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RNA Complementary to a Herpesvirus α Gene mRNA Is Prominent in Latently Infected Neurons

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In initial attempts to define the molecular events responsible for the latent state of herpes simplex virus, in situ hybridization was utilized to search for virally encoded RNA transcripts in latently infected sensory neurons. The use of cloned probes representing the entire viral genome indicated that transcripts encoded within terminal repeats were present. When the α genes encoding ICP-0, ICP-4, and ICP-27 and the γ_1 gene encoding VP-5 were employed, only RNA transcripts hybridizing to the ICP-0 probe were detected. In latently infected cells, the ICP-0–related transcripts were localized principally in the nucleus; this was not the case in acutely (productively) infected neurons or in neurons probed for RNA transcripts coding for actin. In Northern blotting experiments, an RNA of 2.6 kilobases was detected with the ICP-0 probe. When single-stranded DNAs from the ICP-0 region were used as probes, RNA from the strand complementary to that encoding ICP-0 messenger RNA (mRNA) was the major species detected. This RNA species may play a significant role in maintaining the latent infection.

HERPES SIMPLEX VIRUS (HSV) establishes latent infections in the neurons of sensory ganglia, and the natural history of recurrent disease is characterized by reactivation of active infection from these cellular reservoirs (*1*). The molecular mechanisms operating during establishment and maintenance of latency and during reactivation from the latent state are not understood. On the basis of information available about other DNA viruses that establish latent infections, including the related Epstein-Barr virus, one would predict that the replication cycle of the latent virus is restricted or blocked at some early point. Although the precise mechanisms are not defined, in these other systems it is generally thought that at least some viral encoded proteins that are detectable in latently infected cells function to maintain the latent state (*2*).

Many laboratories, including our own, have searched for HSV transcripts and proteins in latently infected sensory neurons. At least three groups have detected transcripts (*3*), and there is one report that a specific protein, the α polypeptide ICP-4, occurs in trigeminal ganglia of latently infected rabbits (*4*). In spite of this latter finding, proteins are generally difficult to reproducibly demonstrate in latently infected cells harboring the HSV genome.

As a first step in defining the molecular nature of the latent state, we have determined which viral genes are transcribed during latent infection in mice. Using the complementary technologies of in situ and RNA blot hybridization, we have shown that transcripts from the genomic region encoding the α protein ICP-0 were present in relative abundance in latently infected sensory neurons. These were the only RNAs readily detected by in situ methods, and they were localized mainly in the neuronal nucleus. ICP-0–related RNA transcripts in acutely (productively) infected neurons and actin gene transcripts in uninfected neurons were not localized in the nucleus. Further investigation showed that the RNA transcripts in the latently infected neurons derived from the DNA strand opposite to that encoding the ICP-0 messenger RNA (mRNA).

To establish which regions of the viral genome were transcribed, we utilized in situ hybridization on frozen sections of murine spinal ganglia latently infected with the HSV-1 strain KOS-M, which does not readily invade neural tissue (*5*). Spinal ganglia from latently infected mice were cut on a cryostat and subjected to in situ hybridization with methods specific for viral RNA. The four groups of probes represented essentially the entire genome; they included Hind III fragments I, O, and J; Hind III fragments A, K, and L; Hind III fragments D+M and Eco RI fragment H; and Eco RI fragment J+K (*6*). All were nick-translated with [^{3}H]nucleotides. A positive signal was obtained with the pools of labeled probes that contained Hind III D+M and Eco RI H, and Eco RI J+K (all probes were positive on acutely infected ganglia) (Fig. 1). These results suggested that genetic information in the terminal repeat regions might be expressed during the latent state. Included in these regions and in adjacent areas in the long and short unique regions are the genes encoding the five α polypeptides (that is, those proteins that are the first to be synthesized during a productive infection). Three of these genes are the ICP-4 gene [0.83 to 0.86 and 0.96 to 0.99 mu (map units)], which codes for a multifunctional regulatory protein that controls the synthesis of both itself and later genes (*7*), and the ICP-0 (0.79 to 0.81 and 0.01 to 0.03 mu) and ICP-27 genes (0.74 to 0.75 mu), which encode proteins that regulate later genes (*8*) by an unknown mechanism.

