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Tumor progression locus 2 (*Tpl-2*) encodes a protein kinase involved in the progression of rodent T-cell lymphomas and in T-cell activation

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ABSTRACT **The *Tpl-2* locus, cloned by provirus tagging from one of three sublines of the Moloney leukemia virus-induced rat thymoma 2769, defines a gene encoding a protein kinase associated with progression in 22.5% of the tumors. *Tpl-2* is expressed primarily in spleen, thymus, liver, and lung. Provirus integration occurs in the last intron of the gene, leading to the expression of a truncated mRNA that terminates in the proviral long terminal repeat and encodes a protein with an altered C-terminal domain. Strong evidence that this genetic change confers growth advantage to affected cell clones was provided by the finding that, during cultivation of all three sublines derived from tumor 2769, cells were selected that harbored independent provirus insertions in the *Tpl-2* locus. Exposure of normal rat spleen cells to Con A induces the expression of enhanced levels of *Tpl-2* within the first 60 min from the time of exposure suggesting that, in normal splenocytes, *Tpl-2* may be involved in the transition from a quiescent to the G_1 phase of the cell cycle.**

Tumors are composed of cells with substantial genetic and phenotypic heterogeneity, which are subject to continuous selection (1, 2). The basis of selection may be the invasive and metastatic properties of the cells or perhaps their proliferative advantage in all or unique microenvironments. The outcome of the selection is tumor progression. To study tumor progression, we used T-cell lymphoma lines established from thymomas induced in rats inoculated with Moloney leukemia virus (MoMuLV) (2, 3). We showed that these cell lines, during their maintenance in culture or passage in nude mice, acquire increasing numbers of integrated proviruses (4). Since insertion mutations caused by provirus integration into the cellular genome play a major role in retrovirus oncogenesis (5, 6), we asked whether these newly integrated proviruses contributed to the selection operating upon the heterogeneous population of tumor cells. To date two of these proviruses were cloned and characterized. The first was shown to define a locus of common integration (*Tpl-1*) located immediately 5′ of the first exon of the *Ets-1* protooncogene (ref. 4; A. Bellacosa and P.N.T., unpublished work). The second provirus also defined a locus of common integration (*Tpl-2*) whose characterization is the subject of this report.†

MATERIALS AND METHODS

Tumors and Cell Lines. T-cell lymphomas induced in rats inoculated with MoMuLV and cell lines derived from them have been described (2).

Southern and Northern Blotting. Southern blotting of genomic cell DNA and Northern blotting of polyadenylylated cellular RNA were carried out as described (2, 3, 7).

Construction of cDNA Libraries and cDNA Cloning. cDNA libraries were constructed by using 5 μg of polyadenylylated liver and spleen RNAs as described (4).

To clone the aberrant 10-kb and 1.6-kb RNA transcripts, an additional cDNA library was constructed by using 5 μg of polyadenylylated RNA from the tumor cell line 2769 carrying the originally detected *Tpl-2* rearrangement. To ensure the isolation of cDNA clones corresponding to specific transcripts, prior to cDNA cloning the RNA was fractionated by using 10–30% (wt/vol) sucrose/0.5% SDS linear gradients. The gradients, in SW-41 Beckman ultracentrifuge tubes, were centrifuged at 210,000 × *g* for 15 hr and were fractionated in 0.25-ml fractions. The fractions containing the known *Tpl-2* mRNA transcripts were identified by Northern blot analysis and hybridization to the *Tpl-2* genomic probe A and the *Tpl-2.3* cDNA probe.

PCR Using Genomic DNA Templates. *Tpl-2*-containing fractions of *Eco*RI-digested tumor cell DNA, isolated by preparative gel electrophoresis, were used as templates for PCR employing oligonucleotide primers from the R and U_5 regions of the proviral long terminal repeat (LTR) (CCTCCGATTGACTGAGTC;GGTCTCGCTGTTCCTTGG) and the last *Tpl-2* exon (CAATGTAGAGGGAACGCT;GAACGCTGTCTCCTGAGC).

