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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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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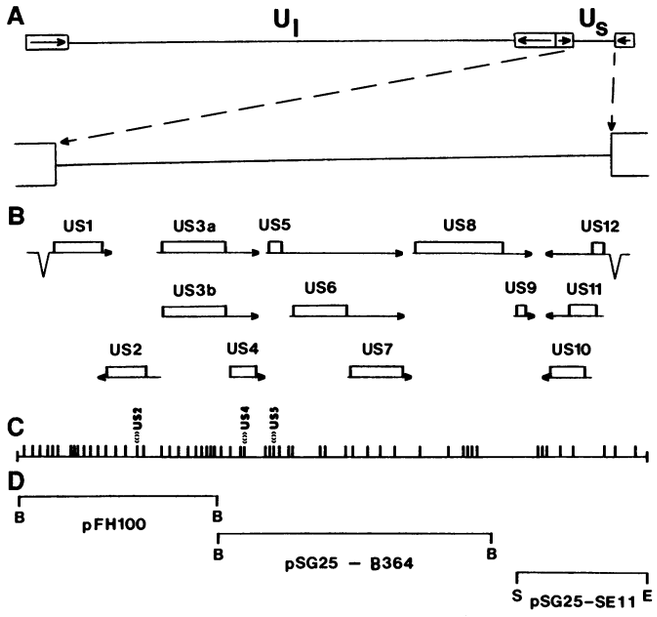

Fig. 1. Tn5 mutagenesis of the short unique region of the HSV-1 genome. (**A**) Expansion of the 13-kb short unique (U_s) region of the HSV-1 genome. (**B**) The 12 genes of U_s, as determined by DNA sequence and RNA transcript analysis (*4*). US1 and US12 encode the immediate early proteins ICP22 and ICP47, respectively (*10*); US4, US6, and US8 encode the glycoproteins gG, gD, and gE, respectively (*11*); and US9 encodes a virion phosphoprotein (*12*). The functions and gene products of the remaining six genes are unknown, although US3 may be a protein kinase (*13*), US5 and US7 may be glycoproteins (*4*), and US11 may be a DNA binding protein (*14*). (**C**) Location of Tn5 insertion mutations within the U_s region. The three Tn5 insertions that were recombined into HSV-1 in this work are indicated. (**D**) Cloned restriction fragments used in Tn5 mutagenesis experiments. pFH100 is the Bam HI n fragment cloned in pBR322, pSG25-B364 is the Bam HI j fragment cloned in pBR325, and pSG25-SE11 is a 3.7-kb Sal I/Eco RI fragment spanning Bam HI fragments x and z cloned in pBR325 (*15*).

fied. A simple method for insertional mutagenesis of these genes by means of the bacterial transposon Tn5 is presented here, so that viral functions that are dispensable for growth in vitro, but that may be essential for replication in the natural host, can be readily discerned.

Tn5, a transposable element carrying a kanamycin resistance marker, has been used extensively for insertional mutagenesis in prokaryotes (*3*). The random insertion of Tn5 into a gene results in truncation of the corresponding gene product, due to the presence of amber codons in all three reading frames of the transposon termini. The short unique (U_s) component of the HSV-1 genome (Fig. 1A) was chosen for study for two reasons: (i) its DNA sequence and RNA transcripts have recently been determined (*4*), and (ii) it is likely to contain a number of nonessential genes, since two deletion mutations in U_s have been mapped (*5*) and no temperature-sensitive mutants in U_s have yet been found. The U_s region consists of 12 genes, most of which have uncharacterized functions (Fig. 1B).

