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Continued Expression of a Poly(A)$^+$ Transcript of Herpes Simplex Virus Type 1 in Trigeminal Ganglia of Latently Infected Mice

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Radioactively labeled cDNAs were prepared by using as the template poly(A)$^+$ mRNA from trigeminal ganglia of mice latently infected with herpes simplex virus type 1. These cDNAs were used as hybridization probes for Southern blots of cloned herpes simplex virus type 1 DNA fragments. Specific hybridization to fragments from the terminal repetition of the L segment was detected with probes derived from mRNAs obtained as early as 3 weeks and as late as 17 months postinoculation. Fine mapping of the region of hybridization showed that the viral transcripts originated from DNA sequences coding for the immediate-early gene IE-1 (alpha-0). These results indicate that IE-1, or an as yet unidentified gene colinear with it, is continuously expressed during latency.

Analysis of the transcriptional status of the latent viral genome is crucial to our understanding of the molecular mechanisms involved in the establishment and maintenance of latent herpes simplex virus (HSV) infections in the nervous system. The regulatory cascade of HSV gene expression that occurs in a lytic infection (9) is interrupted during neuronal latency (12), suggesting that the latent state results from a neuron-specific silencing of viral genes required for the expression of this regulatory cascade. The immediate-early viral genes, essential for the expression of early and late functions (19), are likely targets for this silencing. However important, the study of viral gene expression during latency has received little attention. Early experiments from our laboratory showed that transcripts from the latent viral genome were undetectable by standard molecular hybridization techniques (12). Since limited transcription could not have been detected in those studies, this apparent silencing need not be absolute. Indeed, HSV type 2 (HSV-2) transcripts from a discrete region of the long unique region were later detected by in situ hybridization in human paravertebral ganglia (3, 4). More recently, evidence suggesting the presence of the viral Vmw 175 (ICP4) polypeptide, coded for by the immediate-early gene IE-3, was obtained in trigeminal ganglia of rabbits in the apparent absence of expression of other HSV proteins (7).

In an attempt to identify viral genes transcribed during latency, we have analyzed RNAs obtained from latently infected mice for the presence of viral transcripts. We report here the finding of an mRNA species that is expressed throughout the latent state. This transcript is either identical to or colinear with the mRNA from gene IE-1 (IEmRNA-1), corresponding to the gene coding for Vmw 110 (ICP0).

Six- to eight-week-old female BALB/cJ mice were inoculated by corneal scarification with approximately 5×10^6 PFU of HSV-1 (F), as previously described (12). Groups of 40 to 60 mice were sacrificed at 3 weeks, 10 months, and 17 months postinoculation, and total RNA was extracted from both trigeminal ganglia of each mouse by the guanidium thiocyanate method (17). To serve as a control, RNA was similarly extracted from a group of uninoculated mice. Poly(A)$^+$ RNA was purified by two cycles of oligo(dT)-cellulose chromatography by using 0.5 M NaCl–0.01% sodium dodecyl sulfate–1 mM EDTA–10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) (pH 7.5) as the binding buffer. Bound RNA was eluted in 10 mM HEPES (pH 7.5), precipitated with 2.5 volumes of ethanol, and kept at −20°C until ready for use. RNA concentrations in the poly(A)$^+$ fractions were estimated by spotting a sample onto an agarose slab and comparing the ethidium bromide fluorescence intensity with that of a dilution series of known standards. Recovery of RNA was 5 ± 1 μg of total RNA per ganglion, in good agreement with prior determinations (12). Approximately 2 to 3% of the total RNA was recovered in the poly(A)$^+$ fraction.

