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A Conserved Open Reading Frame That Overlaps the Herpes Simplex Virus Thymidine Kinase Gene Is Important for Viral Growth in Cell Culture

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Of 18 mutants containing clustered point mutations within UL24 (an open reading frame that overlaps the herpes simplex virus thymidine kinase gene on the opposite strand), 15 formed small plaques and were substantially impaired for virus growth in cell culture. Mutations conferring the small plaque phenotype disrupt regions of UL24 that share considerable sequence similarity with open reading frames common to herpesviruses of mammals and birds. We infer that UL24 is expressed and important for virus growth in cell culture and suggest that possible effects on UL24 should be considered in studies of thymidine kinase-deficient mutants.

Herpes simplex virus (HSV) types 1 and 2 encode a thymidine kinase (tk) that is able to phosphorylate thymidine and a variety of other pyrimidine deoxynucleosides and various nucleoside analogs (2, 8, 10, 13, 15, 24). The *tk* gene and its mRNA and polypeptide products have been mapped onto the HSV genome at approximately 0.3 map unit, and the sequence of this region has been determined from several strains (5, 11, 14, 16, 17, 23, 25; A. F. Irmiere, M. M. Manos, J. G. Jacobson, J. S. Gibbs, and D. M. Coen, Virology, in press) (Fig. 1). During productive infection of cells in culture under ordinary conditions, tk is not essential for virus growth (8), although tk-deficient mutants have been shown to be impaired for growth in serum-starved cells (9, 13). Many such mutants are also impaired in their ability to mount acute and latent infections of mammalian hosts (for a review, see reference 20).

To study the regulated expression of the *tk* gene during HSV infection, a number of linker-scanning (LS) mutations—clustered point mutations created by replacement of wild-type sequences with a restriction enzyme linker—were introduced into the HSV-1 strain KOS chromosome at the *tk* gene promoter (4). Studies of the resulting HSV LS mutants identified promoter domains required for efficient *tk* expression during HSV infection (4). During construction of the HSV LS mutants, we observed by visual inspection that many of them appeared to form small plaques. The studies reported here document these observations and correlate the plaque size phenotypes of the HSV LS mutants with alterations in an open reading frame (ORF), conserved among many herpesviruses, that overlaps the *tk* gene on the opposite strand.

Certain HSV LS mutants form small plaques. To determine whether the apparent differences in plaque size were significant and to test whether there was a correlation between plaque sizes and levels of *tk* expression, crystal violet-stained plaques from HSV mutants PKG7, LS −16/−6, LS −21/−12, and LS −105/−95 were measured with a standard plaque assay (3). PKG7 (Irmiere et al., in press), like the LS mutants, contains a *tk* mutation conferring temperature-dependent drug resistance derived from mutant KG111 and a DNA polymerase mutation derived from antimutator strain PAA^{r}5. It does not contain an LS mutation and thus it serves as a control virus. These four viruses vary in terms of *tk* expression, with HSV LS −21/−12 and LS −105/−95 expressing roughly half as much tk (4; unpublished results) as the other two viruses (Table 1). Wells containing plaques from the various viruses were coded, and the plaques were measured blindly. Table 1 shows the results of these measurements in terms of mean plaque diameter. On average, HSV LS −21/−12 and LS −16/−6 had plaque diameters 2 to 3 times smaller than those of the other two viruses. The Student *t* test showed that this difference was significant, validating our conclusions from visual inspection. However, there was no correlation between plaque sizes and levels of *tk* expression. Similar results were obtained in repeat experiments.

Also included in Table 1 are the peak virus yields per cell after infection of 2.5×10^5 Vero cells by each mutant at a multiplicity of infection of 5 PFU/cell at 37°C. Inocula were titrated to verify this multiplicity of infection. The yields of HSV LS −16/−6 and LS −21/−12 were between 40- and 200-fold lower than those of the other two viruses, consistent with the differences in plaque sizes. Such reductions in virus yield probably explain why it was difficult to obtain stocks with titers of $>2 \times 10^7$ PFU/ml of the LS mutants that by visual inspection form small plaques, while stocks of those that form large plaques routinely gave titers of $>10^8$ PFU/ml (unpublished results).

