IMMUNOLOGICAL ANALYSIS OF SV40 EARLY REGION PRODUCTS

Ximena Montano,

Cancer Research Campaign,

Eukaryotic Molecular Genetics Research Group,

Department of Biochemistry,

Imperial College of Science and Technology,

London SW7 2AZ.

Submitted for the degree of Ph.D. April 1985

ABSTRACT

An immunological analysis of the SV40 early region gene product, large T and small t was carried out in order to understand more about their role in cellular transformation.

Small t was purified, by immunoaffinity chromatography using a monoclonal antibody that recognizes the common N terminus of large T and small t, from bacterial cells containing plasmids encoding the protein. The isolated small t monoclonal antibody complex was used to immunize Balb/c mice. The spleens of these animals were used to prepare monoclonal antibodies specific to small t. Whilst most of the antibodies recognized the N terminus common region shared by large T and small t, one antibody denominated PAb280 exclusively recognized small t. The specificity of PAb280 for small t was analyzed by immunoprecipitation of labelled cell extracts and Western blotting. This reagent was used to study the synthesis and cellular location of small t in SV40 infected and transformed cells. Small t was found to be present in the nucleus and cytoplasm of the cells and it was more readily detected at 72hrs post infection.

By immunoprecipitation of different cellular fractions with PAb280 and a monoclonal antibody that recognizes the amino terminus common region of both early proteins, small t was found to be present in two immunochemically distinct

forms one located in the nucleus and the other in the cytoplasm.

This reagent was used to reevaluate previous work involving small t in the role of SV40 transformation.

Large T produced by SV40 temperature sensitive mutant (tsA mutants) transformed cells was analyzed using a variety of monoclonal antibodies that recognize different epitopes within the molecule. Some antibodies did not distinguish between wild type and mutant molecules whilst one PAb203 was able to do so. Careful titration of large T present in tsA transformed cell lines using PAb203 has given a new insight into the role of large T in the maintenance of transformation.

ACKNOWLEDGEMENTS

I would like to thank David Lane for his supervision, advice and encouragement during the course of this work. I would also like to thank the members of the CRC Group for their assistance and for making this a very interesting experience. Finally I am grateful to Sue Hayman for her typing of this thesis.

C O N T E N T S

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LIST OF ABBREVIATIONS

BSA	Bovine Serum Albumin
bp	base pairs
cpm	counts per minute
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl Sulphoxide
DTT	DL - Dithiotreitol
EDTA	Ethylene Diamine Tetraacetate
FCS	Fetal Calf Serum
IPTG	Isopropyl ß-D Galactoside
kb	kilobases
kd	kilodaltons
MES	2[N-Morpholino]ethanesulfonic Acid
moi	multiplicity of infection
NP40	Nonidet P40
OD	Optical Density
pfu	plate forming units
mRNA	messenger RNA
SDS	Sodium Dodecyl Sulphate
SV40	Simian Virus 40
TCA	Trichloroacetic Acid
TEMED	N,N,N^{1},N^{1}, tetramethyl-1,2-diaminoethane
Tris	Tris(hydroxymethyl)-aminomethane
WT	Wild Type

C H A P T E R O N E

I N T R O D U C T I O N

1.1 Introduction

The aim of this Chapter is to describe transformation by two papovaviruses polyoma and SV40 within the context of our current knowledge of cellular transformation by dominant oncogenes.

1.2 Transformation by Polyoma Virus

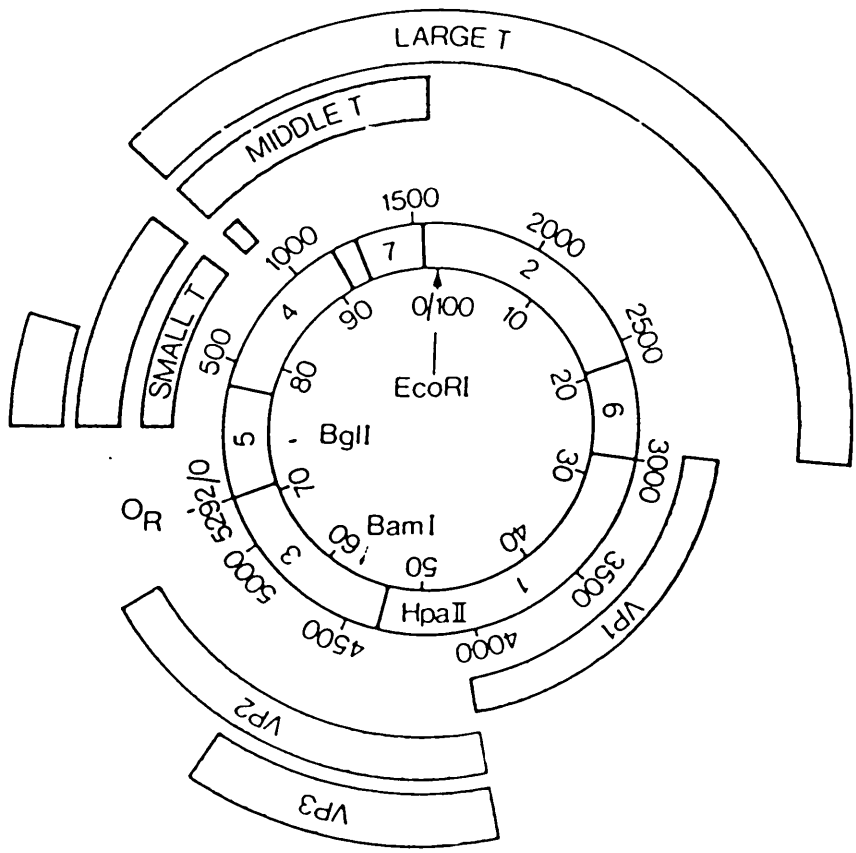
Polyoma is characterized by having a small genome of a double stranded circular molecule of DNA. The location of the protein coding sequences for the early and late region as well as other important features can be seen in Figure (1.1). The unique EcoRI site has been designated as map position zero and the genome is divided clockwise into 100 map units (Reviewed in Tooze, 1981; Reviewed in Smith and Ely, 1983).

Three distinct proteins large T, middle T and small t are encoded by alternate translational reading frames in the early region of the genome of polyoma virus. The splicing events which produce three distinct mRNA species from a common precursor transcript (Reviewed in Tooze, 1981). This region is continuously expressed in virus transformed cells. The three proteins of apparent molecular weight 105Kd (large T), 56Kd (middle T) and 22Kd (small t) can be detected by immunoprecipitation with serum from tumor bearing animals (Reviewed in Tooze, 1981).

i) The Role of the Early Proteins in Transformation

Because of the genomic organisation of the virus it was very difficult to elucidate the role of each protein

Figure 1.1. Diagram of the Polyoma Genome Showing Early (Clockwise) and Late (Counterclockwise) Coding Regions


LARGE T
MIDDLE T
SMALL T
1500
2000
2500
3000
3500
4000
4500
5000
500
1000
0/100
EcoRI
BglI
BamI
HpaII
O_R
VP1
VP2
VP3

during initial studies of transformation by polyoma. It was with the application of cDNA cloning that this problem was overcome. Treisman et al., 1981 constructed a modified viral early region encoding exclusively the middle T protein. They started with a recombinant plasmid containing full length wild type viral DNA and replaced a small genomic restriction fragment spanning the middle t intervening sequence with the equivalent fragment from a cloned partial cDNA copy of the middle T protein mRNA. Transfection of the modified viral DNA into cultured Fisher rat cells (F2408 cells) efficiently induced the formation of transformed cell foci which gave rise to cell lines that grew as tumours after injection into Fisher rats. By immunoprecipitation of extracts from transformed cells it was shown that the only viral early region antigen synthesized by the cells was the middle T protein. Therefore it was concluded that middle T is sufficient to establish and maintain the transformed state.

S1 mapping analysis of the polyoma mRNA produced by two of the transformed cell lines showed that it was structurally indistinguishable from the normal middle T mRNA.

However, it was found that the plasmid had a slightly reduced transformation efficiency when compared to wild type virus and also it was noted that the transformants grew poorly when seeded at low densities in 5% FCS. This final observation was pursued further by Rassoulzadegan et

al., 1982. They showed that cell lines derived from FR3T3 cells and transfected with the middle T containing plasmid, grew to high density in 10% serum but ceased growth at confluence in 0.5% serum. After two weeks the morphology of the cells grown to confluence in 0.5% serum was reminiscent of that of normal cells. All lines exhibited high cloning efficiency in 10% serum containing medium but could not form microcolonies at 0.5% serum.

Plasmids encoding only small t or large T or the N terminus of large T (40Kd truncated protein product), were constructed in a similar manner to the middle T plasmid (Rassoulzadegan, 1981). Cell lines transformed with the middle T containing plasmid were fused with bacterial protoplasts carrying the large T or large T truncated product containing plasmids and colonies were allowed to develop in agarose medium.

When these transformants were allowed to grow into cell lines they were shown to have growth properties characteristic of WT transformants i.e. the cells grew efficiently in media containing 10% and 0.5% serum. These results support the hypothesis that the activity of a 40Kd truncated large T (i.e. the N terminus) or the whole large T protein can reduce the serum dependence of middle T transformants.

No efficient complementation was observed in the progeny of middle t transformants fused to bacterial protoplasts carrying the small t coding plasmid, but prolonged incubation of these plates produced a significant

number of very small colonies in agarose medium and as foci on plastic plates, but these cell lines have not been studied further.

Normally cells taken directly from an animal into culture are referred to as primary cells and they have a limited life span. Only a small proportion of such cells survive the crisis that occurs after a certain number of passages in culture and go on to become established or immortalized as permanent cell lines.

Polyoma virus is known to be able to transform primary rodent cells. In addition to the transformation characteristics observed in transformed derivatives from established cell lines, it is recognized that virus transformation enables primary cells to grow in culture for an unlimited period (Vogt and Dulbecco, 1960).

Rassoulzadegan et al., 1982 showed that when plasmids containing middle T, large T, small t or the wild type early region were transferred into primary rat fibroblasts transformation resulted only with the wild type early region DNA containing plasmid. The plasmid encoding middle T did not transform primary cells even in medium containing 10% serum but when the plasmids containing large T and middle T were cotransferred transformation of primary cells took place. From these results it was concluded that middle T and large T have independent effects on cellular physiology and when brought together are responsible for the phenotype of wild type transformants.

Rassoulzadegan et al., 1982 have also demonstrated that transfer of the full size or truncated large T alone was sufficient to promote growth of normal FR3T3 cells at low serum concentrations without inducing the expression of other properties of transformation.

These results have been studied further by transferring into middle T expressing FR3T3 cells a viral genome encoding only large T carrying the tsa mutation. The cells displayed a serum dependent transformed phenotype at 40°C but not at 33°C. This result provides further evidence for the continuous requirement for a large T gene product to maintain transformation in low serum medium.

The results described above suggest that the phenomenon of 'immortalization' is not conferred on cells by expression of middle T only but requires the presence of large T, Rassoulzadegan et al., 1983 asked whether the recombinant DNAs separately encoding large T or small t could promote the establishment of permanent cell lines.

Small t or large T coding plasmids were transferred into primary rat embryo cells and it was shown that only large T expression conferred the ability to form colonies on plating at low density.

The plasmid coding for the truncated 40Kd large T product was also transferred into primary rat embryo cells by protoplast fusion and shown that it was as efficient as whole large T in inducing colony formation.

Seven rat lines and three mouse lines independently established by this procedure were studied. The growth

properties in medium with 10% serum were in all cases similar to those of established normal lines such as FR3T3 i.e. in attached culture cell division was arrested at a low saturation density corresponding to a complete monolayer and cells seeded in suspension in agarose medium did not divide.

However unlike FR3T3 cells these cells could grow in medium supplemented with 0.5% serum.

This result strongly indicates that the function of large T in induction of colony formation by primary cells can be mediated by the amino-terminal domain of the protein.

Representative lines were maintained for more than 120 generations in culture without any apparent decrease in cell viability or in growth rate . To determine whether the presence of large T protein is continuously required for growth in culture a recombinant DNA was constructed that carries the tsa mutation and thus encodes an altered form of large T unstable at high temperature. After transfer into primary rat embryo fibroblasts, selection by colony formation at 33^{o}C produced the same proportion of established clones as with the plasmid containing the cDNA sequence for wild type large T at 33^{o}C. The cells grew with the same phenotype as the cells established after transfer of a wild type cDNA large T plasmid but when grown at 40^{o}C their growth was arrested. After one residual doubling the viability of the cells at 40^{o}C progressively

decreased as shown by growth measurements after replating at 33°C. The fact that the tsa mutation maps within the carboxy terminus of large T is not contradictory with the attribution of the immortalization function to an amino-terminal domain of the protein because the whole tsA mutant protein is unstable and turning over rapidly at high temperature.

All these experiments imply that the inability of middle T to transform primary cells was due to the lack of an 'immortalising' function. This appears to be a large T function.

The role of polyoma small t in transformation has been more difficult to elucidate. As already mentioned Rassoulzadegan et al., 1982 and 1983 could not detect differences in the growth patterns of cultured cells showing transformation properties expressed by all three proteins (i.e. the wild type early region) and cell lines that lack a functional small t.

The only effect of the small t gene alone that could be observed in cells of an established line was a low efficiency of complementation of the serum requirement of middle T transformants, selection either by growth in suspension or by focus formation in the presence of 0.5% serum after transfer of the small t coding plasmid into middle T expressing cells yielded very small colonies or foci of slow growing cells which could not be cultivated successfully.

Asselin et et., 1984 have evidence of a possible role for polyoma small t. In order to determine whether complementation could be exerted by polyoma small t they assessed the tumorigenic properties of a mutant bc1051, a mutant that because of a base change at nucleotide 410 prevents the splicing of the large T antigen but produces small and middle T antigens. When injected into newborn rats they induced tumors with an efficiency comparable to that of wild type DNA and with a construct containing middle T alone. The tumors were removed and established into cell lines. The cells were labelled with ^{35}S methionine and the proteins immunoprecipitated with anti T serum and found that all tumors induced by either wild type polyoma DNA or bc1051 DNA expressed the polyoma middle T and small t. These results provide some evidence that the large T or its aminoterminus can be dispensible in the tumorigenic process when assessed in vivo.

From these results it would seem that only large T and middle T are required to produce full transformation but controversial results were obtained when employing (Rat Embryo Fibroblasts) REFS cells as the assay system (Cuzin et al., 1984). When middle T and large T were simultaneously transferred into the cells no stably transformed line was isolated. Combinations of middle T and small t or large T and small t genes were similarly inefficient. Transformation could only be achieved by transfer of a wild type viral genome or of a combination of modified genomes that could allow the simultaneous

synthesis of the three early proteins. These results demonstrate a requirement for the small t protein for transformation of the REF cells.

This group also observed cellular changes induced by transfer of small t coding plasmid into normal rat cells (REF and FR3T3 cells). After one to five days of transfer a large proportion of the recipient cells were floating in the medium.

These cells were not dead but maintained an organized structure and an extended flat morphology. These islands were growing but at a reduced rate and they reattached to the plastic plates after the first five to ten days. These observations are likely to correspond to the transitory expression of the gene after transfer. They have been confirmed and extended by experiments based on the selection of permanent lines by transfer of a plasmid carrying the small t gene linked to the geneticin resistant gene. After transfer resistant G418 clones were selected; the cells were loosely attached and did not reattach after trypsinization. Addition of fibronectin allowed attachment and further growth of the resistant cells.

They also noticed that cells expressing either only middle T (in high serum medium) or middle T and large T proteins (in both low and high serum medium) exhibited the characteristic compact morphology of wild type transformants. By contrast cells expressing only the large T protein remained flat and extended like embryonic

fibroblasts or 3T3 cells. This observation has been confirmed by indirect immunofluorescence visualization of the cytoskeleton and indicates that middle T protein induces a complete disruption of actin cables.

Liang et al., 1984 have arrived at similar conclusions using hr-t mutants. These mutants have lesions affecting both middle T and small t antigens, and are defective in transformation and in replication in certain cell types. They have constructed a modified hr-t virus carrying a single base substitution at the 3' splicing site for middle T mRNA, thus achieving separation of the hr-t gene products.

Immunoprecipitation of ^{35}S methionine labelled baby mouse kidney cell extracts showed the presence of large T and small t but not middle T. A consequence of the altered splice site for middle T was a 3 to 5 fold increase in the ratio of large T/small t.

The mutant was found to be totally defective when tested for its ability to transform F-111 rat cells assayed by dense focus formation in monolayers and by clonal growth in soft agar.

The presence or absence of actin cables and the definition of extracellular fibronectin was determined by immunofluorescence in NIH3T3 cells infected with the mutant virus. It was observed that actin cables could still be detected. The distribution of fibronectin was also as in mock infected cells. Only the wild type virus caused loss of actin cables and reduction of the fibronectin matrix.

This data indicates that middle t mediates these changes and has been corroborated by transferring the cDNA middle T coding plasmid into F-111 cells.

Enhanced agglutination by plant lectins is associated with cell transformation brought about by viruses as well as by other means. To examine the role of small t and large T in this process NIH 3T3 cells were infected with the mutant virus. Cells were harvested and tested for agglutinability. The result was positive indicating that small t and large T proteins in the absence of middle T can bring about cell surface change.

Therefore it seems that although polyoma small t has 30% homology to SV40 small t antigen and has similar cell distribution i.e. nuclear and cytoplasmic (Zhu et al., 1984, Montano and Lane, 1984), these two proteins exhibit different functional properties since SV40 small t dissociates actin cables.

Complementation in transformation is not a situation only found in the phenomenon of transformation by polyoma virus, it can also be observed with other oncogenes.

Land et al., 1983 transfected a clone containing the activated Ha-ras gene from the EJ bladder carcinoma into secondary rat embryo fibroblasts (REFs) and found that in the conditions of the focus assay no foci of morphologically altered cells were obtained; but if they transfected an established cell line such as Rat-1 cells, foci were formed in conditions closely resembling those

which did not permit focus formation of the secondary REFs. The transformed Rat-1 cells yielded rapidly growing cell lines whereas the REFs entered cell crisis immediately on repassaging. These results showed that the EJ ras oncogene had only limited power to induce the transformed state i.e. certain cellular functions supplied by the Rat-1 cells were needed for the expression of its oncogenic potential or alternatively the REF cells alone contain factors able to suppress this potential.

A variety of genes were assayed for complementation of the activated Ha-ras gene.

One choice was c-myc. Because rearrangement of this gene and an activated Ha-ras gene have been observed to coexist in human tumor cells (Murray et al., 1983). When c-myc and EJ ras were introduced into secondary REFs a dramatic alteration in the cell phenotype to a transformed state was observed (this change was not observed with the c-myc transfectant alone). When the transformed foci were picked they gave rise to rapidly growing cultures of morphologically transformed cells; these cells were tumorigenic when introduced into nude mice or 12 day old Fisher rats but the tumors reached a static size whereas activated Ha-ras transfected Rat-1 cells tumors that grew progressively until they killed the host.

They also tested cotransfection of REFs with EJ Ha-ras and a clone containing polyoma middle T and found that transformants were not produced, but transformants were obtained where polyoma middle T was cotransfected with c-

myc. The transformants were found to induce tumours in only two animals, the remainder had only small nodules at the site of innoculation. Thus middle T behaved similarly but not identically to activated EJ Ha-ras.

Transfection of the truncated polyoma large T clone or c-myc had no obvious change in morphology of the REF monolayer. However, when the former was cotransfected with EJ Ha-ras dense foci were observed. These transformants induced tumours that killed the host.

Ruley 1983 observed the same phenomenon when transfecting T24 Ha-ras gene into primary cultures of baby rat kidney cells (BRK). T24 Ha-ras or polyoma middle T were unable to transform these cells, but when cotransfected with plasmids coding for the Ad-2 Ela region products foci were produced with a similar frequency to when BRK cells were cotransfected by Ad-2 Ela and Elb. The requirement of Ela suggests that this region of Ad2 expresses an activity required for transformation of primary cells.

Newbold and Overell, 1983 have tested transformation of primary hamster fibroblasts immortalised by chemical carcinogens. They transfected cloned EJ Ha-ras into the cells and found that it induced foci. All foci possessed a characteristic morphology consisting of rapidly dividing cells which did not shown density inhibition. No foci resulted from transfection of EJ Ha-ras into untreated primary hamster fibroblasts.

1.3 Transformation by SV40

The SV40 virion has a diameter of approximately 45 nm. The virion is made of a single double stranded covalently closed DNA molecule surrounded by an icosahedral capsid made of three virally encoded proteins.

The nucleotide sequence of the SV40 DNA has been determined (Fiers et al., 1978; Reddy et al., 1978, Tooze, 1981) and known to be 5243 base pairs long.

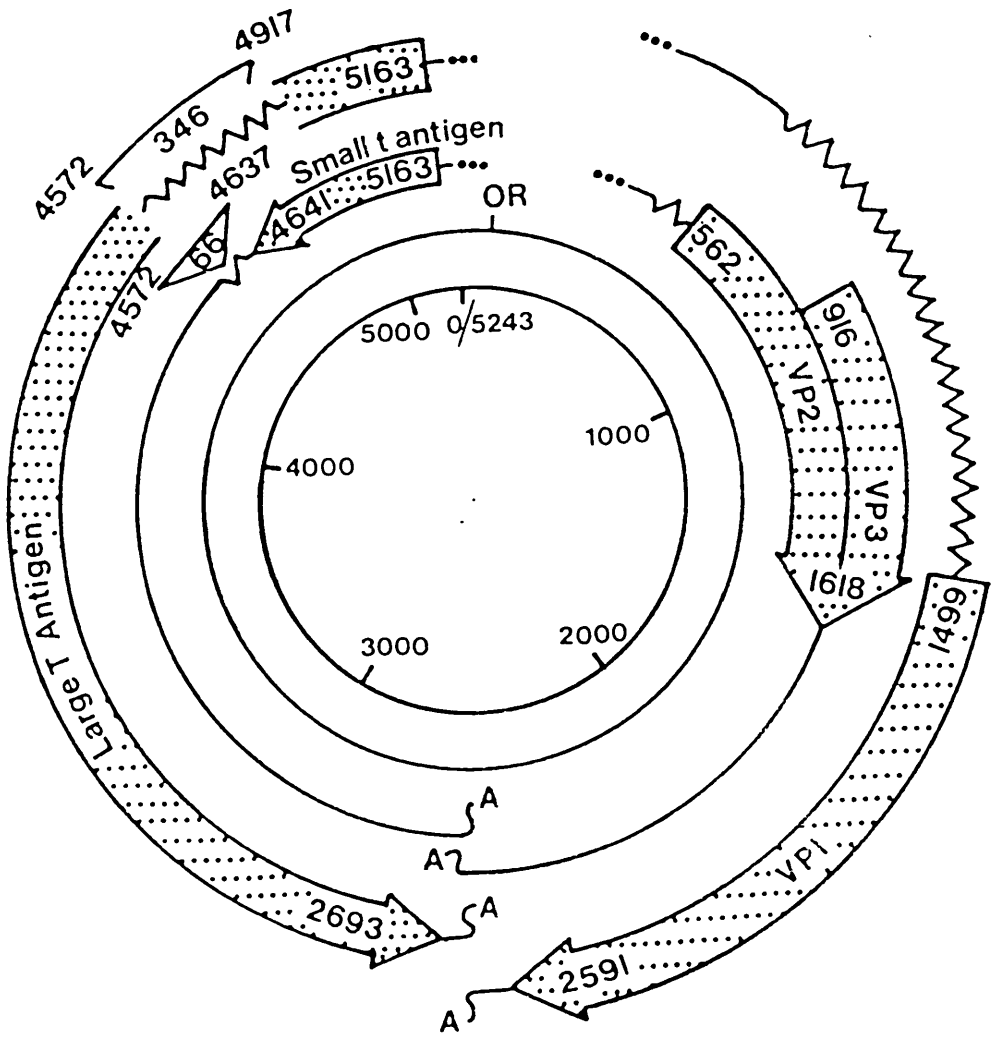
The early and late regions and other important features can be seen in Figure (1.2). The early mRNA become translated to produce two proteins designated large T and small t. These two proteins have a common amino-terminus of 82 amino acids but unique carboxy termini ; this is due to differential splicing of the mRNAs (Reviewed in Tooze, 1981).

a) The Role of the Different Early Region Proteins in Transformation.

i) The Role of Large T

To analyse the contribution that the early viral gene products make to the establishment and maintenance of the transformed state temperature sensitive mutants (tsA) were employed. These mutants result from alterations in the base sequence of DNA that cause changes in the amino acid sequence of a protein in such a way that the protein can function at one temperature (permissive) but not at another, usually higher, (non permissive) temperature. Ts mutants of SV40 have been separated into five groups. In

Figure 1.2. Diagram of the SV40 Genome Showing the Early (counterclockwise) and Late (clockwise) Coding Regions.


4917
5163
346
4572
4637
Small t antigen
4641
5163
OR
66
4572
562
916
VP2
VP3
1618
1499
5000
0/5243
1000
4000
3000
2000
A
A
Large T Antigen
2693
A
VP1
2591
A

the context of transformation complementation Group A has been employed, these mutants are defective in replication (Reviewed by Tooze, 1981; Reviewed by Martin, 1981).

Many laboratories have carried out experiments using these mutants which strongly suggested that large T had a clear role in the induction of transformation. However there was some disagreement about the role of large T in the maintenance of transformation.

Most of the problems in trying to elucidate the role of large T using tsA mutants come from the variety of parameters that were employed to assay its function. Some of which are a) cell types employed; b) actual procedure employed to transform the cells which include the m.o.i. of virus used for infection; c) the actual permissive and non-permissive temperatures employed to grow the transformants.

Martin and Chou (1975) using secondary chinese hamster lung cells infected at 37^{o}C with six different tsA mutants showed that when transformed clones were selected and examined at both restrictive and permissive temperatures the transformed phenotype was lost at the restrictive temperature. In this case two criteria for transformation were employed. The ability to overgrow a monolayer and the ability to form colonies in medium with low serum concentrations.

Brugge and Butel (1975) made a more extensive study. They used several cell lines (human skin cells, hamster embryo fibroblasts, and Balb/c 3T3 cells). They transformed them at the permissive temperature and assayed

the morphology saturation density, colony formation on plastic on cell monolayers and in soft agar. They found that the cell lines tested were temperature sensitive for these parameters but when grown at the restrictive temperature they were phenotypically similar to to nontransformed cells.

Tegtmeyer (1975) obtained results which were in some disagreement with those found by Brugge and Butel and Chou and Martin. The same tsA mutant viruses were employed and used to infect Swiss 3T3 cells, rabbit kidney cells and Syrian hamster cells. Although 33^{o}C was used as the permissive temperature for all cell lines the approach to select a non-permissive temperature was determined by the viability of each cell line at elevated temperatures 39^{o}C being the limit for Swiss 3T3, 41.5^{o} for the rabbit kidney cells and 40.5^{o} for hamster embryo cells. The only restriction placed on the cells was the selection of rapid growth at 33^{o}C. Tegtmeyer found that none of the mouse lines he obtained were temperature sensitive for colony formation in high serum. Hamster lines were temperature sensitive for colony formation at low cell density but not at high cell density. The lines that grew at non-permissive temperature retained their transformed morphology. Therefore it was concluded that the temperature sensitivity of the different transformed phenotypes may depend on the particular combination of virus and cell.

It was Brockman, 1978 who presented a clearer study of the temperature dependence and independence phenomenon.

Six tsA mutants that map in H-I and B HindII and III fragments (see Figure (1.3)) were used to infect Balb/c 3T3 cells. Then 2 to 3 colonies transformed by each virus were isolated. Transformants were tested for temperature sensitivity of the transformed phenotype by plating the cells on confluent 3T3 cell monolayer and comparing the ability to form colonies in soft agar at permissive (33°C) and non-permissive temperatures (39.5°C). Comparison of the efficiency of plating and a colony forming ability at the permissive (P) and non-permissive temperature (NP) for WT transformant gave a ratio of 1 but a ratio of 10^2 greater for the vast majority of tsA transformants. Two tsA transformants were exceptional in that they were nearly as temperature resistant as wild type transformants.

The possibility of leakiness by the mutants was taken into account and the two temperature independent clones were assayed for growth at 39.8°C. It was found that at higher temperature only one line was still temperature independent. Virus rescue from this line was still ts. However, the thermolability of large T assayed by complement fixation in all the tsA transformed lines was shown to be six times greater than wild type large T.

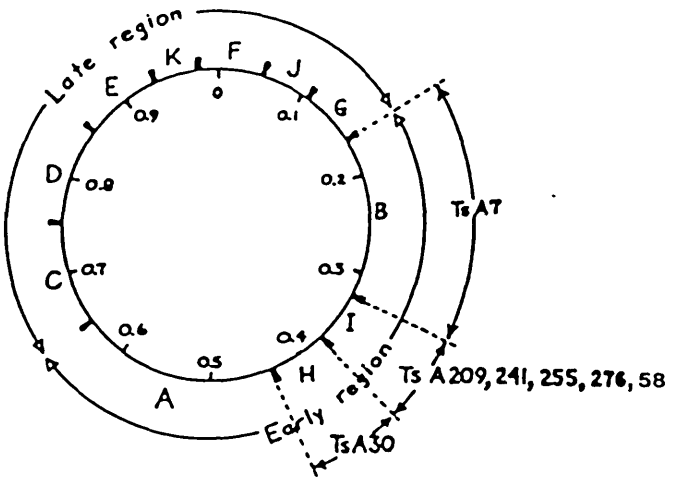
The intracellular concentrations of large T were observed by an immunocomplex assay and it was found that the temperature independent cell line had 1.7 times more large T than a temperature dependent line at 33°C. This

Figure 1.3. Diagram Showing a HindII and III Cleavage Map of the SV40 Genome.

The positions of the tsA mutants are indicated.

HIND II + III CLEAVAGE MAP OF THE SV40 GENOME

SHOWING REGIONS IN WHICH THE TSA MUTATIONS MAP



difference was increased to 4.8 times when the cells were grown at 39.3°C. This result suggested that temperature independence is due to a protein dosage effect. However, there are two criticisms of this work only one type of cell line was assessed and the growth state of the cells was not taken into account.

Seif and Martin (1979a) carried out experiments using FR3T3 cells but taking into account the growth state of the cells.

Growing rat cells were either infected with tsA or WT virus. Immediately after absorption the cells were seeded at different cell densities then dense foci were counted. It was clearly observed that the growth state of the cells postinfection affected the frequency with which temperature insensitive foci were found. When the ratio of resting to growing cells was very low the transformants were temperature sensitive. When the ratio of resting to growing cells was larger the transformants were temperature resistant. They found that the choice between the two transformed states was limited to the initial period after infection i.e. temperature sensitive transformants when passaged at different densities and at 40°C did not acquire a temperature resistant phenotype.

When immunoprecipitation of labelled cell extracts of both temperature dependent and independent lines was carried out they did not see any systematic differences in T antigen at 33°C at 40°C. However, whilst they discussed

the possibility that the rate of degradation is higher in temperature sensitive transformants than in the resistant transformants, they did not show data to support this hypothesis.

Based on this data the authors proposed: the normal cell contains a growth control mechanism leading to a resting stage. When culture conditions are suboptimal the mechanism fails at a restriction point and does not function. When conditions are optimal for cell division the virus interacts with this cellular control mechanism. When the mechanism is on in resting cells, viral DNA integrates into the host DNA, leading to the distruction of the control mechanism by mutation. This process requires T antigen and predicts that there is site specificity to the integration of viral DNA in resistant transformants. When the mechanism is off i.e. when the cells are growing, integration cannot take place at the same sites and thus cannot destroy the control mechanism by mutation. However, T antigen can still prevent the cell from entering the resting state under restrictive conditions by inactivating the control mechansism at the RNA or protein level. In this case the integrated viral DNA is mainly used as a source of T antigen. Thus resistant transformants that are derived from resting cells do not require T antigen for their maintenance whereas sensitive transformants that are derived from growing cells do.

The same authors (Chepelinsky et al., 1980) also studied the pattern of integration of SV40 DNA into

cellular DNA of both temperature sensitive and resistant transformants to see if they could verify this hypothesis.

This study was undertaken to determine what if any correlations exist between temperature sensitivity of the tsA mutants transformed FR3T3 and CHL cell lines and the number of copies of the SV40 genome integrated. Analysis of the cellular DNA using restriction endonucleases that cut cellular but not viral DNA followed by Southern hybridization showed no correlation between copy number and phenotype since the CHL sensitive transformant tested have fewer than two copies and resistant transformants may have more than two copies. Whereas for rat cell lines the sensitive transformants have fewer than two copies but the resistant transformants may have a single copy. Therefore it was concluded a) sensitive transformants do not arise from the failure of SV40 to become integrated; b) resistant transformants do not arise by gene amplification or by a single gene dosage effect; c) Neither sensitive or resistant transformants have a unique integration pattern which is a result found with WT virus (Botchan et al., 1976; Ketner and Kelly, 1976; Sambrook et al., 1968, Clayton and Rigby, 1981). Thus they could find no experimental support for their initial model.

The same laboratory (Chepelinsky et al., 1983) then put forward two new explanations for temperature dependence and independence. a) temperature independence may arise by large T leakiness possibily due to overproduction of T

antigen (already proposed by Brockman, 1978; Christensen and Brockman, 1982) or b) that a cellular mutation has taken place that produces these temperature independent phenotypes. A temperature independent FR3T3 cell line was used to select flat revertants using fluorodeoxyuridine. The isolation was performed at the non-permissive temperature so as not to select against temperature sensitive transformants. All the revertants examined had lost their ability to express T antigen by immunofluorescence. They conclude that if T antigen was not required for the maintenance of the transformed state in temperature resistant cell lines then revertant cell lines should not be T antigen negative. This would be the case because the selection used in this experiment was for the loss of the transformed phenotype, not for the loss of T antigen. Therefore, the results strongly indicate that temperature resistance is due to large T leakiness and does not require a cellular component for this effect. These results agree with those obtained by Brockman.

Defendi's group (Hiscott and Defendi, 1979; Hiscott et al., 1980, 1981) still pursued the possibility of temperature phenotype being associated with a gene dosage effect. They used mouse embryo fibroblasts transformed by a tsA mutant. Transformed clones were selected in agar at 32°C and from these a representative line was selected (A21). This line is characterized by having integrated as well as episomal SV40 DNA and a strong temperature dependence when assayed for saturation density focus

formation in plastic and growth in agar.

From this line a subline was selected (J78) from a G_1 arrested population. This line was temperature sensitive for saturation density and focus formation but was intermediate for colony formation in agar. From this subline a clone (J78/11/(40)) was selected from cells grown in agar and subjected to 40°C (i.e. a positive pressure was used to select this subline). This line is characterized by being temperature independent using all three criteria employed to assess the previous cell lines.

When restriction endonuclease analysis of both J78 and J78/11(40)) was carried out it was noticed that some viral DNA amplification and rearrangement had taken place in the temperature resistant line.

Further restriction endonuclease analysis showed a marked structural similarity of the integration sites between the parental clone and the temperature resistant derivative suggesting that the sites may have arisen through a common mechanism either from a single integrative precursor or by unequal crossing over.

This group suggests that temperature independence arose through a gene dosage effect but only when a positive selection pressure was used to select this type of transformant. It is important to indicate that this group did not analyse the concentration and stability of large T protein.

Thus although a comprehensive analysis carried out using tsA mutants to elucidate the role of large T in

transformation has been attempted in this section there are still discrepancies. A conservative view is that the continuous expression of large T is necessary for transformation but other factors such as cell types used in the assaying system have been shown to play an important role in the phenomenon.

O'Neil et al., 1980 used primary mouse embryo fibroblasts and Balb/c 3T3 cells transformed by WT and tsA mutants. Clones of transformed cells were obtained in soft agar and in liquid medium by focus formation. The mouse embryo fibroblasts transformants generated with the tsA were temperature sensitive transformation. In contrast to Brockman's results transformants generated in the Balb/c 3T3 cells were often temperature resistant exhibiting the transformed phenotype at both temperatures. This result shows that the condition of a transformant being temperature dependent or independent not only depends on the growth state of the cell, T leakiness or gene dosage effect but also on the cell type used for the infection.

It has been known for sometime that SV40 transformed cells often contain T antigens distinct from the small t and large T antigen that are found during infection of permissive cells. These large T products have been divided into two classes super T antigens and truncated T antigens.

In order to study the role of these proteins in transformation Clayton et al., 1981, 1982a and 1982b using recombinant DNA techniques have isolated viral DNA

sequences from WT SV40 transformed Balb/c 3T3 cells.

They have isolated a sequence of DNA containing the coding region of a 45Kd protein from SV3T3 Balb/c Cl20. By DNA sequencing it was shown that the viral region in this construct is from the EcoRI site to nucleotide 3713 (D. Murphy, personal communication). Analysis of the cloned DNA demonstrated that the virus host junction occured at nucleotide 3713. When this clone was used to transfect Rat-1 cells it was noticed that some transformants were induced. These foci were fewer in number and appeared ten days later than those induced by wild type SV40 early regions. By immunofluorescence it was shown that the truncated product was present in both the nucleus and cytoplasm. This result very strongly suggested that the N terminus of large T alone can mediate transformation.

The same laboratory (Lovett et al, 1982) have analysed a super T antigen of 145Kd using the same cloning techniques and from the same cell line the template of this super T was analysed by sequencing and shown that the integrated viral early transcription unit contains an in phase perfect tandem duplication of 1212 base pairs. The duplication of the super T extends from nucletoide 4103 to 2892 (Clayton et al., 1982b). When this DNA was transfected into Rat-1 cell colonies became visible after 2 weeks. When the transforming capacity of the cloned DNA as well as the cloned WT SV40 early region was titrated it was shown that both transformed with equal efficiency (Clayton et al., 1982b). An interesting result was obtained when

this clone was transfected into permissive CV1 cells and it was shown that the super T was incapable of initiating DNA replication at the viral origin. The duplication thus defines a mutation which separates the transformation and DNA replication functions of T antigen.

A similar result was obtained by Stringer, 1982. He identified a mutant that separates the transformation and replication function of large T. In this case the lesion has been mapped by marker rescue and localized to a 214 base pair segment of the viral genome bounded by nucleotide numbers 4100 and 4314. DNA sequence analysis showed the mutation to be an adenine to guanine transition at nucleotide 4178. This change predicts a lysine to glutamic acid in residue number 214.

A series of large T mutants have been obtained by various laboratories (Gluzman and Ahrens, 1982; Prives et al., 1983, Manos and Gluzman, 1984, 1985; Pipas et al., 1983, Soprano et al., 1983; Clark et al., 1983; Kalderon et al., 1984a, 1984b) with the aim of investigating the biological functions of the protein. Many of these mutant proteins are still only partially characterised and therefore only limited references can be made at this time.

Firstly, it has been possible to isolate mutants that are replication defective but that can transform. Secondly, mutations located at various sites throughout the protein have been isolated that do not destroy the transforming capacity of large T. A problem has arisen

when assaying for transformation since different authors have used different cell systems and therefore the results obtained are difficult to compare.

Sompayrac and Danna, 1984 produced two SV40 deletion mutants one at position 0.41mu and a second that spans 0.536 to 0.168mu. These mutants were used to infect mouse C3H 10T1/2 cells and assayed for transformation using a focus assay. Both mutants induced the formation of transformed foci but the efficiency of transformation was only 0.2% of that of cloned WT SV40. By immunoprecipitation of cell extracts they did not detect large T but could detect a band comigrating with small t. All transformants grew in low serum and to high saturation density.

If these results are compared with those of Pipas et al., 1983 (in which mutants containing the same deletion sites did not transform REF cells) it can be seen that there is some cell type specificity for transformation.

Pipas et al., 1984 proved this hypothesis. They examined the ability of T antigen mutants (Pipas et al., 1983) to transform different cell lines. Their initial experiments using REF 52 cells indicated that mutations near the carboxy terminus left the transformation activity intact, but in frame deletion mutants near the amino-terminus as well as in the central portion of the molecule were inactive, as were frame shift mutants that synthesized altered T antigens of 590 amino acid residues or less.

A parallel transformation assay was carried out on REF 52 cells and on C3H 10T1/2 cells.

It was shown that WT SV40 transforms the two cell types with comparable frequencies but the deletion mutants that synthesize the amino-terminus or are internally deleted transform C3H 10T1/2 but not REF 52.

From these results it can be concluded that different activities of large T are needed to transform different cell types. There is a need to identify cell types that by virtue of their genetic background, state of differentiation or passage history require only a subset of T antigen activities to be transformed. This will permit the elucidation of the different functions of large T utilised in cell transformation and allow a proper characterisation of the functional deficiencies of the various large T mutations.

Sompayrac and Danna, 1983 have also investigated transformation of different cell lines but using a mutant that lacks sequences between 0.168-0.424mu. This mutant encodes a normal small t and several truncated large T proteins. When this mutant was used to infect F111 cells and Balb/c 3T3 A31 it was noticed that the mutant was as efficient in transforming these cell lines as the WT DNA.

When assayed in C3H 10T1/2 cells the transformation frequency was only 1% that of WT DNA. Immunoprecipitation of labelled cell extracts showed the presence of the truncated large T protein products suggesting that one or more of these truncated products are required for

transformation. It was proposed that the levels of these products was not high enough to induce the full transformed phenotype. The levels of truncated products were compared to those of mutant transformed F111 cells and it was observed that a truncated product of 34Kd was present at a lower level in C3H 10T1/2 than in F111 cells. This supports the hypothesis that the truncated proteins are functionally important in transformation.

Kalderon et al, 1984a, 1984b have used a different approach to produce mutants. They have used mixed oligonucleotide mutagenesis to produce point mutants into a region coding for a putative DNA binding domain of the protein. Within this domain there are five basic residues (Lys, Lys, Lys, Arg, Lys). The transforming potential of the mutants was assayed by transfection into Rat1 cells. Individual foci were picked up, the cell population expanded and the subcellular location of large T assessed by indirect immunofluorescence. Surprisingly, mutants that encode Thr instead of a Lys at position 128 showed cytoplasmic fluorescence. The exclusion of large T from the nuclei of transformed Rat-1 cells was not observed for mutations of individual adjacent amino acids but when simultaneous mutation at position 129 and 131 (Lys to Meth and Lys to Thr) were produced, a nuclear and cytoplasmic pattern was observed. Therefore it seems that mutations close to Lys 128 modulate that accumulation of large T in the nucleus.

The transforming activity of this mutant was similar to wild type. The latent period before foci became visible was not significantly different from WT.

Lanford and Butel, 1984 and Cold Spring Harbor Tumor Virus Meeting, 1984 have constructed an SV40 mutant utilizing the sequences from an SV40-adenovirus hybrid (PARA (CCT)). The mutant is defective in transport of T antigen to the nucleus. The mutation has been identified and results in a change at amino acid 128 of large T from Lys to Asn.

The transforming potential of this mutant was examined in primary cells and established cell lines of both mouse and rat origin. By transfection using the calcium phosphate technique the mutant was able to transform immortalized cells at efficiency approaching that of wild type SV40 (as shown by foci formation on plastic in high and low serum concentration and colony formation in methocel).

Incubation under stringent culture conditions (low serum or semi-solid medium) reduced the transforming frequency of immortalized cells by about 2 fold compared with wild type.

This mutant was unable to transform primary rat kidney cells but did induce foci in primary mouse embryo fibroblasts at about 10 fold lower efficiency than WT virus, but these transformed cells were found to transport small amounts of T antigen to the nucleus.

Although complementation of large T and middle T has been seen in transformation by polyoma this phenomenon has been more difficult to elucidate in the case of SV40 i.e. do large T and small t complement each other to produce the transforming phenotype? There are some experiments indicating that large T is responsible for cell immortalisation as well as transformation.

Petit et al. 1983 have studied the immortalization function of SV40 large T by using a tsA mutant, a deletion mutation which does not express small t and a double tsA/small t deletion mutant. These mutants were used to infect Balb/c mouse or Fisher rat embryo secondaries and the cells were grown at the permissive temperature at low density. After infection by these viruses clones of actively growing cells were observed with a frequency of 0.2 to 0.8%. Rat clones were passaged for at least 30 times and were referred to as immortalized cell lines, these clones synthesised large T. The fact that the double mutant and the small t deletion mutant could induce immortalization indicated that small t is not required for this event. This conclusion was strengthened by the observation that the tsA mutant is defective in the immortalization assay at 39^{o}C suggesting an active requirement for functional large T antigen. However, this assay does not tell whether it is initiation or a maintenance defect. The role of large T antigen in maintenance of immortalisation was addressed by studying the effect of temperature on the growth properties of cell

lines established at the permissive temperature by the various mutants.

The immortalized cell lines were selected by their capacity to clone on plastic at low cell density.

