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Synthesis of Bovine Growth Hormone in Primates by Using a Herpesvirus Vector

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A strain of herpesvirus saimiri containing a bovine growth hormone (bGH) gene under the control of the simian virus 40 (SV40) late-region promoter was constructed. This strain, bGH-Z20, was replication competent and stably harbored the bGH gene upon serial passage. Nonpermissive marmoset T cells persistently infected with bGH-Z20 produced a 0.9-kilobase RNA which contained all of the bGH exon sequences and appeared to initiate within the SV40 promoter region. However, in permissively infected owl monkey kidney cells, RNAs containing growth hormone sequences appeared to initiate from herpesvirus saimiri promoters positioned upstream from the SV40-growth hormone gene. Persistently infected T cells in culture secreted 500 ng of bGH protein per 10^6 cells per 24 h during the several months of testing. The secreted protein was 21 kilodaltons, the size of authentic bGH. New World primates experimentally infected with bGH-Z20 produced circulating bGH and developed immunoglobulin G antibodies directed against bGH. Because herpesviruses characteristically remain latent in the infected host, these observations suggest a means for replacing gene products missing or defective in hereditary genetic disorders.

One area of investigation made possible by modern molecular biotechnology is gene therapy for inherited metabolic disorders. Experimental approaches to gene transfer in animals have included germ line and somatic cell manipulations. Alteration of genetic information in the germ line has been achieved by microinjection of DNA and by the use of retrovirus vectors. Alteration of genetic information in somatic cells of animals has been achieved by retrovirus vectors and by incubation with Ca^{2+} DNA precipitates. Successful introduction of cellular genes has been limited so far to mice and *Drosophila* spp. (for a review, see reference 2).

The power of large DNA viruses as vectors for expressing cloned genes has been forcefully demonstrated by the use of vaccinia virus to express a variety of antigens (22, 25, 27). Vaccinia virus is ideally suited for this purpose. It has been long used and well studied as the vaccine for smallpox, and its flexible structure allows the stable incorporation of large amounts of foreign DNA (33). Herpesviruses are also genetically complex, with virion DNA of 140 to 240 kilobase pairs (kbp). Transient expression of cloned genes has been achieved by using herpes simplex virus as vector (13, 19, 32). The ability of herpesviruses to remain latent in the infected host may have utility for the stable expression of a desired gene product in that host. Insertion of DNA fragments into a region not required for replication results in a virus that is replication competent; replication competence eliminates complications arising from the use of defective virus and the need for helper virus (18). Because the herpesvirus genome is large, DNA fragments of reasonable length can be accommodated; however, the upper limit for the amount of inserted DNA will more than likely be lower than with vaccinia virus. To date we have inserted as much as 7.2 kbp (K. Briggs and R. C. Desrosiers, unpublished data).

Herpesvirus saimiri, a lymphotropic virus of New World primates, naturally infects squirrel monkeys, producing no signs of disease in this species. Infection of other species of New World primates, however, results in a rapidly progressing, malignant, T-cell lymphoma (for a review, see reference 10). New World primate monolayer cell lines, e.g., owl monkey kidney (OMK), are generally permissive for herpesvirus saimiri replication. Infection of such cell lines leads to cell lysis, with viral titers frequently exceeding 10^7 infectious particles per ml. Infection of lymphoid cells, however, is semipermissive at best. The structure of virion DNA resulting from permissive infection has been studied in considerable detail (3, 4, 10, 11, 16). Nondefective, infectious virion DNA contains a high degree of intramolecular heterogeneity in guanine-plus-cytosine (G+C) content. The centrally located unique DNA region is about 110 kbp long, contains probably all the coding sequences, and is 36% G+C (light- or L-DNA). Covalently attached to each end of L-DNA is repetitive DNA with 71% G+C content (heavy- or H-DNA). Each repeat unit of 1.4 kbp is oriented in the same direction, and approximately 30% of an infectious virion DNA molecule is H-DNA.

A region of the herpesvirus saimiri strain 11 genome has recently been identified that is required for its lymphoma-inducing capacity in vivo and for immortalization of T cells in vitro; this region of viral DNA is not required for replication of the virus (4, 5, 16). These sequences are located within the first 4.5 kbp of L-DNA at the left H-L-DNA border. New World primates inoculated with herpesvirus saimiri mutants with deletions in this region become latently infected but develop no signs of disease. Such nonpathogenic vaccine strains of herpesvirus may be suitable as vectors for achieving stable expression of a gene in an animal host. In the work described here, we used the

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nonpathogenic S4 deletion mutant strain of herpesvirus saimiri to express bovine growth hormone (bGH) in cultured cells and in New World primates.

MATERIALS AND METHODS

Cells and virus. OMK line 637 cells were grown in complete minimal essential medium with 10% fetal calf serum, penicillin, and streptomycin. Purification of herpesvirus saimiri virions from lytically infected OMK cells has been described previously (4). The SgO21 cell line was established from the thymus of a cotton-top marmoset (*Saguinus oedipus*) that died from natural causes. The thymus was minced to a single-cell suspension, and the cells were incubated for 48 h in RPMI 1640 medium with 20% fetal calf serum, penicillin, and streptomycin with 1 μg of phytohemagglutinin per ml. The T cells were then grown continuously in complete RPMI 1640 medium with 20% fetal calf serum, penicillin, and streptomycin supplemented with T-cell growth factor (IL-2; Electro-Nucleonics, Inc.). SgO21 cells persistently infected by herpesvirus saimiri produced low levels of virus, approximately 100 infectious virus particles per ml of cell-free supernatant.

