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Deregulated expression of *TCL1* causes T cell leukemia in mice

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ABSTRACT The *TCL1* oncogene on human chromosome 14q32.1 is involved in the development of T cell leukemia in humans. These leukemias are classified either as T prolymphocytic leukemias, which occur very late in life, or as T chronic lymphocytic leukemias, which often arise in patients with ataxia telangiectasia (AT) at a young age. The *TCL1* oncogene is activated in these leukemias by juxtaposition to the α or β locus of the T cell receptor, caused by chromosomal translocations t(14:14)(q11:q32), t(7:14)(q35:q32), or by inversions inv(14)(q11:q32). To show that transcriptional alteration of *TCL1* is causally involved in the generation of T cell neoplasia we have generated transgenic mice that carry the *TCL1* gene under the transcriptional control of the $p56^{lck}$ promoter element. The *lck-TCL1* transgenic mice developed mature T cell leukemias after a long latency period. Younger mice presented preleukemic T cell expansions expressing *TCL1*, and leukemias developed only at an older age. The phenotype of the murine leukemias is $CD4^-CD8^+$, in contrast to human leukemias, which are predominantly $CD4^+CD8^-$. These studies demonstrate that transcriptional activation of the *TCL1* protooncogene can cause malignant transformation of T lymphocytes, indicating the role of *TCL1* in the initiation of malignant transformation in T prolymphocytic leukemias and T chronic lymphocytic leukemias.

Nonrandom translocations, involving either the Ig or the T cell receptor (TCR) loci, juxtapose cellular protooncogenes to strong enhancer elements, leading to oncogene deregulation and tumor initiation (1–3). Overexpression of the *TCL1* gene in humans has been implicated in the development of mature T cell leukemia, in which chromosomal rearrangements bring the *TCL1* gene in close proximity to the TCRα or TCRβ regulatory elements (4, 5). T cell leukemias associated with *TCL1* activation are most commonly classified as T prolymphocytic leukemia (T-PLL), a very aggressive mature T cell proliferation, and sometimes as T chronic lymphocytic leukemia (T-CLL) (6). T cell leukemias arising in patients with ataxia telangiectasia often carry *TCL1* rearrangements. These rearrangements and *TCL1* activation are already present in T cell clonal expansions of these patients at the preleukemic stage (7). These findings suggest that *TCL1* activation may be the first step in the neoplastic process and might be present at the preleukemic stage for an extended period of time before the occurrence of secondary genetic events, which would then result in the onset of overt leukemia. It has already been shown that the development of aggressive leukemia or lymphoma is a multistep process. For example, follicular lymphoma with BCL2 rearrangements can progress to a more malignant form by acquiring a c-MYC rearrangement (8, 9). Another example is the transition from the chronic to the acute phases in chronic myelogenous leukemia (CML), in which p53 mutations and deletions are observed in 20–30% of the cases of CML in blastic crisis (10).

TCL1 codes for a protein of 114 aa that is expressed in the cytoplasm and in the nucleus of lymphoid cells (5). In normal T cells *TCL1* is expressed in $CD4^-CD8^-$ cells, but not in cells at later stages of differentiation (5). We have also shown that the Tcl1 protein has a high similarity to $p13^{MTCP1}$ (11), a protein encoded by one of the ORFs of the MTCP1 gene and involved in the t(14:X)(q32:q28) chromosome translocation in T cell proliferative diseases (12). To understand the effect of *TCL1* overexpression in the lymphoid compartment and whether the *TCL1* deregulation produces lymphoid tumors in experimental models, we generated transgenic mice carrying the *TCL1* gene under the control of the T cell-specific gene promoter $p56^{lck}$ whose expression specifically targets gene expression in the thymus (13). The development of *TCL1* transgenic mice represents an optimal system to study the multiple genetic events involved in the leukemogenic process of T-PLL and T-CLL.

METHODS

Constructs and Transgenic Mice. A schematic representation of the construct used to generate *lck-TCL1* transgenic mice is given in Fig. 1. The original plasmid containing the *lck* proximal promoter (lck^{pr}), the 3′ untranslated region, and the poly(A) site for the human growth hormone gene (hGH) was kindly provided by Roger Perlmutter, University of Washington, Seattle (13). The construct was obtained by inserting a 350-bp fragment containing the entire human *TCL1* coding sequences into a *Bam*HI restriction site downstream to the lck promoter. The construct was freed from vector sequences by digestion with *Not*I, purified from agarose gel, and injected in fertilized mouse oocytes essentially as described (14). The fertilized mouse oocytes were derived from superovulated $(B6C3)F_1$ animals. Successful integration of the injected DNA was monitored by Southern analysis of tail tip DNA as described (14), by using as probe the same DNA fragment used to inject the oocytes. Founder mice were backcrossed with C57BL/6J mice.