DNA Sequencing. DNA sequence analysis of genomic and cDNA *Tpl-2* clones was carried out as described (7, 8).

Con A Stimulation of Rat Splenocytes. The spleens of six (4–6 weeks old) F344 rats were minced, and the cell suspension was washed twice in 50 ml of RPMI 1640 medium supplemented with 10% fetal bovine serum, 10% horse serum, penicillin (50 units/ml), streptomycin (50 μg/ml), and kanamycin (100 μg/ml). The cells were pelleted at 400 × *g* for 10 min, resuspended in the above medium at a concentration of 6×10^5 mononuclear cells per ml, and cultured at 37°C in aliquots of 9×10^7 cells. All the cultures with the exception of one were exposed to Con A (4 μg/ml), and they were harvested after 1, 3, 22, and 30 hr. The unstimulated spleen cells were harvested at time 0. The response of the spleen cells to Con A was measured at the 48-hr time point by [^{3}H]thymidine incorporation (7). Polyadenylylated RNA isolated from cells harvested from these cultures was electrophoresed in denaturing 1% agarose/2.2 M formaldehyde gels, and after transfer to nylon membranes it was hybridized to the *Tpl-2.3* cDNA probe.

RESULTS

Southern blot analysis of *Eco*RI-digested genomic DNA from the MoMuLV-induced T-cell lymphoma 2769 and three derivative cell lines (2) revealed that the pattern of provirus

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Abbreviations: MoMuLV, Moloney leukemia virus; LTR, long terminal repeat.

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†The sequence reported in this paper has been deposited in the GenBank data base (accession no. M94454).

integration, in agreement with earlier observations (4), was unstable (Fig. 1*A*). To determine whether the newly integrated proviruses observed during progression were causally involved in the growth selection operating upon the heterogeneous population of proliferating tumor cells, we cloned the provirus indicated by the arrowhead in cell line 2 (Fig. 1*A*). A restriction endonuclease map of this clone is shown in Fig. 1*B*. The single-copy probe A flanking the integrated provirus was hybridized to *Eco*RI-digested genomic DNA from primary tumors and cell lines derived from 40 independent MoMuLV-induced rat T-cell lymphomas. The results revealed that 9 (22.5%) of the tumors carried a rearrangement in *Tpl-2* (Fig. 1*C*). In some cases, provirus insertion in *Tpl-2* was detected in tumor cells established and maintained in culture. In the majority of cases, however, provirus insertion occurred during tumor growth in animals. This suggests that provirus insertion in *Tpl-2* confers growth advantage to affected clones both *in vivo* and in culture. Restriction endonuclease digestion with multiple enzymes and hybridization to probe A revealed that the transcriptional orientation of the integrated provirus was the same in all the tumors carrying *Tpl-2* rearrangements (data not shown).

Northern blot analysis of polyadenylylated RNA (7) from multiple normal rat tissues and hybridization to probe A revealed an ≈3-kb-long RNA transcript, which was expressed primarily in spleen, thymus, liver, and lung. Lower levels of expression of the same RNA were detected in brain and testis (data not shown). A cDNA library was constructed from oligo(dT)-primed normal rat liver mRNA (4) in λZapII (Stratagene), and, prior to amplification, it was screened by hybridization to probe A. This analysis yielded two 2.7-kb cDNA clones (*Tpl-2.3* and *Tpl-2.4*), which were identical by DNA sequence analysis. To confirm the 5′ end of the *Tpl-2* gene, we constructed a second cDNA library from random-primed polyadenylylated RNA from normal rat spleen. The library was screened by hybridization to a 269-bp *Eco*RI–*Sst* I probe derived from the 5′ end of the cDNA clone *Tpl-2.3* (Fig. 2). Eleven clones were isolated. DNA sequence analysis revealed that none of them extended beyond the 5′ end of the *Tpl-2.3* cDNA. The 5′ end of 7 clones mapped in clusters of 150 bp and 20 bp 3′ of the 5′ end of clone *Tpl-2.3*. Primer extension and RNase protection assays confirmed that these sites may represent alternate sites of transcriptional initiation (data not shown).

