Identification of the SL3-3 Virus Enhancer Core as a T-Lymphoma Cell-Specific Element

ANTHONY L. BORAL,¹ SHARON A. OKENQUIST,¹ AND JACK LENZ^{1,2*}

Departments of Genetics¹ and Microbiology and Immunology,² Albert Einstein College of Medicine, Bronx, New York 10461

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Transient expression assays were used to determine the sequences within the long terminal repeat (LTR) that define the high activity in T-lymphoma cells of the leukemogenic SL3-3 virus in comparison with that of the nonleukemogenic Akv virus. Each of these viruses contains sequences related to the consensus element, the enhancer core. The SL3-3 and Akv enhancer cores differ at a single base pair. Substitution of the Akv core element into the SL3-3 LTR decreased expression in T-lymphoma cells but not in other cell types. Likewise, substitution of the SL3-3 core sequence into the Akv LTR increased expression in T-lymphoma cells but not in other types of hematopoietic cells. These data indicate that the SL3-3 enhancer core sequence functions better than that of Akv in T-lymphoma cells, but in other hematopoietic cell types the two are approximately equivalent. Competition DNA-protein binding assays were used to assess what nuclear factors from T-lymphoma lines and non-T lines bound to the SL3-3 and Akv core elements. Factors were detected that bound specifically to either the SL3-3 or Akv core but not to the other. Another factor was detected that bound equally well to both. However, none of these factors was specific to T-lymphoma cells.

The leukemogenicity of murine leukemia viruses (MuLVs) is determined by sequences within the U3 region of the viral long terminal repeats (LTRs). Recombinant viruses that contain the U3 tandem direct repeats of the T-lymphomagenic virus SL3-3, placed in the genetic background of the nonleukemogenic, non-T-lymphotropic murine retrovirus Akv, are both lymphomagenic and T-lymphotropic (19, 28). The corresponding LTR sequences of other MuLVs also determine viral leukemogenicity and tissue tropism (3, 5, 6, 16, 20, 24, 39). The use of transient and stable expression assays has established that expression of reporter genes driven by MuLV LTRs reflects the tissue specificity of viral leukemogenicity (1, 2, 32, 36, 37, 43). Specifically, the SL3-3 LTR is on the average about eightfold more active than the Akv LTR in a variety of T-lymphoma cell lines, while both are approximately equally active in several non-T-lymphoma cell lines (2, 32).

Except for a single base pair substitution to the 5' side of the direct repeats (position -419 in Fig. 1A), sequences composing the LTRs of SL3-3 and Akv viruses differ solely within the repeats (19). These differences include single base pair changes, substitutions, and deletions of longer stretches of nucleotides (19). Some of the differences that distinguish SL3-3 from Akv are also present in the LTR of thymomagenic Gross passage A virus (19, 38). One that is common to both SL3-3 and Gross passage A virus resides in a block of nucleotides related to the consensus sequence element known as the enhancer core, which was first identified in the simian virus 40 (SV40) enhancer (18, 40) and is important for its activity (15, 40, 44). The position of the core elements within the SL3-3 and Akv LTRs and the location of the single base pair difference, a T on the coding strand of SL3-3 and a C on that of Akv, are shown in Fig. 1A.

In this report, we have undertaken an analysis of the importance of this single base pair difference for T-lymphocyte specificity. Recombinant LTRs were constructed in which the enhancer core sequences of the two viruses were

exchanged. These were linked to the chloramphenicol acetyltransferase (CAT) gene and tested for cell type specificity. In addition, DNA-protein binding assays were used to assess whether any proteins bind specifically to either of the viral core elements.

MATERIALS AND METHODS

Cell lines. L691-6 (21) and WEHI 7.1 (13) are T-lymphoma cell lines derived from radiation-induced thymic lymphomas of a C57L mouse and a BALB/c mouse, respectively. BW5147.3 is a T-lymphoma line derived from a spontaneous thymic lymphoma in an AKR/J mouse (26). SL3B is a T-lymphoma line derived from a thymic lymphoma that was induced in an AKR mouse by injection with SL3-3 virus (14). J558 (12) and MOPC315 (31) are immunoglobulin A (IgA)-secreting myeloma cell lines which were derived from mineral oil-induced plasmacytomas from different BALB/c mice. The murine erythroleukemia line MEL DS19 is an erythroid cell line derived from a subcutaneous implant of Friend virus-infected cells from a DBA/2 mouse (4). J774 is a macrophage cell line derived from a histiocytic tumor that arose in a BALB/c mouse (27). SL3B cells were grown in RPMI 1640 medium supplemented with 10% fetal calf serum, 100 U of penicillin per ml, 10 µg of streptomycin per ml, and 2 mM glutamine. All other cells were grown in Dulbecco modified Eagle medium with the same supplements. All cells were grown at 37°C in 100% humidity and 7.5% CO₂.

