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Characterization of a Temperature-Sensitive Mutant of the U_L15 Open Reading Frame of Herpes Simplex Virus 1

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The U_L15 gene of herpes simplex virus 1 consists of two exons and is highly conserved among the herpesviruses sequenced to date. Other than its homology to a phage protein involved in the packaging of DNA, nothing is known of its function. This report concerns the isolation of a temperature-sensitive mutant with a mutation mapping in the U_L15 open reading frame. Cells infected with the parent, mutant, and rescued viruses all make DNA at the nonpermissive temperature. Direct analyses of the DNA and electron microscopic studies indicate that although viral DNA is made, it is not packaged into capsids present in nuclei. These studies suggest that U_L15 may be involved in the packaging of viral DNA.

In this article, we report the isolation and phenotype in infected cells of a temperature-sensitive (*ts*) mutant of the herpes simplex virus 1 (HSV-1) gene U_L15. Relevant to this report are the following.

(i) Most isolates of HSV-1 passaged a limited number of times in cells in culture exhibit a *ts* phenotype. This is the case for HSV-1 strain F [HSV-1(F)] passaged a limited number of times in cells in culture. To develop *ts* mutants, it is first necessary to select viruses which do multiply at higher temperatures to serve as genetic wild types. For this purpose, we have in the past used a strain of virus which is passaged serially in cell culture many times and which multiplies well at 39°C, the nonpermissive temperature. In the course of plaque purifications of viruses following transfection of cells with intact DNA and fragments of a gene mutagenized at a specific site, we isolated numerous *ts* mutants not relevant to the mutagenized gene. One of these *ts* mutations turned out to map specifically in the U_L15 open reading frame. The phenotype of this mutant is described in this report.

(ii) The U_L15 open reading frame is of interest from several points of view. First, it is one of the very few genes known to yield spliced RNAs (20). It is particularly noteworthy that exons 1 and 2 are separated by two open reading frames, U_L16 and U_L17, arranged antisense to U_L15. U_L16 is not essential for viral replication in cells in culture (1). Attempts in this laboratory to delete U_L15 or U_L17 were not successful (2a). However, a recent report from this laboratory showed that a cDNA copy of the U_L15 gene yielding a single, unspliced RNA can replace U_L15 exons 1 and 2 without affecting the capacity of the virus to replicate in cells in culture (2).

(iii) A recent publication by Davison (6) drew attention to the facts that the U_L15 open reading frame is highly conserved among herpesviruses and that the separation of the two exons by antisense open reading frames is also conserved. A homolog of the U_L15 open reading frame exists in the channel catfish herpesvirus. Davison pointed out that the U_L15 protein may be related to protein 17 of the T4 phage (6). This protein is known to be involved in packaging DNA (15, 16).

We report that the *ts* mutant at the nonpermissive temperature synthesizes capsids and DNA but packaging of DNA does not take place.

MATERIALS AND METHODS

Virus and cells. HSV-1(mP) was the name given to a strain initially designated strain NT and isolated from a human infection in embryonated eggs (10). From the NT strain, two viruses were isolated in cell culture. One, designated the microplaque strain [HSV-1(mP)], causes infected cells to aggregate in a fashion typical of most HSV-1 strains. The other, designated the macroplaque strain [HSV-1(MP)], causes cells to fuse into polykaryocytes (7). A characteristic of HSV-1(mP) is that it grows as well at 39°C as at 34°C. Unlike the HSV-1(F) strain used in this laboratory as the prototype HSV-1 strain and passaged a limited number of times in cells in culture, HSV-1(mP) has been passaged at least 80 times in FL and HEp-2 cell cultures.

The sources and procedures for the propagation of Vero and rabbit skin cells have been described elsewhere (2, 14).

Plasmid libraries. The HSV-1(F) plasmid library was described elsewhere (14). The *Hin*dIII J fragment was cloned as pRB208 in pACYC184 (13a) and was derived from that plasmid. The derivation of the fragments shown in Fig. 1 was as follows. DNA fragment *Eco*RI-2 was obtained by digestion of *Hin*dIII-J. *Bam*HI-*Bgl*II 4, *Sal*I 6, and *Mlu*I 1 DNA fragments were obtained by digestion of *Eco*RI-2 with the appropriate enzymes. The *Xba*I fragments of HSV-1 DNA (4) were the kind gift of Mark Challberg.

