Journal of Supramolecular Structure 12:127–137 (1979)
Tumor Cell Surfaces and Malignancy 493–503

Polyoma T (Tumor) Antigen Species in Abortively and Stably Transformed Cells

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Stable neoplastic transformation of cells by polyoma virus requires the participation of two viral genes, designated *ts-a* and *hr-t*. The effects of mutations in these two genes on the patterns of T-antigen synthesis during productive infection have been previously described: ts-a mutants are affected in the "large" (100K) nuclear T antigen, and hr-t mutants are affected in the "middle" (36K, 56K, 63K) and "small" (22K) T antigens. The latter are associated predominantly with the plasma membrane (56K) and cytosol fractions, respectively.

Here we examine the expression of the various forms of polyoma T antigen in nonproductive infection (abortive transformation) as well as in stably transformed cell lines of different species. The results on abortive transformation are essentially the same as those described above for productive infection. In stably transformed cells, the middle and small T antigens are seen to various extents. The large T antigen, however, is often absent or present below the level of detection. Clones lacking the large T antigen are found most often among mouse transformants, but are also seen among rat transformants. Retention of the 100K species in transformed cells therefore appears to be, at least in part, an inverse function of the level of permissivity of the host toward productive viral infection. These findings indicate that the induction of the transformed phenotype in both abortively and stably transformed cells generally does not require the large T antigen, but rather the products of the hr-t gene.

Key words: **polyoma virus; large, middle, and small tumor antigens; ts-a; hr-t; abortive transformation; transformed cells.**

Mouse cells undergo predominantly a productive infection by polyoma virus, although occasionally they may become transformed. The reverse is true for rat and hamster cells, which undergo predominantly a nonproductive infection. The latter kind of infection leads first to an abortive transformation in which up to 50% of the cells become transformed within 24–48 h after infection. The majority of these altered cells revert to a normal phenotype after a few cell divisions [1]. A few percent, however, will remain stably transformed. Stable transformants contain integrated viral DNA and express virus-

Received June 26, 1979; accepted June 26, 1979.

0091-7419/79/1201-0127$02.30

specific proteins called T, or tumor, antigens [2]. Although direct evidence is lacking, it is plausible that a failure of stable integration of viral DNA in abortive transformants leads to a loss of the transformed phenotype.

The hr-t and ts-a mutants define two viral gene functions essential for stable transformation [3,4]. While neither type of mutant can stably transform cells, ts-a mutants are capable of abortively transforming cells at the nonpermissive temperature [5,6]. Various cellular parameters of transformation have been used to monitor abortive transformation, such as growth in soft agar, morphologic changes, lectin agglutinability, and loss of stress fibers [3]; hr-t mutants fail to induce these changes [6,7–9], while ts-a mutants induce them in a manner essentially identical to the abortive transformation phase of a wild-type viral infection [5,6,8,10]. These results show a requirement for the hr-t gene function in the induction of abortive transformation. They also suggest a persisting role for these proteins in maintaining the stably transformed state.

The present study describes the T-antigen species induced by wild-type and mutant strains of polyoma virus during abortive transformation, and shows them to be the same as previously described for productive infection. A survey of stably transformed cell lines shows that the hr-t products continue to be expressed, while the 100K product of the ts-a gene may or may not persist, depending in part on the permissiveness of the host for viral DNA replication.

MATERIALS AND METHODS

Cell Lines and Viruses

A derivative of the Pasadena small-plaque strain of polyoma virus was used to transform cells from various established lines of mouse or rat origin. Py-3T3-6 was derived from Swiss mouse 3T3 cells [11]; five polyoma transformants and four SV-40 transformants were obtained from Balb-3T3 clone A-31 cells. Rat cells of the NRK, BN, and F-111 cell lines were transformed by polyoma virus. All transformants were either directly isolated or eventually cloned in soft agar.

De novo infections of rat F-111 cells were carried out with different virus strains at multiplicities of 5–50 plaque-forming units (PFU) per cell to study T-antigen species made during the period of abortive transformation. Wild-type polyoma, hr-t mutants NG-18 and NG-59 [11], and ts-a mutants ts-616 and ts-25D [12,13] were used.

