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Genetics

# Provirus insertion in *Tpl-1*, an *Ets-1*-related oncogene, is associated with tumor progression in Moloney murine leukemia virus-induced rat thymic lymphomas

**(T-cell lymphoma/thymoma/common region of provirus integration)**

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ABSTRACT **T-cell lymphomas induced in rats by Moloney murine leukemia virus acquire increasing numbers of proviruses in their genome during tumor progression *in vivo* and passage of tumor cells *in vitro*. To determine whether the proviruses progressively acquired during tumor progression play a causal role in this process, we cloned one of them from a cell line derived from the primary tumor 2772. A probe from the cellular DNA flanking the provirus was used to analyze 79 DNA samples from primary tumor tissues of 28 tumor-bearing rats and 80 DNA samples from 30 independent tumor cell lines. This analysis revealed a rearrangement in this region in the primary tumor derived from the thymus of one animal but not in a clone of the same tumor segregating in the spleen. Of the cell line DNA samples, three carried a provirus in this region. Two of these integration events had occurred independently in two clonally related sublines derived from tumor 2772, and they were followed by rapid selection in culture. On the basis of these findings this locus was named *Tpl-1* (tumor progression locus 1). The *Tpl-1* locus was mapped to rat chromosome 8 and to mouse chromosome 9 at a genetic distance of 1.2 ± 0.9 centimorgans from the *Ets-1* protooncogene. Although the genetic distance between *Tpl-1* and *Ets-1* indicates that they are different genes, analysis of *Tpl-1* cDNA clones revealed that the two are closely related.**

Tumor progression is the result of growth selection among continuously arising variants of the clonally derived tumor cells. The generation of these variants is the result of genetic and epigenetic changes occurring in the growing tumor cell population (1, 2). The genetic changes could be point mutations or amplifications of genes that directly or indirectly regulate cell growth or could be DNA recombination events such as chromosomal translocations, deletions, inversions, or insertions of movable genetic elements (3–5). We had reported previously that individual tumor cells from independent lymphomas induced by Moloney murine leukemia virus (Mo-MuLV) may carry proviruses inserted in multiple common regions of integration (6). Furthermore, it has been shown that the late stages of oncogenesis as well as the passage of tumor cells in syngeneic animals are associated with the selection of cell populations carrying newly integrated proviruses (7, 8). These data, combined with the finding that at least 30% of Mo-MuLV-induced rat T-cell lymphoma cell lines have no detectable karyotypic abnormalities (G.L. and P.N.T., unpublished data), suggested to us that in retrovirus-induced neoplasms, provirus integration may be a major factor responsible for the generation of genetic heterogeneity among tumor cells and tumor progression.

The work presented in this report was designed to address the question of whether the appearance of additional proviruses in subpopulations of tumor cells was due to sequential provirus integration into the genome of infected cells. Furthermore, it was designed to determine whether the acquisition of these proviruses could be linked to tumor progression. The data we present indicate that the emergence of tumor cell variants carrying novel integrated proviruses is due to sequential provirus integration and not to selection of preexisting cell clones. One of these late provirus integration events had occurred in a locus of common integration (*Tpl-1*), which is genetically linked and closely related to the *Ets-1* protooncogene, suggesting that provirus insertion into the genome of infected tumor cells may play an important role in tumor progression.

## MATERIALS AND METHODS

**Thymic Lymphomas and Cell Lines.** Thymic lymphomas were induced in Osborn–Mendel and Long–Evans rats by i.p. inoculation with $5 \times 10^5$ XC plaque-forming units of Mo-MuLV at birth (6, 9). The cell lines were established from primary tumors. The detailed characterization of these cell lines is the subject of a separate report to appear elsewhere.

**Isolation of Genomic DNA/Southern Blotting.** Genomic DNA isolation and Southern blotting were carried out by using standard procedures as described (6, 9). Hybridization of the tumor progression locus probe to mouse and mouse–rat hybrid genomic DNA was carried out under conditions of reduced stringency [40% formamide/6× SSC (1× SSC is 0.15 M NaCl/15 mM sodium citrate) at 37°C]. The filters hybridized under these conditions were washed three times with 2× SSC/0.1% $NaDodSO_4$ at room temperature and once with 1× SSC/0.1% $NaDodSO_4$ at 65°C for 30 min.

