A Protein-Binding Site with Dyad Symmetry in the Long Terminal Repeat of the MCF13 Murine Leukemia Virus That Contributes to Transcriptional Activity in T Lymphocytes

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We have previously identified regions in the long terminal repeat (LTR) of the MCF13 murine leukemia virus (MLV) that contribute to transcriptional activity in different cell types. We have observed that enhancer sequences and a region that resides 3' of the enhancer make significant contributions to transcriptional activity in T lymphocytes (T. Hollon and F. K. Yoshimura, *J. Virol.* 63:3353–3361, 1989). In this report, we have focused on the region of the MCF13 LTR that is 3' of the enhancer to identify binding sites for proteins that may play a role in the regulation of transcription in T cells. By gel shift and DNA footprint analyses, we have identified a single protein-binding site (MLPal) that includes a nucleotide sequence with dyad symmetry. A synthetic double-stranded oligonucleotide corresponding to this protein-binding site formed a specific protein-DNA complex. Deletion of this protein-binding site from the wild-type LTR decreased transcriptional activity in T lymphocytes but not in fibroblasts as determined by a transient expression assay. The MLPal sequence by itself cannot augment transcription in T cells but is able to do so in conjunction with enhancer sequences.

Retroviral transcription initiates in the long terminal repeat (LTR) region that contains multiple elements responsible for the regulation of this activity. To identify regions that control transcription in T cells, we have created deletions in the LTR of the MCF13 murine leukemia virus (MLV) that induces thymic lymphoma in mice (12). These studies have identified two regions of the MCF13 LTR that are important for transcriptional activity. One of these regions consists of 23 bp within the enhancer element. The second is a 119-bp region corresponding predominately to sequences located between the enhancer and viral promoter elements (12). It has been demonstrated by several laboratories that sequences in both of these regions of the LTR are important for the enhancer activity and pathogenicity of various MLVs (1, 8, 10, 11, 14, 18, 19, 21, 32, 33, 35, 36).

Speck and Baltimore (31) have identified binding sites for six different proteins in the enhancer of the Moloney MLV. We have observed that the nucleotide sequences of all of these sites are highly conserved in the MCF13 LTR. Three of these sites fall partly or completely within the 23-bp region of the MCF13 enhancer that we have observed to be important for transcription in T cells. One of these sites is the viral core consensus sequence, which has been shown to be an important determinant for T-cell-specific transcription (1, 21, 33, 35, 36). The viral core consensus sequence and a second protein-binding site (LVb) within this 23-bp region also have been shown to determine the disease specificity of leukemogenic MLVs (10, 32). The LVb site, however, contributes to transcription in a variety of cell types, as determined by transient expression assays (33).

To our knowledge, no protein-binding sites that specifically function in the regulation of transcription in T lymphocytes have been identified in the region between the enhancer and promoter of MLVs that induce T-cell leukemias. Because we have observed that this region makes a significant contribution to transcription in T cells, we have sought

to identify protein-binding sites within it and to determine their effect on transcription in this cell type. As described in this report, we have identified a protein-binding site, which we refer to as the MCF13 LTR palindrome (MLPal), in this region. The MLPal site was identified by gel shift and DNA footprint analyses. We have, furthermore, tested the functional activity of this site by deletion studies and by testing the effect of MLPal sequences on the activity of the MCF13 promoter.

MATERIALS AND METHODS

Nuclear extract and cell lines. Nuclear extract from the murine T-cell line L691 was prepared according to the method of Fujimura (6), except that 0.5% Nonidet P-40 was used for the preparation of nuclei. Protein was measured by the Bradford method (2). L691 cells were grown in RPMI 1640 (GIBCO Laboratories, Grand Island, N.Y.) containing 10% fetal bovine serum (FBS) from HyClone Laboratories, Logan, Utah. NIH 3T3 fibroblasts were grown in Dulbecco's modified Eagle medium and 10% FBS.

