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Human CD3-ε gene contains three miniexons and is transcribed from a non-TATA promoter

(miniexons/alternative splicing/pulsed-field electrophoresis/CD3 locus)

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ABSTRACT **The antigen receptor of the T lymphocyte consists of two variable T-cell receptor chains (either TCR-α, TCR-β or TCR-γ, TCR-δ) noncovalently linked to four different invariant membrane proteins (CD3-γ, CD3-δ, CD3-ε, and the CD3-ζ homodimer). The CD3 genes are expressed early in thymocyte development, preceding the rearrangement and expression of the T-cell receptor genes. Here we report the isolation and structural analysis of the human CD3-ε gene. The gene consisted of nine exons. Three exons, encoding the junction of leader peptide and mature protein, were extremely small (21, 15, and 18 base pairs, respectively). The murine gene contained only two such miniexons, the sequences of which were not homologous to those of the three human miniexons. But from comparisons of intron sequences the regions surrounding the human miniexons III and IV appeared to be closely related to those surrounding the murine miniexons III and IV. The most-3′ miniexon in the human gene (IVa) had no murine counterpart and appeared not to duplicate any of the other miniexons. Sequence analysis of CD3-ε cDNA clones isolated from four independent libraries gave no evidence for alternative use of these miniexons. Like CD3-δ, the CD3-ε gene was transcribed from a weak, nontissue-specific, TATA-less promoter. Pulsed-field electrophoresis showed that the human CD3-ε gene was separated from the CD3-γ, CD3-δ gene pair by at least 30 kilobases, but by no more than 300 kilobases.**

The T-cell antigen receptor complex consists of a sulfhydryl-linked T-cell receptor (TCR) heterodimer (α chain and β chain or γ-chain and δ chain) noncovalently associated with the invariable CD3 chains. Four different CD3 chains take part in the formation of the mature TCR–CD3 complex: two glycoproteins, CD3-γ and CD3-δ; and two non-N-glycosylated proteins, CD3-ε and CD3-ζ (1). CD3-ζ appears as a sulfhydryl-linked homodimer. cDNA clones representing all four CD3 chains have been isolated (2–5). The expression of the CD3 genes is restricted to cells of T-lymphocyte lineage. CD3-γ, CD3-δ, and CD3-ε mRNA can be detected in the earliest thymocytes and leukemias before rearrangement and expression of the TCR-encoding genes (6, 7).

Sequence comparisons have revealed that CD3-γ and CD3-δ are highly homologous. CD3-ε appeared to be more distantly related, whereas CD3-ζ showed no apparent homology with any of the other CD3 chains (2–5). Genetic mapping of CD3-γ and CD3-δ led to the unexpected finding that they are encoded on opposite strands in a head-to-head configuration, separated by 1.4 kilobases. These observations strongly suggested the possibility of a gene-duplication event (8, 9). Comparison of the sequence and gene organization of the murine CD3-ε gene with that of the CD3-γ, CD3-δ gene pair revealed that two exons, those encoding the extracellular and the transmembrane regions of the CD3 chains, are conserved in these three genes (3, 29, 30). In addition, the CD3-γ, CD3-δ gene pair and the CD3-ε gene have been mapped to band q23 of chromosome 11 in humans (10–12). From these findings the existence of a small CD3 gene family has been postulated.

Here we report the isolation and characterization of the human CD3-ε gene, as a first step toward understanding its T-cell-specific expression. The gene comprised nine exons, including three miniexons. CD3-ε cDNA clones obtained from four different T-cell sources were isolated to determine whether these miniexons were subject to alternative splicing. The promotor of the CD3-ε gene was characterized in *in vitro* gene-regulation studies. Pulsed-field electrophoresis mapped the CD3-ε gene within 300 kb from the CD3-γ, CD3-δ gene pair.

MATERIALS AND METHODS

Gene Isolation and Characterization. DNA was isolated from peripheral blood mononuclear cells obtained from a healthy volunteer. A genomic library was constructed from this DNA by cloning ≈15-kb partial *Sau*3A1 fragments into the *Bam*H1 site of bacteriophage λEMBL3. Recombinant bacteriophages (3×10^6) were screened with the human CD3-ε cDNA clone pDJ4 (3). Nine individual positive bacteriophages were further characterized. The gene was found to be contained in two overlapping phages, pEH1 and pEH2. The exons were identified by restriction mapping, by probing with fragments of pDJ4, and by double-stranded dideoxy chain-termination sequencing in pGEM4. The miniexons III, IV, and IVa were identified with oligonucleotide probes (TTGGTGTTTGGGGGCAAG, GTAATGAAGAAATGGGT, and GGTATTACACAGACACGT, respectively).

