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The Major Site of Tyrosine Phosphorylation in Polyomavirus Middle T Antigen is Not Required for Transformation

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The induction of tumors and cellular transformation mediated by polyomavirus requires the action of middle T antigen. Accordingly, we have begun to define the domains of the viral protein important for these processes to learn more about its site and mechanism of action. One of the domains of middle T antigen which is thought to be important for its function includes a stretch of acidic amino acids and a vicinal tyrosine residue (tyrosine 315), the major site of tyrosine phosphorylation in vitro. To determine whether these acidic amino acids and tyrosine 315 are required to maintain the transforming activity of middle T antigen, we constructed deletions within the DNA sequences encoding these amino acids and measured the capacity of the resulting mutants to transform Rat-1 cells in culture. This was accomplished by using in vitro mutagenesis techniques with molecularly cloned polyomavirus DNA. Seven mutants were isolated. Five of these proved incapable of transforming Rat-1 cells and were found to contain deletions which altered the reading frame for middle T antigen. However, two mutants, pPdl1-4 and pPdl2-7, retained the capacity to transform Rat-1 cells at high frequencies. The middle T antigen encoded by one of these mutants, pPdl1-4, lacks part of the acidic string of amino acids but not tyrosine 315 (amino acids 304 through 310 are deleted), whereas the middle T antigen encoded by the other mutant, pPdl2-7, lacks the entire acidic amino acid stretch as well as tyrosine 315 (amino acids 285 through 323 are deleted). Rat-1 cells transformed by one or the other mutant DNA displayed a fully transformed phenotype, including the capacity to form tumors in animals. These results prove that the major site of tyrosine phosphorylation in middle T antigen and the acidic amino acids which precede it are not essential for its transforming activity.

Polyomavirus, one member of the papovaviruses, causes tumors in rodents and transforms cells in culture. The tumorigenic and transforming potentials of the virus are mediated by proteins encoded by the early transcription unit. Three early proteins have been identified to date and named large, middle, and small tumor (T) antigen. The precise function of each T antigen in bringing about transformation has not been completely elucidated, but it is the subject of intensive investigation. Middle T antigen alone is sufficient to cause transformation of established cell lines (30) and to cause tumors in hamsters (W. Topp and J. A. Hassell, unpublished data; C. Gelinas and M. Bastin, personal communication). However, transformation of primary cultures of rat embryo cells requires not only middle T antigen but also large T antigen (17, 18). DNA-mediated tumor formation in newborn rats also requires the concerted action of middle T antigen and large or small T antigen (1). Therefore, whereas middle T antigen may not be sufficient to transform certain cell types (rat embryo fibroblasts) nor sufficient to induce tumors in some experimental animals (newborn rats), the protein nonetheless is required for both processes. Because middle T antigen plays such a pivotal role in oncogenesis, we have attempted to elucidate its mechanism of action by genetic means (13).

Middle T antigen is composed of 421 amino acids, and its predicted molecular weight is 48,506. Two species of middle T antigen with apparent molecular weights of 56,000 and 58,000 have been detected in lytically infected and transformed cells. It is thought that the minor, 58,000-molecular-weight (58K) species is a posttranslational modification product of the 56K form because the in vitro translation product of middle T antigen mRNA has a molecular weight of 56,000 (9, 21). Both forms of the protein are predominantly phosphorylated in vivo at serine residues and to a much lesser extent at threonine and tyrosine residues (20, 21, 24).

Middle T antigen shares several characteristics with the products of other oncogenes. One of these is its association with the plasma membrane (11, 22, 25). Another is its capacity to become phosphorylated at tyrosine residues in vitro in immunoprecipitates (7, 19, 26). The principal site of phosphorylation in vitro is tyrosine residue 315 (20). This tyrosine residue is preceded by a cluster of six glutamic acid residues, a sequence which is reminiscent of the amino acid sequences surrounding sites of tyrosine phosphorylation in the transforming proteins of several retroviruses (2, 16). A number of deletion mutants of polyomavirus have been isolated which lack tyrosine 315 and part or all of the glutamic acid stretch (6, 8, 14, 27). Invariably, each of these viruses is incapable of transforming cells in culture, and these observations have led to the conclusion that tyrosine 315 is indispensible for the transforming activity of middle T antigen.

We previously reported the isolation of deleted polyomavirus genomes cloned in plasmid DNA whose mutations were introduced by in vitro mutagenesis techniques (13). Many of these mutant DNAs are deleted near the region encoding tyrosine 315 about a *Pvu*II site at nucleotide 1144. Although most of these mutant DNAs with deletions of the *Pvu*II site are nontransforming, two DNAs, pPdl1-4 and pPdl2-7, are capable of transforming established Rat-1 cells at relatively high frequencies. Here, we report the sequences surrounding the deletions in these various mutant DNAs, the

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phenotype of transformed Rat-1 cells which harbor transforming pPdl1-4 and pPdl2-7 DNA, and the sizes and activities of middle T antigens encoded by these DNAs. One of the transforming mutant DNAs, pPdl2-7, lacks DNA sequences which encode tyrosine 315 and the entire glutamic acid stretch which precedes it. Rat-1 cells transformed by pPdl2-7 DNA display a completely transformed phenotype, including the capacity to form tumors in animals. These transformed cells contain a novel, truncated middle T antigen which is relatively inactive as a substrate in the in vitro kinase reaction. These observations lead us to conclude that tyrosine 315 and many of the surrounding amino acids, including the entire glutamic acid stretch, are not absolutely required to maintain the transforming activity of middle T antigen.

