Analysis of the Significance of Two Single-Base-Pair Differences in the SL3-3 and Akv Virus Long Terminal Repeats

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Two single-base-pair differences between the long terminal repeats (LTRs) of the T-lymphomagenic murine retrovirus SL3-3 and nonleukemogenic Akv virus were tested for effects on activity of the LTRs. Evidence was obtained from electrophoretic mobility shift assays for the presence of at least one factor in both T and non-T cells that bound to the region of the viral enhancers that contained the differences. However, no significant differences in activity in expression assays were detected when the two base-pair differences were exchanged between the two LTRs. Therefore, they do not contribute to the higher activity of the SL3-3 LTR in T-lymphoma cell lines.

Transcriptional enhancer sequences in the long terminal repeats (LTRs) of murine leukemia viruses (MuLVs) are major genetic determinants of viral leukemogenicity (4, 8, 13, 15). MuLV enhancers generally comprise tandemly repeated units that are situated about 170 bp upstream of the transcriptional initiation site in the 5' LTR. Multiple sequences within each repeat unit contribute to enhancer activity (1, 3, 6, 7, 10, 11, 16–22, 26–28, 30, 33). Sequences flanking the tandem repeats also contribute to activity (11, 19). The relative levels of activity of the MuLV enhancers in cultured cells that were derived from various hematopoietic lineages reflect the tissue specificity of viral leukemogenicity (2, 6, 24, 29, 32). Presumably, the viral enhancers have evolved so that elements within them are recognized by the transcription factors that are present in the target cells for disease.

The tandem repeats of T-lymphomagenic SL3-3 and nonleukemogenic Akv viruses differ by four single-base-pair transitions and by more complex sequence rearrangements (Fig. 1). We have used a number of approaches to elucidate which elements are important for the high activity of the SL3-3 enhancer relative to that of Akv virus in T lymphocytes. One approach has been to determine the contribution of each difference independently. We previously reported that the single-base-pair difference between the enhancer core elements (Fig. 1) was responsible for differential binding of cellular factors and was important for the higher activity of the SL3-3 enhancer in T lymphocytes (1, 17, 18).

In this study, we examined the importance of two additional single-base-pair differences. Figure 1 outlines the organization of the enhancer regions of the SL3-3 and Akv LTRs. The SL3-3 enhancer contains two and a half copies of a 72-bp unit, while the Akv enhancer contains two copies of a 99-bp unit. The repeated sequences comprise the binding sites for numerous transcription factors, including glucocorticoid response elements (3, 7), nuclear factor 1 binding sites (21, 22, 26); enhancer core consensus sequences (CORE),

which bind multiple factors (1, 26); LVb binding sites (26–28); and, specifically in the SL3-3 enhancer, a second core sequence homology, INV CORE (18). The positions of the single-base-pair transitions (C to T) between the Akv and SL3-3 cores are also shown in Fig. 1.

The Akv enhancer contains two copies of a sequence that was previously termed A (15), one in each 99-bp repeat (Fig. 1). In the SL3-3 LTR, there is only a single A component, which is situated immediately 5' to the tandem repeats (Fig. 1). The A region sequences differ between Akv virus and SL3-3 virus by two tandemly situated G-to-A transitions (Fig. 1). The same G-to-A transitions are also present in the LTRs of the T-lymphomagenic viruses Gross passage A and SL3-2 (6, 31). Therefore, it was important to examine

FIG. 1. Organization of the U3 regions of the SL3-3 and Akv LTRs. The two maps show the positions of the repeated sequences within the U3 components of the viral LTRs. The positions of the A regions (15) are marked above the maps. Shown at the top of the figure are the sequences of the probes (I and II) from the SL3-3 A region that were used for the binding assays. The differences in the sequences of the equivalent probes from Akv virus are indicated below the arrows. Shown below the maps are the single-base-pair differences between the A regions (AA versus GG) and the cores (T versus C) of SL3-3 virus and Akv virus, respectively, and the positions of the *EcoRV* sites. GRE, Glucocorticoid response element; NF-1, nuclear factor 1.

