JOURNAL OF VIROLOGY, Mar. 1980, p. 1215-1220
0022-538X/80/03-1215/06$02.00/0

Vol. 33, No. 3

Viable Deletion Mutant in the Medium and Large T-Antigen-Coding Sequences of the Polyoma Virus Genome

MARY M. BENDIG,† THRESIA THOMAS, AND WILLIAM R. FOLK*

Department of Biological Chemistry, The University of Michigan, Ann Arbor, Michigan 48109

A polyoma virus mutant that maps in the early region between the known *hr-t* and *ts-a* mutants has been isolated. Its 66-base-pair deletion results in structural changes in both medium and large T-antigens but causes no substantial alterations in viral replication or cell transformation.

The early region of the polyoma virus genome contains two known complementation groups defined by mutants, the host-range-nontransforming (*hr-t*) mutants and the temperature-sensitive *ts-a* mutants. The *hr-t* mutants map in the proximal portion of the early region. They induce an altered host range for lytic growth and are defective in virus-induced cell transformation and tumorigenesis (3, 8). The *ts-a* mutants map in the distal portion of the early region and are defective, at the nonpermissive temperature, in DNA replication, control of viral RNA transcription, and virus-induced cell transformation (6, 7, 9, 10, 25, 26). In this report we describe the isolation and partial characterization of a deletion mutant, mutant 45, that maps in the early region between the *hr-t* and *ts-a* mutants.

Mutant 45 was detected while screening for mutants with deletions at the endo *BglI* site near the origin of replication. The isolation procedure has been described elsewhere (1). In brief, form I DNA from unmutagenized Pasadena large-plaque polyoma virus was digested with endo *BglI* (which cuts polyoma DNA at one site) and then electrophoresed through a 1% agarose gel. The resulting form III linear DNA was extracted from the gel and digested first with S1 nuclease (14 U of enzyme per 2.5 μg of DNA for 30 min at 25°C) and then with exonuclease III (0.4 U of enzyme per 1.2 μg of DNA for 60 min at 37°C). Secondary whole mouse embryo cells were infected with the digested DNA, and from the resulting virus stock individual plaques were picked and tested for alterations in their endo *HpaII* digestion patterns. Mutant 45 gave a wild-type size *HpaII* fragment 5 (*HpaII-5* contains the endo *BglI* site, Fig. 1) but was missing wild-type size *HpaII* fragments 4 and 8. A new fragment slightly smaller in size than that expected of a composite *HpaII-4/8* fragment was observed (Fig. 2), thus suggesting that mutant 45 has a deletion removing the endo *HpaII-4/8* site. The altered virus was plaque purified once more before being further characterized.

Digestion of mutant 45 DNA with endonucleases *AluI* (4) and *HaeIII* (12) indicated that a 60- to 100-base-pair deletion had occurred between (but not including) the *AvaI* site and the *PvuII* site located on either side of the *HpaII-4/8* junction. Accordingly, mutant 45 DNA was cleaved with endonuclease *AvaI* and incubated

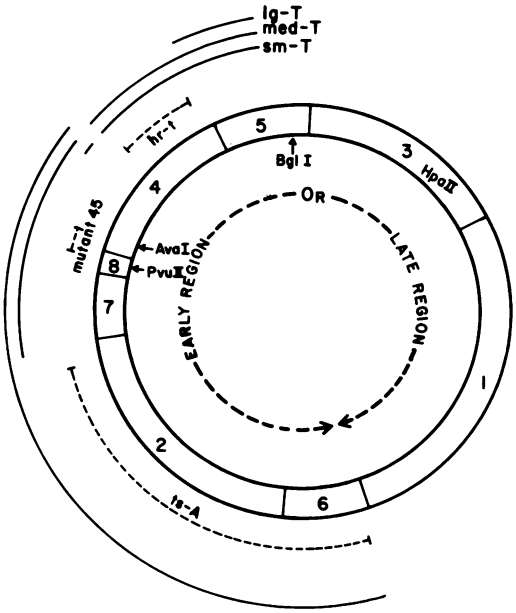

FIG. 1. *Map of the polyoma virus genome. The circular map is divided into the the eight HpaII fragments (12). The single endo BglI site and the endo AvaI and endo PvuII sites mapping near the endo HpaII-4/8 site are indicated by arrows (11). On the outer edge of the map, dotted lines define the location of the hr-t mutants (14), the ts-a mutants (25), and mutant 45; solid lines define the predicted coding regions for the small, medium, and large T-antigens (11, 30; R. Kamen, personal communication). On the inside of the map, the origin of replication and the early and late regions of the genome are indicated (19, 21).*

† Present address: Institut für Molekularbiologie II der Universität Zürich, 8093 Zürich, Switzerland.

