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A Polyoma Mutant That Encodes Small T Antigen But Not Middle T Antigen Demonstrates Uncoupling of Cell Surface and Cytoskeletal Changes Associated with Cell Transformation

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The *hr-t* gene of polyoma virus encodes both the small and middle T (tumor) antigens and exerts pleiotropic effects on cells. By mutating the 3′ splice site for middle T mRNA, we have constructed a virus mutant, Py808A, which fails to express middle T but encodes normal small and large T proteins. The mutant failed to induce morphological transformation or growth in soft agar, but did stimulate postconfluent growth of normal cells. Cells infected by Py808A became fully agglutinable by lectins while retaining normal actin cable architecture and normal levels of extracellular fibronectin. These properties of Py808A demonstrated the separability of structural changes at the cell surface from those in the cytoskeleton and extracellular matrix, parameters which have heretofore been linked in the action of the hr-t and other viral oncogenes.

The early region of polyoma encodes three proteins detected as tumor (T) antigens. These polypeptides of 100,000 daltons (100K; large T), 56K (middle T), and 22K (small T) are encoded as overlapping products by alternate splicing of early region transcripts. They play important roles in both productive infection and cell transformation by the virus (5, 56). Studies with conditional growth mutants have shown two early genes encoding the three T antigens. Mutants of the "*ts*-a" class are defective in the large T protein. They are unable to initiate viral DNA replication or stable transformation at the nonpermissive temperature; they can, however, abortively transform cells, giving a transient induction of a full range of transformation-related cellular changes. Mutants of the "hr-t" class have alterations affecting both the middle and small T antigens; they are uniformly defective in all aspects of cell transformation and show a partial host-dependent block in capsid protein modification and virion assembly (27).

Other approaches have been used to study the roles of individual T antigens. Transfection with middle T cDNA has shown that this protein alone is sufficient to transform rat embryo fibroblasts of an established line (73). Similar studies with primary cells showed a requirement for large T, or its N-terminal half, along with middle T for transformation (53). One report indicates no effect by small T (53), whereas another shows this protein to cause a decrease in cell-substratum adhesion (18a). Along with large and middle T, small T may contribute to "one-step" transformation of primary cells (18a). In vivo studies have suggested a role of small T in tumorigenesis (1, 2). A different approach has involved site-directed mutagenesis in which specific alterations are introduced into middle T. Virus mutants encoding "tailless" forms of middle T that fail to associate with cell membranes also fail to transform (14, 70). Interestingly, a middle T protein made by gene fusion and containing the hydrophobic C-terminal segment of the vesicular stomatitis virus glycoprotein becomes membrane bound and acquires associated kinase activity, but fails to transform (71). The substitution of phenylalanine for tyrosine at position 315 in middle T has been made and studied in two laboratories, with different results. By plasmid transfection, the altered middle T was shown to transform well and to possess normal levels of associated kinase activity (50). Studied in a virus mutant, the same defect gives only low levels of middle T-associated kinase activity and weak transformation (15).

To help evaluate the contribution of the small T protein to the hr-t mutant phenotype, we have constructed a virus carrying a single-base substitution at the 3′ splice site for middle T mRNA. This virus mutant, Py808A, expresses the large and small T proteins, but no detectable middle T. Py808A is unable to induce complete transformation of established rat embryo fibroblasts, although it has a mitogenic effect on resting cells. The mutant behaves like wild-type virus in inducing agglutinability by lectins. Small T alone can thus induce changes of the cell surface associated with transformation; this occurs without detectable alteration of the microfilament system or extracellular fibronectin, parameters that are coordinately affected by an intact hr-t gene.

MATERIALS AND METHODS

Materials. Enzymes and the synthetic octadecamer 5′-d(GCTCTCCCCCTAAAACGG)-3′ were from New England Biolabs. [^{35}S]methionine (400 to 600 Ci/mmol) and [^{3}H]thymidine (20 Ci/mmol) were from New England Nuclear Corp. α-^{32}P-labeled deoxynucleotide triphosphates (400 Ci/mmol) and [γ-^{32}P]ATP (2,000 Ci/mmol) were from ICN Pharmaceuticals. 2-[^{3}H]deoxyglucose (5 Ci/mmol) and [α-^{14}C]aminoisobutyrate (58 mCi/mmol) were from Amersham Corp. Electrophoresis reagents were obtained from Bio-Rad Laboratories. Wheat germ agglutinin was obtained from Calbiochem.

Culture techniques. Cells were grown on plastic dishes, using Dulbecco modified Eagle medium containing 5% calf serum and antibiotics. Passage of cultures was carried out with a trypsin (0.025%, wt/vol)-Versene (0.2%, wt/vol) solution prepared in phosphate-buffered saline (PBS; 144 mM NaCl, 2.7 mM KCl, 15 mM sodium phosphate buffer [pH 7.4], 0.92 mM $CaCl_2$, 0.49 mM $MgCl_2$).

Cells and viruses. NIH 3T3 mouse cells and F-111 rat cells have been described before (25). Primary baby mouse kid-

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ney cells (78) were used to grow viral stocks. The hr-t mutant NG59 and the marker rescued 59RA wild type have been described previously (24). Plaque assays were done on mouse UC1-B cells. Viruses were concentrated by extractions with Genetron, ultracentrifugation, and suspension in Dulbecco modified Eagle medium without serum (57).

Phage and bacteria. Wild-type polyoma virus DNA (59RA) was digested with *Pst*I and *Eco*RI. The 1,076-base pair (bp) fragment from nucleotides 484 to 1560 was isolated and cloned into phage M13mp8, with *Escherichia coli* strain JM103 as the host. This recombinant was referred to as PR3 (15).

