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# THE TUMOR ANTIGENS AND THE EARLY FUNCTIONS OF POLYOMA VIRUS

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**Summary**

Polyoma virus (Py) tumor (T) antigens are the proteins specified by the early region of the viral genome. They are responsible for most biological effects caused by this oncogenic virus, i.e. induction of tumors, cell transformation and most of the virus-induced events observed in productive and transforming infection. By immunoprecipitation with antitumor serum followed by gel electrophoresis three major Py T-antigens have been characterized: large T-antigen (lT) with an apparent $M_r$ of about 100 000, middle T-antigen (mT) of about 55 000 $M_r$ and small T-antigen (sT) of about 23 000 $M_r$. In addition, there may exist one or more minor species by Py T-antigens. Analysis of the tryptic peptides showed that lT, mT and sT have a common N-terminal amino acid sequence, but differ from each other in the size and the sequence of the C-terminal part of the molecule as a consequence of different splicing of their mRNAs. With the nucleotide sequence of the Py genome being known, the coding regions for each of the Py T-antigens have been identified and consequently the amino acid sequence of lT, mT and sT was deduced. Cell fractionation experiments showed that the major part of 1T is located in the nucleus, mT was found in plasma membranes and sT is mainly present in the cytoplasm. Large T is a phosphoprotein and undergoes posttranslational modification. Two-dimensional gel electrophoresis of Py T-antigens revealed considerable charge heterogeneity particularly for mT and sT.

All Py transformed cell lines analyzed contained mT and sT. Large T was not detected in virtually all Py transformed mouse cell lines and in about one third of Py transformed rat and hamster cell lines. Instead of lT often new immunoreactive proteins were found which are probably truncated forms of lT. These and other recent results suggest that lT is required neither for initiation nor for maintenance of cell transformation. For tumor induction in hamsters, similar conclusions were reached from analysis of Py T-antigens and viral DNA sequences in cell lines derived from tumors that had been induced either by virus or by viral DNA digested with various restriction enzymes. Experiments done with several deletion mutants indicated that mT is required for cell transformation by Py. In a protein kinase assay done *in vitro* with Py T-antigen immunoprecipitates, a kinase activity associated with Py mT was found which phosphorylates tyrosine residues mainly of mT and less frequently of lT and of rat immunoglobulins. In all transformation defective mutants, kinase activity measured by this assay was absent or strongly reduced.

In a concluding chapter I discuss the events occurring in wild-type virus and mutant infected cells trying to attribute specific functions to each of the three Py T-antigens. At least two functions are known for lT, one is initiation of viral DNA replication, the other induces a mitotic response of the host cell, i.e. the events leading to and including host chromatin duplication. Middle T-antigen is certainly involved in cell transformation, possibly by its presence in the membrane. No function has been defined

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yet for sT. Since there are more virus-induced events observed in infected cells than Py T-antigens at least one of them must be a multifunctional protein.

## Introduction

This review summarizes the present knowledge on polyoma virus (Py) tumor (T) antigens, the proteins coded by the early region of the viral genome. The discussion of these proteins is preceded by a concise introduction on structure and biology of Py and on mutations that affect early viral functions. For detailed description and documentation of Py as well as comparisons with the closely related simian virus (SV) 40 and BK virus, the reader should consult the new edition of 'Molecular Biology of Tumor Viruses' (J. Tooze, ed., Cold Spring Harbor Laboratory) which appeared early this year. In the conclusion of this review, an attempt is made to correlate the Py T-antigens and the two genetically defined classes of early mutants with the biological effects of Py on the host cells. However, even at a time when the amino acid sequence of the T-antigens can be deduced from the known nucleotide sequence of viral DNA, it is impossible to attribute defined functions to each of the Py T-antigens and only few hints exist as to possible interactions of the viral proteins with host cell constituents. During the last year a considerable amount of new results on Py T-antigens became available which indicate new approaches for studying interactions of the early proteins of oncogenic viruses with various host cells. Therefore, I hope that this article may be of interest to a broader range of scientists working in cellular and molecular biology and biochemistry, since these studies should yield important information on regulation of mitosis and proliferation of mammalian cells.

## Polyoma Virus

*History*

Polyoma virus (Py) was discovered by GROSS[1] in 1953. In his studies on cell free transmission of leukemia in mice he found besides the leukemia agent, another, 'parotid tumor agent' which caused tumors in the salivary glands and differed in many respects from the leukemia agent. Later STEWART *et al.* showed that this agent could be propagated in mouse embryo cell cultures and, because of its capacity to induce a great variety of tumors in different tissues of mice and other rodents, proposed the name 'polyoma virus'[2,3]. The host of Py is the mouse where under natural conditions the virus causes no detectable disease. Injection of virus into newborn mice at relatively high dose, however, leads to tumor formation.

*Structure*

The genome of Py consists of circular, double-stranded DNA of about $3.5 \times 10^6$ daltons which is infectious[4,5]. Together with mouse histones H 2A, H 2B, H 3 and H 4 it forms a circular minichromosome with 20–25 nucleosomes[6]. The nucleoprotein core is surrounded by an icosahedral capsid consisting of 72 capsomeres[7] built up of 3 virus-coded proteins designated as VP 1, VP 2 and VP 3. The interactions between capsid proteins and the core to build up the virion are still under investigation[8,9]. VP 1 is the major capsid protein and accounts for 75% of the total viral proteins. The diameter of the virus particle is about 45 nm.

The structure of the virus particle and of its DNA, as well as the protein to DNA ratio are characteristic for the papovaviruses. Closely related to Py in size, genome organisation and biology are the monkey virus SV40 (reference 10, for a recent review see 11) and the human BK virus[12].

During the last year the analysis of the nucleotide sequence of Py DNA has been completed independently in two laboratories[13,14,15,16]. Based on the physical map established by GRIFFIN *et al.*[17], Figure 1 shows a schematic representation of the coding potential of the viral genome. From the origin of replication ($O_R$) at 71 map units the early and late regions extend clockwise and counterclockwise, respectively, to about 25.5 map units, the early and late mRNAs being transcribed from opposite strands in opposite directions[18]. The presence of termination codons for protein synthesis in each reading frame is indicated by black boxes. Crosses show the position of the initiation codons: for the early proteins at 74 map units, for VP 2 at 65 map units, for VP 3 at

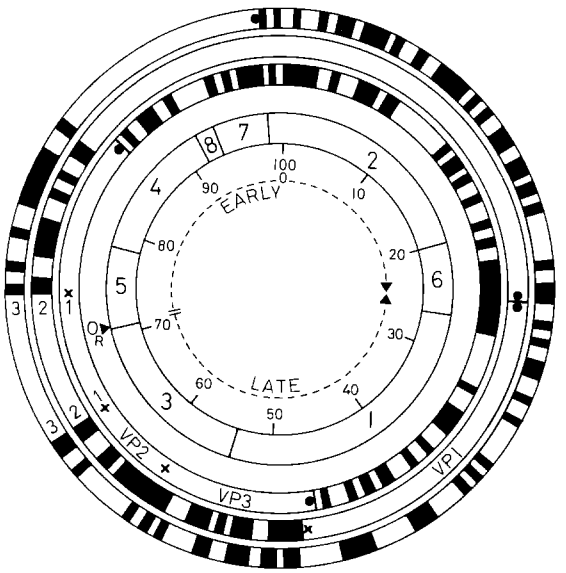


Fig. 1. The coding regions of the Py genome. The inner circle shows the physical map of Py DNA with the fragments generated by Hpa II. The map is divided into 100 units starting at the EcoRI site[17]. Close from the origin of replication ($O_R$) transcription of early and late mRNAs starts in opposite directions, thus dividing the genome into an early and a late region[18]. The three outer circles show the coding regions in the three reading frames numbered according to SOEDA *et al.*[15]. Crosses show the positions of AUG initiation codons, dots represent stop codons after the coding regions. Black boxes indicate presence of termination codons preventing synthesis of polypeptides.

58.5 map units and for VP 1 at 47.5 map units. Dots indicate the first stop codons after the coding region. The early region will be discussed in detail below. In the late region the related proteins VP 2 and VP 3 are read in the same frame and overlap with their carboxy-terminus the initiation codon of VP 1 by 29 nucleotides. The capsid proteins are translated from 3 differently spliced mRNAs[19,20] all having a common leader sequence mapping at 66 units and the same polyadenylated 3'-terminal sequence at 26 units[21,22].

The sequences published by the two groups are with few exceptions identical. Some minor differences which might be due to different strains or variants of Py exist in the noncoding region around the origin of replication as well as in some parts of the coding regions. The starting point for the nulceotide numbering is slightly different and as a consequence also the numbering of the reading frames. In this article I shall use the numbering proposed by SOEDA *et al.*[23] which starts clockwise from the Hpa II 3/5 cleavage site and has 5292 base pairs[16], while the sequence found by FRIEDMANN *et al.* has 5295 base pairs[13,14].

*Biology*

The limited coding potential of the viral genome and the dramatic biological effects of Py make it, together with the closely related SV40, a favorite tool to study host-virus interactions both *in vivo* and in cell cultures[10].

The induction of tumors by Py has been reviewed by EDDY[24] and recently also by WEIL[11]. Subcutaneous injection of newborn hamsters with Py ($1\times10^4$–$5\times10^4$ plaque forming units (pfu) per animal) leads to multiple subcutaneous tumors at the site of injection, first detectable by 4–8 weeks after inoculation. Tumor bearing animals develop humoral antibodies against Py tumor antigens and capsid proteins. Upon injection of higher doses of virus ($1\times10^7$–$5\times10^7$ pfu per animal) multifocal outgrowth of mesenchymal cells is observed as the beginning of tumor formation in heart and kidneys as early as 4 days after inoculation[11].

With cells in culture Py interacts in two ways. 'Permissive cells', i.e. mouse cells, support a lytic or productive infection leading to production and release of progeny virus and destruction of the host cell. The early phase of a productive infection comprises all the events occurring before onset of viral DNA replication. The late phase starts with the synthesis of viral DNA which is followed by synthesis of viral capsid proteins, virus assembly and cell lysis. In 'nonpermissive cells', i.e. hamster or rat cells, little if any virus is produced. In a majority of infected cells Py transiently induces a small number of cell divisions, while a few percent of these cells stably acquire new morphological and growth characteristics. This process is called 'transformation' and the infection of nonpermissive cells is designated as abortive or transforming. *In vitro* transformed cells are in some instances tumorigenic in a syngeneic host. Both cells derived from Py induced tumors and *in vitro* transformed cells contain viral DNA sequences stably integrated, i.e. covalently joined to host chromosomal DNA, they synthesize virus-specific RNA and viral tumor (T)-antigens. In general, virus-transformed or tumor cells differ from their parent cells (embryo fibroblasts or

'untransformed' cell lines) by growth to higher saturation density, lower requirement for serum growth factors, proliferation in semi-solid medium (agar or methylcellulose), production of higher amounts of plasminogen activator, stronger agglutination by plant lectins, a less ordered structure of the cytoskeleton (actin fibers) and reduced amounts of fibronectin on the cell surface. However, none of these characteristics is a unique property of transformed cells. From some, but not all transformed cells, infectious virus can be rescued upon fusion with permissive cells[10].

Soon it became obvious that the early events in productive and abortive infection were closely similar, because a striking analogy was observed when quiescent mouse kidney cells were infected with either Py (productive infection) or SV40 (abortive infection)[25,26]. Synthesis of small amounts of virus-specific RNA was followed by the appearance of intranuclear virus-specific T-antigen detectable by indirect immunofluorescence. Shortly after, infected cells went into S-phase (chromatin duplication) leading in abortive infection to mitosis and in lytic infection to production of progeny virus and destruction of the host cell (for details see last chapter). These findings and the observation that *in vivo* stimulation of mitosis also occurred in some tissues after injection of Py led to the proposition that viral T-antigens are multifunctional (pleiotropic) proteins which act primarily as inducers of a mitotic reaction in the host cell[25].

### *Mutants affected in early functions*

#### *Temperature-sensitive (ts) mutants*

Already in 1965 the first ts mutants were isolated on the basis of differential growth at low (32 °C) and high (39 °C) temperatures[27]. Analysis of a number of individual isolates by complementation experiments revealed that there is only one group of early ts mutants which is designated 'tsA' in analogy to a similar group of SV40 mutants[28]. By marker rescue experiments with different fragments of viral DNA generated by restriction enzymes, the Py tsA mutants were mapped between 1 and 26 map units (reference 29, see Fig. 2). At the high temperature tsA mutants are defective in initiating new rounds of viral DNA replication[30]. They are, however, able to induce cellular DNA replication, while intranuclear T-antigen determined by immunofluorescence is either absent[31] or its appearance is delayed[32]. Recent results showed that early viral mRNA is produced in tsA-infected cells at both permissive and non-permissive temperature in significantly larger

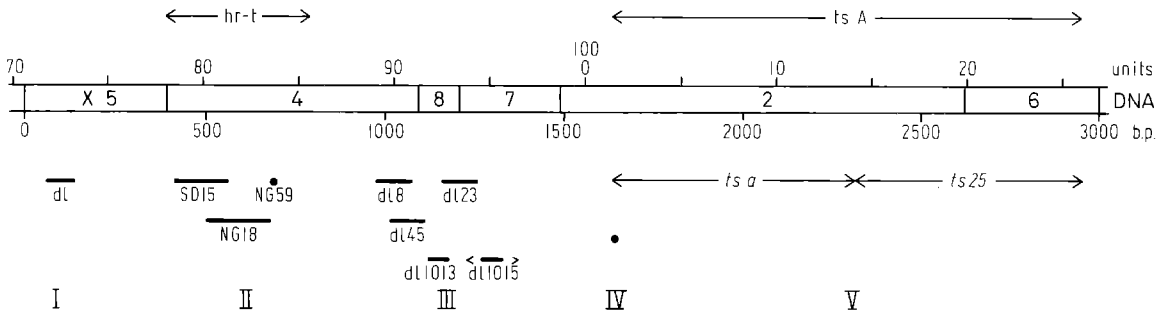


Fig. 2. Mutants mapping in the early region. On the linear physical map of the early region are indicated map units (above) and nucleotide numbers (below). The cross shows the position of the initiation codon at nucleotides 173–175[23]. The mutants can be subdivided into five groups:

(i) deletion mutants mapping in the noncoding region[49,50,51,52].

