JOURNAL OF VIROLOGY, Feb. 1994, p. 638–645
0022-538X/94/$04.00+0

Vol. 68, No. 2

The Human Foamy Virus Internal Promoter Directs the Expression of the Functional Bel 1 Transactivator and Bet Protein Early after Infection

MARTIN LÖCHELT,[1] ROLF M. FLÜGEL,[1]* AND MORDECHAI ABOUD[2]

Abteilung Retrovirale Genexpression, Forschungsschwerpunkt Angewandte Tumorvirologie, Deutsches Krebsforschungszentrum, 69009 Heidelberg, Germany,[1] and Department of Microbiology and Immunology, Faculty of Health Sciences, Ben-Gurion University of the Negev, Beersheva, Israel[2]

Received 2 August 1993/Accepted 8 November 1993

The human foamy virus or spumaretrovirus (HFV) is a complex retrovirus that has the capacity to code not only for the three retroviral genes *gag*, *pol*, and *env* but, in addition, for at least three *bel* genes. The HFV provirus contains two different and functionally active promoters: the classical retroviral promoter in the 5′ long terminal repeat and a recently identified second promoter in the *env* gene upstream of the *bel* genes. Both promoter/enhancers are strongly dependent on the HFV transcriptional transactivator protein Bel 1. Here we report that the internal promoter directs the synthesis of viral transcripts that code for functionally active Bel 1 and for Bet proteins that appeared early after HFV infection. The viral mRNAs of the internal promoter have a 112-nucleotide-long leader exon and were spliced predominantly at the first splice donor site in the 5′ untranslated region. The data were obtained by transient expression assays, transactivation experiments, and RNA analyses of transcripts derived from HFV-infected cells. The results provide strong evidence for the crucial role the internal promoter plays during HFV infection in generating *bel*-specific transcripts.

The human foamy virus or spumaretrovirus (HFV) is an exogenous retrovirus that was isolated from the lymphoblastoid cells of a nasopharyngeal carcinoma patient (1). One of the hallmarks of the HFV genome is that it encodes *bel* genes that are located 3′ of *env* (8, 17). A number of groups have tried to correlate HFV infections in humans with defined clinical diseases (10, 15, 21). It is quite interesting that HFV transgenic mice developed brain-specific lesions (2, 3). The *bel 1* gene was identified as a transcriptional transactivator for the long terminal repeat (LTR)- and internal promoter-directed transcription (9, 11, 13, 23, 26). *bel 1* is absolutely required for viral replication and gene expression (14). Similar results for the *taf* transactivator of the closely related simian foamy virus type 1 were reported by the laboratory of Luciw and coworkers (18–20).

Recently, a novel internal promoter/enhancer element was detected in the HFV genome just upstream of the *bel* genes in the 3′ end of the *env* gene (13). The internal cap site at HFV nucleotide (nt) 9196 is located in the noncoding exon 6 within the *env* gene (Fig. 1). The presence of a second, internally located transactivator-dependent promoter and the great number of differentially spliced HFV transcripts (reference 22a; updated in reference 7) indicate that HFV gene expression is complex and comparable in structural and functional complexity to that of the other human retroviruses (5, 7). In addition, the basal activity of the internal promoter was increased by the presence of the LTR promoter in *cis*, indicating complex interactions between the two promoter/enhancers in the HFV provirus (12).

In this report, defined HFV expression plasmids and RNA from HFV-infected cells were used to demonstrate that the internal start site of HFV transcription within the 3′ end of the *env* gene directs the expression of the Bel 1 transactivator and the Bet protein early after infection. As the HFV internal and the LTR promoters are both dependent on Bel 1, the dual capacity of Bel 1 expression points to an essential role of the internal promoter in HFV retroviral gene expression. The analysis of *cis*- and *trans*-acting factors that allow foamy viruses to utilize the LTR and the internal promoter efficiently may give insight into the mechanisms that determine the activity and use of internal promoters in retroviral vectors designed for gene transfer experiments.

MATERIALS AND METHODS

Plasmids used. The infectious HFV DNA clone pHSRV13 served as a source of viral DNA (14); nucleotide numbering starts at the first base of the 5′ LTR of the HFV provirus. The eukaryotic Bel 1 expression clones pBCbel1, pBCbel, and bel1s described recently (12, 14, 27) direct Bel 1 gene expression from the cytomegalovirus immediate-early (CMV-IE) promoter. The plasmids used are compiled in Table 1. Indicator gene plasmids pNNCAT+ and pNNSEAP+ contain the internal promoter/enhancer (HFV nt 8971 to 9253) in the sense orientation upstream of the chloramphenicol acetyltransferase (CAT) gene and the gene for the secreted form of the human alkaline phosphatase, SEAP, respectively (13).

Construction of recombinant clones. Molecular cloning was performed according to standard techniques (24). Recombinant DNA clones designed to express the Bel 1 or the complete *bel* coding region under the control of the HFV internal promoter were constructed as follows: plasmid pNNSEAP+, which contains the SEAP gene under the control of the internal promoter, was digested with *Sac*I, which cuts directly at the internal HFV cap site, and *Sma*I, which cleaves between the end of the SEAP gene and the poly(A) addition site. The deleted SEAP coding region was replaced by HFV DNA fragments extending from the *Sac*I site at the internal promoter to the end of the *bel 1* gene (blunt-ended *Acc*I site at nt

* Corresponding author. Mailing address: Abteilung Retrovirale Genexpression, Forschungsschwerpunkt Angewandte Tumorvirologie, Deutsches Krebsforschungszentrum, Im Neuenheimer Feld 242, 69009 Heidelberg, Germany. Phone: 49-6221-424611. Fax: 49-6221-424852.

