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Transcriptional Activity of Core Binding Factor α (AML1) and β Subunits on Murine Leukemia Virus Enhancer Cores

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Core binding factor (CBF), also known as polyomavirus enhancer-binding protein 2 and SL3 enhancer factor 1, is a mammalian transcription factor that binds to an element termed the core within the enhancers of the murine leukemia virus family of retroviruses. The core elements of the SL3 virus are important genetic determinants of the ability of this virus to induce T-cell lymphomas and the transcriptional activity of the viral long terminal repeat in T lymphocytes. CBF consists of two subunits, a DNA binding subunit, CBF α, and a second subunit, CBF β, that stimulates the DNA binding activity of CBF α. One of the genes that encodes a CBF α subunit is *AML1*, also called *Cbfα2*. This locus is rearranged by chromosomal translocations in human myeloproliferative disorders and leukemias. An exogenously expressed *Cbfα2*-encoded subunit (CBFα2-451) stimulated transcription from the SL3 enhancer in P19 and HeLa cells. Activity was mediated through the core elements. Three different isoforms of CBF β were also tested for transcriptional activity on the SL3 enhancer. The longest form, CBF β-187, increased the transcriptional stimulation by CBFα2-451 twofold in HeLa cells, although it had no effect in P19 cells. Transcriptional activation by CBF β required binding to the CBF α subunit, as a form of CBF β that lacked binding ability, CBF β-148, failed to increase activity. These results indicated that at least in certain cell types, the maximum activity of CBF required both subunits. They also provided support for the hypothesis that CBF is a factor in T lymphocytes that is responsible for recognition of the SL3 cores. We also examined whether CBF could distinguish a 1-bp difference between the enhancer core of SL3 and the core of the nonleukemogenic virus, Akv. This difference strongly affects transcription in T cells and leukemogenicity of SL3. However, no combination of CBF α and CBF β subunits that we tested was able to distinguish the 1-bp difference in transcription assays. Thus, a complete understanding of how T cells recognize the SL3 core remains to be elucidated.

Core binding factor (CBF) is a transcription factor initially identified as a cellular factor that binds to enhancers of several viruses and cellular genes. CBF binds to an element termed the enhancer core (4, 18, 41, 46, 48, 51) within the genomes of mammalian type C retroviruses (10). It also binds to similar elements within the enhancers of polyomavirus and several cellular genes, including those encoding the T-cell receptor (TCR) β, γ, and δ chains, the immunoglobulin heavy chain, CD3-ε, and CD3-δ, interleukin-3, and myeloperoxidase (6, 14, 15, 17, 35, 37, 45, 49, 51). CBF is also called polyomavirus enhancer-binding protein 2 (PEBP2) (17), SL3 enhancer factor 1 (46), and SL3/Akv core binding factor (4).

CBF is composed of two heterologous subunits, CBF α and CBF β. cDNAs encoding both subunits of CBF have recently been cloned. There are at least three related mammalian genes that encode CBF α subunits. One, called *AML1* or *Cbfα2*, was originally cloned from a common translocation breakpoint in patients with acute myelogenous leukemia (AML) of the M2 subtype (28). The reciprocal 8;21 translocation results in the 5′ portion of *AML1* being fused in frame to sequences on chromosome 8 termed *ETO* or *MTG8* (9, 27). Subsequently, 3;21 translocations that occur in therapy-related AML, myelodysplastic syndrome, or blast crisis of chronic myelogenous leukemia were also found to result in rearrangments at the *AML1* locus (31, 38). One type of t(3;21) results in the 5′ portion of *AML1* being fused to a gene encoding a ribosomal protein (EAP/L22) on chromosome 3 such that *EAP* sequences in the fusion protein are read out of frame (30). Another type of t(3;21) results in fusion of the 5′ portion of *AML1* to the entire *EVI-1* coding sequence (26). cDNA clones encoding the mouse homolog of *AML1*, called *PEBP2αB*, were shown to encode proteins that bound to the polyomavirus enhancer (2). In addition, the human *AML1*-encoded protein was shown to bind to the enhancer core consensus sequence (25, 34). These results confirmed that *AML1*/*PEBP2αB* encodes a CBF α subunit.

Two additional genes encode CBF α subunits: *Cbfα1* (*Pebpα2a*, *AML3*) and *Cbfα3* (*Pebpα2c*, *AML2*) (1, 2, 20, 34, 52). Each of the three CBF α-subunit genes encodes a stretch of 128 amino acids called the Runt domain that is highly homologous to a portion of the *Drosophila* segmentation pair-rule gene, *runt* (8, 9, 16, 34). The Runt domain mediates DNA binding (16, 25, 34). As a result of alternative splicing, multiple forms of the CBF α proteins are encoded by both the *Cbfα2* and *Cbfα1* genes (2, 16, 34). A third gene, *AML2* or *Cbfα3*, that encodes a CBF α subunit was identified on the basis of sequence homology (20).

While the transcriptional properties of CBF α have been studied, less is known about the in vivo function of the CBF β subunit. CBF β was initially identified as a protein that copurified with CBF α (17, 33, 50). Unrelated to any known genes, CBF β is encoded by a single gene called *CBF* β (*Pebp2*β) (33, 50). There are at least four forms of CBF β due to alternative splicing (33, 50). None are capable of binding DNA. However, some forms of CBF β have been shown to interact with CBF α in vitro and increase its affinity for DNA (33, 50). The interaction of the two heterologous subunits is mediated through

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the Runt domain of CBF α (16, 25, 34). Interestingly, the *CBF* β gene has also been implicated in AML (21). A pericentric inversion of chromosome 16 [inv(16)(p13q22)] that is characteristically observed in humans with AML of the M4 eosinophil subtype results in fusion of *CBF* β to a smooth muscle myosin heavy-chain gene, *MYH11*. This inversion results in a transcript encoding a fusion protein in which the first 165 amino acids of CBF β are linked to a variable length portion of the carboxyl tail of the myosin chain.

