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An enhancer located in a CpG-island 3′ to the TCR/CD3-ε gene confers T lymphocyte-specificity to its promoter

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The gene encoding the CD3-ε chain of the T cell receptor (TCR/CD3) complex is uniquely transcribed in all T lymphocyte lineage cells. The human CD3-ε gene, when introduced into the mouse germ line, was expressed in correct tissue-specific fashion. The gene was then screened for T lymphocyte-specific *cis*-acting elements in transient chloramphenicol transferase assays. The promoter (−228 to +100) functioned irrespective of cell type. A 1225 bp enhancer with strict T cell-specificity was found in a DNase I hypersensitive site downstream of the last exon, 12 kb from the promoter. This site was present in T cells only. The CD3-ε enhancer did not display sequence similarity with the T cell-specific enhancer of CD3-δ, a related gene co-regulated with CD3-ε during intrathymic differentiation. The CD3-ε enhancer was unusual in that it constituted a CpG island, and was hypomethylated independent of tissue type. Two HTLV I-transformed T cell lines were identified in which the CD3-ε gene was not expressed, and in which the enhancer was inactive.

Key words: CD3-ε gene/CpG-island/DNase I hypersensitive site/T cell-specific enhancer

Introduction

Transcription of eukaryotic genes by RNA polymerase II is controlled by two broad categories of *cis*-acting elements: (i) promoters typically consist of a TATA box and multiple upstream promoter elements. The TATA box is involved in the correct initiation of transcription. Upstream promoter elements control the efficiency of transcription. The architecture of a promoter is relatively rigid and the activity of individual elements is dependent on position and orientation; (ii) enhancers, by definition, control transcription initiation from homologous as well as from heterologous promoters and are independent of distance and orientation. A wealth of evidence gathered in recent years indicates that *cis*-acting elements exert their effects through binding of sequence-specific *trans*-acting transcription factors (Reviewed in Ptashne, 1986; Maniatis *et al.*, 1987; Atchison, 1988).

One of the modes of transcription control involves the expression of particular genes in a tissue-specific fashion. From a number of 'model' genes, a general picture has emerged in which tissue-specific transcription is controlled by discrete *cis*-acting elements, interacting with tissue-specific *trans*-acting factors. Such elements may be part of promoters as well as enhancers, and can control tissue-specific transcription of heterologous sequences in hybrid gene constructs (Maniatis *et al.*, 1987; Atchison, 1988).

The antigen receptor complex on the surface of T lymphocytes consists of at least six different protein chains. The T cell receptor (TCR) occurs either as a TCRα/β or a TCRγ/δ disulfide-linked heterodimer. All four TCR chains are the products of rearranging genes that resemble the immunoglobulin genes in structure (reviewed in Toyonaga and Mak, 1987; Davis and Bjorkman, 1988). The CD3 complex is non-covalently associated with the TCR heterodimer, and consists of the invariable integral membrane proteins CD3-γ, CD3-δ, CD3-ε, and the CD3-ζ homodimer (reviewed in Clevers *et al.*, 1988a). CD3-γ, CD3-δ, and CD3-ε form a small gene family as a evidenced by similarities in sequence and gene structure (van den Elsen *et al.*, 1986; Gold *et al.*, 1986; Tunnacliffe *et al.*, 1987). Furthermore, they are tightly linked on chromosome 11q23 in man (Gold *et al.*, 1987; Clevers *et al.*, 1988b; Tunnacliffe *et al.*, 1988). CD3-γ and CD3-δ occur as a divergently transcribed gene pair, their transcription start sites being 1.6 kb apart (Saito *et al.*, 1987; Tunnacliffe *et al.*, 1987). The CD3-ε gene is located 26 kb downstream from the CD3-δ gene, and is transcribed in the direction of the CD3-γ/CD3-δ gene pair (Tunnacliffe *et al.*, 1988). CD3-ζ appears unrelated to the other CD3 genes, and maps to chromosome 1 in man (Weissman, 1988).

We have recently described the human and mouse genes encoding the CD3-ε chain (Clevers *et al.*, 1988b-c). This single-copy gene does not rearrange and gives rise to one species of mature message, precluding such complicating mechanisms as alternative promoters, alternative splicing or cryptic polyadenylation signals. As is the case with the other CD3 genes, the CD3-ε gene is expressed uniquely in all cells of T lymphocyte lineage (Furley *et al.*, 1986; van Dongen *et al.*, 1987; van Dongen *et al.*, 1988). Once switched on early in the thymus-dependent differentiation pathway, the CD3-ε gene appears not to be subject to major changes in its level of expression. Therefore, this gene represents an excellent model system in which to study the mechanisms by which T cell-specific gene expression is controlled.

The human CD3-ε gene contains nine exons and spans 12 kb (Clevers, 1988b). Here we report the characterization of *cis*-acting elements that control the T cell-specific expression of this gene. A human genomic fragment comprising 24 kb of intragenic and flanking sequences of the CD3-ε gene was used to create a transgenic mouse strain. This strain expressed the transgene in correct tissue-specific fashion. The 24 kb fragment was then screened for the presence of promoter and enhancer elements by transient DNA transfer of hybrid CD3-ε/chloramphenicol transferase (CAT) constructs into cells of T- or non T-lymphoid origin. It was thus shown that the CD3-ε promoter was functional