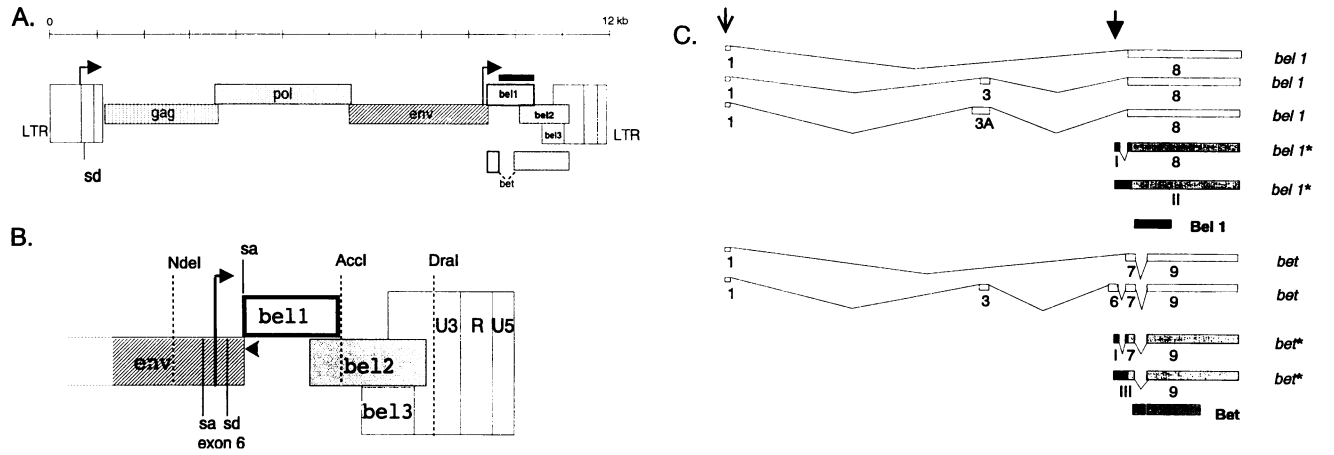

FIG. 1. (A) Schematic presentation of the genomic organization of the HFV provirus, the location of the LTR and the internal promoter, and some splice sites used in this report. The start site and the direction of transcription in the 5′ LTR and the novel promoter II in the 3′ part of *env* directly upstream of *bel 1* are indicated by bent arrows above the LTR and *env*, respectively. The HFV regions coding for the 56-kDa Bet protein are shown below the *bel* genes. The chimeric, abundantly expressed Bet formed by splicing of a *bel 1* exon to a *bel 2* exon is shown (14, 22a). The solid bar on top of *bel 1* shows the Bel 1 domain that was bacterially expressed in plasmid pETbel1 and that is not present in the Bet protein sequence. sd, splice donor. (B) Blown-up presentation of the *bel* gene region and the location of internal promoter II (bent arrow as in panel A) in the noncoding exon 6 in the *env* gene. The locations of the exon 6 splice acceptor (sa) and donor (sd) sites and that of the *bel 1* splice acceptor are given. Cleavage sites for the restriction enzymes (*Nde*I, *Acc*I, and *Dra*I) used to construct recombinant clones pPLNdeAcc and pPLNdeDra are shown by broken lines. The arrowhead below *bel 1* indicates the localization and direction of the antisense primer 9485a. (C) HFV *bel 1* and *bet* transcripts generated from the 5′ LTR cap site (7, 22a) and postulated structures of *bel 1** and *bet** mRNAs initiated at internal promoter II. The two vertical arrows in the top line mark the two cap sites. Solid boxes indicate the regions of those promoter II transcripts that were determined in this report; those parts that were deduced from protein expression data are marked by stippled boxes. Open rectangles mark exons described previously (22a) and are numbered consecutively with arabic numerals (from 1 to 9). Roman numerals (I to III) indicate those exons that start at the internal promoter. Transcripts starting at the internal cap site are marked by asterisks at the right margin. Introns are indicated by inverted carets. Exon 3A was recently determined (22). Exon I was found to be 112 nt in length. Exon II starts at nt 9196 and ends at the poly(A) site; exon III extends from nt 9196 to nt 9699. Bel 1 and Bet proteins are shown by hatched boxes.

10372) or to the end of the *bel* coding region (*Dra*I site at nt 11318). Recombinant clones were designated pPLNdeAcc and pPLNdeDra, referring to the restriction sites flanking the HFV DNA inserts (Table 1; Fig. 1B).

A bacterial expression clone that contains only that part of the HFV *bel 1* open reading frame that is unique to the Bel 1 transactivator and not part of the Bet protein was constructed. The HFV DNA fragment between the *Stu*I (nt 9754) and the *Ssp*I (nt 10386) sites was inserted into the blunt-ended *Bam*HI site of the prokaryotic pET3b expression vector (25). Recombinant clone pETbel1 expressed a fusion protein of about 24 kDa consisting of 13 amino acid residues of pET3b fused in frame to the HFV *bel 1* gene. The 24-kDa Bel 1-specific protein was expressed and purified on a preparative scale by repeated preparative polyacrylamide gel electrophoresis (PAGE) as described previously (16). The monospecific polyclonal Bel 1 antiserum, anti-bel1ss, that does not recognize the Bet protein was obtained by immunizing rabbits with PAGE-purified pET3b Bel 1 fusion protein.

Cell culture, transfection, and expression assays. COS7 and human embryonic lung (HEL) cells were grown, and virus infections were performed as described previously (14). Transfections by electroporation of 10 μg of DNA were performed as described previously (13); for cotransfections, Bel 1 expression clones or the parental pBC12CMV vector was used. CAT assays were performed and normalized to coexpressed β-galactosidase activity as described elsewhere (13). For quantitation, cellular extracts were serially diluted and analyzed by thin-layer chromatography, and separated radioactivity was measured with a thin-layer chromatography linear analyzer. SEAP activity was quantitated as described elsewhere and expressed in milliunits of SEAP activity (9, 13).

RNA extraction and primer extensions. Total RNA was harvested by cell lysis in guanidinium thiocyanate and CsCl sedimentation (4). When indicated, total RNA was selected for poly(A)$^+$ mRNA with oligo(dT)-containing magnetic beads as described by the manufacturer [Dynabeads oligo(dT)$_{25}$; Dynal, Hamburg, Germany]. Primer extensions were performed as described elsewhere (13) with 5′-^{32}P-labeled primer oligonucleotide 9485a, 5′-CTGAATTCCAGAGGTGGAGGCTACT GAT-3′, which is complementary to the 5′ part of the *bel 1*

TABLE 1. Comparison of different eukaryotic Bel 1 expression clones used

Bel 1 expression plasmid	Promoter	HFV insert	Bel 1/Bet expression	Reference(s)
pBCbel	CMV-IE	nt 9250 to 11955	Bel 1, Bet	12, 14
pBCbel1	CMV-IE	nt 9250 to 10372	Bel 1, truncated Bet	12
pbel1s	CMV-IE	nt 9339 to 10386	Bel 1	27
pPLNdeAcc	HFV internal	nt 8971 to 10372	Bel 1, truncated Bet	This report
pPLNdeDra	HFV internal	nt 8971 to 11318	Bel 1, Bet	This report

gene and located 59 nt downstream of the splice acceptor of the *bel 1* and *bet* exons 8 and 7 (Fig. 1B and C).

