Activation of Multiple Genes by Provirus Integration in the *Mlvi-4* Locus in T-Cell Lymphomas Induced by Moloney Murine Leukemia Virus

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Moloney murine leukemia virus-induced rat T-cell lymphomas harbor proviruses integrated near *c-myc* and near *Mlvi-1/Mis-1/Pvt-1*, another locus of common integration which maps 270 kilobases 3' of *c-myc*. In this report, we present the characterization of a new locus of common integration in Moloney murine leukemia virus-induced T-cell lymphomas (*Mlvi-4*) which maps 30 kilobases 3' of *c-myc*, between *c-myc* and *Mlvi-1*. The *Mlvi-4* locus, whose chromosomal map location is conserved in rats, mice, and humans, is also the target of chromosomal rearrangements in a variety of animal and human tumors. Evidence presented elsewhere shows that provirus integration in *Mlvi-4* enhances the expression of *c-myc* and *Mlvi-1* by *cis*-acting mechanisms operating over long distances of genomic DNA. In this manuscript, we show that provirus integration in the *Mlvi-4* locus activates, by promoter insertion, one additional gene which maps immediately 3' to the cluster of the *Mlvi-4* proviruses and which is transcribed in the same orientation as *c-myc*, giving rise to 3- and 10-kilobase mRNA transcripts. The *Mlvi-4* gene is also expressed in normal thymus and spleen at very low levels, giving rise to 3- and 5.5-kilobase messages. Although *Mlvi-4* is expressed in normal thymus, it is not expressed in Moloney murine leukemia virus-induced T-cell lymphomas corresponding to several stages of T-cell differentiation, but lacking a provirus in this locus. This suggests that *Mlvi-4* may be expressed only in a subpopulation of T cells. We conclude that provirus insertion in *Mlvi-4* activates *c-myc* and two additional genes, *Mlvi-1* and *Mlvi-4*, whose expression is restricted to, and may be developmentally regulated in, T cells. Since *Mlvi-4* is the target of genetic changes in a great variety of human and animal neoplasms, these results are critical for our understanding of oncogenesis.

The main factor in oncogenesis by retroviruses that lack an *onc* gene is insertional mutagenesis mediated by provirus integration (P. A. Lazo and P. N. Tschlis, *Curr. Top. Microbiol. Immunol.*, in press). The integration of the provirus into the cellular genome is responsible for the transcriptional activation or enhancement of neighboring genes due to promoter (19) or enhancer (21) insertion. Alternatively, provirus insertion alters the structure of the mRNA transcripts and the protein products coded by neighboring genes (20, 24, 35). In all of these cases, the integrated provirus is known to activate a single gene which is located in its immediate vicinity.

Moloney murine leukemia virus (MoMuLV), a retrovirus that lacks an *onc* gene, induces T-cell lymphomas in rats and mice. Tumor induction is associated with provirus insertion in multiple common regions of integration (Lazo and Tschlis, in press). The work we present was undertaken to determine the mechanism by which provirus integration in the *Mlvi-4* locus, a new common region of provirus insertion which maps 30 kilobases (kb) 3' of *c-myc*, may be involved in tumor induction. Our data revealed that the integration of the provirus in *Mlvi-4* enhanced, by promoter insertion, the expression of a neighboring gene which is transcribed in the same orientation as *c-myc* and is expressed at very low levels in normal thymus and spleen. Furthermore, evidence

presented elsewhere showed that provirus integration in *Mlvi-4* also activates *c-myc* (15a) and *Mlvi-1* (31, 32) by *cis*-acting mechanisms operating over long distances of genomic DNA. Provirus insertion in this locus, therefore, affects the expression of at least three genes, *c-myc*, *Mlvi-1*, and *Mlvi-4*, the first two of which are located at a significant distance from the site of integration.

MATERIALS AND METHODS

T-cell lymphomas and cell lines. The origin of the T-cell lymphomas used in this report has been described previously (32, 33). Briefly, they were induced by intraperitoneal inoculation of newborn Osborn-Mendel and Long-Evans rats with 50,000 PFU of MoMuLV. The characterization of the cell lines established from these tumors, and used in our experiments, is presented in a separate report (P. A. Lazo, A. Klein-Szanto, and P. N. Tschlis, submitted for publication).

