The oncogenic T cell LIM-protein Lmo2 forms part of a DNA-binding complex specifically in immature T cells

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The LIM-only protein LMO2 is expressed aberrantly in acute T-cell leukaemias as a result of the chromosomal translocations t(11;14) (p13;q11) or t(7;11) (q35;p13). In a transgenic model of tumorigenesis by Lmo2, T-cell acute leukaemias arise after an asymptomatic phase in which an accumulation of immature CD4+ CD8+ double negative thymocytes occurs. Possible molecular mechanisms underlying these effects have been investigated in T cells from Lmo2 transgenic mice. Isolation of DNA-binding sites by CASTing and band shift assays demonstrates the presence of an oligomeric complex involving Lmo2 which can bind to a bipartite DNA motif comprising two E-box sequences ~10 bp apart, which is distinct from that found in erythroid cells. This complex occurs in T-cell tumours and it is restricted to the immature CD4+ CD8+ thymocyte subset in asymptomatic transgenic mice. Thus, ectopic expression of Lmo2 by transgenesis, or by chromosomal translocations in humans, may result in the aberrant protein interactions causing abnormal regulation of gene expression, resulting in a blockage of T-cell differentiation and providing precursor cells for overt tumour formation.

Keywords: chromosomal translocation/leukaemia/LIM/LMO2/TAL1

Introduction

Chromosomal translocations are found as recurring features in human tumours and have led to the identification of novel oncopgenes whose functions in tumour development frequently involve transcriptional regulation (Rabbitts, 1994). One such oncogene is LMO2 (formerly called RBTN2 or TTG2) which is activated in T-cell acute leukaemias (T-ALLs) by the chromosomal translocations t(11;14) (p13;q11) or t(7;11) (q35;p13) (Boehm et al., 1991a; Royer-Pokora et al., 1991). This gene encodes a nuclear LIM-domain protein which is necessary for embryonic erythropoiesis (Warren et al., 1994) and for adult haemopoiesis (Yamada et al., 1998).

The models of Lmo2-driven T-cell tumours have involved expression of the gene in thymocytes using the promoter/enhancer of the T-cell gene CD2 (Lang et al., 1988) to drive Lmo2 expression (Fisch et al., 1992; Larson et al., 1994) or the metallothionein promoter (Neale et al., 1995). A similar situation has been reported for the family member Lmo1 (Fisch et al., 1992; McGuire et al., 1992). The period before Lmo2-induced tumours arise is long, the first ones appearing after ~6 months of age, implying that mutations (in other genes) need to occur before overt disease appears. During this asymptomatic phase, there are marked changes in the thymus T-cell population, principally an increase in immature T cells lacking CD4 and CD8 surface markers (herein referred to as DN thymocytes) (Larson et al., 1995; Neale et al., 1995). The number of T cells in the DN population varies between individual transgenic mice, but increases can be seen in mice soon after birth. Thus it appears that the primary effect of the Lmo2 transgene is exerted on these immature T cells (Larson et al., 1995), suggesting that this is also the target population in patients with the chromosomal translocation t(11;14) or t(7;11).

The molecular function of the LIM protein Lmo2 in this tumorigenesis is as yet unclear. It is known that in normal erythroid cells, the Lmo2 protein interacts with the basic-helix–loop–helix protein (bHLH) Tal1/Scl (Valge-Archer et al., 1994; Wadman et al., 1994) (herein referred to as Tal1). In addition, while Tal1 transgenic mice (in which the Tal1 gene is driven by the same CD2 promoter as used for the Lmo2 transgene) do not develop leukaemias (Robb et al., 1995b; Larson et al., 1996), nor show any perturbation of T-cell development, Lmo2–Tal1 double transgenic mice exhibit an accelerated tumour development profile compared with Lmo2-only mice (Larson et al., 1996). However, in transgenic mice with Tal1 driven by the lck promoter, a proportion of mice developed tumours but there was no reported alteration of surface phenotype of thymocytes prior to tumour induction (Condorelli et al., 1996; Kelliher et al., 1996). Further, double transgenic mice (co-expressing Lmo2 and Tal1) have high levels of thymic DN immature T cells at non-leukaemic stages, even as soon as they are born, showing a marked change in the differentiation pathway resulting in a partial block in the progression to mature CD4+ CD8+ CD3+ T cells (Larson et al., 1996). Thus, the interaction of Lmo2 and Tal1 proteins seems to have a synergistic effect on tumour formation as well as on T-cell differentiation. Such a situation may also occur in some human T-ALLs which acquire both a chromosomal translocation involving LMO2 and also a chromosomal abnormality involving TAL1 (Wadnan et al., 1994).

