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Immediate-Early Transcription of *Herpesvirus saimiri*

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Transcription of *Herpesvirus saimiri* was characterized during the initial phases of productive infection by Northern blot analyses and hybridizations of radioactive cDNA with cloned fragments of virion L-DNA. Under conditions of immediate-early transcription, e.g., blocking of viral protein synthesis by cycloheximide, a single cytoplasmic polyadenylated viral RNA of 2.7 kilobases was found in infected cells. The sequence coding for this RNA was between map units 0.89 and 0.93; it was transcribed from right to left in prototype arrangement of M-DNA. The immediate-early mRNA of lytically infected cells appeared to be very similar, if not identical, to the single viral RNA species found in lymphoid cells transformed by *H. saimiri*.

Herpesvirus saimiri induces various types of lymphoproliferative diseases in New World primates and rabbits (5, 8, 12, 13, 22). Tumor cell lines with characteristics of T-lymphocytes have been established from leukocytes and infiltrated organs of diseased animals, and the virus is capable of immortalizing these lymphocytes in vitro (23; H. Rabin, personal communication). *H. saimiri*-transformed cells contain multiple copies of nonintegrated circular viral genomes. Some of these DNA molecules appeared to be generated by circularization of monomeric linear virion M-DNA (ca. 150 kilobase pairs [kbp]); other circular viral DNA molecules from tumor cells revealed duplications, sequence rearrangements, and deletions when compared with the linear DNA of virus particles (7, 8, 23, 29). Despite the high complexity of viral DNA in the transformed cells, transcription appeared to be restricted to a single gene. Tumor cell line 1670 was found to contain a single polyadenylated [poly(A)+] viral RNA of ≅2.7 kilobases (kb) which is encoded by DNA corresponding to the virion L-DNA between map units 0.89 and 0.93 (19). This fact led us to search for the function of this viral gene during productive infection of *H. saimiri*. As far as is known for herpesviruses, RNA and protein syntheses are coordinately regulated in three phases during replication (1, 3, 14, 21, 26, 27). At the initial stage of infection a few immediate-early (IE) genes are expressed. The respective proteins are required to initiate the synthesis of multiple types of mRNA and proteins that are characteristic of the early phase of replication. This phase is followed by the onset of viral DNA replication and by a third (or late) phase of virion polypeptide synthesis. Some herpesviruses, such as pseudorabies virus, seem to encode a single IE RNA (10, 11); other viruses, such as human cytomegalovirus (6, 15, 21, 24) and herpes simplex virus (3, 26, 28), are transcribed from several genomic regions during the IE phase of replication. In this study we describe the single IE gene of *H. saimiri*, which appears identical to the region that was found to be predominantly transcribed in transformed lymphoid cells.

MATERIALS AND METHODS

Virus and cell culture. *H. saimiri* 11 was isolated by cocultivation of squirrel monkey lymphocytes with permissive monolayers (9). The virus was grown in owl monkey kidney (OMK) cell lines 210 and 637 (4, 25). The cells were cultured in minimal essential medium with 5% (vol/vol) fetal calf serum by standard procedures. Infectious virus was titrated in OMK cell cultures by endpoint dilution or plaque assays under 2% (wt/vol) methylcellulose.

Preparation of RNA. To obtain IE RNA, we infected subconfluent OMK cell cultures at a relatively high multiplicity of infection (0.1 to 1.0) in the presence of 100 μg of cycloheximide per ml. Isolation of total cellular RNA and fractionation by oligodeoxythymidylate [oligo(dT)] cellulose chromatography were done as described before (19). Cytoplasmic and nuclear RNAs were isolated after cells were lysed in the presence of 1% Nonidet P-40 in a buffer containing 0.15 M KCl, 0.01 M magnesium acetate, 0.001 M dithiothreitol, and 0.01 M Tris-hydrochloride (pH 8.0). After centrifugation at 3,000 × *g* for 5 min, the cytoplasmic extract and the nuclei were suspended separately in 5 M guanidine isothiocyanate (19). The RNA was centrifuged through a 5.7 M CsCl cushion as described before (2, 19).