**Genomic DNA Cloning.** Genomic DNA cloning was done by using standard procedures as described (6, 9). Briefly, restriction endonuclease-digested genomic DNA from a cell line derived from tumor 2772 was fractionated by agarose gel electrophoresis. The DNA fraction enriched for the desired DNA fragment was ligated to the *Eco*RI site of the bacteriophage λ vector λgtWES·λB′ (6, 9). Following *in vitro* packaging, the resulting mixture of recombinant phage particles was plated in the *Escherichia coli* strain LE392. The library was screened with a Mo-MuLV long terminal repeat (LTR) probe (6, 9). A 600-bp *Eco*RI/*Taq* I fragment from the clone

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Abbreviations: Mo-MuLV, Moloney murine leukemia virus; RFLP, restriction fragment length polymorphism.

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representing the new provirus integration in tumor 2772 was subcloned in plasmid PUC18 digested with *Eco*RI and *Acc* I. The subclone (pSB-1) contained only single-copy cellular DNA and lacked both proviral and repeated cellular sequences.

**Construction and Screening of cDNA Libraries.** Total RNA was isolated from tumor 2772 cells containing a provirus in the *Tpl-1* locus, using the guanidinium isothiocyanate/cesium chloride method (10). Poly(A)$^+$ RNA was selected by using affinity chromatography in oligo(dT)-cellulose (11). cDNA was synthesized from 5 $\mu$g of poly(A)$^+$ RNA by using oligo(dT) primers and reverse transcriptase. To obtain double-stranded DNA, we used the method of Gubler and Hoffman (12). Following the addition of *Eco*RI linkers, the double-stranded cDNA was ligated to the *Eco*RI arms of the bacteriophage $\lambda$ZapII purchased from Stratagene. The constructed cDNA library was screened by using single-copy genomic *Tpl-1* clones.

**Genetic Mapping.** The chromosomal map location of *Tpl-1* in the rat genome was determined by Southern blot analysis of genomic DNA from mouse–rat somatic cell hybrids segregating rat chromosomes (13). To determine the map position of *Tpl-1* into the mouse genome, we took advantage of restriction fragment length polymorphisms (RFLP) to examine the frequency of recombination between this locus and known genetic markers in the genome of (*Mus musculus* × *Mus spretus*) × *Mus musculus* interspecific back-cross mice (14).

## RESULTS

Mo-MuLV-induced T-cell lymphomas carry multiple proviruses integrated in their genome (6–9). To determine whether these proviruses integrate sequentially during oncogenesis, we inoculated rats heterozygous for a RFLP in the *Mlvi-4* locus (unpublished data) with Mo-MuLV. Since the two alleles of the *Mlvi-4* locus could be distinguished by Southern blot analysis, the allele rearranged by provirus integration could be determined. In the example in Fig. 1, the tumors derived from different lymphoid organs of one animal (no. 21) were found by this analysis to be monoclonal with regard to provirus integration in *Mlvi-4*, while they were distinctly different in the overall pattern of provirus integration. We conclude that novel provirus insertions distinguishing the tumor cells segregating in two different organs of animal no. 21 occurred subsequently to provirus insertion in the *Mlvi-4* locus.

Because of the apparent selection of cells containing an increasing number of integrated proviruses during oncogenesis, we hypothesized that the secondary provirus insertions play an active role in the selection process. To investigate this possibility, we cloned a secondary provirus that arose in a subline derived from a tumor (2772) during passage in culture (Fig. 2). A DNA probe (pSB-1) containing unique cellular and no viral sequences was obtained from this clone and used to generate a restriction map of this region (Fig. 3*B*). The dashed arrow, indicating a *Sac* I site in the map, means that this site was polymorphic and therefore was frequently absent in normal rat DNA. This was followed by the analysis of 79 DNA samples from 28 primary tumor-bearing animals and 66 DNA samples from 29 independent tumor cell lines for rearrangements in this locus. This analysis revealed a rearrangement in this locus in the primary tumor derived from the thymus of one animal. However, no rearrangement was detected in the clonal metastasis of this tumor in the spleen (Fig. 3*A*). A rearrangement in this locus was also detected in the subline 5675CL1 of the cell line derived from tumor 5675. The parental primary tumor tissue (5675T) as well as a

