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Transcription factors in early T-cell development

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Characterization of the transcription control regions for most genes encoding the TCR-CD3 complex has been followed by identification of DNA-binding factors, some of which are restricted to lymphoid or T-lineage cells. The sequential expression of these factors is linked with the appearance of stage-specific T-cell markers, thus controlling the commitment of the elusive lymphoid progenitor cell and its progression along the T-lineage. Here, Hans Clevers, Mariëtte Oosterwegel and Katia Georgopoulos discuss recent progress made in the recognition of transcription factors that regulate these early events in T-cell ontogeny.

Commitment to and early progression along the T-cell lineage remains a poorly understood process, both at the cellular and at the molecular level. This is partly due to the lack of stem cell or precursor T-cell lines that can differentiate *in vitro* to the various stages of this differentiation pathway. Nevertheless, studies on the rearrangement and expression of the genes of the T-cell receptor (TCR)/CD3 complex, have led to the description of three stages in early T-cell ontogeny: (1) The prothymocyte stage, represented by committed T-cell precursors around the moment of entry into the thymus. These prothymocytes are Thy-1⁺ and contain CD3- γ mRNA, but have not undergone TCR gene rearrangements, and do not express the CD3- δ , CD3- ϵ or TCR genes (Refs 1, 2 and A. Kruisbeek, unpublished). Recent studies suggest that some of these progenitor cells retain the potential to differentiate to non-T-cell lineages³. (2) The prethymocyte stage, represented by intrathymic cells that express all CD3 genes and that have initiated, but not completed, TCR rearrangements in an orderly fashion. These cells are surface-TCR/CD3 negative. (3) The thymocyte stage, represented by intrathymic cells that express the TCR/CD3 complex on their surface, generally coincident with CD4 and/or CD8. The maturation pathway of thymocytes to immunocompetent T cells, which involves either positive or negative selection, is now well documented^{4,5}.

Studies on the expression of the TCR/CD3 complex have elucidated the molecular make-up of early T cells as well as the control mechanisms that mediate T-cell specific gene expression. Transcriptional enhancers have been described for the TCR- α , - β , - γ and - δ genes, as well as for the CD3- δ , - ϵ and the CD2 genes^{6,7}. The activity, specificity and composition of these enhancers have been extensively studied using *in vitro* gene regulation assays. Interacting nuclear factors have been described in protein-DNA-binding assays and a number of these factors have been cloned (Fig. 1). As the nature and mode of action of these proteins are now being documented, the ill-defined early stages of T-cell

differentiation become amenable to molecular approaches. T-cell differentiation can be manipulated *in vitro* or *in vivo* by altering the physiological expression of the factors and thus their target genes. Below, we discuss recent achievements in the recognition of transcription factors that are promising candidates to control T-cell commitment and differentiation by virtue of their restricted expression patterns. We will not address the role of more widely expressed transcription factors or factors implicated in T-cell activation, such as Rel-related proteins.

The HMG box proteins Tcf-1, Lef-1 and Sox-4

Studies on the CD3- ϵ and TCR- α enhancers have led to the cloning of three related transcription factors: Tcf-1 (Refs 8, 9), Lef-1 (Refs 10, 11) and Sox-4 (Ref. 12) (Fig. 1). The DNA-binding domains of these factors belong to the High Mobility Group-1 (HMG) box family. The HMG box is an unusual DNA-binding domain as it interacts with the minor groove and induces strong bending in the DNA helix^{13,14}. The most closely related member of this family is SRY, the gene that specifies the male phenotype in mammals¹⁵. Two other related genes, *matMc* and *MatA1*, determine sexual differentiation (mating type) in the fission yeast gene and in the fungus *Neurospora crassa*, respectively^{16,17}. It is tempting to speculate that Tcf-1, Lef-1 and Sox-4 exert similarly dramatic effects on the differentiation status of lymphoid cells.