Plasmids. Experiments were performed with plasmids containing the SL3-3 or Akv LTRs linked to the *cat* gene. These contain the U3 and R sequences of the viral LTRs that extend on the coding strand from a position 36 nucleotides from the 5' end of the LTR to a position 32 nucleotides to the 3' side of the U3-R boundary, as shown in Fig. 1A. The 5' end of the LTR sequences was a junction of a *Pst*I site in the LTR to an *Nde*I site in the CAT plasmid (19, 32). Flush ends were created at each of the two sites by using T4 DNA polymerase prior to ligation. The 3' end of the LTR sequences was created at an *Ava*I site in the viral LTR, which was converted to a flush end with T4 DNA polymerase and

* Corresponding author.

FIG. 1. (A) Structure of the plasmids used to measure LTR-driven CAT expression. Positions of the LTR, *cat* gene, and ampicillin resistance gene (Ap^r) are shown. The arrow originating in the LTR sequences indicates the direction of transcription. The expanded schematic view of the LTRs shows the organization of the LTR sequences; numbers indicate the distance (in base pairs) from the U3-R boundary. The open boxes show the positions of the tandem direct repeats in each LTR. SL3-3 contains two and a half copies of the 72-bp sequence. Akv contains two 99-bp repeats. The solid arrow under the diagram of each LTR indicates the 31-bp *EcoRV* (RV)-*BstNI* (BNI) fragments that were radiolabeled and used in band shift assays. The sequence on the coding strand of each 31-bp fragment is shown, and the enhancer core-related sequences of each virus are indicated by a solid bar. The T-A to C-G transversion between the SL3-3 and Akv cores is indicated by an arrow and a T or a C as appropriate. The positions of the oligomers used in competition experiments, S-CORE, A-CORE, and CON, are indicated below the corresponding sequences. S-CORE and A-CORE differ only at the single base pair, while the CON sequence is identical in both viruses. (B) Schematic organization of the U3 direct repeat sequences in the plasmids used in these experiments. Numbers refer to the distance from the transcription initiation site. RV, *EcoRV*; BNI, *BstNI*. The position of the difference between the SL3-3 and the Akv cores is indicated with an arrow, and the appropriate coding strand base is shown. The top shows the organization of the repeat sequences in the native LTRs. The U3 regions of SL3-del and Akv-del each had a single copy of the direct repeat removed by cleavage with *EcoRV* and recircularization of the plasmid. In SL3-del, 72 bp have been removed with respect to SL3; in Akv-del, 99 bp have been removed with respect to Akv. The only difference to the right-hand side of the *EcoRV* site between SL3-del and Akv-del LTRs is the single base transversion in the core; all other differences are to the left of the site. SA and AS are recombinants between SL3-del and Akv-del at the *EcoRV* site.

ligated to an 8-base-pair (bp) *Hind*III linker (New England Biolabs). This was joined to an *Hind*III site in the CAT plasmid as described previously (2, 32).

All plasmids were purified from *Escherichia coli* lysates by two rounds of equilibrium density gradient centrifugation in CsCl. Before being loaded onto the second CsCl gradient, the plasmids were treated with RNase A (Sigma) and proteinase K (Boehringer Mannheim).

Transfection and CAT assay. Transient transfections of plasmid DNA were performed by the DEAE-dextran method (35). Cells (5×10^6) were suspended in 10 ml of serum-free medium containing 5 μ g of the appropriate plasmid and 250 μ g of DEAE-dextran per ml. The cells were incubated for 1 h at 37°C, 100% humidity, and 7.5% CO₂ in a 100-mm tissue culture dish. The cells were pelleted, suspended in 10 ml of medium containing serum and all other supplements, and incubated for 48 h at 37°C, 100% humidity, and 7.5% CO₂. The cells were suspended in 200 μ l of 0.25 M Tris hydrochloride (pH 7.8), and lysates were made by three cycles of freezing on dry ice for 3 min and thawing in a 37°C water bath for 3 min, followed by centrifugation for 20 min at 4°C in an Eppendorf microfuge. The concentration of protein in the lysates was determined by the Bio-Rad protein assay.

CAT enzymatic activity was determined as previously described (11, 32). Briefly, transfections of a particular cell line were always performed with all six CAT plasmids in parallel cultures. Cells were harvested and extracts were prepared. An equal amount of protein from each of the six cultures was incubated with 0.15 μ Ci of [¹⁴C]chloramphenicol (Amersham), 3 mM acetyl-coenzyme A (Pharmacia), and 0.25 M Tris hydrochloride (pH 7.8) in a final volume of 150 μ l at 37°C. The amount of extract and the length of the incubation were adjusted according to the cell line used so that the most active plasmid in that particular line acetylated 5 to 40% of the chloramphenicol in the reaction. In this manner, sufficient acetylation was obtained to be easily quantitated, and the amount of chloramphenicol acetylated in the reaction mix was a linear function of time. Reactions were extracted with 0.8 ml of ethyl acetate. Extracts were dried under vacuum, suspended in 20 μ l of ethyl acetate, and analyzed by thin-layer chromatography on silica gel plates (Eastman Kodak) in a solution of 95% chloroform–5% methanol.