S1 nuclease analysis. S1 nuclease protection assays were performed with 30 μg of total RNA as described elsewhere (6, 13, 28). The hybridization probe was prepared by PCR with human spuma retrovirus sense-primer 8971s (5′-TATGTTC CTAGCATCGTGACTG-3′), 5′-^{32}P-end-labeled antisense primer 9382a (5′-GGATAGGCTTTAAGTATCCCAAGAG AC-3′), or antisense primer 9307a (13) and pHSRV13 DNA. The gel-purified probes were annealed to RNA at 46°C overnight, and protected DNA was separated on sequencing gels with a dideoxy sequencing reaction of pHSRV13 DNA run in parallel.

Immunological techniques. The preparation of cell-associated antigen, PAGE, Western blotting (immunoblotting), and indirect immunofluorescence (IIF) of cells grown on microscopic slides were performed as recently described (14).

RESULTS

Preparation of a persistently HFV-infected COS7 cell culture. COS7 cells are not permissive for infection with wild-type HFV; they are, however, capable of synthesizing infectious HFV particles upon transfection with the infectious HFV DNA clone pHSRV13 (14). To prepare persistently infected COS7 cells, pHSRV13 DNA was transfected into COS7 cells and the transfected cell culture was passaged serially. Directly after transfection, HFV *gag*, *env*, and *bel 1*- and *bel 2*-specific antigens were detectable by IIF in about 10% of the transfected cells. The number of HFV antigen-positive cells decreased gradually upon serial passages below the detection limit. During the first 10 passages, a typical HFV cytopathic effect concomitant with the formation of large syncytia was not detectable. After the 10th passage, some syncytia appeared and their number increased steadily. In these late cell cultures designated COS-HFV, HFV-specific antigens (*gag*, *env*, *bel*) were detectable by IIF or Western blotting (data not shown) and the pattern of HFV mRNAs was indistinguishable from that of HFV-infected HEL cells (see below). The capacity for syncytium formation was passageable to uninfected COS7 cells with a low efficiency as cell-free COS-HFV supernatant. However, cocultivation of COS-HFV with COS7 cells resulted in a rapid spread of cytopathic effect and expression of HFV mRNAs and proteins.

Detection of *bel 1*/*bet*-specific transcripts in HFV-infected HEL and COS-HFV cells by primer extension. To determine whether the internal HFV promoter (13) directs the synthesis of *bel 1*/*bet*-specific transcripts, primer extension experiments were performed with the HFV antisense oligonucleotide 9485a located in the 5′ part of the *bel 1* gene (Fig. 1B, arrowhead). By using primer 9485a, it should be possible to identify *bel 1*/*bet*-specific transcripts that start either at the internal cap site or at the cap site in the 5′ LTR (Fig. 1C). The RNA preparations used were from HFV- or mock-infected HEL cells harvested 72 h postinfection (p.i.) (Fig. 2, lanes 3 and 1, respectively) or from the persistently HFV-infected COS-HFV cells (lane 2) described above. The reaction products of the primer extensions were loaded on a sequencing gel next to those of a dideoxy sequencing of pHSRV13 DNA primed with the same 9485a oligonucleotide (lanes G to C). Two specific reaction products were detectable in primer extensions with HFV RNAs but not in that of mock-infected cells.

The predominant reaction product had a size of 171 nt (Fig. 2, solid arrow); the minor bands were 290 nt in size (open arrow). The size of the 290-nt DNA bands when aligned with the sequencing ladder is equivalent to the 3′ terminal C base in

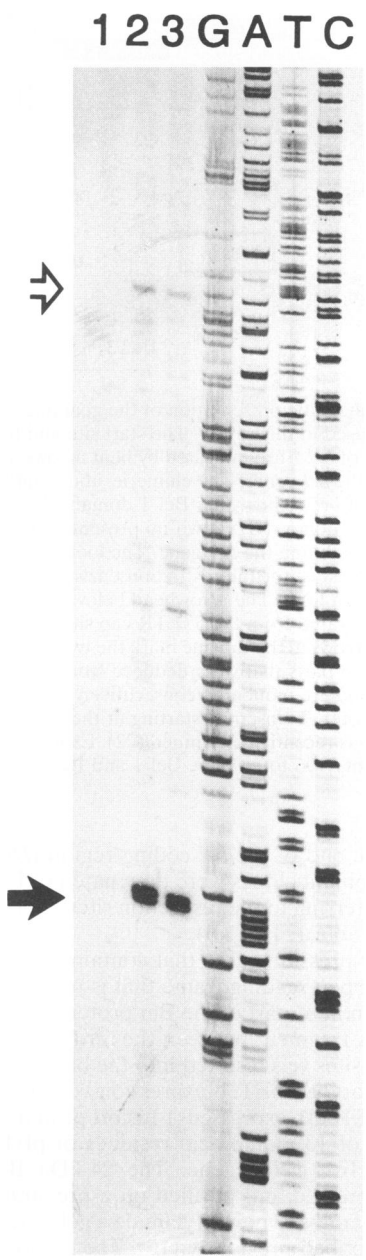

FIG. 2. Detection of spliced and unspliced *bel 1**/*bet**-specific transcripts derived from promoter II by primer extension. Primer extensions were performed with RNA from mock-infected (lane 1) and HFV-infected (lane 3) HEL cells harvested 72 h p.i. and RNA from the persistently HFV-infected COS-HFV cells (lane 2). The antisense primer 9485a used is located in the 5′ end of the *bel 1* gene, 59 nt downstream of the splice acceptor of exons 8 and 7. The reaction products of the primer extensions were analyzed parallel to a sequencing reaction of pHSRV13 DNA with primer 9485a (four lanes marked G, A, T, and C indicate the dideoxynucleoside triphosphate used). The open arrow points to the 290-nt-long extension product that represents unspliced *bel 1**/*bet**-specific transcripts directed by promoter II. The solid arrow indicates the position of the spliced promoter II transcripts that extend into the *bel 1*/*bet* genes.

the antisense sequence 5′-AAGGAGCTC-3′. The corresponding G base residue at HFV provirus nt 9196 was recently shown to be the internal start site of transcription on the sense strand (13). The 290-nt cDNA bands therefore correspond to HFV mRNAs that start at the internal cap site and extend into the *bel 1* gene. The predominant band of 171 nt represents an internally initiated HFV mRNA that was spliced from the splice donor at the end of the noncoding exon 6 (nt 9307) to the splice acceptor of the *bel 1/bet* exons 7 and 8 at nt 9427 (Fig. 1B and C). As predicted, this cDNA is exactly 119 nt shorter than the unspliced internally initiated transcript. Some minor reaction products were also visible (Fig. 2). Surprisingly, we were not able to identify unambiguously an LTR-derived singly spliced *bel 1*-specific transcript of 110 nt that was recently detected in HFV-infected cells (22a). However, the LTR-derived HFV mRNAs were detected by the extremely sensitive reverse transcription-PCR technique. To improve the sensitivity of the primer extension reaction, total RNA from HFV-infected HEL cells harvested 72 h p.i. was selected for poly(A)$^+$ RNA with magnetic beads containing oligo(dT) and again subjected to primer extension experiments with primer 9485a. The pattern of reaction products corresponding to spliced and unspliced internally initiated *bel 1/bet* transcripts was indistinguishable from that obtained with unselected RNA (data not shown). This demonstrates that the internally initiated HFV transcripts are also polyadenylated like the LTR-derived transcripts (22a). However, LTR-derived *bel 1/bet* transcripts were again not detectable. This result implies that at least the *bel 1/bet* transcripts originate predominantly from the internal promoter and not from the HFV 5′ LTR promoter. Figure 1C schematically presents an overview of multiply spliced *bel 1/bet* transcripts expressed from the 5′ LTR and the internal promoter.

