The EMBO Journal vol.9 no.1 pp.109 – 115, 1990

Tissue-specific nuclear factors mediate expression of the CD3δ gene during T cell development

Katia Georgopoulos, Deborah Galson[1] and Cox Terhorst

Laboratory of Molecular Immunology, Dana Farber Cancer Institute, Harvard Medical School and [1]Department of Biology and Center for Cancer Research, Massachussetes Institute of Technology, MA, USA

Communicated by M.F.Greaves

An obligatory step towards T cell maturation is expression of the CD3 gene products which occurs very early during thymic differentiation and may even precede migration to the thymus. Delineation of the transcriptional mechanisms that determine expression of the CD3 complex in immature and mature T cells will help us understand the molecular events that govern T cell development. We have previously reported that a 400 bp region 3′ of the CD3δ gene functions as a transcriptional enhancer with strong specificity for T cells. Here we identify two elements in the CD3δ enhancer which mediate its T cell restricted function. Element δA can function as an independent enhancer while element δB has no independent function but augments the activity of element δA. Together, δA and δB are sufficient to reconstitute the activity of the CD3δ enhancer. Nucleoprotein complexes found in mature T cells have been identified whose presence correlates with activity of these two elements. Since these protein binding sites are conserved in other genes of the TCR–CD3 complex, elements δA and δB and their cognate nuclear factors may play an important role in T cell development.

Key words: CD3δ enhancer/T cells/transcription factors

Introduction

The cells of the immune system arise from a common precursor stem cell. The earliest definitive signal of commitment to the T cell lineage is expression of the CD3 genes. As members of the TCR–CD3 complex these proteins appear on the cell surface relatively late in development after all of the component chains are synthesized (von Boehmer, 1988). Transcription of the CD3 genes, however, begins much earlier; it precedes TCR rearrangement and may even occur prior to migration to the thymus (Haynes *et al.*, 1989). Early thymocytes and mutant thymoma lines with an immature phenotype express low levels of CD3δ mRNA while more mature T cells express higher levels. This suggests that transcription of CD3δ is controlled by the activities of nuclear regulatory proteins which are modulated during T cell development.

We have previously reported that a 400 bp region 3′ of the CD3δ gene functions as a transcriptional enhancer with strong specificity for T cells (Georgopoulos *et al.*, 1988). In this report we have identified two *cis*-acting elements δA and δB in this region responsible for mediating the enhancer function. Element δA is a T cell specific enhanson which in the context of the CD3δ enhancer requires element δB for high levels of activity. We have studied the nuclear protein factors that bind to these elements and found that their concentration level correlates with enhancer activity and expression of the endogenous gene.

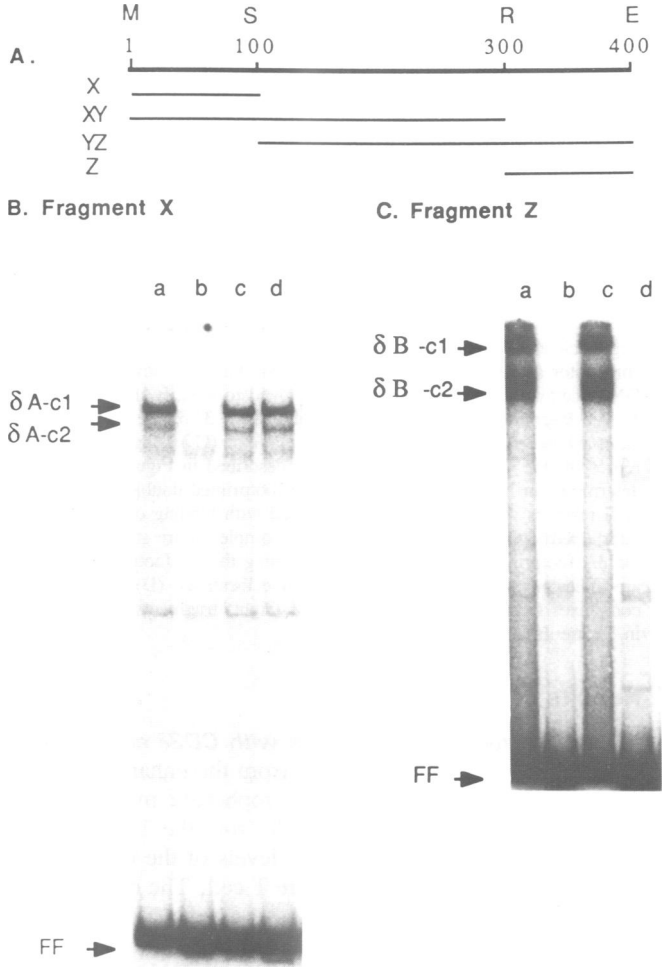

Fig. 1. Specific nuclear complexes bind to the CD3δ enhancer. (**A**) Map of the CD3δ enhancer. Shown below the bar that represents the genomic map of the enhancer region characterized previously (Georgopoulos *et al.*, 1988) are the four fragments used for the gel mobility shift assay. M, *Msp*I; S, *Ssp*I; R, *Rsa*I, E, *Eco*RI.(**B**,**C**) Fragments X (100 bp) and Z (100 bp) were end-labelled and were assayed in a DNA binding assay with nuclear extract prepared from the leukaemic T cell line EL4. A 50-fold molar excess of DNA competitors was included in the assay in order to test for sequence specificity of the complexes. These were: **a**, none; **b**, XYZ (400 bp enhancer); **c**, non-specific DNA from λ phage cut with *Pvu*II (400 bp); **d**, CD3δ promoter (450 bp *Bcl*I–*Bcl*I, Georgopoulos *et al.*, 1988). (**B**) δA–c1 and δA–c2, specific nucleoprotein complexes observed with fragment X. (**C**) δB–c1 and δB–c2, specific nucleoprotein complexes observed with fragment Z. FF denotes the unbound DNA.