DNA analyses. Procedures for DNA preparation, agarose gel electrophoresis of DNA, transfer of DNA to nitrocellulose by the procedure of Southern (34), and hybridization have been described previously (6). Procedures for transfection of virion DNA into OMK cells and the screening of progeny virus by limiting dilution and spot hybridization have also been described (5). Infectious S4 virion DNA was introduced into permissive OMK cells by the calcium-precipitation procedure of Graham and van der Eb (12), together with *Eco*RI-linearized pBGH-Z20. After complete destruction of the monolayer, virus was diluted (6×10^{-7}) in complete minimal essential medium, and 150 μl was added to individual wells of OMK cells growing in 96-well Linbro trays. Forty-eight percent of the wells yielded virus, which corresponds to a multiplicity of infection of 0.65 infectious virus particles per well. Samples (2 μl) from virus-positive wells were spotted onto nitrocellulose, and the DNA was denatured in situ and hybridized with a ^{32}P-labeled fragment of bGH DNA (the 4.0-kbp *Eco*RI fragment of pSVB3). After being rinsed, the filter was exposed to film. Dark spots in the array were indicative of recombinant herpesvirus saimiri strains containing bGH DNA. Approximately 10% of the progeny virus contained bGH DNA sequences. Cloned DNA manipulations were performed by the method of Maniatis, Fritsch, and Sambrook (23).

RNA analyses. Northern blot hybridization was performed as previously described (14). For the RNase protection experiments, the *Bam*HI-*Eco*RI fragment of pSVB3 (Fig. 1) was cloned into pSP65 vector (24). This *Bam*HI-*Eco*RI fragment contained genomic sequences starting at the approximate cap site and extending approximately 400 base pairs 3′ to the polyadenylation site (38). Complementary ^{32}P-labeled RNA transcripts were prepared by transcription of *Bam*HI-linearized pSP65-bGH plasmid by the method of Melton et al. (24). After hybridization of the complementary ^{32}P-labeled RNA with cellular RNA, the RNA was digested with RNase A (40 μg/ml) and T1 RNase (2 μg/ml), and resistant hybrids were electrophoresed through a 40-cm denaturing 7 M urea polyacrylamide gel.

Protein analysis. Infected or uninfected SgO21 cells (10^6) were pelleted and suspended in 1 ml of RPMI 1640 medium lacking methionine and containing 20% fetal calf serum, penicillin, streptomycin, and T-cell growth factor, and 50 μCi of [^{35}S]methionine (5 to 15 Ci/mmol) was added. After 40 h, the cells were pelleted, and portions of the cell-free supernatant were stored at −70°C before immunoprecipitation or direct sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (7). Amino-terminal sequencing of the ^{35}S-labeled 21,000-molecular-weight protein (21K protein) immunoprecipitated from cell-free supernatants of bGH-Z20-infected Sg021 cells was performed on an Applied Biosystems model 470A Protein Sequencer.

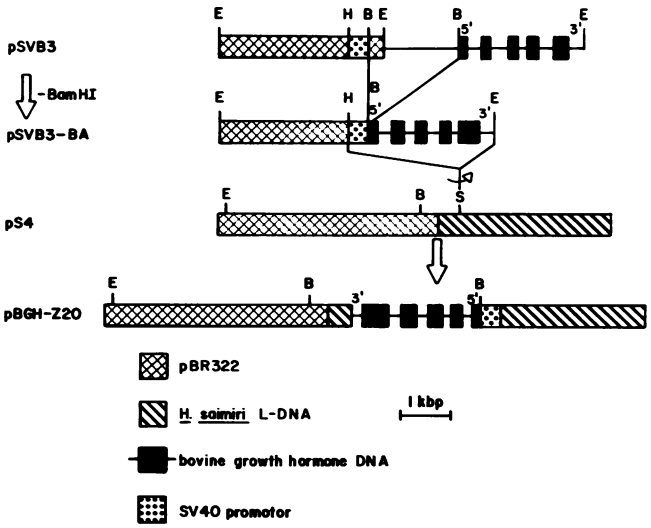

FIG. 1. Construction of hybrid plasmid with bGH DNA flanked by herpesvirus saimiri L-DNA. The structure of pSVB3-BA has been described previously (39). Briefly, a deleted pBR322 derivative was first constructed containing SV40 *ori* region sequences (the *Hin*dIII to *Hpa*II fragment of SV40 DNA from 0.648 to 0.725 map units). A genomic bGH *Eco*RI fragment was then inserted into the *Eco*RI site to form pSVB3. Excision of the 1.8-kbp *Bam*HI fragment from pSVB3 to form pSVB3-BA resulted in the fusion of the SV40 *ori* region sequences to nucleotide 2 of the bGH mRNA sequence. The SV40 region (400 base pairs) contains 72-base-pair repeat enhancer elements and promoter sequences in the orientation of late transcription. The structure of the pS4 plasmid also has been described previously (6). It is a deletion derivative of a plasmid containing 7.4 kbp from the left end of herpesvirus saimiri strain 11 unique sequence L-DNA. The 4.0 kbp that have been deleted are not required for replication of the virus, but they are required for the transforming ability and oncogenicity of the virus (5). The bGH gene under SV40 promoter control was placed within herpesvirus saimiri L-DNA sequences by inserting the *Hin*dIII plus *Eco*RI fragment of pSVB3-BA into the *Sst*I site of pS4. DNA fragments were made blunt ended before ligation. E, *Eco*RI; H, *Hin*dIII; B, *Bam*HI; S, *Sst*I.

