Phosphorylation of Polyomavirus Large T Antigen: Effects of Viral Mutations and Cell Growth State

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Phosphorylation is responsible for the shift in electrophoretic mobility of polyomavirus large T antigen observed in pulse-chase or continuous-labeling experiments. Phosphorylated forms migrated more slowly than newly synthesized [³⁵S]methionine large T antigen, and alkaline phosphatase treatment reversed the mobility shift. Analysis of phosphopeptides with *Staphylococcus aureus* V8 protease showed that large T antigen forms of intermediate mobility were enriched in peptides 1 to 4, 8, and 9, while the slower migrating species had all nine phosphopeptides, including peptides 5 and 7. The phosphorylations represented by phosphopeptides 5 and 7 were of particular interest. These phosphopeptides were entirely lacking in large T antigen from *tsa* mutants such as *ts616* labeled at the nonpermissive temperature. Also, the phosphorylation of peptides 5 and 7 depends on the growth state of the cell. Early in infection of quiescent cells intermediate mobility forms of large T antigen with little or no phosphorylation, particularly of peptides 5 and 7, were seen, whereas peptides 5 and 7 were well represented at the same time in patterns from growing cells. Later in infection of growth-arrested cells, these phosphorylations were observed, suggesting that infection stimulates the relevant kinase. Because large T antigen of *hrt* mutants, which lack middle and small T antigens, showed phosphorylation of peptides 5 and 7, large T antigen was apparently responsible for the stimulation. Because some differences in the distribution of phosphopeptides were noted between *hrt* mutants and the wild type, middle T antigen, small T antigen, or both may play a modulating role in large T antigen phosphorylation.

Large T antigen is one of three early gene products of polyomavirus (Py) (41). This multifunctional DNA-binding protein (8) is important for both productive infection and transformation (10, 15, 18, 39). Py large T antigen is required for the initiation of viral DNA replication (16), and by analogy to simian virus 40 (SV40) large T antigen, it may be involved in elongation as well (50). It is also involved in stimulating host cell DNA synthesis (47, 53). Large T antigen negatively autoregulates early viral transcription and positively regulates transcription of late viral genes (7, 12, 13) and probably some host cell genes as well (31). In the transformation of primary cells, Py large T antigen functions in immortalization (1, 40) and is important in integration and excision of the viral genome from that of the host (2, 9).

How these different biological functions are carried out at the molecular level by this single molecule is of great interest. The assortment of functions to different structural domains is one way. Considerable evidence points to a domain structure for the homologous SV40 large T antigen (36). For Py large T antigen, the N-terminal 40 kilodaltons are sufficient for immortalization, while sequences that are more C terminal are required for viral DNA replication (1, 25, 38-40). Posttranslational modifications may also serve to determine the functions of the large T antigen molecule. SV40 large T antigen has been demonstrated to be phosphorylated (46), acylated (32), glycosylated (30), adenylated (5), and ADP ribosylated (21). Py large T antigen is known to be phosphorylated (24, 44), and multiple forms have been detected by alterations in electrophoretic mobility (26, 27, 29, 44, 52). The relationship between the different forms and functions of large T antigen is not known, although the electrophoretic conversion has been correlated with viral and cellular replication (52, 53).

We found that phosphorylation is the basis for the conversion. In quiescent cells the kinase activity responsible to complete this conversion must be induced. While phosphorylations in the N-terminal region can occur to some extent before the induction of cell replication, phosphorylation of the more distal V8 peptides 5 and 7 does not occur until later. These specific phosphorylations may well be related to viral DNA replication. They are not found when replication-defective, *tsa* mutant large T antigens are labeled at the nonpermissive temperature.

MATERIALS AND METHODS

Cell lines. 3T6 and NIH 3T3 cell lines were maintained in Dulbecco modified essential medium (DME) containing 5% calf serum. F111 rat embryo fibroblast cells (17) were maintained in DME plus 10% calf serum. Baby mouse kidney cells (BMK) were isolated from CRW baby mice (age, 8 to 10 days; Charles River Breeding Laboratories, Inc., Wilmington, Mass.) by the method described by Winocour (54). NIH 3T3 cells were made quiescent by serum starvation in 0.4% calf serum for 48 h; 3T6 cells were made quiescent by serum starvation for 72 h.

