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Differences in Activities of Murine Retroviral Long Terminal Repeats in Cytotoxic T Lymphocytes and T-Lymphoma Cells

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Transcriptional activities of the long terminal repeats (LTRs) of various murine leukemia viruses were tested in the cytotoxic T-cell lines CTLL-1 and CTLL-2. In contrast to T-lymphoma cells, in which the LTRs of T-lymphomagenic virus SL3-3 and Moloney murine leukemia virus are more active than those of other viruses, transcriptional activity in these mature, interleukin-2-dependent cells is not correlated with the specificity of viral leukemogenicity. Several approaches were used to investigate the molecular basis for LTR activity differences in lymphoma cells and mature cytotoxic T cells. Deletion analysis of the Moloney virus LTR showed that the direct repeats associated with enhancer activity have, at most, a slight effect on expression in CTLL-1 cells, whereas they stimulate expression six- to eightfold in T-lymphoma cells. This suggests that the mature T-cell line lacks one or more factors present in T-lymphoma cells that function to augment transcription from the Moloney murine leukemia virus LTR. We also used recombinant viral LTRs to investigate the role of the enhancer core element of SL3-3 in CTLL-1 and CTLL-2 cells. A one-base-pair difference between the core sequences of SL3-3 and nonleukemogenic Akv virus, which is important for SL3-3 activity in T-lymphoma cells, had no effect in these cells. The inability to distinguish the single-base-pair difference in expression assays was correlated with the absence of binding of a cellular factor, S-CBF, to the SL3-3 enhancer core in extracts of CTLL-1 and CTLL-2 nuclei. These studies may have implications for identification of the target cells for viral leukemogenesis, as well as for tracing of changes in the transcriptional machinery during T-lymphocyte differentiation.

Viral recombination studies have established that sequences that make up the U3 component of the murine leukemia virus (MuLV) long terminal repeats (LTRs) are the primary genetic determinant of viral leukemogenicity (6–8, 14, 17, 37). The critical sequences are tandem repeats within the U3 region that are about 50 to 100 base pairs (bp) long and function as transcriptional enhancers (5, 15, 17, 18, 36). MuLV enhancers, like those of cellular genes, appear to have a modular construction of tandemly arranged components, which have been called "enhansons" (24). Each component is a sequence that is roughly 10 to 15 bp long and is the binding site for one or more cellular proteins which act in concert to augment transcription.

Tissue specificity of reporter gene transcription driven by MuLV LTRs reflects the specificity of viral leukemogenicity (4, 5, 28, 34, 35, 39). In cultured T-lymphoma cell lines, LTRs of the T-lymphomagenic viruses SL3-3 and Moloney MuLV (Mo-MuLV) are more active than are the nonlymphomagenic Akv virus and erythroleukemogenic Friend MuLV (Fr-MuLV) LTRs (5, 28, 34). Multiple elements within the MuLV enhancers appear to contribute to tissue specificity (3, 15, 18, 35). In particular, the consensus sequence element, the enhancer core, which was first identified in the simian virus 40 (SV40) enhancer (16, 38), has been shown to be important for the high activity of SL3-3 virus in T-lymphoma cells (3).

Unlike normal T cells, lymphoma cells are transformed, form tumors in syngeneic mice, and do not require interleukin-2 (IL-2) for maintenance in tissue culture. The two cytotoxic T-lymphocyte (CTL) lines used in our study were originally isolated as cytotoxic cells from the spleens of immunized animals. Although they have lost their cytotoxic ability, they retain their absolute requirement for IL-2. These cells represent a more mature, or at least different, stage in T-cell differentiation than do the thymus-derived lymphomas (9, 23), because they were isolated from a peripheral lymphoid organ and presumably had already undergone thymic passage and selection. Therefore, we decided to examine the activity of the SL3-3, Akv, Mo-MuLV, and Fr-MuLV LTRs in differentiated, IL-2 dependent CTL lines to determine whether the relative activities of the LTRs are comparable to those seen in lymphoma cells. Since the enhancer core of SL3-3 plays a significant role in transcriptional specificity in cells of the T lineage, we also tested whether it is recognized in CTL cells as it is in lymphoma cells.

MATERIALS AND METHODS

Cell lines. CTLL-1 was isolated as as CTL line that was derived from mixed allogeneic tumor cell-lymphocyte cultures of spleen cells from a C57BL/6 mouse (11). CTLL-2 was also derived as a CTL line from the spleen of a C57BL/6 mouse that had been immunized with allogeneic Friend erythroleukemia cells (1). 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 from a spontaneous thymic lymphoma in an AKR/J mouse (25). CTLL-1 and CTLL-2 cells were grown in RPMI 1640 supplemented with 10% fetal bovine serum, 100 U of penicillin per ml, 10 μg of streptomycin per ml, and 10% of the IL-2-containing supplement Rat T Cell Monoclone (Collaborative Research, Inc.). The T-lymphoma cell lines were maintained in Dulbecco modified Eagle medium with 10% fetal bovine serum–100 U of

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penicillin per ml–10 μg of streptomycin per ml–2 mM glutamine. All cells were grown at 37°C in 100% humidity and 7.5% CO_2.

Plasmids. LTRs from clones of murine retroviruses Mo-MuLV, Fr-MuLV, SL3-3, and Akv were previously used to construct plasmids containing viral LTRs linked to the chloramphenicol acetyltransferase (CAT) gene (5, 28) (see Fig. 1).