Quantitation of spliced versus unspliced promoter II transcripts. To distinguish between internally initiated and LTR-derived transcripts, internally initiated transcripts are marked by an asterisk (e.g., *bel 1** RNA). The noncoding leader exon of the internally initiated transcripts from nt 9196 to 9307 was designated exon I (roman numeral) to distinguish it from the 5′ LTR leader exon 1 (arabic numeral) as shown in Fig. 1C. Accordingly, exons that start at the internal cap site at nt 9196 will be indicated by roman numerals (Fig. 1C). The 5′ LTR promoter is promoter I, and the internal or intragenic promoter is named promoter II.

S1 nuclease protection assays were performed to confirm that the spliced *bel 1*/bet** transcripts are much more abundant than their unspliced counterparts. To this end, two DNA probes were synthesized and labeled with the same specific activity. The two probes spanned the entire exon 6 and upstream sequences from nt 8971 to 9307 (Fig. 3, lanes 1 and 2) or extended further downstream into the following intron (nt 8971 to 9382, lanes 3 and 4). The probe 8971-9382 is expected to be protected exclusively by unspliced promoter II transcripts, whereas probe 8971-9307 should be protected by both spliced and unspliced *bel 1*/bet** mRNAs. RNA from HFV- (lanes 1 and 3) and mock-infected (lanes 2 and 4) HEL cells harvested 72 h p.i. was subjected to S1 nuclease analysis to identify the RNA species. For length determination, the reaction products of a dideoxy sequencing of pHSRV13 DNA with primer 9307a (left lanes G to C) and primer 9382a (right lanes G to C) were loaded in parallel. The protection of probe 8971-9307 by HFV RNA confirmed that the internal start of promoter II is at nt 9196 (Fig. 3, arrow). The comparison with the reaction products with probe 8971-9382 revealed that the spliced transcripts are much more abundant than the unspliced promoter II transcripts that were capable of protecting probe 8971-9382 (Fig. 3, faint band in lane 3, arrowhead).

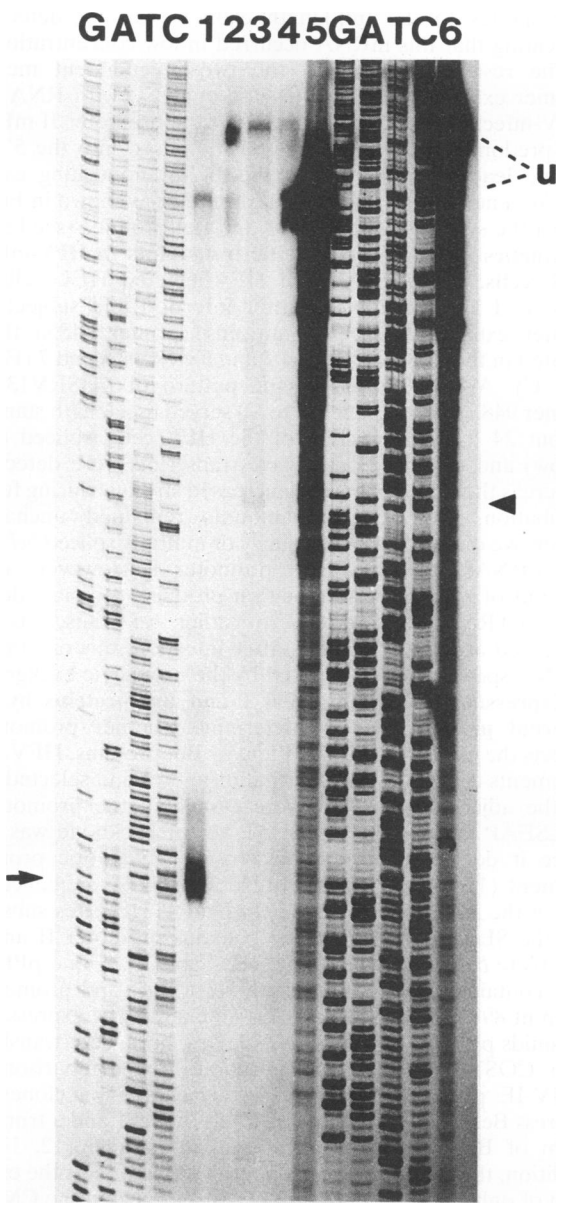

FIG. 3. Quantitation of internally initiated, spliced, and unspliced HFV transcripts in HFV-infected HEL cells by S1 nuclease assays. S1 nuclease assays were performed with total RNA from HEL cells harvested 72 h after HFV (lanes 1 and 3) or mock (lanes 2 and 4) infection. RNA from HFV-infected cells protected DNA fragments of about 112 nt with probe 8971-9307 (arrow) and a very faint band of 187 nt with the labeled probe 8971-9382 (arrowhead). RNA from mock-infected cells did not specifically protect any DNA fragments; "u" indicates the position of undigested hybridization probes loaded as a control on lane 5 (probe 8971-9307) and lane 6 (probe 8971-9382). Lanes G through C show the dideoxy sequencing patterns of pHSRV13 DNA with primer 9307a (left-hand side) and primer 9382a (right-hand side) separated in parallel for length determination.

Probe 8971-9307 should also be protected by a *bet* mRNA species, since it was shown by reverse transcription-PCR that it carries exon 6 (22a) (Fig. 1C). However, a protected probe that

corresponds to the full-length exon 6 was not detectable, indicating that this mRNA occurred in low concentrations.

The results obtained by the two independent methods (primer extension and S1 nuclease analysis) with RNA from HFV-infected cells clearly prove that the promoter II mRNAs are predominantly (more than 95%) spliced into the 5′ noncoding leader, giving rise to the 112-nt noncoding exon I. RNAs generated by the internal promoter as shown in Fig. 1C direct the synthesis of the Bel 1 and Bet proteins (see below).

