

Complex Alternative RNA Splicing of ϵ -Immunoglobulin Transcripts Produces mRNAs Encoding Four Potential Secreted Protein Isoforms*

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In RNA from human IgE-producing lymphocytes, we previously discovered two alternatively spliced ϵ -immunoglobulin mRNA isoforms that encode a novel secreted form of IgE and a membrane-bound species. Further analysis using ϵ -specific reverse transcriptase polymerase chain reactions has elucidated several additional alternatively spliced species of ϵ mRNA. One RNA isoform is generated by splicing the CH4 exon to a novel distal splice acceptor site, forming an ϵ RNA species (designated CH4-M2') that encodes a secreted ϵ protein 6 amino acids larger than the classic secreted ϵ protein. The other three novel ϵ RNAs are generated by splicing from within CH4 to a new exon structure (designated CH5) that is located between CH4 and the membrane exons. Since the three new mRNAs using CH5 share the same stop codon in CH5, they all encode the same novel protein, which is 10 amino acids shorter than the classic secreted ϵ heavy chain. The new alternatively spliced ϵ mRNAs reported here, in addition to the previously reported forms encoding membrane and larger secreted IgE, appear to reflect the normal splicing pattern in humans, as we have detected all these ϵ RNAs in all the human IgE-secreting cells and cell lines tested.

Humans have only one functional ϵ -immunoglobulin gene (1), which produces ϵ heavy chains for IgE, the immunoglobulin class that mediates immediate hypersensitivity diseases (2). We (3) and others (4, 5) previously reported that human IgE-secreting cells produce, in addition to the "classic" secreted and membrane forms of the ϵ heavy chain, RNAs encoding a novel membrane and secreted form generated by alternative RNA splicing of downstream "membrane" exons. The ϵ mRNA coding for the dominant membrane IgE form has the structure of the ϵ constant region exons (CH1-CH4) plus two membrane exons (M1' plus M2), in which M1' includes the human sequence homologous to the murine M1 counterpart as well as a unique segment of 156-nucleotide sequence upstream of the M1 exon that encodes an additional 52 amino acids (3-5). This structure

we designate CH4-M1'-M2. The second membrane form is encoded by a very minor RNA isoform with the structure CH4-M1-M2, i.e. using M1 instead of M1'. The other novel ϵ RNA containing membrane exon sequence was found to have the structure CH4-M2', in which M2' is read in an alternative reading frame from that of M2. This species of RNA, because of its lack of a hydrophobic transmembrane segment, encodes a secreted ϵ heavy chain that is 134 amino acids larger than the classic secreted protein (3). Western blot analysis of human polyclonal IgE from serum and cell lysates showed a secreted ϵ protein of the size predicted for the translation product of this large form of RNA (3).

However, our Western blot analysis always showed that, in addition to the species discussed above, human polyclonal IgE exhibits a heterogeneity of protein bands around the size of the classic secreted ϵ protein. These findings suggested the existence of yet additional forms of ϵ proteins resulting from alternative ϵ mRNA splicing, and our previous studies also lent themselves to such an interpretation. In this report, we describe five more novel species of ϵ RNA that are generated by alternative splicing and that encode two more novel secreted ϵ proteins. Functional differences among the various resulting IgE proteins may be relevant to diseases involving immediate hypersensitivity.

MATERIALS AND METHODS

Cell Lines and Cell Cultures—The human IgE-secreting cell line AF-10 (a derivative of human myeloma U266) was maintained in complete RPMI 1640 medium as described previously (6). Epstein-Barr virus-transformed interleukin-4-induced IgE-secreting human B cell lines 2C4/F3 and 2C10/F9 (7) (kindly provided by Dr. Fred D. Finkelman) were maintained in complete RPMI 1640 medium supplemented with 50 mM Hepes and 3×10^{-5} M 2-mercaptoethanol (Sigma). Fresh purified human B cells were isolated from tonsil and purified by the methods described previously (8). For induction of productive ϵ transcripts, purified B cells (1×10^6 cells/ml) were cultured for 7 days in the presence or absence of 100 units/ml recombinant interleukin-4 (a kind gift of Dr. Eugene Medlock, Amgen Inc., Thousand Oaks, CA) plus 0.1 μ g/ml anti-CD40 monoclonal antibody G28-5 (9). Human B cells spontaneously producing IgE were obtained from patients with the hyper-IgE immunodeficiency syndrome or atopic dermatitis.

Western Blot Analysis—Western blot analysis was performed as previously described (3). IgE was first immunoprecipitated from culture supernatant and patient sera by anti-IgE monoclonal antibody (clone CIA-E-7.12), followed by treatment with recombinant endoglycosidase H (Boehringer Mannheim) as described (10).