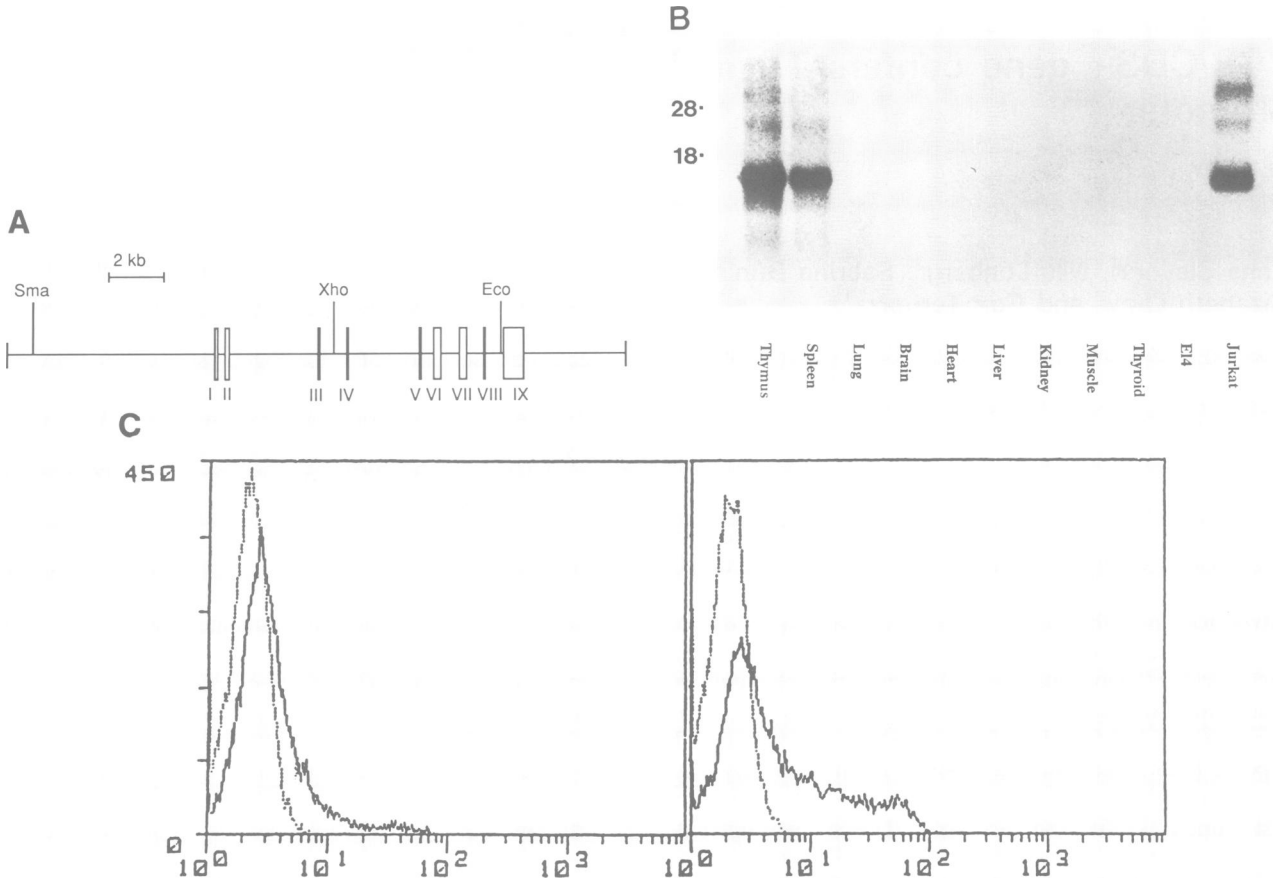

Fig. 1. Expression of the human CD3-ϵ gene in a transgenic mouse strain. The human CD3-ϵ gene and flanking sequences were reconstructed from two overlapping bacteriophage inserts using the central, unique, *Xho*I site. The resultant plasmid pL12, depicted in **A**, was used to generate a transgenic mouse strain. **B** demonstrates Northern blot analysis of RNA isolated from a number of tissues from this mouse strain. In each lane 20 μg of RNA was run. Inspection of the ethidium bromide-stained gel revealed the presence of equal amounts of RNA in each sample. The blot was probed with a 3′ untranslated human CD3-ϵ cDNA probe (bp 840−1300 of pDJ4 (Gold *et al.*, 1986)). This probe does not hybridize with mouse CD3-ϵ mRNA under stringent hybridization conditions. RNA from the human T cell line Jurkat served as a positive control; RNA from the CD3-ϵ expressing murine cell line EL4 was used as a negative control. **C** shows that a fraction of thymocytes (49%, left panel) and splenocytes (31%, right panel) of transgenic mice expressed the human CD3-ϵ chain on the cell surface as evidenced by direct immunofluorescence staining with the Leu-4 antibody. Cells stained with an irrelevant antibody served as a negative control. No Leu-4 positive lymphocytes were detectable in non-transgenic littermates (not shown).

in a broad spectrum of cell lines. A T cell-specific enhancer satisfying all classical criteria (see above), was found to be located directly downstream of exon IX, and coincided with a CpG-island and a T cell-specific DNase I hypersensitive site.

Results

Expression of the human CD3-ϵ gene in a transgenic mouse

First it was determined, if the human genomic CD3-ϵ sequences described elsewhere (Clevers *et al.*, 1988b) contained a complete set of *cis*-acting elements required for appropriate T cell-specific expression. To this end, a line of transgenic mice was generated using a 24 kb *Sma*I−*Sal*I fragment of the human CD3-ϵ gene (Figure 1A). This mouse line contained 10−50 copies of the human gene inserted as a single tandem array into its genome. Tissue-specificity of the expression of the transgene was investigated in transgenic offspring. RNA prepared from various tissues was analyzed by Northern blot analysis using a 3′ untranslated fragment from a human CD3-ϵ cDNA clone that does not cross-hybridize to the endogenous mouse transcript (Gold *et al.*, 1986). As shown in Figure 1b, the transgene was only expressed in the thymus, and to a lesser extent in the spleen, consistent with the tissue distribution of endogenous CD3-ϵ expression in human and mouse. In addition, a substantial fraction of thymocytes (49%) and spleen lymphocytes (31%) expressed the human CD3-ϵ chain on their surface, as evidenced by positive staining with the anti-human CD3-ϵ monoclonal antibody Leu-4 (Figure 1C). It could thus be concluded that a complete set of *cis*-acting elements was present in the human CD3-ϵ construct.

The CD3-ϵ promoter

Tissue-specificity of the CD3-ϵ promoter was next examined. Mapping of the transcription start site of the CD3-ϵ gene has been presented elsewhere (Clevers *et al.*, 1988b-c). Homology comparisons of 1 kb of sequence directly upstream of the transcription start site of the human and mouse CD3-ϵ genes suggested that functional activity of the promoter was restricted to the most proximal 200 bp. Therefore, a fragment from the human gene (bp −228 to +27), extending into the first exon, was tested for promoter activity in a quantitative CAT assay (Seed and Sheen, 1988). Unless otherwise stated, constructs were tested by transient

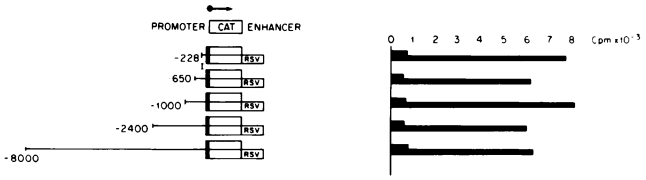

Fig. 2. Characterization of the CD3-ϵ promoter and assaying of upstream sequences for regulatory elements by transient transfer of CAT constructs into Jurkat T cells. Fragments from +27 and extending in an upstream direction were cloned in front of the CAT open reading frame (ORF) in pCAT3 (Luckow and Schutz, 1987). Each construct was tested with and without the RSV-enhancer cloned downstream of CAT. Promoter activity was restricted to the smallest fragment (bp −228 to +27). No additional regulatory elements were detected up to 8000 bp upstream from this promoter.

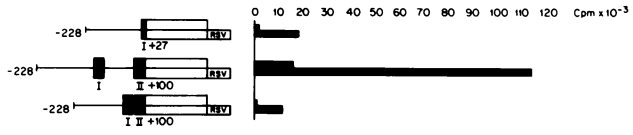

Fig. 3. Effect of the first intron on measured promoter activity. The short version of the CD3-ϵ promoter was extended into exon II (middle constructs), and activities in Jurkat were compared with the short CD3-ϵ promoter (upper constructs) plus and minus the RSV-enhancer. An approximately 10-fold increase in CAT activity was measured both with and without the RSV-enhancer (note that in this experiment 120 000 c.p.m. equals 100% conversion of [^{14}C]chloramphenicol). Removal of intron I (lower constructs) resulted in levels of CAT that equalled those of the short CD3-ϵ promoter constructs.

DEAE-mediated transfection in the T lymphocytic cell line, Jurkat.

As demonstrated in Figure 2, this fragment had very low activity. However, when a retroviral enhancer with broad tissue-specificity (Roux sarcoma virus (RSV)-LTR, bp −491 to −51 (Gorman *et al.*, 1982) was introduced downstream from CAT, activity was readily detectable. Insertion of the RSV enhancer in a promoterless CAT construct resulted in background CAT values (data not shown). Stepwise extension of the promoter fragment to −650, −1000, −2400 and −8000 did not affect the measured activities of the CAT constructs, with or without the RSV enhancer (see Figure 2). Qualitatively similar results were obtained in HeLa cells and in HepG2 hepatoma cells (data not shown). It was therefore concluded that the fragment −228 to +27 constituted a non-tissue specific promoter, and that no additional enhancing or attenuating elements were located upstream from bp −228.

Since the first exon of the CD3-ϵ gene is not translated, it was feasible to extend the promoter fragment into exon II, thus investigating the effect of the presence of the first (120 bp) intron. As shown in Figure 3, extension of the promoter fragment into exon II increased CAT activity ~10-fold both in the presence and absence of the RSV enhancer. Activity of the extended CD3-ϵ promoter was now readily measurable in the absence of an enhancer. This intron I-dependent increase in CAT expression was found to be independent of cell type (data not shown). In order to map the sequences responsible for the increase, the intron was removed by replacing bp 27 in exon I to bp 100 in exon II by the corresponding cDNA fragment (Figure 3). The activities of the resulting constructs were of the same order as those with just exon I-sequence (compare upper and lower

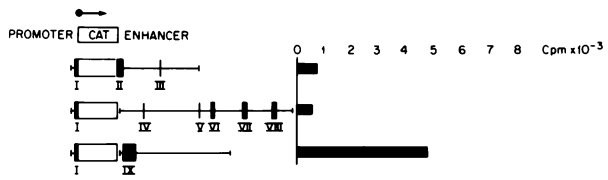

Fig. 4. Characterization of the CD3-ϵ promoter and enhancer by transient transfer of CAT constructs into Jurkat T cells. Genomic CD3-ϵ- DNA, located downstream from the CD3-ϵ promoter was tested for enhancer activity in Jurkat cells. The indicated fragments were cloned in two orientations behind CAT, driven by the CD3-ϵ promoter. The results obtained with the fragments in positive orientation are shown; negative orientations gave essentially identical results. Both orientations of the fragment encompassing exon IX reproducibly resulted in ~10-fold enhancement of CAT activity.

constructs in Figure 3), indicating that indeed the presence of the intron I-sequence was responsible for the 10-fold increase in CAT activity. Essentially identical observations were made with mouse CD3-ϵ promoter constructs (H.Clevers and C.Terhorst, unpublished).

