Two Unusual Forms of Human Immunoglobulin E Encoded by Alternative RNA Splicing of ϵ Heavy Chain Membrane Exons

By Ke Zhang,* Andrew Saxon,* and Edward E. Max‡

*From *The Hart and Louise Lyon Laboratory, Division of Clinical Immunology/Allergy, Department of Medicine, University of California, Los Angeles, School of Medicine, Los Angeles, California 90024; and the ‡Center for Biologics Evaluation and Research, Food and Drug Administration, Bethesda, Maryland 20892*

Summary

We present evidence for RNA transcripts encoding two forms of human ϵ immunoglobulin (Ig) heavy chain that differ significantly from those of other isotypes. We previously demonstrated three human ϵ mRNA species, instead of the two, corresponding to membrane and secreted proteins, seen with other heavy chain transcripts. In human genomic DNA downstream of the Cϵ gene, we identified sequences homologous to the two putative murine exons M1 (encoding a hydrophobic, presumably transmembrane region) and M2 (encoding hydrophilic residues). To determine the structures of ϵ transcripts containing these sequences, we amplified ϵ-related RNAs with the reverse transcriptase polymerase chain reaction. RNA was examined from fresh human B cells stimulated to IgE production by interleukin 4 plus anti-CD40, as well as from the human IgE-producing line AF10. Instead of the single CH4-M1-M2 splice product predicted for murine membrane IgE, we found two other RNA species. One form has the structure CH4-M1′-M2, in which M1′ includes the human sequence homologous to the murine M1 as well as a unique segment of 52 codons further upstream in the genomic sequence; this RNA species apparently encodes the IgE expressed on the membrane of IgE-producing lymphocytes. The other RNA has the structure CH4-M2′, in which M2′ is spliced in an alternative reading frame that includes an additional 109 codons downstream of the termination codon of the CH4-M1′-M2 form. Because the CH4-M2′ mRNA form does not encode a hydrophobic segment, its translated product should be secreted. A secreted ϵ protein of approximately the size predicted for this form was identified by Western blotting. This novel IgE protein could play a significant and distinctive role in allergic disorders.

Downloaded from www.jem.org on October 23, 2005

IgE was recognized as the antibody mediating immediate allergic reactions approximately 25 yr ago (1). The structure of the human secreted IgE protein was defined through the amino acid sequencing of secreted myeloma proteins (2) and through analysis of the genomic gene and cDNAs encoding the human ϵ-secreted heavy chain (3). However, we were interested in the structure of membrane-bound ϵ chain and the genetic elements encoding this form, both previously unknown for human IgE. Furthermore, the nature of the membrane and secreted ϵ chains in human polyclonal IgE has not been elucidated.

We previously demonstrated that in RNA isolated from human B lymphocytes and from an IgE-secreting myeloma, human Cϵ probes hybridize to three different sizes of mRNA: 2.1, 3.0, and 3.8 kb (4). Three bands of very similar sizes were also reported on Northern blot analysis of RNA from a murine IgE-secreting hybridoma (5). Similar analyses of RNA species encoding other Ig isotypes generally reveal only two bands, corresponding to forms encoding either a membrane-bound or secreted protein. These two RNA species result from alternative splicing either including or excluding "membrane exons" that in germline DNA lie downstream of the corresponding CH gene. The finding of three bands in the Cϵ system suggested that the splicing of membrane exons for this isotype might be more complex than for other heavy chain RNAs.

To explore this possibility we began our investigation by sequence analysis of the germline DNA encoding exons downstream of the Cϵ gene. Our sequence data allowed us to design oligonucleotides that were used to reverse transcribe and amplify the relevant regions of the ϵ-related mRNAs from human cells by the PCR. In this paper we report the DNA sequence of the two exons of the the human ϵ membrane locus and the structure of three mature ϵ mRNAs that uti-

lize this coding information. Only one of these mRNAs contains the sequence for a hydrophobic peptide segment that would anchor the protein to the membrane, and this form encodes an unusually long stretch of amino acids between this transmembrane region and the last Ig domain. The second ϵ mRNA is generated by an alternate splice to the second membrane exon, a splice that would lead to translation of this exon in a second reading frame yielding a secreted protein with a 134–amino acid COOH-terminal addition compared with the "classical" secreted form. The third RNA form may represent a rare splice variant. The existence of an unusual membrane protein and a second secreted form of ϵ heavy chain would both have potential implications for our understanding of the function of IgE in allergic reactions. As this manuscript was being prepared, results similar in several aspects were reported by Peng et al. (6) based on analysis of two IgE-producing cell lines.

Materials and Methods

Sequence Analysis of Human ϵ Membrane Exons. The membrane exons of the human ϵ gene were subcloned from cosIg10 (7), a kind gift from T. Rabbitts (MRC Laboratory of Molecular Biology, Cambridge, England). The location of the exons was known from previous analysis of sequence downstream of the highly homologous ϵ pseudogene (E. E. Max. and C. Moulding, unpublished results). A 10.6-kb BamHI-XhoI subclone (plasmid p20R) of cosIg10 was further subcloned into Bluescript (Stratagene, La Jolla, CA), and sequence analysis was performed on both strands by the dideoxy termination method using "universal" primers from vector sequence and several internal primers. Some regions of the sequence were consistently difficult to read, probably owing to high GC content and secondary structure, requiring dITP substitution or high temperature (TAQuence; U. S. Biochemical Corp., Cleveland, OH) for elucidation. Differences between our sequence and a sequence of part of the same region that was published during the course of this work (8) were given special scrutiny.

Cell Line and Cell Cultures. The IgE (ϵ/λ)-secreting human cell line AF-10, a subclone derived from the myeloma U266, was maintained as described previously (9). Fresh B cells were isolated from tonsil mononuclear cells. Briefly, after tonsil disruption, mononuclear cells were prepared by Ficoll-Hypaque density centrifugation. T cells were depleted by two cycles of rosette formation with sheep red blood cells treated with 2-aminoethylisothiouronium bromide (Sigma Chemical Co., St. Louis, MO); and monocytes/macrophages were then removed by two cycles of adherence to plastic dishes in the presence of serum. These methods have been described in detail previously (10, 11). The resulting cell populations were analyzed by flow cytometry and consisted of 99% of B cells (CD19/CD20$^+$) and of <1% T cells (CD3$^+$) or monocytes (CD14$^+$). For ϵ mRNA induction, purified B cells (10^6 cells/ml) were stimulated for 5 d in culture in complete RPMI 1640 with IL-4 (200 U/ml; Amgen Biologicals, Thousand Oaks, CA) and the anti-CD40 mAb G28-5 (0.1 μg/ml; the kind gift of Dr. Ed Clark, University of Washington, Seattle, WA).

RNA Isolation and Reverse Transcription. Total cytoplasmic RNA was isolated from both AF-10 cells and induced/uninduced B cells. Briefly, a pellet was lysed with 0.5% NP-40 lysis buffer at 4°C. The nuclei were removed by centrifugation, and the lysate was treated with proteinase K. The resulting cytoplasmic RNA-containing supernatant was extracted with phenol/chloroform followed by precipitation of the RNA in EtOH (12). For isolation of RNA from B cells, *Escherichia coli* rRNA (2 μg/10^6 cells; Boehringer-Mannheim Co., Indianapolis, IN) was added as carrier. cDNA was reverse transcribed from the isolated RNA using oligo$(dT)_{15}$ (Boehringer-Mannheim Co.) as primer and mouse Moloney leukemia virus reverse transcriptase (Bethesda Research Laboratories, Gaithersburg, MD). The cDNA mixture was heated to 95°C for 5 min and then immediately used as a PCR substrate.