Amplification—RNA was isolated from the various cells and reverse-transcribed by standard methods as described (3). Pairs of PCR¹ primers were designed to detect different forms of ϵ mRNA containing membrane exon sequences (see Fig. 2 and Table I). Some primers were designed with terminal enzyme restriction sites to facilitate cloning and

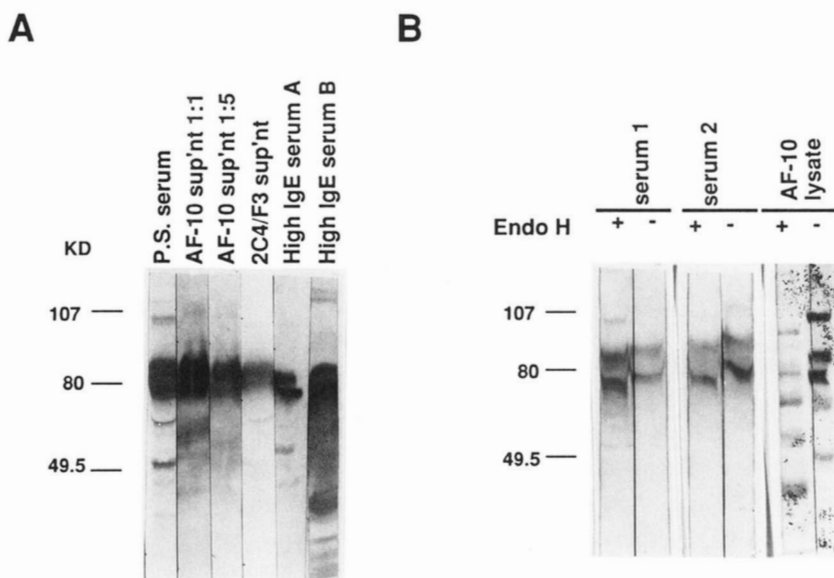
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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EMBL Data Bank with accession number(s) X63693.

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¹ The abbreviation used is: PCR, polymerase chain reaction.

FIG. 1. Western blot analysis of polyclonal IgE. IgE-containing culture supernatant (*sup'nt*) and patient sera (*P.S.*) were immunoprecipitated by anti-IgE monoclonal antibody and stained with alkaline phosphatase-labeled goat anti-human IgE. A, shown is the exhibition of heterogeneity of polyclonal IgE from different samples; B, the heterogeneity of polyclonal IgE cannot be eliminated by treatment with endoglycosidase H (*Endo H*). The reduction in size of the visualized bands in lanes treated with endoglycosidase H (+ lanes) compared with the lanes representing untreated controls demonstrates that the enzyme was active.



sequencing of PCR products. PCR amplifications were performed for 40 cycles under the conditions described (3).

Southern Blot Analysis of PCR Products—PCR products were electrophoresed on 1.5% agarose gel. DNA was transferred to nylon membranes (Nytran, Schleicher & Schuell) in 0.4 M NaOH. Blots were analyzed by probing with end-labeled oligonucleotides corresponding to various regions from the genomic sequence as shown in Table I. Hybridization with phosphorylated oligonucleotide probes was performed as described (3).

Analysis of Amplified ϵ cDNAs—PCR products were digested with *Cla*I and *Sal*I (to uncover the sticky ends designed into the PCR primers), purified by agarose gel electrophoresis, and then ligated into Bluescript® (Stratagene, La Jolla, CA) vector that had been prepared by *Cla*I plus *Sal*I digestion and treatment with alkaline phosphatase. Plasmid minipreps were analyzed by restriction digestion and hybridization with internal probes. The inserts of the selected clones were sequenced on both strands by the dideoxy termination method (11) using a sequencing kit purchased from United States Biochemical Corp.

RESULTS

Splicing of CH4 to Novel Splice Acceptor Site inside M2' Exon—Multiple ϵ bands appeared on Western blots of human secreted IgE both from human serum and from human cells producing IgE. This heterogeneity could not be eliminated by treatment with endoglycosidase H to remove N-linked glycosylation (Fig. 1). Although other types of glycosylation could contribute to the heterogeneity, we considered the possibility that splicing of ϵ -immunoglobulin heavy chain RNAs might be more complex than previously described. In our earlier reverse transcriptase PCR studies of alternative ϵ RNA splicing, we had observed a small PCR product that we had considered a possible artifact since it did not hybridize with several probes internal to exon M1 or M2 (Fig. 2D in Ref. 3). To examine the possibility that this band reflected a genuine splice form containing sequence downstream of the previously tested M2 probes, we subjected RNA from the IgE-secreting cell line AF-10 to reverse transcriptase PCR using the primer pair 3-8 (Fig. 2 and Table I) and included a far downstream probe in our hybridization analysis. As shown in Fig. 3A, amplifications with this primer pair consistently generated five bands with RNA derived from all IgE⁺ cells tested. All five of these products hybridized with a probe located downstream of the M2' exon (Table I, oligonucleotide 7), but only the larger (upper) four bands hybridized with an M2' internal probe located in the beginning of the M2' exon (Table I, oligonucleotide 6). These results suggested that one form of ϵ mRNA, represented by band 5, results from splicing to a novel acceptor site inside the M2' exon between oligonucleotides 6 and 7.