We then tested whether specific HSV transcripts were detectable in latently infected cells by employing probes for the three α genes and the γ_1 gene (0.24 to 0.27 mu) VP-5. Transcripts from the VP-5 gene (en-

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coding the major capsid protein) are among the most abundant in productively infected cells (*9*). Although in situ hybridizations of productively infected ganglia were positive in all cases, the only positive signals in latently infected tissues were detected with the ICP-0 probe.

During these studies we repeatedly observed a unique cellular distribution of the silver grains in latently infected neurons. Thus, transcripts detected by both the genomic probes that showed positive signals (Fig. 1) and the ICP-0 gene probe were compartmentalized in the neuronal nucleus. An example with the ICP-0 probe is shown in Fig. 2A. This observation has also been made by Stroop *et al.* (*3*) in the brains of latently infected mice with a complete HSV-1 genomic probe. In contrast to the situation in latently infected cells, we did not see nuclear restriction of grains in the acutely infected controls (Fig. 2B); these neurons commonly had a significant number of grains in the cytoplasm, often at a density equivalent to or exceeding that in the nucleus. From these results, it seemed possible that there was a restriction of RNA transcripts to the nuclei, and that this might somehow be related to the latent state.

However, we had no information concerning the distribution of transcripts in neurons not burdened by acute or latent viral infection. To investigate this, we asked whether transcripts for a neuronal "housekeeping" gene also showed a relative restriction to the nucleus. To analyze these transcripts, we probed for actin mRNA in the sensory neurons of spinal ganglia and found that there was no restriction of this cellular transcript to the nucleus (Fig. 2C).

As a final characterization of the transcripts detected in latently infected cells, we performed Northern blotting experiments to determine (i) the size of the transcripts homologous to the ICP-0 region, and (ii) the DNA strand within the ICP-0 gene region that encoded these transcripts. An RNA species corresponding to the published size [2.6 kb (*7*)] of ICP-0 mRNA was detected in blots from latently infected spinal ganglia probed with the ICP-0 gene (Fig. 3A, lanes L and P).

It has been reported that HSV nucleotide sequences (particularly those from the repeat region) cross hybridize to mouse and human DNA and to human 28*S* ribosomal DNA and RNA (*10*). Our results (Fig. 3A, lane L) indicate that the signal is not due to the hybridization of ICP-0 DNA to either the large or small ribosomal RNA subunits (5 and 2 kb). As an additional control, RNA from uninfected mouse ganglia was also fractionated and hybridized with the same ICP-0 probe. No bands were seen in the gel (Fig. 3A, lane U), although background hybridization was equivalent to that seen in the RNA samples from latently infected ganglia.

In the final group of experiments, strand-specific probes were used to establish the sense of the transcripts detected in the latently infected neurons. We unexpectedly found that virtually all the RNA was complementary to the DNA strand opposite to

