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Transcription of T cell receptor β-chain genes is controlled by a downstream regulatory element

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To characterize *cis*-acting elements controlling the expression of T cell receptor β-chains we generated a number of transgenic mouse lines harboring a rearranged T cell receptor β-chain with different extensions of 5′ and 3′ flanking sequences. Transcriptional analysis of transgenic mice carrying these clones showed that sequences located downstream of the polyadenylation signal of the Cβ2 region are indispensable for expression in transgenic mice. The sequences conferring enhancer activity in this fragment were further defined by transient CAT assays. Strong enhancer activity was found to reside in a 550 bp fragment located 5 kb downstream from Cβ2. The nucleotide sequence of this fragment revealed a number of oligonucleotide motifs characteristic for enhancer elements.

Key words: T cell receptor/transgenic mice/regulatory element/CAT assay

Introduction

T cell receptors (TCRs) recognize foreign antigens in the context of self major histocompatibility complex (MHC) molecules. They resemble immunoglobulins (Igs) in many respects. Molecular genetic analysis has demonstrated that T lymphocytes generate diversity in receptor molecules in much the same way as B cells (for review see Tonegawa, 1983; Kronenberg *et al.*, 1986). The genes encoding the α- and β-chains of the TCR are composed of segments which rearrange during T cell development thereby generating a T cell population with clonally distributed receptors.

Somatic rearrangement of TCR and Ig genes can occur by deletion of intervening DNA. It has been shown for the TCR α and β genes that the excision products can be detected as circular DNA (Okazaki *et al.*, 1987; Fujimoto and Yagamashi, 1987; for review see Alt and Yancopoulos, 1987). TCR and Ig chains consist of N-terminal variable (V) and C-terminal constant (C) regions. The variable region is encoded by three gene segments, called variable (V), diversity (D) and joining (J) gene segments, which exist in multiple copies in germline DNA. A functional variable region gene is formed by the apparently random fusion of one of each of the three gene segment families. Analogous to Ig gene segments, TCR gene segments are flanked by conserved nonamer and heptamer motifs involved in rearrangement events (Hood *et al.*, 1985; Kronenberg *et al.*, 1986). The presumption that T and B cells use a common mechanism for the assembly of TCR and Ig genes, respectively, is strongly supported by studies which showed that TCR gene segments transfected into B cells are rearranged in the same way as transfected Ig gene segments (Yancopoulos *et al.*, 1986).

Expression of Ig genes is controlled by various regulatory elements. An enhancer element shown to be essential for Ig gene expression is located within the intron in front of the Cμ and Cκ genes (Banerji *et al.*, 1983; Gillies *et al.*, 1983; Queen and Baltimore, 1983; Picard and Schaffner, 1984). This region is retained in all rearranged heavy and κ light chain genes.

Elements regulating the expression of human TCR α genes have recently been identified in front of the C gene at a similar location as in Ig genes (Luria *et al.*, 1987). However, initial studies with TCR β-chain genes suggested that some regulatory elements might be positioned differently (McDougall and Calame, 1987). Using transgenic mice with a rearranged β-chain gene we show that a region 3′ of the polyadenylation signal of Cβ2 is essential for TCR β-chain expression. A strong enhancer element was identified in this region by transient CAT assays. Nucleotide sequencing of this region revealed the presence of a number of characteristic enhancer motifs.

Results

DNA fragments used for transgenesis

The strategy to localize regulatory elements necessary for the expression of TCR β-chain genes was based on the production of transgenic mice harboring a rearranged TCR β-chain gene with different extensions of 5′ and 3′ flanking sequences. The cytotoxic T cell clone, B6.2.16, served as a source for a functionally rearranged TCR β-chain gene. From this clone cDNA and genomic libraries were made in λgt11 and EMBL3 vectors respectively. Restriction map and DNA sequence analysis indicated that two genomic clones (clones 8 and 9) contained a functional rearranged V gene composed of the Vβ5.1 leader, Vβ8.2, Dβ2, Jβ2.3 and the Cβ2 gene segments. A more detailed description of these clones is presented elsewhere (Uematsu *et al.*, 1988). In clone 8 the β gene has 2 kb 5′ and 2 kb 3′ flanking sequences, while clone 9 carries 9 kb 5′ flanking sequences. As these clones were inactive in transgenic mice (see below), a fusion construct was prepared containing a larger extension at the 3′ end. Therefore, the unique *Sac*II site in the Jβ2 cluster was used to fuse clone 9 to a cosmid clone derived from BALB/c liver DNA containing the Cβ2 and downstream region. The insert of the resulting clone, cos HYβ9-1.14-5, harbors a functionally rearranged TCR β-chain gene flanked by 9 kb of upstream and 18 kb of downstream sequences (Figure 1) (note that in this construct the

