

Early Mutants of Polyoma Virus (*dl8* and *dl23*) with Altered Transformation Properties: Is Polyoma Virus Middle T Antigen a Transforming Gene Product?

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The questions raised by Dulbecco (1975)—“Is transformation the result of provirus integration . . . or is it the consequence of the expression of genes present in the provirus?”—have not yet been satisfactorily answered. However, several lines of evidence from recent experiments on many different tumor viruses, as presented in this volume, strongly suggest that viral gene expression is required for transformation, the best evidence still arising from studies on the avian RNA tumor viruses (see Vogt 1977; Hanafusa 1977). With regard to polyoma virus, evidence also exists which indicates that viral gene expression is needed. Studies by Seif and Cuzin (1977) on transformation induced in rat cells by temperature-sensitive *tsA* mutants of polyoma virus suggest that the transformed phenotype is a complex interplay between various types of controls, including expression of a virus-coded protein, as well as cellular changes induced by the virus or selected for during growth in culture. Data from other laboratories (Eckhart 1977; Fluck et al. 1977) show that the ability to transform cells is restored to the otherwise nontransforming host-range transformation (*hr-t*) mutants when they are complemented with another class of polyoma virus mutants, the *tsA* mutants. Further circumstantial evidence is provided by experiments which indicate that although integration between the DNA of an *hr-t* mutant of polyoma virus and cellular DNA does occur, there is no expression of the transformed phenotype (Lania et al. 1979). These data all support the concept that expression of a polyoma virus gene product is needed in order to initiate and maintain cellular transformation.

In an attempt to probe deeper into the question of whether viral gene expression plays a role in transformation by polyoma virus and, if so, what is the relevant gene product (or products), two new types of polyoma virus mutants (designated *dl8* and *dl23*) (Griffin and Maddock 1979) have been studied. Polyoma virus appears to code for three early proteins which are recognized by their antigenic properties and have been designated large T, middle T, and small

T antigens (Ito et al. 1977b). One or more of these proteins is the best candidate for a transforming gene product. The *dl8* and *dl23* mutants are early-region mutants with deletions that affect the sizes of the viral large T and middle T antigens but not the small T antigen (Y. Ito et al., in prep.). In this respect, they differ from previously described polyoma virus mutants. Both the *dl8* and *dl23* mutants produce in cells phenotypic changes that differ from those seen following infection with wild-type polyoma virus. Considered together with data obtained from the other polyoma virus early mutants, i.e., *tsA* and *hr-t* mutants, the properties of the new mutants allow us to make a number of predictions about the roles of the various viral T antigens and to implicate middle T antigen in playing a cardinal role in transformation.

MATERIALS AND METHODS

Virus stocks. Large-plaque strains (A2 or A3) of polyoma virus were used as representative wild-type (*wr*) viral strains (Fried et al. 1975). The viable deletion mutants *dl8* and *dl23* have been previously described (Griffin and Maddock 1979). They were plaque-purified and grown either on whole mouse embryo cells or in primary cultures of baby mouse kidney cells at 32°C in Dulbecco's modified Eagle's medium (E4) containing 10% horse serum. NG-18 virus was a gift of T. Benjamin (Harvard Medical School, Boston, Mass.); NG-18 virus stocks were made following infection of primary cultures of baby mouse kidney cells with this mutant.

Cells. Mouse 3T6 cells were used as permissive cells for the production of viral proteins and DNA. Established lines of rat cells from the Fischer strain F2408 (Freeman et al. 1973) were used for transformation studies. These are designated Rat-1 cells throughout the text.

Isolation of viral proteins. Viral proteins were labeled with [³⁵S]methionine, isolated, and separated

essentially as previously described (Ito et al. 1977a; Ito 1979).

Isolation and analysis of whole-cell DNA. Whole-cell DNA was isolated essentially as described by Gross-Bellard et al. (1973). The DNA solution was treated with proteinase K (Merck) and RNases T1 and T2 to digest protein and RNA. The DNA was phenol-treated between proteinase treatments. The final solution was dialyzed extensively against 10 mM Tris-HCl, 1 mM EDTA (pH 8.0). DNA was cleaved with restriction endonucleases by standard procedures and fragments were separated by electrophoresis on 1% agarose horizontal slab gels (15 cm × 20 cm) using Tris-acetate buffer (Sharp et al. 1973). DNA from the gels was transferred to nitrocellulose paper (BA 85; Schleicher and Schull) and hybridized to a ³²P-labeled polyoma virus DNA probe using the procedure of Southern (1975). High-specific-activity, ³²P-labeled polyoma virus DNA was made by the nick-translation method (Rigby et al. 1977) using [α -³²P]dCTP and [α -³²P]TTP (sp. act. 3000 Ci/mM; Radiochemical Centre, Amersham).

Transformation. The soft-agar assay used was essentially carried out as described by Macpherson and Montagnier (1964). Subconfluent or just confluent Rat-1 cells (low passage) on 90-mm plastic dishes (NUNC) were infected with 1–5 pfu/cell with either wild-type or mutant (*dl8* and *dl23*) viruses for 90 minutes at 39°C. E4 media (10 ml) containing 10% fetal calf serum (FCS) was added, and the cells were left for 2–12 hours at 37°C. The medium was removed and the cells were isolated from the dishes with 0.05% trypsin plus 0.5 mM EDTA. Cells were resuspended in growth medium and plated into soft agar (0.35%). The cells from one 90-mm dish were replated on ten 50-mm dishes, each dish containing a bottom agar layer (6 ml of 0.52% agar) onto which 1.5 ml of soft agar plus cells was added. After about 2 weeks of incubation at 37°C (with occasional feeding), colonies were observed and picked from wild-type and *dl8*-infected cells, the latter giving rise to both large (L) and small (S) colonies and the former only to small colonies. No colonies were ever observed in *dl23*-infected cells. Individual cell clones were grown in E4 media supplemented initially with 10% FCS to establish growth and changed to 5% FCS once the cells began to grow well. Individual cell lines were regrown in soft agar, essentially as described above.

The dense-focus assay was carried out as previously described (Y. Ito et al., in prep.). Cell lines established from dense foci were replated and grown in soft agar as described above.

In vivo experiments. Young adult Fischer rats were injected subcutaneously with about 10⁶ transformed cells per animal. The rats were examined for tumors after 7 days, and subsequently twice weekly. Animals were sacrificed when large tumors (2–3-cm diameter) had developed, and cell lines were established by standard procedures. For animals injected with cells

transformed by *dl8* virus, the time for production of a large tumor was usually about 2 weeks; with cells transformed by wild-type virus, the time required was usually more than 1 month. Experiments on tumors induced slowly by cells transformed by *dl23* are still in progress.

Anchorage-independent growth. Clones of polyoma-virus-transformed Rat-1 cells were obtained as described above. Cultures were removed from dishes (Falcon) with 0.05% trypsin plus 0.5 mM EDTA. Cells were resuspended in growth medium, spun in a refrigerated Sorvall RC3 at 1500 rpm in an HL 8 rotor, resuspended, and diluted in growth medium. Suspensions of each clone were further diluted in growth medium to 10⁵, 10⁴, or 10³ cells/ml. Just 1 ml provided the inoculum for a 60-mm dish (Falcon) for both growth in agar (see above) or on plastic.

For adherent growth on plastic, cultures were set up on 60-mm tissue-culture petri dishes with an inoculum identical to that used in the agar experiments. Cultures were refed with the appropriate media every 3–4 days and the experiment was terminated after 12 days. Adherent cultures were fixed in 10% Formalin and stained with hematoxylin (Harleco), and the total number of colonies was scored. Plating efficiency was between 10% and 40% for all clones.

