Contributions to Transcriptional Activity and to Viral Leukemogenicity Made by Sequences within and Downstream of the MCF13 Murine Leukemia Virus Enhancer

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We have identified nucleotide sequences that regulate transcription in both a cell-type-specific and general manner in the long terminal repeat of the MCF13 murine leukemia virus. Besides the enhancer element, we have observed that the region between the enhancer and promoter (DEN) has a profound effect on transcription in different cell types. This effect, however, was dependent on the copy number of enhancer repeats and was detectable in the presence of a single repeat. When two enhancer repeats were present, the effect of DEN on transcription was abrogated except in T cells. DEN also makes a significant contribution to the leukemogenic property of the MCF13 retrovirus. Its deletion from the MCF13 virus dramatically reduced the incidence of thymic lymphoma and increased the latency of disease in comparison with the wild-type virus. This effect was most marked when one rather than two enhancer repeats was present in the mutant viruses. We also observed that the removal of one repeat alone remarkably reduced leukemogenicity by the MCF13 virus. A newly identified protein-binding site (MLPal) located within DEN affects transcription only in T cells, and its deletion attenuates the ability of an MCF13 virus with a single enhancer repeat to induce thymic lymphoma. This observation suggests that the MLPal protein-binding site contributes to the effect of the DEN region on T-cell-specific transcription and viral leukemogenicity. This study identifies the importance of nonenhancer sequences in the long terminal repeat for the oncogenesis of the MCF13 retrovirus.

The class I mink cell focus-forming (MCF) murine leukemia viruses (MLVs) cause thymic lymphoma (5, 20, 36, 51) by a mechanism referred to as insertional mutagenesis (21, 37). Evidence suggests that proviral insertion enables viral regulatory signals to alter the normal transcriptional level of an adjacent cellular proto-oncogene (6, 8, 21, 37, 38). Insertional mutagenesis is thought to be one step in the cascade of events which ultimately result in tumorigenesis. Therefore, viral transcriptional signals have been the focus of much attention in an attempt to understand their involvement in this mechanism. Studies have identified the viral long terminal repeat (LTR) as containing sequences that regulate transcription (50). In addition, the LTR has been shown to play a role in determining disease specificity, thought to be a consequence of its regulation of transcription in a cell-type-dependent manner (3, 4, 10, 24, 25, 52).

Gel mobility shift analysis and DNA footprinting involving the LTRs of various MLVs that cause thymic lymphoma have resulted in the detection of an array of protein-binding sites within and directly downstream of the enhancer repeat region (1, 29, 35, 45, 48, 58, 59). These sites have been shown to bind tissue-specific and ubiquitous proteins. The Moloney MLV (Mo-MLV) enhancer has been shown to contain binding sites for leukemia virus factors LVA, LVB, and LVC, in addition to sites identified for other viruses, i.e., nuclear factor 1 (NF1), the glucocorticoid response element (GRE), and a conserved core element identified originally in simian virus 40 and polyomavirus enhancers (45, 54). Functional analyses employing transient expression assays of the LTRs of Mo-MLV and SL3-3 MLV (a leukemogenic isolate

from an AKR spontaneous lymphoma [39]) have demonstrated that the LVB and core sites are significant for transcriptional activity in T cells (1, 29, 47, 49). In SL3-3 MLV, the protein-binding site that overlaps with both the LVB and core sites is referred to as SEF 1 (48). The LVB and core sites were also shown to determine disease specificity in studies examining the effects of mutated LTRs upon viral pathogenicity (17, 46). Other protein-binding sites which affected transcription in various cell types and increased the latency of leukemogenesis included the Mo-MLV NF1 and GRE sites (29, 35, 46, 47). In addition, a GC-rich sequence downstream of the Mo-MLV enhancer has been shown to contribute to enhancer activity and to the type of leukemia induced by this virus (19, 27). The notion that the activity of this downstream region is mediated by transactivating factors is supported by the observations that protein binding occurs to analogous sites in the Friend and MCF13 MLV LTRs (32, 58).

MCF13 MLV is an example of a class I MCF virus that is able to induce thymic lymphoma (5, 20, 36, 51). A nucleotide sequence comparison revealed that the MCF13 MLV LTR contains sequences highly homologous to the protein-binding sites identified in the Mo-MLV LTR. The LVB and GRE sites are identical for these two MLVs, and the LVA, LVC, core, and NF1 sites are highly homologous. Moreover, these sites are similarly located in the two LTRs. In addition, we have identified by gel mobility shift and DNA footprinting analyses a 25-bp protein-binding site, MLPal, starting 15 bp downstream of the MCF13 MLV enhancer repeat (58, 59).