DNA sequence analysis of the *Tpl-2.3* cDNA clone revealed a 468-aa open reading frame beginning with an in-frame ATG codon at nucleotide position 318. The predicted amino acid sequence indicates that *Tpl-2* encodes a protein kinase (Fig. 2). Starting at amino acid 145 from the ATG codon, the predicted protein contains the sequence PRGAFGKVYLAQDMKTKKRMACK (see Fig. 2), which is similar to the consensus motif of the ATP-binding domain of protein kinases (9). Moreover, starting at aa 253, it contains sequences (underlined in Fig. 2) that are characteristic of serine/threonine protein kinases (9–18). The Tpl-2 protein is closely related to the protein encoded by the previously identified oncogene *Cot*, which was cloned from transformed foci induced in SHOK cells by transfection of human genomic DNA (10). The predicted amino acid sequences of the two proteins are 87% identical. Some of the differences are scattered throughout the length of the two proteins, whereas others are clustered. One such cluster of dissimilar sequences extends between aa 129 and 151 and encompasses the ATP-binding domain. On this basis, we conclude that *Tpl-2* and *Cot* represent two closely related genes, although we cannot exclude the possibility that they may represent homologs of the same gene in two different species. The Tpl-2 protein also carries a unique 43-aa C-terminal tail. The tail contains five serine, three threonine, and three tyrosine residues, which represent potential targets for phosphorylation. The 318-bp region between the 5′ end of the cDNA clone *Tpl-2.3* and the ATG codon is open and contains three in-frame CTG codons (Fig. 2). These potential translation initiation sites (19) suggest that theoretically the *Tpl-2* mRNA may be translated into more than one protein isoforms.

Expression of *Tpl-2* in MoMuLV-induced rat T-cell lymphomas, with or without rearrangements in this locus, and in normal rat tissues was examined using the genomic DNA