BAL 31 exonuclease deletions were made with SL3-3 and Mo-MuLV LTR-CAT plasmids that contained additional sequences 5′ to the *Pst*I or *Afl*II sites (see Fig. 1). An SL3-3 plasmid that had an *Xho*I linker ligated adjacent to the *Pst*I site was used (5). A Mo-MuLV plasmid that contained additional sequences was used so that the recognition site for *Afl*II was intact. The plasmids were linearized at the unique *Xho*I or *Afl*II site and then digested with BAL 31 exonuclease (New England BioLabs) at 30°C for 15 to 100 s (19). The deleted LTR-CAT fragment was removed by digestion with *Bam*HI, which cuts downstream of the CAT gene. The deleted LTR-CAT fragment was isolated by electrophoresis, ligated to a fragment containing the remainder of the vector (*Nde*I to *Bam*HI), and used to transform *Escherichia coli*. The structures of all of the deletions were determined by Maxam-Gilbert sequencing (20).

Pairs of deletions from the LTRs of SL3-3 and Mo-MuLV which correspond to similar points on aligned genetic maps were chosen for further analysis. Deletions extending to the *Eco*RV sites (pSDRV and pMDRV; see Fig. 1) were constructed by digesting SL3-3 and Mo-MuLV LTR-CAT plasmids with *Nde*I and *Eco*RV, forming flush ends with T4 DNA polymerase, and ligating them together.

Plasmids containing deletions of one tandem repeat and recombinant plasmids were constructed as previously described (3). All plasmids for mammalian cell transfection were purified by two rounds of equilibrium density gradient centrifugation in CsCl.

Transfection and CAT assay. Transfections were performed by the DEAE-dextran method (32) as previously described (3, 28). Cells were used at a density of 5×10^5/ml with 0.5 μg of plasmid DNA per ml for all of the lines except CTLL-2. These grow at lower densities, so a total of 5×10^5 cells was transfected with 5 μg of the appropriate plasmid in 5 ml of serum-free medium containing 250 μg of DEAE-dextran per ml. After 1 h of incubation, the cells were pelleted and suspended in 20 ml of medium containing serum and Rat T Cell Monoclone. These modifications were determined to be optimal for CAT expression in this cell line, which under our standard transfection procedure exhibits very low levels of expression.

For each experiment, parallel cultures of a particular cell line were tested with the appropriate plasmids. CAT enzymatic activity was determined as described previously (3, 12, 28). Protein amounts were determined empirically and ranged from 6 to 150 μg for particular cell lines. Activity was normalized to that of the most active LTR for that trial, and averages were calculated from multiple trials as previously described (3).

Nuclear extracts and binding assay. Nuclear extracts were prepared as described by Dignam et al. (10). L691-6 extracts were previously prepared (3). Extracts from CTL cells were made from 2×10^8 cells growing in the log phase. Plasma membranes were lysed with pestle A in a 1-ml Dounce homogenizer, and lysis was monitored microscopically with trypan blue staining. Extracts from 2×10^8 cells were prepared in a final volume of 0.75 ml.

Nuclear-protein-DNA-binding assays were performed as described by Singh et al. (30). Briefly, 5,000 Cerenkov cpm of labeled SL3-3 or Akv DNA probes, prepared as described previously (3), was brought to a 16-μl volume with 10 mM Tris hydrochloride (pH 7.5)–50 mM NaCl–1 mM dithiothreitol–1 mM EDTA (pH 8.0)–5% glycerol–3.2 μg of poly(dI-dC) · poly(dI-dC) (Pharmacia). Oligonucleotide competitors and 1 mM $ZnCl_2$ were added as indicated in the figure legends. Finally, 4 μl of the CTL or 8 μl of L691-6 extract was added. Binding reactions were incubated for 15 min at room temperature. Reactions were loaded onto a prerun 5% polyacrylamide gel containing 6.7 mM Tris hydrochloride (pH 7.5), 3.3 mM sodium acetate, and 1.0 mM EDTA (pH 8.0). Gels were run at 110 V for 193 V-h with buffer recirculation. They were dried and autoradiographed for about 16 h with an intensifying screen.

RESULTS

LTR activity in T cells. To test the transcriptional activities of retroviral LTRs, transient expression assays were performed with plasmids containing viral LTRs linked to the CAT gene, as previously described (3, 5, 28). The LTRs tested were from the thymomagenic viruses SL3-3 and Mo-MuLV, nonleukemogenic Akv virus, and erythroleukemogenic Fr-MuLV. Each CAT plasmid contains about 450 bp of the LTR, including the U3-R boundary, promoter elements, and the tandem direct repeat sequences associated with enhancer activity (Fig. 1). The LTRs were linked to the 5′ side of the CAT gene as previously described (5, 28). The relative activities of the different LTRs were ascertained by transfection of the plasmids into various T-lymphocyte lines.

Correlation between LTR transcriptional activity and the leukemogenicity of SL3-3 virus and Mo-MuLV has been demonstrated previously (5, 28, 34) and is reiterated in Table 1. In the three T-lymphoma lines tested, L691-6, WEHI 7.1, and BW5147.3, LTRs from the T-lymphomagenic viruses always exhibited a higher level of expression than did the LTRs of Akv and Fr-MuLV.

