In agreement with our results shown here for voltage-gated ion channels analyzed by patch-clamp techniques, transfection of BC_3H1 cells with the Val^{12} *ras* gene (but not the c-*myc* vector) was sufficient to prevent the induction of muscle differentiation products measured at the level of mRNA abundance. It is possible that the coordinate regulation of multiple channels, whose induction is contingent on mitogen withdrawal and is prevented by a single missense mutation, is interpreted most simply in the context of models for the control of muscle-specific gene induction via the recognition of shared, *cis*-acting DNA sequences (*24*). Since the regulatory events underlying the expression of voltage-gated channels during development are likely to be complex, it should prove advantageous to exploit a clonal system such as the present one, in which genetic manipulations produce clear-cut phenotypic effects (*25*).

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Clustering of Genes Dispensable for Growth in Culture in the S Component of the HSV-1 Genome

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The herpes simplex virus 1 genome consists of one long and one short stretch of unique sequences flanked by inverted repeat sequences. The nucleotide sequence and RNA map predict 12 open reading frames designated as US1 through US12 within the short stretch of unique sequences. This paper reports the construction of virus mutants from which US2, US3, or US4 had been deleted that are capable of growth in cell culture. One of the three deleted genes, US4, specifies the viral envelope glycoprotein G. Mutants with deletions in US1, US8, US9, US10, US11, and US12 have been previously reported. The nine genes deleted from this region form two clusters, US1 through US4 and US8 through US12, and encode at least two and possibly more structural proteins. The presence of so many genes dispensable for growth in cell culture suggests several hypotheses regarding their function and evolution.

THE 150–KILOBASE PAIR (KBP) HERpes simplex virus 1 (HSV-1) genome consists of two covalently linked components (L and S). Each component consists of unique sequences (U_l or U_s) flanked by inverted repeats (*1–3*). The inverted repeats of the L component, designated as *ab* and *b'a'* (*4*), each contain in their entirety two genes (α0 and γ34.5) (*5–7*); those of the S component, designated as *a'c'* and *ca*, each contain a copy of the α4 gene (5, 6) and an origin of viral DNA synthesis (*8–10*). The nucleotide sequence of the U_s sequence predicts the presence of 12 open reading frames designated as US1 to US12 (*11*). The products of several of these open reading frames have been identified as those of the α proteins α22 (US1) and α47 (US12) and of the glycoproteins G (US4), D (US6), and E (US8). Other studies have shown that virus mutants with deletions in the α22 gene or from which the entire domain of genes specifying gE (US8), US9, US10, US11, and US12 had been deleted are viable and multiply in cells in culture (*12, 13*). In this paper we report the deletion of DNA sequences encoding the open reading frames US2, US3, and US4. These genes are contiguous, mapping within the Bam HI N and Bam HI J fragments of HSV-1 strain F [HSV-1(F)] viral DNA (Fig. 1). On the basis of analyses of the nucleotide sequences, it was suggested that US2 specifies a membrane-translated protein (*11*) because of the predicted hydrophobic NH_2-terminal region, which could be a signal for membrane-bound translation. The predicted amino acid sequence for US3 is homologous to members of a protein kinase family in eukaryotes and retroviruses (*11, 14*). US4 encodes glycoprotein gG (*15, 16*).

The procedures used for construction of the deletion mutants were similar to those described previously (*12, 17*). Specifically, a chimeric thymidine kinase gene consisting of the promoter-regulatory domain of the α27 gene fused to the transcribed domain of

