The Human Foamy Virus Internal Promoter Is Required for Efficient Gene Expression and Infectivity

MARTIN LÖCHELT, SHUYUARN F. YU,* MAXINE L. LINIAL,* AND ROLF M. FLÜGEL[1]

Abteilung Retrovirale Genexpression, Forschungsschwerpunkt Angewandte Tumorvirologie, Deutsches Krebsforschungszentrum, Im Neuenheimer Feld 242, 69009 Heidelberg, Germany; and *Division of Basic Sciences, Fred Hutchinson Cancer Research Center, 1124 Columbia Street, Seattle, Washington 98104-2092

The human foamy or spumaretrovirus (HFV) is a complex retrovirus that codes for the three retroviral genes *gag, pol,* and *env* and the regulatory and accessory *bel* genes. A particular feature of HFV gene expression was recently described: not only does the HFV provirus contain the classical retroviral long terminal repeat promoter, a second functionally active promoter is present in the *env* gene upstream of the *bel* genes (M. Löchelt, W. Muranyi, and R. M. Flügel, 1993, *Proc. Natl. Acad. Sci. USA* 90, 7317–7321). Both, the HFV long terminal repeat promoter I and internal promoter II depend upon the HFV transcriptional transactivator Bel 1 for efficient gene expression. The internal promoter directs the synthesis of functionally active Bel 1 transactivator and Bet proteins that are expressed early after HFV infection. In this report, it is shown that mutation of the promoter II TATA box resulted in HFV proviral clones with a reduction in infectivity by a factor of approximately 100. Gene expression by promoter II TATA box mutant HFV proviruses was reduced. HFV proviruses with the mutated promoter II TATA box used cryptic start sites of transcription upstream of the original promoter II TATA box, resulting in an inefficient and less accurate transcriptional initiation. The reduced HFV structural gene expression by the mutated HFV proviruses was relieved by providing Bel 1 protein in *trans.* This demonstrates that HFV promoter II-directed Bel 1 expression is important for producing the high levels of Bel 1 that increases virus replication. © 1995 Academic Press, Inc.

INTRODUCTION

The human foamy or spumaretrovirus (HFV) is an exogenous and complex retrovirus isolated from the lymphoblastoid cells of a nasopharyngeal carcinoma patient (Achong *et al.,* 1971). The HFV genome (Fig. 1A) encodes regulatory and accessory *bel* genes that are located 3' of *env* (Flügel *et al.,* 1987; Maurer *et al.,* 1988; Muranyi and Flügel, 1991; Weissenberger and Flügel, 1994). The Bel 1 protein is a transcriptional transactivator for the long terminal repeat (LTR) promoter I and the internal promoter II and is absolutely required for viral replication and gene expression (Keller *et al.,* 1991; Lee *et al.,* 1993; Rethwilm *et al.,* 1991; Venkatesh *et al.,* 1991; Löchelt *et al.,* 1991, 1993a, 1994; Baunach *et al.,* 1993; Yu and Linial, 1993). A complex genomic organization, the existence of the *taf* transactivator, and the presence of an internal promoter corresponding to that described for HFV were recently reported for the closely related simian foamy virus type 1 (Mergia *et al.,* 1990, 1991; Mergia and Luciw, 1991; Mergia, 1994; Campbell *et al.,* 1994). Internal promoters are not restricted to foamy viruses, since an internal promoter for Tax transactivator expression was re-

cently reported for human T-cell lymphotropic virus type I (Nosaka *et al.,* 1993).

The existence of the internal promoter II adds another level of complexity to HFV gene expression. Both, singly and multiply spliced transcripts are derived from the LTR promoter I and the internal promoter II (Muranyi and Flügel, 1991; Löchelt *et al.,* 1993a, 1994). The internal cap site at HFV nucleotide (nt) 9196 is located upstream of the *bel* genes in the 3' end of the *env* gene (Löchelt *et al.,* 1993a) (Figs. 1A and 1B). The promoter II is strongly Bel 1 transactivator-dependent like the HFV LTR promoter I. It is likely that there is a mutual interdependence of both HFV promoters in the provirus, since the basal activity of the internal promoter is increased by the presence of the LTR promoter in *cis* in reporter gene constructs containing both promoters (Löchelt *et al.,* 1993b). In this study, the HFV promoter II TATA box was mutated and the effects on gene expression and infectivity were analyzed. The resulting proviral DNA clone exhibited a greatly reduced infectivity when compared to the wild-type provirus. Furthermore, the expression of the HFV genes was reduced. The reduction of HFV structural gene expression by promoter II TATA box mutant proviruses could be compensated by coexpression of Bel 1. The experiments presented demonstrate that the HFV promoter II plays an important role early in HFV gene expression and infectivity by providing Bel 1 transactiva-

[1] To whom reprint requests should be addressed. Fax: 49-6221-424852.

tor protein necessary for viral gene expression and infectivity.

MATERIALS AND METHODS

Plasmids used

The infectious HFV DNA clone pHSRV13 served as source of viral DNA (Löchelt et al., 1991); nucleotide numbering starts at the first base of the 5' LTR of the HFV provirus. Plasmids pCMVβgal, pCMVSEAP, and pCMVluc (Butz and Hoppe-Seyler, 1993; Löchelt et al., 1993a,b) direct the expression of β-galactosidase (β-gal), the secreted form of the human alkaline phosphatase (SEAP), and the firefly luciferase (luc) gene from the cytomegalovirus immediate−early (CMV-IE) promoter. Indicator gene plasmid pNNSEAP+ contains the internal promoter/enhancer (HFV nt 8971 to 9253) in the sense orientation upstream of the SEAP gene, respectively (Löchelt et al., 1993a). Bel 1 expression clones pBCbel, pBCbel1, and pbel1s and the parental pBC12CMV vector were described recently (Keller et al., 1991; Venkatesh et al., 1993; Löchelt et al., 1994).