By a deletion analysis of the MCF MLV LTR, we demonstrated that a region in the enhancer that includes the LVB, core, and LVC sites was responsible for a large amount of transcriptional activity in both T cells and fibroblasts (23).

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We also identified a region encompassing sequences predominantly downstream of the enhancer, which includes the MLPal site, that was important for activity primarily in T cells (23). To further identify sequences in these two regions of the MCF13 MLV LTR that control transcription, we have examined the contributions made by different individual protein-binding sites as well as longer stretches of sequences containing multiple protein-binding sites involving these regions. These studies, performed by deletion mutagenesis, are described in this report. Also, we have determined the effects of some of the deletions upon the leukemogenicity of MCF13 MLV and established whether their effects on transcription in transient expression assays correlate with their effects on viral pathogenicity.

MATERIALS AND METHODS

Cell culture. NIH 3T3 and *Mus dunni* mouse fibroblast cell lines were grown in Dulbecco's modified Eagle's medium (GIBCO Laboratories, Grand Island, N.Y.) containing 10% fetal bovine serum (FBS) (HyClone Laboratories, Logan, Utah). Murine T-cell lines L691 and Ti6 and murine B-cell line P3 were grown in RPMI 1640 (GIBCO) containing 10% FBS. Jurkat cells, a human T-cell line, were grown in RPMI 1640 containing 10% FBS and 5×10^{-5} M β -mercaptoethanol.

Construction of plasmids. The wild-type MCF13 MLV LTR plasmid (WT-LTR) was constructed from plasmid M2 (23) in order to use MCF13 MLV LTR sequences from the *Pst*I site to the *Sma*I site (Fig. 1), which are situated immediately upstream of the chloramphenicol acetyltransferase (CAT) gene. The plasmid representing the wild-type LTR minus one repeat, WT Δ 1R, was derived by digestion of WT-LTR with *Eco*RV and subsequent religation. MOM was generated by digesting WT Δ 1R with *Eco*RV and *Apa*I, resulting in the deletion of 23 bp. OOM was generated by deleting the 208 bp between the *Pst*I and *Apa*I sites from WT Δ 1R. We used oligonucleotide-directed mutagenesis based on a procedure described by Sayers et al. (42) to construct plasmids Δ DEN, Δ LVb, Δ LVc, Δ core, Δ MLPal, WT Δ DEN, and WT Δ MLPal.

Oligonucleotides of 32 to 36 bp were synthesized on an Applied Biosystems 380B synthesizer by a Howard Hughes Medical Institute core facility at the University of Washington. Oligonucleotides were purified by polyacrylamide gel electrophoresis, phosphorylated with T4 polynucleotide kinase, and annealed to the plus strand of either WT Δ 1R or WT-LTR. Minus-strand synthesis, incorporating deoxycytidine 5'-(α -thio)triphosphate (dCTP α S) into the mutant DNA, was done with the Klenow fragment of DNA polymerase I and T4 DNA ligase. The template strand was then digested with *Nci*I and exonuclease III. For second-strand synthesis from the remaining mutant template, the Klenow fragment and T4 ligase were used to generate a mutant double-stranded DNA molecule. Bacterial colonies were generated by transformation of competent TG1 cells (Amersham), and their DNAs were screened by restriction analysis to identify the desired mutations. The identified mutations were sequenced by the dideoxy sequencing method (40) to verify that the selected mutations were correct.

Construction of viruses. Wild-type and mutant viruses were constructed using plasmid PMSL, which contains a permuted MCF13 MLV having a single LTR. The viral genome is permuted at the single *Eco*RI site in the *env* gene as a result of cloning unintegrated closed circular viral DNAs into the λ WES vector (56). An *Eco*RI fragment containing

the viral DNA was subsequently subcloned into plasmid pBS+. Virus stocks were generated by digesting PMSL with *Eco*RI and ligating the linearized plasmids into concatemers with T4 ligase. Twenty micrograms of viral DNA was transfected into *M. dunni* cells, using 33 μ g of Lipofectin reagent (GIBCO), and the transfected cells were passaged at high density every other day for approximately 2 weeks. Supernatants were harvested, centrifuged to remove cells, and either frozen at -70°C for subsequent injection into neonatal AKR mice or used for determination of virus titer.

Virus titers were determined by infecting *M. dunni* cells at various dilutions and performing a focal immunofluorescence assay (43). For this assay, we used monoclonal antibody 83A25 (a kind gift from L. H. Evans, Rocky Mountain Laboratories, Hamilton, Mont.), which reacts with the glycoproteins of all MLVs except Friend MLV, Rauscher MLV, and some MCF MLVs derived from those ecotropic MLVs (12). Mutant viruses were constructed from plasmid PMSL by site-directed mutagenesis as described above.

