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Identification and Functional Characterization of a High-Affinity Bel-1 DNA Binding Site Located in the Human Foamy Virus Internal Promoter

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The transcription of genes carried by primate foamy viruses is dependent on two distinct promoter elements. These are the long terminal repeat (LTR) promoter, which regulates expression of the viral structural proteins, and a second internal promoter, located towards the 3′ end of the *env* gene, that directs expression of the viral auxiliary proteins. One of these auxiliary proteins is a potent transcriptional transactivator, termed Bel-1 in human foamy virus (HFV) and Tas or Taf in the related simian foamy viruses, that is critical for foamy virus replication. Previously, it has been demonstrated that the LTR promoter element of HFV contains a DNA binding site for Bel-1 that is critical for transcriptional activation (F. He, W. S. Blair, J. Fukushima, and B. R. Cullen, J. Virol. 70:3902–3908, 1996). Here, we extended this earlier work by using methylation interference analysis to identify and characterize the Bel-1 DNA binding sites located in the HFV LTR and internal promoter elements. Based on these data, we propose a minimal, 25-bp DNA binding site for Bel-1, derived from the HFV internal promoter element, and show that this short DNA sequence mediates efficient Bel-1 binding both in vitro and in vivo. We further demonstrate that, as determined by both in vitro and in vivo assays, the Bel-1 target site located within the HFV internal promoter binds Bel-1 with a significantly higher affinity than the cap-proximal Bel-1 target site located in the LTR promoter. This result may provide a mechanistic explanation for the observation that the internal promoter is activated significantly earlier than the LTR promoter during the foamy virus life cycle.

Primate retroviruses belonging to the foamy virus, or spumavirus, subfamily encode not only the structural proteins Gag, Pol, and Env but also a potent transcriptional transactivator and at least two auxiliary proteins of currently unknown function (7, 11, 26, 29). The transcriptional transactivator, which is termed Bel-1 in the case of human foamy virus (HFV) and Taf or Tas in the case of simian foamy viruses (SFV), has been shown to be essential for foamy virus replication in culture (1, 24). Foamy viruses contain at least two promoter elements that are highly responsive to the Bel-1/Tas protein. The first is the long terminal repeat (LTR) promoter, which may contain as many as three Bel-1/Tas DNA target sites and which is responsible for transcription of genome-length viral transcripts (8, 18, 20, 28, 30, 33). A second, internal promoter element is located towards the 3′ end of the viral envelope gene and directs transcription of mRNAs encoding the viral auxiliary proteins, including Bel-1/Tas (5, 22, 25). The internal promoter element is thought to activate expression of these auxiliary proteins early in the viral life cycle and is clearly critical for their efficient expression (21, 23, 25). Therefore, the internal promoter element is required for effective virus replication in culture.

Research into the mechanism of action of the HFV Bel-1 protein has identified an acidic transcription activation domain located within the carboxy-terminal ~40 amino acids (aa) of this 300-aa viral regulatory protein and has also defined a DNA targeting domain occupying ~120 aa in the core of Bel-1 (3, 12, 16, 32). While the domain organization of the related SFV type 1 (SFV-1) Tas protein appears to be very similar to that observed in Bel-1 (27), Tas and Bel-1 both fail to activate transcription directed by promoters containing functional DNA target sites specific for the other protein (5, 12).

Although several DNA target sites for Bel-1 have been mutationally defined, these have little evident sequence homology (8, 17, 18, 20, 22, 33). Nevertheless, it has been demonstrated that Bel-1 can directly and specifically bind to the major, cap-proximal Bel-1 response element (BRE) located in the viral LTR promoter and also to sequences present in the HFV internal promoter element (15). Similarly, specific Tas binding to the SFV-1 internal promoter, and to a proposed Tas-dependent enhancer element located in the SFV-1 *gag* gene, has also been reported (4, 34). Surprisingly, for both Tas and Bel-1, DNA sequences that are sufficient for DNA binding in vitro were found to be necessary but not sufficient for Tas or Bel-1 function in vivo (15, 34). This observation raises the possibility that other, cellular DNA binding proteins may play a critical role in mediating Bel-1 and Tas function in vivo.

Although target sequences for the Bel-1 protein have been loosely defined both based on functional criteria and by in vitro DNA binding (8, 15, 17, 18, 20, 22, 33), the actual DNA target specificity of Bel-1 remains far from clear. In this study, we attempted to shed further light on the DNA binding specificity of Bel-1 by comparing the interactions of Bel-1 with the HFV internal and LTR promoter elements. These experiments demonstrate that Bel-1 binds to the BRE present in the internal promoter with significantly higher affinity than to the major LTR promoter BRE. Using modification interference, we have identified individual bases within both the internal and LTR promoters that are critical for Bel-1 DNA binding in vitro. This analysis has permitted the definition of a minimal, 25-bp DNA

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sequence that is sufficient to mediate Bel-1 binding both in vitro and in vivo.

MATERIALS AND METHODS

Construction of molecular clones. The full-length HFV *bel-1* gene was amplified by PCR with primers that introduced flanking *Xba*I sites and then ligated into the *Xba*I-digested plasmid pYCplacIII (13). pYCplacIII is a single-copy, ARS-CEN based yeast (*Saccharomyces cerevisiae*) expression plasmid that contains ADH1 promoter and terminator sequences. Similarly, Tas coding sequences from SFV-1 were amplified and cloned between the *Xba*I and *Eco*RI sites of pYCplacIII. We have previously described (15) a high-copy-number yeast expression plasmid for Bel-1, based on the 2μm origin of replication, in which Bel-1 expression is directed by the potent phosphoglycerate kinase promoter.