Both the *Cbfα1*- and *Cbfα2*-encoded forms of CBF α have been shown recently to have transcriptional activity on the TCR β enhancer in cotransfection assays (2, 34). We wished to determine whether it is also active on the enhancer core sequences that are present in the long terminal repeats (LTRs) of the murine leukemia virus (MuLV) family of retroviruses. MuLV enhancer cores are critical genetic determinants of viral leukemogenicity. Mutation of 2 bp in the core of Moloney MuLV changed the leukemogenic specificity of the virus from thymic lymphoma to erythroleukemia (42). Mutation of 3 bp in each of four enhancer cores of SL3-3 (SL3) strongly reduced the capacity of this virus to induce T-cell lymphomas (13). A 1-bp difference between the enhancer cores of SL3 and a highly homologous, nonleukemogenic MuLV, Akv, also significantly reduced viral leukemogenicity (29) and the transcriptional activity of the SL3 LTR specifically in T lymphocytes (4). As both the *Cbfα1* and *Cbfα2* genes are expressed in T lymphocytes (3, 34, 39), we tested whether the CBF α subunit encoded by the mouse *Cbfα2* gene is transcriptionally active on the MuLV enhancer cores. In particular, we asked whether it could distinguish the 1-bp difference between the SL3 and Akv cores. We also tested the activity of CBF β in transcription assays.

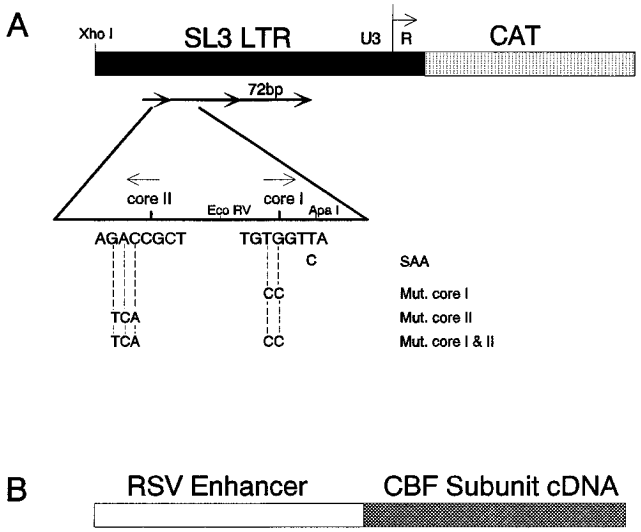

FIG. 1. Schematic representation of sequences within plasmids used in these studies. (A) The reporter plasmid used in cotransfection assays. SL3 LTR sequences, shown in black, include 443 bp of the U3 region upstream of the transcriptional initiation site at the U3/R junction plus 32 bp of the R region. These are positioned upstream of the *cat* gene. The full-size arrows under the enhancer denote the tandem, 72-bp repeats that comprise the enhancer. The small arrow denotes a third partial repeat. The expanded view of part of one repeat shows the sequences and orientations of the two core elements within a single repeat. Underneath the core I sequence is the single nucleotide difference found in the core element of Akv virus (SAA). In addition, the nucleotides that were changed in the core element mutations are shown. (B) General schematic diagram of all constructs used to express the multiple subunits of CBF. The cDNA encoding a particular subunit was placed downstream of the RSV LTR.

MATERIALS AND METHODS

Cell lines. HeLa cells were grown in minimal essential medium supplemented with 10% fetal calf serum, 100 U of penicillin per ml, 10 μg of streptomycin per ml, and 2 mM glutamine. P19 is a mouse embryonal carcinoma cell line. P19 cells were grown in Dulbecco's modified Eagle medium with the same supplements. Both lines were grown at 37°C in 100% humidity. HeLa cells were grown under 5% CO_2, while P19 cells were grown with 7.5% CO_2. L691 cells were grown in the same conditions as P19 cells.

Plasmids. The reporter plasmids SL3 and SAA have been previously described (19, 29, 40). The mutated reporter plasmids Mut. core I, Mut. core II, and Mut. core I & II were constructed by using PCR-based mutagenesis. Primers containing the appropriately altered bases (Fig. 1) and convenient restriction sites were synthesized and purified. The upstream primer, 5′ AGTATCCTCGAGGCTG CAGTAACGCCATTTTGCAAGGCAT 3′, was derived from the 5′ end of the U3 region of SL3. This primer contained an *Xho*I site corresponding to the *Xho*I site in the SL3-chloramphenicol acetyltransferase (CAT) plasmid (Fig. 1). The downstream primer was derived from the SL3 enhancer in a region spanning the corresponding core elements (Fig. 1). The sequence of the primer for Mut. core I was 5′ TGGGCCGGGGCCCTAGTGCTTAACGGCAGATATCCTGTCGT TAGCGGTCTGGGGACCATC 3′. The sequence of the primer used to make Mut. core II and Mut. core I & II was 5′ GCTTAACGGCAGATATCCTGTC GTTAGCGTGATGGGGACCATCTGTTCTTGGCCCTGGG 3′. The underlined bases indicate the mutated nucleotides relative to wild-type SL3. The downstream primer for Mut. core I contained an *Apa*I site, while that for Mut. core II and Mut. core I & II had an *Eco*RV site corresponding to sites present in the SL3 enhancer. PCR was then performed with these purified primers on a wild-type SL3 LTR or a core I mutant LTR template. As the enhancer had two tandem 72-bp repeats, conditions were optimized to obtain a high percentage of products in which the sites were mutated in both repeats of the enhancer. PCR cycling conditions were 1 cycle at 95°C for 2 min followed by 30 cycles at 95°C for 1 min and 72°C for 2 min. The PCR products were then resolved by electrophoresis and cut with either *Xho*I-*Apa*I or *Xho*I-*Eco*RV. *Apa*I and *Eco*RV cut the enhancer once in each 72-bp repeat. Thus, the digestion cleaved the PCR product into two major fragments, an *Xho*I-*Apa*I 162-bp piece or *Xho*I-*Eco*RV 141-bp piece and an *Apa*I-*Apa*I or *Eco*RV-*Eco*RV 72-bp piece. The larger piece was inserted into a subclone of the SL3 LTR in pSP73 (Promega), creating an LTR with a single 72-bp unit. The 72-bp fragment was then inserted into the subclone, yielding tandem 72-bp repeats. The modified LTRs were subsequently excised and inserted into the CAT plasmid. The clones were sequenced at every step to ensure that they were identical to the parental sequence except for the mutated bases.

A 2,047-bp fragment of CBFα2-451 containing the entire open reading frame was subcloned between the *Hin*dIII and *Bgl*II sites of plasmid pRSV-β-globin (obtained from the American Type Culture Collection), replacing the β-globin gene. CBFα2-451 was expressed from the Rous sarcoma virus (RSV) promoter and enhancer. cDNAs encoding the CBF β subunits (CBF β-187, CBF β-182, and CBF β-148) were also subcloned into the modified RSV expression vector.