Construction of recombinant clones

Molecular cloning was performed according to standard techniques (Sambrook et al., 1989). Indicator gene constructs containing a mutated TATA box were constructed by polymerase chain reaction (PCR)-directed mutagenesis as described (Butz and Hoppe-Seyler, 1993). Plasmid pNNSEAP+ was used as template for PCRs with primers 8971s (Löchelt et al., 1993a) plus ΔTATAa (5'-TGCTCCTTGATCGATTCT-CCAGAGGATGTG-3') plus SEAPan (Löchelt et al., 1993a) plus ΔTATAs (5'-GAGAATCGATCAAGGAGCAGATTGAAA-GAG-3'). The reaction products corresponding to HFV nt 8971 to the mutated TATA box and those starting at the mutated TATA box and extending to the SEAP gene were gel-purified and identical concentrations were combined for PCR amplification with primers 8971s and SEAPan. The reaction product of 321 nt was blunt-ended with T4 DNA polymerase and digested with SphI, which cleaved at the initiator codon of the SEAP gene. The gel-purified DNA fragment from HFV nt 8971 to the SEAP leader sequence was inserted between the EcoRV and the SphI sites of plasmid pPLSEAP (Keller et al., 1991), resulting in the pNNSEAP+ derivative pNNSEAPmTATA that contains a mutated TATA box (mutated from 5'-AAAGTATAAAA-3' to 5'-AATCGATCAAG-3', TATA box underlined, mutations are shown in bold face letters; Figs. 1B and 1D). Plasmid pNNSEAPmTATA was sequenced, demonstrating that the HFV insert corresponded to the original sequence except for the mutations given above. The mutated HFV DNA was also transferred into plasmid pBLCAT6 (Boshart et al., 1992) to yield clone pNNCATmTATA.

The mutated TATA box of plasmid pNNSEAPmTATA

was inserted into the infectious HFV DNA clone pHSRV13. Plasmid pNNSEAPmTATA was digested with MaeII (HFV nt 9004) and CelII (HFV nt 9194) and the 190-nt HFV DNA fragment containing the mutated TATA box was inserted into the corresponding restriction sites in pHSRV13. The identity of two independent recombinant clones, pHSRVmTATA7 and pHSRVmTATA19, was confirmed by restriction analysis and DNA sequencing.

A deletion mutant of pHSRV13 lacking the central domain of env was constructed by digesting pHSRV13 DNA with MroI and NdeI. The 13.4-kb DNA fragment lacking HFV env sequences from nt 6962 to 8970 was blunt-ended and religated. The resulting clone pHSRVΔMN lacked 2011 nt of the env gene but contained the intact internal promoter II (Fig. 1F).

Cell culture

COS7 and human embryonic lung (HEL) cells were grown and virus infections were done as described (Löchelt et al., 1991). The HFV indicator cell line FAB was cultured as described (Yu and Linial, 1993). Cell-associated and cell-free HFV particles were harvested and HFV titrations on FAB cells were performed as described except that FAB cells were grown on 24-well plates (Yu and Linial, 1993).

DNA transfection and expression assays

Transfections by electroporation of 10 μg DNA and reporter gene assays were performed and quantitated as described previously (Keller et al., 1991; Löchelt et al., 1991, 1993a). For luc assays, cells were harvested 42 hr after transfection in luc buffer (25 mM Tris/HCl, pH 7.8, 4 mM EGTA, 10% glycerol, and 1% Triton X-100) and insoluble material was sedimented at 12,000 g at 4° (Hoppe-Seyler et al., 1991). The supernatant was used for luc, β-gal, and radioimmunoprecipitation assays (RIPA) or precipitated with ethanol for immunoblotting (Western blotting).

Immunological techniques

The preparation of cell-associated antigen, protein blotting (Western blots), and sera used were described previously (Bartholomä et al., 1992; Löchelt et al., 1991, 1994; Mahnke et al., 1990). RIPAs were performed using the cleared luc lyates that were adjusted with the same volume of 10 mM Tris/HCl, pH 7.5, 300 mM NaCl, 1% Triton X-100, 1% deoxycholate, 2 mM EDTA, and 0.2% SDS to standard RIPA conditions (Sambrook et al., 1989). Antigen lysates (250 μl each) were preadsorbed with preimmune serum and reacted with 2−5 μl of rabbit hyperimmune sera overnight at 4°. Specifically bound antigen was separated on denaturing polyacrylamide gels in the presence of prestained (Gibco BRL) or ^{14}C-labeled (Amersham Buchler) marker proteins, incubated with the

intensifyer Amplify (Amersham Buchler), dried, and exposed to X-ray films (Kodak) or analyzed with a phosphoimager (Molecular Dynamics).

Quantitation of proteins detected by Western blotting and RIPA

Western blots were directly scanned using an Apple scanner with a Biosoft software program and the background value was subtracted. Similarly, radioactive proteins specifically precipitated by HFV-specific antisera were quantitated for each HFV-specific gene product with a phosphoimager (Molecular Dynamics) and the background activity of pUC18-transfected cells was subtracted. For Western blots and RIPAs, viral protein expression of pHSRV13-transfected cells was set to 100. The relative values for viral proteins expressed in pHSRVmTATA-transfected cells are given compared to those of pHSRV13-transfected cells.

RNA extraction and purification

Total RNA was harvested by cell lysis in guanidinium thiocyanate, sedimented through CsCl (Chirgwin et al., 1979), and digested with RNase-free DNase (Boehringer, Mannheim, FRG) or selected for poly(A)$^+$ mRNA as described recently (Löchelt et al., 1994). Primer extensions were done with 5'-^{32}P-labeled primer oligonucleotides 9307a and 9485a (Löchelt et al., 1993a, 1994).

RESULTS

Characterization of promoter II TATA box mutations in the HFV provirus

To study the biological function of the internal promoter, the TATA box of HFV promoter II was changed from 5'-TATAAA-3' to 5'-GATCAA-3' in parental plasmid pNNSEAP+ by PCR-based mutagenesis to obtain pNNSEAPmTATA (Figs. 1B and 1D). The mutated promoter II TATA box in plasmid pNNSEAPmTATA was inserted into the infectious HFV DNA clone pHSRV13 (Löchelt et al., 1991). Two independent clones, pHSRVmTATA7 and pHSRVmTATA19 were found to contain the expected mutations (Fig. 1D) that did not change the env reading frame (Figs. 1C and 1E). Promoter II TATA box mutants were used in transient transfection assays along with the wild-type proviral clone pHSRV13. Deletion mutant pHSRVΔMN was included in this study since it lacks most of the env gene but retains the intact promoter II and should therefore not be capable for virus propagation (Figs. 1A and 1F).

Comparative studies were performed using COS7 and FAB cells. COS7 cells of primate origin were used for the detection and characterization of the HFV internal promoter II and are efficiently transfectable by electroporation. They are capable of synthesizing infectious HFV

particles upon transfection with pHSRV13 (Löchelt et al., 1991, 1993a). FAB cells are baby hamster kidney cells that contain the stably integrated HFV LTR promoter I upstream of the β-gal gene. FAB cells are suitable for HFV infections, titrations and transfections as described recently (Yu and Linial, 1993).

Characterization of promoter II TATA box mutants in COS cells

Initially, COS7 cells were transfected with 10 μg pHSRV13, pHSRVmTATA7 and -19, or pHSRVΔMN DNA (Fig. 1) along with 1 μg of the β-gal expression plasmid pCMVβgal. Cellular extracts were harvested and analyzed for β-gal expression and normalized aliquots were subjected to Western blotting using different HFV-specific polyclonal antisera.