Three cloned restriction fragments spanning the entire U_s region (Fig. 1D) were mutagenized by infecting plasmid-carrying *Escherichia coli* DH1 cells with the defective phage λ467::Tn5 and selecting for kanamycin resistance as described (*3*). The positions of Tn5 insertions within a plasmid can be accurately and unambiguously determined with only two restriction enzymes (Fig. 2). About 60 random Tn5 insertions from three mutagenesis experiments were mapped throughout U_s in this manner (Fig. 1C). Mutations near the 5′ ends of individual genes were selected for incorporation into the viral genome by homologous recombination between mutant plasmid and HSV-1 genomic DNA. The procedures used for cotransfection of DNA into Vero cells, as well as for detection of viral recombinants by an in situ plaque hybridization assay (with radiolabeled restriction fragments of Tn5 as a probe) were described previously (*6*). DNA was then isolated from plaque-purified recombinant viruses to verify the presence of Tn5 in the correct position in the HSV-1 genome. The initial characterization of three such viral mutants, US2::Tn5, US4::Tn5, and US5::Tn5 is described.

Since accurate mapping of the Tn5 insertions had already been carried out on the

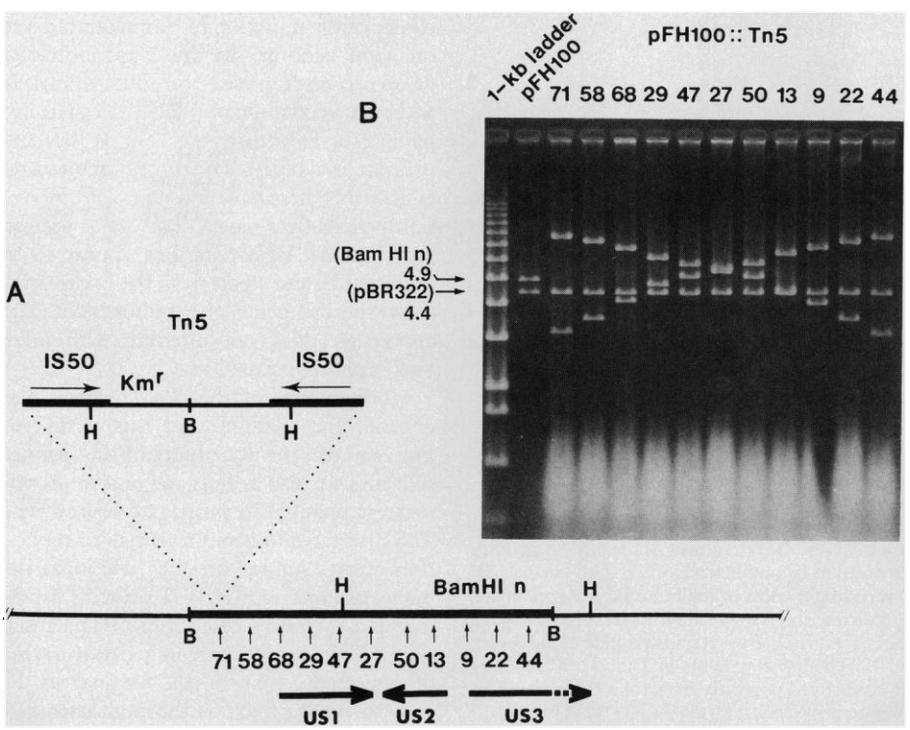

Fig. 2. Mapping of Tn5 insertions within the Bam HI n fragment of the HSV-1 U_s region. pFH100 consists of the 4.9-kb Bam HI n fragment cloned into pBR322. The exact locations of 11 Tn5 insertions within Bam HI n (**A**) were determined by digesting pFH100::Tn5 isolates with Bam HI and Hind III. These represent a sample of the total number of Tn5 insertions isolated within pFH100 (Fig. 1). Digestion with Bam HI indicated whether Tn5 had inserted into the pBR322 vector (causing a loss of the 4.4-kb Bam HI fragment of pFH100), or into Bam HI n (causing a loss of the 4.9-kb Bam HI fragment of pFH100, as illustrated in the agarose gel in **B**). Bam HI digestion was also useful in the initial mapping of the Tn5 insertions, since a single Bam HI site maps approximately in the center of the transposon (that is, 2.7 kb from the end of Tn5). More accurate and unambiguous mapping of the Tn5 insertions was obtained by performing Hind III digests on pFH100::Tn5 DNA, since Hind III sites map exactly 1.2 kb from each end of the transposon. (IS50 and Km^r are the inverted insertion sequences and the kanamycin resistance gene present in Tn5, respectively; B, Bam HI site; H, Hind III site; US1, US2, and US3 are open reading frames within the U_s region.