T-cell hybridomas. The MoMuLV-induced T-cell lymphoma 6889, containing a provirus in the *Mlvi-4* locus, was adapted to grow intraperitoneally in nude mice while it lacked the ability to grow in culture. These tumor cells were fused to the hypoxanthine-guanine-phosphoribosyl transferase-negative murine T-cell lymphoma line BW5147 (8), using polyethylene glycol (7). The generated hybrid cells were selected in the presence of hypoxanthine-aminopterin-thymidine medium. Since the parental 6889 cells lacked

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the ability to grow in culture and the BW5147 cells were hypoxanthine-aminopterin-thymidine sensitive, this procedure selected for hybrid clones. After five to six passages to allow chromosomal segregation, hybrid cell DNA was analyzed by Southern blotting to determine whether individual hybrids harbored the germ line or rearranged *Mlvi-4* locus.

Isolation of cellular DNA, Southern blotting, and genomic DNA cloning. High-molecular-weight cellular genomic DNA cloning and Southern blot analysis were performed by using standard techniques as described previously (32, 33). Genomic clones of the *Mlvi-4* locus were obtained by using standard procedures (30, 33). Briefly, genomic DNA from tumor 6889 was digested with *EcoRI* and size fractionated by agarose gel electrophoresis. The fraction enriched for the desired DNA fragment was ligated to the *EcoRI* site of the bacteriophage lambda vector λ gtWES $\cdot$ λ B'. The library generated following in vitro packaging was screened with a MoMuLV long terminal repeat probe. The clone we obtained identified a new common region of provirus integration in MoMuLV-induced rat T-cell lymphomas which we named *Mlvi-4* (MoMuLV integration-4). A 0.9-kb *EcoRI*-*HindIII* cellular DNA fragment from the *Mlvi-4* clone (pLE18) was subcloned in plasmid vector pUC-18. Additional *Mlvi-4* clones were generated by screening a rat genomic DNA library with the pLE18 probe.

Isolation of polyadenylated RNA, Northern (RNA) blotting, and construction and characterization of cDNA libraries. Total cell RNA was isolated by the guanidinium thiocyanate-cesium chloride method (2). Polyadenylated RNA was selected by using affinity chromatography in oligo(dT)-cellulose (13). A 5- μ g portion of polyadenylated RNA was electrophoresed in denaturing formaldehyde-agarose gels and, following transfer to nylon membranes (Hybond-N; Amersham Corp.), hybridized to the *Mlvi-4* probe pLE18. cDNA was synthesized from 5 μ g of polyadenylated tumor cell RNA, using oligo(dT) primers and reverse transcriptase. To obtain double-stranded cDNA, we used the method of Gubler and Hoffman (10). Following the addition of *EcoRI* linkers, the cDNA was ligated to the dephosphorylated *EcoRI* arms of the bacteriophage lambda vector λ ZapII purchased from Stratagene. The constructed library was screened with *Mlvi-4* probe pLE18. The cDNA clones were analyzed by DNA sequencing, using the dideoxy chain termination method (23).

Chromosomal mapping of the *Mlvi-4* locus. The chromosomal map location of *Mlvi-4* in rats was determined by Southern blot analysis of genomic DNA from mouse/rat somatic cell hybrids segregating rat chromosomes (30). The map position of *Mlvi-4* in the mouse genome was determined by examination of the frequency of recombination among *Mlvi-4*, *c-myc*, and *Mlvi-1* in the genome of (*Mus musculus* $\times$ *Mus spretus*) $\times$ *Mus musculus* interspecific backcross mice (1a, 3, 14). Finally, the precise map position of *Mlvi-4* in the human genome was determined by Southern blot analysis of genomic DNA from mouse/human hybrid cell lines containing either an intact or a rearranged terminally deleted chromosome 8 with breakpoints precisely mapped in relation to *c-myc* (5, 6).

