JOURNAL OF VIROLOGY, Jan. 1999, p. 234–241 Vol. 73, No. 1
0022-538X/99/$04.00+0

Sequence-Specific and/or Stereospecific Constraints of the U3 Enhancer Elements of MCF 247-W Are Important for Pathogenicity

NANCY L. DIFRONZO AND CHRISTIE A. HOLLAND*

Center for Virology, Immunology, and Infectious Disease Research, Children's National Medical Center, Washington, D.C. 20010

Received 19 June 1998/Accepted 29 September 1998

The oncogenic potential of many nonacute retroviruses is dependent on the duplication of the enhancer sequences present in the unique 3′ (U3) region of the long terminal repeat (LTR). In a molecular clone (MCF 247-W) of the murine leukemia virus MCF 247, a leukemogenic mink cell focus-inducing (MCF) virus, the U3 enhancer sequences are tandemly repeated in the LTR. We mutated the enhancer region of MCF 247-W to test the hypothesis that the duplicated enhancer sequences of this virus have a sequence-specific and/or a stereospecific role in enhancer function required for transformation. In one virus, we inserted 14 nucleotide bp into the novel sequence generated at the junction of the two enhancers to generate an MCF virus with an interrupted enhancer region. In the second virus, only one copy of the enhancer sequences was present. This second virus also lacked the junction sequence present between the two enhancers of MCF 247-W. Both viruses were less leukemogenic and had a longer mean latency period than MCF 247-W. These data indicate that the sequence generated at the junction of the two enhancers and/or the stereospecific arrangement of the two enhancer elements are required for the full oncogenic potential of MCF 247-W. We analyzed proviral LTRs within the c-*myc* locus in tumor DNAs from mice injected with the MCF virus with the interrupted enhancer region. Some of the proviral LTRs integrated upstream of c-*myc* contain enhancer regions that are larger than those of the injected virus. These results are consistent with the suggestion that the virus with an interrupted enhancer changes in vivo to perform its role in the transformation of T cells.

Oncogenesis by nonacute murine leukemia viruses (MuLVs) requires multiple steps to disrupt the complex processes of cellular differentiation. Each of these steps is likely to be affected by one or more viral genes or gene products (4, 53). The unique 3′ (U3) region of the long terminal repeat (LTR) contains the viral promoter and transcriptional enhancers. In general, highly oncogenic retroviruses contain two or more tandem copies of the enhancer sequences (9, 10, 15, 16, 21, 29, 32, 35, 43, 54).

The enhancer sequences are important determinants of both the pathogenic potential and disease specificity of MuLVs and other nonacute retroviruses (9, 16, 21, 27–32, 40). It has been suggested that enhancers affect pathogenicity by modulating the level of transcription of viral and cellular genes in a tissue-specific fashion. Transient transfections of a number of different fibroblast and hematopoietic cell lines with MuLV-derived enhancer and promoter regions driving reporter genes have shown that LTR-mediated transcriptional activity correlates with the tissue specificity and disease-inducing potential of the virus (8, 38, 46, 48, 49, 51, 57). The MuLV U3 enhancer sequences consist of a densely packed series of motifs which contain actual or putative binding sites for cellular proteins and transcription factors (22). The organization of several of these motifs, including the LVb, AML-1 (core), NF-1, and GRE/E box constellation, is highly conserved within the nonacute retroviruses and has been thought to provide a "framework" important for enhancer function (22, 49). A number of cellular proteins have been shown to bind to the MuLV enhancer sequences (24, 34, 44, 47, 52, 55, 58, 59). Importantly, mutational analysis of the Moloney, SL3-3, and MCF13 MuLV enhancer elements has revealed that several of these protein binding sites are critical for pathogenesis (3, 25, 36, 37, 41, 48, 54). The complex structural organization of these protein binding sites and the existence of conserved sequence motifs in the MuLV enhancer elements are features reminiscent of the eukaryotic enhanceosome, described by others (7, 23, 50) as a nucleoprotein complex containing a clustered group of binding sites that promotes the assembly of a higher-order enhancer complex, resulting in enhanced transcription.

The molecular mechanisms that require enhancer duplication and sequence redundancy in pathogenic MuLVs are not known. One possible explanation is that the duplicated enhancer sequences function together as an enhanceosome. This suggests that the cellular proteins present in the U3 nucleoprotein complex are positioned in a three-dimensional, stereospecific fashion. This structure is mediated by the interactions between DNA-binding proteins and the enhancer sequences themselves, and facilitated cooperatively by protein-protein interactions. This interpretation predicts that the pathogenic potential of MCF 247-W is dependent upon the stereospecific relationship between the two copies of enhancer sequences. Alternatively, it is possible that the novel sequence created by the junction of the two copies of the enhancer sequences is important for enhancer function. This sequence may either play a spatial role essential for cooperative function between binding sites within the enhancer elements or, alternatively, provide a binding site for a unique protein that affects enhancer function either directly or indirectly through its interactions with other proteins in the enhancer sequences.

As a first step toward addressing these possibilities, we have

* Corresponding author. Mailing address: Center for Virology, Immunology, and Infectious Disease Research, Children's National Medical Center, 111 Michigan Ave., N.W., Washington, D.C. 20010. Phone: (202) 884-3981. Fax: (202) 884-3985. E-mail: holland@gwis2.circ.gwu.edu.

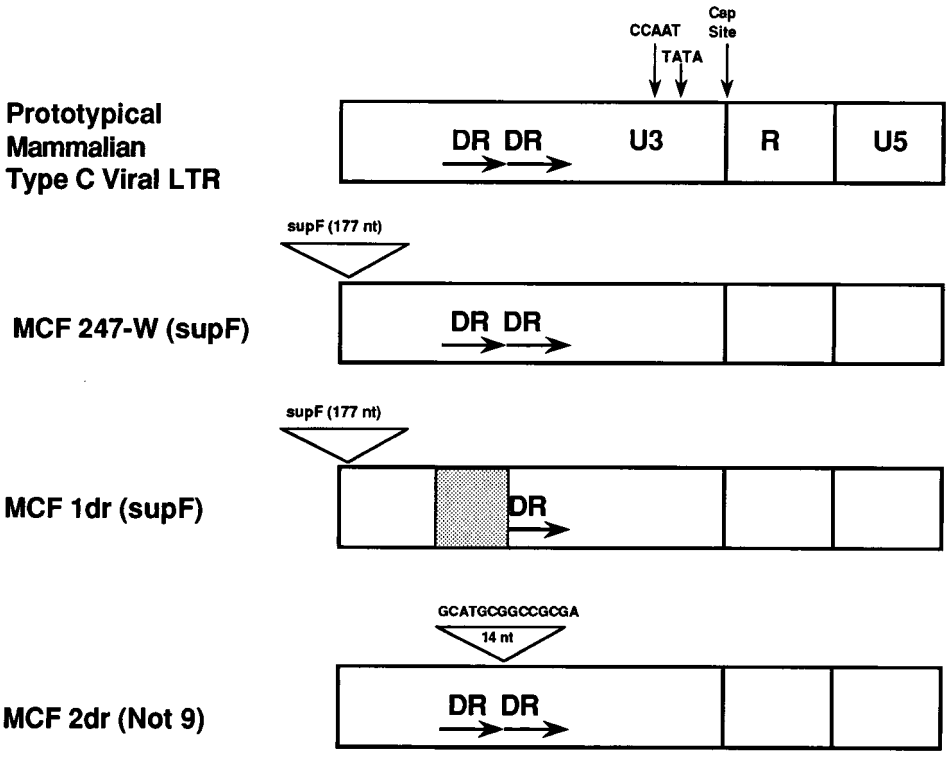

FIG. 1. Diagrammatic representation of the structural features of the proviral LTRs of the prototypical mammalian type C retrovirus and the MCF viruses used in this study. The enhancer sequences, or direct repeats (DR), and the promoter region (CCAAT and TATA) are indicated in U3. The locations of the inserted sequences (*supF* and Not9) in MCF viruses with altered U3 regions are indicated by triangles above the LTRs of MCF 1dr (supF) and MCF 2dr (Not9). The stippled box in the U3 region of MCF 1dr (supF) indicates the absence of the first copy of the enhancer sequences in this molecular clone. nt, nucleotides.

