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Small and Middle T Antigens Contribute to Lytic and Abortive Polyomavirus Infection

HANS TÜRLER AND CONSUELO SALOMON

Department of Molecular Biology, University of Geneva, 1211 Geneva 4, Switzerland

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Using three different polyomavirus *hr-t* mutants and two polyomavirus *mlT* mutants, we studied induction of S-phase by mutants and wild-type virus in quiescent mouse kidney cells, mouse 3T6 cells, and FR 3T3 cells. At different times after infection, we measured the proportion of T-antigen-positive cells, the incorporation of [^{3}H]thymidine, the proportion of DNA-synthesizing cells, and the increase in total DNA, RNA, and protein content of the cultures. In permissive mouse cells, we also determined the amount of viral DNA and the proportion of viral capsid-producing cells. In polyomavirus *hr-t* mutant-infected cultures, onset of host DNA replication was delayed by several hours, and a smaller proportion of T-antigen-positive cells entered S-phase than in wild-type-infected cultures. Of the two polyomavirus *mlT* mutants studied, *dl*-23 behaved similarly to wild-type virus in many, but not all, parameters tested. The poorly replicating but well-transforming mutant *dl*-8 was able to induce S-phase, and (in permissive cells) progeny virus production, in only about one-third of the T-antigen-positive cells. From our experiments, we conclude that mutations affecting small and middle T-antigen cause a reduction in the proportion of cells responding to virus infection and a prolongation of the early phase, i.e., the period before cells enter S-phase. In *hr-t* mutant-infected mouse 3T6 cells, production of viral DNA was <10% of that in wild-type-infected cultures; low *hr-t* progeny production in 3T6 cells was therefore largely due to poor viral DNA replication.

The three early proteins of polyomavirus designated large (lT), middle (mT), and small (sT) tumor (T)-antigens are responsible for the biological effects of the virus: tumor induction in hamsters and rats, cell transformation, and most of the virus-induced events taking place during productive and abortive infection of cells in culture (for reviews, see references 18, 22, 41, 45, 47). In quiescent cells, polyomavirus induces, among other events, increased host RNA (34) and protein synthesis (25) and host chromatin duplication (S-phase) (41, 47). In abortive infection of hamster or rat cells, S-phase is followed by mitosis and eventually by establishment of stably transformed cell lines. In productive infection of mouse cells, chromatin duplication is paralleled by viral DNA replication and followed by synthesis of viral capsid proteins, assembly of virus particles, and cell lysis (41, 47).

Polyomavirus lT, mT, and sT comprisè 785, 421, and 195 amino acid residues, respectively; they share a common aminoterminal region, but because of differently spliced mRNAs the coding sequences end at different sites on the genome (Fig. 1). For definition of functions specified by the T-antigens, a number of mutants have been isolated and studied. Genetic analysis of the early region of the viral genome defined two complementation groups: host-range, transformation negative (*hr-t*) mutants (2, 3), and temperature-sensitive early (*tsA*) mutants, which at the restrictive temperature are defective for viral DNA replication and have a strongly reduced capacity to transform cells (9, 10). *hr-t* mutations map in the intron region of lT and change mT and sT in the same way (3), whereas *tsA* mutations cause single amino acid changes in the carboxy-terminal region of lT (7, 40). A third class of viable early mutants (*mlT*) has deletions between 89 and 95 map units and therefore has altered mT and lT (Fig. 1). Individual *mlT* mutants show different phenotypes (1, 8, 19, 27). Studies with *hr-t* and *mlT* mutants have indicated that the mT protein is required for stable cell transformation and for tumor induction (3, 24), results that have been confirmed by various approaches (5, 15, 29, 31, 43). The lT protein is required for viral DNA replication (11), binds to defined sequences at the replication origin of viral DNA (14), and may regulate synthesis of early viral mRNA (6). Virtually nothing is known on the role of sT.

We are interested in the mitogenic function of polyomavirus, i.e., its capacity to induce S-phase in quiescent cells. According to earlier results, both *tsA* and *hr-t* mutants maintain the mitogenic function (3, 10, 12). So far, no mutations affecting for this function have been described, possibly because they are lethal, and also because no conditional mutants have been found. To learn more about the mitogenic function of polyomavirus, we studied the induction of S-phase in quiescent permissive and nonpermissive cells after infection with different *hr-t* and *mlT* mutants. All mutants tested were able to induce host DNA synthesis, but compared with wild-type virus we found significant differences with respect to time course and the proportion of cells going into S-phase.

MATERIALS AND METHODS

Cell cultures. Primary cultures of mouse kidney (MK) cells were prepared as described previously (47, 50) and grown to confluency in culture medium (Dulbecco modified Eagle H-21, GIBCO, Basel, Switzerland) supplemented with 10% newborn calf serum (GIBCO).

Quiescent cultures of mouse 3T6 cells (Flow Laboratories, Irvine, Scotland) were obtained by plating cells from a low-passage-number culture (between 3 and 10) at 5×10^4 cells per ml of culture medium containing 2% fetal bovine serum (GIBCO). Three days after plating, shortly before the cultures became confluent, the serum concentration was reduced to 0.5%, and the cultures were kept in this medium for 3 more days before they were infected.

Quiescent cultures of rat FR 3T3 cells (37; kindly provided by F. Cuzin, Université de Nice) were made by plating cells

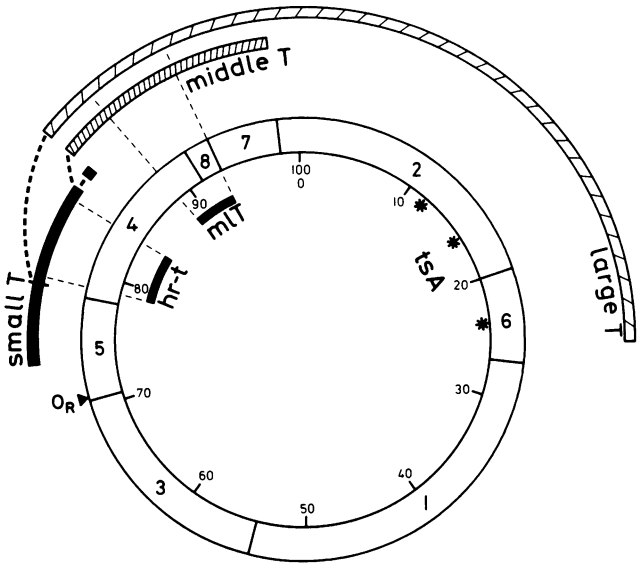

FIG. 1. Early mutants of polyomavirus. The circle shows the map of polyomavirus DNA with the *Hpa*II fragments. The early region extends from the origin of replication (O_R) at 71 units to 26 units. The coding regions and the splice sites for the three T-antigens are indicated by bars that are differently shaded for the three reading frames used. The regions where *hr-t* and *mlT* mutants map are shown inside the circle, and the point mutations in different *tsA* mutants are indicated by asterisks.

from a low-passage-number culture (between 3 and 10) at 3 $\times$ 10^4 cells per ml of culture medium containing 5% fetal bovine serum. Cultures were incubated with this medium for 8 days before they were infected.

