Towards a molecular understanding of T cell differentiation

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REVIEW

Towards a molecular understanding of T-cell differentiation

Hans C. Clevers and Michael J. Owen

Lymphoid differentiation is one of the best studied examples of mammalian development. Here Hans Clevers and Michael Owen describe how the cloning of the genes that encode T-cell-specific membrane proteins allows the identification of transcription factors that control the expression of these T-cell genes. Such transcription factors play a key role in the development of the mature T-cell phenotype by functioning as 'master regulators of T-cell differentiation'.

No scientist can escape being fascinated by the complexity of the processes that allow a single fertilized oocyte to grow into an adult organism. One of the earliest events in the development of a multicellular metazoan is the organization of the body plan, generally termed 'pattern formation'. A key feature of this process is the division of the developing embryo along the anterior–posterior axis into progressively smaller units (segments), after which the identity of each segment is established. Later in development, each segment forms unique structures (for example wings, antennae and legs).

Most of our understanding of these processes has come from studies on the development of the fruitfly *Drosophila melanogaster* and the worm *Caenorhabditis elegans*. In particular, the systematic screening of the *Drosophila* genome for mutations in genes involved in pattern formation has been highly successful. Large numbers of developmental mutants (given such esoteric names as *Wingless, Kruppel, Zerknüllt* and *Hairy*) have been obtained and analyzed at the molecular level. Most of the genes identified by these studies can be classified as members of a few gene families. Three major gene families (Fig. 1), as defined by similarities at the protein level, are the 'homeodomain' genes, the 'zinc-finger' genes and the 'leucine-zipper' genes[1]. These genes encode proteins that bind to specific DNA sequences, and are regulators of gene transcription, or transcription factors (see below).

From these studies a general picture has emerged in which the appearance of the body plan occurs through the highly ordered succession of activation and repression of these 'pattern formation' genes (Fig. 2). Each gene acts as a molecular control switch, whose activity in a given cell depends on the position of that cell in the organism, and on the time point in the development of the organism. Through these gene switch pathways, cells become more and more limited in their potential to give rise to various tissues, and are targeted towards a particular state of differentiation.

The transcription of a eukaryotic gene by RNA polymerase II is controlled by promoter, enhancer and silencer elements, collectively termed *cis*-acting elements. By definition, a promoter is a stretch of DNA that is required for correct initiation of transcription. Promoters are thus located at the transcription start site and usually consist of a TATA box and a set of upstream promoter elements. Enhancers are DNA elements located anywhere in or around a gene that increase the level of transcription initiated by a promoter, independently of their position and orientation. Silencers, like enhancers, can act at a distance, but negatively affect transcription initiation from a linked promoter. Sequence-specific transcription factors, such as the zinc-finger and homeodomain gene products, bind to promoters and enhancers and mediate the transcription control. Transcription factors are made up of at least two domains: one domain recognizes a particular DNA sequence (motif) in a given promoter or enhancer and a second domain subsequently interacts