Kinetics of *bel 1*/*bet*-specific transcripts in HFV-infected HEL cells. Total RNA from HFV-infected HEL cells was harvested in 12-h intervals after infection and subjected to primer extension with the antisense primer 9485a that is located in the 5′ end of the *bel 1* and *bet* exons 8 and 7 (Fig. 1B and C). Again, the sequencing pattern of pHSRV13 with primer 9485a (Fig. 4, lanes G to C) served as a length standard. About 24 h after infection of the HEL cells, spliced (open arrow) and unspliced *bel 1*/bet** transcripts were detectable. Whereas their concentrations increased strongly during further incubation, their relative amounts remained unchanged. Again, we did not detect the singly or multiply spliced *bel 1* and *bet* mRNAs from the LTR promoter I. However, as the amount of premature termination products (possibly derived from LTR-derived *gag*, *env*, or other *bel* transcripts) was increased at late time points after infection, this or other *bel* mRNA species may be masked by the unspecific background.

Expression of authentic Bel 1 and Bet proteins by HFV internal promoter II. To determine whether promoter II directs the expression of Bel 1 and/or Bet proteins, HFV DNA fragments containing the internal promoter and selected parts of the adjacent *bel* genes were cloned in the promoterless pPLSEAP vector. The pPLSEAP vector backbone was used, since it does not contain any cryptic eukaryotic promoter element (12a). In plasmid pPLNdeDra, HFV sequences between the *Nde*I (nt 8971) and *Dra*I (nt 11318) sites substitute for the SEAP gene; this clone contains promoter II and the complete *bel* gene region (Fig. 1B; Table 1). Clone pPLNdeAcc contains only the full-length *bel 1* gene and promoter II from nt 8971 to 10372 (Table 1). To analyze the expression of plasmids pPLNdeAcc and pPLNdeDra, both were transfected into COS7 cells by electroporation. For comparison, the CMV-IE promoter-based eukaryotic expression clones that express Bel 1 and Bet (clone pBCbel) or Bel 1 and a truncated form of Bet (pBCbel1) were used in parallel (12, 14). In addition, the expression clone pbel1s, which directs the expression of only the Bel 1 protein (27) (Table 1), and the CMV-IE expression vector backbone pBC12CMV (negative control) were used. Cellular extracts were prepared 72 h after electroporation of 10 μg of plasmid DNA, and defined amounts of antigen were subjected to protein blotting with two different monospecific polyclonal antisera that were raised against bacterially expressed HFV antigen (for details, see the legend to Fig. 5). The Bel 1/Bet-specific serum is directed against amino-terminal epitopes that are present on both the Bel 1 and the Bet proteins (14). The newly generated Bel 1-specific serum, termed bel1ss, was raised against central and carboxy-terminal sequences of the *bel 1* gene that are not part of the Bet protein (black bar in Fig. 1A). This serum therefore detects only Bel 1 but not Bet, which is a chimeric protein generated by splicing (Fig. 1) (22a).

The Bel 1-specific serum bel1ss detected similar amounts of the 36-kDa Bel 1 protein in transfections with pbel1s, pBCbel, and pBCbel1 (Fig. 5A, lanes 1, 3, and 4). A protein band, comigrating with Bel 1 and most likely representing Bel 1, was also expressed by plasmids pPLNdeAcc and pPLNdeDra (lanes 5 and 6). As additional protein bands were not detectable in either of these samples or in the pBC12CMV negative control (lane 2), the novel Bel 1 antiserum bel1ss seems to be Bel 1 specific. Upon scanning of the corresponding Western blot, the amount of Bel 1 expressed by either HFV promoter

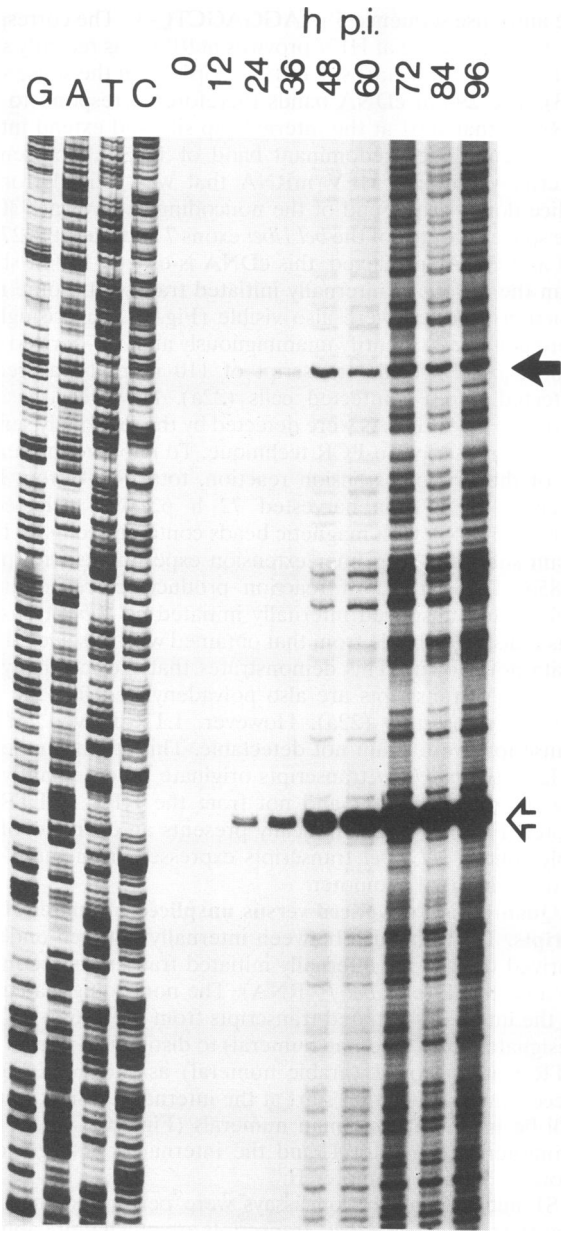

FIG. 4. Kinetics of spliced and unspliced *bel 1*/bet*-specific transcripts in HFV-infected HEL cells. RNA from HFV-infected HEL cells grown in 75-cm^2 flasks was harvested in 12-h intervals after infection (as indicated above the corresponding lanes). Aliquots of 15 μg of total RNA were subjected to primer extensions with the antisense primer 9485a located in the 5′ part of *bel 1*. Aliquots of the reactions were loaded on a 5% sequencing gel together with the dideoxy sequencing pattern of pHSRV13 DNA primed with oligonucleotide 9485a (lanes G to C). The positions of unspliced (solid arrow) and spliced (open arrow) *bel 1*/bet*-specific promoter II transcripts are given. Since the autoradiogram was intentionally overexposed to demonstrate spliced and unspliced promoter II transcripts at 24 h p.i., unspecific termination products became visible in reactions from samples harvested late after infection.