MATERIALS AND METHODS

Cell culture and transformation. All cell lines were propagated in Dulbecco modified Eagle medium (DMEM) supplemented with antibiotics and maintained at 37°C in a humidified CO_2 incubator. The medium for growth of Rat-1 cells was supplemented with 10% fetal bovine serum (FBS), whereas that for the transformed cell lines was supplemented with 5% FBS.

A modification of the calcium phosphate technique (31) was used to transfect Rat-1 cells with cesium chloride density gradient-purified supercoiled plasmid DNA. To establish the various transformed cell lines, we isolated independent foci from separate plastic dishes 14 to 18 days posttransfection and recloned them (13).

Construction of mutant recombinant plasmids. The mutagenesis protocol used to generate deletions in and around the polyomavirus *Pvu*II site at nucleotide 1144 has been described previously (13). Briefly, the recombinant plasmid pPH1-8 (Fig. 1) was digested with *Pvu*II in the presence of ethidium bromide. There are four *Pvu*II sites in pPH1-8 DNA. Permuted linear molecules, generated by this protocol, were then subjected to Bal 31 nuclease digestion and then circularized with T4 ligase. Plasmid DNA was isolated from individual drug-resistant colonies and was tested for the loss of the *Pvu*II site in polyomavirus sequences at nucleotide 1144. Nine such recombinants were found, and their capacity to transform Rat-1 cells was measured. The two largest deletions were found to be of little interest; nucleotides 1147 through 1592 are deleted in pPdl1-11, thus removing the termination codon for middle T antigen; and nucleotides 804 through 1258 are deleted in pPdl2-2, which effectively removes the distal splice acceptor site for middle T antigen mRNA at position 809 (Fig. 1).

Nucleotide sequence determination. The 5′ ends of restriction endonuclease fragments were phosphorylated with [γ-^{32}P]ATP and T4 polynucleotide kinase. The primary cleavage was at the *Ava*I site at nucleotide 1016, and the secondary cleavage was at the *Eco*RI site at nucleotide 1560. The appropriate fragments were isolated from an agarose gel and sequenced by the method of Maxam and Gilbert (12).

Analysis of polyomavirus tumor antigens. Immunoprecipitations of the viral tumor antigens in Rat-1 cells transformed by either pPdl1-4 or pPdl2-7 DNA were performed as follows. Cells were plated on plastic dishes (60 mm) at a density of 5×10^4 cells per cm^2 in DMEM containing 5% FBS. After 24 h, the medium was removed, and the monolayers were washed twice with 5 ml of phosphate-buffered salt solution (PBS). Cells were then incubated for 1 h at 37°C in methionine-free DMEM, and the monolayers were subsequently washed twice with PBS. This was followed by a 4-h incubation in methionine-free DMEM (1 ml) containing 300 μCi of [^{35}S]methionine. Labeling of cellular proteins was terminated by removing the medium and washing the cell monolayers twice with 5 ml of ice-cold PBS. Extracts were prepared and immunoprecipitated with either antitumor or normal serum as described (10). The resulting immunoprecipitates were electrophoresed through 12.5% sodium dodecyl sulfate (SDS)–polyacrylamide gels, and the [^{35}S]methionine-labeled proteins were visualized by autoradiography.

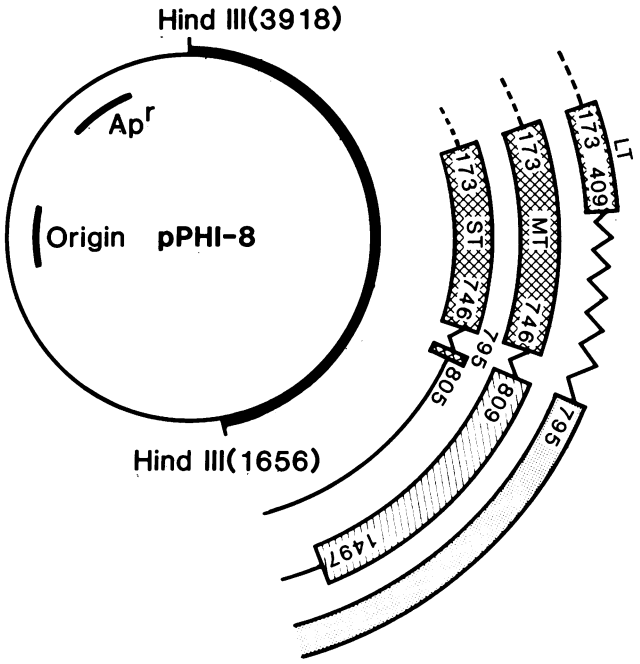

FIG. 1. pPH1-8 contains the *Hin*dIII-1 fragment of polyomavirus DNA (thick line), inserted into the plasmid pBR322 (thin line). The figure also shows the coding sequences for large, middle, and small T antigens which are present in pPH1-8. Note that the amino-terminal portion of the three T antigens (before the proximal splice) as well as the distal portion of small T antigen are in the same reading frame. The carboxy-terminal portion of middle and large T antigen (after the distal splice) are in the two remaining reading frames and are different from each other. Polyomavirus nucleotide numbers are as described by Soeda et al. (28).

