STUDIES OF TEMPERATURE SENSITIVE MUTANTS OF POLYOMA VIRUS

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ABSTRACT

Polyoma virus can undergo two different types of interactions with susceptible cells; one type of interaction leads to the production of new infectious virus and eventual cell death while the other leads to a neoplastically transformed cell which is able to continue to divide under conditions that inhibit the multiplication of uninfected normal cells. In order to study the viral genes involved in both of these virus-cell interactions the isolation of temperature sensitive mutants of polyoma virus was undertaken.

Two strains (TS-a, TS-b) which were temperature sensitive in their plaque forming ability at 38.5°C, but not 31.5°C, were isolated from a mutagenized stock of the polyoma wild type virus (PY). TS-a was studied in further detail.

TS-a grown at 31.5°C was found to be indistinguishable from PY in a number of physical characteristics including the heat sensitivity of the completed viral components. TS-a was inhibited in its ability to produce infectious virus in mouse cells when incubated at 38.5°C; this inhibition could be overcome by infection with high multiplicities.

The nature of the intracellular temperature sensitive step of TS-a was analysed to some degree. It was found that this step occurs after uncoating of the infecting virus particles and about the time of new viral DNA synthesis. New infectious viral DNA does not appear to be made at the nonpermissive temperature; in contrast non-infectious capsids are made at 38.5°C, but in amounts smaller than a full yield, such as made by TS-a at 31.5°C or by PY at both the high and low temperature.

TS-a has also been found to be temperature sensitive in its transforming ability in vitro. Cells transformed at 31.5°C by TS-a retain their transformed characteristics upon cultivation at 38.5°C. Thus the temperature sensitive function seems to be important for the initiation of transformation, but not essential for the maintenance of the transformed state. TS-a also appears to be temperature sensitive in the production of tumours in newborn hamsters.

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GENERAL INTRODUCTION

In recent years there has been an increased interest in the study of tumour viruses. This has been caused in part by the isolation and characterization of several new viruses of this type, but primarily by advances in methodology which permit the study of these viruses in vitro; simple model systems built around some of these viruses allow a reproducible and controllable investigation of the mechanism by which a normal cell is converted to a cancer cell by a known causative agent.

The conversion of normal cells to the neoplastic state (transformation) can be induced by both RNA and DNA containing viruses. Though as Dulbecco[1] has pointed out there is a difference in the type of transformed cells produced by these viruses according to the type of nucleic acid they contain; DNA viruses cause undifferentiated transformed cells while RNA viruses usually cause transformed cells of a differentiated type.

In general, all types of cells transformed by viruses have a number of characteristics in common. These include a changed morphology, loss of contact inhibition[2], and the acquisition of new cellular antigens. The new cellular antigen is the same in cells transformed by the same tumour virus, but different in cells transformed by different tumour viruses.

Reviewed below are some of the current theories regarding the mode of viral carcinogenesis and the properties of two of the better studied tumour viruses, Rous sarcoma virus and polyoma virus. The main emphasis will be on polyoma virus.

A. Theories of Viral Carcinogenesis

If we consider the relationship of the transformed cells to the viral genetic material two hypotheses can be formulated. According to one hypothesis the viral genetic material persists in the transformed cells and is the cause of the neoplastic state; according to the other hypothesis the viral genetic material is needed for the induction of transformation, but is not required to maintain the neoplastic state.

1) Carcinogenesis Caused by Viral Genes Persisting in the Cells

This hypothesis implies that at least part of the viral material is continually present in the transformed cells, either integrated in a host chromosome and thus replicated together with it by mechanisms under cellular control, or as a free episomal element replicating extrachromosomally either by cellular or viral mechanisms. The neoplastic nature of the transformed cells would be the consequence of the effects of the viral genetic material on normal cell functions. The viral genetic information could cause this effect in many ways. For instance, a protein of viral specification could become incorporated into the transformed cell surface and alter it in such a way that the cell would no longer recognize the regulatory signals which normally govern its multiplication; the cell would then continue to multiply under conditions where cells with unaltered cell surfaces stop dividing. A second possibility is that a viral gene product interferes with a cellular gene product needed for the regulation of cell multiplication. According to another form of this hypothesis the neoplastic state could arise as a consequence of insertion of virus genes in the host chromosome by inactivating adjacent host genes which are needed for the regulation of cell multiplication. This could

occur either if a viral gene can act as an operator gene[3] or if the insertion of the viral genes shifts the phase of reading of the adjacent genes resulting in nonsense gene products[4].

Detection of the presence of any viral genetic material in the transformed cells can only suggest the validity of this hypothesis, but does not prove it. To prove it the necessity of the persisting viral genes for the maintenance of the transformed state should be demonstrated.

2) Carcinogenesis Caused by a Transient Action of the Virus

Two types of mechanisms can be considered under this heading. One is that the virus infection causes a specific type of cell mutation and the other that the virus selects a pre-existing neoplastic cell mutant. Although viral genetic material may be found in the transformed cells, it would not be necessary to maintain the transformed state.

a) Viral induced mutagenesis

Either by a direct or indirect mechanism the tumour virus causes, in the cell's genetic material, a specific type of mutation which then confers to the cell the heritable neoplastic property; after this mutation has been produced no portion of the viral genetic material is needed to maintain the transformed state.

A direct action of the virus could be mediated by one of the components of the mature virus particle or one of the substances specified by viral genes in the infected cells. It could also be independent of viral genes: if the viral DNA undergoes reciprocal recombination with specific parts of the host chromosomes replacing

some regulatory cellular genes, the neoplastic state would be the consequence of the deletion of these host genes. Viral genes might still function in the transformed cells to make specific products but they would not be relevant in maintaining transformation.

An indirect mutagenic action would be due to the induction by the virus of some substance specified by cellular genes which has a carcinogenic effect on the infected cell or a neighboring cell.

Some of these various possibilities could be tested. The mutagenic hypothesis of viral carcinogenesis would be strongly supported if transformation could be produced by an isolated non-selfreplicating viral specified component or a component specified by the cell, but induced by the virus. The replacement of host regulatory genes by viral DNA could be tested by the somatic cell hybridization technique[5]. The deletion causing the neoplastic property would be recessive in a hybrid cell derived from the fusion of a normal and transformed cell; the hybrid cell would be normal. If on the contrary the addition of viral genes was necessary for maintenance of the transformed state, such a hybrid cell, in all likelihood, would be transformed.

b) Selection of a pre-existing transformed cell

The following hypothesis is now considered: the virus by means of infection selects for a pre-existing mutant cell with unexpressed properties of a transformed cell. The cell mutant remains unexpressed in the uninfected cell population either because the surrounding normal cells repress it or because it needs some stimulatory effect; virus infection of the surrounding cells eliminates the repressing influence or of the cell mutants supplies the stimulation. Under this hypothesis the presence of different new cellular

antigens in transformed cells induced by different tumour viruses is explained by the selection of different types of cell mutants by each of these viruses. Once the selective action of the virus is accomplished the viral genetic material is no longer needed to maintain the transformed state.

The existence of transformed cells, suppressed by normal cells, in the uninfected population would be suggested by large differences in the frequency of transformation of clonal lines isolated from the same parental population.

B. Findings with Rous Sarcoma Virus (RSV)

1) General Characteristics

RSV is related to the Avian Leucosis Complex (ALC) which includes a group of seriologically related viruses[6]. The virus consists of roughly spherical particles, 70 μ in diameter, enclosed in an envelope and is made up of RNA, lipid and protein[7, 8]. RSV does not form plaques, but can be assayed in vitro by its transforming ability on chicken embryo cells[9].

RSV induces tumours in chickens; although most of the tumours contain virus, some are virus free[10]. Variants of RSV produce tumours in rodents[11].

2) Transformation

Transformed cells are detected in vitro by their ability to form a colony or focus of morphologically altered cells[9]. The number of foci produced is proportional to the virus concentration, suggesting that transformation can be initiated by a single particle.

Under optimal conditions, and with large virus inputs, almost all the infected cells can become transformed[12]. Different viral mutants have been shown to be able to induce different morphological types of transformed cells[13]. Virus free cells, obtained by transformation in vitro, under special precautions, produce tumours upon transplantation into chickens[14].

3) Detection of Viral Genetic Material in Virus Free Transformed Cells

It was originally thought that all RSV transformed cells spontaneously produce infectious virus[15]. Recently it has been found that production of virus, at least with the Bryan strain, of RSV, is due to contamination of the RSV virus stocks with other viruses of the ACL. In most stocks the contaminating virus is in a several fold excess to the RSV[16]. By infecting the cells at very low input multiplicities and by plating the infected cells under agar in the presence of antiviral antibody, virus free transformed cells can be isolated. The production of RSV from these transformed cells is induced by superinfecting them with a related virus of the ALC; other unrelated RNA viruses are ineffective[17]. All the cells derived from a single virus free transformed cell produce virus upon superinfection, showing that the RSV genome replicates with the cells[16]. The RSV released from virus free transformed cells, after superinfection, have at least part of the envelope of the superinfecting virus, since they possess similar antigenicity, host range, and interfering properties[18]. This indicates that the RSV genome may be defective in the production of at least one of the constituents of its envelope. The production of infectious RSV cannot be induced in virus free transformed cells by radiobiological or chemical means[17]. It

is likely that the early reports of Temin[19] of such induction were due to an enhancement by these agents of a low level contamination of other members of the ALC.

The production of RSV can be inhibited by a number of DNA antagonists[20]. These findings have led Temin to postulate that the RSV genome may exist in the form of a DNA provirus in the transformed cells[21]. In support of this hypothesis Temin has attempted to hybridize RNA extracted from RSV preparations with DNA of both the normal and RSV transformed cells[22]. Though the results of these experiments indicate that the transformed cell DNA has a greater affinity than the normal cell DNA for the RNA obtained from the viral preparations, the difference is quite small. The results of these experiments are open to criticism since the viral RNA preparation used was not shown to be pure.

4) New Cellular Antigens in the Transformed Cells

Virus free transformed cells do not contain detectable viral antigen of either RSVor the related member of the ALC that was present in the transforming virus stock[23]. Huebner et al.[24] have reported that hamster cell tumours induced by a variant of RSV (Schmidt-Ruppin), contained a complement fixing antigen that was cross reactive with crude lysates of several viruses of the ALC. From this report, however, it is not clear whether these tumour cells were actually free of viruses.

5) Considerations about the Mode of Carcinogenesis Induced by RSV

It is clear that all virus free cells transformed by RSV, at least with the Bryan strain, contain genetic information of the

original transforming virus which can be detected by superinfection with the appropriate helper virus. RSV appears to exist in a defective state in the transformed cells, possibly owing to the inability to make a normal envelope. The mere presence, however, of the viral genome in these cells does not show that it is needed to maintain the transformed state. The observation that the morphological alteration of the transformed cells can be governed by different viral mutants is the strongest evidence that the viral genome has an influence on the state of the transformed cell. A more conclusive evidence for the controlling role of the viral genome on transformation would be obtained if transformed cells could be cured of the RSV genome and then were found to have lost their transformed properties and to have reverted to normal cells.

Since a major proportion of the cells can be transformed by RSV, it does not seem at all likely that transformed cells pre-exist as unexpressed morphologically altered cell mutants in the uninfected population.

C. Findings with Polyoma Virus

Polyoma virus is an icosohedral particle of a diameter of 45 $\mu\mu$ with 72 morphological capsomeres in its coat[25]. It is a simply constructed virus containing a double stranded DNA surrounded by a protein coat. The viral DNA is relatively small (3×10^6 molecular weight units[26]) and could only specify a maximum of 7 to 10 proteins (assuming that only one of the two strands is used to determine the amino acid sequence, that all the nucleotides of this strand are used for amino acid specification,

and a comma free triplet code[4]). So far only one protein, the capsid protein, has been identified as being virus specified.

An infectious DNA extracted from the virus particles can be assayed by the production of plaques on mouse embryo cells[27]. The DNA extracted from purified virus preparations is composed in the main part of ring molecules without any single strand breaks which are probably in a twisted configuration (Component I)[28]. The viral DNA molecules can also exist in two other configurations; one is a nontwisted circular molecule containing at least one single strand break (Component II) and the other is the linear molecule (Component III). These three forms can be separated by their different sedimenting properties.

Polyoma virus is able to induce a wide variety of tumours in rodents[29]. The tumours are usually solid, localized and non-invasive. Most tissues are susceptible to being transformed in vivo, but especially the parotid gland in some strains of mice and the kidney in hamsters.

1) The Cytolytic Virus Cell Interaction

The multiplication of polyoma virus in monolayers of mouse embryo cells and mouse kidney cells results in characteristic cytopathic effects; a plaque assay for the quantization of the cytolytic effect of the virus has been developed using mouse embryo cells[30, 31].

New virus can usually be detected 18 to 24 hours after infection of mouse embryo cells at 37°C[32]. New infectious viral DNA can be extracted from mouse cells about 12 hours after infection[33], and viral capsids start being completed after about

18 hours[34]. Sheinin[34] has reported the production of non-infectious viral capsids, presumably non DNA containing, in the absence of DNA synthesis.

In mouse kidney cells, which are in a resting stage and do not synthesize much DNA, infection with polyoma virus causes an increase both in DNA synthesis and in the activity of certain enzymes concerned with DNA synthesis (thymidine kinase, DNA dependent DNA polymerase, cytidine deaminase)[35]. The newly synthesized DNA is composed of both host and viral molecules; it is not known whether the increased enzyme activities are specified by host or viral information. Although this "induction" of DNA synthesis in the infected cells seems to be a consequence of polyoma virus infection it has not been shown that it is relevant to the replication of the viral DNA. But since polyoma virus only has a limited amount of genetic information it would not be surprising if it were dependent on the host cell for some of the functions needed for its replication.

Weisberg[36] has shown that after exposure of mouse cells to high concentrations of polyoma virus a variable fraction of the cell population is converted to virus producers. The proportion of cells infectable, as measured by infective center formation and cell survival, is in agreement with that expected from the Poisson Distribution at low input multiplicities (0. 1 PFU/cell and below). At higher input multiplicities the proportion of infected cells is lower than expected. Weisberg observed that the resistance of the mouse embryo cells to infection is due neither to the production of interferon[37] nor to the presence of genetically resistant cells. The existence of "carrier cultures" with polyoma virus[38], in

which at any one time only a small proportion of the cells are virus producers, is consistent with Weisberg's findings.

Both circular components of the polyoma viral DNA (Components I and II) have been shown to be infective[28, 39, 40]. The linear form (Component III) contains little if any infectivity[39, 40]. Single strands of polyoma DNA in a ring configuration have also been found infective while single stranded linear molecules cause little if any plaque formation[39].

2) Transformation

Vogt and Dulbecco[41] using embryonic mouse and hamster cells in mass culture were first to show that polyoma virus could induce transformation in vitro. This observation was followed by the development of an assay of the transforming ability of polyoma virus. This can be done either with a continuous line of hamster cells (BHK/C13)[42] or with secondary cultures of rat embryo cells[43]. This assay is based on the formation of colonies of morphologically altered transformed cells. Recently a new assay has been developed which distinguishes the transformed cells by their enhanced ability to form colonies in agar[44, 45]. The cells transformed in vitro give rise to growing tumours when transplanted to the appropriate host in vivo[41, 46]. In all the transformation assays the number of transformed colonies is proportional to the concentration of the input virus up to multiplicities of about 500 PFU/cell. This suggests that transformation is initiated by a single particle. Above 500 PFU/cell the number of transformed colonies produced levels off and may even decrease at very high input multiplicities[46]. On the average it takes about 10^4 PFU to produce one transformed colony[44, 45].

