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Protein dimerization between Lmo2 (Rbtn2) and Tal1 alters thymocyte development and potentiates T cell tumorigenesis in transgenic mice

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The *LMO2* and *TAL1* genes were first identified via chromosomal translocations and later found to encode proteins that interact during normal erythroid development. Some T cell leukaemia patients have chromosomal abnormalities involving both genes, implying that *LMO2* and *TAL1* act synergistically to promote tumorigenesis after their inappropriate co-expression. To test this hypothesis, transgenic mice were made which co-express *Lmo2* and *Tal1* genes in T cells. Dimers of Lmo2 and Tal1 proteins were formed in thymocytes of double but not single transgenic mice. Furthermore, thymuses of double transgenic mice were almost completely populated by immature T cells from birth, and these mice develop T cell tumours ~3 months earlier than those with only the *Lmo2* transgene. Thus interaction between these two proteins can alter T cell development and potentiate tumorigenesis. The data also provide formal proof that *TAL1* is an oncogene, apparently acting as a tumour promoter in this system.
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Introduction

Chromosomal translocations are recurrent features of tumours and underlie the origin of these malignancies (Rabbitts, 1994). A wealth of data from both leukaemia/lymphoma tumours and solid tumours has led to two concepts about the role of translocation-activated genes in tumour aetiology, namely involved in transcription, which in turn disrupts normal development, giving rise to abnormal cells that are targets for malignant transformation.

There are many different chromosomal translocations associated with human T cell acute lymphoblastic leukaemia (T-ALL) (Rabbitts, 1994), even though it is a disease characterized by common histology, prognosis and disease course. Principle among the T-ALL translocations are t(1;14)(p32;q11) and t(11;14)(p13;q11) which activate the *TAL1* gene (previously known as *tal, SCL* or *TCL5*) (Begley *et al.*, 1989; Bernard *et al.*, 1989; Finger *et al.*, 1989; Carroll *et al.*, 1990; Chen *et al.*, 1990) and the *LMO2* gene (previously known as *RBTN2* or *TTG2*) (Boehm *et al.*, 1991; Royer-Pokora *et al.*, 1991) respectively. Almost 10% of T-ALL cases have t(1;14) or t(11;14) and ~25% of T-ALL patients have a specific deletion near the 5′ end of the *TAL1* gene, resulting in its activation in T cells via the promoter of a second gene called *SIL* (Brown *et al.*, 1990; Aplan *et al.*, 1991, 1992; Bernard *et al.*, 1991; Breit *et al.*, 1993). In addition, microchanges may also occur in *TAL1*, as judged by studies of *TAL1* expression in T-ALL patients lacking detectable chromosomal aberrations of 1p32 (Bash *et al.*, 1995). Finally, T-ALL samples from some patients can contain both a rearrangement of *LMO2*, due to t(11;14)(p13;q11), and a *TAL1* promoter deletion (Wadman *et al.*, 1994), implying activation of both *LMO2* and *TAL1* in the same cell. This suggests complementary effects of *LMO2* and *TAL1* activation in those tumours.

The *LMO2* gene encodes a short protein of 156 amino acids which has two cysteine-rich LIM domains (Boehm *et al.*, 1990, 1991; Royer-Pokora *et al.*, 1991) (hence the new nomenclature *LMO2* for LIM-only). The LIM domains are structurally homologous to the GATA DNA binding zinc finger-like domain (Omichinski *et al.*, 1993; Perez-Alvarado *et al.*, 1994; Sanchez-Garcia and Rabbitts, 1994), but there is no evidence that the LMO2 LIM domains bind to DNA. TAL1 is a basic helix–loop–helix domain protein which interacts *in vivo* with other basic helix–loop–helix proteins (e.g. E12 and E47) to form a DNA binding heterodimer (Hsu *et al.*, 1991, 1994a,b). Both Lmo2 and Tal1 are naturally co-expressed in a variety of locations, including the erythroid lineage (Warren *et al.*, 1994), where the two proteins interact to form a complex (Valge-Archer *et al.*, 1994; Wadman *et al.*, 1994). This molecular interaction probably underlies the biological role of the proteins, because gene targeting experiments introducing null mutations into *Lmo2* (Warren *et al.*, 1994) and *Tal1* (Robb *et al.*, 1995a; Shivdasani *et al.*, 1995) have shown that both are essential for erythroid development and the phenotype of both null mutants are very similar. These studies suggest that the complementary effect of LMO2 and TAL1 in tumours may also involve protein dimerization.

A transgenic model in which Lmo2 is ectopically expressed in the thymus has shown that clonal T cell tumours arise with a long latency period of ~9–10 months (Fisch *et al.*, 1992; Larson *et al.*, 1994, 1995). Tumour appearance is preceded by an asymptomatic period when the thymuses of transgenic mice accumulate cells with little or no CD4 and CD8 surface markers (Larson *et al.*, 1995). Paradoxically, transgenic mice with ectopic expression of Tal1 in the thymus failed to develop any tumours

(Robb *et al.*, 1995b). This latter finding is particularly puzzling because the most common abnormalities in T-ALL tumours involve the *TAL1* gene (Baer, 1993).

