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Tal-1 induces T cell acute lymphoblastic leukemia accelerated by casein kinase IIα

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Ectopic activation of the *TAL-1* gene in T lymphocytes occurs in the majority of cases of human T cell acute lymphoblastic leukemia (T-ALL), yet experiments to date have failed to demonstrate a direct transforming capability for *tal-1*. The *tal-1* gene product is a serine phosphoprotein and basic helix–loop–helix (bHLH) transcription factor known to regulate embryonic hematopoiesis. We have established a transgenic mouse model in which *tal-1* mis-expression in the thymus results in the development of clonal T cell lymphoblastic leukemia/lymphoma. Thus, overexpression of *tal-1* alone can be transforming, verifying its pathogenic role in human T-ALL. In addition, leukemogenesis is accelerated dramatically by transgenic co-expression of *tal-1* and the catalytic subunit of casein kinase IIα (CKIIα), a serine/threonine protein kinase known to modulate the activity of other bHLH transcription factors. Although *tal-1* is a substrate for CKII, the synergy of the *tal-1* and *CKIIα* transgenes appears to be indirect, perhaps mediated through the E protein heterodimeric partners of *tal-1*. These studies prove that dysregulated *tal-1* is oncogenic, providing a direct molecular explanation for the malignancies associated with *TAL-1* activation in human T-ALL.

Keywords: CKII/leukemogenesis/*Tal-1*

Introduction

Over the past decade, significant progress has been made in understanding the molecular basis of hematopoietic malignancies. Studies of non-random chromosomal translocations associated with leukemia and lymphoma have identified genes that are critical regulators of normal hematopoietic cell proliferation and differentiation. Often dysregulated expression of these genes seems to lead to cell transformation. One of these genes, identified by analysis of the (1;14)(p34;q11) translocation in ~3% of patients with T cell acute lymphocytic leukemia (T-ALL), is *tal-1* (Begley *et al.*, 1989; Finger *et al.*, 1989; Bernard *et al.*, 1990; Carroll *et al.*, 1990; Chen *et al.*, 1990a). The same gene was also found to be expressed in the 'stem cell' leukemia blast crisis of chronic myelogenous leukemia, where it was termed *SCL* (Begley *et al.*, 1989).

Tal-1 encodes a basic helix–loop–helix (bHLH) protein that has been shown to regulate embryonic hematopoiesis (Shivdasani *et al.*, 1995; Porcher *et al.*, 1996). In the erythroid cell, the tal-1 protein heterodimerizes with the E2A gene products (E47/12) and recognizes the E box canonical sequence (CANNTG) (Hsu *et al.*, 1991). Once bound to either E47 or E12, tal-1 transactivates transcription *in vivo* (Hsu *et al.*, 1991). More recently, the so-called 'LIM' protein, rbtn2 or LMO2, has been shown to bind to the tal-1–E2A complex (Valge-Archer *et al.*, 1994). *LMO2*- and *tal-1*-deficient mice generated by homologous recombination exhibit a similar phenotype of mid-embryonic lethality, due to an inability to develop erythroid cells, suggesting that *LMO2* and *tal-1* are involved in a common pathway (Warren *et al.*, 1994; Shivdasani *et al.*, 1995).

Aberrant expression of *TAL-1* occurs in most patients with T-ALL (Bash *et al.*, 1995). Although inter-chromosomal translocations involving *TAL-1* are relatively rare (Begley *et al.*, 1989; Finger *et al.*, 1989; Bernard *et al.*, 1990; Carroll *et al.*, 1990; Chen *et al.*, 1990b), activation of *TAL-1* can occur by two other mechanisms. Twenty five percent of patients undergo a precise interstitial 90 kb deletion of a portion of the 5′ region of chromosome 1 which brings the promoter of a T cell-specific gene of unknown function, termed *SIL* (*SCL/TAL*-interrupting locus), into proximity with *TAL-1*, resulting in expression of *TAL-1* in the T cell (Aplan *et al.*, 1990; Brown *et al.*, 1990). More recently, an additional 30% of T-ALL patients negative for the deletion and chromosomal translocations were found to ectopically express *TAL-1* (Bash *et al.*, 1995). This study suggests that mutations of *cis*- or *trans*-acting factors controlling *TAL-1* expression contribute to *TAL-1* deregulation in leukemia.

Thus, *tal-1* appears to be a critical regulator of early hematopoiesis in normal cells, and correlative evidence suggests that it might be the gene most frequently activated in human T cell leukemia. Surprisingly, attempts to demonstrate the oncogenic activity of *tal-1* directly have been largely unsuccessful. In cell culture, *tal-1* has been shown to enhance the tumorigenicity of v-*abl* (Elwood *et al.*, 1993). In two studies in transgenic mice, CD2–*tal-1* constructs were expressed in T cells, yet failed to induce lymphoma or leukemia (Robb *et al.*, 1995; Larson *et al.*, 1996). In one of these studies, the CD2–*tal-1* transgene was unable to accelerate the development of lymphomas caused by Moloney murine leukemia virus (Robb *et al.*, 1995). These studies questioned the hypothesis that aberrantly expressed *tal-1* is oncogenic, and raised the possibility that perturbation of other genes near the *tal-1* locus might be responsible for T-ALL. In the more recent study, *tal-1* by itself had no oncogenic effects in transgenic mice; but, when this transgene was bred into a background also carrying the *LMO2* oncogene, it did accelerate tumor formation (Larson *et al.*, 1996).

