VIROLOGY 196, 256–268 (1993)

BEL-1 Transactivator Responsive Sequences in the Long Terminal Repeat of Human Foamy Virus

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Received February 4, 1993; accepted June 3, 1993

Cis-regulatory elements in the long terminal repeat (LTR) of human foamy virus (HFV) were identified by using LTR mutants to transiently express the chloramphenicol acetyl-transferase gene after co-transfection with an expression plasmid for the virus bel-1 (transactivator) gene. The R-U5 region and an element in the 5′ U3 region were found to negatively influence HFV gene expression. The complete BEL-1 responsive region was mapped to extend from nucleotide position −471 to position −93 relative to the start of transcription. Within this region, three elements were identified that in the homologous or a heterologous (SV40) promoter context can, independently and irrespective of their orientation, act as targets for BEL-1. These elements are located between nucleotide positions −413/−378, −361/−291, and −124/−93. The target elements do not share obvious sequence homologies. The mechanism of HFV transactivation appears to be novel among the complex retroviruses and is likely to involve, as yet, undiscovered cellular DNA binding factors.

INTRODUCTION

Human foamy virus (HFV) belongs to the retroviruses which regulate their gene expression by a transacting activator of viral transcription (Cullen, 1991). The viral bel-1 gene has been identified to encode for a 36 k nuclear phosphoprotein that augments HFV long terminal repeat (LTR) directed transcription in a variety of mammalian and in avian cells (Rethwilm *et al.*, 1991; Keller *et al.*, 1991; Venkatesh *et al.*, 1991, Venkatesh *et al.*, 1993). Transcript mapping experiments revealed three mRNAs, two of which are multiply spliced, that may give rise to BEL-1 protein (Muranyi and Flügel, 1991). BEL-1 is the only regulatory HFV protein which is essential for virus replication *in vitro* (Baunach *et al.*, 1993). No further activity than the transcriptional effects of BEL-1 could be attributed to this protein (Baunach *et al.*, 1993).

BEL-1 (or TAF_{HFV}, for transactivator of foamy virus) acts on a U3 DNA element of the viral LTR (Rethwilm *et al.*, 1991; Keller *et al.*, 1991; Venkatesh *et al.*, 1991). The foamy virus U3 regions are among the longest compared to other retroviruses; HFV U3 is 777 bp long, and the U3 regions of simian foamy virus (SFV) type 1 and 3 are 1296 and 1332 bp in length, respectively (Mergia *et al.*, 1990; Renne *et al.*, 1992).

In previous studies on the mapping of BEL-1 responsive elements in HFV U3, the BEL-1 target region was located upstream of positions −94 (Keller *et al.*, 1991) and −62 (Venkatesh *et al.*, 1991) respectively, relative to the start of transcription. A more definitive analysis has been performed on the TAF response region of SFV-1 and SFV-3. Two regions in the SFV-1 LTR between positions −1196 and −880 and between −403 and −125 were identified to respond to TAF_{SFV-1}; the latter element was also found to act as an inducible enhancer in an orientation independent manner in the context of a heterologous promoter (Mergia *et al.*, 1992). In the SFV-3 LTR, two regions (−637/−496 and −496/−180, respectively) that confer TAF_{SFV-3} responsiveness to a heterologous promoter, irrespective of their orientation, have been mapped (Renne *et al.*, 1993). HFV and SFV U3 regions are highly divergent compared to R and U5 sequences (Renne *et al.*, 1992). This is reflected by a low degree of HFV and SFV TAF amino acid homology compared to the env and pol proteins (Mergia *et al.*, 1991; Renne *et al.*, 1992). Thus, TAF_{SFV-1} does not stimulate the HFV LTR (Mergia *et al.*, 1992). Enhancement of SFV-3 LTR activity by TAF_{SFV-1} has been described (Renne *et al.*, 1993) which might reflect the closer relatedness of these two viruses (Renne *et al.*, 1992). However, TAF_{SFV-3} does not stimulate reporter gene expression driven by the SFV-1 LTR (Renne *et al.*, 1993).

While the results of these experiments suggest a highly specific mechanism of foamy virus transactiva-

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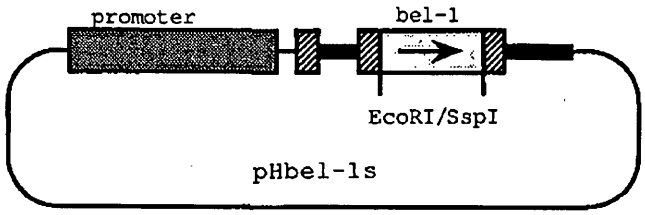


FIG. 1. pHbel-1s expression vector, which was co-transfected with the indicator plasmids in all experiments. The bel-1 coding sequence was cloned as an *Ssp*I fragment derived from plasmid pHSRV (Rethwilm *et al.*, 1990) into the *Eco*RI site in the third exon of the rabbit β-globin gene of pKCR3 (O'Hare *et al.*, 1981) in which the SV40 promoter was replaced by the mouse MHC class I H-2K^b enhancer/promoter (Kimura *et al.*, 1986). Intron and polyadenylation sequences are indicated as bold lines.

tion, TAF_{HFV} and TAF_{SFV} proteins were found to augment lentivirus LTR directed gene expression, which may also indicate some pleiotropic effects of foamy TAF proteins (Keller *et al.*, 1991; Mergia *et al.*, 1992). The human immunodeficiency virus type 1 (HIV-1) U3 element between positions −158/−118 was found to be essentiell for transactivation by TAF_{HFV} (Lee *et al.*, 1992; Keller *et al.*, 1992). Within this element is contained a nonamer motif which shares eight out of nine nucleotides with an HFV U3 motif between positions −135/−127. This sequence has therefore been considered as the BEL-1 responsive element (BRE).

Since knowledge of the TAF target site(s) in the HFV LTR is crucial in elucidating the mechanism of HFV specific transactivation, we constructed a series of LTR mutants and assayed their responsiveness to BEL-1 in both homologous and heterologous promoter contexts. Our results support the view that the mechanism of foamy virus transactivation is novel among complex retroviruses.