The number of colonies per field and the diameters of the agar colonies were measured using a calibrated eyepiece reticle (Steinberg and Pollack 1979). The average number of colonies per dish, using a minimum of 50 colonies, was calculated by multiplying the average number of colonies per field by the number of fields per dish. Using individual diameter measurements, the average colony diameter was calculated and thus the total cell volume was determined. Estimating the volume of a single cell to be 3×10^{-5} mm³, the colony volume increase was calculated (Steinberg and Pollack 1979). Colonies that grew to 0.2 mm or larger in diameter were scored separately in the standard agar growth assay.

Plasminogen-activator assay. Cells grown in tissue-culture flasks were assayed for total-cell-associated plasminogen activator (PA) as described by Rifkin and Pollack (1977).

Cytoskeleton. Cells were trypsinized as previously described and resuspended in growth media. The cells were then used to inoculate 35-mm dishes containing coverslips at 5×10^4 cells per dish. After 8 hours, the cultures were refed with growth media supplemented with 0.1% FCS. After a 24–48-hour incubation, the coverslips were removed and the cells were fixed in 3.8% formaldehyde in PBS for 25 minutes. At the end of this period, the coverslips were immersed in 1% NP-40 in PBS for 25 minutes to extract membranes. The coverslips were rinsed twice in PBS for 1 minute. The individual coverslips were then placed on a piece of moist filter paper in a 15-cm petri dish and 8 μ l of rabbit anti-actin IgG (Burrige 1976) was placed on each coverslip. The covered petri dish was then placed

in a 37°C humidified incubator for 45 minutes. At the end of this period, the coverslips were washed by dipping individual coverslips three times into each of three beakers of PBS solution. The coverslips were blotted after each series of three washes. Rhodamine-conjugated goat anti-rabbit IgG (10 μ l; Cappel Labs) was placed on each coverslip, and a second incubation at 37°C for 45 minutes was performed. At the end of this period, the coverslips were again washed by three successive immersions in each of three PBS solutions followed by blotting after the third immersion. The coverslips were placed cell side down on a drop of Aquamount (Lerner Labs) on microscope slides, and the mounting agent was allowed to harden overnight.

Immunofluorescence. Coverslips were observed with a Leitz automicroscope using epifluorescence and a 63X Zeiss oil-immersion objective. One hundred cells on each coverslip were scored as containing organized or disorganized cytoskeletons as described previously (Pollack and Rifkin 1975).

RESULTS

dl8 and *dl23* Mutants Make Truncated Versions of Large T and Middle T Antigens

The viable early mutant of polyoma virus, *dl8*, has a deletion that lies between 89.8 and 91.5 map units on the physical map of the viral genome; *dl23*, another viable early mutant, has a deletion that lies between 92.5 and 94.4 map units (Griffin and Maddock 1979; N. Smolar and B. E. Griffin, in prep.) (see Fig. 1). The prediction from DNA sequencing and other data (Fig. 2) is that these mutants should produce truncated versions of large T and middle T antigens, whereas their small T antigens should be indistinguishable from that produced by wild-type virus. In lytically infected cells, this has been shown previously to be the case (Y. Ito et al., in prep.). That is, the reduction in size of the mutant proteins relative to the corresponding proteins of the wild-type virus, in general, correspond roughly to those predicted from the DNA deletions themselves. However, the size of the middle T antigen induced by *dl23* appears to be slightly smaller than predicted. This has been discussed elsewhere (Y. Ito et al., in prep.). When the sizes of the various T antigens were investigated in Rat-1 cells transformed by *dl8*, the middle T and large T antigens (but not small T antigen) were also found to be smaller than the corresponding proteins observed in cells productively infected by wild-type virus. In many cell lines, they resembled in size, and presumably were identical with, the proteins in cells lytically infected by the *dl8* mutant (see Figs. 3 and 10a). In some transformed cell lines, large T antigen was even more reduced in size than expected. This, for example, was observed in the transformed cell line *dl8/L3* (a cell line established from a large colony grown in soft agar; see Fig. 10b). Cell lines established from foci produced by infection of Rat-1 cells with mutant *dl23* virus had T antigens that were indistinguishable from the cor-

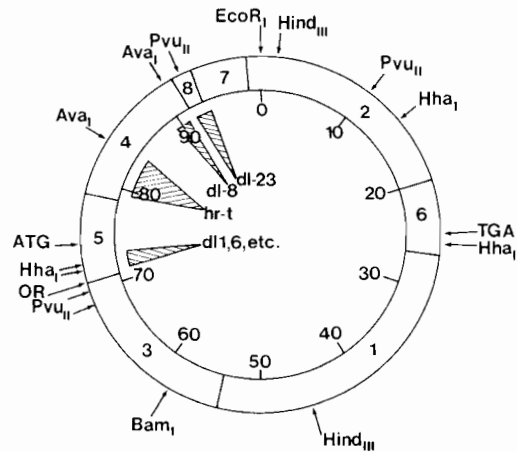


Figure 1. Location of viable deletion mutants on the physical map of polyoma virus (wild-type A2 strain). The standard *Hpa*II physical map of polyoma virus is divided into 100 units (Griffin et al. 1974), with the single *Eco*RI site arbitrarily assigned the value of zero. Some other restriction endonuclease sites are indicated. Mutant *dl8* is missing 90 bp relative to wild-type (A2 strain) polyoma virus DNA, and mutant *dl23* is missing 102 bp. The deletion in the former lies between 89.8 and 91.5 map units and in the latter between 92.5 and 94.4 map units. The *hr-t* mutants have been found to have deletions between about 79.5 and 84 map units (Feunteun et al. 1976; Soeda and Griffin 1978; Hattori et al. 1979), as indicated. Other early viable deletion mutants (*dl1*, *dl6*, etc.) have been found to have deletions between the origin of replication (OR) and the presumed initiation codon (ATG) for the polyoma virus early proteins, as indicated (Griffin and Maddock 1979). Also shown is the presumed termination codon (TGA) for large T antigen (E. Soeda et al., in prep.). Data on the exact location of the deletions in the mutants (*dl1*, *dl6*, *dl8*, and *dl23*) are from N. Smolar and B. E. Griffin (in prep.).

responding proteins found in lytically infected cells.

