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RNA Complementary to Herpes Simplex Virus Type 1 ICP0 Gene Demonstrated in Neurons of Human Trigeminal Ganglia

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Recent studies with mice have demonstrated abundant RNA transcripts which are complementary (antisense) to the herpes alpha gene ICP0 in latently infected ganglia. We investigated the situation in unselected human trigeminal ganglia. Strand-specific 2.7-kilobase herpes simplex virus type 1 (HSV-1) ICP0 RNA probes were prepared, and their sense was determined in productively infected cells. Although in situ hybridization demonstrated ICP0 antisense RNA transcripts in the nuclei of neurons in 46% of the ganglia, ICP0 messenger RNA was not found in any of the ganglia. We conclude that HSV-1 antisense ICP0 RNA is present in humans during ganglionic latency.

Latently infected ganglia from humans (1) and mice (17) have yielded infectious herpes simplex virus (HSV) after cocultivation with sensitive indicator cells. HSV type 1 (HSV-1) RNA transcripts have been demonstrated by in situ hybridization in latently infected sensory ganglia in humans (4) and animals (3, 14, 19, 20), and the HSV-1 proteins, thymidine kinase (21) and ICP4 (7, 13), may also be present. Recently reported animal studies demonstrated abundant RNA transcripts complementary to the herpes alpha gene ICP0 (IE-1) in latently infected sensory ganglia (15, 16, 18). The transcripts have been described as "antisense RNA" because they are complementary to messenger RNA for the ICP0 gene (2, 18).

We sought to determine whether RNA transcripts that arise from the ICP0 gene of HSV-1 were present during latency in unselected human trigeminal ganglia (TGs) and, if they were present, to characterize them as messenger RNA or antisense RNA, i.e., complementary to messenger RNA. Human TGs were removed from 13 unselected autopsied subjects (mean age, 65.9 years) between 2.5 and 27 h after death (mean time, 11.8 h). Table 1 provides additional demographic data: age, race, sex, clinical diagnosis, and HSV-1 serum antibody titers. Antibody titers to HSV-1 were determined by neutralization studies using serum obtained by cardiac puncture at autopsy. The TGs were removed and immediately fixed in periodate-lysine-paraformaldehyde (11) or modified Carnoy's fixative (10), dehydrated in serial alcohols, and embedded in paraffin before being processed for in situ hybridization (5, 8, 19).

Paraffin-embedded tissue specimens were sectioned (5 to 7 μm) on a microtome, and the sections were applied to Denhardt-treated glass slides cleaned with HCl. The sections were then deparaffinized and pretreated with 0.2 N HCl (20 min at 20°C), washed, and incubated in proteinase K (5 mg/ml) for 15 min at 37°C to promote diffusion of the probe. After acetylation to reduce nonspecific background, the sections were dehydrated in graded alcohols and incubated in prehybridization buffer (2 h at 45°C). [^{3}H]Uridine- or [^{35}S]uridine-labeled T7 and SP6 ICP0 Riboprobes (specific activity, 10^6 cpm/μl) were applied to the sections and sealed under plastic caps with rubber cement (20 h at 45°C). Excess probe was removed from sections by repeated 2× SSC (1× SSC is 0.5 M NaCl plus 0.015 M sodium citrate) and 45% formamide washes over 3 days. Dehydrated sections were then dipped in NTB-3 emulsion for autoradiography, exposed 1 to 4 weeks at 4°C, developed with Kodak D-19, and stained with hematoxylin and eosin. Five sections of each specimen were evaluated by light microscopy and scored by an observer who did not know which probe had been applied to the section being evaluated.

Positive controls to demonstrate HSV-1 antisense ICP0 RNA transcripts in latently infected rabbit TGs were obtained from New Zealand albino female rabbits (1.0 to 1.5 kg) 5 months after inoculation of both unscarified corneas with 50 μl of 10^6 PFU of HSV-1 McKrae strain per ml per eye. The rabbit TGs were processed in the same manner as the human TGs.