RESULTS

Cloning and characterization of the *Mlvi-4* locus. Tumor cell DNA isolated from a panel of 30 independent MoMuLV-induced rat T-cell lymphomas was digested with *EcoRI*; following agarose gel electrophoresis and transfer to nylon membranes, it was hybridized to a MoMuLV long terminal

repeat probe. Since *EcoRI* does not cleave the MoMuLV genome (16), each integrated provirus was detected as a single band. Furthermore, the size of the detected band depends on the size of the genomic *EcoRI* fragment within which the provirus integrated and it is independent of the precise site of provirus insertion within this fragment. Therefore, comigrating *EcoRI* bands in two independent tumors may identify common regions of provirus integration. To test this hypothesis, we cloned a 12-kb band detected with the long terminal repeat probe in tumor 6889 and comigrating with a similar size band in tumor 6890 (shown by arrow, Fig. 1A). A 0.9-kb *HindIII*-*EcoRI* genomic DNA fragment isolated from this clone (pLE18, Fig. 1C) was hybridized to *EcoRI*-digested genomic DNA from a panel of 30 MoMuLV-induced rat thymomas. This analysis revealed four tumors with a rearrangement (Fig. 1B) and defined a common region of integration which was named the *Mlvi-4* locus. The same analysis showed that the normal *Mlvi-4* locus was polymorphic. The two *Mlvi-4* alleles detected by this analysis gave rise to 3- and 3.2-kb *EcoRI* fragments. The allele giving rise to the 3.2-kb fragment was generated by the insertion of an approximately 0.2-kb element at the site indicated by the arrow (Po-1, polymorphism 1) in Fig. 1C (data not shown). Both alleles were targeted by the provirus in different tumors. The allele targeted by the provirus in three tumors (6890, 2764, and B1) was revealed by the detection of trace amounts of the unrearranged form of that allele, due to contamination of the tumor by small numbers of normal cells (Fig. 1B). To confirm these data and to extend them by determining the allele targeted by the provirus in tumor 6889, DNA from the same normal and tumor samples was digested with *KpnI* and hybridized to the pLE18 probe (Fig. 1B). No rearrangement was detected in tumors 6889 and 6890, which indicates that provirus integration in both cases had occurred outside the *KpnI* fragment. Since only the allele giving rise to the 3.2-kb *EcoRI* band contains a *KpnI* site within the *EcoRI* DNA fragment, these data confirmed the identity of the allele targeted by the provirus in these tumors. The same experiment revealed the allele targeted by the provirus and the exact site of provirus insertion in tumors 2764 and B1. To confirm the sites of provirus integration and to determine the transcriptional orientation of the provirus, the same normal and tumor cell DNA samples were digested with *HpaI*, which cleaves the MoMuLV genome asymmetrically (Fig. 1D), and they were hybridized to the pLE18 probe (Fig. 1B). A compilation of these data showing the restriction endonuclease map of *Mlvi-4*, the sites of provirus integration, and the orientation of the integrated proviruses is shown in Fig. 1C.

Chromosomal localization of the *Mlvi-4* locus. To determine whether *Mlvi-4* was related to any previously identified oncogenes or loci of common integration, we mapped its chromosomal location in rats, mice, and humans. The mapping of *Mlvi-4* in the rat genome was done by Southern blot analysis of genomic DNA isolated from mouse/rat hybrid cell lines segregating rat chromosomes (30) and hybridization to the pLE18 probe. The results presented in Table 1 indicate that *Mlvi-4* maps to rat chromosome 7, to which the rat *c-myc* gene has also been mapped (28). To determine the chromosomal map location of *Mlvi-4* in the mouse, we used an interspecific backcross mapping panel derived from crosses of C57BL/6J and *M. spretus* mice which has been typed for over 260 loci distributed among all mouse chromosomes (1a, 3, 14). Digestion of C57BL/6J and *M. spretus* genomic DNA with *BamHI* produced fragments of 17 and 5.0 kb or 22 and 5.0 kb, respectively. The *BamHI* restriction

TABLE 1. Correlation of rat chromosomes and *Mlvi-4* in 21 hybrid clones

Chromosome	No. of hybrid clones with <i>Mlvi-4</i> /chromosome retention ^a				% Discordant
	+/+	-/-	+/-	-/+	
1	3	10	8	0	43
2	10	7	1	3	19
3	8	10	3	0	14
4	9	4	2	6	38
5	4	10	7	6	33
6	4	7	7	3	48
7	11	9	0	1	5
8	2	10	9	0	43
9	3	10	8	0	38
10	6	7	5	3	38
11	7	7	4	3	33
12	7	8	4	2	29
13	11	6	0	4	19
14	4	8	7	2	43
15	7	9	4	1	24
16	8	3	3	7	48
17	8	9	3	1	19
18	8	4	3	5	40
19	7	10	4	0	19
20	3	10	8	0	38
X	11	4	0	6	29