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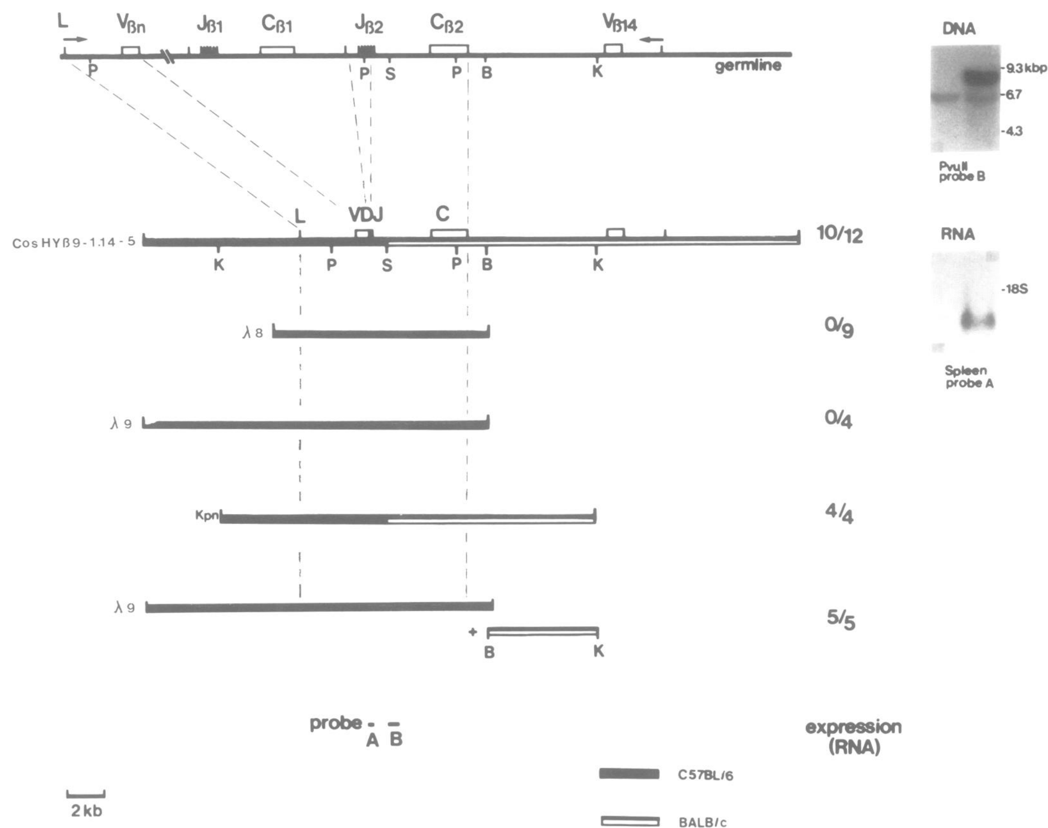

Fig. 1. The TCR β gene fragments used to generate transgenic mice. The upper line shows the germline organization of the TCR β gene locus. Lines below represent the gene fragments that were injected to produce transgenic mice. Fragment 1: the 36 kb insert of cosmid clone cos HYβ9-1.14-5; using the unique *Sac*II site in the Jβ2 region the insert of λ clone 9 (pTCF-9-D) was fused to the insert of (BALB/c liver derived) cosmid clone cosH-2^{d}II 1.14T harboring the Cβ2 and downstream regions (Uematsu *et al.*, 1988). Fragment 2: the insert of clone 8 (TS6.2.16). Fragment 3: the insert of clone 9 (pTCF-9-D). Fragment 4: *Kpn*I fragment of cos HYβ9-1.14-5. Fragments 5: an equimolar mixture of the insert of clone 9 and the 5.5 kb *Bam*HI−*Kpn*I fragment. The following restriction sites are indicated: *Pvu*II (P), *Kpn*I (K), *Sac*II (S) and *Bam*HI (B) (only the relevant *Bam*HI site is indicated). Probes used in this study: probe A: a 30mer specific for the VDJ joining region of the transgenes; probe B: a *Hinc*II−*Eco*RI fragment located downstream from the Jβ2 region. On the right typical examples of DNA and RNA analysis are shown. For Southern blot analysis tail DNA was digested with *Pvu*II and hybridized with probe B. For Northern blot analysis total splenic RNA was hybridized with probe A. For further details see Materials and methods.