Virus stocks. Virus stocks were prepared on 3T6 or BMK cells. Wild-type virus NG59RA (14) was derived from *hrt* mutant NG59 (3) by marker rescue. For some experiments the deletion *hrt* mutant NG18 was used (3). The *tsa* class mutants used in this study were *ts616* and *ts25* (11).

T-antigen extraction. The procedures for T-antigen extraction have been described previously (42-45). Briefly, T antigens were extracted in Nonidet P-40 (NP-40)-containing buffer (0.137 M NaCl, 0.01 M Tris hydrochloride [pH 9.0], 0.001 M MgCl₂, 0.001 M CaCl₂, 10% [vol/vol] glycerol, 1% [vol/vol] NP-40). In phosphate-labeling experiments the solution contained 0.02 M sodium fluoride to minimize phosphatase activity in the extracts. Cell extracts were clarified for 10 min at 10,000 rpm in an 1154 centrifuge (Eppendorf).

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Antibodies. Antibodies were either polyclonal anti-T ascites fluid from Brown Norwegian rats bearing mixed solid ascites tumors (TBR) (48) or polyclonal rabbit sera to Py small T antigen (19-2) (38a). Py small T and large T antigens share 79 amino acids (41), and this serum efficiently immunoprecipitated large T antigen. No qualitative distinctions were seen when anti-small T-antigen serum was compared with anti-T serum.

Immunoprecipitation. Clarified cell extract was added to 50 μ l (50% [vol/vol]) *Staphylococcus aureus* Sepharose (Pharmacia Fine Chemicals, Piscataway, N.J. [Div. Pharmacia, Inc.]) plus 2 μ l of 19-2 or 10 μ l of anti-T ascites fluid from tumor-bearing rats. Immunoprecipitation was carried out at 4°C for 60 min. Immune complexes were then washed twice with phosphate-buffered saline, twice with 0.5 M LiCl–0.01 M Tris hydrochloride (pH 8.0), and once with distilled water. Immune complexes were then boiled for 2 min in sodium dodecyl sulfate (SDS) dissociation buffer (0.0625 M Tris hydrochloride [pH 6.8], 5% [wt/vol] SDS, 5% [vol/vol] 2-mercaptoethanol, 20% [vol/vol] glycerol, 0.15% [wt/vol] bromphenol blue).

Metabolic labeling. Short-term labeling with [³⁵S]methionine (up to 2 h) at concentrations of 25 to 50 μ Ci/ml (>4.0 Ci/mmol; New England Nuclear Corp., Boston, Mass.) was in Hanks saline buffered with sodium bicarbonate. Short-term labeling with ³²PO₄ (0.9 to 1.1 Ci/mmol; New England Nuclear) at 100 to 200 μ Ci/ml was carried out in phosphate-free minimal essential medium (Flow Laboratories, Inc., McLean, Va.). Long-term labelings (>2 h; [³⁵S]methionine or ³²PO₄) were performed in DME supplemented with 5% calf serum.

Alkaline phosphatase treatment. Immunoprecipitated large T antigen was treated directly with bacterial alkaline phosphatase (Sigma Chemical Co., St. Louis, Mo.) for 30 min at room temperature followed by a second incubation with enzyme for 15 min. Alternatively, immunoprecipitated large T antigen was subjected to a single freeze-thaw cycle before an initial enzyme treatment. The immunoprecipitates were then treated for 2 min at 100°C with 0.1% SDS–0.1% 2-mercaptoethanol–0.0625 M Tris hydrochloride (pH 6.8). After complexes had cooled to room temperature, a second dose of alkaline phosphatase was added for 15 min. Complexes were then treated with SDS dissociation buffer for 2 min at 100°C.

Cell fractionation. Cells were fractionated by using a slight modification of the protocol described by Silver et al. (48). Following a rinse in phosphate-buffered saline, cells were suspended in a hypotonic solution consisting of 0.003 M KCl–0.003 M Tris (pH 8.0)–0.0015 M MgCl₂. Swollen cells were disrupted by Dounce homogenization until greater than 95% of the cells were broken, as viewed by phase-contrast microscopy. Nuclei were removed by centrifugation in IEC rotor 269 (International Equipment Co., Div. Damon Corp., Needham Heights, Mass.) at 2,000 rpm for 2 min. Membranes were removed from the cytoplasmic fraction by centrifugation at 10,000 $\times$ g for 10 min.

