Mutation of the core or adjacent LVb elements of the Moloney murine leukemia virus enhancer alters disease specificity

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Transcriptional enhancers of replication-competent mouse C-type retroviruses are potent determinants of the distinct disease-inducing phenotypes of different viral isolates and can also strongly influence the incidence and latent period of disease induction. To study the contribution of individual protein-binding sites to viral pathogenicity, we introduced mutations into each of the known nuclear factor-binding sites in the enhancer region of the Moloney murine leukemia virus and injected viruses with these mutations into newborn NFS mice. All viruses induced disease. Viruses with mutations in both copies of the leukemia virus factor a (LVa) site, leukemia virus factor c (LVc) site, or in just the promoter proximal copy of the glucocorticoid response element (GRE) had a latent period of disease onset and disease specificity indistinguishable from that of the wild-type Moloney virus. Viruses with mutations in two or three of the GREs, in both copies of the leukemia virus factor b (LVb) site, in two of the four nuclear factor 1 (NF1) consensus motifs, or in both copies of the conserved viral core element showed a significant delay in latent period of disease induction. Strikingly, viruses with mutations in the core element induced primarily erythroleukemias, and mutations in the LVb site also resulted in a significant incidence of erythroleukemias. These and other genetic and biochemical studies suggest models for how subtle alterations in the highly conserved structure of mouse C-type retrovirus enhancers can produce a dramatic effect on disease specificity.

[*Key Words*: Moloney murine leukemia virus; transcriptional regulation; enhancer; retroviral pathogenesis]

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Replication-competent mouse C-type retroviruses comprise a large family of closely related viruses, some of which can induce leukemias and lymphomas following injection into newborn mice (for review, see Weiss et al. 1982, 1985). The mechanism of oncogenesis by nondefective mouse C-type viruses that lack their own oncogenes involves a rare integration of proviral DNA in the vicinity of one or more cellular proto-oncogenes in an appropriate target cell (Tsichlis et al. 1983, 1984, 1985; Corcoran et al. 1984; Cuypers et al. 1984; Lemay and Jolicoeur 1984; Li et al. 1984; Shen-Ong et al. 1984; Steffan 1984; O'Donnell et al. 1985; Selten et al. 1985; Weinstein et al. 1986; Mucenski et al. 1988). In most cases, critical integrations appear to perturb gene expression by placing the cellular proto-oncogene under the influence of viral transcriptional signals (Corcoran et al. 1984; Cuypers et al. 1984; Shen-Ong et al. 1984; George et al. 1986; Selten et al. 1986; Morishita et al. 1988). Aberrant gene expression presumably initiates a chain of events that leads to transformation of the infected cell.

A fascinating aspect of mouse retrovirus biology is the striking difference in the incidence and latent period of disease induction and disease specificity of even very closely related replication-competent viruses. The majority of oncogenic mouse C-type viruses induce exclusively T-cell lymphomas, but some isolates induce tumors of B cells, myeloid cells, or erythroid cells (Weiss et al. 1982, 1985). The type of tumor induced often depends on the inbred mouse strain infected, but in many cases, disease specificity is a property of the viral genome.

Genetic studies have shown that multiple viral genes contribute to the pathogenic phenotype of nondefective

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C-type viruses (Lung et al. 1983; Oliff et al. 1984; Holland et al. 1985). A particularly potent viral genetic determinant in many cases is the U3 region and, more specifically, the transcriptional enhancer of the viral LTR, which strongly influences not only the ability of a virus to induce disease but also the latent period of disease induction and, perhaps most surprisingly, the disease specificity (Chatis et al. 1983, 1984; DesGroseillers et al. 1983a,b, 1984a,b; Lenz et al. 1984; Ishimoto et al. 1985; Vogt et al. 1985; Li et al. 1987). Small differences in enhancer sequences can be sufficient to confer distinct biological properties to the virus (Ishimoto et al. 1987; Li et al. 1987; Golemis et al. 1989).

The enhancer region of nondefective mouse C-type retroviruses is usually present as a direct repeat of a sequence 50–120 nucleotides in length (Levinson et al. 1982; Laimins et al. 1984) and contains binding sites for a variety of cellular transcription factors (Speck and Baltimore 1987; Thornell et al. 1988; Boral et al. 1989; Manley et al. 1989). On the Moloney virus (or the closely related Moloney murine sarcoma virus) enhancer (See Fig. 1), there are three glucocorticoid response elements (GRE) (Yamamoto 1985; DeFranco and Yamamoto 1986; Miksicek et al. 1986), four binding sites for nuclear factor 1 (NF1) (DeVries et al. 1985; Speck and Baltimore 1987; B. Graves, pers. comm.), and two copies of a conserved viral "core" element (Weiher et al. 1983), which is a binding site for both activating protein 3 (AP-3) (Mitchell et al. 1987; Mercurio and Karin 1989) and the CCAAT/enhancer-binding protein (C/EBP; Johnson et al. 1987). Whether any of these proteins associate with the Moloney virus enhancer in cells in which the virus normally replicates in the animal is not known.