Marker rescue. Rabbit skin cells grown in 25-cm^2 flasks were transfected with 0.6 to 0.8 μg of HSV-1(mP)*ts*66.4 DNA with the appropriate fragment of HSV-1(F) DNA. The cells were grown at 37°C and were harvested when almost all cells exhibited cytopathic effects, usually 7 to 8 days post-transfection. Titers of the viruses obtained from each transfection were determined at 34 and 39°C.

Efficiency of plating at 34 and 39°C. Replicate cultures of Vero cells in 25-cm^2 flasks were exposed in duplicate to various dilutions of the wild-type, mutant, or rescued virus and incubated at either 34 or 39°C. After 3 days, the cultures were stained and plaques were counted as previously described (7).

Efficiency of replication at 34 and 39°C. Replicate cultures

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of Vero cells in 25-cm^2 flasks were exposed in duplicate to 1.0 or 0.1 PFU of wild-type, mutant, or rescued virus per cell and incubated at either 34 or 39°C. At 18 h postinfection, the cells were harvested and the titers of the viruses were determined at 34°C in Vero cells.

Extraction and analysis of HSV-1 DNA. In all of these studies, Vero cells grown in replicate 25-cm^2 flasks were exposed to 10 PFU of wild-type, mutant, or rescued virus per cell and incubated at 34 or 39°C. At specified times, the cells were harvested, and DNA was extracted and analyzed, as described in Results.

Labeling and electrophoretic separation of infected-cell proteins. Replicate cultures of Vero cells in 25-cm^2 flasks were infected in duplicate with wild-type, mutant, or rescued virus and incubated at either 34 or 39°C. The cells were labeled by incubation with 1 ml of methionine-free medium supplemented with 50 μCi of [^{35}S]methionine (1,000 Ci/mmol; Amersham, Arlington Heights, Ill.) for 1 h at 13 h postinfection and then harvested, lysed, and electrophoretically separated on 11% polyacrylamide gels and subjected to autoradiography as previously described (12, 18).

RESULTS

Isolation of the mutant HSV-1(mP)*ts*66.4. The experiments which led to the isolation of the mutant were as follows. Rabbit skin cell monolayers in 25-cm^2 flasks were transfected with intact HSV-1(mP) DNA and DNA fragments containing the open reading frame U_L48, which encodes the protein designated the α *trans*-inducing factor (αTIF) by Pellett et al. (13) or virion protein 16 (VP16) by Spear and Roizman (18). These fragments were modified by in situ mutagenesis in order to replace selected *cys* codons. The progeny of the transfection was plated at approximately 50 PFU per flask. Plaques were selected, purified, and plated in replicate 24-well dishes at 34 and 39°C. Isolates which failed to cause cytopathic effects at 39°C were selected, plaque purified again, and retested. The isolates which reproducibly failed to multiply at 39°C were studied further.

Although a large number of *ts* mutants were isolated, none of the mutations mapped in the target gene. Some of the mutations mapped in genes whose phenotypes were well characterized in other studies. The mutation in the mutant which we selected for further studies and designated HSV-1(mP)*ts*66.4 mapped in a gene whose function was not known.

Mapping of the mutation in HSV-1(mP)*ts*66.4. In these series of experiments, rabbit skin cells were transfected with intact mutant DNA and wild-type HSV-1 fragments. The progeny of transfection was plated at 34 and 39°C. Several series of experiments were done to narrow the exact location of the mutation responsible for the *ts* phenotype. In the first series, we tested individual fragments from an *Xba*I fragment library, kindly donated by Mark Challberg, which encompass the entire genome. The fragment which rescued the mutation was *Xba*I-C (Fig. 1, line 2; Table 1). The next series of transfections were done with DNA fragments derived from HSV-1(F) DNA with the region spanned by *Xba*I-C. Of the fragments tested, the ones which rescued the mutation are shown in Table 1 and Fig. 1. The two smallest fragments which individually rescued the mutation span exon 2 of U_L15 (coding for the carboxyl terminus of the protein) and the U_L18 open reading frame, and a fragment which encodes all of U_L15 also rescued the mutation (Table 1). Several plaques produced at 39°C were harvested, and the viruses obtained were plaque purified and studied further as representatives of rescued viruses. The two viruses studied extensively were designated HSV-1(mP)Ra, which was obtained by rescue with the *Sal*I 6 fragment, and HSV-1(mP)Rb, which was obtained by rescue with the *Bam*HI-*Bgl*II 4 fragment.

