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Selectable Recombinant Herpesvirus Saimiri Is Capable of Persisting in a Human T-Cell Line

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Strategies were developed to insert the *neo* resistance marker into the junction between L DNA and the right terminal repetitive H DNA of herpesvirus saimiri. Recombinant viruses were selectable in permissive epithelioid cultures. The human T-cell line Jurkat could be infected persistently with the Neo^r virus; the cells contained episomal viral DNA in high copy number. This selectable vector should be generally applicable for gene expression in human T cells.

The genomes of several herpesviruses have been used as eucaryotic expression vectors with the capacity to incorporate long stretches of foreign DNA (15–17, 22). For herpesviruses of the alpha and beta subgroups, satisfactory cell cultures have not been established to obtain long-term persistence. Persisting vectors, however, have been constructed with subgenomic elements of the B-lymphotropic Epstein-Barr virus (gamma-1-herpesvirus [24, 27]); this system, in contrast, did not yield infectious cell-free virus. Herpesvirus saimiri, prototype of the gamma-2 group, is capable of multiplying lytically in epithelioid owl monkey kidney (OMK) cells and of persisting nonlytically in T lymphocytes (reviewed in reference 9), suggesting that herpesvirus saimiri can be developed into an useful expression

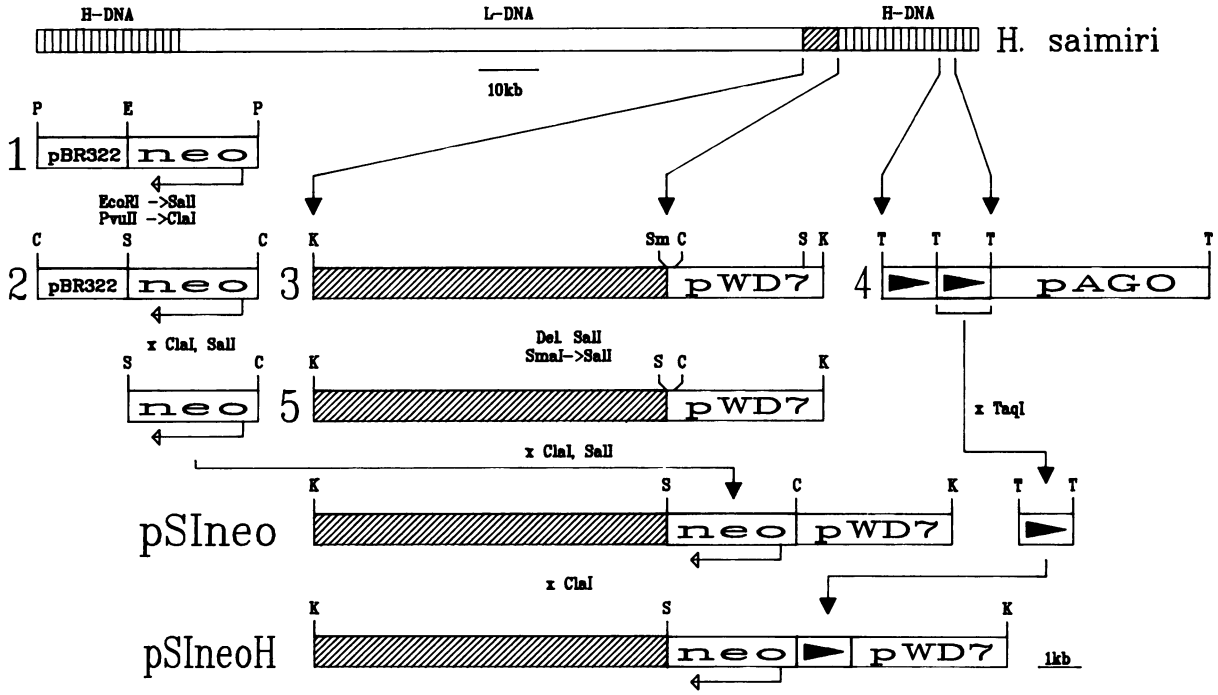

FIG. 1. Construction of plasmids designed for recombination with herpesvirus saimiri. Plasmid 1 (pSV2neo) (20) contains the Neo^r gene under transcriptional control of simian virus 40 signals. To obtain plasmid 2 (pSV2neoCS), we inserted a *Sal*I linker into the *Eco*RI site and a *Cla*I linker into a *Pvu*II site of pSV2neo. The herpesvirus saimiri 11 *Kpn*I E fragment (hatched) is a constituent of plasmid 3 (pWD11) (13). Plasmid 4 (pFG24) contains two H DNA repeat units (black triangles) in the vector pAGO (1). Plasmid 5 (pSI3) was derived from pWD11 by deletion of a *Sal*I site and insertion of a *Sal*I linker into the *Sma*I recognition sequence. To create pSIneo, the Neo^r gene was removed from pSV2neoCS as a *Cla*I-*Sal*I fragment and ligated into pSI3. For the construction of pSIneoH, an H DNA repeat unit was isolated from pFG24 as a *Taq*I fragment and cloned into the *Cla*I site of pSIneo. C, *Cla*I; E, *Eco*RI; K, *Kpn*I; S, *Sal*I; Sm, *Sma*I; T, *Taq*I. kb, Kilobase.

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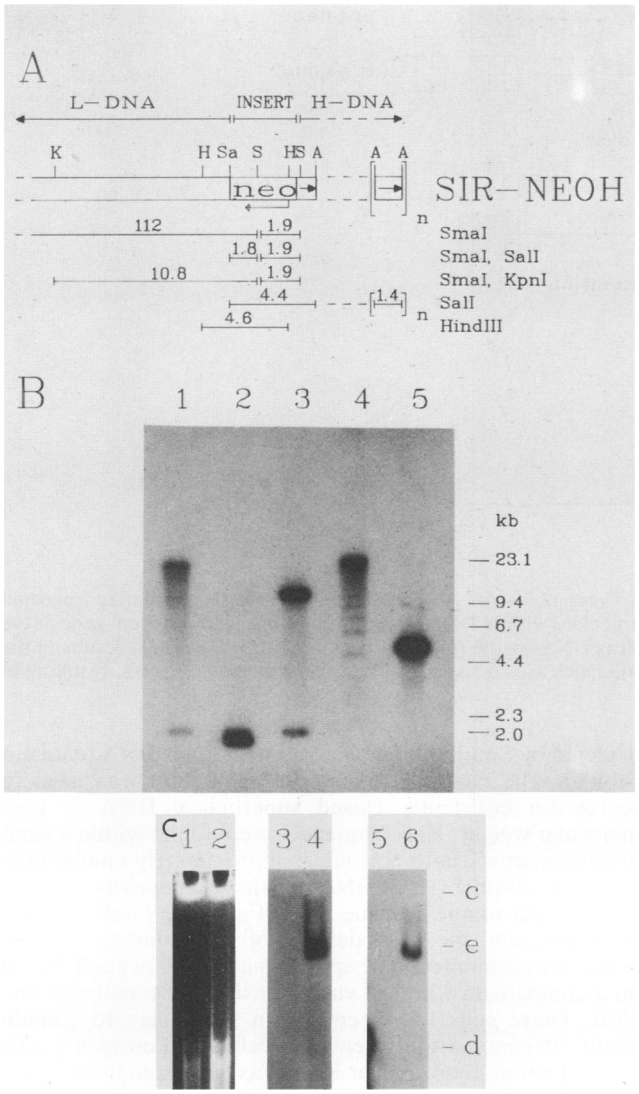