In vitro mutagenesis and virus reconstruction. Procedures for in vitro mutagenesis and virus reconstruction have been described in detail elsewhere (14, 15) . Single-stranded PR3 phage DNA was purified. The oligonucleotide was used to prime the synthesis of double-stranded covalently closed DNA in the presence of deoxynucleotide triphosphates, ATP, *E. coli* DNA polymerase large fragment, and T4 DNA ligase. The reaction mixture was electrophoresed on an agarose gel containing ethidium bromide, and the band migrating at the position of closed circular DNA was excised and purified. The closed circular DNA was then transfected into JM103 cells. Plaques were picked and grown. The cultures were centrifuged to pellet bacteria, and the phage-containing supernatants were mixed with lysis buffer (10× Tris-acetate buffer, 1% sodium dodecyl sulfate, 100 μg of RNase per ml, 0.01% bromophenol blue) and electrophoresed on an agarose gel. The phage DNAs were transferred onto a nitrocellulose paper (68). The octadecamer oligonucleotide was kinased at the 5′ end with [γ-^{32}P]ATP and hybridized to the nitrocellulose paper at a concentration of 10^5 cpm per ml of 5× SSC (1× SSC is 150 mM NaCl plus 15 mM sodium citrate) overnight at 40°C. Candidate phages containing the desired mutation were screened by autoradiography (75). Phage DNAs were purified for dideoxy DNA sequencing. One of the candidates confirmed to have the G-to-A transition at position 808 was selected. The small *Ava*I fragment (map units 83 to 90) from the replicative form of the phage was purified and ligated to the large *Ava*I fragment of 59RA RF. The ligation mixture was used to transfect baby mouse kidney cells by the calcium phosphate procedure (29). The reconstructed virus mutant was plaque purified and referred to as Py808A.

T-antigen analysis. Procedures for [^{35}S]methionine labeling of cells, immunoprecipitation, in vitro kinase reaction, and analysis of T antigens have been described before (58, 64). T antigens were resolved on sodium dodecyl sulfate–10% acrylamide gels. Electrophoresis was carried out at 50 V for 20 h.

S1 nuclease mapping of viral mRNA. The procedure for S1 nuclease mapping used here was described by Favaloro et al. (21). NIH 3T3 cells were infected at a multiplicity of infection of 5 to 10 PFU per cell. After 30 h, the cells were harvested for preparation of polyadenylated RNAs. Wild-type polyoma RF DNA was digested with *Pst*I. T4 polymerase was then added in the absence of nucleotide triphosphates to obtain a 3′-to-5′ exonuclease action. After a predetermined length of digestion, [α-^{32}P]ATP and the other three cold nucleotides were added to reverse the action of T4 polymerase to one predominantly of 5′-to-3′ polymerization. Cold dATP was added later to chase the reaction to completion. The mixture was extracted with phenol and digested with *Ava*I enzyme. The radioactive 359-bp *Ava*I fragment was isolated from polyacrylamide gel and used in the S1 mapping reaction. About 1 ng of radioactive labeled DNA fragment and 200 to 1,000 ng of polyadenylated RNAs were used.

Immunofluorescence staining. A total of 10^5 3T3 cells were plated onto 12-mm glass cover slips in 35-mm dishes and infected the next day with virus at a multiplicity of infection of 10 PFU per cell. At 24 h postinfection, cover slips were rinsed in PBS and fixed with acetone at −20°C for 20 min. The cover slips were air dried. A 10-μl portion of mouse monoclonal antiactin antibody or rabbit antifibronectin antibody was added to cover slips which were then placed in moist chambers and incubated at 37°C for 45 min. The cover slips were rinsed three times with PBS. A 10-μl amount of fluorescein-conjugated sheep anti-mouse or anti-rabbit globulin was applied, and the cover slips were incubated for an additional 45 min at 37°C. Subsequent incubations were carried out with rat anti-T ascites serum and rhodamine-conjugated rabbit anti-rat globulin sequentially to visualize polyoma T antigen. The cover slips were finally mounted on a glass slide with a 9:1 mixture of glycerol-PBS and examined with a Zeiss epifluorescence microscope, utilizing UV illumination and appropriate interference filters to allow selective visualization of rhodamine and fluorescein. Selective photographs were taken.

Lectin agglutination. Wheat germ lectin was dissolved in PBS at 1 mg/ml. Cells were plated at least 3 days before the experiment and were approximately 25% confluent on the day of the experiment. Cells were infected at a multiplicity of infection of 10 PFU per cell. At about 24 h postinfection, they were harvested for the experiment. The agglutination procedure has been described previously (6). The inhibitory effect of *N*-acetylglucosamine on wheat germ agglutinin-induced agglutination was tested by incubating *N*-acetyl-glucosamine at 2×10^{-2} M for 10 min with the lectin solutions before addition of cells.

Uptake of 2-deoxyglucose and α-aminoisobutyrate. The procedures for uptake of 2-deoxyglucose and α-aminoisobutyrate were essentially the same as those described previously (26, 39). Cells were seeded at 4×10^4 NIH 3T3 cells per well in a 24-well Linbro culture tray containing cover slips 12 mm in diameter. On the next day, cells were infected at a multiplicity of infection of 10 PFU per cell. Cells were washed twice with PBS 24 h later and were then incubated with 1 ml of PBS with 0.1% glucose for 30 min at 37°C. The cells were then washed twice with warm PBS, and 0.2 ml of PBS containing 4 μCi of [^{3}H]deoxyglucose and 1 μCi of [^{14}C]-aminoisobutyrate per ml were added. After 10 min of incubation at 37°C, the cover slips were removed and rinsed rapidly (10 s) by dipping them serially through PBS at 37°C. After they were dried, the cover slips were transferred to vials containing Aquasol universal liquid scintillation fluid (New England Nuclear. Protein measurements (Bio-Rad protein assay) were made in duplicate on cells attached to the cover slips.

RESULTS

Py808A has a G-to-A transition at nucleotide 808, a mutation predicted to alter the 3′ splice site for middle T mRNA. The goal of this study was to construct and characterize a mutant polyoma virus which produces functional large and small T proteins, but no middle T protein. The approach utilized site-directed mutagenesis with a synthetic oligonucleotide. The mutation was chosen based on empirical rules of conservation of nucleotide sequences at splice junctions. The general rule for these "consensus" sequences (9) is that each intron begins with the dinucleotide GT and ends with AG. All splice junctions sequenced so far conform to the

consensus rule, including those for the polyoma T antigens (41, 43, 62).