The fragment +27 to +100 containing the first intron did not behave as a classical enhancer: insertion of this fragment downstream from CAT in a construct with the short CD3-ϵ promoter had no effect on its activity. Similarly, insertion of the intron fragment upstream from the herpes thymidine kinase promoter in pBLCAT2 did not affect CAT activity (data not shown). In an attempt to map more closely the sequences involved in the intron I effect, small *Bal*31 deletions were made in the 3′ direction starting from the *Mst*II site in the first exon (bp +27). It was noted that all deletions that went past the splice donor sequence lost the 10-fold increase in CAT activity (not shown). These experiments indicated that either i) the effect was transcriptional and a promoter element was located at the exon I−intron I boundary, or ii) the measured effect was post-transcriptional and was linked to the presence of a spliceable intron in the transcript.

A T cell specific enhancer is located downstream from the last exon

The promoter of the CD3-ϵ gene was not tissue-specific, and no regulatory elements were detected upstream from bp −228. Next, sequences downstream from exon II were screened for enhancer activity. To this end, downstream fragments of the CD3-ϵ gene were subcloned in both orientations behind CAT, driven by the short CD3-ϵ promoter (bp −228 to +27). Upon transfection into Jurkat cells, a fragment containing the last exon and 4 kb of downstream sequence enhanced CAT activity by ~10-fold (see Figure 4). Full enhancer activity resided in a *Kpn*I−*Eco*RV subfragment (see Figure 5A). Serial deletions were then prepared from the *Eco*RV site in an upstream direction. All deletion constructs were inserted in a negative orientation downstream from CAT. As shown in Figure 5A, a precipitous drop in enhancer activity occurred close to the indicated *Pst*I site. Sequencing revealed the shortest fully active construct (pI-9) and the longest inactive one (pI-11) to differ by 110 bp only. In addition to mapping the 3′ boundary of the enhancer, these deletion constructs show that the enhancing effect is independent of distance: in the shortest fully active construct, pI-9, the enhancer was 3 kb closer to the promoter than in the longest construct. Yet the observed enhancement was of a similar magnitude.

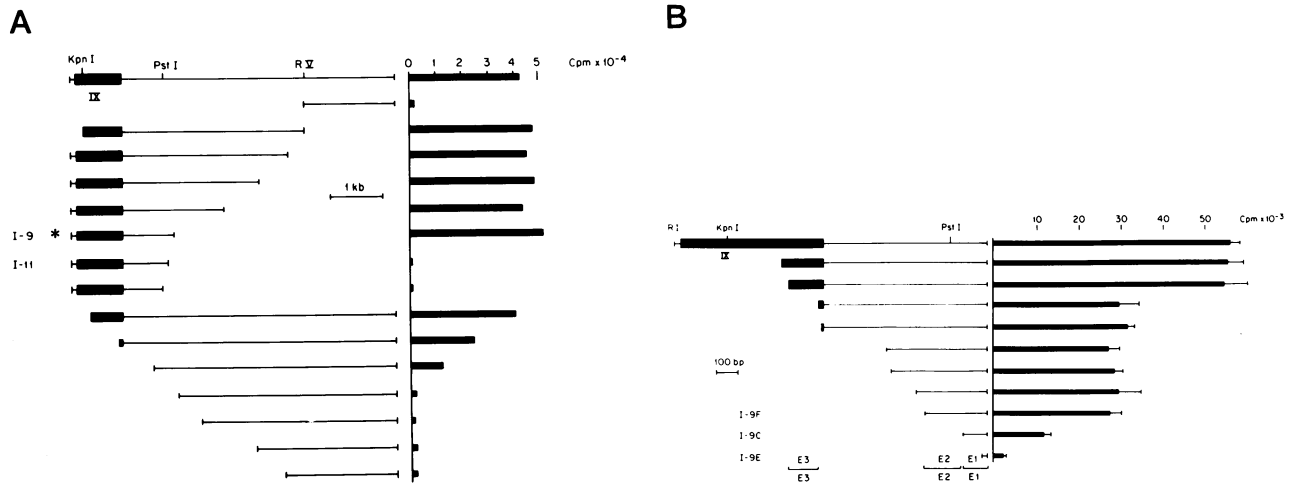

Fig.5. Deletion mapping of the CD3-ϵ enhancer. **A)** Full enhancer activity was first located to a *Kpn*I−*Eco*RV subfragment of the enhancer depicted in Figure 4. *Bal*31 deletions were made from the *Eco*RV site in an upstream direction. The enhancer activity was completely lost in a 120 bp step from clone pI-9 to clone pI-11. A separate set of deletions was produced to map the 5′ boundary of the enhancer, starting from the *Kpn*I site on exon IX. A gradual loss of enhancer activity was noted over a stretch of ~1000 bp. All fragments were cloned in a positive orientation behind CAT, driven by the short CD3-ϵ promoter and tested by transfection into Jurkat. **B)** Fine-mapping of the 5′ boundary of the CD3-ϵ enhancer. A new series of deletions was made starting from the *Kpn*I site in pI-9, the shortest fully active 3′ deletion clone (see A). In order to recognize minor differences in activity, CAT assays were performed in triplicate. Three domains could be identified. 'E1' by itself was a 5-fold enhancer. 'E2' and 'E3' each enhanced enhancer activity 2-fold. All fragments were cloned in a negative orientation behind CAT, driven by the short CD3-ϵ promoter and tested by transfection into Jurkat.

Serial deletions in a downstream direction, starting from the *Kpn*I site in the last exon, resulted in a gradual drop in enhancer activity over a stretch of ~1000 bp (Figure 5A). A new series of 5′ deletions starting from the *Kpn*I site was then prepared from the shortest active 3′ deletion clone (pI-9). These 5′ deletion clones were then tested by triplicate transfection into Jurkat cells, Figure 5B shows that three spatially separate domains could be identified in this experiment. The most 3′ 125 bp ('E1') of this fragment conferred 5-fold enhancement to the short CD3-ϵ promoter. DNA sequences indicated by 'E2' and 'E3' each amplified the enhancer activity 2-fold.

From these experiments it was concluded that a transcriptional enhancer was located at the 3′ end of the CD3-ϵ gene. This enhancer contained a small (<125 bp) core, which we termed 'E1' and which was indispensable for its activity.

The 3′ enhancer was then tested for tissue-specificity by transfection into the human T cell lines REX, HPB-ALL, the mouse T cell lines DO11.10, BW5147, and EL4; into the human B cell lines Raji and JY; and into HeLa cells and the human hepatoma cell line HepG2. 5 to 10-fold enhancement of CAT activity was found in all T cell lines tested, both on the short and on the extended CD3-ϵ promoter. No enhancement was observed in the B cell lines and in HeLa and HepG2 (see Table I).

It was then tested whether T cell-specificity could be conferred onto heterologous promoters. Constructs were prepared by cloning the enhancer downstream from CAT, driven by a linker insertion mutant of the herpes thymidine kinase (tk) promoter (pBLCAT2, Luckow and Schutz, 1987), or from CAT driven by the promoter of the proto-oncogene *pim*-1 (pR3, Krimpenfort *et al.*, 1988) in both orientations. Again, ~5 to 10-fold enhancement was seen in T lymphocytes only. Furthermore, the 125 bp 'E1' domain cloned behind pBLCAT2 resulted in 5 to 10-fold enhancement in T cells only (Table I).