Only about 1 to 6% of the BHK/C13 hamster cells can be transformed at any one time[46]. This low efficiency may derive from a physiological resistance of the hamster cells similar to that observed by Weisberg[36] for the cytolytic interaction of the virus with mouse embryo cells. In fact, cells remaining untransformed after an initial exposure to high concentrations of virus can be transformed upon re-exposure to virus at a later time; these transiently resistant cells show the same frequency of transformation as the cells of the primary infection[48]. The transformation rate of BHK/C13 can be effected somewhat by magnesium concentration[46] and pH[49].

Crawford and Abel[50] have separated the virus into full and empty capsids in CSC1 density gradients and have demonstrated that only the DNA-containing particles can induce transformation. As has been found in the cytolytic virus cell interaction the two circular forms of the viral DNA are able to transform, while little or no transforming activity is induced by the linear molecules[40]. Single strands, presumably the circular strands, can also induce transformation[51].

3) New Cellular Antigens in the Transformed Cells

A new cellular antigen can be demonstrated by transplantation tests in cells transformed by polyoma virus both in the animal and in tissue culture[52, 53]. This antigen is revealed by the lower frequency with which virus free transformed cells produce tumours when transplanted to animals previously immunized with live polyoma virus. Presumably during the immunization some cells are transformed by the virus; these cells contain the new

cellular antigen (transplantation antigen) and cause the investigation of the animal against the new antigen. Tumour formation is thus prevented in this immune animal after the injection of a number of transformed cells sufficient to produce a tumour in an unimmune animal. That rejection of the transformed cells by the immunized animals is not caused by antibody specific for the virus particle has been shown by several lines of evidence[47, 54].

Whether the transplantation antigen is specified by viral or cellular information has not yet been determined. Recently Hare[55] has reported that there is a difference in the transplantation antigen induced by two different strains of polyoma virus. Immunization of animals by one strain of virus did not induce a high resistance to the transplantation of cells transformed by the other strain; high resistance was achieved, however, if the animals were immunized with the same strain of virus that was used to transform the cells used in the test.

Recently Habel[56] has reported that cells transformed by polyoma virus contain a specific complement fixing antigen, which is apparently unrelated to the transplantation antigen. Too little, however, is known about this phenomenon to try to assess its importance. Only very few lines of transformed cells were tested for the presence of the complement fixing antigen and the titers of the complement fixing antibody were very low. Also since an uncloned strain of virus was used to induce the transformation of the cells tested, it is not certain at this time whether the complement fixing antigen is really the result of polyoma virus infection.

4) Detection of Viral Genetic Material in Transformed Cells

a) Marker rescue by superinfection

Since most polyoma transformed cells are not resistant to superinfection by polyoma virus marker rescue experiments can be performed as a means of trying to detect evidence of the presence of the original transforming genome. Dulbecco and Vogt[57] superinfected virus free transformed cells induced by one plaque type of the virus with a mutant of another plaque type. They could not detect any plaque forming units of the transforming plaque type in the progeny virus. This test was not very sensitive and would have only detected the transforming virus plaque type had it been present in proportions of at least 1% in the progeny population. The gene these workers were attempting to rescue is probably concerned with the virus capsid protein.

Ting[58] attempted to demonstrate rescue of the transplantation antigen by using the difference in specificity of the transplantation antigen discovered by Hare[55] as a genetic marker. It will be remembered that mice only show transplantation resistance to transformed cells when pre-immunized by the same (homologous) virus strain that was used to induce the transformation, but not by another (heterologous) virus strain. Ting superinfected virus free transformed cells with the heterologous virus strain and then used the progeny of this infection to immunize animals. These animals were then challenged with the transformed cells induced by both the homologous and heterologous virus strains. His results though suggestive of the rescue of the ability to induce a specific transplantation antigen remain unclear because of the small number of animals used in the experiment.

b) Induction of viral constituents from transformed cells

Attempts have been made to induce viral constituents from virus free transformed cells by methods that have been successful in the induction of temperate phage from lysogenic bacteria. Habel and Silverberg[87] failed to detect any infectious virus after exposure of transformed cells to x-irradiation, ultraviolet light, and cortisone.

Vogt and Dulbecco[59] could not detect any infectious virus from transformed cells after x-irradiation, exposure to mitomycin, deprivation of thymidine by administration of aminopterin, different conditions of starvation and treatment with azoguanine and chloronphenicol. Aminopterin treatment of transformed cells did not induce any viral capsid protein detectable by antiviral antibody conjugated to fluorescent dye or serum blocking power (the serum blocking assay would have detected 300 physical particles per cell), or any noncytocidal transforming particles. Neither aminopterin nor mitomycin treatment induced phenol extractable infectious viral nucleic acid. If one molecule of infectious nucleic acid per cell had been present it would have been detected.

c) Detection of viral DNA by nucleic acid hybrids

Recently the use of hybridization of nucleic acids has been used in the search for polyoma DNA in virus free transformed cells. These methods have been shown to be successful in the detection of prophage DNA in lysogenic bacteria[60, 61].

Winocour[62] employed synthetic RNA produced in vitro by the DNA dependent RNA polymerase using unfractionated polyoma

DNA as a primer, and DNA from normal and polyoma mediated transformed cells; the method of Nygaard and Hall[63] was used for detection of the hybrids. He could not find any significant differences between the hybridizing ability of this synthetic RNA with DNA from virus free transformed cells or normal cells of mice and hamsters. From reconstruction experiments in which known mixtures of polyoma DNA and cellular DNA were hybridized with the synthetic polyoma RNA Winocour estimated that 20 complete genomes per transformed cell would have been detected in his experiments. Whether the synthetic RNA was complementary to all the sequences of the polyoma DNA was not determined.

Axelrod et al.[64] have performed similar experiments by DNA-DNA hybrids using the agar column technique[61]. These workers reported a greater affinity of polyoma DNA for the DNA of cells transformed by polyoma virus than of normal cells or cells transformed by other agents. But hybridization of polyoma DNA with human, hamster and especially mouse cell DNA was significantly higher than with nonmammalian DNA. Assuming a 70% efficiency of hybridization it can be calculated that there are on the average 69 complete genomes of polyoma DNA per transformed mouse cell; but surprisingly there is the equivalent of 31 complete polyoma genomes per normal mouse cells. Another surprising result is that the difference in the amounts of hybridization produced by the DNA of normal hamster cells and hamster cells transformed by polyoma virus is very slight and both are much smaller than that observed with the DNA of normal mouse cells. The DNA of transformed hamster cells shows on the average 11 complete genomes of polyoma DNA per cell while the DNA of normal hamster cells, of SV-40 mediated transformed hamster cells, and of human cells show

on the average 8 complete genomes per cell. Hybridization of E. Coli DNA with polyoma RNA shows on the average 1.44×10^{-3} genomes of polyoma DNA per cell. These results might be explained in part if the unfractionated polyoma DNA preparations used in the hybridization contained some host (mouse) cell DNA which also has some affinity for hamster and human cell DNA. It is interesting to note that a portion of Component III of fractionated polyoma DNA differs in density from the other polyoma DNA fractions[65] and could be host DNA. Even so it is not clear why the polyoma transformed mouse cells should contain more than normal cells of the DNA present as a contaminant in the virus preparations; the difference may derive from the aneuploid nature of the cells used. It must be pointed out that only one line of transformed cells was tested in the experiments of Axelrod et al.[64] and it is not known therefore whether the result is general for all transformed cell lines.

5) Considerations About the Mode of Viral Carcinogenesis by Polyoma Virus

No convincing evidence for the function of polyoma virus genes in the transformed cells has been obtained to date. All attempts to demonstrate the presence of viral genetic material in cells transformed by polyoma virus by methods that have been successful with lysogenic bacteria (induction, marker rescue of known viral genes, and nucleic acid hybridization) have either given negative or unconvincing positive results.

Although at least one specific new cellular antigen is known to be present in all cells transformed by polyoma virus it has not

yet been established whether this antigen is specified by cellular or by viral genes. Hare's results[55] that the type of antigen can be determined by the strain of virus used suggests that the antigen is virus specified. This finding does not necessarily prove the presence of viral genes in the transformed cells, since it could also be explained if the virus acts as a mutagen or selects different pre-existing cell mutants; different virus strains could produce slightly different types of transformed cells. Ting's experiments[58], in which this transplantation antigen appears to be rescued by superinfection, could be conclusive in demonstrating the viral specificity of this gene if experimentally satisfactory; unfortunately, however, these experiments are in a preliminary stage at this time. Even if viral genes are proved to be present in the transformed cells they cannot be considered as the cause of the transformation unless they are shown to be relevant in maintaining the transformed state.

Since transformation is not induced by empty viral capsid protein, but only by DNA containing particles[50] and since furthermore it can be induced by protein free viral DNA[40], the possibility that the completed capsid protein induces a cell mutation causing transformation is clearly ruled out. The possibility of specific mutations by other viral specified products has not been examined. Transformation cannot be attributed to a factor released from a neighboring cell after infection because the efficiency of transformation per PFU of virus is not dependent on the concentration of cells in the assay dish[46], and especially because single cells can be transformed in microdrops[66].

The selection of pre-existing transformed cell mutants which remain unexpressed until viral infection is unlikely; morphologically transformed cells could not be detected in large populations of uninfected mouse and hamster cells, when plated at low cell concentration, by the criteria used in the two types of transformation assays[42, 45, 36]. It has also been observed that the transformation frequency of clonal cell lines are similar to each other and to that of the uncloned parental population[67, 68]. This tends to exclude that transformation is caused by the enhancement of a pre-existing cell mutant by virus infection.

D. Basis for the Present Studies

Conditional lethal mutants of a number of different viruses have been isolated[69-76]. Such mutants have been shown to be extremely useful in the study of viral reproduction and maturation[77, 78]. Since conditional lethal mutations can be distributed over most of the genetic map[77, 78] they could prove to be valuable in the study of tumour viruses. They may be used to "tag" almost all the viral genes, a property of obvious physiological and genetical implication; they can also be useful for studying the mechanisms by which these viruses transform normal cells to cancer cells. For instance, it may be possible to determine which viral functions are needed either for the initiation of cell transformation or for the maintenance of the transformed state. This knowledge would be useful for understanding the mechanisms of viral carcinogenesis. It could be proved that carcinogenesis is caused by the function of persisting viral genes if cells transformed by a mutant of this type lost one or all of the

transformed properties under nonpermissive conditions. Furthermore, conditional lethal mutants allow the use of selective techniques and thus provide a sensitive tool for performing marker rescue experiments, which could show whether any of the genes of the transforming virus persists in the transformed cells.

For the above reasons the isolation of temperature sensitive mutants of polyoma virus was undertaken. This thesis describes the isolation and characterization of two such mutants.

MATERIALS AND METHODS

A. Materials

1) Media

For routine culture of cells and for virus assays a reinforced Eagle's medium (REM)[79] was used. Serum, either calf or horse, was included with the REM in concentrations ranging from 5 to 20%. In the transformation assays a solution of tryptone phosphate broth[9] was included in the medium at a final concentration of 10%.

2) Buffers

For dispersal and subculture of cells, 0.05% trypsin dissolved in tris buffered saline[57] without divalent cations was used. Tris buffered saline (TBS) was usually used as a diluent for virus and also to wash the cells before virus infection.

3) Cells

a) Primary cultures

Mouse embryo cultures: Primary cultures were obtained from 12 to 14 day old embryos of random bred Swiss mice according to the method of Dulbecco and Freeman[30]. Cells from these primary cultures were subcultured to secondary cultures 3 to 7 days later. The secondary cultures were used for virus growth and plaque assays. The cultures obtained from embryos are heterogeneous since they contain cells derived from all parts of the body.

Mouse kidney cultures: Primary cultures were prepared from the kidneys of 13 day old Swiss mice according to the method of Winocour[80]. The primary cultures were used when confluent for the preparation of virus stocks. These cells are predominantly composed of epithelial cells.

b) Continuous lines

BHK/C13: This is a continuous line of Syrian hamster kidney cells which were obtained from the Institute of Virology in Glasgow Scotland. The BHK/C13 cells were grown to a large number on receipt in the laboratory and then frozen in REM containing 10% calf serum, 10% tryptone phosphate, and 10% dimethyl sulfoxide at -70°C. When needed they were thawed and maintained in REM containing 10% calf serum and 10% tryptone phosphate. Thawed cells were discarded after a month of continuous culture since they tend to become inhomogeneous owing to the accumulation of cell variants.

4) Virus

PY was originally obtained from Dr. Rowe of the National Institutes of Health. It is the large plaque type whose properties have been described elsewhere[57]. The temperature sensitive lines were isolated from nitrous acid treated lysates of PY as described in the test.

5) Receptor Destroying Enzyme (RDE)

RDE, an enzyme which destroys the affinity of receptor sites on the surface of erythrocytes and host cells for polyoma

virus[81], was obtained as a lyophilized powder from Behringwerke AG. The powder, supplied in serum bottles, was dissolved in 2.5 ml. of TBS plus 0.5 ml. of REM. When used, RDE was added directly to the culture medium (final concentration 1/40). The preparation of RDE used was effective in inhibiting the agglutination of guinea pig erthrocytes by PY at a concentration of 1/500 under similar conditions to those described by Burnet and Stone[82].

B. Methods

1) Virus Stocks

Virus stocks were prepared according to the method of Winocour[80] on uninfected confluent primary mouse kidney cells. The crude lysates were clarified from cell debris by low speed centrifugation and used directly. In some cases the crude lysates were purified by density gradient centrifugation in CSC1[80].

2) Virus Assays

a) Plaque assay

The plaque assay was essentially that described by Dulbecco and Freeman[30] and employed secondary mouse embryo cultures. The virus was adsorbed for 1 3/4 hours before the cells were overlaid with agar. Infected cells were plated for infective centers, usually after being treated with antiviral antibody and RDE, according to the method of Weisberg[36].

The assay plates were incubated at either 31.5°C or 38.5°C. At 38.5°C polyoma plaques usually start to appear about the seventh day after infection; new plaques continue to appear until

day 16. At 31.5°C plaques start to appear after about 14 days and continue to appear until 32 days. Final counts of plaques were usually made after 13 to 16 days at 38.5°C or after 26 to 32 days at 31.5°C.

b) Transformation assay

The transformation assay was essentially that described by Montagnier and Macpherson[44, 45], which distinguishes the cells transformed by polyoma virus from normal cells by their enhanced ability to form colonies in agar. Hamster cells (BHK/C13) were infected in suspension at a concentration of 10^6 cells/ml. in REM for one hour at 38.5°C. The infected cells were washed three times with REM to remove the unabsorbed virus and then suspended in 0.33% agar containing 10% calf serum and 10% tryptone phosphate in REM at a concentration of 10^4 cells/ml. One and one-half ml. of the 0.33% agar cell suspension was plated onto a preset base layer of 5 ml. of 0.5% agar in 60 mm. plastic petri dishes. After the agar had hardened, the dishes were incubated at 31.5°C and/or 38.5°C in well humidified incubators containing 7% CO_2.

It should be pointed out that under these conditions the seeding of more than 2-3 x 10^4 cells/dish, as originally suggested by Montagnier and Macpherson[44, 45], resulted in the production of many normal cell colonies when incubated at 38.5°C.

Incubation conditions: The assay dishes were divided into four equal groups which were each incubated in one of the following fashions before scoring for transformation.

1) High temperature incubation--Incubation for 16 to 18 days at 38.5°C.