Viral transcripts could not be detected when Northern blots of poly(A)$^+$ or poly(A)$^-$ RNA were hybridized with labeled cloned fragments of HSV-1 DNA representing >95% of the viral genome (data not shown). This result suggested that viral transcripts were either absent or present in very low abundance. As an alternative approach, we prepared radioactively labeled cDNA probes from the poly(A)$^+$ samples and used these probes to hybridize to Southern blots of cloned HSV-1 DNA fragments. Oligo(dT)-primed cDNA synthesis reactions contained 1 μg of poly(A)$^+$ RNA, 5 μg of oligo(dT)$_{12-18}$, 7 μM each [^{32}P]dCTP and [^{32}P]dGTP (each at 50 Ci/mmol), 100 μM each dATP and dTTP, 2 U of RNasin per ml, and 400 U of cloned Moloney murine leukemia virus reverse transcriptase, in 20 μl of the reaction buffer specified by the manufacturers (Bethesda Research Laboratories, Inc.). Incubation was at 37°C for 1 h, with an additional 200 U of reverse transcriptase added at 30 min. The reaction was terminated by sequential addition of EDTA to 20 mM, NaOH to 0.3 M, and 30 μg each of sonicated calf thymus and *Escherichia coli* DNAs. The mixture was boiled for 10 min, loaded onto Sephadex G-75, and eluted in 0.05 M Na_3PO_4. The efficiency of the reverse transcriptase reaction was 0.20 to 0.25 μg of cDNA per μg of RNA, as determined from the total radioactivity incorporated into the product and the specific activity of the precursors. The average size of the cDNA was 1.5 kilobases (kb), ranging from 0.2 to 5 kb, as measured in alkaline agarose gels.

*Eco*RI HSV-1 DNA fragments cloned in pBR325 (6) were released from the plasmid vector by cleavage with *Eco*RI and further digested with *Bam*HI, and the individual digestion products were separated by electrophoresis in 1% agarose gels. Not included were fragments O (1.8 kb) and N

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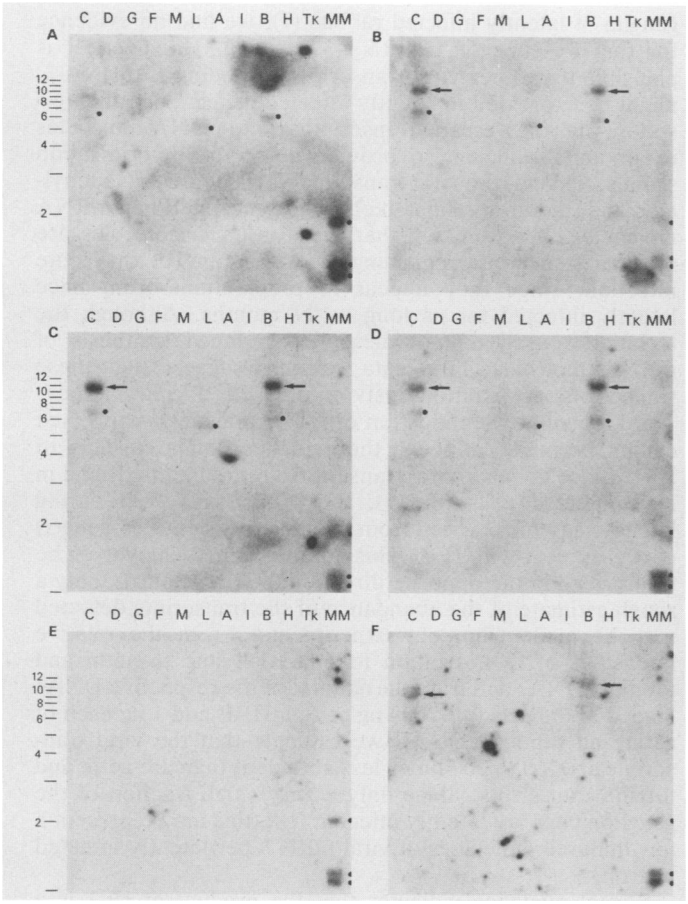