Preparation of DNAs for the gel shift assay. A pUC 18 plasmid clone containing the MCF13 LTR with a single copy of the enhancer element (12) was digested with *RsaI* and *BamHI* to generate a DNA fragment we refer to as dMCF (37). Enzymes were used according to the manufacturer's specifications. The oligonucleotide for the top strand was 41 bp long and contained the MLPal binding site and an additional 8 bp on either end, consisting of 3 bp of LTR sequence extending beyond each end of the MLPal site and 5 bp each at the 5' and 3' ends corresponding to the cleavage sites for *HindIII* and *PstI*, respectively. Its sequence was 5'-AGCTTCAGATAAAGCGAACTAGCAGCAGTTTC TGGCTGCA-3'. A complementary oligonucleotide was synthesized for the bottom strand. Both oligonucleotides were synthesized with an Applied Biosystems 380B synthesizer at a Howard Hughes Medical Institute core facility at the University of Washington. Double-stranded (ds) oligonucleotides and the dMCF fragment were labeled with [α -³²P]

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dATP and [α - 32 P]dCTP with the Klenow fragment of *Escherichia coli* DNA polymerase I (Boehringer Mannheim) at room temperature for 30 min. Specific activities ranged from 1×10^7 to 5×10^7 cpm/ μ g of DNA. Labeled dMCF DNA was isolated by electrophoresis through a 4% polyacrylamide gel. After autoradiography, the DNA was excised from the gel and eluted by overnight incubation in 10 mM Tris-Cl (pH 7.5)–1 mM EDTA at 37°C. The DNA was ethanol precipitated with yeast tRNA as the carrier before further use.

Gel shift assay. The gel shift assay is based on the method devised by Garner and Revzin (7). Briefly, 1 ng of 32 P-labeled dMCF DNA or 8 ng of 32 P-labeled MLPal ds oligonucleotide (approximately 20,000 Cerenkov cpm) was reacted with 0.5 to 6 μ g of nuclear extract and 1 μ g of poly(dI-dC) (Boehringer Mannheim) in 12 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid [pH 7.9])–25 mM NaCl–0.5 mM EDTA–1 mM dithiothreitol for 30 min at room temperature. Samples were electrophoresed through a 4% polyacrylamide gel in a low-salt Tris-acetate running buffer (6.7 mM Tris-Cl [pH 7.5], 1 mM EDTA, 3.3 mM Na acetate) at 180 V for various lengths of time. Gels were dried and exposed to Kodak X-Omat film with a DuPont Lightning Plus intensifying screen at -70°C overnight.

Chemical footprinting. DNA footprints for the dMCF fragment were obtained by the 1,10-phenanthroline-copper ion [(OP) $_2$ Cu $^{+}$] chemical cleavage reaction described by Sigman and coworkers (17, 30). Plasmid DNA was digested with *Bam*HI and end-labeled on the coding strand with [α - 32 P]dATP and the Klenow fragment of DNA polymerase I. The labeled DNA was then digested with *Rsa*I and ethanol precipitated. For labeling of the noncoding strand, the *Bam*HI-digested DNA was end-labeled with [γ - 32 P]ATP and T4 polynucleotide kinase. It was then digested with *Rsa*I and precipitated. The end-labeled dMCF fragments were isolated as described above. Binding reactions were scaled up 20-fold before polyacrylamide gel electrophoresis. The binding gel was submerged in 10 mM Tris-Cl, pH 8.0, to which were added (OP) $_2$ Cu $^{+}$ to 0.045 mM CuSO $_4$ and 0.2 mM OP and 3-mercaptopropionic acid to 5.2 mM. Gels were incubated for 8.5 min at room temperature. Digestions were quenched with the addition of 2,9-dimethyl-1,10-phenanthroline to 3.1 mM for 2 min. Gels were exposed to X-ray film at room temperature. Bands were excised from the gels, and DNA was eluted as described above. Isolated DNAs were treated with phenol-chloroform before ethanol precipitation. DNA samples were resuspended in 50 mM Tris-Cl (pH 8.3)–80% formamide–1 mM EDTA–0.1% xylene cyanol–0.1% bromophenol blue, heated at 70°C for 10 min, and electrophoresed through an 8% polyacrylamide gel containing 8 M urea. The gels were dried and exposed to X-ray film at -70°C with an intensifying screen.