investigated whether the novel junction sequence present in U3 of MCF 247-W is a feature critical to the leukemogenic phenotype of this virus. We generated a mink cell focus-inducing (MCF) virus that contains an interrupted enhancer region, MCF 2dr (Not9), and compared its leukemogenic potential to that of an MCF virus with a single copy of the enhancer, MCF 1dr (supF), and to that of the prototypic leukemogenic MCF virus, MCF 247-W. The data presented in this report demonstrate that disruption of the novel sequence present at the junction of the enhancer sequences reduces the leukemogenic phenotype of the virus, as does removal of one copy of the enhancer sequences. Furthermore, these data demonstrate that when the junction between the two enhancer sequences of MCF 247-W is disrupted, the U3 sequences are altered in tumors.

MATERIALS AND METHODS

Construction of viruses with altered LTRs. We inserted 14 nucleotide bp between the two copies of the enhancer sequences of MCF 247-W (29), an infectious molecular clone of MCF 247 cloned into pBR322, by using overlap extension PCR (26). We named this virus MCF 2dr (Not9) (Fig. 1) and we refer to the inserted 14-bp sequence as Not9. To introduce the Not9 sequence, we separately amplified the first copy of the enhancer sequences and the second copy of the enhancer sequences plus downstream U3 sequences. We used two primers, one homologous to the vector (A, 5′-CGCAACGTTGTTGCC-3′) and second homologous to the extreme 3′ end of the first enhancer (B, 5′-TCGCG GCCGCATGCAGGGTGGGACTCTCC-3′), to generate an amplification product containing the first enhancer copy. The second amplification product was generated by using one primer homologous to the extreme 5′ end of the second enhancer (C, 5′-GCATGCGGCCGCGATCCCAAACAGGATATCT-3′) and a second primer homologous to LTR sequences downstream of U3 (D, 5′-CTATCGGAGGACTGG-3′). The 5′ ends of primers B and C were complementary and contained the Not9 sequence. To generate the entire U3 region containing the Not9 insertion between the two copies of the enhancer sequences, the two U3 amplification products were denatured, annealed, and reamplified in a third reaction using the two external primers (A and D). The altered U3 sequence was molecularly cloned and used to replace the U3 sequence of MCF 247-W. Except for the Not9 sequence, MCF 2dr (Not9) is identical to MCF 247-W.

We generated a second infectious molecular clone containing an altered U3 region lacking the first copy of the enhancer sequences present in MCF 247-W. This clone, MCF 1dr (supF), was constructed by replacing the LTR of MCF 247-W with that from MCF 247-1b (28) and inserting a bacterial suppressor tRNA gene, *supF*, into the *Pst*I site upstream of the enhancer sequences (Fig. 1). We also inserted the *supF* sequence into the *Pst*I site upstream of the enhancer sequences in MCF 247-W to create MCF 247-W (supF). We have shown previously that the *supF* sequence is stable in MCF viruses, does not change the leukemogenic phenotype of the virus, and can be used to track *supF*-tagged viruses in injected mice (17). To generate virus stocks, the clones were transfected (11) separately into *Mus dunni* cells, and culture supernatants were collected. The titers of the virus stocks used for injections were determined by using an assay for reverse transcriptase (5); the stocks differed in titer by less than one-half of a log unit.

Leukemogenicity assays. Newborn (1- to 3-day-old) AKR/J mice were injected, intraperitoneally and intrathymically, with 0.1 ml of a virus stock. Mice were monitored for 6 months for signs of frank leukemia (ruffled fur and labored breathing). All morbid mice were sacrificed and examined for gross pathological changes (enlargement of the thymus, spleen, liver, and lymph nodes) indicative of MCF virus-induced leukemia. To evaluate the pathogenic potential of these viruses, we compared the incidence of leukemia in mice injected with MCF viruses having altered LTRs prior to 180 days with the incidence of leukemia in mice injected with MCF 247-W (supF) by using chi square analyses (60).

Genomic Southern blots. Twenty-microgram samples of high-molecular-weight DNAs prepared from thymic lymphomas isolated from morbid mice were digested separately with either *Eco*RI, *Xba*I, or *Asp*718, separated by electrophoresis through 1% agarose, and transferred to nylon membranes. The membranes were hybridized with a ^{32}P-labeled, 1.2-kb *Sac*I to B*am*HI c-*myc* fragment isolated from pB5′-myc, a plasmid containing the first exon of c-*myc* and approximately 7 kb of upstream cellular sequences (19), prior to exposure of the filter to X-ray film with intensifying screens at −70°C for 48 h.

Analysis of genomic DNA by PCR. Thymoma DNAs containing the Not9 sequence were identified by PCR using primers that span the U3 region (LTR5′ and LTR3′) of the proviral LTR. The LTR5′ and LTR3′ primers recognize sequences located at the beginning of U3 and sequences located at the end of the

