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Herpes Simplex Virus 1 Mutant Deleted in the α22 Gene: Growth and Gene Expression in Permissive and Restrictive Cells and Establishment of Latency in Mice

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R325-βTK$^+$, a herpes simplex virus 1 mutant carrying a 500-base-pair deletion in the α22 gene and the wild-type (β) thymidine kinase (TK) gene, was previously shown to grow efficiently in HEp-2 and Vero cell lines. We report that in rodent cell lines exemplified by the Rat-1 line, plating efficiency was reduced and growth was multiplicity dependent. A similar multiplicity dependence for growth and lack of virus spread at low multiplicity was seen in resting, confluent human embryonic lung (HEL) cells. The shutoff of synthesis of β proteins was delayed and the duration of synthesis of γ proteins was extended in R325-βTK$^+$-infected HEL cells relative to cells infected with the wild-type parent, but no significant differences were seen in the total accumulation of viral DNA. To quantify the effect on late (γ_2) gene expression, a recombinant carrying the deletion in the α22 gene and a γ_2-TK gene (R325-γ_2TK) was constructed and compared with a wild-type virus (R3112) carrying a chimeric γ_2-TK gene. In Vero cells, the γ_2-TK gene of R325-γ_2TK was expressed earlier than and at the same level as the γ_2-TK gene of R3112. In the confluent resting HEL cells, the expression of the γ_2-TK gene of the α22$^-$ virus was grossly reduced relative to that of the α22$^+$ virus. Electron microscopic studies indicated that the number of intranuclear capsids of R325-βTK$^+$ virus was reduced relative to that of the parent virus in resting confluent HEL cells, but the number of DNA-containing capsids was higher. Notwithstanding the grossly reduced neurovirulence on intracerebral inoculation in mice, R325-βTK$^+$ virus was able to establish latency in mice. We conclude that (i) the α22 gene affects late (γ_2) gene expression, and (ii) a host cell factor complements that function of the α22 gene to a greater extent in HEp-2 and Vero cells than in confluent, resting HEL cells.

In cells lytically infected with herpes simplex virus 1 (HSV-1), the first genes to be expressed are a set of five genes designated α0, α4, α22, α27, and α47 (10, 11, 16, 22, 23, 32). Numerous studies have established that a functional α4 product is essential for the transition from α to β and γ gene expression (3, 11, 20, 40). The function of other α genes is not well understood, largely because conditional mutants in those genes have been lacking. On the assumption that the function of at least some α genes may not be necessary in cultured cells, Post and Roizman (28) devised a two-step procedure for the generation of deletions in nonessential domains of large DNA genomes and applied this procedure to the construction of recombinant genomes carrying deletions in the α22 gene. The first step of the procedure consisted of cotransfecting cells with the genome of a recombinant [HSV-1(F)Δ305] carrying a 700-base-pair (bp) deletion in the thymidine kinase (TK) gene, and a chimeric fragment in which the TK gene was inserted into the middle of the α22 gene. The progeny of the transfection were selected for TK$^+$ phenotype and screened for recombinants carrying the TK gene inserted in the α22 gene. In the second step, the recombinant genomes were cotransfected with DNA fragments carrying the α22 gene from which 100 or 500 bp were deleted at the site of the TK insertion. The progeny were selected for TK$^-$ phenotype. Both the recombinant carrying the 100-bp deletion (R328TK$^-$) and the recombinant carrying the 500-bp deletion (R325TK$^-$) grew as well as the parent virus in two cell lines of human (HEp-2) and nonhuman primate (Vero) cells. However, it was noted that these viruses grew poorly in a continuous line of rabbit skin cells in which all the transfections were routinely done (L. E. Post and B. Roizman, unpublished data).

The R325TK$^-$ and R328TK$^-$ recombinants described initially carried two deletions, one in the TK gene and one in the α22 gene. To investigate the function of the α22 gene and especially the differential growth of these viruses in fully permissive HEp-2 and Vero cells and in restrictive human embryonic lung (HEL) cells, the deleted sequences in the TK gene were restored by transfecting the genomes of these recombinants with DNA fragments containing the intact TK gene. The resulting TK$^+$ recombinants carrying deletions in the α22 gene only were designated as R325TK$^+$ and R328TK$^+$, respectively (Post and Roizman, unpublished data). In this paper we report on the characterization of R325TK$^+$ in several cell lines in which its growth is restricted and in experimental animals.

Relevant to the results presented in this report is the general pattern of gene expression in HSV-1-infected cells. The HSV-1 genes form three groups, α, β, and γ, whose expression is coordinately regulated and sequentially ordered in a cascade fashion (10, 11). The β and γ groups are heterogeneous. Whereas β_1 genes (e.g., $\beta_1$8, specifying the major DNA binding protein) and β_2 genes (e.g., $\beta_2$36, specifying the TK gene) differ in the kinetics of synthesis of their proteins (26, 35), the γ_1 (designated also as βγ; 8) and γ_2 (designated also as true γ genes; 8) are more readily

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TABLE 1. Efficiency of plating in various cell lines of viruses grown in Vero cells[a]

Cell line[b]	HSV-1 (F) (A)	R325-βTK+ (B)	Ratio A/B
Vero	4.2×10^8	1.9×10^8	2.2
HEp-2	5.3×10^8	0.95×10^8	5.6
RSC	2.9×10^8	0.23×10^8	13
BHK	2.2×10^8	6.5×10^5	15
Rat-1	1.0×10^6	1.9×10^4	53

[a] Cell cultures were infected at low multiplicities and incubated for several days in medium 199-O. Infected monolayers were fixed and stained with Giemsa, and plaques were counted. The apparent titer of each virus in each cell line is shown. A/B is the ratio of the titers of HSV-1(F) to R325-βTK+ in the cell line indicated.

differentiated on the basis of their dependence on viral DNA synthesis (2, 12, 17). Unlike the γ_1 genes (e.g., $\gamma_1$5, the major capsid protein), the γ_2 genes, exemplified by the glycoprotein C gene, are stringently dependent on viral DNA synthesis for their expression.