Electrophoresis. Discontinuous buffer electrophoresis was carried out as described by Laemmli (33). Gels containing ³²PO₄-labeled samples were dried and visualized with Kodak XAR-5 film by using intensifying screens (Du Pont Co., Wilmington, Del.) at –70°C. Gels containing [³⁵S]methionine-labeled samples were treated with 2,5-diphenyl-oxazole–dimethyl sulfoxide (34).

Mapping and enzymatic digestions. The procedure for partial proteolysis has been described previously (42, 43). Large T antigens were separated on cylindrical gels which

were then placed on top of second-dimension slab gels and overlaid with enzyme solution. *S. aureus* V8 protease (Miles Laboratories, Inc., Elkhart, Ind.) was dissolved at 100 μ g/ml in digestion solution containing 0.125 M Tris hydrochloride (pH 6.8), 0.001 M EDTA, 20% (vol/vol) glycerol, 0.015% (vol/vol) bromphenol blue, and 50 μ g of bovine serum albumin per ml. Fragments were sized by comparison with stained proteins of known molecular weight, including bovine serum albumin (67,000), ovalbumin (45,000), glyceraldehyde-3-phosphate dehydrogenase (36,000), carbonic anhydrase (29,000), phenylmethylsulfonyl fluoride-treated trypsinogen (24,000), soybean trypsin inhibitor (20,100), α -lactalbumin (14,200), pancreatic trypsin inhibitor (6,500), and insulin (2,900 and 3,300).

RESULTS

Phosphorylation is responsible for changes in electrophoretic mobility of Py large T antigen. As expected from results of previous experiments (27, 29), pulse-chase experiments with [³⁵S]methionine in infected mouse cells showed a decrease in electrophoretic mobility of large T-antigen molecules after the chase period (Fig. 1A, lanes A and B). Similar results were observed by increasing the time of continuous [³⁵S]methionine labeling (Fig. 1A, lanes B and C). Most of the decrease in mobility was complete by 2 h, although a slight difference was noticeable between large T antigen labeled for 2 h and that labeled for 19 h. The shift in mobility was not a consequence of productive infection; large T antigen from infected F111 rat embryo fibroblast cells also showed the conversion (see below) (52).

Close examination of large T antigen at different labeling times showed that at least three species could be distinguished. Large T antigen labeled for 5 min migrated more rapidly than that labeled for 2 h. Large T antigen labeled for 30 min had an intermediate mobility (Fig. 1B). Labeled cells were fractionated into nuclei and cytoplasm. For large T antigen the fractionations were not entirely satisfactory. Nuclear leakage is a known large T-antigen problem (51), and cytoplasmic large T antigen has been seen in previous fractionations (48). Whether nuclei were prepared this way or in NP-40-containing buffer, however, the same result was obtained: the unmodified form labeled for 5 min could be found in the nucleus (Fig. 1B). This demonstrated, first, that transport to the nucleus could occur within 5 min and, second, that the alterations in electrophoretic mobility principally result from modifications that occur in the nucleus.

The slower [³⁵S]methionine bands labeled for 2 h comigrated with the major Coomassie blue-stained large T antigen bands (Fig. 1C). The Coomassie blue pattern showed two discrete bands, indicating that at steady state there is more than one large T antigen species.

Although Py large T antigen is a known phosphoprotein (24, 44), the connection between phosphorylation and this electrophoretic conversion has never been clear. Any of the number of modifications reported for SV40 large T antigen, for example, could be responsible. A variety of evidence shows that the connection is quite direct. Large T antigen continuously labeled with ³²PO₄ for 10 or 30 min in growing cells showed only the slower migrating forms matching the 2-h [³⁵S]methionine band (Fig. 1C). Even at 10 min no ³²PO₄ band corresponding to the fastest migrating [³⁵S]methionine band could be detected. The lack of a phosphorylated form corresponding to the fastest migrating [³⁵S]methionine band suggested that phosphorylation may be responsible for the conversion of Py large T antigen.