Polyomavirus, mutants, and infections. Our polyomavirus wild-type strain (Py) originated from G. Attardi, California Institute of Technology, Pasadena, and had the designation "wild-type no. 132." It was kept free of contaminating variants by plaque purifications. The A2 wild-type strain (39) and the *mlT* mutants *dl*8 and *dl*23 (19) were received from B. E. Griffin (ICRF Laboratories, London, England). The *hr-t* mutants NG59 and SD15 were given to us by T. Benjamin (Harvard Medical School, Boston, Mass.), and NG18 was provided by Y. Ito (Recombinant DNA Research Unit, National Institutes of Health, Bethesda, Md.). The original stocks of A2, *dl*23, NG59, and SD15 were plaque purified on MK cells, and new stocks were prepared by infecting MK cell cultures with 0.01 to 0.05 PFU per cell. Virus titers of the stock suspensions were between 4 $\times$ 10^8 and 10 $\times$ 10^8 PFU/ml. With NG18 and *dl*8, we did not obtain clear plaques on MK cultures within 2 weeks. Clean stocks of these mutants were prepared by infecting MK cells with serial dilutions of the original stocks; the lysate from cultures that had been infected with the highest dilution and showing signs of cell lysis within 2 weeks was used for production of stocks as described above. Virus titers determined by endpoint titrations on MK cells were 5 $\times$ 10^8 infectious units per ml for NG18 and 3 $\times$ 10^7 infectious units per ml for *dl*8.

MK cells were infected 2 days after confluency by using 0.4 ml of the virus stock suspensions for 90-mm plates, 0.2 ml for 50-mm plates, and 0.1 ml for cultures grown on glass cover slips (22 by 22 mm) in 33-mm plates. After an adsorption period of 2 h at 37°C, these cultures were covered with 10, 5, and 2 ml of medium without serum, respectively. Infection of mouse 3T6 cells was done in the same way, but after the adsorption period, cultures were covered with medium containing 0.5% fetal bovine serum. For infection of FR 3T3 cells, partially purified virus stocks were used. Virus particles were sedimented by centrifugation at 35,000 rpm for 2 h, and the virus pellet was suspended in phosphate-buffered saline and sedimented again under the same conditions. Finally, the virus pellet was suspended in half of the starting volume of medium containing 5% newborn calf serum. Infected cultures of FR 3T3 cells were covered after the adsorption period with medium containing 0.5% fetal bovine serum. For all mock infections, an equal volume of medium containing 5% newborn calf serum was used instead of the virus suspensions.

Methods used in time course experiments. The percentages of T-antigen- and capsid protein (V-antigen)-containing cells were determined on cover slip cultures by indirect immunofluorescent staining, using anti-polyomavirus T serum from hamsters (45) and a commercial preparation of polyomavirus mouse antiserum (Microbiological Associates, Bethesda, Md.).

For measurement of DNA synthesis, cover slip cultures were pulse-labeled for 1 h with 10 μCi of [^{3}H]thymidine (20 Ci/mmol, New England Nuclear, Dreieich, West Germany) in 1 ml of culture medium. Trichloroacetic acid-insoluble radioactivity was determined on duplicate cover slips. Thereafter, the cover slips were processed for autoradiography with Ilford L4 liquid emulsion and exposed for 1 week.

Total DNA, RNA, and protein per 50-mm petri dish were extracted in duplicates as described earlier (34) and determined by colorimetric reactions with diphenylamine (17) and orcinol (36), and according to Lowry et al. (26), respectively.

Production of viral DNA was measured by labeling, for each time point, two 85-mm cultures with 10 μCi of [^{3}H]thymidine in 10 ml of culture medium from 2 h after infection to the time of extraction. Viral DNA was selectively extracted (21) and purified by phenol extractions and cesium chloride-ethidium bromide density gradient centrifugation (16). The viral DNA was quantitated by spectrofluorometry in the presence of ethidium bromide (44) and by counting radioactivity.

RESULTS

Polyomavirus mutants tested. For our study, we chose three different types of *hr-t* mutants, namely, NG59, an insertion mutant, SD15, a deletion mutant, and NG18, a frame shift deletion mutant; and two *mlT* mutants, *dl*23 and *dl*8, with different phenotypes. The changes in the T-antigens that are expected from the published sequence data (4, 38) and the known phenotypes (3, 19) are listed in Table 1. Wild-type virus A2 (39) and our own wild-type strain (Py) served as references.

To test the quality of our virus and mutant stocks, we isolated the viral DNA by 40 to 48 h after infection of MK cell cultures and digested it with *Hpa*II. The patterns obtained by gel electrophoresis (Fig. 2) agreed with the previously published sequence data and showed that the stocks were free of variant and defective molecules. Fragment *Hpa*II-5 showed the known size differences between A2 and A3 strains (39). Fragment *Hpa*II-3 revealed the size variations due to internal sequence repeats of variable lengths observed in the noncoding part of the late region of wild-type and mutant viruses (33). SD15, with an additional *Hpa*II site in fragment 1 (20), had in our gels a somewhat slower migrating *Hpa*II-2 fragment; the reason for this remains unknown, since in sodium dodecyl sulfate-polyacrylamide

TABLE 1. Early mutants of polyomavirus: mutations and phenotypes

Mutant	Mutation	Changes in T-antigens (amino acid residues)[a]	Phenotype
NG18	Frame shift	Small: − (114–195) Middle: − (114–421)	*hr-t*.
SD15 NG59	Deletion Insertion	Small: − (83–129) Middle: − (83–129) Small: + Ile (179) Middle: + Ile (179)	Lytic, host range; abortive and stable transformation, negative (3)
*dl*8	Deletion	Middle: − (253–282) Large: − (145–174)	Lytic, poor virus yield; transformation, as or better than wild-type (19, 23)
*dl*23	Deletion	Middle: − (302–335) Large: − (145–174)	Lytic, as wild-type; transformation, poor or negative (19, 23)

[a] For numbering of amino acid residues, see reference 45.

gel electrophoresis, lT of SD15 migrated like wild-type lT (data not shown).