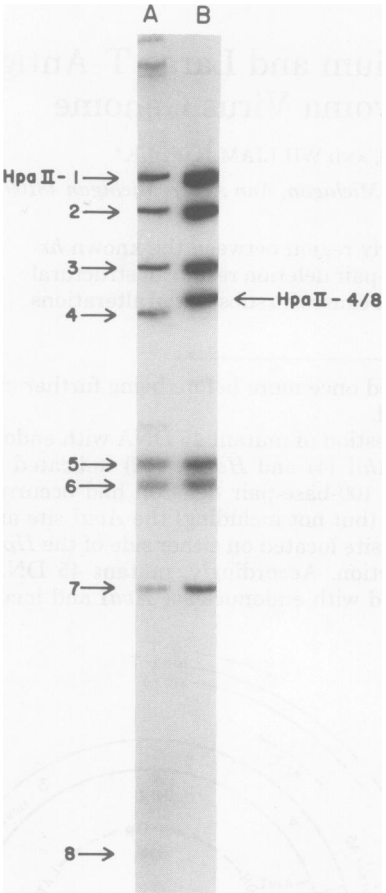

FIG. 2. *Autoradiogram of polyoma DNA digested with endo HpaII. Endo HpaII digests of [^{32}P]DNA from the wild type (slot A) and mutant 45 (slot B) were analyzed by electrophoresis in a 6% polyacrylamide gel (2). Arrows on the left point to the wild-type HpaII fragments. The arrow on the right points to the composite HpaII-4/8 fragment found in mutant 45.*

with [γ-^{32}P]ATP and polynucleotide kinase and sequenced by the chemical degradation procedure of Maxam and Gilbert (24). Comparison of its sequence with that of the wild-type parent revealed that a 66-nucleotide deletion had occurred between nucleotides 1090 and 1157 (Friedmann numbering system [11]; Fig. 3). Because the bases deleted are a multiple of three, the reading frame is not altered.

Inspection of the sequence in this region suggests a possible reason for the susceptibility of polyoma DNA to S1 nuclease. Between nucleotides 1132 and 1183 occurs a partial palindrome which could form a hairpin (Fig. 4). This might provide a region of single-stranded DNA susceptible to S1 nuclease.

The early region of the polyoma virus genome

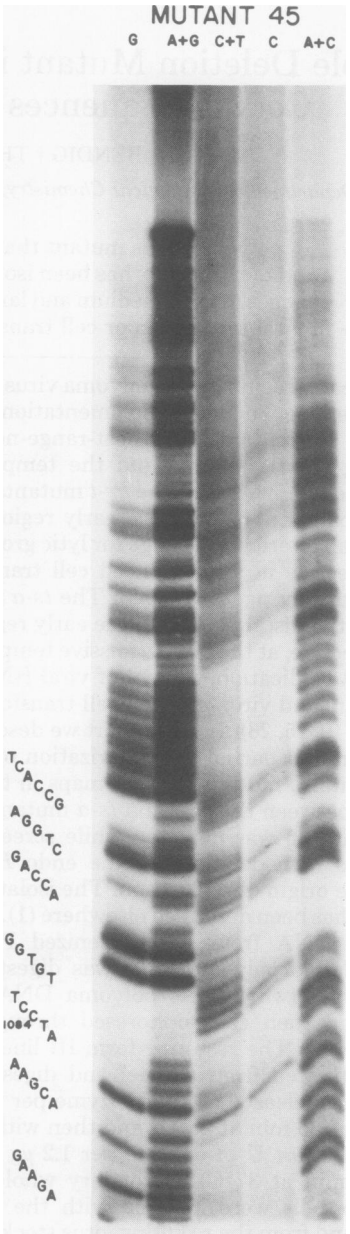

FIG. 3. *Autoradiogram of polyoma DNA subjected to chemical degradation and gel electrophoresis. Polyoma DNAs (mutant 45 and its wild-type parent) were cloned in Escherichia coli with pBR322 (Bendig et al., manuscript in preparation). Endonuclease AvaI digestion of the purified chimeric DNA generated a fragment containing the majority of the polyoma early region. This was labeled with [γ-^{32}P]ATP and polynucleotide kinase and then digested with endonuclease EcoRI. The asymmetrically labeled DNAs were separated and subjected to chemical degradation (10 min). The products were separated on 15% polyacrylamide gels.*