DNA transfection and CAT assay. DNA transfection was done by using a procedure modified from that of Dorman and Yong (11) in which cells were washed twice with serum-free medium, RPMI 1640 for lymphoid cells and Dulbecco's modified Eagle's medium for NIH 3T3 and *M. dunni* cells. For each T-cell transfection, 12.5 μ g of plasmid DNA and 85 μ g of Lipofectin reagent were added to 1.5 ml of medium. The DNA and Lipofectin were mixed, vortexed, and added to 10^7 cells. Cells were incubated for 6 h at 37°C , at which time 7 ml of medium containing 20% FBS was added. Cells were collected 16 h later by centrifugation and added to 30 ml of medium containing 10% FBS. Cells were grown for another 24 h, harvested, and processed for CAT assays as described below. For P3 cells, we used 7 μ g of plasmid DNA, 50 μ g of Lipofectin, and 10^7 cells. For NIH 3T3 and *M. dunni* cells, transfections were done with either a 10-cm (2×10^6 to 3×10^6 cells) or 6-cm (10^6 cells) plate. Cells were washed as described above and incubated for 1 h in 50 μ g of Lipofectin in 1.5 ml of Optimem (GIBCO), at which time 36 μ g of DNA in 1.5 ml of Optimem was added. The rest of the protocol was the same as described above. Transfection conditions were optimized for each cell line such that CAT activities fell on the linear part of the titration curve.

To extract cellular protein, harvested cells were rinsed with 5 ml of phosphate-buffered saline (2 mM Na_2HPO_4 , 2 mM KH_2PO_4 , 150 mM NaCl), resuspended in 0.2 ml of 0.25 M Tris (pH 7.8), and lysed by freezing and thawing four times each. Protein concentrations were determined by the Bradford assay (2). Nonchromatographic CAT assays were performed as described by Sleight (44), with a few modifications. Briefly, for each CAT assay, we combined 100 μ g of protein extract with 1 μ l of 0.5 M EDTA plus deionized water to a total volume of 67 μ l. This mixture was incubated at 65°C for 10 min to denature cellular enzymes interfering with the CAT assay (7). The reaction mixture was brought to a volume of 100 μ l by the addition of 5 μ l of 32 mM chloramphenicol in ethanol, 7.5 μ l of 1 M Tris (pH 7.8), 20 μ l of 0.43 mM acetyl coenzyme A solution, and 0.1 μ Ci of ^{14}C -acetyl coenzyme A (New England Nuclear). The reaction mixture was briefly vortexed and then incubated at 37°C for 1 h. The reaction was terminated with the addition of 1 ml of ethyl acetate and vortexed for 30 s at maximum speed. Organic and aqueous layers were separated by centrifugation for 2 min in an Eppendorf centrifuge. A 0.8-ml sample of the organic layer containing the ^{14}C -acetylated chloramphenicol was added to 5 ml of Biofluor (New England

FIG. 1. WT-LTR and a derivative with one enhancer repeat. (A) Schematic representation of the 600-bp MCF13 MLV LTR. U3, R, and U5 regions and CAT and TATA boxes are indicated; the arrow marks the transcription initiation site. Numbering is relative to the start site of initiation. The diagram below depicts a *Pst*I-*Sma*I 446-bp fragment of the LTR which was inserted upstream of the CAT reporter gene and used for transient expression assays. Boxes with arrows indicate the 69-bp repeats of the enhancer. (B) Structure of WTΔ1R, the one-repeat version of WT-LTR.

Nuclear). Radioactivity was measured by a United Technologies Packard Minaxi Tricarb 4000 scintillation counter. Each experiment included a negative control consisting of a CAT assay performed with protein extracted from mock-transfected cells. Scintillation counts from the negative control were subtracted from those of assayed protein extracts of transfected cells.

Inoculation of mice. Neonatal (1- to 5-day-old) AKR mice were injected intraperitoneally with 70 to 100 μ l of inoculum containing 2×10^4 to 8×10^4 infectious units of virus. Control mice were inoculated with medium. Necropsy was performed on moribund mice. Animals with the diagnosis of thymic lymphoma showed a massive enlargement of the thymus with fusion of thymic lobes. In most mice, there was also enlargement of liver, spleen, and peripheral lymph nodes and the characteristic sausage-shaped enlargement of the mesenteric lymph node. Microscopic examination of selected tumors showed the thymus and lymph nodes to be replaced by and organs infiltrated with large blast cells with a high nuclear/cytoplasmic ratio. These are the typical features of spontaneous and virus-accelerated Thy 1.1-positive lymphoma of AKR mice (36).