2) "Shift up" incubation--Incubation first for four days at 31.5°C and then for 16 to 18 days at 38.5°C.

3) "Shift down" incubation--Incubation first for two days at 38.5°C and then for 32 to 36 days at 31.5°C.

4) Low temperature incubation--Incubation for 32 to 36 days at 31.5°C.

Scoring of transformation: Transformation was usually scored by counting the macroscopic colonies arising after the various incubation periods. Corrections were made for the background of normal colonies by subtracting the number of colonies appearing on the uninfected dishes. In order to verify the transformed nature of a colony, it was picked out of the agar and plated under fluid medium in a plastic petri dish. After being pipetted free of the agar, the colony usually stuck to the bottom of the dish. Within one day at 38.5°C or two days at 31.5°C, cells began to migrate out from the colony and started to divide. The transformed colonies grew in a non-orientated criss-cross manner in a multilayered mat (loss of contact inhibition)[42]. The cells from the normal colonies retained their contact inhibition and grew in an aligned parallel fashion in a single layer. The transformed cells, either as the whole colony or as individual cells, had a great tendency to become dislodged from the substrate under conditions of the slightest agitation. This led to the production of satellite colonies from the parental single colony each time the dish was disturbed. For the

most part, the normal colonies did not produce satellite colonies under conditions of slight agitation.

Dishes kept under the low temperature incubation had a higher background of normal colonies; with these dishes the following procedure was used to estimate the number of transformed colonies. The whole top agar from each dish was removed in medium and transferred to two large plastic petri dishes (100 mm). The agar was broken up by pipetting in order to free the colonies. These dishes were then incubated at 31.5°C for three and one-half days without being disturbed in order to keep the production of satellite colonies to a minimum. After this incubation period, the colonies were fixed by the gentle addition of formaldehyde (final concentration 4%) directly to the medium in the plate. The colonies were then stained with methylene blue and scored for the number of transformed colonies by microscopic examination[42]. By this method too high an estimate can be obtained by counting colonies derived from single cells or pieces of broken colonies. To avoid counting colonies initiated by single cells, only large colonies were scored. This method can also tend to give a low estimate of transformation due to colonies remaining embedded in the agar. When plating single colonies in individual dishes (see above) if perchance a colony is not adequately freed from the agar at the time of the initial transfer, it can be freed at a later time with no ill consequences. When many colonies are transferred into the same dish, it is important that the colonies should not be disturbed until after fixation to avoid the production of satellite colonies. Thus, colonies embedded in the agar after the transfer are lost.

c) Hemagglutination

Hemagglutination was performed according to the method of Rowe et al.[83]. To 0.4 ml. of 0.4% fresh guinea pig red blood cells (usually about 4×10^7 cells/ml) in tris buffered saline without divalent ions (TD) 0.4 ml. of serial two fold virus dilutions in TD were added. The virus-red blood cell mixture was allowed to settle in the cold for three hours before being read. The reciprocal of the highest dilution (before addition to the red blood cells) which caused positive agglutination was taken as the titer of the stock in hemagglutination units (HAU). Since only serial two fold dilutions of the virus were used in this test, the titers have an uncertainty of a factor of two.

Usually each HAU was equivalent to $1\text{-}2 \times 10^5$ PFU of crude virus stock. Assuming that at the hemagglutination endpoint there is one physical particle per red blood cell[84] one can estimate that one out of every 200 to 400 physical particles is a plaque forming unit.

Hemagglutination inhibition tests were performed according to the method of Rowe et al.[83].

3) Virus Growth Experiments

Confluent secondary mouse embryo cultures were washed with either TBS or REM to remove debris and serum before infection. A volume of either 0.2 ml. on 60 mm. dishes or 0.6 ml. on 100 mm. of virus in either TBS or REM was added to the cells and allowed to adsorb for 1 3/4 hours at 38.5°C in a humidified atmosphere. After adsorption the cells were washed with either TBS or REM to remove the unadsorbed virus. Medium was then added (REM plus 10% horse serum) and the cultures were incubated at either 31.5°C or 38.5°C

in a well humidified incubator in the presence of about 7% CO_2. In some experiments RDE was incorporated into the medium to destroy the cell receptors; this cuts down the readsorbtion of released virus and prevents further cycles of virus growth. After various times of incubation the cultures were removed from the incubator and harvested with a rubber policeman. The cells were then broken open by either sonication or three cycles of freeze thawing before being assayed. Such preparations of broken cells will be referred to as lysates.

4) Infectious DNA

Infectious viral DNA was extracted from virus particles and infected cells by the phenol method of Weil[27]. The DNA was assayed for plaque formation on confluent mouse embryo cultures in the presence of hypertonic saline which facilitates the uptake of the DNA by the cells[27]. Usually the extraction of 2 to 5 x 10^5 PFU of whole virus yielded one plaque forming unit of DNA.

5) Nitrous Acid Treatment

Crude virus lysates were treated with 0.67 M $NaNO_2$ in 1 M acetate buffer at pH 4.6. At various times after the start of the treatment an aliquot of the reaction mixture was added to three volumes of 2.0 M tris buffer (pH 8.0) to stop the reaction and then dialysed overnight against 0.02 M phosphate-buffered saline (pH 7.2) in the cold. The treated samples were assayed for plaque formation at 31.5°C.

6) Inoculation of Hamsters

One to three-day old Syrian hamsters were inoculated either subcutaneously (SQ) or inter peritoneally (IP) with 0.1 or 0.3 ml. of virus. The young animals were kept as a litter with the mother until they were 18 days old, at which time they were placed in separate cages. The animals were checked twice a day, when kept as a litter, and once a day when separated, for death or the production of tumours. Tumours were detected by macroscopic examination only.

C. Abbreviations Used in this Thesis

DNA, deoxyribonucleic acid; RNA, ribonucleic acid; PY, polyoma virus; RSV, Rous sarcoma virus; ALC, Avian Leucosis Complex; PFU, plaque forming unit; HAU, hemagglutination unit; RDE, receptor destroying enzyme; REM, reinforced Eagle's medium; TBS, tris buffered saline; TD, tris buffered saline without divalent ions; IP, interperitoneally; SQ, subcutaneously

PART I. ISOLATION OF THE TEMPERATURE SENSITIVE LINES

Nitrous acid treatment (Materials and Methods) inactivates polyoma virus with single hit kinetics down to a survival of about 10^{-5} (Figure 1). In order to obtain a maximum frequency of mutants among the survivors, virus was inactivated to a survival of between 10^{-4} to 10^{-5}; a test for temperature sensitive mutants was carried out in two ways. One method was to first grow the treated virus in mouse embryo cultures at 31.5°C for two weeks before plaquing at 31.5°C. One hundred well isolated plaques were picked at 31.5°C and replaqued at 38.5°C and 31.5°C. All hundred plaques tested had similar plaquing efficiencies at these two temperatures. The failure to detect mutants by this method may be due to a selective advantage of the wild type over the mutants during the initial two week passage at 31.5°C; the mutants may be more sensitive to interfering substances, such as interferon, produced by the inactive particles in the treated stock, or may not grow as well as the wild type virus at 31.5°C.

In the second method the virus was plaqued directly at 31.5°C after nitrous acid treatment. After 32 days incubation, well isolated plaques were picked at random from these plates and replaqued at 31.5°C and 38.5°C. Of the 40 plaques tested two showed a greater plaquing efficiency at 31.5°C than at 38.5°C; plaques were picked from these low temperature plates and replaqued at the two temperatures. The two lines thus isolated showed at least a 100-fold greater plaquing efficiency at 31.5°C than at 38.5°C. The parental wild type (PY), on the contrary, had a similar plaquing efficiency at these two temperatures.

FIGURE 1

Nitrous Acid Inactivation of Polyoma Virus. ●--Polyoma virus treated with 0.67 M $NaNO_2$ in 1 M acetate buffer at pH 4.6. O--Polyoma virus treated with 0.67 M NaCl in 1 M acetate buffer at pH 4.6.

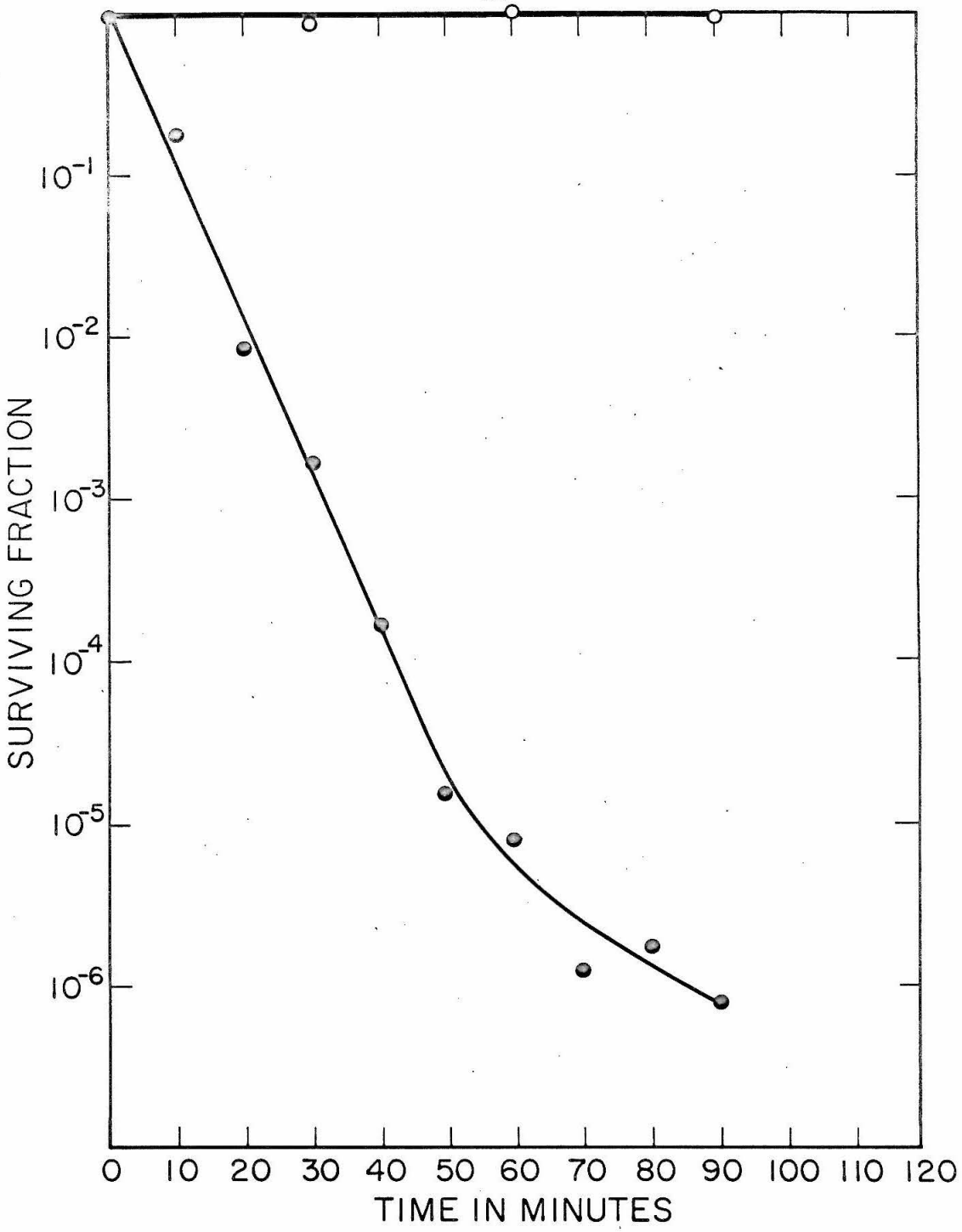

SURVIVING FRACTION
10^{-1}
10^{-2}
10^{-3}
10^{-4}
10^{-5}
10^{-6}
0 10 20 30 40 50 60 70 80 90 100 110 120
TIME IN MINUTES

Figure 1

One of the two temperature sensitive virus lines (TS-a) arose from a plaque which had at least a 100-fold better plaquing efficiency at 31.5°C than 38.5°C in the first plaquing. The other line (TS-b) was isolated from a plaque which originally only showed a two-fold higher plaquing efficiency at the low temperature. When four plaques were picked from this first replaquing of TS-b at 31.5°C and retested at the two temperatures, the virus of one of the plaques was found to have at least a 100-fold better plaquing efficiency at 31.5°C than at 38.5°C; the virus of the other three plaques had similar plaquing efficiencies at the two temperatures.

The virus lines obtained from this temperature sensitive plaque of TS-b as well as that of the TS-a plaque were found to retain their temperature sensitive plaquing character on subsequent passages in mouse cells. Both viral lines were plaque purified three times from plates containing only one plaque after 40 days incubation at 31.5°C. Stocks were grown at 31.5°C and kept frozen at -20°C.

The morphology of the plaques produced at 31.5°C, on mouse embryo cells, by both the temperature sensitive lines is similar to that of PY. The plaques contain mostly degenerated cells of a "sandy" appearance with a small number of prominent surviving cells. The plaques produced by TS-a were usually slightly smaller and appeared slightly later than those of PY. TS-b plaques were clearly smaller and appeared 4 to 6 days later than those of PY. After sufficient incubation of the assay plates both lines produced large plaques, showing that they retained the large plaque character of the parental strain.

The relative plaquing efficiency of PY on mouse cells at 38.5°C compared to 31.5°C is about unity. The relative plaquing efficiency of TS-a at these temperatures was usually about 10^{-4}. The number of plaques formed by high concentrations of TS-a at the high temperature is difficult to evaluate owing to an interference phenomenon and also because the assay plates become very acid and "chewed up". The addition of TS-a at high titers interferes with the detection of plaque formation of PY; this effect becomes noticeable if more than about 3×10^4 PFU of TS-a are added to an assay plate containing about 2.5×10^6 cells. The plaquing efficiency of TS-b was not studied in detail.

In most cases plaques formed by TS-a at 38.5°C were due to virus that was not temperature sensitive, since it could be replaqued with a high plaquing efficiency at 38.5°C. Whether this virus was due to revertants or contaminants carried along with each stock, which were not prepared from single plaques, was not determined. Occasionally plaques formed at 38.5°C were composed of temperature sensitive virus as determined by its plaquing efficiency at 31.5°C compared to 38.5°C.

PART II. COMPARATIVE PROPERTIES OF THE TEMPERATURE SENSITIVE LINES AND WILD TYPE POLYOMA VIRUS

These studies had two objectives: first to show that TS-a and TS-b were mutants of polyoma virus and not merely some contaminating viruses present in the original polyoma stock; second to try to determine why TS-a and TS-b were temperature sensitive in their plaque forming ability. For both these purposes TS-a and TS-b were compared to PY in a number of physical and biological properties. Experiments will first be described in which the characteristics of the structural components of the virus particles (capsid protein and DNA) grown at 31.5°C are compared. Then the characteristics of the viral growth at the high and low temperature will be examined. In all these respects TS-a was studied in much greater detail than TS-b.

A. Characteristics of the Capsid Protein

1) Hemagglutination

The ability to hemagglutinate guinea pig red blood cells resides in the capsid protein of the polyoma virus, since viral capsids not containing DNA hemagglutinate with the same efficiency, in terms of numbers of physical particles, as DNA containing capsids[88].