^a +/+, Hybrids containing *Mlvi-4* and the indicated chromosome; -/-, hybrids lacking *Mlvi-4* and the indicated chromosome; +/-, hybrids containing *Mlvi-4* and lacking the indicated chromosome; -/+, hybrids lacking *Mlvi-4* and containing the indicated chromosome.

fragment length polymorphism was used to follow the segregation of *Mlvi-4* alleles in 151 (C57BL/6J × *M. spretus*) F₁ × C57BL/6J backcross mice. No recombinants were observed among *Mlvi-4*, *c-myc*, and *Mlvi-1* in any backcross animals analyzed. This placed *Mlvi-4* on mouse chromosome 15 with 2.0 centimorgans (upper 95% confidence interval) of these two loci.

On the basis of these results, we predicted that the *Mlvi-4* locus would map to human chromosome 8 in close proximity to the *c-myc* protooncogene. To test this prediction, DNA from several human tumor cell lines including Colo320, which has an amplified *c-myc* gene (1), was hybridized to the pLE18 probe. The detection of an amplified *Mlvi-4* locus in Colo320 (see Fig. 3A, lane 5) suggested that, indeed, *Mlvi-4* maps to human chromosome 8 in close proximity to *c-myc*.

We then determined the precise map position of *Mlvi-4* relative to *c-myc* in rats, mice, and humans. To this end, we cloned a DNA fragment which extends approximately 15 kb 3' of the rat *c-myc*. A subclone (pM3E/B), derived from the distal end of this clone, was hybridized to *HpaI*-digested DNA from normal rat and three tumors containing a provirus in *Mlvi-4* (6889, 6890, and 2764). The same blot, following removal of the probe, was hybridized to the *Mlvi-4* probe,

pLE18. The results in Fig. 2A₁ and 2A₂ revealed that the pLE18 and pM3E/B probes detected identical size *HpaI* fragments in normal rat DNA. Furthermore, both probes detected rearranged *HpaI* fragments. The sum of the sizes of the rearranged fragments in each tumor equaled the size of the unrearranged fragment plus 6.3 kb (Fig. 1D), confirming that the two probes detected the same *HpaI* DNA fragment. To determine the precise map position of *Mlvi-4* in the mouse, a cosmid clone (*c-Myc-1*) containing mouse genomic DNA sequences extending to approximately 30 kb 3' of *c-myc* (4) was digested with *EcoRI*, *BamHI*, or *SacI* and it was hybridized to the pLE18 probe. The results in Fig. 2B allowed us to map *Mlvi-4* approximately 30 kb 3' of *c-myc* (Fig. 2D). To determine the precise map position of *Mlvi-4* in the human genome, a 3-kb *EcoRI* fragment containing sequences homologous to the pLE18 probe was cloned from the human cell line Colo320, which contains amplified *Mlvi-4* sequences. An *EcoRI*-*BamHI* subclone, which was free of repeated DNA sequences, was hybridized to *HindIII*-digested genomic DNA from several human cell lines containing normal or amplified *Mlvi-4* sequences. The same probe was hybridized to *HindIII*-digested DNA from several mouse/human hybrid cell lines containing either an intact or a rearranged terminally deleted chromosome 8 with breakpoints at positions precisely mapped in relation to *c-myc* (5, 6). The results (Fig. 3A) allowed us to map *Mlvi-4* in the position shown in Fig. 3B.

