Relative Importance of Elements within the SL3-3 Virus Enhancer for T-Cell Specificity

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Elements within the enhancer of T-lymphomagenic SL3-3 virus were examined for their contributions to transcriptional activity in T lymphocytes and non-T cells. A region containing two sequences homologous to the enhancer core consensus sequence and a sequence homologous to the binding site for factor LVb was found to have the largest effect on activity. Evidence was obtained that suggests that the activity of this region was greater in T lymphocytes than in non-T cells and that multiple elements within it were necessary for activity. A second region, containing sequences homologous to the binding site of factor NF-I and the glucocorticoid response element, had about a twofold effect on transcription in both T lymphocytes and non-T cell lines. The twofold effect was seen whether the region containing the cores and LVb site was present or not. These results indicate that the most important region for the specificity of SL3-3 enhancer activity and, presumably, for viral leukemogenicity comprises the core elements and the LVb site. DNA-protein-binding studies demonstrated that one cellular factor, S/A-CBF, bound to both core elements, while a second cellular factor, S-CBF, bound to only one of them. In combination with earlier studies, this indicates that cells contain multiple factors that bind to the critical region.

Experiments using recombinant murine leukemia viruses (MuLV) demonstrated that the tandem direct repeats in the U3 component of the long terminal repeats (LTRs) are the primary genetic determinant of viral leukemogenicity (5, 6, 9, 14, 21, 23, 24, 39, 40, 43). The tandem repeats function as transcriptional enhancers which drive expression of reporter genes in various types of cells in a manner that parallels viral disease specificity (2, 3, 33, 36, 42). For example, the LTR of T-lymphomagenic SL3-3 virus is more active than that of nonleukemogenic Akv virus in T-lymphoma cells, while both exhibit about the same activity in non-T hematopoietic cells (33).

MuLV enhancers, like those of cellular genes and other viruses, comprise a series of tandemly arranged components that combine to induce transcription (1, 11, 25, 26, 34, 37). The combination of cellular transcription factors that bind to the components in particular cells presumably determines specificity. We hypothesize that the appropriate combination of factors renders a cell a target for viral leukemogenicity. Thus, to understand the basis of virus-host cell interactions that underlie leukemogenicity, it is necessary to identify the important functional elements within the viral enhancers and the cellular factors that interact with them.

We previously reported that the enhancer core, a consensus sequence element initially described in the simian virus 40 enhancer (41), is necessary for the high relative activity of the LTR of SL3-3 virus in T-lymphoma cells (1, 25). The sequence of the SL3-3 core differs from that of the nonleukemogenic Akv virus by a single base pair. Exchange of this base pair altered LTR specificity in T-lymphoma cells but not in other types of cells. Multiple cellular factors that bind to the core elements were identified, including factors that were specific for either the SL3-3 or Akv elements.

MuLV enhancers contain several other elements in addition to the core. Among these are a glucocorticoid response element (GRE) (4, 8), a binding site for NF-I (29, 34), and

binding sites for LVa, LVb, and LVc (34). In this study, we determined the relative contributions of various sequences to the activity of the SL3-3 enhancer in T and non-T cells. We also extended the analysis of factors that bind to the region determined to be critical for expression.

MATERIALS AND METHODS

Cell lines. L691-6 (28) and WEHI 7.1 (18) are T-lymphoma cell lines derived from radiation-induced thymic lymphomas of a C57L mouse and a BALB/c mouse, respectively. SL3B is a T-lymphoma line derived from a thymic lymphoma from an AKR mouse injected with SL3-3 virus (19). BW5147.3 is a T-lymphoma line derived from a spontaneous thymic lymphoma in an AKR/J mouse (32). CTLL-1 was isolated as a cytotoxic T-lymphocyte line that was derived from a mixed allogeneic tumor cell-lymphocyte culture of spleen cells from a C57BL/6 mouse (13). The murine erythroleukemia line DS19 is an erythroid cell line from leukemic DBA/2 mice (31). NIH 3T3 is a mouse fibroblast cell line (22). J558 is an immunoglobulin A-secreting myeloma cell line from a BALB/c mouse (17). SL3B cells were grown in RPMI 1640 supplemented by 10% fetal bovine serum, 100 U of penicillin per ml, and 100 µg of streptomycin per ml. CTLL-1 was grown in the same medium with an additional 10% concentration of the interleukin 2-containing supplement Rat T Cell Monoclonal (Collaborative Research, Inc.). The remaining cell lines were maintained in Dulbecco modified Eagle medium with the same supplements as SL3B, with the exception of NIH 3T3, which was grown in 10% calf serum instead of fetal bovine serum. All cells were grown at 37°C in 100% humidity and 7.5% CO₂.