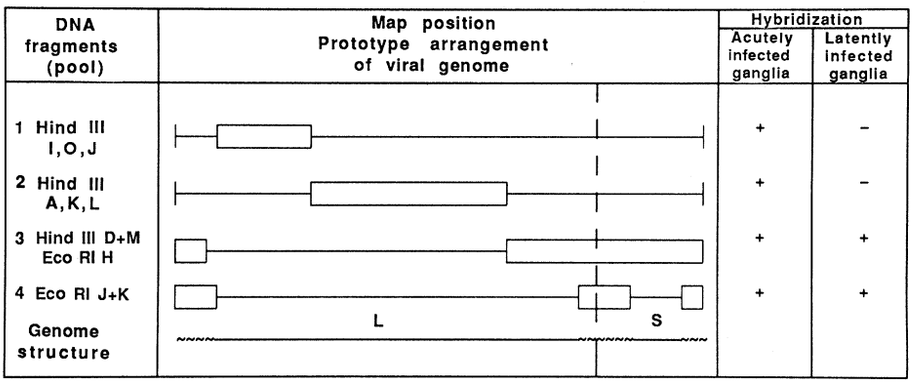

Fig. 1. Position of the probes in the viral genome and in situ hybridization of HSV-1 DNA fragments to RNA in acute and latently infected murine spinal ganglia. L, long; S, short. Cloned DNA fragments described in the text and in (*6*) were used in in situ hybridization. They were nick-translated with [^{3}H]nucleotides to specific activities of 2×10^7 to 3×10^7 cpm/μg of DNA as described by Maniatis *et al.* (*11*). Each group of probes was employed in in situ hybridization experiments on sections from acutely (productively) (4 days after mouse inoculation) and latently (more than 14 days after inoculation) infected murine spinal ganglia taken from infected mice (*5*). The methods for preparation of slides and the in situ method itself, including use of nucleases, were basically that presented by Haase *et al.* (*12*) with conditions of probe "prehybridization" and increased stringency during hybridization and washing taken from Stroop *et al.* (*3*). All slides scored for RNA were treated with deoxyribonuclease (*12*) before hybridization. After exposure times varying from 6 days to 1 month, slides were developed, stained with Giemsa, and observed. Specificity of the hybridization was established by (i) a negative result observed when uninfected ganglia were probed; (ii) removal of a significant portion of silver grains from acutely but not latently infected neurons with deoxyribonuclease; (iii) removal of a significant portion of grains from acutely infected and all grains from latently infected neurons with ribonuclease; and, (iv) removal of all grains from acutely infected ganglia with deoxyribonuclease followed by ribonuclease.

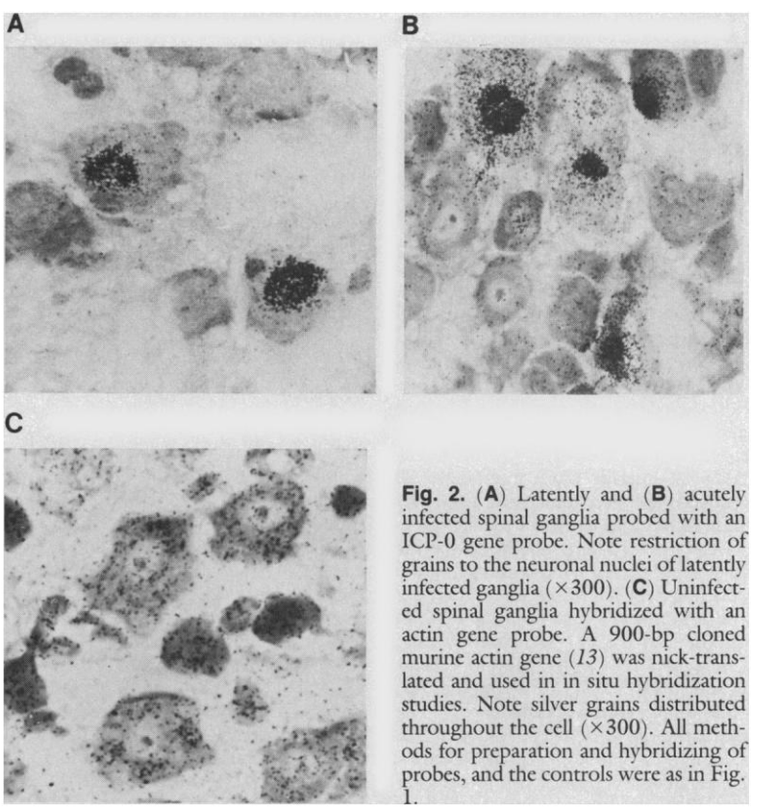

Fig. 2. (**A**) Latently and (**B**) acutely infected spinal ganglia probed with an ICP-0 gene probe. Note restriction of grains to the neuronal nuclei of latently infected ganglia (×300). (**C**) Uninfected spinal ganglia hybridized with an actin gene probe. A 900-bp cloned murine actin gene (*13*) was nick-translated and used in in situ hybridization studies. Note silver grains distributed throughout the cell (×300). All methods for preparation and hybridizing of probes, and the controls were as in Fig. 1.

that encoding ICP-0 mRNA (Fig. 3B). This antisense ICP-0 transcript is essentially the same size as that for the ICP-0 gene, and its extent of complementarity with the ICP-0 gene is presently unknown. Prolonged radioautographic exposure of the Northern blot prepared from productively infected cells and hybridized with the mp-9 probe demonstrated a band of RNA of equivalent size, but this species was present at no more than 5% of the level of authentic ICP-0 mRNA.