All plasmids for transfections of mammalian cells were purified by using Qiagen columns.

Transfections and CAT assays. Transient transfections of P19 and HeLa cells were performed by using an optimized Lipofectin (GIBCO BRL) protocol. Cells were plated at a density of 10^6/60-mm^2 plate 24 h before transfection. The manufacturer's protocol was then followed, using a total of 8 μg of DNA (1.5 μg of CAT reporter, 1.5 μg of a simian virus 40-luciferase internal control, and up to 5 μg of CBF subunit) plus 25 μl of Lipofectin per transfection. When lower amounts of plasmid were used, pBluescript DNA was included so that the amount of DNA in every culture was the same. The medium was changed after a 6-h incubation. The cells were harvested after 30 h, and lysates were prepared as previously described (4, 40). Transfections of L691 cells were performed by the DEAE-dextran method as previously described (43).

The concentration of protein in the lysate was then determined by the Bio-Rad protein assay kit. An aliquot of the lysate was then used to measure CAT enzymatic activity as previously described (11, 40). In addition, another aliquot of lysate was used to determine the luciferase activity from the internal control plasmid (5). In HeLa cells, CAT activity was normalized to these values. In P19 cells, we were unable to obtain usable measurements of luciferase activity. With the simian virus 40 early enhancer/promoter driving luciferase expression, activity was found to decrease about 10-fold at the highest concentration of CBFα2-451 expression plasmid used. Two other luciferase constructs, one with the mouse mammary tumor virus LTR with the negative regulatory element in U3 deleted and the other with a sea urchin embryonic histone gene promoter, resulted in no detectable activity. Therefore, to avoid overestimating the stimulation by CBFα2-451, the luciferase internal control was not used in comparing different transfections in P19 cells. CAT activities were determined by PhosphorImager analysis of thin-layer chromatography plates, and the percentage of chloramphenicol acetylated was calculated. The level of transactivation by CBF was then calculated by dividing the activity of the LTR-CAT plasmid (normalized to luciferase activity in HeLa cells) in the presence of CBF by the level of activity in a parallel sample without CBF. Multiple trials were performed for each

experiment. The means were calculated and plotted. Standard deviations were calculated and plotted for each mean, using the STDEVP program of Microsoft Excel.

Expression and partial purification of the DNA binding domain of CBFα2-451. A DNA fragment encoding a 17-kDa DNA binding (Runt) domain from the mouse CBFα2-451 protein (amino acids 51 to 202) was amplified by PCR using a 5′ (sense) primer (CGGAATTCCCATATGGTGGAGGTACTAGCT) and a 3′ (antisense) primer (CGGATCCTTACTCCAATTCACTGAGCCG). The amplified fragment was digested with *Nde*I and *Bam*HI and subcloned into the *Nde*I-*Bam*HI sites of a modified pET3 vector lacking the codons of T7 gene 10 (32). This synthetic DNA contains a methionine codon in the context of the *Nde*I site in frame with the first amino acid of the DNA binding domain.

For expression and purification, the plasmid was transformed into *Escherichia coli* BL21(DE3) containing plasmid pLysS (1). Expression was induced with 1 mM isopropylthiogalactopyranoside (IPTG) for 3 h at 37°C (A_{600} = 0.7 to 0.9 at time of induction and 1 to 1.5 at time of harvest). The bacterial cell pellet from a 1-liter culture was resuspended in equal-weight lysis buffer (10% sucrose, 50 mM Tris-HCl [pH 7.5]), protease inhibitors [1 mM Pefabloc, 1 mM phenylmethylsulfonyl fluoride, 50 μM calpain inhibitor 1, 1 μg of leupeptin per ml, 1 μg of pepstatin A per ml, 2 μg of aprotinin per ml]). Cells were frozen by being dropped directly into liquid nitrogen, thawed in a room temperature water bath, incubated on ice in the presence of 400 μg of lysozyme per ml and 0.1 M ammonium sulfate for 30 min, and then lysed with gentle agitation for 3 min at 37°C. Samples were centrifuged at 40,000 × *g* for 1 h at 4°C. Soluble proteins were dialyzed against 200 volumes of buffer A (150 mM KCl, 20 mM imidazole [pH 7.0], 20 mM β-mercaptoethanol). After dialysis, samples were applied to a DEAE-Sephacel column (24 ml) equilibrated in buffer A. The column was washed with 1.5 column volumes buffer A, and approximately 2-ml fractions were collected. Flowthrough fractions containing core binding activity were pooled and dialyzed against 200 volumes buffer B (50 mM NaCl, 20 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid [HEPES; pH 7.5], 20 mM β-mercaptoethanol, 10% glycerol, 0.05% Triton X-100) and then applied to a P-11 cellulose phosphate column (3 ml; Whatman) equilibrated in buffer B. Core binding activity was eluted with a 30-ml linear gradient of NaCl from 50 mM to 1.0 M in buffer B. Approximately 1-ml fractions were collected. Fractions with peak activity were pooled, exchanged into buffer C [50 mM NaCl, 20 mM 2-(*N*-morpholino) ethanesulfonic acid (pH 6.5), 20 mM β-mercaptoethanol, 10% glycerol, 0.05% Triton X-100] on a Sephadex G-25 column, and then applied to a Mono S column (Pharmacia). Bound protein was eluted with a 60-ml linear gradient from 50 mM to 1.0 M NaCl. Active fractions were pooled, and glycerol was added to a final concentration of 20%. The DNA binding domain 51-202 was estimated to be 20 to 30% pure by Coomassie brilliant blue staining of a sodium dodecyl sulfate-polyacrylamide gel.

Protein-DNA binding analysis. Complementary 18-bp oligonucleotides containing the SL3 and Akv core site (4), the high-affinity core site deduced by Thornell et al. (47) and Wang and Speck (49), and a mutant core site (see Fig. 4C for sequences) were synthesized on a Biosearch Cyclone DNA synthesizer at Dartmouth Medical School and purified by electrophoresis through 20% polyacrylamide–7 M urea gels (not shown).

^{32}P-labeled DNA probes and electrophoretic mobility shift assays (EMSAs) were performed as described previously (49, 50). Binding reaction mixtures contained 20,000 cpm of ^{32}P-end-labeled high-affinity-site probe, 0.5 μg of poly(dI-dC) · poly(dI-dC), and 0.5 μl of protein sample, in the presence of various concentrations of the SL3, Akv, or high-affinity mutant core site competitor. The data were scanned and analyzed with the NIH Image program.

