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Sequences of Herpes Simplex Virus Type 1 That Inhibit Formation of Stable TK$^+$ Transformants

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We have identified two regions of the herpes simplex virus type 1 (HSV-1) genome that inhibit DNA-mediated transformation of thymidine kinase-less L (Ltk$^-$) cells by the cloned HSV-1 *tk* gene. When plasmids containing the *Eco*RI fragments EK or JK were mixed at 30 fmol/ml with the *tk* gene and transfected into Ltk$^-$ cells, the frequency of transformation was inhibited 80 to more than 90% relative to the control. Of the remaining 10 *Eco*RI fragments of the HSV-1 genome, 8 were inactive and 2 were weakly active. A 6.1-kilobase *Pst*I subclone between 0.743 and 0.782 map units was isolated from pEK. This clone, pEK-P3P4, exhibited antitransformation activity toward HSV-1 *tk* and also the bacterial genes *gpt* and *neo*. pEK-P3P4 contains the alpha 27 gene, and restriction endonuclease inactivation and subcloning studies established that alpha 27 alone did not inhibit transformation. However, alpha 27 plus sequences both upstream and downstream of alpha 27 did inhibit transformation. In addition, alpha 0 or alpha 4 could substitute for alpha 27 in effecting antitransformation with these sequences. Therefore, an alpha gene and two additional loci in pEK-P3P4 are required for antitransformation. A second antitransforming locus in the reiterated sequences common to EK and JK and distinct from those in pEK-P3P4 was also identified but not characterized in detail. How antitransformation may be an expression of regulation of viral and host cell gene expression is discussed.

Herpes simplex virus types 1 and 2 (HSV-1 and HSV-2) are important human pathogens and are the causative agents of a wide range of clinical syndromes (40). Infection of susceptible cells can result in productive infection, transformation (32, 38), or the establishment of latency (13). In productive infection, the cell invariably dies, in contrast to the interaction in transformed or latently infected cells.

Multiple viral genes may contribute to achieving disruption of the host cell and to the complex positive and negative regulation of the infecting genome. It is of interest in this regard that temperature-sensitive (*ts*) mutants of HSV-1 defective in alpha 4 or alpha 27 overproduce both alpha 4 and alpha 27 at the nonpermissive temperature and are defective in late shutoff of host polypeptide synthesis (25, 34). This indicates a role, possibly indirect, for both of these genes in this process. Also, the temporal cascade of HSV-1 polypeptide synthesis (14, 15) is both positively and negatively regulated (2, 7, 8, 10, 17, 26, 27, 31, 34, 36).

In this report, we describe an assay based on interference of DNA-mediated gene transfer to identify regions of the HSV-1 genome that may regulate host or viral metabolism. In this assay, subgenomic fragments of HSV-1 were cotransfected into thymidine kinase-less L (Ltk$^-$) cells along with the HSV-1 *tk* gene. Reduced numbers of TK$^+$ clones, relative to appropriate controls, identified inhibitory HSV-1 fragments. Two *Eco*RI fragments, EK and JK, were found to be quite inhibitory. Subcloning experiments located the EK-containing activity to a 6.1-kilobase (kb) *Pst*I fragment at 0.743 to 0.782 map units (m.u.). This fragment lies partly in the unique sequences of E and contains the gene for alpha 27. Additional studies revealed that alpha 27 alone was inactive in the antitransformation assay and that sequences both 5′ and 3′ to alpha 27, along with alpha 27, were required for interference.

MATERIALS AND METHODS

Cells and virus. Ltk$^-$ cells were cultured in a modified Eagle medium containing 5% calf serum (EM5C) as described previously (1). DNA from a TK-deficient mutant of HSV-1 (KOS), 1093 (16), was prepared by the method of Pignatti et al. (30).

Plasmids. Bacteria harboring plasmids containing *Eco*RI fragments representing the entire genome of HSV-1 (KOS) (11) were a gift from Bette Pancake. The HSV-1 *tk* plasmid, pHSV106 (22), was a gift from Steven McKnight. pSV2-*gpt* (23) and pSV2-*neo* (37) were gifts from Joel Huberman. The alpha 27-containing clone pKHX-BH (3) was a gift from Stanley Person. The alpha 0-containing clone pIGA-15 (10) and the alpha 4-containing clone pRHP6 (10) were gifts from Saul Silverstein. The Vmw65-containing clone pMC1 (4) and pMC7, an inactive frameshift mutant of pMC1 (C. Preston, personal communication), were gifts from Chris Preston. pEK-P3P4 (P3P4) (0.743 to 0.782 m.u.) was obtained from pSG28 (11), the plasmid bearing the EK insert (pEK), by *Pst*I digestion, electroelution of the 6.1-kb fragment after agarose gel electrophoresis, and insertion into the *Pst*I site of pBR325 by standard methods (21). pEK-K1K2 (0.739 to 0.794 m.u.), containing an 8.6-kb *Kpn*I-*Kpn*I fragment; pEK-K2K3 (0.794 to 0.812 m.u.), containing a 2.8-kb *Kpn*I-*Kpn*I fragment; pEK-K3E2 (0.812 to 0.867 m.u.), containing an 8.6-kb *Kpn*I-*Eco*RI fragment; pEK-P3S1 (P3S1) (0.743 to 0.756 m.u.), containing a 2.0-kb *Pst*I-*Sal*I fragment; and pEK-S1P4 (S1P4) (0.756 to 0.782 m.u.), containing a 4.1-kb *Sal*I-*Pst*I fragment, were similarly isolated and inserted into the multiple cloning site of pUC18 (24) as described previously (6). The locations of the subclones are shown in Fig. 2. All restriction endonuclease digestions were carried out as specified by the manufacturer (Boehringer Mannheim), and