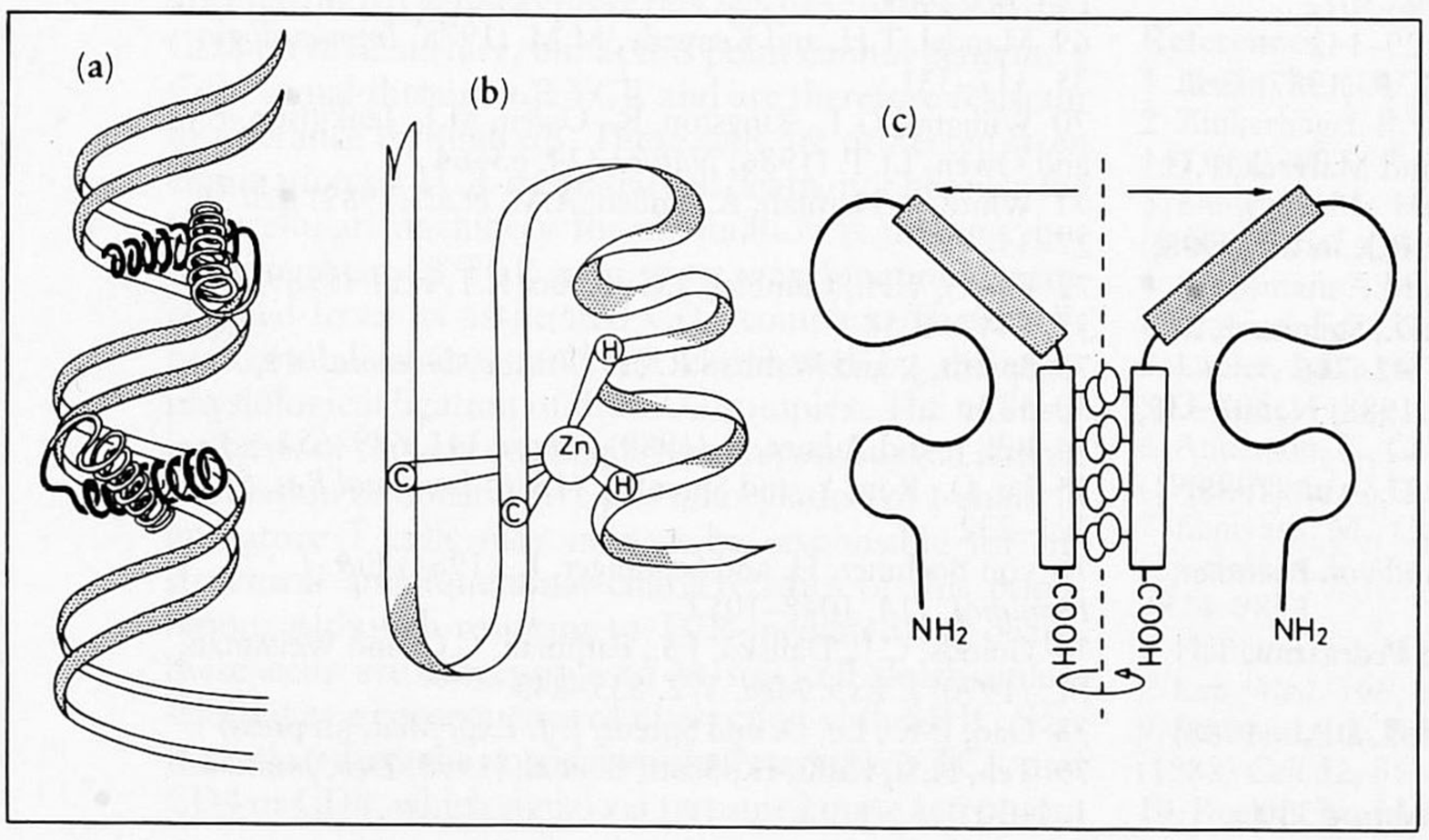

Fig. 1. *Three types of DNA-binding transcription factors (from Ref. 57). (a) A helix-turn-helix protein folds in the major groove of the DNA double helix, where it recognizes its sequence motif. Dimerization with another helix-turn-helix protein bound nearby stabilizes the interaction. (b) A zinc-finger protein binds a zinc ion through paired cysteine and histidine residues. The cysteines reside in antiparallel β sheets; the histidines are located in an α-helical stretch of the protein. The base of the finger presumably recognizes a specific DNA motif. (c) A leucine-zipper protein dimerizes via hydrophobic interactions between regularly spaced leucine residues (open ovals) residing in α helices. The dark rectangles represent the basic regions that recognize the sequence motif, usually of dyad symmetry.*