Earlier results had shown that the T-antigens synthesized in mutant-infected cells corresponded to the DNA sequence alterations. All *hr-t* mutants had unaltered lT; a normal-sized mT was found for NG59, a smaller mT was found for SD15, and no detectable mT was found for NG18. Synthesis or stability of *hr-t* mutant sT was abnormal, since only small amounts of sT were detected for NG59 and no truncated or smaller sT of the expected size was observed for NG18 and SD15 (3; and our unpublished data). Unaltered sT, but smaller lT and mT, were produced by mlT mutants, and mT of *dl*23 migrated faster than expected in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (24).

All of the mutants used in our experiments were expected to have a wild-type origin of replication and wild-type control regions for early and late transcription. At least for *hr-t* mutants, the rate of T-antigen synthesis should be the same in wild-type and mutant-infected cells.

Parameters measured in time course experiments. At 18, 30, and 42 h after infection of quiescent permissive or nonpermissive cells with wild-type and mutant viruses, the following parameters were measured: percent T-antigen-positive cells (by immunofluorescence), incorporation of [^{3}H]thymidine during 1-h pulse-labeling, percent DNA-synthesizing cells (by autoradiography), and total DNA, RNA, and protein contents of the cultures (by colorimetric quantitations). In permissive cells, we also determined the proportion of viral capsid-containing cells by immunofluorescence and the amounts of viral DNA by spectrofluorometry and by counting [^{3}H]thymidine incorporation into viral DNA during a continuous labeling of the cultures. The results are shown as diagrams for selected parameters, which are more instructive than large tables. For each figure, the results were taken from the same experiment.

Lytic infection of MK cells. The results obtained with *hr-t* mutant and wild-type (Py) infection of primary MK cell cultures are shown in Fig. 3. At 18 and 30 h after infection, a similar proportion of infected, i.e., T-antigen-positive, cells was detected for Py, NG59, and SD15; however, all other parameters measured were considerably lower at the corresponding time points in mutant-infected than in Py-infected cultures. There was a delayed time course of *hr-t* mutant infection and a delayed onset of viral DNA synthesis that occurred ca. 10 h later than in Py-infected cultures (Fig. 3). Induction of S-phase measured by the percentage of DNA-synthesizing cells and the increase in RNA content were both delayed and reduced as compared with Py. The same conclusions were reached for NG18, although in this case, the number of T-antigen-positive cells—and consequently the mitotic response of the culture and the production of viral DNA—was lower than for the other mutants.

Figure 4 shows the same analysis done with *mlT* mutants *dl*8 and *dl*23 in comparison with wild-type A2. Confirming earlier observations (19), *dl*23 induced a lytic infection similar to A2 with respect to infected cells, DNA-synthesizing cells, and viral capsid-containing cells (data not shown). However, increases in total RNA, DNA, and protein (data not shown) and production of viral DNA were lower for *dl*23 than for A2. For mutant *dl*8, which yields low titers of progeny virus, immunofluorescent staining of T-antigen at 42 h after infection indicated that 40% of the cells were infected, but most of them showed weak fluorescence. Only 15% had an intensity similar to that observed with *dl*23; this proportion corresponded roughly to the fraction of DNA-synthesizing cells (18%) and of viral capsid-containing cells (10%; data not shown). Increases in DNA, RNA, and protein were <10%, and only small amounts of viral DNA were produced. When cells were infected with a 20-fold-concentrated preparation of *dl*8, 60% of the cells showed weak T-antigen staining, 20% showed medium to strong staining, and 20% of the cells contained capsid antigens at 42 h after infection. From these results, we concluded that in *dl*8-infected MK cultures, only ca. one-third of the infected cells

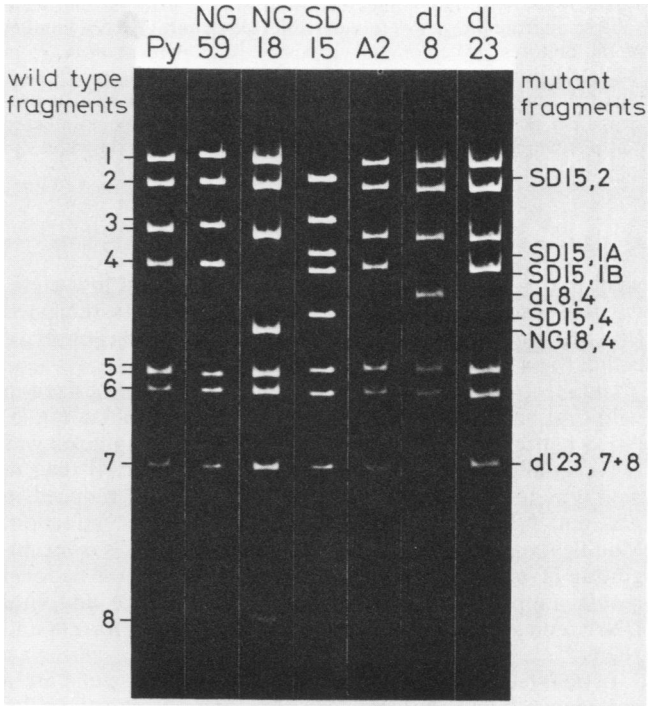

FIG. 2. Restriction enzyme cleavage analysis of wild-type and mutant polyomavirus DNAs. Viral DNA was extracted from infected MK cells 40 to 48 h after infection and purified as described in the text. Fragments generated by *Hpa*II digestion were separated by electrophoresis on a 5% polyacrylamide gel with 89 mM Tris-borate–2 mM EDTA (pH 8.3) and revealed by staining with ethidium bromide. The patterns are discussed in the text.