Labeling of nucleic acids and hybridization techniques. Purification procedures for viral DNA fragments cloned in plasmids and bacteriophage lambda Charon 4A were reported previously (20). Nick-repair labeling of cloned viral DNA, synthesis of oligo(dT)-primed and randomly primed cDNAs, and Northern and Southern blot hybridizations were carried out as described before in detail (19).

RESULTS

Localization of the IE gene region. *H. saimiri* replicates slowly in cell culture, and the maximum titers of infectious virus remain mostly below 10^6 PFU/ml. Synchronous infection of permissive monolayers has not been achieved, even when seed virus was concentrated in an ultracentrifuge. Thus, the amount of IE RNA presumably present within the first hours after infection was usually below the level of detection. To accumulate IE RNA, we kept infected OMK cells under 100 μg of cycloheximide per ml for 16 h. For mapping procedures, total cellular RNA was fractionated by oligo(dT) cellulose chromatography, and ^{32}P-labeled cDNA was synthesized from the poly(A)$^+$ RNA with oligo(dT) as a primer. Figure 1 shows the Southern blot hybridization of labeled IE cDNA with cloned *Eco*RI and *Kpn*I fragments representing the entire L region of *H. saimiri* virion DNA. It became apparent that the cDNA probe hybridized with the *Eco*RI C fragment exclusively. This fragment (17.5 kbp)

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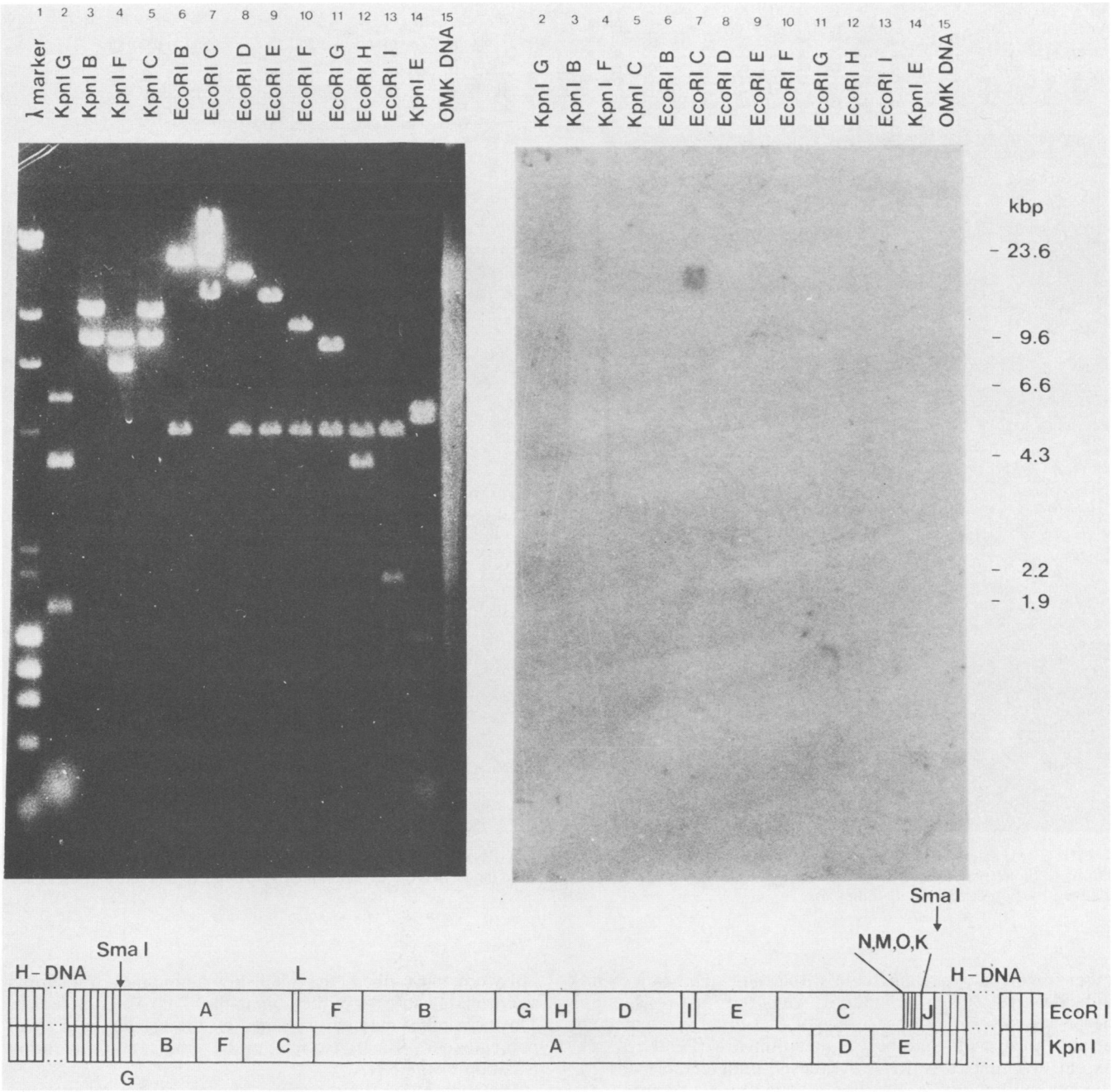