Both temperature sensitive lines were indistinguishable from the wild type polyoma virus (PY) in their hemagglutinating properties. In all three cases, guinea pig red blood cells could

only be agglutinated in the cold ($4^{o}C$) and not at room temperature or $37^{o}C$; the agglutinated complexes could be dissociated upon transfer from $4^{o}C$ to $37^{o}C$ and then reagglutinated when transfered back to $4^{o}C$. In crude virus stocks, produced at $31.5^{o}C$, the ratio of plaque forming units to hemagglutination units was $1\text{-}2 \times 10^{5}$ for all three virus strains. Since the hemagglutination titer is indicative of the number of physical particles, the specific infectivity in terms of plaque forming units to physical particles is therefore similar for both the temperature sensitive lines, produced at $31.5^{o}C$, and the polyoma wild type strain.

The temperature sensitive lines were also found to be similar to PY in the dissociability of their particles from agglutinated red blood cells when the temperature was raised from $4^{o}C$ to $22^{o}C$. On the contrary, a small plaque mutant of polyoma virus which differs from PY in adsorption properties, owing to differences in its capsid protein, is not easily eluted from agglutinated red blood cells when the temperature is shifted from $4^{o}C$ to $22^{o}C$[89]. Thus the TS-a and TS-b lines seem to be indistinguishable from the large plaque polyoma wild type virus strain in all the hemagglutination properties tested.

2) Antigenic Specificity

The antigenic specificity of whole virus particles also resides in the capsid protein. In order to test whether the temperature sensitive lines and PY share common antigens, the antibody sensitivity of the plaque forming and hemagglutinating abilities of these virus strains was tested in a qualitative manner.

Rabbit antiserum prepared against density gradient purified PY virus neutralizes the infectivity of both temperature sensitive lines and PY as tested by plaquing at 31.5°C as shown by the following test. Crude lysates of the three virus lines were incubated with the anti-PY antiserum in TBS for three hours at 37°C. The antibody-virus mixtures were then diluted and plaqued at 31.5°C on mouse embryo cells. Controls, in which no antibody was added, were treated in a similar fashion. It was found that the concentration of anti-PY antiserum used inhibited TS-a plaque formation by 97%, TS-b plaque formation by 96% and PY plaque formation by 85%.

Using the hemagglutination inhibition test[83], in which the virus is incubated with different concentrations of antibody for one hour in TBS and then tested for its hemagglutinating ability, without removing the antibody, it was found that TS-a, TS-b and PY were inhibited to the same degree using either a rabbit anti-PY serum (made against density gradient purified PY) or a hamster anti-TS-a serum (made against crude plaque purified TS-a lysates) (Table 1).

The above results show in a qualitative way that both temperature sensitive lines share common antigenic sites with PY, at least as far as their infectivity and hemagglutinating abilities are concerned. Inactivation rates which would allow the detection of possible quantitative differences were not performed.

The rest of the experiments described in this thesis were performed with TS-a and PY.

TABLE 1

DILUTION OF RABBIT ANTI-PY ANTISERUM

	4000	8000	16000	32000	64000	128000	256000	512000	NO SERUM
8 HAU TS-a	-	-	-	-	-	+	+	+	+
8 HAU TS-b	-	-	-	-	-	+	+	+	+
8 HAU PY	-	-	-	-	-	+	+	+	+
NO VIRUS	-	-	-	-	-	-	-	-	-

DILUTION OF HAMSTER ANTI-TS-a ANTISERUM

	200	400	800	1600	3200	6400	NO SERUM
8 HAU TS-a	-	-	-	+	+	+	+
8 HAU TS-b	-	-	-	+	+	+	+
8 HAU PY	-	-	-	+	+	+	+
NO VIRUS	-	-	-	-	-	-	-

8 hemagglutination units (HAU) of each of the three virus lines (TS-a, TS-b, PY) were mixed with different dilutions of either rabbit anti-PY antiserum or hamster anti-TS-a antiserum and they were incubated at 37°C for 1 hour in 13 mm. glass test-tubes (total volume was 0.4 ml.). After incubation 0.4 ml. of guinea pig red blood cells (0.4%) were added to each tube. The tubes were shaken and placed at 4°C for 4 hours. All dilutions were in TD.

(-) signifies that the red blood cells were not agglutinated after 4 hours at 4°C.

(+) signifies that the red blood cells were agglutinated after 4 hours at 4°C.

3) Buoyant Density, Size and Structure of the TS-a Viral Particles

Since PY virus only contains 13% DNA[80] the density of the virus particle depends for the most part on the capsid protein, and less on the DNA; the density of capsids not containing DNA depends on the capsid protein. The density of TS-a virus grown in the presence of H^3-thymidine at 31.5°C was determined by equilibrium centrifugation in a CSC1 density gradient. As can be seen from Figure 2A two discrete hemagglutinating components could be resolved. One component has a peak at a density of 1.325 and contains DNA, whereas the other component peaked at a density of 1.285 and did not contain any DNA. These results are in agreement with the densities of the full and empty capsids reported by Winocour for PY[80]. Thus, it can be concluded that the densities of the capsid proteins of TS-a and PY as well as the amounts of DNA contained in the full virus particles are similar.

The size and structure of the TS-a virus particles were examined in the electron microscope. Negative staining, using sodium phosphotungstate, of partially purified preparations of TS-a grown at 31.5°C revealed spherical particles of the same diameter (45 μ) as have been reported by other workers for PY[93]. These preparations (Figure 3) also contained elongated structures and larger particles, seemingly composed of the same subunits as the 45 μ particles. Such aberrant forms have also been noted by other workers[94].

Figure 2

The Density Distribution of the Hemagglutinating Activity and Radioactivity of TS-a Virus Grown in the Presence of H^3-Thymidine

Lysates of TS-a virus grown on mouse kidney cells at either 38.5°C or 31.5°C in the presence of H^3-thymidine were concentrated and centrifuged to equilibrium in a CSCl density gradient[80]. Drops were collected by puncturing the bottom of the tube and were assayed for their hemagglutinating activity and radioactivity. The density of every tenth drop was calculated from its observed refractive index.

The cells were infected at an input multiplicity of about 70 PFU/cell; RDE was incorporated in the growth medium to prevent a second cycle of viral multiplication.

O--counts per minute (CPM).

●--hemagglutination units (HAU)

A. Lysate of TS-a infected mouse kidney cells incubated for 192 hours at 31.5°C.

B. Lysate of TS-a infected mouse kidney cells incubated for 96 hours at 38.5°C.

C. Lysate of TS-a infected mouse kidney cells incubated for 12 hours at 38.5°C; this gradient gives in indication of the distribution of the hemagglutinin present before new viral multiplication.

FIGURE 2

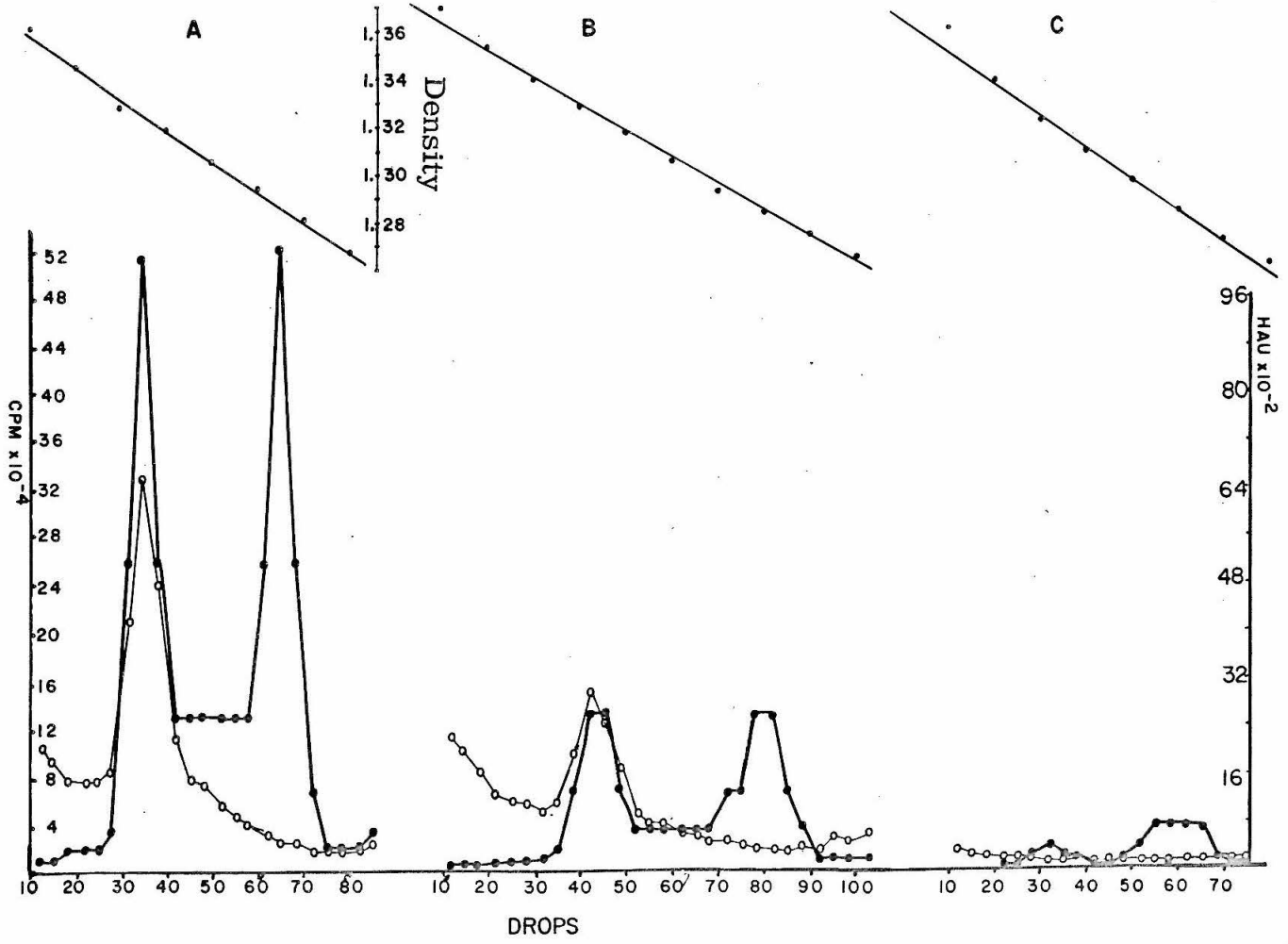

A
B
C
Density
1.36
1.34
1.32
1.30
1.28
CPM x10⁻⁴
52
48
44
40
36
32
28
24
20
16
12
8
4
HAU x10⁻²
96
80
64
48
32
16
10 20 30 40 50 60 70 80
10 20 30 40 50 60 70 80 90 100
10 20 30 40 50 60 70
DROPS

FIGURE 3

An electron micrograph of negatively stained partially purified TS-a virus particle grown at 31.5°C. Magnification x 251,600. Note the full and empty particle and also the elongated structures.

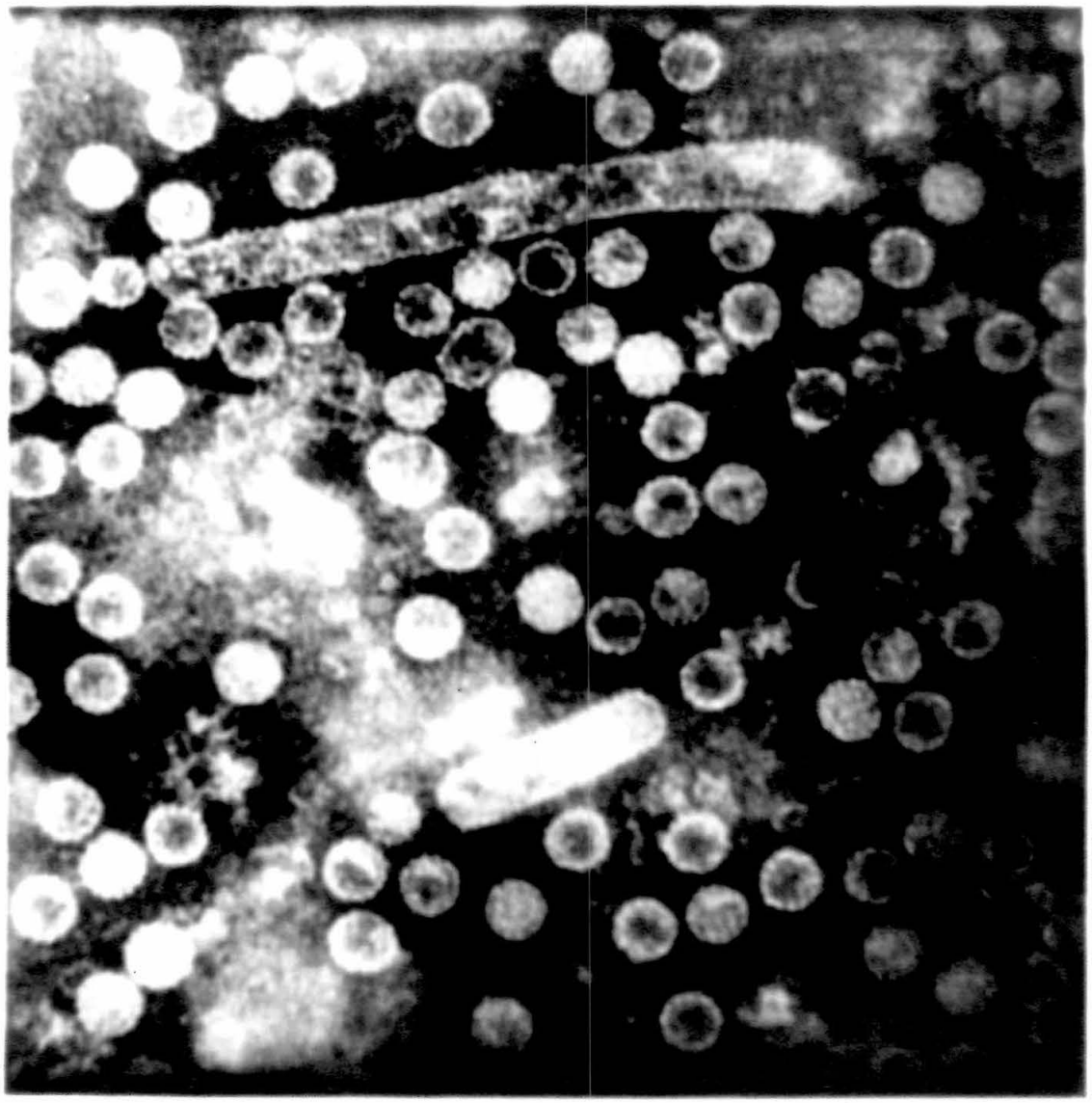


Fig. 3

4) Heat Inactivation of the Plaque Forming Abilities of TS-a and PY

Loss of infectivity of PY on heating at 70°C is due to denaturation of the capsid protein, which abolishes either the attachment of the virus particles to the host cell or the release of the viral DNA from the capsid within the cell. This conclusion is derived in part from the observation that heating destroys the hemagglutinating ability of PY[90] and in part from the finding that infectious viral DNA can be extracted from heat inactivated virus[91]. It was of interest to see whether TS-a was more sensitive to heat inactivation than PY, since, if so its temperature sensitivity could be explained.