Three additional binding sites for nuclear factors, called leukemia virus factors a-, b-, and c-binding sites (LVa, LVb, and LVc sites), have been identified on the

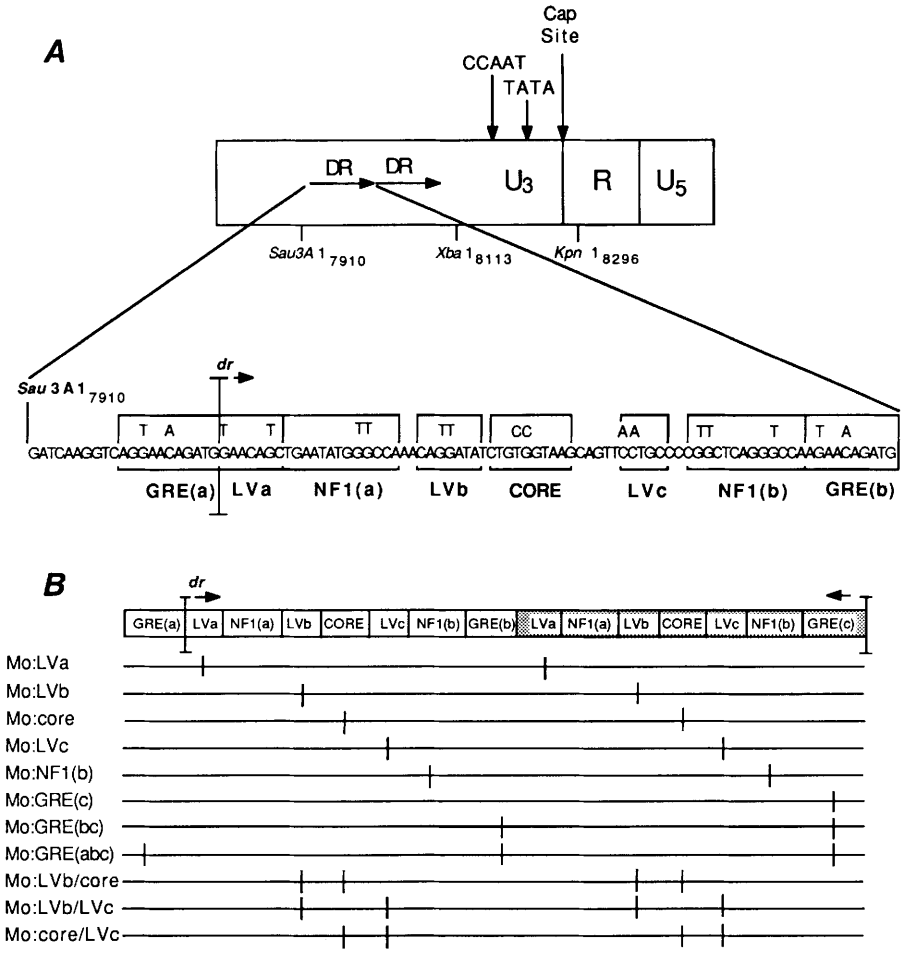

Figure 1. Mutations in the Moloney enhancer. (*A*) A schematic representation of the Moloney LTR is shown at *top*. The sequence at *bottom* of *A* represents the promoter distal copy of the direct repeat of the Moloney enhancer from the *Sau*3A1$_{7910}$ site 21 nucleotides, 5′ to the first copy of the repeat, to the 3′ end of the first copy of the direct repeat. (Numbering is relative to the cap site at the 5′ end of the Moloney viral genome; see Weiss et al. 1985.) The binding sites for nuclear factors, as defined by Speck and Baltimore (1987), are shown in boxes. The mutations are shown above the sequence; multiple base changes were introduced into each site. The notations a, b, or c [e.g., GRE(c), GRE(bc), and GRE(abc)] refer to the location of these sites within the enhancer repeat. (*B*) A diagram of the 75-bp repeat and the corresponding enhancer mutations. Stippled area indicates the position of the mutations in the enhancer for each virus.

Moloney virus enhancer by mobility shift and methylation interference assays (Speck and Baltimore 1987). Very little is known about the proteins that bind these sequences. Evidence is emerging, however, that multiple proteins can associate with many of the binding sites on the Moloney enhancer, and in some cases, both tissue-specific and ubiquitous factors can bind the same site (Speck and Baltimore 1987; N. Manley, M. O'Connell, and N. Hopkins, unpubl.).

Although many studies have addressed the effect of individual enhancer-binding proteins on overall enhancer function and tissue specificity, few have studied the role of these factors in shaping a viral disease. Mouse retroviruses provide an excellent model system to directly investigate the contribution of individual protein-binding sites to disease phenotypes. Thus, we constructed a set of mutated Moloney viruses, each with two or three point mutations in each of the six types of identified binding sites for nuclear factors on the Moloney enhancer, and tested the disease-inducing properties of the viruses.

Results

Generation of viruses with mutations in the enhancer sequence

The sequence of one copy of the Moloney virus direct repeat, its enhancer region, is shown in Figure 1A (Shinnick et al. 1981). Point mutations were introduced at two or three nucleotides in each of the identified binding sites on the Moloney enhancer by site-directed mutagenesis (Zoller and Smith 1984). In some cases, mutations were introduced into both copies of two different types of binding sites. The specific nucleotides that were mutated in each site were chosen from among those determined previously by methylation interference assays to be critical contacts for protein/DNA complex formation (Speck and Baltimore 1987).