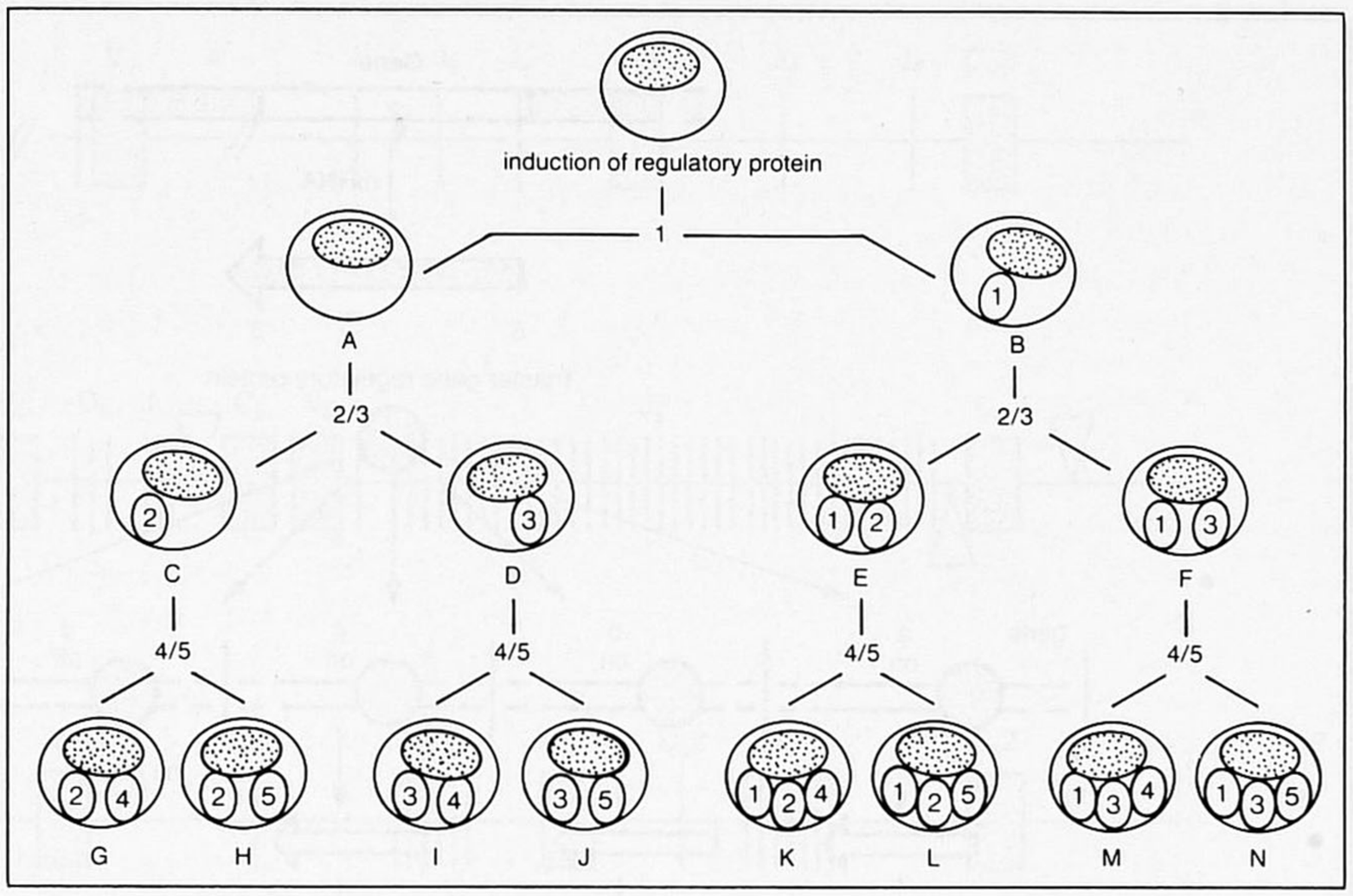

Fig. 2. *A schematic representation of transcription factors acting as molecular switches that control cell fates in a genetic differentiation tree. The numbers represent individual transcription factors that regulate expression of structural genes and thus control the differentiation status ('phenotype', indicated with letters) of the cells. Different combinations of transcription factors result in differences in phenotype, which limits the number of factors necessary to generate the full spectrum of cell types that build an adult organism (from Ref. 58).*

with other proteins to regulate levels of transcription[2,3].

The expression of many genes is limited to a particular tissue or cell type. Transcription of such genes is usually controlled by promoters and/or enhancers that allow binding of a transcription factor (or of a particular combination of factors) present only in that tissue. Thus, the homeodomain protein, the zinc-finger protein and the leucine-zipper protein are transcription factors present in particular cells of a developing organism. They activate selected sets of genes by binding to sequences present in the promoters/enhancers of those genes. In effect, this results in control of the differentiation status, or phenotype, of a cell[3,4].

Unfortunately, it has so far been exceedingly difficult to obtain information in vertebrates, especially in mammals, on 'pattern-control' genes, owing to severe limitations in the techniques of genetic manipulation. However, development and differentiation in mammals can be studied from a different perspective. Rather than looking at the earliest stages of development, much attention has been focussed on its end stages – the terminal differentiation of precursor cells into functionally mature cells. With the advent of molecular biology techniques it has become feasible to clone genes that are expressed only in those end stage cells and to define their promoters and enhancers. The method most commonly used to identify such elements is to use a reporter gene which produces a readily detectable gene product. Promoter regions can be cloned into vectors containing a promoterless reporter gene. Enhancer trap vectors contain a reporter gene associated with a promoter that, by itself, possesses only minimal activity. The cell-type-specific activity of the studied promoter or enhancer is subsequently tested by transient transfection of the reporter gene construct into appropriate cell lines. The most popular reporter genes encode bacterial chloramphenicol acetyl transferase (CAT) and firefly luciferase. This type of *in vitro* assay is quick and convenient; however, it does suffer from inherent limitations. Once promoter and enhancer regions have been characterized, their *in vivo* regulatory capacity should be confirmed by generating transgenic mice with appropriate constructs.

The interaction of putative transcription factors with functionally defined promoters and enhancers can subsequently be visualized by gel retardation; various DNA footprinting assays can reveal the actual sequence motifs recognized by individual transcription factors. Crude or partially purified nuclear extracts prepared from relevant cell lines can be used as the source of such factors[5,6].

Recently, some mammalian transcription factors have been cloned that are present only in particular tissues, and that are responsible for the specific expression of genes in those tissues. Oct-2 is present in B cells and drives expression of immunoglobulin genes[7,8]. Pit-1 is a transcription factor present in cells of the pituitary gland which regulates expression of pituitary hormones[9,10]. Another cloned factor, EryF1, controls expression of the hemoglobin genes in cells of erythroid lineage[11,12]. LF-B1 regulates transcription of liver-specific genes[13]. A surprising discovery was made when the sequences of these genes were compared with data bank sequences. Pit-1 and Oct-2 are highly homologous to the *Drosophila* homeodomain genes; LF-B1 is a more distant relative of the homeodomain gene family; and EryF1 is a zinc-finger gene.