(ii) hr-t mutants: NG 18 is a representative of several different deletion mutants where the deletion changes the reading frame leading to a termination codon shortly after the deletion[41,47]; SD 15 is the only in-phase deletion mutant; NG 59 is one of three identical point mutants[41,48].

(iii) deletion mutants mapping between 89 and 99 units: so far five mutants have been described, dl 8 and dl 23[49], dl 45[53] dl 1013 and dl 1015[51].

(iv) mutants with an altered Hind III site at 1.8 map units without a detectable deletion[54].

(v) early ts mutants forming one complementation group (tsA): ts a and ts 25 are two representatives of several tsA mutants[28,29].

For further description of the mutants, see text.

amounts than in wild-type infected cells[33] and that the large T-antigen is unstable at 39 °C (see below).

In nonpermissive cells stable transformation by tsA mutants is reduced to 1% or less at high temperature, as compared to the permissive temperature[34,35]. However, at 39 °C these mutants can induce a limited number of cell divisions leading to small colonies in agar, so called 'abortive transformation', about half as efficient as wild type virus[36]. T-antigen present in tsA-transformed cells was shown to be unstable at 39 °C by immunofluorescence and also by complement fixation assays with cell extracts[37]. Already the very early experiments with tsA mutants indicated that cells transformed at permissive temperature remained transformed after shift to the high temperature suggesting that the tsA function is not required for the maintenance of the transformed phenotype[28,34].

Finally, it should be noted that ts mutants have never been isolated which map in the other half of the early region, i.e. between 71 and 100 map units (see Fig. 2).

*Host range, transformation defective (hr-t) mutants*

BENJAMIN isolated several mutants which produced in a permissive mouse cell line (3T3 cells) only 2–5% as much infectious virus as wild-type, but grew as well as wild-type virus in Py-transformed 3T3 cells[38]. Subsequent studies with various mouse cell lines and primary mouse cell cultures showed that the virus yield obtained with these host range mutants varied considerably with the type of cells used, but remained, with few exceptions, always below that observed with wild-type virus in the same cells. As judged from immunofluorescence, the percentage of T-antigen positive cells was in all cell lines tested similar for wild-type virus and mutant infected cells[39]. Apparently, these mutants replicate viral DNA, produce capsid proteins and kill the permissive cells to a similar extent as does wild-type virus[40,41]. These observations led to the suggestion that an intracellular block at some stage prevents the production of infectious mutant virions[40]. All the mutants selected by this approach proved to be defective in transformation and tumor formation, and were therefore designated 'hr-t' mutants. In nonpermissive rat cells these mutants synthesize T-antigen and induce host DNA replication, but fail to induce characteristic morphological changes and disappearance of actin fibers that are observed with wild-type virus[42]. All hr-t mutants form a single complementation group and map in Hpa II fragment 4 between 79 and 86 map units (reference 43, see Fig. 2). Hr-t and tsA mutants can complement each other for productive infection and for stable transformation[44,45,46]. The majority of hr-t mutants have deletions varying in size between 2.5 and 5% of total genome length, but three of the isolated mutants showed a normal size Hpa II fragment 4. Recently, the DNA sequence of different hr-t mutants has been determined[41,42]. With one exception (mutant SD 15) all deletion mutants have deletions producing a shift in the reading frame leading to a termination codon shortly after the deleted sequence (see Figs. 1 and 2). Mutant SD 15 has a deletion of 141 base pairs and thus remains in the same reading frame. Finally the three 'point mutants' (e.g. NG 59) all showed the same change in the DNA sequence at 84 map units, an insert of three base pairs followed by a single base pair change[48].

*Other viable mutants in the early region*

Several viable mutants have been isolated which have deletions of about 0.1–1% of the viral DNA in Hpa II fragment 5. More precise mapping placed the deletions between 71.8 and 73.4 map units or between nucleotides 60 and 160 (see Fig. 2)[49,50,51,52]. This corresponds to the noncoding region between the origin of replication at about 71 map units and the initiation codon for the T-antigens at 73.8 map units i.e. nucleotides 173–175. The DNA region covered by these mutants is thought to be important for initiation of transcription and translation of the early viral gene products. Some of these mutants give lower yields of progeny virus and smaller plaques, but no differences were observed in transforming infections.

Other mutants with deletions of 1–2% were found to map in the coding region for the T-antigens between 89 and 99 map units (see Fig. 2). For dl 45 no substantial differences to wild-type virus were observed so far both in productive and transforming infections[53]. Mutant dl 8 gives a significantly lower yield of

progeny virus i.e. <10% in productive infection, but transforms as well or even better than wild-type virus, whereby the transformed colonies show somewhat different morphology[49]. Mutant dl 23 grows as well as wild-type virus in productive infections, however transforms poorly. Very small colonies were formed in the dense focus assay and no colonies were observed in soft agar[49]. Two other mutants mapping in this region showed decreased transforming ability: mutant dl 1013 has a deletion of 15 base pairs and maps in Hpa II fragment 8 and mutant dl 1015 with a deletion of 30 base pairs maps in Hpa II fragment 7[51].

Another group of mutants has an altered Hind III site at 1.8 map units which renders their DNA resistant to the restriction enzyme at this site. One of these mutants without a detectable deletion grows slower and produces less progeny virus than wild type virus[54].

## Polyoma Tumor Antigens

*History*

Tumor (T) – antigens were first detected by complement fixation in cell free extracts prepared from tumors induced by polyoma and other viruses[55,56,57]. The T-antigens were found to be virus-specific and independent of the animal species. Subsequently, Py and other viral T-antigens were also found in virus-transformed cells and in cells undergoing productive infection[58]. Using immunofluorescent staining[58] or immunological methods combined with electron microscopy[59] T-antigens could be located in the nucleus of infected or transformed cells. So far, they have never been detected in virions. Time course analyses in Py-infected mouse kidney cells showed that intranuclear T-antigen detected by immunoflurorescence appears before onset of cellular and viral DNA replication and that there is a correlation in the kinetics of appearance of T-antigen and the induction of host chromosome replication in individual cells[60]. Sedimentation analysis of early virus-specific RNA in sucrose density gradients showed that it formed a rather sharp band at about 19–20 S[25,61]. Hybridization of this RNA to restriction enzyme fragments of Py DNA revealed that this RNA was the transcript of practically the entire early region[61,62]. At a time when processing of eukaryotic mRNAs by RNA splicing remained to be discovered, it was proposed that the early region of the viral genome directed synthesis of one rather large, pleiotropic protein, the T-antigen which was thought to have a mitogenic effect, to carry out the early viral functions for productive and transforming infections, to initiate and maintain the transformed phenotype and to induce tumors in animals[25,62,63]. Experimental evidence that T-antigens are virus-coded proteins was first reported by GRAESSMANN *et al.* After microinjection into mouse cells of RNA synthesized *in vitro* with RNA polymerase of *E. coli*, using as templates either SV40 or Py DNA, synthesis of intranuclear virus-specific T-antigens could be detected by immunofluorescence[64,65]. Direct proof that SV40 T-antigen is coded by the early region of the viral genome yielded the coupled transcription and translation of SV40 DNA in a cell free system derived from *E. coli*. GREENBLATT *et al.* obtained immunoprecipitable T-antigen fragments which comprised all but one $^{35}$S-methionine labeled tryptic peptides found in large SV40 T-antigen isolated from infected monkey cells[66]. Finally, synthesis of large and small SV40 T-antigens indistinguishable by electrophoresis and peptide analysis from the corresponding proteins extracted from infected monkey cells could be demonstrated in *Xenopus* oocytes after microinjection of SV40 DNA[67].

*Tumor antigens in productively infected cells*

*Characterization by gel electrophoresis*

During the last three years several groups reported results of the analysis of Py T-antigens by SDS-polyacrylamide gel electrophoresis. To extract T-antigens from Py-infected, $^{35}$S-methionine-labeled cultures the cells were at first simply frozen and thawed[68], but now, all groups use a buffer of slightly alkaline pH (pH 7.0 to pH 9.0) containing 0.5–1% of a nonionic detergent (e.g. NP 40)[69,70] or a combination of ionic and nonionic detergents[71,72]. The T-antigens were isolated and purified from the cell extracts by means of antisera and immunoadsorbance either to protein A carrying *Staphylococcus aureus* bacteria[73] or to protein A-Sepharose[74]. Antisera were produced in rats by injecting syngeneic Py-transformed cells[68,70],

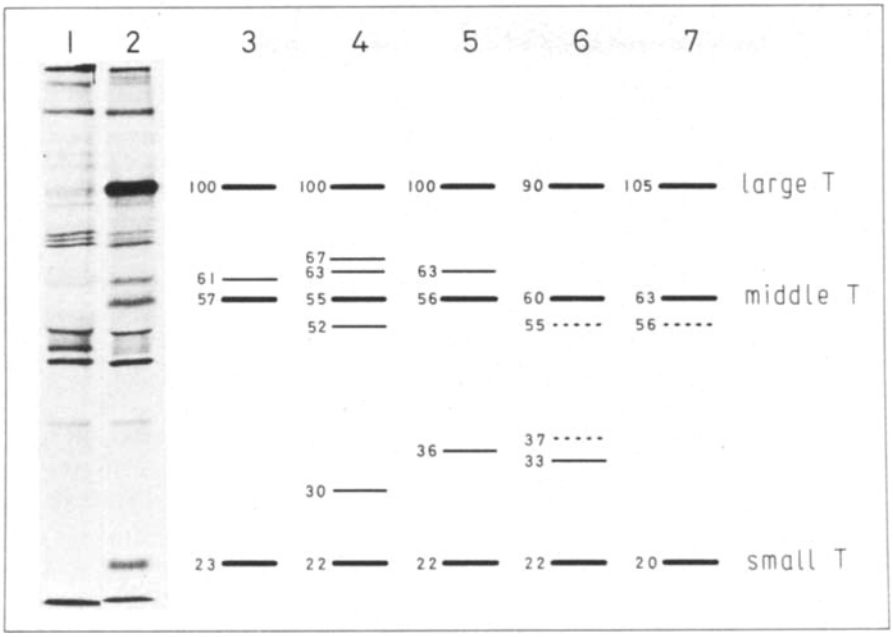


Fig. 3. Proteins precipitated by anti Py tumor serum. 1 and 2: immunoprecipitates of extracts from Py-infected mouse kidney cells labeled with $^{35}$S-methionine 24–28 h after infection with anti SV40 (control) and anti Py tumor serum, respectively separated by electrophoresis on a 10% SDS-polyacrylamide gel[69]. 3: schematic representation of the autoradiograph 2. 4–7: schematic representation of results reported by different groups, i.e. references 68, 70, 71, 72, respectively. Large T, mT and sT are marked by thick lines, minor proteins by thin lines and proteins recognized as cellular components by dashed lines. Numbers indicate $M_r$ ($\times 10^{-3}$) estimated by the different authors.

or in hamsters by injecting either Py into newborn hamsters or hamster tumor cells into 3–4 week old animals[69]. Sera from individual animals may differ considerably in affinity towards the different T-antigen proteins[69].

The results reported so far on the analysis of Py T-antigens are summarized in Figure 3. The main component is a protein with about 100 000 $M_r$. In addition, a small protein of about 22 000 $M_r$ was regularly detected. In analogy to similar results obtained with SV40, the two proteins were designated large T-antigen (lT) and small T-antigen (sT), respectively. Several bands were observed in the range between 67 000 to 52 000 $M_r$, the most evident being a protein estimated by the different groups to have an $M_r$ of 55 000–63 000. Since it was found to be related to sT and lT, it was called middle T-antigen (mT). Hutchinson *et al.* were the first to publish analysis of the $^{35}$S-methionine-labeled tryptic peptides of Py T-antigens and some of the minor immunoreactive proteins[71]. Their results showed that while lT, mT and sT have some peptides in common, lT and mT also have unique peptides (Table 1).

Similar results on the relationship of the three Py T-antigens were obtained by Smart and Ito[75] and by Simmons *et al.*[72]. These results suggested that lT, mT and sT are three distinct proteins sharing a common sequence, probably at the amino-terminal end. This hypothesis has now been confirmed by nucleotide sequence analysis of the early region and by analysis of the early mRNAs (see below). The origin of the other minor immunoreactive proteins is not yet entirely clear. The different results obtained (see Fig. 3) may be due to differences in the extraction procedure and serum specificity. Hutchinson *et al.* showed that the 55 000 $M_r$

Table 1
Common and unique $^{35}$S-methionine-labeled tryptic peptides in Py T-antigens (according to Hutchinson *et al.*[71]).

| | common to lT, mT, sT | common to mT, sT | unique |
|---|---|---|---|
| small T | 1–4 | A–E | none |
| middle T | 1–4 | A–E | U–Z |
| large T | 1–4 | | 5–25 |

and the 37 000 $M_r$ proteins observed in their gels (Fig. 3, column 6) are not related to the T-antigens and are probably cellular proteins, while the 33 000 $M_r$ protein contained some of the unique peptides of mT[71]. The 61 000 $M_r$ protein observed in our gels (Fig. 3, columns 2 and 3) has several peptides in common with lT. We suspect that it is in part a degradation product of lT which might be contaminated with a cellular protein often observed in gel electrophoresis at the same position as a faint band. The minor bands observed by SCHAFFHAUSEN *et al.* at 63 000 and 36 000 $M_r$ (Fig. 2, column 5) were always absent in hr-t mutant infected cells. Complete and partial proteolytic digestion revealed that these two proteins are at least partially related to lT[41,76]. Recently, ITO and SPURR proposed that there is a fourth Py T-antigen component[77]. They observed two minor bands with electrophoretic mobilities corresponding to 43 000 $M_r$ and 39 000 $M_r$. The two proteins gave similar tryptic peptide patterns showing the peptides common to lT, mT, sT and the unique peptides of mT. They argue that these proteins give minor bands because the antibody titers of the sera were low against this particular T-antigen component[77].

*In vitro* translation of virus-specific RNAs isolated from the cytoplasm of productively infected cells and selected by hybridization to Py DNA yielded lT, mT and sT, thus confirming that there are three distinct T-antigens. Middle T and sT were indistinguishable from the respective proteins synthesized in infected cells both by electrophoresis and tryptic peptide analysis. Large T synthesized *in vitro* migrated slightly faster than lT found in infected cells and one $^{35}$S-methionine-labeled peptide showed altered mobility in the chromatographic analysis[78]. With $^{35}$S-methionine-labeled fMet–$tRNA_f^{Met}$ as the only labeled precursor, all three T-antigens could be labeled, which indicates that lT, mt and sT are all primary translation products. Similarly to what was observed with SV40 T-antigens[79], the N-terminal amino group is acetylated both in T-antigens synthesized in infected cells and in the reticulocyte lysate[78].