Isolation of CD3-ε cDNA Clones. cDNA libraries constructed from mRNA from activated human peripheral blood T cells (in pCD1), from the leukemic T-cell line HPB-ALL (in λGT10), and from a population of TCR-γ and TCR-δ T cells (in λGT10) were screened with pDJ4. Inserts of positive phages were subcloned in pGEM4 and characterized by restriction enzyme analysis and by sequencing.

Chloramphenicol Acetyltransferase (CAT) Assays. A 253-base pair (bp) *Pst* I–*Mst* II (blunted) fragment representing bp −209 to +44 of the CD3-ε gene was ligated into pCAT3 (13), digested with *Bam*HI, blunted, and digested with *Pst* I. Into the *Sma* I site of the resultant plasmid, pεCAT, the enhancer from the Rous sarcoma virus (RSV)-long terminal repeat [*Nde* I–*Eco*RI, blunted (14)] was ligated, generating pεCATRSV. A deletion of the promoter of pεCATRSV was made by digestion with *Stu* I and *Xho* I, followed by blunting and religation (deletion from bp −101 to +44: pε**RSV). pεCAT was extended to bp −2400 ($p\varepsilon CAT_2$) and to bp −8000 ($p\varepsilon CAT_3$) by the stepwise insertion of upstream fragments taken from λE1. These constructs were transiently

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Abbreviations: TCR, T-cell receptor; CAT, chloramphenicol acetyltransferase; RSV, Rous sarcoma virus.

transfected into the human T-cell leukemic Jurkat cells and into HeLa cells, with DEAE-dextran used essentially according to Stafford and Queen (15). After 44 hr the cells were harvested, resuspended in 100 μl of 50 mM NaCl/10 mM Tris, pH 7.4/1 mM EDTA; a lysate was obtained through three cycles of freeze/thawing. Fifty microliters of these lysates were added to 125 μl of CAT assay buffer [2.5% glycerol/= 0.25 M Tris, pH 7.5/3 mM butyryl-CoA) at 1 μCi/ml [^{14}C]chloramphenicol (60 mCi/mM; 1 Ci = 37 GBq)], and the samples were incubated for 2 hr at 37°C. Butyrylated [^{14}C]chloramphenicol was then extracted with 400 μl of pristane/xylene (2:1, vol/vol) and counted (16).

Pulsed-Field Electrophoresis. Whole cells from the human Epstein–Barr virus-transformed B-cell line JY were embedded in 0.5% low-melting point agarose in Hanks' medium at 1×10^7 cells per 50-μl aliquot. Individual aliquots were allowed to solidify at 4°C. The agarose blocks were then treated with 1% sodium dodecyl sulfateproteinase K at 50 μg/ml 0.4 M EDTA at 50°C. Next, the blocks were extensively washed and stored in 0.5 M EDTA. Before restriction enzyme digestion the agarose blocks were equilibrated with the appropriate restriction enzyme buffer. Digestion was done in a total volume of 200 μl in the presence of 30–50 units of restriction enzyme for 4 hr at 37°C. The plugs were then transferred to fresh tubes, melted at 65°C, and carefully applied to an 0.8% agarose gel (in 0.5× TBE buffer, where 1.0× TBE = 90 mM) Tris/64.6 mM boric acid/2.5 mM EDTA, pH 8.3). The samples were electrophoresed for 16 hr at 7 V/cm at room temperature. Field inversion was controlled with a PPI-100 computer (MJ Devices, Cambridge, MA). (Program specifications were as follows: initial reverse time, 0.1 sec; reverse increment, 0.01 sec; initial forward time, 0.3 sec; forward increment, 0.03 sec; number of steps/per cycle, 45; reverse increment increment, 0.01 sec; forward increment increment, 0.03 sec.) Transfer of DNA to nitrocellulose and probing with pDJ4 and with the CD3-δ cDNA clone pPGBC-9 (2) was done according to standard procedures.