MATERIALS AND METHODS

Cells and viruses. Vero, HEp-2, and HEL cells were obtained from the American Type Culture Collection. Rat-1 cells were originally obtained from W. C. Topp (39). Resting HEL cells were infected 12 to 15 days after reaching confluency on the dish; the medium was not replenished during this period.

The properties of HSV-1(F), the isolate used as a prototype HSV-1 strain, have been described elsewhere (4). R325TK− contained a 500-bp deletion in the α22 gene and a 700-bp deletion in the TK gene (28). R325-βTK+ was constructed by restoring the DNA sequences deleted in the TK gene. R3112 contains a chimeric γ_2-TK gene constructed by the insertion of the γ_2 promoter in *Bam*HI D′ (5) into the *Bgl*II site of the TK leader sequence such that TK and the γ_2 promoter are in the same transcriptional orientation (37). R325-γTK+ was constructed by coinfecting Vero cells with both R325-βTK+ and R3112 each at a multiplicity of 5 PFU per cell. The cells were harvested 24 h after infection, and individual plaques were picked from infected Vero cell monolayers. DNA of these progeny was screened by restriction enzyme analysis for the presence of both the insertion of γ_2 regulatory sequences into the TK gene present in R3112 and the deletion in the α22 gene of R325-βTK+. An isolate with the expected characteristics was further plaque purified before use.

Growth assays. (i) Vero or HEL cells. Confluent monolayers were infected at multiplicities of 0.001, 0.01, or 1.0 PFU per cell. After adsorption for 1 h at 37°C, the cells were washed three times with medium 199-O (mixture 199 supplemented with 1% calf serum and 0.01% human immunoglobulin G) to neutralize and remove unadsorbed virus, then washed three times with medium 199-V (mixture 199 supplemented with 1% calf serum) to remove residual antibody, and incubated in medium 199-V at 34°C. At 3, 24, 48, or 72 h after infection, the infected cells and media were harvested, and the virus was titrated on Vero cell monolayers.

(ii) Rat-1 cells. Confluent monolayers were infected at multiplicities of 0.02 or 20 PFU per cell. After adsorption for 1 h, the cells were washed three times with medium 199-V and incubated at 37°C in 199-V. Cells infected at high multiplicity were harvested 24 h after infection. Cells infected at low multiplicity were harvested 4 days after infection, when infected with α22+ viruses, or 6 days after infection when infected with α22− mutants. Virus stocks were titrated on the cells indicated.

Staining of plaques by peroxidase-coupled antibody. Plaques were stained as described (21) using anti-HSV(MP) rabbit antiserum and peroxidase-coupled goat anti-rabbit immunoglobulin G (Miles Laboratories, Naperville, Ill.).

TK assays. The assays for TK activity were performed on cytoplasmic extracts of infected and uninfected cells as described (27). The HSV-TK-specific substrate (38) consisted of 0.5 μCi of [^{125}I]iododeoxycytidine (New England Nuclear Corp., Boston, Mass.; 2,200 Ci/mmol) per reaction, with no cold substrate added. Tetrahydrouridine (30 μg/ml) was included in the assays to inhibit cellular deaminases (38).

Labeling and electrophoresis of infected-cell polypeptides. Confluent monolayers of Vero or HEL cells were infected at a multiplicity of 5 PFU per cell. After adsorption for 1 h at 37°C, the inoculum was removed and replaced with medium 199-V, and incubation was continued at 34°C. Labeling was for 1 h at the times indicated in medium 199-V containing 1/20 the normal amount of methionine and 80 μCi of [^{35}S]methionine per ml of medium (800 Ci/mmol; New England Nuclear Corp.). Infected-cell lysates were prepared and subjected to electrophoresis on denaturing 9.25% sodium dodecyl sulfate-polyacrylamide gels as described (24).

DNA dot-blot hybridizations. Whole-cell DNA from infected and uninfected L314 and HEL cells was extracted, alkali treated, and spotted onto nitrocellulose as described (25). After baking for 2 h at 80°C, the filters were hybridized to nick-translated probes.

Infection of mice and assays for latent virus. Four- to 5-week-old BALB/C AnN mice were inoculated after anaesthetization by intraperitoneal injection of sodium pentobarbitol. For eye inoculation, 10 μl of virus suspension was dropped onto the eye after scarification of the cornea. For ear inoculation, approximately 10 μl of virus suspension was injected subcutaneously into the pinna of the ear. Ipsilateral trigeminal ganglia or cervical dorsal root ganglia were harvested 4 weeks after infection, incubated in medium for 4 to 5 days to reactivate latent virus, and then homogenized and plated on Vero cell monolayers. Cervical dorsal root ganglia were pooled for recovery of virus. Negative cultures were frozen and replated on Vero cells to recover low quantities of virus. At no time could replicating virus be recovered from ganglia during this period.

RESULTS

Growth of α22 deletion mutants in primate and nonprimate cell lines. At the time of isolation of the α22 deletion mutants

TABLE 2. Dependence of virus growth on multiplicity of infection[a]

Virus	20 PFU		0.02 PFU		Ratio; (B × 100)A[c]
	Yield[b] (A)	% Yield	Yield[b] (B)	% Yield	
HSV-1(F)	1.5×10^8	100	1.2×10^6	100	0.80
R325-βTK+	1.1×10^7	7	1.3×10^3	0.11	0.01

[a] Rat-1 cells were infected with HSV-1(F) or R325-βTK+ at multiplicities of 0.02 or 20 PFU per cell as described in the text. The infected cell cultures were harvested and titrated in Vero cells.

[b] Relative to the yield of HSV-1(F) at an input multiplicity of 20 or 0.02 PFU per cell.

[c] Yield of virus at a multiplicity of 0.02 PFU per cell × 100, divided by the yield at a multiplicity of 20 PFU per cell.