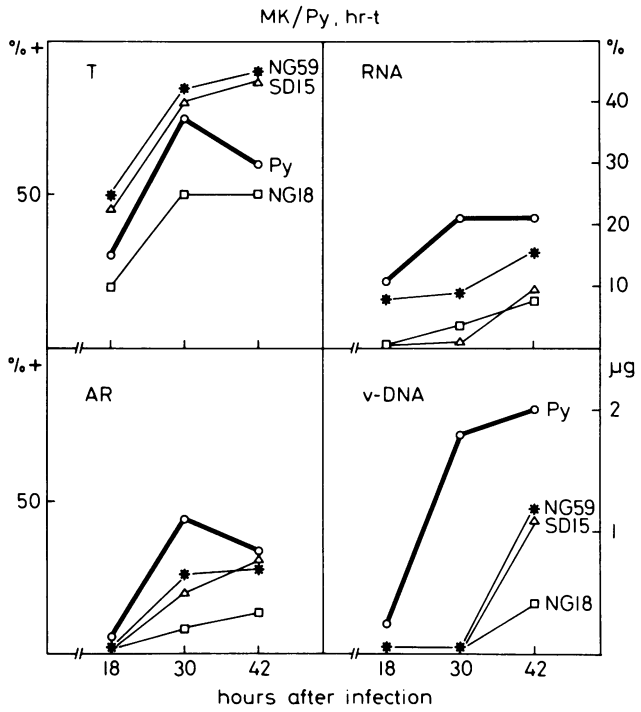

FIG. 3. Wild-type (Py) and *hr-t* mutant infection of MK cells. Cultures were infected with Py, NG59, SD15, and NG18, or mock infected. At 18, 30, and 42 h after infection, various parameters were determind: percent T-antigen-positive cells, by immunofluorescence (T); percent DNA-synthesizing cells, by autoradiography of cultures that had been pulse-labeled with [^{3}H]thymidine for 1 h (AR; the values found with mock-infected cultures were 2 to 3% and were subtracted from the values found in infected cultures); RNA content of the cultures, by colorimetry, expressed as percent increase in infected cultures as compared with mock-infected cultures (RNA); viral DNA, determined by spectrofluorometry, expressed as micrograms per petri dish (v-DNA; background values in mock-infected cultures were below 0.2 µg and were subtracted from the values in infected cultures).

went into S-phase and produced progeny virus at low titers, whereas in the others, T-antigen concentrations remained low and infection apparently aborted before the onset of cellular and viral DNA replication.

Gel electrophoretic analyses of T-antigens synthesized in wild-type and mutant-infected MK cells are shown in Fig. 5. At 18 h after infection, the rate of T-antigen synthesis was lower in all mutant-infected cultures (lanes 2 to 4) than in wild-type-infected cultures (lane 1); it was still reduced in *dl*8- and NG59-infected cultures at 30 h after infection. Modification of lT, i.e., appearance of a second, slower-migrating lT band previously shown to be a posttranslational modification coinciding with the onset of cellular and viral DNA replication (45), was also clearly delayed for *dl*8 and NG59.

Lytic infection of mouse 3T6 cells. Mouse 3T6 cells are a continuous mouse fibroblast cell line and fall into the category of nonpermissive cells for *hr-t* mutants (3). Microscopic observation of *hr-t* mutant-infected 3T6 cultures revealed that lysis of the cultures was delayed and less pronounced than with Py. By hemagglutination assays, production of *hr-t* mutant progeny was <10% of that found for Py (unpublished data).

The results of a time course experiment with *hr-t* mutants are shown in Fig. 6. The fraction of T-antigen-positive cells was lower in this experiment with all *hr-t* mutants than with Py. All *hr-t* mutant-infected cultures showed a slow increase in DNA-synthesizing cells and in DNA content, and a delayed increase in viral capsid-containing cells as compared with Py-infected cultures. The most striking observation, however, was that viral DNA (v-DNA) production, as measured either by radioactivity or by spectrofluorometry, was extremely low. Similarly low yields of viral DNA were obtained when growing 3T6 cells were infected with *hr-t* mutants and also when quiescent cultures were stimulated with serum after *hr-t* mutant infection. These observations differ from results obtained with *hr-t* mutant-infected NIH 3T3 cells, in which viral DNA production was 30 to 50% of that found for wild-type virus and low progeny virus yield was attributed to a defect in virus assembly (3, 13).

We have not done a complete time course analysis with quiescent 3T6 cell cultures infected by *mlT* mutants. Microscopic observations, immunofluorescent staining of T- and V-antigens, and determination of viral DNA production indicated that there were no significant differences between *mlT* mutant infections of 3T6 cells and of MK cells.

Abortive infection of FR 3T3 cells. In preliminary experiments, we observed that some of our mutant stocks, particularly *dl*8, induced in quiescent FR 3T3 cells DNA synthesis in a higher proportion of cells than that showing positive T-antigen staining. The presence of growth factors stimulating quiescent FR 3T3 cells in crude viral lysates was further suggested by the finding that a *dl*8 lysate that had been depleted of virus particles by ultracentrifugation induced

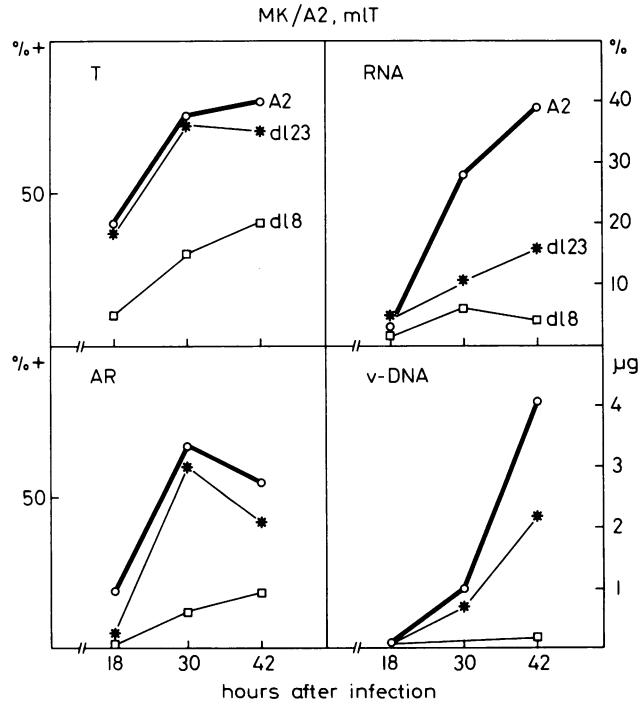

FIG. 4. Wild-type (A2) and *mlT* mutant infection of MK cells. Cultures were infected with A2, *dl8*, and *dl23*, or were mock infected, and various parameters were determined at 18, 30, and 42 h after infection. For presentation and calculation of results, see the legend to Fig. 3. The background values of DNA-synthesizing cells in mock-infected cultures were below 2%.