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the thymidine kinase (α27-TK) gene was inserted in place of a small Nco I fragment within US3 (Fig. 1). The chimeric gene was inserted in a 4.89-kbp DNA fragment of HSV-1(F) cloned as pRB3446; the fragment consisted of a 4.47-kbp Hind III–Sac I fragment (Fig. 1 line 7, arrows) plus the adjacent 0.42-kbp Sac I–Hind III fragment. The DNA of the resulting plasmid, pRB3635, was co-transfected into rabbit skin cells (*18*) with intact DNA of the TK^- recombinant HSV-1(F)Δ305, which has been previously described (*12*). The progeny of the transfection were then used to infect $143TK^-$ cells in HAT (hypoxanthine, aminopterin, thymidine) medium, in which only TK^+ viruses can grow (*13*). The DNA of the selected TK^+ recombinant, designated as R7035, was then analyzed by restriction endonucleases to verify the insertion of the α27-TK gene in its entirety in the Bam HI subfragment N and the Hind III subfragment N. Bam HI digests of R7035 DNA indicated the loss of the wild-type Bam HI N fragment and the appearance of a new band designated as 1 in Fig. 2. As expected, the novel band hybridized with labeled DNA sequences containing the thymidine kinase gene (Fig. 2, probe pRB103, band 1). The smaller Bam HI Q fragment designated ΔQ in Fig. 2 contains the 700-bp deletion within the thymidine kinase gene present in the parental HSV-1(F)Δ305 virus. Hind III digests of R7035 DNA also indicated the loss of wild-type Hind III N and the appearance of a new band designated as 1 in Fig. 2. As expected, the novel band hybridized with labeled DNA of Bam HI J (pRB123), which contains sequences from both the Hind III subfragments N and G, and with labeled Bam HI Q (which contains the thymidine kinase gene). These experiments indicated that the selectible marker gene α27-TK was inserted at the predicted site shown in Fig. 1, line 8.

To delete sequences from US2, US3, and US4, we constructed plasmids with specific deletions in the 4.47-kbp Hind III–Sac I fragment (Fig. 1, line 7, arrows) cloned as pRB425. In pRB3695 the deletions extend from the Kpn I site in US2 to the Bam HI