While no effect on plating efficiency of the elevated temperature was detected in cells carrying the wild type large T the lines which carried the tsA allele showed a 33/39°C plating ratio greater than 1 irrespective of whether a small t antigen was present. Whereas no effect of the elevated temperature can be detected in cells carrying the WT large T. The kinetics of this temperature insensitivity were studied on one tsA immortalized rat line. After 24hrs resting period at 33°C the cells were exposed for 24, 48 and 72hrs to 39°C. Their cloning efficiency was then measured at 33°C. Results showed that the cloning efficiency was reduced to nontransformed cell levels by a 48hr exposure at 39°C. From this experiment it was concluded that high temperature severely impairs the cells ability to grow at low density. The effect of the tsA allele is remarkably stringent in this assay since 7 out of 7 lines showed a clear ts phenotype.

The rat cells maintained at 33°C are small and make a dense and overlapping network. Exposure to 39°C for 10 days caused enlargement; the cells tend to spread and showed an organized skeleton, all characteristics which make them look like pre-crisis cells.

The role of large T in immortalization has been more clearly established by the work of Colby and Shenk (1982).

They have set up an assay using secondary rat embryo cells and three recombinant plasmids of the following characteristics 1) with a deletion at the carboxy terminus of large T (0.37 to 0.17 map units) which removes a 1067bp HpaI cleavage fragment; 2) With a deletion mutant at the 0.54 to 0.59 map units a derivative of dl1440 which has a 268bp deletion between nucleotides 4771 to 4503 and 3) a double mutant containing both deletions present in the previous plasmids. The plasmids were transfected and it was found that the first plasmid immortalized cells to high frequency as well as the plasmid containing both mutations. These results showed that an immortalisation activity was present in the aminoterminus of large T and that this function could take place in the absence of small t. When these plasmids were transfected into REF 52 cells the small t deletion plasmid produced transformed foci but the other two constructs did not.

When the growth properties of the immortalized rat embryo cells were observed it was shown that cell lines established with the double mutant grew more slowly than those established with the wild type virus. These lines also showed serum dependence and failed to form colonies in agar. Whereas cell lines transfected with the 0.54 to 0.59 mutants and wild type virus grew rapidly, had little serum dependence and readily formed colonies in agar.

From these results it can be concluded that segments from the SV40 early region that code for the N terminus of

large T can immortalize rat embryo cells.

More recently Sugano and Yamaguchi (1984) have constructed two mutants of SV40 by introducing a three base pair duplication at 0.335 map units and a second at 0.636 map units (AvaII restriction sites at 3538 and 5118 base pairs respectively).

The mutant at position 0.636 was constructed to have a three base insertion and this was expected to direct the synthesis of a mutated T antigen having an extra aspartic acid between amino acid residues 16 and 17. The mutant with the insertion at 0.335 was expected to code for a T antigen having an extra arginine between amino acids 425 and 426. Neither of the mutants was replication positive. The defect was complemented with wild type T antigen expressed in Cos-1 cells, but both mutants retained their origin binding activity.

The transforming abilities of these mutants was examined by transfecting the established rat cell line 341. The results showed that the mutant at position 0.335 could transform these cells but the 0.636 mutant could not.

When primary brain cells from newborn rats were assayed in the same manner it was observed that both mutants could not transform the cells but could rescue them from senescence.

The results of the transformation assay show that the mutation at 0.636mu destroyed the activity of T antigen required for the transformation of both primary and established cells. The mutation at 0.335mu inactivates

only the activity required for transformation of primary cells.

They have thus identified three activities of T antigen being affected differently by the mutations i) activity indispensable for transformation of primary and established cells; ii) activity essential for transformation of primary cells but not for transformation of established cells; iii) activity sufficient to induce immortalization of primary cells in culture. But the biochemical nature of these activities still needs to be characterized. These experiments show that large T has multiple functions that can be separated by mutations

Large T has been shown to be complexed to a cellular protein p53 (Lane and Crawford, 1979). This complex is tight and non-covalent.

As already stated large T is involved in oncogenesis and it has been suggested that this complex might play some specific role in cellular transformation.

The p53 protein in primary cells and established non-transformed cells has a very short half-life and is present in minute concentrations with each cell containing only a few hundred molecules of the protein. But in the case of SV40 infected or transformed cells i.e. when the protein is bound to large T the half-life is increased and the protein accumulates (approximately 10,000 molecules per cell) Linzer et al., 1979.

A variety of mechanisms by which large T increases the levels of p53 can be proposed. Large T might stimulate the

de novo synthesis of p53 as it does for several enzymatic activities and the cellular ribosomal and tRNA. Alternatively large T might act as a post-translational step to stabilize and prevent the rapid turnover of p53 in infected or transformed cells.

Oren *et al*. 1981 have produced some experiments demonstrating that post-translational turnover of p53 does play a role in regulation of the differential levels of this protein in SV40 transformed murine cell lines.

They purified mRNA that codes for p53 from Balb/c 3T3 transformed line SV40 T-2 or Balb/c 3T3 cells and translated it *in vitro*. The *in vitro* products were immunoprecipitated with anti tumor serum and peptide mapping of the products showed that the p53 product detected was similar but not identical to the *in vivo* p53 product. It was found that the levels of translatable p53 message were roughly equivalent for both the transformed cell line and Balb/c 3T3 cells. Pulse chase experiments of p53 from both cell lines have demonstrated that the p53 protein once synthesized, was rapidly degraded in 3T3 cells but was stable in the transformed cell line. This result was confirmed when a tsA transformed line was employed and it was shown that p53 was stable at the permissive temperature but was rapidly turned over at the non-permissive temperature, whereas the levels of p53 mRNA at both temperatures was roughly similar.

These results point out to a post-translational regulation of p53.

Recently evidence has been obtained that indicates that p53 is an 'immortalizing' but not a transforming gene. This data has been obtained in complementation experiments.

Parada et al. 1984 have carried out transfection experiments with plasmids encoding p53 which they have transfected into Rat-1 cells or into cultures of secondary rat embryo fibroblasts (2^{o}REFS).

The p53 encoding plasmids were unable to induce foci in any of the transfected Rat-1 cultures even when such cultures were surveyed weeks after transfection. In the same experiments an activated ras oncogene elicited foci that were observable in Rat-1 cells within eight days after transfection. Therefore, the results indicate that the p53 gene has no apparent ability to readily induce morphological transformation in established rodent fibroblast cells.

When p53 was tested for the ability to complement ras or myc oncogenes in the transformation of 2^{o}REFS, it was found that it did not induce foci when cotransfected with the myc oncogene alone. However, when the p53 clone was tested in conjunction with the EJ/T24 Ha ras oncogene they were found to cooperate in focus formation. These ras-p53 foci appeared with 10 fold lower frequency than the ras-myc foci.

The transformants were assayed for tumorigenicity. Nude mice were injected with the transfected cells. Ten days after injection tumours had appeared at all of the

sites injected with ras-myc transfectants. Whereas only small nodules were visible in the sites carrying the ras p53 cells. However, by 18 days all sites injected with ras p53 transfectants exhibited large, aggressively growing tumours. Thus it appears that p53 is able to function analogously to myc in that it cooperates with ras oncogenes to induce tumorigenic conversion of primary rat embryo fibroblasts. It is likely that the reason for the delayed appearance of ras-p53 tumor lies in the smaller initial number of transformed cells present in the inocula.

It is concluded that the p53 oncogene function is analogous to those of the cellular myc oncogene.

Eliyahu et al. 1984 using different constructions obtained similar results. c-myc, p53 and Ha-ras were used to transfect secondary Fisher rat embryo fibroblasts. However, it was also noticed that the p53 + Ha ras foci differed from the myc + Ha ras foci in that the former tended to grow more slowly and often appeared to stop spreading after reaching a size of 5 to 7mm, whereas the latter could expand continuously. However, when Chinese hamster embryo fibroblasts were cotransfected with p53 although foci were seen with transformed morphology the cells had a high tendency to undergo lysis. Upon passaging the cells only underwent a few divisions and then gradually lysed.

It is clear that as in the case of large T the cell system used to assay the biological function of p53 has to be assessed carefully.

It can thus be concluded that p53 can act like an oncogene it that it can transform embryo fibroblasts when cotransfected with Ha ras but unlike myc the resultant transformants do not necessarily possess an unlimited proliferative potential.

Jenkins et al. 1984 have reported that the expression of p53 can result in the rescue of primary cells from senescence.

They used Wistar adult rat Xiphisternum chondrocytes and transfected with the p53 gene.

These cells undergo approximately 30 doublings in vitro before senescence with characteristic changes in morphology and cessation of growth. When transfected with the p53 gene the cells were maintained in vitro for over 200 doublings. They did not show morphological evidence of senescence and continued to double every 18 to 20 hours. These cells were non-tumorigenic when injected into syngeneic rats.

These immortalized cells were transfected with cloned activated Ha ras and transformed foci were scored. These transformed cells showed high anchorage independent growth and rapid growth in low serum. Whereas the immortal but untransformed cells were anchorage dependent and only grew in medium containing a relatively high serum concentration.

It was concluded that p53 expression extends the cellular life span and it has no detectable effects on other phenotypes known to be associated with

transformation.

ii) The Role of Small t Antigen

SV40 small t was initially detected when studying cell free translation systems of early SV40 mRNA (Prives et al., 1977; Paucha et al., 1978).

Tegtmeyer et al (1979) carried out biochemical studies in an attempt to characterize this protein. By Dounce homogenization and separation of nuclear and cytoplasmic fractions (this distinction is somehow arbitrary because cellular membranes would remain associated with the nuclear fraction) it was found that small t was preferentially in the cytoplasm.

The state of aggregation of the protein was assayed by gel separation chromatography and it was found that between 60 to 70% was present in a monomeric state and between 30 to 40% behaved like aggregates of molecular weight as large as 200Kd.

During early studies of the organization of the SV40 genomes deletions were made in the early region which still allowed the virus to be viable (Shenk et al., 1976; Cole et al., 1977; Topp, 1980). These viable mutants have deletions ranging from 10 to 250bp and are located at 0.54 to 0.59mu. that is, at a position that affects small t expression.

Some of these mutants have been mapped by marker rescue and others by DNA sequencing and it was noticed that there were two types of mutants, splicing mutants and mutants with internal deletions (in frame and out of

frame).

The use of these mutants has allowed a study of the role of small t. The clearest results have been obtained by Graessmann et al. 1980, they have microinjected rat embryo fibroblasts with intact SV40 DNA, DNA fragments of the early region of wild type SV40 or of 0.54 to 0.59 deletion mutants, and a purified SV40 large T antigen related protein D2 (isolated from cells infected with the adenovirus SV40 hybrid virus $Ad2^{+}D2$). Using immunofluorescence it was observed that injection of WT DNA or of fragments containing an intact early region induced the disruption of the actin cable network within 24hrs of injection. Whereas when D2 protein or fragments from the deletion mutants were injected the network was not disrupted. Similar results have been obtained by Rubin et al. 1982; but conclusive data was obtained by Bikel et al. 1983. They injected purified small t (produced using a small t coding plasmid in E. coli) into Rat-1 cells and when the presence of actin was assayed by immunofluorescence it was noticed that the infection led to actin cable dissolution. Whereas when injecting only the buffer used to purify small t the network was kept intact.

The role of small t in transformation has been more difficult to elucidate. During initial work carried out by Sleigh et al. 1978 using small t deletion mutants, they observed that the mutants had a different ability to transform mouse, hamster and rabbit cell lines. The extent

of transformation observed was also dependent upon the assay.

The deletion mutants failed to induce any foci in Rat-1 cells even at the level of 10μg of viral DNA per plate, while cultures of rat embryo fibroblasts, Balb/c mouse and Fisher rat embryo secondaries were transformed equally well by mutants and wild type virus as assessed by plating cells at low density. In contrast however, a clear difference was observed between mutants and wild type SV40 when rat embryo fibroblasts were scored for their ability to form dense foci on a monolayer of untransformed cells.

The observation that the ability of SV40 deletion mutants to induce transformation varies with the assay used, suggests that the mutants are not able to induce in cells all those changes generally considered to be characteristic of the transformed state. This was confirmed by examining the properties of 19 clones from among the rat embryo fibroblast transformants scored by the criterion of low density plating. The transformants derived from different mutants had similar morphology but showed striking differences in their growth properties. Only one out of twelve was able to grow in methyl cellulose whereas 5 out of 7 SV40 WT transformants did form colonies. In addition transformants derived from the mutants grew to and held a saturation density only 2-3 times higher than that achieved by the parent line of rat embryo fibroblasts, while all the WT transformants lifted off the plate before their saturation densities could be measured. There was no

cloned efficiently in methocel compared to only one of the deletion mutant lines.

These results indicate that for high passage cells, the development of the fully transformed phenotype has a strong dependence on small t protein activity. Whereas freshly explanted cultures do not show such a dependence.

Martin et al 1979 have also used SV40 deletion mutants to transform actively growing CHL cells.

When transformants of WT SV40 were isolated as well as transformants produced by the deletion mutants no obvious differences in the distribution of colony sizes in agar were found between the set of cell lines transformed by WT virus and the sets of cell lines transformed by the deletion mutants.

Several of the cell lines transformed by the deletion mutants were tumorigenic in nude mice. When 10^6 cells were injected into weaning mice, tumours of up to 3cm in diameter were observed within 10 weeks. All lines that grew well in agar produced tumours, whereas those that grew poorly did not. This shows that there is some correlation between these properties.

The mutants failed to transform resting CHL cells. This might be due to a defect in their ability to stimulate resting cells into a growing state because once cells were in a growing state, the deletion mutants transformed at normal frequency. When CHL cells were rapidly growing before infection there was no difference in the frequency of transformation between WT SV40 and the mutants. When

the cells were in a resting state before infection an approximately 50 fold reduction in the relative transforming ability of the deletion virus was observed. This reduction depended upon keeping the cells after infection in conditions that maintain normal cells in a resting state. Thus the defect in the deletion mutants appeared to be in a function required for the establishment of the transformed phenotype in resting CHL cells. Whether this function is required because the cells are in a resting state or whether this function is required because the medium is depleted of some essential serum factor or whether some inhibitor is produced by the cells or present in the medium was not determined. This explanation could also account for the different transformation frequencies observed by the dense focus versus colony assay methods as reported by Sleigh et al. 1978.

A more probable explanation derives from the work of Pledger et al., 1977, 1978. They have demonstrated that there are arrest points from mitosis to the next round of DNA synthesis. Serum factors such as platelet derived growth factor, PDGF, stimulate mouse 3T3 cells from the first arrest point, G_0, to the second, i.e. the competence point G_1. These growth factors are referred to as competence promoting factors. A second set of progression factors for example EGF is required to stimulate cells from the competence point to DNA synthesis. The results obtained with the deletion mutants are consistent with the

hypothesis that the role of small t is to stimulate cells from G_o to G_1, i.e. small t seems to mimic the action of certain normal serum factors, the competence promoting factors. Therefore they proposed that in sera that are rich in progression factors but are limiting in competence promoting factors cells transformed by the viable deletion mutants will fail to grow as efficiently as WT transformed cells.

In order to test this hypothesis (Seif and Martin, 1979b) examined transformation of confluent resting FR3T3 cells using deletion mutants. They infected the cells using either partially purified virus or crude lysate. When the cells were infected and suspended in agar the induction of colony formation by the deletion mutants was significantly reduced relative to wild type virus. This effect was less apparent when using the unpurified virus preparation (crude lysate).

When growing cells were infected and then immediately plated on plastic at different cell densities, the defect was shown only with high cell densities and only when partially purified virus was used. When resting cells were infected with partially purified virus and then suspended in agar and plated on plastic at low, moderate, or high cell densities the defect was again apparent when the cells were plated at high densities only, but this effect was not apparent when crude lysate was employed.

In an attempt to determine how the CV1 lysate was able to complement the defect of the mutants, resting FR3T3

cells were incubated with an uninfected CV1 cell lysate it was shown that most of the cells went through at least one division. Therefore it seems that there are some cellular proteins present in the lysate that had some mitogenic activity.

Taken together these results strongly suggest that small t is needed for the transformation of resting cells and might act as a growth factor.

When the transformation state of the cells was assessed it was shown that cells transformed by the deletion mutants grew in agar, grew over a monolayer of normal cells and gave rise to foci. From these results it seems that small t is needed at the establishment level rather than at the maintenance level.

Seif and Martin, 1979b have indicated that small t might act as a promoter or cocarcinogen during establishment of transformation in resting FR3T3 cells.

In an attempt to determine the role of small t in the establishment and/or the maintenance of temperature sensitive or resistant transformants (see Chapter 5). They used SV40 0.54 to 0.59 deletion mutants and double mutants containing both a deletion of the small t gene and a tsA lesion in large T gene.

Rat cells grown at high densities in plastic were infected with the double mutant and left for three days at 33^{o}C and then suspended in agar. After six weeks approximately five times as many colonies were found in the

cultures infected with the double mutant as with the control tsA mutant. Several colonies were isolated in agar and their growth properties were studied at both permissive and non-permissive temperatures. It was shown that the tsA mutant gave rise to both temperature sensitive and resistant transformants but under these conditions double mutant only induced temperature sensitive transformants. Therefore, they concluded that small t is needed to produce temperature resistant transformants.

Rubin et al, 1982 have used a different approach. They have constructed an SV40 virus which contains a deletion between nucleotides 4212 and 2713 (see Figure (4.10)). This virus contains the whole of the small t coding region. When this virus (SV402) was used to transform NIH 3T3 cells or Balb/c A31 cells together with an SV40 small t deletion mutant they found that, as assayed by growth in soft agar, transformation occured. Whereas when SV402 was used alone far lower numbers of transformants were detected. The small t deletion mutant alone also led to only low level acute transforming effects at very high multiplicities.

However, if the SV402 construction is observed carefully it can be seen that a truncated large T of an estimated molecular weight of 24.2Kd could also be produced. There have been reports (Colby and Shenk, 1982, Chang et al., 1984) of SV40 mutants producing such truncated large T products which can confer immortalization or transformation functions. The results described in

Chapter 4 will clearly show that the findings of Rubin et al have to be reconsidered.

Small t has also been assayed for tumorigenicity.

Lewis and Martin, 1979 have used Syrian hamsters. When hamster pups were innoculated subcutaneously with a series of deletion mutants, tumours developed by 180 days. Meanwhile, hamsters injected with WT SV40 developed tumours 90 to 120 days after in oculation. Histological study of the tumours showed that there were no consistent differences in the general appearance of WT induced tumours compared with mutant induced neoplasms.

To determine the role of the mutant viral genome in tumour induction the expression of the early protein was examined. Immunoprecipitation of representative tumor lines showed that large T was present in all lines examined, whereas small t was detected in extracts of cells obtained only from WT SV40 induced tumours.

When the state of the viral DNA was observed by restriction endonuclease characterization of rescued viral DNA, the digestion patterns of the DNA of the rescued virus was the same as of the parental mutant.

Therefore it was concluded that small t is not necessary for oncogenesis but it appears to augment the rate of tumor development.

Topp et al. 1981 obtained similar results and again it was emphasized that the deletion mutants induced tumours with only 50% the efficiency of WT virus and with a longer

latent period.

Dixon et al, 1982 have obtained data of the possible metastatic potential of the 0.54 to 0.59 SV40 deletion mutants.

They studied the metastic tumours induced in hamsters by this type of deletion. They initially compared the ability of WT virus and the dl884 mutant to induce tumours following subcutaneous injection in newborn hamsters. It was found that, as before (Lewis and Martin, 1979; Topp et al, 1981) the deletion mutant induces tumours with similar frequencies to WT viruses but the tumours appear after a longer latent period. Interestingly animals injected with the deletion mutants developed tumour foci outside the subcutaneous injection site. In some cases tumour foci were only found in the abdominal cavities. In all cases histological analysis showed these tumours to be fibrosarcomas.

In order to observe if distant metastasis derived from deletion mutant transformed cells, they rescued virus by cocultivating tumor cells with CV1 cells and found that the sequences of the rescued virus were exactly similar to that of the injected mutant.

When they analyzed the integration patterns of the primary tumour and the metastatic tumours they found that it was identical and therefore concluded that the tumours that arose away from the site of injection are true metastatic tumours. Finally they cultured cells from a metastatic tumour and these were found to retain their

ability to metastise when reinjected into animals.

The increased metastases seen with the small t deletion mutant induced tumor may have a trivial explanation in that the animals were older by the time the tumors developed.

As in the case of large T, the results relating small t function to transformation are confused due to the range of mutants employed, and the variety of cell systems and assays used to assist transformation. But it is possible that the role of each protein will become clearer with the experiments carried out by Kriegler et al, 1984. They have used an infectious retroviral vector to separate the overlapping early genes (large T and small t) of SV40. The minimal retroviral vector contains LTRs and cis-acting signals required for infectious RNA virus propagation. They have cloned the SV40 early region within this DNA and after transfection of Rat-2 cells producing helper Moloney murine leukaemia virus, SV40 retroviruses were rescued.

Pure SV40 large T or small t retroviruses were cloned from the heterogeneous stocks by secondary transformation of NIH 3T3 cells.

NIH3T3 transformants were assayed for large T presence by immunofluorescence and those transformants that gave a positive signal (T^+ in the nucleus) were subjected to genomic analysis by restriction mapping. They found that 3 of 5 mouse transformants contained a single copy of a provirus approximately 350bp smaller than the parental

virus. The reduction in size is close to the size of the intron of large T. Double digestion of the genomic DNA from all 5 transformants with SmaI and TaqI demonstrated that the TaqI site between the two exons of the SV40 early region have been removed from the proviruses.

Another line contained two proviral DNAs one of whose size indicated either unit length copies of large T and small t spliced messenger RNAs. Purified viruses from the cell lines which only expressed large T (MV40-4) and the small t/large T expressing cell lines (MV40-1) were analysed by Northern blotting and S1 mapping and it was found that MV40-4 only produces viral RNA of the spliced desired size of large T, whereas MV40-1 produced messenger RNA coding for large T and small t.

Immunoprecipitation of SV40 T antigens from these lines verified the messenger RNA studies, and also showed that the retroviruses produce up to ten times more viral products than wild type SV40 transformed lines.

Because it was possible to select only large T producing transformants were which showed similar characteristics as WT SV40 transformants it was strongly emphasized that a small t was not necessary for this process. This was proved by measuring the transformation efficiencies of the different retroviruses in contact inhibited Rat-2 and NIH 3T3 cells. It was found that the absence of small t coding capacity from MV40-4 did not have any effect upon the ability of the stock to transform either rat or mouse lines in focus assays but it was

slightly lower when inducing formation of colonies in soft agar.

These results indicate that small t itself does not cooperate in the initiation and maintenance of transformation and when combined with large T (MV40-1 line) does not appear to confer a selective advantage for transformation.

The studies described in this thesis were directed towards a clear understanding of the role of SV40 large T and small t in transformation. In order to study small t more precisely the first monoclonal antibody that uniquely recognises small t was isolated allowing the discovery of discrete subsets of the protein. A detailed investigation of the concentration and conformation of large T in temperature dependent and temperature independent transformed cells was carried out using monoclonal antibodies to different sites in large T.

CHAPTER TWO

MATERIALS AND METHODS

2.1 Mammalian Cells

The following mouse cell lines transformed by SV40 were employed SV3T3 C138, SV3T3 C120, (Clayton and Rigby, 1981), E7 (Lane and Crawford, 1979). These cell lines were derived following the infection of confluent monolayers of Balb/c 3T3 clone A31 cells with WT SV40.

Cos-1 cells are an African green monkey kidney cell line, CV1, transformed by an SV40 mutant with a defective origin of replication (Gluzman, 1981).

A7B4b, A209B4a and A255B1a are Balb/c 3T3 cells transformed by SV40 tsA7, tsA209 and tsA255 temperature sensitive mutants (Brockman, 1978).

J78, A21 and J78/11(40) are mouse embryo fibroblasts transformed by the SV40 temperature sensitive mutant tsA58 (Hiscott et al., 1980, 1981).

BK virus transformed lines are pBKM, pBKD, both derived from C3H mouse cells and BKHK a Hamster kidney derived cell line. These cell lines were a gift from Dr. Linda Gooding (Emory University, Atlanta, Georgia).

SV3T3 C138, SV3T3 C120 (Clayton and Rigby, 1981) and E7 (Lane and Crawford, 1979) cell lines were grown in DMEM pH7.3, supplemented with 5% FCS, 500 units/ml penicillin and 100µg/ml streptomycin. In the case of Cos-1 cells, BK and SV40 tsA transformed cells, the media was supplemented with 10% FCS.

Cells transformed by WT SV40 and BK virus were grown in 9cm tissue culture dishes (Nunclon) at 37°C and 8% (v/v) CO_2 in air (Leec Incubators).

Cells transformed by SV40 temperature sensitive mutants were grown at 32°C under the same conditions stated above.

2.2 Hybridomas, Untransformed Cell Lines and Myelomas

PAb419, PAb416 and PAb423 are hybridomas which have been made by the fusion of splenocytes with P3-NS1-1-Ag4-1 Balb/c plasmacytoma cells. These lines were a gift from Dr. E. Harlow (Cold Spring Harbor Laboratory).

PAb203 and PAb204 are hybridomas which have been produced by the fusion of splenocytes to SP20/Ag14 Balb/c myeloma cells (Lane and Hoeffler, 1980; Montano et al., manuscript in preparation).

All these lines were grown in DMEM supplemented with 10% FCS, 500 units/ml penicillin and 100µg/ml streptomycin at 37°C and 8% (v/v) CO_2 in air.

CV1 cells, an African green monkey kidney cell line (kindly given by Lionel V. Crawford, Imperial Cancer Research Fund), and mouse Balb/c 3T3 clone A31 (from Dr. W.A. Nelson-Rees, Naval Biomedical Research Laboratory, Oakland, California) were grown in the same manner.

2.3 Freezing of Cells

Cells were grown in 9cm tissue culture dishes to 70 or 80% confluency.

i) Cells that attach to the surface of the plates.

Cells were washed once with 2ml of TD, and then removed from the plate by incubating with 1ml of TD

containing 0.25mM trypsin and 2.5mM EDTA for 1 to 4 min. at room temperature.

To each cell suspension 5mls of DMEM were added to stop trypsinisation and to wash the cells, then the suspension was centrifuged at 1000rpm for 5 min. at room temperature (MSE Bench Centrifuge) and the supernatant discarded. This step was repeated twice.

To each pellet, 1ml of 5% DMSO in foetal calf serum (Flow Laboratories) was added, the pellet resuspended, and 0.5ml aliquoted into 2.5ml freezing vials (Sterilin).

The vials were stored in polystyrene boxes, cushioned with cotton wool, sealed, and left at -70°C overnight. They were then transferred to liquid nitrogen tanks.

ii) Cells that Grow in Supension

Media containing cells was spun at 1000rpm for 5 min., the supernatant discarded and the cells washed with 5mls of DMEM and the cell pellet treated as in (i).

2.4 Cell Extracts

Cells grown to 70 to 80% confluency in 9cm dishes were washed twice with 2ml of TD. Then 1ml of NET containing 1% NP40 and 10mM PMSF was added and incubated at 4°C for 1hr. The cells were scraped from the plates and the extracts centrifuged for 2 min. (Eppendorf Microfuge). The supernatants thus obtained were used immediately or stored at -20°C.

2.5 Sequential Extraction

The method used is as described by Staufenbiel and

Deppert (1983).

Cells were grown to 70-80% confluency, and then washed twice with 2ml of TD. The following sequence of extractions was performed (all extracts were saved, stored at 4°C and used immediately). For the first extraction, 0.5ml KM buffer (10mM MES, pH6.2, 10mM NaCl, 1.5mM $MgCl_2$, 1mM EGTA, 5mM DTT and 1% NP40) was added and incubated at 0°C for 30 min. The second extraction, consisted of two steps. First, 0.25ml of KM buffer (lacking DTT and EGTA), containing 50µg/ml DNase was added, and the plates incubated at 37°C for 15 min. Next, 0.25ml of 1M NaCl was added, and further incubated at 4°C for 30 min. For the final extraction, 0.5ml of TK buffer [40mM Tris HCl pH9, 25mM KCl and 1% Empigen BB, (Marchon Division, England)] was added and incubated at 4°C for 60 min.

2.6 Viruses

SVdl883, SVdl884, SVdl886, SVdl888, SVdl889, SVdl890, SVdl891 and SVdl1264 are deletion mutants of the SV40 early region located in the large T intron. They code for a wild type large T but, in accordance with the extent of the deletion, they either produce small t fragments or no detectable small t at all (Shenk *et al.*, 1976; Cole *et al.*, 1977). These viruses and WT SV40 strain 776 were the kind gift of Lionel V. Crawford (Imperial Cancer Research Fund).

SV402 is an SV40 virus which only encodes small t. This virus was given by D.M. Livingston (Danna Farber Cancer Institute, Harvard).

i) Viral Infection

CV1 cells were grown in either 9cm or 3.5cm plates, or 96 well trays to 60% confluency, washed twice with 2ml of TS and then infected with virus diluted in TS to give 10pfu/cell or TS with no virus for mock infections. The plates were incubated at 37°C for 2hrs and tilted every 20 min. Then DMEM supplemented with 10% fetal calf serum and 500 units/ml penicillin, 100µg/ml streptomycin was added and incubated again at 37°C. The infection was allowed to continue for periods ranging from 12 to 72hrs.

ii) Growth of Viruses

CV1 cells were grown in 9cm plates to 60% confluency, washed twice with 2ml of TS and infected with 1ml of virus diluted in TS to give 0.01pfu per cell.

The plates were returned to a 37°C incubator for 3hrs and swirled at 20 min. intervals. Then 10ml of DMEM supplemented with 10% FCS, 500 units/ml penicillin and 100µg/ml streptomycin was added and the infection allowed to continue for 10 to 14 days until 90% of the cells were detached from the plate surface. The virus preparation plates were subjected to 3 freeze and thaw cycles and the virus harvested.

iii) Plaque Assay

The virus grown as indicated in (ii) was harvested and the titre of the crude lysate was assessed to be between 10^7 to 10^8 pfu/ml in the case of SV40 deletion viruses and between 10^8 to 10^9 pfu/ml for WT SV40.

A serial dilution of the virus was made in TS to give 10 to 20 plaques per dish.

0.2ml of dilutions 10^{-4} to 10^{-9} were used to infect 6cm dishes with 80% confluent CV1 cell monolayers (the infections were done in duplicate and included two mock infected plates). After 1hr at 37°C, 4.5mls of previously warmed autopow DMEM containing 2% FCS, 500 units/ml penicillin, 100µg/ml streptomycin, 4mM Glutamine and 0.9% agar (SEA Plaque low gelling temperature) was layered on top of the infected cell monolayer and allowed to harden for 30 to 60 min, and then the plates were incubated at 37°C.

After 3 to 4 days the plates were overlayed with an additional 4ml of the same agar mixture. 10 days later plaques were counted over a period of 3 days, that is, days 10, 11, and 12 after infection, but no plaques were counted after this because of the probable formation of secondary plaques.

iv) Abortive Infections

Nonpermissive cells were grown in 9cm or 3.5cm plates to 50% confluency. The plates were washed twice with prewarmed DMEM pH7.3. Then 0.5ml of virus diluted in DMEM pH7.3 to 50 pfu/cell was added followed by 0.5mls of DMEM with 20% FCS. The plates were incubated at 37°C for 2hrs and tilted at 20 min. intervals. Then 2mls of DMEM with 10% FCS was added, the plates returned to the 37°C incubator and left for 60 to 72hrs.

2.7 Antisera

Normal rabbit serum, goat anti rabbit serum, rabbit anti D2 serum and rabbit anti T/t serum was the kind gift of Mr. Julian Gannon (Imperial College).

2.8 Bacteria

i) E. coli Strains

294 cells (Thummel et al., 1981) endo I, $_k^-$, m_k^+, Bl^- which was made lac i^q by conjugation with a strain of E. coli which carries this gene on an F factor) were given by Dr. R. Tjian (University of Berkeley, California).

X-90 cells (Bikel et al., 1983) (a $_k^-$, m_k^+ and lac i^q) a high level lac repression producing strain was the gift of Dr. D.M. Livingston (Danna Farber Cancer Institute, Harvard).

HB101 cells (Boyer et al., 1969) are F^- hsd S20 (r_b^- m_b^-), recA, ara, proA, lacY, galK, rpsL20 (Sm^R) xyl5, supE were a gift of V. Simanis (Imperial College).

ii) Growth of Bacterial Strains Transformed by Small t Coding Plasmids

a) Plating Bacteria

L plates supplemented with 25µg/ml ampicillin were prepared, and bacteria from stab cultures streaked to obtain single colonies. Cells were allowed to grow overnight at 37°C.

b) Stab Cultures

Single colonies grown in L plates were used to inoculate stabs; these were grown at 37°C overnight and

were viable when stored at 4°C for 5 to 6 months.

c) X-90

A strain transformed by pTR865 was grown as described by Bikel et al (1983).

Single colonies were grown in 10ml of L broth (containing 25µg/ml ampicillin) at 37°C to saturation. This culture was then added to 500ml L-broth supplemented with the same amount of ampicillin, and at 37°C with constant shaking until an OD_{550} of 0.7 was reached. Isopropyl-ß-D- thiogalactopyranoside (IPTG; Sigma) was then added to 5mM final concentration and the culture left to grow further for 2hrs.

d) 294, HB101

These strains were transformed by HP1 (Thummel et al., 1981). Single colonies were grown in 10ml of L broth (supplemented with 25µg/ml of ampicillin) at 37°C to saturation. Then this culture was added to 500ml of L broth (which contained the same amount of ampicillin) and grown at 37°C under constant shaking for 6hrs. Isopropyl-ß-D-thiogalactopyranoside (IPTG: Sigma) was then added to 1mM final concentration and incubated further for 1hr.

When bacterial strains with no plasmids were grown the same procedure was followed and the ampicillin was omitted from the culture.

iii) Bacterial Transformation

5ml of broth were innoculated with a single bacterial colony and allowed to grow at 37°C to an OD_{550} of 0.3. Then this culture was added to 100ml of broth and

left to grow to OD_{550} of 0.47-0.48 at 37°C with constant shaking. The cells were kept on ice for 15 min. and centrifuged at 5000rpm for 5 min. at 4°C in a SS34 rotor in a Sorvall centrifuge. The supernatant was discarded and the pellet carefully resuspended in 1/3 of the culture volume in TFb_1. The cells were incubated on ice for 5 to 10 min. and centrifuged as before. The supernatant was discarded and the pellets resuspended in 1/25 of the culture volume in TFb_2. The cells were incubated on ice for 10 min., 200µl aliquots made, incubated on ice for an additional 15-20 min. and stored at -70°C. To one aliquot 40ng of DNA was added, mixed and incubated on ice for 30 min. The mix was then heat shocked at 42°C for 2 min. with constant agitation and returned to ice for 2 min. Finally 1ml of broth at room temperature was added, and the culture grown for 1hr at 37°C. The cells were diluted in broth to give 50 to 100 colonies/plate and plated on L amp plates.

iv) Extraction of Small t Protein From Bacteria

Bacterial cultures were grown as described in c) and d). Cells were harvested by centrifugation at 10000rpm for 10min. at 4°C in a Sorvall GSA rotor. The pellet was resuspended in 10ml of NET containing 1% NP40 and 10mM PMSF and left on ice for 1hr so that lysis could take place. The suspension was sonicated for 5 min. and centrifuged at 10,000rpm for 30 min. at 4°C in an SS34 rotor in a Sorvall centrifuge and the supernatant stored

at 4°C.

2.9 Isolation of the PAb419-Small t Complex

10ml of bacterial cell extract were mixed with 1mg of purified PAb419 and incubated on ice for 2hrs. so that a complex between antigen and antibody could form. 0.33g of Protein A Sepharose (Sigma) was added and incubated overnight at 4°C. The mixture was then poured into a 5ml column (Biorad) and allowed to settle. The column was washed with 100ml of 10mM Tris HCl pH8 containing 150mM NaCl and then with 100ml of phosphate buffer pH8. Finally the complex was eluted with citrate buffer pH3 as 1ml fractions into 1ml aliquots of 1M Tris HCl pH8.8 for neutralization.

2.10 Purification of Monoclonal Antibodies from Hybridoma Supernatant

500ml of hybridoma supernatant from cell lines producing IgG_1 subclass antibody was dialysed against 5 two litre changes of phosphate buffer pH8.3 for 48 to 72hrs at 4°C, to allow the supernatant to reach pH8. This step was omitted when purifying monoclonal antibodies of the IgG_2 subclass.

1.65g of protein A Sepharose beads were suspended in phosphate buffer pH8 and poured into a 10ml column (Biorad), and thoroughly washed with 100ml of the same buffer. 500ml of hybridoma supernatant were poured over the column and a pumping system was set up to recycle the supernatant through the column for 48hrs at 4°C.

The column was then washed thoroughly with 100ml phosphate buffer pH8 and the antibody eluted as 1ml fractions with citrate buffer pH3 into 1ml 1M Tris HCl pH8.8. The protein concentration of the samples was measured by reading the absorbance at 280nm on a Cecil spectrophotometer.

2.11 High Specific Activity Iodination of Purified Monoclonal Antibodies

i) Lactoperoxidase Iodination

50µg of purified monoclonal antibody and 500µCi ^{125}I (Amersham) was added to 10µl of lactoperoxidase coupled beads (0.2mg solid phase coupled to 5µg lactoperoxidase). 10µl of 100 vol. H_2O_2 (BDH) diluted 1:1000 in distilled H_2O was added at times 0, 10 and 20 min. with thorough mixing after each addition. After 30 min. the reaction was centrifuged for 1 min. (Eppendorf microfuge) and made up to 500µl in NET containing 0.5% NP40 and 1% BSA (Buffer C). The supernatant was layered onto a Sephadex G50 column previously washed with 100ml of buffer C. 200µl fractions were collected; 5µl of each fraction was taken (in duplicate) and to one set 100µl of buffer C was added followed by 100µl of 20% TCA. After precipitation the samples were centrifuged (Eppendorf microfuge) and both sets of samples counted on a Gamma counter for 10 sec.

ii) Iodogen Catalysed Iodination

25µg of purified monoclonal antibody was added to 2µg or 50µg of iodogen (Previer Chemical Company) and 500µCi of

^{125}I (Amersham) and mixed thoroughly. The reaction was left at room temperature for either 5 min (2µg of iodogen) or 1 min (50µg of iodogen). The reaction mix was added to 100µl of 10% BSA in PBS and the mixture carefully layered into a 10ml Sephadex G50 column which was previously washed with 10% BSA in PBS.

200µl fractions were collected. Then 5µl aliquots of each fraction were taken in duplicate. To one set, 100µl of 10% BSA in PBS was added, followed by 100µl of 20% TCA. The tubes were spun for 2 min. (Eppendorf Microfuge), the supernatant discarded and the pellet and the unprecipitated aliquots counted on a Gamma counter to determine the % incorporation of ^{125}I.

2.12 Immunization.

Five to six week old female Balb/c mice were immunized twice interperitoneally with 10µg of small t-PAb419 complex mixed with 500µl of Freunds incomplete adjuvant at the start and end of a three week period. The mice were boosted intravenously with the same concentration of complex in 500µl of DPBS every three days. Serum samples were monitored for the presence of antibodies to small t by staining cultures of CV1 cells infected with WT SV40 and SV d1883 and by a solid phase radioimmunoassay.

2.13 Hybridoma Production

i) Fusion

The method employed was basically that described by

Kennett et al, (1978). All fusions were carried out $2^1/2$ days after the last boost.

The spleen of a mouse was obtained and macerated in 5ml of DMEM to obtain a large number of cells in suspension. After maceration any large pieces of tissue were discarded.

SP2O/Ag14 cells (10^7) grown to mid log phase at 37°C were spun down for 4 min. at 1000rpm. The supernatant was discarded and the cells were resuspended in 5ml of DMEM.

The spleen cells and SP2O/Ag14 cells were added to a round bottom tube (a total of 10ml) and were spun down at 1000rpm for 4 min. at room temperature. The complete removal of the supernatant was critical, as any dilution of the sterile PEG, added subsequently, resulted in complete failure of the fusion process.

Stock sterile PEG was previously melted at 65°C and 0.3ml was mixed with 0.6ml of previously warmed DMEM and kept at 37°C. After mixing, 0.2ml was carefully added to the cell pellet and thoroughly mixed, and then spun at 1000rpm for 7 min at room temperature. The supernatant was discarded and the pellet resuspended in 5ml of DMEM. This suspension was added to 60ml of DMEM with 20% FCS, 500 units/ml penicillin and 100µg/ml streptomycin. The fusion was plated as 150µl aliquots over 96 well trays (Costar) and incubated at 37°C.

ii) Selection and Growth of Hybridomas

24hrs after the fusion was carried out, 150µl of DMEM with 15% FCS, 500 units/ml penicillin, 100µg/ml

streptomycin and 2 x HAT was added to each well, to allow hybrid selection. Every 3 to 4 days 100µl of DMEM supplemented with 15% FCS, 500 units/ml penicillin, 100µg/ml streptomycin and 2 x HT was added to each well to prevent excessive evaporation. The hybrids were allowed to grow for 10 to 15 days until colony sizes of at least 200 cells were obtained, and then the supernatants were screened.

Cells from wells that gave a positive response in the screening assays were carefully transferred to 24 well trays (Costar) with 0.5ml of DMEM with 15% FCS plus 2 x HT and 500 units/ml penicillin and 100µg/ml streptomycin and allowed to grow to 80% confluency.

iii) Cloning of Hybridomas in Soft Agarose

Two methods were employed:

a) This procedure is as described by Kennett et al. (1978) with some modifications. A 10ml stock of 3.3% agarose in 0.15M NaCl was made and autoclaved. 1:10, 1:100 and 1:1000 cell dilutions were made of the hybridoma line in cloning media A (DMEM with 10% FCS, 10% CS, 2 x HT and 500 units/ml penicillin and 100µg/ml streptomycin) to give 0.6ml aliquots.

The agarose stock was diluted $^{1}/_{10}$ in DMEM with 11% FCS, 11% CS, 2 x HT, 4mM glutamine, 500 units/ml penicillin, 100µg/ml streptomycin to give a final agarose concentration of 0.33% and the mix was kept at 45°C.

6 well trays (Costar) were layered with 2ml of the agarose mixture and allowed to harden at 4°C for 5 min;

0.3ml of each cell dilution was mixed with 1.7ml of the agarose mixture, mixed thoroughly and poured immediately over the first layer. This layer was allowed to harden at room temperature, then a final 2mls of agarose mix was layered onto the cell containing layer and allowed to set at room temperature. Colonies were grown at 37°C for 8 to 10 days, then single colonies were picked up, grown in cloning media A, subcloned again by the same method, and finally grown as a continuous cell line in DMEM with 10% FCS, 500 units/ml penicillin and 100µg/ml streptomycin.

b) Method as described by Civin and Banquerigo (1983).

A 10ml stock of 3% agarose (low gelling temperature SEA KEM) in double distilled H_2O was made, autoclaved and kept at 37°C.

2 x DMEM supplemented with 20% FCS, 1000 units/ml penicillin, 200µg/ml streptomycin and 4mM glutamine (cloning media B) was prepared and also kept at 37°C.

A serial dilution of the cell line to be cloned was made in DMEM giving 300µl final volume.

The agarose stock and the cloning media B were mixed 1:1 and 2ml of this mix were added to 5ml tubes (Falcon) containing 150µl of each cell dilution, mixed thoroughly, allowed to set at room temperature and incubated at 37°C. Clones were allowed to develop for 7 to 8 days. Single colonies were picked up, grown in cloning media B, recloned using the same method and then grown in DMEM supplemented

with 10% FCS, 500 units/ml penicillin and 100µg/ml streptomycin as an established cell line.

2.14 Solid Phase Radioimmunoassay

25µl of a bacterial extract prepared from E. coli cells synthesising small t or control cells was added to each well of a 96 well flexible plastic microtitre plate (Flow Laboratories), and allowed to dry overnight at 37°C. The plates were washed once with PBS, and 25µl of hybridoma supernatant was added to each well and incubated for 2hrs at room temperature. The plates were then washed 5 times with PBS, and 25µl (50000cpm) of iodinated sheep anti mouse immunoglobulin (Amersham) previously diluted in PBS containing 5% FCS was added to the wells. After 1.5hrs. incubation at 4°C, the plate was washed 5 times with PBS, dried and cut up using a hot wire machine. The individual wells were counted in a Gamma counter.

2.15 Sandwich Assay

A serial dilution of mammalian cell extract (prepared as indicated in Section 2.4) in NET containing 0.5% NP40 was made.

100µl of each dilution was mixed with 100µl testing hybridoma supernatant, as well as 100µl of high specific activity iodinated monoclonal antibody, which was previously diluted in NET with 0.5% NP40 to give 20000cpm/sample. The reaction mixtures were incubated overnight at 4°C, then 100µl of a 10% formalin fixed suspension of Staphylococcus aureus Cowan 1 (BRL) was added

and incubated for 2hrs at 4°C. Finally, to each mixture, 800µl of NET containing 0.5% NP40 was added and centrifuged for 2 min. (Eppendorf Microfuge). The supernatant was discarded and the pellet counted on a Gamma counter.