Previous studies demonstrated that removal of one copy of the direct repeat of the Moloney enhancer dramatically increased the latent period of disease onset and attenuated the leukemogenicity of the virus (Li et al. 1987). We therefore opted to retain both copies of the direct repeat, and identical mutations were introduced into each binding site in both copies of the repeat. The nomenclature of the viruses indicates the binding site that is mutated, for example, Mo : LVb contains identical mutations in its two LVb sites. For viruses with mutations at two different binding motifs, Mo : LVb/core would designate a virus that contains mutations in both the LVb and core sites. The panel of mutated enhancers is illustrated schematically in Figure 1B. The vertical lines indicate the two or three point mutations introduced into each binding site. A more detailed description of the mutagenesis and characterization of the transcriptional activity of the mutated enhancers is reported elsewhere (Speck et al. 1990).

A 386-bp *Sau*3A1–*Kpn*I DNA fragment from U3 containing the mutated enhancer was substituted into a plasmid containing a circularly permuted Moloney virus genome, using the cloning strategy illustrated in Figure 2. The virus genome was excised from the pMo : (*) vector with *Hin*dIII, ligated to form concatamers, and transfected into NIH-3T3 cells to obtain viral stocks (Graham and Van der Eb 1973). The presence of infectious virus was determined by XC plaque assay onto transfected cells 3–4 days following transfection (Rowe et al. 1970) and by the presence of reverse transcriptase activity in culture supernatants at 12–14 days post-transfection (Baltimore 1970). All of the mutated viral DNAs produced plaques on the first XC plaque assay and reverse transcriptase activity within 12–14 days following transfection.

The structure of the viruses obtained from transfections was confirmed by Southern analyses (Southern 1975) of unintegrated DNA from Hirt supernatants (Hirt 1967) prepared from infected BALB/c 3T3 cells (data not shown). The correct size of the long terminal repeat (LTR) fragment in the viruses was confirmed by polymerase chain reaction (PCR) analysis of the Hirt DNA (Saiki et al. 1988), using probes that hybridized to highly conserved sequences at the 5′ end of U3 and in the R region to amplify a 485-bp fragment that contained the U3 sequence plus 45 bp from the R region. For some viruses, including Mo : core, the PCR-amplified U3 fragments were subcloned into pUC13, and the sequence of the enhancer from *Sau*3A1$_{7910}$ to *Xba*I$_{8113}$ was confirmed.

Latent period of disease induction by viruses with mutated enhancer sequences

Virus stocks generated from two independent molecular clones for each enhancer mutation were injected into newborn NFS mice. Because there were no significant discrepancies in the disease-inducing phenotypes of independently derived virus stocks, data for viruses obtained from independent clones containing the same mutations are pooled in Table 1 and Figure 3.

Figure 3 shows the latent period of disease induction for viruses with mutations in one type of binding site, along with previous data showing disease induction by the wild-type Moloney virus. All of the viruses were leukemogenic, but there was variability in the latent period of disease onset. Several mutations did not affect the latent period of disease onset, for example, mutation of either the LVa, LVc, or the promoter proximal GRE site [viruses Mo : LVa, Mo : LVc, Mo : GRE(c)] resulted in viruses with latent periods of disease onset indistinguishable from that of the wild-type Moloney virus. In contrast, mutation of the LVb, core, NFl(b), and either two or three GRE sites [Mo : LVb, Mo : core, Mo : NF1(b), Mo : GRE(bc), Mo : GRE(abc)] increased the average latent period of disease onset from 81 days to ~130–170 days. We note that mutation of the promoter proximal GRE site [virus Mo : GRE(c)] did not alter the latent period of disease onset compared to the wild-type Moloney virus. In contrast, mutation of two or three GRE sites extended the latent period significantly, although both types of mutations had similar effects.