Table I. Tissue-specific activity of the CD3-ϵ enhancer on the CD3-ϵ promoter and on heterologus promoters

Promoter	enhancer	Jurkat	HepG2	Raji	C8166
CD3-ϵ	–	6780	4010	1100	2350
	RSV	40 420	56 130	12 080	7190
	I-9	39 780	3420	910	1850
tk	–	1200	2080	880	3110
	I-9	9600	980	910	2780
	I-9c	8200	2040	ND	3550
pim-1	–	10 270	1830	1210	1610
	I-9(pos)	45 680	2420	1450	2050
	I-9(neg)	43 460	ND	1230	1850

Cells of different lineages were transfected with CAT constructs driven by the extended CD3-ϵ promoter (bp −28 to 100), by the tk promoter (Luckow and Schutz, 1987), or by the pim-1 promoter (pR3, Krimpenfort *et al.*, 1988). The indicated enhancers: I-9 is the CD3-ϵ enhancer taken from the deletion clone pI-9; I-9c is the shortest (125 bp) version of the CD3-ϵ enhancer taken from the deletion clone pI-9c. The CD3-ϵ enhancer was tested in both orientations on the pim-1 promoter [I-9(pos) and I-9(neg)]. The RSV-enhancer is a fragment from the RSV-LTR. All constructs were tested at least three times. The results (c.p.m. of butyrylated [^{14}C]chloramphenicol) of a representative experiment are shown. Qualitatively similar results to the activities measured in Jurkat were obtained for all other human (REX, HPB-ALL) and mouse (DO11.10, EL4, BW5147) CD3-ϵ$^+$ T cell lines. In addition to the observations made in the B cell line Raji and the hepatoma cell line HepG2, the CD3-ϵ enhancer was found to be inactive in the B cell line JY, in HeLa, and in an SV40-transformed human fibroblast cell line. C8166 is an HTLV-I transformed T cell line which does not express CD3-ϵ mRNA. As shown, the transfected CD3-ϵ enhancer was inactive in C8166, whereas the CD3-ϵ promoter functioned normally.

The CD3-ϵ enhancer is located in a hypomethylated CpG-island

Next, the sequence of the 3′ enhancer was determined. The sequence of region 'E3' located in the last exon has been

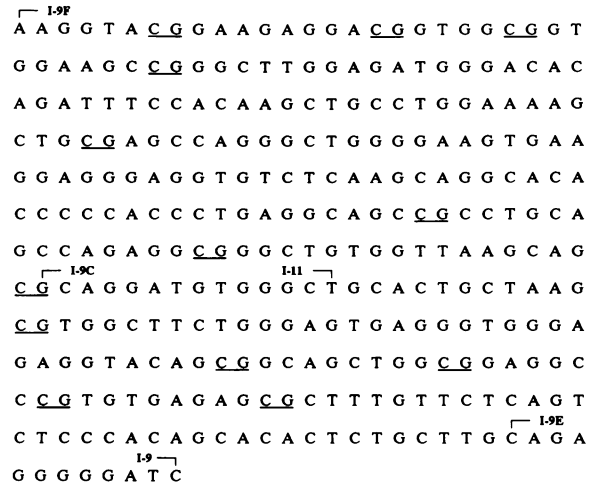

Fig. 6. Sequence of the domains 'E2' and 'E1' of the human CD3-ϵ enhancer. Boundaries of the 3′ deletion clones pI-9 (fully active) and pI-11 (inactive), and of the 5′ deletion clones pI-9F, pI-9C, and pI-9E are indicated by arrows. Domain 'E2' is defined as the region between the 5′ boundaries of pI-9F and pI-9C; domain 'E1' is defined by the sequence of pI-9C. The presented sequence consists of 64% C + Gs, and contains 13 CpG pairs (underlined).

published elsewhere (1157−1295 bp of the cDNA clone pDJ4 (Gold *et al.*, 1986). The functionally inactive stretch of DNA located between 'E3' and 'E2' was found to consist almost exclusively of G's and A's (positive strand). The sequence of 'E2' and 'E1' is given in Figure 6 and constituted a CpG-rich island, with 64% C+G's and 13 CpG pairs. No strong matches to consensus motifs of *cis*-acting elements (Wingender, 1988) were found. However, many *trans*-acting factors can interact functionally with degenerate versions of their optimal sites. Therefore, it remains possible that some of the transcription factors involved in CD3-ϵ enhancer function have already been studied in other genes.

In a number of reported instances demethylation of CpG pairs has been associated with active transcription in genes (reviewed in Bird, 1986). We therefore examined the state of methylation of the 3′ enhancer in cells that did, or did not, actively transcribe the CD3-ϵ gene. Three of the CpG pairs were located in recognition sites of restriction enzymes that are inhibited by methylation at C:*Hpa*II (one site) and *Hha*I (two sites). A restriction map relevant for this experiment is given in Figure 7A. The *Hpa*II site was not polymorphic, based on a control experiment with its methylation-insensitive isoschizomer *Mst*I (not shown). No such enzyme exists for *Hha*I. *Hpa*II and *Hha*I sites located downstream from the enhancer were considered irrelevant; their location and possible polymorphisms were not mapped.

No correlation existed between expression of the CD3-ϵ gene and methylation of its enhancer. DNA was prepared from two T cell lines [Jurkat (T1), Molt4 (T2)], four lymphoblastoid B cell lines [BL2, (B1), ER (B2), JY (B3) and Raji (B4)] and HepG2 (H). DNA was digested with *Kpn*I alone or by *Kpn*I−*Hpa*II or *Kpn*I−*Hha*I double digest. Southern blots were probed with the *Kpn*I−*Pst*I fragment indicated in Figure 7A. *Kpn*I digestion resulted in the expected 5.5 kb band. For the unmethylated *Hpa*II and *Hha*I sites, bands of ~1 kb were predicted in the double digests. As shown in Figure 7B, the 5′ most *Hha*I site was completely demethylated in three of the B cell lines and in the T cell line Molt4 (see lanes 2). In cell lines where the first site was partially methylated, the second *Hha*I site was demethylated (HepG2, Jurkat, Raji). Methylation of the *Hpa*II site occurred at a low level, and no specific differences between T cells and non T cells were detected (see Figure 7B, lanes 3).

The CD3-ϵ enhancer coincides with a DNase I hypersensitive site in T cells

Hypersensitivity of regulatory sequences to DNase I treatment of whole nuclei is thought to reflect the accessibility of such sequences for *trans*-acting factors (Stalder *et al.*, 1980; Wu, 1980; McGhee *et al.*, 1981; Parslow and Granner, 1982). Nuclei were prepared from Jurkat cells, from the B cell line Raji, and from HeLa cells. Samples of these nuclei were incubated with a range of DNase I concentrations. Subsequently, DNA was prepared, digested with *Eco*RI, and Southern blot analysis performed. As a probe, a fragment from the CD3-ϵ cDNA covering most of the last exon was used (see Figure 7A). *Eco*RI digestion resulted in the expected 5.5 kb band. With increasing amounts of DNase I a second band appeared of ~1.3 kb in the Jurkat DNA (see Figure 7C). This band was not observed in DNA from the B cell line Raji (Figure 7C), nor in HeLa (not shown). The location of this hypersensitive site in Jurkat (indicated in Figure 7A by a hatched bar) coincided with the 'E1' domain, indicating that in T cells the enhancer is indeed accessible for soluble factors.

The CD3-ϵ enhancer is inactive in two CD3-ϵ⁻ T cell lines

As evidence for the physiological relevance of the CD3-ϵ enhancer, it was investigated whether activity of transfected CD3-ϵ enhancer constructs correlated with active transcription of the endogenous CD3-ϵ gene in cells of T lymphocyte lineage. In an extensive screen for $CD3^-$ variants in our laboratory, two cell lines have been identified that do not express the CD3 genes; One cell line, 827-p19-1, was derived from a functional T cell clone by HTLV-I immortalization (Yssel *et al.*, 1989). 827-p19-1 is surface $TCR/CD3^-$, does not express CD3-γ, CD3-δ, CD3-ϵ and CD3-ζ, but does express TCR-α and -β. C8166 is a $CD4^+$ T cell line obtained by HTLV-I transformation of human peripheral blood lymphocytes. C8166 is surface $TCR/CD3^-$, and does not express CD3-δ and CD3-ϵ (see Materials and methods).

