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Herpes Simplex Virus Virion Host Shutoff Function

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Herpes simplex virus (HSV) virions contain one or more functions which mediate the shutoff of host protein synthesis and the degradation of host mRNA. HSV type 1 (HSV-1) mutants deficient in the virion shutoff of host protein synthesis (*vhs* mutants) were isolated and were found to be defective in their ability to degrade host mRNA. Furthermore, it was found that viral mRNAs in cells infected with the *vhs*1 mutant have a significantly longer functional half-life than viral mRNAs in wild-type virus-infected cells. In the present study we have mapped the *vhs*1 mutation affecting the virion shutoff of host protein synthesis to a 265-base-pair *Nru*I-*Xma*III fragment spanning map coordinates 0.604 to 0.606 of the HSV-1 genome. The mutation(s) affecting the functional half-lives of host mRNA as well as the α (immediate-early), β (early), and γ (late) viral mRNAs were also mapped within this 265-base-pair fragment. Thus, the shutoff of host protein synthesis is most likely mediated by the same function which decreases the half-life of viral mRNA. The shorter half-life of infected-cell mRNAs may allow a more rapid modulation of viral gene expression in response to changes in the transcription of viral genes. Interestingly, the *vhs*1 mutation of HSV-1 maps within a region which overlaps the *Bgl*II-N sequences of HSV-2 DNA shown previously to transform cells in culture. The possible relationship between the transformation and host shutoff functions are discussed.

Infection of cells with herpes simplex virus types 1 and 2 (HSV-1 and HSV-2) results in the shutoff of host protein synthesis and the sequential expression of several coordinately regulated groups of viral genes (20). The inhibition of host macromolecule synthesis in HSV-infected cells is a multiphase process (reviewed in reference 7). A primary phase of the shutoff of host protein synthesis is mediated by a virion component. It occurs in cells infected in the presence of actinomycin D (to prevent viral gene expression) and in cells infected with UV light-irradiated virus (8, 10, 24, 39–41, 44, 56–58). A late (secondary) shutoff function reduces the remaining levels of host protein synthesis and requires the expression of viral genes (9, 40, 44).

HSV-1 and HSV-2 reduce the abundance of host mRNAs in a variety of cell types (3, 10, 21, 24, 34, 36, 38, 40–42, 48, 54–56). However, the mechanism by which this occurs has yet to be elucidated. In Vero cells, both the shutoff of host protein synthesis and the degradation of host mRNA are observed in the absence of viral gene expression (3, 10, 21, 24, 41, 44, 48, 56). Host mRNA degradation may be responsible for the dissociation of host polyribosomes (56). In contrast, in HSV-infected Friend erythroleukemia cells, the synthesis of globin is arrested by a virion function, whereas host mRNA degradation appears to require the expression of viral genes (38–40).

A set of virion host shutoff (*vhs*) mutants were previously isolated in our laboratory (44). Unlike the wild-type (wt) virus, all of these mutants were deficient in their ability to degrade preexisting β-actin and α-tubulin mRNA in the absence of viral gene expression (24, 56). However, the *vhs* mutants are not altered with respect to the secondary shutoff function. When viral gene expression is allowed, the synthesis of host proteins is turned off, albeit in a delayed and incomplete manner (24, 44).

Viral protein synthesis begins concomitantly with the shutoff of host protein synthesis. The program of expression involves the sequential turning on of the transcription of several groups of viral genes, including the α (immediate-early), β (early), and γ_1 and γ_2 (late) genes (20, 61). As the later viral genes are turned on, the synthesis of earlier viral proteins ceases, implying the existence of a mechanism to turn off the translation of the previously synthesized mRNAs (20, 24). The mechanism of this shutoff is unknown.

We have recently proposed that HSV encodes a function which indiscriminately shortens the half-life of host as well as viral α, β, and γ mRNAs (24). We have also proposed that the shutoff of host protein synthesis is a consequence of this function. This hypothesis is based on the finding that both host and viral mRNAs are significantly more stable in cells infected with the *vhs*1 mutant virus than in cells infected with the wt virus. However, because this mutant was derived by general bromodeoxyuridine mutagenesis, it could contain mutations in separate genes which might be responsible for these different phenotypes. In fact, as already noted, in infected Friend erythroleukemia cells the shutoff of host protein synthesis was mediated by a virion function whereas the degradation of host mRNA required the expression of viral genes (40).

In this report we have mapped the *vhs*1 mutation affecting the shutoff of host protein synthesis and the degradation of host mRNA within a 265-base-pair (bp) region spanning map coordinates 0.604 to 0.606 of the HSV genome. The mutation(s) affecting the functional destabilization of the α, β, and γ mRNAs was also mapped within the same 265-bp region. Thus, all of these phenotypes are most likely mediated by a single viral gene.

MATERIALS AND METHODS

Cells and virus. Rabbit skin cells and mouse Ltk^- cells were obtained from B. Roizman (University of Chicago, Chicago, Ill.). Vero monkey cells were obtained from S. Bachenheimer (University of North Carolina, Chapel Hill), and human epidermoid-2 (HEp-2) cells were from the American Type Culture Collection. The *vhs*1 mutant was derived by bromodeoxyuridine mutagenesis of HSV-1 (KOS) as previously described (44). The KOS strain was originally

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obtained from P. A. Schaffer (Harvard Medical School, Boston, Mass.).

Preparation of viral and plasmid DNAs. Viral DNAs were prepared from infected cells by CsCl density centrifugation as described previously (29). The *Eco*RI clones of HSV-1 strain KOS (16) were a gift from M. Levine (University of Michigan, Ann Arbor). Transfections were done using CsCl density-purified plasmid DNA prepared by the method of Clewell and Helinski (5). Restriction enzymes and other enzymes used in the cloning work were obtained from New England BioLabs, Inc. (Beverly, Mass.), or Bethesda Research Laboratories (Gaithersburg, Md.). The enzymes were used as recommended by the suppliers.