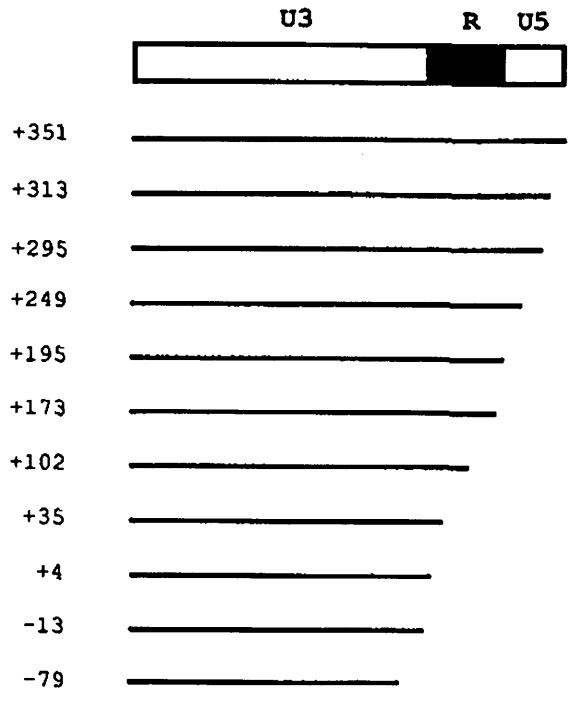
MATERIALS AND METHODS

Plasmid constructions

All DNA manipulations were done by established molecular cloning techniques (Sambrook *et al.*, 1989). Recombinant plasmids were characterized by dideoxy sequencing (Sanger *et al.*, 1977).

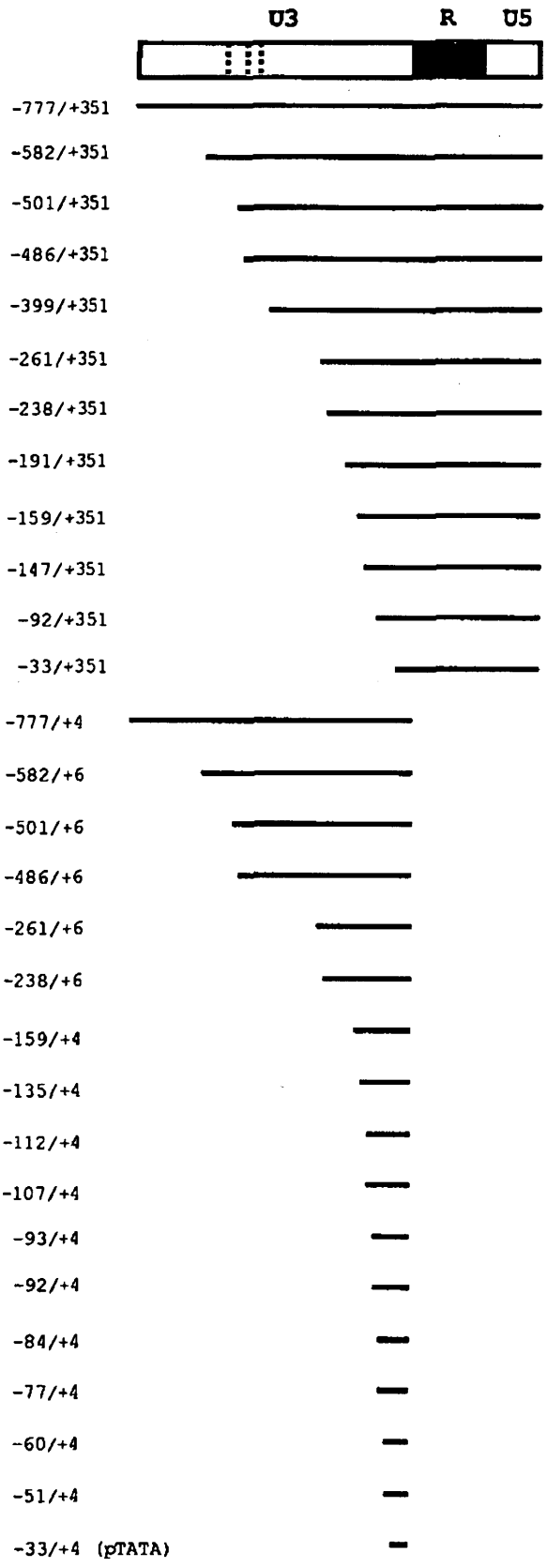
The BEL-1 expression plasmid, pHbel-1s, was constructed by inserting the bel-1 coding sequence as an *Ssp*I fragment derived from pHSRV (Rethwilm *et al.*, 1990) into the *Eco*RI site of pH2EV after filling in with Klenow enzyme. pH2EV is a derivative of the pKCR eukaryotic expression vector (O'Hare *et al.*, 1981). The vector contains the mouse MHC class I H-2K^b promotor (Kimura *et al.*, 1986) and was a generous gift from I. Horak, Würzburg (I. Horak, unpublished). This plasmid is shown in Fig. 1. pHbel-1a is a non-expressing control plasmid harboring the bel-1 gene in reverse orientation with respect to the promoter.

p5'LTR and p5'LTRinv were the source of all further LTR mutants and were generated by excising the *KpnI/NarI* fragment, spanning the complete 5'LTR, from the infectious molecular clone pHSRV (Rethwilm *et al.*, 1990), treatment with Klenow enzyme, and insertion



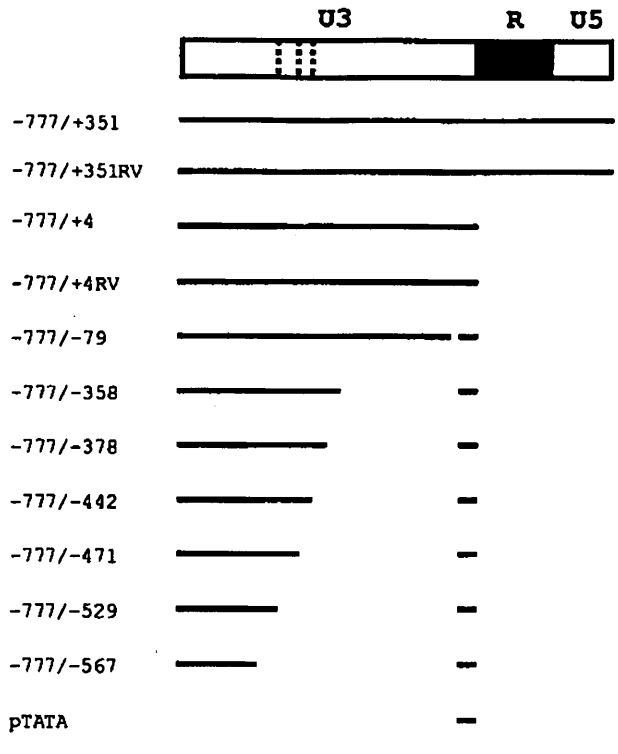
- TAF	+ TAF	fold activation
1.0	10.1	10
0.7	61.7	88
0.3	38.9	129
0.3	69.1	230
0.3	86.7	289
0.3	94.4	314
0.3	84.3	281
0.3	92.9	310
0.3	92.9	310
0.8	84.9	106
0.5	2.8	6

FIG. 2. Positive effect of nested deletions of the R-U5 region on HFV LTR promotor activity. Assay conditions: 50 μg of protein lysate was incubated for 60 min.