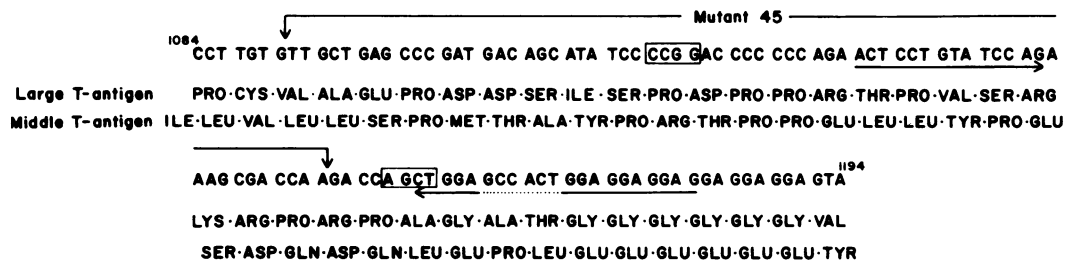

FIG. 4. *Sequence of the region of wild-type polyoma DNA including the mutant 45 deletion. The nucleotide sequence of the parent of mutant 45 was in complete agreement with that determined by Friedmann et al. (11) and Soeda et al. (30). Arrows point to the region deleted in mutant 45. Underlined sequences indicate a partial palindrome which could form an intrastrand loop. The HpaII-4/8 and PvuII sites indicated in Fig. 1 are enclosed in boxes. Amino acid sequences for middle and large T-antigens are indicated (11, 30).*

codes for at least three tumor antigens of approximately 95,000 (95K), 60K, and 22K molecular weight (15, 16, 18, 29). In *ts-a* mutants, large T-antigen (95K) is thermolabile, whereas the 60 and 22K T-antigens remain unaffected (16, 18, 26, 29). In contrast, the *hr-t* mutations affect the 60 and 22K antigens but not the 95K T-antigen (16, 17, 27, 29). To determine whether mutant 45 synthesized early viral proteins of normal size and amounts, 3T6 cells were infected, the proteins were labeled with [^{35}S]methionine, and the T-antigens were immunoprecipitated and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. As seen in Fig. 5, large and medium T-antigens from mutant 45-infected cells migrate ahead of those from wild type. Small T-antigen, however, is the same in both mutant 45- and wild-type-infected cells. (The 37 and 62K protein bands are believed to be analogous to the 37 and 55K species that Hutchinson et al. [16] found by tryptic peptide analysis to be unrelated to small, medium, or large T-antigen.) The protein band labeled VP1 corresponds in size to the major polyoma virus capsid protein, VP1. Cells infected wth a small-plaque strain of polyoma virus have an altered VP1 (Fig. 5B). Size estimates of the mutant 45 T-antigens indicate that large T-antigen is 3,000 to 4,000 daltons smaller than wild type and that medium T-antigen is approximately 2,000 daltons smaller. The size of the deletion in mutant 45 DNA predicts a 2,600-dalton reduction in protein molecular mass. Chasing the ^{35}S-labeling medium for 6 or 12 h with medium containing an excess of unlabeled methionine revealed no significant differences between mutant 45 and wild-type virus in the amounts and stability of the T-antigens (Fig. 5).

Schaffhausen et al. have observed in vivo phosphorylation of large and medium T-antigens and no phosphorylation of small T-antigen, when infected cells are incubated with [^{32}P]-