RESULTS

Transcriptional activity of CBF α on an MuLV enhancer. We tested the ability of the CBF α subunit encoded by the murine *Cbfα2* gene to transactivate the LTR of the T-cell lymphomagenic MuLV, SL3. The CBFα2-451 cDNA encodes a 451-amino-acid polypeptide identical to PEBP2αB1 (2, 3, 7). A schematic representation of this cDNA is shown in Fig. 2.

The CBFα2-451 cDNA was cloned into an expression vector downstream of the RSV LTR and cotransfected with a plasmid containing the SL3 LTR upstream of the bacterial reporter gene *cat* (Fig. 1). The SL3 LTR enhancer is composed of two 72-bp repeats. As shown in Fig. 1A, there are two different core elements to which CBF binds in each repeat, making a total of four CBF binding sites in the SL3 enhancer. The two cores within each repeat are not identical and are in inverse orientation relative to each other, separated by 14 bp. Thornell et al. (47) termed these sites I and II (Fig. 1A) and estimated that CBF from mouse T cells bound about five times more strongly to core I.

The activity of CBFα2-451 was tested in two cell lines, HeLa and P19 (Fig. 3). P19 cells were chosen because the *Cbfα2* gene is not expressed in these cells and both *Cbfα1* and *Cbfα2* have been shown to transactivate the TCR β enhancer when expressed exogenously in this cell line (2). HeLa cells were chosen empirically as another type of cells which also supported CBF activity. In both lines, CBFα2-451 stimulated transcription from the SL3 LTR. Maximum stimulation was about eightfold in HeLa cells and sixfold in P19 cells. As a control, the empty RSV vector was tested. The RSV vector had no effect on the level of LTR activity in either cell line. In P19 cells, maximum stimulation was seen with about 0.5 μg of CBFα2-451. Higher levels resulted in decreased levels of transactivation. Perhaps excess CBFα2-451 squelched transcription (2, 36). It is unclear whether saturating levels of CBF α were attained in the experiments in HeLa cells.

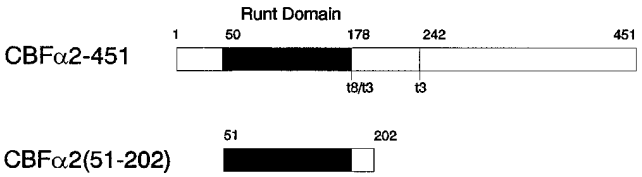

FIG. 2. Schematic representation of CBFα2-451. t8 and t3 indicate the positions of the fusion of sequences from chromosomes 8 and 3 in the translocated forms. Numbers at the top refer to amino acid positions. The black box represents the Runt domain. CBFα2(51-202) is the portion of CBFα2-451 used in the binding studies.

Activity of CBFα2-451 on different core sequences. Core I in SL3 is conserved in other MuLVs and related type C retroviruses, while core II is not (10). The sequences of the core elements in different viruses often vary slightly (10). Notably, the core of nonleukemogenic Akv virus differs from core I of SL3 by 1 bp (Fig. 1) (19). This T-C difference has a large effect on the ability of SL3 to induce T-cell lymphomas (29). It also affects transcription in T lymphocytes but not other types of cells (4, 29). CBF from mouse thymocytes was previously shown to bind both the SL3 and Akv cores (4, 47). Competition binding experiments by Thornell et al. (47) indicated that CBF bound preferentially to the SL3 core, although quantitation of the difference has not been reported. Selected and amplified binding sequence footprint analysis showed that calf thymus CBF preferred a T over a C at the corresponding position, but it preferred C to other bases (24). Like the Akv core, core II of SL3 has a C at the equivalent position as the T-C difference between SL3 and Akv (Fig. 1).

To quantify the relative binding of CBF α to the SL3 and Akv cores, a 17-kDa portion of CBFα2-451 from amino acids 51 through 202 [CBFα2(51-202); Fig. 2] containing the DNA binding (Runt) domain was PCR amplified and expressed in *E. coli* by using a modified pET3 vector system. The protein was chromatographically enriched to about 20 to 30% purity. EMSAs were performed with a high-affinity binding site as a probe (49). Competition experiments were performed with increasing amounts of the high-affinity site, the SL3 core, the Akv core, and a mutated core element as competitors (Fig. 4). The SL3 core element competed for binding about twice as well as the Akv core, in that approximately twice as much Akv competitor was needed to decrease binding to the same extent as the SL3 core site. The high-affinity site competed for binding about four times more effectively than the SL3 core. The mutated element that contained a crucial C-for-G substitution in the two adjacent G nucleotides present in the functional CBF binding sites failed to compete. Thus, CBF can bind to core sequences that contain a T or C at the position that distinguishes SL3 from Akv, but there is a preference for a T.