TABLE 3. Growth of HSV-1(F) and R325-βTK$^+$ in actively growing HEL cells[a]

Multiplicity (PFU per cell)	Time (h) postinfection	Titer of HSV-1(F)	Titer of R325-βTK$^+$
0.01	3	$<10^1$	$<10^1$
	24	2.6×10^4	1.1×10^3
	48	4.2×10^5	6.8×10^2
	72	4.3×10^6	6.3×10^3
1.0	3	1.9×10^3	2.3×10^2
	24	2.1×10^7	1.8×10^5
	48	1.3×10^5	5.0×10^3
	72	1.1×10^5	1.8×10^3

[a] HEL cell monolayers were infected as soon as they reached confluency, harvested 3, 24, 48, or 72 h postinfection, and titrated in Vero cells.

R325TK$^-$ and R328TK$^-$, it was noted that the plaque production and yields in rabbit skin cells were significantly lower than those observed in infected Vero cell cultures. To facilitate assessment of the defect in growth attributable directly to the deletion in the α22 gene, we have focused primarily on the R325 recombinant converted to TK$^+$ phenotype by marker rescue (Post and Roizman, unpublished data). This recombinant is designated as R325-βTK$^+$ to differentiate this virus, carrying the natural (β) TK gene, from other constructs described later in the text.

The conclusion to be drawn from the results obtained to date is that R325-βTK$^+$ has an impaired capacity to grow and form plaques in some cell lines but not others.

The efficiency of plating of R325-βTK$^+$ was reduced relative to that of the parent HSV-1(F) in nearly all cell lines tested, with the exception of Vero and HEp-2 cells (Table 1). When both the parent virus and R325-βTK$^+$ showed a reduced plating efficiency, as in the case of Rat-1 cells, the reduction was greater for R325-βTK$^+$ than for the HSV-1(F) parent.

The growth impairment is best illustrated in the restrictive Rat-1 cells. In these cells, the virus yield was dependent on the multiplicity of infection. A 100-fold decrease in the multiplicity of infection with HSV-1(F) was reflected in a nearly 100-fold decrease in yield (Table 2). Whereas the yield of R325-βTK$^+$ was lower than that of the HSV-1(F) parent at both multiplicities of infection, the difference was considerably greater at the lower multiplicity. These results suggest that in some cell lines the yield of R325-βTK$^+$ is multiplicity dependent, i.e., it reflects a cooperativity among infecting virions, and that in the absence of such an effect, as in the

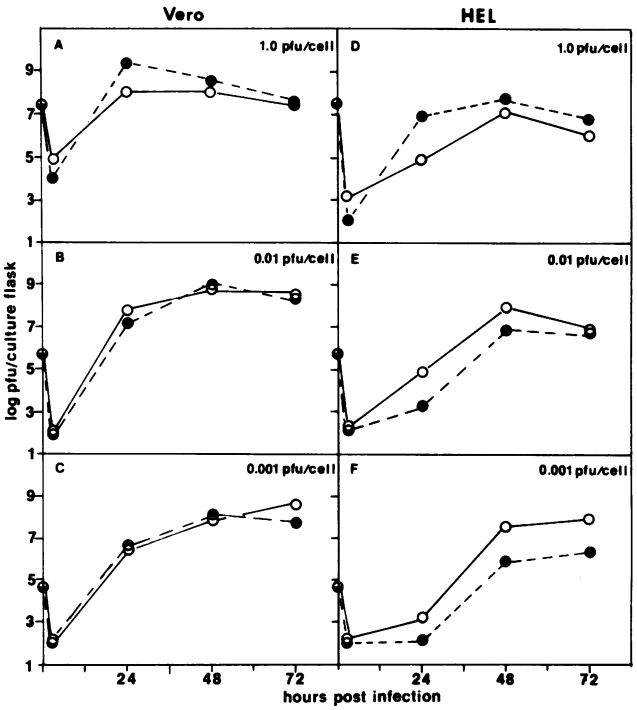

FIG. 2. Growth curves of R325-βTK$^+$ and HSV-1(F) in Vero and confluent, resting HEL cells. HEL cells were infected with parental or recombinant virus at multiplicities of 0.001, 0.01, or 1.0 PFU per cell and incubated at 34°C. At 3, 24, 48, or 72 h after infection, the cultures were harvested and titrated in Vero cells. Solid circles, R325-βTK$^+$; open circles, HSV-1(F).

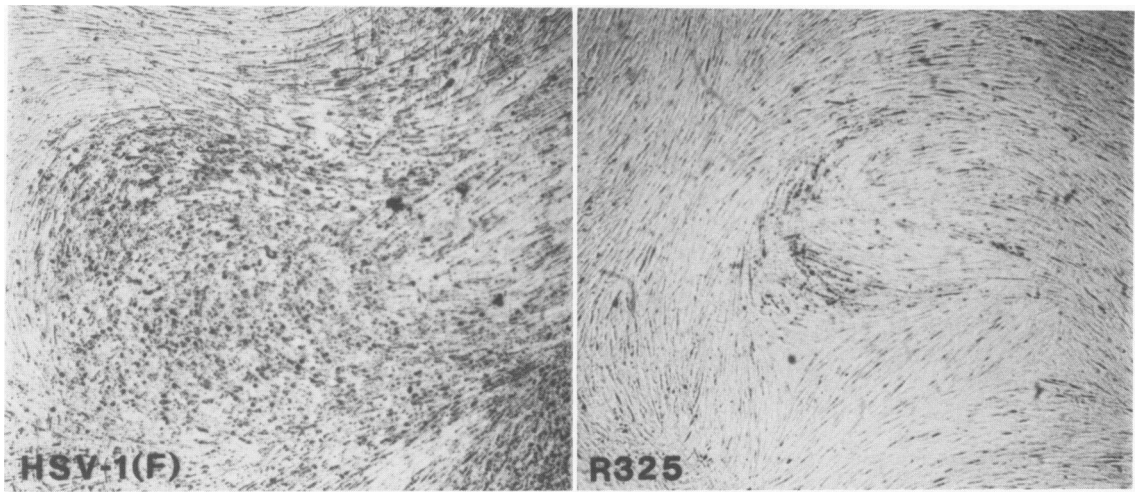

FIG. 1. Confluent, resting HEL cells infected with R325-βTK$^+$ or HSV-1(F) and stained by a peroxidase-coupled immune assay. HEL cells were infected at low multiplicities and incubated for 5 to 6 days at 34°C. The monolayers were then washed, incubated with an anti-HSV(MP) rabbit antiserum, and stained as described (21), using peroxidase-coupled goat anti-rabbit immunoglobulin G in an immune staining reaction.