Reaction products were visualized by autoradiography at –80°C, and the radioactivity in acetylated and unacetylated spots was quantified by cutting the spots out of the plate and liquid scintillation counting. The percentage of chloramphenicol that was converted to the acetylated form was calculated. The absolute percent conversion of the most active plasmid in a particular experiment involving six parallel cultures varied somewhat each time the experiment was performed. This is due to uncontrollable variation in cell culture or transfection conditions, since experiments performed simultaneously gave identical results while those performed on different days often yielded different levels of activity. Nonetheless, the relative activities of the different LTRs in parallel cultures were essentially constant regardless of the absolute levels obtained. Therefore, to calculate averages obtained in multiple experiments, the percentages of chloramphenicol acetylated in each experiment were normalized to that with the most active LTR in each experiment, arbitrarily set at 100. Means were then calculated from these normalized values and are reported here.

Preparation of nuclear extracts. Extracts of cultured cells were prepared as described by Dignam et al. (7). Extracts were made from 10⁹ cells growing in log phase. Plasma

membranes were lysed with pestle B in a Dounce homogenizer. Cells were always checked for lysis microscopically with trypan blue staining, and if necessary, additional homogenization was performed until virtually all nuclei were stainable. Phenylmethylsulfonyl fluoride (0.5 mM) and dithiothreitol (DTT) (0.5 mM) were added to buffers just before use. Extracts from 10⁹ cells were prepared in a final volume of 3 ml. Concentrations ranged from 1.5 to 5 mg/ml.

DNA-binding assay. DNA fragments containing the enhancer core sequences were obtained from plasmid clones of the viral LTRs. SL3-3 or Akv DNA (5 μ g) was digested with *Bst*NI and *Eco*RV (New England BioLabs) in a reaction volume of 25 μ l. In each case, this yielded a 31-bp fragment containing the core sequences (Fig. 1A). dCTP, dGTP, and dTTP (1 μ l of each at 1 mM), 100 μ Ci of [³²P]dATP (Amersham), and 10 U of Klenow fragment (New England Biolabs) were added. The reaction mix was incubated for 20 min on ice, and the fragments were resolved by electrophoresis on a 12% polyacrylamide gel (29:1 acrylamide-bisacrylamide) for 3.5 h at 300 V. The gel was autoradiographed, and the band corresponding to the SL3-3 or Akv 31-bp fragment was cut out of the gel, total Cerenkov cpm in the gel slice were measured, and the slice was soaked overnight in 0.6 ml of 10 mM Tris hydrochloride (pH 7.4)–1 mM EDTA (pH 8.0)–0.3 M sodium acetate (pH 5.2). The supernatant along with an additional 0.2-ml wash of the gel slice was phenol-chloroform extracted, ethanol precipitated, and suspended in 50 μ l of TE (10 mM Tris hydrochloride [pH 7.4], 1 mM EDTA). The 31-bp fragments were labeled to specific activities of 1×10^7 to 4×10^7 Cerenkov cpm/ μ g.

DNA binding to nuclear protein was carried out according to the method of Singh et al. (33) with various modifications. Briefly, 8 μ l of protein extract in buffer D (6) was brought to 25 μ l with 10 mM Tris hydrochloride (pH 7.5), 50 mM NaCl, 1 mM DTT, 1 mM EDTA (pH 8.0), 5% glycerol, 3.2 μ g of poly(dI-dC) · poly(dI-dC) (Pharmacia), and 2,500 to 5,000 Cerenkov cpm of end-labeled DNA. Oligomer competitor and 1 mM ZnCl₂ were added as indicated in the figure legends. Binding reactions were carried out for 15 min at room temperature. We tested the effect of time of incubation on the complexes formed in extracts from one cell line, L691-6. No differences in the complexes were detected in incubations as short as 1 min to as long as 30 min (data not shown). After incubation, reaction mixes were loaded onto prerun 5% polyacrylamide gels (29:1 acrylamide-bisacrylamide) and run at 110 V for 193 V-h with an integrating power supply. The electrophoresis buffer consisted of 6.7 mM Tris hydrochloride (pH 7.5), 3.3 mM sodium acetate, and 1.0 mM EDTA (pH 8.0); it was recirculated. The gel was dried onto Whatman 3mm paper and autoradiographed with an intensifying screen at –80°C. Binding was quantified by scanning with an LKB Ultrosan XL laser densitometer.