2.16 Enzyme Linked Immunoabsorbant Assay (ELISA)

25µl of bacterial extract prepared from E. coli strains synthesising small t or control cells was added to each well of a 96 well flexible plastic microtitre plate (Flow Laboratories) and allowed to dry overnight at 37°C. The plates were washed once with PBS and incubated with 50µl of 10% BSA in PBS for 24 hrs at 4°C. This blocking mixture was discarded and the plates rinsed thoroughly with PBS. 25µl of hybridoma supernatant was added and incubated for 2hrs at room temperature. The plates were washed 5 times with PBS. 25µl of horseradish peroxidase coupled rabbit antimouse immunoglobulin (DAKO), diluted 1:100 in PBS containing 5% FCS, was added to each of the wells. After 2hrs incubation at 4°C the plates were washed 3 times with PBS.

4mg of o-phenylenediamine (Miles) was dissolved in 1ml of DMSO (Sigma). A 1:100 dilution of this substrate was made in ELISA buffer pH6 and H_2O_2 (100 vols) (BDH) was added to a 1:5000 dilution. 50µl of this substrate solution was added to each well, and left for 5 min. or until the coloured reaction appeared. The reaction was stopped by adding 50µl of 2M H_2SO_4/well. Finally the colorimetric reaction was measured in a Titertek

instrument at 450nm.

2.17 Immunocytochemistry

i) Cell Fixation

a) Acetone/Methanol Method

Cells infected or transformed by SV40 were rinsed twice with 2 ml of TD and fixed on the plate for 3 min. with 5 ml of 50% acetone:50% methanol and then rinsed with PBS.

b) Formaldehyde Method

Cells infected or transformed by SV40 were rinsed twice with prewarmed DMEM pH7.3 and fixed on the plate with 3% formaldehyde in 85% DPBS for 20 min. on ice. The cells were then rinsed twice with 85% DPBS and incubated further with 50mM NH_4Cl in 85% DPBS for 15 min. and again rinsed twice with 85% DPBS. Two techniques were employed to prepare the formaldehyde fixed cells for staining of internal structures.

1) Freeze Thaw Technique

PBS from the plate was drained carefully and then the plate was placed on a dry ice:ethanol bath until frozen. The plate was thawed at room temperature, and 85% PBS was immediately added.

2) Triton Technique

0.1% Triton x100 (BDH) in DPBS was added to the plate and left for 4 min. at room temperature. The plate was washed twice with 85% PBS.

c) Glutaraldehyde Method

Cells infected or transformed by SV40 were rinsed twice with DMEM pH7.3. 4% glutaraldehyde in DPBS was added and the plate kept on ice for 20 min. Then rinsed twice with 85% DPBS. NH_4Cl in 85% DPBS was added and the plate kept on ice for 10 min. This quenching step was repeated twice and the cells rinsed twice with 85% DPBS between steps. Finally cells were exposed for internal staining by the techniques stated above.

ii) Cell Staining

a) Horse Radish Peroxidase Staining (Immunoperoxidase Staining)

After fixation cells were rinsed with 85% DPBS and incubated with 1ml of hybridoma supernatant or 10µg/100µl of purified monoclonal antibody diluted in PBS with 5% FCS at 4^oC overnight. The cells were rinsed three times with 85% DPBS and further incubated with 100µl of rabbit anti mouse immunoglobulin horse radish peroxidase conjugate (DAKO) at a 1:50 dilution in PBS with 5% FCS for 4hrs at 4^oC.

A saturated solution of o-dianisidine (Sigma) was prepared in ethanol and from this a 1:100 dilution was made in DPBS, filtered, and H_2O_2 (100 vols BDH) was added at a 1:5000 dilution. The plates were incubated in this substrate solution for 1hr, rinsed in distilled water and mounted in gelvatol (Monsanto Chemicals).

b) Fluorescence Staining

After fixation cells were rinsed with 85% DPBS and

incubated with purified monoclonal antibody at 10μg/100μl, and/or serum diluted 1:1000 in DPBS with 5% FCS at 4°C overnight. The cells were rinsed three times with 85% DPBS and further incubated with sheep antimouse immunoglobulin texas red conjugate (Amersham) at a 1:10 dilution, or with swine anti rabbit immunoglobulin fluorescein conjugate (DAKO) diluted to 1:20 in DPBS with 5% FCS, at 4°C for 2hrs. Cells were rinsed twice with 85% DPBS and once with distilled water and mounted in gelvatol (Monsanto Chemicals).

2.18 SDS Polyacrylamide Gel Electrophoresis

Proteins were electrophoresed in vertical SDS polyacrylamide gels as described by Laemmli (1970).

The separating gels were made of either 15% or 20% acrylamide with a 5% acrylamide stacking gel.

All gels were run at 4V/cm for 16hrs and were stained in 25% (^{v}/v) isopropanol, 10% (^{v}/v) coomassie brilliant blue (Biorad) for 1hr at room temperature. The gels were destained in 25% (^{v}/v) methanol and 7.5% (^{v}/v) glacial acetic acid for 2hrs at room temperature.

When ^{35}S methionine labelled proteins were electrophoresed, the gels were subsequently washed for 1hr in 100ml of EN^{3}HANCE (New England Nuclear) and then in water for 1hr. Finally the gels were dried and autoradiographed at -70°C using a Fuji RX X-ray film with a Fuji Mach II intensifying screen as described by Laskey and Mills (1977). Molecular weight markers used when running

polyacrylamide gels were the following: prestained low molecular weight marker (BRL), insulin 3kd, bovine trypsin inhibitor 6.2kd, cytochrome c 12.3kd, lysozyme 14.3kd, ß lactoglobulin 18.4kd, α chymotrypsinogen 25.7kd, ovalbumin 43kd.

High molecular weight markers (BioRad): cytochrome c 12.3kd, ß lactoglobulin 18.4kd, α chymotropsinogen 25.7kd, ovalbumin 43kd, bovine serum albumin 68kd, phosphorylase b 92.5kd, myosin (H chain) 200kd.

2.19 In vivo Labelling Extraction and Immunoprecipitation of Mammalian Proteins

Cells grown in 9cm dishes were washed twice with prewarmed DMEM pH7.3 and incubated with 2mls of DMEM minus methionine (Flow Laboratories) containing 10% FCS (methionine minus) and 10% DMEM at 37°C for 30 min. The cells were washed twice with 2ml of TD and further incubated with 2ml of DMEM minus methionine containing 10% DMEM, 10% FCS (methionine minus) and 50µCi of ^{35}S methionine (Amersham) for 3hrs at 37°C (with swirling at 20 minute intervals).

The cells were washed three times with 2mls of TD and lysed with 0.5ml of NET containing 1% NP40 and 10mM PMSF, then scraped from the plates and centrifuged for 30 min. at 10000rpm in an SS34 rotor in a Sorvall centrifuge. The supernatant was incubated with 15µl of normal rabbit anti mouse serum at room temperature for 1hr. 50µl of a 10% solution of formalin fixed Staphylococcus aureus Cowan 1

(BRL) was added, incubated on ice for 15 mins. and centrifuged for 1 min. (Eppendorf Microfuge). This last step was repeated twice and the supernatant was divided in two and and each aliquot was incubated with 5µg/100µl of different purified monoclonal antibodies previously reconstituted in DMEM with 10% FCS, at 4°C overnight. Then 50µg of rabbit anti mouse immunoglobulin (DAKO) was added, and further incubated for 2hrs at room temperature. Finally 50µl of 50% formalin fixed Staphylococcus aureus Cowan 1 was added and incubated on ice for 1hr and centrifuged for 30 secs. (Eppendorf Microfuge). The supernatant was discarded and the pellet was washed with (i) 50mM Tris HCl (pH7.4), 1% NP40, 0.1% SDS, 100mM NaCl; (ii) 50mM Tris HCl (pH8), 1% NP40, 600mM NaCl; (iii) 50mM Tris HCl (pH8), 1% NP40, 0.1% SDS, 0.5M LiCl; (iv) 50mM Tris HCl (pH7.9), 150mM NaCl, 5mM EDTA, 0.05% NP40, 1% BSA; (v) 50mM Tris HCl (pH7.9), 150mM NaCl, 5mM EDTA.

The pellet was resuspended in 40µl of 2 x sample buffer, boiled for 2 min. and run on a 20% SDS polyacrylamide gel.

2.20 In Vivo Labelling and Extraction of Bacterial Proteins

Single bacterial colonies were grown for 5hrs at 37°C in 4ml of M9 medium supplemented with all amino acids except methionine at a concentration of 20µg/ml. Isopropyl-ß-D-thiogalactopyranoside (IPTG; Sigma) was added to 1mM final concentration and induction allowed to take place for 10 min. at 37°C. The culture was divided in two

and to each half 50μCi of ^{35}S methionine (Amersham) was added, and incubated for 2 and 10 min at 37°C. Cells were harvested by 5 min. centrifugation at 4°C (Eppendorf Microfuge) and lysed in 500μl of NET containing 1% NP40 and 10mM PMSF, left on ice for 1hr, followed by 3 min. sonication. The extract was centrifuged for 5 min. at 4°C (Eppendorf Microfuge) and the supernatant stored at 4°C or used for immunoprecipitation as in the previous section.

2.21 Western Blotting

30 or 40μl of whole cell extracts made in 2 x sample buffer were run on a 15% polyacrylamide gel with a 5% stacking gel at 4V/cm for 16 hrs.

Protein bands from the gel were transferred onto nitrocellulose paper (Schleicher and Schull) for 2hrs at 1 amp using an E.C. Electroblot Apparatus.

Three types of molecular weight markers were employed:

i) Radioactive markers (High Molecular Weight Markers)

In this case the nitrocellulose papers were dried on filter paper and exposed to an RX Fuji X ray film with a Fuji Mach II intensifying screen for 6hrs at -70°C.

ii) Non Stained High Molecular Weight Marker (Biorad).

The paper was stained in Ponceau-S (Sigma) and destained in 3 washes of PBS.

iii) Prestained Low Molecular Weight Markers (BRL)

Markers were directly observed in the nitrocellulose paper.

After either of these procedures, the blot was incubated in 200ml of PBS with 10% BSA (blocking solution) overnight at 4°C. It was then incubated overnight with 5ml of hybridoma supernatant at 4°C. After washing 5 times with PBS containing 1% NP40 the blot was incubated further with horse radish peroxidase conjugated rabbit anti mouse immunoglobulin (DAKO) diluted 1:100 in PBS containing 5% FCS overnight at 4°C. Finally the blot was washed twice in PBS containing 1% NP40, once in PBS with 0.1% NP40 and 10% SSC for 5 min. and rinsed twice in PBS alone. The blot was developed using a 4-chloro-1-naphthol saturated solution in ethanol, diluted 1:100 in PBS, filtered through Whatman paper and H_2O_2 (100 vols BDH) added as a 1:5000 dilution. The reaction was stopped by discarding the substrate solution and rinsing the paper in PBS with 0.05% sodium azide.

2.22 Silver Staining of SDS Polyacrylamide Gels (Sammons et al., 1981)

30µl protein samples prepared in 2 x sample buffer were run on a 15% polyacrylamide gel with a 5% stack for 16 hrs. at 4V/cm.

The gel was fixed by washing twice for 2hrs with 50% ethanol (96% $^{V}/v$) and 10% glacial acetic acid, then once for 2hrs in 25% ethanol (96% $^{V}/v$) and 10% glacial acetic acid, and finally once for 2hrs. in 10% ethanol (96% $^{V}/v$) and 5% glacial acetic acid. The gel was then incubated in 0.2% silver nitrate for 2hrs. and rinsed for 20sec. in

degassed distilled water. Finally it was developed in reducing reagent (3% NaOH, 0.09% $NaBH_4$ and 0.8% HCHO - the $NaBH_4$ was added just before use). When the reaction was completed the reducing reagent was discarded and the gel stored in 7.5% glacial acetic acid and 25% methanol.

2.23 Measurement of Protein Concentration

i) Modified Lowry Assay (Peterson, 1977)

A serial dilution of a known concentration of BSA was made in distilled H_2O in the range of 1 to 100µg of protein/ml. The protein standards were made up to 1ml with distilled H_2O and 1ml of reagent A [2% SDS, 160mM NaOH, copper sulfate solution (0.08% cupric sulfate, 0.24% potassium tartrate) and 10% sodium carbonate] followed by 0.5ml of 0.2N Folin and Ciocalteau phenol reagent (Sigma) were added. The reaction was vortexed immediately and 30 min. later the absorbance read at 750nm in a Gilford 250 Spectrophotometer.

Unknown protein samples were serially diluted in distilled H_2O to obtain a concentration which could be read in the mid log position of the standard curve. Unknowns were tested as described above.

ii) Spectrophorometric Assay

Purified monoclonal antibodies or PAb419-small t complex concentrations were measured by reading the absorbance at 280nm in a Gilford 250 spectrophotometer. A 1:1 mixture of 1M Tris HCl pH8.8 and citrate buffer pH3 was used as the blank.

2.24 Gel Diffusion

1% agarose (SEA KEM) was dissolved in PBS and poured onto a 1.5mm microscope slide. The agarose was allowed to harden for 15 min. at 4°C. A hexagonal array of holes was punched, 5µl of the test antibody was added to the middle well and 5µl of the different subclass anti serum at different dilutions was placed in each of the wells in the hexagonal array.

The slides were incubated at 4°C until the immunoprecipitation reaction was visible.

2.25 Ascites Fluid Production

Five to six week old female Balb/c mice were injected intraperitoneally with 0.5ml of pristane oil. 14 days later 10^6 hybridoma cells were intraperitoneally injected into the primed mice. Ascites fluid was extracted 10 to 14 days after hybridoma innoculation.

TABLE 2.1

Solutions Used for Eukaryotic Cell Growth

TD	25mM Tris HCl pH7.4
	136mM NaCl
	5.7mM KCl
	0.7mM Na_2HPO_4
Ca^{2+}/Mg^{2+} Solution	21mM $MgCl_2$
	18mM $CaCl_2$
TS	TD Containing 5% Ca^{2+}/Mg^{2+} and 2% FCS
2 x HAT Stocks	Hypoxanthine 680mg/100ml dH_2O use $^1/250$
	Aminopterine 92.8mg/500ml dH_2O use $^1/250$
	Thymidine 1.45g/100ml dH_2O use $^1/250$
2 x HT Stocks	Hypoxanthine 680mg/100ml dH_2O use $^1/250$
	Thymidine 1.45g/100ml dH_2O use $^1/250$

TABLE 2.2

Media for Bacterial Growth

L-Broth	1% ($^{W}/v$) Difco Bacto Tryptone, 0.5% ($^{W}/v$) Difco Bacto Yeast Extract 0.5% ($^{W}/v$) NaCl, 0.1% ($^{W}/v$) Glucose Autoclave and store at $4^{o}C$
L-Agar	L-Broth containing 1.5% ($^{W}/v$) Difco Bacto Agar Autoclave and store at $4^{o}C$
-Broth	2% ($^{W}/v$) Difco Bacto Tryptone 0.5% ($^{W}/v$) Difco Bacto Yeast Extract 20mM $MgSO_4$, 10mM NaCl, 5mM KCl adjusted to pH7.5 with KOH Autoclave and store at $4^{o}C$
-Agar	Broth containing 1.5% ($^{W}/v$) Difco Bacto Agar Autoclave and store at $4^{o}C$
M9 Minimal Salts	28g/litre Na_2HPO_4, 12g/litre KH_2PO_4, 2g/litre NaCl, 4g/litre NH_4Cl Autoclave and store at $4^{o}C$
Amino Acids & Vitamins	Added as 20µg/ml Filter sterilised

TABLE 2.3

Buffers for Bacterial Transformation

TFB_I	10mM RbCl, 50mM $MnCl_2$, 10mM $CaCl_2$, 35mM CH_3 COONa, 15% (^{v}/v) Glycerol adjusted to pH5.8 with Acetic Acid and then filter sterilized.
TFB_{II}	10mM RbCl, 75mM $CaCl_2$, 10mM MOPS, 15% (^{v}/v) Glycerol pH6.8 adjusted with KOH and then filter sterilized.

TABLE 2.4

Solutions for Protein Gel Electrophoresis

Stocks	30% (w/v) Acrylamide
	40% (w/v) Acrylamide
	2.5% Bis Acrylamide
	1M Tris HCl pH8.8
	1M Tris HCl pH6.8
	20% SDS
	10% Ammonium Persulphate (freshly prepared)
	Temed (BDH)
15% Gel	15ml of 30% Acrylamide Stock
	11.20ml of 1M Tris HCl pH8.8
	1.04ml of 2.5% Bis Acrylamide
	0.15ml of 20% SDS
	2.6ml of dH_2O
	0.15ml of 10% Ammonium Persulphate
	0.015ml of Temed
20% Gel	15ml of 40% Acrylamide
	11.2ml of 1M Tris HCl pH8.8
	1.38ml of 2.5% Bis Acrylamide
	0.15ml of 20% SDS
	2.26ml of dH_2O
	0.15ml of 10% Ammonium Persulphate
	0.015ml of Temed

5% Stacking Gel	1.67ml of 30% Acrylamide
	1.25ml of Tris HCl pH6.8
	0.65ml of 2.5% Bis Acrylamide
	6.43ml of dH_2O
	0.05ml of 20% SDS
	0.04ml of 10% Ammonium Persulphate
	0.04ml of Temed
Running Buffer	25mM Tris Base, 192mM Glycine, 0.1% ($^{W}/v$) SDS
Sample Buffer	62.5mM Tris HCl pH6.8
	2% ($^{W}/v$) SDS, 100mM DTT
	10% ($^{V}/v$) Glycerol
	0.01% ($^{W}/v$) Bromophenol Blue (BioRad)
Western Transfer Buffer	0.25mM Tris base, 0.37mM Glycine adjust to pH8.3 with HCl

TABLE 2.5

Buffers for Antibody Purification

Phosphate Buffer pH8	0.01M Na_2HPO_4
	0.01M NaH_2PO_4
	Mix both to pH8
Citrate Buffer pH3	0.1M Sodium Citrate
	0.1M Citric Acid
	Mix both to pH3

TABLE 2.6

List of General Buffers

1 x SSC	Standard Saline Citrate
	0.15M Sodium Chloride
	0.015M Trisodium Citrate
NET	150mM NaCl
	5mM EDTA
	50mM Tris HCl pH7.9
Elisa Buffer	0.2M Dibasic Sodium Phosphate
	0.1M Citric Acid
	Mix to pH6
PBS	150mM NaCl
	37mM NaH_2PO_4
	Adjust to pH7.2 with NaOH
DPBS	2.6mM KCl
	1.4mM KH_2PO_4
	136mM NaCl
	8mM Na_2HPO_4
	Adjust to pH7.3 with NaOH

CHAPTER THREE

PRODUCTION AND CHARACTERIZATION OF PAB280

3.1 Introduction

This Chapter will describe: a) The extraction and purification of small t from a prokaryotic source in order to use it as (i) an antigen to immunise animals (ii) as a source to develop a hybridoma screening assay, b) The production of monoclonal antibodies, the subsequent selection of PAb280 and the specificity for small t of PAb280 in immunoprecipitation and Western blotting analysis of infected cells; c) The location of the epitope recognized by PAb280 within the primary structure of small t determined by exploiting a range of small t deletion mutant viruses; d) The relative affinity of PAb280 and PAb419 for small t.

3.2 Extraction and Purification of Small t from E. coli

Protein purifications are long processes in which two problems often arise: (a) the yield of the purified protein can be very low and (b) during the purification process the protein can become denatured. Therefore a method had to be devised that could overcome these particular problems.

There was however, another critical aspect to the development of this particular purification protocol; the necessity of using the purified protein as an antigen. SV40 large T and small t share 82 amino acids at their amino-terminus. Most antibodies (monoclonal antibodies or anti T/t serum) are directed to the common region of these proteins or the unique region of large T (Tooze, 1981) and none to the unique region in small t, indicating

the highly immunogenic nature of the amino-terminus.

Therefore it was decided that an antibody such as PAb419 (Harlow et al., 1981) which recognises an epitope in the amino-terminus common region shared between large T and small t could be used to immunopurify small t from cell extracts, and that this antibody-antigen complex could be used to immunise animals. Because the PAb419 is bound to this common region, thereby masking it, the immune response from the animal might be stronger towards exposed sites, that is, the carboxy unique terminus of small t.

This work was initiated using E. coli 294 cells transformed by HP1 plasmid (Thummel et al., 1981). In order to test if the plasmid expressed small t, a single colony was obtained, the cells grown as indicated in section (2.7(ii)(d)) and pulse labelled for 2 min. and 10 min. ^{35}S methionine labelled proteins were extracted as indicated in section (2.20) and mixed with either PAb416, PAb419, PAb2D1 (hybridoma supernatants) or rabbit anti T/t serum. The samples were treated as indicated in section (2.19) and run on a 15% SDS polyacrylamide gel with a 5% stack. Also included were two tracks showing whole cell lysates labelled for 2 min and 10 min.

The results are shown in figure (3.1). Tracks 1 and 2 show whole cell lysate protein labelled for 10 and 2 min respectively. Tracks 3, 4, 5 and 6, are cell lysates labelled for 2 min. and immunoprecipitated with PAb2D1 (acting as the negative control, PAb2D1 is a monoclonal

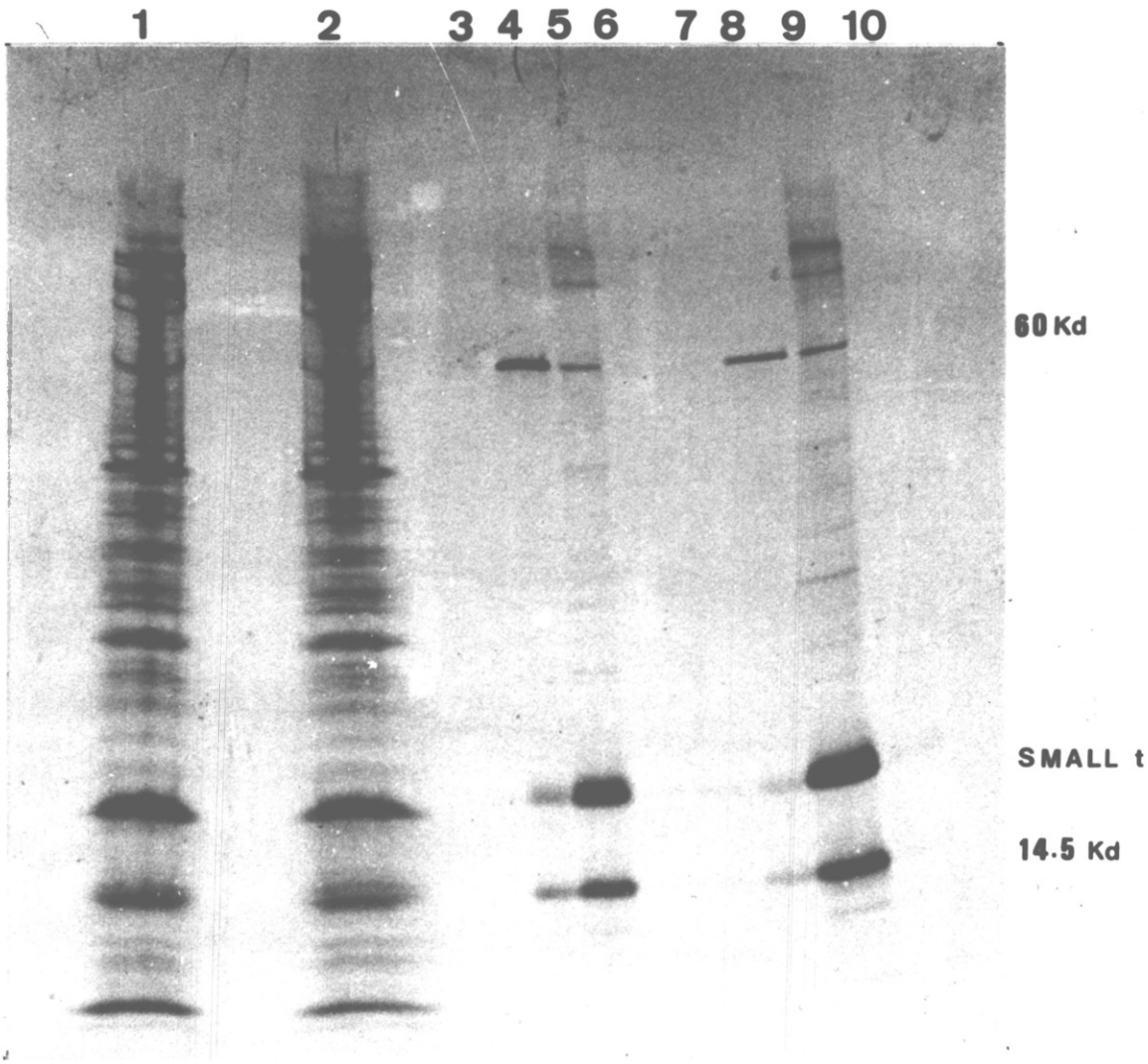
Figure 3.1. Immunoprecipitation of SV40 Small t From E. coli 294 Cells Transformed by HP1.

Cells were grown (Section 2.1(ii)(d)) and pulse labelled for 2 min and 10 min with ^{35}S methionine. Bacterial proteins were extracted (Section 2.20) and immunoprecipitated with PAb416, PAb419, PAb2D1 or rabbit anti T/t serum. The samples were treated as indicated in Section (2.19) and run on a 15% SDS polyacrylamide gel with a 5% stack (24hr exposure).

Tracks 1 and 2 are whole cell lysates induced with IPTG and labelled for 10 min and 2 min.

Tracks 3, 4, 5 and 6 are cell lysates labelled for 2 min and immunoprecipitated with PAb2D1, rabbit anti T/t serum, PAb419 and PAb416.

Tracks 7, 8, 9 and 10 are cell extracts labelled for 10 min and immunoprecipitated with the same antibodies (the order of antibodies is the same as for tracks 3, 4, 5 and 6).


1
2
3 4 5 6
7 8 9 10
60 Kd
SMALL t
14.5 Kd

antibody that recognizes enkephalin Jones et al., 1983). Rabbit anti SV40 T/t serum, PAb419 and PAb416 (the second negative control which recognises large T, see Appendix). Tracks 7, 8, 9 and 10 are cell extracts labelled for 10 min and immunoprecipitated with the same antibodies. This figure shows that PAb419 and rabbit anti SV40 T/t can recognise small t and the 14.5Kd product reported by Thummel et al., 1981 (see Figure (3.2)). As detected by PAb419 the amounts of small t in extracts labelled for 2 min or 10 min are similar. Therefore, the half life of the protein is not long enough for detection but the protein appears to be stable as no degradation products have been seen.

Although there seems to be more small t at 2 min. than at 10 min labelling using rabbit anti SV40 T/t serum, this is actually due to a loading error and not to any real differences in synthetic levels.

It can also be seen that PAb419 and the T/t antiserum recognise a protein of approximately 60kd which might be a cross reactive bacterial protein.

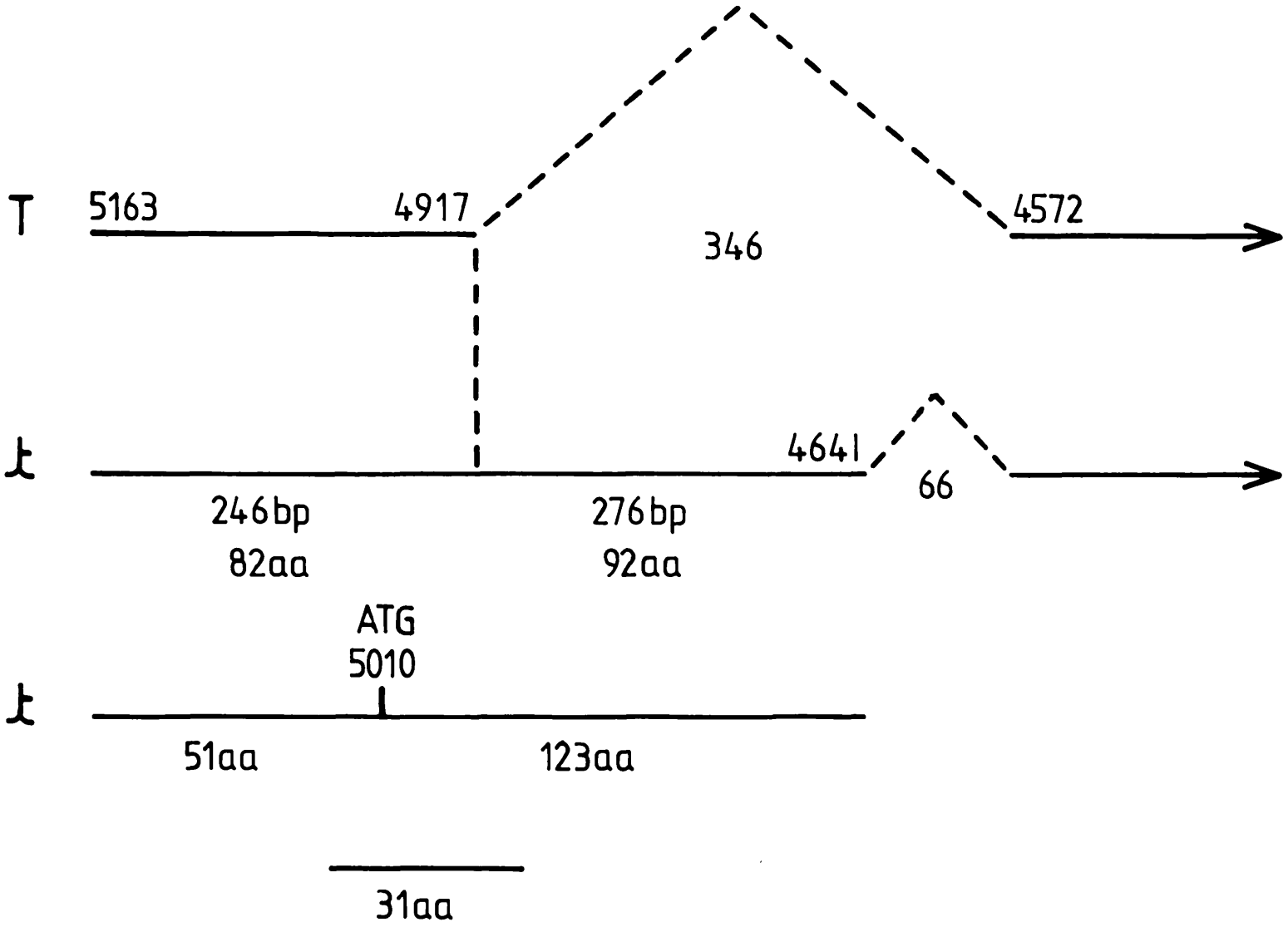
PAb2D1 and PAb416 act as negative controls, that is, they do not recognise small t.

Finally, when whole protein extracts are observed it can be seen clearly that there are two bands which comigrate with small t and the 14.5kd product respectively immunoprecipitated by PAb419 and rabbit anti T/t serum.

This experiment clearly indicated that small t was produced by this plasmid and therefore a large scale

Figure 3.2. Diagram Showing the Region of Small t that is Translated to Give a 14.5Kb Product

Thummel et al., 1981; Derom et al., 1982; Bikel et al., 1983 and Queen, 1983 have reported the production of a 14.5Kd protein product which contain common amino acid sequences to small t. This product is thought to be expressed from an internal ATG codon at position 5010. (Nucleotide numbering system of Buchman, Burnett and Berg; Tooze, 1981).


T
5163
4917
4572
346
上
4641
66
246bp
82aa
276bp
92aa
ATG
5010
上
51aa
123aa
31aa

immunoprecipitation was carried out as indicated in section (2.9). Small t from 294 bacterial lysate was allowed to bind to purified PAb419 monoclonal antibody under prolonged incubation at 4°C. The complex was then allowed to bind to Sepharose 4B beads coated with Staphylococcus aureus protein A (Sigma) and finally eluted.

When samples of each fraction as well as samples of IPTG induced and non-induced HP1 transformed bacterial cell extracts were run on a 15% SDS polyacrylamide gel with a 5% stack, small t was only detectable when silver staining was employed. (This procedure is 50x more sensitive than coomassie staining; amounts in the order to 20ng of protein can be detected).

Figure (3.3) shows this result. Tracks 1 and 2 are whole cell extracts of IPTG induced and non-induced HP1 transformed 294 cells. The appearance of these tracks suggests protein overloading but this is just the result of the sensitivity of the stain. Tracks 3, 4, 5, 6 and 7 are eluted fractions from the protein A sepharose column. PAb419 antibody heavy and light chains are clearly observed, as well as decreasing amounts of small t (Tracks 3, 4 and 5). Tracks 6 and 7 also show heavy and light chains of the monoclonal antibody but no small t can be detected.

This result indicated that the purification procedure did work (no background proteins are observed) but small t was found to be present in very limited amounts.

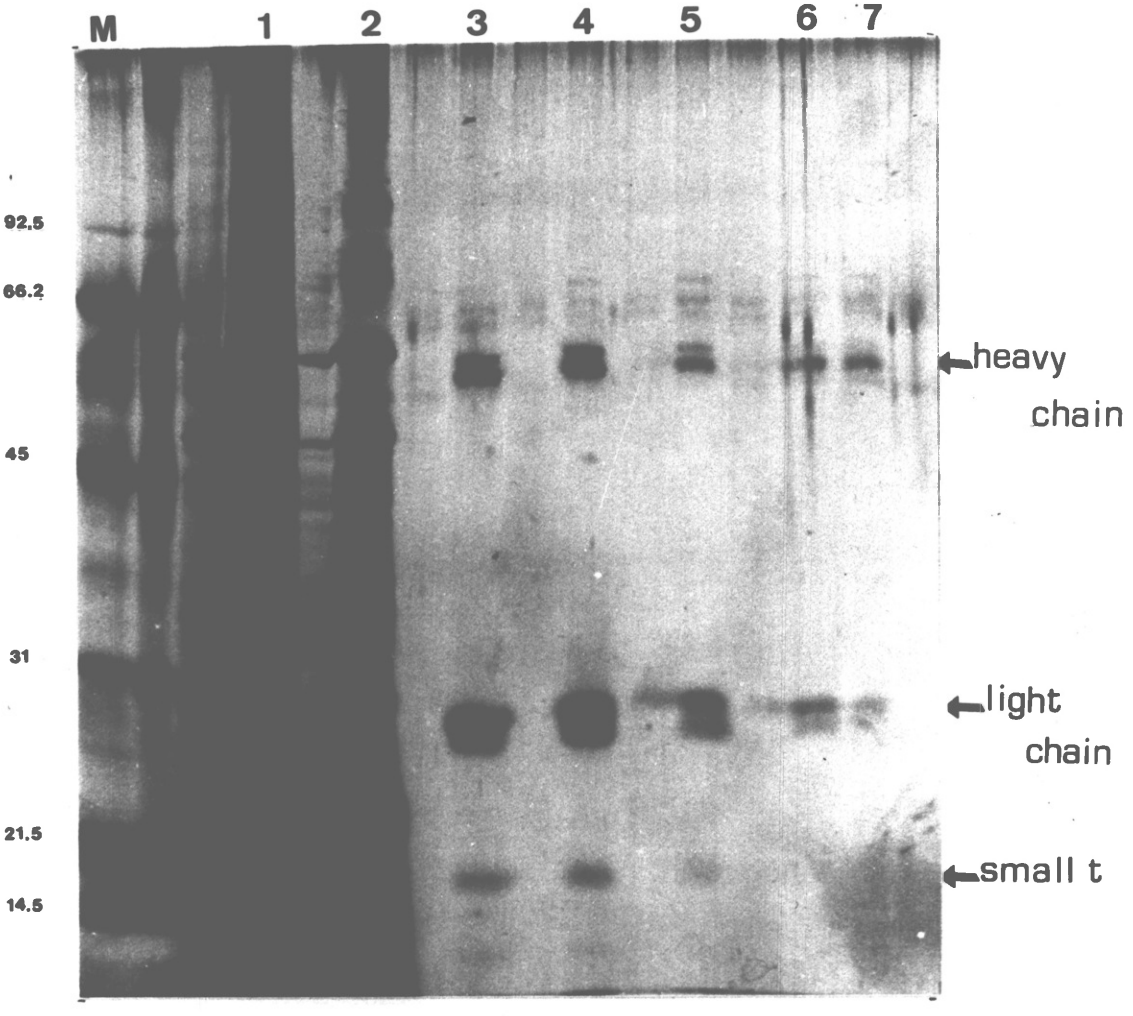
Figure 3.3. Immunoprecipitation of Small t From HPl Transformed E. coli 294 Cells (Silver Staining Technique).

A large scale immunoprecipitation of small t using purified PAb419 was carried out (section 2.9) and a sample from each fraction as well as samples of IPTG induced and non-induced cultures were run on a 15% SDS polyacrylamide gel with a 5% stack and silver stained (Section 2.22).

Track M shows the molecular weight markers.

Tracks 1 and 2 are whole cell extracts which have been IPTG induced and non-induced.

Tracks 3, 4, 5, 6 and 7 are eluted fractions from the protein A sepharose column.


M
1
2
3
4
5
6
7
92.5
66.2
45
31
21.5
14.5
heavy chain
light chain
small t

The small t yield under these extraction conditions was not increased even when larger bacterial cultures were employed. It was also noticed that different yields were obtained from extraction to extraction. This indicated an instability of the plasmid in this bacterial strain as further evidenced by the failure of 1 month old stab cultures to produce colonies on L-amp plates. Therefore purified HP1 plasmid was used to transform the following bacterial strains 7118, DH1 and HB101 (see section 2.8(iii)).

When these strains were tested for small t production it was clear that HB101 gave the highest yields.

In order to optimise small t extraction from HB101 cells transformed by HP1 the procedure described in section (2.8(iv)) was carried out using the following buffers: NET with 1% NP40 and 10mM PMSF, NET with 10mM PMSF, NET with 1% NP40 and NET alone.

With these extracts a radioimmunoassay for small t was devised (see section 2.14) and small t titrated with PAb419 and PAB423 (PAb423 is a monoclonal antibody that recognizes a unique sequence at the carboxy terminus of SV40 large T and therefore does not recognize small t (see Appendix).

Results obtained in this experiment showed that PAb419 detected more efficient extraction of small t when using NET with 1% NP40 and 10mM PMSF. The control PAb423 gave low background binding indicating that the assay is specific for small t (see Figure (3.8) showing the radioimmunoassay for small t using PAb280). In order to

improve further the amounts of small t extracted from HB101 transformed by HP1, cells were grown for times 4, 6 and 17hrs and induced with IPTG for 15 min. Proteins were extracted as indicated in section (2.8(iv)) and tested for small t using a radioimmunoassay (section (2.14)). The results showed that there is more small t produced when the cells are grown for 4hrs (followed by IPTG induction). At this time the culture is in mid log phase.

Finally HB101 cells transformed by HP1 were grown for 4hrs, induced with IPTG to 1mM final concentration, a cell extract made as indicated in section (2.8(iv)) and a large scale immunoprecipitation performed using PAb419. The small t-PAb419 complex was purified as indicated in section (2.9).

Samples from eluted fractions as well as IPTG induced whole bacterial extract were run on a 15% SDS polyacrylamide gel with a 5% stack and stained with coomassie blue.

Figure (3.4) shows the result of this experiment. Tracks 1 and 2 show the heavy and light chains of immunoglobulin of PAb419 as well as small t, whereas track 3 shows heavy and light chains of PAb419 only. Track 4 is IPTG induced whole cell extract. All of these results show that HP1 produces more small t in HB101 cells than in 294 cells. Therefore small t produced in HB101 was employed during initial stages of this project as will be described in sections (3.3 and 3.5). However, the amounts

Figure 3.4. Immunoprecipitation of Small t From E. coli HB101 Cells Transformed by HP1.

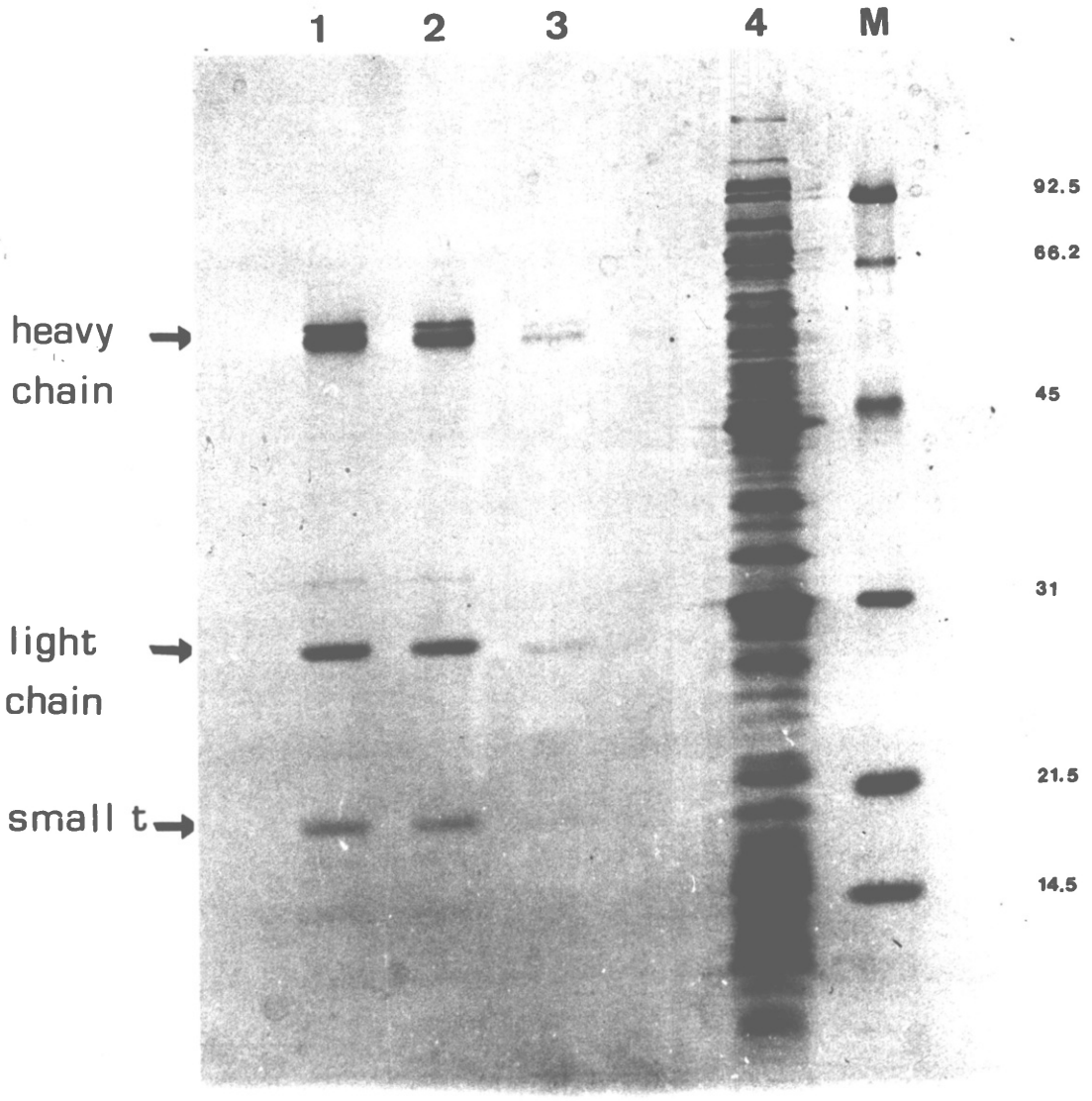
HP1 transformed HB101 cells were grown for 4hrs, induced with IPTG to 1mM final concentration. The proteins were extracted (section 2.8(iv)) and a large scale immunoprecipitation using purified PAb419 was carried out (section 2.9).

Samples from eluted fractions as well as IPTG induced whole cell extract were run on a 15% SDS polyacrylamide gel with a 5% stack and stained with coomassie blue.

Tracks 1, 2 and 3 are samples eluted from the protein A sepharose column.

Track 4 shows IPTG induced whole cell extract.

Track M shows the molecular weight markers.


1
2
3
4
M
heavy chain
light chain
small t
92.5
66.2
45
31
21.5
14.5

small t, which in this case is a very prominent band (only one representative fraction eluted from the protein A Sepharose column is shown in this gel). Track 3 shows IPTG induced cell extracts in which both small t and the 14.5kd product are clearly visible. Track 4 shows whole cell extracts of cells containing pTR865 but are not IPTG induced; therefore, no small t or 14.5kd products are observed.

The expression of this plasmid in X-90 cells proved to be sufficient for all experiments; large amounts of small t were obtained which optimised the immunisation process (section (3.3)) and resulted in the development of a very sensitive screening assay which was critically important for the successful completion of this work.

