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Comparison of Phosphorylation of Two Polyoma Virus Middle T Antigens In Vivo and In Vitro

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Two species of polyoma virus middle T antigen were detected in both lytically infected and transformed cells by in vitro kinase assay of immunoprecipitates. A minor species with an apparent molecular weight of 58,000 (58K) represented less than 10% of the total middle T protein. This species was roughly 10 times more active as a phosphate acceptor than was the predominant 56K form. Partial proteolytic mapping experiments showed that the same site was phosphorylated in both species. Mapping of the middle T antigens from a series of deletion mutants suggested that the major site of phosphorylation is tyrosine residue 315. Phosphorylation occurred on both middle T species in vivo, involving sites predominantly other than the tyrosine labeled in vitro. The 56K and 58K middle T forms differed from each other in their in vivo phosphorylation patterns. Some phosphate was incorporated into the 58K species in a region of the molecule to which at least part of the apparent molecular weight difference could be mapped. *hr-t* mutant NG-59, which codes for a slightly altered middle T, produced only a single species (56K) which was inactive in the in vitro kinase reaction. Moreover, no 58K species appeared in vivo with this mutant. *hr-t* mutants are therefore defective in both aspects of phosphorylation. Phenotypically normal revertant cells of a polyoma transformed line failed to express any middle T antigens or associated kinase activity.

In at least two well-studied tumor virus systems, a single viral gene is ultimately responsible for inducing a full array of changes associated with malignant transformation. In the case of polyoma virus, this gene is the *hr-t* gene: its products are the middle and small T antigens. In the case of Rous sarcoma virus, it is the *src* gene and pp60^{src} product. For both viruses, a second gene (*ts-a* for polyoma and *pol* for Rous sarcoma virus) is needed to bring about a stable association of the viral genome with the cell. Although the two viruses are profoundly different in their structures, life cycles, and natural histories, the pattern of cellular changes caused by the two "oncogenes" is strikingly similar. Dramatic morphological alterations occur; these are accompanied by decreased cell-substratum adhesion and by structural changes at the cell surface (lectin agglutinability) and in the cytoplasm (stress fibers). Growth control is relaxed, leading to decreased regulation by cell density, by serum concentration, or by the availability of a solid substrate.

Protein modification reactions controlled directly or indirectly by the products of these viral genes have been implicated in the biochemical mechanisms underlying their pleiotropic action in cells. Expression of the *hr-t* gene leads to hyperacetylation of histones H3 and H4 encapsidated in virions (32), a finding consistent with a pleiotropic regulatory model of *hr-t* gene action proposed on the basis of the host range and transformation properties of *hr-t* mutants (3, 39). Immune precipitates containing pp60^{src}, the product of the *src* gene, show a kinase activity leading to phosphorylation of pp60^{src} and of the heavy chain of immunoglobulins (8, 25, 29, 31). The activity is absent or reduced when mutants defective in the *src* gene are used. Analogous experiments with polyoma virus T antigens show a similar activity, with the 56,000-dalton (56K) middle T antigen being the major phosphate acceptor (9, 33, 37). *hr-t* mutants are uniformly negative in this reaction (33). In mutant NG-59, an alteration from Asp to Ile-Asn at a specific site within the sequence shared by the middle and small T proteins (6) is sufficient to abolish the in vitro kinase activity (33). Specificity for tyrosine as the phosphate acceptor is one feature shared by the kinases associated with the transforming proteins of polyoma virus (9), Rous (21), Fujinami (10), and Y73 (26) sarcoma viruses, and Abelson leukemia virus (42). Another common feature is the association of the viral gene products with plasma membranes (22, 36, 40, 43).

Understanding the mechanism of action of these viral oncogenes will require further detailed study of the protein modification reactions under their control. Here, we report a new species of polyoma virus middle T antigen which differed from the previously recognized 56K species in having a slightly higher apparent molecular weight (58K) and a different pattern of phosphorylation in vivo. The 58K species was present in small amounts, but had a much higher specific activity in the in vitro kinase reaction than did the major 56K species. Mapping of the site of in vitro phosphorylation indicated a single tyrosine residue in the carboxy half of the proteins as the major site in both species. No 58K protein was detected with *hr-t* mutant NG-59 by either [^{35}S]methionine or $^{32}PO_4$ labeling in vivo. The altered 56K protein in this mutant shows a normal pattern of phosphorylation in vivo, despite being inactive in the in vitro kinase reaction.

MATERIALS AND METHODS

Cells and viruses. Primary cultures of baby mouse kidney (BMK) cells were made by the method of Winocour (41). 3T3-Py6 was derived from Swiss mouse 3T3 cells (2). 3T3-Py6-R1 is a "flat revertant" of 3T3-Py6 (15).

Wild-type viruses were derived from *hr-t* mutants by marker rescue (11) or were derivatives of Pasadena small plaque virus. *hr-t* mutants included NG18 and NG59 (2) and B2 and SD15 (39). Deletion strain 45 (1) was kindly provided by Bill Folk. *dl*8 and *dl*23 (16, 17) were gifts of Beverly Griffin. *dl*1013, *dl*1014, and *dl*1015 (30) were gifts of Gorin Magnusson.

Reagents and materials. Anti-polyoma tumor sera (anti-T sera) came from brown Norwegian rats bearing a syngeneic polyoma-induced tumor or from the ascites of a brown Norwegian rat bearing a mixed ascites-solid tumor as described previously (36). Preimmune serum was obtained from normal brown Norwegian rats. *Staphylococcus aureus* protein A coupled to Sepharose was obtained from Pharmacia Fine Chemicals. [γ-^{32}P]ATP was synthesized by the procedure of Glynn and Chappel (14). The specific activity ranged from 1,000 to 1,500 Ci/mmol. Carrier-free $^{32}PO_4$ and [^{35}S]methionine (400 Ci/mmol) were produced by New England Nuclear Corp. Chymotrypsin was from Worthington Diagnostics, and *S. aureus* protease V8 was from Miles Laboratories. Other chemicals were of the highest reagent grade available.

T antigen preparation. The procedures for T antigen preparation have been previously described in detail (33, 34, 36; B. Schaffhausen and T. L. Benjamin, *In Eighth Cold Spring Harbor Conference on Cell Proliferation*, in press). Briefly, cells were labeled with [^{35}S]methionine (20 to 100 μCi/ml, 400 to 600 Ci/mmol, New England Nuclear) in Hanks balanced salt solution or $^{32}PO_4$ (100 to 400 μCi/ml) in Dulbecco modified Eagle medium lacking phosphate for periods up to 2 h. Labeled or unlabeled cells were washed with cold phosphate-buffered saline and rinsed with washing buffer (0.137 M NaCl, 0.02 M Tris-hydrochloride [pH 9], 0.001 M $MgCl_2$, 0.001 M $CaCl_2$). T antigens were extracted for 20 min at 4°C with the same buffer containing 10% (vol/vol) glycerol and 1% (vol/vol) Nonidet P-40, 1.1 ml per 90-mm dish. Extracts were cleared by centrifugation in an Eppendorf microcentrifuge for 10 min at 4°C. Anti-T ascites or preimmune serum (10 μl) was added to the extract from each dish; 80 μl of a 50% suspension of *S. aureus* protein A-Sepharose was then added, and the mixture was gently agitated for 20 min. The precipitates were collected and washed with cold phosphate-buffered saline. They were then washed twice more with 0.5 M LiCl-0.1 M Tris-hydrochloride (pH 6.8) and finally with distilled water.