Amplification and Analysis of ϵ cDNAs. Pairs of PCR primers were designed for detecting different forms of ϵ mRNA containing membrane exon sequence (Table 1). Primer oligonucleotides were synthesized by Genesys Biotechnologies Inc. (The Woodlands, TX). Some primers were designed with terminal enzyme restriction sites to facilitate cloning and sequencing of PCR products. Table 1 shows the oligonucleotide primers and probes used in this study. PCR amplifications were performed for 40 cycles in a volume of 100 μl/reaction. The reaction mixture contained 10% DMSO, 50 mM KCl, 20 mM Tris-HCl (pH 8.4), 2.5 mM $MgCl_2$, and 100 μg/ml nuclease-free BSA. The reactions were carried out with 1 min of melting at 94°C, 1 min of annealing at 65°C, and 1 min of extension at 72°C, except that PCR was carried out with annealing at 72°C when primer D was used. Primer concentration was 50 pM. Template DNA for each reaction consisted of the total cDNA reverse transcribed from 1 μg of RNA. PCR products were digested with ClaI and SalI (to uncover the sticky ends designed into the PCR primers), purified by agarose gel electrophoresis, and then ligated into a Bluescript (Stratagene) vector that had been prepared by ClaI + XhoI digestion and treatment with alkaline phosphatase. Plasmid minipreps were analyzed by restriction digestion and the inserts of selected clones were sequenced as described above.

Southern Blot Analysis of PCR Products. PCR products were extracted with phenol/chloroform, and then 15 μl of each reaction mixture was subjected to electrophoresis on a 2% agarose gel (NuSieve and Seakem agarose 1:1 mixture; FME Bioproducts, Rockland, ME) in TBE buffer. DNA was transferred to nylon membranes (Nytran; Schleicher & Schuell, Inc., Keene, NH) in 0.4 M NaOH, and blots were analyzed by probing with end-labeled oligonucleotides corresponding to an internal region of each putative ϵ membrane exon. Blots were prehybridized for 2 h at 60°C in 5× SSPE, 0.5% SDS, and 250 ng/ml of salmon sperm DNA. Hybridization with kinased oligonucleotide probes was performed overnight at 60°C. The blots were washed for 20 min at room temperature with 2× SSC plus 0.1% SDS, and then twice more at 60°C with 0.2× SSC plus 0.1% SDS for 20 min.

Northern Blot Analysis. 20 μg of total RNA from AF-10 cells was denatured with glyoxal and subjected to electrophoresis on 1% agarose gels using 10 mM sodium phosphate running buffer and then transferred by capillary transfer to nylon membranes (Nytran). The filters were prehybridized for 2 h at 42°C in 50% formamide, 5× SSC, 50 mM sodium phosphate, pH 7.0, 5× Denhardt's solution, 250 μg/ml denatured salmon sperm DNA, and 0.1% SDS. Three reverse transcription PCR-generated products were used as probes, as shown in Fig. 5. The PCR products were excised from agarose gels and purified by using a Geneclean kit (Bio101, La Jolla, CA). The specificities of the PCR products were verified by hybridization with a kinased internal oligonucleotide probe specific for each amplified fragment. The PCR products were then labeled with [^{32}P]αATP by random priming and hybridized to the filter-bound RNA as described (4).

Western Blot Analysis. Three protein samples were analyzed: a 1:200 dilution of serum from a patient (PS) with an IgE myeloma, a 10-fold concentrated supernatant of AF-10 cells grown in HL-1 serum-free medium (Ventrex Lab. Inc., Portland ME), and a lysate

Downloaded from www.jem.org on October 23, 2005

of AF-10 cells. These samples were subjected to electrophoresis on an SDS-PAGE gel (10% acrylamide) under denaturing conditions. Transfer to nitrocellulose membranes (S&S NC; Schleicher & Schuell, Inc.) was carried out in buffer containing 25 mM Tris, 192 mM glycine, and 20% (vol/vol) methanol for 2 h at 50 V. After blocking, the blot was incubated with mouse human ε-specific mAb (clone CIA-E-7.12, 20 μg/ml) for 2 h followed by incubation with alkaline phosphatase–labeled sheep anti-mouse IgG antiserum (Sigma Chemical Co.) for >2 h. Color development was performed with an AP-conjugate kit (Bio-Rad Laboratories, Richmond, CA) as described by manufacturer.

Results

Genomic DNA Sequence of Putative Human ε Membrane Exons. As a basis for understanding alternative ε RNA species involving membrane exons, we determined the nucleotide sequence of human germline genomic DNA containing these exons. Initial sequence analysis identified a DNA segment, located ~1.8 kb downstream from the 3′ end of the ε CH4 domain, that showed strong sequence similarity to the ε membrane exons previously described in murine DNA at a similar location (Fig. 1 *A*) (5). Of the 72 amino acid residues encoded in the two murine exons M1 and M2, 33 (46%) are conserved in the homologous human sequence. In particular, the hydrophobic residues in M1 that are thought to play a role in anchoring the protein to the lipid membrane of the B lymphocyte are well conserved. These include the LFLLSV segment found in most murine IgH membrane regions and the COOH-terminal alanine residue. Although deletion/insertion differences are scattered in the intron sequences, in the exons they occur in only two places, both with maintenance of reading frame: two compensating deletions in the human sequence between nucleotides 220 and 230, and a 9-bp deletion in the human sequence between nucleotides 180 and 190. The latter probably resulted from an event involving repeated GACCT sequences that in the mouse are separated by 9 bp. An additional deletion just upstream in the human sequence may also be related to repeated sequences (CCCA). The nucleotide sequence of CH4 and 3′ flanking sequence is shown in Fig. 1 *B*, while a 1-kb region including the human membrane ε exons is shown in Fig. 1 *C*. The evidence supporting the exon boundaries defining the translated regions is presented below.

PCR Amplification of RNA Sequence Spanning the Putative ε Membrane Exons. To define the exon structure of the membrane regions of ε mRNAs from human IgE-secreting cells, cDNA copies of extracted RNA were amplified by PCR. As sources of ε RNA we used both fresh human B lymphocytes cultured with IL-4 and anti-CD40, and the IgE myeloma cell line AF10. Three primer pairs were used in the amplifications. For one set of experiments the cDNA was amplified using the primer pair IVm-C (see Fig. 2, *bottom*). When amplification mixtures from the two RNA samples were run on routine TBE agarose gels, both RNAs gave similar patterns of four bands (Fig. 2 *A*). All four bands hybridized to an M2 probe, but only the upper two bands hybridized to either of two probes for M1. This suggested that some ε RNA (represented by the smaller PCR products) is spliced to yield species that contain M2 but not M1 sequence.

