 17% of p53 whilst negative control antibodies bound only

5% of each probe. Thus the PAb1104 determinant on large T was

completely destroyed by SDS denaturation. In contrast, a small

proportion of the p53 probe was rebound by PAb1104, suggesting that

the relevant structure elements in this protein can at least partly

refold under the assay conditions employed. The sensitivity to

denaturation of the PAb1104-reactive determinant on T-antigen is

further evidence that the dual reactivity of PAb1104 does not

represent a chance cross-reaction of the type previously described.
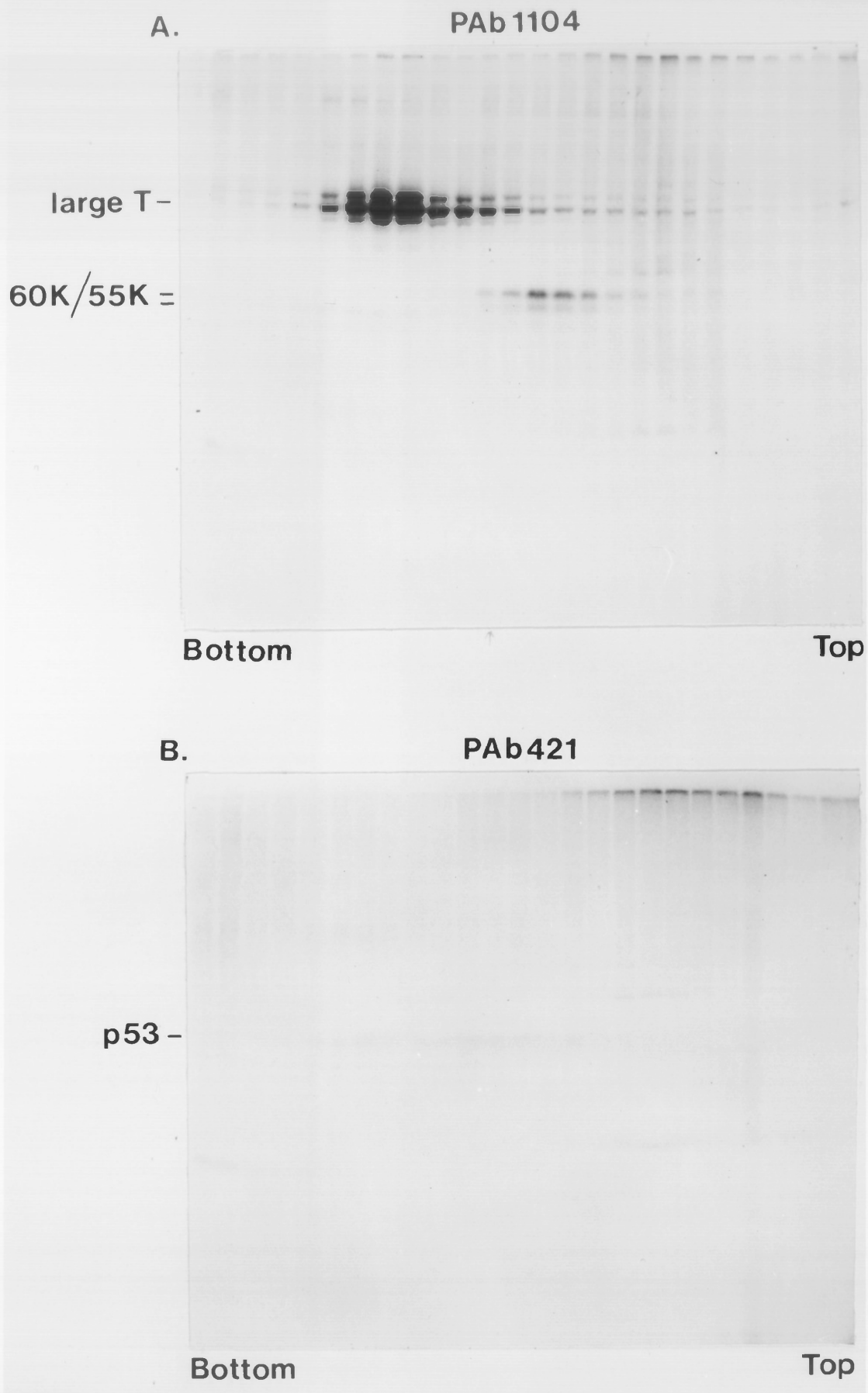

PAb1104 Recognises a Particular Size-Class of Large T-Antigen

It was clearly of interest to determine whether the entire

populations of large T and p53 in infected and transformed cells

shared an immunological determinant, or whether this was an attribute

of only a subset of one or both proteins. I therefore decided to

compare the size distributions of the PAb1104-reactive antigens with

those of the total large T and p53 populations in extracts of SV40-

infected CV1 cells and C33I cells respectively.

Proteins in lysates prepared from SVS-infected CV1 cells,

labelled 48 hours after infection with $^{32}$P-phosphate were separated

by fractionation on 5-20% w/v sucrose gradients. Fractions from two

identical gradients run in parallel were divided into two equal parts,

creating four sets of fractions which were immunoprecipitated with

PAb1104 (Figure 3.13, panel A), PAb421 (panel B), PAb416 (panel C) and

Figure 3.13 (A and B, opposite; C and D, overleaf) Proteins in

lysate from SV40-infected CV1 cells labelled 48 hours after infection

with $^{32}$P-phosphate were separated by velocity sedimentation through

5 to 20% $^{w/v}$ sucrose gradients and gradient fractions immunoprecipitated

with 20µl PAb1104 (panel A), 20µl PAb421 (panel B), 20µl PAb416

(panel C) or 20µl PAb402 (panel D). Each panel displays proteins

derived from about 5 x $10^6$ infected cells. Precipitated proteins

were separated on 10% polyacrylamide - SDS gels and detected by

autoradiography.

$^{32}$P-SVS-inf.CV1

A.                    PAb 1104

large T–

60K/55K =

Bottom                     Top

B.                  PAb 421

p53 –

Bottom                     Top

$^{32}$P-SVS-inf.CV1

C.                PAb 416

– large T

Bottom                Top

D.                PAb 402

– large T
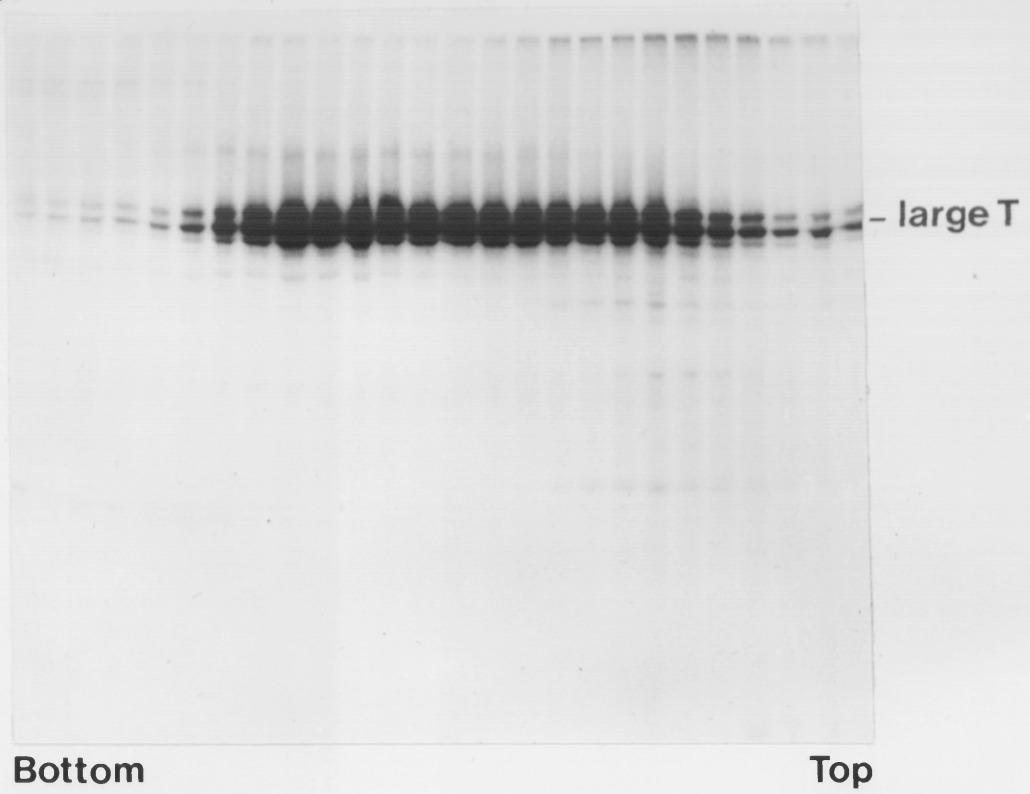
Bottom                Top

PAb402 (panel D). Each panel shows, from left to right, the

precipitation profile from bottom to top of the gradient. The

complex of monkey p53 with SV40 T-antigen is known to be very weak

(Harlow et al., 1981a) and, as expected, PAb421 precipitated virtually

no T-antigen via p53 (panel B). Anti-p53 activity would therefore

not be expected to make any significant contribution to the

precipitation of T-antigen by PAb1104 observed in panel A. When this

PAb1104-precipitated T-antigen was compared with that precipitated

by the classical anti-T antibody, PAb416, it was clear that PAb1104

reacted with a discrete size-class of T-antigen. This T-antigen

migrated with a velocity of approximately 16S relative to marker

proteins. Furthermore, a comparison of the results in panels A, C

and D suggested that the PAb1104-reactive and PAb402-reactive T-

antigen might be mutually exclusive subsets of the T-antigen

recognised by PAb416. An identical experiment with SV80 cell lysate,

precleared of p53 with PAb421 prior to sucrose gradient analysis

showed that PAb1104 recognised a similar size class of T-antigen in

these cells. Although the PAb1104-reactive T-antigen could be

associated with other proteins in a hetero-oligomer, no such proteins

have been detected by metabolic labelling with either $^{32}$P-phosphate,

$^{35}$S-methionine or $^{3}$H-leucine. The PAb1104-reactive material is

therefore likely to be a homo-oligomer of SV40 large T-antigen.

In similar experiments, C33I cells were labelled with $^{32}$P-

phosphate and proteins in the cell extract separated by velocity

sedimentation through sucrose. Each gradient fraction was divided

into two parts and sets of fractions immunoprecipitated with PAb1104

or PAb421 as shown in Figure 3.14, panels A and B. This analysis

revealed that the p53 recognised by these two antibodies was

indistinguishable by the criterion of sucrose gradient mobility. It

appears therefore that PAb1104 recognises the same spectrum of p53

Figure 3.14 (opposite) Proteins in lysates of C33I cells labelled with $^{32}$P-phosphate were separated by velocity sedimentation through a 5 to 20% $^{w/v}$ sucrose gradient and gradient fractions immunoprecipitated with 20μl PAb1104 (panel A) or 20μl PAb421 (panel B). Precipitated proteins were separated on 10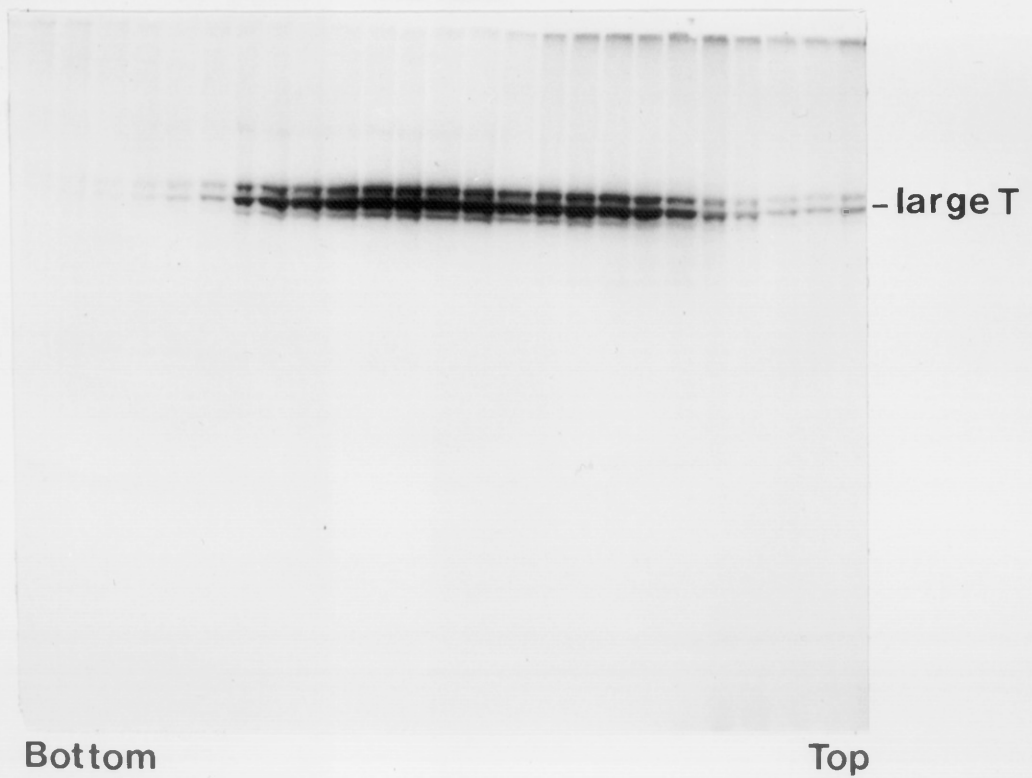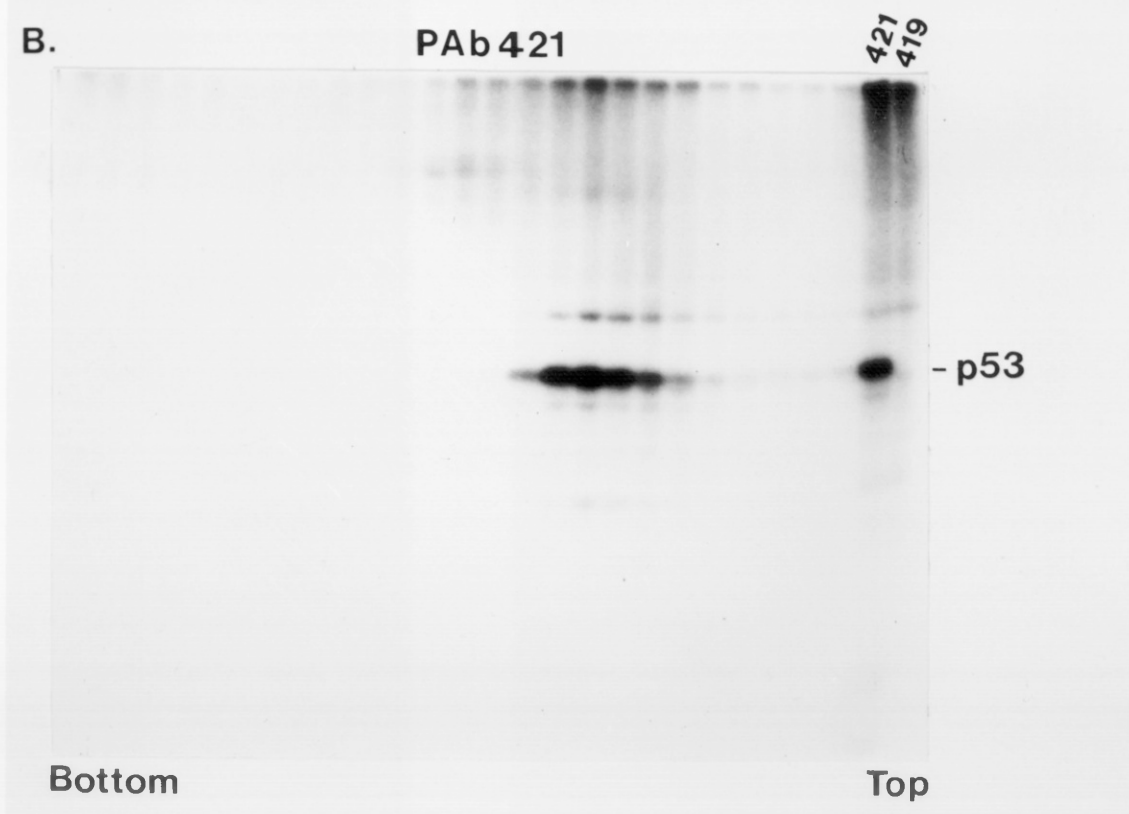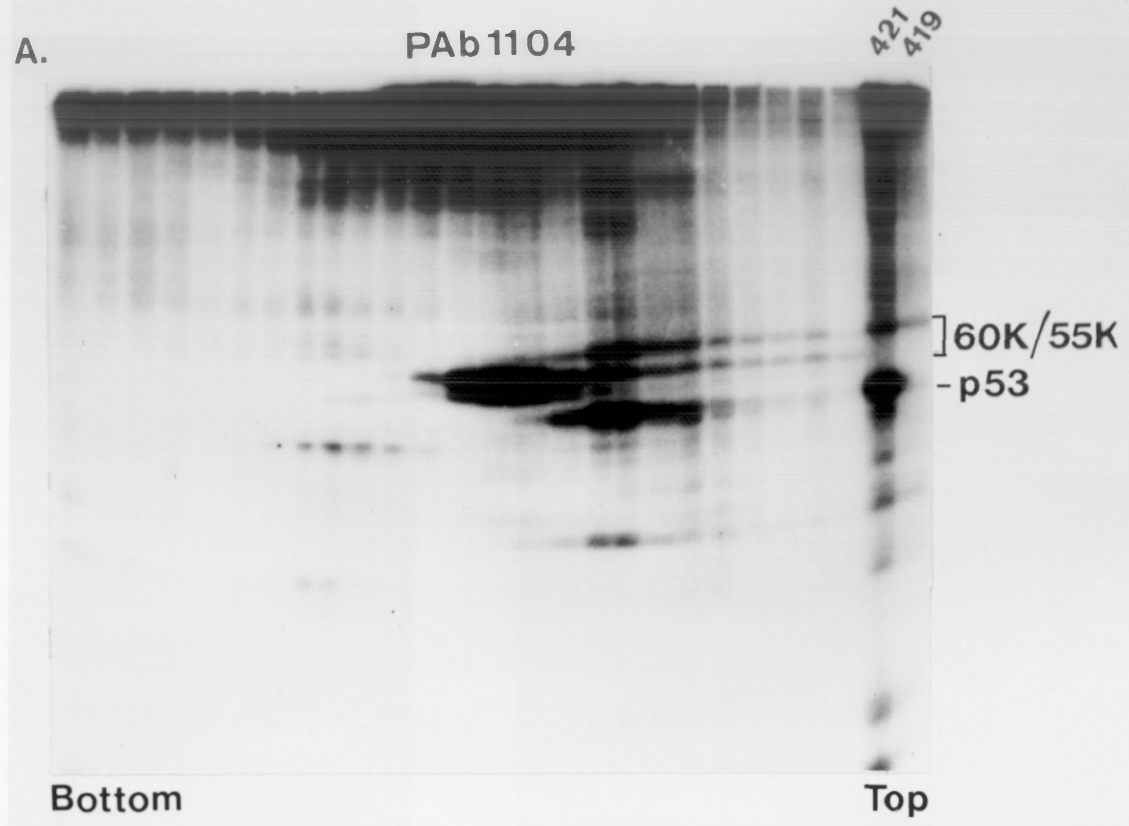% polyacrylamide - SDS gels and detected by autoradiography. Tracks labelled 421 and 419 at the right of each panel show the proteins precipitated in positive and negative control reactions from unfractionated C33I extract.