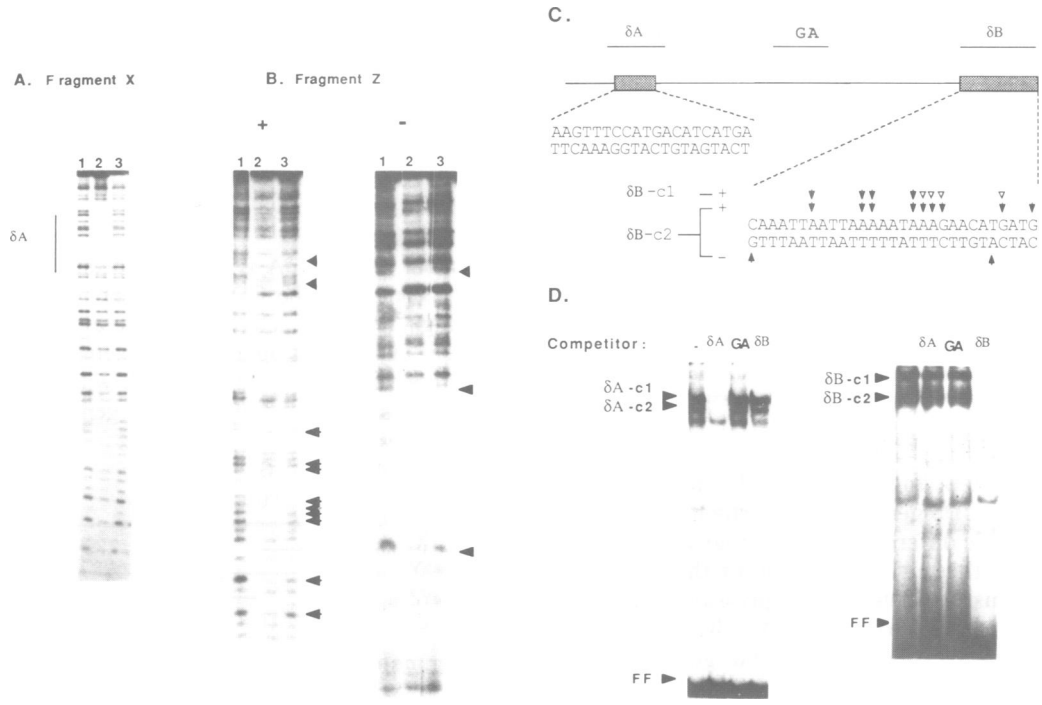

Fig. 2. Recognition sites for the nuclear complexes formed on the CD3δ enhancer. (**A**) The sequence specificity of the nuclear protein complexes binding to fragment X was determined by DNase I footprinting in solution with nuclear protein from the T cell line EL4 (**lanes 2** and **3**). A 40 bp oligonucleotide spanning the 51−91 bp region of the enhancer (Georgopoulos *et al.*, 1988) and containing the protected sequence was used as competitor at 50-fold molar excess (**lane 3**). The bar shows the protection of the δA element by nuclear extract made from the EL4 T cell line. (**B**) DNA methylation interference was employed to identify the binding domain for the δB−c1 and δB−c2 complexes formed on the Z fragment. **Lane 1**, free fragment; **lane 2**, δB−c2 complex; **lane 3**, δB−c1 complex. Both the + and − strands were analysed for binding. Arrows indicate G and A bases whose methylation interfere with binding. (**C**) Diagrammatic representation of the protein binding domains in the CD3δ enhancer. The line represents the CD3δ enhancer region as described in Figure 1(A). The shaded boxes on this line represent the specific nuclear protein footprints determined in Figure 2(A) and (B). The footprinted double-stranded sequences are indicated below this diagram. Solid arrows indicate the specific A/G residues whose methylation interfered with binding of the δB−c1 and δB−c2 factors. Hollow arrows indicate residues whose methylation interferred with formation of the δB−c2 complex more strongly than for the δB−c1. The three oligonucleotides, made to the 51−91 bp (containing the δA footprint), to 343−382 bp (containing the δB footprint) and to the 203−234 bp (containing a GA-rich motif) regions of the CD3δ enhancer are indicated as lines above their respective locations. (**D**) The three oligonucleotides described above (Figure 2C) were used as competitors for the complexes formed on the X (left) and Z (right) fragments. A 50-fold molar excess of competitor DNA was used in the DNA binding assay described in Figure 1(B).

Results

Nuclear protein binding studies with CD3δ enhancer

Overlapping genomic fragments from the enhancer region (Figure 1A) were tested in an electrophoretic mobility shift assay. Nuclear extracts were made from the T lymphoma cell line EL4 that expresses high levels of the CD3δ gene and has the phenotype of a mature T cell. The overlapping enhancer fragments X and XY gave similar mobility shift patterns, while the YZ and Z fragments gave another (Figure 1B and C and data not shown). DNA fragments covering the enhancer and the promoter region of the CD3δ gene and unrelated DNA of the same size were used as competitors in this assay to test for the specificity of the detected binding. The nuclear complexes δA−c1 and δA−c2 formed on the X fragment and the nuclear complexes δB−c1 and δB−c2 formed on the Z fragment were specifically competed away by the enhancer (XYZ) but not by an unrelated fragment from λ phage (Figure 1B and C). The promoter DNA did not compete for binding to element δA but competed for element δB revealing the presence of additional binding site(s) for this factor(s) in the promoter of the CD3δ gene (Figure 1C, lane d). Sequence and footprint analysis of the promoter region has identified sites which resemble the δB element and which are presumably responsible for the competition (data not shown and Table IIIB). Several other non-specific complexes were observed for both fragments and were not competed away at the concentration of specific cold DNA used.