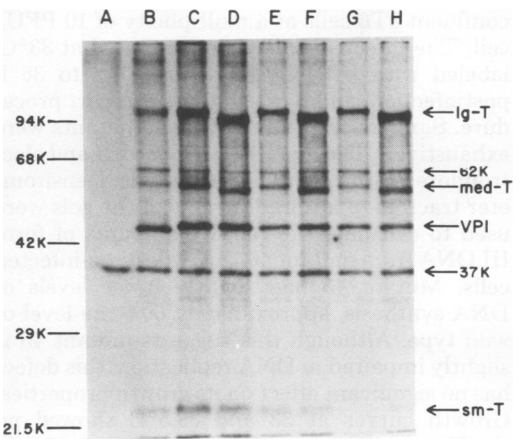

FIG. 5. *Fluorogram of T-antigens labeled with [^{35}S]methionine. Cultures of 3T6 cells were infected with Crawford small-plaque polyoma virus (multiplicity of infection = 10). The infected cells were incubated at 33°C for 43 h, labeled for 2 h with [^{35}S]methionine (100 μCi/ml), and harvested by the procedure of Hutchinson et al. (16). In pulse-chase experiments, cells were washed and chased with medium containing 10 times the normal concentration of methionine before harvesting. Immunoprecipitation of radiolabeled cell extracts was carried out by a modified Staphylococcus protein A procedure (16). Anti-polyoma tumor serum was kindly provided by T. Benjamin. The immune precipitates were electrophoresed through a discontinuous sodium dodecyl sulfate-polyacrylamide gel (5 and 12% acrylamide) (20) which was then stained and prepared for fluorography (5). The protein molecular weight markers, phosphorylase b (94K), bovine serum albumin (68K), ovalbumin (43K), carbonic anhydrase (29K), and soybean trypsin inhibitor (21.5K), are indicated on the left. Slot A is from mock-infected cells, slot B is from Crawford small-plaque-infected cells, slots C, E, and G are from wild-type large-plaque-infected cells, and slots D, F, and H are from mutant 45-infected cells. Samples in slots E and F were chased for 6 h before harvesting, and those in slots G and H were chased for 12 h.*

phosphate (27). To determine whether the altered medium and large T-antigens from mutant 45 retain their sites for phosphorylation, 3T6 cells were infected and labeled wth [^{32}P]phosphate, and the T-antigens were immunoprecipitated. Figure 6 shows that in both mutant 45 and wild-type viruses, large T-antigen is heavily phosphorylated, whereas medium T-antigen has a low level of phosphorylation.

Mutant 45 was tested for defects in known early gene functions. As mentioned previously, one class of early gene mutants, the *ts-a* mutants, affect a viral function required for the initiation of viral DNA replication (9). The relative amounts of viral DNA synthesis in cells infected with either mutant or wild-type virus was determined by infecting duplicate plates of confluent 3T6 cells at a multiplicity of 10 PFU/cell. The infected cells were incubated at 33°C, labeled with [^{32}P]phosphate from 12 to 36 h postinfection, and harvested via the Hirt procedure. Samples from the Hirt supernatants were exhaustively digested with endo *Eco*RI and electrophoresed through 1% agarose gels. Densitometer tracings of autoradiograms of the gels were used to calculate the relative amounts of form III DNA from mutant 45- and wild-type-infected cells. Mutant 45 had slightly lower levels of DNA synthesis, approximately 60% the level of wild type. Although this suggests mutant 45 is slightly impaired in DNA replication, this defect has no significant effect on its growth properties. Growth curves at 33 and 39.5°C showed no significant differences between mutant 45 and wild-type viruses (data not shown).

Both classes of previously characterized early region mutants are defective in virus-induced cell transformation (3, 10). Mutant 45 was tested for its ability to transform BHK cells or Fisher rat 3T3 cells to anchorage independence as determined by growth in soft agar. From the results of these transformation assays (Table 1), it appears that mutant 45 transforms as efficiently as wild-type virus. In addition, preliminary experiments indicate that mutant 45 induces angiomatous lesions of the liver and lungs of Syrian hamsters when injected into newborns.

Although mutant 45 is still able to carry out important early gene functions such as the initiation of viral DNA replication and the transformation of cells, two early gene products, medium and large T-antigens, have reduced molecular weights. DNA sequencing of the early region, mapping of the early mRNA's, and tryptic peptide analysis of early gene products have permitted the identification of the approximate coding regions for the three T-antigens (11, 30; R. Kamen, personal communication). As illustrated in Fig. 1, the deletion in mutant 45 lies within the coding regions for medium and large T-antigens and thus would be expected to alter medium and large T-antigens but not to affect small T-antigen.

