JOURNAL OF VIROLOGY, Feb. 1984, p. 343–348
0022-538X/84/020343-06$02.00/0

Vol. 49, No. 2

# Construction of Replication-Competent *Herpesvirus saimiri* Deletion Mutants

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Received 20 June 1983/Accepted 12 October 1983

DNA fragments derived from the left end of *Herpesvirus saimiri* 11 L-DNA were cloned in *Escherichia coli* by using vector pBR322. Deletions were introduced within a cloned 7.4-kilobase-pair sequence by using restriction endonucleases that cut once or twice within this sequence. Permissive owl monkey kidney-cultured cells were transfected with parental strain 11 viral DNA plus cloned DNA with specific sequences deleted. By screening the progeny of these transfections with a limiting-dilution spot hybridization assay, we isolated recombinant viruses containing deletions in this region. A contiguous 4.5-kilobase-pair sequence representing 4.1% of the coding capacity of the virus was found to be unnecessary for virus replication in cultured cells. These deletion mutants will allow us to test whether sequences in this region are required for the lymphoma-inducing capacity of *H. saimiri*. These same procedures should also allow us to introduce foreign DNA sequences into this region for studying their expression.

*Herpesvirus saimiri* offers a number of advantages for study of oncogenic transformation in a herpesvirus system. Lymphomas are induced rapidly and reproducibly in a variety of New World primate species (for reviews, see references 8, 9, and 19). The ability to grow *H. saimiri* lytically in cultured New World primate monolayer cells furnishes distinct advantages over other lymphotropic viruses such as Epstein-Barr virus for which no truly permissive system is known. Numerous lymphoid tumor cell lines are available, and these may be used to study the persistence of viral DNA and the factors contributing to growth transformation (3, 4, 15).

Passage of *H. saimiri* 11 at 39 and 34°C and repeated plaque purification yielded an unusual nononcogenic variant, 11att (20). This isolate has not produced lymphomas in normally susceptible species: cotton-top marmoset (*Saguinus oedipus*), white-lip marmoset (*Saguinus fuscicollis-nigricollis*), common marmoset (*Callithrix jacchus*), and owl monkey (*Aotus* sp.) (7, 22, 23; L. Falk personal communication). Strain 11att grows as well as parental virus in vitro and produces persistent infection of lymphocytes in vivo; New World primates persistently infected with strain 11att do not, however, develop disease (6). Restriction endonuclease analysis of virion DNA has indicated that about two kilobase pairs (kbp) have been deleted from the left junction of unique (L-) and repetitive (H-) DNA in the generation of 11att (J. Koomey, C. Mulder, R. Burghoff, B. Fleckenstein, and R. Desrosiers, submitted for publication) (see Fig. 1 for a schematic of the structure of virion DNA). It is currently not known whether the deletion in 11att is responsible for the loss of oncogenicity; it is conceivable that point mutations or other undetected changes in other regions of the 11att genome are responsible for or contribute to the inability to induce lymphomas.

In this communication, we report the cloning of DNA fragments from the L-DNA left end of *H. saimiri* 11 and describe procedures we used for constructing replication-competent deletion mutants.

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## MATERIALS AND METHODS

**Cells, virus, and virion DNA.** *H. saimiri* 11 and 11att were originally provided by L. Falk. Virus was propagated in owl monkey kidney (OMK) cell line 637; cells were used between passages 10 and 30. Purified virion M-DNA was prepared as previously described (1). Virus was titered by limiting dilution.

**Molecular cloning.** The 7.4-kbp *Taq*I fragment of strain 11 was purified from an agarose gel by electroelution in a dialysis bag and cloned into the *Cla*I site of plasmid vector pBR322. *Cla*I-cleaved pBR322 was incubated with the 7.4-kbp *Taq*I fragment and T4 DNA ligase for 16 h at 16°C, and this was introduced into competent *Escherichia coli* K-12 (HB101). Cells were spread on Luria broth plates containing 50 μg of ampicillin per ml. A tetracycline-sensitive colony that hybridized to strain 11 DNA was selected for further analysis. *Hpa*II fragments of the pT7.4 clone (see below) were partially purified by sucrose gradient sedimentation and cloned similarly into the *Cla*I site of pBR322. Cloning of *Taq*I (T ↓ CGA) fragments into the *Cla*I (AT ↓ CGAT) site regenerates *Taq*I but not *Cla*I recognition sequences at the insertion site. Cloning of *Hpa*II (C ↓ CGG) fragments into the *Cla*I site generates neither *Hpa*II nor *Cla*I recognition sequences at the insertion site.