RIA. Cell culture supernatants and sera were monitored for bGH protein by radioimmunoassay (RIA) with fixed *Staphylococcus aureus* cells (Pansorbin; Calbiochem-Behring) to separate bound from free antigen (40). Purified bGH was obtained from Miles Laboratories, Inc., and A. Parlow (NIAMDD-GH-B18). Guinea pig anti-bGH antiserum was a gift from The Upjohn Co. The titers of monkey serum anti-bGH were determined by using the same reagents.

Animal inoculations. Animals were inoculated intramuscularly with 1 ml of undiluted virus (approximately 10^7 infectious virus particles). Animals were kept in individual cages in a specially designed biosafety room for housing experimentally infected animals.

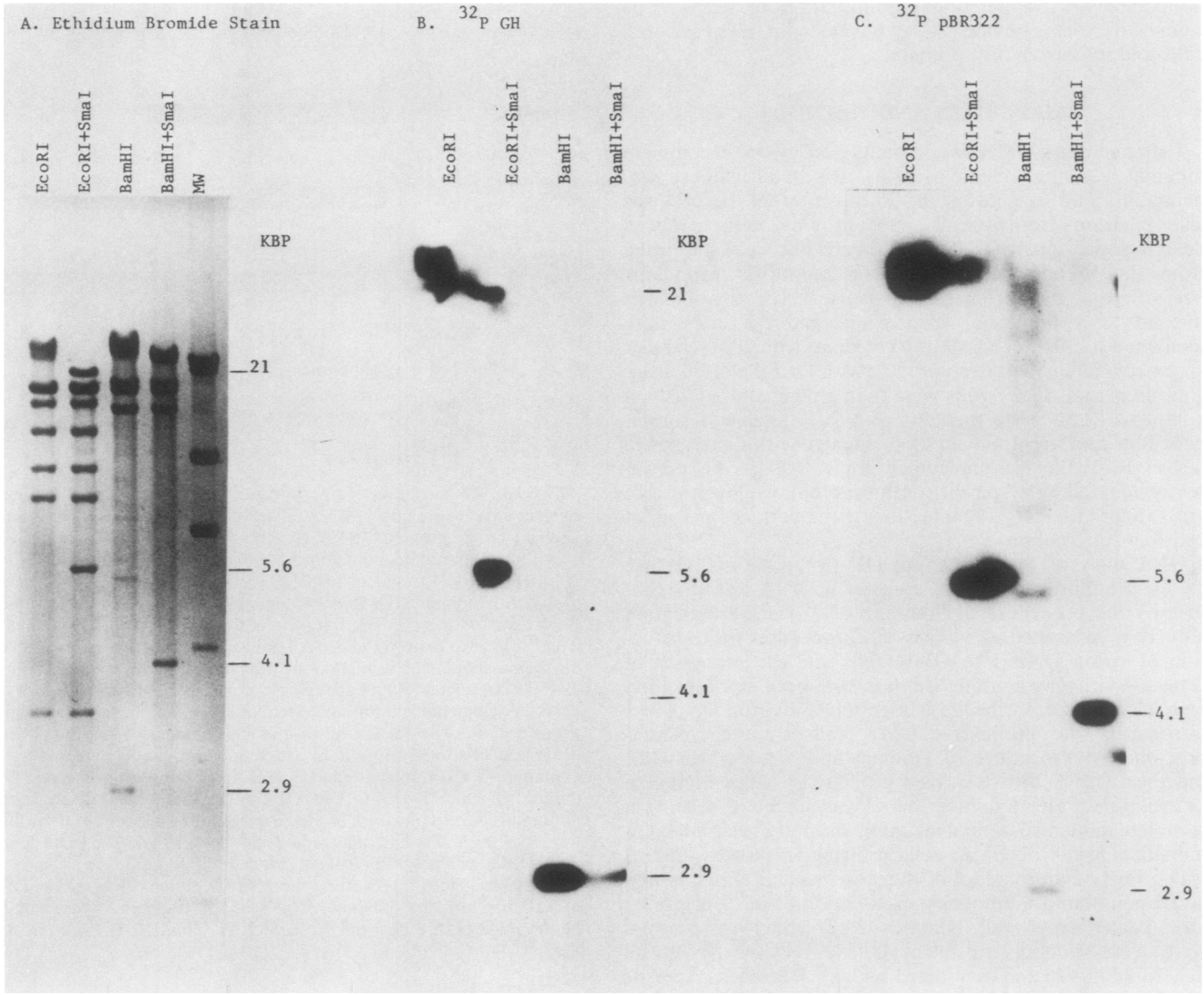

FIG. 2. Analysis of virion DNA from the bGH-Z20 strain of herpesvirus saimiri. Virus stocks were prepared from a single well positive for bGH DNA sequences, and virion DNA was isolated. DNA was cut with restriction endonucleases and fractionated by agarose gel electrophoresis. After ethidium bromide staining and photography, DNA was transferred to nitrocellulose and hybridized as indicated. The 4.0-kbp *Eco*RI fragment of pSVB3 was used for the hybridization in panel B.