The amino acids deleted from the middle T and large T antigens in each of the two mutants can be predicted and compared with the sequence predicted for the proteins induced by the wild-type virus (Soeda et al. 1979). Table 1 summarizes the nature of the amino acids that should be missing in the mutants. Notably, a deletion of 13 acidic amino acids, including six glutamic acids in a row (from a total of 34 amino acids), occurs in the middle T antigen of *dl23*. Nonetheless, it has been found that the ability to associate with cellular plasma membranes, characteristic of wild-type middle T antigen (Ito et al. 1977a), is retained by this protein (see Fig. 4). However, it would be surprising if this deletion did not alter some property of the middle T antigen of *dl23*, and this may indeed be the case (see Discussion). The middle T antigen of *dl8* also associates with membranes, as shown in Figure 4.

dl8 and *dl23* Mutants Have Altered Replication and Transformation Properties

Mutants *dl8* and *dl23* appear to be altered in their ability to replicate relative to wild-type virus, the former being poorer and the latter slightly better than the wild-type species. In a study of plaquing efficiency

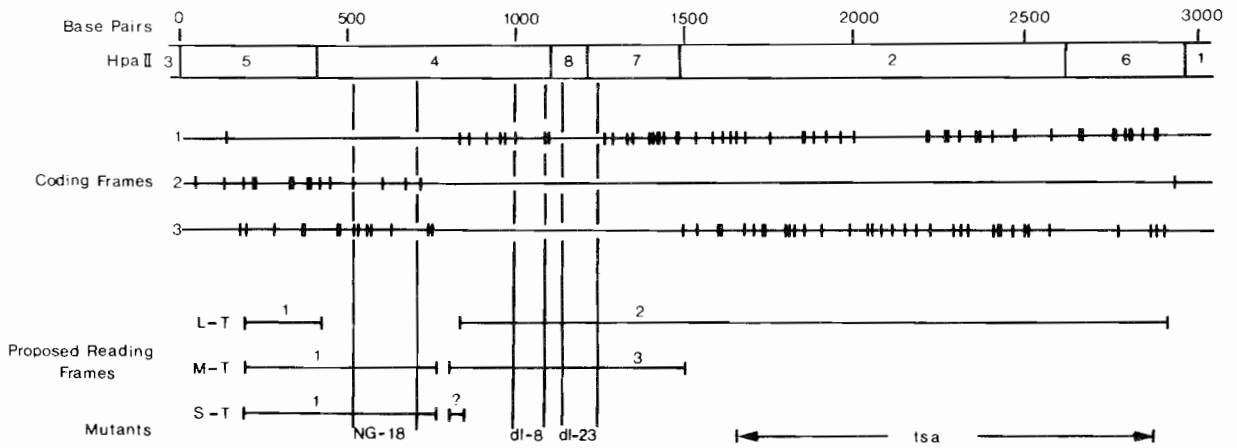


Figure 2. Location of mutants and early proteins on the physical map of the polyoma virus early region. The early region of polyoma virus consists of 2900–3000 bp and encompasses *Hpa*II restriction fragments 5, 4, 8, 7, 2, and (most of) 6. The initiation codon (ATG) for the early proteins is presumed to lie at nucleotide 173. Beginning at this site, the DNA sequence of polyoma virus (A2 strain) has been divided into its three potential coding frames, frame 1 beginning at position 173, frame 2 at 174, and frame 3 at 175. Wherever a potential termination codon (TGA, TAA, or TAG) appears within any frame of the sequence, a vertical bar is drawn. The location of the three early proteins, the large T (L-T), middle T (M-T), and small T (S-T) antigens, as predicted by protein chemistry (Smart and Ito 1978), DNA sequence, and nuclease-S1-mapping of early mRNAs (R. Kamen, pers. comm.), is shown. The locations of mutants *dl8* and *dl23*, as well as the *hr-t* mutant NG-18 and the *tsA* mutants, are indicated. DNA sequence data are taken from Soeda et al. (1979) and E. Soeda et al. (in prep.).

using standard assay conditions, it can be seen that *dl23* virus forms higher-titer stocks and possibly slightly larger plaques than wild-type (A2 strain) virus, whereas *dl8* gives lower-titer stocks and definitely smaller plaques than wild-type virus (see Fig. 5).

In transformation studies in soft agar, the properties

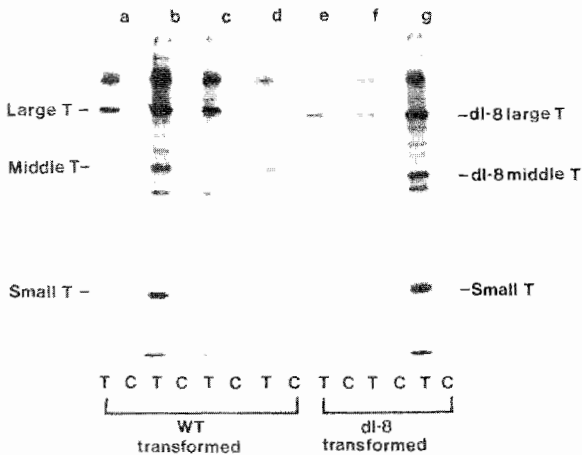


Figure 3. Analysis (by gel electrophoresis) of proteins from polyoma-virus-transformed cell lines. Lines were established from soft-agar colonies formed from Rat-1 cells infected with either wild-type (A2 strain) or mutant (*dl8*) viruses. In the latter case, analysis of small colonies only are shown. Proteins were immunoprecipitated using either rat anti-T serum (T) or control serum (C). The positions of the three early proteins (large T, middle T, and small T antigens) found in wild-type-transformed cells (shown at the left) were indistinguishable from proteins induced in lytically infected cells (Y. Ito et al., in prep.). Cells transformed by *dl8* had truncated versions of large T and middle T antigens (as indicated at the right); small T antigen was not affected by the deletion in the mutant.

observed for the two mutants are entirely different. For example, no colonies have ever been observed in Rat-1 cells infected with *dl23* virus. Since cells infected with *dl23* can form foci, although very slowly (see below), this failure may merely reflect the fact that cells cannot normally be maintained beyond a month, which may be too short a time to observe colonies (see data below) with this mutant. Cells infected with *dl8* virus, on the other hand, appear more extreme in the expression of their transformed properties than corresponding cells infected with wild-type virus (Griffin and Maddock 1979; Y. Ito et al., in prep.). In further studies, it has been consistently shown in soft-agar assays that rat cells transformed by *dl8* virus form two sizes of colonies, which have been rather arbitrarily divided into large (L) and small (S) colonies (see Fig. 6). The L colonies appear to burst out of confinement in agar and essentially grow on top of the agar layer. Some L colonies have diameters about 2 to 4 times larger

Table 1. Predicted Deleted Amino Acids in *dl8* and *dl23* Mutants

| Mutant | T antigen ^a | Amino acids | | | |
|-------------|------------------------|-------------|-------|--------|-----------|
| | | hydrophobic | basic | acidic | uncharged |
| <i>dl8</i> | middle | 16 | 5 | 4 | 5 |
| | large | 13 | 4 | 3 | 10 |
| <i>dl23</i> | middle | 17 | — | 13 | 4 |
| | large | 11 | 4 | — | 19 |

The predicted alterations in the amino acid compositions of two of the early proteins (middle and large T antigens) found in the deletion mutants *dl8* and *dl23*. These deletions do not affect the amino acid composition of small T antigen. Protein sequence predicted from DNA sequence. DNA sequence data of N. Smolar and B. E. Griffin (in prep.) on mutants are compared with the corresponding wild-type sequence of Soeda et al. (1979).

^aNo changes in small T antigen.

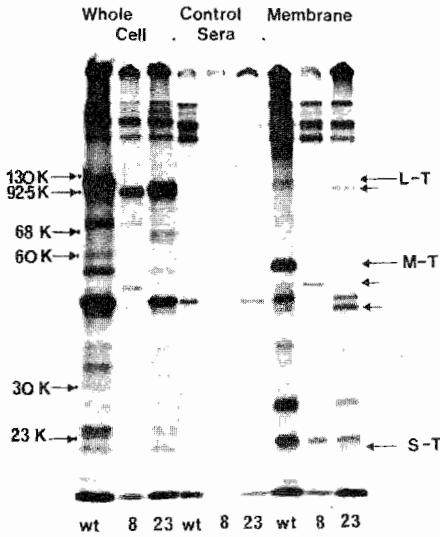


Figure 4. Immunoprecipitation of early proteins (T antigens) of polyoma virus. For this experiment, 3T6 cells were infected with either wild-type or mutant (*dl8* or *dl23*) viruses. [³⁵S]Methionine was added between 24 hr and 26 hr after infection. Proteins from whole cells were precipitated using rat anti-T serum, or preimmune rat (control) serum. Membrane-associated proteins were isolated as described elsewhere (Ito et al. 1977a). Proteins were separated by electrophoresis on SDS-10%-polyacrylamide gels. The positions of proteins used as size markers are indicated at the left, and positions of wild-type large T (L-T), middle T (M-T), and small T (S-T) antigens at the right. Unmarked arrows at the right-hand side indicate the positions of the truncated large T and middle T antigens induced by the mutants *dl8* and *dl23*.

than those of S colonies. *dl8* S colonies correspond in size more nearly to wild-type-transformed colonies.