- TAF	+ TAF	fold activation
0.9	12.7	14
0.3	22.5	75
0.3	43.7	146
0.3	43.3	145
0.3	33.3	111
0.3	11.4	38
0.3	7.5	25
0.3	10.5	35
0.3	8.6	29
0.3	10.1	34
0.3	2.5	8
0.3	0.6	2
1.0	67.9	68
0.8	60.7	61
0.8	83.3	104
0.6	74.9	125
0.6	49.8	83
0.6	52.1	87
0.6	50.4	84
1.2	57.7	48
1.7	19.8	12
0.7	10.8	15
1.0	2.9	3
1.7	3.0	2
2.4	10.0	4
2.2	11.6	5
2.0	4.8	2
2.4	4.0	2
2.4	5.9	2

FIG. 3. The BRE in the HFV U3 region is located upstream of nucleotide position −93 relative to the start of transcription. Nested 5′ deletions of the HFV LTR were analyzed to determine the 3′ border of the BRE. The dotted vertical lines in the LTR represent the three binding sites for the transcription factor AP-1. Assay conditions: 50 μg protein of lysates from cells transfected with constructs bearing the R-U5 region and 25 μg from cells transfected with constructs bearing only U3 sequences was incubated for 60 min.



- TAF	+ TAF	fold activation
1.0	10.1	10
0.8	11.6	15
0.3	92.9	310
0.4	89.6	224
0.5	91.6	183
0.3	66.5	222
0.5	93.0	186
0.5	38.0	76
0.4	8.1	20
0.2	0.7	4
0.4	0.8	2
0.5	1.3	2

FIG. 4. The BRE is located downstream of nucleotide position −471. Nested 3′ deletions of the U3 region were analyzed to determine the 5′ border of the BRE. The dotted lines represent the three binding sites for the transcription factor AP-1. Assay conditions: 50 μg protein was incubated for 60 min.

into the *Sma*I site of the pUC19 vector in either orientation.

Deletion mutagenesis of R-U5 sequences from the 3′ end was performed by Bal31 digestion of *Eco*RI linearized p5′LTR, Klenow treatment and religation with a *Bgl*II linker. *Bam*HI/*Bgl*II fragments were excised from the resulting plasmids and inserted into the chloramphenicol acteyl-transferase (CAT) expression vector p0cat-Bg (Rethwilm *et al.*, 1990), resulting in the constructs shown in Fig. 2.

Similarly deletion mutagenesis of U3 sequences from the 5′ end was carried out by Bal31 digestion of *Eco*RI linearized p5′LTRinv. p5′cat constructs obtained this way are depicted in Fig. 3. For removal of the R-U5 region in these constructs a Bal31 3′ digestion product deleting sequences up to base +4 (start of transcription is +1) was used, alternatively R-U5 was deleted with *Sac*I which cuts at position +6 and in the pUC polylinker.

To facilitate deletion mutagenesis of the U3 region from the 3′ end, an *Eco*RV site was introduced immediately upstream of the TATA box at position −34 relative to the start of transcription by in vitro mutagenesis, using the oligonucleotide 5′-CTTATATACGATATC-CATCATTCTTAAAAC-3′ (mutated nucleotides are in bold face) after subcloning a *Hin*dIII/*Xba*I fragment of p5′LTR into the m13mp19 vector. The mutated *Hin*dIII/*Xba*I fragment was then substituted for the respective fragment of p5′cat (−777/+351) and p5′cat (−777/+4) leading to p5′cat (−777/+351)RV and p5′cat (−777/+4)RV, respectively. 3′ U3 deletions were introduced into p5′cat (−777/+4)RV by cutting with *Eco*RV and appropriate restriction enzymes in U3 and religation after Klenow enzyme treatment. Alternatively, *Hin*dIII (position −362) cut plasmid was digested with Bal31 and religated after *Eco*RV cleavage so that only the truncations in the 5′ direction from the *Hin*dIII site became effective. Plasmids obtained this way are shown in Fig. 4.

The pTATA (−33/+4) vector was created by deleting the U3 region upstream of the TATA box as a *Bgl*II/*Eco*RV fragment from p5′cat (−777/+4)RV and religation with a *Bgl*II linker. LTR fragments or chemically synthezised oligonucleotides introduced into this vector are depicted in Figs. 5–8.

The enhancer-less vector pA_{10}cat (Laimins *et al.*, 1984) was used to test LTR motifs for enhancer function in a heterologous (SV40) promoter context. DNA fragments of the HFV LTR, or chemically synthesized and annealed oligonucleotides, were introduced into the *Bgl*II site 5′ to the CAT gene. These constructs are shown in Fig. 9.

Transfections and CAT-assays

Baby hamster kidney cells (BHK-21) and dog thymus Cf2Th fibroblasts were obtained from Dr. Neumann-Haefelin (Freiburg, Germany) and the ATCC, respec-

- TAF	+ TAF	fold activation
1.6	86.5	54
1.6	86.0	54
1.4	82.3	59
1.4	79.7	57
1.2	59.1	49
1.4	21.5	15
1.4	16.8	12
2.0	12.3	6
1.7	13.0	8
1.5	7.2	5

FIG. 5. BRE I is located between nucleotide positions −413 and −358. Nested 5′ deletions of the −534/−358 sequence of U3 introduced into the pTATA (−33/+4) vector were assayed to map BRE I. The dotted lines represent the AP-1 binding sites. Assay conditions: 50 μg protein were incubated for 60 min.