The CTL lines were tested to examine the breadth of this correlation. These cells were isolated as mature functional T cells (11). They have a doubling time of about 1 day and are strictly dependent on IL-2 for survival. They no longer require stimulation by allogeneic cells or antigen presentation to propagate. This renders them amenable to analysis by CAT plasmid transfection.

LTR activity in cytotoxic lines CTLL-1 and CTLL-2 was quite distinct from that seen in the lymphoma lines (Table 1). The most striking difference was that expression from the LTRs of the non-T-lymphotropic viruses was about the same as or higher than that of the thymomagenic viruses. In CTLL-1 cells, the SL3-3 LTR was the most active; however, it was only slightly more active than the Akv LTR. The Mo-MuLV LTR was the least active. In the CTLL-2 line, the Akv LTR was the most active, since it exhibited about three times the level of transcription of any of the others tested (Table 1). Activity from the SL3-3 LTR was relatively low in CTLL-2 cells. These data showed no correlation between the leukemogenic potentials of the viruses tested and the relative levels of transcription from their LTRs in the CTL lines.

Deletion analysis of sequences responsible for activity in CTLL-1 cells. To investigate the basis of the altered relative activity, we constructed a set of LTR deletion mutants and tested them in CTLL-1 cells. We were interested in elucidating the reasons for the relatively low activity of the Mo-MuLV LTR in these mature T lymphocytes. As a