Transient transfection of CAT constructs revealed that the CD3-ϵ enhancer was inactive in both cell lines. In contrast, the CD3-ϵ promoter functioned normally. All constructs were tested at least three times in each line. Table I demonstrates the results of a representative experiment obtained with C8166. Essentially identical results were obtained with 827-p19-1. Thus, at least in the two cell lines tested, absence of endogenous CD3-ϵ mRNA correlated with inactivity of the transfected CD3-ϵ enhancer, but not of the CD3-ϵ promoter. Therefore, it was concluded that the CD3-ϵ gene was not transcribed in these two variant T cell lines due to a *trans*-acting phenomenon acting through the CD3-ϵ enhancer.

Discussion

The CD3-ϵ gene represents a critical element in the intrathymic differentiation pathway of cell committed to the

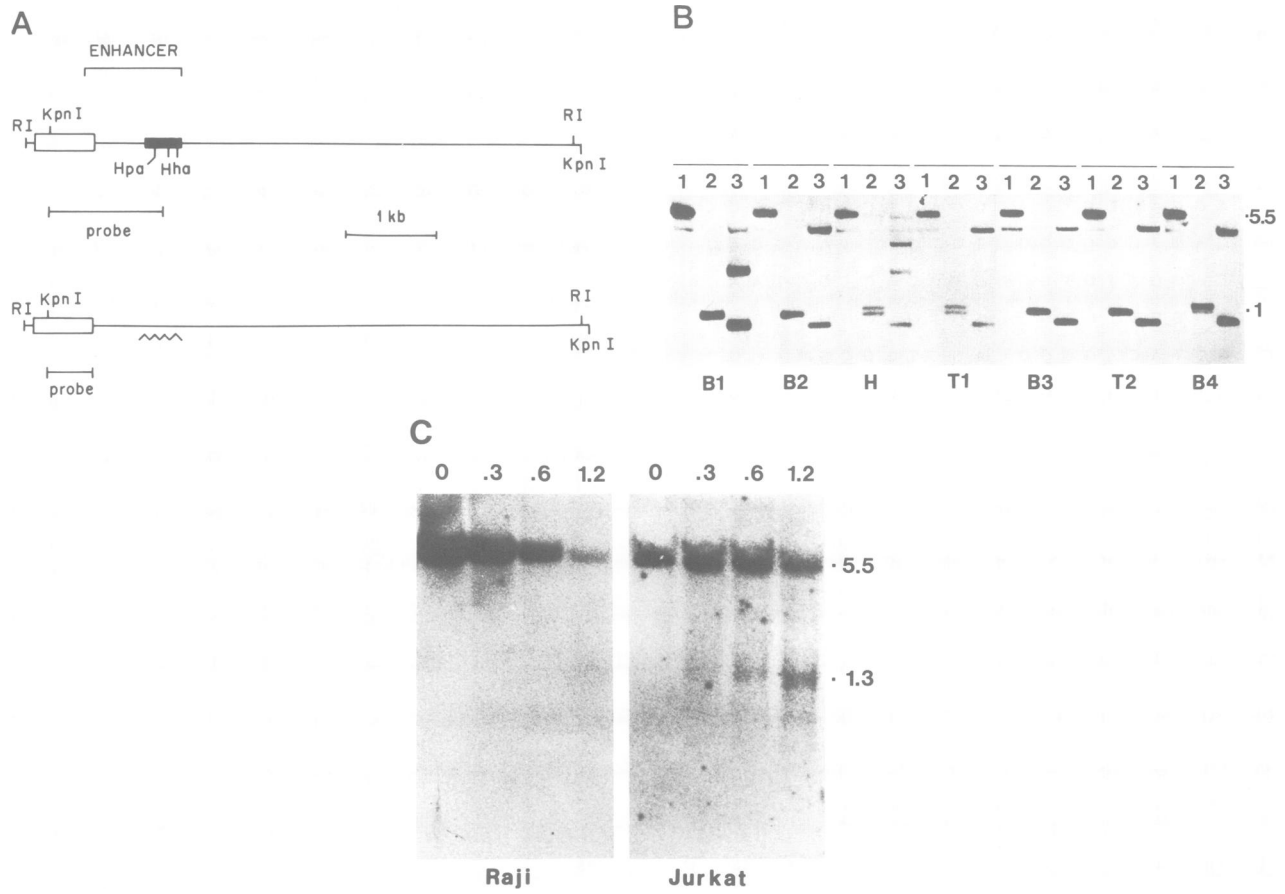

Fig. 7. Methylation of three restriction sites in the CD3-ϵ enhancer, and mapping of a T cell-specific DNase I hypersensitive site. **A**) Restriction maps relevant to the methylation experiment (upper map), and the DNase I hypersensitive experiment (lower map). Exon IX is an open box; a black box indicates the CPG-island. The hatched bar indicates the location of the DNase I hypersensitive site. Restriction enzymes used: *Eco*RI (RI), *Kpn*I, *Hpa*II (Hpa) and *Hha*I (Hha). The probes used in each experiment are indicated by a bar. **B**) CpG-methylation. DNA from four B cell lines [BL2 (B1), ER (B2), JY (B3) and Raji (B4), two T cell lines (Jurkat (T1), Molt4 (T2)] and HepG2 (H) was digested with *Kpn*I alone (**lanes 1**), *Kpn*I and *Hha*I (**lanes 2**), and *Kpn*I and *Hpa*II (**lanes 3**). Digestion with *Kpn*I alone gives the expected 5.5 kb band. From the double digests it can be concluded that the enhancer is only moderately methylated, and, more importantly, that levels of methylation do not correlate with CD3-ϵ expression (see text). **C**) DNase I hypersensitivity from Nuclei were prepared from Raji B cells and from Jurkat T cells, and treated with the indicated amounts of DNase I (0, .3, .6 or 1.2 units) for 10 min, after which DNA was prepared. Next a standard Southern blot was performed after *Eco*RI digestion. As shown, in addition to the expected 5.5 kb band, a second band of 1.3 kb appeared with increasing amounts of DNase I in Jurkat cells (right panel). The predicted hypersensitive site coincided with the 'E1' domain in the enhancer, and was observed in two separate batches of Jurkat nuclei. The site was not observed in Raji (left panel), nor in HeLa cells (not shown).

T lymphocyte lineage. Expression of the CD3 genes sets the stage for TCR gene rearrangements, and for subsequent expression of functional TCR/CD3 complexes at the surface of T lymphocytes.

The experiments presented in this paper address the characterization of sequences in and around the human CD3-ϵ gene that are involved in controlling its T lymphocyte-specific expression. When a 24 kb genomic fragment containing the CD3-ϵ gene was introduced into a mouse germ line, correct tissue-specific expression was observed. This experiment indicated that the *cis*-acting sequences that regulate CD3-ϵ expression were confined to the 24 kb genomic fragment. By transient DNA transfer it was subsequently shown that the promoter of CD3-ϵ was active in a variety of cell types, and could be driven by a heterologous enhancer. Inclusion of the first intron in the promoter constructs resulted in a 10-fold increase in the measured translation product, CAT. A T cell-specific enhancer, independent of orientation and distance, and capable of controlling its own as well as heterologous promoters, was found at the 3′ end of the gene. By deletion mapping the enhancer could be divided into at least three functional domains. The most 3′ 125 bp of the enhancer were indispensable for its activity, and by itself functioned as a 5 to 10-fold T cell-specific enhancer. Two domains located 5′ to this core each acted as a 2-fold 'amplifier'; they were by themselves inactive. Sequencing revealed the enhancer to be a CpG-island. A T cell-specific DNase I hypersensitive site coincided with the CD3-ϵ enhancer. In addition, two T cell lines were identified in which CD3-ϵ mRNA was undetectable, and in which the CD3-ϵ enhancer did not function.