Lmo2 is expressed normally in cells of erythroid/myeloid origin (Warren et al., 1994), central nervous system, lung, kidney, liver and spleen, but not in thymus (Foroni et al., 1992). Recent data on the function of Lmo2...
have shown that it plays a crucial role in the development of primitive erythroid cells (Warren et al., 1994) and in adult haematopoiesis (Yamada et al., 1998). These functions seem to be mediated through protein–protein interactions involving the LIM-domains of Lmo2, which bind to Tal1 (Valge-Archer et al., 1994; Wadman et al., 1994), GATA-1 (Osada et al., 1995) and to the LIM-domain-binding protein Ldb1/Nli (Agulnick et al., 1996; Jurata et al., 1996). Altogether, they form an oligomeric DNA-binding complex in erythroid cells, which also includes the ubiquitously expressed class A bHLH proteins E47/bHLH complex, which operates either via binding to DNA or to participate in a complex which depletes the intracellular concentrations of the DNA-binding protein(s) and thereby affects their normal function. This suggests that Lmo2 functions in haematopoiesis via its ability to carry out protein contacts. Thus the mode of action may be to facilitate formation of a DNA-binding complex or to participate in a complex which depletes the intracellular concentrations of the DNA-binding protein(s) and thereby affects their normal function. This suggests that the most obvious model for the role of LMO2 in T-ALL is that the protein binds to factors in T cells after the chromosomal translocation, creating an aberrant complex which operates either via binding to DNA or by a sequestration mechanism. This proposal has been investigated utilizing Lmo2 transgenic mouse T cells to study molecular complexes involving Lmo2. We have detected a DNA-binding complex involving Lmo2 in transgenic tumours and thymi of asymptomatic mice which binds to a bipartite site consisting of two E-boxes separated by an ~10 bp spacer. The complex has some striking similarities to the erythroid-specific Lmo2-containing DNA-binding complex but recruits a second bHLH heterodimer instead a GATA factor into the oligomeric complex. Tal1 can be part of the oligomeric complex. This complex is present in primary tumours from transgenic mice and it seems to be restricted to the immature DN thymocyte subpopulation in asymptomatic transgenics.

Results

Characteristics of a transgenic T-cell line

CD2-Lmo2 transgenic mice develop T-cell tumours from ~6 months of age onwards (Fisch et al., 1992; Larson et al., 1994). We isolated cell lines from these tumours in order to study the biochemical function of Lmo2 protein. One such line, designated 2114, was made from a transgenic tumour which contains multiple copies of the transgene as judged by filter hybridization of genomic DNA extracted from 2114 cells (Figure 1A, right panel), and lacked expression of CD4 and CD8 surface markers, which is a characteristic feature of immature T cells. The derived cell line 2114 corresponded to this early differentiation stage of thymocytes as fluorescence activated cell sorting (FACS) analysis of 2114 cells (Figure 1C) showed no detectable CD3, CD4 or CD8. But expression of the Thy1.2 T-cell marker as well as CD25 and CD44, which are typical markers for early DN thymocytes (i.e. CD4+ CD8+) undergoing T-cell receptor (TCR) β-gene rearrangement were detected. The status of TCRβ genes in 2114 was examined by filter hybridization with a TCR β2 probe (Figure 1A) showing one major rearrangement presumably because one allele of TCRβ has undergone correct V–D–J joining. In addition, two bands with weaker hybridization are present, presumably reflecting the breakage within the TCRβ probe itself on a second rearranged allele. Labelling of these cells with [35S]cysteine showed that the Lmo2 protein was detectable by immunoprecipitation with anti-Lmo2 antiserum (Figure 1B). The 2114 cells, therefore, appear to originate from a transformed DN immature thymocyte expressing Lmo2.

DNA-binding site selection (CASTing) with anti-Lmo2 antiserum

As a direct means to assess if the Lmo2 protein, ectopically expressed in the transgenic T cells, was present in a DNA-binding complex with other proteins, in vitro DNA-binding site enrichment (CASTing; Pollock and Treisman, 1990; Wright et al., 1991) was carried out using cellular extracts from 2114 cells and a random pool of oligonucleotides. After seven rounds of CASTing, the final selected oligonucleotide pool was cloned and 57 clones were sequenced. When these sequences were analysed in detail, it was found that 24 clones (42%) contained an E-box motif (recognition sequence for bHLH proteins) (Figure 2), of which 14 clones had two E-boxes in the same sequence. Of the clones with two E-boxes, nine had a 10 bp spacing between the two E-boxes and other spacings occurred [3 bp (1); 9 bp (1); 11 bp (1) and 12 bp (2)]. The most common E-box was CATCTG or, in the complementary reversed form, CAGAGT (63% of all E-box motifs), followed by CAGGTG (24% of all E-box motifs). Unlike the situation found with similar experiments using erythroid extracts (Wadman et al., 1997), no clones with an E-box adjacent to a GATA site were isolated. Interestingly, the most common selected E-box (CAGAGT) resembles the preferential binding site for E2A–Tal1 heterodimers (Hsu et al., 1994a) including the flanking regions, suggesting an involvement of Tal1 or a Tal1 homologue in the DNA-binding complex.

Lmo2 is involved in a complex in 2114 cells which recognizes a dual E-box sequence

The involvement of Lmo2 protein in the recognition of the selected binding sites from the CASTing experiments (Figure 2) was assessed further by electrophoretic mobility shift assays (EMSAs) and antibody-mediated supershifts using the same 2114 cellular extracts employed for the CASTing experiments. In these experiments, it was found
that only the sequences with two E-boxes separated by 10 bp (herein called the dual E-box oligo) showed an Lmo2-specific involvement (Figure 3). A number of bands were observed in the EMSAs, shown in Figure 3A using oligonucleotides with either one (E-box/mE-box, designated single E-box oligo) or two E-box motifs (E-box/E-box, designated dual E-box oligo). A low mobility band (herein called band I, Figure 3A, lane 2) appeared in mobility shifts with labelled dual E-box compared with labelled single E-box oligo (Figure 3A, lane 4). This low mobility band I was the only one involving the Lmo2 protein as it is supershifted by addition of anti-Lmo2 antiserum and Staphylococcus aureus protein A (Figure 3A, lane 1).