Protein Extracts from Mouse Tissues. Tissues were homogenized with a hand homogenizer for 30–60 sec in a solution containing 50 mM Tris·HCl (pH 7.6), 150 mM NaCl, 1% NP40, and 1 mM phenylmethylsulfonyl fluoride. The samples were kept on ice for 15 min followed by centrifugation at 12,000 rpm for 15 min at 4°C. Supernatants were analyzed by Western blot.

Flow Cytometry. Single-cell suspensions were made at the time of autopsy from spleen, thymus, and lymph nodes of transgenic and control mice in PBS supplemented with 1%

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Abbreviations: AT, ataxia telangiectasia; ATL, adult T cell leukemia; *TCL1*, T cell leukemia-1; T-CLL, T chronic lymphocytic leukemia; T-PLL, T prolymphocytic leukemia; TCR, T cell receptor; WBC, white blood cell.

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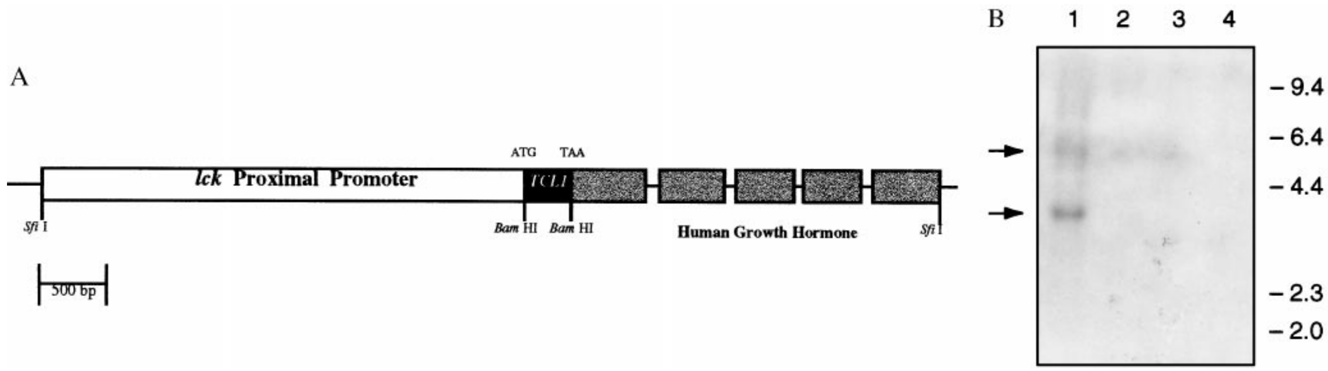

FIG. 1. (*A*) DNA construct used in generating human *lck-TCL1* transgenic mice. A 0.35-kb cDNA fragment containing the entire human *TCL1* coding region was inserted into the *Bam*HI site of a vector containing the lck proximal promoter and the hGH 3′ untranslated region and poly(A) addition site. (*B*) Southern blot analysis of DNA from tail clips from the first transgenic progeny. Blots were hybridized with the same DNA fragment used for injection of zygotes. Lane 1, DNA from a positive founder mouse; lanes 2–4, DNA from nontransgenic progeny.

bovine calf serum and 0.01% sodium azide (staining solution). Cells were washed in this solution, and for direct labeling 1×10^6 cells were resuspended with 100 μl of fluorescein isothiocyanate (FITC)- or phycoerythrin (PE)-conjugated antibodies diluted 1:100 in staining solution and incubated for 30 min at 4°C. Cells were washed three times with cold PBS, fixed with 1% paraformaldehyde for 15 min, and washed once with PBS. Conjugated FITC anti-CD4, FITC anti-TCR, PE anti-CD8, and T cell receptor Vβ region-specific antibodies (Vβ2–8, Vβ8.2, Vβ8.3, Vβ9, Vβ10b, and Vβ11–14, PharMingen) were used according to manufacturer protocols. Cells were analyzed on a Coulter Profile II flow cytometer. Dyes were exited at 488 nm, and the emissions were collected at 525 nm (FITC) and 575 nm (PE).