RESULTS

Construction of virus. A restriction fragment containing the bGH gene under the control of the simian virus 40 (SV40) late-region promoter was cloned into the *Sst*I site of pS4, a plasmid which contains 3.4 kbp from the unique L-DNA region of the herpesvirus saimiri genome (Fig. 1). The single *Sst*I site of pS4 arose by deletion of the 4.0-kbp *Sst*I fragment from pT7.4; pT7.4 contains DNA derived from the left end of L-DNA of the parental, oncogenic virus strain 11 (6). The resultant plasmid, pBGH-Z20, contains the SV40-bGH gene situated between two stretches of herpesvirus saimiri DNA. Permissive OMK cells were transfected with infectious S4 virion DNA plus pBGH-Z20 that had been linearized with *Eco*RI. Ten percent of the progeny virus contained bGH DNA sequences (data not shown). Progeny virus containing bGH DNA sequences were expected to arise by homologous recombination in the infected cell. Because virion DNA from 10 recombinants appeared to have the same sequence arrangement, 1 was selected for more detailed analysis.

Virion DNA maps of wild-type herpesvirus saimiri strains have been derived for *Eco*RI and *Bam*HI (11, 16), and these restriction endonucleases were used for analyzing virion DNA of the recombinant virus (Fig. 2). Additionally, *Sma*I cuts four times in each terminal H-DNA repeat unit of herpesvirus saimiri, once in bGH DNA, not at all in SV40 DNA, and not at all in the internal 110-kbp L-DNA region of herpesvirus saimiri. The isolated recombinant virus (bGH-Z20) was homogeneous, with unimolar fragments of bGH DNA. bGH-Z20 virus also contained pBR322 sequences; the origin of pBR322 sequences in this recombinant virus is considered in the Discussion. The DNA analyses shown in Fig. 2 and other results not shown demonstrated the bGH-Z20 virion DNA sequence arrangement shown schematically in Fig. 3.

The bGH-Z20 strain of herpesvirus saimiri was fully replication competent in OMK and Vero cells. It grew at

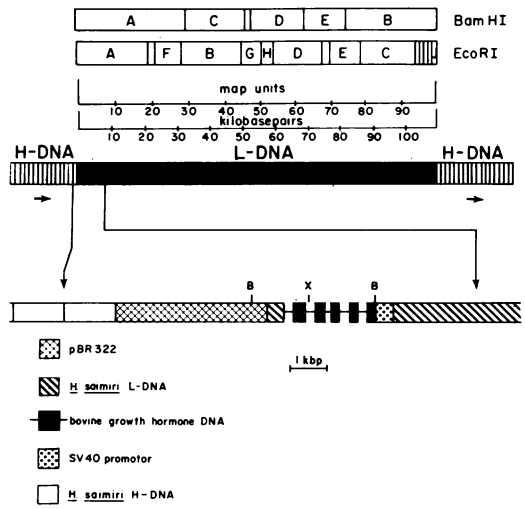

FIG. 3. Virion DNA sequence arrangement in the bGH-Z20 strain of herpesvirus saimiri. Herpesvirus saimiri contains a 110-kbp stretch of unique sequence DNA called L-DNA (light, 35% G+C). This is flanked at each end by a variable number of 1.4-kbp H-DNA repeat units; H-DNA is 71% G+C (3, 4). The bottom portion of the figure shows on an expanded scale the region containing the bGH gene in the bGH-Z20 strain of herpesvirus saimiri. B, *Bam*HI; X, *Sma*I. In addition to the indicated *Sma*I site, each H-DNA repeat unit contains four *Sma*I sites. Although the ordering of *Sma*I sites in the first H-DNA repeat unit at the border has been determined for the parental strain 11 (17), it was not specifically determined for the bGH-Z20 strain; thus, the *Sma*I sites in each H-DNA repeat unit are not specifically indicated in the diagram. Because there are a variable number of H-DNA repeat units at each terminus (4), cleavage with *Bam*HI alone or *Eco*RI alone resulted in a ladder of terminal fragments differing in size by 1.4 kbp (Fig. 2). *Sma*I did not cut within internal L-DNA sequences of herpesvirus saimiri strain 11 (3, 4, 11, 16).

approximately the same rate as parental strains 11 and S4, and it grew to the same high titers ($10^{7.0}$ to $10^{7.5}$ infectious virus particles per ml in OMK cells). Furthermore, serial passage of the bGH-Z20 strain 10 times in OMK cells did not detectably alter the virion DNA sequence arrangement (data not shown).

RNA synthesis. RNA was prepared from OMK cells permissively infected with bGH-Z20 24 and 48 h after infection and from the SgO21 marmoset T-cell line persistently infected with bGH-Z20. Northern blot hybridization of RNA from the persistently infected T cells with a ^{32}P-labeled DNA fragment containing bGH sequences revealed that more than 90% of the bGH RNA was approximately 0.9 kilobase, the size of authentic bGH RNA (Fig. 4). However, in permissively infected OMK cells, at least five species of RNA containing bGH sequences were detected, and all of these were longer than 0.9 kilobase (Fig. 4).

Nuclease protection analyses were performed to determine whether bGH RNA was spliced and terminated normally in SgO21 and OMK cells. RNA from bGH-Z20-infected OMK and SgO21 cells, as well as control RNA from bovine pituitaries, was hybridized with negative-sense ^{32}P-labeled RNA made in the SP6 system. RNA-RNA hybrids were digested with RNase, and the nuclease-resistant fragments were separated on polyacrylamide gels (Fig. 5). Autoradiography of the gel revealed fragments estimated to be 302, 161 (doublet), 117, and 71 bases long. The lengths of the protected fragments corresponded to the sizes of the five exons of the bGH gene (38). Most importantly, the RNA extracted from both the OMK and SgO21 cells protected fragments of the same size as the control growth hormone RNA extracted from bovine pituitaries. Because the ^{32}P-labeled negative-strand RNA used for the analysis contained 400 bases 3′ to the polyadenylation site of the growth hormone gene, we concluded from this analysis that the growth hormone transcripts in infected OMK and SgO21 cells were properly spliced and were correctly processed at their 3′ termini. These results thus indicated that transcription of bGH sequences in permissively infected OMK cells initiated from upstream herpesvirus saimiri promoters. The ^{32}P-labeled RNA used for hybridization in this experiment (see Materials and Methods) did not allow mapping of the 5′ ends of the transcripts.

