

## Mapping of RNA Transcribed from a Region of the *Herpesvirus saimiri* Genome Required for Oncogenicity

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**A region of the *Herpesvirus saimiri* genome that is not essential for replication of the virus has recently been shown to be required for its oncogenicity in New World primates. We have examined the RNAs derived from this region of the genome in permissively infected cells. Two polyadenylated RNAs, of 4.9 and 2.3 kilobases, were the major species coded for by this region of the genome. These two RNAs, as well as a much less abundant RNA of 6.5 kilobases, were specifically altered in two different nononcogenic deletion mutants of *H. saimiri*. The 4.9- and 2.3-kilobase RNAs were mapped by S1 nuclease and exonuclease VII digestion of DNA-RNA hybrids. The transcripts were found to be spliced, overlapping, and transcribed from right to left on the genetic map, with their 3' termini each ca. 150 base pairs from the left border between the unique and repetitive DNA regions. These RNAs were not detected at immediate early times after infection. The possible role of these RNAs in the origin of the malignant T-cell lymphoma caused by this virus is discussed.**

*Herpesvirus saimiri* naturally infects most squirrel monkeys (*Saimiri sciureus*) and produces no signs of disease in this species; however, the virus causes a rapidly progressing malignant T-cell lymphoma or leukemia when it is inoculated into other New World primates such as marmosets (*Saguinus* spp.) and owl monkeys (*Aotus trivirgatus*) (5, 7). In the case of cotton-top marmosets (*Saguinus oedipus*), tumor induction occurs in 100% of the inoculated animals even at a dose of less than 10 infectious virus particles, and death occurs rapidly and predictably (25 to 35 days). Unlike Epstein-Barr virus, *H. saimiri* can be grown in monolayer cells such as owl monkey kidney (OMK) cells, producing a lytic infection with a high virus titer. Serial passage of *H. saimiri* 11 and repeated plaque purification resulted in the generation of a nononcogenic variant termed 11att (18). Although 11att virus has unaltered growth properties in OMK cells and appears to be identical to parental strain 11 by physical, biochemical, and immunological criteria, inoculated marmosets remain healthy and develop no signs of lymphoma or leukemia, even though the virus persists and can be recovered from the lymphocytes of the infected animals (6, 21, 22). Comparison of the genomes of strains 11 and 11att by restriction endonuclease mapping has defined a single alteration in the form of a 2.3-kilobase-pair (kbp) deletion at the left junction of the terminal repeat sequences and the unique sequence DNA (14). Recently, by using restriction endonucleases to introduce specific deletions into a cloned DNA fragment, Desrosiers et al. (4) constructed a second replication-competent deletion mutant virus, termed S4. The S4 variant has a 4-kbp deletion that partially overlaps the 11att deletion and has also been shown to be nononcogenic in susceptible New World primates. Furthermore, the oncogenic capacity of both of these deletion mutants was restored by reinserting the deleted sequences into the viruses, unambiguously showing that these sequences were required for the oncogenic capacity of *H. saimiri* (Desrosiers et al., submitted for publication).

We used Northern blot hybridization to analyze the RNAs

produced from this region of the genome in lytically infected OMK cells in culture. We have identified two major polyadenylated RNAs of 4.9 and 2.3 kb and mapped their 5' and 3' termini as well as their exon-intron topography on the *H. saimiri* genome. The identification of these RNAs and their presumed protein products should aid in understanding the role this region of the genome plays in the oncogenicity of the virus.

### MATERIALS AND METHODS

**Viruses.** *H. saimiri* strains 11 and 11att were originally provided by L. Falk and propagated in OMK cells. The construction of the S4 and KH deletion mutants has been previously described (4). Briefly, the 7.4-kbp *TaqI* fragment of strain 11 virus was cloned into the *ClaI* site of plasmid vector pBR322, and the deletions in pS4 and pKH were generated by restriction endonuclease digestion and religation. After cotransfection of OMK cells with parental strain 11 DNA, recombinant viruses containing the appropriate deletion were isolated.

**RNA isolation.** OMK cell line 637 was grown in 150-cm<sup>2</sup> flasks containing minimal essential medium (GIBCO Laboratories) supplemented with 10% heat-inactivated fetal calf serum. When the cells reached confluence, virus was added at the indicated multiplicity of infection; at the indicated time after infection, RNA was isolated by guanidine thiocyanate extraction (10). Portions (30-ml) of lysis buffer (4 M guanidine thiocyanate [Eastman Chemical Products, Inc.], 14%  $\beta$ -mercaptoethanol, 0.1 M Tris-hydrochloride [pH 7.5]) were used to lyse cells from six 150-cm<sup>2</sup> tissue culture flasks. Lysates were then homogenized in a Sorvall Omnimixer at full speed for 2 min. To the solution was added 2.4 ml of 1 M acetic acid, 1.5 ml of 20% sodium acetate (pH 5.5), and 15-ml of ethanol. After 1 h at -70°C, the precipitate was collected by centrifugation at 10,000  $\times g$  for 20 min. The pellet was then dissolved in 15 ml of guanidine hydrochloride reagent (6 M guanidine hydrochloride [Bethesda Research Laboratories], 25 mM sodium EDTA, 10 mM dithiothreitol [pH 7]), and the RNA was precipitated with 0.75 ml of 20% sodium acetate (pH 5.5) and 7.5 ml of ethanol. The guanidine hydrochloride extraction was repeated once. The final ethanol precipitate was dissolved in 10 ml of 25 mM EDTA (pH

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7) and extracted with 20 ml of chloroform-*n*-butanol (4:1, vol/vol). Finally, the aqueous phase was made 0.15 M NaCl, and RNA was precipitated with 2 volumes of ethanol.

To separate the 4.9-kb viral RNA from the 2.3-kb viral RNA, total cellular RNA was sedimented through a 5 to 20% sucrose gradient containing 10 mM Tris-hydrochloride (pH 7.4), 100 mM NaCl, and 5 mM EDTA. RNA (1 mg) from strain 11 virus-infected OMK cells was heat denatured at 70°C for 2 min, quick-cooled, layered on the gradient, and centrifuged at 41,000 rpm (4°C) for 5 h in an SW-41 rotor (Beckman Instruments, Inc.). Portions of each gradient fraction were analyzed by Northern blot hybridization with radiolabeled probe, and the appropriate fractions were pooled.