Although we detected four bands in the gels of Fig. 2, *A* and *B*, evidence from further investigation suggests that there are only two PCR products, corresponding to bands 1 and 4 of these gels. When the DNA was extracted from bands 2 and 3 and rerun on TBE agarose, the extracted DNA fragments comigrated with bands 1 and 4, respectively (data not shown). Furthermore, when DNA corresponding to all four bands was subcloned and subjected to sequence analysis, material from band 2 always gave subclones identical to those

Table 1. *Oligonucleotide Sequences*

Name	Sequence (5′ to 3′)*	Position
IVb	ggccatcgatAAGTCTATGCGTTTGCGACGCCGG	19–42 (Fig. 1 *b*)
IVm	ggccatcgatGACGCCCGGCACAGCACGACGCAG	147–170 (Fig. 1 *b*)
A	ACAGAGCCTCCTGCTGCTCT	126–107 (Fig. 1 *c*)
B	ggccgtcgacTGTAGCTCACGCTCAGCAGG	281–262 (Fig. 1 *c*)
C	ggccgtcgacGGCTGGAGGACGTTGGTGTA	462–443 (Fig. 1 *c*)
D	ggccgtcgacTGGGTGCCGGGCCCTCCTTGGC	837–816 (Fig. 1 *c*)
G	ATTCCAACCCGAGAGGGGTG	CH3 domain
H	CTGTCCACTGTTGCAATGACC	401–421 (Fig. 1 *b*)
1	TCTGCCACTCCGGACAGCAG	75–94 (Fig. 1 *c*)
2	CTGGACGTGTGCGTGGAGGA	185–204 (Fig. 1 *c*)
3	TGCAGCGGTTCCTCTCAGCC	390–409 (Fig. 1 *c*)
4	CTCCTCGATGACTCTGTTGA	714–733 (Fig. 1 *c*)

* Capital letters correspond to genomic sequence while lower case letters represent added nucleotides. Underlined sequences represent introduced restriction sites for ClaI (atcgat) or SalI (gtcgac).

Downloaded from www.jem.org on October 23, 2005

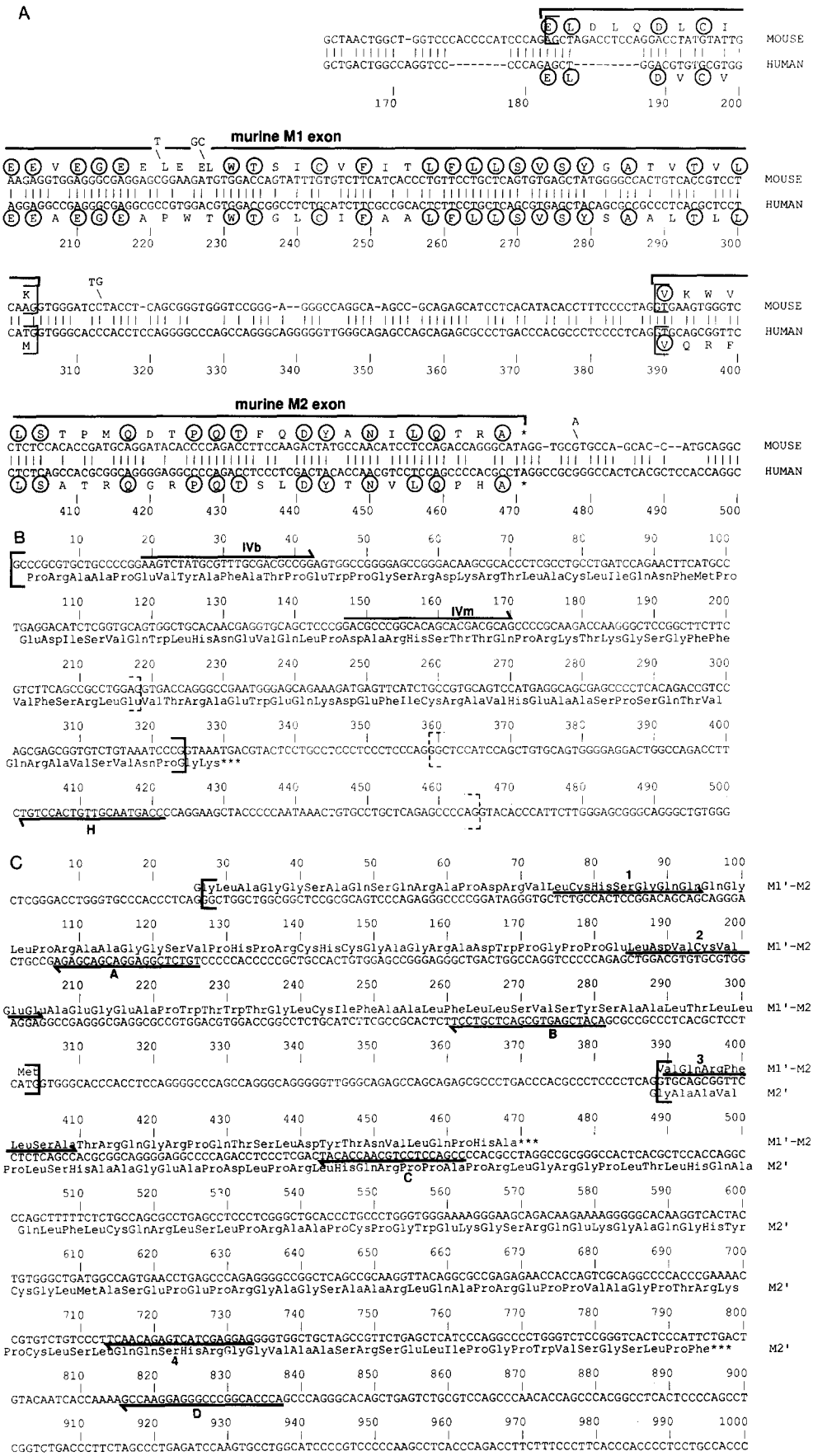

Figure 1. Nucleotide sequence of human genomic clone containing Ig ϵ membrane exons. (*A*) A portion of the human sequence obtained in the present investigation is compared with the previously published murine sequence (5), in which exons M1 and M2 were identified on the basis of homology to the membrane exons of other isotypes. The amino acid translation (one-letter code) is given, with conserved residues circled. (*B*) The genomic sequence of human Cϵ4 (3) is presented (translation below) along with some downstream sequence. The bold bracket after position 324 identifies the splice donor site determined for two of the three RNA species described here that include one or more of the downstream "membrane" exons. The dashed brackets define the splice sites of the unusual CH4'-I-M1'-M2 form. The locations of genomic sequences used to design oligonucleotides used in the present study (IVb, IVm, and H) are shown; the arrow under the sequence indicates that the oligo was designed based on the strand complementary to that shown here. (*C*) The sequence of the human membrane exons is presented. Bold brackets identify the boundaries of the M1' exon and the 5' end of the M2 or M2' exon as defined by the cDNA structures characterized in the present work. Amino acid translation is provided for the M1'-M2 form (above the nucleotide sequence) and the M2' reading frame (below the nucleotide sequence). The locations of genomic sequences used to design oligonucleotides are shown as in *B*. These sequence data are available from EMBL/Genbank/DDBJ under accession number X63693 HSIGEHCA.