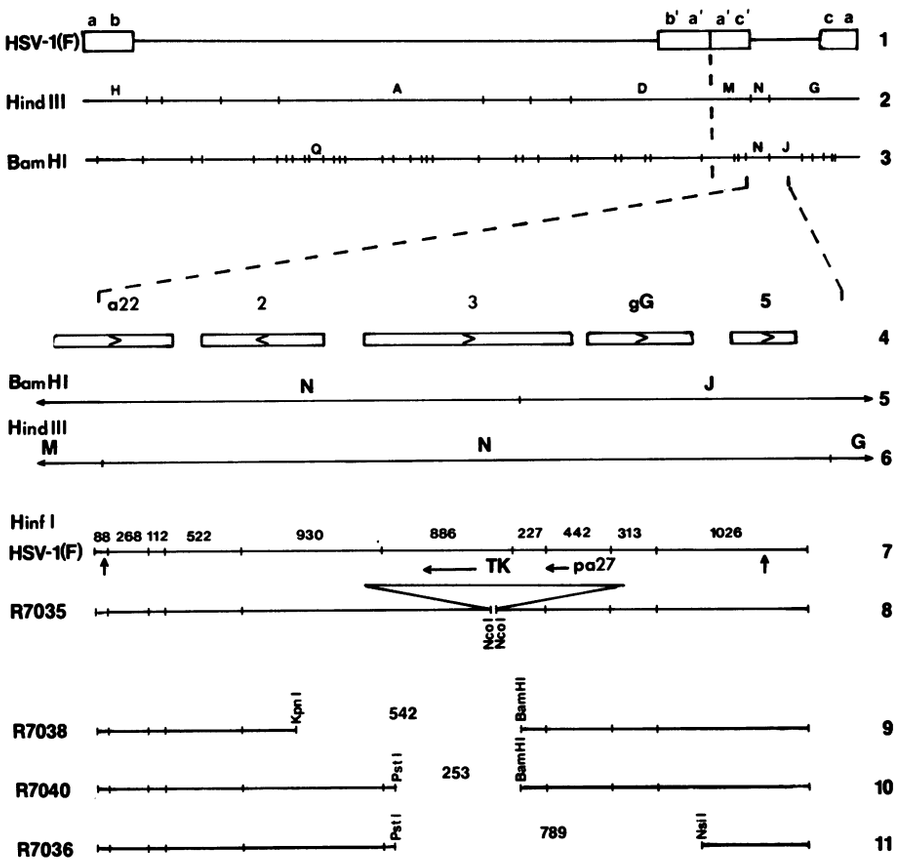

Fig. 1. Schematic representations of the DNA sequence arrangements in HSV-1(F) and the HSV-1(F) recombinants R7035, R7036, R7038, and R7040. The boxes in line 1 represent the inverted repeat sequences that are repeated internally and divide the HSV-1(F) genome into the long (L) and the short (S) components. Lines 2 and 3 show the Hind III or Bam HI restriction endonuclease maps of HSV-1(F) DNA. The fragments pertinent to this report are shown with their letter designation. Line 4 shows an expanded representation of the coding regions that traverse the junction between the Bam HI N and Bam HI J fragments of HSV-1(F) DNA. Numbers correspond to the open reading frames that have been identified by sequence analysis. Lines 5 and 6 show the Bam HI and Hind III maps for the region shown in line 4. Line 7 shows the Hinf I map that corresponds to the region shown in lines 4, 5, and 6. The arrows in line 7 indicate the Hind III–Sac I fragment of HSV-1(F) DNA cloned as pRB425 and used in the construction of the deletion plasmids and as a probe in Fig. 3. Lines 8, 9, 10, and 11 show the sequence arrangement and Hinf I maps of the HSV1(F) recombinants R7035, R7036, R7038, and R7040 in the region shown in line 4. Numbers correspond to the lengths of the expected fragments (in base pairs) predicted by the sequence of the region (*14*).

Table 1. Multiplication of HSV-1(F) and of deletion mutants in Vero cells (*27*); PFU, plaque forming units.

Virus	PFU per cell	Titer* ($\times 10^5$)	Genotype
HSV-1(F)	5	11	Wild type
R7036	5	12	$US3^-$, gG^-
R7038	5	8	$US2^-$, $US3^-$
R7040	5	9	$US3^-$
HSV-1(F)	0.05	1.0	Wild type
R7036	0.05	1.2	$US3^-$, gG^-
R7038	0.05	0.9	$US2^-$, $US3^-$
R7040	0.05	0.9	$US3^-$

*The 48-hour virus yields were titered on Vero cell monolayer cultures and are expressed as plaque-forming units per milliliter.

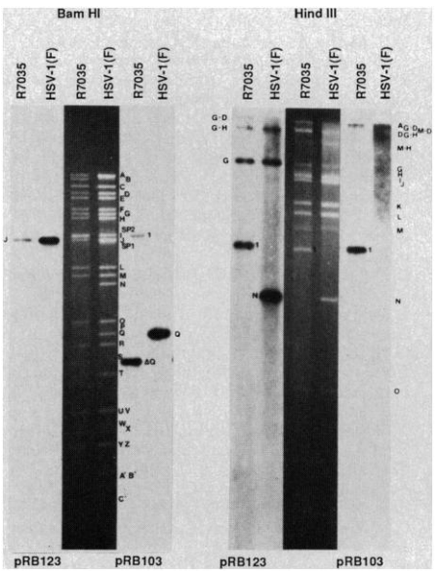

Fig. 2. Photographic and autoradiographic images of electrophoretically separated Bam HI or Hind III digests of HSV-1(F) and R7035 viral DNAs. The viral DNA was prepared as described (*24*); cleaved, separated, and stained (*25*); and transferred to nitrocellulose (*15*). The cloned fragments pRB103 [Bam HI Q fragment from HSV-1(F)] or pRB123 [Bam HI fragment J from HSV-1(F)] were labeled with [^{32}P]dCTP by nick translation with a kit from New England Nuclear. Hybridization conditions were as described (*26*). Letters refer to either the Bam HI fragments or Hind III fragments of HSV-1(F) DNA. The bands designated as "1" are the novel bands generated by the insertion of the chimeric α27-TK gene into the R7035 viral genome. The band ΔQ identifies the Bam HI Q fragment of HSV-1(F)Δ305 from which 700 bp had been deleted.

site located in US3 (Fig. 1, line 9). The deleted sequences include the promoter-regulatory domains, the first 47 amino acids of US2, and the first 357 amino acids of US3. In pRB3696, the deletions extend from the Pst I site located 69 amino acids in from the 5′ end of US3 to the Bam HI site located at amino acid 357 of US3 (Fig. 1, line 10). In pRB3697, the deletions extend from the Pst I site 69 amino acids from the 5′ end of US3 to Nsi I site located 3 nucleotides downstream from the 3′ co-terminus of the messenger RNAs (mRNAs) specifying the proteins encoded by US3 and the glycoprotein G (Fig. 1, line 11).