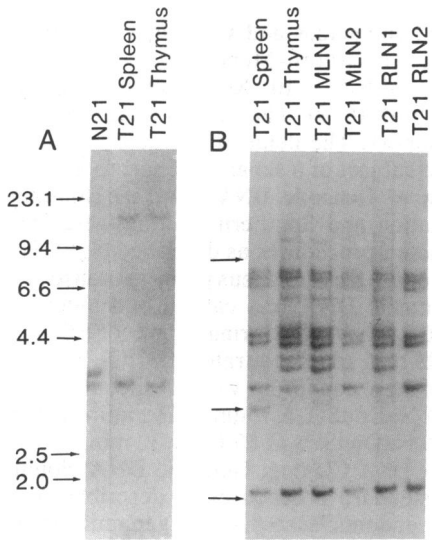


FIG. 1. The detection of increasing numbers of proviruses in subpopulations of tumor cells is due to provirus integration in the genome of already infected tumor cells. (*A*) Southern blot analysis of *Eco*RI-digested normal, and spleen and thymus tumor-cell DNA from rat 21 and hybridization to an *Mlvi-4* probe. Lanes: N21, normal genomic DNA from rat 21; T21 Spleen and T21 Thymus, tumor cell DNA from spleen and thymus of rat 21. (*B*) Southern blot analysis of *Eco*RI-digested DNA from tumors in different lymphoid organs of rat 21 and hybridization to a Mo-MuLV long terminal repeat probe. MLN and RLN refer to mesenteric and retroperitoneal lymph nodes, respectively. The arrows on the left of each panel indicate the migration of DNA fragments generated by digestion of wild-type phage $\lambda$ DNA with *Hin*dIII. The size of the fragments is shown in *A*.

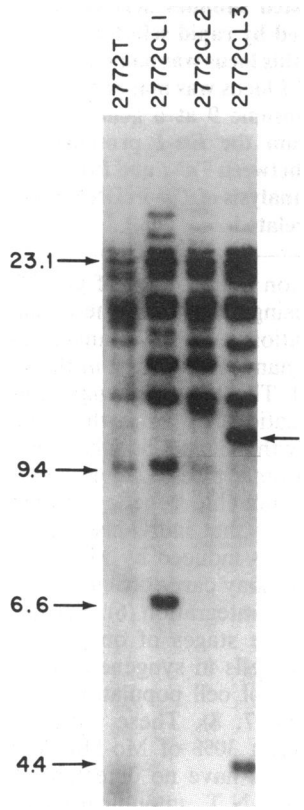


FIG. 2. Establishment of tumor cells in culture is associated with the acquisition of additional integrated proviruses. Southern blot analysis is shown of *Eco*RI-digested genomic DNA from the thymic lymphoma 2772 and from three tissue culture lines derived from this tumor hybridized to a Mo-MuLV long terminal repeat probe. The arrow on the right indicates the integrated provirus we cloned.