Representative protein blots (Fig. 2) developed with a Bel 1/Bet-specific antiserum that recognize the 36-kDa Bel 1 and the 56-kDa Bet proteins and a Bel 2/Bet-specific antiserum that recognize the 43-kDa Bel 2 and the Bet proteins showed that pHSRV13 expressed high amounts of Bet (solid arrow). The bands of 56 kDa (lanes 3 and 4) that represent two independent electroporations were strongly expressed, whereas Bel 1 and Bel 2 (open arrow) were detectable at lower levels. Bet is specifically recognized by both antisera since Bet consists of an amino-terminal Bel 1 and a central and carboxy-terminal Bel 2 domain. An expression pattern similar to that of wild-type was found for plasmid pHSRVΔMN (lane 2). However, the amount of Bet expressed by pHSRVmTATA7 and pHSRVmTATA19 (lanes 5 and 6) was close to the limit of detection, whereas neither Bel 1 nor Bel 2 were visible even after prolonged staining. An analogous reduced antigen expression was also observed using Bel 1- or gag-specific sera showing an approximately 10-fold reduction in antigen expression by plasmids pHSRVmTATA when compared to pHSRV13 and pHSRVΔMN (data not shown). This expression pattern was also observed when transfected COS7 cells were subjected to indirect immunofluorescence using the antisera mentioned above (data not shown). The differences in HFV gene expression were also detectable at the level of HFV mRNA by Northern blot analysis of poly(A)$^+$-selected RNA isolated from transfected COS7 cells (data not shown).

We next sought to find out whether the promoter II TATA box mutation affected virus infectivity. Cell-associated and cell-free infectivities were harvested 42 hr after transfection with plasmids pHSRV13, pHSRVmTATA7 and -19, and pHSRVΔMN. Infectivity was assayed by titration on FAB cells using the β-gal assay or by titration on HEL cells and subsequent counting of syncytia. Infectivity was substantially higher when preparations of cell-associated virus were used as recently described (Yu and Linial, 1993).
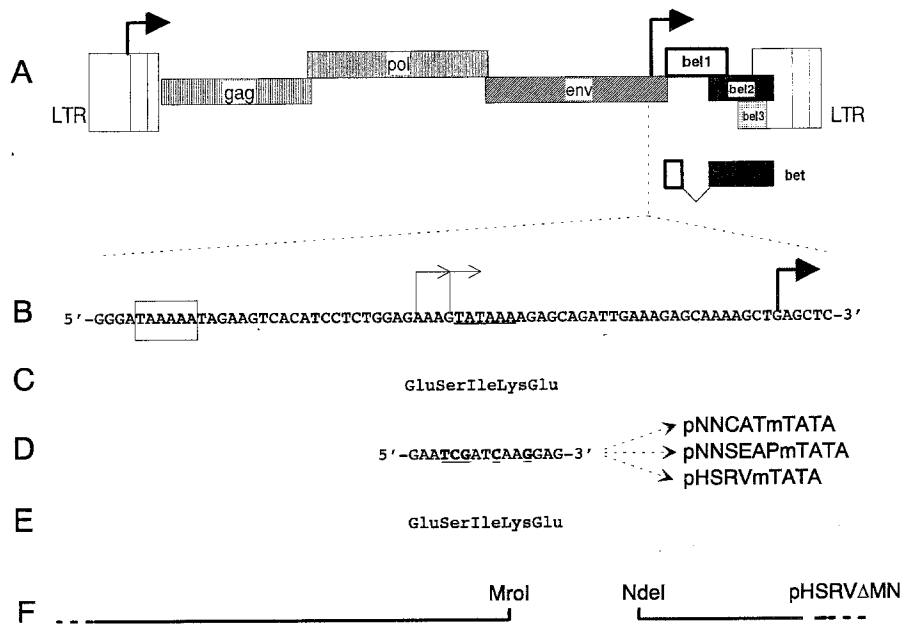
FIG. 1. Schematic diagram of the HFV DNA genome. In A, the bent arrows mark the two HFV promoters, I and II; the differently shaded boxes represent the HFV genes. The Bet protein is formed by splicing. Part of the env sequence is enlarged to emphasize the precise location of the HFV internal promoter (B). HFV sequence between nt 9130 and 9201 in the 5' to 3' sense orientation. The HFV promoter II TATA box is underlined; the internal cap site at nt 9196 is indicated by a bent arrow (in bold). The cryptic start sites of transcription are shown by bent arrows (thin lines); a TATA box homolog upstream of these initiation sites is boxed. In C, part of the deduced protein sequence of the HFV env reading frame is shown in the three-letter code. (D) Sequence of the mutated promoter II TATA box introduced into plasmids pNNCATmTATA, pNNSEAPmTATA, and pHSRVmTATA. Mutated base residues are underlined. In E, the deduced amino acid sequence of the env reading frame of promoter II TATA box mutants is given in the three-letter code. (F) The deletion in the HFV env between the MroI and NdeI sites (HFV nts 6962 to 8970) in plasmid pHSRVΔMN is indicated by the corresponding restriction sites and the gap within the env gene.

Plasmid pHSVR13 resulted in the synthesis of about 1 × 10⁴ blue cells/ml of cell-free HFV particles when titrated on FAB cells. Both plasmids pHSRVmTATA7 and pHSRVmTATA19 yielded an average of about 2 × 10² blue cells/ml. The env deletion mutant pHSRVΔMN did not direct the synthesis of infectious HFV particles as expected due to the large deletion in the env gene (Fig. 1F). The titration described above was repeated several times with similar results: the infectivity of plasmid pHSRVmTATA is 50- to 100-fold reduced in comparison to wild-type plasmid pHSRV13. The difference in titer was not dependent on the mode of titration (FAB versus HEL cells) or the source of infectivity (cell-free or cell-associated virus).

Characterization of promoter II TATA box mutants in FAB cells

For further characterization of HFV promoter II TATA box mutants, FAB cells were used. FAB cells are permissive for HFV infection and capable of forming HFV-specific syncytia. Furthermore, the transfection efficiency is comparable to COS7 cells and FAB cells allow an easy and efficient assay for HFV infectivity and expression of the Bel 1 transactivator. Since FAB cells express β-gal

activity in the presence of Bel 1, the CMV-IE promoter-directed luc construct pCMVluc was employed in order to standardize the transfection efficiency and extraction procedure.