RESULTS

Specificity of the SL3-3 and Akv core sequences. A set of altered LTRs in CAT plasmids was constructed and tested in transient expression assays to assess whether the single base difference between the SL3-3 and Akv enhancer core sequences was important for cell type specificity. The core sequences were present in two copies in each of the viral LTRs (Fig. 1A). One copy was located in each of the tandem direct repeats within the LTRs. To simplify analysis of the role of this element in specificity, LTRs were constructed that contained only a single copy of the core element. This was accomplished by digesting the SL3-3 and Akv CAT

TABLE 1. LTR activity in T-lymphoma cells

Cell line	n ^b	Relative CAT activity ^a (%)					
		SL3-CAT	Akv-CAT	SL3-del	Akv-del	SA	AS
L691-6	4	100	15	73	17	21	71
WEHI7.1	2	100	10	50	8	16	26
BW5147.3	2	100	28	36	12	10	22
SL3B	5	95	25	82	39	55	71

^a Activity is expressed as normalized percent conversion. The percentage of the [¹⁴C]chloramphenicol converted to the acetylated form was determined, and the value of the most active plasmid was arbitrarily set at 100%.

^b Number of times the cell line was transfected. On each occasion, the six plasmids were transfected in parallel cultures.

plasmids with a restriction enzyme that cleaves once within each repeat (Fig. 1B). The resulting plasmids, SL3-del and Akv-del, each contained a single copy of the core.

The activities of the deleted LTRs were compared with those of the native LTRs by transfection into various types of hematopoietic cells. In T-lymphoma lines, the SL3-del LTR was always less active than the intact SL3-3 LTR (Table 1). In all cases, the deleted SL3-3 LTR was more active than the deleted Akv LTR. The activity of the Akv-del LTR relative to that of intact Akv was somewhat variable in these cells. It was decreased in the BW5147.3 line but was consistently slightly increased in the SL3B line (Table 1). Nonetheless, in all the T-lymphoma lines tested, the Akv-del LTR was less active than the SL3-del LTR.

Other types of hematopoietic cells were also used to compare the activities of the deleted and native LTRs (Table 2). Cells tested included an erythroid line (MEL) and two plasmacytoma lines (J558 and MOPC315). In all of these cell lines, the SL3-3 and Akv LTRs were approximately equally active. Likewise, the deleted LTRs expressed the *cat* gene to about the same levels. We also introduced the LTRs into the macrophage cell line J774; however, the levels of CAT activity were too low to measure in this line. We do not know if this low activity reflects an inherent property of the LTRs in this line or if these cells are refractory to the transfection procedure.

Thus, the deleted LTRs resembled the intact LTRs in that SL3-3 was more active than Akv in T-lymphoma cells but not in other types of cells, although the deleted SL3-3 LTR was less potent than the intact SL3-3 LTR. Therefore, we used the deleted versions to analyze the single base difference in the core element. Recombinant LTRs were constructed in which the cores were exchanged (Fig. 1B). The SA construct was identical to SL3-del except that it contained the Akv core sequence, while the AS construct was identical to Akv-del except that it contained the SL3-3 core.

When SA was transfected into various T-lymphoma cell lines, its activity was always lower than that of SL3-del

(Table 1). Except for SL3B cells, a decrease of approximately threefold was evident. Thus, changing the SL3-3 core sequence to the Akv sequence decreased expression of the *cat* gene in T-lymphoma cells. In other types of hematopoietic cells, this effect was not evident (Table 2). Thus, the effect of changing the SL3-3 core sequence to that of Akv was apparent only in T-lymphoma cells.

The importance of the core element was also evident when the core in the Akv-del LTR was changed to that of SL3-3 in the recombinant AS. This resulted in increased expression of the *cat* gene in T-lymphoma cells that ranged from small increases in BW5147.3 and SL3B cells to levels comparable to that of the SL3-del LTR in L691-6 cells. In other hematopoietic cells, the change resulted at most in only a small increase (Table 2). Although the 70% increase detected in the MOPC315 line was comparable to that seen in the T-cell lines BW5147.3 and SL3B, the increases seen in the other non-T lines were lower.

Thus, two lines of evidence argue that there is preferential recognition of the SL3-3 core sequence in T-lymphoma cells. Conversion of the SL3-3 core to the Akv sequence decreased LTR activity. Changing the Akv core to that of SL3-3 increased it at least slightly. The same changes had at most a marginal effect in other types of hematopoietic cells.

In the L691-6 and SL3B lines, the increase due to insertion of the SL3-3 core into an Akv background was to levels comparable to those seen with the SL3-del LTR (Table 1), although the latter line was peculiar in that the activity of the Akv-del LTR was greater than that of intact Akv. In the other T-lymphoma lines, this change yielded levels of activity that were lower than those seen with SL3-del. We interpret these data to mean that at least in some T-lymphoma lines, other differences between SL3-3 and Akv in addition to the core element are also important for the preferential activity of SL3-3. Comparison of the SA LTR to the Akv-del LTR showed that the other differences by themselves had at most only a marginal effect in the absence of the SL3-3 core (Table 1). Thus, they only exhibited their full effect in the presence of the SL3-3 core. The SL3-3 core sequence was important and perhaps essential for the high relative activity of the SL3-3 LTR in T-lymphoma cells.