Plasmid constructs. To generate plasmid constructs containing deletions of the MLPal site or the region between the enhancer and promoter (DEN) of the MCF13 LTR, we used oligonucleotide-directed mutagenesis based on the procedure of Sayers et al. (28). Thirty-six-base-pair oligonucleotides consisting of sequences flanking either the MLPal site or the DEN region were synthesized and purified by electrophoresis through an 18% denaturing polyacrylamide gel. After phosphorylation with T4 polynucleotide kinase, the oligonucleotide was annealed to the single-stranded DNA form of a pBS (Stratagene) clone containing the MCF13 LTR with a single enhancer element linked to the chloramphenicol acetyltransferase (CAT) gene (12). The mutant DNA

strand was synthesized in the presence of dCTP α S with the Klenow fragment of DNA polymerase I and T4 DNA ligase. After removal of single-stranded DNA, the heteroduplex plasmid was treated with *Nci*I. The nicked DNA was then exposed to exonuclease III followed by treatment with Klenow enzyme and ligated as before. Competent TG1 cells (Amersham) were transformed with the reaction mixture, and colonies were screened by restriction endonuclease analysis of miniplasmid DNAs. The LTRs of positive clones were sequenced by the dideoxynucleotide method (Sequenase; United States Biochemical) to verify the mutations.

To create a plasmid construct containing one copy of MLPal sequences in the MCF13 LTR region between the enhancer element and promoter region, we inserted a synthetic ds oligonucleotide containing MLPal sequences into the *Sph*I and *Pst*I sites of the PrM clone. PrM contains MCF13 LTR promoter sequences between the *Dra*I and *Sma*I sites ligated to the CAT gene (12). MCF13 LTR sequences between the *Pst*I and *Rsa*I sites (169 bp long) were amplified from an LTR clone containing a single enhancer element by the polymerase chain reaction technique (27). This LTR fragment with a *Hind*III site at the 5' end and an *Sph*I site at the 3' end was inserted into the MLPal-containing clones at the *Hind*III and *Sph*I sites. The clone containing a mutated MLPal sequence was constructed similarly. Both clones were verified by dideoxynucleotide sequencing.

DNA transfection and CAT assay. Lymphocytes were washed twice with serum-free medium (RPMI 1640; GIBCO). Plasmid DNA (12.5 μ g) and Lipofectin reagent (85 μ g) (Bethesda Research Laboratories) were each added to 1.5 ml of medium. The DNA and Lipofectin were combined and added to 10^7 cells. After incubation for 6 h at 37°C, 7 ml of medium containing 20% heat-inactivated FBS (HyClone Laboratories) was added to the cells. The following day, cells were collected by centrifugation and placed in 30 ml of medium plus 10% FBS. Transfection of NIH 3T3 fibroblasts was performed by incubating 2×10^6 to 3×10^6 cells on a 10-cm plate with 1.5 ml of Optimem (Bethesda Research Laboratories) containing 50 μ g of Lipofectin for 60 min at 37°C. Thirty-six micrograms of DNA in 1.5 ml of Optimem was subsequently added to each plate of cells, which was incubated at 37°C for an additional 6 h. Dulbecco modified Eagle medium (7 ml) containing 20% FBS was then added to each plate. Fresh medium containing 10% FBS was added to the cells the next day. The cells were harvested 24 h later and processed for CAT assays as detailed by Hollon and Yoshimura (12).

RESULTS

Identification of the MLPal protein-binding site by gel mobility shift and DNA footprint analyses. We previously determined that MCF13 LTR sequences predominantly 3' to the enhancer element and 5' to the promoter made a significant contribution to transcriptional activity in T cells (12, 36). Because of the importance of this region for transcription, we searched for binding sites for nuclear proteins that might function as transactivators. No known protein-binding sites in this region of the LTR of MLVs that induce thymic lymphoma have been previously identified. We initially used the gel mobility shift assay (7) to identify potential DNA protein-binding sites (37). For these assays, we used a 103-bp DNA fragment (dMCF) consisting of MCF13 LTR sequences located between the *Rsa*I and *Dra*I sites (Fig. 1) and 10 bp of the multiple cloning site of pUC 18 into which