To clarify the pathogenic role of *TAL-1* in human

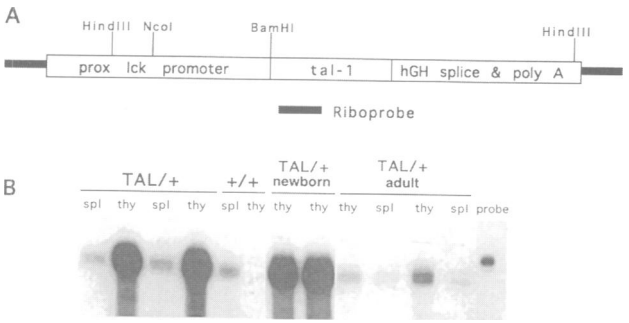

Fig. 1. Structure and expression of a *tal-1* transgene. (**A**) Diagrammatic representation of the *lck–tal-1* fusion construct used to create transgenic mice. The murine *tal-1* cDNA subcloned into a vector with the proximal *lck* promoter and human growth hormone (hGH) splice and poly(A) addition sequences was used to establish six lines of transgenic mice designated 4709, 4727, 4723, 4726, 4719 and 4722. (**B**) Expression of the *tal-1* transgene. RNA prepared from thymus and spleen of wild-type (+/+) and transgenic (tal/+ from lines 4709 and 4727 are shown) was subjected to RNase protection analysis with the antisense riboprobe indicated in (A). The endogenous (spl) and transgenic *tal-1*(thy) mRNA protect a band of 270 bases. To monitor *tal-1* mRNA levels throughout thymic development, RNA was isolated from newborn and adult transgenic pups and the RNase protection assay performed.

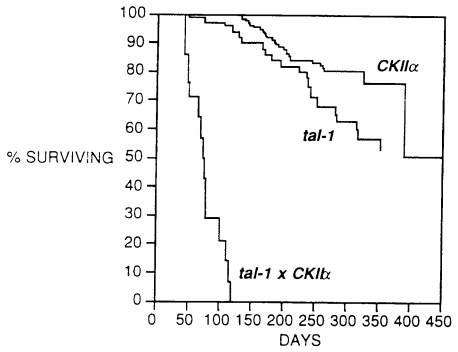

Fig. 2. Kaplan–Meier survival plot of *tal-1*, *CKIIα* and *tal-1/CKIIα* transgenic mice. Survival plot for *tal-1* transgenic lines (*tal-1*), CKII transgenic lines (*CKIIα*) and one arbitrary bi-transgenic line (*tal-1/CKIIα*). The cohort of *tal-1*-only mice consisted of $n = 75$ animals. The *CKIIα*-only cohort consisted of $n = 127$ animals and the *tal-1/CKIIα* bi-transgenic cohort consisted of $n = 14$ animals. All animals were monitored daily for any evidence of disease. Upon onset of disease the mice were sacrificed and a post-mortem examination was done.

T-ALL and to attempt to establish a murine model for the disease, we chose another T cell-specific promoter for a *tal-1* transgene, the promoter of the *src* family kinase, *lck*, which directs high level expression of other transgenes to thymocytes (Garvin *et al.*, 1990; Abraham *et al.*, 1991; Sentman *et al.*, 1991; McGuire *et al.*, 1992). Furthermore, in an attempt to activate the *tal-1* transgene, we mated *lck–tal-1* transgenic mice with casein kinase IIα (CKIIα) transgenic mice. CKII is a ubiquitous serine/threonine kinase that regulates the activity of a number of bHLH transcription factors (Hunter and Karin, 1992) and has been postulated to phosphorylate the tal-1 protein *in vivo* (Goldfarb *et al.*, 1992). Through these strategies, we have been able to provide direct evidence that mis-expression of a *tal-1* transgene can be transforming and, furthermore, that there is marked synergy between the *tal-1* and *CKIIα* transgene products, suggesting that CKII may regulate tal-1 transcriptional activity in vivo.

Results

Tal-1 transgenic mice

A transgenic construct was generated by placing the mouse *tal-1* cDNA under the control of the *lck* proximal promoter (Figure 1A). The 3′ untranslated region of this construct contains introns, exons and the poly(A) addition site of the human growth hormone gene (Abraham *et al.*, 1991). The *lck–tal-1* construct was microinjected into the pronuclei of fertilized FVB/N oocytes (Taketo *et al.*, 1991). Six transgenic founders were identified initially, and three subsequently were studied in detail. Two other founders developed thymomas and died; a third line did not develop disease and therefore was excluded from the analysis. The three *tal-1* lines expanded for study expressed high levels of *tal-1* mRNA as shown by a ribonuclease protection assay (Figure 1B and data not shown); however, mice from these lines differed with respect to the incidence and rate of tumor formation. Transgenic thymus expressed abundant levels of *tal-1* mRNA, whereas no *tal-1* message was detected in wild-type thymus. The transgene was expressed at highest levels at birth and persisted at low levels throughout adult life. *Tal-1* levels were much lower in the spleen than that observed in the thymus. This expression pattern is consistent with published reports noting greater activity of the proximal *lck* promoter in immature thymocytes (Wildin *et al.*, 1991).