FIG. 1. Changes in electrophoretic mobility of Py large T antigen (LT). (A) Actively growing NIH 3T3 cells were infected with wild-type virus and labeled with [35 S]methionine. Infected cells were labeled starting at 22 h postinfection continuously for 30 min (lane B) or 2 h (lane C), or were labeled continuously for 30 min and chased for 2 h with complete medium supplemented with $10\times$ cold methionine (lane A). Immunoprecipitated large T antigen was separated by 5% acrylamide discontinuous buffer SDS-polyacrylamide gel electrophoresis. The positions of large T antigen are indicated by the parentheses. (B) Actively growing 3T6 cells infected with wild-type virus were labeled with [35 S]methionine for 5, 30, or 120 min at 22 h postinfection. Nuclear (N) and cytoplasmic (C) fractions were obtained as described in Materials and Methods. Immunoprecipitated large T antigen was separated on a 5% acrylamide discontinuous buffer SDS gel. The positions of large T antigen are indicated by the parentheses. (C) Actively growing 3T6 cells infected with wild-type virus were labeled for 10 min, 30 min, or 2 h with [35 S]methionine or 32 PO $_4$. Immunoprecipitated 1T was separated on 5% SDS-polyacrylamide gels and stained with 0.25% Coomassie blue. Autoradiography and detection were carried out on the same gel. [35 S]methionine-labeled large T antigen (lane 10 min, left); 32 PO $_4$ -labeled large T antigen (lane 10 min right); [35 S]methionine-labeled large T antigen (lane 30 min, left); 32 PO $_4$ -labeled large T antigen (lane 30 min, right); [35 S]methionine-labeled large T antigen (lane 120 min); and Coomassie blue-stained material (CB) are shown. Arrowheads indicate the position of the two stained forms of large T-antigen. (D) Immunoprecipitated large T antigen from actively growing 3T6 cells labeled for 2 h with 32 PO $_4$ was separated first on a 5% cylindrical gel and analyzed by *S. aureus* V8 protease (100 μ g/ml), as described in the text. The arrow indicates the direction of electrophoresis for the cylindrical gel; the most rapidly migrating species are to the right. The line at the top of the figure indicates the median position of large T antigen in the first dimension and the position of undigested large T antigen in the second dimension. Molecular weights of the peptides are as follow: 1, 22,500; 2, 20,500; 3, 19,000; 4, 18,000; 5, 16,000; 6, 15,000; 7, 14,000; 8, 13,500; 9, 11,500.

An additional demonstration of a relationship between phosphorylation and mobility comes from analysis of phosphopeptides. The phosphorylated large T antigen was partially resolved in the first dimension on a 5% acrylamide gel and then digested with 100 μ g of *S. aureus* V8 protease per ml. Digestion of 32 PO $_4$ -labeled large T antigen resulted in a pattern that generally consisted of nine major fragments (Fig. 1D). A more complete discussion of these fragments and their localization within the intact molecule can be found in the accompanying paper (4). Peptides 1 to 4 were derived from the N-terminal portion of the molecule, while peptides 5 and 7 were more distal and were derived from sequences closer to the middle of the molecule. Peptide 6 was an intermediate digestion product and often was not observed even in initial digestions. Digestion of partially resolved large T antigen in the second dimension revealed that peptides 5 and 7 were preferentially located with the slower migrating large T-antigen species, while the N-terminal peptides were distributed more evenly among the different forms (Fig. 1D).

Digestion of immunoprecipitated large T antigen with alkaline phosphatase demonstrated a direct correlation between phosphorylation and mobility (Fig. 2). Denaturation with 0.1% SDS in the presence of 1% mercaptoethanol followed by treatment with low doses of alkaline phosphatase

resulted in a partial loss of phosphate (Fig. 2, lane 9) and a partial shift of the [35 S]methionine-labeled large T antigen (Fig. 2, lane 4). Removal of most of the phosphate from large T antigen (Fig. 2, lane 8) resulted in a dramatic shift in electrophoretic mobility of [35 S]methionine-labeled protein (Fig. 2, lane 3). Such doses resulted in a band that migrated faster than the intermediate 30-min band (Fig. 2; compare lanes 1 and 3) at the position of the large T antigen labeled for 5 min. Because alkaline phosphatase caused the entire [35 S]methionine band to shift, there was no substantial large T-antigen population that was not phosphorylated. It should be noted that alkaline phosphatase treatment of non-denatured large T antigen also reversed the electrophoretic shift. Therefore, most of the phosphates are accessible on the protein surface (data not shown).