tively. Both cells are susceptible to HFV infection (Rethwilm *et al.*, 1990; and our unpublished observation). Cells were kept in MEM supplemented with 5–10% fetal bovine serum, glutamine, and antibiotics. Transfections of 5×10^5 cells were carried out by the calcium phosphate co-precipitation method as described (Rethwilm *et al.*, 1991). The transfection cocktail contained 5 μg of indicator plasmid and 1 μg of effector plasmid, either pHbel-1s or pHbel-1a. The DNA concentration was adjusted to 20 μg/ml with herring sperm DNA. Cells were harvested after 48 hr by scraping and lysates were prepared as described (Rethwilm *et al.*, 1991). The protein concentration of the lysates was measured with a commercial assay (Bio-Rad). The amount of protein assayed and the incubation time were kept constant within a series of tests but differed from series to series (as specified in the figure legends) to be in the linear range. Lysates were analyzed in a total volume of 200 μl 250 m*M* Tris–HCl, pH 8.0 containing 0.5 m*M* acetyl-CoA (Sigma) and 0.1 μCi of [^{14}C]chloramphenicol (Amersham) at 37°. Non-acetylated chloramphenicol was separated from acetylated forms by ascending thin-layer chromatography and the spots were quantitated with a Molecular Dynamics PhosphorImager. For each construct 5–6 independent transfections with DNA from two preparations were performed. Variation of CAT values for a given construct was less than 20%.

RESULTS

Negative effect of R-U5 sequences on HFV gene expression

Previous studies on the role of R-U5 sequences on HFV LTR directed gene expression have led to controversial results. While a moderate increase in activity has been reported for U5 and R-U5 deleted LTR constructs in one study (Rethwilm *et al.*, 1991), no such effect was observed by others (Keller *et al.*, 1991). For SFV-1 and SFV-3 a marked increase in reporter gene

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AAGCTTTCACATACTCAGTAGCTGTTTCACAATCAACAAAACAATGATGATGTAATCATAAGGAAGTAGTTT   -361/-291
AAGCTTTCACATACTCAGTAGCTGTTT                                                 OLIGO TJ
 AGCTTTCACATACTCAGTAGCTGTTTCACAATCAA                                        OLIGO SO
                    TCACAATCAACAAAACAATGATGATG                               OLIGO MM
                          CAAAACAATGATGATGTAATCATAAGGAAGTAGTTT               OLIGO BG
                                TAATCATAAGGAAGTAGTTT                        OLIGO HH
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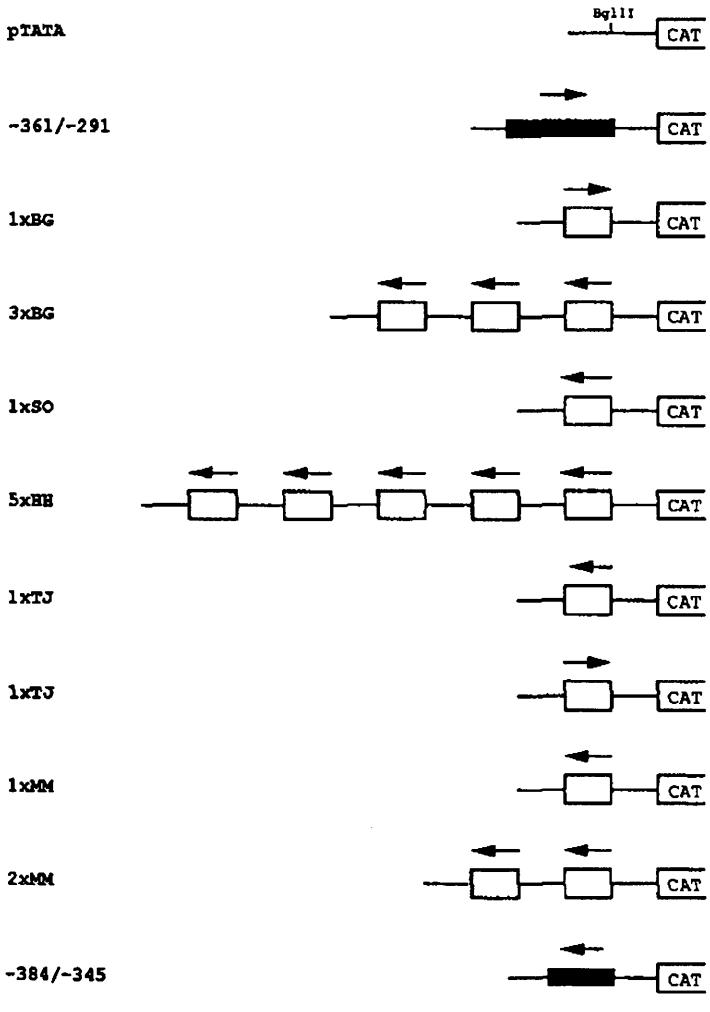
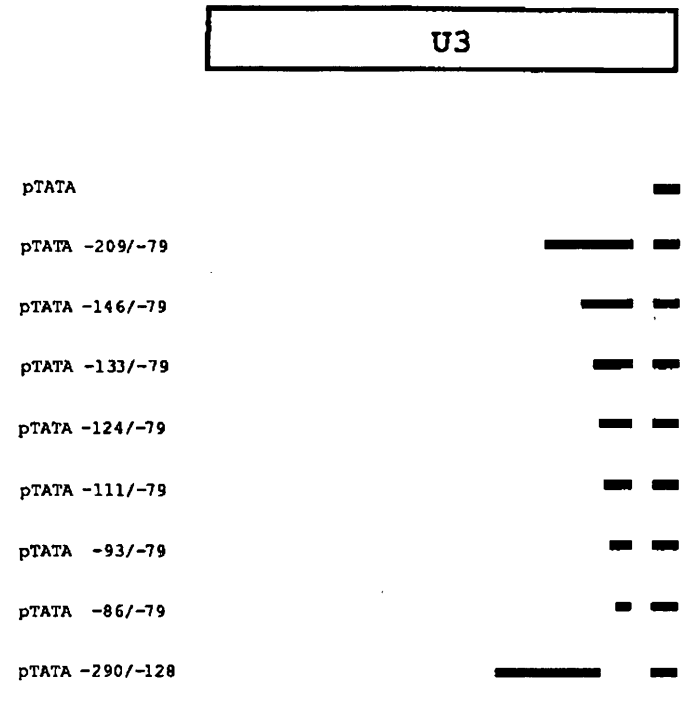