A yeast indicator plasmid containing the complete cap-proximal BRE present in the HFV LTR (positions −112 to −31) linked to the *cyc* promoter and *lacZ* gene present in the yeast indicator plasmid pJLB has been described previously (10, 15). A similar construct containing the extended internal promoter BRE (−191 to −105) was generated by PCR with primers that inserted *Xho*I sites at each end of this DNA sequence, followed by insertion into the *Xho*I site present in pJLB. Wild-type and mutant forms of the HFV internal promoter Bel-1 minimal DNA binding site (−164 to −140, −169 to −140, or −164 to −135), as well as the minimal DNA binding site for Tas in the SFV-1 internal promoter (−69 to −45), were synthesized as oligonucleotides with flanking *Xho*I sites, annealed to make them double stranded, and then either cloned into the *Xho*I site present in the yeast indicator plasmid pJLB (10) or used directly in in vitro binding experiments. The internal promoter with a mutation from −149 to −144 substitutes 5′-CTCCCT-3′ in place of residues 5′-AGAAAG-3′. The orientation of inserts was screened by PCR and confirmed by DNA sequencing.

The construction of single-copy, ARS-CEN-based yeast expression plasmids encoding the GAL4-VP16(413–490) and GAL4-Bel-1(260–290) fusion proteins has been described (3). An expression plasmid encoding a GAL4-Tas(264–308) fusion protein was constructed in the same manner. Briefly, the ADH1 promoter region as well as the coding sequences of GAL4-Tas(264–308) was amplified from plasmid pY-GAL4-Tas(264–308) (3) by PCR with primers that introduced flanking *Bgl*II sites. The resultant DNA fragments were then digested with *Bgl*II and inserted into the *Bam*HI site present in the pYCplacIII expression plasmid polylinker.

Gel retardation analysis. A fusion protein consisting of glutathione *S*-transferase (GST) linked to residues 1 to 228 of Bel-1 was expressed in the protease-deficient XA90 strain of *Escherichia coli* by using the pGST/Bel-1(1–228) plasmid described previously (15). The fusion protein was purified to homogeneity by glutathione affinity chromatography followed by chromatography with a Bio-Rex 70 anion-exchange column (Bio-Rad).

The LTR probe used for gel retardation and methylation interference analysis was generated by PCR and extends from an *Xba*I site introduced at −108 to a *Bam*HI site introduced at −35 in the HFV LTR. The internal promoter probe extends from a *Bam*HI site introduced at −191 to an *Xba*I site introduced at −105 relative to the HFV internal promoter cap site (15). The anti-GST monoclonal antibody used for supershift experiments was obtained from Santa Cruz Biotechnology.

DNA probes were labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase, and the total isotope incorporation was determined by scintillation counting after column purification. The binding reaction was carried out with ~10^4 cpm (~0.2 ng) of the probe and various amounts of GST–Bel-1(1–228) fusion protein in 40 μl of binding buffer as described previously (15). Binding was allowed to proceed for 30 min at 4°C before the reaction products were resolved on a 5% native polyacrylamide gel and visualized by autoradiography. For competition experiments, competitor DNAs were incubated with GST–Bel-1 for 10 min prior to addition of the probe. A 154-bp DNA fragment containing the Mason-Pfizer monkey virus constitutive transport element (9) was amplified by PCR and served as a nonspecific competitor DNA. Quantitation of results of competition experiments was performed with a PhosphorImager and the Image QuaNT program (Molecular Dynamics).

Methylation interference analysis. The LTR probe was uniquely end labeled on the coding strand by filling in the recessed 3′ end at the introduced *Bam*HI site with [α-^{32}P]GTP by using avian myeloblastosis virus reverse transcriptase. The internal promoter probe was similarly labeled on the coding strand at an *Xba*I site with [α-^{32}P]CTP. About 10^6 cpm of each probe was methylated with 1 μl of dimethylsulfide for 1 min at room temperature in 200 μl of methylation buffer (50 mM sodium cacodylate, pH 8.0; 1 mM EDTA, pH 8.0) (31). The methylation reaction was stopped by adding 40 μl of stop buffer (1.5 M sodium acetate, pH 7.0; 1 M 2-mercaptoethanol), and the probe was isolated by ethanol precipitation. The probe was then incubated with 200 ng of the GST–Bel-1(1–228) fusion protein in 200 μl of binding buffer for 20 min at 4°C, and the unbound and bound DNA fractions were resolved by native polyacrylamide gel electrophoresis, located by autoradiography, excised, and eluted. The purified DNA was dissolved in 30 μl of 10 mM sodium phosphate (pH 6.8)–1 mM EDTA and incubated for 10 min at 92°C, and then 3 μl of 1 M NaOH was added to the solution and the incubation was continued for 30 min at 92°C. The DNA was precipitated and dissolved in sequencing buffer. The resultant end-labeled DNA fragments were resolved on an 8% sequencing gel.