Protein synthesis. The continued growth of infected SgO21 cells allowed us to measure the synthesis and secretion of bGH over prolonged periods and in the absence of significant amounts of cell lysis. SgO21 cells persistently infected with bGH-Z20 were labeled with [^{35}S]methionine, and the proteins secreted into the medium were analyzed by SDS-PAGE. bGH-Z20-infected SgO21 cells secreted a prominent 21K protein (Fig. 6 and 7); 21K is the size of authentic bGH (38). Secretion of the prominent 21K protein was not detected in uninfected SgO21 cells, nor was it detected in SgO21 cells persistently infected with the S4 strain of virus (Fig. 6). Sera from New World primates infected with bGH-Z20 (see below) specifically immunoprecipitated the 21K protein, and this immunoprecipitation could be blocked

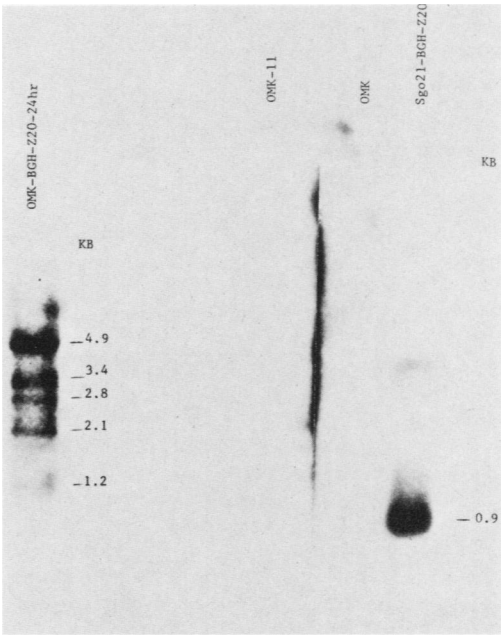

FIG. 4. Sizes of RNA containing bGH sequences in bGH-Z20-infected cells. Total cell RNA (5 μg) was separated by electrophoresis through agarose, transferred to nitrocellulose, and hybridized with the 4.0-kbp *Eco*RI fragment of pSVB3 by using conditions previously described (14). Sizes of bGH-containing RNA in infected OMK cells at 24 and 48 h postinfection (multiplicity of infection, 5) were not distinguishable, but approximately threefold more bGH-containing RNA was present in the 48-h sample. OMK-11 is RNA prepared similarly at 24 h postinfection of OMK cells with herpesvirus saimiri 11. Size markers used were viral RNAs (1.4, 2.3, and 4.9 kilobases) present in infected OMK cells as detected by subsequent hybridization with cloned herpesvirus saimiri DNA fragments (14).

by purified bGH (Fig. 7). Sera from New World primates before experimental infection, as well as sera from New World primates infected with strains S4 and 11att, failed to immunoprecipitate the 21K protein (Fig. 6 and 7 and data not shown).

The level of bGH secreted into the medium of infected SgO21 cells was quantitated by RIA 6 months after original infection and continuous growth (Table 1). The secretion of bGH by the cells was linear for 3 days and amounted to approximately 500 ng/10^6 cells per day. Other determinations indicated that the high level of bGH production was maintained during the 6 months in culture.

^{35}S-labeled 21K protein secreted from infected SgO21 cells was immunoprecipitated with infected monkey sera, and the purified 21K protein was subjected to 10 cycles of sequential amino acid degradation from the amino terminus. [^{35}S]methionine was released in approximately equal amounts in cycles 4 (55%) and 5 (45%). These results of amino-terminal sequence analysis suggest ambiguity in cleavage of the signal peptide before or after an alanine residue, leaving methionine at position 4 or 5 (38). bGH is the classic example of ambiguous signal peptide cleavage; in bovine pituitaries, 65% of bGH-signal peptide cleavage leaves methionine at position 4, and 35% leaves methionine at position 5 (20, 36). These results indicate that processing of bGH in marmoset T cells is very similar to that which occurs in bovine pituitaries.

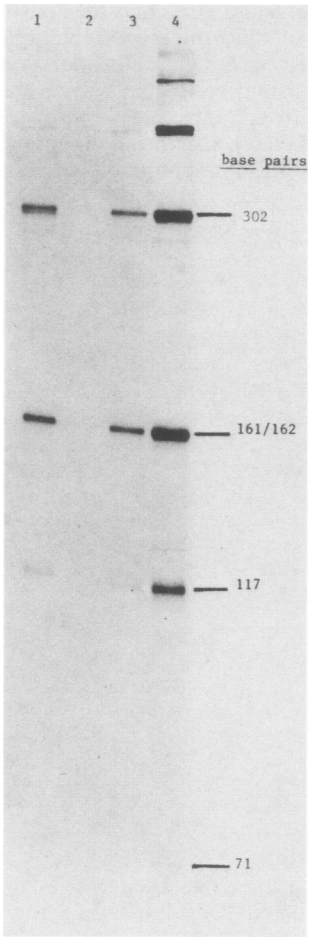

FIG. 5. Nuclease protection analysis of bGH RNAs in bGH-Z20-infected cells. ^{32}P-labeled cRNA, prepared as described in Materials and Methods, was hybridized with 2.5 ng (lane 1) and 0.25 ng (lane 2) of poly(A)$^+$ RNA from a bovine pituitary, with 5 μg of total cell RNA from infected SgO21 cells (lane 3), and with 5 μg of total cell RNA from infected OMK cells 24 h after infection (lane 4). After digestion of hybridized samples with RNase, the protected fragments were electrophoresed in a sequencing gel. The sizes of the protected fragments were estimated by using ^{32}P-labeled *Hpa*II fragments of pBR322.