Correct tissue-specific expression of a transgene is generally regarded as strong evidence for the presence of a complete set of regulatory elements in the introduced construct. In the present study a human CD3-ϵ mouse strain was generated carrying a relatively high copy number of the transgene. Currently, more transgenic mouse strains are being generated to determine the effect of gene dosage and of integration site on CD3-ϵ expression. We have recently obtained four independent transgenic mouse strains that carry a fragment of human DNA comprising the CD3-δ gene. The introduced CD3-δ fragment does not overlap with the CD3-ϵ

construct reported here (N.Lonberg and H.Clevers, unpublished). All four strains expressed the human CD3-δ gene in correct T cell-specific fashion. These observations indicate that the tightly linked CD3-ε and CD3-δ genes are not controlled by shared *cis*-acting elements; each of the two genes has its individual set of T cell-specific regulatory elements. This confirms an earlier report from our laboratory describing a T cell-specific enhancer in the murine CD3-δ gene (Georgopoulos *et al.*, 1988). The levels of human CD3-δ expression between the transgenic mouse strains varied widely, and expression per gene copy was much lower than that of the endogenous gene. It appears plausible, therefore that the overall level of expression of the 50 kb CD3 locus, comprising CD3-γ, CD3-δ and CD3-ε, is controlled by elements similar to the recently described DNase I supersensitive sequences in the β-globin locus. These sequences stimulate position-independent, high-level expression of β-globin genes that are otherwise expressed at low, albeit erythroid-specific levels in transgenic mice (Grosveld *et al.*, 1987). In order to define such locus-activating elements, the CD3-locus will need to be screened for T cell-specific DNase I supersensitive sites. Since a detailed restriction map has recently become available (Tunnacliffe *et al.*, 1988), such a study is certainly feasible at present.

The CD3-ε promoter as defined here functionally, does not contain a TATA-box, nor could any of the growing number of consensus sequences for transcription factors such as the CAAT-box or SP1 (Wingender, 1988) be identified (Clevers *et al.*, 1988b). It is possible that transcription factors that have already been characterized interact functionally with degenerate verions of their optimal motifs in the CD3-ε promoter. Future experiments involving specific protein−DNA interactions might reveal the existence of novel *trans*-acting factors involved in promoter function of the CD3-ε gene.

Here we report that the presence of intron I sequences in the CD3-ε promoter resulted in a 10-fold increase in measured promoter activity. Intron-dependent increase of mature mRNA or translation products has been observed in several other systems, both as a result of transcriptional and post-transcriptional mechanisms. In the c-*myc* gene the presence of a transcriptional activator is located at the boundary between exon I and intron I (Yang *et al.*, 1986). Similarly, enhancer-like elements have been reported in the first intron of several collagen genes (Bornstein *et al.*, 1988), and tissue-specific enhancers have been found further downstream of their respective promoters in introns of a number of genes, including the IgH chain gene, the Igκ light chain gene, and the TCR-α gene (Banerji *et al.*, 1983; Gillies *et al.*, 1983; Queen and Baltimore, 1983; Bergman *et al.*, 1984; Picard and Schaffner, 1984; Potter *et al.*, 1984; Luria *et al.*, 1987). On the other hand, a recent study of the effect of β-globin introns on expression of transfected gene hybrids reiterates the original proposal that introns can act at a post-transcriptional level to increase functional gene expression (Buchman and Berg, 1988). Our experiments indicate that the CD3-ε intron I does not contain enhancer elements. Furthermore, nuclear run-off experiments performed on transiently transfected T cells indicate that the presence of the intron does not affect the rate of transcription (H.Clevers and C.Terhorst, unpublished). Therefore it appears likely that the intron-dependent increase in CAT expression is the result of a post-transcriptional mechanism. However, the presence of a downstream promoter element in intron I has not been formally ruled out at present.

The deletion experiments performed on the CD3-ε enhancer gave some insight into its architecture. The core ('E1') of the enhancer was constituted by 125 bp located at its 3′ end. Deletion of the core rendered the remaining part functionally inactive. On the other hand, the core by itself behaved as a classical, though fairly weak, enhancer with a strict T cell-specificity. It is attractive to hypothesize that the tissue-specificity of the CD3-ε enhancer indeed resides completely in one, or a few, elements located in this dominant core. In this scheme, the more 5′ elements located in 'E2' and 'E3' need not be tissue-specific since they serve only to amplify the effect of the tissue-specific core. Such a situation is not unprecedented. A single site binding a tissue-specific transcription factor plays a dominant role both in the IgH and in the Igκ gene enhancer. In the IgH gene enhancer, the 'octamer' site is the only known site to bind a B cell-specific DNA binding protein (Atchison, 1988).In a truncated version of this enhancer, the octamer is indispensable for functional activity (Lenardo *et al.*, 1987). More clearly,the Igκ gene enhancer is critically dependent on NF-κB for its constitutive activity in B cells (Lenardo *et al.*, 1987).

The C+G content of bulk genomic DNA is ~40%. The CpG pair is particularly rare and occurs at roughly one-fifth of the expected frequency. Between 60 and 90% of CpG pairs are methylated on the cytosine ring. It is assumed that the rarity of CpG is the direct result of C-methylation, since methylated cytidine can be oxidized to thymidine. However, throughout the genome 500−2000 bp clusters of high (65%) C+G content exist, and in these CpG-islands the CpG pair occurs at normal frequency. Many polymerase II housekeeping genes and a number of tissue-specific genes have sequences with characteristics of CpG-islands surrounding their transcription start sites. These areas are generally undermethylated; and it has been suggested that undermethylation of genes and their CpG-islands correlates with active transcription (reviewed in Bird, 1986).

The T cell-specific CD-ε enhancer has all the characteristics of a CpG-island: 64% C+G's, many CpG pairs and low methylation grade. We are not aware of other tissue-specific enhancers with these characteristics. In contrast, the promoter area of the CD3-ε gene is low in C+G's and CpG pairs. Undermethylation of the enhancer was found in T as well as in non T cell lines, suggesting that methylation plays no role in the T cell-specific expression of the CD3-ε gene. Similarly, the CpG rich α-globin locus has been reported to be undermethylated both in cells that do, and that do not express the genes involved (van der Ploegh and Flavell, 1980).

Expression of the genes encoding the constituent chains of the TCR/CD3 complex is restricted to cells of T lymphocyte lineage (Furely *et al.*, 1986; van Dongen *et al.*, 1987; van Dongen *et al.* 1988). Recently, similar studies to the one presented here have been reported for the CD3-δ gene and the TCR-α and TCR-β genes. The CD3-δ gene, like the related CD3-ε gene, is controlled by a non-tissue-specific, non-TATA promoter, and by a T cell-specific transcriptional enhancer located 3′ to the last exon in a DNase I hypersensitive site present in T cells only (Georgopoulos *et al.*, 1988). Thus not only are the CD3-δ and CD3-ε genes similar in coding sequence and exon−

intron organization, but also in some characteristics of their regulatory sequences. Somewhat disappointingly however, sequence comparison of the enhancers of CD3-δ and CD3-ϵ revealed no significant similarities. In addition, the CD3-δ enhancer was not a CpG-island.

A rearranged TCR-α gene was controlled by a T cell-specific promoter, and by a lymphoid-specific enhancer located in the $J_\alpha - C_\alpha$ intron (Luria *et al.*, 1987). This situation is reminiscent of the organization of the rearranging Ig heavy chain and κ light chain gene (Banerji *et al.*, 1983; Gillies *et al.*, 1983; Queen and Baltimore, 1983; Bergman *et al.*, 1984; Picard and Schaffner, 1984; Potter *et al.*, 1984), and it is suggested that an enhancer placed near the C_α region will activate a V_α region promoter only after approximation through $V_\alpha J_\alpha$ joining. In two separate studies, a T cell-specific enhancer was defined 5–7.5 kb downstream from the $C_\beta 2$ region of the TCR-β gene (Krimpenfort *et al.*, 1988; McDougall *et al.*, 1988). The area containing this enhancer was reported to be required for T cell-specific expression in transgenic mice (Krimpenfort *et al.*, 1988). It is not known whether T cell-specific DNase I hypersensitive sites coincide with the TCR enhancers.