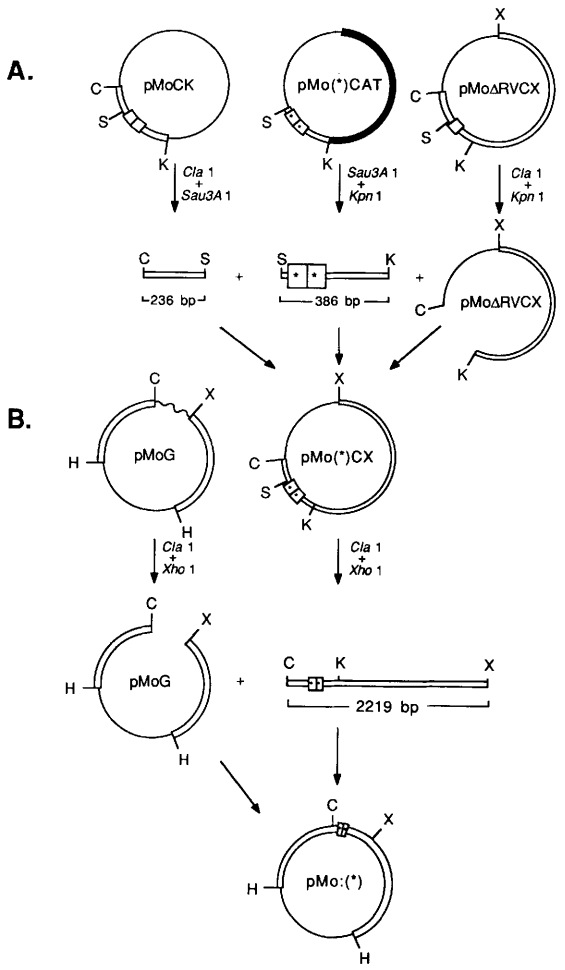

Figure 2. Subcloning strategy for viruses. White areas represent sequences from the Moloney virus genome. Black areas represent sequences from the bacterial CAT gene. Black lines represent bacterial vector DNA sequences. The DNA fragments used for subcloning were derived from the following plasmids. (pMoCK) A 3.2-kb plasmid containing the $ClaI_{7674}$–$KpnI_{8296}$ fragment from the p15E/U3/R region of the Moloney virus genome, subcloned into the *Cla*I–*Kpn*I sites of the pcD vector (Okayama and Berg 1983). [pMo(*)CAT] A 5.6-kb plasmid containing the $Sau3A1_{7910}$–$KpnI_{8296}$ fragment from the U3/R region of Moloney virus, subcloned into a pUC13-based vector with the bacterial CAT gene (Gorman et al. 1982; Speck et al. 1990). The asterisk denotes a mutation in the enhancer. [pMo(ΔRV)CX] A 4.5-kb plasmid containing the 2144-bp $ClaI_{7674}$–$XhoI_{1560}$ fragment from the Moloney virus genome, including sequences from p15E through the 5′ end of the *gag* region (including U3 sequences), subcloned into the pcD vector at the *Cla*I–*Xho*I sites. ΔRV denotes deletion of the 75-bp $EcoRV_{7957}$–$EcoRV_{8032}$ fragment containing one copy of the 75-bp enhancer repeat. (pMoG) The 8300-bp Moloney virus genome, circularly permuted at the $HindIII_{4894}$ site (in the *pol* region) and cloned into the *Hin*dIII site in the polylinker of pUC13. Moloney virus sequences have been deleted from $ClaI_{7674}$–$XhoI_{1560}$ and replaced by a *Cla*I–*Xho*I fragment from pcD to avoid any possible contamination with wild-type Moloney enhancer sequences during subcloning steps. (*A*) The $Sau3A1_{7910}$–$KpnI_{8296}$ fragments from the pMo(*)CAT constructs, containing mutations in the enhancer, were isolated and ligated along with a $ClaI_{7674}$–$Sau3A1_{7910}$ fragment from pMoCK, containing sequences in the U3 region 5′ to the direct repeat, into a *Cla*I–*Kpn*I site of the pMo(ΔRV)CX vector to generate pMo(*)CX. Successful substitution of the mutated U3 regions containing both copies of the direct repeat sequence into pMo(ΔRV)CX was confirmed by restriction digests. (*B*) The 2219-bp *Cla*I–*Xho*I fragment of the plasmid derived from the first step, pMo(*)CX, containing the mutated enhancer sequences, was isolated and ligated into the *Cla*I–*Xho*I sites of the vector pMoG to generate pMo:(*). Abbreviations: (C) *Cla*I; (K) *Kpn*I; (H)*Hin*dIII; (S) *Sau*3A1; (X) *Xho*I.

The average latent period of disease onset for viruses containing mutations in two different binding sites (Mo : LVb/core, Mo : LVb/LVc, Mo : core/LVc) is summarized in Table 1. All viruses with mutations in two binding sites are still leukemogenic.

The average latent period for lymphomas and erythroleukemias is given separately in Table 1 for viruses that induce a mixture of these types of diseases (see below). For most viruses, the latent periods for lymphomas and erythroleukemias are fairly evenly distributed on the latency curves. The latent period for lymphomas is shorter than that for erythroleukemias for Mo : core; however, in the case of Mo : LVb/core and Mo : core/LVc, this pattern is reversed. Thus, no consistent correlation can be made between average latent period and disease specificity.

Stocks of viruses containing mutations at three GRE sites or two different binding sites had an ~10-fold lower titer ($10^{6.3}$–$10^{6.5}$ PFU/ml, as measured by XC plaque assay in NIH-3T3 cells) than the other mutant viruses ($10^{7.2}$–$10^{7.6}$ PFU/ml). As found in an earlier study (Li et al. 1987), these differences in titer do not explain the differences in average latency as stocks of two mutant viruses with long latent periods, Mo : LVb and Mo : core, had titers of $10^{6.7}$ PFU/ml, and the disease latency data for wild-type Moloney virus were obtained using an input multiplicity equivalent to or less than the lowest dose of mutant virus ($10^{4.9}$ PFU/ml).

Disease specificity of viruses containing mutations in enhancer sequences

Identification of hematopoietic neoplasms was carried out according to criteria outlined previously (Chatis et al. 1984). Briefly, diagnosis of lymphoblastic lymphoma was based on gross findings of enlarged thymus, lymph nodes, or both and histological observation of diffuse infiltration with lymphoblasts of splenic white pulp, liver, thymus, and lymph nodes. Mice with erythroleukemia showed severe anemia and had enlarged spleens with erythroblast infiltration of expanded red pulp and enlarged livers with blast infiltration in sinusoids; the thymus was never enlarged and lymph nodes were normal or showed enlarged germinal centers. Although the T- or B-cell lineage of the lymphomas was not formally determined, histopathologically, the thymus appeared to be the site of origin in the great majority of cases.