	- TAF	+ TAF	fold activation
pTATA	2.7	8.3	3
-361/-291	2.5	83.2	33
1xBG	3.0	26.9	9
3xBG	2.9	75.5	26
1xSO	2.9	56.9	20
5xHH	2.9	13.6	5
1xTJ	3.2	23.6	7
1xTJ	3.2	24.9	8
1xMM	3.1	11.6	4
2xMM	3.1	11.3	4
-384/-345	3.0	18.3	6

FIG. 6. BRE II is located between nucleotide positions (−361/−291). Sequences of the upper strands of the *Hin*dIII/*Dra*I (−361/−291) fragment of U3 spanning the BRE II and of chemically synthesized oligonucleotides that were used to dissect element II are shown. The arrows above the open boxes indicate the orientation of the oligonucleotides with respect to the direction of transcription. The filled boxes represent the longer DNA fragments introduced into pTATA. Assay conditions: 50 μg protein was incubated for 60 min.

expression of R-U5 deleted LTRs has been described (Mergia *et al.*, 1992; Renne *et al.*, 1993). We therefore wanted to analyze the effect of R-U5 on reporter gene expression by measuring the CAT activity of LTR mutants with nested deletions introduced from the 3′ end after co-transfection with pHbel-1s (Fig. 1). As shown in Fig. 2, up to 30-fold increase in TAF_{HFV} mediated CAT gene expression was obtained with mutants deleting U5 (construct +195) compared to a construct that extends into the primer binding site (construct +351). Further deletions of R sequences did not result in higher levels of expression. However, it is likely that



- TAF	+ TAF	fold activation
2.7	14.0	5
0.8	80.0	100
0.6	88.8	111
1.1	78.3	71
1.6	88.6	55
1.5	53.3	36
2.4	35.6	14
2.5	23.9	10
1.6	10.7	7

FIG. 7. BRE III is located between nucleotide positions −124 and −93. Nested 5′ deletions of the fragment −209/−79 introduced in the pTATA vector were assayed to map BRE III. Assay conditions: 50 μg protein was incubated for 60 min.

the assays with further R truncations were beyond the linear range in this assay series. Interestingly, the viral cap site and adjacent 5′ sequences were not found to be required for TAF_{HFV} augmented gene expression (construct −13 in Fig. 2). A significant reduction in activity was only noted with construct −79 which deletes the HFV TATA box.

Mapping of the BEL-1 responsive region

To map TAF_{HFV} responsive sequences in the U3 region of the HFV LTR we first investigated a set of nested 5′ deletions which are depicted in Fig. 3. An increase in activity was observed when the 5′ 280 bp of U3 were deleted regardless of whether or not the constructs contained the R-U5 region. However, this effect was more pronounced with constructs bearing R-U5. Using the deletion mutants of both construct types, a first reduction in activity was found at position −261 and the second reduction was observed at position −93. These results indicate that the major BEL-1 responsive region is located in U3 5′ of position −93 and that there is at least one further element which may be located upstream of position −261.

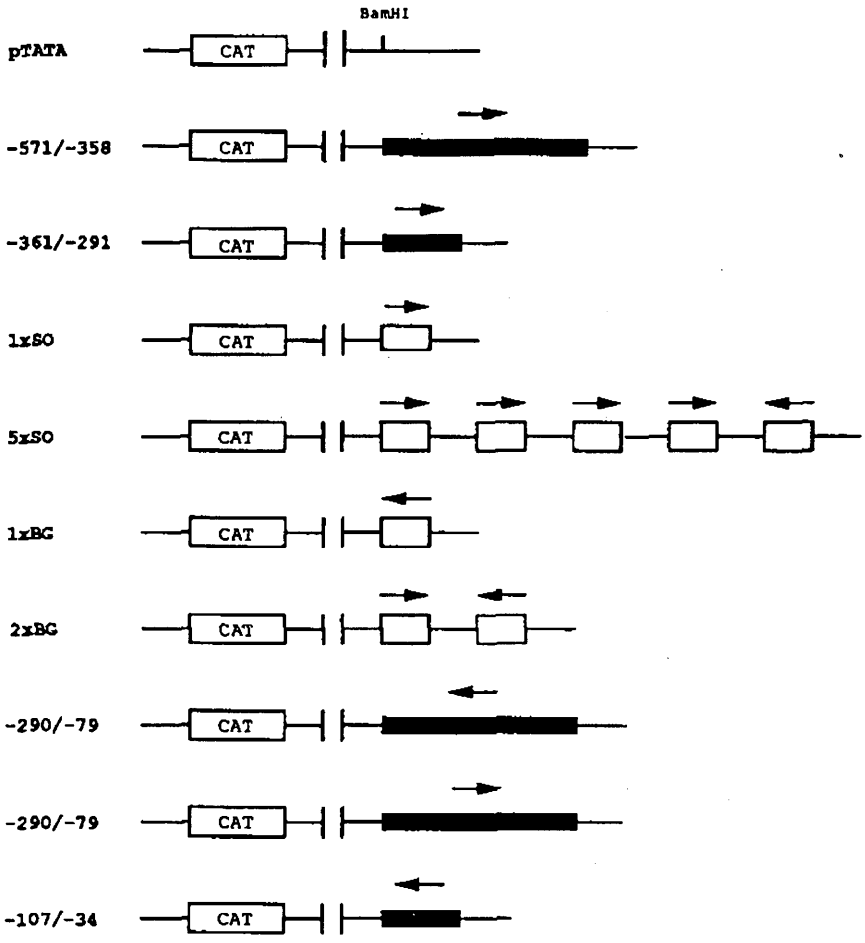
Truncations of U3 from the 3′ end were then analyzed. To facilitate this an *Eco*RV restriction site was introduced by in vitro mutagenesis at position −34 immediately 5′ to the TATA box. As shown in Fig. 4, the introduction of the *Eco*RV site does not significantly alter the activity of the HFV LTR, either with or without R-U5, in response to the transactivator. Using the 3′ deletion mutants, the 5′ border of the BEL-1 response region was determined to be located at approximately position −471, since sequences extending from −471 to −777 did not respond to TAF_{HFV}. These experiments also revealed two reductions in activity between positions −378 and −442 and between positions −442 and −471 indicating major responsive sequences downstream of positions −442 and −471.

Taken together, the results obtained with the 5′ and 3′ deletion mutants of U3 show that the complete BRE is located between positions −93 and −471 relative to the start of transcription. There is also evidence for several target sites of TAF_{HFV} within this element.