T-cell acute lymphoblastic leukemia/lymphoma in tal-1 transgenic mice

Five of the six *tal-1* lines developed disease with a median survival of 350 days (Figure 2). Twenty one of 75 (28%) *tal-1* mice developed disease. These animals exhibited respiratory distress, ruffled coat and weight loss. Necropsy revealed the presence of a thymic mass, often accompanied by hepatosplenomegaly. Histological examination of the thymus revealed effacement of normal thymic architecture by a monomorphic infiltrate of lymphoblastic cells with prominent nucleoli and scant cytoplasm (Figure 3A and B). Similar cells invaded the surrounding para-sternal muscle, pericardium and other organs, such as the spleen, liver and kidney (Figure 3C and D). Clusters of apoptotic cells with pyknotic nuclei and reddish cytoplasm could be observed throughout the tumor. Lymphoblasts were variably present in the peripheral blood of diseased animals. The histologic appearance of these thymic tumors as well as the leukemic blood profiles share several features with human T-ALL or T lymphoblastic lymphoma.

Despite the high levels of *tal-1* expression in the transgenic thymus, and the relatively high penetrance of malignant disease, we have not observed any evidence of a pre-malignant perturbation of T cell development. Fluorescence-activated cell sorting (FACS) analysis of transgenic mice and their wild-type littermates did not reveal any alterations in thymocyte subpopulations. In addition, cell cycle analysis of transgenic thymocytes showed a normal $G_1/S/G_2/M$ distribution. The *in vivo* experiments demonstrate that ectopic expression of *tal-1* in T cells can be transforming, and provide a model for further studies of the role of *TAL-1* in human leukemia.

Casein kinase II accelerates tal-1-induced lymphocyte transformation in mice

CKII has been shown to modulate the activity of several transcription factors *in vitro* and to synergize dramatically with *myc* in inducing lymphocytic leukemia in bi-transgenic mice (Seldin and Leder, 1995). The presence of multiple CKII consensus phosphorylation sites in the tal-1 serine phosphoprotein and the fact that CKII has been shown to phosphorylate tal-1 *in vitro* (Goldfarb *et al.*, 1992) prompted us to test whether CKII might potentiate tal-1 transforming activity in mice. To test this, one *tal-1* transgenic line (4727) was mated with mice which expressed the catalytic subunit of CKII in lymphocytes via an immunoglobulin heavy chain promoter–enhancer (Seldin and Leder, 1995). The *CKIIα* transgenic animals develop clonal T cell lymphomas with a median survival of 400 days (Figure 2). When mated to the *tal-1* transgenic mice, a dramatic acceleration of disease onset and an increase in disease penetrance was observed. All bi-transgenic animals developed aggressive disease with a median survival of 74 days (Figure 2). In the *tal-1/CKIIα* animals, the disease was characterized by thymic enlargement, often accompanied by splenomegaly and lymphadenopathy. The thymic architecture was obliterated by neoplastic cells and, as in *tal-1* mice, numerous clusters of apoptotic cells were observed. In contrast to the *tal-1* tumors, where the mitotic rate appeared low to moderate, histologic examination of bi-transgenic tumors revealed a higher mitotic index.

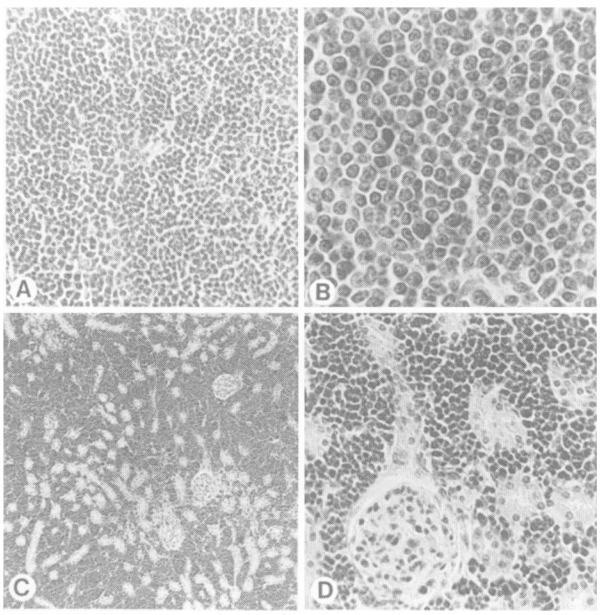

Fig. 3. Histology of the lymphoproliferative disease in *tal-1* transgenic mice. A thymus from an adult *tal-1* transgenic mouse that developed a thymoma shows the effacement of the normal thymic architecture (**A**; 50×) and the proliferation of large lymphoblasts with prominent nucleoli (**B**; 1000×). Similar cells invade visceral organs such as the kidney (**C**; 50× and **D**; 400×).