For preparation of cloned plasmid DNA, plasmids were amplified in minimal (M9) medium with 170 μg of chloramphenicol per ml and plasmid DNA was prepared by the Triton lysis procedure of Herschfield et al. (14). Plasmid DNA was purified by cesium chloride-ethidium bromide gradient centrifugation, the ethidium bromide was removed by repeated extractions with isoamyl-alcohol, and the DNA was dialyzed first versus 0.5 M NaCl–10 mM Tris-hydrochloride (pH 7.5) and then versus 10 mM Tris-hydrochloride (pH 7.5). The DNA was precipitated with ethanol and suspended and stored at 4°C in 1 mM EDTA–10 mM Tris-hydrochloride (pH 7.5).

**Isolation of replication-competent deletion mutants.** Preconfluent monolayers of OMK cells were mix-transfected with 0.2 μg of parental strain 11 DNA plus 2 μg of linearized plasmid DNA containing a deletion within the viral se-

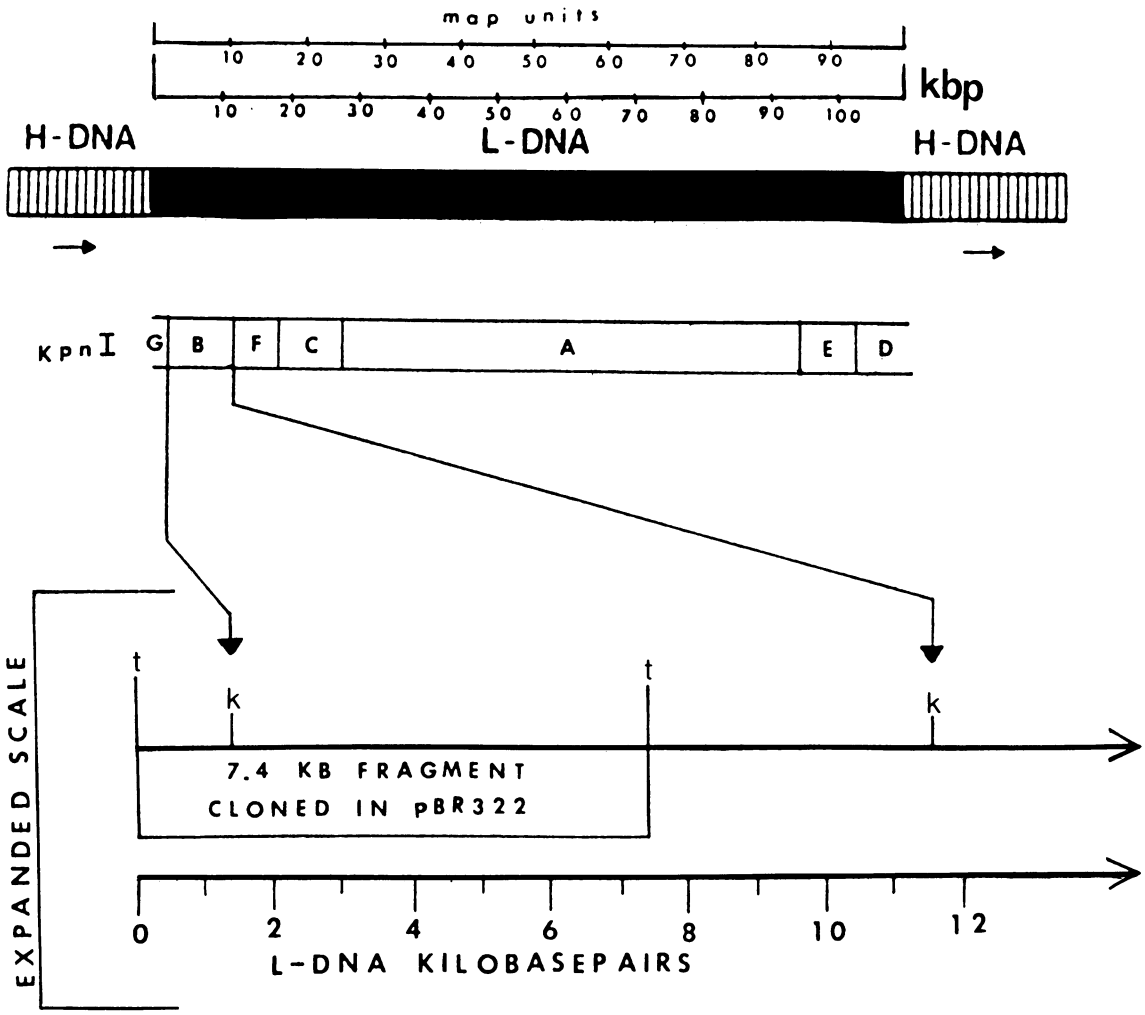


FIG. 1. Structure of *H. saimiri* virion DNA and the origin of pT7.4. The left border of H- and L-DNA occurs within 100 base pairs leftward from the *Taq*I site shown as 0.0 L-DNA kilobase pairs. Symbols: t, *Taq*I; k, *Kpn*I.

quences by the procedure of Graham and van der Eb (12). A 25-s glycerol shock of cells was sometimes used (11). Plasmid DNA was linearized with *Eco*RI, and the *Eco*RI was heat inactivated at 70°C for 10 min before transfection. These amounts of DNA represent about a 200-fold molar excess of the fragment. Transfected cells were incubated in complete minimal essential medium (MEM) with 10% heat-inactivated fetal calf serum at 37°C until virally induced cytopathic effect was complete, usually 7 to 11 days.

Resultant virus was diluted in minimal essential medium, and 150 μl was added to OMK cells growing in individual wells of 96-well Linbro trays. Trays were incubated until virally induced cytopathic effect was complete, usually about 8 days. Viral dilutions were chosen such that 30 to 60% of wells