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POLYOMA VIRUS TUMOUR ANTIGENS

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ABSTRACT

The tumour (T) antigens encoded by polyoma virus have been studied by a variety of immunological and biochemical techniques. The existing methods using anti-tumour serum were investigated and found to be limited in their applicability, so a procedure for the production of monoclonal antibodies directed against the T-antigens was developed. Ten cell lines secreting different antibodies were isolated using this protocol. These antibodies were shown to have varying activities against the T-antigens and to recognize different regions of the molecules. Varying quantities of the T-antigens were recognized by individual antibodies, indicating what appeared to be antigenic, and possibly functional, heterogeneity among the large and middle T-antigens. The monoclonal antibodies were also used to establish a sensitive radio-immuno assay for these two antigens.

The specificity provided by the monoclonal antibodies allowed the sub-cellular distribution of large and middle T-antigens to be investigated by indirect immunofluorescence. The nuclear location of large T-antigen was confirmed, but contrary to the location previously reported, the middle T-antigen was not found predominantly associated with the plasma membrane. Instead, it was found to reside in cytoplasmic membranes, including that of the nucleus.

The protein kinase activity associated with middle T-antigen prepared using anti-T serum, was shown to be present in some monoclonal antibody immunoprecipitates. This activity was demonstrated to be similar to that previously reported in that it resulted in the phosphorylation of a tyrosine residue in middle T-antigen molecules. Distinct antigenic forms of the middle T-antigen were observed to differ in their associated protein kinase activity. Particularly, middle T-antigen recognized by antibodies to its N-terminus did not contain the polypeptide that was phosphorylated *in vitro*. The position on the molecule to which the precipitating antibody binds was also observed to influence the level of the protein kinase activity.

The interpretation of these data, and the future uses of the monoclonal antibodies, are discussed.

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ABBREVIATIONS

The following abbreviations are used throughout the text :

BSA	-	bovine serum albumin
DOC	-	sodium deoxycholate
DTT	-	di thio threitol
EDTA	-	diamino ethane tetra acetic acid (di-sodium salt)
E4	-	Dulbecco's modified Eagle's medium
FCS	-	foetal calf serum
IgG	-	immunoglobulin fraction G
K	-	$x10^3$
Kd	-	$x10^3$ Daltons
MWtapp	-	apparent molecular weight
PEG	-	polyethylene glycol
pfu	-	plaque forming units
PMSF	-	phenyl methyl sulphonyl fluoride
RIA	-	radio-immuno assay
SAC	-	Staphylococcus aureus Cowan I; formaldehyde fixed and heat inactivated
SDS	-	sodium dodecyl sulphate
SDS-PAG	-	sodium dodecyl sulphate containing polyacrylamide gel
SDS-PAGE	-	sodium dodecyl sulphate containing polyacrylamide gel electrophoresis
T-Ag	-	tumour antigen
LT	-	large T-antigen of polyoma virus
MT	-	middle T-antigen of polyoma virus
ST	-	small T-antigen of polyoma virus
TCA	-	trichloroacetic acid
TCF	-	tissue culture fluid

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INTRODUCTION

Polyoma virus was first discovered and described by Gross (1953a) as a cell free contaminant in tissue extracts which were being examined for agents causing murine leukaemia. It was soon isolated as a separate agent and shown to cause tumours in mice (Gross, 1953b). Subsequently, it was found to be easily propagated in tissue culture (Stewart et al., 1957; Stewart et al., 1958; Eddy et al., 1958) in mouse embryo cells. Virus propagated in vitro was shown to transform a variety of cell types in vitro (Dawe and Law, 1959; Sachs and Winocour, 1959: Vogt and Dulbecco, 1960; Sachs and Medina, 1961) and to cause a number of different types of tumours when inoculated into newborn mice. It was therefore designated polyoma virus (Stewart and Eddy, 1959). As a result of its ease of propagation in vitro, its small genome size and its ability to transform cells both in vitro and in vivo, polyoma virus has been used as a model system for the study of both eukaryotic lytic viral infection, and viral transformation. The data obtained from such studies have amply justified its choice. These studies have been extensively reviewed in Eddy (1969), Fried and Griffin (1977), Ito (1980), Türler (1980), Eckhart (1981), and chapters in Tooze (1981).

Polyoma virus is a member of the papovavirus group. The viral particle consists of only protein and DNA. The genome consists of a small, double-stranded, closed-circular DNA molecule that exists in virions as a complex with cellular histones. This "minichromosome" is surrounded by the capsid proteins which consist of 72 capsomeres, making an icosahedral capsid. The virion has a diameter of about 45nm and a sedimentation co-efficient of 240S. The genome consists of about 5,300 base pairs of double-stranded DNA.

Most of the work on polyoma virus has been conducted in tissue culture, where rodent cells have been arbitrarily, and somewhat simplistically, classified into two types depending on their ability to support a productive infection by the virus. Permissive cells are those that can be infected by the virus and produce new infectious particles whereas non-permissive cells do not. Only mouse cells fall into the former class. There is relatively little information available on the adsorption, penetration and uncoating of the virus in cells. Up to 50% of labelled virus adsorbs to cell monolayers in 1 to 2 hours (Bourgaux, 1964); this adsorption probably involves the saccharide moiety NeuAcα2,3Galβ1,3GalNAc and the capsid protein VP-1 (Cahan and Paulson, 1980; Fried et al., 1981).

Virions are observed by electron microscopy to be within pinocytotic vesicles in the cytoplasm soon after infection (Mattern et al, 1966); these presumably ferry the particles to the nucleus where they are uncoated. Little is known of this uncoating process except that it probably occurs within the nucleus (MacKay and Consigli, 1976; Chlumecka et al., 1979). The minichromosome, released in the nucleus, is trancribed and the RNA translated into viral proteins.

Lytic Infection

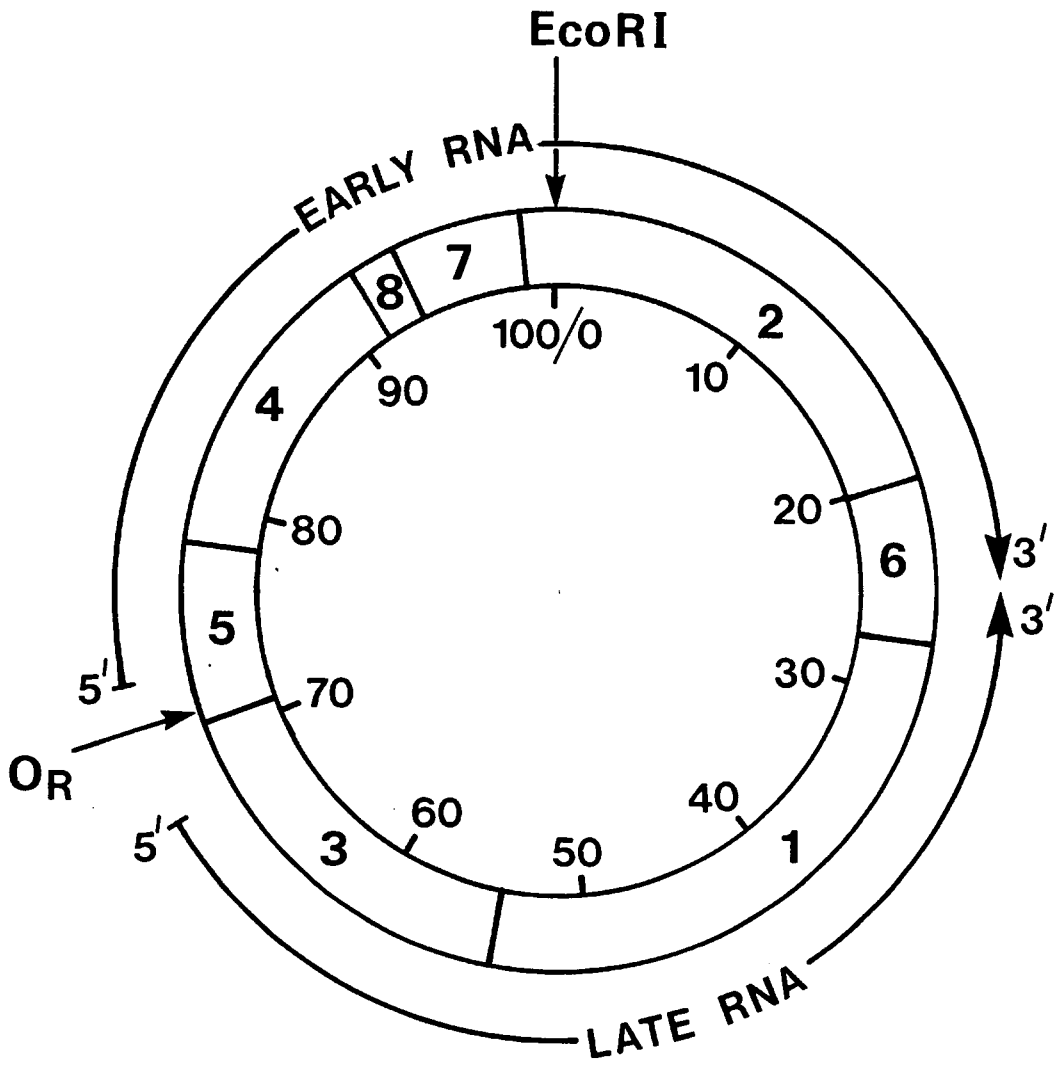
With the advent of restriction endonuclease enzymes, a physical map of the polyoma virus genome was constructed (Griffin et al., 1974) with the genome divided into 100 map units, beginning at the single cleavage site for the enzyme EcoRI. Using this map it was found by pulse labelling experiments (Crawford et al., 1973; Crawford et al., 1975) and electron microscopy (Griffin et al., 1974) that the synthesis of viral DNA was initiated at a single site on the molecule ("the origin of DNA replication") and proceeded bi-directionally around the genome until two daughter molecules were formed. Careful analysis of the RNA synthesised at varying times during the lytic cycle (Hudson et al., 1970; Glover, 1974; Kamen et al., 1975; Kamen et al., 1976; Kamen and Shure, 1976; Türler et al., 1976) showed that there was a difference in the RNA expressed prior to, and after, the initiation of viral DNA synthesis, the terms early and late being applied to separate these events. Both early and late RNA's initiate near the origin of DNA replication, with transcription then proceeding in opposite directions (off different strands of the DNA, the E and L strands). Transcripts in general appear to terminate about 180° from the initiation site(s), see Figure 1.1.

The "early RNA" codes for the proteins which are responsible for many of the effects that the virus has on cells (see later). After the start of viral DNA synthesis, the late proteins are made off corresponding late mRNA's. These mRNA's code for the viral capsid proteins (Smith et al., 1976; Siddell and Smith, 1978; Hunter and Gibson, 1978). As extensive viral DNA and capsid proteins accumulate new virions are assembled in the nucleus by a process which is essentially undefined. When a large number of virions are evident, cell lysis occurs and progeny virus are released. The precise events leading to cell lysis remain to be determined, although a number of membrane permeability changes have been observed for the closely related virus, SV40 (Girard et al., 1973; Norkin, 1977).

On infection with polyoma virus, mouse cells undergo a number of changes in their behaviour and properties. The most obvious change is

Figure I.1 - A Physical Map of the Polyoma Virus Genome.

The circular DNA genome of polyoma virus, divided (inside) into 100 units, starting at the site cleaved by the restriction endonuclease EcoRI (marked at top). The eight fragments generated by cleavage with HpaII are shown (taken from Griffin et al., 1974). The approximate position of the origin of DNA replication (^{O}R) is also indicated. On the outside, the regions of the genome from which RNA is transcribed early and late in infection are noted. The 5' and 3' ends of these RNA molecules are also marked (taken from Kamen and Shure, 1976).


EcoRI
EARLY RNA
8
7
100/0
2
10
90
4
20
80
6
3′
5
3′
5′
30
70
O_R
40
60
50
5′
3
1
LATE RNA

associated with the stimulation of the cells (even when in a confluent non-dividing monolayer) to pass from G1 into the S phase of DNA synthesis. This mitogenic effect of polyoma virus occurs early in infection, and is therefore probably in response to early gene products (Dulbecco et al., 1965; Weil et al., 1965; Winocour et al., 1965). The overall level of synthesis of RNA and proteins is dramatically increased (Salomon et al., 1977, Khandjian et al., 1980; Pockl and Wintersberger, 1980), together with an increase in the level of the enzymes involved in DNA synthesis, particularly the DNA polymerase α which is believed to be involved in the replication of viral and cellular DNA's (Wintersberger and Wintersberger, 1976). These events, with the exception of the increase in histone synthesis, occur in the absence of DNA synthesis (that is, uninfluenced by the addition of 5-fluorodeoxyuridine) (Hancock and Weil, 1969). The increased rate of histone synthesis continues as long as viral DNA synthesis is occurring, even after cellular DNA synthesis has subsided, presumably to provide histones for the viral chromatin. A number of other changes occur to the cell morphology and cellular properties. These are most easily studied in "abortive infections" (see below), but those amenable to analysis are also observed during the lytic cycle on mouse cells.

Transforming Infection

Rodent cells other than those from mice vary in their degree of permissivity. Hamster cells are to a certain extent "semi-permissive", with a limited number of cells producing low yields of virus following infection; rat cells are almost "non-permissive" with little or no virus production. Mouse, rat and hamster cells are the main species that have been widely studied as hosts for polyoma virus, although many other mammalian cells appear to have receptors for the virus (Pontén, 1971). The degree of permissivity appears to be governed by genetic factors which provide a positive influence on viral DNA synthesis, since fused permissive and semi-permissive cells are capable of being productively infected (Basilico et al., 1970). Upon infection of non-permissive cells with polyoma virus, the virus enters the cell, is uncoated and the early region transcribed. The host cell is stimulated to undergo DNA synthesis and division with the concomitant increase in RNA and protein synthesis levels, all properties associated with the lytic cycle. A number of new cellular phenotypes become apparent during this stage (discussed below). Little or no viral DNA is synthesised, however, and most of the infected cells soon cease to transcribe viral RNA, lose viral DNA and revert to the properties common to their uninfected state. This process has been termed "abortive

transformation" (Stoker and Dulbecco, 1969). A small proportion of the infected cells, however, acquire and stabilize the viral genome in such a way that they continue to transcribe and translate viral mRNA, mainly that which gives rise to the "early" proteins. Little synthesis of "late" proteins is observed. These cells permanently acquire the properties expressed during abortive transformation and are said to be "transformed".

The most obvious changes observed in transformed, abortively transformed and lytically infected cells are phenotypic ones. In the early studies on polyoma virus, research was carried out on primary heterogeneous cultures of cells which, if left, would pass through a "crisis" stage and the majority die. With the development of clonable lines, the cell type being studied could be controlled more rigorously and most of the later data have been acquired using such lines. (However, it must be borne in mind that these cell lines even though not tumourigenic when injected back into syngeneic animals are not truly "normal"). Transformation by polyoma virus results in a major alteration in the phenotypic properties of cloned rodent fibroblast cells. The individual cell appearance changes, with the cells becoming less "flattened" and elongated, and more "stellate" in appearance. "Normal" cell lines show orderly growth, cease dividing when confluent, and are said to be "contact inhibited". Transformed cells, on the other hand, grow haphazardly, continue to grow past confluence and show no contact inhibition of movement. They usually require smaller quantities of serum growth factors than normal cells, and are capable of growing in semi-solid medium, that is, without anchorage. Generally, they appear to lose many of the controls on growth and movement exerted among normal cells. On being injected into a syngeneic animal, transformed cells form tumours.

At the biochemical and cellular level, the cells are often heteroploid, with increased nuclear and nucleoli sizes. The ordered cytoskeletal array is frequently disrupted. The surface membrane properties of the cells are also considerably altered, exhibiting an increased agglutinability by plant lectins, a change in the composition of glycoproteins, an absence of tight junctions, and increased rates of transport of nutrients and secretion of proteases, or activators. (All the above events are reviewed in Topp et al., 1980, and references given therein). The broad spectrum of these events implies a considerable disruption of the control processes exerted in normal cells, and since they are nearly all observed also in abortively transformed cells, it is probable that they result from the expression of early gene products.

Early Mutants

To investigate further the roles of the early region products of polyoma virus, attempts were made to construct genetic mutants in this region. Until a few years ago, there were only two classes of mutants known which had an affect upon "early" gene functions of polyoma virus. These were the ts-a and hr-t classes, exemplified by the mutants ts-a-a (Fried, 1965a) and NG-18 (Benjamin, 1970), respectively.

Ts-a:- The ts-a mutants are temperature sensitive for growth; most of these mutants were isolated following nitrous acid treatment of the polyoma virus genome (Fried, 1965a; Eckhart, 1969; Di Mayorca et al., 1969). They form a single complementation group (Fluck et al., 1977; Eckhart, 1977; Fluck and Benjamin, 1979). All of the ts-a mutants examined to date appear to be defective in the initiation of viral DNA synthesis at the non-permissive temperature, and some are also defective in the induction of cellular DNA synthesis (Francke and Eckhart, 1973; Eckhart, 1975). The mutants are usually temperature sensitive for initiating tranformation (Fried, 1965b), although one mutant at least, (tsP155), retains this ability at both temperatures (Eckhart, 1965; Bourgaux et al., 1978). Initiation at the permissive temperature results in transformed cells which generally remain transformed when raised to the non-permissive temperature (with a few notable exceptions, Kimura, 1965; Seif and Cuzin, 1977). Although the ts-a mutants are defective in initiating stable transformation at the non-permissive temperature, they are normal with respect to the initiation of abortive transformation at all temperatures (Stoker and Dulbecco, 1969). From such studies, it was concluded that the ts-a gene is responsible for the integration and stabilization of the polyoma virus genome within the cellular chromosomal DNA, but is probably not involved in the maintenance of the transformed state. There is more direct evidence that the ts-a gene product is involved in both the integration into and excision of polyoma virus genomes from chromosomal DNA in transformed cells (Colantuoni et al., 1980, 1982; Della Valle et al., 1981), in that variations in both properties are temperature dependent when a ts-a mutant is used. This is not the case with a wild type virus experiment. When ts-a-a or ts-25 DNA is used for transfection (rather than infection) of cells, a much smaller temperature dependence of transformation frequency is observed. Transformation occurs in a way which is qualitatively, if not quantitively, indistinguishable from that observed with wild type virus. (Novak and Griffin, 1981; Della Valle et al., 1981). Since at the non-permissive

temperature viral DNA replication does not occur, the late proteins are not synthesised. However, an over-production of early RNA is observed (Cogen, 1978). This suggests that the ts-a gene product can act to repress early RNA synthesis, and hence its own synthesis (autoregulation), and could possibly be involved in the switch from early to late gene synthesis.

The lesions in the ts-a mutants that have been mapped have all been shown to be located within the C-terminal portion of large T-antigen (Feunteun et al., 1976; Miller and Fried, 1976). The lesions in four mutants have been determined at the DNA sequence level (Deininger et al., 1981; Thomas et al., 1981); their positions are indicated in Figure I.2.

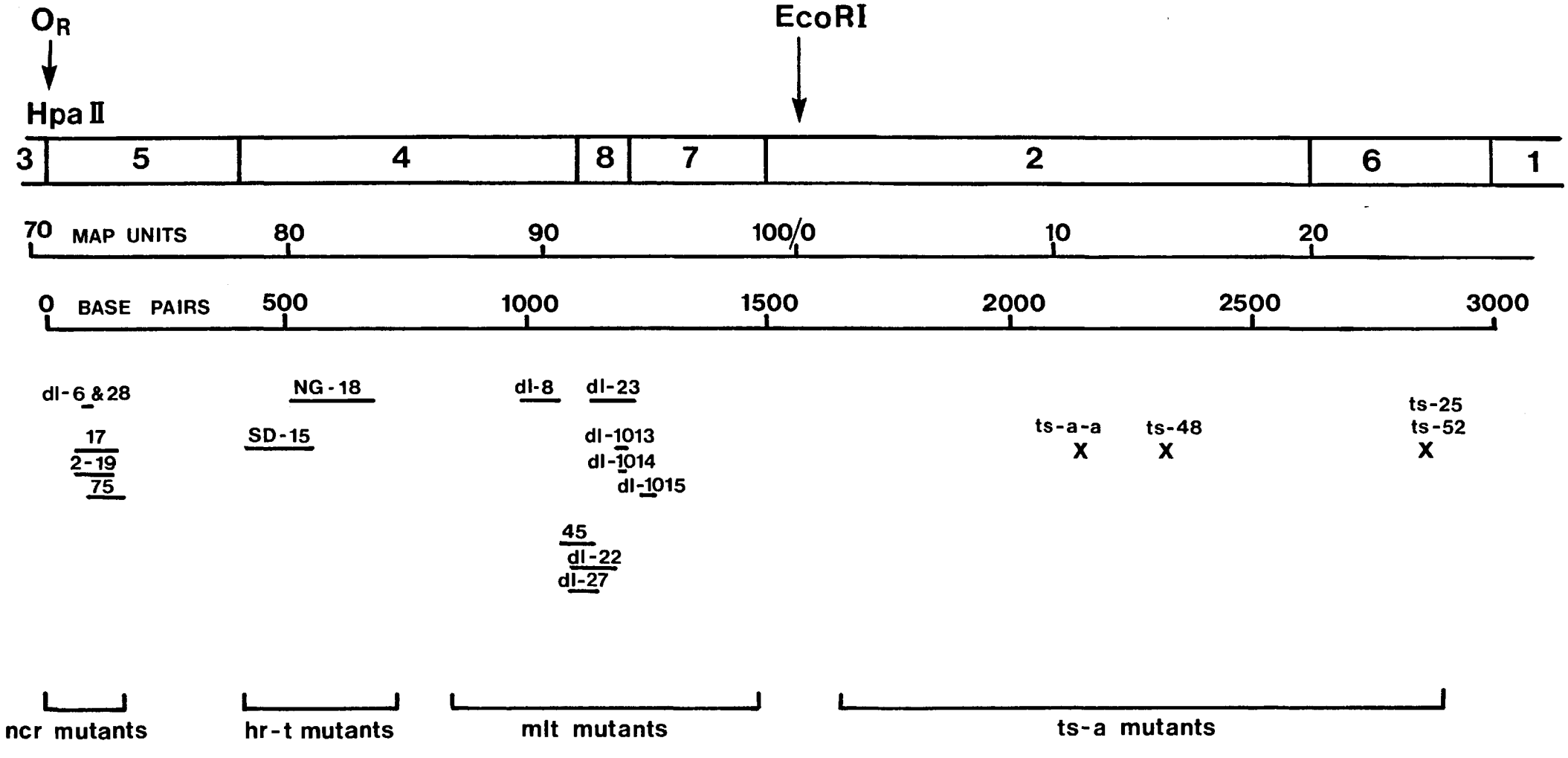
Cells can also be transformed by transfection with a fragment of polyoma viral DNA from the BclI restriction enzyme site to the EcoRI site (see Appendix One), which does not encompass the sites of the ts-a mutations. It appears likely, therefore, that the ts-a gene is not directly involved in the expression of the transformed state induced by polyoma virus, but is probably responsible for its initiation by promoting integration and possibly expression of the viral genome. Although some anomalies still exist, possibly as a result of the use of different cell lines, this overall generalization appears to hold.

Hr-t:- These mutants are classed as host-range, non-transforming mutants. They were originally isolated by their ability to grow in polyoma virus transformed mouse 3T3 cells, but not in normal mouse 3T3 cells (Benjamin, 1970). All these mutants constitute a single complementation group and seem to have identical biological properties, even when isolated by alternative procedures (Lania et al., 1979; Feunteun and Benjamin, 1982). It was subsequently found that cells which contained no integrated polyoma virus could also support growth of the mutant virus (Goldman and Benjamin, 1975; Staneloni et al., 1977; Goldman et al., 1979) so the original rational behind the isolation of the mutants, that of providing a polyoma expressed transforming function to complement a mutant virus, was only in part correct. A wide variety of cells have now been discovered that support the growth of the virus, including baby mouse kidney epithelial cells. This has led to the hypothesis that the hr-t gene elicits factors from the cell that are required for productive infection, as well as characteristic functions of the transformed state. Such factors must be at least partially distinct as some untransformed cells can support growth. Moreover, the defect in productive infection in "non-permissive" cells is not total, as a burst size of 2-5% is often observed (Benjamin,

Figure I.2 - Position of Early Mutants on the Polyoma Virus Genome.

The early region of the polyoma virus genome is shown linearized. The sites of cleavage by the restriction enzyme HpaII are indicated together with that of EcoRI, and the origin of DNA replication (^{O}R). The divisions into map units are shown, together with the number of nucleotides from the HpaII 3/5 junction (described in Soeda et al., 1980a). The sequences missing in various deletion mutants are indicated by a horizontal line together with the name of the appropriate mutant. The positions of the nucleotides altered in four ts-a mutants are indicated by crosses. The four classes of early mutants have sequence alterations within defined regions of the genome. The areas in which these lesions lie are indicated underneath. These mutants are the ncr (non-coding region) mutants, the hr-t (host range, nontransforming) mutants, the mlt (middle and large T-antigen) mutants, and the ts-a (temperature sensitive a gene) mutants.

The sites of these lesions are taken from Soeda and Griffin (1978); Carmichael and Benjamin (1980); Bendig et al., (1980a); Bendig et al., (1980b); Smolar and Griffin (1981); Magnusson et al., (1981); Thomas et al., (1981); Ding et al., (1982).


O_R
Hpa II
EcoRI
3
5
4
8
7
2
6
1
70 MAP UNITS
80
90
100/0
10
20
0 BASE PAIRS
500
1000
1500
2000
2500
3000
dl-6 &28
17
2-19
75
NG-18
SD-15
dl-8
dl-23
dl-1013
dl-1014
dl-1015
45
dl-22
dl-27
ts-a-a
X
ts-48
X
ts-25
ts-52
X
ncr mutants
hr-t mutants
mlt mutants
ts-a mutants

1970). The mutant defect may in part lie in assembly of the virion, since substantial amounts of viral DNA and capsid antigen are made in "non-permissive" infections, but the virus yield is low (Staneloni et al., 1977; B.S. Schaffhausen, personal communication). The precise nature of the defect therefore, remains to be determined. However, the histones H3 and H4 from hr-t virions are known to be less highly acetylated than those from wild type virions (Schaffhausen and Benjamin, 1976).

Hr-t mutants are completely defective in inducing stable transformation (Benjamin, 1970; Fluck et al., 1977; Staneloni et al., 1977), as assayed in vitro both by focus formation and growth in soft agar. They are equally defective in vivo, being incapable of inducing tumours in newborn hamsters (Siegler and Benjamin, 1975) or growing lytically in mice (McCance, 1981). They also do not induce abortive transformation (Schlegel and Benjamin, 1978) with all its concomitant changes, but are capable, to a limited extent, of stimulating cellular DNA and histone synthesis in both permissive and non-permissive cells; only one round of division is usually observed, however (Schlegel and Benjamin, 1978). It was possible that the hr-t mutants did not transform because the mutant DNA failed to become integrated, or integrated in such a manner that cell death resulted. Lania et al., (1979) showed this was not the case in that the genome of a hr-t mutant could be stably integrated into a recipient cell and be transcribed in untransformed cells. It appears likely therefore, that the expression of the product of the hr-t gene is required to cause cells to become transformed by polyoma virus. All of the hr-t mutants complement ts-a mutants for both growth and transformation (Eckhart, 1977; Fluck et al., 1977).

The positions of the lesions in the hr-t mutants have been localized by marker rescue experiments onto the HpaII fragment 4 of the polyoma virus genome (Feunteun et al., 1976; Miller and Fried, 1976), and more recently, many have been directly sequenced (Soeda and Griffin, 1978; Hattori et al., 1979; Carmichael and Benjamin, 1980) (see Figure I.2). All data support the notion that the hr-t gene encodes the function necessary for cell transformation by polyoma virus and induces a cellular factor that allows polyoma virus to replicate in certain cells.

Other Early Mutants:- Many other mutants of the polyoma virus early region have been isolated, but these do not appear to fall into any well defined complementation groups. To some extent, each mutant is unique in its properties. Also, being more recently isolated, the exact influences

these mutants have upon cells are not well characterized. The mutants can be divided into two groups depending upon where the lesion lies. Those with lesions between map units 85 and 100 on the polyoma virus genome have been designated mlt (middle and large T-antigen mutants) (Griffin and Maddock, 1979; Smolar and Griffin, 1981; Magnusson and Berg, 1979; Magnusson et al., 1981; Bendig et al., 1980; Ding et al., 1982), and those between map units 70 and 74 designated ncr (non-coding region) mutants (Griffin and Maddock, 1979; Smolar and Griffin, 1981; Magnusson and Berg, 1979; Wells et al., 1979; Bendig and Folk, 1979), see Figure I.2.

The complete DNA sequence of the polyoma virus genome has been determined (Soeda et al., 1980a; Friedmann et al., 1979; Deininger et al., 1979; and references therein). This allowed the coding potential of the early region of the genome to be assessed. Figure I.2 shows a linearised representation of the early region of the polyoma genome, together with the positions of some of the early region mutants, relative to the physical map. The mutants are divided into four groups on the basis of their properties (the ts-a and hr-t mutants), and the position on the genome of their lesion (the ncr and mlt mutants - see above).

The T-Antigens

The T(tumour) antigens were first recognized and defined by the presence of antibodies directed against them in the sera of animals bearing polyoma virus induced tumours (Habel, 1965). The use of syngeneic virally transformed cells to induce a tumour ensures an immune response to antigens that are related to the transformed state of the cells. Moreover, since the viral proteins expressed in transformed cells are likely to be foreign, antibodies should be raised against them. This has been shown in many cases to be the major antibody response of animals that have virus-induced tumours. However, a number of antibody responses against non-viral proteins have also been observed (Ito et al., 1977a; Hutchinson et al., 1978; Schaffhausen et al., 1978; Simmons et al., 1979). This may be a consequence of cellular alterations during the transformation process that result in their becoming immunogenic. This response could be the result of the presentation to the animal of a normal cellular protein in an abnormal form (for example, modified in some way, or complexed to another protein), or in an unusually large quantity, or even possibly presentation of a protein not usually encountered by the immune system of the animal (for example, an embryonic antigen). It is not clear at present whether any of these alternatives account for some of the properties observed with anti-tumour sera.

Most polyoma virus anti-tumour sera are now raised against rat or hamster fibroblast cells that have been transformed by the virus in vitro and subsequently cloned, propagated and introduced into syngeneic hosts. These cells do not normally express the late proteins, so under these conditions the predominant antibody response is against the "early" proteins encoded by polyoma virus. Thus, the terms "T-antigens" and "early proteins" have become almost interchangeable. Usually "anti-T sera" contain only low levels of antibodies directed against non-viral proteins. In addition to the three T-antigens so far identified (see below), other viral proteins may exist, but because of their poor immunogenicity in tumour cells, possibly as a result of their production in low quantitites or their presence in lytically infected cells only, they have not yet been observed.

The T-antigens were originally identified by the detection of complement fixing activity in anti-tumour serum which reacted with both polyoma virus-induced tumours and transformed cells (Habel, 1965). This activity was separate from anti-virion activity, and its major component was located within the nuclei of lytically infected cells by immunofluorescent analysis (Takemoto et al., 1966). More recent characterization of the T-antigens has involved the use of anti-T sera to immunoprecipitate T-antigens from lysates of cells labelled with radioactive amino acids. Precipitated polypeptides are separated by electrophoresis on polyacrylamide gels containing sodium dodecyl sulphate (SDS-PAGE). Three main polypeptides have been identified by this approach and have been designated large, middle and small T-antigens .

The large T-antigen has a MWtapp of 100Kd on SDS-PAGE and is the product of the ts-a gene. It is found to be thermolabile in cells infected with ts-a mutants (Ito et al., 1977a; Silver et al., 1978; Hutchinson et al., 1978), and is present in a truncated form in cells transformed by a portion of the polyoma virus genome that lacks the sequences altered in ts-a mutants (see Appendix One). Large T-antigen is phosphorylated in vivo (Ito et al., 1977a; Schaffhausen et al., 1978; Schaffhausen and Benjamin, 1979) and increases in size during pulse chase experiments (Ito et al., 1977a; Hutchinson et al., 1978). It can be resolved into at least two species on SDS-PAGE (Türler and Salomon, 1977; Schaffhausen et al., 1978; Türler, 1980), but the difference between these is not clear. The middle T-antigen has a MWtapp of 55Kd on SDS-PAGE (Ito et al., 1977b), and is one of the proteins affected by lesions in the

hr-t gene. The small T-antigen has a MWtapp of 22Kd on SDS-PAGE. Immunoprecipitates from cells infected with deletion hr-t mutants lack both middle and small T-antigens. All three of these T-antigen species are virally-coded as can be shown by their synthesis in an in vitro protein synthesizing system from polyoma virus specific early mRNA's (Hunter et al., 1978).

An analysis of the peptides obtained from each T-antigen after cleavage with trypsin (Smart and Ito, 1978; Hutchinson et al., 1978; Hunter et al., 1979; Simmons et al., 1979) showed that all three T-antigens share a sequence in common (probably at their N-terminus), middle and small T-antigens share a further sequence in common that is not present in large T-antigen, and large and middle T-antigens contain protein sequences that are unique to themselves. It was subsequently found that the deletion mutants dl-8 and dl-23 (Griffin and Maddock, 1979) induced truncated versions of both large and middle T-antigens (Ito et al., 1980) which suggested that a single area of the genome encoded both large and middle T-antigens. Finally, the characterization by S1 analysis of the splice positions within the early messenger RNA molecules from polyoma virus lytically infected and transformed cells (Kamen et al., 1980; Favaloro et al., 1980) and cDNA cloning (Treisman et al., 1981a) enabled the data to be correlated with the open reading frames predicted by the DNA sequence to produce a possible coding scheme for the early region of polyoma virus (Figure I.3) (Ito et al., 1980). Also shown in Figure I.3. are physical positions on the genome of the four classes of early region mutants. It is seen that the ncr mutants lie outside the coding regions, the hr-t mutants lie in the region coding for both middle and small T-antigens, the mlt mutants are located in the region coding for the middle and large T-antigens, and the ts-a mutants lie in the region coding for large T-antigen only. This scheme fits all the data available and allows complete amino acid sequences to be predicted from the DNA sequence for each of the T-antigens (Figure I.4).

The predicted molecular weight for large T-antigen is 88Kd. The difference between this and the MWtapp from SDS-PAGE can probably be attributed to post-translational modifications, particularly phosphorylation, of the large T-antigen molecules, since in vitro translated large T-antigen has a lower MWtapp. The molecular weight predicted for middle T-antigen is 49Kd, considerably less than the 55Kd measured on SDS-PAGE. This difference is probably not a consequence of post-translational modification, since in vitro translated middle T-antigen has the same MWtapp as that synthesised in vivo. Ito et al., (1980) have observed that the MWtapp of

Figure I.3 - Coding Scheme for the Early Region of the Polyoma Virus Genome.

The linearized early region of the polyoma virus genome is shown together with a portion of the HpaII restriction enzyme map. The nucleotide numbering system described in Soeda et al., (1980a) is used. The complete DNA sequence of the early region of the A2 strain was taken and the strand known to be the same sense as early RNA divided into its three possible coding frames. Wherever a potential termination codon appears a vertical line is drawn. The proposed reading frames for each T-antigen are shown. These start at the initiation codon ATG, nucleotide 173. The positions of the splice points in the mRNA are taken from Kamen et al., (1980) and enable the reading frame used for a particular protein to be changed, as indicated. Also shown are the positions of the four classes of early mutants (see Figure I.2) to illustrate the T-antigen species affected by these lesions. Note the large and middle T-antigens are in part encoded by the same region of the genome, translated in different reading frames.

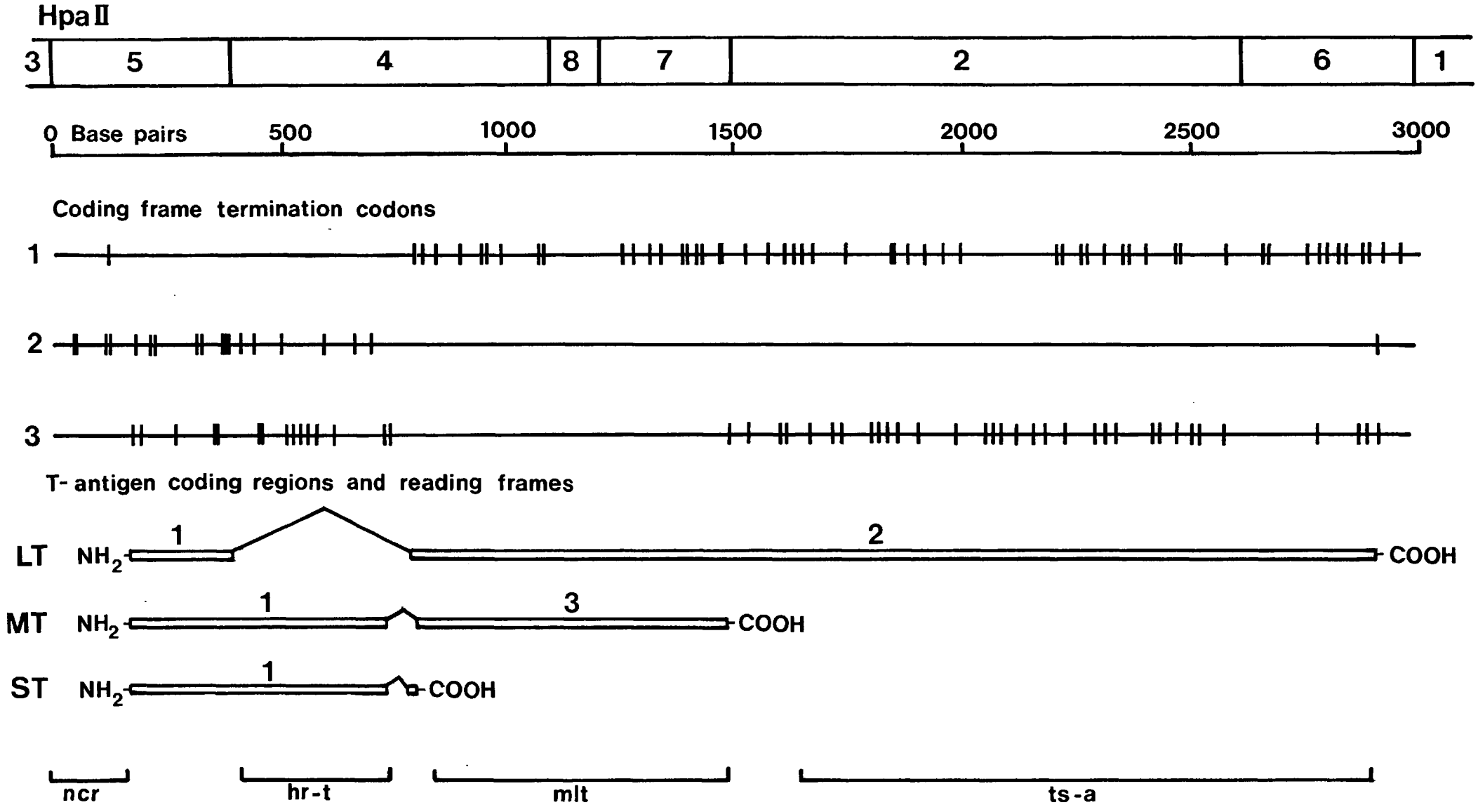

HpaII
3
5
4
8
7
2
6
1
0 Base pairs
500
1000
1500
2000
2500
3000
Coding frame termination codons
1
2
3
T-antigen coding regions and reading frames
LT
NH2
1
2
COOH
MT
NH2
1
3
COOH
ST
NH2
1
COOH
ncr
hr-t
mlt
ts-a

Figure I.4 - Predicted Amino Acid Sequences of the Polyoma Virus T-Antigens.

The complete DNA sequence of the polyoma virus genome (Soeda et al., 1980a) and the postulated coding scheme shown in Figure I.3 were taken and used to predict the amino acid sequences of the three T-antigens.

A. The predicted amino acid sequence of the large T-antigen. The N-terminal (NH_2-) and C-terminus (-COOH) are indicated. The position of the splice in the mRNA coding for large T-antigen is indicated by the solid arrow. The amino acids altered in ts-a mutants are indicated by an open arrow: amino acid 539 is altered in the ts-a-a mutant from Ala to Thr; amino acid 588 is altered in the ts-48 mutant from Ser to Ile; amino acid 789 is altered in the ts-25 and ts-52 mutant from Gly to Cys.

Large T-Antigen Predicted Amino Acid Sequence

NH_2-Met.Asp.Arg.Val.Leu.Ser.Arg.Ala.Asp.Lys.Glu.Arg.Leu.Leu.Glu.Leu.Leu.Lys.Leu.Pro. 20
Arg.Gln.Leu.Trp.Gly.Asp.Phe.Gly.Arg.Met.Gln.Gln.Ala.Tyr.Lys.Gln.Gln.Ser.Leu.Leu. 40
Leu.His.Pro.Asp.Lys.Gly.Gly.Ser.His.Ala.Leu.Met.Gln.Glu.Leu.Asn.Ser.Leu.Trp.Gly. 60
Thr.Phe.Lys.Thr.Glu.Val.Tyr.Asn.Leu.Arg.Met.Asn.Leu.Gly.Gly.Thr.Gly.Phe.Gln.Gly. 80
Ser.Pro.Pro.Arg.Thr.Ala.Glu.Arg.Gly.Thr.Glu.Glu.Ser.Gly.His.Ser.Pro.Leu.His.Asp. 100
Asp.Tyr.Trp.Ser.Phe.Ser.Tyr.Gly.Ser.Lys.Tyr.Phe.Thr.Arg.Glu.Trp.Asn.Asp.Phe.Phe. 120
Arg.Lys.Trp.Asp.Pro.Ser.Tyr.Gln.Ser.Pro.Pro.Lys.Thr.Ala.Glu.Ser.Ser.Glu.Gln.Pro. 140
Asp.Leu.Phe.Cys.Tyr.Glu.Glu.Pro.Leu.Leu.Ser.Pro.Asn.Pro.Ser.Ser.Pro.Thr.Asp.Thr. 160
Pro.Ala.His.Thr.Ala.Gly.Arg.Arg.Arg.Asn.Pro.Cys.Val.Ala.Glu.Pro.Asp.Asp.Ser.Ile. 180
Ser.Pro.Asp.Pro.Pro.Arg.Thr.Pro.Val.Ser.Arg.Lys.Arg.Pro.Arg.Pro.Ala.Gly.Ala.Thr. 200
Gly.Gly.Gly.Gly.Gly.Gly.Val.His.Ala.Asn.Gly.Gly.Ser.Val.Phe.Gly.His.Pro.Thr.Gly. 220
Gly.Thr.Ser.Thr.Pro.Ala.His.Pro.Pro.Pro.Tyr.His.Ser.Gln.Gly.Gly.Ser.Glu.Ser.Met. 240
Gly.Gly.Ser.Asp.Ser.Ser.Gly.Phe.Ala.Glu.Gly.Ser.Phe.Arg.Ser.Asp.Pro.Arg.Cys.Glu. 260
Ser.Glu.Asn.Glu.Ser.Tyr.Ser.Gln.Ser.Cys.Ser.Gln.Ser.Ser.Phe.Asn.Ala.Thr.Pro.Pro. 280
Lys.Lys.Ala.Arg.Glu.Asp.Pro.Ala.Pro.Ser.Asp.Phe.Pro.Ser.Ser.Leu.Thr.Gly.Tyr.Leu. 300
Ser.His.Ala.Ile.Tyr.Ser.Asn.Lys.Thr.Phe.Pro.Ala.Phe.Leu.Val.Tyr.Ser.Thr.Lys.Glu. 320
Lys.Cys.Lys.Gln.Leu.Tyr.Asp.Thr.Ile.Gly.Lys.Phe.Arg.Pro.Glu.Phe.Lys.Cys.Leu.Val. 340
His.Tyr.Glu.Glu.Gly.Gly.Met.Leu.Phe.Phe.Leu.Thr.Met.Thr.Lys.His.Arg.Val.Ser.Ala. 360
Val.Lys.Asn.Tyr.Cys.Ser.Lys.Leu.Cys.Arg.Ser.Phe.Leu.Met.Cys.Lys.Ala.Val.Thr.Lys. 380
Pro.Met.Glu.Cys.Tyr.Gln.Val.Val.Thr.Ala.Ala.Pro.Phe.Gln.Leu.Ile.Thr.Glu.Asn.Lys. 400
Pro.Gly.Leu.His.Gln.Phe.Glu.Phe.Thr.Asp.Glu.Pro.Glu.Glu.Gln.Lys.Ala.Val.Asp.Trp. 420
Ile.Met.Val.Ala.Asp.Phe.Ala.Leu.Glu.Asn.Asn.Leu.Asp.Asp.Pro.Leu.Leu.Ile.Met.Gly. 440
Tyr.Tyr.Leu.Asp.Phe.Ala.Lys.Glu.Val.Pro.Ser.Cys.Ile.Lys.Cys.Ser.Lys.Glu.Glu.Thr. 460
Arg.Leu.Gln.Ile.His.Trp.Lys.Asn.His.Arg.Lys.His.Ala.Glu.Asn.Ala.Asp.Leu.Phe.Leu. 480
Asn.Cys.Lys.Ala.Gln.Lys.Thr.Ile.Cys.Gln.Gln.Ala.Ala.Ala.Ser.Leu.Ala.Ser.Arg.Arg. 500
Leu.Lys.Leu.Val.Glu.Cys.Thr.Arg.Ser.Gln.Leu.Leu.Lys.Glu.Arg.Leu.Gln.Gln.Ser.Leu. 520
Leu.Arg.Leu.Lys.Glu.Leu.Gly.Ser.Ser.Asp.Ala.Leu.Leu.Tyr.Leu.Ala.Gly.Val.Ala.Trp. 540
Tyr.Gln.Cys.Leu.Leu.Glu.Asp.Phe.Pro.Gln.Thr.Leu.Phe.Lys.Met.Leu.Lys.Leu.Leu.Thr. 560
Glu.Asn.Val.Pro.Lys.Arg.Arg.Asn.Ile.Leu.Phe.Arg.Gly.Pro.Val.Asn.Ser.Gly.Lys.Thr. 580
Gly.Leu.Ala.Ala.Ala.Leu.Ile.Ser.Leu.Leu.Gly.Gly.Lys.Ser.Leu.Asn.Ile.Asn.Cys.Pro. 600
Ala.Asp.Lys.Leu.Ala.Phe.Glu.Leu.Gly.Val.Ala.Gln.Asp.Gln.Phe.Val.Val.Cys.Phe.Glu. 620
Asp.Val.Lys.Gly.Gln.Ile.Ala.Leu.Asn.Lys.Gln.Leu.Gln.Pro.Gly.Met.Gly.Val.Ala.Asn. 640
Leu.Asp.Asn.Leu.Arg.Thr.Thr.Trp.Asn.Gly.Ser.Val.Lys.Val.Asn.Leu.Glu.Lys.Lys.His. 660
Ser.Asn.Lys.Arg.Ser.Gln.Leu.Phe.Pro.Pro.Cys.Val.Cys.Thr.Met.Asn.Glu.Tyr.Leu.Leu. 680
Pro.Gln.Thr.Val.Trp.Ala.Arg.Phe.His.Met.Val.Leu.Asp.Phe.Thr.Cys.Lys.Pro.His.Leu. 700
Ala.Gln.Ser.Leu.Glu.Lys.Cys.Glu.Phe.Leu.Gln.Arg.Glu.Arg.Ile.Ile.Gln.Ser.Gly.Asp. 720
Thr.Leu.Ala.Leu.Leu.Leu.Ile.Trp.Asn.Phe.Thr.Ser.Asp.Val.Phe.Asp.Pro.Asp.Ile.Gln. 740
Gly.Leu.Val.Lys.Glu.Val.Arg.Asp.Gln.Phe.Ala.Ser.Glu.Cys.Ser.Tyr.Ser.Leu.Phe.Cys. 760
Asp.Ile.Leu.Cys.Asn.Val.Gln.Glu.Gly.Asp.Asp.Pro.Leu.Lys.Asp.Ile.Cys.Asp.Ile.Ala. 780
Glu.Tyr.Thr.Val.Tyr.-COOH

Figure I.4 -

B. The predicted amino acid sequence of the middle T-antigen. The position of the splice in the mRNA coding for middle T-antigen is indicated by the solid arrow. The position of the premature termination seen for middle T-antigen encoded by the mutant NG-18 is indicated by an open arrow. The amino acids deleted by the deletion mutants dl-8 and dl-23 are also shown (open arrows). The hydrophobic amino acid sequence possibly involved in the association of middle T-antigen with membranes is underlined.

C. The predicted amino acid sequence of the small T-antigen. The position of the splice in the mRNA coding for small T-antigen is indicated by the solid arrow.

Middle T-Antigen Predicted Amino Acid Sequence

NH_2-Met.Asp.Arg.Val.Leu.Ser.Arg.Ala.Asp.Lys.Glu.Arg.Leu.Leu.Glu.Leu.Leu.Lys.Leu.Pro. 20
Arg.Gln.Leu.Trp.Gly.Asp.Phe.Gly.Arg.Met.Gln.Gln.Ala.Tyr.Lys.Gln.Gln.Ser.Leu.Leu. 40
Leu.His.Pro.Asp.Lys.Gly.Gly.Ser.His.Ala.Leu.Met.Gln.Glu.Leu.Asn.Ser.Leu.Trp.Gly. 60
Thr.Phe.Lys.Thr.Glu.Val.Tyr.Asn.Leu.Arg.Met.Asn.Leu.Gly.Gly.Thr.Gly.Phe.Gln.Val. 80
Arg.Arg.Leu.His.Ala.Asp.Gly.Trp.Asn.Leu.Ser.Thr.Lys.Asp.Thr.Phe.Gly.Asp.Arg.Tyr. 100
NG-18
Tyr.Gln.Arg.Phe.Cys.Arg.Met.Pro.Leu.Thr.Cys.Leu.Val.Asn.Val.Lys.Tyr.Ser.Ser.Cys. 120
Ser.Cys.Ile.Leu.Cys.Leu.Leu.Arg.Lys.Gln.His.Arg.Glu.Leu.Lys.Asp.Lys.Cys.Asp.Ala. 140
Arg.Cys.Leu.Val.Leu.Gly.Glu.Cys.Phe.Cys.Leu.Glu.Cys.Tyr.Met.Gln.Trp.Phe.Gly.Thr. 160
Pro.Thr.Arg.Asp.Val.Leu.Asn.Leu.Tyr.Ala.Asp.Phe.Ile.Ala.Ser.Met.Pro.Ile.Asp.Trp. 180
Leu.Asp.Leu.Asp.Val.His.Ser.Val.Tyr.Asn.Pro.Lys.Arg.Arg.Ser.Glu.Glu.Leu.Arg.Arg. 200
Ala.Ala.Thr.Val.His.Tyr.Thr.Met.Thr.Thr.Gly.His.Ser.Ala.Met.Glu.Ala.Ser.Thr.Ser. 220
Gln.Gly.Asn.Gly.Met.Ile.Ser.Ser.Glu.Ser.Gly.Thr.Pro.Ala.Thr.Ser.Arg.Arg.Leu.Arg. 240
dl-8
Leu.Pro.Ser.Leu.Leu.Ser.Asn.Pro.Thr.Tyr.Ser.Val.Met.Arg.Ser.His.Ser.Tyr.Pro.Pro. 260
Thr.Arg.Val.Leu.Gln.Gln.Ile.His.Pro.His.Ile.Leu.Leu.Glu.Glu.Asp.Glu.Ile.Leu.Val. 280
dl-8
Leu.Leu.Ser.Pro.Met.Thr.Ala.Tyr.Pro.Arg.Thr.Pro.Pro.Glu.Leu.Leu.Tyr.Pro.Glu.Ser. 300
dl-23
Asp.Gln.Asp.Gln.Leu.Glu.Pro.Leu.Glu.Glu.Glu.Glu.Glu.Glu.Tyr.Met.Pro.Met.Glu.Asp. 320
dl-23
Leu.Tyr.Leu.Asp.Ile.Leu.Pro.Gly.Glu.Gln.Val.Pro.Gln.Leu.Ile.Pro.Pro.Pro.Ile.Ile. 340
Pro.Arg.Ala.Gly.Leu.Ser.Pro.Trp.Glu.Gly.Leu.Ile.Leu.Arg.Asp.Leu.Gln.Arg.Ala.His. 360
Phe.Asp.Pro.Ile.Leu.Asp.Ala.Ser.Gln.Arg.Met.Arg.Ala.Thr.His.Arg.Ala.Ala.Leu.Arg. 380
Ala.His.Ser.Met.Gln.Arg.His.Leu.Arg.Arg.Leu.Gly.Arg.Thr.Leu.Leu.Leu.Val.Thr.Phe. 400
Leu.Ala.Ala.Leu.Leu.Gly.Ile.Cys.Leu.Met.Leu.Phe.Ile.Leu.Ile.Lys.Arg.Ser.Arg.His. 420
Phe.-COOH

Small T-Antigen Predicted Amino Acid Sequence

NH_2-Met.Asp.Arg.Val.Leu.Ser.Arg.Ala.Asp.Lys.Glu.Arg.Leu.Leu.Glu.Leu.Leu.Lys.Leu.Pro. 20
Arg.Gln.Leu.Trp.Gly.Asp.Phe.Gly.Arg.Met.Gln.Gln.Ala.Tyr.Lys.Gln.Gln.Ser.Leu.Leu. 40
Leu.His.Pro.Asp.Lys.Gly.Gly.Ser.His.Ala.Leu.Met.Gln.Glu.Leu.Asn.Ser.Leu.Trp.Gly. 60
Thr.Phe.Lys.Thr.Glu.Val.Tyr.Asn.Leu.Arg.Met.Asn.Leu.Gly.Gly.Thr.Gly.Phe.Gln.Val. 80
Arg.Arg.Leu.His.Ala.Asp.Gly.Trp.Asn.Leu.Ser.Thr.Lys.Asp.Thr.Phe.Gly.Asp.Arg.Tyr. 100
NG-18
Tyr.Gln.Arg.Phe.Cys.Arg.Met.Pro.Leu.Thr.Cys.Leu.Val.Asn.Val.Lys.Tyr.Ser.Ser.Cys. 120
Ser.Cys.Ile.Leu.Cys.Leu.Leu.Arg.Lys.Gln.His.Arg.Glu.Leu.Lys.Asp.Lys.Cys.Asp.Ala. 140
Arg.Cys.Leu.Val.Leu.Gly.Glu.Cys.Phe.Cys.Leu.Glu.Cys.Tyr.Met.Gln.Trp.Phe.Gly.Thr. 160
Pro.Thr.Arg.Asp.Val.Leu.Asn.Leu.Tyr.Ala.Asp.Phe.Ile.Ala.Ser.Met.Pro.Ile.Asp.Trp. 180
Leu.Asp.Leu.Asp.Val.His.Ser.Val.Tyr.Asn.Pro.Arg.Leu.Ser.Pro.-COOH 195

the dl-23 encoded middle T-antigen is close to that predicted from the DNA sequence and have suggested that it is the sequence of the polypeptide within the region deleted by the dl-23 mutant that causes the abnormal migration of the wild type polypeptide on SDS-PAGE (see Chapter One). The predicted molecular weight of 23Kd for small T-antigen agrees well with the measured 22Kd. All of the data obtained since this coding scheme (Figure I.3) was proposed agree with it (Hunter et al., 1979; Chapter One). The unusual features present in these predicted sequences have been well reviewed (for example, see Eckhart, 1981); of particular interest is the stretch of 22 continuous hydrophobic amino acids near the C-terminus of middle T-antigen. This is characteristic of sequences found in many transmembrane proteins. That is, it lacks charged amino acids, histidine, or proline, and is flanked by lysine or arginine residues (reviewed in Warren, 1981).

Properties of the T-Antigens

Large T-Antigen:- There are very few biochemical activities that can be precisely attributed to the T-antigens of polyoma virus. At best, the term "associated with" still applies to most activities, even the better characterized ones. This is a consequence of the absence of pure proteins with which to conduct biochemical studies. Most of the activities of the large T-antigen have been studied by comparison with the related large T-antigen of SV40. Polyoma virus large T-antigen has been shown to bind to double-stranded DNA, with a low affinity for non-specific sequences and a high affinity for specific sequences, near the polyoma viral origin of DNA replication (Paulin et al., 1973; Paulin et al., 1975; Clertant and Blangy, 1977; Gaudray et al., 1977; Gaudray et al., 1981; F. Chaudry and A. Cowie, personal communication). An ATP phosphohydrolase activity has also been described for partially purified polyoma virus large T-antigen (Gaudray et al., 1980). The relationship between this activity and the DNA binding activity (if any) is not clear. The large T-antigen is found in the nucleus of lytically infected and transformed cells when purified sub-cellular fractions are examined (Ito et al., 1977b; Silver et al., 1978), and a newly synthesised fraction is found in the nuclear matrix (Buckler-White et al., 1980).

In vivo, a number of cellular functions can be deduced for the large T-antigen from the study of the hr-t and ts-a mutants. Since the hr-t mutants are capable of growing lytically in primary mouse kidney cells, it appears that large T-antigen alone is sufficient for inducing viral replication and virion assembly. The hr-t mutants are also capable of

inducing a single round of cellular DNA synthesis. Consequently, large T-antigen is to a certain extent mitogenic (Schlegel and Benjamin, 1978). Cells infected with ts-a mutants and subsequently shifted to the non-permissive temperature "overproduce" early viral mRNA relative to cells infected with wild type virus (Cogen, 1978) and do not produce late mRNAs. Therefore, large T-antigen is probably responsible for the "early" to "late" gene switch, and acts as an "auto-repressor" (Kamen et al., 1975). Cells infected with ts-a mutants and shifted to the non-permissive temperature complete an already initiated round of viral DNA replication but do not initiate any new ones (Francke and Eckhart, 1973), suggesting that large T-antigen is crucial for the initiation of viral DNA synthesis. It also seems likely that large T-antigen regulates the integration, excision, and amplification of polyoma virus DNA in the host chromosome and the reversible generation of polyploid cells (Colantuoni et al., 1982; Dailey et al., 1982). It may be this property that makes it necessary for the initiation of transformation (Fried, 1965). All these data are deduced from the temperature sensitivity of these functions in ts-a infected cells. It is tempting to speculate that these functions are related to the DNA binding activity of large T-antigen, but there is no evidence for this. Undoubtedly other functions of the polyoma virus large T-antigen remain to be discovered.

Middle T-Antigen:- Immunoprecipitates of the polyoma virus T-antigens contain an activity that transfers phosphate from the γ- position of ATP onto either middle T-antigen (Eckhart et al., 1979; Schaffhausen and Benjamin, 1979; Smith et al., 1979) or the heavy chain of rat immunoglobulin (Smith et al., 1979), in an assay similar to that devised by Collett and Erikson (1978). This activity appears to be associated with the middle T-antigen since it is absent from lysates of cells that have been infected with hr-t mutants (Schaffhausen and Benjamin, 1979; Smith et al., 1979; Eckhart et al., 1979), and can be shown to be altered by the deletions in mutants dl-8 and dl-23 (Smith et al., 1979) which affect middle and large T-antigens. It is also absent in mutants that have premature termination codons inserted into the middle T-antigen coding frame (Templeton and Eckhart, 1982; Carmichael et al., 1982). This middle T-antigen associated protein kinase activity has the unusual property of transferring phosphate onto the amino acid, tyrosine, (Eckhart et al., 1979) rather than onto the more usual phosphate acceptors serine or threonine. Sub-cellular fractionation studies have localized at least part of

middle T-antigen to membrane fractions in cells, particularly the plasma membrane (Ito et al., 1977b; Silver et al., 1978; Ito, 1979).