A single base substitution in the 5′ dinucleotide sequence is sufficient to abolish correct splicing and RNA maturation (22, 46, 49, 74, 77). Deletions or alterations of the 5′ sequence do not necessarily abolish splicing altogether, but may lead to aberrant splicing involving sequences resembling a consensus structure (cryptic splice sites) instead of the normal site (22, 74, 77). Alteration of a 3′ splice sequence has not been reported thus far. The oligonucleotide 5′-d(GCTCTCCCCCTAAAACGG)-3′ was used to introduce a single-base pair change into a polyoma DNA restriction fragment containing the small and middle T introns (map units 80 to 100) and was cloned into an M13 vector, as described in Materials and Methods. The predicted 3′ splice site for middle T mRNA is altered by this oligonucleotide (Fig. 1). In the large T reading frame, an arginine codon (A*G*A) is replaced by a lysine codon (A*A*A). No change in small T is expected, as one termination codon (TA*G*) is replaced by another (TA*A*). Recombinant phages with the desired mutation were screened by blot hybridization, and the presence of adenine at nucleotide position 808 was confirmed by DNA sequencing. One of the candidates was chosen to reconstruct a complete polyoma genome by excising the small *Ava*I fragment (map units 83 to 90, containing the G-to-A base change) from RF phage DNA and ligating it to the large *Ava*I fragment from cloned wild-type polyoma virus DNA. This ligation mixture was then transfected into baby mouse kidney cells, and the resulting progeny virus was plaque purified to give the desired mutant Py808A. A wild-type virus, Py808G, was similarly constructed by using the small *Ava*I fragment from the unmutated polyoma-M13 recombinant phage DNA. Py808A and Py808G were recloned into M13 for dideoxy sequencing. The single G-to-A difference at position 808 was confirmed. No other difference was found from map units 80 to 90, which contain all of the sequences derived from the polyoma-M13 recombinant phage.

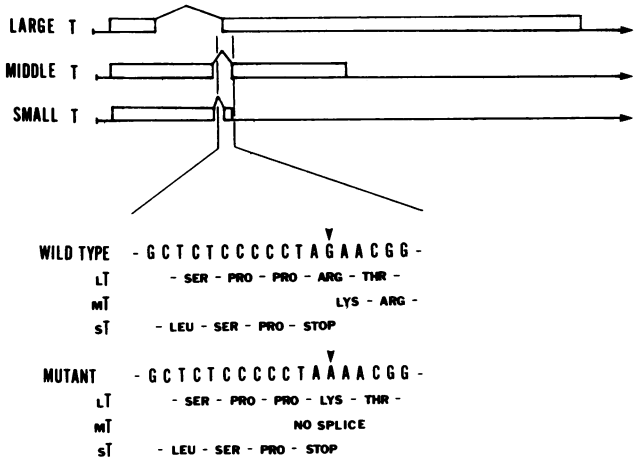

FIG. 1. Early region of polyoma virus and the alteration in Py808A. The three major early mRNAs, differing in splicing patterns, are denoted by horizontal arrows. Boxed areas represent coding sequences for wild-type T antigens. Below is shown an 18-nucleotide DNA sequence around the 3′ splice site for middle T mRNA. Vertical arrowheads point to nucleotide 808; the 18-base mutant sequence is that of the oligonucleotide used for mutagenesis. lT, Large T; mT, middle T; sT, small T.

Py808A fails to direct the synthesis of middle T antigen by metabolic labeling and in vitro protein kinase assay. The typical pattern of [^{35}S]-methionine-labeled wild-type T antigens, the viral large T (100K), middle T (56K), and small T (22K) antigens as well as the nonviral 63K and 36K species, is shown in Fig. 2 (right, lane 5). The viable wild-type deletion mutant *dl*45 (lane 4) encodes a normal-sized small T antigen and truncated large and middle T antigens (4). Py808G (lane 3) shows a wild-type pattern, whereas mutant Py808A (lane 2) induces the same pattern except that no 56K middle T species is observed. The two nonviral T antigens of 63K and 36K, previously shown to be missing in hr-t mutant immunoprecipitates (58, 64), are present with both Py808A and Py808G. No new aberrant species of virus-specific protein were detected in the Py808A immunoprecipitate. Since the standard polyclonal anti-T ascites used in this experiment should recognize products from any open reading frame, it would appear that no secondary or cryptic splice site(s) is utilized efficiently by the mutant.

The in vitro kinase reaction is several hundred times more sensitive than metabolic labeling in detecting the presence of normal middle T. Incubation of standard wild-type and Py808G immunoprecipitates with [γ-^{32}P]ATP results in the labeling of 56K and 58K middle T antigen species (Fig. 2,

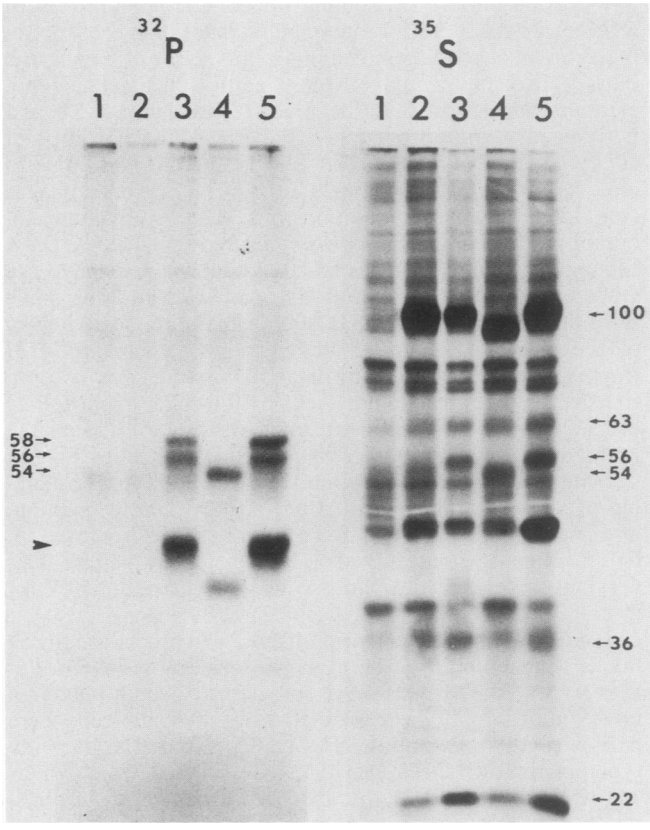

FIG. 2. Immunoprecipitates of wild-type and mutant T antigens. In vitro kinase reaction (left) and [^{35}S]methionine labeling (right) were performed on extracts of baby mouse kidney cells: mock (lane 1), Py808A (lane 2), Py808G (lane 3), *dl*45 (lane 4), and 59RA (lane 5). Numbers on the right indicate positions and sizes of various T antigens. Arrowhead in the left panel points to the degradation products of phosphorylated middle T protein. Electrophoresis was on a 10% polyacrylamide gel in the presence of sodium dodecyl sulfate.