yielded virus, usually $2 \times 10^{-6}$ dilution. Using the equation $B_0 = Be^{-vpw}$, where B is the total number of wells, $B_0$ is the number of wells not showing virally induced cytopathic effect, and vpw is infectious virus particles per well, 30 to 60% of wells yielding virus represents 0.36 to 0.91 infectious virus particles per well.

A spot hybridization assay was used to identify replication-competent virus containing a deletion (2). Samples (2 μl) of viral lysates from positive wells were spotted onto sheets of nitrocellulose and air dried. An 88-$cm^2$ filter can easily accommodate 100 such spots. Virions were disrupted, and DNA was denatured in situ by placing nitrocellulose filters (spot side up) on a piece of 3 MM paper soaked with 0.5 M NaOH (7 min). Nitrocellulose filters were then sequentially placed on 3 MM paper soaked with 6 M NaCl–1 M Tris-hydrochloride (pH 6.8) (twice for 1 min), and 1.5 M NaCl–0.5 M Tris-hydrochloride (pH 6.8) (twice for 5 min). Nitrocellulose filters were then air dried (about 20 min), soaked in chloroform, and air dried again. Filters were then completely soaked in 2× SSC (1× SSC = 0.15 M NaCl plus 0.015 M sodium citrate) for 20 min; submerged filters were hand rubbed with gloved fingers to remove debris and particulate material. Filters were baked at 80°C for 2 h and hybridized with $^{32}$P-labeled DNA as described below.

**Gel electrophoresis.** DNAs cut with restriction endonucleases were electrophoresed through horizontal agarose gels in 1× Peacock buffer (2.8 mM EDTA–0.089 M Tris-borate [pH 8.3]). Gels were soaked in 1 μg of ethidium bromide per ml for 1 h and destained in 1× Peacock buffer for a few hours. Stained DNA fragments were photographed over UV (302 nm) light. Lambda DNA × *Hin*dIII fragments were used for molecular size markers.

**Filter hybridizations.** DNA fragments in agarose gels were transferred to nitrocellulose filters according to the proce-