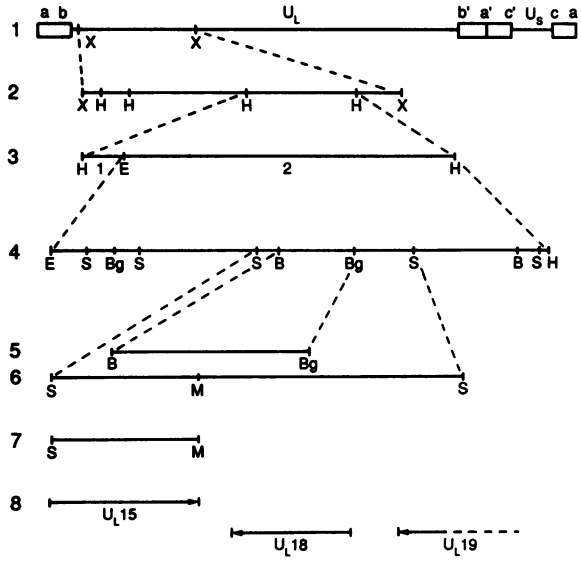

FIG. 1. HSV-1 DNA sequence arrangement and location of DNA fragments which rescued HSV-1(mP)*ts*66.4. Line 1, sequence arrangement of HSV-1 DNA (rectangles represent the inverted repeats *ab*, *b'a'*, *a'c'*, and *ca*, which flank the long and short unique sequences, U_L and U_S); line 2, *Xba*I C fragment; line 3, *Hin*dIII J fragment; line 4, *Eco*RI 2 fragment; line 5, *Bam*HI-*Bgl*II 4 fragment; line 6, *Sal*I 6 fragment; line 7, *Mlu*I 1 fragment; line 8, map positions of the U_L15, U_L18, and U_L19 genes relative to the *Sal*I 6 fragment. The positions of the U_L15, U_L18, and U_L19 transcriptional units were drawn according to the study of Costa et al. (5). B, *Bam*HI; Bg, *Bgl*II; E, *Eco*RI; H, *Hin*dIII; M, *Mlu*I; S, *Sal*I; X, *Xba*I.

Plating and replication efficiencies of the wild-type, mutant, and rescued viruses at the nonpermissive temperature. The results of the studies done with wild-type, mutant, and rescued viruses are summarized in Table 2. All of the assays were done with Vero cells incubated in a 34°C incubator or submerged in a 39°C water bath. The results are internally consistent: they show that the parent virus replicates and plates better at 39 than at 34°C, that the mutant virus

TABLE 1. Results of marker rescue of HSV-1(mP)*ts*66.4

Fragment or plasmid used	Virus titer (PFU/ml)		Ratio (39/34°C)
	34°C	39°C	
None	3.5×10^7	$<10^2$	$<2.9 \times 10^{-6}$
*Xba*I C[a]	3.2×10^8	1.9×10^7	5.9×10^{-2}
	3.4×10^8	7.9×10^7	2.3×10^{-1}
*Hin*dIII J	1.5×10^8	5.6×10^7	3.7×10^{-1}
*Eco*RI 2	3.0×10^7	1.4×10^6	4.7×10^{-2}
*Sal*I 6	7.7×10^7	3.6×10^7	4.7×10^{-1}
*Bam*HI-*Bgl*II 4	2.5×10^7	7.4×10^6	3.0×10^{-1}
*Mlu*I 1[a]	2.4×10^8	1.8×10^7	7.5×10^{-2}
	9.5×10^7	3.0×10^7	3.2×10^{-1}
pRB4121[b]	4.7×10^7	2.4×10^7	5.1×10^{-1}

[a] Data for *Xba*I-C and *Mlu*I-1 represent repeat experiments.
[b] This plasmid contains an intact cDNA copy of the U_L15 gene (2).

