

Characterization and Expression of Alternatively Spliced IgE Heavy Chain Transcripts Produced by Peripheral Blood Lymphocytes

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We have investigated the IgE heavy chain isoforms produced in vivo by analyzing the ϵ mRNA species present in unstimulated PBL and expressing them individually in a myeloma cell line. Seven ϵ mRNA species were identified by using reverse transcription-PCR, cloning, and sequencing analysis. These species included the classical secreted (ϵ CH4-S) and membrane-bound (ϵ CH4-M1'-M2) IgE and five alternatively spliced ϵ transcripts. At the protein level, the five alternatively spliced ϵ transcripts (ϵ CH4*, ϵ CH4-M2', ϵ CH4'-I, ϵ CH4'-I-M2, and ϵ CH3-I3-CH4) corresponded to four ϵ heavy chain isoforms, in which various parts of the CH4 domain were replaced by new stretches of amino acids at the carboxyl termini. The same ϵ mRNA species also were present in the IgE producing myeloma cell line U266. However, except for the classical membrane and secreted IgE, the corresponding proteins could not be identified. To further characterize the ϵ CH4-S, ϵ CH4*, ϵ CH4-M2', ϵ CH4'-I, and ϵ CH4-M1'-M2 species, we expressed them as chimeric mouse/human anti-4-hydroxy-5-iodo-3-nitrophenacetyl Abs in a mouse myeloma cell line. Only the classical secreted and membrane isoforms were found to be secreted or expressed on the cell surface, respectively, and the other forms were retained within the cells and degraded. These data suggest that some of the ϵ mRNA isoforms produced by PBL are aberrantly spliced mRNAs, the protein products of which are eliminated by post-translational events. *The Journal of Immunology*, 1995, 154: 209–218.

The ϵ H chain² gene consists of four C region exons (CH1 to CH4) and two membrane exons; the latter encode the transmembrane and intracytoplasmic parts of the membrane-bound IgE (1–3). The sequence of the human ϵ membrane exons was recently reported and allowed identification of two types of membrane transcripts that differ in the length of the first membrane exon (M1 and M1') (4–6). These two ϵ mRNA species arise by alternative splicing between a donor splice site at the 3' end of the CH4 exon and two acceptor splice sites separated by 150 bp and located several hundred nucleotides downstream of the CH4 exon. The RNA species that contained the longer variant of the first membrane exon (ϵ CH4-M1'-M2) was found to be at least 100 times more abundant than the species that contained the short mem-

brane exon (ϵ CH4-M1-M2) in IgE-producing myeloma (SKO-007) and transfectoma cell lines (5). Furthermore, the ϵ CH4-M1'-M2 form was the only membrane protein that could be detected by immunoprecipitation in these cell lines (5). An ϵ mRNA species that lacks both the M1' and M1 exon but contains the second membrane exon (M2) in a different reading frame also has been described (5, 6). This RNA species seems to be rather abundant because it can be detected, together with the classical secreted (ϵ CH4-S) and membrane (ϵ CH4-M1'-M2) transcripts, by Northern blot analysis in the U266 cell line and its subclones, SKO-007 and AF-10 (5–8). A secreted ϵ protein of corresponding size has been identified by Western blotting in the AF-10 cell line and in the serum of a patient with IgE myeloma, and it has been suggested that this ϵ CH4-M2' form might play an important and distinct role in allergic reactions (6). Several other alternatively spliced forms, which lack different parts of the CH4 exon or contain additional sequences 3' to it, have been identified by RT-PCR analysis of the U266 and AF-10 cell lines and in PBL stimulated with IL-4 and an anti-CD40 mAb (6, 8). The presence of their putative protein products has not been as yet investigated, and it is unclear whether these ϵ transcripts have any functional significance or are only

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² Abbreviations used in this paper: H chain, heavy chain of Ig; CDR, complementarity-determining region; RT, reverse transcription; ER, endoplasmic reticulum; WGA, wheat germ agglutinin; nt, nucleotide; NIP, 4-hydroxy-5-iodo-3-nitrophenacetyl; L, light chain of Ig; SSPE, saline sodium phosphate EDTA.

byproducts of alternative splicing in the ϵ locus. Furthermore, this ϵ mRNA splicing pattern could be a characteristic of transformed plasma cell lines or a consequence of in vitro culture conditions and might not be representative of the splicing pattern that occurs in vivo.

In this paper, we have addressed these issues by analyzing the ϵ mRNAs produced by unstimulated PBL, which recently were shown to provide a convenient source for the analysis of the ϵ H chain repertoire of the IgE-producing B cell subset (9). By using RT-PCR and sequence analysis, we identified seven alternatively spliced ϵ transcripts, which also were present in the U266 cell line. The properties of the protein products of the different ϵ transcripts were investigated by constructing IgE H chain expression vectors and examining their expression in transfected cell lines.