Tcf-1 was cloned because of its affinity for the AACAAAG motif in the enhancer of the gene encoding CD3- ϵ (Ref. 8). Human Lef-1 was originally identified as a T-cell specific protein binding to the TTCAAAG motif in the TCR- α enhancer¹⁰. Murine Lef-1 was simultaneously cloned from pre-B lymphocytes by a subtraction hybridization strategy and was shown to bind to the same TCR- α motif¹¹. Tcf-1 and Lef-1 derive from a recent gene duplication event as evidenced by the cloning of a single chicken homologue, chTCF (Ref. 18). Their HMG boxes are virtually identical and probably display indistinguishable DNA-binding

	Overall structure	MW	DNA binding domain	Consensus	Target	Motif
TCF-1		27 kDa	HMG box	A/TA/TCAAAG	CD3- ϵ TCR- α TCR- β TCR- δ CD4	MW56 T α 2 T β 5 T δ E7 CD4-2
LEF-1		55 kDa	HMG box	as above	as above	as above
Sox-4		44 kDa	HMG box	AACAAAG	CD3- ϵ	MW56
GATA-3		47 kDa	C4 Zinc finger	A/TGATAG/A	TCR- α TCR- β TCR- δ	T α 3 T β 2 T δ E4
Ets-1		63 kDa	ETS-domain	AGGATGTG	TCR- α TCR- β	T α 2 T β 3 ³⁷ T β E2, T β E3 ³⁸
Elf-1		68 kDa	ETS-domain	A/CGGAAA/G	IL-2	NFAT-1 IL-2B
Ikaros		48 kDa	'Hunchback' Zinc finger	G-box	CD3- δ	δ A
LH-2		43 kDa	LIM/ Homeodomain	?	?	?

Fig. 1. Schematic representation of candidate regulators of T-cell differentiation and their characteristics. Indicated are: overall structure; molecular weight (MW); type of DNA-binding domain (HMG; ZF, zinc finger; the Ets domain consists of an α -helical and a basic domain (BD); LIM domain; HD, homeodomain); consensus binding site (if determined); potential target genes; and the names of tested binding sites in the regulatory elements of those genes (see text).

specificities. Tcf-1/Lef-1 binding motifs have since been found in several other lymphoid-specific enhancers, including those of the TCR- β and TCR- δ genes¹⁹ and the CD4 gene^{11,20}. Within the hemopoietic system, Tcf-1 is uniquely expressed by all T-lineage cells beyond the prothymocyte stage, as detected by immunohistochemistry (J. Castrop and H.C. Clevers, unpublished) and Northern blot analysis² (Figs 2 and 3), whereas Lef-1 is expressed by all T-lineage cells as well as by pre-B-cells (Fig. 3)^{2,11}. Additionally, Tcf-1 and Lef-1 exhibit a partially overlapping expression pattern in several non-hemopoietic tissues during embryogenesis (Table 1)². This contrasts with their lymphoid-specific expression in the postnatal mouse^{9,11}. Tcf-1 and Lef-1 do not transactivate transcription from a reporter CAT gene that carries multiple copies of their minimal binding motif¹¹ (M. van de Wetering, unpublished). However, single Lef-1, binding the TCR- α enhancer, has been shown to mediate transactivation. This indicates that Lef-1 contains an activation domain that functions only in a specific context of multiple factor binding sites^{21,22}. More recently, a third related factor was cloned from T-cells, Sox-4. Again, this factor is preferentially expressed in the T-lineage and recognizes the AACAAAG motif, but in contrast to Tcf-1 and

Lef-1, it strongly transactivates transcription through its multimerized cognate sites¹². The possibility of sequential occupancy of the AACAAAG binding sites by Lef-1/Tcf-1/Sox-4 nuclear proteins, in combination with subsequent interactions with other bound factors, adds an unexpected level of complexity to the role of the HMG box binding site in T-cell enhancers.

The GATA family

Another group of transcription factors important for tissue-specific gene expression in the hemopoietic lineage is the GATA family. These proteins contain a highly conserved amino-acid domain which binds the GATA sequence motif through a unique C4 zinc-finger structure²³.

GATA-1, the prototype member of this family, was cloned from murine erythroid cells as a result of its affinity for the GATA motif in the β -globin gene²⁴. Its expression is restricted to erythroid cells, mast cells, megakaryocytes and testis^{25,26}. Subsequently, three homologous genes (GATA-1, GATA-2 and GATA-3) were cloned from chicken erythrocytes under low-stringency hybridization conditions²⁷. Their human, murine and *Xenopus* orthologues have since been isolated²⁸⁻³³. GATA-2 and GATA-3 polypeptides are very

homologous throughout their entire sequence and are more related to each other than to GATA-1 (Ref. 33). All three proteins have been demonstrated to transactivate transcription from a minimal TATA-box promoter adjacent to a multimerized GATA motif²⁹⁻³¹.