Plasmids. Plasmids that contained the SL3-3 or Akv LTR linked to the gene for chloramphenicol acetyltransferase (CAT) were previously constructed (1, 3, 33). Deletions of the SL3-3 LTR were made by using BAL 31 exonuclease and internal restriction sites as previously described (25). Deletions to the *EcoRV* site (ΔRV) and beyond [Δ273 and Δ300] were constructed by digestion with *EcoRV*, *ApaI*, and

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*Ava*II, respectively, which cut at the corresponding locations in the enhancer. Flush ends were formed with T4 DNA polymerase and linked to a blunted *Nde*I site which cuts in vector sequences immediately upstream of the LTR. Internal deletions were made by removing restriction fragments from the appropriate exonuclease-deleted SL3-3 LTR plasmid. Plasmids containing deleted versions of the SL3-3 LTR with the Akv core element were assembled as previously described (1) by replacing a restriction fragment containing the core with the corresponding fragment from an Akv LTR CAT plasmid. The Akv fragment was identical to SL3-3 except for the single-base-pair difference in the core. Sequences of the Akv core recombinants were confirmed by using Maxam-Gilbert chemical sequencing (27).

Transfection and CAT assay. Transfections were performed as previously described (1, 33), by using the DEAE-dextran method (35). Cells (5×10^5 /ml) were transfected in 5 ml of serum-free medium containing 250 μ g of DEAE-dextran per ml with 0.5 μ g of plasmid DNA per ml for 1 h at 37°C. J558 cells do not express the gene for CAT efficiently under these conditions, so the transfection was performed by using 2.5×10^6 cells in 1 ml of serum-free medium with 250 μ g of DEAE-dextran per ml and 2.5 μ g of plasmid DNA per ml. In each experiment, a set of the plasmids was tested in parallel transfections and CAT activity was determined as previously described (15, 33). Activity was normalized, as previously described (1, 33), to the most active LTR in the trial. Averages were calculated by using the normalized numbers from multiple trials as indicated.

Protein-DNA-binding assays. Nuclear extracts from cultured cells were prepared by the method of Dignam et al. (10), with slight modifications, as described previously (1). Electrophoretic mobility shift assays were performed as previously described (1). 32 P-3'-end-labeled, 31-base-pair (bp) probes containing either the SL3-3 or Akv core were prepared as *Eco*RV-to-*Bst*NI restriction fragments from plasmids containing the SL3-3 or Akv LTR as previously described (1). Each binding reaction contained 2,500 to 5,000 Cerenkov cpm of an end-labeled probe, 3.2 μ g of poly(dI-dC) · poly(dI-dC) (Pharmacia), 10 mM Tris hydrochloride (pH 7.5), 50 mM NaCl, 1 mM dithiothreitol, 1 mM EDTA (pH 8.0), an oligonucleotide competitor as indicated in the legend to Fig. 3, and 8 μ l of nuclear extract. Each reaction was fractionated on a 5% polyacrylamide gel in a buffer consisting of 6.7 mM Tris hydrochloride (pH 7.5), 3.3 mM sodium acetate, and 1.0 mM EDTA (pH 8.0). The gel was dried and autoradiographed.