Cβ2 region is of BALB/c origin while the Vβ region is of C57BL/6 origin). The *Kpn*I fragment depicted in Figure 1 was derived from cos HYβ9-1.14-5 and contains 5 kb of flanking sequences at the 5′ and 7 kb flanking sequences at the 3′ end.

Using the large insert of cos HYβ9-1.14-5 12 transgenic mice were obtained, nine transgenic mice were produced with the 13 kb insert of clone 8 whereas four transgenic mice were obtained for each of the two other fragments (the 20 kb insert of clone 9 and the 20 kb *Kpn*I fragment of cos HYβ9-1.14-5). The transgenic mice contained 1−50 copies of the transgene, mostly integrated in head-to-tail concatemers (data not shown).

Transcriptional activity of the TCR β transgenes

To determine whether the transgenic TCR β gene was transcribed, Northern blot analysis was performed on RNA isolated from the spleens of founder mice. A ^{32}P end-labeled 30mer covering the VDJ join and therefore specific for the transgene was used as probe. Ten out of 12 transgenic mice carrying the insert of cos HYβ9-1.14-5 showed a high, although variable level, of mature transgenic mRNA (1.3 kb) in splenic lymphocytes. A similar high level of transgene transcripts was observed in splenocytes from all mice that harbored the 20 kb *Kpn*I fragment from cos HYβ9-1.14-5. No transgene-specific mRNA could be detected in splenocytes from any of the transgenic mice containing either the 13 kb insert of clone 8 or the 20 kb *Sal*I insert of clone 9. These results strongly suggest that, within the 7 kb of 3′ flanking DNA sequences in the 20 kb *Kpn*I fragment from the cosmid clone, regulatory elements are present which are indispensable for transcription of the transgenic β-chain gene.

The transgenic TCR β gene is expressed in a tissue-specific manner

Transgenic mice carrying either the complete insert from cos HYβ9-1.14-5 or the 20 kb *Kpn*I fragment contained high levels of HYβ9-specific mRNA in their spleens. To determine the tissue-specific expression of the different transgenes, Northern blot analysis was performed with RNA obtained from tissues of offspring of mice heterozygous for the different transgenes (Figure 2). The results show that both the large cosmid clone and the 20 kb *Kpn*I fragment are expressed at high levels in T lymphocytes. To determine whether the TCR β transgene was also expressed in B cells, splenic B and T cells were stimulated for 4 days with LPS and ConA respectively, RNA was isolated and analyzed by Northern blot analysis. The expression pattern varied among the different transgenic strains (results not shown). The most

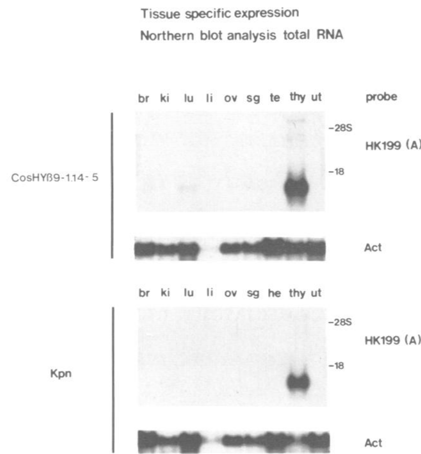

Fig. 2. Tissue specific expression of transgenic TCR β genes. Total RNA was isolated from tissues, electrophoresed on agarose formaldehyde gels and transferred to nitrocellulose filters; filters were hybridized to the ^{32}P end-labeled 30mer (probe A) and to a probe specific for actin (Act) as a measure for the quantity and integrity of the RNA. The upper panel shows a Northern blot of RNA isolated from tissues of a transgenic mouse line containing the insert of cos HYβ9-1.14-5, in the lower panel a Northern blot with RNA from tissues of a transgenic mouse line harboring the *Kpn*I fragment of cos HYβ9-1.14-5. Abbreviations: br, brain; ki, kidney; lu, lung; li, liver; ov, ovary; sg, salivary gland; te, testes; thy, thymus; ut, uterus.

