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Identification of a Region of a Murine Leukemia Virus Long Terminal Repeat with Novel Transcriptional Regulatory Activities

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The 93-bp region downstream of the enhancer (DEN) in the long terminal repeat (LTR) of the mink cell focus-forming virus (MCF13) has been shown to be important for transcriptional activation and viral lymphomagenicity (J. C. Tupper, H. Chen, E. F. Hays, G. C. Bristol, and F. K. Yoshimura, J. Virol. 66:7080–7088, 1992). In this report, we have further explored the role of the DEN region in transcriptional activation. We observed that it has enhancer-like abilities as well as some unique LTR properties. Transcriptional activation by the DEN region involved interactions with enhancer sequences that were either synergistic or additive, depending on the cell type. The most intriguing property of the DEN region is its ability to induce transcription in activated T cells. This activity is unique for the LTR in that no other LTR region can do this. We also examined the role of the DEN region in retroviral lymphomagenesis. We cloned and sequenced proviral LTRs integrated upstream of the cellular c-*myc* gene from DNA obtained from thymic tumors induced by DEN region deletion mutant viruses in AKR mice. We determined that for transcriptional activation of the c-*myc* proto-oncogene, enhancer sequences can substitute for the DEN region. This study identifies the significance of non-enhancer sequences in the LTR for the oncogenesis of the MCF13 retrovirus.

Replication-competent murine leukemia viruses (MLVs) that do not contain an oncogene within their genome induce diseases in a tissue-type-specific manner. For example, the mink cell focus-forming (MCF) MLVs cause thymic lymphomas (7, 17, 38, 58), and the Friend MLV induces erythroleukemias (5, 6, 55). The most relevant mechanism involved in disease induction by these MLVs is referred to as insertional mutagenesis (18, 39, 42). Studies have shown that integration of proviruses in the vicinity of cellular proto-oncogenes results in the deregulation of the normal transcription level of these proto-oncogenes (8, 10, 18, 39, 42). Aberrant gene expression presumably initiates a chain of events that leads to transformation of the infected cells and, ultimately, to tumorigenesis. Numerous studies have shown that in addition to its role in the regulation of viral gene expression, the long terminal repeat (LTR) region of the viral genome is responsible for the aberrant transcription of host cellular proto-oncogenes during insertional mutagenesis (8, 10, 18, 39, 42, 57). Studies of recombinant retroviruses generated by combining LTR regions from MLVs with different disease properties have mapped the major determinants of disease specificity to the U3 region (11–13, 20, 23, 24, 30, 32). Within these U3 regions are tandem direct repeat sequences which have been shown to be transcriptional enhancers (28–30). We and others have demonstrated that certain protein-binding sites within the enhancers of these MLVs regulate cell-type-specific transcription, which in turn determines tissue tropism and disease specificity (3, 21, 33, 36, 50, 53, 56, 64).

MCF13 MLV is an example of a class I MCF virus that is able to induce thymic lymphoma (7, 17, 38, 58). To identify viral sequences important for disease induction, we previously demonstrated that in addition to the enhancer, the entire region between the enhancer and promoter (DEN, for downstream of enhancer) of the MCF13 LTR plays an important role in transcriptional activation and viral lymphomagenicity (21, 56). When we deleted the DEN region from the LTR with only a single enhancer repeat, we observed a significant reduction in transcription regardless of cell type. This effect, however, was dependent on the copy number of enhancer repeats. In the presence of two repeats, the effect of the DEN region on transcription was restricted to lymphocytes. Deletion of the DEN region from the LTR containing either one or two enhancer repeats in the MCF13 genome severely reduced the disease incidence and increased the latency of thymic lymphoma induction by the MCF13 MLV (56). Our observation is supported by the work of others which showed that a GC-rich sequence located in the region analogous to the DEN region of the Moloney LTR contributes to enhancer activity and to the disease specificity of this virus (16, 28). In addition, Ishimoto et al. (24) showed that the Moloney virus enhancer alone could not exert a dominant effect over that of the Friend MCF virus in disease specificity, but sequences in the Moloney DEN region were also required (24). We identified a single protein-binding site—MCF13 LTR palindrome (MLPal)—within the DEN region by gel mobility shift assays and DNA footprinting (62). The MLPal site starts 14 bp downstream of the enhancer and contains a nearly perfect 18-bp palindrome. The presence of this palindrome is intriguing because it has frequently been observed that sequences with dyad symmetry bind proteins that regulate transcription (22, 27, 41). We have further demonstrated that the MLPal site contributes to T-cell-specific transcription and requires enhancer sequences in *cis* for its activity (62). The effect of the MLPal site on MCF lymphomagenicity is modest (56). This observation suggests that there are other *cis*-acting elements in the DEN region that are responsible for its role in lymphomagenesis.

In this report, we have further explored the role of the DEN region in transcriptional activation. We observed that it has enhancer-like abilities as well as other properties. The most intriguing property of the DEN region is its ability to induce

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transcription in activated T cells. No other region of the LTR has this activity. We also examined the role of the DEN region in retroviral lymphomagenesis and determined that for transcriptional activation of the c-*myc* proto-oncogene, enhancer sequences can substitute for the DEN region.

MATERIALS AND METHODS

Cell lines. Murine T-lymphoma cell lines L691 and Ti6 were grown in RPMI 1640 (GIBCO Laboratories, Grand Island, N.Y.) containing 10% fetal bovine serum (FBS) (GIBCO). Murine B-cell lines P3 and S194 and the human Jurkat T-cell line were grown in RPMI 1640 containing 10% FBS and 5×10^{-5} M β-mercaptoethanol. NIH 3T3 mouse fibroblasts were grown in Dulbecco's modified Eagle's medium (GIBCO) containing 10% FBS.