The control of differentiation by tissue-specific transcription factors is probably best illustrated by the study of myogenic cell determination. Several gene products have been described that can convert 10T1/2 mouse fibroblasts to a skeletal muscle phenotype. One of these gene products, MyoD1 (Ref. 14), binds to a specific sequence in the muscle-specific creatine kinase enhancer, implying that it is a transcription factor controlling myogenesis by regulating the expression of muscle-specific genes[15]. Factors, like MyoD1, that can control the phenotype of a cell have been termed 'master regulators of differentiation' (Fig. 3). MyoD1 does not belong to one of the two gene families mentioned above, but is homologous to a group of genes including the *Drosophila* development genes *achaete-scute, daughterless* and *twist*, the *myc* genes, and two proteins, E12 and E47, that bind to the Ig κ enhancer[16].

Thus, development and differentiation are regulated by very similar genes throughout the animal kingdom, in animals as diverse as insects and mammals, and in processes as different as embryonic pattern formation and bone marrow differentiation. Another important conclusion can be drawn from the studies discussed above:

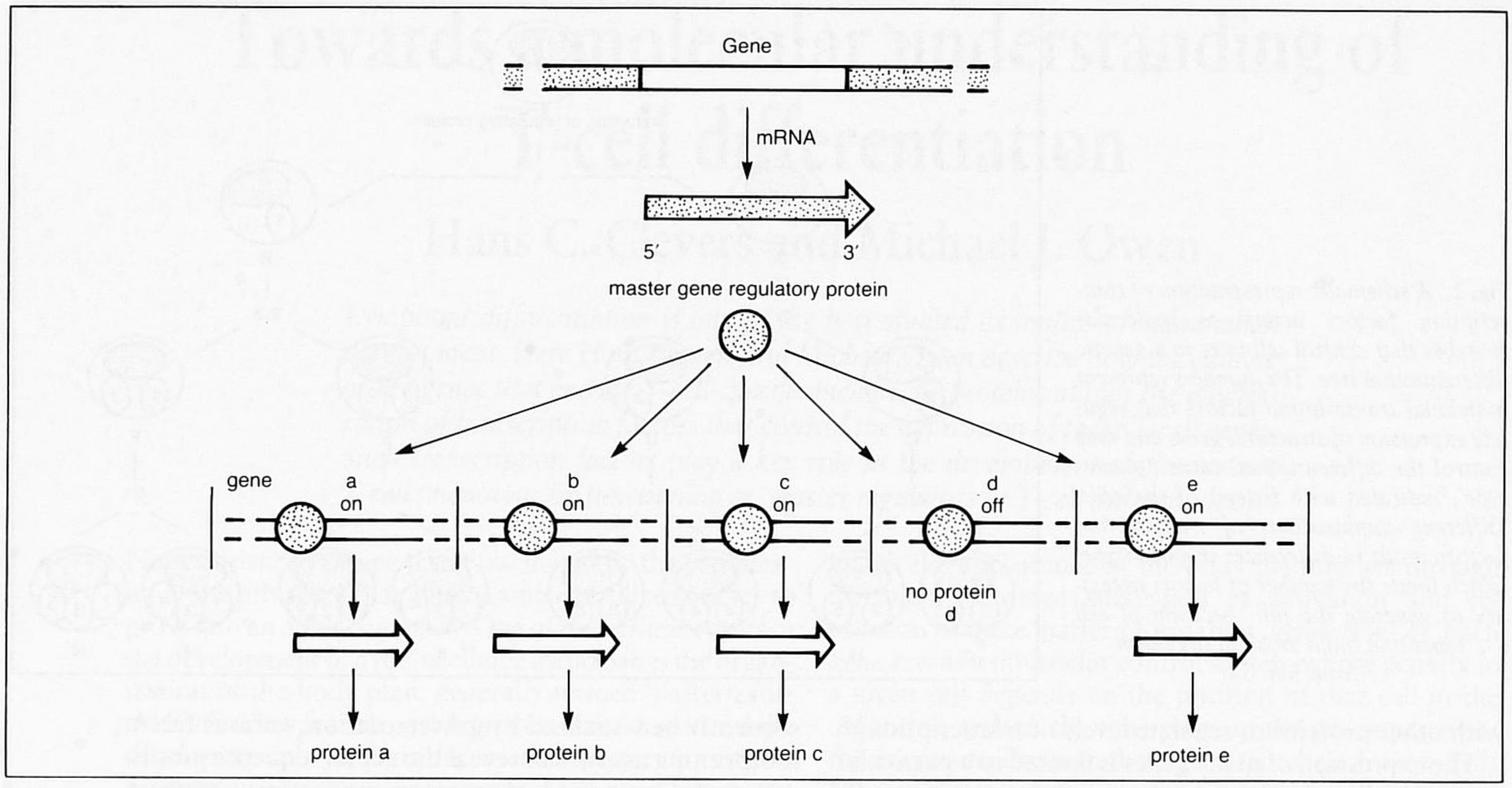

Fig. 3. *A 'master regulator of differentiation' is defined as a gene which encodes a transcription factor (the 'master gene regulatory protein') controlling the phenotype of a cell. The master regulator can exert both positive and negative effects on the transcription of structural genes (from Ref. 58).*

the approach of describing mammalian differentiation processes by characterizing tissue-specific transcription factors is a legitimate alternative to the study of *Drosophila* developmental mutants.

Transcription regulation studies in immunology

The mammalian bone marrow is one of the most widely studied model systems of vertebrate differentiation. It represents a multi-branched differentiation pathway that persists in the adult animal. A number of cell lineages are generated from pluripotent stem cells: the megakaryocyte–platelet lineage, the erythroid lineage, the granulocyte–monocyte lineage and the lymphoid lineage. The field of immunology, through its extensive knowledge of lymphocyte differentiation, provides a unique opportunity for the identification of tissue-specific (that is T-cell-specific and B-cell-specific) transcription factors. B-cell-specific transcription control, in particular of the abundantly expressed Ig heavy and Ig κ genes, has been analyzed with great success[17]. More recently, similar studies of genes encoding molecules of lower abundance have become feasible. Below we discuss advances made in the study of transcription regulation of membrane molecules expressed uniquely by cells of the T-cell lineage: the T-cell antigen receptor (TCR)–CD3 genes and the CD2 gene.

