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The Potent Enhancer Activity of the Polycythemic Strain of Spleen Focus-Forming Virus in Hematopoietic Cells Is Governed by a Binding Site for Sp1 in the Upstream Control Region and by a Unique Enhancer Core Motif, Creating an Exclusive Target for PEBP/CBF

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The polycythemic strain of the spleen focus-forming virus (SFFVp) contains the most potent murine retroviral enhancer configuration known so far for gene expression in myeloerythroid hematopoietic cells. In the present study, we mapped two crucial elements responsible for the high activity of the SFFVp enhancer to an altered upstream control region (UCR) containing a GC-rich motif (5′-GGGCGGG-3′) and to a unique enhancer core (5′-TGCGGTC-3′). Acquisition of these motifs accounts for half of the activity of the complete retroviral enhancer in hematopoietic cells, irrespective of the developmental stage or lineage. Furthermore, the UCR motif contains the major determinant for the enhancer activity of SFFVp in embryonic stem (ES) cells. Using electrophoretic mobility shift assays, we show that the UCR of SFFVp, but not of Friend murine leukemia virus, is targeted by the ubiquitous transcriptional activator, Sp1. The core motif of SFFVp creates a specific and high-affinity target for polyomavirus enhancer binding protein/core binding factor (PEBP/CBF) and excludes access of CAAT/enhancer binding protein. Cotransfection experiments with ES cells imply that PEBP/CBF cooperates with the neighboring element, LVb (the only conserved Ets consensus in the SFFVp enhancer), and that the Sp1 motif in the UCR stimulates transactivation through the Ets-PEBP interaction. Putative secondary structures of the retroviral enhancers are proposed based on these data.

The polycythemic strain of spleen focus-forming virus (SFFVp) is a replication-incompetent murine leukemia virus (MuLV) encoding the truncated *env* glycoprotein gp55, which stimulates the cellular receptor for erythropoietin. When propagated by with the replication-competent Friend MuLV (FMuLV), it induces a highly acute bistage erythroleukemia in susceptible mice. The host range specificity of SFFVp-induced erythroblastosis is due to the activity of gp55 on EpoR (13, 16, 31, 64, 67), whereas the enhancer of SFFVp, closely related to that of endogenous polytropic retroviruses belonging to the Friend mink cell focus-forming (FMCF) virus family, has outstanding activity throughout all stages and lineages of hematopoietic development (8). Retroviral vectors utilizing the SFFVp enhancer are thus excellent tools for gene transfer and expression in hematopoietic cells (8, 15) and enable sustained multilineage gene expression in vivo (62). Definition of the molecular mechanisms by which SFFVp exerts its potent enhancer functions is expected to contribute significantly to our understanding of retroviral enhancer evolution. It might also create a more rationally defined basis for the design of retroviral vectors mediating elevated gene expression efficiencies in transduced hematopoietic progenitor cells, applicable for both experimental hematology and gene therapy.

The retroviral enhancer resides in the U3 region of the long terminal repeat (LTR). Here, a battery of *cis*-acting elements is condensed in a complex array, part of which is often found duplicated as a direct repeat (20). The tissue tropism of the enhancer is mostly responsible for the disease specificity of slowly transforming retroviruses like the lymphotropic Moloney MuLV (MoMuLV) or the more erythrotropic FMuLV (32). Little changes in the enhancer often have striking consequences for the host range. An example is the myeloproliferative sarcoma virus (MPSV), which is closely related to MoMuLV but shows increased activity in primitive myeloerythroid cells and in embryonic stem (ES) cells. This is largely explained by a high-affinity binding site for the ubiquitous transcription factor, Sp1, in the GC-rich 3′-flanking region of the direct repeat (1, 21, 55). Enhancer alterations can also influence the disease dynamics of retrovirus-induced murine leukemias. Thymic lymphomas caused by MoMuLV, for example, develop with increased latency when point mutations are introduced in the enhancer core or cooperating sequences (53). Sequence comparison of the SFFVp enhancer with the corresponding region of FMuLV (Fig. 1) and other MuLVs reveals at least three intriguing features which might contribute to its wide activity in hematopoeitic cells.

(i) We observed that many FMCF-type viruses, including the SFFVp strain studied here, show a consensus for the ubiquitous transcription factor Sp1 at the 5′ basis of the direct repeat. The corresponding region in MoMuLV and FMuLV is known

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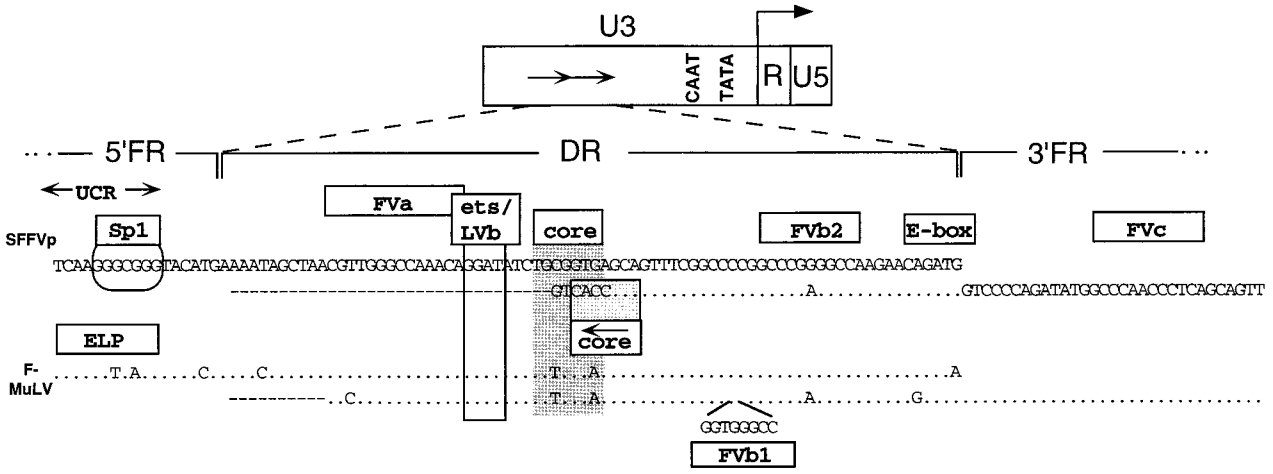