**Northern blot hybridization.** For analysis of RNA on agarose gels, RNA was first denatured with glyoxal previously deionized with AG 501-X8 mixed-bed ion exchange resin (Bio-Rad Laboratories) (16). The RNA was then electrophoresed on horizontal 1.1% agarose gels and transferred to nitrocellulose paper by the procedure of Thomas (19). Northern blots were prehybridized (12 h), and then the transferred RNA was hybridized for 12 h to radiolabeled DNA at 42°C in a 50% formamide solution containing 5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 1× Denhardt solution, 50 mM sodium phosphate (pH 6.5), and 200 µg of sheared and heat-denatured salmon sperm DNA per ml as described by Wahl et al. (20); 10% dextran sulfate (Pharmacia, Inc.) was included in the hybridization solution along with the <sup>32</sup>P-labeled DNA probe prepared as described by Rigby et al. (17) with [α-<sup>32</sup>P]dATP and [α-<sup>32</sup>P]TTP (2,000 to 3,000 Ci/mmol; Amersham Corp.). The specific activity of the probes was 2 × 10<sup>8</sup> to 4 × 10<sup>8</sup> dpm/µg of DNA. Blots were washed three times for 10 min each at room temperature in 2× SSC-0.1% sodium dodecyl sulfate, followed by two 30-min washes at 50°C in 0.1× SSC-0.1%

sodium dodecyl sulfate. For autoradiography, blots were exposed at -70°C to Kodak X-OMAT film with intensifying screens. To remove radiolabeled probe for sequential hybridizations, the blots were stripped by incubating them in 50% formamide for 1 h at 60°C; the cycle of prehybridization and hybridization to radiolabeled probe was then repeated.

**S1 nuclease mapping.** For 5' end labeling, CsCl-gradient-purified pT7.4 plasmid DNA was linearized with *Xba*I (Bethesda Research Laboratories) and then extracted twice with phenol-chloroform-isoamyl alcohol (1:1:0.04). Before end labeling, the DNA was dephosphorylated with calf alkaline phosphatase (grade I; Boehringer Mannheim) as follows. DNA ends (3.5 pmol) were incubated at 37°C for 30 min in a 10-µl assay solution containing 0.13 U of calf alkaline phosphatase [furnished as an (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> suspension, pelleted, and dissolved in water], 50 mM Tris-hydrochloride (pH 9), 1 mM MgCl<sub>2</sub>, 0.1 M ZnCl<sub>2</sub>, and 1 mM spermidine. The calf alkaline phosphatase was then inactivated by heating at 68°C for 15 min in the presence of 0.5% sodium dodecyl sulfate, 1 mM EDTA, followed by two extractions with phenol-chloroform-isoamyl alcohol. To de-salt the solution, the extract was passed through a Sephadex G50 (Pharmacia) column, and the DNA was ethanol precipitated before end labeling. The 30-µl kinase reaction contained 3.5 pmol of dephosphorylated 5' ends of DNA, 50 mM Tris-hydrochloride (pH 7.6), 10 mM MgCl<sub>2</sub>, 5 mM dithiothreitol, 0.1 mM EDTA, 125 pmol (250 µCi) of 5'-[γ-<sup>32</sup>P]ATP (>2,000 Ci/mmol; Amersham), and 3.5 U of T4 polynucleotide kinase (Bethesda Research Laboratories). After 1 h at 37°C, the reaction mixture was extracted twice with phenol-chloroform-isoamyl alcohol, and the DNA was precipitated at -70°C with 2 volumes of ethanol.

For 3' end labeling, 10 µg of linearized (*Xba*I) pT7.4 DNA was incubated at 37°C for 1 h in a 50-µl reaction volume containing 50 mM Tris-hydrochloride (pH 7.2), 10 mM

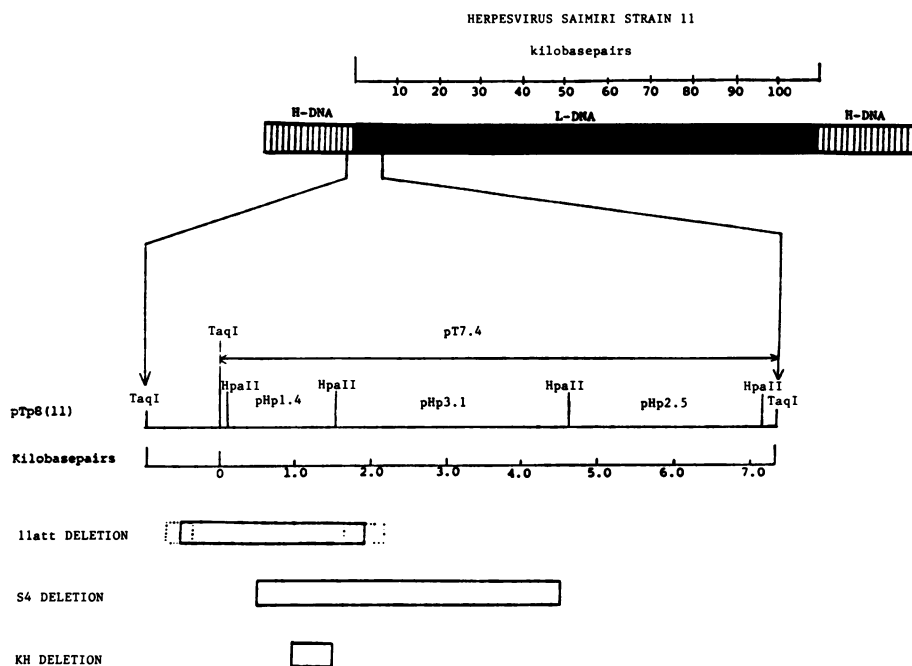


FIG. 1. Genetic map of *H. saimiri* and location of the 11att, KH, and S4 deletions. The pT7.4 clone and its three subcloned derivatives, pHp1.4, pHp3.1, and pHp2.5, were used as radiolabeled probes in this experiment. The dotted lines indicate the region of uncertainty in the 11att deletion.

MgSO<sub>4</sub>, 0.1 mM dithiothreitol, 50 µg of bovine serum albumin per ml, 25 pmol (50 µCi) of 5'-[α-<sup>32</sup>P]dCTP, and 8 U of the Klenow fragment of *E. coli* DNA polymerase I (Bethesda Research Laboratories). The reaction mixture was extracted once with phenol-chloroform-isoamyl alcohol, and the DNA was ethanol precipitated.

The end-labeled DNA (either 5' or 3') was then digested with *Bam*HI (Bethesda Research Laboratories), and the 10.2- and 1.6-kbp end-labeled fragments were separated on 1% agarose gels. DNA was electroeluted from gel slices, purified by chromatography on Sephacel (Pharmacia), extracted once with phenol-chloroform-isoamyl alcohol, and ethanol precipitated.