*T-antigens synthesized in mutant-infected cells*

In tsA mutant-infected cells synthesis of lT, mT and sT occurs both at low and high temperature (reference 68 and own unpublished observations). During a chase at 39 °C mT and sT show no detectable change, but lT is clearly unstable[68,71,76]. Within 2–6 h the amount of radioactivity in the lT band decreases 4–20 fold for tsA mutants, but remains practically unchanged for wild-type virus[71]. SMART and ITO observed that in tsA mutant-infected cells which had been infected at 32 °C for about 40 h, then shifted to 39 °C and labeled 4 h after the shift, the incorporation of radioactivity into lT, mT and sT was significantly increased when compared to wild-type infected cells (reference 75, see Fig. 4A). This is a useful procedure to obtain higher amounts of radioactivity in Py T-antigens for structural studies.

The results of the analysis of T-antigens synthesized in cells infected by different hr-t mutants[76] can be summarized as follows: (i) large T remains unchanged with all hr-t mutants, suggesting that the region which is deleted in some hr-t mutants does not serve for the synthesis of lT. (ii) middle T is not detected in cells infected with hr-t deletion mutants with the exception of mutant SD 15 which has an in-phase deletion. This mutant induces synthesis of a shortened mT of about 50 000 $M_r$, while the hr-t 'point mutants' (e.g. NG 59) show a normal size mT. (iii) small T is never detected in cells infected by hr-t deletion mutants. Several very small proteins of 6000–9000 $M_r$ were found to react with anti tumor serum. However, no correlation between location and size of the deletion and the size of these proteins could be made. Normal size sT was found in cells infected with 'point mutants', but radioactivity incorporated into mutant sT was lower than that in wild-type sT. (iv) the two additional bands (63 000 and 36 000 $M_r$) observed in wild-type infected cells by SCHAFFHAUSEN *et al.* (Fig. 3, column 5) were never observed by these authors in hr-t mutant-infected cells. They think that synthesis of these T-antigen related proteins is blocked, because of aberrant processing of the mRNAs of hr-t mutants[41,76].

Figure 4B shows T-antigens extracted from wild-type and NG 18 infected primary mouse kidney cells. The bands corresponding to mT and sT are clearly absent in NG 18 infected cells. The 61 000 $M_r$ band, if present at all is very weak in sample 1 extracted 25 h after infection. However, in cells extracted 45 h after

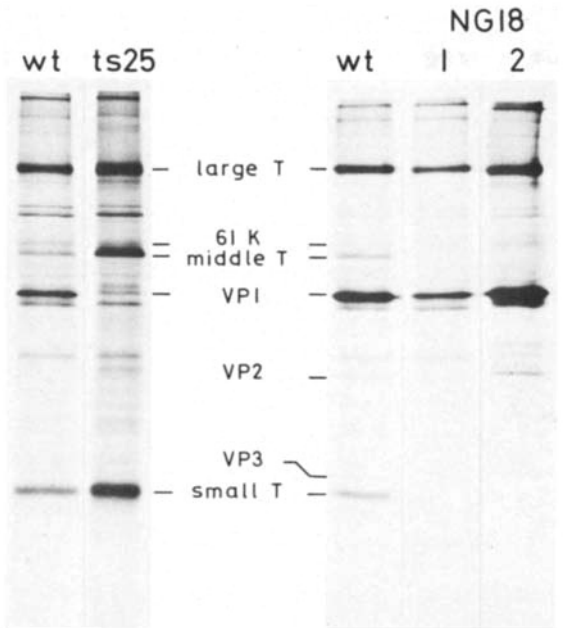


Fig. 4. Py T-antigens in mutant-infected cells. (A) shift-up with tsA mutants: wild-type and ts 25 infected mouse kidney cells were incubated for 42 h at 32 °C, then shifted to 39.5 °C; they were labeled with $^{35}$S-methionine from 7–10 h after the shift.

(B) hr-t mutants: wild-type and NG 18 infected mouse kidney cells were incubated at 37 °C and labeled with $^{35}$S-methionine 22–25 h after infection (wt and NG 18,1) and 46–49 h after infection (NG 18,2).

Py T-antigens were extracted and analyzed as described earlier[69] on 12.5% gels.

infection (sample 2) a band similar to that found in wild-type infected cells was observed at this position.

*Alignment of the T-antigens to the nucleotide sequence of the early region*

It was a fortunate coincidence that both in London and in San Diego groups studying the T-antigens and their tryptic peptide patterns were working in close vicinity to groups establishing the nucleotide sequence of the Py genome. These cooperations and the simultaneous fine structure analyses of early viral mRNAs done by KAMEN *et al.* rapidly yielded a clear picture how the early region of the viral genome was organized. Using a modified BERK and SHARP S1-nuclease mapping procedure[80,81], KAMEN *et al.* detected three major species of early mRNAs in cells undergoing lytic infection. All major mRNAs have their 5′-ends at about 73.3 map units and a polyadenylated 3′-end at about 25.8 map units, but differ in the length of internal nucleotide sequences that have been removed by RNA splicing. The smallest RNA lacks about 390 nucleotides from 78.3 to 85.6 map units and amounts to about 50% of the early mRNAs found in infected cells. The two other mRNAs lack about 65 and 50 nucleotides between 84.6 and 85.8 or 85.6 map units, respectively[82]. Sizing of early mRNAs by gel electrophoresis followed by *in vitro* translation showed that the mRNA coding for lT is smaller than the mRNAs coding for mT and sT[78]. The comparison of the sequences of several spliced mRNAs of different origin with their DNA template sequences led to the formulation of general rules for the donor and acceptor nucleotide sequences at the splice junction. Applying these rules to the results obtained in the mRNA analysis and the known nucleotide sequence KAMEN *et al.* proposed that splicing occurs between the nucleotide numbers indicated in Figure 5. These nucleotide junctions allow the three mRNAs reentry in the different reading frames that are required for synthesis of lT, mT and sT[82].

The organization of the early region of Py DNA, is schematically represented in Figure 5. This scheme agrees with the DNA nucleotide sequence, the structural analysis of the early viral mRNAs and the tryptic peptide analysis of the T-antigens. Large T, mT and sT all start at the initiation codon formed by nucleotides 173–175 and share a common amino acid sequence up to 78.3 map units. The region between 78.3 and 85.6 map units is not used for synthesis of lT and corresponds to that part of the genome where the hr-t mutants map. This region serves for synthesis of sT and mT being read in frame 1 up to 84.6 map units. Between 84.6 and 85.6 map units there are about 50 nucleotides that are not translated at all. The splice in the mRNA for sT is not compulsory since no change in reading frame is required to reach the stop codon at 85.8 map units (TAG, nucleotides 806–808). The existence of a deleted sequence in sT was suggested from the absence of any continuous mRNA in the cytoplasm of infected cells and by the observation that RNA complementary to Py DNA directed in the reticulocyte lysate synthesis of only one immunoreactive protein which migrated slightly

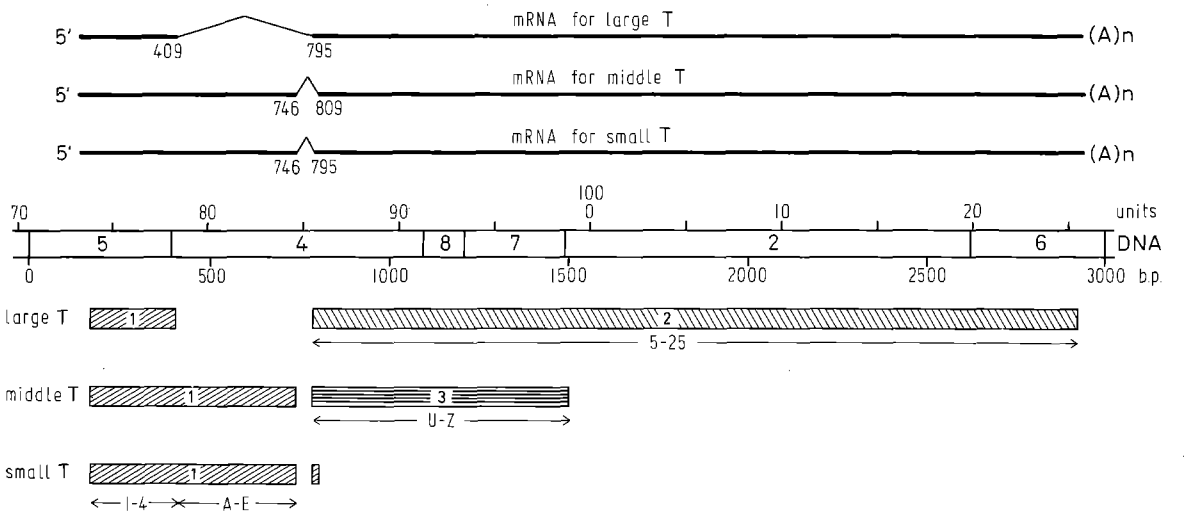


Fig. 5. Organization of the early region of Py DNA. Above the linear physical map of the early region the structures of the early mRNAs are shown with numbers indicating the nucleotides joined together by RNA splicing as proposed by KAMEN *et al.*[82]. Below the DNA map, the regions of the genome and the reading frames used for synthesis of Py T-antigens are indicated by bars. Figures and letters indicate the $^{35}$S-methionine labeled tryptic peptides assigned to each region according to HUTCHINSON *et al.*[71].

slower than sT in gel electrophoresis but had the same methionine containing tryptic peptides as sT[78]. From 85.6 to 98.8 map units lT and mT are read in different frames from the same DNA sequence. Middle T read in frame 3 stops at nucleotides 1498–1500 (TAG). Finally, lT is read in frame 2 which is open up to 25.5 map units and covers the region where the ts A mutants map. In fact, only lT is altered in these mutants. Large T is terminated by a TGA codon at position 2913–2915. Between the initiation codon and the EcoR1 site (100 map units) the nucleotide sequences reported by FRIEDMANN *et al.*[13] and SOEDA *et al.*[15] are identical except for one base pair (nucleotide 1217). Several differences exist however in the remaining part of the early region (0–26 units) leading to a slightly altered position of the termination codons. The early coding region comprises 2731 nucleotides according to FRIEDMANN *et al.*[13] and 2740 nucleotides according to SOEDA *et al.*[15].

The S1 gel-mapping experiments by KAMEN *et al.* revealed several minor species of early mRNAs in addition to the three major ones. They all have the same splice junctions as the three major mRNAs. One group has polyadenylated 3'-ends mapping at about 99 units which is at or very close to the termination codon for mT. In productively infected cells this group amounts to less than 10% of the early mRNAs, but in some transformed cells it constitutes the major early mRNAs. Another group of minor early mRNAs shows an additional S1 nuclease sensitive site at about 93.2 units which could be either a second very small splice or an unknown RNA modification which destabilizes the hybrid structure[82].

The organization of the early region as shown in Figure 5 is also supported by the observed changes in the T-antigens caused by different deletion mutants. With hr-t deletion mutants, full size lT is always observed, but sT and mT are either absent or reduced in size. Large T and mT are both reduced in size by dl 8 and dl 23 as is expected from the location of these deletions (49, see below).

The region between the Hpa II 3/5 site and the initiation codon contains termination codons on both strands and in every frame and is therefore designated as noncoding. Several variations in the nucleotide sequence of different Py strains have been detected in this part of the genome. Some interesting characteristics revealed by the sequence analyses have been discussed in detail[13,23]. It might be pointed out here that Py, SV40 and BK virus all show extensive sequence homology around their origin of replication, including a stretch of 8 identical AT base pairs[83,84,85,86]. The DNA of

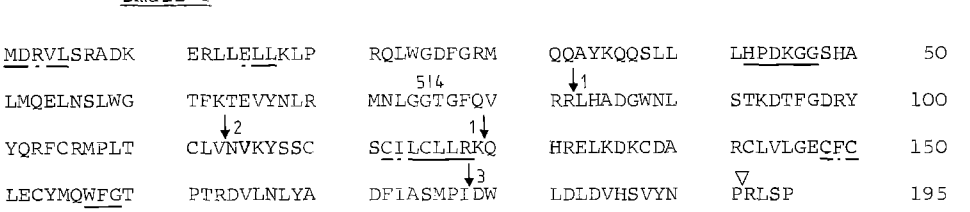


Fig. 6. Amino acid sequence of sT. Amino acids are designated by one-letter abbreviations: A = Ala, C = Cys, D = Asp, E = Glu, F = Phe, G = Gly, H = His, I = Ile, K = Lys, L = Leu, M = Met, N = Asn, P = Pro, Q = Gln, R = Arg, S = Ser, T = Thr, V = Val, W = Trp, Y = Tyr. 5/4 indicates the Hpa II 5/4 site, ∇ the location of the splice in the mRNA. Mapping of some hr-t mutants is indicated by arrows: 1, beginning and end of the deletion in SD 15; 2, beginning of the deletion in NG 18; 3, site of sequence change in point mutants. Main homologies to SV40 sT: Underlined amino acid sequences are also found in SV40 sT at same positions, dots indicate residues replaced by closely related amino acids.

this region can either be folded into a double, cruciform hairpin structure or alternatively into a fourstranded conformation[23].

*The amino acid sequences of polyoma T-antigens*

From the DNA nucleotide sequence and the proposed splice junctions in viral mRNAs the amino acid sequences of the three Py T-antigens can be deduced. They are given with the one-letter abbreviations in Figures 6, 7 and 8. Since the sequences of the mRNAs around the splice junctions have not been determined yet, the amino acid sequences given here may still be subject to changes near the splice sites. Comparison of the amino acid sequences of Py sT and lT to the corresponding proteins of SV40 showed a rather high degree of homology[13,15,87]. This was quite unexpected, because no homology of the early region was found by annealing of the two viral DNAs[88] and furthermore no or only very weak immunological crossreactions were observed between the T-antigens (see Fig. 3, columns 1, 2). To simplify the presentation of the sequences only the main homology regions to SV40 T-antigens are indicated in Figures 6, 7 and 8. For direct comparison consult references 13 and 15.