Tissue Immunohistochemistry. Tissues isolated from transgenic mice and control mice were fixed in 4% paraformaldehyde and paraffin-embedded. Five-micron-thick serial sections were cut from formalin-fixed, paraffin-embedded specimens and mounted on Plus slides (Fisher Scientific). These sections were deparaffinized and rehydrated through graded xylene and alcohol series and placed in citrate-buffered solution (pH 6.0), and then heated at 100°C by microwave oven for 20 min for antigen retrieval as previously described (15). Endogenous peroxidase was blocked with 3% hydrogen peroxide, and nonspecific binding was blocked with 10% normal serum. Polyclonal rabbit anti-mouse antibodies specific for recombinant human Tcl1 were used at 1:200 dilution. Immunohistochemical staining was performed with Vectastain ABC kit (Vector Laboratories). Diaminobenzidine was used for coloration, and nuclei were counterstained with hematoxylin. Secondary antibody alone was consistently negative on all sections. Normal goat serum was substituted for the primary antibody in negative controls.

TCR Rearrangements. DNA isolated from splenocytes and thymocytes was digested with *Eco*RI restriction enzyme, and Southern blots were obtained as described (16). The blots were hybridized with a DNA probe specific for the Jβ_2 segment of the TCR gene described by Kronenberg *et al.* (17), followed by autoradiography.

RESULTS

lck^pr^-*TCL1* Transgenic Mice. The *TCL1* gene is normally expressed in $CD4^-CD8^-$ thymocytes and in pre-B/IgM^+ cells (5). Based on *TCL1* tissue specificity and its involvement in leukemias of T cell origin, the lck proximal promoter was used to target *TCL1* expression in the T cell compartment of transgenic mice. A transgenic construct was generated by introducing the coding sequences of human *TCL1* cDNA into the *Bam*HI cloning site, which is located downstream of the $p56^{lck}$ promoter region (Fig. 1*A*). The 3′ untranslated portion of this construct provided introns, exons, and the poly(A) site for the human growth hormone gene (hGH). Purified DNA of the construct was microinjected into zygotes and transplanted into pseudopregnant females. Founder mice were identified by screening of the transgenic progeny tail DNA by Southern hybridization with a human *TCL1* DNA probe, which does not cross-hybridize with mouse sequences (Fig. 1*B*). Transgenic lines were successfully established and analyzed by Western blot by using antibodies specific for human Tcl1 protein. Fig. 2 shows the immunoblot result of total protein extracts from the thymus, liver, spleen, and kidney from a representative transgenic (TG) and nontransgenic mouse (non-TG). High expression of the human Tcl1 protein was observed in the thymus of the transgenic mouse. No expression was visible in the spleen whole extracts by immunoblot analysis; however, a very low level of expression could be detected in the spleen after enrichment for lymphocytes (data not shown).

lck^pr^-*TCL1* Transgenic Mice Develop Lymphocytic Leukemia with Very Late Onset. Transgenic animals were analyzed at 1–3 months of age to determine the presence of developing malignancies. No pathological signs of tumor development were evident, and flow cytometry of the thymocytes showed normal distribution of $CD3^+$, $CD4^+CD8^-$, $CD4^-CD8^+$, and $CD4^+CD8^+$ populations. Splenic populations were not analyzed because the organs appeared normal in size and color. However, peripheral blood of some mice showed increases in the percentage of lymphocytes (75–90%) and in the cell counts compared with normal mice (60%). The transgenic mice were

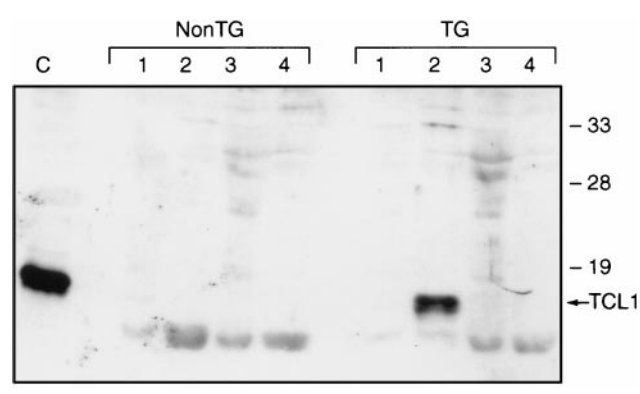

FIG. 2. Western blot analysis of protein extracts from transgenic (TG) and nontransgenic (non-TG) mouse tissues. Proteins (100 μg) were loaded per lane: 1, kidney; 2, thymus; 3, liver; and 4, spleen. The control lane, C, contains extracts from the pre-B leukemic cell line 697, which expresses high levels of the Tcl1 protein (5).