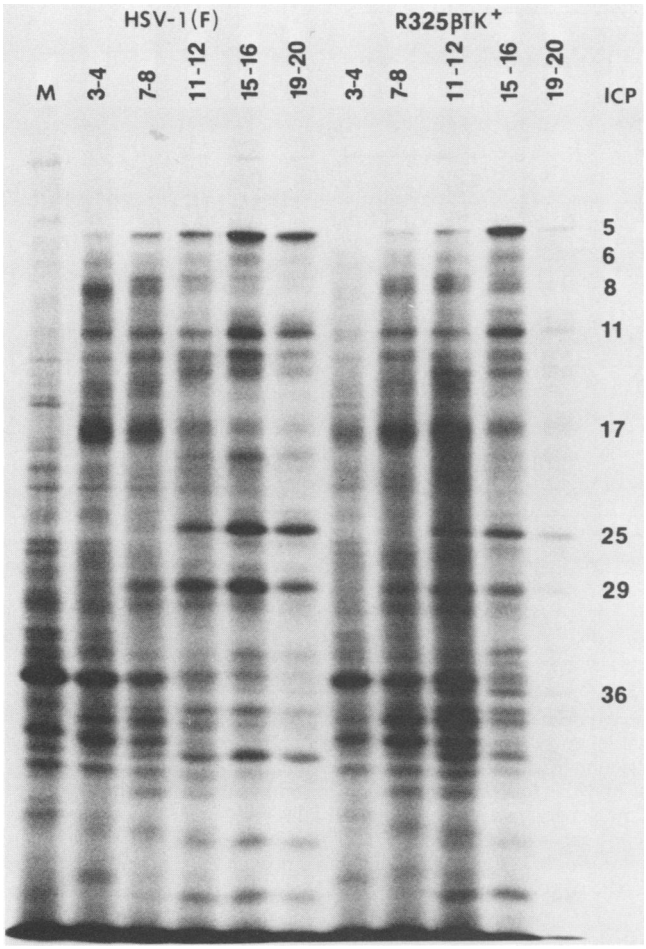

FIG. 3. Autoradiographic image of lysates of Vero cells infected with R325-βTK$^+$ or HSV-1(F), electrophoretically separated on a denaturing 9.25% polyacrylamide gel. Cells were infected at a multiplicity of 5 PFU per cell, labeled with [^{35}S]methionine for 1 h at 3, 7, 11, 15, or 19 h after infection, and harvested immediately after labeling. Infected-cell polypeptides (ICP) are numbered on the right according to the nomenclature of Honess and Roizman (9). Labeling periods are indicated at the top of the figure. M, Mock-infected cells.

case of plaque assays, the probability that sufficient viral progeny will emerge to initiate a plaque is lower for R325-βTK$^+$ than for the wild-type parent.

The example cited above involved cells relatively restrictive even for wild-type virus. Decreased yields and decreased plating efficiency were also noted in permissive cells such as rabbit skin cells, baby hamster kidney cells, and human MRC5 cells.

Growth of R325-βTK$^+$ virus in resting, confluent HEL cells. The experiments described in the preceding section suggested that the primate cell lines HEp-2 and Vero contained factors that complemented the function of the α22 gene, whereas the nonprimate lines tested lacked such factors. To determine whether the putative factors were present only during active cell growth, we tested the growth of the virus in HEL cell cultures. Actively growing HEL cells were found to be somewhat restrictive for the growth of R325-βTK$^+$ (Table 3); however, the results were not consistent from one experiment to the next and seemed to depend to some degree on the age of the cell cultures. To eliminate variability and obtain consistent results, in all of the experiments described below the HEL cell cultures were infected 12 to 14 days after reaching confluency. In this resting state the cells were consistently and reproducibly permissive for the wild-type parent HSV-1(F) but restrictive for the growth of the R325 virus recombinants tested in these studies.

The striking feature of the results of infection of confluent, resting HEL cells with HSV-1(F) and R325-βTK$^+$ was that whereas the parent HSV-1(F) formed plaques and grew in the resting cultures, no discernible plaques were seen in cultures infected with R325-βTK$^+$ virus and stained with Giemsa stain. When stained by the peroxidase-coupled antibody technique using anti-HSV antiserum, small clusters of infected cells could be seen, but the virus spread very slowly upon continuous incubation (Fig. 1).

Figure 2 shows growth curves obtained by the infection of Vero and confluent, resting HEL cells with HSV-1(F) and R325-βTK$^+$. At multiplicities of 1 PFU per cell in both Vero and HEL cultures, R325-βTK$^+$ virus attained slightly higher titers than HSV-1(F) by 24 h after infection. This was consistent with previous observations that R325-βTK$^+$ virus grew as well as, if not better than, wild-type virus in Vero