The CD3-ϵ enhancer, as defined in this paper, is inactive upon transient transfection into each of two CD3-ϵ^-, HTLV-I tranformed T cell lines. This observation implies that these two cell lines either lack an essential transcription factor or contain a *trans*-acting factor with a negative effect on the enhancer. This notion has several implications. First, it confirms the pivotal role of the enhancer in the control of endogenous CD3-ϵ transcription. Second, since both cell lines are also CD3-δ^-, the CD3-ϵ and -δ genes appear to be regulated by at least one shared transcription factor. Utilization of a transcription factor by individual *cis*-acting elements in both genes would elegantly explain the simultaneous appearance of CD3-δ and CD3-ϵ mRNA early in the thymic differentiation pathway. Third, since both cell lines are HTLV-I transformed, products of the retroviral genome might play a causative role in the specific downregulation of CD3 gene transcription. Fourth, the availability of these cell lines will provide valuable tools in the study of *trans*-acting factors that drive the CD3-ϵ enhancer.

In conclusion, the CD3-ϵ gene was found to be regulated by a non-tissue-specific promoter, and by a T cell-specific enhancer located 12 kb downstream. Within this enhancer, a small core was found to exhibit qualitative characteristics similar to those of the full-length enhancer. It is expected that the key to the T cell-specific expression of the CD3-ϵ gene is enclosed within this 125 bp sequence.

Materials and methods

Generation of transgenic mice

A 24 kb *Sma*I–*Sal*I fragment from the human CD3-ϵ gene (Clevers *et al.*, 1988b) cloned into pGEM-4 (Promega Biotec, Madison, WI), and termed pL12, was purified away from vector sequences by agarose gel electrophoresis and injected into (C57BL/6J × CBA/J)F2 embryos as described elsewhere (Hogan *et al.*, 1986).

Cells

T cell lines Jurkat, REX, HPB-ALL, DO11.10, BW5147, EL4, C8166, 827-p19-1 and the B cell lines Raji and JY were grown in RPMI1640 supplemented with 5% FCS and antibiotics. Jurkat, REX and HPB-ALL are human leukaemic T cells. DO11.10 (a kind gift form Dr. P.Marrack) is an ovalbumin-responsive murine T cell hybridoma. BW5147 and E14 are murine thymona cell lines. C8166 is an HTLV-I transformed CD4$^+$ T cell line which does not express CD3-ϵ and CD3-δ as assessed by Northern blot anlaysis (H.Clevers and C.Terhorst, unpublished). 827-p19-1 is an HTLV-I immortalized line derived from a tetanus-toxoid responsive T cell clone, which lost expression of all CD3 genes but still expresses TCRα and -β. Raji is a Burkitt lymphoma B lymphoblastoid cell line; JY is an EBV-transformed B lymphoblastoid cell line. HeLa cells, SV40-transformed human fibroblasts, and HepG2 human hepatoma cells were maintained in DME supplemented with 5% FCS and antibiotics.

Flow cytometry

Cells were prepared and stained with FITC-conjugated Leu-4 monoclonal antibody as described by the manufacturer (Becton-Dickinson, Mountain View, CA). FITC-conjugated Leu-3 (anti-human CD4) served as a negative control. Stained cells were analyzed on a FACScan flow cytometer (Becton-Dickinson).

Plasmid constructs

pL12 was constructed by fusing a 13 kb *Sal*I–*Xho*I fragment from bacteriophage ϵH1 (exons I–III) to an 11 kb *Xho*I–*Sal*I fragment from ϵH2 (exons IV–IX) into pGEM-4 (Clevers *et al.*, 1988b). All CAT vectors were derived from pCAT2 (CAT driven by the Herpes simplex tk promoter in pUC18) or pCAT3 (promoterless CAT in pUC18) (Luckow and Schutz, 1987), with the exception of pR3 (CAT driven by the pim-1 promoter with a unique *Bam*HI site downstream of CAT (Krimpenfort *et al.*, 1988). The short CD3-ϵ promoter is a *Pst*I–*Mst*II blunted fragment (−228 to +27), cloned upstream from CAT in pCAT3 in *Pst*I–*Bgl*II blunted. The extended CD3-ϵ promoter was created by cloning a *Pst*I–*Sfa*NI blunted fragment (−228 to +100) in the same fashion into pCAT3. Deletion of the intron from the extended promoter was obtained by replacing the *Mst*II–*Sfa*NI fragment (+27 to +100) by the relevant *Mst*II–*Sfa*NI fragment taken from the CD3-ϵ cDNA clone pDJ4 (Gold *et al.*, 1986). All promoter constructs were sequenced. The short CD3-ϵ promoter was extended to bp −650 by insertion of a *Pst*I–*Pst*I fragment (−650 to −228) in a unique *Pst*I site. For extension to the *Sac*I site at −1000, genomic *Sac*I fragment was first subcloned in pUC19 and the relevant fragment taken out by *Mst*II digest (site at +27), blunting, and subsequent *Hin*dIII digest. This leaves all pUC-polylinker sites from *Hin*dIII to *Sac*I upstream of the genomic fragment. The resulting fragment was subcloned in pCAT3, which was digested with *Bgl*II, blunted, and digested with *Hin*dIII. Further extension to −2400 (*Hin*dIII) was performed by cloning the relevant *Hin*dIII–*Sac*I fragment (−2400 to −1000) into the −1000 -extention plasmid which was digested with *Hin*dIII and partially digested with *Sac*I. Extension to −8000 was similarly performed by subcloning the relevant *Sal*I–*Sac*I fragment (−8000 to −1000) into the −100 extension plasmid, which was digested with *Sal*I and partially with *Sac*I. The RSV-enhancer (taken from RSV–CAT (Gorman *et al.*, 1982), 492 bp *Nde*I–*Eco*RI fragment, blunted) was cloned in positive orientation into the downstream *Sma*I site. Constructs from Figure 2b were made by cloning a 4 kb *Sal*I–*Xho*I fragment, a 6 kb *Xho*I–*Eco*RI fragment or a 5 kb *Eco*RI–*Sal*I fragment, all from ϵH2 and all blunted, in both orientations into the *Sma*I site of the short CD3-ϵ promoter construct. Three series of *Bal*31 enhancer deletions were made. One series was made in the 5′ direction, by opening the *Eco*RV site (Figure 5), taking several *Bal*31 deletion time points and subsequently digesting with *Hin*dIII. The CD3-ϵ promoter/CAT were the cloned into the pUC/deleted enhancer backbone as a *Hin*dIII–*Sma*I fragment taken from the short CD3-ϵ promoter construct. Two series were made in the 3′ direction by similar strategies, staring from the upper construct in Figure 5 or from pI-9 respectively. The *Kpn*I site was opened and deletions were performed, followed by a *Hin*dIII digest. Next, the CD3-ϵ promoter/CAT/deleted enhancer fragment was cloned into pUC18 digested with *Hin*dIII and *Sma*I. The enhancer deletions were tested on the extended CD3-ϵ promoter, or on the tk promoter, simply by exchanging promoters as *Hin*dIII–*Xho*I fragments. The complete enhancer from pI-9 was cloned in both orientations into the unique *Bam*HI site of pR3, as a blunted *Cla*I–*Eco*RI fragment with *Bam*HI–linkers.

CAT assays

Described in detail elsewhere (Seed and Sheen, 1988). In short, 5×10^6 cells were transfected with plasmid DNA, equimolar to 10 μg of pCAT2, for 60 min in 3 ml of RPMI with 50 μg DEAE–dextran/ml (non-adherent cells), or for 240 min at 250 μg DEAE–dextran/ml (adherent cells). 48 h later cells were harvested and freeze–thawed in 100 μl of 50 mM NaCl/10 mM Tris pH 7.4/1 mM EDTA. 50 μl of the centrifuged lysate was added to 125 μl of CAT reaction cocktail [^{14}C]chloramphenicol, 1 μCi/ml (60 mCi/mmol)/2.5% glycerol/250 mM Tris pH 7.5/3 mM butyryl-CoA), and incubated for 2 h at 37°C. Pristane/xylene (2:1)

extractable c.p.m. were determined by liquid scintillation counting. Initially, transfection efficiency was standardized by co-transfection with an RSV-LTR driven β-galactosidase construct. Since the transfection efficiencies of individual samples consistently differed by <15%, these co-transfections were discontinued. As evidence in Figure 5B, transfections of CAT constructs performed in triplicate routinely resulted in standard deviations of 5–15%.

Genomic methylation assay

DNA preparations, restriction enzyme digests and Southern blots were according to standard procedures (Ausubel *et al.*, 1988).