Partial purification of core-binding factor S/A-CBF. All of the following manipulations were performed at 4°C. Nuclear extract from 2×10^9 L691-6 cells in 12 ml of buffer D (20 mM HEPES [N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid] [pH 7.9], 20% [vol/vol] glycerol, 0.2 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, 0.5 mM dithiothreitol) containing 50 mM KCl was loaded onto a 4-ml Bio-Rad Affi-gel heparin-agarose column and washed with 8 ml of buffer D with 50 mM KCl. Adsorbed proteins were then eluted with a step gradient of 3-ml samples of buffer D containing a KCl concentration of 0.1, 0.2, 0.3, 0.4, 0.5, 0.7, or 1.0 M. A 25- μ l sample of each column fraction was dialyzed into buffer D containing 100 mM KCl and tested for SL3-3 and Akv core-binding activity using electrophoretic mobility shift assays. S/A-CBF, which bound both the SL3-3 and Akv probes, and SL3-3 core-binding factor (S-CBF), which bound only to the SL3-3 probe, eluted simultaneously at 0.2 to 0.4 M KCl. Fractions containing the binding activity were pooled and fractionated by Mono Q fast-protein liquid

chromatography (Pharmacia). The sample was loaded onto the column in buffer D with 50 mM KCl, washed with 3 ml of buffer D containing 50 mM KCl, and eluted with a 10-ml linear KCl gradient from 0.05 to 0.70 M in buffer D. Samples of fractions were dialyzed into buffer D with 100 mM KCl and tested for core-binding activity. S/A-CBF eluted at 0.4 to 0.6 M KCl; S-CBF eluted in the column flowthrough, the 0.05 M KCl wash, and the gradient fractions through 0.1 M KCl. Fractions containing S/A-CBF activity were combined in no. 2 dialysis tubing (Spectrapor) and concentrated by covering the tubing with polyethylene glycol 20000. The sample was then dialyzed against buffer D with 100 mM KCl, frozen on dry ice, and stored at -85°C.

DNase I protection assays. A 72-bp *Bst*NI restriction fragment that encodes a single copy of the 72-bp SL3-3 direct repeat was derived from a plasmid clone of the LTR. A 10- μ g sample of the plasmid was digested with *Bst*NI, 3' end labeled with [32 P]dATP, which labeled only the coding strand, and isolated by polyacrylamide gel electrophoresis. Partially purified S/A-CBF was serially diluted 1:2 four times with buffer D with 100 mM KCl, and 20 μ l of each dilution was incubated with 10,000 Cerenkov cpm of the 72-bp probe-0.8 μ g of poly(dI-dC) · poly(dI-dC)-10 mM Tris hydrochloride (pH 7.5)-1 mM dithiothreitol-1 mM EDTA in a total volume of 50 μ l for 10 min at room temperature. Each sample was treated by addition of 5 μ l of 75- μ g/ml DNase I (Bethesda Research Laboratories, Inc.) in 55 mM MgCl₂ and 45% glycerol for 1.0 min at room temperature. Reactions were stopped by addition of 6 μ l of 100 mM EDTA, followed by 65 μ l of phenol saturated with TE (10 mM Tris hydrochloride [pH 7.4], 1 mM EDTA). The aqueous phase was removed and extracted with 65 μ l of CIA (24:1 chloroform-isoamyl alcohol). The DNA was ethanol precipitated and suspended in 6 μ l of loading buffer (80% formamide, 10 mM NaOH, 1.0 mM EDTA, 0.1% bromophenol blue, 0.1% xylene cyanol). For a marker lane, the G-specific reaction of Maxam and Gilbert (27) was performed on the 32 P-labeled 72-bp probe. A portion (1,500 Cerenkov cpm) of each sample was heated at 100°C for 2 min, chilled on ice, electrophoresed through a 0.4-mm-thick 12% polyacrylamide gel containing 8.3 M urea, and autoradiographed.