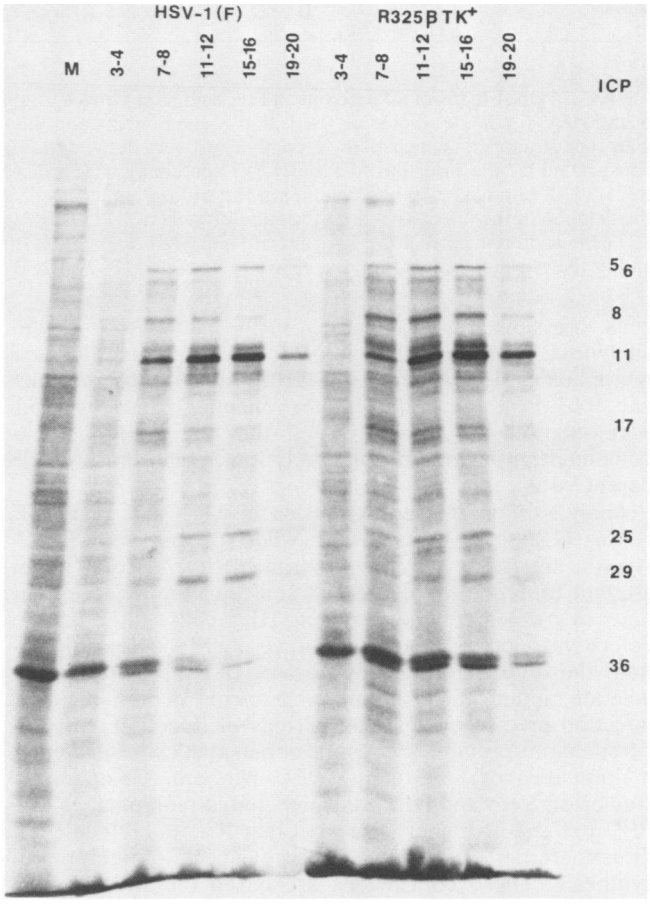

FIG. 4. Autoradiographic image of lysates of resting, confluent HEL cells infected with R325-βTK$^+$ or HSV-1(F) at a multiplicity of 5 PFU per cell. Infected cell cultures were labeled with [^{35}S]methionine for 1 h at 3, 7, 11, 15, or 19 h after infection, harvested immediately after labeling, and electrophoresed on a denaturing 9.25% polyacrylamide gel. Infected-cell polypeptides (ICP) are numbered according to the nomenclature of Honess and Roizman (9). Labeling periods are indicated at the top of the figure. M, Mock-infected cells.

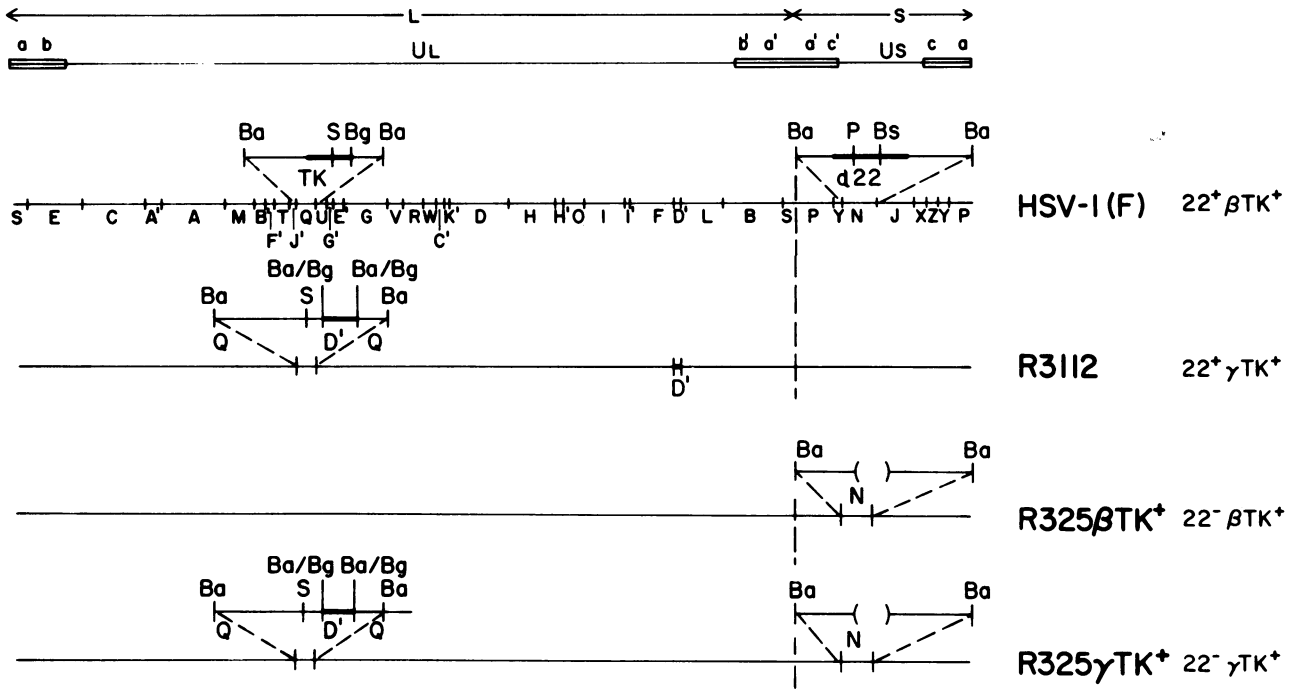

FIG. 5. DNA maps of all parental and recombinant viruses used in these experiments. The top two lines detail the sequence arrangement of the HSV-1 genome, including the L and S components and the terminal inverted reiterated sequences (ab-b'a'a'c'-ca). HSV-1(F) is the wild-type strain from which all the recombinants were derived. *Bam*HI restriction fragments are labeled. R3112 carries a chimeric γ_2-TK gene constructed by the insertion of *Bam*HI D', containing a γ_2 promoter (5), into the *Bgl*II site in the leader sequence of the TK gene (37). R325-βTK$^+$ contains a 500-bp deletion in the coding sequences of the α22 gene and a wild-type β-regulated TK. R325-γTK$^+$ contains both the deletion in the infected-cell polypeptide 22 gene of R325-βTK$^+$ and the γ_2-regulated TK gene of R3112. Presence or absence of an intact α22 gene and regulation of the TK gene are indicated on the right of the figure. *Bam*HI N and D' are indicated both in their normal positions and in the expanded diagrams. Ba, *Bam*HI; Bg, *Bgl*II; P, *Pvu*II; Bs, *Bst*EII; S, *Sac*I.

cells. No substantial differences in the patterns of growth and virus yield were seen in Vero cells infected at lower multiplicities. In HEL cells infected at low multiplicities (0.1 and 0.01 PFU per cell), the exponential phase of virus accumulation was delayed, and the final yields of recombinant virus were considerably lower than those of the parent virus.

Analysis of viral gene expression in Vero and confluent, resting HEL cells infected with wild-type and R325-βTK$^+$ viruses. Several series of experiments were done in an attempt to define the decreased ability of the deletion mutants to grow in confluent, resting HEL cells. Although the defect was more pronounced at lower multiplicities of infection, the differences in growth rates of the wild-type and deletion mutants made it impossible to control multiplicity of infection precisely over long periods of infection and rendered uninterpretable the results obtained in cells infected at multiplicities of less than 1 PFU per cell. Comparative studies on Vero and HEL cells infected at multiplicities of 5 PFU per cell or higher suggested that R325β-TK$^+$ deletion viruses are defective in one or more aspects of late protein synthesis. These conclusions are based on the following experiments.