FIG. 1. MCF13 LTR. Open boxes correspond to 69-bp direct repeats; hatched box delineates downstream *RsaI*-*DraI* DNA fragment (dMCF) used in this study; CAT and TATA boxes are indicated; horizontal arrow marks transcription initiation site; the MLPal site is indicated by the darkly stippled box.

this LTR was molecularly cloned. We have referred to this region of the LTR as downstream of the enhancer (DEN), which corresponds to nearly all of the sequences located between the enhancer element and the promoter as indicated by the hatched box in Fig. 1. We have also included in Fig. 1 the MCF13 enhancer element, which is defined as the 69-bp tandem repeat shown by open boxes, and the start of transcription, which is denoted by the horizontal arrow. Experiments involving the gel mobility shift assay, which were detailed in a previous report (37), resulted in the detection of five protein-DNA complexes for the dMCF fragment by using nuclear extract from the murine T-lymphocyte cell line L691 for binding (Fig. 2A, lane 1). Competition experiments revealed that only three of these complexes (I, II, and III) resulted from specific binding of protein to the dMCF DNA fragment (37).

To identify the nucleotide sequences of the site bound by protein for each of these three specific complexes, we performed DNA footprint analysis with the chemical reagent $(\text{OP})_2\text{Cu}^+$ for strand scission (16, 30). This footprinting technique was chosen because of the finer resolution of cleavage sites that this chemical reagent generates compared with that generated by the enzyme DNase I. ^{32}P -labeled dMCF was reacted with nuclear extract from the T-cell line L691 and electrophoresed through a 4% polyacrylamide gel. DNA complexed with protein while still in the gel was subjected to cleavage by $(\text{OP})_2\text{Cu}^+$ and isolated from complexes I, II, and III. Isolated DNA was subsequently electrophoresed through an 8% polyacrylamide sequencing gel. Results from the footprint analysis of the coding strand of dMCF complexes I through III are presented in Fig. 3A. We observed that DNA from dMCF protein-DNA complexes I, II, and III produced identical footprint patterns, as shown in lanes 2, 4, and 5, respectively. For each of these footprints, we observed that the same nucleotides extending from positions -146 to -163 with respect to the transcription start site were protected by bound protein (Fig. 3C). We also observed the appearance of two hypersensitive sites at nucleotides -146 and -157 (indicated by arrows in Fig. 3C). The footprint we obtained for unbound DNA that comigrated with free DNA on a mobility shift gel is presented in lanes 3 and 6 of Fig. 3A. Lane 1 corresponds to a G tract of the coding strand.

The footprints we obtained for the noncoding strand of DNA present in protein-DNA complexes II and III are shown in Fig. 3B. The footprints for DNA bound in these two complexes are also identical to each other (lanes 2 and 4). We observed protection of nucleotides from positions -147 to -170 with hypersensitive cleavage sites at positions -148, -149, and -159 (marked by arrows in Fig. 3C). The pattern for unbound DNA is shown in Fig. 3B, lane 3. Lane 1 displays the G tract of this strand. The protected bases on

both strands are identified by brackets in Fig. 3C. We observed that the nucleotides protected by protein in dMCF complexes I, II, and III include an 18-bp sequence with dyad symmetry (nucleotides underlined in Fig. 3C). We refer to this site of protein binding in the dMCF region of the MCF13 LTR as MLPal (MCF LTR palindrome) because of the presence of this nearly perfect palindromic sequence. The MLPal protein-binding site, indicated by the darkly stippled