FIG. 1. Alignment of the enhancer regions of SFFVp and FMuLV (modified as in references 9, 18, and 49). Shown are the direct repeats (DR) and their 5′- and 3′-flanking regions (5′FR and 3′FR). Dots indicate sequence homology with SFFVp, and dashes represent deletions. Putative transcription factor-binding sites in the enhancers are shown above or below the sequences. Shaded boxes accentuate the presence of the enhancer core. The open box delineates the conserved *ets* site, LVb. The open rounded box delineates the putative Sp1 site. For abbreviations of binding sites, check Introduction and references therein. FVa, FVb, FVc, Friend virus factors a, b, and c, respectively (36, 37).

as the upstream control region (UCR). It contains a binding site for the putative embryonic repressor activity (embryonal LTR binding protein [ELP]) (60), which is the mouse homolog of the *Drosophila* FTZ-1 protein (61). Disruption of the ELP consensus by a single point mutation might contribute to the elevated activity of MPSV and its derivative, PCMV (for PCC4-cell passaged MPSV), in embryonic cells (1). Of note, the two mutations found in the UCR of SFFVp create a putative Sp1 motif while disrupting the ELP consensus (Fig. 1).

(ii) The enhancer core of FMCF-type viruses is almost invariably altered in comparison to MoMuLV and FMuLV. A second copy of this core is located in the junction to the truncated copy of the second direct repeat (54). The core motif of MuLV is shared with simian virus 40 (SV40) and mouse polyomavirus (65). It represents the anatomical and functional center of the direct repeat region (20). This motif is target for several transcription factors, including CAAT/enhancer binding protein (C/EBP) and core binding factor (CBF), also known as polyomavirus enhancer binding protein (PEBP). Distinct C/EBP family members contribute to tissue-specific gene expression in the liver, in adipocytes, and in myeloid cells (reviewed in reference 42). PEBP/CBF is of outstanding importance for the regulation of both retroviral and endogenous genes in lymphoid and myeloid precursor cells (26, 44, 47, 57, 58).

(iii) When compared with the more lymphotropic MoMuLV, the leukemia virus b (LVb) element is the only conserved recognition site for Ets transcription factors in both FMuLV- and FMCF-type enhancers. Ets factors binding to LVb cooperate with PEBP/CBF in transactivation of the MoMuLV enhancer (56). Similar mechanisms are involved in lineage-specific regulation of a variety of endogenous genes expressed in hematopoiesis (26, 44, 47, 57, 58).

In this study, we have determined the functional significance of the sequence alterations in the UCR and enhancer core of SFFVp and identified the cognate transcriptional activators as Sp1 and PEBP/CBF, respectively. We show that the Sp1 motif in the UCR supports transactivation through the LVb/core region in the ES cell system.

MATERIALS AND METHODS

Plasmid construction. Chloramphenicol acetyltransferase (CAT) vectors for deletion mapping of U3 responsive elements were based on SF-CAT (8) as follows: ΔB-CAT, deletion up to the *Bsp* 143I site (position +96 according to LTR sequence numbering in reference 54); ΔE-CAT, deletion up to the *Eco*RV site (+144); ΔS-CAT, deletion up to the *Sma*I site (+174); ΔES-CAT, deletion between *Eco*RV (+144) and *Sma*I (+174). ΔX-CAT is derived from MP-CAT (8) by *Nhe*I-*Xba*I digestion and religation, which removes the complete enhancer, leaving only the extended retroviral promoter.

SSS-CAT was constructed by introducing an *Nhe*I site into the 5′ part of the SFFVp U3 by mutagenizing PCR with oligonucleotides Nhe+ and pp13 and ligating the amplified fragment after *Nhe*I-*Sma*I digestion with the 270-bp *Sma*I fragment of the SFFVp LTR into an MP-CAT backbone opened with *Nhe*I and *Sma*I. SSS-CAT lost the incomplete second direct repeat of the original SFFVp clone 5.7 by recombination at the plasmid level. In FFS-CAT, FFF-CAT, and SSF-CAT, SSS-CAT sequences between *Pst*I (+41) and *Eco*RV (+144), *Pst*I (+41) and *Ava*I (+172), and *Eco*RV (+144) and *Ava*I (+172), respectively, were replaced by the corresponding fragments derived from FMuLV c157. SFS-CAT, SFa5-CAT, and SFa6-CAT were generated on the basis of SSS-CAT by mutagenizing PCR with oligonucleotides SFa4, SFa5, and SFa6, respectively. Sequences of oligonucleotides used for PCR were as follows: Nhe+, 5′-CCAAG TTGCTTAGCCTGCTAGCTGCAGTAACGC-3′; pp13, 5′-ACCGCATCTGG GGACCAT-3′; SFa4, 5′-TTCAGATCAAGGGCGGGTACACGAAAACAGC TAACG-3′; SFa5, 5′-CCGCAGATATCTTGTTTGGCCCA-3′; SFa6, 5′-AACAGGATATCAGCCGTAAGCA-3′.

pRc/Fli-I was kindly provided by C. Bartholomew and R. Nibbs, The Beatson Institute for Cancer Research, Glasgow, Scotland; it contains the cDNA of the murine Ets factor Fli-I under control of the cytomegalovirus promoter in pRC/CMV (Invitrogen). pEF-BOSαB1, an expression vector for the cDNA of murine PEBP2αB1, has been described previously (4).

Cell lines. The indicator cell lines for mapping of responsive elements and preparation of nuclear extracts were as follows: FDCP-mix 15S and CFU-mix, equivalent murine cell lines; WEHI-3B D−, macrophage progenitor cell line; F4-12-B2, erythroid murine progenitor and Friend cell line; COS-7, ape kidney epithelium-derived cell line; CCE, murine ES cell line. The maintenance and sources of these cell lines have been reported previously (7, 8, 21, 28). Experiments with ES cells (CCE) were performed in medium supplemented with recombinant leukemia-inhibitory factor in the absence of feeder cells.

Transfections and reporter gene assays. The cells were transfected by a very efficient electroporation method (7). Reporter gene assays were performed essentially as described previously (8), by using the CAT enzyme-linked immunosorbent assay (Boehringer, Mannheim, Germany) to detect CAT protein and the *o*-nitrophenyl-β-D-galactopyranoside (ONPG) assay (50) to detect cotransfected β-galactosidase activity in cell extracts harvested 24 to 48 h after electroporation in the presence of 10 μg of CAT plasmid per assay plus 10 μg of pCMVβ (Stratagene, Heidelberg, Germany) per assay. As indicated in Results, in some experiments 5 μg/assay of control plasmid pUC19 or transcription factor expression plasmids was also added.