If it is assumed that the RNA species detected plays a role in HSV pathogenesis, three possible roles can be envisioned. First, the RNA may be an mRNA and encode a protein that is important in initiating or maintaining the lytic cascade in infected cells. In this case, nuclear retention of the mRNA for this protein in latently infected neurons would block translation and the subsequent lytic cascade. Second, a protein encoded by the message could be present in latently infected cells and function to maintain the latent state. However, the nuclear distribution of the RNA transcripts that we report here, and the inability to demonstrate viral proteins in latently infected neurons, would argue against this alternative. Third, the "anti–ICP-0" transcript may not be an mRNA, but may function as a natural antisense RNA and could regulate expression of the ICP-0 gene product by lowering or eliminating the pool of functional ICP-0 mRNA. This latter possibility is especially attractive when one considers the lytic cascade of HSV and the requirements for early expression of the α genes during this process.

Finally, it should also be clear that no matter what the function of this "anti–ICP-0" transcript in latent and lytic infection, the metabolic and physiological state of the neuron itself plays a crucial role in viral genetic expression. Analyses of the properties and kinetics of expression of the RNA transcript that we have detected should help define the nature of the latent state.

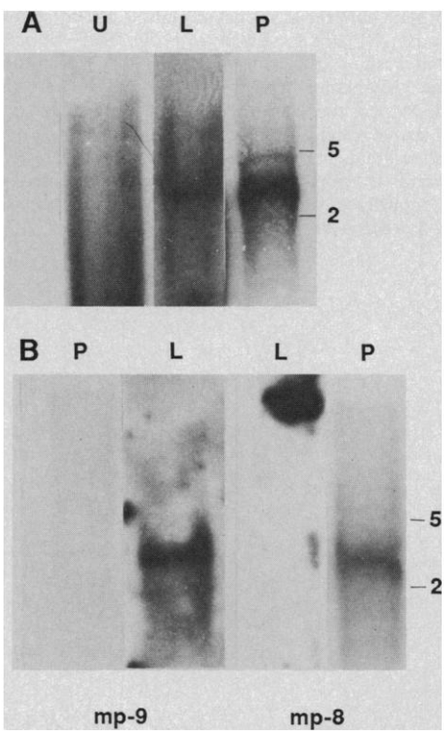

Fig. 3. Northern blot analysis of RNA from latently infected mouse sensory ganglia. Total RNA was isolated from (U) uninfected mouse ganglia, (L) latently infected ganglia, and (P) infected cultured rabbit skin cells to provide a size standard for ICP-0 RNA. RNA was extracted with guanidinium-isothiocyanate (*11, 14*). Four milliliters of the denaturing guanidinium solution was used for 35 to 40 ganglia. The material was then homogenized by a 30-second sonication at a setting of 5 with a microtip on a Branson model 185 Sonifier. After homogenization, 100 μg of translation grade transfer RNA (tRNA) (Sigma) was added as a carrier. For samples to be hybridized with strand-specific probes, the RNA was adjusted with 5 U of ribonuclease-free deoxyribonuclease (U.S. Biochemicals) in the presence of 200 U of RNasin (Promegma) for 30 minutes at 30°C. Northern blot analysis was carried out by fractionation of total RNA from 20 ganglia on 1.4% agarose gels containing 5 m*M* methyl mercury hydroxide as a denaturant (*15*). One-microgram aliquots of polyadenylated RNA from rabbit skin cells (that had been infected with 10 PFU of HSV-1 per cell 6 hours after initiation of a productive infection) were fractionated in parallel as a control. The migration of the large and small subunits of rRNA (2 and 5 kb) are indicated as size markers. RNA was electrophoretically transferred to Gene-Screen membranes (New England Nuclear) and hybridized with ^{32}P-labeled DNA probes prepared by nick translation of electroeluted, cloned HSV-1–specific DNA fragments. All these procedures have been described (*14*). (**A**) RNA hybridized with a double-stranded nick-translated ICP-0 probe. This probe had a specific activity of ~4 × 10^7 cpm/μg and consisted of the Sal I–Bam HI fragment J-B (0.79 to 0.81 mu; 2.2 kbp). Hybridization was for 40 hours at 48°C in 50% formamide and 0.4*M* Na^+. After rinsing, the blots were exposed with Dupont Cronex intensifying screens at −70°C for 72 hours. (**B**) RNA hybridized with strand-specific probes. The two strands of the Sal I–Bam HI fragment J-B were cloned in mp-8 and mp-9 phage vectors, respectively. Radioactive probes complementary to the cloned DNA fragments were synthesized from a sequencing primer (*14*), and the specific activity was ~5 × 10^7 cpm/μg. The sense of the probes was established by specific labeling of ICP-0 RNA from productively infected cells (lanes labeled P). The viral DNA in the mp-9 clone has the opposite sense as ICP-0 mRNA, and therefore directs the synthesis of a probe that will hybridize to "anti–ICP-0" RNA (that is, it indicates the presence of "antisense" RNA) (lanes mp-9, P and L). The viral DNA in the mp-8 clone has the same sense as ICP-0 mRNA and directs the synthesis of a probe that will hybridize to ICP-0 mRNA or "sense" RNA (lanes mp-8, L and P). Specificity was further confirmed by direct sequencing of probes.