*Small T-antigen* (see Fig. 6)

This protein consists of 195 amino acids and its calculated $M_r$ of 22785 is close to the values found in gel electrophoresis. The sequence suggests that there are 7 methionine containing tryptic peptides. HUTCHINSON *et al.* detected 9 spots of which 3 were rather weak[71]. On the basis of net charge and hydrophobicity calculated for the expected peptides HUNTER *et al.* have tentatively identified all 7 peptides[89,90]. Further correlations between the peptide analysis and the deduced amino acid sequence were found by comparing the number of expected and observed cysteine containing peptides and the sensitivity of the peptides to

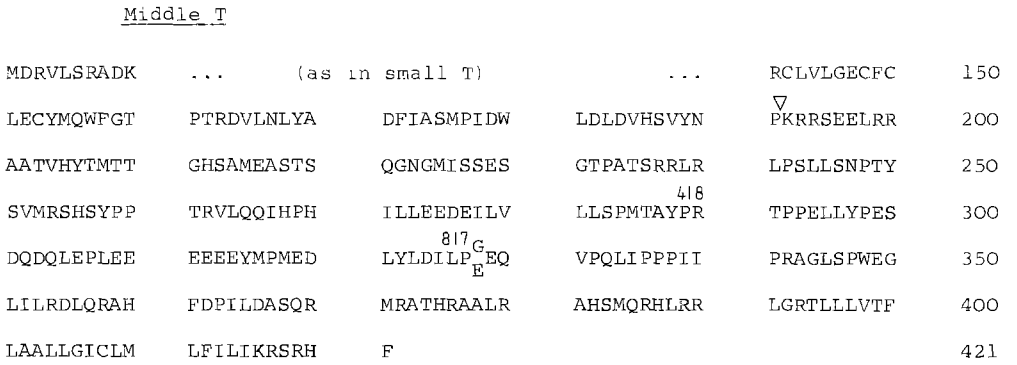


Fig. 7. Amino acid sequence of mT. For symbols see legend to Figure 6. The first 191 amino acids are the same as in sT. Close to the Hpa II 8/7 site (base pair 1217) there is a difference in the sequence reported by the two groups coding a different amino acid at position 328[13,15]. Mutant dl 8 lacks amino acid residues 253–282, dl 45 residues 281–302 and dl 23 residues 302–335 (53 and B. E. GRIFFIN, personal communication).

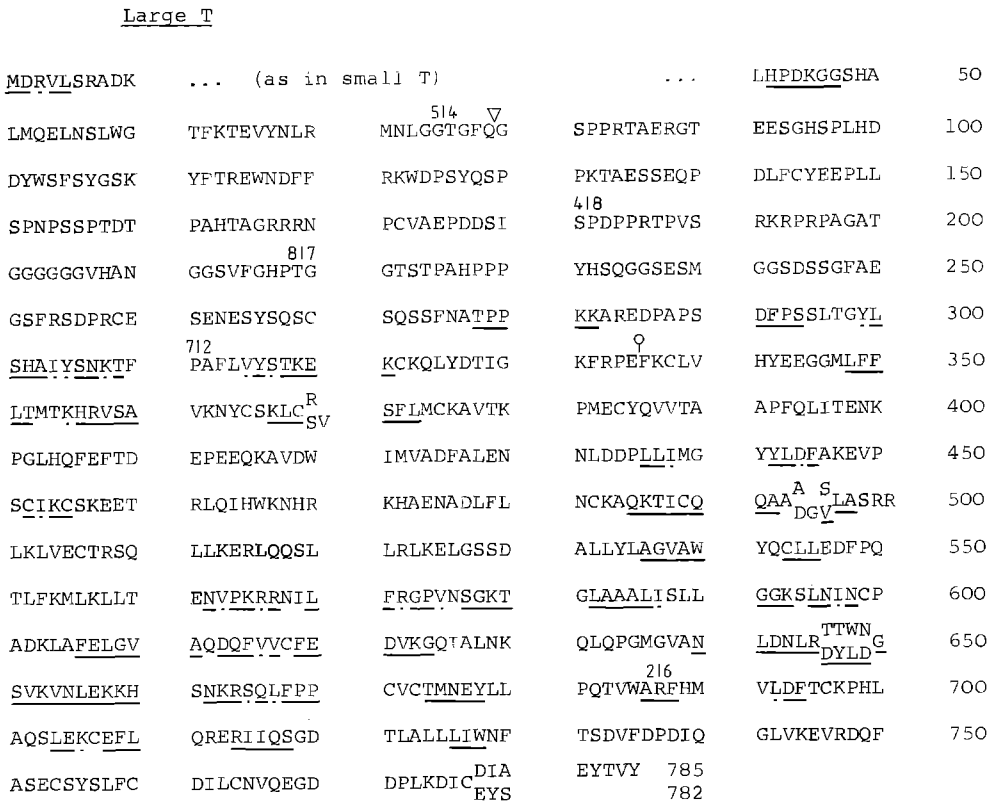


Fig. 8. Amino acid sequence of lT. For symbols see legend to Figure 6. O indicates the EcoRI site. The first 75 amino acids are the same as in sT and mT. Where the reported sequences differ, the top letter gives the amino acid coded by the sequence of SOEDA *et al.*[15] the bottom letter that derived from the sequence of FRIEDMANN *et al.*[13]. Residues 145–174 are missing in mutant dl 8, residues 173–194 in mutant dl 45 with arginine changed to glycine at position 195 and residues 195–228 are absent in mutant dl 23 (53 and B. E. GRIFFIN, personal communication).

digestion with chymotrypsin and V8 protease[89,90]. The N-terminal methionine is most likely acetylated as has been demonstrated for sT and lT of SV40[79]. The expected N-terminal tryptic peptide of all three Py T-antigens is N-AcMDR and has been compared to chemically synthesized N-AcMDK, the N-terminal peptide of SV40 T-antigens. The electrophoretic mobilities were found to be identical and only a slight difference was found in chromatography[89,90]. The sT of SV40 is slightly smaller and has 174 amino acids. Homology between Py and SV40 was found both in the N-terminal and the C-terminal portions[87]. In residues 1–58 which are common to all three Py T-antigens 25 amino acids were found at identical positions, in residues 105–159 22 amino acids were found at same positions, among them 8 cysteine residues[87]. The cysteine rich region or part of it is deleted in all hr-t deletion mutants. Mutant SD 15 is deficient in amino acids 83–129[48]. Mutant NG 18 lacks residues 114–176. Thereafter the reading frame is changed and protein synthesis stops shortly after[47]. The hr-t point mutation causes the insertion of isoleucine after position 177 (isoleucine) and changes the following aspartic acid to asparagine. The remaining sequence of sT from hr-t point mutants is the same as in wild-type virus[48].

*Middle T-antigen* (see Fig. 7)

With 421 amino acids, mT has a calculated $M_r$ of 48 566. This is considerably less than was found in gel electrophoresis where the estimates were between 55 000 and 63 000 $M_r$. Up to position 191 the sequence is identical to that of sT, i.e. only the last four amino acids of sT are not represented in mT. This region comprises 6 methionine containing tryptic peptides. The 7th methionine containing peptide of sT (residues 164–192) undergoes by RNA splicing only a change from arginine to lysine in the last amino

acid. This change should not alter the electrophoretic mobility and the change in chromatography (second dimension) is expected to be small, since it is a rather large peptide. Indeed, HUTCHINSON *et al.* did not find a unique peptide in sT[71]. In the remaining portion of mT which is read from the same DNA sequence as lT, 7 unique methionine-labeled tryptic peptides are expected. HUTCHINSON *et al.* found 6 of which 2 have been identified by comparison with chemically synthesized peptides, i.e. MR at positions 371–372 (peptide W) and AHSMQR at positions 381–386 (peptide V)[89,90]. In this way they confirmed the reading frame for mT. Three other peptides have been tentatively assigned on basis of net charge and hydrophobicity[89,90].

The amino acid sequence of mT has some unusual features. Residues 274–320 comprises 17 acidic amino acids, four in a row at positions 274–277 and a run of 6 glutamic acids at positions 309–314. These latter are flanked by proline rich sequences containing 6 prolines within 25 residues. Towards the C-terminal end of the molecule is an uninterrupted sequence of 22 hydrophobic amino acids which may be important for the association of mT with membranes (see below).

No virus-coded mT has been detected in SV40-infected cells or by *in vitro* translation of SV40 mRNAs. Also, no sequences homologous to Py mT between residues 192 and 421 are found in SV40lT. There is however a cluster of 6 acidic amino acids towards the C-terminal end of SV40 lT and a proline rich sequence at the very end[83].

Middle T of hr-t mutants undergoes the same changes as sT, however mutant SD 15 has in addition to the deletion several single basepair changes in Hpa II fragment 4 leading to a change of the amino acid at 4 positions in mT and at 2 positions in lT[48]. The three deletion mutants dl 8, dl 23[49] and dl 45[53] mapping between 88 and 94 units increase the migration of both mT and lT in SDS-gel electrophoresis[91,53]. Mutant dl 8 has a deletion of 30 amino acids deleting residues 253–282 (B. E. GRIFFIN, personal communication) and affects two methionine containing peptides at positions 241–254 and 262–290. Tryptic peptide analysis of mT specified by dl 8 showed the disappearance of one and a different migration of another of the wild-type peptides[91]. This is further evidence that the proposed reading frame for mT is correct. Mutant dl 45 has a deletion of 22 amino acids removing residues 281–302[53]. Middle T of mutants dl 8 and dl 45 shows by gel electrophoresis a reduction in $M_r$ of about 5 000 and 2 000 respectively[53,91] corresponding roughly to the size of the deletions, but the resulting values of their $M_r$ are still much higher than expected from the amino acid composition. In mutant dl 23, 34 amino acids, i.e. residues 302–335 including the run of 6 glutamic acids are deleted (B. E. GRIFFIN, personal communication). No change in methionine containing tryptic peptides was observed, despite the presence of 2 methionines at positions 316 and 318[91]. However, these are present in a very large acidic peptide (291–342) which is probably not detected. In SDS-gel electrophoresis mT of dl 23 migrates with an apparent $M_r$ of 43 000 which is close to the calculated value[91].

The question remains why wild-type mT migrates slower than predicted in SDS-gel electrophoresis. Comparison of the migration of mT from dl 8 and dl 23 suggests that structural features inherent to the amino acid sequence between positions 302 and 335 may be the cause. Since wild-type mT synthesized *in vitro* has the same or a closely similar apparent $M_r$ as mT extracted from cells[78], extensive post-translational modification seems unlikely, although it can not be excluded.

*Large T-antigen* (see Fig. 8)

According to the sequence and splicing data obtained in London, large T-antigen is a protein of 785 amino acids with a calculated $M_r$ of 87 991. This is again less than was estimated from SDS-gel electrophoresis. The few differences in the nucleotide sequence reported by the two groups[13,15] between 0 and 26 map units lead to some changes in the amino acid sequence which are indicated in Figure 8. Where there is a divergence, the top letter indicates the amino acid derived from the nucleotide sequence reported by SOEDA *et al.* (15) the bottom letter that coded by the sequence of FRIEDMANN *et al.* (13). The latter sequence leads to an insertion of an amino acid after position 370 and of another after position 495. Other differences occur at the C-terminal end of lT, leading to a protein with 782 amino acids. Analysis of


Fig. 9. Intracellular localization of Py T-antigens. Mouse kidney cells were infected with Py and labeled with $^{35}$S-methionine 22–26 h after infection. They were fractionated using the procedure shown schematically on the left. The aqueous two-phase system has been described by BRUNETTE and TILL[96]. The fractions obtained (PP, PM, SP, SM and SS) were extracted, immunoprecipitated and analyzed on a 12.5% gel as described earlier[69]. The autoradiograph of the gel is shown on the right. A: actine.

the C-terminal region of lT should clarify at least some of the divergence in the sequence data.

The first 79 amino acids of lT yielding 3 methionine containing tryptic peptides are in common with sT and mT. The splicing occurs within another methionine containing tryptic peptide. In the remaining part of lT (positions 80–785) there are only 11 methionines which give rise to 9 tryptic peptides. The total of 13 expected methionine containing peptides is considerably less than the 25 spots, i.e. 21 unique and 4 in common with sT and mT, observed by HUTCHINSON *et al.*[71]. ITO found by a similar technique 10 peptides of which two are shared with mT and sT[91,92]. One of the unique peptides of lT, MLK at positions 555–557 (peptide 14), was found to be identical to the chemically synthesized compound by HUNTER *et al.*[89,90]. Of the remaining unique peptides, five could be labeled with cysteine and methionine as was expected from the derived amino acid sequence[89,90]. These results thus confirm the proposed reading frame and large parts of the deduced amino acid sequence for lT.

Comparison of the amino acid sequence of Py lT and SV40 lT shows besides the homology in the N-terminal region which was discussed above rather large homology from position 278 corresponding to about 96.5 map units throughout the rest of Py lT[13,15]. The most extensive homology region extends from position 562 to position 688 with 82 identical and 14 closely related amino acids at the same positions. In the corresponding region of the SV40 genome several tsA mutants have been mapped[93]. They have characteristics similar to Py tsA mutants[28]. So far no precise mapping data for polyoma tsA mutants are available, but it would not be surprising if at least some of them map in this region. Despite the high degree of homology between Py and SV40 lT, it had never been possible to complement tsA mutants of one virus by wild-type virus of the other

No sequence homology to SV40 T-antigens was found in the region between amino acids 80

to 240, i.e. the sequence specified by the DNA between 86 and 96 map units. Instead of the 6 glutamic acids found in mT there are 6 glycines, but most of the proline residues flanking this sequence have been conserved. The region between residues 81 and 280 clusters more than 50% of all serines and prolines present in lT.

The lT specified by mutants dl 8 and dl 23 migrate faster than wild-type lT with an estimated $M_r$ of about 95 000. But, unlike mT where a rather large difference was observed between dl 8 and dl 23, the two mutated lT show a closely similar electrophoretic mobility. For both proteins no difference was observed in the methionine tryptic peptides as compared to wild type lT[92]. The deletion of dl 8 removes residues 145–174 containing no methionine (B. E. GRIFFIN, personal communication). In mutant dl 45, amino acids 173–194 are missing[53] and in mutant dl 23 residues 195–228 are deleted (B. E. GRIFFIN, personal communication). This latter deletion affects one large, quite hydrophobic tryptic peptide with one methionine at position 240.