For each S1 nuclease or exonuclease VII digestion, 20 µg of RNA was ethanol precipitated along with  $10 \times 10^3$  to  $60 \times 10^3$  dpm of end-labeled DNA. Twenty microliters of hybridization buffer (40 mM PIPES [pH 6.4], 1 mM EDTA, 400 mM NaCl, and 80% formamide) was then added to the dried pellets, and the samples were incubated at 74°C for 15 min and then rapidly transferred to a second water bath set at the desired hybridization temperature (15). The 1.6- and 10.2-kbp end-labeled DNAs had different optimum temperatures for RNA-DNA hybridization: the optimum hybridization temperature for the 1.6-kbp DNA was 50°C, whereas 43°C was the optimum hybridization temperature for the 10.2-kbp DNA fragment. After 4 h, 200 µl of ice-cold nuclease buffer (at the indicated concentration of nuclease) was added, and digestion was continued for 45 min at 37°C. Nuclease S1 buffer contained 0.28 M NaCl, 0.06 M sodium acetate (pH 4.6), 4.5 mM ZnSO<sub>4</sub>, and 20 µg of denatured and sonicated salmon sperm DNA per ml; exonuclease VII digestion buffer contained 67 mM potassium phosphate (pH 7.9), 8.3 mM EDTA, and 10 mM β-mercaptoethanol. Nuclease S1 was purchased from Boehringer-Mannheim Biochemicals; 1 U of

S1 nuclease is the amount which catalyzes the formation of 1 µg of acid-soluble deoxynucleotides in 30 min with denatured DNA at 37°C, whereas one U of exonuclease VII (Bethesda Research Laboratories) produces 1.0 nmol of acid-soluble oligonucleotides in 30 min at 37°C with linear, denatured simian virus 40 DNA as substrate. After digestion, 50 µl of 4 M ammonium acetate and 5 µl of 0.33 M EDTA were added to all assays. Chromatography on Sephadex G50 was used to desalt the exonuclease VII digestions. All reaction mixtures were extracted once with phenol-chloroform-isoamyl alcohol, and the nucleic acids were ethanol precipitated along with 20 µg of yeast tRNA. Each precipitate was dissolved in 40 µl of 10 mM Tris-hydrochloride (pH 7.4) buffer and adjusted to 10% glycerol and 0.04% bromocresol green. Half of the sample was electrophoresed on 1% neutral agarose gels containing Peacock buffer (2.8 mM EDTA, 0.089 M Tris-borate [pH 8.3]), and the other half was adjusted to 0.05 N NaOH and electrophoresed on alkaline agarose gels containing 30 mM NaOH and 1 mM EDTA (15). The *Hae*III fragments of φX174 replicative-form DNA and the *Hind*III fragments of lambda DNA (Bethesda Research Laboratories) were used as molecular size markers. After electrophoresis, the agarose gels were treated with 7% trichloroacetic acid, dried, and directly exposed to X-ray film with intensifying screens at -70°C.

## RESULTS

The genome of *H. saimiri* is composed of three regions: an internal 110-kbp region of unique sequence DNA low in G+C content (37%), termed L-DNA, flanked at each end by repetitive DNA with a high G+C content (71%), termed H-DNA (2, 5); H-DNA is composed of variable numbers of direct tandem repeat units, each 1.4 kbp in length. Fig. 1 shows an expanded scale of the left H/L-DNA border, with

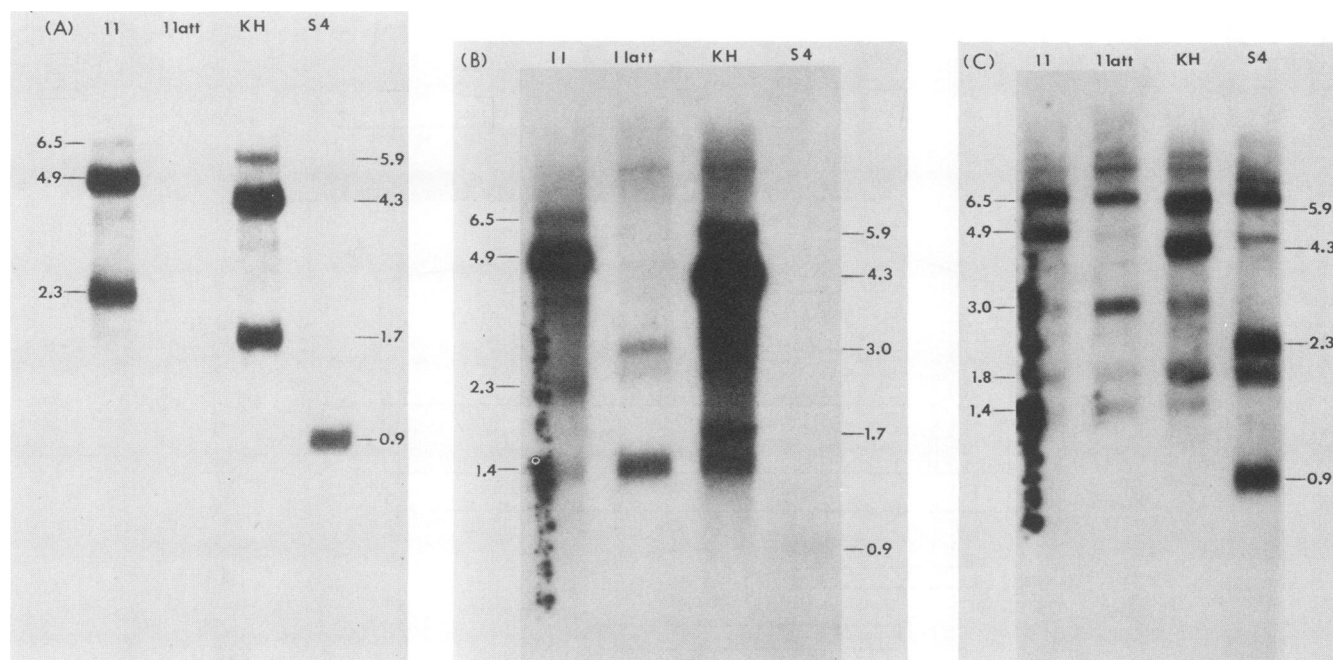


FIG. 2. Northern blot analysis of RNAs from OMK cells infected with either strain 11 of *H. saimiri* or the 11att, KH, or S4 deletion mutants. Confluent cultures of OMK cells were infected with virus at a multiplicity of infection of 1. At 30 to 48 h postinfection, when the cells showed a uniform cytopathic effect, RNA was isolated as described in the text;  $10^7$  dpm of each probe was used sequentially on the same blot. (A) pHp1.4; (B) pHp3.1; (C) pHp2.5. Each slot contained 6 µg of RNA; 28S (4.9 kb) and 18S (1.8 kb) rRNAs were used as molecular size standards.

TABLE 1. Virus-specific RNAs in strain 11 virus-infected OMK cells detected with each of the *HpaII* subcloned DNAs

RNA (kb)	RNA detected with subclone:		
	pHp1.4	pHp3.1	pHp2.5
6.5	+	+	+
4.9	+	+	+
2.3	+	+	-
6.5	-	-	+
3.0	-	-	+
1.8	-	-	+
1.4	-	+	+

the map positions of sequences that have been cloned in plasmids and the sequences that have been deleted from different mutant viruses. The pT7.4 plasmid clone contains 7.4 kbp of L-DNA but little or no H-DNA (4). The 2.3-kbp deletion in the 11att strain, which spans the left H/L-DNA border, arose spontaneously after serial passage of strain 11 virus (14, 18). The 4-kbp S4 deletion was constructed in strain 11 viral DNA by first excising a 4-kbp *SstI* fragment from pT7.4; the resultant plasmid was introduced into permissive OMK cells along with parental strain 11 virion DNA by cotransfection. A recombinant virus (S4) containing the 4-kbp deletion was selected by colony hybridization (4). The 0.6-kbp KH deletion was constructed in the same manner, with elimination of sequences between the *KpnI* and *HpaI* sites. (4).