Footprinting analysis of the CD3δ enhancer

In order to identify the binding domains within the enhancer, we employed an *in vitro* DNase I protection assay for fragment X and methylation interference footprinting for fragment Z. A 20 bp sequence motif was protected on the X fragment, which we designated as element δA (Figure 2A). An excess of unlabelled oligonucleotide made to this protected sequence abrogated binding in both the footprinting and gel-retardation assays. The protein complex protecting this region is tissue specific since nuclear extracts made from other cell types either did not footprint (MPC11, F9) or footprinted very weakly (NIH3T3) under the protein concentration conditions used to footprint with the EL4 extract (data not shown).

The sequence specificity of the δB−c1 and δB−c2 complexes was analysed by methylation interference since the *in vitro* DNase I protection assay did not yield clear results. Methylation of bases in a 29 bp AT-rich region interferred with binding of both the δB−c1 and δB−c2

Table I. Functional dissection of the CD3δ enhancer

Construct	Fold stimulation				
	EL4	BW	SAK8	SL12.1	3T3
XYZ	15	1.8	1	1	1
XY	6	1	1	1	1
YZ	1	1	1	1	1
Z	1	1	–	–	1
2Ad	10	1.5	1	1	1
5Au	29	2.9	1.5	1	3
5Au + 3Bu	36	2	–	–	–
5Au + 3Bd	28	2	–	–	–
4Bu	1	1	–	–	–
4Bd	1	1	–	–	–
4B'u	1	1	–	–	–
4B'd	1	1	–	–	–
SL3	45	40	50	30	10
tkCAT	1	1	1	1	1

The CD3δ enhancer fragments and elements were introduced upstream and downstream of the chloramphenicol acetyl transferase (CAT) gene driven by the basal level herpes thymidine kinase promoter in the pBLCAT2 plasmid (Luchow and Shutz, 1987). The first series place the fragments used for the binding assays downstream of the tkCAT gene (in the *Sma*I site). These are designated XYZ, YZ, Z and YZ respectively. The next series employed multiple copies of oligonucleotides spanning the identified binding sites (see Figure 2). 2Ad contains two copies of δA downstream of tkCAT (*Sma*I site), 5Au contains five copies of δA upstream (*Sal*I site), 5Au + 3Bu and 5Au + 3Bd contain an additional three copies of the B site upstream (*Hin*dIII site) or downstream (*Sma*I site) in the 5Au construct. 4Bu and 4Bd contain four copies of the δB site upstream and downstream (*Sal*I and *Sma*I sites respectively) of the tkCAT gene. 4B'u and 4B'd contain four copies of an oligonucleotide spanning the δB site and including 30 additional nucleotides which were involved in the methylation interference of the δB−c1, −c2 complexes. The SL3 construct contains a single copy of SL3 enhancer downstream (*Sma*I site) of the tkCAT gene. The recombinant plasmids were analysed for transcriptional activity in a transient transfection assay as previously described (Georgopoulos *et al.*, 1988 and modified according to the Brian Seed protocol). The absolute level of CAT activity from the enhancerless tkCAT gene in the pBLCAT2 plasmid varied between 400 and 1000 c.p.m. The level of CAT expression of the individual CAT constructs was calculated as fold stimulation relative to that of the enhancerless pBLCAT2 plasmid. The data given in this table represent the average of at least four experiments. Transfections were done in triplicates and efficiencies were corrected by RSVβgal cotransfections.

complexes. Differences in the interference pattern obtained for these two complexes (δB−c1 and δB−c2) indicated their distinct identities (Figure 2B and C). The interference pattern for the δB−c2 complex extended further upstream than that of δB−c1. This implies that either there is a distinct upstream binding site for δB−c2 or that binding of a different factor to this region relies on occupancy of the AT-rich region. Synthetic oligonucleotides made to the AT-rich motif abolished binding of both δB−c1 and δB−c2 complexes (Figure 2D). A summary of the binding domain data obtained for elements δA and δB is presented in Figure 2(C).

Functional analysis of the enhancer elements

The role of elements δA and δB in the enhancer function and specificity was studied in transient transfection assays (Table I). The cell lines used in this study were the phenotypically mature T cell lymphoma EL4 that expressed high levels of the endogenous CD3δ gene, a low expressor variant of the AKR thymoma line BW5147 (J.Versteegen, unpublished results), the AKR thymoma lines SAK8 and SL12.1, which were CD3δ negative (Gasson and Bourgois, 1983; MacLoed *et al.*, 1983), and the NIH3T3 fibroblasts. As previously shown (Georgopoulos *et al.*, 1988), the CD3δ enhancer (fragment XYZ) stimulated transcription of a heterologous promoter to moderate levels (15-fold) in the T cell line EL4. Deletion of the Z fragment including the δB site from the enhancer decreased its activity by 2- to 3-fold (fragment XY). Deletion of the X fragment and removal of the δA site abrogated the activity of the enhancer (fragment YZ). A fragment (Z) of the enhancer containing only the δB site did not exhibit any activity. Therefore, element δA is essential for enhancer function and its effect is amplified by the presence of element δB in the enhancer. We then reiterated elements δA and δB and tested their function as independent enhancers. A dimer of δA (2Ad) when introduced 3′ of the tkCAT gene stimulated transcription to a higher level than the monomer present in the enhancer fragment XY. A pentamer of element δA introduced 5′ of the reporter gene (5Au) functioned as a strong enhancer stimulating transcription by 30-fold; 2-fold higher than the intact CD3δ enhancer itself. A tetramer of δB had no effect on transcription whether introduced upstream (4Bu) or downstream (4Bd) of the tkCAT reporter gene. A δB′ tetramer with upstream sequences corresponding to the whole region involved in methylation interference of δB−c2 complex also had no effect (Table I). Element δB reiterated and juxtaposed to an element δA pentamer stimulated its activity to a small degree (5Au + 3Bu). When separated from element δA by placing δB downstream of the tkCAT gene in the 5Au + 3Bd construct the multimerized element δB had no effect on transcription. Although the effect of multimerized δB on multimerized δA was not as dramatic as the effect of the δB monomer on the δA monomer in the enhancer (constructs XYZ and XY, EL4, Table I) it was consistently detected. The inactivity of δB alone and the requirement for proximity of δA suggest that this element acts by stabilizing the interaction of element δA with its cognate factor(s). Multimerization of the δA element may functionally stabilize the δA complex and partially replace the requirement for element δB. This could explain the relatively small effect of δB in the 5Au + 3Bu construct.