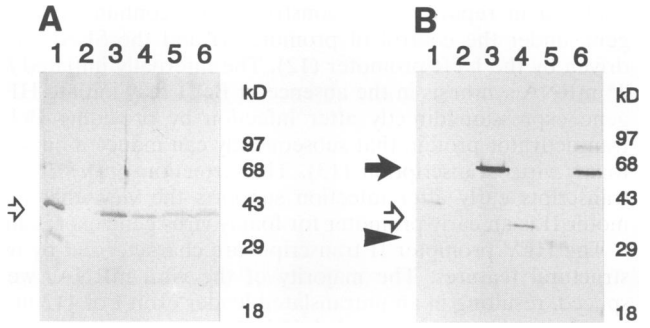

FIG. 5. Detection of Bel 1 and Bet proteins expressed from HFV promoter II in plasmids pPLNdeAcc and pPLNdeDra. COS7 cells were transfected with 10 μg of plasmid pbel1s (lane 1), pBC12CMV (lane 2), pBCbel (lane 3), pBCbel1 (lane 4), pPLNdeAcc (lane 5), or pPLNdeDra (lane 6). Extracts of cell-associated proteins were prepared 3 days after transfection and analyzed by Western blots with the monospecific polyclonal rabbit serum bel1ss, which is Bel 1 specific (A), and the Bel 1/Bet-specific serum (B). The amount of protein loaded onto each lane was as follows: panel A, lanes 1 to 4, 12 μg, and lanes 5 and 6, 36 μg; part B, lanes 1 to 4, 2.5 μg, and lanes 5 and 6, 7.5 μg. The positions and sizes (in kilodaltons) of marker proteins (prestained high-molecular-weight markers) are given. An open arrow marks the position of the 36-kDa Bel 1 protein; a solid arrow marks that of 56-kDa Bet; and the arrowhead points to the position of the truncated Bet versions of 30 and 33 kDa.

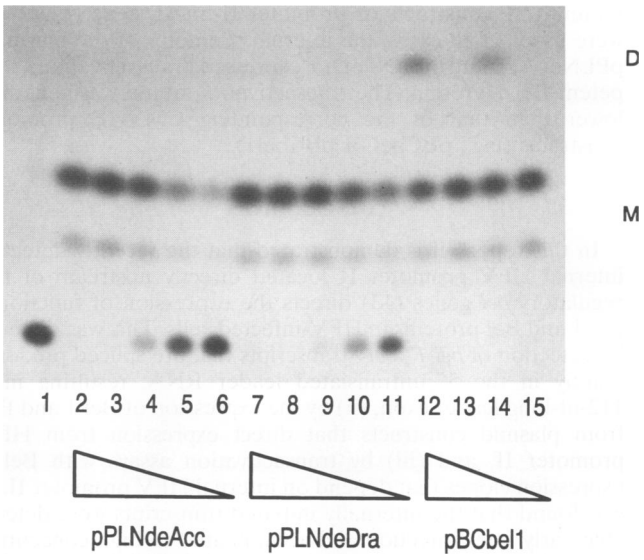

FIG. 6. Transient expression assays of COS7 cells transfected with constructs containing the HFV internal promoter. Shown are CAT assays of COS7 cell extracts after cotransfection with 1.5 μg of indicator gene construct pNNCAT+ *trans*-complemented with 8 μg of pBC12CMV (lane 1) and decreasing amounts of pPLNdeAcc (lanes 2 to 6), pPLNdeDra (lanes 7 to 11), and pBCbel1 (lanes 12 to 15). In transfections, 8.0 μg (lanes 2 and 7), 4.0 μg (lanes 3 and 8), 2.0 μg (lanes 4 and 9), 1.0 μg (lanes 5 and 10), 0.5 μg (lanes 6, 11, 14, and 15), and 5 μg (lanes 12 and 13) of the corresponding *trans*-complementing Bel 1 expression plasmid were used. For CAT assays, the amount of cell extract used was normalized to coexpressed β-galactosidase activity. The reactions corresponding to lanes 13 and 15 were performed with 1/10 of normalized cell extracts to allow better quantitation. Therefore, extracts from cells containing high amounts of coexpressed Bel 1 showed CAT reaction kinetics that were out of linear range. The positions of both monoacetylated (M) and the diacetylated (D) chloramphenicol derivatives are indicated.

II-based expression clone was at least sixfold lower than that expressed by one of the CMV-IE promoter-based expression clones (a threefold-higher amount of protein was loaded in lanes 5 and 6). The specificity of the antiserum bel1ss was furthermore confirmed by Western blot analysis of antigen extracts of HFV- and mock-infected HEL cells and IIF of HFV-infected HEL cells and found to react exclusively with Bel 1 present in HFV-infected cells (data not shown).

As a control, the Western blot was developed parallel to the Bel 1/Bet-specific antiserum. As expected, plasmid pbel1s again exclusively produced the 36-kDa Bel 1 protein (Fig. 5B, lane 1, open arrow). Clones pBCbel and pPLNdeDra (lanes 3 and 6) directed the synthesis of huge amounts of full-length 56-kDa Bet protein (solid arrow), whereas the 36-kDa Bel 1 protein was detectable only as a faint band. Plasmids pBCbel1 and pPLNdeAcc also produced low concentrations of Bel 1 and substantially higher amounts of truncated versions of Bet ranging from 30 to 33 kDa (lanes 4 and 5). The different sizes of the truncated Bet proteins (arrowhead) are due to different termination sites in the vector backbones used to construct pPLNdeAcc and pBCbel1 and are consistent with the calculated values.

The expression of Bel 1- and Bet-specific antigen was also demonstrated by IIF of COS7 cells transfected with pPLNdeAcc and pPLNdeDra (data not shown). Primer extension experiments performed with antisense primer 9485a and total RNA from COS7 cells transfected with pPLNdeAcc and pPLNdeDra confirmed that the *bel 1/bet*-specific transcripts started at nt 9196 of the HFV promoter II (data not shown). Furthermore, these transcripts were predominantly spliced from exon 6 to exons 7 and 8 as was also found for *bel 1*/bet** transcripts derived from HFV-infected HEL cells. The results demonstrate that the internal promoter is capable of directing the expression of Bel 1 and Bet proteins.