FIG. 1. Hybridization of oligo(dT)-primed radioactive cDNA (synthesized from IE RNA) with cloned DNA fragments representing the entire L region of *H. saimiri* DNA. Lane 1, Size markers. Radioactive cDNA was hybridized with the cloned fragments shown in lanes 2 through 13, with the cloned *Kpn*I E fragment cleaved by *Eco*RI (lanes 14), and with OMK DNA cleaved by *Eco*RI (lanes 15).

corresponds to map units 0.81 to 0.96 in virion L-DNA. When cDNA was synthesized, however, from non-polyadenylated IE RNA with fragmented calf thymus DNA as a random primer, no hybridization with any fragment of *H. saimiri* L-DNA was detected (data not shown). This finding indicated that IE transcription in *H. saimiri*-infected cells is restricted to a single genomic region and that essentially all transcripts are poly(A)$^+$.

In some experiments, cycloheximide was omitted, and replication was allowed to proceed into the early phase of replication. Poly(A)$^+$ RNA was isolated at 20 h postinfection and used as a template for oligo(dT)-primed synthesis of ^{32}P-labeled cDNA. Hybridization of the radioactive probe with DNA fragments representing the entire L region indicated that all large (>1.8-kb) *Eco*RI and *Kpn*I fragments are transcribed during this stage (Fig. 2). However, the poly(A)$^+$ RNA transcribed from the *Eco*RI C fragment appeared to be more abundant than any other class of viral RNA. The cDNA did not hybridize with virion H-DNA, indicating that the repetitive DNA is not appreciably transcribed during the IE or early phase of virus replication. As expected, radioactively labeled cDNA made from RNA isolated at 9 h postinfection also hybridized with the *Eco*RI C fragment or its *Hin*dIII subfragments; however, no hybridization with