Transcription regulation of T-cell-specific genes

The TCR–CD3 complex consists of a TCR heterodimer (occurring either as αβ TCR, or as γδ TCR) non-covalently linked to the CD3 complex[18,19]. CD3 is constituted by at least five invariable chains: the glycoproteins γ CD3 and δ CD3, the non-glycosylated ϵ CD3 chain and the ζ CD3 homodimer or ζη CD3 heterodimer. The expression of the TCR genes of γ, δ and ϵ CD3 is restricted to T cells. ζ CD3 is probably more broadly expressed; it occurs in natural killer cells as a component of the surface antigen CD16 (Ref. 20).

Structure and T-cell-specific regulation of the TCR genes

The regulation of the individual TCR genes is not only T-cell-specific, but poses two extra constraints. First, the phenomenon of allelic exclusion dictates that T cells should express only one functional allele of a given TCR gene. Little is known about the molecular mechanism of allelic exclusion. Second, in any given T cell, the α and β, and γ and δ genes are expressed in a mutually exclusive fashion.

The organization of the TCR genes is reminiscent of that of the immunoglobulin genes[21]. They consist of *V*(variable), *J*(joining), sometimes *D*(diversity) and *C*(constant) gene segments and undergo rearrangement, presumably by the same molecular machinery as do the Ig genes in the B-cell lineage. As in B cells, the process of rearrangement appears to be linked to transcriptional activity of a particular gene. Each *V* region is associated with a promoter which becomes active after *V(D)J* rearrangement. By analogy with the Ig gene enhancers, putative enhancer elements in the TCR genes are likely to be located near their *C* gene segments.

The four TCR genes map to three loci. β TCR and γ TCR each occur as single transcription units (Fig. 4). The recently characterized δ TCR gene is unusual in that it is located within the α TCR gene: any attempt to rearrange α TCR will result in deletion of the δ TCR gene (Fig. 4). This mechanism ensures that one αδ TCR allele transcribes either α or δ. However, additional regulatory mechanisms are needed to inhibit expression of the reciprocal α or δ TCR gene from the other allele.

T-cell-specific elements in the α TCR gene

The promoter of a rearranged human α TCR gene was T-cell-specific when tested in CAT assays[22]. In the same study an enhancer was detected in the *JC* α intron that is active in lymphoid but not in HeLa cells. Two more recent studies describe the presence of a T-cell-specific enhancer several kilobases downstream from C_α in