In vitro kinase reaction. Washed immunoprecipitates were suspended in 0.4 ml of 0.02 M Tris-hydrochloride (pH 7.5)-0.005 M $MgCl_2$. [γ-^{32}P]ATP (20 to 100 μCi) was added, and the mixture was incubated for 15 min at room temperature. The precipitates were collected and washed as above.

Gel electrophoresis. Immunoprecipitates were boiled for 2 min in 30 to 80 μl of dissociation buffer containing 5% (wt/vol) sodium dodecyl sulfate-5% (vol/vol) 2-mercaptoethanol-0.0625 M Tris-hydrochloride (pH 6.8)-0.03% (wt/vol) bromophenol blue-20% (vol/vol) glycerol. Precipitates were electrophoresed on discontinuous buffer sodium dodecyl sulfate-polyacrylamide gels of 10 and 12.5% acrylamide (28). Gels were dried and exposed directly with or without a Lightning-Plus (Du Pont Co.) intensifying screen or fluorographed as described by Bonner and Laskey (5).

Partial proteolysis. Partial proteolysis was done as described previously (33, 34; Schaffhausen and Benjamin, in press). Cylindrical gels (10 cm by 1.5 mm, 10% acrylamide) were run at 50 V for 12 to 14 h. The cylinders were placed head to head on top of a slab gel, and 2 ml of digestion solution (0.125 M Tris-hydrochloride [pH 6.8], 0.01 M EDTA, 20% [vol/vol] glycerol, 50 μg of bovine serum albumin per ml, and 20 μg of either chymotrypsin or *S. aureus* V8 protease per ml) was layered on top of the gels. Electrophoresis was carried out at 50 V for approximately 16 h.

RESULTS

Three gene products are encoded in the early region of polyoma virus DNA: large (100K), middle (56K), and small (22K) T antigens. The large T antigen is affected in *ts-a* mutants, whereas both the middle and small T antigens are affected in all *hr-t* mutants (4). Figure 1 shows the position within middle T antigen of two *hr-t* mutants. SD15, an in-phase deletion mutant, codes for a truncated (50K) middle T species (36); NG59 has an in-phase insertion-substitution (Asp to Ile-Asn [6]). Both SD15 and NG59 are totally defective in transformation (39) and in the in vitro kinase reaction (33). The sequences coding for the C-terminal half of middle T antigen are read in an overlapping frame to specify a portion of the large T antigen (24). Deletion mutants in this overlap region have been isolated (Fig. 1) (1, 16, 17, 30). These mutants, showing truncated large and middle T

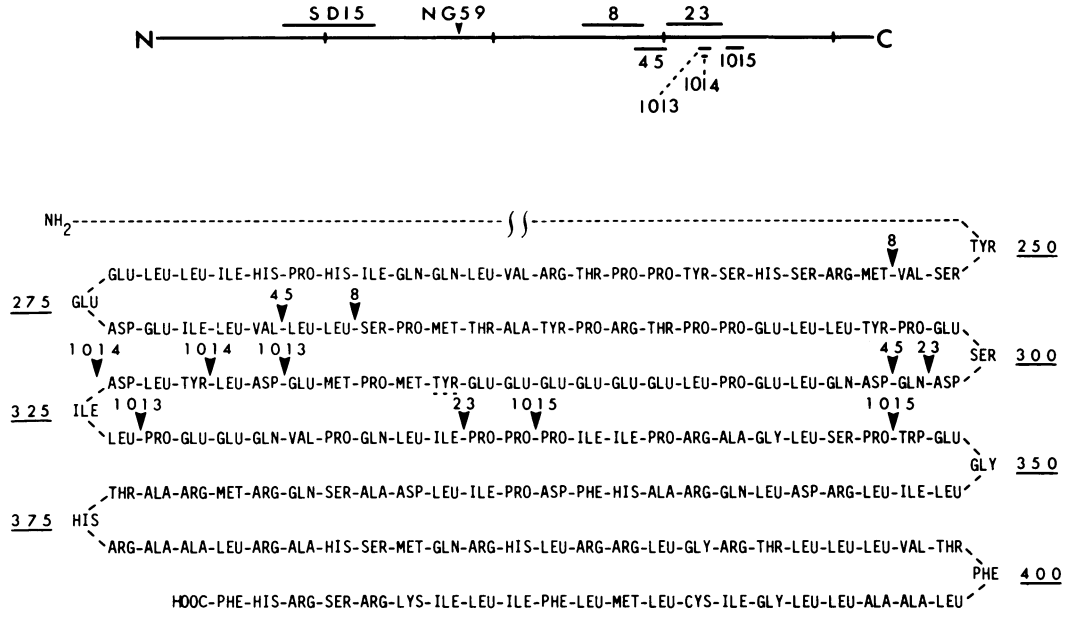

FIG. 1. *Sites of mutations affecting middle T antigens. Top, Line drawing showing the alterations in middle T antigens in various mutants. Hr-t mutant SD15 has a deletion eliminating amino acids 83 to 129; hr-t mutant NG59 has a change from Asp to Asn with the insertion of an additional isoleucine at amino acid 179 (6). Sizes and positions of the deletions are shown for the dl mutants. Vertical tickmarks are placed 100 amino acids apart along the molecule. Bottom, Amino acids in the C-terminal region of middle T antigen. The sequence is deduced from DNA sequencing results of Friedmann et al. (13). The exact positions of the deletions are shown for dl45 (1), dl8, and dl23 (Smolar and Griffin, submitted for publication), and dl1013, dl1014, and dl1015 (Magnusson, personal communication).*

antigens, are not uniform in their biological properties. All but *dl*23 retain significant transforming activity; this mutant is negative or strongly diminished in kinase activity (37). Mutants *dl*45 (33), *dl*8 (37), and *dl*1013, *dl*1014, and *dl*1015 (see below) have readily detectable levels of kinase activity.

Two species of middle T antigen are phosphorylated in the in vitro kinase reaction. Figure 2A (lane 2) shows an anti-T immune precipitate of [^{35}S]methionine-labeled wild-type virus-infected cells. Large (100K), middle (56K), and small (22K) T antigens are seen, along with two additional bands, at 63K and 36K, as previously reported (32). (The latter species do not appear to be primary viral translation products as judged from cell-free translation of viral mRNA [20; R. Shaikh, E. Ozkaynak, and T. Benjamin, unpublished data].) When similarly prepared wild-type immune precipitates from unlabeled cells were incubated with [γ-^{32}P]ATP two major phosphorylated species were seen in the middle T region of the gel (Fig. 2B, lane 2). One comigrated with the 56K T antigen detected by metabolic labeling. The other slower-migrating species had an apparent molecular weight ranging from 58K to 63K depending on gel conditions. In earlier work this protein migrated close to the major [^{35}S]methionine-labeled 63K protein. On gels such as those shown here, this species was clearly resolved from the major 63K band and did not comigrate with any major [^{35}S]methionine-labeled species. This species will be subsequently called 58K. Small amounts of a 58K [^{35}S]methionine-labeled middle T antigen could be detected by using a second dimension with partial proteolysis (see below).