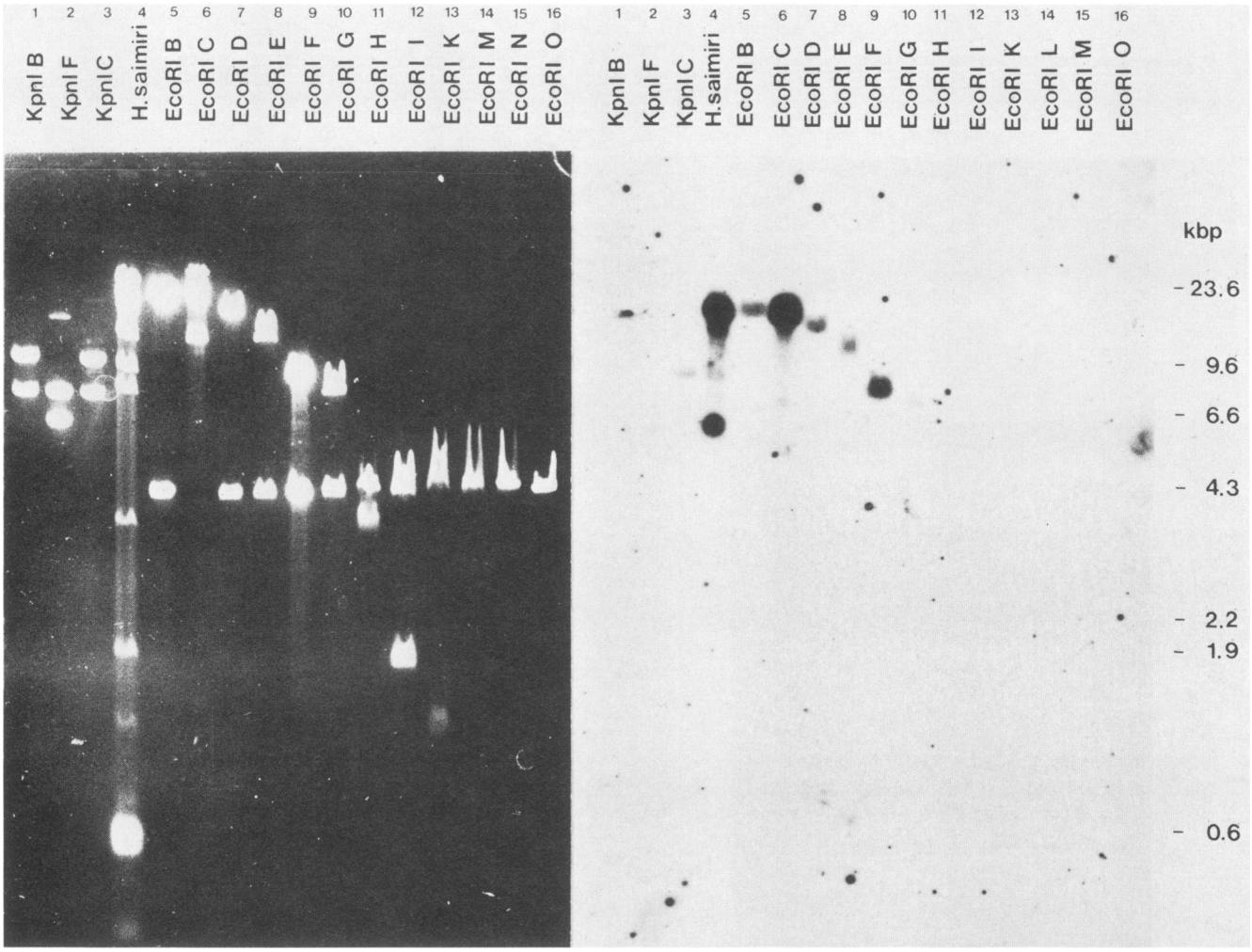

FIG. 2. Hybridization of oligo(dT)-primed cDNA (synthesized from early RNA) with cloned fragments of *H. saimiri* L-DNA and virion DNA. Radioactive cDNA was hybridized with the cloned fragments shown in lanes 1 through 3 and lanes 5 through 16 and with virion DNA cleaved by *Eco*RI and *Sma*I (lanes 4).

other fragments was detectable at that time. This fact could indicate that, when compared with the fast-replicating herpesviruses, early transcription of *H. saimiri* is initiated at a late time point. Another explanation may be the small extent of initial early RNA synthesis, undetectable due to the low multiplicity of infection reached by *H. saimiri*.