FIG. 2. Gel shift assay for dMCF and MLPal DNAs with L691 nuclear extract (A) and binding competition for MLPal (B) and dMCF (C) in a gel shift assay. (A) Lane 1, dMCF (five different protein-DNA complexes are indicated by Roman numerals); lane 2, ds MLPal oligonucleotide. DNAs were ^{32}P -labeled with T4 DNA polymerase and the large fragment of *E. coli* DNA polymerase I by using $[\alpha\text{-}^{32}\text{P}]\text{dATP}$ and $[\alpha\text{-}^{32}\text{P}]\text{dCTP}$ and purified by polyacrylamide gel electrophoresis. One nanogram of dMCF or 8 ng of MLPal ^{32}P -labeled DNA was reacted with 0.5 to 6 μg of L691 nuclear extract and 1 μg of poly(dI-dC) (Boehringer Mannheim) in 12 mM HEPES (pH 7.9)-25 mM NaCl-0.5 mM EDTA-1 mM dithiothreitol for 30 min at room temperature. Samples were electrophoresed through a 4% polyacrylamide gel in Tris-acetate buffer (6.7 mM Tris-Cl [pH 7.5], 1 mM EDTA, 3.3 mM Na acetate) at 180 V for 80 min. Gels were dried and exposed to Kodak X-Omat film with a DuPont Lightning Plus intensifying screen at -70°C overnight. (B) Lanes: 1, ^{32}P -labeled MLPal with no cold competitor DNA; 2, 100 $\times$ MLPal cold competitor; 3, 100 $\times$ HLA cold competitor. Binding reactions with 8 ng of ^{32}P -labeled MLPal ds oligonucleotide were performed as described for panel A, except that unlabeled ds DNAs were added to the reaction mixture prior to incubation. (C) Lanes: 1, ^{32}P -labeled dMCF with no cold competitor; 2, 3, and 4, 100 $\times$, 1,000 $\times$, and 10,000 $\times$ unlabeled MLPal ds oligonucleotide, respectively. Competition experiments were performed as described for panel B.

FIG. 3. Identification of protected nucleotides in protein-DNA dMCF complexes I, II, and III by DNA footprinting. DNA strand scission by $(\text{OP})_2\text{Cu}^+$ was used for footprinting analysis of both the coding (A) and noncoding (B) strands of dMCF. dMCF DNA (100 ng) that was ^{32}P -labeled on one strand was used in a binding reaction and electrophoresed through a 4% polyacrylamide gel as described for Fig. 2A. Gels were treated with $(\text{OP})_2\text{Cu}^+$ as detailed in Materials and Methods and were exposed to X-ray film at room temperature overnight. Bands were excised from the gels, and the eluted DNA was treated with phenol-chloroform before electrophoresis through an 8% urea-polyacrylamide sequencing gel, which was dried and exposed to X-ray film with an intensifying screen at -70°C . (A) Lanes: 1, G tract; 2, complex I; 3, unbound DNA treated identically to complexed DNAs; 4, complex II; 5, complex III; 6, unbound DNA. (B) Lanes: 1, G tract; 2, complex II; 3, unbound DNA; 4, complex III. Protected nucleotides for both strands are shown to the right of the lanes. (C) Nucleotide sequence of dMCF DNA with residues protected from $(\text{OP})_2\text{Cu}^+$ cleavage indicated by brackets and hypersensitive sites by arrows. A sequence with dyad symmetry is underlined. (D) Nucleotide sequences for various CAT reporter gene constructs at the site in the wild-type M3 clone where either MLPal or DEN sequences have been deleted and MLPal or Mut-1 sequences have been substituted into the ΔDEN clone. Deleted bases are indicated by black dots. The CAT box has been delineated as a reference point for all of the clones.

box in Fig. 1, starts 14 bp downstream of the enhancer element.

Protein-binding assays of MLPal oligonucleotide. To test the ability of the MLPal site to specifically bind protein, we have used synthetic ds oligonucleotides corresponding to the MLPal sequence in gel mobility shift assays. ^{32}P -labeled MLPal ds oligonucleotide was reacted with nuclear extract from L691 cells and electrophoresed alongside the dMCF DNA fragment that was treated similarly. This oligonucleotide generated only one major band that comigrated with

dMCF complex III (Fig. 2A, lane 2). Fainter bands that comigrated with the other dMCF complexes were also detectable. However, the intensities of these bands varied between experiments (compare Fig. 2A and B).

