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Increase in the basal transcriptional activity of the human foamy virus internal promoter by the homologous long terminal repeat promoter in *cis*

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ABSTRACT

The human foamy or spumaretrovirus HFV is a complex and exogenous retrovirus that encodes several *bel* genes besides the three classical retroviral genes *gag*, *pol*, and *env*. HFV was recently reported to contain two functionally active promoters that are both strongly trans-activated by the HFV trans-activator protein Bel 1. The occurrence of a second internal cap site underscores the complexity of the HFV genome. We have analysed whether there is interference between the HFV long terminal repeat promoter and the internal promoter located in the 3′ end of *env* upstream of the *bel* genes. Recombinant clones were constructed that carry two different indicator genes, one under the control of the U3 promoter, the other under the control of the internal promoter. The portion of the basal transcriptional activity of the internal promoter that is not trans-activated by Bel 1 was increased two- to threefold in the presence of the long terminal repeat promoter. The rate of trans-activation by Bel 1 of both HFV promoters was not altered in these constructs.

INTRODUCTION

The human foamy or spumaretrovirus (HFVor HSRV) is an exogeneous retrovirus that was isolated from the lymphoblastoid cells of a nasopharyngeal carcinoma patient (1). The HFV genome was determined by nucleotide sequencing and contains accessory *bel* genes which are located 3′ of *env* (2, 3). The complexity of the HFV genome is reflected in the complex splicing pattern as illustrated by the occurrence of singly and multiply spliced mRNA species of the *bel* genes (4). Recently, a second HFV promoter was identified at the 3′ end of *env* directly upstream of the *bel* genes (5). This internal promoter and the promoter in the 5′ long terminal repeat (LTR) are both strongly dependent on the presence of the Bel 1 trans-activator (5, 6, 7, 8). Bel 1 was shown to be absolutely required for infectivity and viral gene expression (9). The presence of an internally located and functionally active promoter in the HFV genome indicates that HFV gene expression is of comparable if different complexity to the other complex human retroviruses, the human T-cell lymphotropic viruses (HTLV-I and -II) and the human immunodeficiency viruses (HIV-1 and −2) (10, 11).

The pathogenic properties of HFV remained obscure until recently (12). Construction and analysis of HFV-transgenic mice revealed that HFV genes induced specific brain lesions (13). Epidemiological studies showed that the prevalence of HFV infection in human patients is relatively small, but that antibodies against HFV occur more often in the sera of patients from African countries (14, 15). It is thus potentially quite interesting that the HFV Bel 1 trans-activator also trans-activates the heterologous HIV-1 LTR in addition to activating the homologous HFV promoters (6, 16).

In this report, defined indicator gene constructs were used to determine whether there is *cis*-interference between both foamy viral promoters when they are contained within the same DNA construct. The results indicate that the internal HFV promoter is not only active in the presence of the LTR promoter, but its basal activity in the absence of Bel 1 is even increased. The LTR promoter is not directly influenced by its homologous intragenic counterpart.

MATERIALS AND METHODS

Plasmids

Plasmid p778-10 SEAP contains the gene for the secreted form of the human alkaline phosphatase (SEAP) under the transcriptional control of the HFV U3 region spanning from nucleotides −778 to +10 relative to the LTR start site of transcription (3, 6). In plasmid pNNCAT+, the 283 bp NdeI to NheI HFV *env* DNA fragment containing the internal promoter/enhancer is located 5′ of the chloramphenicol acetyl transferase (CAT) gene (5). The eukaryotic expression clone pBCbel1 harbouring the complete HFV bel region 3′ of the cytomegalovirus (CMV) promoter in plasmid pBC12/CMV was

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recently described (9) and is herein designated pBCbel. Plasmid pβGal contains the bacterial β-galactosidase (β-gal) gene downstream of a CMV promoter and was kindly provided by A.Alonso. Nucleotide numbering is as in the infectious clone pHSRV13 (9).