When cloned cells are regrown in soft agar, those that were originally derived from large colonies again form predominantly L colonies. Those that were originally derived from small colonies yield a mixture of S and L colonies. Analysis by Southern blotting (Southern 1975) of the physical state of the viral DNA in cell lines derived from both S and L lines shows that most of the lines contain free and/or tandemly in-

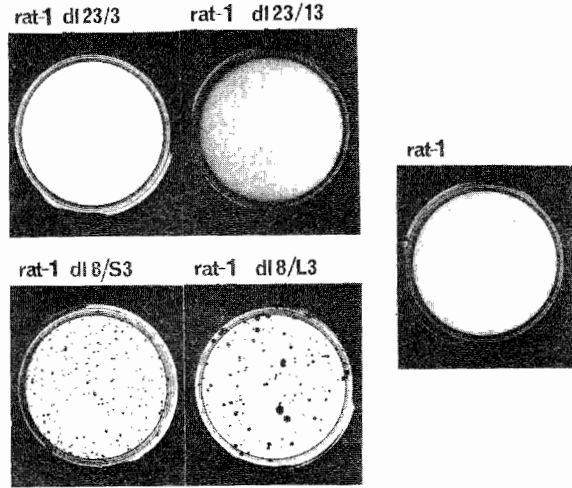


Figure 6. Growth in soft agar of Rat-1 cells transformed by deletion mutants *dl8* and *dl23*. Cell lines *dl23/3* and *dl23/13* were established from dense foci; cell lines *dl8/S3* and *dl8/L3* were established from colonies grown in soft agar. S specifies a small colony and L, a large colony (see Table 4). Control Rat-1 cells are shown. Data (not shown) from cells transformed by wild-type virus (A2 strain) were indistinguishable from that seen with Rat-1-*dl8/S3*.

tegrated viral DNAs, which give restriction enzyme patterns characteristic of the virion DNA of the original *dl8* mutant (see Fig. 7a,b). For example, when the total cellular DNA is cleaved with *PvuII*, the second largest viral restriction endonuclease fragment (1173 bp in wild-type DNA) is reduced in size (Fig. 7a), as would be expected from the position of the deletion in *dl8* (Fig. 1). The patterns observed indicate that the transformation phenotypes are a property of infection with the mutant itself, rather than of some other viral species, and protein data (above) suggest that the mutation is being expressed. The DNA patterns in the transformed lines are complex; integrated sequences are present, but the viral sequences adjacent to host DNA have not yet been characterized. Both S and L lines will grow easily in suspension culture (K. Osborn et al., unpubl.). This may be

Plaque Formation

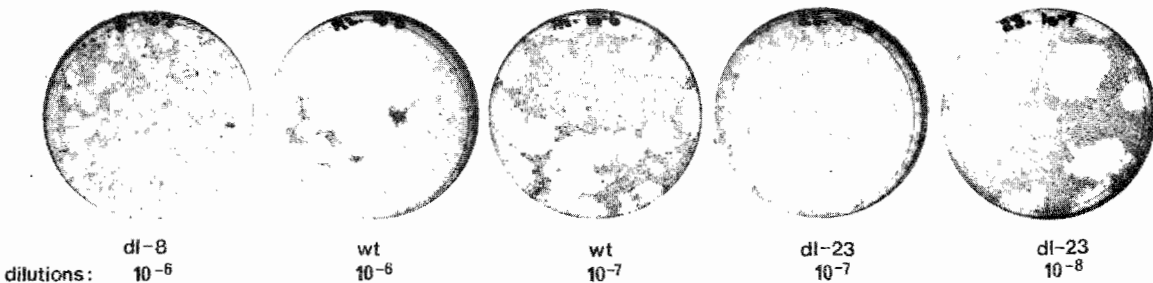


Figure 5. Plaques observed on whole mouse embryo cells following infection with wild-type (A2 strain) and mutant (*dl8* and *dl23*) viruses. Plaques formed with wild-type or *dl23* virus stocks were mainly large, whereas those with *dl8* stocks were consistently small. *dl23* repeatedly yielded two- to fivefold higher titer stocks, and *dl8* about five- to tenfold lower titer stocks, than wild-type virus.

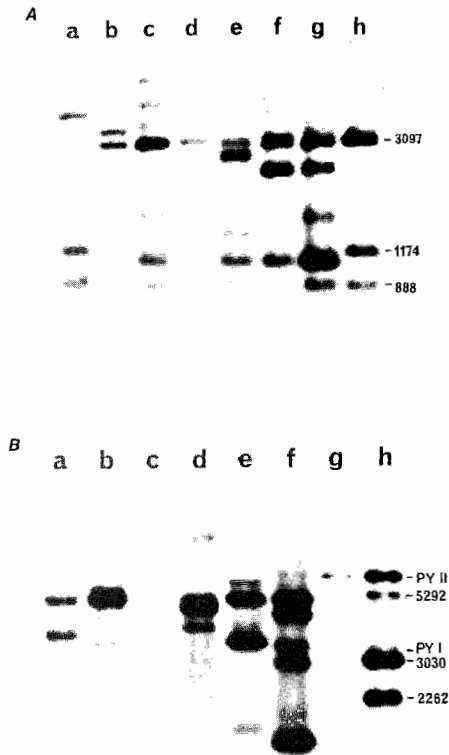


Figure 7. Analysis of viral DNA from Rat-1 cells transformed by wild-type (A2 strain) and mutant (*dl8*) polyoma viruses. Whole-cell DNA was cleaved with restriction endonucleases, separated by gel electrophoresis, and hybridized to ^{32}P -labeled polyoma virus DNA using the blotting technique of Southern (1975). (A) DNA cleaved with *Pvu*II (see Fig. 1). (a) DNA from wild-type-transformed cell line (A2-2); (b,c,d) DNA from cell lines established from large colonies (*dl8*/L1, *dl8*/L2, *dl8*/L3, and *dl8*/L4, respectively) isolated from soft agar following infection with mutant *dl8*; (e,f,g) as above, except established from small colonies (*dl8*/S1, *dl8*/S2, *dl8*/S3, and *dl8*/S4, respectively); (h) three largest *Pvu*II fragments of polyoma virus (wild type) DNA. The number of base pairs in each fragment, given at the right, is taken from DNA sequence data (E. Soeda et al. 1979, and in prep.). (B) DNA cleaved with *Eco*RI (see Fig. 1). (a,b,c) DNA from cell lines established from large colonies (*dl8*/L1, *dl8*/L2, *dl8*/L3, and *dl8*/L4, respectively) isolated from soft agar, following infection of Rat-1 cells with mutant *dl8*; (d,e,f) as above, except established from small colonies (*dl8*/S1, *dl8*/S2, *dl8*/S3, and *dl8*/S4, respectively); (g) marker polyoma virus DNA, form I (PyI) and form II (PyII); (h) marker polyoma virus DNA, forms I, II, and III and the two fragments obtained by cleavage with *Hind*III (see Fig. 1). The numbers of base pairs for form-III DNA (5292) and the two *Hind*III fragments (3030 and 2262) are taken from DNA sequence data.

related to the ability of these cells to produce more PA than is generally seen with cells transformed by wild-type virus (see below).