Construction of plasmids. The Prm plasmid containing the MCF13 promoter alone was constructed by placing the LTR *Dra*I-to-*Sma*I fragment immediately upstream of the chloramphenicol acetyltransferase (CAT) gene. To generate the DENf (forward orientation) and DENb (backward orientation) plasmids, the DEN fragment (*Rsa*I to *Dra*I) was isolated from the wild-type MCF13 MLV LTR plasmid, blunt ended with Klenow DNA polymerase (Boehringer Mannheim GmbH, Mannheim, Germany), and inserted in either orientation upstream of the *Dra*I site of the Prm plasmid. To construct the 1R and 2R plasmids, we used PCR to amplify sequences between the enhancer repeat(s). The PCR products are 69 bp for one enhancer repeat (1R) and 140 bp for two enhancer repeats (2R). Oligonucleotide primers were synthesized on an Applied Biosystems 380B synthesizer by a Howard Hughes Medical Institute core facility at the University of Washington. We inserted the PCR products at the *Pst*I site of the polylinker region which is immediately upstream of the *Dra*I site in the Prm plasmid. The 1R+DEN clone was derived by digestion of the wild-type MCF13 LTR plasmid with *Eco*RV and subsequent religation, resulting in the deletion of a 69-bp enhancer repeat. All LTR derivatives were sequenced by the dideoxynucleotide method (46) to verify that the constructs were correctly made. To assay the transcriptional activities of proviral LTRs isolated from thymic tumors, we molecularly cloned LTRs c, d1, and d2 into the promoterless pCAT expression vector (25) (a kind gift from Jean Buskin, Department of Biochemistry, University of Washington). We performed PCR to amplify the entire LTR region of the c, d1, and d2 proviral LTRs and inserted the PCR products at the *Bam*HI-*Xba*I sites of the polylinker region which is immediately upstream of the CAT reporter gene of the pCAT plasmid.

DNA transfection and CAT assay. DNA transfection was done by performing lipofection by a modified procedure of Dorman and Yang (14). Cells were washed twice with serum-free medium—RPMI 1640 for lymphoid cells and Optimem (GIBCO) for NIH 3T3 cells. For each T-cell transfection, 12.5 μg of plasmid DNA and 85 μg of Lipofectin reagent (GIBCO) were each added to 1.5 ml of serum-free medium. The DNA and Lipofectin solutions 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 24 h later by centrifugation and added to 30 ml of medium containing 10% FBS. For studies of activated T cells, 25 ng of 12-*O*-tetradecanoyl-phorbol 13-acetate (TPA) per ml and 1.4 ng of ionomycin per ml were added to Jurkat cells at this step. Cells were grown for another 24 h, harvested, and processed for CAT assays as described below. For P3 cells, we used 7 μg of plasmid DNA, 50 μg of Lipofectin, and 10^7 cells. For S194 cells, we used 20 μg of plasmid DNA, 50 μg of Lipofectin, and 10^7 cells. For NIH 3T3 cells, transfections were done with 10-cm plates (2×10^6 to 3×10^6 cells per plate). Cells were washed as described above and incubated for 1 h in 50 μg of Lipofectin in 1.5 ml of Optimem, at which time 36 μg 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-HCl (pH 7.8), and lysed by freezing and thawing three times each. Protein concentrations were determined by the Bradford assay (4). Nonchromatographic CAT assays were performed as described by Sleigh (49), with a few modifications as detailed by Hollon and Yoshimura (21). CAT activities of plasmid constructs were compared in at least two different experiments in which each plasmid was tested in duplicate. More than one plasmid preparation was tested for each construct.

Tissue samples and DNA preparation. Thymic tumors from AKR/J mice inoculated with 2×10^4 to 8×10^4 infectious units of 1RΔDENv or 2RΔDENv were excised, frozen in liquid nitrogen, and stored at −70°C prior to DNA extraction. Genomic DNA was prepared by homogenizing the tissue and then shaking it for 6 h at 37°C in the presence of 0.1 M NaCl–1× SET (1% sodium dodecyl sulfate [SDS], 5 mM EDTA, 10 mM Tris-hydrochloride [pH 7.5])–0.5 mg of proteinase K per ml. DNA was extracted with phenol-chloroform two or three times and precipitated with 1 M ammonium acetate, to which was added 2.5 volumes of ethanol, and left at −20°C overnight. DNA was subsequently treated with DNase-free RNase A at 250 μg/ml for 4 h at 37°C, followed by phenol-chloroform extractions and ethanol precipitation overnight. Purified cellular DNA was resuspended in TE buffer (10 mM Tris-HCl [pH 7.4], 5 mM EDTA) and stored at 4°C for further analysis.

Genomic Southern blots. Ten micrograms of genomic DNA was digested with either *Kpn*I or *Eco*RI (Boehringer Mannheim GmbH) according to the manufacturer's instructions, electrophoresed through 0.7 to 1% agarose gels, transferred to nitrocellulose, and probed with the 4.8-kb *Xba*I-*Bam*HI fragment isolated from the pSVc-*myc* plasmid (Robert Weinberg, Massachusetts Institute of Technology). This *myc*-specific fragment was labeled with ^{32}P by random priming to specific activities of 2×10^8 to 8×10^8 cpm/μg of DNA. Hybridizations were performed with 25 mM KH_2PO_4 (pH 7.4)–5× SSC (SSC is 150 mM NaCl and 15 mM sodium citrate)–5× Denhardt's solution–50 μg of salmon sperm DNA per ml–50% deionized formamide at 42°C overnight. Filters were washed with 0.25× SSC–0.1% SDS at 42°C for 1 h. Filters were dried and exposed to X-ray film with an intensifying screen at −70°C overnight.