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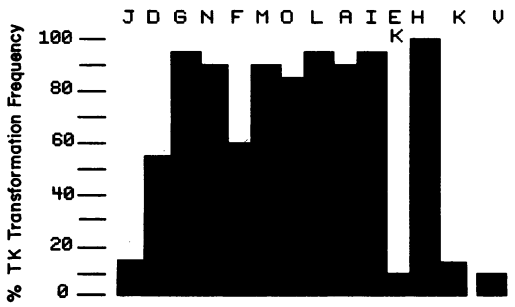

FIG. 1. Specific fragments from an *Eco*RI library of HSV-1 inhibit *tk* transformation. Precipitates containing 200 ng of HSV-1 *tk* plasmid, pHSV106 (22), and 30 fmol of cloned HSV *Eco*RI fragment (11) per ml were transfected into Ltk$^-$ cells as described in Materials and Methods. Each determination was carried out at least twice. Bars of the graph do not reflect differences in sizes of the *Eco*RI fragments. Transformation is relative to that in precipitates containing pHSV106 alone. The fragments are arrayed in the prototype arrangement of the genome in which fragments E and K are present as the junction fragment EK and fragments J and K are at the termini. The data for fragments J and K, although depicted as terminal fragments, were derived from the junction fragment JK cloned from the I_L or I_{SL} arrangements of the genome (11). As a control for the entire genome, DNA from a TK-deficient mutant, 1093 (16), was digested with *Eco*RI and transfected at 30 fmol/ml of precipitate; these results are under the letter V.

completeness of digestion was verified by agarose gel electrophoresis.

Transformation assay. DNA coprecipitates containing 200 ng of pHSV106 per ml, the desired HSV-1 plasmid, and 20 μg of Ltk$^-$ DNA per ml were prepared as described by Wigler et al. (42). Precipitates were diluted 10-fold in EM5C. Cultures were treated with 10 to 20 ml of diluted precipitate per 10^6 cells. After 4 h at 37°C, cells were treated for 3 min at room temperature with 4% dimethyl sulfoxide (Fisher Scientific) in EM5C. Selection was applied the following day. For selection of TK$^+$ cells, EM5C was made 10^{-4} M hypoxanthine, 6×10^{-7} M methotrexate, and 1.6×10^{-5} M thymidine (HAT). For *gpt* selection, EM5C was made 1.4 mM xanthine (NBCo Biochemicals) and 6 μg of mycophenolic acid per ml. For *neo* selection, 400 μg of geneticin (G418 sulfate; Gibco Laboratories) was added.

RESULTS

Specific *Eco*RI fragments of HSV-1 inhibit transformation. The cloned HSV-1 *tk* gene can efficiently transform Ltk$^-$ cells to TK$^+$ (1). We asked whether specific segments of the HSV-1 genome could interfere with this process. In initial studies, DNA from a TK-deficient mutant of HSV-1 (KOS), 1093, was used because it does not transform Ltk$^-$ cells to TK$^+$ in standard HAT medium (16). Genomic DNA from 1093, when coprecipitated with the cloned HSV-1 *tk* gene, pHSV106 (22), and transfected into Ltk$^-$ cells inhibited transformation in a concentration-dependent manner (data not shown). Since intact HSV DNA is known to be infectious (12) and the production of progeny virus results in the death of permissive cells, inhibition of transformation could have resulted from cell death due to a complete infectious cycle mediated by the transfected viral genome as well as to one or more HSV genes acting singly or in concert to inhibit transformation in some other way.

To determine whether inhibition of transformation could be attributed to specific regions of the genome, 1093 DNA was digested with different restriction endonucleases and assayed for loss or retention of the antitransformation phenotype. Digestion with *Hin*dIII, *Xba*I, or *Eco*RI had no effect on the ability of the DNA to interfere with transformation, while digestion with *Bam*HI inhibited its antitransforming activity (data not shown). These results suggested that the antitransformation effect could be localized to a specific set of restriction fragments of the genome.

Since *Eco*RI did not inactivate antitransformation by genomic DNA, individual restriction fragments from an *Eco*RI library of HSV-1 (KOS) (11) were assayed. Figure 1 shows the results obtained by mixing plasmids bearing the appropriate inserts, at 30 fmol/ml of coprecipitate, with pHSV106. Two of the plasmids, pSG28 (pEK) and pSG1 (pJK), containing the fragments EK and JK, respectively, inhibited transformation by 80 to 90% relative to transformation effected by pHSV106 alone. *Eco*RI-restricted 1093 DNA, at the same molarity as pEK, inhibited to a similar degree. Control experiments with pBR325, into which the HSV fragments were cloned, showed that the vector itself did not inhibit transformation at concentrations up to 400 fmol/ml (data not shown). Plasmids containing fragments A, G, H, I, L, M, N, and O did not significantly inhibit transformation. Although plasmids containing fragments D and F consistently exhibited measurable (40 to 50%) inhibition, and pJK clearly had antitransforming activity, we focused on pEK. The remaining studies in this report were directed to a more detailed analysis of its inhibitory properties. The pJK-directed activity and how it related to that in pEK will be considered in the Discussion.