Thymomas exhibit diverse immunophenotypes

Tumors from the *tal-1* and *tal-1/CKIIα* mice were examined by flow cytometry to assess the cellular origin of the malignant cells. All *tal-1* tumors expressed the T cell lineage cell surface markers CD3, Thy1 and αβ T cell receptor (TCR). None of the *tal-1* or *tal-1/CKIIα* tumors expressed the B-cell specific antigen B220 or surface immunoglobulin heavy chain.

These T cell tumors were of varying stages of thymocyte maturity (Table I). Three of 10 tumors consisted of predominantly CD4- and CD8-positive cells and most likely developed from the immature thymocyte population (Figure 4A). An additional three tumors contained primarily CD4-negative, CD8-positive cells that also expressed the interleukin-2 receptor (IL-2R) and the hematopoietic marker heat-stable antigen (HSA) or J11d, indicative of a single positive thymocyte of intermediate maturity (Figure 3B). Interestingly, this tumor phenotype ($CD4^{-}8^{+}$) was also observed frequently in *LM01/rbtn1* transgenic mice, suggesting that this thymocyte population may somehow be sensitive to transformation by both *tal-1* and *LMO1* (McGuire *et al.*, 1992). Another two *tal-1* tumors exhibited a more mature phenotype, consisting of CD3-positive, CD4-positive, CD8-negative cells (Figure 4C) that did not express J11d or IL-2R (data not shown).

Two other *tal-1* tumors consisted of transformed cells that seemed to be capable of ongoing differentiation *in vivo*. In both of these cases, the tumor cells formed thymomas that filled the entire thorax and had no residual histologically normal thymic tissue, yet their FACS profiles revealed a mixture of immature and mature thymocytes. One of these is illustrated in Figure 4D. Half of the tumor contained double-negative cells, whereas the remaining

Table I. Immunophenotypes of *tal-1* and *tal-1/CKII* tumors

Animal	Genotype	Age (days)	% of cells with the following phenotype				Growth in culture
			CD3	CD4	CD8	CD4,CD8	
4862	*tal-1* (4709)	285	95	99	98	98	+
1341	*tal-1* (4709)	219	76	87	96	78	+
1444	*tal-1* (4709)	105	40	71	99	64	+
1330	*tal-1* (4722)	320	42	2	99	5	+
1011	*tal-1* (4709)	75	4	25	99	24	+
4789	*tal-1* (4709)	225	8	2	95	1	+
1469	*tal-1* (4709)	195	93	98	15	ND	+
1413	*tal-1* (4709)	167	25	84	1	0.1	–
4709	*tal-1* (4709)	128	85	81	37	47	+
1022	*tal-1* (4709)	49	99	22	31	6	+
1323	*tal/CKII* (4727)	42	74	96	98	95	+
1337	*tal/CKII* (4727)	42	36	56	97	56	+
4867	*tal/CKII* (4723)	223	95	83	78	62	+

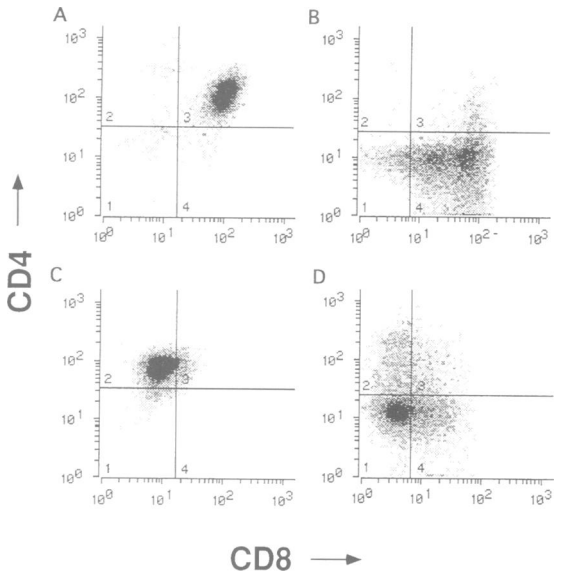

Fig. 4. Two-color flow cytometry analyses of tumors arising in the *tal-1* transgenic mice. Single cell suspensions were prepared from the tumors and stained with fluorescent antibodies recognizing CD4 and CD8 as indicated in Materials and methods. Four representative *tal-1* tumor phenotypes are shown.

tumor cells expressed CD4 only (22%) or CD8 only (31%). Only 6% of the tumor cells expressed both CD4 and CD8.

In contrast to the heterogeneity of *tal-1* tumors, the bi-transgenic *tal-1/CKIIα* tumor cells exhibited a uniform immunophenotype. Similar to what was observed for *CKII*-only tumors, the bi-transgenic tumor cells expressed CD3, CD4 and CD8 and therefore were derived from the immature double-positive thymocyte population (Table I). The consistency in tumor phenotype observed in the *tal-1/CKIIα* bi-transgenic tumors may indicate that the double-positive cell is the first developmental stage at which this transgene combination is transforming, or perhaps merely where both transgenes are first expressed. Alternatively, expression of *tal-1* and *CKIIα* transgenes may inhibit further thymocyte development.