TABLE 2. Plating efficiency and yield of parent, *ts* mutant, and rescued viruses at 34 and 39°C

Virus	Plating efficiency (39/34°C)		Yield[a] at 1 PFU/cell		Ratio (39/34°C)	Yield[a] at 0.1 PFU/cell		Ratio (39/34°C)
	Expt 1	Expt 2	34°C	39°C		34°C	39°C	
HSV-1(mP)	0.97	0.98	3.7×10^6	3.1×10^7	8.4	1.8×10^5	6.4×10^6	35.6
HSV-1(mP)*ts*66.4	$<6.7 \times 10^{-7}$	$<8.7 \times 10^{-7}$	6.3×10^5	1.1×10^3	1.7×10^{-3}	5.8×10^4	1.0×10^2	1.7×10^{-3}
HSV-1(mP)Ra	1.1	0.7	4.9×10^6	2.2×10^7	4.5	2.7×10^5	4.3×10^6	15.9
HSV-1(mP)Rb	0.77	0.81	6.0×10^6	2.1×10^7	3.5	3.9×10^5	5.6×10^6	14.4

[a] Yield of virus at 18 h postinfection.

replicates and plates with much lower efficiency at 39 than at 34°C, and that the rescued viruses resemble the parent virus.

The synthesis of proteins by the parent, mutant, and rescued viruses at permissive and nonpermissive temperatures. In this series of experiments, replicate 25-cm^2 cultures of Vero cells were infected in duplicate with the parent, mutant, or rescued virus (5 PFU per cell) and incubated in a 34°C incubator or submerged in a 39°C water bath. At 13 h postinfection, the cells were labeled for 1 h with [^{35}S]methionine and then harvested, solubilized, and subjected to electrophoresis in polyacrylamide gels. The results shown in Fig. 2 indicate that electrophoretic profiles of labeled proteins from cells infected and maintained at the nonpermissive temperature could not be differentiated from those extracted from cells infected and maintained at the permissive temperature.

Electron microscopic studies. The purpose of this series of experiments was to attempt to define the phenotype of the virus in cells infected and maintained at the nonpermissive temperature. Replicate Vero cell cultures were infected with the parent, mutant, or repaired virus (5 PFU per cell) and maintained at 39°C. The cells were fixed at 18 h postinfection and then sectioned, stained, and examined in a Siemens 102 electron microscope. As shown in Fig. 3, cells infected with HSV-1(mP) and maintained at 39°C contained numerous enveloped capsids containing DNA in the space between cells and in the cytoplasmic vesicles (Fig. 3A and C, respectively). In contrast, the nuclei of cells infected with HSV-1(mP)*ts*66.4 contained numerous capsids with scaffolding proteins (which form a thin ring inside the capsid) but lacking DNA. These capsids were scattered in the nucleus, and some were in apposition to the nuclear membrane (Fig. 3B and F). Empty capsids within envelopes and vesicles were also seen occasionally (Fig. 3B) but infrequently. A striking feature of cells infected with the HSV-1(mP)*ts*66.4 virus was the presence of numerous cytoplasmic vesicles lacking virus particles (Fig. 3D). Some of these vesicles resembled fragmented Golgi recently reported to be present in infected Vero cells (3). Electron micrographs of cells infected with HSV-1(mP)Ra and maintained at 39°C could not be differentiated from those of cells infected with the parent virus (Fig. 3A and E). Similar results were obtained with HSV-1(mP)Rb-infected cells. We should note that at the permissive temperature (34°C), the mutant virus could not be differentiated from wild-type virus with respect to the presence in infected cells of virions containing DNA (data not shown).

Synthesis and packaging of viral DNA. The electron microscopic studies indicated that in cells infected with HSV-1(mP)*ts*66.4 and maintained at the nonpermissive temperature capsids containing the scaffolding proteins were made and that some of the empty capsids were enveloped but that none of the capsids contained DNA. To determine whether the defect at the nonpermissive temperature was due to the absence of viral DNA or a failure to package the DNA, three series of experiments were done.