Because PCR-generated heteroduplex formation between alternative spliced products may cause artifactual bands (3, 12), we investigated whether any of the observed bands represented heteroduplexes. When DNA corresponding to bands 2 and 4 was recovered and subcloned, DNA from band 2 always gave subclones identical to those of band 1 or 3, whereas band 4 always gave subclones identical to those of band 3 or 5. No subclones with inserts the size of those of band 2 or 4 were ever obtained in these experiments (data not shown). On the other hand, bands 2 and 4 could be generated on gels by reamplifying the DNA templates from mixtures of the subcloned bands 1, 3, and 5 (Fig. 3B). Amplification of templates corresponding to a single subclone never produced band 2 or 4. These experiments indicated that bands 1, 3, and 5 represent true individual PCR products, whereas bands 2 and 4 are PCR-generated heteroduplexes.

Of the three true PCR products amplified with primer pair 3-8, bands 1 and 3 had the correct size and hybridization characteristics expected for two RNA splice forms that we previously described: CH4-M1'-M2 and CH4-M2', respectively. These identities were confirmed by sequence analysis. Sequencing further showed that the band 5 products derived from all of our IgE⁺ cell RNAs were identical, reflecting an RNA spliced from the usual donor site near the 3'-end of CH4 to a novel acceptor site in the terminal portion of the M2' exon (nucleotide 421 in Fig. 2B). The sequence at this position is a typical splice acceptor site (Fig. 2B and Table II). This novel spliced RNA (designated CH4-M2'') has a one-nucleotide reading frame shift compared with the frame of the same DNA sequence in the previously described CH4-M2' form. As a result, translation of the new CH4-M2'' mRNA should terminate at the TAG codon at position 444 in Fig. 2B. The protein encoded by this RNA was secreted (as it lacks the membrane anchor sequence of the M1 exon), and in its structure, the two COOH-terminal acids of the classic secreted form were replaced with a novel 8-residue COOH terminus (Fig. 2B).

Three Novel ϵ RNA Transcripts That Define New ϵ Exon: CH5—In our previous analysis of ϵ RNA isoforms (3), we described a single reverse transcriptase PCR subclone representing an RNA that spliced from a novel donor site within CH4 (nucleotide 218 in Fig. 2A) to a novel splice acceptor site located in the 3'-untranslated region sequence downstream of CH4 (nucleotide 359 in Fig. 2A). This clone, whose structure was designated CH4'-I-M1'-M2 (3), was originally isolated from the same PCR band as the abundant CH4-M1'-M2 splice product.