(i) General patterns of protein synthesis in HEL and Vero cells infected with HSV-1(F) and R325-βTK$^+$ differed in two significant ways (Fig. 3 and 4). First, while infection was essentially complete 24 h after infection of either cell line with HSV-1(F), there was still considerable synthesis of viral proteins in R325-βTK$^+$-infected HEL cells at that time. Second, there was also a delay in the shutoff of synthesis of β proteins (β8, 17, 36) in HEL cells infected with R325-βTK$^+$. This delay was less pronounced in infected Vero cells.

(ii) The delay in shutoff of β protein synthesis and the extended synthesis of viral gene products late in infection could have been a reflection of a decreased rate of viral DNA synthesis by the recombinant virus. Dot-blot analysis of DNA extracted from confluent, resting HEL cells infected with parental HSV-1(F) and recombinant R325-βTK$^+$ failed to establish a significant difference in the levels of accumulation of viral DNA (data not shown).

(iii) To test directly and quantitatively the effectiveness of late protein synthesis in the absence of functional α22, we made use of a recombinant, R3112, which carries a TK gene linked to a γ_2 promoter-regulator sequence (37). The TK gene resident in this virus was expressed as a γ_2 gene in that the accumulation of the enzyme in infected cells was blocked by inhibitors of DNA synthesis. To measure the effect of the deletion in the α22 gene on the expression of an exemplary γ_2 gene, the R3112 recombinant was crossed with R325-βTK$^+$, and recombinant R325-γTK$^+$, carrying the γ_2-TK gene and the deletion in α22 gene, was isolated (Fig. 5). The parent virus, HSV-1(F), and recombinants R3112, R325-βTK$^+$, and R325-γTK$^+$ were used to infect Vero and resting, confluent HEL cells at multiplicities of 5 PFU per cell. TK assays of the infected cells are shown in Fig. 6. Several significant differences can be seen. First, whereas βTK was expressed equally well in Vero cells by the α22$^+$ and the α22$^-$ viruses, γ_2-TK was expressed earlier by the α22$^-$ virus in these cells both in this and in a repeated experiment. Second, βTK was overexpressed in HEL cells by the α22$^-$ mutant, which shut off synthesis of βTK later and accumulated a higher final

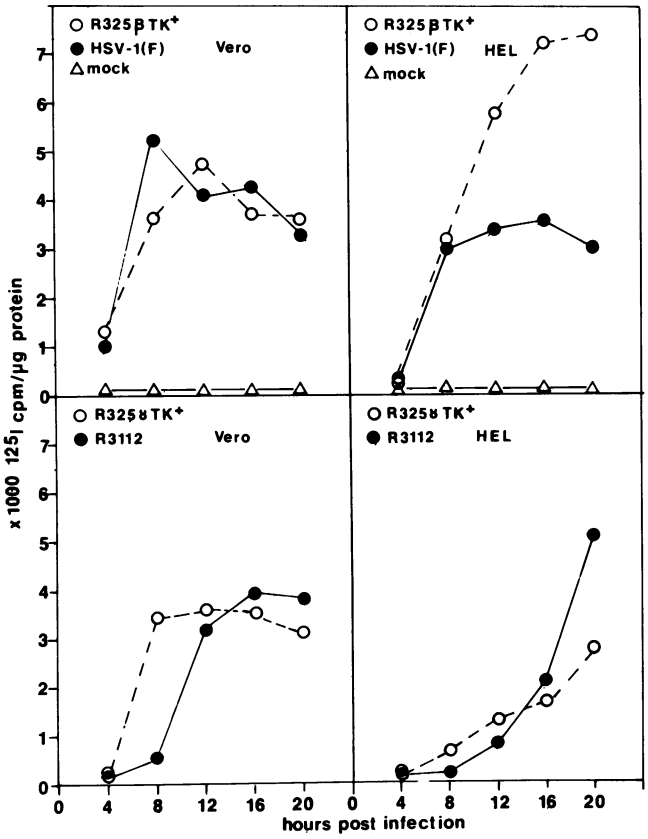

FIG. 6. TK assays of Vero and confluent, resting HEL cells infected with HSV-1(F), R325-βTK+, R3312, or R325-γ2TK+. Cells were infected at a multiplicity of 5 PFU per cell and harvested 4, 8, 12, 16, or 20 h after infection. TK assays were done on cytoplasmic extracts of infected and mock-infected cells, using [125I]iododeoxycytidine as an HSV-TK-specific substrate (38).

level of TK than did wild-type HSV-1(F). Finally, γ2-TK was underproduced by the α22− recombinant R325-γ2TK+ as compared to its α22+ counterpart R3112. Thus, in the experiment shown, the α22− mutant had accumulated only 50% of the level of TK of the α22+ virus 20 h postinfection. This underproduction of γ2-TK by R325-γ2TK+ was even more pronounced in a repeated experiment (data not shown) in which R3112 accumulated four times as much TK as R325-γ2TK+ 24 h after infection.

Electron microscopic studies of infected resting, confluent HEL cells. The experiments described in the preceding section indicated that in confluent, resting HEL cells infected with recombinants carrying a deletion in the α22 gene there was a reduction in the expression of an exemplary γ2 gene, γ2-TK, as compared to the levels produced by α22+ virus. Since this effect could have been specific for the γ2-TK gene, due to a manifestation of some selective effect on the promoter-regulatory domain used in the construction of the surrogate γ2 gene, we examined thin sections of resting, confluent HEL cells infected with HSV-1(F) or with R325-βTK+ for evidence of obvious defects in replication.