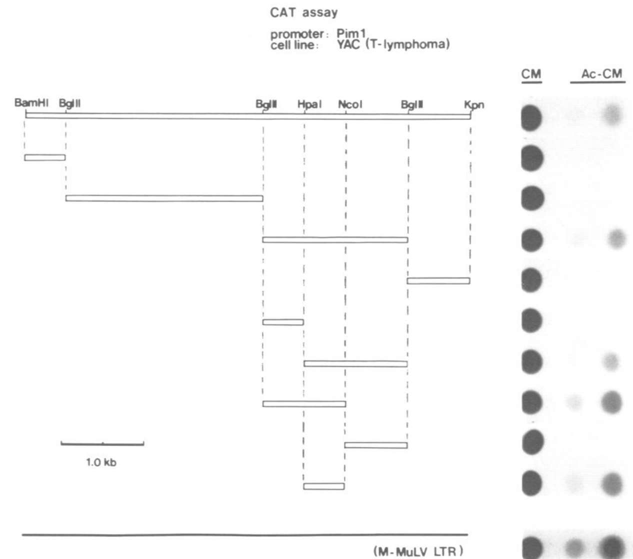

Fig. 3. Activation of *pim*-1/CAT fusion genes by fragments located downstream from the TCR β transcription unit. YAC-1 cells were transfected with 30 μg of plasmid DNA and cultured for 42 h. Protein extracts were prepared from the cells and analyzed for CAT activity. The amount of protein used in the reaction was the same for all samples. The upper line represents the 5.5 kb *Bam*HI–*Kpn*I fragment, which is located downstream from the TCR β gene. The relevant restriction endonuclease sites are indicated. The fragments used to further delineate the enhancer element are depicted below.

pronounced expression was generally found in T cells. The expression in B cells varied from very low levels to levels comparable with the levels seen in T cells. In one of the strains analyzed also some aberrant expression was observed in non-lymphoid tissues. Apparently the site of integration or the structural organization of the transgenic copies affects the expression patterns. No difference was found between the transcription patterns of cosmid HYβ9-1.14-5 and the smaller 20 kb *Kpn*I fragment (data not shown). Therefore, the transgenic TCR β gene carrying 5 kb of 5′ and 7 kb of 3′ flanking sequences is expressed in a lymphoid specific manner with a preference for T cells.

The 5.5 kb 3′ BamHI–KpnI fragment can restore transcriptional activity of inactive clones

The results described above strongly suggest that a regulatory element located between 2 and 7 kb downstream of the Cβ2 gene is essential for the expression of the TCR β gene in transgenic mice (Figure 1). However, in the constructs which showed transcriptional activity, both the Cβ2 and 3′ flanking sequences were of BALB/c origin. Therefore, one could argue that the smaller C57BL/6-derived clones were inactive because of a defect (e.g. cloning artefact) in the region replaced by BALB/c sequences in the cosmid. To check this possibility transgenic mice were raised harboring a mixture (molar ratio 1:1) of the 20 kb *Sal*I fragment of clone 9 and the 5.5 kb *Bam*HI–*Kpn*I fragment located downstream from Cβ2 (Figure 1). Coinjected DNA fragments usually integrate at the same chromosomal location in host cell DNA. The arrangement of the cointegrate is mostly complex with concatemeric regions of each fragment (Folger *et al.*, 1985). Five transgenic mice containing both fragments within the same chromosomal locus were obtained (Figure 1). All these mice expressed the transgene in the spleen at a significant level. These results indicate that the C57BL/6-derived clones lack an essential *cis*-acting sequence, which is present in the 5.5 kb *Bam*HI–*Kpn*I fragment.

The 3′ region contains an enhancer which can activate heterologous promoters

To identify the sequences within the 5.5 kb *Bam*HI–*Kpn*I fragment conferring enhancer activity, various subfragments were tested for their capacity to stimulate transcription from heterologous promoters in a transient CAT assay. We used the pR3 plasmid containing an enhancer-dependent hybrid gene consisting of the *pim*-1 promoter fused to the CAT gene. The YAC T cell lymphoma cell line served as recipient. The *pim*-1 promoter was chosen because of its low background responsiveness to various enhancers in lymphoid cell lines (M.Van Lohuizen and A.Berns, unpublished results). The unique *Bam*HI site downstream of the *pim*-1/CAT hybrid gene was used as an 'enhancer insertion site'. A physical map of the 5.5 kb *Bam*HI–*Kpn*I fragment with the relevant restriction endonuclease cleavage sites is given in the upper line of Figure 3.