The HSV-1 ICP0 RNA probes were derived from a 2.7-kilobase *Sal*I-*Bam*HI fragment subcloned from the *Bam*HI B fragment of HSV-1 strain F (Fig. 1) and then inserted into the plasmid pGEM-1 (gift of D. L. Rock, Lincoln, Nebr.). Using the Riboprobe Gemini System Vectors (Promega Biotec, Madison, Wis.), strand-specific ICP0 probes were transcribed by using the T7 promoter (leftward strand) and SP6 promoter (rightward strand) and radiolabeled with [^{3}H]uridine or [^{35}S]uridine (6). HSV-1 strain W in infected Vero cells was probed 20 h postinfection with strand-specific ICP0 probes. Cells treated with the SP6 ICP0 RNA probe were intensely positive and presumably hybridized with messenger RNA, whereas the T7 ICP0 RNA probe was weakly positive and presumably hybridized with antisense RNA (data not shown). The observed weak hybridization of T7 ICP0 during productive infection is consistent with a previous report (18) that ICP0 antisense RNA was present in small amounts (less than 5%) compared with ICP0 messenger RNA during productive infection. The specificity of both probes to HSV-1 RNA was further demonstrated by the elimination of hybridization when the sections were pretreated with RNase before hybridization (not shown).

Additional controlled studies, using the T7 and SP6 ICP0 probes, ruled out homology to lower mammalian and human RNA. No hybridization was observed between these probes and the following uninfected samples: Vero cells, rabbit TGs, human A549 cells, human liver, and human cerebellum (data not shown). Homology to RNA transcribed from another DNA virus, adenovirus type 8 (data not shown), was ruled out when no hybridization was demonstrated during

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TABLE 1. In situ hybridization with strand-specific ICP0 RNA probes

Subject no.	Age (yr)	Race and sex[a]	Diagnosis	No. of positive TGs/no. of TGs tested		% Positive neurons for each positive TG	HSV-1 antibody titer
				SP6 (mRNA)	T7 (antisense RNA)[b]		
1	56	WM	Heart transplant	0/1	1/1	0.86	>1:256
2	47	BF	Pulmonary emboli	0/2	0/2	0	NA[c]
3	76	WF	Subdural hematoma	0/2	1/2	0.17	NA
4	75	WF	Myocardial infarction	0/1	0/1	0	>1:256
5	93	WM	Ischemic heart disease	0/2	0/2	0	>1:256
6	73	BM	Septicemia	0/2	2/2	0.95, 1.17	>1:256
7	29	WM	Pulmonary hypertension	0/2	0/2	0	>1:256
8	68	WM	Lung cancer	0/2	2/2	1.22, 0.71	>1:256
9	33	BM	Hepatic necrosis	0/2	2/2	0.29, 0.29	>1:256
10	65	BM	Aortic aneurysm	0/2	0/2	0	NA
11	86	WF	Alzheimer's disease	0/2	1/2	0.57	>1:64
12	69	WF	Alzheimer's disease	0/2	0/2	0	NA
13	84	WM	Subdural hematoma	0/2	2/2	0.44, 0.63	NA

[a] W, White; B, black; M, male; F, female.
[b] The percentage of TGs positive by T7 was 46% ($P < 0.001$, chi-square analysis).
[c] NA, Serum not available to test.

acute infection of A549 cell monolayers with adenovirus type 8.

The results of strand-specific ICP0 probes applied to TGs from 13 autopsied subjects are summarized in Table 1. Using the method of in situ hybridization (5, 8, 19), the T7 ICP0 probe demonstrated dense nuclear, neuronal hybridization (Fig. 2A) in 11 of 24 TGs (46%) in 7 of 13 (54%) autopsied subjects. In general, the percentage of positive neurons per TG section was low, ranging from 0.17 to 1.22%. In striking contrast, there was no nuclear hybridization (Fig. 2B) with the SP6 ICP0 probe in any of the 24 TGs ($P < 0.001$) from the 13 autopsied subjects ($P < 0.005$). Table 1 also demonstrates a correlation between positive hybridization for ICP0 antisense RNA (T7 probe) and a prior exposure to HSV-1. Five of five subjects (100%) for whom serum samples were available had ICP0 antisense RNA transcripts in their TGs and a documented prior exposure to HSV-1 as demonstrated by high serum antibody titers. Two subjects were positive for antisense transcripts in their TGs, but sera from them were not available for testing. There were no proven sero-