RESULTS

In our previous study, we identified two regions of the MCF13 MLV LTR that contributed to transcription in T cells (23). These regions consisted of a 23-bp nucleotide sequence within the enhancer element and 119 bp located between the enhancer and promoter. While the 23-bp region was important for transcription in both T cells and fibroblasts, the 119-bp region was important primarily in T cells. In this study, we have defined these two regions further by a finer deletion mapping of each. To do this, we have deleted individual protein-binding sites within each region as well as regions encompassing multiple protein-binding sites within the MCF13 MLV LTR. Plasmid constructs containing deleted LTRs linked to the CAT reporter gene were tested in transient expression assays. To determine the contribution of the LTR sequences which were deleted to cell-type-specific transcription, we tested the constructs in lymphoid and nonlymphoid cells. Lipofectin was used to transfect

DNA into cells. Although in our previous study we had used electroporation for this purpose, we have observed that the transfection variability of CAT assays is lower with this liposomal reagent.

Deletions of protein-binding sites within a 23-bp region of the enhancer of the MCF13 MLV LTR. WT-LTR (Fig. 1A) consists of 600 bp comprising the U3, R, and U5 regions. Within the U3 region lie two 69-bp direct repeats, which we have defined as the enhancer element (57), located approximately 100 bp upstream of the CAAT and TATA promoter elements. Deletions of protein-binding sites were initially made from an LTR in which a single enhancer repeat had been removed by digestion with the restriction endonuclease *Eco*RV (WTΔ1R) (Fig. 1B). WTΔ1R was made to facilitate the subsequent deletion of single protein-binding sites from the 23-bp region within the enhancer. In a previous study, we had shown that the deletion of one repeat caused only a slight reduction in transcriptional activity in T cells (23).

Locations of the individual protein-binding sites that we have deleted within the MCF13 MLV LTR are illustrated in Fig. 2A. The LVb, core, and LVc sites were originally identified in the enhancer of Mo-MLV as sequence motifs that bound nuclear proteins (45). Furthermore, the core and LVb sites were subsequently shown to be important for transcriptional activation and viral pathogenicity for Mo-MLV and SL3-3 MLV (1, 9, 17, 29, 46, 47). The LVb sequence is the most highly conserved in retroviruses from different species (14) and is identical for Mo-MLV and MCF13 MLV. The MCF13 MLV core site is identical at 6 of the 8 bp of the site in Mo-MLV. The MCF13 MLV and Mo-MLV LVc sites share 4 of 5 bp. The NF1b and GRE sites are identical for MCF13 MLV and Mo-MLV.

Construct MOM, in which the 23-bp region between the *Eco*RV and *Apa*I restriction endonuclease sites in the enhancer repeat was deleted, was used to originally identify the contribution of this region to transcriptional activity (23). This deletion removed the entire core and LVc sites and a single nucleotide from the 3' end of the LVb site. We had previously shown that MOM reduced transcriptional activity by three- to fivefold compared with WTΔ1R in a murine T-cell line and in NIH 3T3 fibroblasts. In this investigation, we have extended our study of MOM to other lymphoid

FIG. 2. Deletion mutants of the MCF13 MLV LTR. (A) Nucleotide sequence of a single-repeat region, denoted by the box, and its flanking sequences. The sequence motifs of identified protein-binding sites are underlined. In the WTΔ1R structure, the relative positions of protein-binding sites are indicated. (B) Deletions of the 123-bp region between the enhancer and promoter. OOM is deleted of all sequences 5' to the *Apa*I site; ΔDEN is deleted of the *Rsa*I-*Dra*I 93-bp region; ΔMLPal is deleted of a 25-bp protein-binding site located between 15 and 39 bp 3' to the repeat.

lines. We observed that the 23-bp deletion within the enhancer repeat decreased transcriptional activity by about twofold compared with WTΔ1R in all cell lines examined except for P3, a murine B-cell line, for which we detected no effect (Table 1). The slightly smaller effect of MOM on transcription in T cells in these studies may have been due to the change in transfection protocol as discussed above. We wished to determine which protein-binding site(s) present in this 23-bp region was responsible for its effect on transcription. Constructs with deletions of the LVb, core, or LVc site were subsequently generated by site-directed mutagenesis (42). We observed that construct ΔLVb had little or no effect in lymphoid cells but reduced transcriptional activity by threefold in fibroblasts (Table 1). On the other hand, the core deletion reduced activity two- to threefold in T cells but had no effect in the B-cell line and only a slight effect in fibroblasts. Deletion of the LVc site had no effect upon transcription in any of the cell types examined.

The data in Table 1 suggest that in lymphoid cells, deletion of the core site could account for the entire effect of removing the 23-bp region (MOM), with no contribution from either LVb or LVc. In fibroblasts, however, deletion of

LVb appeared mainly to be responsible for the decrease in transcriptional activity detected with MOM.