left, lanes 3 and 5). *dl*45 induces a truncated (~54K) middle T species which is also active as a phosphate acceptor (lane 4). However, the immunoprecipitate of Py808A (lane 2) contains no detectable phosphorylated middle T species.

Cells infected by Py808A show no middle T mRNA. To investigate the effect of the mutation on RNA splicing, mRNAs from NIH 3T3 cells infected with mutant or wild-type virus were isolated and analyzed by S1 nuclease mapping. Labeled small *Ava*I fragment (map units 83 to 90) from wild-type DNA was used as the hybridization probe (see Materials and Methods). The results are shown in Fig. 3. The S1 digestion products expected from Py808G early mRNA should include a 226-nucleotide fragment specific for the small and large T mRNAs, a 212-nucleotide fragment specific for the middle T mRNA, and an 85-nucleotide upstream fragment specific for the middle and small T mRNAs. These three fragments are seen in lanes 3 and 4. Mutant Py808A clearly shows the presence of the 226- and 85-nucleotide fragments, but no detectable 212-nucleotide fragment (lanes 5, 6, and 7) unique for middle T mRNA. The full-size *Ava*I fragment of 359 bp, seen with both Py808A and Py808G, represents either reanealed cDNA strands or DNA fully protected by unspliced transcripts. Longer exposure of this gel revealed the presence of minor bands (<5% of total) in both the Py808A and Py808G lanes, probably representing nonspecific actions of nuclease S1 or degraded fragments of the probe (lane 2).

The mutation in Py808A leads to an increase in the ratio of large/small T mRNA and protein. An unexpected consequence of the altered splice site for middle T is a three- to fourfold increase in the ratio of large T/small T observed at both protein and RNA levels. Densitometer scanning of the [^{35}S]-methionine autoradiogram in Fig. 2 reveals ratios of large T/small T proteins of 5 for Py808G and 15 for Py808A. Densitomer analysis of the S1 mapping gel (Fig. 3) is more complicated, since the 226-nucleotide fragment represents both large and small T mRNAs and the 85-nucleotide fragment represents the middle and small T mRNAs. However, for Py808A, the 85-nucleotide fragment should derive solely from small T mRNA. Assuming that the radioactivity is evenly distributed in the DNA probe, ratios of large T to small T mRNAs can be calculated. Ratios of 4 for Py808G and 16 for Py808A were obtained, similar to those calculated for the proteins. The most likely explanation for these data is that mutant transcripts are preferentially spliced into large T mRNA.

Py808A is defective in transformation of F-111 rat fibroblasts. Comparisons of Py808A with Py808G provide an opportunity to study the contribution of small T in the absence of middle T to various parameters of cell transformation. The two viruses were first tested for their ability to transform F-111 rat cells. Assayed by dense focus formation in monolayers or by clonal growth in soft agar, the mutant is totally defective, whereas its wild-type counterpart Py808G is similar in its transforming ability to the standard wild-type virus (Table 1).

Wild-type polyoma virus has been shown to cause F-111 rat cells to retract from their normal side-to-side and end-to-end orientation and to assume a disordered overlapping arrangement of cellular processes; infected cells change from a bipolar, fusiform appearance to a more retracted, multipolar, and stellate shape. Enlarged nuclei and prominent nucleoli are characteristic of these cells; their cytoplasms also show increased basophilia and absence of stress fibers (59). A careful morphological study of F-111 cells infected by Py808A showed them to be indistinguishable