Downloaded from www.jem.org on October 23, 2005

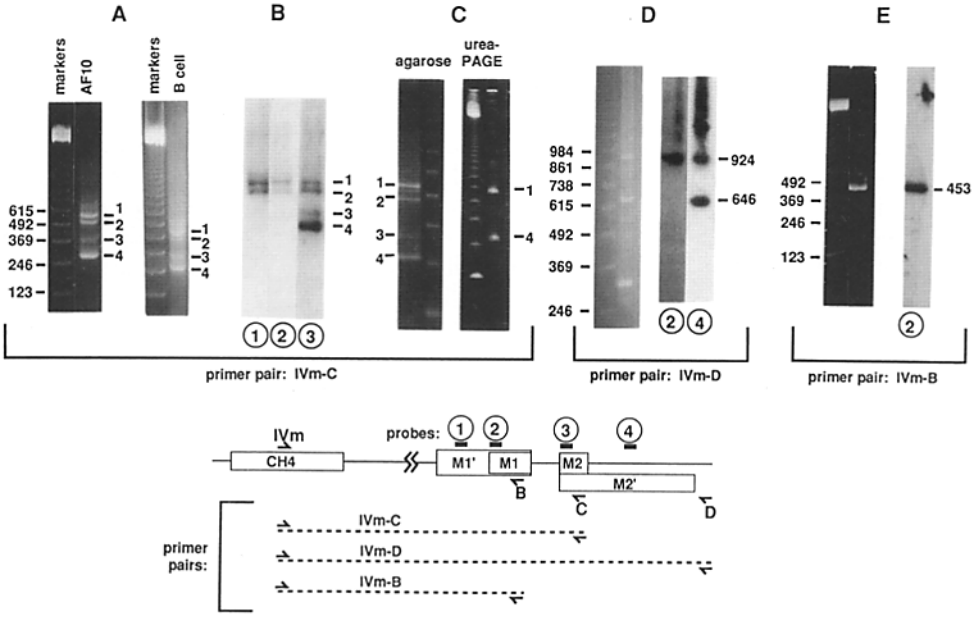

Figure 2. Gel electrophoresis of RT-PCR products. Size estimates of the products were based on comparison with oligomers of 123-bp markers. The products of three primer pairs are shown, along with a map that diagrams the position of the pairs with respect to the exons. The circled numbers identify the four oligonucleotide probes used in the Southern blots shown. (*A*) RT-PCR bands derived from RNA of AF10 cells or from B cells cultured with IL-4 and anti-CD40 show a nearly identical pattern. Essentially, identical patterns were seen with RNA from both sources in all the PCR experiments shown here. (*B*) The four bands obtained with AF-10 RNA hybridize differentially with three oligonucleotide probes from the locus. (*C*) Aliquots of the same amplification mixture were subjected to electrophoresis on agarose or on a denaturing 8 M urea-12 acrylamide gel. The middle two bands seen on agarose are absent on the denaturing gel and may represent heteroduplex artifacts. (*D*) The IVm-D primer pair was used to determine the 3′ end of the coding exon M2′. Sequence analysis of the PCR products ruled out the possibility of introns interrupting the coding sequence downstream of oligonucleotide C. (*E*) The IVm-B primer pair generates a single band of ~453 bp, consistent with a CH4-M1′ splice.

from band 1, and similarly, band 3 subclones were identical to band 4. It has been reported that some PCR products produce anomalous bands on agarose gels because of heteroduplex formation (13). This is a likely explanation for the occurrence of bands 2 and 3 because these bands were absent when the PCR mixtures were run on a denaturing urea gel (Fig. 2 *C*).

In another set of experiments, designed to explore the more 3′ region of the ε mRNA structure, cDNAs were amplified by the primer pair IVm-D. In these experiments two major PCR products were observed (Fig. 2 *D*). Again, the both bands hybridized with the M2 probe, but only the larger PCR product hybridized with the M1 probe. This observation independently confirms the existence of an RNA form containing M2 but no M1 sequence.

Sequence Analysis of the ε mRNA Species Containing Membrane Exon Sequence. To determine the exact structure of the PCR products, PCR mixtures amplified from both of the cell sources were cloned into a plasmid vector for sequence analysis. The two sets of primer pairs used were IVb-C and IVm-D.

Products corresponding to three types of RNA splicing were observed (Fig. 3). One product, isolated from the larger of the two PCR products from each primer set, includes both

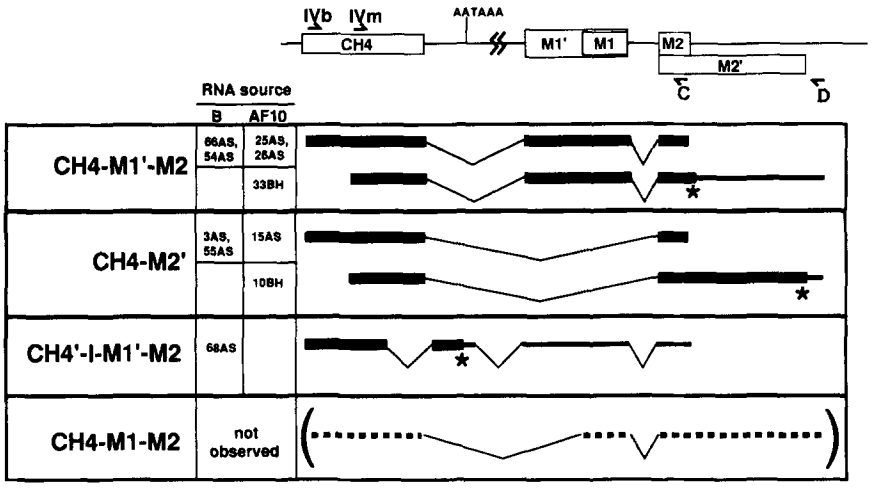

Figure 3. RNA species defined by PCR products analyzed in this study. The three species demonstrated by PCR amplification and nucleotide sequence analysis are diagrammed. The names of the corresponding clones used for sequence analysis are given under the cell source of the RNA (stimulated B cells or AF10). The bold horizontal bars represent translated exon sequence. The asterisks show the positions of in-frame termination codons, with the thinner horizontal lines representing 3′ untranslated sequence. The fourth RNA splice form (CH-M1-M2) was not observed in the present experiments.

Downloaded from www.jem.org on October 23, 2005

M1 and M2 sequences. However, the 5′ end of the human M1 defined by this PCR product is 156 bp 5′ of the glutamate residue (at nucleotide 182; Fig. 1, *A* and *C*), which is homologous to the 5′ end of murine M1 exon. We refer to this human exon as M1′ to distinguish it from the smaller murine exon M1. The M2 exon of this splice product encodes 27 amino acids and ends at position 470 in Fig. 1 *C*. The reading frame of this RNA splice product is defined by the splice donor site at the 3′ end of the ϵ CH4 exon. Our sequence analysis of the PCR products defines the end of the membrane form of the CH4 exon to be at nucleotide 324 in Fig. 1 *B*. This pattern of splicing for membrane ϵ utilizes the reading frame that encodes an amino acid sequence homologous to that reported for the murine M1 and M2 exons (as shown in Fig. 1 *A*).

The second RNA form, corresponding to the smaller PCR product in each amplification, splices from the same position of CH4 directly to the M2 exon (diagramed in Fig. 3). The omission of M1 in this form causes the reading frame of M2 to be shifted from that used in the CH4-M1′-M2 form. The position of the first termination codon in the new reading frame, at nucleotide 796 in Fig. 1 *C*, defines a much longer coding exon, which we designate M2′. This exon encodes 136 amino acids in contrast to the 27 residues of M2. This RNA form is designated CH4-M2′ in Fig. 3. Because the conserved hydrophobic region encoded by M1 is absent from this RNA, it should encode a secreted protein; this protein would be 134 amino acids longer than the conventional ϵ heavy chain. To explore whether such a protein could be detected, Western blot analysis was performed on serum from a patient with IgE myeloma and on AF10 cell supernatant and lysate (Fig. 4). In each sample, a band of ∼97 kD was observed after development of the blot with specific anti-ϵ antibody. This band likely represents the large ϵ IgE coded for by the CH4 to M2′ exon splice, as that protein should be 134 amino acids (∼15 kD) larger than the classic secreted form.

