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Identification and Characterization of a Herpes Simplex Virus Gene Product Required for Encapsidation of Virus DNA

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A mutant of herpes simplex virus type 1, 17*ts*VP1201, has a temperature-sensitive processing defect in a late virus polypeptide. Immunoprecipitation studies with monoclonal antibodies showed that the aberrant polypeptide in mutant virus-infected cells was the nucleocapsid polypeptide known as p40. Since a revertant, TS^+ for growth, processed the polypeptide normally under conditions restrictive for the mutant, the processing event must be essential for virus replication. Electron microscopic analysis of mutant virus-infected cells grown at the nonpermissive temperature revealed that the nuclei contained large aggregations of empty nucleocapsids possessing some internal structure. Therefore, although the mutant synthesized virus DNA at the nonpermissive temperature, the DNA was not packaged into nucleocapsids. When mutant virus-infected cells were shifted from 39 to 31°C in the presence of cycloheximide, the polypeptide p40 was processed to lower-molecular-weight forms, and full nucleocapsids were detected in the cell nuclei. The aberrant polypeptide of the mutant, however, was not processed in cells mixedly infected with 17*ts*VP1201 and a revertant at the nonpermissive temperature, suggesting that the defect of the mutant was in the gene encoding p40 rather than in a gene of a processing enzyme.

The herpesvirus virion consists of an icosadeltahedral capsid surrounded by an envelope. Within the capsid is the core, a cylindrical structure, around which the virus DNA is wound (7). Nucleocapsids are assembled in the cell nucleus and normally acquire their envelope by budding through the nuclear membrane (5, 18). Full herpes simplex virus type 1 (HSV-1) nucleocapsids isolated from cell nuclei contain two polypeptides, VP21 (apparent molecular weight, 44,000) and VP22a (apparent molecular weight, 38,000), which are not present in nucleocapsids lacking DNA. Of these two polypeptides, VP22a is thought to be on the surface of the capsid, leaving VP21 as a possible candidate for the core protein (8). Polypeptide VP22a does not appear to be a constituent of the mature virus particle (8). It has been proposed, however, that VP22a undergoes some modification, possibly cleavage or phosphorylation, to form VP22, a virion polypeptide that is loosely bound to the nucleocapsid (9). Monoclonal antibodies against a nucleocapsid polypeptide known as p40 have been produced by Zweig et al. (27). This polypeptide is thought to correspond to VP22a (11).

In HSV and pseudorabies virus, DNA is present in the nucleus in the form of concatemers at late times in infection (2, 12). This high-molecular-weight DNA is cleaved as it is packaged into preformed nucleocapsids (1, 6, 15, 24, 25). Sequences from the "short" region of HSV DNA are thought to contain a recognition signal(s) for cleavage and encapsidation of the concatemeric virus DNA (25). Although DNA shorter than virion size can be packaged, only capsids that contain approximately full-size DNA are transported into the cytoplasm (25).

In this study, we report the characterization of an HSV-1 mutant that has a temperature-sensitive processing defect in the nucleocapsid structural polypeptide p40 and fails to encapsidate virus DNA at the nonpermissive temperature (NPT).

MATERIALS AND METHODS

Cells. BHK-21 clone 13 cells (17) were used for virus titrations and propagation. In all other experiments, human fetal lung (Hfl) fibroblasts were used. This cell line, established by B. Carritt, Medical Research Council Biochemical Genetics Unit, University of London, was grown in Eagle medium supplemented with 10% fetal calf serum and 1% nonessential amino acids.

Viruses. The wild-type HSV-1 stock was strain 17 (3). From a UV-mutagenized stock of this strain, the mutant 17*ts*JC116 was isolated. Since this virus had several temperature-sensitive lesions, the *Eco*RI F fragment, which contained one of the mutations, was

cloned into the plasmid vector pACYC184 and recombined into HSV-1 TS^+ strain 17 DNA. From this transfection experiment the mutant 17 *ts*VP1201 was isolated. This virus has a relative efficiency of plating (efficiency at 39°C/efficiency at 31°C) of $<2 \times 10^{-5}$. Plaque-purified stocks of the mutant were screened for the presence of revertants able to grow at the NPT. The revertant, 17*ts*VP1201 rev1, was selected for this study. For brevity, in this paper the mutant and the revertant are referred to as *ts*1201 and *ts*1201rev1, respectively.