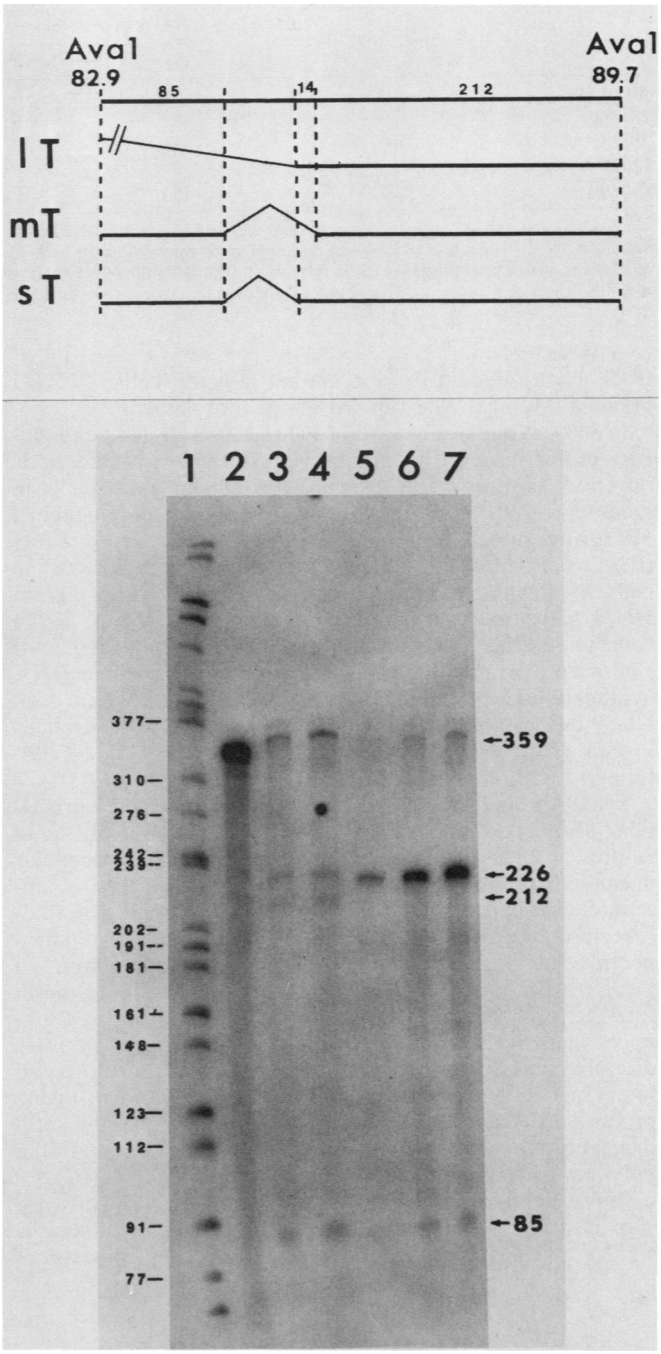

FIG. 3. S1 nuclease mapping of early RNAs of Py808A- and Py808A-Infected cells. (Top) Schematic diagram of mRNA-DNA hybrids. Map positions of the *Ava*I cleavage sites are indicated. The dotted vertical lines represent the expected sites of nuclease action on the hybrids formed between the labeled DNA probe and the early polyoma mRNA species. The expected sizes of protected DNA fragments are shown. lT, Large T; mT, middle T; sT, small T. (Bottom) S1 nuclease mapping reactions were carried out as described in the text. A 4% acrylamide–7 M urea denaturing gel was used. Lane 1 shows size markers. Lane 2 is the DNA probe alone. Lanes 3 and 4 represent 0.2 and 0.8 mg of total polyadenylated mRNAs of Py808G virus. Lanes 5, 6, and 7 represent 0.2, 0.8, and 2.0 μg of mRNAs of Py808A mutant. Numbers on the right indicate the sizes of protected fragments generated in the reaction.

TABLE 1. Test for transformation of rat cells by Py808A[a]

Virus	Input (PFU)	No. of transformants	
		A	B
Mock	0	0	0
NG59	10^6	0	0
59RA	10^6	43	29
Py808A	10^6	0	0
Py808G	10^6	38	31

[a] A total of 10^5 F-111 rat embryo fibroblasts were infected with 10^6 PFU of virus. In A, 10^4 infected cells were replated in 60-mm plates, grown to monolayer, and scored for dense focus formation. In B, 10^4 infected cells were replated in soft agar and scored for development of macroscopic colonies (25).

from mock-infected or hr-t mutant-infected cells; Py808G induced a typical wild-type response (not shown).

Py808A stimulates growth of F-111 cells arrested at confluence in low serum. Table 2 shows results of viral infections on DNA synthesis and growth of confluent F-111 cells in medium with 0.5% serum. Cells were grown to confluence in 5% serum, infected, and incubated with 0.5% serum. Virus titers were adjusted to achieve infection of 50 to 60% of the cells by T-antigen immunofluorescence. The hr-t mutant NG59 can stimulate DNA synthesis and growth of F-111 cells under these conditions, as previously reported (59). Followed over a 96-h period, mutant Py808A induces DNA synthesis and cell division to a greater extent than does NG59 but less than does wild-type virus. These differences in cell growth stimulation (Py808G > Py808A > NG59), though small, are reproducible and significant ($P < 0.01$).

Py808A fails to elicit changes in actin cables and extracellular fibronectin matrix. The presence or absence of "actin cables" (stress fibers) and the deposition of extracellular fibronectin were scored in infected cells by double immunofluorescence. Infected cultures were stained with fluorescein-conjugated antiglobulin to visualize actin or fibronectin and with rhodamine-conjugated antiglobulin to visualize the viral T antigens. Individual cells could therefore be scored simultaneously for T-antigen expression, microfilament architecture, and fibronectin distribution. Loss of actin cables has previously been shown to depend on an intact hr-t gene (59), but whether one or both of the hr-t gene products is required is not known. Extracellular fibronectin distribution has not been analyzed in detail in relation to the polyoma early gene products.

Representative results with infected NIH 3T3 cells are shown in Fig. 4 and 5. The pattern of fibronectin staining is similar to that reported earlier (16, 37). It consists of punctate patches generally in the central region of the cell, often arranged in roseate formation or fibrils or both. Occasional fibrils can be seen aligned along the long axes of the cells. Since the cells have been permeabilized, it is not clear that all the structures are external to the plasma membrane. However, by careful focusing it was observed that virtually

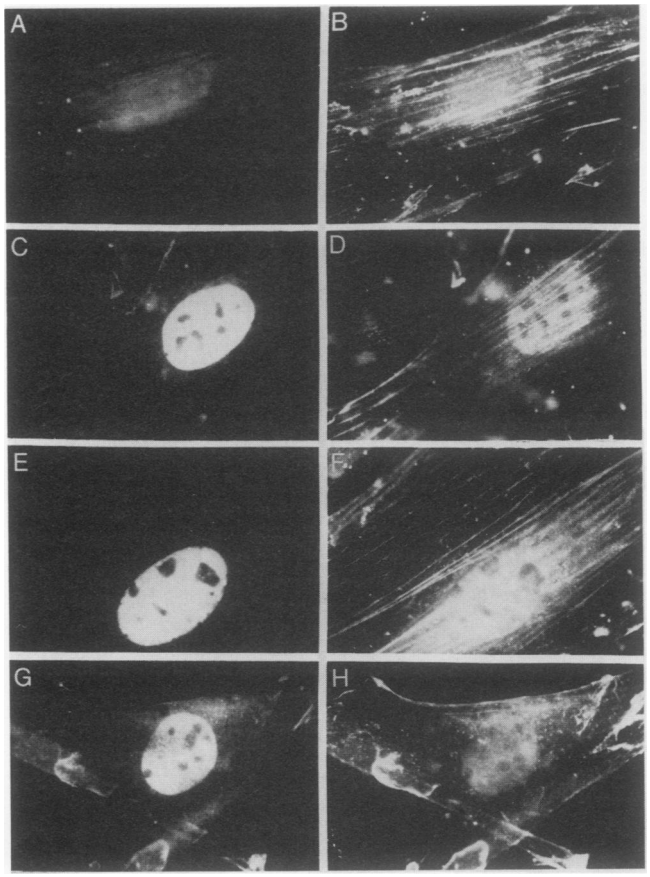

FIG. 4. Actin immunofluorescence photographs of infected mouse 3T3 cells. Mouse NIH 3T3 cells were plated sparsely onto glass cover slips. (A, B) Mock infected; (C, D) NG59; (E, F) Py808A; (G, H) Py808G. The cells were fixed 24 h postinfection and stained with antiactin and anti-T antibodies. Actin was visualized with fluorescein-conjugated antiglobulin, and T antigen was visualized with rhodamine-conjugated antiglobulin. (A, C, E, G) T-antigen staining; (B, D, F, H) actin staining of some cells. 800.