$^{32}$P-C33I

A.  PAb1104

]60K/55K

-p53

Bottom                                                        Top

B.  PAb421

-p53

Bottom                                                        Top

molecules as is recognised by PAb421, whilst recognising only a certain size-class of large T-antigen oligomers that have a sedimentation coefficient of around 16S in sucrose gradients.

The level of cross-reactivity shown by PAb1104 has already been discussed. In this connection, it should be noted that $^{32}$P-phosphate labelling reveals a further PAb1104 cross-reaction, to the 60K /55K protein complex indicated in Figures 3.13 and 3.14, panels A. However, titration experiments, similar to those shown for PAb1101 etc. in Figure 3.8, indicated that essentially all the p53 or large T/p53 complex in an extract was precipitated by PAb1104 before any precipitation of 60K/55K complex could be detected. The low affinity of PAb1104 for this phosphoprotein complex which these results imply makes it likely that the interaction represents a chance cross-reaction of the type previously characterised and probably does not indicate the existence of any significant structural homology between the 60K/55K complex and either large T or p53.

A very large number of monoclonal antibodies reacting with SV40 large T-antigen have now been described. Through the generosity of their respective isolators who made them available to me, I was able to screen 44 anti-T antibodies of the PAb200-series (Lane and Hoeffler, 1980; Clark et al., 1981), PAb400-series (Harlow et al., 1981b) and PAb600-series (L. Gooding, unpublished) for ability to block binding of PAb1104 to large T-antigen. For this I used a radioimmune blocking assay described in Chapter 2. In an initial screen, only one antibody, PAb427, showed any blocking of PAb1104 binding to T-antigen from an extract of SV40-infected CV1 cells cleared of p53. The results of a subsequent titration experiment, in which increasing amounts of PAb427 were used to block the binding of either PAb1104 or PAb416 to T-antigen, are shown in Figure 3.15. PAb416 has a binding site on T-antigen that is completely separate

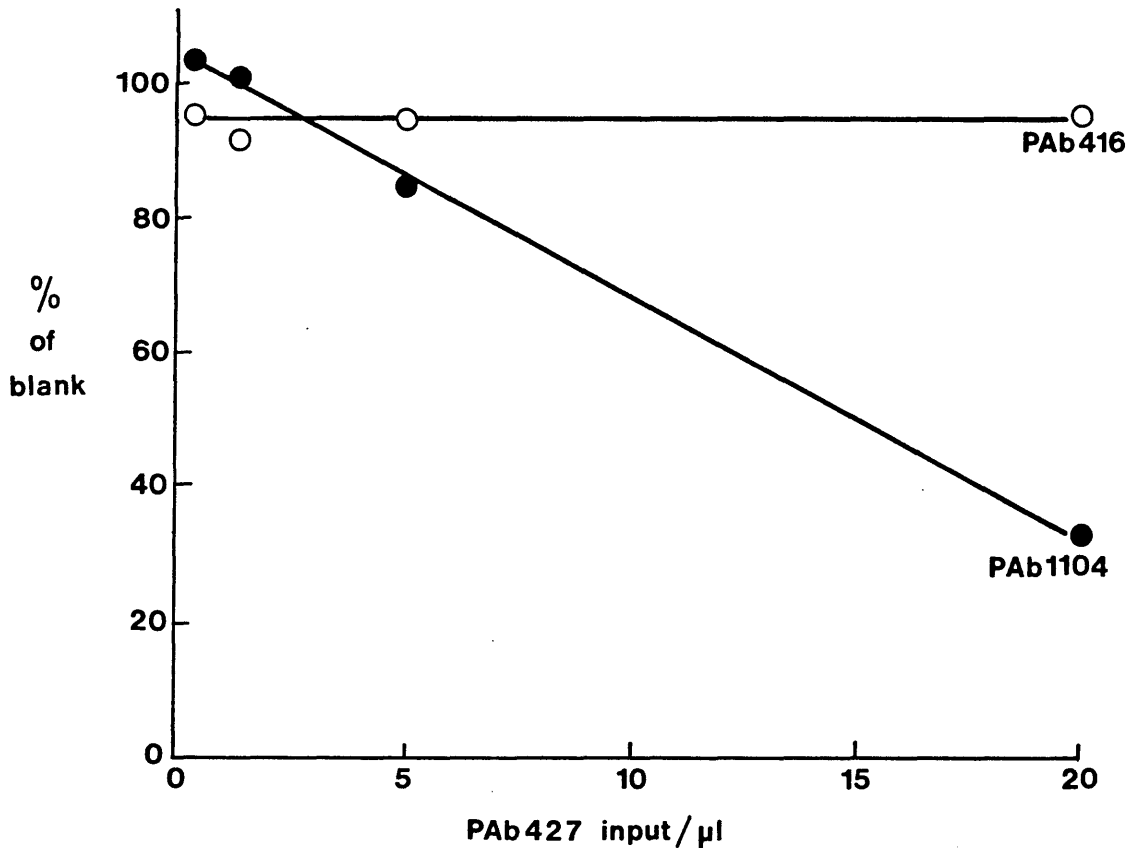## The blocking of PAb 1104 anti-T activity by PAb 427



Figure 3.15  PVC microtitre wells precoated with anti-T antibody PAb419

were allowed to adsorb T-antigen from 20μl of an extract of SVS -

infected CV1 cells, prepared 48 hours after infection at around $2 \times 10^7$

cells/ml lysate, over a period of 2 hours. Wells were washed and

treated with a mixture of $^{125}$I-labelled antibody (either PAb416 or

PAb1104 in 10μl of NET/gel buffer) and unlabelled PAb427 antibody

(from 0 to 20μl of tissue culture supernatant in a total volume of 20μl

made up with unused RPMI tissue culture medium) for a period of 2 hours

after which the bound counts were in each case determined. These data

are expressed as a percentage of the counts bound from the appropriate

probe in the absence of unlabelled antibody and plotted against the

input of PAb427. The blank determination in each case gave a bound

count of around 200cps.

from that of PAb427 (Harlow et al., 1981b) and, as expected, PAb416

binding to T-antigen was not affected by any input of PAb427. In

contrast, PAb1104 binding was reduced to only 30% of the value

observed in the absence of blocking antibody.

This result suggests that the PAb1104 and PAb427-reactive

determinants on large T-antigen are adjacent or overlapping, such

that occupancy of the PAb427 determinant hinders access to the PAb1104

determinant. Since the PAb427 determinant was mapped to the C-terminal

quarter of large T-antigen by Harlow et al.(1981b) on the basis of its

ability to precipitate the $Ad2^+ND1$ hybrid virus 28K T-antigen-related

protein, my results imply that the PAb1104-determinant also incorpor-

ates elements from this C-terminal region of the T-antigen polypeptide.

However, unlike the PAb1104 determinant, the PAb427 determinant on

large T-antigen is apparently stable to denaturation (Harlow et al.,

1981b). Thus the PAb1104 and PAb427 determinants are clearly distinct

and, because of the stability of its determinant, PAb427 would not be

expected to show the same specificity for oligomeric large T-antigen

that is shown by PAb1104. Unfortunately, further experiments to

investigate the nature of the relationship between these large T-

antigen determinants were prevented by lack of PAb427 antibody, this

hybridoma cell line being no longer available.

The simplest and most plausible interpretation of the available

data is that monoclonal antibody PAb1104 has dual reactivity towards

p53 and an oligomeric form of SV40 large T-antigen, implying that

these antigens have some element of structure in common. There are

however at least two alternative explanations of the data that, whilst

less likely, cannot be excluded. Firstly, it is known that rapidly

sedimenting T-antigen can be associated with p53. The evidence that

the PAb1104-reactive T-antigen is not a T/p53 complex rests on the

fact that this oligomeric form can be precipitated from an extract

under conditions where no material reactive with anti-p53 antibody

PAb421 remains. In practice, immunoreactivity with PAb421 defines

p53, but it cannot be excluded that this material is only a subset

of total p53 and that PAb1104 defines a wider subset. However this

putative PAb1104-reactive, PAb421-unreactive p53 could not be

labelled either with phosphate or with leucine and I therefore

regard this explanation as unlikely. Secondly, it is possible that

p53 and an oligomeric subset of T-antigen have in common the ability

to bind to some non-protein component of the cell, either ligand or

macromolecule. If so, the available data could be explained by

PAb1104 having direct activity against such a cellular component,

although this could not be present at very high levels in cells

otherwise the precipitation of p53 and T-antigen would require much

higher inputs of PAb1104 than of genuine anti-T or anti-p53 antibodies

and this is not the case.


## The Distribution on p53 of Determinants Characterised by the
## PAb1100-Series Antibodies

Having studied in some detail the specificities of the PAb1100-

series antibodies, it was clear that the activity of each one had

certain novel features when compared with those anti-p53 antibodies

previously characterised. It was likely therefore that they defined

one or more new antigenic determinants. I therefore examined the

topological distribution of the determinants on human p53 recognised

by the PAb1100-series antibodies and compared them with those

previously defined, using blocking assays which were analogous to

those used to study the PAb1104 determinant on T-antigen and which are

also described in Chapter 2.

Applying these procedures to the study of the PAb1100-series

anti-p53 determinants was not straightforward. One would ideally use

Table 3.2

Competition Between Anti-p53 Antibodies (I)

- SV80 Antigen

| Blocking Antibody | Probe Antibody | | | | | |
|---|---|---|---|---|---|---|
| | PAb1101 % | PAb1102 % | PAb1103 % | PAb1104 % | PAb421 % | PAb122 % |
| PAb1101 | [O] | [O] | 69 | 91 | 87 | 86 |
| PAb1102 | 66 | [O] | 99 | 93 | 92 | 94 |
| PAb1103 | 95 | 59 | [O] | 89 | 74 | 93 |
| PAb1104 | 40 | 36 | 20 | [O] | 53 | 76 |
| PAb421 | 95 | 65 | 87 | 98 | [O] | 41 |
| PAb122 | 79 | 62 | 90 | 91 | [1] | [O] |
| RA3-2C2 | 97 | 64 | 89 | 96 | 99 | 91 |

Antibodies as indicated in the left-hand column, in 25µl of 50mM tris/HCl pH7.4, 150mM NaCl were allowed to compete with $^{125}$I-labelled antibodies as indicated across the Table, in 10µl of the same buffer containing 1.0mg/ml bovine serum albumin, for binding to large T/p53 complex from SV80 cell extract, immobilised via PAb419 on PVC microtitre wells and the counts remaining bound after 2 hours determined. The amount of each probe bound in the presence of an excess of the same blocking antibody was taken as the nonspecific background for the probe and was deducted from each result. Data was then expressed as a percentage of the corrected zero blocking control in which probe was diluted with 25µl of buffer containing no antibody. Under conditions of zero blocking, the bound counts from each probe were ~200cps. Boxed results indicate a strong blocking interaction and underlined results a partial blocking interaction. Each result is the mean of two determinations which differed by not more than 10%.

free p53 rather than T/p53 complex as the antigen for this assay

because the anti-T activity of PAb1104 would make interpretation of

experiments employing T/p53 complex rather complicated. However,

PAb1101 and PAb1102 recognise free p53 with very low efficiency,

making it difficult to obtain a sufficient signal in the assay when

probing with these antibodies. Also, since the molecular basis for

this low efficiency of recognition is not known, it is arguable

whether data on the blocking of binding of these antibodies to free

p53 are in fact representative of the situation for the bulk of p53

or just for an antigenically distinct subset of it. I have therefore

used both SV80 T/p53 and C33I p53 as antigens in blocking assays, the

results of which are presented in Tables 3.2 and 3.3 and drawn

conclusions from both sets of data that reflect these considerations.

PAb421 and PAb122 were used as probes only with the T/p53 complex

as antigen, since the antigenic region they recognise was used to

mediate the binding of free p53 to the PVC wells in these experiments.

In the examination of antibody binding to SV80 T/p53 complex,

PAb122 completely blocked PAb421 binding, as previously shown

(Benchimol et al., 1982) and PAb1101 completely blocked PAb1102

binding indicating that these two antibodies recognise close or

overlapping determinants. However, in neither case were these

interactions completely reciprocal since the reverse combinations

showed only partial blocking. The possible basis and significance

of partial blocking is considered further below. When analysed on

C33I antigen PAb1103 and PAb1104 showed a reciprocal partial blocking

effect, but this combination of antibodies behaved non-reciprocally

when assayed on SV80 antigen, presumably because of PAb1104's

additional anti-T activity. The binding of probes PAb1101 and PAb1102

to C33I p53 appeared to be enhanced in the presence of either PAb421

or PAb122 but, given the weakness of the signal from these two probes,

Table 3.3

Competition Between Anti-p53 Antibodies (II)

- C33I Antigen

| Blocking Antibody | Probe Antibody | | | |
|---|---|---|---|---|
| | PAb1101 % | PAb1102 % | PAb1103 % | PAb1104 % |
| PAb1101 | ☐O | ☐O | 104 | 92 |
| PAb1102 | 86 | ☐O | 110 | 95 |
| PAb1103 | 46 | 109 | ☐O | 70 |
| PAb1104 | ☐12 | 37 | 65 | ☐O |
| PAb421 | ⬭130 | ⬭220 | 114 | 98 |
| PAb122 | 117 | ⬭162 | 106 | 116 |
| RA3-2C2 | 96 | 99 | 99 | 92 |

These experiments were performed as described in Table 3.2 except that antigen was derived from C33I cells and immobilised on PVC microtitre wells via PAb421 antibody. Under conditions of zero blocking, the bound counts from probes PAb1103/4 were ∿100cps but from probes PAb1101/2 were only 60cps and 30cps respectively. Circled results suggest enhanced probe binding in the presence of the blocking antibody.

the significance of these results is doubtful. A further observation was that PAb1104 appeared to reduce the binding of each of the other anti-p53 antibodies to p53, whether or not T-antigen was present. Taken together, these data clearly show that each of the PAb1100-series determinants is distinct from those recognised by PAb421 and PAb122. However, whilst they are all distinguishable, the PAb1100-series of antibodies itself divides into two antibody pairs, PAb1101/PAb1102 and PAb1103/PAb1104, the blocking interaction between members of a pair being greater than between members of different pairs.

Non-reciprocality of blocking and partial rather than complete blocking have been observed between certain antibodies, both here and during the study of anti-T antibodies (E. Harlow, pers. comm.). The initial observation of partial blocking was surprising since it had been expected that for any determinant pair, dual occupancy would either be possible or completely inhibited. One explanation for the existence of a "partial blocking" situation is that the antibody: antigen interactions occurring in the PVC well do not reach equilibrium. In this event, the amount of probe bound would reflect more the rate of association of antibody with determinant than the total number of determinants available. This would not affect the validity of the assay as a measure of determinant proximity but would explain how "partial blocking" might arise. Since it is present in vast excess, the unlabelled antibody must rapidly occupy all its available determinants. Occupancy of these determinants could reduce the rate of association of a labelled probe antibody with the antigen by sterically hindering its approach or by altering the conformation of the antigen in the vicinity of the probe determinant to lower the association constant for its interaction with the probe antibody.

Non-reciprocality of a blocking interaction can be envisaged most easily as an imposition of directionality on the steric hindrance

occurring between antibodies occupying adjacent determinants. Thus, if an antigen is fixed so that determinant "a" is closer to the PVC surface than determinant "b", anti-"b" will exhibit greater blocking of anti-"a" binding than vice versa. Such effects would be particularly marked if bulky IgM antibodies were involved. This theory makes the clear prediction that if the same antigen can be fixed in the opposite orientation, then the effect will be reversed but this is difficult to test without some knowledge of the shape of the antigen and the distribution on it of at least some antigenic determinants.


## Conclusions

The results of this work can be summarised as follows. Five hybridomas capable of secreting anti-p53 antibodies have been isolated and cloned. Four of these antibodies have been characterised in detail. One of the four antibodies displays what is so far a unique pattern of reactivity, being able to bind both to human p53 and to a particular oligomeric subset of T-antigen in SV40-transformed or infected cells. The other three antibodies have anti-p53 activity only. All of these three antibodies react with human p53, two with monkey p53 and only one, at low efficiency, with mouse p53. Surprisingly, these three antibodies showed a strong preference for binding SV40 large T-associated rather than uncomplexed p53. It has not been possible to determine whether the other antibody, which has anti-T and anti-p53 activities, displays a similar preference or indeed whether it has activity to p53 from cells of non-human origin since its dual reactivity makes experimental interpretation difficult.

The determinants recognised on human p53 by the four new antibodies were compared with those recognised by previously characterised anti-p53 antibodies to determine whether or not any new determinants had been defined. On the basis of competitive

blocking experiments, all four new antibodies were found to recognise previously uncharacterised p53 determinants which themselves fell into two groups. The determinant on T-antigen recognised by the antibody having dual reactivity mapped in the C-terminal quarter of T-antigen and was, as expected from its restriction to oligomeric T-antigen, sensitive to denaturation.

Chapter 4


THE PURIFICATION AND PARTIAL AMINOACID SEQUENCE ANALYSIS OF p53


Introduction

As I have discussed in Chapter 1, there are grounds for believing

that p53 may be altered in some way in transformed cells and tumours

as compared with normal cells. The changes in the metabolism and

behaviour of p53 in different transformed cells are however varied

and it is not known which, if any, of the observed differences between

p53 in normal and transformed cells are directly linked to the

difference in phenotype. There are data to suggest that transformed

cell p53 is hyper-phosphorylated and this could be due to a number of

factors, including mutation of the p53 gene to generate a more readily

modified protein structure. Equally, alterations to the structure of

p53 may be closely linked to the transformed phenotype, whether or not

they are the cause of altered phosphorylation levels.

At the time of initiating this project, there were no data

available on the aminoacid sequence of p53 in any cell type. Since

the essential difference between normal and transformed cell p53 could

potentially be at the aminoacid sequence level, it was clear that a

complete description of p53 structure in different cell types would

be necessary before the relevance of structural changes to any

alteration of p53 function in transformation could be properly assessed.

Recently such data have been most easily acquired by cloning DNA

complementary to the mRNA for the protein of interest and predicting

a protein sequence from the sequence of the DNA. However, considerable

difficulty was being experienced in cloning p53 cDNA since its mRNA

was apparently a member of the lowest abundance class of cytoplasmic

mRNA and no cell type overproducing p53 mRNA was known.