FIG. 2. Structure of the recombinant herpesvirus saimiri SIR-NEOH14 and its persistence in human T cells. (A) Cleavage map of the right H-L DNA junction. The lines below the map represent restriction fragments hybridizing with the Neo^r probe. Fragment sizes are in kilobases (kb). A, *Apa*I; H, *Hin*dIII; K, *Kpn*I; S, *Sma*I; Sa, *Sal*I. The packaging cleavage signal of H DNA coincides with the unique *Apa*I restriction site in each H DNA repeat unit. (B) Southern blot analysis of DNA from SIR-NEOH14. Viral DNA was harvested by the quick lysis protocol and cleaved with *Sma*I (lane 1), *Sma*I-*Sal*I (lane 2), *Sma*I-*Kpn*I (lane 3), *Sal*I (lane 4), and *Hin*dIII (lane 5). DNA fragments were separated on a 0.8% agarose gel, blotted, and hybridized to a Neo^r gene-specific probe. (C) Episomal persistence of recombinant viral DNA in human T cells, demonstrated by the procedure of Gardella et al. (10). Jurkat cells, both persistently infected with SIR-NEOH14 and mock-infected controls, were lysed on the top of an agarose gel. DNA was fractionated and stained with ethidium bromide. Positions of chromosomal (c) DNA, degraded linear (d) DNA, and high-molecular-weight episomes (e) are indicated. Lanes 1 and 2, Ethidium bromide-stained gel with DNA from uninfected cells (lane 1) and from SIR-NEOH14-infected Jurkat cells (lanes 2). Lanes 3 and 4, A Southern blot of this gel was hybridized with a Neo^r probe: lane 3, uninfected controls; lane 4, SIR-NEOH14-infected cells. Lanes 5 and 6, DNA from the same cells (lane 5, uninfected cells; lane 6, SIR-NEOH14-infected Jurkat cells) was fractionated by Gardella gel electrophoresis and hybridized with the *Kpn*I D fragment contained in plasmid pSS53 (13).

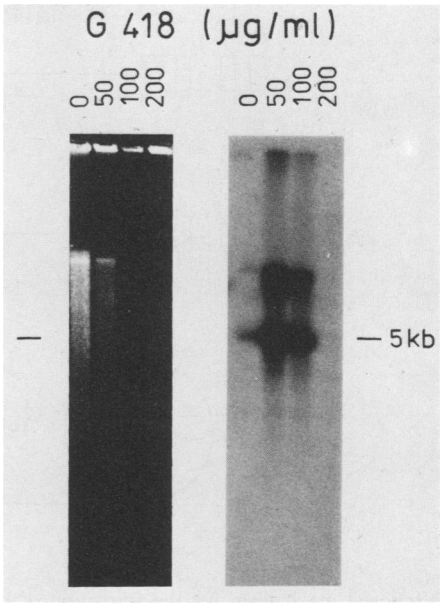

FIG. 3. Selection of recombinant virus SIR-NEOK in lytically infected cells. To demonstrate enrichment of Neo^r sequences, virus particles were harvested from aliquots of infected cell culture after selection with geneticin. Purified viral DNA was digested with *Sma*I, run on a 0.8% agarose gel, stained with ethidium bromide (left panel), and hybridized with pSV3neoCS (Fig. 1, plasmid 2) (right panel). kb, Kilobases.

vector. Herpesvirus saimiri induces rapidly progressing T-cell lymphomas in various New World primates (reviewed in reference 9) and is capable of transforming marmoset monkey lymphocytes in vitro to permanent growth (7, 18, 25). Viral genomes persist in lymphoid T-cell lines as episomes in multiple copies (12, 26). The linear genomic DNA (M DNA) of herpesvirus saimiri consists of an unique 112-kilobase region of low G+C content (L DNA), flanked at both ends by variable numbers of 1.4-kilobase noncoding tandem repeats that are termed H DNA (1, 2, 8, 23). Here we show that recombinant herpesvirus saimiri containing the Neo^r gene near the right junction between H DNA and L DNA (4) could be efficiently selected by geneticin (G418) in OMK cell cultures and that the virus was capable of persistently infecting simian and human T-cell lines.

For the insertion of the Neo^r gene into the herpesvirus saimiri genome, a *Sma*I cleavage site was chosen which is located close to the right H-L DNA junction (2), 35 base pairs within the first H repeat unit (1, 23); this region appears not to be transcribed (1) and does not overlap with a major open reading frame (23). Plasmids were designed that allowed insertion of the *neo* resistance marker (4) by homologous recombination. The Neo^r gene was under the transcriptional control of simian virus 40 DNA elements (20). The plasmid pSIneo was constructed by placing the Neo^r gene adjacent to the right-terminal *Kpn*I-*Sma*I L DNA fragment of herpesvirus saimiri strain 11 (Fig. 1). In the recombinant plasmid pSIneoH, the resistance marker gene was flanked by the same *Kpn*I-*Sma*I L DNA fragment and by a single H DNA repeat unit in genuine orientation (Fig. 1).