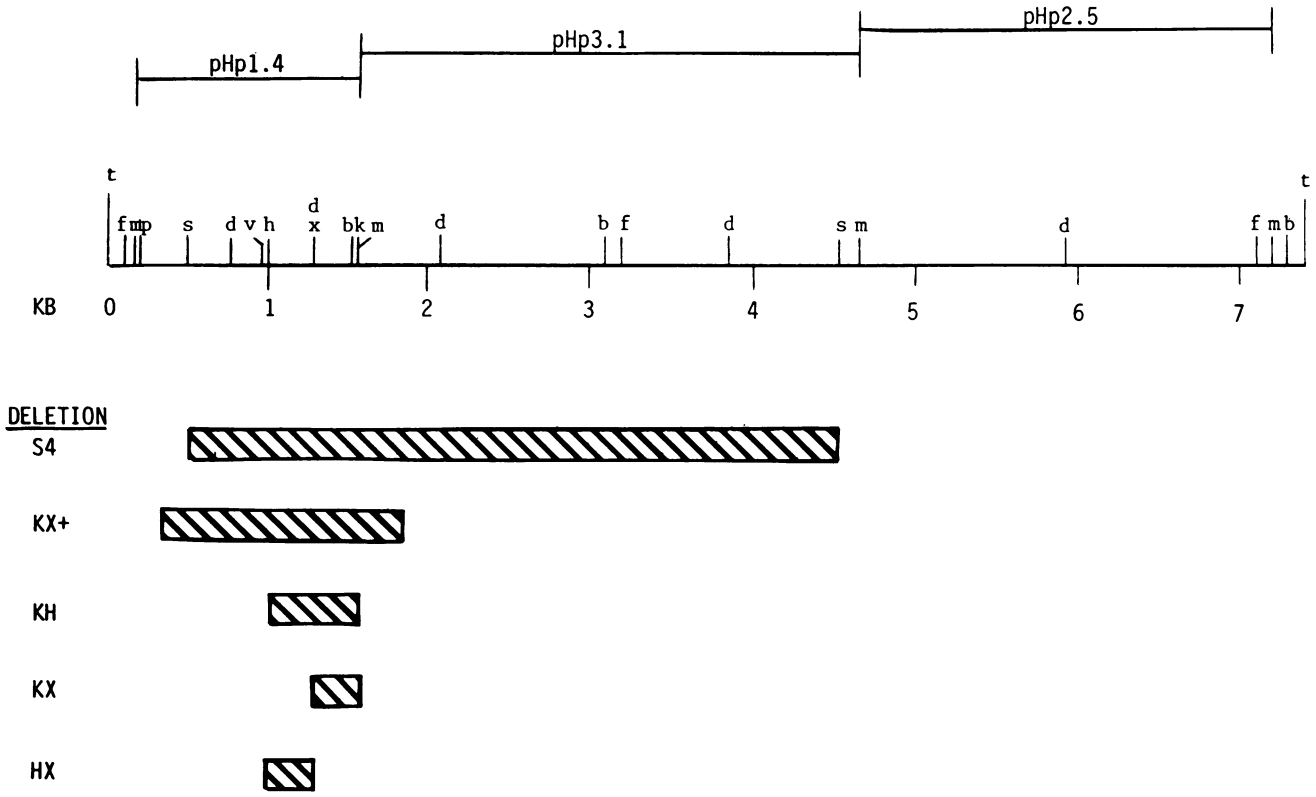


FIG. 2. Restriction endonuclease maps of the 7.4-kilobase-pair sequence of pT7.4 and of its constructed deletions. The right border of the deletion in 11att is located between the *Kpn*I and *Hin*dIII sites (1.5 to 2.05 kbp). The left border of the 11att deletion is located in H-DNA (submitted). The *Hpa*II site at 7.2 kbp and the *Fnu*dII site at 7.1 kbp are undermethylated in the 1670 tumor cell line (4). Symbols: m, *Msp*I (*Hpa*II); t, *Taq*I; f, *Fnu*dII; p, *Pst*I; s, *Sst*I; h, *Hpa*I; x, *Xba*I; k, *Kpn*I; b, *Bgl*II; d, *Hin*dII; and v, *Pvu*II.

dure of Southern (21). DNA to be used as hybridization probe was labeled in vitro with $^{32}P$ by nicking with DNase and incubation at 16°C with nucleoside triphosphates (at least one of which was alpha-$^{32}P$ labeled) and *E. coli* DNA polymerase I (17, 18). Nitrocellulose filters were soaked in 4× SSC–0.1% sodium dodecyl sulfate, rolled end to end, and incubated with denatured [$^{32}P$]DNA in 4× SSC–0.1% sodium dodecyl sulfate at 67°C for Southern blot hybridizations or 56°C for virus or bacterial colony hybridization (13). Filters were rinsed for autoradiography as described previously (4).