Detailed mapping and size determination of IE RNA. Northern blot analysis of IE viral RNA was done with the *Eco*RI C fragment and cloned subfragments thereof. Hybridization of the entire labeled *Eco*RI C fragment with total poly(A)$^+$ IE RNA from infected cells revealed a single dominant transcript of 2.7 kb. The same RNA was seen after Northern blot hybridization with the labeled *Kpn*I D fragment (Fig. 3A). Additional Northern blots with the cloned *Hin*dIII subfragments indicated that the region transcribed into cytoplasmic poly(A)$^+$ RNA is confined to four *Hin*dIII fragments of 1,270, 940, 820, and 375 base pairs (bp) (Fig. 3B–E, respectively). Northern blot analysis with the cloned *Kpn*I D fragment as a radioactive probe revealed two low-abundancy RNA molecules of 2.0 and 1.6 kb (Fig. 3A). Since cDNA hybridizations had not indicated that regions other than these four *Hin*dIII fragments (1,270, 940, 820, and 375 bp) were transcribed, the small low-abundancy transcripts are probably subsets of the dominant 2.7-kb RNA molecule. This possibility may be explained by the splicing of a small percentage of IE transcripts or by fragmentation during isolation of the RNA.

Direction of IE transcription. The orientation of the predominant IE transcript was determined by comparing hybridization patterns of oligo(dT)-primed cDNA with those of randomly primed cDNA. The method has been applied in our laboratory to analyze the IE transcripts of human cytomegalovirus (15) and the RNA in *H. saimiri*-transformed cells (19). As oligo(dT)-primed cDNA is primarily transcribed from the 3′-terminal sequence of poly(A)$^+$ RNA, hybridization is less intense with DNA coding for the 5′ end of the message. Figure 4A shows that short stretches of oligo(dT)-primed cDNA (≦1.0 kb) hybridized preferentially with the *Hin*dIII fragment of 940 bp at a higher intensity than with the 1,270-bp fragment. A similar pattern was seen when oligo(dT)-primed cDNA from early poly(A)$^+$ RNA was hybridized with the *Hin*dIII fragments of the *Kpn*I D fragment (Fig. 4C). On the other hand, clearly stronger hybridization of the 1,270-bp fragment became visible when a