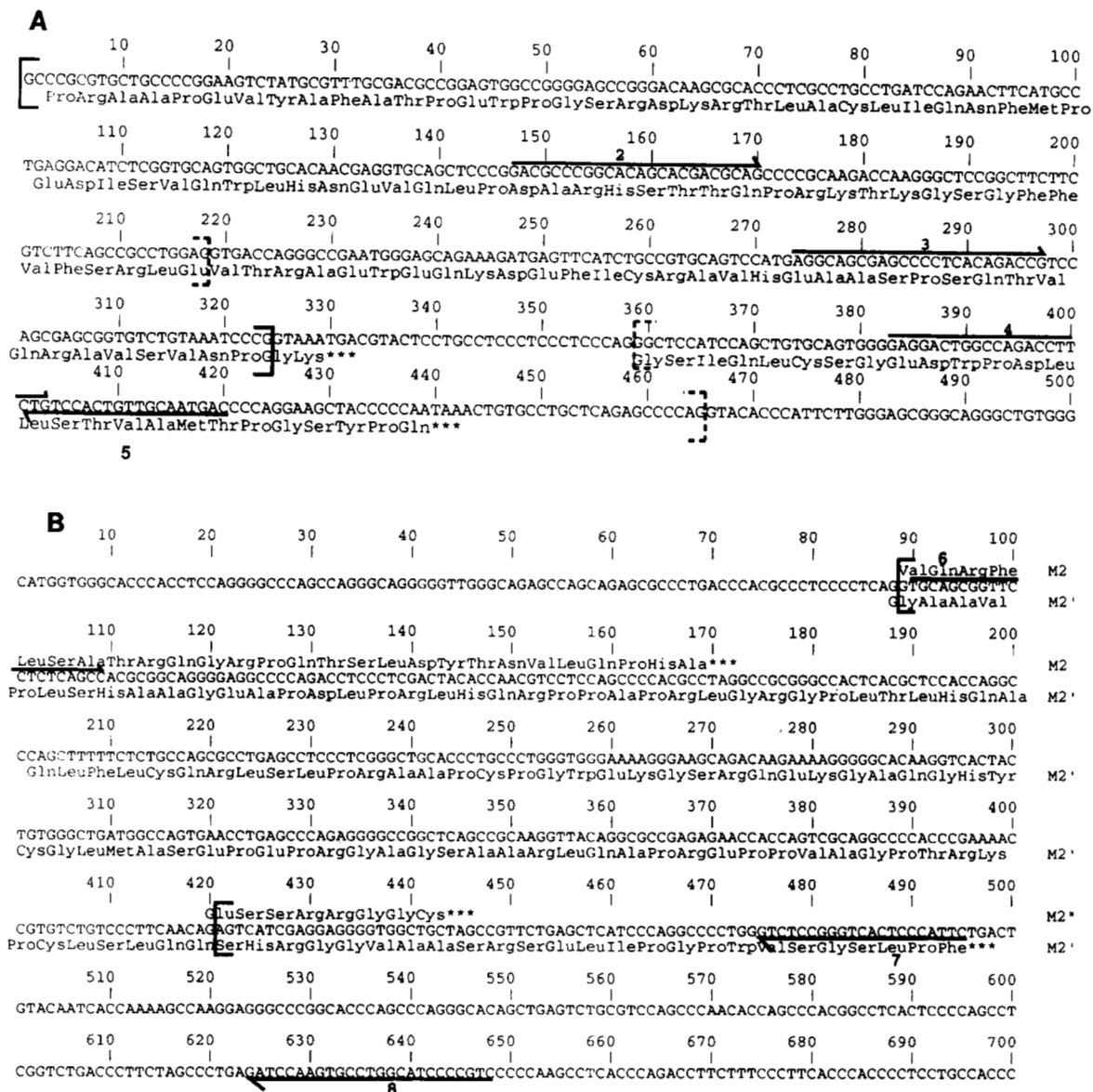


Fig. 2. ϵ gene nucleotide sequences related to various alternative splices investigated in this study. A, the genomic sequence of human CH4 (nucleotides 1-324) and its 3'-flanking sequence is presented, and the amino acid translation for the CH4 domain and CH4'-CH5 splicing product is given below the nucleotide sequence. The solid brackets represent splice donor or acceptor sites, and the dashed brackets define the boundary of CH4'-CH5 splicing. The oligonucleotides used for primers or probes are also shown; the half arrow under the sequence indicates that the oligonucleotide was designed based on the strand complementary to that shown here. B, the sequence of human ϵ membrane exon M2' (nucleotides 89-498) and its 3'-untranslated region is shown. The amino acid sequences generated from the various alternative splicing products in different frames are also given. The solid brackets define the boundary of the splice acceptor sites described. The amino acids shown above nucleotide sequence 89-169 represent the translation of the M2 exon, whereas those below nucleotides 89-495 represent the translation of the M2' reading frame. The amino acids shown above nucleotides 420-443 represent the translation of the M2' exon defined in this paper. The locations of oligonucleotides used as primers or probes are shown as described for A.

The existence of two PCR products in the same band was explained by the nearly identical sizes of these two products, as the length of the segment designated "I" (107 base pairs) almost exactly compensates for the DNA lost from the 3'-end of truncated CH4' (106 base pairs). Because this CH4'-I-M1'-M2 splice form was found in only one subclone in our earlier studies, its significance was uncertain. Our current evidence (described below) indicates that this novel splice is found in all IgE-producing cells tested, defining a new exon that we have renamed CH5 (to avoid confusion with "I" exon upstream of the ϵ switch region).