## RESULTS

**pT7.4.** We set out to clone DNA fragments derived from the left end of *H. saimiri* 11 L-DNA that included sequences contained within the 11att deletion. Cloning of DNA fragments that span the left L-DNA–H-DNA border is not straightforward because of the extreme differences in the G+C content of L- and H-DNA. Many restriction endonucleases useful for cloning (e.g., *Eco*RI, *Bam*HI, *Sal*I) cleave L-DNA very infrequently and do not cleave within H-DNA at all (10). We compared digestions of 11 and 11att DNAs with a number of enzymes and chose to clone the 7.4-kbp *Taq*I fragment of strain 11; the corresponding 11att fragment was 6.0 kbp, and other differences among *Taq*I fragments were not detected.

Cloning of *Taq*I fragments into the *Cla*I site of pBR322 was found to interrupt the tetracycline resistance gene with high efficiency; greater than 90% of colonies with *Taq*I inserts in the *Cla*I site were resistant to ampicillin (50 μg/ml) but sensitive to tetracycline (50 μg/ml).

The 7.4-kbp *Taq*I fragment of strain 11 was purified from an agarose gel and ligated into the *Cla*I site of pBR322. We chose one ampicillin-resistant, tetracycline-sensitive colony (pT7.4) for larger scale growth and detailed analysis.

*Taq*I digestion of pT7.4 produced a fragment that comigrated with the 7.4-kbp *Taq*I fragment of virion DNA, and $^{32}P$-labeled pT7.4 hybridized strongly to this fragment. Plasmid DNA pT7.4 contained sequences derived from the left end of L-DNA since it hybridized to the following virion DNA fragments on Southern transfers: *Eco*RI + *Sma*I fragment A, *Kpn* fragment B and *Kpn*I + *Sma*I fragment G. The map locations for these fragments can be found in reference 10. The relative locations of the *Kpn*I sites in L-DNA and pT7.4 shown in Fig. 1 were determined by mapping and Southern blot hybridization. Comparative analysis of pT7.4 with virion DNA and with other clones recently obtained in our laboratory has localized the left junction of H- and L-DNA to within 100 base pairs to the left of the left *Taq*I site in the 7.4-kbp sequence (Koomey et al., submitted for publication).

Restriction endonuclease cleavage sites within the 7.4-kbp sequence were mapped by using a strategy of single and double digestions (Fig. 2). The enzymes *Hpa*I, *Xba*I, and *Kpn*I each cleaved the 7.4-kbp sequence only once and none of these enzymes cleaved within pBR322 sequences. *Sst*I, which also does not cleave within pBR322, cleaved the 7.4-kbp sequence twice. Four *Hpa*II sites spanned most of the

length of the 7.4-kbp sequence producing fragments of 1.4, 3.1, and 2.5 kbp. These *Hpa*II fragments were conveniently subcloned again into the *Cla*I site of pBR322 and were useful for mapping restriction sites of greater frequency (Fig. 2).

**Construction of deletions within pT7.4.** Restriction endonucleases which cut the 7.4-kbp sequence once or twice but which did not cut pBR322 sequences provided a convenient means for introducing deletions into the cloned DNA. A 4.0-kbp deletion derivative (pS4) was constructed by digestion with *Sst*I, ligation, and transfection back into *E. coli*; selected ampicillin-resistant colonies contained the expected 4.0-kbp deletion (Fig. 3; other mapping data not shown). Similarly, deletions were constructed by double digestion with *Kpn*I + *Xba*I (pKX), with *Kpn*I + *Hpa*I (pKH), and with *Hpa*I + *Xba*I (pHX). After double digestion, DNA was treated with S1 nuclease, ligated, and transfected into *E. coli*. Plasmid DNA from selected ampicillin-resistant colonies was characterized for the expected deletion (Fig. 3; other mapping data not shown). The larger-than-expected KX+ deletion probably arose from overdigestion with S1 nuclease.