Construction of recombinant clones

Recombinant DNA techniques were performed according to Sambrook *et al.* (17). Indicator gene constructs that contain the SEAP gene under the control of the HFV U3 region and the CAT gene under the control of the HFV NdeI−NheI DNA were constructed by digesting plasmid p778-10SEAP with BglII and subsequent blunt ending. The blunt-ended XbaI to SmaI DNA fragment from plasmid pNNCAT+ that contains the HFV NdeI to NheI DNA fragment fused in sense 5′ to the CAT gene with the SV40 termination signals was inserted into this site (Fig. 2). The recombinant clone that contains the 3′ end of the CAT gene in direct vicinity to the 5′ end of the HFV U3 was designated pCATSEAPu; the clone that carries the HFV NdeI site closest to the U3 region as pCATSEAPb. The symbols u and b indicate that the transcription of both genes is unidirectional (u) or bidirectional (b).

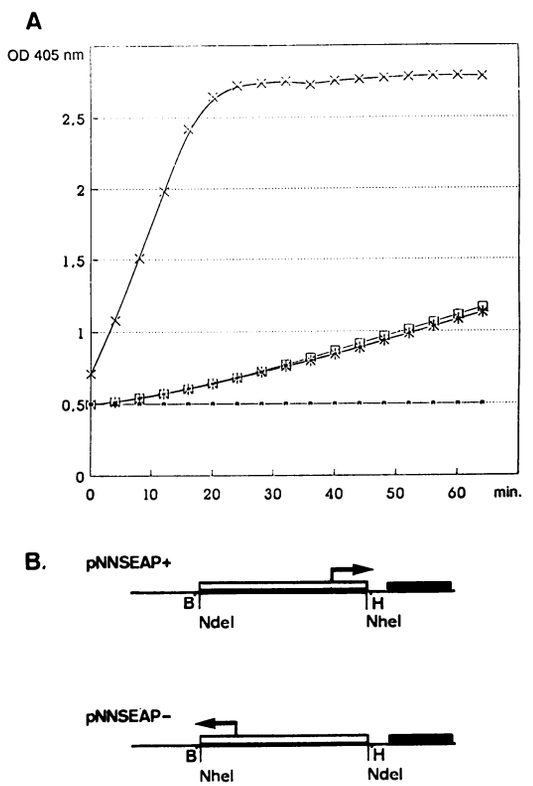

Figure 1. (A). Kinetics of SEAP assays of cells transfected with the following constructs: squares, pNNSEAP+; open boxes, pNNSEAP+ plus pBCbel1; asterisks, pNNSEAP+ plus pBCbel and crosses, a CMV promoter SEAP construct. The change of optical density at 405 nm (OD 405 nm) is plotted versus the incubation time. Supernatants of COS cells were withdrawn 70 h after transfection. (B). the orientation of the HSRV NdeI−NheI insert (open box, HSRV nucleotides 8971 to 9253) in pPLSEAP relative to the 5′-end of the SEAP gene (black box) in pNNSEAP+ (sense orientation) and pNNSEAP− (antisense orientation) is shown. The flanking BglII (B) and HindIII (H) sites of the polylinker are indicated, arrows mark the start and direction of transcription.

The eukaryotic expression clone that encodes exclusively Bel 1 was constructed from plasmid pBCbel that expressed the Bel 1 and, in addition, the Bet protein (9). To truncate Bet, all HSRV sequences downstream from *bel 1* were deleted by digestion of pBCbel with AccI (nucleotide position 10370 in the HFV DNA sequence), and blunt-ended with the Klenov enzyme followed by digestion with PvuI that cuts the vector backbone 3′ of the poly (A) site. The excised AccI to PvuI fragment was substituted by the SmaI to PvuI fragment from pBC12CMV harbouring the poly (A) site. This plasmid was designated pBCbel1.

Cell culture and transfection

The cultivation of COS7 cells and DNA transfections of 10 μg DNA by electroporation were performed as described (9). Transactivation studies were performed with equimolar amounts of indicator gene construct and Bel1 expression clones. As negative controls, the pBC12CMV expression vector backbone was used. For CAT assays, 1 μg pβGal was cotransfected as an internal control.

Transient expression assays

Extracts of transfected cells were harvested 48 h after transfection by three cycles of freezing and thawing; the β-gal activity of each extract was quantitated as described previously (17) and expressed in arbitrary units. Equal amounts of β-gal activity were used to determine the expressed CAT activity in one hour reactions. Reaction products were analysed by thin-layer chromatography and subsequent autoradiography or by direct measurement of the radioactivity with a Berthold scanner. For quantitative analyses, equal amounts of β-gal activity were diluted 1:10 and 1:100 and used for standard CAT assays. SEAP assays were done using 100, 10 and 5 μl aliquots of COS7 cell culture supernatants taken 48 and 70 h after transfection (18). SEAP activity was expressed in milli-units alkaline phosphatase activity as described (18).