RESULTS

Structure of the SL3-3 LTR. The organization of the SL3-3 LTR is shown in Fig. 1. The promoter elements CCAAT and TATAAAA lie in the 173 bp between the direct repeats and the transcription initiation site (U3/R boundary [Fig. 1]) (23). Upstream of the promoter, there are 2.5 copies of a 72-bp sequence that has enhancer activity. Starting from the promoter-proximal end, the enhancer contains consensus sequences for the GRE and the NF-I-binding site (4, 29). The enhancer core lies 5' to the NF-I site. Multiple factors that bind to MuLV cores have been identified (1, 25, 34, 37). Next, there is a sequence that matches the LVb-binding site that was identified in Moloney MuLV (34). The LVb site partially overlaps a second element that has homology to the core but is situated in the opposite orientation (Fig. 1, INV CORE). We arbitrarily designated this sequence the inverse core for convenience in distinguishing it from the other core. It differs from the core at two positions TGTGGTTAA versus TGTGGTTAG [Fig. 1]. The SL3-3 tandem repeats lack sequences closely homologous to the binding sites for LVa and LVc that were identified by using Moloney MuLV (34). The 72-bp structure is repeated tandemly. The portion of the repeat unit containing the NF-I-GRE region is re-

FIG. 1. Organization of the SL3-3 LTR and activities of LTR-CAT plasmids. The SL3-3 LTR-CAT plasmid (SL3) contains sequences extending from a *Pst*I site located 36 bp from the 5' end of the LTR. The 2.5 72-bp enhancer repeats are situated another 95 bp further downstream of the 5' end of the LTR. There are 173 bp from the 3' end of the repeats to the U3/R boundary. Starting from the promoter-proximal end, each repeat contains a sequence homologous to the GRE and the binding site for NF-I in the black rectangles. The positions of the consensus sequence for the core (CORE), the binding site for LVB, and a copy of the core sequence in the inverse orientation (INV CORE) are shown by white rectangles. The sequence of the region containing the core, LVB site, and inverse core is shown above the map. The *Eco*RV cleavage site is depicted by the dot within the LVB sequence (this enzyme generates flush ends). The single-base-pair difference (T-A versus C-G) between the cores of the SL3-3 and Akv viruses is also displayed. Each deletion is depicted below the SL3-3 LTR map. The number below each deletion refers to the number of base pairs deleted from the 5' end of the LTR. The activity of each deletion, normalized in each of six cell lines to intact SL3-CAT, which gave the highest activity, is shown below the 5' extent of the LTR sequences present in each. For each of the four T-cell and two non-T-cell lines, the number of trials performed with each deletion is shown in parentheses next to the relative activity. RV, *Eco*RV; ND, plasmid not tested in this cell line.

peated a third time directly upstream to complete the 2.5-repeat structure. Upstream of the enhancer region, there is another 131 bp to the 5' end of the LTR.

Mapping of sequences responsible for transcriptional activity of the SL3-3 LTR in various types of cells. We previously reported that the core is important for the activity of the SL3-3 enhancer. Specifically, substitution of the core of nonleukemogenic Akv virus, which differs from the SL3-3 core at a single base pair (Fig. 1), decreased activity in T-lymphoma cells but not in other types of cells (1). To investigate the role of the other elements within the enhancer of the SL3-3 LTR, we constructed a set of 5' deletion mutations that removed the entire 131 bp upstream of the repeats, as well as a portion of the direct repeats (Fig. 1). These were linked to the gene for CAT and tested for activity.

It was previously demonstrated that deletion of the region upstream of the tandem repeats resulted in a progressive decrease in expression; removal of the entire upstream region resulted in, at most, about a 60% reduction in the level of expression in T lymphocytes and cytotoxic T cells (16, 25). Deletion of the 131 bp upstream of the repeats plus

the adjacent copy of the NF-I-GRE motif also resulted in about a 50% reduction in the level of expression (Fig. 1, Δ154). Thus, most of this decline was due to removal of the upstream region; further deletion through the upstream NF-I-GRE region resulted in only a slight further decrease. Progressive deletion leaving a single copy of a 72-bp repeat unit resulted in an additional decrease in activity of about two- to threefold (Fig. 1, Δ199 and Δ226).