Examination of the kinetics of virus growth of the four viruses from yield experiments such as those reported in Table 1 revealed little difference in the times at which the mutants entered into and came out of the eclipse phase (data not shown). In addition, no correlation was found between plaque size and the ratio of extracellular virus to intracellular virus or between plaque size and thermal stability of virus stocks (data not shown).

Of the 18 HSV LS mutants (4), 15—all except HSV LS −105/−95, LS −95/−85, and LS +5/+15—exhibited a small

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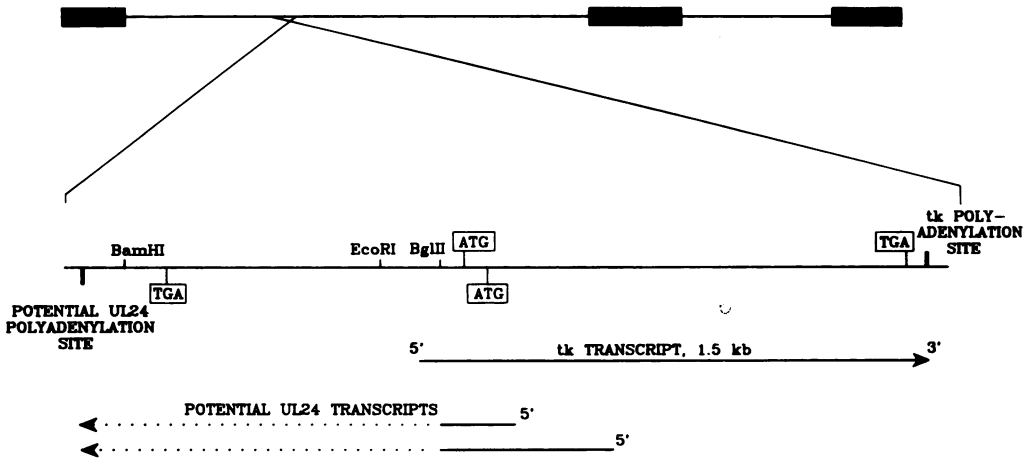

FIG. 1. A map showing the region of the HSV-1 genome which contains the *tk* and UL24 genes. The top line is a schematic representation of the HSV genome in the prototype arrangement. ■, Major repeat sequences. The next line shows the location of the restriction sites for *Bam*HI, *Eco*RI, and *Bgl*II in the region of the *tk* gene. The ATG and TGA marked above the line indicate the beginning and end of the *tk* coding region, and the locations of the *tk* transcript start site and polyadenylation site are shown (5, 11, 16, 17, 25; Irmiere et al., in press). The ATG and TGA marked below the line indicate the beginning and end of the UL24 ORF (16). The 5′ ends of two potential UL24 transcripts shown are those mapped in vivo by Read et al. (21) and Wilkie et al. (26). These transcripts have only been mapped to the right of the *Bgl*II site. The transcripts are continued as dotted lines between the *Bgl*II site and the potential UL24 polyadenylation site (16), as no intervening polyadenylation signals have been identified.

plaque phenotype by visual inspection. The 15 small plaque HSV LS mutants, but not LS −105/−95, LS −95/−85, and LS +5/+15, formed syncytial plaques at 39°C. All 18 LS mutants formed nonsyncytial plaques at 34°C (data not shown). At 37°C, the plaque morphology varied from assay to assay, which we ascribe to incomplete penetrance at the intermediate temperature. Both the small plaque phenotype and the syncytial plaque morphology could be ascribed to the 15 LS mutations because they were observed in at least two independent viruses derived from each original LS plasmid. Thus, the LS mutations define a function that is required for normal virus growth and plaque morphology in cell culture, at least in the genetic background of the HSV LS mutants, and map it to a region that overlaps the 5′ end of the *tk* gene.