The 20 kb *Kpn*I fragment was subcloned into pUC18. Taking advantage of the restriction endonuclease sites in the polylinker of pUC18, the 5.5 kb 3′ region could now be isolated as a *Bam*HI fragment and inserted directly into pPR3. In either orientation this 5.5 kb *Bam*HI fragment was capable of inducing CAT activity at low levels (Figure 3). This suggests that the *pim*-1 promoter responds to an enhancer-like element located in the 5.5 kb downstream region. Subsequently, a series of smaller fragments from this region were inserted—in both orientations—into the *Bam*HI site of pR3. The location of the fragments and their enhancer activity is shown in Figure 3. The highest activity was observed with the *Bgl*II–*Nco*I fragment, whereas the overlapping *Hpa*I–*Bgl*II fragment was somewhat less active. The most relevant enhancer sequences appeared to be contained within the overlapping 550 bp *Hpa*I–*Nco*I fragment (Figure 3). The latter fragment showed a similar high activity both

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001  AGATCTCAGC TCGATATGGA GCACATGTTC TCCAAACTCT GCCTGGACTC CAGGATCTGC TAAAACTCAG AGAATCAGTT CTTCCCCCTC CTCTCTCCCC
101  AGCTGCAGAA TGCATTTACC ATCTCCAGCT TTCCAGATCC CTTCAATTCC TGTGAACCAA GGTGCTCCAT AAGGAGCCTC AGACCCTAAC TTAGTGGTTT
201  GTTGATGAAA ATTCTGCAGG CCTTGTAGCA CTGACAGAAT GGTAGTGGGG AAATCTAAGT CCTTCCCAGC TCCAGACACA CACATTGTGA TATATCTTAC
301  AAGGCATCTG ACCTGCAAAG GGACAGTGCT GATCTTGAGT CCCATAGTTA ACCAGGCACA GTAGGACCCT TAATACTTGT TTCCCTGACC GATTCCAGCA
401  AAGAGATATA GGAACATGAT AAAAAAAATA ATCTTAAGAA GAACTGTTTT TCATCAAACA TCAGCAAGTG AAGAGATGCA TTCCTGGGAC TTTTCGGTTC
501  CTGAAGACAA TGGGGGAAGG GGTGGAAGCA TCTCACCCCA GGTCTGGGTG TTTATCTGTA AGTAACATCA GCACCAAGTA AGAATGGCCA CCTGCCATAG
601  CTCCATCTCC AGGAGTCACA ACAGGATGTG GTTTGACATT TACCAGGTCC TACATCTGGG GTGCCTGTGA ATGCTCCCCC ACTCACTCAC ATTCTGAGCA
701  TTTTGGGAAC CACACTTGCC ACATCCTGTC TTCAAACCCT TCTCATGCAG CCCTTTCTAC CTCAGCCTCT AGCTATCACC GTGACAGTGA GAACAACCAA
801  GAGGAGGGTC TCTAAAGGGG AACCCATATA TCTCATCTCA AGCCCCCCAT CTAAACTATC TTACATCTGT TAGAAGTCAC ACAGGAAACA GAAGCCTTCA
901  GTATGCACCA TGG
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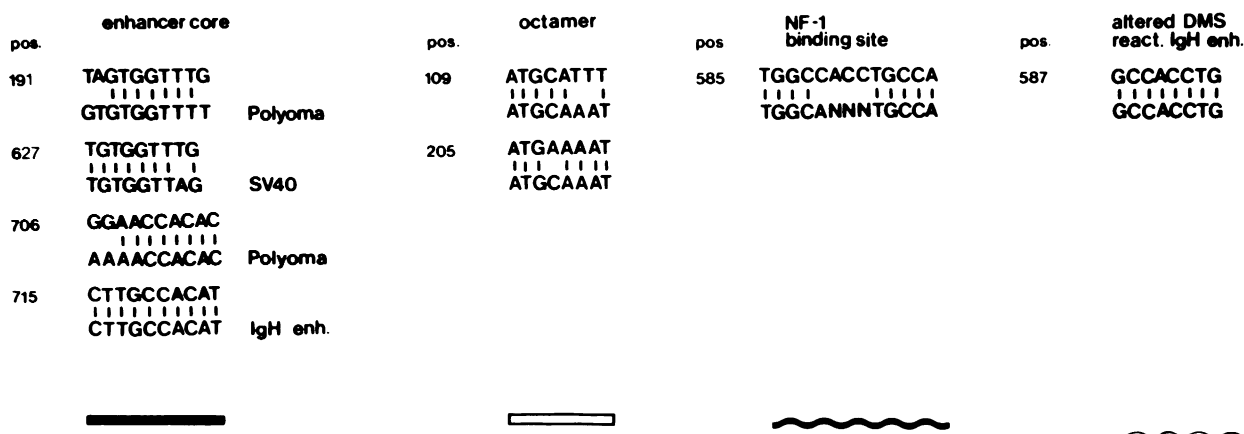

Fig. 4. Sequence of the 913 bp *Bgl*II–*Nco*I fragment. The nucleotide stretches that show significant homology with known enhancer motifs are marked and presented below with their position (pos.) next to the most homologous enhancer motif.

in T and B cells, whereas no activity was observed in NIH-3T3 fibroblast (data not shown). These experiments were repeated with the δMoCAT construct in which various fragments were inserted in the *Xba*I site in front of the enhancerless M-MuLV LTR promoter (Linney *et al.*, 1984). The results of the CAT assays with the δMoCAT plasmids were essentially the same as with the pR3 construct, although transcription from the M-MuLV promoter was activated to a lower extent (data not shown).