Virus-infected cell polypeptide synthesis. Cells (1.5×10^6 per dish) were infected at a multiplicity of infection (MOI) of 20 PFU/cell, washed twice with cell culture medium at 1 h postinfection, and overlaid with 2 ml of medium per dish. Virus-infected cell polypeptides were radiolabeled by incubation in phosphate-buffered saline containing 100 μCi of [^{35}S]methionine per ml. After 20 min, samples were harvested either immediately as described by Marsden et al. (18) or after incubation for various times in medium lacking [^{35}S]methionine from virus or mock-infected cells. When used, cycloheximide was added to a final concentration of 200 μg/ml. Virus-infected cell polypeptide samples were analyzed on 8.0% sodium dodecyl sulfate (SDS)-polyacrylamide gels, with a 5% polyacrylamide stacking gel, as described by Marsden et al. (18).

Polypeptide nomenclature. Virus-infected cell polypeptides were labeled according to their estimated molecular weight on SDS-polyacrylamide gels (18). Thus, the virus-induced polypeptides of molecular weight 40,000 and 39,000 were described as Vmw40 and Vmw39, respectively. The polypeptides, including Vmw40 and Vmw39, that were immunoprecipitated by monoclonal antibody against p40 were collectively referred to as p40 in this paper.

Immunoprecipitation. Protein samples were prepared in RIPA buffer (0.1% SDS, 1% sodium deoxycholate, 1% Nonidet P-40, 1 mM EDTA, 150 mM NaCl, 10 mM Tris-hydrochloride [pH 7.4]). Two immunoprecipitation methods were used, one based on the procedure described by Kessler (14) and the other on the method of Sutcliffe et al. (22).

Electron microscopy. Cells (2×10^6 per dish) were infected with virus at an MOI of 10 PFU/cell. At various times after infection, samples were harvested. The cells were washed with phosphate-buffered saline, scraped into 0.5 ml of buffer, pelleted by low-speed centrifugation, and suspended in phosphate-buffered saline containing 2.5% glutaraldehyde. The samples were then treated as described by Atkinson et al. (1), except that hydroxypropylmethacrylate was used instead of propylene oxide.

Synthesis of virus DNA in infected cells. Cells (4×10^6 per dish) were infected with virus at an MOI of 5 PFU/cell. At 1 h postinfection, samples were washed once with cell culture medium and incubated in 2 ml of medium at the appropriate temperature for 18.5 h. [^{3}H]thymidine (15 μCi/ml) was added to each dish at 3 h postinfection. Virus-infected cell lysates were prepared essentially as described by Halliburton and Timbury (10). The virus DNA was separated from host DNA by isopycnic density gradient centrifugation in CsCl in a Beckman 40 Ti rotor at 40,000 rpm at 18°C for 56 h. The initial density of CsCl in the sample was 1.72 g/ml. Fractions were collected from the gradient, and the radioactivity was determined by scintillation counting (10).

Thermal inactivation of virus. Virus, diluted to approximately 2×10^5 PFU/ml in Tris-buffered saline containing 0.05% calf serum, was incubated in a water bath at 39°C. At appropriate times, samples were removed and diluted in ice-cold phosphate-buffered saline containing 10% calf serum, and the virus was titrated.

Marker rescue assay. Marker rescue was performed as described by Stow et al. (21), with the modifications of Preston (20).

Construction of recombinant plasmid DNA. *Bam*HI fragments U and D′ from wild-type HSV-1 strain 17 DNA were cloned into the plasmid pAT153 by direct ligation of *Bam*HI cohesive ends (23). Cloned HSV-1 *Xho*I-F was a gift from B. Matz, Institut für Virologie, University of Freiburg. The plasmids were referred to as pGX56 (*Bam*HI-U insert), pGX27 (*Bam*HI-D′ insert), and pGX96 (*Xho*I-F insert). The genome location of each of the cloned HSV-1 fragments is shown in Fig. 8. Plasmid DNA was prepared as described previously (20).

Preparation of restriction endonuclease DNA fragments. Fragments were separated by agarose gel electrophoresis in the presence of ethidium bromide as described by Wilkie and Cortini (26). The DNA bands were visualized under long-wave UV light and excised from the gels. The DNA was eluted from the gel slice by high-voltage electrophoresis in 5 mM sodium acetate–1 mM EDTA–40 mM Tris-hydrochloride (pH 7.8) and concentrated by ethanol precipitation.