Phosphorylation and the complete conversion of large T antigen is related to the growth state of the cell. Other investigators (52, 53) have noted a connection between viral and cellular replication and the electrophoretic conversion of large T antigen. Viral DNA replication and late transcription are not required for this conversion. Infection of F111 rat embryo fibroblasts, which do not permit any significant viral DNA replication, resulted in large T antigen with altered mobility (Fig. 3A). Mapping of large T antigen from infected F111 cells demonstrated a pattern that was qualitatively

FIG. 2. Dephosphorylation of Py large T antigen reverses the electrophoretic mobility shift. 3T6 cells were infected with Py large T antigen and labeled 22 h postinfection with [35 S]methionine (lanes 1 to 5) or 32 PO $_4$ (lanes 6 to 10). All samples were labeled for 2 h, except for those in lanes 1 and 6, which were labeled for 30 min. The samples in lanes 3 and 8 were treated with 8 U of alkaline phosphatase, and those in lanes 4 and 9 were treated with 1 U of alkaline phosphatase as described in the text. Lanes 5 and 10 show incubation controls without enzyme. Lanes 1, 2, 6, and 7 show control samples labeled for 30 min (lanes 1 and 6) or 2 h (lanes 2 and 7). The bottom one-third of the 5% acrylamide SDS gel is shown.

similar to that seen in the infected mouse cell (Fig. 3B). V8 phosphopeptides 5 and 7, however, were quantitatively underrepresented (Fig. 3B).

The alteration in electrophoretic mobility appears instead to be related to some aspect of cellular growth. The complete conversion of large T antigen could be detected early in the infection of growing cell cultures, but not in infection of growth-arrested cells. Infection of confluent BMK cells or serum-starved 3T6 cells did not result in complete conversion when labeling was carried out from 7 to 9 h after infection (Fig. 4, lane C). This was a time when cellular DNA synthesis was not yet significantly induced, as measured by

FIG. 3. Comparison of phosphorylations of Py large T antigen in mouse 3T6 and rat F111 cells. (A) Actively growing rat F111 cells were infected with wild-type virus and labeled with [35 S]methionine. Infected cells were labeled starting at 22 h postinfection continuously for 30 min (lane B) or 2 h (lane C), or labeled continuously for 30 min and chased for 2 h with complete medium supplemented with 10 $\times$ cold methionine (lane A). The position of large T antigen (LT) on this 5% acrylamide SDS gel is indicated by the arrowheads. (B) Rat F111 or 3T6 cells were infected with NG59RA (RA) wild-type virus and labeled with 32 PO $_4$ for 2 h at 22 h postinfection. Large T antigens were analyzed with V8 protease as described in the text. Arrowheads indicate positions of V8 peptides 5 and 7.

[3 H]thymidine incorporation. The complete conversion to significantly slower forms could be seen 30 to 32 h after infection (Fig. 4, lane D). By contrast, large T antigen from actively growing 3T6 or NIH 3T3 cells did convert when examined 7 to 9 h after infection (Fig. 4, lane E).

In vivo 32 PO $_4$ labeling of infected cells showed the expected difference in the mobility of phosphorylated species from arrested versus growing cells (Fig. 4; compare lanes K and M). From arrested cells only the faster migrating, intermediate form of large T antigen was seen (Fig. 4, lane K); from actively growing cells shifted to low serum at the time of infection the intermediate and slowest forms were obtained (Fig. 4, lane M). When actively growing cultures were fed with medium containing 5% serum at the time of infection, only the slowest migrating form was observed (Fig. 1C).

Mapping of the phosphorylated forms of large T antigen observed under these conditions is shown in Fig. 5. V8 phosphorylation patterns from quiescent cells labeled 7 to 9 h after infection lacked phosphopeptides 5 and 7 (Fig. 5A and B). The patterns of the N-terminal peptides were similar to those from growing cells, with some differences such as an increase in the intensity of peptide 8. Such results are consistent with the mapping of partially resolved large T-antigen forms shown above (Fig. 1D). It suggests that phosphorylation of peptides 5 and 7 is part of the process of complete conversion of large T antigen. The presence of phosphopeptides 5 and 7 later in infection (Fig. 5C and D)