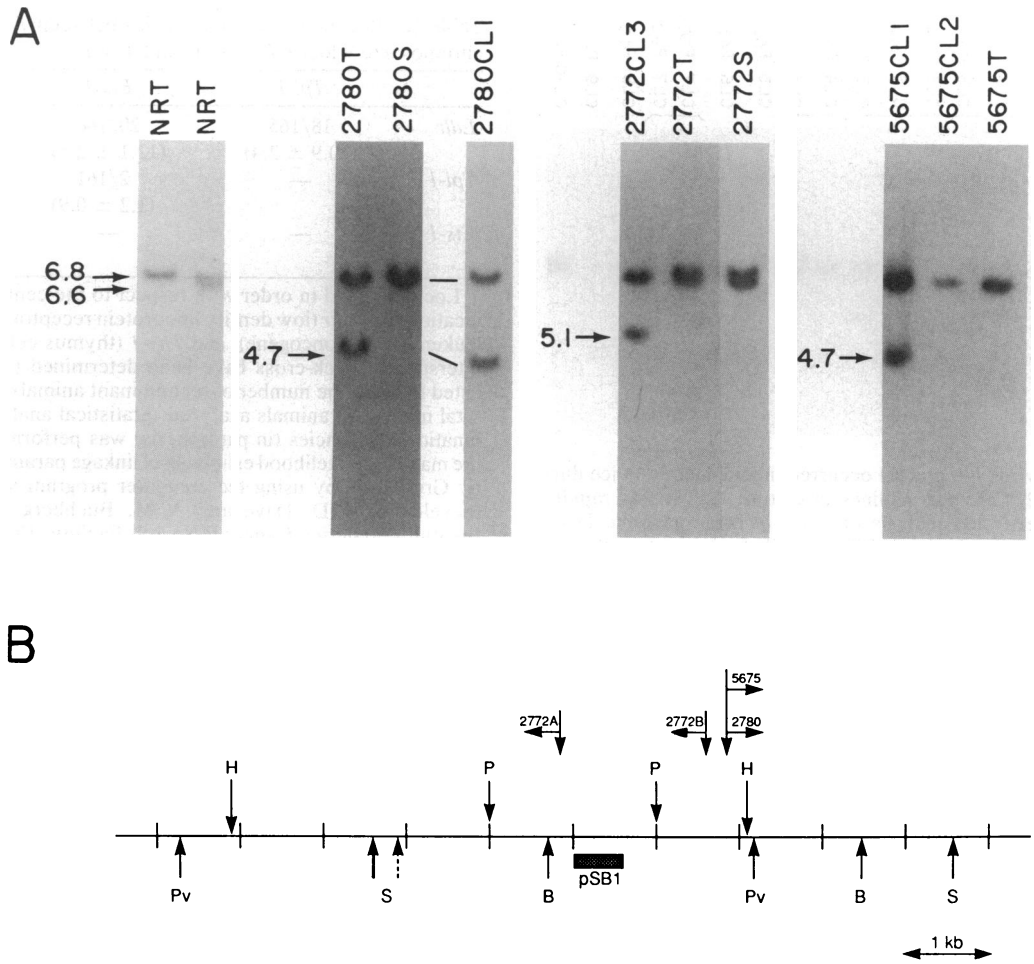


FIG. 3. Distribution of genetic rearrangements in the *Tpl-1* locus among primary tumors and cell lines. (*A*) Southern blot analysis is shown of *Sac* I-digested genomic DNA from normal rat thymus (NRT) and primary tumors and cell lines derived from the animals 2780, 2772, and 5675 hybridized to the *Tpl-1* probe pSB-1 (see *B*). A RFLP due to the presence or absence of the *Sac* I site indicated by the dashed arrow in *B* distinguishes animals that are homozygous or heterozygous in *Tpl-1*. The rearrangement in 2780 was observed only in the thymus (lane 2780T) and the cell line derived from the thymus (lane 2780CL1), but it was absent in the spleen. Similarly the 2772 and the 5675 rearrangements were observed in a single tumor subline each, but they were absent in other sublines as well as in the primary tumors. (*B*) Restriction map of the *Tpl-1* locus, indicating the origin of the pSB-1 probe as well as the sites of provirus integration and the orientation of the integrated proviruses. One of the two *Sac* I sites indicated by the dashed arrow is frequently missing in normal rat DNA.

separate subline (5675CL2) contained no provirus in this locus (Fig. 3*A*).

To determine the timing of provirus insertion in this locus, we examined 14 DNA preparations from different passages of eight sublines derived from tumor 2772. This experiment revealed that during passage of the 2772 cells in culture, rearrangement of this locus occurred twice, independently. The independent nature of the two events is indicated by the different size of the rearranged bands generated by these events and by the observation that a different allele of this locus was rearranged each time (Fig. 4). This experiment also showed that the rearrangement of this locus was followed by rapid selection of the cells in which it took place (Fig. 4).

