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Genetics

Activation of the *Mlvi*-1/*mis*1/*pvt*-1 locus in Moloney murine leukemia virus-induced T-cell lymphomas

(thymic lymphoma/common region of provirus integration/chromosomal translocation/amplification/oncogenesis)

PHILIP N. TSICHLIS*, BARBARA M. SHEPHERD, AND SUSAN E. BEAR

Department of Medicine, Fox Chase Cancer Center, Philadelphia, PA 19111

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ABSTRACT **The *Mlvi*-1/*mis*-1/*pvt*-1 locus, located ≈270 kilobase pairs 3′ of the c-*myc* protooncogene, was originally discovered as a common region of provirus integration in Moloney murine leukemia virus-induced rat T-cell lymphomas. The same locus was shown subsequently to be coamplified with c-*myc* and to be involved in chromosomal translocations in a variety of human and animal neoplasms. Provirus integration in *Mlvi*-1 in Moloney murine leukemia virus-induced rat T-cell lymphomas activates the c-*myc* protooncogene. The studies reported here were aimed to determine whether, in addition to the activation of c-*myc*, provirus integration affected the expression of other neighboring genes. Provirus integration was shown to occur in three clusters separated by regions of uninterrupted DNA. The proviruses in all three clusters had integrated in a single-transcriptional orientation, and they appeared intact. Systematic hybridization of *Mlvi*-1 clones to rat, mouse, and human genomic DNA revealed three patches of evolutionarily conserved sequences. Two of them were mapped in regions targeted by the provirus, and the third was mapped immediately 5′ to the provirus clusters. A probe derived from the conserved sequences 5′ of the integrated proviruses detected a tumor-specific RNA transcript in tumors carrying a provirus in *Mlvi*-1 or in the neighboring *Mlvi*-4 and c-*myc* loci. The highest level of RNA transcript expression, however, was seen in a CD4$^+$ CD8$^+$ tumor cell line that was not carrying a provirus in this region. We conclude that provirus insertion in this region activates both c-*myc* and another gene that is located in the immediate vicinity of the integrated *Mlvi*-1 proviruses and may be developmentally regulated in T cells.**

Tumor induction and progression by retroviruses that lack an oncogene depend on provirus integration in particular genomic DNA regions (1–3); the importance of provirus insertion was deduced from the fact that these regions were either known oncogenes (1) or they served as substrates for provirus integration in multiple tumors (2, 3).

The integrated provirus influences tumor induction by deregulating the expression of neighboring genes. This influence occurs in a variety of ways. Originally it was thought to be the result of promoter insertion (1, 4–6). However, it was subsequently shown that in many cases the deregulated gene was transcribed in an orientation opposite to that of the integrated provirus, indicating that deregulation could be the result of enhancer insertion (7–10). In contrast to the cases where the deregulated gene remains intact, there are additional instances in which the cellular gene is truncated as a result of proviral insertion. The normal and truncated (or hybrid) proteins may differ in their ability to induce transformation. Examples of this phenomenon include the activation of c-*erb*^{B} in avian leukosis virus-induced erythroblastosis (11) and c-*myb* in several murine myeloid cell lines (12, 13).

At least 15 common regions for provirus integration have been identified in murine retrovirus-induced hematopoietic neoplasms (14). However, only a small number of these common integration events have been shown to affect the expression of neighboring cellular oncogenes. The first common proviral integration detected in rodent hematopoietic neoplasms induced by murine retroviruses occurred in a locus that was named *Mlvi*-1 [Moloney leukemia virus integration locus (Mo-MuLV) 1] (3). Two additional independently identified loci, one of them a locus of common integration (*mis*-1) (15) and the other a locus involved in the 6;15 variable translocation in mouse plasmacytomas and similar translocations in human lymphoid neoplasms (*pvt*-1) (16–18), were shown recently to be identical to *Mlvi*-1 (36). In three species, rats, mice, and humans, the *Mlvi*-1/*mis*-1/*pvt*-1 locus has been mapped 3′ of c-*myc* in close cytogenetic distance from this protooncogene (refs. 16–19 and unpublished data). In humans this distance has been shown to be at least 270 kilobase pairs (kbp) (ref. 18; M. Lipp, personal communication). Amplification of c-*myc* is frequently associated with amplification of the *Mlvi*-1/*mis*-1/*pvt*-1 locus in a variety of human and animal neoplasms (20, 21).