Marker transfer tests. Mixtures of *vhs*1 mutant viral DNA and cloned KOS test DNA fragments were used to transfect rabbit skin cells in 25-cm^2 dishes by the calcium phosphate precipitation method (17, 47). Approximately 5 μg of intact viral DNA, 5 μg of salmon sperm carrier DNA (Sigma Chemical Co., St. Louis, Mo.), and 0.1 to 2.0 μg of test fragment DNA (excised from the plasmid vector) were gently mixed in 0.5 ml of HeBs buffer (21 mM HEPES [*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid], 0.702 mM Na_2HPO_4, 0.137 M NaCl, 5 mM KCl, 5.6 mM D-glucose, pH 7.05) and precipitated for 20 min at room temperature by the addition of 30 μl of 2 M $CaCl_2$. Meanwhile, the cells were washed twice with Versene (137 mM NaCl, 2.68 mM KCl, 8 mM Na_2HPO_4, 1.47 mM KH_2PO_4, 0.537 mM EDTA) and then twice with HeBs solution. The precipitated DNA was added to the cells, and after 30 min at room temperature, 4 ml of Dulbecco modified Eagle medium supplemented with 2% heat-inactivated fetal calf serum was added and the transfected cells were incubated at 37°C. After 4 to 5 h, the cells were rinsed three times with Dulbecco modified Eagle medium supplemented with 6% heat-inactivated fetal calf serum and were then incubated at 37°C until the viral infection had spread through the entire culture (4 to 5 days). Virus from the transfection (passage 0) was harvested by three cycles of freeze-thawing and was serially propagated (at 1:3 dilution) in 25-cm^2 cultures of HEp-2 cells. Working stocks were prepared in 150-cm^2 cultures of HEp-2 cells which were inoculated with 4/5 the yield of P2 or P3 virus.

Trichloroacetic acid assay for the host shutoff function. Vero cells (24-well cultures) were preincubated for 30 min with medium 199 plus actinomycin D. This medium consists of medium 199 (KC Biologicals, Lenexa, Kansas) containing 1% heat-inactivated calf serum and 5 μg of actinomycin D (Calbiochem-Behring, La Jolla, Calif.) per ml. The cells were infected with the test virus in medium 199 plus actinomycin D. After 2 h of virus adsorption at 37°C, the cells were rinsed three times and were incubated in medium 199 plus actinomycin D. Eight hours after infection, the cells were washed three times with medium 199 lacking methionine and containing 1% dialyzed calf serum and 5 μg of actinomycin D per ml. The cells were then labeled for 2 h in labeling medium containing 5 μg of actinomycin D per ml. Labeling medium consisted of medium 199 with 1/20 the normal concentration of unlabeled methionine, 1% dialyzed calf serum, and 50 μCi of [^{35}S]methinonine (New England Nuclear Corp., Boston, Mass.) per ml. The protein samples were prepared as previously described (44).

Analyses of the functional stability of viral mRNAs. For the α mRNA stability assays (20, 24), Ltk^- or Vero cells in 24-well cultures were preincubated for 30 min in medium 199 plus cycloheximide. This medium consists of medium 199 containing 1% inactivated calf serum and 50 μg of cycloheximide (Sigma) per ml. The cells were then infected in medium 199 plus cycloheximide as described above. Thirty minutes before the cycloheximide reversal, actinomycin D was added to the medium at a final concentration of 5 μg/ml. After a 30-min incubation, the cycloheximide block was reversed by three washes with medium containing actinomycin D, and the cells either were labeled with [^{35}S]methionine at that point or were incubated in the presence of actinomycin D for 4 h further before the addition of the label. The proteins were labeled as described above.

For the analyses of the functional stability of β and γ mRNAs (24), Vero cells were infected in medium 199 containing 1% heat-inactivated calf serum. At the indicated time, the cells were washed three times with medium containing 5 μg of actinomycin D per ml. The cells were labeled with [^{35}S]methionine either at that point or after further incubation in the presence of actinomycin D. The preparation of the protein samples and gel electrophoresis were done as previously described (44).

Analyses of total infected-cell RNA. The procedures used in the RNA preparation and RNA blot hybridization are detailed elsewhere (24, 56).

RESULTS

Design of the marker transfer assay. The *vhs*1 mutation is not lethal to virus growth, although the yield of infectious *vhs*1 virus per cell is two- to fivefold lower than the yield of wt virus (44). We reasoned that the slight growth advantage of the wt virus might be employed in the mapping of the *vhs* lesion. By this method, cells would be transfected with *vhs*1 mutant virus DNA and cloned DNA fragments of the wt virus. Putative recombinants are expected to constitute a small proportion of the initial virus population. However, recombinants in which the *vhs* mutation has been repaired may have a growth advantage and take over the population during sequential virus propagation. We could thus test for the transfer of the *vhs* phenotype by analyzing the population en bloc rather than by laboriously screening hundreds of plaque isolates in the original transfection.

The study summarized in Fig. 1 was designed to test whether the wt virus exhibited a growth advantage during the propagation of mixed virus populations. Specifically, replicate cultures of rabbit skin cells were infected with wt virus alone, with the *vhs*1 mutant virus alone, or with different mixtures of the wt and mutant viruses. A total of 100 PFU were inoculated per culture so as to approximate the number of plaques produced during routine DNA transfections. Plaques were allowed to progress for several days, and the harvested virus stocks (passage 0) were serially propagated in HEp-2 cells. Passages 0 through 3 were then assayed for the *vhs* phenotype to determine whether this protocol resulted in selection of the wt virus.

In the assay, Vero cells were infected with test viruses in the presence of 5 μg of actinomycin D per ml, preventing viral gene expression. Parallel cultures were mock infected in the presence of the drug. The cells were labeled with [^{35}S]methionine from 8 to 10 h postinfection in the presence of actinomycin D. The amount of trichloroacetic acid-precipitable counts in the infected cells was then compared with that in the mock-infected cultures which were similarly treated with actinomycin D. The findings (Fig. 1) can be summarized as follows.

(i) As expected, infections with the wt KOS virus yielded virus stocks which exhibited the virion-associated host shutoff phenotype. In cells infected with the KOS passage 0

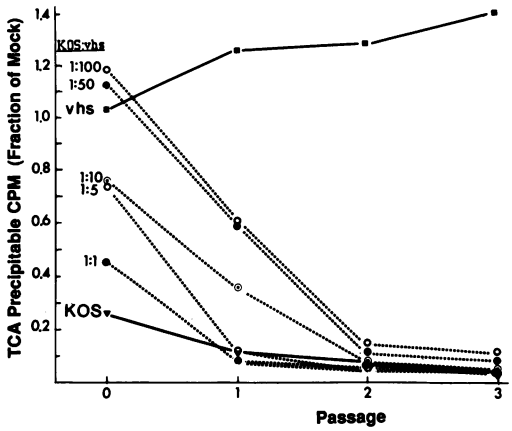

FIG. 1. Advantageous growth of the wt virus. Duplicate cultures of rabbit skin cells (25-cm^2 dishes) were infected with a total of 100 PFU of HSV-1 (KOS) wt virus, the *vhs*1 mutant virus, and mixtures containing the specified ratios of wt to *vhs*1 mutant viruses. The infected cells were harvested, yielding the passage 0 stocks which were further propagated in HEp-2 cells, as described in the text. Two independent series were derived from each virus mixture and were assayed separately for the virion-associated host shutoff phenotype as described in the text. Each point on the graphs represents an average of data obtained from the two separate series. The variations between the duplicate series were less than 5% of the values in the mock-infected cells.

virus, host protein synthesis was reduced to 25% of the synthesis in the mock-infected cells. The incomplete (75%) shutoff reflected the fact that passage 0 virus was low in titer (not shown). The level of shutoff increased to greater than 95% with successive passaging due to increasing virus titers.