The LS mutations disrupt a conserved ORF that overlaps the *tk* gene. Gompels and Minson reported a partial ORF in the 5′ region of the *tk* gene from HSV-1 strain HFEM in a head-to-head orientation to, and overlapping, the *tk* gene (11). The complete version of this ORF has been sequenced from HSV-1 strain 17 by McGeoch et al., who have designated it UL24 (16). While determining the sequence of the *tk* gene of herpesvirus of turkeys (HVT) (S. L. Martin, D. I. Aparisio, and P. K. Bandyopadhyay, submitted for publication), we found an ORF, similar to UL24, in a head-to-head orientation with respect to the HVT *tk* gene. We therefore asked whether similar ORFs were present in other herpesviruses. We learned that such ORFs were present in human cytomegalovirus (P. Tomlinson, C. M. Brown, A. T. Bankier, and B. G. Barrell, personal communication), Epstein-Barr virus (1), equine herpesvirus (G. Robertson, personal communication), herpes simplex virus type 2 (HSV-2) (14, 23), herpesvirus saimiri (U. Gompels, personal communication), and varicella-zoster virus (6). In all these viruses, except cytomegalovirus, the UL24-like ORF is also in a head-to-head orientation with respect to the viral *tk* gene. However, no such strong sequence similarity was observed between UL24 and an ORF with a similar orientation to the viral *tk* gene, deduced from the sequence of the region of marmoset herpesvirus encoding its tk (19).

The predicted amino acid sequences of ORFs from the eight herpesviruses, arranged so that alignment to UL24 is maximal, were compared by using the algorithm of Needleman and Wunsch (7; see also reference 18) (Fig. 2). The alignment reveals five regions of strong sequence similarity. Of the 15 small plaque HSV LS mutants, 14 have mutations altering—either by substitution or by causing a frameshift—one or more of the amino acids contained in the five regions strongly conserved among the eight herpesviruses. The small plaque virus that is the exception, HSV LS −84/−74, contains a mutation immediately adjacent to one of these regions; perhaps this mutation leads to some disruptive effect on the adjacent conserved region. None of the mutations in the three LS viruses retaining the large plaque phenotype alters any conserved amino acids (Fig. 2). Thus, the small plaque phenotype is highly correlated with alterations in regions of UL24 that are strongly conserved among the herpesviruses. We infer that UL24 is expressed during HSV infection and is a function that is important, although not absolutely required, for virus growth in cell culture.

TABLE 1. Expression of *tk*, viral plaque size, and growth

Virus	*tk* expression[a]	Mean plaque size (mm)[b]	Burst size[c]
PKG7	1	0.6	58
HSV LS −16/−6	1	0.3	1
HSV LS −21/−12	0.5	0.2	0.4
HSV LS −105/−95	0.6	0.6	42

[a] The value given under *tk* expression is the relative level of *tk* expression as measured by primer extension of *tk* RNA (4).

[b] Mean plaque size represents the average width of crystal violet-stained plaques at their widest points after a standard plaque assay (3). The Student *t* test yields a probability, $P < 0.05$, that the mean plaque sizes of HSV LS −16/−6 and HSV LS −21/−12 represent the same plaque size population as the mean plaque sizes of PKG7 and HSV LS −105/−95. The number of plaques counted was between 32 and 56 for each sample.

[c] Burst size indicates the peak yield, in PFUs per cell, of a single-cycle virus growth experiment.