In a first series of experiments, the plasmid pSIneoH was linearized with *Kpn*I, mixed at 80-fold molar excess with purified virion DNA (8), and cotransfected into the permissive OMK cell line 637 (ATCC CRL1556) (5) by calcium

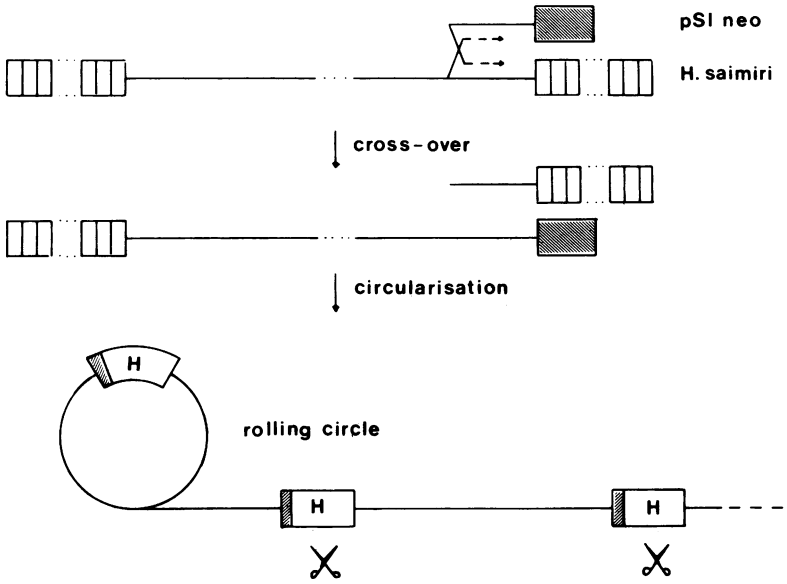

FIG. 4. Proposed mechanism for the formation of herpesvirus saimiri recombinants. A single crossover between linearized plasmid (pSIneo) and herpesvirus saimiri genomic DNA will result in a linear intermediate with H DNA at the left terminus and the Neo^r gene at the right end. The molecule is assumed to circularize. Replication of herpesvirus DNA by the rolling circle mechanism (3, 14, 21) results in the formation of long concatemers. Those are cut by the action of specific endonucleases (scissors) during the packaging process, resulting in monomers that contain the gene to be cloned (hatched boxes).

phosphate precipitation (8, 11), followed by a 20% (wt/vol) glycerol shock after 4 h. When cytopathic changes resulted in destruction of monolayers within 3 weeks, cell culture fluids were checked for the recombinant viruses by blot hybridization (18). For this purpose, virus was concentrated from 1 ml of cell-free supernatant and lysed with 2% (wt/vol) sodium *N*-lauroyl sarcosine; the DNA was extracted with phenol and ether, ethanol precipitated, and digested with restriction enzymes prior to blot hybridization. Recombinant viruses could always be detected without resort to selection procedures. Viruses were plaque purified from OMK cells under 20% (wt/vol) methylcellulose, and Neo^r recombinants were identified by dot-spot hybridization. In six of nine recombinant virus clones that were tested by Southern blotting, the inserted pSIneoH DNA was interspersed between H DNA repeats. Two virus clones, one of them termed SIR-NEOH14, had the Neo^r sequence placed near the right H-L junction (Fig. 2A and B). The recombinant SIR-NEOH was transferred into Sg021 cells, a persistently infectable marmoset T-cell line (6). The cell line became resistant against geneticin in concentrations up to 750 μg/ml; the cells contained about 140 viral genome equivalents and expressed Neo^r-specific transcripts of the expected size (2.5 kilobases) (data not shown).