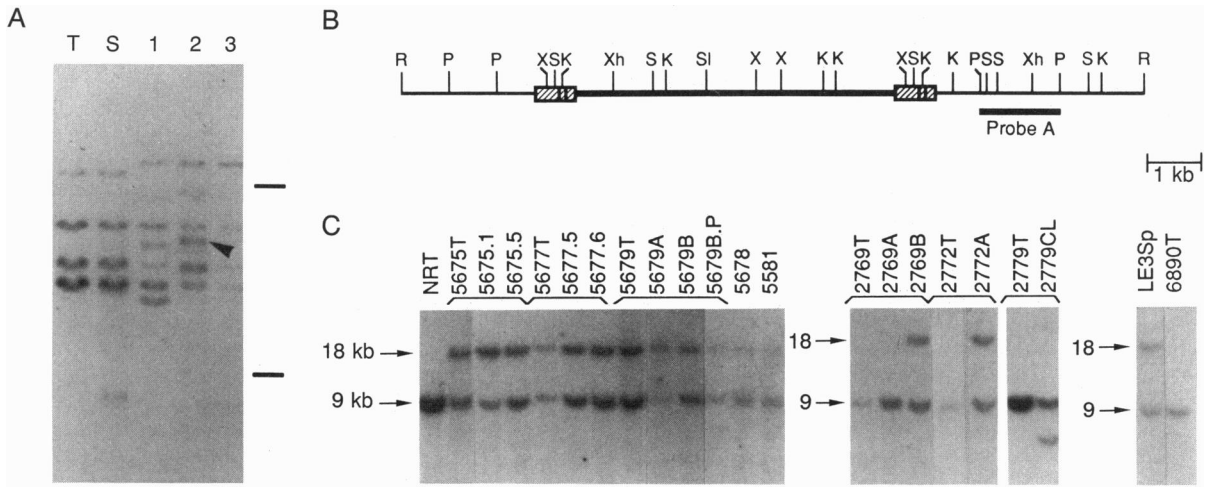

FIG. 1. (*A*) Southern blot analysis of *Eco*RI-digested DNA of the MoMuLV-induced T-cell lymphoma 2769 and cell lines derived from it in culture. The blot was hybridized to a MoMuLV LTR probe. T and S refer to tumor tissue derived from thymus and spleen of the primary tumor-bearing animal, respectively. Lanes 1–3, three tumor cell lines established in culture from the primary tumor. The arrowhead indicates the newly integrated provirus whose characterization we report. The positions of 9-kb and 18-kb λ/*Hin*dIII are indicated at the right. (*B*) Provirus integration in the *Tpl-2* locus in MoMuLV-induced rat T-cell lymphomas. The restriction endonuclease map of the *Eco*RI genomic DNA clone of the newly integrated provirus (thick black line) detected in subline 2 derived from tumor 2769 is shown. The map shows the site of provirus insertion and the orientation of the integrated provirus. The code for the restriction endonuclease sites is as follows: R, *Eco*RI; P, *Pst* I; X, *Xba* I; S, *Sst* I; K, *Kpn* I; Xh, *Xho* I; Sl, *Sal* I. The solid bar marked probe A indicates a *Pst* I genomic DNA fragment that was used as a probe to screen rat thymomas for *Tpl-2* rearrangements. (*C*) Southern blot analysis of *Eco*RI-digested genomic DNA from normal rat thymus (NRT) and multiple primary tumors (samples ending with the letter T) and cell lines derived from them (all other samples) hybridized to the *Tpl-2* genomic probe A. In three cases (tumors 2769, 2772, and 2779) provirus insertion was detected only after establishment in culture. The integrated provirus in the cell line 2779CL is a MoMuLV/VL30 recombinant (8) giving rise to a rearranged band that is smaller than the germ-line one.