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FIG. 2. Electrophoretic mobility shift assays using the A region oligonucleotide probes. The double-stranded probes and the sources of nuclear extracts (lane 0, no extract; lane 3T3, NIH 3T3 fibroblasts; lane L691-6, L691-6 T lymphocytes; lane BW5147, BW5147 T lymphocytes) that were used in each assay are indicated. The assays whose results are shown at the top of the figure were performed with Tris-glycine gels (22, 25), while those whose results are shown at the bottom were performed with Tris-acetate gels (1).

whether these single-base differences contribute to the higher activity of the SL3-3 LTR in T cells.

Initially, we tested whether factors that bound to the A region could be detected in extracts of the nuclei of murine cells. Electrophoretic mobility shift assays (EMSAs) were used for this purpose. Oligonucleotide probes corresponding to the A regions of the SL3-3 and Akv viruses (Fig. 1) were synthesized and radiolabeled with either $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ and polynucleotide kinase or $\alpha\text{-}^{32}\text{P}$ -deoxynucleoside triphosphates and the Klenow fragment of *Escherichia coli* DNA polymerase I. These were incubated with nuclear extracts that were prepared as described previously (9, 23) from the murine T-cell lines L691-6 and BW5147 and the fibroblast line NIH 3T3. Binding took place in the presence of the nonspecific competitor DNA poly(dI-dC) · poly(dI-dC) as described previously (1, 22, 25). The reaction products were resolved by electrophoresis on 5% polyacrylamide gels and detected by autoradiography.

Binding was detected in both the T-cell and the fibroblast lines whether the reaction products were analyzed on Tris-glycine or Tris-acetate nondenaturing gels (Fig. 2). The SL3-3 and Akv probes yielded similar patterns. To test the specificity of the binding, EMSAs were performed with SL3-3 or Akv probes (probe II [Fig. 1]) and NIH 3T3 nuclear extract in the presence of excess unlabeled SL3-3 or Akv competitor DNAs. After autoradiography, the bound com-

FIG. 3. Effects of unlabeled competitor oligonucleotides on binding by radiolabeled SL3-3 (top) and Akv (bottom) A-region probes. EMSAs were performed as described in the legend to Fig. 2 with NIH 3T3 fibroblasts, Tris-glycine gels, and double-stranded probe I. The amount of bound probe was quantified by scintillation counting of excised bands from binding assays that were performed in the presence of the amounts of unlabeled, double-stranded oligonucleotide (probe I) indicated.

plex was excised and the radioactivity was quantified. The effects of the competitor DNAs are shown in Fig. 3. Binding to the SL3-3 probe was blocked equally well by increasing concentrations of either SL3-3 or Akv competitor DNAs. This indicated that the same factor bound to both probes. When the Akv probe was tested, binding was inhibited more efficiently by the Akv competitor DNA than by the SL3-3 DNA. This observation might indicate that the binding factor had a higher affinity for the Akv sequence. An alternative hypothesis is that there might be two factors, one that binds equally to SL3-3 virus and Akv virus and another that binds only to Akv virus. In this case, both factors would have the same mobility in EMSAs. As a negative control, competition experiments were also performed with competitor oligonucleotides derived from the core regions of either SL3-3 virus or Akv virus (1, 17, 18). When present at a 1,000-fold molar excess, these oligonucleotides did not compete for binding to the A region (data not shown). This demonstrated that the A-region binding activity was specific for sequences within the A-region oligonucleotide probes and that it is distinct

TABLE 1. Relative activity of A-sequence recombinants in T-lymphoma and non-T cells

Cell type and line	<i>n</i> ^a	Relative CAT activity ^b					Ratio		
		SΔ	AΔ	AS	SΔ123	AS122	SΔ/AΔ	SΔ123/SΔ	AS122/AS
T cell									
L691-6	4	67	10	39	81	55	6.7	1.2	1.4
WEHI 7.1	2	100	17	63	49	71	5.8	0.5	1.1
SL3B	3	81	17	33	98	60	4.6	1.2	1.8
Jurkat	2	87	4	6	88	11	21.8	1.0	2.0
Non-T cell									
MEL	3	75	68	88	83	80	1.1	1.1	0.9
NIH 3T3	3	44	39	37	62	81	1.1	1.4	2.2

^a Number of independent experiments.^b All five plasmids were transfected into parallel cultures for each experiment. CAT activity was quantified and normalized to the most active LTR plasmid in each experiment as previously described (1, 17, 18). The numbers shown are averages of these values for all the experiments in each cell line.

from the previously identified SL3-3 and Akv core binding factors (1, 17, 18).