Labeling and Immunoprecipitation

Polyoma anti-T serum was obtained as ascites fluid of brown Norwegian rats inoculated with a syngeneic polyoma virus-transformed cell adapted to grow as a transplantable ascites tumor. Procedures for labeling of cells, extraction, T-antigen immunoprecipitation, and analysis by gel electrophoresis have all been described [14,15]. Briefly, exponential cells were grown in Dulbecco's modified Eagle's medium plus 5% calf serum, and labeled in methionine-free medium with [^{35}S] methionine (20–100 μCi/ml, 400 Ci/mmole) for 2–4 h at 37°C, or as indicated in the figure legends. Labeled cells were washed and extracted with a pH 9 buffer containing NP-40. The clarified extracts were incubated with 5–10 μl of anti-T serum and the immune precipitates collected on Staphylococcus protein A-Sepharose beads. Washed immunoprecipitates were dissociated and electrophoresed on 12.5% acrylamide gels in the presence of sodium dodecyl sulfate (SDS).

TABLE I. Test for the Induction of Abortive and Stable Transformation by Transformation-Defective Mutants of Polyoma Virus*

	Abortive transformation[a]		Stable transformation[b]		T antigen[c]	
Virus 5–10 PFU/cell	33°C	39.5°C	33°C	39.5°C	33°C	39.5°C
Polyoma, wild-type	20	23	120	152	80	80
Polyoma TS-616	17	29	25	0	80	3
Polyoma NG-18	0.3	0.3	0	0	80	80
Control F-111 cells	0.3	0.2	0	0	--	--

*Data from Fluck M, Benjamin T [6].
[a]Percentage of population undergoing more than one division, scored after 10 days at 33°C or 5 days at 39.5°C.
[b]Macroscopic clones per 10^4 infected cells, scored after 30 days at 33°C or 18 days at 39.5°C.
[c]Percentage of population with nuclear immunofluorescence.

RESULTS

Abortive Transformation

Table I shows results of tests for abortive and stable transformation of F-111 rat cells by wild-type polyoma virus, hr-t mutant NG-18, or a ts-a class mutant, ts-616 [6]. The mutant ts-616 induces abortive transformation in a temperature-independent way, as shown by the early proliferation of cells in soft agar at the high as well as at the low temperature. Similar results for ts-a were reported earlier by Stoker and Dulbecco using BHK (hamster) fibroblasts [5]. When the same F-111 cultures are scored later for macroscopic clones corresponding to stable transformants, the temperature-sensitivity of the ts-616 infected cells is evident. Thus, the ts-a function is not required to *induce* anchorage-independent growth, but rather to *secure* that property in a stably inherited fashion. A failure of stable integration of the viral DNA in cells abortively transformed by ts-a mutants has been suggested as the basis for these findings [6]. In the same experiment, hr-t mutant NG-18 is unable to induce even transient growth in soft agar, showing that the hr-t function is required for anchorage-independent growth [6,7].

T-Antigen Expression During Abortive Transformation Compared With Productive Infection Infection

Sera from rats or hamsters carrying tumors derived from syngeneic polyoma-transformed cells have been used to immunoprecipitate T antigens from cells productively infected by wild-type polyoma virus and various mutants. Labeling of infected cells with ^{35}S methionine, followed by immune precipitation and SDS-polyacrylamide gel electrophoresis, has led to the identification of a large T antigen (100K), a small T antigen (22K), and one or more middle-sized antigens (36K–63K) [14–17]. Work from several laboratories has shown that the 100K T antigen, localized predominantly in the nucleus, is thermolabile in pulse-chase experiments in ts-a mutant-infected cells [15,16]. The hr-t mutants of the deletion type, such as NG-18, make normal amounts of the 100K species, but fail to make any of the middle or small T antigens [14]. The hr-t mutants of the nondeletion type, such as NG-59, again induce normal 100K, but also induce a 56K