3.3 Immunisation and Monitoring of Immunisation

PAb419 is a monoclonal antibody that was obtained by the fusion of splenocytes from Balb/c mice with NS1/Ag cells (Harlow et al., 1981). As was previously described the aim of the purification was to produce a small t-PAb419 complex which was to be used as the antigen. 5 to 6 week old Balb/c female mice were used for immunisation. Other mouse strains were not suitable as antiallotype antibodies could be formed and any response by the mice to small t might be obscured.

The actual procedure for the immunisation has been described in section (2.12).

produced were still not optimal for the preparation of PAb419-small t complex for use as an immunogen, or for the development of a very sensitive screening assay.

After improving small t yields obtained by HP1 on HB101 cells a second plasmid pTR865 was obtained (Bikel et al., 1983). X-90 cells transformed by pTR865 were reported to produce small t at levels approaching 5 to 10% of the total bacterial protein.

Small t production from this plasmid was tested by growing the cells as indicated by I. Bikel et al., (1983) (section (2.8(ii)(c)). Cell extracts were made as indicated in section (2.8(iv)) and tested for small t amounts using the same radioimmunoassay employed for HP1 transformed HB101 cells.

Results of this assay clearly indicated that larger amounts of small t were produced by this plasmid (in the neighbourhood of 5% total bacterial protein).

The next step was to see how much small t was able to complex with PAb419 in the previously described immunopurification (section (2.9)).

Figure (3.5) shows the result of this protocol. A sample from one eluted fraction as well as a control, followed by IPTG induced and noninduced cell extracts were run on a 15% SDS polyacrylamide gel with a 5% stack and coomassie blue stained.

Track M shows the molecular weight markers, track 2 the heavy and light chains of PAb419 immunoglobulin and track 1 shows heavy and light chains of PAb419 as well as

Figure 3.5. Immunoprecipitation of Small t From X-90 Cells Transformed with pTR865.

pTR865 transformed X-90 cells were grown (Section 2.8(ii)(c)) and extracts made as indicated in Section (2.8(iv)) and a large scale immunoprecipitation using purified PAb419 was carried out (section 2.9).

Samples were run on a 15% SDS polyacrylamide gel with a 5% stack and the gel was stained with coomassie blue.

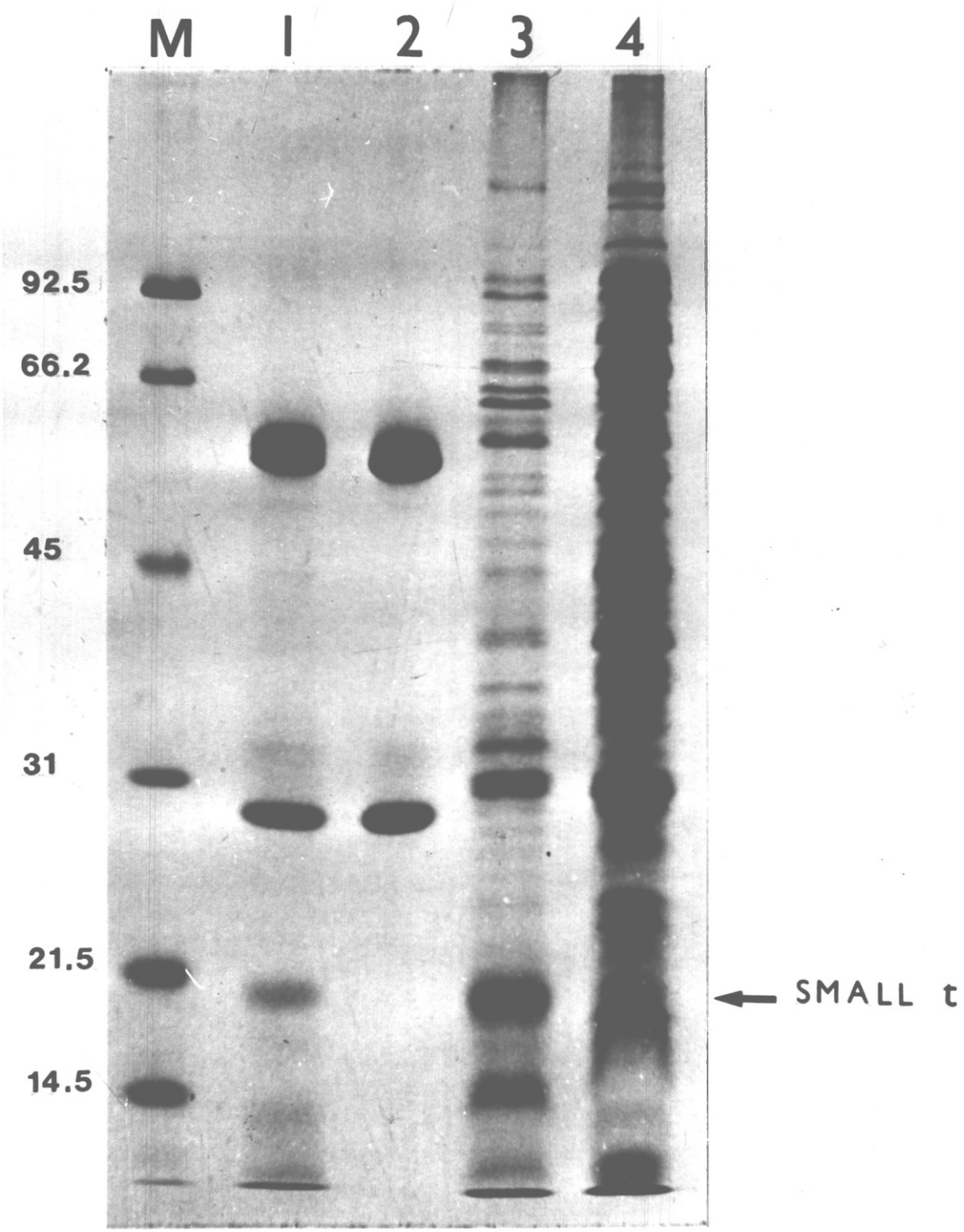
Track M shows molecular weight markers.

Track 1 shows a sample from an eluted sample.

Track 2 shows the heavy and light chains of PAb419.

Track 3 shows IPTG induced cell extract.

Track 4 shows non-induced cell extract.


M
1
2
3
4
92.5
66.2
45
31
21.5
14.5
SMALL t

During initial stages of this project mice were immunised twice interperitoneally with less than 1µg of smallt-PAb419 complex (obtained from 294 cells transformed by HP1) mixed with Freund's complete adjuvant. Subsequently the mice were intravenously boosted with less than 1µg of complex mixed with DPBS as a 1:1 mixture. In this case optimal immunisation was only possible when extended over periods of up to 5 months.

When spleens from these mice were obtained and cells fused to SP2O/Ag14 myeloma cells the hybrids produced secreted antibodies against bacterial proteins and not against small t. This was due to the usage of Freund's complete adjuvant. Therefore in subsequent immunisations Freund's incomplete adjuvant was employed.

As the production of small t was improved by transforming HB1O1 cells with HP1, it became possible to inject 2µg of small t-PAb419 complex per intraperitoneal injection.

During the boosting stages X-90 cells transformed by pTR865 became available and were used as the source of small t. This dramatically increased the amounts of small t thus allowing the use of 1Oµg of PAb419-small t complex for each injection.

To monitor the immunisation procedure individual mice were tail bled, the serum diluted $^{1}/_{10}$, $^{1}/_{100}$ and $^{1}/_{1000}$ in DPBS and used in the 'spot' staining assay described below.

CV1 cells were grown in 9cm plates to 60% confluency and infected with either WT SV4O or SVd1883 or mock

infected for 60hrs. The cells were fixed by the acetone:methanol method described in section (2.17(i)(a)). The plates were dried at room temperature and the serum spotted onto the cells. Positive reactions were detected using horseradish peroxidase coupled to rabbit anti mouse immunoglobulin and o-dianisidine as the substrate as indicated in section (2.17(ii)(a)).

3.4 Fusion

Fusions were carried out as described by Kennett et al., (1978) with the modifications indicated in section (2.13(i)). For all fusions SP20/Ag14 myeloma cells were employed.

Fusions were performed two and a half days after the mouse received the final boost (intravenous injection). Single fusions were made every 10 days; each fusion gave between 100 to 400 hybrids, and 12 fusions were done, giving a total of 3500 hybrids.

For an initial fusion NSI/Ag plasmacytoma cells were employed. Unfortunately, hybrid yields were very poor (between 50 and 100) and those retained were extremely fragile. It has also been found (D.P. Lane, personal communication) that fusions carried out with this myeloma line produce a higher rate of revertant hybrids. Therefore SP20/Ag14 cells were used in all subsequent fusions.

The fusions were distributed over 96 well trays in order to separate the hybrid cells and to allow the formation of single clones.

Clones of up to 200 cells were obtained after 8 to 10 days of growth.

3.5 Screening

As this was to be the first monoclonal antibody to recognise only small t, a screening assay had to be devised that could fulfil the following requirements:

a) An assay that could allow binding to small t with high specificity;

b) A method that could discriminate between binding to the amino or carboxy terminus of small t;

c) The assay had to be simple and fast as hundreds of hybridoma supernatants were going to be screened.

As has been previously stated the main limitation in all these experiments was the low yields of small t.

A series of screening assays such as ELISA were devised (section 2.16), but these did not give satisfactory results with the exception of the radioimmunoassay which was initially developed in order to test small t production by the bacterial cells.

The radioimmunoassay described in section (2.14) was developed further in order to increase its sensitivity to be used as a screening assay. Although there was a considerable difference between small t detection by PAb419 and background binding as indicated by PAb423, there was still the need to increase this difference, as there are differences in levels of antibody production among hybrid colonies of the same size. Therefore a PAb419 hybridoma

supernatant titration was carried out to determine the sensitivity of the assay with respect to the monoclonal supernatant.

Results obtained showed that small t was detected at dilution up to $^{1}/_{8}$ PAb419 with low background reading as indicated with PAb423.

The results of this experiment and the experiment shown in section 3.2 established that this assay was the method of choice, but it is important to note that the efficiency of this assay reflected the quantity of small t produced and reached its maximum using the plasmid pTR865 in X-90 cells (Bikel et al., 1983).

Although a screening assay that allowed the recognition of small t by the monoclonal antibodies was developed, it was extremely important to prepare an assay that could discriminate between antibodies recognising either the amino-terminus or the carboxy-terminus of small t.

As has been previously discussed, SV40 large T and small t share 82 amino acids at their amino-terminus, and all previously prepared monoclonal antibodies that bind small t map in this region, therefore recognising both large T and small t.

Fortunately SV40 deletion mutants have been constructed which produce truncated or non-detectable small t (Shenk et al., 1976; Cole et al., 1977). These deletions occur in the SV40 region 0.54 to 0.59 map units, produce a

wild type large T and are viable in monkey cells. One deletion mutant in particular, SVd1883, (which does not produce any detectable small t) was used to develop a secondary screening assay, to clearly establish the unique nature of a small t recognising antibody. The following assay was developed:

CV1 cells were plated as indicated in section (2.3(i)) and infected with WT SV40, SVd1883 or mock infected for 60hrs. They were fixed as indicated in section (2.17(i)(a)) and stained with PAb419 or the testing hybridoma supernatant as indicated in section (2.17(ii)(a)). The desired result of this experiment was the detection of an antibody that would stain CV1 cells infected with WT SV40 (i.e. both large T and small t are present) but would not stain cells infected with SVd1883 (in which only large T is produced).

This assay proved to be successful and therefore was the method of choice.

3.6 Growth of Clones

Hybridomas which looked promising in the previous screening assays were allowed to grow to confluency in 96 well trays, then were carefully transferred to 24 well trays and grown to 80% confluency (see section 2.13(ii)). Because hybridomas are very fragile and susceptible to contamination as a result of the handling procedure, half of these cells were then used to inject mice to obtain ascites fluid (see section 2.25), and the other half

transferred to a 3.5cm plate and the cells grown in DMEM supplemented as indicated in section 2.13(ii). When cells reached 70 to 80% confluency half of the cells were cloned using the soft agarose method (section 2.13(iii)(a)). Cells left on the plate were frozen down as indicated in section (2.3(ii)).

3.7 Cloning of Hybrids

After a fusion, hybrid cells are extremely fragile. Two factors determine their successful development: (i) the media and the combination of supplements and (ii) the handling of the cells. Although contamination is always a problem, extra care is needed with these cells. During the development of hybrid colonies some of the supernatant has to be regularly harvested and replaced by fresh media, but at the same time it is essential that the colonies should not be disturbed until at least 100 cells are obtained, as the media must be conditioned by the cells in order to provide the proper growing conditions.

Even after colonies start to develop, revertant cells may grow. It is therefore essential to clone these cells before they are passaged, as these revertants may overgrow the cells which are producing the antibodies.

(i) Soft Agarose Cloning

Soft agarose cloning as described by Kennett et al., (1978) (section (2.13(iii)(a)) was employed.

During trial stages a range of commercially available agarose was employed to see which one gave the highest

cloning capacity. Ultimately SEAKEM agarose was employed.

The system was set up as follows: 6 well trays (Costar) were layered with the agarose prepared in cloning media and allowed to harden, then agarose containing the hybrid cells was layered on top, followed by a third layer of agarose prepared in cloning media. The purpose of this layering was to allow hybrid colonies to develop individually embedded between the agarose-media layers, rather than at the bottom of the well where they tend to spread, hampering the selection of single colonies.

This method was used throughout as it was successful and produced colonies of up to 200 cells in 8 to 10 days of incubation.

Single colonies were picked and grown in 24 well trays to 70-80% confluency (see section 2.13(iii)(a)). 200µl of hybridoma supernatant was removed and screened by staining CV1 cells infected with WT SV40, SVd1883 or mock infected.

Hybrids that were positive were transferred to 9cm plates, passaged 3 times, and cloned again as indicated in (section 2.13(iii)(b)).

Single colonies were picked and grown in 24 well trays to 80% confluency and 100µl was removed and tested for small t binding by a radioimmunoassay using cell extracts from X-90 cells transformed with pTR865 (see section 2.14).

Finally the cells were transferred to 9cm plates and grown as an established cell line.

A monoclonal antibody that uniquely recognizes small t was obtained using the above procedures. This antibody was

designated PAb280 according to Crawford and Harlow, 1982.

3.8 Typing of PAb280

The immunoglobulin class and subclass of PAb280 was determined using a simple gel immunodiffusion test (Section 2.24). PAb280 gave a positive immunoprecipitation reaction with rabbit anti mouse IgG_1 antibody but no reaction with anti IgG_{2a}, IgG_{2b} or IgG_3 typing reagents. PAb280 is therefore a mouse IgG_1 subclass antibody.

3.9 Specificity of PAb280 for SV40 Small t

The specificity of PAb280 for small t was assessed by immunoprecipitation, Western blotting and by a solid phase radioimmunoassay.

i) Immunoprecipitation

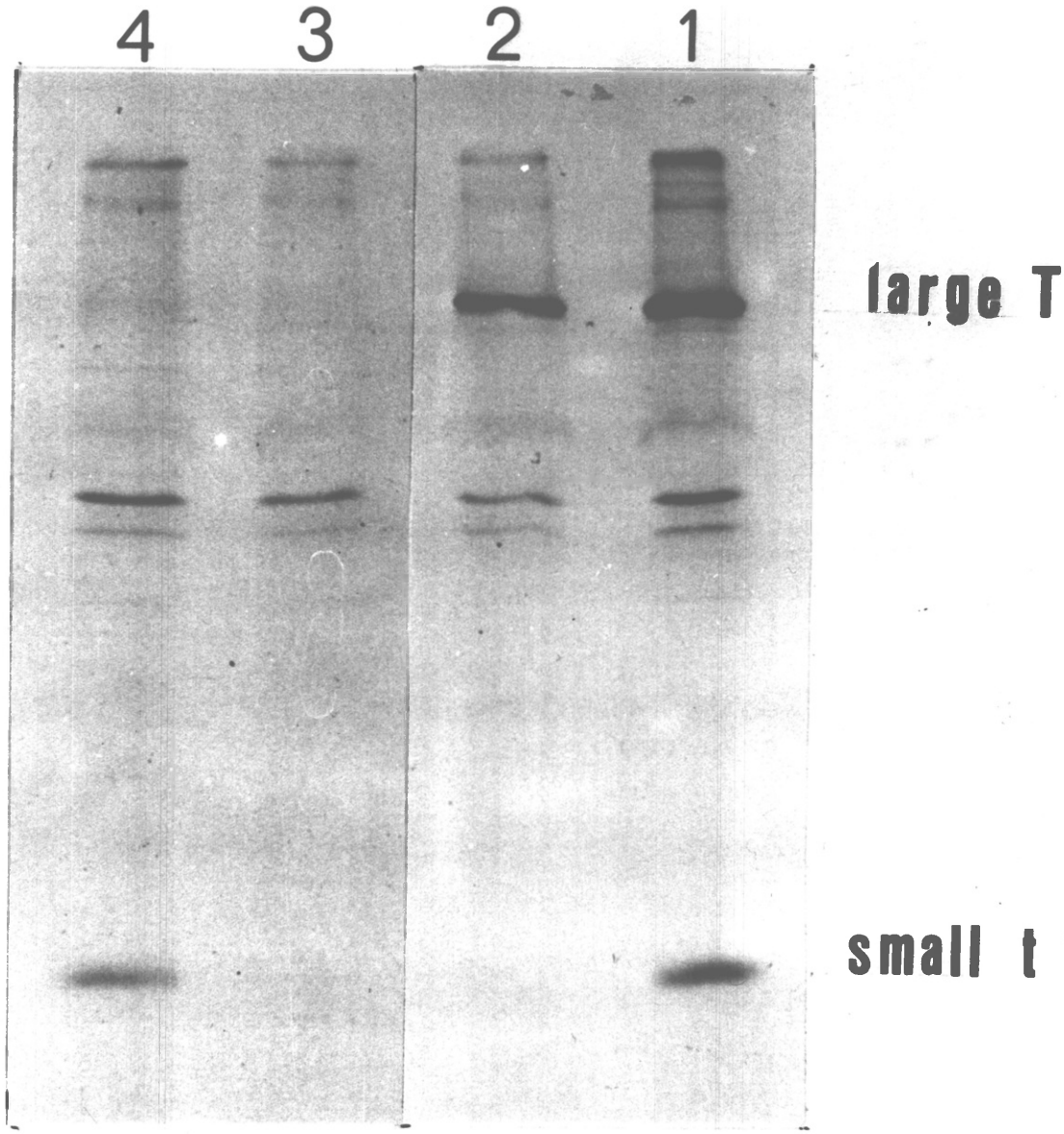
^{35}S methionine labelled cell extracts of CV1 cells infected with WT SV40 and SVdl883 were immunoprecipitated with PAb419 or PAb280 (Section 2.19), the samples run on a 20% SDS polyacrylamide gel with a 5% stacking gel.

As shown in figure (3.6) PAb280 specifically immunoprecipitates a protein of 17kd from WT SV40 infected cell extracts, which comigrates with small t immunoprecipitated by PAb419. PAb280, unlike PAb419, does not immunoprecipitate large T.

It can be concluded that PAb280 is specific for small t and in this assay does not cross-react with either SV40 large T or any other cellular or viral protein present in the extract.

Figure 3.6. Immunoprecipitation of Small t From SV40 Infected CV1 Cells with PAb280 and PAb419

60% confluent CV1 cells were infected for 72hrs with WT SV40 or SVdl883 virus, labelled with ^{35}S methionine for 3hrs and the cell extracts immunoprecipitated with PAb280 and PAb419 (Section 2.19) and the samples run on a 20% polyacrylamide gel with a 5% stack. Track 4: WT SV40 infected cell extract immunoprecipitated with PAb280 and rabbit anti mouse immunoglobulin; Track 3: SVdl883 infected cell extract immunoprecipitated with PAb419 and rabbit anti mouse immunoglobulin; Track 2: SVdl883 infected cell extract immunoprecipitated with PAb419 and rabbit anti mouse immunoglobulin; Track 1: WT SV40 infected cell extract immunoprecipitated with PAb419 and rabbit anti mouse immunoglobulin.


4
3
2
1
large T
small t

It has been reported (Rundell and Cox., 1979; Rundell et al., 1979; Yu-Chang Yang et al., 1979; Rundell, 1982) that in lytic infection small t is complexed with two cellular proteins of 56kd and 32kd. This complex has not been observed during immunoprecipitation of cell extracts of WT SV40 infected cells with PAb280.

ii) Western Protein Transfer

The specificity of PAb280 for small t was assessed by Western blotting.

Whole cell extracts of CV1 cells, mock infected or infected for 72hrs with WT SV40 or SVdl883, were prepared for gel electrophoresis (Section 2.18) and the samples run on a 15% SDS polyacrylamide gel with a 5% stacking gel. The protein bands were electrophoretically transferred to nitrocellulose, incubated with PAb280 or PAb419 hybridoma supernatant and the blots developed using peroxidase coupled rabbit anti mouse immunoglobulin (Section 2.17 (ii) (a)).

Figure (3.7) shows that PAb419 (A) clearly binds to large T and small t from WT SV40 infected cell extracts but only large T from SVdl883 infected cell extracts. PAb280 (B) binds to a 17kd band from WT SV40 infected CV1 cell extract. This band is absent from SVdl883 infected cell extracts and from uninfected cells whether PAb280 or PAb419 is used as the testing antibody.

It should be noted that PAb419 also binds to a 36kd cellular protein which is only seen in uninfected cell

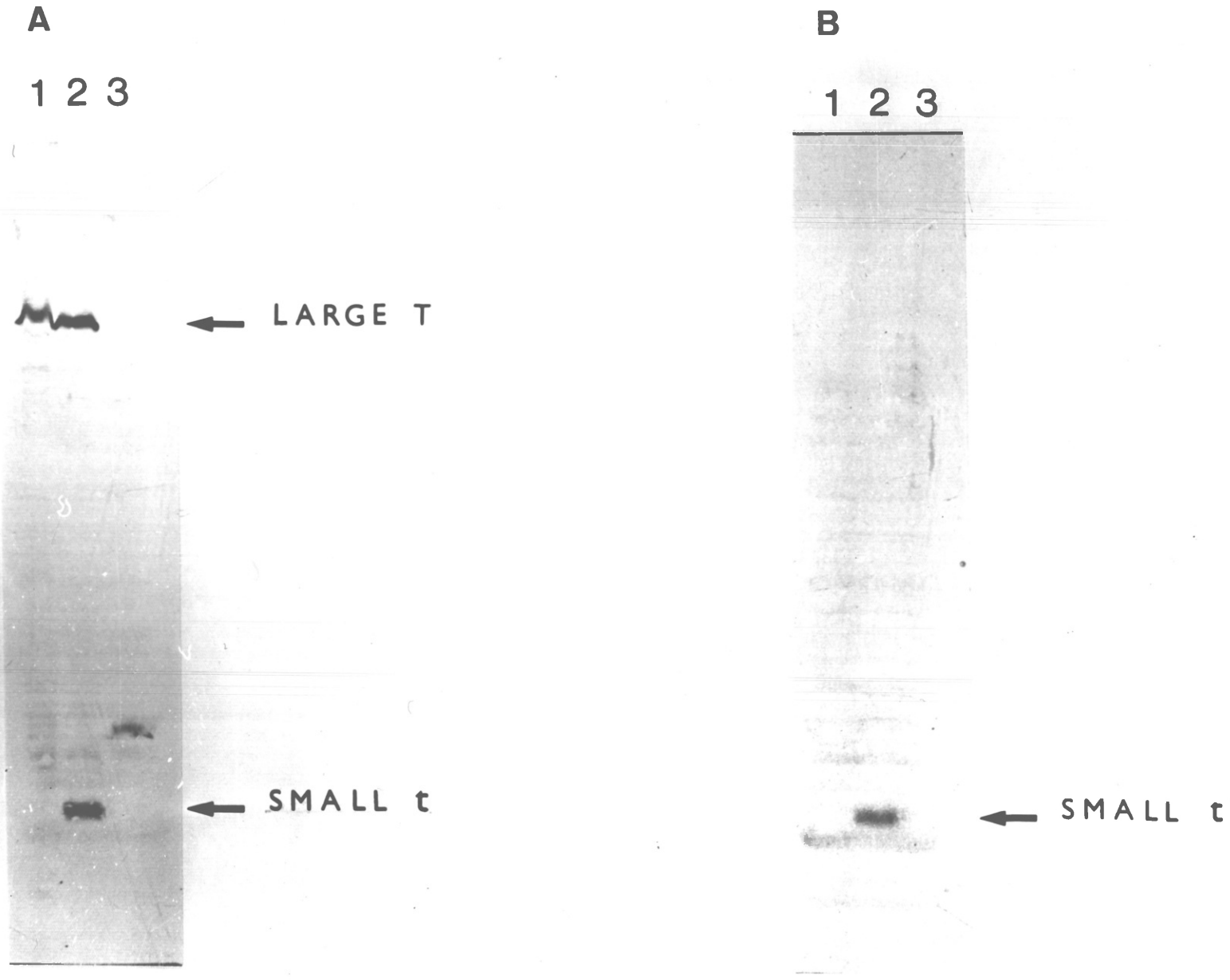
Figure 3.7. Western Blot Analysis Showing the Specificity of PAb280

60% confluent CV1 cells were infected with WT SV40, SVdl883 virus or mock infected. Cell extracts were made (Section 2.21) and 30µl of each sample run on a 15% polyacrylamide gel with a 5% stack and the protein bands transferred to nitrocellulose paper (Section 2.21).

Track 1, extract of SVdl883 infected cells; Track 2, extract of WT SV40 infected cells; Track 3, extract of mock infected cells.

In A the blot was incubated with PAb419.

In B the blot was incubated with PAb280.


A
1 2 3
LARGE T
SMALL t
B
1 2 3
SMALL t

extracts. This protein or a protein of similar molecular weight has also been reported to be synthesised during lytic infections, as shown by immunoprecipitations of labelled cell extracts (Crawford et al., 1982a, 1982b).

The result of this experiment shows that PAb280 specifically recognises an SDS resistant epitope on small t and does not cross-react with any cellular or SV40 proteins.

3.10 A Comparison of the Binding of PAb419 and PAb280 to Small t in a Solid Phase Radioimmunoassay

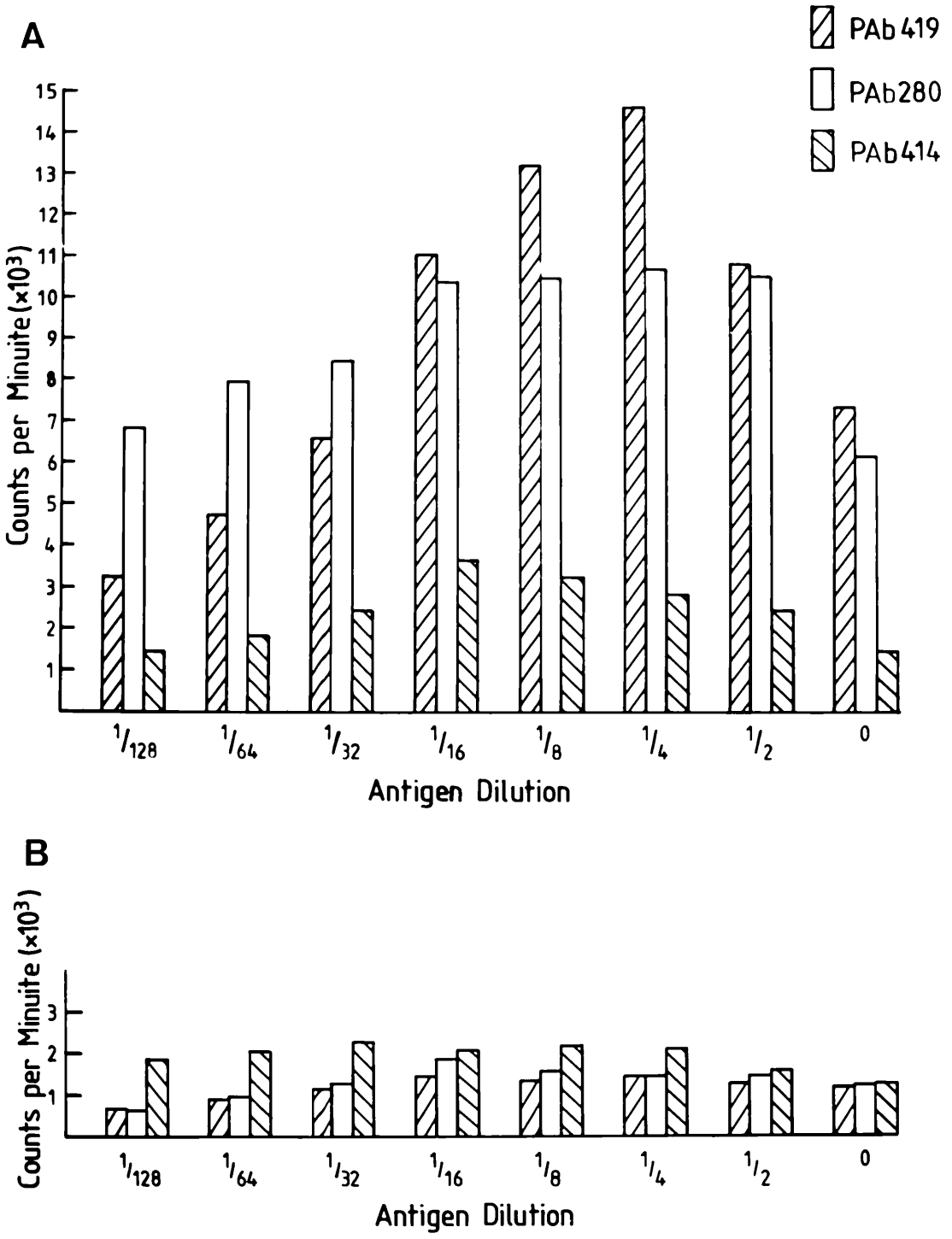
The specificity of PAb280 was analysed by a radioimmunoassay.

A serial doubling dilution was made with IPTG induced extracts of X-90 cells transformed or untransformed by the small t producing plasmid pTR865. 25µl of each dilution was added in duplicate to a 96 well tray left to dry overnight at 37°C and the extracts titrated with PAb419, PAb280 and PAb414 (an antibody that binds large T but not small t) (see Appendix).

Figure (3.8) shows that PAb419 and PAb280 can detect small t in a specific manner. At high antigen concentrations (0 and $^{1}/_{2}$ dilutions) (Figure A) both antibodies show a prozone effect. Although PAb419 (A) detects small t throughout the entire dilution series the peak of detection occurs at dilution $^{1}/_{4}$ (14500cpm) and then steadily decreases. PAb280 detects small t throughout the dilution series and its binding plateaus between

Figure 3.8. Solid Phase Radioimmunoassay Demonstrating the Specificity of PAb280 for Small t

Bacterial cell extracts of E. coli X-90 cells containing pTR865 induced with IPTG (A) or from X-90 cells with no plasmid and induced with IPTG (B) were prepared (Section 2.8(iv)). A serial dilution was plated on a 96 well tray and allowed to dry overnight at 37°C and hybridoma supernatant from PAb419, PAb280 and PAb414 [an antibody that recognizes large T but not small t (see Appendix 1)] followed by iodinated sheep anti mouse immunoglobulin was used to titrate small t from the bacterial extracts (Section 2.14).


A
PAb 419
PAb280
PAb414
Counts per Minuite (x10³)
15
14
13
12
11
10
9
8
7
6
5
4
3
2
1
1/128
1/64
1/32
1/16
1/8
1/4
1/2
0
Antigen Dilution
B
Counts per Minuite (x10³)
3
2
1
1/128
1/64
1/32
1/16
1/8
1/4
1/2
0
Antigen Dilution

dilutions $^{1}/2$ to $^{1}/16$ (10200cpm) and then decreases. Background levels as indicated by PAb414 binding to extracts with and without small t are low; the maximum occurs at dilution $^{1}/16$ (3500cpm). In (B) the background levels are defined by the 3 antibodies, therefore PAb419 and PAb280 recognise small t in the context of E. coli proteins.

The results indicate that if the binding of PAb419 and PAb280 for small t are compared it can be seen that PAb280 can bind small t when present at lower concentrations but the explanation of this effect is unclear.

3.11 Mapping of the PAb280 Epitope on small t

The approximate site to which PAb280 binds was determined by immunoprecipitating CV1 cell extracts infected with SV40 small t deletion mutants [deletions at 0.54 to 0.59 map units (Shenk et al., 1976; Cole et al., 1977)]. The diagram in figure (3.9) shows the mutants employed and the location of the mutations.

Cell extracts were immunoprecipitated with PAb419 or PAb280 (section 2.19). The results are shown in Figure (3.10).

Tracks 1 to 8 show CV1 cell extracts infected with deletion mutants (the order is indicated in the figure legend) and immunoprecipitated with PAb419. Tracks 9 to 16 show immunoprecipitation of the same infected cell extracts with PAb280.

Figure 3.9. Summary of the SV40 Deletion Mutants Employed in the Mapping of THE PAb 280 EPITOPE

This diagram summarizes the SV40 deletion mutants used to map PAb280 (Khoury et al., 1979, Shenk et al., 1976, Cole et al., 1977).

The amino-terminus large T/small t is from nucleotide 5081 to 4838.

The filled in boxes correspond to deletions determined by DNA sequencing.

The hatch lines indicate approximate location of the deletion and have been determined by restriction mapping.

The nucleotide numbering is as for Reddy et al., 1978.

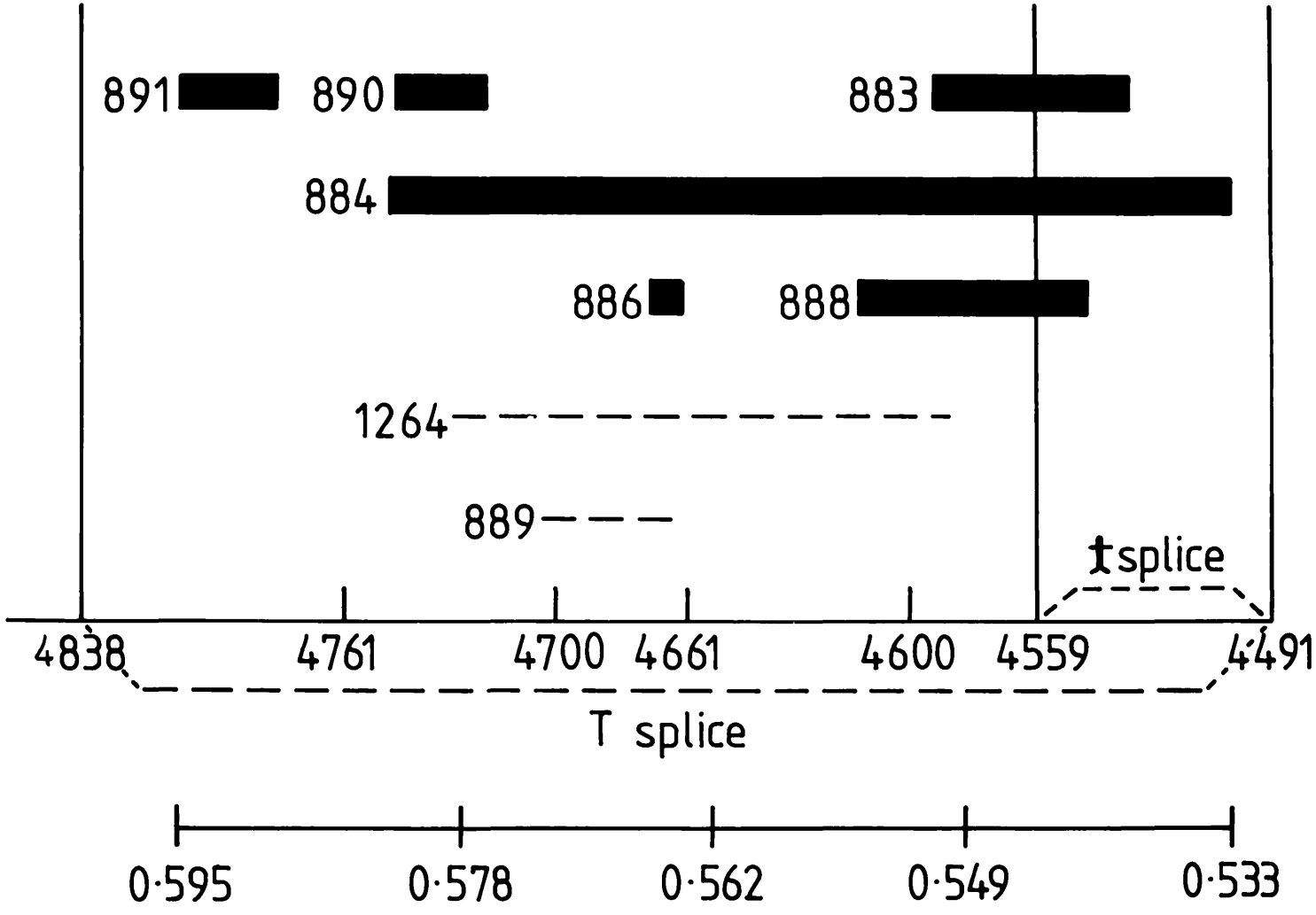
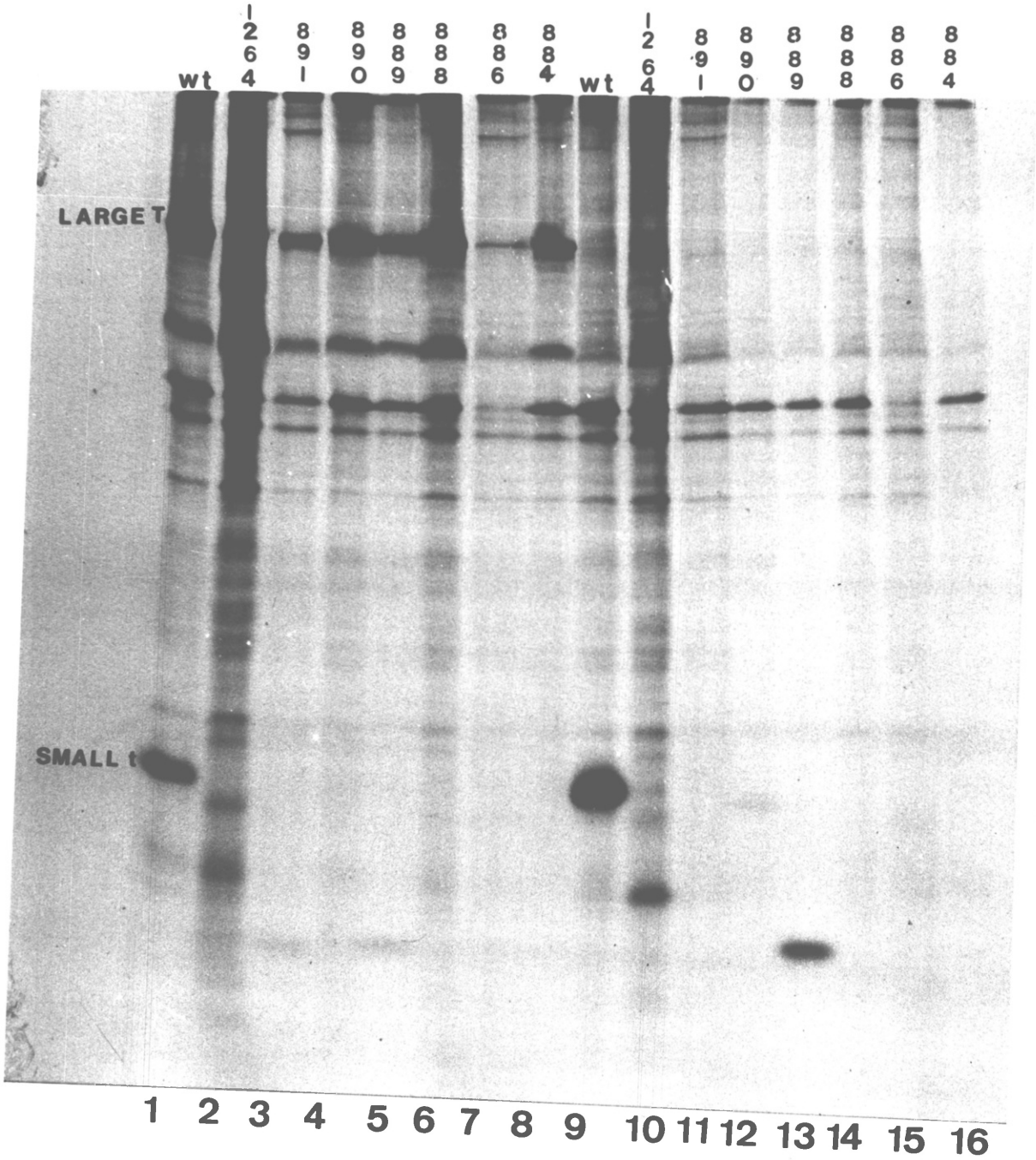

891
890
883
884
886
888
1264
889
t splice
4838
4761
4700
4661
4600
4559
4491
T splice
0·595
0·578
0·562
0·549
0·533

Figure 3.10. Immunoprecipitation of Small t from CV1 Cells Infected with 0.54 to 0.59 Deletion Mutants Using PAb280

CV1 cells were infected with WT SV40, SVdl1264, SVdl891, SVdl890, SVdl889, SVdl888, SVdl886 and SVdl884 for 72hrs and labelled for 3hrs with ^{35}S methionine. Cell extracts were prepared (Section 2.4) and immunoprecipitated with PAb419 (tracks 1 to 8) and with PAb280 (Tracks 9 to 16) (60 day exposure).


wt
I 264
891
890
889
888
886
884
wt
I 264
891
890
889
888
886
884
LARGE T
SMALL t
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16

The immunoprecipitation with PAb419 indicates that all deletion viruses went through EARLY EXPRESION .. This is evidenced by the presence of a 94kd protein band present in all tracks which comigrates with large T from WT SV40 infected cell extracts immunoprecipitated with PAb419 (track 1).

The position of wild type small t can be observed in WT SV40 infected cell extracts immunoprecipitated with PAb419 (track 1) and PAb280 (track 9).

The results obtained in this experiment and a summary of the characteristics of the SV40 deletion mutants are listed in table (3.1).

It can be observed that PAb280 specifically immunoprecipitates truncated small t proteins from cell extracts infected with SVdl890, SVdl889 and SVdl1264, with molecular weights similar to those described by Khoury et al., 1979 for small t truncated proteins (see Table 3.1).

If the immunoprecipitation results with PAb280 and PAb419 are compared it can be seen that PAb419 can detect an 11kd small t fragment from SVdl891; this fragment is not detected by PAb280.

When SVdl884, SVdl886 and SVdl888 infected cell extracts were used, no immunoprecipitation of any specific band was observed by PAb280 or PAb419.

To verify and extend these results immunoperoxidase staining of CV1 cells infected with all these mutants was also carried out. The results are shown in Table 3.1 (PAb419 staining was used as a control).

TABLE 3.1

Summary of the Characteristics of the SV40 small t Deletion Mutants

Strain	Size of deletion (nucleotides)	Mol. wt. of Small t (Kd) Predicted by Khoury et al (1979)	Observed by PAb280	Observed by PAb419	Detection of small t by PAb280 staining	Position of deletion Proximal	Distal	Type of Deletion
SVdl884	-247	12	ND	ND	+	4748	4502	Splice Junction
SVdl886	-10	15	ND	ND	+	4672	4663	Frame Shift
SVdl888	-68	ND	ND	ND	+	4611	4544	Splice Junction
SVdl889	-46*	14.5	11.3	11.3	+	4707*	4665*	Frame Shift
SVdl890	-27	16	17	17	+	4746	4720	In Frame
SVdl891	-25	11	ND	11	-	4809	4785	Frame Shift
SVdl1264	-120*	16.5	13	13	+	4730*	4590*	Frame Shift
WT SV40	-	-	17.5	17.5	+	-	-	-

PAb280 stains cells infected with all the deletion mutants with the exception of SVdl891 and of course SVdl883. A positive result with the splicing mutants SVdl888 and SVdl884 was surprising. SVdl884 has been described as producing a detectable small t fragment of 12kd (Khoury et al., 1979; Crawford and o'Farrel, 1979) but no fragment has been detected from cell extracts infected with SVdl888 (Khoury et al., 1979). It is important to point out that the frequency of cells stained with PAb280 and PAb419 in all the deletion infected cultures was the same.

As PAb280 is specific for small t only and does not cross-react with any other protein (see Sections 3.9 (i) and (ii)) the staining results suggest the possibility of PAb280 recognising the small t products from the two splicing site mutants.

Based on the immunoprecipitation data it can be deduced that the PAb280 binding epitope is located either between nucleotides 4809 to 4747 or nucleotides 4580 to 4559 (approx). The staining data is concordant with the immunoprecipitation data and the results obtained with SVdl888 and SVdl884 show that the epitope is found between nucleotides 4809 to 4747. (Nucleotide numbering according to Reddy et al., 1978).

It is important to stress that the sensitivity of the staining assay is higher than the immunoprecipitation.

All these results show that PAb280 maps between nucleotides 4809 to 4747, that is, amino acids 93 to 114.