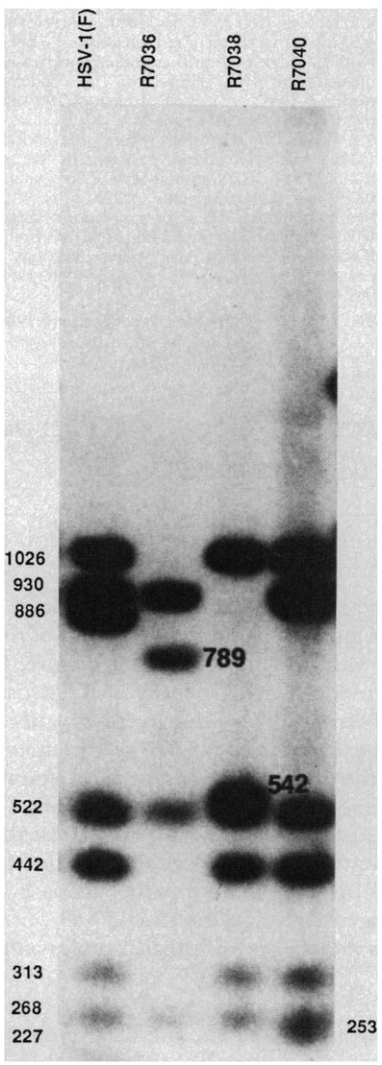

Fig. 3. Autoradiographic image of electrophoretically separated Hinf I digests of HSV-1(F), R7036, R7038, and R7040 DNAs. The ^{32}P-labeled pRB425 was hybridized to viral DNAs transferred to nitrocellulose and hybridized as described in the legend to Fig. 2. Probe pRB425 corresponds to the 4.47-kbp Hind III–Sac I fragment shown in Fig. 1, line 7. Numbers adjacent to HSV-1(F) correspond to the sizes of the bands in base pairs hybridizing to the probe and are schematically shown in Fig. 1, line 7. The numbers identify the novel bands formed by fusion of remaining portions of Hinf I fragments generated by the deletions shown in Fig. 1, lines 9, 10, and 11.

The deletion plasmids were each co-transfected with intact viral DNA from recombinant R7035 and TK$^-$ progeny were selected in 143TK$^-$ cells in the presence of bromodeoxyuridine (*13*). The recombinants R7038, R7040, and R7036 were then isolated; schematic representations of the deletions contained in each are shown in Fig. 1 (lines 9, 10, and 11, respectively). Confirmation of the deletions in the recombinant viruses is based on hybridization of labeled Hind III–Sac I DNA probe (pRB425) to electrophoretically separated Hinf I digests of the recombinant genomes. As shown in Fig. 3, in recombinant R7036 DNA (Fig. 1, line 11) the 886-, 227-, 442-, 313-, and 1026-bp Hinf I fragments (whose order is shown in Fig. 1, line 7) were replaced with a novel band of 789 bp predicted to be the product of fusion of the remaining portions of the 886- and 1026-bp bands. In recombinant R7038 DNA (Fig. 1, line 9), the 930-, 886-, and 227-bp Hinf I fragments were replaced by a novel band of 542 bp predicted to be the product of the fusion of the remaining portions of the 930- and 227-bp bands. In recombinant R7040 DNA (Fig. 1, line 10), the 886- and 227-bp Hinf I fragments were replaced by a novel band of 253 bp predicted to be the product of the fusion of the remaining portions of the two bands.