Table 1. Phenotype of leukemias from lck-*TCL1* transgenic mice

Age	No. mice/total	Phenotype
1–12	0/5	Leukemia*
12–14	9/13	T cell expansion†
	2/13	Lymphocytic leukemia‡
15–20	6/10	Lymphocytic leukemia

*Mice were analyzed for clonal expansions, increased lymphoid count, or other abnormalities characteristic of leukemia.
†Characterized by an increase in $CD3^+$ cells and skewed CD8/CD4 ratio compared with normal mice and no detectable clonal TCR rearrangement (see text for details).
‡Leukemia was determined by clonal rearrangement of the T cell receptor as detected by a Jβ2 probe, splenomegly, increased WBC count, and any lymph node or thymic abnormalities (increased size or altered architecture).

kept under close supervision and checked biweekly for signs of illness. Mice became visibly ill around the age of 15–20 months. These mice presented with enlarged spleens, enlarged lymph nodes and thymus, and high white blood cell (WBC) counts (Table 1). An example of a representative enlarged spleen is shown in Fig. 3. The weight of the transgenic spleen of a leukemic animal (TG 39) shown in this figure was 2 g, approximately 30 times the weight of a normal spleen (normal splenic weight was 0.07 ± 0.01 g). Immunohistochemical analysis of tumorigenic spleens with anti-Tcl1 polyclonal antibodies showed staining in numerous clusters of lymphocytes showing a uniform morphology, indicating that leukemic cells express Tcl1 (Fig. 4). Flow cytometric analysis of splenocytes indicated that the phenotype of the leukemic cells is $CD3^+$ and $CD4^-CD8^+$ in all the cases analyzed (Table 2). This observation contrasts with the situation in humans where T cell leukemias with *TCL1* involvement are most often $CD3^+$ and $CD4^+CD8^-$. Pathological examination of peripheral blood smears classified the malignancies as lymphocytic leukemias with the predominant cell type represented by a large (10–15 μ in diameter) lymphoid cell.

Lymphocyte Populations in Transgenic Mice. Transgenic mice between the age of 12 and 14 months were also analyzed for pathological abnormalities. Some of the animals presented slightly enlarged spleens and a high white blood cell count but with no hyperplasia of the thymus or lymph nodes (Table 2).

FIG. 3. Whole spleen specimen isolated from a leukemic mouse (*Left*, TG 39) and a control mouse (*Right*).

In two of the mice, TG71 and TG82, the presence of full-blown malignancy was observed (Table 1). Flow cytometric analysis of the T cell populations in transgenic spleens indicated that the $CD3^+$ population is high in all the animals examined, ranging between 50 and 70% (Table 1). The percentage of $CD8^+$ T cells is nearly doubled, 21–34%, compared with control mice (nontransgenic), whereas the percentage of $CD4^+$ cells shows only a slight increase in some specimens. The ratio $CD4^+/CD8^+$ in the spleens of the transgenic mice was 1.0 compared with 1.7 in control mice. Immunocytochemical analysis of the spleens with *TCL1* antibodies showed the presence of large numbers of lymphoid cells expressing *TCL1* (Fig. 4). It is possible that an early or preleukemic stage exists in these mice, denoted by the increased white blood cell count, expansion in the $CD3^+$ and $CD8^+$ populations, and increased numbers of *TCL1*$^+$ cells clustered within the spleen (Fig. 4 *E* and *F*). We predict that these mice are at increased risk for leukemia as they age. Future experiments will be needed to determine the relationship between each stage of disease.