Figure (3.11) shows the amino acid sequence of SV40, BK and polyoma virus. By comparison of the sequence of the site of the 3 viruses at which the PAb280 epitope maps it can be seen that there is little homology; the only conserved sequence is between BK and SV40 at amino acids 95 to 102. The possibility of BK small t binding to PAb280 was investigated experimentally by immunoperoxidase staining of cells transformed by 3 strains of BK virus (Section 2.1). In all 3 cases no staining was detected. This result suggests that the binding site of PAb280 is probably between amino acids 103 to 114 but this must be investigated further.

The binding of PAb280 to polyoma small t was also tested by a solid phase radioimmunoassay (Trevor Dale, personal communication) and shown to be negative.

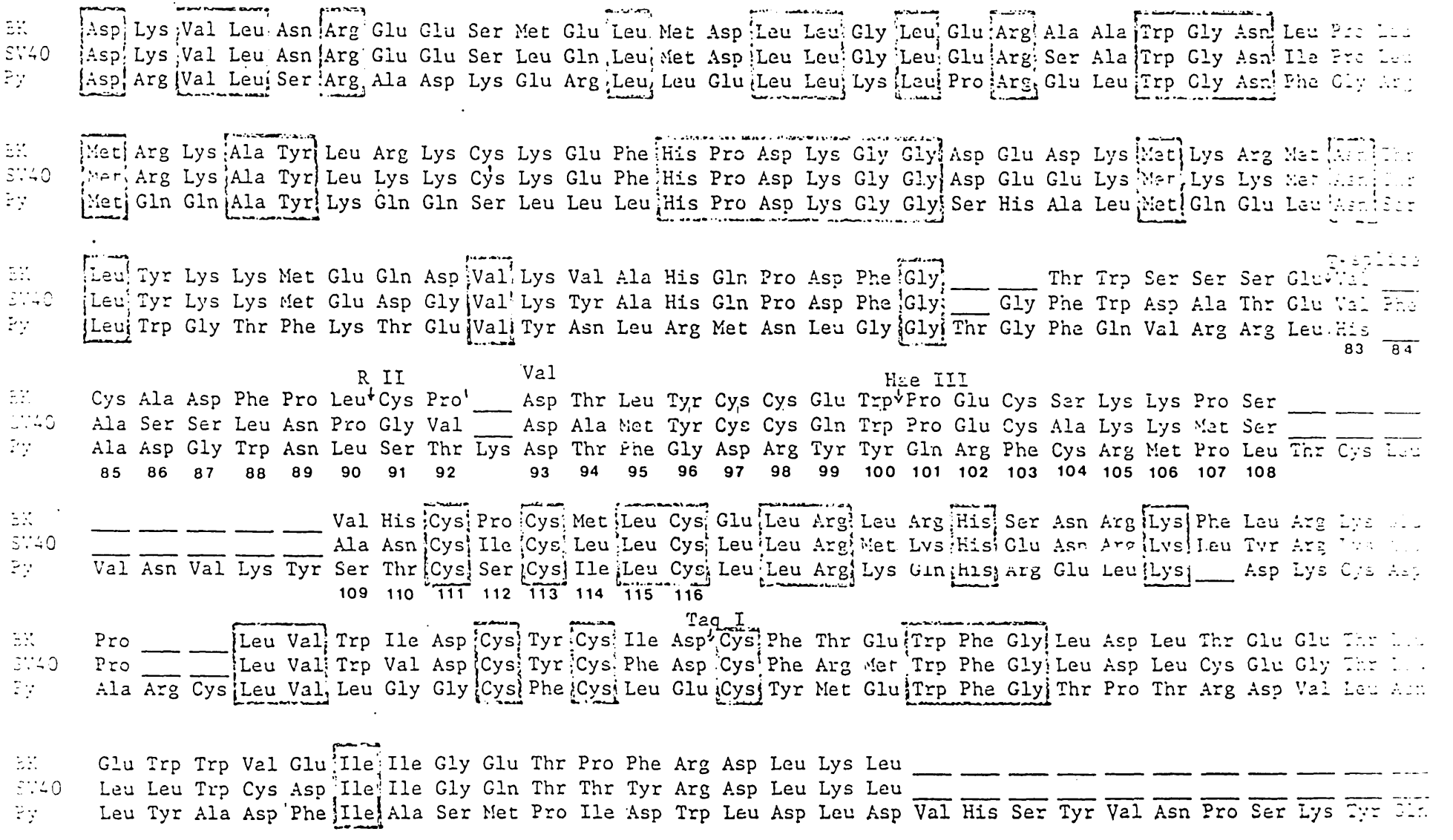
3.12 Discussion

The production of a monoclonal antibody to SV40 small t proved to be a difficult task. During initial experimental stages two problems were encountered: a) the lack of large amounts of small t and b) the antigenicity of the amino-terminus of the small t protein.

Although the HP1 plasmid (Thummel et al., 1981) was obtained during initial stages of this work, improvement in extraction and purification did not sufficiently enhance the levels of small t. It was only with pTR865 (Bikel et al., 1983) which is a plasmid that expresses up to 5% of the total bacterial protein as small t, that this problem

Figure 3.11. Comparative Amino Acid Sequence of SV40, BK and Polyoma Small t

Small-t Protein Sequence



was solved.

The amino-terminus of large T/small t was seen to be very immunogenic, from each fusion between 70% to 80% of the hybrid colonies did express antibodies of the PAb419 type. Even when amounts of less than 1μg of small t-PAb419 complex were used to immunize the animals.

At a molecular level it is still not known if the PAb419-small t complex successfully masks the whole of the 82 amino acid region shared by large T and small t. The binding sites of the PAb419 like antibodies (obtained from different fusions) have not yet been mapped. Therefore it is not known if the complex has hindered the response to any part or all of the 82 amino acid sequence.

PAb280 is an IgG_1 immunoglobulin that can specifically recognise SV40 small t in its native form as shown by immunoprecipitation. Western blotting analysis proves that PAb280 can detect small t after SDS denaturation.

The monoclonal antibody shows no cross-reaction with cellular protein in either of these assays nor in solid phase radioimmunoassay or immunocytochemistry. This makes it an ideal reagent to study the synthesis, cellular location and hopefully the function of small t in eukaryotic cells.

The approximate mapping site of PAb280 is at nucleotides 4809 to 4747, that is, it has been mapped down to 21 amino acids (amino acids 93 to 114) in the unique carboxy-terminus of small t. The mapping location of

PAb280 could be studied further by using fusion proteins and peptide synthesis.

CHAPTER FOUR

SV40 SMALL t CHARACTERISATION

4.1 Introduction

In this chapter a preliminary characterisation of SV40 small t using PAb280 will be described. It will start by showing the localisation of small t in lytically infected cells as well as the time of appearance of the protein. The localisation of small t in four characterised SV40 transformed cell lines and the synthetic levels of the protein in this system will also be shown. Evidence will be given that strongly indicates the presence of two immunologically distinct subsets of small t. Finally studies with the small t producing mutant virus SV402 (Rubin et al., 1982) will be presented.

4.2 Immunocytochemical Localisation of SV40 small t in Lytically Infected Cells.

The quantities of small t present in lytically infected or transformed cells is small; therefore three fixation methods were examined in order to determine which detected small t with most sensitivity. These are acetone:methanol (Section 2.17(i)(a)), glutaraldehyde (Section 2.17(i)(c)) and formaldehyde (Section 2.17(i)(b)). With glutaraldehyde fixation it was not possible to detect small t, whereas with both the acetone:methanol and the formaldehyde procedure this was possible, but the former method was less sensitive than the latter. Therefore in the experiments described below as well as in subsequent experiments the formaldehyde method was employed.

CV1 cells infected with WT SV40, SVdl883 or mock

infected were formaldehyde fixed and the cells opened by a freeze thaw cycle (Section 2.17(i)(b)(1)) then stained with PAb280 or PAb419 using the immunoperoxidase method (Section 2.17(ii)(a)).

Figure (4.1) shows the result of this experiment. PAb280 stains the nucleus and cytoplasm of WT SV40 infected cells (A) but does not stain SVd1883 (B) or mock infected cells (data not shown).

PAb419 stains very strongly the nuclei of both WT SV40 (C) and SVd1883 (D) but not mock infected cells (data not shown). The localisation of small t was investigated further by immunofluorescence staining (Section 2.17(ii)(b)). In this case WT SV40 infected cells were stained with PAb280 followed by Texas Red conjugated to sheep anti mouse immunoglobulin. Figure (4.2(A)) shows that fluorescence staining is seen in the nucleus and cytoplasm. No staining was observed in SVd1883 infected cells (data not shown).

The differential distribution of large T and small t within a given cell was observed by a double immunofluorescence staining procedure. Figure (4.2(B)) shows CV1 cells infected with WT SV40, stained with PAb280 followed by Texas red conjugated to sheep anti mouse immunoglobulin and rabbit anti D2 serum, followed by fluorescein conjugated swine anti mouse immunoglobulin. (Rabbit anti D2 serum is a serum raised against a purified native adenovirus type 2-SV40 fusion protein which contains

Figure 4.1. Cellular Localization of Small t

CV1 cells infected with WT SV40 (A) and (C) or SVdl883 (B) and (D) for 72hrs were formaldehyde fixed (Section 2.17(i)(b)) and stained with PAb280 (A) and (B) or with PAb419 (C) and (D) using the immunoperoxidase method (Section 2.17(ii)(a)).

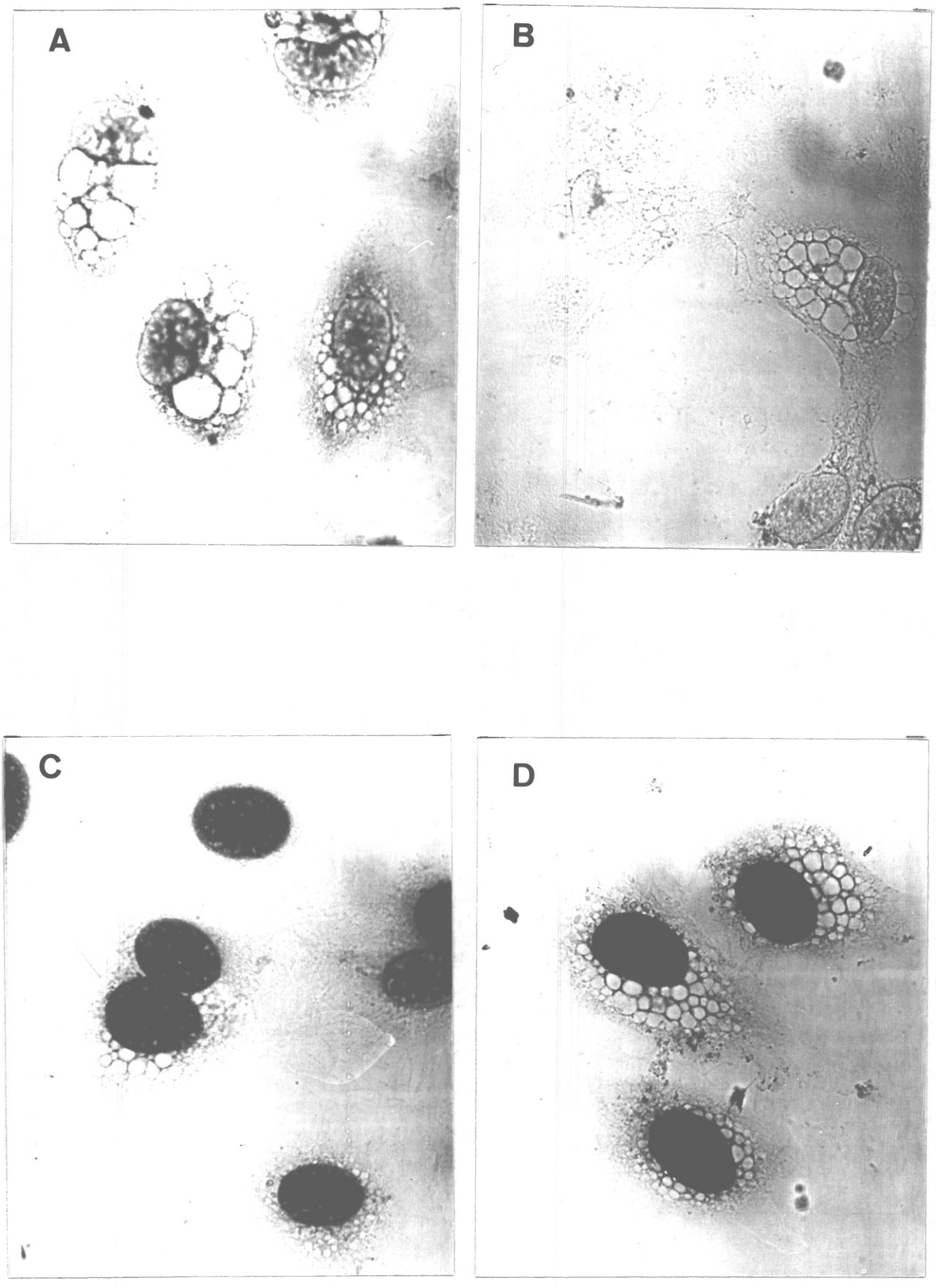
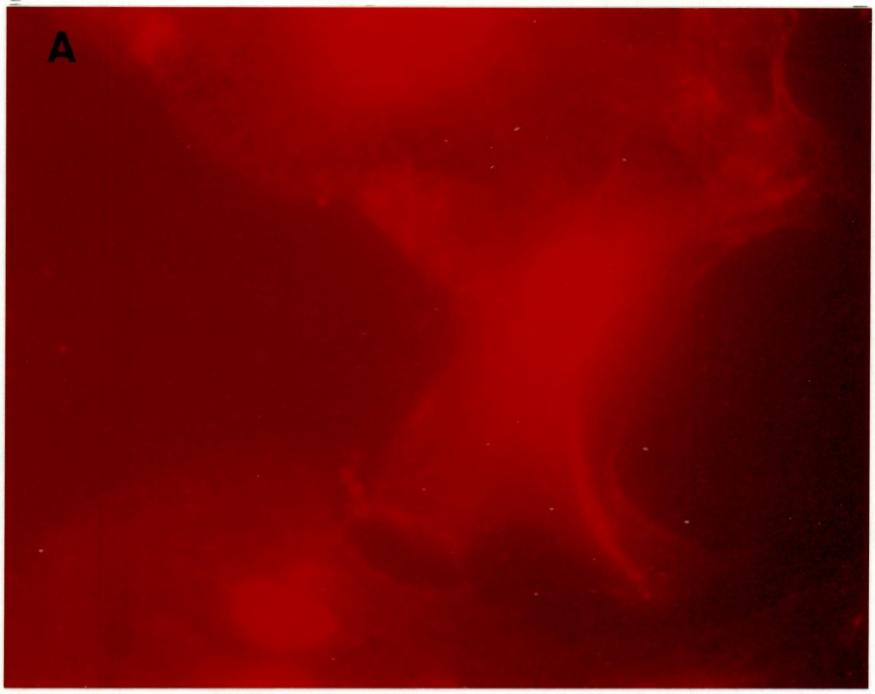
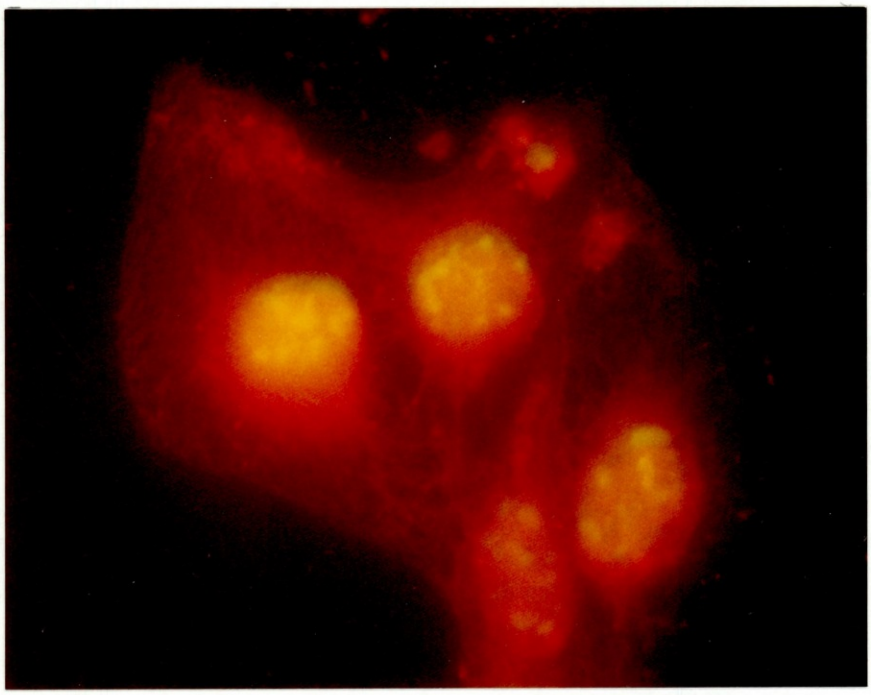

A
B
C
D

Figure 4.2. Cellular Localization of Small t by Immunofluorescence Staining

CV1 cells infected with WT SV40 virus for 72hrs were formaldehyde fixed (Section 2.17(i)(b)) and stained with PAb280 (A) or with PAb280 and rabbit anti D2 serum (B) followed by texas red conjugated to sheep anti mouse immunoglobulins (A) or texas red conjugated to sheep anti mouse immunoglobulins and fluorescein conjugated swine anti mouse immunoglobulins (B).


A



the SV40 large T antigen unique region and therefore recognises epitopes of large T which are not common with small t). Again it can be clearly seen that small t is in the nucleus and cytoplasm as indicated by the Texas red fluorescence. Meanwhile large T can only be detected in the nucleus (as indicated by the fluorescein fluorescence) with no detectable leakage into the cytoplasm.

4.3 Time of Appearance of Small t During Lytic Infection

CV1 cells were infected with WT SV40 (Section (2.6 (i)) and labelled with ^{35}S methionine for 3hrs prior to harvesting.

Cells were harvested at 12, 24, 48, 72 and 78hrs after infection and aliquots of the extracts containing the same number of counts were immunoprecipitated with PAb280 or a rabbit anti T/t serum (Section (2.19)). The samples were run on a 20% polyacrylamide gel with a 5% stack.

Figure (4.3) shows the results of this experiment. It can be seen that small t is detected at 24hrs post infection (Track 2); the synthetic levels increase up to 72hrs post infection (see Tracks 3 and 4) and then stay the same (see Track 5).

In the case of large T (Tracks 6 to 9) high levels are detected from times 24 to 72hrs and then the amount decreases (Track 10).

These results confirm those obtained by Crawford et al., (1980).

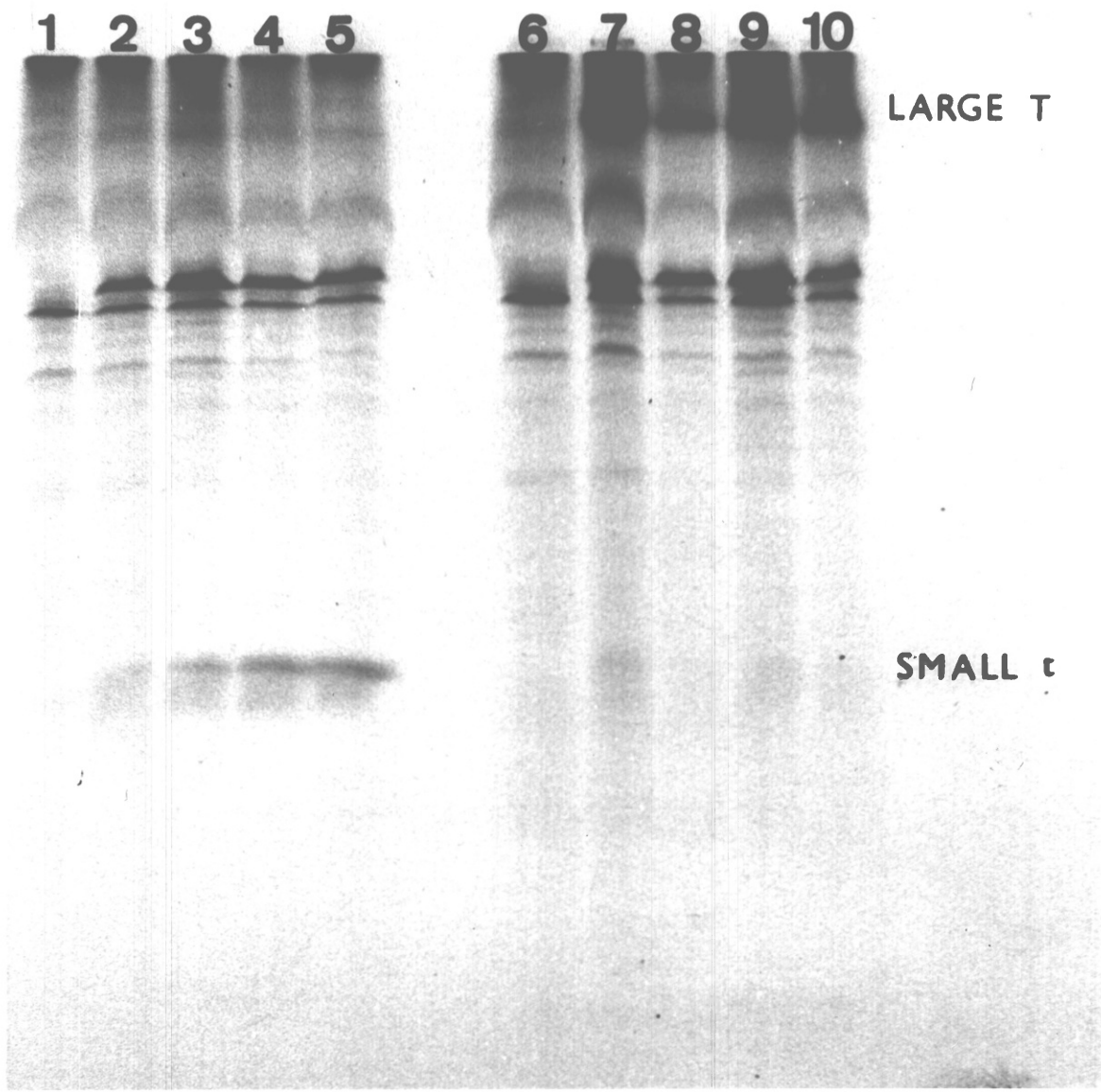
Although small t is a protein encoded by the SV40

Figure 4.3. Time of Appearance of Small t During Lytic Infection.

CV1 cells were infected with WT SV40 for 12, 24, 48, 72 and 78hrs labelled with ^{35}S methionine for 3hrs and cell extracts prepared (Section 2.4). Aliquots with the same number of counts were immunoprecipitated with PAb280 or rabbit anti T/t serum (Section 2.19).

Tracks 1 to 5 are times 12, 24, 48, 72 and 78hrs post-infection immunoprecipitated with PAb280.

Tracks 6 to 10 are times 12, 24, 48, 72 and 78hrs post-infection immunoprecipitated with rabbit anti T/t serum.


1 2 3 4 5
6 7 8 9 10
LARGE T
SMALL t

early region these results show that the peak of expression is fairly late during lytic infection.

The fact that higher synthetic levels of large T are detected early during infection and that small t is synthesized at a later time indicates that there must be some mechanism involved in the selective expression of these genes. There are several possibilities:

a) Post-transcriptional processing *via* a selective splicing mechanism; during early stages of lytic infection, the mRNA, could be preferentially spliced to produce messages that code for large T rather than those that code for small t.

b) It is possible that after processing the stability of the mRNA is involved in the selective mechanism, that is, the processed mRNA encoding for large T is more labile during later stages of the lytic cycle giving an overall result of having less large T being produced.

c) It is also possible that post-translational processing is taking place, that is, at later stages during lytic infection large T antigen is more labile than small t.

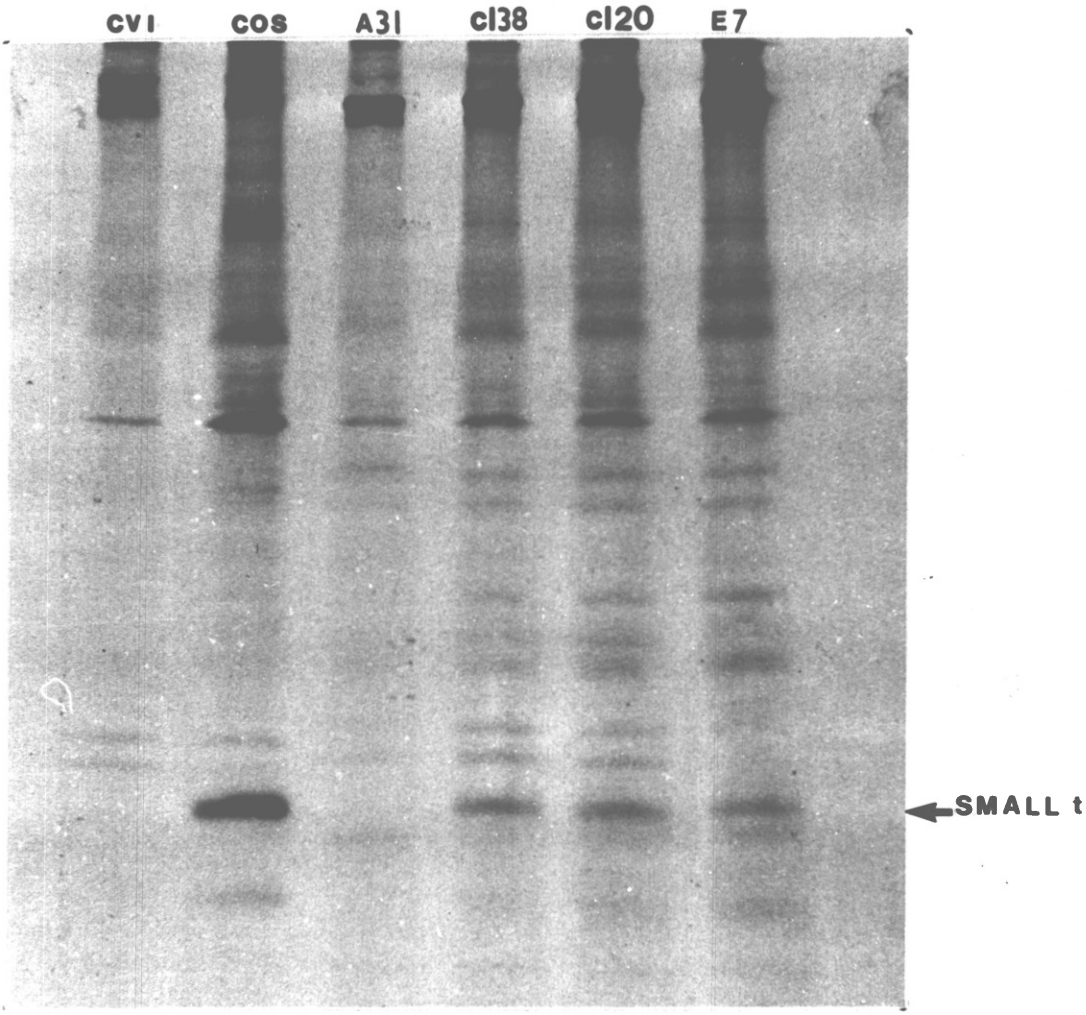
4.4 SV40 Small t in Transformed Cell Lines

i) Immunoprecipitation

Small t synthetic levels were detected by immunoprecipitation of ^{35}S methionine labelled cell extracts (3hr label) of SV3T3 Cl2O, SV3T3 Cl38 (Clayton and Rigby, 1981), E7 (Lane and Crawford, 1979) and Cos-1 cells (Gluzman, 1981) with PAb28O. Figure (4.4) shows the result

Figure 4.4. Immunoprecipitation of Small t From SV40 Transformed Cells.

CV1, Cos-1, Balb/c 3T3 A31, SV3T3 C138, SV3T3 C120 and E7 cells were grown to 80% confluency and labelled for 3hrs with ^{35}S methionine. Cell extracts were prepared (Section 2.4) and aliquots with the same number of counts were immunoprecipitated with PAb280 (Section 2.19). The samples were run on a 20% polyacrylamide gel with a 5% stack (14 days exposure).


CV1
COS
A31
cl38
cl20
E7
SMALL t

of this experiment.

If the immunoprecipitation reactions of nonpermissive cells transformed by WT SV40 such as E7, SV3T3 C120 and SV3T3 C138 are observed, it can be seen that the levels of small t immunoprecipitated by PAb280 are similar in all these cell lines (the control line Balb/c 3T3 A31 was immunoprecipitated with PAb280 and no small t was observed).

The level of small t present in Cos-1 cells was found to be higher than from the other WT SV40 transformed cell lines (no small t was observed in the control cell line CV1).

E7, SV3T3 C120 and SV3T3 C138 are WT SV40 transformed cell lines characterised by having multiple SV40 DNA insertions in their genome. Meanwhile Cos-1 cells, which are the permissive CV1 cell line transformed by an origin of replication defective SV40 virus has a single copy of the SV40 genome. Therefore it is interesting to note that the synthetic levels of small t do not reflect the number of copies of the SV40 genome present in the cell.

A possible explanation of this result could be the fact that CV1 cells have the correct milieu for SV40 viral functions while Balb/c 3T3 do not; but it has also to be pointed out that in this experiment two different cell systems are under observation: epithelial cells (CV1) and fibroblasts (Balb/c 3T3 A31). Therefore a direct comparison cannot be carried out.

ii) Cellular Localisation

The same cell lines used in the previous experiment were formaldehyde fixed (Section 2.17(ii)(b)) the cells opened by a freeze thaw cycle (Section 2.17(ii)(c)(2)) and stained with PAb280 or PAb419 (as a control antibody) using the immunoperoxidase method (Section 2.17(ii)(a)).

Figure (4.5) shows that small t is detected by PAb280 in the nucleus and cytoplasm of the E7 cells (A). The distribution is similar to lytically infected cells.

In the case of cells stained with PAb419 only the nucleus is stained (data not shown) and the control cell line Balb/c 3T3 A31 cells show no staining with PAb280 (B).

The same result was observed for all cell lines examined: SV3T3 C138, SV3T3 C120 and Cos-1. Therefore it seems that the nuclear and cytoplasmic distribution of small t is ubiquitous in WT SV40 transformed cell lines.

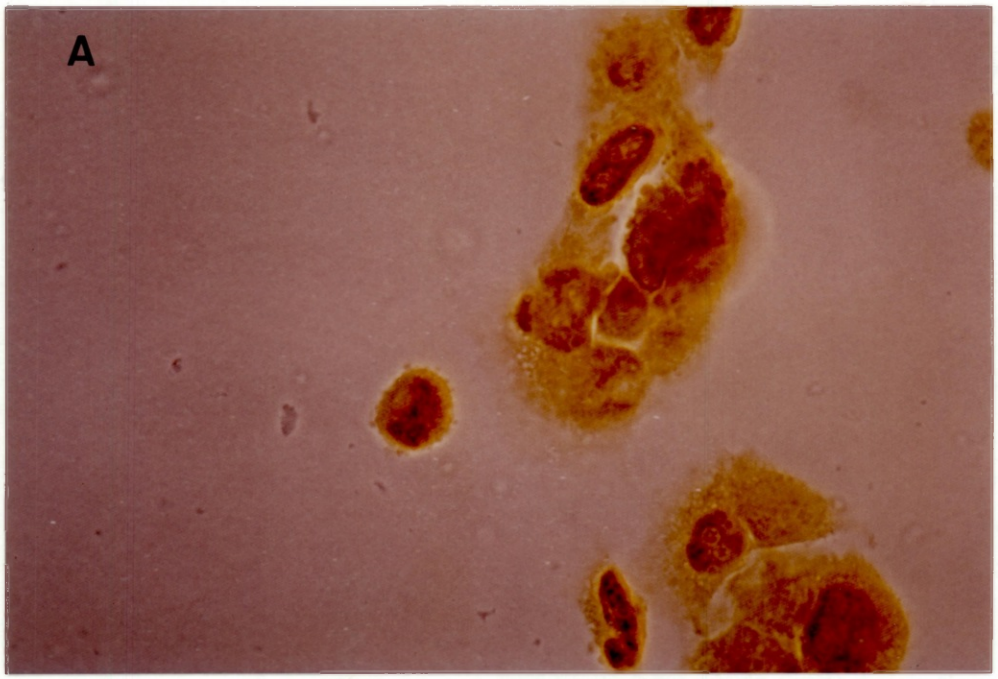
4.5 Detection of Two Immunologically Distinct Subsets of Small t

i) Cellular Localisation of Small t in Lytically Infected Cells Using PAb280 and PAb419 Simultaneously

When the cellular localisation of small t was determined by staining with PAb280 to be present in the nucleus as well as in the cytoplasm, it was interesting to see that PAb419, an antibody that recognises large T and small t through the amino-terminus common region, could not recognise the fraction of small t localised in the cytoplasm.

Figure 4.5. Small t Localization in SV40 Transformed Cells Using PAb280.

E7 cells (A) and Balb/c 3T3 A31 cells (B) were formaldehyde fixed (Section 2.17 (i)(b)) and stained with PAb280 using the immunoperoxidase method (Section 2.17 (ii)(a)).


A

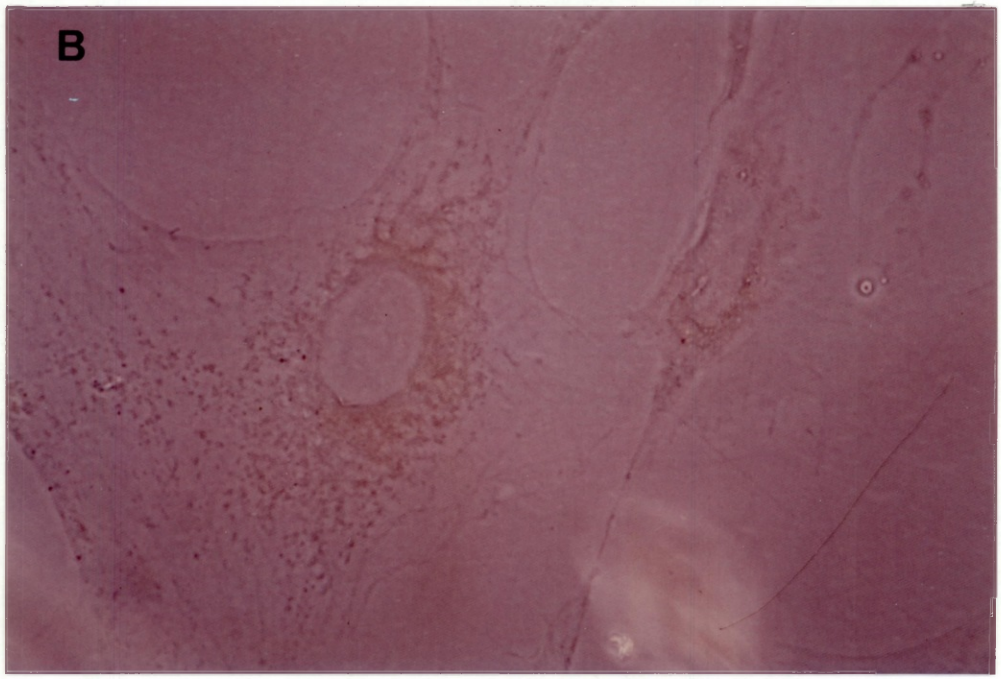
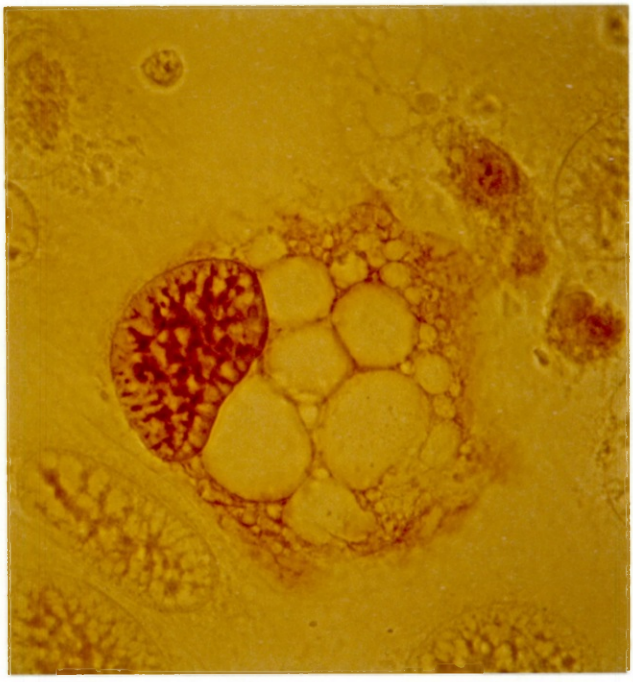

B

Figure 4.6. Simultaneous Staining of Small t and Large T

CV1 cells were infected with WT SV40 for 72hrs. formaldehyde fixed (Section 2.17 (i)(b)) stained with PAb280 and PAb419 using the immunoperoxidase method (Section 2.17 (ii)(a)).



There is a potential problem. It is possible to argue that there is not enough PAb419 antibody; therefore the fraction of small t present in the cytoplasm cannot be detected. When PAb419 concentrations of up to 40µg/3.5cm plate were used the same result was obtained and the intensity of the nuclear stain reached a plateau above 1µg/plate. Accessibility of the antibody to the antigen is not a problem since PAb280 can detect the cytoplasmic fraction under the same conditions.

Figure (4.6) shows a double staining experiment of CV1 cells infected with WT SV40, stained with PAb419 and PAb280 using the immunoperoxidase method (Section 2.17(ii)(a)). It can be seen that both the nucleus and cytoplasm are stained. The result of this experiment suggests that there are at least two subsets of immunologically distinct small t molecules.

ii) Sequential Extraction of Cellular Fractions and Immunoprecipitation

The presence of two subsets of small t was investigated further by carrying out the sequential extraction described by Staufenbiel and Deppert (1983). Figure (4.7) shows the result of this experiment.

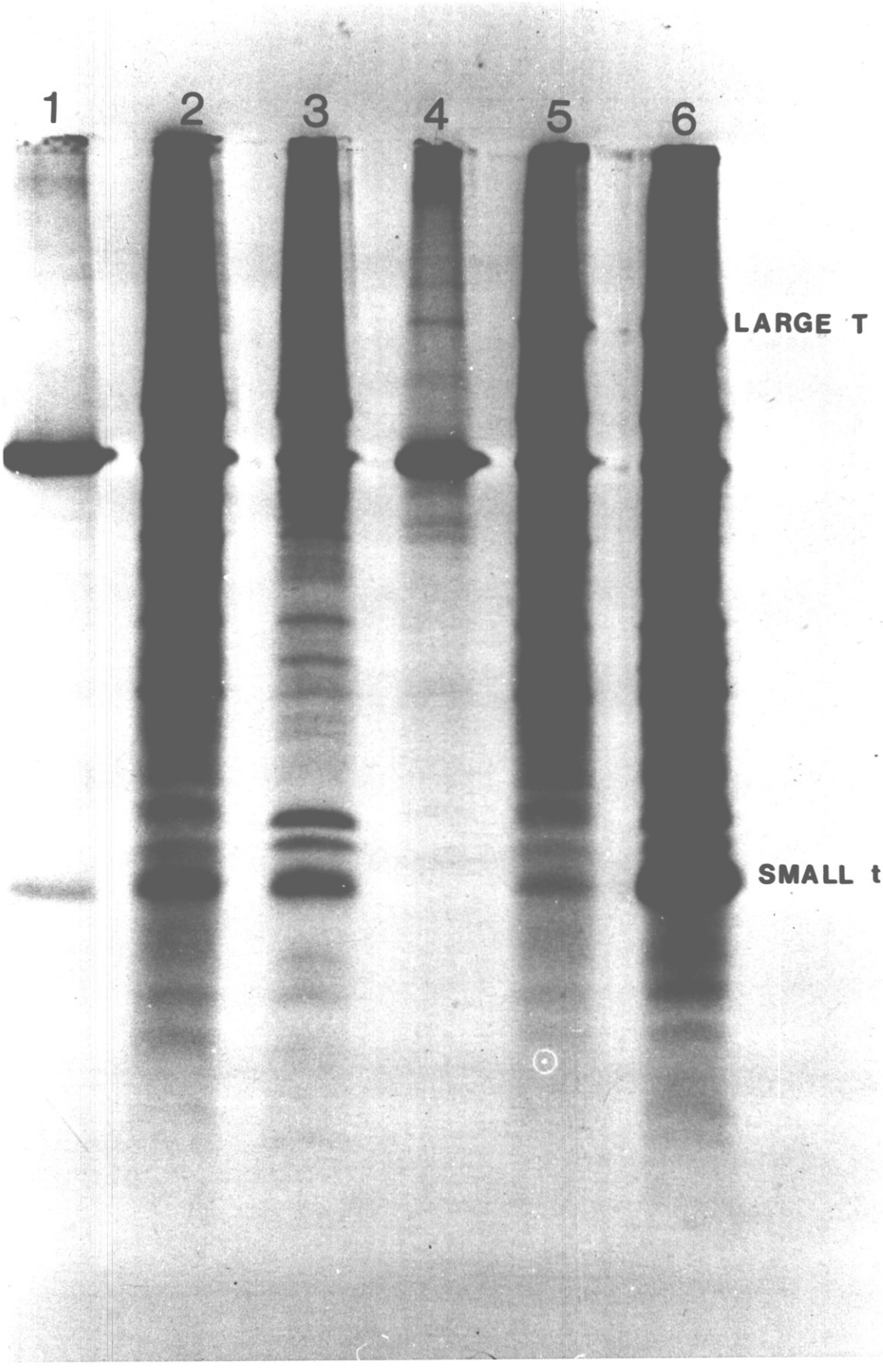
During this three step procedure, cells are first lysed in situ using a hypotonic buffer containing NP40. This fraction yields cytoplasmic and nuclear proteins as well as solubilised membranes (Tracks 6 and 3). Then a second extraction is carried out using DNAse and high salt, in order to solubilise chromatin and associated structures

Figure 4.7. Immunoprecipitation of Subfractions of WT SV40 Infected CV1 Cells (Staufenbiel and Deppert (1983))

CV1 cells were infected with WT SV40 for 60hrs and then first lysed *in situ* with a hypotonic buffer containing NP40 (Tracks 6 and 3). Then a second extraction was carried out using DNAse and high salt (Tracks 5 and 2). Finally the cells were extracted with a buffer containing Empigen BB (Tracks 4 and 1).

Tracks 6, 5 and 4 show fractions immunoprecipitated with PAb 419.

Tracks 3, 2 and 1 show fractions immunoprecipitated with PAb 280.


1
2
3
4
5
6
LARGE T
SMALL t

(tracks 5 and 2). Finally the last extraction uses Empigen BB, a detergent that solubilises the nuclear matrices attached to a residual cytoskeleton (tracks 4 and 1).

Tracks 1 to 3 are immunoprecipitated with PAb419 and tracks 4 to 6 with PAb280.

It can be seen that there is more small t accessible to PAb419 than to PAb280 in the NP40 extract (tracks 6 and 3). There is some small t accessible to PAb419 but more to PAb280 in the extract containing the chromatin and associated structures (tracks 2 and 5). Meanwhile only PAb280 recognises small t associated with the nuclear matrix and cytoskeletal fraction (Tracks 1 and 4).

It is possible to observe that in the presence of the detergent Empigen BB PAb419 recognises large T. Therefore if small t is not detected by PAb419, it cannot be due to inactivation of the antibody and is unlikely to be due to modification of the small t epitope since large T is still detectable.

This experiment shows the existence of two subsets of small t. There are molecules that can be recognised by PAb419 and a second set of molecules that can be recognised by PAb280. The experiment strongly suggests that a fraction of small t is associated with the cytoskeleton and is present in the cytoplasm and detected by PAb280 but not PAb419.

iii) Sequential Immunoprecipitation of Small t

In order to see if these two subsets of small t were two different molecular species, ^{35}S methionine labelled

extracts of CV1 cells infected with WT SV40 were immunoprecipitated with PAB280 followed by PAb419 or vice-versa.

The results of this type of experiment are observed in Figures (4.8) and (4.9).

In Figure (4.8) the experiment was carried out for 9 days. Tracks 1 to 5 show immunoprecipitation with PAb280 followed by PAb419 (tracks 6 to 9). Tracks 10 to 14 show immunoprecipitation with PAb419 followed by PAb280 (tracks 15 to 18).

In Figure (4.9) the experiment was carried out for 14 days. That is the cell extract was immunoprecipitated eight times with PAb280 (tracks 1 to 4) and six times with PAb419 (tracks 5 to 7) and vice-versa (tracks 8 to 14). See figure legend.

Both experiments show that it is not possible to deplete small t totally with either PAb280 or PAb419 (see tracks (5) and (14) from Figure (4.8) and tracks 4 and 11 from Figure (4.9) even during extended periods of incubation.