In light of the suggestion of differential binding to the SL3-3 and Akv A regions, we tested the effects on transcriptional activity of exchanging these sequences between the two LTRs. A set of recombinant recombinant LTRs (Fig. 4) was engineered and tested for the ability to direct expression of the chloramphenicol acetyltransferase (CAT) gene in transient expression assays. To simplify the generation and analysis of the recombinant LTRs, the GG and AA nucleotides in the A region were exchanged in LTRs from which one U3 tandem repeat unit was deleted. This is the same approach that was used previously to analyze the core mutations (1). The deleted LTRs, SΔ and AΔ (Fig. 4), were generated by removal of the 72- or 99-bp *EcoRV* fragment (Fig. 1) from the SL3-3 or Akv LTR, respectively. In addition, we utilized the previous observation that the single-base-pair difference in the SL3-3 core was necessary, although not sufficient, to raise the activity of the AΔ LTR to the level exhibited by the SΔ LTR in T cells (1, 18). The mutation of the Akv A-region sequence was performed with an LTR plasmid (AS) in which the SL3-3 core sequence was present in an AΔ background (Fig. 4). Mutations were introduced by site-directed mutagenesis (14) of LTR-CAT

sequences that had been cloned in M13mp18, and replicated in the *dut ung* *E. coli* mutant strain CJ236. Synthetic oligonucleotides that contained the two tandem base differences were used to prime DNA synthesis on the single-stranded templates. The mutated LTR-CAT sequences were excised from the M13 vector and subcloned. The resulting plasmids (SΔ123 and AS122, representing mutations in the SΔ and AS backgrounds, respectively [Fig. 4]) were then assayed for transcriptional activity by transfection into various cell lines in culture as previously described (1, 6, 17, 18). Duplicate isolates of each mutated LTR were tested, and the data obtained were combined (Table 1). As previously reported (1), the SΔ LTR was more active than the AΔ LTR in various T-cell lines, while in the non-T-cell lines, the two exhibited about the same activity. No substantial effects on expression were detected when the AA sequence in SL3-3 virus was changed to the GG of Akv virus. Similarly, no significant effects were seen when the reciprocal mutation was introduced into the AS LTR. The largest effects that were seen were about twofold (SΔ123 in WEHI 7.1 lymphocytes and AS122 in NIH 3T3 fibroblasts [Table 1]). However, these effects were only detected in a few cell lines. Usually, the differences were even less apparent.

We conclude that the two base-pair changes between the A regions are, at most, of minor significance for differences in the activities of the SL3-3 and Akv LTRs in expression assays in cultured T-lymphoma cells. Therefore, the high activity of the SL3-3 LTR in T cells is due primarily to the core mutation and the sequence rearrangements between the LTRs. It remains formally possible that the two differences in the A region of SL3-3 virus are involved in viral leukemogenicity. Previous deletion analyses of the SL3-3 and Akv LTRs showed that modest decreases in activity occurred when deletions extended through the A regions and neighboring sequences (12, 17, 19). Thus, it remains possible that these sequences are among the many components that contribute to the overall activity of the LTRs and that the factor(s) that we have identified here are important for this effect. It is interesting to note that factors that bind to very similar sequences in other enhancers have been identified (5).

FIG. 4. Structures of the enhancer regions of the LTR-CAT plasmids. Arrows show the positions of the repeated sequences and the CAT transcripts. The small boxes show the positions of the A regions. Positions of the single-base-pair-differences between the A regions (AA versus GG) and between the cores (T versus C) are shown.

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