Even after specific cDNA has been cloned, there are still problems in obtaining an unequivocal aminoacid sequence for the protein in question by prediction from the cDNA nucleotide sequence. It is not possible to locate the N-terminus of the translation product of a cDNA with certainty and any predicted N-terminus must be validated by independent protein sequence analysis. It is also difficult to prove that a selected cDNA clone actually encodes the protein expected without having independently obtained aminoacid sequence data for comparison. The standard test of identity, checking for hybridisation between the cloned cDNA and specific mRNA, can only show that some homology exists between the cloned and correct sequences. I therefore decided to obtain some of the p53 polypeptide sequence by direct analysis. Such information would allow the production of sets of synthetic oligonucleotides, corresponding to the various DNA sequences that would specify the determined aminoacid sequence, which could then be used as specific probes for p53 sequences in cDNA clone banks. If in the meantime p53-specific clones had been isolated by some other screening procedure, then aminoacid sequence information would provide an independent direct validation of such clones, would identify the reading frame in those clones and perhaps bear on the correct location of the site of initiation of translation.

## Protein Purification

The first problem was to successfully purify sufficient p53 for sequence analysis. The automated sequencing facilities available to me, by collaboration with Dr. M. Waterfield, had an absolute limit of detection of around 5pmol of liberated PTH-aminoacid. Amounts of protein in the order of 0.1-0.5nmol would be sufficient therefore to determine sequence from the N-terminus of the protein. However, many proteins are post-translationally modified at their N-termini so that
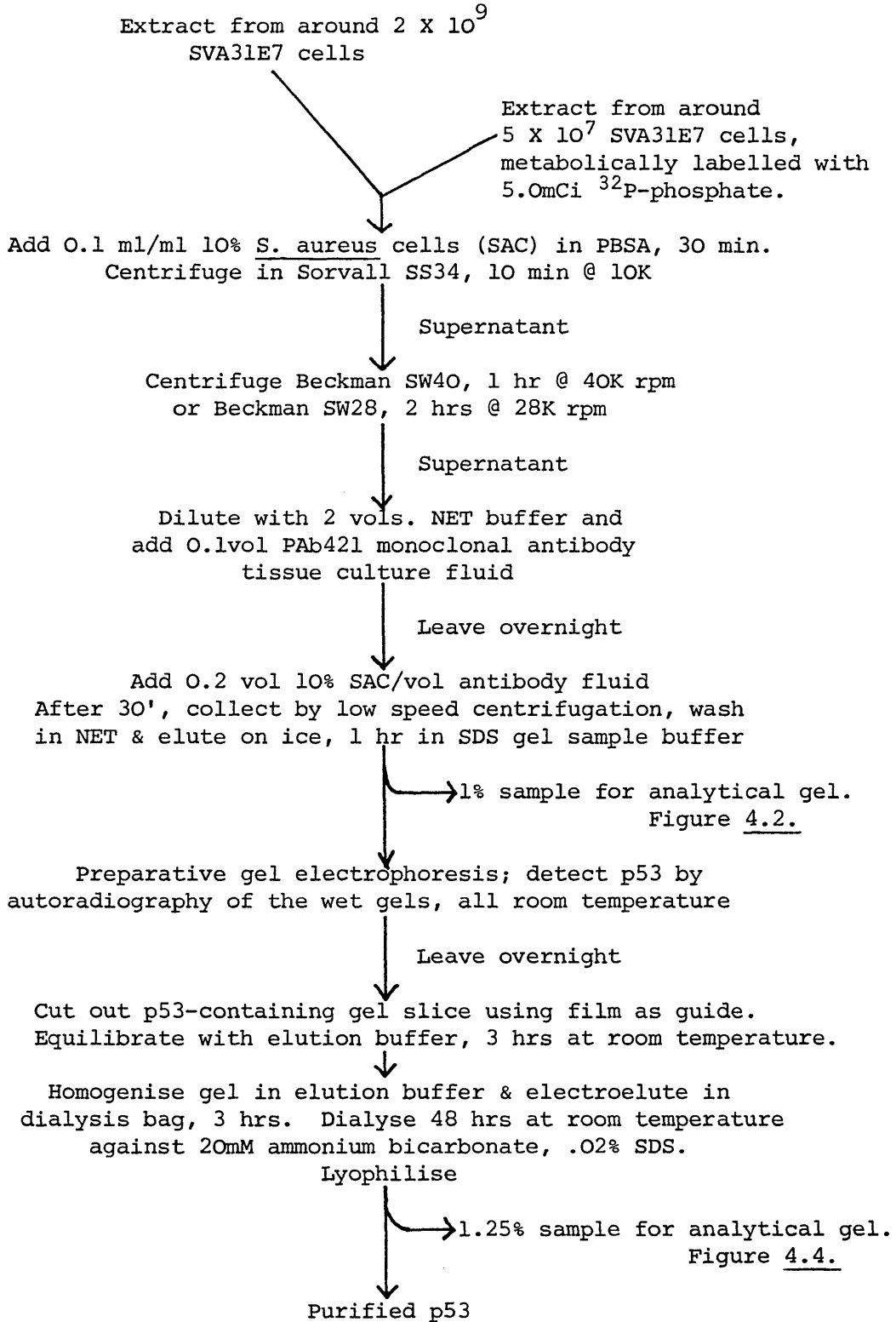
they are not amenable to sequential chemical degradation. In the event that p53 was so modified, it would be necessary to prepare internal fragments from the protein for sequence analysis. These procedures would require larger amounts of protein, perhaps 1-2nmol, to allow for inevitable losses during the production and purification of fragments for analysis. I selected the SV40-transformed mouse 3T3 cell line SVA31E7 (E7) as a source of p53, since, of the series of mouse cell lines tested by Benchimol et al. (1982), it contained the most p53 protein. In a pilot experiment, Coomassie G-stainable p53 equivalent to about 0.5μg protein was obtained from $10^7$ E7 cells by immunoprecipitation and gel electrophoresis. It appeared therefore to be possible in principle to purify the required amounts of p53 from E7 cells by a direct scale-up of standard monolayer cell culture, immunoprecipitation and gel electrophoresis techniques following which the protein would have to be recovered from the polyacrylamide gel and concentrated.

It was clearly essential to minimise losses of protein during purification whilst still achieving maximum purity and certain features of my purification protocol were incorporated to achieve this. These included the use of a high speed supernatant of cell extract as a source of p53, the omission of any background protein from the buffers used to dilute the extract for immunoprecipitation, the use of tris/ borate/sulphate rather than tris/glycine buffering for gel electrophoresis and the recrystallisation of SDS and precycling of dialysis tube used in the elution of p53 from gel slices. Two alternative procedures were used to obtain a p53-containing extract of E7 cells. These involved either extraction of cell monolayers in NP40 lysis buffer or the homogenisation of a washed cell pellet in hypotonic buffer containing neutral detergent and subsequent fractionation of the homogenate. These methods are described fully in Chapter 2 and

Figure 4.1

## PURIFICATION OF MOUSE p53

(All procedures at $+4^{\circ}$ unless stated otherwise)

Extract from around $2 \times 10^9$
SVA31E7 cells

Extract from around
$5 \times 10^7$ SVA31E7 cells,
metabolically labelled with
5.OmCi $^{32}$P-phosphate.

Add 0.1 ml/ml 10% <u>S. aureus</u> cells (SAC) in PBSA, 30 min.
Centrifuge in Sorvall SS34, 10 min @ 10K

Supernatant

Centrifuge Beckman SW40, 1 hr @ 40K rpm
or Beckman SW28, 2 hrs @ 28K rpm

Supernatant

Dilute with 2 vols. NET buffer and
add 0.1vol PAb421 monoclonal antibody
tissue culture fluid

Leave overnight

Add 0.2 vol 10% SAC/vol antibody fluid
After 30', collect by low speed centrifugation, wash
in NET & elute on ice, 1 hr in SDS gel sample buffer

→1% sample for analytical gel.
Figure <u>4.2.</u>

Preparative gel electrophoresis; detect p53 by
autoradiography of the wet gels, all room temperature

Leave overnight

Cut out p53-containing gel slice using film as guide.
Equilibrate with elution buffer, 3 hrs at room temperature.

Homogenise gel in elution buffer & electroelute in
dialysis bag, 3 hrs. Dialyse 48 hrs at room temperature
against 20mM ammonium bicarbonate, .02% SDS.
Lyophilise

→1.25% sample for analytical gel.
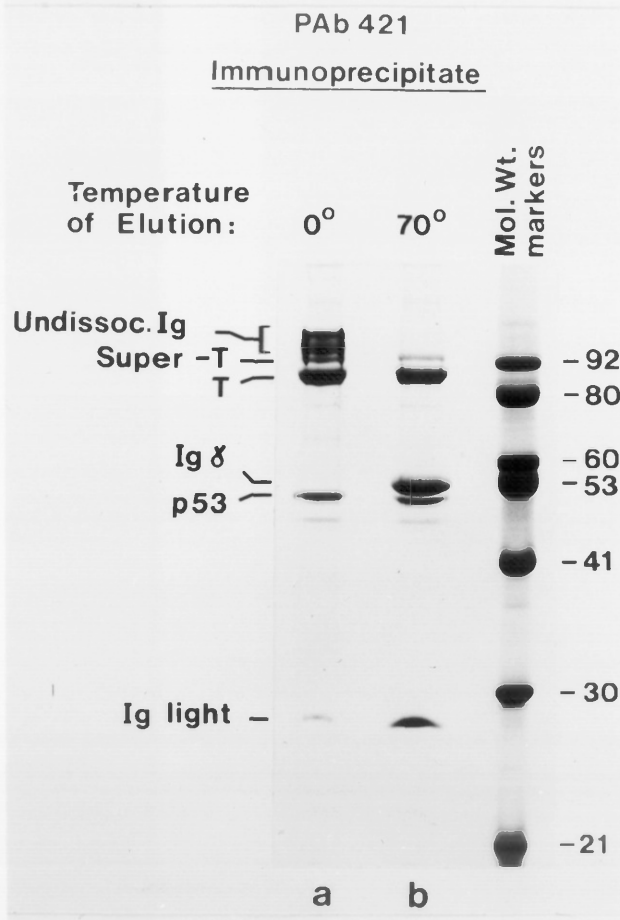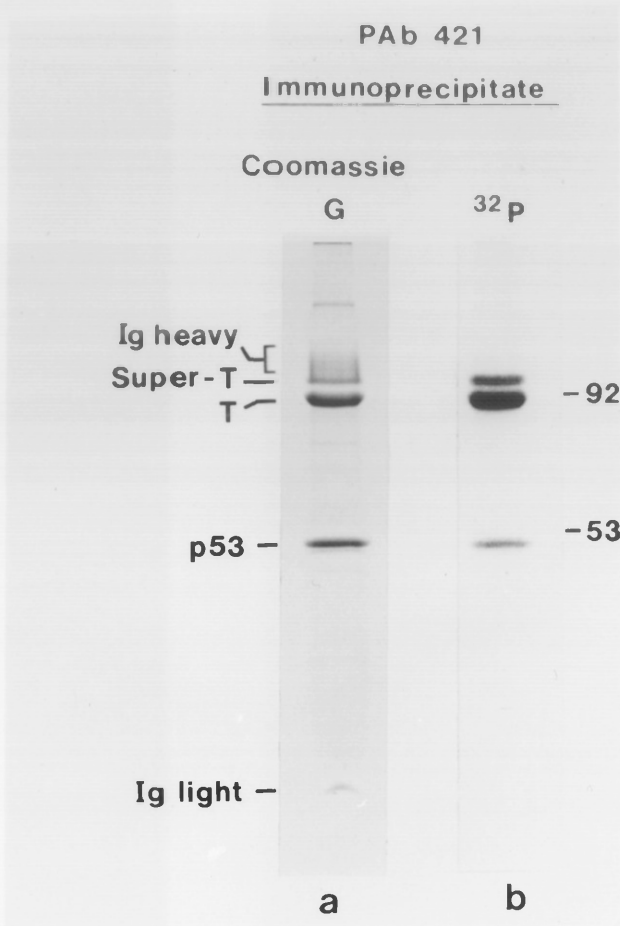Figure <u>4.4.</u>

Purified p53

appeared equally successful in providing a source of E7 large T/p53

complex in soluble form.

The purification protocol employed is summarised in Figure 4.1

and described in detail below. Extract from $\sim 2 \times 10^9$ E7 cells was

pooled with an extract of about $5 \times 10^7$ $^{32}$P-phosphate-labelled E7

cells, prepared in NP40 lysis buffer, to provide a labelled tracer for

p53. Before use, this extract was precleared of any material capable

of being nonspecifically precipitated in the subsequent immunoprecipi-

tation by incubation with 0.1ml of 10% S. aureus strain Cowan 1 (SAC)

per ml of lysate for 30 minutes on ice, and clearance by centrifugation

firstly at 10,000rpm for 10 minutes in a Sorvall SS34 rotor and then

either at 28,000rpm for 2 hours in a Beckman SW28 rotor or at 40,000rpm

for 1 hour in an SW40 rotor depending on the total volume to clear any

remaining denatured protein aggregates. The p53/large T-antigen

complex in the extract was then immunoprecipitated with an anti-p53

monoclonal antibody, PAb421 (Harlow et al., 1981b). Precleared extract

was diluted at $+4^{\circ}$C with 2 volumes of NET buffer per volume of

supernatant and immunoprecipitated overnight with 0.1 volumes of

tissue culture fluid from hybridoma PAb421 (containing $\sim 30$ μg/ml anti-

p53 antibody). Immune complexes were collected with 200μl 10% SAC per

ml of antibody fluid (Kessler, 1975) for 30 minutes at $+4^{\circ}$, washed

twice in 10ml NET buffer per 100μl 10% SAC and finally eluted by

resuspending in 100μl SDS gel sample buffer per 100μl 10% SAC at $+4^{\circ}$

and holding at that temperature for 1 hour. Bacteria were than removed

by centrifugation and the supernatant either used directly or stored

at $-20^{\circ}$.

Figure 4.2 shows the Coomassie G-stained proteins (lane a) and

the $^{32}$P-labelled proteins (lane b) from a 1% aliquot of the total

sample for gel electrophoresis typically produced by these procedures,

separated on an analytical gel. $^{32}$P-labelled species characteristic of

Figure 4.2 (opposite) The large T/p53 complex was immunoprecipitated from an extract of $\sim 1.6 \times 10^9$ SVA31E7 mouse cells (of which a small proportion had been metabolically labelled with $^{32}$P-phosphate) using anti-p53 monoclonal antibody PAb421 and eluted into 2.5ml of SDS gel sample buffer at $0^\circ$. 25μl of this sample was analysed on a 10% polyacrylamide - SDS gel, which was then stained with Coomassie blue stain (lane a) before being dried and autoradiographed (lane b). The positions of components of the immunoprecipitate and molecular weight marker proteins are shown on the left and right respectively.

Figure 4.3 (opposite) Samples of immunoprecipitated proteins in SDS gel sample buffer as in Figure 4.2 were either loaded directly (lane a) or heated to $70^\circ$ for 5 minutes before loading (lane b). The components of the immunoprecipitates are identified on the left and molecular weight marker proteins on the right of the figure.

PAb 421

Immunoprecipitate

Coomassie

G          $^{32}$P

Ig heavy
Super-T                        − 92
T

p53 −                          − 53

Ig light −

a          b

PAb 421

Immunoprecipitate

Temperature
of Elution:    0°    70°    Mol. Wt. markers

Undissoc. Ig
Super -T                              − 92
T                                    − 80

Ig γ                                 − 60
                                     − 53
p53

                                     − 41

Ig light −                          − 30

                                     − 21

a    b

p53 and large T from this cell line comigrate with heavily stained

protein bands. A super T-antigen, similar to that described by

Kress et al. (1979), produced by these cells is also present in the

immunoprecipitate. Polypeptides with molecular weights of 25,000 and

110,000 (lane a) correspond to immunoglobulin light and heavy chains

respectively. The latter migrate as dimers, some of which retain

bound light chains, because the sample was prepared without heating

and as a result the dissociation of IgG into subunits is minimal

(McCormick and Harlow, 1980). To check that there was no significant

contamination of p53 with $\gamma$ chain monomer, aliquots of immunoprecipitate

were, on a separate occasion, either kept at $0^\circ$ or heated to $70^\circ$ for

5 minutes before loading. The pattern of Coomassie stained proteins

derived from these samples is shown in Figure 4.3. A comparison of

lanes a and b shows that on heating, the $\gamma$ chains run as a heavily

stained band of $\sim$50K, clearly distinguishable from the p53 band present

in both lanes. No monomer heavy chain was detected in the unheated

sample and the light chain band was of much reduced intensity. A set

of protein species of 100-120K are seen only in the unheated sample.

These represent the various possible partially dissociated IgG molecules.

Proteins in the bulk immunoprecipitate were separated by

preparative gel electrophoresis using the tris/borate/sulphate buffer

system described by Neville (1971). This system was used in preference

to the tris/glycine system described by Laemmli (1970) to avoid a

carry-over of glycine into the purified sample which could have

affected the results of aminoacid composition analysis. p53 was

located in the wet gels by overnight autoradiography and the p53-

containing slices of gel excised. These slices, about 1 $cm^3$ swollen

gel volume for a typical experiment, were incubated for 3 hours at

room temperature with agitation in 2.0ml elution buffer (1% $^{w/v}$SDS, 0.2M

tris/acetate pH 8.0, 0.1% $^{w/v}$dithiothreitol) and then broken by passage

through a 5ml syringe. The gel suspension was confined in precleaned

dialysis tubing and subjected to a 100 V, 100 mA electric field for

3 hours, immersed in 0.1% $^{w/v}$ SDS, 50mM tris/acetate pH 7.4, 0.5mM sodium

thioglycolate in a flat-bed electrophoresis tank. At the end of this

period, the current was reversed for 30 seconds and the material then

transferred to dialysis against 4 litres of 20mM ammonium bicarbonate,

0.02% $^{w/v}$ SDS. After 48 hours with one buffer change the dialysed sample

was cleared of gel fragments by filtration through a GF-C filter and

lyophilised.

The purity of this eluted material and the approximate yield

were tested by rerunning a 1.25% aliquot on an analytical gel, as

shown in Figure 4.4. Single bands of both stained (lane a) and

labelled (lane b) protein are apparent which precisely comigrate with

an apparent molecular weight of $\sim$48,000, estimated from the mobility

of marker proteins. Based on the amount of bound Coomassie stain,

the final yield of p53 protein from 2 X $10^9$ cells was in this case

about 75μg, 50% of the p53 initially immunoprecipitated.

p53 is defined by its reactivity with antibodies such as PAb421

and by this definition the $^{32}$P-labelled material of 48,000 molecular

weight that had been purified from cell extract by immunoprecipitation

with PAb421 was p53. To ensure that the Coomassie blue - stainable

protein that had copurified with the labelled p53 shared its

immunological and chemical properties, I tested the ability of

stainable protein to rebind either to PAb421 or to the control anti-

large T antibody PAb419. As shown in Figure 4.5, PAb421 (lane b)

rebound a significant amount of the input purified p53 whereas PAb419

(lane d) showed no specific precipitation of this material. Since

PAb421 rebinds no more than 50% of $^{35}$S-methionine-labelled p53

purified in this way in direct binding assays (Chapter 3, Figure 3.12

and E. Harlow, pers. comm.), it appears that the purified Coomassie-

Figure 4.4 (opposite)  p53 protein from E7 cells, some of which had

been labelled in vivo with $^{32}$P-phosphate, was purified by electro-

phoretic separation of components of an anti-p53 antibody immuno-

precipitate and subsequent electroelution from the appropriate

slice of polyacrylamide gel. An aliquot of this material was then

re-electrophoresed through an SDS-polyacrylamide gel alongside

proteins of known molecular weight. The gel was firstly stained

with Coomassie blue (lane a) and then dried and autoradiographed

(lane b). The sizes of the marker proteins are indicated on the left

of the figure.