Analysis of genomic DNA by PCR. Oligonucleotide primers were designed to hybridize specifically to the first exon of the c-*myc* gene (MYC) and the MCF13 LTR region (LTR5′ or LTR3′). The LTR5′ primer recognizes sequences located at the beginning of the U3 region, and the LTR3′ primer recognizes sequences at the end of the R region. MYC (5′-GCTGGAATTACTACAGCGAG-3′), LTR5′ (5′-GCCATTTTGCAAGGCATGG-3′), and LTR3′ (5′-TATCGGATGACTGGCGCG-3′) primers possess restriction site tails at their 5′ termini to facilitate subsequent directional cloning (sequences of the restriction sites not shown). PCR was carried out with 2.5 U of Replitherm DNA polymerase (Epicentre Technolo-

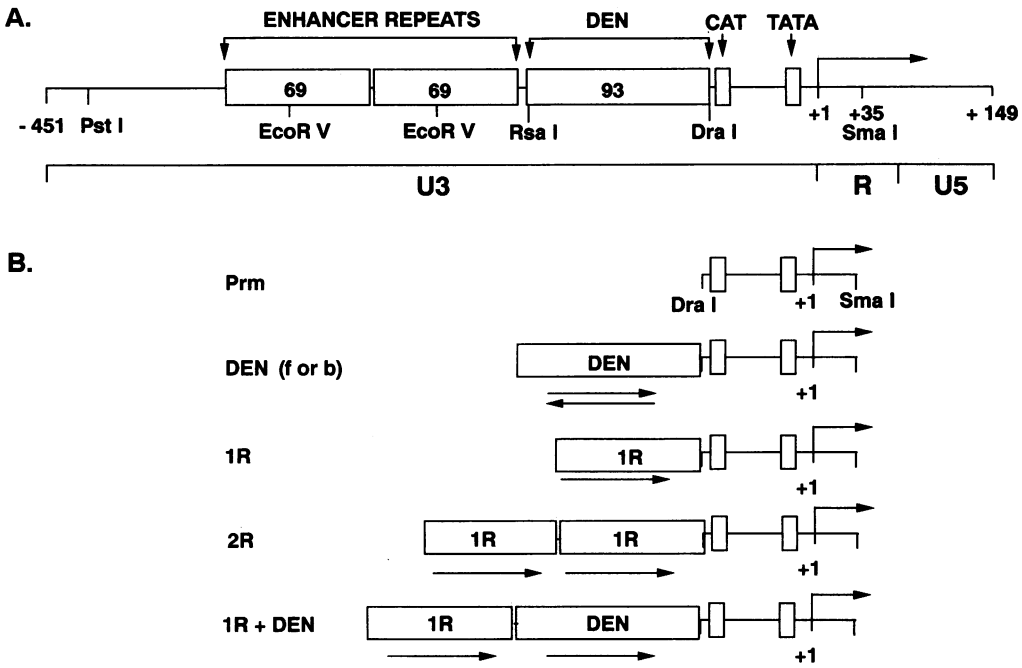

FIG. 1. (A) Schematic representation of the 600-bp MCF13 MLV LTR. The U3, R, and U5 regions and CAT and TATA boxes are indicated; horizontal arrows mark the transcription initiation sites. Numbering is relative to the start site of transcription. Enhancer repeats are shown as two 69-bp boxes. The DEN region is the 93-bp *Rsa*I-*Dra*I region between the enhancer and promoter. (B) MCF13 LTR constructions used for transient expression CAT assays. All LTRs were cloned into a pUC18-derived plasmid containing the CAT gene. Prm is the promoter-alone plasmid, which contains MCF13 promoter sequences from *Dra*I to *Sma*I. DEN (f or b) plasmids were generated by inserting a 93-bp *Rsa*I-to-*Dra*I fragment of the MCF13 LTR in either orientation upstream of the *Dra*I site of Prm. 1R and 2R correspond to one or two copies of the enhancer repeat inserted upstream of the Prm plasmid in the forward orientation. The 1R+DEN clone contains one enhancer repeat and the DEN region upstream of the Prm promoter.

gies, Madison, Wis.) in a buffer containing 2.5 mM $MgCl_2$, 0.2 mM (each) of four deoxynucleoside triphosphates, 150 ng of each oligonucleotide primer, and 1 μg of genomic DNA template. Optimized thermal cycling conditions consisted of a 10-min initial denaturation at 95°C, followed by 30 cycles of 1-min denaturation at 95°C, 1-min annealing at 60°C, and 2-min extension at 72°C. To confirm the specificity of the reaction product and the lack of amplification in the negative control reaction (no DNA), a portion of each PCR product mixture was electrophoresed through a diagnostic 1% agarose gel and analyzed by Southern blot with the ^{32}P-labeled MYC primer as probe. For the first PCR step, we used primers that were a combination of either MYC and LTR5′ or MYC and LTR3′ to detect MCF13 proviral LTRs that were integrated upstream of c-*myc*. Tumor-specific bands detectable on an agarose gel were purified and used as templates in the second PCR step, in which we used only the LTR primers LTR5′ and LTR3′ to obtain sequences corresponding to the entire LTR. Each analysis was done multiple times, and positive and negative controls for PCRs were performed in parallel.

Molecular cloning and nucleotide sequence analysis of proviral LTRs. For each tumor DNA sample, the products of multiple PCRs were pooled to obtain a representative population of proviral sequences and treated with the Klenow fragment of *Escherichia coli* polymerase I to facilitate completion of product termini. Product DNA was precipitated, treated with Klenow enzyme a second time, and electrophoresed through a 1% agarose gel, and DNA fragments containing LTRs involved in c-*myc* rearrangement in thymic tumors were gel purified with standard methods. These DNA fragments were digested with the restriction endonucleases *Eco*RI and *Bam*HI; the DNA was precipitated and ligated to the similarly digested Bluescript plasmid (Stratagene, La Jolla, Calif.) at a 50:1 molar ratio of insert to vector. Nucleotide sequence analysis of these clones was performed with the dideoxynucleotide double-stranded sequencing method (Sequenase; United States Biochemical, Cleveland, Ohio). We used either LTR5′ or LTR3′ primers for the sequencing reactions. Sequences were analyzed with the Genetics Computer Group program available through the Locke Computer Center at the University of Washington.

RESULTS

The DEN region behaves as a transcriptional enhancer and interacts with the LTR enhancer differently in various cell types. To further elucidate the mechanism by which the DEN region is involved in transcriptional regulation, we created a series of constructs containing different regions of the MCF13 LTR linked to the CAT reporter gene. The wild-type MCF13 LTR, which is 600 bp long, is shown in Fig. 1A. Earlier work defined the two 69-bp direct repeats as transcriptional enhancer elements (21). The DEN region is located between the enhancer and viral promoter.