The T cell leukemias/lymphomas derived from the *tal-1* and *tal-1/CKIIα* transgenics were all clonal. DNA was isolated from cell lines derived from the tumors, restricted with *Hin*dIII and examined by filter hybridization with the TCR $J_\beta 2$ probe. Clonal rearrangements were detected in both the mono- and bi-transgenic tumor cells analyzed and, in most cases, both TCR β alleles were rearranged (Figure 5 *tal-1/+* lanes; *tal-1/CKIIα* lanes). The rearrangement patterns observed in the cell lines were confirmed by analyzing DNA isolated directly from the tumor (data not shown). In animals where disease involved multiple organs such as thymus, spleen and kidney, the specific βTCR bands were evident in all tissues. The immunoglobulin heavy chain locus was retained in its germline configuration, as would be expected for T lineage tumors (data not shown).

Tal-1 and CKII synergy in leukemia

Among other possibilities, the oncogenic cooperativity observed in the mice suggested that *tal-1* and *CKIIα* might interact directly. To determine whether *CKIIα* transgene

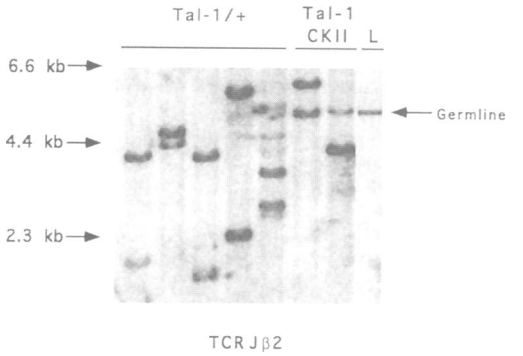

Fig. 5. The *tal-1* and *tal-1/CKIIα* tumors are clonal. DNA prepared from tumor cell lines and wild type liver tissue was digested and subjected to Southern blot analysis. T cell receptor J_β chain rearrangements were detected with a probe that identifies a single 5 kb band in the germline configuration of genomic liver DNA digested with *Hin*dIII (lane L). *Tal-1/+* lanes contain DNA from tumor cell lines derived from *tal-1* mice, whereas lanes designated Tal-1/CKII contain DNA isolated from *tal-1/CKIIα* tumor cell lines.

expression might affect *tal-1* mRNA levels, we used an RNase protection assay to examine RNA from the *tal-1* and *tal-1/CKIIα* thymomas. Using a *tal-1*-specific riboprobe, we detected high levels of *tal-1* expression in the mono-transgenic tumors. We observed no further up-regulation of *tal-1* mRNA levels in the *tal-1/CKIIα* bi-transgenic tumor cells (Figure 6A), nor was *tal-1* expression activated in *CKIIα* mono-transgenic tumors (data not shown). Transgenic *tal-1* expression does not seem to influence expression of endogenous *CKIIα*. In the bi-transgenic tumors, modest *CKIIα* transgenic mRNA expression was seen, consistent with what is observed with the *CKIIα* transgene alone (Figure 6A) (Seldin and Leder, 1995). Endogenous *CKIIα* mRNA was unaffected in *tal-1*-only tumors (Figure 6A). These experiments argue against any co-regulation of *tal-1* and *CKIIα* expression at the RNA level. Furthermore, activation of endogenous *CKIIα* does not appear to be a common mechanism of *tal-1*-mediated transformation.

An attractive alternative hypothesis is that CKII phosphorylates tal-1 directly, leading to its activation. To compare the phosphorylation status of the tal-1 protein in the mono- versus the bi-transgenic tumor cell, we immunoprecipitated tal-1 from ^{32}P-labeled lysates of leukemic cell lines. As shown in Figure 6B, the anti-tal-1 antisera (lanes I) (generously provided by Dr Richard Baer), but not the corresponding pre-immune serum (P), immunoprecipitated ^{32}P-labeled $pp42^{tal\text{-}1}$ from control murine erythroleukemia cells (MEL-lane C) and from all the *tal-1* and *tal-1/CKIIα* leukemic cell lines tested (Figure 6B and data not shown). We did not detect a significant increase in the overall phosphorylation of the tal-1 protein in the *tal-1/CKIIα* leukemic cell lines. Furthermore, we have been unable to co-immunoprecipitate tal-1 and CKIIα (data not shown). While we cannot exclude the possibility of subtle or site-specific differences in tal-1 phosphorylation in the presence of transgenic CKIIα, these data make it more likely that the synergy of the two transgenes is indirect.