In vivo, similar to the deductions for large T-antigen, a number of induced cellular changes can be attributed to the middle and small T-antigens. Abortive transformation occurs at the non-permissive temperature with ts-a mutants, so large T-antigen may not be primarily responsible for the transformed phenotype. Middle or small T-antigens probably are. The hr-t mutants are totally non-transforming and are incapable of inducing abortive transformation (Schlegel and Benjamin, 1978). They no longer induce a morphological change in cells, disrupt the sub-cellular actin cable network, increase nuclei or nucleoli size, increase lectin agglutinability of the cell surface, or increase the uptake of glucose. They are, however, capable of integrating into the host chromosome without inducing any of these changes (Lania et al., 1979). The hr-t mutants also fail to induce the cellular factors necessary for permissivity of these mutants in established mouse fibroblast cell lines, leading to a host range phenotype, and are deficient in the ability to stimulate cells to highly acetylate the histones H3 and H4 (Schaffhausen and Benjamin, 1976).

The effects on cells of other mutants have not been studied in as much detail at the present time, but a comparison of the transforming abilities of dl-8 and dl-23 (Griffin and Maddock, 1979), (where large and middle T-antigens are affected) and the hr-t mutants (where middle and small T-antigens are affected) suggests that it is the middle T-antigen that is responsible for the observed transforming effects. The presence of middle and small T-antigens, but not large T-antigen, in all of the transformed cell lines examined (Ito and Spurr, 1980; Lania et al., 1980), provides further weight to this argument. Also, mutants that only affect expression of middle T-antigen (Carmichael et al., 1982; Templeton and Eckhart, 1982) do not transform cells, which further suggests a crucial role for middle T-antigen in transformation. Finally, the demonstration by Treisman et al., (1981b), that a plasmid which is only capable of expressing middle T-antigen when transfected into an established rat fibroblast cell line transforms it, shows clearly that middle T-antigen is capable on its own of inducing most of the properties of transformed cells, including that of tumourigenesis.

Small T-Antigen:- No function, either *in vitro* or *in vivo* has been found associated with the small T-antigen of polyoma virus. It is present in the cytoplasm of the cell (Türler, 1980). The fact that it possibly has only four amino acids (at its C-terminus) that are not present in middle T-antigen, may suggest that it does not possess any function unique from a middle T-antigen function. However, its different sub-cellular location may result in it having a role separate to that of middle T-antigen. Friedmann *et al.*, (1978) have pointed out the sequence relationship between small T-antigen and a number of hormones such as the glycoprotein hormones, thyroid stimulating hormone, luteinizing hormone and chorionic gonadotropin.

The above data are summarized in Table I.1.

It can be seen, therefore, that much is known about the structural organization of polyoma virus. Unfortunately, there is still little known about the biochemical changes in the cell that accompany viral infection and transformation. A major achievement of the molecular biological studies has been to provide overwhelming evidence in support of a virally-coded "transforming gene" and has thus enhanced the usefulness of polyoma virus as a model system for tumourigenesis. Further study of the tumour antigens, however, requires new techniques and approaches to determine the biochemical properties and functions of the proteins both *in vitro* and *in vivo*. The application of one of these new techniques to the study of polyoma virus is described in this thesis.

Table I.1. Properties of the Polyoma Virus T-Antigens.

Large T-Antigen: nuclear location.	
In vitro	In vivo
Polyoma DNA "origin region" binding.	Induction of cellular DNA synthesis.
ATPase activity	Initiation of viral DNA synthesis
	Repression of early mRNA synthesis.
	Raised levels of late mRNA synthesis.
	Lower cellular serum requirement.
	Establish cell line permanency.
Middle T-Antigen: membranal location.	
In vitro	In vivo
Tyrosine specific protein kinase activity.	Cytoskeletal disruption.
	Induction of:
	Cell growth to high saturation density.
	Growth in soft agar:anchorage independence.
	Tumour formation.
	Lower cell contact inhibition.
	Phenotypic changes.
	Increased lectin agglutinabilit
	Increased nutrient transport.
	Increased protease secretion.

RESULTS

CHAPTER ONE

Section 1.1 - Aims

The protocols published for extracting the T-antigens from cells use a variety of different procedures (Ito et al., 1977a,b; Türler and Salomon, 1977; Schaffhausen et al., 1978; Huchinson, et al., 1978; Simmons et al., 1979). These are all based on the lysis of cell membranes with mainly non-ionic detergents, which leave the nuclei essentially intact. An alkaline pH is used to disrupt large T-antigen-DNA interactions and allow the protein out of the nucleus. After lysis, centrifugation removes nuclei and other large cell debris leaving a preparation of solubilized cell proteins. A number of different protease inhibitors have been added to the lysis buffer to prevent the proteolytic degradation of proteins by cellular proteases released during the lysis. The efficiency of some of these methods were tested in parallel in this Chapter to ensure that maximum protein yields were achieved in later experiments. For a similar reason, and to investigate the possibility that the three T-antigens are expressed at differing times during the lytic infection process, as reported for the SV40 T-antigens (Crawford et al., 1980), the rate of synthesis of the T-antigens at various times after infection was also determined.

The coding scheme described in the Introduction Section (Figure I.3), for the early region of the polyoma virus genome makes a number of predictions about the expected size of the T-antigen molecules and the effects mutants will have on individual T-antigens. Since this was proposed, a number of physically defined deletion mutants have been isolated. To verify the accuracy of the proposed coding scheme of polyoma virus, the T-antigens encoded by a number of the mutants were studied. The transforming properties of two of them (dl-8 and dl-23; Griffin and Maddock, 1979) are radically altered. It is possible that this is a reflection of an altered membrane association of the middle T-antigen (Ito et al., 1977b) expressed by these two mutants. To investigate this, cells lytically infected with these viruses were fractionated, and the middle T-antigen present in the plasma membrane fraction determined.

The experiments described in this Chapter are illustrative of the use of anti-tumour serum in the study of the polyoma virus T-antigens. They define some physical characteristics of the T-antigens and describe the extraction conditions used for future experiments. The experiments are limited but illustrate the type of studies that can be achieved with these sera.

Section 1.2 - Extraction Procedural Tests.

To investigate the efficiency of T-antigen extraction by various detergent solutions of varying character and concentration, comparable dishes of polyoma virus infected, radio-labelled 3T6 Swiss Albino mouse cells were lysed with the appropriate solution, the T-antigens extracted, and immunoprecipitated with an (predetermined) excess of anti-T serum. The resulting polypeptides were separated by SDS-PAGE. Figure 1.1 shows the T-antigens obtained in this manner with three different detergent solutions which were all made up to a final concentration of 100mM NaCl, 100mM TrisHCl, pH8.8. The mixed detergent solution shown in lysate (c) was the strongest detergent tested; the addition of the ionic detergent, SDS, at low concentration considerably increased its solubilizing power, whilst the denaturing effect of this detergent is limited by the formation of mixed micelles with the non-ionic detergents. It can be seen that this solution does not significantly alter the quantity of T-antigens extracted relative to the other detergent solutions. To quantitate this procedure, the gel pieces containing individual T-antigen species were excised, dissolved with NCS (Amersham, Searle) and the amount of (^{35}S) present determined by scintillation counting. No significant variation in extraction efficiency was observed. It appears, therefore, that 0.5ml of 0.5% Nonidet P40/50mm dish of cells is sufficient to solubilize most of the detectable T-antigen molecules and, this is preferred to the other detergents because of its mild, non-denaturing action.

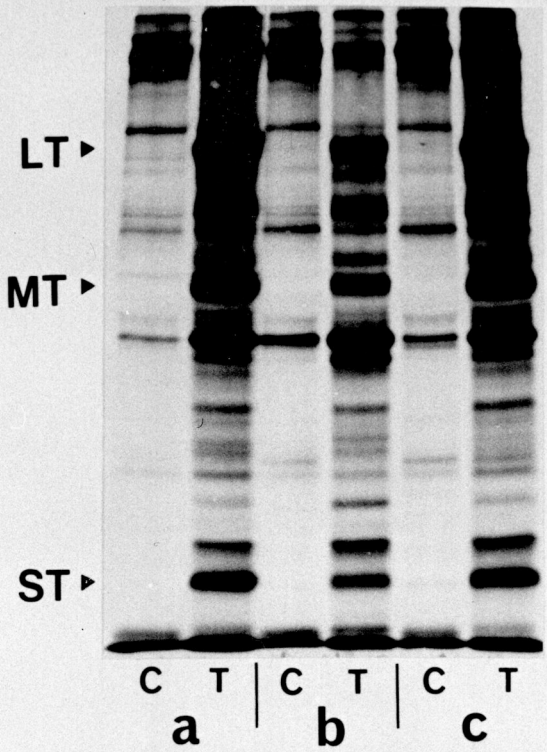
A similar experiment was conducted to investigate the effect of pH on the solubilization of the T-antigens. Extraction was conducted with 0.5% Nonidet P40, 100mM NaCl and 100mM TrisHCl at varying pH's. Little alteration was observed in the quantities of middle and small T-antigen solubilized between pH6 and 8. However, only approximately 10% of the large T-antigen molecules were solubilized at pH6, and this extraction efficiency increased until a maximum was reached at pH8.5. Increasing ionic strength was also observed not to have a significant effect on the solubilizing efficiency of any of the T-antigens. However, at high ionic strengths the nuclear envelope was disrupted and the cellular DNA released. This made it more difficult to remove unsolubilized material by centrifugation and so reduced the quantity of the T-antigen obtained. Therefore, for future studies the extraction buffer consisting of 100mM NaCl, 100mM TrisHCl, pH8.8, 0.5% Nonidet P40 was used..

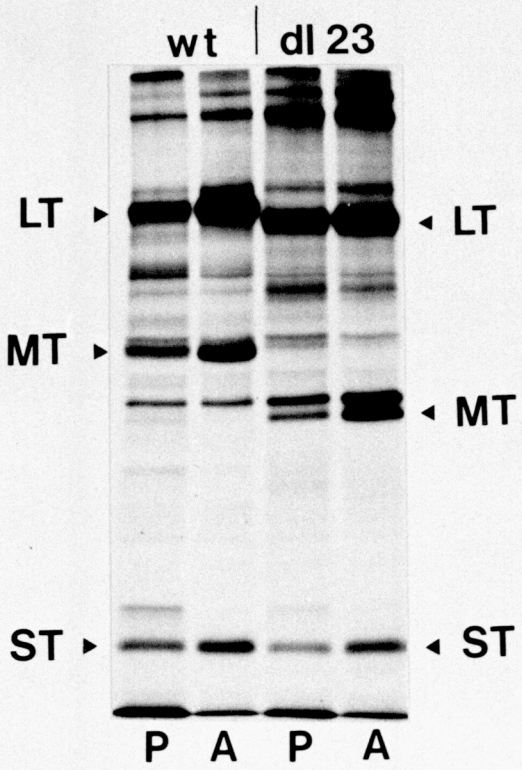
Figure 1.1 - The T-Antigens Extracted By Various Detergent Solutions

Wild type polyoma virus infected 3T6 Swiss Albino mouse cells were labelled with (^{35}S)-methionine for 4 hours at 37°C at 24 hours post infection. The cells were then lysed with a detergent solution, the lysate immunoprecipitated, the resulting polypeptides separated by SDS-PAGE on a 10% acrylamide gel, and then located by fluorography, as described in Materials and Methods. Each lysate was divided in two and immunoprecipitated with either pre-immune rat serum (lanes C) or polyoma anti-T serum (lanes T). Lysate (a) was extracted with 0.5% Nonidet P40; lysate (b) with 1% Nonidet P40, 1% DOC; and lysate (c) with 1% Nonidet P40, 1% DOC, 0.1% SDS. All three detergent solutions were made up in 100mM NaCl, 100mM TrisHCl, pH 8.8. The positions of large (LT), middle (MT) and small T-antigens (ST) are indicated on the left.

Figure 1.2 - Efficiency Test on Two Protease Inhibitors.

Polyoma virus infected cells were labelled with (^{35}S)-methionine for 4 hours at 37°C, 24 hours post infection. The cells were infected with wild type virus (wt) or the deletion mutant dl-23 (dl23), as indicated. The cells were then lysed with Extraction Buffer containing either PMSF (lanes P) or Aprotinin (lanes A) and the T-antigens immunoprecipitated with anti-T serum. The resulting polypeptides were separated by SDS-PAGE on a 10% gel and located by fluorography. The position of the wild type virus encoded T-antigens are indicated on the left and those encoded by the deletion mutant dl-23 on the right.


LT
MT
ST
C T C T C T
a b c


wt dl 23
LT
MT
ST
LT
MT
ST
P A P A

Eukaryotic cells contain considerable quantities of proteolytic enzymes that are usually isolated in membrane enclosed vesicles, the lysosomes, to separate them from normal cellular proteins. On detergent lysis, however, these vesicles are disrupted and leave the solubilized proteins susceptible to proteolytic cleavage. The T-antigens, being only a minor proportion of the proteins in cell lysates, are protected to a certain extent from proteolysis but inhibitors of protease action are usually added to the lysis buffers, however, to prevent their cleavage. The two most commonly used inhibitors, PMSF (a serine protease inhibitor) and Transylol (aprotinin, an inhibitor from bovine lung), were added to separate lysis buffers, and these then used to lyse comparable radio-labelled, infected cells. The resulting lysates were immunoprecipitated with anti-T serum and the polypeptides separated by SDS-PAGE (Figure 1.2). It can be seen that the T-antigen preparation obtained after lysis with buffers containing aprotinin contained considerably more intact sized T-antigen polypeptides and lower quantities of other polypeptides than that where PMSF was used. The other (unidentified) polypeptides probably represent proteolytically cleaved T-antigen molecules, as their appearance could be correlated with the disappearance of intact-sized polypeptides. These data imply, therefore, that aprotinin is a better protease inhibitor to use than PMSF in the isolation of the T-antigens, and it was used in future experiments.

It has been suggested that the large decrease in the MWtapp of middle T-antigen encoded by the dl-23 mutant of polyoma virus (Ito et al., 1980) could be a result of a rapid proteolytic cleavage of the protein as a result of it being significantly less stable than the wild type encoded protein. Figure 1.2 shows the polypeptides immunoprecipitated from radio-labelled lysates of dl-23 infected cells, with lysis buffer containing either aprotinin or PMSF. A similar increase, described for wild type infected cells, is observed in the yield of T-antigens when aprotinin is used instead of PMSF. This would not be expected if truncated middle T-antigen were the result of proteolytic cleavage. The observed polypeptide, therefore, probably reflects the full-sized middle T-antigen encoded by this mutant and the large decrease in MWtapp is a result of the deletion alone.

Section 1.3 - Time Course of T-antigen Synthesis.

It has been reported that the large and small T-antigens of SV40 virus are expressed at different times during the lytic infection process (Crawford et al., 1980).

To investigate whether a similar situation occurs during polyoma virus infection, comparable dishes of cells were infected with wild type polyoma virus and then labelled for two hours with (^{35}S)-methionine at varying times post-infection. The cells were lysed, immunoprecipitated with a saturating amount of anti-T serum, and the polypeptides separated by SDS-PAGE. Figure 1.3 shows the polypeptides obtained in this manner,and Figure 1.4 shows a quantitation of the (^{35}S) incorporated into the T-antigens at different times. It can be seen that, in contrast to the observation with SV40, there is no time differential in the expression of any of the polyoma virus T-antigen species. The difference in the time of expression of the early proteins (T-antigens) and late proteins (VP-1) can be clearly seen, however. The rate of synthesis of the T-antigens is found to reach a maximum between 24 and 30 hours post infection, and to decrease (probably due to the start of cell lysis) subsequently.

Section 1.4 - T-Antigens Expressed By Polyoma Virus Deletion Mutants.

A number of deletion mutants of polyoma virus have recently been isolated (Griffin and Maddock, 1979; Magnusson and Berg, 1979; Ding et al., 1982) that do not belong to either of the two previously defined classes of polyoma mutants that is, the ts-a or hr-t mutants. The biology of these new mutants varies considerably and this has led to some difficulties in naming them. Smolar and Griffin (1981) designated them mlt, based on their genotype (described in Introduction). The physical location of many of these deletions on the polyoma virus genome has been determined (Smolar and Griffin, 1981; Magnusson et al., 1981; Ding et al., 1982) and are shown in Figure I.2. The T-antigens encoded by dl-8 and dl-23 have been studied (Ito et al.,1980) and were used for proposing the coding region map for the T-antigens, shown in Figure I.3 (see Introduction). This makes the prediction that mutations between nucleotides 810 and 1495 (Soeda et al.,1980a) will affect both the large and middle T-antigen polypeptides. Mutants in the region of the origin of DNA replication for example (dl-1, dl-6, and dl-28; Smolar and Griffin, 1981) would be expected to have no effect on any of the T-antigens, but as this region is thought to contain regulatory signal elements, may alter the level of T-antigen expression.

The polypeptides immunoprecipitated by anti-T serum from cells infected with these deletion mutants are shown in Figure 1.5, together, for comparison, with those expressed by wild type, dl-8, dl-23 and the hr-t mutant, NG-18.

Figure 1.3 - Time Course of T-Antigen Synthesis: Autoradiograph of SDS-PAGE.

3T6 Swiss Albino mouse cells were infected with about 50 pfu/cell of wild type polyoma virus and then pulse labelled with (^{35}S)-methionine for 2 hours at 37°C at various times post infection. The cells were lysed, the T-antigens immunoprecipitated with anti-T serum, separated by SDS-PAGE on a 10% gel and located by autoradiography. The time (post infection) at which the cells were lysed is indicated underneath each lane. The positions of the three T-antigens are indicated on the left and of the capsid protein VP-1 on the right.

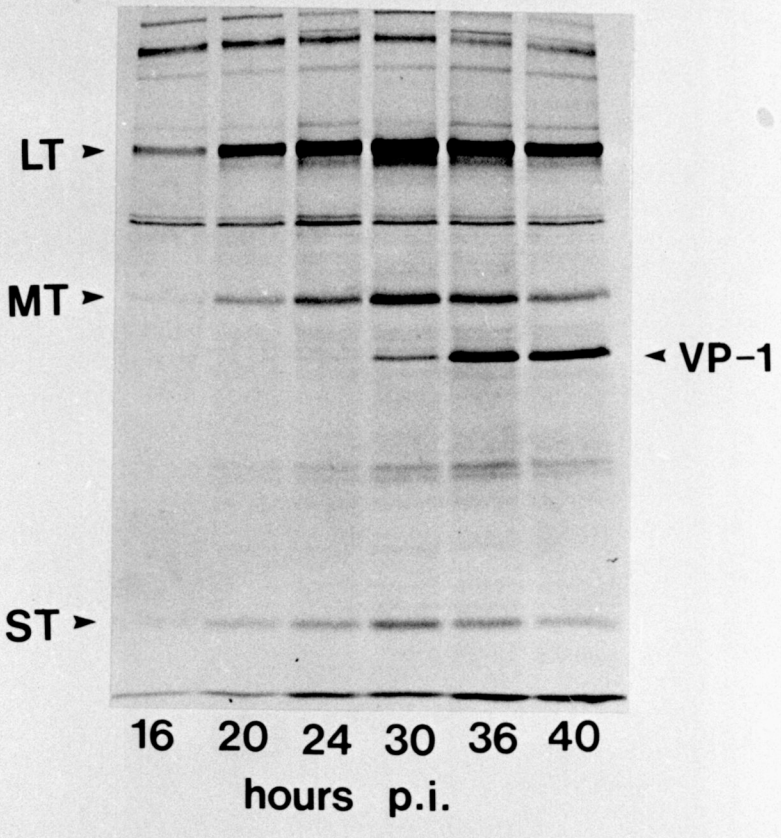
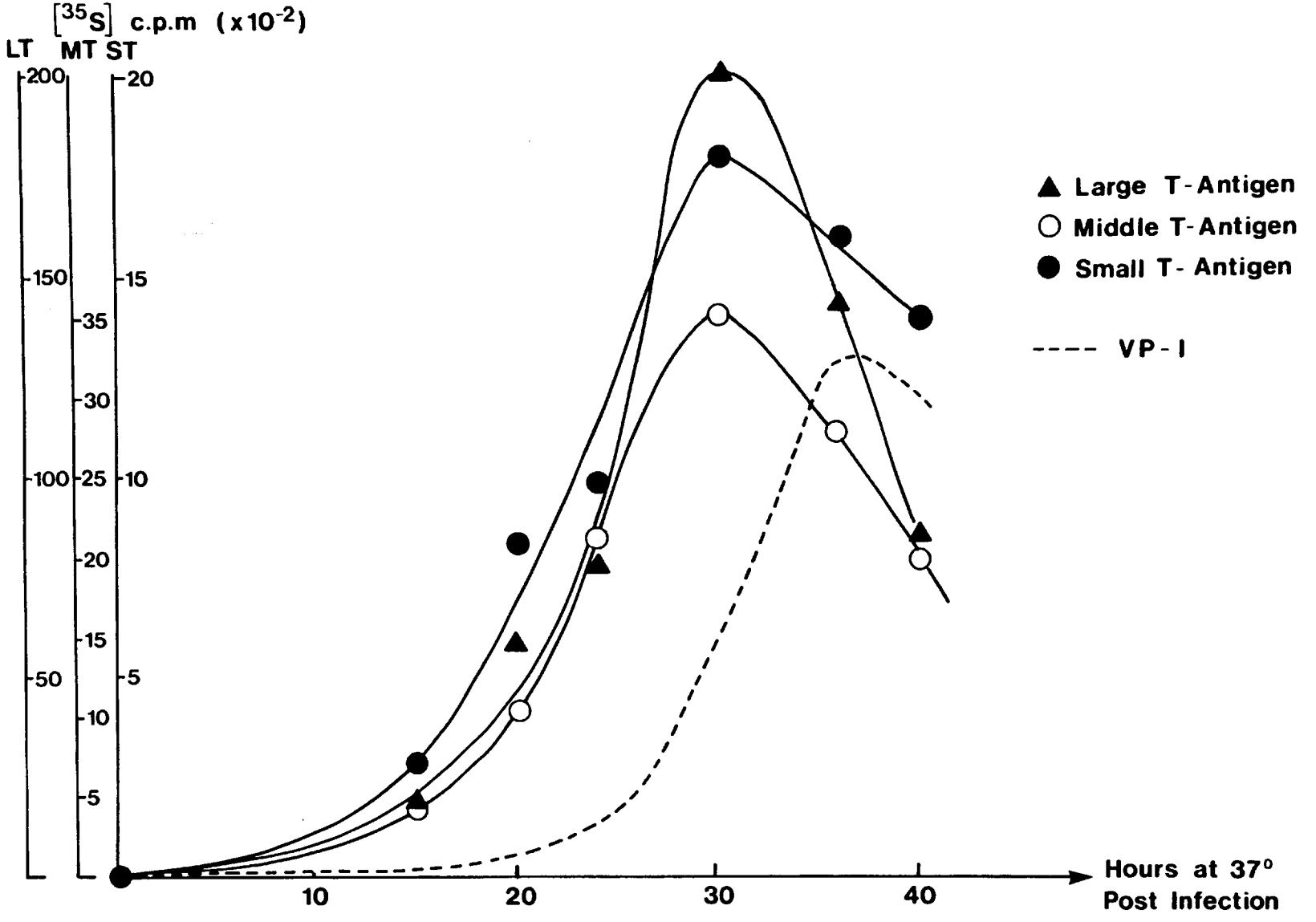

LT
MT
ST
VP-1
16
20
24
30
36
40
hours p.i.

Figure 1.4 - Time Course of T-Antigen Synthesis: Quantitation of the Rate of Synthesis.

The T-antigen polypeptides shown in Figure 1.3 were quantitated by excising the gel piece containing the polypeptide, dissolving it in NCS, and determining the (^{35}S) present by scintillation counting. The counts per minute (c.p.m.) obtained for each polypeptide are plotted against the time post infection at which the cells were lysed. This gives an indication of the rate of T-antigen synthesis at these various times. The rates of synthesis of large, middle and small T-antigens are plotted independently.


[35S] c.p.m (x10-2)
LT MT ST
200
150
100
50
20
15
10
5
35
30
25
20
15
10
5
10
20
30
40
Hours at 37°
Post Infection
Large T-Antigen
Middle T-Antigen
Small T- Antigen
VP-I

Two control reactions are always conducted to ensure that the polypeptides observed are the result of specific immunoprecipitations. Each sample is divided and one is reacted with non-immune rat serum and the other with anti-T serum; only polypeptides present in the latter, and not in the former, are considered specific. A non-infected cell lysate was immunoprecipitated with anti-T serum to ensure that the polypeptides observed were virally-induced. It can be seen that similar to mutants dl-8 and dl-23, the mutant viruses with deletions lying between nucleotides 810 and 1495 (dl-1013, dl-1014, dl-1015, dl-22, and dl-27) all affect the MWtapp of both large and middle T-antigens, but leave unaltered the small T-antigen. This provides further evidence that the proposed coding scheme is correct. The deletions around the origin of DNA replication (dl-1, dl-6, and dl-28) do not appear to alter the level of the T-antigen polypeptide expression.

It has been reported (Ito et al., 1980; see Introduction) that the deletion in mutant dl-23 affects the MWtapp of the middle T-antigen to a greater extent than predicted by the size of the deletion. A decrease of 12Kd is observed for a 34 amino acid deletion (dl-23 mutant), compared with only 5Kd decrease found for the 30 amino acids deleted by the dl-8 mutant. A similar result for mutants dl-22, dl-1013 and dl-1014 is observed (Figure 1.5), where a deletion of only 3 amino acids in middle T-antigen (dl-1014) results in a detectable truncation of the protein and the removal of 33 amino acids by mutant dl-22 reduces the MWtapp by 12Kd. It therefore appears likely that the region of the middle T-antigen molecule involved in these lesions is responsible for the abnormal migration of the wild type protein on SDS-PAGE; its removal results in an apparent large decrease in MWtapp and the restoration of a migration more appropriate to its real size, as predicted by the DNA sequence. Alternative explanations are obviously possible.

Section 1.5 - Membrane Location of Truncated Middle T-Antigens.

The deletion mutants dl-8 and dl-23 show a marked difference in their ability to transform rat cells in culture (Griffin and Maddock, 1979). Middle T-antigen expressed by wild type polyoma virus has been reported to be associated with the membrane fractions of cells (Ito et al., 1977b). It is possible that the middle T-antigens encoded by the deletion mutants dl-8 and dl-23 have an altered sub-cellular distribution and this in turn is responsible for the altered biological properties observed with these viruses. To examine this possibility, the membrane fractions from radio-labelled 3T6 mouse cells lytically infected with deletion mutants dl-8 and dl-23 were prepared and examined for the presence of T-antigens (Figure 1.6). The

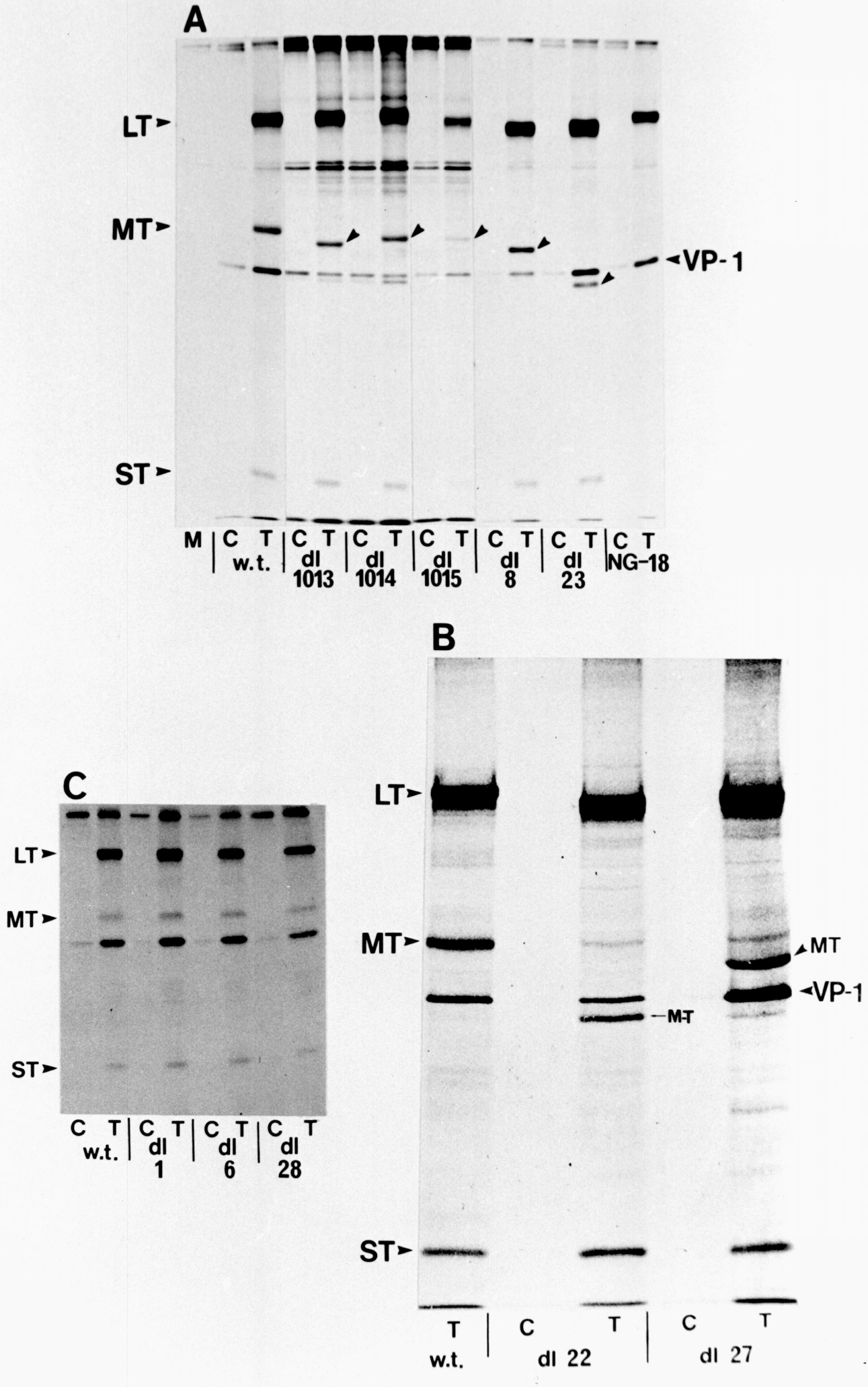
Figure 1.5 - T-Antigens Expressed by Deletion Mutants of Polyoma Virus

3T6 Swiss Albino mouse cells were infected with various deletion mutants of polyoma virus, and labelled with (^{35}S)-methionine for 4 hours at 37°C, 24 hours post infection. The cells were then lysed, the lysate divided, and immunoprecipitated with either pre-immune serum (lanes C) or polyoma anti-T serum (lanes T). The polypeptides were separated by SDS-PAGE on a 10% gel and located by fluorography. The positions of the wild type polyoma virus (w.t.) T-antigens are indicated on the left of each gel.

A. The T-antigens expressed by deletion mutants described by Magnusson and Berg, (1979) and Griffin and Maddock, (1979). The deletion mutant used is indicated underneath. Lane M contains polypeptides immunoprecipitated from mock infected cells with anti-T serum. The position of the truncated middle T-antigens encoded by the deletion mutants are indicated by the unlabelled arrows.

B. The T-antigens expressed by deletion mutants isolated by Ding et al., (1982). The deletion mutant used to infect the cells is indicated underneath. The position of the truncated middle T-antigen encoded by these deletion mutants are indicated on the right of each lane.

C. The T-antigens expressed by the deletion mutants described by Griffin and Maddock (1979) with deletions near the origin of DNA replication. The deletion mutant used is indicated underneath.


A
LT
MT
ST
VP-1
M
C T
w.t.
dl 1013
dl 1014
dl 1015
dl 8
dl 23
NG-18
B
LT
MT
MT
VP-1
MT
ST
T
w.t.
C T
dl 22
C T
dl 27
C
LT
MT
ST
C T
w.t.
dl 1
dl 6
dl 28

procedure adopted was identical to that described by Ito et al., (1977b), but no attempt was made to determine the purity of the membranes obtained, or the necessity of detergent for solubilizing the middle T-antigens. It can be seen that the expected truncated forms of these middle T-antigens were considerably enriched within the membrane fraction. The data suggest, therefore, that there is no alteration in the membrane association of the truncated middle T-antigens induced by these deletion mutants.

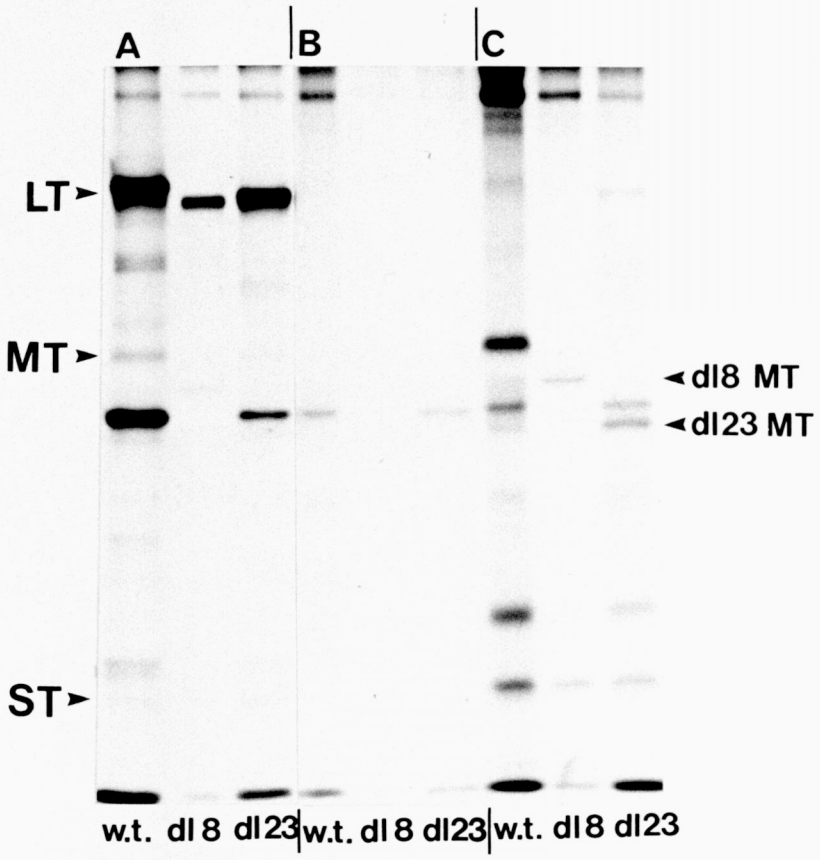
Section 1.6 - Discussion.

The conditions used by a number of groups for the lysis and extraction of the T-antigens from cells have been examined in parallel experiments. It was found that 0.5% Nonidet P40 was sufficient to extract all of the T-antigens from infected cells, and with 100mM NaCl, 100mM TrisHCl pH8.8, most of the detectable molecules were solubilized (see Figure 1.1). These are reasonably mild conditions and it is expected that they will not cause excessive disruption of protein structure. Therefore, these conditions have been adopted for future preparations of the T-antigens. The use of aprotinin as a protease inhibitor relative to PMSF has also been shown to increase significantly yields of the T-antigens (see Figure 1.2). The time course analysis for the production of T-antigen molecules during the lytic cycle has shown that there is no differential expression of these proteins. It has provided data on the appropriate time to label cells in order to produce the maximum incorporation of radio-label into the T-antigens (see Figure 1.3). Although not exhaustive, all these investigations have helped optimise conditions for future studies.

The T-antigens expressed by a number of physically defined deletion mutants have been studied and the results are all found to agree with the coding scheme proposed for the early proteins (see Introduction). The four mutants with deletions in the non-coding region around the origin of viral DNA replication do not alter T-antigen synthesis. The mutants with deletions in the genome between nucleotides 810 and 1495 (Soeda et al., 1980a) all result in a decrease in the MWtapp of both the large and middle T-antigens (see Figure 1.5). This region of the genome is therefore extremely likely to encode two separate polypeptides using different coding frames. The anomalies observed between the MWt as predicted for the middle T-antigen by the DNA sequence (49Kd) or the MWtapp on SDS-PAGE (55Kd) has also been investigated with these mutants. The deletion of 30 amino acids in mutant dl-8 has been observed to produce a

Figure 1.6 - Membrane Association of Truncated Middle T-Antigens.

3T6 Swiss Albino mouse cells were infected with wild type, dl-8 and dl-23 mutant polyoma viruses and labelled with (^{35}S)-methionine for 16 hours, 24 to 40 hours post infection, at 32°C. 1/5 of the cells were lysed normally with Extraction Buffer and immunoprecipitated with anti-T serum (lanes A). The rest of the cells were disrupted by nitrogen cavitation and a plasma membrane fraction prepared, as described in Materials and Methods. The solubilized membrane polypeptides were then immunoprecipitated with either pre-immune serum (lanes B) or anti-T serum (lanes C), separated by SDS-PAGE on a 10% gel and located by fluorography. The virus used is indicated beneath each lane. The position of wild type polyoma virus encoded large (LT), middle (MT) and small T-antigens (ST) are indicated on the left and the positions of the truncated middle T-antigens encoded by the deletion mutants on the right.


A
B
C
LT
MT
ST
dl8 MT
dl23 MT
w.t. dl 8 dl23 w.t. dl 8 dl23 w.t. dl 8 dl23

truncation in the MWtapp of middle T-antigen by approximately 5Kd (Ito et al., 1980), whereas the deletion of 33 amino acids in mutant dl-22, or 34 amino acids in dl-23, results in approximately 12Kd being removed from the middle T-antigen (see Figure 1.5). The region deleted in mutant dl-23 appears to be responsible for this variation, since all the mutants in this region have a middle T-antigen truncated to an apparently larger extent than expected. This region is therefore probably responsible for the abnormally low mobility observed for middle T-antigen on SDS-PAGE. The reason for this is unclear; it has been reported that middle T-antigen has little detectable post-translational modification (Schaffhausen et al., 1978), and the same MWtapp on SDS-PAGE when synthesised in vitro (Hunter et al., 1978). It seems likely, therefore, that post-translational modification cannot account for the abnormal migration. The large number of proline residues in this region of the polypeptide sequence is a possible cause for the anomalous mobility. Proline is an unusual amino acid in that it imposes an abnormal angle on the polypeptide chain, and it is feasible that these "kinks" influence the mobility on SDS-PAGE. That the deletion of this region could release this constraint, is suggested by the observation that middle T-antigen expressed by mutants dl-22 and dl-23 have the MWtapp on SDS-PAGE predicted by the DNA sequence and coding scheme.

The middle T-antigen polypeptides expressed by mutants dl-8 and dl-23 have been shown to be associated with cellular membranes, similar to the wild type virus encoded protein (see Figure 1.6). It appears, therefore, that the altered transforming ability of these viruses is not a direct result of an altered sub-cellular distribution of the proteins induced by these mutants. Explanations other than membrane association must therefore be sought to explain their phenotypic behaviour.

CHAPTER TWO

Section 2.1 - Aims

Anti-tumour (T) serum has been used extensively in the isolation and characterization of the early gene products of polyoma virus. However, the use of anti-T serum has a number of disadvantages. The response to challenge with a polyoma virus induced tumour varies considerably between individual animals. Although techniques have been developed to ensure as much reproducibility as possible, the antibody response still shows inconsistencies (see for example, Ito and Spurr, 1980). There is also the possibility of antibodies being raised to proteins, other than the virally-coded ones, which are altered by the transformation process (for example, the expression of embryonic antigens by transformed cells that are not normally presented to the animal's immune system and are therefore recognized as "foreign"). Even the antibody response to the T-antigens of polyoma virus lacks specificity in that it recognizes all three T-antigens; it is thus not possible to differentiate among these T-antigens by the use of anti-T serum. This lack of specifity, together with the low levels of antibodies directed against the T-antigens in anti-T serum (probably a reflection of the small quantities of T-antigens expressed in transformed cells), has meant that alternative procedures are required to study further the properties of these proteins.

The fact that the functions of the T-antigens are so little understood has meant that there is no enzymic assay for the proteins that can be reliably used in their purification. Ironically, the inability to purify the proteins is the main reason for this lack of understanding of their function. Moreover, the low levels of T-antigen expressed in both lytically infected and transformed cells have further hampered purification. Because of these problems, immunological techniques are still the main means available for the study of the T-antigens. High specific activity immunological probes were therefore required that recognize individual T-antigens and could be used for the assay and analysis of each protein. Ideally, the probes should be able to be produced in sufficiently large quantities to allow them to be used in affinity isolation techniques for purifying the T-antigens. The fact that there are no purified proteins means that conventional immunization techniques could not be used to produce these probes.

This Chapter describes the development of a protocol for the production of monoclonal antibodies directed against the polyoma virus T-antigens. The procedure was used to isolate ten cell lines which secrete antibodies with differing specifities for the T-antigens. The polypeptides recognized by these antibodies have been characterised and shown to be the same as the T-antigens recognized by anti-T serum. Some of the other polypeptides immunoprecipitated have also been characterised.

Section 2.2 - Protocol for the Production of Monoclonal Antibodies Directed Against the T-Antigens.

To produce monoclonal antibody secreting hybrid cell lines (hybridomas), an immunized animal is required that has the correct cell types in the spleen. The techniques currently used for producing anti-T serum (Ito et al., 1977a) were used to produce such an immunized animal. A number of modifications were developed in order to increase the yield of anti-T-antigen antibody secreting hybridomas. The yield of antibody producing cells from an individual immunized animal was determined by intravenously boosting animals that bore tumours of a similar size (see Materials and Methods), preparing spleen cells 3 days later, and fusing these cells with a similar ratio (spleen cells : NS-1) of NS-1 cells under identical fusion conditions. The resulting fused cells were plated out into approximately ~100 tissue culture wells and screened for anti-T-antigen antibody production after sufficient cell growth had occurred. The screening procedure adopted involved immunoprecipitation of labelled polypeptides (see Materials and Methods); it enabled up to 200 different tissue culture fluids (TCFs) to be examined in less then 48 hours and was therefore sufficiently quick and easy to be used for all future screenings. The particular T-antigen recognized was also easily determined after the SDS-PAGE step. The screening procedure, which utilised a SAC precipitation step, gives an artificially low level of the number of anti-T-antigen antibody secreting hybridomas, since it only assesses protein A-binding antibody production As further experiments, in general, require protein A-binding antibodies, this was considered acceptable.

Throughout the immunization tests, considerable variations between individual immunized animals were observed, even when using the same protocol. However, one method gave consistently better yields than any other and so is described in detail. This procedure involved growing PyREWA5/T1A1 polyoma virus transformed cells as a tumour in syngeneic Wistar rats and transplantating a small piece of it intra-peritoneally into another Wistar rat.

This protocol was previously used for SV40 T-antigens by Harlow et al., (1981) to remove any immunogenic proteins present in cells derived from tissue culture as a result of contamination with FCS; this method was considerably better in the induction of anti-T-antigen antibody production than tumours formed by cells taken directly from tissue culture. An intra-peritoneal site of tumour formation was also observed to give higher yields than a sub-cutaneous site. The new tumour was left for 3 to 4 weeks until a palpable growth had formed. 5×10^7 PyREWA5/T1A1 cells from tissue culture were then lysed in 1 ml Extraction Buffer, the lysate cleared by a 5 minute centrifugation in an Eppendorf microfuge, and the supernatant injected into the tail vein of the immunized rat. This intravenous injection was essential for the production of anti-T-antigen antibody secreting hybridomas. After 3 days the animal was sacrificed and its spleen removed and fused with the mouse plasmacytoma line P3-NS-1/1-Ag4-1, as described in Materials and Methods. The procedure involving slow speed centrifugation in PEG (Kennet, 1979) was observed to be considerably more efficient for the formation of hybridomas than other methods. This procedure is represented schematically in Figure 2.1.

This protocol was used to isolate ten hybridoma cell lines that secreted antibodies against polyoma virus T-antigens. Figure 2.2 shows an autoradiograph of the polypeptides that were immunoprecipitated by supernatants from 48 tissue culture wells that contained hybrid cells which had been growing for 10 days subsequent to fusion. (This particular fusion showed a considerably higher number of anti-T-antigen antibody secreting hybridomas than usually found.) It can be seen that there is considerable variation in the polypeptides immunoprecipitated by TCF of different wells, although only a few wells failed to show any anti-T-antigen activity. In total, 90 out of 96 wells tested in this experiment were to some extent positive. Figure 2.2 also demonstrates the use of the adopted screening procedure. The relatively high level of non-specific precipitation was due to insufficient washing, necessary if the screening was to be conducted quickly. In spite of this, it is possible to identify TCF that contains antibodies which immunoprecipitate polypeptides of the same MWtapp (on SDS-PAGE) as with the T-antigens. The cells from ten of the wells shown in Figure 2.2 were cloned three times as single cells, by limiting dilution, over spleen feeder cells before being considered as established monoclonal antibody secreting hybridomas.

Figure 2.1 - Schematic Representation of Protocol for Production of Monoclonal Antibodies Directed Against the Polyoma Virus T-Antigens.

Production of Monoclonal Antibodies to the Polyoma T-Antigens

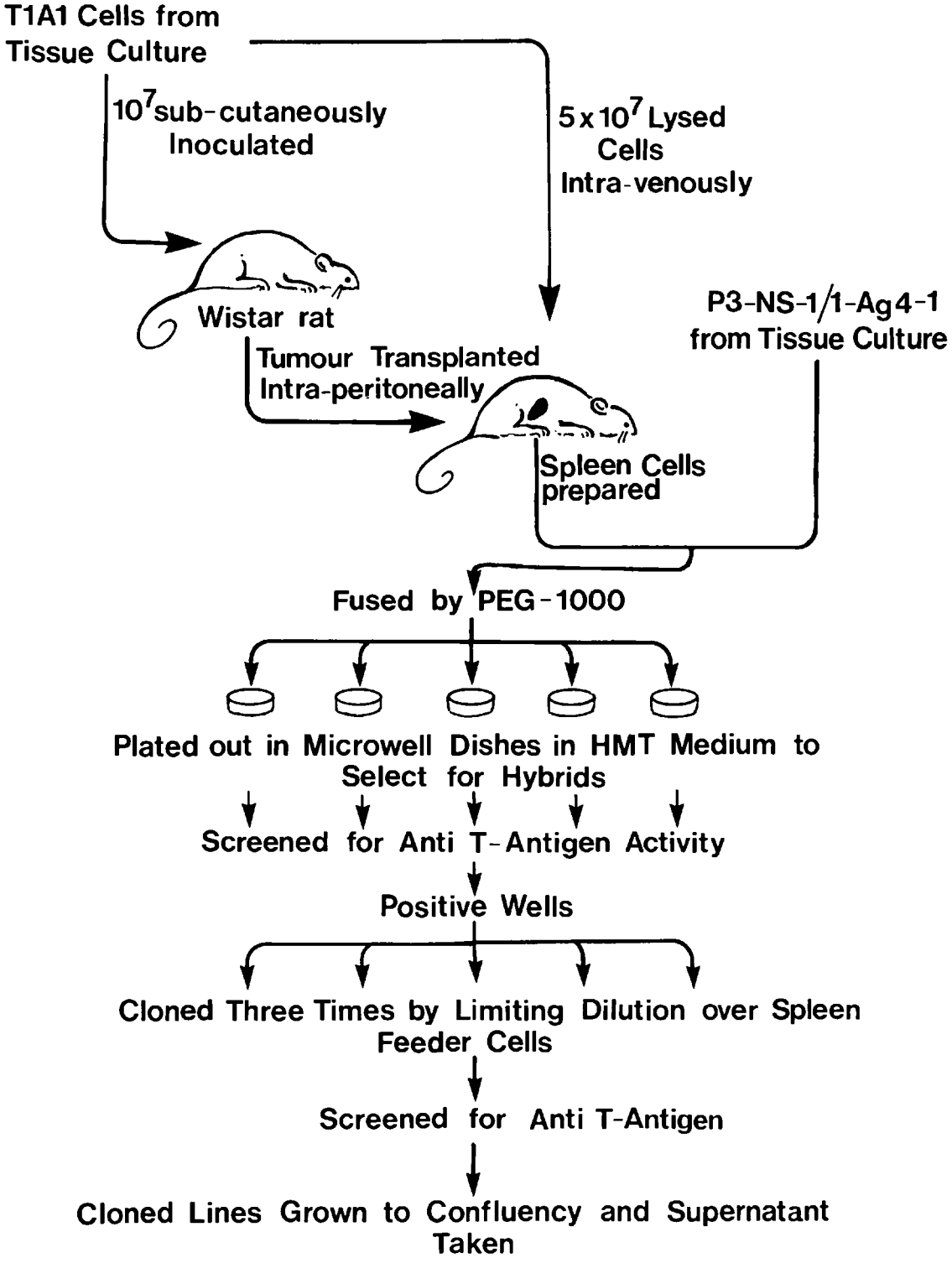
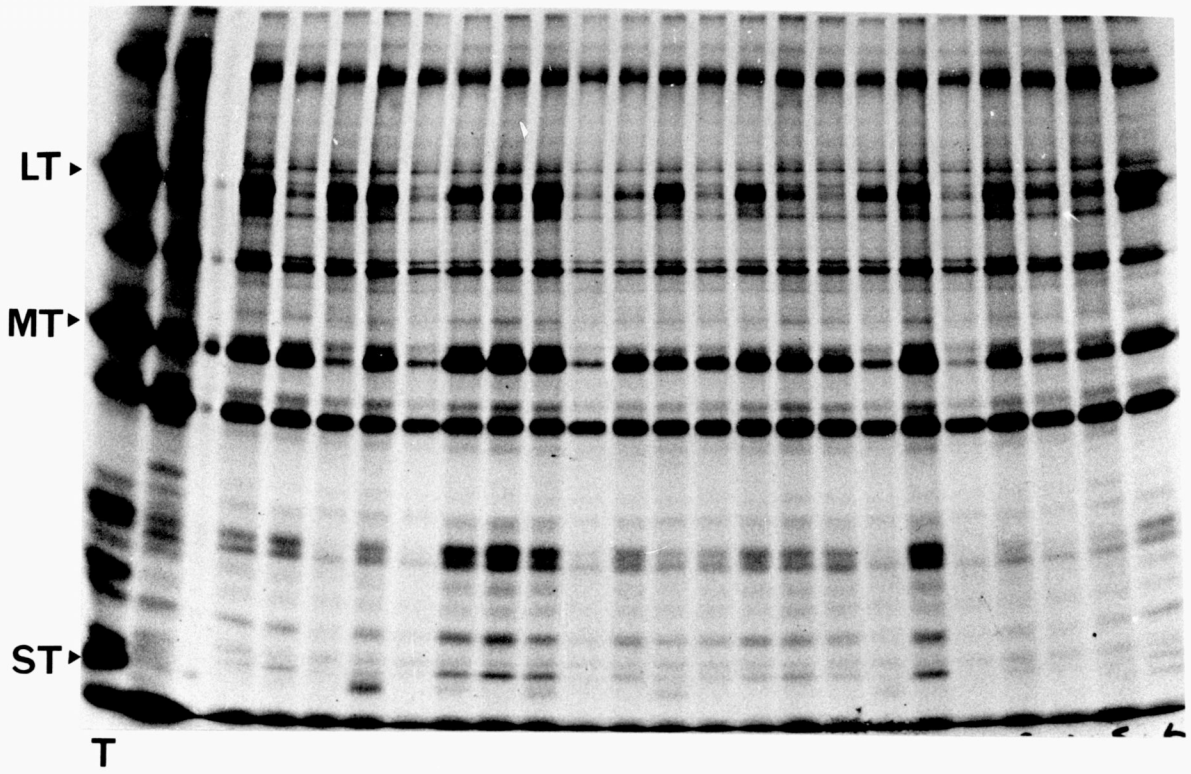


Figure 2.2 - Screen for The Production of Anti-T-Antigen Antibodies by Hybrid Cell Lines.

Ten days after fusion, the supernatants from 48 tissue culture wells were used to immunoprecipitate polypeptides from a (^{35}S)-methionine labelled lysate of ts-a-a infected cells (see Materials and Methods). The polypeptides were then separated by SDS-PAGE on a 10% gel and localized by autoradiography. The lanes labelled T show proteins that were immunoprecipitated with anti-T serum. The positions of the T-antigens are indicated on the left.


LT
MT
ST
T

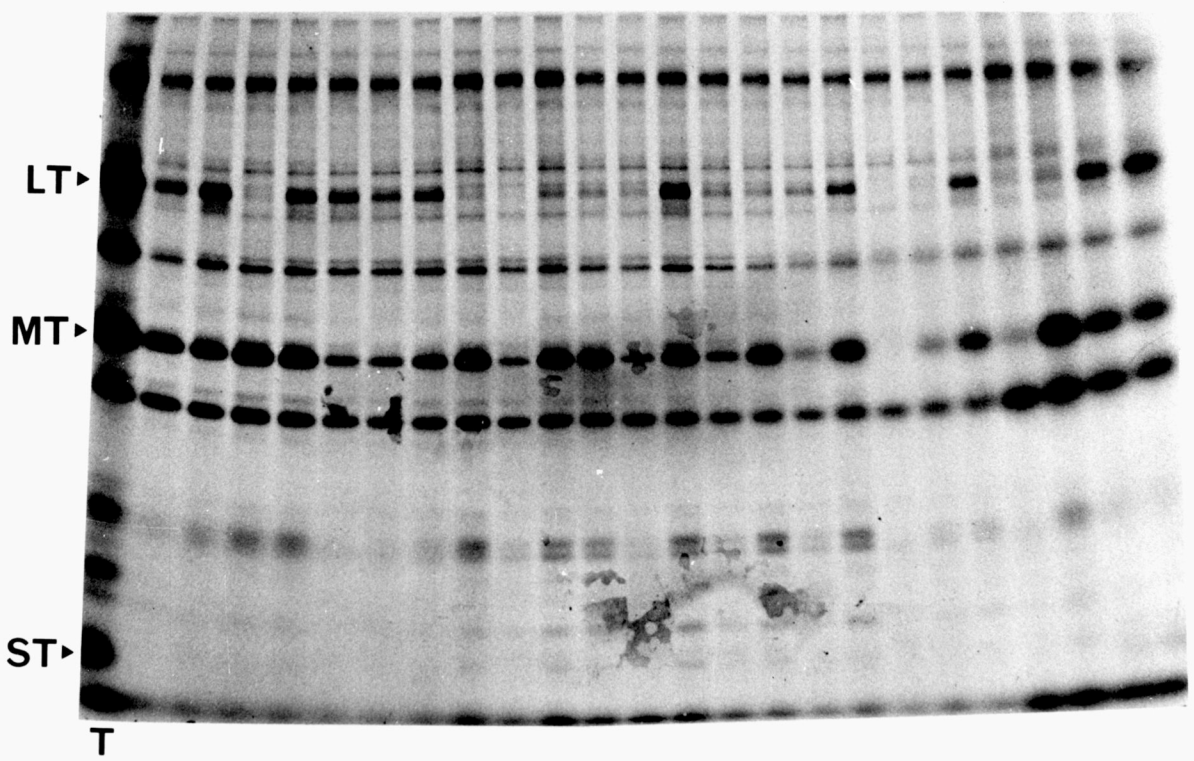

LT
MT
ST
T

Figure 2.3 shows a representative screen from the first and second cloning stage of one of these hybridomas. It can be seen that many of the cells isolated from the original well do not secrete anti-T-antigen antibody (first cloning), but in a second cloning of positive cells, over 90% were antibody secreting. At the third cloning, all the wells that were tested contained antibody. The cells from a single well after a third cloning were allowed to grow in order to produce antibody TCF for future use. Hybridoma cells were not propagated for more than 20 passages before they were recloned in order to avoid the selection of non-secreting variants, although tests on many of the lines showed there was little tendency to cease secreting antibody.

Section 2.3 - T-Antigens Immunoprecipitated by Ten Monoclonal Antibodies.

The polypeptides immunoprecipitated from a lysate of (^{35}S)-methionine labelled 3T6 Swiss Albino mouse cells infected with wild type (A2 strain) polyoma virus by the ten isolated monoclonal antibodies are shown in Figure 2.4. To aid immunoprecipitation with the monoclonal antibodies, an anti-rat immunoglobulin antibody was added to the SAC before use in this experiment.

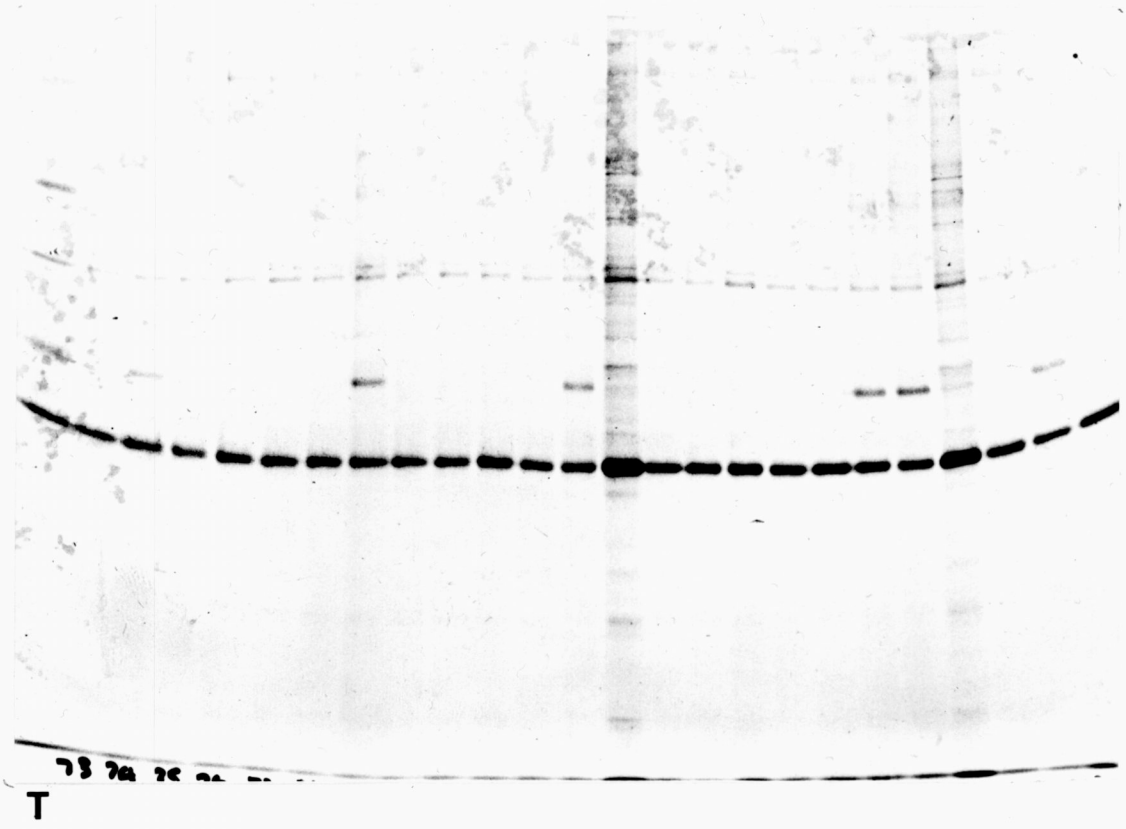
The antibodies have all been assigned the prefix αPy to signify their anti-polyoma virus antigen activity, and have been divided into three groups according to their ability to immunoprecipitate the various T-antigens. The αPyC group of antibodies, of which there are two members, αPyC1 and αPyC4, recognize all three T-antigens and must therefore react with the N-terminal region which is common to all three T-antigens (see Figure 1.3). The αPyLT group of antibodies, of which there are three members, αPyLT1, αPyLT4 and αPyLT7, react only with the large T-antigen, and not with middle or small T-antigens. The αPyMT group of antibodies of which there are five members, αPyMT5, αPyMT7, αPyMT10, αPyMT13 and αPyMT16, immunoprecipitate only the middle T-antigen, and not large or small T-antigens. The antibodies have also been assigned PAb numbers to fit into a generalised method of designating monoclonal antibodies to the papova virus antigens, as suggested by Crawford and Harlow (1982), see Table 2.1.

The polypeptides immunoprecipitated by the monoclonal antibodies were provisionally identified by their MWtapp on SDS-PAGE.