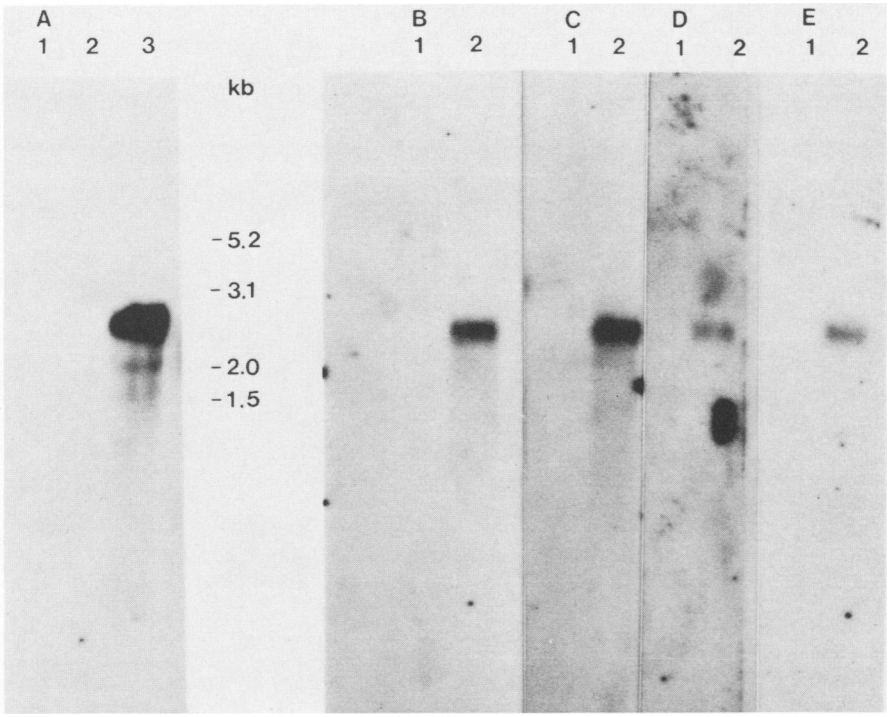

FIG. 3. Size determination of IE transcripts by Northern blot analyses. (A) Hybridization of the ^{32}P-labeled cloned *Kpn*I D fragment with 20 μg of total cellular RNA from uninfected OMK cells (lane 1), 10 μg of rRNA from *Escherichia coli* (lane 2), or 20 μg of total cellular RNA isolated from OMK cells 20 h after infection with *H. saimiri* (lane 3). (B) Hybridization of the ^{32}P-labeled cloned 940-bp *Hin*dIII subfragment with 10 μg of RNA from uninfected OMK cells (lane 1) or 2 μg of poly(A)$^+$ IE RNA (lane 2). (C) Hybridization of the ^{32}P-labeled cloned 1,270-bp *Hin*dIII subfragment with 10 μg of RNA from uninfected OMK cells (lane 1) or 2 μg of poly(A)$^+$ IE RNA (lane 2). (D) Hybridization of the ^{32}P-labeled cloned 820-bp *Hin*dIII subfragment with 10 μg of RNA from uninfected OMK cells (lane 1) or 2 μg of poly(A)$^+$ IE RNA (lane 2). (E) Hybridization of the ^{32}P-labeled cloned 375-bp *Hin*dIII subfragment with 10 μg of RNA from uninfected OMK cells (lane 1) or 2 μg of poly(A)$^+$ IE RNA (lane 2). Clear hybridization was only obtained with a reduced reaction temperature (35 instead of 43°C), suggesting that the 375-bp fragment is very rich in A+T.

longer (≅1.5-kb) oligo(dT)-primed cDNA or randomly primed cDNA was used as a probe (Fig. 4B and D). The randomly primed cDNA also hybridized with the small *Hin*dIII fragment of 375 bp and the adjacent *Hin*dIII fragment of 820 bp. This hybridization indicated that the direction of transcription for the dominant poly(A)$^+$ IE RNA of *H. saimiri* is from right to left. Apparently, the terminus of transcription lies in the internal part of the 940-bp *Hin*dIII fragment; the promoter is located in the 820-bp fragment when short leader sequences are not located upstream (Fig. 5).

DISCUSSION

The experiments described in this paper have shown that IE gene expression in *H. saimiri*, as far as it is detectable by hybridizations with radioactive cDNA and by Northern blot analysis, is confined to a single viral genomic region which is localized in the L region of virion DNA between map units 0.89 and 0.93. The cells contain a single dominant species of poly(A)$^+$ transcripts of 2.7 kb that are found in the cytoplasmic RNA pool. Two smaller RNA molecules (2.0 and 1.6 kb) of very low abundancy, probably arising by splicing or artificial fragmentation, are instructed by the same genomic region. The direction of RNA synthesis is from right to left. There was no indication of any type of virus-specific nonpolyadenylated RNA during the IE phase. When replication continued beyond the start of the early phase, most parts of L-DNA were transcribed; however, the 2.7-kb poly(A)$^+$ IE RNA still appeared to be a dominant fraction among virus-specific transcripts. Additional experiments will be required to determine, by analysis with single-strand-specific DNases, whether the 2.7-kb IE transcripts and the homologous RNA found during the early phase are identical with regard to their coding regions and possible small introns. *H. saimiri* replicates slowly in cell cultures, and the final titers of infectivity are never very high. Thus, synchronous replication was not achieved, and IE RNA could not be found during the initial stage (≦60 min) after infection. Detection of IE transcripts has remained dependent on their accumulation in the presence of 100 μg of cycloheximide per ml. Since with a cycloheximide block only a single transcript was found within 20 h, it seems very likely that this RNA is identical to the IE mRNA made in productive *H. saimiri* infection.

Some herpesviruses, such as pseudorabies virus (11) and murine cytomegalovirus (18), have a single dominant IE gene. Other viruses, such as human cytomegalovirus (15, 27) and herpes simplex virus (3, 16, 26, 28), code for multiple (four or five) distinct classes of IE RNA, several of them instructing proteins (24, 26). Since *H. saimiri*, a representative of lymphotropic herpesviruses, possesses a single IE genomic region and is far distant in phylogeny from both herpesvirus subgroups mentioned above, the organization of IE genes does not seem to be a useful criterion for herpesvirus taxonomy.