To determine further the nature of this Lmo2-containing low mobility band I, we performed competition assays with non-labelled dual E-box oligo or non-labelled single E-box oligo (Figure 3B). The cold single E-box oligo inhibited the appearance of band II, also seen in the band shift with the labelled single E-box oligo (this band must be due to the binding of a bHLH dimer to the single E-box), but it had no effect on the low mobility band I.

Fig. 1. Characterization of the T-cell line 2114. The T-cell line 2114 was established in culture from a thymic tumour which arose in a CD2-Lmo2 transgenic mouse. (A) Southern hybridization analysis of 2114 genomic DNA. HindIII-digested DNA from the cell line 2114 was probed with a \( \beta 2 \) probe (Malissen et al., 1984) (left panel) and compared with a control liver DNA and lymphoid cell lines [J 558L (B-cell line), BW 5147 (T-cell line)]. The same blot was reprobed with a Lmo2 probe (right panel) to confirm the presence of the transgenic construct. (B) Immunoprecipitation of Lmo2 from \( 35S \)-labelled 2114 cellular extracts using anti-Lmo2 antiserum (Warren et al., 1994) compared with rabbit anti-Lmo2 antiserum blocked by peptide antigen (Lmo2 residues 2–17). (C) Cultured cells from mid-log phase were examined by FACS analysis. Cell surface markers tested for in the upper panel are isotype control with rat IgG2a\( \kappa \)–FITC and IgG2a\( \kappa \)–PE or CD4–FITC/CD8–PE double staining. Lower panel: double staining with anti-CD25–FITC and anti-CD44–PE or single staining with anti-Thy1.2–PE compared with isotype control (dotted line).
Fig. 2. Oligonucleotide sequences obtained by Lmo2-binding site selection (CASTing) using cellular extracts from the 2114 T-cell line. Cellular extracts from 2114 cells were mixed with a random oligonucleotide pool (R76; Pollock and Treisman, 1990) and specific complexes immunoprecipitated with anti-Lmo2 antiserum. After seven rounds of binding and immunoprecipitation, the oligonucleotides were cloned and sequenced. (A) Sequences containing one E-box are aligned by their E-box. (B) Sequences containing two E-boxes. The sequences are aligned by their E-box, and the variation in the spacer (9–12 bp) adjusted by adding bars, with the exception of the first sequence containing a 3 bp spacer. Upper case letters represent the random part of the selected sequence, whereas lower case letters represent the start of constant flanking regions. The E-box motifs are presented in bold and separated by a space from the rest of the sequence. (C) Representation of the consensus sequence as a consensus sequence as a percentage for nucleotides at each position. The E-boxes and the two flanking positions which had a non-random pattern of nucleotide frequency are shown. The consensus sequence generated from a nucleotide frequency >43% is shown in the last row. In the case of an equal nucleotide frequency, the standard IUPAC code has been used (M = A or C, R = A or G).

Lmo2, E47, Tal1 and Ldb1 are part of the complex in 2114 cells binding to the dual E-box motif

The low mobility of the band I which binds to the dual E-box motif must be due to an oligomeric complex involving other proteins in addition to Lmo2, as Lmo2 does not seem to bind to DNA. Indeed, an analogous complex found in erythroid cells which binds to an E-box–GATA motif appears to comprise at least five different proteins (Wadman et al., 1997). Candidates for involvement in the low mobility band I were assessed with antibody-mediated supershifts. Anti-Lmo2 antiserum supershifts the dual E-box oligo-specific band I especially in the presence of *S. aureus* protein A, which binds to the exception at the very highest concentration of competitor (lane 19) when a small inhibition begins to occur. Conversely, cold competitor dual E-box oligo inhibited the formation of three bands, including the low mobility band I, in the same concentration-dependent way (Figure 3B, lanes 8–13). These results show that the dual E-box site is bound by synergistically interacting bHLH dimers, causing an avidity effect, rather than by independently binding bHLH dimers. Thus, while the intermediate mobility band II seems to be due to a complex binding to a single E-box, not including Lmo2, the low mobility band I is due to an Lmo2-containing complex with two DNA-binding elements each binding to one of the E-boxes.
Fig. 3. Band shift assays using cellular extracts from the 2114 T-cell line. Cellular extracts from the transgenic mouse-derived T-cell line 2114 were used in EMSAs with various oligonucleotides. (A) The labelled oligonucleotides were dual E-box (lanes 1–3; CATCTG–N10–CAGATG) or single E-box (lanes 4–6; CATCTG–N10–CCGATC). Antibody supershifts were performed as indicated with antiserum recognizing Lmo2 (lanes 1 and 4) or Tal1 (lanes 3 and 6). (B) Labelled dual E-box oligo (CATCTG–N10–CAGATG) was competed with unlabelled oligonucleotide corresponding to itself or to the single E-box oligo. The right part of the gel (lanes 8–20) shows competition experiments on mobility shifts of 2114 cellular extracts with the 32P-labelled dual E-box oligo. Lanes 8, 14 and 20 show the mobility shift without addition of competitor. In lanes 9–13, increasing amounts (5×, 10×, 50×, 100×, 300×) of cold dual E-box oligo were added. In comparison, increasing amounts (5×, 10×, 50×, 100×, 300×) of cold single E-box oligo were added to lanes 15–19. For clarity, only the upper part of the gel is presented in the figure. Diagrams of protein complexes which may cause the particular mobility shifts of interest are indicated as symbols at the side of the gel.