Discussion

We have demonstrated that *tal-1*, a major gene associated with human T cell leukemia, induces T cell malignancies

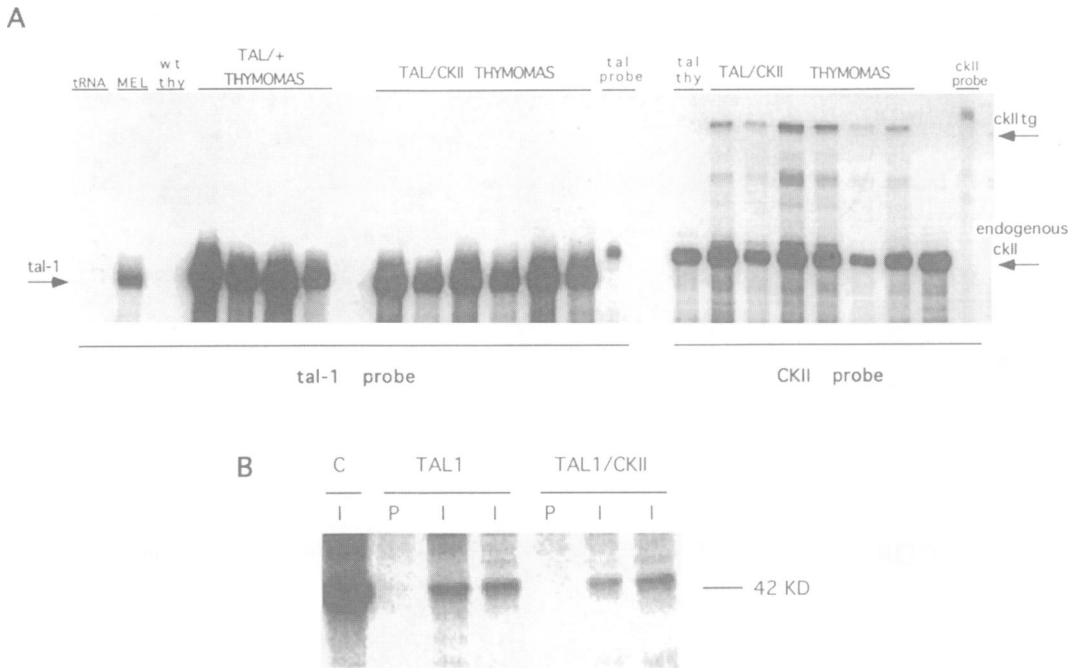

Fig. 6. The tumor cells express the trangenes. (**A**) RNA prepared from *tal-1* and *tal-1/CKIIα* tumors was subjected to RNase protection analysis with an antisense *tal-1* probe or an antisense *CKIIα* riboprobe. The *tal-1* mRNA protects a band of 270 bases present in all the *tal-1* and *tal-1/CKIIα* tumors tested as well as the murine erythroleukemic (MEL) cell line known to express endogenous *tal-1*. The endogenous *CKIIα* mRNA protects a band of ~300 bases, whereas the *CKIIα* transgenic mRNA protects a band of ~400 bases. The transgene-specific *CKIIα* band (designated ckII tg) was evident in all the bi-transgenic tumors tested, but was not present in the *tal-1* tumors. (**B**) Immunoprecipitation of the pp42^{tal1} phosphoprotein from leukemic cell lines. *Tal-1* and *tal-1/CKIIα* leukemic cell lines were labeled metabolically with [^{32}P]orthophosphate and each cell lysate was immunoprecipitated with an anti-mouse tal-1 antiserum (labeled I for immune) or the corresponding pre-immune serum (labelled P for pre-immune). A murine erythroleukemic cell line (designated here as C for control) was used as a positive control.

in transgenic mice. Twenty eight percent of *tal-1* transgenic mice in three lines and two founders died of clonal T lymphoblastic lymphoma or leukemia, reminiscent of the pathology of the human disease. This provides direct evidence that *tal-1* is transforming: in contrast to other transgenic *tal-1* mice that failed to develop disease (Robb *et al.*, 1995; Larson *et al.*, 1996). Mice expressing *tal-1* from a CD2 enhancer and a hybrid viral promoter [HTLV long terminal repeat (R-U5) segment/SV-40 promoter–enhancer] failed to develop leukemia (Robb *et al.*, 1995). Similarly, CD2–*tal-1* transgenic mice did not develop disease; yet, when mated with CD2–*LM02* transgenic mice, a modest disease acceleration in bi-transgenic offspring was induced (Larson *et al.*, 1996). The reason for the lack of tumors in other transgenic lines is unclear, but might be due to differences in the promoter–enhancer combinations used or to the genetic backgrounds of the mouse strains in which the experiments were done.

Tal-1 encodes a putative transcription factor that is required for embryonic hematopoietic lineage development (Shivdasani *et al.*, 1995; Porcher *et al.*, 1996). In T-ALL, it has been hypothesized that *TAL-1* transforms cells through an arrest of thymocyte differentiation (Voronova and Lee, 1994; Goldfarb and Lewandowska, 1995). However, in our mice, massive transgenic *tal-1* mRNA does not perturb thymic development or T cell cycling, with normal numbers of cells occupying the various thymocyte subsets. Furthermore, the T lymphomas in the *tal-1* mice are of a variable immunophenotype, from immature thymocytes to a relatively mature, single-positive phenotype. One tumor even appeared to be capable of differentiation *in vivo* from a double-negative to a single-positive phenotype, strongly arguing against a *tal-1*-induced arrest of differentiation.