Figure 2.3 - Anti-T-Antigen Antibody Screening at Different Cloning Stages of a Hybridoma Line.

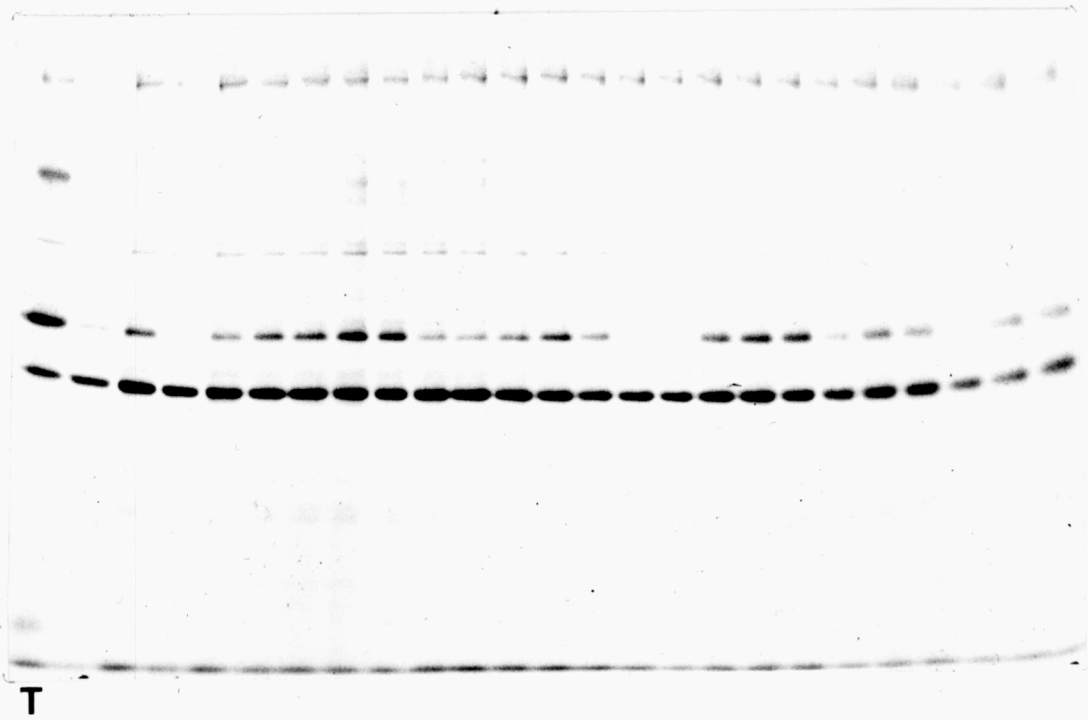
Cells from a tissue culture well that contained anti-T-antigen antibody were cloned as single cells and the supernatant from 24 tissue culture wells that contained growing cells was screened for antibody production after 14 days (upper gel). One of the wells showing strong anti-middle T-antigen activity was chosen and the cells from it re-cloned by single cell dilution, and tested for antibody production (lower gel). One of these wells was chosen, and the cells cloned again (not shown) before being considered to be an established hybridoma cell line (see text).

First Cloning



T

Second Cloning



T

Figure 2.4 - T-Antigens Immunoprecipitated by Ten Monoclonal Antibodies.

Wild type polyoma virus infected cells were labelled with (^{35}S) methionine for 4 hours at 37°C, 24 hours post infection. The cells were then lysed and the T-antigens immunoprecipitated with the monoclonal antibody-containing TCF, using MRC Ox12 as a second antibody (see Materials and Methods). The polypeptides were separated by SDS-PAGE on a 10% gel and localized by autoradiography. The monoclonal antibody used is indicated underneath; the track designated N contains polypeptides non-specifically immunoprecipitated by monoclonal antibody negative TCF. The positions of large (LT), middle (MT) and small (ST) T-antigens are indicated on the left.

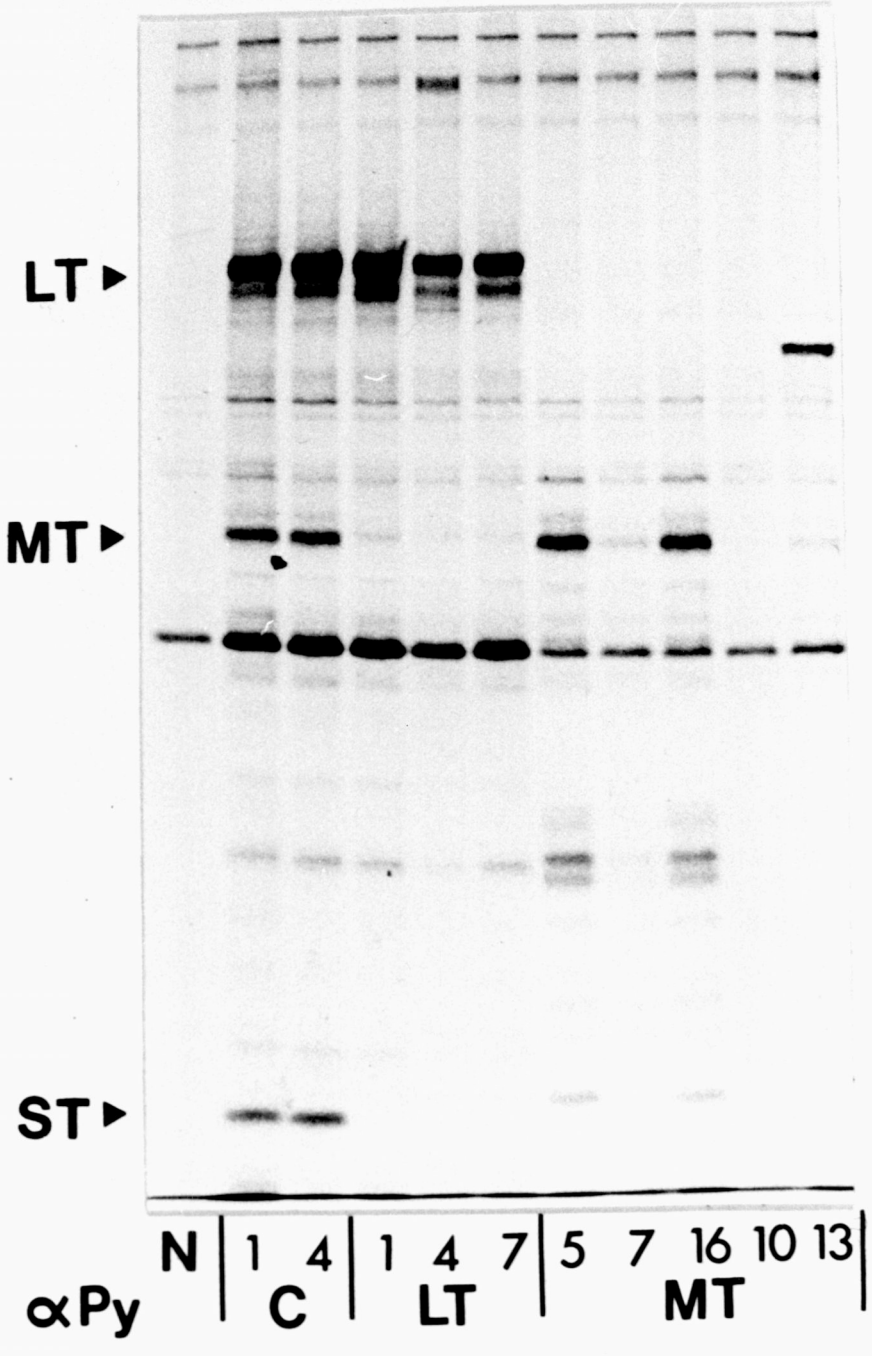

LT
MT
ST
N
1
4
1
4
7
5
7
16
10
13
αPy
C
LT
MT

Table 2.1. Numbering and Properties of Ten Monoclonal Antibodies.

Antibody	PAb No.	T-Antigen Recognized	Other Polypeptides Recognized
αPyC1	701	Large,Middle,Small	-
αPyC4	702	Large,Middle,Small	-
αPyLT1	711	Large	-
αPyLT4	712	Large	>200 Kd
αPyLT7	713	Large	-
αPyMT5	721	Middle	-
αPyMT7	722	Middle	-
αPyMT10	723	Middle	-
αPyMT13	724	Middle	80 Kd
αPyMT16	725	Middle	-

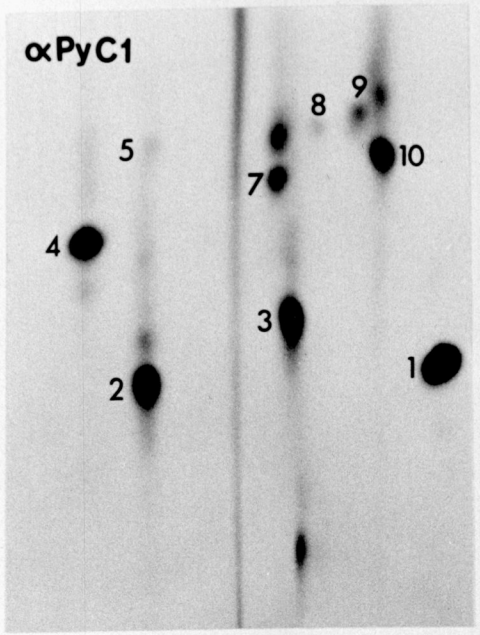
To ensure that they were identical to the polyoma virus T-antigens, two-dimensional separations of the peptides that were obtained after trypsin digestion of (^{35}S)-methionine labelled polypeptides (2D tryptic maps) were examined. Figure 2.5 shows the 2D tryptic maps from the MWtapp 100Kd polypeptides immunoprecipitated by the individual monoclonal antibodies, together with an appropriate mixture of these polypeptides. Figure 2.6 shows the 2D tryptic maps from many of the MWtapp 55Kd polypeptides immunoprecipitated by individual monoclonal antibodies, shown also together with appropriate mixtures of these polypeptides. It can be seen that there is no significant difference between the peptides from different monoclonal antibody immunoprecipitated polypeptides, and all the 2D tryptic maps appear to be identical to those obtained with anti-T sera (Ito, 1979). The antibodies were therefore positively confirmed to react with the T-antigens of polyoma virus.

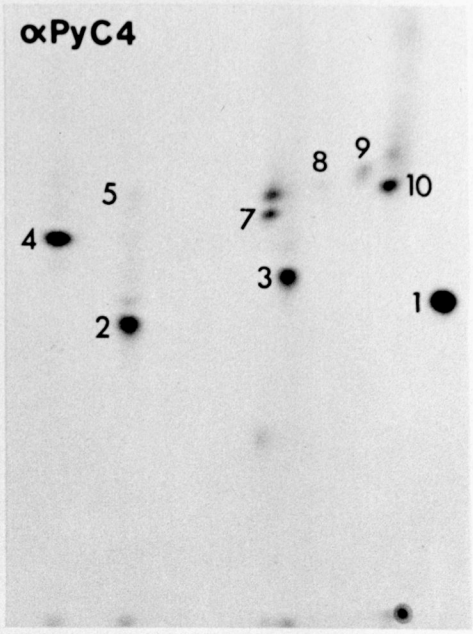
It can further be seen (Figure 2.4) that the intensity of the (^{35}S) labelled polypeptides immunoprecipitated varies considerably with the monoclonal antibody used, particularly so in the case of the middle T-antigen specific antibodies. Therefore, to confirm the identify of the polypeptides and to examine any differences that might exist between those reacting with different monoclonal antibodies, the polypeptides were subjected to limited proteolytic cleavage analysis, similar to that described by Cleveland et al., (1977). This technique is more dependent upon higher orders of structure than on primary sequence and so could possibly detect differences not detected in the tryptic peptide analysis. Figure 2.7 shows some of the partial digestion products obtained by this method. It can be seen that by this method also there are no significant differences in the polypeptides immunoprecipitated by different monoclonal antibodies.

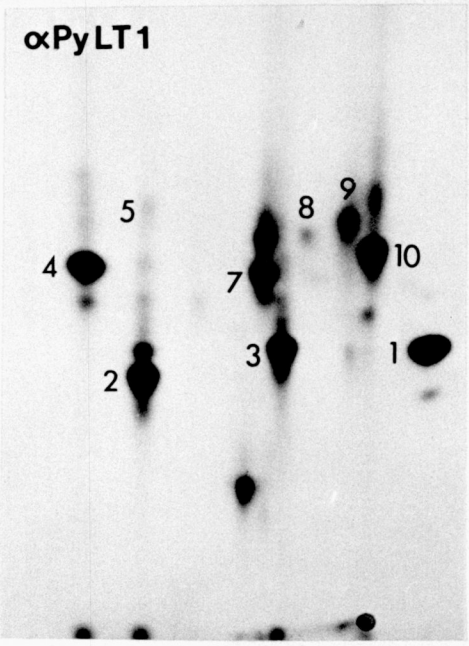
The large T-antigen, reported to separate into two MWtapp polypeptides on low percentage SDS-PAGE (Türler and Salomon, 1977, Ito et al., 1977a; Schaffhausen et al., 1978), was immunoprecipitated by the monoclonal antibodies and analysed by SDS-PAGE on a 5% gel (Figure 2.8). Both MWtapp forms of the protein were found to be immunoprecipitated by the five monoclonal antibodies that recognize the large T-antigen.

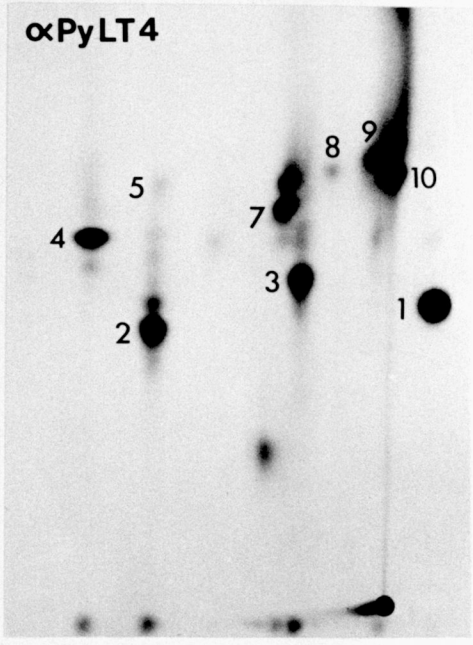
Figure 2.5 - Two Dimensional Tryptic Maps of 100Kd Polypeptides Immunoprecipitated by Monoclonal Antibodies.

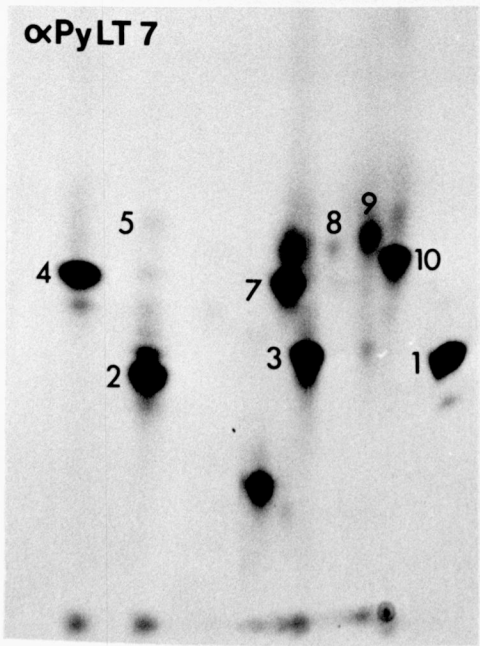
The (^{35}S)-methionine labelled 100Kd polypeptides immunoprecipitated by different monoclonal antibodies from wild type polyoma virus infected cells were isolated, digested with trypsin, the resulting peptides separated on t.l.c. plates, and localized by autoradiography (see Materials and Methods). Electrophoresis was carried out in the horizontal direction, and ascending chromatography in the vertical direction. The antibody used to immunoprecipitate the polypeptide is indicated in the top left corner of each separation. Where two antibodies are indicated, similar quantities of (^{35}S) labelled peptides were mixed following trypsin digestion. The peptides are numbered according to the system adopted by Ito, (1979).


αPyC1
5
8
9
10
7
4
3
2
1


αPyC4
8
9
10
5
7
4
3
2
1


αPyLT1
5
8
9
4
10
7
3
1
2


αPyLT4
8
9
5
10
4
7
3
1
2


αPyLT7
5
8
9
4
10
7
3
1
2

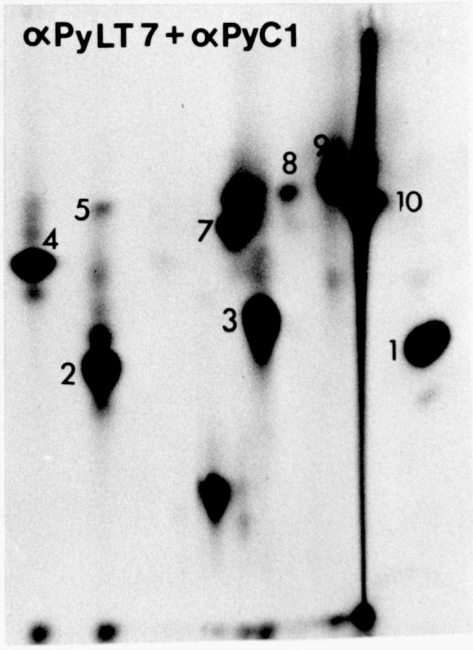
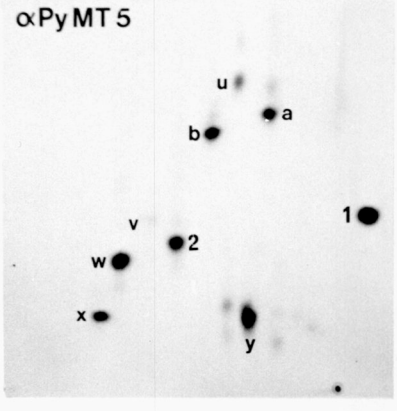
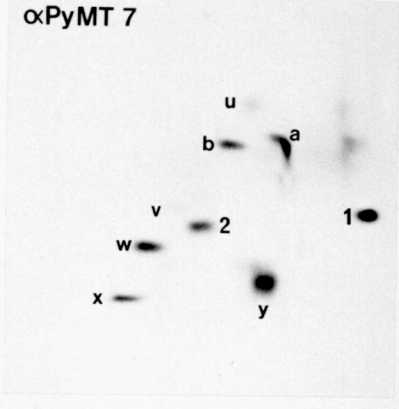
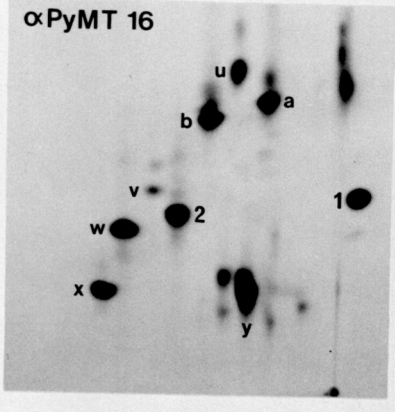

αPyLT7+αPyC1
8
9
5
10
4
7
3
1
2

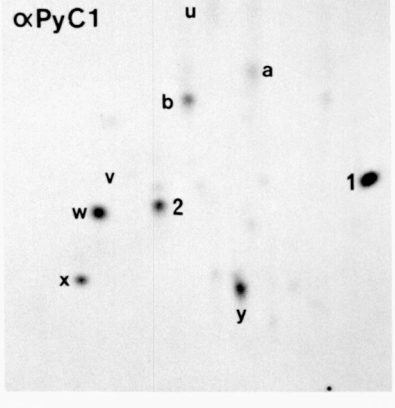
Figure 2.6 - Two Dimensional Tryptic Maps of 55Kd Polypeptides Immunoprecipitated by Monoclonal Antibodies.

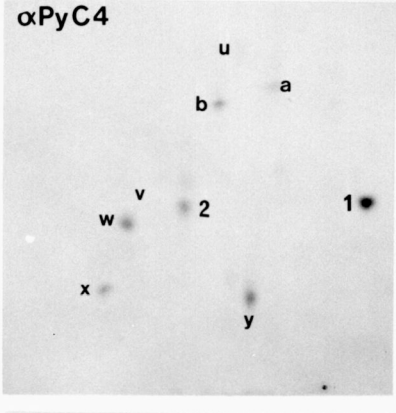
Tryptic peptides obtained from (^{35}S)-methionine labelled 55Kd polypeptides immunoprecipitated from wild type polyoma virus infected cells with various monoclonal antibodies (indicated in top left corner of each separation) and separated in two dimensions, as described in Figure 2.5. The peptide numbering system used is that of Ito, (1979).

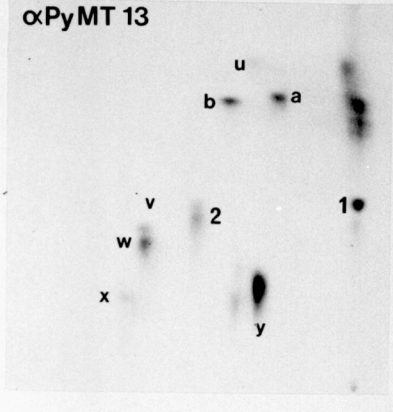

αPyMT 5
u
a
b
v
1
2
w
x
y

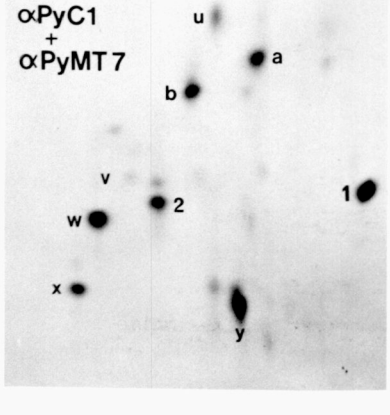

αPyMT 7
u
b
a
v
2
1
w
x
y


αPyMT 16
u
a
b
v
1
2
w
x
y


αPyC1
u
a
b
v
1
2
w
x
y


αPyC4
u
a
b
v
1
2
w
x
y


αPyMT 13
u
b
a
v
1
2
w
x
y


αPyC1
+
αPyMT 7
u
a
b
v
1
2
w
x
y

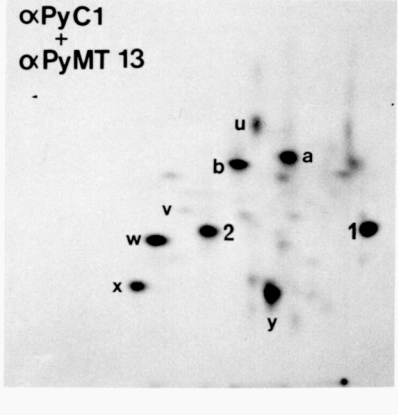

αPyC1
+
αPyMT 13
u
b
a
v
1
2
w
x
y

Figure 2.7 - Limited Proteolytic Cleavage Analysis of Polypeptides Immunoprecipitated by Monoclonal Antibodies.

(^{35}S)-Methionine labelled polypeptides were immunoprecipitated from lysates of wild type polyoma virus infected cells with various monoclonal antibodies. The polypeptides were then isolated, cleaved with S.aureus V8 protease, separated by SDS-PAGE on a 15% gel and localized by autoradiography (see Materials and Methods). The concentrations of protease used (in mg/ml) are indicated beneath each lane. The polypeptides analysed are :-

(A) the 100Kd polypeptide immunoprecipitated by αPyC1 antibody;

(B) the 100Kd polypeptide immunoprecipitated by αPyLT7 antibody;

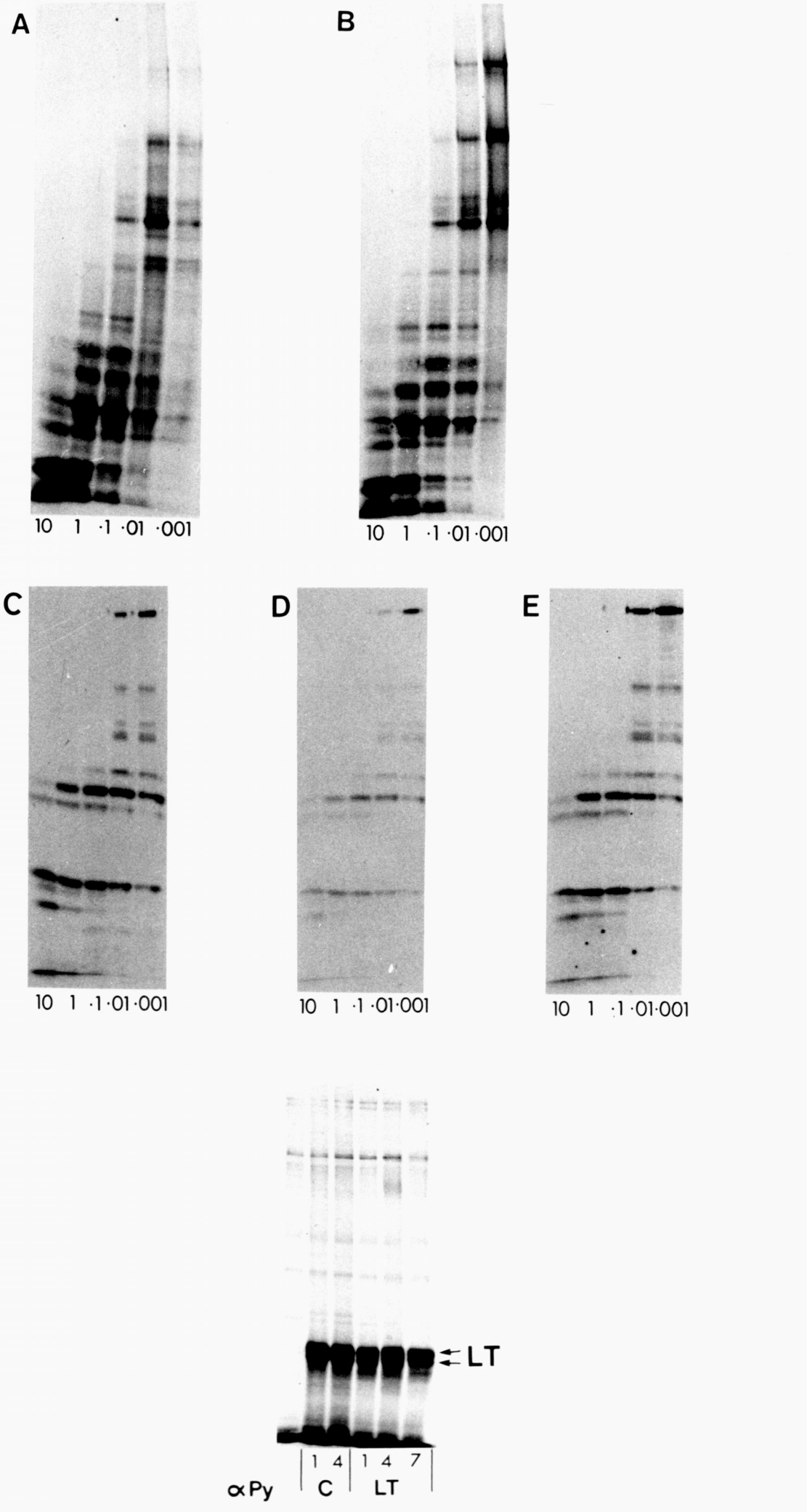
(C) the 55Kd polypeptide immunoprecipitated by αPyMT5 antibody;

(D) the 55Kd polypeptide immunoprecipitated with αPyMT7 antibody; and

(E) the 55Kd polypeptide immunoprecipitated by αPyMT13 antibody.

Figure 2.8 - Separation of Large T-Antigen Polypeptides on 5% SDS-PAGE.

The large T-antigens from (^{35}S)-methionine labelled, wild type virus infected cells were immunoprecipitated with monoclonal antibody-containing TCF, using MRC 0x12 as a second antibody. The polypeptides were then separated by SDS-PAGE on a 5% gel. The monoclonal antibody used is indicated beneath each lane. The position of the two MWtapp forms of large T-antigen are indicated on the right.


A
10 1 ·1 ·01 ·001
B
10 1 ·1 ·01 ·001
C
10 1 ·1 ·01 ·001
D
10 1 ·1 ·01 ·001
E
10 1 ·1 ·01 ·001
LT
1 4 1 4 7
∝Py
C
LT

Section 2.4 - Other Polypeptides Immunoprecipitated by the Monoclonal Antibodies.

The monoclonal antibodies immunoprecipitate polypeptides other than the known T-antigens, as indicated in Figure 2.9. Whenever large T-antigen is immunoprecipitated (whether by the ∝PyC or ∝PyLT antibodies), the viral capsid protein VP-1 (and probably VP-2) is also precipitated. This appears to be a quantitative phenomenon as similar quantities of large T-antigen and VP-1 are precipitated, despite the presence of much larger quantities of VP-1 in infected cell lysates. The fact that VP-1 is always immunoprecipitated together with the large T-antigen, and not with middle T-antigen, indicates that VP-1 is bound to the large T-antigen in a cell lysate. It has previously been reported (Walter and Deppert, 1975) that disulphide bonds are involved in maintaining the structure of polyoma virus. To investigate the role of disulphide bonds in the large T-antigen-VP-1 interaction, lytically infected cells labelled with (^{35}S)-methionine were lysed in Extraction Buffer containing 5mM dithiothreitol (DTT) and then immunoprecipitated. Figure 2.10 shows the polypeptides obtained in this manner. It can be seen that the specific interaction between large T-antigen and VP-1 has been disrupted by the presence of the reducing agent. The significance of disulphide bond formation between large T-antigen and VP-1, and whether this interaction is present within cells or occurs on cell lysis, is unclear. It is tempting to speculate that large T-antigen could be involved in the assembly of polyoma virions.

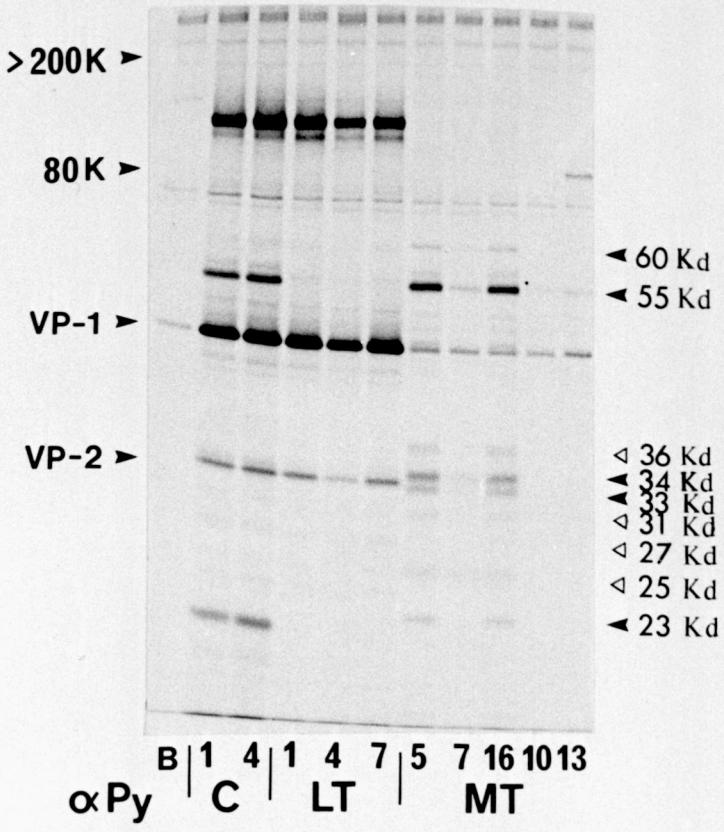
The polypeptides, other than the T-antigens, that are specifically immunoprecipitated by the monoclonal antibodies can be divided into two categories: (a) Polypeptides that are specifically immunoprecipitated by one monoclonal antibody only. There are two example of this. In one case, a polypeptide with a very high MWtapp on SDS-PAGE is present in ∝PyLT4 immunoprecipitates. This product has proved difficult to isolate free from other contaminating polypeptides and therefore has not been characterised in any detail. A second polypeptide, with a MWtapp of 80Kd, is specifically immunoprecipitated by the ∝PyMT13 monoclonal antibody. The two dimensional tryptic maps of this polypeptide, together with that from middle T-antigen, are shown in Figure 2.11. It can be seen that there is little similarity between the peptides obtained from these two species.

Figure 2.9 - Other Polypeptides Immunoprecipitated by Monoclonal Antibodies.

(^{35}S)-Methionine labelled T-antigens were immunoprecipitated by the ten monoclonal antibodies, as in Figure 2.4. The monoclonal antibodies used are indicated underneath. On the left are shown the position of the MWtapp 200Kd polypeptide (>200K) precipitated by ∝PyLT4 antibody, the MWtapp 80Kd polypeptide (80K) precipitated by ∝PyMT13 antibody, and the viral proteins VP-1 and VP-2. On the right are shown members of the "middle T-antigen family", together with their MWtapp. Those marked with a solid arrow are analysed by 2D tryptic maps in Figure 2.13.

Figure 2.10 - Effect of DTT on Large T-Antigen/VP-1 Interaction.

Cells infected with wild type polyoma virus were labelled with (^{35}S)-methionine, lysed and immunoprecipitated with ∝PyC1 antibody, lanes (a) and (b), or ∝PyLT1 antibody, lanes (c) and (d). Lanes (a) and (c) contained 5mM DTT in the Extraction Buffer used for lysis. The polypeptides were separated by SDS-PAGE on a 10% gel and detected by autoradiography. Indicated on the left are the positions of the viral capsid proteins, VP-1 and VP-2.


>200K
80K
VP-1
VP-2
60 Kd
55 Kd
36 Kd
34 Kd
33 Kd
31 Kd
27 Kd
25 Kd
23 Kd
B 1 4 1 4 7 5 7 16 10 13
αPy
C
LT
MT

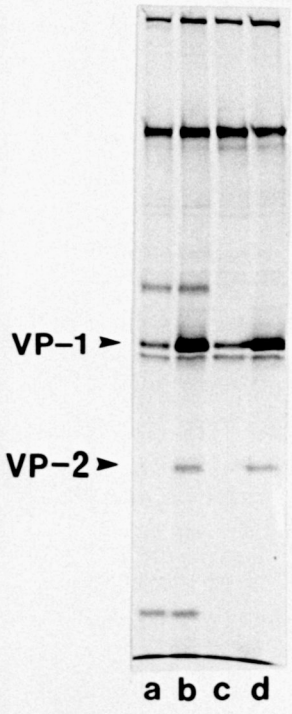
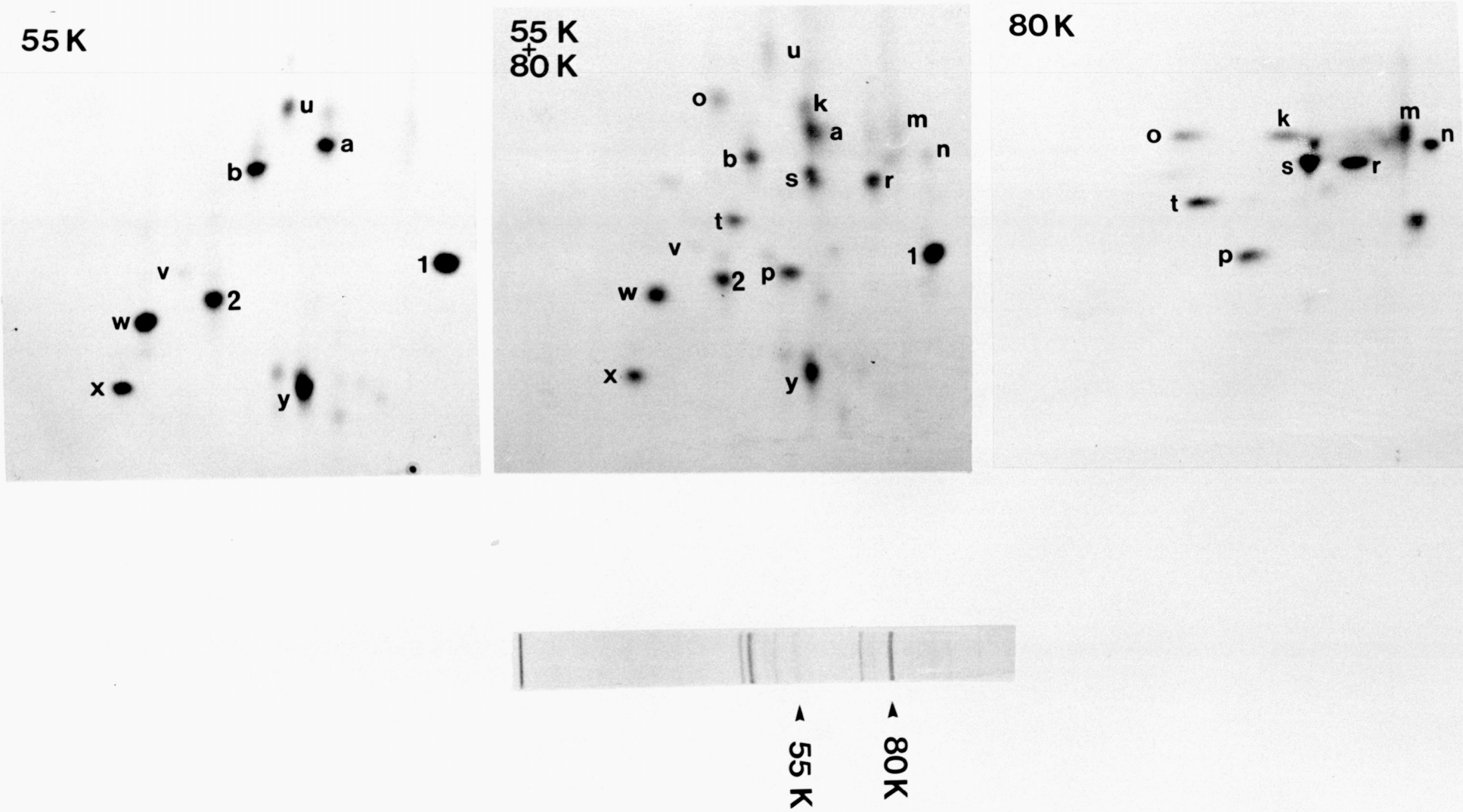

VP-1
VP-2
a b c d

Figure 2.11 - Two Dimensional Tryptic Maps of the Polypeptides Immunoprecipitated by the ∝PyMT13 Monoclonal Antibody.

The polypeptides immunoprecipitated by the ∝PyMT13 antibody from polyoma virus infected 3T6 Swiss Albino mouse cells, after separation by SDS-PAGE, are shown on the right. The position and MWtapp of the two polypeptides specifically immunoprecipitated are indicated on the right. The (^{35}S)-methionine labelled polypeptides were eluted from the SDS-PAGE, digested with trypsin, and separated on t.l.c. plates (see Materials and Methods). Tryptic maps shown are derived from the 55Kd and 80Kd polypeptides singly, or from a mixture of the two species.

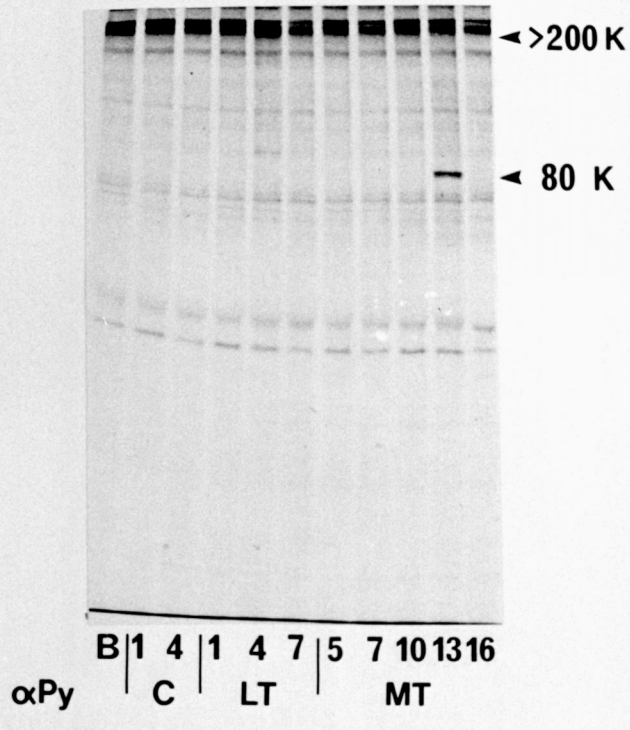

55 K
u
a
b
1
v
2
w
x
y
55 K
+
80 K
u
o
k
a
m
n
b
s
r
t
v
1
p
2
w
x
y
80 K
o
k
m
n
s
r
t
p
55 K
80K

Both of the novel polypeptides are also immunoprecipitated from uninfected cells, as shown in Figure 2.12, and so are neither virally-coded nor precipitated in association with a T-antigen molecule. Figure 2.12 also shows that no other monoclonal antibody immunoprecipitates any polypeptides from uninfected cells. The significance, therefore, of the immunoprecipitation of these two polypeptides by monoclonal antibodies directed against the T-antigens is unclear. They could have antigenic (and possibly functional) similarity to the T-antigens and so be host counterparts of the virally-coded enzymes. Alternatively, they could be a result of fortuitous cross reactions that are thought to occur with monoclonal antibodies. This phenomenon has also been observed with monoclonal antibodies to T-antigens of the related virus, SV40 (Lane and Hoeffler, 1980; Harlow et al., 1981; Crawford et al., 1982; reviewed in Lane and Koprowski 1982).

(b) Polypeptides that are specifically immunoprecipitated by a number of αPyMT antibodies. There are a number of these polypeptides; the easily identified ones are indicated in Figure 2.9. Although others are present, they have been difficult to isolate pure enough for further studies. Figure 2.13 show the 2D tryptic maps obtained from some of these polypeptides, together with appropriate mixes with peptides obtained from middle T-antigen. The spots are designated according to the numbers used by Ito, (1979) and have all been tentatively assigned, by a number of approaches (Hunter et al., 1979; Y. Ito, personal communication), to specific methionine-containing tryptic peptides that are predicted by the amino acid sequence of middle T-antigen (see Introduction, Figure I.4). Peptides 1 and 2 are present in all three T-antigens, so must be encoded before the large T-antigen mRNA splice site. Peptide 1 is probably the N-terminal peptide, Met. Asp. Arg, which is thought to be N-acetylated as it is resistant to amino-peptidase treatment (A. Mellor, personal communication). Peptide 2 is probably Met. Gln. Gln. Ala. Tyr. Lys. (amino acids numbers 30 to 35, inclusive, Figure I.4) as suggested by its mobility in the two dimensional separation. Peptides a and b are present in middle and small T-antigens only and so must be present between amino acids 79 and 191. Peptide a is probably Met. Pro. Leu. Thr. Cys. Leu. Val. Asn. Val. Lys. (amino acids numbers 107 to 116, inclusive), as it can also be labelled with (^{35}S) cysteine (Hunter et al., 1979), and peptide b is probably Met. Asn. Leu. Gly. Gly. Thr. Gly. Phe. Gln. Val. Arg. (amino acid numbers 71 to 81, inclusive).

Figure 2.12 - Polypeptides Immunoprecipated From Uninfected Cells By Monoclonal Antibodies.

3T6 Swiss Albino mouse cells were labelled with (^{35}S)-methionine for 4 hours at 37°C, the cells lysed, and polypeptides immunoprecipitated with the ten monoclonal antibody -containing TCF's, using MRC Ox12 as a second antibody (see Materials and Methods). The polypeptides were then separated by SDS-PAGE on a 10% gel and localized by autoradiography. The monoclonal antibody used is indicated underneath; track B contains polypeptides non-specifically immunoprecipitated by monoclonal antibody negative TCF. The positions of the >200Kd polypeptide immunoprecipitated by αPyLT4 antibody, and the 80Kd polypeptide immunoprecipitated by αPyMT13 antibody are indicated on the right.


>200 K
80 K
B 1 4 1 4 7 5 7 10 13 16
αPy
C
LT
MT

Peptides u, v, w, x and y are present in middle T-antigen only and therefore must be encoded after the mRNA splice site for this protein. Peptide w co-migrates with chemically synthesised Met. Arg. (Hunter et al., 1979; A Mellor, personal communication) (amino acids 372-373) and x with chemically synthesised Ala. His. Ser. Met. Gln. Arg. (Hunter et al., 1979) (amino acids 382 to 387, inclusive). The identifications of peptides u, v and y are less clear. However, u and v are altered as a result of the deletion in the mutant dl-8 (Ito et al., 1980), and so lie in the region that corresponds to the lesion in this mutant; it is thought that peptide y lies on the N-terminal side of this lesion (Hunter et al., 1979). The peptide x therefore probably lies 33 amino acids from the predicted C-terminus of middle T-antigen. Although these identifications are tentative, they do allow certain conclusion to be drawn from the 2D tryptic maps shown in Figure 2.13. Polypeptides smaller than the middle T-antigen all possess peptides w and x and then as their relative size increases, peptides u, v and y are also observed to be present. This suggests that these polypeptides all share significant sequence homology with middle T-antigen, containing the C-terminal end and varying amounts of sequence from the N-terminus. Peptides not found in middle T-antigen are observed to be present in some of the species. These could arise from novel N-termini of these molecules or could be a reflection of the existence of other polypeptide sequences not normally present in middle T-antigen. The 60 Kd MWtapp polypeptide provides an intriguing problem. It can be seen that peptides 1, 2 and b from the N terminus of middle T-antigen are present, as are peptides w and x from the C-terminal end. In addition, a number of peptides not found in any of the T-antigens are also present (Figure 2.13). The results from these analyses are represented schematically, relative to the polyoma virus genome, in Figure 2.14. The exact positions of N-termini of polypeptides smaller than middle T-antigen have not been determined, so the positions indicated are only estimates. It is not known whether the extra sequence present in the MWtapp 60Kd polypeptide is sited between the two sequences it shares with middle T-antigen, or whether some more complex arrangement exists. As this "family" of polypeptides is notpresent in uninfected cells (Figure 2.12) and the peptides are immunoprecipitated by a number of monoclonal antibodies, it appears likely that they are virally-coded and possess antigenic sites in common with middle T-antigen (as suggested by the tryptic peptide maps) and are not immunoprecipitated as a result of monoclonal cross reaction (see above).

Figure 2.13. - Two Dimensional Tryptic Maps of Members of the "Middle T-Antigen Family" of Polypeptides.

The polypeptides immunoprecipitated with αPyMT5 antibody from wild type polyoma virus infected 3T6 mouse cells are shown at the top left. Some members of the "middle T-antigen family" of polypeptides, together with their MWtapp on SDS-PAGE, are indicated on the right. These (^{35}S)-methionine-labelled polypeptides were eluted from the gel, digested with trypsin, and the peptides separated in two-dimensions on t.l.c. plates (see Materials and Methods). In the corner of each tryptic map is indicated the MWtapp of the appropriate polypeptide. The peptides from each species are separated either singly or mixed with those from middle T-antigen (55K) (underneath) to verify the identity of the peptides present. The peptide numbering system used is that of Ito, (1979).

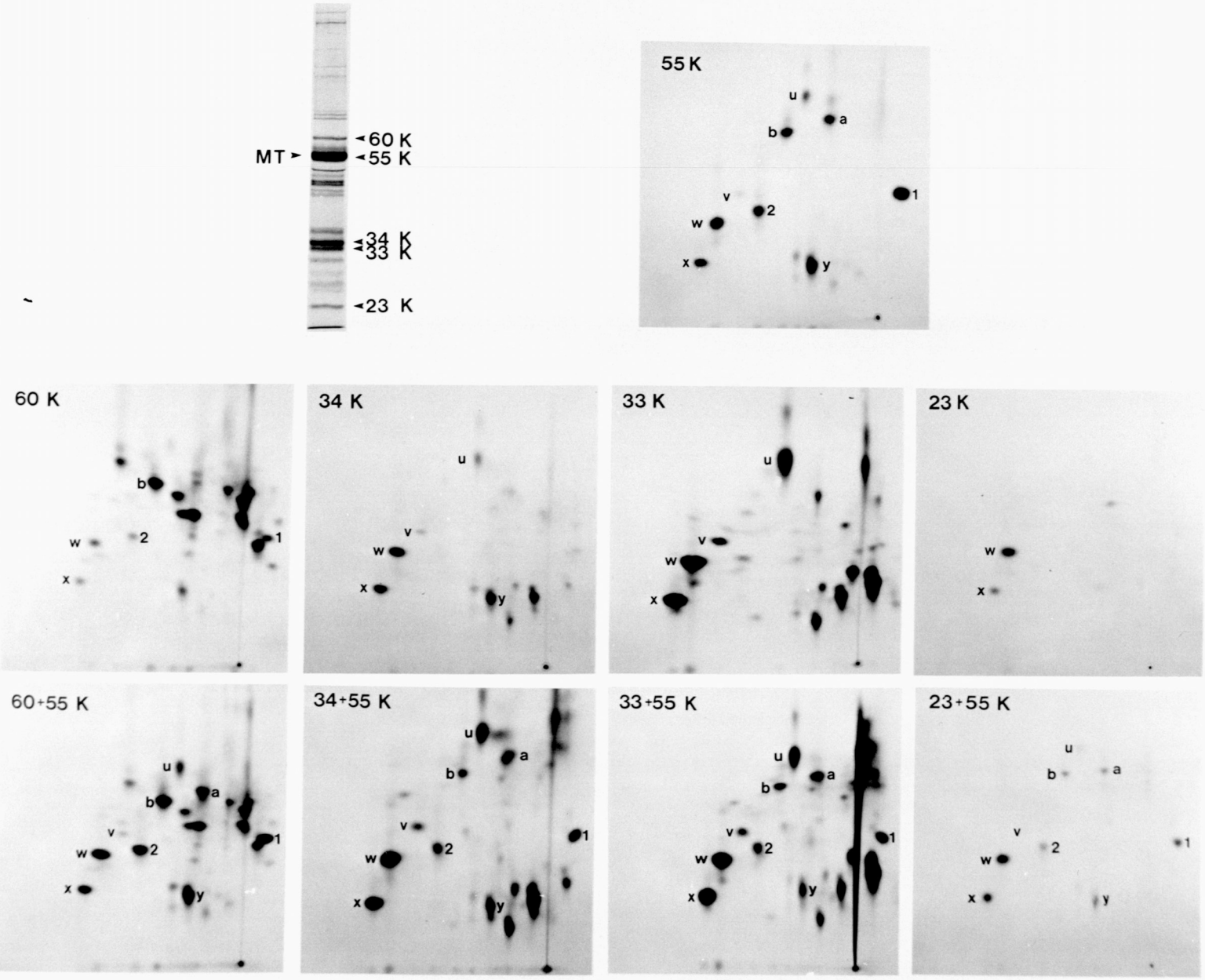
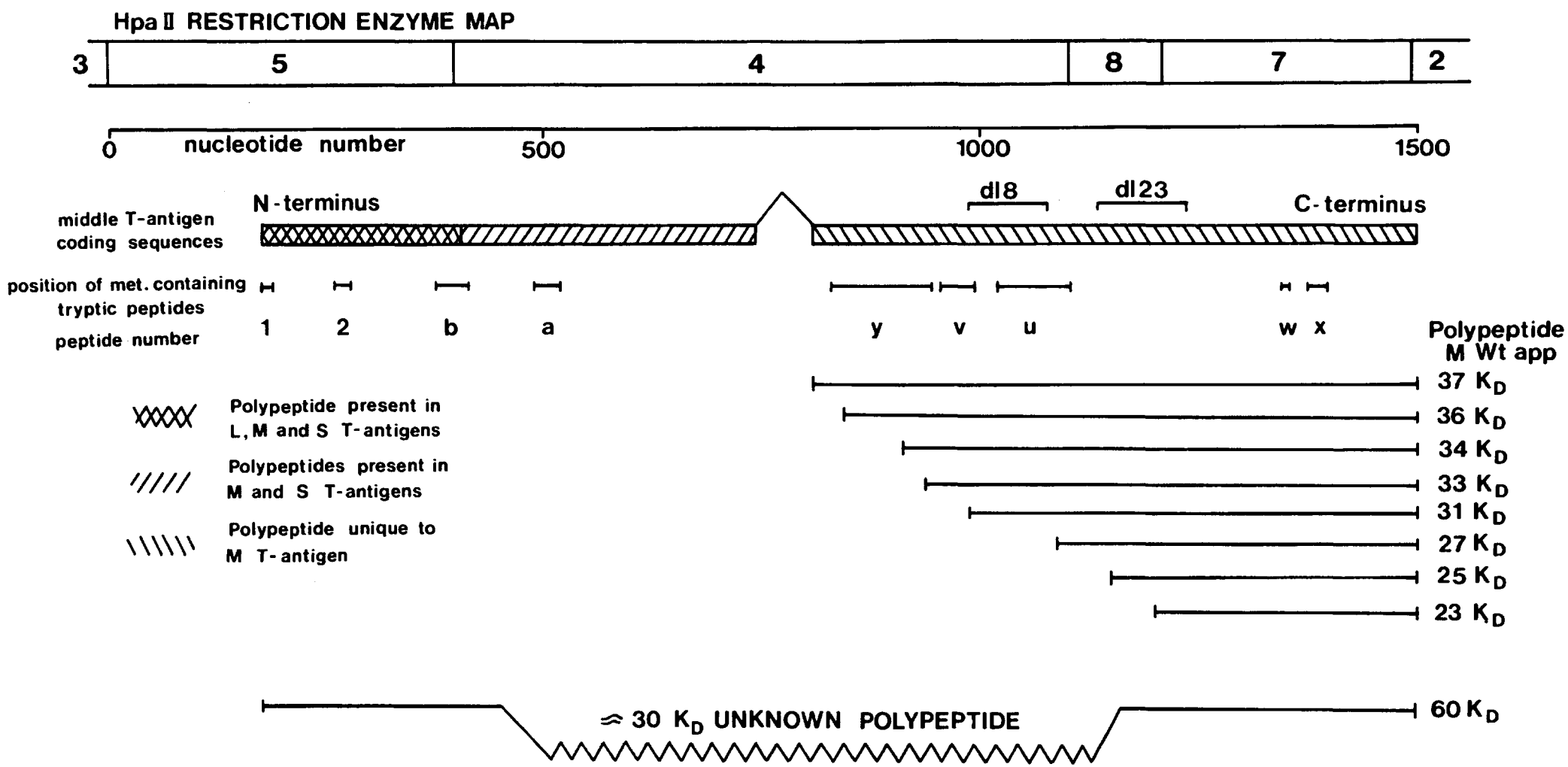

MT
60 K
55 K
34 K
33 K
23 K
55 K
u
a
b
v
1
2
w
x
y
60 K
34 K
33 K
23 K
60+55 K
34+55 K
33+55 K
23+55 K

Figure 2.14 - Schematic Representation of the "Middle T-Antigen Family" Coding Region.

The regions of the polyoma virus genome coding for the middle T-antigen are shown relative to a portion of the HpaII restriction enzyme map and the nucleotide sequence (see Figure I.3). The sequences deleted in the mutants dl-8 and dl-23 are indicated. The DNA sequences thought to encode the methionine containing tryptic peptides marked in Figure 2.13 are also indicated (see text). The sequence of middle T-antigen probably present in polypeptides of the "middle T-antigen family" are indicated below, together with their MWtapp. The exact position of the N- and C-termini have not been determined, and those shown are only approximations. The origin of the extra 30Kd of polypeptide sequence present in the MWtapp 60Kd polypeptide is unclear.

POLYOMA VIRUS GENOME



It is possible that the members of the "middle T-antigen family" are expressed at times during the lytic cycle different from the other T-antigens. To examine this possibility, infected 3T6 Swiss Albino mouse cells were radio-labelled and examined for the presence of these polypeptides at varying times after the infection; the results are shown in Figure 2.15. It can be seen that no significant difference occurs between the times of expression of any of the T-antigens (see Chapter One) or of the "middle T-antigen family" of polypeptides during the lytic cycle. The ratio between the various polypeptides remains similar with time. It is further possible that these polypeptides arise as a result of defective viral molecules present in the virus stock used to infect the cells. Were this the case it would be expected that the ratio of the polypeptides to the other T-antigens would vary as the multiplicity of infection is varied. This was not found to be so. Figure 2.16 shows the results of such an experiment where the viral stock used to infect the cells was diluted to varying extents; no significant alteration in the ratio between the various polypeptides was observed.

Section 2.5 - Discussion.

The protocol described in this Chapter (see Figure 2.1) has enabled monoclonal antibodies directed against the polyoma virus T-antigens to be produced. The antibodies isolated provide for the first time highly active, well-defined, and reproducible probes for the study of the T-antigens. The specificity of these antibodies has been examined by the immunoprecipitation of labelled proteins from the lysates of cells (see Figure 2.4) and have been positively confirmed to be the T-antigens of polyoma virus by 2D tryptic peptide mapping of immunoprecipitated polypeptides (see Figure 2.5 and 2.6). Using different antibodies from cloned hybridoma lines, specificity for either large, middle, or all three T-antigens can be obtained free from contaminating antibody activity, such as is often present in anti-tumour serum. No hybridoma lines that secrete antibodies specific for small T-antigen, or for the region common to small and middle T-antigen (but not large T-antigen), have yet been produced.

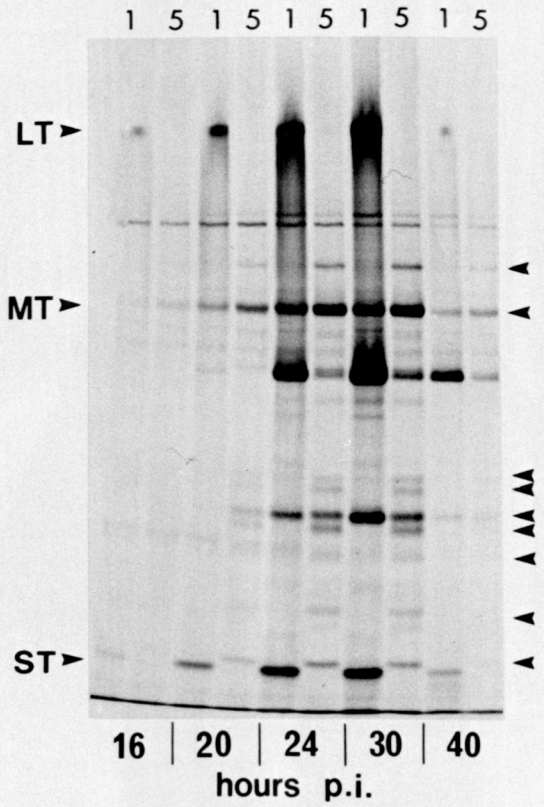
The monoclonal antibodies that have been produced, provide some interesting "side products", such as the high MWtapp polypeptide recognized by antibody αPyLT4 and the MWtapp 80Kd polypeptide recognized by αPyMT13 antibody.

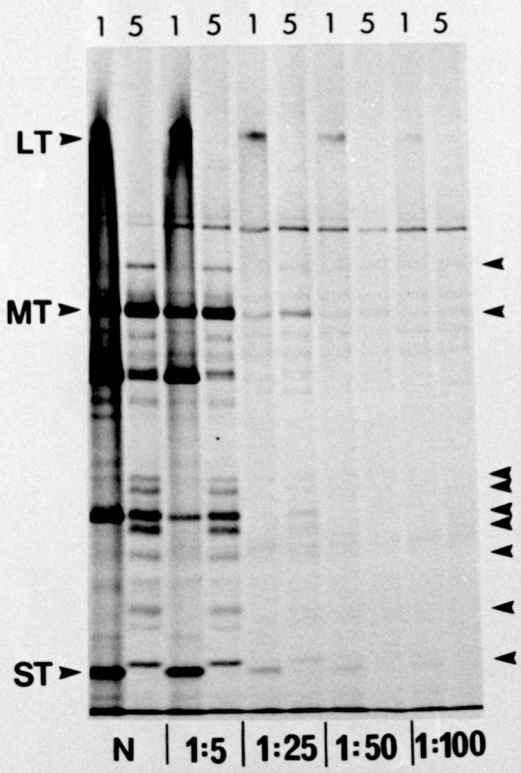
Figure 2.15 - Time Course of Synthesis of the "Middle T-Antigen Family" of Polypeptides.