To target the foreign DNA more efficiently to the H-L junction, we also followed a second approach that aimed at formation of recombinants by a single crossover in L DNA. Virion DNA was cotransfected with the plasmid pSIneo, which is devoid of H sequences (Fig. 1). All resulting virus (SIR-NEOK) had the Neo^r gene and plasmid vector DNA inserted between L DNA and the H DNA repeats. The recombinant viruses could be enriched by subsequent passage in OMK cells in the presence of geneticin (Fig. 3). To investigate whether the selectable herpesvirus saimiri can persist in human T cells, we infected the Jurkat cell line (19) with the recombinant SIR-NEOH14 and cultured it under increasing (from 200 to 900 μg/ml) concentrations of geneticin. After more than 8 weeks, the complete Neo^r gene was detected by Southern blot analysis of cellular DNA (data not shown). The method of Gardella et al. (10) was used to search for covalently closed superhelical DNA of high molecular weight. Neo^r sequences were found within a band of herpesvirus saimiri episomes, approximately comigrating with the circular viral DNA of the herpesvirus saimiri-transformed tumor cell line 1670 (Fig. 2C). Twelve weeks after infection, the copy number of recombinant viral genomes was estimated to be approximately 100 per cell, based on a comparison with the virus-transformed tumor cell line 1670. These cells had been shown previously to contain about 210 genome equivalents per cell (12). Fourteen weeks after infection, total cellular RNA was harvested from Jurkat cells infected with SIR-NEOH14 infection; Northern (RNA) blot hybridization revealed a single Neo^r-specific transcript of the expected size (data not shown).

In summary, we showed that recombinant Neo^r-expressing herpesvirus saimiri can persistently infect human cells of hematopoietic lineage. Recombinant viruses were selected in lytically infected epithelioid cells and persisted under selective pressure in lymphoid cells as large episomes of high copy number. Two strategies, both relying on homologous recombination of the herpesvirus saimiri genome with plasmid-cloned virus-specific sequences, led to Neo^r recombinants. If the Neo^r marker was cloned between right-terminal L DNA and one H repeat unit and was cotransfected with M DNA, a minority of recombinants had the resistance marker positioned at the desired site between H and L sequences. Most viruses contained the entire plasmid within H DNA; this is easily explained as the consequence of recombination of H DNA exclusively. When Desrosiers and co-workers (6) cloned the bovine growth hormone gene into herpesvirus saimiri by replacing a transformation-relevant sequence at the left end of L DNA, they observed that DNA of the plasmid vector (pBR322) was incorporated into the recombinants. This suggests that recombinants can arise by a single crossover event in L DNA, followed by circularization and subsequent DNA replication

according to the model of the rolling circle (3, 14, 21). We used this as a working hypothesis to develop the alternative strategy. Since the Neo^r-containing plasmid (pSIneo) lacks H DNA, it could only recombine by crossover in L DNA, thus avoiding the insertion into the H repeats of virion genomes (Fig. 4). Although this mechanism has not been proven yet, the procedure yielded the expected recombinants and should provide a generally applicable means of cloning into gamma-2-herpesvirus genomes.

Herpesvirus saimiri may prove to be a versatile vector for expression of foreign genes in human cells. The particular genome structure of herpesvirus saimiri, with about 30% highly repetitive DNA (8), might provide a large capacity to insert foreign DNA, as the insertion can possibly be compensated by loss of terminal repeated sequences. Foreign DNA can be inserted near the H-L junction without interrupting or replacing viral genes. Recombinant virus replicates without helper virus and is able to infect human and simian T-cell lines, thus it can shuttle between lytic and persistent infection. Since the recombinant DNA is packaged into infectious particles, foreign genes can be transferred into cells, such as primary lymphocytes, that are not particularly susceptible to DNA transfection. Herpesvirus saimiri recombinants persist in T-cell lines as high-copy-number episomes; the recombinant DNA appeared not to be integrated, at least not within the limits of sensitivity of the detection method. For these reasons, the study of certain aspects of gene regulation in human T cells may be facilitated, for instance, investigations of *trans*-acting genes, avoiding *cis* effects by the integration into the host cell genome. If a transformation-negative variant of herpesvirus saimiri is used as a vector, genes involved in T-cell activation could be functionally analyzed.

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