To understand the functional role of the *Mlvi*-1 locus in oncogenesis we conducted studies that revealed that provirus integration in this locus had a long-range *cis* effect on the expression of c-*myc* (P. Lazo, J. Lee, and P.N.T., unpublished data). This finding, however, did not exclude the possibility that the integrated provirus may have activated an additional gene in its immediate vicinity. Intensive studies conducted in many laboratories and aiming at the identification of a transcriptional unit in the vicinity of the *Mlvi*-1/*mis*-1/*pvt*-1 locus had been negative to the present time. This report presents evidence for a tumor-specific RNA transcript whose expression depends on provirus integration in *Mlvi*-1, c-*myc*, or *Mlvi*-4, another common region of provirus insertion that maps ≈30 kilobases (kb) 3′ of the c-*myc* protooncogene (P. Lazo, J. Lee, and P.N.T., unpublished data). Because provirus insertion in c-*myc*, *Mlvi*-1, and *Mlvi*-4 exerts a long-range effect on the expression of the c-*myc* protooncogene (P. Lazo, J. Lee, and P.N.T., unpublished data) we conclude that provirus integration in these loci may be responsible for effects on the expression of multiple neighboring genes.

MATERIALS AND METHODS

T-Cell Lymphomas. The origin of the Mo-MuLV-induced T-cell lymphomas used in these experiments has been described (refs. 3 and 22). They were induced by i.p. injection

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Abbreviations: Mo-MuLV, Moloney murine leukemia virus.

*To whom reprint requests should be addressed at: Fox Chase Cancer Center, 7701 Burholme Avenue, Philadelphia, PA 19111.

of newborn Osborn–Mendel, Long–Evans, and Fischer 344 rats with 50,000 XC plaque-forming units (pfu) of Mo-MuLV. Normal tissues were obtained from 4- to 5-month-old uninjected rats. Cell lines established from these tumors and their characteristics will be described in a separate report (P. Lazo and P.N.T., unpublished data).

Preparation of Cellular DNA and Southern Blotting. Genomic DNA was prepared by using standard procedures as described (3). Southern blot analyses were done by using standard procedures as described (3). In the experiments addressing the evolutionary conservation of the *Mlvi*-1 locus, cloned rat *Mlvi*-1 DNA was hybridized to filter-immobilized genomic DNA from various species under the following reduced stringency conditions: 40% (vol/vol) formamide, 6× SSC (1× SSC is 0.15 M sodium chloride/0.015 M sodium citrate)/20 mM sodium phosphate, pH 6.5/5× Denhardt's solution (1× Denhardt's solution is 0.02% polyvinylpyrrolidone/0.02% Ficoll/0.2% bovine serum albumin)/sonicated salmon sperm DNA at 200 μg/ml/10% (wt/vol) dextran sulfate at 37°C for 16–24 hr. Filters were first washed several times in 2× SSC and 0.5% SDS at room temperature; the final wash was in 1× SSC/0.1% SDS at 65°C for 30 min.

Isolation of Polyadenylylated RNA and Northern (RNA) Blotting. Total cell RNA was isolated from normal and tumor tissues and cell lines using the guanidinium thiocyanate/cesium chloride method (23). Polyadenylylated RNA was selected by affinity chromatography in oligo(dT) cellulose (24). Five micrograms of polyadenylylated RNA was electrophoresed in denaturing formaldehyde/agarose gels, and after transfer to nylon membranes (25) this RNA was hybridized to random-primed rat *Mlvi*-1 probes under the following high-stringency conditions: 50% formamide/5× SSC/20 mM sodium phosphate, pH 6.5/5× Denhardt's solution/sonicated salmon sperm DNA at 10 mg/ml/10% dextran sulfate/0.5% SDS at 42°C for 16–24 hr. Filters were washed in 2× SSC/0.1% SDS at room temperature and then in 0.1× SSC/0.1% SDS at 65°C for at least 60 min. To confirm the presence of equal amounts of RNA in each lane the same filters were hybridized to a mouse actin probe (26).