We have utilized the failure of *Tal1* transgenic mice to develop tumours (Robb *et al.*, 1995b) and the high frequency of tumour occurrence in *Lmo2* transgenic mice (Larson *et al.*, 1994, 1995) to test a number of hypotheses relating to the function of these genes in tumorigenesis. By generating transgenic mice expressing both *Lmo2* and *Tal1* transgenes from the same promoter cassette (herein called double transgenic mice), we have found that abnormalities of T cell differentiation and tumours appear at a faster rate than *Lmo2*-only transgenics. These effects appear to be mediated through Tal1–Lmo2 protein interaction, since *in vivo* association between the ectopically expressed Lmo2 and Tal1 proteins was observed in both the pre-leukaemic and malignant T cells of the mice.

Results

Lmo2 and Tal1 dimerize in transgenic T cells

The normal expression of *LMO2* and *TAL1* in erythroid cells results in the formation of a complex between their protein products (Valge-Archer *et al.*, 1994; Wadman *et al.*, 1994). This observation raises the possibility that Lmo2–Tal1 dimers also occur in the leukaemic cells of T-ALL patients with both *LMO2* and *TAL1* gene abnormalities (Wadman *et al.*, 1994). The double transgenic mice provided an opportunity to study the ability of these two proteins to form a complex after their aberrant expression in T cells.

Transgenic mice expressing *Lmo2* mRNA from the T cell-specific CD2-promoter cassette have been described (mice designated CD2-rbtn2) (Fisch *et al.*, 1992). The same CD2-promoter was used to generate mice expressing *Tal1* (mice herein called CD2-tal1). Extracts prepared from transgenic thymocytes were examined by Western blotting with a monoclonal anti-Tal1 antibody (Pulford *et al.*, 1995) which preferentially recognizes human rather than mouse Tal1 protein (Figure 1A). This antibody can therefore distinguish transgenic Tal1 (whose origin was a human cDNA) from endogenous mouse protein. The data shown in Figure 1A demonstrate that Tal1 protein is detectable in thymus cells (age 6 weeks) from CD2-tal1 transgenic mice and CD2-rbtn2×CD2-tal1 double transgenic mice but not CD2-rbtn2-only mice. Immunocytochemistry of CD2-tal1 thymocytes showed uniform expression of Tal1 protein (data not shown). Tal1 protein was also detected in tumours arising in double transgenic mice (Figure 1A). Four tumours and five asymptomatic thymuses have been similarly analysed from double transgenic mice. Previous studies showed that Lmo2 protein is detectable in the thymuses of CD2-rbtn2 transgenic mice (Larson *et al.*, 1994) but not those of normal mice (Warren *et al.*, 1994).

A two-step immunoprecipitation–Western blotting procedure was used to assess whether Lmo2–Tal1 dimers were present in transgenic thymocytes. If Tal1 is associated with Lmo2 in the double transgenic mice, it will be co-precipitated along with Lmo2 by anti-Lmo2 antiserum; co-precipitated Tal1 protein can then be visualized by Western blotting of the immunoprecipitated material using anti-Tal1 antibody (Pulford *et al.*, 1995). Equal amounts