The objective of the first two series of experiments was to determine whether cells infected with the HSV-1(mP)*ts*66.4 and maintained at the nonpermissive temperature synthesize viral DNA. In the first series (Fig. 4), replicate Vero cell cultures were exposed to 10 PFU of parent, mutant, or rescued virus per cell and maintained at 34 or 39°C. The cells were harvested at 15 h postinfection. Total DNA was

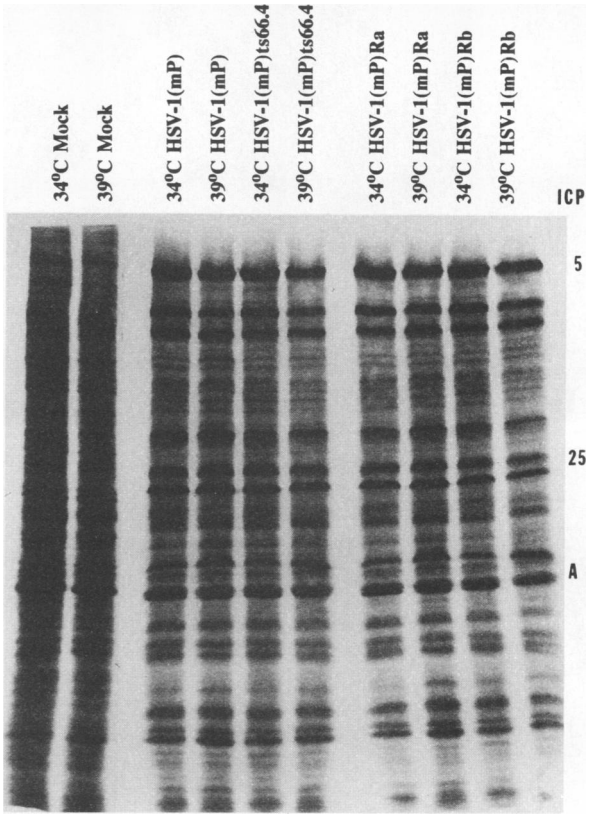

FIG. 2. Autoradiographic images of [^{35}S]methionine-labeled proteins from replicate cultures of Vero cells infected with parent, mutant, and rescued viruses and from mock-infected cells maintained at 34 or 39°C. The cells were labeled for 1 h at 13 h after infection, harvested, solubilized, electrophoretically separated on an 11% denaturing polyacrylamide gel, and subjected to autoradiography. To help identify the proteins, the major capsid protein VP5 and the tegument protein VP16 were identified by their infected-cell protein (ICP) designations (5 and 25, respectively) as described by Honess and Roizman (11) and by Morse et al. (12). A, actin.