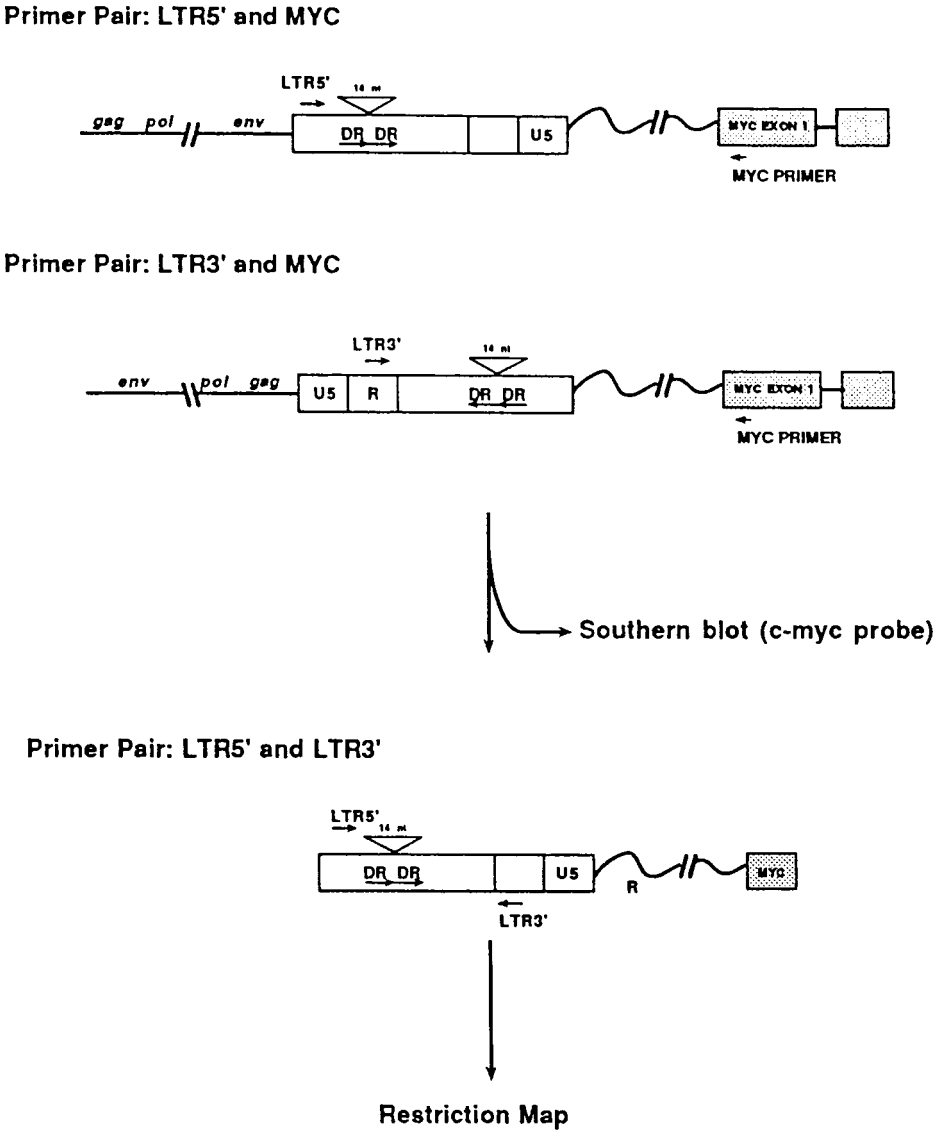

FIG. 2. Approach used for analysis of proviral LTRs inserted into the c-*myc* locus. Primers that hybridize to the first exon of c-*myc* (MYC) and the proviral LTR (LTR5′ or LTR3′) were used to amplify tumor DNAs. Primer pairs were selected to detect proviruses integrated upstream of c-*myc* in the same (LTR5′ and MYC) or the opposite (LTR3′ and MYC) transcriptional orientation. Amplification products were analyzed by Southern blot using the c-*myc* probe. DNAs that hybridized with the c-*myc* probe were gel purified and reamplified by using primers that span U3 (LTR5′ and LTR3′). The U3 amplification products were analyzed by Southern blotting with the 4Not9-2 probe and by limited restriction analysis. nt, nucleotides.

R region, respectively (12). PCR amplifications were performed in 50 μl with 10 mM Tris-HCl (pH 8.3), 2.5 mM $MgCl_2$, 0.2 mM each deoxynucleoside triphosphate, 0.25 mM each primer, 0.025 U of Ampli*Taq* polymerase (Perkin-Elmer Cetus) per ml, and 25 ng of genomic DNA. PCR mixtures were heated to 94°C and then subjected to a 30-cycle program (1 min at 94°C, 30 s at 56°C, and 1 min at 72°C). Aliquots of the amplification reaction mixtures were electrophoresed through agarose, transferred by Southern blotting to nylon membranes, and hybridized with a ^{32}P-labeled oligonucleotide probe specific for the Not9 sequence, 4Not9-2 (5′-ccctgcatgcggccgcgatc-3′), in the context of the adjacent enhancer sequences.

Proviral LTRs integrated near exon 1 of c-*myc* were identified by amplifying tumor DNAs with a primer specific for c-*myc* in conjunction with a primer complementary to sequences in the MCF LTR (Fig. 2). The primer pairs were designed to detect viruses integrated in the same or the reverse transcriptional orientation relative to c-*myc* exon 1 (12). The sequences of the six c-*myc*-specific primers used in this study (mycA, mycB, mycC, mycD, and mycE [36]; mycM [12]) were derived from the nucleotide sequences at positions 122 to 95, 296 to 269, 627 to 600, 1688 to 1659, 1342 to 1316, and 1674 to 1655 relative to the *Xba*I site located 1.5 kb 5′ of c-*myc* exon 1 (13). PCR amplifications were performed in 50 μl with 10 mM Tris-HCl (pH 8.3), 2.5 mM $MgCl_2$, 0.2 mM each deoxynucleoside triphosphate, 0.25 mM each primer, 0.025 U of Ampli*Taq* polymerase (Perkin-Elmer Cetus) per ml, and 25 ng of genomic DNA. PCR mixtures were heated to 94°C and then subjected to a 32-cycle program (1 min at 94°C, 1 min 64°C, and 3 min at 72°C) and a 10-min extension step. Amplification products were resolved in agarose gels, transferred by Southern blotting to nylon membranes, and hybridized with a ^{32}P-labeled c-*myc* probe. The blots were stripped and subsequently rehybridized with the 4Not9-2 oligonucleotide probe. Amplification products that hybridized to both probes were gel purified, reamplified by using the LTR3′ and LTR5′ primers, and analyzed by Southern blotting with the 4Not9-2 oligonucleotide probe.

RESULTS

Generation of MCF viruses with altered enhancers. The U3 region of MCF 247-W contains two copies of a 105-bp sequence considered to be the viral enhancer (22, 28). The sequence generated by the 3′ end of the first enhancer and the 5′ end of the second enhancer contains a novel nucleotide se-