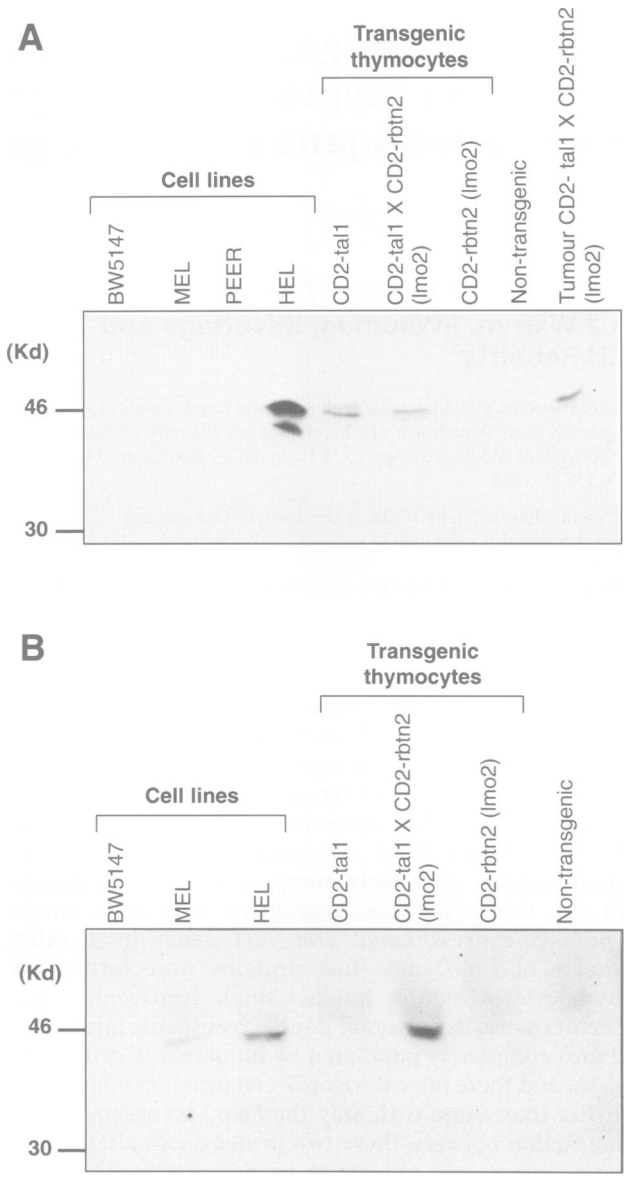

Fig. 1. Interaction of Lmo2 and Tal1 protein in transgenic T cells. (**A**) Western analysis of proteins isolated from transgenic thymocytes. Proteins were extracted from 10^6 tissue culture cells (BW5147; Peer; MEL or HEL) or 10^7 thymocytes of 6-week-old mice, and separated on 12% SDS–PAGE followed by transfer to a nitrocellulose membrane. This filter was incubated with anti-Tal1 monoclonal antibody followed by anti-mouse horseradish peroxidase and developed by ECL detection. The transgenic mice used for each lane are indicated at the top of the diagram. Cells from a tumour arising in a CD2-rbtn2×CD2-tal1 double transgenic mouse were also fractionated (right-hand lane). (**B**) Two-step Western analysis of transgenic thymocytes. Proteins were prepared from three cells lines (BW5147, MEL and HEL) or from thymocytes of 6-week-old transgenic mice indicated at the top of the figure. Proteins from 10^7 cultured cells and 10^8 thymocytes were first immunoprecipitated with polyclonal rabbit antisera recognizing Lmo2. The immune complex was recovered, boiled in SDS loading buffer prior to fractionation by 12% SDS–PAGE and transferred to nitrocellulose filters. The membrane was incubated with monoclonal anti-Tal1 antibody and the proteins detected by ECL.

of proteins from thymocytes or from control cell lines were immunoprecipitated with antiserum recognizing Lmo2 [anti-rbtn2 (Warren *et al.*, 1994)], and the precipitated material was solubilized and analysed by Western blotting with the anti-Tal1 antibody. As anticipated, Tal1

protein associated with Lmo2 was detectable in the human erythroid cell line HEL but only faintly in the mouse line MEL (due to the preferential recognition of human TAL1 by the antibody) (Figure 1B). In the transgenic thymus samples, Tal1 protein was not detected by the two-step procedure in the CD2-tal1-only thymocytes (Figure 1B) since these do not express Lmo2. Conversely, Tal1 protein was detected on the second-step Western blot of thymocyte proteins from the double transgenic mice (Figure 1B). This shows that when the anti-Lmo2 antiserum was used for the immunoprecipitation step, Tal1 associated with Lmo2 was co-precipitated, allowing detection in the Western step of the procedure. Therefore, as a result of the ectopic co-expression of these proteins in T cells, a complex is formed between them, akin to that naturally present in erythrocytes.

Altered thymocyte development in double transgenic mice

Although tumours develop in single transgenic mice (CD2-rbtn2 or thy1.1-rbtn2) which express high levels of the T cell markers CD4 and CD8 (Fisch *et al.*, 1992; Larson *et al.*, 1994, 1995), the surface phenotype of an abnormal proportion of thymocytes in pre-symptomatic CD2-rbtn2 and thy1.1-rbtn2 mice expressed little or none of these markers (and were operationally designated double negative or 'DN' cells) (Larson *et al.*, 1995). An investigation of pre-symptomatic, double transgenic mice was undertaken to ascertain if there was any effect of co-expression of Lmo2 and Tal1 on this phase. Thymocytes prepared at the pre-symptomatic stage (5.6 months) from littermates of a CD2-rbtn2 and CD2-tal1 inter-cross were analysed by FACS using antibodies recognizing CD4 and CD8 surface proteins (Figure 2). The thymocytes of non-transgenic and CD2-tal1 littermates expressed normal levels of CD4 and CD8, as previously reported (Robb *et al.*, 1995b). In contrast, a double transgenic littermate showed a marked increase in the so-called DN cells compared with a CD2-rbtn2-only littermate (Figure 2). For example, the asymptomatic thymus from the CD2-rbtn2-only mouse contained ~45% DN cells compared with ~95% in the double transgenic thymus. Thus, prior to tumour formation, Tal1 synergizes with Lmo2 during the pre-symptomatic phase to exaggerate the effect on T cell differentiation evident with Lmo2 alone.

The consistency of this synergistic effect was investigated by comparing cohorts of double and single transgenic mice (Figure 3). In accordance with previous data (Larson *et al.*, 1995), there is a variable increase in the DN cells within the thymuses of individual CD2-rbtn2 mice but no significant alterations in the CD2-tal1 mice (Robb *et al.*, 1995b). There was, however, a striking and consistent increase in the number of DN cells in the double transgenic mice since DN cells comprised >80% of the total thymocyte population in most animals. Moreover, it is of interest that this perturbation of thymus differentiation begins *in utero* because newborn mice display the high DN cell content seen in the older asymptomatic animals. Thus, the synergistic effect of Lmo2 and Tal1 on T cell differentiation occurs very early in thymus development.