We have tested the specificity of binding of the major protein-DNA complex detected with the MLPal oligonucleotide by competition experiments. We have used cold ds oligonucleotides corresponding to either MLPal or 34 bp from the 5' regulatory region of the HLA-DR α gene promoter (34) to compete with protein binding to ^{32}P -labeled

TABLE 1. Relative transcriptional activity of MLPal deletion mutant^a

CAT clone	Relative transcriptional activity of MLPal deletion mutant in cell line		
	L691	Jurkat	NIH 3T3
M3	1.00	1.00	1.00
ΔMLPal	0.43 ± .04	0.34 ± .02	1.12 ± .09

^a Values are expressed as a fraction of the activity of the wild-type clone M3 for each cell line. The activity of M3 has been assigned the arbitrary value of 1.00. Each ΔMLPal value represents the mean ± standard error of at least two independent transfections performed in at least two different experiments. The range was 5×10^3 to 1×10^5 cpm for each CAT assay; the values have been corrected for background (less than 10^3 cpm). More than one plasmid preparation for each clone was tested. L691 and Jurkat are a mouse and human T-cell line, respectively, and NIH 3T3 is a mouse fibroblast line.

MLPal in a gel mobility shift assay (Fig. 2B). As seen in lanes 1 and 3, besides the major band that we consistently observed for the MLPal oligonucleotide (shown by arrow), other additional bands were present on this gel. One of these bands did not correspond to any of the dMCF complexes, and we have not characterized it further because it was not consistently detectable (Fig. 2A). With a 100-fold excess of cold competitor ds oligonucleotides, we observed that the MLPal (Fig. 2B, lane 2) but not the HLA (lane 3) oligonucleotide effectively competed with binding. The results from these competition experiments indicated that the protein binding we observed with the MLPal oligomer is indeed specific. Using cold MLPal DNA, we also have successfully inhibited the binding of dMCF in the generation of complexes I through III (Fig. 2C). We have also observed that MLPal competed with two other dMCF complexes (IV and V) that resulted from nonspecific protein binding (37).

Effect of MLPal deletion on MCF13 LTR transcriptional activity. We wished to determine the importance of the MLPal protein-binding site for the transcriptional activity of the MCF13 LTR in different cell types. To do this, we deleted the MLPal site from a plasmid clone of the MCF13 LTR (MMM, which is abbreviated M3 in this paper [12]) that contained a single enhancer element by oligonucleotide-directed mutagenesis (28). The rationale for using a clone of the MCF13 LTR with a single enhancer was to facilitate the construction of this deletion mutant. Furthermore, we had previously demonstrated that the deletion of one enhancer element did not significantly affect the transcriptional activity of the MCF13 LTR in T cells (12). The reporter gene we have used for transient expression assays of LTR activity was the CAT gene (9). The transcriptional activity of the MLPal deletion mutant ΔMLPal (Fig. 3C) was compared with that of the wild-type clone M3 in different cell types (Table 1). Because we and others have observed that the removal of a single protein-binding site in a potent enhancer can have only a moderate effect on transcriptional activity (1, 33, 35, 36), it was essential that we use a method of transfection that would yield CAT values with little variability to assess the significance of potentially small differences in CAT enzyme activity. A transfection technique that resulted in little variability involved the use of a liposome reagent (Lipofectin [36]). Plasmid DNAs for M3 and ΔMLPal were transfected into T cells (L691 and Jurkat) and fibroblasts (NIH 3T3). Data from these transfections demonstrated that deletion of the MLPal site resulted in a two- to threefold decrease in CAT activity in the T-cell lines compared with that when M3 was deleted, but this effect was

TABLE 2. Effect of the MLPal sequence on transcriptional activity^a

Clone	Transcriptional activity in cell line	
	L691	NIH 3T3
M3	1.00	1.00
ΔDEN	0.11 ± .01	0.23 ± .04
ΔDEN+MLPal	2.05 ± .15	0.25 ± .03
ΔDEN+Mut-1	0.92 ± .10	ND ^b

^a Values in this table were calculated as described in Table 1.

^b ND, not determined.

only in the T cell lines. MLPal deletion had no effect on transcription in murine fibroblasts (Table 1).