RESULTS

Polypeptides induced by *ts*1201, a revertant of *ts*1201 (*ts*1201rev1), and wild-type strain 17 virus in Hfl cells at 31 and 39°C. Virus-infected cells incubated at 39°C were pulse-labeled with [^{35}S]methionine at 5 h postinfection. After 20 min, the radioactive medium was removed, and the infected cells were harvested either immediately or after incubation for a further 1, 2, or 4 h at 39°C in virus-infected cell growth medium lacking [^{35}S]methionine. Figure 1 shows an autoradiogram of electrophoretically separated virus-infected cell polypeptides. In the pulse-labeled wild-type virus-infected cells, a polypeptide of about 40,000 molecular weight (Vmw40) was synthesized. The intensity of this polypeptide band decreased with increasing length of chase, concomitant with the appearance of a polypeptide band of approximate molecular weight 39,000 (Vmw39), which was not detectable in pulse-labeled virus-infected cell samples. A pattern of polypeptide synthesis similar to that of the wild-type virus was observed in *ts*1201rev1-infected cells. In the mutant-infected cells, however, the Vmw40 band did not decrease in intensity during the chase, and the Vmw39 band was not seen. This result suggests that Vmw40 is related to Vmw39. In wild-type virus-infected cells, the conversion of Vmw40 to

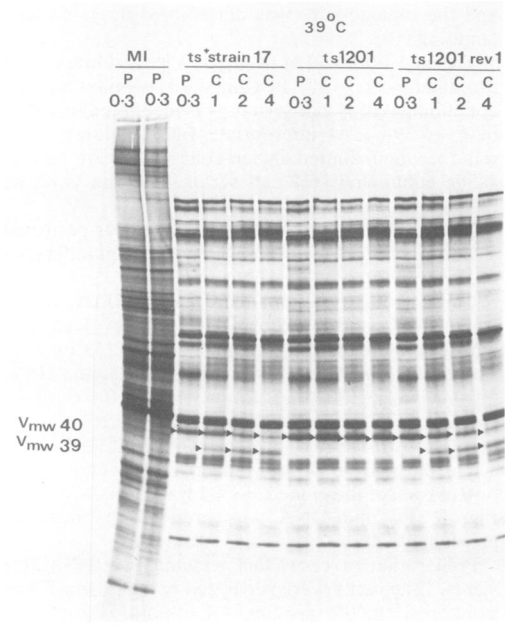

FIG. 1. Autoradiogram of polypeptides induced by HSV-1 strain 17, *ts*1201, *ts*1201rev1, and in mock-infected cells at 39°C. Virus-infected cells were pulse-labeled with [^{35}S]methionine at 5 h postinfection. After 20 min, the radioactive medium was removed, and the cells were harvested either immediately or after a chase of 1, 2, or 4 h. Protein samples were analyzed on an 8% SDS-polyacrylamide gel, with a 5% polyacrylamide stacking gel. P, Pulse-labeled; C, chase; MI, mock infected. The numbers at the top of the autoradiogram refer to time in hours.

Vmw39 was slow, since after a 4-h chase small amounts of Vmw40 were still detectable.

At the permissive temperature (PT) of 31°C, *ts*1201-infected cells processed Vmw40 at a rate comparable to that in cells infected with wild-type virus (Fig. 2). The modification of Vmw40 must be essential for virus growth, because the revertant of *ts*1201, TS$^+$ for growth, processed Vmw40 normally at both 31 and 39°C.

Immunoprecipitation studies. Since Vmw40 is synthesized late in the virus growth cycle, it is likely to be a structural component of the virion, probably the nucleocapsid polypeptide known as VP22a (8), p40 (11, 27), or NC-3 (4). To investigate this possibility, we obtained mouse monoclonal antibody specific to p40 from M. Zweig, Frederick Cancer Research Center (27). When wild-type virus, mutant, or *ts*1201rev1-infected cells were pulse-labeled with [^{35}S]methionine late in infection, at least three virus-specific polypeptides, ranging in approximate molecular weight from 39,000 to 45,000, were detected in immune precipitates (Fig. 3). After a 4-h chase, two new polypeptide bands, both lower in molecular weight, were observed in wild-type virus- and *ts*1201rev1-infected cells. These species, however, were not present in significant amounts in the mutant virus-infected cells at the NPT. The results indicate that Vmw40 corresponds to p40. The data also suggest that there was multiple processing of p40 and that some modification of p40 occurred in the mutant virus-infected cells at the NPT, because the three higher-molecular-weight forms were present.