(ii) The passaged *vhs*1 virus stocks exhibited a stable mutant phenotype. In fact, in cells infected with passages 1 through 3 of the duplicate *vhs*1 series, the level of host protein synthesis was higher (130 to 140%) than the amount synthesized in mock-infected cells. We have repeatedly noted that host protein synthesis increases in cells infected with high multiplicities of infection of the *vhs*1 mutant virus in the presence of actinomycin D. Tentatively, this increase may be attributed to the ability of the mutated *vhs*1 function to protect host mRNA from normal turnover (56; A. D. Kwong and N. Frenkel, manuscript in preparation).

(iii) The mixed (KOS plus *vhs*1) virus series exhibited increasing levels of wt host shutoff with successive passaging, revealing the selection of virus possessing the wt shutoff function. In the series derived from coinfections with equal amounts of *vhs*1 and KOS viruses (1:1; Fig. 1), significant shutoff (54%) was attained upon infection of cells with passage 0 virus. Furthermore, the shutoff of the host was almost complete (92%) in cells infected with passage 1 virus. No shutoff was observed in cells infected with passage 0 of the series, derived by infection of the initial cultures with 100 PFU of *vhs*1 and 1 PFU alone of KOS virus (1:100; Fig. 1). However, by passage 2, the ability to shut off the host approached that of the wt virus. The advantageous growth of the wt KOS virus was used in the marker transfer experiments described below.

Mapping of the *vhs* mutation(s) using *Eco*RI DNA fragments. The *Eco*RI enzyme cleaves the 150-kilobase (kb) HSV-1 genome into several large fragments. The wt KOS *Eco*RI fragments were cloned by Goldin et al. (16) into pBR325. The *vhs*1 mutation was first mapped within these large fragments. Specifically, duplicate rabbit skin cell cultures were cotransfected with *vhs*1 mutant virus DNA and each of several cloned *Eco*RI fragments. Control transfections received *vhs*1 DNA alone or *vhs*1 DNA along with plasmid DNA (pBR322). Virus stocks derived from these transfections were passaged to enrich for putative recombinant viruses in which the *vhs* mutation has been repaired. The various transfections were done in duplicate (designated a and b in Fig. 2), and the resultant stocks were passaged separately. The passage 2 stocks were then assayed for their ability to shut off host protein synthesis in the presence of actinomycin D. Specifically, 24-well cultures of mouse Ltk$^-$ cells were infected with samples of the passage 2 test viruses in the presence or absence of actinomycin D. Additional cultures were mock infected or were infected with standard HSV-1 wt KOS and *vhs*1 viruses (not derived from the transfection experiments). The proteins were labeled from 8 to 10 h postinfection in the presence or absence of actinomycin D, respectively, and were analyzed by electrophoresis in sodium dodecyl sulfate-polyacrylamide gels.

Lanes 1 through 14 of Fig. 2 show the pattern of protein synthesis in the cultures which were infected in the presence of actinomycin D. Comparison of the level of protein synthesis in the wt virus-infected cells (lane 2) with that in the mock-infected culture (lane 1) shows the pronounced shutoff of host protein synthesis in the presence of the drug. Lane 3 (Fig. 2) shows the corresponding lack of shutoff by the *vhs*1 mutant virus. Lanes 5 and 6 represent the duplicate independent transfections (a and b) with the *Eco*RI-A clone and show that this clone was capable of transferring the wt host shutoff phenotype to the mutant virus. Lanes 7 through 14 reveal that the other tested *Eco*RI clones were not capable of this transfer.

Lanes 15 through 28 of Fig. 2 contain protein samples from cultures infected in the absence of actinomycin D. They show that high amounts of viral proteins were produced in cells infected with viruses derived from all of the *Eco*RI cotransfections. This result ascertains that sufficiently high multiplicities of infection were used in the actinomycin D tests shown in lanes 5 through 14.

These results show that the *vhs*1 mutation resides within the 22-kb KOS *Eco*RI A fragment, spanning map coordinates 0.493 to 0.633 of the HSV-1 genome. It is noteworthy that Read and co-workers have also mapped the *vhs*1 mutation within the *Eco*RI A fragment (C. R. Krikorian and G. S. Read, unpublished results, cited in reference 41).

Location in *Eco*RI-A of the mutation affecting the functional stability of α and β/γ mRNAs. We next determined whether the *Eco*RI-A recombinant viruses resembled the wt or the *vhs*1 mutant viruses with respect to the functional stability of viral mRNAs (24, 41, 44).

Lanes 1 through 14 of Fig. 3 show the experiment designed to test the functional stability of α mRNA. Specifically, Ltk$^-$ mouse cells were infected with the second passages of two independently derived *Eco*RI-A recombinant stocks. Control cultures were mock infected or were infected with passage 2 virus derived from transfections which received KOS wt virus DNA alone, KOS plus pUC19 plasmid, *vhs*1 DNA alone, or *vhs*1 plus pUC19 plasmid. Infections were done in the presence of cycloheximide, allowing the transcription of α genes. At 8.5 h postinfection, actinomycin D was added to the medium. Thirty minutes later the cycloheximide was removed, and incubation was continued in the presence of actinomycin D, blocking further transcription, but allowing the translation of proteins from the preformed α mRNA (20). Proteins were labeled with [^{35}S]methionine in the continued presence of actinomycin D either immediately