Fig. 4. Antibody supershift of dual E-box oligonucleotide-specific complex. Cell extracts from 2114 cells (lanes 1–14) and EL4 T cells (Lmo2 negative; lanes 16–20) were incubated with 32P-labelled dual E-box oligo (CATCTG–N10–CAGATG) in the absence or presence of specific antisera (with or without S. aureus protein A). Left panel: 2114 extract. The mobilities of complexes binding to the dual E-box oligo in the absence of antibody are shown in lane 4. Addition of protein A on its own (lane 11) or the appropriate pre-immune sera (lanes 3, 5, 10 and 12) does not alter the pattern of the bands, with the exception of pre-immune serum of the anti-Tal1 antiserum (lane 5). Mobility shifts in the presence of antisera (plus or minus protein A) are as indicated. Right panel: EL4 cell extract. The right part of the gel (lanes 16–20) shows the mobility shift pattern based on incubating 15 μg of EL4 cellular extracts with 32P-labelled dual E-box oligo without antibody (lane 7) or with antibody (lanes 16 and 11–20). For clarity, only the upper part of the gel is presented in the figure. Complexes thought to cause the particular mobility shifts of interest are indicated at the side of the gel.

Fc part of the anti-Lmo2 antiserum, whereas the pre-immune serum does not have any influence on the mobility of any band (Figure 4, lanes 1–3). The same supershift behaviour can be produced by a second, independent anti-Lmo2 antiserum from another rabbit (data not shown). The low mobility complex also contains the recently identified Ldb1/Nli (Agulnick et al., 1996; Jurata et al., 1996) since cellular extracts from 2114 cells were subjected...
to band shifts with the dual E-box oligo and supershifted with anti-Ldb1 antiserum with or without the presence of protein A (Figure 4). While the band due to the single E-box-binding complex (band II) was not affected by the anti-Ldb1 serum, that of the low mobility complex (band I) was supershifted.

A second binding partner of Lmo2 is the bHLH protein Tal1 (Valge-Archer et al., 1994; Wadman et al., 1994). Thus, Tal1 might also be part of the T-cell-specific dual E-box oligo-binding complex. Therefore, we tested the ability of anti-Tal1 antiserum to alter dual E-box oligo mobility shifts and found that the low mobility band I was supershifted (Figure 3A, lane 3), suggesting the presence of Tal1 protein in this complex. Band III is also affected by the Tal1 antiserum, but this is non-specific as the pre-immune serum also causes a supershift of this band (Figure 4, lanes 5). This unexpected presence of Tal1 in the 2114 cell complex was confirmed by the ability of anti-Tal1 serum (Figure 4, lane 6) or antiserum with S.aureus protein A (lane 7) specifically to supershift only the low mobility complex. It was surprising to find Tal1 protein in a T-cell line derived from a CD2-Lmo2 transgenic thymoma since thymus cells do not express Tal1 (Kallianpur et al., 1994; Pulford et al., 1995). The presence of Tal1 in the 2114 cell line was confirmed by RT–PCR of 2114 cDNA employing several sets of Tal1 primers (data not shown) and sequencing of the RT–PCR products [the largest was a 516 bp fragment spanning position 348, which covers part of Tal1 protein used for antiserum preparation (Hsu et al., 1994a), to 864, which corresponds to the end of bHLH region].

The E-box–GATA binding complex in erythroid cells includes the class A bHLH proteins E47/E12, products of the E2A gene (Murre et al., 1989; Sun and Baltimore, 1991; Roberts et al., 1993). Accordingly, the involvement of these molecules in the 2114-specific T-cell complex was tested using an E2A-specific immune serum. This antiserum was found to reduce the mobility of two specific E-box-binding complexes identified by competition experiments (Figure 3B), namely the low mobility complex containing Lmo2 (band I) and the band due to protein(s) binding to a single E-box [band II, which has the same mobility as the E47 homodimer band in the erythroid cell line MEL (Wadman et al., 1997) and, therefore, is presumably due to the E47 homodimer]. Thus the Lmo2-containing low mobility complex (band I) also contains E47/E12, analogous to the oligomeric E-box–GATA binding complex in erythroid cells (Wadman et al., 1997). This dual E-box-binding complex would therefore appear to have a Tel1–E2A bHLH heterodimer bound to each E-box, with Lmo2 and Ldb1/Nli bridging the two heterodimers.