Figure 4.5 (opposite)  The ability of anti-p53 antibody PAb421 to

rebind the purified Coomassie - stainable p53 material was tested

by diluting what was estimated to be between 1µg and 5µg of purified

protein into NET/gel buffer and immunoprecipitating with either

1.5µg PAb421 (lane b) or 1.5µg PAb419, a control anti-SV40 large T-

antigen antibody (lane d). After 30 min. at room temperature, immune

complexes were collected on 20µl 10% SAC, washed twice in NET/gel

buffer and eluted into 25µl SDS gel sample buffer by incubation for

1 hour at 0°. Components in the sample were then separated on a 10%

polyacrylamide - SDS gel and detected by Coomassie - blue staining.

Lanes a and e show control precipitations with 1.5µg PAb421 or

PAb419 in the absence of added p53 and lane c shows the amount of

p53 input into the precipitations shown in lanes b and d, loaded

directly onto the gel.

Purified
Protein
_____

Coomassie

G    ³²P

92 –

80 –

60 –
53 –                              – p53

41 –

30 –

a    b
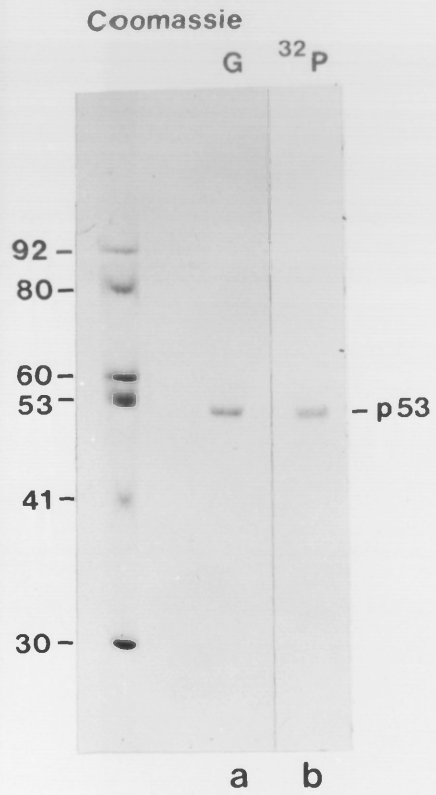
Specific Rebinding

of Purified p53

Antibody :    421        419
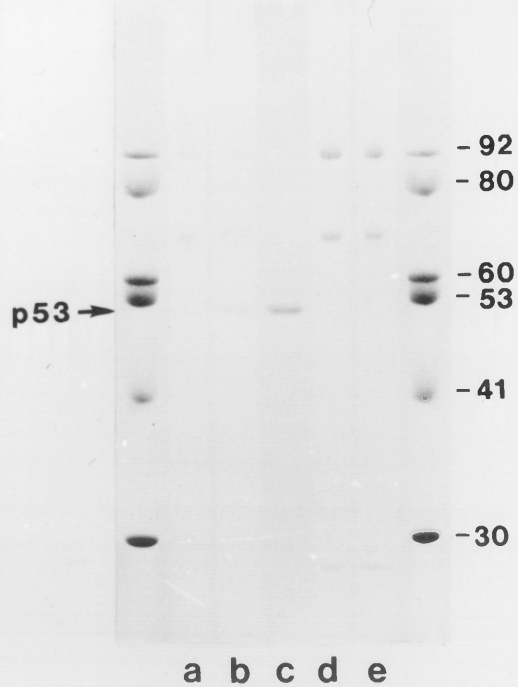Purified p53 :   – + +  + –

p53 →

– 92
– 80

– 60
– 53

– 41

– 30

a b c d e

Table 4.1

Aminoacid Composition of p53

| Aminoacid | Description | Mouse p53 mol%* | TA3 mouse homologue of EBNA-assoc. 53K mol%** |
|---|---|---|---|
| D/N | charged/polar | 5.9 | 11.2 |
| E/Q | "  /  " | 10.6 | 14.7 |
| S | polar | 8.1 | 7.1 |
| T | " | 3.7 | 3.1 |
| G | " | 6.2 | 15.9 |
| C | " | N.D | 0.9 |
| Y | " | 5.0 | 0.9 |
| F | hydrophobic | 3.7 | 3.4 |
| W | " | N.D | N.D |
| V | " | 5.5 | 4.5 |
| I | " | 2.8 | 2.5 |
| L | " | 10.1 | 4.6 |
| A | " | 7.7 | 7.0 |
| M | " | 2.9 | 1.2 |
| P | " | N.D | 5.8 |
| R | charged | 6.9 | 6.8 |
| K | " | 6.9 | 8.2 |
| H | " | 1.4 | 2.3 |
| Total | | 87.4 | 100.1 |

Approximately 0.1nmol of p53 was hydrolysed in 50µl 6N HCl with 0.5µl 1.4-butanedithiol for 22hr. at $110^{\circ}$ under $N_2$, and the products analysed by HPLC. Data here and subsequently are presented using the single letter aminoacid code (A-Ala; C-Cys; D-Asp; E-Glu; F-Phe; G-Gly; H-His; I-Ile; K-Lys; L-Leu; M-Met; N-Asn; P-Pro; Q-Gln; R-Arg; S-Ser; T-Thr; V-Val; W-Trp; Y-Tyr). N.D signifies 'not determined'. *P, C, W were not detectable by the procedures employed. These aminoacids were assumed to contribute 12.5% of the residues of p53 for calculation of mol% figures for the other 17 aminoacids (see text). **Data taken from Jörnvall et al. (1982) and calculated excluding any contribution from tryptophan.

stainable protein is essentially homogeneous p53, as usually defined.

## Aminoacid Composition of p53

p53, prepared as described, was further concentrated and the level of SDS in the sample reduced, either by electrophoresis of the protein from one well to another of a commercial electroelution apparatus in low SDS buffer, or by precipitating the protein with trichloracetic acid (TCA) and redissolving in 98% $v/v$ formic acid. Protein prepared by either method, or further subjected to performic acid oxidation (Hirs, 1967) was then hydrolysed and its aminoacid content determined (Darbre and Waterfield ,1983). I am indebted to G.Scrace for performing HPLC analyses of the liberated aminoacids. Data from an experiment using between 0.1 and 0.2 nmol of TCA precipitated, unoxidised p53 protein are shown in Table 4.1. Since proline, cysteine and tryptophan could not be determined by the method employed, these aminoacids were assumed to contribute 12.5mol% of the protein as a basis for calculation of the contributions of the other 17 aminoacids. This assumption was based on the observed frequency of these aminoacids in the predicted translation product of a 277 codon open reading frame in cDNA clones recently derived by others in the laboratory (see below). The composition data reveal that p53 contains no excess of negatively charged residues; its pI of between 5.5 and 6.5 is therefore probably due to the observed phosphorylation of several serine and threonine residues along the length of the polypeptide chain (van Roy et al., 1981). One third of the residues (excluding proline) are hydrophobic and one eighth are hydroxy-aminoacids. The estimated methionine content of 2.9% would imply 11 or 12 methionines per 400 residues (the predicted length of p53, based on its apparent molecular weight and a typical mean residue molecular weight of 120).

It is worth noting that there are major differences between the

composition of p53 determined here and the published composition of

the 53,000 dalton EBNA-associated protein which is reproduced in

Table 4.1 for comparison (Luka et al., 1980; Jörnvall et al., 1982).

Most notable are the disparities between the figures for aspartic

acid plus asparagine, glycine, leucine and tyrosine. My data are

therefore in good agreement with the suggestion (Luka et al., 1983)

that the 53,000 dalton EBNA-associated protein is not related to the

SV40 large T-associated cellular protein, p53.


## Aminoacid Sequence of p53

If only part of a protein sequence is to be obtained it is

clearly of advantage to derive that information from the N-terminus

of the intact protein since the data will then relate to a known

position in the protein. Unfortunately, many proteins are not amenable

to direct sequence analysis because the N-terminal residue has been

modified so as to leave the first peptide bond in the protein stable

to Edman degradation reactions. Two common blocking modifications of

proteins in eukaryotes are N-acetylation and formation of pyroglutamate

at the N-terminus by cyclisation of glutamine. When 0.5nmol of p53

was subjected to N-terminal sequence analysis, no aminoacid release

could be detected, even at a sensitivity of 10pmol, indicating that

p53 has some form of blocked N-terminus.

Since N-terminal sequencing was not possible, I proceeded to

isolate internal fragments of p53 that could be successfully subjected

to sequential Edman degradation. Approximately 1nmol of performic

acid oxidised p53 was digested with trypsin and the peptides separated

by HPLC. The absorbance of the column eluate was monitored continuously

at 206nm and the resulting eluate profile is shown in Figure 4.6. The

peptides designated K2, K8 and K9 have been analysed further and in

each case I am indebted to N. Totty for operating the sequence analyser

Figure 4.6 (opposite) Tryptic peptides derived from 1 nmol of

purified p53 were redissolved in 50μl of 0.1% $v/v$ trifluoracetic acid

(TFA), 6M urea, loaded onto a Synchropak RP-P column and eluted

with a linear 0 to 60% $v/v$ acetonitrile gradient in 0.1% $v/v$ TFA. The

optical density of the eluate was monitored at 206nm and plotted

against the cumulative eluate volume. Full scale on the ordinate

represents an optical density of 0.2. The positions of the peaks

designated K2, K8 and K9 are indicated.

SEPARATION OF p53 TRYPTIC PEPTIDES BY HPLC
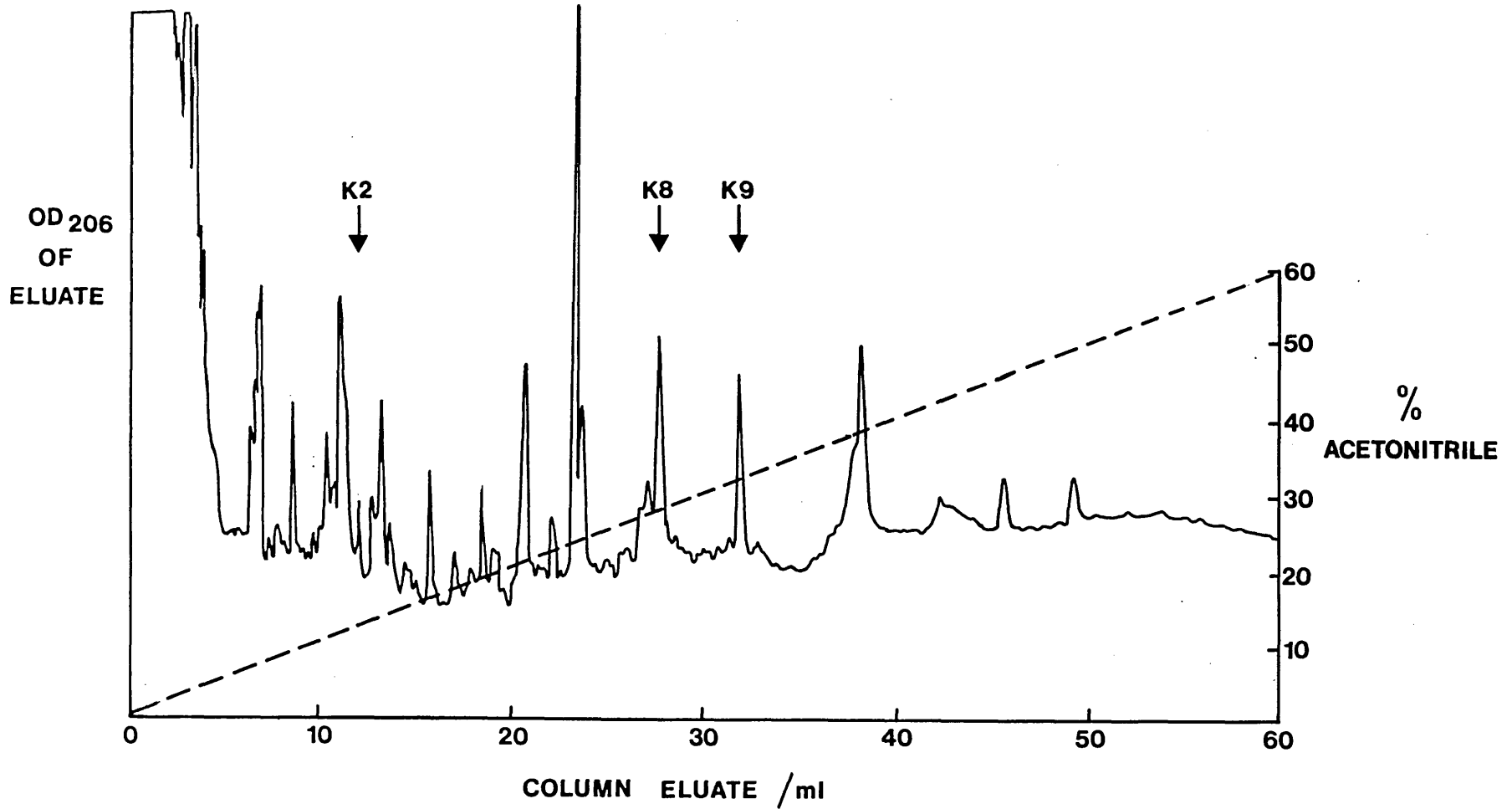
on my behalf.

For each of the peptides K2, K8 and K9, 250µl (50%) of the appropriate column fraction was used for sequence determination by the method described. The HPLC traces derived from analysis of the first 18 sequence fractions of peptide K9 are shown in Figure 4.7 as an example of the data obtained. Each PTH-aminoacid has a characteristic elution time from the HPLC column allowing liberated residues to be identified. The relevant peaks identified for K9 in Figure 4.7 are shaded and the aminoacid they represent shown arrowed below each trace.

The amount of each aminoacid derivative detected during HPLC analysis is automatically quantitated by reference to a calibration mixture run in the same set of samples. The yield of aminoacid derivative detected at each degradation cycle of K9 and K2 and the aminoacid sequences deduced in each case are shown in Figures 4.8 and 4.9 respectively. It was not possible to quantitate the recovery of serine and threonine derivatives due to their instability. No derivative could be identified in the analysis of K9 at either cycle 14 or from cycle 18 onwards and in the analysis of K2 at either cycle 4 or from cycle 9 onwards. In both cases, the repetitive yield fell as sequencing progressed, especially in the last few cycles in which residues could be identified. This phenomenon is usually due to an increasing loss of residual peptide from the solid support during the reagent cycle as the peptide becomes shorter and particularly affects detection of the final residue of a peptide. It is therefore likely that neither K2 nor K9 extended much beyond the last residue detected.

HPLC analysis of sequential fractions from peptide K8 identified two aminoacid derivatives in several cases (Figure 4.10), suggesting that K8 was, in fact, a mixture of two peptides. Presumably, at the positions where only one residue was detected, either the two peptides

Figure 4.7 (overleaf)  The components of sequenator fractions arising

from the sequential N-terminal degradation of peptide K9 were

analysed by high pressure  liquid chromatography (HPLC), to

determine the identity of the aminoacid liberated at each step. The

traces of optical density versus elution time (increasing left to

right) for the first 18 degradation fractions of K9 are shown here.