Thus, a provirus integration event that originally was detected in tumor cells passaged in culture may also be observed in primary tumor cells derived directly from tumor-bearing animals. However, all provirus insertions in this locus shared at least two common features: (*i*) they occurred late and (*ii*) they were followed by rapid selection. We conclude that rearrangements of this locus are involved in tumor progression. Because of its association with tumor progression, this locus was named *Tpl-1* (tumor progression locus 1). When we consider that the cells were maintained by passaging at a dilution of ≈1:50, each passage was associated with 6 cell divisions; therefore, there are ≈24 cell divisions in the four passages sufficient for the selection of clone 3 following provirus insertion in *Tpl-1*.

Digestion of tumor cell DNA with multiple restriction endonucleases and hybridization to the probe pSB-1 revealed the site of integration as well as the orientation of the integrated provirus in all four specimens carrying a provirus in the tumor progression locus (Fig. 3*B*).

To determine the possible relationship of the *Tpl-1* locus to any known oncogenes, we proceeded to map its chromosomal location in the rat and mouse genomes. To map this locus into the rat genome, we analyzed a panel of mouse–rat somatic cell hybrids that segregate the rat chromosomes (13). This analysis revealed that the *Tpl-1* locus maps to rat chromosome 8 (Table 1). To determine the map position of *Tpl-1* into the mouse genome, we examined the frequency of recombination between this locus and known genetic markers in the genome of (*Mus musculus* × *Mus spretus*) × *Mus musculus* interspecific back-cross mice (14). Gene order was determined by minimizing the number of double recombinants between all relevant loci. This analysis, a summary of which is shown in Table 2, revealed that *Tpl-1* maps to the murine chromosome 9 between *Ldlr* (low density lipoprotein receptor) and *Ets-1*. The map distances (centimorgans) and the gene orders in the murine chromosome 9 are as follows: centromere *Ldlr*-(10.9 ± 2.4)-*Tpl-1*-(1.2 ± 0.9)-*Ets-1*-(10.2 ± 2.3)-*Thy-1*.

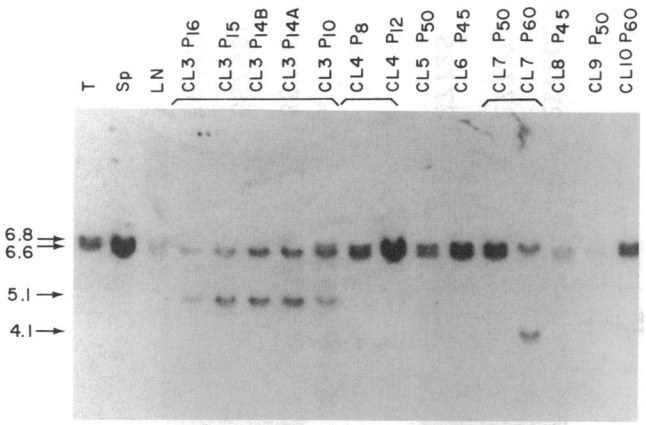


FIG. 4. Provirus integration occurred independently twice during passage of 2772 tumor sublines in culture, and it was rapidly selected. Southern blot analysis of *Sac* I-digested genomic DNA isolated from the primary tumor 2772 and different passages of eight 2772 tumor sublines hybridized to the pSB-1 probe. The 2772 rat was heterozygous for the *Tpl-1* RFLP. The two events occurred independently because (*i*) the rearranged band generated by each event was different in size and (*ii*) a different allele was rearranged by each event. The rearrangement was followed by rapid selection as suggested by the fact the cells carrying the rearrangement dominated the culture within a few passages.

*Tpl-1* cDNA clones were isolated by screening a cDNA library constructed from poly(A)$^+$ RNA from tumor 2772 with *Tpl-1* genomic DNA probes. Comparison of the sequence of these clones to the DNA sequences in the GenBank data base revealed that *Tpl-1* is strongly homologous to the *Ets-1* protooncogene (data not shown). This was confirmed by hybridization of a viral v-*ets* probe to a cDNA clone of the *Tpl-1* locus (Fig. 5). In the chromosome mapping experiments, *Tpl-1* was detected by using a genomic DNA probe derived from the 5′ end of the *Tpl-1* gene, while *Ets-1* was detected with a genomic DNA probe spanning exon VII. If the two probes were derived from the same gene, the distance between them would be less than 60 kilobases (kb) (16). This makes it unlikely that the observed recombination frequency between these probes was due to intragenic recombination events. We conclude that *Tpl-1* and *Ets-1* are two independent genes that are genetically linked and closely related.