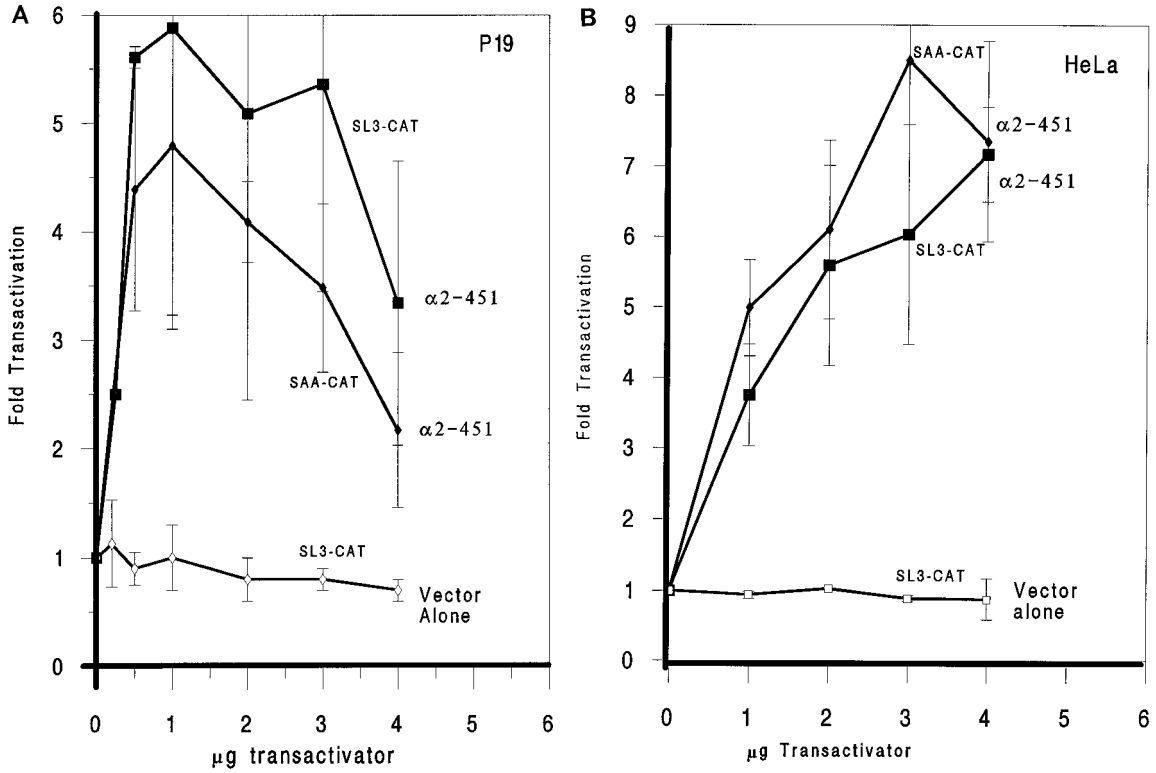

FIG. 3. Titration of CBFα2-451. Increasing amounts of CBFα2-451 were cotransfected with 1.5 μg of the indicated reporter plasmid, and the fold transactivation was plotted. Datum points are the means of at least two independent trials for each experiment, and error bars show the standard deviations. The total amount of DNA was kept constant at 8 μg in all experiments by the addition of pBluescript DNA. Transfections were performed in P19 (A) cells and HeLa (B) cells.

The ability of CBFα2-451 to distinguish the SL3 and Akv cores was then tested in transcription assays. The activity of CBFα2-451 was tested by using a recombinant enhancer (termed SAA; Fig. 1) that was identical to that of SL3 except for the single base pair found in the core of Akv. This mutation had no effect on the basal activity of the LTR reporter plasmid in either P19 or HeLa cells (53). Cotransfection of CBFα2-451 resulted in equivalent levels of stimulation of the viral LTR whether the core was that of SL3 or Akv (Fig. 3). The simplest interpretation of this observation is that this form of CBF α is not solely responsible for the capacity of T cells to distinguish the SL3 and Akv cores.

One possible explanation for the equivalent activation of the SL3 and Akv cores by CBF was that the transient expression assays may have resulted in overexpression of the factor. It was conceivable that at low, limiting levels of CBF, the difference in activity between the two cores might be greater. We tested this possibility by transfecting increasing amounts of CBFα2-451 into L691 T lymphocytes, a cell line in which the SL3 LTR is about fivefold more active than the SAA LTR. Cotransfection of increasing amounts of CBFα2-451 failed to alter the activity of either LTR in transient transfections (Fig. 5). This finding indicated that the level of this subunit of CBF was not limiting for the transcriptional activity of these MuLV LTRs in this T-cell line. It was also consistent with the idea that the equivalent activities of the SL3 and Akv cores in cotransfection assays were not due to overexpression of CBF that resulted in driving the SAA LTR activity to that of the SL3 LTR.

It was important to confirm that CBF α was acting through the core elements in the SL3 enhancer. Mutations were introduced into both copies of core I, both copies of core II, or all four CBF binding sites simultaneously (Fig. 1A). The 2-bp mutation in core I was previously shown to block CBF binding (49), as was the 3-bp mutation in core II (47). These mutations had no effect on the basal activity of the LTR reporter plasmids in either P19 or HeLa cells (53). LTRs with these mutations were tested in cotransfection experiments with CBFα2-451 (Fig. 6). In P19 cells, mutation of core I eliminated all of the activity of CBFα2-451. This result confirmed that the factor acts through this site. Mutation of core II also reduced the level of transactivation by CBFα2-451, but the effect was less than that seen with core I. The smaller contribution of core II to the total effect of the SL3 enhancer was consistent with lower binding affinity for CBF reported by Thornell et al. (47) and is analogous to the relative effects of mutation of each of the cores on leukemogenicity of SL3 (13). Simultaneous mutation of the core I and core II sites resulted in lack of transactivation in P19 cells.

In HeLa cells, mutation of either core I or core II reduced the level of transactivation to about threefold (Fig. 6). Simultaneous mutation of all four sites reduced the level of transactivation to about twofold. Thus, most of the activity of CBFα2-451 in HeLa cells is indeed through the core elements. However, there was a residual level of stimulation that was independent of the cores. To investigate the basis of this, we also tested a mutated SL3 LTR in which the 5′ 300 bp including essentially the entire enhancer repeat region was deleted (22). CBFα2-451 also transactivated this LTR about twofold (53). Thus, a small component of CBF activity on the SL3 reporter construct in HeLa cells was independent of the enhancer. It could be due to the factor acting through an unknown CBF binding site elsewhere in the SL3-CAT plasmid. However, an equivalent effect was not seen in P19 cells. An alternative explanation is that CBFα2-451 may have induced