The sensitivity of detection was increased after analysis of fraction

11. The aminoacid identified in each fraction by the characteristic

elution time of its phenylthiohydantoin (PTH) derivative is indicated

below each trace. PTH-threonine is unstable and two derivatives of

it are detected with low yield as a pair of characteristic peaks (as

indicated at positions 5 and 8). No characteristic peaks could be

identified at positions 14 or 18 (see text). For a description of

the single letter aminoacid code see Table 4.1.
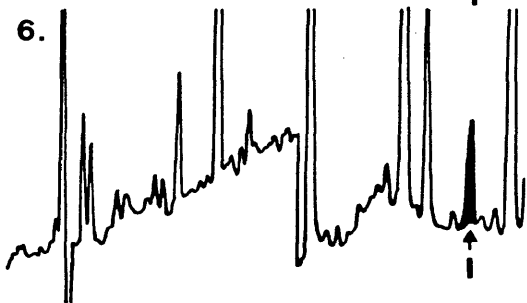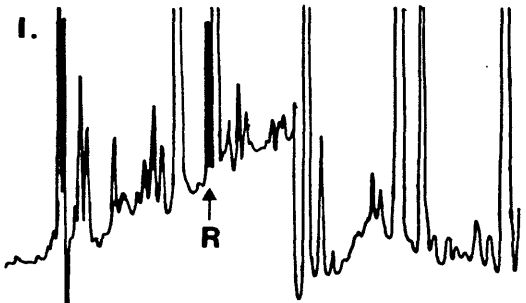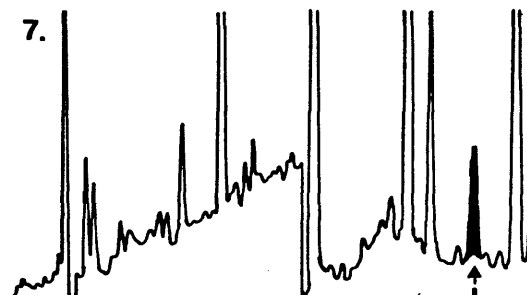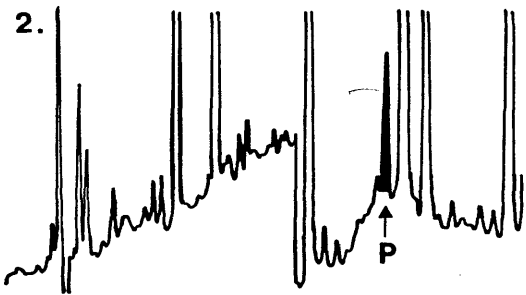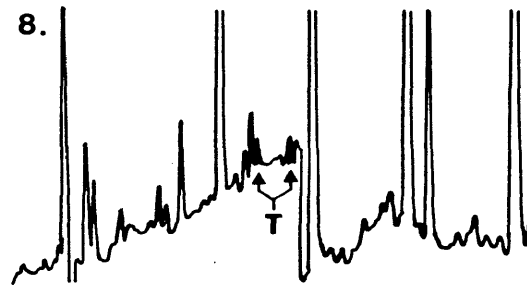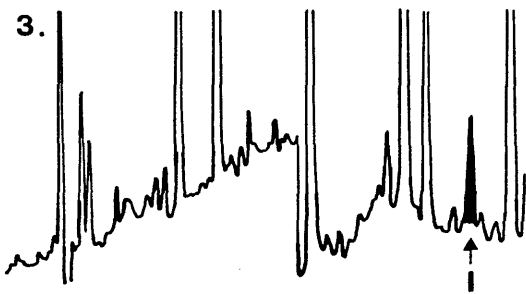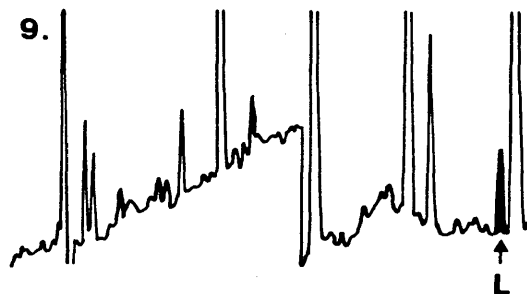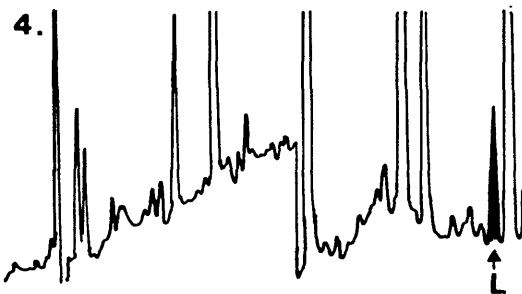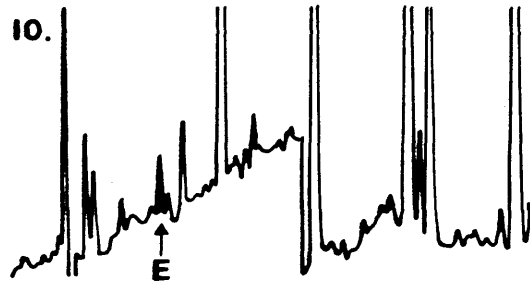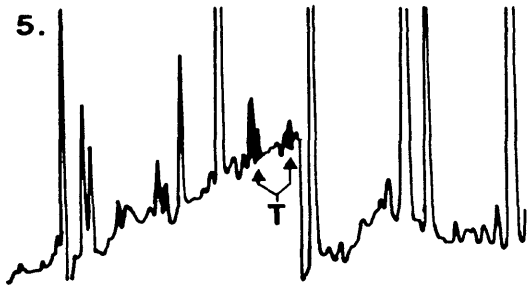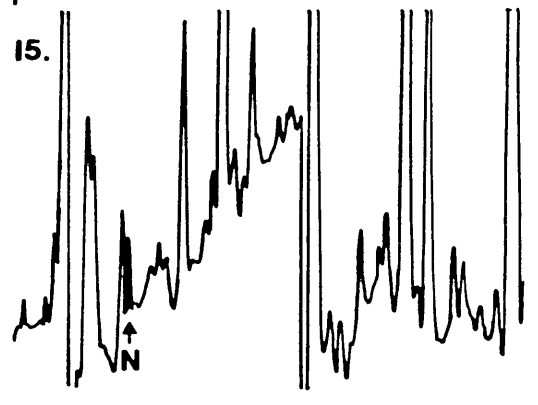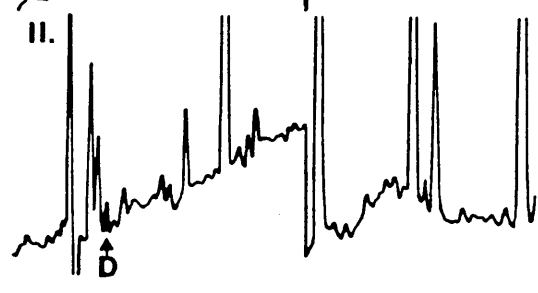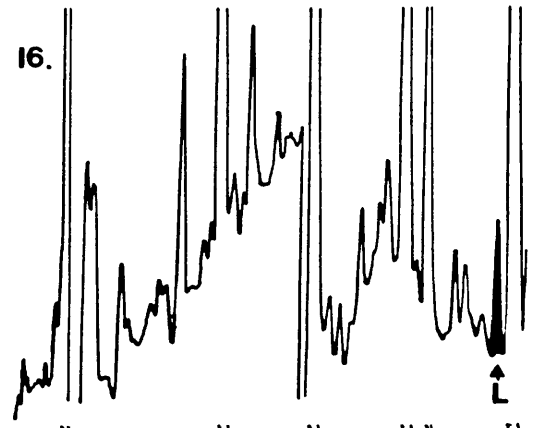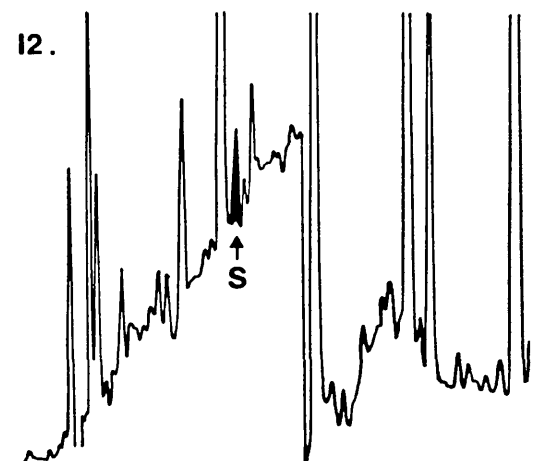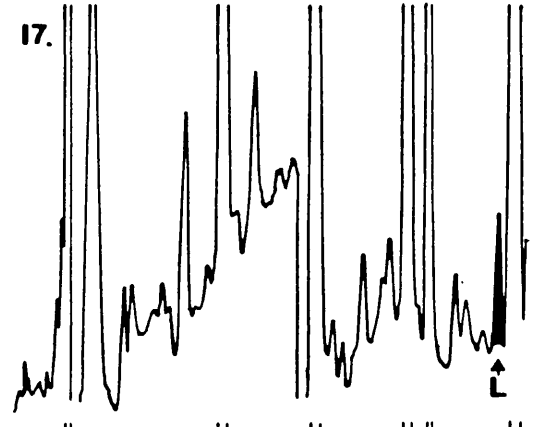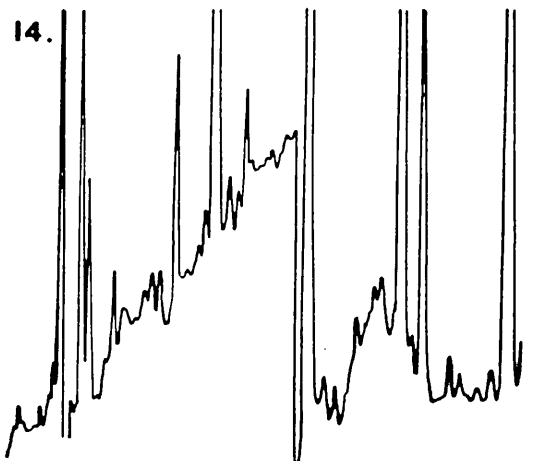
Figure 4.8 (opposite)   From the HPLC analysis of K9 degradation

fractions shown in Figure 4.7 the recovery of each PTH-aminoacid was

automatically quantitated and these recoveries plotted against the

degradation cycle number. The residue identified at each cycle is

indicated beneath the appropriate cycle number. PTH-serine and PTH-

threonine could not be quantitated due to their instability but,

where indicated, were unambiguously identified on the basis of their

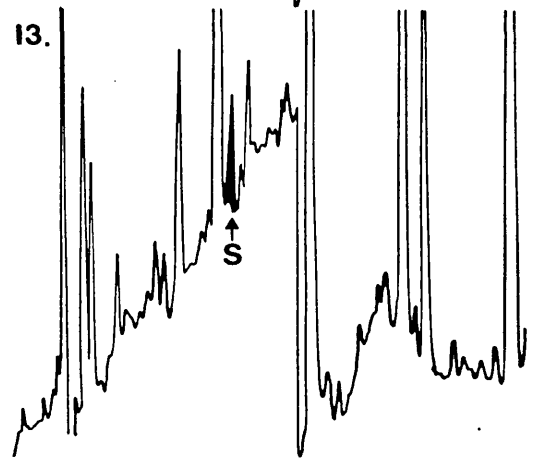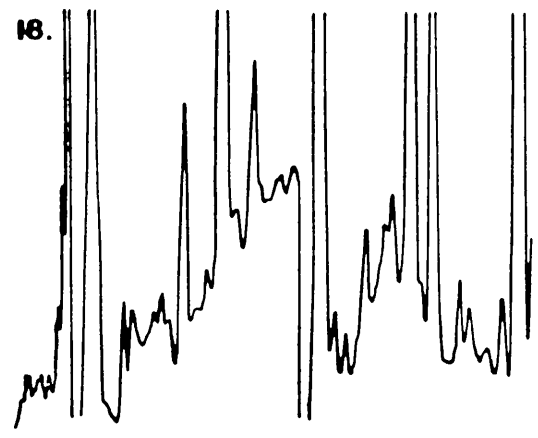characteristic elution times from the column (Figure 4.7). For a

description of the single letter aminoacid code see Table 4.1.

Figure 4.9 (opposite)   The recovery of PTH-aminoacids from sequence

analysis of peptide K2 was determined as described for peptide K9 in

Figures 4.7 and 4.8. These data are plotted against cycle number.

PTH-threonine (cycle 5) could not be quantitated and no characteristic

peak was identified at cycle 4. For a description of the single

letter aminoacid code see Table 4.1.

PTH-AMINO-ACID YIELD AT EACH DEGRADATION OF

PEPTIDE K9



PTH-AMINO-ACID YIELD AT EACH DEGRADATION

OF PEPTIDE K2

## PTH-AMINO-ACID YIELD AT EACH DEGRADATION
## OF PEPTIDE MIXTURE K8



| Residue number | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Amino-acids detected | V | Y | Q | A | N | Y | A | F | H | L | V | F | L | Q |
| | (T) | (S) | | | P | A | | Q | D | P | | | | |
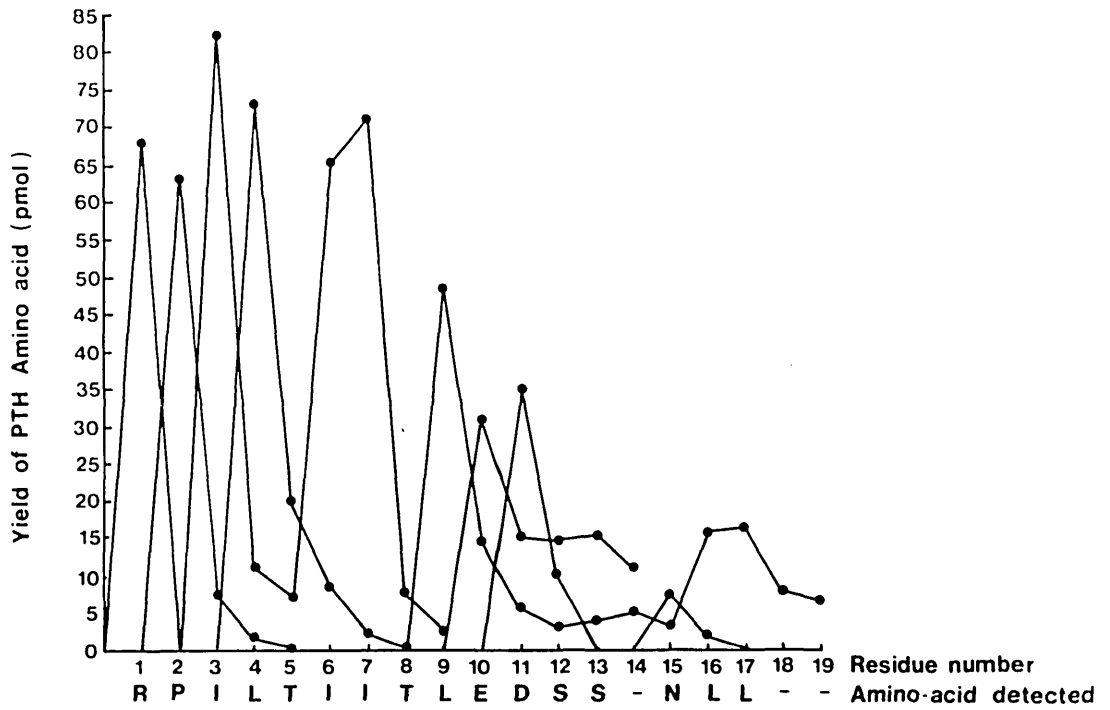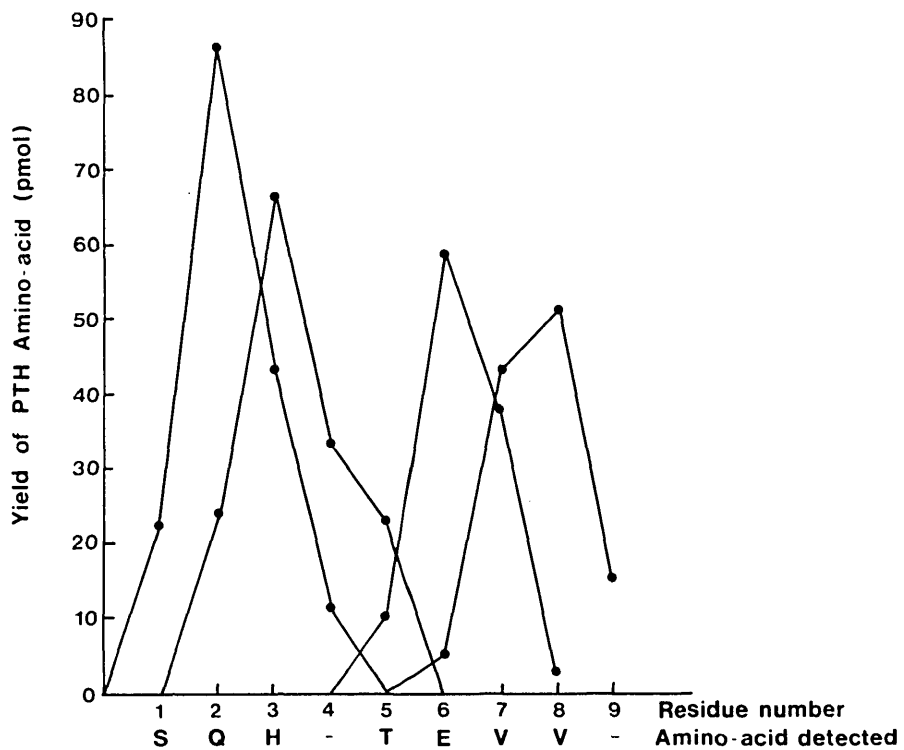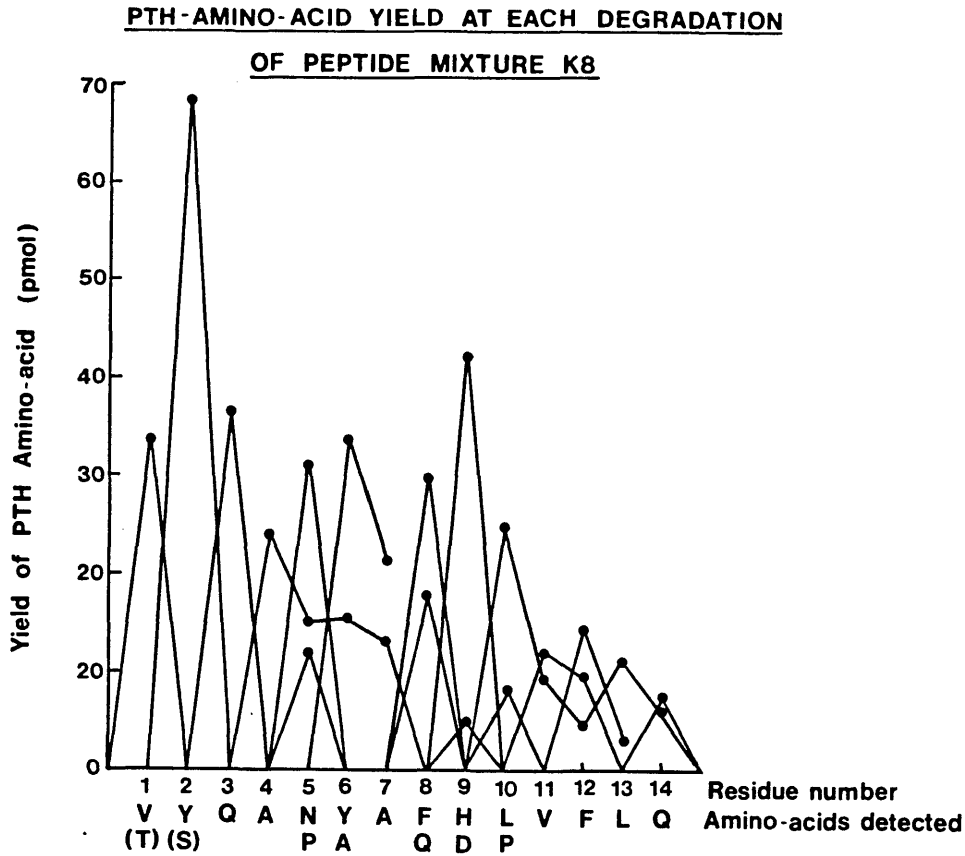
Figure 4.10  The recovery of PTH-aminoacids from sequence analysis
of peptide K8 was determined  as described for peptide K9 in
Figures 4.7 and 4.8. These data are plotted against cycle number.
PTH-serine (cycle 2) and PTH-threonine (cycle 1) could not be
quantitated due to their instability. Two residues were identified
at each of cycles 1, 2, 5, 6, 8, 9 and 10 indicating that K8 was
a mixture of peptides. For a description of the single letter
aminoacid code see Table 4.1.

COMPARISON OF PEPTIDES K2 AND K9 WITH PREDICTED p53 SEQUENCE

| | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Peptide K2 | | | S | Q | H | - | T | E | V | V | - | | | | | | | |
| Predicted p53 sequence | | ·········· K | S | Q | H | M | T | E | V | V | R ···· / 74 codons in frame /····· | | | | | | | |
| cDNA sequence * | | ············· AAG | TCA | CAG | CAC | ATG | ACG | GAG | GTC | GTG | AGA ··/222 nucleotides /········ | | | | | | | |

| | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Peptide K9 | | R | P | I | L | T | I | I | T | L | E | D | S | S | - | N | L | L | - - |
| Predicted p53 sequence | ···R | R | P | I | L | T | I | I | T | L | E | D | S | S | G | N | L | L | G R ···· |
| cDNA sequence * | ···CGC | CGA | CCT | ATC | CTT | ACC | ATC | ATC | ACA | CTG | GAA | GAC | TCC | AGT | GGG | AAC | CTT | CTG | GGA CGG··· |

Figure 4.11 Alignment of p53 peptide sequences K2 and K9 with the sequence of a polypeptide predicted from cDNA sequence. *This cDNA sequence is taken from the work of S.Benchimol, L.Crawford, E.Harlow and J.Jenkins (Benchimol et al., in preparation), and I acknowledge the generosity of the authors in allowing me access to their data prior to publication. For a description of the single letter amino-acid code see Table 4.1.

contained the same aminoacid or one of the two peptides contained a

residue that was difficult to detect. As discussed further below,

under the conditions employed such residues include glycine (high

background), threonine (unstable derivative) and the sulphur

containing aminoacids methionine and cysteine which were modified

in the course of isolating these peptides. It was not, therefore,

possible to deduce an aminoacid sequence for either of the K8 peptides

from this data alone.