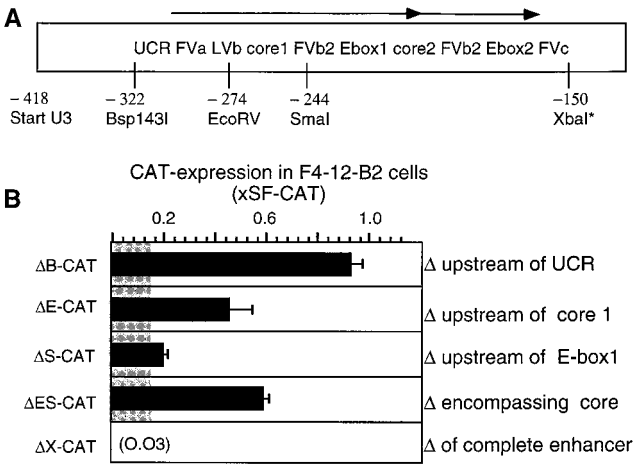

FIG. 2. Deletion analysis of responsive elements within the SFFVp U3 region. (A) Localization of the restriction sites used for construction of the mutant U3 regions. (B) Transfections and reporter assays were performed as described in Materials and Methods. CAT expression values obtained with the deletion-mutated vectors are given with respect to those obtained with SF-CAT containing the complete SFFVp U3. Mean values of three transfections in F4-12-B2 cells ± standard deviation (error bars) are shown. The grey zone delineates the activity of the MoMuLV U3 region in these cells (8). *, in MPSV.

Band shift assays. Nuclear extracts from mammalian cell lines were prepared as described in reference 2 but with Pefabloc (Boehringer) at 5 mM (final concentration) as a protease inhibitor. Double-stranded oligonucleotide probes were labeled with T4 polynucleotide kinase and [γ-^{32}P]ATP under standard reaction conditions. The specific activity was determined by the method in reference 50. Labeled probes were purified from free radioactivity on NAP-10 columns (Pharmacia). The gel shift assays were performed as described in reference 3. Unless otherwise indicated, the binding reaction mixture (20 μl) contained 2 μg of nuclear extract, 20,000 cpm of end-labeled oligonucleotide (corresponding to 1.5 fmol), 20 μg of bovine serum albumin, 1 mM Pefabloc, 2 μg of poly(dI-dC), 12% glycerol, 10 mM HEPES-KOH (pH 7.9), 4 mM Tris · Cl (pH 7.9), 80 mM NaCl, 1 mM EDTA and 1 mM dithiothreitol. The incubation time was 20 min at 25°C. For competition assays, unlabeled specific oligonucleotides were added at molar excesses as indicated in Results. In some experiments, antisera were added as indicated in Results, with 20-min preincubation on ice before addition of the probe. Antisera to Sp1 (PEP2) and C/EBP β (Δ198) were obtained from Santa Cruz Biotechnology, Heidelberg, Germany. Antisera to PEBP/CBF were described previously (34).

Oligonucleotide sequences were as follows: MOcore, 5′-TATCTGTGGTAA GCA-3′ and 3′-ATAGACACCATTCGT-5′; SFcore, 5′-TATCTGCGGTGAG CA-3′ and 3′-ATAGACGCCACTCGT-5′; FRUCR, 5′-CAGATCAAGGTCA GGTACACGAA-3′ and 3′-GTCTAGTTCCAGTCCATGTGCTT-5′; SFUCR, 5′-CAGATCAAGGGCGGGTACACGAA-3′ and 3′-GTCTAGTTCCCGCC CATGTGCTT-5′.

RESULTS

Responsive elements of the SFFVp U3. Recent work in our laboratory has revealed that SFFVp contains the most potent retroviral enhancer configuration known so far for gene expression in hematopoietic progenitor cells (8, 15). To define the molecular mechanisms by which the enhancer of SFFVp exerts its powerful activity, we started with CAT expression vectors containing defined deletions in the retroviral enhancer. By using convenient restriction sites, serial 5′-to-3′ deletions were introduced into the U3 region of SFCAT, removing the region upstream of the UCR (ΔB-CAT), of the enhancer core (ΔE-CAT), or of the inverted second core motif (ΔS-CAT) or the region encompassing the first core element (ΔES-CAT). As shown by transient-transfection assays with erythroid F4-12-B2 cells, sequences located upstream of the UCR were responsible for less than 10% of the U3 activity (ΔB-CAT) (Fig. 2). Close to half of the activity was lost by deleting either the region upstream of the first enhancer core including the UCR (ΔE-CAT) or the region surrounding and including the first core element (ΔES-CAT). Deletion of both regions reduced the activity to about 20% (ΔS-CAT), a level still above that achieved with the complete MoMuLV-U3, which includes two direct repeats (Fig. 2). Similar results were obtained with human erythroleukemic K562 cells (data not shown).

The UCR and core of SFFVp contain essential sequence alterations. A new series of CAT vectors was cloned that lacked the truncated second copy of the direct repeat and thus the second core element, a situation found in several SFFVp isolates (49). Using these constructs, we concentrated on the UCR and on the core, because these regions were likely to contain crucial *cis*-acting elements based on sequence comparison (Fig. 1) and the deletion analysis described above.

By exchanging SFFVp sequences with those derived from FMuLV, which differ in only three regions from the SFFVp enhancer (Fig. 1 and 3A), a limited number of mutations was introduced (Fig. 3B). The SSS-CAT construct displays the UCR, the pyrimidine (Py) residues downstream of the UCR and the core motif of SFFVp. In FFF-CAT, these regions are derived from FMuLV; this enhancer configuration is found in SFFVa (anemic strain of SFFV) and those FMuLV variants that have lost the second copy of the direct repeat (49). FFS-CAT is homologous to FFF-CAT but contains the core of SFFVp. SFS-CAT is homologous to FFS-CAT with the exception of the UCR, which contains the putative Sp1 element. SSF-CAT is homologous to SSS-CAT but has the core of FMuLV and MoMuLV.