To characterize the effect of the DEN region on transcription, we cloned the 93-bp *Rsa*I-*Dra*I fragment into the Prm expression plasmid. Prm contains the MCF13 viral promoter (Fig. 1B, *Dra*I to *Sma*I) linked to the CAT reporter gene. We initially examined whether the DEN region has the characteristics of an enhancer element. To do this, we inserted the DEN region upstream of the viral promoter in both orientations, forward (DENf) and backward (DENb) (Fig. 1B). To compare

TABLE 1. Effect of MCF13 LTR elements on cell-type-specific transcription

LTR construct	CAT activity in murine[a]:				
	T cells		B cells		NIH 3T3 fibroblasts
	L691	Ti6	S194	P3	
Prm	1	1	1	1	1
DENf	5.5 ± 0.02	2.5 ± 0.01	11.2 ± 1.15	9.0 ± 0.49	2.9 ± 0.64
DENb	2.4 ± 0.02	ND[b]	17.7 ± 1.56	14.6 ± 0.12	ND
1R	5.3 ± 0.93	5.5 ± 0.67	1.3 ± 0.11	0.58 ± 0.08	5.1 ± 0.88
2R	19.6 ± 1.57	9.1 ± 1.68	2.8 ± 0.02	0.93 ± 0.03	15.1 ± 1.25
1R+DEN	27.1 ± 3.08	8.2 ± 1.07	13.8 ± 0.07	8.7 ± 0.89	21.9 ± 0.12

[a] CAT values are expressed as fold activation over that of the Prm clone for each cell line. The activity of Prm has been assigned the arbitrary value of 1. Each value represents the mean of at least four independent transfections performed in at least two different experiments. Standard errors of the mean are included. The range of counts per minute was 5×10^3 to 1×10^5 for each CAT assay. The counts per minute have been corrected for background (less than 10^3 cpm). More than one plasmid preparation was used for each construct tested.

[b] ND, not determined.

the activity of the DEN region with that of the viral enhancer repeat, we inserted either one copy (1R) or two copies (2R) of the 69-bp repeat in the forward orientation upstream of the viral promoter. The transcriptional activity of these constructs was tested with L691 mouse T-lymphoma cells in which the MCF13 LTR is transcriptionally active (60). We observed that DENf activated transcription by sixfold over the basal promoter level, which was equivalent to the activation by 1R alone (Table 1). In addition, we observed that the DEN region in the backward orientation (DENb) was also able to activate transcription over the promoter alone (Prm), but to a lesser degree than DENf.

To examine potential interactions between the DEN region and the enhancer repeat in transcriptional activation, we constructed the 1R+DEN clone, which contains both 1R and the DEN region upstream of the viral promoter (Fig. 1B). When 1R+DEN was tested in L691 cells, we observed a 27-fold increase in transcriptional activity over that of the Prm clone (Table 1). Two enhancer repeats (2R) generated a 20-fold increase.

To test whether the transcriptional activity of the DEN region and its interactions with the enhancer repeat differ in different cell types, we compared the CAT activities of the LTR elements in various cell lines. In addition to L691 T cells, we tested another murine T-cell line, Ti6. In Ti6 cells, DENf and 1R activated transcription to a similar degree, i.e., three- to fivefold over that of the Prm clone (Table 1). However, unlike L691 T cells, the DEN region and 1R acted additively in Ti6 cells. In mouse B cells (P3 and S194), we observed that both 1R and 2R are relatively inactive (Table 1). In contrast, DENf augmented transcription about 10-fold over the basal level in both B-cell lines. DENb was also able to activate transcription 14- to 17-fold over that of Prm. Thus, the activity of the DEN region accounted for the entire effect of the LTR in B cells.

In addition to lymphoid cells, a murine fibroblast cell line (NIH 3T3) was tested (Table 1). We observed that both DENf and 1R augmented transcription to approximately the same extent, i.e., three- to fivefold over that of the Prm clone. The 1R+DEN clone increased transcription 22-fold over the basal activity, which was similar to the effect of 2R, suggesting that the interaction between the DEN region and 1R is synergistic in fibroblasts.

The DEN region but not 1R induces transcription in activated T cells. Because the DEN region is transcriptionally active in T cells, we wished to determine whether the activation state of these cells influences its activity. This question is germane to the role of the DEN region in tumorigenesis that requires T-cell proliferation, which in turn is dependent on activation (2, 9, 45). We assessed the inducibility of DEN region activity by transfecting the DENf construct into stimulable Jurkat T cells. We activated these cells by treating them with TPA and the calcium ionophore ionomycin. It has been demonstrated that the combination of these two reagents mimics T-cell activation by antigen presentation (45, 59). We observed a 10- to 13-fold induction of transcription by the DEN region when T cells were activated (Fig. 2). The enhancer elements (1R and 2R) showed no change in activity after activation (Fig. 2A). The sevenfold induction of a complete LTR containing 1R (1R+DEN) in activated Jurkat cells indicated that all of its inducibility was attributable to the DEN region (Fig. 2A).

As a control, we examined whether TPA or ionomycin alone could induce the transcriptional activity of the DEN region. We observed that ionomycin alone did not induce this activity (Fig. 2B). TPA by itself led to a modest induction. However, our data indicated that most of the increase in transcriptional induction by the DEN region requires both TPA and ionomycin.

Role of the DEN region in MCF13 MLV lymphomagenesis. In our previous study, we showed that, in addition to its regulation of transcription, the DEN region has a significant effect on lymphomagenesis (56). To investigate the role of the DEN region in tumorigenesis, we asked whether proviruses with the DEN region deletion are present in tumor DNA and whether these proviruses have any changes in their LTR configuration compared with the input mutant virus. To answer these questions, we analyzed thymic tumors induced by both 1RΔDENv and 2RΔDENv mutant MCF13 viruses. To analyze the LTRs of proviruses most directly involved in tumorigenesis, we isolated LTRs involved in cellular proto-oncogene activation. Since MCF integrations occur most frequently near c-*myc* in thymic tumors (1, 8, 31, 44, 47, 63), we isolated proviral LTRs involved in c-*myc* rearrangement.