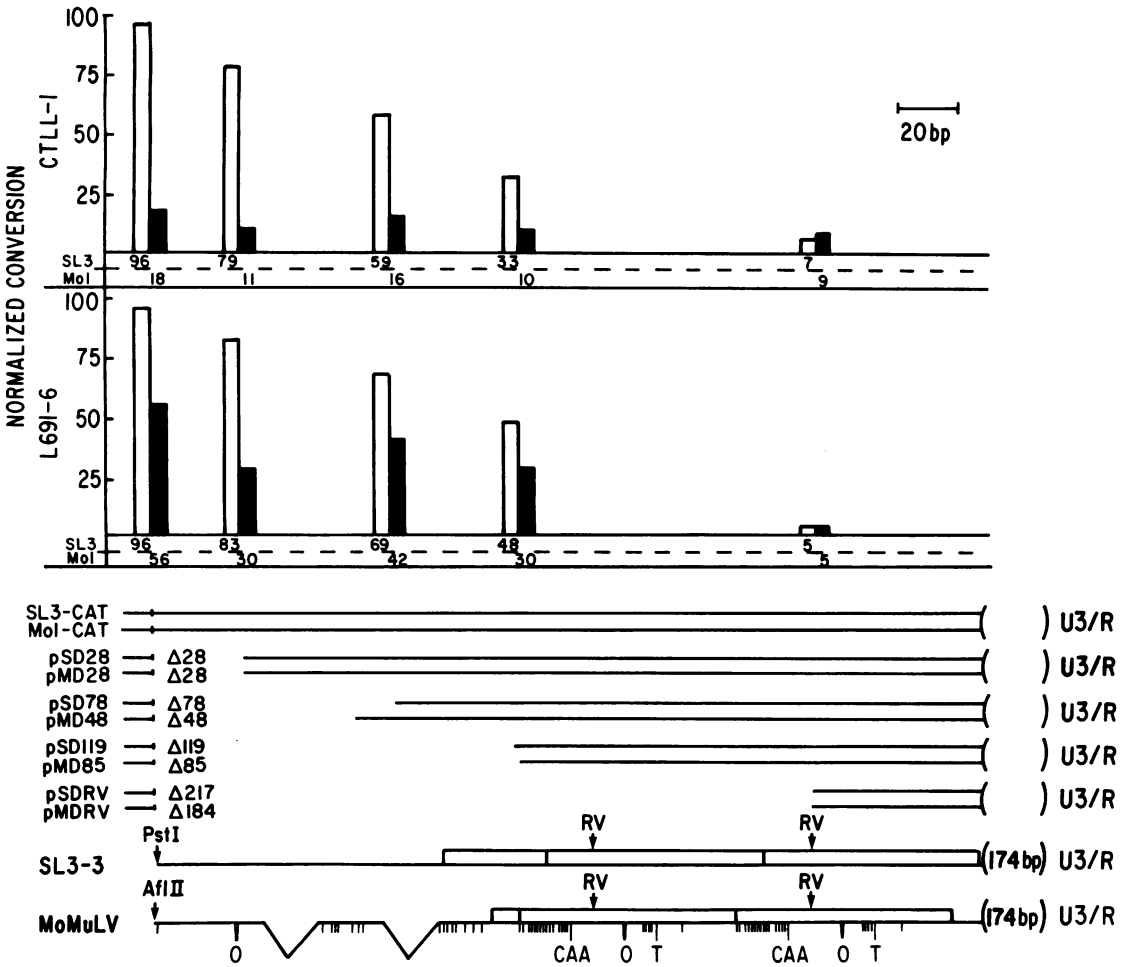

FIG. 1. LTR-CAT activities of intact and deleted versions of SL3-CAT (white bars) and Mol-CAT (black bars) in CTLL-1 and L691-6 cell lines. The bar graph represents the average percent conversion of [^{14}C]chloramphenicol to the acetylated form normalized so that that of the most active plasmid is 100. Numbers below the bars are the values represented on the graph. They are averages of a minimum of three trials performed with each set of constructs. Each pair of bars comes from a pair of corresponding deletions in the SL3-3 and Mo-MuLV LTRs. Sequences present in the deletions are diagrammed below; the 5′ ends of the deleted LTRs align with the corresponding activity data. SL3-CAT and Mol-CAT are plasmids containing LTR sequences extending to the *Pst*I and *Afl*II sites of the respective LTRs. These sites are located at precisely equivalent positions in the two LTRs, 36 bp from the 5′ ends of the LTRs; 5′ and 3′ refer to sequences on the viral coding strand. pSD28, pMD28, pSD78, pMD48, pSD119, and pMD85 are pairs of BAL 31-digested LTR-CAT plasmids from the *Pst*I site in SL3-CAT and the *Afl*II site in Mol-CAT; the length of each deletion is delineated by the name of the plasmid. pSDRV and pMDRV are deletions to the *Eco*RV sites of SL3-CAT and Mol-CAT, respectively. Genetic maps of the U3 regions of the two LTRs are shown at the bottom. The direct tandem repeats are represented by boxes. SL3-3 has two and one-half 72-bp repeats (17), while Mo-MuLV has two 75-bp repeats (27). SL3-3 contains 35 bp of the third copy of the repeat, while Mo-MuLV contains 9 bp of a third copy. The lines under the Mo-MuLV represent base pairs in Mo-MuLV that are different from those found in SL3-3. The diagonal lines in the Mo-MuLV map represent short deletions relative to sequences in SL3-3. Insertions are denoted by the nucleotides, and 1-bp deletions are indicated by zeros. Distances in base pairs to the U3-R boundary are shown in parentheses. RV, *Eco*RV.

control, we also tested an equivalent set of deletions in the SL3-3 LTR, since it was the most active in these cells. A comparison of the organizations of sequences in the two LTRs showing the positions of differences is shown in Fig. 1. Each LTR contains tandem direct repeat sequences that function as enhancers. The roles of sequences within and 5′ to the repeats were examined.

In CTLL-1 cells, deletion of the Mo-MuLV LTR to the *Eco*RV site in the promoter-proximal 75-bp repeat resulted in only a twofold reduction in activity (Fig. 1, pMDRV). A smaller deletion of the Mo-MuLV LTR, which left both of the repeats intact, except for the 5′-most 3 bp (Fig. 1, pMD85), did not yield a significant increase in activity over the larger deletion. In contrast, deletion of the SL3-3 LTR to the *Eco*RV site decreased its activity about 14-fold in these cells. The level of activity was equivalent to that of the corresponding deletion in Mo-MuLV. Deletion pSD119, which contains sequences from SL3-3 corresponding to sequences from Mo-MuLV deletion pMD85, exhibited about five times as much activity as the *Eco*RV deletion. Inclusion of additional SL3-3 or Mo-MuLV sequences further upstream resulted in only a two- to threefold increase in activity for either LTR. Thus, in CTLL-1 cells, there appears to be recognition of multiple *cis*-acting elements in the SL3-3 LTR both upstream and within the repeats. In contrast, comparable elements within the repeats of the Mo-MuLV LTR have little or no effect on transcription.

We also tested the same set of deleted LTRs in the

TABLE 1. LTR activity in T-cell lines[a]

Cell line (no. of trials)	% [^{14}C]chloramphenicol converted			
	SL3-CAT	Mol-CAT	Akv-CAT	Fr-CAT
L691-6 (5)	100	63	25	5
WEHI7.1 (3)	100	57	4	5
BW5147.3 (3)	67	100	7	15
CTLL-1 (12)	95	21	80[b]	60[c]
CTLL-2 (3)	31	34	100	38[d]

[a] Each trial involved testing each of the LTR-CAT plasmids in parallel cultures. Activity was determined by the percentage of [^{14}C]chloramphenicol converted to the acetylated form. The value of the most active plasmid in each trial was normalized to 100, and the averages of the multiple trials were calculated.
[b] Six trials were performed with Akv-CAT in CTLL-1.
[c] Three trials were performed with Fr-CAT in CTLL-1.
[d] One trial was performed with Fr-CAT in CTLL-2.

T-lymphoma cell line L691-6. Deletion to the *Eco*RV site decreased the activities of the Mo-MuLV and SL3-3 LTRs about 10- and 20-fold, respectively (Fig. 1). Plasmids containing shorter deletions exhibited higher activity. Inclusion of repeat sequences increased activity about 6- to 10-fold. Sequences upstream of the repeats also increased activity an additional twofold. Therefore, each LTR contains *cis*-acting elements both upstream and within the repeats that are recognized in these lymphoma cells.

Comparison of the activities of the various deletions in the two cell lines provided insight concerning the basis for the low relative activity of Mo-MuLV in CTLL-1 cells. Since inclusion of the entire sequence upstream of the repeats in Mo-MuLV (Fig. 1, pMD85 versus Mol-CAT) resulted in a twofold increase in activity in both CTLL-1 and L691-6, the explanation for the relatively low activity of the Mo-MuLV LTR in CTLL-1 must involve the repeats. This suggests that these cells lack one or more activities that stimulate transcription via the Mo-MuLV repeats in lymphoma cells.