Transient-transfection assays carried out with these constructs in a series of hematopoietic and ES cells revealed that the SFFVp-type core and UCR present in SSS-CAT were needed for full activity of the SFFVp U3, whereas the Py exchange had no significant effect on enhancer function (Fig. 3B). When compared with SFFVp, the FMuLV enhancer present in FFF-CAT allowed 80% of the activity in COS cells, but was reduced to <20% in ES cells (CCE) and to 40 or 60% in hematopoietic cells FDCP-mix, F4-12-B2, or WEHI-3B, respectively. Most of this loss was attributable to the exchange of the UCR, since Py exchange alone (SFS-CAT) had no influence on enhancer activity. Introducing only the FMuLV-MoMuLV core in the context of the SFFVp U3 (SSF-CAT) reduced transcription to about 60 to 80% of the original levels in ES (CCE) and hematopoietic cells but not in COS cells. The presence of the SFFVp UCR aggravated the effect of the core exchange (compare SSS-CAT and SSF-CAT) in hematopoietic cells, since the resulting loss of activity was smaller in the presence of the FMuLV-type UCR (compare FFF-CAT and FFS-CAT).

Taken together, these data revealed that the SFFVp core motif needed the altered UCR motif for full activity. These effects were not restricted to certain lineages or stages within the myeloerythroid compartment. The influence of these elements was found to be dominant in the context of the complete retroviral enhancer.

The upstream control region of SFFVp is a target for Sp1. Band shift data obtained with ES and hematopoietic cells revealed that Sp1 binds to the UCR of SFFVp. Oligonucleotides that span the UCR of SFFVp (SFUCR) or FMuLV (FRUCR), differing only in two point mutations creating the putative Sp1 site in the SFUCR and the ELP site in the FRUCR, were designed. The sequence represented in the SFUCR is conserved in a number of SFFVp/FMCF virus isolates (49), strongly suggesting functionality. Indeed, SFUCR specifically bound two complexes shared by all cell lines studied, whereas no specific factors were detected with FRUCR as the probe (Fig. 4A). The specific complexes detected with SFUCR were

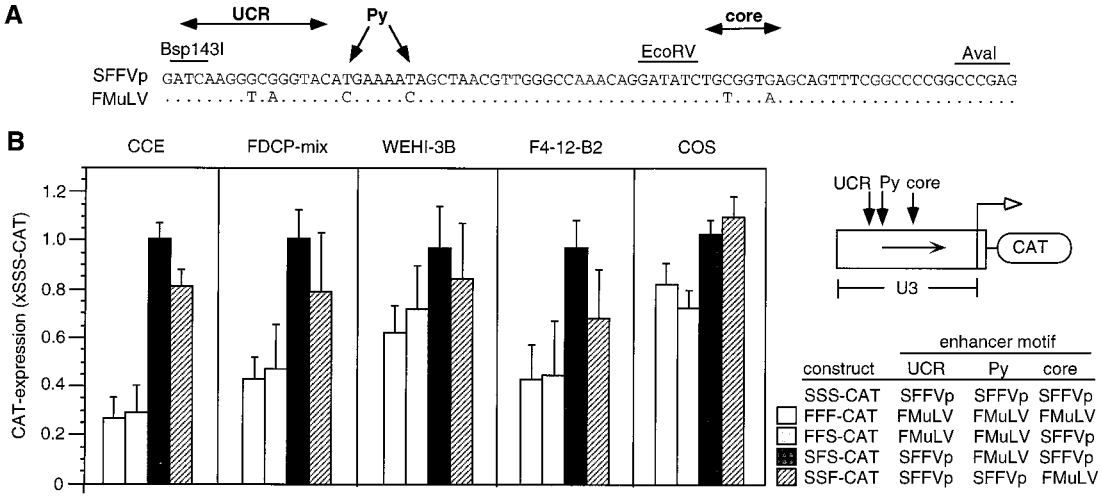

FIG. 3. The SFFVp UCR and enhancer core potentiate transactivation in hematopoietic cells in a lineage-independent manner. (A) Sequence alignment of SFFVp and FMuLV within the region between restriction sites *Bsp*143I and *Ava*I used for vector construction. The UCR, the Py exchange, and the core are indicated. (B) CAT expression values relative to those obtained with the SFFVp enhancer contained in SSS-CAT. Mean values of at least three transient transfections in each cell line ± standard deviations (error bars) are shown. A schematic representation of the CAT expression vectors containing one direct repeat (horizontal arrow) is given beneath the graph.

competed with unlabeled SFUCR or an oligonucleotide representing six putative Sp1-binding sites derived from the SV40 enhancer (22) but not with FRUCR (Fig. 4B). Finally, incubation with an Sp1-specific antiserum resulted in a supershift, thus formally identifying Sp1 (Fig. 4B). Similar results were obtained in FDCP-mix cells; complete inhibition of SFUCR-specific complexes with Sp1 antiserum was also achieved (data not shown). Consistent with the anticipated ubiquitous expression of Sp1, the specific complexes were detected in a wide range of additional cell lines (COS-7, murine fibroblasts and lymphoid cells, and human erythroleukemia cells [data not shown]).

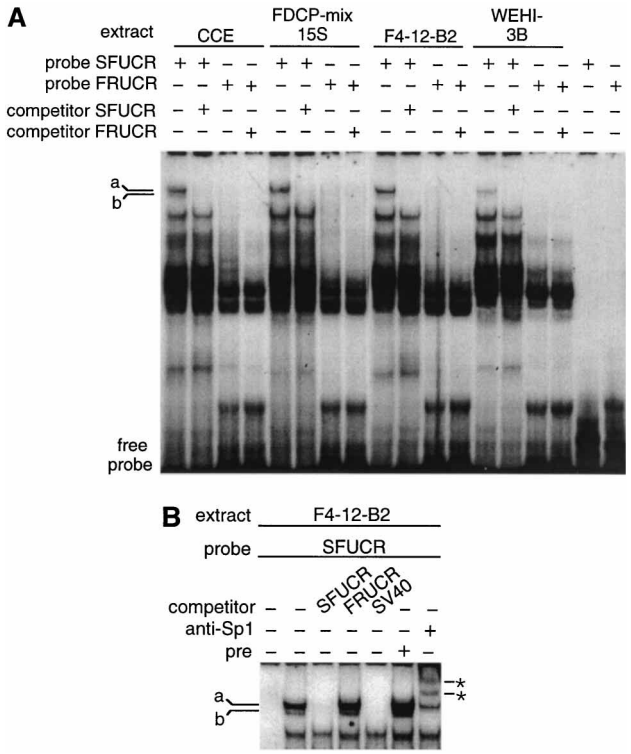

FIG. 4. The altered UCR of SFFVp is a target for Sp1. (A) SFUCR, but not FRUCR, specifically binds two complexes that are expressed in a lineage-independent manner. Band shift assays were performed as described in Materials and Methods. The contents of the reaction mixtures are given above each lane. Specific complexes a and b are indicated. (B) SFUCR-specific complexes contain Sp1. Competition is achieved with an SV40 enhancer fragment containing multiple Sp1-binding sites. Incubation with Sp1 antiserum (anti-Sp1) but not with the preimmune control serum (pre) results in a supershift (asterisks).