GATA-3 was originally reported to be expressed specifically in T-cells. Functional GATA binding sites have indeed been identified in the TCR- α , TCR- β and TCR- δ enhancers³⁰⁻³². During murine embryogenesis, expression of GATA-3 is detectable in fetal thymus as well as in erythroid cells and outside the hemopoietic system, for example in the collecting tubuli of the kidney (Table 1)³⁴.

Gene knock out experiments have demonstrated that the *GATA-1* gene is indispensable for normal erythroid differentiation³⁵. Indeed, none of the erythroid genes analysed to date are independent of GATA-motifs in their promoters or enhancers²³. GATA-3 is likely to be of similar importance for T-cell lineage differentiation.

The *Ets* gene family

The *Ets* family of nuclear proteins is defined by an 85 amino-acid region of homology that mediates DNA-binding to a purine-rich core motif (GGAA/T)³⁶. The first member of this family, *Ets-1*, was identified as the cellular counterpart of *v-ets*, one of the proteins encoded in the *gag/v-ets/myb* oncogene of the avian retrovirus E26 (*E*-twenty six), responsible for leukemic transformation of chicken erythroblasts³⁷. Multiple *Ets*-like proteins have since been cloned and have been shown to interact with the regulatory domains of genes in a variety of cell types³⁶.

T cells express at least five members of this family: *Ets-1*, *Ets-2*, *GABP α* , *Elf-1* and *Fli-1* (Refs 7, 38). *Ets-2*, *GABP α* and *Fli-1* are expressed ubiquitously. *Ets-1* is expressed in a variety of murine neonatal organs, but becomes restricted to lymphoid tissues by six weeks of age³⁹. Within a panel of hemopoietic cell lines, *Ets-1* expression was limited to lymphoid cells⁴⁰. Limited data indicate that *Elf-1* is expressed in the murine thymus, spleen and kidney, as well as in T- and B-cell lines⁴¹.

The role of *Ets*-like proteins in T-cell specific gene regulation was first addressed when Leiden and colleagues (re)cloned *Ets-1* in a screen for proteins binding to the Ta2 motif in the TCR- α enhancer⁴². Since then, a variety of *Ets* binding sites have been detected in the enhancers of genes encoding TCR/CD3 and interleukin 2 (IL-2)⁷, suggesting a regulatory role for *Ets* family members both in maturing thymocytes, as well as in activated T cells. Binding of *Ets-1* to the Ta2 binding site is not sufficient for transcriptional activation through this element, which probably relies on additional interactions with other nuclear factors tethered to the Ta2 motif⁷. *Elf-1* binds to consensus *Ets*-binding sites in the IL-2-enhancer. Significantly, *Ets-1* and -2 do not bind these sites, indicating that important differences in fine-specificity exist among *Ets* family members. *Elf-1* (another *Ets*-family member) might participate in a protein complex termed NF-AT, formed after T-cell activation, to mediate inducible expression of T-cell-specific genes such as the IL-2 gene⁴¹. More recently, it has been demonstrated that

Fig. 2. Immunohistochemistry performed with a monoclonal anti-TCF-1 antibody (J. Castrop and H.C. Clevers, unpublished). (a) Nuclear staining is readily detectable in the cortex (left half) and the medulla (right half) of the thymus. (b) Similarly, nuclei of T cells in the peri-arteriolar lymphatic sheath (P) of the spleen stain brightly. F, follicle; A, artery; R, red pulp.

Elf-1 can also interact with the underphosphorylated form of the tumor suppressor protein Rb. After T-cell activation, *Elf-1* is released from Rb, resulting in activation of *Elf-1* mediated transcription⁴³. Two consensus *Ets*-binding sites in the TCR- β enhancer control its activity and phorbol ester inducibility⁴⁴. The interaction of *Ets* proteins with a DNA-binding protein complex termed core binding factor (CBF) appears crucial for high-affinity binding to these sites (M. Owen, unpublished). Indeed, it appears to be a general characteristic of *Ets*-factors, that they interact with other DNA-binding proteins to control gene expression efficiently³⁶.