Processing of p40 after downshift of *ts*1201-infected cells from 39 to 31°C. Cells were infected with *ts*1201, *ts*1201rev1, or wild-type virus and incubated at 39°C. At 5 h postinfection, the virus-infected cells were pulse-labeled with [^{35}S]methionine for 20 min and harvested either immediately or after incubation at 31 or 39°C in the presence of cycloheximide to prevent further protein synthesis. Figure 4A shows the polypeptides that were immunoprecipitated by monoclonal antibody against p40. When *ts*1201-infected cells were shifted from the NPT to the PT, p40 was processed to the lower-molecular-weight forms detected in cells infected with wild-type virus or *ts*1201rev1. Therefore, new protein synthesis is not necessary for the processing of p40 after a temperature shift down. This result suggests that the function of the gene product

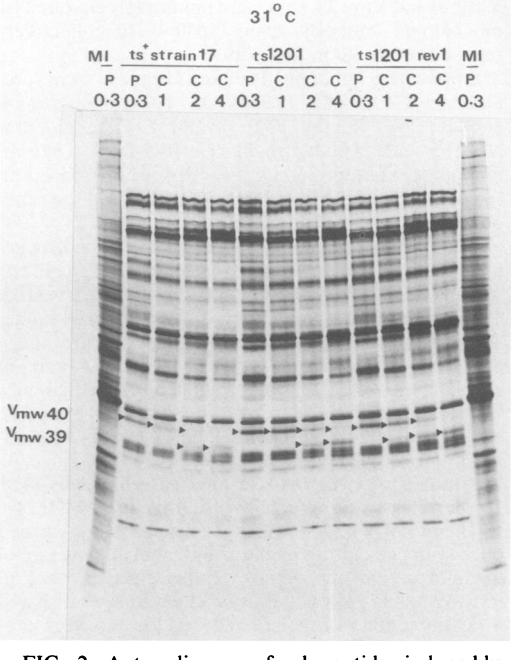

FIG. 2. Autoradiogram of polypeptides induced by HSV-1 strain 17, *ts*1201, *ts*1201rev1, and in mock-infected cells at 31°C. Virus-infected cells were pulse-labeled with [^{35}S]methionine at 7.25 h postinfection. For details and explanations, see the legend to Fig. 1.

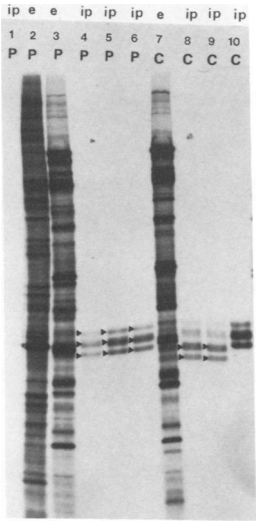

FIG. 3. Autoradiogram of immune precipitates of p40 from virus-infected cells. Virus-infected cell polypeptides were labeled with [^{35}S]methionine as described in the legend to Fig. 1. The samples were suspended in RIPA buffer and incubated at 37°C with ascites fluid containing monoclonal antibody against p40. Immunoprecipitation was carried out by the method of Kessler (14). The immunoprecipitated polypeptides were analyzed by SDS-polyacrylamide gel electrophoresis. P, Pulse-labeled; C, chase of 4 h; ip, immune precipitate; e, virus- or mock-infected cell extract. Tracks: 1 and 2, mock-infected cells; 3, 4, 7, and 8, HSV-1 strain 17-infected cells; 5 and 9, *ts*1201rev1-infected cells; 6 and 10, *ts*1201-infected cells.

defective in *ts*1201-infected cells at the NPT was restored after a downshift of the cells to the PT.

Processing of p40 at the NPT in mixed infections of *ts*1201 and *ts*1201rev1. Cells infected with *ts*1201, *ts*1201rev1, or a mixture of the two viruses were incubated at 39°C, pulse-labeled with [^{35}S]methionine at 5 h postinfection, and harvested either immediately or after a chase of 4 h at 39°C. The virus-infected cell polypeptides immunoprecipitated by monoclonal antibodies to P40 were analyzed by SDS-polyacrylamide gel electrophoresis (Fig. 4B). In mixed infections of *ts*1201 and *ts*1201rev1 harvested after a 4-h chase, p40 gave a pattern of processing similar to that in cells infected with *ts*1201 alone. Similar data were obtained when wild-type virus was used in the mixed virus infection instead of *ts*1201rev1 (data not shown).

Assembly of capsids at the PT and NPT in cells infected with *ts*1201, *ts*1201rev1, and wild-type virus. Thin sections of virus-infected Hfl cells were examined by electron microscopy. The nuclei of mutant virus-infected cells grown at the NPT contained large aggregations of empty nucleocapsids possessing some internal structure (Fig. 5). These large aggregations of empty nucleocapsids were not observed in wild-type HSV-1- or *ts*1201rev1-infected cells at this temperature. After the mutant virus-infected cells were shifted from the NPT to the PT in the presence of cycloheximide, a protein synthesis inhibitor, full nucleocapsids were detected in the nuclei of cells (Fig. 5), and enveloped virions were present in the cytoplasm and on the cell surface. The correct processing of p40 described in the previous section therefore correlates with the assembly of full nucleocapsids.