The core of SFFVp is less promiscuous than that of FMuLV and MoMuLV. The elevated activity of the core of SFFVp compared to that present in FMuLV or MoMuLV suggested either binding of transactivators at increased affinity, exclusion of inhibitors, or binding of factors that cannot bind to or can bind to but cannot transactivate the FMuLV and MoMuLV core. To study the reactivity of the core in the absence of the adjacent LVb site, minimal 15-bp oligonucleotides were designed. The motif of SFFVp is represented in SFcore, and that of FMuLV and MoMuLV is represented in MOcore, differing at positions 3 and 7 of the core consensus (Fig. 5A). Band shift assays were performed with nuclear extracts from a series of ES and hematopoietic cells to study the differentiation-dependent regulation of core-binding activities (CBAs).

Five distinct CBAs were recognized by these probes. They were distinguishable according to mobility pattern, substrate affinity, and developmental distribution when the band shift assays were performed with mutual competition of the two core oligonucleotides (Fig. 5B). SFcore bound three factors, termed CBA1, CBA2, and CBA3. These were also detected with MOcore, but with reduced sensitivity. Specific competition of CBA1 to CBA3 was possible with both probes. CBA1 to CBA3 were absent or extremely faint in ES cells (CCE). CBA1 and CBA2 were observed in FDCP-mix cells, in erythroid cells (F4-12-B2), and in lymphoid cells and fibroblasts (data not shown). In WEHI-3B cells, CBA1 and CBA2 were faint, possibly at the expense of CBA3, which was also found in FDCP-mix cells but was very faint in erythroid cells (F4-12-B2).

MOcore also bound two additional factors, termed CBA4 and CBA5, which were neither bound nor competed by using SFcore. The mobility of CBA4 detected in ES (CCE), erythroid (F4-12-B2), and FDCP-mix cells only marginally differed from that of CBA3. These two complexes were more

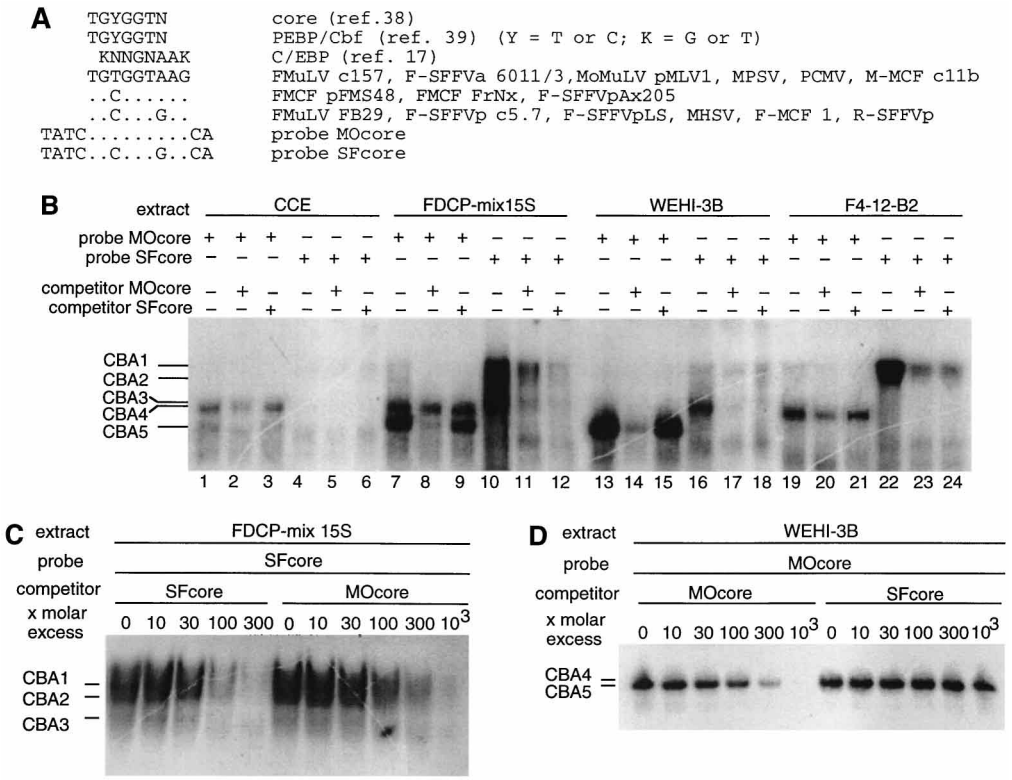

FIG. 5. Alterations of the core result in differential binding specificities. (A) Sequence conservation of the core among various families of MuLV (49) and oligonucleotides used for band shifts. Dots indicate sequence homology to FMuLV c157. Alterations in position 3 of the core almost invariably occur in FMCF-related viruses, including SFFVp. Many of these strains also exhibit a purine switch at position 7. Consensus motifs for PEBP/CBF and C/EBP are aligned. (B to D) Band shift experiments with the ^{32}P-labeled oligonucleotides representing the core of SFFVp (SFcore) or FMuLV/MoMuLV (MOcore) performed as described in Materials and Methods. The contents of the reaction mixtures are indicated above each lane. (B) Mutual competition of CBAs with MOcore and SFcore. The probes were 1.5 fmol of MOcore and 3 fmol of SFcore. The amount of probe MOcore was reduced to avoid overexposure caused by the abundance of CBA4 and CBA5. SFcore is an exclusive target for CBA1 to CBA3, which are regulated in a lineage-specific manner (lanes 10, 16, and 22); these CBAs also bind to MOcore, as evident from competition assays (lanes 11, 17, and 23) and visible in lanes 7, 13, and 19 after prolonged exposure (data not shown). Complexes CBA4 and CBA5 are specific for MOcore (lanes 1, 7, 13, and 19); they are not competed with SFcore (lanes 3, 9, 15, and 21). Free probe was allowed to leave the gel for higher resolution. (C) SFcore shows enhanced affinity to CBA1 to CBA3. Reactions were performed with constant amounts of probe SFcore and molar excesses of unlabeled SFcore or MOcore as indicated. Quantifying the levels of bound probe in repetitive assays with the FujiBAS PhosphorImager system revealed that the dose needed for half-maximal competition was 1.5- to 3-fold higher with MOcore than with SFcore (data not shown). (D) CBA4 and CBA5 are competed by MOcore but not by SFcore. Resolution of CBA4 and CBA5 in WEHI-3B cells is shown in Fig. 6C.