Table 1. *Latent period of disease induction by wild-type and mutant Moloney viruses and relationship to disease specificity*

Virus	Number of positive/ number of inoculated[a]	Average latent period[b] (days)	Average latency[c] (days)	
			lymphoma	erythroleukemia
Moloney	44/44	81 (19)	81 (19)	—
Mo : LVa	22/22	75 (14)	75 (19)	—
Mo : LVb	36/37	149 (37)	147 (30)	155 (59)
Mo : core	19/24	162 (26)	140 (23)	170 (22)
Mo : LVc	11/11	94 (24)	94 (24)	—
Mo : NF1(b)	18/18	128 (42)	118 (43)	—
Mo : GRE(c)	10/10	84 (16)	84 (16)	—
Mo : GRE(bc)	16/16	173 (57)	137 (44)	—
Mo : GRE(abc)	20/24	147 (38)	147 (39)	146
Mo : LVb/core	19/21	198 (49)	235 (60)	187 (39)
Mo : LVb/LVc	13/15	153 (35)	156 (19)	168 (38)
Mo : core/LVc	8/13	178 (42)	212 (31)	144 (13)

[a]Number positive by 9 months postinoculation, at which point the experiment was terminated.
[b]Average latent period is calculated from animals sacrificed with disease or found dead.
[c]Any discrepancies between numbers listed under average latent period and numbers for lymphomas or erythroleukemias is attributable to occurrence of another category of hematopoietic neoplasm or to deaths without definitive diagnosis. Deaths without gross or histopathological diagnosis represented 5.5% of total. Range of latent periods is indicated in parentheses.

Table 2 shows the frequency of each type of leukemia induced by viruses containing mutations in the enhancer sequence. The most striking result was that several viruses had altered disease specificity. Mo : core induced a large percentage of erythroleukemias (61%) or mixture of lymphoblastic lymphomas and erythroleukemias (18%). Erythroleukemia is a disease that is rarely, if ever, seen with the parent Moloney virus. Mutation of the adjacent LVb site (Mo : LVb) resulted in a virus that induced a small but significant number (25%) of erythroleukemias or a mixture of lymphoblastic lymphomas and erythroleukemias (3%). Mutation of LVb + core, core + LVc, or LVb + LVc (Mo : LVb/core, Mo : core/LVc, Mo : LVb/LVc) resulted in an incidence of erythroleukemias comparable to that of viruses with mutations in either the core or the LVb sites alone.

Viruses containing mutations in the LVa, LVc, NF1(b), and GRE sites retained the disease specificity of the parent Moloney virus; Mo : LVa, Mo : LVc, Mo : NF1(b), Mo : GRE(c), Mo : GRE(bc), and Mo : GRE(abc) induced >90% lymphomas in inoculated mice. Several of these viruses, Mo : GRE(bc), Mo : GRE(abc), and Mo : NF1(b), had latent periods similar to those of Mo : core and Mo : LVb, yet their disease specificities were different and indistinguishable from wild-type Moloney virus. Therefore, the alteration of disease specificity of Mo : core and Mo : LVb, was not an indirect result of the extended latent period of disease onset.

The Mo : LVb, Mo : core, and Mo : GRE(abc) viruses were purified biologically by limiting dilution, starting with the original virus supernatants from transfected cells. These biologically cloned viruses were also injected into NFS mice, and the disease specificity was compared to that of uncloned stocks. In all cases, the disease specificity of the biologically cloned viruses was similar to that of the uncloned viral stocks [data pooled

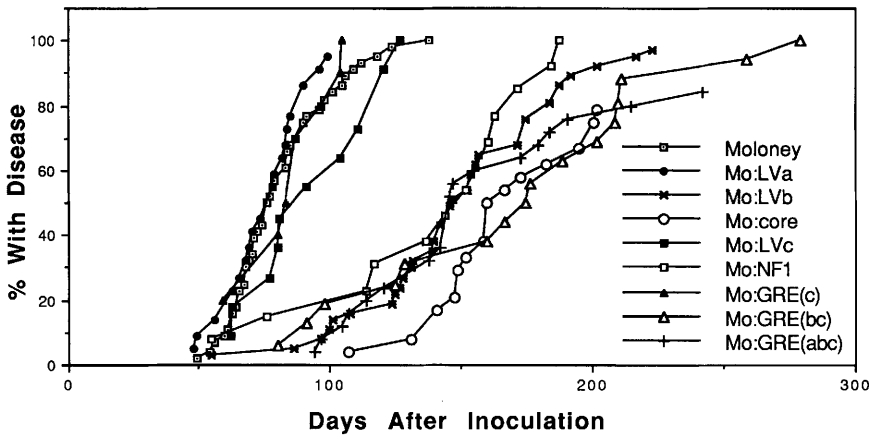

Figure 3. Leukemia induction in NFS mice by Moloney virus and viruses containing mutations in their enhancers. Leukemia induction, as a function of time, following injection of newborn mice with the wild-type Moloney virus or viruses with mutations in both copies of one type of binding site in the enhancer.