When dense-focus assays were used as a measure of transformation, the results with the mutants roughly paralleled the soft-agar assays. With Rat-1 cells (or with Balb/c-3T3-A31 cells; Y. Ito et al., in prep.), dense foci of transformed cells appeared in wild-type- and *dl8*-infected cultures within 2 weeks. In *dl23*-

infected cultures, small, slowly growing foci became visible much later, generally after another 1 to 2 weeks of incubation. With the *hr-t* mutant NG-18, foci were not observed. Foci in *dl8*-infected cultures were generally more dense than those in wild-type-infected cultures, and frequently the foci centers became detached from the plastic dish (Griffin and Maddock 1979). Foci in *dl23*-infected cultures remained small. The latter growth pattern is an intrinsic property of the mutant itself and is not due to some cytotoxic effect, as seen in Figure 8. In the experiment shown (Fig. 8), Rat-1 cells were infected with wild-type and mutant viruses, and the transformed cells were replated at different concentrations. Among the cells replated at low concentrations, the number of surviving cells were found to be essentially the same in each case. At higher cell concentrations, easily visible foci become apparent in the cases of wild-type and *dl8*-infected cultures, but not with *dl23* or NG-18. With time, small foci appeared among Rat-1 cells infected with *dl23* virus. Cell lines established from these foci have been further analyzed in the soft-agar assay by replating transformed cells (*dl23* foci) at various concentrations in agar. Although dense, visible colonies were not observed, as was the case with wild-type-transformed or *dl8*-transformed cells, limited growth could be detected by microscopic examination (see Fig. 6). In the case of the Rat-1 *dl23*/13 line, minute visible colonies eventually were seen, but not in the case of the *dl23*/3 line (Fig. 6; see also Table 2). At higher magnification (not shown), colonies were also seen in the latter case.

Other Phenotypes of Rat-1 Clones Transformed by *dl8* and *dl23* Mutants and by Wild-type Virus

For these studies, colonies were isolated as dense foci of rat cells arising after infections by polyoma wild-type, *dl8*, and *dl23* viruses. Additional cell isolates were obtained from agar colonies directly after infection of Rat-1 cells by wild-type and *dl8* viruses. As noted above, transformation with *dl8* leads to a mixture of L and S colonies in agar; only S colonies were cloned and analyzed in the following studies.

Rat-1 cells transformed by different mutant strains of polyoma virus were examined for their expression of a set of in vitro phenotypic changes that have been correlated with tumorigenicity in the SV40-rodent-fibroblast system (Steinberg et al. 1979; Pollack et al., this volume). Properties of cells transformed by mutant virus were compared with cells transformed by wild-type virus; these properties include anchorage-independence, PA production, and alteration of cytoskeletal actin patterns.

Anchorage independence. Anchorage-independent growth is the in vitro marker which appears to correlate best with cellular tumorigenicity in a number of systems (Shin et al. 1975; Barrett et al. 1979; Shin et al., this volume). Control cells (Rat 1) and 13 cell lines

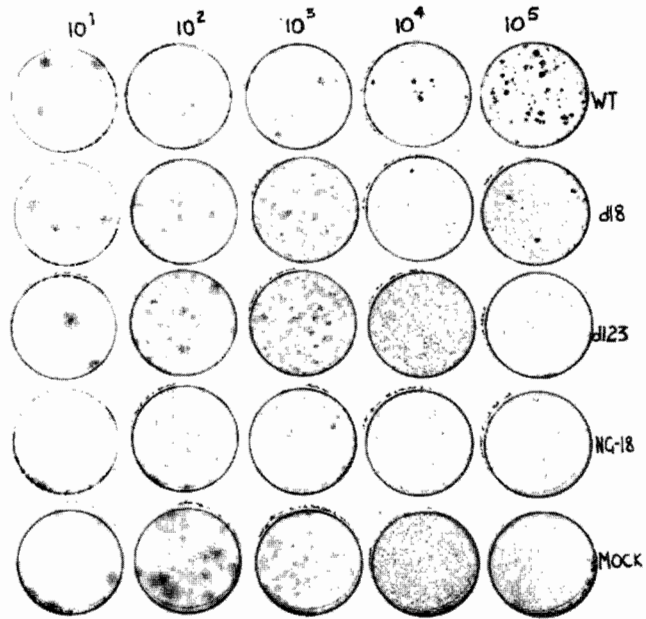


Figure 8. Dense-focus assays. Rat-1 cells were infected with wild-type (A3) and mutant (*dl8*, *dl23*, and NG-18) polyoma viruses, replated at different cell concentrations (10^1 to 10^5), and allowed to grow. Foci are easily discernible only with wild-type and mutant *dl8* at the higher concentrations.

transformed by wild-type or mutant polyoma virus were examined for their ability to multiply in the absence of a solid support. The standard anchorage-independent growth assay does not score cell multiplication unless visible colonies result. Since each visible colony contains 10^3 to 10^4 cells, multiplication up to 10^3 , or about ten divisions, is not scored. The average colony volume increase (CVI) is a measure of total cell division in agar. Both visible colonies and average CVI in agar were measured and the results are summarized in Table 2.

Uninfected Rat-1 cells grew very poorly in agar. Fewer than 1 in 10^4 cells formed a large colony, and the average colony volume was equivalent to less than four divisions. In contrast, cells transformed with wild-type polyoma virus grew very well in agar. The average colony volume was equivalent to more than 10^3 cells and more than half of the colonies were visible. The wild-type-transformed colonies isolated from soft agar were slightly less able to grow when subcultured in agar than were the wild-type-transformed colonies that were picked as dense foci. This

Table 2. Anchorage-independent Growth of Cell Lines Derived from Rat-1 Cells after Transformation by Wild-type and Mutant Polyoma Viruses

| transforming virus | Clone | | Growth in agar | |
|--------------------|---------------------|------------------------|---------------------------|--|
| | origin ^a | name | CVI (n-fold) ^b | visible colonies ^c (% of cells) |
| None | — | Rat 1 ^d | 13 | 0.007 |
| Wild type | focus | Rat-1-wtT | 3100 | 58 |
| | | Rat-1-wtY | 2450 | 140 |
| | | Rat-1-wtZ | 6520 | 30 |
| | agar | Rat-1-wtA1 | 3750 | 6.5 |
| | | Rat-1-wtA2 | 1130 | 7.5 |
| | | Rat-1-wtA6 | 1760 | 6.5 |
| <i>dl8</i> | focus | Rat-1- <i>dl8</i> -T | 1445 | 33 |
| | | Rat-1- <i>dl8</i> -Y | 25 | 10 |
| | | Rat-1- <i>dl8</i> -Z | 2350 | 63 |
| | agar | Rat-1- <i>dl8</i> -A4 | 590 | 7.5 |
| | | Rat-1- <i>dl8</i> -A3 | 6650 | 16 |
| | | | | |
| <i>dl23</i> | focus | Rat-1- <i>dl23</i> /13 | 2000 | 0.8 |
| | | Rat-1- <i>dl23</i> /14 | 105 | 0.01 |

^aFocus: clone picked as dense focus from transformation on plastic; agar: clone picked as sphere from transformation in soft agar.