Immature T cell tumours appear in the double transgenic mice

Mice carrying only the *LMO2* transgene (CD2-rbtn2 and thy1.1-rbtn2) develop T cell tumours (Fisch *et al.*, 1992;

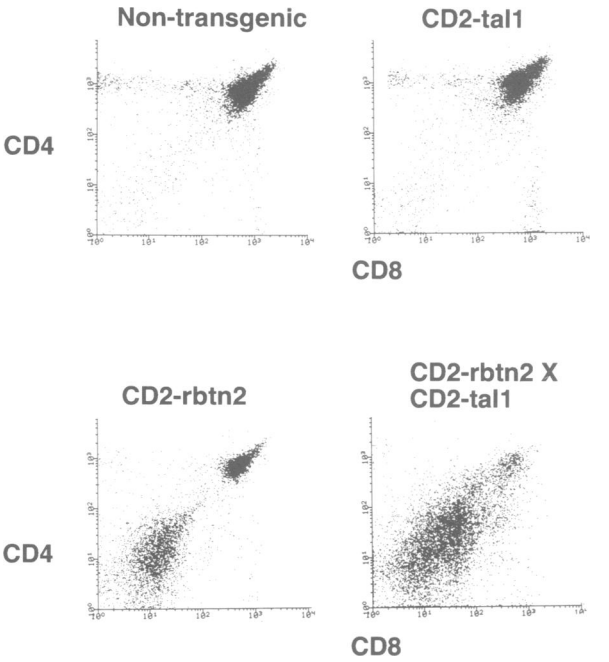

Fig. 2. Surface phenotype of thymocytes in the transgenic mice. Single cell suspensions were prepared from asymptomatic thymuses from the indicated transgenic mice or non-transgenic littermates at 5.6 months of age. The cells were stained with fluorescent antibodies recognizing CD4 or CD8. Fluorescence profiles were obtained using a FACScan flow cytometer.

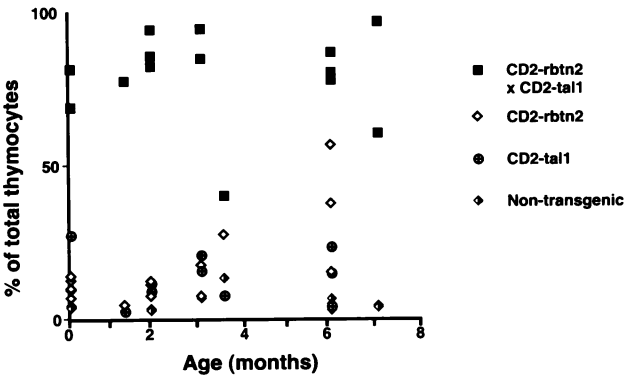

Fig. 3. Age relationship of DN T cell expansion in asymptomatic thymus. Cells were prepared from thymuses of the indicated transgenic mice or non-transgenic littermates and stained with fluorescent antibodies recognizing CD4 or CD8. Fluorescence profiles were obtained using a FACScan flow cytometer and the graphical analysis indicates the percentage of the total thymocytes at a given point with DN ($CD4^{lo/-}/CD8^{lo/-}$) phenotype. Seven litters were analysed. Each vertical column of points represents the data for a single litter, and each point represents one mouse.

Larson *et al.*, 1994, 1995). In accordance with this, double transgenic mice also developed tumours which invariably presented as thymomas with splenomegaly and hepatomegaly (see Table I in Materials and methods) typically with circulating lymphoblasts evident in blood smears (data not shown). Histological examination of tissue showed extensive malignant cell infiltration of peripheral organs such as liver and kidney, and complete disruption of splenic and thymic architecture (data not shown). In addition, cells from these transgenic tumours are able to divide *in vitro*.

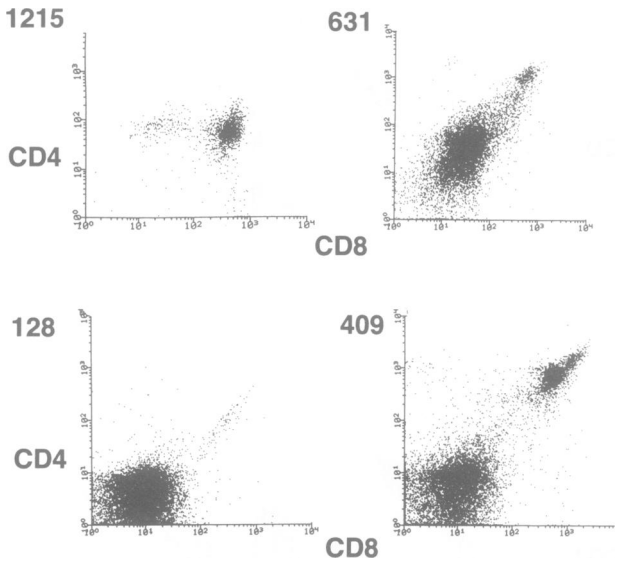

Fig. 4. Surface phenotype of tumours arising in the transgenic mice. Tumours which arose in a CD2-rbtn2-only transgenic mouse (1215) and in CD2-rbtn2×CD2-tal1 double transgenic mice (631; 128; 409) were removed for analysis. Single cell suspensions were prepared from neoplastic thymus (mice 1215; 409; 128) or spleen (mouse 631) of mice bearing tumours and stained with fluorescent antibodies recognizing CD4 or CD8. Fluorescence profiles were obtained using a FACScan flow cytometer.