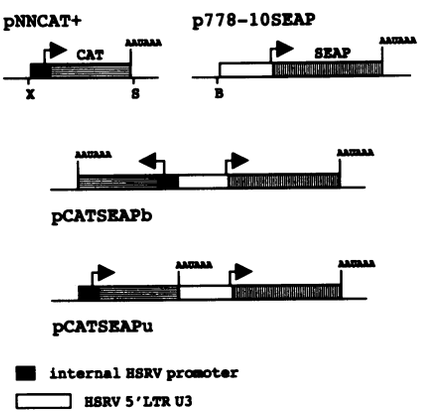

Figure 2. The construction of reporter gene plasmids pCATSEAPb and pCATSEAPu is schematically shown. The blunt-end XbaI (X) to SmaI (S) DNA fragment of pNNCAT+ (top, left) with the sense-oriented internal promoter and the CAT gene was inserted in either direction into the blunt-ended unique BglII site (B) of plasmid p778-10SEAP clone (top, right) carrying the SEAP gene under the control of the HSRV U3 promoter. The direction of transcription of the internal promoter in the NdeI−NheI DNA fragment (solid box) and the U3 LTR promoter (open box) is marked by rectangular arrows. The indicator genes CAT and SEAP are indicated by horizontally and vertically hatched boxes, resp.. Polyadenylation signals are indicated by AAUAAA. Plasmid pCATSEAPb (middle) contains both transcription units in opposite directions, whereas both promoters in pCATSEAPu (bottom) direct transcription in the same direction.

RESULTS

Transactivation capacities of different eukaryotic Bel 1 expression plasmids

A recombinant eukaryotic Bel 1 expression clone pBCbel1 was constructed from the plasmid pBCbel (9). The novel pBCbel1 clone contains only the *bel 1* gene under the control of the CMV promoter and should not express full-length *bet* gene products. In fact, pBCbel1 expressed low amounts of Bel 1 and and high concentrations of a truncated version of Bet of approximataly 33kDa. This expression profile was similar to that of the parental plasmid clone that had yielded low amounts of Bel 1 but abundant amounts of the full-length Bet protein (9).

To test whether both recombinant plasmids share the same capacity to trans-activate the recently detected internal and Bel 1-dependent HFV promoter/enhancer, reporter plasmids were used that carry the internal promoter upstream of an indicator gene (Fig. 1B). In plasmids pNNSEAP+ and pNNSEAP− the HSRV NdeI−NheI DNA fragment that contains the Bel 1-dependent promoter/enhancer element was placed in the sense and antisense orientation 5′ of the secreted alkaline phosphatase (SEAP) gene in the promoterless pPLSEAP vector (Fig. 1). Upon transfection in COS7 cells, pNNSEAP+ and pNNSEAP− (with sense and antisense orientation of the insert, Figure 1B) alone did not show any activity. After trans-complementation with pBCbel and pBCbel1, SEAP activity was detectable in supernatants from pNNSEAP+ cotransfections (Fig. 1A) but not in those from pNNSEAP−. The rate of trans-activation of plasmid pNNSEAP+ by either pBCbel or pBCbel1 was similar in independent experiments and was in general less than 10% of the activity of a CMV promoter SEAP construct.The ability to trans-activate the HFV internal promoter/enhancer is comparable for the plasmids pBCbel and pBCbel1 and is obviously not changed when high amounts of either full-length or truncated version of Bet are co-expressed.

Transcriptional interference between the HFV LTR promoter and the internal promoter

To analyse transcriptional interference between the 5′ LTR promoter and the internal HFV promoter, indicator genes were placed under the control of either promoter in a single vector plasmid. The constructs carry the HFV NdeI−NheI DNA fragment with the internal promoter/enhancer in sense orientation 5′ of the CAT gene and, in addition, the SEAP gene 5′ of the HFV U3 region (nt −778 to +10 relative to the LTR cap site). In plasmid pCATSEAPu (Fig. 2, bottom line) transcription of both CAT and SEAP genes has unidirectional orientation, whereas in plasmid pCATSEAPb transcription is bidirectional as the SEAP gene expression starts from the U3 region and runs clockwise; CAT gene transcription starts from the internal promoter and is counter-clockwise (Fig. 2, middle part).