FIG. 1. Hybridization of [^{32}P]cDNA probes of poly(A)$^+$ RNA from trigeminal ganglia of uninfected and latently infected mice to Southern blots of cloned HSV-1 *Eco*RI DNA fragments digested with *Bam*HI. A 500-ng amount of each *Eco*RI HSV-1 DNA fragment cloned in pBR325 (6) was digested with *Eco*RI and *Bam*HI and separated in 1% agarose gels. Radioactive labeling of probes and hybridization conditions are described in the text. cDNA probe from uninfected mice (A). cDNA probes from mice latently infected for 3 weeks (B), 10 months (C), and 17 months (D). Repetition of the experiments shown in panels A (E) and D (F). In panels A to D hybridization was at 72°C, and blots were washed at 70°C; in panels E and F hybridization was at 75°C, and blots were washed at 72°C. The letters above the lanes correspond to the different *Eco*RI fragments (Fig. 3). Tk is the *Bam*HI Q fragment containing the viral thymidine kinase gene. Lanes labeled MM contained 1 ng each of the inserts in plasmids pA1 (ca. 2-kb chicken beta-actin cDNA), pT1 (1.4-kb chicken alpha-tubulin cDNA), and pT2 (1.7-kb chicken beta-tubulin cDNA) (2). Dots mark the positions of fragments that hybridize with all the probes; arrows indicate fragments that hybridize specifically with probes from latently infected animals. The scale at the left (in kilobases) was determined from the migration of ^{32}P-labeled 1-kb ladder DNA markers (Bethesda Research Laboratories) included in all the gels. Autoradiographic exposure was for 1 week with intensifying screens.

(2.6 kb), the latter being replaced by *Bam*HI-Q, which contains the viral thymidine kinase gene. In total, over 98% of the viral genome was represented in the gels. As positive controls we used a mixture of chicken beta-actin and alpha- and beta-tubulin cloned cDNAs, which show extensive cross-hybridization with their rodent counterparts (2). DNA fragments were transferred to nitrocellulose and hybridized to the cDNA probes from uninfected and latently infected mice described above. Hybridization was for 16 h at 72 or 75°C in 6× SET (0.9 M NaCl, 0.18 M Tris hydrochloride [pH 8.0], 12 mM EDTA), 5× Denhardt solution (0.1% each bovine serum albumin, polyvinylpyrrolidone, and Ficoll [Pharmacia Fine Chemicals]), 0.1% sodium dodecyl sulfate, 25 μg each of sonicated, denatured *E. coli* and calf thymus DNAs per ml, and 30 × 10^6 to 40 × 10^6 cpm of ^{32}P-labeled cDNA. After hybridization, blots were washed in 0.1× SET–0.1% sodium dodecyl sulfate at 70 or 72°C.

Figure 1 shows the results of these experiments. Hybridization of cDNA probes from all the latently infected animals is clearly evident with the 11-kb *Bam*HI B fragment in *Eco*RI-B and with the 10.5-kb *Bam*HI E fragment in *Eco*RI-C (Fig. 1B to D and F). These two *Bam*HI fragments are derived from the diploid IR_L/TR_L region of the viral genome and also include U_L sequences (see Fig. 3 for a schematic diagram of the HSV-1 genome). In contrast, neither of these two fragments hybridized with the control cDNA probe from uninfected mice (Fig. 1A and E). The blot in Fig. 1E, free of the high background smudges of Fig. 1A, more clearly demonstrates this point.

All the probes used, regardless of source, hybridized weakly with *Bam*HI-K and *Eco*RI-L (Fig. 1A to D, lanes B and C). These weak signals with viral fragments resulted from nonspecific hybridization of host sequences transcribed in both latently infected and uninfected ganglia, since they could be eliminated by increasing the stringency conditions for both the control probe (Fig. 1E) and the probe from mice latently infected for 17 months (Fig. 1F), leaving unaffected the specific hybridization of the latter to *Bam*HI fragments B and E. Regardless of stringency, all the cDNA probes hybridized with the cloned actin and tubulin fragments used as positive controls.