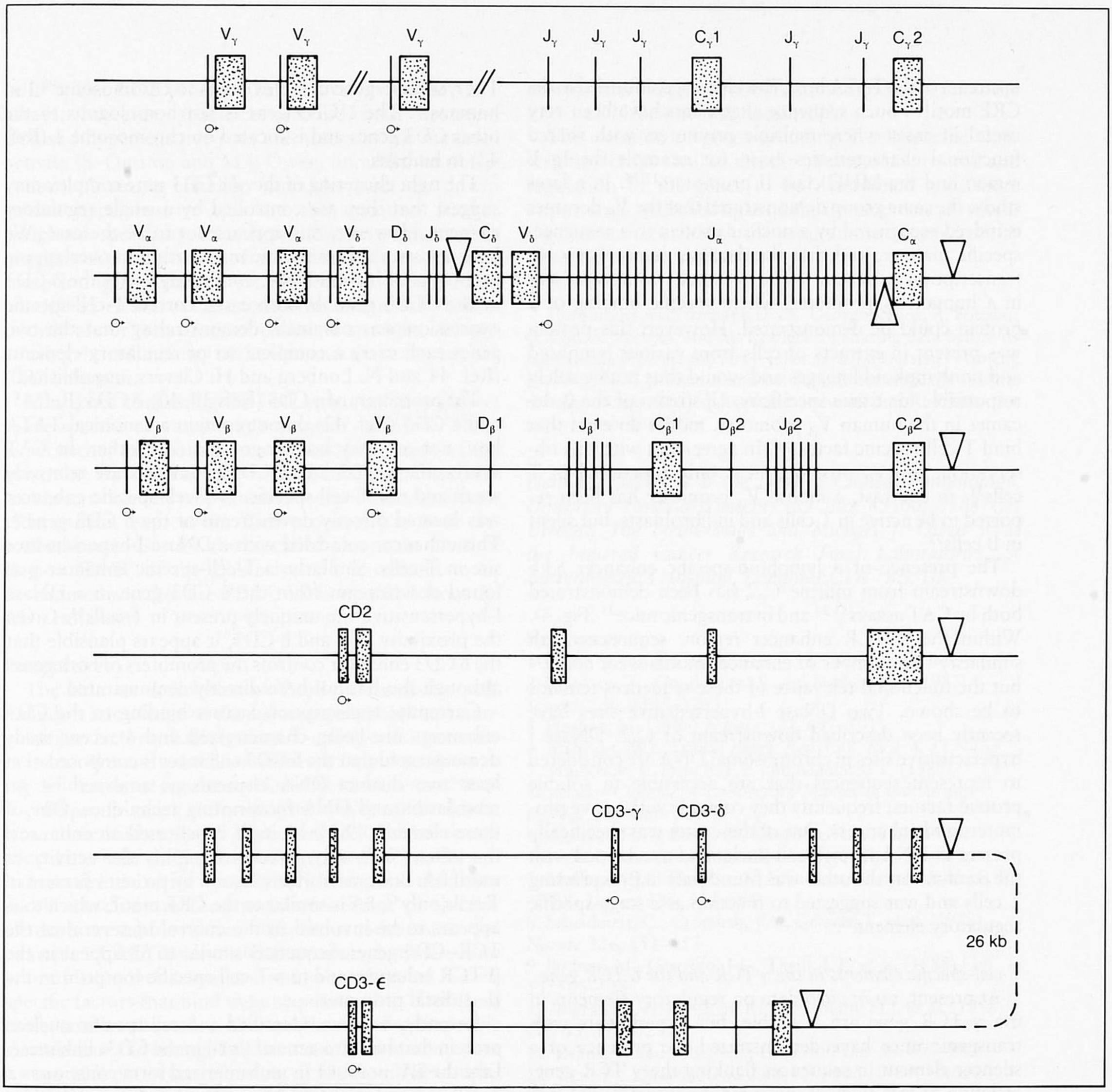

Fig. 4. *Schematic representation of T-cell-specific genes and their regulatory sequences as discussed in the text. From top to bottom: the γ TCR gene, the αβ TCR gene complex, the β TCR gene (from Ref. 21), the CD2 gene and the CD3 gene complex. Vertical bars and rectangles represent gene segments and exons. Arrows indicate the location of promoters; open triangles indicate tissue-specific enhancers (see text).*

humans[23] and mice[24] (Fig. 4). Sequence comparison of these two enhancers demonstrates a virtually complete homology over a stretch of approximately 120 base pairs. The presence of an enhancer in the JC_α intron could not be confirmed in these later studies. DNase I footprinting of the mouse α TCR enhancer detected two DNA-binding proteins uniquely present in T cells; two others were also found in other cell lineages[24]. In the human enhancer, two footprints were found at homologous positions[23]. One of these footprints contains the consensus of a cyclic AMP responsive element (CRE), a motif which can mediate the induction of transcription by cyclic AMP. The relevance of this finding is not clear at present. A recent report describes the purification of one of the T-cell-specific DNA-binding proteins interacting with the α TCR enhancer. This protein can interact with motifs in several T-cell-specific regulatory elements and might thus represent a 'master regulator of T-cell differentiation'[25].