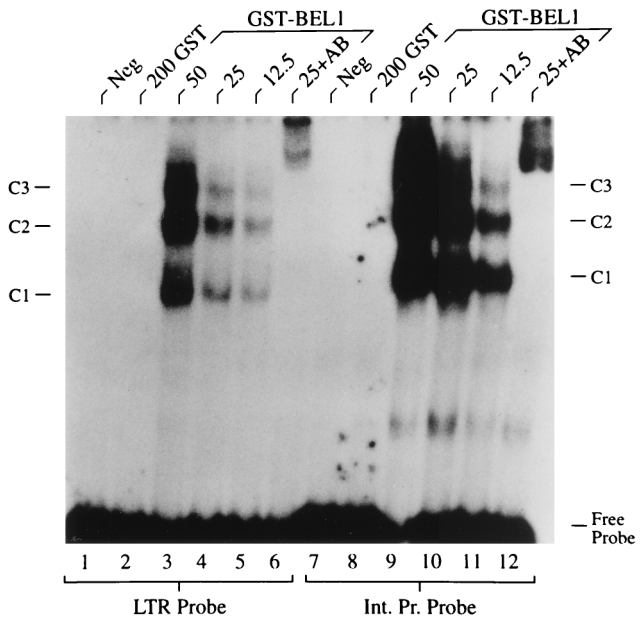

FIG. 1. Bel-1 binds to target DNA sites located in both the LTR and internal promoters of HFV. EMSA showing binding of increasing levels of a purified, recombinant GST–Bel-1(1–228) fusion protein to an HFV LTR and internal promoter (Int. Pr.) probe. Numbers above the lanes indicate the amounts, in nanograms, of fusion protein used. Controls include no added protein (Neg.) or addition of 200 ng of purified GST. Lanes 6 and 12 show a supershift obtained by addition of an anti-GST monoclonal antibody (~50 ng). C1, C2, and C3 are retarded complexes displaying different electrophoretic mobilities.

Yeast transformation and analysis. The Bel-1 and Tas yeast expression plasmids and pJLB-derived *lacZ* indicator plasmids were cotransformed into the yeast strain PSY 316 (2). After 3 days of growth selection in media lacking uracil and leucine, yeast cell extracts were prepared and assayed for β-galactosidase (β-Gal) activity as described previously (3). Yeast plasmids expressing fusion proteins consisting of the GAL4 DNA binding domain and the various viral activation domains were transformed into the yeast strain Y190, which harbors an integrated *lacZ* reporter construct (14). After 3 days of growth selection in medium lacking leucine, β-Gal assays were carried out as described previously (3).

RESULTS

Using DNase I protection analysis, we have previously mapped DNA binding sites for the HFV Bel-1 protein to between −84 and −32 relative to the LTR promoter cap site and between −171 and −135 relative to the internal promoter cap site (15). The ability of these HFV DNA sequences to bind the Bel-1 protein specifically in vitro was confirmed in the electrophoretic mobility shift assay (EMSA) shown in Fig. 1, which shows that a recombinant GST–Bel-1 fusion protein is able to interact with both an LTR DNA probe (LTR sequences −108 to −35) (lanes 3 to 5) and an internal promoter DNA probe (residues −191 to −105) (lanes 9 to 11). Of interest, under identical assay conditions, and with similar levels of LTR and internal promoter probes labeled to closely comparable specific activities, a significantly higher percentage of the internal promoter probe than of the LTR probe was bound by GST–Bel-1 (compare lanes 5 and 11). The observed probe shifts were due to the Bel-1 protein, in that GST itself failed to bind either probe (lanes 2 and 8). All three observed protein-DNA complexes were supershifted by addition of an anti-GST monoclonal antibody (Fig. 1, lanes 6 and 12), thus demonstrating that these were due to the GST–Bel-1 fusion protein and not to a contaminating DNA binding activity. The fact that at least three distinct protein-DNA complexes, labeled C1, C2,

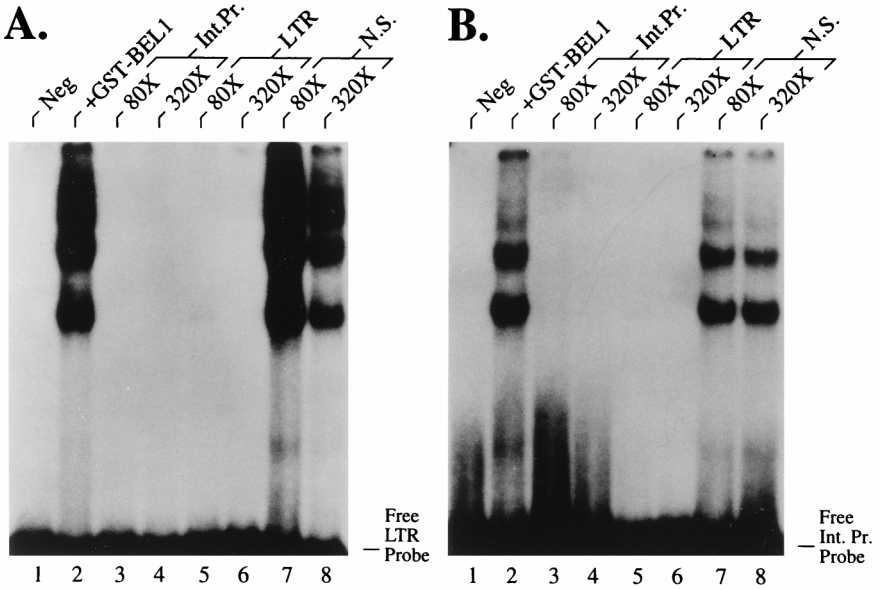

FIG. 2. DNA binding by HFV Bel-1 is specific. The direct interaction of GST–Bel-1 (25 ng) with an HFV LTR (A) or internal promoter (Int. Pr.) DNA probe (B) was specifically blocked by preincubation with an 80- or 320-fold molar excess of unlabeled forms of these same two DNA probes but was not blocked by preincubation with a similar excess of a nonspecific (N.S.) DNA competitor.

and C3, were observed in this EMSA is of interest, given the previous report that Bel-1 is able to form multimers in vivo (6). However, it should be noted that the linked GST protein can also form dimers.