Comparison of the roles of tandem repeat sequences in lymphoma and CTL lines. If factors that distinguish the Mo-MuLV tandem repeats were in fact missing in CTLL-1 cells, it would be expected that deleting one of them would not change expression. Versions of SL3-3, Mo-MuLV, Akv, and Fr-MuLV CAT plasmids that were each deleted of one tandem repeat were constructed by digestion with *Eco*RV, which cuts once in each repeat (Fig. 1), and joining of the sites together. In L691-6 cells, removal of one of the repeats resulted in a drop in LTR activity of at least 1.6-fold for all of the plasmids tested (Table 2). This demonstrated that both copies of the repeats are needed for maximal activity in these cells. Similar results were found in CTLL-1 cells, with the exception of the Mo-MuLV CAT plasmid (Table 2). Deletion of one repeat element of Mo-MuLV had no appreciable effect. This also suggests the absence of transcription factors in CTLL-1 that act via the Mo-MuLV direct repeats.

Similar experiments were performed with CTLL-2 cells. With the exception of Akv, deletion of one repeat unit from any LTR resulted in no decrease in expression (Table 2). In fact, expression from the deleted version of SL3-3 actually increased slightly. This indicates that, with the exception of Akv, both repeat elements of these LTRs are not necessary for full activity in these cells.

Test of enhancer core function in CTL lines. Since LTR activity differed between CTLs and thymoma cells, we also compared the activity of the SL3-3 and Akv enhancer core elements. The SL3-3 core differs from the Akv core by 1 bp (17). This difference is critical for the high activity of the SL3-3 LTR in T-lymphoma cells (3).

TABLE 2. Activities of native, deleted, and recombinant LTRs[a]

LTR-CAT plasmid transfected	% [^{14}C]chloramphenicol acetylated		
	L691-6[b]	CTLL-1[c]	CTLL-2[d]
SL3-CAT	99	91	29
SL3-del	68	73	44
Mol-CAT	45	21	32
Mol-del	27	26	30
Akv-CAT	27	76	97
Akv-del	16	53	69
Fr-CAT	6	42	38
Fr-del	3	28	39
MS-CAT	21	11	30
MA-CAT	4	9	28
FS-CAT	39	9	45
FA-CAT	10	8	60

[a] LTR activities were determined as described for Table 1.
[b] Four trials were performed with each plasmid in L691-6 cells, except for recombinants MS-CAT and MA-CAT, which were tested twice.
[c] Four trials were performed with each plasmid in CTLL-1 cells, except for the plasmids containing Fr-MuLV sequences, which were tested once. Data from the four native LTR plasmids presented here were also included in calculating the averages for this cell line in Table 1.
[d] Two trials were performed with each plasmid in CTLL-2 cells, except for the plasmids containing Fr-MuLV sequences, which were tested once. Data from the four native LTR plasmids presented here were also included in calculating the averages for this cell line in Table 1.

The approach taken was to construct recombinants among the various LTRs. One copy of the core is present in each of the tandem repeats in the LTRs of all of the viruses used in these studies. To simplify the experiments, the LTRs containing a deletion of one tandem repeat were used, since each of these contains a single core sequence. Recombinant LTRs were constructed, as previously described (3), at the *Eco*RV site (Fig. 2). They were then tested for the ability to drive CAT expression.

The core sequences are situated at precisely equivalent positions just to the 3′ side of the *Eco*RV site (Fig. 2). The 1-bp difference between the SL3-3 and Akv cores is the only difference between the two LTRs in the entire segment located 3′ of the *Eco*RV site. Thus, by comparing the activities of LTRs containing the 3′ LTR segment from SL3-3 or Akv in an otherwise isogenic background, the relative activities of the two cores can be assessed.

Two pairs of chimeric LTR-CAT plasmids were tested. One pair was designated MS and MA, and the other was designated FS and FA, where each letter designates the source of LTR sequences to the 5′ and 3′ sides of the *Eco*RV site (Fig. 2). Each plasmid pair is genetically identical, except for the 1-bp difference in the core. In L691-6 lymphoma cells, the LTRs containing the SL3-3 core were four to five times as active as the respective LTRs containing the Akv core (Table 2). However, when the same LTRs were tested in CTLL-1 and CTLL-2 cells, no significant difference was seen (Table 2). These data show that the CTL lines lack a factor that is essential for distinguishing between the two core elements, supporting the conclusion that these lines are distinct from T-lymphoma cells in terms of transcriptional activity.

Binding studies with the SL3 and Akv enhancer cores. DNA-protein-binding studies were undertaken to investigate