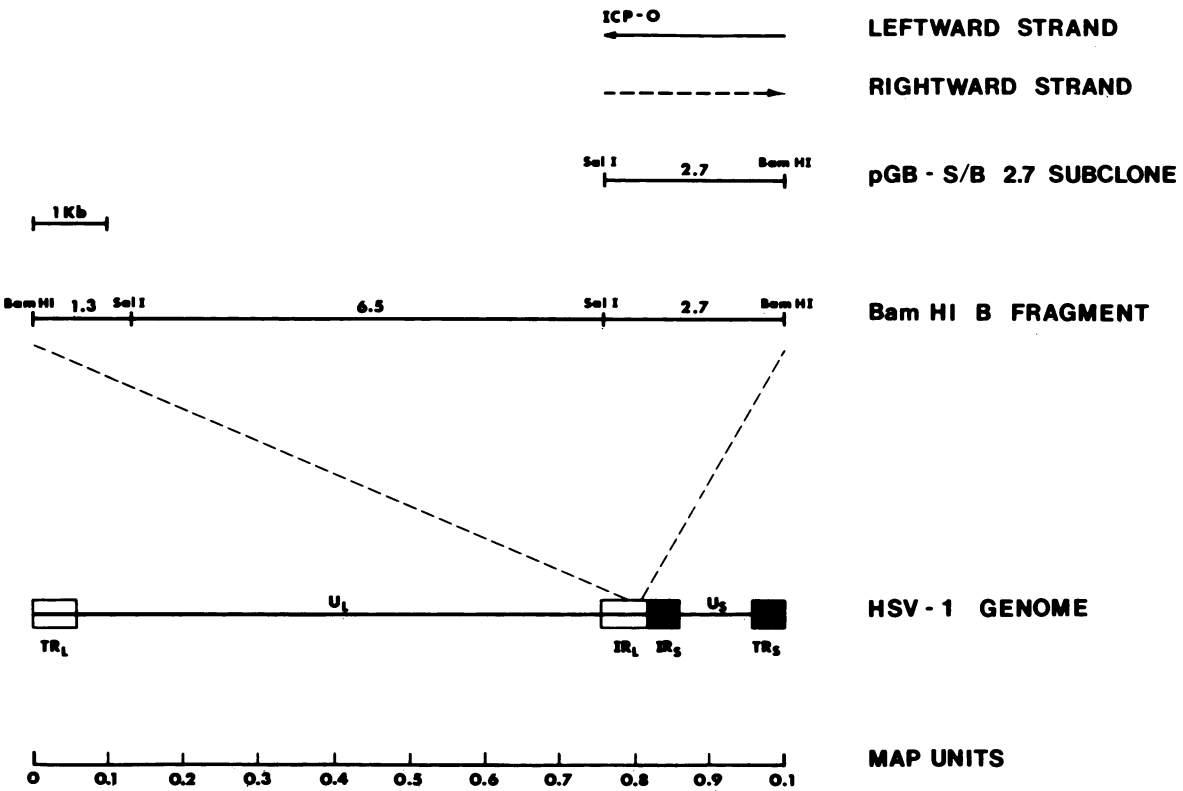

FIG. 1. Source of ICP0 probes. The ICP0 region of the HSV-1 genome which served as the source of the probe was located in the inverted-repeat long segment (0.79 to 0.81 map units). The HSV-1 ICP0 probes were derived from a 2.7-kilobase *Sal*I-*Bam*HI fragment subcloned from the *Bam*HI B fragment of HSV-1 strain F and then inserted into the plasmid pGEM-1.