For the results presented above as well as for initial trial experiments the aliquot employed came from the same cell extract pool. Therefore it was interesting to note that in the first immunoprecipitation reaction (track 1 in Figure (4.8) and Figure (4.9) different amounts of small t have been immunoprecipitated by PAb280.

These observations indicate that there is a single small t molecular species but that the molecule is in

Figure 4.8. Sequential Immunoprecipitation of Small t with PAb280 and PAb419 (9 days).

CV1 cells were infected with WT SV40 for 72hrs and labelled with ^{35}S methionine for 3hrs. Cell extracts were made (Section 2.4).

Immmunoprecipitations were carried out as follows: to 150µl of cell extract 5µg of monoclonal antibody was added and incubated overnight at 4°C. Then 50µg of rabbit anti mouse immunoglobulin was added and incubated for 1hr at room temperature. Finally 100µl of Staphylococcus aureus Cowan 1 was added and incubated for 1hr at 4°C. The sample was spun for 2 min (Eppendorf microfuge) the pellet kept and treated as noted in Section 2.19. The supernatant obtained was mixed with 5µg of monoclonal antibody and the procedure repeated.

This method was carried out for 9 days. For 5 days the cell extract was immunoprecipitated with PAb280 (Tracks 1 to 5) and 4 days with PAb419 (Tracks 6 to 9) or viceversa (Tracks 10 to 14 and Tracks 15 to 18) (7 days exposure).

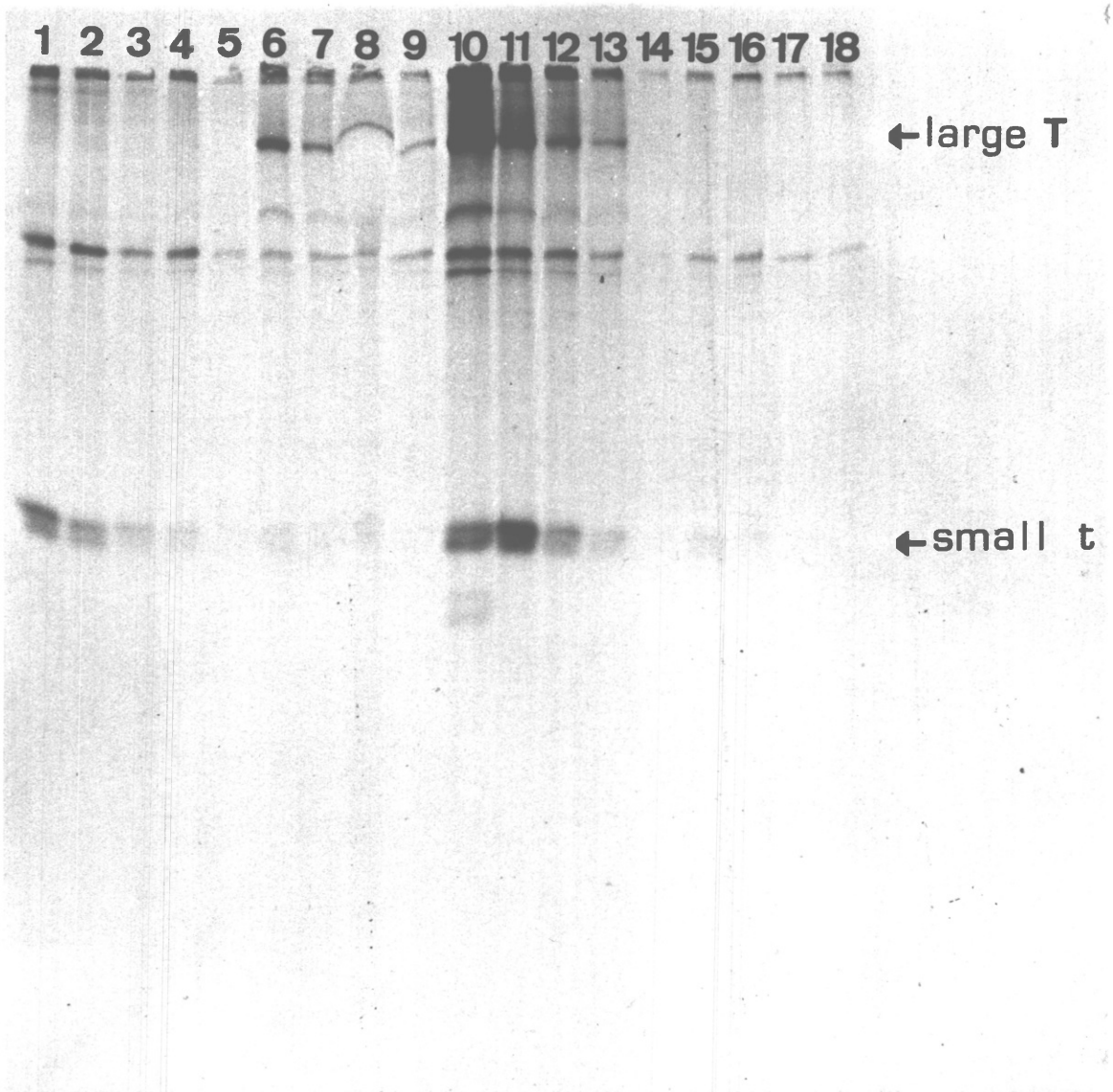

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
large T
small t

Figure 4.9. Sequential Immunoprecipitation of Small t with PAb280 and PAb419 (14 days).

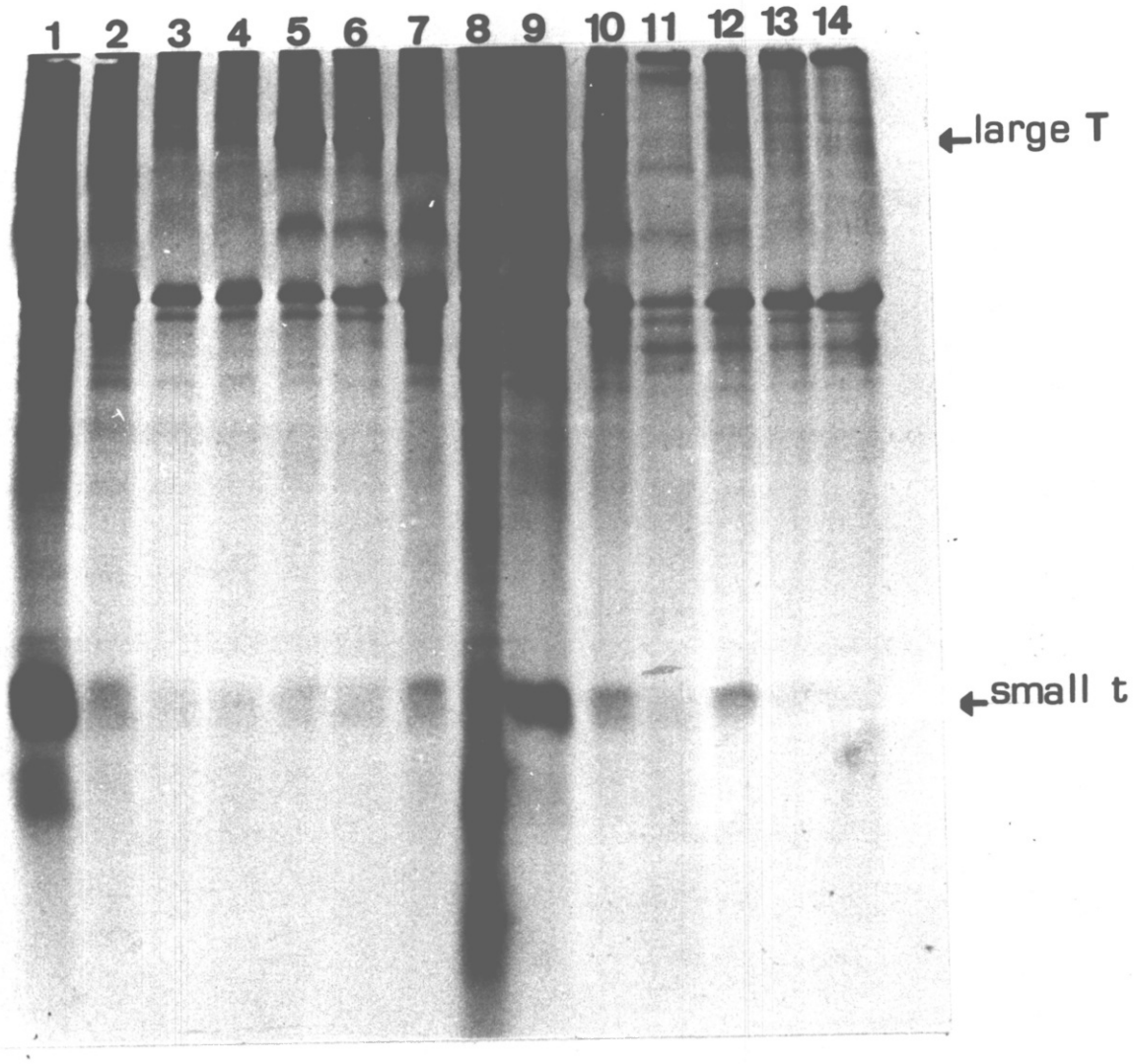
CV1 cells were infected with WT SV40 for 72hrs and labelled with ^{35}S methionine for 3hrs and cell extracts were made (Section 2.4).

Immunoprecipitations were carried out as follows: to 150µl of cell extract 5µg of monoclonal antibody was added and incubated overnight at 4°C, then 50µg of rabbit anti mouse immunoglobulin was added and incubated for 1hr at room temperature. Finally 100µl of Staphylococcus aureus Cowan 1 was added and incubated for 1hr at 4°C. The sample was spun for 2 min. (Eppendorf microfuge). The pellet kept and treated as noted in Section 2.19. The supernatant obtained was mixed with 5µg of monoclonal antibody and the procedure stated above repeated.

This method was carried out for 14 days. 8 days with PAb280 (Tracks 1 to 4) and 6 days with PAb419 (Tracks 5 to 7) and viceversa (Tracks 8 to 11 and 12 to 14).

The samples run for this figure are from immunoprecipitation days 1, 3, 5, 7, 9, 11 and 14.

Samples were run on a 20% polyacrylamide gel with a 5% stack (14 days exposure).


1 2 3 4 5 6 7 8 9 10 11 12 13 14
large T
small t

constant 'motion' in such a way that the epitope recognised by PAb280 at times becomes hidden and therefore there are less small t molecules that can be recognised by this antibody.

It should be pointed out that there is a small population that only show either the PAb280 or PAb419 epitopes; this is noted by an increase in levels immunoprecipitated by PAb419 (tracks (6) in Figure (4.8) and 5 in Figure (4.9) after the extract has been immunoprecipitated with PAb280 and vice-versa.

During preliminary trials of this experiment both PAb419 and PAb280 were present at saturation levels, so that it cannot be argued that there are insufficient antibody molecules of PAb419 to bind small t because large T molecules are competing with this process.

4.6 Small t in Cells Infected with SV402

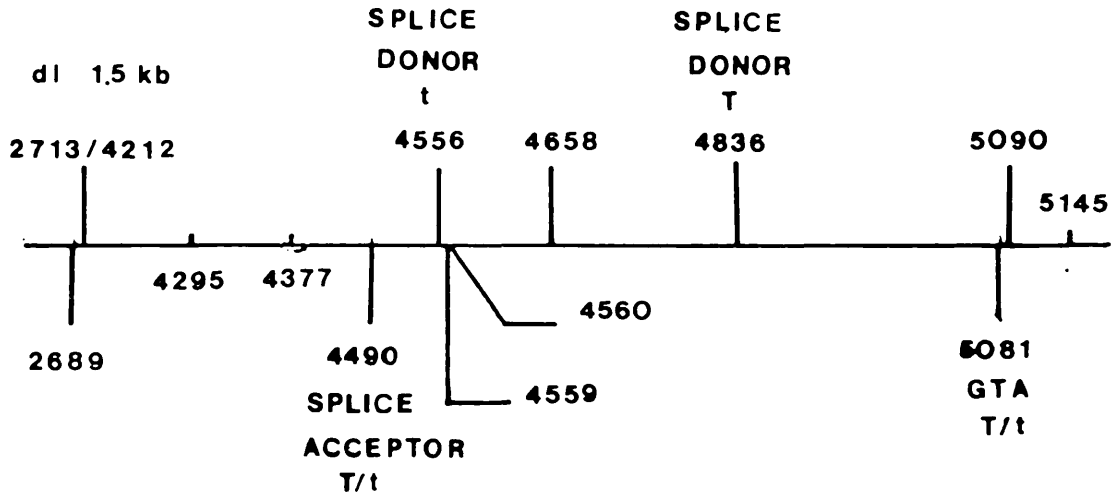
i) Cellular Localisation of Small t

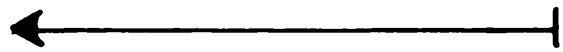
SV402 (Rubin et al., 1982) is an SV40 mutant virus that only produces small t. Figure (4.10) shows the physical map of the SV402 early region. This virus is said to produce 5 times more small t than WT SV40. When CV1 and NIH-3T3 cells were infected with SV402, and small t distribution observed by immunofluorescence staining using rabbit antiserum raised against SDS gel purified t, it was noticed that small t was localised in the nucleus and cytoplasm of the infected cells (Ellman et al., 1984).

The distribution of small t in CV1 cells infected with

Figure 4.10. Sequence Organisation of the SV402 Early Region as Indicated by Rubin et al. 1982.

SEQUENCE ORGANIZATION OF THE SV402 EARLY REGION





SMALL t CODING REGION

SV402 was investigated by using PAb280. Figure (4.11) shows the result. It can be observed that PAb280 stains the nucleus and the cytoplasm of the cell but in this case the staining in the nucleus is significantly stronger than that found in the cytoplasm.

In order to observe if only the small t present in the nucleus is detectable with PAb419 as it is found in WT SV40 infected CV1 cells, SV402 CV1 infected cells were stained with PAb419. Figure (4.11) shows this result. It is interesting to note that in this case the nucleus as well as the cytoplasm of the cell was stained. This result suggested that a) the distribution of the two immunologically different subsets of small t is dependent on the presence of large T or, b) a truncated large T, is expressed by the virus and is transported to the cytoplasm of the cell, where it is detected by PAb419.

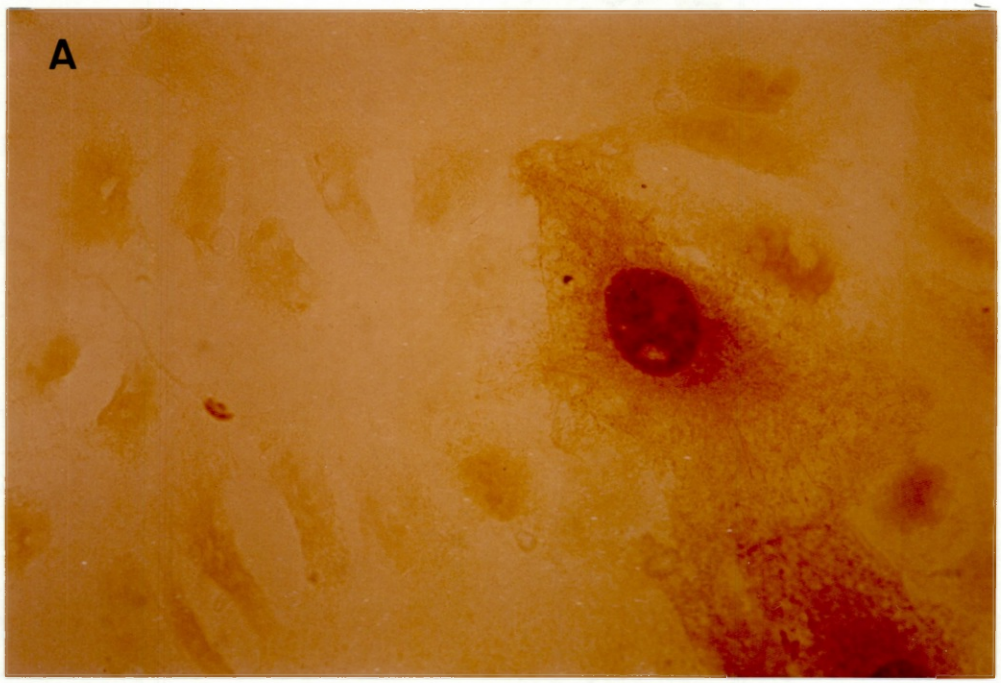
ii) Detection of a Large T Truncated Protein in SV402 Infected Cells

If the SV402 construction is observed in figure (4.10) it can be seen that the segment comprising nucleotides 5081 to 4212 can express small t and a truncated large T of expected m.w. 24.2kd.

To see if this truncated fragment can be detected, CV1 cells were infected with SV402 and stained with PAb416, a monoclonal antibody that recognises an epitope in large T absent from small t. The site has been mapped between amino acids 1 to 130 (Schwyzer et al., 1983).

Figure 4.11. Localization of Small t in CV1 Cells Infected with SV402 Virus

CV1 cells were infected with SV402 for 72hrs. formaldehyde fixed (Section 2.17 (i)(b)) and stained with PAb280 (A) or PAb419 (B) using the immunoperoxidase method (Section 2.17 (ii)(a).


A

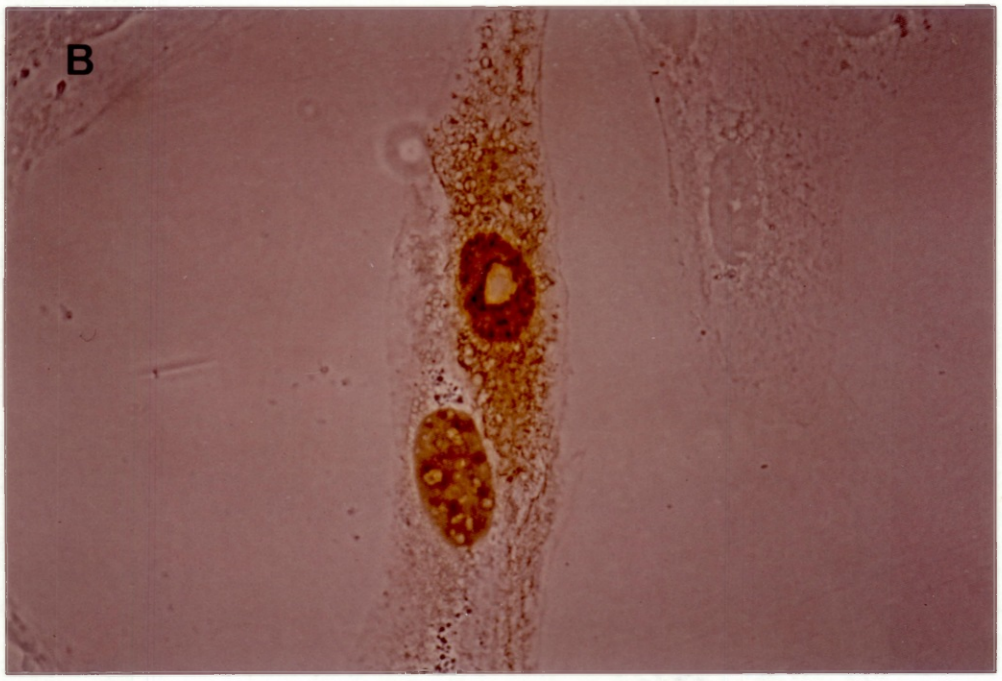

B

Figure (4.12) shows that PAb416 stains the nucleus and cytoplasm of the infected cells, indicating the presence of a large T truncated product. The frequency of stained cells was the same as those stained with PAb280 and PAb419 (70% of cells are stained with any of the mentioned antibodies). Immunoprecipitations to see the truncated product are in progress.

These results suggest that SV402 produces two products: small t and a truncated large T. Both products are detectable by immunoperoxidase staining. The presence of a truncated large T might interfere with the investigation of the role of small t, therefore any biological studies carried out using this virus have to be considered carefully.

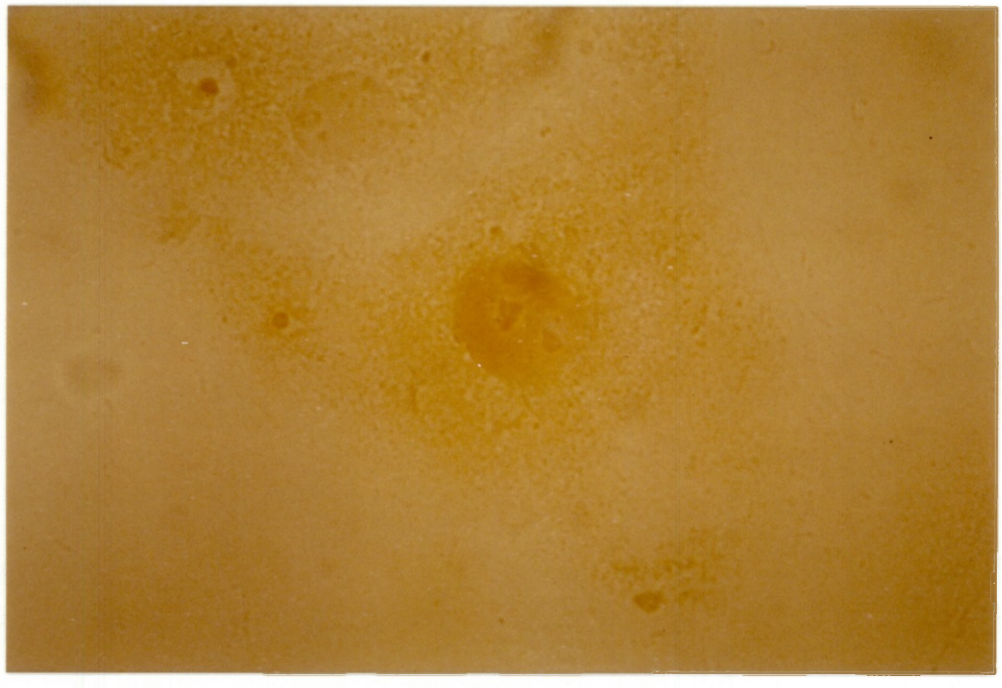
4.7 Discussion

A preliminary characterisation of SV40 small t has been carried out using PAb280. By staining, small t has been found to be located in the nucleus and cytoplasm of lytically infected cells. During lytic infection small t is detected at times 24 to 78hrs after infection, but 72 hrs is the time in which the largest amount of small t is detected.

Small t is also found in the nucleus and cytoplasm of SV40 transformed cells and results are obtained by immunoprecipitation of cell extracts from the same transformed cell lines indicate that small t levels synthesised do not reflect the number of SV40 DNA copies

Figure 4.12. Localization of a Truncated Large T Produced During Infection with SV402 Virus.

CV1 cells were infected with SV402 for 72hrs formaldehyde fixed (Section 2.17 (i)(b)) and stained with PAb416 using the immunoperoxidase method (Section 2.17 (ii)(a)).



found in the cell genome.

When using PAb419 (an antibody that recognises large T and small t through a common epitope at the amino-terminus common region) and PAb280, two subsets of immunologically distinct small t molecules have been detected. These subsets are clearly seen by immunoprecipitation with PAb419 and PAb280 of sequential extraction (a procedure by Staufenbiel and Deppert, 1983) from lytically infected cells.

Sequential immunoprecipitation experiments show that these sets cannot be separated totally. Instead it can be inferred that the small t molecule is in constant motion in such a way that either PAb419 or PAb280 epitopes are expressed with a subpopulation probably presenting both simultaneously.

When small t produced by SV402 is studied using PAb280, it can be seen that it is localised in the nucleus and cytoplasm of the infected cells. When PAb419 was used to stain the same infected cells it was surprisingly found to be nuclear as well as cytoplasmic. Two hypotheses can be proposed from this result. First it is possible that large T is needed for the correct distribution of subsets of small t. The second hypothesis relates to the possible presence of a truncated large T detected with PAb419 in the cytoplasm of the cell. This possibility was deduced from the construction of the virus (see Figure 4.10).

These hypotheses were assessed by staining SV402 infected CV1 cells with PAb416 (a monoclonal antibody that

recognises large T between amino acids 1 to 130 (Schwyzer et al., 1983) (see Appendix) but does not recognise small t). Using the immunoperoxidase method the nucleus and cytoplasm of these cells was stained strongly, indicating the presence of a truncated large T protein. Therefore the cytoplasmic stain seen with PAb419 in SV402 infected cells is probably due to the recognition of this truncated large T and not necessarily small t. Therefore any previous result obtained using this virus which relates to the role of small t has to be considered carefully in view of the presence of this additional viral product.

CHAPTER FIVE

AN IMMUNOCHEMICAL INVESTIGATION OF LARGE T FROM SV40 tsA TRANSFORMED CELL LINES

5.1 Introduction

During early investigation of the genetics of SV40, temperature sensitive mutants (ts) were isolated. These ts mutants have been separated into five groups (A, B, C, BC and D) on the basis of their ability to complement each others defects at restrictive temperature during lytic infection. Temperature sensitive mutants result from alterations in the DNA sequence that, in turn, cause changes in the amino acid sequence of a protein. The structure of the protein is altered in such a way that it can function at one (permissive) temperature, but not at another, usually higher (restrictive) temperature.

In productive infection, the A gene protein initiates viral DNA replication, represses early transcription, and promotes late transcription. The requirement for A gene function in DNA replication and early transcription appears to be continuous and direct because both processes are rapidly affected by a shift from permissive to restrictive temperature (Reviewed in Tooze, 1981; Reviewed in Martin, 1981).

tsA mutants were initially characterized by synthesizing large T (A gene protein) at a faster rate than by WT SV40. The protein specified by the mutants is much more labile than the WT protein (Tooze, 1981, Brockman, 1978). The net result is a decreased accumulation of large T in cells infected by tsA mutants (Tegtmeyer, 1975).

The expression of the SV40 A gene encoding large T has also been shown to be necessary and sufficient for

transformation of non-permissive cells. tsA mutants were employed to analyze the role of large T in this process (Reviewed in Tooze, 1981; Reviewed in Martin, 1981).

Transformation studies with tsA mutants suggested that expression of large T can influence the behaviour of cells after stable transformation has been established. A number of the growth properties of cells transformed by tsA mutants are affected by a shift to the restrictive temperature. These properties include: growth rate, saturation density, growth in medium with a reduced serum concentration, and colony formation a) on a plastic or glass surface, b) on monolayers of non-transformed cells, or c) in soft agar. Therefore continuous production of large T may be necessary to maintain at least some of the phenotypic characteristics of SV40 transformed cells.

In an attempt to define the role of SV40 in the maintenance of the transformed state several laboratories have examined the temperature sensitivity of the transformed phenotype of cells transformed by several tsA mutants. There is some disagreement between the results obtained by different groups. Brugge and Butel (1975) found tsA transformants to be temperature sensitive, however Tegtmeyer (1975) found some tsA transformants to be temperature independent. Other authors have also reported the isolation of temperature independent tsA lines (Reviewed in Martin, 1981) and found that the growth state of the cells at the time of infection, multiplicity of

infection, as well as inherent variabilities within different cell types may influence whether the resultant transformed phenotype is temperature dependent or independent and may also indicate whether it is regulated by large T expression (Brugge and But el, 1975, Noonan et al., 1976; Brockman, 1978; Gaudray et al., 1978; Rassoulzadegan et al., 1978; Fluck and Benjamin, 1979; Imbert et al., 1983; Seif and Martin, 1979a).

Recently (James Pipas, personal communication) a variety of the tsA mutants have been characterized further by DNA sequencing and from these data the aminoacid sequence of the site of the mutation has been obtained. These include tsA255 and tsA209 (Chou and Martin, 1974), tsA58 and tsA7 (Tegtmeyer et al, 1970, 1971; Tegtmeyer, 1972, 1975).

For the studies described in this chapter, six cell lines transformed by the tsA mutants indicated above were employed. A209 B4a, A255 B1a, A7B4b (Brockman, 1978). A21, J78 and J78/11 (40) (Hiscott et al., 1980, 1981; Hiscott and Defendi, 1980). These cell lines were chosen because they are well characterized.

The approach to the isolation of both sets of cell lines by Brockman is based on previous results (discussed above) obtained by Brugge and But el (1975) and Tegtmeyer (1975). The former group found that the transformed phenotype shifted to a normal phenotype at the non-permissive temperature whereas the latter did not detect such reversion. It is important to note that Brugge and But el used the tsA7 mutant which maps at the HindII and

HindIIIB fragment (see Figure (1.3)). Meanwhile Tegtmeyer used ts28 and tsA30 which map in the I fragment. Therefore it was assumed that tsA mutants that differ in the location of their defect might also differ in the temperature sensitivity of their ability to maintain transformation. This hypothesis was tested by Brockman. Balb/c 3T3 cells were transformed at 33°C by tsA mutants whose mutation sites map at 0.25 to 0.5 in the early region (see Figure (1.3)). Individual colonies were selected using the focus formation assay. The role of large T in the maintenance of the transformed state of the cells was tested for temperature sensitivity by the focus formation assay and by growth in soft agar. Colonies growing at the permissive (P) and non-permissive temperature (NP) were scored and the P/NP ratio obtained. It was shown that 15 cell lines including A209 B4a, and A255 B1a were temperature sensitive whereas A7 B4b is a cell line which is marginally affected by temperature shift. However, other cell lines transformed by other tsA mutants (e.g. tsA30) were temperature independent for the transformed phenotype.

Brockman also showed that large T antigen extracted from tsA transformed cell lines when assayed by complement fixation was more thermolabile (irrespective of whether the large T comes from a temperature dependent or independent cell line) than large T antigen extracted from a WT transformant.

Finally in order to determine whether the difference in the temperature sensitivity of the transformed phenotype

among temperature dependent and independent might be due to intracellular concentrations of large T, the levels of large T were quantitated (Crawford and Lane, 1977) this was carried out by monitoring the formation of immune complex where increasing amounts of cell extract were added to a fixed amount of anti T serum and ^{125}I-Protein A. It was found that temperature independent lines have more large T than temperature dependent lines implying very strongly that large T is required to maintain the transformed phenotype and that a cell line is temperature independent due to a protein dosage effect i.e. higher amounts of large T have to be produced to maintain the transformed phenotype at the non-permissive temperature.

The investigation of Defendi's group used a different approach. During preliminary work using mouse embryo fibroblasts transformed by SV40 tsA mutants they noted that the viral DNA integrated into the mouse genome to a precise pattern, and that this pattern had to be rearranged for the conversion from temperature dependent to a temperature independent phenotype (Hiscott and Defendi, 1980). They examined more closely the hypothesis that changes in viral DNA arrangement occured frequently or indeed were responsible for the selection of the temperature independent phenotype.

A21 is a temperature sensitive mouse embryo fibroblast cell line transformed by tsA58 which was selected by the capacity of growing in soft agar at 33^{o}C (Hiscott et al.,

1981) and is characterized by having a) multiple copies of SV40 DNA integrated into the mouse genome at discrete sites, b) covalently closed SV40 DNA molecules which comigrate with SV40 DNA form I. J78 a temperature sensitive line which was subcloned from A21 is free from episomal SV40 DNA and is characterized by containing two SV40 integration sites contained within BglII restriction fragments with MW 10Kb and 14Kb respectively. A clonal derivative from J78 was also obtained by growing cells in soft agar at 40°C and the arrangement of viral DNA sequences was examined (Hiscott et al., 1981). J78/11 (40) in addition to the 10Kb and 14Kb BglII fragments also contains a 25Kb BglII fragment which hybridized to SV40. The authors suggested that this last fragment has been produced due to an amplification mechanism. Finally it was hypothesized that the acquisition of temperature independence is due to the increased number of viral templates which in turn results in higher levels of transcription.

The aim of the experiments presented in this chapter was to determine the different levels of large T in the tsA transformed cell lines described above. Large T was quantitated using a two site sandwich radioimmunoassay and a range of anti T monoclonal antibodies. The assay revealed that certain epitopes on large T were altered in in tsA mutant T molecules.

5.2 In vivo Experiments

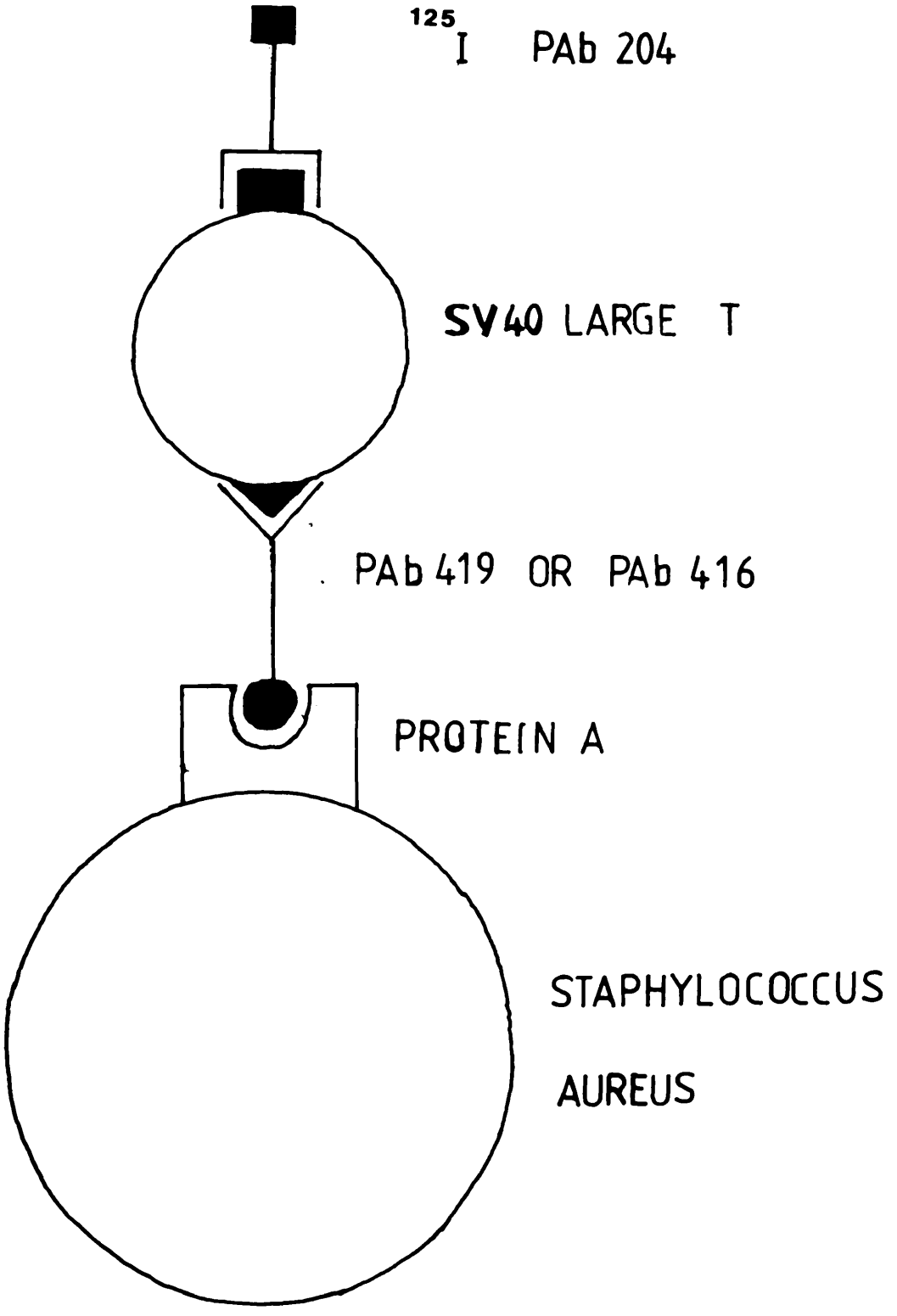
The principle of the assay employed in this set of experiments uses the differential binding of different subclasses of mouse immunoglobulin to Protein A on formalin fixed Staphyloccus aureus. It has been determined that monoclonal antibodies of the IgG_1 subclass do not bind significantly to heat killed formalin fixed Staphylococcus aureus cells in NET pH8 containing 0.5% NP40; whereas monoclonals of other lgG subclasses bind very tightly under the same conditions. Therefore high specific activity iodinated IgG_1 monoclonal antibodies will only bind to Staphylococcus aureus cells in the presence of the specific antigen they can recognize and a second monoclonal antibody of non-IgG_1 subclass which can recognize a non-overlapping epitope.

For these experiments the following reagents were used: PAb204 (Lane and Hoeffler, 1980) an IgG_1 monoclonal antibody, PAb419 and PAb416 (Harlow et al., 1981) and PAb203 (Montano, Gannon and Lane, manuscript in preparation) which are IgG_{2a} subclass monoclonal antibodies as well as SV40 large T antigen from tsA transformed cell lines.

Figure (5.1) shows a molecular representation of the principle of the assay (Sandwich assay).

It is important to state that all the components, that is, PAb204, large T containing cell extracts and PAb419, PAb416 or PAb203, were added in solution and therefore all molecular species have an equal chance to bind. Similarly

Figure 5.1. Diagramatic Representation of the Binding of Large T to PAb204 (IgG_1) and PAb416 or PAb419 (IgG_2a) In a Sandwich Assay


^{125}I PAb 204
SV40 LARGE T
PAb 419 OR PAb 416
PROTEIN A
STAPHYLOCOCCUS
AUREUS

the antibodies are present at saturation concentrations so that all the antigen within a given reaction mixture can be bound by the antibody.

Figure (5.2) shows a typical titration of large T from WT SV40 transformed cell line SV3T3 C138 (Clayton and Rigby, 1981) using the sandwich assay. The titrations were carried out using cells grown at 32°C and 39.5°C.

In (A) titrations with PAb419, PAb416 and PAb203 of large T from cells grown at 32°C are observed. It can be seen that the levels of large T as detected by all three antibodies are the same (see slopes of the curves) although at higher concentrations of large T there seems to be more large T detected by PAb203 than by PAb419 and PAb416. Similarly when extracts of cells grown at 39.5°C were titrated (B) it was possible to see that the binding trend of the antibodies was the same as for 32°C.

If the titrations at 32°C and 39.5°C are compared it can be noted that there is a slight decline in the level of large T at 39.5°C detected by all three antibodies. This is probably due to changes in cell metabolism which are induced by an increase in temperature (from 32°C to 39.5°C) which in turn slightly alters the levels of large T.

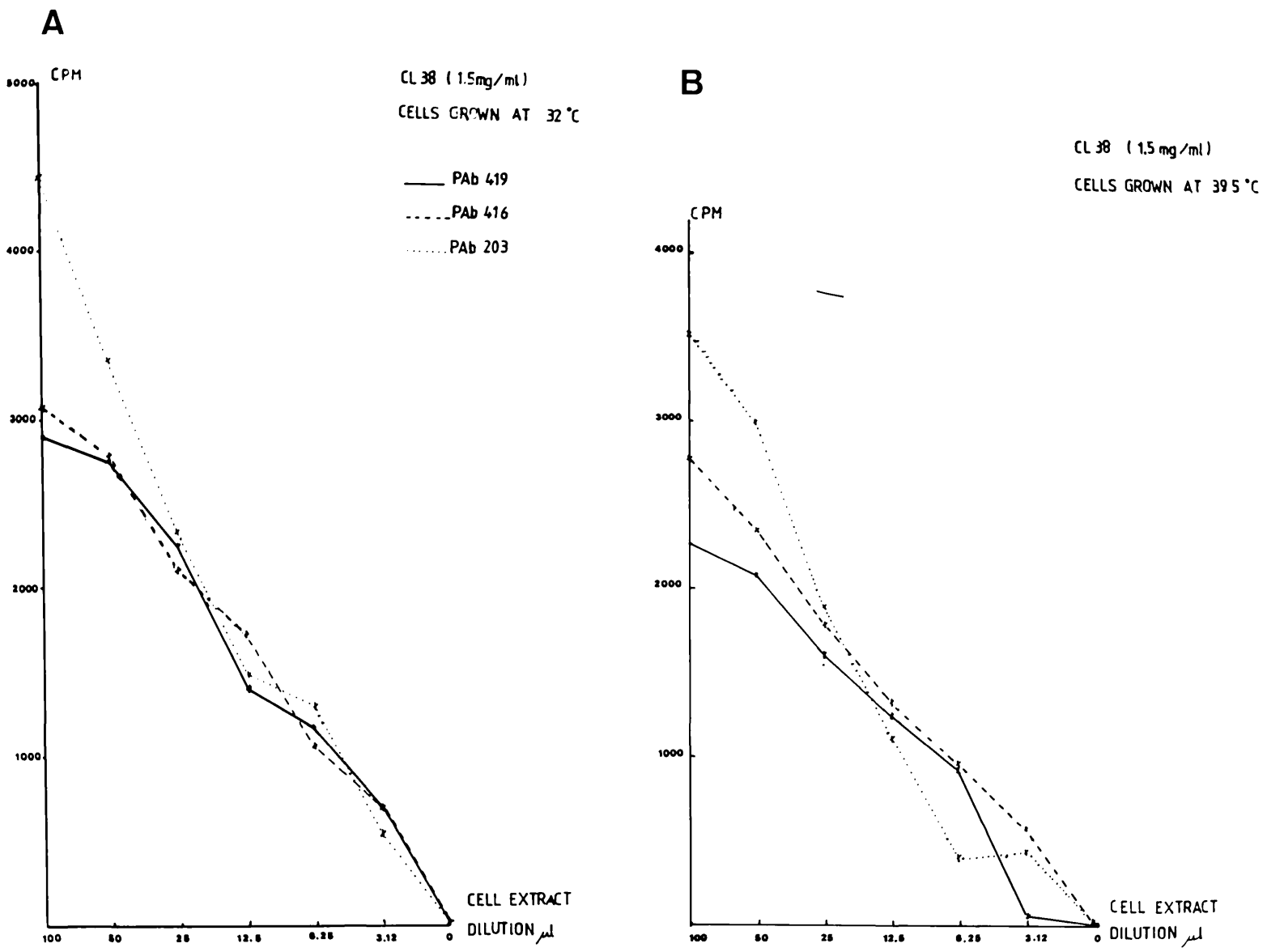
The experiment shown in figure (5.2) indicates that the assay is very sensitive and specific for the component to be assayed, in this case SV40 large T antigen. When controls in which PAb204, cell extract but not the second antibody (i.e. PAb419 or PAb416) were present the

Figure 5.2. Titration of Large T Antigen From SV3T3 C138 Cells Grown at 32°C and at 39.5°C

SV3T3 C138 cells were grown to 80% confluency at 32°C or 39.5°C. Cell extracts were made (Section 2.4) and large T titrated with PAb416, PAb419 or PAb203 using the sandwich assay (Section 2.15).

A. Titration of large T with all 3 antibodies from cell extracts of cells grown at 32°C.

B. Titration of large T with all 3 antibodies from cell extracts of cells grown at 39.5°C.


A
CPM
5000
4000
3000
2000
1000
CL 38 (1.5mg/ml)
CELLS GROWN AT 32 °C
PAb 419
PAb 416
PAb 203
CELL EXTRACT
DILUTION µl
100
50
25
12.5
6.25
3.12
0
B
CL 38 (1.5 mg/ml)
CELLS GROWN AT 39.5 °C
CPM
4000
3000
2000
1000
CELL EXTRACT
DILUTION µl
100
50
25
12.5
6.25
3.12
0

background levels were extremely low; the same results were obtained when both first and second antibodies were mixed in the absence of the cell extract containing large T.

(i) Experiments With tsA Transformed Cell Lines

Cells were plated in 9cm dishes to 40% confluency and allowed to settle overnight at 32°C. One set of plates was then transferred to 39.5°C. Cells were left to grow for 3 days (to 80-90% confluency) at both temperatures. Cell extracts were made (Section 2.4) and the protein concentration determined (Section 2.23(i)) and standardized to 1.5mg/ml. A serial doubling dilution of the extracts was made and large T was titrated with PAb419, PAb416 and PAb203 hybridoma supernatant (Section 2.15). Three different antibodies were chosen: PAb419 and PAb416 which recognize SDS denaturation resistant epitopes in large T and PAb203 which recognizes an SDS denaturation sensitive epitope (See Appendix).

An example of the results obtained using tsA transformed cell lines is given in Figure (5.3) using cell extracts of A209 B4a a temperature sensitive cell line (Brockman, 1978).