Table 2. *Types of leukemia induced by wild-type and mutant Moloney viruses*

Virus	Number of diagnosed tumors				Percent of diagnosed tumors[a]		
	lymphoma	erythroleukemia	lymphoma + erythroleukemia	other	lymphoma	erythroleukemia	other
Moloney	44	—	—	—	100	—	—
Mo : LVa	20	—	—	—	100	—	—
Mo : LVb	27	8	1	—	76	24	—
Mo : core	5	11	2	—	35	65	—
Mo : LVc	11	—	—	—	100	—	—
Mo : NF1(b)	13	—	—	1[b]	93	—	7
Mo : GRE(c)	10	—	—	—	100	—	—
Mo : GRE(bc)	9	—	—	1[c]	90	—	10
Mo : GRE(abc)	19	1	—	—	95	5	—
Mo : LVb/core	4	14	1	—	25	75	—
Mo : LVb/LVc	9	3	—	1[b]	69	23	9
Mo : core/LVc	4	4	—	—	50	50	—

[a]Percentages were calculated as the number of a given type of leukemia divided by the sum of all leukemias with histopathological diagnosis. For this purpose, mice diagnosed as having two distinct types of leukemias were scored twice.
[b]Myelogenous leukemia.
[c]Megakaryocytic leukemia.

with uncloned stocks for Mo : LVb; data not shown for Mo : core and Mo : GRE(abc)].

Discussion

We introduced a series of point mutations into known protein-binding sites in the enhancer from the Moloney murine leukemia virus and determined the effects of these mutations on the latent period of disease onset and specificity of disease induced by the virus. We found that mutations in several binding sites, the LVa, LVc, and promoter proximal GRE site, do not affect the latent period nor the disease specificity of the Moloney virus. In contrast, mutation of the LVb, core, NF1(b), or two to three GRE sites increases the latent period of disease onset significantly. Furthermore, viruses with mutations of the core element and, to a lesser extent, the LVb site induce a significant percentage of erythroleukemias. This is the first demonstration that an important determinant of viral pathogenesis can be localized to a single protein-binding site in an enhancer.

The LVb, core, NF1(b), and GRE motifs are present in the enhancers from a large number of mammalian C-type retroviruses, in the same 5′ to 3′ orientation and with essentially the same spacing between adjacent motifs as seen in the Moloney virus (Golemis et al. 1990). Highly leukemogenic viruses usually contain multiple tandem copies of short sequences within U3, and the repeated region invariably includes the LVb and core motifs and frequently the NF1 and GRE sites as well. This constellation of binding sites has been called the enhancer 'framework' for the C-type viruses (Golemis et al. 1990). We note that mutations introduced into any of these sites, LVb, core, NF1(b), or GRE, affect pathogenesis of the Moloney virus significantly.

Subtle alterations in the sequences of the conserved framework sites, as well as in the more variable regions flanking these sites, can have striking effects on disease. In the experiments reported here, mutating the core sequence (TGTGGTAA → TGCCGTAA) alters the disease specificity of the Moloney virus to erythroleukemia. Interestingly, the enhancers from several murine viruses that are generally found associated with nonthymic lymphomas, including the Rauscher, Soule, a variant of the nondefective Friend viruses, and the Rauscher and Friend mink cell focus-forming (MCF) viruses have a similar substitution in the core sequence (TGCGGT/GAA) (Clark et al. 1985; Golemis et al. 1990; M. Sitbon, pers. comm.). Thus, the highly conserved LVb site and certain core sequences may encode the enhancer determinant for the thymic disease specificity of many C-type viruses.

There are, however, viruses with core sequences identical to that found in the Moloney enhancer that nonetheless cause nonthymic lymphomas or erythroleukemias. The most well-studied example is the enhancer from the clone 57 Friend murine leukemia virus which, when substituted into the Moloney virus genome, causes the resultant recombinant virus to induce erythroleukemias (Chatis et al. 1983, 1984; Li et al. 1987). The determinants for erythroid disease specificity for the Friend enhancer have been genetically mapped to variable sequences flanking the LVb/core sequence (Golemis et al. 1989). These variable flanking sequences in the Moloney and Friend enhancers result in a distinctly different array of nuclear factor-binding sites (Manley et al. 1989). Presumably, several of the binding sites specific to the Friend enhancer are preferentially used by nuclear proteins that directly activate transcription from the Friend enhancer in erythroid cells. Alternatively, proteins that bind to sequences flanking the LVb/core motifs in the Friend enhancer may prevent access of lymphocyte-specific proteins to the LVb and core sites.

Several tissue-specific and ubiquitously expressed nuclear proteins have been characterized that bind to the core consensus sequence (Johnson et al. 1987; Mitchell

et al. 1987; Mercurio and Karin 1989). Two groups have reported a core-binding activity in nuclear extracts prepared from T cells that appears to be distinct from either AP-3 or C/EBP (Thornell et al. 1988; Serfling et al. 1989). The factor described by Serfling et al. (1989) also binds to a core consensus sequence in the interleukin-2 enhancer, in a region that contributes to the T-cell-specific transcriptional activity of the interleukin-2 gene. The thymic disease specificity of the Moloney virus may therefore be mediated by protein(s) that are important transcriptional activators of T-cell-specific genes.