Enhancer core-binding factors. These expression assays indicated that one or more cellular factors differentially interacted with the SL3-3 and Akv core elements. They also suggested that such factors might be different among various types of hematopoietic cells. To investigate the nature of the factors present in cells, we analyzed nuclear protein binding to core sequences by using mobility shift assays (33).

Nuclear extracts were prepared as described by Dignam et al. (7) from the same T-cell lymphoma and non-T lines used in the expression assays. The 31-bp ³²P-end-labeled DNA fragments that extend from the *EcoRV* site downstream to the *Bst*NI site and encompass the enhancer core were prepared from the SL3-3 and Akv LTRs (Fig. 1A). These two probes differ at only a single base pair, the T-A/C-G transversion in the core. Radiolabeled probe, nonspecific competitor DNA [poly(dI-dC) · poly(dI-dC)], and other supplements as indicated were mixed, and then nuclear extract was added. Reaction mixes were incubated at room temperature, and the products were resolved by electrophoresis in 5% nondenaturing polyacrylamide gels.

Binding to the SL3-3 core probe. Binding of factors to the SL3-3 probe was tested by using extracts from four T-lymphoma lines and three non-T lines (Fig. 2). Several binding activities were evident from this analysis. Some bands of the same mobility were detectable in multiple cell

TABLE 2. LTR activity in non-T hematopoietic cells

Cell line	n ^b	Relative CAT activity ^a (%)					
		SL3-CAT	Akv-CAT	SL3-del	Akv-del	SA	AS
MEL	5	76	95	67	54	60	73
J558	4	88	75	67	57	51	61
MOPC315	2	76	86	80	50	72	86

^a Activity is expressed as normalized percent conversion. The percent of the [¹⁴C]chloramphenicol converted to the acetylated form was determined, and the value of the most active plasmid was arbitrarily normalized to 100%.

^b Number of times the cell line was transfected. On each occasion, the six plasmids were transfected in parallel cultures.

FIG. 2. Binding assays of end-labeled SL3-3 enhancer core probe. For comparison, binding of L691-6 nuclear proteins to the Akv probe is also shown. Nuclear extracts from hematopoietic cell lines were tested; the origin of each extract is shown above the autoradiogram. No extract was added to the last lane (-). Also shown is the hematopoietic lineage of the lines used: T-lym, T-cell lymphoma; E, erythroid; M, macrophage; B, plasmacytoma. All binding reaction mixes contained 8 μ l of nuclear extract and 2,500 Cerenkov cpm of labeled DNA, and reactions were performed for 15 min. Labels on the left side of the figure indicate the positions of the bands detected with the SL3-3 probe. Those on the right show bands detected with the Akv probe.

lines, while others appeared to be unique to particular lines. The positions of two complexes that appeared to be common among several lines are shown in Fig. 2. The complex labeled S/A was present in extracts from all the T-cell lines tested, MEL cells, and J774 cells at low levels. The complex labeled S was detected in all the lines tested. In the non-T lines J774 and J558, this complex was only detected after prolonged autoradiography, suggesting that the nuclear factor responsible for it either is present in lower amounts, is less active, or is degraded in extracts from these cells. Comparison of the four T-lymphoma lines with the non-T lines showed that neither the S/A nor the S complex was specific to the T cells (Fig. 2). The amount of both binding activities detected in one non-T line, MEL, was comparable to that seen in the T-lymphoma lines.

Binding to the Akv core probe. When the same panel of nuclear extracts was tested with the Akv probe, a different pattern was detected (Fig. 3). A slow mobility complex was seen at the same position as the slowest mobility complex (S/A) formed with the SL3-3 probe and was present in all lines in which the same complex was detected with the SL3-3 probe. The equal mobility of the Akv and SL3-3 S/A complexes is shown in Fig. 2. Thus, the factor responsible for this binding activity appeared to recognize both viral probes.

No complex equivalent to the band labeled S in Fig. 2 was detected with the Akv probe (Fig. 3). Even after prolonged autoradiography, no evidence of a complex of this mobility was evident (data not shown). Thus, the factor that forms the S complex appears to be capable of distinguishing the single base pair difference between the SL3-3 and Akv probes and binds specifically to SL3-3.