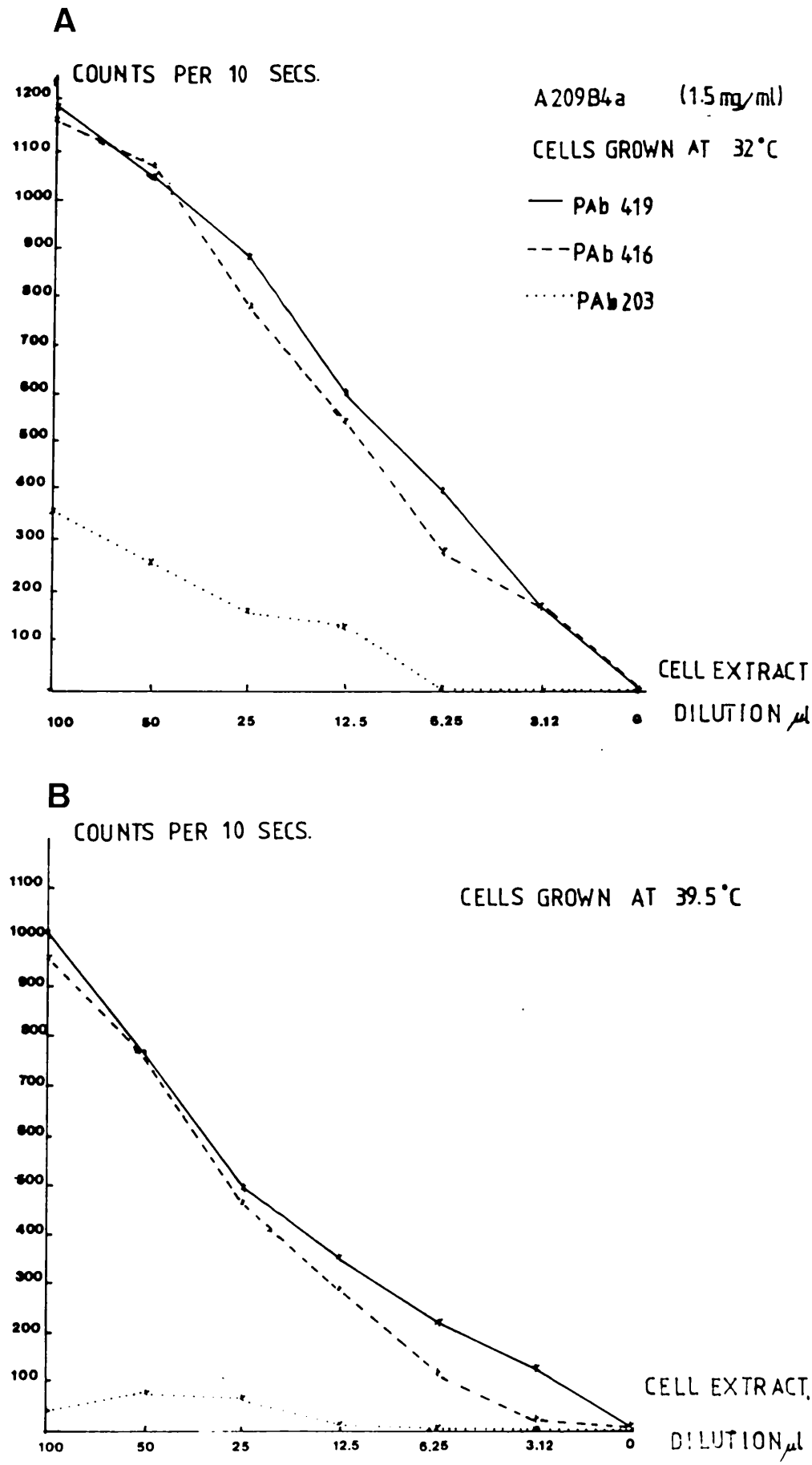
It can be seen that when cell extracts of cells grown at the permissive temperature (32°C) are used, PAb416 and PAb419 bind practically identical amounts of large T (see slopes of the curves), that is PAb419 and PAb416 relative binding trends are similar to PAb416 and PAb419 large T binding in SV3T3 C138 (see Figure (5.2)). Whereas this is not the case for PAb203; large T amounts detected by PAb203

Figure 5.3. Titration of Large T Antigen from A209 B4a Cells Grown at 32°C and at 39.5°C

A209 B4a cells were grown to 80% confluency at 32°C or 39.5°C and cell extracts made (Section 2.4).

A. Titration of large T with all 3 antibodies from cell extracts of cells grown at 32°C.

B. Titration of large T with all 3 antibodies from cell extracts of cells grown at 39.5°C.


A
COUNTS PER 10 SECS.
1200
1100
1000
900
800
700
600
500
400
300
200
100
A209B4a (1.5 mg/ml)
CELLS GROWN AT 32°C
PAb 419
PAb 416
PAb 203
100
50
25
12.5
6.25
3.12
0
CELL EXTRACT
DILUTION µl
B
COUNTS PER 10 SECS.
1100
1000
900
800
700
600
500
400
300
200
100
CELLS GROWN AT 39.5°C
100
50
25
12.5
6.25
3.12
0
CELL EXTRACT
DILUTION µl

in A209 B4a are much smaller than when PAb203 is employed to assay large T from SV3T3 Cl38.

When large T is titrated from A209 B4a cells grown at the non-permissive temperature (39.5°C), only a small drop in T bound by PAb419 and PAb416 can be observed (see slopes) but when tested with PAb203 a further pronounced decrease in binding capacity is clearly detected. This last observation is once again different to that found with the titration of large T with PAb203 in SV3T3 Cl38 grown at 39.5°C (See Figure (5.2)).

It is important to stress that these assays measure the relative binding by PAb203, PAb416, PAb419 of PAb204 labelled molecules through SV40 large T.

A series of conclusions can be drawn from this experiment. First it seems obvious that PAb203 can clearly recognize differences between large T from a WT SV40 transformant and a tsA209 transformant. Secondly, PAb203 recognises a difference in large T produced by A209 B4a at 32°C and at 39.5°C.

tsA mutations induce a change in molecular configuration of large T which can be detected by PAb203 but not by PAb416, PAb419 or PAb204. This occurs either because most but not all of the large T molecules lose the epitopes recognized by PAb203 or because PAb203 has a much lower affinity for all the tsA molecules.

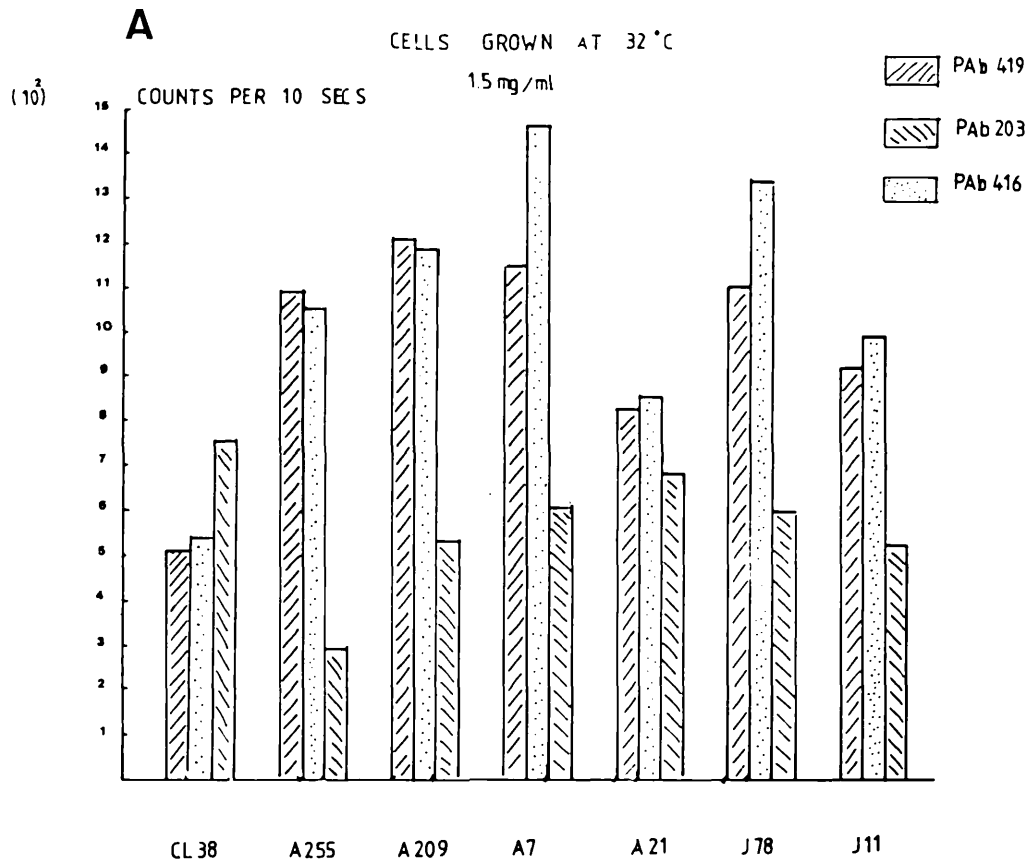
Figures (5.4) (A) and (B) show a summary of the results obtained with all six tsA transformed cell lines introduced

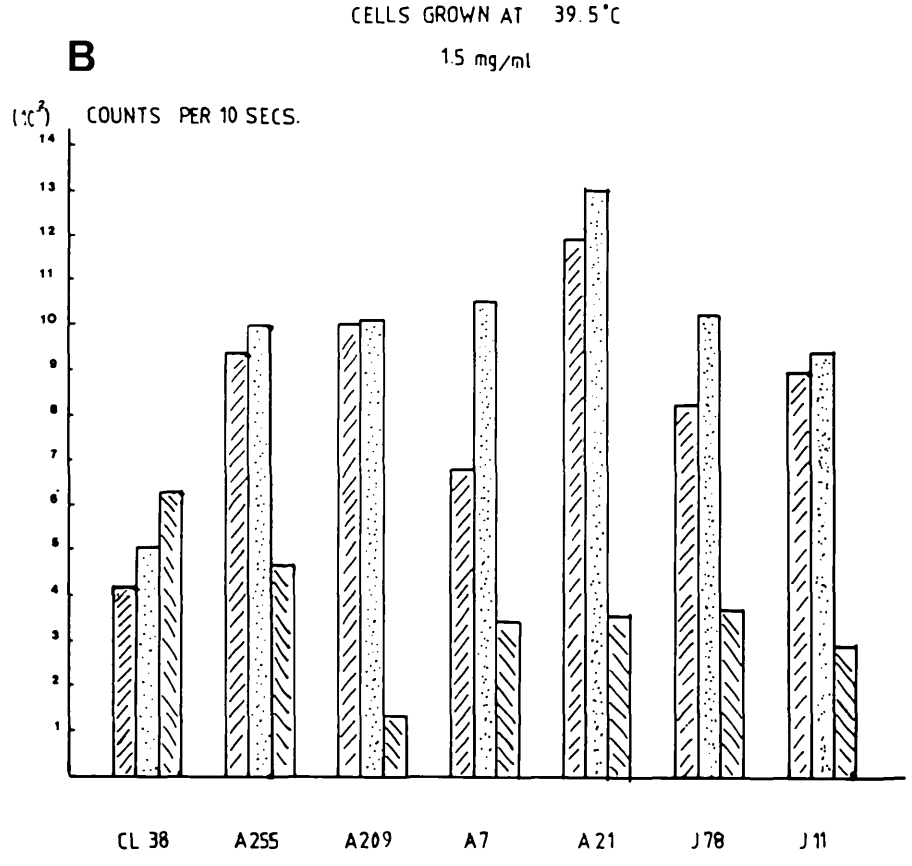
Figure 5.4. Histogram Presenting a Summary of the Results Obtained by Titrating Large T with PAb416, PAb419 and PAb203 at 32°C and at 39.5°C.

A full titration of large T from SV3T3 C138 and tsA transformed cell lines grown at 32°C and at 39.5°C using PAb416, PAb419 and PAb203 was carried out. A linear regression for each curve was calculated and an equal point obtained and used to plot a histogram.

A. Histogram of large T titrated with PAb416, PAb419 and PAb203 from cell extracts of cells grown at 32°C.

B. Histogram of large T titrated with PAb416, PAb419 and PAb203 from cell extracts of cells grown at 39.5°C.


A
CELLS GROWN AT 32 °C
1.5 mg/ml
(10²)
COUNTS PER 10 SECS
PAb 419
PAb 203
PAb 416
CL 38
A255
A209
A7
A21
J78
J11


CELLS GROWN AT 39.5 °C
1.5 mg/ml
B
COUNTS PER 10 SECS.
CL 38
A255
A209
A7
A21
J78
J11

in Section 5.1.

A full titration of cell extracts from cells grown at 32°C and at 39.5° as for A209 B4a was carried out for each cell line. A linear regression was plotted and an equal point obtained from the linear range.

Figure (5.4) (A) shows the quantitation of large T from cell extracts of cells grown at 32°C. It can be seen that in all six tsA cell lines, less large T is bound by PAb203. Table (5.1) shows the ratios of PAb203 to PAb416 and PAb419 and it can be clearly noted that for all six cases, PAb419 and PAb416 bind more large T molecules than does PAb203, whereas for the control SV3T3 C138 the opposite is the case (see also figure (5.2)). When large T from SV3T3 C138 is titrated with PAb416 and PAb419 and compared with large T from tsA transformed lines titrated with the same antibodies it can be seen, that the levels of large T in C138 are slightly lower than from tsA transformed lines. This seems to be an innate property of SV3T3 C138 (G. Winchester, personal communication).

When cell lines were subjected to 39.5°C and large T titrated from the cell extracts (see Figure (5.4) (B)), it was possible to see that in all five cell lines with exception of A255 B1a there is a decrease in the number of large T molecules bound by PAb203. In addition the ratios of PAb416 and PAb419 to PAb203 bound molecules is higher in each case with the exception of A255 B1a (see table (5.1)). Whereas in the control SV3T3 C138 only a small change is observed.

TABLE 5.1

Ratios of Large T Molecules Bound by Monoclonal Antibodies

Cell Line	Ratio at 32°C		Ratio at 39.5°C	
	PAb416 / PAb203	PAb419 / PAb203	PAb416 / PAb203	PAb419 / PAb203
A255 B1a	3.63	3.76	2.10	1.97
A209 B4a	2.22	2.27	7.25	7.21
A7 B4b	2.40	1.88	3.03	1.95
A21	1.26	1.21	3.63	3.31
J78	2.25	1.84	2.74	2.20
J78/11(40)	1.89	1.759	3.186	3.050
C138	0.71	0.68	0.80	0.67

When comparing the amounts of large T detected at 32°C and at 39.5°C a decrease is observed as judged by PAb419 and PAb416 for A255 B1a, A209 B4a, A7 B4b, J78 and J78/11 (40).

It should be noted that in A7 B4b, J78 and SV3T3 C138 cell extracts, the overall steady state of large T has dropped as detected by all three antibodies. When using A255 B1a, A209 B4a and J78/11 (40) cell extracts there is also a small drop in large T detected by PAb416 and PAb419 but there is a large increase in the case of A21.

Thus by examining the ratio of PAb203 to PAb416 and PAb419 at both temperatures three distinct phenotypes can be observed: a) cells that show a ts effect when shifted to 39.5°C giving a reduced population of large T molecules with the PAb203 epitope such as A209 B4a, A21 and J78/11 (40); b) cells that show a negative ts effect and have a large population of large T molecules with the PAb203 epitope such as A255 B1a; c) cells that are marginally affected by the ts effect such as A7 B4a, and J78.

5.3 In vitro Experiments

The aim of these experiments was to see if the results obtained by the in vivo experiments could be mimicked by an in vitro temperature shift of the tsA and WT SV40 transformed cell line extracts.

Cell extracts of the temperature sensitive cell lines as well as SV3T3 C138 grown at 32°C were made (Section (2.4)), their protein concentrations determined (Section

(2.23 (i)) and standardized to 1.5mg/ml. Cell extracts were subjected to 39.5°C for various times and titrated with PAb203 and PAb416.

It has to be noted that the design of the experiment is clearly different to that used *in vivo* in that the cell extracts are subjected to 39.5°C for a relatively short time, while *in vivo* large T is being synthesized and processed at the non-permissive temperature.

Figures (5.5) (A and B) show an example of the results obtained. In the case of the control SV3T3 C138, a steady decrease in detection of large T by PAb416 can be seen. It decreases with cell extract dilution as well as with an increase of time of incubation at 39.5°C. A similar result can be seen with A7B4b cell extracts.

The same experiments were carried out with all cell lines using both PAb416 and PAb203.

The half life of large T was calculated, and the results obtained can be seen in Table (5.2).

On average it can be seen that the half life of large T from the tsA transformed cell lines is 10 min (with the exception of A7B4b) whereas large T from WT SV40 transformed cell lines has a half life of 30 min regardless of which antibody has been used in the assay. These results agree with those obtained by Brockman, 1978 using complement fixation assays.

The large T amounts obtained reflect the *in vivo* experiments.

Figure 5.5. In vitro Experiments: Titration of Large T with PAb416 from SV3T3 Cl38 and A7B4b

A. Large T titration with PAb416 from SV3T3 Cl38 cell extracts of cells grown at 32°C and extracts subjected to 39.5°C for times 0, 10, 30 and 60 min.

B. Large T titration with PAb416 from A7B4b cell extracts of cells grown at 32°C and extracts subjected to 39.5°C for times 0, 10, 30 and 60 min.

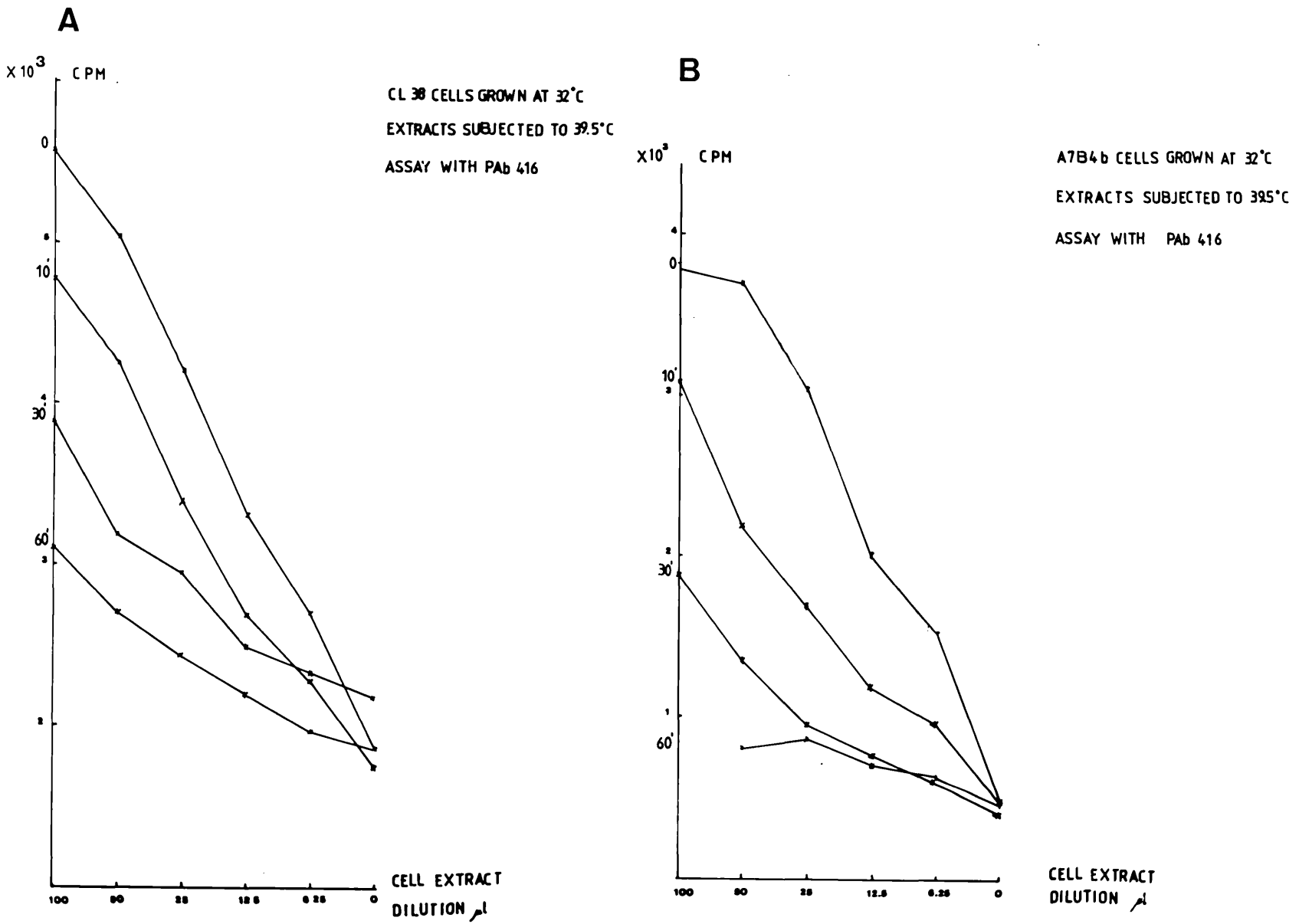

A
X 10³
CPM
0
10'
30'
60'
CL 38 CELLS GROWN AT 32°C
EXTRACTS SUBJECTED TO 39.5°C
ASSAY WITH PAb 416
100
50
25
12.5
6.25
0
CELL EXTRACT
DILUTION µl
B
X10³
CPM
0
10'
30'
60'
A7B4b CELLS GROWN AT 32°C
EXTRACTS SUBJECTED TO 39.5°C
ASSAY WITH PAb 416
100
50
25
12.5
6.25
0
CELL EXTRACT
DILUTION µl

TABLE 5.2

Half Life of Large T Antigen

Cell Line	PAb419	PAb203
A255 B1a	7'20"	5'40"
	PAb416	
A7 B4b	11'40"	31'20"
A209 B4a	8'	6'40"
A21	5'40"	5'41"
J78	7'40"	11'
J78/11(40)	9'40"	8'20"
C138	26'	30'

The fact that tsA large T is more thermolabile than WT large T seems to be a property of the protein (as this set of experiments prove). Therefore its detection is highly dependent upon this characteristic and not upon which epitope within the protein has been looked at.

If the early region of the tsA mutants employed to transform the set of cell lines used in these experiments are observed at the nucleotide and aminoacid sequence level the changes noted in Table (5.3) can be seen (when comparing to WT SV40).

5.4 Discussion.

This chapter describes the measurement of large T levels using a set of monoclonal antibodies which recognize epitopes in different sites of the large T molecule.

The *in vivo* experiments show that when large T is titrated from the tsA transformed cell lines grown at 32°C there is more large T detected by PAb419 and PAb416 than by PAb203 (if compared to WT transformant SV3T3 C138). This implies that PAb203 is recognizing an epitope altered by the mutation in large T.

When cells are subjected to 39.5°C and large T titrated with the monoclonal antibodies it can be seen that: in five cases (A209 B4a, A7 B4b, A21, J78, J78/11 (40)) there is a decrease in the amount of large T bound to PAb203; A255 B1a is exceptional since an increase can be detected. PAb419 and PAb416 reveal a decrease in large T concentrations in A255 B1a, A209 B4a, A7 B4b and J78 but

TABLE 5.3

Base Pair and Amino Acid Changes of the tsA Mutants

tsA Mutant	bp Change	Aminoacid Change	HindII and III Cleavage Fragments
tsA 255	G(3552)-C	trp(422)-lys	I
tsA 209	C(3538)-T	pro(427)-leu	I
tsA 58	C(3505)-T	ala(438)-val	I
tsA 7	3480 1708*	-	B to H

* indicates that the map position of the mutation has been estimated from marker rescue data.

not in A21 and J78/11 (40). In the case of A7 B4b and J78 all three antibodies detect a drop in large T concentration at the non-permissive temperature. If the binding of PAb203 for large T obtained from cells grown at 39.5°C is observed and compared to 32°C three distinct phenotypes can be observed: a) SV40 tsA transformed cells that were grown at 39.5°C have large T molecules showing a ts effect, therefore have a small population of large T molecules which can bind to PAb203 such as A209 B4a, A21 and J78/11 (40); b) SV40 tsA transformed cells that when grown at 39.5°C have large T molecules showing a negative ts effect, therefore have a large population of large T bound by PAb203 such as A255 B1a; c) SV40 tsA transformed cells that when grown at 39.5°C show a population of large T marginally affected by the ts effect such as A7 B4b and J78.

The in vitro results do not resemble the in vivo results. The only conclusion that can be obtained is that large T from tsA transformed cell lines is more thermolabile than from WT transformants such as SV3T3 C138. These results agree with those obtained by Brockman (1978) and with those for large T using tsA lytic infection (Kuchino and Yamaguchi (1975)), although in these laboratories this measurement has been carried out using polyclonal serum rather than monoclonal antibodies.

From this data it is not possible to conclude if the temperature independent phenotype is due to a protein dosage effect. A7 B4b is a temperature semi-sensitive cell

line and J78/11 (40) is a temperature independent cell line. In the case of the former the overall steady state of large T is lower as detected by all three antibodies whereas for the latter there is a drop in large T detected by PAb203 but not by PAb419 and PAb416. But if the levels of large T in A21 a temperature dependent cell line are observed it can be seen that as detected by PAb419 and PAb416 there is a large increase in T levels which is not detected by PAb203. Therefore the data indicates that there is probably another property of large T that confers the temperature independent phenotype.

It is also possible that the site recognized by PAb203 has some biological relevance and that it is not the overall amounts of large T which are important to acquire the temperature independent phenotype instead it is the number of molecules in the correct molecular configuration.

Initially there was some discrepancy among the findings by different laboratories. Brockman (1978) and Tennen et al., (1977) working with mouse cells transformed by SV40 tsA mutants indicated that a temperature independent phenotype is manifested due to an overproduction of T antigen (that is leakiness of T antigen), whereas Martin's group working with rat cells transformed by SV40 tsA mutants (Seif and Martin, 1979, Chepelinsky et al., 1980) indicated that this is not the case. In more recent investigations Chepelinsky et al (1983) have isolated flat revertants of FR 3T3 SV40 tsA

mutant transformed cell lines by selectively killing with fluorodeoxyuridine cells which continue replication at high cell densities. This selection was performed at the non-permissive temperature. It was observed that all of the flat revertants examined had lost their ability to express T antigen at both temperatures and all had the non-transformed phenotype at both temperatures. As the selection was carried to correct for the transformed phenotype and not for the loss of T antigen, it was concluded that large T is required for the maintenance of the transformed state at the non-permissive temperature and that this is due to leakiness of large T.

The work described in this chapter indicates that large T has to be present in the correct conformation i.e. WT conformation, and that if leakiness occurs as a result of overproduction of large T, then this has to be expressed in terms of leakiness of large T molecules which are in the correct molecular configuration, i.e. a configuration that can confer the transformed phenotype to the cells.

Results obtained with A255 Bla cannot be explained within this context and will have to be investigated further.

C H A P T E R S I X

D I S C U S S I O N

Discussion

The aim of the work described in this thesis was the use of monoclonal antibodies to investigate the role of SV40 small t and large T in transformation. In order to achieve this purpose the first monoclonal antibody that uniquely recognizes small t has been produced (Montano and Lane, 1984) and used to characterize small t in lytically infected cells as well as in transformed cells.

The production of a monoclonal antibody of this specificity was a difficult task. A large source of small t was needed to devise an immunization procedure as well as a hybridoma screening method, this problem was overcome by the use of bacterial expression vectors that direct the synthesis of small t through inducible promoters giving yields of up to 5% small t from total bacterial protein.

Large T and small t share 82 amino acids at the amino-terminus and this region is extremely immunogenic, giving rise to a variety of antibodies that recognize epitopes within this region. In an attempt to hinder the antigenic activity of the amino-terminus, small t extracted from a bacterial source was bound to a monoclonal antibody that recognizes this region (PAb419 see Appendix) and this complex was employed as the antigen.

Fusions of splenocytes from immunized mice with plasmacytoma cells (SP20/Ag14) gave rise to approximately 3500 hybrids. Large numbers of the hybrids produced antibodies that did recognize the common amino-terminus of

large T and small t.

Only one hybridoma produced antibodies that could uniquely recognize small t. This hybridoma was designated PAb280 in accordance to the nomenclature by Crawford and Harlow, 1982.

The specificity of PAb280 for small t was established by immunoprecipitation of ^{35}S methionine labelled cell extracts from CV1 cells infected with WT SV40 and SV dl883 and shown that PAb280 immunoprecipitates small t but not large T from WT infected cells. No detection of any protein reacting with PAb280 was observed from extracts of cells infected with the deletion mutant.

The specificity of PAb280 was also assessed by Western blotting giving the same results and proving that this antibody recognizes an SDS denaturation resistant site in the molecule.

The binding site of PAb280 has been mapped using a series of mutants at the 0.54 to 0.59 m.u. (Shenk et al., 1976; Cole et al., 1977). The mapping has been carried out by immunoprecipitation of ^{35}S methionine labelled cell extracts from cells infected with these mutants as well as by immunoperoxidase staining and shown that PAb280 binds between nucleotides 4809 to 4747, i.e. between amino acids 93 to 114. Staining of BK virus transformed cells have indicated that the antibody might recognize a site between amino acids 103 to 114 but this result needs to be investigated further by using fusion proteins and peptide synthesis.

The above experiments identifying fragments of small t synthesised by the deletion mutants have verified results obtained previously by other laboratories (Khoury et al., 1979; Crawford and O'Farrell, 1979).

The situation with regards to the splicing mutant dl884, as the literature shows, was less clear. Khoury et al., 1979 and Crawford and O'Farrell, 1979 have detected, by immunoprecipitation with anti T serum, fragments of 12Kd or 15Kd after alkylation. Whereas Paucha and Smith, 1978 did not find putative fragments related to small t. But experiments carried out using CV1 cells infected with dl884 incubated with PAb280 followed by immunoperoxidase staining (see section (2.17(ii)(a)) have shown that this product can be detected in the nucleus and cytoplasm of infected cells, confirming the reports of Khoury et al and Crawford and O'Farrell.

This finding is very important. Many laboratories have carried out experiments using this mutant and stating that it is a non small t producing source.

Sugano et al., 1982 have infected Fisher rat embryo 3Y1-K cells with either wild type SV40 or dl884 and found that the foci induced by the deletion mutant were smaller and the cells showed only partial piling up when compared to wild type transformants. Infection of actively growing cells instead of confluent monolayers, or of confluent cells followed by replating gave a similar result. They concluded that small t is required for dense

focus formation in this particular rat cell line.

Dixon et al., 1982 as already described in Chapter 1 have indicated that tumours induced by the mutant dl884 tend to metastasize whereas this is not the case for WT SV40 induced tumours.

These results have to be considered carefully in view of the fact that a fragment of small t is produced by dl884. Although produced in low amounts (i.e. it cannot be easily detected by immunoprecipitation) it is possible that it can have some biological action. Therefore in the work described in this thesis dl883 has been used which previous reports (Khoury et al., 1979, Crawford and O'Farrell, 1979) showed produced no small t or truncated product. This finding has been confirmed in this thesis.

Immunocytochemical staining of WT SV40 and SV dl883 infected cells with PAb280 have shown that small t is located in the nucleus and cytoplasm of the cells (Montano and Lane, 1984). The differential distribution of small t and large T within a given cell was also observed using immunofluorescence staining and it was found that small t is in the nucleus and cytoplasm of the cells and large T is located in the nucleus; no leakage of large T to the cytoplasm was observed. Similar results have been obtained by Ellman et al., 1984 but in this case they have used a small t producing virus (SV402) and rabbit serum raised against SDS denatured gel purified small t.

The time of appearance of small t during lytic infection was determined by immunoprecipitating ^{35}S

methionine labelled cell extracts of WT infected cells for times ranging from 12 to 78hrs and it was shown that small t is detected at 24hrs post infection. The synthetic levels increase up to 72hrs post infection and stay the same up to 78hrs. Interestingly large T was detected at times 24 to 72hrs at high levels which decrease at time 78hrs post-infection. These results confirm those obtained by Crawford, Pim and Lane, 1980.

There seems to be a selective mechanism that favours the synthesis of small t, rather than large T, at later times during infection. There are several possibilities: a) Post-transcriptional processing via a selective splicing mechanism; b) The stability of the mRNA after splicing can also account for this phenomenon, i.e. the mRNA coding for large T could be more labile at late times post-infection than the mRNA coding for small t; c) The lability of the proteins per se, i.e. large T could be more labile at later stages than small t. Interestingly this experiment shows that although small t is an 'early' protein it is expressed quite late in the lytic cycle, possibly indicating that it has some important role at this time during infection.

In the staining results obtained with CV1 cells infected with WT SV40 it was very surprising to note that PAb280 could recognize small t in the nucleus and cytoplasm but PAb419, an antibody that recognizes the common N terminus of both large T and small t only recognized a

nuclear fraction. This observation was studied further. It is possible to argue that the concentration of PAb419 was not high enough to detect cytoplasmic small t, the nuclear small t and large T, but when concentrations of PAb419 up to 400µg/ml were employed the same result was obtained (the nuclear stain reached a plateau above 10µg/ml). The experimental conditions used for staining with PAb280 and PAb419 were exactly the same. The cell was permeable to all antibody molecules. Therefore if PAb419 cannot detect small t in the cytoplasm is not due to cellular exclusion from the cytoplasmic compartment.

In a double staining experiment (using both PAb419 and PAb280) both the nucleus and the cytoplasm of the cell were stained confirming the possibility that two immunologically distinct subsets of small t were present in the infected cell.

This observation was investigated using the sequential extraction procedure described by Staufenbiel and Deppert (1983). They have devised a method by which cellular subfractions can be separated. They used a hypotonic solution containing NP40 to obtain an extract containing nuclear and cytoplasmic proteins, then a second extraction was carried out using DNAse and high salt in order to solubilise the chromatin and associated structures. Finally, they have used an zwiterionic detergent, Empigen BB, to solubilize the nuclear matrices attached to a cytoskeleton.

This sequence of extractions was carried out in CV1 cells infected with WT SV40 and the extracts were immunoprecipitated either with PAb280 or with PAb419. The results obtained showed that a) there was more small t accessible to PAb419 than to PAb280 in the NP40 extract; b) there was some small t accessible to PAb419 but more to PAb280 in the extract containing solubilized chromatin and associated structures; c) finally only PAb280 recognized small t associated with the cytoskeletal structures. It was important to note that PAb419 could still recognize large T in extracts made with Empigen BB indicating very strongly that if small t is not detected by PAb419 in these extracts is not due to inactivation of the antibody by the detergent Empigen BB.

This result shows that there are at least two subsets of immunologically distinct small t molecules, one set recognized by PAb280 (found in the nucleus and cytoplasm of the cell) and a second set recognized by PAb419 (and detected in the nucleus of the cell). It is also interesting to note that some small t is associated with a cellular insoluble fraction which contains the cell cytoskeleton.

In order to determine if there were various small t molecular species a sequential immunoprecipitation of small t using PAb419 and PAb280 or vice versa was carried out. Results obtained in these experiments showed that although two molecular species can be separated this separation never reached completion suggesting that it is possible

that more than two molecular species are present alternatively it is possible that it is a single molecular species present in at least three different configurations which are in equilibrium.

The results obtained in this experiment show that it is possible that at certain times there are molecules presenting the PAb419 epitope that can at other times present the PAb280 epitope.

The immunoprecipitation procedure used for this experiments employed only the cellular soluble fraction of small t. It is possible to argue that most of small t recognised by PAb280 is present in the pellet as the insoluble fraction, therefore the sequential immunoprecipitation does not include all the small t species present in the infected cell.

During preliminary trials of this experiment both PAb419 and PAb280 antibody concentrations were adjusted to saturation levels, therefore it cannot be argued that large T is competing with small t for antibody binding sites.

Walter et al, 1980 have used two synthetic peptides representing the amino-terminal 7 amino acids of large T (and small t) and the carboxy terminal 11 amino acids of large T antigen to induce an immune response in rabbits. Where the antisera was assayed for their content of antibodies against large T it was found that both antisera precipitated large T but neither sera precipitated small t.

Competition experiments between large T and the synthetic peptides demonstrated that the amino acid

sequence in T antigen recognized by the antibodies was the same as that of the peptide used for immunization.

These sets of experiments and the experiments described in Chapter 4 show that it is possible that the amino-terminus of small t antigen is in a different conformational state than that of large T antigen or it may be sterically inaccessible to the antibodies due to interactions with other parts of the polypeptide chain. This possibility could be investigated by a sequential extraction of SV40 infected CV1 cells followed by Western blotting. Small t can then be detected with PAb419 or PAb280. Both antibodies are capable of recognizing SDS denaturation resistant epitopes. Therefore PAb419 has a similar chance as PAb280 to detect SDS denatured small t. This experiment is currently in progress.

Another interpretation of these results relates to the possibility of some molecules of small t being modified (e.g. by acylation) in such a way that this modification excludes the presence of the PAb419 epitope and therefore the cytoplasmic fraction of small t cannot be recognized.

Although there has been studies on the protein chemistry and amino acid sequence of small t (Paucha *et al.*, 1978; Prives *et al.*, 1977). These have been done on *in vitro* translation products.

It would prove interesting to make small t tryptic 2D maps of *in vivo* and *in vitro* protein products to investigate if there are any modifications in the protein

synthesized by either system. Following this analysis it could be important to immunoprecipitate the in vitro translation product with PAb280 and PAb419 to observe if subsets of small t can be detected this system.

A more important experiment would be to produce a 2D peptide map of both the PAb280 and PAb419 small t subsets from the in vivo system to detect differences among the protein as identified by both antibodies.

SV402 is an SV40 mutant virus characterized by producing up to five times more small t than wild type SV40 (Rubin et al., 1982). This virus was used to infect CV1 cells and small t was found to be present in the nucleus and cytoplasm of the cells as detected by PAb280. When PAb419 was used to detect the distribution of small t it was surprising to find the same pattern of detection as with PAb280. If the construction of SV402 is observed (see Figure (4.10)) it can be seen that a large T truncated fragment of estimated molecular weight 24.2Kd can also be expressed. Expression of this fragment was tested by staining CV1 cells infected with SV402 with PAb416 an antibody that recognizes large T but not small t (see Appendix) and it was found that it could detect the putative truncated large T fragment in the nucleus and cytoplasm of the cells. There HAVE been reports indicating that truncated large T of this approximate size can be detected in the nucleus and cytoplasm of cells (Clayton et al., 1982).

Studies carried out using this virus have to be reconsidered in the light of this observation. Rubin et al., 1982 have reported that small t is needed in transformation by SV40. The authors have used SV402, SV40, dl884 or dl883 to infect Balb/c A31, NIH3T3 or CHL cells and scored for micro-colony formation in semi-solid medium. The results showed that SV402 alone failed to induce acute transformation at m.o.i. of 800 and dl884 or dl883 gave only low levels of acute transforming effects at a m.o.i. of 1000 to 3000. By contrast mixtures of various concentrations of SV402 and dl884 or dl883 in which one or the other or both were applied at a fixed concentration and the other was titrated, displayed prominent acute transforming activity. Furthermore a mixture used to transform Balb/c A31 cells in which dl884 m.o.i. was kept constant and the input concentration of SV402 was varied, showed an increasing frequency of transformation reaching ultimately levels as nearly as high as wild type SV40 alone which at a m.o.i. of 3000 transformed 73% of the culture.

These experiments are difficult to interpret. The results presented in this thesis indicate that it is possible that SV402 when mixed with dl883 or dl884 achieve transformation due to complementation with the 24.2Kd (estimated m.w.) truncated large T protein rather than by small t per se.

Shenk and Colby, 1982 have shown that only the N terminus of large T (a 40Kd truncated product) is needed for immortalization of secondary rat embryo cells. Clayton

et al., 1982 have shown that a 45Kd amino-terminus fragment of large T can induce transformation of Rat-1 cells at a frequency of 1% of that obtained by wild type SV40. Chang et al., 1984 have made a construct containing sequences of the 0.725 to 0.373 m.u. which can transform BHK cells with a frequency of 2% of wild type SV40 and have indicated that this transforming activity can be due to the truncated large T protein expressed from this clone.

Colby and Shenk, 1982 have shown that the truncated product expressed by the deletion mutant has a half life of 15 mins or less. It is possible that the truncated large T expressed by SV402 has a very short half life.

Pulse label experiments were carried out in CV1 cells infected with SV402 (data not shown). Cells were labelled for 1, 10 and 15 mins. Extracts were immunoprecipitated with PAb419, PAb416 and PAb280 and run on a 20% SDS polyacrylamide gel, and a 27Kd fragment was observed in immunoprecipitates of PAb416, PAb419 but not PAb280. The fragment was present in larger amounts after a 10 min labelling period. Although this fragment has a short half life (similar to the truncated large T described by Shenk and Colby) it is possible to speculate that it can have a biological activity.

Studies described in Chapter 1 and results shown in Chapter 4 show that there is still a need to elucidate the role of small t in the phenomenon of transformation. Previous results are not clear in view of the different

cell systems as well as the different assays of transformation that have been used. Results described in this thesis also show that virus constructions have to be assessed critically.

It is possible that the study of the biology of small t will be clearer when using the system of Kriegler et al., 1984. They have produced retroviruses that separately encode large T and small t and are currently assessing for transformation activity (M. Kriegler, personal communication).

Kaufman and Sharp, 1982 have constructed a vector which can be used to amplify specific sequences and see their effects when expressed. They have used the dihydrofolate reductase gene (DHFR) (DHRF confers methotrexate resistance due to amplification of the gene. This amplification can be induced by increasing sequentially the methotrexate concentration) and cloned with the SV40 early region. This vector was used to transform Chinese hamster ovary cells deficient in DHFR. They selected a clone that due to integration followed by amplification lost viral DNA sequences in such a way that only small t was detected in the transformants. Small t represented greater than 10% of the total protein synthesis and it was found to be toxic to the cells.

D. Livingston (personal communication) has cloned small t into a vector containing Molony Leukaemia virus LTR and found it to have a toxic effect on NIH3T3 cells.

These experiments suggest that small t cannot accomplish its biological activity when over-expressed. It is possible that a non-toxic system can be obtained if small t is expressed by the SV40 promoter. The construction of such a vector using a small t cDNA clone is in progress.

Experiments using the tsA transformed cell lines show that changes in the levels of large T can be detected at the permissive and non-permissive temperatures when using PAb203. This monoclonal antibody is characterized by detecting an SDS denaturation sensitive epitope within the protein; therefore it can be inferred that the tsA mutation induces structural conformational changes that can be detected by this antibody.

As already stated these studies have distinguished three phenotypes in base of the PAb203/PAb416, PAb419 ratios obtained a) cells phenotypically affected by the ts mutation and containing low amounts of large T in the wild type conformation (as detected by PAb203); b) cells that are marginally affected by the ts mutation and show similar levels of large T in the PAb203, PAb416 and PAb419 configuration and c) cells that do not show any ts effect and have high levels of large T in the PAb203 configuration.

Previous reports (described in Chapter 1) have shown that temperature resistant cell lines can be due to either of two phenomena a) over-production of large T or b) gene dosage effect.

Data obtained and described in Chapter 5 show that the level of protein is not important but rather the number of molecules that can be detected in the correct steric conformation (i.e. the wild type conformation).

These results need to be confirmed carrying out similar studies using tsA virus in lytic infection (i.e. tsA7, tsA58, tsA255, tsA209) at both permissive and non-permissive temperatures to see if the levels of large T as detected by all three antibodies are similar to those of transformed lines especially in the case of tsA58, which is the virus that has been used to produce J78, A21 and J78/11(40) and that as already indicated show a temperature dependent (A21), temperature resistant (J78/11(40), an intermediate (J78), phenotype for transformation.

It would also prove interesting to see if the same phenomenon can be detected upon transient expression of the HindII/III I and B fragments carrying the tsA mutation.

The work described in this thesis emphasises the importance of careful study of the conformation, concentration and cellular location of the oncogene product within the transformed cells. Without precise knowledge of these factors it is difficult to assess the role that the different viral (or cellular) oncogenes play in the transformation process. Conclusions obtained by comparisons of apparent protein function that rely simply on introduction of the gene encoding the product in question could easily be deceptive.