T Cell Receptor Rearrangements in *lck-TCL1* Mouse Tumors. Southern blot analysis of DNA isolated from leukemic cells with probes for the TCRβ chain gene joining region Jβ2 showed clonal rearranged bands (Fig. 5*A*). DNA from healthy transgenic spleens showed the TCRβ gene in its germ-line configuration, whereas DNA from leukemic splenocytes show the presence of extra rearranged bands, indicating the presence of clonal T cell populations. As expected, DNA from spleen, thymus, and lymph nodes from the same leukemic animals all present the same TCR gene rearrangements (Fig. 5*A*). In one representative case the expressed Vβ gene was found to be the Vβ6 gene by flow cytometry (Fig. 5*B*). The presence of clonal TCR gene rearrangements in conjunction with the phenotypic data confirms the diagnosis of T cell leukemia. DNA from spleens of asymptomatic transgenic mice (mice <1 year old) showed expansions of the $CD3^+$ and $CD8^+$ cells although clonal TCR gene rearrangements were not detected (Fig. 5*A* and data not shown), suggesting that these T cell expansions were not clonal at this stage.

Table 2. Cell surface phenotype of transgenic lymphocytes

Animal number	Percent of spleen cells positive for CD3	CD4	CD8	WBC*, × 10^6
N 1	36.0	21.0	12.0	1.8
N 2	35.0	24.0	12.0	1.8
N 3	36.0	22.0	16.0	ND
TG 37	63.0	17.0	29.0	2.6
TG 54	57.0	21.0	34.0	6.2
TG 60	54.0	33.0	17.0	11.6
TG 68	55.0	34.0	21.0	ND
TG 72	64.0	37.0	22.0	ND
TG 73	67.0	31.0	27.0	5.6
TG 74	70.0	32.0	30.0	2.4
TG 83	49.0	23.0	30.0	ND
TG 108	65.0	27.0	24.0	2.4
TG 109	63.0	22.0	35.0	1.8
TG 116	52.0	27.0	26.0	24.8
TG 117	55.0	24.0	22.0	ND
TL 25	65.0	1.0	60.0	ND
TL 39	99.0	1.4	97.0	168.0
TL 47	65.0	20.0	40.0	ND
TL 53	75.0	3.0	74.0	ND
TL 71	63.0	21.0	40.0	ND
TL 82	65.0	10.0	73.0	ND

N, normal; TG, transgenic; TL, T cell leukemic mice; ND, not done.
*The mean WBC/ml blood for normal adult mice (10–18 months old) was 2.6 ± 0.4 × 10^6 cells/ml (n = 10).