pEK inhibits DNA-mediated transfer of neomycin and mycophenolic acid resistance. Since the HSV-1 *tk* gene is negatively regulated by certain HSV functions (2, 36) during productive infection, inhibition of *tk* transformation could be due to expression of a *trans*-acting factor suppressing *tk* expression. Therefore, it was of interest to determine whether pEK could inhibit transformation by genes other than HSV-1 *tk*. Transformation of Ltk$^-$ cells with the bacterial genes *neo* (37) and *gpt* (23) was also inhibited by pEK (Table 1). The plasmid bearing fragment G had little effect. Since these *neo* and *gpt* constructs contain simian virus 40 (SV40) early promoters, the results indicate that the

TABLE 1. Inhibition by HSV *Eco*RI fragments of transformation by *neo* or *gpt* genes

Selectable gene[a]	HSV DNA	Avg no.[b] of transformed clones/flask ± SD	% Inhibition
neo	None	146 ± 9	0
	pG	130 ± 10	11
	pEK	7 ± 4	95
	P3P4	4 ± 1	97
gpt	None	126 ± 17	0
	pG	125 ± 15	1
	pEK	7 ± 1	94
	P3P4	11 ± 6	91
tk	None	505 ± 22	0
	pG	430 ± 24	15
	pEK	28 ± 13	94
	P3P4	26 ± 1	95

[a] Precipitates containing 100 ng of pSV2-*neo*, 1 μg of pSV2-*gpt*, 200 ng of pHSV106, and 30 fmol of pG, pEK, or P3P4 per ml were transfected into Ltk$^-$ cells as described in Materials and Methods. Duplicate precipitates were prepared for each HSV DNA class, and each was used to transfect three 75-cm^2 flasks containing 3×10^5 Ltk$^-$ cells. The following day, selection for *neo*, *gpt*, or *tk* was applied.

[b] Average number of transformants in two flasks.

TABLE 2. Inhibition of *tk* transformation by endonuclease-digested HSV-1 *Eco*RI fragment EK[a]

Treatment (digestion)	Avg no.[b] of TK+ clones/dish ± SD	% Inhibition
Expt I		
No EK	107 ± 5	0
No digestion	0.3 ± 0.4	>99
*Eco*RI	1.7 ± 0.5	98
*Kpn*I	5 ± 1	95
*Pst*I	9 ± 2	92
*Xho*I	2 ± 1	98
*Sma*I	82 ± 8	23
Expt II		
No EK	71 ± 8	0
No digestion	6 ± 1	92
*Pvu*II	5 ± 2	93
*Sal*I	48 ± 8	33
*Bam*HI	51 ± 6	28

[a] Precipitates containing 200 ng of pHSV106 and 30 fmol of pEK per ml were transfected into Ltk⁻ cells as described in Materials and Methods.

[b] Average number of transformants based on one precipitate per sample and three 9.6-cm² dishes per precipitate.

inhibitory effect of pEK is not restricted to the HSV-1 *tk* promoter or gene product. pEK was capable of interfering with functions other than those coded for by HSV-1 *tk*.

Sensitivity of antitransforming activity of pEK to digestion with certain restriction endonucleases. To more precisely localize the regions essential for antitransformation, pEK was digested with several restriction enzymes prior to assay. pEK-mediated antitransformation was resistant to digestion with *Eco*RI, *Kpn*I, *Pst*I, *Xho*I, and *Pvu*II (Table 2), indicating that regions essential for antitransformation did not contain these restriction enzyme recognition sites. Digestion with *Sma*I, *Sal*I, or *Bam*HI lessened the antitransforming activity of pEK (Table 2), suggesting that these sites were critical for antitransformation.

A map indicating the location of these sites and the location of the alpha gene transcripts of pEK is shown in Fig. 2. Interestingly, *Xho*I and *Pvu*II each cut the HSV-1 sequences of pEK only once, in the genes for alpha 0 and alpha 4, respectively. These cuts did not inactivate antitransformation, suggesting that neither of these genes is essential for that activity.

Subcloning an antitransformation region from pEK. Since *Pst*I and *Kpn*I cut pEK into a number of fragments yet did not affect antitransformation (Fig. 2 and Table 2), these enzymes were used to obtain subclones of pEK.

Three subclones were obtained initially: pEK-K1K2 (0.739 to 0.794 m.u.), pEK-K2K3 (0.794 to 0.812 m.u.), and pEK-K3E2 (0.812 to 0.867 m.u.). pEK-K1K2 was active in antitransformation (data not shown), while the remaining two clones were inactive (Table 3).

Next, a *Pst*I subclone was isolated which contained sequences lying wholly within pEK-K1K2. This subclone, pEK-P3P4 (0.743 to 0.782 m.u.), abbreviated P3P4, also had antitransformation activity (Table 3). Significantly, P3P4 contained *Bam*HI and *Sal*I sites. These sites were expected to be within the antitransforming sequences, based on the data in Table 2. An antitransforming function was specified by sequences located entirely within P3P4. Also, P3P4, like pEK, inhibited transformation by *neo* and *gpt* (Table 1).