RNAs transcribed in OMK cells infected with *H. saimiri* 11 or one of the replication-competent variants, 11att, S4, or KH, were analyzed by Northern blot hybridization. The blotted RNA was sequentially probed with the three *HpaII* subclones of pT7.4. After each exposure, the blot was stripped of the hybridization probe and exposed before the next probe was used, to verify that all of the previous probe had been removed.

With the radiolabeled pHp1.4 DNA, three RNAs were detected in strain 11-infected cells (Fig. 2A): two major RNAs of 4.9 and 2.3 kb plus a less abundant RNA of 6.5 kb. These RNAs were determined to be polyadenylated by virtue of their binding to oligodeoxythymidylate-cellulose (data not shown). The pHp1.4 subclone contains sequences close to the H/L-DNA border, and part or all of its sequences are deleted in each of the three deletion mutants. All the pHp1.4 sequences are deleted from the 11att strain, and as expected, OMK cells infected with this virus had no detectable RNA containing these sequences; however, cells infected with the KH and S4 viruses did have RNAs containing these sequences. In each case, the RNAs were shortened precisely by the size of the deletion. The RNAs from the KH-infected cells were 0.6 kb shorter than their strain 11 counterparts: 5.9 versus 6.5 kb, 4.3 versus 4.9 kb, and 1.7 versus 2.3 kb. The S4 RNA samples contained a 0.9-kb RNA, 4 kb shorter than its 4.9-kb counterpart in strain 11-infected cells, and a 2.2-kb RNA, 4.3 kb shorter than the 6.5-kb counterpart.

When blotted RNA was hybridized to the radiolabeled middle *HpaII* fragment (pHp3.1; Fig. 2B), a 1.4-kb RNA was observed in RNA from strain 11-infected cells in addition to the 6.5-, 4.9-, and 2.3-kb RNAs. As with the pHp1.4 probe, RNA from cells infected with the S4 and KH deletion mutants showed precisely shortened RNAs as well as the new 1.4-kb RNA. In addition, the 11att sample had an RNA doublet of ca. 3 kb; this RNA could represent a shortened version of the 4.9-kb RNA seen in strain 11-infected cells.

The radiolabeled pHp2.5 subclone did not detectably hybridize to an RNA of 2.3 kb from strain 11-infected cells, but it did hybridize to a 6.5- and a 4.9-kb RNA (Fig. 2C). In KH virus-infected cells, the 5.9- and 4.3-kb RNAs were still observed, as was the 0.9-kb RNA in the S4 RNA sample. In addition, both a 3- and a 1.8-kb RNA were observed in the RNA samples from the 11, 11att, and KH virus-infected cells. The S4 sample lacked this 3-kb RNA. The 1.8-kb RNA was also detected in the S4 samples as well as a 2.3-kb RNA unrelated to the 2.3-kb RNA coded for by the pHp1.4 and pHp3.1 *HpaII* fragments. Another RNA of 6.5 kb that appeared in all RNA samples represented a new RNA, since neither the 11att nor the S4 samples contained an RNA of this size with sequences complementary to the pHp1.4 and pHp3.1 probes.

The RNAs detected with each of the three *HpaII* subclones are summarized in Table 1. Of these RNAs, only the 4.9- and 2.3-kb RNAs and the minor 6.5-kb RNA were specifically altered by the deletions in the noncogenic variants 11att and S4.

To determine when the 4.9- and 2.3-kb RNAs were synthesized during the course of infection, OMK cells were infected at a multiplicity of 10 in the presence or absence of cycloheximide (200  $\mu$ g/ml). At 4, 8, and 19 h after infection, RNA was prepared from the cycloheximide-treated and untreated cultures. The RNAs were then transferred from an agarose gel to nitrocellulose, and radiolabeled pHp1.4 DNA was used to identify the 4.9- and 2.3-kb RNAs on the blot (Fig. 3). No virus-specific RNA was detected at 4 or 8 h postinfection with or without cycloheximide; however, at 19 h postinfection in the absence of cycloheximide, both the 4.9- and the 2.3-kb RNAs were detected. Neither of these RNAs was detected in cultures that had been treated with cycloheximide. Since the transcription of these RNAs

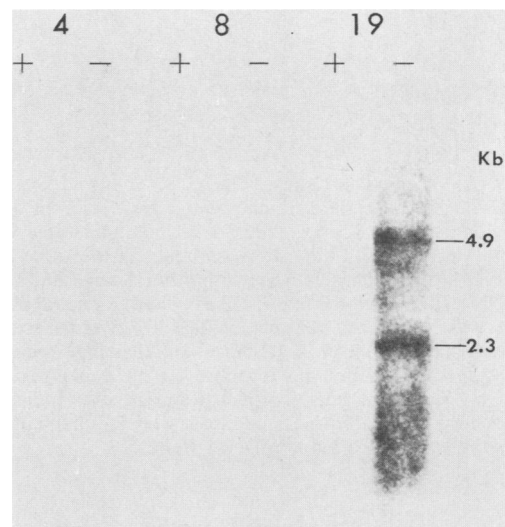


FIG. 3. Time course of the accumulation of virus-specific RNA during infection. Eighteen 150-cm<sup>2</sup> flasks of confluent OMK cells were infected with virus at a multiplicity of 10. Cycloheximide (Sigma Chemical Co.) at 200  $\mu$ g/ml was added to nine of the flasks at the time of infection. At 4, 8, and 19 h postinfection, RNA was extracted from three of the cycloheximide-treated (+) and control cultures (-), and RNA was prepared by guanidine thiocyanate isolation as described in the text. RNA (5  $\mu$ g per slot) was applied to a 1.1% agarose gel. After Northern transfer, 10<sup>7</sup> dpm of <sup>32</sup>P-labeled pHp1.4 DNA was used as radiolabeled probe to detect the 4.9- and 2.3-kb RNAs.