To measure the capacity of middle T antigen to become phosphorylated in vitro, we used the procedures described by Schaffhausen and Benjamin (21). Briefly, transformed cells were grown to 60% confluence on plastic dishes (100 mm) in DMEM supplemented with 5% FBS, and the cells were washed three times with PBS and rinsed in washing buffer (0.135 M NaCl, 0.020 M Tris-hydrochloride [pH 9], 0.001 M $MgCl_2$, 0.001 M $CaCl_2$). Cells were lysed, and the T antigens were extracted for 20 min at 4°C with 1.1 ml of washing buffer supplemented with 1.0% Nonidet P-40. The extracts were cleared by centrifugation and reacted with either normal or antitumor serum in the presence of Formal-in-fixed *Staphylococcus aureus* for 30 min at 4°C. The precipitates were washed three times with cold PBS, twice with 0.5 M LiCl–0.1 M Tris-hydrochloride (pH 6.8), and finally once with distilled water. These washed immunoprecipitates were then suspended in 0.4 ml of 0.02 M Tris-hydrochloride (pH 7.5)–0.005 M $MgCl_2$ containing 5 μCi of [γ-^{32}P]ATP. The reaction was allowed to proceed for 15 min at 22°C, after which the immunoprecipitates were collected

and washed as described above. The proteins released from the immunocomplexes were then electrophoresed through 10% SDS–polyacrylamide gels, and ^{32}P-labeled proteins were visualized by autoradiography.

The technique of partial proteolysis has been described previously (19, 20, 23). In short, the T antigens to be compared were first resolved in cylindrical 10.0% SDS–polyacrylamide gels. The cylinders were then placed head to head on top of a 12.5% SDS–polyacrylamide gel. Two milliliters of a digestion solution (0.0125 M Tris-hydrochloride [pH 6.8], 0.01 M EDTA, 0.0075% [wt/vol] bromphenol blue, 20% [vol/vol] glycerol containing 50 μg of bovine serum albumin per ml and 30 μg of *S. aureus* V8 protease per ml) were layered on top. Electrophoresis was carried out at 50 V for ca. 16 h, until the bromphenol blue reached the bottom of the gel.

In all immunoprecipitates, ascites fluids from brown Norwegian rats which had been injected with PyB4a cells (25) were used as a source of anti-polyomavirus T antigen immune serum.

Growth in agar and tumorigenicity of transformed cell lines. To test the capacity of cells to grow without a solid support, we suspended 10^5 cells in 0.33% agarose in DMEM containing 5% FBS and then plated them over a layer of 0.60% agarose in the same medium. After 1 week, the plates were scored for the presence of colonies.

To test the tumorigenicity of the various transformed cell lines, we injected 10^6 cells subcutaneously into 3-week-old Fisher rats. After 21 days, rats were examined for the presence of solid tumors at the site of injection, and the number of animals with tumors was scored.

RESULTS

We determined the transforming activity and primary DNA structure of seven deleted polyomavirus recombinant plasmids. Each of these DNAs bears a deletion of polyomavirus sequences about the *Pvu*II site at nucleotide position 1144 in wild-type (wt) viral DNA. The composition and transforming properties of these seven deleted recombinant plasmids are summarized in Table 1. Five DNAs (pPdl1-8, pPdl1-10, pPdl2-3, pPdl2-5, and pPdl2-12) were completely incapable of transforming Rat-1 cells even when large quantities of DNA were used to transfect the cells (1 μg per 3×10^5 cells). We sequenced the DNA surrounding the deletions in each mutant genome and found that in all cases the deletion in these DNAs caused a shift in reading frame (Table 1). Out-of-phase deletions in this area of the genome would result in the synthesis of hybrid proteins containing sequences coding for middle T antigen up to the 5′ boundary of the deletion, whereas the remainder of the protein would be derived from sequences which normally do not code for proteins (the small T antigen reading frame) or from sequences encoding large T antigen (Fig. 1).

Two mutant DNAs, pPdl1-4 and pPdl2-7, retained the capacity to transform Rat-1 cells (Table 1). pPdl1-4 DNA transformed Rat-1 cells at the same frequency at which the wt parental plasmid pPH1-8 did, whereas pPdl2-7 DNA transformed Rat-1 cells at an average frequency of 20% of that of wt DNA. In three separate experiments, the mutant DNA transformed Rat-1 cells at a frequency between 15 and 30% of that of wt DNA. These comparisons were made by transfecting nanogram quantities of recombinant DNA over a range of DNA concentrations (Table 1). The response to DNA was linear and directly proportional to DNA concentration over this range (13).

We sequenced both DNAs to determine the exact boundaries of the deletion in each. pPdl1-4 DNA is deleted of 21 base pairs by comparison with wt DNA. The middle T antigen encoded by this DNA should lack amino acids 304 through 310 (Fig. 2). These amino acids include the first two of six glutamic acid residues which precede tyrosine 315. pPdl2-7 contains a 117-base-pair deletion. The middle T antigen encoded by pPdl2-7 DNA should be deleted of amino acids 285 through 323 (Fig. 2). This large deletion results in the loss of 39 amino acids from middle T antigen, including all six glutamic acid residues as well as tyrosine 315 and tyrosine 322 (Fig. 2).