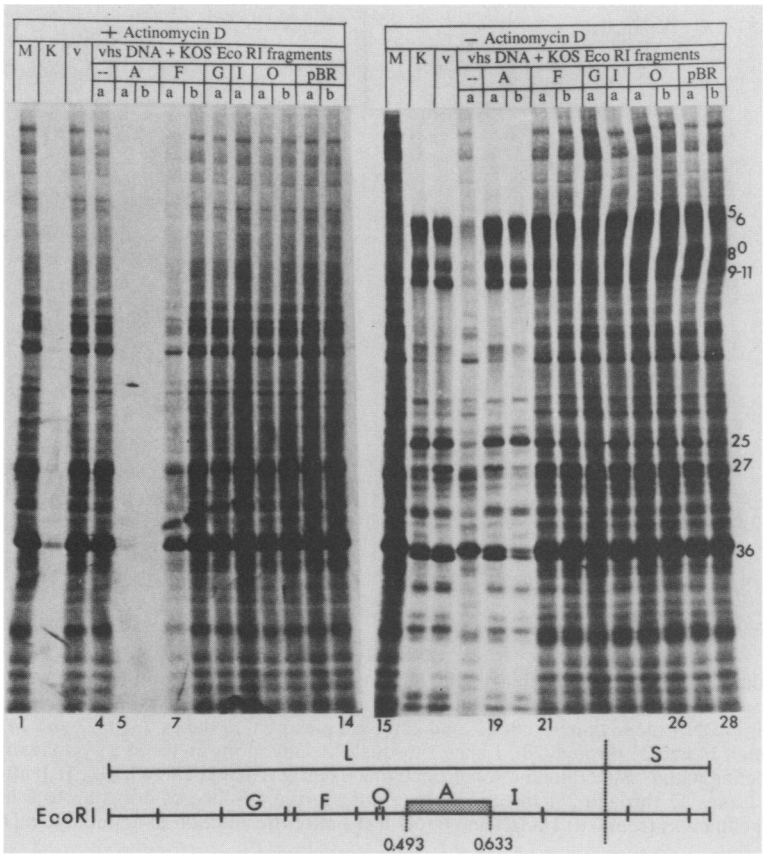

FIG. 2. The wt *Eco*RI A fragment transfers the host shutoff phenotype. Ltk$^-$ mouse cells were mock infected (lanes 1 and 15) or were infected with (per cell) 10 PFU of wt HSV-1 KOS virus (lanes 2 and 16), the *vhs*1 mutant virus (lanes 3 and 17), or virus stocks propagated from transfected cultures receiving the *vhs*1 virus DNA along with the test *Eco*RI clones (16), as indicated. Lanes 1 through 14, Infection was done in the presence of actinomycin D, and proteins were labeled from 8 to 10 h postinfection in the presence of the drug. Lanes 15 through 28, Infection was done in the absence of actinomycin D, and proteins were labeled from 8 to 10 h postinfection. The map shows the location of the *Eco*RI wt KOS fragments (51) tested in this experiment. Numbers to the right of the gel designate ICPs.

(0 to 2 h) or after further incubation (2 to 4 h) after the cycloheximide reversal.

The functional stability of the α mRNAs can be determined by comparing the level of α protein synthesis during the early (0 to 2 h) and late (2 to 4 h) pulses. In the mock-infected cells (Fig. 3, lanes 1 and 2) equivalent amounts of proteins were made during the early (E) and late (L) pulses, indicating that the bulk of the host mRNAs were stable for at least 2 to 4 hrs. In cells infected with the wt KOS virus (lanes 3 and 4) or with the KOS plus pUC19 control virus (lanes 5 and 6), the synthesis of the α proteins 4, 0, 22, and 27 decayed significantly 2 to 4 h after the α mRNA was made. In these cells the α proteins were expressed from functionally labile mRNAs. In contrast, α-protein synthesis was stable in the cells which were infected with the *vhs*1 virus (lanes 7 and 8) or with the *vhs*1 plus pUC19 control virus (lanes 9 and 10). The *Eco*RI A recombinant virus stocks clearly exhibited the wt α-synthesis phenotype (lanes 11 through 14). Thus, the *Eco*RI A fragment was capable of transferring both the virion-associated host shutoff function and the α destabilization function.

The assay for the transfer of the β/γ mRNA destabilization function (24) is shown in lanes 15 through 28 of Fig. 3. Specifically, Vero cells were infected in the absence of added drugs with virus stocks which were generated as described above. Actinomycin D was added to the cells at 8 h postinfection. Proteins were labeled either immediately (0 to 1 h after actinomycin D addition) or after further incubation (6 to 7 h) in the presence of the drug. This design allowed us to determine the stability of the β and γ mRNAs which were made prior to actinomycin D addition.

The synthesis of β infected cell proteins (ICPs) (e.g., 6, 8, 36) and γ ICPs (e.g., 5, 9, 11, 25) decayed in the cells which were infected with the KOS and KOS plus pUC19 viruses (Fig. 3, lanes 17 through 20). In contrast, the synthesis of these proteins continued at undiminished rates during the late pulse in the *vhs*1 and the *vhs*1 plus pUC19 infections (lanes 21 through 24). The synthesis of the β and γ proteins decayed rapidly in the cells that were infected with the two virus stocks derived from the *Eco*RI-A cotransfections (lanes 25 through 28). These results show that the mutation which affects the functional destabilization of the β and γ mRNAs also resides within the *Eco*RI A fragment.

Fine mapping of the host shutoff mutation. To fine map the *vhs*1 mutation, the wt KOS *Eco*RI A fragment was further subcloned. The subclones containing the fragments shown in Fig. 4 were each tested for the ability to transfer the wt host shutoff trait to the *vhs*1 mutant. The fragments which failed to transfer the wt *vhs* phenotype are shown as solid black bars; those with which transfer was obtained are shown as hatched bars (Fig. 4). Representative protein profiles are shown in Fig. 5.

On the basis of these results, the *vhs*1 mutation resides within a 265-bp *Nru*I-*Xma*III fragment spanning map coor-