Figure 2 also shows the patterns of in vitro phosphorylation obtained for various mutants affected in the middle T antigen. No phosphorylation of middle T antigens of *hr-t* mutants NG59 or SD15 was observed. Mutant *dl*23, which is most affected in transformation, is largely inactive in the kinase reaction (37). The other *dl* mutants all show active but truncated products. Like wild-type virus, *dl*1013, *dl*1014, and *dl*1015 each show two phosphorylated species, whereas *dl*8 and *dl*45 each show only a single species in the in vitro reaction. Interestingly, *dl*1014 shows truncated species even though the deletion is predicted to remove only three amino acids (G. Magnusson, personal communication).

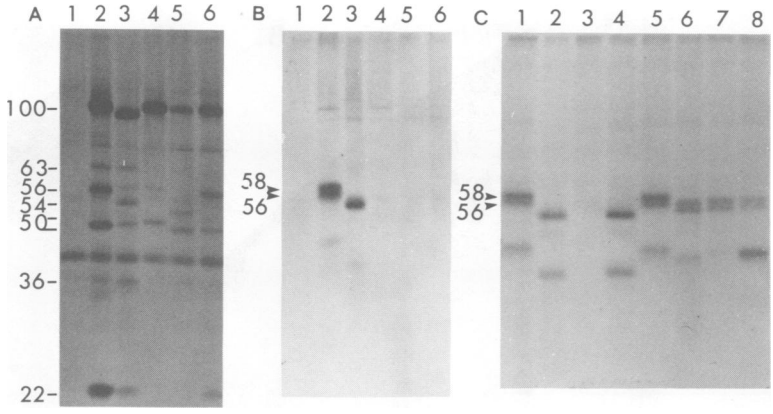

FIG. 2. *T Antigens labeled in vivo with [^{35}S]methionine or in vitro with [γ-^{32}P]ATP. The conditions of labeling were as described in the text. Approximately 10^6 cells are used for each immunoprecipitation. Washed immunoprecipitates were electrophoresed on discontinuous buffer sodium dodecyl sulfate gels of 10% acrylamide. Panels A and B are parts of the same gel, so that the mobilities are directly comparable. A, [^{35}S]-methionine. Lanes: 1, mock-infected cells; 2, wild type; 3, dl45; 4, hr-t mutant NG18; 5, hr-t mutant SD15; and 6, hr-t mutant NG59. The positions and apparent molecular weights of wild-type T antigens are indicated. The truncated middle T antigens of dl45 (54K) and SD15 (50K) are also shown. The horizontal line indicates capsid protein VP1 in the immunoprecipitates. B, [γ-^{32}P]ATP-labeled T antigens in the same order as in A. C, [γ-^{32}P]ATP-labeled T antigens. Lanes: 1, wild type; 2, dl8; 3, dl23; 4, dl45; 5, wild type; 6, dl1013; 7, dl1015; and 8, dl1014.*

56K and 58K middle T antigens are closely related species differing in the C-terminal region. Partial proteolytic mapping with chymotrypsin showed that the 56K and 58K species are related. T antigens were first resolved on cylindrical gels and then digested with protease during electrophoresis on a slab gel (33, Schaffhausen and Benjamin, in press). Figure 3A shows the chymotryptic fragments of wild type 56K and 58K species phosphorylated in vitro. Undigested material remains on the diagonal, and chymotryptic fragments appear below. A series of five pairs of fragments differing by a constant molecular weight between the two forms was found. The intense doublet pairs at 39K/33K derived from the 58K species and at 37K/31K derived from the 56K species are especially diagnostic. This digestion pattern is expected for two closely related species that differ in one portion of the molecule and are cleaved at common sites. A smaller fragment at 18K (shown by the line) was common to both 56K and 58K. This suggests that a common site is phosphorylated in the two species and that the difference between the 58K and 56K forms is not localized in this 18K phosphopeptide. Figure 3B shows that fragments at 39K and 33K arising from the 58K species could be detected by [^{35}S]methionine labeling with a sufficiently long exposure of the gel. Unlike the results with in vitro ^{32}P-labeled T antigens, the intensity of the ^{35}S 39K/33K spots was very much reduced relative to the intensity of the 37K/31K fragments. Continuous labeling with [^{35}S]methionine resulted in a similar high ratio of 56K to 58K, indicating that 58K was present in low concentrations. The 58K species is therefore much more active than the 56K species in the kinase reaction, perhaps by an order of magnitude. Pulse-chase experiments suggested that the bulk of the 56K middle T antigen is not converted to the 58K form. *hr-t* mutant NG-59 did not appear to make a 58K species detectable by chymotryptic digestion (Fig. 3C). A small amount of the minor species cannot be ruled out because of the background. However, independent experiments based on in vivo labeling with $^{32}PO_4$ also indicated the absence of a 58K species in NG-59 (see below).

The 37K/31K chymotryptic fragments containing the major phosphorylation site in 56K can be localized within the C-terminal half of the molecule. *dl*45, which has a deletion in the distal portion of middle T (Fig. 1), gave ^{32}P-labeled fragments shorter than those of the wild type (Fig. 3A). Figure 4 shows a comparison of ^{35}S-labeled middle T antigens from wild type with *dl*45 and SD15. SD15, with a deletion in the N-terminal region, gives the normal-sized 37K/31K fragments. These fragments therefore arise from the C-terminal portion of the molecule. Because the difference between the 56K and 58K forms of middle T is retained in the fragments, it must derive from some alteration in that portion of the molecule.