FAB cells were transfected with plasmids pHSRV13, pHSRVmTATA7 and -19, and pHSRVΔMN in the presence of 1 μg of pCMVluc DNA. Cells were harvested 42 hr after transfection and aliquots of the cell culture supernatants were used for titrations. At the time of harvest, virtually all cells except those transfected with plasmid pHSRVΔMN showed the formation of syncytia. Both the size and the number of syncytia in cultures transfected with wild-type pHSRV13 DNA were substantially larger than after transfection with either of the pHSRVmTATA clones.

The titration of cell-free culture supernatants on either HEL or FAB cells showed again that the infectivity obtained with pHSRV13 DNA was about 50-fold higher than that derived from transfections with pHSRVmTATA7 or -19. The titers for a representative titration of cell-free supernatants on FAB cells were as follows: pHSRV13, 1.7 × 10⁴ blue cells/ml; pHSRVmTATA, 3.0 × 10² blue cells/ml; pHSRVΔMN, blue cells not detectable.

In parallel, the transfected cells were harvested, lysed, and assayed for luc activity. Luc activity varied only mini-
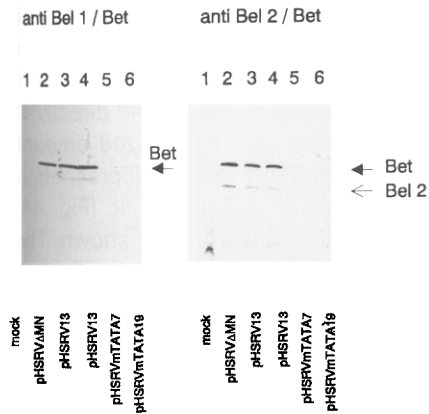
FIG. 2. Protein blot analysis of Bel 1/Bet- and Bel 2/Bet-specific proteins with different plasmids. Proteins expressed by plasmid pHSRVΔMN (lane 2), pHSRV13 (lanes 3 and 4, representing two independent transfections), pHSRVmTATA7 (lane 5), and pHSRVmTATA19 (lane 6). COS7 cells were harvested 42 hr after transfection and the amount of protein loaded was normalized to coexpressed β-gal activity. Proteins from nontransfected COS7 cells served as negative control (lane 1). The position of the 56-kDa Bet protein is given by a solid arrow, that of the 43-kDa Bel 2 protein by a thin arrow. The size of the proteins was calculated from the migration of the same protein markers as in Fig. 5.

mally between different transfections, indicating that the transfection efficiency for each of the plasmids used was comparable. We then analyzed to which degree each of the different HFV proviruses was capable of transactivating the integrated, Bel-1-dependent HFV-LTR-β-gal construct of the FAB cells; i.e., the analysis should reveal the level of Bel 1 expression by wild-type and mutant proviruses. To this end, regular aliquots of luc extracts were subjected to β-gal assays and the β-gal activity was normalized to coexpressed luc activity. The following relative values for each construct were found: pHSRV13, 1.0; pHSRVΔMN, 0.95; and pHSRVmTATA, 0.19. These experiments were repeated three times with similar results.

The pattern of gene expression was analyzed on the level of HFV-specific transcripts derived from each of the HFV provirus clones (data not shown). Plasmid pHSRV13 expressed high amounts of bel/bet transcripts and much lower amounts of the other HFV mRNAs. Promoter II TATA box mutants pHSRVmTATA7 and -19 showed reductions in expression of the viral mRNAs.

To study directly the utilization of the internal promoter II, transcripts directed by wild-type and mutant proviruses were analyzed by primer extension experiments (Fig. 3). RNA was harvested 42 hr after transfection of FAB cells with pHSRV13 (lanes 2 and 3 and 9 and 10, each pair representing two independent electroporations), pHSRVΔMN (lanes 1 and 8), and pHSRVmTATA19 (lanes 4 and 5 and 11 and 12, two independent transfections). HFV anti-sense oligonucleotide 9307a (lanes 1 to 6) is located at the 3′-end of exon 6/exon I and primer 9485a

(lanes 7 to 12) is located in the 5′ part of bel 1. Primer 9307a should allow the detection of most, if not all promoter II transcripts, whereas primer 9485a should result in cDNAs of spliced and unspliced promoter II bel 1*/bet* transcripts (internal promoter II-derived transcripts are marked by an asterisk, Löchelt et al., 1993a, 1994).
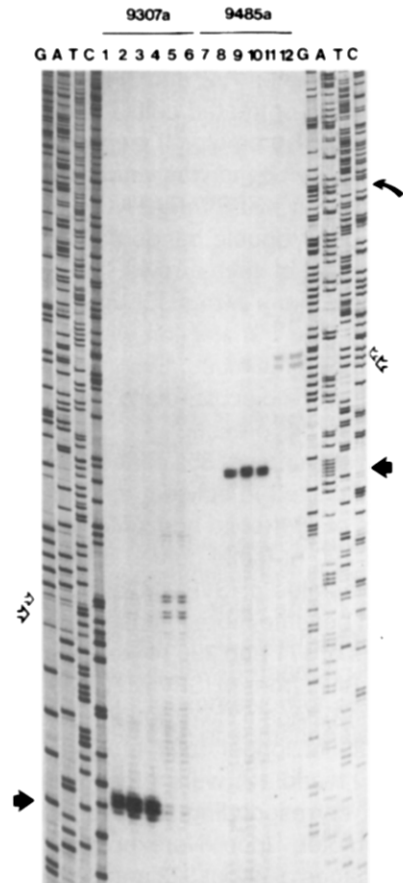

FIG. 3. Detection of spliced and unspliced bel 1/bet-specific transcripts from various plasmids 42 hr after transfection. Plasmids pHSRVΔMN (lanes 1 and 8), pHSRV13 (lanes 2, 3, 9, and 10; two independent transfections), and pHSRVmTATA19 (lanes 4, 5, 11, and 12; two independent transfections) were transfected into FAB cells. RNA from untreated FAB cells was used as control (lanes 6 and 7). Primer extensions were done with antisense primer 9307a located at the 3′-end of exon 6, and primer 9485a located in the 5′ end of the bel 1 gene, 59 nt downstream of the major bel 1/bet splice acceptor. The reaction products of the primer extensions were analyzed in parallel to a sequencing reaction of a yeast gene (left hand lanes G to C, indicating the ddNTP used) and of pHSRV13 DNA with primer 9485a (right hand lanes G to C). The open arrows in the left margin point to the 5′ end of cryptically initiated transcripts detected with primer 9307a (lanes 4 and 5). The solid arrow on the left points to cDNAs 112 nt in length (lanes 1, 2, and 3), corresponding to promoter II transcripts. The curved and short solid arrows in the right margin mark unspliced (only faintly visible) and spliced HFV promoter II bel 1*/bet* transcripts detected with primer 9485a (lanes 8, 9, and 10). The open arrows in the right margin indicate the position of the spliced cryptic promoter II transcripts for bel 1/bet (lanes 11 and 12). The location of the primers were as described previously (Löchelt et al., 1993a, 1994).