Two additional complexes were detected with the Akv probe. These are identified as A-1 and A-2 in Fig. 3. The mobilities of these complexes were distinct from those of S/A and S, and they were not detected with the SL3-3 probe (Fig. 2). Thus, the factors that are responsible for these binding activities appear to be capable of distinguishing the single base pair difference between the SL3-3 and Akv probes and are specific for Akv. Complex A-1 was detected

FIG. 3. Binding assays of end-labeled Akv probe. Nuclear extracts from the cell lines were tested for binding to the Akv probe. The hematopoietic lineage of each line is shown as described in the legend to Fig. 2. The positions of the band common to both the SL3-3 and the Akv 31-bp fragments, S/A, and of the bands specific to the Akv 31-bp fragment, A-1 and A-2, are indicated. All binding reaction mixes contained 8 μ l of nuclear extract, 1 mM ZnCl_2 , and 2,500 Cerenkov cpm of end-labeled Akv 31-bp fragment, and reactions were performed for 15 min.

in extracts from all the T-lymphoma cells. It was also detected in the MEL line, although at a lower level. The complex A-2 was detected in three of the four T-cell lines tested, but was absent from BW5147 cells.

Formation of complexes A-1 and A-2 was stimulated by Zn^{2+} ions (Fig. 4). Under our standard binding conditions in the absence of any supplemental ZnCl_2 , complex A-1 was barely detectable while complex A-2 was undetectable. This is illustrated with nuclear proteins from L691 cells in Fig. 4 and was the case for all cell lines in which these factors were present. Addition of 1 mM ZnCl_2 increased the level of both complexes (Fig. 4). Supplemental ZnCl_2 had no effect on the levels of any other complexes detected with either probe (data not shown). We also tested whether Mg^{2+} affected binding. No alteration in the complexes that formed with either probe was detected with MgCl_2 at up to 5 mM.

Specificity of binding factors that form complexes S/A, S, A-1, and A-2. The 31-bp probes used in these binding reactions contained enhancer core consensus-related se-

FIG. 4. Effect of Zn^{2+} on binding to the Akv probe. Binding of L691-6 nuclear factors to the Akv probe was tested in the absence (-) and presence (+) of 1 mM ZnCl_2 . Each reaction mix contained 8 μ l of L691-6 nuclear extract and 5,000 Cerenkov cpm of end-labeled Akv 31-bp fragment, and the reaction was performed for 15 min. Positions of the bands are shown.

FIG. 5. Binding of nuclear factors to the SL3-3 probe performed in the presence of specific competitor oligonucleotides. Radiolabeled SL3-3 probe was incubated with nuclear extracts from the T-lymphoma lines L691-6 and WEHI7.1 as indicated. A 1,000-fold molar excess of the competitor oligonucleotide indicated above each lane was included as indicated: O, no competitor; S, S-CORE; A, A-CORE; CON; S+A, S-CORE and A-CORE. Sequences of the competitor oligonucleotides are shown in Fig. 1A. The positions of the bands S/A and S are indicated. All binding reaction mixes contained 8 μ l of nuclear extract and 2,500 Cerenkov cpm of end-labeled fragment, and reactions were performed for 15 min.

quences that were situated in the 5' portion of the sequence of the strand shown in Fig. 1A. Since the factors that formed complexes S, A-1, and A-2 can distinguish the single base pair difference between the core sequences in the SL3-3 and Akv probes, we presume that they are binding to this region. Complex S/A formed about equally well with either DNA. This could be due to binding of a factor to a region of the probes containing identical sequences or to binding of a factor to the core region. If the latter is the case, then the factor would not distinguish between the SL3-3 and Akv cores. To determine more precisely where the various factors bound, the mobility shift experiments were performed in the presence of 1,000-fold molar excesses of unlabeled competitor oligonucleotides. The competitors used were 16-bp double-stranded oligonucleotides encompassing the core region, called S-CORE and A-CORE, and a 15-bp oligonucleotide, CON, encompassing the remainder of the 31-bp probes (Fig. 1A).

Binding of the SL3-3 probe was tested in T-cell extracts, since both the S/A and S complexes formed with these extracts. Results obtained in competitor assays are shown in Fig. 5. Addition of the S-CORE competitor greatly reduced the levels of both the S and the S/A complexes in both extracts. These data indicate that both complexes are formed by binding of proteins to the core region of the probe. The A-CORE competitor also reduced the level of the S/A band to near background levels. This provides further evidence that the S/A complex formation is due to a core region-binding factor and indicates that the factor recognizes both SL3-3 and Akv. The A-CORE competitor also slightly reduced the level of the S complex. Densitometric quantification of the levels of the S complex in the presence of the 1,000-fold excess of A-CORE competitor showed that binding in the L691 extract was decreased to about 25% of the control levels in the absence of competitor. In WEHI7.1 cells, the decrease was to about 75% of control levels. Based on the inability of the Akv 31-bp probe to form the S complex and the relatively inefficient competition by the A-CORE oligonucleotide, we conclude that the factor that forms the S complex is SL3-3 specific. When the CON oligonucleotide, which does not contain the core homology, was used as the competitor, at most a slight inhibition of