Contribution of the 119-bp region between the enhancer and promoter to transcriptional activity. Our results from a previous study suggested that sequences predominantly 3' to the enhancer were transcriptionally active in T cells but not in fibroblasts (23). For this study, we used a deletion mutant (OOM) generated by removing 137 bp from the 5' end of the MCF13 MLV LTR in WTΔ1R (Fig. 2B). This deletion removed five protein-binding sites previously identified in the Mo-MLV enhancer, i.e., the LVa, LVb, LVc, core, and upstream NF1a sites (45). OOM retains the downstream NF1b and GRE protein-binding sites within the MCF13 MLV enhancer repeat (Fig. 2A). The NF1b and GRE sites have been shown to bind nuclear proteins in the Mo-MLV enhancer (45) and to contribute to transcription in a manner that was not cell type specific (47). In this investigation, we have further examined the contribution to transcription made by the LTR sequences retained in OOM. We have extended the study of this clone to other cell lines to determine whether this region could confer activity in a cell-type-specific manner.

TABLE 1. Effects of deletions involving the 23-bp region within the MCF13 MLV enhancer on relative transcriptional activity

LTR construct	% CAT activity $\pm$ SEM ^a				
	T cells		P3 mouse B cells	NIH 3T3 mouse fibroblasts	
	Mouse	Human Jurkat			
	L691	Ti6			
MOM	55 $\pm$ 1.4	53 $\pm$ 13.3	52 $\pm$ 5.3	97 $\pm$ 7.2	46 $\pm$ 3.7
Δ LVb	96 $\pm$ 2.9	81 $\pm$ 1.7	81 $\pm$ 4.6	115 $\pm$ 3.5	32 $\pm$ 0.8
Δ core	28 $\pm$ 1.2	55 $\pm$ 0.6	58 $\pm$ 2.3	116 $\pm$ 7.2	73 $\pm$ 3.5
Δ LVc	90 $\pm$ 8.9	105 $\pm$ 1.4	108 $\pm$ 11.0	88 $\pm$ 16.7	105 $\pm$ 7.2

^a Expressed as percentage of the activity of WT Δ 1R, which has been assigned the arbitrary value of 100. Each value represents the mean of 4 to 14 independent transfections performed in two to four different experiments. The range of counts per minute was 5×10^3 to 1×10^5 for each CAT assay; the values have been corrected for background (less than 10^3 cpm). More than one plasmid preparation was used for each construct tested.

We observed that the 119-bp region represented by construct OOM conferred more than 25% of the activity of WT Δ 1R in T cells (Table 2). We observed that WT Δ 1R augmented the transcriptional activity of the MCF13 MLV promoter by 25-fold (data not shown). In B cells, OOM was as active as WT Δ 1R. The least amount of OOM activity was detected in fibroblasts. The results for T and B cells were surprising because most of the enhancer is absent from OOM, and they indicated that the NF1b and GRE sites in conjunction with sequences 3' to them could confer a substantial amount of activity to this viral LTR in these types of cells.

Given our results for OOM, we wished to determine the effect on transcription of removing only the sequences between the enhancer and promoter. Accordingly, we constructed Δ DEN, from which sequences downstream of the enhancer (DEN) between *Rsa*I and *Dra*I have been deleted (Fig. 2B). We observed that deletion of DEN reduced transcriptional activity in all of the cell lines examined by 2- to 20-fold (Table 2). These results indicated that the DEN region confers a significant amount of activity upon the MCF MLV LTR with a single enhancer repeat in various cell types.

By gel mobility shift and DNA footprinting analyses, we have detected a single protein-binding site (MLPal) within the DEN region (58, 59). This site, which is 25 bp in length, contains a sequence of dyad symmetry (Fig. 2A) (58). We deleted the MLPal site to examine its effect on transcription (Fig. 2B). We observed that Δ MLPal decreased transcription two- to threefold in T cells alone, having little or no effect in other cell types (Table 2). Thus, in T cells, removal of the

TABLE 2. Relative transcriptional activities of LTRs with deletions involving the DEN region

LTR construct	% CAT activity $\pm$ SEM ^a				
	T cells		P3 mouse B cells	NIH 3T3 mouse fibroblasts	
	Mouse	Human Jurkat			
	L691	Ti6			
OOM	26 $\pm$ 1.7	28 $\pm$ 2.3	27 $\pm$ 1.1	104 $\pm$ 7.0	15 $\pm$ 4.5
Δ DEN	11 $\pm$ 0.9	56 $\pm$ 4.9	6 $\pm$ 0.8	5 $\pm$ 0.9	23 $\pm$ 4.1
Δ MLPal	43 $\pm$ 4.0	63 $\pm$ 11.1	34 $\pm$ 2.0	89 $\pm$ 6.9	112 $\pm$ 8.8

^a See Table 1, footnote a.