Substantial attenuation of expression occurred when deletions extended into the promoter-proximal 72-bp repeat. Removal of an additional 26 bp by deletion to the *Eco*RV site (Fig. 1, ΔRV) decreased activity from about 2.5-fold in MEL and WEHI 7.1 cells to 14-fold in SL3-B cells. This effect was more pronounced in T cells. The deleted region included the overlapping inverse core sequence and LVB-binding site but not the core. These data suggest that one or both of these elements are necessary for maximal activity. Further deletion of the core, the NF-I site, and the GRE resulted in, at most, an additional 40% decline in activity (Fig. 1, Δ300). In light of our previous observation of the importance of the core, this suggested that the core operates only in the context of sequences upstream of the *Eco*RV site.

TABLE 1. Comparative LTR activities of SL3-3 and Akv core-containing enhancers

Cell type and line (no. of expts)	Avg percentages of conversion (ratio) of transfected plasmids ^a		
	ΔS^b -SA	S $\Delta 226$ -S $\Delta 226$ /A	S ΔRV -A ΔRV
T			
L691-6 (2)	87-22 (4.0)	26-8.1 (3.2)	3.7-2.3 (1.6)
SL3-B (2)	100-32 (3.1)	31-9.5 (3.3)	4.7-4.8 (1.0)
BW5147.3 (2)	42-13 (3.2)	29-14 (2.1)	7.4-7.1 (1.0)
Non-T			
MEL (2)	58-59 (0.98)	21-24 (0.88)	13-12 (1.1)
NIH 3T3 (1)	57-44 (1.3)	17-11 (1.5)	4.7-5.2 (0.90)
J558 (1)	82-71 (1.2)	21-14 (1.5)	7.5-7.4 (1.0)

^a The first two numbers are the average normalized percentages of conversion of the two CAT plasmids relative to the intact SL3-3 LTR. The three columns represent activities from pairs of a deleted enhancer derived from SL3-3 and a construct that was isogenic except that the core element was changed to that of Akv.

^b An SL3-3 LTR CAT plasmid from which one 72-bp unit was deleted.

Effects of upstream sequences on the function of the SL3-3 and Akv cores. Recombination experiments previously established that the core element was necessary for the high activity of the SL3-3 LTR relative to the Akv LTR in T-lymphoma cells (1, 25). In light of the new evidence that sequences just upstream of the core were also important, we tested whether the SL3-3 and Akv cores could be distinguished in the context of various deletions of the SL3-3 enhancer. Table 1 reiterates the previous observation that substitution of the Akv core into an SL3-3 LTR from which one 72-bp unit had been deleted resulted in a three- to fourfold decrease in activity in T-lymphoma cells (ΔS and SA) (1). In non-T-cell lines, these two plasmids were about

equally active. We also tested whether the SL3-3 and Akv core elements could be distinguished in the context of the SL3-3 LTR deletions. A 2.1- to 3.3-fold difference was seen in the T-cell lines when the Akv core was substituted in the $\Delta 226$ construct, which contained essentially one 72-bp unit. These plasmids exhibited, at most, a 1.5-fold difference in non-T cells. However, when the Akv core was tested in the context of deletion to the *EcoRV* site that removed the inverse core and LVb region but not the core consensus sequence, essentially no difference was seen (ΔRV [Table 1]). In one T-cell line (L691-6), the SL3-3 core-containing plasmid was 1.6-fold more active; in the other two T-cell lines and the non-T lines, the two plasmids were about equally active. Thus, the SL3-3 and Akv cores could be distinguished in T-lymphoma cells only in the context of sequences 5' to the *EcoRV* site. Specifically, the 26 bp directly upstream of the *EcoRV* site were sufficient to confer most of this difference.

Effects of the inverse core-LVb-core and NF-I-GRE motifs. Additional deletions within the promoter-proximal 72-bp unit were tested in various cell lines to assess the importance of regions within it. Figure 2 shows activities relative to that of a deletion ($\Delta 300$) that extends into the promoter-proximal GRE. Addition of sequences containing the NF-I-GRE motif resulted in a twofold increase in expression (Fig. 2, $\Delta 273$). Similar effects were seen in all of the cell lines tested. The inverse core-LVb-core region, on the other hand, stimulated expression 1.9- to 8.9-fold in various T-cell lines (Fig. 2, CLC). No comparable effect was seen in the non-T-cell lines tested.