MLPal lacks enhancer activity. To define the role of the MLPal protein-binding site in transcriptional activation in T cells, we first determined whether this sequence by itself had enhancer activity. We tested a single copy of the MLPal sequence or six tandem copies of it for transcriptional activation of either the MCF13 or human β-globin promoter. A single or multimerized synthetic ds oligonucleotide of the MLPal sequence was inserted upstream of either MCF13 promoter sequences linked to the CAT gene or those linked to the β-globin promoter (24). We were not able to detect any increase in transcriptional activity for the MLPal-containing CAT or β-globin constructs in either lymphocytes or fibroblasts compared with the promoter-alone clones (data not shown).

Transcriptional activation by the MLPal site in conjunction with the enhancer element. Because we have observed that the MLPal sequence by itself is unable to activate transcription, we tested the idea that this sequence may be active when it is juxtaposed with the MCF13 enhancer. We hypothesized that enhancer sequences may interact with MLPal because of their proximity to each other (Fig. 1). To perform this analysis, we first deleted the region between the *Rsa*I and *Dra*I sites in the MCF13 LTR (Fig. 3D) of the clone containing a single enhancer (M3). We refer to this deletion mutant as ΔDEN. The deletion eliminated 93 of the 108 bp located between the MCF13 enhancer and promoter and reduced CAT activity by 5- and 10-fold compared with the wild type in a fibroblast cell line and a T-cell line, respectively (Table 2).

We subsequently inserted a single copy of the MLPal sequence between the enhancer and promoter of the ΔDEN clone (Fig. 3D). This clone, referred to as ΔDEN+MLPal, increased CAT activity over ΔDEN by 19-fold in T cells and had no effect in NIH 3T3 fibroblasts (Table 2). Our observation that ΔDEN activity was twice that of the M3 clone suggests that there may be sequences in the DEN region that can repress MLPal transcriptional activity. Because our results for the ΔDEN+MLPal clone could also be due to the alteration of spacing between the enhancer and promoter by insertion of the MLPal sequence, we tested the effect of inserting the Mut-1 ds oligonucleotide, which consists of random sequences of approximately the same length as MLPal, into the same site of the ΔDEN clone (Fig. 3D). Furthermore, because we wanted to test whether the secondary structure was important for activity, the random sequences of this oligonucleotide corresponded to a palindrome as in MLPal. When clone ΔDEN+Mut-1 was tested in a transient CAT assay, we observed that its activity was eightfold greater than that of ΔDEN (Table 2). Thus, the transcriptional activation we have detected by inserting the

MLPal sequence between the enhancer and promoter appears to be the result of the combination of two effects. The first is the result of inserting a specific protein-binding site adjacent to the enhancer, and the second is due to increasing the spacing between the enhancer and promoter of the LTR. Secondary structure does not appear to be a requirement for transcriptional activity, because a ds oligonucleotide containing random sequences that are not a palindrome has the same effect on transcription as the oligonucleotide in the Δ DEN+Mut-1 clone (data not shown).

DISCUSSION

We have identified two regions of the LTR of MCF13 MLV, a murine retrovirus that causes thymic lymphoma (3, 23), that are important for its transcriptional activity in T lymphocytes (36). One of these regions includes the core consensus sequence. The other region, DEN, corresponds to the sequences located between the enhancer and promoter regions (Fig. 1). Besides its importance for transcriptional activity, we have also demonstrated that the DEN region makes a significant contribution to the pathogenicity of the MCF13 virus (36). Because of the importance of this region to both disease and T-cell transcription, we have searched for protein-binding sites within DEN that could account for its activity.

By gel mobility shift and DNA footprinting analyses, we have detected a single protein-binding site (MLPal) that contains a nearly perfect 18-bp palindrome within the DEN region. Our ability to detect only a single protein-binding site within this 93-bp region of the LTR could be due to the different abundances of proteins which can bind to this fragment or to the different stabilities of the protein-DNA complexes that may potentially form. Unlike our gel shift results with the dMCF fragment that includes the entire DEN region, we were able to detect only one major protein-DNA complex with an oligonucleotide consisting of the MLPal sequence. An explanation for this difference may be that protein-DNA complexes formed with the oligonucleotide are less stable than those formed with the larger DNA fragment. If this is the case, it would suggest that nucleotides flanking the MLPal site present in the dMCF DNA fragment may stabilize its other two faster-migrating protein-DNA complexes. The identification of a sequence with dyad symmetry within the MLPal site is intriguing because of the frequency that palindromic sequences play in binding proteins that regulate the transcription of both eukaryotic and prokaryotic genes (13, 16, 25, 26).