RNA from untreated FAB cells was used as negative control (lanes 6 and 7). The reaction products of the primer extensions were loaded on a sequencing gel next to dideoxy sequencing reactions (lanes G to C) of pHSRV13 DNA primed with oligonucleotide 9485a (right-hand side) or another sequencing reaction (left-hand side).

Reactions with primer 9307a yielded predominant extension products of about 112 nt in length with RNA from plasmids pHSRVΔMN and pHSRV13 (Fig. 3, left-hand side, solid arrow in lanes 1, 2, and 3) that was not detectable with RNA from untreated cells (lane 6). This cDNA corresponds to HFV promoter II transcripts (Löchelt et al., 1993a). The 112-nt band was virtually absent in reactions with RNA from pHSRVmTATA19 (lanes 4 and 5). However, two minor double bands of 143/144 and 146/147 nt in length (pair of open arrows, lanes 4 and 5) were present in transfections with pHSRVmTATA19 that also appeared faintly after transfection with nonmutated promoter II TATA box proviruses. Even upon longer exposure, bands of lower molecular mass specific for a particular plasmid were not present.

Extensions with primer 9485a (lanes 7 to 12, right-hand side of Fig. 3) resulted in spliced and unspliced bel 1*/bet* transcripts of 171 (solid horizontal arrow in lanes 8, 9, and 10) and 290 nt (solid curved arrow in lanes 8, 9, and 10) with plasmids pHSVRΔMN and pHSRV13. The bands of 290 nt became more intense upon longer exposure. The bands of 171 and 290 nt were almost absent in reactions with RNA from pHSRVmTATA19 transfections (lanes 11 and 12). Again, two double bands that were 31/32 and 34/35 nt longer (pair of open arrows, right margin, lanes 11 and 12) were present in much lower concentrations corresponding to spliced transcripts of the cryptic promoter. Upon overexposure of the autoradiogram, faint bands of about 322 and 325 nt appeared in lanes 11 and 12 that most probably represent unspliced transcripts derived from the cryptic promoter. It is noteworthy that bel 1/bet transcripts from the 5'-LTR promoter (e.g., the 110-nt-long singly spliced LTR-derived bel 1 transcript) were clearly not detectable.

Close examination of DNA sequences upstream of the promoter II cap site at HFV position 9196 (Fig. 1B, bold arrow) revealed that a TATA box homology exists at HFV nt 9134 (boxed sequence in Fig. 1B). The transcriptional start sites directed by this cryptic TATA box are marked by thin arrows that map to a HFV DNA sequence bordered by two G base residues at HFV nt 9160 and nt 9164 (Fig. 1B).

The expression of HFV proteins in transfected FAB cells was subsequently analyzed by RIPA and immunoblotting with different HFV specific antisera. In all cases, the amount of antigen used was normalized to coexpressed luc activity.

To compare directly the kinetics of antigen expression,

cells transfected with the different DNA constructs were split into halves and labeled with [^{35}S]methionine and [^{35}S]cysteine for RIPA from 24 to 29 hr and from 29 to 34 hr after transfection. Cells were lysed directly at the end of the labeling period and normalized amounts of cell extracts were subjected to RIPA. Representative RIPAs performed with the Bel 1/Bet-specific (Fig. 4A) and an env-specific (Fig. 4B) antiserum are shown. The amount of Bet protein synthesized by pHSRV13 (lanes 1, 9, and 4; two transfections were performed in parallel) was substantially higher than that expressed by clones pHSRVmTATA7 and 19 (lanes 2, 10, and 5, respectively). The quantitation of specifically immunoprecipitated HFV proteins by means of a phospoimager revealed that pHSRVmTATA clones expressed 14 and 19% of Bet protein synthesis of wild-type pHSRV13 (Table 1) upon labeling from 24 to 29 and from 29 to 34 hr after transfection, respectively. The relative amount of Bet expression from clones pHSRVmTATA was lower for the sample labeled from 24 to 29 hr after transfection than for that labeled later. This holds true also for the env-specific proteins: the relative expression of the env precursor gp130 by pHSRVmTATA increased from 35 to 42%; that of TM even increased from 9 to 17%. Similar results were also obtained with Bel 1- and gag-specific antisera (Table 1). When FAB cells were labeled for 15 hr from 27 to 42 hr after transfection, HFV antigen expression was still higher in pHSRV13-transfected cells. However, HFV gene expression for pHSRVmTATA-transfected cells was in general significantly increased (Table 1), indicating that the mutation of the promoter II TATA box in clones of pHSRVmTATA negatively affected gene expression predominantly early after transfection. Furthermore, the amount of HFV particles released from such cultures during this labeling period was almost identical or maximally twice in pHSRV13-transfected culture (Table 1).

To determine the net synthesis of HFV-specific proteins, immunoblots were performed with antigen preparations harvested 42 hr after transfection (Fig. 5). Proteins from luc extracts of transfected cells were analyzed for the expression of Bel 1/Bet-specific (Fig. 5A) and gag-specific antigens (Fig. 5B). The reduction in Bel 1 expression (open arrow in A) by clone pHSRVmTATA7 (lane 2) was concomitant with a reduced bet gene expression (solid arrow in A) in comparison to pHSRVΔMN and pHSRV13 (lanes 1 and 3). The reduction of Bel 1 expression was about 12-fold, and that of Bet 8-fold when comparing the intensities of bands obtained from transfections with pHSRV13 and pHSRVmTATA7. The expression of the HFV gag gene precursor molecules of 74 and 78 kDa (Fig. 5B) was reduced about 10-fold in the promoter II TATA box mutant (compare lane 2 versus lane 3). The reduction in gag and bel genes was also detectable for protein samples derived from the high-speed pellet fraction (data not shown).
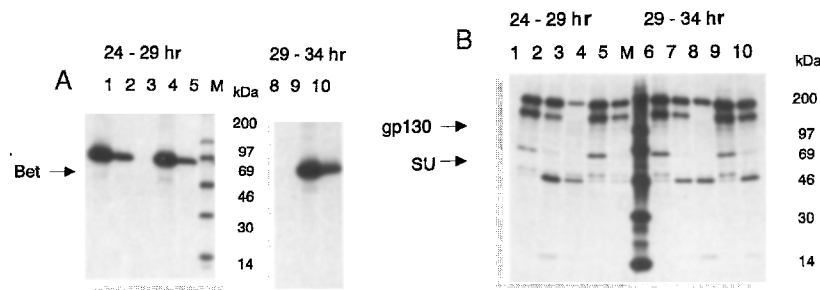
FIG. 4. RIPA experiments performed with Bel 1/Bet- (A) and *env* SU- and TM-specific sera (B). FAB cells were transfected with pHSRV13 (lanes 1 and 6 and 4 and 9, each pair representing one independent electroporation), pHSRVmTATA7 (lanes 2 and 10), pHSRVmTATA19 (lanes 5 and 7), and pUC18 (lanes 3 and 8). The cells were split directly after transfection into halves and labeled with [³⁵S]methionine and [³⁵S]cysteine from 24 to 29 hr or from 29 to 34 hr after transfection as indicated above the lanes. Normalized amounts of cellular extracts were used for specific precipitation of HFV proteins. The positions of the Bet protein in A and those of the *env* gp130 precursor and the *env* SU proteins in B are given by arrows in the left margin. In lanes M, a ¹⁴C-labeled protein marker was separated; the molecular masses of the proteins are given in kDa.