FIG. 4. Py large T antigen (LT) conversion is related to the growth state of the cell. Exponentially growing or quiescent 3T6 cells were infected with wild-type virus, except where the *hrt* mutant NG59 is indicated below. All cells were fed 2 h after the addition of virus with 0.4% calf serum-DME. Cells were labeled beginning 7 and 28 h after the addition of DME with [35 S]methionine or 32 PO $_4$. Large T antigen was separated on a 5% acrylamide SDS-polyacrylamide gel. Quiescent 3T6 cells were labeled for 2 h with [35 S]methionine beginning at 7 h (lane C) or 28 h (lane D) after infection. Actively growing 3T6 cells were similarly labeled at 7 h (lane E) or 28 h (lane F) after infection. For comparison with the wild type, quiescent 3T6 cells were infected with *hrt* mutant NG59 and labeled at 7 h (lane G) or 28 h (lane H) after infection. Quiescent 3T6 cells were labeled for 2 h with 32 PO $_4$ beginning at 7 h (lane K) or 28 h (lane L) after infection. Actively growing 3T6 cells were similarly labeled at 7 h (lane M) or 28 h (lane N) after infection. Marker lanes show large T antigen labeled for 15 min (lanes A and I) or 2 h (lanes B and J) with [35 S]methionine.

suggests that the virus induces the kinase activity that is responsible for these phosphorylations.

Large T antigen appears sufficient to induce the phosphorylation and conversion, but both *tsa* and *hrt* mutations can affect large T antigen phosphorylations. The unusual mobility of *tsa* mutant large T antigen at 39°C has been known for some time (29). *ts616* is one of the *tsa* mutant class (10, 11). Although the large T antigen shows a mobility shift similar to that of the wild type at 32°C, at 39°C the large T antigen migrated more rapidly even when labeled for 2 h (Fig. 6A). Higher resolution (Fig. 6B) showed a limited conversion to the intermediate mobility position on the gel, while most (>75%) of the large T antigen remained unmodified. Given the relationship between phosphorylation and conversion, it is not surprising that the specific activity (^{32}P to ^{35}S) measured for the *ts616* molecules was less than 10% that of wild type at 39°C (data not shown).

The V8 maps of the phosphorylations of *ts616* were quite striking. There was essentially the complete absence of V8 phosphopeptides 5 and 7 at 39°C (Fig. 7). The N-terminal peptides 1 to 4, 8, and 9 all seemed to be present in relative ratios similar to that of the wild type. Amounts of all these phosphopeptides were reduced compared with that of the wild type; this was reflected in the specific activity measurements described above. At 32°C the pattern matched that of the wild type (Fig. 7). These same results were also obtained with *ts25*, another member of the *tsa* class of mutants (data not shown).

Studies with *hrt* mutants indicated that large T antigen is responsible for inducing the kinase(s) that is responsible for the complete conversion but that middle T and small T antigens may play a modulating role. The large T antigen from quiescent cells infected with *hrt* mutant NG59 showed a reduced mobility, similar to that of the wild type, when examined later in infection (Fig. 4, lane H). Similar results were obtained with 31F cells (a cell line that produces only large T antigen from the bovine papillomavirus promoter) and LT 3T3 cells (an NIH cell line that produces only large T antigen from a metallothionein promoter) (data not shown). Mapping of large T-antigen molecules from *hrt*-infected cells showed a phosphopeptide pattern that was quite similar to that of the wild type (Fig. 8A). These results

FIG. 5. Py large T antigen labeled early in infection of quiescent cells shows differences in phosphorylation. Actively growing (A and C) or quiescent (B and D) cells were infected with wild-type virus. Large T antigen molecules were labeled for 2 h with $^{32}\text{PO}_4$ starting 7 h (A and B) or 30 h (C and D) postinfection. The absence of peptide 7 in panel B is indicated by the arrowhead.