The significance of the MLPal site is supported by our observation that deleting it from the LTR reduced both transcriptional activity specifically in T cells as monitored by transient expression assays (Table 1) and the incidence of thymic lymphoma in mice compared with the incidence of wild-type MCF13 virus (36). In addition, deletion of this site increased the latency of the disease (36). We have observed that the MLPal sequence is highly conserved (a 1- or 2-bp mismatch) in the LTRs of other murine retroviruses which cause thymic lymphoma (4, 15, 22). Our nucleotide sequence search also revealed similar sequences in the LTRs of human T-cell leukemia virus I (HTLV-I), a human leukemia virus which is transcriptionally active in T cells (29), and several feline immunodeficiency virus isolates that cause diseases involving T cells (5). The presence of the MLPal protein-binding site in the LTRs of these retroviruses suggests that it may have an important role in the development of these other types of T-cell diseases. Furthermore, the deletion of

sequences from an analogous site of the Moloney MLV LTR altered the type of hematopoietic disease caused by this virus (11). Hence, *cis*-acting determinants of disease specificity are present both within and directly downstream of the enhancer and may interact with each other as discussed below.

It could be argued that the reason we detected a decrease in transcriptional activity in T cells for the MLPal mutant is that the deletion has juxtaposed sequences that bind a transcriptional repressor present only in this cell type. However, this possibility cannot account for our results where we have demonstrated that the MLPal sequence has a positive effect on transcription in T cells. Our data have suggested that the MLPal sequence can activate transcription specifically in T cells by 19-fold only in the presence of adjacent enhancer sequences. Although nearly half of this effect is due to an effect on the spacing between the promoter and enhancer, this observation suggests that a protein or proteins that bind to the MLPal site can interact with those that bind to the enhancer region. This interaction could occur between either a single protein or multiple proteins that bind to the enhancer. The MLPal site is separated from the enhancer by only 14 bp, and this proximity could enable protein-protein interactions to occur. This idea is supported by pathogenicity studies of recombinant Moloney and Friend MLVs that have implicated a region analogous to the MLPal site that interacts with enhancer sequences, presumably via protein interactions, in determining disease specificity (8). There are other examples of enhancers that by themselves are unable to augment transcription but are active in the context of additional regulatory elements (20, 24).

Our observation that the addition of 33 random bases between the enhancer and promoter increased transcription argues that proper spacing must be maintained between these two transcriptional elements for optimal transactivation to occur. Because 33 bases correspond to approximately three helical turns, alignment of transactivating factors on the same surface of the DNA may be required for proper interactions between the enhancer and promoter of the MCF13 LTR. We are presently performing experiments to test this hypothesis.

It appears that no single protein-binding site is responsible for the transcriptional activity of retroviral LTRs, including T-cell-specific transcriptional activity. This idea is supported by studies, including ours, that have shown that the viral core consensus site in the enhancer also partially contributes to this cell-type-specific activity (1, 21, 33, 35, 36). The proteins which bind to the MLPal and core consensus sites may perhaps interact with each other and with additional T-cell-specific proteins binding elsewhere in the LTR to generate the full transcriptional activity in T cells observed for these LTRs. The idea that multiple sequence motifs acting in a combinatorial manner are responsible for enhancer activity in any one cell type has been most fully examined for the simian virus 40 enhancer (24, 38). It has been shown for this enhancer that individual sequence motifs composing it have different cell-type-specific activities which, when present in toto, confer upon the simian virus 40 enhancer its activity in multiple cell types. It is not surprising that the enhancer of the MCF13 LTR, which is active in many different cell types but has its greatest activity in T cells, appears to be similarly constructed.

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