Table 1. Correlation of rat chromosomes and the tumor progression locus (*Tpl-1*) in 17 hybrid clones

| Chromosome | No. of hybrid clones with *Tpl-1*/chromosome retention* | | | | % discordance |
|---|---|---|---|---|---|
| | +/+ | −/− | +/− | −/+ | |
| 1 | 0 | 12 | 2 | 3 | 29.4 |
| 2 | 2 | 6 | 0 | 9 | 52.9 |
| 3 | 2 | 9 | 0 | 6 | 35.3 |
| 4 | 2 | 5 | 0 | 10 | 58.8 |
| 5 | 0 | 12 | 2 | 3 | 29.4 |
| 6 | 2 | 11 | 0 | 4 | 23.5 |
| 7 | 2 | 6 | 0 | 9 | 52.9 |
| 8 | 2 | 15 | 0 | 0 | 0 |
| 9 | 0 | 13 | 2 | 2 | 23.5 |
| 10 | 1 | 8 | 1 | 7 | 47.0 |
| 11 | 2 | 7 | 0 | 8 | 47.0 |
| 12 | 1 | 7 | 1 | 8 | 52.9 |
| 13 | 2 | 5 | 0 | 10 | 58.8 |
| 14 | 1 | 12 | 1 | 3 | 23.5 |
| 15 | 2 | 10 | 0 | 5 | 29.4 |
| 16 | 2 | 6 | 0 | 9 | 52.9 |
| 17 | 2 | 9 | 0 | 6 | 35.3 |
| 18 | 0 | 5 | 2 | 10 | 70.6 |
| 19 | 2 | 11 | 0 | 4 | 23.5 |
| 20 | 1 | 11 | 1 | 4 | 29.4 |
| X | 2 | 2 | 0 | 13 | 76.5 |

*+/+, Containing *Tpl-1* and the indicated chromosome; −/−, lacking *Tpl-1* and the indicated chromosome; +/− containing *Tpl-1* and lacking the indicated chromosome; −/+ lacking *Tpl-1* and containing the indicated chromosome.

Table 2. Recombination frequencies between *Tpl-1* and the chromosome 9 loci *Ldlr*, *Ets-1*, and *Thy-1*

| | *Tpl-1* | *Ets-1* | *Thy-1* |
|---|---|---|---|
| *Ldlr* | 18/165 (10.9 ± 2.4) | 20/166 (12.1 ± 2.5) | 39/170 (22.9 ± 3.2) |
| *Tpl-1* | — | 2/161 (1.2 ± 0.9) | 20/165 (12.1 ± 2.5) |
| *Ets-1* | — | — | 17/167 (10.2 ± 2.3) |

Loci are listed in order with respect to the centromere. The map locations for *Ldlr* (low density lipoprotein receptor), *Ets-1* (E26 avian leukemia virus oncogene), and *Thy-1* (thymus cell antigen 1) in the interspecific back-cross have been determined (14). The numbers listed indicate the number of recombinant animals detected over the total number of animals analyzed. Statistical analysis of the recombination frequencies (in parenthesis) was performed by calculating the maximum likelihood estimates of linkage parameters as described by Green (15) by using the computer program SPRETUS MADNESS (developed by D. Dave and A. M. Buchberg, National Cancer Institute–Frederick Cancer Research Facility, Frederick, MD).

## DISCUSSION

During tumor progression and during passage of tumor cells in syngeneic animals and in culture, there is selection of tumor cells carrying an increasing number of integrated proviruses (refs. 7 and 8 and this report). The findings presented in this report show that the acquisition of these proviruses is due to sequential provirus reintegration in the genome of infected cells. Preliminary data suggest that the mechanism of this phenomenon may be reinfection of virus-infected tumor cells (P.N.T., unpublished data).