easily recognized by their substrate specificity: CBA4 did not bind to SFcore. Complete competition with unlabeled MOcore was possible only in the presence of a large excess of oligonucleotide, possibly due to the reduced length of the oligonucleotides. The same binding specificity was observed for CBA5 of WEHI-3B cells, which was also detected in lymphoid cells (data not shown). At higher resolution, CBA4 clearly separated from CBA5 in WEHI-3B cells (see Fig. 6C). Furthermore, a group of low-mobility complexes were seen, probably corresponding to degradation products of CBA1 to CBA5 (data not shown).

Competition with increasing amounts of unlabeled oligonucleotide revealed that the affinity of CBA1 to CBA3 to SFcore was 1.5- to 3-fold higher than that to MOcore (Fig. 5C). In contrast, CBA4 and CBA5 were specifically blocked in the presence of excess unlabeled MOcore competitor only, whereas SFcore had no effect (Fig. 5D).

SFcore is thus a specific target for a subset of CBAs which are regulated in a stage- and lineage-dependent manner. These complexes are bound at increased affinity when compared with MOcore. Furthermore, SFcore excludes the binding of the predominant complexes detected with MOcore, presumably contributing to its increased reactivity with CBA1 to CBA3.

SF core is an exclusive target for PEBP/CBF, whereas the core shared by MoMuLV and FMuLV allows competitive access of PEBP/CBF and C/EBP. Sequence alignment of the core motifs of SFFVp and of FMuLV and MoMuLV with the consensus for PEBP/CBF and C/EBP revealed that the PEBP/CBF consensus is present in both motifs whereas the purine switch at position 7 of SFcore destroys the consensus for C/EBP (Fig. 5A). Thus, we assumed that CBA1 to CBA3 were members of the PEBP/CBF family of transcription factors while CBA4 and CBA5 might be distinct and related to C/EBP. To test these hypotheses, we performed band shift experiments in the presence of specific antisera raised against PEBP/CBF or C/EBP.

CBA1 and CBA2 were inhibited by polyclonal antisera raised against an N-terminal fragment of PEBP2αA1, anti-αA1N35, or against a C-terminal fragment of PEBP2αA1, anti-αA1C17 (Fig. 6A). Likewise, CBA1 and CBA2 were competed by an antiserum raised against the β subunit of PEBP/CBF, anti-β2, indicating the presence of a PEBP2 heterodimer (Fig. 6A) (34). In contrast, CBA3 was inhibited by anti-αA1N35 or anti-β2 but not by anti-αA1C17 (Fig. 6B). This pattern was compatible with PEBP3, which lacks the C terminus of the α polypeptide (34). Thus, all complexes detected with SFcore

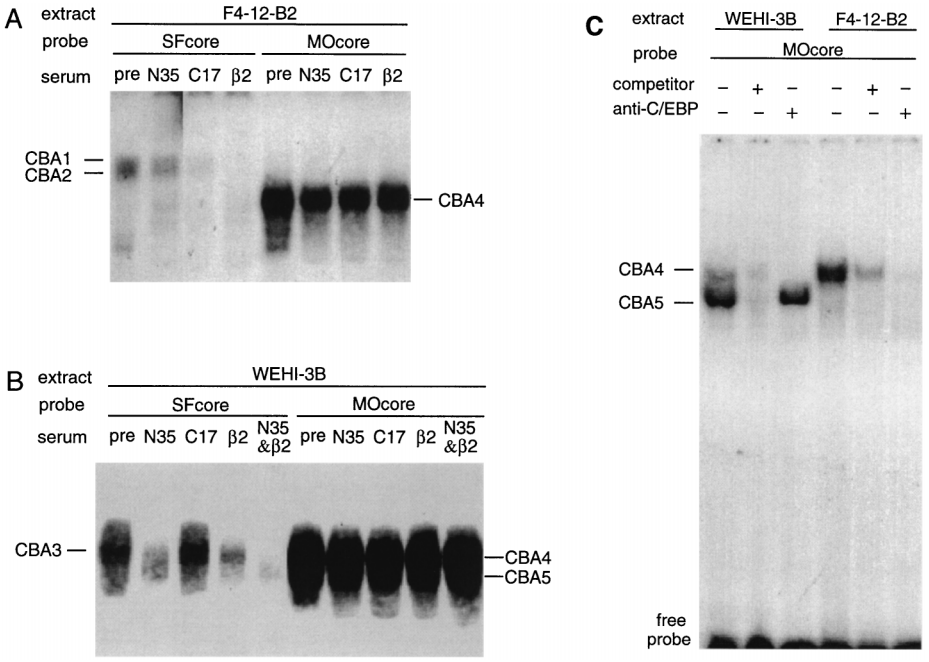

FIG. 6. CBA1 to CBA3, targeting the SFFVp core motif, are PEBP/CBF family members. Band shift assays were performed as described in the legend to Fig. 5 but after a 30-min preincubation on ice with polyclonal antisera raised against PEBP subunits (34). The assay composition and detected complexes are indicated. pre, preimmune serum; N35, anti-αA1N35; C17, anti-αA1C17; β2, anti-β2. (A) F4-12-B2 nuclear extracts. Recognition of CBA1 by the antisera resulted in a decrease of complex intensity. MOcore-specific CBA4 was not affected. (B) WEHI-3B nuclear extracts. CBA3 was recognized by N35 and by β2 but not by C17, compatible with PEBP3 (34). CBA5 was not affected. (C) Effect of anti-C/EBPβ antiserum on CBA4 and CBA5. CBA4 was completely abolished upon preincubation with anti-C/EBPβ, while CBA5 migrated with marginally reduced motility. The use of 0.5 μg of nuclear extract enabled complete resolution of CBA4 and CBA5. Preimmune serum had no effect on these CBAs.

were identified as PEBP/CBF. Identical results were obtained in FDCP-mix cells (data not shown).