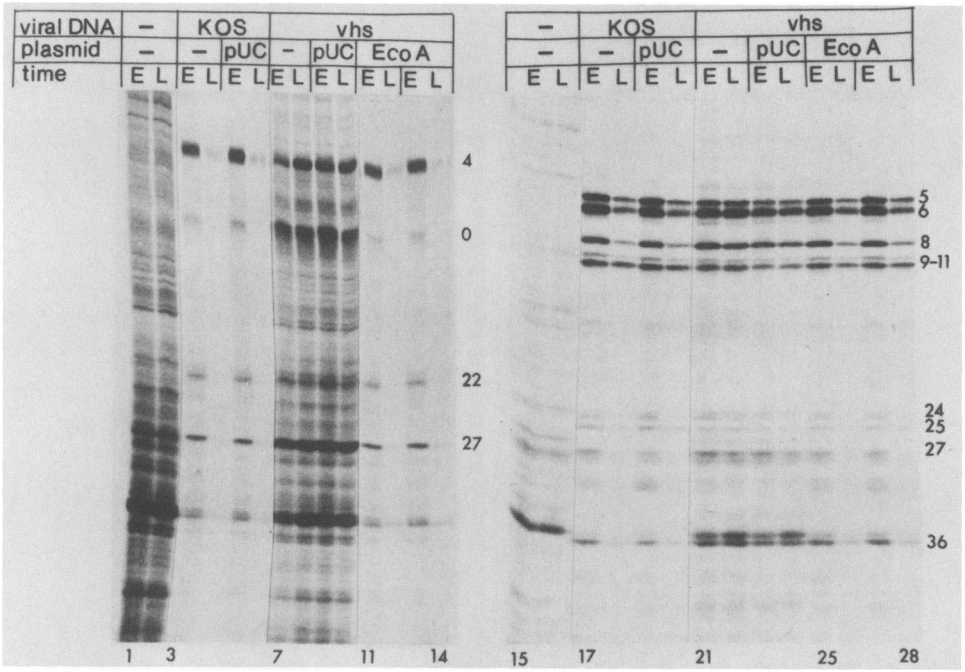

FIG. 3. *Eco*RI-A contains the function which destabilizes the α, β, and γ mRNAs. Ltk⁻ mouse cells were mock infected (lanes 1, 2, 15, and 16) or were infected with stocks propagated from transfections receiving wt KOS virus DNA alone (lanes 3, 4, 17, and 18); wt plus pUC19 (lanes 5, 6, 19, and 20); *vhs*1 virus DNA alone (lanes 7, 8, 21, and 22); *vhs*1 plus pUC19 (lanes 9, 10, 23 and 24); or *vhs*1 plus the wt *Eco*RI-A plasmid pSG124 (lanes 11 through 14 and 25 through 28). Lanes 1 through 14, Infections involved a cycloheximide reversal into actinomycin D as described in the text. Labeling with [^{35}S]methionine was from 0 to 2 h (early [E]) and 2 to 4 h (late [L]) after the cycloheximide reversal. The α proteins are indicated. Lanes 15 through 28, Infections were done in the absence of drugs up to 8 h postinfection, at which point actinomycin D was added. Labeling was from 0 to 1 h (E) and 6 to 7 h (L) after the addition of actinomycin D. Exemplary β and γ ICPs are indicated.

dinates 0.604 to 0.606. This region contains sequences common to all the clones which successfully transferred the wt phenotype. Most notably, positive transfer was attained with the *Hpa*I-to-*Nru*I clone (pNF630) (Fig. 5, lanes 17 through 20) and the *Bam*HI-to-*Xma*III clone (pNF633) (lanes 21 and 22). The results showing transfer or lack of transfer of the wt *vhs* trait by the remaining fragments were all consistent with this map assignment.

Mapping of the function responsible for the physical degradation of host mRNA. Our previous studies have shown that host mRNA was rapidly degraded in cells which were infected with wt virus in the presence of actinomycin D. In contrast, host mRNA was stable in the cells which were infected with the *vhs*1 mutant in the absence of viral gene expression (24, 56).

The passaged virus stocks described above were used to determine whether the mutation affecting the stability of host mRNA also mapped within the *Nru*I-*Xma*III fragment. Duplicate series (a and b) were tested for each of the plasmids. Cells were mock infected or infected with the test viruses in the presence of actinomycin D for 6.5 h. The cells were then harvested, and the nucleic acids were prepared and transferred to Nytran (Northern [RNA]) blots. Before transfer of the RNA to the blot the gel was stained with ethidium bromide to ascertain that the amounts of RNA loaded were similar in all the lanes. The blots were probed with β-actin (18) and α-tubulin (6) probes. The results obtained with the two probes were similar, and only the β-actin hybridizations are shown (Fig. 6). The data revealed host mRNA instability in the cells which were infected with the wt KOS propagated virus (Fig. 6, lanes 2 and 3). In contrast, host mRNA was stable in cells infected with the stocks derived from the transfections receiving *vhs*1 DNA or *vhs*1 plus the pUC8 plasmid vector (Fig. 6, lanes 4 through 7). Some of the recombinant stocks transferred the wt host mRNA degradation trait. Most notably, the *Bam*HI-to-*Xma*III clone (pNF633; Fig. 6, lanes 14 and 15) and the *Hpa*I-to-*Nru*I clone (pNF630; lanes 12 and 13) rescued the *vhs*1 defect. These results demonstrate that the mutation affecting the ability of *vhs*1 to degrade the β-actin and α-tubulin mRNAs also resides within the 265-bp *Nru*I-to-*Xma*III fragment.

Tests for transfer of the viral mRNA destabilization function. The virus stocks derived from the cotransfections described above were next assayed with regard to the functional stability of the α and β/γ viral mRNAs. As described above, to test the functional stability of the α mRNAs, cells were infected with the test viruses in the presence of cycloheximide, allowing α mRNA transcription. At 7 h postinfection the cycloheximide was removed, and incubation was continued in the presence of actinomycin D. The amount of α proteins made immediately after addition of actinomycin D (Fig. 7, lanes E) was compared with that made 4 h after addition of the drug (lanes L). The same clones which transferred the trait of destabilizing host mRNA also transferred the functional instability of the α mRNA (Fig. 7, lanes 1 through 18). Therefore, the *vhs*1 mutation which affects the stability of the α mRNA also maps within the 265-bp *Nru*I-to-*Xma*III fragment.

Finally, the functional stability of β and γ mRNA was assayed by allowing infected-cell mRNA to accumulate in the absence of drugs. At 11 h postinfection, actinomycin D was added to the cultures and β and γ protein synthesis was