At least some of the proviruses detected in tumor cells in the late stages of oncogenesis appear to be involved in tumor progression. This was suggested by the finding that one integrated provirus that was first detected in a cell population emerging from the passage of a tumor cell line in culture defined a new common region of integration. This region was named tumor progression locus 1 (*Tpl-1*). Rearrangement of the *Tpl-1* locus occurred both in primary tumors and during passage in culture. However, all of the integration events we observed shared the common feature that they occurred late during tumor progression. Analysis of multiple passages from eight sublines derived from tumor 2772 from which the *Tpl-1* locus was cloned revealed that provirus integration occurred

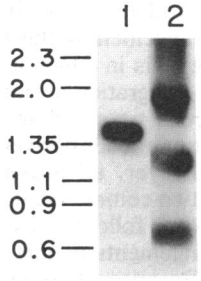


FIG. 5. Hybridization of a v-*ets* probe to a murine *Ets-1* cDNA probe (kindly provided by J. H. Chen, M. D. Anderson Hospital and Tumor Institute, Houston) and to an *Eco*RI-digested *Tpl-1* cDNA clone (lane 2). The size of the markers is given in kb.

twice independently. Furthermore, after provirus integration there was rapid selection of the affected cells.

Chromosome mapping followed by sequence analysis of *Tpl-1* cDNA clones revealed that the tumor progression locus is genetically linked and strongly homologous to the *Ets-1* protooncogene. The linkage between these two closely related genes is reminiscent of the linkage between two other *Ets-1*-related genes (*ETS2* and *ERG*) in human chromosome 21 (17). The functional and evolutionary significance of these genetic linkages is unknown.

The frequency of provirus integration in the *Tpl-1* locus among primary thymomas was low. Analysis of 79 DNA samples derived from tumor tissues isolated from different lymphoid organs of 28 independent animals revealed only 1 that was carrying a *Tpl-1* rearrangement. Similarly, when we exclude the two independent *Tpl-1* rearrangements observed in two sublines derived from tumor 2772, analysis of 66 DNA samples derived from multiple sublines of 29 independent cell lines revealed only 1 *Tpl-1* rearrangement. The low frequency of provirus integration in the *Tpl-1* locus in primary tumor tissues (1 of 79) and cell lines (1 of 66 DNAs tested) is in sharp contrast with the high frequency of provirus integration in this locus in cells derived from tumor 2772 (2 of 10). The difference in frequency of provirus integration in *Tpl-1* between all the thymic lymphomas we tested and 2772 was significant at the $P = 0.02$ level. This suggests that the growth-promoting effects of provirus insertion in the *Tpl-1* locus may be restricted to cells derived from some but not all of the tumors. Since it is known that these T-cell lymphomas express a wide spectrum of differentiation phenotypes (unpublished data), we propose that the *Tpl-1* locus may exert its growth-promoting effect only in cells that exist within a narrow developmental window. Alternatively, *Tpl-1* may interact with the product of another gene that is activated only in some tumors such as tumor 2772 (6).

The importance of provirus integration in tumor progression suggested by our data is supported by reports from other laboratories. In one such report murine hematopoietic cells transformed by a *Myc*-containing retrovirus gave rise to a clonal myelomonocytic cell line. This cell line expresses c-*fms* [colony-stimulating factor 1 (CSF-1) receptor] (18) and contains a provirus-mediated rearrangement in CSF-1 (19). Abelson MuLV-induced lymphomas are clonal, and frequently they carry a copy of the helper provirus in a common region of integration (20). Finally, provirus insertion in *Pim-2*, a region of common integration in retrovirus-induced murine T-cell lymphomas, was observed in tumor cells passaged in syngeneic animals (21). Collectively, these data suggest that provirus tagging can be used as a method to identify genes involved in specific aspects of tumor progression, such as tumor cell homing to different lymphoid organs and tumor dissemination. Furthermore, they suggest that placement of tumor cells under well-defined selective pressures in culture may allow the selection of cells whose defined phenotypes are due to newly acquired integrated proviruses. This approach may allow the direct identification of genes involved in T-cell function.

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