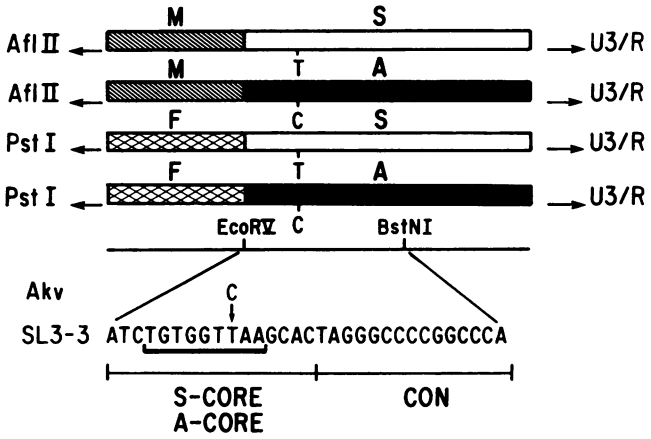

FIG. 2. Recombinant LTRs used to test the SL3-3 enhancer core. The structures of the enhancer regions of recombinant viral LTRs for all of the recombinants are shown (M, Mo-MuLV; F, Fr-MuLV; S, SL3-3; A, Akv). All were constructed at the *Eco*RV site. The sequence of the *Eco*RV-*Bst*NI fragment of virus SL3-3 that was used as a probe in the binding studies is shown. The enhancer core consensus sequence is underlined, and the position of the 1-bp difference in Akv is shown. The sequences of the competitor oligonucleotides (S-CORE, A-CORE, and CON) are indicated.

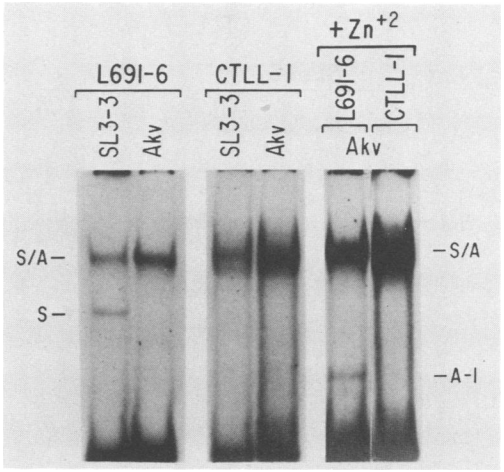

FIG. 3. Binding of nuclear factors to the enhancer core probes from SL3-3 and Akv. Nuclear extracts from L691-6 and CTLL-1 cells were mixed with 5,000 Cerenkov cpm of the indicated probe for 15 min and 1 mM $ZnCl_2$ was included as indicated. The positions of the complexes that formed are labeled. S/A is a core-binding complex that appears with the SL3-3 and Akv cores, S is found specifically with the SL3-3 probe, and A-1 is an Akv-specific complex.

the molecular basis of the inability of the CTL lines to distinguish the SL3-3 and Akv cores. Previously, we identified nuclear factors from a number of T-lymphoma lines and other types of hematopoietic cell lines that bind the SL3-3 and Akv enhancer cores (3). Using an electrophoretic binding assay and DNA probes from the SL3-3 and Akv LTRs that differ at only the 1 bp within the core, we determined that three distinct factors bind the viral cores. One factor forms a complex with either core probe, another exclusively interacts with the SL3-3 probe, and the other binds only to Akv. The three core-binding factors, S/A-CBF, S-CBF, and A-CBF, presumably work individually or in concert to yield the higher activity of the SL3-3 core in T-lymphoma cells (3).

To investigate the molecular basis for the lack of difference in activity between the SL3-3 and Akv cores in CTL cells, we used previously described probes (3) to perform binding studies on the SL3-3 and Akv enhancer cores with nuclear extracts from the CTL lines. The LTR plasmids were digested with *Eco*RV and *Bst*NI, which cleaves 31 bp downstream (Fig. 2). The 5′ half of each of the 31-bp fragments includes the core consensus sequence. The probes differ from one another only by the 1-bp T-A/C-G transition in the core (3, 17).

The end-labeled SL3-3 or Akv probe and nonspecific competitor DNA [poly(dI-dC) · poly(dI-dC)] were incubated with the nuclear extract from L691-6 or CTLL-1 cells and electrophoresed on a 5% polyacrylamide gel. As previously reported (3), two binding activities were detected with the SL3-3 probe in extracts from L691-6. One was the slow-mobility complex that can form with either probe (S/A), and the other was the SL3-3-specific complex (S) (Fig. 3). Binding assays performed with the Akv probe also detected S/A-CBF (Fig. 3). Under our standard reaction conditions, binding of A-CBF to form the complex A-1 is very low or nonexistent. However, when the binding reactions are supplemented with $ZnCl_2$, formation of the complex is detected (3). Both S/A-CBF and A-CBF bound the Akv probe when L691-6 extract was supplemented with $ZnCl_2$ (Fig. 3).

Binding of these same probes with nuclear extract from CTLL-1 cells yielded a single binding activity that had the same mobility as the S/A core-binding protein in L691-6 extracts (Fig. 3). Since the S/A complex was detected in CTLL-1 cells with either probe, it was possible that the factor either bound the core without specificity for the single-nucleotide difference or that it bound to the downstream region, which was identical in the two probes. To distinguish these possibilities, competition binding assays were done with a 1,000-fold molar excess of each unlabeled oligonucleotide. The competitors used were 16-bp oligonucleotides encompassing the 5′ half of the 31-bp fragment, and thus the SL3-3 or Akv core (S-CORE, A-CORE), and a 15-bp control oligomer (CON) from the downstream half of the fragment (Fig. 2).

Results from the competition binding assays with CTLL-1 nuclear extract are shown in Fig. 4. Addition of either the S-CORE or A-CORE competitor reduced the formation of the S/A complex to near-background levels, while the CON oligonucleotide had no visible effect. These data demonstrate that the S/A complex is formed by a core region binding factor that recognizes both SL3-3 and Akv sequences. Presumably, it is equivalent to the S/A-CBF seen in other cell lines.