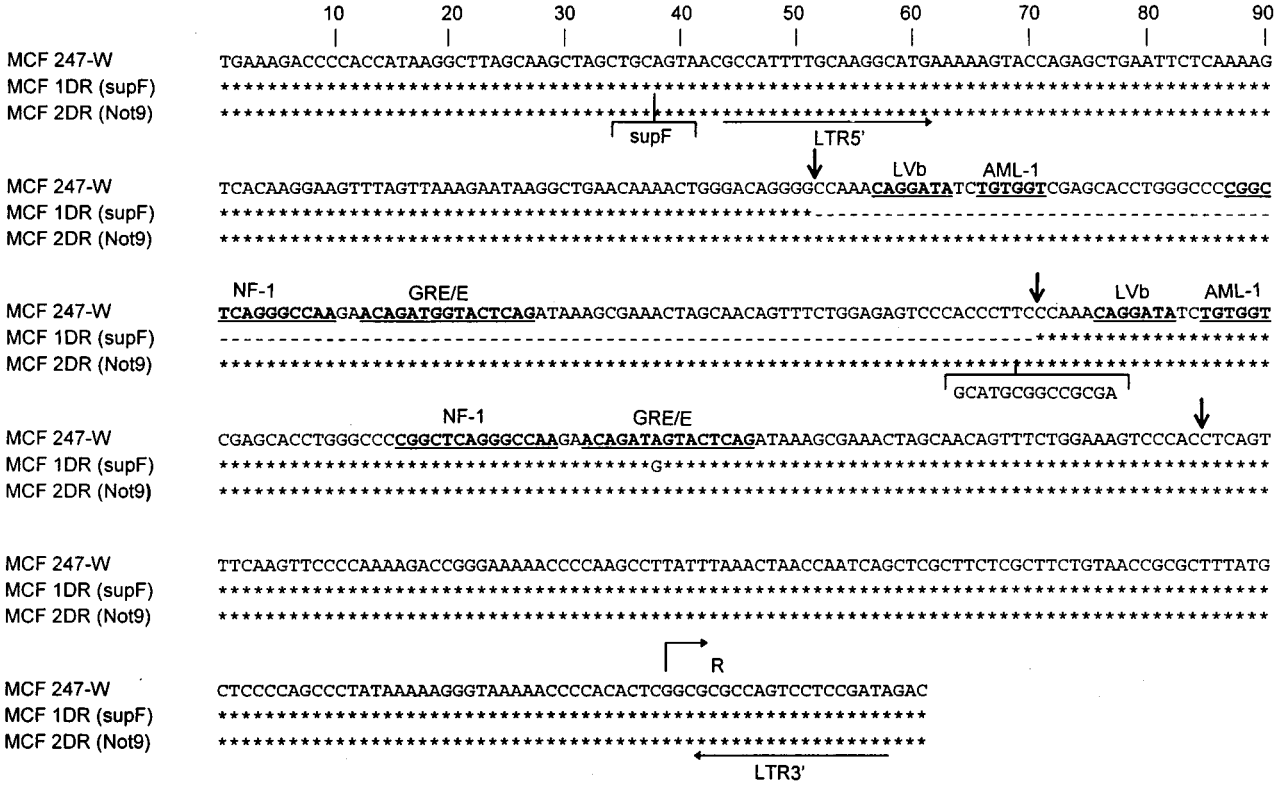

FIG. 3. Alignment of the nucleotide sequences of the U3 regions of MCF 247-W (supF), MCF 1dr (supF), and MCF 2dr (Not9). The bold arrows above the sequence of MCF 247-W indicate the beginning and end of each enhancer sequence. Shown in brackets beneath the sequences of the U3 regions of MCF 1dr (supF) and MCF 2dr (Not9) are the locations of the *supF* and Not9 insertions, respectively. The asterisks indicate sequence identity with MCF 247-W, and the dashes indicate deleted nucleotides. The positions of the enhancer "framework" sequences (22) LVb, AML-1 (core), NF-1, and GRE/E are indicated by the boldface and underlined nucleotides in the MCF 247-W sequence. The locations of primers LTR5′ and LTR3′ are indicated by the horizontal arrows.

quence not present elsewhere in either copy of the 105-bp enhancer sequences. This junction sequence does not exist in the enhancer sequences of either MCF 247-1b or MCF 30-2, two molecular clones of mildly pathogenic MCF viruses that contain only one copy of the enhancer sequence present in the leukemogenic virus MCF 247-W (28).

To test the hypothesis that this novel junction sequence is important to the function of the enhancers and viral pathogenesis, we generated molecular clones of MCF viruses with altered LTRs. The organization of the prototypical mammalian type C proviral LTR is illustrated at the top of Fig. 1. The enhancer sequences (DR, direct repeat), the promoter (CCAAT), and the site of transcriptional initiation (TATA box) are located in U3. The LTR of MCF 247-W is organized similarly to the prototypical proviral LTR and contains two copies of the enhancer sequences. The LTR of MCF 1dr (supF) contains only one copy of the 105-bp sequence that is repeated in MCF 247-W. The LTR of MCF 2dr (Not9) is identical to that of MCF 247-W but contains the Not9 sequence inserted between the two copies of enhancer sequences in U3. This insertion disrupts the novel sequence located between the enhancer sequences of MCF 247-W, as well as alters both the spacing and the helical orientation of the enhancer sequences relative to each other. We replaced the LTR of the infectious clone of MCF 247-W with each of these altered LTRs. We confirmed the structure of the resected clones by restriction mapping and by hybridizing the appropriate clones with probes specific for the inserted sequences.

The U3 sequences of MCF 247-W, MCF 1dr (supF), and MCF 2dr (Not9), confirmed by DNA sequence analysis, are shown aligned in Fig. 3. MCF 247-W contains two enhancer elements. The U3 region of MCF 247-W (supF), not shown, is derived from MCF 247-W and contains a 177-bp insertion, *supF*, upstream of the duplicated enhancer sequences. MCF 1dr (supF) contains a single copy of the enhancer sequences present in MCF 247-W and *supF* inserted into the identical location as in MCF 247-W (supF). The enhancer sequences of MCF 1dr (supF) are identical to the second direct repeat of MCF 247-W (supF), except for a single nucleotide difference (A to G) in the GRE/E box. The A-to-G nucleotide change creates a consensus Ephrussi (E) box-like motif (14) identical to that present in the first enhancer of MCF 247-W. The U3 region of MCF 2dr (Not9) is identical to MCF 247-W, except that it contains the Not9 sequence inserted two nucleotides from the end of the first direct repeat, thus disrupting both the novel sequence between the two direct repeats and the spatial relationship between the two enhancer copies. Thus, both MCF viruses containing altered U3 regions have unique stereospecific constraints on their enhancer sequences that differ from that of MCF 247-W (supF).

Pathogenicity of MCF viruses with altered enhancer sequences. We injected newborn AKR/J mice with MCF viruses having altered U3 regions and compared the leukemogenicity of these viruses with that of MCF 247-W (supF). Since the incidence of spontaneous T-cell lymphoma in AKR/J mice prior to 6 months of age is extremely rare but increases dramatically after that time, we monitored the injected mice for 180 days. The results are summarized in Table 1. In contrast to

TABLE 1. Leukemogenicity of MCF viruses in AKR/J mice

Virus[a]	No. of diseased mice/ no. injected[b]	Mean latency period (days)[c]	Incidence of leukemia (%)[d]
Control[e]	0/26	NA[f]	0
MCF 247-W (supF)	31/32	117 ± 27	97
MCF 1dr (supF)	16/33	140 ± 17	48
MCF 2dr (Not9)	38/55	135 ± 29	69

[a] Virus stocks were prepared from *M. dunni* cells transfected with cloned viral DNAs. Mice were injected with virus stocks having titers that varied less than one-half of a log unit, as determined by reverse transcriptase activity assay.

[b] The number of mice with disease is compared with the total number of mice injected with a specific virus.

[c] The mean latency period calculated is expressed as the mean of all mice observed with advanced disease ± the standard error.

[d] Percent incidence was calculated as (number of mice with advanced disease at 180 days)/(number of mice injected) × 100.

[e] Control mice indicate the incidence of spontaneous leukemia prior to 180 days.

[f] NA, not applicable.

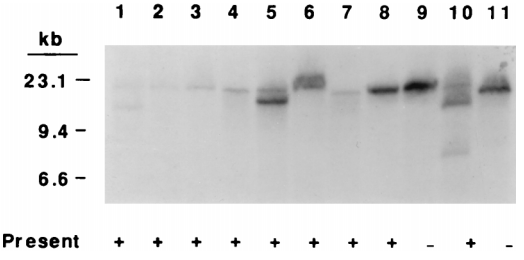

FIG. 4. Southern blot analysis demonstrating rearrangements of the c-*myc* locus in thymic lymphomas induced with MCF 2dr (Not9). Tumor DNAs were digested with *Eco*RI and probed with c-*myc* sequences. Thymic lymphomas containing rearrangements of the c-*myc* locus (lane 1, 92105; lane 5, 92130; lane 10, 92143) were harvested from morbid mice at 88, 142, and 120 dpi. Tumor DNAs containing proviral LTRs with the Not9 sequence are indicated by a plus sign beneath the lane. Tumor DNAs lacking proviral LTRs with the Not9 sequence are indicated by a minus sign beneath the lane.

that of MCF 247-W (supF), the leukemogenic potential of MCF viruses having altered enhancers was reduced. The percentages of mice injected with MCF 1dr (supF) and MCF 2dr (Not9) that developed disease (48 and 69%, respectively) were significantly reduced compared to that of mice injected with MCF 247-W (supF) (97%) (chi square, $P < 0.05$). In addition, viruses with these altered LTRs induced leukemia with longer latency periods than MCF 247-W (supF). We considered the possibility that insertion of the *supF* tag upstream from the enhancer sequences in MCF 247-W (supF) and MCF 1dr (supF) altered the pathogenic potential of these *supF*-tagged viruses. This is unlikely, however, as the incidence of leukemia in mice injected with these viruses was similar to that of their parent viruses, MCF 247-W (27) and MCF 1dr (28), respectively (chi square, $P > 0.05$). Taken together, these data are consistent with the interpretation that either sequence-specific and/or stereospecific constraints on the U3 enhancer sequences are required to achieve the full oncogenic potential of MCF 247-W.