*Intracellular localization of T-antigens*

ITO *et al.* demonstrated the presence of virus-specific proteins reacting with anti tumor serum in plasma membrane preparations from Py-infected or transformed cells[94]. The plasma membranes were obtained from pressure disrupted cells by sedimentation in a discontinuous sucrose dextrane gradient. The main component of the immunoreactive proteins found in the plasma membranes was a 55 000 $M_r$ protein which was absent in NG 18 (hr-t deletion mutant) infected cells. This protein was later identified as mT by tryptic peptide analysis[92]. Large T was found mainly in the nuclear fraction and was not detected in membrane preparations[94].

Using a simple cell fractionation procedure by differential centrifugation, SILVER *et al.* showed that lT was predominantly located in the nuclear fraction together with some mT. In the high speed pellet of the cytoplasmic fraction which among other cell organelles comprised the membranes, mainly mT and some 63 000 $M_r$ protein was found. All five T-antigen proteins (see Fig. 3, column 5) were detected in the high speed supernatant of the cytoplasmic fraction, but lT and mT were present in relatively decreased amounts[76].

In our own cell fractionation experiments we have obtained similar results[95]. The procedure used is shown in Figure 9. Both from the low speed nuclear pellet and from the high speed cytoplasmic pellet we isolated plasma membranes by centrifugation in a two phase aqueous system described by BRUNETTE and TILL[96]. With this procedure rather clean plasma membranes band at the interphase and can easily be isolated. The analysis of the different fractions after detergent extraction and immunoprecipitation of the T-antigens is shown in Figure 9. All four immunoreactive proteins usually detected in our experiments were found in the nuclear pellet (PP) which was probably due to the presence of undisrupted cells in this fraction. Both plasma membrane preparations (PM and SM) contained mT, but this protein was also found in material that sedimented through the two phase system (SP). All these fractions had only traces of lT. The high speed supernatant of the cytoplasmic fraction showed sT and a 61 000 $M_r$ protein. At present we do not know whether this protein is identical to the 61 000 $M_r$ protein observed in total cell extracts. The observation that exposure of cell monolayers to a hypotonic buffer of pH 6 containing 0.1% NP 40 at 0 °C for 15 min leads to extraction of sT and again a 61 000 $M_r$ protein suggests that the majority of sT is present in the cytosol. Under these conditions the monolayer remained intact and no lT or mT was detected in the hypotonic buffer.

The cell fractionation experiments reported by the different groups show that at least some mT is associated with plasma membranes. On the other hand it has not as yet been possible to show the presence of mT in the membrane by surface labeling procedures[77].

*Modification of large T-antigen during lytic infection*

Earlier we reported that Py lT isolated from productively infected cells resolves into a double band in 5% or 6% polyacrylamide gels with apparent $M_r$ of 102 000 and 99 000, while in Py-transformed rat cells only the smaller species is found[69]. Moreover, we could show that in Py-infected mouse kidney cells the slightly larger species of lT appeared only after onset of

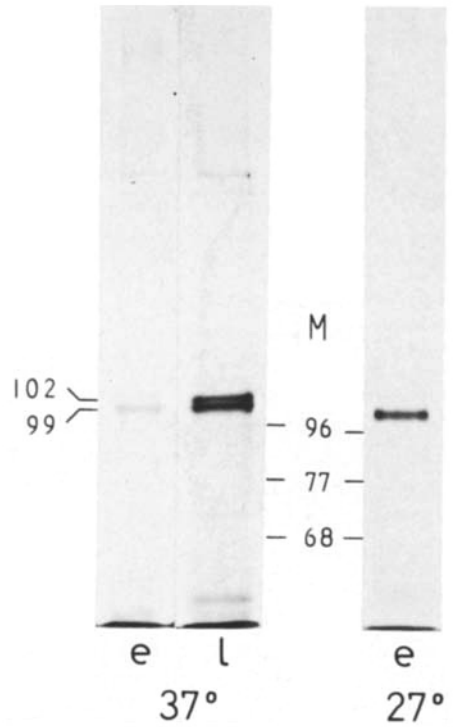


Fig. 10. Modification of IT during lytic infection. Py IT labeled with $^{35}$S-methionine before onset of cellular and viral DNA replication (e, 'early') forms on 6% gels a single band with an apparent $M_r$ of 99 000. Cells infected at 37 °C were labeled 10–13 h after infection, cells infected at 27 °C, 40–46 h after infection. Py IT labeled after onset of cellular and viral DNA replication (l, 'late'), i.e. 22–26 h after infection at 37 °C, resolves into a double band with $M_r$ of 99 000 and 102 000. M, marker proteins: phosphorylase a (96 000), transferrin (77 000) and bovine serum albumine (68 000).

cellular and viral DNA replication (reference 69 and Fig. 10). The results of more detailed experiments on the formation of the modified IT will be presented elsewhere but can be summarized briefly as follows.

The formation of the larger species of IT coincides with onset of cellular and viral DNA replication. This has been shown with cultures of mouse kidney cells incubated up to 40 h after infection at 27 °C. Most cells of these cultures synthesized IT forming a single band (99 000 $M_r$) and no detectable increase of $^3$H-thymidine incorporation over mock-infected cultures was observed[63,97]. After shifting these cultures to 37 °C pulse labeling and pulse chase experiments showed that the larger species (102 000 $M_r$) appeared between 5 to 7 h after the temperature shift which coincided with an increased incorporation of $^3$H-thymidine into cellular and viral DNA. Coordinate formation of the larger IT species with onset of cellular and viral DNA replication was also observed in NG18 (hr-t deletion mutant) infected mouse kidney cells. In these cells both events were delayed by about 10 h as compared to wild-type infected cultures, while at 13 h after infection wild-type and NG 18 infected cells contained similar amounts of 99 000 $M_r$ IT. In tsA mutant infected cells the double band is observed only at permissive temperature (32 °C). At 40 °C tsA mutants direct synthesis of the smaller IT species only which in addition is unstable during a chase of a few hours (Fig. 11). When tsA IT was labeled at high temperature then chased after shifting the cultures to 32 °C, the larger species appeared within 2 hr even in the presence of 20 $\mu$g cycloheximide per ml culture medium. These results strongly suggest that Py IT is modified after its synthesis. However, we have never observed complete conversion of the smaller into the larger species. Results obtained by HUNTER *et al.* suggest that the 99 000 $M_r$ species is already a modified form of IT. From cells pulse labeled for 5 min only, they isolated a form of IT which represented probably the unmodified translation product, because it migrated faster than the 99 000 $M_r$ species and comigrated precisely with IT synthesized *in vitro*. After a chase of 60 min, the pulse labeled IT migrated slower and resolved into a double band (89, and T. HUNTER, personal communication). Both the 99 000 and 102 000 $M_r$ IT can be labeled with $^{32}$P-orthophosphate (70 and own unpublished results). While the amount of $^{32}$P-labeling was considerably greater late in infection, we found so far no qualitative difference in the $^{32}$P-labeled tryptic peptides of IT synthesized before or after onset of DNA replication. By two dimensional thin layer chromatography we found one major and a few minor spots in both cases. At least nine phosphopeptides present in roughly equimolar amounts have been found by HUNTER *et al.* in IT synthesized late in lytic infection (T. HUNTER, personal communication). Our attempts to label the larger species of IT with $^3$H-mannose of $^3$H-acetate during the period of its formation were not successful. A possible site for modification is indicated by

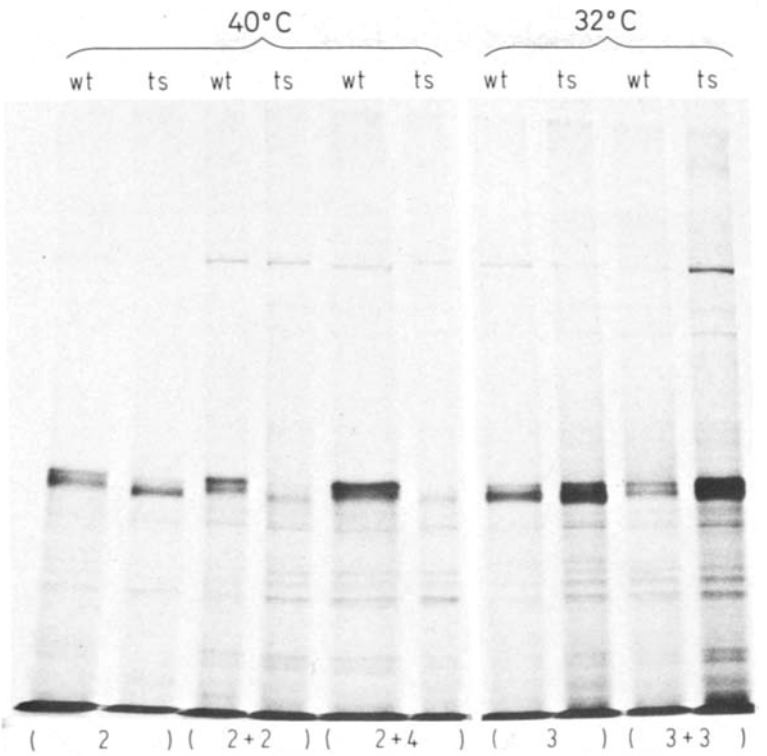


Fig. 11. Large T of tsA mutants. Mouse kidney cells were infected with wild-type virus (wt) or mutant ts a and incubated at 32 °C for 40 h. At this time part of the cultures was shifted to 40 °C and labeled with $^{35}$S-methionine 0–2 h after the shift. Some of these cultures were extracted immediately (2), others were chased for 2 h (2+2) and 4 h (2+4). The other part of the cultures was maintained at 32 °C and labeled 40–43 h after infection. Half of these cultures were extracted immediately (3), the others after a chase of 3 h (3+3). T-antigens were extracted, immunoprecipitated and analyzed on a 6% gel as described earlier[69].

the results of HUNTER *et al.* who observed different migration of one tryptic, methionine containing peptide in lT synthesized *in vitro* as compared to lT extracted from cells undergoing productive infection[71]. Previously we proposed that lT is modified in permissive cells and that this modification is required for initiation of viral DNA replication[98], a hypothesis that is supported by the results obtained with tsA mutants. However, we also observed the appearance of the larger species of lT after onset of host DNA replication in secondary cultures of hamster embryo cells and in rat 3T3 cells (isolated and kindly provided by F. CUZIN, Université de Nice) where only a minor fraction of cells allows viral DNA replication. These latter observations suggest that modification is not the only factor provided by the host cell for viral DNA replication.

*Analysis of polyoma T-antigens by two-dimensional gel electrophoresis*

To obtain further information on the Py T-antigens and to look for charge differences between the two species of lT, we analyzed immunoprecipitated T-antigens by two-dimensional gel electrophoresis according to O'Farrell[99]. Figure 12 shows typical gels for Py T-antigens extracted from wild-type and ts a mutant infected mouse kidney cells. Cultures were first infected at 27 °C for 42 h and then shifted to 39.5 °C. They were labeled with $^{35}$S-methionine from 6–10 h after the shift. Samples were loaded once on the basic end of the isoelectric focusing gel (left) and once on the acidic end (right). A small amount of the same sample was applied for SDS-gel electrophoresis only. While VP 1, actin (A) and other proteins behaved normally and migrated to the

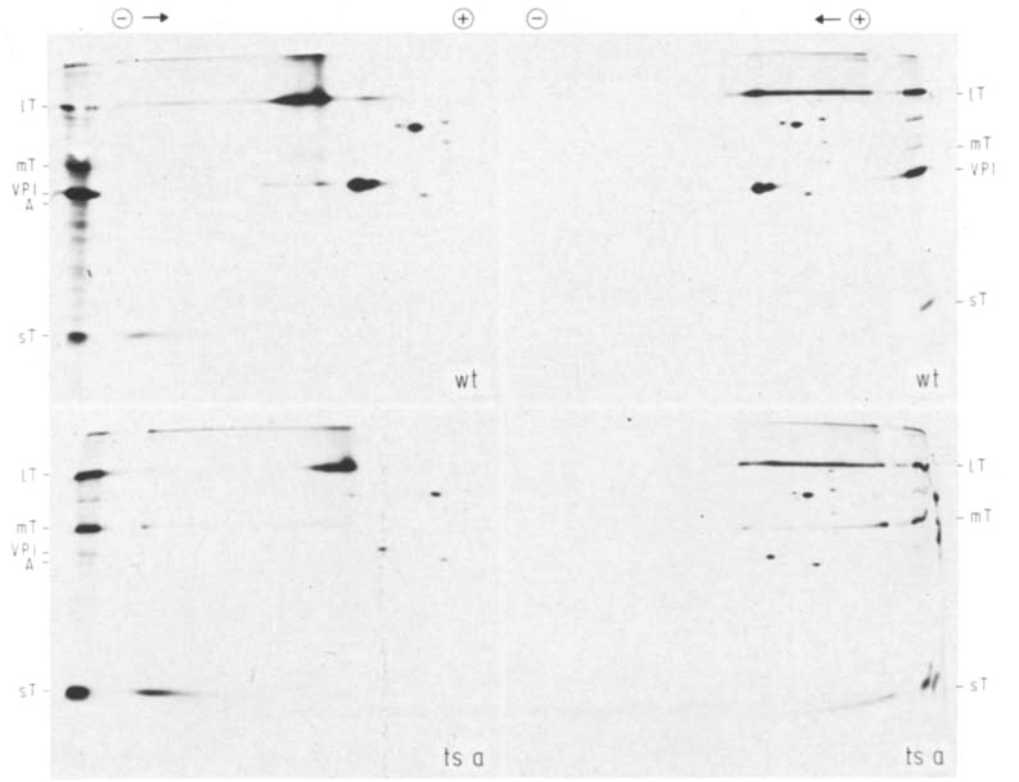


Fig. 12. Analysis of Py T-antigens by two-dimensional gel electrophoresis[99]. Mouse kidney cells were infected with wild-type virus (wt) and mutant ts a at 27 °C for 42 h. Then, they were shifted to 39.5 °C and labeled with $^{35}$S-methionine 6–10 h after the shift, T-antigens were extracted and immunoprecipitated as described earlier[69]. The immunocomplexes bound to protein A-Sepharose were eluted with 9.5 M urea containing 5% mercaptoethanol and 2% NP 40. Samples were loaded on tubular 4% acrylamide gels prepared in 9.5 M urea, 2% NP 40, 2% ampholytes pH 3.5–10, either at the basic (gels on the left) or the acidic end (gels on the right). Isoelectric focusing was done at 300 V for 15 h. The second dimension was electrophoresis on a 12.5% SDS-polyacrylamide gel. Part of the same samples was run in the second dimension only. The positions of the main capsid protein (VP I) and of actine (A) are also indicated.

same position irrespective of the site of loading, all three Py T-antigens showed abnormal migration in the isoelectric focusing gel. When loaded on the basic end, the bulk of lT appeared as 2–3 spots on the basic side of VP 1. When loaded on the acidic side, lT formed a streak from the end of the gel to a position corresponding to the isoelectric point of VP 1. Middle T and sT remained mainly at the site of loading and entered the isoelectric focusing gel only as a faint streak under both conditions.