No binding activity that could distinguish the 1-bp difference between the SL3-3 and Akv cores was detected with either probe whether or not the reactions were supplemented with $ZnCl_2$ (Fig. 3). Thus, the CTLL-1 line lacks both S-CBF and A-CBF, and there is a correlation between the absence of these factors and the inability to distinguish the SL3-3 and Akv cores in transient expression assays.

Binding studies were also performed with nuclear extracts prepared from CTLL-2 cells. As in the CTLL-1-binding assays, SL3-3 and Akv enhancer probes formed an S/A complex that was competed by both S-CORE and A-CORE but not by CON (Fig. 5). The band had a mobility greater than that observed with CTLL-1 extract but exhibited the same core-binding specificity (Fig. 5). Most likely, this complex is formed by a proteolytic fragment of the same S/A-CBF that is present in CTLL-1 and other types of hematopoietic cells (3). Proteolytic fragments of DNA-

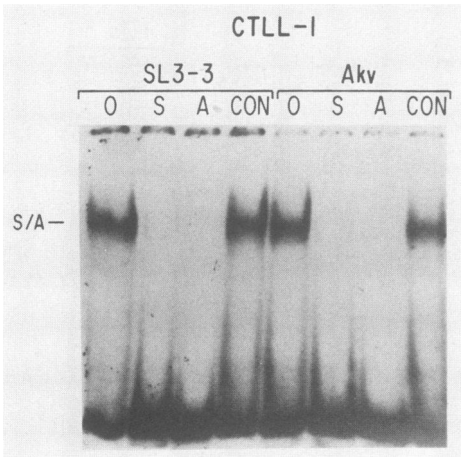

FIG. 4. Effects of competitor oligonucleotides on SL3-3- and Akv-binding factors in CTLL-1 extracts. The SL3-3 or Akv core probe was used as indicated. A 1,000-fold molar excess of one of the specific competitor oligonucleotide was included as indicated: 0, no competitor; S, S-CORE; A, A-CORE; CON, a control oligonucleotide from a sequence common to both the SL3-3 and Akv probes (Fig. 2). S/A marks the only core-binding complex formed with the CTLL-1 extract.

binding factors can retain the same sequence specificity as the native proteins (33, 35). A similar-mobility complex was sometimes detected in extracts from other cell lines and was always accompanied by a decrease in the S/A complex of the mobility seen with L691-6 and CTLL-1 extracts (3). Alternatively, it could be a distinct factor.

In addition, an Akv-specific complex, A-1, is formed with the CTLL-2 nuclear extract supplemented with $ZnCl_2$ (Fig. 5). It runs with the same mobility as the A-1 complex in L691-6-binding assays (Fig. 3). It is not competed by S-CORE or CON but is competed by A-CORE, proving that it is indeed an Akv core-specific complex (Fig. 5). In the absence of supplemental $ZnCl_2$, formation of complex A-1

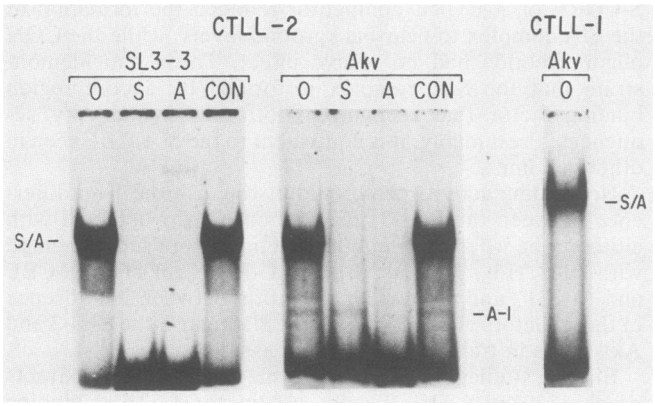

FIG. 5. Binding of factors in CTLL-2 nuclear extract to the SL3-3 or Akv probe in the absence or presence of specific competitors. A 1,000-fold molar excess of each specific oligonucleotide competitor was included as described in the legend to Fig. 4, and 1 mM $ZnCl_2$ was included in the binding reactions. The positions of S/A and A-1 complexes are indicated. One lane with CTLL-1 extract bound with the Akv probe is provided for comparison of the mobilities of the S/A complexes. The abbreviations are as defined in the legend to Fig. 4.

was not observed in CTLL-2 cells (data not shown). Thus, we presume that the factor that forms this complex is equivalent to the A-CBF seen in other cell lines (3). The fact that an Akv-specific core-binding factor was found in CTLL-2 cells without a significant difference in expression assays (Table 2) indicates that this factor plays no significant functional role in distinguishing the SL3-3 and Akv cores in these cells. In addition, the fact that S-CBF was not detected in these cells demonstrates that there is a correlation between the absence of this factor and the inability to distinguish the cores in expression assays.

DISCUSSION

These experiments show that the transcriptional activities of murine retroviral LTRs in CTL lines are distinct from those in T-lymphoma cell lines. In the lymphoma cells, LTRs of thymomagenic viruses are more active than those of other viruses (5, 28, 34, 39). In CTLL-1 and CTLL-2 cells, no such correlation with viral disease specificity exists.