Transactivation of promoter II by Bel 1 protein expressed from HFV promoter II. To determine whether the Bel 1 protein expressed by either pPLNdeAcc (Fig. 6, lanes 2 to 6) or pPLNdeDra (lanes 7 to 11) is capable of transactivating the HFV promoter II, cotransfections with indicator gene construct pNNCAT+ were performed. As controls, the CMV-IE promoter Bel 1 expression clone pBCbel1 (lanes 12 to 15) and the parental vector pBC12CMV (lane 1) were used. To quantitate the expression of the Bel 1 transactivator, transfections with 8.0, 4.0, 2.0, 1.0, and 0.5 μg of plasmids pPLNdeAcc and pPLNdeDra and 5.0 and 0.5 μg of pBCbel1 and the constant amount of 1.5 μg of pNNCAT+ DNA were carried out. CAT assays were performed with normalized amounts of COS7 cell extracts; however, in lanes 13 and 15 only 1/10 of the corresponding extract was used. The autoradiogram showed that the plasmids pPLNdeAcc and pPLNdeDra (lanes 2 to 11) are capable of strongly transactivating promoter II above the level of the basal activity shown in the absence of Bel 1 (lane 1). The CAT activities induced by Bel 1 expressed from HFV promoter II were about 10% of that expressed by the CMV-IE promoter in plasmid pBCbel (compare lanes 3 and 8 with lane 13). Furthermore, concentrations of pBCbel decreasing from 5 to 0.008 μg resulted in a gradually decreasing transactivation of pNNCAT+. In contrast, only pPLNdeAcc or pPLNdeDra concentrations above 0.2 μg resulted in a significant Bel 1-mediated transactivation of promoter II (data not shown). Lower amounts of these Bel 1 expression clones did not increase CAT expression over the basal, Bel 1-independent expression level.

Similar results were obtained when either HFV LTR pro-

moter CAT constructs or promoter II-SEAP gene constructs were used. In all cases, the internal promoter of the plasmids pPLNdeAcc and pPLNdeDra expressed transactivation-competent Bel 1 protein. The transactivation found was generally lower than that of the corresponding CMV-IE promoter constructs (e.g., pBCbel or pBCbel1).

DISCUSSION

In this report, it is demonstrated that the recently detected internal HFV promoter II located directly upstream of the regulatory *bel* genes (13) directs the expression of functional Bel 1 and Bet proteins in HFV-infected cells. This was done (i) by detection of *bel 1*/bet** transcripts that are spliced predominantly in the 5′ untranslated leader RNA, resulting in a 112-nt-long leader exon, (ii) by the expression of Bel 1 and Bet from plasmid constructs that direct expression from HFV promoter II, and (iii) by transactivation assays with Bel 1 expression clones that depend on internal HFV promoter II. It was found that the internally initiated transcripts were detectable early after infection and accumulated to high concentrations during further incubation. This finding indicates that HFV promoter II is active during most, if not all, phases of viral gene expression. Since the synthesis of the HFV *gag*, *pol*, and *env* gene products is directly dependent on a functional 5′ LTR promoter, this result implies that the two HFV promoters are simultaneously active during certain phases of foamy virus replication. Whereas primer extension experiments starting in the 5′ part of *bel 1* produced high amounts of internally initiated *bel 1*/bet** transcripts, corresponding LTR-dependent mRNA species were not unambiguously detectable. However, LTR-derived *bel 1/bet* mRNAs were present, since they were detected by the highly sensitive reverse transcription-PCR technique (Fig. 1C) (7, 22a). It is therefore possible that most *bel 1/bet*-specific transcripts were initiated at internal promoter II. Consequently, the internal promoter should play a critical role in HFV replication. This assumption is strongly supported by the observation that HFV DNA clones with deletions in the internal promoter showed reduced Bel 1 and Bet expression (unpublished data).

Plasmids pPLNdeAcc and pPLNdeDra, which contain *bel 1* under the control of HFV promoter II, induced a clear and significant transactivation of the Bel 1-dependent reporter gene construct pNNCAT+. Both plasmids, however, transactivated at a lower level than the CMV-IE promoter-based plasmid pBCbel1. The fact that only high concentrations of pPLNdeAcc or pPLNdeDra induced considerable transactivation indicates that a critical threshold level of these expression vectors has to be present in the transfected cells. This may be related to the fact that HFV promoter II in pPLNdeAcc and pPLNdeDra is itself strongly Bel 1 dependent (13); the low Bel 1-independent expression of Bel 1 directly after transfection of sufficient amounts of pPLNdeAcc or pPLNdeDra resulted in the transcriptional transactivation of HFV promoter II in these plasmids. Subsequently, this Bel 1-transactivated expression provides thereafter sufficient Bel 1 protein for transactivation of the HFV reporter gene construct pNNCAT+. The results indicate an autoactivation mechanism for Bel 1 expression for HFV promoter II.

It is assumed that an analogous Bel 1-independent gene expression can take place in naturally infected cells directly after provirus synthesis (7). Therefore, the interplay of both HFV transcription units may be of crucial importance for Bel 1-independent gene expression. The level of the basal, Bel 1-independent activity of HFV promoter II was increased in the presence of the HFV U3 promoter of the LTR in *cis* as analyzed in reporter gene constructs that contain the CAT gene under the control of promoter II and the SEAP gene driven by the LTR promoter (12). The internally initiated *bel 1** mRNA synthesis in the absence of Bel 1 may initiate HFV gene expression directly after infection by providing Bel 1 transactivator protein that subsequently can induce a burst of foamy virus transcription (13). The detection of *bel 1*/bet** transcripts early after infection supports the view that promoter II is an early promoter for foamy virus gene expression.

The HFV promoter II transcripts are characterized by two structural features. The majority of the viral mRNAs were spliced, resulting in an untranslated leader exon I of 112 nt in length. In the case of the *bel 1*/bet** transcripts analyzed, a 119-nt-long intron is spliced out in more than 95% of these mRNA molecules. Thus, HFV LTR promoter I and promoter II transcripts have two features in common besides their differences in sequence and localization (Fig. 1). Transcripts from either promoter were predominantly spliced, and the leader exon is in both cases relatively small with 51 nt (LTR) or 112 nt (promoter II). It remains to be determined whether these structural similarities have biological implications, e.g., in the regulation of gene expression.

ACKNOWLEDGMENTS

We thank G. Chinnadurai for providing plasmid pbel1s, Jennifer Reed and Walter Muranyi for critically reading the manuscript, and Harald zur Hausen for support.

M. Aboud and R. M. Flügel are jointly supported by grants from The Joint Israeli-German Research Program of The Ministry of Science and Technology, NCRD, Israel, and the Bundesministerium für Forschung und Technologie, Germany. This work was partly supported by grants from the Bundesministerium für Forschung und Technologie (01KI 8901/6).