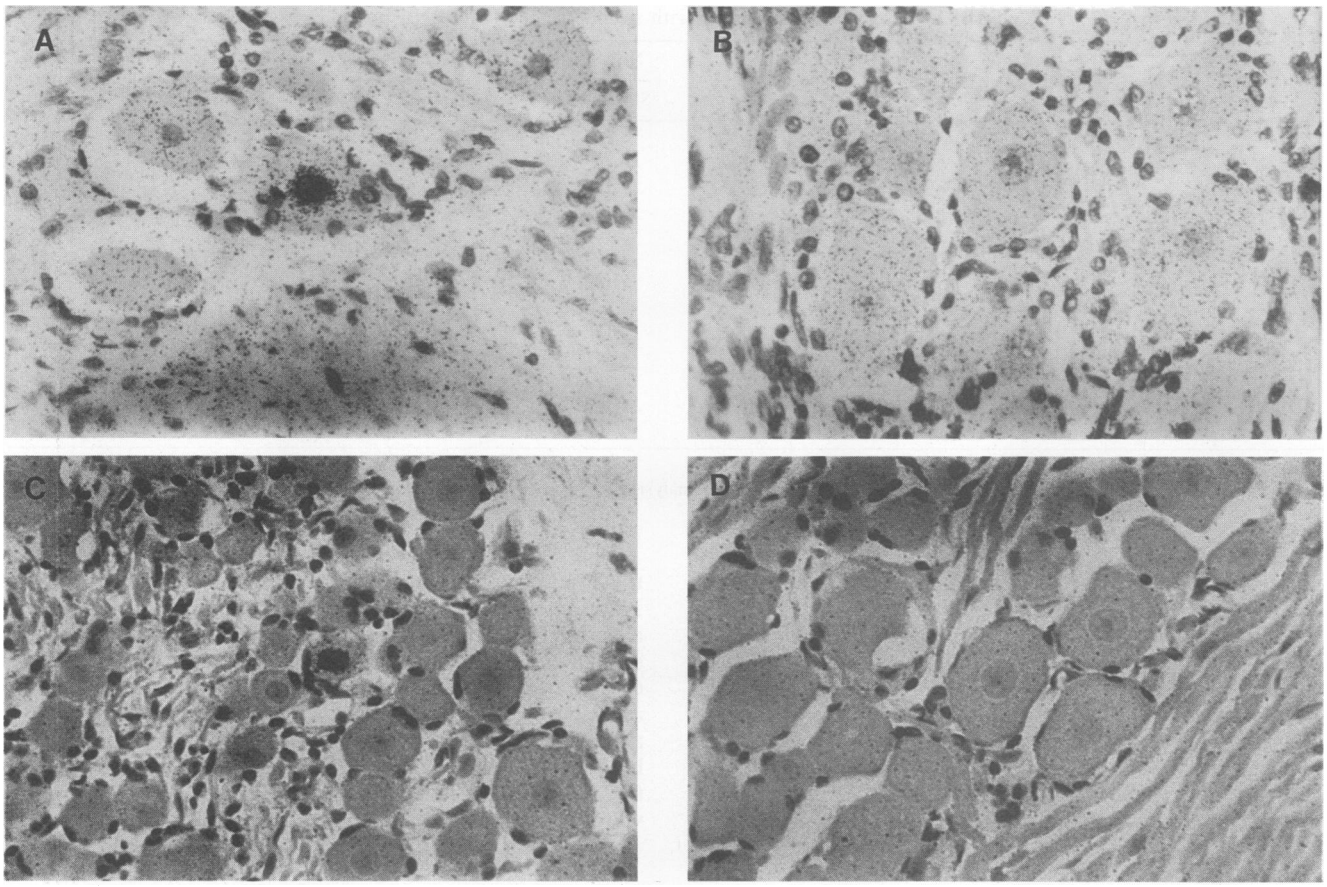

FIG. 2. In situ hybridization with specific ICP0 RNA probes. (A) Positive nuclear hybridization of T7 ICP0 probe in neuron of human TG; (B) negative nuclear hybridization of SP6 ICP0 probe in neuron of human TG; (C) positive nuclear hybridization of T7 ICP0 probe in neuron of rabbit TG; and (D) negative nuclear hybridization of SP6 ICP0 probe in neuron of rabbit TG. Magnification, ×125.

negative subjects in the study population. Serum samples were available for 8 of 13 subjects.

The results from control rabbit TGs were comparable. The T7 ICP0 probe showed nuclear neuronal hybridization (Fig. 2C) in 5 of 14 TGs (36%) in 3 of 7 rabbits (43%), whereas the SP6 ICP0 probe did not hybridize (Fig. 2D) with any of the 14 TGs (0%) ($P < 0.02$) in the 7 control rabbits.

These results demonstrate that HSV-1 ICP0 antisense RNA transcripts were present in human TGs removed at autopsy from seropositive subjects. Our results are further supported by the findings of two additional groups who independently demonstrated, by in situ hybridization, the exclusive presence of ICP0 antisense RNA in TGs removed from seropositive cadavers (2; L. Haarr, personal communication). Therefore, we conclude that the ICP0 antisense transcript appears to be a major transcript present in latently infected human TGs. Additional evidence favoring ICP0 antisense RNA expression during latency include the following: (i) comparable results in our HSV-1 latently infected rabbit controls and previous animal studies (15, 16); (ii) the absence of a hybridization pattern suggesting a productive infection following reactivation, i.e., hybridization in both nucleus and cytoplasm of the same neuron; and (iii) the absence of hybridization with probes designed to detect transcription of a beta gene (thymidine kinase) and a gamma gene (glycoprotein B), which would have been present in a productive infection following reactivation (data not shown).

Therefore, we interpret the cumulative data as evidence that ICP0 antisense RNA transcripts are present in human TGs during herpetic latency, whereas ICP0 messenger RNA has not been detected. However, this finding does not preclude the possibility that other RNA transcripts may also be found in future latency studies.

The role of ICP0 antisense RNA during latency is unknown. While a possible regulatory role has been suggested (2, 18), there is currently no proof that an endogenous antisense mechanism is operative during human ganglionic latency. Recent experimental studies have demonstrated that exogenous antisense RNA can regulate gene expression by an antisense mechanism in the myosin heavy-chain system (9) and the globin gene (12).

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