TABLE 3. Relative transcriptional activities of deletion mutants of an LTR with two enhancer repeats

LTR construct	% CAT activity $\pm$ SEM ^a			
	T cells		P3 mouse B cells	NIH 3T3 mouse fibroblasts
	Mouse L691	Human Jurkat		
WT Δ 1R	51 $\pm$ 7.8	56 $\pm$ 3.5	90 $\pm$ 1.4	72 $\pm$ 7.3
WT Δ DEN	54 $\pm$ 8.1	52 $\pm$ 2.0	14 $\pm$ 5.7	110 $\pm$ 6.9
WT Δ MLPal	85 $\pm$ 5.0	ND ^b	ND	ND

^a See Table 1, footnote a (data expressed here as percentages of the activity of WT-LTR, which has been assigned the arbitrary value of 100).

^b ND, not determined.

MLPal site can either partially or entirely account for the effect observed with the DEN deletion. As this was not the case for B cells and fibroblasts, these data suggest that there are other protein-binding sites in DEN that may regulate transcription in these cell types.

Deletions from WT-LTR. Studies done with various MLV LTRs indicated that sequences located downstream of the enhancer were dispensable for transcriptional activation in fibroblasts (15, 18, 31). In these studies, deletion of this region was made from LTRs with enhancer elements constituted by two repeats. To determine whether this was the case for the MCF13 MLV LTR, we deleted the DEN and MLPal sequences from the wild-type LTR with two repeats. These deletion mutants are called WT Δ DEN and WT Δ MLPal, respectively.

Similar to the results of others (15, 18), WT Δ DEN no longer had an effect in fibroblasts (Table 3). WT Δ 1R had a slight effect. On the other hand, the DEN deletion from this LTR continued to affect transcription in lymphocytes, albeit at a reduced level compared with its deletion from WT Δ 1R (Tables 2 and 3). In T cells, WT Δ DEN produced only a moderate reduction in transcriptional activity (Table 3). Comparison of the activities of WT Δ DEN and WT Δ 1R revealed that the two constructs were equally active in T cells (Table 3). These data suggest that the DEN region can substitute for one repeat of the enhancer for transcriptional activation in this cell type. Unlike our observations for T cells and fibroblasts, deletion of DEN dramatically reduced transcription in B cells regardless of whether Δ DEN or WT Δ DEN was used (Tables 2 and 3). These results indicate that in B cells, the DEN region is more critical than the enhancer for transcriptional activity.

WT Δ MLPal had little effect on transcriptional activity in T cells. In contrast, when MLPal was removed from the one-repeat LTR construct, T-cell activity was reduced two- to threefold (Table 2).

Leukemogenicity of mutant MCF13 viruses with deletions in the LTR. Our deletion mutagenesis studies have identified two LTR regions that contribute to the control of transcription and which have not previously been implicated in this regulatory role. We have identified the DEN region as being important for transcriptional activity in lymphocytes and the MLPal protein-binding site within this region for its contribution to T-cell-specific activity. To determine whether these LTR sequences were important for viral leukemogenicity, we constructed viruses that contained deletions of these two sites within their LTRs.

Because we observed that the MLPal and DEN deletions had different effects on transcription depending on whether the LTR contained one or two enhancer repeats, we con-

FIG. 3. Induction of thymic lymphoma in AKR mice by wild-type MCF13 virus and mutant viruses containing deletions in their LTRs. Thymic lymphoma induction is shown as a function of time following inoculation of newborn mice with wild-type MCF13 virus or viruses with mutations in the enhancer or downstream of the enhancer. Inoculation of mice and diagnosis of thymic lymphoma are described in Materials and Methods. Symbols: ●, WT-LTRv; △, WTΔMLPalv; □, WTΔDENv; ○, WTΔ1Rv; ▲, ΔMLPalv; ■, ΔDENv.

structed mutant viruses that contained one or two repeats. Viral constructs were derived from a molecular clone of MCF13 MLV that was permuted at the *EcoRI* restriction endonuclease site and that contained a single LTR. Plasmid DNA was restricted with *EcoRI* and ligated prior to transfection into *M. dunni* fibroblasts. Viral supernatants were titered before inoculation into neonatal AKR mice. We subsequently examined the incidence of thymic lymphoma and the latency of onset of disease induced by the different MCF13 viruses. For these assays of viral lymphomagenicity, we tested the ability of each virus to accelerate the onset of disease, which normally begins at about 6 months of age in a population of AKR mice (36). We refer to the wild-type virus as WT-LTRv. Mutant viruses with deletions of the MLPal site and DEN region in the context of two repeats were named WTΔMLPalv and WTΔDENv, respectively. Viruses with a single repeat were named WTΔ1Rv, ΔMLPalv, and ΔDENv.