Cell growth and differentiation decisions in many cell types appear to be determined by the equilibrium between hetero- and homodimeric complexes of bHLH transcription factors, some of which are tissue specific and some of which are ubiquitously expressed. In the erythroid cell, tal-1 heterodimerizes with E2A proteins and the complex transactivates transcription (Hsu *et al.*, 1991). In human T-ALL cells, ectopically expressed *TAL-1* and the E2A gene product, E47, also appear to form stable heterodimers (Hsu *et al.*, 1994). We hypothesize (Figure 7, first line) that the formation of these complexes may have multiple effects that lead to transformation of T cells. First, TAL-1 might compete for E proteins in human T-ALL and murine *tal-1* transgenic cells, leading to the formation of tal-1–E protein heterodimers not normally present in T cells. These tal-1–E complexes may transactivate genes normally silent in thymocytes, perhaps activating genes involved in expansion of the hematopoietic lineages in the developing embryo. Second, the formation of tal-1–E protein heterodimers may decrease the quantity of E proteins available to form functional homodimers, resulting in decreased transcription of genes involved in lymphoid differentiation (see Figure 7, second line).

Furthermore, we show that *tal-1*-induced leukemia/lymphoma is accelerated dramatically by expression of the catalytic subunit of the serine–threonine kinase CKII. Although tal-1 is a serine phosphoprotein that appears to be constitutively phosphorylated by CKII *in vivo*, we were

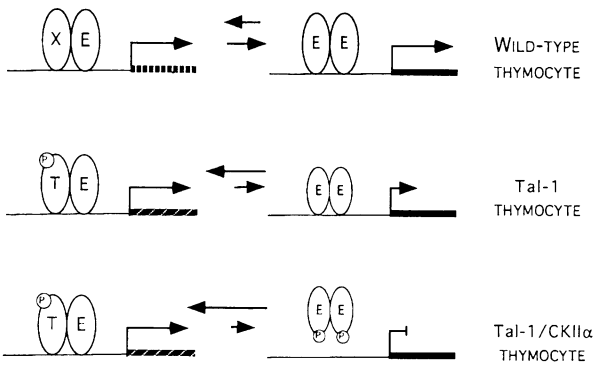

Fig. 7. Diagrammatic representation of a model describing the putative mechanism of tal-1 action. In the wild-type thymocyte, E protein homodimers form and transactivate target genes. The E proteins may also form heterodimers with unknown bHLH proteins and these may be active in T cells. However, in the normal wild-type thymocyte, no tal-1–E complexes form because *tal-1* is not expressed. In the *tal-1* transgenic thymocyte and potentially in human T-ALL cells, the concentration of E homodimers is reduced and the formation of tal-1–E heterodimers is favored. The bHLH equilibrium is shifted further to the left in favor of tal-1–E complexes in the *tal-1/CKIIα* bi-transgenic thymoctye. In these leukemic cells, CKII phosphorylation inactivates E homodimers and therefore more E protein is available to bind tal-1. The dashed and the solid dark lines represent target genes normally expressed in T cells and regulated by bHLH proteins, while the hatched line represents putative tal-1 target genes normally found inactive in T cells. The length of the equilibrium arrows indicates the relative amounts of tal–E heterodimer and E homodimer available in the various genetic backgrounds.

unable to demonstrate a direct interaction of transgenic tal-1 and transgenic CKII, either at the level of gene regulation or at the level of the protein. Recently, CKII has been shown to markedly enhance the transcriptional activity of the related bHLH myogenic regulatory factors (MRF) through phosphorylation of its E protein partners. CKII phosphorylation inactivates the inhibitory E homodimers, thereby favoring binding of activating MRF–E heterodimers (Johnson *et al.*, 1996). Such a mechanism would fit well with the model we propose for the *in vivo* synergy observed between tal-1 and CKIIα in T cell transformation. In the bi-transgenic *tal-1/CKIIα* mice, CKIIα phosphorylation of E proteins in T cells may inhibit E homodimeric DNA binding, thus favoring formation of tal-1–E heterodimers that activate genes potentially involved in hematopoietic lineage expansion (Figure 7, last line). If this model is correct, then overexpression of E proteins should inhibit transformation mediated by tal-1 and tal-1/CKIIα.

Materials and methods

Generation of transgenic mice

Using oligos specific for mouse *tal-1* (5′-GCC-TCA-CTA-GGC-AGT-GGG-TTC-3′ and 5′-GTG-CTT-TCC-CCC-AAC-TCC-AGC-3′), the *tal-1* cDNA was isolated from a cDNA library made from a murine erythroleukemic cell line (generously provided by Dr Stuart Orkin, Boston, MA). The cDNA was subcloned into pKS Bluescript and confirmed as *tal-1* cDNA by sequencing. A 1.4 kb fragment was excised from pKS as a *Bam*HI–*Bgl*II fragment and cloned into the *Bam*HI cloning site of p1017, a plasmid cassette containing the proximal *lck* promoter, the human growth hormone splice and poly(A) addition sites (Abraham *et al.*, 1991). After being checked for proper orientation, the plasmid was digested with *Spe*I and the linear insert DNA was prepared and microinjected into the pronuclei of a fertilized FVB/N egg and implanted into the oviduct of a pseudopregnant Swiss mouse. Transgenic mice were identified by probing Southern blots of *Eco*RI-digested tail DNA with a ^{32}P-labeled random-primed 585 bp *Eco*RI *tal-1* partial cDNA fragment. Southern blots were hybridized and washed as described previously (Krane and Leder, 1996). Transgenic-positive lines were propagated by crossing founder animals with FVB/N animals.