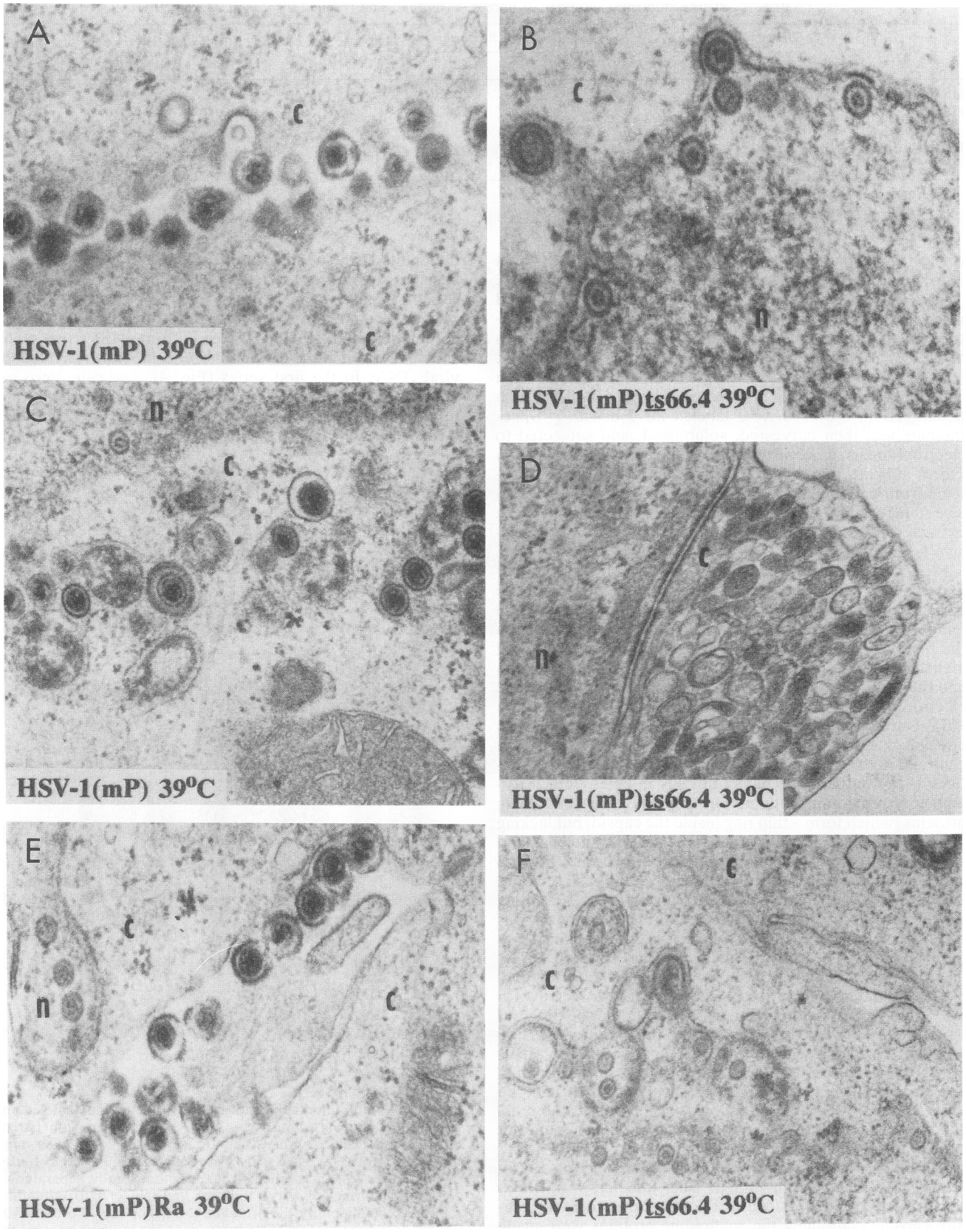

FIG. 3. Electron micrographs of replicate cultures of Vero cells infected with HSV-1(mP), HSV-1(mP)*ts*66.4, and HSV-1(mP)Ra and maintained at 39°C. The cells were harvested at 18 h postinfection. The diameter of the HSV capsid is 105 nm. c, cytoplasm; n, nucleus.

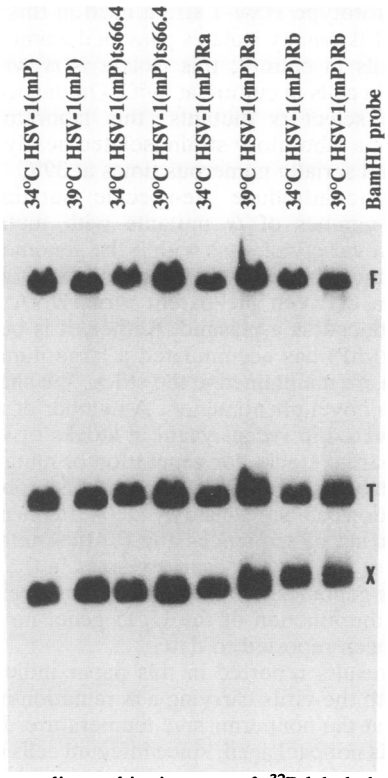

FIG. 4. Autoradiographic images of ^{32}P-labeled *Bam*HI fragments F, T, and X hybridized to electrophoretically separated *Bam*HI digests of total DNAs extracted from replicate cultures of Vero cells infected with HSV-1(mP), HSV-1(mP)*ts*66.4, HSV-1(mP)Ra and HSV-1(mP)Rb and maintained at 34 or 39°C. The infected replicate cell cultures were harvested at 15 h after infection.