The third RNA form detected in our PCR products is represented by a single clone derived from the larger PCR band amplified from the RNA of B lymphocytes stimulated by IL-4 and anti-CD40. In this RNA a splice occurred from a donor site within CH4 (position 218 in Fig. 1 *B*). The sequence of the clone then jumps to a short segment derived from the 3′ untranslated region downstream of CH4, represented by nucleotides 359–465 in Fig. 1 *B*. The 3′ end of the clone includes exons M1′ and M2 spliced as in the CH4-M1′-M2 form. The translation of the RNA corresponding to this clone would be terminated by the TAA at position 440 of Fig. 1 *B*, leading to a protein that lacks a membrane anchor and is almost identical in size to the classic secreted form of ϵ heavy chain. This RNA structure (designated CH4′-I-M1′-M2 in Fig. 3) is probably not an RT-PCR artifact in that the splice donor and acceptor sites are reasonably consistent with consensus sites; but it may represent an atypical splice product and is not considered further.

Search for a Splice Product Homologous to the Murine CH4-M1-M2 Form. Because of the high degree of sequence similarity between the human and murine genomic DNA sequences in the ϵ membrane region, we initially expected to detect evidence of an RNA species similar to the CH4-M1-M2 product suggested for the mouse membrane ϵ structure. Indeed, another laboratory has described such a form for human ϵ mRNA (6). Since no PCR products of the size expected for such an RNA form were detected in our experiments described above, amplification with a third primer set was undertaken specifically to look for such an RNA form. In this experiment RNA derived from both AF10 cells and IL-4 plus CD40-stimulated human B cells was reverse transcribed and the cDNA amplified with the primer pair IVm-B; this pair was chosen to simplify the pattern of PCR products by eliminating any contribution of the CH4-M2 splice form. With these primers the PCR product expected for the CH4-M1′-M2 splice form should be 453 bp (including the lengths of the primer "tails" containing restriction sites), while the product of the corresponding CH4-M1-M2 form should be 286 bp. The amplification yielded only one band, of ∼453 bp (Fig. 2 *E*); no 286-bp band was seen even on long exposure of a Southern blot. Therefore, our data provide no evidence for the existence of any RNA splice form that utilizes the splice acceptor site (position 183) that is homologous to that at the 5′ end of the murine M1 exon.

Identity of ϵ mRNA Species Detected on Northern Blots. To determine the relationship between the three RNA species detected on Northern blots and the RNA splice forms re-

Downloaded from www.jem.org on October 23, 2005

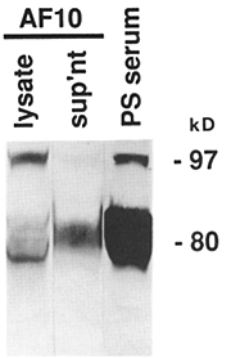

Figure 4. Western blot identification of a large secreted ϵ protein. The indicated protein samples were detected in a Western blot developed by binding to a mouse anti-ϵ mAb CIA-E-7.12 and a labeled anti-mouse IgG. The 80-kD band represents the classic secreted IgE protein, which shows some heterogeneity probably resulting from alternative glycosylation. The band marked 97 kD is close to the size expected for the large secreted protein resulting from the CH4-M2′ splice. Similar results were obtained using a rabbit anti-human IgE antiserum. The size estimates are approximate.

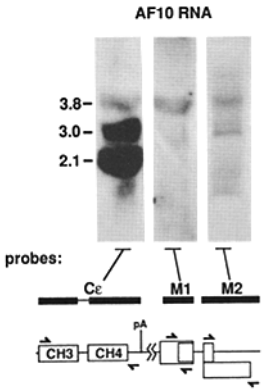

Figure 5. Identification of RNA species visualized by Northern blot analysis. Total RNA (20 μg) from AF10 cells was subjected to electrophoresis and blotted in triplicate. The three probes were generated by RT-PCR using primer pairs G-H (for Cϵ), 1-B (for M1′), and 3-D (for M2-M2′). The 3.8-kb band hybridizes with all three probes; but the 3.0 hybridizes only with Cϵ and M2, while the 2.1 hybridizes with Cϵ alone.

vealed by sequence analysis of our PCR products, a blot of RNA from AF-10 cells was hybridized to three different probes. As shown in the first lane in Fig. 5, a Cϵ probe identifies the three bands previously described in RNA from human IgE-secreting cells (4) of 3.8, 3.0 and 2.1 kb. The 2.1-kb band does not hybridize to either of the two probes derived from the membrane locus and therefore should represent the mRNA encoding the "classical" secreted ϵ heavy chain. The 3.0-kb band hybridizes to the M2 probe but not the M1; it therefore corresponds to the CH4-M2′ splice form that we detected encoding a new, large secreted form of ϵ. Finally, the 3.8-kb band, as well as hybridizing to the M2 probe, is the only band that hybridizes to the M1 probe; this would be consistent with its identification with the CH4-M1′-M2 form encoding the membrane ϵ heavy chain. Because the size differences between these three bands are much greater than can be accounted for by the presence or absence of exons identified in our PCR products, it is evident that other factors (such as differing poly(A) addition sites or differing lengths of poly(A) tails) contribute to the length of the mature RNAs.

Discussion

An Unusual Membrane Ig. Igs exist in two forms (membrane bound or secreted) depending on alternative splicing and polyadenylation patterns of primary RNA transcripts. Indeed, the alternative splicing of the μ heavy chain (14) was one of the early examples of this mechanism by which two proteins can be encoded by a single gene. For the secreted Ig form, the COOH-terminal amino acids of the heavy chain are encoded contiguously with the final Ig domain. For the membrane Ig form, RNA splicing eliminates the COOH-terminal residues of the secreted form and joins the remaining part of last Ig domain exon to one or two exons that encode amino acids characteristic of membrane Ig. The features of these membrane peptide segments are shown in Fig. 6, which includes the sequences of human and murine Ig membrane segments published to date. The most characteristic feature is a segment of uncharged, mostly hydrophobic amino acids that presumably anchors the protein in the cell membrane lipid. With respect to this transmembrane segment, we find the human ϵ membrane sequence to be typical, including most of the consensus amino acid residues found in other isotypes as well as a typical number of hydroxyl amino acids and a single cysteine. Some of these residues may play a role in interactions with other membrane-bound proteins that form part of the antigen receptor signal transduction machinery on the cell surface. Despite conservation of these features of the transmembrane region, it is clear that the degree of human-murine sequence similarity is lowest for the ϵ membrane exons as compared with the membrane exons of all other available isotypes. The relatively poor sequence conservation between human and mouse is also seen in the Ig domains of Cϵ. On the COOH-terminal cytoplasmic side of the transmembrane region, all of the sequences show at least one positively charged residue that is presumably important for establishing the orientation of the protein in the membrane (15) and may also play a role in directing the protein to appropriate posttranslational processing pathways (16). The length of the cytoplasmic domain of the human ϵ sequence is identical to that of the murine homologue and to all of the published γ membrane forms of both species (27 residues, counting from the conserved valine just beyond the transmembrane segment).

On the extracellular side near the transmembrane segment, the human ϵ sequence is typical in having a high density of negatively charged residues. These are also thought to play a role in orienting the protein with respect to the membrane topology.