***H. saimiri* deletion mutants S4 and KH.** OMK cells were transfected with strain 11 virion DNA and linearized pS4 DNA as described above. To identify recombinant virus with the 4-kbp deletion, we used a limiting-dilution, spot hybridization assay. Virus resulting from the mixed transfection was diluted and added to OMK cells growing in 96-well Linbro trays such that each well received, on the average, less than one infectious virus particle. Aliquots from wells exhibiting virally induced cytopathic effect were spotted on nitrocellulose, and the DNA was denatured in situ and hybridized to a $^{32}$P-labeled, purified 4.0-kbp fragment resulting from *Sst*I digestion of pT7.4. Results from hybridization of aliquots from 24 wells are shown in Fig. 4. Of these 24 samples, 1 did not hybridize detectably to the 4.0-kbp fragment in this assay, indicating a virus lacking the 4.0-kbp fragment. Of 113 wells examined, 7 (6.2%) did not hybridize detectably to the *Sst*I 4.0-kbp fragment in the spot hybridization. Three of the isolates (S4-A9, S4-E4, and S4-G8) were grown to larger volumes, and virion DNA was prepared for

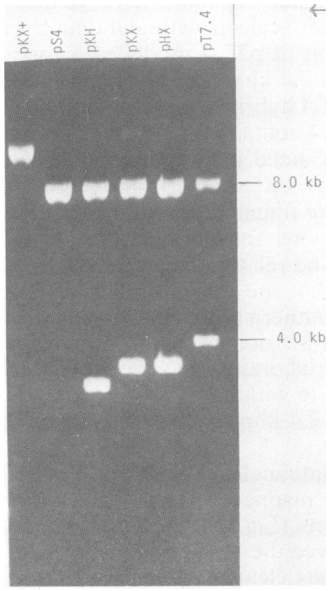


FIG. 3. Agarose gel electrophoresis of pT7.4 and constructed deletion plasmid DNA cut with *Sst*I.

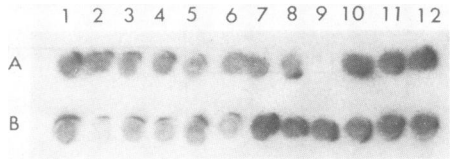


FIG. 4. Limiting-dilution, spot hybridization assay to detect virus with S4 deletion.

restriction endonuclease analysis (Fig. 5). The 7.4-kbp *Taq*I fragment was not observed in the ethidium bromide-stained gel with any of these three isolates; a new *Taq*I fragment of 3.4 kbp was observed with these S4 isolates, consistent with the expected 4.0-kbp deletion. Similarly, an *Sst*I fragment of 4.0 kbp was not observed with the three S4 isolates. Also, the *Kpn*I + *Sma*I fragments B (10.2 kbp) and G (1.5 kbp) were not observed in the ethidium bromide-stained gel, but a new fragment of 7.7 kbp was observed, further confirming the 4.0-kbp deletion. Hybridization of Southern blots to $^{32}$P-labeled pT7.4 was also consistent with these observations (Fig. 5). The Southern blot hybridizations did reveal a slight contamination of the S4-E4 isolate with parental strain 11 sequences; the S4-E4 isolate was not further used.

Similar results were obtained after cotransfection with strain 11 virion DNA and pKH DNA. In this case, the purified 300-base-pair *Kpn*I + *Xba*I fragment of pT7.4 served as hybridization probe. About 5% recombinant virus was obtained. One of the recombinant viruses, KH-G4, was shown to lack the appropriate 0.6-kbp fragment. Isolates S4-A9 and KH-G4 were again clonally purified by limiting dilution, and stocks derived from these clonally purified strains were used in all subsequent experiments. The rate of growth and yield of these deletion derivatives on OMK cells was not detectably different from those of the parental strain 11 virus.

Virion DNA of clonally purified *H. saimiri* isolates S4-A9 and KH-G4 was compared with DNA of strains 11 and 11att. The results of *Taq*I, *Sst*I, and *Kpn*I + *Sma*I digestions confirmed the expected 4.0- and 0.6-kbp deletions (Fig. 6; other data not shown). The Southern blot hybridizations in Fig. 6 represent only a 1-h exposure. Exposure for several days did not reveal any parental strain 11 left-end L-DNA sequences. Thus, no parental left-end L-DNA sequences were detected under conditions where a 1% level of contamination would easily have been detected. $^{32}$P-labeled pBR322 did not hybridize detectably to viral deletion derivative DNA, indicating that these isolates did not contain sequences derived from pBR322.

The presence of naturally occurring deletions in the L-DNA left end of strain 11 was examined by spot hybridization of 180 individual clonal isolates with $^{32}$P-labeled pHp1.4 and by Southern blot hybridization of virion DNA digests from 24 selected isolates; deletions were not detected.