A novel type of regulatory element has recently been found near the α TCR enhancer. This element downregulated transcription of test promoters in γδ but not in αβ T cells and was termed 'silencer'[26]. The presence of this silencer, together with the deletion of δ TCR in the rearranged α TCR gene, elegantly accounts for the exclusion of δ TCR expression in αβ T cells.

T-cell-specific elements in the β TCR gene

Several studies have addressed the issue of tissue specificity of V_β promoters. Alignment of 14 murine V_β promoters revealed the presence of a strongly conserved decamer motif (AGTGAT/CG/ATCA) 10–40 bp

upstream of the TATA box. This element conformed to the CRE motif[27]. Such sequence alignments have been very useful in cases where multiple promoters with shared functional characteristics exist, for example the Ig *V* region and the MHC class II promoters[17,28]. In a later study, the same group demonstrated that the V_β decamer is indeed recognized by a nuclear protein in a sequence-specific manner, and that the decamer is necessary for transcription initiation[29]. The decamer was also present in a human V_β promoter, where specific binding of a protein could be demonstrated. However, this protein was present in extracts of cells from various lymphoid and nonlymphoid lineages and would thus not be solely responsible for tissue specificity. Upstream of the β decamer in the human V_β promoter, motifs do exist that bind T-cell-specific factors[30]. In agreement with this observation, the V_β promoter was only functional in T cells[31]; in contrast, a mouse V_β promoter has been reported to be active in T cells and in fibroblasts, but silent in B cells[32].

The presence of a lymphoid-specific enhancer 5 kb downstream from murine $C_\beta 2$ has been demonstrated both by CAT assays[33,34] and in transgenic mice[33] (Fig. 4). Within the β TCR enhancer region, sequences with similarity to a number of enhancer motifs were noted[33] but the functional relevance of these sequences remains to be shown. Two DNase I-hypersensitive sites have recently been described downstream of $C_\beta 2$. DNase I hypersensitive sites in chromosomal DNA are considered to represent sequences that are accessible to soluble protein factors; frequently they coincide with active promoters and enhancers. One of these sites was specifically present in DNA from T-cell nuclei and overlapped with the β enhancer; the other was found only in β-expressing T cells and was suggested to function as a stage-specific regulatory element[35].

T-cell-specific elements in the γ TCR and the δ TCR gene

At present, no *in vitro* data on regulatory elements in the γ TCR gene are available, but experiments with transgenic mice have demonstrated the presence of a silencer element in sequences flanking the γ TCR gene. Deletion of this element, presumably operative in αβ T cells to repress γ TCR gene transcription, resulted in expression of γ TCR in cells that would otherwise express the TCR αβ heterodimer. Consequently, the generation of αβ TCR cells was seriously impaired in these mice[36].

Studies on tissue specificity of V_δ promoters have not appeared as yet. However, two groups have described a T-cell lineage-specific enhancer in the $J_\delta 3$–C_δ intron (Fig. 4). A number of sequences homologous to known regulatory motifs were noted but their functional significance is unclear[37,38].

Structure and T-cell-specific regulation of the CD3 genes

The genes encoding γ CD3 and δ CD3 are highly homologous and appear in a head-to-head configuration, which is strongly suggestive of a recent gene duplication event (Fig. 4)[39,40]. The human ε CD3 gene is located 26 kb downstream from δ CD3 and displays limited sequence homologies with the γδ CD3 gene pair, particularly in the extracellular and transmembrane exons[41,42]. The γδε CD3 gene complex maps to chromosome 11 in humans[41]. The ζ CD3 gene is not homologous to the other CD3 genes and is located on chromosome 1 (Ref. 43) in humans.

The tight clustering of the γδε CD3 gene complex may suggest that they are controlled by a single regulatory element. However, this appears not to be the case. We have constructed transgenic mice with non-overlapping fragments of human DNA, containing either the δ CD3 or the ε CD3 gene. In both cases, correct T-cell-specific expression was obtained, demonstrating that the two genes each carry a complete set of regulatory elements (Ref. 44 and N. Lonberg and H. Clevers, unpublished).

The promoters of γ CD3 (Refs 39, 40), δ CD3 (Ref. 45) and ε CD3 (Ref. 42) do not contain a canonical TATA box, nor are they homologous to each other. In CAT assays, the δ CD3 and ε CD3 promoters are relatively weak and not T-cell-specific. A T-cell-specific enhancer was located directly downstream of the δ CD3 gene[46]. This enhancer coincided with a DNase I-hypersensitive site in T cells. Similarly, a T-cell-specific enhancer was found downstream from the ε CD3 gene in a DNase I-hypersensitive site uniquely present in T cells[44]. Given the proximity of γ and δ CD3, it appears plausible that the δ CD3 enhancer controls the promoters of both genes although this has not been directly demonstrated.