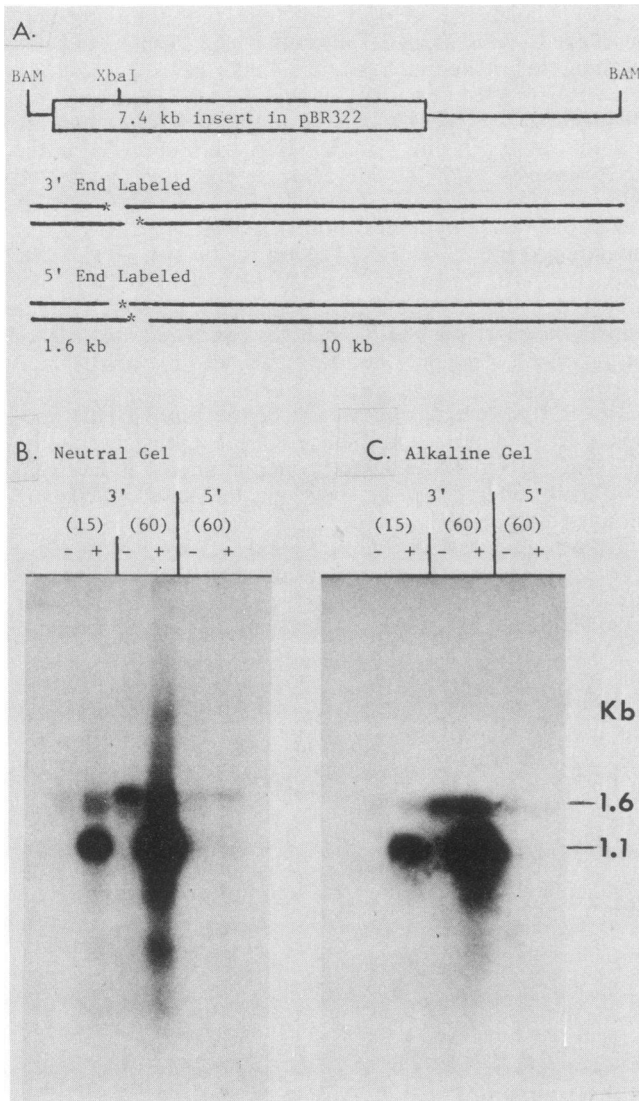


FIG. 4. (A) Mapping strategy. The pT7.4 plasmid DNA was linearized with *Xba*I and then either 3' end labeled with the Klenow DNA polymerase I or 5' end labeled with polynucleotide kinase. The orientation of the 7.4-kbp DNA sequence is the same as that shown in Fig. 1. Secondary cleavage with *Bam*HI generated 1.6- and 10.2-kbp DNA fragments. (B and C) The 3'- and 5'-end-labeled 1.6-kbp DNA was hybridized to total cellular RNA from strain 11-infected OMK cells (+) or yeast RNA (-) and then digested with S1 nuclease (2,500 U/ml) and electrophoresed on a neutral (B) or alkaline (C) 1% agarose gel. Numbers in parentheses indicate  $10^3$  dpm of labeled 1.6-kbp DNA. The nuclease-resistant band at 1.6 kb represents the reannealed 1.6-kbp DNA probe.

seemed to require cellular protein synthesis, they were not immediate early transcription products. The infection of these cultures followed a time course that involved a nearly complete viral cytopathic destruction of the cell monolayer by 36 h postinfection. Therefore, it would appear that these RNAs are produced fairly late during the viral replication cycle.

To map the 5' and 3' ends of the 4.9- and 2.3-kb RNAs on the *H. saimiri* genome, we used S1 nuclease and exonuclease VII digestions of RNA- $^{32}$ P]DNA hybrids and subsequent analysis of the protected radiolabeled DNAs on agar-

ose gels. The length of the protected fragment indicated how far the 3' or 5' end (or splice site) of the RNA was from the radiolabeled restriction site. Fig. 4A illustrates the strategy used for the nuclease mapping experiments. The pT7.4 clone was linearized with *Xba*I and then either 5' end labeled with [ $\gamma$ - $^{32}$ P]ATP by polynucleotide kinase or 3' end labeled with  $\alpha$ - $^{32}$ P-deoxynucleotide triphosphate by the Klenow fragment of DNA polymerase I. After digestion with *Bam*HI, the 1.6- and 10.2-kbp digestion products were isolated by agarose gel electrophoresis. The end-labeled DNAs were then hybridized to the indicated RNA, digested with nuclease, and electrophoresed on agarose gels.

Total cellular RNA from strain 11-infected cells was hybridized to the 3'-end-labeled and the 5'-end-labeled 1.6-kbp DNA (Fig. 4B and C). The RNA from cells infected with strain 11 virus protected a 1.1-kb DNA fragment from the 3'-end-labeled DNA strand on both neutral and alkaline gels. Even this overexposed autoradiogram did not reveal any DNA fragments protected by yeast RNA, nor was a DNA fragment from the 5'-end-labeled strand protected. This implied that there was only one 3' terminus located ca. 1.1 kbp from the *Xba*I site and that transcription was from right to left on the *H. saimiri* genetic map. To confirm this, total cellular RNA was first fractionated on a sucrose gradient to separate the 4.9-kb RNA from the 2.3-kb RNA (Fig. 5). Aliquots containing the 4.9- and 2.3-kb RNA were then hybridized to either the 5'- or 3'-end-labeled 1.6-kbp DNA and digested with S1 nuclease. With both of the RNAs, a 1.1-kb DNA fragment was protected from the 3'-end-labeled DNA strand on both the neutral and alkaline gels (Fig. 6). This indicated that the 3' end of each of the RNAs was 1.1 kbp from the *Xba*I site, ca. 150 base pairs from the left H/L-DNA border.

Since the orientation of the RNAs was now known, the 5' ends of the RNAs were mapped by hybridizing the 5'-end-labeled 10.2-kbp restriction fragment to RNA from strain 11-infected cells (Fig. 7). At low concentrations of S1 nuclease, the RNA from *H. saimiri*-infected cells protected DNA fragments of 3.6 and 1 kb. As the S1 nuclease concentration

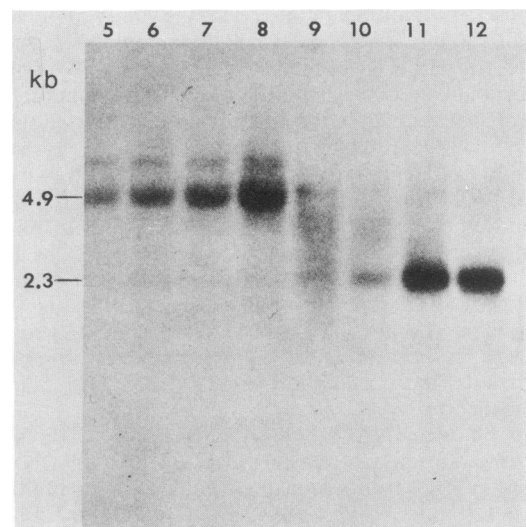


FIG. 5. Sucrose gradient separation of the 4.9- and 2.3-kb RNAs. A 5 to 20% sucrose gradient was used to separate the 4.9-kb RNA (pooled fractions 7 and 8) from the 2.3-kb RNA (pooled fractions 11 and 12). Portions of each fraction were analyzed on a Northern blot with the  $^{32}$ P-labeled pHp1.4 probe.