TABLE 2. Test for mitogenic effect of viral infections on confluent rat cells[a]

Virus	Cell no. per dish × 10^4			% of cells with labeled nuclei		
	48 h	72 h	96 h	22–28 h	46–52 h	70–76 h
Mock	26.4 ± 2.1	27.6 ± 2.0	26.6 ± 2.0	5.3 ± 0.5	2.6 ± 0.3	2.2 ± 0.3
NG59	33.9 ± 2.9	34.8 ± 2.8	34.2 ± 2.9	11.1 ± 0.9	10.0 ± 0.8	9.6 ± 0.7
Py808A	33.7 ± 2.8	39.1 ± 3.3	42.2 ± 3.4	10.1 ± 0.7	12.2 ± 0.9	12.8 ± 0.9
Py808G	34.5 ± 3.1	48.3 ± 3.8	56.4 ± 4.6	12.9 ± 0.9	15.7 ± 1.1	19.0 ± 1.4

[a] F-111 cells were grown to confluence in 35-mm culture dishes, infected with Genetron-purified virus, and fed with medium containing 0.5% calf serum. To determine cell number per dish, at the times indicated cells were trypsinized and counted. The numbers given are averages of cells per culture from triplicate cultures ± standard error of means. To determine percentage of cells with labeled nuclei, parallel cultures were labeled with [^{3}H]thymidine for 6 h at the indicated times and processed for autoradiography (59). Five fields chosen at random (total, approximately 5,000 cells) were scored for nuclear labeling. The average percentages of labeled cells are given ± standard error of the means. T-antigen immunofluorescence showed that 54% of NG59-, 50% of Py808A-, and 56% of Py808G-infected cells were T antigen positive at 24 h postinfection.

all of the fibronectin staining occurred at either the bottom or the top of the cells. The photographs in Fig. 5 were taken of fibronectin staining at the bottom of cells. The viruses tested, Py808A, Py808G, and hr-t mutant NG59, all induce large T antigen in the nuclei. Only the wild-type virus Py808G causes a loss of actin cables and reduction of fibronectin matrix. (Compare adjacent infected and uninfected cells in Fig. 5 G and H, for example.) Wild-type-infected NIH 3T3 cells also undergo the typical morphological changes described previously, whereas cells infected with the hr-t mutant NG59 or Py808A retain a normal appearance. F-111 rat cells transformed by polyoma virus or by a middle T cDNA-containing plasmid also show diminished fibronectin staining and few actin cables, indicating that middle T alone is sufficient to bring about these changes (not shown).

Quantitation of changes in actin and fibronectin was performed by scoring the percentage of cells lacking organized actin cables and reduced fibronectin network in a population of T-antigen-positive cells infected by each virus. Greater than 80% of the mock-infected cells show actin cables as well as extracellular fibronectin matrix. Cells infected with Py808A and NG59 showed a percentage similar to that of mock-infected cells. However, with wild-type viruses Py808G and 59RA, about 70 and 50% of T antigen-positive cells clearly showed loss of actin cables and substantial reduction of fibronectin matrix, respectively.

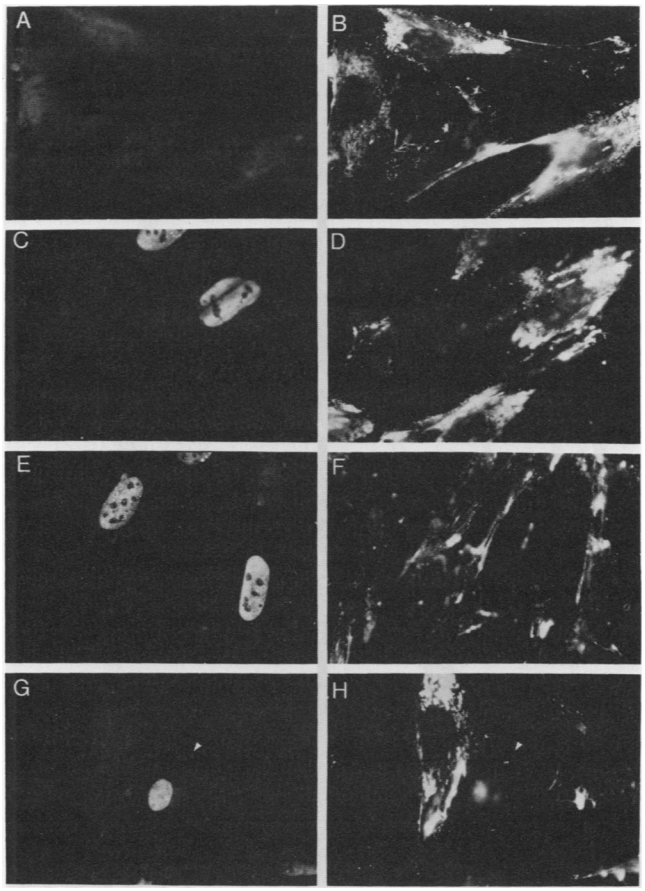

FIG. 5. Fibronectin immunofluorescence. Procedures were the same as for Fig. 4, except that antifibronectin antibodies were used instead of antiactin. (A, B) Mock infected; (C, D) NG59; (E, F) Py808A; (G, H) Py808G. (A, C, E, G) T-antigen staining; (B, D, F, H) fibronectin staining. Arrowheads in (G) and (H) point to the same cell infected by Py808G.