The IE mRNA of *H. saimiri* was found to be very similar to the major type of virus-specific RNA in transformed

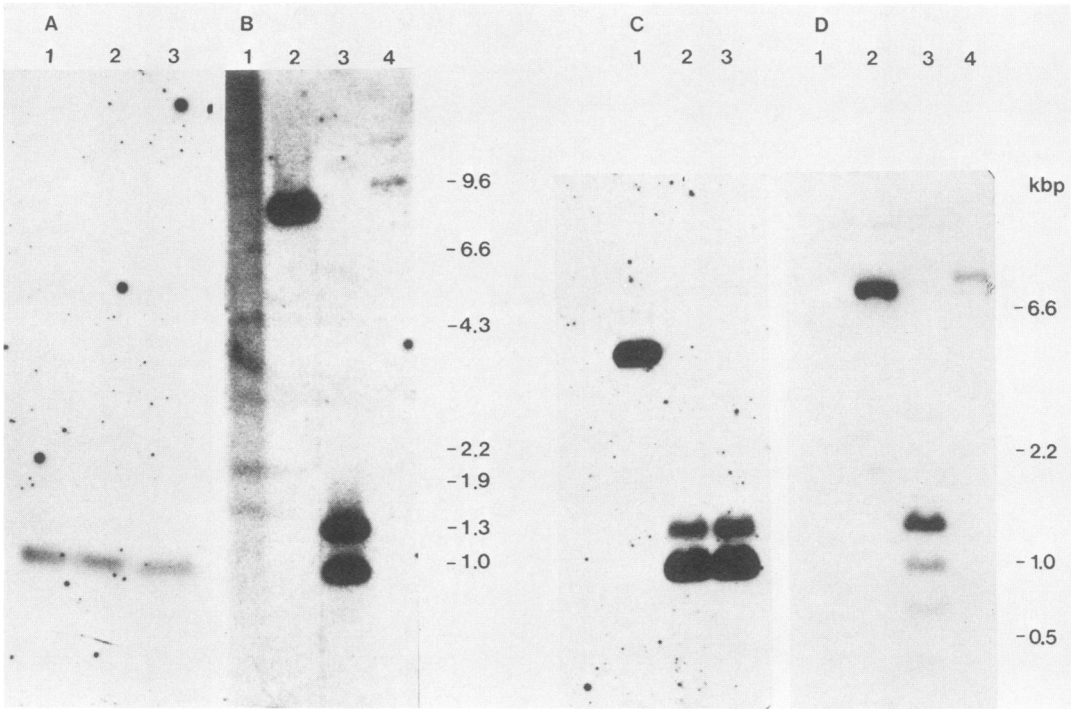

FIG. 4. Determination of the direction of transcription within the IE region. (A) ^{32}P-labeled cDNA (~500 bases) was synthesized from IE RNA with oligo(dT) as primer and hybridized with the cloned KpnI D fragment cleaved by *Hin*dIII and *Kpn*I (lane 1), the cloned *Eco*RI C fragment cleaved by *Hin*dIII and *Kpn*I (lane 2), or the cloned *Eco*RI C fragment cleaved by *Hin*dIII (lane 3). (B) ^{32}P-labeled cDNA (~1,500 bases) was synthesized from IE RNA with oligo(dT) as primer and hybridized with OMK cell DNA cleaved by *Hin*dIII (lane 1), the cloned *Eco*RI C fragment cleaved by *Hpa*II (lane 2), the cloned *Kpn*I D fragment cleaved by *Hin*dIII (lane 3), or the cloned *Kpn*I E fragment cleaved by *Kpn*I and *Sma*I (lane 4). (C) ^{32}P-labeled cDNA was synthesized from early RNA (20 h postinfection) with oligo(dT) as primer and hybridized with the cloned *Eco*RI C fragment cleaved by *Kpn*I and *Hpa*II (lane 1), the cloned *Kpn*I D fragment cleaved by *Kpn*I, *Hpa*II, and *Hin*dIII (lane 2), or the cloned *Kpn*I D fragment cleaved by *Kpn*I and *Hin*dIII (lane 3). (D) ^{32}P-labeled cDNA was synthesized from early RNA (20 h postinfection) with calf thymus DNA as a random primer and hybridized with OMK cell DNA cleaved by *Hin*dIII (lane 1), the cloned EcoRI C fragment cleaved by *Hpa*II (lane 2), the cloned KpnI D fragment cleaved by *Hin*dIII (lane 3), or the cloned KpnI E fragment cleaved by *Kpn*I and *Sma*I (lane 4).