^bAverage increase in total cellular volume in agar after 12 days; 10^4 , 10^3 cells/plate inoculated.

^cVisible colonies were more than 0.2 mm in diameter.

^dRat-1 data were taken from Steinberg et al. (1979).

result may possibly depend on the fact that only small agar colonies were studied in this experiment, and small colonies may differ in their properties from the larger colonies.

Two of the transformed clones, recovered from foci isolated following infection of Rat-1 cells with *dl8* virus, grew well according to both agar assays, although one (Rat-1-*dl8*-Y) had a very small average colony volume and a reduced fraction of cells that were able to make big colonies. Also in this case the *dl8*-transformed clones isolated directly from agar had a somewhat lower capacity for anchorage-independent growth than cells from dense foci.

Although Rat-1 cells transformed by *dl23* virus could be recovered as dense foci, none arose in agar. The two *dl23* clones examined here were both very inefficient at growing to visible colonies in agar. Fewer than 10^{-2} of the Rat-1-*dl23*/13 cells could be detected as visible colonies, and none were detected with Rat-1-*dl23*/14 (see also Fig. 6 for data obtained with other clones). However, the *dl23* clones did grow in agar, but only to average colony sizes of 2000 and 100 cells, respectively (Table 2). Thus, as with the possibly analogous SV40-*dl54*/59-transformed lines (Steinberg and Pollack 1979), the Rat-1 lines produced by transformation with *dl23* virus can grow in agar, but only much more slowly than transformed cells isolated following infection with wild-type or *dl8* viruses.

Plasminogen activator. Normal fibroblasts produce

little, if any, PA. Many, but not all, transformed cell lines synthesize and secrete this protease, which converts plasminogen to plasmin (Ossowski et al. 1973; Unkeless et al. 1973; Christman and Acs 1974). In a study of a set of SV40-transformed rat cell lines, the production of PA was shown to correlate well with the capacity to form large colonies in agar (Pollack et al. 1975, Rifkin and Pollack 1977).

Total cell-associated PA was measured for the cell lines transformed by wild-type and mutant polyoma viruses described above. The results are given in Table 3. In general, PA activity correlated well with anchorage independence for this set of lines. In particular, the *dl8*-transformed lines established from foci synthesized much more PA than any wild-type clones. Dense foci produced following infection with *dl8* virus attach poorly to plastic, and colonies show hollow centers (Griffin and Maddock 1979). In previous work with SV40-transformed cell lines, this colony morphology has been linked to high PA production (Pollack et al. 1975). Once again, the *dl8*-transformed colonies picked from agar were not as high producers as the isolates of *dl8*-transformed cells from dense foci. Since the colonies derived from soft agar also were slow growing on subculturing in agar (Table 2), they quantitatively extend the correlation between these parameters.

Isolates of *dl23*-transformed cells from dense foci produced a small amount of PA, comparable to that produced by Rat-1 cells (control) and significantly

Table 3. Plasminogen-activator Production and Cytoskeletal Actin Organization of Cell Lines Derived from Rat-1 Cells after Transformation by Wild-type and Mutant Polyoma Viruses

| transforming virus | Clone | | Cytoskeletal actin cables (% in cells) | Plasminogen activator ^a (% fibrin counts released/hr) | |
|--------------------|--------|------------------------|--|--|----------------------------|
| | origin | name | | clone | mean \pm S.D. |
| None | — | Rat 1 ^b | 59 | 1.5 | 1.5 \pm 0.5 |
| Wild type | focus | Rat-1-wtT | 12,9 ^c | 5.5 | 5.8 \pm 2 |
| | | Rat-1-wtY | — | 2.0 | |
| | | Rat-1-wtZ | — | 7.7 | |
| | agar | Rat-1-wtA1 | — | 0.6 | 1.2 \pm 0.8 |
| | | Rat-1-wtA2 | — | 0.8 | |
| Rat-1-wtA6 | | 57,71 | 2.2 | | |
| <i>dl8</i> | focus | Rat-1- <i>dl8</i> -T | — | 16.4 | 12.3 \pm 3 |
| | | Rat-1- <i>dl8</i> -Y | 17,23 | 10.8 | |
| | | Rat-1- <i>dl8</i> -Z | — | 9.8 | |
| | agar | Rat-1- <i>dl8</i> -A4 | 82,79 | 1.5 | 1.2 \pm 0.5 |
| | | Rat-1- <i>dl8</i> -Z | — | 0.8 | |
| <i>dl23</i> | focus | Rat-1- <i>dl23</i> /13 | 76,73 | 2.4 | 2.2 \pm 0.2 ^d |
| | | Rat-1- <i>dl23</i> /14 | 84,84 | 2.0 | |

^aTotal cell extract (about 5 ng of protein) was incubated with iodinated insoluble fibrin for 5 hr as described elsewhere (Crow et al. 1979); average of percent radioactive material released/hr.

^bRat-1 data were taken from Steinberg et al. (1979).

^cScores made by separate observers indicate percent of cells with more than two actin cables seen by immunofluorescence at the plane of adhesion to run the length of the cell.

^dThe production of PA by the clones of *dl23*-transformed cells is approximately equal to the level of PA production observed in uninfected rat primary fibroblasts.

lower than *dl8*- or wild-type-transformed foci (Table 3).

Cytoskeletal actin pattern. Cytoskeletal organization is disrupted in anchorage-independent-transformed cells (Pollack et al. 1975; Goldman et al. 1977; Tucker et al. 1978). Tumor promoters and proteases, especially plasmin, can specifically disrupt the cytoskeleton of normal cells (Pollack and Rifkin 1975; D. Rifkin and R. E. Pollack, in prep.). The cytoskeleton of some of the transformed lines studied above was examined by immunofluorescence with antibody to actin. All cell lines that grew well upon subculturing in agar had disrupted cytoskeletons. Other lines, in particular those established from foci isolated following infection of Rat-1 cells with *dl23* virus and from the clones transformed with wild-type and *dl8* viruses isolated from soft agar, as well as control (Rat-1) cells, had more ordered cytoskeletons as shown in Table 3.

Tumorigenicity of *dl8* and *dl23* Mutant and Wild-type Viruses

Colonies isolated from soft agar (wild-type- and *dl8*-Rat-1-infected cells) or from dense foci (*dl23*-Rat-1-infected cells) were grown on plastic surfaces as described above. Approximately 10^6 cells derived from individual colonies or foci were then injected into young adult, syngeneic (Fischer) animals and tumor formation was followed. Tumors were visible in animals injected with three of the four transformed cell lines derived from *dl8* infection on first examination, i.e., within 7 days following the injection. A fourth line (*dl8/L1*) produced tumors slightly more slowly. In corresponding experiments using transformed cell lines derived from wild-type-infected cells, the average time for tumor formation to be observed was 3 to 4 weeks. Animals injected with one transformed cell line derived from *dl23* (*dl23/13*) produced no tumor within 3 months, whereas another line (*dl23/3*) produced small tumors slowly. (Cell lines have not yet been established from the latter tumors.) The data are summarized in Table 4. Figure 9 shows characteristic tumors produced in rats following injection with transformed cell lines derived from mutant *dl8*. Preliminary analysis of DNA and proteins from a number of individual tumors indicates that tumor formation can be associated with the individual viruses (wild type or mutant) used in the transformation of the cells, which were injected into the animals. These in vivo data, consequently, correlate well with the in vitro phenotypic changes observed for the same cells.