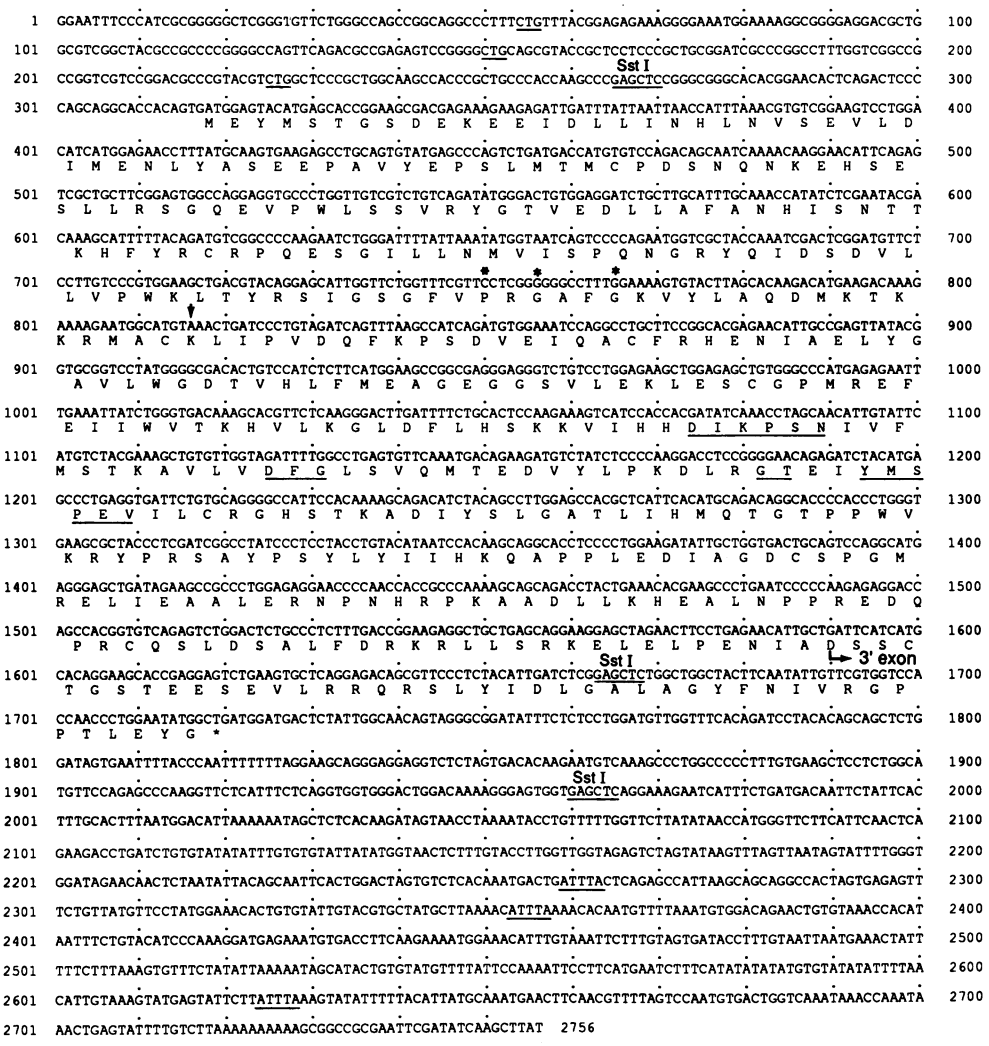

FIG. 2. Nucleotide and predicted amino acid sequence of the cDNA clone *Tpl-2.3* and direct biochemical analyses (C.P. and P.N.T., unpublished results) showed that *Tpl-2* encodes a serine/threonine protein kinase. The characteristic amino acid residues defining the ATP-binding site are indicated by three asterisks followed by a vertical arrow. Highly conserved or invariant amino acids characteristic of the catalytic domain of serine/threonine protein kinases are underlined. Also underlined are three in-frame CTG codons in the 5′ region of the cDNA clone and three copies of the putative destabilizing sequence ATTTA in the 3′ untranslated region. The boundary between the seventh and eighth exons is indicated by a bent arrow. The *Sst* I sites have been marked.

probe A from the region flanking the integrated provirus. This analysis revealed that *Tpl-2* was silent in all tumors lacking a provirus in this locus with only one exception (tumor 6890; data not shown). Tumors containing a provirus in *Tpl-2* expressed 1.6- and 10-kb RNA transcripts but lacked the 3-kb transcript detected in normal cells (Fig. 3*A*). Hybridization of a *Tpl-2.3* cDNA probe to the same RNA blot detected an ≈3-kb tumor-specific RNA transcript, which was expressed at high levels and which had not been detected with the genomic probe (Fig. 3*B*). Comparison of the sequence of cDNA clones derived from these transcripts with the sequence of the genomic DNA flanking the integrated provirus in the tested tumors revealed that: (*i*) The provirus always integrates in the last intron of *Tpl-2* and in the same transcriptional orientation as the gene. (*ii*) The 1.6- and 10-kb RNA transcripts are the products of promoter insertion-mediated transcription of the 3′ end of the gene and do not encode protein products. (*iii*) The 3-kb tumor-specific RNA is a truncated *Tpl-2* transcript that terminates in the proviral LTR. This transcript is predicted to encode a protein product with an altered C-terminal domain (Fig. 3*C*). The steady-state level of the 3-kb tumor-specific RNA in tumors harboring a provirus in *Tpl-2* is markedly enhanced compared to the level of the normal *Tpl-2* transcript in spleen cells (Fig. 3*B*). The enhanced steady-state level of the tumor-specific RNA could be the result of stabilization due to the removal of potentially destabilizing sequences in the last exon of the gene (20, 28). Analysis of the *Tpl-2.3* cDNA clone indeed revealed three copies of the putative destabilizing sequence ATTTA (21) in the 3′ untranslated region (Fig. 2). Alternatively, the enhanced levels of this RNA transcript could be due to enhancer insertion.