We used PCR to amplify and isolate proviral LTRs integrated upstream of c-*myc* from thymic lymphoma DNAs. We concentrated on the upstream region of c-*myc* because other investigators have observed that the majority of thymic tumors involved proviral integration upstream rather than downstream of the c-*myc* gene (1, 8, 31, 44, 47, 63). Our strategy included the use of a PCR primer that hybridized specifically to the first exon of the c-*myc* gene (MYC) and a second primer that hybridized to either the 5′ (LTR5′) or 3′ (LTR3′) region of the MCF13 LTR (Fig. 3). The use of two different LTR primers allowed us to determine the orientation of proviral

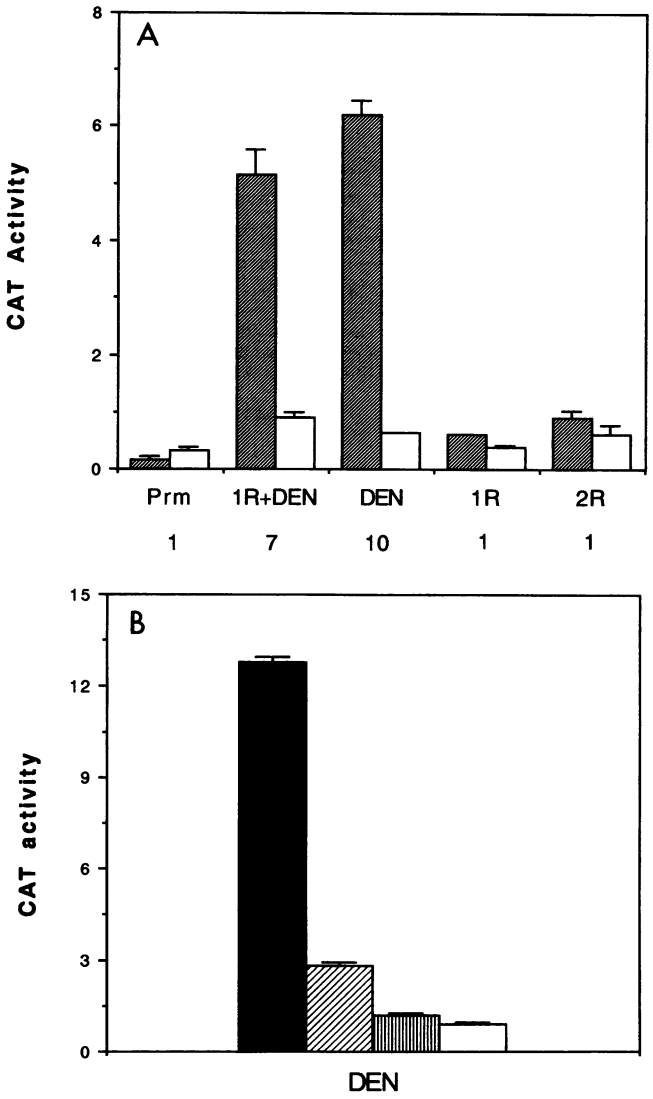

FIG. 2. (A) Transcriptional induction by LTR elements in activated T cells. Plasmid DNAs were transfected by lipofection into Jurkat cells either with or without 25 ng of TPA per ml and 1.4 ng of ionomycin per ml. Shaded bars indicate CAT activity for activated cells, and open bars indicate CAT activity for nonactivated cells. CAT activity measurements are expressed as 10^3 cpm of ^{14}C-acetylated chloramphenicol as detailed in Table 1. Error bars indicate the standard errors of the mean. The fold induction (ratio of CAT activity of the activated cells over that of the nonactivated cells) is shown at the bottom. (B) Transcriptional induction by the DEN region in Jurkat cells. DENf DNA was transfected into Jurkat cells treated with both 25 ng of TPA per ml and 1.4 ng of ionomycin per ml (solid bar), 25 ng of TPA per ml (diagonally hatched bar), 1.4 ng of ionomycin per ml (vertically hatched bar), or without any additional reagents (open bar). CAT activity measurements are described in Table 1.

integration with respect to the direction of transcription of c-*myc*. At each step of our analysis, we compared DNA from a normal thymus as a control. We subsequently cloned and sequenced the LTR PCR products and compared their sequences with the LTRs of the inoculated mutant viruses.

Using PCR analysis, we examined 11 tumors induced by the 2RΔDENv virus and 13 tumors induced by the 1RΔDENv virus. We determined that 2 of 11 2RΔDENv tumors (a, b),

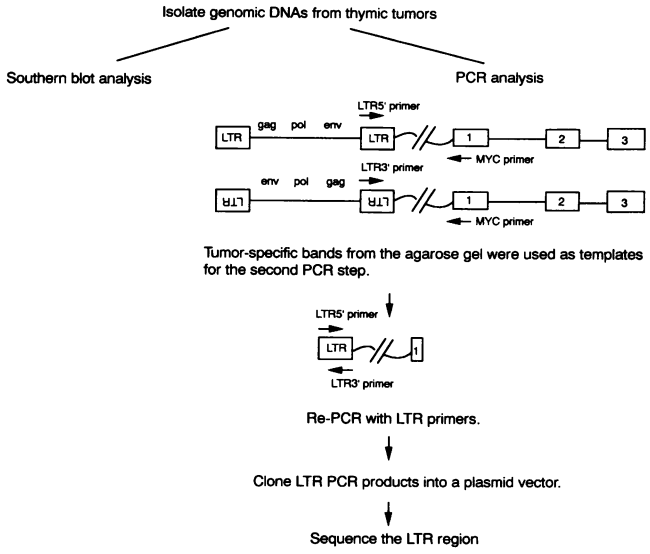

FIG. 3. Outline of the strategy for cloning and sequencing proviral LTRs involved in c-*myc* rearrangement in thymic lymphomas. Exons of c-*myc* are shown by open boxes marked 1, 2, and 3.