We next sought to confirm that the observed Bel-1–DNA interactions are specific. As shown in Fig. 2, the ability of GST–Bel-1 to bind to either an LTR DNA probe (Fig. 2A) or an internal promoter probe (Fig. 2B) was efficiently competed by an excess of either the unlabeled internal promoter probe (lanes 3 and 4) or the LTR probe (lanes 5 and 6) but was essentially unaffected by the same level of a nonspecific DNA competitor (lanes 7 and 8). Therefore, this DNA binding event is clearly specific and the interactions of Bel-1 with the DNA target sequences are likely to be mechanistically similar.

Bel-1 binds the internal promoter more effectively than the LTR promoter. As noted above, the GST–Bel-1 fusion protein used in these assays bound to a higher percentage of the internal promoter probe than of the LTR promoter probe when incubated under the same assay conditions (Fig. 1). This observation suggests that the internal promoter may contain a higher-affinity binding site for Bel-1 than the one present in the LTR. To address this issue in more detail, we performed a quantitative EMSA with the internal promoter probe and several different levels of unlabeled internal promoter, LTR, or nonspecific competitor DNAs. As shown in Fig. 3, the internal promoter-derived competitor DNA (lanes 3 to 6) proved a far more effective competitor of the internal promoter probe–Bel-1 binding reaction than did the LTR-derived competitor (lanes 7 to 10). Therefore, it is clear that this internal promoter target sequence is a significantly higher-affinity in vitro DNA binding site for Bel-1 than is the tested HFV LTR target sequence.

Modification interference analysis of Bel-1 DNA binding. We next wished to identify specific residues within the internal promoter and LTR DNA probes required for Bel-1 binding by modification interference analysis. For this purpose, the −108 to −35 HFV LTR probe and the −191 to −105 internal promoter probe were each uniquely end labeled and then incubated with dimethylsulfide, which specifically methylates G and A residues (31). The modified probes were then used for EMSA, and the C1 and C2 complexes (Fig. 1) observed with the internal promoter probe and the C1 complex observed with the LTR probe were excised, purified, and chemically cleaved. The pattern of DNA modification observed in these shifted DNA complexes was then compared to the pattern seen in the free-DNA probes.

As shown in Fig. 4, this experimental approach identified several bases whose modification resulted in an inhibition of interaction with the GST–Bel-1 fusion protein. In the internal promoter DNA probe, marked inhibition of binding was observed upon modification of residues −158G, −157G, −148G, −144G, and −143A (Fig. 4). More modest interference with binding was noted upon modification of residues −152G, −151G, and −149A. No additional modification interference was observed in the C2 complex compared to the C1 complex (Fig. 4, compare lanes 2 and 3), thus suggesting either that the lower-mobility C2 complex results entirely from a protein-protein interaction or that any second Bel-1 DNA binding event is relatively nonspecific. Analysis of LTR probe binding by Bel-1 by using modification interference showed significant inhibition upon modification of residues −59G and −55G and modest interference upon modification of residue −65G (Fig. 4, lanes 4 and 5).

The analysis presented in Fig. 4 identifies several residues critical for Bel-1 binding and localizes these between −158 and −143 in the internal promoter and between −65 and −55 in the LTR promoter. Therefore, for both the internal promoter and the LTR promoter, the mapped residues are centered in the Bel-1 binding sites previously mapped to between −171 and −135 in the internal promoter and to between −84 and −32 in the LTR promoter, using DNase I footprinting. An alignment of these two Bel-1 binding sites, shown in Fig. 5, suggests that several purine residues identified as important for Bel-1 DNA binding in Fig. 4 are conserved between the high-affinity internal promoter and lower-affinity LTR Bel-1 binding sites.

Identification of a minimal Bel-1 DNA binding site. We next wished to determine if the minimal, ~25-bp internal promoter