The results presented here were obtained with COS7 and FAB. As gene expression and infectivity of mutant pHSRVmTATA were also severely impaired in HEL cells (data not shown), the dependence on a functional promoter II is not restricted to a certain cell type although it showed some minor differences between the cells used.

Trans-complementation of HFV promoter II TATA box mutant provirus with *bel 1*

We next analyzed whether the reduced expression of the Bel 1 transactivator is responsible for the decreased HFV antigen expression by HFV proviruses containing the mutated TATA box. We transfected plasmids pUC18,

pHSRV13, and pHSRVmTATA19 in the presence or absence of a Bel 1 expression clone into FAB cells. Cells were labeled with [³⁵S]cysteine and methionine 24 to 32 hr after transfection, lysed, and processed for RIPA assays. The amounts of cell lysates used for RIPAs were normalized to coexpressed luc activity. RIPAs were performed with Bel 1/Bet-, integrase (Int)-, and Env-specific antisera.

The RIPA analysis with the Bel1/Bet-specific antiserum showed that in the absence of Bel 1 the concentration of the Bet protein (Fig. 6, solid arrow) synthesized by plasmid pHSRVmTATA19 (Fig. 6, lane 5) was about 5% of that expressed by pHSRV13 (lane 3) under the same conditions (Table 2). Coexpression of plasmid pBCbel1 that directs the expression of authentic Bel 1 and a trun-

TABLE 1

RELATIVE AMOUNTS OF HFV-SPECIFIC PROTEINS OBTAINED AFTER TRANSFECTION OF FAB CELLS WITH PROMOTER II TATA BOX MUTANTS COMPARED TO pHSRV13 WILD-TYPE TRANSFECTED FAB CELLS[a]

	RIPA, labeling period after transfection[b]		
	24–29 hr (%)	29–34 hr (%)	27–42 hr (%)
Bel 1	8.3	7.1	50.0
Bet	14.5	18.9	71.4
p74/78*gag*	15.6	22.2	33.3
gp130*env*	34.5	41.7	83.3
gp70/p65*env*	9.3	16.9	27.0
gp41*env*	nd[c]	nd	8.5
virion gp70*env*	nd	nd	76.9
virion gp41*env*	nd	nd	76.9
virion p33*gag*	nd	nd	50.0

[a] Data were quantitated as described under Materials and Methods and represent the relative expression of promoter II TATA box mutant pHSRVmTATA; the expression of wild-type pHSRV13 DNA was set to 100%.

[b] Data represent mean values of two transfections of pHSRV13 and pHSRVmTATA DNA done in parallel.
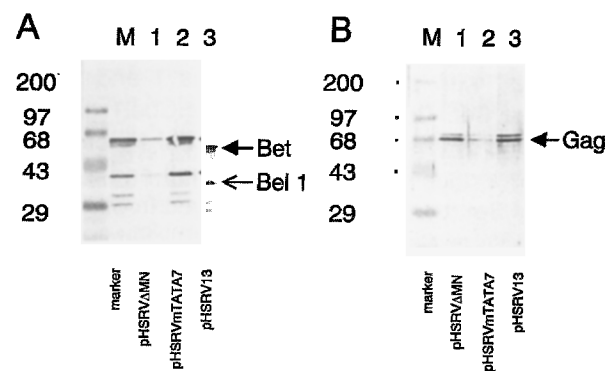
[c] nd, not determined.

FIG. 5. Western blot analysis of FAB cell extracts harvested 42 hr after transfection with pHSRVΔMN (lanes 1), pHSRVmTATA7 (lanes 2), and pHSRV13 (lanes 3). The amount of protein analysed was normalized to coexpressed luc activity. The transferred proteins were reacted with a Bel 1/Bet-specific (A) and a *gag*-specific polyclonal antiserum (B). In A, the solid arrow points to the 56-kDa Bet, the thin arrow to the 36-kDa Bel 1 protein. The arrow in B marks the position of the 74/78-kDa *gag*-precursors. The molecular masses of prestained proteins separated in lanes M are given in the left margin, consisting of the following proteins (from top): myosin (heavy chain), 200 kDa; phosphorylase b, 97.4 kDa; bovine serum albumin, 68 kDa; ovalbumin, 43 kDa; carboanhydrase, 29 kDa; β-lactoglobulin, 18.4 kDa; lysozyme, 14.3 kDa.
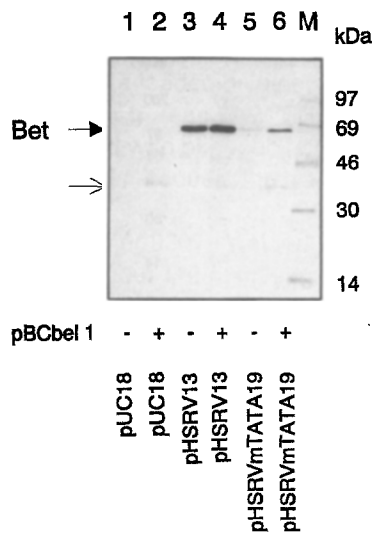
FIG. 6. Detection of *bel 1/bet*-specific proteins expressed in FAB cells transfected with the following plasmids: pUC18 plus pBC12CMV (lane 1), pUC18 plus pBCbel1 (lane 2), pHSRV13 plus pBC12CMV (lane 3), pHSRV13 plus pBCbel1 (lane 4), pHSRVmTATA19 with pBC12CMV (lane 5), and pHSRVmTATA19 plus pBCbel1 (lane 6). Cells were labeled with [^{35}S]cysteine and methionine 24 to 32 hr after transfection, lysed, and normalized aliquots of the cell extracts were subjected to RIPAs with a Bel 1/Bet-specific antiserum. The solid arrow points to the 56-kDa Bet, the thin arrow to the truncated form of the Bet protein expressed from cotransfected plasmid pBCbel1. [^{14}C]-labeled protein marker were separated in parallel (lane M); their molecular masses are given in kDa.