DNase I hypersensitivity assay

Log-phase cells (Jurkat, Raji, JY, HeLa) were washed in PBS and resuspended in 60 mM KCL, 15 mM NaCl, 1 mM EDTA, 0.15 mM spermine, 0.5 mM spermidine, 15 mM Tris pH 7.4, 250 mM sucrose, 0.25% NP-40. The suspension was layered over the same buffer without EDTA, containing 1.8 M sucrose and spun for 5 min at 1000 *g*. The nuclei were then resuspended in digestion buffer (60 mM KCl, 15 mM NaCl, 15 mM Tris pH 7.4, 0.5 mM DTT, 250 mM sucrose, 0.05 mM $CaCl_2$, 3.0 mM $MgCl_2$ and incubated with the indicated units of DNase I (Worthington) for 10 min at 37°C. Next DNA was prepared from the nuclei, and Southern blot analysis performed, according to standard procedures (Ausubel *et al.*, 1988).

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References

Atchison,M.L. (1988) *Annu. Rev. Cell Biol.*, **4**, 127–153.

Ausubel,F.M., Brent,R., Kingston,R.E., Moore,D.D., Smith,A.A., Seidman,J.G. and Struhl,K. (eds) (1988) *Current Protocols in Molecular Biology*. Wiley Interscience, Greene.

Banerji,T., Olson,L. and Schaffner,W. (1983) *Cell*, **33**, 729–740.

Bergman,Y., Rice,D., Grossschedl,R. and Baltimore,D. (1984) *Proc. Natl. Acad. Sci. USA*, **81**, 7041–7045.

Bird,A.P. (1986) *Nature*, **321**, 209–213.

Bornstein,P., McKay,J., Liska,D.J., Apone,S. and Devarayalu,S. (1988) *Mol. Cell. Biol.*, **8**, 4951–4857.

Buchman,A.R. and Berg,P. (1988) *Mol. Cell. Biol.*, **8**, 4395–4405.

Clevers,H., Alarcon,B., Wileman,T. and Terhorst,C. (1988a) *Annu. Rev. Immunol.*, **6**, 629–662.

Clevers,H.C., Dunlap,S., Wileman,T.E. and Terhorst,C. (1988b) *Proc. Natl. Acad. Scil. USA*, **85**, 8156–8160.

Clevers,D., Dunlap,S., Georgopoulos,K., Wileman,T. and Terhorst,C. (1988c) *Proc. Natl. Acad. Sci. USA*, **85**, 8623–8627.

Davis,M.M. and Bjorkman,P.J. (1988) *Nature*, **334**, 395–402.

Furley,A.J., Mizutami,S., Weilbaecher,K., Dhaliwal,H.S., Ford,A.M., Chan,L.C. Molgaard,H.V., Toyonaga,B., Mak,T., van den Elsen,P., Gold,D., Terhorst,C. and Greaves,M.F. (1986) *Cell*, **46**, 75–78.

Georgopoulos,K., van den Elsen,P., Bier,E., Maxam,A. and Terhorst,C. (1988) *EMBO J.*, **7**, 2401–2407.

Gillies,S.D., Morrison,S.L. Oi,V.T. and Tonegawa,S. (1983) *Cell*, **33**, 717–728.

Gold,D.P., van Dongen,J.J.M., Morton,C.M., Bruns,G.A.P., van den Elsen,P., Geurts van Kessel,A.H.M. and Terhorst,C. (1987) *Proc. Natl. Acad. Sci. USA*, **84**, 1664–1668.

Gold,D.P., Puck,J.M., Pettey,C.L., Cho,M., Colligan,J., Woody,J.N. and Terhorst,C. (1986) *Nature*, **321**, 431–434.

Gorman,C.M., Merlino,G.T., Willingham,M.C., Pastan,I. and Howard,B.H. (1982) *Proc. Natl. Acad. Sci. USA*, **79**, 6777–6781.

Grosveld,F., Blom van Assendelft,G., Greaves,D.R. and Kollias,G. (1987) *Cell*, **51**, 975–985.

Hirt,B. (1967) *J. Mol. Biol.*, **26**, 365–369.

Hogan,B., Constantini,F. and Lacy,E. (eds), (1986) *Manipulating the Mouse Embryo*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Krimpenfort,P., de Jong,R. Uematsu,Y., Dembic,Z., Ryser,S., von Boehmer,H., Steinmetz,M. and Berns,A. (1988) *EMBO J.*, **7**, 745–750.

Lenardo,M., Pierce,J.W. and Baltimore,D. (1987) *Science*, **236**, 1573–1577.

Luckow,B. and Schutz,G. (1987) *Nucleic Acids Res.*, **15**, 5490.

Luria,S. Gross,G., Horowitz,M. and Givol,D. (1987) *EMBO J.*, **6**, 3307–3312.

Maniatis,T., Goodbourn,S. and Fischer,J.A. (1987) *Science*, **236**, 1237–1245.

McDougall,S., Peterson,C.L. and Calame,K. (1988) *Science*, **241**, 205–208.

McGhee,J.D., Wood,W.I., Dolan,M., Engel,J.D. and Felsenfeld,G. (1981) *Cell*, **27**, 45–55.

Parslow,T.G. and Granner,D.K. (1982) *Nature*, **299**, 449–451.

Picard,D. and Schaffner,W. (1984) *Nature*, **307**, 80–82.

Potter,H., Weir,L. and Leder,P. (1984) *Proc., Natl. Acad. Sci. USA*, **81**, 7161–7165.

Ptashne,M. (1986) *Nature*, **322**, 697–701.

Queen,C. and Baltimore,D. (1983) *Cell*, **33**, 741–748.

Saito,H., Koyama,T., Georgopoulos,K., Clevers,H., Haser,W.G., LeBien,T., Tonegawa,S. and Terhorst,C. (1987) *Proc. Natl. Acad. Sci. USA*, **84**, 9131–9134.

Seed,B. and Sheen,J.-Y. (1988) *Gene*, **67**, 271–277.

Stalder,J., Groudine,M., Dodgson,J.B., Engel,J.D. and Weintraub,H. (1980) *Cell*, **19**, 973–980.

Toyonaga,B. and Mak,T. (1987) *Annu. Rev. Imunol.*, **5**, 585–620.

Tunnacliffe,A., Buluwela,L. and Rabbitts,T.H. (1987) *EMBO J.*, **6**, 2953–2957.

Tunnacliffe,A., Olsson,C., Buluwela,C. and Rabbits,T.H. (1988) *Eur. J. Immunol.*, **18**, 1639–1642.

van der Ploegh,L.H.T. and Flavell,R.A. (1980) *Cell*, **19**, 947–958.

van den Elsen,P., Georgopoulos,K., Shepley,B., Orkin,S. and Terhorst,C. (1986) *Proc. Natl. Acad. Sci. USA*, **83**, 2944–2948.

van Dongen,J.J.M., Quertermous,T., Bartram,.C.R., Gold,D.P., Wolvers-Tettero,I.L.M., Comans-Bitter,W.M., Hooykaas,H., Adriaansen,H.J., de Klein,A., Raghavachar,A., Ganser,A., Duby,A.D., Seidman,J.G., van den Elsen,P. and Terhorst,C. (1987) *J. Immunol.*, **138**, 1260–1269.

van Dongen,J.J.M., Krissansen,G.W., Wolvers-Tettero,I.L.M., Comans-Bitter,W.M., Adriaansen,H.J., Hooykaas,H., van Wering,E.R. and Terhorst,C. (1988) *Blood*, **71**, 603–612.

Weissman,A.M. Hou,D. Orloff,D.G., Modi,W.S., Seanez,H., O'Brien,S.J. and Klausner,R.D. (1988) *Proc. Natl. Acad. Sci. USA*, **85**, 9709–9713.

Wingender,E. (1988) *Nucleic Acids Res.*, **16**, 1879–1902.

Wu,C. (1980) *Nature*, **286**, 854–860.

Yang,J.-Q., Remmers,E. and Marcu,K.B. (1986) *EMBO J.*, **5**, 3553–3562.

Yssel,H., de Waal Malefyt,R., Duc Dodon,M., Blanchard,D., Gazzolo,L., de Vries,J.E. and Spits,H. (1989) *J. Immunol.*, (in press).

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