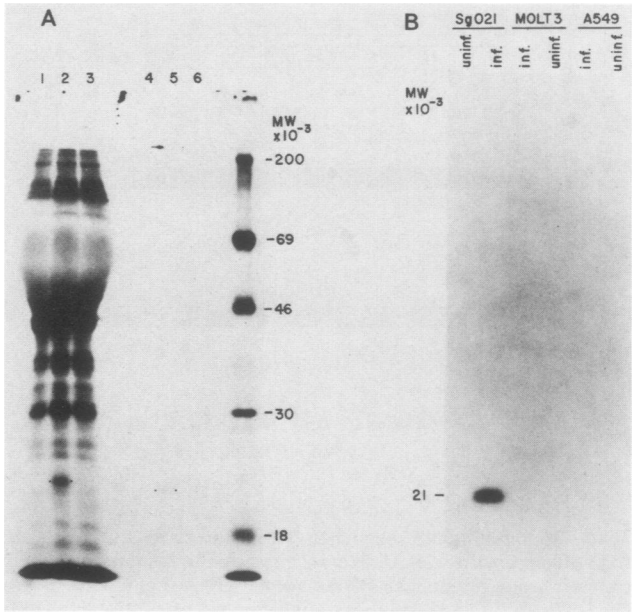

FIG. 6. Protein of 21K secreted by bGH-Z20-infected SgO21 cells. (A) Uninfected SgO21 cells, as well as SgO21 cells infected with S4 and bGH-Z20, were labeled with [^{35}S]methionine, and labeled proteins secreted from the cells were analyzed by SDS-PAGE. Lanes: 1 to 3, total secreted proteins; 4 to 6, proteins immunoprecipitated by Sgf 138-82 sera; 1 and 4, uninfected; 2 and 5, bGH-Z20 infected; 3 and 6, S4 infected. ^{14}C molecular weight standards (New England Nuclear Corp.) are myosin (200,000), albumin (69,000), ovalbumin (46,000), carbonic anhydrase (30,000), and lactoglobulin A (18,000). (B) SgO21 cells, MOLT-3 cells (a human T-cell tumor line), and A549 cells (a human lung carcinoma cell line) were infected with bGH-Z20, and seven days later infected and uninfected cells were labeled with [^{35}S]methionine. Proteins secreted into the medium were analyzed by SDS-PAGE after immunoprecipitation with Sgf 138-82 sera. The size standards used were the same as in panel A.

Experimental infection of New World primates. One white-lipped marmoset (*Saguinus fuscicollis*) and two owl monkeys (*Aotus trivirgatus*) were inoculated with the bGH-Z20 strain of herpesvirus saimiri. Three parameters were monitored from weekly blood samples from these animals: (i) ability to isolate virus from peripheral blood lymphocytes (PBL) and from cell-free plasma, (ii) immunoglobulin G antibodies directed against bGH by immunoprecipitation and SDS-PAGE, and (iii) the level of bGH in the serum by RIA.

Virus was never recovered from the white-lipped marmoset (Sgf 138-82) when bled weekly for 3 months from the time of inoculation. We recovered virus from the PBL of one owl monkey (Aot 222-83) on weeks 1 through 7 and 9 postinoculation. In addition, virus was recovered from cell-free plasma on weeks 2 and 4 postinoculation, indicating that Aot 222-83 was viremic at these times. However, virus was not recovered from the PBL or plasma of Aot 222-83 from week 10 onward (eight additional bleedings). Virus was recovered