The zinc finger protein Ikaros

Studies on the CD3- δ enhancer have led to the cloning of the Cys2-His2 DNA-binding protein Ikaros⁴⁵. It is comprised of five zinc finger modules organized in two clusters, strongly reminiscent of the *Drosophila* patterning gene *Hunchback*. Ikaros was cloned for its ability to bind to a multimerized version of the G-rich site TGGGGGT present in the δ A element of the CD3- δ enhancer⁴⁶. Binding sites also occur in the CD3- δ promoter at previously described T-cell specific footprints (K. Georgopoulos and J. Molnar, unpublished). The identification of functional binding

Fig. 3. A schematic representation of the differential expression of the transcription factors during early lymphoid commitment and differentiation. The expression patterns are based on available data from Northern blot analysis of a panel of lymphoid (precursor) cell lines (see text). Expression data are not available for the lymphoid progenitor (?), but the factors that are present in pre-B cells and prothymocytes are likely to be expressed in this cell type. Stage-specific expression of TCR and CD3 genes is discussed in the text. clg-h, cytoplasmic Ig-heavy chain expression; mlg, membrane Ig expression.

sites in other T-cell specific regulatory regions awaits further analysis. Expression of Ikaros is restricted to thymic and splenic T cells in the adult mouse. Within a panel of hemopoietic cell lines, appreciable levels of Ikaros were only detected in T- and some B-lineage cells⁴⁵. *In situ* hybridization on mouse embryos revealed a restricted expression pattern (Table 1)⁴⁵: Ikaros mRNA is first expressed in the fetal liver at the onset of hemopoiesis and shifts to the thymus during mid- to late gestation.

The highly restricted Ikaros mRNA distribution and its ability to transactivate transcription suggest a determining role in regulating phenotypic changes during T-cell commitment and differentiation. High expression in the early fetal liver may be a prerequisite for commitment of the hemopoietic stem cell to the lymphoid lineage and for further differentiation along the T-lineage.

Homeobox proteins

Genes encoding proteins with a homeobox DNA-binding domain have been described in many systems as master regulators of cell fate and tissue identity. Members of the mammalian Hox-clusters (involved in determining metamere identity in the early embryo) are later expressed in a variety of tissues, where they exert pleiotropic effects. Expression of the Hox genes of clusters 1, 2 and 3 has been detected in a variety of hemopoietic lineages⁴⁷⁻⁴⁹. One example of the influence

of Hox genes on hemopoietic differentiation is that of Hox2.2 overexpression, which has profound effects on myeloid *versus* erythroid lineage commitment⁵⁰. A diverged homeobox gene, *HB24*, is reported to be expressed in bone marrow progenitor cells (CD34 positive) as well as activated lymphocytes and monocytes⁵¹. Ectopic expression of this gene under control of the TCR-β promoter/enhancer in transgenic mice revealed an increase in activated thymocytes and peripheral T cells, a decrease of CD4 positive T cells and an impaired involution of the ageing thymus⁵². No putative target genes have been identified for this homeobox gene yet.

Recently, a novel member of the LIM-homeobox family, termed LH-2, was cloned from a pre-B-cell library by subtracted hybridization⁵³. LH-2 is homologous to *Apterous*, a *Drosophila* homeobox gene involved in formation of the central nervous system and wings. In the mouse embryo, expression of LH-2 mRNA was restricted to the fetal liver and the central nervous system, but was not detected in the thymus. In addition, LH-2 was expressed in pre-B, pre-T, and B-cell lines, and in mature γδ T cells. LH-2 mRNA was also found in the subdermal region of the embryonic skin from day 16 onwards, where intra-epithelial lymphocytes (mostly γδ T cells) reside. DNA-binding specificity and transcriptional properties of LH-2 have not yet been determined. Nevertheless, its restricted expression suggests a determining role as a cell type-

Table 1. Expression of Tcf-1, Lef-1, GATA-3 and Ikaros during mouse embryogenesis as assayed by *in situ* hybridization^{2,34,45}

Tcf-1	Lef-1	GATA-3	Ikaros
Limb buds	Limb buds	Kidney	Fetal liver
Neural crest	Neural crest	Thymus	Thymus
Pharyngeal arches	Pharyngeal arches	Lens fibers	Corpus striatum
Nasal process	Nasal process	Whisker follicles	
Urogenital system	Urogenital system	Epidermis	
Lung	Lung	Primary pallate	
Tooth buds	Tooth buds	Inner ear	
Thymus	Thymus	Medulla adrenal gland	
Ribs	Hip bone	Spinal cord	
Thoracic prevertebrae	Tail prevertebrae	Mesencephalon	
Cranio-facial structures	Inner ear	Pons	
Cortex adrenal gland	Pituitary gland	Diencephalon	
Choroid plexus	Choroid plexus		
	Mesencephalon		
	Telencephalon		
	Dorsal thalamus		
	Hypothalamus		

specific regulator in early lymphoid progenitors and in the $\gamma\delta$ T cell.