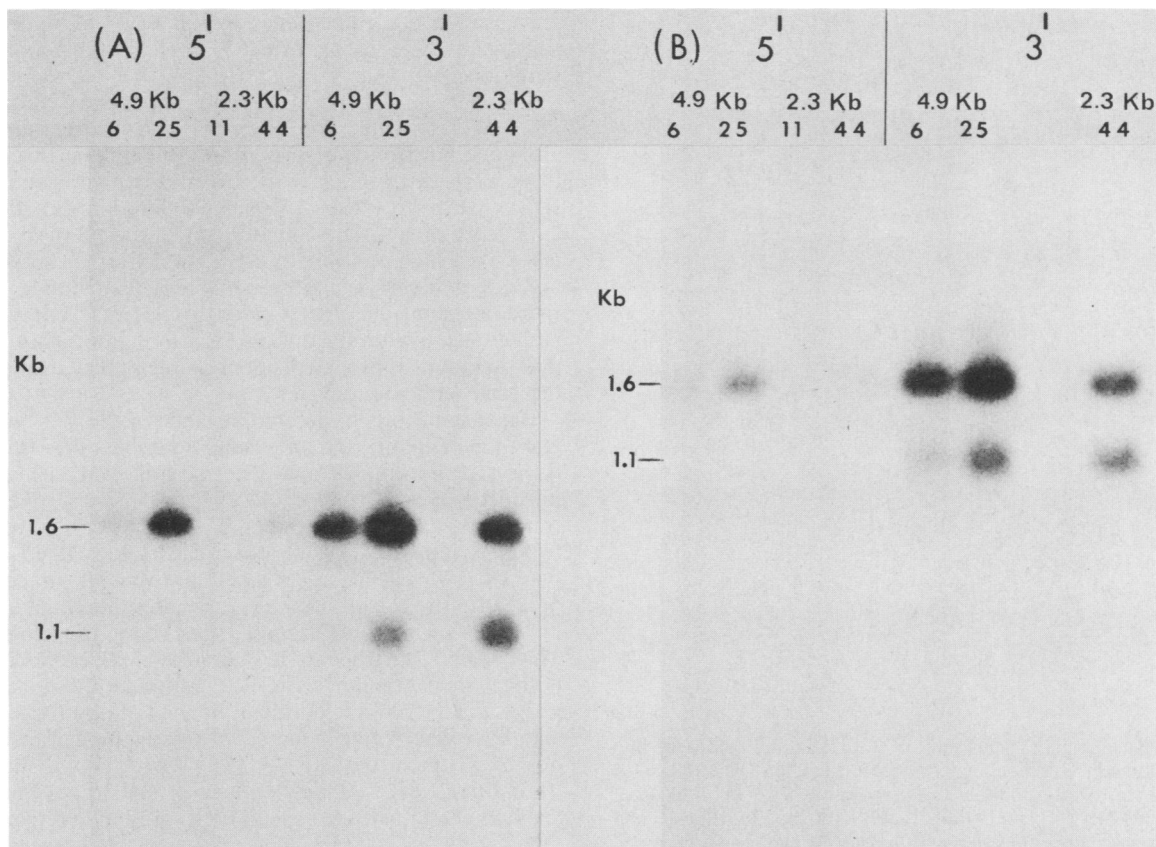


FIG. 6. S1 mapping of the 3' ends of the 4.9- and 2.3-kb RNAs. The 4.9- and 2.3-kb RNAs were hybridized to  $40 \times 10^3$  dpm of either the 3'- or 5'-end-labeled 1.6-kbp DNA and digested with S1 nuclease. (A) Neutral gel; (B) alkaline gel. Numbers indicate the amounts of RNA (in micrograms) per assay.

was increased to 2,500 U/ml, the amount of 3.6-kb DNA decreased and the amount of 1-kb DNA increased along with a new DNA band at 0.8 kb. The 3.6-kbp band observed at low S1 nuclease concentrations was the size expected for protected DNA from the *Xba*I site to the 5' terminus of the 4.9-kb RNA, and the 1-kbp band was the size expected for protected DNA from the *Xba*I site to the 5' end of the 2.3-kb RNA. To determine the origin of the 0.8-kb fragment, the 4.9- and 2.3-kb RNAs were each hybridized to 5'-end-labeled 10.2-kbp DNA and digested with S1 nuclease. With the 4.9-kb RNA, the alkaline agarose gel of the digestion products showed that as the S1 nuclease concentration was increased to 20,000 U/ml, the 3.6-kb band completely disappeared and two fragments of 1 and 0.8 kb were generated and that the 1-kb fragment, in turn, was converted to the 0.8-kb fragment. The 2.3-kb RNA also protected 1 kb of DNA that was converted to a 0.8-kb DNA fragment as the S1 nuclease concentration was increased (Fig. 8). These results indicated that the 4.9-kb RNA was spliced at 0.8 and 1 kbp from the *Xba*I site and that the 2.3-kb RNA was also spliced at the 0.8-kbp location. To determine the location of the 5' ends of the RNAs, exonuclease VII was used to digest the hybrids formed between the 5'-end-labeled DNA and either the 2.3-kb RNA or the 4.9-kb RNA. The digestion products were then analyzed on alkaline agarose gels along with their S1 nuclease-treated counterparts (Fig. 9). Exonuclease VII does not degrade RNA, single-stranded regions of duplex nucleic acids, or intron loops, but instead trims single-

stranded DNA ends. Therefore, the length of the exonuclease VII digestion products in this case should be a measure of the distance from the *Xba*I site to the 5' end of the RNA. With the 4.9-kb RNA, exonuclease VII protected a single DNA band of 3.6 kb, the same size as the longest S1 nuclease product observed on either alkaline (Fig. 8) or neutral (Fig. 7) agarose gels, and no bands of 1 or 0.8 kb were observed. This indicated that the 5' end of the 4.9-kb RNA was 3.6 kbp from the *Xba*I site and that the splices that generated the 0.8- and 1-kb bands on these gels had very short introns. The 3.6-kbp DNA from the right side of the *Xba*I site plus the 1.1-kbp S1 nuclease fragment from the left side of the *Xba*I site combined to make a 4.7-kb transcript length, which would be just long enough to code for a 4.9-kb RNA containing a 200-nucleotide polyadenylate tail. The close agreement in size between the 4.9-kb RNA found in infected cells and the RNA measured by nuclease mapping of the 5' and 3' termini on the *H. saimiri* genome also substantiated our claim that the lengths of the intron segments lost from the mature RNA were quite small, probably less than 100 nucleotides. With the 2.3-kb RNA, exonuclease VII produced one DNA fragment of 1 kb and no smaller fragments. Therefore, the 5' end of this transcript was 1 kbp from the *Xba*I site, with a splice point at 0.8 kbp. This 1-kbp DNA plus the 1.1-kbp DNA to the left side of the *Xba*I site combined for a transcript length of 2.1 kb. As with the 4.9-kb RNA, the intron spliced out of the RNA at the 0.8-kbp splice point must be very small since the S1 nuclease product on