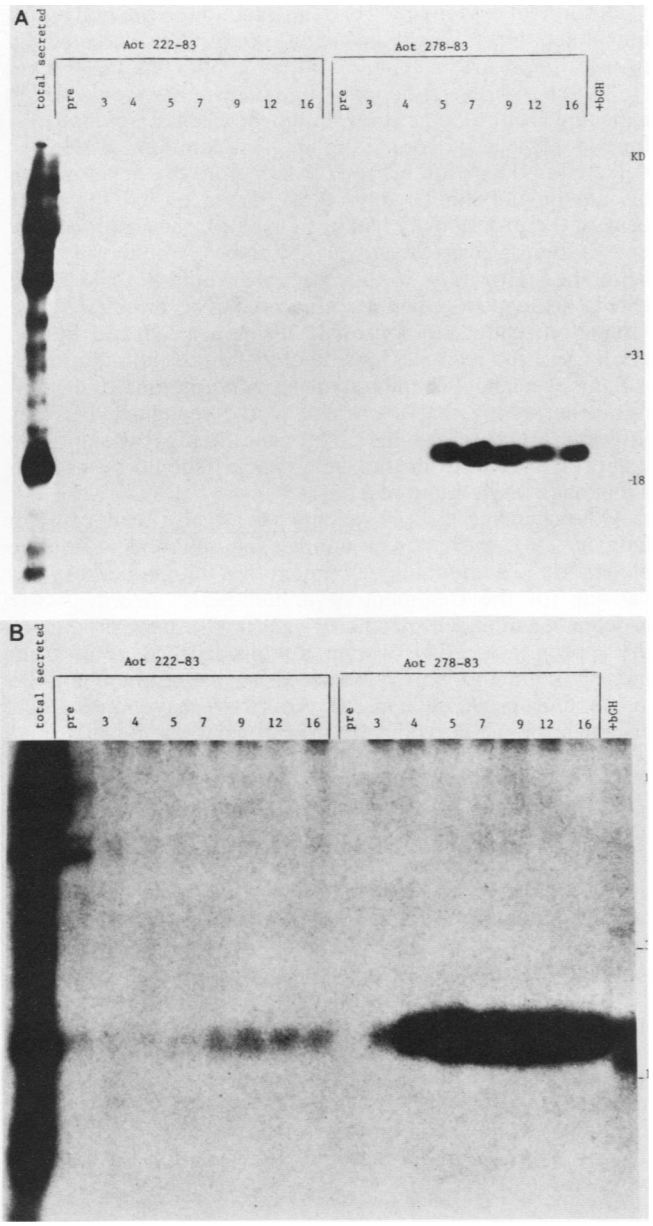

FIG. 7. Antibody response of two owl monkeys inoculated with the bGH-Z20 strain of herpesvirus saimiri. Sera from inoculated owl monkeys Aot 222-83 and 278-83 were tested for their ability to immunoprecipitate the ^{35}S-labeled 21K bGH protein secreted from bGH-Z20-infected SgO21 cells. pre is preinoculation sera, and the numbers indicate weeks postinoculation. The procedure used for immunoprecipitation has been described previously (7). Here, 16 μl of ^{35}S-labeled supernatant was incubated with 5 μl of test serum in a total volume of 90 μl for 30 min at 30°C. +bGH indicates the inclusion of 10 μg of purified bGH (Miles) in the immunoprecipitation reaction with 5-week postinoculation sera of Aot 278-83. ^{35}S-labeled total secreted proteins (3 μl) from bGH-Z20-infected SgO21 cells were used without immunoprecipitation in lane 1. (A) 4-day exposure; (B) 4-week exposure.

from the PBL of the other owl monkey (Aot 278-83) on weeks 1 through 6 and 9, 11, and 14. These results contrast with the recovery of strains S4 and 11att from experimentally infected owl monkeys (5). Strains S4 and 11att can be repeatedly recovered from owl monkeys many months after infection. We recently recovered virus from S4-infected owl monkeys 16 months after infection, and 11att has been recovered from the PBL of marmosets as long as four years after experimental infection (9).

TABLE 1. Secretion of bGH from SgO21 cells infected with bGH-Z20[a]

Time (h)	bGH concn (ng/ml)	
	Infected	Uninfected
24	256	2.2
48	487	2.2
72	750	2.2

[a] SgO21 cells were pelleted and suspended in RPMI 1640 medium with 20% fetal calf serum and 10% T-cell growth factor at a concentration of 0.5×10^6 cells per ml. Samples were taken at each time point, and cells were removed by centrifugation. The level of bGH in the cell-free supernatant was determined by RIA. The cell concentration at 72 h was 1.1×10^6/ml. The infected cells used in this experiment were infected 6 months previously and grown continuously in culture until the time of the experiment. The 2.2-ng/ml concentration reflects the level of bGH in 20% fetal calf serum.

Serum samples from inoculated animals were used for the immunoprecipitation of [^{35}S]methionine-labeled proteins secreted from bGH-Z20-infected SgO21 cells. Antigen-antibody complexes were bound to fixed *Staphylococcus aureus* A protein and analyzed by SDS-PAGE (Fig. 7). Preinoculation sera of Aot 278-83 did not precipitate the 21K bGH protein, but a strong antibody response directed against the 21K bGH protein developed in this animal in the weeks after infection. Precipitation was effectively competed out by purified bGH (Miles). Longer film exposures revealed a very weak antibody response of Aot 222-83 to the 21K bGH protein. Sgf 138-82 also developed a strong antibody response to bGH (Fig. 6).

Serial dilutions of the monkey sera were also tested for their ability to immunoprecipitate ^{125}I-labeled bGH in the RIA. The titers of bGH antibody in sera from the weekly bleedings were expressed as the reciprocal dilution required to precipitate 50% of the ^{125}I-labeled bGH. Antibodies directed against bGH were first detected 2 to 4 weeks after inoculation. Antibody titers reached maximal levels of 1:20 at 7 to 8 weeks postinoculation of Aot 222-83, 1:500 at 6 weeks postinoculation of Aot 278-83, and >1:1,000 at 10 weeks postinoculation of SgF 138-82. Antibody levels decreased thereafter.

The measurement of free bGH in the sera of inoculated animals was complicated by the strong antibody response. Nevertheless, free bGH was detected by radioimmune competition assay in the sera of Aot 222-83, the animal with the weakest antibody response, in the initial weeks postinoculation. Circulating levels of bGH in Aot 222-83 were 2.4, 6.6, and 3.4 ng/ml at weeks 1, 2, and 3 postinoculation. Thereafter, free bGH was not detected.