Perspectives

An increasing number of transcription factors that may serve as 'master genes' for lymphocyte differentiation are being cloned, but how can the detailed involvement of such factors in differentiation control be studied?

In recent years, gene targeting in embryonic stem cells has attempted to assess the role of genes in intact animal systems ('knock out' mice). The creation of knock out mice for most of the factors discussed here is well underway in several laboratories. Inactivation of genes which regulate the fate of cells in the lymphoid lineage should lead to differentiation arrest. As this is not expected to be lethal for the developing mouse, one can study the effect of such gene inactivation in adult animals. Moreover, these mice can serve as a source of 'arrested' cells which can be explored further *in vitro*.

However, most of the genes discussed are more widely expressed in the mouse embryo and may exert pleiotropic actions (e.g. Table 1). Consequently, their inactivation may result in embryonic lethality, precluding a direct functional assessment in lymphopoiesis. It should be realized that the importance of factors in early embryogenesis is not at variance with a crucial role in T-cell development. To circumvent embryonic lethality, embryonic stem (ES) cells homozygous for the introduced mutation ('double knock-out') can be used to create chimeric mice. In these mice, contribution of the ES cells to the various tissues can be addressed. If the gene of interest is indispensable for establishment of a particular cellular phenotype (such as the T cell), the double knock-out ES cells will not contribute to that cell type. A refinement on this approach involves the use of the recombinase activating gene (*Rag-1* or *-2*) knock out blastocysts as recipients for the mutant ES cells. Lymphoid cells in the resulting chimeric mice can only derive from the donor ES cells, thus allowing rapid evaluation of the role of

the disrupted gene in the later stages of B- and T-cell differentiation.

The multipotential of ES cells can also be exploited *in vitro*, as these cells can differentiate to blood islands populated with hemopoietic progenitors. Several groups have documented the appearance of lymphoid progenitors in such cultures (Refs 54–56, E. Dzierzak and A. Müller, unpublished). These culture systems allow the manipulation of expression of putative 'master genes' by means of knock out or transfection technology. The easy access to hemopoietic progenitors at different stages of their development makes this a highly promising system for future lymphocyte differentiation studies.

Functional redundancy of transcription factors in the lymphoid lineage, e.g. between the various HMG box- or Ets-proteins, may preclude their evaluation in gene targeting studies. A complementary approach involves altering the orderly expression of transcription factors in transgenic mice, e.g. by overexpression or by ectopic expression. In particular, the expression of 'dominant-negative' mutant variants (that act as functionally inactive competitors of endogenous transcription factors) circumvents several of the problems inherent in gene knock out systems. First, unlike the effects of gene knock out, the expression of dominant-negatives can be targeted to particular issues (i.e. the T cell), thus avoiding developmental problems. Second, a dominant-negative mutant might affect multiple, functionally redundant genes and, unlike the knock out mutation, will then generate a mutant phenotype. Third, since the expression levels of the mutant protein will vary between transgenic strains, a range of phenotypes (from partial to null) can be analyzed, allowing the study of an otherwise lethal mutation.

It has become apparent that commitment and differentiation of a hemopoietic stem cell to the T-cell lineage involves a cascade of transcription factors which control cellular phenotype at each stage of this process. This molecular cascade in the nucleus is unlikely to evolve in an autonomous, stochastic fashion. Activation of transcription factors in a differentiating

T cell may be triggered by signals transduced from cell-surface receptors acting as ligands for growth factors, adhesion molecules or extracellular matrix. Such signals will be integrated into the transcriptional cascade. Many of the players in these intricate processes remain to be identified. The discovery of a number of (relatively) T-cell specific transcription factors, followed by their functional characterization in knock out and ectopic expression studies will soon expand our insight into the field of T-cell differentiation.

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