TABLE 3. Test for agglutination of virus-infected cells by wheat germ agglutinin[a]

Virus	Wheat germ lectin concn at half-maximal agglutination (μg/ml)	
	Absence of hapten	Presence of hapten (*N*-acetylglucosamine)
Mock	>800	>800
NG59	>800	>800
59RA	50	>800
Py808A	50	>800
Py808G	50	>800

[a] Subconfluent NIH 3T3 cells were infected. Cells were harvested gently by EDTA treatment 24 h later, washed, and tested for lectin agglutinability. Wheat germ agglutinin was used at concentrations of 0, 10, 50, 100, and 400 μg/ml, and percentages of cells agglutinated were determined. Concentrations at half-macimal agglutination were determined by interpolation. The results are representative of three separate experiments.

Infected cells were also examined for focal adhesions, using interference reflection microscopy (40). Infection by wild-type viruses Py808G and 59RA led to reduced numbers of adhesions, whereas cells infected by mutants Py808A and NG59 were indistinguishable from uninfected controls (K. Ballmer-Hofer, T. J. Liang, and T. L. Benjamin, unpublished data).

Py808A induces increased agglutination of cells by plant lectins. Enhanced agglutination by plant lectins is associated with cell transformation brought about by viruses as well as by other means. The dependence of this cell surface change on viral oncogene expression has been shown for both polyoma virus (6) and Rous sarcoma virus (42). To examine the role of the small T polypeptide in this aspect of transformation, NIH 3T3 cells were infected with the following virus strains: Py808A, Py808G, NG59, and 59RA. Cells were harvested 24 h after infection and tested for agglutinability with wheat germ agglutinin. The results (Table 3) demonstrate that the half-maximal agglutination concentrations for cells infected with Py808A, Py808G, or 59RA are more than an order of magnitude less than that for mock- or NG59-infected cells. Agglutination of both wild-type- and Py808A-infected cells was inhibited by addition of the hapten *N*-acetylglucosamine. Similar results have been obtained by using concanavalin A with a-methyl mannoside specificity. Small T protein in the absence of middle T can thus bring about this cell surface change.

Py808A exerts no effect on uptake of 2-deoxyglucose and α-aminoisobutyrate. Stimulation of uptake of glucose, amino acids, phosphate, and other small molecules has been found consistently in a variety of virus-transformed cell systems (26, 30, 39). These changes in transport properties may well reflect a cell surface change. Whether this functional change is linked to structural changes involved in the development of agglutinability is unclear. A previous study with polyoma *ts*-a mutants suggested that the large T polypeptide is not responsible for the observed alterations in transport (20). To assess the role of small T, the effects of Py808G, Py808A, and NG59 on uptake of 2-deoxyglucose and α-aminoisobutyrate in NIH 3T3 cells were examined. The results are shown in Table 4. Py808G-infected cells showed three- to fourfold increases in uptake of both the amino acid and sugar analogs compared with mock-infected cells. Neither Py808A nor NG59, however, exerted an effect on the uptake of these molecules.

TABLE 4. Test for enhanced uptake of small molecules in virus-infected cells[a]

Virus	Uptake (cpm/10 min per μg of protein)	
	[2-^{3}H]deoxyglucose	[α-^{14}C]aminoisobutyrate
Mock	1,520 (1.0)	330 (1.0)
NG59	2,080 (1.4)	390 (1.2)
Py808A	1,860 (1.2)	370 (1.1)
Py808G	5,740 (3.8)	920 (2.8)

[a] NIH 3T3 cells were infected with viruses and examined 24 h later. Numbers shown are averages of five cultures; percent increases relative to mock-infected cultures are given in parentheses.

DISCUSSION

Previous studies have defined a role of the polyoma hr-t function in transformation of cells in vitro and tumorigenicity (5). All parameters of transformation so far tested require an intact hr-t gene. These include anchorage-independent growth, dense focus formation on monolayers, and morphological transformation, as well as concomitant structural changes affecting the cytoplasm and cell surface. The hr-t gene also performs functions in productive viral infection, in particular the induction of post-translation modifications of VP-1, which appear to be essential for virion assembly (5a, 27). Single genetic defects in hr-t mutants coordinately affect their growth and ability to transform. Thus, marker rescue of the host range defect simultaneously restores transforming ability (24), and selection for dependence in growth on primary mouse embryo cells that constitutively express a permissive factor(s) yields mutants that are nontransforming (23).

Efforts to understand the pleiotropic effects of the hr-t gene must reckon with the fact that two proteins, the small and middle T antigens, are products of this viral gene. These proteins are encoded as in-phase overlapping products, and they share a common 5′ splice site. In addition, small T utilizes the same 3′ splice site as large T. Thus, alteration of the 3′ acceptor site for middle T is the only way of achieving a clean separation of the hr-t gene products. This has been accomplished by using an oligonucleotide which directs a G-to-A transition at nucleotide 808. The primary effect of this transition is the abolition of splicing for middle T mRNA. As anticipated, mutant Py808A produces large and small T proteins but no middle T detectable by either [^{35}S]methionine labeling or in vitro kinase reaction. These results are the first to deal with the effects of alteration of a 3′ splice site, and they confirm the assignment of nucleotide 808 in polyoma DNA as the 3′ junction for middle T predicted from T-antigen peptide fingerprints (34, 67) and S1 maps (41).

An unexpected consequence of the mutation in Py808A is a three- to fourfold increase in the ratio of large T/small T mRNA and protein in mutant versus wild-type-infected cells. Most likely this effect takes place at the level of splicing, although it is possible that the stabilities of large and small T mRNAs are differentially affected in the absence of middle T. In contrast to observations on utilization of secondary or cryptic 5′ splice sites (22, 74, 77), we do not detect utilization of any such 3′ splice site within map units 83 to 90 (about 360 bp). Examination of the DNA sequence in this region reveals a sequence of TTTCTTC*AG*/A around map unit 88, conforming closely to the consensus sequence. If this site were used, a protected fragment of 101 bp would be observed in the S1 mapping experiment; however, no such fragment was observed even in prolonged exposure of the gel. Northern blots of total RNAs of Py808A- and Py808G-infected cells show no additional species of early RNA with the mutant. Whatever minor aberrantly processed RNAs might be present as a result of the mutation in Py808A, no new or aberrant T antigen species could be detected by immune precipitation.