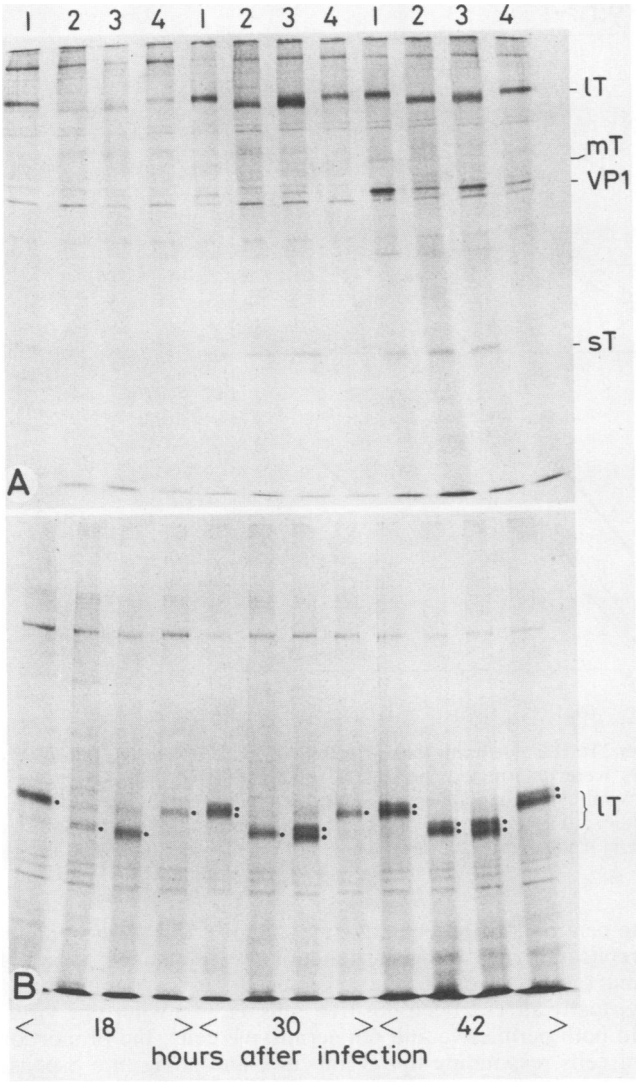

FIG. 5. T-antigens synthesized in wild-type and mutant-infected MK cells. Wild-type and mutant-infected MK cell cultures were labeled with 150 μCi of [^{35}S]methionine in 2 ml of methionine-free medium at 16 to 18, 28 to 30, or 40 to 42 h after infection. After the labeling periods, T-antigens were extracted and immunoprecipitated with anti-polyomavirus T serum, using protein A-Sepharose. Samples were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis on 12.5% acrylamide gels (A) and 6% acrylamide gels (B). The gels were dried and autoradiographed. Lanes: 1, A2; 2, *dl8*; 3, *dl23*; 4, NG59. The same stocks of A2, *dl8*, and *dl23* were used as in the experiment shown in Fig. 4. For NG59, another stock was used which gave 5 and 47% of T-antigen-positive cells by 18 and 30 h after infection, respectively. In B, dots indicate lT bands.

DNA synthesis in 13% of the cells by 30 h after "infection," whereas less than 1% of the cells contained detectable T-antigen and only 3% of DNA-synthesizing cells were found in parallel cultures that had been mock infected with medium containing 5% serum. To measure the virus-induced mitotic response in FR 3T3 cells, we therefore used partially purified virus preparations (see above).

Figure 7 shows the results obtained with *hr-t* mutants. With respect to the rather large proportion of T-antigen-positive cells, *hr-t* mutant-infected cultures showed a slow and rather small increase in DNA-synthesizing cells, in DNA content, and particularly in RNA content when compared with Py-infected cultures. Although the proportion of T-antigen-positive cells was similar for Py- and *hr-t* mutant-infected cultures, the intensity of staining was always weaker in mutant-infected cells.

Infection of quiescent FR 3T3 cell cultures with *mlT* mutants is shown in Fig. 8. The nontransforming mutant *dl*23 induced the mitotic response with a slight delay in time course and with a relatively small increase in RNA content as compared with A2. Infection with mutant *dl*8, which has a more proficient transforming capacity than A2 (19), showed characteristics similar to those reported above for *dl*8-infected MK cells. Only a fraction of T-antigen-positive cells exhibited strong immunofluorescent staining, i.e., comparable in intensity to *dl*23-infected cells; apparently only this fraction of cells went into S-phase, as judged by autoradiography. However, at 42 h after infection, DNA and RNA contents of *dl*8-infected cultures had increased significantly and reached values close to those found in *dl*23-infected cultures, which had a higher proportion of T-antigen-positive cells. By 3 days after infection, *dl*8-infected cultures showed morphological changes, i.e., contraction and rounding of the cells, which was typical for wild-type-infected cultures but did not occur in cultures infected with nontransforming mutants.

DISCUSSION

The mitogenic function of polyomavirus, i.e., the capacity to push quiescent cells into S-phase, was described some time ago (48), but it is not yet defined in terms of coding sequence, nor is its mode of action known. To get more information on this function, we compared the mitotic host responses induced by different viable T-antigen mutants in permissive mouse and nonpermissive rat cells with that induced by wild-type virus. For this study, we chose three different *hr-t* mutants and two *mlT* mutants of different phenotypes.

The capacity of *hr-t* mutants to induce one cycle of host DNA replication has been reported earlier (35). In our experiments, the induction of S-phase by *hr-t* mutants was delayed by several hours in both productive and abortive infections as compared with the wild type. Onset of viral DNA replication and of viral capsid synthesis in permissive cells was also coordinately delayed. In nonpermissive FR 3T3 cells, we observed not only a delay but also a smaller proportion of responding cells: as judged by autoradiography, not all T-antigen-positive cells participated in the mitotic reaction. *hr-t* mutant infection of permissive mouse 3T6 cells also led to a delayed and reduced mitotic response. In these cells, very low levels of viral DNA were produced, and consequently the yield of progeny *hr-t* mutants was low. A similar observation was made by others with NG18-infected 3T6 cells (30). Our results differ from those reported by Garcea and Benjamin (13) on *hr-t* mutant infection of NIH 3T3 mouse cells. They found that viral DNA present in infected cultures amounted to 30 to 40% of that found in wild-type-infected cultures and that low progeny mutant yields were mainly the consequence of inefficient virus assembly due to the absence of posttranslational acylation of the main viral capsid protein. In our experiments, we did not find significant differences between the phenotypes of the three *hr-t* mutants studied, confirming earlier observations (3).