### The Lmo2-containing complex is present in primary T-cell tumours arising in transgenic mice

The representative nature of the 2114 cell line was evaluated by examining primary tumours derived from CD2-Lmo2 transgenic mice or from CD2-Lmo2×CD2-TAL1 double transgenic mice for the presence of the Lmo2-containing band I complex. Crude nuclear extracts from lymphomas of several transgenic mice were incubated with the 32P-labelled dual E-box oligo, and mobility shift and supershift patterns were studied. An example is shown in Figure 5 using nuclear extracts of thymus cells from a CD2-Lmo2-only mouse (2122) which developed a T-cell lymphoma at 12 months of age. The EMSA pattern was compared with the pattern seen with extract from non-transgenic mouse thymus (2 months of age) and 2114 cells. Gel retardation bands obtained with 2122 extracts were similar to those seen in 2114 (Figure 5). The low mobility band I observed with 2122 nuclear extracts contained Lmo2, Tal1, E2A and Ldb1 proteins as judged...
by antibody supershifts (Figure 5, lanes 4–7 and 9 respectively). The specificity of band I for transgenic T cells was confirmed since non-transgenic thymus has no complex that binds to the dual E-box motif (Figure 5, lanes 12–16), consistent with the lack of Lmo2 expression in thymus (Foroni et al., 1992). The complex is also absent in cellular extracts from the non-transgenic T-cell thymoma line EL4 (Figure 4). E2A antibodies did, however, cause in both cases a supershift of band II which corresponds to the binding of an E47 homodimer to a single E-box. This same band II is also evident when EL4 (Figure 4) or 2122 extract was used (Figure 5) and, therefore, can be used as an internal standard.

**The dual E-box-binding complex is restricted to the CD4− CD8− double negative transgenic thymocytes**

We have observed a variation in intensity of the Lmo2-containing band I relative to the intensity of the E47 homodimer band II in the panel of transgenic lymphomas tested (data not shown). One difference between the lymphomas tested was the differentiation stage of thymocytes, ranging from CD4+ CD8− DN to CD4+ or CD8− single positive (SP) (Larson et al., 1994). Thus, the T-cell differentiation stage might be a key determinant in the ability of Lmo2 to enter the complex which can bind the dual E-box site. This possibility was investigated by analysis of T cells from transgenic mice prior to the development of recognizable disease.

During the early stages of thymus development, the transgenic mouse thymus were found to contain nearly normal numbers of T cells but with an increase in CD4+ CD8− DN thymocytes (Larson et al., 1994), particularly apparent in Lmo2− Tal1 double transgenics (Larson et al., 1996). This suggested that the DN T-cell precursors might be the target cells in which the oncogenic effect of Lmo2 protein is exerted. Therefore, proteins from thymi of transgenic mice at the age of two months were assessed for the presence of the Lmo2-containing band I. Crude cellular extracts from the whole thymi of these mice, in which the level of DN T cells was assessed by FACS analysis, were subjected to EMSAs using the dual E-box oligo. In the case of a pool of T cells extracted from Lmo2− Tal1 double transgenic mice (comprising ~22% DN cells in the pool), we observed band I which was supershifted with Lmo2 antiserum (Figure 6A, lanes 1–3). No such complex could readily be seen after EMSA with extracts from a pool of T cells from an Lmo2-only transgenic (~6% DN cells) (Figure 6A, lanes 11–13), suggesting that the Lmo2-containing complex was only present in the DN cells of asymptomatic mice.

The presence of band I in CD4− CD8− DN T cells was verified by purification of this cell population from asymptomatic transgenic mice. A positive selection approach was used to prepare the DN thymocyte subpopulation by taking advantage of the presence of the CD25 (IL2Rα chain) surface marker, which is expressed on a subset of DN thymocytes. Two cell populations were compared, one selected with anti-CD25 beads and one selected with anti-CD8 beads. Successful selection was assessed by FACS analysis of the depleted fraction (data not shown). EMSA analysis of the unfraccionated thymocytes from the Lmo2− Tal1 double transgenic mice (comprising ~23% CD25+ cells) produced a weak band I corresponding to the low mobility complex which could be supershifted with both anti-Lmo2 and anti-Tal1 antiserum (Figure 6A, lanes 1–3), whereas the single transgenic T cells (6% DN T cells) did not exhibit this complex. However, when the cell population was enriched for CD25-expressing cells, both single and double transgenic T cells exhibit the oligomeric Lmo2-containing complex (band I; Figure 6A, lanes 4–6 and 14–16). Interestingly, Tal1 was detected in band I of both single and double asymptomatic transgenic mice (according to supershifts with anti-Tal1 antiserum). The CD8+ cells [i.e. CD4+ CD8+ double positive (DP) or CD4+ CD8+ SP thymocytes] do not have this complex in detectable amounts but the single E-box-binding complex (band II) appeared in all samples analysed (acting as an internal standard). We conclude, therefore, that the low mobility oligomeric complex is found predominantly in the immature DN T-cell precursors.

One reason for the observed difference between the two thymocyte subpopulations might be different expression levels of Lmo2, and Tal1 in the case of the double transgenic mice, in the DN compared with DP T cells, but this seems unlikely given the use of the CD2 promoter cassette to control Lmo2 and Tal1 transgene expression. However, we carried out Northern hybridization analysis of RNA prepared from CD25+ DN T cells and CD8+ DP T cells, which revealed that both RNA populations contain high levels of transgenic Lmo2 as well as RNA coding for the ubiquitously expressed Lmo2-binding partner Ldb1/Nli (Figure 6C). We cannot exclude differences in expression levels of other unidentified component(s) of the dual E-box-binding complex, which might be restricted to the CD25+ DN cells.

In summary, the dual E-box-binding complex which contains Lmo2 is present in asymptomatic thymocytes of the two transgenic mouse lines, either expressing Lmo2 alone or in combination with Tal1 under control of the CD2 promoter (Larson et al., 1996). It seems to be restricted to the CD25+ CD4− CD8− DN thymocyte subset, which represent early thymocytes undergoing TCRβ gene rearrangement.