RESULTS

Nature of DNA Rearrangements in the *Mlvi*-1 Locus. The restriction endonuclease map of ≈30 kbp of cloned *Mlvi*-1 sequences was determined by single and double enzyme digestions, electrophoresis in agarose gels, and hybridization to *Mlvi*-1 probes (Fig. 1*D*). Based on this map normal rat and tumor-cell DNA was digested with *Sac* I or *Kpn* I, which cleave the Mo-MuLV long-terminal repeat, and after agarose gel electrophoresis and transfer to nylon membranes, this DNA was hybridized to probes B, D, and F (Fig. 1; data not shown). This analysis revealed that 40% of the Osborn–Mendel rats, 20% of the Long–Evans rats, and 25% of the Fischer 344 rats contained a provirus in this locus. The same analysis allowed us to localize the integrated *Mlvi*-1 proviruses within three clusters separated by uninterrupted cellular sequences (Fig. 1*D*). The next step was to determine the orientation of the integrated proviruses in both *Mlvi*-1 clusters. Based on the *Mlvi*-1 restriction endonuclease map and on the localization of the integrated proviruses within this map, tumor-cell DNA from groups of tumors was digested with *Hpa* I, *Pst* I, or *Bam*HI, and it was hybridized to probes F and D or B, respectively. Because *Hpa* I, *Pst* I, and *Bam*HI cleave the Mo-MuLV genome asymmetrically (Fig. 1*E*), this analysis allowed us to determine the transcriptional orientation of the provirus. A representative sample of the results shown in Fig. 1 *A*, *B*, and *C* indicates that the transcriptional orientation of the integrated provirus was the same in all tumors.

The provirus integrated in the genomic DNA of two thymic lymphomas (A2 and A7) was cloned. Restriction endonuclease digestion of the cloned DNA revealed that the integrated provirus was intact, and its transcriptional orientation was that predicted from Southern blot analysis of genomic tumor-cell DNA. Transfection into NIH 3T3 cells revealed that both provirus clones were biologically active and identical to Mo-MuLV (data not shown).

Evolutionary Conservation. Normal rat, mouse, and human DNA as well as genomic DNA from two human colon carcinoma cell lines carrying an amplified c-*myc* gene (Colo228 and Colo320) was digested with *Eco*RI, and the DNA was hybridized systematically under conditions of reduced stringency to single-copy DNA probes representing all the cloned sequences of the rat *Mlvi*-1 locus. Because Colo228 and Colo320 carry large amplification units extending to at least 300 kbp 3′ to the c-*myc* protooncogene (20, 21), these cell lines were used to determine the authenticity of hybridization between the rat and human *Mlvi*-1 loci. This analysis revealed three regions that were conserved between rats and humans (Fig. 2); two of these map within the two 3′ clusters of the *Mlvi*-1 proviruses, whereas the third maps upstream of all the known provirus insertions. A probe from the upstream conserved region (probe A) was hybridized to *Eco*RI-digested genomic DNA derived from mouse, hamster, rabbit, dog, cat, and chicken. This analysis revealed that probe A was conserved in all mammals. However, it did not hybridize to chicken DNA (Fig. 2*F*).

Expression of the *Mlvi* Locus. Polyadenylylated RNA from several tumors and tumor cell lines, as well as from a variety of normal rat tissues, was electrophoresed in denaturing formaldehyde/agarose gels and after transfer to nylon membranes, the RNA was hybridized to the conserved *Mlvi*-1 probe A (Fig. 1*C*). Three tumors (6889, B8L, and D1) (refs. 22 and 27; J. Lee, personal communication) carrying a provirus in *Mlvi-4*, *Mlvi-1*, and *c-myc*, respectively, showed expression of a ≈10-kb mRNA transcript hybridizing to the probe. However, the normal tissues, which included thymus, spleen, and liver, expressed undetectable levels of the *Mlvi*-1 transcript (Fig. 3*A*). Analysis of tumors and tumor cell lines carrying no detectable proviruses in the DNA region between c-*myc* and *Mlvi*-1 revealed a mixed picture. Fig. 3*B* shows two tumor cell lines (D0 and B4) that do not carry a provirus in this region and do not express detectable levels of the *Mlvi*-1 transcript. The same figure shows one tumor (915) and one cell line (LE3Sp), which also lack a provirus in this region and express either low (915) or high (LE3Sp) levels of this transcript. The low-level expression detected in tumor 915 could be due to the integration of an undetected provirus in the large chromosomal domain between c-*myc* and *Mlvi*-1. The unusually high level of expression of the *Mlvi*-1 transcript in LE3Sp correlates with the unique phenotype of this cell line. LE3Sp is the only one out of 27 independent tumor cell lines established in this laboratory, with a $CD4^+$, $CD8^+$, interleukin 2 $receptor^+$, and T-cell receptor α-chain/β-$chain^+$ phenotype (P. Lazo and P.N.T., unpublished data).