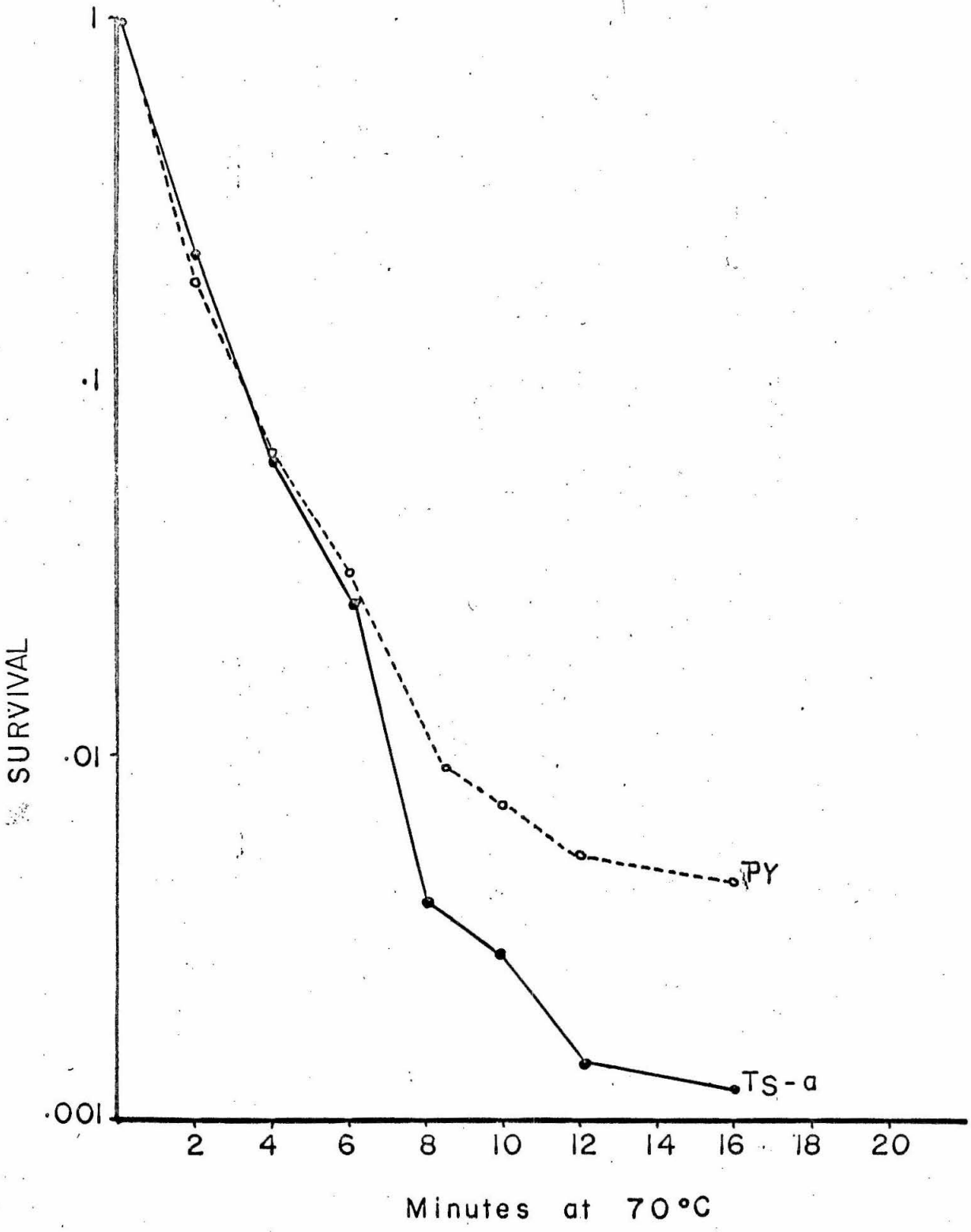
Crude lysates of TS-a and PY were diluted to approximately the same titer in TD and placed in a water bath at 70°C. At different times aliquots were removed, quickly diluted into cold TD and then assayed for their plaque forming ability. The results of the experiment are presented in Figure 4; they show that both TS-a and PY have similar kinetics of inactivation for the first six minutes of heating, during which inactivation seems to follow first order kinetics. The differences in time at which the inactivation curves, of the two strains, start to level off is probably due to different amounts of protective debris or clumping of virus particles in the two virus stocks. Thus the TS-a virus particle is not noticeably more heat sensitive than the PY virus particle; the temperature sensitivity of TS-a is not due to the heat lability of the capsid protein of the completed virus particles.

FIGURE 4

Heat Inactivation of PY and TS-a at 70°C

TS-a or PY virus was heated at 70°C in TD in a water bath. At different times aliquots were removed and cooled by immersion into ice. The unheated 0 time sample and the heated samples were then assayed by plaquing at 31.5°C.

FIGURE 4



B. Characteristics of the Viral DNA

1) Infectivity

An infectious DNA could be extracted from TS-a virus, grown at 31.5°C, by using phenol, as previously described by Weil[27]. Whereas the infectious DNA of PY formed plaques on mouse cells both at 31.5°C and 38.5°C, the DNA extracted from TS-a formed plaques only at 31.5°C; thus the plaque forming ability of the DNA had the same temperature dependence as the plaque forming ability of the whole virus particle. The plaquing efficiency for TS-a and PY DNA was similar; about 3000 plaques were produced at 31.5°C for every microgram of DNA extracted from either virus.

2) Sedimentation Properties

Vinograd et al.[28] have shown that careful extraction of PY DNA produces only two sedimenting forms. The major form (component I) has a $S_{20,w}$ of 20 and is probably a ring with inborn twists, while the other minor form (component III) has a $S_{20,w}$ of 14.5 and is probably composed on linear molecules. If at least one single strand break is introduced into component I another form (component II) is produced which has a $S_{20,w}$ of 16.

Fractionation of carefully extracted DNA from TS-a by band centrifugation in CSC1 according to the method of Wiel and Vinograd[26] revealed two discrete bands (Figure 5). One band had a $S_{20,w}$ of 20.3 and the other of 14.2. These two bands correspond very well to the I and III components of PY.

Figure 5

A densitometer tracing of a band centrifugation of TS-a DNA in CSCl (ρ = 1.35, sedimentation coefficients corrected to standard conditions and the sodium salt, ($S_{20,w}$)) in a 30 mm cell. The run was performed in a Spinco model E analytical ultracentrifuge at 44,770 RPM. The base line is obscured on both the top and the bottom of the tracing owing to the high refractive gradient in the 30 mm cell. Sedimentation precedes from left to right.

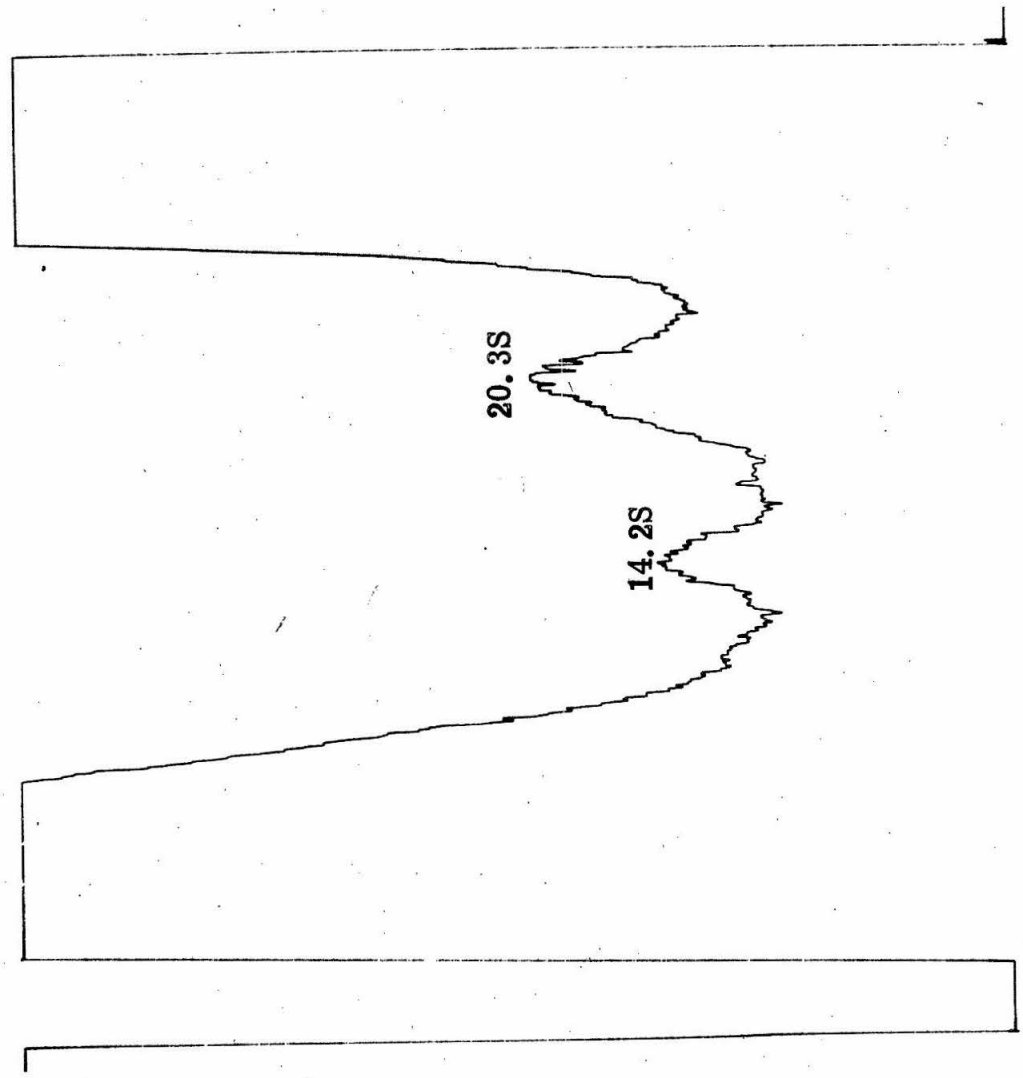


Figure 5

3) Heat Sensitivity of the Infectious DNA

The possibility was considered that the DNA of TS-a is heat sensitive, but when protected by the capsid protein it loses its temperature sensitivity. Therefore, the heat sensitivity of the infectivity of the extracted TS-a DNA was compared to that of PY DNA.

DNA extracted from either TS-a or PY was heated at 100°C in 0.55 M NaCl, 0.05 M tris (pH 8.0). After heating for 10 minutes the DNA samples were quickly cooled in ice and assayed for their plaque forming ability. Control samples of DNA were treated in a similar manner except they were not heated at 100°C. The results of this experiment presented in Table 2 show that the heating and rapid cooling did not destroy the infectivity of the DNA from either virus strain. Thus, it can be concluded that the infectious DNA of TS-a is not noticeably more heat sensitive than the infectious DNA of PY.

C. Characteristics of Growth of TS-a at 38.5°C and 31.5°C

Before analyzing the results of the viral growth experiments, two pecularities of the multiplication of PY that were observed by Weisberg[36] should be pointed out. The proportion of infected cells do not follow the expected Poisson Distribution at input multiplicities greater than 0.1 PFU/cell; in addition, the virus yields and the proportion of cells infected at the same input multiplicities vary from experiment to experiment, but are usually constant within the same experiment. Therefore, the yields of PY and TS-a virus presented in Table 3 are only comparable within a single experiment

TABLE 2

Heating at 100°C of TS-a and PY DNA

	Titer without heating	Titer after heating
TS-a DNA	1.1×10^4	1.3×10^4
PY DNA	2.1×10^4	2.3×10^4

PY or TS-a DNA in 0.55 M tris buffer (pH 8.0) was heated for 10 minutes at 100°C; the samples were then quickly cooled by immersion in ice. Control samples were treated in the same fashion but were not heated. The DNA of the heated and unheated samples were assayed for their plaque forming ability at 31.5°C.

TABLE 3

TS-a and PY Growth Characteristics

Experiment	Virus	Temperature of incubation	Input Multiplicity PFU/cell	Yield per Culture after Incubation for (time in hours) 0	21	33	39	45	69
A	PY	38.5°C	1000	2.7×10^{7}			6.5×10^{8}		
			100	2.3×10^{6}			6.2×10^{8}		
			10	3.6×10^{5}			3.6×10^{8}		
			1	1.8×10^{4}			1.2×10^{8}		
			0.1	3.6×10^{3}			2.4×10^{7}		
B	PY	38.5°C	10	2.5×10^{5}			6.0×10^{8}		
			1	3.1×10^{4}			1.8×10^{8}		
			0.1	3.4×10^{3}			4.0×10^{7}		
			0.01	1.5×10^{2}			3.4×10^{6}		
C	PY	38.5°C	10	4.5×10^{5}	5.5×10^{5}	6.0×10^{6}		3.4×10^{7}	7.0×10^{7}
	TS-a	38.5°C	100	6.0×10^{6}	5.0×10^{6}	5.5×10^{7}		1.4×10^{8}	1.9×10^{8}
D	PY	38.5°C	10	2.6×10^{5}	4.7×10^{5}	1.0×10^{7}		2.4×10^{7}	7.0×10^{7}
	TS-a	38.5°C	10	5.0×10^{5}				3.7×10^{6}	1.2×10^{7}
E	PY	38.5°C	1	7.0×10^{4}	2.5×10^{4}	9.0×10^{6}		5.0×10^{7}	2.0×10^{8}
	TS-a	38.5°C	1	1.9×10^{5}	1.3×10^{5}	1.0×10^{5}		2.5×10^{6}	6.7×10^{6}

TABLE 3 continued

Experiment	Virus	Temperature of incubation	Input Multiplicity PFU/cell	Yield per Culture after Incubation for (time in hours) 0	21	33	39	45	69
F	PY	38.5°C	0.1	6.5×10^{3}	2.5×10^{4}	2.8×10^{6}		1.2×10^{7}	7.5×10^{7}
	TS-a	38.5°C	0.1	7.9×10^{3}	7.6×10^{3}	7.4×10^{3}		3.2×10^{4}	6.5×10^{4}
G	PY	38.5°C	0.01	4.9×10^{2}	7.4×10^{3}	2.0×10^{5}		2.4×10^{6}	1.4×10^{7}
	TS-a	38.5°C	0.01	5.4×10^{2}	1.8×10^{2}	4.9×10^{2}		4.1×10^{3}	4.9×10^{3}
H	PY	38.5°C	0.1	2.5×10^{3}		3.0×10^{6}			
	TS-a	38.5°C	0.1	3.0×10^{3}	2.5×10^{3}	2.4×10^{3}			
	PY	31.5°C	0.1	4.0×10^{3}				1.0×10^{4}	7.0×10^{5}
	TS-a	31.5°C	0.1	4.0×10^{3}				5.0×10^{3}	9.0×10^{5}
I	TS-a	31.5°C	1	1.5×10^{4}					6.2×10^{6}
			0.1	1.8×10^{3}					2.0×10^{6}
J	TS-a	31.5°C	1	4.8×10^{4}	5.0×10^{4}			1.4×10^{5}	4.6×10^{6}
K	PY	31.5°C	0.01	4.1×10^{2}	2.2×10^{2}	1.1×10^{3}	3.2×10^{3}	8.0×10^{3}	3.5×10^{5}

Mouse embryo cells were inoculated with the indicated input multiplicities at 38.5°C. After washing (no antiviral antibody or RDE was used) to remove unabsorbed virus, REM was added and the cells were placed at the indicated temperatures. After various times the cells were harvested, lysed and assayed for plaques at 31.5°C. The start of the incubation period is about 2 hours after the adsorption of the virus. As mentioned in the text the results within a single experiment are comparable, but the results between experiments are not necessarily comparable.

and not necessarily between experiments. It should also be noted that the yields from the later times (45 to 69 hours) at 38.5°C in Table 3 especially at the lower input multiplicities, may contain virus from a second cycle of virus growth. The possibility of a second cycle, however, was minimized in the experiments referred to in Figure 6 by using a very high input multiplicity, which causes the infection of a high proportion of cells, and by treating the cells with RDE after infection to destroy the virus receptors of the cells. These conditions reduce both the loss of virus due to readsorption as well as the occurrence of a second viral growth cycle.