DISCUSSION

The experiments described above consistently indicate that the polyoma virus middle T antigen may play an important role in transformation; the involvement of the large T and small T antigens appears less certain. Middle T antigen was first described as a

Table 4. In Vivo Experiments to Study Tumorigenicity of Mutant and Wild-type Polyoma-virus-transformed Cells

| Cell line | Days after injection when tumors detected | | | | |
|------------------------------|---|----|----|----|----|
| | 21 | 19 | 19 | 26 | 19 |
| Rat-1-PywtA1 | 21 | 19 | 19 | 26 | 19 |
| Rat-1-PywtA6 ^{a,b} | 39 | — | 32 | 49 | — |
| Rat-1-Pydl8/S2 | 7 | 7 | 7 | 7 | 7 |
| Rat-1-Pydl8/S3 | 7 | 7 | 7 | 7 | 7 |
| Rat-1-Pydl8/L1 | 12 | 15 | 12 | 12 | 12 |
| Rat-1-Pydl8/L3 | 7 | 7 | 7 | 7 | 7 |
| Rat-1-Pydl23/3 | 48 | 31 | — | — | 38 |
| Rat-1-Pydl23/13 ^b | — | — | — | — | — |
| Rat 1 | — | — | — | — | — |
| Animals per experiment | 1 | 2 | 3 | 4 | 5 |

Cells (10^6) isolated from in-vitro-transformed lines were injected into young adult Fischer rats. Five animals were used per experiment. The wild-type (*wt*) and *dl8* (S denotes small colony and L denotes large colony) lines were established from soft-agar colonies grown on a continuous Fischer rat cell line, F2408 (Rat 1) (Freeman et al. 1973). *dl23* lines were established from dense foci from mutant-infected Rat-1 cells. No tumors were seen after 3 months in the control (Rat 1) experiment or in animals injected with one of the *dl23* lines (Pydl23/13).

^aAnimals were injected with 6×10^5 cells isolated from this in-vitro-transformed line.

^bOne animal sacrificed for control.

polyoma-virus-induced antigen with a molecular weight of about 55,000 (Ito et al. 1977a) and it was found to be associated with cellular plasma membranes. Membrane association may conceivably be due

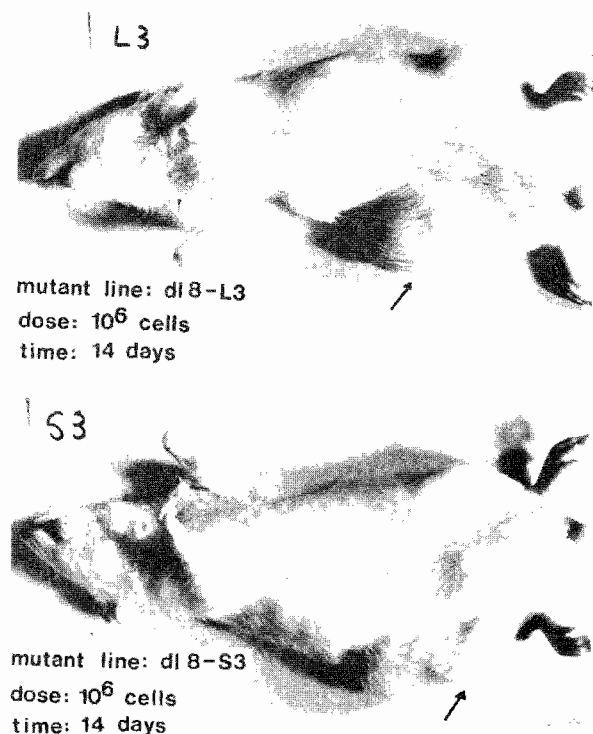


Figure 9. Fischer rats injected with two different transformed cell lines (*dl8/L3* and *dl8/S3*; see Fig. 10a,b and Table 4) derived from *dl8* virus. Tumors seen after 14 days are indicated by arrows. In similar experiments with cell lines derived from wild-type or *dl23* virus, no tumors were visible.

to the presence of a long sequence of hydrophobic amino acids, which has been predicted from the DNA sequence (Soeda et al. 1979) to lie near the C terminus of middle T antigen. This particular sequence is retained in the *dl8* and *dl23* mutants investigated here. The significance of the membrane association is still far from clear, and the transformation properties observed with these early mutants do not seem to be directly related to the actual association, since the middle T antigens induced by mutant and wild-type viruses apparently associate equally well (see Fig. 4), but the viruses transform unequally. The present assignment of a role in transformation to middle T antigen comes from a comparison of properties of the previously described *hr-t* mutants (Benjamin 1970) with the properties observed for the novel mutants *dl8* and *dl23*. The prototype of the nontransforming *hr-t* mutants, NG-18, produces alterations in middle T and small T antigens, but not in large T antigen (Ito et al. 1977b; Schaffhausen et al. 1978). Mutant *dl23* is a very poor transforming mutant, whereas *dl8* appears superior to wild-type polyoma virus in *in vitro* transformation assays. The new mutants have truncated middle T and large T antigens, but they have a small T antigen of normal size. Therefore, the only protein altered in all types of transformation mutants is middle T antigen.

On the polyoma virus genome, the region that appears to code for middle T antigen extends from about 74 to 99 map units (see Fig. 1) and includes about 1300 bp (see Fig. 2). Its sequence can be predicted from the corresponding DNA sequence (Soeda et al. 1979). Support for the assignment of the whole of middle T antigen to this region comes from studies with polyoma virus DNA fragments. Thus, the *BclI-EcoRI* restriction fragment, which extends clockwise from 67 to 0 map units (see Fig. 1), has recently been shown to be effective in the transformation of rat cells. This is the smallest polyoma virus DNA fragment so far shown to be sufficient for the establishment of cellular transformation. Cells transformed by this DNA fragment express a middle T antigen that is indistinguishable from wild-type middle T antigen (U. Novak, unpubl.).

The effect on the amino acid compositions caused by the deletions in the mutants *dl8* and *dl23* is shown in Table 1. The most dramatic changes are observed in the middle T antigen of *dl23*. It may be predicted that the loss of 13 acidic and 17 hydrophobic residues, out of a total of 34 deleted amino acid residues, should affect protein conformation, and thereby function. Whether this structural alteration in the protein is directly related to the appreciable loss of ability to transform cells by the *dl23* mutant remains to be seen.