Role of MCF 2dr (Not9) in MCF virus-induced leukemogenesis. To characterize the U3 regions of MCF viruses that play a role in tumorigenesis, we analyzed thymoma DNAs from mice injected with MCF 2dr (Not9). Since insertional mutagenesis of the c-*myc* locus is considered to be a common and critical step in the induction of tumors by MuLVs (1, 12, 13, 33, 36, 39, 42, 45, 56), we analyzed tumor DNAs for rearrangement of this cellular proto-oncogene and characterized the proviral LTRs in the c-*myc* locus. We limited our analysis to LTRs integrated upstream of c-*myc* exon 1, as previous studies, as well as our own data, indicate that this location is the predominant insertion site of MCF viruses and other nonacute retroviruses (13, 33, 36, 39, 42, 45).

We first examined MCF 2dr (Not9)-induced tumor DNAs for the presence of the Not9 sequence by PCR. The Not9 sequence was present in 77% (24 of 31) of the tumor DNAs tested. We next analyzed the tumor DNAs for rearrangement of the c-*myc* locus. Figure 4 shows a representative Southern blot containing 11 *Eco*RI-digested thymic lymphoma DNAs hybridized with the c-*myc* probe. Three of these tumor DNAs (lane 1, 92105; lane 5, 92130; lane 10, 92143) contain a rearranged c-*myc* locus, as confirmed by using two additional restriction enzymes (*Xba*I and *Asp*718). We screened the remainder of the tumor DNAs from animals injected with MCF 2dr (Not9) for c-*myc* rearrangements similarly. In total, 25% (6 of 24) of the tumor DNAs containing MCF 2dr (Not9) proviral LTRs had rearrangements in the c-*myc* proto-oncogene, as confirmed by at least two separate restriction enzyme analyses. Two additional tumors showed evidence of c-*myc* rearrangement when digested with one of the three restriction enzymes used; these tumors were not further characterized. In addition, we screened seven tumors that did not contain a Not9-tagged provirus and determined that none contained rearranged c-*myc* loci.

Using PCR and a series of primer pairs where one primer hybridizes to sequences in the first exon of c-*myc* and the second hybridizes to sequences in the LTR of MCF 247-W, we evaluated the c-*myc* loci for the presence of integrated proviral LTRs. Primer pairs were selected to amplify proviruses near the first exon of c-*myc* which are either in the same transcriptional orientation (i.e., LTR5′-mycM) or the opposite transcriptional orientation (i.e., LTR3′-mycM) with respect to c-*myc* (Fig. 2). These analyses demonstrated that the tumors containing rearrangements of the c-*myc* locus had proviruses upstream of and in the opposite transcriptional orientation from c-*myc*. These results are consistent with previous reports (33, 42, 45) and suggest that insertions of the MCF proviral sequences activate c-*myc* by enhancer insertion rather than by use of the promoter in the LTR.

To determine whether the U3 regions of proviruses integrated upstream of c-*myc* were derived from the injected virus and contained the Not9 sequence, the six tumor DNAs containing rearrangement of the c-*myc* locus were amplified with the LTR3′ primer and a series of *myc* primers and analyzed by Southern blotting; six different *myc* primers were used to ensure the amplification of the U3 region from the integrated provirus. As expected, the six tumors contained multiple amplification products that hybridized with the *myc* probe. These blots were subsequently stripped. Probe removal was confirmed by exposing the blots for 1 week prior to hybridization of the blots with the 4Not9-2 probe. Amplification products from five of the six tumor DNAs hybridized with the 4Not9-2 probe. The results for two tumors are shown in Fig. 5. For tumor 92143, the primer pair LTR3′-mycA amplifies a DNA fragment that hybridizes to both the c-*myc* and 4Not9-2 probes. Likewise for tumor 92145, several amplification products hybridize to both probes. For each tumor, a greater number of amplification products hybridized to the *myc* probe than to the 4Not9-2 probe. This is due to both the LTR3′ primer employed for amplification, as well as the specificity of the 4Not9-2 probe for the injected virus. In particular, the sequence of the LTR3′ primer is present in many MuLVs, including the replication-competent endogenous MuLV present

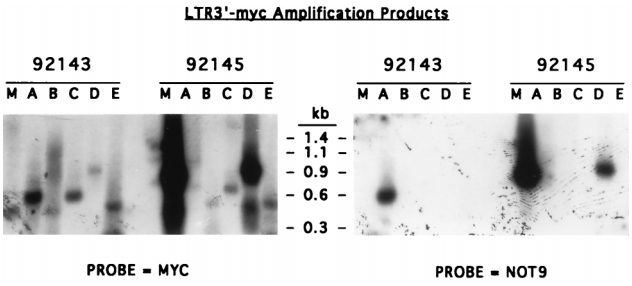

FIG. 5. Southern blot analysis of amplification products generated by PCR using LTR3′ and six *myc*-specific primers. Genomic DNAs from thymic lymphomas (92143 and 92145) of mice injected with MCF 2dr (Not9) were amplified in separate reactions using primer LTR3′ and primer mycM (M), mycA (A), mycB (B), mycC (C), mycD (D), or mycE (E) and analyzed by Southern blotting. The membrane on the left was hybridized with a *myc*-specific probe, stripped, and rehybridized with a probe specific for the Not9 sequence (4Not9-2).

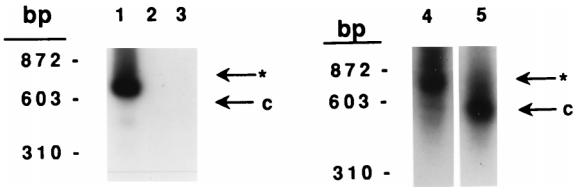

FIG. 6. Southern blot analysis of U3 regions of proviral LTRs upstream of c-*myc*. Amplification products generated by using the primer pair LTR3′-MYC that hybridized with the *myc*-specific and 4Not9-2 probes were gel purified from parallel gels and reamplified across the U3 region by using the primer pair LTR3′-LTR5′. Amplification products were separated by electrophoresis and hybridized with the 4Not9-2 probe. The predicted size of U3 from MCF 2dr (Not9) is indicated by the C at the right of each blot. Lanes 1 (92145) and 4 (92143) contain U3 regions larger than the injected virus, MCF 2dr (Not9) (lane 5). Negative controls include MCF 2dr (Not9)-induced tumor DNA that lacked rearrangement of c-*myc* (lane 2) and AKR/J normal thymus DNA (lane 3).

in AKR/J mice, whereas the 4Not9-2 probe is not homologous with published MuLV or murine cellular sequences (2). Moreover, it is significant that DNA fragments that amplify and hybridize with the *myc* probe are the same size as those that hybridize with the 4Not9-2 probe.