Currently, transcription factors binding to the CD3 enhancers are being characterized and a recent study demonstrated that the δ CD3 enhancer is composed of at least two distinct DNA elements as analyzed by gel retardation and DNA footprinting techniques. One of these elements ('δA') by itself constituted an enhancer; the other ('δB') only served to amplify the activity of motif δA. Both motifs were bound by proteins present in T cells only[47]. δA is similar to the CRE motif, which thus appears to be involved in the control of several of the TCR–CD3 genes. Sequences similar to δB appear in the β TCR enhancer and in a T-cell-specific footprint in the IL-2 distal promoter.

Recently, we have identified a T-cell-specific nuclear protein that binds to a motif ('ε1') in the CD3ε enhancer. Like the δA motif, ε1 in multimerized form constitutes a T-cell-specific enhancer. Using the ε1 motif as a double-stranded probe, a protein T-cell factor 1 (TCF-1) that specifically binds to the ε1 motif and that is expressed uniquely in T cells was cloned[48]. TCF-1 is a member of a new family of proteins containing the so-called HMG box, a DNA-binding domain with homology to High Mobility Group I proteins. TCF-1 appears to be a good candidate for a 'master regulator of T-cell differentiation'.

Structure and regulation of the CD2 gene

CD2 is expressed by most thymocytes, by virtually all mature T cells and by about 20% of mouse splenic B cells. CD2 has not been detected on human B cells and the different murine and human expression patterns presumably reflect the spectrum of regulatory elements controlling each gene.

The human CD2 gene (see Fig. 3) is associated with two DNAse I hypersensitive sites at its 5′ end, and one at its 3′ end. The upstream sites are present only in cells expressing CD2 but the downstream site is found in all T

cells[49]. The CD2 promoter is relatively weak in CAT assays; however, sequences around both upstream DNase I-hypersensitive sites are necessary for maximal activity (S. Outram and M.J. Owen, unpublished observation). A strong enhancer coincides with the downstream hypersensitive site and this enhancer confers lymphoid specificity to a heterologous promoter but requires its own promoter for T-cell specificity. DNase I footprinting has defined six elements in the CD2 enhancer[50]. Deletion of these footprints individually results in only minor reductions of enhancer strength, demonstrating the redundancy of the elements that comprise the CD2 enhancer.

Minigenes containing the CD2 enhancer gave correct tissue-specific expression in transgenic mice[51,52]. These experiments established an additional property of the enhancer: in contrast to most cellular and viral enhancers, it confers copy number-dependent, position-independent, expression. This property has been described previously for the so-called Dominant Control Region (DCR) of the globin locus[53]. It remains to be determined whether the CD2 DCR is separable from the CD2 enhancer.

Perspectives

The ultimate description of T-cell differentiation will include a knowledge of the *cis*-acting elements and transcription factors that regulate T-cell-specific gene expression. Recent years have witnessed rapid progress in the delineation of regulatory elements within a number of T-cell-specific genes and some generalities can be drawn from these studies. T-cell promoters are often weak and contribute little to T-cell specificity. In contrast, the enhancers defined so far are generally strong and do confer T-cell specificity to their promoters. Some of the *cis*-elements resemble other ubiquitous sequences, for example CCAAT boxes or CRE elements, but there is no scarcity of novel sequences and, therefore, of T-cell-specific factors that bind these sequences. It is clear from these studies that even the simplest of T-cell-specific genes is likely to be regulated in a combinatorial manner by upwards of ten factors, many of which will be ubiquitous, others perhaps lymphoid-specific, or T-cell-specific; the latter possibly qualifying as master regulators of T-cell differentiation.

Having defined protein factors that establish and maintain a gene in a transcriptionally active state in a T cell, the problem becomes one of cloning the genes encoding these factors in order to determine their mechanisms of action and their function in T-cell differentiation. Three different strategies have been used. The first relies on affinity chromatography, using the specific oligonucleotide motif to purify the binding protein. Partial amino acid sequence can then be used to design a probe for library screening. This approach has been used successfully for, among others, the Serum Response Factor gene[54,55]. The other strategies both involve generating cDNA expression libraries. Double-stranded oligonucleotide motifs have been used to screen λ GT11 expression libraries. Phage plaques containing the DNA-binding protein bind the radiolabeled motif[56]. A more elaborate approach uses transiently transfected COS-1 cells. Pools of these cells are analyzed in gel retardation assays; after successive subdivisions of positive pools the EryF1 cDNA clone was obtained[12].

The isolation of genes encoding T-cell-specific transcription factors will enable the pattern of expression and regulation of the genes to be determined and, more importantly, will shed light on the developmental switching events that establish the programme of gene activity observed in mature T cells.

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Hans C. Clevers is at the Dept of Clinical Immunology, University Hospital Utrecht, PO Box 85500, 3508 GA Utrecht, The Netherlands and Michael J. Owen is at the Imperial Cancer Research Fund Laboratories, St Bartholomew's Hospital, London EC1A 7BE, UK.

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