To investigate RNA forms including this splice, amplification products obtained using primer pair 2(IVm)-8 were analyzed by Southern blotting and hybridization with oligonucleotide

probes specific either for CH5 (oligonucleotide 4) or for the 3'-end of CH4, which is absent from CH4' (oligonucleotide 3). Since the primer 2(IVm) sequence is 5' to the splice donor site at the 3'-end of CH4', this primer should amplify RNA forms with an intact CH4 exon as well as the CH4'-CH5 RNAs (which could not have been amplified with primer pair 3-8 used in the experiments of Fig. 3). As in the amplification described above, three major bands were produced (labeled 1, 3, and 5 in Fig. 4), along with intermediate-sized bands that appear to be heteroduplex artifacts on the basis of experiments similar to those described above. Strikingly, all five bands hybridized with both oligonucleotides 3 and 4, suggesting that each band might represent a mixture of CH4 and CH4'-CH5 amplification products. This interpretation was supported by cloning PCR bands 1, 3,

TABLE I
Oligonucleotide sequences of primers and probes employed

No.	Sequence (5' to 3') ^a	Positions
2(IVm) ^b	ggccatcgatGACGCCCGGCACAGCAGCAGCAG	147-170 (Fig. 2A)
3	ggccatcgatAGGCAGCGAGCCCTTCACAGACCG	274-297 (Fig. 2A)
4	ggccatcgatGAGGACTGGCCAGACCTTCTG	383-403 (Fig. 2A)
5	GTCATTGCAACAGTGGACAG	420 to 401 (Fig. 2A)
6	TGCAGCGGTTCTCTCAGCC	90-109 (Fig. 2B)
7	GAATGGGAGTGACCCGGAGAC	495 to 475 (Fig. 2B)
8	ggccgtcgacGACGGGGATGCCAGGCAGTTGGATC	648 to 624 (Fig. 2B)

^a Upper-case letters correspond to genomic sequence, whereas lower-case letters represent added nucleotides. Underlined sequences represent introduced restriction sites for *Cla*I (atcgat) or *Sal*I (gtcgac).