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6. The probes were made by pooling equimolar amounts of cloned HSV-1 DNA fragments. The nomenclature, cloning, and use of map locations (mu) on the prototype genomic arrangement of HSV-1 to describe cloned HSV-1 DNA fragments has been discussed elsewhere [P. G. Spear and B. Roizman, in *Molecular Biology in Tumor Viruses*, J. Tooze, Ed. (Cold Spring Harbor Laboratories, Cold Spring Harbor, NY, 1980), part 2, pp. 615–746]. Based on a genome size of 150 kilobase pairs (kbp), 0.1 mu is equivalent to 15 kbp. The location of the cloned fragments encompassing only unique sequences of the HSV-1 genome used were: Hind III O, 0.087 to 0.1 mu; Hind III I, 0.1 to 0.182 mu; Hind III J, 0.182 to 0.261 mu; Hind III A, 0.261 to 0.527 mu; Hind III K, 0.527 to 0.592 mu, and Hind III L, 0.592 to 0.647 mu. Clones spanning both unique and repeat regions of the HSV-1 genome were: Hind III D, which contains unique sequences spanning 0.647 to 0.763 mu and long repeat sequences spanning 0.763 to 0.825 mu (this corresponds to 0.0 to 0.062 mu because the repeat region between 0.763 mu and the joint at 0.825 mu is equivalent to the long terminal repeat from the left end of the HSV-1 genome); Hind III M, which contains short repeat sequences from 0.825 to 0.870 mu (equivalent to 0.956 to 1.0 mu) and the unique region from 0.870 to 0.876 mu; Eco RI H, which contains short repeat sequences from 0.863 to 0.870 mu (0.956 to 0.963 mu) and the entire short unique region (0.870 to 0.956 mu); and Eco RI J+K, which contains the long repeat region from 0.0 to 0.062 mu (0.763 to 0.825 mu), the short repeat region from 0.825 to 0.863 mu (0.963 to 1.0 mu), and the long unique region from 0.062 to 0.085 mu.
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Construction of Synthetic Immunogen: Use of New T-Helper Epitope on Malaria Circumsporozoite Protein

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The circumsporozoite (CS) protein of *Plasmodium falciparum* is the focus of intense efforts to develop an antisporozoite malaria vaccine. Localization of sites for T-cell recognition on this molecule is critical for vaccine design. By using an algorithm designed to predict T-cell sites and a large panel of H-2 congenic mice, a major nonrepetitive T-cell site was located. When a synthetic peptide corresponding to this site was covalently linked to the major B-cell site on the molecule, an immunogen capable of eliciting a high-titer antibody response was formed. This peptide sequence could prime helper T cells for a secondary response to the intact CS protein. The new helper T-cell site is located outside the repetitive region of the CS protein and appears to be the immunodominant T site on the molecule. This approach should be useful in the rational design and construction of vaccines.