Virus DNA synthesis. Electron microscopic studies of *ts*1201-infected cells grown at the NPT revealed that the mutant failed to make full nucleocapsids. This phenotype could result either from a block in virus DNA synthesis or from a failure to package virion DNA. The DNA phenotype of *ts*1201 at 31 and 39°C was determined to distinguish between these two possi-

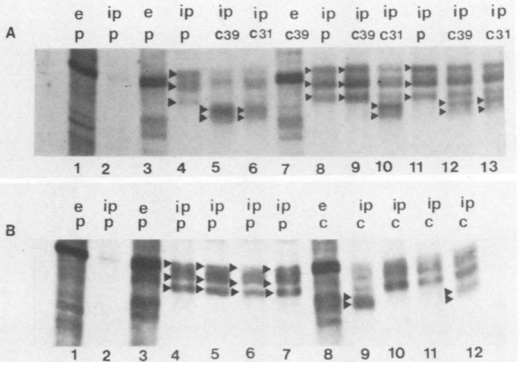

FIG. 4. Autoradiograms of immune precipitates of p40 from (A) virus-infected cells shifted down from 39 to 31°C and (B) mixed virus infections at 39°C. In A, virus-infected cells incubated at 39°C were pulse-labeled with [^{35}S]methionine at 5 h postinfection and harvested either immediately or after a chase of 4 h at 31 or 39°C in the presence of cycloheximide. In B, cells infected with a total MOI of 20 PFU/cell (10 PFU of each virus in the mixed infection) were incubated at 39°C and pulse-labeled at 5 h postinfection with [^{35}S]methionine. Samples were harvested either immediately or after a chase of 4 h at 39°C. Immunoprecipitation with monoclonal antibody against p40 was carried out by the method of Sutcliffe et al. (21). Immune precipitates were analyzed by SDS-polyacrylamide gel electrophoresis. P, Pulse-labeled; C, chase; ip, immune precipitate; e, virus or mock-infected cell extract. (A) Tracks: 1 and 2, mock-infected cells; 3, 4, 5, 6, and 7, *ts*1201rev1-infected cells; 8, 9, and 10, *ts*1201-infected cells; 11, 12, and 13, HSV-1 strain 17-infected cells. (B) Tracks: 1 and 2, mock-infected cells; 3, 4, 8, and 9, *ts*1201rev1-infected cells; 5 and 10, *ts*1201-infected cells; 6 and 11, cells mixedly infected with *ts*1201 and *ts*1201rev1; 7, a mixture of the immune precipitates shown in tracks 4 and 5; 12, a mixture of the immune precipitates shown in tracks 9 and 10.