plasmids, only one restriction enzyme digest of the mutant viral DNA was necessary to confirm the proper recombination of Tn5 into the viral genome. If the Tn5 insertions in genes US2, US4, and US5 recombined correctly into the viral genome, then the Hind III n fragment (HSV-1 map units 0.867–0.905) should be replaced by three new fragments, because of the presence of two Hind III sites in Tn5 (Fig. 3A). The Hind III digests of each of the three viral recombinants (Fig. 3B) confirmed the absence of the Hind III n fragment and the presence of three novel Hind III fragments whose sizes matched those predicted. The identity of these novel fragments with Tn5 was confirmed by hybridization with radiolabeled Tn5 restriction fragments (Fig. 3C). Thus, the Tn5 insertion mutations that had been created in *E. coli* were transferred to the HSV-1 genome at their homologous positions, and no additional alterations in the viral genome were apparent from these restriction enzyme digests.

The fact that the three recombinant viruses undergo productive infection in vitro demonstrates that the US2, US4, and US5 genes are not essential for HSV-1 replication in Vero cells. Studies on the infectivity of these viruses (Table 1) show that their replication was only slightly impaired at both high and low multiplicities, although this effect was more pronounced at a low multiplicity of infection for US2::Tn5. This mutant also consistently produced smaller plaque sizes. However, only the US4::Tn5 mutant revealed any defects in the ability to replicate in the central nervous system of mice (Table 1). It will be necessary to assess the ability of these viruses to establish and maintain latent infections in the sensory ganglia and to identify missing gene products encoded by US2, US4, and US5. Of these three genes, only US4 has a well-defined gene product, which is apparently the HSV-1 equivalent of the HSV-2 glycoprotein gG (*7*). The availability of mutations in these and other U_s genes should greatly facilitate the study of the role these functions play in the molecular biology of HSV-1 infections.

Table 1. Growth of HSV-1::Tn5 mutants in Vero cells and the mouse central nervous system.

Virus	MOI = 1.0		MOI = 0.01		Titer (LD_{50})†
	Titer (PFU/ml)*	Fold reduction in yield	Titer (PFU/ml)*	Fold reduction in yield	
HSV-1(KOS)	1.0×10^8		1.0×10^8		$10^{2.3}$
US2::Tn5	2.6×10^7	3.9	1.1×10^7	9.1	$10^{2.9}$
US4::Tn5	3.3×10^7	3.0	4.3×10^7	2.3	$10^{3.9}$
US5::Tn5	3.9×10^7	2.6	4.3×10^7	2.3	$10^{2.9}$

*Titers represent the yield of virus from a 60-mm dish of Vero cells infected at the indicated multiplicity. (MOI, multiplicity of infection; PFU, plaque-forming units.) †Titers represent the median lethal dose (LD_{50}) of virus after intracerebral inoculation into DBA/2 mice, determined by the methods of Kumel *et al.* (*16*). Briefly, each of five mice was injected intracerebrally with virus doses ranging from 10^1 to 10^6 PFU in half-$\log_{10}$ increments. The animals were observed daily for signs of neurologic disease. Death due to viral encephalitis was confirmed by histopathological examination and occurred between 3 and 5 days for all viruses except for the US4::Tn5 mutant (between 4 and 7 days).