^b Oligonucleotide is the same as primer IVm in Ref. 3.

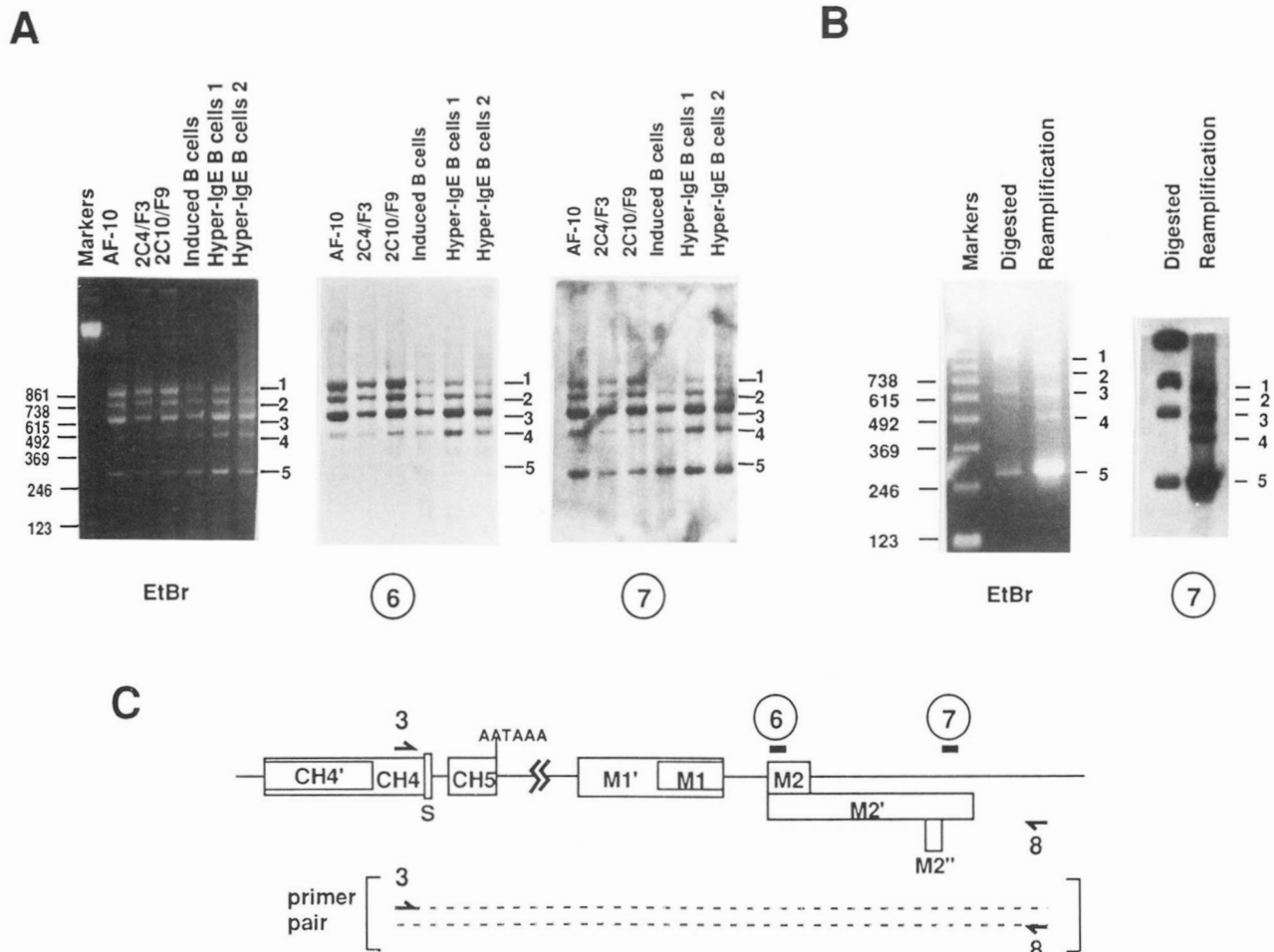


FIG. 3. Analysis of reverse transcriptase PCR products amplified with primer pair 3-8. Circled numbers identify oligonucleotide probes used in the Southern blots. A, reverse transcriptase PCR products from the cell sources shown were electrophoresed and visualized by EtBr staining (as shown in the left panel) or by hybridization of Southern blots with oligonucleotide 6 or 7. The sizes of markers (in base pairs) are shown to the left of the EtBr panel. B, plasmid subclones representing bands 1, 3, and 5 were digested with *Cla*I plus *Sal*I and then mixed together (lanes marked *Digested*); an aliquot of the mixture was then reamplified with primer pair 3-8 (lanes marked *Reamplification*). As shown by EtBr staining or hybridization with oligonucleotide 7, bands 2 and 4 were created by reamplification of cloned templates representing a mixture of bands 1, 3, and 5 and therefore do not derive from distinct templates. C, map of the exons at the 3'-end of the human ϵ heavy chain showing relationship of the oligonucleotides used as PCR primers (oligonucleotides 3 and 8) and those used as hybridization probes (oligonucleotides 6 and 7).

and 5; each band yielded some clones hybridizing with oligonucleotide 3 and others hybridizing with oligonucleotide 4, but no clones were obtained that hybridized with both oligonucleotides. Sequence analysis of representative clones containing CH5 showed that this exon always terminated with the same 5'- and 3'-ends and that the sequences downstream of CH5 reflected the same three splice acceptor sites observed in the RNA forms that contain intact CH4 and no CH5. These CH5-containing clones thus defined three new RNA splice forms: CH4'-CH5-M1'-M2 (band 1), CH4'-CH5-M2' (band 3), and CH4'-CH5-M2'' (band 5). All of these encoded the same protein

because translation of all terminated within CH5 at the TAA codon at position 440 in Fig. 2A. Clones hybridizing with oligonucleotide 3 were shown by sequence analysis to reflect the intact CH4 exon spliced to the same three downstream splice acceptor sites, representing the splice forms reported earlier (CH4-M1'-M2 and CH4-M2') or described above (CH4-M2''). Neither the rare CH4-M1-M2 form (3, 4) nor a CH4'-CH5-M1-M2 counterpart was observed in these experiments, and we did not detect any products splicing from the donor site at the 3'-end of M1 or M1' to M2'.

Additional RNA Isoform Encoding Classic Secreted ϵ Protein

TABLE II
Splice junctions of human ϵ mRNA

Splice donors			Splice acceptors		
Consensus	NNNN ^C _A AG	GT ^A _G AGTAN ^a	Consensus	TTT ^T _{CCC} NCAG	G ^A _{NNNNNNN}
CH4'-3'	CCTGGAG	GTGACCAG	5'-CH5	CTCCCAG	GGCTCCAT
CH4-3'	AATCCCG	GTAAATGA	5'-M1'	CCCTCAG	GGCTGGCT
CH5-3'	GCCCCAG	GTACACCC	5'-M1	CCCCCAG	AGCTGGAC
M1-3'	CCTCATG	GTGGGCAC	5'-M2, M2'	CCCTCAG	GTGCAGCG
			5'-M2''	TCAACAG	AGTCATCG

^a N represents any nucleotide, and where two nucleotides are given, the superior is more common (20, 21). Boldface letters indicate exon nucleotides, whereas lightface letters represent introns.