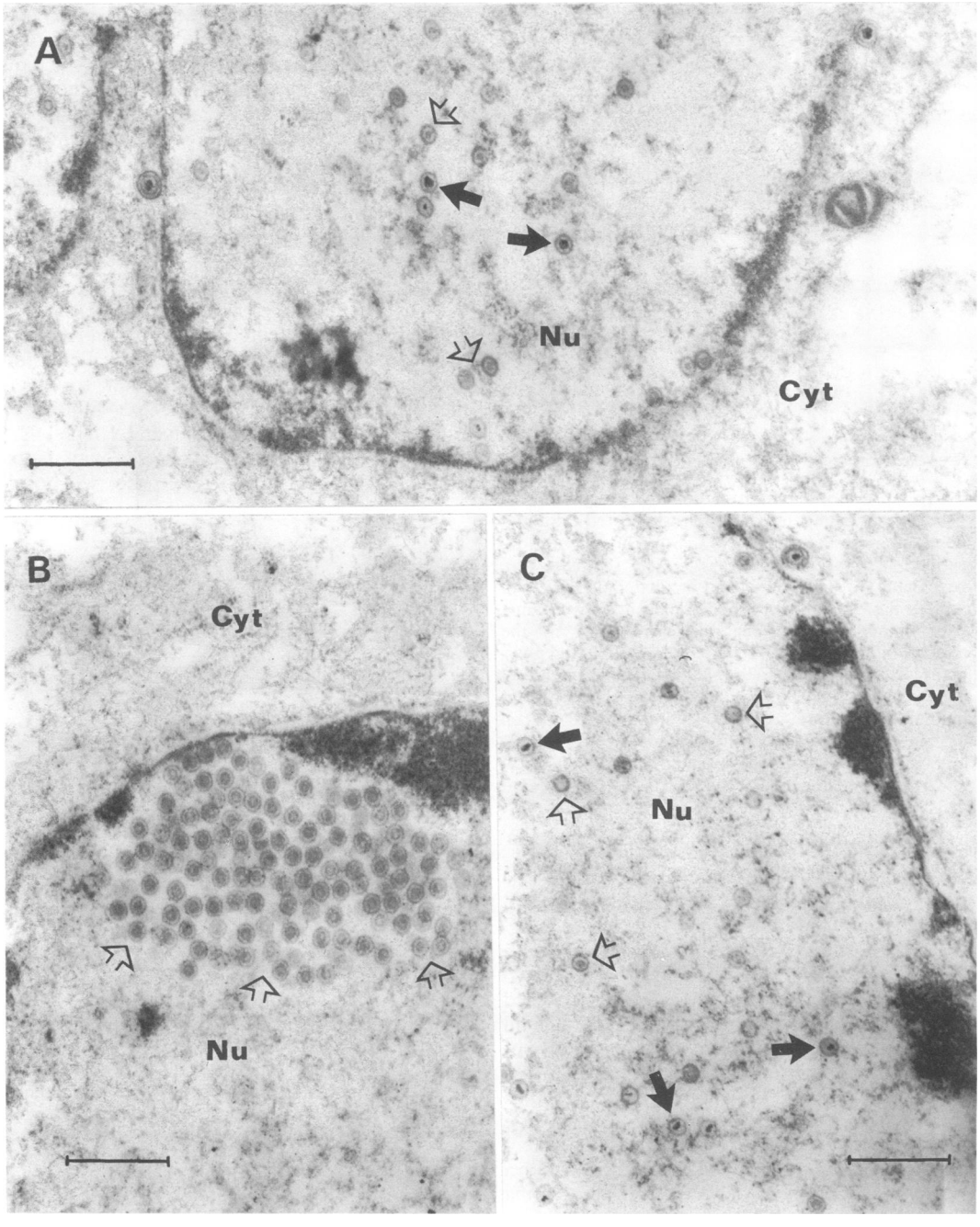

FIG. 5. Electron mirographs of thin sections of virus-infected cells. Cells infected with *ts*1201rev1 (A) or *ts*1201 (B) were incubated at 39°C and harvested at 9 h postinfection. (C) Cells infected with *ts*1201 were incubated at 39°C for 6 h. The virus-infected cells were then shifted down to 31°C and incubated in the presence of cycloheximide for 4 h. Nu, Nucleus; Cyt, cytoplasm; open arrows, nucleocapsids with electron-translucent cores; solid arrows, nucleocapsids with electron-dense cores. Bar, 0.5 μm.

bilities. Figure 6 shows the results of CsCl equilibrium density gradient centrifugation of [^{3}H]thymidine-labeled DNA extracted from mutant- and wild-type virus-infected cells grown at 31 or 39°C. Since *ts*1201 synthesized virus DNA at the NPT, the mutant must have a block in packaging virus DNA at this temperature.