To determine whether cells transformed by these mutant DNAs exhibited the full complement of transformed-cell characteristics, we isolated Rat-1 cell lines transformed by either pPdl1-4 DNA (dl1-4:1A and dl1-4:3A) or pPdl2-7 DNA (dl2-7:5B and dl2-7:6A) and measured their final saturation density, their capacity to form colonies in semisolid medium, and their capacity to induce tumors in isogenic rats. All four cell lines grew to an indefinite saturation density (data not shown) and were capable of forming colonies in semisolid medium to the same extent (Table 2). Moreover, all four experimental cell lines induced tumors when injected subcutaneously into 3-week-old Fisher rats and did so at frequencies and latencies comparable with those for Rat-1 cells transformed with a wt control plasmid DNA (Table 2). Therefore, the phenotypes of cells transformed by pPdl1-4 DNA, pPdl2-7 DNA, or wt DNA were not significantly different when compared in these assays.

TABLE 1. Description of mutations affecting middle T antigen-coding sequences

DNA source	Boundaries of deletion	No. of base pairs deleted	Avg. no. of foci per μg of DNA[a]	Predicted amino acid composition of proteins produced from mRNAs having middle T antigen donor and acceptor splice sites[b]
pPH1-8		0	3,000	wild-type mt
pPdl1-4	1144–1164	21	3,000	303 NH_2-mt + 111 COOH-mt
pPdl1-8	1147–1150	4	<1.0	304 NH_2-mt + 39 n.c.st
pPdl1-10	1137–1162	26	<1.0	300 NH_2-mt + N + 165 LT + 4p
pPdl2-3	1110–1186	77	<1.0	292 NH_2-mt + 157 LT + 4p
pPdl2-5	1147–1157	11	<1.0	304 NH_2-mt + 167 LT + 4p
pPdl2-7	1087–1203	117	600	284 NH_2-mt + 98 COOH-mt
pPdl2-12	1106–1190	86	<1.0	290 NH_2-mt + N + 155 LT + 4p

[a] Cells (3.5×10^5) on 100-mm plastic dishes were transfected with 5, 10, or 20 ng of DNA in triplicate, and the foci were counted 14 days posttransfection. The results are given as calculated number of foci per microgram of transfected DNA and represent the average of three separate experiments.

[b] Abbreviations: NH_2-mt, number of amino acids from the amino portion of middle T antigen before the deletion; COOH-mt, number of amino acids from the carboxy portion of middle T antigen after the deletion; n.c.st., new amino acids coded for by sequences normally forming part of small T antigen mRNA 3′ noncoding sequences; N, novel amino acids formed at the junction of the deletion; LT, amino acids that correspond to those normally found in large T antigen; and p, amino acids derived from pBR322 sequences at the polyomavirus-plasmid junction in the recombinant plasmid.

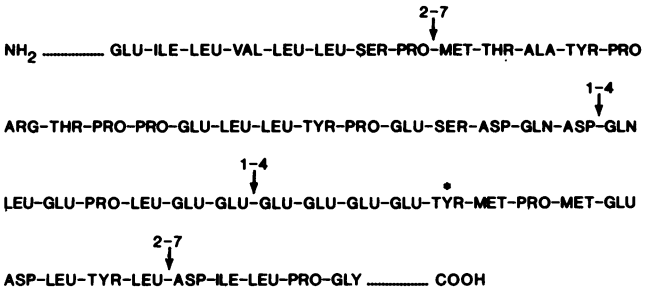

FIG. 2. Partial amino acid sequence of the polyomavirus middle T antigen protein extending from amino acid 277 through 328. Shown by the vertical arrows are the amino acids predicted to be deleted from the middle T antigens encoded by the mutant recombinant plasmids pPdl1-4 and pPdl2-7. Tyrosine 315 is indicated by the asterisk.

To ensure that the cell lines transformed with the mutant DNAs expressed altered forms of middle T antigen, we immunoprecipitated the T antigens within them with antitumor serum and compared their sizes with those T antigens synthesized by cells transformed with a wt polyomavirus genome (T1A1). The middle T antigen expressed by cells transformed with pPdl1-4 DNA (dl1-4:1A and dl1-4:3A) was slightly smaller than the wt middle T antigen synthesized by the T1A1 cell line (Fig. 3). The relative molecular weight of the pPdl1-4-encoded middle T antigen was estimated to be 55,000, and that of the wt middle T antigen was estimated to be 56,000. The measured molecular weight of the truncated pPdl1-4-encoded middle T antigen is that which would be expected if seven amino acids were deleted from the wt protein. Expectedly, the small T antigens expressed by these same cell lines were the same size as the wt small T antigen synthesized by the T1A1 cell line (Fig. 3).

The middle T antigens expressed by cells transformed with pPdl2-7 DNA (dl2-7:5B and dl2-7:6A) were much smaller than that encoded by the T1A1 cell line, and the measured molecular weight of the pPdl2-7-encoded middle T antigen (47,000) was much smaller than expected of a 39-amino-acid deletion (Fig. 3). The latter observation is consistent with reports of other investigators who have previously shown that the removal of acidic amino acids from middle T antigen has an effect that is much greater than expected on the mobility of the protein in SDS-polyacrylamide gels (6, 8). Like others (20, 24), we also observed two forms of middle T antigen in lysates prepared from the T1A1 cell line (Fig. 3). However, we could not detect two closely migrating forms of middle T antigen in any of the cell lines transformed with mutant DNA (Fig. 3). As expected, the small T antigens encoded by the pPdl2-7 genome in the two cell lines examined were the same size as that encoded by the wt genome in the T1A1 cell line (Fig. 3).