APPENDIX

Map of Monoclonal Antibodies Binding Site to SV40 Large T

PAb419 (Harlow et al., 1981)

PAb416 (Schwyzer et al., 1983)

PAb414 (Harlow et al., 1981)

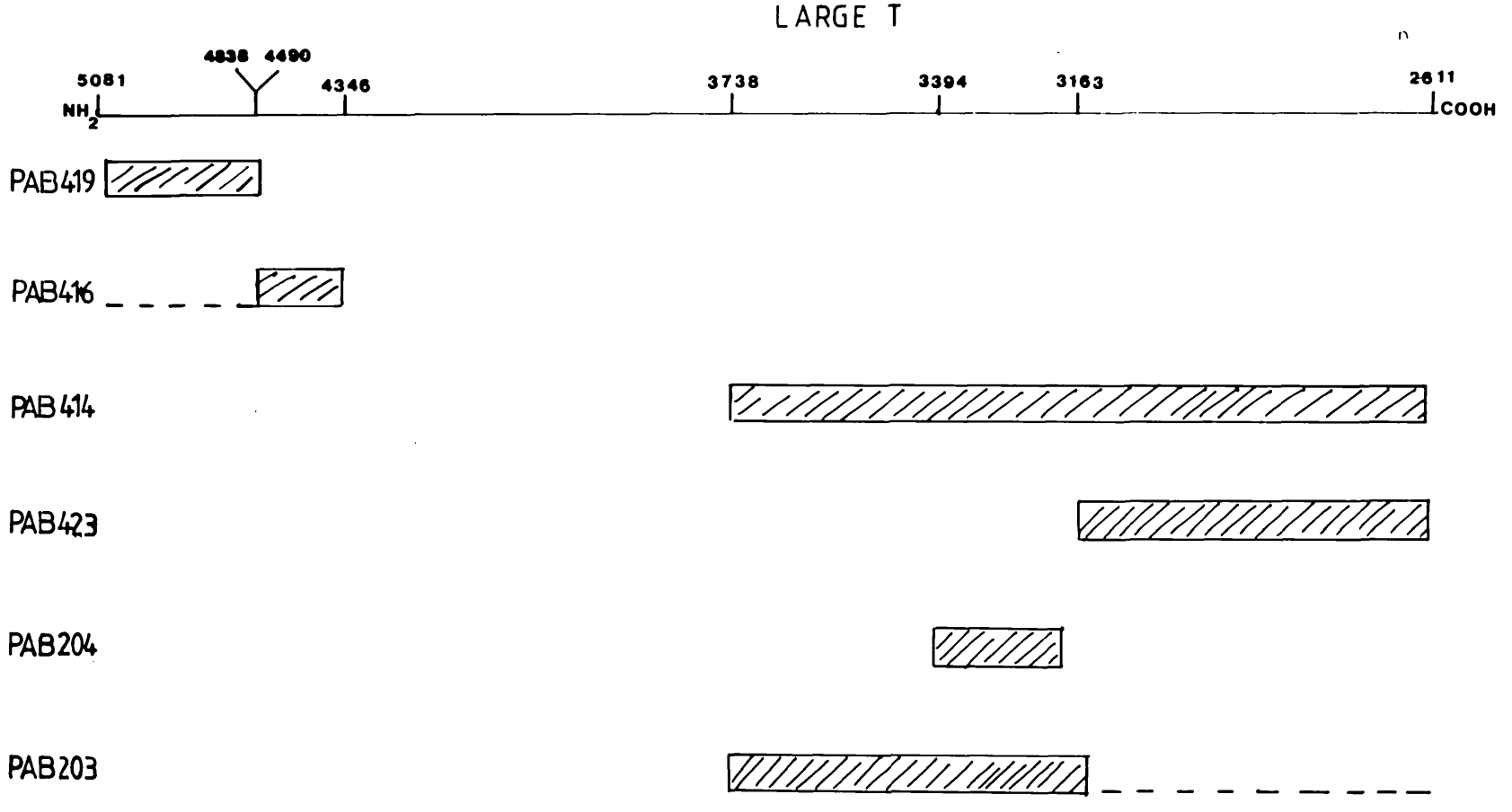
PAb423 (Mole and Lane, in press)

PAb204 (Mole and Lane, in press)

PAb203 (Montano, Gannon and Lane, manuscript in preparation)

Nucleotide numbering of Reddy et al., 1978

Only the approximate binding sites are shown in the Figure. Therefore it is important to state that PAb204 and PAb203 do not cross-react.


LARGE T
5081
4838
4490
4346
3738
3394
3163
2611
NH2
COOH
PAB419
PAB416
PAB414
PAB423
PAB204
PAB203

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JOURNAL OF VIROLOGY, Sept. 1984, p. 760–767
0022-538X/84/090760-08$02.00/0

Vol. 51, No. 3

Monoclonal Antibody to Simian Virus 40 Small t

XIMENA MONTANO* AND DAVID P. LANE

Cancer Research Campaign, Eukaryotic Molecular Genetics Research Group, Department of Biochemistry, Imperial College, London SW7 2AZ, England

Received 16 January 1984/Accepted 16 May 1984

A monoclonal antibody, PAb280, was produced that recognizes simian virus 40 (SV40) small t but does not react with SV40 large T. The specificity of the antibody was analyzed by immunoprecipitation of labeled cell extracts, Western blotting, and immunocytochemistry. Small t was found to accumulate late in the SV40 lytic cycle and was localized in both the cytoplasm and the nucleus of cells infected with wild-type SV40. Importantly, antibodies against determinants common to SV40 large T and small t did not appear to be able to recognize the cytoplasmic form of SV40 small t at the immunocytochemical level. The localization of small t within the nucleus appeared to be distinct from that of large T.

The genome of simian virus 40 (SV40) encodes six known proteins: small t, large T, VP1, VP2, VP3 (30), and agnoprotein (19). Of these proteins, only two, small t and large T, are normally synthesized in abortively infected or transformed cells (30). In lytic infection, synthesis of small t and large T is detected before the onset of viral DNA synthesis and persists throughout the infection (6, 30). The mRNAs that code for small t and large T are thought to be derived from the same precursor RNA transcript which is differentially spliced to produce the two mRNAs such that small t and large T share an amino-terminal region of 82 amino acids but differ in their carboxy-terminal amino acid sequences. A very large number of biochemical and physical properties of large T have been described, and compelling genetic evidence exists to indicate the pivotal role of large T in both the viral lytic cycle and cell transformation (20, 30). The functions and properties of small t are far less clearly defined. Deletion mutants that affect splicing of the small t transcript, but not of the large T transcript, have been isolated. These viruses, although they synthesize a normal large T, do not synthesize any detectable small t (14). Other mutants, reflecting deletions within the large T intron, produce truncated small t antigens but, again, synthesize normal large T (5, 14). All of these mutants are viable in permissive tissue culture cells. Topp (31), however, has demonstrated that the small t deletion mutants are slightly defective in that they produce a smaller virus burst at low multiplicities of infection. This implies that small t does play some role in lytic infection in in vitro conditions. In in vivo infections, the role of small t may be more important, but this has not yet been assessed.

A large body of work has addressed the crucial issue of the role of small t in cell transformation. Initial reports have indicated that small t is extremely important, since small t deletion mutants have been shown to be partially defective for transformation (26). Subsequent studies have shown considerable variation in the different transforming potentials of wild-type and small t deletion mutant viruses (7, 18, 21, 23, 27). A somewhat simplified, consensus viewpoint is that small t is required in situations in which the normal growth of untransformed recipient cells is restricted soon after virus infection, or at the time of virus infection, by conditions that permit only the growth of transformed cells, for example, growth in semisolid media or overgrowth of the monolayer. Actively growing cells are usually transformed with near-equal efficiency by both wild-type and deletion mutant viruses. A particularly clear recent study by Rubin et al. (21) exploited transfection of a small t-encoding, but non-large T-encoding, plasmid in the presence or absence of infection with small t deletion mutant viruses to demonstrate complementation between small t and large T in both abortive and permanent transformation of BALB/c 3T3 cells as assessed by growth in semisolid medium.

The biochemical properties of small t are also less well defined than those of large T. Purified small t, which is derived from the expression of the small t gene in *Escherichia coli*, will induce partial dissociation of the actin cable network when it is microinjected into the cell cytoplasm of RAT 1 cells (2). This is a direct result mirroring the earlier observations that wild-type SV40 DNA microinjected into rat embryo fibroblasts induces actin cable disruption but that deletion mutant DNA does not (8). In this latter study, it was observed that DNA fragments capable of encoding intact small t, but only a grossly truncated large T, also induce actin cable dissociation.

Small t has been shown to specifically associate with two host proteins; one is 56 kilodaltons and one is 32 kilodaltons (22). It is also shown to be involved in the stimulation of the appearance of a host centriolar antigen (25), but the correlation of these properties to its functions is not yet clear.

Many characteristics of small t, e.g., cellular location and precise quantitation, have been hard to elucidate because of the absence of a specific antibody to the protein. It has been demonstrated originally by Lane and Robbins (17) that small t and large T share common antigenic determinants; more recently, monoclonal antibodies reactive to both large T and small t have been isolated by a number of groups (10, 11; J. Yewdell and D. P. Lane, manuscript in preparation). No antibody specific to small t has been derived from these fusions, however, despite the demonstration that antibodies of such specificity do occur, at least in hamster immune response to SV40-induced tumors (9). We set out, therefore, to exploit the existence of plasmids that direct the synthesis of small t in abundant amounts in *E. coli* to produce a monoclonal antibody which is specific to small t.

MATERIALS AND METHODS

Viruses and monoclonal antibodies. Monoclonal antibodies PAb419, PAb423, and PAb414 were a kind gift from Ed Harlow (Cold Spring Harbor Laboratory [11]).

* Corresponding author.

SV40 *dl* 883 (24) was a gift from L. V. Crawford (Imperial Cancer Research Fund). Large-plaque wild-type SV40 stock was grown in CV1 cells to a final titer of 5×10^8 PFU/ml.

CV1 and SP20/AG14 cells, as well as the hybridomas, were grown in Dulbecco modified Eagle minimum essential medium supplemented with 10% fetal calf serum and 500 U of penicillin and 100 μg of streptomycin per ml.

Extraction of small t. Small t was isolated from *E. coli* cells containing plasmids in which the small t gene had been placed under the control of strong bacterial promoters. The plasmid HP1 with the small t gene controlled by the *lac* UV-5 promoter was a gift from R. Tjian (29), and the plasmid pTR865 with the small t gene controlled by the *tac* promoter was a gift from D. Livingston (2).

Bacterial cells were grown to mid-log phase in L broth supplemented with 25 μg of ampicillin per ml, and then isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 5 mM, and the cells were allowed to grow to late log phase. The cells were then harvested, and the pellet was resuspended to 1/50 of the original volume in NET (150 mM NaCl–5 mM EDTA–10 mM Tris-hydrochloride [pH 8.0]) containing 1% Nonidet P-40 (NP-40) and 1 mM phenylmethylsulfonyl fluoride. This suspension was sonicated for 5 min, and then the extract was centrifuged at 10,000 rpm for 30 min by using a GS34 rotor in a Sorvall centrifuge; the supernatant was kept.

Purification of PAb419. Monoclonal antibody PAb419 was purified by protein A-Sepharose chromatography from the tissue culture supernatant of the hybridoma culture. The antibody was bound to the column (packed volume, 5 ml) by recycling 500 ml of the supernatant through the column for 48 h at 4°C. After the column was washed extensively with 150 mM NaCl–10 mM Tris-hydrochloride (pH 8.0), followed by sodium phosphate buffer (0.01 M [pH 8]), the antibody was eluted with citrate buffer (0.1 M citric acid–0.1 M trisodium citrate [pH 3]) as 1-ml fractions which were neutralized immediately with 1 ml of 1 M Tris-hydrochloride (pH 8.8) and stored at 4°C.

Isolation of PAb419-small t complex. Bacterial cell extract (10 ml) was mixed with 1 mg of purified PAb419 and 1 ml of protein A-Sepharose and allowed to incubate at 4°C overnight. The mixture was then poured into a 5-ml column (Bio-Rad Laboratories) and allowed to settle. The column was washed first with 50 ml of 150 mM NaCl–10 mM Tris-hydrochloride (pH 8.0), and then with 100 ml of sodium phosphate buffer (0.01 M, pH 8). Finally, 0.1 M citrate buffer (pH 3) was used to elute the complex (1-ml fractions) into 1 ml of 1 M Tris-hydrochloride (pH 8.8) which neutralized it.

Immunization. Female BALB/c mice (5 to 6 weeks old) were immunized with 10 μg of the purified PAb419-small t complex. The first two injections were given intraperitoneally in Freund incomplete adjuvant. The mice were boosted before fusion by intravenous injection of the purified complex in saline. Serum samples were monitored for the presence of antibodies to small t by staining cultures of CV1 cells infected with wild-type or *dl* 883 viruses.

Hybridoma fusion. The method for hybridoma fusion was basically that described by Kennett et al. (13). The spleen cells of the immunized mice were suspended in serum-free medium and fused to 10^7 SP20/AG14 myeloma cells by using polyethylene glycol. The fusions were plated out among 800 to 1,000 separate wells, and growing hybrids were obtained at frequencies of between 100 to 500 events per fusion.

Cloning and growth of hybridoma cells. The hybridoma cells from wells that gave a positive signal in the screening assays were transferred to 24-well Costar trays, and after growing for 3 to 4 more days, the cells were cloned by using the soft agarose method (13). Agarose colonies were picked after 8 to 10 days; individual colonies were rescreened, recloned, retested, and finally grown in bulk and frozen at 10^7 cells per ml in liquid nitrogen in a freezing mixture consisting of 5% dimethyl sulfoxide–95% serum. Recovery of frozen cells was usually excellent under these conditions. For growth as ascitic tumors, 10^6 hybridoma cells were inoculated intraperitoneally into pristane-primed BALB/c mice (0.2 ml of pristane oil was given intraperitoneally 10 days earlier).

Solid-phase radioimmunoassay for hybridoma screening. Of bacterial extracts prepared from *E. coli* synthesizing small t or control cells, 25 μl was added to each well of a 96-well, flexible, plastic microtiter plate (Flow Laboratories, Inc.) and allowed to dry overnight at 4°C. Of each hybridoma supernatant tested, 25 μl was added to each well, and the plate was incubated for 2 h at room temperature. The plates were washed five times in phosphate-buffered saline (PBS), and then 25 μl (50,000 cpm) of iodinated sheep anti-mouse immunoglobulin (Amersham Corp.), diluted in PBS containing 5% fetal calf serum, was added to the wells. After 1.5 h of incubation at room temperature, the plate was washed as described above, dried, and cut up with a hot wire machine. The individual wells were counted in a gamma counter.

Gel electrophoresis and Western blotting. Polyacrylamide gels (15%) were prepared by the method of Laemmli (16). The amounts of protein loaded in each gel are as described in the figure legends.

For Western blotting (32), protein bands from a sodium dodecyl sulfate (SDS)–15% polyacrylamide gel were transferred onto nitrocellulose paper (Schleicher & Schuell, Inc.) for 2 h at 1 A by using an electroblot apparatus (E-C Apparatus Corp.). The papers were exposed on RX Fuji X-ray film with a Fuji Mach II intensifying screen for 6 h at −70°C to detect the radioactive markers run with every gel.

The blot was first incubated overnight at 4°C with 5 ml of antibody-containing tissue culture supernatant from the hybridoma cells. After being washed five times over a period of 50 min in PBS (150 mM NaCl, 30 mM NaH_2PO_4 [pH 7.2]) containing 1% NP-40, the blot was incubated overnight at 4°C in 5 ml of a 1:100 dilution of horseradish peroxidase-conjugated rabbit anti-mouse immunoglobulin. Finally, the blot was washed twice in PBS–1% NP-40, once in PBS–0.1% NP-40, and three times in PBS alone. The blot was developed with a 4-chloro-1-naphthol substrate solution (saturated solution in ethanol diluted 1/100 in PBS and filtered through a Whatman no. 1 filter paper, and then H_2O_2 [30 volumes of stock] was added at a 1/5,000 dilution).

Immunoprecipitation. CV1 cells (10^7) infected with wild-type SV40 or with *dl* 883 for 72 h were labeled for 3 h with 50 μCi of [^{35}S]methionine in methionine-free medium to give a specific activity of 10^7 cpm per ml of cell extract. The cells were extracted in 0.5 ml of buffer (0.1 M NaCl, 0.05 M Tris [pH 7.9], 5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1% NP-40) and centrifuged at 10,000 rpm for 30 min. To the supernatant, 15 μl of normal rabbit serum was added and left to incubate for 1 h at room temperature; then 50 μl of 10% Formalin-fixed *Staphylococcus aureus* was added, and the mixture was incubated at 4°C for 15 min more. The last step was repeated twice. One-third of the final cell extract volume was mixed with 2.5 μg of PAb280 or PAb419 and incubated overnight at 4°C, and then 50 μg of rabbit anit-mouse immunoglobulins (DAKO Corp.) was added, and the mixture was incubated further for 2 h at room temperature. Finally, 100 μl of 10% Formalin-fixed *S. aureus* was added,

and the mixture was incubated for 1 h at 4°C. After this period, each immunoprecipitation reaction was centrifuged for 30 s (Eppendorf microfuge). The supernatant was discarded, and the pellet was then washed once with each of the following buffers: (i) 50 mM Tris (pH 7.4)–1% NP-40–0.1% SDS–100 mM NaCl, (ii) 50 mM Tris (pH 8)–1% NP-40–600 mM NaCl, (iii) 50 mM Tris (pH 8)–1% NP-40–0.1% SDS–0.5 M LiCl, (iv) 150 mM NaCl–5 mM EDTA–50 mM Tris (pH 7.9)–0.05% NP-40–1% bovine serum albumin, (v) 150 mM NaCl–5 mM EDTA–50 mM Tris (pH 7.9). After the last wash, the pellet was resuspended in 2× sample buffer (16), boiled for 2 min, and centrifuged for 1 min, and the supernatant was run on an SDS–20% polyacrylamide gel.

Horseradish peroxidase staining. CV1 cells, 72 h after infection with wild-type SV40 or *dl* 883 or after mock-infection, were fixed on the plates for 15 min with 3% paraformaldehyde in 85% PBS (in water) (pH 7.4) with 1 mM Ca^{2+}-Mg^{2+} at 4°C, rinsed twice with 85% PBS, and further incubated with 50 mM NH_4Cl in 85% PBS for 20 min at 4°C. Finally, they were permeabilized by a freeze-thaw cycle by using a dry ice–ethanol bath, rinsed again twice with 85% PBS, and incubated with purified monoclonal antibody (100 μg/ml; diluted with 85% PBS–10% fetal calf serum) overnight at 4°C. Cells were rinsed twice with 85% PBS and further incubated with rabbit anti-mouse immunoglobulin–horseradish peroxidase conjugate (DAKO) at a 1:50 dilution in PBS–10% fetal calf serum for 3 h at 4°C; the plates were rinsed as described above. An oversaturated solution of *o*-dianisidine was prepared in ethanol, and from this, a 1:100 dilution was made in PBS and filtered; H_2O_2 (30 volumes; BDH) was added to attain a 1:5,000 dilution. The plates were incubated in this substrate solution for 30 min, rinsed in distilled water, and mounted in Gelvatol (Monsanto Chemicals).

RESULTS

Isolation of small t for immunization. Although immunization of mice or hamsters with syngeneic SV40-transformed cells induces a good immune response to SV40 large T and to the common antigenic determinants of small t and large T antibodies specific to small t arise at very low frequencies (9), and no monoclonal antibodies specific for small t have been isolated from the spleens of mice immunized in this manner (10, 11; J. Yewdell and D. P. Lane, unpublished data).

To prepare a suitable immunogen, we decided therefore to take advantage of plasmids which direct the expression of small t in *E. coli*, since the quantity of small t that could be obtained from this source was expected to be much greater than that isolated from lytically infected cells. Initially, we used the plasmid HP1 described by Thummel et al. (29) in which small t is under the control of the UV-5 *lac* promoter. In our hands, the yields of small t that could be obtained from this source were disappointing since, although the bacteria transformed by HP1 synthesized small t very efficiently as judged by ^{35}S-pulse-labeling of cultures, the steady-state level of small t was low. To overcome this problem and hopefully to enhance the immunogenicity of the small t unique epitopes, we isolated the small t from soluble extracts by using a monoclonal antibody (PAb419) directed against the common determinants of small t and large T (11); we then immunized mice with the purified antibody-antigen complex. Such complexes appeared highly immunogenic, since amounts (less than 1 μg) of small t detectable only by silver staining of gels induced good antibody responses.

When plasmid pTR865 was made available to us, we were able to isolate much larger quantities of small t, since *E. coli* X90 cells transformed by pTR865 synthesize and accumulate large quantities of small t on induction with IPTG. Although Bikel et al. (2) have purified small t from pTR865-transformed *E. coli* cells by using conventional protein chemical methods, we retained our immunoaffinity protocol because of the high immunogenicity of the PAb419-small t complexes and because we hoped that it would direct the immune response to the unique epitopes of small t. The induction of small t in *E. coli* X90 cells containing pTR865 and the isolation of small t-PAb419 complexes are illustrated in Fig. 1.

Immunizations and fusions. A total of 10 fusions were carried out in mice immunized with the complex. In each fusion, between 100 and 1,000 hybrid clones arose and were screened. Although many hundreds of clones producing antibody to small t were detected by their ability to bind small t in the solid-phase radioimmunoassay, subsequent analysis of these antibodies by immunoperoxidase staining of wild-type or *dl* 883-infected CV1 cells proved that all of these clones, with only three exceptions, were directed to epitopes shared by SV40 small t and large T in that the antibodies stained the nuclei of *dl* 883-infected cells. Of the three exceptions, one antibody was successfully cloned and characterized. In accordance with the proposals of Crawford and Harlow (3), this antibody is designated PAb280.

Characterization of PAb280. PAb280 was originally identified as a putative anti-small t-specific antibody since it scored strongly in the solid-phase radioimmunoassay with extracts of *E. coli* containing small t, but not with control extracts. It shares this property with the many antibodies isolated that recognize epitopes common to large T and small t, but unlike these reagents, PAb280 failed to stain *dl* 883-

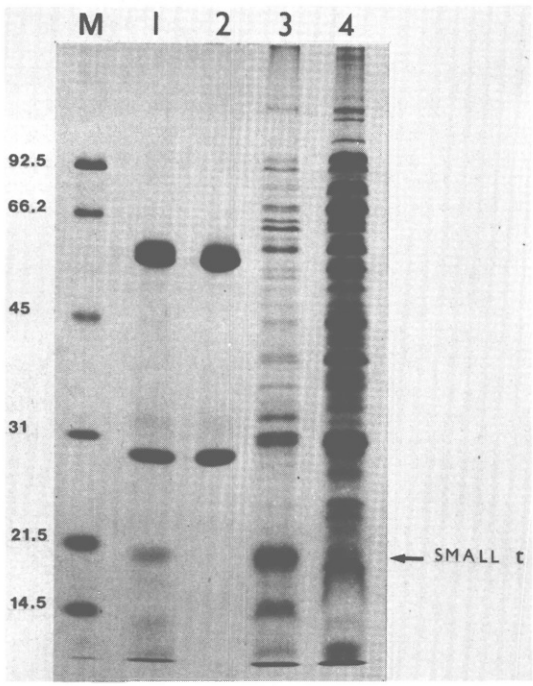

FIG. 1. Isolation of the small t PAb419 complex. Samples were analyzed by electrophoresis in an SDS–15% polyacrylamide gel. The separated proteins were visualized by staining the gel with Coomassie blue. Lane M, molecular weight markers, lane 1, 10 μg of the PAb419-small t complex; lane 2, 10 μg of PAb419 alone; lane 3, 20 μg of a total cell extract of *E. coli* X90 cells containing pTR865 after induction with IPTG; lane 4, 20 μg of a whole-cell extract of *E. coli* X90 cells containing pTR865 in the absence of induction.

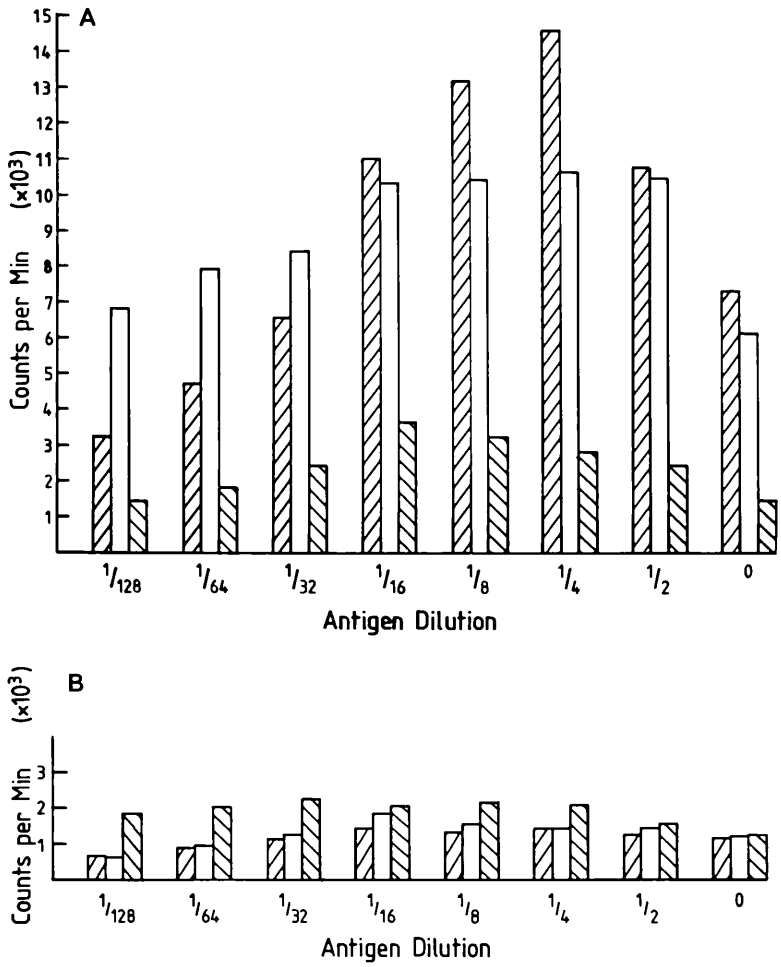

FIG. 2. Solid-phase radioimmunoassay demonstrating the specificity of PAb280 for small t. Bacterial cell extracts were prepared from *E. coli* X90 cells containing pTR865 induced with IPTG (A) or from X90 control cells containing no plasmid (B). Serial dilutions (25 μl) of the two extracts were plated into 96-well microtiter plates and dried onto the plates by overnight incubation at 37°C. The binding of PAb280 (□), PAb419 (▨), and PAb414 (▩) (11) (an antibody that binds large T but not small t) to the cell extracts was determined with iodinated sheep anti-mouse immunoglobulin as described in the text. The hybridomas were used as undiluted tissue culture supernatants.

infected CV1 cells. The cells producing PAb280 were therefore cloned twice through soft agarose, and the individual clones were analyzed for reactivity to small t. All the clones picked after both the first and second rounds of cloning retained the two properties of the parent cells. PAb280 binding to small t-containing extracts of *E. coli* is illustrated in Fig. 2. It is clear that PAb280, like the control anti-large T-small t-reactive antibody PAb419, recognized something present in an extract of induced X90 cells containing pTR865 but showed no specific binding to the same cells lacking the plasmid. The solid-phase assay proved to be very reliable and ideally suited to screening large numbers of samples. It was found to be important that mice were immunized with the complex in Freund incomplete adjuvant, as the mycobacteria present in the complete adjuvant induced a strong antibacterial response which gave rise to a large number of hybridomas which secrete antibodies that react with all *E. coli* extracts. When the solid-phase assay was used at a fixed-antigen concentration (1/4; Fig. 2) to determine titers in tissue culture supernatants from both PAb419 and PAb280 cells, both antibodies had a titer (50% binding of plateau level) of approximately 1/128. Ouchterlony gel diffusion analysis established that PAb280 was an immunoglobulin of the immunoglobulin G1 subclass.

Immunoprecipitation analysis and Western blot analysis. We further analyzed the specificity of PAb280 in both immunoprecipitation and Western blot procedures. In the presence of goat anti-mouse immunoglobulin, PAb280 specifically immunoprecipitated a band of 17,000 molecular weight from extracts of wild-type SV40-infected cells (Fig. 3). This band comigrated with the small t band precipitated by PAb419. Unlike PAb419, however, PAb280 did not immunoprecipitate SV40 large T. Furthermore, in the immunoprecipitation of *dl* 883-infected cell extracts, PAb419 clearly precipitated large T but not small t, whereas PAb280 did not immunoprecipitate anything.

By the Western blot procedure, PAb280 was shown to bind directly to the small t polypeptide rather than to any associated species. The antibody bound strongly to a 17-kilodalton band present only in wild-type SV40-infected CV1 extracts but absent from *dl* 883-infected or uninfected CV1 cell extracts (Fig. 4B). The PAb419 control (Fig. 4A) clearly reacted with large T in both infected cell extracts and to small t in the wild-type SV40 but not in the *dl* 883-infected

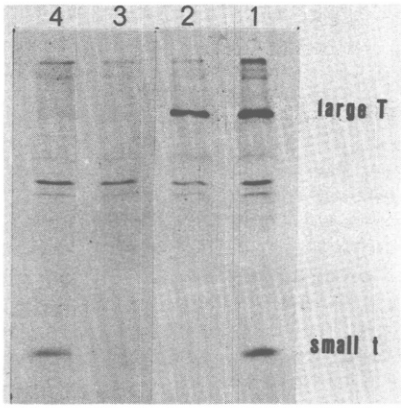

FIG. 3. Immunoprecipitation of small t from SV40-infected CV1 cells with PAb280. CV1 cells (10^6) were infected with wild-type SV40 or *dl* 883 virus and labeled with [^{35}S]methionine, and cell extracts were prepared at 72 h postinfection as described in the text. Lane 1, Wild-type virus-infected cell extract immunoprecipitated with PAb419 and goat anti-mouse immunoglobulin serum; lane 2, *dl* 883 virus-infected cell extract immunoprecipitated with PAb419 and goat anti-mouse immunoglobulin serum; lane 3, *dl* 883 virus-infected cell extract immunoprecipitated with PAb280 and goat anti-mouse immunoglobulin serum; lane 4, wild-type virus-infected cell extract immunoprecipitated with PAb280 and goat anti-mouse immunoglobulin serum.

cell extracts. PAb419 has previously been shown to cross-react with a 36,000-molecular-weight host protein (4). This reaction is clearly detected with the uninfected cell extract but is not detected in the infected cells, although the earlier study, using immunoprecipitation of labeled cell extract, has shown the host protein to be synthesized during lytic infection. This is a provocative observation which we are currently investigating further.

The fact that PAb280 recognizes small t in native form, as demonstrated by the immunoprecipitation, and also in Western blots, means that the reagent will be particularly useful for further analysis of small t.

Immunocytochemical localization of small t in lytically infected cells. We have performed an extensive series of experiments directed towards localizing small t in infected cells by using the PAb280 reagent. Wild-type SV40-infected CV1 cells, *dl* 883-infected CV1 cells, and mock-infected CV1 cells were stained by using the immunoperoxidase method with both PAb280 and PAb419 at various times after infection. PAb280 did not stain uninfected CV1 cells (data not shown) or *dl* 883-infected cells (Fig. 5B); no trace of reaction could be detected even under the highly sensitive conditions we developed with prolonged incubations and the use of the immunoperoxidase rather than the immunofluorescence technique. PAb419 also failed to stain uninfected CV1 cells (data not shown) but gives an intense nuclear stain on *dl* 883-infected cells (Fig. 5D) and on wild-type SV40-infected cells (Fig. 5C). When used to stain wild-type SV40-infected cells, PAb280 shows a clearly positive reaction with staining of both the nucleus and the cytoplasm readily apparent (Fig. 5A). The nuclear stain showed a different localization from that seen with PAb419. Although PAb419 staining of the nuclei was readily detected by 15 to 20 h postinfection and remained at a maximum level after 30 to 40 h postinfection, the PAb280 staining did not appear until 40 h and reached maximum intensity very late in infection (72 to 80 h). Even at these very late time points, the intensity of stain varied considerably from cell to cell. At these very late times, most of the cells showed a considerable cytopathic effect. Importantly, when PAb280 and PAb419 were mixed together and used to stain wild-type SV40-infected cells at 72 h postinfection (Fig. 6), stain was apparent intensively in the nucleus but was also clearly present in the cytoplasm.

DISCUSSION

PAb280 is the first monoclonal antibody to be isolated that recognizes the small t protein of SV40 and not large T. As such it represents a unique tool to investigate the properties of small t, particularly in situations when large T is also present in the material under investigation. The specificity of PAb280 was established in immunoprecipitation of [^{35}S]methionine-labeled extracts from CV1 cells infected with either wild-type SV40 or *dl* 883 mutant. It is clear that PAb280 efficiently immunoprecipitated small t but not large T from extracts of wild-type SV40-infected cells even though large T was present in the cell extract in a form accessible to PAb419. No protein specifically reacting with PAb280 was found in the deletion mutant-infected cells. Importantly, PAb280 also worked very efficiently in the Western blot procedure, allowing unique and ready detection of small t in both lytically infected and transformed cells (manuscript in preparation). Since in this latter technique total cellular proteins were analyzed rather than a soluble fraction, the specificity of PAb280 was very firmly established.

Immunocytochemical staining of wild-type and deletion mutant-infected cells with PAb280 gave some striking results. Strong staining with PAb280 was not detected until

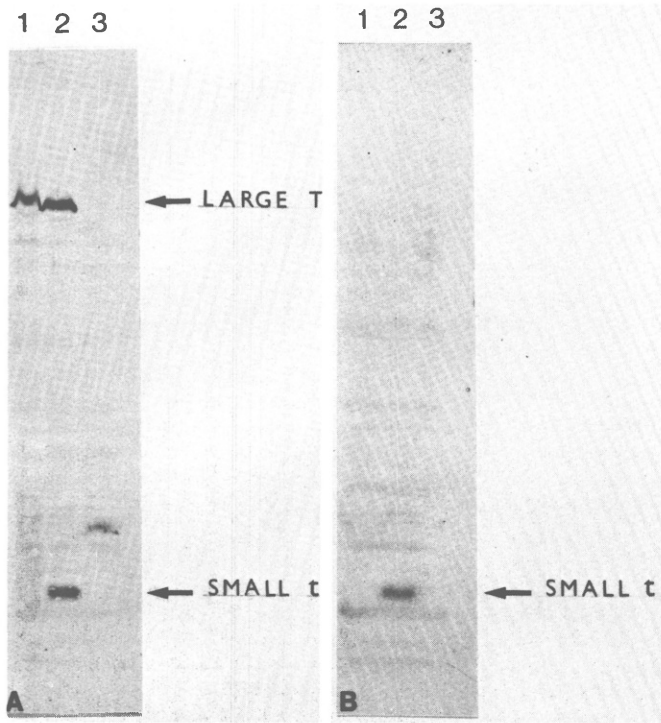

FIG. 4. Western blot analysis demonstrating the specificity of PAb280. CV1 cells (10^6) were infected with *dl* 883 virus (lane 1) or wild-type SV40 (lane 2) or mock infected (lane 3) and extracted in 200 μl of sample buffer. After sonication, 30 μl of the sample was run in each track. Blotting and antibody incubations were as described in the text. Iodinated molecular weight markers were used. Lane 1, extract of *dl* 883-infected cells; lane 2, extract of wild-type-infected cells; lane 3, extract of mock-infected cells. In (A) the blot is developed with PAb419 and in (B) it is developed with PAb280.

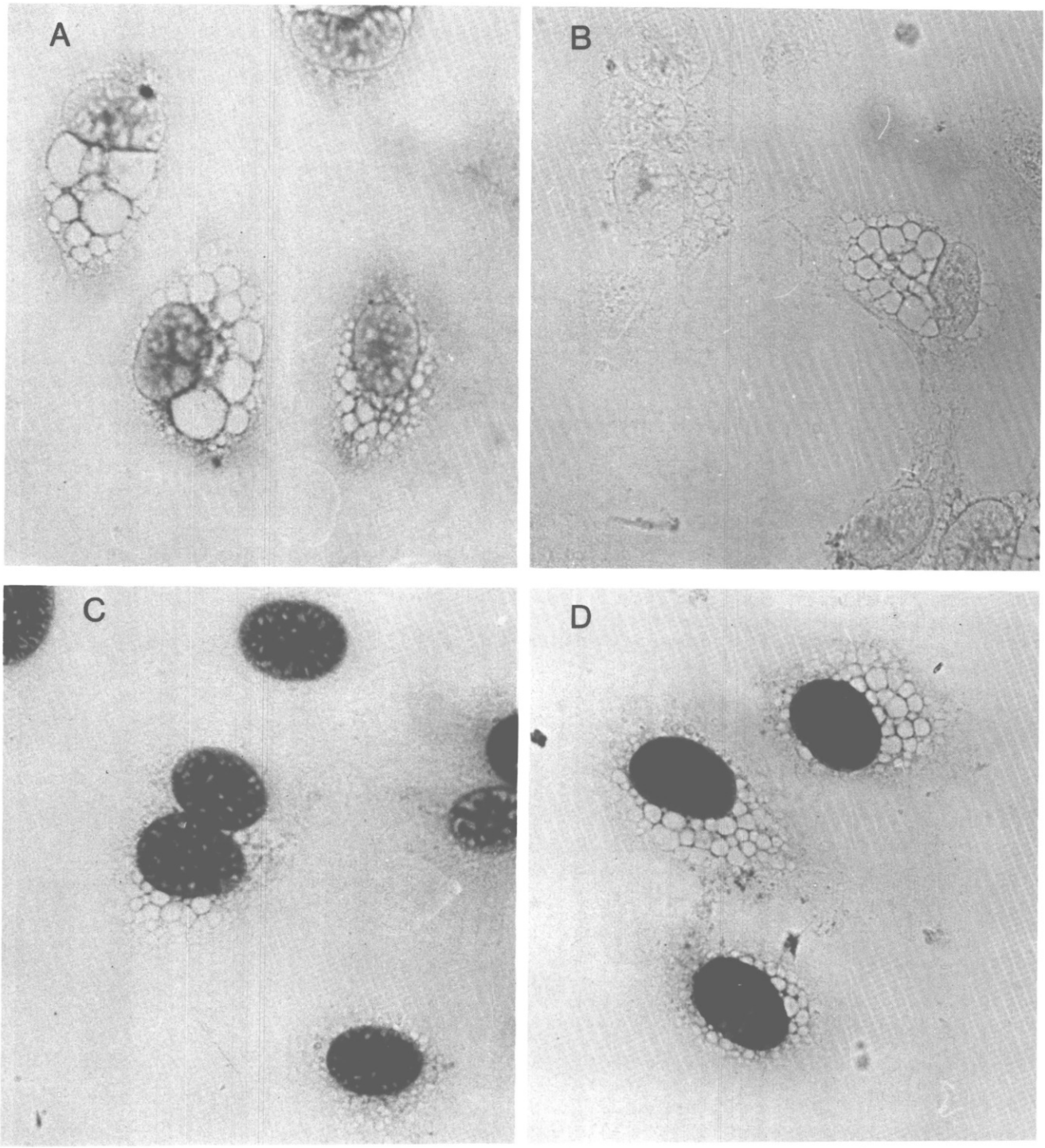

FIG. 5. Immunocytochemical localization of small t. CV1 cells infected with wild-type SV40 (A and C) or *dl* 883 virus (B and D) were fixed at 72 h postinfection and stained either with PAb280 (A and B) or PAb419 (C and D) by using the immunoperoxidase procedure described in the text. The stained cells were photographed with a Zeiss photomicroscope 3 and bright-field optics.

quite late in the lytic cycle, and the intensity of staining increased strongly at very late times in infection, i.e., between 60 and 72 h. This late onset of small t accumulation coincided with a switch in the rate of small t synthesis relative to that of large T that had been observed earlier (6). The observation may imply that small t plays some specific role late in the lytic cycle, but it could perhaps more simply reflect a disruption of the splicing apparatus at very late times, such that the precursor RNA is more readily spliced to remove the intron in the small t rather than in the large T message. The small t splice apparently is a thermodynamically favored reaction (1). Preliminary Western blot analysis of the stages of the lytic cycle supports the late accumulation of small t (data not shown). It should be

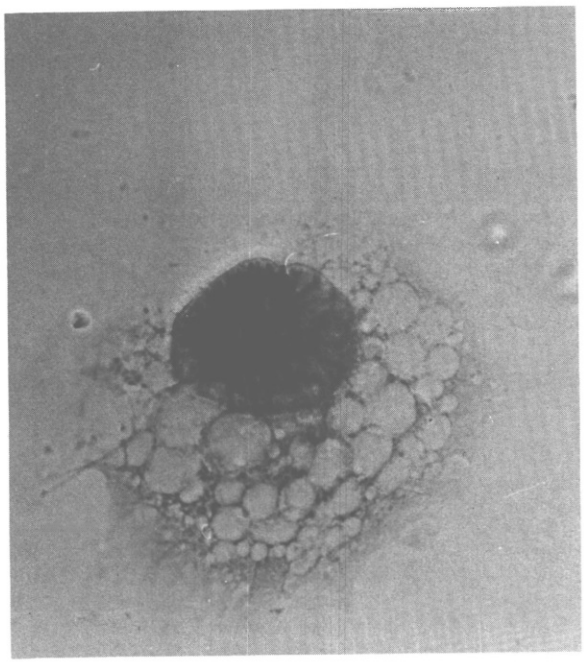

FIG. 6. Simultaneous staining of small t and large T. CV1 cells were infected with wild-type SV40, fixed, and then stained with a mixture of PAb280 and PAb419 as described in the text.

emphasized that some small t was synthesized early in lytic infection, and our failure to immunocytochemically detect this either reflects the very low concentrations accumulated or could imply that the PAb280 epitope is sequestered at early times of infection.

The cellular location of small t determined in these experiments was also unexpected since biochemical fractionation had implied a cytoplasmic localization for the protein (28). Our data indicated that small t resides in both the nucleus and the cytoplasm of infected cells. It is of interest whether small t performs distinct functions in these two cellular compartments and whether the nuclear and cytoplasmic forms can be distinguished biochemically. A suggestion that this is possible is provided by comparison of the staining patterns of PAb419, which recognized a common epitope of small t and large T, and PAb280. PAb419 gave an exclusively nuclear stain in both wild-type and *dl* 883-infected CV1 cells, yet was clearly capable of recognizing small t in native form since, like PAb280, it efficiently immunoprecipitated small t. Further, the epitope recognized by PAb419 is like that recognized by PAb280; it is resistant to denaturation, surviving boiling in 2% SDS and gel electrophoresis. The question then is why at late times in infection did PAb419 fail to stain the cytoplasm of wild-type-infected CV1 cells. A trivial explanation, that the strong reaction of PAb419 with nuclear large T in some way chelated all of the PAb419 antibody or the second detecting antibody can be ruled out. Careful determination of PAb419 titers by the immunoperoxidase test showed the antibody to be in gross excess, since dilution of the hybridoma supernatant by a factor of 1/100 still gave as intense a staining reaction. Chelation of the second antibody can be dismissed since, as shown, in Fig. 6, when PAb280 and PAb419 were used together, both the strong nuclear stain of PAb419 and the cytoplasmic stain of PAb280 were clearly visible.

Two possible explanations can be put forward to account for these findings, either there is a new molecular species produced in lytic infection and located in the cytoplasm that bears the PAb280 epitope but not the PAb419 epitope or the cytoplasmic form of small t adopts some particular conformational structure that prevents the binding of PAb419. We will be able to distinguish these possibilities by cell fractionation and more detailed biochemical studies. PAb280 can provide the basis for a quantitative assay of small t in infected and transformed cells, and this will allow us to correlate levels of small t expression with the biological properties of individual transformed cell lines. Such quantitative data are essential for a true understanding of the role of small t in transformation. PAb280 should also prove useful in investigation of the biochemical properties of small t. Although, in our initial immunoprecipitations with the antibody, we have failed to detect the two small t-associated host proteins described by Rundell (22), further work is necessary, since our conditions for washing the immunoprecipitates may have disrupted the complex.

Experiments to localize the PAb280 epitope precisely on small t, with deletions in the range of 0.54 to 0.59 map units, are in progress. Initial results indicated that the site lies just beyond the carboxy side of the 82 amino acids common to small t and large T. The only other antibodies specific to small t that have been described are those of hamster serum as described by Greenfield et al. (9) and a polyclonal rabbit antibody to a synthetic peptide corresponding to the six amino acids at the extreme carboxy terminus of the protein (12). Neither of these studies has reported on the cellular location of small t in infected cells, and so it is hard to compare our results with theirs. If our initial localization of the PAb280 determinant is correct, then small t can display at least two unique epitopes absent from large T, the carboxy-terminal epitope and the PAb280 epitope. It is unclear why the carboxy-terminal-unique 92 amino acids of small t should be so very poorly immunogenic compared with the amino-terminal 82 amino acids of shared sequence. Other examples do exist of small proteins showing such behavior, and at least in the case of the 27-amino-acid hormone glucagon (15), evidence exists that the area of the molecule recognized by the majority of the antibodies synthesized is discrete from that recognized by the majority of the helper T cells. It may thus be that the carboxy terminus of small t is preferentially recognized by T cells. Our efforts to present the carboxy terminus of small t via the antibody PAb419 do not seem to have been especially successful, but it will be of interest to determine whether the many hundreds of anti-small t-large T-common monoclonal antibodies induced will compete with PAb419 for binding to T or whether that epitope at least was excluded from the repertoire of the antibody response.

PAb280, in conjugation with the construction of viruses only producing small t (2), will hopefully allow a clear picture to emerge of the role of small t in the transformation process. We are particularly intrigued by the suggestion of Martin et al. (18) that small t may act in a manner analogous to known peptide growth factors, and we are now using the antibody to investigate that hypothesis.

ACKNOWLEDGMENTS

This research was funded by the Cancer Research Campaign grant to the Eukaryotic Molecular Genetics Research Group.

We thank Jon Yewdell for his generous help and advice at all stages of the project. The project would not have been possible without the provision of plasmid HP1 from the laboratory of R. Tjian and plasmid pTR865 from the laboratory of David Livingston. We are indebted to both groups.

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