Cells undergoing provirus insertion in *Tpl-2* exhibit growth advantage. This was suggested by the demonstration that *Tpl-2* is a locus of common integration in our panel of MoMuLV-induced thymomas. Additional evidence supporting this conclusion was obtained by monitoring three sublines derived from tumor 2769 during passage in culture for provirus insertion in *Tpl-2*. After ≈50 passages, independent provirus insertions in this locus were detected in the cells selected from all three sublines (Fig. 4).

In normal T cells, *Tpl-2* contributes to the early events associated with mitogen-induced activation. Therefore, it may be involved in the entrance from a quiescent state to the G_1 phase of the cell cycle. This was suggested by Northern blot analysis of polyadenylylated RNA isolated from normal rat spleen cells before and at multiple time points after exposure to Con A (4 μg/ml). The results showed that Con

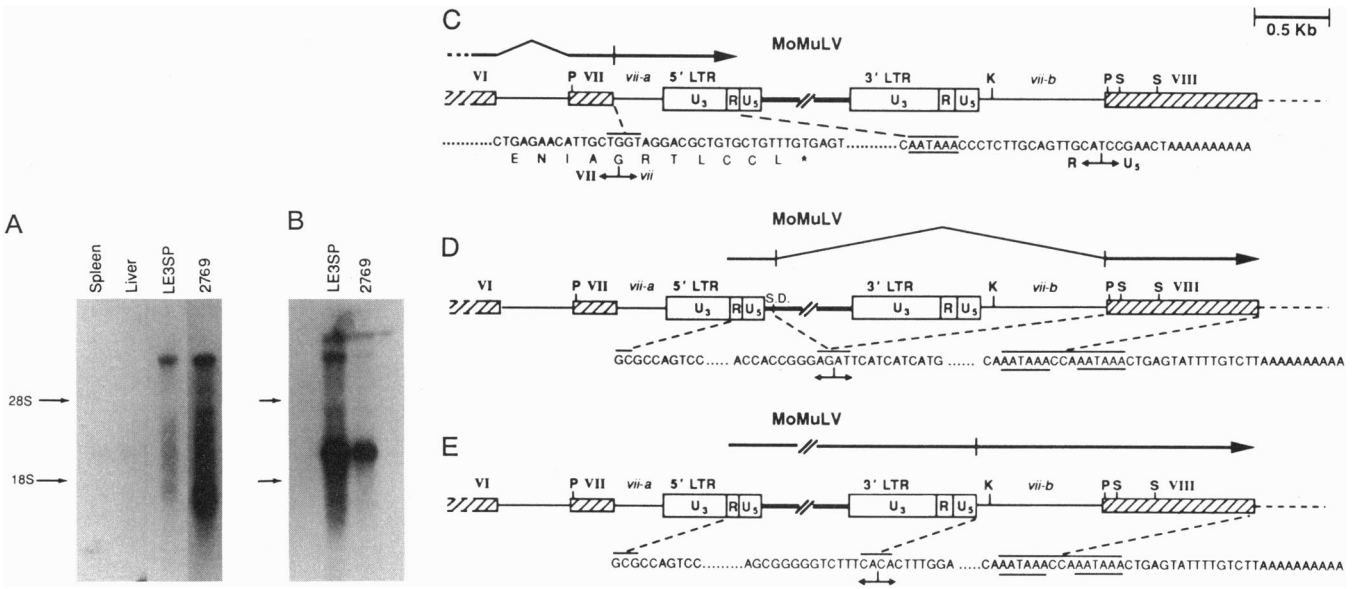

FIG. 3. Expression of *Tpl-2* in normal tissues and tumors harboring a provirus in *Tpl-2*. The structures of the tumor-specific RNA transcripts and predicted protein products are shown. (*A*) Northern blot analysis of poly(A)-selected RNAs derived from normal rat spleen and liver and from tumor cell lines LE3Sp and 2769, which carry a provirus in the *Tpl-2* locus. The blot was hybridized with the genomic *Tpl-2* probe A (Fig. 1*B*). (*B*) Hybridization of the tumor RNAs in *A* to the *Tpl-2.3* cDNA probe. (*C*) The 3-kb truncated RNA transcript in *B* (see text for details) is predicted to encode a protein product that contains 7 additional aa derived from truncated intron sequences but lacks the 43 C-terminal aa encoded by exon VIII. (*D* and *E*) Structure of the 1.6- and 10-kb RNA transcripts, respectively (see text for details). K, *Kpn* I; P, *Pst* I; S, *Sst* I. Hatched bars indicate *Tpl*-2 exons VI, VII, and VIII.

A induces enhanced expression of *Tpl-2* within the first 60 min from the time of exposure (Fig. 5).

DISCUSSION

The data presented in this report support the hypothesis that additional provirus insertions occurring spontaneously during tumor growth contribute to tumor progression. Moreover, they suggest that the process of acquisition of additional provirus insertions during cellular proliferation, which we call spontaneous insertional mutagenesis, can be used as a genetic tool to identify genes associated with selectable phenotypes. On the basis of these data, spontaneous insertional mutagenesis was used recently, with success, to identify genes involved in interleukin 2 signal transduction (24).