Tyrosine 315 is a major site of phospho-

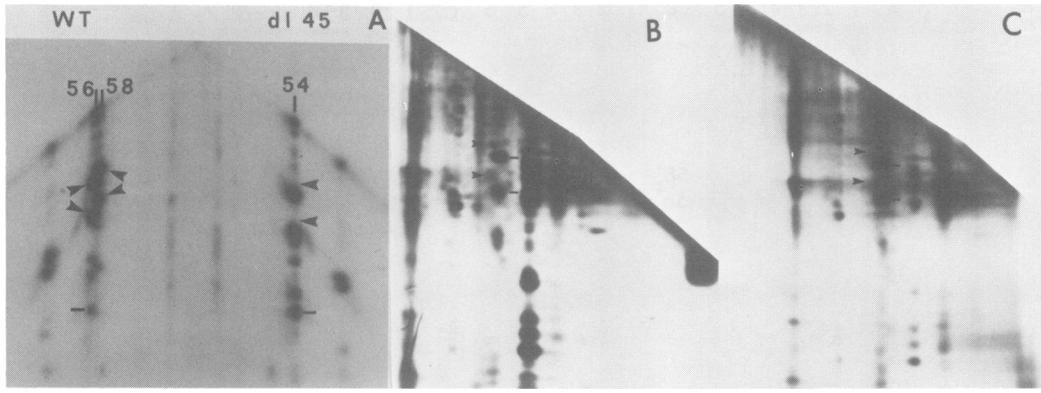

FIG. 3. *Partial chymotryptic digestion of polyoma T antigens. A, Digestion of wild-type (WT) and dl45 T antigens labeled in vitro with [γ-^{32}P]ATP. T antigens were isolated from BMK cells, labeled and run on 10-cm cylindrical sodium dodecyl sulfate gels of 10% acrylamide. Digestion was carried out on sodium dodecyl sulfate-12.5% acrylamide gels with chymotrypsin. The arrowheads in the wild-type pattern indicate the position of the 39K/33K and 37K/31K pairs. The arrowheads in the dl45 pattern indicate where the full-length 37K and 31K species of full-length wild type would run in the second dimension. The line indicates an 18K chymotryptic fragment common to both 56K and 58K middle T antigens. B, Digestion of [^{35}S]methionine-labeled wild-type T antigens. [^{35}S]methionine-labeled T antigens were electrophoresed on 20-cm sodium dodecyl sulfate gels of 10% acrylamide. Digestion was carried out in the second dimension. The positions of the 39K/33K fragments derived from the 58K T antigen are shown by the arrowheads; the 37K/31K fragments derived from the 56K T antigen are shown by the lines. C, Digestion of [^{35}S]methionine-labeled hr-t mutant NG59 T antigens. [^{35}S]methionine labeled T antigens were digested as in B. The positions of the 37K and 31K species deriving from the 56K species are indicated by the lines. The region where the 39K/33K fragments would be found for the wild type is shown by arrowheads.*

rylation in vitro in the 56K and 58K middle T antigens. Figure 5A shows the cleavage of [^{35}S]methionine-labeled wild-type T antigens with *S. aureus* V8 protease. For the 56K middle T antigen, a composite spot at ~24K and another at 18K were obtained. The patterns for the in vitro ^{32}P-labeled material also showed fragments at 24K and 18K (Fig. 5B). Double-digestion experiments showed that the 18K fragment can be generated from the 24K fragment, indicating that these two fragments overlap (not shown). Differences between the 58K and the 56K species were not as obvious with V8 as with chymotryptic digestion. The ~24K spot could be resolved on 10% acrylamide gels into two species, one derived from 56K and the other from 58K (Fig. 5B). The 18K V8 fragment remained as a single component common to both species. These results suggest that at least part of the difference between the 56K and 58K species resides in the 6K region unique to the 24K fragment.

Results of V8 mapping of the various truncated middle T antigens are shown in Fig. 6. For the most distal mutants, *dl*1013, *dl*1014, and *dl*1015, both the 24K and the 18K species were truncated. This localizes these fragments to the C-terminal portion of the molecule. For *dl*45, the 18K fragment was of normal length, indicating that the cleavage generating the 18K must occur distal to the *dl*45 deletion. The "24K" fragment of *dl*45 was shortened; the cleavage generating this fragment must therefore be proximal to the *dl*45 deletion. For *dl*8, the 18K fragment was again of normal size, whereas the "24K" species was larger than that observed for the wild-type virus, implying that the cleavage generating the 24K species lies within the *dl*8 deletion. Interestingly, *dl*23, which has very poor kinase activity, gave a diffuse pattern.

The above results show that the major site of in vitro phosphorylation of both 56K and 58K middle T species lies within the C terminal 18K V8 fragment. This kinase reaction phosphorylates middle T antigen predominantly on tyrosine (9). Based on the DNA sequence, only two tyrosines (residues 315 and 322) are present downstream of the *dl*45 deletion (Fig. 1). Tyrosine 322 is deleted in *dl*1013 and *dl*1014, and yet both mutants were normal in the in vitro kinase reaction (Fig. 2). Therefore, tyrosine 315 is most likely the major site of in vitro phosphorylation. This assignment is consistent with the defect in kinase activity in *dl*23, the only *dl* mutant in which tyrosine 315 is missing.

Two middle T species with associated kinase activity are present in polyoma-transformed 3T3 cells, but not in a phenotypi-

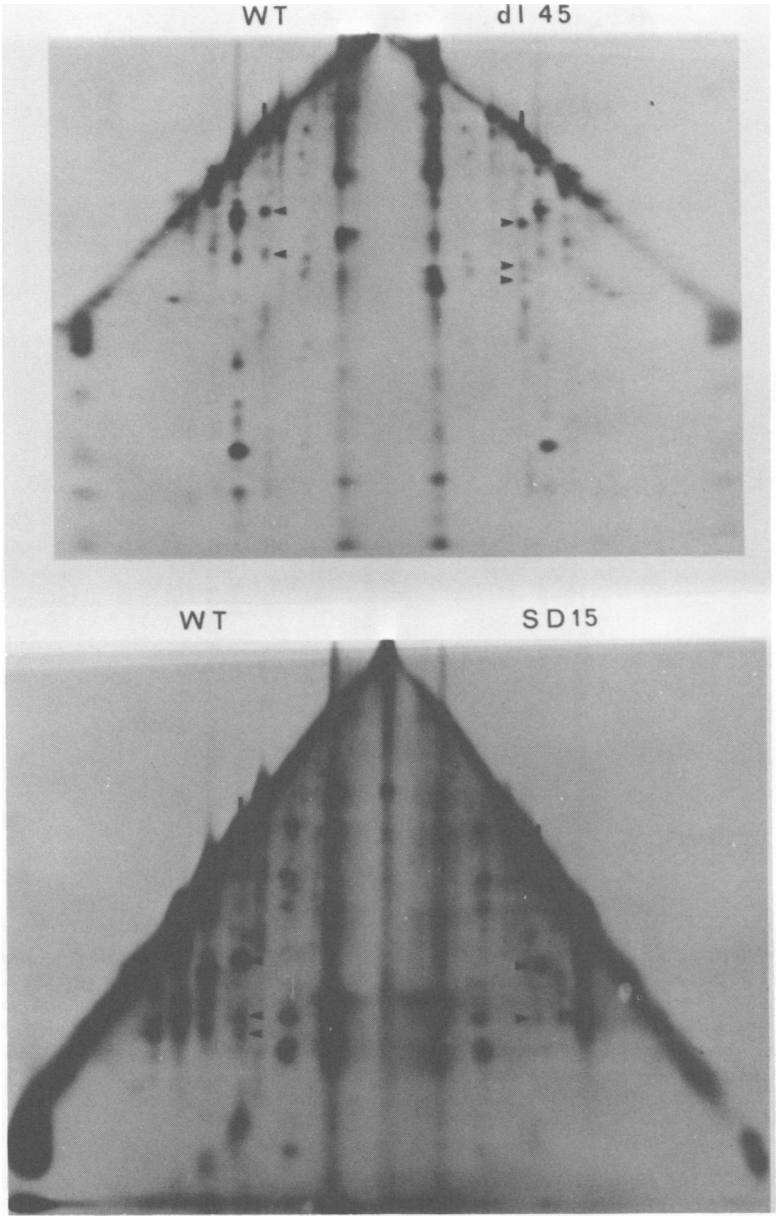

FIG. 4. *Partial chymotryptic digestion of wild-type, dl45, and SD15 T antigens labeled with [^{35}S]methionine. T antigens were extracted from infected BMK cells labeled with [^{35}S]methionine (100 μCi/ml) for 90 min at 24 h postinfection. Digestion and gel conditions were as in Fig. 3A, except that the second dimension of the SD15 gel was 10% acrylamide. The positions of the 37K/31K fragments are shown for the wild type. The lowest arrow in the wild-type pattern in the bottom panel indicates a 30.5K fragment that does not resolve from the 31K on 12.5% acrylamide gels. This fragment arose from internal cleavage of middle T: it was affected by both the dl45 and SD15 mutations. The full-length 37K/31K fragments of SD15 and the truncated dl45 products are also indicated by arrowheads.*