Of the two *mlT* mutants used in our experiments, *dl23* behaved in productive and abortive infections very similarly to wild-type virus with respect to most, but not all, parameters studied. The other mutant, *dl8*, was originally thought

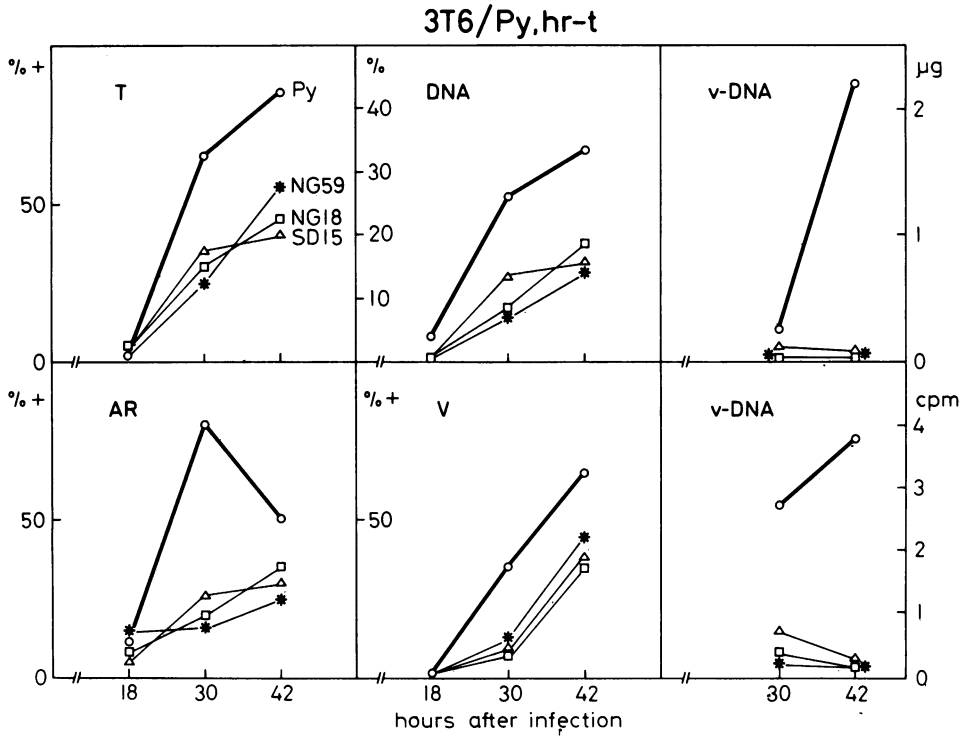

FIG. 6. Py and *hr-t* mutant infection of 3T6 cells. Quiescent cultures of 3T6 cells were infected with Py, NG59, SD15, or NG18, or were mock infected. At 18, 30, and 42 h after infection, various parameters were determined: percent T-antigen-positive cells (T); percent DNA-synthesizing cells (AR); increase in DNA content (DNA); percent viral capsid-containing cells (V); amounts of viral DNA (v-DNA), determined by spectrofluorometry as micrograms per culture and by radioactivity (10^4 cpm). Background values found in mock-infected cultures for DNA-synthesizing cells (<2%) and for viral DNA (<0.2 μg and <500 cpm) were subtracted. For details, see the legend to Fig. 3 and the text.

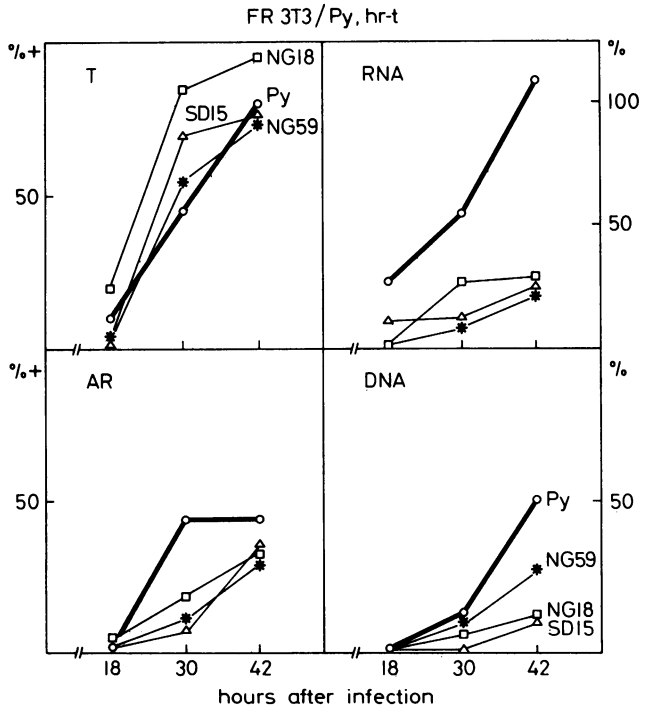

FIG. 7. Py and *hr-t* mutant infection of rat FR 3T3 cells. Quiescent cultures of FR 3T3 cells were infected with Py, NG59, SD15, or NG18, or were mock infected. At 18, 30, and 42 h after infection, various parameters were determined: percent T-antigen-positive cells (T); percent DNA-synthesizing cells (AR), and increase in RNA and DNA content. The background values for DNA-synthesizing cells in mock-infected cultures (<4%) were subtracted. For details, see the legend to Fig. 3 and the text.

to be a replication-defective mutant (19). If it were so and if replication and mitogenic functions were distinct, as in *tsA* mutants, we would have expected a mitotic stimulation in virtually all T-antigen-positive cells as with wild-type virus. In both permissive and nonpermissive cells, the proportion of cells responding to *dl8* infection and going into S-phase was only ca. one-third of all T-antigen-positive cells. As judged by immunofluorescence, the larger part of the infected cells had low concentrations of early viral proteins and did not incorporate [^{3}H]thymidine. In permissive cells, only small amounts of viral DNA were produced, and the proportion of viral capsid-containing cells corresponded closely to the proportion of cells synthesizing DNA. This indicates that low titers of progeny *dl8* were due to a low proportion of cells induced to synthesize cellular and viral DNA.