Provirus insertion in the *Tpl-2* locus, described in this report, is associated with the activation of a gene encoding a serine/threonine protein kinase. The activation of *Tpl-2* is characterized by the enhanced expression of a truncated RNA transcript that encodes a protein with an altered C-terminal domain. The biological outcome of the C-terminal alteration and/or enhanced expression of *Tpl-2* appears to be the promotion of cellular proliferation or the prevention of apoptosis. The detection of independent provirus insertions occurring during passage in culture in all three sublines of a single tumor provided strong evidence supporting this conclusion.

Comparison of Tpl-2 with the cAMP-dependent protein kinase revealed that the two proteins are identical in structurally critical residues. Therefore, similar to the cAMP-dependent protein kinase (22, 25), Tpl-2 is predicted to consist of two structurally distinct lobes: a small lobe consisting of the N-terminal portion of the kinase domain and containing the MgATP binding site and a large lobe containing the substrate-binding site. The two lobes should be linked by a loop that extends between amino acid residues Ser-221 and Lys-247 (Fig. 2). The Tpl-2 kinase also contains a unique 43-aa C-terminal tail, which is encoded by the last exon of the *Tpl-2* gene. This tail is of particular interest because it is deleted in the protein encoded by the activated *Tpl-2* gene, and it is replaced by a 7-aa stretch encoded by the intron sequences in the truncated *Tpl-2* RNA. Interestingly, similar to *Tpl-2*, the activation of the *Cot* gene induced as an artifact of the transfection process appears to have been the result of a 3′ end rearrangement (10). Most kinases contain unique C-terminal tails, which may contribute to the regulation of their enzymatic activity. In the case of the Src kinase, phosphorylation of a tyrosine residue at position 527 in the Src C-terminal tail promotes the intramolecular interaction between the tail and the Src homology 2 region (26). The