3T6 Swiss Albino mouse cells were infected with wild type polyoma virus and labelled with (^{35}S)-methionine for 2 hours at 37°C at various times post infection (p.i., indicated underneath). The cells were then lysed and protein immunoprecipitated with either αPyC1 antibody (1) or αPyMT5 antibody (5) (indicated above each lane), using MRC Ox12 as a second antibody, separated by SDS-PAGE on a 10% gel, and detected by autoradiography. The positions of large (LT), middle (MT), and small (ST) T-antigens are indicated on the left, and the "middle T-antigen family" of polypeptides on the right.

Figure 2.16. Effect of Virus Dilution on Expression of "Middle T-Antigen Family" of Polypeptides.

3T6 Swiss Albino mouse cells were infected with wild type polyoma virus diluted with E4 to varying degrees (indicated underneath); N shows data with undiluted virus. The cells were labelled with (^{35}S)-methionine for 4 hours at 37°C, between 24 and 28 hours post infection. The cells were then lysed and proteins immunoprecipitated with either αPyC1 antibody (1) or αPyMT5 antibody (5), as indicated, using MRC Ox12 as a second antibody, separated by SDS-PAGE on a 10% gel, and detected by autoradiography. The positions of large (LT), middle (MT) and small (ST) T-antigens are indicated on the left, and the "middle T-antigen family" of polypeptides on the right.


1 5 1 5 1 5 1 5 1 5
LT
MT
ST
16 | 20 | 24 | 30 | 40
hours p.i.


1 5 1 5 1 5 1 5 1 5
LT
MT
ST
N | 1:5 | 1:25 | 1:50 | 1:100

Monoclonal antibodies can often recognize proteins not present in the immunizing preparation, as previously noted (Dulbecco et al., 1981; Pillemer and Weissman, 1981; Lane and Hoeffler, 1980; Harlow et al., 1981; Crawford et al., 1982). This phenomenon has been reviewed by Lane and Koprowski, (1982) and may be relevant to the cross reactions observed with αPyLT4 and αPyMT13 antibodies (see Figure 2.9). The possibility exists that they are fortuitous in that monoclonal antibodies may recognize small regions of primary amino acid sequence that are present in proteins of unrelated structure or function. Alternatively, the cross reactions could reflect shared antigenic and functional sites on a host counterpart of a viral function. Viral proteins must necessarily interact closely with host cell proteins in performing their functions. They achieve this by mimicking normal cellular protein interactions. It is therefore not inconceivable that these cross reactions are a reflection of viral functional resemblance to cellular proteins. It has previously been suggested that the region of the polyoma virus genome encoding parts of the middle and large T-antigens may be of host origin (Soeda et al., 1980b). The cross reactions could also be a reflection of the expression of polypeptides that have originally been derived from the host genome. The MWtapp 80Kd polypeptide and middle T-antigen lack peptide similarity (see Figure 2.11), so any relationship would presuppose that extensive evolutionary drift of the amino acid sequence of this region of DNA in the viral genome has occurred, while at the same time maintaining a small amount of antigenic (and perhaps functional) similarity to the "parental" polypeptide. To choose among these alternatives and elucidate the cause of the cross reaction will require further study. The availability of monoclonal antibodies that react with these polypeptides makes such study possible.

The αPyMT antibodies have revealed the presence in polyoma virus lytically infected cells of a series of polypeptides that are related to the middle T-antigen. This "middle T-antigen family" appears to possess varying amounts of amino acid sequences and antigenic sites from the C-terminal portion of the middle T-antigen (see Figure 2.13). The origin of these polypeptides remains to be determined. Those with a lower MWtapp than middle T-antigen could possibly arise by polyteolytic cleavage of the antigen, either within the cell, or upon cell lysis. Novel peptides not normally detected in the middle T-antigen would therefore arise as a result of the new N-termini or possibly also as a result of altered post-translational modifications. This proteolytic cleavage would necessarily have to be very specific, since the polypeptides observed are of a discrete MWtapp and reproducible. Moreover, if they arise after cell lysis, the

protease must not be susceptible to either PMSF or aprotinin. Proteolytic cleavage cannot account for the MWtapp 60Kd polypeptide which apparently is larger than the middle T-antigen. This polypeptide appears to have an unusual structure in that it possesses sequences from both the N-terminus and C-terminus of middle T-antigen, but not sequences normally present in between; in addition, it has tryptic peptides that are not present in any of the other T-antigens. It is not clear from where this extra polypeptide sequence originates although a number of possibilities exist. Defective viral DNA molecules are known to exist in polyoma virus stocks and it is possible that transcription and translation off a reorganised viral genome could account for the production of either the MWtapp 60Kd polypeptide or the whole "middle T-antigen family". However, no variation in the ratio of middle T-antigen to its related polypeptides is observed during the course of the polyoma virus infection (see Figure 2.15), or with different dilutions of the infecting virus (see Figure 2.16) as would probably be expected if defective viral DNA molecules were the cause of the expression of the "middle T-antigen family". Also none of the known, and characterised, defective viral DNA molecules could account for the structure of any of the "middle T-antigen family" of polypeptides (Griffin and Fried 1975, Lund et al., 1977 a and b). Another possibility is that the extra sequences present in the MWtapp 60Kd polypeptide are of host origin. This species might arise from the integration of polyoma virus DNA into its host genome during the lytic infection (an occurrence postulated from data of Türler, 1977) followed by transcription and translation of the join viral-host sequences or, alternatively, by the integration of an extrachromosomal piece of host DNA, similar to bacterial transposon sequences, into the polyoma virus genome. As the same polypeptides are expressed with each viral infection, the events responsible for any of the "middle T-antigen family" must be extremely specific and occur on every infection. Yet another explanation for the presence of the "middle T-antigen family" of polypeptides is aberrant RNA transcription and processing (possibly even intermolecular splicing of RNA molecules), or protein translation.

Further investigation is obviously required to determine the origin and significance of the family of polypeptides related to middle T-antigen, as none of the obvious explanations can account for all of the observed facts, or be positively discounted. Indeed it is not clear if a single mechanism is responsible for the presence of all the members of the "middle T-antigen family". Neither is it clear why the middle T-antigen sequences, and not those of large T-antigen, should be expressed in this manner. This series of polypeptides was not previously detected, presumably because of the numerous other polypeptides that are immunoprecipitated by anti-tumour sera, which make the identification of "genuine" minor polypeptides difficult. Polypeptides in the MWtapp 30-40Kd region have been reported by Hutchinson et al., (1978) and by Ito and Spurr (1979) and probably represent similar species. The specificity provided by the monoclonal antibodies have allowed this series of polypeptides to be identified and characterised properly for the first time.

CHAPTER THREE

Section 3.1 - Aims

The monoclonal antibodies described in Chapter Two have provided for the first time very specific probes for the T-antigens of polyoma virus. They have been shown to react with the T-antigens and in some cases, with other polypeptides. For further investigations of functions of the antigens, it will be of obvious advantage to have probes for different regions of the proteins. The monoclonal antibodies can provide such probes. To determine the region of the T-antigen molecule to which each of the ten monoclonal antibodies described in Chapter Two binds, the polypeptides expressed by well-defined deletion mutants of polyoma virus, as well as some defined truncated T-antigens expressed in several polyoma virus transformed cell lines, were immunoprecipitated with each of the antibodies. It has been observed by Harlow et al., (1981) that a negative reaction in such experiments does not necessarily indicate that the antigenic site has been deleted, since it is possible that the site could be masked by folding of the polypeptide chain in an unusual manner in the truncated polypeptide, or the deleted region of the polypeptide may have been required for expression of an antigenic site elsewhere on the molecule. Therefore, negative data have to be viewed with caution. Positive data could provide significant information and it was anticipated that the reaction of the monoclonal antibodies with the truncated T-antigens would help characterise the binding site of the antibodies on the wild type T-antigen molecules.

It has been reported (Gurney et al., 1980; Harlow et al., 1981) that the large T-antigen(s) of SV40 can be sub-divided into classes according to its ability to be recognized by some monoclonal antibodies. The antibodies described in Chapter Two also appear to immunoprecipitate varying quantities of T-antigen molecules, particularly of the middle T-antigen. To examine this further, a "cascade" immunoprecipitation approach was adopted in order to determine whether various antigenic classes of T-antigen exist. The physical properties of the T-antigens recognized by individual monoclonal antibodies were also studied to determine whether differences exist among varying forms of T-antigens.

One of the aims in producing monoclonal antibodies to T-antigens was to enable reasonable quantities of these proteins to be purified. The effectiveness of the antibodies in achieving this was investigated by an immunoprecipitation technique. For conventional protein purification, the advantage of a quick, accurate assay for individual T-antigens is obvious. The monoclonal antibodies were therefore employed in developing a radio-immuno assay for large and middle T-antigens. For many of the proposed future uses of the antibodies a knowledge of the physical properties of the molecule produced by individual hybridoma cells is desirable. To determine the immunoglobulin subclass to which the antibodies belong, Ouchterlony double diffusion assays against type specific antisera were carried out.

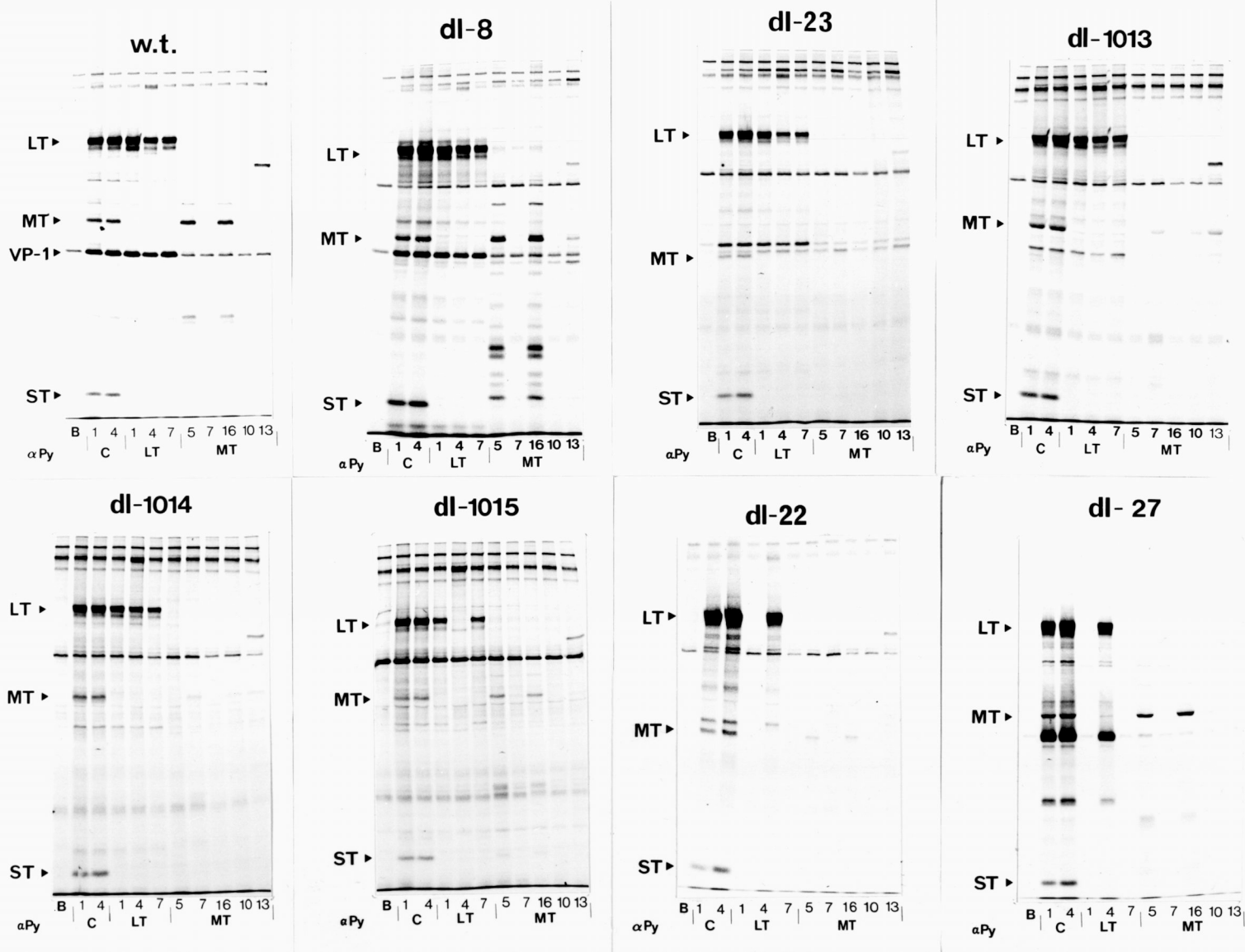
All of the experiments described in this Chapter characterise the monoclonal antibodies further in regard to their physical properties and the properties of the antigen that they recognize. An understanding of these properties is important for the future use of the antibodies as it is necessary to know which forms of T-antigen are being studied with individual monoclonal antibodies and how they are being bound.

Section 3.2 - Monoclonal Antibody Binding to Truncated T-Antigens.

3T6 Swiss Albino mouse cells were infected with various deletion mutants of polyoma virus (described in Chapter One, and Figure I.2), labelled with (^{35}S)-methionine, lysed and immunoprecipitated with the ten monoclonal antibodies described in Chapter Two. Figure 3.1 shows the SDS-PAGE separations of the polypeptides obtained in this manner. It can be seen that the antibodies vary in their ability to recognize and immunoprecipitate different truncated forms of the T-antigens. There are obvious difficulties associated with detecting a reaction with αPyMT10 and αPyMT13 antibodies since they recognize such low levels of middle T-antigen. With certain deletion mutants, where the non-specific precipitation is high relative to the quantity of middle T-antigen expressed, it becomes impossible to determine whether a reaction has occurred or not. Therefore, only where a clear reaction has occurred are the αPyMT10 and αPyMT13 antibody reactions considered.

Figure 3.1 - Immunoprecipitation of Truncated T-Antigens with Monoclonal Antibodies

3T6 Swiss Albino mouse cells were infected with various deletion mutants of polyoma virus and labelled with (^{35}S)-methionine for 4 hours at 37°C between 24 and 28 hours post infection. The cells were then lysed and immunoprecipitated with monoclonal antibody-containing TCF, using MRC Ox12 as a second antibody. The polypeptides were then separated by SDS-PAGE on a 10% gel and detected by autoradiography. The specific mutant virus used is indicated above each gel, w.t. being the wild type A2 strain of polyoma virus, and the monoclonal antibody indicated below each lane. Track B contains the polypeptides non-specifically immunoprecipitated by monoclonal antibody negative TCF. The positions of the large (LT), middle (MT) and small (ST) T-antigens expressed by these mutants are indicated on the left of each gel.


w.t.
dl-8
dl-23
dl-1013
dl-1014
dl-1015
dl-22
dl- 27
LT
MT
VP-1
ST
B 1 4 1 4 7 5 7 16 10 13
αPy
C
LT
MT

With the caution noted in Section 3.1 concerning negative reactions, a number of features can be seen in the immunoprecipitations. The αPyC antibodies, as expected, react with all the truncated T-antigens; the deletions are sited away from the N-termini of the proteins where the binding site of these antibodies must lie. The αPyLT antibodies must recognize at least two separate antigenic sites since αPyLT1 and αPyLT7 antibodies do not react with the truncated large T-antigen expressed by mutants dl-22 and dl-27, whereas αPyLT4 does; the reverse is observed in the case of mutant dl-1015. The αPyMT antibodies similarly show at least two different antigenic sites, with αPyMT7 antibodies recognizing the truncated middle T-antigen expressed by mutant dl-23 and not that of mutant dl-8, and the reverse situation being observed with αPyMT5 and αPyMT16 antibodies. It is interesting that the loss of only three amino acids from the middle T-antigen polypeptide expressed by mutant dl-1014 results in the loss of expression of the antigenic site recognized by αPyMT5 and αPyMT16 antibodies. It is probably the absence of these same amino acids that results in the failure of these antibodies to react with the truncated middle T-antigen encoded by mutants dl-1013 and dl-23. The absence of any reaction between αPyMT7 antibody and the truncated middle T-antigen encoded by mutants dl-8 and dl-27 is also interesting in that the deletions in these two mutants do not overlap. This could mean that the antigenic site for the αPyMT7 antibody spans the region where the two deletions meet, or that this region is necessary for the expression of an antigenic site which is elsewhere on the T-antigen molecule.

Figure 3.1 also shows some other notable features in that the MWtapp 80Kd polypeptide is immunoprecipitated in an unaltered form by αPyMT13 antibody regardless of the deletion mutant used to infect the cells. Some members of the "middle T-antigen family" of polypeptides are visible in the immunoprecipitates. It can be seen that some of the polypeptides related to, but smaller than, the middle T-antigen are also further truncated by the deletions in some of the mutants, particularly mutants dl-8 and dl-27, which clearly demonstrates that they are virally-coded species. (The MWtapp 23Kd polypeptide, unaffected by these deletions in the mutants, probably contains sequences C-terminal to them, see Chapter Two.) The MWtapp 60Kd polypeptide is also visible in immunoprecipitates from dl-8 and dl-27 mutant infected cells and appears to be unaltered in MWtapp, so it seems likely that it does not contain the region of the polypeptide deleted in these mutants, a fact consistent with the 2D tryptic peptide analysis shown in Chapter Two.

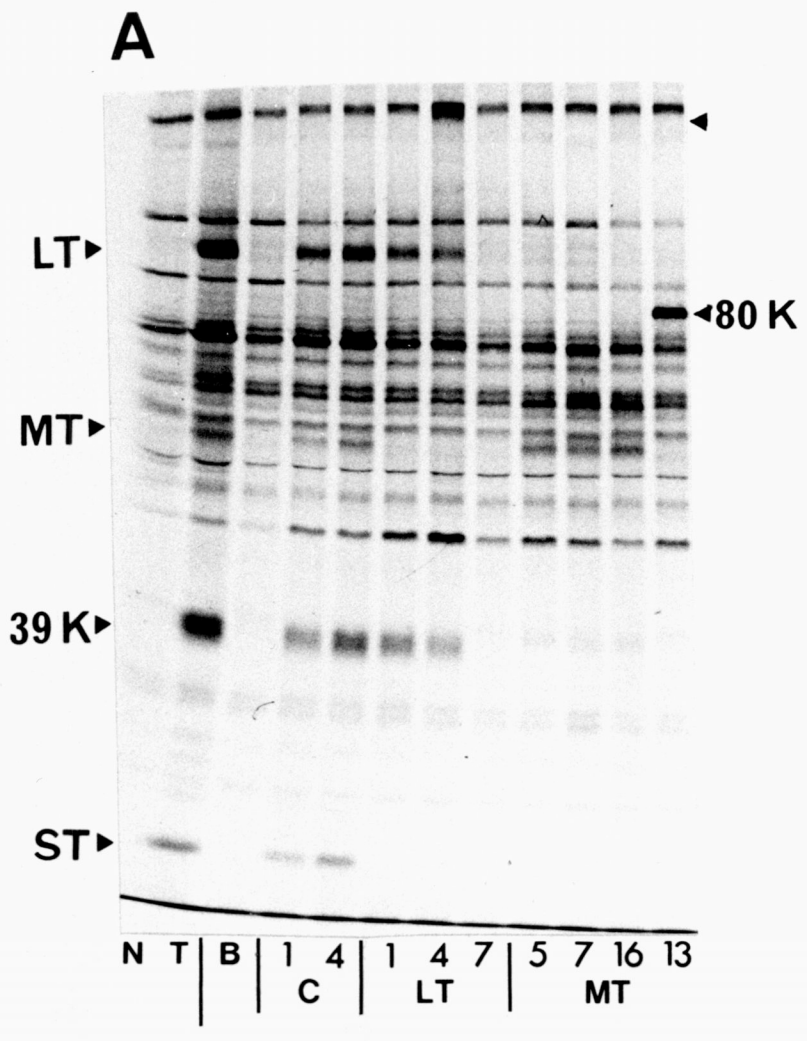
To characterize further the monoclonal antibody binding sites on the T-antigen molecules, viral proteins expressed by two well-defined polyoma virus transformed rat cell lines were immunoprecipitated with the monoclonal antibodies. The PyREWA5/T1A1 transformed cell line was initially used in the generation of anti-T serum; it was derived by Ito et al., (1977b) from primary Wistar rat embryo cells, transformed with wild type polyoma virus and selected for high tumourigenicity. This line has been shown to express normal-sized large, middle and small T-antigens, and in addition, a MWtapp 39Kd polypeptide which is related to large T-antigen, and probably contains the N-terminal region of the molecule (Ito and Spurr, 1979). The BE-1 line is derived from Rat-1 cells that were transformed with a 35% fragment of the polyoma virus genome and selected for growth in soft agar (see Appendix I). This line expresses normal-sized middle and small T-antigens, together with a MWtapp 34Kd polypeptide that contains the N-terminus of the large T-antigen. Figure 3.2 shows the (^{35}S)-methionine labelled polypeptides immunoprecipitated by the monoclonal antibodies from lysates of these two cell lines. It can be seen that the αPyC antibodies recognize all of the T-antigens immunoprecipitated by the anti-T serum. The αPyMT antibodies immunoprecipitate the middle T-antigen only. The MWtapp 80Kd polypeptide is also immunoprecipitated from both lines with the αPyMT13 antibody and the species expressed in rat cells appears to be of the same MWtapp as that from mouse cells. The full-sized large T-antigen expressed by PyREWA5/T1A1 cells is recognized by both αPyLT1 and αPyLT4 antibodies but, interestingly, not by the αPyLT7 antibody. The reason for this is not clear. However, the apparent absence of the antigenic site could be due to a mutation in the normal large T-antigen coding sequence, or to an abnormal post-translational modification reaction (either a lack of modification, or the occurrence of one not normally found in lytically infected cells) in the non-permissive rat cells. The MWtapp 39Kd polypeptide expressed in PyREWA5/T1A1 cells is again recognized by αPyLT1 and αPyLT4 antibodies, but not by αPyLT7. The αPyLT1 antibody is the only member of the αPyLT series to recognize the MWtapp 34Kd polypeptide expressed by BE-1 cells. This means that the binding site for the αPyLT1 antibody must lie in the N-terminal 35% of the large T-antigen molecule, and that for αPyLT4 in the N-terminal 40%. These binding data are summarized in Table 3.1.

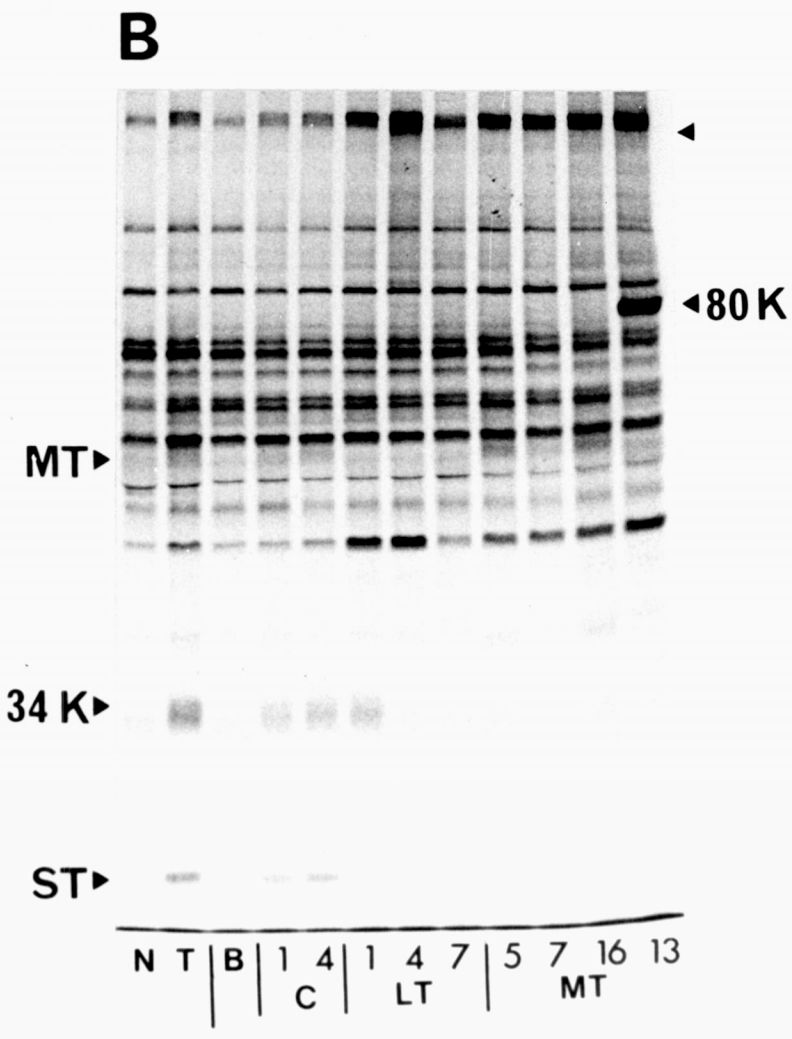
Figure 3.2 - Immunoprecipitation of the T-Antigens Expressed by Two Polyoma Virus Transformed Rat Cell Lines By Monoclonal Antibodies.

Semi-confluent transformed cells were labelled with (^{35}S)-methionine for 16 hours at 32°C, as described in Materials and Methods. The cells were then lysed and proteins immunoprecipitated with monoclonal antibody-containing TCF, using MRC Ox12 as a second antibody. The polypeptides were then separated by SDS-PAGE on a 10% gel and detected by autoradiography. The monoclonal antibody used is indicated beneath each lane; track N contains the polypeptides immunoprecipitated by non-immune rat serum, T, those immunoprecipated with anti-T serum, and B those immunoprecipitated by monoclonal antibody negative TCF. The position of large (LT), middle (MT) and small T-antigens (ST) are indicated on the left, together with the position and MWtapp of the truncated large T antigen also expressed by these cells (see text). On the left are indicated the positions of the MWtapp 80Kd polypeptide recognized by αPyMT13 antibody and the 200Kd polypeptide recognized by αPyLT4 antibody (unmarked arrow).

A. Polypeptides immunoprecipitated from lysates of PyREWA5/T1A1 cells.

B. Polypeptides immunoprecipitated from lysates of BE-1 cells.


A
LT
MT
39 K
ST
80 K
N T B 1 4 1 4 7 5 7 16 13
C
LT
MT


B
MT
34 K
ST
80 K
N T B 1 4 1 4 7 5 7 16 13
C
LT
MT

Section 3.3 - Antigenic Sub-Populations of the T-Antigens.

The quantity of the T-antigens (particularly the middle T-antigen) immunoprecipitated by different monoclonal antibodies was observed to vary considerably. To eliminate the possibility that the low quantities of T-antigen precipitated by some monoclonal antibodies was due to a low antibody-antigen affinity a "cascade" approach, similar to that developed by Harlow et al., (1981), was used. This involves sequential immunoprecipitations, conducted on the same cell lysate until no further antigen is precipitated on addition of a further quantity of antibody. Any T-antigen molecules remaining in the lysate are then precipiated by the addition of anti-T serum. Figure 3.3 shows the polypeptides that were precipitated in this manner by each of the ten monoclonal antibodies. For each antibody, five sequential immunoprecipitations were carried out with the specific antibody being examined, followed by one with anti-T serum. In each case it can be seen that by the fourth or fifth immunoprecipitation, no significant quantity of T-antigen is being precipitated. Therefore, the anti-T serum track represents a reliable estimate of the T-antigen molecules that remain unbound by the monoclonal antibody.

It can be seen in Figure 3.3 that both αPyC1 and αPyC4 antibodies react with all of the detectable (^{35}S)-methionine labelled T-antigens, there being no T-antigen precipitated by anti-T serum from the lysate after reaction with either of these monoclonal antibodies. (For each of the other antibodies, the antigens that were not recognized by the monoclonal antibody were left unaffected by the "cascade" reaction and available for reaction with anti-T serum. For example, normal levels of small and middle T-antigen remained in lysates after reaction with the αPyLT antibodies, etcetera). αPyLT4 antibody was found to immunoprecipitate all of the large T-antigen, whereas αPyLT1 and αPyLT7 antibodies left a small amount of the large T-antigen molecules unrecognized. The amount of large T-antigen not recognized by αPyLT1 antibodies was always a small proportion only of the total T-antigen population. However, that remaining after αPyLT7 precipitation was observed to vary considerably between different lysates, often approaching 50% of the large T-antigen. The reason behind this variability is not clear, but could reflect the fact, as suggested in Section 3.2, that αPyLT7 antibodies recognize a modified form of the large T-antigen, the extent of the modification varying among lysates.

Table 3.1. Summary of Monoclonal Antibody Binding to Truncated T-Antigens.

T-Antigen Source	Large T-Antigen			Middle T-Antigen				
	αPyLT1	αPyLT4	αPyLT7	αPyMT5	αPyMT7	αPyMT16	αPyMT10	αPyMT13
Wild Type Virus	+	+	+	+	+	+	+	+
Mutant dl-8	+	+	+	+	-	+		+
Mutant dl-23	+	+	+	-	+	-		
Mutant dl-22	-	+	-	+	-	+	+	+
Mutant dl-27	-	+	-	+	-	+	+	+
Mutant dl-1013	+	+	+	-	+	-	+	+
Mutant dl-1014	+	+	+	-	+	-	+	+
Mutant dl-1015	+	-	+	+	+	+		+
PyREWA5/T1A1:100 Kd	+	+	-					
PyREWA5/T1A1: 39 Kd	+	+	-					
BE-1 : 34 Kd	+	-	-					

Figure 3.3 - "Cascade" Immunoprecipitations of the T-Antigens Using Monoclonal Antibodies.

3T6 Swiss Albino mouse cells were infected with wild type polyoma virus, and labelled with (^{35}S)-methionine for 4 hours at 37°C between 24 and 28 hours post infection. The cells were then lysed and subjected to sequential immunoprecipitations with a monoclonal antibody-containing TCF, using MRC Ox12 as a second antibody, as described in Materials and Methods. Any remaining T-antigens were then immunoprecipitated with anti-T serum and are shown in lanes marked T (underneath). The polypeptides were separated by SDS-PAGE on a 10% gel and localized by autoradiography. On the left of each gel, the positions of the T-antigens immunoprecipitated by the first antibody are marked (solid arrows), and on the right, the positions of those immunoprecipitated by the anti-T serum are marked (open arrows).

The monoclonal antibody used initially for immunoprecipitation is:

A αPyC1

B αPyC4

C αPyLT1

D αPyLT4

E αPyLT7

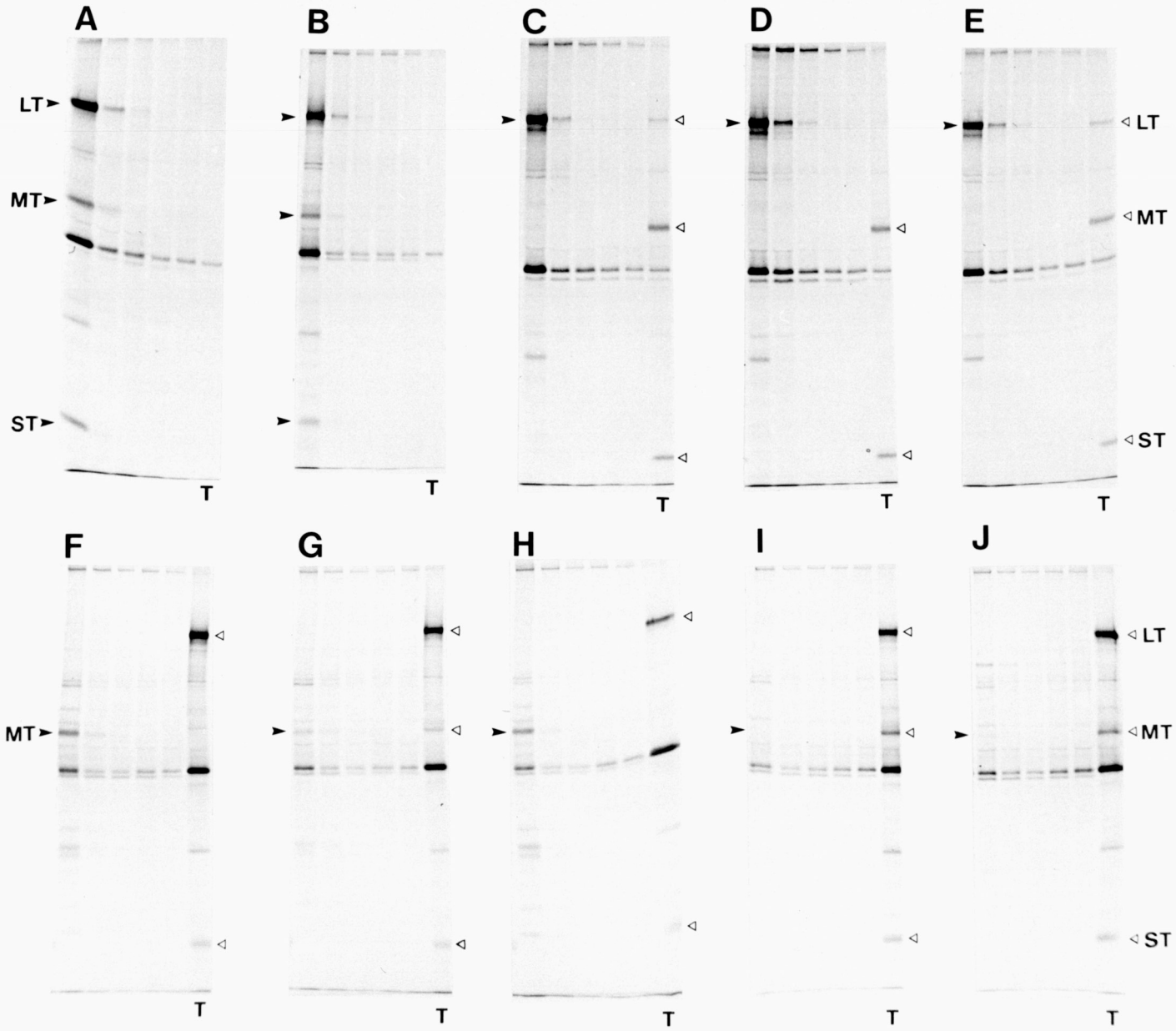
F αPyMT5

G αPyMT7

H αPyMT16

I αPyMT10

J αPyMT13


A
B
C
D
E
LT
MT
ST
T
F
G
H
I
J

∝PyMT5 and ∝PyMT16 antibodies are shown, Figure 3.3, to recognise all of the detectable middle T-antigen from lysates of wild type polyoma virus infected 3T6 Swiss Albino cells labelled with (^{35}S)-methionine. ∝PyMT7 antibodies, however, appear to immunoprecipitate only 50% of the middle T-antigen molecules. Similarly, there is a considerable fraction of the middle T-antigen that is not immunoprecipitated by ∝PyMT10 and ∝PyMT13 antibodies. The fraction recognised by these two antibodies is quite small and the simplest conclusion is that they bind only minor populations of this antigen. As support for the concept that this is not a question of low antibody-antigen affinities but that these monoclonal antibodies are only detecting antigenic sub-populations of the total available T-antigen molecules in a cell lysate, the precipitations can be seen to reach an end point where no further detectable T-antigen is precipitated on the further addition of antibody.

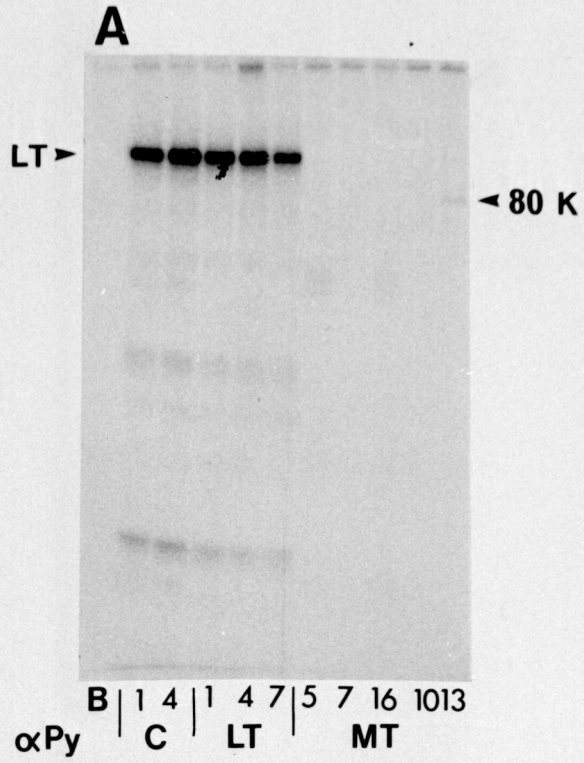
The differences among the antigens, that give rise to specific sub-groups of large and middle T-antigens which are recognised by individual monoclonal antibodies, were investigated by a number of methods. The modification of the T-antigens to varying extents could easily account for various antigenic forms of the proteins. The T-antigens are known to contain phosphate groups covalently linked to them. To investigate the phosphorylation state of the proteins recognized by the various monoclonal antibodies, wild type polyoma virus infected 3T6 Swiss Albino mouse cells and the PyREWA5/T1A1 transformed rat cell line were labelled with (^{32}P)-orthophosphate, lysed, and proteins immunoprecipitated with the ten monoclonal antibodies, see Figure 3.4. From these data, no significant difference appears to exist in terms of phosphate content between the T-antigens immunoprecipitated with different monoclonal antibodies. The large T-antigen is heavily phosphorylated as previously reported (Ito et al., 1977a; Schaffhausen et al., 1978) and only small amounts of phosphate are incorporated into middle T-antigen (Schaffhausen et al., 1978; Schaffhausen and Benjamin 1979; Smith et al., 1979). Interestingly, the MWtapp 80Kd polypeptide immunoprecipitated by ∝PyMT13 antibody is phosphorylated to a low extent. Similar patterns are seen with polypeptides immunoprecipitated from PyREWA5/T1A1 cells. Here again, the ∝PyLT7 antibody does not appear to immunoprecipitate the large T-antigen.

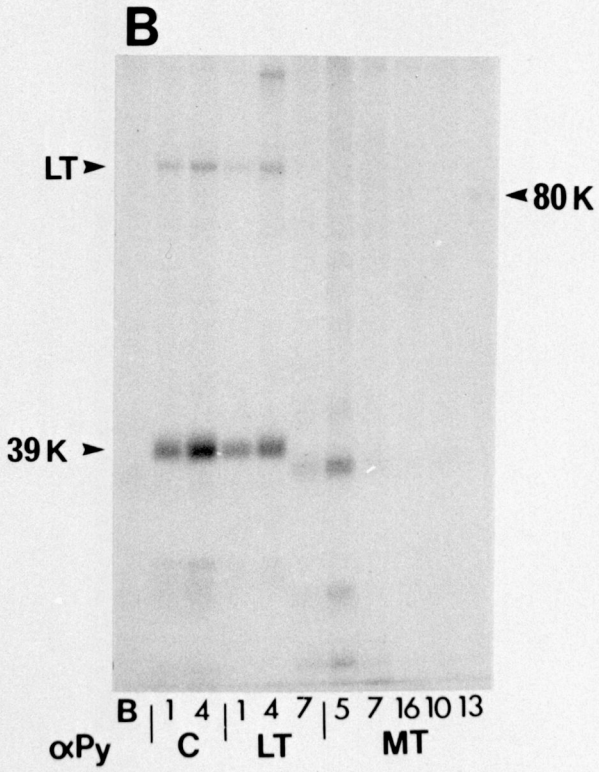
Figure 3.4 - Immunoprecipitation of Phosphorylated T-Antigens with Monoclonal Antibodies.

Cells were labelled with (^{32}P)-orthophosphate, lysed, and proteins immunoprecipitated with monoclonal antibody-containing TCF, using MRC Ox12 as a second antibody, as described in Materials and Methods. The polypeptides were then separated by SDS-PAGE on a 10% gel and detected by autoradiography. The monoclonal antibody used is indicated beneath each lane; track B contains polypeptides immunoprecipitated by monoclonal antibody negative TCF.

A. Polypeptides from wild type polyoma virus infected 3T6 Swiss Albino mouse cells. The position of large T-antigen (LT) is indicated on the left, and the MWtapp 80Kd (80K) polypeptide immunoprecipitated by αPyMT13 antibody on the right.

B. Polypeptides from PyREWA5/T1A1 transformed rat cells. The positions of large T-antigen (LT) and the MWtapp 39Kd (39K) polypeptide related to large T-antigen are indicated on the left. On the right is indicated the position of the MWtapp 80Kd (80K) polypeptide immunoprecipitated by αPyMT13 antibody.


A
LT
80 K
B 1 4 1 4 7 5 7 16 1013
αPy C LT MT


B
LT
80K
39K
B 1 4 1 4 7 5 7 16 10 13
αPy C LT MT

Another possible explanation for the antigenic sub-groups could lie in the existence of a number of tertiary and quaternary conformations for the T-antigens. The interactions involved in such structures could mask antigenic sites, or alternatively, be responsible for expressing them. To investigate this possibility, (^{35}S)-methionine labelled lysates of lytically infected cells were separated by sedimentation velocity analysis on sucrose gradients, followed by immunoprecipitation with various monoclonal antibodies of the fractions across the gradient. Some of the results of such analyses are shown in Figure 3.5. It can be seen with αPyC1 antibody immunoprecipitates that the sedimentation peak of both large and middle T-antigen is of considerably greater velocity than would be expected were the molecules present in a monomeric form, whereas the small T-antigen is much closer to the expected sedimentation velocity. Middle T-antigen molecules are immunoprecipitated over the whole gradient with either αPyC1 or αPyMT5 antibodies. Some middle T-antigen molecules have a sedimentation velocity of greater than 28S and so probably have a very high molecular weight. No significant variation was observed in the sedimentation size of the large T-antigens immunoprecipitated by any of the different monoclonal antibodies (for example, αPyLT7 antibody). However, the αPyMT13 antibodies appear to recognize preferentially the fast sedimenting form of middle T-antigen, which interestingly, separates from the MWtapp 80Kd polypeptide on sedimentation. This demonstrates that immunoprecipitation of either of the polypeptides is due to direct antibody binding and not to their being associated, and so co-precipitated. It therefore appears that the sub-group of middle T-antigen molecules defined by reaction with αPyMT13 antibodies is present as a much higher molecular weight form of protein than the rest of the molecules. Data with the other αPyMT antibodies are indistinguishable from those obtained on middle T-antigen molecules recognized by the αPyC1 antibody.

Section 3.4 - Antibody Characterization

To investigate the feasibility of using these monoclonal antibodies to purify T-antigens, the products from five 90mm tissue culture dishes of polyoma virus lytically infected cells were immunoprecipitated with the αPyC1 antibody, the polypeptides separated by SDS-PAGE and then stained with Coomassie Brilliant Blue. Figure 3.6 shows the stained polypeptides obtained from uninfected, wild type virus, d1-8, d1-23 and ts-a-a infected 3T6 Swiss Albino mouse cells as described in Materials and Methods.

Figure 3.5 - Sucrose Gradient Sedimentation Velocity Analysis of T-Antigens Recognized by Monoclonal Antibodies.

Wild type polyoma virus infected 3T6 cells were labelled with (^{35}S)-methionine for 4 hours at 37°C, 24 hours post infection, and the cells then lysed. Two hundred ul of this lysate were layered onto the top of a 5% to 20% sucrose gradient and separated by sedimentation, as described in Materials and Methods. The gradient was then fractionated, the individual samples immunoprecipitated with monoclonal antibody-containing TCF, using MRC Ox12 as a second antibody, the resulting polypeptides separated by SDS-PAGE on a 10% gel, and detected by autoradiography. In each case the direction of sedimentation is indicated underneath, and an immunoprecipitate from an unfractionated lysate shown in lane M. The sedimentation locations of protein standards are indicated above, together with their molecular weights. The positions of the T-antigens are indicated on the left. The monoclonal antibody used for immunoprecipitation is :

A αPyC1

B αPyMT5

C αPyLT7

D αPyMT13

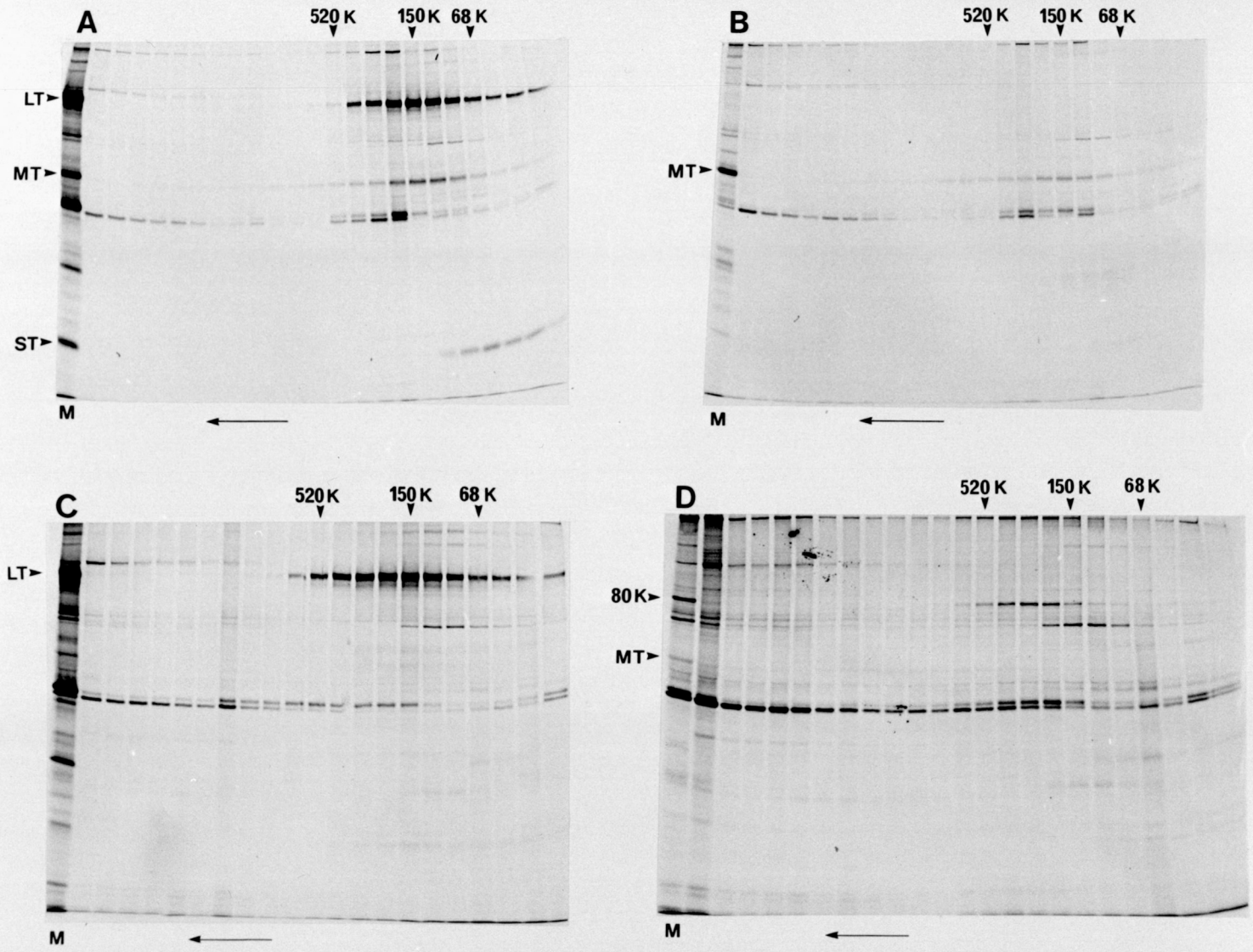

A
520 K
150 K
68 K
LT
MT
ST
M
B
520 K
150 K
68 K
MT
M
C
520 K
150 K
68 K
LT
M
D
520 K
150 K
68 K
80 K
MT
M

Figure 3.6 - Coomassie Blue Stained Preparation of T-Antigens.

3T6 Swiss Albino mouse cells (5 x 90mm dishes) were infected with wild type, or mutant, polyoma viruses and incubated for 42 hours at 32°C. The cells were lysed and immunoprecipitated by ∝PyC1 antibody as described in Materials and Methods. The polypeptides were then separated by SDS-PAGE on a 10% gel and localized by staining with Coomassie Blue.

Lane (a) Polypeptides immunoprecipitated from uninfected (u) cell lysates.

Lane (b) Polypeptides immunoprecipitated from wild type polyoma virus infected cell lysates.

Lane (c) Polypeptides immunoprecipitated from mutant d1-8 infected cell lysates.

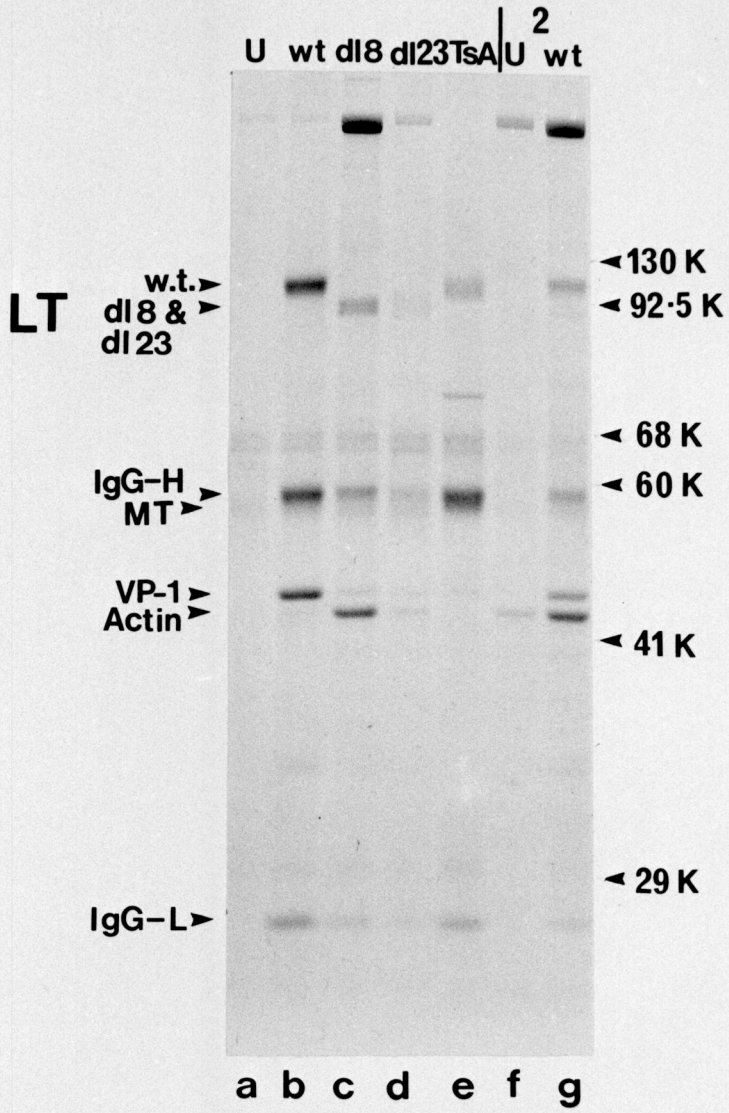
Lane (d) Polypeptides immunoprecipitated from mutant d1-23 infected cell lysates.

Lane (e) Polypeptides immunoprecipitated from mutant ts-a-a infected cell lysates after shifting up to the non-permissive temperature (39°C) for 4 hours, following incubation for 42 hours at the permissive temperature (32°C).

Lane (f) Polypeptides immunoprecipitated from the lysate used for track (a) when retreated with SAC.

Lane (g) Polypeptides immunoprecipitated from the lysate used for track (b) when retreated with SAC.

The positions of wild type (wt), d1-8 and d1-23 large T-antigens (LT) are indicated on the left, together with the positions of the immunoglobulin G heavy (IgG-H) and light (IgG-L) chains, the middle T-antigen (MT), the viral capsid protein VP-1 and cellular actin. The positions and M.Wt. of protein standards are indicated on the right.

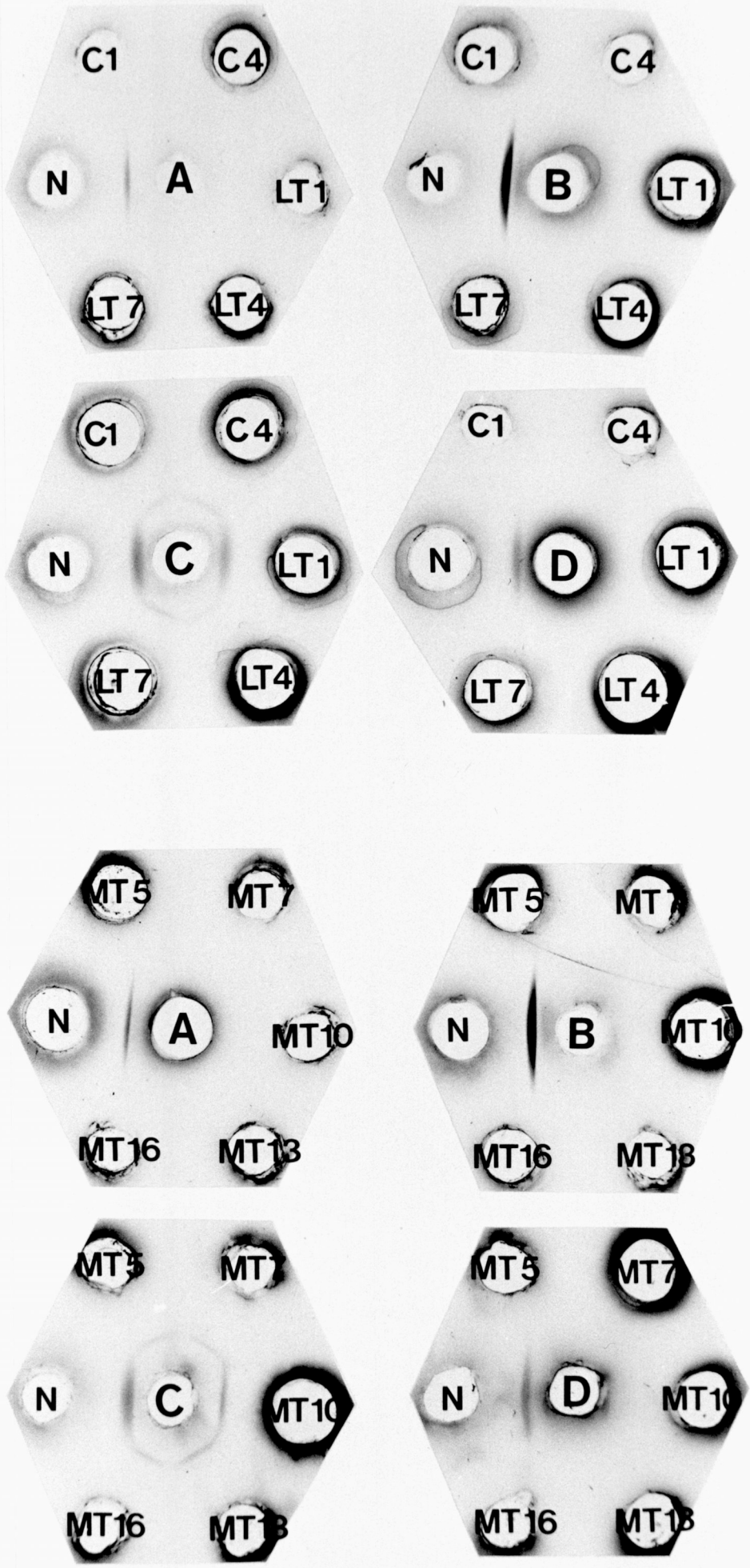

U
wt
dl8
dl23
TsA
U
2
wt
LT
w.t.
dl 8 &
dl 23
IgG-H
MT
VP-1
Actin
IgG-L
130 K
92·5 K
68 K
60 K
41 K
29 K
a b c d e f g

In this experiment, the large T-antigen from wild type polyoma virus infected cells is clearly visible; its identity is substantiated by the mobility change observed in the product obtained from cells infected with polyoma virus deletion mutants dl-8 and dl-23. Figure 3.6 also demonstrates an interesting property of the αPyC1 antibody (subsequently shown to apply to the other nine monoclonal antibodies). That is, in uninfected cell lysates there was no immunoprecipitation of the antibody, despite its presence in the same quantity as in infected cell lysates. Quantitatively, it can be seen that the amount of immunoglobulin bound to the protein A of the SAC in lytically infected cell lysates is approximately equal to the amount of antigen immunoprecipitated. This suggests that the monoclonal antibody only binds to the protein A of SAC when it is complexed with its antigen. This reaction is also seen to be incomplete; a further addition of SAC to the supernatant following immunoprecipitation results in further T-antigen being immunoprecipitated without any further addition of antibody. This occurs even when the quantities of SAC added were increased four-fold and so was not a result of insufficient SAC being used. The observation that large T-antigen can be immunoprecipitated in sufficient quantity to be easily stained with Coomassie Blue demonstrates that these antibodies should be extremely useful in purifing the T-antigens of polyoma virus.

The unusual protein A binding properties observed with these rat monoclonal antibodies (described above) led to an interest in the immunoglobulin sub-class to which they belong. Knowledge of this would also be of use where information about the physical properties of the antibodies become important for further investigations. To determine the antibody type, the proteins present in monoclonal antibody-containing TCF were concentrated 50-fold by ammonium sulphate precipitation and then analysed by Ouchterlony double diffusion assay against rat immunoglobin type specific antisera (Bazin et al., 1974) The results obtained are shown in Figure 3.7 where it can be seen that all ten antibodies react only with the anti-rat IgG2b sub-class specific antisera to form a precipitin line. The rat IgG2b sub-class of immunoglobulin has been reported to bind only very weakly to protein A from SAC (Medgyesi et al., 1978; Rousseaux et al., 1981). This agrees with the poor binding to SAC observed with these antibodies in the absence of antigen.

Figure 3.7 - Antibody Subclass Analysis of Monoclonal Antibodies Secreted by Hybridomas.