In contrast, the MOcore-specific activities, CBA4 and CBA5, were not recognized by PEBP/CBF antisera (Fig. 6A and B). Instead, CBA4 was inhibited by an antiserum raised against C/EBPβ but was cross-reactive with other C/EBPs. CBA5 of WEHI-3B cells was only slightly altered in the presence of anti-C/EBP (Fig. 6C). The strong reactivity of CBA4 with anti-C/EBP suggests the presence of C/EBPβ, whereas CBA5 might be a functionally related yet distinct member of the C/EBP family.

The UCR of SFFVp stimulates the cooperation of Ets proteins with PEBP/CBF through the LVb/core region. Studies with the MoMuLV enhancer have shown that transactivation by PEBP/CBF requires cooperation with Ets factors targeting the neighboring LVb site (56). To elucidate whether the enhancer motifs in the UCR, LVb, and core of SFFVp act synergistically, cotransfection experiments were performed with ES cells (CCE) (Fig. 7). These cells express Sp1 endogenously (Fig. 5) but do not express PEBP/CBF (Fig. 6). PEBP2αB1 and/or the Ets factor Fli-I was supplemented by cotransfection of expression vectors pEF-BOSαB1 and pRc/CMVFli-I, respectively. To analyze whether transactivation is dependent on the UCR-located Sp1 site, LVb, or core, we used reporter gene vectors SSS-CAT containing all three elements, FFS-CAT lacking the Sp1 consensus in the UCR, SFa5-CAT with a destroyed LVb site, and SFa6-CAT with a mutated core element which destroys the consensus for PEBP/CBF but not for C/EBP (as confirmed by band shift assays [data not shown]). Expression of Fli-I only slightly raised the activity of the SFFVp enhancer, possibly due to competition with Ets factors endogenously present in ES cells (CCE). This effect was dependent on the LVb site. Addition of PEBP2αB1 led to a significant increase of CAT expression but only in constructs containing an intact sequence both in LVb and the core. Importantly, cooperative superstimulation by Fli-I and PEBP2αB1 was highly significant (threefold transactivation with respect to the control [$P < 0.02$]) only in the presence of the Sp1 motif in the UCR (construct SSS-CAT). Considering the reduced input of the expression vectors (2.5 μg each versus 5 μg for single use), cooperative stimulation by PEBP2αB1 and Fli-I might also have occurred in the absence of the Sp1 site in the UCR (construct FFS-CAT) but at a lower level.

DISCUSSION

In this study, we have identified two dominant *cis*-active elements of the retroviral enhancer of SFFVp which contribute to its potent and lineage-independent activity in myeloerythroid progenitor and ES cells and have identified the cognate transcription factors. These elements map to the UCR and the enhancer core, already known to be major determinants of viral host range and pathogenicity in lymphotropic viruses such as MoMuLV. Two point mutations in the UCR of SFFVp destroy the consensus for the putative repressor, ELP, and simultaneously create a target for the ubiquitous transactivator, Sp1, as confirmed here by band shift experiments. The UCR motif is the major determinant of the enhancer activity of SFFVp in ES cells and, together with the alterations in the core, also accounts for half of the activity of the complete retroviral enhancer in myeloerythroid progenitor cells. Furthermore, we observed that the core motif of SFFVp creates a specific and high-affinity target for PEBP/CBF and excludes the binding of C/EBP. As suggested for MoMuLV-related

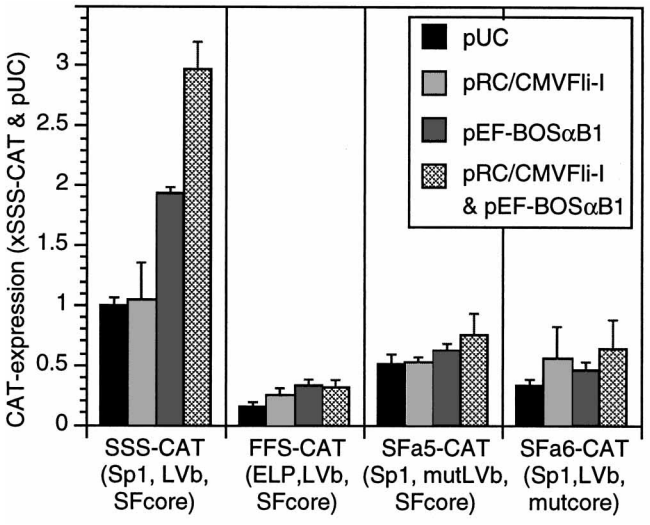

FIG. 7. Cooperation of the Ets factor Fli-I and PEBP2αB1 is superstimulated by the presence of the Sp1 site in the UCR. Transient transfections were performed as in the experiments in Fig. 3 but with the addition of 5 μg of pUC (control), pRc/CMVFli-I, or pEF-BOSαB1 or 2.5 μg of pRc/CMVFli-I or pEF-BOSαB1. β-Gal values were not significantly affected by cotransfecting these plasmids. A 25-μg quantity of input DNA was not exceeded, to prevent excessive mortality during electroporation. Mean values ± standard deviations of three transfections are given. mutLVb, mutated LVb; mutcore, mutated core.

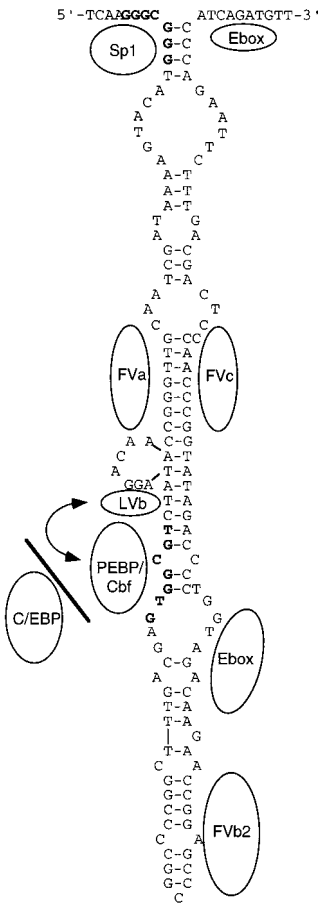

FIG. 8. Model for an extended secondary structure formed by the SFFVp enhancer and stabilized by access of transcription factors. The structure proposed in reference 14 was used as a template for plotting hitherto defined transcription factors targeting their cognate binding sites. The Sp1 site and the core motif are shown in boldface type. This structure may be formed in vivo to allow physical interaction of cooperating enhancers that appear to be separated when aligned on the linear scale, e.g., Ets, PEBP, and Sp1 (Fig. 1). C/EBP, according to the results of the present study, is unable to participate in transactivation of the SFFVp enhancer.