In previous analyses of CD2-rbtn2 mice, most of the clonal T cell tumours expressed high levels of surface CD4 and CD8 (Fisch *et al.*, 1992; Larson *et al.*, 1994, 1995). The surface phenotypes of tumours from the double transgenic mice were also examined with these markers, and three examples are shown in Figure 4, compared with a tumour from a CD2-rbtn2-only mouse. The tumour cells from this mouse expressed both CD4 and CD8 (Figure 4, mouse 1215) while the double transgenic mice exhibited tumours with $CD4^{lo/-}/CD8^{lo/-}$ or $CD4^{-}/CD8^{-}$ phenotypes reminiscent of those detected during the asymptomatic phase in the CD2-rbtn2 and thy1.1-rbtn2 mice (Larson *et al.*, 1995). For example, the tumour cells of mouse 631 expressed low levels of CD4 and CD8, whilst those of mice 409 and 128 lacked expression of these markers (Figure 4).

The lack of CD4 and CD8 expression and the associated splenomegaly in the tumours in double transgenics made it formally possible that these might be B lymphocyte rather than T lymphocyte in origin. Accordingly, T cell receptor (TCR) and immunoglobulin gene rearrangement status was analysed. Tumour DNA from double transgenic mice was compared with DNA from a single transgenic mouse, from a T cell line (BW) and a plasma B cell line (J558L) and from non-transgenic thymus or spleen. These samples were cleaved with restriction enzymes and examined by filter hybridization with Cβ1 (Figure 5A) or Jβ2 (Figure 5B) probes. Clonal rearrangements were detected, with either TCR Cβ1 or Jβ2, in all the transgenic tumours analysed. For example, the tumour from mouse 631 did not have any rearranged restriction fragments when hybridized with Cβ1 probe but exhibited two rearranged fragments when the Jβ2 was used (Figure 5A and B), indicating that these were T cell tumours.

Final confirmation of this was obtained by hybridization with an immunoglobulin H chain enhancer probe. No rearranged fragments were detected with this probe except in DNA from tumour 128 which had a rearranged fragment of ~5 kb (Figure 5C), which is consistent with the size of DQ52-JH aberrant joining sometimes seen in T cells (Kurasawa *et al.*, 1981), rather than a productive immunoglobulin V_H–D_H–J_H join. We therefore conclude that these are clonal T cell tumours. Moreover, judging from the surface phenotype of these tumours, they arise from within the DN thymocytes of the pre-symptomatic phase.

Accelerated T cell tumour development in double transgenic mice

Transgenic mice expressing *Lmo2* controlled by either the CD2 or thy1 promoters develop T cell malignancies with a mean occurrence of 9–10 months (Larson *et al.*, 1994, 1995). A construct has been reported in which the *Tal1* gene is expressed from the CD2 promoter but no tumours developed in large cohorts of mice up to 15 months of age (Robb *et al.*, 1995b). In the transgenic mice used in the current study, both transgenes were expressed from a similar CD2 promoter to obtain coincident expression of the transgenes. The incidence of malignancy was compared between cohorts of CD2-rbtn2-only mice, CD2-tal1-only mice and double transgenic mice carrying both CD2-rbtn2 and CD2-tal1 constructs. While the CD2-rbtn2 mice developed T cell malignancy with a mean age of 9.8 months (Figure 6), none of the CD2-tal1 mice developed tumours in a period of 16 months (Figure 6).

A distinct difference in the pattern of tumour development was, however, found in the double transgenic mice. In this group, all the mice developed disease, and the average time of onset of observable disease was ~7 months (Figure 6). The general features of the tumours (histology and organomegaly) were indistinguishable from those arising in CD2-rbtn2-only mice. The average tumour appearance in the double transgenic mice was around 3 months faster than the average time for tumour formation in CD2-rbtn2-only mice. This altered time course of tumour development in Lmo2-expressing double transgenic mice (Figure 6) must therefore be due to the presence of *Tal1* gene expression, and demonstrates that the two transgene products synergize in the aetiology of the T cell tumours, even though CD2-tal1 mice do not develop tumours.

Discussion

Lmo2 and Tal1 co-operation mediated by protein interaction

The transgenic co-expression of Lmo2 and Tal1 has two obvious biological effects that can be observed by comparing double with single transgenics. First, there is an increased occurrence of DN cells in their thymuses and secondly there is an increased rate of tumour occurrence. Our molecular data show the presence of Lmo2 and Tal1 heterodimers in the T cells of double transgenic mice. Thus, the biological effects on tumorigenesis and T cell differentiation are mediated through these heterodimers since CD2-tal1-only mice exhibit neither biological phenomenon.