Whilst this work was in progress, cDNA clones capable of hybrid-

selecting p53 mRNA were isolated without the use of specific oligo-

nucleotide primers (Oren and Levine, 1983; Oren et al., 1983;

Benchimol et al., in preparation) and through the generosity of

S. Benchimol, L. V. Crawford, E. Harlow and J. Jenkins in allowing me

access to their data in advance of publication, I have been able to

compare my peptide sequences with the translation product predicted

from an open reading frame in their clones. As shown in Figure 4.11,

there is an exact match between both K2 and K9 and the predicted cDNA

translation product. This finding confirms that the cDNA clones were

derived from mRNA capable of encoding the p53 protein and fixes the

correct reading frame in these clones. It also permits a prediction

of the complete sequences of K2 and K9, respectively 9 and 19 aminoacids

in length. Positions 14 and 18 in K9 are predicted to be glycine,

position 4 in K2 to be methionine and positions 9 in K2 and 19 in K9

to be arginine. The C-terminal residues were probably not detected

because of cumulative losses of material in the preceding cycles,

whilst methionine is converted to its undetectable sulphone derivative

by performic acid. There is a particular difficulty with glycine

detection at such a high level of sensitivity. Glycylglycine, added

to the sequencer to remove potential N-terminal blocking agents that

contaminate the reagents used, gives rise to a high background release

**Separation of Peptide Sequences K8a & K8b**

| Position No: | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| pseudogene prediction: | V | S | G | A | P | A | A | Q | D | P | V | – | – | – | – | – | – | – | – |
| K8 a: | V | S | – | A | P | A | A | Q | D | P | V | – | – | – | – | – | – | – | – |
| b: | T | Y | Q | – | N | Y | – | F | H | L | – | Ⓕ | L | Q | – | – | – | – | – |
| pseudogene prediction: (confirmed by cDNA from position 13 ) | T | Y | Q | G | N | Y | G | F | H | L | G | L | L | Q | S | G | T | A | K |

Figure 4.12  The mixed sequence of peptide K8, shown in Figure 4.10, can be separated into two sequences K8a and K8b by comparison of the data for K8 with the translation product predicted for an open reading frame in the sequence of a p53 pseudogene that has been cloned by others in the laboratory (Benchimol et al., in preparation). This open reading frame lies upstream of and extends into the open reading frame in the cDNA clones already aligned with peptides K2 and K9 (Figure 4.11). At the positions where only one aminoacid was detected by sequence analysis of K8, one or other of the two predicted sequences contains a glycine residue. At position 12 in K8b there is a difference between the determined and predicted sequences that probably reflects a mutation in the pseudogene at this position.

of glycine at each cycle. The N-terminal arginine of K9, unusual in

a tryptic peptide because of the enzymes's known specificity, is

explained by the presence of a second arginine codon immediately

upstream of the K9 sequence in the cDNA. Trypsin is known to cut

solely between adjacent target residues.

S. Benchimol and N. Williamson have now employed cDNA probes

to clone mouse genomic DNA fragments containing p53 sequences and

these clones have been sequenced by D. Pim and myself. One clone

represents the 3' half of the p53 gene whilst the other contains a

region that has all the characteristics of a p53 pseudogene. In the

regions where comparison is possible, the sequence of the pseudogene

does not appear to have diverged greatly from that of the active gene.

It was therefore used to predict the aminoacid sequence of the N-

terminal portion of p53, which was not available directly from cDNA

or genomic sequences. On comparison of the aminoacid sequence data

for the K8 peptide mixture with this predicted polypeptide, it was

possible to separate out two peptide sequences, K8a and K8b, from

the mixed sequence of K8 as shown in Figure 4.12. The peptide

designated K8b appeared to be present in the mixture in larger amounts

than K8a, on the basis of the comparative yields of aminoacid

derivatives from the two sequences. As a result, the yield of K8a

derivatives reached the limit of detection after 11 cycles whereas

derivatives from K8b could be detected as far as cycle 14. It is not

possible to predict the position of the C-terminus of K8a having made

this sequence alignment since the pseudogene may carry frame-shift

mutations. K8b, however, runs into known cDNA sequence at residue 13

and so can be safely predicted to be 19 aminoacids in length. At each

of the positions where only one derivative was detected, the DNA

sequences predict a glycine residue for one or other peptide. At

position 12 in K8b a phenylalanine residue was detected whereas the

pseudogene sequence predicts a leucine residue. Codons for leucine can be generated from phenylalanine codons by a single base pair substitution in either the first or third position. The K8b peptide sequence determined here would therefore predict that the p53 pseudogene has suffered a missense point mutation in this codon.

## Conclusions

The results of this work can be summarised as follows. p53 has been purified from mouse SV40-transformed 3T3 fibroblasts in nanomole quantities. The identity of the purified protein was verified by rebinding to anti-p53 antibodies. Using this source of purified p53, its aminoacid composition was determined. Since the protein did not prove amenable to N-terminal sequence analysis, presumably due to some blocking modification, tryptic peptides were prepared and purified by high pressure liquid chromatography. Three peptides were subjected to sequence analysis and two of them produced interpretable sequence data. The third peptide gave ambiguous aminoacid assignments at several positions, suggesting it was a peptide mixture. Subsequently, the two unambiguous peptide sequences were located in the predicted translation products of putative p53 cDNA clones isolated and sequenced by others. Similarly the sequences of two components of the peptide mixture were separated by comparison with the predicted translation products of p53 genomic DNA clones, also isolated by others in the laboratory.

Chapter 5


DISCUSSION AND FUTURE PROSPECTS


Many studies of transformed cells and tumours have tentatively

linked expression of the transformed phenotype with some alteration

in the expression, metabolism or function of p53 whilst there are

limited data from studies of normal cells which suggest that p53 is

essential for the movement of quiescent cells out of the resting

state ($G_o$) back into the cell cycle. It is not difficult to accept

that the aberrant expression of such a protein might indeed contribute

to the expression of a transformed phenotype, given that uncontrolled

division is a principle feature of transformed cells, but so far a

precise function for p53 has not been defined, either in the control

of cell division or in any other cellular process. However, compelling

indirect evidence of the importance of p53 has been sufficient to

sustain continuing efforts to describe the parameters of p53 expression

in different cell types with greater precision in the hope of defining

any features of p53 expression that are absolutely linked with

transformation.

Studies of p53 at present depend on immunological reagents, in

particular monoclonal antibodies, for detection of the protein.

However, p53 might be expected to carry many antigenic determinants

and p53 isolated from different cell types and species might have

sets of determinants that, whilst mostly common to all p53 proteins,

contain certain subset-specific or species-specific members. If the

properties of p53 are to be inferred from experiments that employ

monoclonal antibodies for p53 detection then it is important to have

as wide an understanding as possible of the immunological complexity

of the protein so that any reservations that the detection method

may impose on the data can be properly assessed.

The four anti-p53 monoclonal antibodies which I have isolated
and characterised add considerably to the range of immunological
probes for p53, not only because they increase the total number of
antibodies available but also because their properties differ in a
number of ways from those of the earlier anti-p53 antibodies. The
determinants recognised by antibodies of the PAb1100-series are
different from those previously defined since their species
distributions are variously primate$^+$, human$^+$ and primate$^+$>mouse$^+$.
These specificities are approximately complementary to those of the
rodent$^+$ and rodent$^+$, primate$^+$ antibodies already described. The
isolation of anti-p53 antibodies having these various species-
specificities is in line with expectations based on the comparative
tryptic peptide analysis of p53 from different species described by
Simmons (1980). That the determinants recognised by the PAb1100-
series antibodies are novel is confirmed by the lack of
competition between them and existing anti-p53 antibodies for
binding to human p53 in a radioimmune blocking assay. Furthermore,
within the PAb1100-series at least two and probably four new
determinants on p53 were defined in the same assay. There is
uncertainty in this figure because the blocking assay necessarily
gives only a minimum estimate of the number of different determinants
recognised. Several antibody pairings showed incomplete or partial
blocking of the binding of one antibody by the other. This was
probably a consequence of considerable steric hindrance occurring
between competing IgM molecules, even when their target determinants
on p53 were well separated. The isolation of the PAb1100-series
antibodies which recognise p53 determinants having a novel species
distribution and occurring in previously uncharacterised antigenic
regions of p53 constitutes a real expansion of the available probes

for human p53.

Whilst the interactions of the PAb1100-series antibodies with human p53 were being examined in detail, it was found that PAb1104 had intrinsic activity not only towards human p53 but also to SV40 large T-antigen, a property not observed in any other anti-p53 antibody so far isolated. This observation was particularly surprising since the possibility that p53 and large T-antigen might share antigenic determinants had been examined and discounted in the course of proving that these two proteins associate in vivo. Clearly the polyclonal antisera and anti-polypeptide sera with anti-p53 and anti-T activities then available did not have detectable activity towards the PAb1104-reactive determinants on either of these antigens implying that this determinant does not contribute significantly to the total antigenicity of either protein.

The significance of the observed dual reactivity of PAb1104 towards p53 and SV40 large T-antigen is still open to question as it is impossible to measure the extent of the structural similarity between these proteins implied by this result. Since no other anti-p53 or anti-T antibodies show such dual-reactivity, this similarity is presumably not extensive. Nevertheless, there are no previous examples of an antibody being capable of reacting with two antigens and yet recognising a determinant which is not only sensitive to disruption of protein tertiary structure but which also depends for its expression in one case on the formation of a polypeptide oligomer. The presence in proteins of regions having similar tertiary structures has in the past been found to correlate with the existence of a common function amongst the proteins involved, for example, ATP or cofactor binding. On this basis one might therefore expect the similarity between large T-antigen and p53 to be functionally significant and one speculation as to how similar

surface structures on p53 and oligomeric T-antigen might be important

is that they could represent the sites of large T/p53 association.

Given this possibility, it will certainly be of interest in the

future to determine which segments of these two polypeptides contribute

to their respective PAb1104 binding sites and to compare the locations

of these segments with the locations of elements of large T-antigen

and p53 required for the association of these polypeptides.

When the anti-p53 activities of PAb1101, PAb1102 and PAb1103

were characterised, it was found that, unlike previously characterised

anti-p53 antibodies, they precipitated large T-associated human p53

much more efficiently than free p53. Three possible explanations

for this observation have been considered. Firstly, the conformation

of p53 in its large T-associated form is almost certain to differ

from that occupied by uncomplexed p53. It is possible that antibodies

PAb1101, PAb1102 and PAb1103 recognise the T-associated p53 conform-

ation more readily than the free p53 conformation although in this

event it would be difficult to explain why these antibodies arose

out of immunisation with purified p53. Secondly, it is possible

that an effect of large T-association is to stabilise p53 both in

the cell and during extraction. The half-life of p53 in SV40-

transformed mouse 3T3 cells is known to be extended to more than

22 hr compared with only 20-30 min in 3T3 cells (Oren et al., 1981)

and Benchimol et al.(1982) have shown that an SV40-transformed 3T3

line, SVA31E7, has at least 100-fold more p53 protein per cell than

does the untransformed parent. If large T-antigen stabilises p53

by associating with it, it would be reasonable to expect this effect

to be reflected at an immunological level in the stabilisation of a

set of determinants on p53 which would therefore be detected more

readily on the large T/p53 complex than on free p53. PAb1101,

PAb1102 and PAb1103 may recognise determinants of this type. Thirdly,

the T-antigen polypeptide may actually contribute to the determinants

recognised by these antibodies. In this case, the ability of these

antibodies to interact with p53 in non-SV40-transformed cells must

be attributed to the existence of a cellular protein capable of

interacting with p53 and creating a structure that is in this respect

equivalent to the T/p53 complex. As previously discussed, there is

some theoretical justification for expecting to find in cells a

"T-equivalent" protein able to bind to p53 (Lane and Hoeffler, 1980)

but there has not yet been any convincing experimental demonstration

of the existence of such a protein.

If one of these explanations of the preference for binding

to T/p53 complex displayed by PAb1101, PAb1102 and PAb1103 is correct

then the suggestion that the phenomenon reflects the stabilisation

of p53 by its association with T-antigen is probably the most

plausible. Of the three hypotheses, this one can most readily

accommodate the fact that three different antibodies have been

isolated which display a preference for binding T-associated p53

but to different degrees. Also, it is implicit in this hypothesis

that each of the three antibodies recognises a determinant which is

destroyed by denaturation and on the basis of experiments similar

to that shown for PAb1104 in Figure 3.12 this appears to be the case.

Nevertheless, the precise basis of this aspect of PAb1101, PAb1102

and PAb1103 anti-p53 activity remains to be determined.

The PAb1100-series antibodies have revealed levels of

immunological complexity in p53 and its complex with SV40 large T-

antigen which could not previously be detected. None of the four

antibodies characterised appear to recognise what might be regarded

as typical p53 antigenic determinants and the scope for using any

of these antibodies as probes for comparative analysis of p53

expression in different cells is probably therefore limited.

Nevertheless, the isolation of these antibodies has raised an

important question about the nature of the effect on p53 of its

association with large T-antigen and the unusual pattern of reactivity

of PAb1104 may be a clue as to the molecular interactions involved

in large T/p53 association. These questions will no doubt be

addressed further in the future.

It is certainly possible that the only intrinsic difference

between normal and transformed cell p53 is at a detailed structural

level. Some cellular "oncogene" products have been shown to have

primary structures that are very slightly different from those of

their normal cell homologues and it is these differences which appear

to confer on the oncogenes their transforming functions. (Reddy et al., 1982; Tabin et al., 1982) Whilst

there is as yet no suggestion that the p53 gene can have oncogene

properties, it is clear that the possibility that systematic

structural differences may exist between normal and transformed

cell p53 will have to be tested before our understanding of p53

function can be complete. This would require the definition and

comparison of the structures of p53 from a number of related cell

lines by cDNA or protein sequence analysis and perhaps also the

mapping of the post-translational modifications of these proteins.

The purification of p53 and the subsequent determination of

aminoacid sequences for peptides derived from this material represents

an important advance as no aminoacid sequence information has

previously been derived from p53. Whilst the directly-derived

protein sequence data I obtained were limited to two p53 tryptic

peptides in which a total of 24 aminoacid residues were identified,

this analysis could obviously be extended, using proteases other

than trypsin to produce overlapping peptides, to generate a virtually

complete sequence of p53 from perhaps 10 nanomoles of material.

However, it is unlikely that many glycine residues could be identified

from these small amounts of material on the basis of my experience

in generating the results described in Chapter 4 and the N-terminal

peptide would be expected to retain the blocked N-terminus of the

intact protein and so to be difficult to sequence. Nevertheless, the

method applied here to p53 could clearly be used generally to obtain

segments of aminoacid sequence for any protein against which an

S. aureus protein-A binding monoclonal antibody is available.

Despite technical advances it remains a lengthy task to obtain

a complete protein sequence by direct analysis and it is usually

easier to obtain a predicted polypeptide sequence from the sequence

of cloned complementary DNA (cDNA). However, the availability of at

least some directly obtained sequence from a protein of interest can

be critical to the isolation of cDNA clones derived from specific

mRNA. The mRNAs for many proteins form only a very small proportion

of the total polyA$^+$ mRNA in a cell and it is consequently very

difficult to isolate cDNA clones derived from these messages. In the

absence of any other assisting feature, the use of a segment of

aminoacid sequence determined from the protein to predict the DNA

sequences able to specify it and subsequently synthesising this

oligonucleotide mixture for use either as a specific primer for cDNA

synthesis or as a specific probe with which to screen libraries of

cDNA clones can be the only way to clone DNA complementary to a rare

mRNA. Whilst it appeared at one time that cloning p53 cDNA might

require this type of approach, the isolation of putative p53 clones

was ultimately achieved by the use of mouse mRNA derived from

polysomes immunoselected with anti-p53 antibody and thus highly

enriched for p53 sequences either as a template for cDNA synthesis

or as a probe for the detection of p53 clones (Oren and Levine, 1983;

Benchimol et al., in preparation). The aminoacid sequences of p53

peptides K2 and K9 could be aligned with the predicted translation

products of the clones isolated by Benchimol et al. to provide

direct proof that their cloned cDNA did indeed derive from p53 mRNA.

This validation was important because, although the cDNA could hybrid-

select p53 mRNA, it was possible that the implied homology with p53

mRNA extended over only part of its length and that the cDNA was

perhaps derived from mRNA for a p53-related protein rather than mRNA

capable of encoding the protein defined immunologically as p53.

Obtaining partial aminoacid sequence data of the type I have

described for p53 also allows the preparation of synthetic peptides

corresponding to the determined sequence which can then be used as

immunogens, ultimately producing antisera with activity towards

defined segments of the intact protein from which the sequence data

derived. Such antisera function much like monoclonal antibodies and

sets of antipeptide antisera have been used to perform a detailed

immunochemical analysis of the influenza virus haemagglutinin

protein (Green et al., 1982). These antisera have the major advantage

over monoclonal antibodies that their target determinants are always

well defined. As a result, it is possible to use them to circumvent

a major problem in the application of immunoaffinity purification of

proteins, namely the elution of purified protein from the adsorbing

antibody without causing its denaturation. For example, polyoma

virus middle T-antigen was purified by Walter et al.(1982) by

competing-out the antigen from a loaded antipeptide immunoadsorbant

column with an excess of free peptide corresponding to the C-terminus

of the intact antigen. Having obtained some protein sequence data

for p53, an attempt to apply this purification technique to this

protein is a logical step forward, since only by studying purified

native-conformation p53 is there any real hope of defining its precise

biochemical function.

There is little doubt that in the near future a combination of

direct protein sequence analysis and cDNA sequence analysis will reveal the complete primary structure of BALB/c mouse p53 from SV40-transformed cells, since this protein is under intensive study in several laboratories. It will then be possible to compare p53 with the many proteins whose sequences and structures have already been determined with a view to identifying any local similarities that exist between them. Using this approach together with computer prediction it will be possible to start to define the secondary and tertiary structure of p53 and to identify in it regions which are potentially antigenic. By the use of synthetic peptides and specific anti-peptide sera, probably in combination with deletion mutant analysis, the antigenic sites recognised by anti-p53 monoclonal antibodies may then be located on the protein. This will associate the individual features of each antibody with particular parts of p53 and will allow any functional sites on p53 that are identified in the future by antibody blocking studies to be similarly located. A rigorous molecular analysis of p53 will thus provide the framework within which an understanding of p53 function can emerge.