When NF-I-GRE sequences were added to the inverse core-LVb-core motif, there was about a two- to fourfold stimulation of expression. This was seen whether the NF-I-GRE sequence was located upstream or downstream of

FIG. 2. Activities of sequences in the promoter-proximal 72-bp repeat of the SL3-3 LTR. A map of the SL3-3 LTR enhancer region is shown at the top. Aligned below it are maps of five constructs that contain various elements from the SL3-3 LTR. $\Delta 300$ extends through most of the GRE homology at the 3' end of the enhancer. $\Delta 273$ has a single copy of the region containing the NF-I site and the GRE sequence (black box). CLC has a single copy of the INV CORE-LVb-CORE motif (white box). NGCLC and CLCNG each contain the INV CORE-LVb-CORE motif flanked either upstream (NGCLC) or downstream (CLCNG) by the NF-I-GRE region. The relative activity of each plasmid was tested in four T-cell and two non-T-cell lines. In each cell line, the activity of the $\Delta 300$ plasmid was assigned a value of 1.0; the fold increase caused by the additional sequence is depicted. RV, *EcoRV*.

FIG. 3. Electrophoretic mobility shift assays with the 31-bp SL3-3 core probe in the presence of specific oligonucleotide competitors. The 3'-end-labeled probe was incubated with nuclear extracts from T-lymphoma cell line WEHI 7.1 or L691-6, as indicated. A 200- or 1,000-fold molar excesses of the indicated oligonucleotide was included in each binding reaction. A zero indicates that no specific competitor was added. The mobilities of complexes formed with S/A-CBF and S-CBF are indicated.

the inverse core-LVb-core region and occurred in all of the cell lines tested (Fig. 2, NGCLC and CLCNG, respectively). The presence of the NF-I-GRE motif on both sides of the inverse core-LVb-core sequence (Fig. 1, $\Delta 199$) resulted in about another twofold increase in activity compared with the presence of one copy in most of the cell lines tested. In general, an NF-I-GRE motif had a two- to fourfold effect in all cells, while the greatest effects of the inverse core-LVb-core region occurred specifically in T lymphocytes.

Cellular factors that bind to the inverse core. The activity of the region encompassing the inverse core, LVb site, and core is presumably mediated by cellular factors that bind to these sequences. Previous work detected several factors that bound to this region of MuLV (1, 25, 37). The SL3-3 core bound to at least two distinct nuclear factors that were present in a variety of hematopoietic cell lines. One, S-CBF, bound preferentially to the SL3-3 core (1). The other, S/A-CBF, bound to both the SL3-3 and Akv cores (1). Speck and Baltimore demonstrated that factor LVb bound to the enhancer of the related virus Moloney MuLV (34). The bases critical for LVb binding, as determined by methylation interference assays, were conserved in both SL3-3 and Akv. However, the inverse core sequence was present in SL3-3 and in the similar virus Gross passage A but not in Akv or Moloney MuLV (23, 34, 38, 39).

To initiate the analysis of whether cellular factors bind to the inverse core, we tested whether a DNA fragment containing the inverse core sequence could compete with a probe containing the SL3-3 core for binding to S/A-CBF and S-CBF. The 31-bp probe (5'-ATCTGTGGTTAAGCAC TAGGGCCCCGGCCCA-3') containing the SL3-3 core (underlined) was prepared with the *EcoRV* cleavage site (Fig. 1) as the upstream end. This fragment was tested in electrophoretic mobility shift assays with nuclear extracts prepared from T-lymphoma lines L691-6 and WEHI 7.1. In each case, complexes S/A and S formed (Fig. 3). In the presence of a