## DISCUSSION

We have described procedures for introducing deletions into nonessential regions of the *H. saimiri* genome for which there are no selectable markers. These procedures may be readily applicable to other herpesviruses. Constructed viral deletions presumably arise through homologous recombination of sequences flanking both sides of the deletion. The relatively high efficiency with which recombinants were generated (5 to 8%) is somewhat surprising (16). Factors

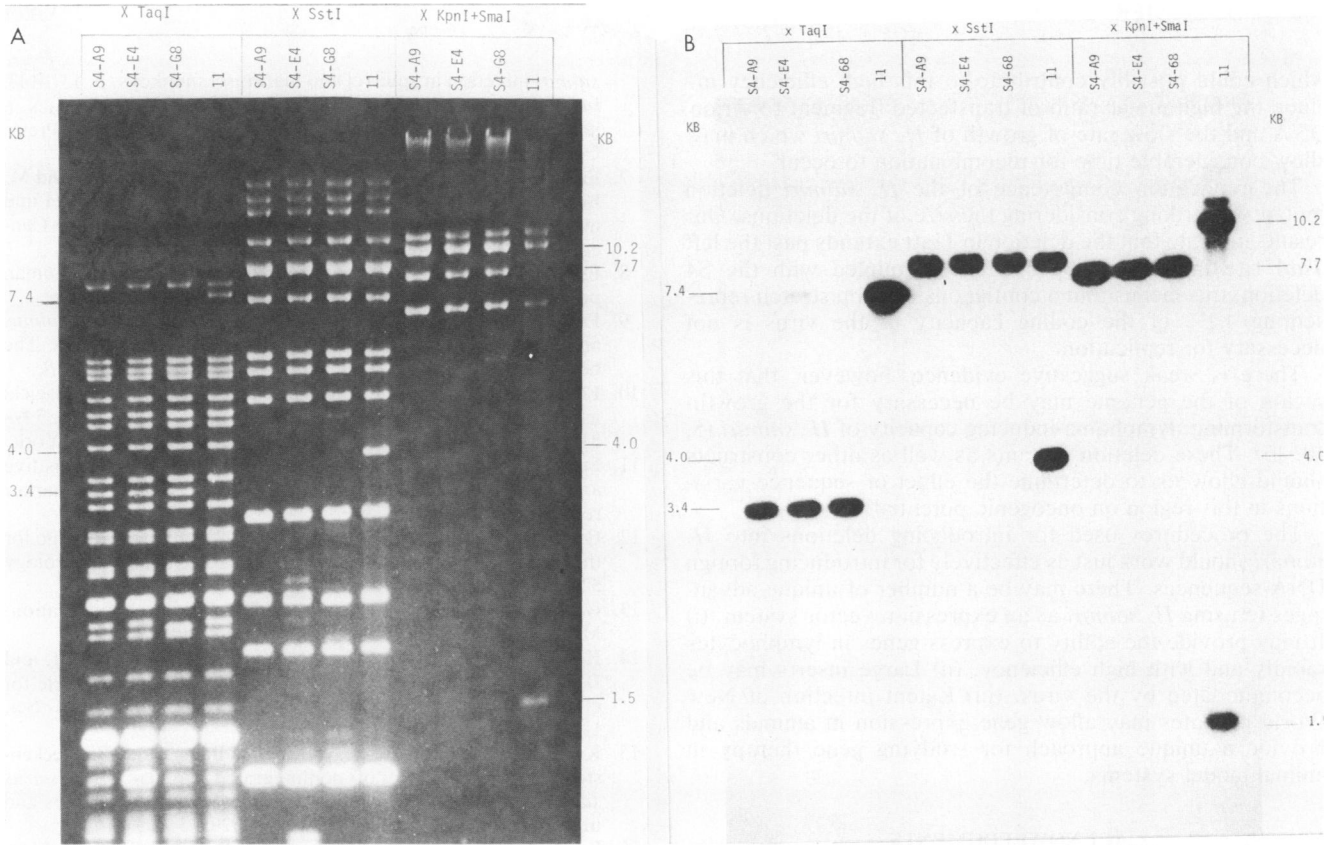


FIG. 5. Analysis of DNA of potential *H. saimiri* S4 deletion mutants. Virion DNA was cut with restriction endonuclease and electrophoresed through an agarose gel. (A) Ethidium bromide stain pattern; (B) Southern transfer hybridized to $^{32}$P-labeled pT7.4.