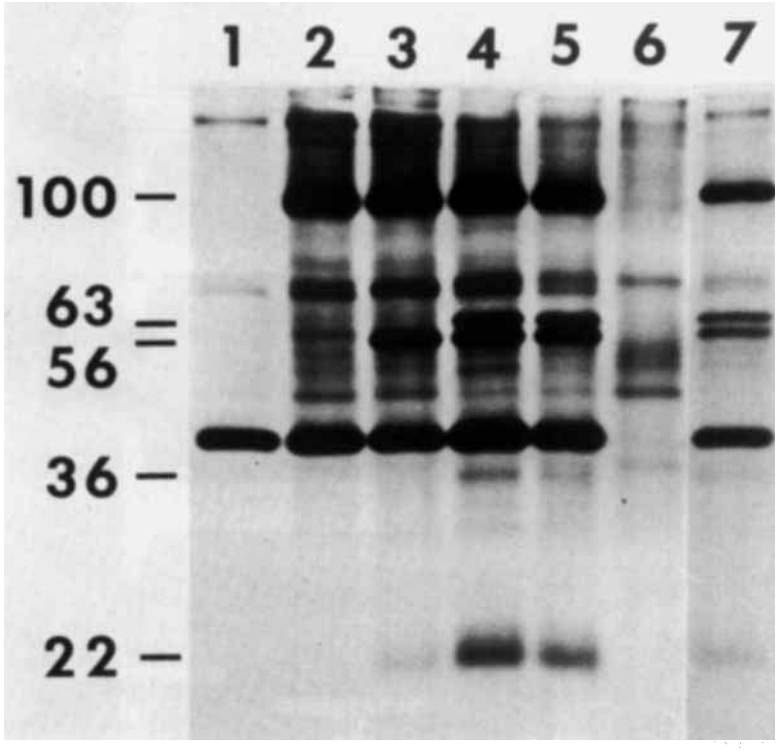

Fig. 1. De novo infection of F-111 rat cells. Confluent F-111 were infected at a multiplicity of infection of 5–20 PFU per cell. Cells at 32°C were pulse-labeled at 44 h after infection for 45 min in Hanks's salts containing 100 μCi/ml[^{35}S]methionine. Cells at 39°C were pulse-labeled at 30 h after infection for 20 min in the same medium. Cells were extracted and T antigens collected as described in Materials and Methods, using either preimmune serum or anti-T ascites. 1, Control cell extracts, 32°C, precipitated with anti-T ascites; 2, NG18-infected cells, 32°C, anti-T ascites; 3, NG-59-infected cells, 32°C, anti-T ascites; 4, ts-25D-infected cells 32°C, anti-T ascites; 5, wild-type-infected cells, 32°C, anti-T ascites; 6, wild-type-infected cells, 32°C, preimmune serum; 7, ts-25D-infected cells, immune serum, 39°C.

middle T species and a 22K small T species, the latter in very reduced amounts [15]. Both NG-18 and NG-59 fail to show two other middle-sized T-antigen species (63K and 36K) made by wild-type virus and ts-a mutants [14,15]. From peptide mapping data, it appears that the hr-t region of the polyoma DNA codes for the C-terminal portion of the small T antigen, and that the same sequences also code for part of the 56K middle T species [18,19]. Cell fractionation experiments have shown the 56K species to be associated with the plasma membrane, and the 22K to be largely in the cytosol fraction [15,17].

To examine the T-antigen patterns in a nonproductive infection, F-111 rat cells were infected by NG-18, NG-59, ts-25D, or wild-type virus. The results of immunoprecipitation followed by gel electrophoresis and autoradiography are shown in Figure 1. Lanes 1–6 are from cells incubated and labeled at 33°C. The results are strictly parallel to those reported earlier for lytic infection: NG-18 (lane 2) induces only 100K; NG-59 (lane 3) induces 100K, a 56K species, and reduced amounts of a 22K species (seen here by overexposing the film); ts-25D (lane 4) and wild-type virus (lane 5) induce all five species – 100K, 63K, 56K, 36K, and 22K. Since ts-25D, like ts-616, abortively transforms F-111 cells at 39.5°C, its T-antigen pattern was also examined at the high temperature; as seen in lane 7, all five T-antigen

species are seen. In pulse-chase experiments, the 100K band of ts-25D proved to be thermolabile, as previously shown for lytic infection [15]. The patterns with wild-type virus and hr-t mutants are independent of the temperature. These results show that under conditions of abortive transformation, hr-t and ts-a mutants induce the same T-antigen species as in lytic infection. Thus, anchorage-independent growth occurs only in cell populations expressing hr-t gene products, ie, middle and small T antigens; a normal large T antigen is neither sufficient nor necessary for abortive transformation.