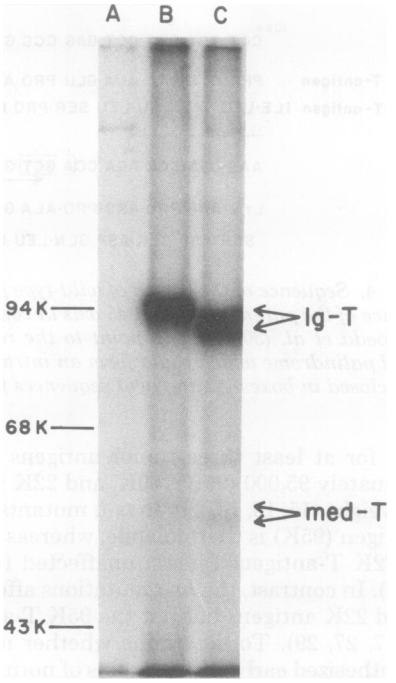

FIG. 6. *Autoradiogram of T-antigens labeled with [^{32}P]phosphate. Cultures of 3T6 cells were infected with wild-type and mutant 45 viruses (multiplicity of infection = 10). After 42 h at 33°C, the cells were washed twice with phosphate-free medium and then labeled for 4 h with [^{32}P]phosphate (100 μCi/ml). Cell extracts were prepared and immunoprecipitated as described for ^{35}S-labeled cells. The immunoprecipitates were analyzed on a 4 and 8% discontinuous sodium dodecyl sulfate-polyacrylamide gel. The gel was stained with Coomassie brilliant blue, destained, dried, and autoradiographed. Molecular weight markers are indicated on the left. Slot A is from mock-infected cells, slot B is from wild type, and slot C is from mutant 45.*

The amino acids within middle and large T-antigens that are deleted in mutant 45 are shown in Fig. 4. The most noteworthy sequence of amino acids, a stretch of six glutamic acid residues in middle T-antigen, is just beyond the limits of the deletion.

Several other viable mutants of polyoma virus with deletions in the middle of the early region have recently been reported. Magnusson and Berg have isolated several mutants with the deletions in *Hpa*II-7 or -8 with lowered capacities for transforming rat 1 fibroblasts (23). The precise location of these deletions is not known, however. Griffin and Maddock (13) have isolated

TABLE 1. *Transformation assays*[a]

Expt	Cell	Virus	MOI	Transformed colonies/infected cells
1	BHK	Mock	0	$0/5.6 \times 10^4$
		Wild type	21	$73/5.6 \times 10^4$
		Mutant 45	61	$143/5.6 \times 10^4$
2	BHK	Mock	0	$0/4.0 \times 10^4$
		Wild type	80	$94/4.0 \times 10^4$
		Mutant 45	70	$56/4.0 \times 10^4$
3	FR3T3	Mock	0	$0/2.0 \times 10^4$
		Wild type	60	$90/2.0 \times 10^4$
		Mutant 45	280	$296/2.0 \times 10^4$

[a] Baby hamster kidney cells (BHK-21/13 from G. DiMayorca) or Fisher rat 3T3 cells (FR3T3 from R. G. Martin) (28) were mock infected or infected with polyoma virus at the indicated multiplicities (MOI). Infected cells were plated in a layer of 0.33% agar medium over a base layer of 0.5% agar medium (22). After 28 days at 37°C for BHK cells or 33°C for FR3T3 cells, large macroscopically visible colonies were scored as transformed.

and characterized two mutants (dl-8 and dl-23) in this region. The dl-8 mutant maps on one side of mutant 45 toward the origin, and its approximately 100-base-pair deletion may slightly overlap the 66-base-pair deletion in mutant 45. The approximately 100-base-pair deletion in dl-23 maps on the other side of mutant 45, but there is likely to be little or no overlap between the deletions in mutants dl-23 and 45. Together, mutant 45, dl-8 and dl-23 delete about 250 contiguous base pairs (approximately 10% of the early region). Like mutant 45, both dl-8 and -23 have truncated medium and large T-antigens and normal-size small T-antigen; however, unlike mutant 45, dl-8 and dl-23 exhibit altered replication or transformation properties. These viable deletion mutants constitute a new class of mutants in the early region of the polyoma virus genome. It is of interest that this region shares no homology with that of simian virus 40 or BK virus (11, 30). It seems that their large T-antigens are functional without such a sequence component.

We thank Patricia Vollmer and Roseann White for help in preparing the secondary whole mouse embryo cells. The gift of polyoma anti-T serum from T. Benjamin is gratefully acknowledged.

Support was provided by Public Health Service grant CA 13978 from the National Cancer Institute, training grant GM 07135 to M.M.B. from the National Institute of General Medical Sciences, and American Cancer Society Faculty Research Award FRA 152 to W.R.F.