1,000-fold molar excess of an oligonucleotide (5'-TGGTC CCCAGACCGCTAACGACAGGAT-3') containing the inverse core (underlined), which also extended from the *EcoRV* cleavage site (Fig. 1), the S/A complex was almost completely eliminated. The S complex was, at most, only slightly reduced when L691-6 extract was used and not at all reduced when WEHI 7.1 extract was used. Parallel control experiments were performed as previously described to demonstrate the specificity of the complexes. An oligonucleotide (5'-ATCTGTGGTTAAGCAC-3') which comprised the SL3-3 core (underlined) was tested as a competitor with L691-6 extract. Both the S/A and S complexes were eliminated (Fig. 3). Conversely, when an oligonucleotide (5'-TAGGGCCCCGGCCCA-3') encompassing the 3' half of the 31-bp probe and not containing the core was used as a competitor (Fig. 3, CON), neither complex was reduced significantly. Therefore, in agreement with our previously published results (1), both S/A-CBF and S-CBF bound to the core region. However, only S/A-CBF bound to the oligonucleotide containing the inverse core. It is interesting that the inverse core shares with the core the T-A base pair that distinguishes the SL3-3 core from the Akv core (Fig. 1). Nonetheless, S-CBF, which binds SL3-3 preferentially to Akv (1), did not recognize the inverse core (Fig. 3). Thus, at least one of the other differences between the inverse core and the SL3-3 core (Fig. 1) was responsible for the lack of S-CBF binding.

To define more precisely the binding sites of S/A-CBF within the SL3-3 enhancer, we performed DNase I footprint analysis. S/A-CBF was enriched approximately 100-fold by fractionation of nuclear extracts from L691-6 cells by using heparin-agarose chromatography, followed by fractionation with Mono-Q fast-protein liquid chromatography as described above. Fractionation of S/A-CBF and S-CBF was monitored by using electrophoretic mobility shift assays with the SL3-3 and Akv 31-bp core probes. Mono-Q fast-protein liquid chromatography separated S/A-CBF from S-CBF. DNase I footprinting using a 72-bp fragment from the SL3-3 enhancer showed that S/A-CBF protected the entire region of the coding strand extending from the 5' end of the inverse core through the 3' end of the core (Fig. 4). The core was protected to a greater extent than the inverse core, suggesting that S/A-CBF had a higher affinity for the core. In combination with the competition experiments (Fig. 3), the footprinting analysis provided evidence that S/A-CBF bound to both the inverse core and the core. Also, the fact that both elements bound the same factor provided justification beyond sequence similarity that it was legitimate to consider the inverse core to be related to the core.

DISCUSSION

The SL3-3 enhancer comprises a modular array of elements that augment transcription. Previous studies showed that deletion of the 131 bp upstream of the tandem repeats resulted in about a 25 to 60% reduction in activity in T cells (16, 25). An internal deletion of a single copy of a 72-bp repeat which left the upstream region intact also reduced activity 20 to 50% in T-lymphoma cells (1). We found that deletion of both the upstream region and the 5'-most 72 bp of the repeats (Fig. 2, $\Delta 199$) resulted in little or no further reduction in activity beyond that caused by deletion of either region alone. Thus, both regions appear to be necessary for full activity of the SL3-3 LTR. Hallberg and Grundström (16) showed that the upstream region by itself could stimulate activity up to severalfold in the context of deletions that removed most of the tandem repeat region.

FIG. 4. DNase I protection assays to identify the S/A-CBF-binding sites on the coding strand of the SL3-3 72-bp repeat. S/A-CBF from T-lymphoma cell line L691-6 was enriched approximately 100-fold by fractionation through heparin-agarose, followed by Mono Q fast-protein liquid chromatography. Samples (20 μ l) of 1:2 serial dilutions were incubated with a 3'-end-labeled 72-bp probe that encoded a single copy of the SL3-3 72-bp repeat. Numbers above the lanes describe the volume of undiluted, partially purified S/A-CBF included in the reaction run in the respective lane. G indicates a Maxam-Gilbert G-reaction marker lane. The positions of the GRE, the inverse core (upward arrow), the LVB-binding site, and the SL3-3 core (downward arrow) are shown. The *EcoRV* cleavage site that lies within the LVB-binding site is also illustrated.