During the multiplication of PY in mouse embryo cells, new virus begins to appear between 18 and 24 hours after infection at 38.5°C, and between 36 and 48 hours after infection at 31.5°C; the virus yield reaches its maximal value after about 72 hours at 38.5°C and 144 hours at 31.5°C (Figure 6). As had been previously noted by Weisberg, it was observed in these experiments that the yield of progeny virus is proportional to the input multiplicity at less than 0.1 PFU/cell. Above 0.1 PFU/cell, the yield of new virus increases less than expected from the input multiplicity and at about 10 PFU/cell levels off. These results suggest that the yield of infectious virus from each individual PY infected cell is independent of the number of virus particles by which the cell is infected.

From the results presented in Table 3, it can be seen that at 38.5°C the ratio of the virus yield of TS-a to that of PY progressively increases at input multiplicities greater than 0.1 PFU/cell. There is, therefore, a marked contrast between TS-a and PY in this multiplicity range; whereas the yield of PY varies little with multiplicity that of TS-a increases markedly as input multiplicity increases. At an input multiplicity of 100 PFU/cell the yield of TS-a at 38.5°C is about

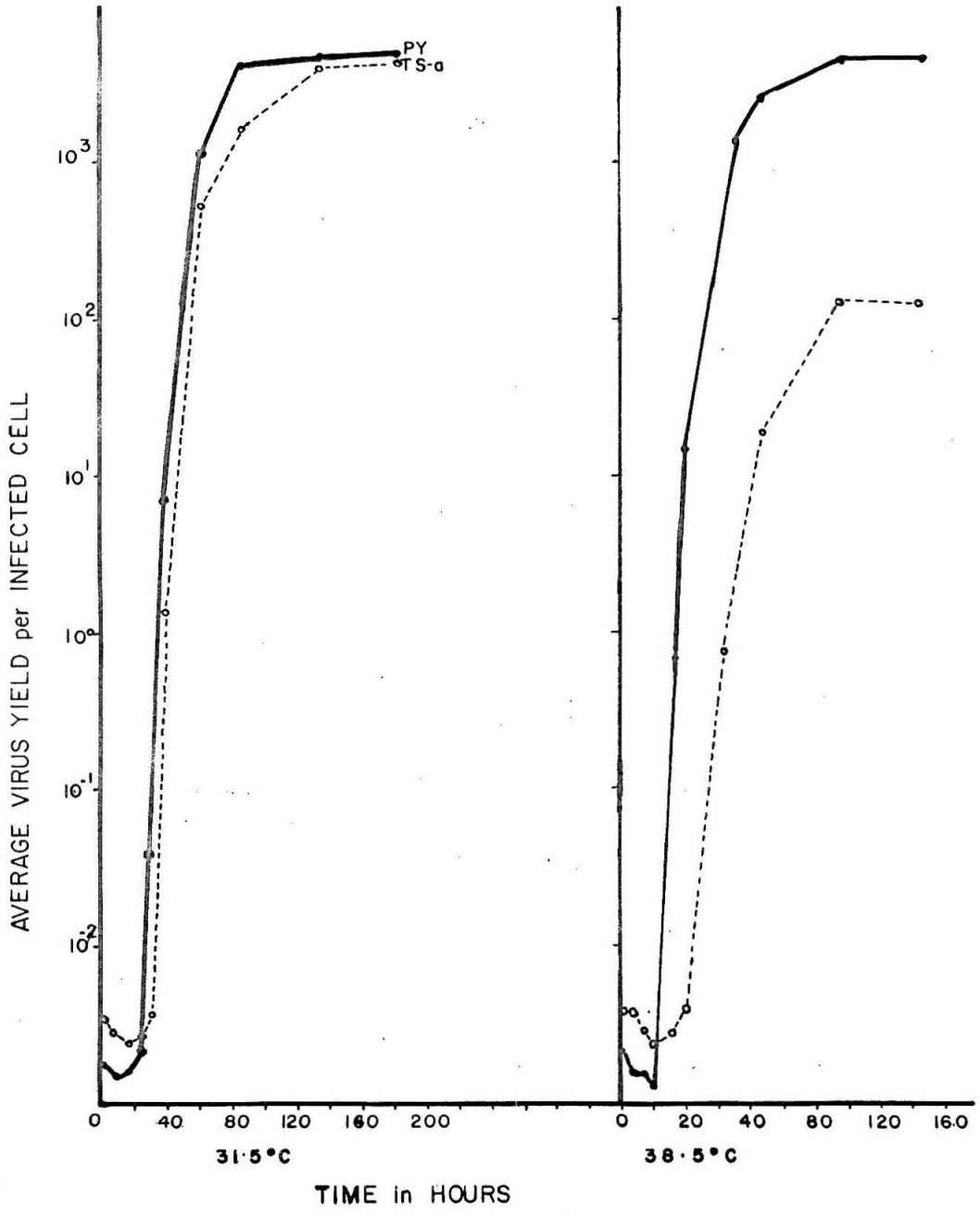
Figure 6

Growth Curves of TS-a and PY at 38.5°C and 31.5°C

Mouse embryo cells were exposed either to 20 PFU of TS-a per cell or 6 PFU of PY per cell for two hours in 0.2 ml. of REM at 38.5°C. The innoculum was removed and the cells were washed with TBS and REM to remove the unadsorbed virus. Anti-PY antibody and RDE were then added to the cells for 20 minutes at 38.5°C to inactivate the superficially adsorbed virus. After removal of the antibody RDE mixture the cells were washed thoroughly with REM to get rid of the residual antibody. Four ml of REM containing 10% horse serum and RDE was then added and the cells incubated at the appropriate temperatures. After various times of incubation the cells were harvested, broken open and assayed for infectious virus at 31.5°C. The number of cells infected was determined by infective center formation as described by Weisberg[36]; cells were incubated for 24 hours at 31.5°C before being plated for infective center formation. It was found by this method that 32.0% of the TS-a infected cells and 12.5% of the PY infected cells registered as infective centers. Since the RDE seemed fairly effective in blocking readsorption and reinfection thus preventing a second growth cycle the growth curves are plotted as a function to the average number of virus PFU produced per infected cell versus time in hours after the cells were incubated. The 0 time is really four hours after the start of the adsorption.

FIGURE 6A

FIGURE 6B



equal to that produced by PY. The virus thus produced is still mainly temperature sensitive in its plaquing ability. This virus contains newly synthesized DNA; this is shown by Figure 2B which reports the results of a fractionation by equilibrium density gradient centrifugation of TS-a virus grown in the presence of H^3-thymidine; the figure shows that TS-a virus grown at 38.5°C gives rise to a DNA containing peak of a similar density (1.325) as the peak generated by TS-a virus grown at 31.5°C (Figure 2A). Thus, particles of TS-a virus grown at 38.5°C contain newly synthesized DNA and not input DNA wrapped in new capsid protein.

Whether increased yields of TS-a at higher input multiplicities are caused by an increase in the amount of infectious virus liberated per infected cell or to an increase in the proportion of cells liberating virus has not been determined.

The growth curves of TS-a and PY at 38.5°C after infection at a relatively high input multiplicity (Figure 6A) differ both in their time course and yield. Some difference in the time course of the two growth curves is also apparent at 31.5°C, where TS-a is produced a little later even though the final yield per infected cell is similar (Figure 6B); this may explain why the plaques produced at the low temperature by TS-a are usually slightly smaller than those of PY.

D. Conclusions

The comparative study of the properties of the temperature sensitive lines and PY lead to the following conclusions:

1) TS-a is a mutant of polyoma virus because it is indistinguishable from PY in a number of physical characteristics and shows antigenic cross reaction with it.

2) The temperature sensitivity of TS-a is a property of some stage of the intracellular viral development because the components of the completed virus particle are not more heat sensitive than those of PY. This conclusion is also supported by the finding that the two virus strains can only be distinguished in a clear way by their biological activity at 38.5°C.

3) The production of infectious TS-a at 38.5°C is multiplicity dependent.

4) The production of TS-a virus at 38.5°C at an input multiplicity of 20 PFU/cell follows a time course different from that of PY.

PART III. NATURE OF THE TEMPERATURE SENSITIVE STEP OF TS-a

In order to try to define the intracellular temperature sensitive step of TS-a, the efficiency of different processes concerned with the growth of TS-a at 38.5°C were investigated. Attempts were also made to try to determine the time during the viral growth cycle at which the temperature sensitive step occurs.

A. Adsorption

Adsorption does not seem to be responsible for the inhibition of the growth of TS-a at 38.5°C as shown by the experiment reported in Figure 6. In this experiment, monolayer cultures of mouse embryo cells were exposed to PY or TS-a for 2 hours at 38.5°C, and then washed thoroughly with anti-PY antiserum and RDE in order to remove unadsorbed virus. They were divided into two sets of cultures of which one was incubated at 38.5°C and the other at 31.5°C. After various periods of time, the cells were harvested, broken open and assayed for virus. As can be seen from Figure 6 after incubation at 31.5°C, the virus yields of PY and TS-a were similar, but after incubation at 38.5°C the yield of TS-a was inhibited while that of PY was normal. If adsorption was solely responsible for the temperature sensitivity of TS-a at 38.5°C, the yield of TS-a should have been inhibited at both temperatures.

B. Release

The release of the newly formed virus from TS-a *infected* cells incubated at 38.5°C cannot be a factor in the inhibition at high

temperature since the cells are forcibly disrupted either by sonication or freeze thawing.

C. Uncoating

In order to test whether a faulty release of the TS-a DNA from its protein coat could be the cause of the temperature sensitivity, viral multiplication at 38.5°C following infection with viral DNA was studied; the need for uncoating was thus eliminated. Mouse embryo cells were infected with either TS-a DNA or PY DNA. The infected cells were divided into two sets of cultures of which one was incubated at 38.5°C and the other at 31.5°C. At various times after infection, the cells were harvested, lysed and assayed for virus. As can be seen from Table 4, the production of TS-a virus was inhibited at 38.5°C, but not at 31.5°C. The production of virus at all temperatures was inhibited by preincubating the DNA with 10 γ of DNAse for 30 minutes at 37°C.

Since the production of infectious virus after infection with TS-a DNA was greatly inhibited at 38.5°C, uncoating is not the function responsible for the temperature sensitivity of TS-a. This experiment also confirms the previous result that adsorption is not the temperature sensitive step since whole virus particles and infectious nucleic acids are taken into the cell by different mechanisms.

D. Production of Infectious Viral DNA

An experiment was performed to see whether TS-a infected cells produced new infectious DNA when incubated at 38.5°C. Mouse embryo cells were infected with either TS-a (0.02 PFU/cell)

TABLE 4

Infection with TS-a or PY DNA

Type of DNA	Temperature of incubation	Yield per Culture after Infection (time in hours) 12	24	36	48	72	96	144
TS-a	38.5°C	<6	<6	<6	<6	<6	5.4×10^3	-
PY	38.5°C	-	-	1.4×10^6	-	2.2×10^8	-	-
TS-a + DNAse	38.5°C	-	-	<24	-	<24	-	-
PY + DNAse	38.5°C	-	-	<24	-	<24	-	-
TS-a	31.5°C	-	<6	-	4.8×10^4	3.4×10^5	1×10^6	4.0×10^7
PY	31.5°C	-	-	-	-	2.0×10^6	-	3.2×10^8
TS-a + DNAse	31.5°C	-	-	-	-	<24	-	<24
PY + DNAse	31.5°C	-	-	-	-	<24	-	<24

Mouse embryo cells were infected with either PY (8.1×10^3 DNA PFU/culture) or TS-a (1.2×10^3 DNA PFU/culture) DNA as described in Materials and Methods. After incubation at the indicated times at the two temperatures, the cells were lysed and assayed for virus by plaquing at 31.5°C. Since no special precautions were taken, the later points may contain virus from a second cycle of virus replication.

or PY virus (0.01 PFU/cell); the infected cells were divided into two equal portions of which one half was incubated at 38.5°C and the other at 31.5°C. After various periods of incubation at the two temperatures, an aliquot of the infected cells was removed to assay for whole infective virus, the remainder was extracted and assayed for infectious DNA as described in Materials and Methods.

From the results presented in Table 5, it can be seen that the production of infectious DNA is inhibited in TS-a infected cells incubated at 38.5°C compared to PY infected cells incubated at either 31.5°C or 38.5°C. The data also suggest that at 31.5°C the production of TS-a infectious DNA is somewhat inhibited compared to PY DNA, especially since twice as many cells were infected by TS-a than PY. The later finding is also consistent with results already reported that TS-a does not grow as well as PY at this temperature (Figure 6A). In the present experiment, the final yield of infectious virus per TS-a infected cell at 31.5°C was approximately 1/2 of the final yield of PY per infected cell.

The question arises as to whether the infectious DNA detected in this experiment was extracted from an intracellular pool of viral DNA or from completed virus particles. This information is important for analyzing the nature of the temperature sensitive function of TS-a. For instance, if infectious viral DNA could only be extracted from completed virus particles, failure to extract it might derive from a fault of the capsid protein. It seems likely that the infectious viral DNA extracted in these experiments did not come from complete virus particles, because the ratio of viral plaque forming units to DNA plaque forming units was 10^3 to 10^4, whereas for DNA extracted from virus particles this ratio usually is $0.5-1 \times 10^6$.

TABLE 5

Production of Infectious DNA

Virus	Temperature of incubation	Time of extraction	DNA PFU after extraction	Virus PFU before extraction
TS-a	38.5°C	7.0	< 3	3.3×10^4
		12.0	< 3	3.3×10^4
		17.5	< 3	2.7×10^4
		22.5	6	2.7×10^4
TS-a	31.5°C	14.0	< 3	3.3×10^4
		24.0	< 3	3.0×10^4
		35.0	15	4.8×10^4
		45.0	321	3.2×10^5
PY	38.5°C	7.0	< 3	1.2×10^4
		12.0	< 3	1.2×10^4
		17.5	93	6.0×10^4
		22.5	885	3.0×10^6
PY	31.5°C	14.0	< 3	1.2×10^4
		24.0	< 3	1.5×10^4
		35.0	120	1.8×10^5
		45.0	820	1.8×10^6

Mouse embryo cells infected with TS-a (0.02 PFU/cell) or PY (0.01 PFU/cell) were extracted for infectious viral DNA as described in Materials and Methods after being incubated at either 31.5°C or 38.5°C for the indicated times. Each number in column 4 represents the total amount of infectious viral DNA extracted from either 1.0×10^6 TS-a infected cells or 5.0×10^5 PY infected cells. Each number in column 5 represents the total amount of infectious virus present in the infected cells at the time of the extraction. Both the infectious viral DNA and virus were assayed by their plaque formation at 31.5°C.

E. Production of Hemagglutinin

In order to determine whether capsid protein is produced under non-permissive conditions, hemagglutination tests were performed on the crude lysates of TS-a infected mouse cells incubated at 38.5°C. From the results of these tests presented in Table 6, it can be seen that under conditions where the production of infectious TS-a infectious virus is inhibited, the production of hemagglutinin is also inhibited at 38.5°C.