Patterns of T Antigen in Stably Transformed Rat Cells

A series of wild-type polyoma virus-transformed rat cells, isolated as macroscopic clones in soft agar, were examined for their T antigens. Figure 2A shows results with five

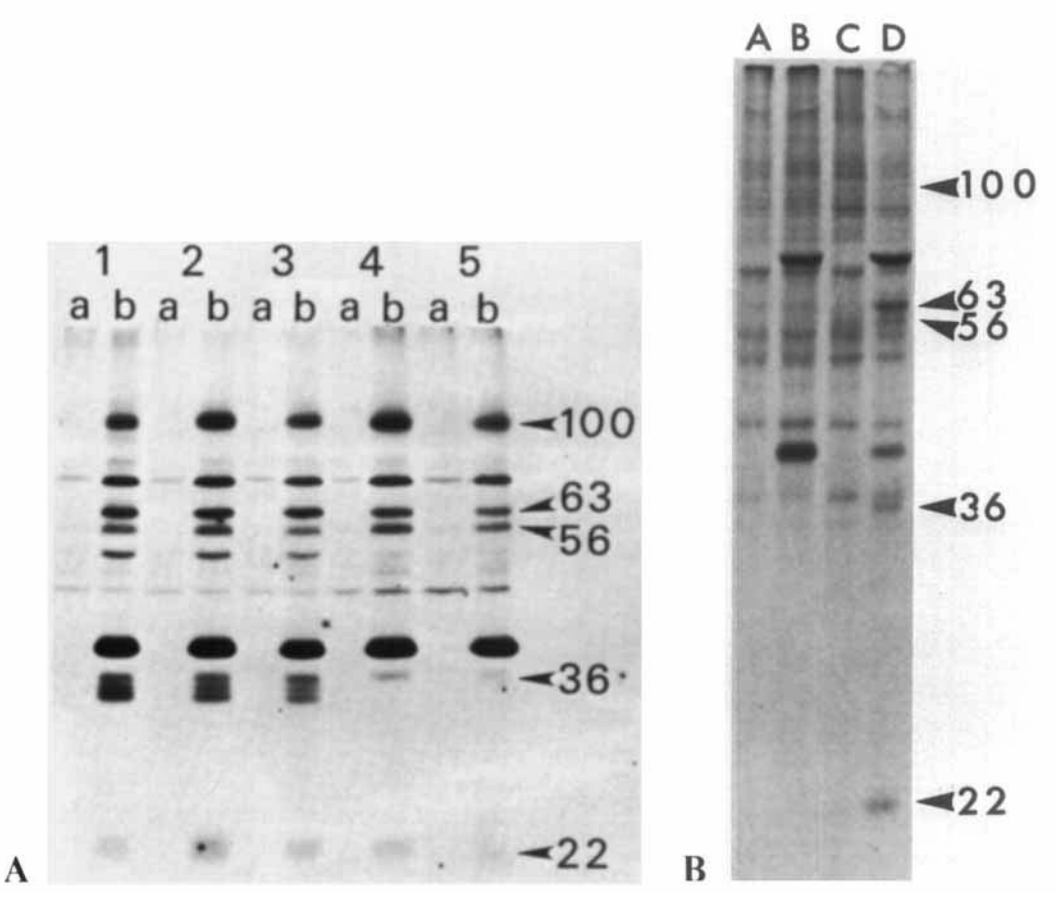

Fig. 2. A: T-antigen patterns of polyoma-transformed rat cells. Polyoma-transformed rat cells were pulse labeled during exponential growth for 1h in phosphate-buffered saline (PBS) containing 20 μCi/ml [^{35}S] methionine. The T antigens were extracted, precipitated, and electrophoresed as described in Materials and Methods. 1, PyB4; 2, PyB4TA; 3, PyB4T; 4, PyF; 5, PyNRK-Cl 1. a, Preimmune serum; b, anti-T ascites. B: Patterns of T antigens in polyoma-transformed normal rat kidney (PyNRK) cells. Cells were labeled for 10 h in Dulbecco's modified Eagle's medium lacking methionine and containing 20 μCi/ml [^{35}S] methionine. T antigens were extracted, precipitated, and separated as described in Materials and Methods. a, NRK, preimmune serum; b, NRK, anti-T ascites. c, PyNRK-Cl 3, preimmune serum; d, PyNRK-Cl 3, anti-T ascites.