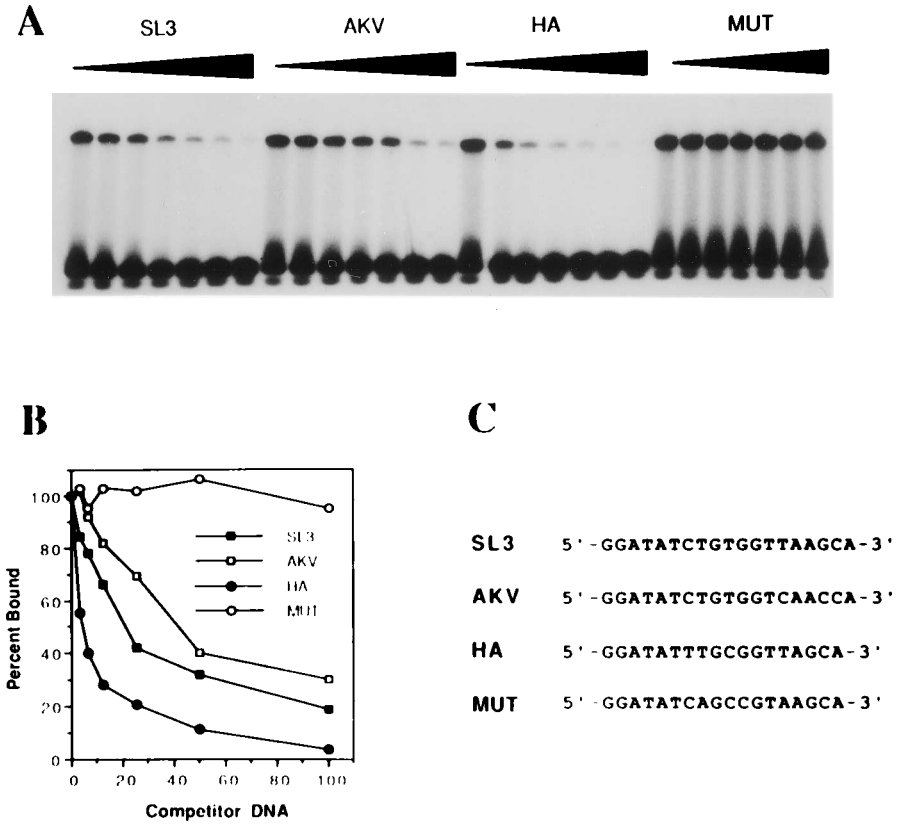

FIG. 4. Competition EMSA with *E. coli*-expressed preparations of the DNA binding domain of CBF α with various enhancer core sequences. (A) A radiolabeled high-affinity-site probe was used in EMSAs. Increasing amounts of each competitor oligonucleotide were added as indicated (HA, high-affinity site; MUT, mutated site). In each set of seven lanes from left to right, the competitors were present at 0-, 3-, 6-, 12-, 25-, 50-, and 100-fold molar excess over probe. (B) Percentage of the protein-DNA complex relative to that with no added competitor DNA versus the amount of competitor DNA. (C) Sequences of one strand of the oligonucleotides used in this experiment.

the activity of some other factor in HeLa cells that in turn increased activity of the SL3 promoter-CAT construct. We note that such a factor would not be active on every transcription unit, as we did not detect an effect on the simian virus 40 early enhancer/promoter-luciferase plasmid that was used as an internal control.

Transcriptional activity of CBF β subunits. There are at least four different spliced forms of CBF β (Fig. 7). These are named on the basis of the predicted number of amino acids in the encoded product. Wang et al. (50) and Ogawa et al. (33) each identified two subunits, CBF β-187 and CBF β-182, that differ at their carboxyl termini. While neither CBF β-187 nor CBF β-182 bound to DNA directly, each could bind to CBF α subunits through the Runt domain and stimulate DNA binding activity. Wang et al. (50) also identified a third CBF β isoform termed CBF β-148. This was identical CBF β-187 except for an internal deletion of the 39 amino acids encoded in exon 3 due to alternative splicing. Ogawa et al. (33) identified another form, here termed CBF β-155, that also differed from CBF β-187 by an internal deletion due to alternative splicing; however, the missing sequences were from exon 5. Interestingly, neither CBF β-148 nor CBF β-155 could bind to CBF α or stimulate its DNA binding capacity, indicating that there is a central domain of CBF β that is important for CBF α binding (12, 33, 50). To examine the effects of CBF β subunits on transcription, plasmids encoding CBF β-187, CBF β-182, and CBF β-148 were tested in cotransfection assays using HeLa and P19 cells. Equal amounts of a CBF β plasmid were co-

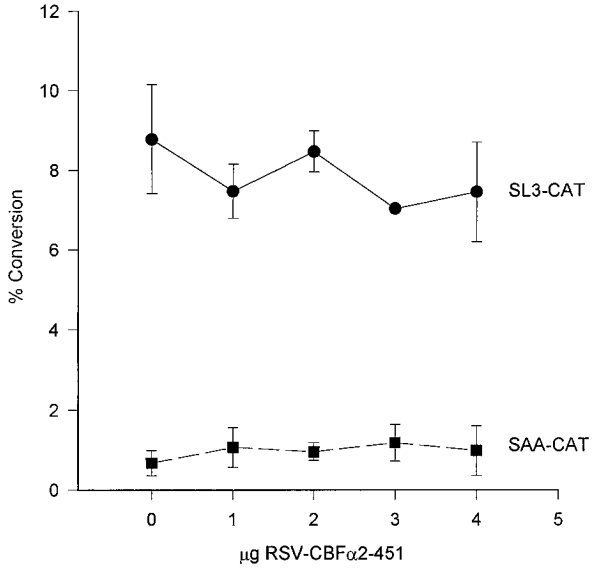

FIG. 5. Effects of cotransfection of CBFα2-451 on activity of the SL3 and SAA LTRs in the T-lymphocyte line L691. LTR-CAT plasmid DNA (3 μg) was transfected into L691 cells by the DEAE-dextran method (43). Increasing amounts of CBFα2-451 were cotransfected, and CAT activity was determined.