To determine transcriptional interference between both HFV promoters, the SEAP and CAT activities expressed from both pCATSEAP constructs were compared to those of the parental p778-10SEAP and pNNCAT+ plasmids (Fig. 2, top line). The indicator gene constructs were transfected in COS7 cells in the presence of either pBC12CMV or the Bel 1 expression clones pBCbel1 or pBCbel. It was previously reported that COS7 cells are capable of synthesizing infectious HSRV particles when transfected with the infectious HFV DNA clone pHSRV13, although COS7 cells are non-permissive to HFV infection (9). About 48 h after transfection, cell culture supernatants were assayed for SEAP activity, i.e. for LTR promoter activity, and cellular extracts were analysed for CAT activity, i.e. for activity of the internal promoter.

Expression of the HFV LTR promoter

The amount of SEAP activity released by cells transfected with plasmids pCATSEAPu, pCATSEAPb, and p778-SEAP in the presence of Bel 1 were determined (Table 1). To quantitate the potential interference of both promoters in the pCATSEAP plasmids, the SEAP expression value of the parental p778-10SEAP clone was set to 100%. The corresponding values

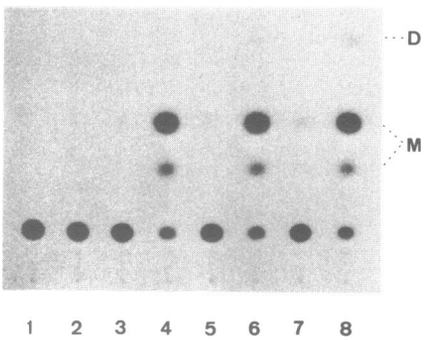

Figure 3. Determination of transcriptional interference between the HSRV U3 promoter and the intragenic promoter. CAT assays were performed with normalized amounts of extracts of COS7 cells transfected with the following indicator gene constructs: p778-10SEAP (lanes 1 and 2), pNNCAT+ (lanes 3 and 4), pCATSEAPb (lanes 5 and 6), pCATSEAPu (lanes 7 and 8). For trans-complementation pBC12CMV (lanes 1, 3, 5, 7) and pBCbel1 (lanes 2, 4, 6, 8) was used. Since the clones transactivated by Bel 1 were out of the linear range of the reaction, samples were serially diluted for quantification (see Table 1). The position of the mono-acetylated (M) and di-acetylated (D) chloramphenicol derivatives is given.

Table 1. SEAP activities expressed by Bel 1-transactivated plasmids pCATSEAPu and pCATSEAPb

	Experiment No.[a]				Average value[b]
	1	2	3	4	
p778-10SEAP 100	100	100	100	100	100
pCATSEAPb	224	304	68	190	196
pCATSEAPu	150	108	62	114	108

[a]in experiments No. 1 and 2 plasmid pBCbel1, in experiments No. 3 and 4 plasmid pBCbel was co-transfected
[b]in percent of Bel 1-transactivated parental plasmid p778-10SEAP

Table 2. Comparison of CAT activities expressed by plasmids pCATSEAPb and pCATSEAPu in the Presence and Absence of BEL 1[a]

	Relative CAT expression[b] −BEL 1	Transactivation[c] BEL 1
pNNCAT+	1.0	576
pCATSEAPb	1.75	447
pCATZSEAPu	3.18	452

[a] Data present are average values of four independent experiments
[b]CAT gene expression by the parental plasmid pNNCAT+ was set to 1.0.
[c]Expressed as increase of CAT gene expression of cotransfections with Bel 1 expression clones relative to pBC12CMV.

of the CATSEAP constructs are given relative to this value (Table 1). Whereas plasmid pCATSEAPu showed no alteration of SEAP gene expression, the level of Bel 1-transactivated SEAP gene expression was twofold higher in pCATSEAPb when compared to parental p778-SEAP. In the absence of Bel 1, none of the constructs expressed detectable SEAP activity (not shown). As expected, pNNCAT+ that carries only the CAT gene did not expresse any SEAP activity in the presence or absence of Bel 1. It is assumed that the failure to detect that part of the basal SEAP gene expression that is not trans-activated by Bel 1 is most probably due to the lower sensitivity of the SEAP assays when compared to that of CAT assays. The results obtained indicate that the activity of the U3 LTR promoter in the presence of Bel 1 is not or only minimally increased in the pCATSEAP constructs as compared to p778-10SEAP.