MUCH EFFORT IS CURRENTLY being devoted to vaccine development as a possible means of preventing malaria. The malaria parasite (*Plasmodium* species) is carried by mosquitoes that inoculate the mammalian host with sporozoites. The sporozoites travel to the liver and commence the exoerythrocytic stage of their life cycle. If they could be blocked before they entered hepatocytes, or if the infected hepatocytes could be destroyed prior to rupture and liberation of merozoites, the disease would be prevented.

Sporozoite-blocking vaccines currently being tested in humans consist of a malarial repeated epitope of the circumsporozoite (CS) protein covalently linked to a sequence unrelated to the parasite. This construction is based on the observations that (i) the central third of the CS protein contains a tandemly repeated epitope that does not differ among the various isolates tested (*1, 2*) and (ii) antibodies to this epitope can prevent invasion of hepatocytes in vitro (*3, 4*) and protect mice in vivo from challenge with murine malaria (*P. berghei*) (*5*). We recently examined the immune response in mice to one of these vaccines, which is referred to as $R32tet_{32}$. It is produced as a fusion protein between 32 tandem repeats $[(NANP)_{15}NVDP]_2$ (single-letter code for amino acid residues; see Fig. 1) derived from the CS protein and part of a sequence (32 residues) encoded by a tetracycline resistance gene read out of frame (*3*). We showed (*6*) that only mice carrying the $I\text{-}A^b$ gene could produce a T-cell response to the malaria-encoded sequence $(NANP)_n$, a finding that has been confirmed by others (*7*). In such mice, $(NANP)_n$ stimulated proliferating T cells as well as helper T cells. The tet_{32} peptide also contained a T-cell site (or sites) but was recognized after immunization with $R32tet_{32}$ by only two of seven congenic mouse strains that differed only at their H-2 loci. If a similar situation occurred in humans, some would not respond to the vaccine. Furthermore, in those humans that did respond, natural boosting from sporozoites would occur only in that subset that responded to $(NANP)_n$, not those who responded only to tet_{32}. Natural boosting is important if constant high levels of antibody are required for protection. A response to a malaria-encoded T-cell epitope is also critical if antibody-independent T-cell immunity is required for protection (*8*).

We therefore searched for other T-cell epitopes on the CS protein of *P. falciparum* that could be included with $(NANP)_n$ in a vaccine for humans. We observed that the immune response to the entire CS protein in mice was under *Ir* gene control. Simultaneously, we analyzed the sequence of the CS protein using an algorithm for predicting T-cell sites (*9, 10*) and found that a potential major site occurred in a region located about 40 amino acids from the tandem repeats, toward the carboxyl terminus. A peptide corresponding to this region elicited an immune response in the same congenic strains that gave the optimal response to the entire CS protein [B10.BR and B10.A(4R)]. We confirmed that this peptide was a helper T-cell site by covalently linking it to the sequence, $NP(NANP)_5NA$. This construct elicited high titers of antibodies in these congenic strains [B10.BR and B10.A(4R)] that did not respond to the repeated tetrapeptide sequence alone.

To determine whether there were T-cell sites other than the NANP repeat on the CS protein, we immunized various congenic strains of mice with an infectious recombinant vaccinia virus encoding the entire CS protein from the 7G8 strain of *P. falciparum* (*1*), and estimated the antibody response to $(NANP)_n$. Because there was no source of purified CS protein, a recombinant vaccinia virus was used for these studies. This recombinant is referred to as CS–vaccinia virus (*11*). Our finding (see Table 1) that the B10 ($H\text{-}2^b$) strain responded to CS–vaccinia virus was expected, since mice carrying the $I\text{-}A^b$ gene have been shown to make antibodies to $(NANP)_n$ after immunization with $(NANP)_n$ (*6, 7*); however, B10.BR and B10.A(4R) mice, whose T cells do not respond to $(NANP)_n$, also produced antibodies to the repeat sequence. The other strains examined responded less well. Control experiments showed that no mouse strain tested produced antibodies to

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