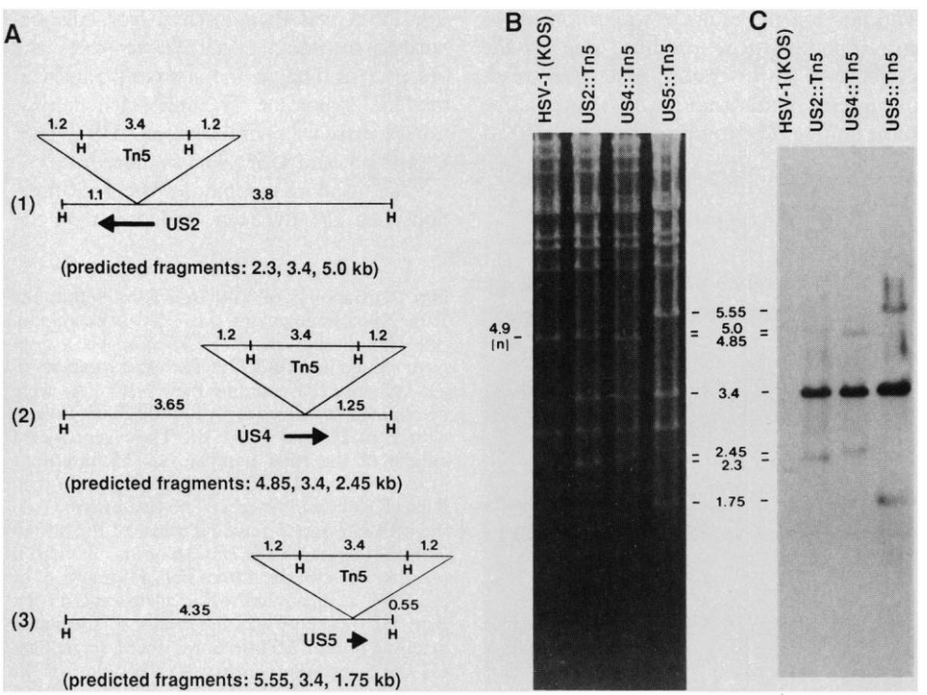

Fig. 3. Mapping of Tn5 insertions within the HSV-1 genome. (**A**) Location of the Tn5 insertions US2::Tn5 (1), US4::Tn5 (2), and US5::Tn5 (3) within the Hind III n fragment of the HSV-1 genome and the predicted sizes of novel Hind III fragments expected in recombinant HSV-1::Tn5 viruses. (**B**) Hind III digests of recombinant HSV-1::Tn5 viruses, revealing the loss of the Hind III n fragment and the appearance of the three novel Hind III fragments predicted in (A) for each virus. (**C**) Hybridization of novel HSV-1::Tn5 Hind III fragments to a Tn5 probe. The gel in (B) was transferred to Gene Screen Plus (NEN/Dupont) and hybridized with the 1.0- and 1.1-kb Pst I fragments of Tn5 (3), which were made radioactive by nick translation. These fragments are derived primarily from, and therefore show the strongest hybridization to, the 3.4-kb Hind III fragment of Tn5.

The Tn5 mutagenesis method offers a number of distinct advantages over other HSV-1 insertional mutagenesis procedures. The site-specific thymidine kinase gene insertion/deletion technique (*8*) involves synthesis of complicated plasmid constructions that must be recombined into the viral genome through a series of cotransfection experiments. The mini-Mu method (*9*) requires a multistep mutagenesis procedure in *E. coli*, and then extensive restriction enzyme mapping of assorted mini-Mu insertions after they have been recombined into the viral genome. In contrast, the Tn5 method involves relatively few steps and requires only two restriction enzymes for the mapping of Tn5 insertions. These insertions are mapped in the plasmid DNA, which allows the choice of the mutation to be introduced into the viral genome. In this way, individual genes can be "targeted" for mutagenesis. In addition, recombinant viruses are detected by in situ hybridization, so that selective pressure is avoided. The three mutant viruses described here have retained their inserted transposons over repeated passages, indicating that the Tn5 mutations appear to be quite stable, and, apart from the inactivation of a specific gene, the presence of Tn5 apparently does not interfere with other viral replicative functions.

Modifications of this Tn5 method may enhance the usefulness of this technique. For example, the availability of transformed cell lines for use as host range systems will make it possible to propagate viruses with Tn5 insertions in genes essential for replication. Also, studies on the functional domains of important HSV-1 proteins can be initiated by means of a series of Tn5 insertions within a given gene to generate a series of truncated gene products for analysis. Finally, a marker such as the β-galactosidase

gene could be inserted into Tn5, which would aid in the detection of recombinant viruses and could be used as a probe for tracing the extent of viral infection in the host. Thus, it appears that the Tn5 mutagenesis procedure described here will be applicable for analyzing large numbers of genes in HSV-1, and in other animal viruses with large complex genomes.