Physical dissection of the BRE

We next investigated to what extent portions of the BRE were able to respond to TAF_{HFV}. To accomplish this, HFV U3 fragments were cloned into the pTATA vector which essentially bears only the core promoter sequences between positions −33 and +4. pTATA itself, however, is not completely silent and is activated approximately two- to sixfold when co-transfected with the BEL-1 expression plasmid (Figs. 3–8). Thus, only those fragments that were stimulated significantly more than 6 fold were regarded as BEL-1 responsive.

The first sequence studied for TAF_{HFV} responsiveness was the portion upstream of position −358. Within this region the HFV LTR bears three recognition sequences for the ubiquitous transcription factor AP-1. It had been shown previously that AP-1 exerts a moderate effect on HFV promoter activity and is dependent on functionally active AP-1 binding sites. However, this effect was found to be distinct from the action of BEL-1

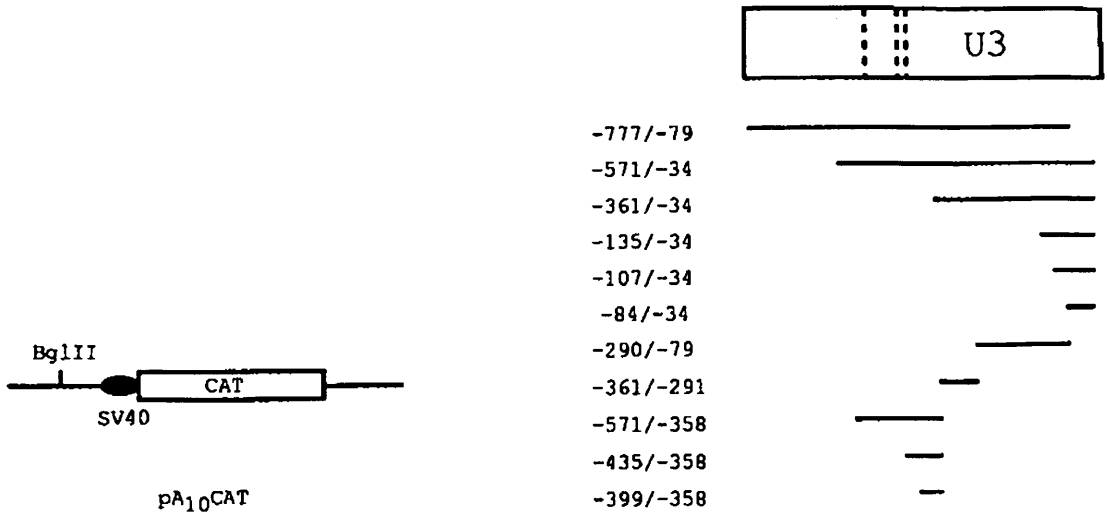


BRE	- TAF	+ TAF	fold activation
	1.6	9.6	6
I+AP-1	1.6	72.3	45
II	1.7	31.6	18
	1.2	24.4	20
	1.3	56.1	43
	1.6	15.7	10
	1.5	9.7	6
III	1.2	6.6	6
III	1.2	5.4	5
	1.9	6.5	3

FIG. 8. Analysis of positional effects of BRE I–III on enhancer activity. DNA fragments and oligonucleotides covering BRE I–III introduced into the *Bam*HI site downstream of the CAT gene in the pTATA vector were measured for BEL-1 response. Filled boxes represent longer DNA fragments and open boxes represent oligonucleotides. The arrows indicate the orientation of the cloned sequences with respect to the CAT gene. Assay conditions: 50 μg lysate was incubated for 60 min.

(Maurer *et al.*, 1991). When 5′ truncations of the −534/−358 fragment (Fig. 5) were analyzed, DNA fragments retained inducibility by TAF_{HFV} until truncations passed position −413 where the first reduction in activity was noticed. A more dramatic decrease in activity was observed between positions −399 and −397 which may indicate that a crucial DNA site for BEL-1 action was affected. Taken together the results from Figs. 4 and 5, the BEL-1 target site I was duly identified to be located in the 36 bp region between positions −413 and −378. The site therefore resides outside the AP-1 binding sites which are located upstream of position −439. We also observed that the 5′ deletions analyzed in this experiment all gave rise to lower stimulative indices than the construct −777/−358 in Fig. 4 that covers the same target element. This may be attributed to the lower basal activity of the latter.

The second portion to be studied was defined by the restriction sites for *Hin*dIII and *Dra*I at positions −361 and −291, respectively. When this fragment was placed in front of the CAT gene in the pTATA vector, a clear response to TAF_{HFV} was noted (Fig. 6). Because of the relatively small size of this element, we attempted to further dissect the BEL-1 target site by using oligonucleotides that were introduced into the pTATA vector (Fig. 6). Oligonucleotide SO spanning the 5′ half of the *Hin*dIII/*Dra*I fragment was activated approximately 20-fold by TAF_{HFV}, but this did not achieve the stimulative index of the complete fragment. The activation of the SO element was found to be orientation independent with respect to the direction of transcription. Oligonucleotide BG spanning the 3′ half of the *Hin*dIII/*Dra*I fragment did not respond to TAF_{HFV} when a single copy was present in the construct. However, three copies of this element gave rise to a measurable response, irrespective of its orientation. This may indicate that the SO and BG sequences are only partially able to fulfill the requirements of a



		BHK-21			Cf_2Th		
	BRE	- TAF	+ TAF	fold activation	- taf	+ taf	fold activation
pA_{10}cat		0.6	3.5	6	0.7	1.3	2
pA_{10}cat -777/-79	I, II, III +AP-1	2.5	60.6	24	1.0	21.7	22
pA_{10}cat -79/-777	I, II, III +AP-1	2.3	75.3	33	1.2	29.3	24
pA_{10}cat -34/-571	I, II, III +AP-1	1.5	37.3	25	1.3	13.8	11
pA_{10}cat -34/-361	II, III	1.8	53.8	30	1.2	26.8	22
pA_{10}cat -135/-34	III	0.8	42.1	53	1.0	22.9	23
pA_{10}cat -107/-34		1.2	31.0	26	0.3	11.5	38
pA_{10}cat -84/-34		1.2	12.6	10	0.7	3.6	5
pA_{10}cat -290/-79	III	0.3	13.0	43	0.5	10.4	21
pA_{10}cat -79/-290	III	0.3	20.8	69	0.5	2.9	6
pA_{10}cat -361/-291	II	1.7	41.8	25	0.7	18.6	27
pA_{10}cat -291/-361	II	1.0	51.4	51	0.9	10.1	11
pA_{10}cat -571/-358	I+AP-1	2.4	54.5	23	0.5	7.9	16
pA_{10}cat -358/-571	I+AP-1	1.8	71.1	40	1.2	15.5	13
pA_{10}cat -435/-358	I	0.8	24.5	30	0.3	2.1	7
pA_{10}cat -399/-358		0.8	3.8	5	0.4	1.4	4