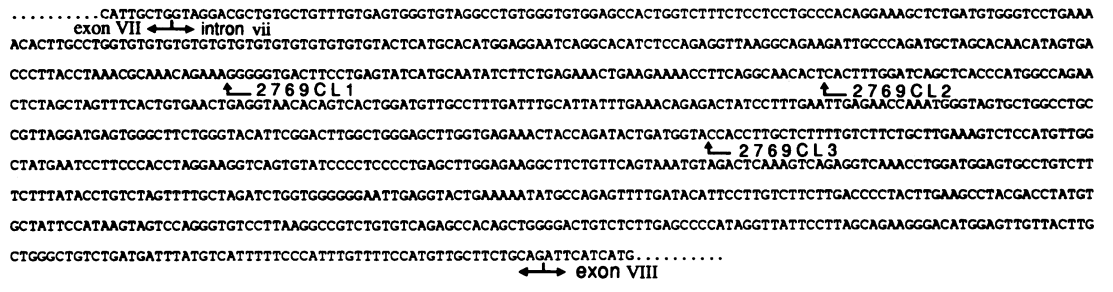

FIG. 4. Sites of provirus integration in the *Tpl-2* locus in three sublines derived from tumor 2769. Precise mapping of the site of integration was determined by PCR using genomic DNA template cloning and sequencing.

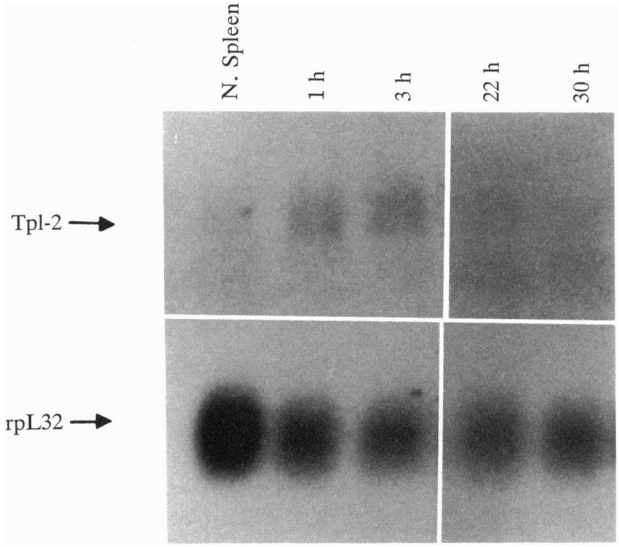

FIG. 5. Northern blot analysis of polyadenylylated RNA from unstimulated and Con A-stimulated rat spleen cells hybridized to the probe *Tpl-2.3*. (*Upper*) Hybridization to the *Tpl-2.3* cDNA probe. (*Lower*) Hybridization to a ribosomal protein L32 probe (23). N. Spleen, normal spleen.

protein folding promoted by this interaction may conceal the catalytic site, thus inhibiting the kinase activity of the protein. A similar form of regulation may also control the enzymatic activity of members of the protein kinase C (PKC) family. It has been shown previously that the C-terminal tail of PKCβII contains two threonine residues, which are targets of intramolecular autophosphorylation, suggesting that the tail folds over the catalytic site, perhaps altering the activity of the kinase (27). The data in this report, combined with the demonstrated or suggested regulatory role of the C-terminal tails of other kinases, represent strong evidence indicating that the C-terminal tail of Tpl-2 plays an important role in regulating the catalytic and biological activities of the protein.

Provirus insertion in the last intron of *Tpl-2* appears to exert a dual oncogenic role: (*i*) it gives rise to a truncated RNA transcript encoding a kinase with an altered C-terminal tail, and (*ii*) it enhances the steady-state level of this RNA in tumor cells. The latter may be secondary to the truncation of the RNA transcript, which deletes the 3′ untranslated region of the gene harboring three copies of the RNA-destabilizing sequence ATTTA. Therefore, provirus insertion may lead to the stabilization of the *Tpl-2* RNA message as seen previously in the case of provirus-mediated activation of *pim-1* (24).

The contribution of *Tpl-2* to the growth selection operating during the progression stage of rodent T-cell lymphomas appears to be an expression of the function of this gene in normal lymphocytes. Stimulation of normal rat spleen cells with Con A induces the expression of enhanced levels of *Tpl-2* within the first hour from the time of exposure. This suggests that the Tpl-2 kinase may be involved in the transmission of mitotic signals regulating the transition of normal lymphocytes from a quiescent state to the G_1 phase of the cell cycle.

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