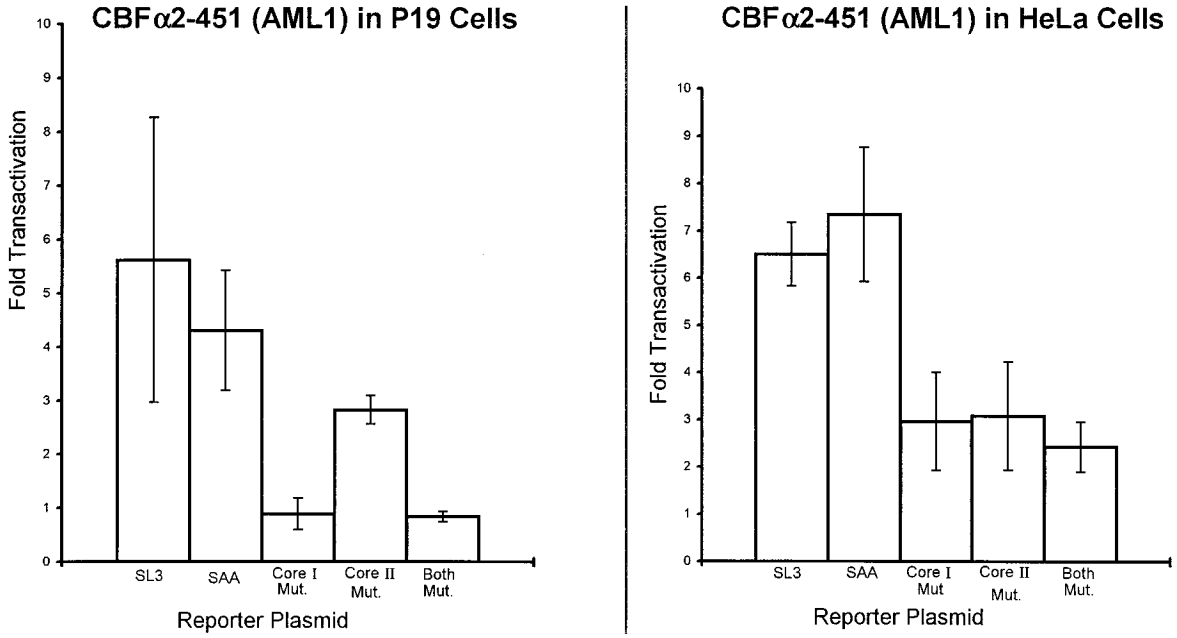

FIG. 6. Effects of mutation of the SL3 cores on the ability of CBFα2-451 to transactivate the reporter construct. The indicated reporters were cotransfected with CBFα2-451. The amount of transactivator used in each cell line was based on the titration in Fig. 3. In P19 cells, 0.5 μg of CBFα2-451 was used; in HeLa cells, 4 μg was used. The means of at least three independent transfections for each experiment are plotted, and the error bars indicate standard deviations.

transfected with the CBFα2-451-encoding plasmid and the LTR-CAT plasmid.

Upon the addition of CBF β-187 in HeLa cells, there was a twofold increase in the level of transactivation over that of CBFα2-451 alone (Fig. 8). This effect required the CBF α subunit, as CBF β-187 did not enhance transcription by itself. In addition, its activity also depended on the presence of both core elements in the SL3 enhancer, as no effects of the CBF β-187 were observed when either core was mutated. Thus, at least in this system, it appeared that CBF β functioned only when CBFα2-451 could bind to both sites in one repeat.

We also tested whether CBF β-187 could affect the ability of CBFα2-451 to distinguish the single base pair difference between the SL3 and Akv cores. Transcription was stimulated about twofold when the T in the SL3 core I was mutated to the C in Akv (Fig. 8). Thus, addition of CBF β-187 did not allow CBFα2-451 to discriminate between the SL3 and Akv core sites in vivo.

The effect of the CBF β-182 form of CBF β was less clear. CBF β-182 appeared to stimulate transcription less well than CBF β-187 but better than CBFα2-451 alone (Fig. 8). This effect was highly reproducible, as it was seen in every trial that we performed, regardless of whether the SL3 or Akv core sequence was present in the reporter construct. However, since the level of stimulation in some experiments was only about 1.5-fold greater than activity of CBFα2-451 alone, we are uncertain whether this effect is significant (Fig. 8). CBF β-148 failed to stimulate transcription relative to CBFα2-451 alone. This is consistent with the inability of this form of CBF β to associate with CBF α in vitro (50).

When CBF β subunits were coexpressed with CBFα2-451 in P19 cells, no stimulation of transcription was seen, even with CBF β-187 (53). Thus, the effects of CBF β differ between the two cell lines. We do not know the basis for this difference. It might be the result of different endogenous levels of the β subunits or differences in the ratios of the different spliced forms in the two lines. Alternatively, the effect may be the result of some additional cofactor that might be required for CBF β activity and is specific for one of the two lines.

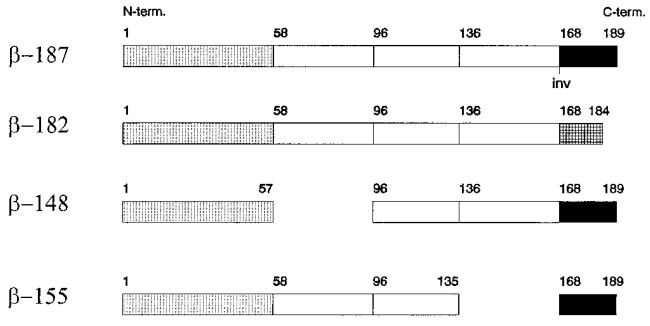

FIG. 7. Schematic diagram of various spliced forms of CBF β. The amino and carboxyl termini are indicated. All forms are compared with CBF β-187. The numbers over the different forms indicate the corresponding amino acid positions in CBF β-187. The empty spaces show the internal deletions found in CBF β-148 and CBF β-155, while the hatched box at the C terminus of CBF β-182 indicates different amino acids at this position.

DISCUSSION

Our results demonstrate the transcriptional activity of *AML1*-encoded CBF α and CBF β on the core elements from two MuLVs, SL3 and Akv. They are consistent with the idea that CBF is a factor in T cells that is responsible for recognition of the viral cores. CBF also binds to the core elements within the LTR enhancers of other mammalian type C retroviruses (47). Therefore, it seems likely that CBF will function on the other MuLV cores.

Mutations within the SL3 core element strongly affect viral leukemogenicity (13, 29). This can be interpreted to mean that