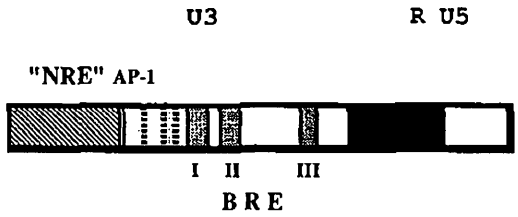


FIG. 10. Schematic drawing of the HFV LTR showing the *cis*-regulatory elements identified in this study. "NRE" represents a proposed weak negative regulatory element in the upstream U3 region, AP-1 marks the region with the three binding sites for the transcription factor AP-1, and BRE I to III indicate the three BEL-1 transactivator responsive elements.

functionally active BEL-1 target site. When subfragments of SO and BG (TJ and HH) or an oligonucleotide spanning the middle of the *Hin*dIII/*Dra*I fragment and overlapping SO and BG (MM) were analyzed, none of these was significantly activated over background values, even when multimerized oligonucleotides were tested. In summary, these results suggest that the complete target site II is located within the 71 bp region between positions −361 and −291. To show that elements I and II are indeed physically distinct, we also tested a DNA fragment that spans the overlap of both elements (construct −384/−345 in Fig. 6). Consistent with the view of two target sites this element was not found to be stimulated by TAF_{HFV}.

The third portion analyzed was the promoter proximal element. As shown in Fig. 7, the region between −209 and −79 responded well to BEL-1. When nested 5′ deletions of this fragment were tested, CAT activity remained high for constructs with truncations up to position −124, then gradually declined and approached values close to background as the deletion reached position −93. Together with the results shown in Fig. 3, target site III was localized within the 32 bp region between positions −124/−93. We also studied the sequence between targets II and III using the construct −290/−128 (Fig. 7) which did not respond to TAF_{HFV}, indicating that there are no further BEL-1 targets in this region. Interestingly, the −290/−128 construct also covers the sequence of HFV which shows homology to an element in the HIV-1 U3 region and has been implicated in cross-transactivation of HIV-1 by HFV BEL-1 (Lee *et al.*, 1992). The irresponsiveness of construct −290/−128 to BEL-1 indicates that the homologous sequence motif is not sufficient to act as a BEL-1 target.

To test whether or not positional effects influence the activity of the identified BREs, we cloned several fragments into the *Bam*HI site of the pTATA vector approximately 1600 bp downstream of the promoter. The results of co-transfections using these constructs with pHbel-1s are shown in Fig. 8. Construct −571/−358 which covers element I and the three AP-1 sites is activated to approximately the same extent as the −534/−358 fragment inserted close to the reporter gene into the *Bgl*II site (Fig. 5). Element II (construct −361/−291) is also activated when cloned downstream of the CAT gene, however, to a lesser extent compared to the respective fragment inserted into the *Bgl*II site (Fig. 6). When the oligonucleotides SO and BG (Fig. 8) were substituted for the complete *Hin*dIII/*Dra*I fragment spanning element II, the BG element was found not to respond to TAF_{HFV} whereas element SO appeared to be an active target site for the transactivator. The increase in reporter gene activity of a construct bearing multimerized copies of the SO sequence is typical for inducible enhancer elements (Serfling *et al.*, 1985). In contrast to the findings with BRE I and II we found that element III did not respond significantly to TAF_{HFV} when located at some distance from the promoter.

The BEL-1 targets are conditional enhancer elements

We finally investigated to what extent the BREs are active in a heterologous promoter context. HFV LTR portions, or oligonucleotides, were inserted into the SV40 promoter bearing CAT expression plasmid pA_{10}cat that had been used previously to identify TAX responsive sequences in the LTR of human T-cell leukemia virus type I (HTLV-I) (Paskalis *et al.*, 1986). And in addition to the BHK-21 cells exclusively used in the experiments reported above constructs were also analyzed in the Cf2Th cell line.

The results of these experiments are summarized in Fig. 9. When U3 fragments covering all BREs were analyzed they were found to respond to BEL-1 irrespective of their orientation to the CAT gene in both cell lines tested. Furthermore, all but one individual elements that were previously found to confer BEL-1 responsiveness to the HFV promoter were also found to be active with the SV40 promoter, although to a lesser extent. The −399/−358 construct, partially active in the pTATA vector (Fig. 5), showed no response in the pA_{10}cat context. All three BREs were active independent of their orientation with respect to the reporter gene. However, some cell type differences were noticed. Generally, transactivation was found to be

FIG. 9. Analysis of the promoter and cell type dependence of BRE I–III. U3 DNA fragments covering BRE I–III were introduced into the BglII site of pA_{10}cat. The orientation of the fragments relative to the reporter gene can be deduced from the position of the numbers after each construct which indicate the fragment borders. The dotted lines represent the AP-1 binding sites. Assay conditions: 25 μg protein of BHK cell lysates were incubated for 40 min, and 50 μg protein of Cf2Th cell lysates was incubated for 60 min.

weaker in dog thymus cells, in particular, construct −435/−358 covering element I and construct −79/−290 bearing element III in reverse orientation with respect to the reporter gene, were barely stimulated.

DISCUSSION

We report here a detailed analysis on the BEL-1 responsive sequences in the LTR of HFV. This was facilitated by analyzing LTR fragments in both homologous and heterologous promoter contexts for responsiveness to the BEL-1 transactivator.