Dose-response analysis of P3P4-mediated antitransformation. To determine the minimum amount of P3P4 required for antitransformation, a dose-response experiment was performed. As little as 10 fmol of P3P4 per ml of coprecipitate had significant antitransforming activity (Fig. 3). The plasmid bearing fragment I, used as a negative control, had no activity even at concentrations as great as 100 fmol/ml. Note that the antitransforming activity of P3P4 was lost after digestion with *Sal*I, implying a role for alpha 27 in antitransformation, since the alpha 27 structural gene contains the only *Sal*I site in P3P4. This possibility is considered further in a later section.

Characterization of restriction enzyme sites in P3P4. P3P4 was digested with *Sma*I, *Sal*I, *Sac*I, or *Bam*HI to determine whether these sites were essential for antitransformation. In each case (Table 4), antitransforming activity was significantly reduced by these cleavages. Since *Bam*HI, *Sal*I, and *Sac*I sites occur only once in P3P4, each of these sites was essential for activity. At least two RNA transcripts pass through the *Bam*HI and *Sal*I sites. As shown in Fig. 2, the transcript for alpha 27 maps through the *Sal*I site (41). Another transcript of unknown function maps through the *Bam*HI site (6). Since no single transcript or gene corresponding to this region is known which contains *Bam*HI, *Sal*I, and *Sac*I sites, several distinct loci may be acting together to achieve antitransformation. To clarify the roles of these loci, a study of the properties of P3P4 was carried out.

Fractionation of P3P4-mediated antitransformation. Since the preceding studies suggested that antitransformation by P3P4 was the result of several genetic loci acting in concert, subcloning of P3P4 was carried out to isolate these regions. P3P4 was divided into three subclones. Two contained contiguous pieces of DNA from P3P4 and were called P3S1 and S1P4. P3S1 contained HSV-1 DNA from 0.743 to 0.756 m.u., while S1P4 contained sequences from 0.756 to 0.782 m.u. Neither contained a functional alpha 27 gene.

pKHX-BH (3), on the other hand, contained HSV-1 strain KOS DNA from the *Bam*HI site at 0.747 m.u. to an *Hpa*I site at 0.764 m.u. This subclone contained the entire, functional alpha 27 gene (20, 34, 41) (also see Fig. 2). The alpha 27-containing clone pKHX-BH did not have antitransformation activity (Fig. 4A, row 5). It was also apparent that the other two subfragments of P3P4 had no activity (rows 3 and 4). Also, no pair of subclones was active (rows 6 to 8). However, when all three subclones (P3S1 plus S1P4 plus pKHX-BH) were added together at 30 fmol/ml each, antitransformation activity comparable to that seen with the parent clone P3P4 at 30 fmol/ml was achieved. This important observation suggests that antitransformation is only achieved when three separate loci act together. Fortuitously, these three loci were contained in P3P4 but could be separated from each other as independent subclones: pKHX-BH, P3S1, and S1P4.

Analysis of the role of alpha 27 in antitransformation. If alpha 27 has a role in antitransformation, we reasoned that stimulation of alpha 27 activity would enhance P3P4's antitransforming activity. To accomplish this, pMC1, a plasmid that codes for Vmw65, a virion polypeptide that specifically stimulates alpha promoters (4, 28), was incorporated into the transfection mixtures.

Use of 3 fmol each of P3S1, S1P4, and pKHX-BH per ml resulted in 52% inhibition of transformation (Fig. 4B, row 5). However, when pMC1 but not pMC7, which bears a frameshift mutation in Vmw65, was included in the transfection with the three component plasmids, increased levels of inhibition were seen (compare rows 6 and 8). No significant antitransformation was seen when pKHX-BH was first digested to completion with *Sal*I, which recognizes a site in the