**Discussion**

**Molecular role of Lmo2 in tumorigenesis of transgenic mice**

In human T-ALL, the LMO2 gene is activated by chromosomal translocations which also involve TCR genes (Boehm et al., 1991a; Royer-Pokora et al., 1991). In transgenic mouse models of Lmo2-induced T-cell tumorigenesis (Fisch et al., 1992; Larson et al., 1994; Neale et al., 1995), there are two features which are outstanding. First, the tumours form after a long latency period (>6 months) during which time secondary mutations in other oncogenes or suppressor genes must occur and are necessary for overt tumour formation (Larson et al., 1994; Neale et al., 1995). Second, there is a impairment of T-cell differentiation manifest in the thymus of transgenic mice as an accumulation of CD4− CD8− DN immature T cells (Larson et al., 1995; Neale et al., 1995). This effect is particularly evident when mice transgenic for both Lmo2 and Tal1 are examined (Larson et al., 1996). This
latter observation raised the possibility that a molecular complex between Lmo2 and Tal1 was causing an effect in the DN precursor T cells in an analogous way to their interaction in erythroid cells (Valge-Archer et al., 1994; Wadman et al., 1994) where an oligomeric complex has been characterized comprising E2A, GATA-1 and Ldb1/Nli in addition to Lmo2 and Tal1 (Wadman et al., 1997). Further investigation of a possible complex in the transgenic T-cell tumours was facilitated by the derivation of tissue culture cell lines from various different tumours; one of these, designated 2114, was found to have a surface marker phenotype typical of the immature T cells expanded in the asymptomatic thymus (i.e. CD4−, CD8−, CD25+, CD44+). DNA-binding site selection with anti-Lmo2 antibodies was used to determine a consensus binding site, and we found that, unlike the site found with analogous experiments with the erythroid Lmo2-containing complex, a dual E-box site was present, with two E-box motifs separated by ~10 bp. The complex which binds this motif involves at least the two bHLH proteins, Tal1 and E47, and the LIM-binding protein Ldb1/Nli in addition to Lmo2. There may also be other bHLH protein combinations involved in the oligomeric complex. A model of how this complex might occur is illustrated in Figure 7. Ldb1/Nli may provide binding sites for two Lmo2 molecules, each of which can bridge to one of the bHLH heterodimers. The potential of Ldb1/Nli as an agent to homo- or heterodimerize LIM-domain-containing proteins has been...
demonstrated recently (Jurata et al., 1998). No GATA-3 protein was found in this complex, despite the fact that Lmo2 and GATA-3 have been shown to interact (Ono et al., 1997).

Since the dual E-box-binding complex contains proteins with transcriptional activation domains, it may well be that this complex recognizes a new specific set of target gene promoters/enhancers (i.e. those with a dual E-box motif) and causes their transcription. In fact, artificial reporter constructs have been described which fortuitously contain two E-boxes, separated by a 10 bp spacer, and these were activated by co-expression of Lmo2 and Tal1 (Ono et al., 1997). Alternative functions must also be considered, such as sequestration of proteins, hindering their normal function after the ectopic expression of Lmo2 in this transgenic model (and, by analogy, in humans, after chromosomal translocation). For instance, Lmo2 may function in this context to sequester Ldb1 and cause an alteration of its function. E2A gene-deficient mice show abnormalities in thymocyte development and develop T-cell lymphoma (Bain et al., 1997; Yan et al., 1997), suggesting that a depletion or inactivation of E2A can lead to T-ALL. The inactivation of E2A can be achieved by heterodimerization with either Tal1 or Lyl1 (both activated by chromosomal translocations in humans) due to changed binding site specificity (Miyamoto et al., 1996) or repression of activation (Hsu et al., 1994c). Thus it may be that Lmo2 acts via the same mechanism by recruiting E2A into a complex with a changed binding site specificity and, therefore, functionally deplete E2A. However, there are differences in abnormalities of thymocyte development between E2A-deficient mice and Lmo2 transgenic mice which indicate a different mode of tumorigenesis. The exact gain or loss of function caused by ectopic Lmo2 expression will necessitate models which selectively affect the individual components of the system.

**Immature T cells are the target for Lmo2 protein function**

The restriction of the dual E-box-binding complex to the CD4^-CD8^- DN immature thymocyte subset corresponds well with the observed increase in the DN thymocyte population in the asymptomatic transgenic thymus (Larson et al., 1995, 1996; Neale et al., 1995), suggesting that the pathogenic effect of Lmo2 is confined to these immature T cells because the complex only occurs there. Recognition of the dual E-box oligo motif by the Lmo2-containing oligomeric complex may lead to abnormal regulation of downstream target genes and, therefore, to a partial differentiation block at this T-cell developmental stage. Loss-of-function models must also be considered. In addition, it cannot be ruled out that the immature DN cells normally express Lmo2 since total thymus cell RNA does have Lmo2 mRNA as judged by RT-PCR (Boehm et al., 1991b). In this circumstance, the transgenic over-expression of Lmo2 would provide the molecular basis for the differentiation block as the Lmo2 gene would, thereby, be constitutively expressed. In this respect, there are similarities to the partial block of erythroid differentiation which has been observed when Lmo2 is over-expressed in a proerythroblast cell line (Visvader et al., 1997). In our transgenic model of T-cell tumorigenesis, the transgene is necessary but not sufficient for tumours to appear (Larson et al., 1994). During the asymptomatic phase, T-cell differentiation is affected and it is possible that additional chromosomal alterations might happen, particularly since CD25+ CD44+ are undergoing TCRβ gene rearrangements (Fehling and von Bohemer, 1997), and the extended expression of RAG recombinase, due to the impairment of T-cell differentiation, might lead to other gene rearrangements. These and other mutations might cause the overt malignancy to appear.