Table 2 summarizes the analysis of LTR3′-myc amplification products from six tumors containing rearranged c-*myc*. All six tumors occurred in less than 180 days postinoculation (dpi) and therefore were accelerated compared to spontaneous leukemia. Five of the six tumors contained amplification products that comigrated and hybridized to both the *myc* and 4Not9-2 probes. Thus, the majority of tumors induced with MCF 2dr (Not9) that have c-*myc* locus rearrangements contain Not9-tagged proviruses integrated upstream of and in the opposite transcriptional orientation with respect to c-*myc*. Interestingly, PCR-derived amplification products from DNA from one tumor (92160, 168 dpi) did not hybridize with the 4Not9-2 probe. This tumor was generated prior to 180 days of age but during the later portion of the preleukemogenic period. Although it was not tested by our studies, it is possible that proviruses from either endogenous MuLVs or spontaneously generated MCF viruses are integrated within the c-*myc* locus in this tumor DNA.

To investigate the structure of the LTRs integrated upstream from c-*myc*, the amplification products shown in Fig. 5 were gel purified and reamplified by using primers (LTR5′ and LTR3′) that span the U3 region of the LTR. The U3 amplification products were analyzed by Southern blot analysis using the 4Not9-2 probe (Fig. 6). Amplification products that contain the Not9 sequence were derived from the injected virus. Two of the five tumors with Not9 sequence-tagged proviruses integrated upstream of c-*myc* (Table 2) contained U3 regions larger than that predicted for the injected virus (Fig. 6). This indicates that the U3 region of the injected MCF 2dr (Not9) virus was altered in vivo in these tumors and yet retained the Not9 sequence.

TABLE 2. Analysis of LTR3′-myc amplification products in accelerated tumors

Not9-induced tumor	No. of dpi	Hybridization with		Comigration
		myc probe	Not9 probe	
92105	88	+	+	Yes
92130	142	+	+	Yes
92143	120	+	+	Yes
92145	120	+	+	Yes
92147	132	+	+	Yes
92160	168	+	−	No

DISCUSSION

We have shown that disruption of the novel sequence present at the junction of the enhancer sequences, as well as the removal of one copy of the enhancer sequences, reduced the leukemogenic phenotype of viruses otherwise identical to MCF 247-W. A number of possible mechanisms may explain the reduced pathogenicity of the MCF viruses having altered enhancer regions. First, the junction sequence in the enhancer region of MCF 247-W may provide essential spacing for cooperative function between the enhancer elements. Precedent for this effect is seen in the simian virus 40 enhancer, where the overall distance between individual motifs affected transcription from a heterologous promoter (20). Second, the junction sequence may be important for enhancer function by maintaining the relative orientation of the two enhancer sequences on the helix. Insertion of 14 bp would increase the distance between the two enhancer elements and introduce one and one-half helical turns between the enhancers. Consequently, the helical phasing of transcription factor binding sites in the two copies of the enhancer sequences would be altered. In the human beta interferon gene enhancer, alteration of the helical phasing is detrimental to transcription (50). Alteration of both the spacing and helical phasing would directly affect the three-dimensional structure of the enhanceosome generated by two copies of the enhancer sequences in the virus containing the 14-bp insertion. Third, the junction sequence may provide a binding site for a unique protein that affects enhancer function either directly or through its interactions with other proteins in the enhancer sequences. Finally, MCF viruses with two copies of enhancer sequences may have greater transcriptionally activity than MCF viruses with one copy of enhancer sequences. This may be due to either the reiteration of transcription factor binding sites provided by the second copy of enhancer sequences in MCF 247-W or the function of the novel sequence formed at the junction between the two enhancer copies. The present experiments do not differentiate between these possibilities but support our hypothesis that the duplicated enhancer sequences in the U3 region of MCF 247-W have a sequence-specific and/or stereospecific role in enhancer function that is required for transformation.

It is possible that the number of enhancers, or the novel junction sequence, affects the ability of these MCF viruses to replicate in T cells in vivo. We previously reported that MCF

viruses containing one or two copies of enhancer sequences replicated to similar titers in primary thymocytes 48 days after virus injection (28). These data suggest that the absence of one copy of enhancer sequences does not negatively affect virus replication in a way that can be detected in a chronic viral infection. Moreover, the MCF virus with one enhancer (28) did not have the novel sequence created by the junction of two enhancers in MCF 247-W. Therefore, neither the presence nor the absence of the novel junction sequence affects MCF virus replication in vivo. We recognize that small or early effects on viral replication would not be evident in this experiment (28) because of the high level of viral replication and the extensive variability in the assay. However, although small effects of the enhancer sequences on transcription may not be reflected in the viral titer, these effects may significantly regulate the expression of cellular genes and, presumably, those genes involved in transformation.

We note that other pathogenic MuLVs do not contain the novel sequence created by the junction of the MCF 247-W enhancers in an analogous position in their U3 regions. In these viruses, the enhancers are variable in both length and sequence, as are the junction sequences themselves (22). If the U3 region of each pathogenic MuLV was selected independently in vivo to optimize its biological role in disease induction, then the functions important to this role may be conserved while the specific nucleotide sequence may vary in these viruses.

Insertional mutagenesis of cellular proto-oncogenes is one general mechanism underlying oncogenesis by nonacute retroviruses (53). Leukemogenesis by MCF viruses is thought to involve activation of c-*myc* (1, 13, 33, 39, 42, 45, 56). We examined proviral integration in the c-*myc* locus in tumors induced by MCF 2dr (Not9), the MCF virus containing enhancer sequences interrupted by a 14-bp insertion. The majority of tumors containing rearranged c-*myc* loci contained a provirus in which the U3 region was derived from the injected provirus. Further analysis of tumor DNAs containing proviruses integrated into c-*myc* showed that the fragment containing the enhancer sequences of these provirus was larger than that of the injected virus in two of the five tumors. These data demonstrate that when the junction between the two enhancer sequences is disrupted, the enhancer sequences in the injected virus are altered in vivo. The presence of additional sequences in the LTRs upstream of c-*myc* suggests that these sequences may be important for transformation. These data are consistent with the findings of others (6, 12, 18, 36) that the process of viral evolution (18) generates and selects viral variants which are better suited to perform a role in the transformation of T cells. Determination of whether the altered LTRs containing the Not9 sequence are more potent regulators of transcription than MCF 2dr (Not9) and whether replication-competent viruses containing the U3 regions altered in vivo are more pathogenic than MCF 2dr (Not9) requires further investigation.

ACKNOWLEDGMENTS

We thank Anamaris Colberg-Poley for critical comments on the manuscript and Eva Tsuda and P. Ann Davis for assistance in the generation of data presented here.

This work was supported by grants to C.A.H. from the National Cancer Institute (CA-41510) and The Children's Cancer Foundation and to N.L.D. from the Board of Lady Visitors of Children's National Medical Center.