Crawford and O'Farrell made similar observations when they analyzed SV40 T-antigens[100]. They found that alkylation with N-ethylmaleimide (NEM) significantly improved the resolution of SV40 T-antigens in isoelectric focusing. The SH-groups of cysteine form with NEM a covalent linkage by addition to the olefinic double bond. When carried out at neutral pH the reaction is specific for cysteine. While not altering the charge of the protein, it increases the $M_r$ by 125 for every cysteine that took part in the reaction[101,102]. After treatment with NEM we observed by gel electrophoresis an increase in $M_r$ of about 2 000 for sT (10 cysteines) and mT (11 cysteines) and of about 3 000 for lT (26 cysteines). On 6% gels both bands of NEM treated lT migrated coordinately slower. As shown in Figure 13 alkylation changed the isoelectric behavior of lT. No more streaking was detected and the majority of lT migrated in the first dimension to a similar position as VP 1, slightly on the basic side of actin. A similar result was found, when the sample was loaded on the acidic side although there was some streaking from the end of the

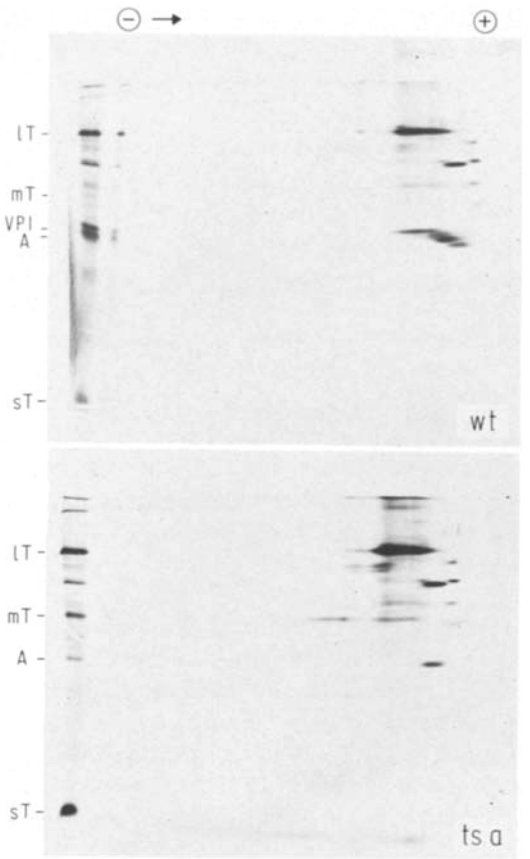


Fig. 13. Two-dimensional gel electrophoresis of Py T-antigens treated with N-ethylmaleimide (NEM). Mouse kidney cells were infected and labeled as described in Figure 12. The immunocomplexes bound to protein A-Sepharose were washed with 100 bed volumes of 0.5 M LiCl, 0.1 M Tris HCl pH 9, 1% mercaptoethanol[74] and subsequently with 100 bed volumes extraction buffer[74]. Ten bed volumes of 0.2 M NEM were added and the suspension was kept in ice for 1 h. The solution of NEM was removed and the immunocomplexes were eluted with urea as described in Figure 12. Samples were loaded on the basic end of the isoelectric focusing gel and two-dimensional electrophoresis was done as described in Figure 12.

gel to the spot of lT (not shown). On the other hand, mT and particularly sT were still heterogeneous with respect to charge. A certain amount of these two proteins seemed to aggregate with lT as judged from the migration in the isoelectric focusing gel. Alkylated sT was detected allover the pH gradient irrespective of the site of loading.

The gels shown in Figures 13 and 14 revealed no difference between wild-type lT (double band) and the ts a lT (not modified) neither with or without alkylation. Nor did we find a charge difference between the two bands of wild type lT when they were resolved in the second dimension on a 6% gel.

*Polyoma T-antigens in transformed cells*

Early analyses already showed that while many Py-transformed cell lines contained lT, mT and sT whereby the last two were often difficult to detect, there were apparently also cell lines where lT was clearly absent[69,71]. Recently, Ito and Spurr did a systematic analysis of a great number of Py-transformed cell lines[77]. In all the lines analyzed mT and sT were detected but presence of lT varied with the origin of the cell line. In 11 out of 16 wild-type virus transformed rat cell lines, lT having the same electrophoretic mobility as that seen in productive infection was present. In the remaining 5

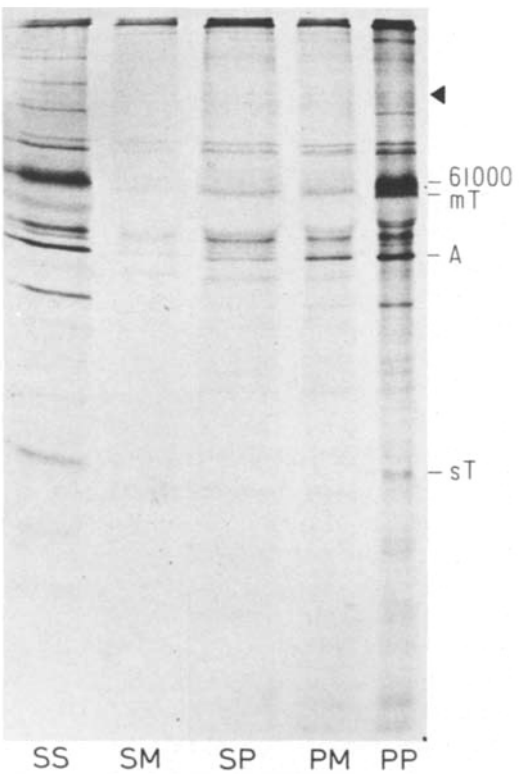


Fig. 14. Intracellular localization of Py T-antigens in hamster tumor cells. Cultures of a cell line derived from a subcutaneous tumor induced by Py were labeled with $^{35}$S-methionine and fractionated as shown in Figure 9. The fractions obtained (PP, PM, SP, SM and SS) were extracted, immunoprecipitated and analyzed on a 12.5% gel as described earlier[69]. The position where lT is expected is marked by a triangle A: actine.

cell lines new immunoreactive proteins were observed instead of the 100 000 $M_r$ lT. In one cell line the new protein migrated more slowly (117 000), while in the other cell lines smaller proteins were found (97 000 to 30 000). Similarly, in 2 out of 3 transformed hamster cell lines lT was missing. While one cell line had a slightly smaller immunoreactive protein of 97 000 $M_r$, the other cell line showed no new species. Finally, in two Py-transformed mouse cell lines new bands of 70 000 $M_r$ and 80 000 $M_r$ were detected instead of lT. Analysis of rat, hamster and mouse cell lines transformed by tsA mutants or the mutant dl 8 gave essentially similar results: mT and sT were always present while lT was sometimes missing and being replaced in some of these cell lines by new proteins of varying electrophoretic mobility. Tryptic peptide analyses of the proteins with the same mobility as lT, mT and sT proved that they were identical to Py T-antigens found in productive infection. Several other prominent proteins found in cell lines without lT ranging from 80 000 to 37 000 $M_r$ were found to be related to lT and seemed to be truncated forms. In these studies Ito and Spurr used a batch of anti tumor serum with rather narrow specificity. Other batches of serum precipitated a considerably larger number of proteins, of which some were unrelated to Py T-antigens[77]. Essentially analogous results were obtained by Benjamin *et al.* when they analyzed Py T-antigens in a series of wild-type virus transformed rat cell lines and several lines of mouse cells transformed by wild-type or partially inactivated virus. In most rat cell lines all forms of Py T-antigens present in productive infection were observed. One rat cell line was lacking lT. However, the mouse cell lines while containing mT and sT had no detectable amounts of lT[103].

Expression of Py T-antigens in transformed cells depends on the structure of the viral DNA sequences and consequently on the vrial mRNAs present in the cell lines. Analyses of viral mRNAs in Py wild-type and tsA mutant transformed mouse cell lines showed that in all cell lines tested viral mRNAs lacked some sequences derived from the 3′-half of the early region, i.e. between 0 and 26 map units[82]. Therefore, none of these cell lines could synthesize full size lT. These results support the notion that the presence of functional lT in permissive mouse cells invariably leads to virus production and cell death[82,103]. Viral mRNAs in transformed mouse cells showed a splicing pattern similar to that observed for early mRNAs in productive infection. In some lines mRNAs with 3′-ends mapping at 99 units were the predominant species and corresponded to the minor species found in permissive cells[82]. The situation was more complex in Py-transformed rat cells. In most cell lines isolated as colonies growing in agar or as dense foci the existence of both free and integrated viral DNA was observed[104,105,106]. The integrated DNA was found to form head to tail tandem repeats of unit length genomes[105]. In these cell lines the same early viral mRNAs were found as in productive infection[82] and full size lT, mT and sT were detected[106,107]. Two cell lines lacked free viral DNA and were shown to have a single insert of viral DNA sequences into rat chromosomal DNA. In both lines the integrated viral DNA showed partial head to tail duplicates of incomplete genomes with discontinuities in the region between 0 and 26 map units resulting from deletion or fusion to host DNA, but comprised at least one copy of the region from the origin of replication to 99 map units coding for mT and sT[106,107]. One line synthesized only these two T-antigens, the other had in addition three other T-antigen related proteins. With this cell line complete correlations were found between the structure of viral DNA sequences, viral mRNAs and the T-antigen related proteins which turned out to be truncated forms of lT and mT[82,106,107].

The intracellular localization of Py T-antigens in transformed cells is similar to that found in productive infection. At least a fraction of mT was found in plasma membrane preparations (reference 77, 95 and own unpublished results). In three independent isolates of Py-transformed rat cells (one mass culture, one dense focus and one colony growing in agar) we analyzed lT by electrophoresis on 6% gels and found only the 99 000 $M_r$ protein corresponding to lT synthesized in productive infection before onset of cellular and viral DNA replication. The same result was obtained with 7 subclones of the mass culture isolated in soft agar.

From all these studies several authors concluded that presence of mT and sT but not of lT is required for maintenance of the transformed

phenotype[77,103,106]. The fact that mT was found to be associated with membranes and also that the ability to transform cells and tumorigenicity were significantly reduced with mutant dl 23 which is altered only in mT and lT, now focus interest on mT as an important factor for cell transformation[77,108]. Using restriction enzyme fragments of Py DNA that had been cloned by insertion into a bacterial plasmid, HASSEL *et al.* were able to transform rat cells with a fragment extending clockwise from 45 to 1.4 units (see Fig. 1); this fragment comprises the origin of replication and the region coding for mT and sT. Furthermore, they did not find a difference in the efficiency of transformation when the entire viral genome was inserted either at the EcoRI site, i.e. in the middle of the early region, or at the Bam Hl site at 58 map units in the late region[109]. In similar transfection experiments, NOVAK *et al.* could transform cells with a Py DNA fragment of 35% extending from 65–100 map units[110]. These results strongly suggest that lT is also dispensable for initiation of cell transformation at least in transfections with DNA. LANIA *et al.* presented evidence that presence of lT is not sufficient to induce in cells characteristic features of transformation. These authors isolated a cell line containing free and integrated DNA sequences of an hr-t deletion mutant A 185 lacking about 50 base pairs at 83 map units. The cells of this line synthesize full size lT but no detectable amounts of mT and sT and behave like normal rat cells with respect to saturation density, anchorage dependent growth and induction of tumors[111]. These results show furthermore that failure of hr-t mutants to transform cells is not due to inability of the viral genome to become inserted into host DNA or to be expressed, but rather to absent or defective mT and sT.

*T-antigens in cell lines derived from polyoma-induced tumors*

Testing the biological activity of circular and linear viral DNA, ISRAEL *et al.* made the observation that interruption of the early region of the genome at certain sites increased the tumorigenicity in newborn hamsters[112,113]. Tumor induction increased 2–5 fold with linear DNA obtained by digestion with restriction enzymes EcoRI (cutting once at 100 map units) or Xba I (cutting twice at 17.3 and 18.2 map units) as compared to intact circular viral DNA[113]. Analysis of T-antigens in several cell lines derived from hamster tumors which had been induced with virus, intact viral DNA or linear DNA obtained by means of EcoRI or Xba I showed only presence of mT (63 000 $M_r$) and sT (20 000 $M_r$, see Figure 3, column 7). In none of the cell lines lT or a new protein which might have represented a truncated form of lT was detected[113]. While this result was expected for cells from tumors induced with EcoRI or Xba I digested DNA, it was quite surprising for tumors induced with virus or intact circular DNA. Analysis of viral DNA sequences present in the tumor cell lines confirmed the T-antigen results. All cell lines tested showed a discontinuity in the early region located most likely between 99 and 9 map units, i.e. none of the cell lines yielded a fragment comigrating with Pvu II fragment 3 (92 to 9 map units) but all lines synthesized full size mT[114].

We obtained similar results with hamster tumor cells. The cell line that we use for antiserum production in hamsters was isolated several years ago by R. WEIL from a subcutaneous tumor induced by inoculation of Py into newborn hamsters. These cells contain mT and sT. No lT was detected but instead a rather well labeled immunoreactive protein of about 61 000 $M_r$. Fractionation of these cells by the same method described in Figure 9 showed that mT was again found in fractions containing plasma membranes, (PM, SP, SM), while sT and a fraction of the 61 000 $M_r$ protein was observed in the cytoplasmic supernatant (SS) (Fig. 14). The pellet fraction (PP) contained all three of these proteins which again was most likely due to presence of a substantial portion of undisrupted cells. *In vivo* passaging of these cells did not change the T-antigen pattern. Two other lines of tumor cells isolated independently in the same way yielded similar results. We suspect that the 61 000 $M_r$ protein is related to lT, since it can be labeled in the cell with $^{32}$P-orthophosphate like lT, but unlike mT in permissive cells. Furthermore, analysis in two-dimensional gels showed that the 61 000 $M_r$ protein had isoelectric characteristics similar to lT.