Computer Analysis of Human CD3-ε Sequence. The sequence from the putative promoter area of the human CD3-ε gene was compared with the murine CD3-ε promoter using the program LOCAL (17) at the Molecular Biology Computer Research Resource (MBCRR)-facilities at the Dana–Farber Cancer Institute. Similar comparisons were performed for the regions surrounding each of the three human miniexons with their two murine counterparts. To minimize the effect of splice consensus sequences at the exon–intron boundaries on similarity scores, we included at least 100 bp of 5′- and 3′-flanking intron sequences in each comparison.

RESULTS AND DISCUSSION

Gene Structure. The human CD3-ε gene comprised nine exons and spanned 13 kb. Fig. 1 shows its exon/intron organization. A small (65-bp) untranslated first exon preceded a larger second exon (109 bp), which contained the translation start site and encoded most of the leader segment. The miniexons III (21 bp), IV (15 bp), and IVa (18 bp) encoded the rest of the leader segment and the extreme N terminus of the mature protein. Exon V (249 bp) encoded the extracellular domain. Exon VI (168 bp) coded for the transmembrane domain. The intracellular segment was encoded by exons VII (54 bp) and VIII (697 bp). A partial nucleotide sequence for the human CD3-ε gene is shown in Fig. 2. As indicated, all exon–intron boundaries conformed to the consensus [Y_nNCAG(–exon–ag)GTAAGT, where Y = C and/or T].

Previously, we determined the transcription start site of the murine CD3-ε gene by means of primer-extension and nuclease S1 protection analysis (30). Based on the virtually complete sequence conservation in this area of the two genes we assume that the transcription start site of the human gene is located at the position indicated in Fig. 2. A sequence