REFERENCES

1. **Adams, J. M., S. Gerondakis, E. Webb, J. Mitchell, O. Bernard, and S. Cory.** 1982. Transcriptionally active DNA region that rearranges frequently in murine lymphoid tumors. Proc. Natl. Acad. Sci. USA **79:**6966–6970.
2. **Altschul, S. F., T. L. Madden, A. A. Schäffer, J. Zhang, Z. Zhang, W. Miller, and D. J. Lipman.** 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. **25:**3389–3402.
3. **Amtoft, H. W., A. B. Sorensen, C. Bareil, J. Schmidt, A. Luz, and F. S. Pedersen.** 1997. Stability of AML1 (core) site enhancer mutations in T lymphomas induced by attenuated SL3-3 murine leukemia virus mutants. J. Virol. **71:**5080–5087.
4. **Athas, G. B., C. R. Starkey, and L. S. Levy.** 1994. Retroviral determinants of leukemogenesis. Crit. Rev. Oncog. **5:**169–199.
5. **Baltimore, D.** 1970. RNA-dependent DNA polymerase in virions of RNA tumor viruses. Nature (London) **266:**1209–1211.
6. **Brightman, B. K., C. Farmer, and H. Fan.** 1993. Escape from in vivo restriction of Moloney mink cell focus-inducing viruses driven by the Mo+PyF101 long terminal repeat (LTR) by LTR alterations. J. Virol. **67:**7140–7148.
7. **Carey, M.** 1998. The enhanceosome and transcriptional synergy. Cell **92:**5–8.
8. **Celander, D., and W. A. Haseltine.** 1984. Tissue-specific transcription preference as a determinant of cell tropism and leukaemogenic potential of murine retroviruses. Nature (London) **312:**159–162.
9. **Chatis, P. A., C. A. Holland, J. E. Silver, T. N. Frederickson, N. Hopkins, and J. W. Hartley.** 1984. A 3′ end fragment encompassing the transcriptional enhancers of nondefective Friend virus confers erythroleukemogenicity on Moloney leukemia virus. J. Virol. **52:**248–254.
10. **Chattopadhyay, S. K., B. M. Baroudy, K. L. Holmes, T. N. Frederickson, M. R. Lander, H. C. Morse, and J. W. Hartley.** 1989. Biologic and molecular genetic characteristics of a unique MCF virus that is highly leukemogenic in ecotropic virus-negative mice. Virology **169:**90–100.
11. **Chen, C. A., and H. Okayama.** 1988. Calcium phosphate-mediated gene transfer: a highly efficient transfection system for stably transforming cells with plasmid DNA. BioTechniques **6:**632–636.
12. **Chen, H., and F. K. Yoshimura.** 1994. Identification of a region of a murine leukemia virus long terminal repeat with novel transcriptional regulatory activities. J. Virol. **68:**3308–3316.
13. **Corcoran, L. M., J. M. Adams, A. R. Dunn, and S. Cory.** 1984. Murine T lymphomas in which the cellular myc oncogene has been activated by retroviral insertion. Cell **37:**113–122.
14. **Corneliussen, B., A. Thornell, B. Hallberg, and T. Grundström.** 1991. Helix-loop-helix transcriptional activators bind to a sequence in glucocorticoid response elements of retrovirus enhancers. J. Virol. **65:**6084–6093.
15. **DesGroseillers, L., and P. Jolicoeur.** 1984. Mapping the viral sequences conferring leukemogenicity and disease specificity in Moloney and amphotropic murine leukemia viruses. J. Virol. **52:**448–456.
16. **DesGroseillers, L., and P. Jolicoeur.** 1984. The tandem direct repeats within the long terminal repeat of murine leukemia viruses are the primary determinant of their leukemogenic potential. J. Virol. **52:**945–953.
17. **DiFronzo, N. L., and C. A. Holland.** 1993. A direct demonstration of recombination between an injected virus and endogenous viral sequences resulting in the generation of mink cell focus-inducing viruses in AKR mice. J. Virol. **67:**3763–3770.
18. **Ethelberg, S., A. B. Sorensen, J. Schmidt, A. Luz, and F. S. Pedersen.** 1997. An SL3-3 murine leukemia virus enhancer variant more pathogenic than the wild type obtained by assisted molecular evolution in vivo. J. Virol. **71:**9796–9799.
19. **Fahrlander, P. D., M. Piechaczyk, and K. B. Marcu.** 1985. Chromatin structure of the murine c-myc locus: implications for the regulation of normal and chromosomally translocated genes. EMBO J. **4:**3195–3202.
20. **Fromental, C., M. Kanno, H. Nomiyama, and P. Chambon.** 1988. Cooperativity and hierarchical levels of functional organization of the SV40 enhancer. Cell **54:**943–953.
21. **Golemis, E., Y. Li, T. N. Fredrickson, J. W. Hartley, and N. Hopkins.** 1989. Distinct segments within the enhancer region collaborate to specify the type of leukemia induced by nondefective Friend and Moloney viruses. J. Virol. **63:**328–337.
22. **Golemis, E. A., N. A. Speck, and N. Hopkins.** 1990. Alignment of U3 region sequences of mammalian type C viruses: identification of highly conserved motifs and implications for enhancer design. J. Virol. **64:**534–542.
23. **Grosschedl, R.** 1995. Higher-order nucleoprotein complexes in transcription: analogies with site-specific recombination. Curr. Opin. Cell. Biol. **7:**362–370.
24. **Gunther, C. V., and B. J. Graves.** 1994. Identification of ETS domain proteins in murine T lymphocytes that interact with the Moloney murine leukemia virus enhancer. Mol. Cell. Biol. **14:**7569–7580.
25. **Hallberg, B., J. Schmidt, F. S. Pedersen, and T. Grundström.** 1991. SL3-3 enhancer factor 1 transcriptional activators are required for tumor formation by SL3-3 murine leukemia virus. J. Virol. **65:**4177–4181.
26. **Ho, S. N., H. D. Hunt, R. M. Horton, J. K. Pullen, and L. R. Pease.** 1989. Site-directed mutagenesis by overlap extension using the polymerase chain reaction. Gene **77:**51–59.
27. **Holland, C. A., J. W. Hartley, W. P. Rowe, and N. Hopkins.** 1985. At least four viral genes contribute to the leukemogenicity of murine retrovirus MCF 247 in AKR mice. J. Virol. **53:**158–165.
28. **Holland, C. A., C. Y. Thomas, S. K. Chattopadhyay, C. Koehne, and P. V. O'Donnell.** 1989. Influence of enhancer sequences on thymotropism and