Finally, the amount of middle T antigen synthesized by the various transformed cell lines was not significantly different (Fig. 3). Only the dl2-7:5B cell line yielded consistently lower amounts of middle and small T antigen (Fig. 3). These results are consistent with the observation that the cell lines transformed by pPdl1-4 and pPdl2-7 contained one or two integrated copies of recombinant plasmid DNA per diploid quantity of cellular DNA (data not shown).

The novel middle T antigens encoded by the pPdl1-4 and pPdl2-7 mutant genomes lack either a part of the acidic amino acid string before tyrosine 315 (pPdl1-4) or the entire acidic amino acid tract as well as tyrosine 315 (pPdl2-7) (Fig. 2). Therefore, we were curious to learn whether these altered middle T antigens could act as substrates for the middle T antigen-associated tyrosine kinase. To this end, we incubated immunoprecipitates prepared from normal Rat-1 cells, the transformed control T1A1 cell line, and the cell lines transformed by pPdl1-4 and pPdl2-7 DNA with [γ-

TABLE 2. Properties conferred on Rat-1 cells by novel middle T antigens

Cell line	Growth in agar[a]	Tumorigenicity (no. of rats with tumors/ no. of rats injected)[b]
Rat-1	−	0/6
BR2(9)[c]	+	3/4
dl1-4:1A	+	3/4
dl1-4:3A	+	4/4
dl2-7:5B	+	3/4
dl2-7:6B	+	3/4

[a] Cell lines were scored positive (+) if >50% of cells formed colonies of ≥16 cells in size after a week in culture; otherwise, cell lines were scored negative (−).

[b] Cells (10^6) were injected subcutaneously into Fisher rats, and the rats were scored for visible tumors at the site of injection 21 days later.

[c] BR2(9) is a transformed cell line derived after transfection of Rat-1 cells with the plasmid pPBR2; it served as a positive control. pPBR2 is composed of the *Bam*HI-*Eco*RI fragment of polyomavirus DNA (which includes the sequences for the amino portion of large T antigen and the entire middle and small T antigen sequences) cloned in the bacterial plasmid pMK16.1 (13).

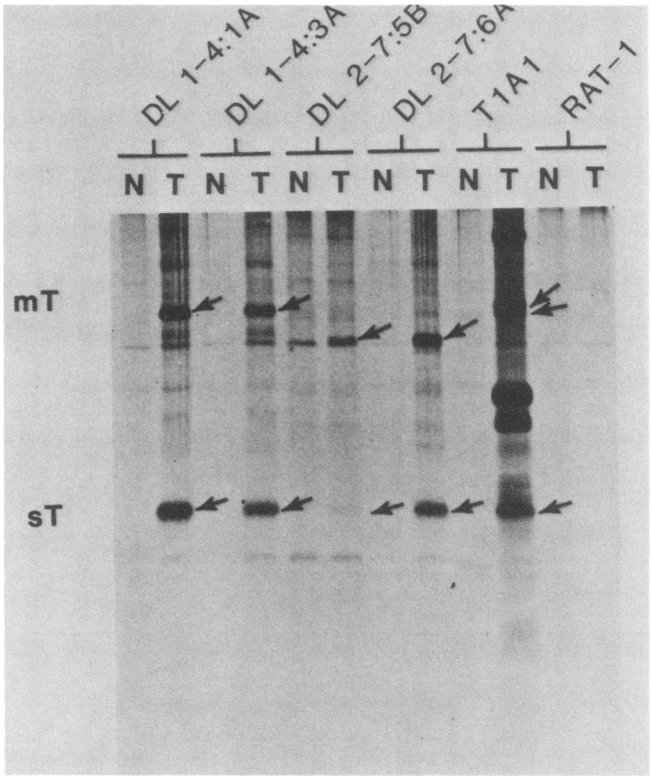

FIG. 3. Autoradiograph of a 12.5% SDS–polyacrylamide gel showing the location of ^{35}S-labeled middle (mT) and small (sT) T antigens immunoprecipitated with antitumor serum (T) from cell lines transformed by wt (T1A1) and mutant (dl1-4:1A, dl1-4:3A, dl2-7:5B, dl2-7:6A) DNA. No T antigens were observed in Rat-1 cells immunoprecipitated with T serum. Tracks to the left of each T lane show similar immunoprecipitation results with normal antiserum (N).

^{32}P]ATP and separated the labeled proteins by electrophoresis through SDS-polyacrylamide gels (Fig. 4).

A 56K protein was phosphorylated in immunoprecipitates prepared from extracts of the T1A1 cell line, whereas no proteins were labeled when extracts from untransformed Rat-1 cells were reacted under identical conditions. Reaction of extracts from the various cell lines with normal rat serum did not lead to the phosphorylation of any proteins (data not shown). Therefore, it is very likely that the heavily phosphorylated 56K protein in the T1A1 immunoprecipitates corresponds to middle T antigen. This contention was bolstered by the observation that the mutant middle T antigens encoded by the pPdl1-4 (55K) and pPdl2-7 (47K) genomes were also labeled with $^{32}P_i$. However, the middle T antigen encoded by pPdl2-7 was phosphorylated to a much lesser extent than either the wt middle T antigen or the protein encoded by the mutant pPdl1-4 (Fig. 4). This was not the result of lesser amounts of middle T antigen in the cells transformed by pPdl2-7 DNA (cf. Fig. 3 and 4).