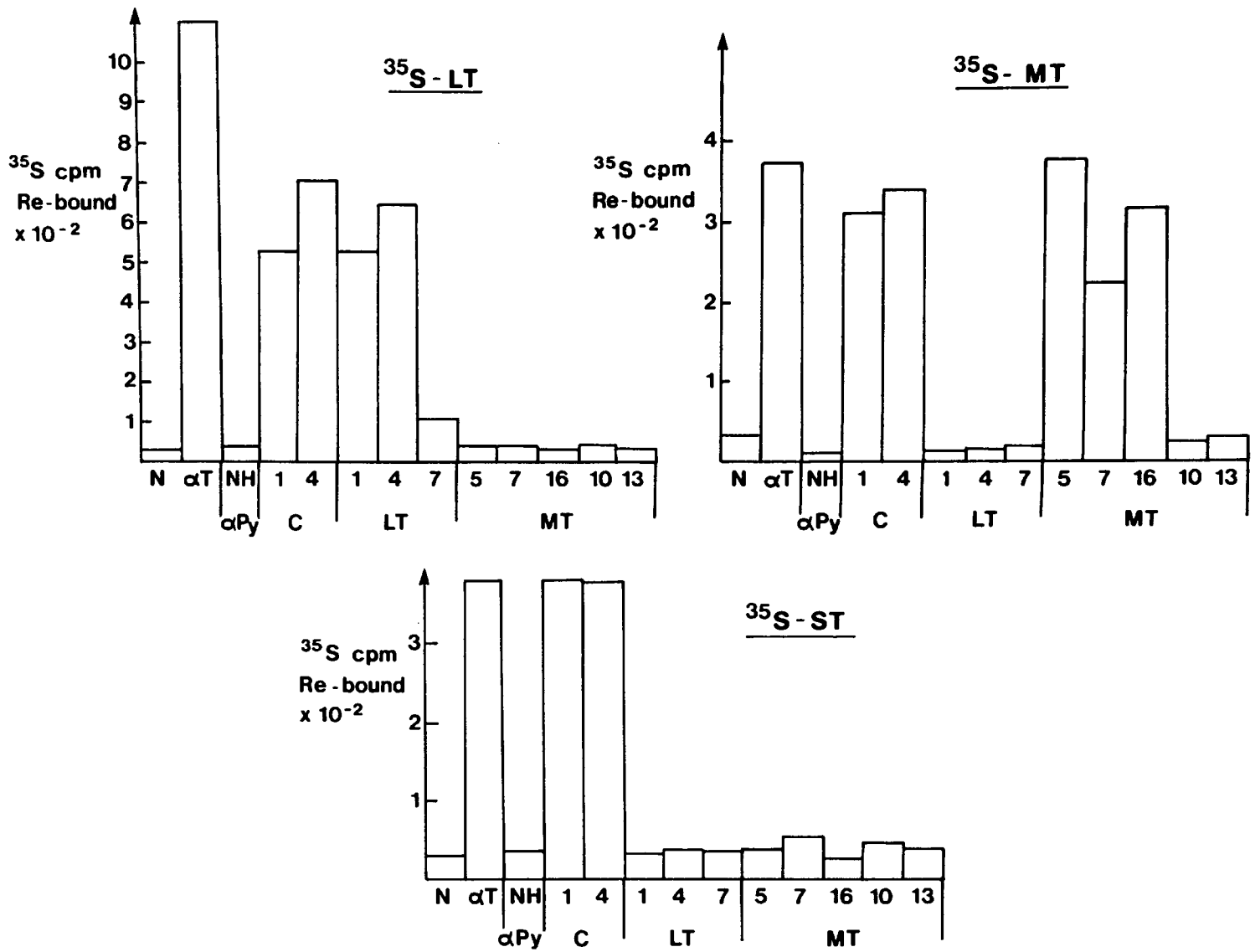
Ouchterlony double diffusion analysis of the proteins precipitated by ammonium sulphate from monoclonal antibody containing TCF, against anti-rat immunoglobulin subclass specific sera (see Materials and Methods). In each case the typing antisera is used in the centre well and the monoclonal antibody in the outer wells. A represents the anti-rat IgG1 sera, B the anti-rat IgG2a sera, C the anti-rat IgG2b, and D the anti-rat IgG2c. In the outer wells, the monoclonal antibody added to the well is designated (omitting the pre-fix ∝Py). N represents rat serum, used as a positive control.


C1
C4
N
A
LT1
LT7
LT4
C1
C4
N
B
LT1
LT7
LT4
C1
C4
N
C
LT1
LT7
LT4
C1
C4
N
D
LT1
LT7
LT4
MT5
MT7
N
A
MT10
MT16
MT13
MT5
MT7
N
B
MT16
MT13
MT5
N
C
MT16
MT5
MT7
N
D
MT16

It has been reported (Rundell et al., 1977; Lane and Robbins, 1978) that many antisera and some monoclonal antibodies, will still recognize and bind to polypeptides that have been denatured by SDS and separated on SDS-PAGE. To investigate the ability of the ten monoclonal antibodies described in Chapter Two to bind SDS-PAGE purified polypeptides, (^{35}S)-methionine labelled T-antigens (prepared by immunoprecipitation with anti-T serum from lytically infected cells) were eluted from a polyacrylamide gel and reacted with the antibodies (see Materials and Methods). Figure 3.8 shows the quantity of the three T-antigens bound in this manner by the various antibodies, as compared with those recognized by anti-T serum. The amount of T-antigen immunoprecipitated by the monoclonal antibodies was always found to be somewhat less than that bound with anti-T serum. Since only one antigenic site on a molecule is recognized by a monoclonal antibody, the probability of loss of this one site is clearly greater than the probability of losing all of those recognized by anti-T serum, which would be required for precipitation to fail to occur in the latter instance. The data obtained show that αPyC1 and αPyC4 antibodies efficiently recognize all three denatured T-antigens. αPyLT1 and αPyLT4 antibodies effectively recognize large T-antigen, but αPyLT7 antibodies recognize small amounts only of the denatured antigen. This is possibly another reflection of the variation observed in the ability of αPyLT7 antibodies to recognize the large T-antigen in different lysates of infected cells (Section 3.3) and its inability to recognize the large T-antigen expressed in PyREWA5/T1A1 cells (Section 3.2). It can also be seen that the αPyMT7 antibody recognizes denatured middle T-antigen less well than the αPyMT5 or αPyMT16 antibodies, so it appears that the SDS denaturation of the molecules does not abolish the distinction between middle T-antigen that is recognized by αPyMT7 antibody, and that which is not. Thus, this sub-group does not appear to be a result of the existence of conformational variants among middle T-antigens, since these are likely to be destroyed by SDS denaturation, but may reflect a more stable difference, such as a post-translational modification. αPyMT10 and αPyMT13 antibodies do not appear to recognize SDS-denatured middle T-antigen. No immunoprecipitation was observed in cases where a reaction was not expected. That is, middle T-antigen was not recognized by the αPyLT antibodies, etcetera.

Figure 3.8 - Monoclonal Antibody Binding of SDS-Denatured T-Antigens.

(^{35}S)-Methionine labelled T-antigens were prepared using anti-T serum and separated by SDS-PAGE. Individual T-antigens were eluted and rebound by a monoclonal antibody, as described in Materials and Methods. The (^{35}S) counts per minute (cpm) are plotted with respect to the antibody being tested. N represents experiments with non-immune rat serum; T represents anti-tumour serum; NH represents monoclonal antibody negative TCF. The T-antigen used is indicated in each case by the usual abbreviations.


35S - LT
35S cpm
Re-bound
x 10-2
10
9
8
7
6
5
4
3
2
1
N αT NH 1 4 1 4 7 5 7 16 10 13
αPy C LT MT
35S - MT
35S cpm
Re-bound
x 10-2
4
3
2
1
N αT NH 1 4 1 4 7 5 7 16 10 13
αPy C LT MT
35S - ST
35S cpm
Re-bound
x 10-2
3
2
1
N αT NH 1 4 1 4 7 5 7 16 10 13
αPy C LT MT

Section 3.5 - Radio-Immuno Assay

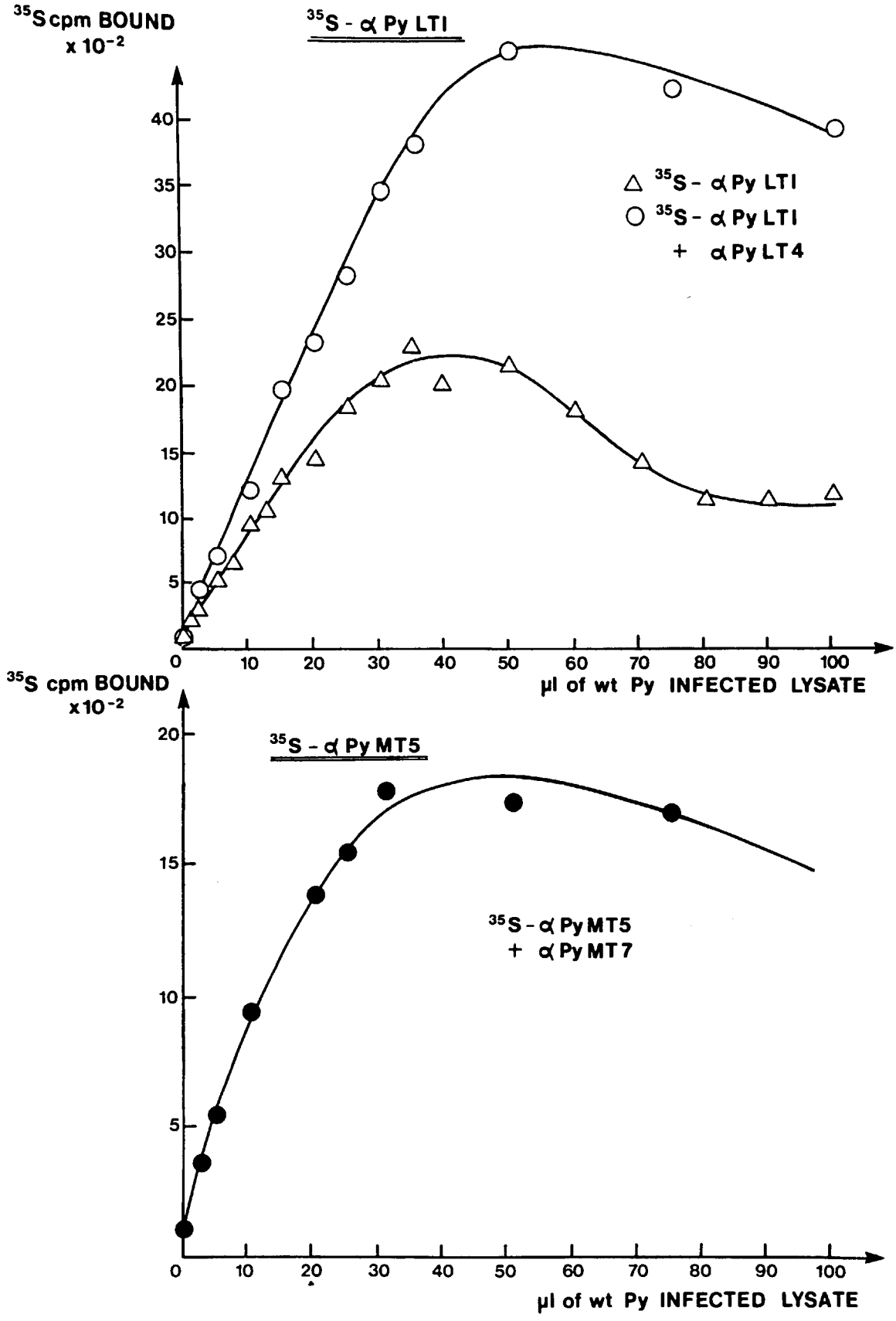
The lack of a specific assay for any of the T-antigens has been one of the hinderances to purifying the T-antigens by conventional protein techniques. It was hoped, therefore, that the specificity and reproducibility provided by the monoclonal antibodies could be used to establish a radio-immuno assay (RIA) for each of the T-antigens. The absence of an antibody recognizing small T-antigen alone has meant that an assay for small T-antigen could not at present be achieved. A RIA for large and middle T-antigen was devised, however. The assay utilizes the property of the monoclonal antibodies to bind to the protein A of SAC only when complexed with their antigen. The antibodies were radio-labelled by incubating the hybridoma cell line secreting a particular antibody with (^{35}S)-methionine, followed by protein precipitation from the TCF with ammonium sulphate (see Materials and Methods).

Figure 3.9A shows the quantitation achieved with the RIA for large T-antigen. In this experiment, a constant amount of the radio-labelled antibody was incubated with varying quantities of a lysate of wild type polyoma virus infected 3T6 Swiss Albino mouse cells. In each case the volume of lysate and the final protein concentration present was corrected by the addition of an appropriate quantity of a lysate from uninfected cells. After an hour's incubation, SAC was added, the immunoprecipitates isolated, and the radioactivity bound (^{35}S) quantitated by scintillation counting. As seen in Figure 3.9A, for this assay there is a linear response of counts bound to antigen input at reasonably low levels of antigen. This response reaches a maximum, probably as a result of saturation of the antibody, and then decreases as further antigen is added. This decrease would not be expected to occur if the antibody bound to SAC, when complexed with the antigen, was a result of a change in the affinity of the interaction between the antibody and the protein A molecule. A more probable explanation for this observation is evident if one postulates the existence of multimeric forms of the large T-antigen. Thus, each of the antigen molecules in the multimer could bind an antibody molecule, and a complex would exist linking a number of antibody molecules together. The effect of this linkage would be to increase the apparent affinity of the antibody for protein A, as only one molecule would need to be bound to protein A at any one time for all of the others associated with it to be effectively bound.

Figure 3.9 - RIA for Large and Middle T-Antigens.

A. Radio-immuno assay for large T-antigen. (^{35}S)-Methionine labelled αPyLT1 antibody was incubated with varying amounts of a lysate of wild type polyoma virus infected 3T6 mouse cells, with or without unlabelled αPyLT4 antibody, as indicated. The immune complexes were then adsorbed onto SAC and the radioactivity determined by scintillation counting (see Materials and Methods). The cpm of (^{35}S) bound were plotted against the quantity of infected cell lysate used.

B. Radio-immuno assay for middle T-antigen. An assay similar to that described for large T-antigen above was devised, except (^{35}S)-methionine labelled αPyMT5 antibody and unlabelled αPyMT7 antibody were used.


^{35}S cpm BOUND
x 10^{-2}
^{35}S - α Py LTI
40
35
30
25
20
15
10
5
0
10
20
30
40
50
60
70
80
90
100
μl of wt Py INFECTED LYSATE
△ ^{35}S - α Py LTI
○ ^{35}S - α Py LTI
+ α Py LT4
^{35}S cpm BOUND
x10^{-2}
^{35}S - α Py MT5
20
15
10
5
0
10
20
30
40
50
60
70
80
90
100
μl of wt Py INFECTED LYSATE
^{35}S - α Py MT5
+ α Py MT7

In the RIA, under the conditions where the antibody is in excess, all of the available antigen has an antibody bound, and the addition of more antigen results in a direct increase in the amount of antibody present in saturated complexes and a concomitant linear increase in the amount of antibody bound to SAC. This increase reaches a maximum when all of the antibody present is bound to antigen. In antigen excess, however, the complexes present are not saturated and therefore the apparent increase in affinity of the antibody for protein A is less. That is, less antibody is bound to SAC. As more antigen is added, the level of saturation of the complexes becomes less, and therefore the apparent affinity of antibody-SAC interaction decreases. The result is that less antibody is bound to the SAC.

The RIA described above, therefore, may be measuring the presence of multimeric forms of large T-antigen and the formation of large immune complexes. If the presence of the latter is responsible for the increased apparent affinity between the antibody and protein A, the addition of another monoclonal antibody that binds to the same antigen without affecting the binding of the first antibody would be expected to raise the apparent antibody-protein A affinity. Figure 3.9A shows the effect of the addition of unlabelled αPyLT4 antibodies on the large T-antigen RIA carried out with radio-labelled αPyLT1 antibody. It can be seen that the assay is still linear at low antigen inputs, but an increased antibody binding is observed. In this assay, the decrease in antigen excess is less pronounced. It is possible that the presence of a large excess of unlabelled antibody allows immune complexes to form that contain monomeric, as well as multimeric, forms of the large T-antigen and so allows the former also to be bound to SAC. Because of this possibility, the addition of a second unlabelled antibody was considered to give a more useful RIA.

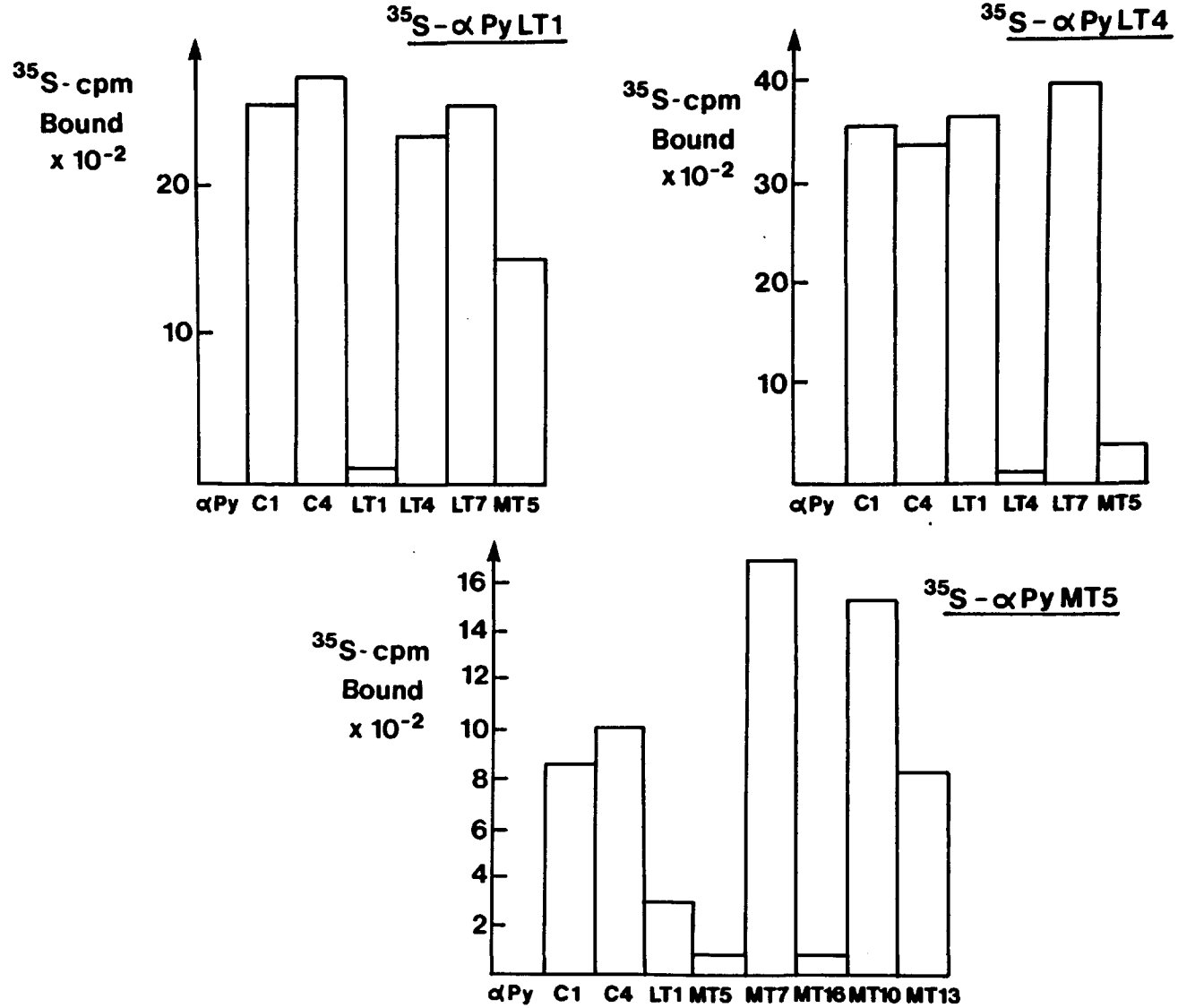
Figure 3.9B shows a similar quantitative assay for middle antigen. The αPyMT5 antibody was used in radio-labelled form, with unlabelled αPyMT7 antibody added in excess. (For the middle T-antigen the assay in the absence of the unlabelled antibody was not reproducible). The characteristics of this assay were precisely those observed for the large T-antigen, so similar considerations concerning the possible existence of multimeric forms of the middle T-antigen may apply. These data further agree with the observation that both large and middle T-antigens sediment on sucrose gradients faster than would be expected were they present as monomeric forms only (Section 3.3).

The RIA procedure described above also provides a means of determining whether the binding of one monoclonal antibody inhibits the subsequent binding of a second, different antibody. Figure 3.10 shows the amount of radio-labelled antibody bound to SAC when a constant quantity of a wild type polyoma virus infected cell lysate is incubated with excess of an unlabelled antibody prior to the addition of the labelled antibody. Although all of the possible combinations of monoclonal antibodies have not been tried, a number of conclusions can be drawn from the data obtained. It appears that the three ∝PyLT antibodies bind to separate antigenic sites and do not inhibit the binding of one another. Thus, the binding of both (^{35}S)-methionine labelled ∝PyLT1 and ∝PyLT4 antibodies is increased (and therefore not blocked) by the addition of the other, or of ∝PyLT7, antibody in unlabelled form. As a control, the effect of adding a large excess of an antibody that does inhibit the binding of the radio-labelled antibody was measured in an experiment where the same monoclonal antibody was first used unlabelled, and then radio-labelled. In this case essentially none of the radio-labelled antibody is bound to the SAC. Using this approach, it could be concluded that the ∝PyC antibodies bind to a separate antigenic site from those recognized by ∝PyLT antibodies. For middle T-antigen, it can be seen that the site recognized by ∝PyMT5 and ∝PyMT16 are the same or close together, as the binding of one inhibits the subsequent binding of the other. The ∝PyMT7, ∝PyMT10 and ∝PyMT13 antibodies recognize another antigenic site, however. Interestingly, the ∝PyC antibodies which recognize yet another antigenic site on the middle T-antigen molecule from that recognized by ∝PyMT5 or ∝PyMT16, are not as effective in forming immune complexes as the ∝PyMT antibodies.

Figure 3.10 - Effect of the Binding of One Monoclonal Antibody on That of Another.

Thirty ul of a wild type polyoma virus infected 3T6 mouse cell lysate were incubated with an unlabelled monoclonal antibody-containing TCF for one hour at 0°C. (^{35}S)-Methionine labelled monoclonal antibody was then added, and the immune complexes adsorbed onto SAC, as described in Materials and Methods. The labelled antibody used is indicated in each case in the caption and unlabelled antibody indicated beneath each sample. The quantity of (^{35}S) bound is plotted.

The control for labelled αPyLT antibodies uses αPyMT5 antibody, an antibody that has no effect on the binding of labelled antibody, and an unlabelled antibody that completely inhibits the binding. Similarly, the control for labelled αPyMT antibodies is αPyLT1 antibody, an antibody that has no effect on the binding of labelled antibody.


^{35}S-αPy LT1
^{35}S-cpm Bound x 10^{-2}
20
10
αPy C1 C4 LT1 LT4 LT7 MT5
^{35}S-αPy LT4
^{35}S-cpm Bound x 10^{-2}
40
30
20
10
αPy C1 C4 LT1 LT4 LT7 MT5
^{35}S-αPy MT5
^{35}S-cpm Bound x 10^{-2}
16
14
12
10
8
6
4
2
αPy C1 C4 LT1 MT5 MT7 MT16 MT10 MT13

Section 3.6 - Discussion

The results presented in this Chapter indicate that the monoclonal antibodies (described in Chapter Two) recognize quite different regions on the T-antigens. The ability of different monoclonal antibodies to bind to the corresponding antigens encoded by defined deletion mutants of polyoma virus, or expressed by polyoma virus transformed rat cells (Table 3.I), indicates that the αPyLT1 antibody recognizes a region of the large T-antigen that is encoded within a sequence that resides between the 3'-splice site for the mRNA of large T-antigen and the EcoRI restriction enzyme site (that is, essentially between amino acids 79 and 327 on the large T-antigen protein sequence, see Figure I.4). This may be further defined as possibly lying between the deletions occurring in mutants dl-8 and dl-23 (that is, between amino acids 169 and 188). The αPyLT4 antibody binding site is, by similar analyses, probably encoded by the DNA sequence surrounding the EcoRI restriction enzyme site. The recognition site for the αPyLT7 antibody is less-well defined, but may involve a higher order structural form of the large T-antigen since it differs from αPyLT1 and αPyLT4 antibodies in only poorly recognizing SDS denatured large T-antigen, (Figure 3.8). The binding sites for all three of these antibodies have been shown to be quite separate. Moreover, the presence of an antibody bound to one site does not inhibit the binding of another antibody to a second site (see Figure 3.10).

The αPyMT antibodies have also been shown to recognize separate regions on the T-antigen molecule (see Table 3.1). The two antigenic sites that have been well characterised are the one recognized by αPyMT7 antibodies, that appears to involve the region of the polypeptide encoded by DNA sequences that are deleted in the mutants dl-8 and dl-27 (that is, between amino acids 253 and 305 on the middle T-antigen protein sequence, see Figure I.4), and that recognized by the αPyMT5 (and possibly also the αPyMT16) antibodies, which appears to involve the region of the polypeptide encoded by DNA sequence that is deleted in the mutant dl-23 (that is, amino acids 302 to 335). Once again these sites are quite separate and an antibody can bind to one without inhibiting binding of another antibody to the other (see Figure 3.10). Both of these sites are retained after denaturation of the polypeptide with SDS. Interestingly, the site recognized by the αPyMT7 antibody does not appear to be expressed on all of the middle T-antigen molecules, even after they have been denatured by SDS. Antigenically middle T-antigen molecules can be divided into a number of sub-groups; the significance of this remains to be determined.

The ability of the αPyC1 antibody to immunoprecipitate the T-antigens, particularly large T-antigen, in sufficient quantities to be readily detected with Coomassie Blue stain indicates that the monoclonal antibodies should have an important role to play in future attempts to purify the polyoma virus T-antigens. Use of the immunoprecipitation technique has already resulted in significant enrichment for the T-antigens (Figure 3.6) and will probably enable them to be available in the near future in pure form. As an aid to purification, the development of a RIA (Figure 3.9) that is specific for either large or middle T-antigens, and moderately quantitative, will be of considerable use. The above experiments have indicated that both large and middle T-antigen exist in multimeric forms in cell lysates, although the exact size and nature of these multimers has not been further defined.

CHAPTER FOUR

Section 4.1 - Introduction

The monoclonal antibodies described in Chapter 2 provide a variety of very specific immunological probes for the large and middle T-antigens of polyoma virus which can be used to investigate directly the sub-cellular location of these T-antigens. Previously, such studies have involved the fractionation of cellular components from lytically infected and transformed cells, followed by immunoprecipitation. This approach was limited by the degree of purity to which the components themselves could be prepared, so it has been difficult to determine the precise location of the T-antigens. Previous data indicated that the large T-antigen was to be found predominantly within the nucleus of the cell (Takemoto et al., 1966; Ito et al., 1977a; Ito et al., 1977b; Silver et al., 1978), the middle T-antigen in the plasma membrane (Ito et al., 1977b; Silver et al., 1978), and the small T-antigen probably in the cytoplasm (Silver et al., 1978). To investigate directly the sub-cellular location, indirect immunofluorescent analysis was conducted using the T-antigen specific monoclonal antibodies. This analysis was conducted on both polyoma virus lytically infected mouse cells and transformed rat cells.

Immunofluorescent studies frequently involve an indirect technique. To avoid the necessity of conjugating each specific antisera with a fluorochrome, unlabelled antisera is used in the assay and the bound antibody then recognized by the addition of a fluorochrome conjugated antibody that reacts with it. In this way, a single fluorochrome conjugated antibody can be used to fluoresce a number of different antigens. This Chapter describes a modification of this approach, with a third unlabelled antibody being used to bridge the monoclonal antibody and the fluorochome conjugated antibody. The rationale behind this modification is that monoclonal antibodies only recognize one antigenic site on a molecule whereas a conventional antisera, with a number of activities, can recognize a number of sites on the same molecule. In the case of the polyoma virus T-antigens, where only small amounts of antigen are expressed in cells, very little specific antibody is bound and the fluorescent intensity is normally low. In an attempt to amplify the specific signal, an unlabelled antibody raised against a rat immunoglobulin was added which recognizes the monoclonal antibody.

This could recognize each monoclonal antibody molecule at a number of sites and so increase the specific signal. A fluorochrome conjugated antisera against the amplifying antibody was then added to achieve the specific desired fluorescence. This technique was found to work well with the individual monoclonal antibodies; probably as a result of the extreme specificity provided by these antibodies, significant amplification was achieved with little increase in non-specific fluorescence. This technique has also been used as a means of recognizing large and middle T-antigens in the same cell, employing different fluorochromes.

During immunofluorescent studies, cells need to be fixed and permeabilised to allow antibody entry, a process which in itself could introduce artefacts with respect to the cellular location of the T-antigens. To ensure that the data observed are a true reflection of antigen location within the intact cell, a number of different fixation techniques were investigated and immunofluorescence subsequently observed, as above. Moreover, the possibility exists that different antigenic sub-groups of the T-antigens (described in Chapter Three) could be a reflection of different sub-cellular locations of the corresponding proteins. Therefore, the T-antigens in lytically infected cells were examined using a number of different monoclonal antibodies, to determine whether any changes could be observed.

Section 4.2 - Immunofluorescence of Lytically Infected Cells.

Immunofluorescence observed with wild type polyoma virus infected 3T6 Swiss Albino mouse cells using αPyLT1, αPyLT4 and αPyLT7 antibodies is shown in Figure 4.1. These cells were fixed with formaldehyde and permeabilised with Nonidet P40 before incubation with the antibody (Griffin et al., 1980). To ensure that the immunofluorescent reactions observed were specific, two control reactions were performed: (i) Lytically infected cells were incubated with TCF from NS-1 cells and the two anti-IgG sera to determine whether the TCF or the two other antibodies stained any specific areas of the cells (Figure 4.1 (g)). (ii) The monoclonal antibodies were used to label uninfected 3T6 cells to ensure that only the T-antigens were being studied, and not host cellular proteins (Figure 4.1(a), (c) and (e)). It can be seen that a significant increase of fluorescence occurs with the lytically infected cells, relative to the controls. The staining is nuclear in location, with the nucleoli prominent but unstained. This pattern is similar to that observed with anti-T serum (Takemoto et al., 1966), a fact which probably reflects a greater quantity of large T-antigen in lytically infected cells and higher levels of antibodies to this protein in anti-T serum since middle or small T-antigens are not normally observed with such sera.

Figure 4.1 - Immunofluorescent Analysis of Lytically Infected Cells with ∝PyLT Antibodies.

Wild type polyoma virus infected 3T6 Swiss Albino mouse cells were fixed with formaldehyde, permeabilised with Nonidet P40, reacted with a monoclonal antibody, rabbit anti-rat IgG and finally a rhodamine labelled goat anti-rabbit IgG (see Materials and Methods). Photographs were taken using epifluorescent optics fitted with rhodamine specific filters and a x63 Planapochromat objective lens. The photomicrographs are :-

(a) Mock infected cells, reacted with ∝PyLT1 antibody, and photographed with a 5 second exposure.

(b) Infected cells, reacted with ∝PyLT1 antibody, 1 second exposure.

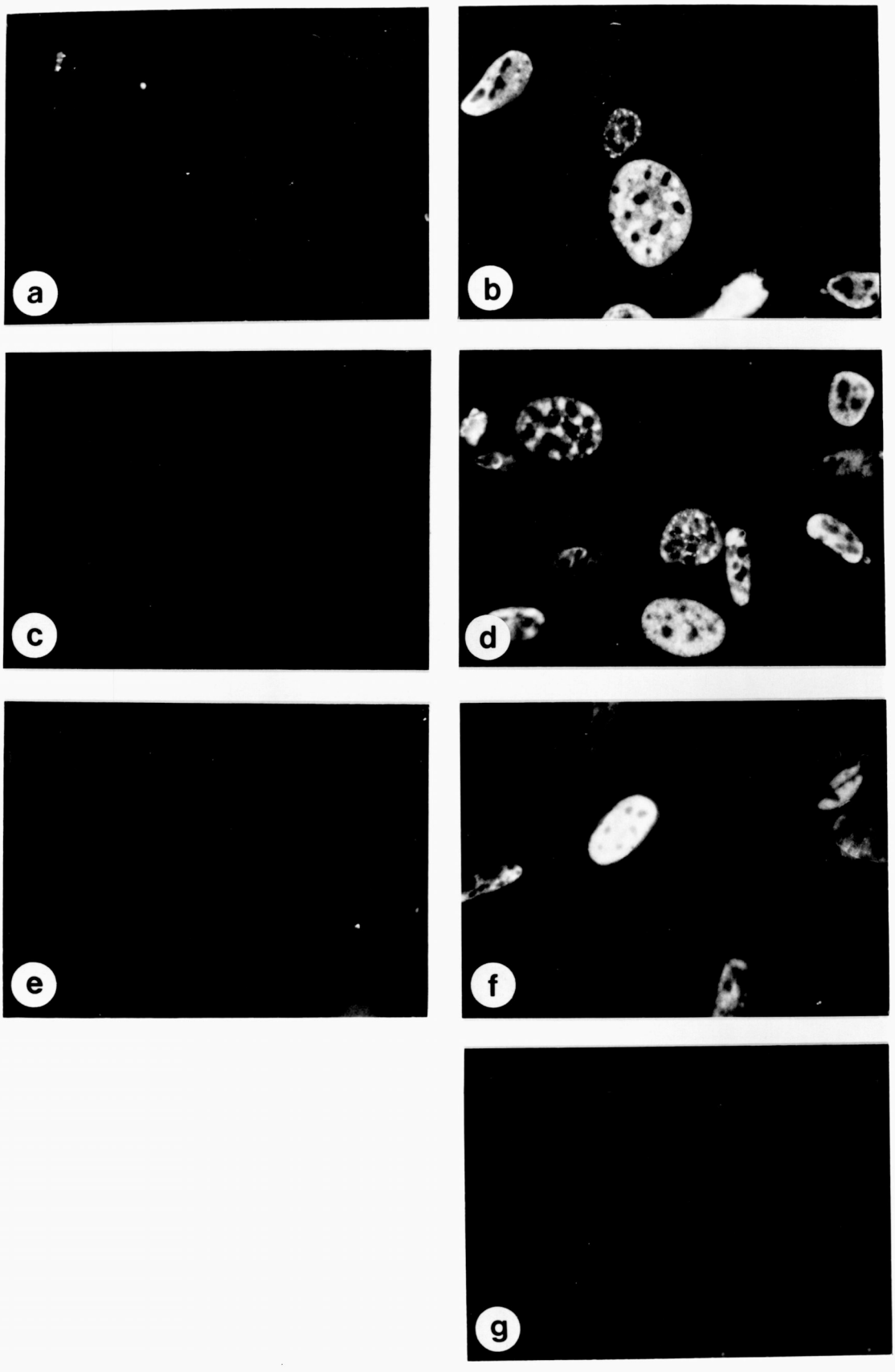
(c) Mock infected cells, reacted with ∝PyLT4 antibody, 5 second exposure.

(d) Infected cells, reacted with ∝PyLT4 antibody, 2 second exposure.

(e) Mock infected cells, reacted with ∝PyLT7 antibody, 10 second exposure.

(f) Infected cells, reacted with ∝PyLT7 antibody, 5 second exposure.

(g) Infected cells, reacted with control TCF, 5 second exposure.


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There was no observable difference in the location of the large T-antigen recognized by any of the three monoclonal antibodies used here. The antigenic sub-groups defined by these antibodies are therefore probably not a result of, nor result in, altered sub-cellular location. However, the intensity of the fluorescent staining with the ∝PyLT7 antibody is reduced relative to the others. (Note in Figure 4.1(f) the higher exposure times used for photography).

Figure 4.2 shows again the immunofluorescence of wild type polyoma virus infected 3T6 Swiss Albino mouse cells, formaldehyde fixed and permeabilised with Nonidet P40, observed using ∝PyMT5, ∝PyMT7 and ∝PyMT16 antibodies. A set of controls similar to those carried out with ∝PyLT antibodies were essentially negative. The specific immunofluorescence appeared to be the same for all three antibodies, so again the antigenic sub-groups appear not to reflect altered sub-cellular locations. The ∝PyMT7 antibody reaction, however, was of a much lower intensity. Data obtained for middle T-antigen were not those expected from earlier studies (Ito et al., 1977b; Ito, 1979). That is, the immunofluorescence appeared to be extranuclear, but with a significantly large amount of staining in the vicinity of the nucleus. There did not appear to be specific localisation to any one area within the cell, and particularly, no staining was observed at the cell edge, where plasma membrane located proteins would be expected. There also appeared to be considerable cell to cell variability in the staining intensity. The same cells used for immunofluorescence are shown in Figure 4.2(g) photographed under phase contrast conditions, rather than epifluorescence, to demonstrate the retention of cell architecture following the fixation process.

The immunofluorescent studies described above were conducted on formaldehyde fixed and Nonidet P40 permeabilised cells. To ensure that the observed sub-cellular distribution was genuine and not altered by the fixation procedure, cells were similarly infected with polyoma virus, fixed by treatment with acetone:methanol and then subjected to immunofluorescent analysis. Figure 4.3 shows some of the results obtained. They were indistinguishable from data obtained for formaldehyde fixed cells. It was therefore concluded that the sub-cellular distributions observed were probably true reflections of the T-antigen locations in live cells; formaldehyde fixation was used for future studies since it appeared preferable for preserving sub-cellular architecture.

Figure 4.2 - Immunofluorescent Analysis of Lytically Infected Cells with αPyMT Antibodies.

Cells analogous to those shown in Figure 4.1 were reacted with αPyMT antibodies and fluoresced in a similar manner. Photographs were taken with a x63 Planapochromat objective lens. The photomicrographs show :-

(a) Mock infected cells, reacted with αPyMT5 antibody and photographed with a 10 second exposure.

(b) Infected cells, reacted with αPyMT5 antibody, 10 second exposure.

(c) Mock infected cells, reacted with αPyMT7 antibody, 10 second exposure.

(d) Infected cells, reacted with αPyMT7 antibody, 10 second exposure.

(e) Mock infected cells, reacted with αPyMT16 antibody, 5 second exposure.

(f) Infected cells, reacted with αPyMT16 antibody, 5 second exposure

(g) Similarly fixed cells photographed under phase contrast conditions to illustrate cellular morphology after fixation.

(h) Infected cells, reacted with a control TCF, 10 second exposure.

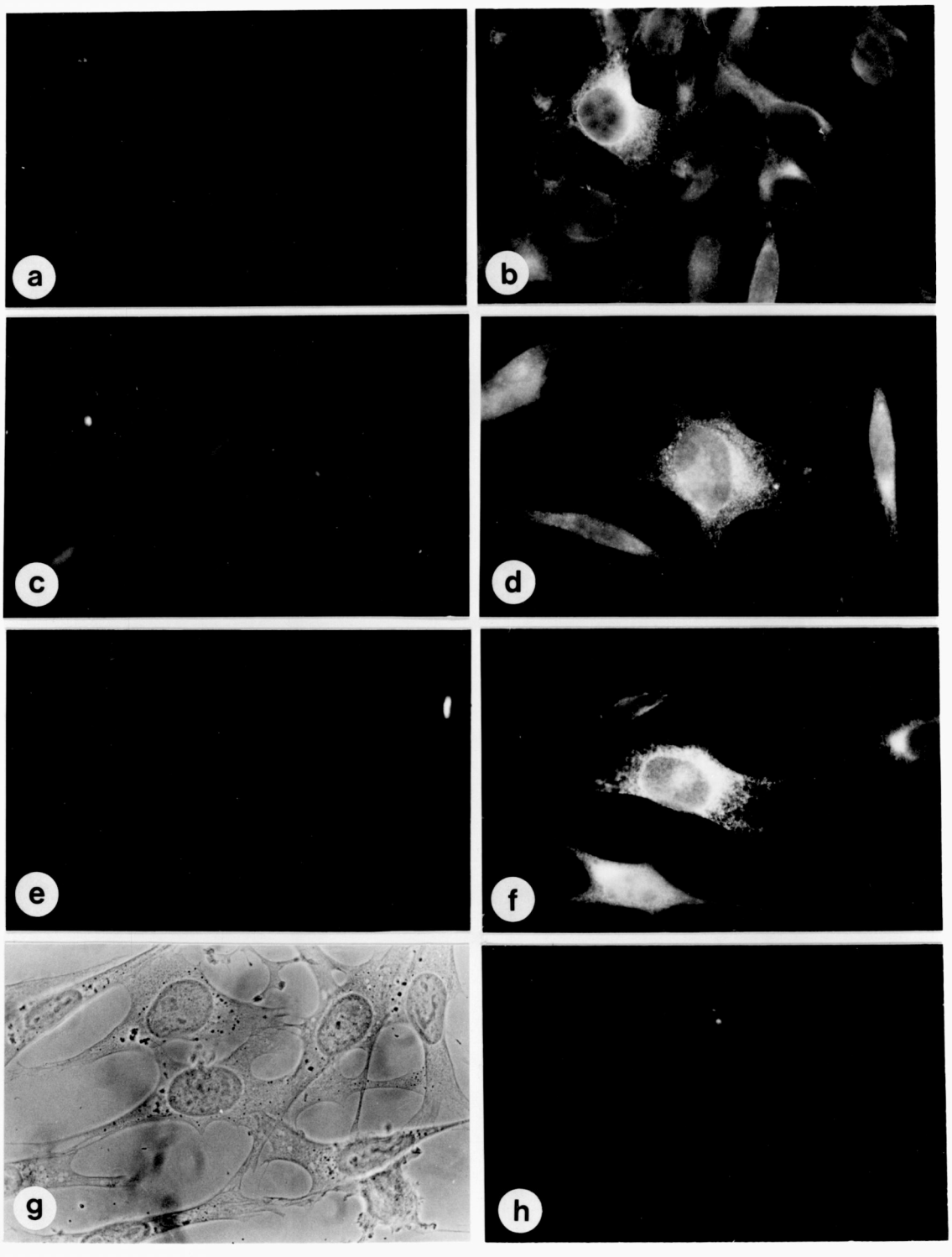

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Figure 4.3 - Immunofluorescent Analysis of T-Antigens in Acetone-Methanol Fixed Cells.

3T6 Swiss Albino mouse cells were infected with wild type polyoma virus, fixed with acetone-methanol and then immunofluoresced (see Materials and Methods). The monoclonal antibodies used, the objective lens and exposure times were as follows :-

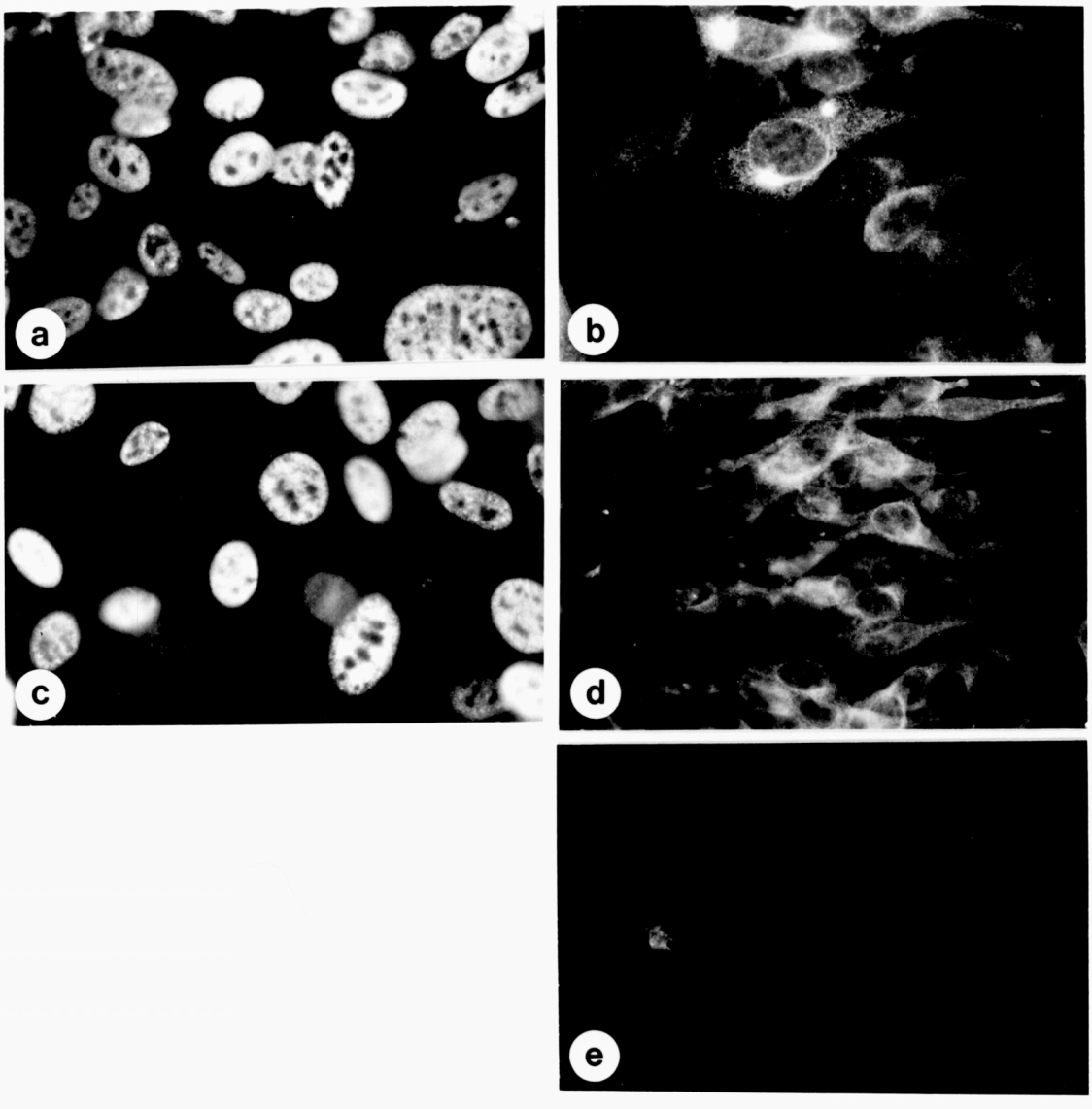
(a) Cells reacted with αPyLT1 antibody, photographed with a x63 objective lens and a 15 second exposure.

(b) Cells reacted with αPyMT5 antibody, photographed with a x63 objective lens, 15 second exposure.

(c) Cells reacted with αPyLT4 antibody, photographed with a x63 objective lens, 10 second exposure.

(d) Cells reacted with αPyMT5 antibody, photographed with a x40 objective lens, 20 second exposure.

(e) Cell reacted with a control TCF, photographed with a x63 objective lens, 20 second exposure.


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The results described above indicated that the three antibodies used individually to recognize either large or middle T-antigen reacted with polypeptides that had a similar location within the cell. To increase the fluorescent intensity, three antibodies for each T-antigen were combined. This allows more than one antibody to bind to each T-antigen molecule and therefore increases the intensity obtained. Anti-LT antibodies for subsequent studies are therefore a mixture of αPyLT1, αPyLT4 and αPyLT7, and anti-MT antibodies a mixture of αPyMT5, αPyMT7 and αPyMT16.

3T6 Swiss Albino mouse cells, lytically infected with wild type polyoma virus, formaldehyde fixed, Nonidet P40 permeabilised, and examined by immunofluorescence with anti-LT and anti-MT antibodies are shown in Figure 4.4. The cells were photographed at different magnifications and appropriate control reactions carried out. The data shown in Figure 4.4, together with those of Figures 4.1 and 4.2, reveal some interesting features about the sub-cellular distributions of large and middle T-antigens as observed by immunofluorescence. Large T-antigen is found to be located predominantly within the nucleoplasm, with the nucleoli and cytoplasm essentially unstained. The nucleoplasmic staining varies considerably between cells. Moreover, in many cells (see arrow in Figure 4.4(b)), there are discrete areas where large T-antigen is concentrated within the nucleoplasm. The cause and significance of such concentrations is not clear, but it is possible that they are either centres of viral DNA synthesis or virion assembly.

The middle T-antigen location observed in Figure 4.4 and 4.2 is in apparent disagreement with recent sub-cellular localization studies. The fluorescence is observed to be extra-nuclear in location, with the nuclear membrane frequently stained. Staining is found throughout the cytoplasm, extending to the cell periphery, but it is more intense in the general region of the nucleus. It is not clear, however, whether this is due to an increased concentration of antigen surrounding the nucleus, or a result of the greater cytoplasmic thickness present in this region. The cytoplasmic staining is not uniform, but "grainy", or reticular, in distribution. There does not appear to be any concentration within particular regions of the cytoplasm, nor any more intense staining at the cell periphery, as would be expected for exclusively plasma membrane associated proteins.

Figure 4.4 - T-Antigen Location In Lytically Infected Cells.

Wild type polyoma virus infected 3T6 Swiss Albino mouse cells, fixed with formaldehyde and permeabilised with Nonidet P40 were stained for immunofluorescent studies with anti-LT and anti-MT antibodies (see Materials and Methods). Photomicrographs (a), (b), (d), (e) and (g) were taken using a x63 Planapochromat objective lens, (c), (f) and (h) using a x40 Planapochromat objective lens. The antibodies used and exposure times are as follows :-

(a) Mock infected cells reacted with anti-LT antibodies, 5 second exposure.

(b) Infected cells reacted with anti-LT antibodies, 2 second exposure.

(c) Infected cells reacted with anti-LT antibodies, 2 second exposure.

(d) Mock infected cells reacted with anti-MT antibodies, 10 second exposure.

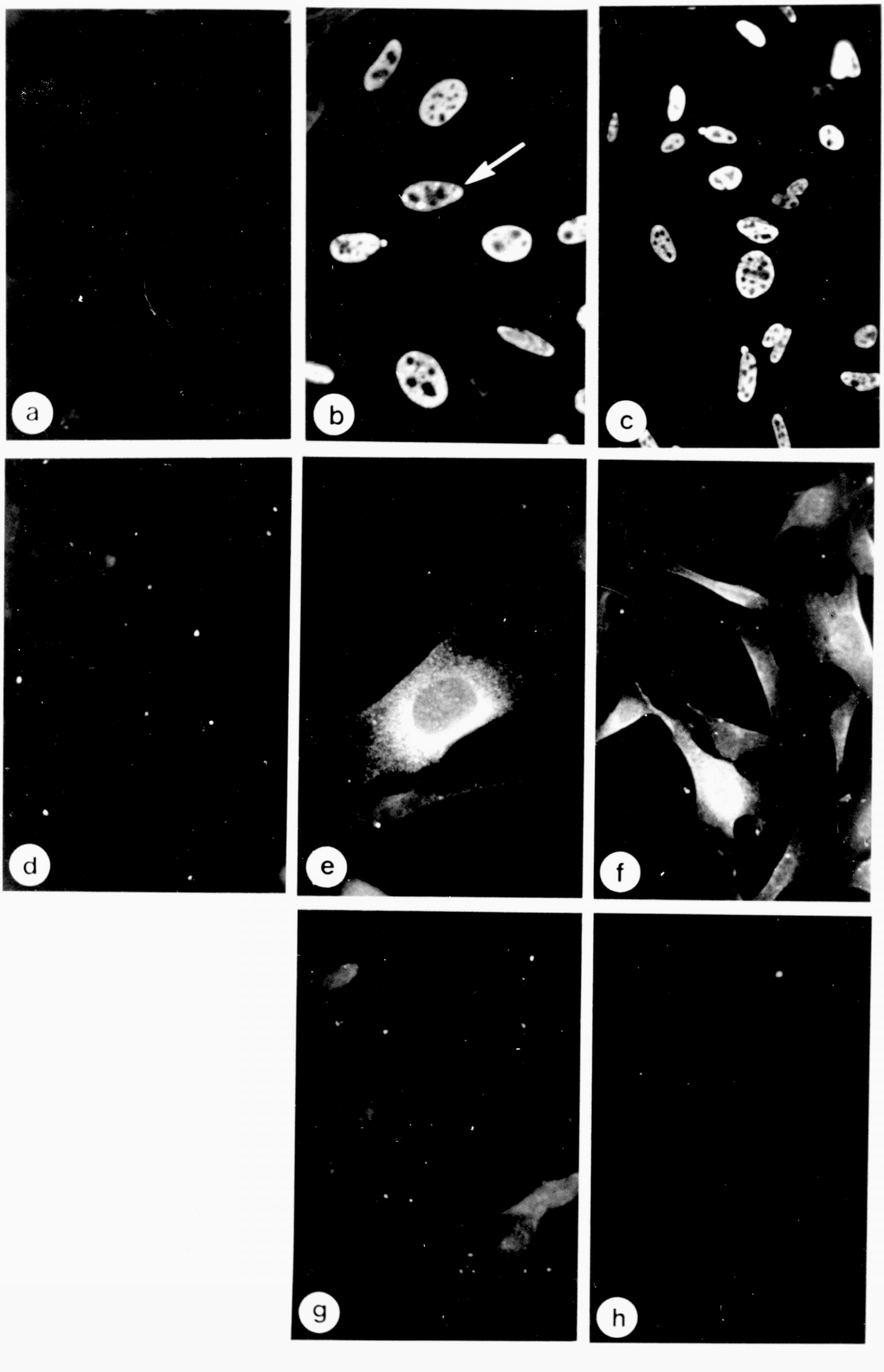
(e) Infected cells reacted with anti-MT antibodies, 10 second exposure.

(f) Infected cells reacted with anti-MT antibodies, 10 second exposure.

(g) Infected cells reacted with control TCF, 10 second exposure.

(h) Infected cells reacted with control TCF, 10 second exposure.

Cell arrowed in (b) shows the frequently observed concentration of LT within the nucleoplasm .


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There also appears to be no concentration at the sites of cell-cell contact. In contrast to the previous studies which suggest that middle T-antigen is mainly associated with plasma membranes and requires detergent for solubilization (Ito et al., 1977b; Ito, 1979), the above data imply that the majority of the middle T-antigen present in lytically infected cells is associated not with the plasma membrane but with cytoplasmic membranes, and probably with the endoplasmic reticulum.

To investigate the detergent solubility of the middle T-antigen that is detected by immunofluorescence, lytically infected cells were treated with Nonidet P40 before fixing with formaldehyde (see Materials and Methods). Under these conditions many membrane proteins are solubilized, but the nuclear membrane remains intact (Osborn and Weber, 1977a). The cytoskeletal network of proteins (Osborn and Weber, 1977 a and b) also remains intact, so any proteins associated with it should also remain. Figure 4.5 shows the immunofluorescent staining obtained with cells treated in this manner. To ensure that the cytoskeletal network was still present, cells similarly treated were reacted with an anti-myosin serum (Weber and Groeschel-Stewart, 1974) and the network analysed by fluorescence (Figure 4.5 (b)). It can be clearly seen that the fibre network remains. The cells used for immunofluorescence were also examined under phase contrast to ensure that the nuclear membrane was still essentially intact (Figure 4.5 (a)). Appropriate controls on uninfected cells and with non-specific TCF are also shown. It can be seen that under these conditions the large T-antigen distribution remained essentially the same as previoiusly found, although the intensity of staining was reduced (Figure 4.5 (e)). This reduction was probably due to the leakage of large T-antigen out of the nuclei at neutral pH, discussed in Chapter One. The middle T-antigen distribution, however, was significantly different from that previously observed. Figure 4.5 (c) shows that the overall diffuse staining was no longer evident and the cytoplasmic staining was completely absent. However, the nuclear membrane staining was still observed, and could be seen as a non-uniform distribution over the membrane surface. These data suggest that middle T-antigen is not tightly associated with the cytoskeleton of lytically infected cells, but the majority of it is bound to cytoplasmic membranes and is easily solubilised by a short incubation with detergent. A small amount of the middle T-antigen in these cells may also be associated with the nuclear envelope.

Figure 4.5 - Effect of Detergent Extraction on T-Antigen In Lytically Infected Cells.

Wild type polyoma virus infected 3T6 Swiss Albino mouse cells, permeabilised by incubation at 0°C for 5 minutes with 1% Nonidet P40 and then fixed with formaldehyde were immunofluoresced with anti-LT and anti-MT antibodies (see Materials and Methods). The photomicrographs are as follows :-

(a) Cells shown under phase contrast conditions to illustrate remaining cellular morphology.

(b) Cells reacted with an anti-myosin antibody to show cytoskeletal structures, 2 second exposure.

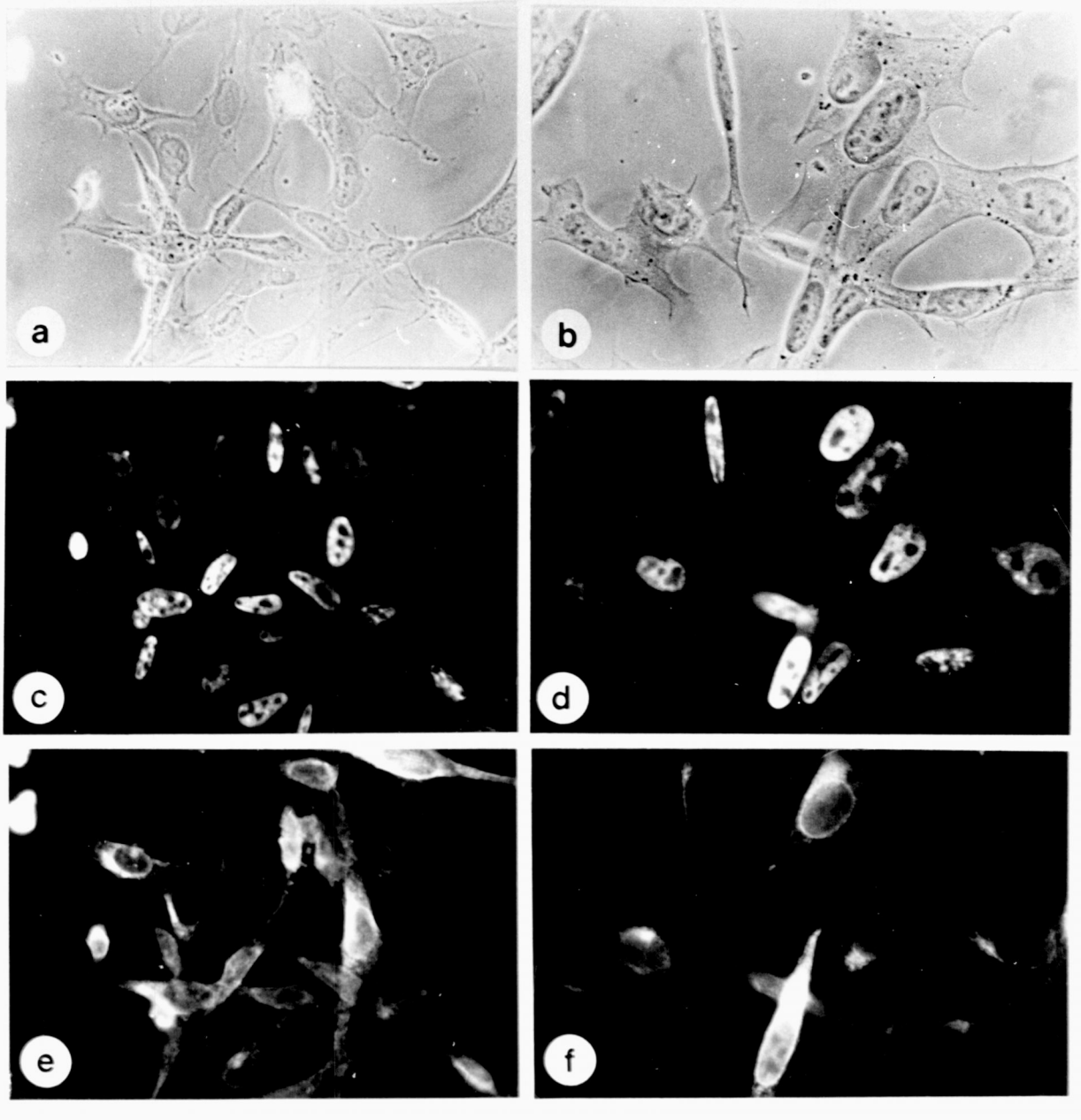
(c) Infected cells reacted with anti-MT antibodies, 15 second exposure.

(d) Mock infected cells reacted with anti-MT antibodies, 15 second exposure.

(e) Infected cells reacted with anti-LT antibodies, 5 second exposure.

(f) Mock infected cells reacted with anti-LT antibodies, 5 second exposure.

(g) Infected cells reacted with control TCF, 15 second exposure.


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f

3T6 Swiss Albino mouse cells lytically infected with the mutant viruses dl-8, dl-23, dl-1013, dl-1014 and dl-1015 were also examined for sub-cellular location of the large and middle T-antigens expressed by these mutants. No difference was observed among any of the mutants, or between mutants and wild type virus infected cells. As suggested in Chapter One, this probably means that the altered properties observed with these viruses are not a result of altered location of the T-antigens.

By incubating with different antisera, it has been possible to detect the same class of antibodies using different fluorochromes. Middle T-antigen has been observed with a rhodamine conjugated goat anti-rabbit IgG antibody via an unlabelled rabbit anti-rat IgG bound to the monoclonal rat anti-MT antibodies, and the large T-antigen in the same cells detected with fluorescein conjugated rabbit anti-rat IgG via the monoclonal rat anti-LT antibodies (see Materials and Methods). Figure 4.6 shows the same field of wild type polyoma virus lytically infected cells examined under phase contrast (Figure 4.6 (a and b)), fluorescein epifluorescent optics for large T-antigen (Figure 4.6 (c) and (d)), and rhodamine epifluorescent optics for middle T-antigen (Figure 4.6 (e) and (f)). All of the features observed for each T-antigen above can also be seen here. Also the fluorescence variability in the intensity of middle T-antigen between cells is clearly visible, and does not correlate completely with the fluorescent intensity of large T-antigen.