cally normal revertant. Some transformed lines show a broad band of middle T antigen labeled in vitro on one-dimensional sodium dodecyl sulfate gels (33). However, analysis of one such transformant (3T3-Py6) by partial proteolysis showed that the chymotryptic fragments of both the 56K and 58K species were present. The intensity of the 39K/33K fragments was

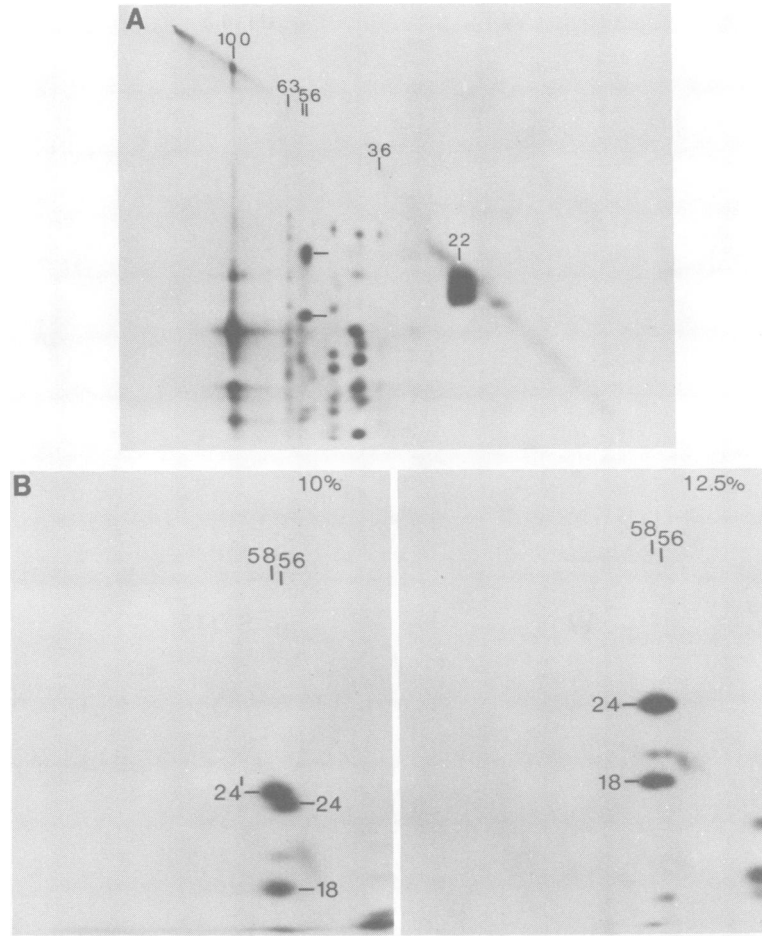

FIG. 5. *Digestion of polyoma T antigens by S. aureus protease V8. A, [^{35}S]methionine-labeled wild-type T antigens were electrophoresed on a 20-cm gel of 10% acrylamide. Digestion was carried out on a sodium dodecyl sulfate-12.5% acrylamide gel with S. aureus protease V8. The positions of the undigested T antigens are indicated on the diagonal line; the extra tickmark indicates the 58K position. The 24K and 18K fragments arising from the digestion of middle T antigens are indicated by the lines. B, [γ-^{32}P]ATP-labeled wild-type T antigens were separated on 10% acrylamide gels in the first dimension. Digestion with V8 protease was carried out in the second dimension on sodium dodecyl sulfate-10% acrylamide (left) or -12.5% acrylamide (right) gels.*

relatively less, and the resolution between 56K and 58K in the first dimension appeared to be not as good compared with T antigens from a wild-type lytic infection (Fig. 7). The importance of middle T antigen-associated kinase to the transformed state can be assessed by examining phenotypically normal revertants of transformed cells. A flat revertant of 3T3-Py6 isolated by FUdR selection (15) was tested by [^{35}S]methionine labeling and in vitro kinase activity. As shown in Fig. 8, the revertant failed to express middle T antigens detectable by either metabolic labeling or by the more sensitive kinase reaction.

56K and 58K middle T antigens are phosphorylated differently in vivo and in vitro. Although the 100K large T is the major phosphorylated species in vivo, the 56K T antigen is also labeled with $^{32}PO_4$ (34). For the wild-type virus, both the 56K and the 58K forms were labeled (Fig. 9); as with the in vitro-labeled products, the exact mobility depended on the gel conditions. Only the 56K band is seen with *hr-t* mutants NG-59 and 3A1 that carry an in-phase insertion (6), whereas no middle T bands are seen with frameshift deletion mutants NG-18 and B-2 (18). When the 58K band was first observed (33), it could largely be removed by a