such clones isolated from three different established rat embryo fibroblast lines. All show large, middle, and small T antigen species, some with multiple bands around 36K. Retention of the complete set of T-antigen species in transformed rat cells is not an invariable rule, as shown by the polyoma-transformed NRK cell in Figure 2B. This clone shows no detectable 100K species but does show middle and small T antigens. Relatively intense bands with an apparent molecular weight around 75K are seen in de novo infections as well as some transformed cell lines (Fig. 1 and Fig. 2A,B). However, such bands are seen in preimmune and normal cell controls so they cannot represent viral products.

It is our experience that transformed rat cells are positive for nuclear T-antigen immunofluorescence immediately after isolation, but may become negative after continued cultivation or subcloning. This loss of nuclear T antigen upon serial passage is not accompanied by any loss or diminution in any parameter of the transformed phenotype as far as we have been able to determine. That the predominant nuclear T antigen is the 100K species [15,16] provides additional support for the idea that the large T antigen is not required after transformation has been established.

Patterns of T Antigen in Stably Transformed Mouse Cells

A similar investigation of polyoma-transformed mouse cells was carried out. Such transformants are most readily obtained by partially inactivating the virus by X-rays or UV irradiation to produce a preferential loss of replicating over transforming ability [20]. Receptor-destroying enzyme and antiviral antiserum are added after infection to protect the rare transformants from being reinfected and killed [11]. Figure 3A shows the results with five independently derived clones of Balb-3T3 clone A-31 transformed by irradiated virus [21]. All are free of infectious virus, and are capable of growth in soft agar. The 100K T antigen is not detected in any of the five clones, while the middle and small T antigens are present in varying amounts in all of them. The same pattern is seen in Py-3T3-6 cells (Fig. 3B), a derivative of Swiss-3T3 cells used in the original isolation of hr-t mutants [11]. This line, and a second one from Swiss 3T3, are negative for T-antigen nuclear fluorescence. These two clones were obtained with unirradiated virus; thus, the absence of 100K T antigen in the Balb-3T3 transformants is not necessarily due to the use of partially inactivated virus.

It is interesting that virus-specific RNA in Py-3T3-6 cells is homologous only to the proximal part of the early region which includes the hr-t gene, and not to the distal or "ts-a" part [22]. Tests for early viral gene functions in some of these polyoma mouse transformants have shown that they are permissive for hr-t mutant growth [21], and fail to complement ts-a mutant growth at the high temperature [3]. These observations taken together point clearly to the persistence and functioning of the hr-t gene in transformed mouse cells, and to the absence of a functioning ts-a gene.

In a survey of 13 independently isolated clones of mouse cells transformed by polyoma virus, 11 showed no evidence of a 100K T antigen species. The higher frequency of