Multiple enhancer motifs are present downstream of Cβ2

Figure 4 shows the nucleotide sequence of the 913 bp *Bgl*II–*Nco*I fragment. This fragment contains a number of typical enhancer consensus motifs. Four stretches show significant homology to the enhancer core sequence of SV40 (Schirm *et al.*, 1987). An exact copy of the Ig heavy chain enhancer core is present at position 715 immediately preceded by a motif that matches the polyoma virus enhancer core sequence at eight of 10 positions. The SV40 core sequence itself is found 88 bp further upstream. Another sequence, which closely resembles the polyoma virus enhancer, is located at position 194. Nearby (at positions 109 and 205) two motifs are found which are homologous to the lymphoid specific octamer sequence ATGCAAAT, present in the promoters of Ig heavy and light chain genes and in the IgH enhancer (Banerji *et al.*, 1983; Falkner and Zachau, 1984; Parslow *et al.*, 1984). A slightly modified version of this sequence is also found in the promoter of the TCR β-chain gene (Royer and Reinherz, 1987). At position 585 a nuclear factor-1 (NF-1) binding site is present. This site consists of the inverted repeat TGGCA, with a 3 nt spacer (Nowock and Sippel, 1982; Rawlins *et al.*, 1984). Within the NF-1 binding site, an 8 nt stretch (CAGGTGGC) is found, which is identical to a motif in the Ig enhancer, which is protected from chemical modification by dimethylsulphate in B lymphocytes (Gimble and Max, 1987).

Discussion

In this paper we show that all the regulatory elements required for lymphoid specific expression of a transgenic TCR β gene are present in a 20 kb *Kpn*I fragment that contains 5 kb of 5′ and 7 kb of 3′ flanking sequences. An enhancer element essential for the expression of the TCR β-chain gene was localized to a 5.5 kb fragment downstream of the polyadenylation signal. High expression levels were observed in T cells. A variable, although generally much lower, expression was observed in B cells, while other cells showed a very low expression in only some transgenic lines. Lymphoid specificity was also observed in a transient CAT assay

which was used to map the enhancer to a 550 bp fragment downstream of the Cβ2 gene segment. Both in the YAC T lymphoma cell line and in the 2M3 pre-B cell line the TCR enhancer can activate the *pim*-1/CAT fusion gene to comparable levels as the M-MuLV LTR enhancer. Sequence analysis showed the presence of typical enhancer motifs. Close resemblance can be observed with the enhancer motifs found in Ig genes. The absence of strict T cell specificity in transgenic mice is reminiscent of the expression behavior of Ig heavy chain genes in transgenic mice, which show significant expression in T cells (Grosschedl *et al.*, 1984).

In the transient CAT assay the enhancer element is as active in T cells as in B cells. This seems somewhat in conflict with the predominant T cell specificity of the TCR β gene. However, one has to realize that the enhancer is only one of the elements conferring tissue specificity. Other elements within the TCR β gene could also contribute to the tissue specificity (Royer and Reinherz, 1987; Luria *et al.*, 1987), as has been shown for the Ig genes (Grosschedl and Baltimore, 1985). Furthermore, for a number of enhancer regions it has been shown that tissue specificity can be imposed by flanking silencer or dehancer elements, which can restrict the activity of the enhancer to distinct cell types (Laimins *et al.*, 1986; Nir *et al.*, 1986; Laimins *et al.*, 1986; Baniahmad *et al.*, 1987; Imler *et al.*, 1987). Such a mechanism could well be operational for TCR β genes. Indeed, a strongly reduced activity is found with larger fragments containing the enhancer in B cells as compared with T cells (data not shown). Alternatively one could argue that TCR-specific transcription is mediated by the rearranged V gene segment. Translocation of a TCR promoter into the proximity of the TCR enhancer might provide the driving force for preferential transcription in T cells, as was also suggested for the B cell specific transcription of Ig genes (Alt *et al.*, 1985).