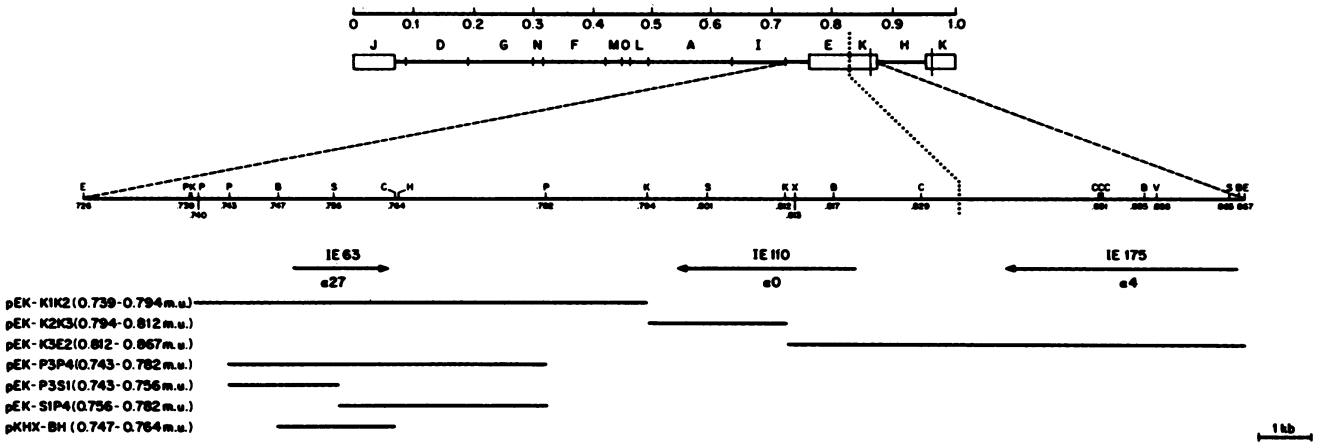

FIG. 2. Subclones of pEK and P3P4. The top line shows the fractional distance (map units) along the HSV-1 genome. The next line shows the genome divided into *Eco*RI fragments (11), with open boxes indicating repetitive sequences found internally and at the termini of the genome. The dotted line indicates the joint about which the four isomeric genomic sequences rearrange. The next line shows an expansion map of EK with relevant restriction sites: *Eco*RI (E), *Bam*HI (B), *Pst*I (P), *Kpn*I (K), *Sal*I (S), *Xho*I (X), *Sac*I (C), *Hpa*I (H) (not all sites shown), and *Pvu*II (V). These sites were determined during the course of this work and are in agreement with those reported previously (3, 19, 26). The alpha transcripts are positioned from previously published studies (20, 29, 33). The remaining lines show the location of subclones of EK, with their names on the left. Details of subcloning are given in Materials and Methods. Splices (29) in the transcript of alpha 0 are not shown.

alpha 27 structural gene (Fig. 4B, row 11). These results strongly imply that an alpha 27 function is necessary but not sufficient to achieve the antitransformation mediated by P3P4.

Substitution of alpha 0 or alpha 4 for alpha 27 in antitransformation. It was formally possible that restoration of antitransformation could have occurred by homologous recombination between the overlapping subclones of P3P4 and that antitransformation was due to some *cis*-acting property of P3P4. To test this possibility, either an alpha 0 clone, pIGA-15, or an alpha 4 clone, pRHP6, was substituted for pKHX-BH in transfection mixtures (see Fig. 2). Neither the alpha 4 nor alpha 0 gene possesses sequence homology to alpha 27. Either alpha 0 or alpha 4 substituted for alpha 27 as necessary components in antitransformation (Table 5).

The data in Table 5 show also that the three separate subclones of P3P4 (P3S1, S1P4, and pKHX-BH) were effective mediators of antitransformation when mixed together. Most significantly, the alpha 27 plasmid pKHX-BH could be replaced with either the alpha 0 (pIGA-15) or alpha 4 (pRHP6) plasmids without a loss in antitransformation activity (compare rows 7, 9, and 11).

Successful substitution of pKHX-BH with alpha 0 or alpha 4 plasmids depended on the structural integrity of the alpha genes. That is, digestion of pIGA-15 with *Xho*I, which cleaves within the alpha 0 gene, or digestion of pRHP6 with *Pvu*II, which cleaves within the alpha 4 gene, eliminated the ability of these plasmids to substitute for pKHX-BH. Similarly, digestion of pKHX-BH with *Sal*I, which cleaves within the alpha 27 gene, prevented it from complementing P3S1 and S1P4 in the assay (Table 5, rows 8, 10, and 12).

Finally, the HSV inserts in P3S1 and S1P4 must have been intact to be antitransforming with pIGA-15 (Table 6). Digestion of P3S1 with *Bam*HI or digestion of S1P4 with *Sac*I abrogated their antitransforming ability. This is consistent

TABLE 3. Inhibition of *tk* transformation by subclones of *Eco*RI fragment EK[a]

HSV DNA	Avg no.[b] of TK$^+$ clones/dish ± SD	% Inhibition
None	49 ± 13	0
pEK	3 ± 1	94
pEK-K2K3	39 ± 5	20
pEK-K3E2	40 ± 3	18
P3P4	8 ± 2	84

[a] Precipitates containing 200 ng of pHSV106 and 30 fmol of pEK, pEK-K2K3, pEK-K3E2, or P3P4 per ml were transfected into Ltk$^-$ cells as described in Materials and Methods.

[b] Average number of transformants based on two to four precipitates per sample and three 9.6-cm^2 dishes per precipitate.

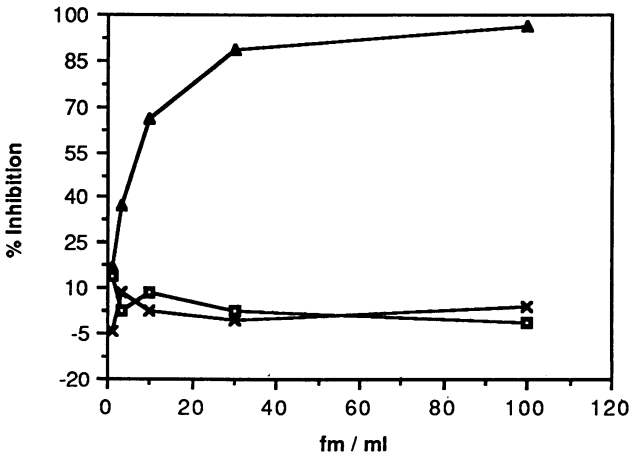

FIG. 3. Dose-response analysis of P3P4. Precipitates containing 200 ng of pHSV106 per ml and a range of concentrations of either P3P4 (▲), *Sal*I-digested P3P4 (×), or pI (□) were transfected into Ltk$^-$ cells in 25-cm^2 flasks, as in Materials and Methods. pBR325 was added as necessary so that each precipitate contained 100 fmol of bacterial sequences per ml. Percentage inhibition is relative to the number of colonies appearing in flasks without HSV sequences other than pHSV106. For this experiment, an average of 149 ± 9 colonies appeared in the control flasks. All values are the average of two flasks, and the standard deviation was not more than ±10 for any point.