LMO2 and *TAL1* normally interact in erythroid cells (Valge-Archer *et al.*, 1994) and they both play a crucial

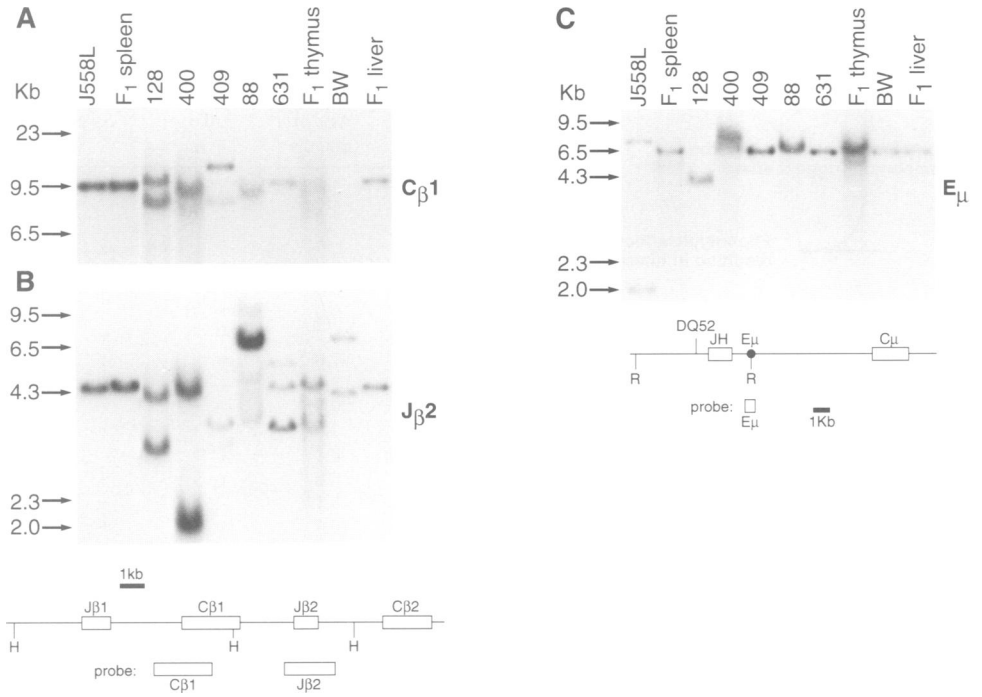

Fig. 5. The tumours in the CD2-rbtn2×CD2-tal1 transgenic mice are clonal T cell tumours. Filter hybridization of DNA prepared from a CD2-rbtn2-only mouse (88), from CD2-rbtn2×CD2-tal1 transgenic mice (128; 400; 409; 631), from cell-lines (J558L and BW) or from non-transgenic mouse liver and thymus. DNA was digested with *Hin*dIII (A and B) or *Eco*RI (C), fractionated on agarose and transferred to nylon membranes for hybridization with a TCR Cβ1 probe (**A**), TCR Jβ2 probe (**B**) or an immunoglobulin Cμ enhancer probe (**C**). The location of enzyme sites and of the probes is indicated below each panel. The size markers, indicated on the left-hand side of each panel, were λ DNA cleaved with *Hin*dIII.

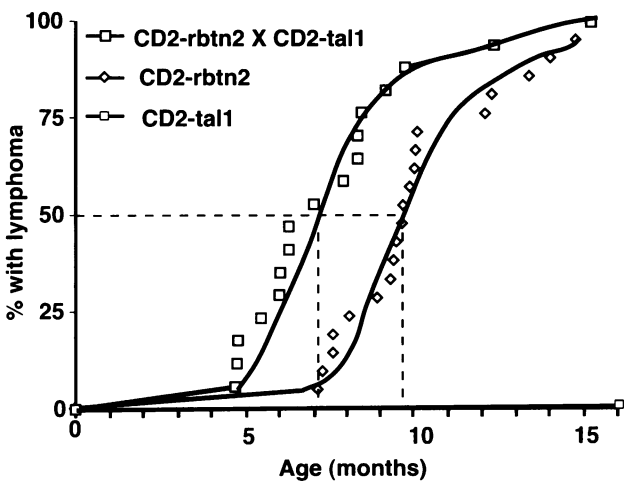

Fig. 6. Occurrence of tumours in CD2-rbtn2×CD2-tal1 transgenic mice. Cohorts of CD2-tal-only (n = 29), CD2-rbtn2-only (n = 20) or CD2-tal1/rbtn2 (n = 17) double transgenic mice were monitored for onset of leukaemia. At the time when symptoms were obvious, the mice were sacrificed, post-mortem examination carried out and in some cases further detailed analyses done. The graphs represent progressive incidence of disease and the dashed lines are the position at which 50% of the mice had developed disease.

role in erythroid differentiation (Warren *et al.*, 1994; Robb *et al.*, 1995a; Shivdasani *et al.*, 1995), presumably via the interaction of the proteins. The results with the double transgenic mice show that totally different outcomes can accrue from Lmo2–Tal1 interaction when they are aberrantly expressed in T cells. The abnormal part played by Lmo2 and Tal1 in human tumorigenesis, following chromosomal translocation, is thus most probably a subversion of the normal role in differentiation, but in a different cellular context. It is becoming increasingly clear that protein–protein interaction is crucial in the control of protein function and of gene expression. The complementation demonstrated for Lmo2 and Tal1 suggest that the same is true for cancer development (Hunter, 1991).