The activities of the CD3δ enhancer and of element δA were also tested in the variant BW5147 AKR thymoma that expresses very low levels of the CD3δ gene (Table I). In this cell line, both the CD3δ enhancer (XYZ) and the 5× δA element (5Au) stimulated transcription by 2- to 3-fold. This level of stimulation was ~10-fold lower than that detected in EL4 cells. No activity was detected for either the CD3δ enhancer or the δA element in the AKR thymoma cell lines Sak8 and SL12.1, which do not express the endogenous CD3δ gene. A low level of stimulation (3×) was detected by the δA pentamer but not by the CD3δ enhancer in the fibroblast cell line 3T3 (Table I). This may be due to distinct enhancer factors present in the fibroblast cell line which bind to the δA element in the CD3δ enhancer with insufficient affinity to allow for stimulation of transcription. When element δA is reiterated, the functional affinity for binding is increased and some stimulation of transcription occurs.

Tissue-specific distribution of nuclear complexes interacting with the CD3δ enhancer elements

In order to correlate the activity of the CD3δ enhancer elements with the presence of specific nuclear factors, we

studied the distribution of the δA−c1, −c2 and δB−c1, −c2 nuclear complexes in the cell lines used to determine the functional specificity of these elements (Figure 3). The δA−c2 complex was detected at similar levels in all cell lines tested (when normalized against non-specific complexes) and did not correlate with enhancer function (Figure 3A). Higher levels of δA−c1 relative to the complex δA−c2 were only detected in the mature T cell line EL4 (10-fold higher than in the other cell lines determined by scanning densitometry, Figure 3A, lanes 1 and 2). In all the other low or non-CD3δ expressing thymoma lines tested, δA−c1 was found at either the same (Figure 3A, lanes 3 and 6) or lower levels than δA−c2 (Figure 3A, lanes 4 and 5). The δA−c1 complex was not detected in the fibroblast line NIH3T3 (Figure 3A, lane 7), but other complexes of higher mobility were observed and could be responsible for the low level of activation detected from this element in fibroblasts. Since higher levels of δA−c1 correlate with CD3δ enhancer activity and the expression of the endogenous gene in the mature T cell line EL4, the levels of δA−c1 detected in the other low or non-expressor cell lines are either quantitatively insufficient to stimulate transcription or represent a distinct nuclear complex which co-migrates with δA−c1 but cannot function as an enhancer. Alternatively if both active enhancer factors (δA−c1) and inactive binders (δA−c2) compete for the δA site their relative concentrations will dictate the activity of the enhancer element in a given cell line.

The nuclear proteins that bind site δB were also found to be differentially expressed (Figure 3B). The δB−c1 complex was present at moderate to high levels in all the lymphoid cells studied and was barely detectable in fibroblasts. In contrast, the δB−c2 nuclear complex was only detectable in the mature T cell line EL4 and the AKR thymoma line SAK8 (Figure 3B, lanes 1, 2 and 5). The CD3δ enhancer and the δA element are not active in SAK8 cells, but this lack of activity may be due to the low levels of δA−c1 complex detected in this line (Table I and Figure 3A, lane 5). The nuclear complexes δB−c1 and δB−c2 may be responsible for mediating a higher level of activity from element δA through interaction with the nuclear factors that mediate its function.

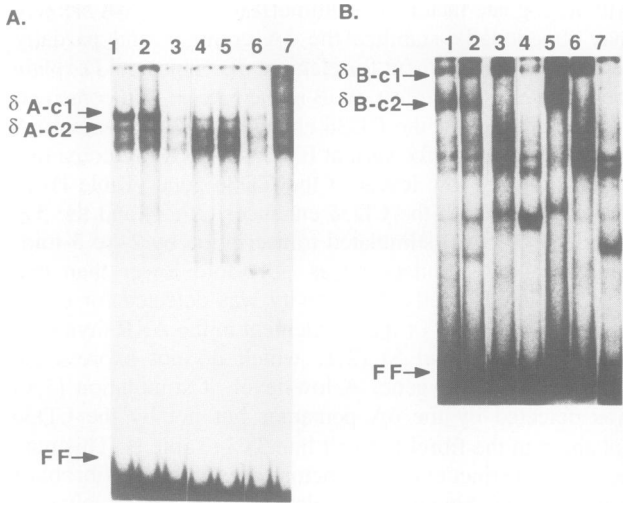

Fig. 3. Tissue-specific distribution of the nuclear protein complexes bound to the CD3δ enhancer. (**A**,**B**) Nuclear extracts were made from the mature T cell line EL4 (**lanes 1** and **2**, extracts from different sources of EL4 cells are compared), a low expressor variant of the thymoma cell line Bw5147 (**lane 3**), the non-expressor thymoma cell lines SL12.1 (**lane 4**), and Sac8 (**lane 5**), the B cell line MPC11 (**lane 6**) and the NIH3T3 fibroblast cell line (**lane 7**). DNA binding assays were performed with fragment X (**A**) and fragment Z (**B**) using 10 000 c.p.m. of DNA probe, 1 μg of nuclear protein and 1 μg of non-specific DNA competitor (dI/dC).