Incubation of SDS-polyacrylamide gels containing the ^{32}P-labeled proteins in alkali (1.0 M NaOH; 1 h at 55°C) did not significantly reduce the intensity of the labeled 56K (T1A1), 55K (dl1-4:1A and dl1-4:3A), and 47K(dl2-7:5B and dl2-7:6A) T antigens (data not shown). By contrast, the $^{32}P_i$ was completely removed from the less intensely labeled proteins shown in Fig. 4. These results suggest that the wt and mutant middle T antigens were predominantly phosphorylated at tyrosine residues (7, 19, 26).

To confirm that the 47K protein encoded by pPdl2-7 was a mutant form of middle T antigen, and to map the site(s) of tyrosine phosphorylation, we compared the in vitro phosphorylated 47K protein and the 56K wt middle T antigen

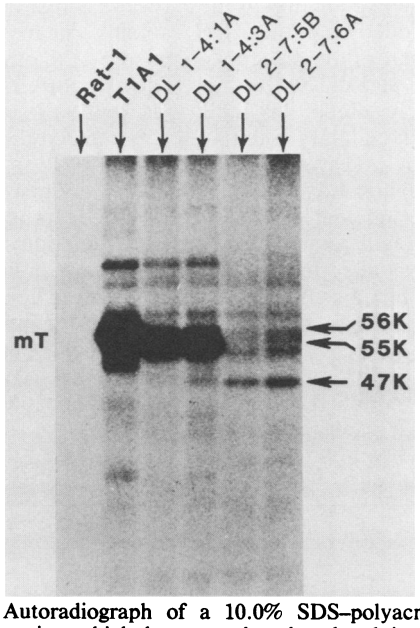

FIG. 4. Autoradiograph of a 10.0% SDS–polyacrylamide gel showing proteins which become phosphorylated in vitro in the presence of [γ-^{32}P]ATP in T antigen immunoprecipitates of cell lines transformed by polyomavirus wt (T1A1) and mutant (dl1-4:1A, dl1-4:3A, dl2-7:5B, and dl2-7:6A) DNA. The top arrow indicates the position of the 56K middle T antigen in T1A1, the middle arrow indicates the position of the 55K truncated middle T antigen in dl1-4:1A and dl1-4:3A, and the bottom arrow indicates the position of the 47K truncated middle T antigen in dl2-75B and dl2-76A cell lines. No ^{32}P-labeled proteins were observed in immunoprecipitates of the Rat-1 cell line.

encoded by the T1A1 cell line by partial proteolysis. Partial digestion of the 47K middle T antigen by *S. aureus* V8 protease yielded three peptides (Fig. 5). These three peptides have the same mobility as three minor spots observed in the wt pattern, but they are distinct from the major carboxy-terminal peptides of the wt middle T antigen (24K, 24K′, and 18K) which contain the major tyrosine acceptor site (residue 315). These results suggest that the 47K protein is an altered form of middle T antigen and that the 47K middle T antigen is phosphorylated in vitro at a residue(s) which is a low-frequency acceptor site(s) in the wt molecule. This minor site is likely located to the amino-terminal side of the Glu-Glu-Asp-Glu sequence (residues 274 through 277). The latter is thought to be the cleavage site of the V8 protease that yields the 24K and 18K carboxy-terminal fragments (23). Attempts to confirm these results by mapping with chymotrypsin and cyanogen bromide have been inconclusive because the peptides from pPdl2-7 middle T antigen comigrate with major peptides of the wt pattern which are known to be derived from phosphorylation at tyrosines 315 and which obscure minor peptides.

DISCUSSION

Using molecularly cloned polyomavirus DNA and in vitro mutagenesis techniques, we isolated a series of deletion mutants whose lesions affect the coding sequences of middle T antigens. These mutations were centered in and around a *Pvu*II site at nucleotide 1144 in polyomavirus DNA. Of the seven deleted recombinant plasmids characterized, five (pPdl1-8, pPdl1-10, pPdl2-3, pPdl2-5, and pPdl2-12) were completely incapable of transforming Rat-1 cells. These five mutants all contain deletions which alter the reading frame for the carboxy-terminal portion of middle T antigen, which is known to be required for its transforming activity (3, 13, 29).

Two deleted recombinant plasmids, pPdl1-4 and pPdl2-7, retained the capacity to transform Rat-1 cells. pPdl1-4 DNA transformed Rat-1 cells at approximately the same frequency at which the wt DNA did, whereas pPdl2-7 DNA transformed cells at a frequency of 15 to 30% of that of wt DNA. Cell lines established after transformation of Rat-1 cells by either pPdl1-4 or pPdl2-7 DNA were indistinguishable from wt transformants as judged by a number of criteria, including the capacity to form tumors in animals. These two deletion mutants are of particular interest as they define portions of middle T antigen which are not essential for transformation of cells in culture.

Polyomavirus middle T antigen has been shown either to be or to be associated with a tyrosine kinase (7, 19, 26). Recent evidence indicates that polyomavirus middle T antigen forms a complex with the product of the c-*src* cellular gene, and it has been suggested that the tyrosine kinase activity measured in vitro in immunoprecipitates of middle T antigen is the activity of the associated pp60$^{c\text{-}src}$ protein (5). Whatever the nature of the tyrosine kinase may be, middle T antigen appears to be a substrate for this activity in vitro, and it has been shown that the major site of phosphorylation in middle T antigen is tyrosine 315 (20). In addition, recent evidence indicates that tyrosine 322 may also become phosphorylated in vitro (15; B. Schaffhausen, manuscript in preparation).