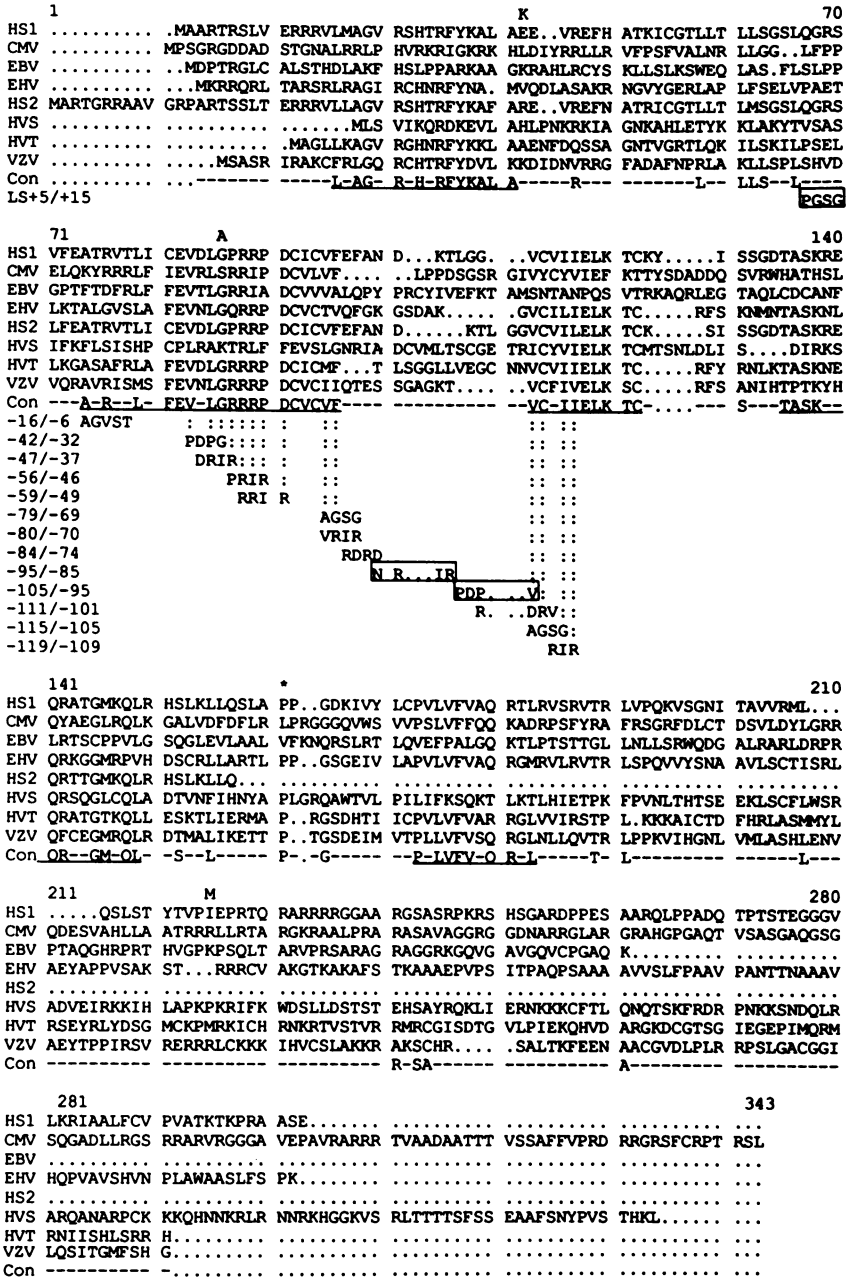

FIG. 2. Optimal alignment of the predicted amino acid sequences of the UL24-like ORFs of seven other herpesviruses (1, 6, 14, 23; Tomlinson et al., G. Robertson, and U. Gompels, personal communications; Martin et al., submitted) to the HSV-1 strain 17 UL24 (HS1) sequence (16). Dots indicate gaps inserted into the sequences to achieve this optimal alignment. Amino acids shown in the consensus line are present at the position in four or more of the aligned sequences. We designated five regions as strongly conserved (shown underlined in the figure). Each of these regions was nine or more amino acids in length and had greater than 65% conserved amino acids. Letters appearing above the HS1 sequence represent amino acids which are different in the predicted amino acid sequence of another HSV-1 strain. The K at position 31 is found in strains KOS and clone 101 (25; Irmiere et al., in press). The A at position 75 is found in strain MP (11). The asterisk at position 150 indicates where the clone 101 (25) nucleotide sequence is frameshifted with respect to the strain 17 sequence. The predicted amino acid sequence for clone 101 continues for only six more amino acids before encountering a stop codon. The M at position 214 is found in strain KOS (Irmiere et al., in press; unpublished results). Available nucleotide sequence data for these other strains were not sufficient to predict the amino acid sequence for the whole UL24 ORF; therefore, there may be more strain-specific differences than are shown here. The HSV-2 (HS2) sequence shown was derived by translation of nucleotide sequence reported by Swain and Galloway (23). The HS2 nucleotide sequence of this region reported by Kit et al. (14) contains a frameshift relative to the Swain and Galloway sequence and, when translated, yields an UL24-like ORF beginning at position 62 on this figure, thereby lacking the most N terminal of the strongly conserved regions. Below the consensus line are the predicted amino acid changes of the LS mutants. The LS changes have been gapped where the HS1 sequence is gapped. The amino acid alterations of the three HSV LS mutants which do not display the small plaque phenotype, HSV LS +5/+15, LS −95/−85, and LS −105/−95, are boxed. All the remaining HSV LS mutants made small plaques. There are four small plaque mutants, HSV LS −7/+3, LS −21/−12, LS −29/−18, and LS −70/−61, that are not shown in this figure. The LS mutations in these viruses resulted in frameshifts within UL24. For ease of reference, dotted lines are drawn from conserved amino acids to LS mutations that alter them. Abbreviations: CMV, human cytomegalovirus; EBV, Epstein-Barr virus; EHV, equine herpesvirus 1; HVS herpesvirus saimiri; VZV, varicella-zoster virus; Con, censensus.