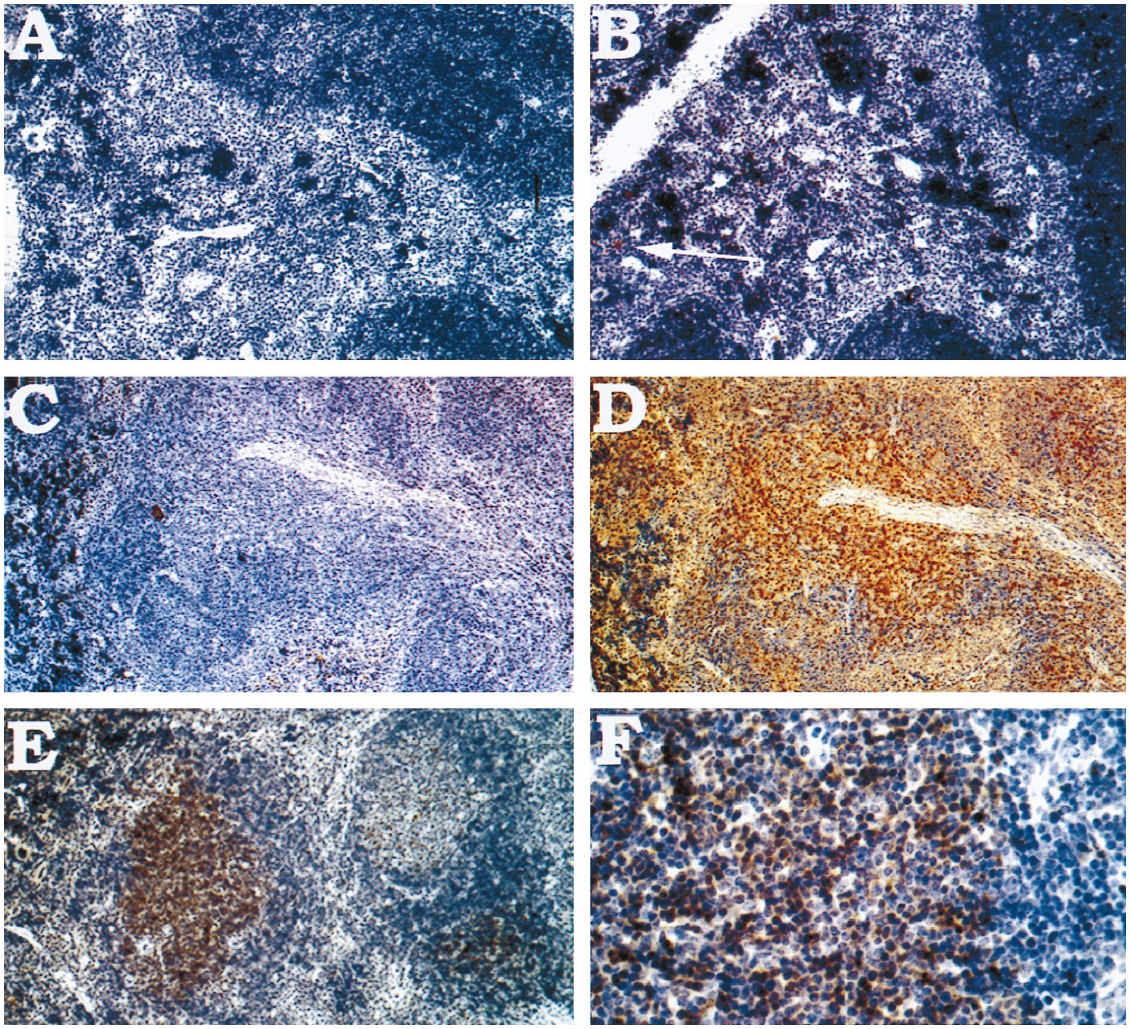

FIG. 4. Immunocytochemical analysis of Tcl1 protein in transgenic mouse spleens. Normal mouse spleen (negative control) stained for Tcl1 protein using polyclonal anti-Tcl1 antibody (*A*). A young transgenic mouse (<1 year old) stained for Tcl1 protein (*B*). Arrow in *B* shows a small cluster of positively stained cells. Small numbers of stained cells can also be observed scattered throughout the specimen. Spleen specimen from a representative animal containing extensive tumor infiltration stained with secondary antibody alone (*C*) or together with anti-Tcl1 polyclonal antibodies (*D*). A low (×10) magnification (*E*) and high (×40) magnification (*F*) depicting a foci of Tcl1-stained cells in pre- (or early) leukemic mice.

DISCUSSION

The *TCL1* gene is involved in the development of T-PLL and T-CLL in humans (5). One T-PLL characteristic is the very late onset in life with a median age of 69 years (6). Leukemias carrying *TCL1* rearrangements also frequently occur in AT patients at a younger age (18, 19). These patients have been reported to carry premalignant T cell expansions coincident with *TCL1* activation (7). It is possible that the same scenario may be true for individuals developing T-PLL, where the premalignant clones may go undetected in asymptomatic individuals. Whereas these individuals may carry the T cell expansions for an extended period before the occurrence of secondary genetic events, AT patients may develop overt leukemia much earlier because of secondary mutations occurring more frequently. lck^{pr}-*TCL1* transgenic mice, which express the transgene specifically in the T cell compartment, develop T cell leukemia after a long latency period closely resembling the leukemia in humans. The leukemias observed in transgenic mice are mature in phenotype, as in humans, but are restricted to the $CD3^+$, $CD4^-CD8^+$ subset. This phenotype is at variance with that of human leukemias with *TCL1* involvement, which are most often $CD3^+$ and $CD4^+CD8^-$ (6). Although we cannot explain why the tumors developing in mice are exclusively $CD8^+$, it cannot be attributed to the promoter specificity because *lck*-BCL2 transgenic mice develop leukemia of the $CD4^+CD8^-$ phenotype after a similarly long latency period (20). Young transgenic mice (<1 year old) already show an increase in the $CD3^+$ and $CD8^+$ populations, suggesting that *TCL1* may influence the ratio of CD4 and CD8 lymphocytes in the spleen. These early expansions could not be considered clonal, because analysis of TCR gene rearrangement failed to detect clonal rearrangements. However, this indicates a selective advantage for the cells expressing *TCL1* resembling the preleukemic clonal expansions in humans. In fact, whereas in human leukemia all T cells overexpressing *TCL1* carry a specific rearrangement juxtaposing the TCRα to the *TCL1* gene and hence are clonal, in lck-*TCL1* transgenic mice *TCL1* expression is forced in all T cells leading to the possible expansion of the entire T cell repertoire. Thus, we hypothesize that additional genetic changes are needed to cause the development of overt leukemia. In this model, *TCL1* overexpression gives the cells a growth advantage increasing the probability for the occurrence of secondary genetic events responsible for the development of aggressive malignancy.