extracted, digested with *Bam*HI, subjected to electrophoresis in an agarose gel, transferred to a Zeta-probe blotting membrane, and probed with ^{32}P-labeled *Bam*HI F, *Bam*HI T, and *Bam*HI X fragments. In the second series of experiments (Fig. 5), replicate Vero cell cultures were exposed to 10 PFU of parent, mutant, or rescued virus per cell and maintained at 34 or 39°C. The cells were harvested at 2 or 15 h postinfection and solubilized in nonionic detergents, and the nuclei were separated from the cytoplasm by centrifugation. The DNA extracted from each compartment was digested with *Bam*HI, subjected to electrophoresis in an agarose gel, transferred to a Zeta-probe blotting membrane, and probed with ^{32}P-labeled *Bam*HI F and *Bam*HI X fragments. The DNA detected at 2 h postinfection represents input viral DNA, since significant viral DNA synthesis does not begin until after 3 h postinfection (17). The results of both series of experiments indicate that cells infected with HSV-1(mP)*ts*66.4 produced significant amounts of viral DNA and that some of the mutant virus DNA was detected in the cytoplasm of cells maintained at the nonpermissive temperature. Moreover, analyses of the results of several experiments (not shown) suggest that the differences in amounts produced by parent, mutant, and rescued viruses seen in Fig. 4 and 5 represent experimental variability and do not reflect the mutant phenotype of HSV-1(mP)*ts*66.4.

The impetus for the third series of experiments emerged from the results of two series of studies described above. Specifically, the electron microscopic studies described above and whose results are shown in Fig. 3 indicated that at the nonpermissive temperature HSV-1(mP)*ts*66.4 DNA does not become encapsidated and enveloped. In the experiments whose results are illustrated in Fig. 5, we detected viral DNA in the cytoplasm of cells infected at the nonpermissive temperature with HSV-1(mP)*ts*66.4. To determine whether the cytoplasmic viral DNA represents DNA contained in capsids, replicate Vero cell cultures were exposed to 10 PFU

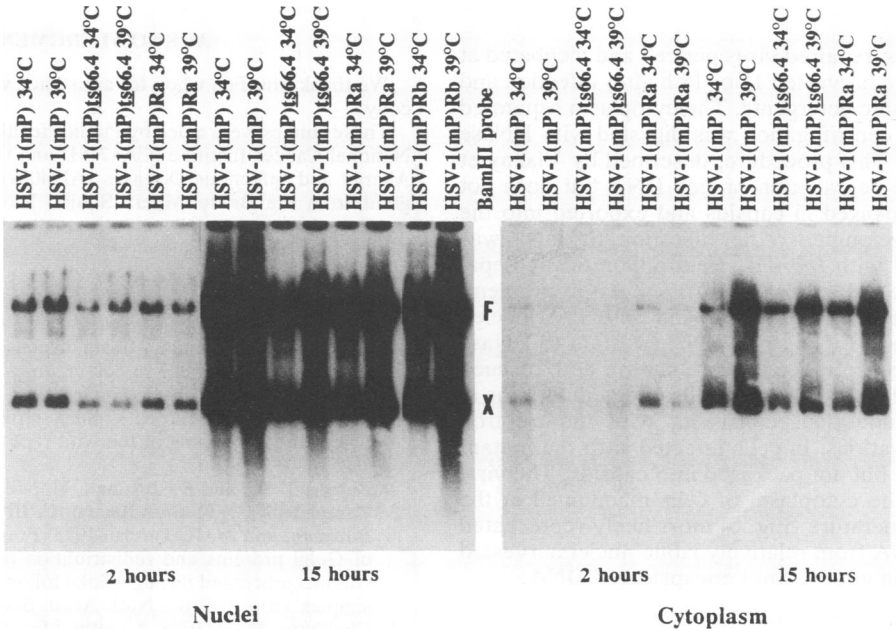

FIG. 5. Autoradiographic images of ^{32}P-labeled *Bam*HI fragments F and X hybridized to electrophoretically separated *Bam*HI digests of nuclear and cytoplasmic DNAs extracted from replicate cultures of Vero cells infected with parent, mutant, or rescued virus and maintained at 34 or 39°C. The infected replicate cell cultures were harvested at 2 and 15 h postinfection.