In general, the pattern of infected resting HEL cells differed from that of infected Vero or HEp-2 cells with respect to several aspects of virus accumulation. The more striking features were the rapid transfer of virus from the nucleus to the infected cell surface and the relative absence of unenveloped nucleocapsids from the cytoplasm of infected cells, even late after infection (Fig. 7). HEL cells infected with HSV-1(F) or R325-βTK+ differed primarily with respect to the total number of capsids and the fraction of DNA-containing capsids in the nuclei of infected cells. Although the average number of capsids per nucleus in R325-βTK+-infected cells was decreased relative to HSV-1(F)-infected cells, the fraction of capsids containing DNA was increased (Table 4). Furthermore, the number of nuclei containing very few (none to five) capsids was considerably higher in R325-βTK+-infected cells than in those cells infected with HSV-1(F) (Fig. 8). These data are consistent with the hypothesis that the defect of the α22 deletion is reflected in late protein synthesis.

Behavior of R325-βTK+ in mice. Studies reported elsewhere (36) have shown that the lethal dose of R325-βTK+ required to kill 50% of BALB/C mice given virus intracerebrally was $10^{6.9}$ to $10^{7.0}$ PFU, as compared to about 70 to 100 PFU of HSV-1(F).

To test for the ability to establish latent infections in mice, BALB/C mice were inoculated with HSV-1(F) or R325-βTK+ either in the pinna of the ear or in the eye as described above. Whereas HSV-1(F) established latent infection when administered by either route, R325-βTK+ was effective only by the eye route (Table 5). Specifically, R325-βTK+ virus was recovered from 50% of trigeminal ganglia ipsilateral to the inoculated eye, but could not be recovered from cervical dorsal root ganglia of any of the animals inoculated in the ear.

DISCUSSION

The 5′ regulatory domain of the α22 gene encompasses one of the two S-component origins of DNA synthesis (31). The gene specifies a protein migrating in polyacrylamide gels with an apparent molecular weight of 72,000 (9, 28). α22 protein accepts ^{32}P and accumulates in the nucleus (41). Beyond this limited set of facts, very little is known regarding its structure and function.

The studies reported in this paper stemmed from an observation that R325 recombinants carrying a deletion of 500 bp (28) grew well in HEp-2 and Vero cells but poorly in a rabbit skin cell line (Post and Roizman, unpublished data). The salient features of the results reported in this paper are as follows.

(i) R325-βTK+ exhibits a diminished plating efficiency and a greater dependence on high multiplicity of infection for optimal yields in rodent cell lines than its wild-type parent, HSV-1(F). The observed disparity of its growth properties in

TABLE 4. Accumulation of capsids in resting HEL cells infected with wild-type and R325-βTK+ virus[a]

Virus	Time (h) postinfection	No. of nuclei counted	No. of total capsids	No. of full capsids	Ratio: capsids/nucleus		% Full capsids
					Total capsids	Full capsids	
HSV-1(F)	24	34	817	349	24	10	43
	48	29	1,004	371	34	13	37
R325-βTK+	24	34	198	108	6	3	55
	48	36	227	11	6	3	53

[a] HEL cells were infected at a multiplicity of 10 PFU per cell and incubated at 34°C. At 24 or 48 h after infection, the cells were fixed, and thin sections were prepared and examined by electron microscopy. Both the total number of capsids and the number of DNA-containing capsids per nucleus were counted in representative samples of nuclei.

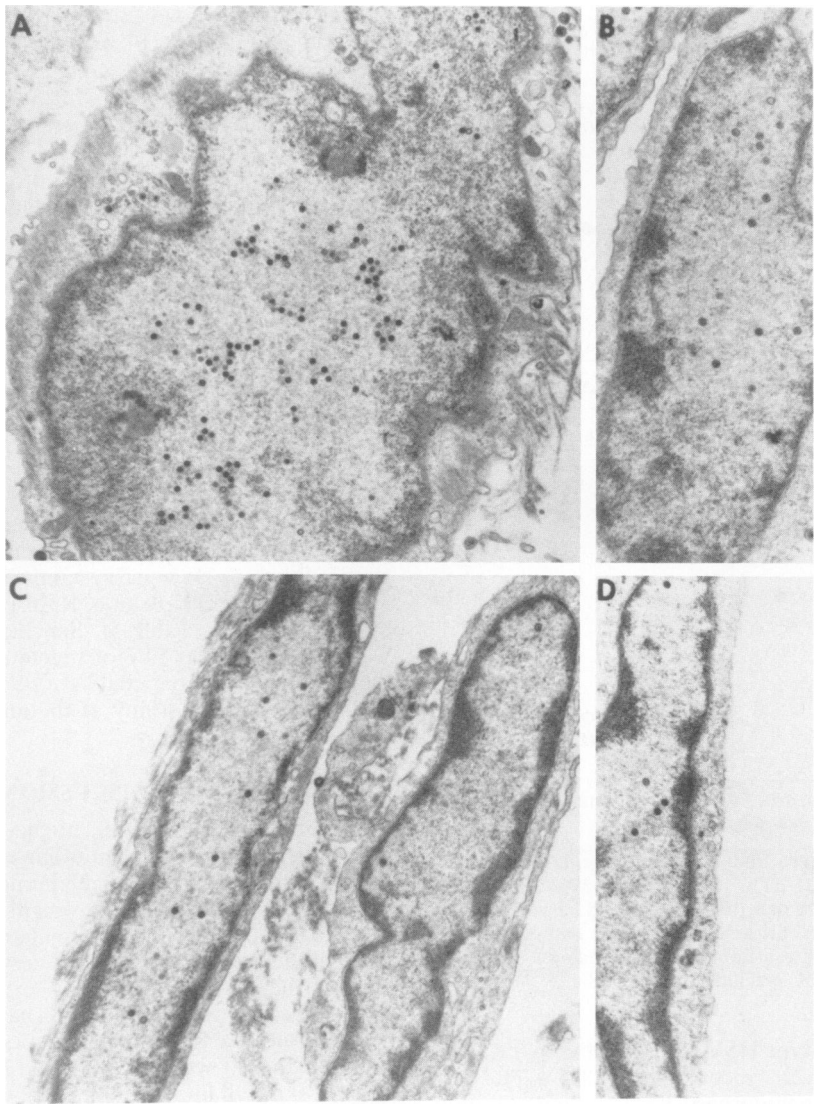

FIG. 7. Electron micrographs of resting, confluent HEL cells infected with R325-βTK$^+$ or HSV-1(F). Cells were infected at a multiplicity of 10 PFU per cell and incubated at 34°C. At 24 or 48 h after infection, the cells were harvested and thin sections were prepared. (A) HSV-1(F), 24 h; (B) HSV-1(F), 48 h; (C) R325-βTK$^+$, 24 h; (D) R325-βTK$^+$, 48 h.

various cell lines and at least one human cell strain (HEL) suggests that a host factor can substitute for gene 22 with various degrees of efficiency.