LITERATURE CITED

1. **Bendig, M. M., and W. R. Folk.** 1979. Deletion mutants of polyoma virus defining a nonessential region between the origin of replication and the initiation codon for early proteins. J. Virol. **32**:530–535.
2. **Bendig, M. M., W. R. Folk, and W. Gibson.** 1979. Polyoma viruses with mutations at endonuclease *Hin*dII site 1: alterations at the COOH terminus of VP1. J. Virol. **30**:515–522.
3. **Benjamin, T. L.** 1970. Host range mutants of polyoma virus. Proc. Natl. Acad. Sci. U.S.A. **67**:394–399.
4. **Berkner, K. L., and W. R. Folk.** 1979. A map of the sites in the polyoma genome cleaved by endonuclease AluI. Virology **92**:682–696.
5. **Bonner, W. M., and R. A. Laskey.** 1974. A film detection method for tritium-labeled and nucleic acids in polyacrylamide gels. Eur. J. Biochem. **46**:83–88.
6. **Cogen, B.** 1978. Virus-specific early RNA in 3T6 cells infected by a tsA mutant of polyoma virus. Virology **85**: 222–230.
7. **Eckhart, W.** 1969. Complementation and transformation by temperature sensitive mutants of polyoma virus. Virology **38**:120–125.
8. **Feunteun, J., L. Sompayrac, M. Fluck, and T. Benjamin.** 1976. Localization of gene functions in polyoma virus DNA. Proc. Natl. Acad. Sci. U.S.A. **73**:4169–4173.
9. **Francke, B., and W. Eckhart.** 1973. Polyoma gene function required for viral DNA synthesis. Virology **55**: 127–135.
10. **Fried, M.** 1965. Isolation of temperature-sensitive mutants of polyoma virus. Virology **25**:669–671.
11. **Friedmann, T., A. Esty, P. LaPorte, and P. Deininger.** 1979. The nucleotide sequence and genome organization of the polyoma early region: extensive nucleotide and amino acid homology with SV40. Cell **17**:715–724.
12. **Griffin, B. E.** 1977. Fine structure of polyoma virus DNA. J. Mol. Biol. **117**:447–471.
13. **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.
14. **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.
15. **Hunter, T., M. A. Hutchinson, and W. Eckhart.** 1978. Translation of polyoma virus T-antigens *in vitro*. Proc. Natl. Acad. Sci. U.S.A. **75**:5917–5921.
16. **Hutchinson, M. A., T. Hunter, and W. Eckhart.** 1978. Characterization of T-antigens in polyoma-infected and transformed cells. Cell **15**:65–77.
17. **Ito, Y., J. R. Brocklehurst, N. Spurr, M. Griffiths, J. Hurst, and M. Fried.** 1977. Polyoma virus wild type and mutant T-antigens. Inst. Natl. Santé Rech. Med. **9**: 145–152.
18. **Ito, Y., N. Spurr, and R. Dulbecco.** 1977. Characterization of polyoma virus T-antigen. Proc. Natl. Acad. Sci. U.S.A. **74**:1259–1263.
19. **Kamen, R., and H. Shure.** 1976. Topography of polyoma virus messenger RNA molecules. Cell **7**:361–371.
20. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
21. **Lund, E., B. E. Griffin, and M. Fried.** 1977. Polyoma virus defective DNAs. II. Physical map of a molecule with rearranged and reiterated sequences (D74). J. Mol. Biol. **117**:497–513.
22. **Macpherson, I.** 1969. Agar suspension culture for quantitation of transformed cells, p. 214–219. *In* K. Habel and N. P. Salzman (ed.), Fundamental techniques in virology. Academic Press Inc., New York.
23. **Magnusson, G., and P. Berg.** 1979. Construction and analyses of viable deletion mutants of polyoma virus. J. Virol. **32**:523–529.
24. **Maxam, R., and W. Gilbert.** 1977. A new method for sequencing DNA. Proc. Natl. Acad. Sci. U.S.A. **74**:560–564.
25. **Miller, L. K., and M. Fried.** 1976. Construction of the genetic map of the polyoma genome. J. Virol. **18**:824–832.
26. **Paulin, D., and F. Cuzin.** 1975. Polyoma virus T antigen.

I. Synthesis of a modified heat-labile T antigen in cells transformed with the ts-a mutant. J. Virol. **15**:393–397.

27. **Schaffhausen, B. S., J. E. Silver, and T. L. Benjamin.** 1978. Tumor antigen(s) in cells productively infected by wild-type polyoma virus and mutant NG-18. Proc. Natl. Acad. Sci. U.S.A. **75**:79–83.
28. **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.
29. **Silver, J., B. Schaffhausen, and T. Benjamin.** 1978. Tumor antigens induced by nontransforming mutants of polyoma virus. Cell **15**:485–496.
30. **Soeda, E., J. R. Arrand, N. Smolar, and B. E. Griffin.** 1979. Sequence from early region of polyoma virus DNA containing viral replication origin and encoding small, middle and (part of) large T-antigens. Cell **17**:357–370.