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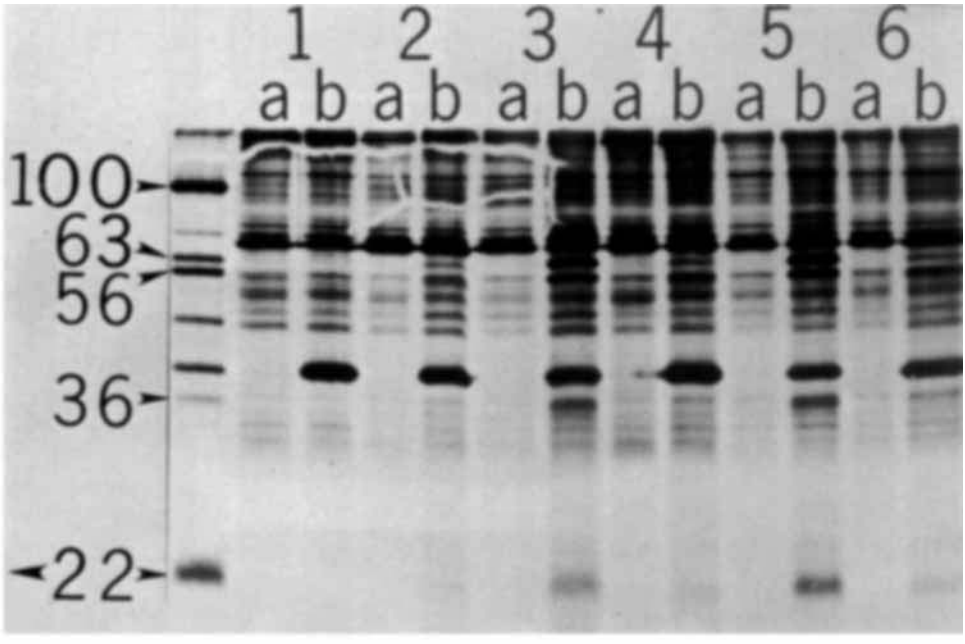
Fig. 3. A: T-antigen patterns of polyoma-transformed Balb/3T3 cells (A31). Cells were labeled for 90 min in Hanks's salt solution containing 5% dialyzed calf serum and 40 μCi/ml [^{35}S]methionine. The T antigens were extracted, precipitated, and electrophoresed as described in Materials and Methods. 1, A31; 2, PyA31-Cl 1; 3, PyA31-Cl 4,PyA31-Cl 6; 5, PyA31-Cl 12; 6, PyA31-Cl 38, a, Preimmune serum; b, anti-T ascites. B: T-antigen patterns of Py-3T3-6 cells. Cells were pulse-labeled for 1 h in Hanks's salt solution containing 10% dialyzed calf serum and 100 μCi/ml [^{35}S]methionine. T antigens were extracted, immune-precipitated, and electrophoresed as indicated in Materials and Methods. a, Preimmune serum; b, Anti-T ascites.



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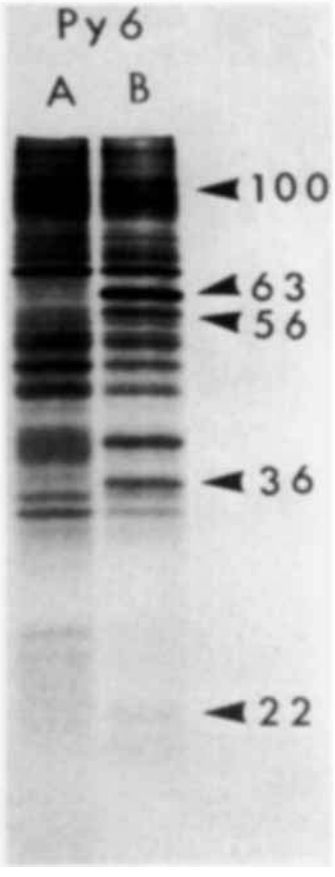

3B

large T antigen-negative clones among mouse compared to rat transformants may be explained by the fact that mouse cells are far more permissive than rat cells to viral DNA replication, a process in which the ts-a gene plays a direct role [23]. Thus, if viral DNA replication were lethal for the cell, one would expect a stronger selection against retention of the 100K T antigen in mouse versus rat transformants.

Retention of the Large T Antigen in SV-40-Transformed Mouse Cells

The argument relating permissivity of the host to selection against expression of large T antigen was tested by examining Balb-3T3 clone A-31 cells transformed by SV-40. These cells are nonpermissive for SV-40 DNA replication. Four out of four clones examined show persistence of the large T antigen (Fig. 4). The 94K protein is the product of the SV-40 "A" gene, and as in the case of the polyoma virus 100K protein, it is required for the initiation of viral DNA synthesis during productive infection [24]. These results lend further support to the idea that the permissivity of the host to viral DNA replication is a major factor in the failure of stably transformed cells to express the large T antigen.