The development of *TCL1* transgenic mice represents a very interesting model to study multiple genetic events involved in leukemogenesis, and we are in the process of establishing cell

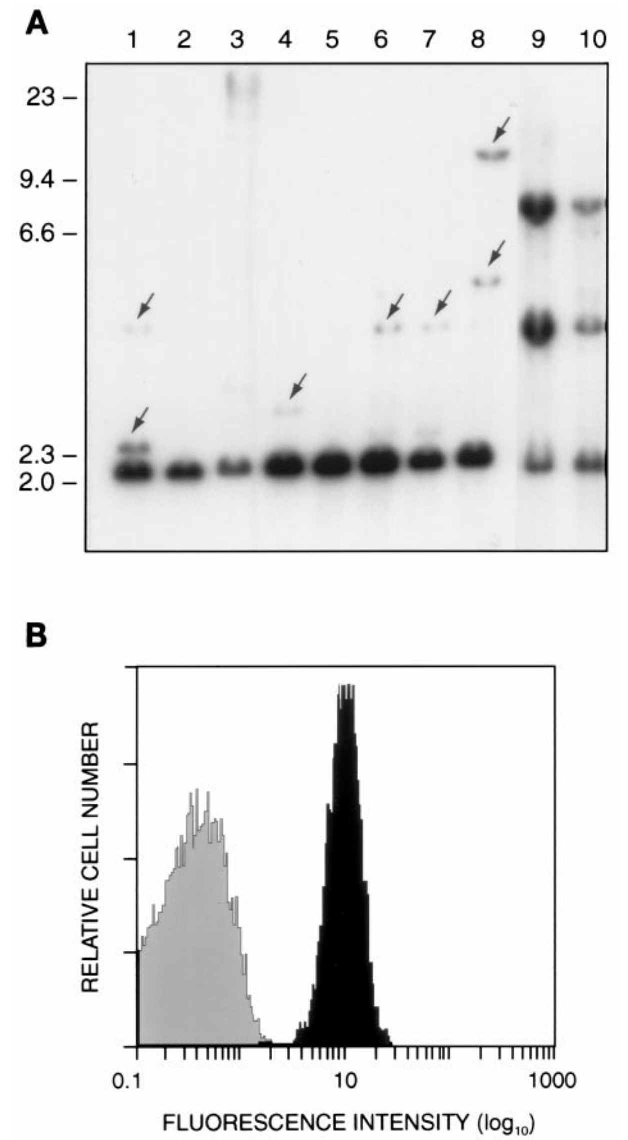

FIG. 5. Southern analysis of TCR gene rearrangements in leukemias from transgenic mice. (*A*) Clonal TCR rearrangements. Southern blot analysis of DNA isolated from the spleens or thymuses of leukemic and nonleukemic mice. DNAs were digested with *Eco*RI. Blots were hybridized with a probe specific for the TCR Jβ_2 gene segment. Lanes: 1, spleen TG25; 2, spleen non-TG littermate; 3, spleen TG28 (partial digest); 4, spleen TG 43; 5, spleen TG 45; 6, spleen TG 47; 7, thymus TG 47; 8, spleen TG 39; and 10, thymus TG39. The strong 2.2-kb bands represent the TCR Jβ_2 gene in its germ-line configuration; arrows point to weaker hybridizing bands indicating the presence of clonal TCR gene rearrangements. (*B*) Expression of a specific TCR-Vβ on the surface of mouse leukemic cells. Single-cell suspensions of splenocytes from a representative tumor were stained with anti-Vβ6 antibodies (black) or with control antibody (FITC control, gray) followed by fluorescence-activated cell sorter analysis.

lines from the leukemic cells to study these events. For these studies secondary genetic events may also be identified by crossing *TCL1* transgenic mice with $Atm^{-/-}$ or $p53^{-/-}$ mice.

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