In conclusion, the results shown in Fig. 1 indicate that transcripts arising from *Bam*HI fragments B and E in the diploid IR_L/TR_L region of the HSV-1 genome are present exclusively in the poly(A)$^+$ RNA preparations from ganglia

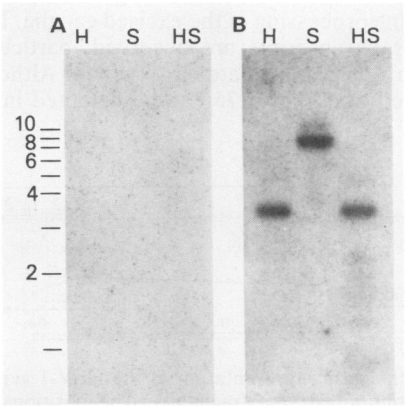

FIG. 2. Hybridization of [^{32}P]cDNA probes of poly(A)$^+$ RNA from trigeminal ganglia of uninfected and latently infected mice to Southern blots of DNA fragments from HSV-1 *Bam*HI-B. A 500-ng amount of the purified insert of a pBR322 clone of *Bam*HI-B were digested with *Hpa*I (lanes H), *Sac*I (lanes S), and *Hpa*I plus *Sac*I (lanes HS). The digestion products were separated by electrophoresis in 1% agarose gels. A blot hybridized with a [^{32}P]cDNA probe synthesized on 1 μg of poly(A)$^+$ RNA from trigeminal ganglia of uninfected mice (A) and a blot hybridized with a [^{32}P]cDNA probe prepared with a pool of 0.33 μg each of poly(A)$^+$ RNA from trigeminal ganglia of mice latently infected for 3 weeks, 10 months, and 17 months (B) are shown. Hybridization and washing conditions were as in Fig. 1A to D. Exposure was for 6 days with intensifying screens. Size markers are as in Fig. 1.

of latently infected mice. These transcripts appear to be expressed throughout the latent period, since they can be detected at 3 weeks, 10 months, and 17 months postinoculation.

The map position in IR_L/TR_L of the gene(s) transcribed was localized more precisely by using subfragments of *Bam*HI-B. The purified insert of a plasmid containing this viral fragment was digested with several restriction enzymes, and the products were separated in 1% agarose gels. Southern blot hybridizations with cDNA probes from uninfected mice and from an equimolar pool of poly(A)$^+$ RNA from the latently infected mice are shown in Fig. 2. The probe from latently infected mice hybridized with the 8.2-kb *Sac*I fragment and with the 3.5-kb *Hpa*I M fragment, proximal to the IR_L/IR_S junction. In contrast, no hybridization was observed with the cDNA probe from uninfected mice.

A schematic diagram of the HSV-1 genome, including the position of the immediate-early genes and of relevant restriction fragments is shown in Fig. 3. Two immediate-early genes, IE-1 (alpha-0) and IE-2 (alpha-27) have been mapped to the *Bam*HI B fragment (10, 14, 20). IEmRNA-2 is completely contained in *Hpa*I-S, whereas IEmRNA-1 terminates 240 bases to the right of the *Hpa*I-R–*Hpa*I-M boundary (14). IEmRNA-1 extends through *Hpa*I-M, and its 5′ end is located 774 base pairs to the right of the *Bam*HI-B–*Bam*HI-K junction (11). The results from our experiments indicate that the viral transcripts detected during latency originate from DNA sequences in *Hpa*I-M, and therefore from IE-1, known to code for the viral protein Vmw 110 (ICP0) (9, 10, 20). In good agreement with these results, transcripts from the same DNA sequences have also been detected by in situ hybridization in at least one other laboratory (N. Fraser, Proc. Natl. Acad. Sci., in press).