Downloaded from www.jem.org on October 23, 2005

```
                                                  (-)          -----TRANSMEMBRANE-----   (+)                                  Ref

μ    Human                                          EGEVSADEE GFENLWATASTFIVLFLLSLFYSTTVTLFKVK                              a
     Mouse                                          ----N-E-- ------T-------------------------                              b
δ    Human                          LAMTPLIPQSKDENSDDYTTFDDV   GSLWTTLSTFVALFILTLLYSGIVTFIKVK                              c
     Mouse                          IVN-IQHSCIM--Q--S-MDLEEE   NG--P-MC------L-------F-------                              d
γ    Human:1                                  ELQLEESCAEAQDG ELDGLWTTITIFITLFLLSVCYSATVTFFKVKWIFSSVVDLKQTIIPDYRNMIGQGA      e
          3                                   -------------- -----------L-------------------------------------------      f
          ψ                                   -------------- ----------------------------------------V-----------      e
     Mouse:1                                  G---D-T------- -------------S----------A--L-----------E----LV-E-K-----AP      g
          2a                                  G-DDV--------- -------------S----------S--L-----------E-----S-----------      g
          2b                                  --E-NGT------- -------------S----------S--L-----------E---K-S---G-------      g
          3                                   --E-NGT------- -------------S----------S--L-----------QV---A-----------      g
α    Human:α1                     gscsvaDWQMPPPYVVLDLPQETLEEETPGANLWPTTITFLTLFLLSLFYSTALTVTSVRGPSGNREGPQY                    f,h
          α2                      ---c-------------------------------------------------------------K------                f,h
     Mouse                                ER-E-LS--L--QS-D-----A---S-----V-------------------T----F-SK-V---                   i
ε    Human      GLAGGSAQSQRAPDRVLCHSGQQQ
                 GLPRAAGGSVPHPRCHCGAGRADWPG PPEL   DVCVEEAEGEAPWT WTGLCIFAALFLLSVSYSAALTLLMVQRFLSATRQGRPQTSLDYTNVLQPHA      j
     Mouse                                   --DLQ-L-I--V---ELEEL--SI-V-IT--------G-TG-V-K-KWV--TPM-DT---FQ--A-I--TR-      k

                                CONSENSUS                          LW     F  LFLL   YS   T   V
```

Figure 6. Amino acid sequences encoded by membrane exons. The available human and murine sequences are aligned to highlight conserved features, including the hydrophobic transmembrane region, flanked by acidic (extracellular) residues and basic (intracellular) residues. Within each isotype a human sequence was chosen as prototype, and other human and murine sequences are shown below by listing the residues that differ from the prototype. The six residues of human α that are listed in lower case letters represent the translation from an alternative RNA splice acceptor site. Consensus residues listed include those that appear in all aligned sequences (*underlined*) or all except one sequence (*not underlined*). References for the sequences are as follows: (*a*) 22; (*b*) 23; (*c*) 24; (*d*) 25; (*e*) 26; (*f*) 27; (*g*) 28; (*h*) 29; (*i*) 30; (*j*) this paper; (*k*) 31.

However, the human ε membrane sequence is unique among all the currently described murine and human membrane Igs in the length of the peptide segment between the membrane and the nearest extracellular Ig domain. The number of residues in this proximal extracellular region is characteristic for each isotype and well conserved between species, ranging from a minimum of 14 in μ (counting to the conserved tryptophan that begins the transmembrane segment) to 33 in the long form of human α1 and α2 heavy chains. A shorter 27-residue form of the human α isotypes is also produced by an alternative splice (17), and the latter form matches the length of the murine homologue. In contrast to the 20-residue length of this region in murine ε, the corresponding human segment is 68 amino acids long. Although the available sequence data in the murine sequence are limiting, from the sequence similarity between the two species upstream of the murine M1 exon (Fig. 1 *C*), it is tempting to speculate that the longer M1′ sequence in humans was derived from the conversion of intron to exon sequence as a consequence of mutations affecting splice acceptor sites. As a hypothetical example of such a mechanism, the 9-bp deletion in the human sequence between nucleotides 177 and 178 (Fig. 1 *A*) and the A at position 172 may combine to "weaken" the splice acceptor activity of the AG dinucleotide at positions 181-182, since these sequence differences (vs. the murine gene) bring another AG dinucleotide (positions 172-173) too close for the optimum sequence configuration of a splice acceptor site (18). As a result, the more upstream splice acceptor site at position 28 (Fig. 1 *C*) may be used preferentially. Alternatively, it is possible that a long exon in the common ancestor of mice and humans was shortened in the mouse line by a converse mechanism.

The amino acid sequence of this long proximal extracellular region reveals a proline-rich peptide that contains four cysteine residues, two of which are separated by only a single amino acid. The biologic function of this extracellular segment of membrane-bound IgE remains to be determined. Besides potentially serving as a site for inter–ε chain binding, it could play a role in binding to specific ligands. It is now appreciated that membrane Ig serves as only the "core" of a signal transducing complex (19). In this perspective one attractive possibility for the function of this new peptide segment may be to bind to other B cell surface molecules (e.g., CD45 in its various isoforms) important in the mIgE signal transducing complex. If this peptide does play a significant functional role, it could potentially serve as a target for manipulation of IgE expression in clinical situations.

Do human ε RNA transcripts ever splice at the position homologous to the murine splice acceptor? This question arose because of a preliminary account from Davis et al. (20) reporting such an RNA form and suggesting the existence of a corresponding translated protein. Our PCR experiments using mRNA from both the AF10 cell line and human lymphocytes did not detect this form using three different primer pairs, including one pair specifically designed to search for this form. It is possible that the difference in results may be accounted for by our use of fresh human B lymphocytes and the AF10 clone rather than the cells lines used by Davis et al. (20). Alternatively, the CH4-M1-M2 short splice product may exist in stimulated normal B cells but at such a low concentration that our PCR amplifications were unable to detect it. The very recent report of Peng et al. (6) indicated that indeed the CH4-M1-M2 product may be present in <1% of the concentration of the species containing the longer M1′ exon. Furthermore, no protein product corresponding to the shorter splice form could be detected by those investigators. Thus, our results and those of Peng et al. (6) support the view that the CH4-M1′-M2 RNA species encodes the overwhelmingly dominant form of membrane IgE.

Transcripts Encoding a New Secreted Form of IgE. The second RNA form we have identified results from a splice that excludes the M1′ exon entirely. The M1′ exon (like the murine M1 exon) has the unusual feature that its length is not a multiple of three; thus, it begins after the first nucleotide of one codon but ends after the third nucleotide of another. Therefore, with its omission, the direct splicing of CH4 to M2 leads to a shift in the reading frame of the latter exon. This means that the nucleotide sequence from positions 389 to 469 (Fig. 1 *C*) has the unusual feature of being used in more than one reading frame. The coding sequence of the shifted reading frame, which we designate M2′, includes 136 amino acids before the stop codon at position 797. Clearly, this splice form lacks the conserved transmembrane segment encoded in exon M1′. To see whether the sequence might contain a hydrophobic region that could serve as an alternative lipid anchor, the sequence was examined using the Kyte-Doolittle algorithm (implemented for the Macintosh in the MacVector software package). As shown in Fig. 7, while the protein encoded by the CH4-M1′-M2 splice form shows a clear hydrophobic peak representing the transmembrane segment (around residue 500), such a hydrophobic peak is lacking from the translated CH4-M2′ form as well as from the classical secreted form. This suggests that the product encoded by the CH4-M2′ form is likely to be a second form of secreted ε heavy chain, 134 amino acids larger than the "classical" secreted protein. On the basis of the relative intensities of the Northern blot bands representing this CH4-M2′ form (3.0 kb) and the "classical" secreted form (2.1 kb), it would appear that the RNAs encoding the two forms are present in a ratio of ∼1:2 (4).