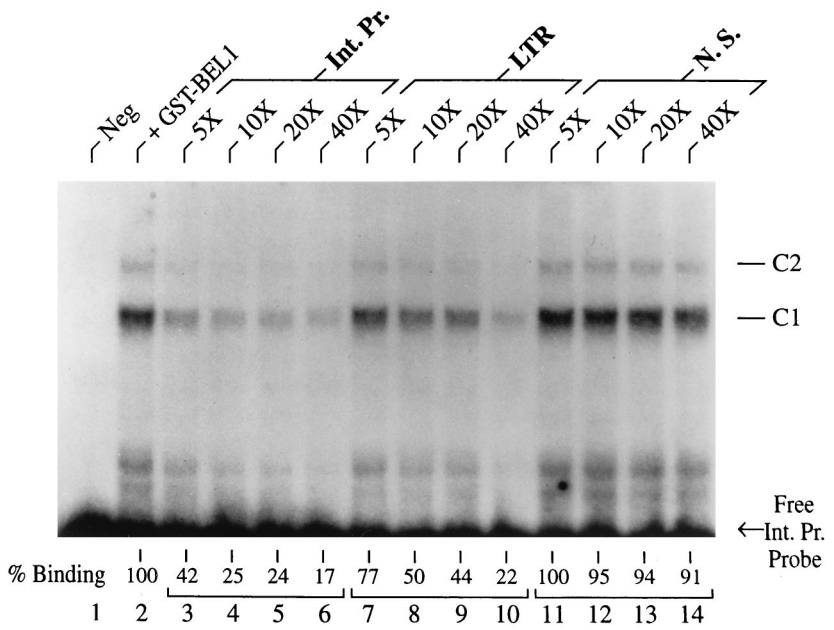

FIG. 3. Bel-1 binds the internal promoter DNA target more effectively than the LTR DNA target. This EMSA measures the degree of inhibition of GST–Bel-1 protein (25 ng) binding to an internal promoter (Int. Pr.) DNA probe observed upon preincubation with a 5-, 10-, 20-, or 40-fold molar excess of unlabeled forms of the internal promoter or LTR Bel-1 binding site or a nonspecific (N.S.) DNA competitor. The degree of inhibition seen with each competitor was measured with a phosphorimager and is given at bottom as percent residual binding, with binding in the absence of competitor (lane 2) set at 100%. As can be seen, the internal promoter probe competes more effectively for Bel-1 binding than does the LTR DNA probe. Little competition is observed with similar levels of the nonspecific DNA competitor. Neg., no added protein (control).

Bel-1 binding site delineated in Fig. 5 is indeed a complete, functional Bel-1 binding site. For this purpose, we prepared synthetic, double-stranded DNA oligonucleotides containing the wild-type 25-bp internal promoter sequence shown in Fig. 4 or containing the same sequence bearing a 6-bp mutation between residues −144 and −149. As shown in Fig. 6, the wild-type oligonucleotide proved able to effectively compete for Bel-1 binding to the full-length (−191 to −105) internal promoter probe (lanes 3 to 5). However, introduction of a mutation into this minimal DNA binding site blocked this competition (lanes 6 to 8).

Previously, we have demonstrated that Bel-1 is able to bind to the HFV LTR BRE in yeast cells and activate a linked minimal yeast promoter element (15). Importantly, this activation was shown to be dependent on LTR sequences that are also critical for Bel-1 DNA binding in vitro but was independent of flanking DNA sequences that, while not essential for DNA binding in vitro, are nevertheless critical for Bel-1 function in mammalian cells. We therefore asked whether Bel-1 would also bind the internal promoter BRE in yeast cells and, in particular, whether the minimal 25-bp internal promoter sequence shown to bind Bel-1 in vitro (Fig. 6) would also suffice to bind Bel-1 in vivo. For this purpose, we inserted the extended HFV internal promoter Bel-1 binding site (−191 to −105), or various truncated versions thereof, in front of the minimal *cyc* promoter element located 5′ to the *lacZ* indicator gene in the yeast indicator plasmid pJLB (10). Shorter sequences were inserted in both the sense and antisense orientations to address the possibility that flanking sequences were contributing fortuitously to any observed Bel-1 binding activity. These indicator plasmids were then introduced into yeast cells along with a previously described (3) high-copy-number Bel-1 expression plasmid or with an appropriate control plasmid.

As shown in Table 1, and also demonstrated previously (15), insertion into the pJLB yeast indicator plasmid of the extended HFV LTR Bel-1 binding site (−112 to −31) results in activation of the linked *lacZ* indicator gene upon expression of the Bel-1 protein in *trans*. Insertion of the extended internal promoter Bel-1 binding site (−191 to −105) also resulted in activation of *lacZ* expression. Remarkably, however, this activation was almost 200-fold higher than the level seen with the equivalent LTR sequence (Table 1). Insertion of the 25-bp candidate minimal internal promoter Bel-1 binding site (−164 to −140) resulted in a level of activation that was ~15% (sense orientation) or ~43% (antisense orientation) of the level seen with the entire (−191 to −105) sequence. We next asked whether a minor, 5-bp extension of the inserted 25-bp internal promoter sequence in either the 5′ (−169 to −140) or 3′ (−164 to −135) direction would enhance the Bel-1 response. As shown in Table 1, extension in the 5′ direction enhanced the level of activation modestly while extension in the 3′ direction had no significant effect. Remarkably, however, all of these short internal promoter-derived DNA sequences, including the minimal 25-bp sequence shown in Fig. 5, generated a level of indicator gene activation that was far higher than seen with the full-length HFV LTR-derived Bel-1 target sequence.

To examine whether the minimal 25-bp internal promoter Bel-1 binding site defined in Fig. 5 could display functional synergy, and to also more fully demonstrate the specificity of Bel-1-mediated gene activation in yeast cells, we next constructed pJLB-based indicator plasmids containing two tandem copies of wild-type and mutant forms of the 25-bp internal promoter sequence shown in Fig. 5. We also constructed an indicator plasmid containing a single copy of a 25-bp SFV-1 internal promoter sequence (Fig. 5) centered on residues previously identified by Zou and Luciw (34) as likely to be important for binding of SFV-1 Tas by DNase I footprinting analysis. In order to maximize the potential for detection of functional synergy, and also because expression of SFV-1 Tas in yeast cells from a multicopy expression plasmid produces significant