and 2 out 13 1RΔDENv tumors (c, d) had c-*myc* rearrangements (Fig. 4A). To confirm our PCR results, we performed genomic Southern blot analysis of DNAs from tumors and normal thymus digested with *Eco*RI or *Kpn*I. Blots were hybridized with a 4.8-kb c-*myc*-specific probe which contains exons 2 and 3 of the *myc* gene. As indicated in Fig. 4B, for *Kpn*I digestion (right side of the panel), we detected an extra band for tumors a, b, c, and d compared with normal thymus (lane N). For *Eco*RI digestion (left side of the panel), we detected an extra band only for tumors a and d compared with normal thymus (lane N). Our PCR analysis revealed that tumor d contained two LTRs (d1, d2) involved in c-*myc* rearrangement. However, this result was not indicated by Southern blot analysis. Since tumor d also contained a band corresponding to a germ line c-*myc* allele detectable by Southern blotting, we concluded that tumor d is oligoclonal in origin.

The sequence of the LTRs of tumors a and b indicated that there was no change in the general LTR configuration for proviruses involved in c-*myc* rearrangement and the input 2RΔDENv virus (Fig. 5). However, for tumor b there is a 6-bp insertion in the promoter region of the LTR. In addition, we observed that the LTRs of tumors a and b were integrated opposite to the direction of c-*myc* transcription. All of the LTRs cloned from the 1RΔDENv tumors, however, had acquired extra sequences during lymphomagenesis. Moreover, their proviruses were integrated in the same direction as c-*myc* transcription (Fig. 5). The LTR cloned from tumor c acquired sequences corresponding to the first two-thirds of an enhancer repeat, i.e., −322 to −278 bp of the MCF13 LTR. For LTR d1, the first four-fifths of an enhancer repeat was regained. For LTR d2, we determined that almost all of the 1RΔDEN LTR, i.e., an intact enhancer repeat, ΔDEN junction, and part of the promoter, was acquired. All three LTRs had acquired, more or less, one extra copy of an enhancer repeat. However, these repeats were no longer direct tandem repeats as is found in the wild-type LTR. All of the LTRs cloned from tumors induced by DEN deletion viruses retained the DEN deletion junction, indicating that they are from the inoculated MCF13 mutant viruses rather than from endogenous proviruses present in the mouse genome.

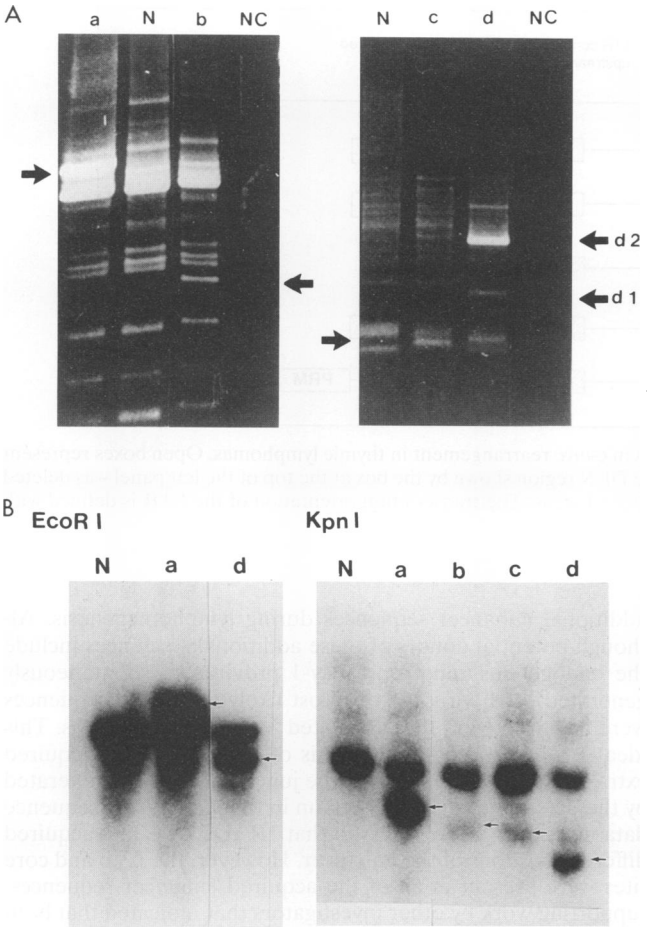

FIG. 4. (A) PCR products from amplification of genomic DNA of thymic tumors isolated from AKR mice inoculated with mutant MCF13 MLVs. Subgenomic portions of MCF13 proviral sequences were amplified by PCR with the primers MYC and LTR5′ (or LTR3′). The DNAs used as templates for PCR were 1 μg of genomic DNA isolated from normal thymus and thymic tumors induced by 2RΔDENv or 1RΔDENv. Ten microliters of reaction product was electrophoresed through a 1% agarose gel and stained with ethidium bromide. Extra bands detectable for the positive tumors are indicated by arrows. a and b are 2RΔDENv tumors, and c and d are 1RΔDENv tumors. d1 and d2 are two different bands identified in tumor d. N indicates DNA from normal thymus. NC indicates the negative control for PCR which does not contain any template DNA. (B) Southern blot of tumor DNAs. Ten micrograms of tumor and normal thymus DNAs was digested with either *Eco*RI or *Kpn*I, electrophoresed through a 0.7% agarose gel, and transferred to nitrocellulose. Filters were hybridized with the ^{32}P-labeled 4.8-kb c-*myc* fragment. Bands corresponding to c-*myc* rearrangements are indicated by arrows.

Transcriptional activity of LTRs cloned from tumors induced by 1RΔDENv mutant virus. We wished to determine what effect the acquired sequences in LTRs c, d1, and d2 have on transcriptional activity compared with the LTR from the inoculated 1RΔDENv mutant virus. To do so, we cloned LTRs c, d1, and d2 into a pCAT expression plasmid (pCAT-c, pCAT-d1, or pCAT-d2) to assay transcriptional activity. pCAT is a plasmid that contains the CAT reporter gene without a promoter. 1RΔDEN LTR is the LTR of the inoculated mutant virus, which was also cloned into pCAT for comparison (pCAT-1RΔDEN). We tested all of the constructs in L691 T-lymphoma cells by transient expression assays. We observed that pCAT-c, pCAT-d1, and pCAT-d2 were approximately twofold greater in CAT activity than the pCAT-1RΔDEN clone (Fig. 6).