FIG. 6. Binding of nuclear factors to the Akv probe in the presence of specific competitor oligonucleotides. Radiolabeled Akv 31-bp fragment was incubated with nuclear extracts from T-lymphoma lines L691-6 and WEHI7.1 and 1,000-fold molar excess of oligomer competitor(s) to labeled fragment as described in the legend to Fig. 5. The positions of the bands S/A, A-1, and A-2 are indicated. All binding reaction mixes contained 8 μ l of nuclear extract, 1 mM ZnCl₂, and 2,500 Cerenkov cpm of end-labeled fragment, and reactions were performed for 15 min.

formation of the S/A and S complexes was detected. These observations provide additional evidence that the S/A and S complexes are formed by factors that bind to the core sequence.

In the presence of the A-CORE competitor, a band of more complex origin was detected. The SL3-3 probe appeared to form a new complex of slower mobility than S/A in both extracts. The new complex was not detected when S-CORE was used as a competitor (Fig. 5). However, when both A-CORE and S-CORE were used simultaneously, the complex was still detectable, as was another complex of higher mobility in the WEHI7.1 extract (Fig. 5). This indicates that the 16-bp S-CORE oligonucleotide does not effectively compete with the radiolabeled 31-bp probe for binding to form these new complexes. The nature of these new complexes is unclear and remains under investigation.

The specificity of the complexes that formed with the Akv 31-bp probe was also tested with L691 and WEHI7.1 nuclear extracts (Fig. 6). The S-CORE decreased the level of the S/A band to near background levels. This provides further evidence that the same cellular factor recognizes either the SL3-3 or Akv core region to form this complex. The S-CORE probe did not affect the levels of the A-1 or the A-2 complex. All three complexes were competed with by the homologous A-CORE oligonucleotide. None of the three was significantly competed with by the CON oligonucleotide. These data indicate that all three complexes that form with the Akv probe in T-lymphoma cell extracts are the result of recognition of the core region of the probe. They also show that complexes A-1 and A-2 are formed by factors that are specific for the Akv sequence, while the S/A complex is formed by a factor that does not distinguish between SL3-3 and Akv.

DISCUSSION

Enhancers comprise an array of sites termed enhansons (25) that bind various proteins. The combination and spacing of proteins that bind in a particular type of cell determine the level of transcription. Our data show that sequences in the lymphomagenic SL3-3 virus that are related to the enhancer core consensus sequence are important for T-lymphocyte specificity. The related element in the nonleukemogenic Akv virus, which differs at a single base pair, lacks this specificity.

Exchange of the SL3-3 and Akv enhancer cores in LTRs containing a single copy of the element had about a threefold effect on activity. It is possible that the difference might be more pronounced in the native LTRs, in which two copies of the core sequence are present. Our data also indicate that other sequences besides the core contribute to the higher activity of SL3-3 in T cells. These sequences only exhibit their full effect in the presence of the SL3-3 core, which suggests that some type of cooperativity is occurring. A definitive test of the importance of the SL3-3 core in leukemogenicity awaits the generation of intact viruses with exchanged cores.

The difference in activity between the SL3-3 and Akv core sequences in T-lymphoma cells indicates that at least one nuclear factor exists in these cells that can distinguish the two elements. This factor may act positively on SL3-3, negatively on Akv, or both. One way to try to distinguish among these possibilities is to cotransfect competitor DNA molecules during the expression assays (8, 29, 30). We have attempted such experiments with plasmids or synthetic oligonucleotides containing viral core sequences as competitors; however, these studies did not distinguish between the two alternatives (A. Boral, M. Short, and J. Lenz, unpublished observations). Thus, we must consider both possibilities.

Binding studies detected the presence of factors that are capable of forming four complexes with the cores of SL3-3 and Akv. Since all the binding activities could be inactivated by heating the extracts to 90°C for 10 min (data not shown), the factors involved are presumably proteins. They might comprise multiple proteins, or they might be proteolytic fragments containing the DNA-binding domains of larger nuclear proteins. The absence or low abundance of a particular complex in any cell line could indicate that the factor is not present in the intact cells or that it is less active in extracts from that line. Alternatively, it could indicate that the factor is highly labile in extracts from that line. The S/A complex was present at low levels or absent in binding assays performed with extracts from SL3B, J558, and J774 cells (Fig. 2 and 3). In those cases, different complexes of higher mobility than S/A were frequently detected (Fig. 2 and 3). When present, these complexes, like S/A, formed with either the SL3-3 or the Akv probe. Thus, we believe that it is likely that they are formed with proteolytic products of the factor that forms S/A in most extracts, although it remains a possibility that they are formed by distinct factors.