FIG. 6. *ts616* (616) large T antigen molecules show little conversion at the nonpermissive temperature. (A) 3T6 cells were infected with wild-type virus or *ts616* at 37°C and shifted 24 h postinfection to 39 or 32°C. At 2 h following the temperature shift, cells were labeled with [^{35}S]methionine for 2 h (lanes A) or 30 min (lanes B). Large T antigen was immunoprecipitated and analyzed on 5% acrylamide SDS gel. (B) Cells were infected as described for panel A. At 2 h after the temperature shift cells were labeled with [^{35}S]methionine for 2 h (lanes A) or 5 min (lanes B). Large T antigen (LT) was immunoprecipitated and analyzed on a 5% acrylamide SDS-polyacrylamide gel.

suggest that large T antigen is sufficient to induce the kinase(s) that is responsible for these phosphorylations.

hrt mutants also appear to differ from the wild type under certain circumstances. For example, the *hrt* infections can lag behind those of the wild type (20, 53). When examined at such intermediate times, the mobility shift of large T antigen of mutant NG59 was less complete than that of the wild type (data not shown), and the phosphopeptide pattern showed some decrease in the amount of phosphopeptide 7 (Fig. 8B). NG59 also showed an additional change in the region of peptide 5. A minor peptide (5') in the wild-type digest became a major fragment in the NG59 pattern (Fig. 8C, bar). Two-dimensional analysis with V8 indicated that this peptide is related to fragment 5 and comes from the central portion of the molecule (4) (data not shown).

FIG. 7. Specific phosphopeptides are absent in *ts616* (616) large T-antigen molecules produced at the nonpermissive temperature. BMK cells were infected with wild-type virus or *ts616* mutant virus as described in the legend to Fig. 5. Large T-antigen molecules were immunoprecipitated and analyzed with *S. aureus* V8 protease as described in the text. The amount of large T antigen and length of exposure were adjusted to equalize the intensity of the peptides.

FIG. 8. Comparison of phosphorylations of *hrt* mutant and wild-type Py large T antigens. Actively growing 3T6 cells were infected with wild-type (WT) or NG59 (59) virus and were labeled with $^{32}\text{PO}_4$ 7 to 9 h postinfection (A) or 30 to 32 h postinfection (B). Actively growing NIH 3T3 cells were infected with wild-type or NG59 virus and labeled with $^{32}\text{PO}_4$ 22 h postinfection (C). Immunoprecipitated large antigen was analyzed with V8 protease as described in the text. Panel C is printed at a higher magnification to increase the resolution of the minor peptide 5' (bar).

DISCUSSION

Large T antigen undergoes a decrease in electrophoretic mobility after synthesis, indicating posttranslational modification (26, 27, 29, 53). Comparisons of $^{32}\text{PO}_4$ - and ^{35}S [methionine]-labeled large T antigen patterns and the effects of alkaline phosphatase treatment suggest that this shift can be explained entirely by phosphorylation. Quantitatively, most of this phosphorylation takes place in the nucleus, although labeling of isolated nuclei with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ suggests a subset of sites could be cytoplasmic phosphorylations (unpublished data). The close connection between mobility and phosphorylation does not mean that there are no other significant *in vivo* modifications of large T antigen. It only means that either they have little effect on the electrophoretic mobility or are present only on minor subpopulations of the protein. We expect that, as for SV40, a lengthy list of modifications will eventually be described.

The pattern of phosphorylation is relatively complicated, as demonstrated by the V8 phosphopeptide analysis. The arrangement of the phosphorylation sites is discussed in the accompanying paper (4). For the current discussion, it is most important to realize that there are two areas of phosphorylation. One of these is near the N terminus (V8 peptides 1 to 4, 8, and 9), and the other is farther toward the center of the molecule (peptides 5 and 7). Both of these areas are important to the conversion of Py large T antigen. However, phosphopeptides 5 and 7 appear preferentially in the slower migrating molecules.

The genetic evidence argues that phosphorylation could be important for large T antigen function. The large T antigen from *tsa* class mutants (*ts616* or *ts25*) is strikingly underphosphorylated at the nonpermissive temperature. Although all of the phosphopeptides were less highly labeled, phosphorylations represented by V8 phosphopeptides 5 and 7 appeared to be entirely absent. Furthermore, they were underrepresented in other situations in which replication did not occur at a high rate, such as infection of nonpermissive

rat cells with wild-type virus or *hrt* mutant infections of some nonpermissive mouse cells. Because there is always uncertainty as to whether a change in phosphorylation is a cause or an effect, this will have to be tested further by site-directed mutagenesis.