Effects of provirus integration in the *Mlvi-4* locus on transcription of neighboring and distant genes. Provirus integration in the *Mlvi-4* locus enhances *c-myc* and *Mlvi-1* expression (1a, 31). To determine whether, in addition to its long-range effects on *c-myc* and *Mlvi-1* expression, provirus insertion in *Mlvi-4* affected the expression of another neighboring gene, polyadenylated RNA from a panel of MoMuLV-induced T-cell lymphomas was electrophoresed in denaturing formaldehyde agarose gels and, following transfer to nylon membranes, hybridized to the pLE18 probe. The results in Fig. 4A indicate that all tumors containing a provirus in the *Mlvi-4* locus, and only these tumors, express two RNA transcripts 3 and 10 kb long, hybridizing to the pLE18 probe. The two transcripts were not detected in tumors containing a provirus in the *Mlvi-1* locus (2780 and 2788) and several T-cell hybrid lines between the tumor 2780 and the murine T-cell lymphoma line BW5147 segregating the normal from the rearranged *Mlvi-1* allele. Furthermore, the *Mlvi-4* transcripts were also not detected in the cell line LE3Spl, which expresses high levels of *Mlvi-1* (31). To determine the orientation of transcription, orientation-specific RNA probes, generated by *in vitro* transcription of pLE18 subcloned in plasmid pGEM3Z (Promega), were hybridized to RNA from tumor B1. The results in Fig. 4B, showing that only one of the two orientation-specific probes hybridized, revealed that *Mlvi-4* is transcribed in the same orientation as the integrated provirus and the *c-myc* protoon-

FIG. 1. Cloning and characterization of the *Mlvi-4* locus. (A) Southern blot analysis of genomic DNA from tumors 6889 (lanes A and B) and 6890 spleen and thymus (lanes C and D) following digestion with *EcoRI* and hybridization to the MoMuLV long terminal repeat probe. The arrows on the left correspond to the migration of *HindIII*-digested phage lambda DNA. The non-numbered arrow indicates the DNA fragment we cloned. (B) Southern blot analysis of DNA from two normal rats (A and B) and tumors 6889 (C), 6890 (D), 2764 (E), and B1 (F) digested with *EcoRI* (left panel), *KpnI* (middle panel), and *HpaI* (right panel) and hybridized to the *Mlvi-4* probe pLE18. The faint bands in lanes D and E in the *EcoRI* and *HpaI* panels were not seen reproducibly in other experiments, and they were interpreted to represent partial digests. (C) Restriction endonuclease map of the *Mlvi-4* locus indicating the sites in which insertion of repeated DNA sequences gives rise to DNA polymorphisms (Po-1 and Po-2) (upper) and the sites of provirus integration and the orientation of the integrated proviruses (lower). (D) Restriction endonuclease map of MoMuLV indicating the sites of *KpnI* and *HpaI* cleavage. Code for restriction endonucleases: E, *EcoRI*; K, *KpnI*; Hp, *HpaI*; B, *BamHI*; S, *SacI*; H, *HindIII*.

FIG. 2. Determination of the distance between *c-myc* and *Mlvi-4* in rats and mice. (A₁) Southern blot analysis of *Hpa*I-digested genomic DNA from normal rat thymus and tumors 2764, 6889, and 6890 and hybridization to the *Mlvi-4* pLE18 probe. (A₂) The same blot hybridized to the pM3E/B probe. (B) Southern blot analysis of cosmid *c-Myc1* DNA digested with *Bam*HI, *Eco*RI, and *Sac*I and hybridized to the *Mlvi-4* probe pLE18. (C) Restriction endonuclease map of the DNA region linking *c-myc* to *Mlvi-4* in the rat. The dotted line represents a region which has not been cloned. The arrows marked Po-1 and Po-2 show the sites in which the insertion of repeated DNA sequences has produced restriction fragment length polymorphisms. (D) Restriction endonuclease map of the DNA region linking *c-myc* to *Mlvi-4* in the mouse. Code for restriction endonucleases: E, *Eco*RI; K, *Kpn*I; Hp, *Hpa*I; B, *Bam*HI; S, *Sac*I; H, *Hind*III.