cated version of Bet increased the expression of the 56-kDa Bet protein by plasmid pHSRV13 (lanes 4) only slightly in comparison to cotransfection with the vector backbone pBC12CMV (lane 3) and Table 2. However, coexpression of Bel 1 increased Bet expression by plasmid pHSRVmTATA19 (lane 6) about sevenfold. Cells transfected with pUC18 DNA (lanes 1 and 2) did not synthesize Bet. Cotransfection of pBCbel1 (lane 2) resulted in the synthesis of the 36-kDa Bel 1 protein (visible only upon longer exposure of the gel) and a truncated version of Bet (thin arrow) as observed in other cotransfections with pBCbel1 (lanes 4 and 6). Similar observations were also obtained with Env- and Int-specific antisera (Table 2). Remarkably, cotransfection of *bel 1* along with either wild-type or promoter II mutant proviruses resulted in a significantly elevated gene expression of LTR-dependent *pol* and *env* gene products, whereas the increase in *bet* gene expression was not as much pronounced.

In summary, the exogenously added Bel 1 transactivator shifted *bet, pol, env,* and *gag* (data not shown) gene expression by promoter II TATA box mutants approximately to wild-type levels and even increased the expression of structural genes by pHSRV13 wild-type provirus. This indicates that the decreased protein expression by plasmids pHSRVmTATA is predominantly due to *bel 1*

gene expression reduced by the mutated internal promoter II.

DISCUSSION

The data presented here show that the internal HFV promoter II that directs the expression of functional Bel 1 and Bet proteins plays an important role during HFV infection (Löchelt *et al.,* 1993a, 1994). Proviruses with a mutated promoter II TATA box expressed reduced amounts of HFV-specific proteins and transcripts and resulted in HFV progeny virus with a reduced infectivity. The reduced expression of HFV structural and accessory genes by plasmid pHSRVmTATA was restored by providing Bel 1 in *trans.* Cryptic start sites of transcription located 32 to 35 nt 5' of cap site II were utilized by promoter II TATA box mutants instead of the original one at HFV nt 9196.

The mutagenesis of the promoter II TATA box virtually abolished the utilization of the original promoter II cap site at HFV nt position 9196 for gene expression. However, since *bel 1* /bet** mRNAs transcribed from cryptic start sites of transcription were detectable and since reporter gene plasmids pNNSEAPmTATA and pNNCAT-mTATA that carry the mutated promoter II TATA box showed a two- to threefold reduced basal and Bel 1-transactivated activity (data not shown), plasmids pHSRVmTATA can be considered only as promoter II "leaky" mutants. Nevertheless, the results obtained with these clones support the assumed critical role of the HFV promoter II for gene expression and infectivity (Löchelt *et al.,* 1993a,b, 1994; Mergia, 1994; Campbell *et al.,* 1994). The increased utilization of cryptic start sites of transcription in promoter II TATA box mutants resulted in *bel 1* / bet** transcripts with a slightly extended mRNA leader sequence. Utilization of the 5'LTR promoter I to generate *bel* transcripts was not observed by means of primer extension experiments even when the promoter II was mutated (Fig. 3). Therefore, wild-type and mutant HFV proviruses directed only miniscule amounts of *bel 1* tran-

TABLE 2

RELATIVE AMOUNTS OF HFV-SPECIFIC PROTEINS OBTAINED AFTER TRANSFECTION OF FAB CELLS WITH PROMOTER II TATA BOX MUTANTS COMPARED TO pHSRV13 WILD-TYPE TRANSFECTED FAB CELLS IN THE ABSENCE OR PRESENCE OF COTRANSFECTED PLASMID pBCbel1[a]

		Bet (%)	Pol p120 (%)	Env gp130 (%)
pHSRV13	plus pBC12CMV	100.0	100.0	100.0
pHSRV13	plus pBCbel1	137.5	268.1	297.6
pHSRVmTATA19	plus pBC12CMV	4.8	4.4	16.8
pHSRVmTATA19	plus pBCbel1	31.4	183.1	146.4

[a] Data were quantitated as described under Materials and Methods; the expression of wild-type pHSRV13 DNA plus pBC12CMV was set to 100%.

scripts from the LTR promoter I, the majority of *bel 1* mRNAs started at the promoter II or at its cryptic start sites (Löchelt *et al.,* 1994).

The limiting step during gene expression of promoter II TATA box mutants seems to be their reduced Bel 1 expression, since cotransfection of *bel 1* and pHSRVmTATA DNA increased *env, pol,* and *gag* (data not shown) gene expression comparable to that of the wild-type HFV provirus. The fact that Bet expression was not completely restored by transcomplementation with *bel 1* supports the view that the *bel* genes are predominantly expressed from the internal promoter II. Since Bel 1 was shown to be absolutely required for HFV gene expression and infectivity (Löchelt *et al.,* 1991; Yu and Linial, 1993; Baunach *et al.,* 1993; Lee *et al.,* 1994) and since HFV *bel 1* /bet** transcripts were detected early after infection (Löchelt *et al.,* 1994), it is likely that the internal promoter II is the early promoter for expressing Bel 1 after provirus synthesis. This conclusion is supported by the RIPA experiments where gene expression by plasmids pHSRVmTATA was shown to be more strongly reduced early after transfection. This indicates that the mutation of the promoter II in plasmids pHSRVmTATA resulted in concentrations of Bel 1 early after infection not sufficient for complete transactivation (Löchelt *et al.,* 1994). This may lead to abortive transfections in HEL cells. Alternatively, the threshold levels of Bel 1 needed for efficient transactivation were reached much later when compared to cells transfected with the infectious pHSRV13 DNA (Löchelt *et al.,* 1994). Consequently, the burst of viral gene expression was delayed in pHSRVmTATA transfected FAB cells and was not as high as that found in wild-type transfected cells. The initial Bel 1-independent transcription of *bel 1* mRNAs by the internal promoter may be accomplished by its elevated basal activity when the LTR promoter I is present in *cis* (Löchelt *et al.,* 1993b).

HFV DNA sequences upstream of nt −226 relative to the promoter II cap site seem not to be important for full activity of the internal promoter, since mutant pHSRVΔMN was virtually indistinguishable in *bel, gag,* and *pol* gene expression compared to wild-type pHSRV13. As expected, this clone was incapable of syncytia formation and not infectious as the *env* gene was deleted. An analogous lack of structural gene expression or defects in packaging or virus maturation by pHSRVmTATA DNA are unlikely to cause its strongly reduced infectivity, since the amounts and composition of viral proteins of HFV particles released late after pHSRVmTATA transfection were similar to that obtained from wild-type transfected cells (Table 1). We therefore assume that the low infectivity of pHSRVmTATA provirus is primarily related to a reduced capacity to initiate gene expression by the mutated promoter II directly after infection.