REFERENCES

1. **Achong, B. G., P. W. A. Mansell, M. A. Epstein, and P. Clifford.** 1971. An unusual virus in cultures from a human nasopharyngeal carcinoma. J. Natl. Cancer Inst. **46:**299–307.
2. **Aguzzi, A., E. F. Wagner, K. O. Netzer, I. Anhauser, K. Bothe, I. Horak, and A. Rethwilm.** 1993. Human foamy virus proteins accumulate in neurons and induce multinucleated giant cells in the brain of transgenic mice. Am. J. Pathol. **142:**1061–1072.
3. **Bothe, K., A. Aguzzi, H. Lassmann, A. Rethwilm, and I. Horak.** 1991. Progressive encephalopathy and myopathy in transgenic mice expressing human foamy virus genes. Science **253:**555–557.
4. **Chirgwin, J. M., A. E. Przybyla, R. J. MacDonald, and W. J. Rutter.** 1979. Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. Biochemistry **18:**5294–5299.
5. **Cullen, B. R.** 1991. Human immunodeficiency virus as a prototypic complex retrovirus. J. Virol. **65:**1053–1056.
6. **Davis, L. G., M. D. Dibner, and J. F. Battey.** 1986. Basic methods in molecular biology, p. 276–284. Elsevier Co., New York.
7. **Flügel, R. M.** 1992. The molecular biology of the human spumavirus, p. 193–214. *In* B. Cullen (ed.), Human retroviruses—frontiers in molecular biology. Oxford University Press, Oxford.
8. **Flügel, R. M., A. Rethwilm, B. Maurer, and G. Darai.** 1987. Nucleotide sequence analysis of the *env* gene and its flanking regions of the human spumaretrovirus reveals two novel genes. EMBO J. **6:**2077–2084.
9. **Keller, A., K. M. Partin, M. Löchelt, H. Bannert, R. M. Flügel, and B. R. Cullen.** 1991. Characterization of the transcriptional transactivator of human foamy retrovirus. J. Virol. **65:**2589–2594.
10. **Lagaye, S., P. Vexiau, V. Morozov, V. Guenebaut-Claudet, J. Tobaly-Tapiero, M. Canivet, G. Cathelineau, J. Peries, and R. Emanoil-Ravier.** 1992. Human spumaretrovirus-related sequences in the DNA of leukocytes from patients with Graves' disease. Proc. Natl. Acad. Sci. USA **89:**10070–10074.
11. **Lee, K. J., A. H. Lee, and Y. C. Sung.** 1993. Multiple positive and negative *cis*-acting elements that mediate transactivation by *bel1* in

the long terminal repeat of human foamy virus. J. Virol. **67:**2317–2326.

12. **Löchelt, M., M. Aboud, and R. M. Flügel.** 1993. Increase in the basal transcriptional activity of the human foamy virus internal promoter by the homologous long terminal repeat promoter in *cis*. Nucleic Acids Res. **21:**4226–4230.

12a. **Löchelt, M., R. M. Flügel, and M. Aboud.** Unpublished observations.

13. **Löchelt, M., W. Muranyi, and R. M. Flügel.** 1993. Human foamy virus genome possesses an internal, Bel 1-dependent and functional promoter. Proc. Natl. Acad. Sci. USA **90:**7317–7321.

14. **Löchelt, M., H. Zentgraf, and R. M. Flügel.** 1991. Construction of an infectious DNA clone of the full-length human spumaretrovirus genome and mutagenesis of the *bel 1* gene. Virology **184:**43–54.

15. **Mahnke, C., P. Kashaiya, J. Rössler, H. Bannert, A. Levin, W. A. Blattner, M. Dietrich, J. Luande, M. Löchelt, A. E. Friedman-Kien, A. L. Komaroff, P. C. Loh, M. E. Westarp, and R. M. Flügel.** 1992. Human spumavirus antibodies in sera from African patients. Arch. Virol. **123:**243–253.

16. **Mahnke, C., M. Löchelt, H. Bannert, and R. M. Flügel.** 1990. Specific enzyme-linked immunosorbent assay for the detection of antibodies to the human spumavirus. J. Virol. Methods **29:**13–22.

17. **Maurer, B., H. Bannert, G. Darai, and R. M. Flügel.** 1988. Analysis of the primary structure of the long terminal repeat and the *gag* and *pol* genes of the human spumaretrovirus. J. Virol. **62:**1590–1597.

18. **Mergia, A., and P. Luciw.** 1991. Replication and regulation of primate foamy viruses. Virology **184:**475–482.

19. **Mergia, A., K. E. S. Shaw, E. Pratt-Lowe, P. A. Barry, and P. Luciw.** 1990. Simian foamy virus type 1 is a retrovirus which encodes a transcriptional transactivator. J. Virol. **64:**3598–3604.

20. **Mergia, A., K. E. S. Shaw, E. Pratt-Lowe, P. A. Barry, and P. Luciw.** 1991. Identification of the simian foamy virus transcriptional transactivator gene (*taf*). J. Virol. **65:**2903–2909.

21. **Muller, H. K., G. Ball, M. A. Epstein, B. G. Achong, G. Lenoir, and A. Levin.** 1980. The prevalence of naturally occurring antibodies to human syncytial virus in east African populations. J. Gen. Virol. **47:**399–406.

22. **Muranyi, W.** 1991. Ph.D. thesis. University of Heidelberg, Heidelberg, Germany.

22a. **Muranyi, W., and R. M. Flügel.** 1991. Analysis of splicing patterns of human spumaretrovirus by polymerase chain reaction reveals complex RNA structures. J. Virol. **65:**727–735.

23. **Rethwilm, A., O. Erlwein, G. Baunach, B. Maurer, and V. ter Meulen.** 1991. The transcriptional transactivator of human foamy virus maps to the bel 1 genomic region. Proc. Natl. Acad. Sci. USA **88:**941–945.

24. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

25. **Studier, F. W., A. H. Rosenberg, J. J. Dunn, and J. W. Dubendorf.** 1990. Use of T7 DNA polymerase to direct expression of cloned genes. Methods Enzymol. **185:**60–89.

26. **Venkatesh, L. K., P. A. Theodorakis, and G. Chinnadurai.** 1991. Distinct *cis*-acting regions in U3 regulate *trans*-activation of the human spumaretrovirus long terminal repeat by the viral *bel 1* gene product. Nucleic Acids Res. **19:**3661–3666.

27. **Venkatesh, L. K., C. Yang, P. A. Theodorakis, and G. Chinnadurai.** 1993. Functional dissection of the human spumaretrovirus transactivator identifies distinct classes of dominant-negative mutants. J. Virol. **67:**161–169.

28. **Weaver, R. F., and C. Weissmann.** 1979. Mapping of RNA by a modification of the Berk-Sharp procedure: the 5′ termini of 15S β-globin mRNA precursor and mature 10S β-globin mRNA have identical map coordinates. Nucleic Acids Res. **7:**1175–1193.