leukemogenicity of mink cell focus-forming viruses. J. Virol. **63:**1284–1292.
29. **Holland, C. A., J. Wozney, P. A. Chatis, N. Hopkins, and J. W. Hartley.** 1985. Construction of recombinants between molecular clones of murine retrovirus MCF 247 and Akv: determinant of an in vitro host range property that maps in the long terminal repeat. J. Virol. **53:**152–157.
30. **Ishimoto, A., M. Takimoto, A. Adachi, M. Kakuyama, S. Kato, K. Kakimi, K. Fukuoka, T. Ogiu, and M. Matsuyama.** 1987. Sequences responsible for erythroid and lymphoid leukemia in the long terminal repeats of Friend-mink cell focus-forming and Moloney murine leukemia viruses. J. Virol. **61:**1861–1866.
31. **Lenz, J., D. Celander, R. L. Crowther, R. Patarca, D. W. Perkins, and W. A. Haseltine.** 1984. Determination of the leukaemogenicity of a murine retrovirus by sequences within the long terminal repeat. Nature (London) **308:** 467–470.
32. **Li, Y., E. Golemis, J. W. Hartley, and N. Hopkins.** 1987. Disease specificity of nondefective Friend and Moloney murine leukemia viruses is controlled by a small number of nucleotides. J. Virol. **61:**693–700.
33. **Li, Y., C. A. Holland, J. W. Hartley, and N. Hopkins.** 1984. Viral integration near c-myc in 10–20% of MCF 247-induced AKR lymphomas. Proc. Natl. Acad. Sci. USA **81:**6808–6811.
34. **Manley, N. R., M. O'Connell, W. Sun, N. A. Speck, and N. Hopkins.** 1993. Two factors that bind to highly conserved sequences in mammalian type C retroviral enhancers. J. Virol. **67:**1967–1975.
35. **Matsumoto, Y., Y. Momoi, T. Watari, R. Goitsuka, H. Tsujimoto, and A. Hasegawa.** 1992. Detection of enhancer repeats in the long terminal repeats of feline leukemia viruses from cats with spontaneous neoplastic and non-neoplastic diseases. Virology **189:**745–749.
36. **Morrison, H. L., B. Soni, and J. Lenz.** 1995. Long terminal repeat enhancer core sequences in proviruses adjacent to c-*myc* in T-cell lymphomas induced by a murine retrovirus. J. Virol. **69:**446–455.
37. **Nieves, A., L. S. Levy, and J. Lenz.** 1997. Importance of a c-Myb binding site for lymphomagenesis by the retrovirus SL3-3. J. Virol. **71:**1213–1219.
38. **Nishigaki, K., M. Okuda, Y. Endo, T. Watari, H. Tsujimoto, and A. Hasegawa.** 1997. Structure and function of the long terminal repeats of feline leukemia viruses derived from naturally occurring acute myeloid leukemias in cats. J. Virol. **71:**9823–9827.
39. **O'Donnell, P. V., E. Fleissner, H. Lonial, C. F. Koehne, and A. Reicin.** 1985. Early clonality and high-frequency proviral integration into the c-*myc* locus in AKR leukemias. J. Virol. **55:**500–503.
40. **Pantginis, J., R. M. Beaty, L. S. Levy, and J. Lenz.** 1997. The feline leukemia virus long terminal repeat contains a potent genetic determinant of T-cell lymphomagenicity. J. Virol. **71:**9786–9791.
41. **Pedersen, F. S., K. Paludan, H. Y. Dai, M. Duch, P. Jorgensen, N. O. Kjeldgaard, B. Hallberg, T. Grundström, J. Schmidt, and A. Luz.** 1991. The murine leukemia virus LTR in oncogenesis: effect of point mutations and chromosomal integration sites. Radiat. Environ. Biophys. **30:**195–197.
42. **Reicin, A., J.-Q. Yang, K. Marcu, E. Fleissner, C. F. Koehne, and P. V. O'Donnell.** 1986. Deregulation of the c-*myc* oncogene in virus-induced thymic lymphomas of AKR/J mice. Mol. Cell. Biol. **6:**4088–4092.
43. **Rohn, J. L., and J. Overbaugh.** 1995. *In vivo* selection of long terminal repeat alterations in feline leukemia virus-induced thymic lymphomas. Virology **206:**661–665.
44. **Satake, M., M. Inuzuka, K. Shigesada, T. Oikawa, and Y. Ito.** 1992. Differential expression of subspecies of polyomavirus and murine leukemia virus enhancer core binding protein, PEBP2, in various hematopoietic cells. Jpn. J. Cancer Res. **83:**714–722.
45. **Selten, G., H. T. Cuypers, M. Zijlstra, C. Melief, and A. Berns.** 1984. Involvement of c-myc in MuLV-induced T cell lymphomas in mice: frequency and mechanisms of activation. EMBO J. **3:**3215–3222.
46. **Short, M. K., S. A. Okenquist, and J. Lenz.** 1987. Correlation of leukemogenic potential of murine retroviruses with transcriptional tissue preference of the viral long terminal repeats. J. Virol. **61:**1067–1072.
47. **Speck, N. A., and D. Baltimore.** 1987. Six distinct nuclear factors interact with 75-base-pair repeat of the Moloney murine leukemia virus enhancer. Mol. Cell. Biol. **7:**1101–1110.
48. **Speck, N. A., B. Renjifo, E. Golemis, T. N. Fredrickson, J. W. Hartley, and N. Hopkins.** 1990. Mutation of the core or adjacent LVb elements of the Moloney murine leukemia virus enhancer alters disease specificity. Genes Dev. **4:**233–242.
49. **Speck, N. A., B. Renjifo, and N. Hopkins.** 1990. Point mutations in the Moloney murine leukemia virus enhancer identify a lymphoid-specific viral core motif and 1,3-phorbol myristate acetate-inducible element. J. Virol. **64:**543–550.
50. **Thanos, D., and T. Maniatis.** 1995. Virus induction of human IFNβ gene expression requires the assembly of an enhanceosome. Cell **83:**1091–1100.
51. **Thiesen, H.-J., Z. Bösze, L. Henry, and P. Charnay.** 1988. A DNA element responsible for the different tissue specificities of Friend and Moloney retroviral enhancers. J. Virol. **62:**614–618.
52. **Thornell, A., B. Hallberg, and T. Grundström.** 1991. Binding of SL3-3 enhancer factor 1 transcriptional activators to viral and chromosomal enhancer sequences. J. Virol. **65:**42–50.
53. **Tsichlis, P. N., and P. A. Lazo.** 1991. Virus-host interactions and the pathogenesis of murine and human oncogenic retroviruses. Curr. Top. Microbiol. Immunol. **171:**95–171.
54. **Tupper, J. C., H. Chen, E. F. Hays, G. C. Bristol, and F. K. Yoshimura.** 1992. Contributions to transcriptional activity and to viral leukemogenicity made by sequences within and downstream of the MCF13 murine leukemia virus enhancer. J. Virol. **66:**7080–7088.
55. **Wang, S., and N. A. Speck.** 1992. Purification of core-binding factor, a protein that binds the conserved core site in murine leukemia virus enhancers. Mol. Cell. Biol. **12:**89–102.
56. **Wirschubsky, Z., P. N. Tsichlis, G. Klein, and J. Sumegi.** 1986. Rearrangement of *c-myc*, *pim*-1 and *Mlvi*-1 and trisomy of chromosome 15 in MCF- and Moloney-MuLV-induced murine T cell leukemias. Int. J. Cancer **38:**739–745.
57. **Yoshimura, F. K., B. Davison, and K. Chaffin.** 1985. Murine leukemia virus long terminal repeat sequences can enhance gene activity in a cell-type-specific manner. Mol. Cell. Biol. **5:**2832–2835.
58. **Yoshimura, F. K., and K. Diem.** 1995. Characterization of a nuclear protein binding site to a site in the long terminal repeat of a murine leukemia virus: comparison with the NFAT complex. J. Virol. **69:**994–1000.
59. **Zaiman, A. L., and J. Lenz.** 1996. Transcriptional activation of a retrovirus enhancer by CBF (AML1) requires a second factor: evidence for cooperativity with c-Myb. J. Virol. **70:**5618–5629.
60. **Zar, J. H.** 1984. Biostatistical analysis, 2nd ed. Prentice-Hall, Inc., Englewood Cliffs, N.J.