DISCUSSION

We undertook studies to understand the mechanism of action of the DEN region of the MCF13 LTR for transcriptional activation and retroviral lymphomagenesis. Our previous work indicated that the role of the DEN region in transcriptional regulation is complex, and our recent data have revealed versatile functions for this LTR region. For example, the DEN region can act as an enhancer element in different cell types with the ability to augment transcription in either orientation. In T cells and fibroblasts, the transcriptional activity of the DEN region was comparable to that of a single enhancer repeat. Surprising results were the lack of activity of either one or two enhancer repeats in B cells and the fact that the DEN region was the only region of the LTR with activity in this cell type.

We observed that the DEN region could interact with enhancer sequences in either a synergistic or additive manner, depending on the cell type. In general, the effect of the interactions between 1R and DEN was similar to that between two enhancer repeats (2R). This result suggested that *cis*-acting elements in the DEN region can substitute for those in the enhancer repeat, and vice versa, to activate transcription to similar levels in different cell types. Possible *cis*-acting motifs in the DEN region that may control its various activities include binding sites for NF-κB, MLPal-binding protein(s), GATA-3, and SEF1 (Fig. 7). The activity of the DEN region in B cells may be regulated by NF-κB, which is known to be required for transcriptional activation of immunoglobulin genes in this cell type (35, 48). The MLPal, GATA-3, and SEF1 sequence motifs have been implicated in T-cell-specific transcription (15, 19, 26, 34, 36, 53, 54, 62). It is likely that interactions between proteins binding to these sites regulate the transcriptional activity of the DEN region in nonactivated T cells. A number of different protein-binding sites have been identified in the enhancer that may be involved in its interactions with the DEN region. The most relevant enhancer sites are the core consensus motif, which regulates T-cell-specific transcription, and the LVb site, which is important for transcription in multiple cell types (52, 56). We propose that the maximal transcriptional activity of the MCF13 LTR in any cell type depends on the levels and interactions of nuclear proteins that interact with DNA-binding sites in the DEN region and the enhancer and the potential interactions of these proteins with coactivators that exist in different cells.

Our observation that the DEN region is the only element of the MCF13 LTR with the ability to induce transcription in activated T cells is intriguing. In an earlier study, we observed that the MCF13 LTR had significant transcriptional activity in primary thymic lymphocytes only after they had been activated with phorbol ester and calcium ionophore (37). Because our data demonstrated that the DEN region accounts for the transcriptional induction of the entire LTR in activated Jurkat cells, we propose that the DEN region plays a similar role in activated thymocytes.

To examine the mechanism for the role of the DEN region in MCF13 lymphomagenicity, we analyzed the proviral LTRs in thymic tumors that were induced by MCF13 mutant viruses with DEN region deletions. We previously observed that deletion of the DEN region significantly reduced the incidence of thymic lymphoma produced by the MCF13 MLV (56). The reduction in disease incidence of this mutant virus was similar

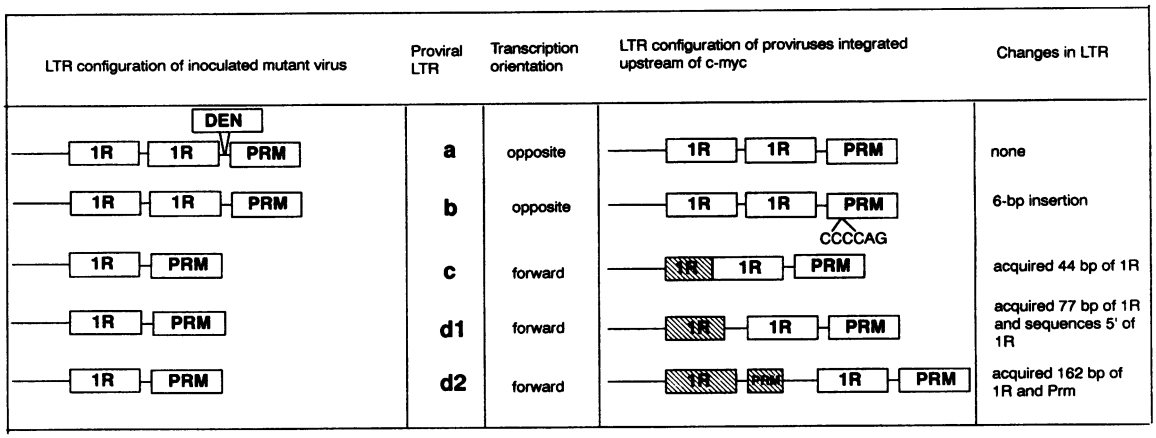

FIG. 5. Summary of analysis of 1RΔDENv and 2RΔDENv LTRs involved in c-*myc* rearrangement in thymic lymphomas. Open boxes represent different LTR elements present in the inoculated MCF13 mutant viruses. The DEN region shown by the box at the top of the left panel was deleted from the inoculated mutant viruses. Acquired sequences are indicated by hatched areas. The transcription orientation of the LTR is defined with respect to the direction of transcription of c-*myc*.