Section 4.3 - Immunofluorescent Analysis of Polyoma Virus Transformed Cells.

The locations of large and middle T-antigen in polyoma virus transformed rat cells (PyREWA5/T1A1, Hae 2, annd BE-1) were examined by immunofluorescence. Figure 4.7 shows some of the results obtained. Two controls were again performed to ensure that specific fluorescence was being observed. That is, the same cells were examined for fluorescence with the TCF from NS-1 cells to ensure that there was no reaction with this or with the two anti-IgG antibodies used, and secondly, the untransformed Rat-1 cells (the parental line for the Hae 2 and BE-1 transformed cells) were examined with anti-LT and anti-MT antibodies to ensure that only the viral T-antigens were being analysed. (It can be seen that these controls produce only a very low intensity fluorescence). The specific fluorescent intensity observed was considerably less than that found with lytically infected cells, probably a reflection of the lower quantities of T-antigen present in these cells.

Figure 4.6 - Double Staining of Lytically Infected Cells.

Wild type polyoma virus infected mouse cells, formaldehyde fixed and permeabilised with Nonidet P40 were stained with rhodamine for anti-MT antibodies and fluorescein for anti-LT antibodies (see Material and Methods). Photomicrographs (a), (c) and (e) show the same field of cells using a x40 objective lens (b), (d) and (f) the same field using a x63 objective lens.

(a) and (b) were photographed under phase contrast conditions.

(c) and (d) were photographed under epifluorescent conditions with fluorescein specific filters.

(e) and (f) were photographed under epifluorescent conditions with rhodamine specific filters.

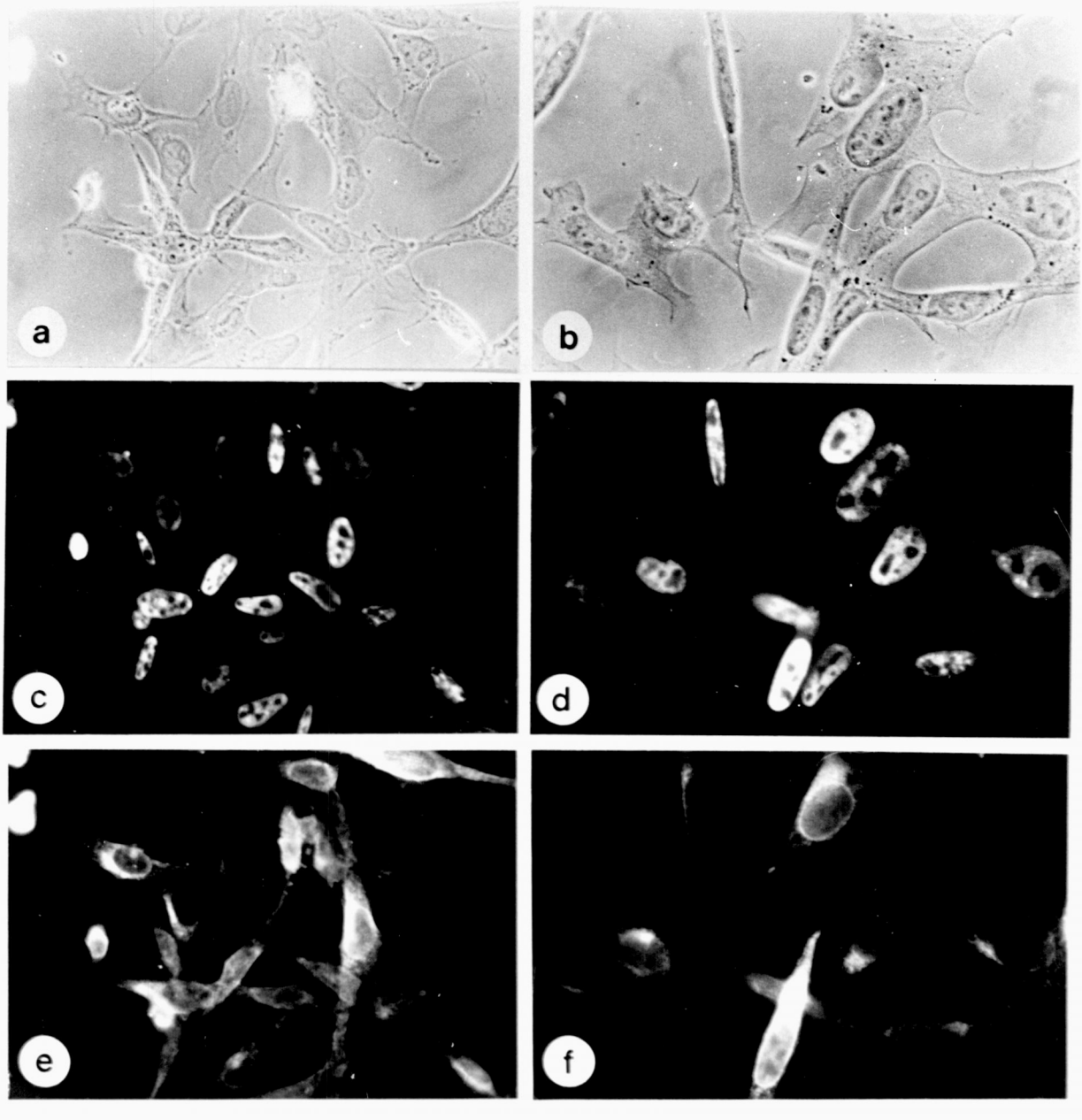

a
b
c
d
e
f

Figure 4.7 - T-Antigen Immunofluorescence of Polyoma Virus Transformed Rat Cells.

Polyoma virus transformed rat cell lines, formaldehyde fixed and Nonidet P40 permeabilised were observed by fluorescence using anti-LT and anti-MT antibodies. All photomicrographs were taken with a x63 objective lens. The cells and antibodies used are as follows :-

(a) Non-transformed Rat-1 cells, reacted with anti-MT antibodies, 20 second exposure.

(b) Non-transformed Rat-1 cells, reacted with anti-LT antibodies, 10 second exposure.

(c) Hae 2 cells, reacted with control TCF, 15 second exposure.

(d) Hae 2 cells, reacted with anti-MT antibodies, 15 second exposure.

(e) Hae 2 cells, reacted with anti-LT antibodies, 10 second exposure.

(f) PyREWA5/T1A1 cells, reacted with control TCF, 20 second exposure.

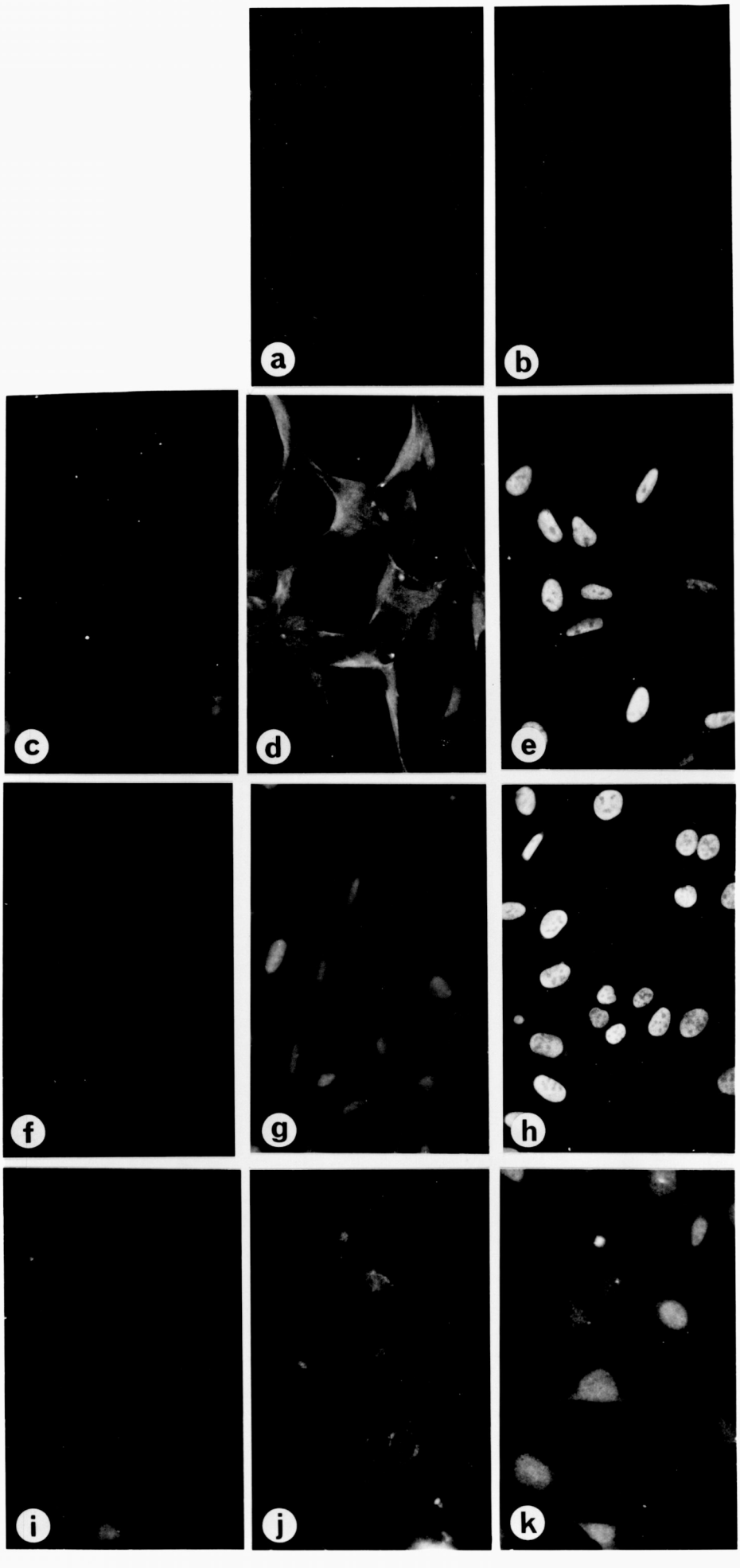
(g) PyREWA5/T1A1 cells, reacted with anti-MT antibodies, 20 second exposure.

(h) PyREWA5/T1A1 cells, reacted with anti-LT antibodies, 10 second exposure.

(i) BE-1 cells, reacted with control TCF, 15 second exposure.

(j) BE-1 cells, reacted with anti-MT antibodies, 10 second exposure.

(k) BE-1 cells, reacted with anti-LT antibodies, 15 second exposure.


a
b
c
d
e
f
g
h
i
j
k

However, a number of conclusions can be made from this study. The Hae 2 and PyREWA5/T1A1 lines express a full-sized large T-antigen (A. Hayday and G. Bjursell, personal communication; Ito and Spurr, 1980; Chapter Three). The antigen is observed to be located within the nucleoplasm. Unlike the result with lytically infected cells, there does not appear to be a concentration of large T-antigen into discrete areas, but the nucleoli are again prominent and unstained. The truncated large T-antigen-related polypeptide present in BE-1 cells (see Appendix 1 and Chapter Three) stains poorly, but appears to be mainly concentrated within the nucleus. For both the Hae 2 and PyREWA5/T1A1 cells, the middle T-antigen staining pattern is of low intensity and diffuse, although there is possibly a concentration in the nucleus (or nuclear membrane) with PyREWA5/T1A1 cells. The middle T-antigen immunofluorescence in BE-1 cells, however, is of a higher intensity and is observed to be very similar to that found in lytically infected mouse cells, particularly with respect to the nuclear membrane location.

Section 4.4 - Discussion

The sub-cellular location of the large and middle T-antigens have been examined directly by immunofluorescent analysis of fixed cells. The results presented in this Chapter confirm the nuclear location of large T-antigen previously obtained from data derived from sub-cellular fractionation studies, and for lytically infected cells, have extended this result to show that the polypeptide is concentrated into discrete complexes within the nucleoplasm. The cytoplasm and the prominent nucleoli are essentially free of large T-antigen. The significance of the concentration of large T-antigen within discrete areas is unclear, but intriguing. Since these complexes do not appear to be present in rat cells transformed with polyoma virus, they may be related to the lytic infection process. It is known that large T-antigen is involved in the initiation of viral DNA synthesis (Francke and Eckhart, 1973) so it is further possible that these antigen concentrations may reflect regions of viral DNA synthesis within the nucleoplasm. It has also been suggested that large T-antigen may be involved in the assembly of virus particles (Chapter Two), they could be regions of virion assembly. The determination of the function of these complexes will require further study.

The middle T-antigen location seen by immunofluorescence appears to conflict with the previous data derived from sub-cellular fractionation studies. No evidence for plasma membrane location is seen in immunofluorescence studies, although it cannot be excluded that some of the middle T-antigen molecules are located at this site.

Provided that the middle T-antigen recognized in both kinds of study is the same, it would appear that the sub-cellular fractionation studies may possibly give misleading results. For example, it has been reported that the amount of middle T-antigen isolated from the plasma membrane is considerably less than that isolated by a detergent lysis of whole cells (Smith et al., 1979), so it is possible that in these experiments only a small fraction of the total middle T-antigen was being studied. Another possibility is that despite measurements of enzyme markers, the sub-cellular fractionation procedures were not sufficiently specific and plasma membrane preparations are contaminated with other membrane components (Ito et al., 1977b). It appears likely that the middle T-antigen is associated with cellular membranes, but in addition to the plasma membrane, is associated with the endoplasmic reticulum and nuclear membranes. This has been confirmed directly by immuno-electron microscopy (G. Bjursell and H. A. Hansson, personal communication), where middle T-antigen was observed to be associated predominantly with the endoplasmic reticulum, with a small amount being also present on the nuclear envelope and the plasma membrane. This location is similar to that observed for the pp60 ^{src} transforming gene product from certain Rous Sarcoma Virus mutants (Garber et al., 1982) which has also been shown to be associated with reticular membranes. Moreover, the fluorescence observed with known endoplasmic reticulum enzymes appears to be similar to that observed here (Garber et al., 1982).

During these studies an indirect technique has been developed to allow two similar antibodies to be detected by different fluorochromes. This technique evolved from a method of amplifying the signal produced by the binding of monoclonal antibodies and was developed to allow large and middle T-antigens to be investigated independently within the same cell, but it is applicable to any other pair of antibodies.

CHAPTER FIVE

Section 5.1 - Aims

It has been shown previously (Smith et al., 1979; Schaffhausen and Benjamin, 1979) that when immunoprecipitates of the polyoma virus T-antigens that contain the middle T-antigen are subjected to an in vitro phosphorylation reaction, similar to that devised by Collett and Erikson (1978), a protein kinase activity is evident. This activity has been demonstrated to transfer (^{32}P) from γ-(^{32}P) adenosine triphosphate (ATP) onto the middle T-antigen polypeptide and under some conditions, onto the heavy chain of the rat immunoglobulin molecule (Smith et al., 1979). The protein kinase activity has the unusual property of transferring the phosphate group onto the amino acid tyrosine, rather than onto the more usual acceptor, serine or threonine (Eckhart et al., 1979). When there is no middle T-antigen present in the immunoprecipitate (for example, in those from uninfected cell lysates, or lysates of cells infected with the hr-t mutant NG-18), this activity is not detected. It is decreased in immunoprecipitates from lysates of cells infected with viral mutants that are defective in their transforming ability (for example, the polyoma virus mutant dl-23). The ability of anti-T serum to immunoprecipitate this activity has been shown to vary greatly among different sera (Smith et al., 1980).

The studies described above were all conducted on immunoprecipitates obtained with anti-T sera, and it could not, therefore, be discounted that this sera contained low level activities against proteins other than the known T-antigens. For example, a number of authors have reported other polypeptides to be immunoprecipitated specifically by anti-T serum (Ito et al., 1977a; Ito et al., 1977b; Schaffhausen et al., 1978; Silver et al., 1978; Hutchinson et al., 1978). It is therefore possible that it is these proteins that are responsible for the protein kinase activity observed in vitro, and that the middle T-antigen is merely a substrate for this activity. The close correlation observed to exist between the level of the reaction and the concomitant transforming ability of the virus has made a further examination and characterization of this activity of considerable interest. The monoclonal antibodies described in Chapter Two provide very specific probes for studying the reaction. Since they are are reasonably well characterised, the polypeptides specifically immunoprecipitated can be used to define the components necessary for the expression of the in vitro protein kinase activity. The antigenic sub-groups of middle T-antigen identified in Chapter Three as a result of

reaction with different monoclonal antibodies, may also reflect an altered enzymic activity among the different polypeptides, so these were also examined for altered in vitro protein kinase activities.

Section 5.2 - Protein Kinase Activities Present in Monoclonal Antibody Immunoprecipitates.

A comparison of the polypeptides immunoprecipitated from lysates of wild type polyoma virus infected 3T6 Swiss Albino mouse cells by the ten monoclonal antibodies (described in Chapter Two) after labelling with (^{35}S)-methionine in vivo, or phosphorylated in vitro by γ(^{32}P) ATP, is shown in Figure 5.1. The conditions used for immunoprecipitation were identical with the two labels; a long period of labelling in vivo was used to give a more genuine reflection of the polypeptides present rather than a bias towards rapidly synthesized proteins that could result from short-term labelling procedures. The (^{35}S) labelled polypeptides are similar to those described in Chapter Two. The polypeptides phosphorylated in vitro show considerable variation with the antibody used in the immunoprecipitation.

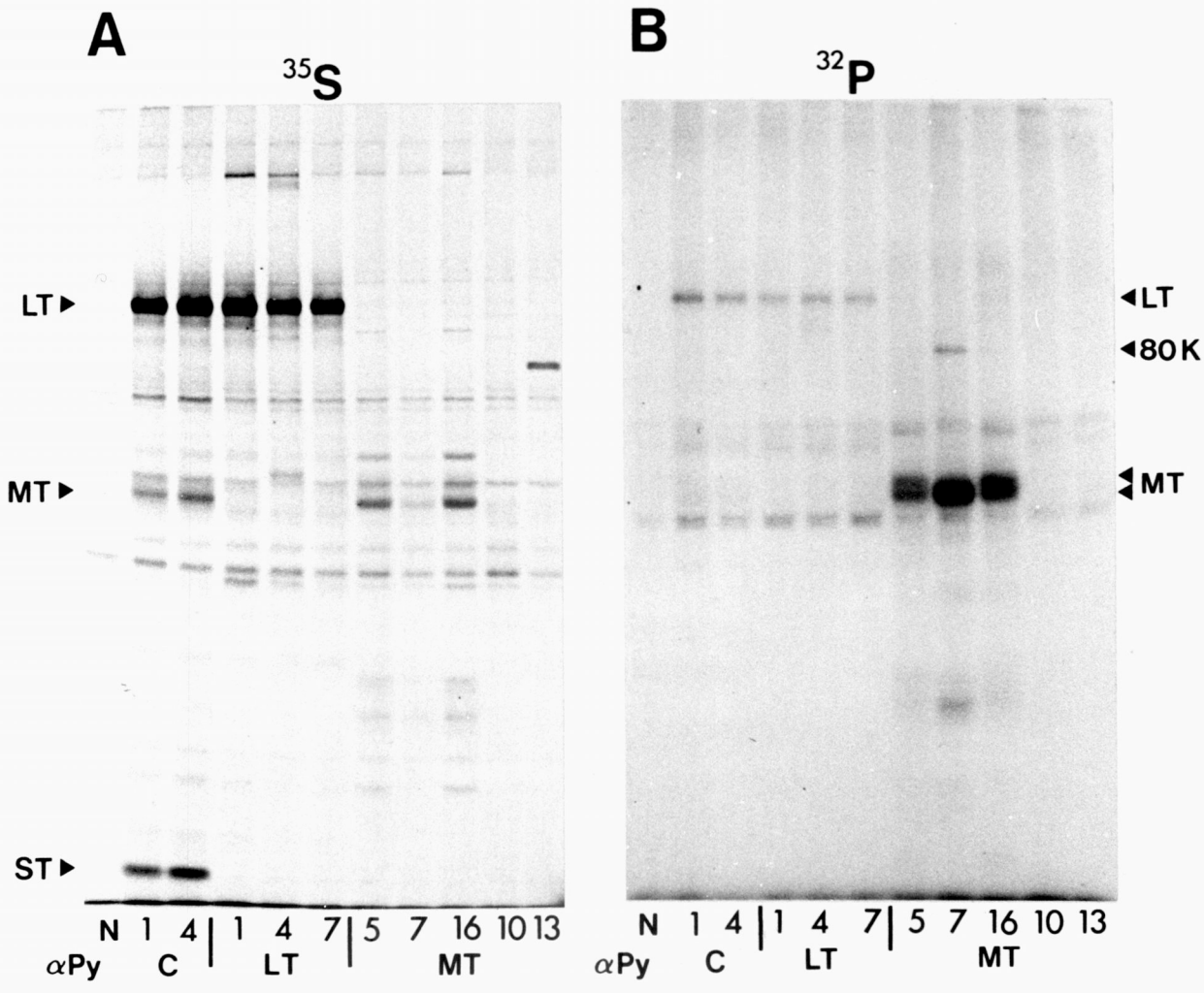
In immunoprecipitates with αPyLT1, αPyLT4 and αPyLT7 antibodies, no phosphorylation of polypeptides of the same MWtapp on SDS-PAGE as the middle T-antigen is observed, as expected. Interestingly, only a small amount of phosphate incorporation into a polypeptide that comigrates with middle T-antigen is observed in immunoprecipitates with either αPyC1 or αPyC4 antibodies, despite the fact that these precipitates have been shown to contain most of the in vivo labelled middle T-antigen polypeptides (see Chapter Three). No phosphorylation of a polypeptide of the same MWtapp on SDS-PAGE as middle T-antigen is observed in immunoprecipitates with αPyMT10 or αPyMT13 antibodies. It is therefore probable that the minor sub-groups of middle T-antigen molecules recognized by these antibodies (see Chapter Three) are not involved in the protein kinase activity expressed in vitro.

Immunoprecipitates with αPyMT5 and αPyMT16 antibodies show significant incorporation of phosphate into a polypeptide of the same MWtapp on SDS-PAGE as middle T-antigen, and also into a polypeptide of a MWtapp 57Kd, an extra 2Kd larger than middle T-antigen. This is similar to the in vitro phosphorylation reaction observed by Schaffhausen and Benjamin (1981). Immunoprecipitates with αPyMT7 antibodies incorporate still larger amounts of phosphate into a polypeptide of the same MWtapp on SDS-PAGE as middle T-antigen. This high level of phosphate incorporation contrasts with results obtained with in vivo labelling, where it was observed that αPyMT7 antibodies only bind

Figure 5.1 - Comparison of T-Antigens Labelled In Vivo or by Protein Kinase Activity In Vitro in Monoclonal Antibody Immunoprecipitates.

A. Polypeptides from 3T6 mouse cells were infected with wild type polyoma virus, labelled in vivo with (^{35}S)-methionine, and immunoprecipitated by the ten monoclonal antibodies described in Chapter Two. The cells were labelled with 1mCi of (^{35}S)-methionine/90mm dish of cells between 24 and 42 hours post infection at 32°C, as described in Materials and Methods. Immunoprecipitates for this experiment and those in B. (below) were conducted in parallel using SAC, plus MRC Ox12 antibody. The immunoprecipitated polypeptides were separated by SDS-PAGE on a 10% gel and visualized by autoradiography. The antibodies used are indicated underneath; lane N shows immunoprecipitates of polypeptides using tissue culture fluid from the growth of the myeloma parental cells, NS-1. Positions of the (^{35}S)-labelled large (LT), middle (MT) and small (ST) T-antigens are indicated on the left.

B. Polypeptides from wild type polyoma virus infected 3T6 cell lysates were immunoprecipitated with monoclonal antibodies, and labelled in vitro by incubation with γ-(^{32}P)-ATP. Protein kinase reactions were conducted as described in Materials and Methods, and the antibodies used are indicated underneath. The positions of the (^{32}P) labelled large (LT) and middle (MT) T-antigens, as well as the apparent 80,000 (80K) molecular weight polypeptides, are indicated on the right.


A
35S
LT
MT
ST
N 1 4 1 4 7 5 7 16 10 13
αPy C LT MT
B
32P
LT
80K
MT
N 1 4 1 4 7 5 7 16 10 13
αPy C LT MT

approximately half of the available middle T-antigen present in a lytically-infected cell lysate. No phosphorylation of a 57Kd MWtapp polypeptide was observed in the immunoprecipitates with this antibody.

Figure 5.1 also shows that polypeptides other than middle T-antigen(s) are phosphorylated in immunoprecipitates that are subjected to the in vitro phosphorylation reaction. In particular, a polypeptide of the same MWtapp on SDS-PAGE as large T-antigen is phosphorylated. This only occurs when large T-antigen is known to be immunoprecipitated, that is, in immunoprecipitates with αPyC or αPyLT antibodies. It is therefore likely that this represents phosphorylation of large T-antigen molecules. Also a MWtapp 80Kd polypeptide is phosphorylated in immunoprecipitates with αPyMT5, αPyMT7 and αPyMT16 antibodies and, similar to results found for middle T-antigen, is observed to be phosphorylated to the greatest extent in immunoprecipitates with the αPyMT7 antibody; phosphorylation of this polypeptide in αPyMT5 and αPyMT16 antibody immunoprecipitates occurs at a low level, and is only detected by autoradiography using a long exposure time. Table 5.1 shows the amount of phosphate incorporated into these polypeptides as assayed by excising the appropriate gel band and determining its radioactivity by the Cerenkov method.

To investigate the role of the second antibody used in these in vitro phosphorylation reactions, an experiment was conducted using five monoclonal antibodies with SAC only, or SAC and MRC 0x12 antibody, or SAC and a rabbit anti-rat IgG antibody. The resulting phosphorylated polypeptides are shown in Figure 5.2. It can be seen that there is no significant differences between the reactions involving the MRC Ox12 antibody or the rabbit anti-rat IgG , but there is no apparent phosphorylation of the 55 and 57Kd polypeptides when SAC alone is used. The low level phosphorylation of the large T-antigen is still observed, however, with SAC only. It has been shown in Chapter Three that some immunoprecipitation of large and middle T-antigens occurs with SAC alone, but that the reaction is incomplete. This precipitation has been postulated to be due to the existence of multimeric forms of both large and middle T-antigens. The phosphorylation data suggest that the form of middle T-antigen phosphorylated in vitro is not represented in this multimeric state, whereas the large T-antigen that becomes phosphorylated is multimeric.

Section 5.3 - Characterization of the Phosphorylated Polypeptides.

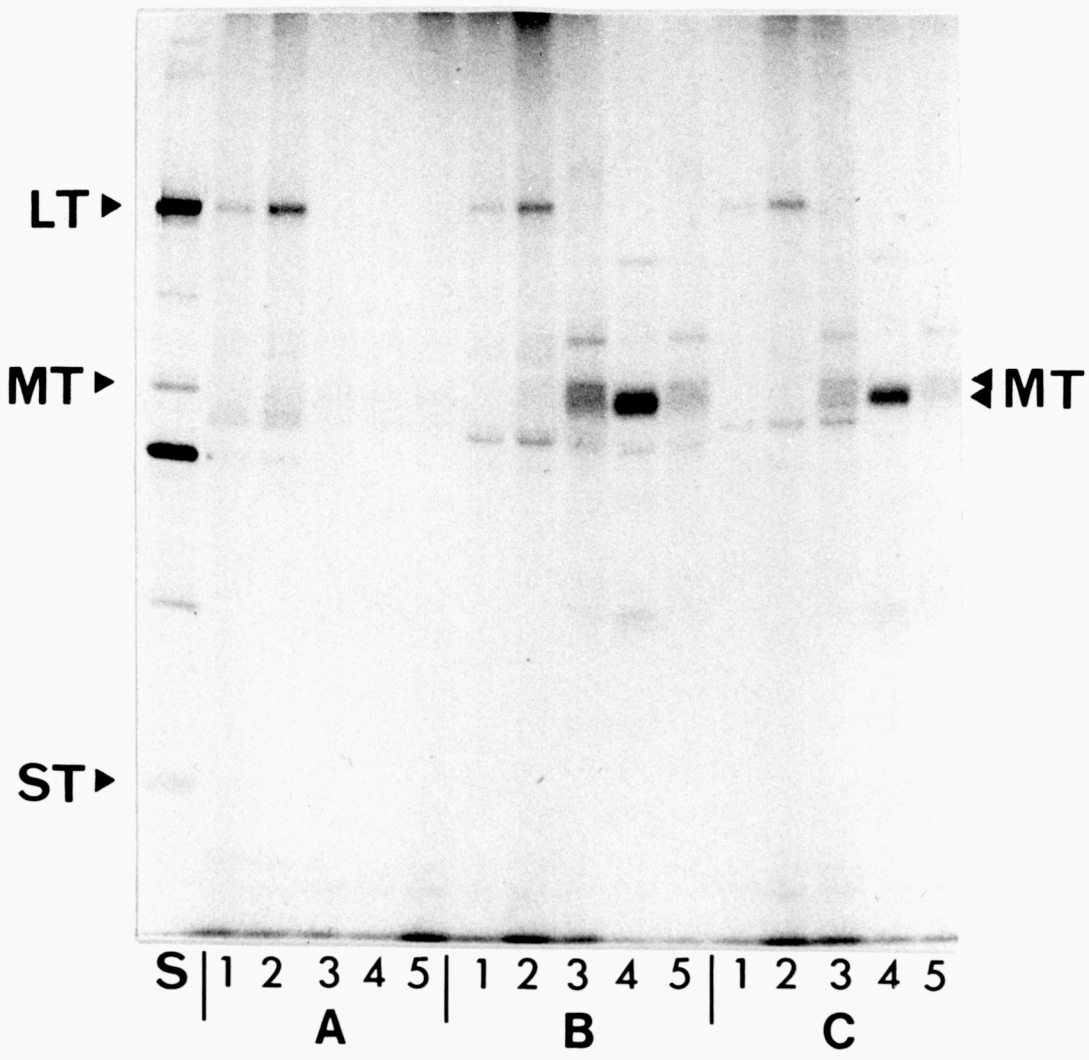
To ensure that the phosphorylation reactions observed in Section 5.2 are similar to those reported previously, the polypeptides phosphorylated in the reactions with monoclonal antibodies have been

Table 5.1. Quantitation of ^{32}P Incorporation into Polypeptides in an *In Vitro* Protein Kinase Reaction

Monoclonal Antibody Used to Effect Immunoprecipitation	Apparent Molecular Weight on SDS-PAGE.	Radioactivity (Cerenkov c.p.m.)
αPyC1	100 Kd	260
αPyLT1	100 Kd	240
αPyMT5	55 Kd	550
αPyMT5	57 Kd	650
αPyMT5	80 Kd	100
αPyMT7	55 Kd	3500
αPyMT7	80 Kd	250
αPyMT16	55 Kd	750
αPyMT16	57 Kd	800
αPyMT16	80 Kd	90

Figure 5.2 - Effect of Second Antibody on the Protein Kinase Activity Expressed In Vitro

Polypeptides from lysates of 3T6 mouse cells were infected with wild type polyoma virus, immunoprecipitated with monoclonal antibodies, and phosphorylated in vitro by γ-(^{32}P)-ATP. (^{35}S)-Methionine containing T-antigens labelled in vivo are shown in track S, and the position of the large (LT), middle (MT) and small (ST) T-antigen are indicated on the left. Polypeptides were separated by SDS-PAGE on a 10% gel and detected by autoradiography. Immunoprecipitation was accomplished by SAC alone in lanes A; SAC plus MRC Ox12 antibody in lanes B; and SAC plus rabbit anti-rat IgG in lanes C (see Materials and Methods). In each case the monoclonal antibody used is indicated underneath: lanes 1 were immunoprecipitated with αPyLT1; lanes 2 with αPyC1; lanes 3 with αPyMT5; lanes 4 with αPyMT7; and lanes 5 with αPyMT16. The two MWtapp phosphorylated forms of middle T-antigen (MT) are indicated on the right.


LT
MT
ST
MT
S 1 2 3 4 5 1 2 3 4 5 1 2 3 4 5
A
B
C

characterised. Figure 5.3 shows an analysis of the amino acids phosphorylated in the various reactions. The phosphorylated polypeptides were eluted from gels and hydrolysed to release amino acids which were then separated by electrophoresis on thin layer cellulose plates. The MWtapp 55Kd and 57Kd phosphorylated species in αPyMT5 and αPyMT7 antibody immunoprecipitates were found to be phosphorylated on a tyrosine residue, as previously reported (Eckhart et al., 1979). Interestingly, the MWtapp 80Kd polypeptide is also phosphorylated on a tyrosine residue. The large T-antigen, however, was phosphorylated on serine and threonine residues.

The phosphorylated polypeptides were analysed by treatment of the appropriate proteins for a limited period with protease, followed by separation of the resulting products by SDS-PAGE. Figures 5.4 and 5.5 show some of the polypeptides obtained by this limited proteolysis analysis. It can be seen in Figure 5.4 that the polypeptides obtained from digestion of the (^{35}S)-methionine labelled middle T-antigen immunoprecipitated by αPyMT5 antibodies are extremely similar to those obtained after digestion of the in vitro phosphorylated MWtapp 55Kd species obtained by immunoprecipitation with αPyMT7 antibody. In the latter reaction, the phosphate group has therefore been transferred to a middle T-antigen molecule. Figure 5.5 shows a comparison of some other phosphorylated polypeptides. No significant difference between the MWtapp 55Kd and 57Kd polypeptides phosphorylated in αPyMT5 antibody immunoprecipitates, or the MWtapp 55Kd polypeptide phosphorylated in αPyMT7 antibody immunoprecipitates, are observed. The MWtapp 80Kd polypeptide phosphorylated in αPyMT7 antibody immunoprecipitates, however, is clearly different.

Section 5.4 - Characterization of Components of the In Vitro Phosphorylation Reaction.

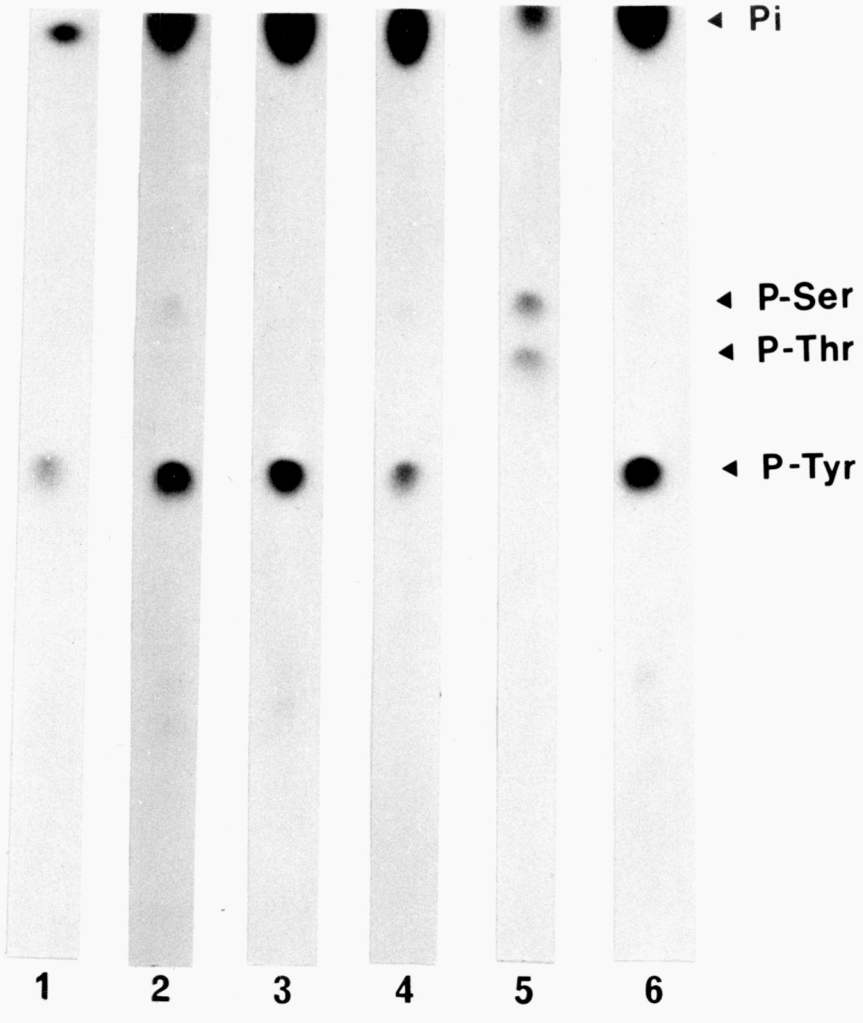
The failure of a middle T-antigen polypeptide to be phosphorylated in immunoprecipitates with αPyC1 and αPyC4 antibodies is surprising when the ability of these antibodies to immunoprecipitate metabolically labelled middle T-antigen is considered (see Chapter Three). A number of possibilities could account for this observation. The obvious possibilities are: (a) The binding of the αPyC1 and αPyC4 antibodies to the middle T-antigen molecule could inhibit the enzymic activity responsible for the phosphorylation. (b) The binding of the αPyC1 and αPyC4 antibodies fails to stimulate the enzymic activity necessary to phosphorylate the polypeptides, whereas αPyMT5, αPyMT7 and αPyMT16 antibodies do stimulate this activity. (c) The components of the in vitro

Figure 5.3 - Analysis of Amino Acids Phosphorylated In Vitro

An analysis of the phosphoamino acids present in the phosphorylated polypeptides are observed in Figure 5.1B. Phosphoamino acids are derived from: (column 1) the MWtapp 55Kd polypeptide precipitated by αPyMT5 antibody; (2), 57Kd polypeptide precipitated by αPyMT5; (3), 55Kd polypeptide precipitated by αPyMT7; (4), 80Kd polypeptide precipitated by αPyMT7; (5), 100Kd polypeptide precipitated by αPyC1; (6), 55Kd polypeptide precipitated by αPyMT5+αPyMT7 using SAC alone (see Figure 5.6.B). The positions of marker phosphoserine (P-Ser), phosphothreonine (P-Thr), phosphotyrosine (P-Tyr) and inorganic phosphate (Pi) are indicated on the right.

Figure 5.4 Limited Proteolytic Cleavage Analysis of Middle T-antigen and the 55Kd In Vitro Phosphorylated Polypeptide.

Fragments produced by limited proteolytic digestion of methionine (^{35}S) labelled middle T-antigen and the (^{32}P) labelled 55Kd species and immunoprecipitated with αPyMT7 antibody. Concentrations (in mg/ml) of S.aureus V8 protease used in the experiment are indicated underneath. Polypeptides were separated by SDS-PAGE on a 15% gel, and detected by autoradiography.


Pi
P-Ser
P-Thr
P-Tyr
1
2
3
4
5
6

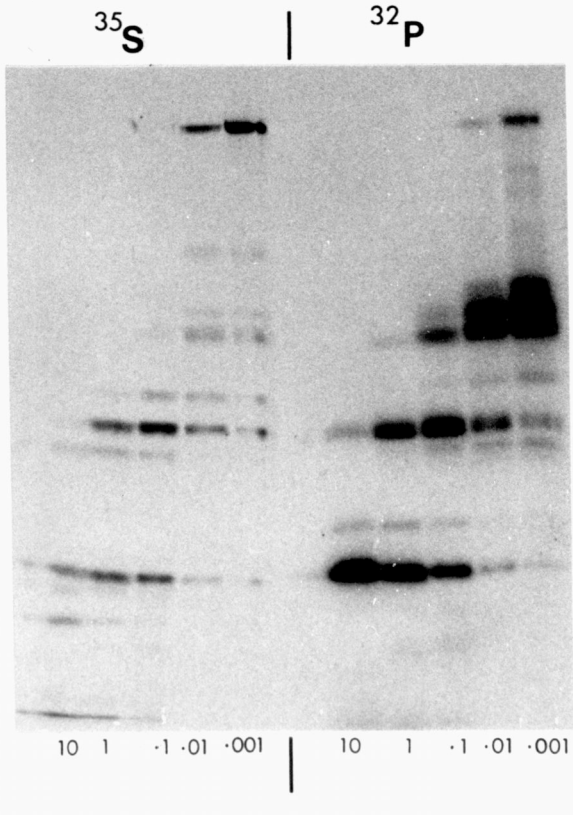
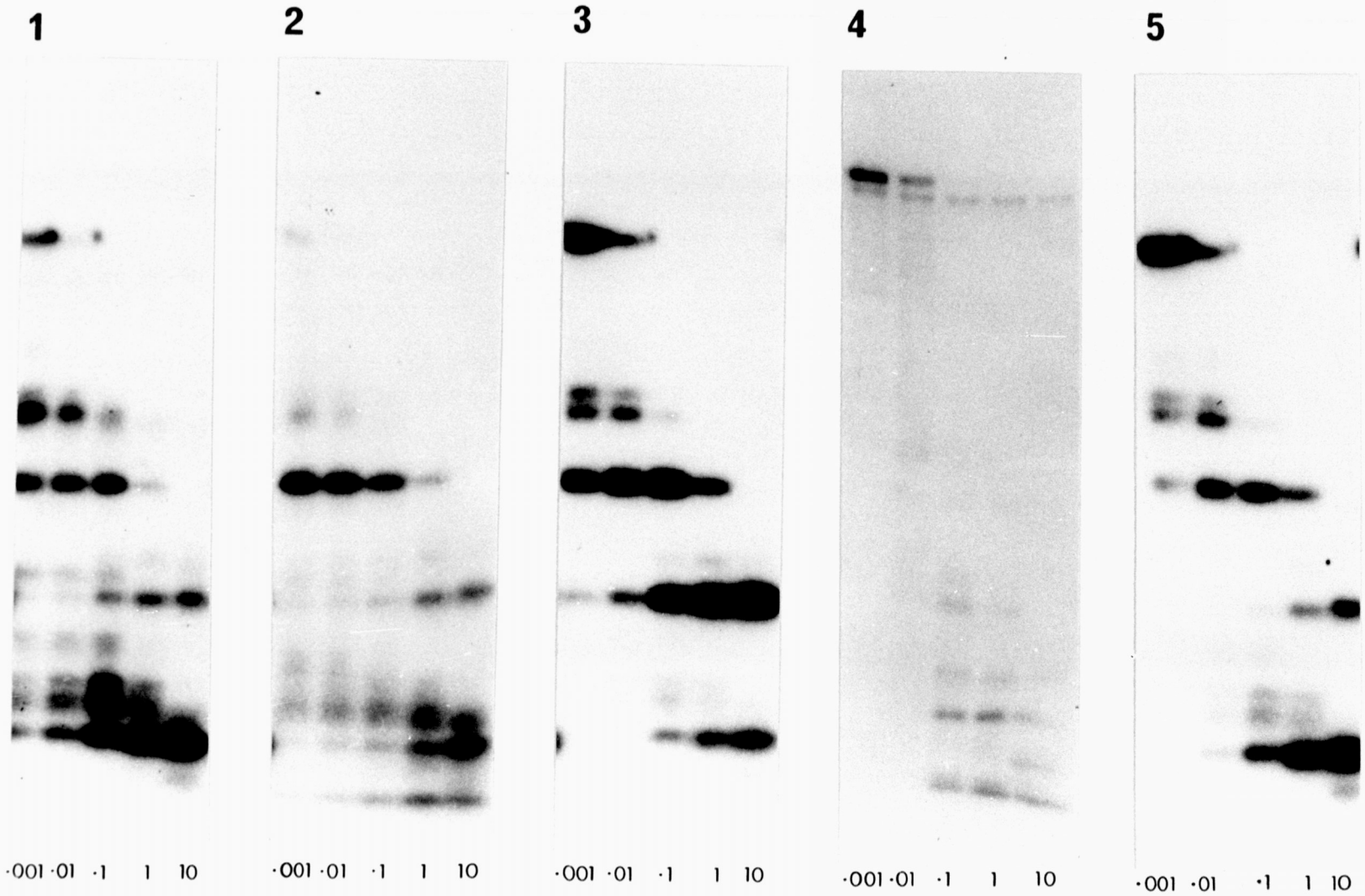

35S
32P
10 1 ·1 ·01 ·001
10 1 ·1 ·01 ·001

Figure 5.5 - Limited Proteolytic Cleavage Analysis of In Vitro Phosphorylated Polypeptides.

A comparison made by limited proteolytic cleavage of the different phosphorylated polypeptides isolated following in vitro protein kinase reactions. The concentrations of S.aureus V8 protease (in mg/ml) are indicated underneath. Panel 1 is the 55Kd polypeptide immunoprecipitated by ∝PyMT5 antibody; panel 2, the 57Kd polypeptide immunoprecipitated by ∝PyMT5; panel 3, the 55Kd polypeptide immunoprecipitated by ∝PyMT7; panel 4, the 80Kd polypeptide immunoprecipitated by ∝PyMT7; and panel 5, the 55Kd polypeptide immunoprecipitated by ∝PyMT5+∝PyMT7 antibodies with SAC alone (see Figure 5.6.B). Polypeptides were separated by SDS-PAGE on a 15% gel, and detected by autoradiography.


1
2
3
4
5
·001 ·01 ·1 1 10
·001 ·01 ·1 1 10
·001 ·01 ·1 1 10
·001 ·01 ·1 1 10
·001 ·01 ·1 1 10

protein kinase reaction require to be correctly orientated in order for the reaction to occur, and this is not achieved with the αPyC1 and αPyC4 antibodies. (d) The antigenic form of the middle T-antigens immunoprecipitated by αPyC1 and αPyC4 antibodies are not components of the in vitro protein kinase activity.

To investigate these possibilities, two approaches were used, one of which involved the simultaneous addition of two monoclonal antibodies, and the other, a sequential immunoprecipitation reaction.

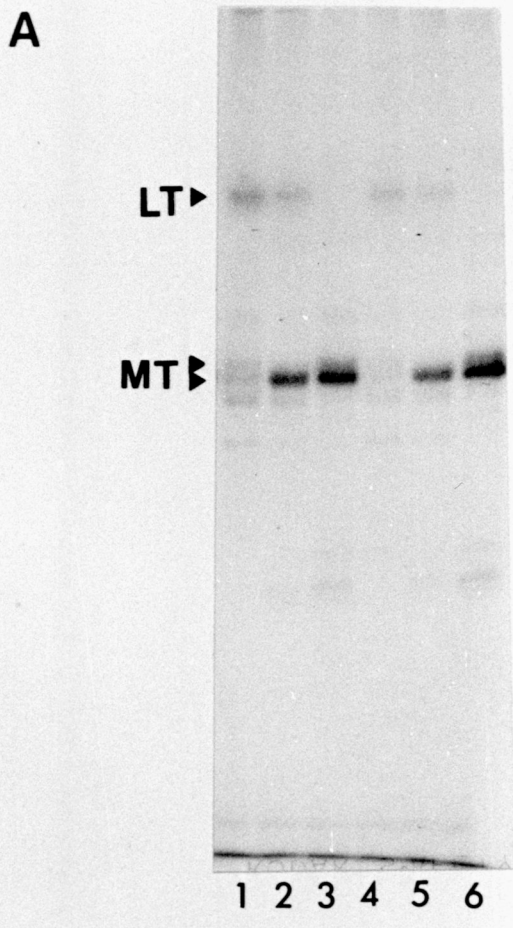
Figure 5.6A shows the polypeptides phosphorylated in immunoprecipitates from lytically infected 3T6 Swiss Albino mouse cells subjected to an in vitro phosphorylation reaction, using two monoclonal antibodies which have been found not to inhibit the binding of one another (see Chapter Three). The data obtained show that the normal level of phosphorylation with αPyMT5 and αPyMT7 antibodies to be observed in cases where αPyLT1 antibodies was also present, the latter being used as a control for the experiment. The presence of αPyC4 antibody did not affect the level of the phosphorylation reaction observed in αPyMT5 or αPyMT7 antibody immunoprecipitates. This indicates that the binding of the αPyC4 antibody does not inhibit the enzymic reaction that is responsible for the protein kinase activity. Interestingly, it can also be seen that the higher level of middle T-antigen phosphorylation observed in αPyMT7 antibody immunoprecipiates was not altered by the presence of either αPyMT5 or αPyMT16 antibodies. Therefore the lower levels of phosphorylation observed with the latter antibodies are probably not a result of an inhibition of an enzymic activity. When the same pairs of antibodies were used to immunoprecipitate the T-antigens with SAC alone (Figure 5.6B), as in Figure 5.2, no phosphorylation was observed, except in the case where αPyMT7 antibody was used in conjunction with either αPyMT5 or αPyMT16 antibodies. It was shown in Chapter Three that the use of two antibodies increased the efficiency of SAC immunoprecipitation, probably as a result of promoting the formation of large immune complexes. It cannot be excluded that the addition of two antibodies immunoprecipitates conformational forms of middle T-antigen molecules other than the multimeric forms immunoprecipitated by a single monoclonal antibody, and these are responsible for the protein kinase activity. The failure of phosphorylation to occur in immunoprecipitates with a single monoclonal antibody and SAC alone may be due to the fact that the "active" form of middle T-antigen is not being precipitated, rather than to any particular effect of a second antibody.

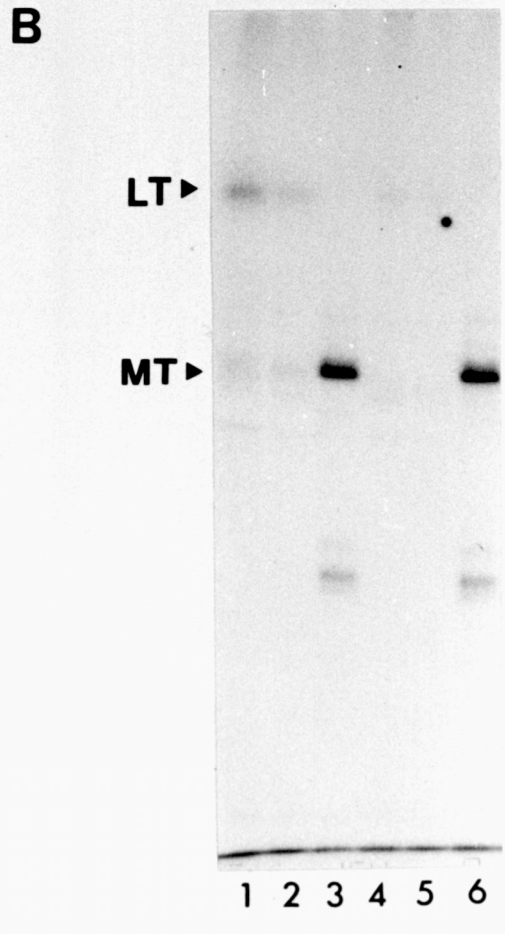
Figure 5.7 shows data obtained on the phosphorylation of

Figure 5.6 - Effect on Protein Kinase Activity of Immunoprecipitation with Two Monoclonal Antibodies.

A. An analysis of the polypeptides from a lysate of wild type polyoma virus infected 3T6 mouse cells phosphorylated in an in vitro protein kinase reaction and immunoprecipitated with two monoclonal antibodies and SAC plus MRC Ox12. The antibodies used were: lane 1, αPyC4 and αPyMT5; 2, αPyC4 and αPyMT7; 3, αPyMT5 and αPyMT7; 4, αPyMT5 and αPyLT1; 5, αPyMT7 and αPyLT1; 6, αPyMT7 and αPyMT16. Polypeptides were separated by SDS-PAGE on a 10% gel and detected by autoradiography (conducted for a shorter time than used for Figure 5.1.B. to allow variations in the level of phosphorylation to be detected). The positions of large (LT) and middle (MT) T-antigens are indicated on the left.

B. As above, but the polypeptides phosphorylated in an in vitro protein kinase reaction and immunoprecipitated with two monoclonal antibodies and SAC alone. The antibodies used are those indicated above, respectively, and the positions of large (LT) and middle (MT) T-antigens are indicated on the left.


A
LT
MT
1 2 3 4 5 6


B
LT
MT
1 2 3 4 5 6

polypeptides in a sequential immunoprecipitation procedure. In this experiment, a lysate of wild type polyoma virus infected 3T6 Swiss Albino mouse cells was subjected to a "cascade" reaction similar to that described in Chapter Three, and the first, fourth and final immunoprecipitates examined for in vitro protein kinase activity. The αPyLT1 antibody was used as a control, and it can be seen that it had no effect on any component of the in vitro protein kinase activity, leaving the level and type of reaction unchanged. Further, it was observed that the αPyC4 antibody did not bind to any component of this reaction, leaving the activity present in immunoprecipitates with αPyMT5 and αPyMT16 antibodies essentially unchanged. These data indicate that the middle T-antigen molecules present in immunoprecipitates obtained with αPyC4 (or αPyC1) antibody are not involved in the in vitro protein kinase activity. This suggests that the N-terminal region of the middle T-antigen molecules that are phosphorylated in vitro are antigenically different from the large proportion of the metabolically labelled molecules which are immunoprecipitated by αPyC4 antibodies (see Chapter Three). Figure 5.7 further shows that αPyMT5 and αPyMT16 antibodies are both capable of binding all of the middle T-antigen that is phosphorylated in αPyMT7 antibody immunoprecipitates. Since the activity in αPyMT5 and αPyMT16 immunoprecipitates is lower than that observed with αPyMT7 antibodies, and the former do not inhibit the reaction (see above), it is likely that the higher activity found with αPyMT7 antibody results from an activation of the protein kinase activity. The αPyMT7 antibody can be shown (Figure 5.7) to recognize most of the MWtapp 55Kd phosphorylated polypeptide precipitated by αPyMT5 antibody, but not to recognise the MWtapp 57Kd polypeptide. The MWtapp 57Kd component must therefore be antigenically distinct from the MWtapp 55Kd polypeptide, but the nature of this difference, or the factors responsible for the 2Kd MWtapp alteration, are unclear.

Section 5.5 - Protein Kinase Activities Associated With T-Antigens From Other Sources.

The polypeptides from lysates of 3T6 Swiss Albino mouse cells infected with deletion mutants of polyoma virus that are phosphorylated in an in vitro phosphorylation reaction following immunoprecipitation with four different monoclonal antibodies are shown in Figure 5.8. Again in all these reactions, immunoprecipitates obtained with the αPyC4 (and αPyC1) antibody show no incorporation of phosphate into the truncated middle T-antigens, despite the fact that these antibodies recognize the antigen expressed by these mutants (see Chapter Three). It was further observed

Figure 5.7 - Protein Kinase Activities Analysed by "Cascade" Immunoprecipitation.

"Cascade" derived immunoprecipitates, were analysed by an *in vitro* protein kinase reaction. Serial immunoprecipitations were performed and precipitates 1 and 4, as well as the second antibody precipitation, were assayed for protein kinase activity, as described in Materials and Methods. The phosphorylated T-antigens precipitated by the first antibody are indicated by solid arrows on the left, and those precipitated by the second antibody by open arrows on the right. Note that in each case the centre track is essentially blank, indicating complete precipitation by the first antibody. The reactions shown are: (A) ∝PyLT1 followed by ∝PyMT7; (B) ∝PyC1 followed by ∝PyMT7; (C) ∝PyMT5 followed by ∝PyMT7; (D) ∝PyMT16 followed by ∝PyMT7; (E) ∝PyLT1 followed by ∝PyMT16; (F) ∝PyC1 followed by ∝PyMT16; (G) ∝PyMT7 followed by ∝PyMT16. Polypeptides were separated by SDS-PAGE on a 10% gel, and detected by autoradiography.

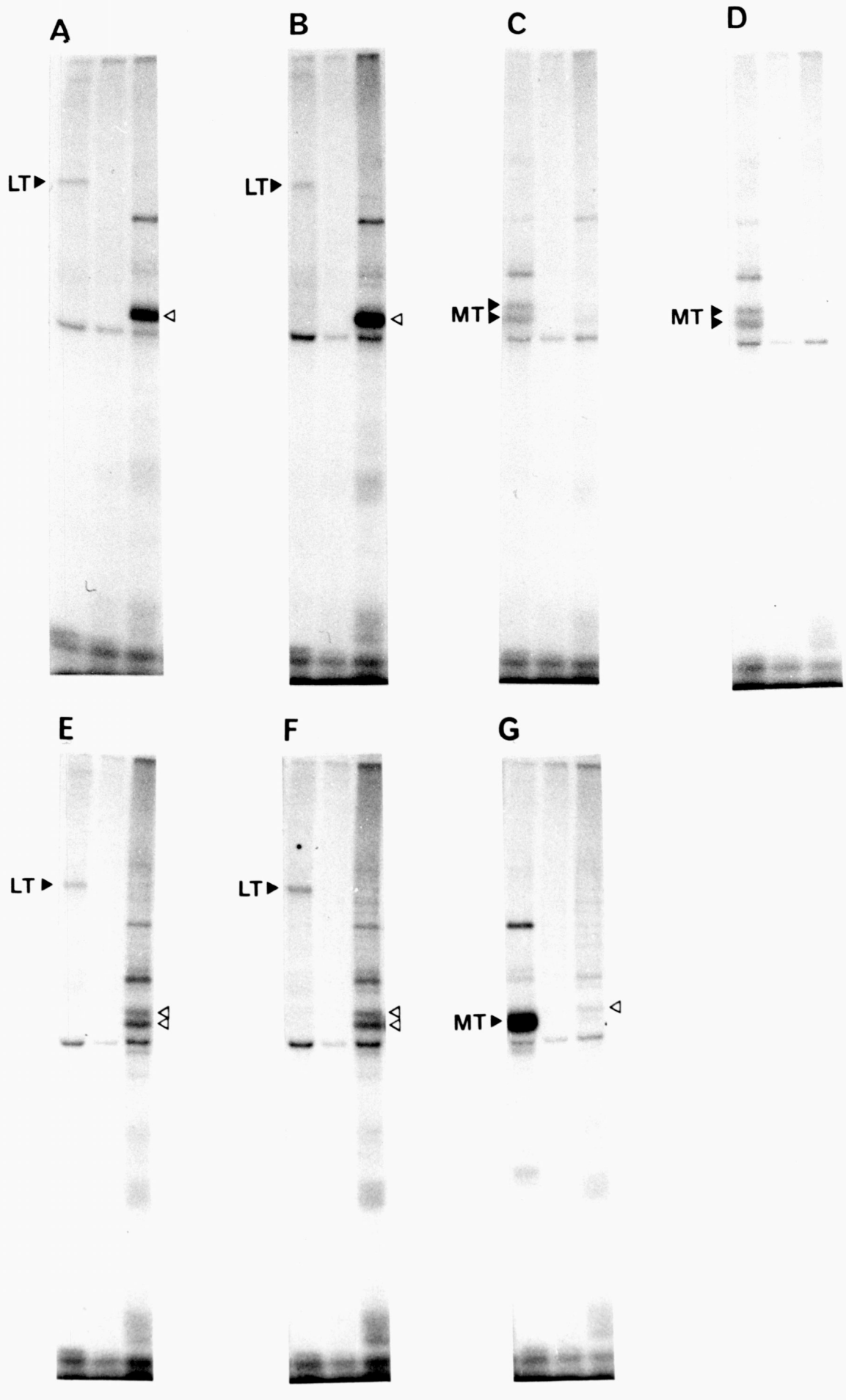
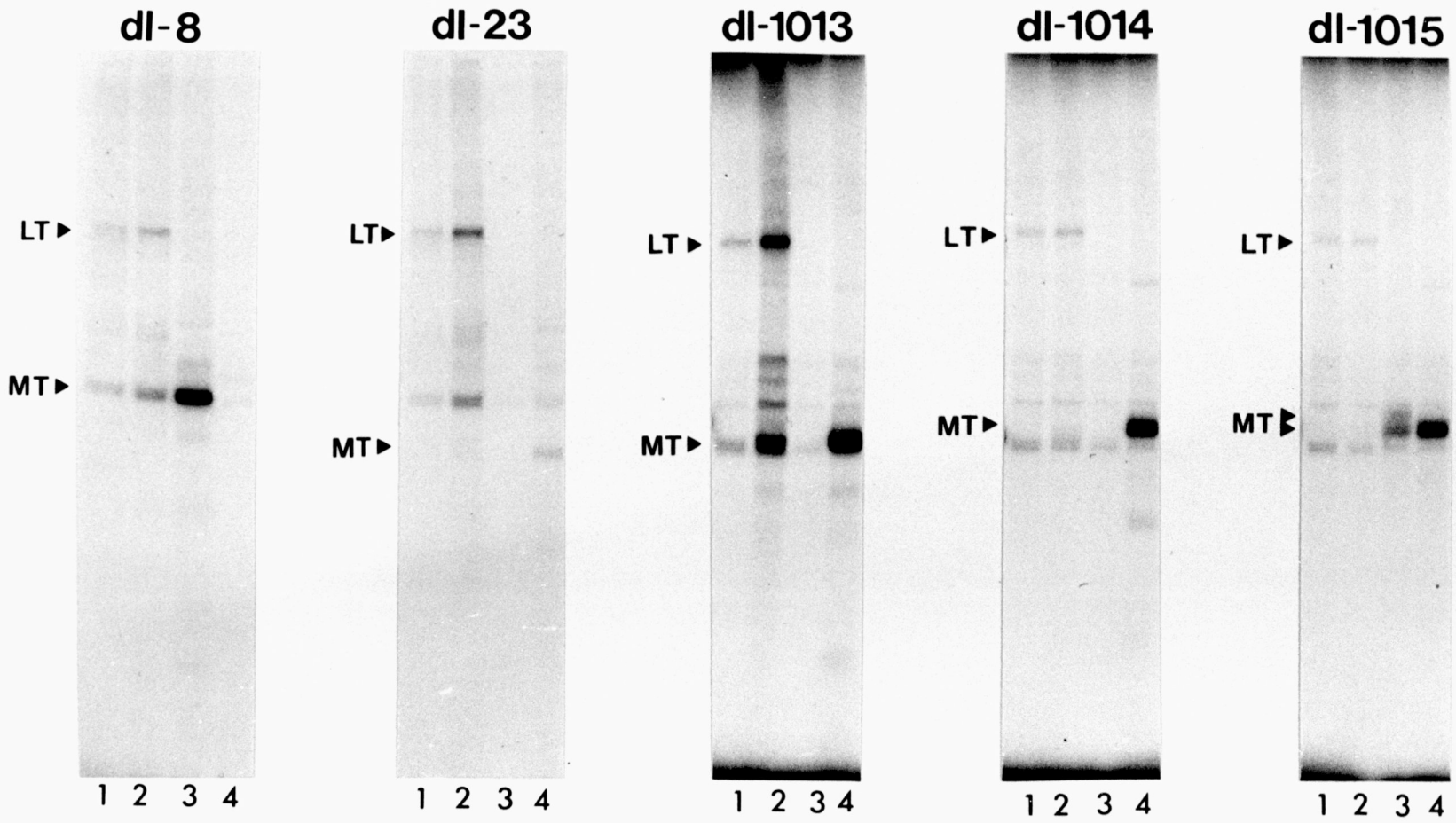

A
LT
B
LT
C
MT
D
MT
E
LT
F
LT
G
MT

Figure 5.8 - Protein Kinase Activities Expressed by Deletion Mutants of Polyoma Virus.