Several pieces of evidence suggest that at least three distinct factors are responsible for the S/A, S, A-1, and A-2 complexes. Foremost is the sequence specificity of binding. The factors had different thermal stabilities, as heating the L691 extract to 60°C for 10 min had no effect on the levels of complex S formed, while the other factors were completely inactivated. Formation of the A-1 and A-2 complexes was stimulated by the presence of supplemental Zn^{2+} ions, while the others were not. The mechanism by which Zn^{2+} stimulates binding is unclear. Many nucleic acid-binding proteins utilize Zn^{2+} as a cofactor (10, 22). While it is uncertain how much Zn^{2+} was present in our extracts, it is possible that the supplemental $ZnCl_2$ was restoring depleted levels of this cofactor.

We have no evidence that the A-1 and A-2 complexes are formed by different proteins. It is quite possible that one is a proteolytic fragment of the other or that a factor binds to form one of the complexes and then a second protein binds to the complex to generate the other. In the absence of any evidence that A-1 and A-2 are formed by different core-

binding proteins, we consider them together as Akv-specific (A) complexes.

Thus, we propose that three different nuclear factors exist, SL3-3 core-binding factor (S-CBF), Akv CBF (A-CBF), and S/A-CBF, that bind the SL3-3 and Akv core sequences. Further studies will be necessary to elucidate precisely which nucleotides in the core region are bound by the various factors.

Proteins that bind to the core sequence motif have been identified and in some cases purified by using SV40, retrovirus, and mouse immunoglobulin heavy-chain enhancer probes (9, 17, 23, 34, 41, 42). The core sequences of SV40, which have also been termed GT-I and GT-II, and Moloney MuLV differ from those of SL3-3 and Akv at the four nucleotides farthest downstream in the sequence shown in Fig. 1A. Whether the SL3-3 and Akv binding factors identified here are the same as any of the previously identified proteins remains to be determined. Thornell et al. (37) have identified a protein factor termed SEF1 that binds to the SL3-3 core sequence. Binding is competed with by an autologous SL3-3-specific sequence but not by an oligonucleotide containing the Akv core sequence. This factor thus may be equivalent to the factor, S-CBF, that we have identified. Binding of factors equivalent to S/A-CBF and A-CBF was not reported (37). This might be due to the different probes that were used and the addition of $ZnCl_2$ to our reaction mixes.

Analysis of factors that bind to the Moloney MuLV enhancer has also shown the presence of a core-binding factor (34). Moloney MuLV has two core sequences that differ at a single base pair (TGTGGTATG and TGTGGTAAG), both of which are different from those in SL3-3 and Akv. Like SL3-3, Moloney MuLV induces T-cell lymphomas, and it will be interesting to test whether the Moloney MuLV core sequence binds the same factors as SL3-3. A factor called LVb binds just upstream of the core region of Moloney MuLV, and another called LVc binds just downstream of it (34). However, cleavage at the *EcoRV* site, as was done with our probes, eliminates LVb binding (34), and we detected no binding in the region downstream of the core, which is where LVc binds to the Moloney MuLV enhancer. Therefore, it is unlikely that we detected binding of LVb or LVc to the SL3-3 and Akv probe that we used.

We propose that one or more of the factors identified here are responsible for the higher activity of the SL3-3 core than the Akv core in T lymphocytes. However, based on the cell type distribution of the three factors, a simple model to explain the specificity of the SL3-3 core is difficult to construct. One possibility is that S-CBF is a positively acting factor in T-lymphoma cells. Another is that A-CBF is a negatively acting factor. A third is that factor S/A-CBF recognizes the SL3-3 core sufficiently better than the Akv core in vivo to yield a threefold difference in activity. However, none of these factors was unique to T-lymphoma cells. S-CBF was detected in all cell lines that we tested. Thornell et al. (37) also found SEF1 in all cell lines tested. In general, more of the SL3-3 core-specific binding activity was found in extracts of T cells than non-T cells. However, the level in the MEL DS19 cells was about the same as that detected in the T-lymphoma lines (Fig. 2). We interpret these data to indicate that S-CBF is not T-cell specific. Likewise, A-CBF was also detected in MEL cells (Fig. 2), although the level of binding was lower than in the T-cell lines.

The cell type specificity of SL3-3 seen in the expression studies could be explained by the critical binding factor either being present at higher levels in T cells than in non-T

cells or being qualitatively different in T than in non-T cells. Higher levels of binding of S-CBF and A-CBF were seen in T-lymphoma cell extracts than in most non-T lines. Perhaps in MEL cells, in which high levels of S-CBF binding also occurred, the factor is different in some way. One possibility is that different posttranslational modifications occur. Another theoretical possibility is that an additional factor exists that is only found in cells of the T lineage that binds to complexes formed between S-CBF and SL3-3 DNA. Extensive biochemical and genetic analyses will be required to distinguish among these possibilities as well as to elucidate how some of the binding factors can distinguish the single base pair difference between SL3-3 and Akv.

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