In the case of the Lmo2 transgenic model, the RNA levels of Lmo2 and Ldb1 are approximately equivalent in both the CD4^-CD8^- DN and CD4^+ CD8^- DP thymocyte subsets (Figure 6). In addition, Tal1 and E47 proteins are present in both subpopulations as judged by band shift data in double transgenic mice. Therefore, it is surprising that the complex is only detectable in the DN T-cell subset. This suggests that there is an additional unidentified component necessary for the formation of the complex and that this component is only present in the DN and not the DP T cells. An alternative explanation is that post-translational modification of any of the identified components of the complex either enables the formation of the complex in the DN T-cell subset or disables it in later stages of differentiation. Whatever the mode of Lmo2 action, our data suggest that the most likely result of Lmo2 ectopic expression in T cells is an alteration of gene expression within the T cell destined to become an overt tumour.

**Materials and methods**

**Transgenic mice and derivation of cell line 2114**

Transgenic mice line 989 (using murine Lmo2) and line 2 (using human TAL1) and the appropriate cross-breedings have been described before (Larson et al., 1996). The CD2^-Lmo2^ transgenic mouse 2114 was sacrificed at 11.5 months and found to have a thymoma with splenomegaly and hepatomegaly. The thymoma was excised and a single cell suspension was established by passing it through a 70 μm cell strainer. After washing with phosphate-buffered saline (PBS), the cells were resuspended in RPMI 1640 + 10% fetal calf serum (FCS) at a density of 10^7 cells/ml. Cell growth was observed after ~2 weeks. Cells were maintained in this medium in the presence of 10 U/ml of interleukin-7 (IL-7; Genzyme) and 20 U/ml of IL-2 (Boehringer Mannheim).
Antibodies and immunoprecipitation

Rabbit anti-peptide antisera directed against the N-terminus (residues 2–17) of Lmo2 have been described earlier (Warren et al., 1994). The rabbit anti-Ldb1 antisera recognizing Tal1 (residues 1–121; Cheng et al., 1993) and E2A (residues 217–371; Hsu et al., 1994a,b) were kind gifts of Richard Baer. The anti-Ldb1 antisera, residues 256–270 (Agulnick et al., 1996), was kindly provided by Alan Agulnick.

Antibodies for FACS analysis, directly coupled to either phycoerythrin (PE) or fluorescein isothiocyanate (FITC), were obtained from Pharmingen, San Diego, CA. Antibodies directed against CD2 (RM2-5), CD3 (e14–21C11), CD4 (RM4-5), CD8a (53–67, 6), CD25 (7D4), CD44 (1M7), Thy1.2 (53–21), TCRβ (β-chain specific, H57-597) and TCRγ (GL3) were used for characterizing the cell line 2114 and to control the quality of thymocyte subset selection. Biotinylated versions of antibodies directed against CD8a and CD25 have been used for the thymocyte selection.

Immunoprecipitation with anti-Lmo2 antisera has been performed as described (Valge-Archer et al., 1994).

Preparation of nuclear and cellular extracts

Nuclear extracts for EMSA were prepared as described recently (Wadman et al., 1997). Cellular extracts were prepared by washing the cells twice with PBS/5 mM EDTA and resuspending at a ratio of 150 ± 5 × 10^6 cells in CE solution (10 mM HEPES pH 7.6, 250 mM NaCl, 0.5% NP-40, 5 mM EDTA) with freshly added 1 mM dithiothreitol (DTT) and protease inhibitors [1 mM phenylmethylsulfonyl fluoride (PMSF), 1 μg/ml pepstatin A, 1 μg/ml aprotinin, 1 μg/ml leupeptin]. The suspension was vortexed vigorously for 30 s and then incubated for 30 min on ice with occasional vortexing. The cell debris was pelleted at 14 000 r.p.m. for 20 min at 4°C in a microfuge and the supernatant (cellular extract) collected. The samples were frozen on dry ice and kept at –70°C. The protein concentration was determined by the Bradford assay (Bio-Rad). Cellular extracts were used either for CASTing experiments or EMSA.

CASTing procedure

The CASTing procedure was performed essentially as described previously (Wadman et al., 1997) with 200 μg of 2114 cellular extracts per reaction. The immunoprecipitated oligonucleotides were used directly for PCR without further purification. After seven rounds of selection, the final product was amplified for 25 cycles with primers and blunt-end cloned into the EcoRI site of the appropriate pre-immune or positive control. The clones were sequenced and a consensus sequence defined.