FIG. 3. Determination of the distance between *c-myc* and *Mlvi-4* in humans. (A) DNA ($\sim 10 \mu\text{g}/\text{lane}$) from a mouse cell line (lane 1), the human T-cell line Jurkat (lane 2), the human lymphoblastoid cell line GM1500 (lane 3), HeLa cells (lane 4), Colo320 cells (lane 5), a rodent/human hybrid retaining human chromosome 8 and other chromosomes (lane 6), a rodent/human hybrid retaining human chromosome 8 only (lane 7), a hybrid retaining 8q24 $\rightarrow$ 8qter (from 5' of *c-myc* to qter) (lane 8), a hybrid retaining chromosome 8 carrying the amplified *c-myc* region from HL60 (lane 9), a hybrid carrying an 8q⁺ (8pter $\rightarrow$ 8q24, to $>50 \text{ kb}$ 3' of *c-myc*) from PA682 (lane 10), a hybrid also carrying an 8q⁺ from PA682 (lane 11), a hybrid with an 8q⁺ (8pter $\rightarrow$ 8q24, to approximately 32 kb 3' of *c-myc*) from JI (lane 12), a hybrid with an 8q⁺ (8pter $\rightarrow$ 8q24, to approximately 10 kb 3' of *c-myc*) from BL2 (lane 13), and two hybrids which do not retain chromosome 8 (lanes 14 and 15) was cleaved with *HindIII*, electrophoresed, transferred to nitrocellulose, and hybridized to the radiolabeled human *Mlvi-4* probe. (B) Map position of the *Mlvi-4* locus within the region 3' of *c-myc* in humans. The restriction endonuclease map of this region has been published (29). Code for restriction endonucleases: E, *EcoRI*; K, *KpnI*; Hp, *HpaI*; B, *BamHI*; S, *SacI*; H, *HindIII*.

cogene (Fig. 4C). To assess the expression of *Mlvi-4* in normal cells, polyadenylated RNA from six normal rat tissues was hybridized to the pLE18 probe. Furthermore, to confirm that *Mlvi-4* expression in tumors carrying a provirus in this locus was the direct result of provirus integration, polyadenylated RNA from the rat and mouse T-cell lymphomas 6889 and BW5147 and from four hybrid lines between these tumors was hybridized to the pLE18 probe. The results of these experiments are shown in Fig. 5. Only the thymus and, to a lesser degree, the spleen of normal animals expressed low levels of *Mlvi-4* RNA transcripts. One of these transcripts is approximately 3 kb long and comigrates with a similar size transcript in tumors carrying a provirus in the *Mlvi-4* locus. The same normal tissues expressed an additional 5.5-kb RNA transcript which is unique to normal cells and lacked the tumor-specific, 10-kb RNA transcript. The results in Fig. 5 also confirm that the expression of *Mlvi-4* in tumors carrying a provirus in this locus is the direct result of the integration of the provirus. Hybrid cells between the tumor cell lines 6889 and BW5147 express *Mlvi-4* when they carry the rearranged locus (Hy4-E9R and Hy4-D4R), and they are negative for *Mlvi-4* expression when they carry the nonrearranged *Mlvi-4* allele (Hy4-D1G and Hy4-E14G). The segregation of the normal from the rearranged *Mlvi-4* allele in these hybrids was presented in a separate report (15a). It should be noted that the expression of *Mlvi-4* correlates with the expression of *c-myc* in the same hybrid lines (15a). Furthermore, evidence presented elsewhere shows that provirus insertion in *Mlvi-4* also affects the expression of *Mlvi-1* (31) approximately 240 kb 3' to the *Mlvi-4* locus (12). Therefore, provirus insertion in the *Mlvi-4*

locus affects the expression of at least three genes within a 300-kb chromosomal domain (Fig. 6). *Mlvi-4* cDNA clones were isolated from a cDNA library constructed from tumor B1 polyadenylated RNA. DNA sequence analysis of one clone revealed that the *Mlvi-4* gene is unrelated to any known genes and that at least one of the *Mlvi-4* mRNA transcripts (most likely the 10-kb, tumor-specific one) is a hybrid containing viral and cellular sequences. The splicing of the viral to the *Mlvi-4* sequences utilized a cryptic splice donor site at position 6397 in the *env* gene of MoMuLV (Fig. 6 and 7).

DISCUSSION

The *c-myc* protooncogene and the chromosomal region extending at least 300 kb 3' of this gene are targeted by gross chromosomal aberrations in a variety of human and animal tumors. These aberrations include chromosomal translocations observed in Burkitt's lymphomas, human T-cell lymphotropic virus type I-induced T-cell lymphomas and murine plasmacytomas (9, 11, 17, 29), and DNA amplifications observed in a great variety of human and animal neoplasms (1). In the case of translocations, the chromosomal break-points, similarly to provirus insertions, cluster in distinct loci within this region. Two of these loci correspond to the loci of common integration *Mlvi-1/Mis-1/Pvt-1* (15, 34) and *Mlvi-4* (12). Chromosomal translocations as well as provirus insertions in these loci enhance the expression of *c-myc* by a *cis*-acting mechanism (11, 15a). However, because of the long distance between *c-myc* and these loci, we asked whether any additional genes mapped between them and