One of the most striking differences in the CTL lines is the low level of expression from the Mo-MuLV LTR. Experiments undertaken to understand the molecular basis of this observation in CTLL-1 cells indicated that sequences in the tandem repeat region of the Mo-MuLV LTR do not stimulate transcription in these cells. They do, however, stimulate expression in L691-6 lymphoma cells. This implies that CTLL-1 cells lack one or more factors that are present in T-lymphoma cells that recognize sequences in the Mo-MuLV tandem repeat region. DNA-protein-binding studies have shown that many factors interact with these sequences (31). Experiments are in progress to test the hypothesis that one or more of these are absent in CTLL-1 cells.

Our data also demonstrate that the enhancer region of the SL3-3 LTR stimulates expression in CTLL-1 cells. This suggests that these cells contain at least one factor that recognizes the SL3-3 sequences but not the Mo-MuLV LTR. Numerous differences exist between the Mo-MuLV and SL3-3 LTRs (Fig. 1). Since even 1-bp differences can have profound effects (3), extensive studies may be required to determine the critical sequences responsible for the difference in activity between the two in these cells.

Other experiments are also necessary to determine whether the same factors recognize the SL3-3 enhancer in both CTLL-1 cells and lymphoma cells. As a first step, we examined the role of the enhancer core sequence, which was previously shown to be important for the difference in activity between the SL3-3 and Akv LTRs in various T-lymphoma lines (3). The data reported here show that neither CTLL-1 nor CTLL-2 cells could distinguish the SL3-3 and Akv cores in expression assays. Both of these cell lines lack a factor, S-CBF (3), that is found in thymoma cells and binds specifically to the SL3-3 core sequence. Thus, we conclude that the molecular basis of the capacity of T-lymphoma cells to distinguish the SL3-3 and Akv cores in transcription assays is most likely due to the presence of S-CBF in these cells.

While CTL lines did not distinguish the core in expression assays, it is possible that they contain factors that functionally interact with the core elements. Factors that bind to the cores of SL3-3 and Akv were detected in the CTL lines. Both lines have a factor, S/A-CBF, that interacts with either core sequence. CTLL-2 cells have an additional factor that binds specifically to the Akv sequence. Thornell et al. showed that a 3-bp mutation introduced into the SL3-3 core (TGTGGTTAA to TGTGTGGAA) resulted in a twofold

decrease in expression in CTLL-2 cells (35). The decrease in activity was attributed to a decrease in binding of a factor, SEF1 (35). Since this was the only binding activity that was observed, it is possible that SEF1 is equivalent to the factor S/A-CBF. Therefore, it is possible that the 3-bp mutation inhibits binding of this factor, with a concomitant decrease in transcription.

Our data indicate that S/A-CBF binds either the SL3-3 core or the Akv core and that binding is competed by oligonucleotides containing either sequence. However, binding of SEF1 to the SL3-3 core in CTLL-2 extracts was reported to be more efficiently competed by oligonucleotides containing the SL3-3 core than by oligonucleotides containing the Akv core (35). Possible explanations for the different observations concerning binding specificity include the different probes and the different concentrations of competitor oligonucleotides used in the studies. Nonetheless, our expression assays comparing the effects of the nucleotide difference between the cores of SL3-3 and Akv indicate that there is no preferential recognition of the SL3-3 LTR enhancer core that has any functional effect on transcription in the CTL lines.

The experiments reported here indicate that differences in transcriptional potential exist between the CTL lines and T-lymphoma cells. Several possibilities might explain the distinctions. One is that these cells are adapted to growth in culture, and changes in cellular transcriptional factors may have occurred during passage. A second is that the CTL lines resemble activated cells in that they constitutively express IL-2 receptors and require IL-2 for survival. Immune activation of T cells has been shown to stimulate expression of IL-2 receptor genes, as well as human immunodeficiency virus type 1 LTR activity, and these changes are correlated with increases in binding factor activity (2, 22, 26, 29). It is possible that murine retroviral LTRs could be coincidently regulated by factors elicited as a consequence of T-cell activation or factors regulated by IL-2 (or both types of factors). We attempted to test the latter possibility by removing the Rat T Cell Monoclone from the medium of CTLs that had been transfected with LTR-CAT plasmids. However, the cells did not survive sufficiently long under these conditions to yield measurable CAT activity.

A third possibility is based on the fact that the CTL lines and the lymphoma lines are derived from cells at distinct stages or pathways in T-lymphocyte development. The CTLs were derived as functional cells from spleens, while the lymphomas were of thymic origin. Enhancer sequences in murine leukemia virus LTRs determine the target cells of virally induced leukemias (5–8, 14, 15, 17, 18, 34, 37). Thus, it is likely that only cells that contain the appropriate transcriptional factors for viral enhancer recognition are targets for leukemogenesis. The CTL lines do not respond to the tandem repeat sequences of Mo-MuLV. Thus, if they accurately reflect a subset of T cells in mice, then it is unlikely that these are targets for leukemogenesis by this virus. Since they also lack factors that distinguish the SL3-3 core from the Akv core, they may not be targets for leukemogenesis by this virus either. Rather, the target cells may represent cells at distinct points in T-cell development that express particular transcriptional factors. If so, then the viral LTRs may be useful probes for investigating changes that occur in the factors during T-lymphocyte differentiation.

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