Inoculation of WT-LTRv resulted in a 100% incidence of thymic lymphoma with an average latency of 98 days, similar to results obtained with a biological clone of MCF13 MLV (Fig. 3 and Table 4) (36). We observed that WTΔDENv decreased disease incidence by 35% and had an increased latency of 130 days compared with the wild-type virus (Table 4). Thus, loss of the DEN region significantly affected the leukemogenicity of the two-repeat wild-type virus. In contrast, WTΔMLPalv was similar in disease incidence and latency to WT-LTRv.

Similar to results obtained for other MLVs (28), deletion of one repeat significantly affected leukemogenicity, with WTΔ1Rv having a twofold decrease in disease incidence and a significant increase in latency (158 days) compared with

TABLE 4. Effects of LTR deletions upon leukemogenicity of MCF13 MLV prior to 190 days postinoculation

Virus	No. of mice inoculated	Disease incidence (%) ^a	Avg latency ± SEM (days) ^b
WT-LTRv	11	100	98 ± 5.7
WTΔDENv	20	65	130 ± 6.0
WTΔMLPalv	21	95	92 ± 4.2
WTΔ1Rv	19	53	158 ± 5.4
ΔMLPalv	20	35	170 ± 3.1
ΔDENv	10	10	128 ^c

^a Percentage of inoculated animals that developed thymic lymphoma. Diagnosis of disease is described in Materials and Methods.

^b Calculated from animals with thymic lymphoma when sacrificed or found dead.

^c Standard error not determined.

WT-LTRv. The removal of one repeat appeared to have an even greater effect on MCF13 virus leukemogenicity than did the removal of the DEN region. We found that deletion of the DEN region from WTΔ1R significantly attenuated the leukemogenicity of this virus. The incidence of disease of ΔDENv was decreased fivefold. Although the data indicated no increase in latency for ΔDENv, this is not an accurate reflection of the pathogenic potential of the virus, since only a single animal had developed lymphoma before 190 days. Experiments were terminated at 190 days because of the occurrence of spontaneous lymphoma beginning at this age in AKR mice (36). Thus, deletion of the DEN region from an LTR with either one or two repeats had a deleterious effect on the leukemogenicity of MCF13 MLV. When we compared ΔMLPalv with WTΔ1Rv, we found that the MLPal deletion resulted in a moderate decrease in incidence and a slight increase in latency (170 days).

Therefore, in contrast to our observations for WTΔMLPalv, the effect of the MLPal deletion was detectable only when the virus contained a single repeat. The effect of MLPal on viral leukemogenicity was similar to its effect on transcription in cultured cells; i.e., it was detectable only in the presence of a single repeat in the LTR. All lymphomas had the typical gross pathologic features of thymic lymphoma as described in Materials and Methods. There were no animals with organomegaly without thymic enlargement, which would represent tumors of nonthymic origin.

DISCUSSION

We previously have identified a 23-bp region in the enhancer repeat of the MCF13 MLV LTR to be important for transcriptional activation in both T cells and fibroblasts (23). Although three protein-binding sites have been identified within this enhancer region, our current studies have indicated that a single site can account for the entire effect of this region on transcription and that this site differs for different cell types. For T cells, the core sequence contributes to transcriptional activation, but for fibroblasts, it is the LVb site that does so. Our identification of the core site for its importance in T-cell-specific transcription corroborates previous work by other laboratories (1, 47, 48). It has also been shown that this protein-binding site is a determinant of viral leukemogenicity (17, 46). A single nucleotide difference between the SL3-3 MLV and Akv virus core sequences is apparently responsible for the different transcriptional activities of these two viral LTRs in T cells (30). Although the MCF13 MLV core consensus sequence also differs from that of Akv virus by one nucleotide, it is a different nucleotide

from the one that differs for SL3-3 MLV. Different core sequences also exist for other murine retroviruses that cause thymic lymphoma (14). Hence, it appears that different but highly related proteins in T cells may be able to recognize different core sequences for transcriptional activation. This notion is supported by the detection of multiple proteins isolated from thymus tissue that are able to bind to the Mo-MLV core sequence (53).

Our results demonstrating that the LVb site was important for transcription in fibroblasts but not in lymphocytes were surprising because it has been reported that this site contributes both to transcription in various cell types (47) and to disease specificity (46). Furthermore, the LVb site contains a consensus sequence for the binding of Ets-1, which belongs to a multigene family of proteins known to activate transcription in various cell types (16). That deletion of the LVb site did not affect transcription in T cells may indicate that this site has only a weak effect on the activity of the MCF MLV LTR in these cells. Our results also could be explained by potentially low levels of Ets-1 in the lymphocyte cell lines that we have used or by the creation of a novel protein-binding site resulting from the deletion of LVb that may activate transcription in lymphocytes. Although the potential creation of novel protein-binding sites is a caveat for the deletion analysis performed in this study, our computer searches have not revealed any known sequence motifs generated by the juxtaposition of sequences flanking each site that we have deleted.