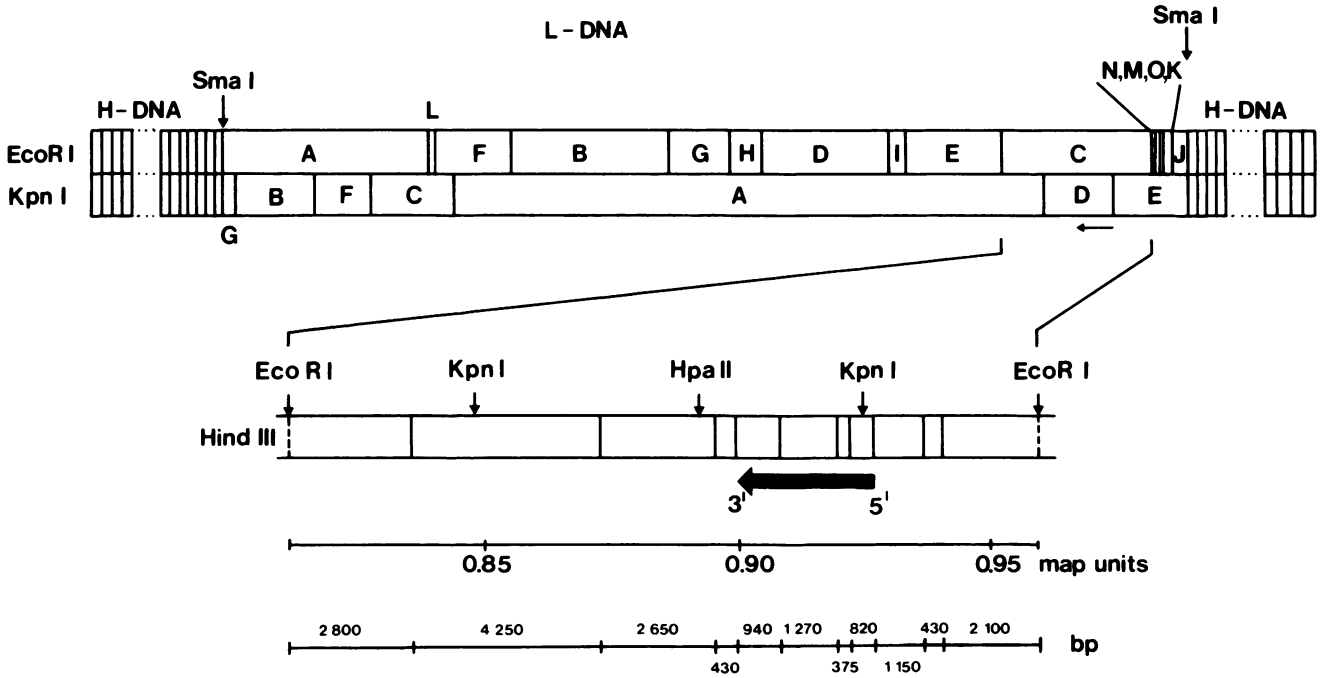

FIG. 5. Mapping of the predominant IE mRNA of *H. saimiri*. Length and direction of the arrow indicate size, orientation of transcription, and approximate initation and termination sites for the RNA.

lymphoid tumor cells (19). This raises the question of whether IE protein of *H. saimiri* plays a role in the maintenance of the immortalized or transformed phenotype. A number of *H. saimiri*-transformed cells carry circular viral genomes from which large portions of L-DNA have been deleted. As far as it has been investigated, the IE region has always been conserved (7, 17, 23), suggesting that it is functionally important in the cell lines.

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