To confirm the tumor-specific expression of the *Mlvi*-1 transcript, polyadenylylated RNA from a larger collection of normal rat tissues and tumor D1 was hybridized to the *Mlvi*-1 probe A. The results shown in Fig. 4 confirmed that *Mlvi*-1 is not expressed in normal rat tissues.

DISCUSSION

It has been shown previously that provirus insertion in c-*myc*, *Mlvi*-1, and *Mlvi*-4 upregulates c-*myc* expression (P. Lazo, J. Lee, and P.N.T., unpublished data). In this manuscript we presented evidence that the reverse is also true—i.e., provirus insertion in c-*myc*, *Mlvi*-4, and *Mlvi*-1 upregulates the expression of an RNA transcribed from the region

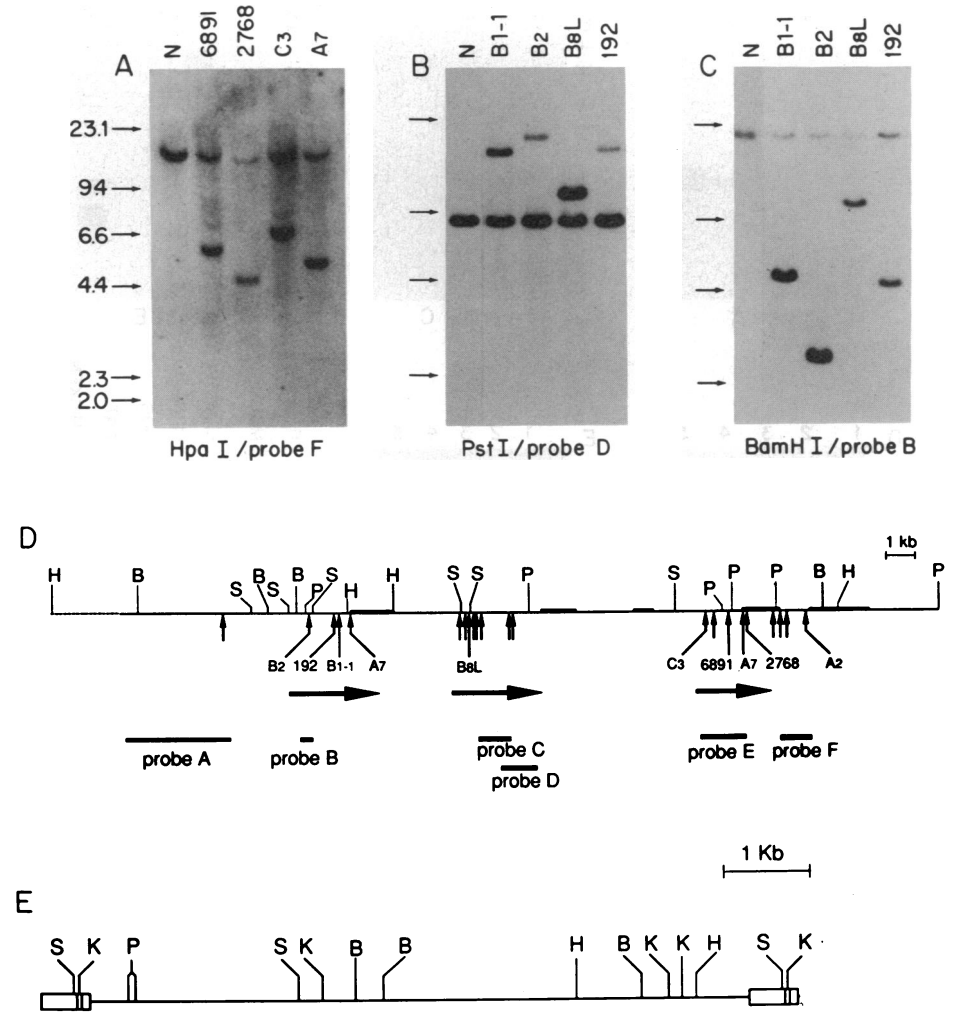

FIG. 1. Sites of provirus integration and orientation of the integrated proviruses in the *Mlvi*-1/*mis*-1/*pvt*-1 locus. (*A*) Southern blot analysis of *Hpa* I-digested genomic DNA from four representative thymic lymphomas (6891, 2768, C3, and A7) carrying a provirus in the right-hand cluster and normal rat thymus DNA (N) hybridized to the *Mlvi*-1 probe F. Given the site of provirus insertion in these tumors and the restriction maps of *Mlvi*-1/*mis*-1/*pvt*-1 (*D*) and Mo-MuLV (*E*), this experiment allowed us to determine the orientation of transcription of these proviruses (*D*). (*B*) Southern blot analysis of *Pst* I-digested genomic DNA from four representative thymic lymphomas (B1-1, B2, B8L, and 192) carrying proviruses in the middle- and left-hand clusters and normal thymus DNA (N) hybridized to the *Mlvi*-1 probe D. Given the sites of provirus insertions in these tumors and the restriction maps of *Mlvi*-1 (*D*) and Mo-MuLV (*E*), this experiment allowed us to determine the orientation of transcription of these proviruses (*D*). (*C*) Southern blot analysis of *Bam*HI-digested genomic DNA from the same normal and tumor tissues shown in *B* hybridized to probe B. The findings in *C* confirm the findings in *B*. The arrows on the left of each panel indicate the migration of DNA fragments generated by digestion of wild-type λ DNA with *Hin*dIII; the size of these fragments is shown in *A*. (*D*) Restriction endonuclease map, mapping of repeated DNA sequences, sites of provirus integration, orientation of the integrated proviruses, and map position of the probes used throughout this study. Sequences that have not been cloned are marked by an interrupted line at the far left. Repeated DNA sequences are marked by thick lines. The arrows pointing toward the linear *Mlvi*-1 map indicate the sites of provirus insertion in individual tumors. Tumors shown in *A*, *B*, and *C* were identified by number in *D*. The heavy arrows pointing from left to right in *D* indicate the transcriptional orientation of the integrated proviruses. Tumor A7 is composed of two populations of tumor cells (22), one of which carried a provirus in the right-hand and the other in the left-hand provirus clusters. The proviruses A2 and A7 from the right-hand cluster were cloned and they were shown to have the predicted orientation, be intact, and be biologically active and identical to Mo-MuLV (data not shown). The code for the restriction endonuclease sites is as follows: H, *Hpa* I, B, *Bam*HI, S, *Sac* I, and P, *Pst* I. Probes A–F represent the following plasmids: probe A, pBS8.1 (*Eco*RI); probe B, pE/B7 (*Eco*RI–*Bam*HI); probe C, pBS8.5 (*Eco*RI); probe D, pBS27A (*Eco*RI–*Sal* I); probe E, pTS26 (*Eco*RI–*Sac* I), which was cloned from tumor C3 and contains viral sequences; and probe F, pTS25E/P (*Eco*RI–*Pvu* II). (*E*) Restriction endonuclease map of Mo-MuLV. The orientation of transcription is from left to right. The code for the restriction endonuclease sites is as for *D*.

immediately 5′ to the *Mlvi*-1 proviruses. These findings indicate that the DNA region between c-*myc* and *Mlvi*-1 represents a domain of coordinately regulated genes. Perturbations within this domain mediated by provirus integration or other chromosomal events may affect the expression of multiple genes. The mechanism(s) of these phenomena is unknown. In this report, however, we presented data that may be relevant in understanding these mechansims. Provirus integration in *Mlvi*-1 clusters in three regions separated by uninterrupted cellular DNA. Two of these regions contain evolutionarily conserved sequences, and they are surrounded by long stretches of nonconserved DNA. The evolutionary conservation of the regions providing the target for provirus insertion suggests that the integrated provirus may exert its effect by interrupting and altering the function of important control elements that influence the activity of neighboring and distant genes and are conserved among species. Sequences involved in the regulation of gene expression from a long distance have been described previously in at least two instances—one of which involves the regulation of expression of the β globin gene (28–31) and the other the expression of the variable genes of the immunoglobulin heavy