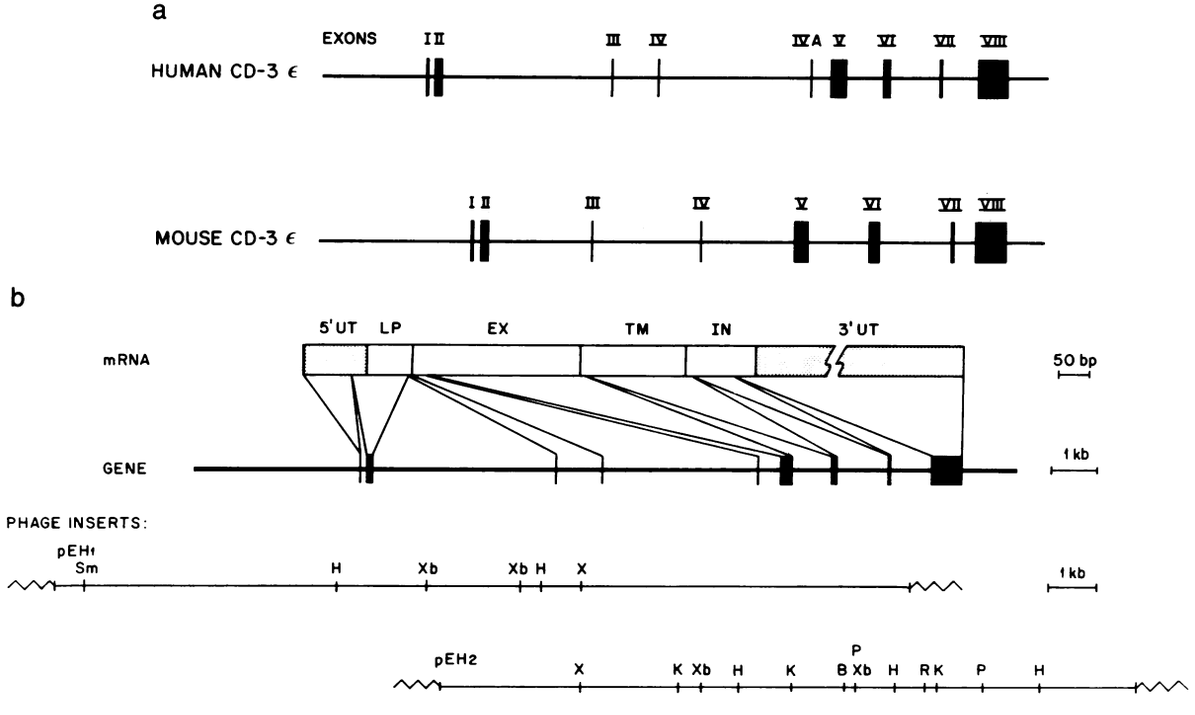

FIG. 1. (*a*) Schematic comparison of the organization of the human and murine CD3-ε genes. The two genes are remarkably similar apart from the miniexon area (exons III, IV, and IVa in the human and exons III and IV in the mouse). (*b*) Partial restriction map of the two recombinant bacteriophages (pEH1 and pEH2) that together contained the human CD3-ε gene. The individual exons are projected on the CD3-ε mRNA. UT, untranslated; LP, leader peptide; EX, extracellular domain; TH, transmembrane domain; IN, intracellular domain. Restriction sites: B, *Bal* I; H, *Hin*dIII; K, *Kpn* I; R, *Eco*RI; X, *Xho* I; Xb, *Xba* I; Sm, *Sma* I.

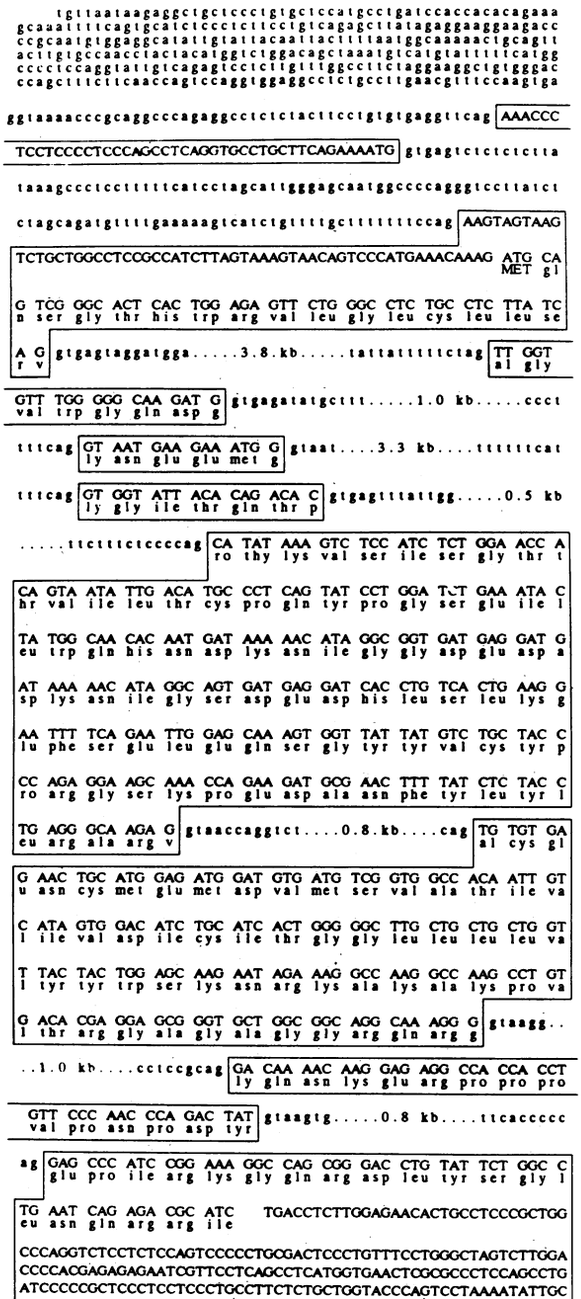

FIG. 2. Partial sequence of the human CD3-ε gene. Exon sequences are boxed. We report here the first-exon sequence (boxed) of the CD3-ε cDNA clone pDJ4 (3).

comparison of the putative promoter of the human gene with that of the mouse is given in Fig. 3. The first, untranslated exon and intron I showed a striking cross-species homology, which extended to bp −200. No significant homology was detected further upstream. Similar to reports describing the CD3-δ and CD3-γ genes (8, 9, 18), neither TATA nor CAAT elements were present in the CD3-ε promoter. Furthermore, we did not find any consensus sequences of generally occurring upstream promoter elements 5′ of the transcription start site (19).