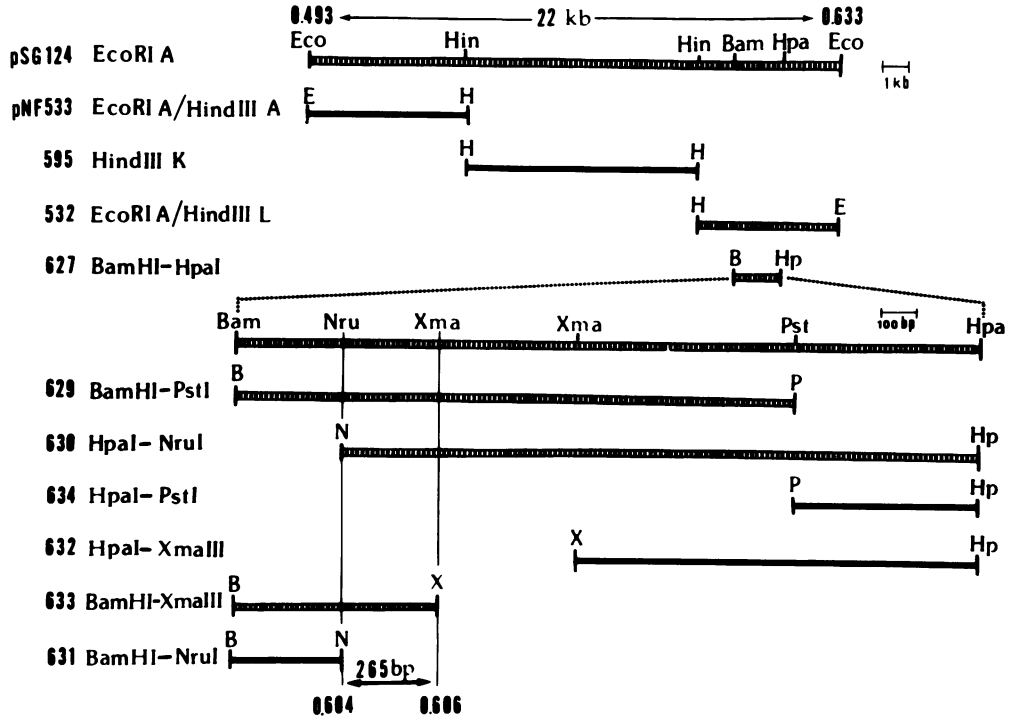

FIG. 4. Maps of the various clones tested in the *vhs* marker transfer tests. The *Eco*RI-A clone pSG124 was derived by Goldin et al. (16) and was further subcloned as follows: pNF533, −595, and −532 were subcloned in pBR325; pNF627 and pNF629 through −633 were subcloned into a modified form pUC9 in which an *Hpa*I linker had been inserted into the *Pst*I site in the polylinker region. Each of the subclones was tested for the ability to transfer the wt host shutoff trait to the *vhs*1 mutant. The fragments which failed to transfer the wt *vhs* phenotype are shown as solid black bars. The subclones with which the wt transfer was obtained are shown by hatched bars. The HSV-1 map coordinates for the 22-kb *Eco*RI A DNA fragment (0.493–0.633) and the 265-bp *Nru*I-*Xma*III DNA fragment (0.604–0.606) are indicated. Restriction enzyme sites: E, *Eco*RI; H, *Hin*dIII; B, *Bam*HI; N, *Nru*I; X, *Xma*III; P, *Pst*I; and Hp, *Hpa*I.

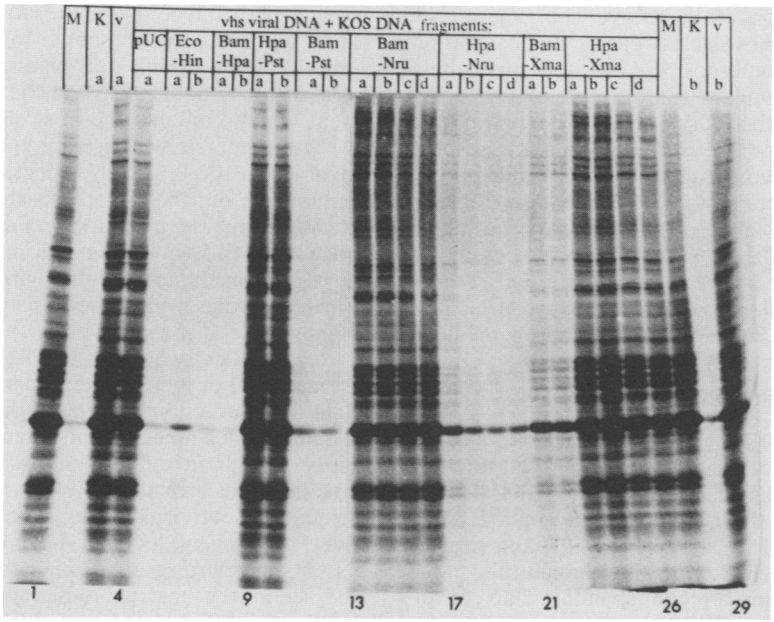

FIG. 5. Fine mapping of the virion host shutoff mutation to a 265-bp *Nru*I-*Xma*III DNA fragment. Vero cells were mock infected (lanes 1 and 27) or infected with passage 4 of virus stocks derived from transfections receiving KOS viral DNA alone (lanes 2 and 28), *vhs*1 viral DNA alone (lanes 3 and 29), *vhs*1 DNA plus pUC8 (lane 4), or *vhs*1 DNA plus the indicated KOS DNA fragments (lanes 5 through 26). Lanes a through d denote separately propagated series from replicate cultures receiving the indicated DNAs. The *Eco-Hin* clone shown in lanes 5 and 6 is the pNF532 subclone of *Hin*dIII fragment L. The infections were performed in the presence of actinomycin D, and the cells were labeled with [^{35}S]methionine from 9 to 12 h postinfection. The protein samples were electrophoretically separated in a 9.25% sodium dodecyl sulfate-polyacrylamide gel.

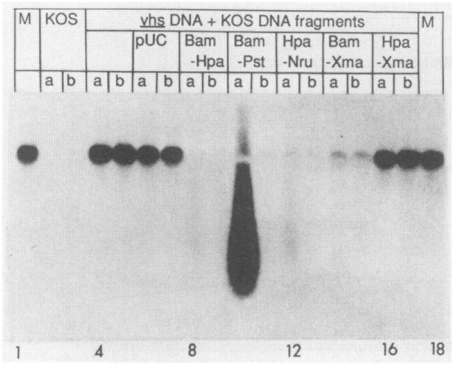

FIG. 6. The mutation affecting the stability of host mRNA maps in the 265-bp *Nru*II-*Xma*III DNA fragment. Vero cells were mock infected (lanes 1 and 18) or infected with passage 4 virus derived from transfections receiving wt KOS virus DNA alone (lanes 2 and 3), *vhs*1 mutant virus DNA alone (lanes 4 and 5), *vhs*1 plus pUC8 vector DNA (lanes 6 and 7), or *vhs*1 virus DNA plus the indicated test KOS DNA fragments (lanes 8 through 17). The cells were infected in the presence of actinomycin D, and the total cell RNA was harvested at 6.5 h postinfection. The RNA was electrophoretically separated in a 1.2% agarose–2.2 M formaldehyde gel and electrotransferred to Nytran (Schleicher and Schuell). The blot was probed with β-actin (18). The smear in lane 10 of the blot represents the hybridization of bacterial plasmid and not actin DNA sequences, inasmuch as a similar pattern of hybridization was seen with a pUC9 plasmid DNA probe (data not shown). Further analyses of this virus stock (data not shown) revealed the presence of defective genomes containing plasmid DNA sequences. These genomes most likely arose by recombination between the pNF629 construct and the *vhs*1 genome during the cotransfection. The transcription of the resultant defective genomes apparently gives rise to RNAs of variable lengths containing plasmid DNA sequences. Studies in progress are designed to characterize these genomes further.