Further delineation of the sequence requirements for binding of the δA complex

Element δA was further dissected starting with a 40 bp oligonucleotide which supported complex formation

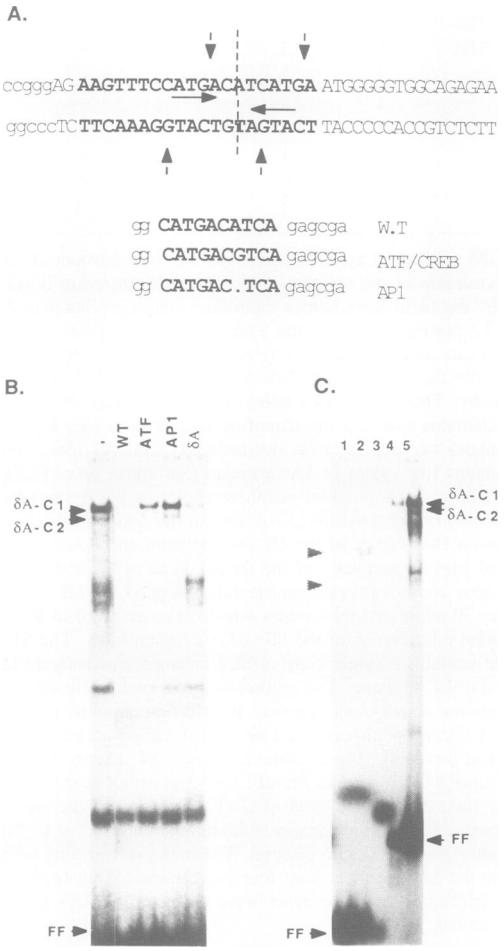

Fig. 4. Delineation of the sequence requirements for binding of the δA−c1 and δA−c2 complexes to element δA. (**A**) The sequence of the 40 bp oligonucleotide (51−91) containing the δA site (bold letters) is analysed. The hyphenated palindrome in this sequence is indicated by the horizontal arrows. The *Nla*III restriction cuts are indicated by vertical arrows. The three 18 bp oligonucleotides made to the wild type (WT), and mutant (ATF/CREB and AP1) palindromes are shown below. The AP1 oligonucleotide deletes 1 bp in the palindrome which binds with high affinity the Jun/Fos heterodimer (data not shown). The ATF/CREB oligonucleotide involves a single A−G transversion. (**B**) The 40 bp oligonucleotide (δA) was end-labelled as described in Figure 1 and used as a probe in the DNA mobility shift assay with nuclear extract from the T cell line EL4. A 50-fold molar excess of the three 18 bp oligonucleotides containing the wild-type (WT) and mutant palindromes (ATF/CREB, and AP1) were multimerized, and respectively used as competitors. The 40 bp (δA) oligonucleotide was also included in this competition experiment as a monomer. (**C**) The 18 bp oligonucleotides were end-labelled and used as probes in the DNA mobility shift assay as described above. **Lane 1**, WT (18 bp oligonucleotide); **lane 2**, ATF/CREB (18 bp oligonucleotide); **lane 3**, AP1 (17 bp oligonucleotide); **lanes 4** and **5**, δA (40 bp oligonucleotide) 10 000−20 000 c.p.m. The unlabelled gray arrows point to unique species detected binding to the oligonucleotide with the ATF/CREB site. The rest of the protein complexes are designated as in previous figures.

(Figure 4). Many transcription factors interact with palindromic sequences and element δA contains a 12 bp hyphenated palindrome. Deletions in the parent oligonucleotide which extended into the palindromic region from either end abolished binding (*Nla*III sites indicated in Figure 4A as vertical arrows were used to generate the truncated palindromes, data not shown). To investigate further the role of this palindromic sequence in nuclear complex formation, we made three 18 bp oligonucleotides which contained either the wild-type or mutations in the 12 bp palindrome (Figure 4A). A monomer of the wild-type palindrome did not support complex formation when used as a probe in a binding reaction, and competed very weakly for δA−c1,−c2 (Figure 4, lane 1 and data not shown). Multimerization of this oligonucleotide, however, resulted in effective competition (Figure 4B, lane WT). Therefore sequences outside this palindrome may be required to increase the binding affinity or to provide a larger surface for the protein DNA interaction to occur. A 1 bp deletion (G) in the palindrome abolished its ability to compete for binding of the δA−c1,−c2 complexes (Figure 4B, lane AP1). This mutation created a consensus AP-1 binding site (Angel *et al.*, 1987; Bohman *et al.*, 1987) which formed a high affinity complex with the Jun/Fos heterodimer (Halazonitis *et al.*, 1988; and data not shown). A single base pair change in the palindrome (A-G) decreased its ability to compete for binding of the δA−c1,−c2 complexes by 5-fold (Figure 4B, lane ATF). Nevertheless, when the oligonucleotide containing this mutant palindrome was used as probe DNA in a gel-shift assay, distinct complexes appeared which were not detected with an oligonucleotide containing the wild-type palindrome (Figure 4C, lanes 2 and 3 respectively). Since this mutant palindrome is the consensus recognition site for the cAMP response factor and the ATF factor (Comb *et al.*, 1986; Lee and Green, 1987; Montimny and Bilezikjian, 1987) we assume that the nuclear proteins bound to this mutant DNA represent *bona fide* CREB or ATF factors in T cells and are distinct from those contained in the δA−c1 and δA−c2 complexes. Given the sequence specificity of the enhancer factor(s) that binds to and mediates the activity of the δA element we conclude that this enhancer complex is a novel T cell specific entity.