Transcripts that potentially could encode UL24 have been reported by several laboratories (12, 21, 26). The 5′ ends of two potential UL24 transcripts are shown in Fig. 1. We are currently investigating the possibility that one or more of these transcripts is involved in expression of the UL24 gene product.

In HVT, the *tk* gene and the UL24-like ORF are head-to-head but nonoverlapping, assuming that the initiator methionines for these reading frames are those indicated by Martin et al. (submitted) and in Fig. 2. The codons for these two methionines are separated by 93 nucleotides. In scanning the 6-kilobase-pair region encompassing the HVT *tk* gene for sites that would allow insertion of the kanamycin resistance (Kan^r) gene from pUC4K, we were unable to recover viruses with inserts in the UL24-like ORF, although recombinant viruses with insertions adjacent to the 3′ end of the *tk* gene were consistently recovered (P. K. Bandyopadhyay, D. I. Aparisio, R. Florkiewicz, D. Doherty, and S. L. Martin, submitted for publication). We were able to recover viruses with insertions of the Kan^r gene in the 5′ end of the *tk* gene near the UL24-like ORF by selecting for resistance to arabinosylthymine. However, these mutant viruses grew poorly, yielding titers <1/1000th those of wild-type HVT and <1/100th those of tk-deficient viruses with mutations in the 3′ end of the HVT *tk* gene such as mutant ATR^o described by Martin et al. (submitted). These results suggest that, like the HSV UL24, the HVT UL24-like ORF is important for virus growth in cell culture.

Sanders et al. (22) have reported that two HSV mutants containing deletions covering much of the *tk* gene and its promoter grew poorly in BHK-21 cells in culture. These deletion mutants, like the LS mutants, also formed small syncytial plaques at 37°C. The deletion mutations would evidently disrupt *tk*, UL24, and several smaller ORFs, the *tk* promoter, and putative promoters for three other transcripts that map (21) to this region. It is possible that the disruption of UL24 is responsible for the phenotype of these mutants. However, Sanders et al. (22) reported alterations in eclipse phase for these two deletion mutants that we did not observe in the LS mutants (data not shown).

Because UL24 overlaps the *tk* gene, many previously reported HSV tk-deficient mutants may also contain mutations that affect UL24 or its expression. Although it is clear that certain UL24 mutations such as LS −105/−95 do not affect HSV growth in cell culture under normal conditions, it is possible that they could affect HSV growth under other culture conditions or during HSV infection in animal models of latency and pathogenesis.

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