(ii) Analyses of the growth properties of the virus in HEL cells suggest that the lack of growth of the α22$^-$ viruses can be attributed to a lack of late viral gene products. This underproduction of the γ_2-TK chimera used as a surrogate late gene was apparent even at the relatively high multiplicities of infection used in these experiments and might be expected to be more pronounced at lower multiplicities. The observed results correlated with the diminished accumulation of capsids in cells infected with the R325-βTK$^+$ recombinant.

(iii) Unlike adenoviruses and papovaviruses, herpesviruses encode many of the enzymes required for viral DNA synthesis (1, 2, 6, 7, 13, 14, 18, 19, 29, 30). Most of these enzymes (e.g., DNA polymerase, TK, and ribonucleotide reductase) are counterparts of cellular enzymes but have different substrate specificities and regulatory properties (14, 15, 33). Some host enzymes (e.g., TK) can complement, in part, viral requirements for these functions. If, for the purpose of developing this model further, we entertain the hypothesis that the α22 function can be replaced by a host factor produced in some cell lines, then it follows that the Vero cell factor which complements the function of α22 may in fact be more efficient or be regulated differently than the authentic α22 protein. This conclusion is based on the observation that in cells which can complement α22, and are presumably producing this putative factor, γ_2-TK was expressed earlier in the absence of α22 than in its presence.

(iv) The site of action of the α22 protein is not clear. The decrease in the production of late (γ_2) proteins may well be a reflection of a defect in some preceding step of the cascade regulating HSV-1 protein synthesis. The failure to document a significant decrease in the amount of viral DNA accumulating late in infection in cells infected with R325-βTK$^+$ does not preclude the possibility that the decrease in late gene expression reflects a defect in viral DNA synthesis. Studies to define the characteristics of R325-βTK$^+$ DNA synthesis in permissive and restrictive cells are in progress.

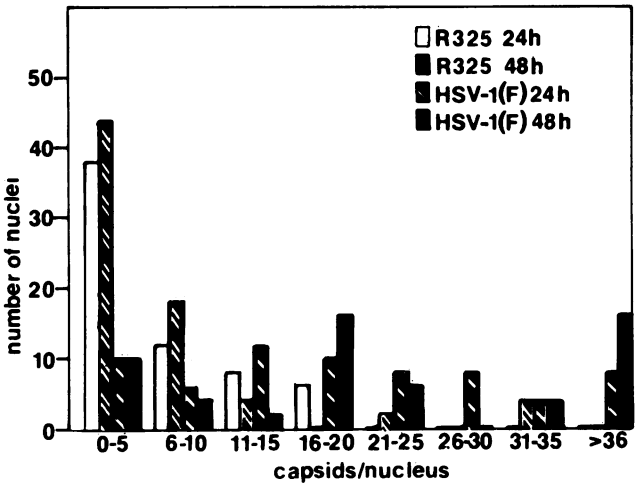

FIG. 8. Frequency distribution of the number of capsid-containing nuclei of confluent, resting HEL cells infected with HSV-1(F) or R325-βTK$^+$. The total number of capsids in the nuclei of cells infected in the experiment described in Fig. 8 were counted. The diagram represents the number of nuclei containing a given number of capsids, e.g., 0 to 5 or 6 to 10 capsids per nucleus.

(v) As cited above, R325-βTK$^+$ virus is highly attenuated for mice even on intracerebral inoculation. The attenuated property of the virus may be related to its inability to grow in mice, as would be predicted from its poor growth in murine cells in culture. Of special interest, however, is the observation that R325-βTK$^+$ was capable of establishing latent infections when inoculated by the eye route but not by the ear route. The interest in the capacity of R325-βTK$^+$ to establish latency stems from two considerations. First, if the establishment of latency requires the expression of a viral function, it could be predicted that such a function would be expressed early in infection, possibly by α genes, inasmuch as expression of β and γ genes signals a commitment to viral DNA synthesis and concomitant irreversible damage to the infected cell (34). That R325-βTK$^+$ was able to establish latency in mice, albeit with a lower efficiency than wild-type virus, suggests that the α22 gene product is not required for this purpose. Second, the difference in the ability of R325-βTK$^+$ to establish latency by the two routes of inoculation suggests an inherent difference between the requirements of the two routes. The higher efficiency obtained by the eye route could reflect the shorter pathway of axonal migration from the site of inoculation to the ganglion, a lower requirement for replication at the site of inoculation, an enhanced ability of R325-βTK$^+$ to multiply in the cornea, or a higher probability of infection of nerve endings because of increased abundance or altered susceptibility. Although we cannot differentiate between these alternatives, the eye route appears to be a suitable model for testing the ability to establish latency of viruses with diminished capacity to multiply in the mouse.

TABLE 5. Latency of R325-βTK$^+$ in mice[a]

Site of inoculation	Virus	Dose (PFU)	No. of positive ganglia/ no. of eyes or ears infected	% Latency
Eye	HSV-1(F)	10^7	5/5	100
	R325-βTK$^+$	10^7	5/10	50
	R325-βTK$^+$	$10^{7.3}$	10/20	50
Ear	HSV-1(F)	10^7	10/10	100
	R325-βTK$^+$	10^7	0/10	0

[a] BALB/C mice were inoculated in either one eye or one ear with approximately 10^7 PFU of virus as described in the text. Ipsilateral trigeminal ganglia or pooled cervical dorsal root ganglia were assayed for latent virus. Latency percentage is given as the number of animals from which virus could be recovered divided by the number of animals infected.

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