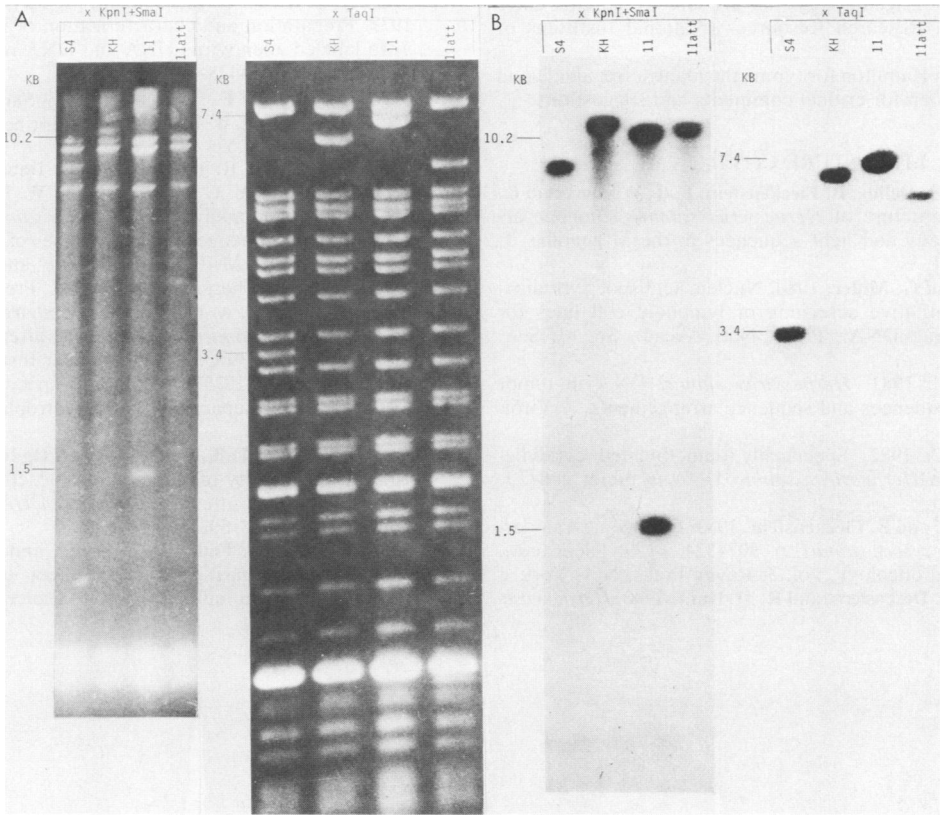


FIG. 6. Analysis of DNA from clonally purified *H. saimiri* deletion mutants. Virion DNA was cut with restriction endonuclease and electrophoresed through an agarose gel. (A) Ethidium bromide stain pattern; (B) Southern transfer hybridized to $^{32}$P-labeled pT7.4.

which could possibly contribute to this high efficiency include the high molar ratio of transfected fragment to virion DNA and the slow rate of growth of *H. saimiri* which may allow considerable time for recombination to occur.

The replication competence of the *H. saimiri* deletion mutants is striking considering the size of the deletions. Our results indicate that the deletion in 11att extends past the left *Taq*I site in the 7.4-kbp sequence; coupled with the S4 deletion, this means that a contiguous 4.5-kbp stretch representing 4.1% of the coding capacity of the virus is not necessary for replication.

There is weak suggestive evidence, however, that this region of the genome may be necessary for the growth-transforming, lymphoma-inducing capacity of *H. saimiri* (5, 10, 15). These deletion mutants as well as other constructs should allow us to determine the effect of sequence variations in this region on oncogenic potential.

The procedures used for introducing deletions into *H. saimiri* should work just as effectively for introducing foreign DNA sequences. There may be a number of unique advantages to using *H. saimiri* as an expression vector system. (i) It may provide the ability to express genes in lymphocytes rapidly and with high efficiency. (ii) Large inserts may be accommodated by the virus. (iii) Latent infection of New World primates may allow gene expression in animals and provide a unique approach for studying gene therapy in animal model systems.

## ACKNOWLEDGMENTS

This investigation was supported by Public Health Service grant 31363 awarded by the National Cancer Institute, by a special fellowship from the Leukemia Society of America, and by grant RR00168 to the New England Regional Primate Research Center from the Division of Research Resources (National Institutes of Health).

We thank Elisabeth Hamilton for typing the manuscript and David Knipe and Donald Coen for critical comments and suggestions.

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