We have also analyzed the transcriptional activity of the Moloney enhancer mutations in vivo, using transient expression [chloramphenicol acetyltransferase (CAT)] assays, to attempt to identify parameters that are predictors for effects on pathogenesis (Speck et al. 1990). Mutations in the LVa or LVc sites did not affect the latent period; neither was the transcriptional activity of the enhancer attenuated in vivo. We found that all of the mutations that result in viruses with an extended latent period of disease onset [LVb, core, NF1(b), and GRE(bc)] modestly attenuated (<2× to 5×) the transcriptional activity of the Moloney enhancer in T cells. Quantitation of virus-positive cells in the thymuses of infected animals is consistent with the correlation of latent period with transcriptional strength. Fewer virus-positive thymocytes could be recovered from mice infected with viruses that induced disease with extended latent periods [$10^{4.2}$–$10^{5.6}$ focus-forming units (FFU)/10^7 cells] than for viruses with latent periods similar to wild-type Moloney virus ($10^{5.9}$–$10^{6.1}$; Moloney = $10^{6.4}$ FFU/10^7 cells; N. Speck, N. Hopkins, and J. Hartley, unpubl.).

Mutation in the core motif specifically decreased transcription of the Moloney enhancer in T cells in transient CAT assays, as compared to fibroblasts or an erythroid cell line. A sequence in the Moloney virus enhancer distinct from the core site has also been found to contribute to the transcriptional activity of the enhancer in T cells (Thiesen et al. 1988). An alteration of 2 bp in the vicinity of the LVc site to the sequence found in the enhancer from the clone 57 Friend virus at that site specifically attenuates transcription from the Moloney enhancer in T cells versus erythroid cells in transient CAT assays. This mutation is distinct from the mutation that we introduced into the LVc site to generate the Mo : LVc virus. A virus constructed in a previous study [Mo : Fr(b1)] that contains the 2-bp substitution in the Moloney enhancer at the site defined by Theisen et al. (1988) to the sequence found in the Friend enhancer induced a 23% incidence of either erythroleukemia, or mixture of thymic and erythroleukemia, with an extended latent period (Golemis et al. 1989). Thus, in two cases, a T-cell-specific attenuation of transcription can be correlated with alterations in disease specificity.

Why would mutations that result in T-cell-specific attenuation of transcription enable the Moloney virus to induce leukemias in other hematopoietic lineages? The answer may lay in the organ tropism of the Moloney virus. Unlike many of the thymotropic murine leukemia viruses that replicate almost exclusively in the thymus, the Moloney virus appears to have a broader organ tropism; Moloney also replicates to high titers in the spleen and can infect multiple hematopoietic lineages (Jaenisch 1979). The Moloney virus may be capable of transforming multiple cell types, but induces thymic leukemias most efficiently. Mutations that ubiquitously attenuate transcription of the Moloney enhancer would presumably affect replication nonselectively in all hematopoietic cells, resulting in disease with an extended latent period, but maintaining specificity for T cells. Mutations that specifically attenuate transcription in T cells may allow transformation of other cell types not normally seen with the wild-type Moloney virus, because leukemias of these cell types are masked by more rapidly developing thymic leukemias. Leukemogenesis by Moloney virus is a complex, multistep process. The enhancer mutations may affect any one step (or several steps) in the disease progression.

Lately, much attention has focused on the transcriptional induction of retroviral gene expression by factors that regulate cell proliferation. Interleukin 1, tumor necrosis factor α, and phorbol esters have all been demonstrated to activate transcription from the human immunodeficiency virus enhancer, and it has been hypothesized that this would contribute significantly to pathogenesis (Nabel and Baltimore 1987; Tong-Starksen et al. 1987; Griffin et al. 1989; Osborn et al. 1989). The Moloney enhancer is also transcriptionally activated by 12-*O*-tetradecanoylphorbol 13-acetate (TPA) (Elsholtz et al. 1986), and the sequences that mediate TPA induction of the Moloney enhancer include the LVb, core, and LVc sites (Speck et al. 1990). Although mutation of the LVb and core sites has a significant effect on the latent period of disease induction and on disease specificity of Moloney virus, a virus with a mutation in the LVc site (Mo : LVc) has a latent period of disease onset and disease specificity indistinguishable from that of the parent Moloney virus. Because mutation of the LVc site does not significantly attenuate the basal level of transcription from the Moloney enhancer but completely attenuates the TPA response, these results imply that the constitutive transcriptional activity of the Moloney enhancer is a better correlate for pathogenesis than transient inducibility in response to extracellular signals.

Methods

Cells and parental virus

NIH-3T3 cells were used for DNA transfections of virus clones. BALB/c 3T3 cells were used for preparation of Hirt DNA. Both cell lines were maintained in Dulbecco's modified Eagle medium, plus 10% calf serum and 10 μg/ml penicillin plus streptomycin. The infectious DNA clone of Moloney virus was obtained from S. Goff (Columbia University).

Oligonucleotides

All oligonucleotides used for mutagenesis, PCR analysis, and sequencing were synthesized on Autogen 6500 DNA synthesizer.

Mutagenesis

Site-directed mutagenesis of the Moloney enhancer was done on a plasmid containing the *Sau*3A1$_{7910}$–*Kpn*I$_{8296}$ fragment from the U3 region of the Moloney genome (numbering of the Moloney viral genome is according to Weiss et al. 1985), which was cloned into a pUC13-based vector containing the bacterial CAT gene (pMoCAT) (Gorman et al. 1982; Speck et al. 1990). The mutagenesis has been described in detail elsewhere (Speck et al. 1990).