Virus particle stability. VP22, which is thought

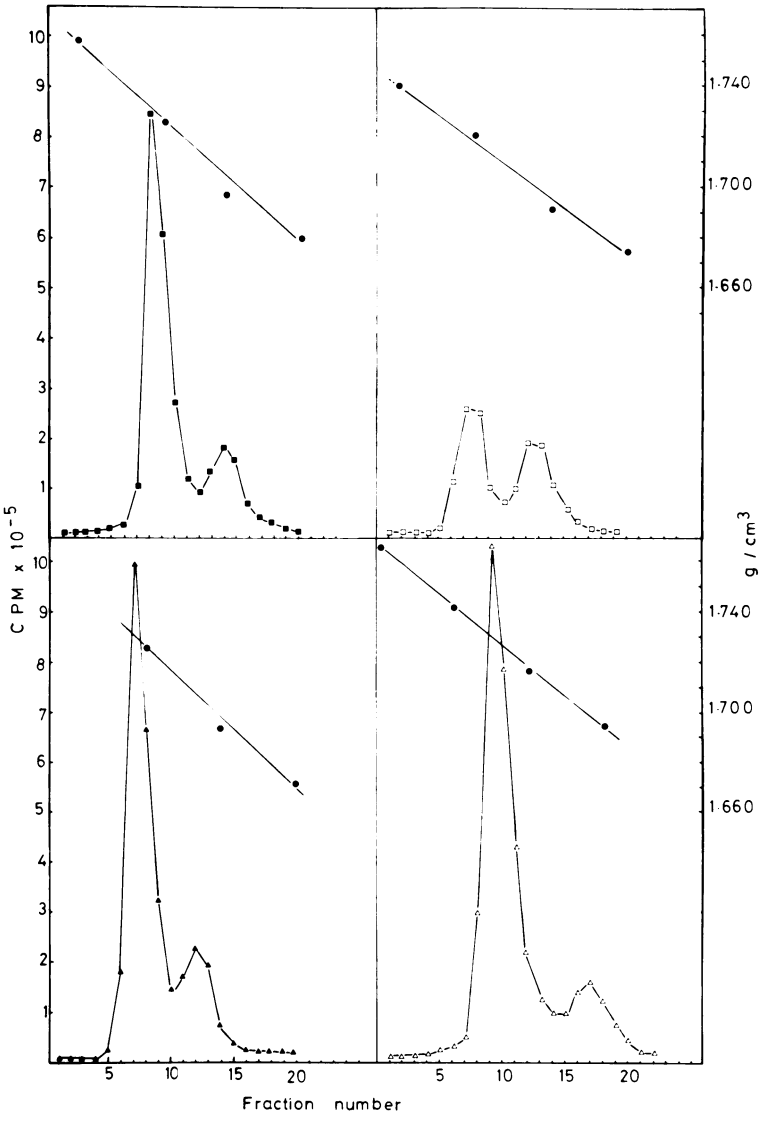

FIG. 6. Isopycnic CsCl gradients of [^{3}H]thymidine-labeled DNA extracted from virus-infected Hfl cells. HSV-1 strain 17- (▲, △) and *ts*1201- (■, □) infected cell DNAs were synthesized in cells incubated at 31 (▲, ■) or (39°C (△, □). The density of the CsCl (●) solution was determined from the refractive index. The virus and cellular DNAs had buoyant densities of 1.725 and 1.700 g/cm^3, respectively.

to correspond to p40 (11), has been reported to be present in the virion (8). For this reason, the particle stability of *ts*1201, *ts*1201rev1, and wild-type virus at 39°C was determined. The results (Fig. 7) revealed that the mutant was inactivated at a rate similar to that of wild-type virus and *ts*1201rev1.

Physical mapping of *ts*1201 lesion. *ts*1201 was rescued by *Xho*I-F cloned from wild-type HSV-1. The mutation was mapped more precisely by DNA transfection experiments in which separated *Sal*I fragments from cloned *Xho*I-F were used to marker-rescue the mutant (Table 1 and Fig. 8). The lesion mapped within *Sal*I-D. Since this fragment contains a *Bam*HI site that delimits HSV-1 *Bam*HI-U and -D', these cloned *Bam*HI fragments were screened for the ability to rescue *ts*1201. Significant levels of TS$^+$ progeny were obtained only with cloned *Bam*HI-U (Table 1 and Fig. 8). The mutation therefore lies within a region of approximately 640 base pairs common to *Bam*HI-U and *Sal*I-D.

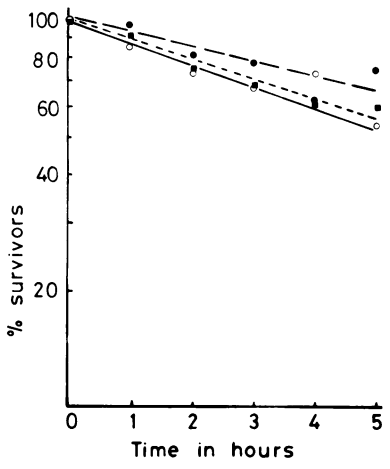

FIG. 7. Thermal inactivation of HSV-1 strain 17, *ts*1201, and *ts*1201revl at 39°C. The results are presented as percent virus survival compared with the virus sample at time zero of incubation at 39°C (100%). Symbols: ■, HSV-1 strain 17; ○, *ts*1201; ●, *ts*1201revl.

TABLE 1. Marker rescue of *ts*1201

DNA	Relative efficiency of plating[a] (×10³)
pGX27 (*Bam*HI-D')[b]	0.15
pGX56 (*Bam*HI-U)[b]	3.30
None	0.10
pGX96 (*Xho*I-F)[c]	1.42
pGX96 (*Sal*I-A)	<0.02
pGX96 (*Sal*I-B)	<0.02
pGX96 (*Sal*I-C)	0.02
pGX96 (*Sal*I-D)	3.39
None	<0.01

[a] Efficiency of plating at 39°C/efficiency of plating at 31°C for progeny virus from transfected cells.
[b] Plasmid digested with *Bam*HI was used.
[c] Plasmid digested with *Sal*I was used.