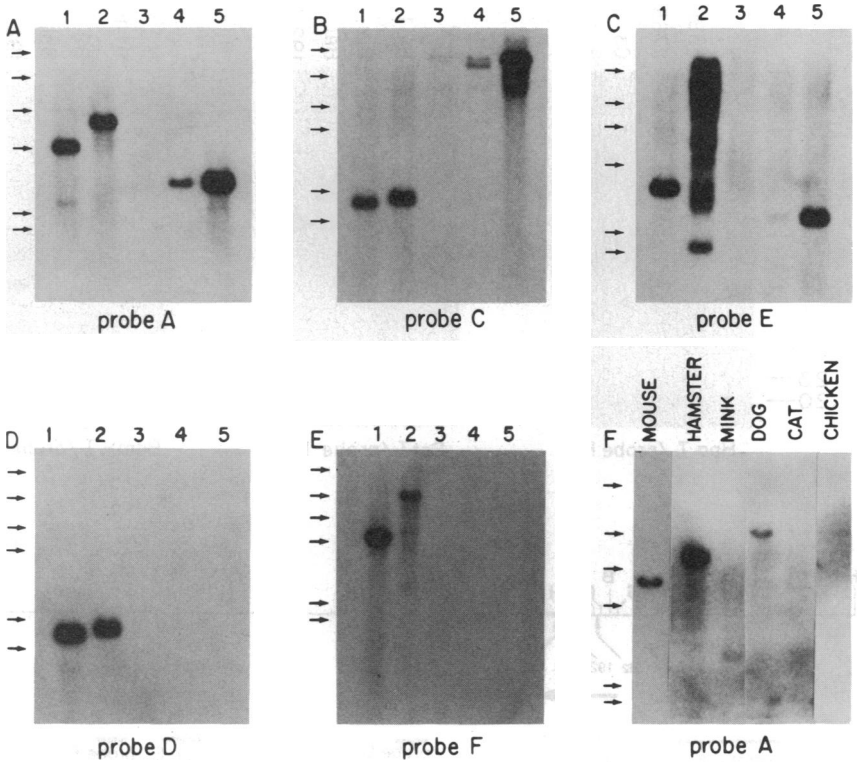

FIG. 2. Evolutionary conservation of single-copy sequences in the *Mlvi*-1/*mis*-1/*pvt*-1 locus. (*A–E*) *Eco*RI-digested genomic DNA from rat (lanes 1), mouse (lanes 2), HTK, a human rhabdomyosarcoma cell line containing normal c-*myc* (lanes 3) (M. Erisman, personal communication), Colo228, a human colon carcinoma cell line containing an amplified c-*myc* (lanes 4) (20, 21), and Colo320, a human colon carcinoma cell line containing an even higher degree of c-*myc* amplification (lanes 5) (20, 21). The probes were A, C, E, D, and F, as indicated. Probe C detects both an amplification, as well as a rearrangement in the cell line Colo228 (*B*). Probe E detects multiple bands in mouse DNA because it contains viral sequences. Arrows at left indicate DNA fragments from digestion of wild-type λ phage DNA with *Hin*dIII; sizes of these fragments are identical to those shown in Fig. 1. Hybridization of probe A to a series of *Eco*RI-digested genomic DNA from several animals (*F*) revealed that probe A is conserved among mammals, but it is not conserved in birds.

chain locus (32). We have no direct evidence to explain why provirus integration in these regions may affect the expression of multiple genes, both neighboring and distant, although effects on the chromatin structure of the region surrounding the insertion should be expected. However, any attempts to address this issue should take into account the finding presented in this report that all the proviruses integrated in the *Mlvi*-1 locus integrate in a single transcriptional orientation.