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Distribution of Cones in Human and Monkey Retina: Individual Variability and Radial Asymmetry

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The distribution of photoreceptors is known for only one complete human retina and for the cardinal meridians only in the macaque monkey retina. Cones can be mapped in computer-reconstructed whole mounts of human and monkey retina. A 2.9-fold range in maximum cone density in the foveas of young adult human eyes may contribute to individual differences in acuity. Cone distribution is radially asymmetrical about the fovea in both species, as previously described for the distribution of retinal ganglion cells and for lines of visual isosensitivity. Cone density was greater in the nasal than in the temporal peripheral retina, and this nasotemporal asymmetry was more pronounced in monkey than in human retina.

THE TOPOGRAPHIC DISTRIBUTION, size, and packing geometry of photoreceptors contribute to the functional grain of the primate retina. Most of what is known about these variables in the human retina comes from the classic study of Østerberg (*1*), but sampling gaps in that study have left our understanding of photoreceptor topography incomplete. In the fovea, the region responsible for acute vision, only a small strip of the temporal horizontal meridian was examined. A large portion of the inferior peripheral retina was not available for analysis. Furthermore, because only one retina was studied, variability in either the overall pattern or absolute values of the photoreceptor map remains unknown. Several estimates exist for the maximum density of cones in the young adult fovea (*2, 3*), but each of these histological studies also included only one eye.

Because the Old World macaque monkey is used widely as a model of the human visual system, it is important to compare macaque and human retinas by similar methods. There is considerable information on behaviorally measured visual development (*4*) in *Macaca nemestrina*, but little information about retinal anatomy. Topographic data are available for the retina of *M. mulatta* and *M. fascicularis*, but most of the data are confined to the fovea (*3, 5*) or the horizontal and vertical meridians (HM and VM) (*6, 7*). As far as we know, no complete topographic description is available for any macaque retina.

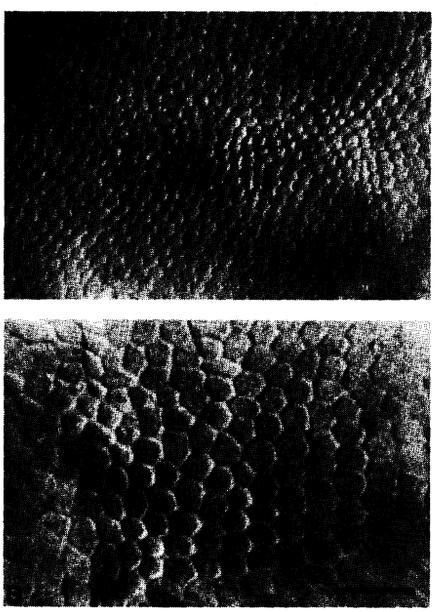

Fig. 1. Optical sections through cone inner segments at the center of the fovea in human retinas with (**A**) high peak density (H1) and (**B**) low peak density (H2). Micrographs are the same dimensions as the counting field. Bar, 10 μm.

We have developed tissue and computational techniques (*8, 9*) to facilitate the collection and analysis of topographic data from four human and two *M. nemestrina* retinas. Our data represent the first photoreceptor maps for the human retina since Østerberg's study and the first ever for the monkey retina. We describe the distribution of cones in these two species, extending the previously described topography and providing new evidence for radial asymmetry and individual variability.

Four human retinas (H1 to H4) were obtained from eye bank donors under 45 years of age without history of eye disease. Eyes were fixed in phosphate-buffered 4% paraformaldehyde and 0.5% glutaraldehyde within 3 hours of death. Eyes were inspected under the dissecting microscope to exclude ocular disease and postmortem retinal folds. Two *M. nemestrina* eyes (M1 and M2), obtained from the Regional Primate Research Center, were enucleated under deep barbiturate anesthesia. The eyes were injected intravitreally with phosphate-buffered 4% paraformaldehyde and immersed in the same fixative within 10 minutes after being removed (*10*).

Eyes were trisected into a belt containing

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