FIG. 4. Expression of *Mlvi-4* in MoMuLV-induced T-cell lymphomas. Northern blot analysis of tumor cell RNA from tumors which contain a provirus in the *Mlvi-4* locus (6890, 2764, 6889, and B1) and tumors which contain *Mlvi-4* in the germ line configuration (all others). Tumors 2780 and 2788 contain a provirus in *Mlvi-1*, and cell lines Hy1-7G, Hy1-19R, Hy1-22R, and Hy1-24R represent T-cell hybrids between 2780 and the murine T-cell lymphoma line BW5147 which are known to segregate the rearranged from the normal *Mlvi-1* alleles (15a). Tumors 2775, 2779, and LE3Sp contain a provirus in *Mlvi-2*. Note that tumors carrying a provirus in *Mlvi-1* and the LE3Sp cell line transcribe *Mlvi-1* but not *Mlvi-4*. In addition to the main transcripts in all tumors expressing *Mlvi-4*, several minor transcripts were also observed. The molecular structure of these transcripts is not known. All lanes were loaded with 5 μ g of polyadenylated RNA. The RNA was transferred on nylon membranes (Hybond-N; Amersham), and it was cross-linked on the membrane by UV light.

whether provirus integration affected their expression. Our data revealed that this region contains at least two genes in addition to *c-myc*. Evidence presented in an earlier manuscript showed that a probe derived from a region immediately 5' to the cluster of *Mlvi-1* proviruses, 270 kb 3' to *c-myc*, detects an RNA transcript which is expressed only in tumors carrying a provirus in this region and in a CD4⁺ CD8⁺ tumor cell line with no detectable provirus in these loci (31). In this manuscript, we present evidence that provirus insertion in the *Mlvi-4* locus results in the expression of two *Mlvi-4* mRNA transcripts, 3 and 10 kb long, which are transcribed in the same orientation as *c-myc* from the region 3' to the provirus. Low-level expression of the *Mlvi-4* gene, giving rise to 3- and 5.5-kb mRNA transcripts, was detected in normal thymus and spleen but not in testis, brain, kidney, and liver. Although *Mlvi-4* was expressed in normal thymus, it was silent in all of the MoMuLV-induced rat T-cell lymphomas, lacking a provirus in this locus. Since these lymphomas correspond to multiple stages in T-cell development (Lazo et al., submitted), these data suggest that

Mlvi-4 may be expressed only in a subpopulation of T cells. Therefore, contrary to *c-myc*, the expression of both the *Mlvi-1* (31) and *Mlvi-4* genes appears to be restricted to subpopulations of T cells. This suggests that both genes may be developmentally regulated during T-cell differentiation. Although it has not been shown formally, *Mlvi-1* and *Mlvi-4* appear to represent two different genes. This is based on the finding that *Mlvi-1* and *Mlvi-4* generate different size RNA transcripts, and they are expressed in different normal and tumor tissues. Finally, cDNA clones of these genes available to date do not cross-hybridize.

Shtivelman et al. presented evidence recently for a hybrid RNA transcript between *c-myc* and additional sequences located 3' of *c-myc* (25, 26). This transcript was expressed in Colo320, a cell line with rearranged and amplified *c-myc*, *Mlvi-1*, and *Mlvi-4* loci. Transcripts of sequences derived from the region 3' of *c-myc* were easily detected in other tumor cell lines carrying DNA amplifications in this region (25). The first exon of these transcripts was located 57 kb 3' of *c-myc* and 30 kb 3' of the first recognized exon of *Mlvi-4*

FIG. 5. Northern blot analysis of polyadenylated RNA from normal tissues, a tumor carrying a provirus in the *Mlvi-4* locus (6889), the murine T-cell lymphoma line BW5147, and T-cell hybrids between 6889 and BW5147 segregating the rearranged from the normal *Mlvi-4* alleles. Hybrids Hy4-E9R and Hy4-D4R contain the rearranged allele, while hybrids Hy4-D1G and Hy4-E14G contain the normal allele.