The CD3-ε Promoter Is not T-Cell-Specific. Next, a fragment, which spans the area of human/mouse homology (−209 to +44, see Fig. 3) was assayed for promoter activity. By *in vitro* gene regulation analysis using CAT as reporter gene, this fragment was found to act as a weak promoter both in the T-cell line Jurkat and in HeLa cells (see Table 1, pεCAT). High expression could be obtained in both cell types by including the enhancer from the RSV long terminal repeat 3′ of the reporter gene in this construct (Table 1, pεCAT-RSV). A small deletion in the promoter area abrogated the CAT activity, indicating that transcription was indeed initiated in the putative promoter fragment (pε**RSV). From these experiments it was deduced that the CD3-ε promoter itself does not govern the T-cell specificity of the CD3-ε gene and that therefore tissue-specific enhancing elements are located elsewhere in the gene. Stepwise extension of this fragment up to bp −8000 did not raise the measured level of CAT activity (constructs $p\varepsilon CAT_2$ and $p\varepsilon CAT_3$). It appears, therefore, most likely that cis-acting elements will be located downstream from the promoter. A similar situation has been found recently in the CD3-δ gene: The CD3-δ promoter is relatively weak and nontissue specific, and a T-cell-specific enhancing element has been identified 3′ of the last CD3-δ exon (20). It is conceivable that a similar enhancer confers T-cell specificity to the CD3-ε gene and human and murine CD3-ε genes need to be scanned for such enhancer activities in *in vitro* gene-regulation assays.

Human/Mouse Miniexon Homologies. Comparison of the human and murine miniexons revealed no cross-species homology. Thus, between the two species the number of miniexons differed (three versus two), as did the number of base pairs per miniexon (21, 15, and 18 versus 18 and 15, respectively) and the actual sequence of the exons. To investigate potential evolutionary relationships between individual miniexons of the human and of the mouse CD3-ε genes we decided to compare stretches of at least 200 bp surrounding each miniexon with similar stretches around all other miniexons in the two genes using the computer program LOCAL (17). Highly significant matches were found only for the exon III areas in human and mouse (similarity score, 37.04) and for the exon IV areas in human and mouse (similarity score, 55.99). We therefore concluded that the exons III and IV in the human and mouse have diverged from

Table 1. *In vitro* analysis of the activity of the CD3-ε promoter using CAT as reporter gene

	Jurkat, cpm	HeLa, cpm
Mock	380	390
pRSVCAT	31,400	37,230
pεCAT (bp −204)	780	600
pεCATRSV	8,320	6,790
pε**RSV	420	ND
$p\varepsilon CAT_2$ (bp −2400)	760	690
$p\varepsilon CAT_2$RSV	7,320	8,990
$p\varepsilon CAT_3$ (bp −8000)	990	780
$p\varepsilon CAT_3$RSV	9,230	7,860

Jurkat T cells and HeLa cells were transfected with the indicated plasmids and 48 hr later CAT activity was measured. The numbers represent the total amount of butyrylated [^{14}C]chloramphenicol in cpm per 2-hr reaction. As shown, the fragment in pεCAT (bp −204 to +44) constitutes a weak nontissue specific promoter. No enhancer activity could be detected up to 8000 bp upstream from this fragment ($p\varepsilon CAT_2$ and $p\varepsilon CAT_3$). Because the RSV enhancer, inserted 3′ of the reporter gene, induces CAT activity equally well in all three constructs, it could be inferred that no repressor element was present in this area. A deletion (bp −101 to +44, pε**RSV) completely abrogates CAT activity. pRSVCAT, in which the RSV long terminal repeat drives transcription of the CAT gene (14), serves as a positive control.

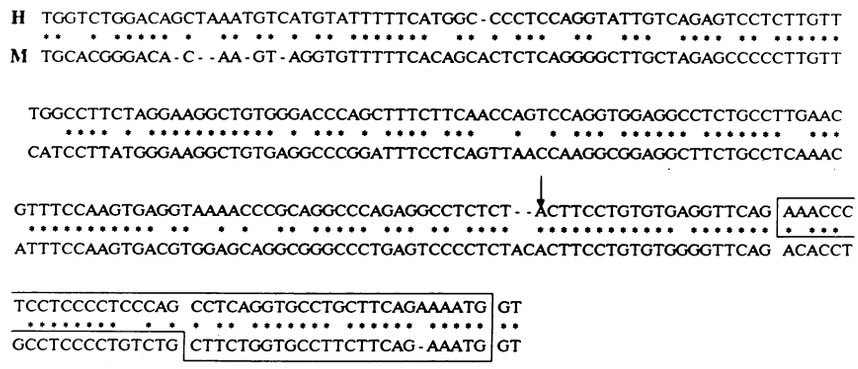

FIG. 3. Sequence comparison of the human and mouse CD3-ε promoter. The similarity value, calculated by LOCAL, was 115.55; asterisks indicate common bases. An arrow indicates the transcription start site of the murine CD3-ε gene. No TATA or CAAT boxes are present. The sequence homology is lost upstream of bp -200, indicating that the promoter is probably confined to bp -200 to $+1$.

two common ancestor exons. Either exon IVa has emerged in the human CD3-ε gene after human/mouse divergence or its murine equivalent has been lost after evolutionary divergence of the two species.