A comparison is made of the polypeptides immunoprecipitated from lysates of 3T6 cells infected with deletion mutants of polyoma virus using four different monoclonal antibodies, and assayed with the in vitro protein kinase reaction. The mutant involved is indicated at the top of each gel. The monoclonal antibodies used were: lanes (1), αPyLT1; lanes (2), αPyC4; lanes (3), αPyMT5; and lanes (4), αPyMT7. The position of the expected truncated forms of large (LT) and middle (MT) T-antigens are indicated on the left of each gel. Polypeptides were separated by SDS-PAGE on a 10% gel, and detected by autoradiography.


dl-8
dl-23
dl-1013
dl-1014
dl-1015
LT
MT
1 2 3 4

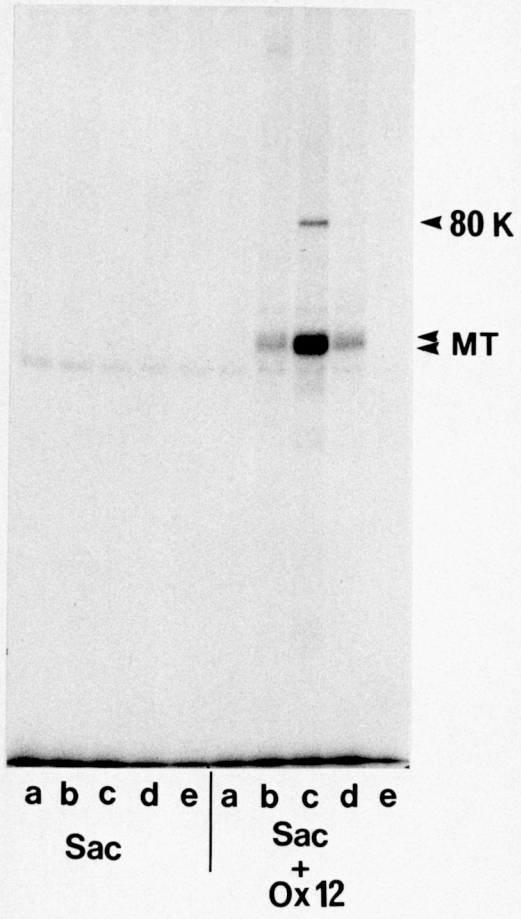
that whenever the monoclonal antibody used did not recognize the middle T-antigen of the mutant (see Chaapter Three), no phosphorylation reaction was observed. Correspondingly, whenever the middle T-antigen encoded by a mutant could be recognized by the αPyMT antibody, it was phosphorylated in the in vitro phosphorylation reaction.

It has been reported that mutant dl-8 does not express the two different MWtapp forms of phosphorylated middle T-antigen (Schaffhausen and Benjamin, 1981); similarly, in αPyMT5 antibody immunoprecipitates, only one polypeptide was found to be phosphorylated. The mutant dl-1015, on the other hand, expressed the two middle T-antigen polypeptides; these are also observed in αPyMT5 antibody immunoprecipitates. Similar to observations on wild type polyoma virus infected cell lysates, only a single polypeptide is found phosphorylated in αPyMT7 antibody immunoprecipitates of dl-1015 infected cell lysates, and phosphorylation occurs to a greater extent than that observed in αPyMT5 immunoprecipitates. The middle T-antigen encoded by mutants dl-23, dl-1013 and dl-1014 are only recognized by the αPyMT7 antibody and phosphorylation of a single polypeptide is observed, with the same MWtapp as the truncated middle T-antigen obtained from metabolically labelled cells. The result obtained on the middle T-antigen encoded by dl-23 is noteworthy since this polypeptide is missing the tyrosine residue reported to be phosphorylated by the protein kinase activity (Schaffhausen and Benjamin, 1981); with αPyMT7, the level of phosphorylation was considerably reduced, but the residue phosphorylated is tyrosine. This implies that the middle T-antigens are either phosphorylated at more than one site, or that an alternative site can be phosphorylated when the normal site is absent, or that the original observations of the site of phosphorylation are in error. (It can be seen in Figure 5.8 that the large T-antigen encoded by each of these mutants is phosphorylated to a slight extent in the in vitro reaction.)

The T-antigens from lysates of the transformed rat cell line PyREWA5/T1A1 immunoprecipitated by five different monoclonal antibodies and phosphorylated in vitro are shown in Figure 5.9. The phosphorylation reactions are similar to those seen with wild type polyoma virus lytically infected cells. That is, phosphorylation of large T-antigen, phosphorylation of middle T-antigen in αPyMT5, αPyMT7 and αPyMT16 antibody immunoprecipitates and a MWtapp 57Kd polypeptide in αPyMT5 and αPyMT16 antibody immunoprecipitates, are observed. No phosphorylation was detected when the anti-rat IgG antibody was omitted.

Figure 5.9 - Protein Kinase Activity in Monoclonal Antibody Immunoprecipitates From PyREWA5/T1A1 Cells.

The polypeptides immunoprecipitated with five monoclonal antibodies from lysates of PyREWA5/T1A1 transformed cells were phosphorylated in vitro by incubation with γ-(^{32}P)-ATP, separated by SDS-PAGE on a 10% gel, and detected by autoradiography. The lanes on the left were immunoprecipitated with SAC alone, those on the right with SAC plus MRC 0x12. The antibodies used were: lane (a), αPyC1; lane (b), αPyMT5; lane (c), αPyMT7; lane (d), αPyMT16; and lane (e), αPyMT13. The positions of the two middle T-antigens (MT) and the MWtapp 80Kd polypeptide (80K) are indicated on the right.


80 K
MT
a b c d e a b c d e
Sac
Sac
+
Ox 12

The data presented here suggest that some of the multimeric forms of middle T-antigen do not take part in the in vitro protein kinase reaction. To investigate this further, a lysate of wild type polyoma virus infected 3T6 Swiss Albino mouse cells was separated according to sedimentation velocity on a sucrose gradient, and individual fractions were immunoprecipitated by αPyMT5 and αPyMT7 antibodies and analysed for protein kinase activity using radioactive γ-(^{32}P) ATP (Figure 5.10). Both of the species of middle T-antigen phosphorylated in αPyMT5 antibody immunoprecipitates, and the single polypeptide phosphorylated in αPyMT7 antibody immunoprecipitates, were found to sediment with a similar velocity that was comparable to the sedimentation velocity observed with metabolically labelled middle T-antigen (see Chapter Three) and greater than that which would be expected were the protein present in monomeric form. The reason for the apparent conflict of these data is unclear, but could reflect the existence of a number of quaternary structural forms for middle T-antigen which have similar sedimentation coefficients, but different enzymic properties.

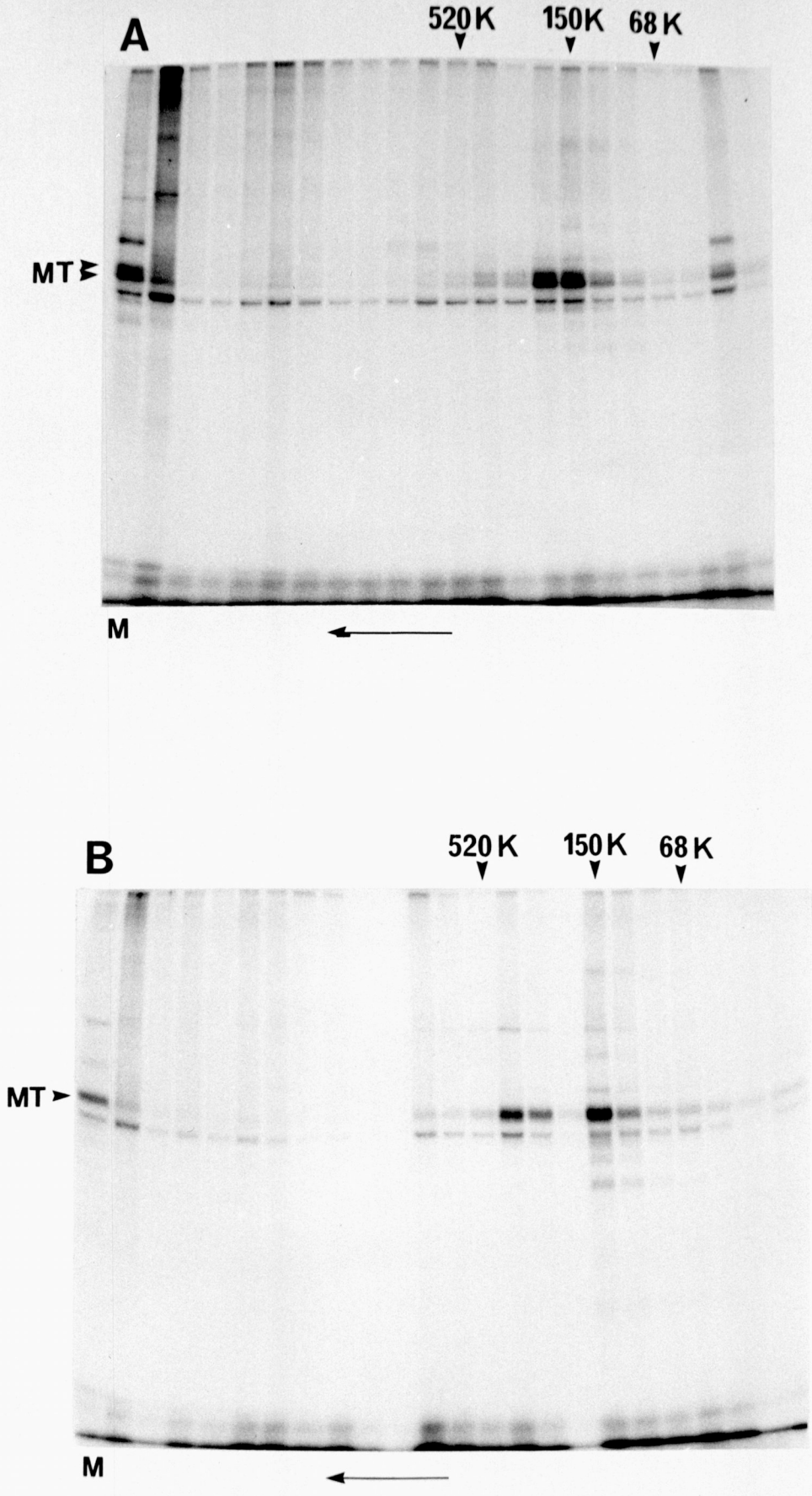
Section 5.6 - Discussion.

This Chapter describes a study of the protein kinase activity that phosphorylates middle T-antigen in vitro in immunoprecipitates of the T-antigens of the polyoma virus obtained with monoclonal antibodies. In these studies a number of surprising results were obtained. Earlier work aimed at defining this activity have involved the use of anti-T serum, which, as previously observed, contains a wide range of antibody activities. With the use of the more specific monoclonal antibodies to immunoprecipitate the polypeptides, it was hoped that some of the ambiguities previously observed could be clarified. The data obtained show that the protein kinase activity can still be observed in immunoprecipitates with antibodies that recognize middle T-antigen only (αPyMT5, αPyMT7 and αPyMT16 antibodies, see Figure 5.1) and is therefore a property of this protein, requiring no other T-antigen or polypeptide for its detection. Since this activity can be separately detected with three middle T-antigen specific monoclonal antibodies, cross reactivity between antigens (see Chapter Two) can be disregarded as the cause of the phosphorylations observed.

An interesting result concerning the middle T-antigen associated protein kinase activity that arose from these studies is the extent of phosphate incorporation observed with the use of the different antibodies. Immunoprecipitates obtained with αPyMT7 antibody incorporate 5 to 10

Figure 5.10 - Sedimentation Analysis of the Middle T-Antigen Molecules that Show Activity in In Vitro Protein Kinase Assay.

A lysate of wild type polyoma virus infected 3T6 mouse cells was separated by sedimentation on a sucrose gradient (the direction of sedimentation is indicated), the gradient fractionated, and proteins immunoprecipitated and subjected to an in vitro phosphorylation reaction (see Materials and Methods). Lane M shows an unfractionated lysate immunoprecipitated in the same manner. The position of the phosphorylated middle T-antigens (MT) are indicated on the left. The sedimentation positions of proteins used as molecular weight markers are indicated above each gel. Panel A, polypeptides immunoprecipitated with αPyMT5 antibody, and panel B, with αPyMT7 antibody. Phosphorylated polypeptides were separated by SDS-PAGE on a 10% gel, and detected by autoradiography.


A
520K
150K
68K
MT
M
B
520K
150K
68K
MT
M

times more phosphate into middle T-antigen than those with αPyMT5 or αPyMT16 antibodies (see Table 5.1), even though the latter appear to precipitate all of the protein kinase activity (see Figure 5.7). The lower level of incorporation observed in αPyMT5 and αPyMT16 antibody immunoprecipitates does not appear to be a result of an inhibition of the enzymic activity by these antibodies (by binding at the active site, for example) since their use, together with the αPyMT7 antibody, results in a high level of incorporation of phosphate (see Figure 5.6). It seems possible, therefore, that the binding of the αPyMT7 antibody actually stimulates the protein kinase activity. This could result from the antibody binding in such a manner as to mimic the binding of a substrate molecule, or alternatively, altering the conformation of the middle T-antigen in such a way that it becomes more susceptible to phosphorylation.

The monoclonal antibodies have also identified a number of forms of middle T-antigen that do not appear to show any associated protein kinase activity. The most obvious case of this is exemplified by the middle T-antigen molecules that are recognized, at their N-termini, by αPyC1 and αPyC4 antibodies. These antibodies immunoprecipitate all of the detectable (^{35}S)-methionine labelled middle T-antigen, but do not react with antigens involved in the protein kinase reaction (see Figure 5.7). The middle T-antigen species phosphorylated in vitro, therefore, appears to be a minor proportion only of the total middle T-antigen present in a cell lysate and differs from the bulk of the protein in some manner that involves the N-terminal region. The precise difference is as yet unknown, but a number of possibilities can be envisaged: (a) The middle T-antigen molecules could be post-translationally modified at their N-termini in some way that alters the antigenic site recognized by the αPyC1 and αPyC4 antibodies, the molecules that are not recognized being the active form in the phosphorylation reaction. (b) Other polypeptides of the "middle T antigen family" (see Chapter Two), which are not recognized by the αPyC1 or αPyC4 antibodies, could be the species responsible for the protein kinase activity in vitro. It is known from studies with many purified protein kinases that they are capable of auto-phosphorylation via an intramolecular mechanism (see Flockhart and Corbin, 1982, for a review), and this activity is frequently stimulated by a limited proteolytic cleavage. Similarly, one or more of the middle T-antigen related polypeptides, of lower MWtapp than intact middle T-antigen but retaining the C-terminal portion, could be responsible for the observed activity. However, as argument against this idea, these polypeptides are only found

phosphorylated to a slight extent in an in vitro phosphorylation reaction. The presence of the unidentified structure in the MWtapp 60Kd polypeptide could also be involved, but exactly how is not clear. (c) The most likely explanation to account for all of the data presented in this Chapter invokes the existence of a number of tertiary and quaternary structural form of middle T-antigen, with only a minority of them having protein kinase activity. The N-terminus of the middle T-antigen moieties could be altered or masked in this form, possibly by protein-protein interactions. This explanation also explains the absence of a protein kinase activity in the multimeric forms of middle T-antigen molecules that are immunoprecipitated by a single monoclonal antibody and SAC (see Chapter Three and Figure 5.6), and for the lack of activity in the minor antigenic forms recognized by αPyMT10 and αPyMT13 antibodies (see Figure 5.1). These different structural forms of middle T-antigen, if they exist, should have a similar sedimentation velocity on sucrose gradients (see Figure 5.10) but the separation system used here would not detect small differences in size. The possibility cannot be excluded that another polypeptide is associated with a small proportion of the middle T-antigen molecules and that it is responsible for the observed phosphorylation of middle T-antigen. The N-terminus of these middle T-antigen molecules could be masked by being bound to this polypeptide. In this regard, the phosphorylation of a tyrosine residue on a MWtapp 80Kd polypeptide is interesting. Whether this polypeptide phosphorylates middle T-antigen molecules, or whether middle T-antigen phosphorylates it, or whether some other molecule phosphorylates both of them is unclear.

The data presented in this Chapter indicate again that a number of forms of middle T-antigen exist in cell lysates, and that these have varying enzymic activities. The recognition by their antigenic properties of different enzymic activities within the same polypeptide indicates the future usefulness of monoclonal antibodies for determining the precise activities expressed by individual T-antigen species and for separating activities expressed by the same polypeptide. The two MWtapp forms of middle T-antigen phosphorylated in vitro have similarly been shown to be antigenically distinct, and to be present as such within a cell lysate prior to immunoprecipitation.

DISCUSSION

The small DNA virus, polyoma, is an extremely attractive model system for the study of eukaryotic virus lytic infection and virally-induced transformation and tumourigenesis processes. Its small size and ease of growth *in vitro* have enabled many of its functions to be studied and manipulated at the cellular and molecular level. The effects the virus has on cellular properties have been previously studied in detail and include a stimulation of host cell DNA synthesis (Dulbecco *et al.*, 1965; Winocour *et al.*, 1965; Weil *et al.*, 1965), an alteration in the level and type of RNAs and proteins synthesized (Salomon *et al.*, 1977; Khandjian *et al.*, 1980), a change in the biochemical properties of the cell surface (Burger, 1969; Benjamin and Burger, 1970; Burger, 1973; Nicholson, 1974) and a considerable modification of the phenotypic properties of the cell (Dulbecco, 1970) (all reviewed in Tooze, 1980). Depending upon the genotype of the infected cell (whether permissive or not) this then leads to either a synthesis of viral DNA, assembly of virions and cell lysis, or in a small percentage of non-permissive cells, to the stable inheritance of many of the unusual properties described above. The acquisition of these properties is called "transformation", and cells become tumourigenic.

The early gene products of polyoma virus have been demonstrated to be responsible for the cellular effects observed subsequent to viral infection; immunological analysis has defined these gene products in terms of the three tumour (T) antigens expressed by the virus (Ito, *et al.*, 1977 a, and b; Türler and Salomon, 1977; Hutchinson *et al.*, 1978; Schaffhausen *et al.*, 1978; Simmons *et al.*, 1979). The ease of manipulation of the genome has enabled many genetic mutants of the virus to be constructed and analysed and a combination of many forms of analysis has resulted in a quite detailed knowledge of the molecular organization and expression of the virus (Soeda *et al.*, 1980). This now enables defined regions of the viral genome to be altered and studied virtually at will. Early studies with virus mutants preliminarily assigned roles for inducing the cellular alterations observed in both lytic and transforming infection to particular T-antigen species (Stoker and Dulbecco, 1969; Schlegel and Benjamin, 1978). More recent analyses have confirmed some of these assignments and shown that the expression of a middle T-antigen alone is in itself sufficient to change an established fibroblast cell line, though not primary cells, into a transformed, tumourigenic line (Tressman *et al.*, 1981).

Hopefully, with continued study the cellular affects produced by each T-antigen will be eventually defined. Interest is now centred on the molecular basis of these affects, particularly that of transformation. To understand these events, the understanding of the properties of the individual T-antigens is necessary.

The T-antigens were originally defined by the presence of specific antibodies against them in the sera of animals that had tumours induced by polyoma virus (Habel, 1965). This anti-T serum has proved useful in the characterization of the proteins expressed by the virus, enabling them to be studied, and to a limited extent, defined (Ito et al., 1977 a and b; Smart and Ito, 1978; Hutchinson et al., 1978; Schaffhausen et al., 1967; Silver et al., 1978; Simmons et al., 1978; Ito, 1979). Studies to date have included an analysis of the proteins expressed by polyoma virus mutants (Ito et al., 1981; Chapter One), determining the relationship that exists among the three T-antigens, and include experiments aimed at defining the distribution of the proteins within the cell (references above; Chapter One). Although such anti-T sera were invaluable for the initial characterisation of the T-antigens, further studies are subject to limitations that arise as a consequence of adverse properties of such sera. Probably as a consequence of the small quantity of T-antigens expressed in transformed cells, the level of antibody activity present in anti-T sera is extremely low. This means that relatively large quantities of serum have to be employed in individual experiments and non-specific precipitation becomes a problem. The raising of antisera against live tumour cells, even though they are derived from a syngeneic animal, poses another problem with these sera, that of specificity. Antibodies to proteins other than the T-antigens can be produced by tumour bearing animals and indeed a number of authors have reported that anti-T serum specifically immunoprecipitate other (non-viral) polypeptides (Ito et al., 1977 a and b; Türler and Salomon, 1977; Schaffhausen et al., 1978; Hutchinson et al., 1978). Causes for this problem can easily be conceived. These include the possible expression of a normal cellular protein in excessive quantities in transformed cells, becoming thereby immunogenic, or the expression during transformation of antigens (for example, an embryonic antigen) not normally presented to the immune system of the animal. Generally, the entirely foreign viral proteins are the major immunogenic species, but other activities can, and do, exist at varying levels. This poses difficulties when attempting to interpret the data obtained with such sera.

This problem is further complicated by the variation between individual animals in their response to a tumour challenge. In addition to variation in the non-viral proteins recognized, variable responses are also observed to the T-antigens (Türler and Salomon, 1977; Ito and Spurr, 1980). This variation makes reproducible studies difficult. The presence of antibodies to varying regions of the T-antigen molecules also poses another problem in using anti-T serum, in that no specificity to an individual T-antigen can be obtained. The study of all three T-antigens simultaneously poses few problems for the physical characterization of the T-antigens, where additional separation steps, such as SDS-PAGE, are also involved. However, for the future determination of enzymic activities, where native preparations are required, this lack of specificity could produce significant difficulties. Therefore, future studies should be greatly simplified by the availability of more specific, high activity immunological probes.

In the absence of a purified T-antigen preparation with which to immunize an animal, two approaches are possible for providing specific immunological probes for the T-antigens. The first of these involves chemical synthesis of peptides against which antisera is raised (Sutcliffe et al., 1980). By predicting a peptide sequence from the known DNA sequence, it is possible to prepare this species and use it to raise antibodies that react with a defined region of the protein. As such antibodies can be affinity purified, they can also be produced with high activity. This approach has been used successfully for the production of antibodies that are specific for the polyoma virus middle T-antigen (Walter et al., 1981), but a number of problems exist with such an approach. That is, the antibodies produced by this method often react with polypeptides other than the expected ones, possibly due to a common primary sequence among proteins or to contamination within the immunizing peptide. Variation between animals also means that reproducibility could still be a problem. Moreover, the chemically synthesised peptides cannot take into account alterations to the amino acid structure that may occur in the native polypeptide, such as post-translational modifications, or conformational change that occur as a result of polypeptide chain folding, etcetra. Therefore with such sera, caution needs to be exercised with regard to the precise polypeptide or activity being studied. The addition of ill-defined variabilities to study unknown properties of the T-antigens can only complicate interpretation of data obtained.

The other possible approach, which was adopted here, uses monoclonal antibody secreting hybrid cell lines (Köhler and Milstein, 1975) to produce defined antibodies. By an adaptation of the method previously adopted to produce anti-T serum, ten monoclonal antibody secreting cell lines have been purified and their antibodies characterized. These allow defined antibodies to be made in unlimited quantities and provide the specificity and high activity necessary for future studies. The technique, moreover, allows a number of different antibodies to be produced, thereby producing immunological probes for different regions on the T-antigens. The generation of antibodies against tumours probably provides a selective set of antibody activities which depends upon the antigen presented by the tumours. However, the monoclonal antibodies which have been produced will in future allow alternative procedures of immunization to be used, and thereby a wide range of antibody activities to be obtained. The ten antibodies described here have been shown to react with the T-antigens and to recognize different antigenic sites on the molecules. Antibodies that are specific for large T-antigen and for middle T-antigen, as well as ones that react with all three T-antigens, have been produced (see Chapter Two). To date, antibodies recognizing the region common to middle and small T-antigen, or unique to small T-antigen, have not been isolated. One problem associated with monoclonal antibodies is that it is difficult to define precisely the location of the antigenic site. Therefore, only approximate positions on the primary sequence have been determined for these antigenic sites. Many interesting properties of the T-antigen molecules have been uncovered, however, by the use of these monoclonal antibodies.

The polypeptides recognized by some of the monoclonal antibodies in addition to the known T-antigens (see Chapter Two), are intriguing. The possibility that an 80Kd MWtapp polypeptide recognized by the αPyMT13 antibody is the cellular counterpart of the viral protein postulated to exist by Soeda et al., (1980b) is obviously interesting, but there is little evidence to confirm or disprove this, although the lack of any obvious peptide homology (Chapter Two) between this polypeptide and middle T-antigen appears to suggest that the cross reaction observed may be fortuitous. Indeed, the discovery of a number of seemingly unrelated cross reactions that are found when employing monoclonal antibodies (Lane and Koprowski, 1982) throws doubt on the significance of any such reaction observed with monoclonal antibodies in general.

The monoclonal antibodies and studies presented here have extended this heterogeneity to antigenic properties, native sizes and enzymic activities (see the protein kinase activity described in Chapter Five). The nature of many of the antigenic variations are not clear, but are probably reflections of the existence of conformational, or other structural, alterations in the antigens. Such variation obviously poses problems when the properties of individual T-antigens are being investigated. In studies on the function expressed by these proteins, it would be easily possible to overlook a property expressed by them through unknowingly studying only a sub-group of the molecules. This consideration must also be borne in mind when purifying the T-antigen proteins prior to determining their activities. That different forms of protein structure may be required for performing the different functions that could be expressed by a single polypeptide is possible.

Some of the advantages of defined immunologicial probes in the study of enzymatic activities are illustrated by the study of the in vitro protein kinase reaction with monoclonal antibodies (see Chapter Five). This activity has previously been shown to be closely linked to the transforming ability of the virus; proteins from transformation defective mutants show lowered protein kinase activities. That this activity is associated with the middle T-antigen (Smith et al., 1979; Smith et al., 1980; Schaffhausen and Benjamin, 1979; Eckhart et al., 1979; Schaffhausen and Benjamin, 1981) and results in its being phosphorylated on a tyrosine residue (Eckhart et al., 1979) has also previously been shown. The results presented here, however, indicate that the activity is associated with only a minor population of middle T-antigen molecules and that they are antigenically altered in their N-terminal region. The exact nature of this alteration is unclear. The minor sub-groups of middle T-antigen molecules recognized by the αPyMT10 and αPyMT13 antibodies appear not to be involved in the protein kinase reaction. The two MWtapp forms of phosphorylated middle T-antigen observed by Schaffhausen and Benjamin (1981) have been observed to be antigenically distinct molecular species, with the MWtapp 55Kd form possessing an antigenic site not present on the MWtapp 57Kd form. That the enzymic reactions are influenced by the position on the molecule to which the antibody is bound has also been demonstrated. All these studies have defined the nature and components of the protein kinase activity to a degree that would have been impossible with anti-T or conventional polyclonal serum.

The specificity provided by the monoclonal antibodies has proved extremely valuable in analysing the sub-cellular distributions of the T-antigens. Using antibodies that recognize either the large or middle T-antigens only, it has been possible to investigate their sub-cellular location directly by immunofluorescence (see Chapter Four). This analysis has confirmed data regarding the nuclear location of large T-antigen (Ito et al., 1977b; Silver et al., 1978) and extended it by demonstrating the presence of discrete concentrated areas of large T-antigen within the nucleoplasm of lytically infected cells. This is not observed in transformed cells and so is possibly a reflection of the lytic growth cycle of the virus. Considering early data, the middle T-antigen immunofluorescent pattern has provided some surprises. Previous sub-cellular fractionation studies suggested middle T-antigen to be located within the plasma membrane (Ito et al., 1977b; Silver et al., 1978). Immunofluorescent data with the monoclonal antibodies indicate that the location of the middle T-antigen is not confined to the plasma membrane. It seems probable that it is membrane bound, but it appears to be located on a number of different cellular membranes, probably including the plasma membrane. Whether the "middle T-antigen family" of polypeptides is responsible for any of these differences is still not clear. Moreover, whether specific functions (for example, the protein kinase activity) of middle T-antigen molecules can be related to different cellular locations remains to be determined. Unfortunately, the immunofluorescent technique does not allow sufficient sensitivity and resolution to be achieved to determine whether the antigenic sub-group of molecules recognized by the αPyMT10 and αPyMT13 antibodies vary in their location. The sub-group recognized by the αPyMT7 antibody, however, does not appear to vary in its location. These findings are being further studied by immuno-electron microscopy. So far, this study has confirmed the conclusions reached by the immunofluorescence studies (G. Bjursell and H.A. Hansson, personal communication). The locations of the middle T-antigen(s) pose extra problems when considering the action of the molecule within the cell. The search for its activity with respect to transformation has been previously concentrated on the plasma membrane. However, the location on many cell membranes, now uncovered, means that alternative sites of action must be considered, and considerably widens the possibilities for the activity of the middle T-antigen protein. The determination of the mechanisms involved in the transformation process induced by this protein therefore becomes inherently more complicated.

Future studies on the activities associated with the T-antigens will require purified protein preparations. The specificity of the monoclonal antibodies, and the ability to radio-label them has enabled a sensitive radio-immunoassay to be established for the large and middle T-antigens (see Chapter Three). Such an assay will be essential when attempting to purify these proteins by normal techniques, and will be very useful in immuno-affinity purification procedures for the assessment of yields and monitoring binding, etcetra. The development of an RIA has also enabled the properties of the monoclonal antibodies to be studied further, and has thus indicated that many of them bind to independent, non-blocking antigenic sites, and that the large and middle T-antigens exist in multimeric forms. That the monoclonal antibodies can be used in affinity techniques to purify the T-antigens is indicated by their ability to immunoprecipitate enough large T-antigen in a highly enriched form to be detected by Coomassie Blue staining after SDS-PAGE (see Chapter Three).

The availability of purified, native T-antigen protein will enable the enzymatic properties of these proteins to be studied in detail. As indicated above, care needs to be exercised in the interpretation of such data to ensure that the heterogeneities observed for the T-antigen molecules do not influence the results obtained. The monoclonal antibodies, however, can be used in these studies to determine precisely the nature of the molecule being studied. Their defined binding affinities, and recognition sites, will also enable different regions of the antigens to be probed for activity, so determining regions of the protein involved in expression of any particular function. This, together with data obtained from studies of viral mutants should help correlate antigen expression with altered cellular properties. The separation of causal reactions and effects resulting from a primary event will be a significant problem for the future. However, it will hopefully soon be possible to determine at the molecular level how the T-antigens bring about alterations in cellular phenotypes. The interaction between cellular and viral proteins will undoubtedly lead to a fuller understanding of the normal control processes exercised in cellular proliferation and perhaps help in understanding transformation processes induced by other agents. The polyoma virus has already justified, and probably will continue to justify, its choice as a model system for the study of these processes.

MATERIALS AND METHODS

Materials

Except where indicated in the text, all chemicals were of Analar purity.

Foetal Calf Serum	:- from Gibco or Flow Laboratories
RPMI 1640 Medium	:- from Flow Laboratories
Dulbecco's Modified Eagle's Medium (E4)	:- From I.C.R.F. Tissue Culture Media Production Unit
Acrylamide	:- from British Drug Houses (BDH)
N.N'-methylene-bis-acrylamide	:- from Bio-Rad, all Electrophoresis Grade Purity.
Ammonium persulphate	:
N.N.N',N' tetramethylethylenediamine (TEMED)	:
Coomassie Brilliant Blue R.250	:- from Hopkins and Williams
Bromophenol Blue	:- from Gurr
Sodium dodecyl sulphate	:- from BDH Biochemicals, Electrophoresis Grade Purity.
Sodium deoxycholate	:- from Sigma
Nonidet P40	:- from BDH Biochemicals
Phenyl methyl sulphonyl fluoride	:- from Sigma
Aprotinin	:- from Sigma
Sucrose	:- from Schwarz/Mann
Bovine Serum Albumin	:- from Armour Pharmaceuticals
Tris Base	:- from Sigma
X-Ray Film	:- from Kodak, SB-5 or XH-1 type.

GENERAL METHODS

Cell Lines

Whole mouse embryo cells and baby mouse kidney cells were supplied by the I.C.R.F. Cell Production Unit.

3T6 Swiss Albino mouse cells were obtained from the American Type Culture Collection.

PyREWA5/T1AI is a primary Wistar rat embryo cell line transformed with polyoma virus as reported by Ito et al., (1977b) and was a kind gift from Y.Ito.

Rat-1 is an established line of rat fibroblast cells from the Fischer strain F2408 (Freeman et al., 1973).

BE-1 is a Rat-1 cell line transformed by transfection with a 35% fragment of the polyoma virus genome (Novak et al., 1980).

Hae 2 is a Rat-1 cell line transformed by transfection with polyoma virus DNA cleaved with the restriction enzyme HaeII (G. Bjursell, A. Hayday and M. Fried, personal communication).

P3-NS-1/1-Ag4-1 is a non-secreting mouse plasmacytoma line selected for HGPRT (hypoxanthine guanine phosphoribosyl transferase) negative expression by growth in 8-azaguanine (Sigma), as reported by Köhler and Milstein (1976).

Fibroblast propagation was achieved by removing the cells from tissue culture dishes by limited treatment with 0.25% trypsin in versene solution. The cells were then diluted and re-plated onto fresh tissue culture dishes at an appropriate dilution in E4 media + 5% FCS.

All fibroblasts were grown in E4 supplemented with 5% FCS in a 10% CO_2: 90% air humidified incubator.

All lymphocyte lines were grown in RPMI 1640 media supplemented with 10% FCS in a 5% CO_2: 95% air humidified incubator.

All cell lines were tested regularly for, and were free of, mycoplasma contamination by aceto-orcein staining (Fogh and Fogh, 1964) and cultivation in mycoplasma agar (Lemcke, 1965) as determined by the ICRF Cell Production Unit.

Virus Stocks

All virus stocks were initially plaque purified (Dulbecco and Freeman, 1959) and then grown by infection at approximately 0.1 pfu/cell in whole mouse embryo cells, or baby mouse kidney cells, followed by incubation at 32°C until an extensive cytopathic effect was observed. The cells and media were then freeze-thawed twice and stored at -20°C until use. Just before infection the virus was briefly sonicated.

Viral stocks with titres $> 10^9$ pfu/ml were routinely obtained although certain poorly replicating mutants (for example, dl-8 and NG-18) were usually below this level.

Polyoma Virus Infection of Cells

The media on subconfluent cell monolayers were removed and virus, diluted in E4 medium, added. The volumes used were usually 0.5ml/50mm dish and 1ml/90mm dish. Adsorption was allowed to continue at 37°C for 90 minutes, with frequent rocking, then E4 + 5% FCS added; this was taken as the zero time point of infection.

Buffers

NET :- 150mM NaCl, 5mM EDTA, 50mM TrisHCl, pH7.4 and 0.05% (vol/vol) Nonidet P40.

TBS :- 150mM NaCl, 50mM TrisHCl, pH7.4 and 0.05% (vol/vol) Nonidet P40.

PBS.A :- Phosphate buffered saline, 10g/l NaCl, 0.25g/l KCl, 1.4g/l Na_2HPO_4, 0.25g/l KH_2PO_4.

PBS Complete:- PBS.A plus 10mM $CaCl_2$ and 6mM $MgCl_2$.

SDS-PAGE Sample Buffer :- 2% (wt/vol) SDS, 10% (vol/vol) glycerol, 80mM TrisHCl, pH6.8, 5% (vol/vol) 2-mercaptoethanol (Sigma) and 0.001% (wt/vol) bromophenol blue.

Anti-Tumour (T) Serum

Anti-T serum was raised by the method of Ito et al., (1977b). Approximately 10^7 PyREWA5/T1A1 cells were sub-cutaneously inoculated in complete PBS into the flank of a young adult male Wistar rat. When the tumours had grown to approximately 5cm in diameter (2 to 3 weeks) the animal was bled out. The blood was allowed to clot for 6 to 8 hours at 4°C, the serum removed, centrifuged at 1000xg for 5 minutes, and then stored at -20°C until use.

Labelling Cells With (^{35}S)-Methionine

Short periods of labelling (up to 4 hours) were conducted in E4 media lacking methionine. Normal cell medium was removed and the cells washed twice with E4 lacking methionine at 37°C. (^{35}S)-Methionine in E4 lacking methionine (Radiochemical Centre, Amersham; > 600 Ci/mmole) was then added to the cells and incubated in a humidified environment. Volumes usually used were 2 mls/50mm dish and 6 mls/90mm dish.

Longer periods of labelling (18-20 hours) were conducted in a similar manner, with the modification that the label was added in E4 containing 10% of the normal methionine concentration, plus 5% dialysed FCS.

Detergent Lysis Solution.

Various detergent solutions were made up in 100mM TrisHCl pH8.8, 100mM NaCl. Nonidet P40 was added at 0.5% and 1% (vol/vol), DOC and SDS were added at 1% and 0.1% respectively (wt/vol).

PMSF was added to the lysis buffer from a stock 20 mg/ml solution made up in absolute ethanol immediately before use. Aprotinin was added at 0.2 to 0.3 trypsin inhibitory units (TIU)/ml.

Cell Lysis

Following the labelling period, the radioactive medium was removed and the cell layer washed once with ice cold complete PBS. The cells were removed from the tissue culture dish by scraping with a silicone rubber policeman, transferred to a centrifuge tube with a large quantity of ice cold complete PBS, and pelleted by centrifugation at 1000xg for 5 minutes. The supernatant was removed and the appropriate detergent solution added to the cell pellet. The cells were resuspended by brief vortexing then incubated at 0°C for 30 minutes. (Other incubation times between 15 and 45 minutes were observed not to make a significant difference in the quantity of T-antigen extracted). The nuclei and unlysed cell debris were then pelleted by centrifugation in an Eppendorf microfuge for 5 minutes at 4°C. The supernatant was removed and either used immediately or stored at -70°C.

Extraction Buffer for further experiments consisted of 100mM NaCl, 100mM TrisHCl pH8.8, 0.5% (vol/vol) Nonidet P40 with 0.2-0.3 TIU/ml aprotinin.

Immunoprecipitation with Anti-Tumour Serum.

Immunoprecipitation with anti-T serum was accomplished by a method similar to that described by Ito (1979). (^{35}S)-Methionine labelled cell lysates were incubated for 18 hours at 0°C with 50 ul of pre-immune rat serum. Two hundred and fifty ul (5 x vol of serum used) of a 20% SAC suspension were then added and incubated for 30 minutes at 0°C. The SAC was then pelleted by centrifugation for 2 minutes in an Eppendorf microfuge, the supernatant removed and divided into two equal samples to which were added either 50ul of pre-immune rat serum or 50ul of rat anti-T serum. After incubation of 2 to 4 hours at 0°C, 100ul (2 x volume of serum used) of a 20% SAC suspension were added and incubated for a further 30 minutes at 0°C. The SAC was then pelleted by centrifugation for 20 seconds and the supernatant discarded.

The pellet was washed three times by resuspension in NET at 0°C followed by centrifugation. The final SAC pellet was resuspended in 100ul of 7M urea, 80mM TrisHCl pH 6.8., and incubated for 15 minutes at room temperature. The SAC was then pelleted by centrifugation for 2 minutes, the supernatant removed, diluted with ice cold NET (1ml) and incubated for 18 hours at 0°C. One hundred ul of a 20% SAC suspension were added and incubated for 30 minutes at 0°C. The SAC was then pelleted by centrifugation for 20 seconds and washed twice with NET at 0°C. The final SAC pellet was resuspended in 100ul of SDS-PAGE Sample Buffer and incubated for 15 minutes at room temperature. The SAC was further pelleted, the supernatant taken and heated in a boiling water bath for 5 minutes. The sample was then loaded onto a SDS-polyacrylamide gel, and the polypeptides separated as previously described.

All volumes given are for a 50mm dish of cells, the volumes were corrected accordingly for other cell quantities. Assays showed the volume of anti-T serum added was significantly greater than the amount necessary to immunoprecipitate all the available T-antigens from a cell lysate.

Fluorography

Electrophoresed gels were stained and subjected to fluorography as described by Bonner and Laskey (1974). This provided a more linear response than autoradiography.

Sub-Cellular Fractionation

Cells infected with deletion mutants of polyoma virus and labelled with (^{35}S) - methionine were fractionated essentially as described by Ito et al., (1977b). This involved the disruption of cells by nitrogen cavitation (equilibration for 15 minutes at 600 lbs/in^2) in 250mM sucrose, 5mM TrisHCl pH7.4, 2mM $MgCl_2$ + 20 ug/ml PMSF. The nuclei were then removed by centrifugation at 500xg for 10 minutes and the supernatant applied to a discontinuous sucrose dextran gradient. The gradient was made of the following layers : 0.8ml of 60% sucrose, 2.8 ml of 35% sucrose, 1.2ml of 22.5% dextran T40, 1.4 mls of 19% sucrose, 2.8 mls of 15% sucrose, 2.8 mls of sample and 1ml of 5% sucrose. All solution concentrations were percent weight/weight and contained 1mM EDTA, 5mM TrisHCl, pH 7.4. The gradients were centrifuged in a SW40 rotor for 45 minutes at 28K rpm and the membranes at the 19% sucrose/22.5% dextran interface taken. The membranes were diluted to 3 times their volume with 1mM EDTA, 5mM TrisHCl, pH7.4 and pelleted by centrifugation at 240,000xg for 30 minutes.

The plasma membranes were resuspended in 0.5ml of 1mM EDTA, 5mM TrisHCl, pH 7.4 and lysed by making the solution 1% with respect to DOC. After incubation for 30 minutes at 0°C, the solution was recentrifuged at 240,000xg for 30 minutes, the supernatant taken and immunoprecipitated as above.

CHAPTER TWO METHODS

Hybrid Cell Line Production

All fusions were conducted with the mouse plasmacytoma line P3-NS-1/1-Ag4-1 (NS-1) and Wistar rat spleen cells. The FCS used was pre-screened for its ability to support the cloning of NS-1 to high efficiency. The polyethylene glycol M.Wt.1000 (PEG1000) (Koch Light Laboratories) was also pre-tested for low toxicity.

Three days after an intravenous antigenic boost, a rat was sacrificed, bled out and the spleen removed by sterile techniques. Spleen cells were prepared in RPMI 1640 at 37°C by repeated teasing apart of the spleen structure with sterile needles. The preparation was allowed to stand for 3 to 4 minutes to allow large debris to separate and the supernatant taken. The spleen cells were then gently centrifuged, washed three times with RPMI 1640 at 37°C and mixed with NS-1 cells that had been washed once. The ratio used was usually 4:1 spleen cells: NS-1, as determined by viable cell counting in a haematocrit chamber. The cells were pelleted by centrifugation at 300xg for 5 minutes and the supernatant removed. Fusion was accomplished either by slow speed centrifugation in 30% (wt/vol) PEG1000 at 37°C in RPMI 1640, followed by incremental dilution, or by slow addition of 50% PEG1000 at 37°C in RPMI 1640 followed by incremental dilution, as described by Kennet (1979). Generally the first procedure produced a higher yield of hybrid cell lines. The resulting hybrid cells were plated out into 24 well dishes (Linbro) in media containing 70% RPMI 1640, 20% FCS, 10% National Cancer Tissue Culture 135 medium (Flow Labs) supplemented with cis-oxaloacetate (150 µg/ml), sodium pyruvate (50µg/ml), and bovine insulin (0.2 units/ml) (all from Sigma). HGPRT positive cells were selected for by the addition of 100µM hypoxanthine, 10µM thymidine and 10µM methotrexate (all from Sigma) (Littlefield, 1964). The cells were fed by replacement of half the medium every three days.

When sufficient growth had occurred, the tissue culture fluid (TCF) was screened for the production of antibodies directed against the polyoma virus T-antigens. The cells in positive wells were cloned by plating out at < 0.5 cells/well over non-immune rat spleen feeder cells (prepared as above) in 96 well dishes (Linbro). After the first cloning, methotrexate was omitted from the medium. Antibody producing cells were cloned three times before being considered as an established hybridoma line. The cells were then allowed to grow to confluency to produce antibody-containing TCF.

Screen for the Production of Antibodies

Directed Against the Polyoma Virus T-Antigens.

3T6 Swiss Albino mouse cells were infected with ts-a-a mutant of polyoma virus (Fried, 1965) and incubated at 32°C for 40 hours. The cells were then shifted to 39°C for 1 hour and labelled for 4 hours at this temperature with 1mCi of (^{35}S)-methionine/90mm dish in methionine free E4 medium, as before. Cells were harvested and lysed in 5mls/90mm dish of Extraction Buffer, as described above. One hundred ul of this lysate were incubated for 18 hours at 0°C with approximately 100µl of TCF from the tissue culture well being tested. Ten µl of a 20% SAC suspension were then added and incubated for 30 minutes at 0°C. The SAC was recovered by centrifugation (20 seconds in an Eppendorf microfuge) and washed once by resuspension in NET buffer. The final SAC pellet was resuspended in 50µl of SDS-PAGE sample buffer and incubated for 15 minutes at room temperature. The sample was then centrifuged for 2 minutes, and the supernatant removed and heated in a boiling water bath for 5 minutes. The polypeptides were separated on a 10% SDS-PAG; the gel was dried and autoradiographed. A sample immunoprecipitated with anti-T serum was electrophoresed in parallel to the unknown TCF and the presence of antibodies to the T-antigens identified by the ability to precipitate a polypeptide of the same MWtapp as the T-antigens on SDS-PAGE.

Immunoprecipitation of T-Antigens With Monoclonal Antibodies

3T6 Swiss Albino mouse cells were infected with approximately 50 pfu/cell of the wild type (A2) strain of polyoma virus and incubated for 24 hours at 37°C. The cells were labelled for 4 hours at 37°C with 500uCi/50mm dish (^{35}S)-methionine and then washed and lysed with 1ml Extraction Buffer/50mm dish, as described above. The lysate was incubated with 50ul of a 20% SAC suspension for 30 minutes at 0°C, the SAC pellet recovered by centrifugation in an Eppendorf microfuge for 5 minutes at 4°C, and the supernatant collected. The purpose of this treatment was to remove non-specific SAC binding proteins. One hundred ul of the lysate were then incubated with 15ul of a monoclonal antibody-containing TCF for 1 to 2 hours at 0°C. Twenty ul of a 20% SAC supension pretreated with either 5ug of a rabbit IgG anti-rat IgG (Miles-Biochemicals) or 1ug of MRC 0x12 monoclonal antibody (a mouse anti-rat K light chain antibody, a gift from Dr. S. Hunt, Oxford) were then added and the mixture incubated for 30 minutes at 0°C.

The resulting immune complexes were pelleted by a 20 second centrifugation in a Eppendorf microfuge and the supernatant discarded. The SAC was then washed by resuspension in NET buffer followed by centrifugation and this process repeated four times. The final SAC pellet was resuspended in 50ul of SDS-PAGE Sample Buffer and incubated for 15 minutes at room temperature. The sample was centrifuged for 2 minutes and the supernatant recovered and heated for 5 minutes in a boiling water bath. The polypeptides were separated on a 10% SDS-PAG, the gel stained, destained, dried and autoradiographed as described above. Preparative samples were prepared in a similar manner with an appropriate increase in the quantities of reagents, and gels dried without staining or fixation.

A negative control was performed by replacing the monoclonal antibody-containing TCF with culture fluid from the parent plasmacytoma, NS-1.

Two Dimensional Tryptic Peptide Fingerprints of Polypeptides

(^{35}S)-Methionine labelled polypeptides were "fingerprinted" by a method similar to that described by Ito (1979). Polypeptides were separated by electrophoresis on a 10% SDS-polyacrylamide gel and the gel dried without fixation. The polypeptide of interest was identified by autoradiography and the appropriate gel piece excised. This was rehydrated with 0.5ml of H_2O and homogenized with a Teflon headed plunger. Eight mls of 50mM $NaHCO_3$, 0.1% (wt/vol) SDS + 200ug BSA were then added and the gel fragments shaken for 18 hours at 37°C. Pieces of gel were then separated from the supernatant by passing them through a glassfibre plug followed by a 0.45um Millipore filter. (Counting the (^{35}S) in the supernatant and in the gel pieces indicated that typically 70-80% of the counts present in a sample were eluted).

The supernatant was placed on ice for 30 minutes, then made up to 20% (wt/vol) with trichloroacetic acid (TCA) from a stock 100% solution and incubated for 1 hour at 0°C. The sample was then centrifuged for 20 minutes at 10K rpm in a SS-34 rotor at 4°C and the supernatant discarded. The precipitated protein was washed three times with ether and air dried. The polypeptides were then oxidized by dissolving them in 200ul of performic acid and incubating for 3 hours at 0°C. (The performic acid was freshly prepared by incubating 1.9ml of formic acid with 100ul of 30% (vol/vol) H_2O_2 for 1 hour at 37°C).

The sample was diluted with 2ml H_2O and lyophilised. The polypeptides were dissolved in 20ul of 100mM NH_4HCO_3 and digested with 10ug of TPCK Trypsin (Worthington) for 1 hour at 37°C. A further 10ug of TPCK Trypsin was then added and the incubation continued for a further 3 hours at 37°C. The sample was lyophilised and the peptides dissolved in 20ul of 100mM acetic acid. An appropriate quantity of the peptide solution was then spotted onto a thin layer cellulose plate (0.1mm thick, MN300 cellulose coated 20cm x 20cm glass plate; Camlab, Cambridge). The sample was electrophoresed for 1 hour at 500V in 2.5% (vol/vol) pyridine, 2.5% (vol/vol) acetic acid, pH4.5 buffer. The plate was dried and then subjected to ascending chomatography at right angles to the electrophoresis direction for 3 hours in butanol: acetic acid: pyridine: H_2O (75:15:60:60 vol/vol). The resulting plate was dried and autoradiographed.

The procedure was modified slightly for later experiments in that 4ml of buffer with 100ug BSA were used to elute the polypeptides, which were washed with 1:1 (vol/vol) ethanol: ether at -20°C following precipitation with TCA. The polypeptides were then digested with 5ug TPCK Trypsin each time. The lower amount of total protein, together with the more efficient removal of SDS and TCA with ethanol: ether, resulted in much improved two-dimensional separations.

Limited Proteolytic Cleavage (Cleveland) Analysis of Polypeptides

(^{35}S)-Methionine labelled polypeptides were subjected to analysis by limited proteolytic cleavage by a method similar to that described by Cleveland et al., (1977), as modified by Dr. A. E. Smith (personal communication). Polypeptides were separated by electrophoresis on 10% SDS-PAG, the gel dried and the polypeptides visualised by autoradiography. The appropriate gel band was excised, rehydrated with 150ul of 10mM $NaHCO_3$, 0.1% SDS, 0.1% 2-mercaptoethanol, 20% (wt/vol) sucrose, homogenized in a small glass homogenizer and the resulting slurry divided into five aliquots. Five ul of Staphyloccus aureus V8 protease (Miles Biochemicals Ltd.) at concentrations of 10, 1, 0.1, 0.01 or 0.001 mg/ml, respectively, were added to each aliquot and it was incubated for 1 hour at 37°C. Twenty-five ul of SDS-PAG Sample Buffer were then added to each sample and the polypeptides separated by electrophoresis on 15% SDS-PAGE. The gel was then stained (to locate size markers), destained, dried and autoradiographed.

Monoclonal Antibody Immunoprecipitation of
T-Antigens Encoded By Viral Deletion Mutants.

Immunoprecipitation was conducted by a method similar to that described for Chapter Two, except that the 3T6 Swiss Albino mouse cells were infected with various deletion mutants of polyoma virus. MRC 0x12 monoclonal antibody was used as a second antibody.

Immunoprecipitation of the T-Antigens
Expressed by Polyoma Virus Transformed Cells

Sub-confluent monolayers of transformed cells were labelled for 18 hours at 37°C with 500 uCi/50mm dish of (^{35}S)-methionine, as described before. The cells were lysed and immunoprecipitated with monoclonal antibodies, as described for lytically infected mouse cells.

Sequential Immunoprecipitations

Sequential immunoprecipitations ("cascade" reactions) were conducted by a method similar to that described by Harlow et al., (1981). One hundred ul of a wild type polyoma virus infected, (^{35}S)-methionine labelled, cell lysate were treated with 15ul of monoclonal antibody-containing TCF and incubated for 3 hours at 0°C. Twenty-five ul of a 20% SAC suspension containing 1.5ug of MRC 0x12 monoclonal antibody were then added and the resultant mixture incubated for 30 minutes at 0°C. The SAC pellet was recovered by centrifugation, the supernatant removed and treated with 15 ul of the same monoclonal antibody-containing TCF, then incubated for 18 hours at 0°C. A further 25 ul of SAC containing 1.5ug of MRC 0x12 were added and the mixture incubated for 30 minutes at 0°C. The SAC pellet was removed by centrifugation and the supernatant treated a further two times with monoclonal antibody-containing TCF (3 hours at 0°C) followed each time by SAC and MRC 0x12 antibody. A further 25ul of SAC containing 1.5ug of MRC 0x12 were added to the final supernatant to complete the removal of monoclonal antibody. Fifteen ul of anti-T serum were then added to the supernatant and it was incubated for 2 hours at 0°C. Twenty-five ul of SAC were then added, the mixture incubated for 30 minutes at 0°C and the SAC isolated by centrifugation. All six of the SAC immunoprecipitates, obtained by this procedure with each monoclonal antibody, were washed three times by resuspension in NET buffer. The resultant SAC pellets were resuspended in 50ul SDS-PAGE Sample Buffer, incubated for 15 minutes at room temperature, and polypeptides in the supernatants isolated and heated in a boiling water bath for 5 minutes. The samples were then separated by 10% SDS-PAGE, the gel stained, destained, dried and autoradiographed, as described above.

Cell Labelling With (^{32}P)-orthophosphate

Cells to be labelled with (^{32}P) were washed once with phosphate free E4 medium at 37°C and incubated for 2 to 3 hours at 37°C with 2 mls/50mm dish of phosphate free E4 medium containing 500 uCi of carrier free (^{32}P)-orthophosphate (Radiochemical Centre, Amersham). Following the labelling period, cells were harvested, lysed and immunoprecipitated as described for (^{35}S)-methionine labelled cells in Chapter Two.

Sucrose Gradient Sedimentation Velocity Analyses
of T-Antigens Recognized by Monoclonal Antibodies.

Sedimentation analyses were conducted on sucrose gradients essentially as described by McCormick and Harlow (1980). The sucrose gradient consisted of a 0.4 ml 50% (wt/vol) sucrose cushion and a 5 to 20% linear sucrose gradient (total volume, 5 ml). All solutions were made to a final concentration of 100mM NaCl, 100mM TrisHCl, pH8.8, 0.5% (vol/vol) Nonidet P40. Two hundred ul of cell lysate were layered onto the top of the gradient and it was centrifuged for 15 hours at 4°C and 25K r.p.m. in a SW50.1 rotor. The gradient was then fractionated into 250ul aliquots (21 to 24). Molecular weight marker markers were run in parallel and consisted of 50ug each of β-galactosidase (molecular weight 520KD; Sigma), rat IgG (150Kd) and BSA (68Kd) and their positions on the gradient located by SDS-PAGE, followed by Coomassie Blue staining.

The samples were diluted to four times the original volume before being immunoprecipitated as above.

Binding of SDS Denatured T-Antigens By Monoclonal Antibodies

The T-antigens encoded by wild type polyoma virus were labelled with (^{35}S)-methionine, immunoprecipitated with anti-T serum and separated by SDS-PAGE, as described above. Proteins were located by autoradiography and the gel pieces containing them excised. The labelled polypeptide was eluted by the same procedure as that used for two dimensional peptide fingerprint analysis, up to the step involving filtration of the gel fragments. The denatured T-antigen solution was mixed with an equal volume of NET Buffer containing 1% (vol/vol) Nonidet P40 and 1% (wt/vol) DOC to reduce the denaturing effect of the SDS in the buffer by the formation of mixed micelles. An aliquot of the T-antigen was incubated for 18 hours at 0°C with 10ul of a monoclonal antibody-containing TCF and the immune complexes collected on 15ul of a 20% SAC suspension containing 1ug MRC 0x12 monoclonal antibody. Incubation

of the mixture was carried out for 30 minutes at 0°C, then NET Buffer (1 ml) at 0°C added, and the SAC pellet isolated by centrifugation in an Eppendorf microfuge. The pellet was resuspended in 100ul of H_2O, added to 1.5 ml of Aquasol (NEN) and the radioactivity determined in a scintillation counter in the (^{35}S) channel.