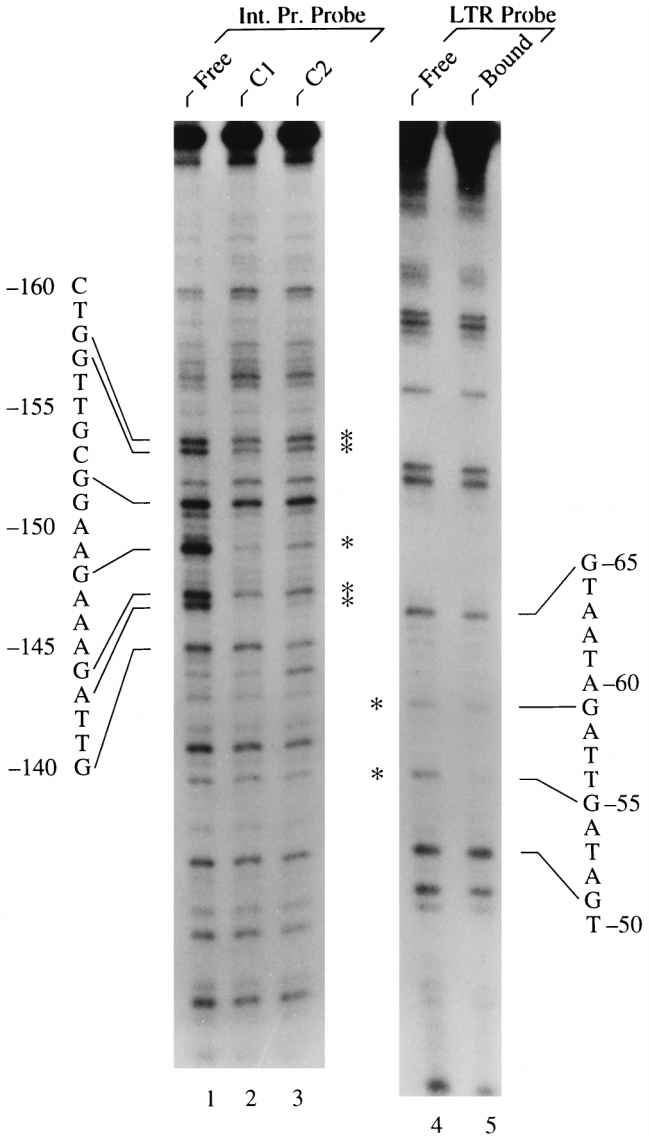

FIG. 4. Identification of critical residues for Bel-1 binding in the internal and LTR promoters. Modification interference analysis was used to identify purine residues present in the internal promoter (Int. Pr.) and LTR promoter that are critical for in vitro binding by the GST–Bel-1 fusion protein. Two DNA-protein complexes of different electrophoretic mobilities that form on the internal promoter probe, labeled C1 and C2 (Fig. 1), were analyzed independently, with comparable results. Residues showing marked interference are indicated by asterisks.

toxicity (reference 3 and data not shown), we introduced these indicator plasmids into yeast cells along with single-copy yeast expression plasmids encoding full-length forms of Bel-1 or Tas.

As shown in Table 2, yeast indicator plasmids containing a single-copy of the minimal internal promoter were again found to give readily detectable levels of β-Gal activity upon expression of the Bel-1 protein in *trans*. The reproducibly lower level of activity reported in Table 2 than in Table 1 likely results from the far lower level of Bel-1 expression generated by the single-copy, ARS-CEN-based Bel-1 expression plasmid used here than by the high-copy-number, 2μm-based Bel-1 expression plasmid utilized in the experiments whose results are reported in Table 1. Insertion of a second copy of this 25-bp sequence resulted in the synergistic activation of the linked *cyc* promoter element, resulting in an ~10-fold-higher level of activation than seen with indicator plasmids bearing only a single copy. This activation was dependent on the integrity of the introduced candidate Bel-1 binding site, because mutation of this site, in the context of the double-copy plasmid, blocked the Bel-1 response (Table 2).

The parental pJLB indicator plasmid, lacking any inserted viral sequence, failed to respond to Bel-1 protein expression, as did also a pJLB-derived indicator plasmid containing a single copy of a potential Tas binding site derived from the SFV-1 internal promoter. However, this SFV-1-based indicator plasmid was strongly activated by expression in *trans* of the homologous SFV-1 Tas protein. While the Tas protein was able to only very modestly activate β-Gal expression directed by plasmids containing a single-copy of the HFV internal promoter sequence (Table 2), it did prove able to activate the indicator plasmids containing two copies of the wild-type, but not mutant, HFV internal promoter sequence (Table 2). Therefore, it appears possible that the HFV and SFV-1 internal promoters may retain some degree of sequence similarity that permits a low-affinity interaction of Tas with the HFV internal promoter sequence. However, these binding sites clearly do not demonstrate any marked homology (Fig. 5).

Comparison of the activation domains of Bel-1 and Tas. A surprising result reported in Table 2 is that the interaction, in yeast cells, of SFV-1 Tas with a single copy of its minimal internal promoter binding site resulted in ~12 times more activation of the linked *lacZ* indicator gene than did the interaction of Bel-1 with a comparable single-copy sequence from the HFV internal promoter. One possible explanation for this phenomenon is that the HFV internal promoter binding site used in these constructs is incomplete. However, the observation that an extended internal promoter Bel-1 binding site is only a slightly more effective target for Bel-1 in vivo (Table 1) makes this explanation unlikely. An alternative possibility is that the transcription activation domain of SFV-1 Tas is significantly more active than the equivalent domain in HFV Bel-1. To examine this possibility, we linked the previously mapped (3, 12, 16, 27, 32) transcription activation domains of Bel-1 and of Tas to the DNA binding domain of GAL4 to determine whether expression of these fusion proteins in yeast cells would induce different levels of activation of a integrated yeast indicator gene bearing GAL4 DNA binding sites. Plasmids expressing fusion proteins consisting of the GAL4 DNA binding domain (aa 1 to 117) linked to various activation domains were introduced into the yeast strain Y190. After 3 days of selection, β-Gal activity was determined as previously described (3). The GAL4 DNA binding domain alone induced a β-Gal activity of ≤1 mOD/ml, while the GAL4-VP16 fusion protein induced had a β-Gal activity of 5,645 mOD/ml. As previously reported (3), the Bel-1 activation domain, while clearly functional, is nevertheless ~90-fold less active (β-Gal activity of 61 mOD/ml) than the potent activation domain present in the VP16 transcription factor when tested either in yeast cells, as in this case, or in mammalian cells. In contrast, the Tas activation domain was found to be ~14-fold more active than the Bel-1 activation domain when tested in this yeast assay system (β-Gal activity of 850 milli-optical density units [mOD]/ml). Therefore, it appears that the ~12-fold difference in the activities of Bel-1 and Tas noted in Table 2 is likely primarily caused by a comparable difference in activation domain function. However, this finding does not exclude the possibility that Tas may also bind its internal promoter target site with a somewhat higher affinity than does Bel-1.