T cell differentiation abnormalities and distinct tumour phenotypes in double transgenic mice

A significant difference was observed in the surface phenotypes of T cell tumours that arose in single and double transgenic mice. In general, the tumours of double transgenic mice appeared more immature in that they expressed little or no CD4 and CD8 markers. In contrast, the single transgenic (*Lmo2*-only) mice primarily developed tumours with a double-positive ($CD4^+$, $CD8^+$) phenotype. It is evident that the tumour cells of double transgenic mice are similar to the previously defined DN cells characterizing the pre-symptomatic phase in *Lmo2* transgenic mice (Larson *et al.*, 1995). Thus, it is possible that a more immature cell is the target for tumour development in the double transgenic mice. Nevertheless, the perturbation in the phenotypes of the asymptomatic thymic T cells is qualitatively similar in single *Lmo2* and double transgenics; the major difference between the two is a quantitative increase in the proportion of DN cells and in the timing of their accumulation of these cells in the thymuses of double transgenic mice. Moreover, the double transgenic tumours seem to arise from within the DN population (e.g. mouse 631, Figure 4). It is plausible, therefore, that the *Lmo2*-only tumours also arise from these immature cells, as suggested (Larson *et al.*, 1995), but that further T cell maturation, accompanied by changes

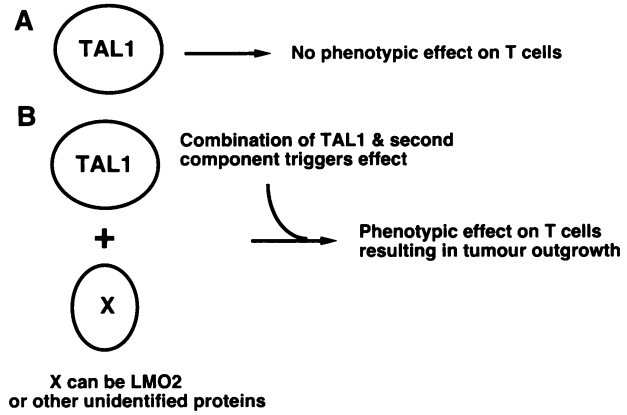

Fig. 7. Tal1 as a tumour promoter. (A) CD2-Tal1-only transgenic mice do not develop tumours nor do they exhibit altered thymocyte phenotype. Thus Tal1 alone does not appear to be able to exert a biological effect in this particular biological context. However (**B**), when Tal1 occurs in combination with a second, complementary component (e.g. Lmo2), there occurs a triggering effect on tumorigenesis. The second component can be Lmo2 (as in some T-ALL) but, in cases lacking Lmo2, other unidentified proteins may serve this function.

in surface phenotype patterns, occurs during the longer latency period to overt disease in these mice (Larson *et al.*, 1995). It remains to be shown formally whether the DN cells reflect abnormal expansion of normal immature thymocytes or of an abnormal type of T cell. This issue can only be resolved by further detailed analysis of phenotype and other features of the DN cells.

Although double transgenic mice develop tumours faster than single transgenics (Figure 6), the latency of tumour formation still suggests that the occurrence of at least one additional mutation (presumably in another oncogene) is necessary for overt disease to appear (Adams and Cory, 1991). Thus, in this particular transgenic context, the *Lmo2* transgene is necessary but not sufficient for tumour development. We have identified one co-operating oncogene, namely *Tal1*; the other(s) is not yet identified.

The Tal1 gene acts as a tumour promoter in this mouse model

The involvement of single transgenic *Lmo2* (Fisch *et al.*, 1992; Larson *et al.*, 1994, 1995) but not *Tal1* mice (Robb *et al.*, 1995b) in T cell tumours is a singular finding, given the prevalence of *TAL1* abnormalities in T-ALL (Baer, 1993). The *TAL1* gene is the most commonly affected in T-ALL, disruption occurring by both translocation and submicroscopic deletions or putative mutations affecting the promoter regions of the gene (Bash *et al.*, 1995). Use of the double transgenic mice described here provides formal evidence that Tal1 can be involved in tumorigenesis and endorses previous evidence of *TAL1* co-operation with v-*abl* in tumorigenecity (Elwood *et al.*, 1993).

The enhancement of tumour rate by the presence of Tal1 in the double transgenics implicates Tal1–Lmo2 co-operation in T-ALL patients that have both the *LMO2* chromosomal translocation t(11;14)(p13;q11) and the *TAL1* promoter deletion abnormality (Wadman *et al.*, 1994). The complementation seen in the double transgenic mice therefore mimics a naturally occurring situation in humans. The curious lack of neoplastic effect by Tal1 alone therefore suggests that Tal1 operates as a tumour promoter in this transgenic model (Figure 7). In this concept, the *TAL1* gene product has no detectable biological effect when expressed alone but promotes tumorigenicity by synergy when in conjunction with an appropriate complementary factor (Figure 7) (which is Lmo2 in the transgenic model but in other cases it could be different proteins). The frequent activation of *TAL1* expression in T-ALL patients is consistent with the notion that it serves as an oncogene which is integral to this disease.