enhancers (56), PEBP/CBF acts through the core of SFFVp in cooperation with Ets factors targeting the adjacent LVb site, which is the only Ets site shared by the enhancers of SFFVp-, FMuLV-, and MoMuLV-related strains. Intriguingly, we observed both in hematopoietic cells and—upon cotransfection with PEBP2αB1 and Fli-I cDNAs—in ES cells that cooperative transactivation through the LVb/core region is supported by the Sp1 site of the UCR.

Coincidence of consensus sites for Sp1 and Ets factors is observed in a number of endogenous genes specifically expressed in early hematopoietic (10, 40, 70, 71), myeloid (24, 25), erythroid (10, 12), megakaryocytic (10), B-lymphocytic (48), and T-lymphocytic (27, 69) cells. Sp1 also cooperates with Ets proteins in transactivating the LTRs of human T-cell leukemia virus type 1 (19) and human immunodeficiency virus type 1 (HIV-1) (52). Cooperative transactivation through interaction of PEBP/CBF and Ets factors has been reported for a variety of endogenous genes expressed in myeloid and lymphoid differentiation (26, 44, 47, 57, 58, 68). The triadic concert of Sp1, an Ets factor, and PEBP/CBF therefore would be expected to generate the basis for lineage-independent enhancer activity in hematopoietic cells, as observed in this study.

Although we detected striking differences in the developmental regulation of PEBP/CBF in band shift assays, the core motif of SFFVp appears to exploit the activity of the different isoforms irrespective of the developmental context. PEBP/CBF factors comprise a complex group of heterodimeric transcription factors. The DNA-binding α subunit of PEBP2 is encoded by at least three distinct genes, the PEBP2αA/Cbfa1/AML3, PEBP2αB/Cbfa2/AML1, and PEBP2αC/Cbfa3/AML2 genes; all are frequently rearranged in acute myeloid leukemias and childhood lymphomas (4–6, 30, 39, 41, 66). Heterodimerization with PEBP2β, encoded by a single gene also frequently disrupted in acute myeloid leukemias, is required for full activity of the α subunit (33, 46, 63). PEBP2α and PEBP2β genes generate multiply spliced RNAs. Posttranscriptional modifications further expand the number of possible variants with as yet unidentified transcriptional properties (4, 5, 41, 45). PEBP2αB/Cbfa2 has been detected in fetal and adult erythroblasts and myeloblasts, whereas PEBP2αA is expressed mostly in lymphoid cells; the detection of PEBP2αC and PEBPβ has been more widespread (5, 46, 51, 63). Guided by our interest in virus-host interactions in myeloerythroid progenitors, PEBP2αB was chosen for the cotransfaction experiments in ES cells. Addition of this α subunit was sufficient to activate transcription through the core element of SFFVp in ES cells, most probably because PEBP2β and Ets factors are expressed endogenously. This also suggests that PEBP2αB, the murine homolog of AML1, is crucial for activating transcription of the SFFVp enhancer in myeloerythroid cells. Moreover, interesting lineage-dependent differences in the posttranscriptional modification of PEBP/CBF were seen in this study, with PEBP3, possibly a cleavage product of PEBP2 (34), being present mainly in myeloid and PEBP2 being present preferentially in erythroid cells.

We assume, by analogy to studies with lymphotropic retroviruses, that the Py switch at position 3 is responsible for the enhanced affinity of the SFFVp core motif to PEBP/CBF (38,

44, 56, 59, 72). This elevated affinity would be sufficient to explain its increased activity. However, the purine switch at position 7 might contribute by excluding the access of factors that are less efficient than PEBP/CBF in cooperation with transactivators targeting neighboring sequences. Such factors might be represented in the complexes formed on the FMuLV- and MoMuLV-type core only, which we identified as C/EBPs. The significance of the exclusion of C/EBP from the retroviral enhancer is a challenging subject for further investigations. According to results obtained with the HIV-1 enhancer, it can be imagined that C/EBP plays a stimulatory role in certain stages of hematopoietic development not analyzed in this study, such as in mature monocytes or T lymphocytes (23). However, the C/EBP family also contains putative negative regulators (reference 23 and references therein). Solving this puzzle requires specific identification of the C/EBPs targeting the FMuLV- and MoMuLV-type core in distinct hematopoietic stages.

Finally, it remains to be determined how the Sp1 site in the UCR stimulates transactivation through the LVb/core region. Sp1 is known to operate in close association with the transcription initiation complex, and thus its recognition sites are usually found close to the promoter (17). The UCR is at least 260 bp apart from the cap site. Interaction with both the LVb/core region and the promoter would require that the enhancer adopt a complex secondary structure. This could occur via bending of the double helix, similar to the regulation of the HIV enhancer, where Sp1 may cooperate with "architectural" transcription factors to activate transcription from chromatin-packaged DNA (52). Alternatively, the retroviral enhancer might be able to adopt the "hairpin" secondary structure proposed by Clark and Mak 15 years ago (14). This hairpin is formed by an unusually large and well-conserved inverted repeat (14, 54). Intriguingly, it covers the majority of the hitherto described retroviral responsive elements, as shown in Fig. 8. Sp1 might stabilize the putative 5′ basis of the hairpin structure (Fig. 8). This might allow interaction of Sp1 with the transcription initiation complex, since the base of the hairpin is exactly one nucleosome winding apart from the cap site (14). Sp1 may thus prevent de novo methylation as described for endogenous genes (11, 35) and support the interaction with Ets factors and PEBP/CBF. Likewise, a higher-order complex with accessory factors targeting the enhancer might be stabilized; these include E-box family members (29, 43) and the FV factors, which contribute to the tissue tropism of FMuLV-related retroviruses in vivo (32, 36, 37). In contrast, binding of ELP to the UCR of MoMuLV or FMuLV could block formation of the secondary structure in ES cells and thus could induce attenuation or even silencing of retroviral activity.

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