We have detected a form of secreted ε protein of about the size expected for the translated product of the CH4-M2′ RNA. In Western blots of AF10 cell line supernatant and of serum from an IgE myeloma patient, a protein was observed that reacted with the anti–human-ε mAb CIA-E-7.12 (9) (as well as with rabbit anti–human ε antiserum) and that is ∼17 kD larger than the major secreted ε protein band (Fig. 4). The latter band corresponds in size to the ε protein encoded by the 2.1-kb classic secreted mRNA while the larger protein is likely the translation product of the 3.0-kb RNA. While the relative amounts of the two protein species seen in the AF-10 supernatant are not the 1:2 predicted from the mRNA levels, the supernatant for the Western blot experiment was generated by growing the cells under the very

Downloaded from www.jem.org on October 23, 2005

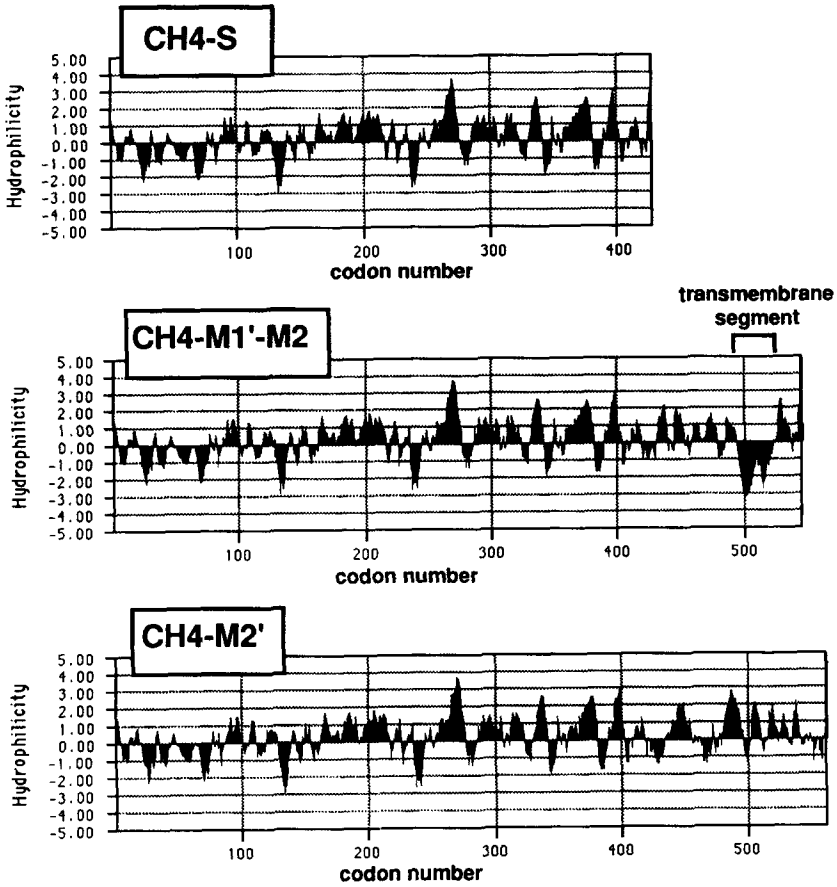

Figure 7. Hydrophilicity plots for three human ε amino acid sequences. Plots of the Kyte-Doolittle hydrophilicity parameter are shown for the Cε sequences corresponding to the 427-residue secreted form (CH4-S), the 545-residue membrane form translated from the CH4-M1'-M2 RNA species described here, and the putative second secreted form (561 residues) translated from the CH4-M2' RNA species. Only the membrane form shows a clear hydrophobic segment, around codon 500.

difficult conditions of serum-free medium for 7 d; these culture conditions may have altered the relative amounts of the two proteins.

In an experiment reported in abstract form, Kim et al. (21) found evidence for a protein larger than the classical secreted ε chain using an antiserum against a synthetic peptide derived from the M2' reading frame. However, it should be noted that the nucleotide sequence initially reported by that group differs from ours by omitting a G at position 473 in Fig. 1 *C* (as well as by several other differences further downstream); the one-nucleotide omission would throw their remaining downstream sequence out of frame, including some residues incorporated into their peptide antigen. Thus, if our sequence is correct (and it is in fact confirmed by the recent paper of Peng et al. [6]), part of the peptide antigen used by Kim et al. (21) would not correspond to the correct sequence of this protein. Nevertheless, their antiserum may contain enough antibodies against the part of their peptide representing the correct sequence that their identification of this new protein is valid. Additional experiments are under way in our laboratory to clarify this point.

The existence of a second form of circulating IgE could have important implications for our understanding of immediate hypersensitivity reactions. One can speculate that the addition of 134 amino acids to the "classical" secreted form might have significant effects on the ability of the protein to bind to the high- and low-affinity Fcε receptors. Furthermore, if the new secreted form has a function, one might expect the RNA splicing pathways leading to the synthesis of these two ε chains to be tightly regulated. Indeed, we have reported that suppression of IgE production by an anti-CD23 monoclonal is associated with decreases in both the 2.1- and 3.0-kb RNAs, while the 3.8-kb species encoding the membrane form is unaffected (5). Abnormalities in the regulation of the alternative splice pathways might be associated with disease states. Further work will be necessary to explore these possibilities. It is interesting to note that Northern blots of murine RNA were also reported too show three bands (2.2, 3.15 and 3.7 kb) similar to those we have found in humans (4). Thus, it is possible that the existence of three RNA splice pathways is a general feature of the ε locus.

Downloaded from www.jem.org on October 23, 2005

We acknowledge the excellent technical assistance of Hai-Kit Cheah, Miheala Kube-Leamer, and Mary Malarkey-Briggs. We thank Dr. Eugene Medlock (Amgen Inc., Thousand Oaks, CA) for the generous gift of IL-4 and Dr. Ed Clark (University of Washington, Seattle, WA) for mAb G28-5 reactive with CD40. We also thank Dr. Randolph Wall for his advice, encouragement, and thoughtful critiques.

This work was in part supported by U.S. Public Health Service grants AI-15251 and AI-15332 (Center for Interdisciplinary Research in Immunologic Diseases and UCLA), a gift from the Clemente Foundation, and a gift from Mr. Robert and Mrs. Sara LeBien.

Address correspondence to Edward E. Max, FDA Center for Biologics, Building 29A, HFB-800, Bethesda, MD 20892.

Received for publication 22 January 1992 and in revised form 3 April 1992.

Note added in proof: We have now confirmed the existence of the rare CH4-M1-M2 splice form reported by Peng et al. (6). In our hands this form was detectable by PCR when a new upstream primer near the 3′ end of CH4 (AGGCAGCGAGCCCCTCACAGACCG, corresponding to positions 274–297 of Fig. 1 *B*) was used with downstream primer B. With these primers the CH4-M1-M2 form appeared as a very minor band on Southern blots of RT-PCR samples derived from both AF10 cells and B cells treated with IL-4 plus CD40. The identity of the band was established by cloning and sequence analysis. This result does not change the conclusion from our paper that this form is quite rare compared with the CH4-M1′-M2 form.