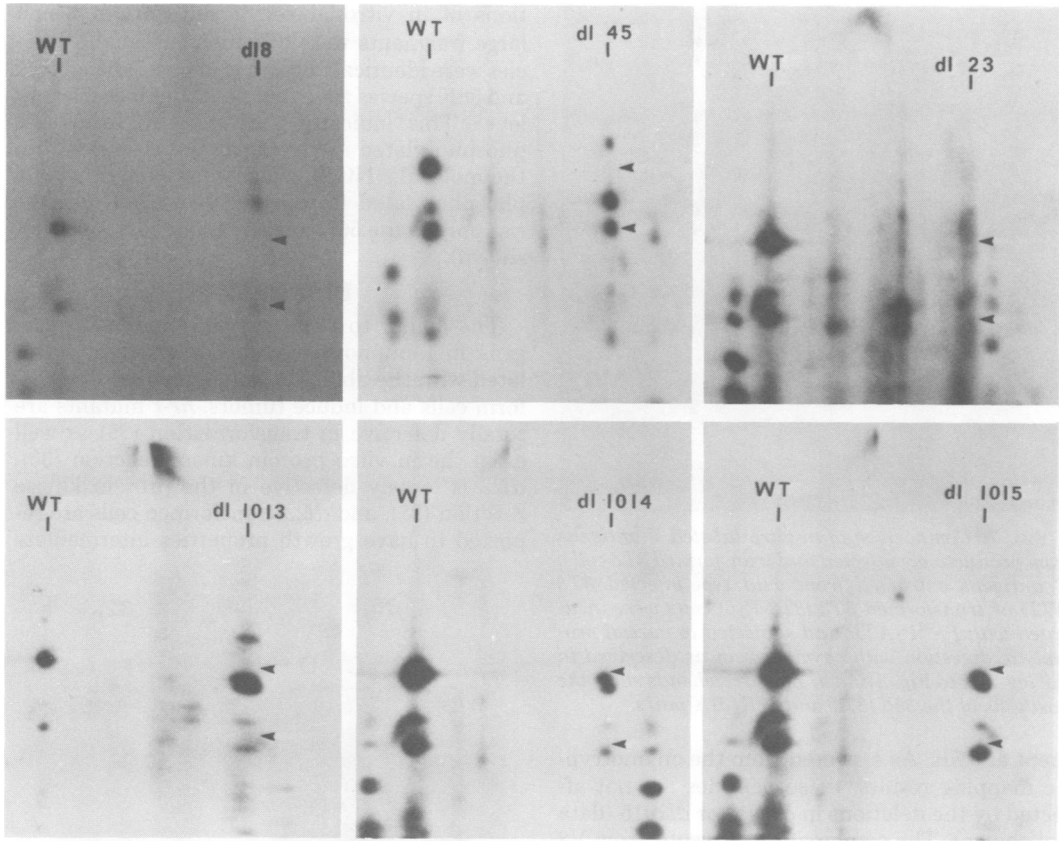

FIG. 6. *S. aureus protease V8 digestion of [γ-^{32}P]ATP T antigens of wild-type and dl viruses. T antigens were isolated from BMK cells, labeled in vitro, and resolved on 10-cm sodium dodecyl sulfate gels. Individual dl mutants were compared with wild-type virus by digestion on second-dimension 12.5% acrylamide gels with V8 protease. The position of middle T is indicated by the line designating wild-type or dl mutant. The arrowheads in the mutant lane indicate where full-length 24K or 18K fragments would be. dl8 does not grow well (17), and the stock used here has a 20-fold-lower titer than do the other viruses.*

complicated washing procedure, making the identification of this phosphoprotein as a T-antigen species problematic. This question has now been directly addressed by mapping the in vivo T antigen phosphorylations.

Figure 10 shows a comparison of the in vivo and in vitro phosphorylated wild-type T antigens by partial proteolysis. In the left panel, the chymotryptic digestion of in vivo ^{32}P-labeled middle T antigens shows the same set of spots (39K/33K and 37K/31K) as were seen for the in vitro ^{32}P-labeled products (Fig. 3A). This result shows that the 58K species labeled in vivo was the 58K middle T antigen and suggests that the variability observed previously (33) is a consequence of lability during extraction. Since these fragments have been mapped to the C-terminal region, the major in vivo phosphorylations also occur in that part of the molecule. Figure 10 also shows that a fragment of 18K was seen in digests of in vitro-labeled middle T antigen, but not in chymotryptic digests of the in vivo-labeled material. This 18K fragment appeared to be derived from the 33K/31K peptides by further digestion. It was truncated in digests of *dl*1015 middle T antigens (not shown), but not in digests of *dl*45 middle T antigen (Fig. 3). The 18K fragment therefore arises from a cleavage between these two deletions and extends toward the C terminus. The absence of in vivo labeling of the 18K peptide indicates that tyrosine 315 is not a major site for in vivo phosphorylation. This conclusion is also supported by analysis with V8 protease. Figure 10B shows that the 24K and 18K fragments characteristic of in vitro-labeled middle T antigens were absent in digests of in vivo-labeled proteins. Instead, different fragments appeared, including one slightly larger and one slightly smaller than the 18K in vitro fragment, a set of smaller peptides (8K to 10K), and a major frag-

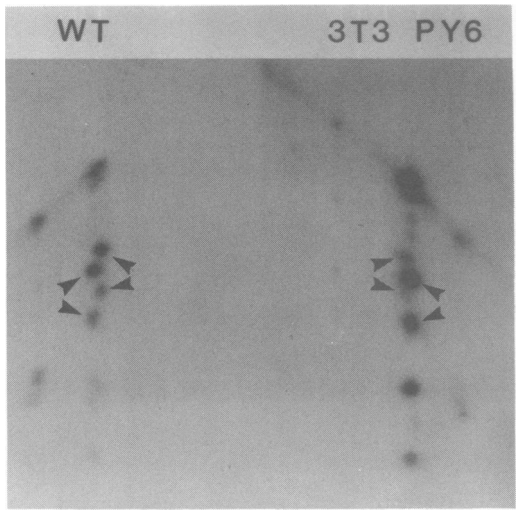

FIG. 7. *Comparison of in vitro-labeled T antigens from productively infected and transformed 3T3 cells. T antigens extracted from wild type-infected 3T3 (WT) or transformed 3T3 (3T3-Py6) cells were incubated with [γ-^{32}P]ATP and subjected to partial proteolytic digestion with chymotrypsin as described in the legends to Fig. 3 and 4. The arrowheads show the positions of the 39K/33K and 37K/31K pairs.*

ment at 6.5K. As expected from the chymotryptic mapping results, these peptides are not affected by the deletions in *dl*1013 or *dl*1015 (data not shown). The combination of results from V8 and chymotryptic mapping strongly suggests that the major in vivo phosphorylations of middle T antigen occur between the cleavage site that generates the 31K chymotryptic fragment and the site that generates the 24K V8 fragment. The in vivo phosphorylation most likely occurs on serine or threonine residues, since alkaline treatment of the gels shows that the in vivo phosphate is more labile than the in vitro tyrosine phosphate. Also, antibody directed against phosphotyrosine will precipitate the in vitro-labeled material, but not the in vivo-labeled middle T antigen (B. Schaffhausen, S. Ju, and T. L. Benjamin, manuscript in preparation).

56K and 58K middle T antigens differ in their phosphorylation sites in vivo. When the 56K and 58K middle T antigen species were resolved on 20-cm gels in the first dimension, proteolytic mapping showed that each species had its own characteristic fragment pattern (Fig. 11). The peptides around 18K and the peptide at 10K generated by V8 were specific to 58K, whereas the series of fragments near 8K and the major fragment at 6.5K arose from the 56K middle T. In a similar fashion, the chymotryptic fragments smaller than the 31K product were also different for the two forms. Unlike digestions of in vitro-labeled T antigens in which large fragments were different but smaller species were identical, digests of in vivo-labeled 56K and 58K species gave fragments distinct at every level. This indicates that the two forms are phosphorylated in vivo at different sites within the molecule. NG59, which shows only the 56K phosphorylated form by in vivo labeling, gave rise only to the 56K-specific fragments (data not shown).