Unlabelled T-Antigen Preparation

5 x 90mm (5 x 10^7 cells) dishes of 3T6 Swiss Albino mouse cells were infected with polyoma virus and incubated for 42 hours at 32°C. The cells were then washed with complete PBS at 0°C, removed by scraping with a silicone rubber policeman and pelleted by centrifugation at 600xg for 5 minutes. Five mls of Extraction Buffer were added, the cells suspended by brief vortexing and incubated for 30 minutes at 0°C. Nuclei and cell debris were removed by centrifugation for 10 minutes at 4°C, 10K r.p.m. in a SS-34 rotor. The supernatant was withdrawn, 100ul of a 20% SAC suspension added to it, and the mixture incubated for 30 minutes at 0°C. The SAC was then removed by centrifugation for 10 minutes at 10K r.p.m. The supernatant was again withdrawn and 1 ml of a monoclonal-antibody containing TCF added, and the mixture incubated for 2 hours at 4 °C. One hundred ul of SAC were then added and incubation continued for 30 minutes at 0°C. The SAC pellet was collected by a brief centrifugation (approximately 1 minute at 10K r.p.m. in a SS-34 rotor) and washed four times by resuspension in NET buffer at 0°C. The final SAC pellet was resuspended in 100ul of SDS-PAGE Sample Buffer and incubated for 15 minutes at room temperature. The SAC was then pelleted, the supernatant removed and heated for 5 minutes in a boiling water bath. The polypeptides were separated by electrophoresis on 10% SDS-PAG and the gel stained with Coomassie Blue, destained as described above, and photographed.

Antibody Subclass Analysis by Ouchterlony Double Diffusion

The antibody subclass of the monoclonal antibodies was determined by Ouchterlony double diffusion analysis against rat immunoglobulin sub-class specific antisera (from Miles Biochemicals) (Bazin et al., 1974). Monoclonal antibody-containing TCF was concentrated by the addition of solid ammonium sulphate (BDH Biochemicals, Aristar grade) to 50% saturation and stirring for 5 hours at 4°C. The precipitate was isolated by centrifugation for 15 minutes at 4°C, 10K r.p.m. in a SS-34

rotor, dissolved in a small volume of PBSA and dialysed against a large volume of PBSA. The final volume was approximately 1/50th of the starting TCF volume. Ouchterlony double diffusion was conducted in 1% (wt/vol) agarose containing PBSA + 5mM EDTA. Ten ul of each sample and antiserum were used and the diffusion was allowed to progress for 18 hours at room temperature. The agarose was then dried, washed for 2 hours in PBSA, stained and destained with Coomassie Blue, essentially as described by Ouchterlony and Nilsson (1978).

Radio-Immuno Assay (RIA)

(^{35}S)-Methionine labelled antibody was made by incubating 2 x 10^6 monoclonal antibody secreting cells for 18 hours at 37°C with 500 uCi of (^{35}S)-methionine in 5 mls of E4 media containing 10% of the normal methionine concentration and 5% FCS. The cells were removed by centrifugation for 10 minutes at 1000xg, the supernatant added to an equal volume of saturated ammonium sulphate solution and incubated for 5 hours at 4°C. The precipitate was then isolated by centrifugation for 10 minutes at 2000xg and washed once with 50% saturated ammonium sulphate solution. The final precipitate was redissolved in a small volume of PBS.A containing 0.1% (wt/vol) NaN_3 and stored at 4°C until use. The antibody was diluted with NET buffer just before use.

For the RIA, a constant amount of (^{35}S)-methionine labelled antibody was added to varying quantities of a lysate of wild type polyoma virus infected cells, and the solutions corrected to contain the same volume and protein concentration using the appropriate quantity of an uninfected cell lysate. The antibody and lysate were incubated for 1 hour at 0°C, then 10ul of a 20% SAC suspension added and the mixture incubated for a further 30 minutes at 0°C. The SAC pellet was isolated by a 20 second centrifugation in an Eppendorf microfuge and washed once by resuspension in NET buffer at 0°C. The final pellet was resuspended in 100ul H_2O, added to 1.5 ml Aquasol (NEN) and the radioactivity determined in a scintillation counter.

Immunofluorescence

Cells in E4 medium with 5% FCS were seeded onto 13mm diameter round glass, sterile coverslips. Where appropriate, the cells were infected with polyoma virus after the cells had attached to the coverslips over a period of 12 hours at 37°C. The cells were infected with polyoma virus (50 pfu/cell) in E4 medium and allowed to absorb for 90 minutes at 37°C with frequent rocking. E4 media plus 5% FCS were replaced on the cells and they were then incubated for 24 hours at 32°C. The media were then changed to E4 media plus 0.5% FCS, and the cells incubated for a further 18 hours at 32°C. Transformed cells were treated similarly, omitting the viral infection.

Formaldehyde Fixation :- Cells attached to coverslips were fixed with formaldehyde and permeabilised with Nonidet P40 by a method similar to that described by Griffin et al., (1980). The attached cells were washed once with complete PBS at 37°C then fixed with 3.8% (wt/vol) formaldehyde in PBS.A for 15 minutes at room temperature, washed twice with PBS.A and permeabilised by incubation for 15 minutes at room temperature with 1% (vol/vol) Nonidet P40 in PBS.A. The cells were then washed twice with PBS.A and used for immunofluorescent studies.

Acetone Fixation :- Cells attached to coverslips were washed once with complete PBS at 37°C, then the coverslips placed into methanol:acetone (7:3,vol/vol) at -20°C for 15 minutes. The coverslips were air dried and washed in PBS.A before use for immunofluorescent studies.

Fluorescent Staining :- Fixed and permeabilised cells on coverslips were incubated for 1 hour at 37°C with 15ul of a monoclonal antibody-containing TCF in an humidified atmosphere. The cells were then washed by immersing the coverslip three times in large volumes of PBS.A and incubated with 15ul of a 100 ug/ml rabbit IgG anti-rat antibody solution (Miles Biochemicals) in PBS.A for 1 hour at 37°C in a humidified atmosphere. They were then washed three times again with PBS.A and incubated for 1 hour at 37°C with 15 ul of a rhodamine isothiocyanate conjugated goat IgG anti-rabbit IgG solution (Miles Biochemicals) diluted 1:100 in PBS.A. The resulting coverslips were washed with PBS.A three times, once with distilled H_2O and mounted on a microscope slide with Aquamount (Hopkins and Williams).

Double Antibody Staining :- Fixed and permeabilised cells on coverslips were incubated with 15 ul of antibodies in the following order: (1) monoclonal antibody-containing TCF number one, (2) 100ug/ml rabbit IgG anti-rat IgG, (3) monoclonal antibody-containing TCF two, (4) rhodamine isothiocyanate conjugated goat IgG anti-rabbit IgG diluted 1:100, (5) fluorescein isothiocyanate conjugated rabbit IgG anti-rat IgG (Miles Biochemicals) diluted 1:20 with PBS.A. With this technique, rhodamine labels the monoclonal antibody one and the fluorescein labels monoclonal antibody two.

All antibody solutions were passed through a 0.45 um Millipore filter before use.

Photography :- Cells were viewed and photographed with a Zeiss microscope fitted with epifluorescent optics. The cells were visualised under ultraviolet light with appropriate filters for the fluorochrome used, using either a x40 or a x63 Planapochromat objective lens. Photomicrographs were taken using an ASA400 black and white print film (Kodak Tri-X or Ilford HP5 film) and a timed exposure, and developed according to the manufacturer's instructions.

CHAPTER FIVE METHODS

In Vitro Phosphorylation Reactions

3T6 Swiss Albino mouse cells were infected with polyoma virus (approximately 50 pfu/cell) and incubated for 42 hours at 32°C. The cells were then washed, scraped off the dish and lysed with 1ml of Extraction Buffer (containing 0.3 TIU/ml aprotinin)/90 mm dish of cells, as described for Chaper Two. Non-specific SAC binding proteins were removed from the cell lysate by adding 50ul of a 20% SAC suspension/ml of lysate, incubating for 30 minutes at 0°C, then removing the SAC by centrifugation at 4°C (5 minute) in an Eppendorf microfuge and collecting the supernatant.

Ten ul of a monoclonal antibody-containing TCF were added to 50ul of lysate and the solution incubated for 1 hour at 0°C. Fifteen ul of a 20% SAC suspension (with or without prior addition of 5 ug of rabbit IgG anti-rat IgG, or 1ug of MRC 0x12 monoclonal antibody) were added and the mixture incubated for 30 minutes at 0°C. One ml of TBS at 0°C was then added and the SAC containing immune complexes pelleted by a 20 second centrifugation in an Eppendorf microfuge. The SAC pellet was resuspended in 20ul of 20mM Tris acetate, pH 7.4, 5mM manganous acetate, containing 5 uCi of γ-(^{32}P)-adenosine triphosphate (ATP) (Radiochemical Centre, Amersham; >5000 Ci/mmole) and incubated for 15 minutes at 30°C. The reaction was terminated by the addition of 1 ml NET buffer at 0°C and the SAC recovered by centrifugation for 20 seconds in an Eppendorf microfuge. The pellet was resuspended in 50ul of SDS-PAGE Sample Buffer and incubated for 15 minutes at room temperature. The SAC was removed by centrifugation and the supernatant heated in a boiling water bath for 5 minutes. The polypeptides were then separated by electrophoresis on 10% PAG, the gel stained, destained, dried and autoradiographed.

(^{35}S)-Methionine Labelling and Immunoprecipitation

3T6 Swiss Albino mouse cells were infected with polyoma virus and incubated at 32°C. Between 26 and 42 hours post infection, the cells were incubated with 1mCi/90mm dish of (^{35})-methionine, as described above. At the end of the labelling period the cells were washed, lysed and immunoprecipitated as described for the in vitro phosphorylation reaction, omitting the incubation with γ-(^{32}P)-ATP.

Analysis of Phosphorylated Amino Acids In Polypeptides

The amino acid(s) phosphorylated in a phospho-polypeptide was determined essentially as described by Hunter and Sefton (1980).

Phosphorylated polypeptides were separated by SDS-PAGE and the gel dried without fixation. Labelled species were identified by autoradiography, and the bands excised and rehydrated with 0.5ml H_2O. The gel fragment was homogenised with a Teflon headed plunger and the polypeptide eluted by agitating the gel with 4 mls of 100 mM $NaHCO_3$, 0.1% (wt/vol) SDS, containing 100ug BSA, for 18 hours at 37°C. Gel fragments were removed by filtration first through a glass fibre plug and then a 0.45 um Millipore filter. The polypeptides were precipitated by the addition of TCA to 20% (wt/vol) and incubation for 1 hour at 0°C. The sample was then centrifuged for 20 minutes at 4°C and 10K rpm in a SS-34 rotor and the supernatant removed. The precipitated material was washed twice with ethanol/ether (1:1) at -20°C and air dried. The polypeptides were then hydrolysed by dissolving them in 200ul of constant boiling point HCl by heating in a boiling water bath for 3 minutes, then heating at 110°C under nitrogen for 2 hours. The HCl was removed by evaporation under vacuum and the resulting amino acids dissolved in 10ul of H_2O, spotted onto a cellulose thin layer plate (0.1mm thick, MN300 cellulose coated, 20cm x 20cm glass plate; Camlab, Cambridge) and separated by electrophoresis for 1 hour at 750 volts in pyridine:acetic acid:H_2O 5:50:950, vol/vol), pH3.5. Two ug of phosphoserine, phosphothreonine (Sigma) and phosphotyrosine (a gift from K. Leppard) were run in parallel tracks and their positions identified by spraying the plate with a ninhydrin solution (BDH Biochemicals) followed by heating for 30 minutes at 80°C. The plate was autoradiographed to locate (^{32}P) labelled amino acids.

Sequential Immunoprecipitations

"Cascade" immunoprecipitations were conducted as described in the methods for Chapter Three, with the following modifications: Fifty ul of an unlabelled lysate were used and the final immunoprecipitation was achieved using a second monoclonal antibody rather that anti-T serum. The first, fourth and final SAC immunoprecipitates were washed and subjected to an in vitro phosphorylation reaction, as described above.

Limited Proteolytic Cleavage Analysis of Polypeptides

Limited proteolytic cleavage analysis was conducted as described in Chapter Two.

Sucrose Gradient Sedimentation Velocity Analyses

Sedimentation analysis on sucrose gradients was carried out as described in Chapter Two. The resulting fractions were diluted with NET buffer, immunoprecipitated and phosphorylated in vitro using the protocol described above.

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Appendix 1

Coding Capacity of a 35% Fragment of the Polyoma Virus Genome is Sufficient to Initiate and Maintain Cellular Transformation.

by Ulrike Novak, Stephen M. Dilworth and Beverly E. Griffin.

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Coding capacity of a 35% fragment of the polyoma virus genome is sufficient to initiate and maintain cellular transformation

(large tumor antigens/DNA integration/transfection/tumors)

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ABSTRACT **Rat-1 cells were transfected with the restriction enzyme fragment of polyoma virus DNA that extends clockwise from the *Bcl* I site (65.4 map units) to the *Eco*RI site (0/100 map units). Six transformed cell lines were obtained and one of them (BE-1) has been investigated in detail. The viral DNA that is integrated into host DNA in this line appeared to consist of two fragments arranged in a "head-to-tail" tandem with no detectable intervening host sequences. BE-1 cells contained polyoma virus small and middle tumor antigens that were indistinguishable from the corresponding tumor antigens from lytically infected cells. No large tumor antigen was detected but a "new" M_r 34,000 protein, which proved to be a truncated version of large tumor antigen, was immunoprecipitated by anti-tumor-antigen antiserum. After injection of 10^6 BE-1 cells into young syngeneic Fischer rats, tumors appeared within 3–4 weeks. Thus, the coding capacity of the *Bcl* I/*Eco*RI fragment of polyoma virus DNA is sufficient to enable the cells to produce all of small and middle tumor antigens and about a third of large tumor antigen, to transform cells stably in culture, and to produce tumors *in vivo*.**

The early region of polyoma virus DNA (67–26 map units) (Fig. 1) has been shown to code for at least three proteins (2–4) that have been designated small, middle, and large tumor antigens (T antigens). These proteins appear in susceptible cells shortly after infection with the virus and are identical to the T antigens found in cells transformed by polyoma virus. The functions of these early proteins have been defined primarily by the study of three different classes of viral mutants.

(*i*) The host-range- and transformation-defective (hr-t) mutants in general have deletions that lie between 78 and 86 units on the physical map of the viral genome (5). They do not transform rat or hamster cells *in vitro* and are not tumorigenic. Infected cells produce a normal large T antigen and may also produce small amounts of truncated versions of the middle and small T antigens (6).

(*ii*) Temperature-sensitive (tsa) mutants, which map in the distal part of the early region (between 0 and 26 map units) (7), transform cells at the permissive temperature and under certain circumstances the cells remain transformed at the nonpermissive temperature (8). tsa mutants code for normal-sized small and middle T antigens; large T antigen usually also is of normal size and is always temperature sensitive in its virus growth function. hr-t and tsa mutants can complement each other for transformation of rat or hamster cells (9, 10).

(*iii*) A new class of polyoma virus early mutants (dl-8 and dl-23), which map between 89 and 95 map units, has been shown to have altered transformation properties (11, 12). These mutants code for a small T antigen of normal size and middle and large T antigens of reduced size.

The properties of the various classes of early mutants indicate that a functional middle T antigen may be essential for cellular transformation. In contrast, large T antigen does not appear to be necessary for the maintenance of transformation and, on the basis of data presented here, may not even be necessary for the initiation of transformation. A function for small T antigen in transformation has not been defined.

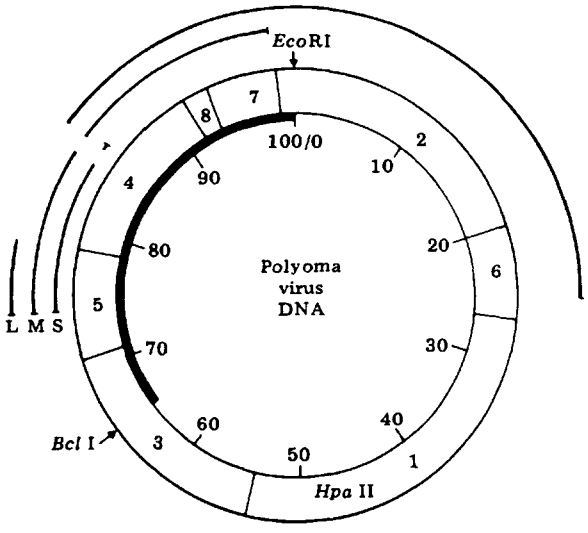


FIG. 1. Physical map of polyoma virus DNA divided into 100 units. The eight sites of cleavage by *Hpa* II (1) are indicated within the circle and the coding locations for the three viral early proteins—large (100,000), middle (55,000), and small (22,000) tumor antigens (2–4)—are indicated outside the circle. The fragment used for transfection of the Rat-1 cells extends from the *Bcl* I site at 65.4 map units clockwise to the *Eco*RI site at 100/0 map units, as indicated by the heavy line. It encompasses the whole coding region of small and middle tumor antigens but only a part of that for large tumor antigen.

We have carried out experiments to define the sequences of polyoma virus DNA that are essential for cellular transformation and to correlate data on transformation with viral gene products.

MATERIAL AND METHODS

Preparation of Viral DNA and DNA Fragments. The large plaque A2-strain of polyoma virus was used as wild-type virus strain (13). Plaque-purified virus was grown on whole mouse embryo cells, and virus-infected mouse 3T6 cells were used for the preparation of viral DNA as described (1, 14, 15).

Supercoiled A2-strain viral DNA (50 μg) was cleaved to completion with *Bcl* I (BioLabs) and then further cleaved with *Eco*RI (MRE Porton) under standard conditions.

The resulting two fragments, about 35% and 65% genome length, were separated by electrophoresis on a 1% agarose slab

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Abbreviations: T antigen, tumor antigen; hr-t, host-range- and transformation-defective; tsa, temperature-sensitive.

gel as described (16). DNA was visualized by staining with ethidium bromide and examination under UV light; bands were excised and isolated by electrophoresis into dialysis bags (15).

Transfection. An established line (Rat-1) of rat cells from the Fischer strain F2408 (17) was used for transformation studies. In a 90-mm dish, slightly subconfluent Rat-1 cells were transfected with either the purified *Bcl* I/*Eco*RI 35% fragment (0.2 μg) or whole viral DNA (1 μg) by using the calcium technique (18). DNA was absorbed at 37°C for 1 hr, 5 ml of growth medium was added, and the cells were incubated for another 4 hr at 37°C, trypsinized, resuspended in 0.35% agar, plated onto 10 50-mm dishes, and fed once weekly as described (12). Three weeks later the colonies were counted. All 10 colonies observed in cells transfected with the fragment and 10 of about 100 colonies of the control cells transfected with polyoma virus form I DNA were picked and grown individually in Dulbecco's modified Eagle's medium with 10% fetal calf serum. After the first passage, the serum concentration was reduced to 5%. Cell lines were established from some of the transformed colonies by standard procedures.

Isolation and Analysis of Whole Cell DNA. Total cellular DNA was extracted and purified as described by Gross-Bellard *et al.* (19). Purified cellular DNA was cleaved under standard conditions with *Bgl* I, *Bgl* II, *Hin*fI, *Hpa* II, *Sac* I, *Pvu* II, *Acc* I, *Taq* I, *Mbo* I, and *Hph* I prepared by established procedures (20). The resulting DNA fragments were separated by electrophoresis (16) in 0.8–2% agarose slab gels (15 × 20 cm), transferred from the gels onto nitrocellulose (BA 85, Schleicher & Schuell) (21), and hybridized to ^{32}P-labeled polyoma virus DNA (10^8 cpm/μg) (22).

Isolation of Viral Proteins and Tryptic Peptide Analysis. Nearly confluent transformed cells (one 50-mm dish per experiment) were washed with methionine-free medium and labeled for 4 hr with [^{35}S]methionine (800 Ci/mmol; 250 μCi/50-mm dish; 1 Ci = 3.7×10^{10} becquerels; Amersham) in 5 ml of methionine-free Dulbecco's modified Eagle's medium. Viral proteins were isolated by immunoprecipitation (23) and separated by electrophoresis on 10% polyacrylamide gels. Tryptic peptide fingerprint analyses were carried out as described (24).

***In Vivo* Experiments.** Young adult syngeneic Fischer rats were injected subcutaneously with 10^6 cells in 1 ml of phosphate-buffered saline per animal. The rats were examined for tumors twice weekly and sacrificed when large tumors (2 cm in diameter) had developed. Cell lines were established essentially as described (25). Control animals were injected with either 10^6 cells transformed with polyoma virus A2 strain or with uninfected Rat-1 cells.

RESULTS

Transformed cells were selected for their ability to grow in soft agar. Colonies were detected 2 weeks after transfection with either whole polyoma virus DNA or the fragment. Uninfected Rat-1 cells formed no colonies in soft agar. Ten colonies of cells transformed with the *Bcl* I/*Eco*RI fragment were isolated and, from these, six cell lines, designated BE-1 to BE-6, could be established. Because all cell lines appeared to contain only polyoma virus DNA sequences from the fragment used for transfection, only one cell line, BE-1, was examined in detail (see below).

Biological Activity of BE-1 Cells. In order to test the tumorigenicity of the BE-1 cell line, 10^6 cells suspended in phosphate-buffered saline were injected subcutaneously into syngeneic young adult Fischer rats. Two different cell lines transformed with polyoma A2-strain virus (Rat-1 A1 and Rat-1 A6) and normal Rat-1 cells were used as controls. Tumor formation was neither drastically enhanced nor retarded with BE-1 cells (Table 1) nor were the tumors morphologically different from those induced by the control cells. After 5 weeks the tumors, which were about 2–3 cm in diameter, were removed and cell lines designated BE-1t1 to BE-1t4 were established. These tumor cell lines were not recloned.

Table 1. Time required for tumor formation in rats injected with transformed cells

Cell line	Days after injection of 10^6 cells				
Rat-1 A1	19	19	19	21	25
Rat-1 A6	25	49	48	59	70
BE-1	19	22	22	29	33
Rat-1	—	—	—	—	—

Analysis of the Integrated Polyoma Virus DNA in the BE-1 Cell Line. The parental cell line BE-1, as well as the four tumor cell lines BE-1t1 to BE-1t4, were examined for the presence of free and integrated polyoma virus DNA because it has been shown that many rat cells transformed with polyoma virus contain both integrated and free copies of the whole viral genome (26, 27). Total cellular DNA was cleaved with a number of restriction endonucleases and the resulting fragments were analyzed. In the cellular DNA, only fragments that corresponded to wild-type virus fragments *Sac* I-3, (81.3–96.5 map units), *Pvu* II-2 (70.0–92.1), *Hpa* II-4 and -5, or *Hin*fI-4 and -5 (66.4–77.8 and 78.8–88.7) were observed (Fig. 2). That is, the integrated viral DNA was found to contain only sequences of the polyoma virus genome that lie clockwise between the *Bc* I and *Eco*RI sites on the physical map (Fig. 1). Data obtained



FIG. 2. Analysis of the polyoma virus DNA integrated within the host chromosome in BE-1 cells. Total cellular DNA (about 30 μg) was cleaved with restriction endonucleases *Pvu* II (lane a), *Sac* I (lane c), *Hpa* II (lane e), *Hin*fI (lane g), or *Taq* I (lane i), and fragments were separated by electrophoresis in a 1.4% agarose slab gel and transferred onto a nitrocellulose filter (21). Virus-specific DNA sequences were hybridized to polyoma virus DNA that had been labeled with [^{32}P]phosphate by nick-translation (22) and bands containing virus were detected by autoradiography. Lanes b, d, f, and h contain fragments of polyoma virus DNA that result from cleavage of the wild-type genome with *Pvu* II, *Sac* I, *Hpa* II, and *Hin*fI, respectively, and provide base-pair size markers for the experiments, as indicated. A fragment present in lanes a and e was too faint to be reproduced in the print it was apparent in the original film.

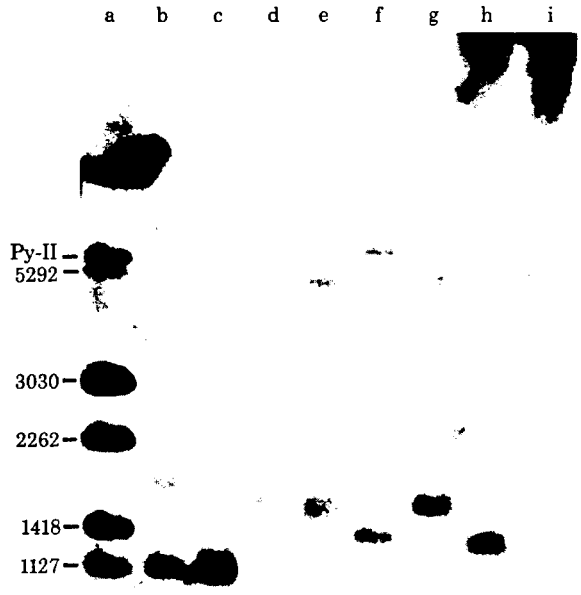


FIG. 3. Mapping of integrated polyoma virus DNA sequences within BE-1 cells. Lanes b to h contain cellular DNA from BE-1 cells cleaved with *Acc* I (b), *Acc* I and *Eco*RI (c), *Bgl* I (d), *Bgl* I and *Eco*RI (e), *Hph* I (f), *Taq* I (g), and *Ava* I (h). Fragments were separated by electrophoresis on 0.8% agarose slab gel. The experimental procedure is described in Fig. 2. The slowly migrating fragment in lane b appears to be due to a partial digestion with *Acc* I, as confirmed by other data (not shown). Lane i contains whole BE-1 cellular DNA. Size markers (given as base pairs, on the left) are derived from whole polyoma virus DNA (forms I and II, 5292) and *Hin*dIII and *Hpa* II fragments of polyoma virus DNA (lane a).

from uncleaved BE-1 DNA (Fig. 3, lane i) show that the cells contain no free viral DNA. When the cellular DNA was cleaved with *Taq* I or *Bgl* I, each of which recognizes only one site within the 35% *Bcl* I/*Eco*RI fragment used in the transfection experiments (at 95.4 and 72.2 map units, respectively), bands with lengths of about 1800 base pairs, which were indistinguishable from each other, were observed on gels (Fig. 3). This length is about the same as that of the initial viral fragment used for transfection. These data suggest that the viral DNA sequences integrated within the cellular genome consist of a tandem "head-to-tail" repeat of the 35% fragment. Cleavage of the cellular DNA with various restriction endonucleases provided further support for this hypothesis (Table 2; Fig. 3).

In order to estimate the number of viral copies in the tandem repeat, the molarities of fragments that resulted from cleavage of the cellular DNA with either *Sac* I or *Acc* I were compared.

Table 2. Unique viral sequences found in integrated DNA

Enzyme	A	B	Enzyme	A	B
Bgl I	1830	1800	*Sac* I	1030	1000
Taq I	1830	1800	*Pvu* II	660	630
Ava I	1475	1450	*Acc* I	700	700
Hph I	1530	1500	*Hin*fI	230	†
Mbo I	1700*	1650	*Hpa* II	345	†

Column A: The expected length (in base pairs) of the unique fragments that would result from cleavage of cellular DNA if it contained a head-to-tail tandem integration of the portion of polyoma virus from 65.4 to 100 map units; fragment sizes are derived from the total sequence of polyoma virus DNA (28). Column B: The observed fragment lengths (in base pairs).

* Assuming that the *Mbo* I site at 65.4 map units (part of the *Bcl* I site) is lost.

† Too small to be detected.

In both cases, two fragments with ratios of about 1:2 were found to hybridize to polyoma virus DNA. The more plentiful fragment, as estimated visually, corresponded to a restriction fragment found in wild-type polyoma virus DNA; the less plentiful one was unique to the tandem viral insert. Data for the *Sac* I cleavage are shown in lane c of Fig. 2, and data from both *Sac* I and *Acc* I digests are illustrated on Fig. 4. These data indicate that the tandem repeat sequence consists of two 35% polyoma virus DNA fragments. For confirmation, the cellular DNA was first cleaved with *Eco*RI, which has no site within the viral insert, and the resulting viral-host fragment (about 10.5 kilobases) then was cleaved with restriction enzymes such as *Pvu* II or *Taq* I. The sizes of the resulting fragments could only be accounted for by a duplicated viral insert.

The proposed map of the integrated polyoma virus DNA in BE-1 cells is shown in Fig. 4. Some uncertainties remain about sequences around the junction of the viral fragments and the junctions of the viral insert with the host DNA. Neither the *Eco*RI nor the *Bcl* I sites within the viral DNA were retained. Cleavage of the cellular DNA with *Eco*RI alone or with *Eco*RI and either *Pvu* II or *Taq* I showed that no *Eco*RI sites remained within the viral fragments. Cleavage with *Mbo* I (which recognizes four of the six base pairs that make up the *Bcl* I site) resulted in a fragment about 1650 base pairs long, different from the *Mbo* I-1 fragment (about 1460 base pairs) that should have been obtained (11) had the *Bcl* I sites been preserved in the viral DNA.

Because both of the terminal viral restriction sites have been lost during integration, it cannot be ruled out that additional viral sequences have been deleted, although sizes observed for the various viral joint fragments (Fig. 4; Table 2) suggest that the deletions must be small. Furthermore, cleavage of BE-1 DNA with *Hin*fI produces equimolar amounts of fragments which correspond in size to wild-type virus *Hin*fI fragments 4 and 5 (11), showing that sequences that lie between 66 and 78 as well as between 79 and 89 map units in wild-type DNA are present in the tandem viral insert. The site at 66 map units is within 50 base pairs of the *Bcl* I site. Comparable studies showed that the *Acc* I site at 99 map units in wild-type virus DNA was present twice in the insert. This site lies within 50 base pairs of the *Eco*RI site in polyoma virus DNA. Therefore, at most 100 base pairs, and possibly less, have been lost from the viral junction sequence in the duplicated fragment. Furthermore, the sizes of the joint fragments, as obtained by cleavage with a number of restriction endonucleases, support the hypothesis of a tandem repeat of the viral DNA fragment without intervening host sequences.

Tumor Cell Lines. The cell lines BE-1t1 to BE-1t4 established from tumors appeared to have the same tandem integration of the *Bcl* I/*Eco*RI fragment found in the parental BE-1 cell line but the flanking host sequence varied (data not shown). These differences may reflect different cellular recombination events *in vivo* during the process of tumor formation. Alternatively, some cellular selection may have occurred during the propagation of the cell lines.

Virus-Specific Proteins. Polyoma virus-specific proteins synthesized in BE-1 cells or in the four tumor cell lines BE-1t1 to BE-1t4 were immunoprecipitated by sera from rats bearing tumors that were induced by injection of polyoma virus-transformed rat cells. This antiserum has previously been shown to precipitate specifically polyoma virus early proteins (23). BE-1 cells were found to contain middle (55,000) and small (22,000) T antigens which were electrophoretically indistinguishable from those derived from lytically infected cells but no protein equivalent to the normal large T antigen (100,000) was found (Fig. 5 *Upper*). BE-1 cells also contained a protein

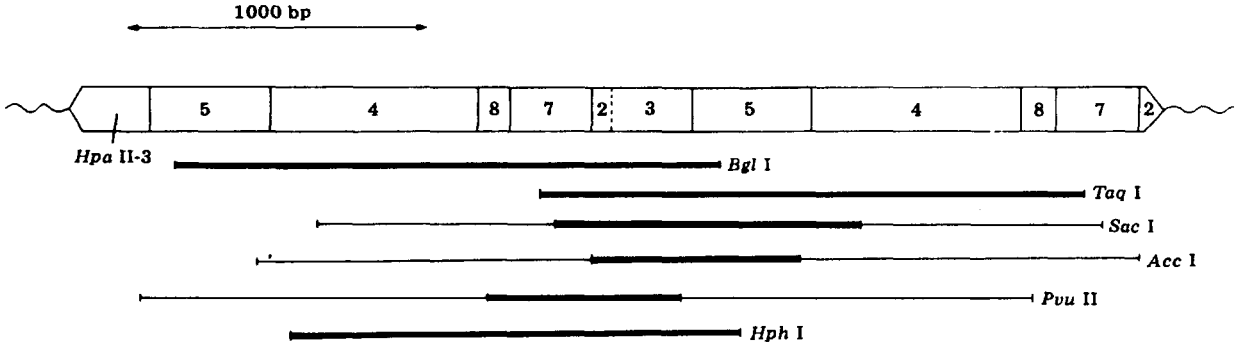


FIG. 4. Map of the portion of cellular DNA from BE-1 cells that contains integrated polyoma virus sequences. Cellular DNA is indicated by wavy lines; viral DNA, including *Hpa* II sites (1), is indicated by double lines. The maximal amount of viral sequence from the *Hpa* II-3 fragments would be that between the single polyoma virus *Bcl* I site (see Fig. 1) and the *Hpa* II-3/5 junction. Similarly, the maximal amount of viral sequence from the *Hpa* II-2 fragments would lie between the *Eco*RI site and the *Hpa* II 7/2 junction. Viral fragments that result from cleavage with a number of restriction endonucleases are indicated. Heavy solid lines, unique fragments which result from a tandem duplication of a portion of the viral DNA (Figs. 2 and 3; Table 2); thin solid lines, fragments which are indistinguishable from those obtained by cleavage of whole wild-type polyoma virus DNA.

of M_r about 34,000 which essentially fits the size that would be predicted if all the large T antigen sequence present in BE-1 cells and very little host DNA sequence were used in coding for this protein. A corresponding protein was not found in lytically infected cells. To characterize these proteins further, they were isolated and their tryptic peptides were analyzed as described by Ito (24). Peptides from the M_r 55,000 and 22,000 proteins derived from BE-1 cells were indistinguishable from peptides derived from middle and small T antigens of cells lytically infected with polyoma virus (Fig. 5 *Lower*). The M_r 34,000 protein from BE-1 cells proved to be related to large T antigen but contained a modification observed only in the corresponding antigen derived from cells infected with the viral mutant tsa and lacked peptides from the COOH-terminal portion of large T antigen.

DISCUSSION

Our results allow us to reach the following conclusions with respect to transformation. A fragment of polyoma virus DNA that contains only half of the early region (the *Bcl* I/*Eco*RI 35% viral fragment) provides sufficient genetic information to initiate and maintain cellular transformation of rat cells, and cells transformed by this fragment produce tumors in syngeneic rats (Table 1). Two of the polyoma virus early proteins, middle T antigen and possibly small T antigen, may be important for transformation but an intact large T antigen, the third early protein, is not required.

Classically, polyoma virus is assumed to play two separate roles in transformation, one in initiating and the other in maintaining transformation. Any role for large T antigen in the latter (maintenance function) now seems extremely doubtful. For example, many transformed cells fail to express a full-sized large T antigen or to produce full-sized viral early mRNAs and do not contain the whole of the early region DNA (refs. 29 and 30; unpublished results). Israel *et al.* (25) found that injection of polyoma virus DNA that had been linearized by cleavage with *Eco*RI, *Bam*HI, or *Xba* I into newborn hamsters caused tumor formation with high efficiency. Cell lines established from these tumors expressed only small and middle T antigens. Furthermore, in studies with temperature-sensitive mutants, Seif and Cuzin (31) showed that transformed rat cells selected for their ability to grow in soft agar were independent of a large T antigen function whereas those selected for their ability to form foci on plastic were mainly larg. T antigen dependent. In our experiments, not only did we select for transformants that grew in soft agar but also the viral information given the cell was less than that required to generate large T antigen (4). The initiation of transformation must therefore have occurred in the absence of a full-sized large T antigen.

In some of our transformed cell lines, notably the BE-1 line, we found that a truncated version of large T antigen was present in the cells (Fig. 5). In several lines, however, no detectable large T antigen-related species was observed. All the transformed cell lines established after transfection with the *Bcl* I/*Eco*RI fragment of polyoma virus DNA appeared to express small and middle T antigens. Moreover, because a normal-sized middle T antigen is expressed in BE-1 cells and the two-dimensional tryptic peptide pattern of middle T antigen in these cells is indistinguishable from the corresponding protein in lytically infected cells, we conclude that the coding region of middle T antigen lies completely within the proximal half of the early region of the polyoma virus genome.

The head-to-tail tandem arrangement of the integrated polyoma virus DNA sequences found in BE-1 cells was somewhat surprising. In general, in transformed cell lines derived from infection of rat cells with polyoma virus, viral DNA has previously been found integrated in head-to-tail tandem sequence arrangements (27, 29). The reason for this is not known. Because a functional large T antigen appears to be necessary for viral DNA replication (32), it appears unlikely in our case that the tandem repeat resulted from a replication of the viral DNA followed by integration of dimers (or multimers) into the host chromosome. In any event, there is no requirement for such a sequence arrangement in the maintenance of transformation because several of our cell lines (BE-2 to BE-5) did not appear to contain a comparable viral insert.

Tumor cell lines derived from animals injected with the BE-1 rat cell line show an identical tandem integration of the 35% polyoma virus DNA fragment found in the parental line. Flanking host sequences, however, were not identical in the various tumor lines. Changes in the state of integrated viral sequences in cells which occur with the passaging of the cells *in vitro* have been described (27, 33). In all cases these changes resulted from the excision of either a full-sized viral genome or a defective variant and appeared to require a functional large T antigen for the excision event. In our case, the apparent stability of the integrated subgenomic viral DNA may be due to the absence of a large T antigen.

Although they have many similarities, the two papova viruses, simian virus 40 and polyoma virus may differ with respect to transformation (12). Our studies, aimed at defining for polyoma virus the minimal amount of genetic information es-

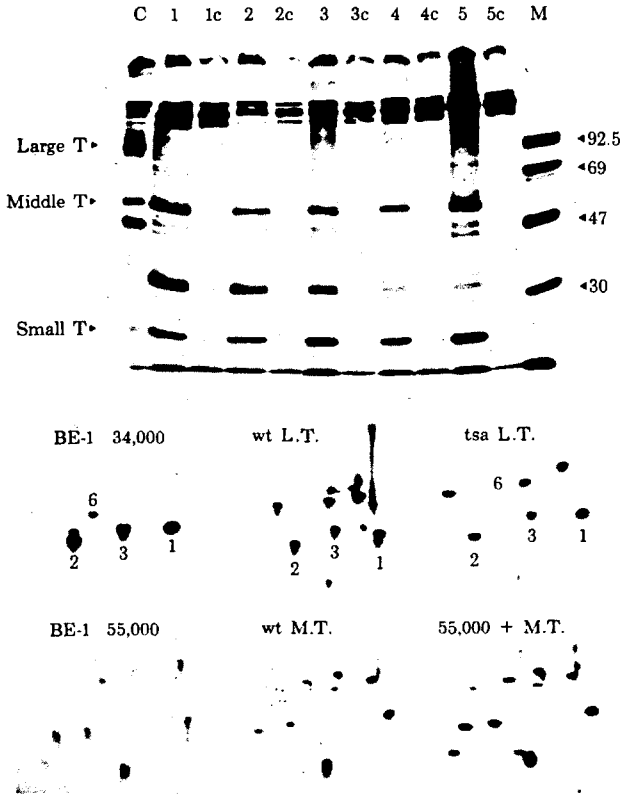


FIG. 5. (*Upper*) Comparison of early viral proteins expressed in the parental cell line BE-1 and in the tumor lines BE-1t1 to BE-1t4. [^{35}S]Methionine-labeled proteins were extracted from cells and immunoprecipitated with anti-T-antigen antiserum. Lane 1 contains the proteins from BE-1 cells precipitated with anti-T-antigen antiserum and lane 1c (control) shows proteins precipitated with serum from normal rats. Similarly, lanes 2c to 5c contain proteins from the tumor cells BE-1t1 to BE-1t4, respectively, precipitated with antiserum and the corresponding controls. Lane C contains polyoma virus early antigens expressed in lytically infected cells (that is, the large, middle, and small T antigens whose positions are indicated by arrows (at left). Lane M contains protein size markers, as indicated ($\times 10^{-3}$). (*Lower*) Autoradiographs of two-dimensional tryptic fingerprints of methionine-labeled proteins (24) immunoprecipitated from extracts of BE-1 cells or of polyoma virus lytically infected cells. Top row, left to right: M_r 34,000 protein from BE-1 cells, large T antigen (L.T.) from 3T6 cells infected with polyoma virus (A2-strain), and the large T antigen from 3T6 cells infected with tsa mutant and grown at the nonpermissive temperature. Spot 1 corresponds to the NH_2-terminal tryptic peptide (A. Mellor, personal communication) and spot 2, to the other methionine-containing peptide that is common to all three T antigens; spot 3 is a large T antigen-specific peptide (2, 24). The M_r 34,000 BE-1 protein contains a peptide (spot 6) that is unique to the large T antigen found in tsa mutant-infected cells but absent from the corresponding protein from wild-type polyoma virus-infected cells. This alteration may reflect a difference in some posttranscriptional modification of the two large T antigens. Bottom row, left to right: The M_r 55,000 protein of BE-1 cells, middle T antigen (M.T.) of wild-type polyoma virus lytically infected cells, and a mixture of the tryptic peptides from both proteins. Minor spots within middle T antigen from lytically infected cells have been shown to be large T antigen-specific species, possibly produced by proteolytic degradation.

sential for transformation of cells, further illustrate this difference. Whereas with polyoma virus, half the early region has proved fully competent for transformation, with simian virus 40 an interruption of early viral DNA sequences produced fragments of DNA that were nontransforming (34). The *Bcl* I/*Eco*RI DNA fragment used in the present study contains only the control region of the viral genome and only about 60 nucleotides more of the coding sequence than is apparently needed to specify middle T antigen (28).

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Appendix 2

Immunological Cross-Reaction Between Large T-Antigens of SV40 and Polyoma Virus.

by Frank McCormick, David P. Lane, and Stephen M. Dilworth.

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Immunological Cross-Reaction between Large T-Antigens of SV40 and Polyoma Virus

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Serum containing a high titer of antibodies against SV40 T-antigen was produced in rabbits, using D2 protein as immunogen. This protein was purified from cells infected with the adenovirus-SV40 hybrid virus Ad2$^+$D2. Anti-D2 serum recognized SV40 large T, but not small T. In addition, it was able to immunoprecipitate polyoma virus large T, demonstrating that these proteins share antigenic determinants. The titer of this antiserum against polyoma virus large T was comparable to the titers of polyoma virus antitumor sera. Anti-D2 serum did not immunoprecipitate polyoma virus middle T or small T antigens. The activity against polyoma virus large T was sensitive to SDS denaturation.

Polyoma virus and simian virus 40 (SV40) are closely related DNA tumor viruses of the papovavirus group. Polyoma virus occurs naturally in mice, SV40 in the rhesus-macaque monkey; both are able to grow in appropriate cell lines *in vitro*, to transform rodent cells *in vitro*, and to induce tumors *in vivo* (see Ref. (*1*) for reviews). SV40 is immunologically related to the other primate papova viruses, JC virus and BK virus, cross-reaction being reported for both the capsid and T (tumor) antigens (*1-3*). The DNA sequences of SV40 (*4, 5*) and polyoma virus (*6, 7*) have been determined and have revealed extensive homologies in the predicted amino acid sequences of the proteins coded for by the viruses (*8*). This has been used to argue for a common evolutionary ancestor for the viruses and similar mechanisms of replication in the host cell. Notwithstanding, only a single common antigen has been detected between polyoma virus and the primate papovaviruses, the VP-1 capsid protein (*3*). Sera from animals bearing SV40-induced tumors and sera raised against gel-purified SV40 large T-antigen have failed to cross-react with the tumor antigens of polyoma virus (*9*). In this communication, however, we have used an antiserum with high levels of antibodies against SV40 large T and show that shared antigenic determinants exist between the two large T proteins.

A high titer antiserum against SV40 large T-antigen has been raised in rabbits using purified adenovirus-SV40 hybrid protein, D2 as immunogen. D2 protein contains all of the sequences of SV40 large T-antigen except those at the N-terminus that large T shares with small T-antigen (Fig. 1). In place of these sequences, D2 protein contains a small portion of an adenovirus-coded protein which has been identified as the product either of gene VIII, or of the adenovirus late 100K protein (*10*). The anti-D2 serum has strong activity against SV40 large T-antigen and has been found to inhibit the ATPase activity associated with SV40 large T-antigen and D2 protein (*11*).

The anti-D2 serum immunoprecipitated a 100K-dalton polypeptide from lysates of 3T6 mouse cells that had been infected with wild-type polyoma virus (Fig. 2a). This polypeptide comigrated with polyoma virus large T-antigen that had been immunoprecipitated from the same lysate

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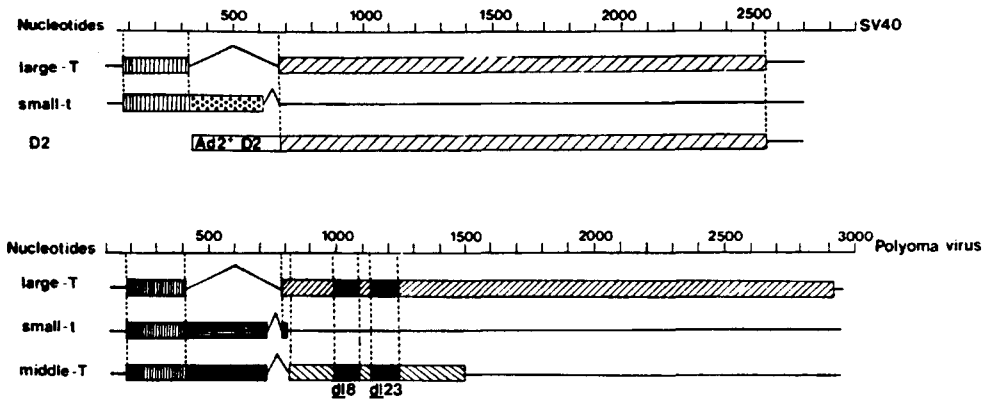
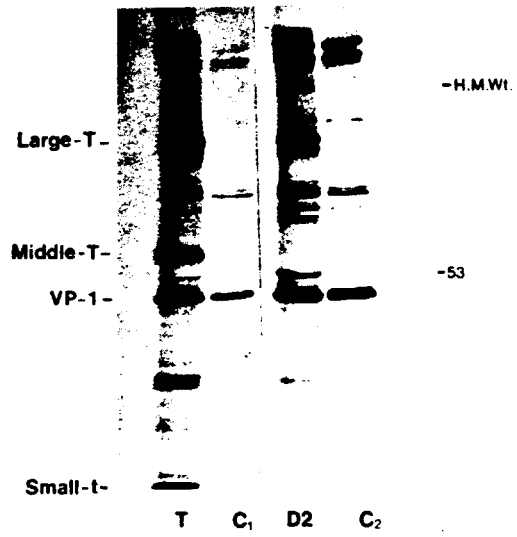


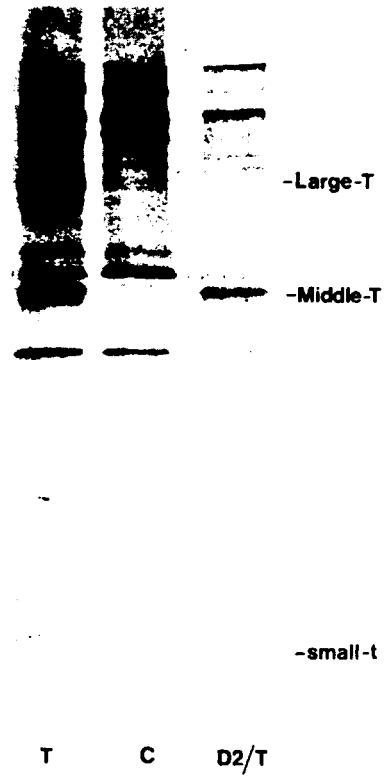
FIG. 1. Schematic representation of the coding sequences of the T-antigens of SV40 and polyoma virus, and of adenovirus-SV40 hybrid proteins, D2. The assignments of the early coding regions of SV40 and polyoma virus are those described in detail by Fiers *et al.* (*4*) and Soeda *et al.* (*6*) and their respective systems for nucleotide numbering used. Splice junctions for polyoma virus are taken from Kamen *et al.* (*15*), and from Reddy *et al.* (*16*) for SV40. Different protein coding frames are indicated by different shading areas. The amino-termini of the individual proteins lie on the left end of each bar. The D2 protein is thought to contain the unique sequences of SV40 large T and a sequence derived from Ad2+D2 (*10*). The regions of polyoma virus DNA deleted in mutants *dl*-8 and *dl*-23 are indicated. The precise sequence of these deletion mutants has been described elsewhere (*17*).

using antiserum raised against polyoma virus-induced tumors, and could be abolished by prior incubation of the anti-D2 serum with purified D2 protein (data not shown). Anti-D2 serum did not, however, immunoprecipitate any polypeptide that comigrated with either polyoma virus middle (55K) or small T-antigen (22K). (The strong 45K protein band present in all four lanes in Fig. 2a has been shown to be the late viral protein VP-1, S. Dilworth, unpublished data.) Also immunoprecipitated specifically by anti-D2 serum is a polypeptide of apparent molecular weight 53K and a high-molecular-weight polypeptide, neither of which is detectable in the antitumor serum track. The presence of the antibodies against these proteins in anti-D2 serum could be the result of contamination in the sample of D2 protein used for immunization. Although these proteins were not detected in our analysis, this possibility cannot be excluded. Another possibility is that purified D2 protein associates with these proteins *in vivo* and renders them immunogenic.

It therefore appears that these additional polypeptides are immunoprecipitated directly by antibodies present in anti-D2 serum rather than through an association with polyoma virus-coded proteins. Figure 2b shows that anti-D2 serum can immunoprecipitate all of the polyoma large T-antigen in a lysate, leaving only middle and small T-antigen to be immunoprecipitated by subsequent addition of antitumor serum. Anti-D2 serum was used to immunoprecipitate polypeptides from lysates of cells infected with the polyoma virus deletion mutants, *dl*-8 and *dl*-23 (*12*). It has been shown (*13*) that as a result of deletions at the positions indicated in Fig. 1 these mutants code for truncated forms of polyoma virus large and middle T-antigens. As shown in Fig. 3, anti-D2 serum immunoprecipitated polypeptides that comigrated with the truncated forms of large T-antigen, but failed to immunoprecipitate polypeptides corresponding to the truncated middle T-antigen species. We have confirmed the identity of the former species by tryptic fingerprint analyses of the polypeptides (data not shown). Anti-D2 serum also immunoprecipitated both forms of the large T-antigen (*14*), the 100K-molecular-weight polypeptide being



a FIG. 2a. Immunoprecipitation of polyoma virus large T-antigen by anti-D2 serum. The latter serum was raised by injecting purified D2 protein (prepared as described by Tjian, 1978, (*8*)) mixed with an equal volume of complete Freund's adjuvant into rabbits, subcutaneously at multiple sites. The injections were repeated three times at two weekly intervals, followed by two injections of D2 protein alone in PBS, again at two weekly intervals. Serum was then collected. 3T6 cells were infected with wild-type polyoma virus, (50 PFU/cell), strain A2, and labeled with [^{35}S]methionine (>600 Ci/mmol, Amersham) in methionine-free medium from 24 to 28 hr postinfection at 37°. Cells are lysed and immunoprecipitated as previously described (*18*). Samples were prepared using 50 μl of the following sera: track T, rat antitumor serum, raised in animals bearing polyoma-induced tumors, track C1, preimmune rat serum, track D2, rabbit anti-D2 serum, track C2 preimmune rabbit serum. The individual samples were then loaded onto a 10% SDS-polyacrylamide gel (*19*), electrophoresed, and the gel dried and autoradiographed by standard procedures. Unidentified polypeptides are marked, high-molecular-weight (H.M.Wt) and 53,000 (53K).



b FIG. 2b. Removal of all large T-antigen from a wild-type infected cellular extract by anti-D2 serum. A wild-type infected, labeled cellular extract was immunoprecipitated with: T, anti-T rat serum; C, preimmune rat serum; D2/T, extract treated with anti-D2 followed by protein A bearing *Staphylococcus aureaus*, repeated, and the supernatant immunoprecipitated by anti-T rat serum. Samples were then electrophoresed on a 10% SDS-polyacrylamide gel, and the gel dried and autoradiographed.

a doublet (data not shown). The 53K and high-molecular-weight polypeptides were also precipitated and their mobilities were not changed by the viral deletions.

These results strongly suggest that anti-D2 serum has activity against polyoma virus large T-antigen, but not against middle or small T-antigens. Anti-D2 serum was found to be only slightly less active than antitumor serum in its ability to immunoprecipitate polyoma large T-antigen (Fig. 4a). As shown in Fig. 4b large T-antigen precipitated either by anti-T serum or by anti-D2 serum and subsequently isolated, could be efficiently rebound in the denatured state by antitumor serum. Anti-D2 serum, on the other hand, was relatively inefficient at rebinding the gel-purified large T-antigen. The more efficient binding of the antigen prepared by antitumor serum probably occurred because of the high background encountered at high inputs of rabbit serum and was not

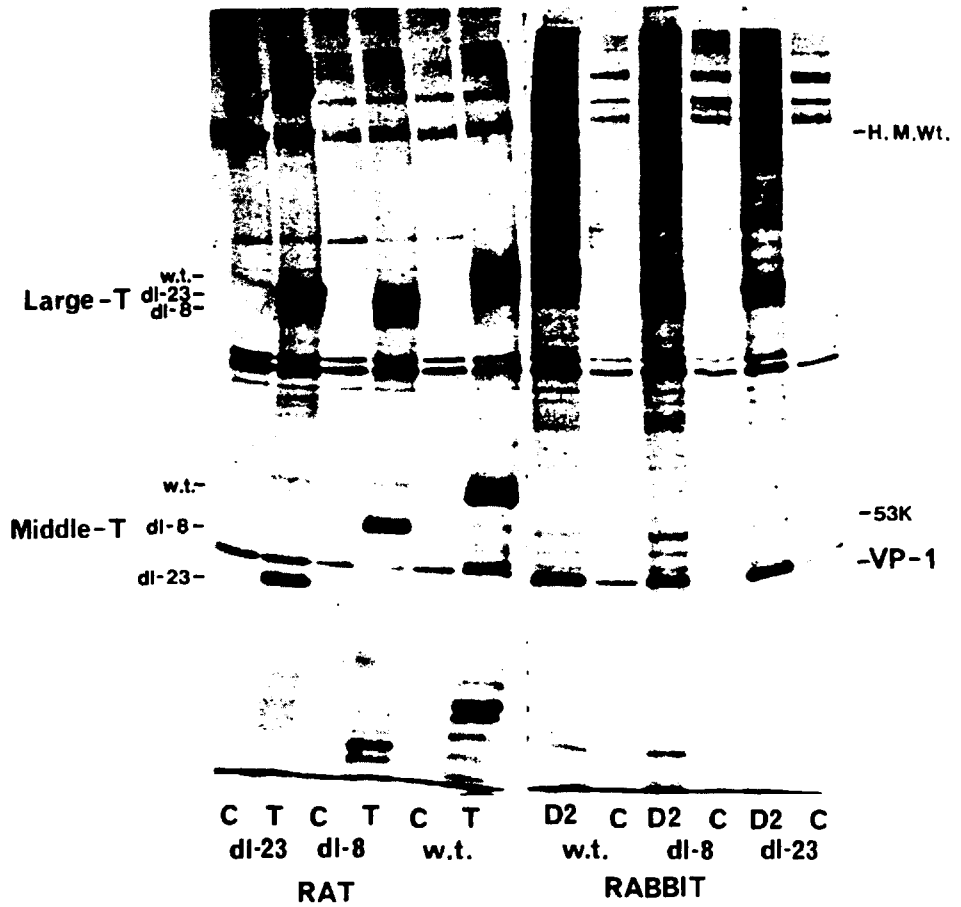


FIG. 3. Immunoprecipitation of truncated forms of large T-antigen from polyoma virus mutants *dl*-8 and *dl*-23 (*13*). Samples were prepared essentially as described above. Mutant proteins were separated from wt species by electrophoresis on a 7% SDS-polyacrylamide gel. In the left-hand lanes are shown samples immunoprecipitated with appropriate rat sera: C, preimmune serum; T, anti-T serum. In the right-hand lanes samples immunoprecipitated with rabbit sera: D2, anti-D2 serum; C, preimmune serum.

reproducible. These results suggest that a majority of the antigenic determinants recognized by anti-D2 serum on polyoma large T-antigen are sensitive to denaturation by SDS, and may only be expressed on the protein in its native state.

These experiments show for the first time that the large T-antigens of SV40 and polyoma virus share common antigenic determinants. This is not a surprising observation in view of the sequence homology of the two proteins that can be deduced from the viral nucleotide sequences. The two proteins are known to share common functions in that both are ATPases (*20–22*) and both are required for the initiation of viral DNA synthesis (*1*). Indeed SV40 large T will bind specifically to both the polyoma and SV40 origins of replication (*23*).

The polyoma and SV40 large T-antigens do differ in one vital respect; the SV40 protein's expression is required to induce and maintain the transformed state in rodent cells, while in the case of polyoma the large T seems to be defective in this func-

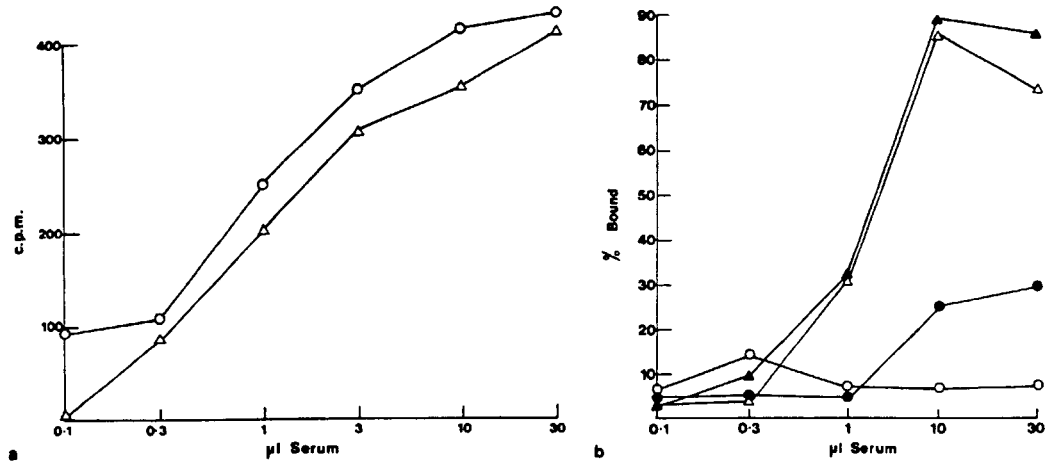


FIG. 4. (a) Comparison of sera potency against polyoma virus large-T antigen. [^{35}S]Methionine-labeled polyoma virus extracts were prepared and immunoprecipitated with varying amounts of rat anti-T serum (○), or rabbit anti-D2 serum (△). The proteins precipitated were separated by electrophoresis and the large T antigen band excised and counted. Radioactivity (cpm) was plotted against volume (µl) of sera used. (b) Binding of sera to gel-purified large T-antigens. [^{35}S]Methionine-labeled large T-antigen was prepared by immunoprecipitation by anti-T or anti-D2 serum, the appropriate band excised and eluted from the gel in 0.1% SDS. The protein was then rebound with antisera, collected on protein A bearing *S. aureus*, and counted. △, Large T antigen prepared with anti-T serum and rebound with anti-T serum; ▲, prepared with anti-D2 serum and rebound with anti-T serum; ○, prepared by anti-T serum and rebound with anti-D2 serum; ●, prepared by anti-D2 serum and rebound with anti-D2 serum. The percentage of rebound counts is plotted against the volume (µl) of sera used.

tion and the virus-coded middle T of polyoma is responsible for the oncogenic potential of this virus.

It is surprising that the cross-reaction between the two large T-antigens has not been detected before, even in the case of a high titer antiserum made against SDS-gel-purified SV40 large T (*9*). It is clear from the relative titer of the anti-D2 against SV40 and polyoma large T that the degree of cross-reaction is low and our results indicate that it is restricted to native (SDS sensitive) determinatnts. This cross-reaction may not have been detected with antitumor sera simply because of their lower overall titer or it may be that the cross-reactive determinants are sequestered from the antitumor response. In this regard it is interesting to note that SV40-induced tumors do not cross-react with polyoma-induced tumors in tumor transplantation assays (*24*).

That a cross-reaction between two proteins of related function and extensively homologous primary sequence should prove so elusive sounds a note of caution in the interpretation of negative results when immunological techniques are used to search for homologies between polypeptides. Further studies, perhaps using monoclonal antibodies, should serve to localize the sites of cross-reaction precisely and allow these sites to be mapped on the linear sequences of the two proteins.

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