DISCUSSION

The procedures used for constructing the bGH-Z20 strain of herpesvirus saimiri should be applicable for inserting a foreign gene into other herpesviruses. In fact, similar techniques have been used in the manipulation of the herpes simplex virus genome (13, 19, 32, 35). Homologous recombination of herpesvirus saimiri L-DNA sequences on both sides of the bGH DNA insert of the hybrid plasmid pBGH-Z20 in the infected cell would generate a virus without pBR322 sequences. However, all 10 virus recombinants that we selected had pBR322 sequences. Two explanations can be given for the presence of pBR322 sequences in the

recombinant virus. (i) A single crossover event occurred in L-DNA sequences to the right of the bGH insert, and this yielded virus with pBR322 sequences at the left end out to the point of linearization (*Eco*RI). Circularization of this DNA in the course of replication would yield the virus DNA structure shown in Fig. 3. (ii) Homologous recombination of L-DNA sequences to the right of the bGH insert and illegitimate recombination between H-DNA and pBR322 sequences to the left of the bGH insert would yield the virus DNA structure shown in Fig. 3.

The former possibility seems more likely, because the length of pBR322 sequences in the recombinant virus is full length or very nearly full length out to the point of plasmid linearization (*Eco*RI), and evidence has been presented for a circular DNA intermediate in the course of herpesvirus saimiri replication (3).

bGH RNA is spliced and terminated properly in bGH-Z20 persistently infected SgO21 cells, and its size, 0.9 kilobase, suggests transcription from the SV40 promoter. RNA containing bGH sequences is also spliced and terminated properly in permissively infected OMK cells; however, in this case, multiple species of larger RNAs are observed. Transcription of bGH sequences in permissively infected OMK cells must initiate from upstream herpesvirus saimiri promoters. One of these promoters has been mapped previously (14). This should not be a surprising result; in the course of permissive infection, herpesvirus saimiri turns the cellular metabolism over to recognition of the herpesvirus saimiri promoter and regulatory signals (26, 31). In persistently infected marmoset T cells, pre-bGH protein is faithfully cleaved and secreted to produce mature bGH.

The results described in this report extend the use of herpesviruses as vectors to the gamma (lymphotropic) class of herpesviruses. Because of their ability to persistently infect lymphoid cells, the gamma herpesviruses have utility for achieving expression of introduced genes for prolonged periods in vitro. Herpesvirus saimiri may be particularly useful for studying genes whose expression is limited to T cells or T-cell subsets. This system may also be useful for producing large amounts of a protein requiring processing in a mammalian cell. Multiple copies of circular DNA are characteristically found in cells latently infected with lymphotropic herpesviruses (1, 37). Multiple copies of viral DNA containing a foreign gene under the influence of a strong promoter and enhancer could conceivably produce large amounts of protein. Although we have made no attempt to maximize output or select for high producers, the level of bGH production by the SgO21 cells in our study appears to have been reasonably high in comparison with other systems (8, 21, 28, 29). A herpesvirus saimiri strain containing the bGH gene with its own promoter was also constructed; infection of SgO21 cells with this strain did not yield detectable levels of 21K bGH protein (data not shown).

We were initially surprised to find that the bGH-Z20 virus was unable to establish a long-lasting latent infection in at least two of the inoculated animals. However, subsequent studies on these infected animals revealed the development of antibodies directed against the foreign protein, bGH. The establishment of latent infection may thus be blocked, since latently infected cells are forced to produce a foreign protein, bGH, because of the constitutive SV40 promoter; these cells can be eliminated by cell-mediated immunity. These results raise the possibility that constitutive expression of a foreign antigen may allow the development of herpesvirus vaccines that will not remain latent.

The strong antibody response directed against bGH is in itself forceful evidence for bGH production in the bGH-Z20-inoculated New World primates. Antibodies detected by using *S. aureus* A protein are of the immunoglobulin G class (15). Such a strong immunoglobulin G response would not be expected from a single inoculation of a small amount of a foreign protein without adjuvant. In addition, bGH was detected in the serum of Aot 222-83 before the development of immunoglobulin G antibodies. If the growth hormone gene of the owl monkey had been used for these studies, we expect that growth hormone antibodies would not have developed. However, in this case, we would not have been able to distinguish growth hormone synthesis directed by the virus from endogenous growth hormone produced by the pituitary of the animal. Considerable amino acid differences exist between human and bovine growth hormones (38), so it is not surprising that a primate would recognize bGH as foreign. In any real somatic cell gene therapy attempt, the appropriate gene from the same species should be used to avoid such an immune response.

Although a herpesvirus vaccine has already been tested in humans (30), there are a number of apparent stumbling blocks to the eventual development of a human herpesvirus vaccine for the treatment of a hereditary disorder. One problem is target cell specificity—getting the gene product to the appropriate cell. However, for products secreted from the cell, it may not always be necessary for production to be in the appropriate cell; in this report, we have shown that growth hormone, a product of the pituitary, can be made and secreted by lymphoid cells. For catabolic enzyme deficiencies, it is possible that latently infected cells anywhere in the body could serve as "factories" turning over the accumulated product. Certainly control of gene expression is another problem. However, not all genes may need to be tightly regulated, and constant constitutive expression may be all that is required in some cases. For genes that do need to be strictly regulated, additional developments in gene technology will likely be needed.

The results described in this report demonstrate the usefulness of herpesviruses as vehicles for introducing genes into animals. The potential for gene transfer in animals and for gene therapy has been stressed because of the rather unique ability of herpesviruses to remain latent in the infected host; however, herpesviruses may also prove useful for the expression of microbial antigens for vaccination purposes.

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