The R-U5 region was found to negatively influence HFV LTR directed gene expression. The negative regulatory function of SFV-1 and SFV-3 R-U5 has been attributed to an extensive secondary structure of the nascent R-U5 mRNA (Mergia *et al.*, 1992; Renne *et al.*, 1993). Analysis of HFV R-U5 sequences, using the Genetics Computer Group Fold Program (University of Wisconsin, Madison, WI), revealed a secondary structure with the free energy of −73 kcal/mol (data not shown). The significance of this finding for the observed effect of R-U5 on HFV gene expression remains in question until studies using *in vitro* mutagenized LTR constructs, which disrupt the postulated RNA secondary structure, have been performed. Furthermore, it is so far not clear whether co-transcriptional or post-transcriptional events, as for example the utilization of the HFV mRNA, are responsible for the observed negative effect of R-U5. However, it is noteworthy that the splice donor of HFV has been mapped to position +51 (Muranyi and Flügel, 1991). A putative negative effect of R-U5 would therefore influence the genomic or gag-pol mRNA alone. Improvement of the transactivation values obtained with the R-U5 deletion mutants presented in Fig. 2 seems not to be the result of splicing events using the HFV splice donor at position +51, since the constructs extending to positions +195 showed similar activation levels as constructs that are lacking the splice donor (+4 and +35 in Fig. 2).

A second element which exerts a weak negative effect on HFV LTR directed gene expression was located in the upstream U3 region. A similar observation has been described for the upstream U3 region of SFV-3 (Renne *et al.*, 1993). A negative regulatory element has also been located in the U3 region of HIV-1 (Rosen *et al.*, 1985). However, to what extent the HFV sequences constitute a true negative regulatory element remains to be determined, for example, by testing its effect on the activity of a variety of constitutive promoters.

Three elements in the HFV LTR were identified that were able to respond independently from each other to BEL-1. Element I was mapped to sequences −413/−378 relative to the start of transcription. It resides outside the binding sites for the transcription factor AP-1 which has previously been shown to exert a moderate effect on HFV gene expression (Maurer *et al.*, 1991). While element I was found to be active with the homologous as well as with the SV40 promoter in hamster kidney cells, it was only weakly stimulated in dog thymus fibroblasts when placed in the heterologous promoter context. Whether or not this indicates any enhancer cell specificity (Serfling *et al.*, 1985) might be shown by a closer analysis using additional cell lines and different promoters.

Element II was located between positions −361/−291. Because element II responded to TAF_{HFV}, irrespective of its position and orientation relative to the promoter and irrespective of the kind of promoter used and cell type analyzed, it behaves like a typical inducible enhancer (Serfling *et al.*, 1985). When fragments of element II were analyzed for enhancer activity (oligonucleotides SO and BG in Fig. 6) they gave rise to a partial response that could be restored by multimerization of the targets. Further subfragments were found to respond only weakly to BEL-1. The dissection of element II into functional partially active fragments may indicate that BEL-1-mediated transactivation is a complex mechanism which might involve DNA sites with different specificities. BREs I and II were found to lay close to each other. However, the inactivity of a DNA fragment partially overlapping both elements (−384/−345 in Fig. 6) is indicative of two separate elements.

Element III was mapped to the positions −124/−93. This element was also found to be active in dog thymus cells with the heterologous promoter, irrespective of its orientation to the reporter gene (Fig. 9). However, it did not act well when placed distant from the promoter (Fig. 8). This might reflect the natural situation in the HFV LTR, where BRE III is located next to the promoter. Figure 10 summarizes the organization of the HFV LTR deduced from our studies.

In a recent study Lee *et al.* (1993) identified five BEL-1 response elements in the HFV LTR that were located at positions −559/−506 (a), −454/−418 (b), −360/−342 (c), −327/−284 (d), and −116/−89 (e). While our mapping experiments are in close agreement with respect to the elements c–e we could not show that fragments covering the region of elements a and b in the study of Lee *et al.* contribute substantially to the BEL-1 mediated transcriptional activity of the HFV LTR.

The HFV BREs I–III do not share sequence homologies, and to our knowledge, do not show obvious homologies to known eukaryotic enhancer sequence motifs. In this respect HFV is similar to SFV type 1 and 3 (Mergia *et al.*, 1992; Renne *et al.*, 1993). In preliminary experiments we were unable to demonstrate direct binding of a prokaryotically expressed βGAL/BEL-1 fusion protein to HFV LTR sequences, although bacterially expressed βGAL/JUN protein did bind to its target sequence (data not shown). Similar observa-

tions have been made recently (Venkatesh *et al.*, 1993). It is therefore likely that BEL-1 mediated transactivation makes use of a novel method to regulate retrovirus gene expression which might also involve, as yet, undiscovered cellular transcription factors. These cellular factors must be ubiquitous since replication and transactivation of HFV have been reported for a wide range of cells from different tissues and species (Hooks and Detrick-Hooks, 1981; Rethwilm *et al.*, 1991; Keller *et al.*, 1991). With respect to the likely involvement of cellular factors in transactivation by BEL-1, HFV is reminiscent of HTLV-I (Marriot *et al.*, 1989; Fujisawa *et al.*, 1991; Zhao and Giam, 1992; Tong-Starksen and Peterlin, 1990). The finding that a sequence distinct from the 21 bp repeat can serve as a target for TAX (Marriot *et al.*, 1989) is a further analogy between HTLV-I and the results reported here for the disparate elements of the HFV LTR responding to BEL-1.

The requirements for BEL-1-mediated transactivation have been analyzed at the protein level by using mutants of BEL-1 (Venkatesh *et al.*, 1993; He *et al.*, 1993). However, the results have been controversially interpreted. While Venkatesh *et al.* (1993) suggested the minimal activation domain to be located in the region of aa positions 56–227, He *et al.* (1993) mapped the activation domain to the 50 c-terminal aa of the 300 aa BEL-1 protein and favored the view of a direct interaction of BEL-1 with its target DNA. This conclusion was mainly drawn from the activation of the human immunodeficiency virus type 1 LTR by chimeras comprising the VP16 activation domain and c-terminally truncated BEL-1.

Fundamental questions in elucidating the molecular mechanism of BEL-1 transactivation of its own LTR are still open, for example, whether or not BEL-1 interacts directly with the target DNAs, or perhaps has DNA binding properties itself, and what additional cellular proteins may be involved in BEL-1 transactivation. The knowledge of the exact target sites for BEL-1 identified in this study will be of use in answering these questions.

ACKNOWLEDGMENTS

We thank Edgar Serfling and Lee Dunster for critical review of the manuscript, Dieter Neumann-Haefelin and Ivan Horak for the gift of reagents, Sandra Bräutigam for comments on the manuscript and help with the computer analysis, and Volker ter Meulen for support. This work was supported by the Deutsche Forschungsgemeinschaft (SFB 165).

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