Constructions

The strategy for substituting the mutated enhancers in the Moloney virus genome is illustrated schematically in Figure 2. The structures of the resultant plasmids were confirmed by restriction digests, followed by electrophoresis through agarose gels. All virus cloning was done with DNA obtained from small-scale plasmid preparations to avoid possible contamination of virus or viral DNA from glassware.

Transfection of virus clones

Three independent molecular clones of each mutated virus [pMo : (*)] were transfected into NIH-3T3 cells, and viruses obtained from two of these clones were subsequently analyzed. The virus genome was excised from the vector by *Hin*dIII. The restricted DNA sample was ethanol-precipitated, and the pellet was rinsed in 70% ethanol, resuspended in 20 μl of 50 mM Tris-HCl (pH 7.6), 10 mM $MgCl_2$, 10 mM dithiothreitol, 1 mM spermidine, 1 mM ATP, and 0.1 mg/ml bovine serum albumin plus T4 DNA ligase (Collaborative Research), and incubated overnight at 15°C to form concatamers. Sheared salmon sperm DNA (8 μg) was added to the ligated samples, the DNA was ethanol-precipitated, and the pellets were rinsed in 70% ethanol, air-dried for 1 hr in a sterile hood, and resuspended in transfection buffer [25 mM HEPES (pH 7.1), 134 mM NaCl, 5 mM KCl, 5 mM glucose, 0.7 mM Na_2HPO_4] for calcium phosphate precipitations (Nelson et al. 1984). $CaCl_2$ was added with mixing to 124 mM, and the precipitate was allowed to form for 15 min before adding to cells. The $CaPO_4$ precipitates were removed 4–6 hr later, and fresh medium was added. The transfected cells were split 1 : 10 into duplicate plates 12 hr following transfection. XC plaque assays were performed directly on one such plate (Rowe et al. 1970); in all cases, XC plaques were present. The remaining plate was maintained for 2 weeks, at which time the culture supernatants were assayed for reverse transcriptase activity (Baltimore 1970). Virus was harvested after 2–3 weeks, by which time the virus had spread throughout the culture, as determined by XC plaque assay.

Mice, tumor induction, and classification of disease

Newborn (<2-day-old) NFS mice, supplied by the Small Animal Production Section of the National Institutes of Health, were inoculated with 0.04 ml of tissue culture-grown virus, representing from $10^{4.7}$ to $10^{6.2}$ PFU/mouse. Viruses from two independent molecular clones for each enhancer mutation were injected. Mice received viruses intraperitoneally and intrathymically and were sacrificed by CO_2 anesthesia when diseased. Animals were autopsied and examined for gross evidence of erythroleukemia or lymphoma. Diagnosis of erythroleukemia, lymphoma, or myelogenous leukemia was based on gross pathology and histology, as described previously (Chatis et al. 1984). For most mice, diagnosis included determination of hematocrit and microscopic examination of histological preparations. Experiments were terminated at 9 months postinoculation.

Confirmation of virus genome structure

The Mo : LVb, Mo : core, and Mo : GRE(abc) viruses were purified biologically by two or three limiting dilution titrations in NIH-3T3 cells, starting with each of the recombinant virus supernatants. These biologically cloned virus stocks were also injected into newborn NFS mice, and the resulting disease was noted and compared with those induced by uncloned stocks. As an additional control, the genomic structure of injected viruses was determined by restriction enzyme analysis and Southern blotting of Hirt supernatants (Hirt 1967; Southern 1975). This analysis was performed for viruses derived from one molecular clone for each of the enhancer mutations by using the initial subcloned stocks obtained from transfection and used for injection. The size of the U3 region in the viruses was confirmed by PCR amplification (Saiki et al. 1988) of a 485-bp fragment from the U3 region using DNA from the Hirt supernatant, followed by electrophoresis through 1.5% agarose gels with appropriate DNA molecular weight markers. The oligonucleotide primers used in the PCR reaction (5′-CCCCACCTGTAGGTTTGG-3′ and 5′-GGATACACGGGTACCCG-3′) hybridized to highly conserved sequences at the inverted repeat region at the 5′ end of U3 (nucleotides 7825–7842) and immediately 5′ to the polyadenylation signal in the R region (nucleotides 8296–8310), respectively [conserved regions were identified by sequence alignment of 35 independent C-type virus isolates (Golemis et al. 1990)]. PCR amplification was done with the kit supplied by Perkin-Elmer Cetus, following the protocol contained in the kit; amplification conditions were 1 μg Hirt DNA, 50 pM primer at 50°C for 20 cycles. The U3 fragment from Mo : core was amplified using primers that were first phosphorylated with T4 polynucleotide kinase (New England Biolabs). The amplified fragment was isolated from a low melting point agarose gel and subcloned into the *Sma*I site of pUC13 for further confirmation of the enhancer sequence. Dideoxy chain termination (Sanger et al. 1977) was done using the reagents and protocols supplied in the Sequenase kit.

Hirt supernatants were also prepared from cells that had been infected with virus reisolated from an erythroid tumor from a mouse inoculated with Mo : core. PCR amplification and sequence analysis of the enhancer from this virus from *Sau*3A1$_{7910}$ to *Xba*I$_{8113}$ confirmed the sequence of the original mutation.

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