The nature of large T-antigen phosphorylation depends on the state of the cell. When growth-arrested cells are infected, large T antigen is less highly phosphorylated. The protein does not undergo the full shift in electrophoretic mobility that is characteristic of the highly phosphorylated forms. Instead, only a slight shift is observed. Analysis of the phosphopeptides at this time showed that the N-terminal region phosphorylation represented by V8 phosphopeptides 1 to 4, 8, and 9 were detected at reduced levels but that the more distal phosphorylations represented by peptides 5 and 7 were largely absent. Later in infection of the arrested cell large T antigen was more highly phosphorylated, and the peptide pattern showed the usual peptide configuration. When growing cells were infected, large T antigen phosphorylation was much less dependent on the time after infection. The most straightforward interpretation of these results is that the virus induces cell growth and that one consequence of induction is the stimulation of the kinase(s) that is responsible for some of the large T-antigen phosphorylation. Py or SV40 infection stimulates cellular DNA synthesis, as well as both histone and nonhistone chromosomal protein synthesis (23, 28). Increased kinase activities have been associated with the S phase of the cell cycle (22, 49). It is likely that the enzyme(s) that is responsible for phosphorylation is among them. The results indicate that phosphorylations represented by peptides 5 and 7 can be separated from those at the N terminus. We favor the interpretation that this means that more than one kinase is involved. An alternative possibility would be that structural changes in large T antigen alter the accessibility of the phosphorylation sites to enzyme.

What function do the viral gene products have in the phosphorylation of large T antigen? *hrt* mutants that produce large T antigen but not middle T or small T antigen stimulate one cycle of cell growth (47). The large T antigen of *hrt* mutants undergoes phosphorylation and shifts in electrophoretic mobility, even in the absence of serum. Large T antigen therefore appears to be responsible for the stimulation of the kinases. *ts616* mutant large T antigen was defective in phosphorylation and showed a partial mobility shift (Fig. 6 and 7). This could be because the mutant large T antigen is incapable of stimulating the appropriate kinase(s) or because it is a poor substrate at the nonpermissive temperature. The latter explanation seems more likely. *tsa* mutants are known to stimulate cell replication even at the nonpermissive temperature (47) and might be expected to induce kinases associated with the S phase. Also, *tsa* large T antigen fails to undergo the electrophoretic conversion in shift-up experiments. In such experiments, cellular factors induced by large T antigen would presumably remain available after shift-up. Preliminary experiments with deletion mutants showed that large T-antigen molecules were converted 9 h after infection of quiescent mouse cells if these cells had been previously infected with *ts616* (at the nonpermissive temperature).

Although the conversion of *hrt* mutant large T antigen seems to rule out an absolute requirement for middle T or small T antigen, results of analysis of the peptide patterns suggest that either or both may play a modulating role. *hrt* mutants show differences in the kinetics with which the conversion becomes possible, so that large T antigen can be converted only later in infection. This is in keeping with the

lag in infections and cell replication by middle T- and small T-antigen mutants noted previously (20, 37, 53). At the peptide level, *hrt* large T antigens also show differences. In general, peptides 5 and 7 were underrepresented. A minor peptide, 5', became more prominent. These effects were somewhat more pronounced in nonpermissive cells such as NIH 3T3 or acutely starved 3T6 cells than in permissive BMK cells. By affecting the response of the cell, middle T and small T antigens may alter the phosphorylation, and hence the efficiency, of large T-antigen function in DNA replication. There have been various reports on the efficiency of DNA replication in *hrt* mutants. Garcea and Benjamin (20) observed levels of DNA replication that were 30 to 40% that of the wild type when NIH 3T3 cells were infected. In 3T6 cells, Turler and Salomon (53) observed less than 10% that of the wild type. If phosphorylations represented by V8 phosphopeptides 5 and 7 are important for function, then *hrt* mutant replication could depend on the physiology of the cell as it affects the particular kinase. Both small T and middle T antigens have been shown to affect cellular phenotype (6, 35), so their relative importance to large T antigen phosphorylation is uncertain.

Garcea and colleagues (19) have shown an underphosphorylation of the major capsid protein VP1 in *hrt* mutant infections of nonpermissive cells, indicating that perhaps an appropriate kinase is lacking. It is conceivable that the VP1 kinase is related to the nuclear kinase phosphorylating large T antigen. Permissivity for *hrt* mutants, however, is not affected by the addition of serum to NIH 3T3 cells. In our hands, such treatment of resting NIH 3T3 cells increased the yield of V8 phosphopeptides 5 and 7 early in infection. Therefore, the enzyme phosphorylating 5 and 7 and that phosphorylating VP1 seem to be distinct.

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