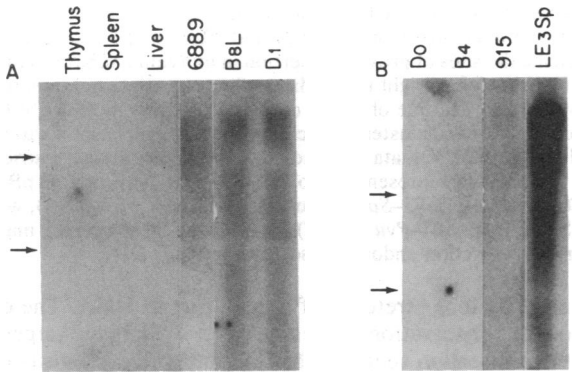

FIG. 3. Northern blot analysis of polyadenylylated RNA from normal thymus, spleen, and liver, and tumor cell lines 6889 (containing a provirus in *Mlvi*-4), B8L (containing a provirus in *Mlvi*-1), D1 [containing a provirus-mediated rearrangement upstream from the first exon of c-*myc* (27)], and D0, B4, 915, and LE3Sp (containing no known provirus in this region). Arrows at left in *A* indicate 28S and 18S ribosomal RNAs and correspond to ≈5.0 and 2.0 kb, respectively.

A very interesting, although puzzling, finding presented in this report is that a cell line (LE3Sp) that lacks a provirus in this region expresses unusually high levels of the *Mlvi*-1 transcript. Interestingly this is the only one out of 27 independent T-cell lymphoma lines we have established in this laboratory with a $CD4^+$, $CD8^+$, interleukin 2 $receptor^+$, and T-cell receptor α chain/β $chain^+$ phenotype (P. Lazo and P.N.T., unpublished data). This fact suggests that the *Mlvi*-1 transcript may be expressed in a developmentally regulated fashion in a subset of $CD4^+$ $CD8^+$ cells. Differentiating this cell line to a more mature phenotype and *in situ* hybridization of the *Mlvi*-1 probe to sections of the thymus should enable us to answer this question.

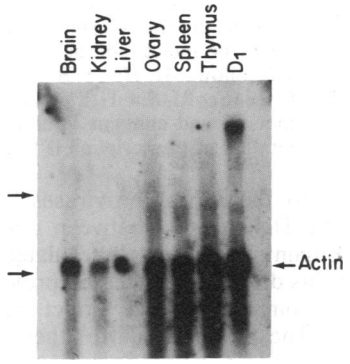

FIG. 4. Expression of *Mlvi*-1 probe A sequences in normal tissues and tumor cell line D1 containing a provirus-mediated rearrangement upstream from c-*myc* (27). Arrows indicate 28S and 18S ribosomal RNAS.

The *Mlvi*-1 locus, in addition to providing the substrate for provirus integration in Mo-MuLV-induced T-cell lymphomas, is also the frequent target of chromosomal translocations in both mouse and human lymphoid neoplasms (16–18). Furthermore, the same locus is frequently amplified in a wide variety of human and animal tumors (18, 20, 21). Although direct evidence is lacking, it is reasonable to expect that all these chromosomal events may affect the expression of the *Mlvi*-1 RNA transcript.

The evidence we presented in this report indicates that clustering of provirus insertion in a set of loci of common integration in the vicinity of c-*myc* may affect the expression of at least two genes. Because one of the genes (c-*myc*) is a known protooncogene the question arises whether the second one plays any role in oncogenesis. We suggest that both genes may be involved in this process. This statement is supported by the fact that genes with related functions frequently cluster within the genome (28–31, 33–35). In addition, in the case presented here c-*myc* and *Mlvi*-1 appear to be deregulated in parallel, suggesting that the two genes respond to related regulatory signals. This in turn indicates that they may be functionally related, in which case the deregulation of both probably plays a role in oncogenesis. Characterization of the gene represented by the *Mlvi*-1-encoded RNA transcript may provide more direct answers to these questions. Furthermore, it may help us to address the issue of potential interaction between *Mlvi*-1 and other loci during oncogenesis. Such an interaction between *Mlvi*-1 and *Mlvi*-2 was suggested by the finding that Mo-MuLV-induced T-cell lymphomas in Osborn–Mendel rats carry provirus-mediated rearrangements of both loci simultaneously (22).

In summary, the data presented in this report show that provirus integration may exert effects on the expression of multiple genes neighboring the site of insertion. In addition, they provide evidence for an unidentified gene that may be developmentally regulated and may be involved in the induction of a wide variety of human and animal tumors.

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