TABLE 4. Inactivation of antitransforming activity of subclone pEK-P3P4[a]

HSV DNA	Digestion	Avg no.[b] of TK+ clones/dish ± SD	% Inhibition
Expt I			
None		49 ± 13	0
pG		34 ± 8	31
pEK		3 ± 1	94
P3P4		8 ± 2	84
P3P4	*Sma*I	33 ± 5	33
P3P4	*Sal*I	31 ± 2	37
P3P4	*Sac*I	31 ± 3	37
Expt II			
None		46 ± 9	0
pG		40 ± 2	13
pEK		0 ± 0	100
P3P4		1 ± 1	98
P3P4	*Bam*HI	34 ± 9	26
P3P4	*Sac*I	36 ± 7	22

[a] Precipitates containing 200 ng of pHSV106 and 30 fmol of pG, pEK, or P3P4 per ml were transfected into Ltk- cells as described in Materials and Methods.

[b] Average number of transformants based on two to four precipitates per sample and three 9.6-cm² dishes per precipitate.

with the results of previous experiments which suggested that *Bam*HI and *Sac*I sites are in essential sequences in P3S1 and S1P4, respectively.

Restriction of plasmids per se did not diminish antitransformation function. *Pst*I or *Sal*I digestion of P3S1 and S1P4 did not affect their ability to cooperate in the antitransformation assay, even though those enzymes linearized both plasmids. Cleavage must be in an essential, specific sequence.

Together, Tables 5 and 6 indicate that an alpha gene acts in *trans*, either directly or indirectly, with P3S1 and S1P4 to achieve antitransformation. The data do not distinguish between a requirement for an alpha gene to *trans*-activate sequences whose products are antitransforming or a requirement for the alpha gene product to interact with other gene products for the effect.

TABLE 5. Substitution of alpha 0 or alpha 4 for alpha 27 in antitransformation

HSV DNA[a]	Digestion[b]	Avg no.[c] of TK+ transformants/ flask ± SD	% Inhibition
None		149 ± 9	0
P3P4		14 ± 3	91
pKHX-BH		139 ± 19	7
pIGA-15		142 ± 21	5
pRHP6		138 ± 11	7
P3S1 + SIP4		151 ± 12	−1
P3S1 + SIP4 + pKHX-BH		20 ± 1	87
P3S1 + SIP4 + pKHX-BH	*Sal*I	124 ± 5	17
P3S1 + SIP4 + pIGA-15		15 ± 6	90
P3S1 + SIP4 + pIGA-15	*Xho*I	145 ± 7	3
P3S1 + SIP4 + pRHP6		15 ± 4	90
P3S1 + SIP4 + pRHP6	*Pvu*II	155 ± 6	−4

[a] Precipitates containing 200 ng of pHSV106 and 30 fmol of each HSV DNA-containing plasmid per ml were transfected into Ltk- cells as described in Materials and Methods. In each case, pBR325 was added as necessary so that each precipitate contained bacterial plasmid sequences at a concentration of 90 fmol/ml.

[b] Plasmid DNA was restricted with the indicated enzyme prior to incorporation into the transfection mixture.

[c] Average number of TK+ transformants based on one precipitate per sample and two 25-cm² flasks per precipitate.

TABLE 6. Restriction enzyme analysis of antitransformation subclones

HSV DNA and digestion[a]	Avg no.[b] of TK+ transformants/ flask ± SD	% Inhibition
None	155 ± 8	0
P3P4	4 ± 2	97
P3S1	147 ± 4	5
S1P4	149 ± 10	4
pKHX-BH	155 ± 6	0
pIGA-15	154 ± 14	1
pI	147 ± 4	5
P3S1 + S1P4	146 ± 7	6
P3S1 + S1P4 + pKHX-BH	9 ± 2	94
p3S1 + S1P4 + pIGA-15	7 ± 3	95
P3S1 (*Pst*I) + S1P4 + pIGA-15	7 ± 3	95
P3S1 (*Bam*HI) + S1P4 + pIGA-15	155 ± 5	0
P3S1 (*Sal*I) + S1P4 + pIGA-15	7 ± 7	95
P3S1 + S1P4 (*Pst*I) + pIGA-15	14 ± 5	91
P3S1 + S1P4 (*Sac*I) + pIGA-15	146 ± 3	6
P3S1 + S1P4 (*Sal*I) + pIGA-15	17 ± 4	89

[a] Precipitates containing 200 ng of pHSV106 and 30 fmol of each of the indicated HSV DNA-containing plasmids per ml were transfected into Ltk- cells as described in Materials and Methods. In each case, pBR325 was added as necessary so that each precipitate contained bacterial plasmid sequences at a concentration of 90 fmol/ml. Plasmid DNA was restricted with the indicated enzyme prior to incorporation into the transfection mixture.