Table II.

Construct	Fold stimulation							
	Control		Forskolin		PMA		PMA + Ca	
	EL4	BW	EL4	BW	EL4	BW	EL4	BW
XYZ	15	1.8	15	1	14	1.8	14	3
2Ad	10	1.6	10	1	11	1.5	10	2.3
5Au	30	3	30	3	32	3	29	7
SL3	45	40	40	40	40	35	40	35
tkCAT	1	1	1	1	1	1	1	1

The constructs tkCAT, XYZ, 2Ad, 5Au and SL3 described in Table I were transfected in EL4 and BW5147 cells. Twenty-four hours later forskolin (10 μm) or PMA (100 ng) or PMA and Ca ionophore (100 ng TPA + 0.025 μm of A23187) were added to the media. Cells were harvested after 12−15 h and processed as described in Table I.

Effect of signal transduction agonists on activity of the CD3δ enhancer elements

Agonists of protein kinases, such as PMA, ionophore and cAMP have profound effects on gene expression in lymphoid cells by mimicking the effect of developmental signals that are transmitted through these effector pathways (Sen and Baltimore, 1986; Crabtree, 1989; Shirakawa *et al.*, 1989). We have studied the effect of these reagents on the CD3δ enhancer in mature T cells (EL4) and in T cells of a more immature phenotype (BW5147). These cells were transfected with constructs containing either the enhancer or δA multimers and were treated with forskolin (protein kinase A agonist), PMA, and PMA and Ca ionophore (protein kinase C agonist). No effect on the enhancer activity was detected upon treatment of the EL4 T cell line with any of these agents (Table II). In contrast, the weak enhancer activity detected in the BW5147 cells was consistently increased 2- to 3-fold upon treatment with PMA and ionophore. This effect seems to be mediated by δA; both the enhancer and element δA alone showed similar levels of stimulation.

Sequence similarities with other T cell-specific regulatory domains

A sequence similarity search was done between the δA and δB elements and the regulatory domains of the other members of the TCR−CD3 complex. No obvious similarities were found with the CD3ε enhancer (Clevers *et al.*, 1989). However, an element similar to δA was found in the TCRα chain enhancer where deletion of this sequence destroyed enhancer activity (Winoto and Baltimore, 1989). A motif similar to δA is also highly conserved in the promoter regions of the various TCRβ chain genes (Anderson *et al.*, 1988; Lee and Davis, 1988). Deletions

Table III. Sequence similarities of the CD3δ enhancer elements with other T cell-specific regulatory elements

(A)		
CD3 δA(63)		G A A G T T T C C A T G A C A T C A T G A A
TCRα enhancer	(124)	c c c a c **T T C C A T G A C** g **T C A** c **G** g c
TCRβ promoter	(−100)	**G A** g g a g **T** a g t **T G A C A T C A T** a t t
(B)		
CD3 δB (356)		T C A T C C T C A A A T T A A T T A A A A A T A A A G A A C A T G A T G A
CD3δ promoter	(−416)	a g c c t t **T C** c t g g **T A A** c a **A A A A A** c **A A A** t **A** g **C A** a t **A** g t c
	(−449)	g **C A** a t a g t c **A** c **T** g g t a a **A** c **A A A** c **A A A G A** g **A T** t t **T G** g
	(−493)	**T** a c a a **C T** a c **A** t **T T A** c **T** c **A** c **A A A** c **A A** g **G A A** g t g c t **T** t g
TCRβ enhancer	(551′)	**T g g T** g **C T** g **A** t g **T T A** c **T T A** c **A** g **A T A A A** c **A** c **C** c a **G A** c c t
IL-2 promoter	(−263)	g t t c a t **T** g t **A T** g **A A T T A A A A** c t g c c **A** c c t a **A** g t g **T** t g

Homology searches performed using the Intelligenetics Seq program. The bold upper-case letters indicate identity while small letters indicate differences in the sequence motif. The sequence motifs in theTCRα and TCRβ enhancer and promoter regions homologous to δA bind nuclear factors (Winoto and Baltimore, 1989; D.Loh, personal communication). Sequences in the CD3δ and IL2 promoters homologous with δB lie within T cell-specific footprints (K.Georgopoulos, unpublished data; Serfling *et al.*, 1989).

through this region abrogated TCRβ expression (D.Loh, personal communication). Sequences similar to the δB element were also found in the TCRβ chain enhancer and in the IL2 promoter (Table III and Fuse *et al.*, 1984; Serfling *et al.*, 1989).