DISCUSSION

The ability to phosphorylate middle T antigens in immunoprecipitates is strongly correlated with the ability of polyoma virus to transform cells and induce tumors. *hr-t* mutants are totally defective in transformation (39) as well as in the in vitro protein kinase reaction (33). *dl*23 is largely defective in the protein kinase reaction (37), and *dl*23-transformed cells are reported to have growth properties intermediate

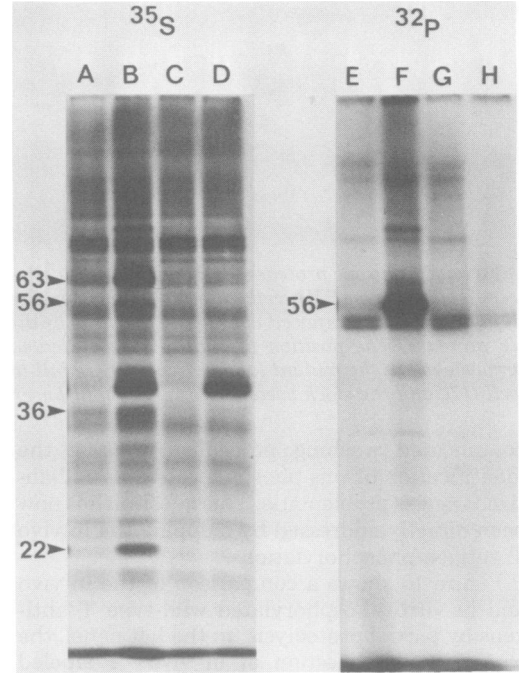

FIG. 8. *Immunoprecipitates of T antigens from transformed (3T3-Py6) and phenotypically normal revertant (3T3-Py6-R1) cells. A through D, T antigens extracted from cells labeled for 90 min with [^{35}S]-methionine: A, 3T3-Py6, preimmune serum; B, 3T3-Py6, anti-T ascites; C, 3T3-Py6-R1, preimmune serum; and D, 3T3-Py6-R1, anti-T ascites. E through H, T antigens from unlabeled cells were incubated in vitro with [γ-^{32}P]ATP: E, 3T3-Py6, preimmune serum; F, 3T3-Py6, anti-T ascites; G, 3T3-Py6-R1, preimmune serum; and H, 3T3-Py6-R1, anti-T ascites.*

between those of normal and transformed cells (16, 23). Immunoprecipitates from polyoma-transformed cells contain middle T kinase activity, whereas those from phenotypically normal revertants of these cells show no middle T-antigen species. No instance is known of a polyoma mutant that is transformation positive and middle T-antigen kinase negative. Instances are known of transformation-defective viruses that show normal in vitro kinase reactions; however, these mutants show *ts-a* defects affecting the large T antigen. *ts-a* mutants have normal *hr-t* function in abortive transformation (12, 35) and in middle T antigen kinase (9, 33, 37). *dl*1015, truncated in large and middle T antigen, has a partial defect in transformation that appears likely to be at least in part *ts-a* related, since in our hands this mutant is partially complemented by *hr-t* mutants (C. Ware and T. L. Benjamin, unpublished results).

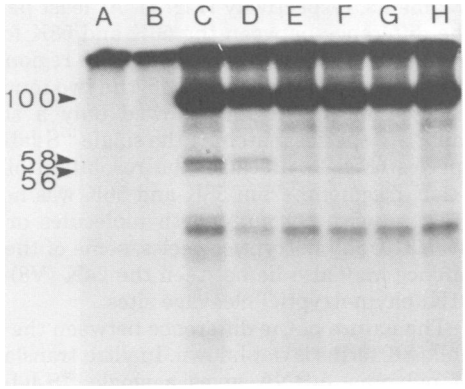

FIG. 9. *In vivo $^{32}PO_4$ labeling of T antigens of wild type and hr-t mutants. Wild type- or hr-t mutant-infected BMK cells were pulse-labeled with $^{32}PO_4$ (100 μCi/ml) for 90 min at 26 h after infection. T antigens were extracted, immunoprecipitated and electrophoresed on a sodium dodecyl sulfate-12.5% acrylamide gel as described in the text. The positions of the large T antigen (100K) and middle T antigens (56K and 58K) are shown. Lanes: A, wild type, preimmune serum; B, mock-infected cells; C, wild type; D, wild type; E, hr-t mutant NG59; F, hr-t mutant 3A1; G, hr-t mutant B2; and H, hr-t mutant NG18.*

Two middle T-antigen species, 56K and 58K, were phosphorylated in the in vitro reaction. Partial proteolytic mapping identified tyrosine 315 as the major phosphorylation site in both species. This tyrosine lies on the C-terminal side of an unusual stretch of six glutamic residues. The identification relies on the known DNA sequence of polyoma and also on the assumption that different mutants do not utilize different phosphorylation sites. However, no single mutant is essential for the identification, and the conclusion can be reached by using mutants (*dl*45 and *dl*1014) that are not strongly affected biologically. These results do not rule out the possibility of other phosphorylation sites. Using

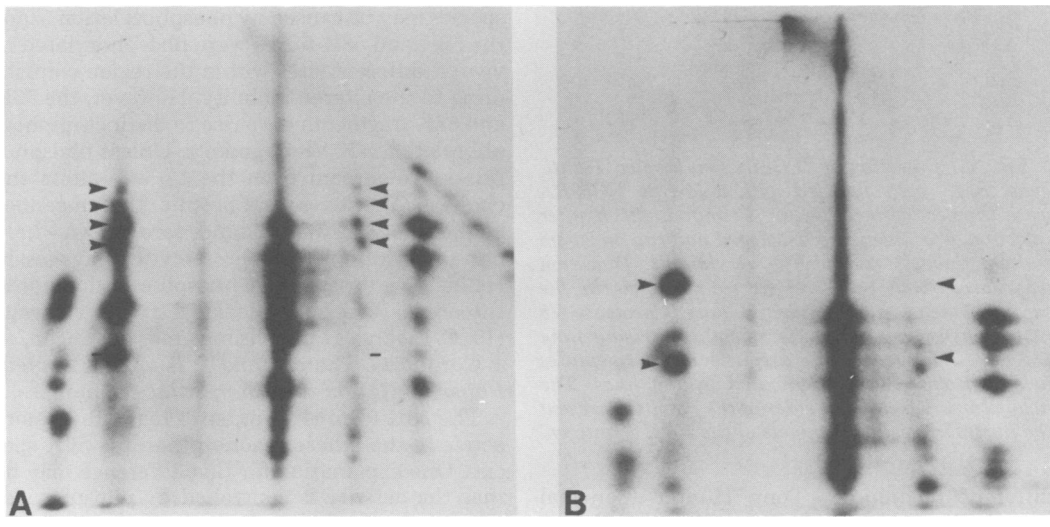

FIG. 10. *Comparison of wild-type T antigens labeled with ^{32}P in vivo and in vitro. Wild type-infected BMK cells were labeled with $^{32}PO_4$ in vivo as described in the legend to Fig. 9. T antigens from unlabeled infected cells were labeled in vitro with [$\gamma^{32}P$]ATP. The labeled T antigens were resolved on 10-cm sodium dodecyl sulfate-10% acrylamide gels and then digested with either chymotrypsin (A) or S. aureus V8 protease (B) on a second-dimension 12.5% acrylamide gel. In each panel the in vitro-labeled T antigens are on the left side, and the in vivo-labeled T antigens are on the right. For chymotryptic digestion (A) the 39K/33K and 37K/31K pairs are indicated by arrowheads. The line indicates the 18K chymotryptic fragment. For V8 digestion (B), the arrowheads indicate the positions of the 24K and 18K fragments.*