Recombinant R7036 was of particular interest because of the deletion of the sequences encoding glycoprotein G. Several experiments confirmed the absence of G from cells infected with this recombinant. First, unlike cells infected with wild-type virus or recombinants R7038 and R7040, the R7036-infected Vero cells did not react with monoclonal antibodies to glycoprotein G in the biotin-avidin–enhanced surface immunoassays as previously described (*15, 19*). Cells infected with wild-type or with mutant virus reacted in these assays with monoclonal antibodies to glycoproteins D and E. In the second series of experiments (Fig. 4), electrophoretically separated cell lysates of mutant virus-infected cells did not react with monoclonal antibody to glycoprotein G, while there was a reaction when lysates from cells infected with wild-type virus were used. Both cell lysates reacted with monoclonal antibody to glycoprotein B.

Studies on the growth properties of the recombinant viruses in Vero cells infected at multiplicities of 0.05 and 5 plaque-forming units per cell (Table 1) indicated that the recombinant virus yields were similar to those of the wild-type parent. The host range and other biological properties of these deletion mutants are under study.

The significance of the results communicated in this report stems from three observations. First, all three open reading frames (US2, US3, and US4) deleted in these studies are adjacent to each other and to the α22 gene (US1) previously shown not to be essential for growth in some cells in culture (*12, 20*). Second, of the 12 open reading

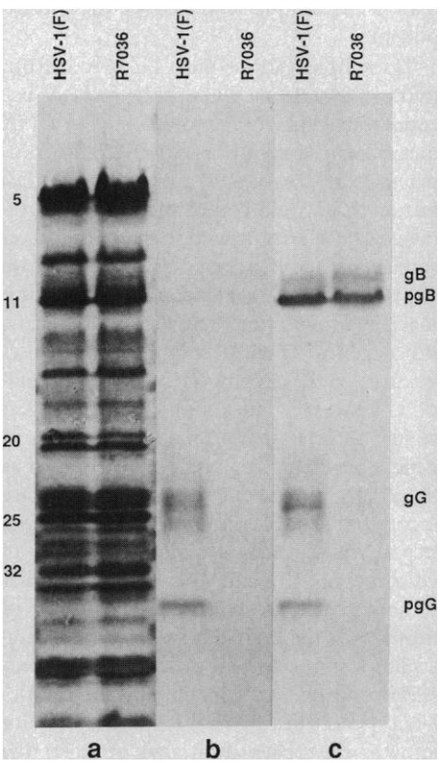

Fig. 4. Autoradiographic images and immune reactivity of infected cell polypeptides electrophoretically separated in a denaturing gel, transferred to nitrocellulose, and reacted with monoclonal antibodies H1379 and H1163. Cultures of HEp-2 cells were infected with 10 plaque-forming units of HSV-1(F) or R7036 per cell and labeled with [^{35}S]methionine (25 μCi/ml, 1.260 Ci/mmol, New England Nuclear) from 12 to 18 hours after infection. At 18 hours after infection cells were harvested and solubilized in denaturing buffer; the lysate was electrophoretically separated in denaturing polyacrylamide gels, transferred to nitrocellulose, and reacted with monoclonal antibodies as described (*15*). (**a**) Autoradiographic images of [^{35}S]methionine-labeled proteins from HSV-1(F)- and R7036-infected cells. (**b**) Immune reactivity of the electrophoretically separated, immobilized proteins shown in (a) with monoclonal antibody H1379 to glycoprotein G in an immunoperoxidase-coupled reaction as described by Ackerman *et al.* (*15*). (**c**) The immune reactivity of the proteins shown in (b) with monoclonal antibody H1163 to glycoprotein B after the reaction with monoclonal antibody to gG shown in (b). The pgG, gG, pgB, and gB designate the precursor and mature forms, respectively, of the glycoproteins G and B recognized by the monoclonal antibodies. The numbers to the left identify selective viral proteins by their infected cell protein (ICP) number designation.

frames reported to be encoded in the unique sequences of the S component of HSV-1 DNA, a total of nine genes in two clusters (US1 to US4 and US8 to US12) have now been shown to be "dispensable" for growth at least in some cell lines in culture (*12, 13, 20*). Third, of the six (B, C, D, E, G, and H) major HSV-1 glycoproteins identified to date, at least three (C, E, and G) appear to be not essential for growth (*13, 21*), and both glycoproteins G (US4) and E (US8) map in the unique sequences of the S component.