Substantial activity was provided when sequences comprising one 72-bp unit were situated upstream of the 173 bp between the tandem repeats and the U3/R boundary. Among the elements within a single 72-bp unit, the core was shown by recombination experiments to be necessary for T cells to distinguish the SL3-3 enhancer from the Akv enhancer (1, 25). Here we tested the activities of combinations of different regions within a 72-bp unit. We found that a region containing the core, the LVB site, and the inverse core was sufficient to stimulate activity substantially in several T-cell lines but not in the other lines that were examined. This observation is consistent with leukemogenicity studies that showed that single repeat units of Moloney or Friend MuLV contained most of the determinants for cell type specificity of viral leukemogenicity (14, 24).

The region containing the NF-I site and the GRE gave about twofold stimulation in expression assays. However, the effect of this region was evident in both T and non-T lines. It also stimulated activity about twofold when the cores and the LVB site were present, although in a few instances the effect approached fourfold (Fig. 2). The NF-I site accounts for at least part of the activity of this region of SL3-3, as Nilsson et al. (29) showed that base substitutions within it decrease activity. The GRE is also functional (4, 16). It remains possible that additional functional sites are present in this region. However, we conclude that the region

containing the two core sequences and the LVB site is the most important determinant of the T-lymphoma cell specificity of SL3-3.

Multiple factors that bind to sequences within this region have been reported. The core element binds S-CBF and S/A-CBF (1). Thornell et al. (37) also reported a factor, SEF1, that binds to the SL3-3 core. Although we do not know whether the LVB site of SL3-3, in fact, binds LVB, the critical nucleotides identified by methylation interference assays (34) are conserved. Thus, it is likely that it does. The inverse core oligonucleotide bound S/A-CBF. Although the inverse core partially overlaps the LVB site, it is unlikely that S/A-CBF is LVB. LVB binding to Moloney MuLV is prevented by cleavage of the DNA probes at the *EcoRV* site (34), and our probe and competitor oligonucleotides were truncated at this site (Fig. 3). We did not determine whether the partially purified preparations of S/A-CBF that were used in the footprinting studies (Fig. 4) also contained LVB. It is also possible that there are additional factors that interact with this region beyond those that have already been reported. Presumably, the complete combination of factors that bind or some subset of them is responsible for viral and cell type specificity.

The single-base-pair difference between the SL3-3 and Akv cores is situated 10 bp 3' to the *EcoRV* cleavage site (Fig. 1), yet sequences 5' to the *EcoRV* site were necessary for the ability of T cells to distinguish the SL3-3 and Akv cores (Table 1). One explanation for this observation is that a single factor in T cells might both recognize sequences 5' to the *EcoRV* site and distinguish the 1-bp difference. The alternative is that activity requires one factor that binds to the core, thereby distinguishing SL3-3 from Akv, plus one or more additional factors whose binding requires sequences 5' to the *EcoRV* site. The latter situation is highly reminiscent of the core-related elements of simian virus 40 (also called GT-I and GT-IIIC) which have been shown to function only as tandemly duplicated elements or when situated adjacent to a binding site for a second factor (7, 12, 20, 30, 44).

One of the potential explanations (1) for the higher activity of the SL3-3 core in T-lymphoma cells is that factor S-CBF might act in concert with a second factor that is preferentially found or active in T cells. S-CBF was shown to bind the SL3-3 core preferentially to the Akv core when probes that were truncated at the *EcoRV* site were used (1, 25). However, it was found in both T and non-T cells (1). One possibility is that it acts in conjunction with one or more factors that bind to sequences across or 5' to the *EcoRV* site. The combination of these factors would then be responsible for viral and cell type specificity. It is interesting that the two core elements in the SL3-3 enhancer are present as a dyad symmetry centered about the *EcoRV* cleavage site. It is also interesting that the SL3-3 and Akv cores could be distinguished in T cells when sequences upstream of the *EcoRV* site were derived from Akv, Moloney, or Friend MuLV (1, 25). These three viruses share the LVB site at this position, but none has the inverse core.

Understanding fully how the SL3-3 enhancer functions will require detailed characterization of the factors that interact with multiple elements within it. Nonetheless, it appears that the factors responsible for viral cell type specificity and, by inference, for differences in viral leukemogenicity interact with the region that comprises the two cores and the LVB site.

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