[b] Average number of TK+ transformants based on one precipitate per sample and two 25-cm² flasks per precipitate.

DISCUSSION

We have determined that two restriction fragments of the HSV-1 (KOS) genome contain sequences that interfere with stable transformation of cells by DNA-mediated gene transfer. These sequences occur in the cloned *Eco*RI fragments EK and JK. Of the remaining 10 *Eco*RI fragments of the genome, 8 were inactive and 2 were weakly active. The antitransforming activity of pEK was subcloned as a 6.1-kb *Pst*I fragment, pEK-P3P4 (0.743 to 0.782 m.u.), called P3P4. Femtomole concentrations of P3P4, when present in calcium phosphate coprecipitates, inhibited biochemical transformation of Ltk- cells by HSV-1 *tk* and bacterial *gpt* and *neo* genes. These genes are driven by viral promoters: an HSV-1 promoter for HSV-1 *tk* and the SV40 early promoter for *gpt* and *neo* (22, 23, 37). It is not yet known whether inhibition is restricted to genes bearing viral promoters or extends to those bearing eucaryotic promoters.

P3P4 contains one of the five alpha genes, alpha 27. The alpha genes are the first viral genes transcribed during productive infection and are responsible for activating additional classes of HSV genes (7, 8, 10, 14, 15, 26, 27, 31, 34). Further study of P3P4 revealed that inactivation of alpha 27 by specific restriction endonuclease cleavage abolished antitransformation. Activity could be restored by adding a functional alpha 27 gene to the transfection mixture, indicating that alpha 27 was necessary for the phenomenon and probably acted in *trans*. Additional evidence for a *trans*-acting role for alpha 27 came from the observation that antitransformation was enhanced by pMC1, which encodes Vmw65, an HSV-1 polypeptide that specifically stimulates alpha promoters (4, 28). Other sequences in P3P4 were also required for activity since the alpha 27 gene alone was inactive and cleavages both upstream and downstream from the alpha 27 gene resulted in loss of antitransforming activity.