Also presented in Table 6 is the specific infectivity of the different samples in terms of plaque forming units per hemagglutination unit. It will be noted that the specific infectivity of every inhibited TS-a sample is low compared to the specific infectivity of either TS-a produced at 31.5°C or PY produced at either temperature. This indicates the production of non-infectious capsids by TS-a at 38.5°C. The amount of non-infectious hemagglutin formed is greater at lower input multiplicities, at which the inhibition of the production of infectious TS-a virus is more pronounced. It has not yet been determined whether these non-infectious hemagglutinating particles contain DNA.

The results presented in Table 6 also suggest that the number of plaque forming units per hemagglutination unit increases with time during the viral growth cycle.

F. Time of the Temperature Sensitive Step as Determined by Temperature Shift Experiments

In order to try to determine at what time during the virus growth cycle the temperature sensitive step occurs experiments were

TABLE 6

Hemagglutination Properties of TS-a and PY

Experiment	Virus	Temperature	Input Multiplicity PFU/Cell	Time in Hours	Virus Yield PFU/ml	Hemagglutination Yield (HAU) Units	Specific Infectivity PFU/HAU
A[a]	TS-a	38.5°C	1.0	36	1.0×10^4	10	1.0×10^3
				48	6.6×10^4	20	3.3×10^3
				72	1.6×10^5	40	4.0×10^3
				102	3.1×10^5	80	3.9×10^3
				120	3.2×10^5	80	4.0×10^3
	TS-a	31.5°C	1.0	48	2.5×10^5	10	2.5×10^4
				72	8.1×10^6	80	1.0×10^5
				102	2.0×10^7	160	1.3×10^5
B[bc]	TS-a	38.5°C	2.7	40	1.1×10^5	80	1.4×10^3
				81	5.0×10^6	320	1.6×10^4
	TS-a	31.5°C	2.7	81	3.5×10^7	640	5.5×10^4
				160	3.5×10^8	2560	1.4×10^5
	PY	38.5°C	1.0	40	4.0×10^7	640	6.3×10^4
				81	5.0×10^8	2560	2.0×10^5
	PY	31.5°C	1.0	81	4.0×10^7	640	6.3×10^4
				160	4.8×10^8	2560	1.9×10^5
C[ac]	TS-a	38.5°C	20.0	44	3.0×10^6	160	1.9×10^4
				68	2.4×10^7	320	7.5×10^4
				92	2.3×10^7	320	7.2×10^4
	TS-a	31.5°C	20.0	65	9.2×10^7	640	1.5×10^5
				89	3.0×10^8	1280	2.3×10^5
				137	6.9×10^8	2560	2.7×10^5

TABLE 6 continued

Experiment	Virus	Temperature	Input Multiplicity PFU/Cell	Time in Hours	Virus Yield PFU/ml	Hemagglutination Yield (HAU) Units	Specific Infectivity PFU/HAU
	TS-a	31.5°C	20.0	185	7.0×10^8	2560	2.7×10^5
	PY	38.5°C	6.0	20	9.2×10^5	80	1.2×10^5
				32	9.5×10^7	320	3.0×10^5
				44	1.8×10^8	640	2.7×10^5
				68	3.0×10^8	1280	2.3×10^5
				92	2.9×10^8	1280	2.3×10^5
	PY	31.5°C	6.0	41	4.5×10^5	40	1.1×10^5
				65	7.5×10^5	320	2.3×10^5
				89	1.5×10^8	640	2.3×10^5
				137	3.0×10^8	1280	2.3×10^5
				189	3.0×10^8	1280	2.3×10^5
D[a]	TS-a	38.5°C	100.0	33	5.5×10^7	160	3.4×10^5
				45	1.4×10^8	640	2.0×10^5
				69	1.9×10^8	640	2.1×10^5
	PY	38.5°C	10.0	33	6.0×10^6	80	7.5×10^4
				45	3.4×10^7	160	2.1×10^5
				69	7.0×10^7	320	2.2×10^5

Hemagglutination tests were performed on crude lysates of TS-a or PY infected mouse cells incubated at either 31.5°C or 38.5°C and harvested at the indicated times. The specific infectivity (column 8) of the samples is given in terms of plaque forming units per hemagglutination unit; this ratio is usually about $1\text{-}2 \times 10^5$ for either TS-a virus stocks grown in 31.5°C or PY stocks grown at either the high or low temperature. A decreased ratio indicates the production of non-infectious hemagglutinating particles. a-Mouse embryo cells b-Mouse kidney cells c-RDE was incorporated into the growth medium

performed in which infected mouse embryo cells were transferred either from low to high temperature or from high to low temperature at different times after infection; after the shift, the cells were incubated either for 96 hours at 38.5°C or for 192 hours at 31.5°C, depending on the direction of the shift,and then were lysed and assayed for infectious virus. In order to avoid the complication of multiple virus growth cycles, high input multiplicities and RDE treatment were used as already described. The growth curves obtained in these experiments, but without temperature shifts, have already been presented in Figure 6. The results of the shift experiments are presented in Figure 7.

In the interpretation of the shift from low to high temperature, or "shift up" experiment, (Figure 7A), it is assumed that all the virus particles whose precursors have passed the temperature sensitive step before the shift will be infectious. Thus, the time at which the virus yield starts to become resistant to the shift at 38.5°C is the time at which the temperature sensitive step begins to be completed at 31.5°C, and the time at which the virus yield is completely insensitive to the up shift, is the time at which the temperature sensitive step is completely finished. From Figure 7A, it can be seen that the virus yield starts to become resistant to the up shift between 15 and 23 hours after infection and is completely resistant at 89 hours. From the growth curve of TS-a at 31.5°C (Figure 6B) it can be seen that at 89 hours about 40% of the final virus yield has been completed.

The temperature shift from high to low temperature, or "shift down" experiment, (Figure 7B) is much harder to interpret than the "shift up" experiment, because of possible complications. If the block to the production of infectious virus at the high temperature

Figure 7

Temperature Shifts

The infection procedure and growth conditions of these experiments have already been described in Figure 6. Either TS-a or PY infected mouse embryo cells were shifted from either 31.5°C to 38.5°C (Figure 7A "Shift Up") or from 38.5°C to 31.5°C (Figure 7B "Shift Down") at different times during the viral growth cycle. The transferred cells were then kept at either 31.5°C for 192 hours or 38.5°C for 96 hours, depending on the direction of the shift, before being broken open and assayed for infectious virus by plaquing at 31.5°C. The growth curves at the two temperatures, which indicate the amount of virus produced before the infected cells were shifted, have already been presented in Figure 6.

A-("Shift UP"). This graph is plotted as the time spent at 31.5°C before being shifted to 38.5°C, versus the final average yield of infectious virus per infected cell; the final average yield of infectious virus per infected cell includes virus produced both during the indicated time spent at 31.5°C and the 96 hours spent at 38.5°C.

B-("Shift Down"). This graph is plotted as the time spent at 38.5°C before being shifted to 31.5°C, versus the final average yield of infectious virus per infected cell; the final average yield of infectious virus per infected cell includes virus produced both during the indicated time at 38.5°C and the 192 hours spent at 31.5°C.

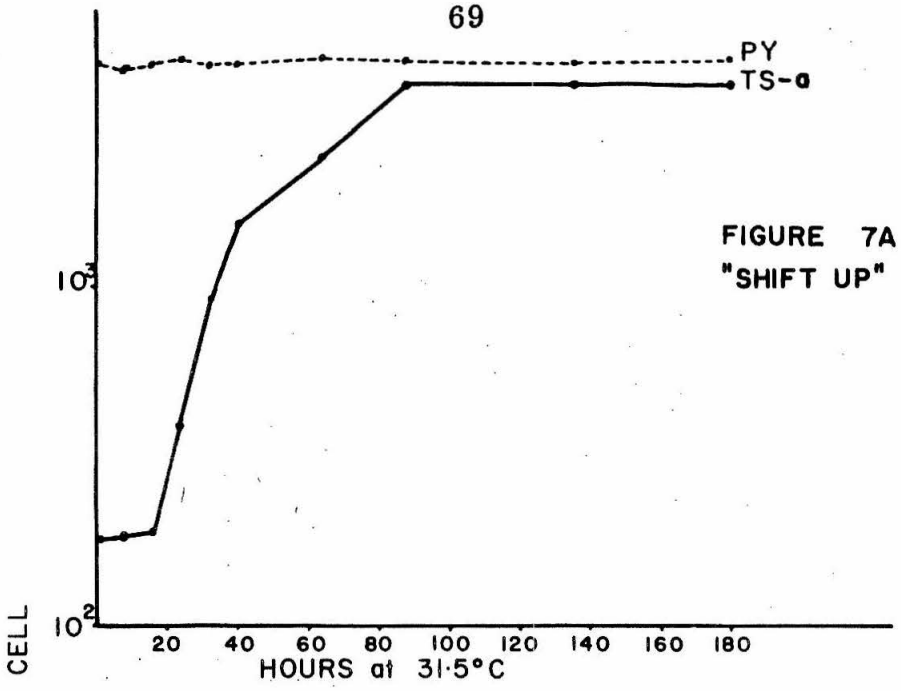


FIGURE 7A
"SHIFT UP"

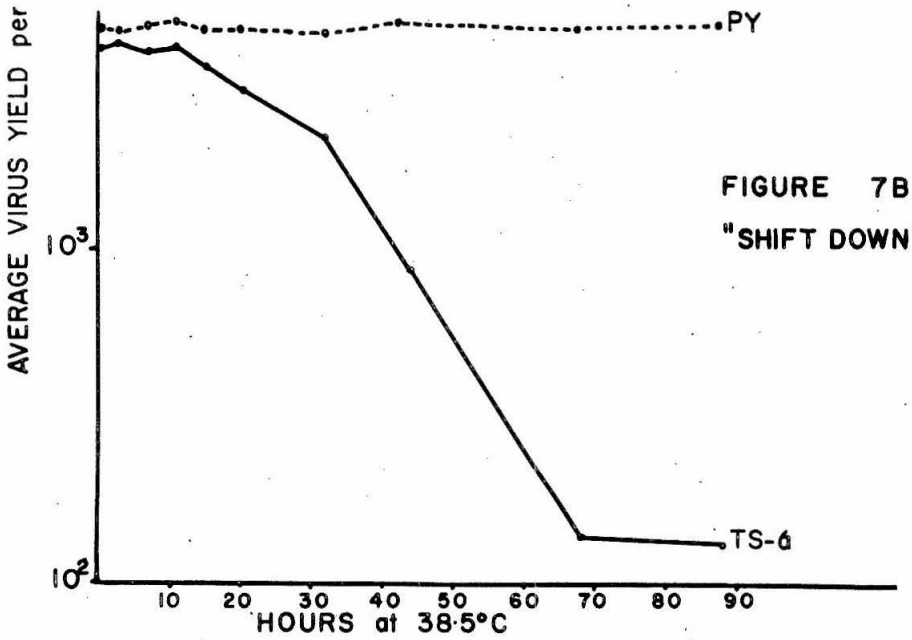


FIGURE 7B
"SHIFT DOWN"

is irreversible after the temperature is lowered, and therefore no complicating circumstances, the time of the down shift at which the virus yield starts declining would be the beginning of the temperature sensitive step; the time of the down shift at which the virus yield is maximally inhibited would represent the end of the temperature sensitive step. This conclusion may, however, be invalid when the multiplicity of infection is high as in this experiment; the time of the temperature sensitive step may be overestimated if, within the same cells, different viral functional units, deriving from the input virus, can act in succession. Under conditions of the "shift down" experiment late acting units may still be functional after the shift and may support viral multiplication although in normal infections their contribution may be unimportant.

The results of the "shift down" experiment would also be difficult to interpret if the block to the production of infectious virus is reversible upon transfer to 31.5°C and, furthermore, the time or amount of virus made in an infected cell is governed by a second later process. Then the time at which the virus yield first begins to become sensitive to the down shift corresponds not to the time of the temperature sensitive step, but to that of the second limiting process; and the time of the occurrence of the temperature sensitive step is again overestimated.

The results of the "shift down" experiment reported in Figure 7B show that the virus yield begins to become sensitive to the shift at about 20 hours after incubation and is completely sensitive at 68 hours. Owing to the reservations outlined above, no attempt will be made to interpret these results.

G. Conclusions

The results presented in this section allow the following conclusions to be made concerning the nature of the temperature sensitive function of TS-a

1. The intracellular temperature sensitive function of TS-a occurs after uncoating of the input virus particles and affects both the production of infectious viral DNA and capsid formation at 38.5°C.

2. An excess of noninfectious hemagglutinating particles are produced in TS-a infected mouse cells incubated at 38.5°C; this amount varies with the input multiplicity.

3. The temperature sensitive function begins to be completed about half way through the 31.5°C eclipse period.

PART IV. TRANSFORMING PROPERTIES OF TS-a

Since TS-a was found to be temperature sensitive in the production of new infectious virus in its cytolytic interaction, it was of interest to see if it also was temperature sensitive in its transforming ability.

A. Transformation In Vitro

Hamster cells that were exposed to five different concentrations of TS-a (1000, 100, 10, 1 and 0 PFU/cell) were plated in agar and incubated under the different temperatures as described in Materials and Methods. The amount of transformation at these different virus concentrations and incubating conditions are presented in Table 7. Each number in Table 7 represents the total number of transformed colonies on 15 replicate dishes.

1) High Temperature and "Shift Up" Incubation

The data are reported in the second and third columns of Table 7. In dishes in which uninfected cells were plated, a background of 1 colony per dish was found by macroscopic examination. In order to determine what proportion of the colonies arising from the platings of the infected cells were transformed all the colonies were picked from the agar in the cases where there was less than 100 colonies per 15 replicate dishes; the colonies were tested for their transformed morphology by plating in fluid medium at 38.5^{o}C as described in Materials and Methods. In the instances where there were more than 100 colonies per 15 replicate dishes, a representative number (60 to 100) of colonies were replated in fluid

TABLE 7

Transformation In Vitro by TS-a

Input Multiplicity PFU Per Cell	Total Number of Transformed Colonies Per 15 Replicate Dishes			
	High Temperature[a] Incubation 16-18 days at 38.5°C.	"Shift Up"[a] Incubation 4 days at 31.5°C. then 16-18 days at 38.5°C.	"Shift Down"[a] Incubation 2 days at 38.5°C. then at 31.5°C.	Low[b] Temperature 32-36 days at 31.5°C.
1000	5	504	3	415
100	1	296	0	225
10	0	69	0	56.7
1	0	7	0	7.5
0	0	0	0	0

a - All the transformed colonies were determined by replating individual colonies under fluid medium as explained in the text.

b - All the numbers of transformed colonies were obtained by plating out the whole top agar of 6-8 plates of each set under fluid medium as explained in the text; the numbers are normalized to 15 dishes.

medium and could be classified as either normal or transformed. In a few cases, colonies were observed which could not be classified. It is not known whether these are normal cell mutants selected by the agar or transformed cells with an unusual morphology.

The results clearly show that the transforming ability of TS-a is inhibited under the conditions of the high temperature incubation when compared to those of the "shift up" incubation. In other experiments, it was found that the transforming ability of PY is similar under the conditions of both the high temperature and "shift up" incubations; the difference in the number of transformed colonies formed under the conditions of the two incubations was less than a factor of two.