The time course of infection and the asynchronous induction of S-phase in individual cells by polyomavirus depend on the multiplicity of infection (49). For this reason, we adjusted our virus and mutant stocks, with the exception of *dl8*, to similar titers. That the delayed induction of S-phase by mutants was not due to variations in multiplicity of infection became evident from *hr-t* infection of FR 3T3 cells,

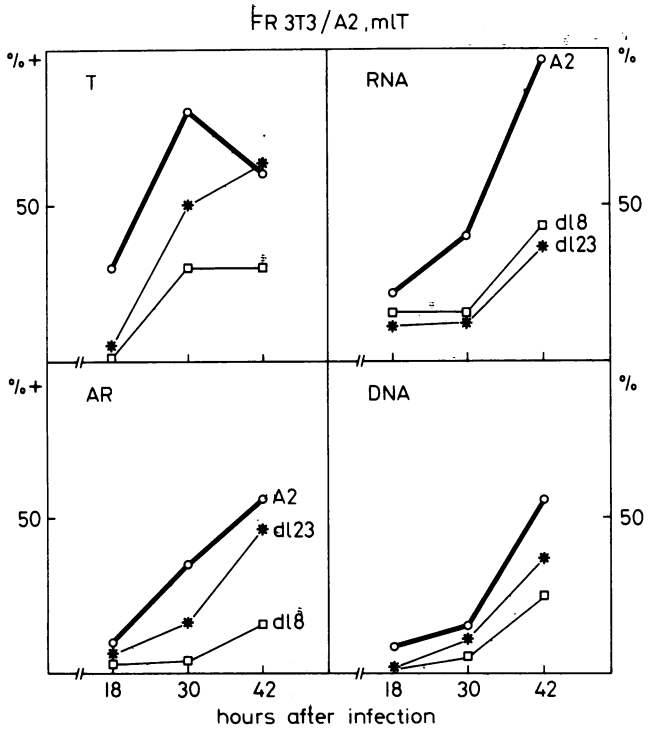

FIG. 8. Wild-type (A2) and *mlT* mutant (*dl8* and *dl23*) infection of quiescent FR 3T3 cells. See the legend to Fig. 7.

in which the proportion of infected (T-antigen-positive) cells was practically the same for wild type and mutants. However, as judged from the intensity of the immunofluorescent staining, the concentrations of intranuclear lT seemed to be lower in mutant-infected than in wild-type-infected cells. This observation, which is difficult to understand because both the control regions for early transcription and the lT protein itself are not affected by *hr-t* mutations, needs confirmation by a quantitative lT assay.

From the experiments with *hr-t* mutants, we can conclude that lT alone is not sufficient to induce a wild-type-like mitotic response of a quiescent host cell. On the other hand, the presence of sT (and mT) is required neither for the mitogenic function nor for the viability of polyomavirus, since a constructed polyomavirus only synthesize variant, LT-v, (42, 46), which can lT, is viable and able to induce host DNA replication only in quiescent cells (unpublished data; the variant was kindly provided by R. Kamen). However, infection of MK cells with LT-v proceded slower than with NG18, and smaller amounts of viral DNA were produced (unpublished data).

Our studies suggest that sT, and possibly also mT, contributes as cofactor to the mitogenic function specified by the lT protein. The observation that *hr-t* mutant-infected cells seem to contain less lT than wild-type-infected cells might suggest that a certain amount of lT protein or of one of its posttranslationally modified forms has to reach a threshold concentration before the cell enters S-phase. Small T (and mT) might be involved in this process by stimulating host cell transcription, leading also to increased synthesis of early viral mRNAs (28, 34); alternatively, they might favor posttranslational modifications of lT.

We consider the most likely candidate for the mitogenic function to be the N-terminal half of the lT protein, despite the fact that lT of *tsA* mutants is unstable at 39°C (23, 45). Rassoulzadegan et al. (32) assigned to this region the "immortalization function" of polyomavirus, i.e., the capacity to confer to cells in primary cultures an unlimited potential for proliferation. Experiments are in progress to determine whether the immortalization and the mitogenic functions are specified by the same domain of the lT protein. We are also testing the alternative hypotheses that the mitogenic function resides in the very N-terminal region common to all three T-antigens and can be provided by any one of the three proteins, or that lT and sT specify two distinct activities capable of inducing S-phase.

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LITERATURE CITED

1. **Bendig, M. M., T. Thomas, and W. R. Folk.** 1980. Viable deletion mutant in the medium and large T-antigen-coding sequences of the polyoma virus genome. J. Virol. **33**:1215–1220.
2. **Benjamin, T. L.** 1970. Host range mutants of polyoma virus. Proc. Natl. Acad. Sci. U.S.A. **67**:394–399.
3. **Benjamin, T. L.** 1982. The hr-t gene of polyoma virus. Biochim. Biophys. Acta **695**:69–95.
4. **Carmichael, G. G., and T. L. Benjamin.** 1980. Identification of DNA sequence changes leading to loss of transforming ability in polyoma virus. J. Biol. Chem. **255**:230–235.
5. **Chowdhury, K., S. E. Light, C. F. Garon, Y. Ito, and M. A. Israel.** 1980. A cloned polyoma DNA fragment representing the 5′ half of the early gene region is oncogenic. J. Virol. **36**:566–574.
6. **Cogen, B.** 1978. Virus-specific early RNA in 3T6 cells infected by a tsA mutant of polyoma virus. Virology **85**:222–230.
7. **Deininger, P. L., P. LaPorte, and T. Friedmann.** 1981. Nucleotide sequence changes in polyoma *ts-a* mutants: correlation with protein structure. J. Virol. **37**:871–875.
8. **Ding, D., S. M. Dilworth, and B. E. Griffin.** 1982. *mlt* mutants of polyoma virus. J. Virol. **44**:1080–1083.
9. **Eckhart, W.** 1974. Genetics of DNA tumor viruses. Annu. Rev. Genet. **8**:301–317.
10. **Eckhart, W.** 1974. Properties of temperature-sensitive mutants of polyoma virus. Cold Spring Harbor Symp. Quant. Biol. **39**:37–40.
11. **Francke, B., and W. Eckhart.** 1973. Polyoma gene function required for viral DNA synthesis. Virology **55**:127–135.
12. **Fried, M.** 1970. Characterization of a temperature-sensitive mutant of polyoma virus. Virology **40**:605–617.
13. **Garcea, R. L., and T. L. Benjamin.** 1983. Host range transforming gene of polyoma virus plays a role in virus assembly. Proc. Natl. Acad. Sci. U.S.A. **80**:3613–3617.
14. **Gaudray, P., C. Tyndall, R. Kamen, and F. Cuzin.** 1981. The high affinity binding site on polyoma virus DNA for the viral large-T protein. Nucleic Acids Res. **9**:5697–5710.
15. **Gélinas, C., L. Bouchard, and M. Bastin.** 1981. Tumorigenic activity of cloned polyoma virus DNA in newborn rats. Experientia **37**:1074–1075.
16. **Germond, J. E., V. M. Vogt, and B. Hirt.** 1974. Characterization of the single-strand-specific nuclease S_1 activity on double-stranded supercoiled polyoma DNA. Eur. J. Biochem. **43**: 591–600.
17. **Giles, K. W., and A. Myers.** 1965. An improved diphenylamine