Downloaded from www.jem.org on October 23, 2005

References

1. Ishizaka, K., T. Ishizaka, and M.M. Hornbrook. 1966. Physiochemical properties of human reagenic antibody V. Presence of a unique immunoglobulin as carrier of reagenic activity. *J. Immunol.* 97:75.
2. Bennich, H., and S.G.O. Johansson. 1971. Structure and function of human immunoglobulin E. *Adv. Immunol.* 13:1.
3. Max, E., J. Battey, R. Ney, I. Kirsch, and P. Leder. 1982. Duplication and deletion in the human immunoglobulin E genes. *Cell.* 29:691.
4. Saxon, A., K. Behle, M. Kurbe-Leamer, E.E. Max, and K. Zhang. 1991. Inhibition of human IgE production via FceR-II stimulation results from a decrease in the mRNA for secreted but not membrane epsilon (ϵ) heavy chains. *J. Immunol.* 147:4000.
5. Ishida, Y., S. Ueda, H. Hayashida, T. Miyata, and T. Honjo. 1982. The nucleotide sequence of the mouse immunoglobulin epsilon gene: comparison with the human epsilon gene sequence. *EMBO (Eur. Mol. Biol. Organ.) J.* 1:1117.
6. Peng, C., F.M. Davis, L.K. Sun, R.S. Liou, Y. Kim, and T.W. Chang. 1992. A new isoform of human membrane bound IgE. *J. Immunol.* 148:129.
7. Flanagan, J.G., and T.H. Robbitts. 1982. Arrangement of human immunoglobulin heavy chain constant region genes implies evolutionary duplication of a segment containing γ, ϵ, and α genes. *Nature (Lond.).* 300:709.
8. Sun, L.K., R.S. Liou, N.C. Sun, L.A. Gossett, C.R.Y. Sun, F.M. Davis, D.W. MacGlashan, Jr., and T.W. Chang. 1991. Transfectomas expressing both secreted and membrane-bound forms of chimeric IgE with anti-viral specificity. *J. Immunol.* 146:199.
9. Hassner, A., and A. Saxon. 1984. Isotype-specific human suppressor T cells for IgE synthesis activated by IgE-Anti-IgE immune complexes. *J. Immunol.* 132(6):2844.
10. Saxon, A., J. Feldhaus, and R.A. Robins. 1976. Single step separation of human T and B cells using AET treated SRBC rosettes. *J. Immunol. Methods.* 12:285.
11. Saxon, A., and J. Portis. 1977. Lymphoid subpopulation changes in regional lymph nodes in squamous head and neck cancer. *Cancer Res.* 37:1154.
12. Maniatis, T., E.F. Fritsch, and J. Sambrook. 1982. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY. 191–193.
13. Zorn, A.M., and P.A. Krieg. 1991. PCR analysis of alternative splicing pathways: identification of artifacts generated by heteroduplex formation. *Biotechniques.* 11:180.
14. Early, P., J. Rogers, M. Davis, K. Calame, M. Bond, R. Wall, and L. Hood. 1980. Two mRNA's can be produced from a single immunoglobulin μ gene by alternative RNA processing pathways. *Cell.* 20:313.
15. Boyd, D., and J. Beckwith. 1980. The role of charged amino acids in the localization of secreted and membrane proteins. *Cell.* 62:1031.
16. Mitchell, R.N., A.C. Shaw, Y.K., Weaver, P. Leder, and A.K. Abbas. 1991. Cytoplasmic tail deletion converts membrane immunoglobulin to a phosphatidylinositol-linked form lacking signaling and efficient antigen internalization functions. *J. Biol. Chem.* 266:8856.
17. Yu, L., C. Peng, S.M. Starnes, R.S. Liou, and T.W. Chang. 1990. Two isoforms of human membrane-bound α immunoglobulin resulting from alternative mRNA splicing in the membrane segment. *J. Immunol.* 145:3932.
18. Self, I., G. Khoury, and R. Dhar. 1979. BKV splice sequences based on analysis of preferred donor and acceptor sites. *Nucleic Acids Res.* 6:3387.
19. Cambier, J.C., and K.S. Campbell. 1990. Signal transduction by B lymphocyte receptors: structure-function relationships of membrane immunoglobulins and associated molecules. *Sem. Immunol.* 2:139.
20. Davis, F.M., Y.M. Kim, L.A. Gossett, and T.W. Chang. 1991. Two isoforms of membrane-bound human IgE. *FASEB (Fed.*

Am. Soc. Exp. Biol.) J. 5:605*a*. (Abstr.)
21. Kim, Y.M., F.M. Davis, C. Peng, L. Yu, L.K. Sun, R.S. Liou, and T.W. Chang. 1991. Secretion of a new IgE subclass by human IgE-producing cell lines. *FASEB (Fed. Am. Soc. Exp. Biol). J.* 5:1086*a*. (Abstr.)
22. Milstein, C.P., E.V. Deverson, and T.H. Rabbitts. 1984. The sequence of the human immunoglobulin μ-δ intron reveals possible vestigial switch segments. *Nucleic Acids Res.* 12:6523.
23. Early, P., J. Rogers, M. Davis, K. Calame, M. Bond, R. Wall, and L.E. Hood. 1980. Two mRNAs can be produced from a single immunoglobulin μ gene by alternative RNA processing pathways. *Cell.* 20:313.
24. White, M.B., A.L. Shen, C.J. Word, P.W. Tucker, and F.R. Blattner. 1985. Human immunoglobulin D: Genomic sequence of the delta heavy chain. *Science (Wash. DC).* 228:733.
25. Cheng, H.L., F.R. Blattner, L. Fitzmaurice, J.F. Mushinski, and P.W. Tucker. 1982. Structure of genes for membrane and secreted murine IgD heavy chains. *Nature (Lond.).* 296:410.
26. Kinoshita, K., A. Shimizu, and T. Honjo. 1991. The membrane exons of the pseudo-γ-chain gene of the human immunoglobulin are apparently functional and highly homologous to those of the γ1 gene. *Immunol. Lett.* 27:151.
27. Bensmana, M., and M.P. Lefranc. 1990. Gene segments encoding membrane domains of immunoglobulin gamma 3 and alpha chains. *Immunogenetics.* 32:321.
28. Yamawaki-Kataoka, Y., S. Nakai, T. Miyata, and T. Honjo. 1982. Nucleotide sequences of gene segments encoding membrane domains of immunoglobulin gamma chains. *Proc. Natl. Acad. Sci. USA.* 79:2623.
29. Yu, L., C. Peng, S.M. Starnes, R.S. Liou, and T.W. Chang. 1990. Two isoforms of human membrane-bound α Ig resulting from alternative mRNA splicing in the membrane segment. *J. Immunol.* 145:3932.
30. Word, C.J., J.F. Mushinski, and P.W. Tucker. 1983. The murine immunoglobulin alpha gene expresses multiple transcripts from a unique membrane exon. *EMBO (Eur. Mol. Biol. Organ.) J.* 2:887.
31. Ishida, M., S. Ueda, H. Hayashida, T. Miyata, and T. Honjo. 1982. The nucleotide sequence of the mouse immunoglobulin epsilon gene: comparison with the human epsilon gene sequence. *EMBO (Eur. Mol. Biol. Organ.) J.* 1:1117.

Downloaded from www.jem.org on October 23, 2005