Expression of the HFV internal promoter

In parallel to the determination of SEAP gene expression, COS7 cells were assayed for CAT gene expression directed by the internal promoter. The autoradiogram of a representative CAT assay with equal amounts of β-gal activity is shown in Fig. 3: expression by plasmid p778-10SEAP co-transfected with pBC12CMV (lane 1) or pBCbel1 (lane 2) was negative as anticipated. pNNCAT+ trans-complemented with the expression vector backbone pBC12CMV (lane 3) resulted in a very low basal activity that was increased 1.6fold in cotransfections with pCATSEAPb (lane 5) and 3.1fold with pCATSEAPu (lane 7); see Table 2. The level of CAT gene expression after Bel 1 transactivation of plasmids pNNCAT+, pCATSEAPb and pCATSEAPu (lanes 4, 6 and 8) reached values high enough that the reactions were out of the linear range. The cell extracts were therefore serially diluted as described and again assayed for CAT activity. The average values of four independent quantitative CAT assays are summarized in Table 2. Plasmids pCATSEAPb and pCATSEAPu had approximately 1.7- and 3.2-fold elevated basal, non-Bel 1-transactivated activity when compared to the parental plasmid clone pNNCAT+. In the presence of Bel 1, the corresponding transactivation rates (pBC12CMV- versus pBCbel1- or pBCbel-cotransfections) for the reporter gene constructs used was in a similar range: about 580fold for pNNCAT+, and 450fold for pCATSEAPb and pCATSEAPu (Table 2).

In summary, the internal promoter has only a minimal effect on the HFV U3 promoter. A transcriptional suppression of the internal promoter by the U3 promoter was not detectable in the constructs used. In contrast, the LTR promoter increased the basal activity of the internal promoter about two- to threefold. However, efficient gene expression was strongly dependent on the expression of Bel 1 and the rate of transactivation was almost unchanged under the conditions used.

DISCUSSION

It is remarkable that HFV has the capacity to transcribe not only genomic RNA from the first cap site in the 5′ LTR, but also generates subgenomic viral transcripts from the internal transcriptional start site located in *env*. The identification of the intragenic HSRV enhancer/promoter reported previously indicates a novel mode of retroviral gene expression for at least the spumavirus group of complex retroviruses (5). To analyse a possible interference between the two promoters, we used indicator gene constructs that carry both promoters 5′ of distinguishable reporter genes. Downstream of each reporter gene poly-adenylation signals were introduced to avoid expression of both reporter genes from only one of the two promoters in constructs with unidirectional transcription. Therefore the experimental system used here cannot detect promoter suppression by run-through transcription as reported for the 3′LTR of avian retroviruses (19). However, as both promoters were active in the context of the HFV proviral DNA (5), the internal promoter is not down-regulated by the 5′ LTR promoter in the genetic context of wild-type viral genome.

The experiments reported here revealed that the basal transcriptional activity of the intragenic promoter was increased when the promoter/enhancer of the U3 part of the 5′LTR was located in *cis*. This result is remarkable in view of the frequently observed promoter suppression in retroviral constructs with one or two internal promoters (20). Due to the lower sensitivity of the SEAP reporter gene system when compared to the CAT gene assay, it was not possible to determine whether the internal promoter/enhancer also stimulated the basal activity of the HFV U3 promoter.

Another important aspect that may influence the activities of the promoters in the pCATSEAP constructs is their relative spatial arrangement to each other. In this respect, the pCATSEAPu plasmid, where both promoters are separated by at least 1650 nucleotides showed the highest differences between them. Whereas the LTR promoter is barely influenced by the intragenic promoter, the internal promoter had a significantly increased basal activity in the sense promoter/enhancer construct. The promoters in the pCATSEAPb construct with the bidirectional transcription and with directly adjacent promoter/enhancers showed only a slight increase in activity for both promoters.

It is presently unclear whether there is also interference between the enhancer elements of the LTR and the internal promoter within the native HFV provirus. However, as it is well established that enhancers function over long distances, an interference of this type is likely to occur during HFV provirus transcription. Thus, expression of the foamy viral genes depends at least to a certain extent on the interplay of similar LTR promoter/enhancers and those of the intragenic promoter/enhancer.

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