Tyrosine 315 is located in a highly acidic portion of middle T antigen. In particular, it is preceded by a string of six glutamic acid residues (amino acids 309 through 314). A number of polyomavirus mutants lacking either a part or all of the DNA encoding this region of middle T antigen have

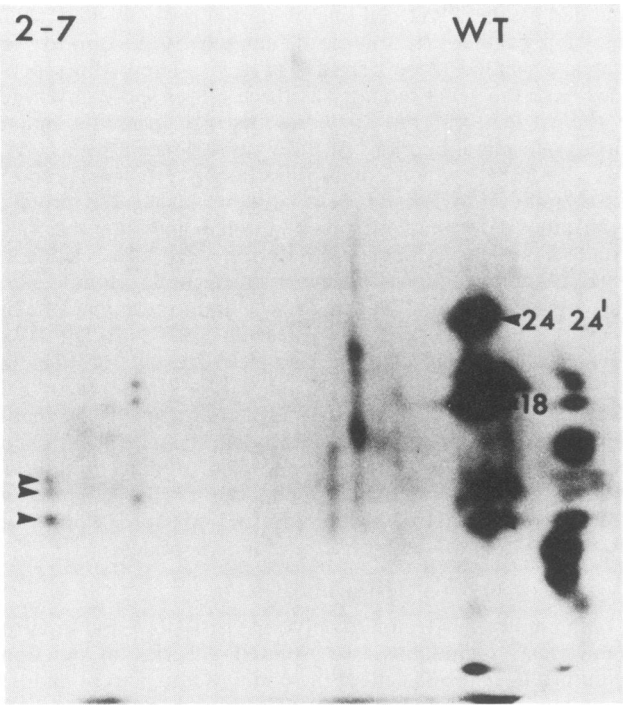

FIG. 5. Partial *S. aureus* V8 protease digestions of wt T1A1 (WT) and pPdl2-7 (2-7) middle T antigens labeled in vitro with [γ-^{32}P]ATP. The major C-terminal peptides (24, 24', 18) of wt middle T antigen are indicated. As well, the arrows indicate the three peptides obtained by cleavage of pPdl2-7 middle T antigen (left) and the corresponding minor spots observed in the wt pattern (right). The labeled spots to the right and left of the major spots in the wt lane are the partial V8 protease products of other proteins which become labeled with $^{32}P_i$ in immunoprecipitates of T1A1 cell lysates (Fig. 4).

been isolated (Table 3). The polyomavirus mutant dl17 contains a deletion which causes the removal of amino acids 300 through 310, thereby deleting the first two glutamic acid residues in the stretch of six (6). dl2210 contains a larger deletion from sequences coding for middle T antigen, which results in the removal of amino acids 296 through 312 from middle T antigen. This amino acid tract includes the first four of six glutamic acid residues which precede tyrosine 315 (14). Both dl17 and dl2210 transform cells at frequencies close to that of wt DNA. These results have led to the suggestion that all the acidic amino acids need not precede tyrosine 315 to preserve the transforming activity of middle T antigen. Our data with pPdl1-4 confirm these results. pPdl1-4 middle T antigen lacks amino acids 304 through 310, thus removing the first two glutamic acid residues, and pPdl1-4 DNA transforms Rat-1 cells at the same frequency at which wt DNA does. The deletion in pPdl1-4 DNA repositions sequences such that the milieu of tyrosine 315 is still generally acidic (Fig. 2). However, these new acidic amino acids do not seem to be required for middle T antigen function, because the deletion in dl2210 removes them with no apparent loss in transforming activity (Table 3).

Three polyomavirus deletion mutants have been isolated whose middle T antigens lack the entire glutamic acid residue stretch, as well as tyrosine 315. They include dl23 (8), dl22 (6), and dl2208 (14); the middle T antigens of these mutants lack amino acids 302 through 335, 287 through 319, and 298 through 316, respectively (Table 3). All three mutants are incapable of transforming cells in culture. These mutations appear to define a region in polyomavirus middle T antigen which is absolutely required for transformation. However, our results with pPdl2-7 contradict this conclusion. In middle T antigen encoded by pPdl2-7, amino acids 285 through 323 are deleted. This deletion includes all six glutamic acid residues as well as tyrosine 315 and tyrosine 322. However, pPdl2-7 DNA transforms Rat-1 cells at a frequency of 15 to 30% of that of wt DNA. These results indicate that amino acids 285 through 323 are not absolutely required for transformation by middle T antigen.

It is difficult to reconcile the difference between our observations and previous results, especially in view of the fact that all of the sequences deleted from dl22 DNA and dl2208 DNA are smaller subsets of those deleted from pPdl2-7 DNA. One explanation of these results is that the middle T antigen encoded by pPdl2-7 DNA retains a nearly wt conformation, whereas the proteins encoded by the other mutants (dl23, dl22, and dl2208) do not. The structure of middle T antigen likely affects its stability as well as its capacity to interact with cellular proteins such as the c-*src* product and perhaps other substrates.