Most enhancers are found upstream of transcriptional units. A few enhancers have been identified within introns and a few downstream of transcriptional units. Examples of genes with enhancers within introns are the α2 collagen gene (Rossi and De Crombrugghe, 1987) and Ig genes (Gillies *et al.*, 1983; Banerji *et al.*, 1983; Picard and Schaffner, 1984), whereas downstream enhancers are found in β-globin (Bodine and Ley, 1987), γ-globin (Kollias *et al.*, 1987) and Histon H5 genes (Trainor *et al.*, 1987). Especially for genes which undergo diverse somatic rearrangements in their 5′ regions, like Ig genes and TCR genes, the positioning of an enhancer into their 3′ regions makes sense. For Ig heavy and κ light chain genes the enhancer is found in the intron between the J and C gene segments in a region which is not deleted during V−D−J joining or heavy chain switching. For TCR β genes one would predict that, if a single enhancer element is used, it should be located either within the intron between Jβ2 and Cβ2, or downstream of Cβ2. Our experiments identify an important enhancer element downstream of Cβ2. Apparently also Ig κ genes contain an important downstream regulatory element which is required for their expression in transgenic mice (Storb *et al.*, 1986). Analysis of human and mouse TCR α genes (Luria *et al.*, 1987; our own preliminary data) suggest that they contain an enhancer in the intron between Jα and Cα.

The TCR β gene can be distinguished from other genes with downstream enhancers by one important aspect: distance of the enhancer to the promoter. The minimal distance between enhancer and promoter is 18 kb. Preliminary data indicate that the region between Cβ1 and Cβ2 does not contain an enhancer (McDougall and Calame, 1987), suggesting that TCR genes which use the Cβ1 region also are dependent on the same enhancer in this case located at least 27 kb downstream of the promoter. We are currently testing whether transcription of a β1 rearranged TCR gene is indeed dependent on the same enhancer element, identified in this study.

Materials and methods

Generation of transgenic mice

The DNA fragments that were used for injection were released from the vectors with the appropriate restriction endonucleases and purified by agarose gel electrophoresis and electroelution. The final DNA concentration was adjusted to 4 μg/ml. Fertilized mouse eggs were recovered in cumulus from the oviducts of superovulated (CBA/BrA × C57BL/LiA) F_1 females that had mated with F_1 males several hours earlier. The DNA fragments were injected into the most accessible pronucleus of each fertilized egg essentially as described (Hogan *et al.*, 1986). After overnight culturing two-cell-stage embryos were implanted into the oviducts of 1 day pseudopregnant F_1 fosters and carried to term. Several weeks after the birth of the animals that had developed from microinjected eggs, total genomic DNA was prepared from tail biopsies as described (Hogan *et al.*, 1986).

DNA analysis

For Southern blot analysis, 8 μg of total genomic DNA of each mouse was digested with restriction enzymes as recommended by the supplier, separated on agarose gels and transferred to nitrocellulose. Filters were hybridized to ^{32}P-labeled probes and washed as described (Cuypers *et al.*, 1984). Final wash was at 0.1 × SSC, 42°C. Probes used for DNA analysis: an *Eco*RI−*Hinc*III fragment located downstream from the Jβ2 region (probe B). This probe recognizes a 6.0 kb *Pvu*II fragment in germline DNA, whereas a 8.5 kb fragment is diagnostic for the transgene. A 913 bp long *Blg*II−*Nco*I fragment (see Figure 3 for location) was used to show the presence of the TCR enhancer region in the transgenic mice.

RNA analysis

5−15 μg of total RNA, prepared by the LiCl−urea method (Auffray and Rougeon, 1980) was separated on 1% agarose formaldehyde gels (Maniatis *et al.*, 1982) and transferred to nitrocellulose. Probes used for RNA analysis: probes for Thy1 (courtesy of Dr R.Evans), Ig light chain, pHBCk (Lewis *et al.*, 1982) and actin (Dodemont *et al.*, 1982); these probes were ^{32}P-labeled by nick translation; hybridization conditions were as described (Cuypers *et al.*, 1984), final wash was 0.1 × SSC, 60°C. For analysis of transgenic β-chain transcription a 30mer oligonucleotide (probe A) (Uematsu *et al.*, 1988), specific for the VDJ join of the transgene was used. The oligonucleotide was end labeled with ^{32}P as described (Maniatis *et al.*, 1982); hybridization conditions were as follows: 20% formamide, 5 × SSC, 5 × Denhardt's, 50 mM sodium phosphate, pH 6.8, 0.1% SDS, 10 mM pyrophosphate, 0.1 mM ATP, 50 μg/ml yeast RNA, 50 μg/ml poly(A) at 42°C overnight. Final wash was 30 min in 3 × SSC, 0.1% SDS at 55°C.