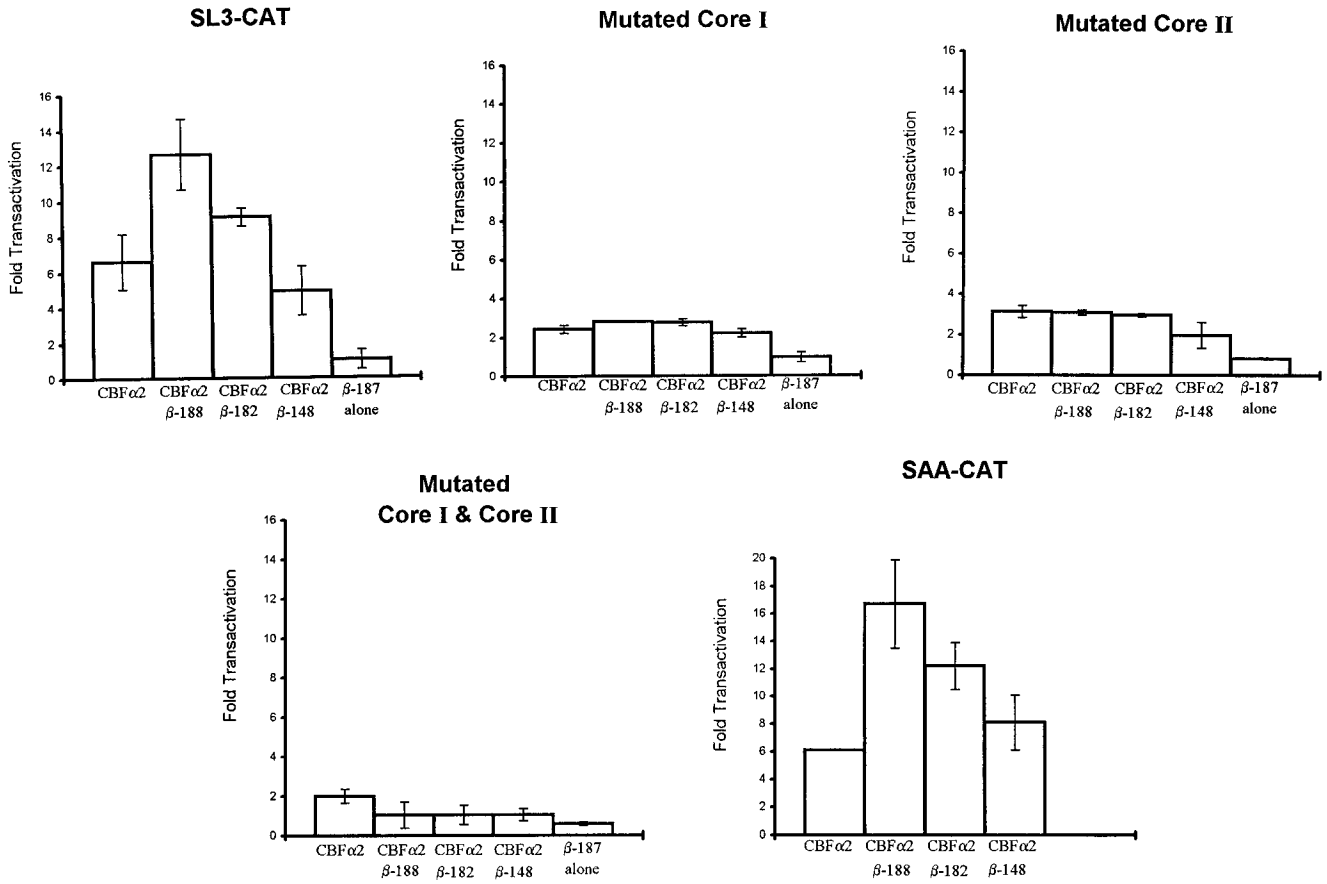

FIG. 8. Effects of the multiple forms of CBF β in cotransfection experiments with each of the reporter plasmids in HeLa cells. Each panel shows a specific reporter construct. The CBF subunits used in each experiment are indicated along the *x* axis of each panel. When a single CBF subunit was tested, 2.5 μg of the plasmid encoding it plus 2.5 μg of pBluescript (SK+) were used. When plasmids encoding both CBF subunits were transfected together, 2.5 μg of each was used. The graphs present the means of at least two independent transfections for each experiment. The error bars indicate standard deviations.

the transcriptional activity of CBF on the SL3 enhancer is important for one or more steps in the viral leukemogenic process in T lymphocytes. However, mutation of the SL3 core I to that of Akv significantly reduced both the transcriptional activity in T cells and the capacity to induce T-cell lymphomas (4, 29). Thus, it is surprising that CBFα2-451 transactivated both viral cores to the same level. Binding assays have indicated that CBF binds the SL3 core better than that of Akv (24, 47). However, it does bind the Akv sequence (4, 22, 24, 47). Binding assays presented here indicated that CBF bound the SL3 core approximately two times better than the Akv core. The key question is whether this difference in binding is important for the differential transcription of the two cores.

CBF may still play an important role in the ability of T cells to distinguish the SL3 and Akv cores, despite the observation that CBFα2-451 transactivated both viral cores to the same extent in the cotransfection assays. It is likely that the cotransfection assays do not fully reflect the conditions in T cells. Thus, additional factors in T cells that bind the SL3 enhancer may modulate the ability of CBF to distinguish the T-C difference between the two viral cores. Indeed, previous data indicated that sequences immediately upstream of the core element in the SL3 enhancer sequences were important for T cells to distinguish the two cores (22). Thus, the absence of one of these factors in HeLa or P19 cells may prevent CBF from differentiating the viral cores.

It is also possible that the preferential recognition of the SL3 core is actually mediated by a transcription factor other than CBF. At least two other factors that bind to MuLV core elements, S-CBF and MCREF-1 (4, 23, 44), have been identified in T-cell nuclear extracts. If either of these factors is actually important for distinguishing the SL3 and Akv cores, it would be necessary to explain how they can act in the presence of CBF. Perhaps CBF somehow works in combination with one or more additional core binding factors.

A full description of how transcription factors in T lymphocytes recognize MuLV enhancers must account for two observations. One is the ability of T cells to distinguish the SL3 and Akv cores. The other is that CBF is present in these cells and at least certain combinations of CBF α and CBF β are transcriptionally active on both the SL3 and Akv enhancer cores.

Our results also showed that the CBF β-187 form of CBF β had transcriptional activity. The CBF β-182 form also reproducibly stimulated transcription to a lower level than CBF β-187, although the effect was small and difficult to measure. The lower activity of CBF β-182 did suggest that the carboxyl-terminal amino acids that distinguish CBF β-187 from CBF β-182 (Fig. 7) have a modest effect on transcription. These amino acids are replaced by smooth muscle myosin heavy-chain sequences in human AML (21).

The core site dependence of CBF β transcriptional activity was consistent with a requirement for it to work by binding to CBF α. CBF β was previously shown to stimulate the ability of CBF α to bind DNA (50). Transcriptional stimulation by CBF

β may simply reflect this property. However, it is curious that the effect of CBF β was seen only when both core elements within each 72-bp repeat in the SL3 enhancer were intact. It is possible that the stimulation by CBF β-187 on enhancers with one of the two cores mutated was too small to be detected. On the other hand, it is also possible that CBF β-187 can stimulate transcription only when all four binding sites are intact. Perhaps it has an additional function beyond the stimulation of CBF α binding. Whatever the mechanism(s), the maximum activity of CBF in HeLa cells required both subunits.

In addition to its activity on the core elements of two MuLV enhancers, CBF has been demonstrated to function on the enhancer of the TCR β chain (2, 34). As similar sequences are present in the enhancers of many lymphoid cell-specific genes, it seems likely that this factor regulates the transcription of many lymphoid genes. CBF binding activity is also present in nonlymphoid cells. Its potential role in these cells remains to be investigated.

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