Table I. Summary of age of disease onset and pathology of CD2-rbtn2×CD2-tal1 transgenic mice

Mouse	Age in months	Enlarged thymus	Splenomegaly	Hepatomegaly
633	4.0	+	–	+
630	4.3	+	+	+
631	4.8	+/–	+	+
406	6.0	+	+	+
409	6.0	+	+	+
639	6.0	+	–	–
539	6.7	+	+	+
491	7.0	+	+	+
472	7.3	–	+	+
544	7.5	–	+	+
414	7.9	+	+	+
400	8.1	+	–	+
413	8.6	+	+	+
495	9.1	–	+	+
478	9.1	+	+	+
417	9.2	+	+	+
128	12.6	+	+	+

A group of 17 double positive transgenic mice was included in a study of age of onset of disease and status of thymus, spleen and liver. 65% of the mice had involvement of the three organs. Age (in months) corresponds to the time when the mouse was sacrificed due to evidence of disease (see Materials and methods).

Materials and methods

Transgenic mice

CD2-rbtn2 transgenic mouse line 989 has been described (Fisch *et al.*, 1992; Larson *et al.*, 1994, 1995). The CD2-tal1 transgenic construct was made by cloning the *Eco*RI–*Hin*dIII *TAL1* fragment from the human *TAL1* clone talM1/pBS (Cheng *et al.*, 1993) into the unique *Xho*I site of the CD2 vector (Lang *et al.*, 1988; Lake *et al.*, 1990). Transgenic mice were generated using a *Not*I fragment encompassing the insert. The mice were monitored for symptoms of distress as a first predictor of disease; generally slow movement, hunched back and ruffled hair indicated the presence of advancing malignancy. Post-mortem examination was carried out as a routine, and some general features of one cohort of mice are illustrated in Table I. FACS analysis was conducted on some tumours as described (Larson *et al.*, 1995). Mice sacrificed prior to onset of disease were considered to be asymptomatic if there were no circulating leukaemic cells, no abnormal histology or absence of any signs of organomegaly.

DNA analysis

DNA was prepared by tail biopsy, from tumours or from tissues by standard methods. For filter hybridization, DNA samples were cleaved with the appropriate restriction enzyme and fractionated on 0.8% agarose gels prior to transfer (Southern, 1975) to Hybond-N nylon membranes. Hybridization of randomly labelled probes (Feinberg and Vogelstein, 1983) was carried out as described (Rabbitts *et al.*, 1993). The TCR probes were the *Hin*dIII–*Bam*HI fragment from clone 1 for TCR Cβ1 and the *Eco*RI fragment from clone 3 for TCR Jβ2 (Malissen *et al.*, 1984). The immunoglobulin enhancer probe was the *Xba*I fragment containing the Eμ enhancer region (Neuberger *et al.*, 1989).

Western protein detection

Single-step Western. Single cell suspensions of 1×10^6 tissue culture cells or 1×10^7 thymocytes from 6-week-old mice were lysed in low stringency buffer [10 mM HEPES pH 7.6, 250 mM NaCl, 5 mM EDTA, 0.5% Nonidet P-40, 0.1% phenylmethylsulfonyl fluoride (PMSF) and 1 μg/ml each of leupeptin, aprotinin and pepstatin] (Wadman *et al.*, 1994). Twenty micrograms of protein were fractionated by 12% SDS–PAGE and transferred to nitrocellulose filters. The filters were washed for 1 h in phosphate-buffered saline with 0.1% Tween 20 and 5% powdered skimmed milk before 1 h incubation with a 1:100 dilution of anti-Tal1 monoclonal antibody (Pulford *et al.*, 1995). The filters were washed three times, incubated with a 1:10 000 dilution of horseradish peroxidase-conjugated anti-mouse antibody (Amersham) for 1 h and washed eight times. Filters were subjected to ECL analysis (Amersham) according to the manufacturer's recommendations and exposed to X-ray film for 2 min.

Two-step protocol. Single cell suspensions of 1×10^7 tissue culture cells or 1×10^8 thymocytes from 6-week-old mice were lysed in low stringency buffer. The preparation was twice cleared by 1 h incubation with normal rabbit serum at 4°C with gentle agitation followed by the addition of 75 μl of 10% PanSorbin cells (Calbiochem), incubation for a minimum of 1 h and centrifugation at 16 000 *g* for 10 min. Ten microlitres of rabbit anti-rbtn2 antiserum (Warren *et al.*, 1994) were added to the resulting supernatants and incubated at 4°C with gentle agitation for 1 h. This was followed by addition of 100 μl of protein A–Sepharose, further incubation for 1 h and centrifugation for 10 min at 16 000 *g*. The pellets were resuspended and washed four times in fresh, low stringency buffer with protease inhibitors before boiling for 5 min in 20 μl of SDS–PAGE sample buffer (10 mM Tris–HCl pH 6.8, 5% glycerol, 2% SDS, 0.1% β-mercaptoethanol). Ten microlitres of sample was separated on 12% acrylamide gels and transferred to nitrocellulose filters. The filters were treated as above with monoclonal anti-Tal1 antibody.

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