Discussion

Two distinct elements, δA and δB, were identified in the CD3 enhancer that interact to stimulate transcription in mature T cells. When reiterated, element δA functions as a strong transcriptional enhancer but as a monomer in the CD3δ enhancer it requires the presence of δB for high levels of activity (Table I, EL4). The levels of transcription mediated by element δA correlate well with the levels of expression of the endogenous CD3δ gene and with the potency of the CD3δ enhancer in the mutant T cell lines studied. The level of transcription mediated by element δA in the low expressor T cell line BW5147 could be weakly stimulated by PMA and Ca ionophore (Table II). Recent studies have shown that certain AKR thymoma cell lines increase the rate of transcription of the endogenous CD3δ gene by 3- to 5-fold when treated with Ca ionophore in response to increase in the level of intracellular Ca (W.Miles *et al.*, submitted). The activity of element δA correlates with the presence of the δA−c1 nuclear complex detected at higher concentration in mature T cells. The low concentration of this complex seen in other lymphoid cells (10-fold less) may be insufficient for transcriptional stimulation, especially if the δA−c1 factor has to compete for binding with other non-activating factors present at similar or higher concentration levels. Alternatively, either δA−c1 is absent from these cells and distinct nuclear complexes which do not function as enhancers may co-migrate at this position, or it is present in a low-affinity form which weakly stimulates transcription. Post-transcriptional modifications taking place during T cell development may confer higher affinity and activity to this factor. Element δA is the major transcriptional element of the CD3δ enhancer responsible for mediating changes in CD3δ gene expression during T cell development.

Element δB is also required for full activity of the CD3δ enhancer. In mature T cells, however, it interacts with element δA to stimulate its enhancer activity. The function of binding sites for the δB factor found in the promoter of the CD3δ gene remains unclear (Table III). These additional δB complexes in the promoter may further stimulate the activity of the δA enhancer. Element δB binds two specific complexes, one of which appears to be tissue specific (complex δB−c2). We cannot eliminate the possibility that complex δB−c1 is involved in transcriptional enhancement because the function of element δB can only be assayed in the presence of an active element δA-factor and we have yet identified a cell line with high levels of δA−c1 and no δB−c2. Nevertheless, the tissue specificity of the δB−c2 complex suggests that it is the active factor. In the absence of these specific nuclear factors, stimulation of transcription by the δA element alone may not be sufficient for level of expression of the CD3δ gene. Mechanistically similar elements have been described in the SV40 enhancer (Fromental *et al.*, 1988).

The CD3δ enhancer elements may also be involved in regulating the expression of the other members of the TCR−CD3 complex. Since the CD3γ gene is closely linked and divergently transcribed (Saito *et al.*, 1987), the CD3δ gene enhancer may be directly involved in regulating expression of this gene. No obvious similarities have been found between the CD3δ and ε enhancers. However, an element similar but not identical to δA was found in the TCRα chain enhancer and deletion of this sequence destroyed enhancer activity (Winoto and Baltimore, 1989). In the promoter region of the TCRβ chain gene a similar motif of δA is found conserved which is important for expression of the gene (D.Loh, personal communication). Although these homologous sites are protected by both T and non-T cell nuclear extracts in the DNase I footprinting assay, this may be due to the presence of distinct non-activating binding factors present in these extracts (Royer and Reinharz, 1987; Winoto *et al.*, 1989). This hypothesis is supported by the subtle differences observed in the footprints obtained with the T and non-T cell extracts. It remains to be determined whether the same nuclear factors interact with these elements to mediate the transcriptional regulation of these genes. Some sequence similarities between the δB element and the TCRβ chain enhancer and the IL2 distal promoter were also found (Table IIIB). Since each member of the TCR−CD3 complex appears at a different time in T cell ontogeny it is possible that these genes share some tissue-specific regulators but that their differential expression is accomplished either by differential affinity to the respective control element found in these genes or by interaction with additional control elements in their enhancer or promoter regions.

We suggest that commitment to the T cell lineage takes place upon activation of the transcriptional complexes that interact with the δA element in the CD3δ enhancer. Activation of the δB nuclear factors and further increase in activity of the δA transcriptional complex may take an early thymocyte through T cell maturation. The concomitant action of these factors appears to be required for the high level expression of CD3−TCR complex necessary in a mature T cell. Isolation of the genes that code for the δA and δB nuclear complexes will enable us to test their role in T cell development.

Materials and methods

Nuclear extracts

Nuclear extracts were prepared from the leukemic T cell EL4, the thymoma cells SL12.1, SAK8 and BW5147, the myeloma MPC11 and the fibroblast NIH3T3 cell lines according to the Dignam protocol (Dignam *et al.*, 1983). The protein concentration was estimated by the Bradford method.

Gel-retardation assay

Fragments X (100 bp) and Z (100 bp) of the enhancer were end labelled by the Klenow (large fragment) fill in reaction. Between 10 000 and 20 000 c.p.m. of DNA probe were incubated with 1 μg of nuclear extract and 1 μg of non-specific DNA competitor for 15 min at room temperature. The binding reactions were then analysed by gel retardation under conditions previously described (Halazonitis *et al.*, 1988). A 50-fold molar excess of DNA competitors was included in the assay in order to test for sequence specificity of the complexes.

In vitro enzymatic and chemical footprinting

DNase I footprinting in solution was performed as previously described (Galson and Housman, 1988). Between 2−3 μg of nuclear protein were used per reaction together with 1 μg of non-specific DNA competitor (dI·dC) and 25 U of DNase I. A 40 bp oligonucleotide spanning the 51−91 bp region of the enhancer (Georgopoulos *et al.*, 1988) and containing the protected sequence was used as competitor at 50-fold molar excess. Nuclear

extracts made from the B myeloma MPC11, the F9 teratocarcinoma and the NIH3T3 fibroblast cell lines were also used.

DNA methylation interference was employed to identify the binding domain for the δB−c1 and δB−c2 complexes formed on the Z fragment as described (Galson and Housman, 1988). Weak acid depurination followed by alkali hydrolysis was employed to determine the effect of adenine methylation on binding of the δB complexes. The positive and negative strands were end-labelled by the Klenow fill in the T4 kinase reactions respectively.