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# Cellular Proteins Reactive with Monoclonal Antibodies Directed Against Simian Virus 40 T-Antigen

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Several recently isolated monoclonal antibodies which reacted with simian virus 40 T antigens also reacted with proteins found in uninfected and untransformed cells. The proteins were different from each other, PAb419 reacting with a 35,000-molecular-weight protein, PAb427 reacting with a 75,000-molecular-weight phosphoprotein, PAb405 reacting with a 150,000-molecular-weight phosphoprotein, and PAb204 reacting with a 68,000-molecular-weight protein. It is suggested that although some of these cross-reactions may be fortuitous, they may, as an alternative, reflect similarities of shape and perhaps function between domains of the viral T antigen and the relevant host proteins.

Monoclonal antibodies are specific probes for protein epitopes and have been used to define the immunochemical anatomy of proteins such as the influenza hemagglutinin antigen (5, 22). Previous studies defining the antigenic determinants of proteins such as myoglobin and lysozyme (1, 2) used polyclonal sera in which immunoglobulins specific for a single epitope were from a heterogeneous, multiclonal family of antibodies. With the development of monoclonal antibodies (17), the problems of studying the structure of antigenic determinants with polyclonal sera were overcome, but concomitant with this advance came other problems caused by the increased sensitivity of monoclonal antibodies. Several authors have noted that, although polyclonal sera may specifically bind only with the immunogen, monoclonal antibodies to the same antigen sometimes cross-react with proteins not present in the immunizing dose (8, 24; see reference 21 for review). Cross-reaction demands that the two proteins share a limited structural homology. This homology may be fortuitous, but a certain fraction of these cross-reactions may detect proteins that are related by function or evolution.

A number of monoclonal antibodies directed against the simian virus 40 (SV40) tumor antigens have been isolated, and several cross-reactions between SV40 large-T antigen and host proteins have been observed (14, 20). The first such observation was by Lane and Hoeffler, who found that monoclonal antibody PAb204 also bound a host 68,000-molecular-weight (68K) protein (20). It was hypothesized that this 68K protein might share some functional property with the viral T-antigen. Although such a

cross-reaction had not been observed in earlier studies with anti-T monoclonal antibodies (12, 23), there was some basis for expecting cross-reactivity of anti-T monoclonal antibodies to a postulated cellular T-equivalent structure (19).

A set of monoclonal antibodies against the SV40 large-T antigen and its associated 53K protein, p53, have recently been isolated, and the determinants which they recognize have been localized (14). In the course of comparative studies on these antibodies, it was found that some of them specifically precipitated host proteins in addition to the T antigens and p53. None of these proteins appeared to be related to the 68K host protein. We have examined some of the properties of these proteins and comment on the possibility that one or more of them may represent T-equivalent molecules in uninfected cells.

## MATERIALS AND METHODS

**Cell lines and virus strains.** The following cell lines were used: CV-1 monkey kidney cells; 3T6, BALB/c 3T3 A31, and Swiss 3T3K mouse fibroblast cells; SVA31E7 SV40-transformed BALB/c 3T3 A31 cells, isolated by Y. Ito and obtained from him; PTK1 and PTK2 lines of marsupial cells from *Potorous tridactylis* (29), obtained from E. B. Lane; C33-I human cervical tumor cell line, isolated by N. Auersperg (3) and obtained from her; Hs578T human mammary carcinosarcoma cell line (13), obtained from J. Taylor-Papadimitriou; Namalwa human lymphoblastoid cells, obtained from N. Finter, Wellcome Laboratories; and SV80 line of SV40-transformed human fibroblasts, obtained from D. Livingston, Harvard Medical School. Cells were grown in Dulbecco modified Eagle medium (E4) with 10% calf serum (CV-1, BALB/c 3T3 A31, and Swiss 3T3K), 10% fetal calf serum (PTK1,

PTK2, Namalwa, and SV80), or 5% fetal calf serum (3T6 and SVA31E7). Human tumor cell lines were grown in RPMI 1640 plus 10% fetal calf serum (Hs578T) or Waymouth medium plus 10% fetal calf serum. The SV-S strain of SV40 (25) was used for infection of CV-1 cells.

**Monoclonal antibodies.** The details of the isolation of hybridoma cells and characterization of the monoclonal antibodies they produce have already been published (14, 20). The nomenclature used has been altered to conform to a recently agreed convention. The correspondence with previously used names is as follows: PAb204 = 3C4; PAb405 = L5; PAb407 = L7; PAb409 = L9; PAb416 = L16; PAb419 = L19; PAb421 = L21; and PAb427 = L27.

**Cell labeling and preparation of extracts.** Confluent cell monolayers were labeled with 0.5 mCi of either [$^{35}$S]methionine or $^{32}$P$_i$ (Amersham) per 50-mm dish in 1.0 ml of E4 medium supplemented with 2% dialyzed calf serum lacking methionine and phosphate, respectively, for 3 h at 37°C.

Extracts (low salt) were prepared by washing the monolayer in Tris-saline at 0°C and lysing for 30 min with 0.5 ml of ice-cold 1% Nonidet P-40 (NP-40), 150 mM NaCl, and 50 mM Tris, pH 8. Dishes were then scraped dry, and the lysate was cleared by centrifugation (microfuge for 1 min). Where indicated, high-salt extracts were prepared from the insoluble residue of normal extraction by suspending the pellet in 0.5 ml of 1% NP-40, 650 mM NaCl, and 50 mM Tris, pH 8, on ice as before.

**Immunoprecipitation and gel electrophoresis.** Lysates prepared as above were either immunoprecipitated directly or pre-precipitated with 20 μl of normal mouse serum per 0.5 ml of extract for 1 h at 20°C. The lysate was then cleared of immunoglobulin G (IgG) by the addition of 0.2 ml of a 10% suspension of Formalin-treated *Staphylococcus aureus* Cowan 1 (16) for 15 min on ice, which was then pelleted by centrifugation.

Portions (0.1 ml) of lysate or precleared lysate were routinely immunoprecipitated with either 1.5 μg of purified antibody or 20 μl of hybridoma tissue culture supernatant, in 400 μl of NET/BSA buffer (150 mM NaCl, 5 mM EDTA, 50 mM Tris, 0.25% gelatin, 0.02% NaN$_3$, and 2 mg of bovine serum albumin [BSA] per ml, pH 7.4), for 1 to 2 h at 20°C. Immune complexes were collected on 20 μl of *S. aureus* Cowan 1 as above, washed three times in NET/gel buffer (NET/BSA lacking BSA), and then eluted into 25 μl of sample buffer (2% sodium dodecyl sulfate [SDS], 100 mM dithiothreitol, 10% glycerol, and 50 mM Tris, pH 6.8) by heating to 70°C for 10 min. *S. aureus* Cowan 1 cells were removed by centrifugation, and samples were loaded onto either 10% or 5 to 15% gradient SDS-polyacrylamide gels. Electrophoresis was for 4 to 5 h at 100 to 200 V. Gels were fixed in 7% acetic acid–45% methanol for at least 30 min before drying and exposing to Kodak SB-5 film.

Purified D2 protein from Ad2$^+$D2-infected HeLa cells (27) and bacterial clones HP1 and pGL101 (26) were the kind gift of R. Myers, C. Thummel, and R. Tjian.

## RESULTS

**Monoclonal antibody PAb419.** Since the Pab419 antibody recognizes an antigenic deter-

minant which is common to the SV40 large-T and small-t antigens (14), its binding site must fall within the N-terminal 82 amino acids of the two polypeptides. In addition to immunoprecipitating these SV40-coded early antigens, PAb419 also immunoprecipitated a 35K protein (p35) from SV40-infected CV-1 monkey cells, SV40-transformed mouse cells, and uninfected human tumor cells (Fig. 1). The presence of p35 in uninfected cells demonstrated that this protein was host coded. The p35 was widely distributed, being found in all the mammalian cells tested and also in two marsupial cell lines. As well as being related by their ability to bind to PAb419, several of the p35 proteins from different cells were shown to be closely related by partial protease digestion (7) (Fig. 2). The p35 was apparently not phosphorylated, since it could be metabolically labeled with [$^{35}$S]methionine but
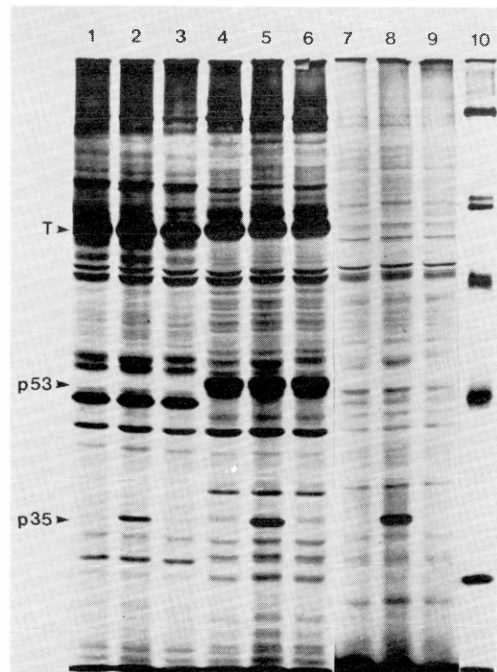


FIG. 1. Detection of p35 in monkey, mouse, and human cells. [$^{35}$S]methionine-labeled extracts of SV40-infected CV-1 cells (tracks 1, 2, and 3), SVA31E7 cells (tracks 4, 5, and 6) or C33-I cells (tracks 7, 8, and 9) were immunoprecipitated as described with each of three anti-SV40 T-antigen monoclonal antibodies: PAb416 (lanes 1, 4, and 7); PAb419 (lanes 2, 5, and 8); and PAb405 (lanes 3, 6, and 9). Samples were analyzed by electrophoresis on a 10% SDS-polyacrylamide gel. Track 10 shows labeled proteins of known molecular weight as markers. From top to bottom these were 200,000, 92,500 (doublet), 69,000, 46,000, and 30,000.
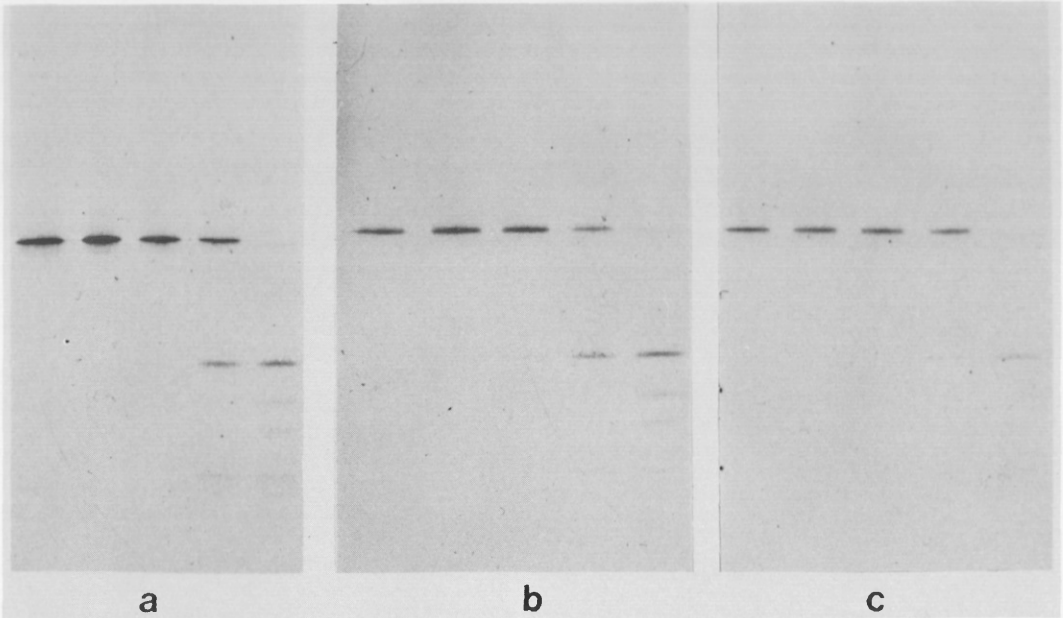
FIG. 2. Comparison by partial proteolysis (7) of p35 from three cell types: (a) SVA31E7 (mouse); (b) C33-1 (human); (c) and Hs578T (human). [$^{35}$S]methionine-labeled extract (300 μl) was immunoprecipitated with 4.5 μg of PAb419 as described, and the precipitated proteins were separated on a 10% SDS-polyacrylamide gel. The gel was dried without fixing under low heat and exposed to autoradiography overnight. The film was used as a guide to cut p35 bands from the gel. These were swollen overnight at 0°C in digestion buffer (1% SDS, 2% sucrose, 0.1% β-mercaptoethanol, and 10 mM sodium bicarbonate) and then minced finely in a ground-glass homogenizer. The resulting slurry was divided in each case into five equal portions S. aureus V8 protease was added as indicated below to each one, and all were then left at 37°C for 1 h. The digested samples were mixed with an equal volume of 2× sample buffer, heated to 70°C for 2 min, and then loaded onto a 7 to 20% gradient SDS-polyacrylamide gel to separate the digestion products. Enzyme additions were, in each case from left to right, 0, 0.005, 0.05, 0.5, and 5 μg.

not with $^{32}$P$_i$. Comparison of p35 and small-t by tryptic peptide analysis showed no common [$^{35}$S]methionine-containing peptides in digests of the two proteins (data not shown). The homology between the virus-coded protein and the host-coded protein is therefore not likely to be extensive.

To ensure that p35 and large-T/small-t antigens were not immunoprecipitated by separate antibody activities present in the PAb419 preparation, small-t antigen from an expressing bacterial clone, HPl (26), was used to inhibit the immunoprecipitation of p35 by PAb419 (Fig. 3). Previous work had shown that PAb419 bound specifically to the small-t antigen produced by HPl (F. McCormick and E. Harlow, unpublished data). Increasing amounts of the small-t antigen-containing bacterial extract (HPl) showed progressively increasing blocking of the activity of PAb419 against p35. The control extracts from the bacterial clone (pGL101) not expressing small-t antigen showed no effect on the precipitation of p35 by PAb419. In a further check on the specificity of this effect, incubation

of PAb421, a monoclonal antibody specific for the host p53, with HPl extract did not affect its ability to precipitate p53 (data not shown). The activity of HPl extract is therefore not a general one against other antibodies but is specific for PAb419. The inhibition of p35 precipitation by small-t demonstrated that these proteins shared similar antigenic determinants. Since the exact conformation of the antigenic determinants of p35 and the SV40-coded antigens was unknown, we compared their structure by a careful titration of antibody concentrations. Large-T required approximately 10-fold less antibody for 50% precipitation than p35. Both large-T and p35 sedimented rapidly on sucrose gradients (data not shown) and may therefore have been in multimeric aggregates. The difference in antibody levels required for precipitation is therefore consistent with PAb419 having a higher affinity for large-T than for p35, but other explanations are possible.

**Monoclonal antibody PAb427.** This antibody recognizes a determinant in the C-terminal one-quarter of SV40 large-T antigen (14). It also
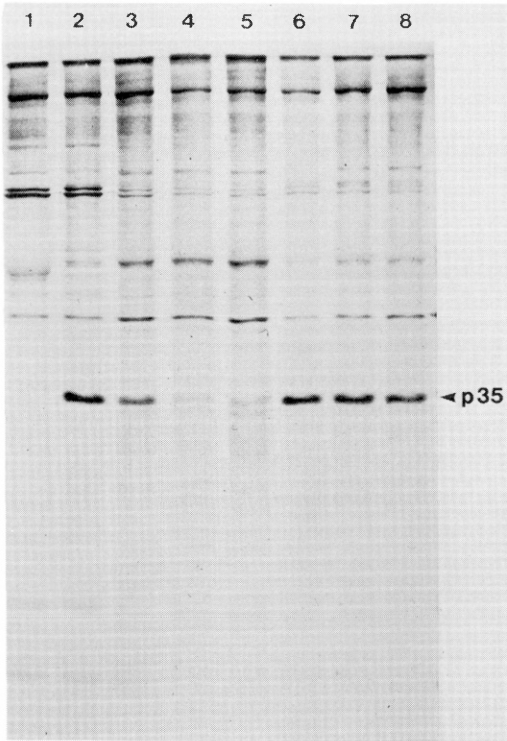
FIG. 3. Precipitation of p35 by PAb419 is blocked by SV40 small-t antigen. Bacterial clone HP1 (26) was used as a source of SV40 small-t that did not contain p35. Sonicated extracts of the small-t-producing clone HP1 and its parental, nonproducing clone pGL101 were cleared by centrifugation, pre-precipitated, and then used to test for blocking of p35 immunoprecipitation. Tracks 3–5: PAb419 incubated with increasing amounts of HP1 extract for 2 h before being used for immunoprecipitation of [$^{35}$S]methionine-labeled CV-1 cell extract. The HP1 extract was a pre-precipitated high-speed supernatant from $10^9$, $2 \times 10^9$, and $3 \times 10^9$ cells, respectively. This was calculated to be equivalent to 2, 4, and 6 μg of small-t. Tracks 6–8: Control incubation of PAb419 with corresponding amounts of pGL101 extract (from $10^9$, $2 \times 10^9$, and $3 \times 10^9$ cells) before immunoprecipitation. Tracks 1 and 2: Direct immunoprecipitation of labeled CV-1 extract with PAb416 and PAb419, respectively. Samples were run on a 15% SDS-polyacrylamide gel.

immunoprecipitated a 75K host protein (p75) from [$^{35}$S]methionine- or $^{32}$P$_i$-labeled mouse 3T3 or 3T6 cells when extracts were made in buffer containing 1% NP-40 and 650 mM NaCl (Fig. 4a). The PAb405 and PAb419 did not precipitate p75. A small amount of p35 was present in the high-salt extract and was precipitated by PAb419 (track 1, Fig. 4a). The p75 was extracted less efficiently when cells were lysed in buffer containing 1% NP-40 and 150 mM NaCl. Sequential extraction of cells first with normal extraction buffer and then with high-salt buffer

showed that p75 was contained mainly in the 650 mM NaCl extract in contrast to the p35, which was mostly in the low-salt extract (Fig. 4b). Precipitation of p75 was inhibited by preincubation of the PAb427 antibody with D2 protein purified from cells infected with the defective adeno-SV40 hybrid virus Ad2$^+$D2 (15, 27). The D2 protein carries the determinant recognized by PAb427 (14), but lacks the determinant recognized by PAb419, since Ad2$^+$D2 does not contain the SV40 sequence which codes for the N-terminal region of large-T. The activity of PAb427 toward p75 was completely blocked by preincubation with the purified D2 protein (Fig. 4c). Incubation of PAb419 with D2 protein did not reduce the antibody's ability to precipitate p35, demonstrating the specificity of the blocking reaction. The amounts of PAb427 required for 50% precipitation of p75 and SV40 large-T were similar, indicating a rather close similarity of the determinants on the two proteins.