to that of MCF13 with a deletion of an enhancer repeat (56). In this study we restricted our analysis to LTRs inserted upstream of c-*myc* which is the most frequently rearranged cellular proto-oncogene in AKR thymic tumors (1, 8, 31, 44, 47, 63). Our analysis of LTRs of mutant viruses with either one (1RΔDENv) or two (2RΔDENv) enhancer repeats demonstrated that 15% of the 1RΔDENv and 18% of the 2RΔDENv tumors had c-*myc* rearrangements. The incidence of c-*myc* rearrangement for these mutant viruses is similar to that of the wild-type MCF13 MLV (20%) (31). We observed that proviruses involved in c-*myc* rearrangement retained the DEN region deletion in their LTRs. Proviral LTRs for 2RΔDENv had the same structure as the LTR of the input virus. In contrast, 1RΔDENv proviruses had LTRs that had acquired additional enhancer sequences during lymphomagenesis. Although potential donors of these additional sequences include the endogenous xenotropic Bxv-1 provirus or spontaneously generated MCF viruses, it is most likely that these sequences were acquired from the inoculated MCF13 mutant virus. This idea is supported by our analysis of LTR d2, which acquired extra sequences that included the junction sequence generated by the deletion of the DEN region in the input virus (sequence data not shown). We observed that 1RΔDENv LTRs acquired different portions of the enhancer. However, the LVb and core sites were present in all of the acquired enhancer sequences, supporting work by other investigators that indicated that both of these sites play important roles in MLV lymphomagenesis (15, 51, 52).

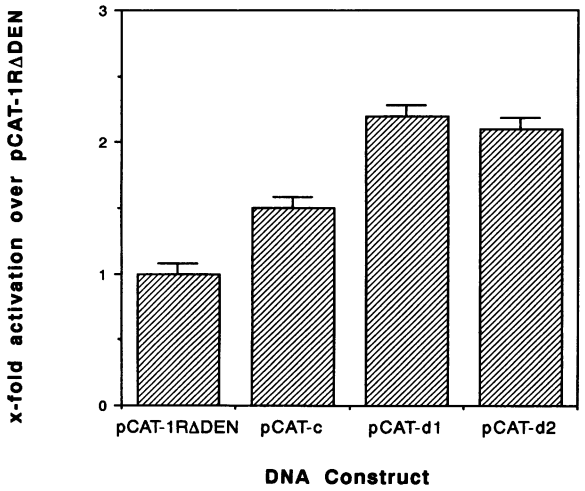

FIG. 6. Comparison of CAT activity of proviral LTRs c, d1, and d2 and the 1RΔDENv LTR. Proviral LTRs c, d1, d2, and the 1RΔDENv LTR were cloned into a pCAT expression vector and assayed for their transcriptional activity in L691 T-lymphoma cells. CAT assays are described in Table 1. The CAT activity of pCAT-1RΔDEN has been set at 1, and all other CAT activity mean values are expressed as fold activation over that of pCAT-1RΔDEN. Error bars show standard errors of the mean.

Our data from transient expression assays indicated that the acquired sequences for LTRs c, d1, and d2 increased transcriptional activity in T cells by twofold compared with the LTR of the inoculated 1RΔDENv mutant virus. Although we have not directly examined the levels of c-*myc* transcripts in these tumors, we and others have shown that c-*myc* message is increased by about twofold in AKR thymic tumors (31, 44). Thus, although the twofold increase in transcriptional activation by these LTRs is modest, it is relevant for c-*myc* activation in MCF13 lymphomagenesis.

Our analysis of the LTRs of proviruses in thymic tumors indicated that transcriptional activation of at least the c-*myc* proto-oncogene can occur without DEN sequences in the LTR. Because we observed that the DEN region is important for MCF13 lymphomagenesis (56), our results from studies of c-*myc* suggest that DEN sequences may be required for the activation of other proto-oncogenes that are rearranged in AKR thymic tumors (10, 42a). To test this notion, analysis of these additional proto-oncogenes similar to that we have performed for c-*myc* will be required. This is especially relevant for our identification of transcriptional activation by the DEN region in activated T cells and the role that this activity may play in MLV lymphomagenesis. It is possible that different proto-oncogenes will require different *cis*-acting regulatory elements that will depend on the stage of T-cell differentiation when their transcriptional activation occurs. On the other hand, the role of the DEN region may be to regulate MCF13 replication in thymocytes. It has been observed that there is a close correlation between titers of MLV in the thymus and

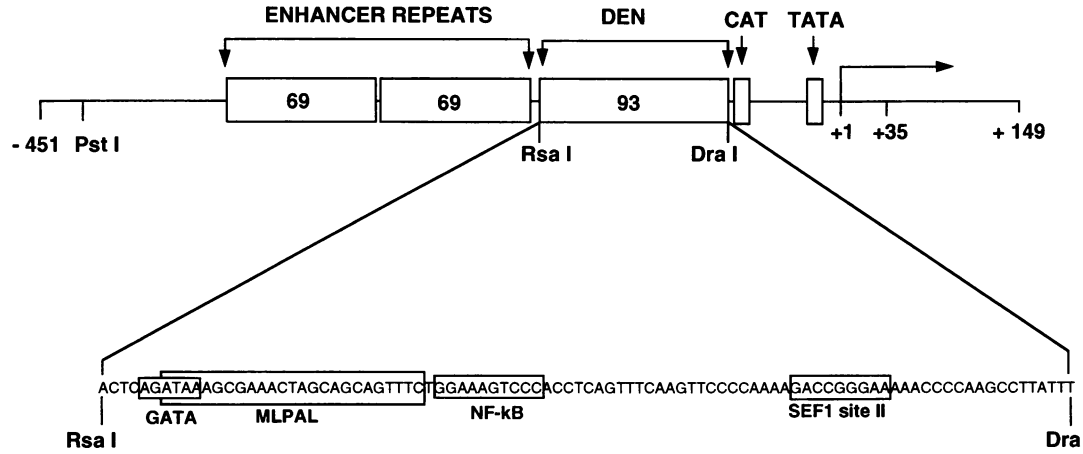

FIG. 7. Protein-binding sites within the DEN region of the MCF13 LTR. The nucleotide sequence of the DEN region is shown along with the sequence motifs of identified protein-binding sites enclosed by boxes. The MLPal and GATA sites partly overlap.

incidence of lymphoma (7, 40). We are currently performing experiments to test these possibilities. In this study, we have identified novel LTR transcriptional regulatory activities for the region between the enhancer and promoter of the MCF13 MLV, some of which may be relevant for the role of this region in retroviral lymphomagenesis.

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