DISCUSSION

We identified the aberrant Vmw40 in cells infected with *ts*1201 at the NPT as the nucleocapsid polypeptide p40. Zweig et al. (28) found in HSV-1-infected cells several polypeptides, ranging in molecular weight from 39,000 to 45,000, that were immunoprecipitated by mouse monoclonal antibody against p40. We detected in virus-infected cells pulse-labeled with [^{35}S]methionine late in infection at least three polypeptides of this class in immune precipitates. During a chase, two new polypeptide bands of lower molecular weight were observed in wild-type- or *ts*1201revl-infected cells, but not in significant amounts in the mutant virus-infected cells. In cells mixedly infected at 39°C with the mutant and *ts*1201revl or wild-type virus, most of the p40 was not processed to the lower-molecular-weight species detected in a chase in wild-type virus- or revertant-infected cells. It therefore appears that the wild-type virus or *ts*1201revl is unable to process the partially modified mutant p40. Furthermore, there was reduced processing of *ts*1201revl or wild-type virus in the mixed virus infection. It is, however, unlikely that *ts*1201 has a dominant lethal temperature-sensitive mutation (13) be-

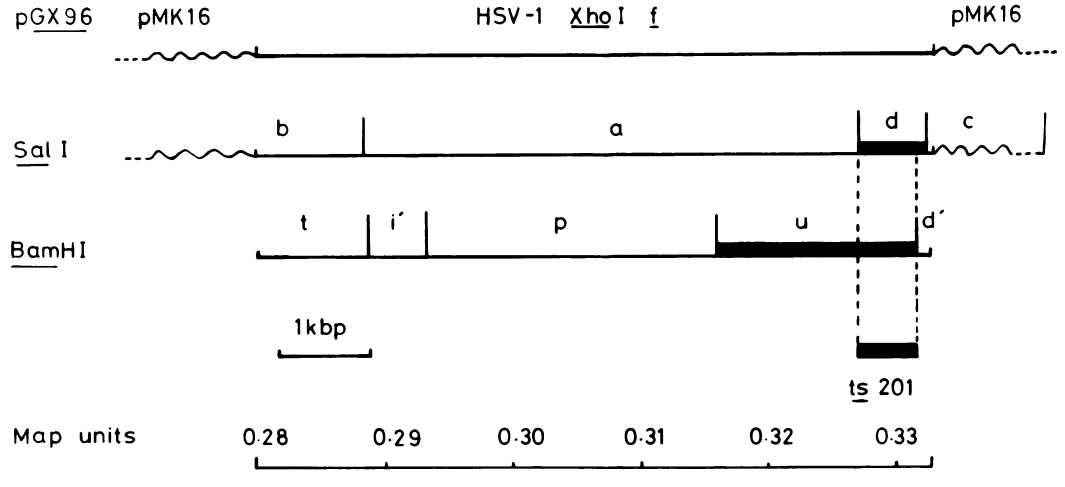

FIG. 8. Summary of the marker rescue data on *ts*1201. The solid lines represent the regions of HSV-1 DNA that rescue the mutant.

cause *ts*1201 complements other HSV-1 temperature-sensitive mutants (for example, *ts*A) very efficiently (V. Preston, unpublished data). The results suggest that the defect in *ts*1201 lies within the gene encoding p40 rather than in a gene of an enzyme that processes p40. The p40 gene has been mapped by analysis of HSV-1/HSV-2 intertypic recombinants to the genome region 0.3 to 0.38 map units (18), which spans the location for the mutation in *ts*1201 reported here.

The failure of *ts*1201 to encapsidate virus DNA at the NPT could be a direct or indirect consequence of the inability of the mutant to modify p40. This processing step could be required for the attachment of other polypeptides to the nucleocapsid, which in turn could be responsible for packaging virus DNA. Alternatively, p40 could be directly required for this function. Gibson and Roizman (8) proposed that processing of VP22a (p40) was necessary for the binding of the high-molecular-weight tegument polypeptides, VP1, VP2, and VP3, to the nucleocapsid and subsequent envelopment of the virus particle. It is not known, however, whether these tegument polypeptides are required for encapsidation of virus DNA. Nevertheless, it is clear from the work on temperature-sensitive mutants of pseudorabies virus (16) that more than one virus gene product is necessary for packaging virus DNA. It is likely that structural polypeptides will be required not only to bind and retain virus DNA in the capsid, but also to cleave the concatemeric DNA as it is packaged. Further investigation into the function of individual structural polypeptides is needed to determine the role p40 has in virus DNA packaging.

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