method for the estimation of deoxyribonucleic acid. Nature (London) **206**:93.
18. **Griffin, B. E., and S. M. Dilworth.** 1983. Polyomavirus: an overview of its unique properties. Adv. Cancer Res. **39**:183–267.
19. **Griffin, B. E., and C. Maddock.** 1979. New classes of viable deletion mutants in the early region of polyoma virus. J. Virol. **31**:645–656.
20. **Hattori, J., G. G. Carmichael, and T. L. Benjamin.** 1979. DNA sequence alterations in hr-t deletion mutants of polyoma virus. Cell **16**:505–513.
21. **Hirt, B.** 1967. Selective extraction of polyoma DNA from infected mouse cell cultures. J. Mol. Biol. **26**:365–369.
22. **Ito, Y.** 1980. Organization and expression of the genome of polyoma virus, p. 447–480. *In* G. Klein (ed.), Viral oncology. Raven Press, New York.
23. **Ito, Y., N. Spurr, and R. Dulbecco.** 1977. Characterization of polyoma virus T antigens. Proc. Natl. Acad. Sci. U.S.A. **74**:1259–1263.
24. **Ito, Y., N. Spurr, and B. E. Griffin.** 1980. Middle T antigen as primary inducer of full expression of the phenotype of transformation by polyoma virus. J. Virol. **35**:219–232.
25. **Khandjian, E. W., J. M. Matter, N. Léonard, and R. Weil.** 1980. Simian virus 40 and polyoma virus stimulate overall cellular RNA and protein synthesis. Proc. Natl. Acad. Sci. U.S.A. **77**:1476–1480.
26. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**:265–275.
27. **Magnusson, G., M.-G. Nilsson, S. M. Dilworth, and N. Smolar.** 1981. Characterization of polyoma mutants with altered middle and large T-antigens. J. Virol. **39**:673–683.
28. **Matter, J. M., J. M. Tiercy, and R. Weil.** 1983. Sequential stimulation of cellular RNA synthesis in polyoma-infected mouse kidney cells. Nucleic Acids Res. **11**:6611–6629.
29. **Mes, A.-M., and J. A. Hassell.** 1982. Polyoma viral middle T-antigen is required for transformation. J. Virol. **42**:621–629.
30. **Nilsson, S. V., and G. Magnusson.** 1983. T-antigen expression by polyoma mutants with modified RNA splicing. EMBO J. **2**:2095–2101.
31. **Novak, U., S. M. Dilworth, and B. E. Griffin.** 1980. Coding capacity of a 35% fragment of the polyoma virus genome is sufficient to initiate and maintain cellular transformation. Proc. Natl. Acad. Sci. U.S.A. **77**:3278–3282.
32. **Rassoulzadegan, M., Z. Naghashfar, A. Cowie, A. Carr, M. Grisoni, R. Kamen, and F. Cuzin.** 1983. Expression of the large T protein of polyoma virus promotes the establishement in culture of "normal" rodent fibroblast cell lines. Proc. Natl. Acad. Sci. U.S.A. **80**:4354–4358.
33. **Ruley, H. E., and M. Fried.** 1983. Sequence repeats in a polyoma virus DNA region important for gene expression. J. Virol. **47**:233–237.
34. **Salomon, C., H. Türler, and R. Weil.** 1977. Polyoma-induced stimulation of cellular RNA synthesis is paralleled by changed expression of the viral genome. Nucleic Acids Res. **5**:1483–1503.
35. **Schlegel, R., and T. L. Benjamin.** 1978. Cellular alterations dependent upon the polyoma virus hr-t function: separation of mitogenic and transforming capacities. Cell **14**:587–599.
36. **Schneider, W. C.** 1957. Determination of nucleic acids in tissues by pentose analysis. Methods Enzymol. **3**:680–684.
37. **Seif, R., and F. Cuzin.** 1977. Temperature-sensitive growth regulation in one type of transformed rat cells induced by the *tsa* mutant of polyoma virus. J. Virol. **24**:721–728.
38. **Smolar, N., and B. E. Griffin.** 1981. DNA sequences of polyoma virus early deletion mutants. J. Virol. **38**:958–967.
39. **Soeda, E., J. R. Arrand, N. Smolar, J. E. Walsh, and B. E. Griffin.** 1980. Coding potential and regulatory signals of the polyoma virus genome. Nature (London) **283**:445–453.
40. **Thomas, T., P. Vollmer, and W. R. Folk.** 1981. Nucleotide sequence changes in polyoma virus *A* gene mutants. J. Virol. **37**:1094–1098.
41. **Tooze, J. (ed.).** 1980. DNA tumor viruses, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
42. **Treisman, R., A. Cowie, J. Favaloro, P. Jat, and R. Kamen.** 1981. The structures of the spliced mRNAs encoding polyoma virus early region proteins. J. Mol. Appl. Genet. **1**:83–92.
43. **Treisman, R., U. Novak, J. Favaloro, and R. Kamen.** 1981. Transformation of rat cells by an altered polyoma virus genome expressing only the middle T protein. Nature (London) **292**:595–600.
44. **Türler, H.** 1975. Interactions of polyoma and mouse DNAs. III. Mechanism of polyoma pseudovirion formation. J. Virol. **15**:1158–1167.
45. **Türler, H.** 1980. The tumor antigens and the early functions of polyoma virus. Mol. Cell. Biochem. **32**:63–93.
46. **Tyndall, C., G. La Mantia, C. M. Thacker, J. Favaloro, and R. Kamen.** 1981. A region of the polyoma virus genome between the replication origin and late protein coding sequences is required in cis for both early gene expression and viral DNA replication. Nucleic Acids Res. **9**:6231–6250.
47. **Weil, R.** 1978. Viral tumor antigens, a novel type of mammalian regulator protein. Biochim. Biophys. Acta **516**:301–388.
48. **Weil, R., and J. Kara.** 1970. Polyoma "tumor antigen": an activator of chromosome replication? Proc. Natl. Acad. Sci. U.S.A. **67**:1011–1017.
49. **Weil, R., E. May, P. May, and H. Türler.** 1974. A study on polyoma and SV40-induced chromosome replication and mitosis, p. 397–419. *In* Molecular studies in viral neoplasia. 25th Annual Symposium on Fundamental Cancer Research. The University of Texas, M.D. Anderson Hospital and Tumor Institute at Houston. The Williams & Wilkins Co., Baltimore.
50. **Winocour, E.** 1963. Purification of polyoma virus. Virology **19**:158–168.