compared early (0 to 4 h; Fig. 7, lanes E) or late (4 to 8 h; lanes L) after the addition of the drug. The β/γ mRNA stability phenotypes were not as clear-cut as the host shutoff and α mRNA stability phenotypes. We attribute this behavior to the fact that the secondary shutoff function (9, 40, 44) was expressed in the cells which were infected in the absence of drugs to allow the synthesis of β/γ mRNA. Nevertheless, all of the constructs which transferred the ability to shut off host protein synthesis were also found to transfer the functional instability of the β and γ mRNAs. These results suggest that the *vhs*1 mutant virus contains a single mutation which affects a single gene product responsible for the destabilization of host, α, and β/γ mRNAs. This mutation maps within the 265-bp *Nru*I-*Xma*III fragment.

DISCUSSION

Fine mapping of the *vhs*1 mutation. Two approaches have been used to map the virion-associated host shutoff function of HSV. The first was based on the finding that HSV-2 inhibited host protein synthesis more rapidly than HSV-1. Using a battery of HSV-1 × HSV-2 recombinant viruses, Morse et al. (35) and Fenwick et al. (8) mapped the "rapid host shutoff function" within a region spanning map coordinates 0.52 and 0.59 of the HSV-2 genome. A second approach, used in the present study, has mapped the *vhs*1 mutation within the 265-bp *Nru*I-*Xma*III fragment spanning map coordinates 0.604 to 0.606 of the HSV-1 genome. The rapid shutoff by HSV-2 and the *vhs* function most likely represent the same gene, since the 265-bp fragment lies at the right-hand border of the 11-kb region identified by Morse et al. (35). The small discrepancy in map coordinates most likely reflects the ways in which the map coordinates have been calculated. It is also noteworthy that Oroskar and Read have recently mapped the *vhs*1 mutation within the *Eco*RI A fragment of HSV-1 DNA (41).

Role of the shutoff function in virus replication. We have found that the same DNA fragment which transferred the ability to shut off host protein synthesis was also capable of transferring the function affecting the physical integrity of host mRNA. This finding suggests that in Vero cells, the shutoff of host protein synthesis is mechanistically linked to the physical degradation of host mRNA. As elaborated elsewhere (56), there are three alternative mechanisms to explain this linkage. First, the *vhs* locus could encode a nuclease, which is brought into the cells as a virion component and which reduces the half-life of mRNA in the infected cell. Second, the *vhs* function could activate a preexisting host nuclease. Finally, the *vhs* function could modify the translational machinery so as to render mRNA more vulnerable to nucleases. At present we cannot exclude any of these hypotheses. However, the virion RNase hypothesis appears unlikely in light of recent studies which have revealed that the mutated *vhs*1 function can irreversibly protect host mRNA from degradation by the wt *vhs* function (Kwong and Frenkel, manuscript in preparation).

We have previously proposed that the virion host shutoff function plays a role in the transience of translation of viral mRNA. This hypothesis is supported by several observations, as follows. (i) Prolonged synthesis of α proteins was observed in cells infected with several independently derived *vhs* mutants (44). (ii) The destabilization of α-protein synthesis coincides with shorter half-life of α mRNA (24, 41, 56). Furthermore, the decreased stability of the α mRNA is mediated by a virion component (24). (iii) The functional stability as well as abundance of β and γ mRNAs are lower in cells infected with the wt virus compared with their counterparts infected with the *vhs*1 mutant virus (24, 56). (iv) Most conclusively, as shown in the present work, the wt virus function which decreases the functional stability of viral mRNAs comaps within the 265-bp *Nru*I-*Xma*III fragment which contains the host shutoff mutation. However, we have not shown directly that a virion component is involved in the β/γ mRNA degradation, and at present we cannot rule out the hypothesis that this phenotype is mediated by a separate mechanism. For example, changes in viral mRNA stability could depend on the prior modification of host cell machinery by the virion host shutoff function. Further studies will be needed to resolve this issue unambiguously.

Regardless of whether the host and viral shutoff functions are identical or merely interdependent, they both operate to limit the expression of genes in the infected cells. The virion function which mediates this effect is clearly not essential for virus growth in cultured cells. However, it does confer advantageous growth to the wt virus, as shown by the rapid selection of wt virus during serial virus propagation. Moreover, a minimal level of host and viral shutoff may be essential for virus growth, since the *vhs*1 mutant has retained the secondary host shutoff function, and the synthesis of host, α, and β proteins is eventually turned off (24, 44, 56). Lastly, the economy implied in the timely transitions in the synthesis of viral proteins may play a more critical role in viral replication in vivo, during which the synthetic machinery may be more limiting. Thus, the initial shutoff of host protein synthesis may allow the maximal translational capacity of the cell to be used in the expression of viral genes.