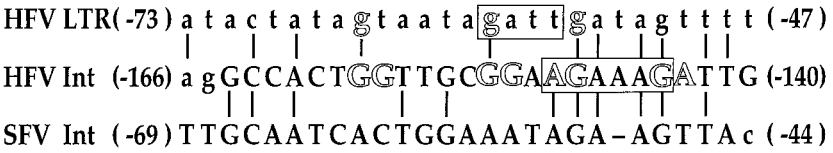

FIG. 5. Alignment of possible Bel-1 and Tas minimal binding sites. Candidate minimal binding sites for HFV Bel-1 located in the LTR and internal promoter (Int. Pr.) elements are indicated and aligned. Purine residues that gave rise to detectable inhibition of Bel-1 binding after methylation are indicated. A candidate minimal SFV-1 Tas DNA binding site, based on the DNase I footprinting studies of Zou and Luciw (34), is also given, although the alignment used is largely arbitrary and does not show significant conservation of purine residues shown to be critical for Bel-1 binding. DNA sequences used for subsequent analyses are in capital letters. Boxes define residues whose mutation blocks Bel-1 binding in vitro and function in vivo (15).

DISCUSSION

The expression of genes contained by the primate foamy viruses is regulated by the interplay of two viral promoter elements, located in the LTR and at the 3′ end of the *env* gene, with the viral Bel-1/Tas transcriptional transactivator (29). Early after infection, the internal promoter is activated, resulting in the synthesis of mRNAs encoding the foamy virus accessory proteins, including Bel-1/Tas (21, 25). Subsequently, the LTR promoter is activated, presumably as a result of Bel-1/Tas expression, and mRNAs encoding the Gag, Pol, and Env structural proteins begin to accumulate. Therefore, although foamy viruses are not known to encode posttranscriptional regulatory proteins equivalent to the Rev and Rex proteins found in other primate complex retroviruses (7, 19), they nevertheless appear to be similar in that they display a temporal regulation of viral gene expression. However, the basis for this temporal order has been unclear, given that the internal promoter does not appear to be significantly more active than the LTR promoter in either the presence or the absence of the relevant Bel-1/Tas protein (5, 22, 25).

In this article, we describe a series of experiments designed to shed further light on the interaction of HFV Bel-1 with both the LTR and internal promoter elements. An interesting result to emerge from this analysis is that the Bel-1 DNA binding site present in the HFV internal promoter element is a significantly higher-affinity binding site for Bel-1 both in vitro and in vivo than is the cap-proximal Bel-1 binding site located in the HFV LTR promoter element. In particular, when analyzed by EMSA under identical assay conditions, Bel-1 was found to shift a higher percentage of an internal promoter probe than of a similar LTR promoter probe (Fig. 1). Similarly, internal promoter sequences also proved able to compete more effectively for Bel-1 DNA binding than did LTR promoter sequences when analyzed in a quantitative EMSA (Fig. 3). Finally, when assayed for in vivo DNA binding in yeast cells by a previously described assay, Bel-1 was able to activate a linked *lacZ* indicator gene ~200-fold more effectively when targeted to the internal promoter Bel-1 DNA binding site than when targeted to an equivalent LTR-derived Bel-1 binding site (Table 1). Taken together, these data suggest that activation of the foamy virus internal promoter may precede activation of the LTR promoter during the foamy virus life cycle because the internal promoter acts as a more effective Bel-1 protein binding site when Bel-1 levels are limiting, as they are predicted to be early in infection. We caution, however, that this analysis only examined binding to the cap-proximal BRE present in the HFV LTR and did not address the affinity of Bel-1 for other proposed LTR BRE elements (8, 20, 33). Although the cap-proximal BRE is fully sufficient to direct essentially wild-type levels of LTR-driven transcription in the presence of the Bel-1 protein in mammalian cells (18), these more 5′ putative BREs could potentially play an important role in modulating the level of response of the LTR promoter to Bel-1, particularly given the observation (Table 2) that Bel-1 binding sites can display functional synergy.

Using modification interference and EMSA, we were able to identify several purine residues within both the LTR and internal promoters that are critical for Bel-1 binding in vitro (Fig. 4). We were then able to use this information to align these two sites (Fig. 5) and to propose a potential minimal, 25-bp DNA binding site for Bel-1. This minimal site was then shown to in fact function as an effective Bel-1 DNA binding site both in vitro (Fig. 6) and in vivo (Tables 1 and 2). The identification of this minimal Bel-1 DNA binding site will permit the future mutational definition of individual bases that form a functional binding site and should also allow the identification of residues that attenuate Bel-1 binding to the LTR promoter compared to the internal promoter. It will clearly be of interest to test whether an enhancement in the affinity of the HFV LTR binding site for Bel-1 results in a disruption of the normal temporal order of HFV gene expression.