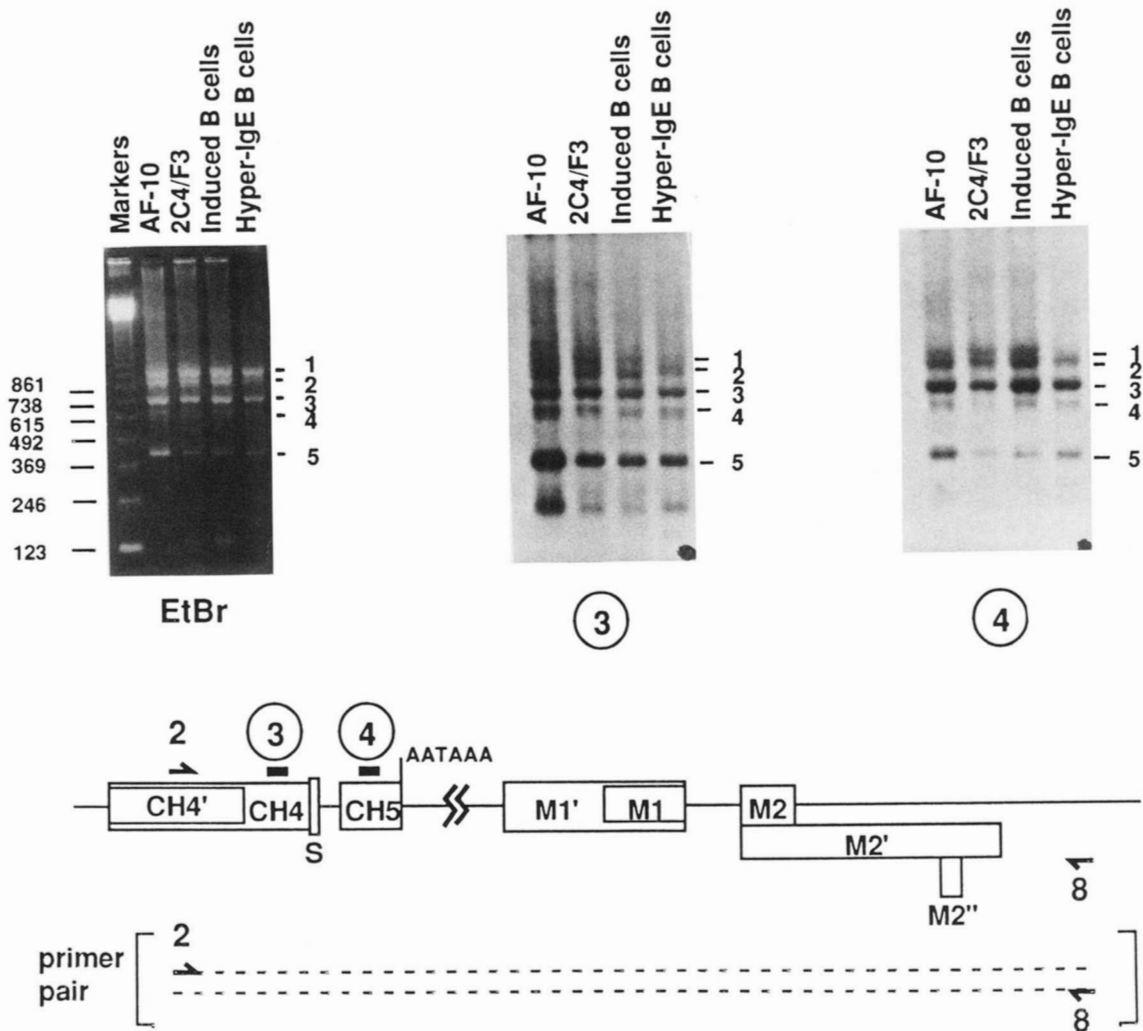


FIG. 4. Demonstration of CH4'-CH5 splice in various IgE-producing cells. Reverse transcriptase PCR products from the cell sources shown were electrophoresed and visualized by EtBr staining (left panel) or hybridization with oligonucleotides 3 and 4. The map below shows locations of primers and probes.

—In an independent amplification of cDNA from AF-10 cells with primer set 3-8, we isolated one clone whose sequence revealed that CH4 and CH5 and the intron sequences between them were processed as a single exon that spliced to M2'. The mRNA corresponding to this clone, designated CH4-3'UT-CH5-M2'' (Fig. 5), encoded the classic secreted IgE protein. Apparently, this species of ϵ mRNA was transcribed past the poly(A) addition site generally used for mRNA encoding the classic secreted IgE.

DISCUSSION

Alternative splicing is a mechanism that enables different proteins to be generated from a single gene. A classic example

is the production of secreted and membrane forms of immunoglobulin from the same DNA sequence; although initially documented for the μ heavy chain (13), similar alternative splicing patterns have been shown for all other heavy chain isotypes examined. Recently, we (3) and others (4, 5) have shown that the alternative splicing of human ϵ mRNA differs from that of other heavy chains in two respects: 1) one splice form (CH4-M1'-M2) encodes a membrane form with an unusually long spacer between the hydrophobic transmembrane residues and the closest immunoglobulin domain, and 2) another form (CH4-M2') involves a splice that excludes one of the membrane exons and encodes a soluble protein. Our present work defines addi-

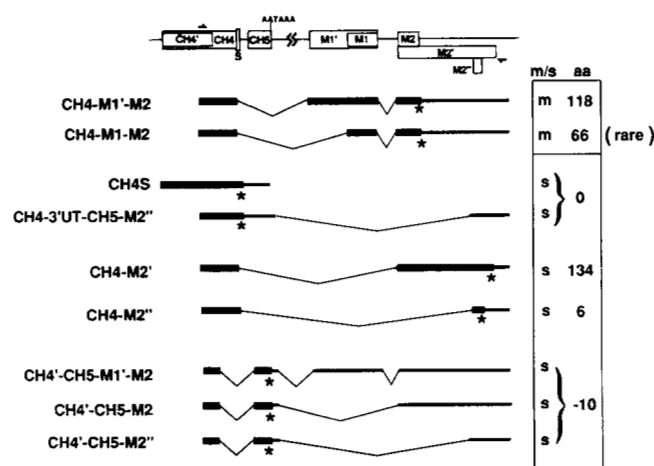


FIG. 5. Diagrammatic summary of 3'-ends of various ϵ mRNA species detected in our reverse transcriptase PCR experiments. A genomic DNA map of CH4 and the ϵ membrane region is shown at the top. All the sequence-confirmed splice products are diagrammed, with their names to the left. Thick horizontal lines represent coding sequence; asterisks show the positions of in-frame termination codons. The table to the right indicates which RNA forms encoded membrane (m) or secreted (s) protein and shows the number of amino acids by which the encoded proteins differed in size from the classic secreted ϵ protein.

tional ϵ mRNAs and their predicted protein products.