Stimulation of B and T cells

Cell suspensions isolated from spleens were grown in Iskove medium supplemented with 10% fetal calf serum (FCS), 2 mM L-glutamin, 100 U/ml streptomycin and penicillin and 20 μM β-mercaptoethanol. B cell growth was stimulated by the addition of lipopolysaccharide added to a final concentration of 2.5 μg/ml and T cells were stimulated by ConA (4 μg/ml). After 96 h of culture at 37°C, B and T cell blasts were isolated by lymphocyte gradient centrifugation.

CAT constructs

The following plasmids were used for the transient CAT assay experiments. pR3: the *Hind*III−*Bam*HI fragment obtained from pSV2CAT harboring the CAT encoding sequence was subcloned in pUC18. Via a *Hind*III linker the promoter of the *pim*-1 gene (Selten *et al.*, 1986), located on a 1720 bp *Sac*I−*Sac*II fragment, was fused to the 5′ of the CAT encoding sequence The unique *Bam*HI site 3′ of the CAT encoding domain was used as insertion site for the various fragments. *Bgl*II fragments were inserted directly into this *Bam*HI site, whereas *Bam*HI linkers were used for the insertion of fragments with non-complementary restriction sites. pR5: essentially the

same construct as pR3, but the enhancer from the M-MuLV LTR isolated from MoCAT as a 490 bp *Hin*dIII−*Xba*I fragment, was inserted into the *Pvu*II site of the pUC vector sequence in front of the *pim*-1 promoter; this construct was used as a positive control. MoCAT: in this construct the 5′ end of the CAT fragment was fused to the *Sma*I site, 30 bp downstream from the cap site in the M-MoLV LTR via a *Hin*dIII linker. δMoCAT: essentially as MoCAT but the enhancer sequences located at −340 to −180 bp in the U3 region of the LTR have been deleted (Linney *et al.*, 1984). As insertion site the *Xba*I site at −150 bp from the cap site was used. This construct was obtained from Dr Linney. Transfection plasmid DNA was prepared as described (Maniatis *et al.*, 1982).

Transfection of tissue culture cells and CAT assays

YAC-1 and 2M3 cells were grown in Iskove medium, supplemented with 10% FCS, 2 mM L-glutamin, 100 U/ml streptomycin and penicillin and 20 μM β-mercaptoethanol. NIH-3T3 cells were grown in DMEM supplemented with 10% FCS, 2 mM L-glutamin, 100 U/ml streptomycin and penicillin. For transfection of YAC-1 or 2M3 cells the DEAE−dextran procedure described by Gorman *et al.* (1982) was used with minor modifications. 10×10^6 cells were washed once with serum-free medium and twice with Tris-buffered saline (TBS: 25 mM Tris−HCl, pH 7.4, 137 mM NaCl, 5 mM KCl, 0.7 mM $CaCl_2$, 0.5 mM $MgCl_2$, 0.6 mM Na_2HPO_4). Cells were resuspended in 0.5 ml DNA/DEAE−dextran solution containing 250 μl TBS including 30 μg plasmid DNA and 250 μl DEAE−dextran (5 mg/ml), followed by 30 min incubation at room temperature. After incubation the cells were washed twice with TBS and twice with medium containing 5% FCS subsequently cultured in medium containing 10% FCS for 42 h. NIH-3T3 cells were transfected using the DNA−$CaPO_4$ precipitation method (Graham and van der Eb, 1972). Cells (0.6×10^6) were plated on a 9-cm culture dish in 5 ml DMEM/10% FCS. At 24 h after plating, 30 μg of plasmid DNA was transfected, and the DNA−$CaPO_4$-containing medium was replaced by fresh medium 7 h later. At 24 h after addition of the DNA−$CaPO_4$ precipitate cells were trypsinized, plated onto two 9-cm culture dishes and cultured for an additional 24 h.

At 42−48 h after transfection, cells were harvested and lysed by four cycles of freeze−thaw. Cell extracts were analyzed for CAT activity by the method of Gorman *et al.* (1982). The amount of cell extract used in this analysis was corrected for the total number of living cells.

Nucleotide sequencing

Appropriate restriction fragments were inserted into M13 mp10/11 vectors. DNA sequencing was performed by the dideoxy nucleotide chain termination method (Sanger, 1980).

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