EMSA

EMSA were performed essentially as described (Wadman et al., 1997). One strand of the oligonucleotide used was labelled with 32P by kinase reaction and annealed to an excess of cold complementary strand. Then 1 μl (4 nM) of this labelled annealed oligonucleotide was used per reaction. In total, 5–18 μg of cellular or nuclear extract were incubated in the presence of 0.8 μg of sonicated Escherichia coli DNA. The samples (10 μl total volume) were incubated for 30 min at room temperature in loading buffer (20 mM HEPES pH 7.6, 50 mM KCl, 1 mM EDTA, 25% v/v glycerol, 1 mM DTT) and then loaded directly onto a native 4% polyacrylamide gel on top of a 10% cushion. The gel was run for 5 h at 12 mA constant current at 4°C in 0.5× TBE (45 mM Tris, 45 mM boric acid, 1.25 mM EDTA), dried and exposed overnight for autoradiography.

For competition assays, both strands of the oligonucleotide were annealed at an equal molar ratio and then simultaneously added together with 32P-labelled oligonucleotide into the samples at various concentrations, leading to a final excess ranging from 3 to 300 times. For antibody-mediated supershift analysis, 1 μl of the appropriate pre-immune or immune serum was added to the sample and, where indicated, 1 μl of S.aureus protein A (2 mg/ml) also added. For clarity, only the upper half of the gels are presented in the figures, but all EMSA experiments were carried out with an excess of free probe.

Sequences of oligonucleotides

For EMSA: E-box/E-box (dual E-box oligo), 5′-AGCCGACATC-TGGTCAGTAAAAACAGATGTGCGCA; E-box/me-box (single E-box oligo), 5′-AGCCGACATCAGTGGTCGAATAACCCGATGTGCA. For Tal-1 RT–PCR: TAL-N2, 5′-GCCGCGGGAGCTTTAACGGGC; Tal-1 (Shivdasani et al., 1995), 5′-GTCCTCAGACCAAAGTAGTG; Tal-1 (Li et al., 1991), 5′-CTGGCGAGAGAACATGATGA.

Hybridization procedures

For filter hybridization, DNA samples were cleaved with HindIII and fractionated on a 0.8% agarose gel prior to transfer (LeFran et al., 1986) to a Hybond-N nylon membrane. Hybridization of randomly labelled probes (Amersham rediprime manual) was carried out as described (Rabbitts et al., 1993). The TCR β1 probe was a HindIII–BamHI fragment from TCR β1 (Malissen et al., 1984) and the Lmo2 probe was a NotI fragment of the murine cDNA clone pMR2A12N (Boehm et al., 1991b).

RNA was prepared by the Trizol method (Gibco-BRL). Cells from total thymi of 2-month-old CD2-Lmo2 transgenic mice (unselected) or C57Bl/6/CBA (wild-type) mice were used after passage them through a cell strainer and washing in PBS. For anti-CD25-positive or anti-CD8a-positive selected samples, 8.8-10^6 thymocytes from 10 CD2-Lmo2 transgenic mice were selected as described below and the magnetic beads directly incubated and vortexed in 1 ml of Trizol solution. Northern analysis was carried out as described (Rabbitts et al., 1993) with 10 μg of each RNA per lane. The Lmo2 probe was the same as used for Southern analysis, the Ldb1-specific probe was an XhoI fragment of a full-length cDNA of mouse Ldb1 (Agulnick et al., 1996) (kind gift of Alan Agulnick) cloned into the pEF-BOS vector (Mizushima and Nagata, 1990).

Selection for thymocyte subsets

Ten thymi of 2-month-old mice were pooled and passed through a cell strainer to generate a single cell suspension. Cells were washed once with PBS, resuspended in two times 750 μl of PBS, transferred into Eppendorf tubes and placed on ice. For FACS analysis of unfractionated cells, 1 × 10^7 cells were washed again with PBS/5 mM EDTA, resuspended in 50 μl of CE solution (see above) and cellular extracts prepared as described above.

The remaining thymus cells (usually 2–6 × 10^6) were used for depletion experiments and were incubated directly with a 1:1 mixture (400 μl each) of anti-CD4 (L3T4+) and anti-CD8 (Lyt2)-coated magnetic Dynabeads (washed before three times with PBS) for 20 min on ice with occasional gentle mixing. The Eppendorf tubes were transferred to a magnetic block and after 2 min the supernatants (non-adherent cells) were collected. The magnetic beads were washed three times with PBS and the last wash directly resuspended in 30 μl of CE buffer in order to lyse the cells bound to the beads. The cells of the collected supernatants were pelleted by centrifugation at 2000 r.p.m., 4°C for 8 min. The cell pellet was resuspended in 1 ml of PBS/5 mM EDTA, and an aliquot taken for FACS analysis.

For positive selection with antibodies directed against either CD25 (7D4) or CD8a (Ly2), the remaining thymic cell suspension (after taking the first aliquot for the unselected sample, see above) was divided into two halves. The cells were incubated with 30 μl of the biotinylated antibodies and incubated for 10 min on ice. Then 400 μl of streptavidin-coated magnetic beads suspension (Dynabeads M280, washed before three times with PBS) was for 20 min on ice with occasional gentle mixing. The Eppendorf tubes were transferred to a magnetic block and after 2 min the supernatants (non-adherent cells) were collected. The magnetic beads were washed three times with PBS and the last wash directly resuspended in 30 μl of CE buffer in order to lyse the cells bound to the beads. The cells of the collected supernatants were pelleted by centrifugation at 2000 r.p.m., 4°C for 8 min. The cell pellet was resuspended in 1 ml of PBS/5 mM EDTA, and an aliquot taken for FACS analysis.

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