Histology

Upon necropsy, all tissue samples were preserved in Optimal Fix (American Histology Reagent Company, Inc.). Four mm sections were cut and stained with hematoxylin and eosin for histologic evaluation in the Transgenic Core Pathology Laboratory at the University of California at Davis.

Fluorescence-activated flow cytometry analysis

Mouse thymomas were gently teased with toothed forceps in order to produce single cell suspensions. The cells were washed and stained with fluorescent-labeled antibodies and subjected to flow cytometry in a Cytofluorograf II flow cytometry machine (Becton-Dickinson, Mountain View, CA). Dead cells were eliminated from the analyses by gating forward and perpendicular scatter. Data were analyzed with software from Ortho Diagnostics Systems Inc. (Raritan, NJ) and Cytomation (Fort Collins, CO).

Antibodies

Antibodies used in flow cytometry included monoclonal rat anti-mouse CD3 29B (Portholes *et al.*, 1989), monoclonal hamster anti-mouse CD3 145.2C11 (Samelson *et al.*, 1987), FITC-conjugated polyclonal goat anti-rat immunoglobulin antibodies (FITC–anti-rat Ig) (Kirkegaard and Perry Laboratories, Gaithersburg, MD), FITC–anti-Thy-1.1, FITC–anti-Thy-1.2 (New England Nuclear, Boston, MA) and phycoerythrin (PE)–anti-B220 (CD45R), PE–anti-CD4, PE–anti-CD8 and FITC–anti-CD3, FITC–anti-T cell receptor αβ, FITC–anti-T cell receptor γδ (PharMingen, San Diego, CA), FITC–anti-CD8 and PE–anti-CD4 (Becton Dickinson).

Tumor DNA analysis

Southern blots of *Hin*dIII-digested DNA (10 μg) obtained from primary tumors and from tumor cell lines were hybridized with a ^{32}P-labeled 2 kb *Eco*RI fragment containing the murine TCR $J_\beta 2B$ exon (Siu *et al.*, 1984). Genomic DNA from tail samples was also digested with *Eco*RI, transferred to Nytran (Dupont) and hybridized to a ^{32}P-labeled 1.5 kb *Pst*I μ fragment (Early *et al.*, 1980). Blots were washed at room temperature at low stringency followed by higher stringency washes (0.1× SSC, 0.1% SDS) at 65°C.

RNase protection analysis

Total RNA was isolated from mouse organs and tissue culture cells by a guanidine isothiocyanate lysis procedure (Sambrook *et al.*, 1989). T3 and T7 antisense probes were synthesized and hybridized to RNA samples as described (Krieg and Melton, 1987). The probe for *tal-1* was derived by linearization of a 770 bp partial cDNA clone with *Pvu*II, resulting in a probe that protects 270 nucleotides. The *CKIIα* riboprobe has been described (Seldin and Leder, 1995). The endogenous *CKIIα* mRNA protects a band of ~300 nucleotides, whereas the transgene mRNA protects a band of ~400 bases.

Radiolabeling of leukemic cell lines

Suspension cultures of leukemic cell lines were harvested at a density of 2×10^6 cells/ml. For each labeling experiment, 1×10^7 cells were pelleted by centrifugation, washed twice with phosphate-free Tris-buffered saline (TBS) and resuspended in 2 ml of phosphate-free RPMI-1640 growth medium (Gibco). After culturing for 1 h, the cells were pelleted by centrifugation and resuspended in 2 ml of phosphate-free RPMI growth medium containing 1 mCi of [^{32}P]orthophosphate. After culturing at 37°C for 2 h, the cells were pelleted, washed twice with phosphate-buffered saline (PBS) containing 5 mM EDTA, and lysed in 1 ml of RIPA buffer containing protease inhibitors.

Immunoprecipitations

Twenty μl of protein A–agarose was added to clear the lysate. The beads were then removed by a brief centrifugation and the supernatant was recovered. Five μl of anti-mouse tal-1 antiserum or pre-immune serum (generously provided by Dr Richard Baer, University of Texas, Dallas, TX) was then added to the lysate. After gentle rocking at 4°C for 1 h, 20 μl of protein A–agarose was added and the mixture was rocked for an additional hour at 4°C. The beads were then pelleted by a brief centrifugation, washed twice with 1 ml of RIPA buffer, resuspended in 2× sample buffer, boiled for 10 min and then pelleted by centrifugation. The supernatants were then fractionated by electrophoresis on an 8%

SDS–PAGE gel. Protein molecular weight standards were obtained from BioRad (Hercules, CA).

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