The results reported by ISRAEL *et al.* and our own results show that lT and most likely also

truncated forms of lT are dispensable to maintain the transformed phenotype and tumorigenicity of hamster tumor cells. The possibility that lT may be synthesized subsequent to inoculation and may play a role in the early phase of tumor induction was also ruled out. ISRAEL *et al.* induced tumors with DNA of a recombinant plasmid (Py PBR322) containing the entire viral genome inserted at the EcoRI site, i.e. the viral genome is interrupted at 100 map units by the plasmid DNA[115]. Cells derived from this tumor contained mT and sT and showed insertion of the entire viral genome into hamster DNA whereby the two EcoRI sites joining viral and plasmid DNA sequences were maintained[114]. It was therefore impossible that lT was synthesized in this experiment at any stage of tumor induction.

Only one hamster tumor cell line, PYT 54, has been described so far which contains besides mT and sT also full size lT[113]. The absence of viral DNA sequences of the region coding only lT (99–25.5 map units) and consequently absence of lT in practically all hamster tumor cells suggest that there is selective outgrowth of such cells. This phenomenon could be due (i) to semipermissivity of embryonal hamster cells where the presence of lT allows viral DNA replication which might prevent outgrowth of these cells or (ii) to immunological rejection of lT containing cells.

At present less data are available for rat tumor cells. ITO and SPURR[77] analyzed two cell lines from rat tumors induced by injection of *in vitro* transformed rat embryo cells. One line which is strongly tumorigenic (PyREW TlAl) had all three T-antigens. The other line had instead of lT a protein migrating with 75 000 $M_r$ and besides mT and sT two smaller immunoreactive proteins[77]. A selective process similar to that observed in hamster tumor cells was observed by LANIA *et al.*[107]. When tumors were induced by inoculation with *in vitro* transformed cells containing multiple inserts of viral DNA sequences and also free viral genomes, the tumor cells had lost the free viral DNA and retained only a single insert of less than one genome. Parallel to these changes lT which was present in the transformed cells was absent in the tumor cells where an immunoreactive protein of about 75 000 $M_r$ besides mT and sT was detected. Apparently, similar changes were observed with other rat cell lines by passaging *in vivo*[107].

*Protein kinase activity in immunoprecipitates of polyoma T-antigens*

When Py T-antigen immunocomplexes bound to formalin fixed *S. aureus* bacteria or to protein A-Sepharose were incubated in the presence of $Mg^{2+}$ with $\gamma^{32}$P-ATP, phosphorylation of mT and sometimes also of lT was observed[116,117,118]. The protein kinase activity (EC 2.7.1.37) was not detected when control serum was used or in immunoprecipitates with anti tumor serum of extracts from uninfected cells. Phosphorylated mT was identified by comigration with $^{35}$S-methionine-labeled mT from infected cells and also by the same changes in electrophoretic mobility observed for mT of certain deletion mutants like dl 8[116] dl 45[118] and of the small plaque Py strain[117]. Besides phosphorylation of mT some authors observed also weaker phosphorylation of lT[118] and of the 63 000 $M_r$ T-antigen related protein (reference 118, see Figure 3 column 5). One group reported phosphorylation of rat IgG heavy chain when rat anti tumor serum was used but no labeling of IgG when hamster anti tumor serum was used for immunoprecipitation[116].

There is good evidence that the kinase activity is associated with mT. Immunoprecipitates of T-antigens extracted from cells infected with hr-t deletion mutants that do not synthesize mT (e.g. NG 18, A 185) do not phosphorylate rat IgG[116] and show no[117] or reduced[118] labeling of lT; residual labeling of lT might be due at least in part to contaminating cellular kinases[118]. The same experiment done with hr-t point mutants (e.g. NG 59) or the inphase deletion mutant SD 15, again gave no or significantly reduced labeling of mT which was in these cases present in the immunoprecipitates[118]. Furthermore, mT of NG 59 or SD 15 not only lacks kinase activity but also can not accept phosphate in mixed immunoprecipitates of wild-type and mutant infected cell extracts or of extracts from cells infected simultaneously with wild-type and mutant virus[118]. Similarly, kinase activity and phosphate labeling were significantly reduced when assayed with mutant dl 23, but were maintained with mutant dl 8[116]. Immunoprecipitates of extracts from cells infected with tsA mutants at non permissive temperature had

similar kinase activity as immunoprecipitates from parallel cultures infected with wild-type virus[117,118]. The kinase activity was also detected in several lines of Py-transformed cells independent of presence or absence of lT[116,117,118]. The rat cell line described by LANIA *et al.*[111] which contains free and integrated sequences of hr-t deletion mutant A 185 and synthesizes full size lT but no mT and sT had no detectable kinase activity in this assay[116]. Cell fractionation experiments showed that kinase activity can be detected like mT in preparations of plasma membranes, however, the activity recovered was less than a third of that found in whole cell extracts[116]. All these results suggest that mT either is a kinase which phosphorylates mainly itself and to a lesser extent lT and heavy chains of rat IgG, or has tightly associated to it a cellular protein kinase. Both, enzyme and substrate activity is strongly reduced in mutants defective in cell transformation, i.e. all hr-t mutants and mutant dl 23, but is maintained in other deletion mutants affecting mT (and lT) like dl 8 and dl 45 which show normal ability to transform cells.

The *in vitro* protein kinase assay is very sensitive. It was estimated that in general less than 1% of mT molecules are labeled in this reaction[117,118]. Furthermore, the reaction is clearly distinct from phosphorylation of Py T-antigens in the cells. In permissive cells the main phosphorylated component is lT and only weak labeling if any was observed for mT and sT[70,117,118]. Under some conditions SCHAFFHAUSEN and BENJAMIN also obtained weak phosphorylation of the 36 000 $M_r$ and 63 000 $M_r$ T-antigen related proteins[70,118]. Apparently, also mT of hr-t mutants NG 59 and SD 15 are weakly labeled when extracted from $^{32}$P-orthophosphate labeled cells[118]. Phosphate label of lT synthesized in permissive cells is found mainly in phosphoserine and in smaller amounts in phosphothreonine[117]. On the other hand, the only amino acid labeled in the *in vitro* assay both in mT and lT, is tyrosine[117] which was until now not known as phosphate acceptor in proteins. This result is particularly interesting with respect to the recent discovery that the phosphorylated product of the src gene, pp60$^{src}$, of avian sarcoma viruses contains besides phosphoserine not phosphothreonine as previously reported[119] but phosphotyrosine[120]. The src gene product has itself kinase activity in a similar assay and phosphorylates rabbit IgG heavy chains of a specific antiserum[121,122,123] at tyrosine residues[120]. In addition, it autophosphorylates itself *in vitro* also at a tyrosine (R. L. ERIKSON, personal communication). The closely related cellular homologues of the viral pp60$^{src}$, the products of the sarc genes which are present in all vertebrate cells[124] also catalyze phosphorylation of tyrosine in immunoprecipitates[120]. Therefore, the possibility has to be considered that functional Py mT instead of having itself protein kinase activity associates specifically with this new type of cellular kinase and serves as a substrate for it.

Using essentially the same *in vitro* assay originally described by COLLETT and ERIKSON[121], protein kinase activities have also been detected in T-antigen immunoprecipitates of adenoviruses 5[125] and 12[126]. On the other and, contradictory results have been reported on protein kinase activity of purified SV40 lT[127,128] or a closely related protein[129]. Although many questions on the *in vivo* role of these kinases remain to be answered, their association in the case of pp60$^{src}$ and Py mT with proteins being probably involved in cell transformation and being present near or in plasma membranes indicates new possibilities for biochemical studies on virus-induced cell transformation.

*Attempts to purify native polyoma T-antigens*

First attempts to purify Py T-antigens in native form were reported by CUZIN and his collaborators. Using complement fixation as assay for Py T-antigens they fractionated cell extracts from a Py-transformed mouse cell line (3T3 Py 6) or from productively infected mouse 3T6 cells. With 20% saturated ammonium sulfate, complement fixing activity remained in solution and was then precipitated quantitatively with 40% saturated ammonium sulfate[130]. In the presence of 0.05 M NaCl complement fixing activity bound to a double-stranded calf-thymus DNA cellulose column. With stepwise increasing salt concentrations T-antigens were eluted between 0.4 and 1.0 M NaCl. Calculated on the basis of complement fixing activity purification of Py T-antigens was from 300 to more than 1 000 fold with recoveries of better than

50%[131]. Recently, the same authors could show by immunoprecipitation that in a protein fraction binding to double-stranded DNA cellulose in the presence of 0.25 M KCl and being eluted with 1 M KCl only lT and the 63 000 $M_r$ T-antigen related protein were present, while mT and sT did not bind to DNA cellulose. Their experiments further indicated that a DNA independent ATP phosphohydrolase (EC 3.6.1.3) of low specific activity copurified with lT. The enzyme activity was inhibited by anti Py tumor serum and was thermolabile in tsA mutant-infected cells (F. CUZIN, personal communication). Previously, an ATPase activity has been described for highly purified lT of SV40[128] and for a closely related protein[129] which was shown to bind specifically to SV40 DNA at the origin of replication[132]. These results suggest that the ATPase activity although being rather low may be intrinsic to lT of Py and SV40 and possibly involved in initiation of viral DNA replication.

## The Early Functions of Polyoma Virus

Before discussing possible functions of Py T-antigens I shall briefly review the main events observed in productive and transforming infection and how they are influenced by different mutations. I shall concentrate on the events occurring simultaneously with or after synthesis of detectable amounts of Py T-antigens. Virus adsorption, penetration into the cell, transport to the nucleus and decapsidation have been discussed in a recent review[8]. Although virus particles are found in the cell nucleus as early as 15 min after infection[133], synthesis of early viral mRNA is not detected until 6–8 h after infection[26,63].

*Productive infection*

In primary cultures of baby mouse kidney cells infected with Py the activities of RNA polymerase I and II increase 2–5 fold between 5 and 8 h after infection. This increase which is one of the first measurable reactions of the cells to Py infection is sensitive to drugs inhibiting either transcription or translation and therefore depends most likely on synthesis of small amounts of T-antigens (E. WINTERSBERGER, personal communication). In these cultures Py T-antigens can be detected by immunofluorescence or by immunoprecipitation by about 10 h after infection (reference 25 and own unpublished results). The main component being lT, only this protein is detected at this time, mT and sT become detectable a few hours later. Besides this finding, no significant differences were observed in the relative amounts of the T-antigens synthesized at any time throughout the productive infection (reference 72 and own unpublished results). With appearance of detectable amounts of T-antigen a number of events happen in the infected cell. They are schematically shown in Figure 15, whereby the 'pathways' of the mitotic response of the host cell and of the events leading to production of progeny virus are separated. In infected cells these processes are tightly linked, virus production depending entirely on the replication, transcription and translation systems of the host cell.

The mitotic response starts with an increased rate of synthesis of all classes of host RNA and of a large part of host proteins. This leads to a gradual increase of total RNA and protein up to about 30% as compared to mock-infected cultures by 25–30 h after infection[26,63]. Simultaneously, an increase in activity of enzymes involved in DNA synthesis is noticed leading to chromatin duplication which starts asynchronously in individual cells between about 12 and 30 h after infection[25]. The events leading to progeny virus production start with increased transcription of early viral mRNA and begin of transcription of late mRNA which are observed before detectable replication of viral DNA[63]. Viral DNA replicates as a nucleoprotein simultaneously with host chromatin duplication[134,135]. By 25–30 h after infection, about 10–12 times more late than early viral mRNAs are produced[18,62,136] and capsid proteins are synthesized[137]. Virus is assembled in the nucleus and finally the cell lyses.

Other events which can not necessarily be correlated with one or the other 'pathway' may also be induced by T-antigens, i.e. integration of viral DNA sequences into host DNA, cell surface changes and possibly other cellular functions. Recombination of cellular and viral DNA could first be detected by about 10 h after infection[138]. It was impossible to distinguish between the two alternatives: (i) T-antigen induces a cellular DNA recombination system

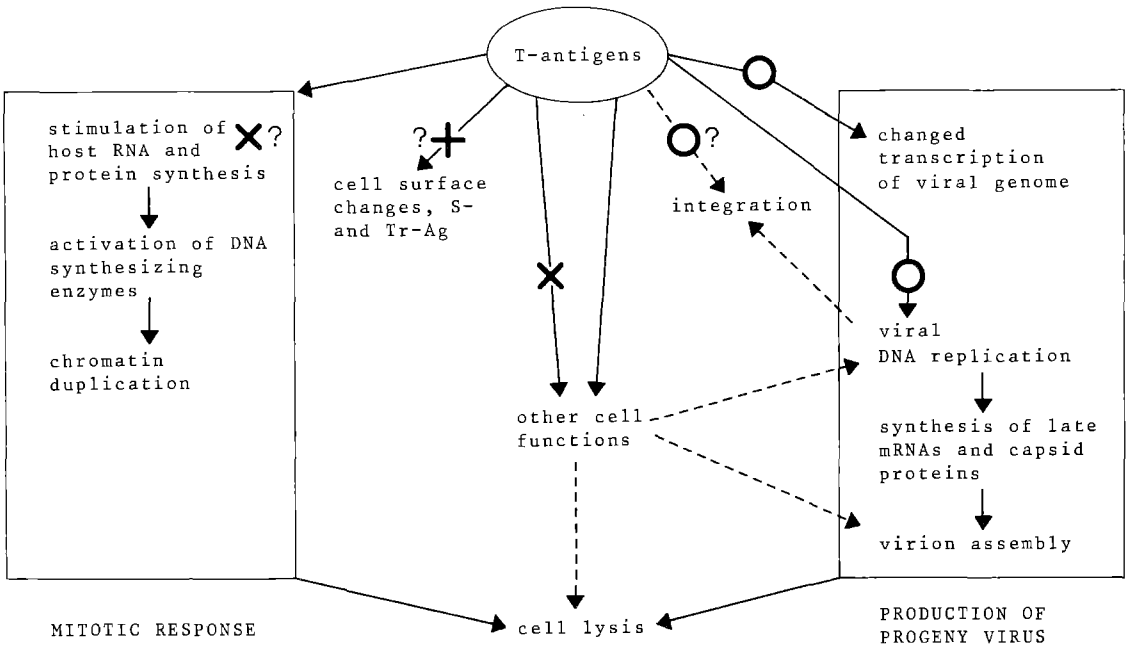


Fig. 15. Events occurring in Py infected cells during productive infection. Events known to be induced by Py T-antigens are indicated by arrows starting from 'T-antigens'. Possible functions of T-antigens are indicated by dashed arrows. Functions of Py T-antigens affected by hr-t mutations are marked with crosses, those affected by tsA mutations by circles. One arrow connecting 'T-antigens' and 'other cell functions' stands for induction of histone acetylation which is changed with hr-t mutants and may be important for virion assembly[145]; the other represents modification of lT by a host cell factor which may be required for initiation of viral DNA replication[98]. For further discussion see text.