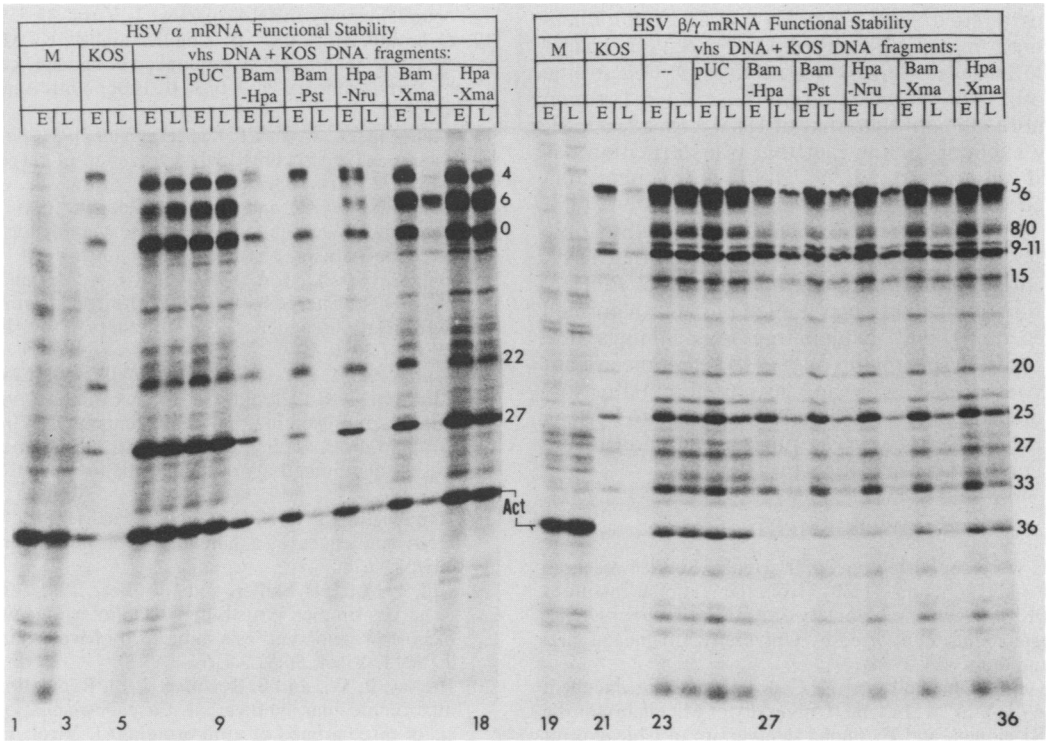

FIG. 7. The mutation affecting the functional stability of α, β, and γ viral mRNAs maps in the 265-bp *Nru*I-*Xma*III DNA fragment. Vero cells were mock infected (lanes 1, 2, 19, and 20) or were infected with passage 4 of virus stocks derived from transfections which received KOS viral DNA alone (lanes 3, 4, 21, and 22), *vhs*1 viral DNA alone (lanes 5, 6, 23, and 24), *vhs*1 plus pUC8 (lanes 7, 8, 25, and 26), or *vhs*1 viral DNA plus the indicated test KOS DNA fragments (lanes 9 through 18 and 27 through 36). Lanes 1 through 18, Test for HSV α mRNA functional stability. Vero cells were infected in the presence of cycloheximide. At 7 h postinfection, actinomycin D was added, and the cells were labeled with [^{35}S]methionine from 0 to 4 (early [E]) or from 4 to 8 (late [L]) after the addition of the drug. The α ICPs are indicated. Lanes 19 through 36, Test for HSV β/γ mRNA functional stability. Vero cells were infected in the absence of drugs. At 11 h, actinomycin D was added, and the cells were labeled with [^{35}S]methionine from 0 to 4 h (E) or from 4 to 8 h (L) after addition of the drug. Representative β and γ HSV ICPs are indicated.

In addition, limiting the expression of α and β genes (when γ transcription is turned on) may allow the maximum synthesis of the structural proteins when virion maturation begins.

Tentative identification of the *vhs* gene product and relationship between host shutoff and malignant cell transformation. Frink et al. (11) have mapped a single mRNA species overlapping the *Bam*HI-to-*Xma*III fragment spanning map coordinates 0.602 to 0.606. This mRNA is 1.9 kb in size and encodes a 58-kilodalton protein. Nucleotide sequence data (D. J. McGeoch, personal communication) are consistent with the transcriptional data. Taken together, these data suggest that the *vhs* function is mediated by the 58-kilodalton-protein. Studies designed to identify the *vhs* protein more directly are currently in progress.

The assignment of the *vhs* function to this region of the HSV-1 genome is especially intriguing inasmuch as this region overlaps the *Bgl*II N fragment of HSV-2 DNA, representing one of several HSV DNA regions exhibiting the ability to transform cells in culture (12, 13, 45). The transforming sequences within the *Bgl*II N fragment were shown to reside in an open reading frame which encodes a 61-kilodalton protein (15). This reading frame of HSV-2 is homologous to that for the 58-kilodalton HSV-1 polypeptide which we have tentatively identified as the *vhs* protein.

The paradoxical comapping of the transforming and host shutoff functions can be interpreted in at least two ways: (i) the two functions could involve different gene products, e.g., if the observed cell transformation involved a promoter insertion mechanism; and (ii) transformation could be an indirect consequence of the transient inhibition of host protein synthesis. By this model, the *vhs* activity could result in transient cell stress alterations leading to amplification, translocation, or other rearrangements of oncogenes or cell cycle regulatory genes. Such gene amplification may in turn lead to cell transformation (reviews in references 1, 27, and 53). Indeed, amplification of simian virus 40 (SV40) DNA sequences was shown to occur in SV40-transformed cells after infection with HSV (32, 33, 50). Furthermore, HSV induces the amplification of cotransfecting SV40 DNA sequences in cells which are nonpermissive for SV40 DNA replication (R. R. Danovich and N. Frenkel, submitted for publication), although it is as yet unknown whether the *vhs* function plays any role in this amplification.

With regard to this model, it is noteworthy that treatment of cells with inhibitors of protein synthesis such as cycloheximide and puromycin was previously shown to be accompanied by the formation of small polydisperse circular DNA structures. Gene amplification and gene rearrangements were shown to be induced by transient treatment of cells with inhibitors of DNA replication such as methotrexate and hydroxyurea (4, 22, 31, 49, 60) and upon stress conditions such as transient hypoxia (46). Furthermore, amplification of specific DNA sequences was induced by physical and chemical carcinogens (2, 23, 25, 26, 37) which interfere with the normal progression of the S phase, as shown by Lavi and co-workers (23). It has been suggested that transient treat-

ment of cells with such agents may lead to DNA amplification by inducing replicon misfiring and the repeated replication of specific DNA sequences (23, 49, 52, 59, 62). In line with this hypothesis is the observation that the *vhs* function of HSV-2 is more efficient than that of HSV-1 (8, 19, 35, 43, 56). This may account for the fact that transformation has been observed only with the HSV-2 *Bgl*II N fragment, and not with the corresponding sequences of HSV-1 DNA (45).

Because the *vhs* function is expected to be incompatible with long-range cell survival, it can be predicted that DNA sequences located in the proximity of the HSV-2 *Bgl*II N transforming function will not be retained in the transformed cells. This reasoning may explain previous findings that DNA sequences from this region of the HSV genome are not retained in transformed cells and are preferentially lost during cell propagation (12, 14, 28, 30). It remains to be seen whether further studies of the *vhs* function can add to the unresolved issue of transformation by HSV.

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