It is unclear why this extensive divergence occurred in two otherwise highly homologous genes. Conceivably, a minor change in one of the CD3-ε genes, such as the loss of a miniexon or a nonconservative point mutation, may have affected the function of the CD3-ε protein. A subsequent correction of this defect then occurred through a series of sequence changes in the miniexon region. Alternatively, a mutation in a chain interacting with the CD3-ε protein—e.g., one of the other members of the TCR–CD3 complex, could have induced sequence alterations in CD3-ε at the site of interaction.

Alternative Splicing. Miniexons as described here occur rarely in higher eukaryotes (21–25), but among the few examples of such genes alternative splicing has been reported rather frequently (24, 25). The structure of the troponin T protein-encoding gene, in particular, resembles that of the CD3-ε gene. The troponin T protein gene contains a series of six small exons (III–IX), all consisting of a multiple of three nucleotides. Alternative usage of these miniexons occurs in individual troponin T mRNA molecules. Thus, a variety of transcripts are generated from one gene with preservation of an open reading frame (24).

To test for the occurrence of alternative splicing in the CD3-ε gene, nucleotide sequences of CD3-ε cDNA clones isolated from four different libraries were compared. These libraries were derived from a $CD4^+$ T-cell clone (3), from activated blood mononuclear cells (pCD11), from a T-leukemic cell line (pHPB), and from a population of TCR-γ/δ-positive T cells (p$\gamma\delta$). Fig. 4 compares the miniexon sequences of these clones with the genomic sequence. A conservative C $\rightarrow$ G base change present in the genomic sequence was also found in the pCD11 clone and represents a polymorphism.

The failure to detect alternative exon usage in these studies does not preclude that alternative splicing involving miniexons occurs at relatively low levels, such as in the CD3-δ gene (26). Nuclease S1 protection analysis has been used for the detection of alternative splicing events (24, 26) but could not be applied in this case, due to the high frequency of retained introns in the CD3-ε message, as determined by Northern (immunologic) blot analysis (data not shown). There is yet no protein-chemical or functional evidence for alternative splicing in CD3-ε (1). From these observations we suspect that alternative splicing does not occur in the CD3-ε gene.

Long-Range Mapping of the CD3 Locus. The CD3-γ, CD3-δ gene pair and the CD3-ε gene map to band q23 of chromosome 11 in humans and to chromosome 9 in mice (10–12). To study the linkage of the CD3 genes more closely, field-inversion gel electrophoresis was used. This recently developed variation of pulsed-field electrophoresis allows the separation of megabase-size DNA fragments on conventional agarose gels (27). The major advantage of field inversion lies in the homogeneity of the applied electric field; relative mobilities of individual lanes are therefore independent of their position in the gel.

High-molecular weight DNA, prepared from the human B cell line JY, was digested with *Eco*RI and *Bam*HI and with the infrequently cutting enzymes *Mlu* I and *Not* I. Fig. 5*a* shows a field-inversion gel blot probed with the CD3-ε probe pDJ4 (*Left*), then stripped and reprobed with the CD3-δ probe pPGBC-9 (*Right*). The probes hybridized to different *Eco*RI and *Bam*HI restriction fragments. No clear signal was obtained for the *Bam*HI digest probed with CD3-δ, probably due to the presence of multiple *Bam*HI sites in the CD3-δ gene (18). CD3-ε hybridized to a 40-kb and a 6-kb *Eco*RI band and to a 40-kb *Bam*HI band. A schematic drawing of the relevant *Eco*RI and *Bam*HI sites in the CD3-ε locus is given in Fig. 5*b*. Both probes detected *Mru* I and *Not* I bands of identical size in the 300-kb range, the *Mru* I fragment being slightly larger than the *Not* I band. We therefore concluded that CD3-ε was separated from the CD3-γ, CD3-δ gene pair by at least 30 kb (Fig. 5*b*) but by <300 kb. Recently, similar results were reported by Tunnacliffe and coworkers (9).