The mutant Py808A is defective in transformation, a result which agrees with other observations that middle T protein is essential (14, 70, 73). Studies of morphological and cytoskeletal changes associated with the transformed phenotype have shown a clear dependence on the hr-t function (59). These changes are not evident in cells infected by Py808A. Changes at the ultrastructural level, however, have not been excluded.

Although incapable of inducing anchorage-independent growth of established rat fibroblasts, Py808A can still stimulate postconfluent growth of these cells on monolayers. A limited mitogenic effect has also been seen with hr-t mutants (59). Py808A is stronger than hr-t mutants but weaker than wild-type virus in inducing cell DNA synthesis and growth. The mitogenic effect of Py808A most likely reflects the presence of small T, although overproduction of large T could also be a factor. Consistent with the latter possibility are the results of Rassoulzadegan et al. (53), who found a strong mitogenic effect of the large T protein but none with small T at low serum concentration. However, a lack of effect of large T in various aspects of the mitogenic response has been shown in other studies with *ts*-a mutants (19, 69). A growth-stimulating effect by small T of Py808A is consistent with earlier results showing abortive transformation by *ts*-a but not by hr-t mutants (25) and would also be in line with similar observations of simian virus 40 showing a requirement for small T in transformation of resting but not of growing fibroblasts (45, 61).

Pollack et al. (52) first demonstrated a correlation between loss of actin cables and the transformed state induced by papovaviruses. In the polyoma system, the loss of actin cables is dependent on the hr-t gene (59). Polyoma and simian virus 40 small T, which share about 30% amino acid sequence homology (72), may also share some functional homology (2). However, contrary to findings in simian virus 40 (7, 28, 55), small T of polyoma does not induce detectable loss of actin cables in either mouse or rat fibroblasts. Middle T thus appears to be essential for restructuring the microfilament system in these cells; this viral protein may indeed be sufficient, since F-111 cells transfected by a middle T cDNA plasmid show very few actin cables (unpublished data).

Fibronectins are large, dimeric glycoproteins that form an extracellular matrix. This protein has been implicated in cell-cell and cell-substratum adhesions, as well as in interactions with other extracellular matrix materials (38). Its disappearance associated with transformation has been studied extensively (16, 35). Mouse 3T3 cells transformed by polyoma or simian virus 40 show reduced quantities of this protein (32). We have shown that de novo infection of mouse 3T3 cells by wild-type polyoma virus leads, within 24 h, to a significant reduction of fibronectin. Py808A and hr-t mutants, however, do not induce detectable loss of this material.

Immunofluorescence studies have shown an association between the termini of microfilament bundles and points of close adhesion to the substratum. Thus, actin (31), vinculin (65, 66), and α-actinin (76) have all been localized to regions of focal contacts. Moreover, deposits of fibronectin occur in

TABLE 5. Parameters of transformation induced by polyoma small T antigen as deduced from studies with Py808A[a]

Parameter	hr-t mutant (lT^+, mT^-, sT^-)	Py808A (lT^+, mT^-, sT^+)	Wild type (lT^+, mT^+, sT^-)
Growth-related changes			
Focus formation	−	−	+
Morphological transformation	−	−	+
Anchorage-independent growth	−	−	+
Loss of density regulation of growth	±	+	+
Structural changes			
Decreased focal adhesions	−	−	+
Loss of actin cables	−	−	+
Loss of fibronectin	−	−	+
Lectin agglutinability	−	+	+

[a] lT, Large T; mT, middle T; sT, small T.

close proximity to actin cables and adhesion sites (8, 17, 37, 65). This linkage in the organization of microfilaments and matrix is unaffected by mutant Py808A, indicating that a functional middle T protein is essential for transformation-related changes in these components.

Though unable to change the cytoskeletal and exoskeletal elements described above, Py808A is fully capable of inducing a transformation-related change of the cell surface itself. This property of lectin agglutinability is closely associated with neoplastic transformation in viral as well as in other transformed cell systems. Whenever nontransforming virus mutants have been studied, the failure to induce lectin agglutination accompanies the failure to transform (6, 42).

Although the molecular basis for agglutination is not understood, there is evidence to indicate that the agglutinable state reflects a cell surface change that is operative in the altered growth behavior of transformed cells. First, exposure of transformed cells to concanavalin A leads to cell death and selection of phenotypic revertants which show normal growth characteristics and are no longer highly agglutinable (51). Second, transformed cells can be made to exhibit normal growth properties by specific and reversible binding of trypsinized or succinylated concanavalin A that is nontoxic and nonagglutinating (3, 12). Third, normal cells can be rendered agglutinable in a controlled and reversible way by mild protease treatment; such treatment of resting 3T3 cells leads to initiation of single rounds of postconfluent growth (10). Interestingly, Py808A demonstrates a coupling of the same two parameters, lectin agglutinability and loss of density regulation of growth (Table 5).

The findings with Py808A do not support models that necessarily couple changes in cytoskeletal organization with those at the cell surface. Linkages of lectin or other cell surface receptors with cytoskeletal elements have been documented (11, 47), and such linkages are thought to play important roles in the processing of signals from outside to inside the cell in many systems. The occurrence of coordinate changes of the cell surface and cytoskeleton induced by the action of single viral-transforming genes has suggested that molecules such as vinculin or other putative linking elements might be logical targets for protein kinases or other effects brought about by the action of the transforming proteins (33, 60, 63). In the case of polyoma, the middle T protein interacting with the c-*src* kinase (18), presumably at the plasma membrane, would be a plausible candidate for effecting coordinate changes in membrane, cytoskeletal, and extracellular components. At present, however, no linking element has been clearly identified as a critical target for a virus-related kinase. Furthermore, the results with Py808A show that changes of the cell surface do not require middle T and that such changes can be dissociated from those that occur immediately inside and outside the cell.

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