As part of this analysis, we also tested whether a 25-bp sequence derived from the SFV-1 internal promoter, first identified as important for Tas binding by DNase I footprinting (34), was sufficient to function as an effective Tas binding site

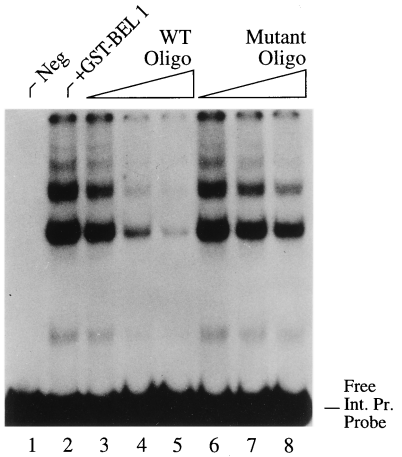

FIG. 6. A 25-bp internal promoter sequence competes for specific Bel-1 DNA binding. A double-stranded, synthetic oligonucleotide consisting of the 25-bp internal promoter (Int. Pr.) sequence (−164 to −140) shown in Fig. 5 specifically competed Bel-1 binding to the full-length (−191 to −105) internal promoter probe (lanes 3 to 5). In contrast, a similar internal promoter-derived oligonucleotide containing mutations at residues −149 to −144 (Fig. 5) failed to compete (lanes 6 to 8). This competition experiment used 4-, 20-, and 80-fold molar excesses of each competitor oligonucleotide. WT, wild type; oligo, oligonucleotide.

TABLE 1. The HFV internal promoter contains a high-affinity in vivo Bel-1 DNA binding site

Promoter	β-Gal activity[a]
pJLB	≤5 (~0)
LTR (−112 to −31)	450 (0.6)
Int. Pr. (−191 to −105)	74,960 (100)
Int. Pr. (−164 to −140)	10,930 (15)
Int. Pr. (−140 to −164)	32,530 (43)
Int. Pr. (−169 to −140)	27,420 (37)
Int. Pr. (−140 to −169)	41,090 (55)
Int. Pr. (−164 to −135)	28,630 (38)
Int. Pr. (−135 to −164)	26,472 (35)

[a] Sequences derived from the HFV LTR or internal promoter (Int. Pr.) were inserted 5′ of the minimal *cyc* promoter element present in the *lacZ*-based yeast indicator plasmid pJLB. Sense or antisense orientation is indicated by the promoter coordinates; e.g., the sequence from −169 to −140 is sense, while that from −140 to −169 is antisense. These reporter plasmids were introduced into yeast cells together with a multicopy Bel-1 expression plasmid (3) or a similar control plasmid. Induced β-Gal activities were measured after selection for cotransformants and are given in mOD/milliliter. No detectable activity was observed with any indicator plasmid in the absence of Bel-1. Activity levels are also given (in parentheses) relative to the extended internal promoter Bel-1 binding site, which is arbitrarily set at 100%. These data are the averages of four independent experiments.

in vivo. As shown in Table 2, this short sequence indeed proved fully sufficient to bind Tas efficiently. This observation allowed us to determine whether the inability of Tas to function effectively via HFV promoter elements in mammalian cells was due to an inability of Tas to bind these HFV DNA sequences or, instead, reflected the absence of a critical Tas cofactor binding site. As shown in Table 2, Tas was able to interact only poorly with the minimal internal promoter Bel-1 target site. It is therefore apparent that Tas and Bel-1 have evolved distinct DNA sequence specificities over time. The future definition of these differences will clearly be of interest.

A final interesting result is that the activation domain present in SFV-1 Tas was found to be significantly more active than the one present in Bel-1. This finding explains the earlier observation (3) that expression of SFV-1 Tas from a multicopy plasmid in yeast cells is significantly more deleterious for yeast growth than is expression of HFV Bel-1, in that it has previously been shown that the level of toxicity observed in yeast upon overexpression of an acidic transcription activation domain is a function of the activity of the tested domain (2). This difference also appears to explain the finding that the level of indicator gene expression induced by the binding of Tas to its minimal binding site in yeast cells is significantly higher than the level observed when using Bel-1 and its minimal DNA binding site (Table 2). The question of whether this difference in activation domain function observed in yeast cells is relevant to the effectiveness of transcriptional activation by Tas and Bel-1 in mammalian cells remains to be explored.

TABLE 2. Binding of Bel-1 and Tas to minimal DNA target sites in yeast cells[a]

Promoter	β-Gal activity with plasmid		
	Control	+Bel-1	+Tas
pJLB	≤5	≤5	≤5
HFV Int. Pr. (1× WT+)	≤5	86	11
HFV Int. Pr. (1× WT−)	≤5	167	17
HFV Int. Pr. (2× WT+)	≤5	701	120
HFV Int. Pr. (2× WT−)	≤5	1,262	54
HFV Int. Pr. (2× M+)	22	17	48
HFV Int. Pr. (2× M−)	22	23	31
SFV-1 Int. Pr. (1× WT+)	≤5	≤5	2,870
SFV-1 Int. Pr. (1× WT−)	≤5	≤5	714

[a] Wild-type (WT) or mutant (M) forms of the minimal 25-bp HFV or SFV-1 internal promoter (Int. Pr.) sequence shown in Fig. 5 were inserted in one (1×) or two (2×) copies into the *lacZ*-based yeast indicator plasmid pJLB. Orientation is indicated by a plus (sense) or minus (antisense) sign. These reporter plasmids were introduced into yeast cells together with single-copy plasmids expressing Bel-1 or Tas or a similar control plasmid. Induced β-Gal activity was measured after selection for cotransformants and is given in mOD/milliliter. These data are representative of three independent experiments.

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