The clustering of the CD3 genes and possibly other T-cell-specific genes in a relatively small locus might play a role in their coordinate tissue-specific expression. A recent report by Grosveld and coworkers (28) has emphasized the importance of a discrete class of regulatory elements flanking the entire β-globin locus. These elements had initially been noticed as tissue-specific DNaseI-supersensitive sites. Conceivably, similar elements regulate the expression of the CD3 locus. Establishment of a long-range map of the CD3 locus by pulsed-field analysis will be crucial in the search for such regulatory elements.

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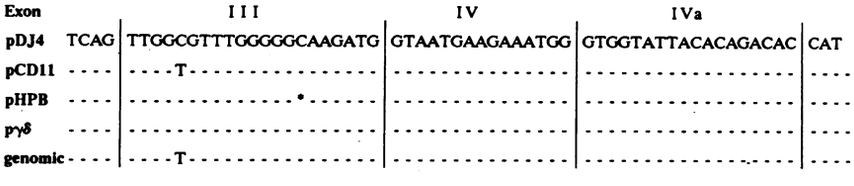

FIG. 4. Sequence comparison of the miniexon region of four CD3-ε cDNA clones and of the genomic exon sequence (see text). The exons are separated by lines and numbered with roman numerals. An asterisk represents a 1-bp gap in one of the clones, most likely a cloning artifact. No evidence for alternative splicing is evident from these data.

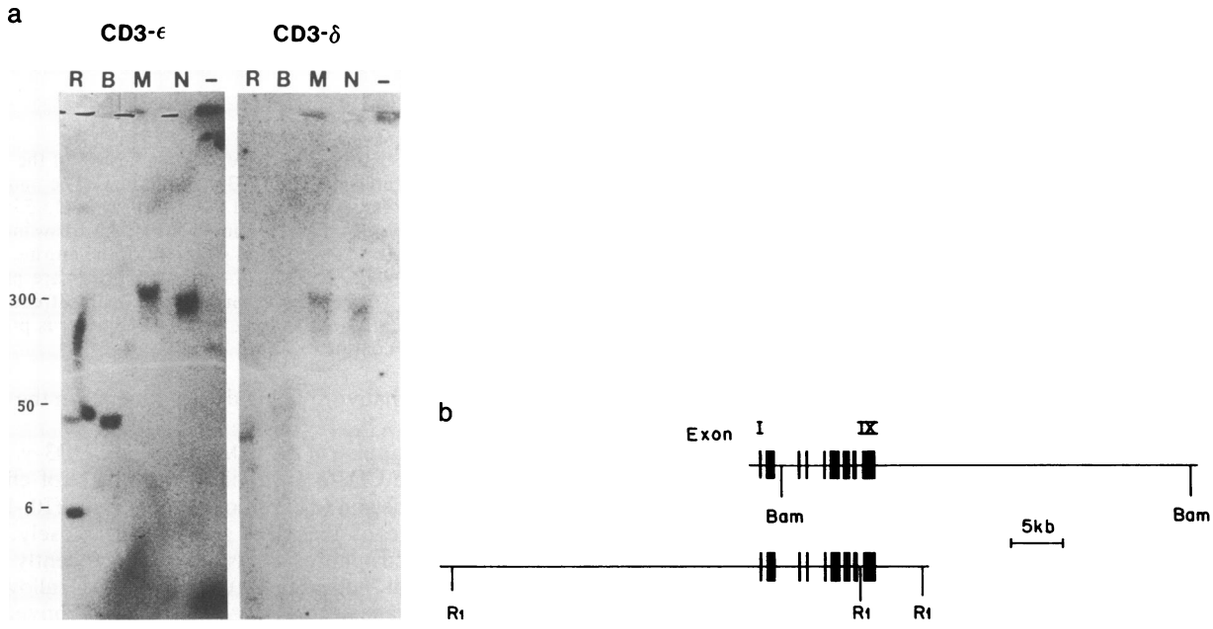

FIG. 5. (*a*) Field-inversion Southern blot of human DNA. Probes were pDJ4 (CD3-ε, *Left*) and pPGBC-9 (CD3-δ, *Right*). R, *Eco*RI; B, *Bam*HI; M, *Mru* I; and N, *Not* I. Sizes are given in kb. (*b*) Location of the *Eco*RI (R1) and *Bam*HI restriction sites in the CD3-ε gene.

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