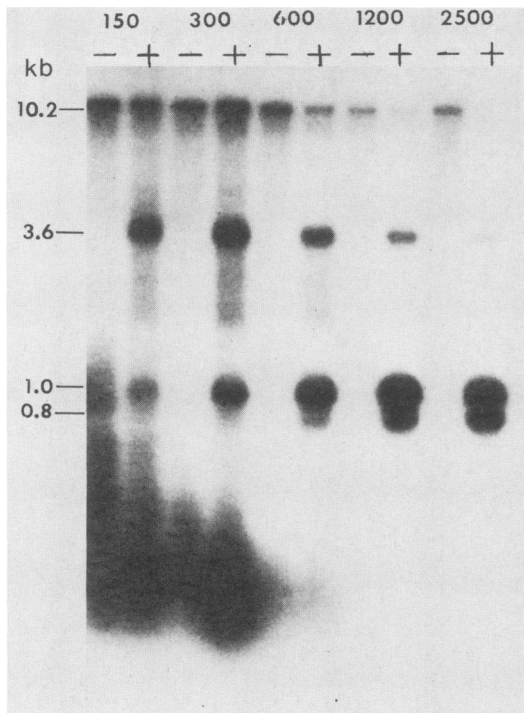


FIG. 7. S1 nuclease digestion of the 5'-end-labeled 10.2-kbp DNA hybridized to total cellular RNA. End-labeled DNA ( $50 \times 10^3$  dpm) was hybridized to either 20  $\mu$ g of yeast RNA (-) or 20  $\mu$ g of total cellular RNA (+) from strain 11 virus-infected OMK cells and then digested at increasing concentrations of S1 nuclease (expressed in Boehringer Mannheim units per milliliter). Neutral 1% agarose gels were used. The nuclease-resistant band at 10.2 kb represents the reannealed 10.2-kbp DNA probe.

both neutral and alkaline gels was approximately the same size as the exonuclease VII product, and the size of the 2.3-kb cellular RNA was close to the overall size of the transcript estimated from the nuclease mapping of the termini of the RNA on the *H. saimiri* genome. From the nuclease mapping data, the overall length of the RNA would be just enough the code for an RNA of 2.3 kb containing a 200-nucleotide polyadenylate tail. The nuclease mapping data are summarized in Fig. 10.

#### DISCUSSION

We have used Northern blot analysis to characterize the RNAs produced from the leftmost 7.4 kbp of unique-sequence DNA of the *H. saimiri* genome. Two major polyadenylated transcripts of 4.9 and 2.3 kb have been identified that are specifically altered in both the 11att and S4 nononcogenic variants. The 11att and S4 deletion mutants, as well as a third mutant containing a deletion in this region, termed KH, whose oncogenic properties have not been determined, all either produce precisely shortened RNAs or lack them altogether. In addition to the 4.9- and 2.3-kb RNAs, a 6.5-kb RNA is detected in much smaller amounts, and this minor RNA is also precisely shortened by these deletions. There are also four RNAs transcribed from sequences just to the right of this region on the *H. saimiri* genetic map, but they are not affected by these deletions. In addition, our results with a cloned probe of the H-DNA repeat unit in conjunction with these Northern blots indicated that H-DNA is not transcribed into RNA (data not shown). Thus, the 4.9-

2.3-kb RNAs and the minor 6.5-kb RNA all appear to be specifically altered by the deletions in the nononcogenic variants.

The 4.9- and 2.3-kb RNAs are both very abundant in infected OMK cells. We have not precisely determined their amounts, but from a comparison of their signal strengths on these blots with other Northern blots of viral and cellular RNAs we have estimated the abundance to be 200 to 800 copies per cell. A time course study of the synthesis of these RNAs at a high multiplicity of infection has shown that they are not made in the presence of cycloheximide and are therefore not immediate early transcripts, but are transcribed relatively late during viral infection. This suggests that their synthesis is dependent on regulatory events during the viral replication cycle.

Because of the possible importance of the 4.9- and 2.3-kb RNAs in mediating the oncogenic potential of *H. saimiri*, we have mapped their 5' and 3' termini as well as their exon-intron topography (Fig. 10). These RNAs are overlapping transcripts that each have their 3' terminus ca. 150 base pairs from the left H/L-DNA border. The 4.9-kb RNA contains two closely spaced splice sites, the first of which is also present in the 2.3-kb RNA. The 5' end of the 2.3-kb RNA is located at or near this second splice site of the 4.9-kb RNA. The 4.9-kb RNA appears to contain no splice sites from this point to its 5' terminus (Fig. 10). Although these introns are very short (<100 nucleotides), introns of approximately this size have also been detected in overlapping transcripts in herpes simplex virus RNA (9). We do not believe that "breathing" of A+T-rich regions of DNA could impart an artifactual S1 nuclease sensitivity to these two regions, since

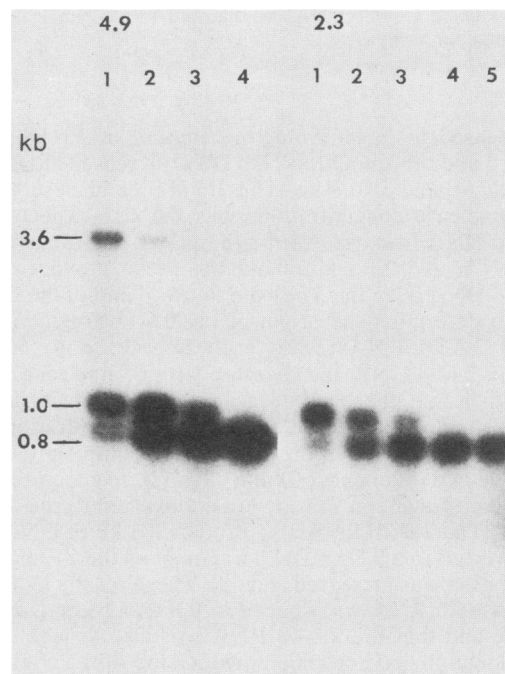


FIG. 8. S1 nuclease digestion of 5'-end-labeled 10.2-kbp DNA hybridized to either the 4.9- or 2.3-kb RNA;  $40 \times 10^3$  dpm of  $^{32}$ P-labeled DNA was hybridized to 20  $\mu$ g of either the 4.9- or 2.3-kb RNA. Digestion with S1 nuclease was carried out at the following concentrations (in Boehringer Mannheim units per milliliter): (lane 1) 1,000; (lane 2) 2,500; (lane 3) 10,000; (lane 4) 20,000; (lane 5) 40,000. Alkaline 1% agarose gels were used.