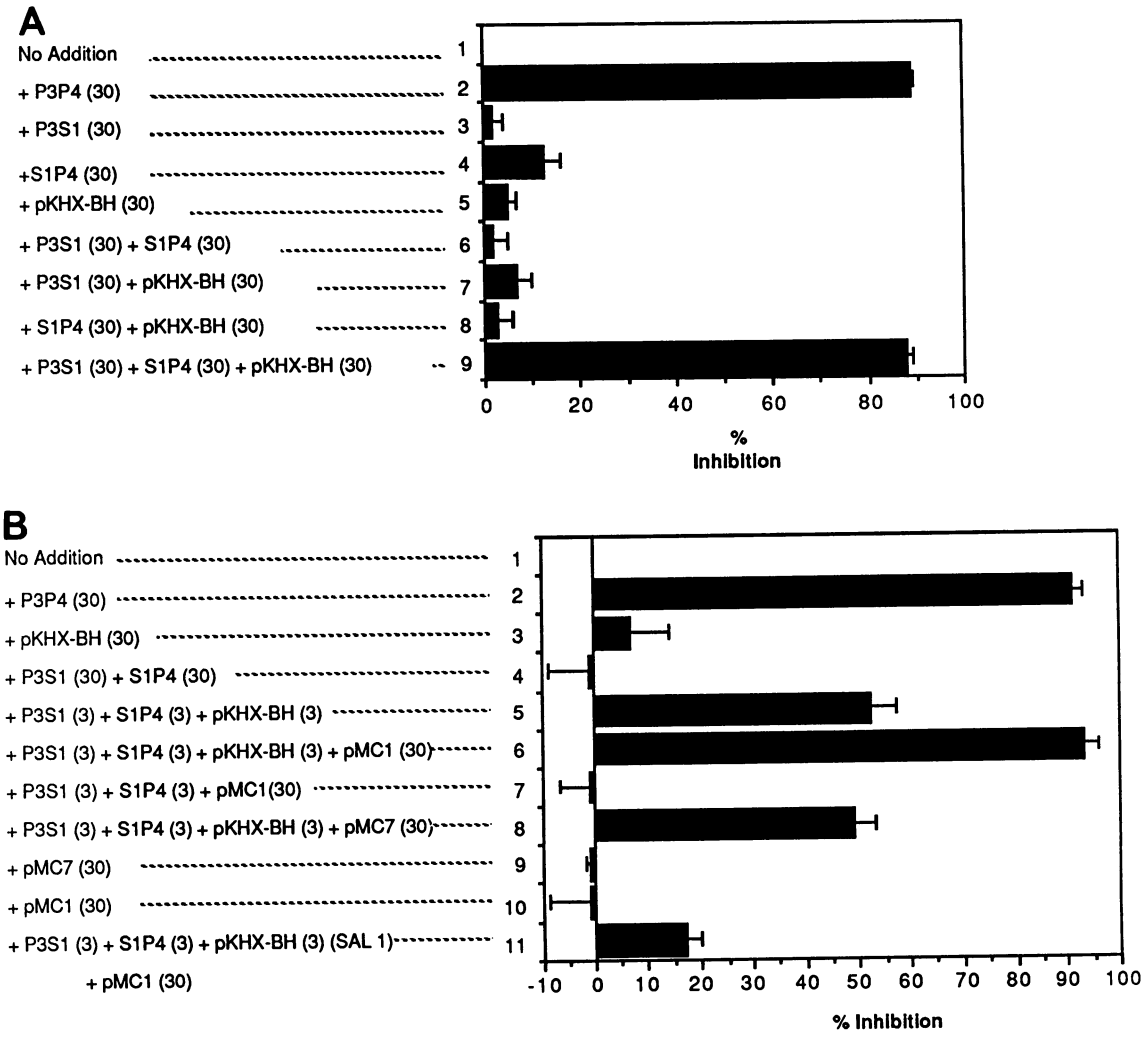

FIG. 4. (A) Antitransformation by subclones of P3P4. (B) Stimulation by Vmw65 of antitransformation by subclones of P3P4. Precipitates containing 200 ng of pHSV106 and 3 or 30 fmol of HSV subclones per ml as indicated were transfected into Ltk$^-$ cells. pBR325 was added as necessary so that each precipitate contained bacterial sequences at a concentration of 90 fmol/ml. Percentage inhibition values were computed as described in the legend to Fig. 3. Control values were 301 ± 7 and 149 ± 9 transformants per flask for panels A and B, respectively. All values are the average of two flasks. Error bars are standard deviations. In the left column, the various plasmids added are given. Values in parentheses show the concentration of each plasmid (in femtomoles per milliliter). Note that in panel B, row 11, pKHX-BH was digested with *Sal*I prior to transfection.

P3P4 was subcloned into two contiguous pieces, P3S1 and S1P4, dividing alpha 27. The combination of these two subclones was inactive in the antitransformation assay, presumably because alpha 27 was destroyed. However, two other alpha genes, alpha 0 and alpha 4, were capable of substituting for alpha 27. These results support the hypothesis that an alpha gene product and two sequences in P3P4 are necessary for antitransformation. Less is known about these sequences of P3P4. Debroy et al. (6) have reported a leftward-reading transcript that spans the *Bam*HI site at 0.747 m.u., but its function is unknown. Since restriction enzyme cleavage of these sequences inactivated antitransformation, it seems likely that these regions of P3P4 also act in *trans* to effect antitransformation. Whether the alpha genes *trans*-activate these sequences or act with the products of genes in P3S1 and S1P4 to effect antitransformation is not known. That is, the alpha gene product may be required, along with other gene products, for inhibition of transformation, or it may be required only indirectly to produce the ultimate antitransforming products.

The antitransforming activity of pJK has not been studied in detail. However, it is clearly distinct from that in P3P4 and is contained entirely within the reiterated sequences common to EK and JK (data not shown). Preliminary studies indicate a requirement for an alpha gene (alpha 0 or alpha 27) as well as additional sequences between the 5′ end of alpha 0 and the 3′ end of alpha 4. Interestingly, a gene of unknown function in this region has recently been described (5). Therefore, pEK has two distinct antitransforming regions, one located in the reiterated sequences which also occur in pJK and another located at 0.743 to 0.782 m.u., which was subcloned as P3P4.

The data presented in this paper represent a genetic analysis of antitransformation and do not suggest a biochem-

ical mechanism. However, because several sequences have antitransforming activity, antitransformation may have multiple mechanisms. There are at least four ways in which HSV genes might interfere with transformation. First, HSV may contain genes that prevent integration of the selectable plasmid into the host genome. Since the plasmids used in these studies did not have suitable origins of replication, they could not be maintained as episomes in mammalian cells and transformed colonies would not be formed.

Second, certain HSV genes may interfere with transcription or translation of the selectable gene. Indeed, in the case of HSV *tk*, control of TK synthesis is at the transcriptional level, with the amount of *tk* mRNA greatest 4 to 5 h after infection and absent by 12 h after infection (2, 36). Since at least one beta-gamma mRNA accumulates throughout infection (17), *tk* mRNA may be specifically degraded. Antitransformation might be due to inhibition of transcription of the selectable marker gene or degradation of its mRNA. Also of interest in this regard is the observation that adenovirus E1a proteins inhibit transcription from the SV40 early promoter (39).

Third, HSV inhibits host cell polypeptide synthesis (9), and even transient inhibition in the face of HAT selection might be lethal to the cell. This inhibition occurs in at least two phases, one induced by a virion polypeptide and the second requiring polypeptide synthesis (9). The overall effect is disaggregation of host cell polyribosomes and degradation of host cell mRNA (9, 35). Since host cell shutoff appears to be complex, multiple genes may participate in the phenomenon.

Last, the activity of some HSV genes may be toxic to cells by other mechanisms. It has been found, for example, that expression of the adenovirus DNA-binding protein gene is toxic to human cells (18). The gene products of the antitransformation region might be similarly inhibiting to host cell growth.

Much has been learned about positive regulation of HSV genes; less is known about negative regulation. The use of this interference assay may reveal a number of classes of HSV genes that manifest themselves by antitransforming activity. Some of these genes may control such seemingly disparate phenomena as cell death during productive infection and the establishment of latency in appropriate cells.

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