2) "Shift down" Incubation

The data are reported in the fourth column of Table 7. Under this incubation condition, a background of three colonies per dish was observed by macroscopic examination of the dishes of uninfected cells. All the colonies arising on all the dishes were picked from the agar and replated in fluid medium at 31.5°C. Only about 35% of these replated colonies could be classified as either normal or transformed. Some of the colonies did not stick to the dish on replating. In some of the colonies that stuck, the cells did not migrate out of the colony; in others the cells migrated but did not divide. Nonclassifiable colonies were observed in approximately the same frequency from the uninfected and infected sets of dishes. Therefore, it is most likely that, for the most part, these non-classifiable colonies were derived from normal cells. But since this was not shown to be the case, the results presented in Table 7 can only be taken as a lower limit of the transformation under the

"shift up" incubating conditions. If one assumes that transformation among the nonclassifiable colonies is in the same proportion as it is among the classifiable colonies, or even if all the nonclassifiable colonies are transformed, it is still found that transformation is greatly inhibited compared with the amounts of transformation obtained in cells kept at the low temperature of "shift up" incubations.

3) Low Temperature Incubation

The data are reported in the fifth column of Table 7. By macroscopic examination, there were about 60 to 80 normal colonies per dish of cells kept at the low temperature incubation. This high background hampered any accurate count of the transformed colonies. An estimate of the general number of transformed colonies was made by plating out the whole top agar from 6 to 8 dishes of each virus concentration in fluid medium at 31.5°C as described in Materials and Methods.

Since it is not known to what effect the errors involved in plating out the whole top agar affected the true number of the transformed colonies, the results presented in Table 7 for the low temperature incubation can only be taken as approximate.

The validity of these data, however, are shown by the presence of transformed colonies when the cells were infected by one PFU per cell: since at this input multiplicity not more than one transformed colony was recognized per original dish, the number cannot be an overestimate.

Since the numbers obtained at the various input multiplicities show the same general relationship to each other as those obtained in the "shift up" experiment, it seems that the dose response to the

virus is similar in the two cases. However, the considerable similarity between the absolute numbers shown by Table 7 may be spurious.

B. Transformation in Vivo

In a preliminary experiment to test the tumour-producing ability of the mutant, five three-day old hamsters were inoculated subcutaneously with 10^6 PFU of TS-a. All five animals developed subcutaneous fibrosarcomas about the site of inoculation 40 to 90 days after inoculation. No tumours were found associated with the internal areas of the body. A hemagglutination inhibition titer[83] of 1280 was detected in the sera of one of the tumour-bearing animals (the only animal so tested) 90 days after inoculation. Usually the wild type (PY) when inoculated under similar conditions causes angiomatous tumours of the liver and lungs and tumours of the internal organs predominantly in the kidneys and heart.

In order to test whether the tumours induced by TS-a were truly restricted to the subcutaneous site, possibly due to the lower temperature in this area, the experiment was repeated on a larger scale, using different routes of inoculation. One to three-day old hamsters were inoculated either subcutaneously or interperitoneally with either 9×10^6 PFU of TS-a or 2.7×10^7 PFU of PY and observed for 70 days.

The results of this experiment are presented in Table 8. All the animals inoculated with PY, either through the SQ or IP route, died from the rupture of angiomatous tumours of the liver or lungs between 12 to 40 days after infection. Very few of the TS-a inoculated animals died as a direct effect of the virus but were sacrificed when

TABLE 8

Production of Tumours in Hamsters

Virus	Inoculation Route	Total Number of Animals Inoculated	Number of Animals With: No Tumours	Subcutaneous Tumours	Internal Tumours
TS-a	SQ	19	0	19	3
TS-a	IP	15	5	8	5
PY	SQ	16	0	1	16
PY	IP	13	0	2	13

The total number of tumours at the different sites for each type of inoculation adds up to more animals than were inoculated since some animals had tumours at more than one site.

their tumours became too burdensome or at the end of the experiment. Except for some PY-infected animals that were lost previous to the 16th day after inoculation, all had, in addition to the angiomatous tumours, macroscopic tumours of the internal organs mainly in the heart and kidneys. Subcutaneous tumours were only detected in those PY animals which survived for the longest time. On the contrary, only three (two SQ and one IP inoculated) TS-a infected animals had any indication of angiomatous tumours of the liver at the time of death. Only one TS-a infected animal had an internal tumour associated with an internal organ (heart). All the other internal tumours were either free or found to be loosely connected to the internal body wall.

The results presented here clearly indicate that there is an inhibition in hamsters infected with TS-a, either by a SQ or IP route, of both the angiomatous tumours of the liver and lungs and the production of tumours of the other internal organs that are found in the early stages of PY infection.

C. Conclusions

The study of the transforming ability of TS-a *in vitro* and *in vivo* lead to the following conclusions:

1) TS-a is temperature sensitive in its transforming ability at 38.5°C.

2) Cells transformed by TS-a at 31.5°C can retain their transformed characteristics upon cultivation at 38.5°C.

3) The inhibition of the transforming ability of TS-a cannot be reversed, at least for the most part, by transferring the infected cells to 31.5°C after they have been incubated for 2 days at 38.5°C.

4) TS-a is inhibited in its ability to form tumours of the internal organs of newborn hamsters.

DISCUSSION

A. The Cytolytic Virus Cell Interaction with the TS-a Mutant

The results presented here show that the inhibition of the production of infectious virus particles by TS-a infected mouse cells, at 38.5°C, is due to the temperature sensitivity of some intracellular process during the viral growth cycle as opposed to the heat lability of a constituent of the mature virus particle produced at 31.5°C.

Concerning the consequence of the temperature sensitive function the most direct result of the reported experiments, is that the formation of infectious viral DNA is inhibited in TS-a infected cells incubated at 38.5°C. The exact nature of the function is not known; a number of processes important for the production of infectious viral DNA could be temperature sensitive. The following processes could be involved: 1) the ability of the input DNA to reach or attach to the intracellular site where it synthesizes new viral DNA molecules; 2) the act of synthesis of new viral DNA molecules; this could be inhibited owing to the temperature sensitivity of the production or activity of a DNA synthesizing enzyme specified by a viral gene; 3) the process needed to confer biological activity to these molecules, for example, by means of methylation or ring closure.

The notion that the temperature sensitive step is involved in the synthesis of infectious viral DNA is also strengthened by its timing. Gershon and Sachs[33] detected the first increase in the production of infectious polyoma DNA in infected mouse embryo cells, incubated at 37°C, at approximately half way through the

eclipse period. The temperature sensitive step, as measured by the shift up experiment (Figure 7A), also begins to become completed at about half way the length of the eclipse period at 31.5°C. It seems that relative timing in terms of fractions of the eclipse period are meaningful for comparing the viral growth cycle at different temperatures, because the multiplication of the viral DNA follows a similar time course at 31.5°C and 38.5°C (Table 5), if referred to such a relative timing. On this basis the time of the temperature sensitive step as measured by the shift up experiment, would correspond to the time of synthesis of infectious viral DNA as determined by Gershon and Sachs[33]. The result of the shift down experiment, on the contrary, (Figure 7B) indicates that the temperature sensitive step occurs at about the end of the eclipse period; it is likely, however, that this is an overestimate of the time of the step, owing to one of the reasons suggested in section F of Part III.

So far the effects of the TS-a mutation on the synthesis of the infectious viral DNA have been considered. The synthesis of competent viral DNA, ie., able to be transcribed into messenger RNA, appears also to be inhibited at 38.5°C, since in cells infected by the mutant at low multiplicity there is little synthesis of viral protein. It is interesting, however, that under these conditions the ratio infectious to hemagglutinating titer of the viral progeny is smaller than normal, indicating the formation of non-infectious virus particles. These findings can be explained as the consequence of the inhibition of the multiplication of viral DNA competent as a template for the production of messenger RNA of the capsid protein, on the basis of two assumptions: 1. the amount of capsid protein ultimately made in a cell depends on the number of biologically

competent viral DNA molecules; 2. the capsid subunits once formed can assemble into the viral protein coat with or without a viral DNA core. The hypothesis is made that a high yield of capsids is formed either if the biologically competent viral DNA is replicated, as in normal polyoma virus infection, or, in the absence of DNA replication, if the cell is supplied by infection with a very large number of biologically competent viral DNA molecules. This hypothesis finds support in two recent known results: 1. Sheinin[34] observed the production of large amounts of non DNA containing capsids after the infection of mouse embryo cells with very large amounts of polyoma virus (1000 PFU/cell) in the presence of FUDR, which is a potent inhibitor of DNA synthesis. 2. Weigle[92] observed that the late protein produced in cells infected by bacteriophage λ in the absence of DNA synthesis is more abundant after infection at high than at low multiplicities.

Under this hypothesis the production of capsids in cells infected by TS-a at 38.5°C at low multiplicity is specified by molecules of biologically competent viral DNA introduced into the cells by the inoculum. The non-infectious capsids produced under these conditions have not yet been analysed to see whether they contain viral DNA. If they don't, it is likely that the production of any type of TS-a DNA is inhibited at 38.5°C.

These considerations do not apply to cells infected by TS-a at high multiplicity of infection, where a characteristic of the mutant, not discussed so far, becomes prominent. This characteristic is the leaky nature of the mutation.

Leakiness is suggested by a strong multiplicity effect on the yield of TS-a at 38.5°C where the ratio: yield of infectious TS-a to yield of PY increases from 10^{-3} to almost unity when the input

multiplicity varies from 0.1 to 100 PFU/cell. This phenomenon could be explained by two different hypotheses.

The first hypothesis is based on four assumptions: 1. the function of the mutated gene is needed for the synthesis of a biologically active viral DNA; 2. the function itself is quantitatively dependent on the number of biologically active DNA molecules present; 3. the maximum amount of virus produced per cell is governed by some other factor which is multiplicity independent; 4. the expression of the function at 38.5°C is quite efficient for molecules of PY DNA, but very inefficient for molecules of TS-a DNA.

Thus, in a PY infected cell at 38.5°C the function and the production of biologically active viral DNA continue until the maximum amount of virus that can be produced per cell is made. Infection of a cell with one or many virus particles will result in the same maximum amount of virus, although the rate at which the function is performed may be faster in the cells infected with many virus particles. In TS-a infected cells incubated at 38.5°C the function is temperature sensitive and is performed with a very low efficiency so that few biologically active DNA molecules are produced under conditions of single infection, before all viral synthesis ceases. Increasing the input multiplicity affords the cell more biologically active viral DNA molecules and thus relatively more function before viral synthesis stops. Finally, at 100 PFU/cell a full yield of infectious TS-a is produced at 38.5°C before viral synthesis is shut off.

The second hypothesis is that at 38.5°C every particle of TS-a virus has a constant, but small probability to cause a normal virus cell interaction with the production of a normal yield of infectious

mutant virus; the other TS-a virus cell interactions produce little or no virus. As the input multiplicity is increased, more and more cells undergo the rare event until finally at 100 PFU/cell every susceptible cell would do so and a normal yield of TS-a would be produced.

An analysis of the distribution of virus from single cells at different input multiplicities would differentiate between these two hypotheses. Under the first hypothesis all the infected cells would give low yields of virus; the yield per cell would increase as the input multiplicity increases. Under the second hypothesis all the cells producing virus would give maximual yields and the number of virus yielding cells would increase with increased input multiplicities.

The leakiness of the TS-a mutation affects the previous discussion of the synthesis of TS-a capsid protein: it explains why a large excess of non-infectious capsids is not produced at high multiplicities. If there were no leakage, a full yield of hemagglutinin, all non-infectious, would be produced at high multiplicities; the large number of biologically competent DNA molecules introduced into the cell by the innoculum would allow synthesis of capsid protein at a high rate. Owing to leakage, however, a full yield of infectious TS-a virus is produced under these conditions. At low multiplicities competent viral DNA molecules are still brought into the cells by the innoculum, although in smaller number. Since leakage is multiplicity dependent, it is minimal at these multiplicities; little infectious DNA is made and a noticeable excess of non-infectious hemagglutinin can be produced. Under these conditions of low multiplicity there are not enough competent viral DNA molecules afforded the cell by the input to

produce a full yield of capsid protein; the total amount of hemagglutinin is inhibited.

B. Transformation by the TS-a Mutant

The results presented here show that TS-a is also temperature sensitive in its transforming ability. It appears that after completion of the temperature-sensitive step at one temperature (either 31.5°C or 38.5°C) the effect on the transformation produced, at least for the most part, cannot be reversed by then shifting the infected cells to the other temperature. The temperature-sensitive gene involved in TS-a is needed for the initiation of the transformed state, but not necessarily for the maintenance of this state. This is true since the cells transformed at 31.5°C retain their transformed characteristics upon cultivation at 38.5°C.

Because of technical difficulties involved in determining the transformation rate at the low temperature, the exact degree of inhibition of transformation caused by the temperature-sensitive gene at 38.5°C is not known. At 1000 PFU/cell there is a 100-fold decrease in the production of transformed colonies in infected cells kept at the high temperature for the whole incubation period, compared to cells exposed to the "shift up" incubation. Since in the "shift up" incubation the infected cells were kept at 31.5°C for only four days, the inhibition factor created by high temperature at 1000 PFU/cell may even be greater than 100-fold. It is not known whether the transformation produced at 38.5°C is due to leakage, revertants or is a consequence of just the initial virus input.

It is interesting to remark that the transformation caused by this mutant in the "shift up" incubation has similar quantitative

characteristics to that caused by a non-temperature sensitive strain of polyoma virus at 37°C[44, 45]: it has a similar efficiency of transformation and dose response curve.

TS-a also appears to be temperature sensitive in the animal. One could postulate that transformation more readily takes place at the subcutaneous site because of the lower temperature of this area compared with the internal organs. The increased resistance to the neoplastic effect of the virus when inoculated by the IP route (30% of the animals inoculated by this route had no detectable tumours) might be due to the slower growth of the virus at the higher internal temperature, allowing the immunological mechanisms of the animal to fight off the oncogenic response.

It is most reasonable to assume that the same viral function is affected by high temperature in TS-a infected mouse and hamster cells. This similarity has a practical consequence for a problem which is usually not adequately answered; whether unquestionably the transformation by polyoma virus is caused by the same agent that causes the cytocidal effect. This question arises since the efficiency of transformation of hamster cells in terms of virus PFU is very low (on the average it takes about 10^4 PFU to induce one transformation event). The fact that the same mutant confers temperature sensitivity to both effects shows that they are caused by the same agent.

C. Relevance of the Results of this Work to the Hypotheses of Viral Carcinogenesis

The correlation of the failure to synthesize biologically active viral DNA and the failure to cause transformation by the TS-a mutant under nonpermissive conditions does not discriminate

between the two hypotheses of viral carcinogenesis presented in the introduction. Under the "persistence of viral genetic information" hypothesis the synthesis of biologically active DNA would be useful for transformation, since the chance of an integration event would increase with the number of active DNA molecules present in the cell. Once integrated the viral DNA would be under the control of host mechanisms and would no longer depend on the temperature sensitive function of TS-a. In this respect the TS-a transformed cells resemble the E. Coli cells carrying the temperature sensitive sex factor; this sex factor is sensitive to high temperature when replicating autonomously, but insensitive when incorporated in the host chromosome[86].

Under the "transient action of the virus" hypothesis the stability at 38.5°C of cells transformed by TS-a at 31.5°C is explainable since the agent causing the transformation is no longer needed after the transformation event has occurred.

D. Conclusion

This thesis is primarily an introduction into the study of temperature sensitive mutants of polyoma virus. The temperature sensitive function of the mutant investigated here was found to be important both for the production of biologically active DNA in the cytolytic virus cell interaction and for the initiation, but not the maintenance of the polyoma induced transformed state. The isolation and analysis of other temperature sensitive mutants of this virus may be very helpful for identifying the viral functions concerned with the process of neoplastic transformation, and may thus provide a new approach for increasing our knowledge of the mechanisms of viral mediated carcinogenesis.

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