We have shown that the region between the enhancer and promoter of the MCF13 MLV LTR, which we refer to as DEN, has a dramatic effect on transcription regardless of cell type only when a single enhancer repeat is present. In the presence of two repeats, on the other hand, DEN activity is restricted to lymphocytes. Lovmand and coworkers have made similar observations for LTR sequences both upstream and downstream of the Akv virus enhancer that can affect the activity of one but not two enhancer repeats (31). Furthermore, investigations of the deletion of this region have used LTRs with two enhancer repeats that have been tested only in fibroblasts and hence have failed to detect the effect of the DEN region that we have observed in T cells. The importance of DEN is further supported by our testing of the leukemogenic potential of MCF13 viruses that are missing this region from LTRs with one or two enhancer repeats. We have observed that both DEN mutant viruses have a significantly decreased incidence of thymic lymphoma compared with their respective wild-type viruses. Although the effect of the DEN deletion may be the result of altering the spacing between the enhancer and promoter, our results with construct OOM suggested that this region with some enhancer sequences can activate transcription in lymphocytes. These data suggest that the DEN region may contain binding sites for proteins that are able to transactivate the viral promoter. This idea is supported by our identification of a protein-binding site in the DEN region that has this potential as discussed below.

By gel mobility shift assay and DNA footprinting, we have been able to detect a single protein-binding site (MLPal) in DEN that contains a sequence with dyad symmetry (58, 59) and partially overlaps a GATA site (22, 55). The MLPal site starts 15 bp downstream of the enhancer (Fig. 2A). We observed that the deletion of MLPal from an LTR with a single enhancer repeat affected transcription only in T cells. Deleting MLPal had no effect, however, when there were two repeats in the LTR. Hence, similar to findings for DEN, the effect on transcription of deleting MLPal was affected by

the number of enhancer repeats. Moreover, we have observed that MLPal by itself is unable to activate the viral promoter and requires the presence of enhancer sequences for its T-cell-specific activity (58). The effect on leukemogenicity of MLPal deletion from MCF13 MLV paralleled our results from the transient expression assays in T cells in that we were able to detect an effect on latency and incidence of disease only when MLPal was removed from the virus with an LTR containing a single repeat. The MLPal site corresponds to a region in Mo-MLV and Friend MLV that has been shown to participate in determining the type of leukemia induced by these viruses via a putative interaction with the viral enhancer (13, 19). This is a GC-rich region in the Mo-MLV LTR that was identified to also be required for enhancer activity (27). It appears, therefore, that interactions between the protein or proteins that bind to this region of the LTR with those that bind to the enhancer of a variety of MLVs are important for enhancer function and viral leukemogenicity. Our comparison of the leukemogenic activities of mutant viruses with deletions of either DEN or MLPal have shown that the effect of deleting the DEN region is greater than that of removing just the MLPal site (Table 4). These results suggest that in addition to MLPal, there are other sequences within DEN that contribute to MCF13 MLV leukemogenicity.

As stated above, the 5' end of the MLPal site overlaps with a GATA site; therefore, all of the MLPal deletion mutants that we have generated necessarily are also deleted of most of the WGATAR consensus sequence. Deletion of this GATA site could conceivably affect transcription in T cells, since it has been demonstrated that this protein-binding site is involved in the transcriptional regulation of genes expressed primarily in T cells, such as the T-cell receptor α , β , and δ genes, by the binding of GATA-3 (22, 26, 33). GATA-3 is one of a multigene family of proteins that bind to this consensus sequence and is present at highest levels in T cells (22, 26, 33). Thus, there is a good likelihood that the effect of the MLPal deletion on our transient transcription assays and viral leukemogenicity is partly contributed by the GATA site. We are presently conducting experiments to determine whether multiple proteins, including GATA-3, bind to the MLPal site.

In this study, we have demonstrated that the region between the enhancer and promoter of the MCF13 MLV LTR is important for both transcriptional activation in T cells and the leukemogenic potential of this murine retrovirus. We have also demonstrated that the MLPal protein-binding site within this region can affect viral leukemogenicity when there is a single enhancer repeat in the LTR. We are currently investigating the mechanism by which the MLPal site and the DEN region can cooperate with viral enhancer sequences and by which the presence of a second enhancer repeat can attenuate or eliminate this cooperativity. An important part of these studies is to compare the relative infectivities of the mutant viruses in the thymus and to determine whether their proviruses are present in tumor DNA and whether these proviruses have undergone any changes in their LTRs compared with the input virus.

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