One new ϵ mRNA species (CH4-M2'') is produced by splicing of CH4 to a novel splicing acceptor site inside the M2' exon, a site that has not been described before. This splice generates an mRNA that encoded a secreted IgE that is 6 amino acids larger than the classic secreted protein. Even though the M2' exon encodes a peptide with only 8 amino acids, it contains a cysteine residue at its COOH-terminal end (Fig. 2B). The consequences of this cysteine are of interest since cysteine residues located at the COOH-terminal end of heavy chains are usually associated with the J chain that is involved in the formation of the dimer or polymers of IgM and IgA1 (14). Those immunoglobulin isotypes that exist solely as monomers all lack this end cysteine. If the terminal cysteine in the M2' exon plays the same role for IgE as the terminal cysteine does in IgM and IgA1 polymers, then dimer or polymer forms of the CH4-M2'' splicing product-encoded IgE isoform may exist. If such IgE forms exist, they may be expected to differ biologically from the classic secreted IgE or other monomer IgE isoforms described in this report.

Three other ϵ RNAs defined in this paper all include the newly defined exons CH4' and CH5, where CH4' is a truncated form of CH4, and CH5 is an exon composed of DNA lying downstream of CH4 in the intron between CH4 and the membrane exons. Of the alternative splicing schemes reviewed by Smith *et al.* (15), the CH4'-CH5 splice pathway represents an example of mutually exclusive internal splicing compared with the corresponding pathway using an intact CH4 exon. Thus, all the observed ϵ RNAs use either intact CH4 and no CH5 or truncated CH4' with CH5. We have not observed an isoform corresponding to splicing of an intact CH4 exon to CH5; such a splice would be unexpected since the theoretical intron between these segments is only 34 nucleotides, below the accepted limit of 66–70 nucleotides (15) for the shortest intron that can be removed by RNA splicing. Furthermore, we have not detected any evidence of splicing of a truncated CH4' exon directly to one of the membrane exons, although such a splice would not seem to violate any known splicing paradigms. The mutually exclusive mechanism for alternative RNA processing has never been reported for other immunoglobulin heavy or light chain

genes, but has been found in the genes for the contractile α - and β -tropomyosins (16), troponin T (17), myosin light chain (18), and pyruvate kinase (19). The mechanism that ensures mutually exclusive splicing in these genes is not understood.

Because of the stop codon in CH5, the three CH4'-CH5 RNA forms all encoded the same protein, which is 10 amino acids shorter than the classic secreted ϵ heavy chain. The replacement of the COOH-terminal 37 residues of the CH4 sequence with the 27 amino acids encoded by CH5 would create a domain that has 1 cysteine residue in a different position from the highly conserved cysteine that forms the interchain disulfide bond. Whether such a domain with 1 cysteine changed in position would affect the typical immunoglobulin domain remains to be determined. Production of recombinant proteins representing these various ϵ molecules will allow the investigation of these possibilities.

Except for the CH4-3'UT-CH5-M2'' form, the pattern of ϵ RNA species we have detected seems to be a general feature of human IgE-secreting cells. These isoforms have been observed in all IgE⁺ cell sources we have examined, including cell lines and fresh peripheral blood leukocytes stimulated by interleukin-4 plus anti-CD40 as well as spontaneous IgE-producing B cells from hyper-IgE immunodeficiency syndrome patients. The isoforms do not appear to be PCR artifacts because of their occurrence in repeated independent amplifications and because all of the observed splice donor and acceptor sequences appear to conform to consensus junction sequences, as shown in Table II. The splicing is not "promiscuous" in that many theoretical splice products that can be considered based on the junctions we have observed are, in fact, not observed, apparently reflecting tight control by mechanisms that remain to be elucidated. Also unknown at present are the relative amounts of the different isoforms produced under various physiological circumstances.

In summary, we have detected eight species of ϵ mRNA in addition to the form encoding the classic secreted ϵ mRNA form (CH4S) (Fig. 5). Two of the eight encode membrane-bound ϵ proteins (3–5), although one of these (CH4-M1-M2) is extremely rare. One of the eight RNA species (CH4-3'UT-CH5-M2'') encodes the same classic secreted ϵ protein as the CH4S RNA. The other five ϵ mRNAs encode three forms of ϵ chain that are secreted because they lack the hydrophobic transmembrane domain (M1 or M1'); these secreted proteins are either 6 or 134 amino acids larger than the classic secreted protein or 10 amino acids shorter. Two of these five forms (CH4-M2' and CH4'-CH5-M2') were also described recently by Hellman (5). We are currently exploring which of these predicted proteins can be detected by isoform-specific anti-peptide antisera and whether any of the predicted new soluble proteins, when produced as products of recombinant DNA clones, function differently from the classic secreted protein.

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