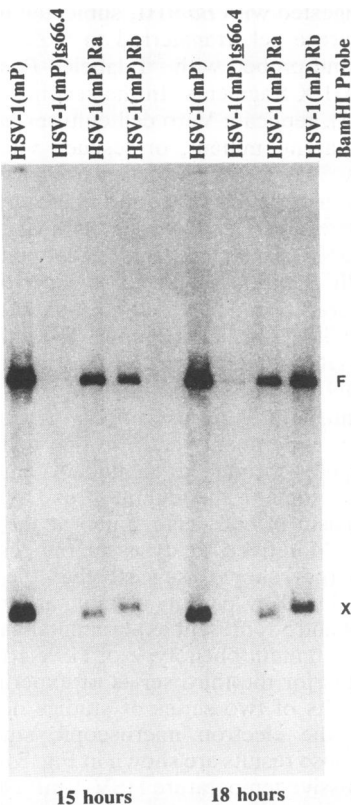

FIG. 6. Autoradiographic images of ^{32}P-labeled *Bam*HI fragments F and X hybridized to electrophoretically separated *Bam*HI digests of DNA extracted from DNase-treated cytoplasms. Replicate cultures of Vero cells were infected with HSV-1(mP), HSV-1(mP)*ts*66.4, HSV-1(mP)Ra, and HSV-1(mP)Rb, maintained at 39°C for 15 or 18 h, and then harvested in a buffer containing Nonidet P-40. The cytoplasm was then separated from the nuclei by centrifugation and digested with DNase prior to the extraction of DNA.

of parent, mutant, or repaired virus per cell and incubated at 39°C. The cells were harvested 15 or 18 h after infection and solubilized in nonionic detergents. The cytoplasm separated from the nuclei by centrifugation was digested with DNase (50 μg/ml; 15 min). This procedure, described by Vlazny et al. (19), causes the degradation of free DNA but does not affect the DNA packaged in capsids and exported into the cytoplasm after envelopment. After digestion, the DNA was extracted, digested with *Bam*HI, electrophoretically separated in an agarose gel, transferred to a Zeta-probe membrane, and hybridized with *Bam*HI-F and *Bam*HI-X labeled with ^{32}P. As shown in Fig. 6, viral DNA resistant to DNase was detected in cells infected with parent and rescued viruses but not in the cytoplasm of cells infected with the *ts* mutant. We conclude that, consistent with the electron microscopic observations, in cells infected with the mutant virus DNA is made but not packaged into capsids. The viral DNA detected in the cytoplasm of cells maintained at the nonpermissive temperature (Fig. 5) most likely represented leakage of viral DNA from relatively labile nuclei harvested at 15 h postinfection and was not encapsidated DNA.

DISCUSSION

The salient features of the results of this study are as follows.

(i) The prototype HSV-1 strain used in this laboratory is HSV-1(F). Like many isolates passaged a limited number of times in cells in culture, this isolate is *ts* with respect to replication in cells in culture at 39°C. On the occasions when we had to select *ts* mutants, this laboratory has used HSV-1(mP), a laboratory strain isolated nearly 40 years ago and passaged serially numerous times at 39°C. In the course of attempts to introduce site-specific mutations, we have isolated a number of *ts* mutants with mutations which mapped at a variety of sites within the genome. We suspect that these mutations do not represent nonhomologous recombination between the parent virus DNA and mutated DNA introduced as a plasmid. Rather, it is our impression that HSV-1(mP) has accumulated a large number of mutations which are maintained in the stock. We should note that this is not a novel phenomenon. A number of mutants have been discovered in recent years in stocks of viruses which served as parent stocks for generation of mutant viruses (8, 9). The relevant issues with respect to this report are that (a) the *ts* mutation can be rescued by DNA fragments containing one specific set of sequences and (b) the mutation maps in U_L15, one of the most conserved genes in the repertoire of herpesvirus genomes. Although there has been much speculation on the function of the U_L15 gene, no experimental data have been reported to date.

(ii) The results reported in this paper indicate that cells infected with the virus carrying a *ts* mutation in U_L15 make viral DNA at the nonpermissive temperature. However, the viral DNA is not packaged, since infected cells maintained at the nonpermissive temperature do not contain capsids with DNA. Moreover, whereas the cytoplasm of cells infected with wild-type and rescued viruses contained DNase-protected viral DNA, such DNA was not present in cells infected with the mutant virus.

We conclude that U_L15 protein as deduced from the phenotype of HSV-1(mP)*ts*66.4 is involved in the packaging of viral DNA. The isolation of this mutant opens the way to detailed studies of its function.

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