**Monoclonal antibody PAb405.** PAb405 recognizes a determinant in the C-terminal one-quarter of SV40 large-T antigen (14). The reaction of PAb405 with a host protein was first observed in the marsupial cell lines PTK1 and PTK2 after labeling with $^{32}$P$_i$ (Fig. 5). The antibody precipitated a phosphoprotein of about 150,000 daltons (p150). A similar protein was also precipitated from extracts of a human tumor cell line, C33-I. It was not clear whether a corresponding protein existed in other mammalian cells. CV-1 monkey cells showed positive nuclear immunofluorescence with PAb405, although we were unable to detect p150 in extracts of the same cells. The same type of blocking experiment already described for PAb427 was used to show that the anti-p150 activity and the anti-T activities of PAb405 were carried by the same antibody molecules. Preexposure of PAb405 to purified D2 protein blocked its anti-p150 activity. Similar experiments with another antibody, PAb407, gave results identical to those obtained with PAb405, indicating that it recognized a similar determinant on large-T (Fig. 5).

**Monoclonal antibody PAb204.** As described earlier, PAb204 recognizes a determinant on the C-terminal half of SV40 large-T (20). It stains the nuclei of a wide variety of different mammalian cell types that do not contain SV40 DNA, and the intensity of the staining reaction is strongly dependent on the growth state of the cell in that only actively growing cultures give a strong positive reaction. The host protein recognized by PAb204 cannot be readily detected by immunoprecipitation, but using protein blots from SDS-gels, the antibody was found to react strongly with a 68K protein (Fig. 6). This protein was highly conserved in molecular weight and was not solubilized from nuclei even by 1 M salt.
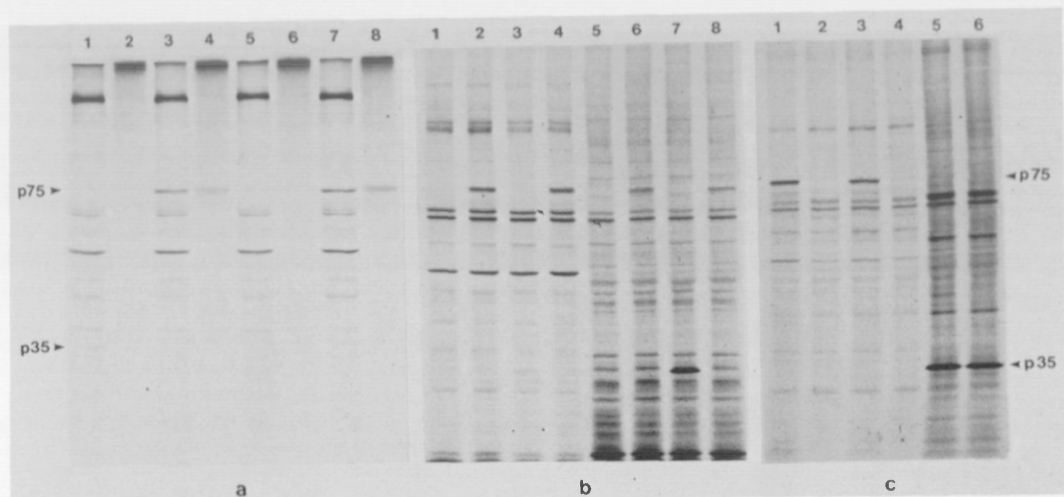
FIG. 4. (a) Host protein p75 is a phosphoprotein. High-salt extracts from [$^{35}$S]methionine (1, 3, 5, and 7)- and $^{32}$P$_i$ (2, 4, 6, and 8)-labeled 3T6 cells were immunoprecipitated with PAb419 (1 and 2), PAb427 (3, 4, 7, and 8), or PAb405 (5 and 6) followed by 0.5 μl of rabbit anti-mouse IgG serum after 1 h of incubation and then left for a further 30 min to equalize collection of immune complexes from the different reactions. Samples were separated on a 7 to 20% SDS-polyacrylamide gel. (b) Extraction of p75 is dependent on salt concentration. [$^{35}$S]methionine-labeled 3T6 cells were first extracted with low salt, and this extract was used in tracks 5 to 8. The pellet was then reextracted with high salt and used for tracks 1 to 4. The extracts were immunoprecipitated with PAb405 (1 and 5), PAb427 (2, 4, 6, and 8), or PAb419 (3 and 7), with 0.5 μl of rabbit anti-mouse IgG serum added as above. Samples were run on a 5 to 15% SDS-polyacrylamide gel. (c) Immunoprecipitation of p75 by PAb427 is blocked by purified D2 protein, whereas the precipitation of p35 is not. [$^{35}$S]methionine-labeled 3T3K high-salt extract immunoprecipitated with PAb427, 1.0 μg (1 and 2) or 0.4 μg (3 and 4), or low-salt extract immunoprecipitated with PAb419, 0.4 μg (5 and 6), after preincubation of the antibody with 1.0 μg of purified D2 protein (2, 4, and 6) or without added D2 protein (1, 3, and 5). Samples were run on 5 to 15% gradient SDS-polyacrylamide gel.

Preliminary experiments indicate that it is tightly bound to DNA and that it rapidly disappears in growth arrested cells (D. Lane, manuscript in preparation).

**Discrimination between determinants.** Figure 7a shows the locations of the sequences in SV40 large-T which contain the determinants for PAb405, PAb419, PAb427, and PAb204. This clearly distinguishes PAb419 and PAb204 from each other and from the other antibodies. PAb405, PAb407, and PAb427 all reacted with determinants in the C-terminal one-quarter of SV40 large-T antigen, since they all precipitated the 28K protein of Ad2$^+$ND1. To give a better discrimination between all of the relevant determinants, we compared the abilities of the antibodies to compete with each other for binding to SV40 large-T antigen. A monoclonal antibody was first bound to the wells of a PVC microtiter tray. This antibody (PAb416) was chosen in preliminary experiments as being one which did not compete with any of the set of antibodies to be tested. This fixed antibody was then used to bind T-antigen from a transformed cell extract. The test antibodies, either labeled with $^{125}$I or unlabeled, were then mixed and allowed to compete for the limited amount of antigen avail-

able (Fig. 7b). This type of test showed that the bindings of PAb419, PAb427, PAb405, and PAb204 were independent of each other (Fig. 7c). Homologous competition inhibited 80 to 95% of binding. The two related antibodies, PAb405 and PAb407, inhibited each other equally well in homologous and heterologous reactions. This is consistent with the observation that they both reacted with p150, and they probably recognized the same determinant on large-T.

## DISCUSSION

The host proteins immunoprecipitated by anti-T monoclonal antibodies show a wide variety of monomer molecular weight, ranging from 35,000 to 150,000, as determined by mobility on SDS-polyacrylamide gels. They do not seem to have any readily identifiable common features. Two of them are phosphoproteins, p75 and p150, whereas p35 is not phosphorylated. Under the conditions used for extraction of T antigen from infected or transformed cells, only p35 and p150 are efficiently extracted. More rigorous conditions of extraction are required for p75, for 68K, and perhaps for the material in monkey cells which gives nuclear immunofluorescence with
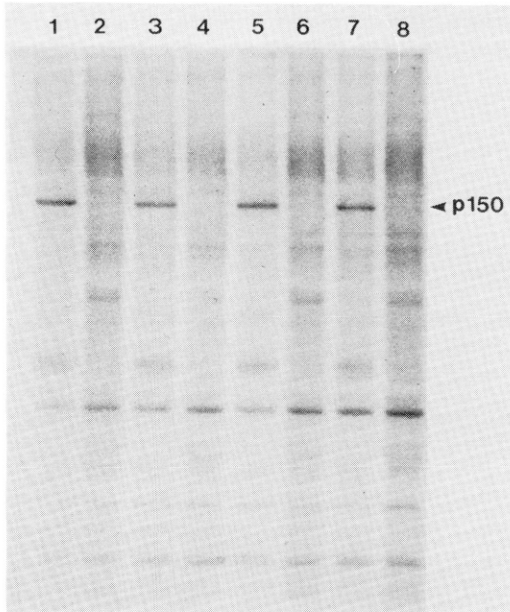
FIG. 5. Immunoprecipitation of p150 by PAb405 and PAb407 is blocked by purified D2 protein. $^{32}P_i$-labeled PTK1 cell extract was immunoprecipitated with either PAb405, 0.8 μg (1 and 2) or 0.2 μg (3 and 4), or PAb407, 0.4 μg (5 and 6) or 0.2 μg (7 and 8), after preincubation of the antibody for 1 h with 1.0 μg of purified D2 protein (2, 4, 6, and 8) or after preincubation without added D2 protein (1, 3, 5, and 7). Samples were run on a 5 to 15% gradient SDS-polyacrylamide gel.

PAb405. The difficulty of extracting the 68K protein and p75 may indicate that these proteins are tightly bound to the chromatin or nuclear matrix (4) in the same way as a fraction of polyoma virus large-T (6). It should be emphasized that we have not detected any sign of association of the cross-reactive proteins p35, p75, and p150 with SV40 T antigen or host p53. These proteins are directly precipitated by the various antibodies from extracts of uninfected and untransformed cells as well as from infected and transformed cells. The situation is therefore different from the precipitation of p53 by anti-T monoclonal antibodies, which is due entirely to the physical association of p53 with SV40 large-T.

The initial observation of a cross-reaction between a host protein and SV40 T-antigen (20) was striking in that it provided experimental support for the idea of a cellular T-equivalent structure. The existence of such a structure had been proposed to account for the retention by mammalian cells of a site on the cellular 53K protein, p53, which allows it to form a highly specific complex with the virus coded large-T

antigen (18, 19). If other monoclonal antibodies had been found which reacted with other determinants on the 68K protein, this would have indicated that the similarity between the 68K and SV40 large-T was extensive. However, the monoclonal antibodies from the 400 series (14) which showed cross-reaction with host proteins were found to react with proteins which were different from each other and from the 68K protein. The frequency with which cross-reactive monoclonal antibodies were found has led us to reexamine the importance of shared determinants on SV40 large-T and host proteins. Three striking facts are evident from the studies presented here. First, the antibodies concerned react with different determinants located in different regions of the T-antigen polypeptide. Second, we do not have any examples of antibodies which recognize different determinants on T-antigen but nevertheless react with the same
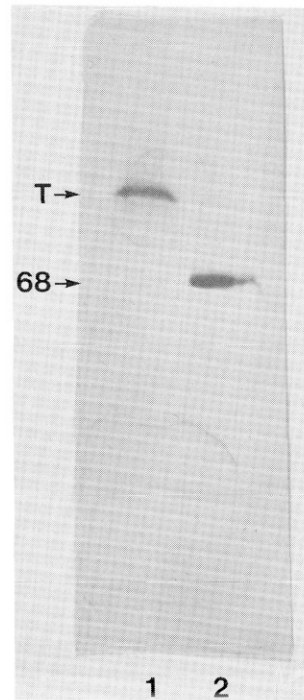


FIG. 6. PAb204 recognizes a 68K host protein. Proteins released from SV80 cells by a low-salt NP-40 extraction (1) and from $10^8$ Namalwa cells by direct incubation in SDS sample buffer (2) were separated on a 15% gel. The separated proteins were transferred to a nitrocellulose sheet by the method of Towbin et al. (28). After incubation of the sheet in PAb204 tissue culture supernatant for 1 h, the location of the bound antibody was determined by a further incubation for 1 h in a 1:100 dilution of peroxidase-conjugated rabbit anti-mouse IgG (Miles Laboratories), followed by a further 10-min incubation in a freshly prepared o-dianisidine substrate solution.

**a**

**b**

No competition        Competition

**c**
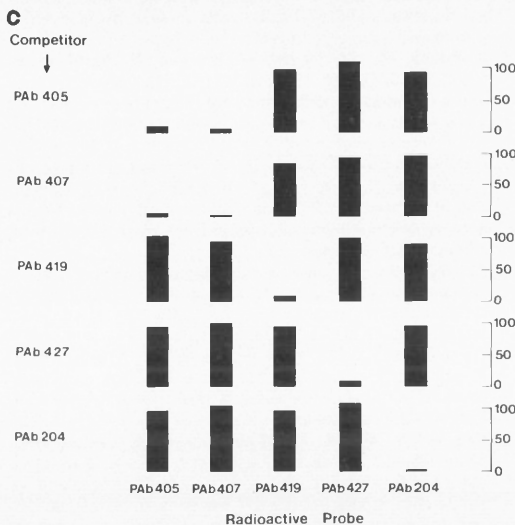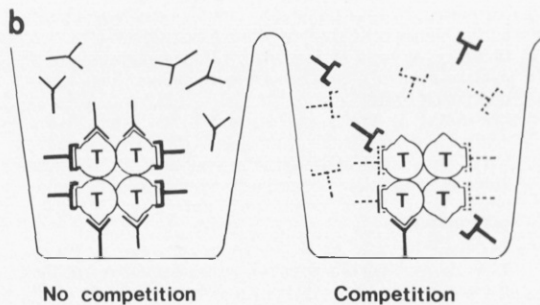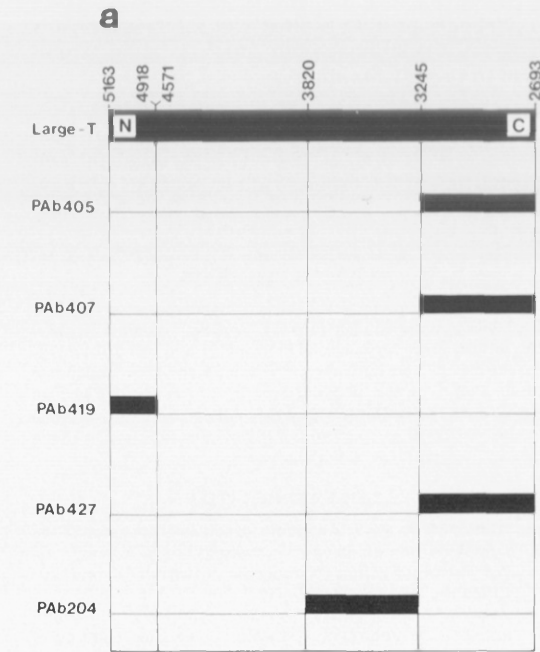
host protein. Third, all of the antibodies which cross-react with host proteins have in common the property of reacting with determinants which survive denaturation (or renature readily after denaturation). This may be an indication that these antibodies recognize the primary sequence of the polypeptide rather than the secondary or tertiary structure of the protein. If these monoclonal antibodies do recognize a primary amino acid sequence, we can calculate what might be the expected frequency of random cross-reaction. Taking the average size of an antigenic determinant to be 4 to 6 amino acids and assuming that all 20 amino acids are randomly distributed, we can calculate that a given sequence of 4 amino acids would appear once in every $1.6 \times 10^5$ tetramers ($20^4$), the same 5 amino acids once in $3.2 \times 10^6$ pentamers ($20^5$), and the same 6 amino acids once in $6.4 \times 10^7$ hexamers ($20^6$). If there are 5,000 proteins detectable by [$^{35}$S]methionine labeling, the average size of these proteins is about 500 amino acids, giving 493 tetramers, 491 pentamers, or 489

FIG. 7. (a) Antibodies were tested for the ability to immunoprecipitate T-antigen-related polypeptides from adeno-SV40 hybrid virus- and SV40 deletion mutant-infected CV-1 monkey cells. Only positive data were used in compiling the diagram, which indicates the maximal length of T-related polypeptide sequence required for immunoprecipitation by each antibody. Data taken from Harlow et al. (14). (b) Schematic representation of the simultaneous binding of two noncompeting antibodies (left) and of the blocking of binding of $^{125}$I-labeled antibody by competing cold antibody (right) onto immobilized T antigen on PVC microtiter wells (see text). The antibody molecules are represented as radioactive probe (⊣), homologous nonradioactive competitor (⊣), and heterologous antibody (⋏). (c) Results of competition assays between the antibodies showing host protein cross-reactivity. Wells were incubated overnight at room temperature with an independent monoclonal antibody having activity against SV40 large-T antigen (1 µg of PAb416 in 50 µl of 10 mM phosphate buffer, pH 7). Antigen was then added in the form of a normal extract of SVA31E7 cells (20 µl) and left for 3 h at room temperature. Wells were then treated with 20 µl of hybridoma tissue culture fluid with $^{125}$I-labeled antibody added (either of the same type as the unlabeled antibody or a second antibody) for 4 h. After each incubation, wells were washed five times in NET/gel buffer and shaken dry. The numbers of counts remaining bound after the final washes are shown as percentage of the counts bound when the appropriate labeled antibody was used mixed with tissue culture fluid containing antibody that did not bind to SV40 T antigen. The input of $^{125}$I-labeled antibody was $5 \times 10^4$ to $10 \times 10^4$ cpm and, in the absence of competition (100% value), $2 \times 10^4$ to $4 \times 10^4$ cpm was bound. The ratio of labeled to unlabeled antibody was between 1:50 and 1:100.

hexamers, and 10% of these sites are available for immunoglobulin binding, each cell would display about $2.5 \times 10^5$ tetramers, pentamers, or hexamers. On this basis, a monoclonal antibody which recognizes a site of four contiguous amino acids would on average find one such site per cell ($2.5 \times 10^5$ displayed sites per cell and $1.6 \times 10^5$ theoretical tetramers). For pentamers, this becomes $2.5 \times 10^5$ displayed sites and $3.2 \times 10^6$ theoretical pentamers, i.e., 1 antibody in 13 that recognize primary sequences will cross-react. For hexamers, this becomes $2.5 \times 10^5$ displayed sites and $6.4 \times 10^7$ theoretical hexamers, i.e., 1 antibody in 256 that recognize primary sequences will cross-react. Thus, the expected frequency is strongly dependent on the number of amino acids comprising an epitope. However, since the sizes of binding sites that have been defined range from four amino acids to six or seven amino acids (1), our assumption that four to six amino acids also comprise an epitope here is not unreasonable. Of the 14 antibodies which have been shown to recognize denaturation-stable determinants on large-T, 4 also react with host proteins. None of the antibodies which recognize denaturation-sensitive determinants was found to cross-react. This numerical argument may serve as a caution against expecting monoclonal antibodies to be totally specific for a single protein, but it does not rule out the possibility that some of the cross-reactions we observe arise in a more interesting way and are related to the function of the proteins. Large-T clearly has control functions and must interact in a very intimate way with cellular proteins in carrying out these functions. In these interactions, it seems reasonable to expect that similar structures will be present in the virus protein and the cellular protein(s) it displaces.

The idea that the several functions of large-T reside in different domains has been put forward to explain the results of injection experiments with fragments of SV40 DNA (9–11). If this is correct, similarities may exist between domains of large-T and host proteins. These similarities could have evolved either by incorporation of the corresponding host sequences into the virus DNA or by convergence of viral and host DNA sequences due to similarity of the functions they encode. Irrespective of the way in which such similarities may have arisen, isolation of monoclonal antibodies reacting with the host proteins would mean that they could be detected and isolated and that their possible functions could be examined. As yet little is known about p35, p75, p150, and the 68K protein, but the fact that antibodies to them are available will make them accessible. Detailed examination of each protein should allow us to decide if they do share functions with T-antigen.

The 68K protein is clearly of interest in that it is a cell cycle-dependent protein, being prominent in rapidly dividing cells and undetectable in stationary cells (20). The syntheses of p35, p75, and p150 appear to be less affected by cell density than that of p68. The metabolism and properties of the cross-reactive proteins are now being studied to see what function they may have in the cell.

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