Transfections and CAT assays

The CD3δ enhancer fragments and elements were introduced upstream and downstream of the chloramphenicol acetyl transferase (CAT) gene driven by the basal level herpes thymidine kinase promoter in the pBLCAT2 plasmid (Luchow and Schutz, 1987). The recombinant plasmids were analysed for transcriptional activity in a transient transfection assay as previously described (Georgopoulos *et al.*, 1988). The CAT assay was modified according to the Brian Seed protocol (Seen and Seed, 1988). The specific activity of the elements was determined by transfections in CD3+ (EL4), CD3+/− (BW5147 note that the BW5147 used in previous studies was a high expressor variant of this cell line) and CD3− (Sac8 and SL12.1) T cell lines and the NIH3T3 fibroblasts.

Stimulation of transfection assays by cAMP and protein kinase C agonists

The constructs tkCAT, XYZ, 2Ad, 5Au and SL3 described in Table I were transfected in EL4 or BW5147 cells. Twenty-four hours later forskolin (10 μm) or PMA (100 ng) or PMA and Ca ionophore (100 ng TPA + 0.025 μm of A23187) were added and cells were harvested 12−15 h later.

Acknowledgements

We thank Thanos Halazonitis for providing us with the Jun/Fos heterodimer, Anita Gupta for providing the 5Au + 3Bd and u plasmids, Bruce Morgan for invaluable help and Ulla Hansen, Thanos Halazonitis and Stephen Ley for critically reviewing the manuscript. K.G. is a Special Fellow of the Leukemia Society of America, D.G. is supported by a Public Health Service grant CA−17575 to David Housman from the National Cancer Institute grant. This work was supported by the NIH, grant AI17651.

References

Anderson,S.J., Chou,H.S. and Loh,D.Y. (1988) *Proc. Natl. Acad. Sci. USA*, **85**, 3551−3554.
Angel,P., Imagawa,M., Stein,C.B. and Karin,M. (1987) *Cell*, **49**, 729−739.
Bohman,T.J., Bos,A.A., Nishimura,T., Vogt,P.K. and Tjian,R. (1987) *Science*, **238**, 1386−1392.
Clevers,H., Dunlop,S. and Terhorst,C. (1989) *EMBO J.*, **8**, 2527.
Comb,M., Birnberg,N.C., Seasholtz,A., Herbert,E. and Goodman,H.M. (1986) *Nature*, **323**, 353−356.
Crabtree,G. (1989) *Science*, **243**, 355−361.
Dignam,J.D., Lebovitz,R.M. and Roeder,R.G. (1983) *Nucleic Acids Res.*, **11**, 1475−1477.
Fromental,C., Kanno,M., Nomiyama,H. and Chambon,P. (1988) *Cell*, **54**, 943−953.
Fuse,A., Fujita,T., Yasumitsu,H., Kashima,N., Hasegawa,?. and Taniguchi,T. (1984) *Nucleic Acids Res.*, **12**, 9323.
Galson,D. and Housman,D. (1988) *Mol. Cell. Biol.*, **8**, 381−390.
Gasson,J. and Bourgois,S. (1983) *J. Cell. Biol.*, **96**, 409−415.
Georgopoulos,K., van den Elsen,P., Bier,E., Maxam,A. and Terhorst,C. (1988) *EMBO J.*, **7**, 2401.
Halazonitis,T., Georgopoulos,K., Greenberg,M. and Leder,P. (1988) *Cell*, **55**, 917−924.
Haynes,B.F., Denning,S.M., Singer,K.H. and Kutzenberg,J. (1989) *Immunol. Today*, **10**, 87−90.
Elsen,P., Georgopoulos,K., Shipley,B., Orkin,S. and Terhorst,C. (1986) *Proc. Natl. Acad. Sci. USA*, **83**, 2944−2948.
Krimpenfort,P., Jong,R., Yashusi,U. and Berns,A. (1988) *EMBO J.*, **7** 745−750.
Lee,K.A. and Green,M. (1987) *EMBO J.*, **6**, 1345−1351.
Lee,N.E. and Davis,M. (1988) *J. Immunol.*, **140**, 1665.
Lin,Y.S. and Green,M.R. (1988) *Proc. Natl. Acad. Sci. USA*, **85**, 3396−3400.
Luchow,B. and Schutz,G. (1987) *Nucleic Acids Res.*, **15**, 500.
MacLoed,C., Hays,E., Hyman,R. and Bourgois,S. (1984) *Cancer Res.*, **44**, 1784−1790.
Montimny,M. and Bilezikjian,L. (1987) *Nature*, **328**, 175−178.
Royer,H.R. and Reinharz,E. (1987) *Proc. Natl. Acad. Sci. USA*, **84**, 232−236.
Saito,H., Koyama,T., Georgopoulos,K., Clevers,H., Haser,W., LeBien,T., Tonegawa,S. and Terhorst,C. (1987) *Proc. Natl. Acad. Sci. USA*, **84**, 9131−9134.
Seen,Y.J. and Seed,B. (1988) *Gene*, **67**, 271−277.
Sen,R. and Baltimore,D. (1986) *Cell*, **47**, 921−928.
Serfling,E., Barthelmas,R. and Karin,M. (1989) *EMBO J.*, **8**, 465.
Shirakawa,F., Chedid,M., Suttles,J., Pollack,B.A. and Mitzel,S. (1989) *Mol. Cell Biol.*, **9**, 959−964.
Von Boehmer,H. (1988) *Annu. Rev. Immunol.*, **6**, 309−326.
Winoto,A. and Baltimore,D. (1989) *EMBO J.*, **8**, 729−733.

Received on September 28, 1989