ACKNOWLEDGMENTS

REFERENCES

ACHONG, B. G., MANSELL, P. W. A., EPSTEIN, M. A., and CLIFFORD, P. (1971). An unusual virus in cultures from a human nasopharyngeal carcinoma. *J. Natl. Cancer Inst.* **46**, 299–307.

BARTHOLOMÄ, A., MURANYI, W., and FLÜGEL, R. M. (1992). Bacterial expression of the capsid antigen domain and identification of native *gag* proteins in spumavirus-infected cells. *Virus Res.* **23**, 27–38.

BAUNACH, G., MAURER, B., HAHN, H., KRANZ, M., and RETHWILM, A. (1993). Functional analysis of human foamy virus accessory reading frames. *J. Virol.* **67**, 5411–5418.

BOSHART, M., KLÜPPEL, M., SCHMIDT, A., SCHÜTZ, G., and LUCKOW, B. (1992). Reporter constructs with low background activity utilizing the *cat* gene. *Gene* **110**, 129–130.

BUTZ, K., and HOPPE-SEYLER, F. (1993). Transcriptional control of human papillomavirus (HPV) oncogene expression: Composition of the HPV type 18 upstream regulatory region. *J. Virol.* **67**, 6476–6486.

CAMPBELL, M., RENSHAW-GEGG, L., RENNE, R., and LUCIW, P. A. (1994). Characterization of the internal promoter of simian foamy viruses. *J. Virol.* **68**, 4811–4820.

CHIRGWIN, J. M., PRZYBYLA, A. E., MACDONALD, R. J., and RUTTER, W. J. (1979). Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. *Biochemistry* **18**, 5294–5299.

CULLEN, B. R. (1991). Human immunodeficiency virus as a prototypic complex retrovirus. *J. Virol.* **65**, 1053–1056.

FLÜGEL, R. M. (1992). The molecular biology of the human spumavirus. *In* "Human Retroviruses Frontiers in Molecular Biology" (B. Cullen, Ed.), pp. 193–214. Oxford Univ. Press, Oxford.

FLÜGEL, R. M., RETHWILM, A., MAURER, B., and DARAI, G. (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.

HOPPE-SEYLER, F., BUTZ, K., RITTMÜLLER, C., and VON KNEBEL DOEBERITZ, M. (1991). A rapid microscale procedure for the simultaneous preparation of cytoplasmic RNA, nuclear DNA binding proteins and enzymatically active luciferase extracts. *Nucleic Acids Res.* **19**, 5080.

KELLER, A., PARTIN, K. M., LÖCHELT, M., BANNERT, H., FLÜGEL, R. M., and CULLEN, B. R. (1991). Characterization of the transcriptional transactivator of human foamy retrovirus. *J. Virol.* **65**, 2589–2594.

LEE, K. J., LEE, A. H., and SUNG, Y. C. (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.

LEE, A. H., LEE, H. Y., and SUNG, Y. C. (1994). The gene expression of human foamy virus does not require a post-transcriptional transactivator. *Virology* **204**, 409–413.

LÖCHELT, M., FLÜGEL, R. M., and ABOUD, M. (1994). The human foamy virus internal promoter directs the expression of the functional Bel 1 transactivator and Bet protein early after infection. *J. Virol.* **68**, 638–645.

LÖCHELT, M., MURANYI, W., and FLÜGEL, R. M. (1993a). Human foamy virus genome possesses an internal, Bel 1-dependent and functional promoter. *Proc. Natl. Acad. Sci. USA* **90**, 7317–7321.

LÖCHELT, M., ABOUD, M., and FLÜGEL, R. M. (1993b). 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.

LÖCHELT, M., ZENTGRAF, H., and FLÜGEL, R. M. (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.

MAHNKE, C., LÖCHELT, M., BANNERT, B., and FLÜGEL, R. M. (1990). Specific enzyme-linked immunosorbent assay for the detection of antibodies to the human spumavirus. *J. Virol. Methods* **29**, 13–22.

MAURER, B., BANNERT, H., D'ARAI, G., and FLÜGEL, R. M. (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.

MERGIA, A. (1994). Simian foamy virus type 1 contains a second promoter located at the 3′ end of the *env* gene. *Virology* **199**, 219–222.

MERGIA, A., and LUCIW, P. (1991). Replication and regulation of primate foamy viruses. *Virology* **184**, 475–482.

MERGIA, A., SHAW, K. E. S., PRATT-LOWE, E., BARRY, P. A., and LUCIW, P. (1990). Simian foamy virus type 1 is a retrovirus which encodes a transcriptional transactivator. *J. Virol.* **64**, 3598–3604.

MERGIA, A., SHAW, K. E. S., PRATT-LOWE, E., BARRY, P. A., and LUCIW, P. (1991). Identification of the simian foamy virus transcriptional transactivator gene (*taf*). *J. Virol.* **65**, 2903–2909.

MURANYI, W., and FLÜGEL, R. M. (1991). Analysis of splicing patterns of human spumaretrovirus by polymerase chain reaction reveals complex RNA structures. *J. Virol.* **65**, 727–735.

NOSAKA, T., ARIUMI, Y., SAKURAI, M., TAKEUCHI, R., and HATANAKA, M.

(1993). Novel internal promoter/enhancer of HTLV-I for Tax expression. *Nucleic Acids Res.* **21**, 5124–5129.

RETHWILM, A., ERLWEIN, O., BAUNACH, G., MAURER, B., and TER MEULEN, V. (1991). The transcriptional transactivator of human foamy virus maps to the bel 1 genomic region. *Proc. Natl. Acad. Sci. USA* **88**, 941–945.

SAMBROOK, J., FRITSCH, E. F., and MANIATIS, T. (1989). "Molecular Cloning: A Laboratory Manual," 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

VENKATESH, L. K., THEODORAKIS, P. A., and CHINNADURAI, G. (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.

VENKATESH, L. K., YANG, C., THEODORAKIS, P. A., and CHINNADURAI, G. (1993). Functional dissection of the human spumaretrovirus transactivator identifies distinct classes of dominant-negative mutants. *J. Virol.* **67**, 161–169.

WEISSENBERGER, J., and FLÜGEL, R. M. (1994). Identification and characterization of the Bel 3 protein of the human foamy virus. *AIDS Res. Hum. Retroviruses* **10**, 595–600.

YU, S. F., and LINIAL, M. L. (1993). Analysis of the role of the *bel* and *bet* open reading frames of human foamy virus by using a new quantitative assay. *J. Virol.* **67**, 6618–6624.