Several relevant conclusions can be drawn from the data presented. First, only transcripts from a very discrete region of the viral genome can be detected, thus ruling out trivial explanations for the results, such as generalized viral reactivation during processing of the excised ganglia. In addition, no other viral transcripts are observed, particularly transcripts from other immediate-early genes. Although it has been reported that Vmw 175 can be detected in trigeminal

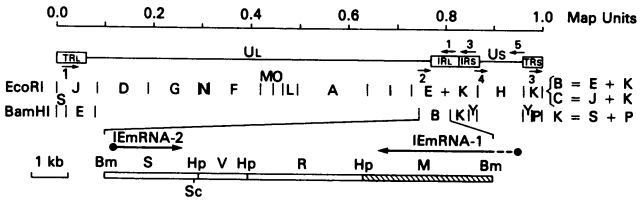

FIG. 3. Schematic representation of the HSV-1 genome and of relevant restriction enzyme fragments. The positions of the five major immediate-early genes are indicated by the numbered arrows. *Eco*RI fragments shown include B (fragments E plus K), the IR_L/IR_S junction fragment in the prototype configuration, and C (fragments J plus K), the corresponding junction fragment in the inverted-L configuration (see reference 16 for a review of the HSV genome structure). Only *Bam*HI fragments relevant to our experiments are indicated. *Bam*HI-Q, not shown in the diagram, is located between 0.306 and 0.318 map units. The expansion of *Bam*HI (Bm) fragment B shows the location of the *Hpa*I sites (Hp) that define fragments S, V, R, and M and of the single *Sac*I site (Sc). The 5′ ends, direction of transcription, and 3′ ends of IEmRNA-1 and IEmRNA-2 are shown, as determined by others (10, 14, 20). The region of *Hpa*I-M contained in *Bam*HI-B is hatched to indicate the localization of viral transcripts detected during latency (see the text).

ganglia of latently infected rabbits (7), we find no evidence for the presence of IEmRNA-3 in mice. However, it is plausible that other viral transcripts, including IEmRNA-3, might be expressed in latently infected ganglia, but at levels so low that they escape transcription into cDNA molecules in amounts sufficient to provide detectable hybridization signals. Second, the viral transcripts present are polyadenylated and, therefore, most likely represent functional mRNA molecules. It is unlikely that these mRNA molecules are residual transcripts remaining from the time of the acute infection, since they appear in preparations from mice latently infected for as long as 17 months. Although the techniques used do not measure continued synthesis of mRNA molecules, the data strongly suggest that these transcripts are continuously expressed during latency. Third, absolute identification of the viral gene(s) expressed cannot be made, although the evidence points strongly to IE-1. Since colinear viral transcripts are frequently found in HSV-infected cells (8, 15), the mRNA detected during latency may have arisen from an as yet unidentified gene(s) partly colinear with IE-1. This putative gene(s) may even be transcribed in the opposite direction to IE-1. Fourth, only a rough estimate of the abundance of the transcripts detected may be made from our experiments. From the relative intensities of hybridization to *Bam*HI-B and to actin and tubulin cDNAs and from the amount of the respective DNAs loaded in the gels (i.e., 500 ng of *Bam*HI-B and 1 ng each of actin and tubulin cDNA), we estimate that the viral transcripts are 100 to 200 times less abundant than are actin and tubulin transcripts. Since only a very small fraction of the ganglion cells are latently infected (18), this may represent a few hundred molecules of viral mRNA per latently infected neuron.

Recent evidence indicates that the product of IE-1 is a *trans*-activating factor that interacts synergistically with Vmw 175 (ICP4), the product of the immediate-early gene IE-3 (alpha-4) (5, 13). It is appealing to hypothesize that latency may result from a neuronally controlled uncoupling of this interaction, perhaps by specifically silencing the transcription of IE-3 and possibly other immediate-early genes. Availability of monoclonal antibodies to different immediate-early viral polypeptides (1) and technical advances for the molecular cloning of low abundance mRNAs should make it possible to unequivocally identify the viral proteins expressed during latency and to test this hypothesis.

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