(26). Tumor cell lines carrying rearranged but not amplified *c-myc*, such as the Burkitt's lymphoma line Daudi, and a variety of other tumor cell lines with no chromosomal rearrangements in this region express invariant low amounts

FIG. 6. Provirus integration in the *Mlvi-4* locus activates *Mlvi-4* by promoter insertion and *c-myc* and *Mlvi-1* by *cis*-acting mechanisms operating over long distances of genomic DNA. The orientation of the arrows indicates the orientation of transcription. Since the orientation of transcription of *Mlvi-1*, relative to *c-myc* and *Mlvi-4*, is not known, the *Mlvi-1* transcript is shown by a bar. The *Mlvi-4* transcript is shown as a hybrid between viral and cellular sequences. The splice donor site used for splicing the viral to the cellular sequences has been mapped in the MoMuLV *env* gene (Fig. 7). The structure of the viral sequences upstream from this site has not been determined.

of a main 4.8-kb transcript and several additional larger and smaller minor transcripts (25). Mouse cells also express indiscriminately low levels of a 4.8- and a 0.5-kb transcript. The size of these transcripts, which is different from the size of the transcripts we detected with the *Mlvi-4* probe, and the invariant pattern of their expression, which is in sharp contrast to the cell-type-specific pattern of expression of *Mlvi-4*, suggest that *Mlvi-4* and the gene defined by Shtivelman et al. are not the same, although they may overlap. Comparison of the cDNA sequences defining these genes should give a definitive answer to the question of their relationship.

The deregulation of multiple neighboring and distant genes following provirus integration may not be a unique property of the *Mlvi-4* locus. Provirus insertion in the *Mlvi-1* locus is also associated with the activation of at least two genes, one of which (*c-myc*) is located approximately 270 kb from the site of provirus integration (12, 15a). Other tumors characterized by clustering of provirus insertions in multiple tightly linked loci may provide additional examples of this phenomenon. Thus, in mouse mammary tumor virus-induced mammary adenocarcinomas, provirus insertion frequently occurs in the vicinity of *Int-2* and *Hst*, two fibroblast growth factor-related genes located within 17 kb of one another (22). Indeed, it was shown recently that, in a small fraction of the tumors carrying a provirus in this region, both genes are activated (22). Two additional tightly linked loci of common integration include *Evi-1* and *Fim-3* which are targeted by provirus insertion in murine myeloid neoplasms (18, 27). The rules that regulate the activation of multiple genes by a single integrated provirus are not known at the present time. It is interesting, for example, that the interaction between *Mlvi-4* and *Mlvi-1* is not reciprocal. Thus, provirus integration in *Mlvi-4* activates *Mlvi-1* (31), while provirus integration in *Mlvi-1* does not affect *Mlvi-4* expression (Fig. 4). Understanding these rules may provide clues on the mechanism of these phenomena.

The identification of multiple genes in close cytogenetic distance to *c-myc* and the ability of the integrated provirus to influence the expression of more than one of them simultaneously provide the biological setting to study the complexity of the effects of provirus integration on gene expression. Furthermore, these experiments provide information which will be critical in understanding the role of provirus integration or chromosomal translocation in the *c-myc*, *Mlvi-4*, and *Mlvi-1* loci in the induction of animal and human tumors.

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1050 TTACTGGAAGCCCTCCTCATCATGGGATTTCATCACAGTAAACAACAATC 1001 cDNA
      |||
6325 TTACTGGAAGCCCTCCTCATCATGGGATTTCATCACAGTAAACAACAATC 6374 MoMuLV
      |||
1000 TCACCTCTGACCAGGCTGTCCAGGATTCTCCTCATGGTTTGTCTGAAGGTC 951 cDNA
      |||
6375 TCACCTCTGACCAGGCTGTCCAGGATGCAAGATAATAAGTGGTGCAAC 6424 MoMuLV
      |||
      splice donor

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FIG. 7. Splicing of viral and cellular sequences in RNA transcripts derived from the activated *Mlvi-4* locus. DNA sequence analysis of a cDNA clone derived from tumor B1 showing the junction between viral (upper line) and cellular sequences. Splicing occurs at a cryptic splice donor site at the *env* gene of MoMuLV. The potential use of additional splice donor sites is under investigation.

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