These observations must be viewed from two considerations. First, because HSV encodes many enzymes involved in viral DNA metabolism, it could be expected that competent cells may supply enzymes and other factors that would complement the requirements of mutants deleted in genes encoding similar factors. This appears to be the case for thymidine kinase and $\alpha 22(US1)^-$ mutants, and might be the case for at least some other genes deleted from the unique sequences of the S component. This explanation, however, does not appear at first glance to fit mutants carrying deletions in genes specifying structural proteins such as the viral glycoproteins E and G, which are incorporated into the viral envelope. One hypothesis that may explain the viability of glycoprotein E^- and G^- mutants is that these proteins are required for survival of the virus in the human host but not for viral replication in cells that do become infected. Although the function of most of the deleted genes in the S component is not known, the observation that at least two (glycoproteins E and G) and possibly more (US3, US10, and US11) genes encode structural proteins raises a question concerning the significance of the clustering of so many of these "dispensable" genes. One hypothesis to explain this clustering is that the genes that evolved in the unique sequences of the S component endow HSV with ability to survive in the environmental niche of its human host rather than to replicate or package its genome. Evolution of a cluster of genes with such functions would be greatly facilitated if the progenitor of HSV had acquired a DNA fragment in which these genes could evolve. The possibility that the evolutionary herpesvirus progenitor arose by insertion of a DNA fragment representing the S component into a larger DNA fragment encoding genes essential for viral replication was postulated elsewhere (*2, 22*).

Note added in proof: Subsequent to the submission of this report we deleted US7 and showed that it specifies a novel glycoprotein designated by the letter I (glycoprotein I) (*23*).

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27. Vero cells (African Green monkey kidney line) were used for the preparation of viral stocks, virus titrations, and the preparation of viral DNA from infected cell lysates. HEp-2 cells (human epidermoid carcinoma line) were used in the preparation of infected cell proteins as described in Ackermann *et al.* (*15*). Growth medium consisted of Dulbecco modified Eagle's minimal essential medium supplemented with 10% calf serum. The maintenance medium, 199-V, consisted of mixture 199 supplemented with 1% calf serum.

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Rapid Identification of Nonessential Genes of Herpes Simplex Virus Type 1 by Tn5 Mutagenesis

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The large genome of herpes simplex virus type 1 (HSV-1) encodes at least 80 polypeptides, the majority of which have no recognized function. A subgroup of these gene products appears to be nonessential for virus replication in cell culture, but contributes to the complex life cycle of the virus in the host. To identify such functions, a simple insertional mutagenesis method has been used for selective inactivation of individual HSV-1 genes. The bacterial transposon Tn5 was allowed to insert randomly into cloned restriction fragments representing the entire short unique (U_s) region of the HSV-1 genome. Of the 12 open reading frames that were mutagenized with Tn5, mutant derivatives of US2, US4, and US5 were recombined into the virus. These three genes proved to be nonessential for HSV-1 replication in Vero (African Green monkey kidney) cells and the US4 gene appeared to be involved in viral pathogenesis in the central nervous system of mice. This rapid mutagenesis procedure should prove useful in exploring the entire HSV-1 genome as well as the genomes of other complex animal viruses.

THE CLINICAL MANIFESTATIONS OF herpes simplex virus type 1 (HSV-1) infection are most often associated with orofacial lesions, although infrequent cases of encephalitis caused by HSV-1 have been reported. Treatment of HSV-1 infections is complicated by the ability of this pathogen to establish a latent infection in the peripheral nervous system of the host, which provides a reservoir of virus for recurrent lesions (*1*). It is probable that specific viral functions are involved in both latency and pathogenesis within the nervous system. Indeed, a number of HSV-1 genes have been described that are not essential for growth in cell culture but that nevertheless play a role in infection in vivo (*2*). Mutants carrying alterations in such nonessential genes are difficult to isolate, due to the absence of identifiable phenotypes. Large-scale DNA sequencing of the 150–kilobase pair HSV-1 genome is currently under way, so that many putative HSV-1 genes with unknown functions have now been identi-

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