S1 nuclease concentrations as low as 300 Boehringer Mannheim units per ml at 30°C are capable of generating the 0.8- and 1-kbp fragments. Likewise, S1 nuclease concentrations from 150 to 40,000 U/ml have failed to generate any additional nuclease-resistant fragments on neutral or alkaline agarose gels; we therefore doubt that there are any additional splice sites within these RNAs. We have also been unable to detect any evidence for alternate splicing between these two sites, since Northern blots of the cellular RNAs do not show multiple bands of the expected sizes.

Nothing is known about the function of these RNAs and the role they might play in oncogenesis by *H. saimiri*. The fact that they are polyadenylated suggests that they code for protein products, but whatever the function of these proteins might be they do not seem to be analogous to retroviral oncogenes for several reasons. First, unlike viral oncogenes whose continual expression is required to maintain the transformed phenotype (1), continued synthesis of the *H. saimiri* 6.5-, 4.9-, and 2.3-kb RNAs does not seem to be necessary to maintain lymphoid cell transformation. We have not observed these RNAs in several *H. saimiri*-induced owl monkey lymphomas or in the 1670 tumor cell line, even though the *H. saimiri* DNA sequences were consistently

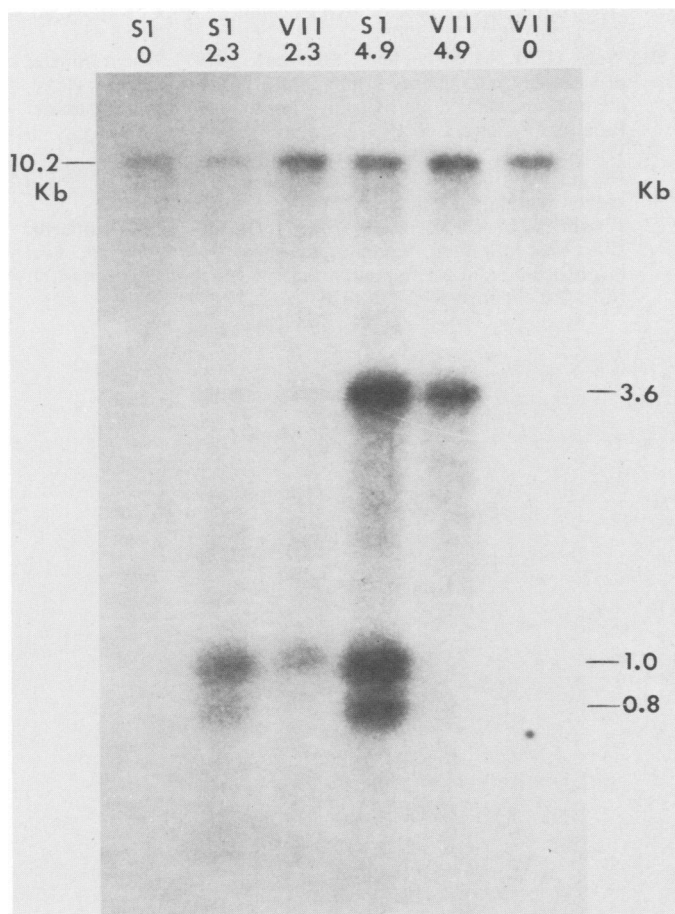


FIG. 9. Exonuclease VII digestion of 5'-end-labeled 10.2-kbp DNA hybridized to either the 4.9- or 2.3-kb RNA. <sup>32</sup>P-end-labeled 10.2-kbp DNA (20 × 10<sup>3</sup> dpm) was hybridized to the 2.3- or 4.9-kb viral RNA or to 20 μg of yeast RNA (lanes 0). S1 nuclease (2,500 U/ml) or exonuclease VII (4.8 U/ml) digestion was followed by electrophoresis on alkaline 1% agarose gels.

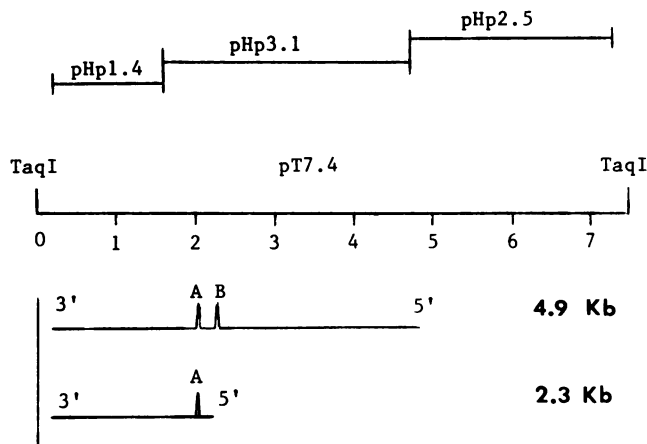


FIG. 10. Genetic map positions of the 5' and 3' termini and the exon-intron topography of the 4.9- and 2.3-kb transcripts. A and B, Points at which splice sites occur along the RNAs.

retained. Second, unlike retroviral oncogenes, which were captured by these viruses from cellular sequences and whose normal cellular homolog is well conserved in vertebrate cells (1), we have not detected cellular DNA sequences which cross-hybridize with pT7.4 sequences on Southern blots at a sensitivity of 0.1 copy per cell. In addition, unlike the transforming regions of the genomes of the papovaviruses simian virus 40 and polyomavirus (11) and the adenoviruses (8), which are all transcribed early in infection, the 4.9- and 2.3-kb RNAs of *H. saimiri* are transcribed late in the viral replication cycle. If these RNAs are essential for transformation by *H. saimiri* it seems likely that they will have a "hit-and-run" type of function in which their continued presence is not required to maintain the transformed phenotype. We are therefore examining the RNAs of owl monkey lymphocytes during the course of their transformation in vitro by *H. saimiri* for the transient expression of these RNAs. Also, since we now know the map location of the termini of these RNAs, it may be possible to delete small regions of DNA at the promoter site to prevent their transcription. It might also be possible to test the oncogenicity of virus strains which do not express one or the other of these two RNAs.

Indirect mechanisms, independent of these RNA and viral protein products, might also be responsible for oncogenesis by *H. saimiri*. Such indirect mechanisms have appeal since it is known that in cells transformed by *H. saimiri*, viral DNA is frequently rearranged with extensive deletions (3, 12); perhaps some rearrangements involve cellular genes with concomitant activation. Although neither we nor others have detected any polyadenylated RNAs derived from the left end of *H. saimiri* L-DNA in the 1670 tumor cell line or in any lymphomas, a polyadenylated RNA that is transcribed from the right end of the *H. saimiri* genome has been found in 1670 cells (13). We have been able to detect large amounts of a 100-nucleotide RNA coded for by the pT7.4 sequences in the 1670 cell line and the five *H. saimiri*-induced tumors we have examined. We have not detected this RNA in uninfected OMK cells or in lytically infected OMK cells. Future work will be directed at testing whether this short, nonpolyadenylated RNA might have a novel activator role in transformation of lymphoid cells by *H. saimiri*.

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