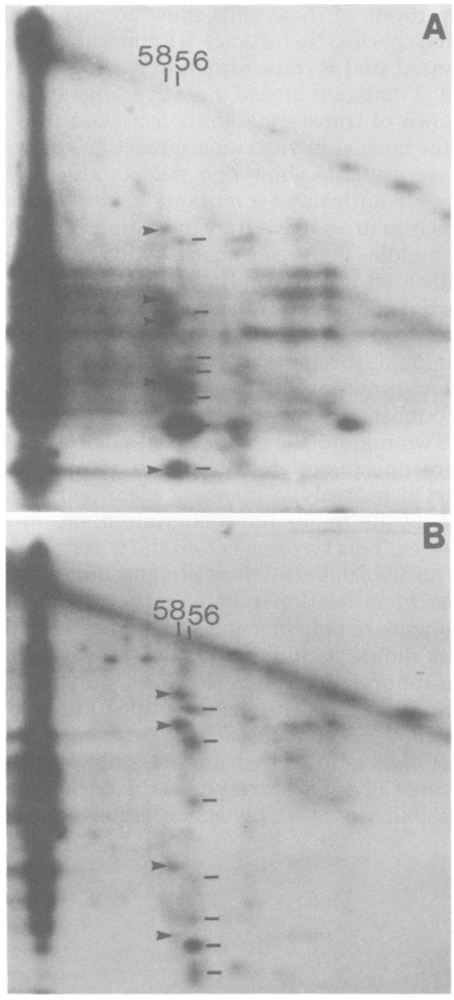

FIG. 11. *Digestion of $^{32}PO_4$ in vivo-labeled T antigens. BMK cells infected with wild-type NG59RA were labeled with $^{32}PO_4$ for 90 min at 24 h after infection. T antigens were isolated and run on 20-cm sodium dodecyl sulfate-10% acrylamide. Digestion was carried out on 12.5% acrylamide gels in the second dimension with either S. aureus protease V8 (A) or chymotrypsin (B). The peptides arising from 58K are indicated by the arrowheads; the peptides arising from 56K are indicated by the lines. The heavy smears at the left sides of the panels represent the partial digestion products of the 100K T antigen.*

different techniques, Tony Hunter (personal communication) has observed phosphorylation of tyrosine residue 250.

The difference between the 56K and 58K middle T antigens lies in the C-terminal region, since the electrophoretic mobility difference between them was retained in the large C-terminal chymotryptic fragments (33K/31K). By V8 cleavage, the two middle T species gave rise to distinct fragments of ~24K and to a common fragment of 18K. The most likely sites for cleavage by the V8 protease are the two *glu*-rich clusters from residues 309 through 314 and from residues 274 through 277, giving rise to 24K and 18K fragments, respectively (Fig. 1). At least part of the difference between the 56K and 58K forms of middle T must reside in the 6K region between these sites. *dl*8 and *dl*45, the two mutants affected in this region, showed only a single middle T species matching the single ^{35}S-labeled species (Fig. 2). Because the resolution of the ~24K fragments from 58K and 56K was not as great as with the full-length molecules or the 33K/31K chymotryptic species, some of the difference may also lie between the 24K (V8) and 31K (chymotryptic) cleavage sites.

The nature of the difference between the 56K and 58K forms is not known. In vitro translation of polyoma mRNA gives a single ^{35}S-labeled species of 56K (20; Shaikh, Ozkaynak, and Benjamin, unpublished data). No minor splice has been reported in early mRNA coming from the region of middle T antigen in which the two species appear to differ (24). Post-translational modification is therefore a likely explanation. Labeling experiments with fucose, glucosamine, and mannose, as well as attempts to affect middle T by treatment with tunicamycin or endoglycosidase H, have all given negative results (unpublished results). The difference in apparent molecular weight between the two middle T species may be caused by phosphorylation, since the 56K and 58K forms were phosphorylated in vivo at different sites within the region contributing to the altered mobility. However, the 56K and 58K fragments gave rise to distinct unphosphorylated 24K V8 fragments. Unless phosphorylation upstream from the V8 site alters the choice of V8 cleavages, phosphorylation cannot be the whole answer. Instances are known where phosphorylation affects proteolytic cleavages; in H1 histone, for example, phosphorylation of the threonine 16 residue blocks a tryptic cleavage (19; C. Zeiling, H. Leichtling, and T. Langan, *in* J. Gordon, G. Thomas, and T. Hunt, ed., *Protein Phosphorylation and Bioregulation*, in press).

The 58K form is an order of magnitude more active in the kinase reaction than the 56K species. One explanation for this difference may be that the activity is controlled by phosphorylation at a second site. A similar suggestion has been made for $pp60^{src}$ which, like polyoma middle T antigen, is phosphorylated at a second site (7). Many cellular enzymes are known to be activated by phosphorylation (27). An alternative explanation may be that the detailed localization of the two middle T antigen forms in the membrane gives rise to differences in both in

vivo and in vitro phosphorylation.

Tyrosine 315, the predominant site of phosphorylation in the in vitro reaction, is not detectably phosphorylated in vivo under the conditions of $^{32}PO_4$ labeling and extraction used here. A small fraction of molecules phosphorylated at this site, or a fraction turning over rapidly, cannot be ruled out. Recent evidence shows that in situ labeling of cellular frameworks labels tyrosine residue 315 (B. Schaffhausen, G. Arakere, and T. Benjamin, manuscript in preparation).

A fundamental question concerns whether either of the middle T forms is indeed a catalytic protein, as opposed to being simply a substrate for one or more cellular kinases or perhaps regulatory subunits thereof. Because the Asp-to-Ile-Asn substitution in *hr-t* mutant NG59 would appear unlikely to alter middle T antigen radically as a substrate, it was earlier argued that the middle T antigen may itself be a kinase (33). However, the present results showing that NG59 fails to induce a 58K form detectable by either ^{35}S or ^{32}P labeling in vivo suggest that this *hr-t* mutation may lead to a defective substrate for some in vivo phosphorylation reactions. Furthermore, efforts to demonstrate a catalytic role for middle T antigen by direct labeling with ATP affinity reagents have proven negative thus far (Schaffhausen, Arakere, and Benjamin, in preparation).

The occurrence of serine-threonine phosphorylations of middle T antigens in vivo and the possibility that they regulate the tyrosine-specific activity of these proteins measured in vitro together suggest a cascade along the lines recently described in Ehrlich ascites tumor cells (38). Interestingly, a mammalian "*src*-like" gene product is interposed in this cascade. The kinases phosphorylating polyoma middle T antigen in vivo along with the tyrosine-specific activity assayed in vitro may conceivably represent a similar or overlapping cascade.

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