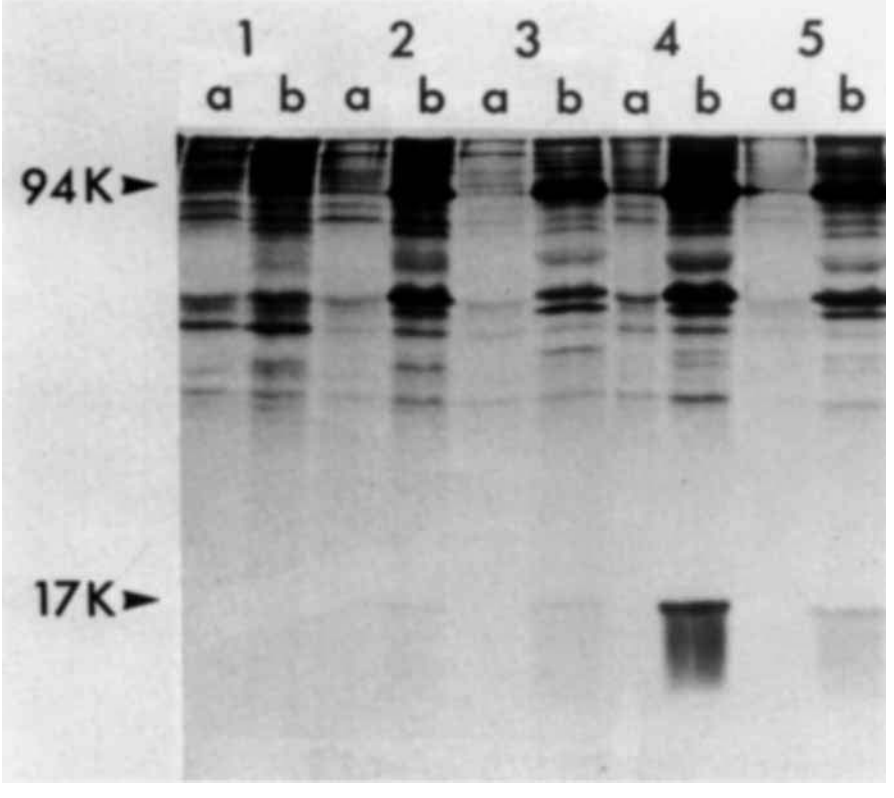

Fig. 4. T-antigen patterns of SV-40-transformed Balb/3T3 cells (A31). Cells were pulse-labeled for 60 min in Hanks's salt solution containing 100 μCi/ml [^{35}S]methionine. T-antigens were collected, immune precipitated, and electrophoresed as described in Materials and Methods. 1, A31; 2, SVA31-Cl 1; 3, SVA31-Cl 5; 4, SVA31-Cl6; 5, SVA31-Cl 8. a, Preimmune serum; b, anti-T hamster serum.

DISCUSSION

Ts-a mutants, which are defective in the large T antigen but make normal middle and small T antigens, induce *abortive* transformation of rat cells at the nonpermissive temperature just as in a wild-type viral infection. The important difference between ts-a mutants and wild-type virus is that ts-a mutants are defective in their ability to induce *stable* transformation. Once transformation is established, the ts-a gene product appears to be no longer required; thus, stable transformants often fail to express large T antigen. The possibility that the 100K protein present in amounts below the level of detection may play a persistent role in transformation cannot be excluded by these data. Although in most 100K antigen-negative clones, there is no indication of fragments of the 100K protein, the existence of such truncated species cannot categorically be ruled out. Among mouse transformants, 100K T antigen-negative clones are the general rule, while in rat transformants such clones occur but less frequently. The difference is most likely related to the greater permissivity of mouse cells to polyoma replication, which brings with it the need for the cells to inactivate the "a" function in order to survive. Hamster (BHK) cells, which are slightly permissive for polyoma replication, also generally fail to show the large T antigen when transformed by polyoma virus [18]. Consistent with this interpretation is the persistence of SV-40 large T antigen in mouse cells, the latter being nonpermissive for SV-40 DNA replication.