simultaneously with host and viral DNA replication, or (ii) integration is the result of higher probability for recombination of replicating structures. Cell surface changes are detected by immunological methods relatively early in infection[139,140]. Surface (S) and transplantation (Tr)-antigens were first detected with tumor cells and transformed cells but could also be revealed in cells undergoing productive infection (for review see reference 11). Neither Tr-antigens nor S-antigens have been identified yet with a polypeptide chain. Experiments with SV40 transformed and tumor cells suggested that at least in some cases S-antigens are cellular proteins[141,142,143]. On the other hand, tumor-specific Tr-antigen which is also on the cell surface was shown to be specific for the tumor inducing virus[11,144]. Finally, from their studies with hr-t mutants BENJAMIN and his collaborators suggested that activation of other cellular genes may be required for progeny virus production[40,41]. One such function could be involved in histone acetylation, since some differences in acetylation of histone H 3 and H 4 were observed between wild-type and hr-t mutant virions[145]. Another function has to be provided by the permissive host cell to allow viral DNA replication, since in nonpermissive cells host chromatin duplication and presence of viral T-antigens are not sufficient for progeny virus production[146,11]. It was proposed[11,98] that a host function modifies chemically lT and that this process is required for initiation of viral DNA replication. The modification of Py lT observed during productive infection apparently depends on a cellular function which is induced after virus infection (see above).

Besides acetylation of histones in viral nucleoprotein, no other cellular or viral function which is defective in hr-t mutants has been defined for productive infection. In analogy to transforming infection (see below) one might expect that hr-t mutants fail to induce surface changes also in permissive cells. Furthermore,

recent experiments done in our laboratory with one hr-t deletion mutant (NG 18) showed that this mutant induces an altered mitotic response of the host cell. In mouse kidney cells infected with similar titers of NG 18 and wild type virus, the mitotic response was delayed by several hours in mutant infected cells despite of a similar percentage of T-antigen positive cells in both cultures. Also, in the NG 18 infected cultures less cells were induced to replicate cellular and viral DNA. Similar observations were made with analogous deletion mutants of SV 40, dl 883 and dl 891[147], which synthesize full size lT but truncated or no sT (R. WEIL, personal communication). These findings and the host range properties of hr-t mutants suggest that sT and in the case of Py possibly also mT play a role in the induction of the mitotic response and in activation of other as yet unknown functions required for progeny virus production.

The Py tsA mutations affect the 'pathway' leading to production of progeny virus by inhibiting initiation of viral DNA replication at nonpermissive temperature[30]. During the early phase of productive infection tsA mutants produce significantly larger amounts of early mRNAs at both permissive and nonpermissive temperatures than does wild-type virus under identical conditions. These results suggest that lT also regulates transcription of early mRNA[33]. The tsA mutants might be defective for recombinations with host DNA either because of altered lT or indirectly because of inhibition of viral DNA replication. On the other hand, Py tsA mutants are able to induce host DNA replication, but no results have been reported yet on other parameters of the mitotic response. However, with tsA mutants of SV40 we observed chromatin duplication and mitosis in monkey kidney cells at nonpermissive temperature[148] which indicates that at least in these cells the viral mitogenic function of SV40 tsA mutants acts normally.

Besides hr-t and tsA mutants there are other mutants which show reduced production of progeny virus, i.e. some deletion mutants mapping in the noncoding region between the origin of replication and the initiation codon, as well as dl 8 and at least one of the Hind III site mutants (see Figure 2 and first chapter). With mutants mapping in the noncoding region virus production is probably reduced because of inefficient viral DNA replication and/or inefficient expression of the early viral genes. For the other mutants there are no data available yet on the mitotic response of the host cell and on individual steps in progeny virus production.

*Transforming infection* (see Figure 16)
The mitogenic effect of Py in nonpermissive resting cells is most likely similar to that observed in productive infection. It leads in most cells to mitosis and in a fraction of the cells, where other requirements are met, to outgrowth of transformed cells. One of these requirements is stable integration of viral DNA into host DNA. The viral DNA sequences have to comprise at least the region from the origin of replication to the EcoRI site, i.e. the complete coding regions of sT and mT. Practically in all Py transformed cell lines analyzed, complete or partial tandem repeats of viral genomes were observed suggesting that dimers or oligomers of viral DNA may be the precursors with the highest probability for integration. Recently, formation of oligomers of viral DNA was observed in nonpermissive mouse cells infected with SV40[149]. The distal part of the early region, where the tsA mutants map is probably required neither for initiation nor for maintenance of transformation (see above). Therefore, tsA mutations affect cell transformation most likely by reducing the probability for stable integration of viral DNA sequences into host chromosomal DNA. Since tsA mutants are able to induce abortive transformation, the other viral functions required for cell transformation are probably maintained. However, some cell lines transformed by Py tsA mutants have been described which show many characteristics of transformed cells only at permissive temperature and loose them at nonpermissive temperature[150]. It could be shown that the phenotype of cells transformed by Py (and SV40) tsA mutants depends on the physiological state and the growth conditions of the cells at the time of infection and during the first days after infection[151,152]. Detailed studies on viral DNA sequences, viral mRNAs and T-antigens in the different types of transformed cells will be required to define the viral functions involved in this phenomenon.

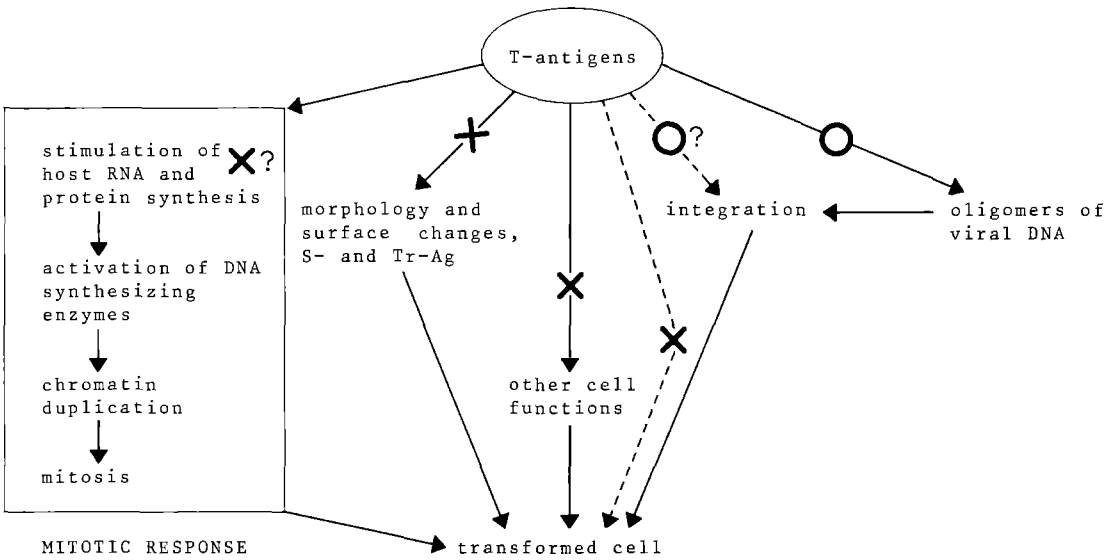


Fig. 16. Events occurring in Py infected cells during transforming infection. For symbols see legend to Figure 15. The arrow connecting 'T-antigens' and 'other cell functions' indicates increased production of plasminogen activator which is not observed with mutant dl 23[108] and possibly also with hr-t mutants. The dashed arrow leading from 'T-antigens' to 'transformed cells' indicates a possible direct action of mT as a transforming gene product[108]. For further discussion see text.

Hr-t mutants which are defective in transformation and tumor formation fail to induce (some) morphological and surface changes of the host cell, but are able to promote a single cycle of host DNA replication and mitosis[42]. There is at least one mutant, dl 23 which maps outside the hr-t region but has some characteristics of hr-t mutants. This mutant is defective for colony formation in agar, for induction of tumors and fails to induce disappearance of actin fibers and increased production of plasminogen activator[108]. This latter phenomenon may be an example of a cellular function that is required for expression of some characteristics of the transformed phenotype, in particular colony formation in agar[153,154]. Since Py mT is the only early viral protein which is altered both in hr-t mutants and in mutant dl 23 and since all these alterations cause loss of capacity to transform cells, it was proposed that Py mT is a transforming gene product similar to the src gene product of avian sarcoma viruses[108].

*Possible functions of Py T-antigens*

*Small T*

With the exception of the last 4 amino acids the complete sequence of sT is also present in mT. This makes it difficult to attribute a discrete function to sT. Defective sT results from all hr-t mutations, but its role in productive or transforming infection remains unknown. Although nucleotide sequence analysis revealed three types of hr-t mutants, no differences were observed so far for specific events occurring in permissive or nonpermissive cells after infection by one of the three types of hr-t mutants. Comparative analyses of the events occurring in permissive and nonpermissive cells after infection with hr-t mutants (having no or altered sT and mT) and mutants dl 8 and dl 23 (having altered mT and lT) might perhaps reveal distinct functions for sT and mT. That these two proteins have different functions might also be indicated by the different subcellular localization of Py sT and mT. As mentioned above, sT might play a role early in the mitotic response of the host cell possibly as a cofactor for the mitogenic function of lT. These observations could be related to the host range properties of hr-t mutants, where absence or alterations of sT (and mT) fail to induce cellular functions required for efficient progeny virus production. Furthermore, the striking heterogeneity of sT observed in two dimensional electrophoresis might indicate that sT

interacts with several host cell constituents. Finally, it was pointed out that the repeated sequence CXCXXC (positions 120–125 and 148–153, see Figure 6) which is also present in similar arrangement in sT of SV40 and BK virus may be important[87,12]. A similar cysteine cluster (CXXCXC) repeated after 19 amino acids is present in a soybean protease inhibitor where each of these sequences is followed by a reactive site for a different protease. By analogy sT might have two sites for protein–protein interactions near the two cystein clusters[12]. Furthermore, CXCXXC has also been found in the $\alpha$ and $\beta$ subunits of all members of the glycoprotein hormone family[87].

*Middle T*

There is now good evidence that mT plays an important role in cell transformation, but its mode of action remains to be elucidated. At least three regions in mT are essential for cell transformation. These are defined by the deletion mutants SD 15 and dl 23 and by the hr-t point mutants (see Figs 6 and 7). Individual parameters that are characteristic for transformed cells, i.e. cell surface changes, increased production of plasminogen activator, colony formation in agar as well as tumorigenicity of the virus seem to be equally affected by absence or alteration of only one of these regions. Similarly, the protein kinase activity assayed in immunoprecipitates requires integrity of all three regions. On the other hand, the region of mT defined by mutants dl 8 and dl 45 (see Figs 2 and 7) is dispensable both for cell transformation and kinase activity. Middle T of all these mutants associates with plasma membranes as does wild-type mT which makes it a good candidate for promotion of cell surface changes and for induction of virus-specific transplantation immunity. In fact, fractionation of Py-transformed cells showed that the Tr-antigen copurified with membrane preparations and was separable from nuclei and lT[155].

Whether Py mT is a transforming protein and has all the functions needed to transform cells and to induce tumors remains to be shown. It should be pointed out, however, that virus-induced cell transformation is not a yes or no event, but reveals a large variability in phenotype (e.g. reference 152). This could be the result of several virus-host interactions taking place to different degrees in individual cells as well as of cell-mediated variations brought about by the procedures used for selection of transformed cell lines. In this respect a certain role of sT and possibly even lT in initiation of cell transformation can not be excluded, even more since SV40 and BK virus are able to transform cells without a virus-coded mT.

*Large T*

One function of lT for production of progeny virus is defined by the tsA mutations which affect initiation of viral DNA replication and map in the C-terminal half of the molecule. Py lT binds to double-stranded DNA and is expected to bind preferentially to Py DNA at the origin of replication in analogy to results obtained with purified lT of SV40[132]. Since all hr-t mutants are able to induce host chromatin replication both in permissive and nonpermissive cells, the mitogenic function of Py which is clearly distinct from the tsA function also has to reside in lT. While it had been possible to demonstrate directly the capacity to induce host DNA replication with purified lT of SV40[157]. no such experiments have been reported yet for Py lT. Because there are no Py mutants affected in their mitogenic action, this function could not be localized in the lT molecule. Further characterization of deletion mutants affecting lT in the N-terminal part of the molecule, in particular those mutants giving low virus yields, might provide more information on this important biological effect.

*Looking forward*

In Figures 15 and 16, I have listed the known and possible functions of Py T-antigens. Now that the three (major) T-antigens have been identified, one would like to know which functions belong to which protein. For lT two functions are known. As for mT and sT, more information may become available in the near future from experiments using viral DNA sequences cloned in recombinant plasmids which can direct the synthesis of only one Py T-antigen. In this manner it should be possible to relate (a) specific event(s) to the presence of a given T-antigen. Following this, the mode of action of T-antigens has to be investigated. From the multiple biological effects of Py it is concluded

that at least one of the T-antigens must be a multifunctional protein. Such a protein could act in different ways: (i) it might interact with regulatory genes of the host cell and trigger a series of reactions, (ii) it might have several active sites each acting on a specific target, (iii) it might interact with different cell constituents thereby undergoing selective modifications and consequently acquire new functions. These problems can be approached thanks to an appreciable number of Py mutants and to several parameters that can be measured in the host cell to follow productive and transforming infections. It is expected that further research on Py and its early proteins will provide important information on the systems and mechanisms regulating proliferation in mammalian cells.


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