Whether phosphorylation of middle T antigen at tyrosine residues is sufficient or even necessary for its transforming activity is open to debate. There are deletion mutants of polyomavirus affecting middle T antigen which fail to transform cells, but their middle T antigens are efficiently phosphorylated in vitro (Table 3) (14, 21, 29). Therefore, the capacity of middle T antigen to become phosphorylated in vitro is not sufficient for transforming activity. Whether the capacity of middle T antigen to become phosphorylated in

TABLE 3. Properties of middle T antigens encoded by mutant polyomavirus

Mutant	Amino acids deleted	Associated kinase assay		Associated kinase activity[a]	Transforming activity (% of wt activity)[b]
		Cell line used	Substrate used[c]		
dl17	300–310			ND	100
dl2210	296–312	3T6	IgG	+	60
pPdl1-4	304–310	Rat-1	mT	+	100
dl23	302–335	3T6	mT	−/+	0
dl22	287–319			ND	0
dl2208	293–316	3T6	IgG	+	<1
pPdl2-7	285–323	Rat-1	mT	−/+	20

[a] ND, Not done; +, positive kinase activity associated with middle T antigen; and −/+, very low, but measurable, kinase activity associated with middle T antigen.

[b] In all such experiments Rat-1 cells were used to assess transforming activity.

[c] IgG, Heavy chain of immunoglobulin G; mT, middle T antigen.

vitro is required for its transforming activity is a more contentious issue. All of the middle T antigens encoded by transforming mutants of polyomavirus with lesions (deletions and base substitutions) in middle T antigen coding sequences are efficiently phosphorylated at tyrosine residues in vitro. Among the latter is the mutant of polyomavirus whose middle T antigen contains a substitution of phenylalanine for tyrosine 315 (15). It is likely that tyrosine 322 serves as the principal acceptor of phosphate in the mutant middle T antigens encoded by these various transforming viruses whose middle T antigens lack tyrosine 315. Therefore it would appear that tyrosine 315 and tyrosine 322 can both act as acceptors of phosphate in vitro. However, Carmichael et al. (4) have very recently reported that substitution of phenylalanine for tyrosine 315 drastically reduces the transforming activity of the virus harboring the mutation (to less than 1% of the transforming activity of the wt virus). Moreover, the middle T antigen encoded by this mutant virus (Py-1178-T) displayed a reduced capacity (20% by comparison with that of the wt protein) to become phosphorylated at tyrosine residues in vitro (4). These results contradict those of Oostra et al. (15) and are inconsistent with our observations. However, Carmichael et al. (4) measured the kinase activity of middle T antigen in extracts prepared from virally infected baby mouse kidney cells, and those authors used rat F-111 cells to determine the transforming activity of the mutant virus. By contrast, Oostra et al. (15) and we used Rat-1 cells and assayed the kinase activity associated with middle T antigen in the resulting transformed cells. It seems likely that the choice of cells (F-111, Rat-1, or baby mouse kidney cells) influences the outcome of the experiments. This could be so because the various cell types may produce differing amounts of the c-*src* product, and it is possible that the concentration of this protein is crucial to effect transformation, to allow association with middle T antigen, and consequently to permit phosphorylation of middle T antigen.

pPdl2-7 DNA is the only mutant which transforms cells at high efficiency (15 to 30% of the frequency of wt DNA) yet encodes a middle T antigen which is a poor substrate for the associated tyrosine kinase. The 47K middle T antigen encoded by pPdl2-7 lacks the acidic amino acid stretch, tyrosine 315, and tyrosine 322. Nonetheless, the 47K middle T antigen is phosphorylated in vitro, albeit very poorly, at tyrosine residues. We believe that the weakly phosphorylated 47K protein is in fact middle T antigen, but we have not been able to definitely rule out the possibility that the 47K species is a cellular protein which becomes phosphorylated at tyrosine residues in immunoprecipitates of the polyomavirus T antigens. If the truncated 47K protein is indeed middle T antigen, then it must be phosphorylated at a tyrosine residue(s) other than tyrosine 315 or tyrosine 322. The V8 protease partial digests of the 47K protein revealed that the site of tyrosine phosphorylation is in the amino-terminal region of middle T antigen. There are 11 tyrosine residues between the amino terminus in middle T antigen and tyrosine 315. We do not know which of these serve as sites of phosphorylation in the 47K middle T antigen. None of these tyrosines are surrounded by amino acids commonly found amino terminal to sites of tyrosine phosphorylation in viral $p60^{src}$ and other $p60^{src}$-related transforming proteins (2, 16). The concensus phosphorylation sequence includes a basic amino acid seven residues to the amino-terminal side of tyrosine and one or more acidic residues between this basic amino acid and tyrosine. Only one but not both of these features precedes the major sites of tyrosine phosphorylation in middle T antigen (315 and 322), thereby making it difficult to choose among the remaining putative, weak sites of tyrosine phosphorylation in the 47K middle T antigen.

Although it is tempting to dismiss the phosphorylation of tyrosine in middle T antigen as an in vitro artifact, especially because elimination of the major sites of phosphorylation does not invariably result in a loss of transforming activity, there are other data worthy of consideration before reaching such a conclusion. First, there is evidence that a small fraction of middle T antigen is phosphorylated at tyrosines in vivo (24). Second, the weak tyrosine phosphorylation site in the 47K middle T antigen is also a minor site of phosphorylation in the wt middle T antigen. One could argue that the phosphorylation of this site(s) (tyrosine 206, 250, 258, or a combination of these) is relevant to the transforming activity of middle T antigen, whereas phosphorylation of tyrosine 315, 322, or both is not. Perhaps the physiological significance of the tyrosine phosphorylation of middle T antigen will only be proven when all putative sites of tyrosine phosphorylation are removed from the protein by mutation.

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