The role of the large T antigen of polyoma virus in transformation thus appears to be one of initiating stable transformation, most likely by carrying out steps which lead to the integration of viral DNA into the host genome [6]. Thereafter, there may be selection against cells expressing large T antigen in order that excision and replication not occur. Two recent findings from other laboratories support this view. First, excision and replication of viral DNA in polyoma rat transformants have been shown to depend on the "a" gene function [25]. Second, inactivation of the "a" gene by endonuclease EcoRI cleavage does not impair the ability of viral DNA to induce tumors in hamsters [26], presumably because the host is competent to take up and incorporate the viral DNA, thus bypassing the need for the "a" function. Although it is clear from the existence of large T antigen-negative stable transformants that the 100K protein is not obligatory for the maintenance of the transformed state, the existence of some ts-a transformants with a temperature-sensitive transformed phenotype [27,28] suggests that the large T may in some cases act to control the transformed state.

A functional hr-t gene is both necessary and sufficient for inducing transformed cell properties following de novo infection of rat cells. The hr-t gene codes for the small and middle T antigens in nonproductive as well as productive infection. A role for these viral products in inducing and maintaining the transformed state is supported by the inability of hr-t mutants to abortively or stably transform cells, and by their persistent expression in transformed cells regardless of the species of origin.

Gene expression from the hr-t region is complicated by multiple RNA splicing events leading to the generation of multiple proteins. Current evidence based on peptide mapping (see Hutchison et al [18] and Smart and Ito [19]; B. Schaffhausen and T. Benjamin, unpublished results), DNA sequencing [29–33], and RNA splicing patterns (R. Kamen, personal communication) gives the following picture of the coding sequences corresponding to the hr-t and ts-a genes: 1) Hr-t mutants map in a region which comprises an "intron" for the 100K T antigen, but which forms part of the mRNAs for both the 56K and 22K T antigens; 2) the 56K protein includes essentially all of the 22K protein in its tryptic peptides, but has additional sequences at the C-terminal end which come from

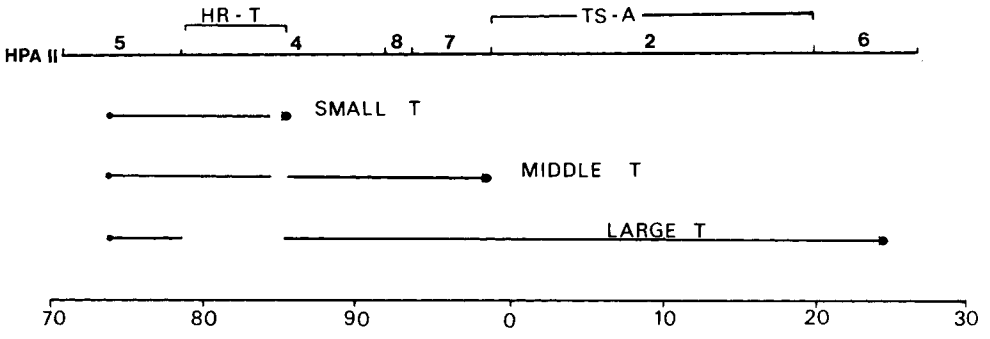

Fig. 5. Schematic diagram of the polyoma virus early region. The coordinates at the bottom refer to the map units defined by the EcoRI cleavage site (0). The map at the top shows the cleavage pattern obtained with HPA II and the positions of the hr-t and ts-a mutations. The sequences coding for small, middle, and large T antigens are shown schematically by the arrows. See Discussion for a more complete description.

reading a portion of the large T coding sequences in a second frame; 3) ts-a mutants map in the distal part of the early region, affecting only the large T antigen in the C-terminal portion downstream from the overlap region with 56K; 4) large, middle, and small T antigens share a common N-terminal sequence which is not directly affected by either hr-t or ts-a mutants. Figure 5 presents these findings schematically.

Besides the normal 56K and 22K products, hr-t mutants fail to induce 63K and 36K species found in wild-type infected cells [14,15]. The relationships of these proteins to the other T-antigen species and to the viral DNA have not yet been determined. An understanding of these relationships may allow the construction of new mutants defective in one but not the other middle or small T antigens, and eventually lead to elucidation of the role of each species in transformation.

ACKNOWLEDGMENTS

The authors gratefully acknowledge the expert technical assistance of Ingrid Lane in the work described here. The work has been supported by grants 19567 and 14723 from the National Cancer Institute.

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