The data obtained with the *dl8* and *dl23* mutants allow us to speculate that there may exist an inverse relationship between DNA replication and transformation. That is, replication may exert some control over transformation. The mutant (*dl8*) that replicates poorly appears to transform well, and the mutant (*dl23*) that replicates well, and possibly better than wild-type virus, transforms poorly (Griffin and Mad-

dock 1979; see Fig. 5). Replication has been studied in a permissive (mouse) host cell, whereas transformation has been studied in an essentially nonpermissive (rat) host cell. To establish an interrelationship between the ability of the virus to replicate and its ability to transform in a more direct fashion, it will be necessary to study viral replication and transformation in parallel in a nonpermissive or semipermissive cell that allows viral replication. The requirement of a functional large T antigen for viral replication is well established. Therefore it is not surprising that the *dl8* and *dl23* mutants have altered replication properties since the deletions affect the structure of the large T antigen. In this connection, it is interesting to note that the small colonies that form in soft agar when rat cells are transformed with *dl8* virus appear to have a large-T-antigen characteristic of this mutant, that is, only slightly smaller than wild-type large T antigen (see Fig. 10a), whereas in at least one case (*dl8/L3*) a large colony

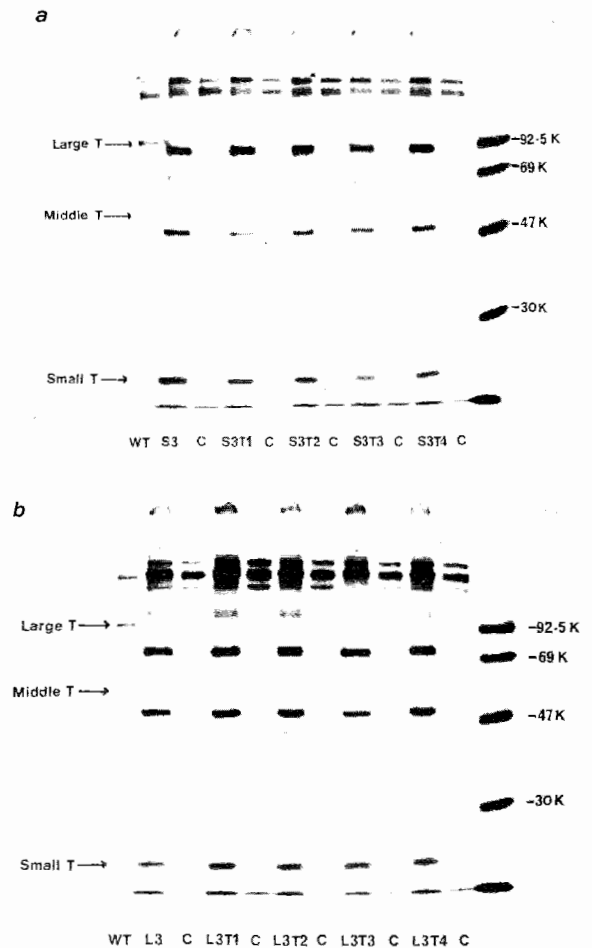


Figure 10. Analysis (by gel electrophoresis) of viral proteins from cell lines derived from tumors. (a) Tumors taken from four separate rats injected with the cell line Rat 1 *dl8/S3* (see Table 4); (b) tumors taken from four separate rats injected with the cell line Rat 1 *dl8/L3* (see Table 4). Proteins in tumor lines (S3T1 to S3T4 and L3T1 to L3T4) are indistinguishable from those seen in the parental lines (S3 and L3, respectively).

contains a further truncated version (about 75K in size) of large T antigen (see Fig. 10b).

To further characterize the transformed state of cells obtained after infection of nonpermissive cells with the *dl8* and *dl23* viruses, three phenotypic markers of oncogenic transformation (CVI, disruption of cytoskeletal actin cables, and increased levels of PA) (see Tables 2 and 3) were studied using colonies and/or foci derived from rat cells infected with the mutants. These markers were found to be expressed very poorly, or not at all, in transformed cells (foci) isolated after infection with *dl23* virus. Cell lines established from foci that formed following infection with *dl8* virus, on the other hand, were at least as able (and in some cases more able) to express these markers than the corresponding wild-type-transformed cells. All these phenotypic markers have already been shown to be well-expressed by cells transformed by wild-type polyoma virus and poorly expressed in cells transformed by the NG-18 *hr-t* mutant (Schlegel and Benjamin 1978). In a further extension of this study, in vivo tumorigenicity studies were carried out in rats and, in general, the results obtained correlated well with the phenotypic markers expressed in vitro. In these experiments (see Table 4), rats injected with *dl8*-transformed cell lines formed tumors faster than rats injected with wild-type-transformed lines. On the other hand, rats injected with *dl23*-transformed (foci) lines formed tumors very slowly, if at all. When all the data are correlated, it is tempting to speculate that polyoma virus middle T antigen may be associated not only with the expression of the in vitro phenotypic markers of transformation but also with in vivo tumor formation.

As discussed above, three early proteins have been described for polyoma virus, which have been designated small T, middle T, and large T antigens. In SV40-infected cells, a small T antigen and a large T antigen are also present, but there appears to be no virus-coded middle T antigen. This notion is supported by recent DNA sequencing studies. Although much (about 80%) of polyoma virus appears, both in organization and sequence, to be similar to SV40 (E. Soeda et al. 1979, and in prep.), the region from about 85 to 0 map units on the physical map of polyoma virus seems to have no counterpart in SV40. This is the region that contains most of the coding sequence for middle T antigen. The possibility may now be considered that, like some of the RNA tumor viruses, polyoma virus has evolved from some more primitive species that lacked the ability to transform, and that through either a reassortment of viral sequence or an incorporation of host or foreign cellular DNA sequence into its genome, became the present transforming virus. The mechanism of transformation by polyoma virus may therefore be rather different from that employed by SV40. Collett and Erikson (1978) showed that the product of the transforming gene of an RNA tumor virus had an associated protein kinase activity. In more recent studies (Collett et al. 1979; Karess et al. 1979), it has been further shown that

uninfected avian cells contain a protein with similar size and properties to the virus-coded transforming protein. However, the normal cell protein, which also had an associated protein kinase activity, was present in substantially smaller amounts than were found for the viral gene product in transformed cells. This led Collett et al. (1979) to speculate that transformation may be a consequence of a quantitative difference in expression of the two genes. In polyoma virus, a protein kinase activity appears to be associated with middle T antigen (A. E. Smith et al., in prep.). In studies with the mutants *dl8* and *dl23* in lytically infected cells, there appears to be a quantitative difference in this activity, i.e., the kinase activity is high in extracts from *dl8*-infected cells and low in extracts from *dl23*-infected cells. Whether the phenotypic properties of transformation observed with these mutants can be correlated with an enzyme activity, such as a kinase, remains to be seen. If so, this may provide further insight into the processes leading to cellular transformation; this might also provide a convenient assay by which to search for transformation-defective polyoma virus strains in wild mice.

This discussion and these studies have raised again many of the problems encountered in attempting to understand transformation by polyoma virus. Studies on the *hr-t* mutants have shown that the presence of a full-sized large T antigen is not enough to allow for either transformation or tumor formation. Studies with deletion mutants *dl8* and *dl23* make it clear that a normal-sized small T antigen is insufficient for the expression of all of the phenotypic markers of transformation, although some role for small T antigen cannot be entirely disallowed. It may be postulated, for example, that foci that form following viral infection result from the expression of a functional small T antigen, and that the latter may possibly act as a promoter of transformation, but not in itself be enough to allow for the full transformation phenotype to be expressed. Alternatively, in polyoma virus, small T antigen may be an anachronism, its role(s) having been taken over by middle T or (in part) large T antigen. These hypotheses will be difficult to test, however, because most, if not all, of the DNA sequence coding for small T antigen also codes for parts of middle T antigen, as well as for some of large T antigen (Soeda et al. 1979). In conclusion, our results do suggest that polyoma virus gene products (or a product) are important in transformation and they point toward an indispensable role for middle T antigen. The latter appears to be necessary for maintaining at least a subset of changes characteristic of transformed cells and, furthermore, appears to be involved in the tumorigenic nature of polyoma virus. Our data lead us to propose that middle T antigen may be to polyoma virus what the *src*-gene product is to the avian sarcoma viruses.

Acknowledgments

We are grateful to Anne Leigh-Brown for technical assistance during part of this study. The work was

partly supported by grants CA-25066, CA-23753, and HL-22266 from the National Institutes of Health.

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