Materials and Methods

Cell lines and transfection

The U266BL cell line (American Type Culture Collection, Rockville, MD) was maintained in RPMI 1640 medium, which was supplemented with 10% (v/v) FCS, penicillin (100 U/ml), and streptomycin (100 μ g/ml). The J558L plasmacytoma cells were obtained from Dr. M. Neuberger (MRC Laboratory of Molecular Biology, Cambridge, U.K.) and grown in DMEM containing 10% FCS, penicillin (100 U/ml), and streptomycin (100 μ g/ml). Transfections were performed by electroporation. Briefly, 2×10^6 cells were washed, resuspended in 1 ml cold PBS, mixed with linearized DNA (15 μ g in 20 μ l H₂O), and electroschocked by using the Bio-Rad Gene Pulser (Bio-Rad, Richmond, CA) at 450 V and 250 μ F. After 5 min on ice, the cells were resuspended in 50 ml DMEM/10% FCS/gentamicin and transferred to five 96-well plates. Selective medium that contained 400 μ g/ml of G418 (Geneticin, Life Technologies, Inc., Gaithersburg, MD) was applied 24 h later. Anti-NIP IgE Ab production was assayed by Western blot analysis, and the cells were cloned by limiting dilution.

RT-PCR analysis of ϵ transcripts

Total cellular RNA was isolated from U266 cells and from PBMC obtained by fractionation of fresh whole blood on a Ficoll/Hypaque gradient, by using the acid guanidinium thiocyanate procedure of Chomczynski and Sacchi (10). Two to six micrograms of each RNA sample were reverse transcribed in duplicate with use of the GeneAmp RNA PCR kit (Perkin-Elmer Cetus, Norwalk, CT) and oligo-dT as primer. Next, 35 cycles of PCR were performed on each RT sample, with either the E3/M2a or E3/S1 oligonucleotide primer pair (E3, 5'-CATGCGGTCCAC GACCAAGAC-3'; S1, 5'-CCACTGCACAGCTGGATGGAG-3'; M2a, 5'-GGCTGGAGGACGTTGGTGTA-3'). The locations of the different oligonucleotides used are given in Figure 1a. The RT and PCR analyses were performed in accordance with the instructions of Perkin-Elmer Cetus. The PCRs were conducted with 1 min of denaturation at 95°C, 1 min of annealing at 62°C, and 2 min of extension at 72°C. The specificity of the obtained PCR products was confirmed by Southern blot analysis with the E4 oligonucleotide (5'-AAGTCTATGCGTTTGCGACGCCGG-3'). One-third of the PCR reaction was subjected to electrophoresis on a 1.2% agarose gel, transferred to a nylon membrane, and hybridized with the ³²P-labeled E4 oligonucleotide for 2 h at 50°C in 5X saline sodium phosphate EDTA (SSPE) plus 5X Denhardt's solution. Membranes were washed two times for 20 min at room temperature with 2X SSPE + 0.1% SDS, followed by a stringent wash at 65°C with 5X SSPE and 0.5% SDS. Semi-nested PCR experiments with internal 5' ³²P end-labeled oligonucleotide primers (S2, 5'-GGAGGCAGGAGTACGTCATT-3'; and M2b, 5'-TGCCGCGTGGCTGAGAGGAA-3') were performed on the first PCR samples to increase the specificity and sensitivity of the assay. Details of this procedure and of the subsequent analysis on denaturing polyacrylamide gels have been reported previously (9).

Cloning and sequencing of ϵ transcripts

The different bands seen on the autoradiograms of the Southern blot experiments were purified by electroelution from 1.2% agarose gels. In the case of the semi-nested PCR experiments, a 30-min autoradiography on nondenaturing, nonfixed, nondried sequencing gels was performed to identify the location of the ³²P-labeled PCR fragments. Slices that contained these fragments were excised from the polyacrylamide gels, and the DNA was eluted by using the "crush and soak procedure" (11). The recovered DNA fragments were ligated in the *Sma*I site of pUC18 (Pharmacia LKB, Uppsala, Sweden) and used to transform *Escherichia coli* strain DH5 α . Clones were picked randomly, and a dsDNA template was prepared and sequenced by the dideoxy chain termination method (12) with use of the T7 Sequencing Kit (Pharmacia LKB).

Construction of chimeric mouse/human ϵ -chains

An *Aat*II/*Kpn*I fragment from Hu-pSVC ϵ (13) (kindly provided by Dr. M. Neuberger), which contained the mouse Ig H chain enhancer and promoter, a mouse anti-NIP V_H segment, and the entire human genomic ϵ gene, was subcloned in a plasmid derived from pRc/CMV (Invitrogen, San Diego, CA), in which the cytomegalovirus promoter and the *fd* portion had been deleted. This new plasmid (pCIG-C ϵ) contained a single *Kpn*I site downstream of the ϵ gene and was used to construct the five alternatively spliced ϵ species (forms ϵ CH4-S, ϵ CH4*, ϵ CH4'-I, ϵ CH4-M2', and ϵ CH4-M1'-M2 in Fig. 4). The polyadenylation site of the bovine growth hormone gene from pRc/CMV was retained in all constructs. Details of each construct are given below.

ϵ CH4-S. A PCR fragment obtained by amplifying cDNA from PBL with the S2 primer, and a CDR3-specific primer was cloned in the *Sma*I site of pUC18 (9). The *Sall*-*Hpa*I fragment from this plasmid, which contained part of the V region, CH1, CH2, and part of CH3, was replaced with the 835-bp fragment between the *Sall* site in exon 1 and the *Hpa*I site in exon 3 of the human genomic ϵ gene from Hu-pSVC ϵ . This plasmid, pUC18 Δ C ϵ S, contains the 3' part of the ϵ CH1 exon, the second intron, the CH2 exon, the third intron, and the CH3 and CH4 exons joined together. The *Bgl*II-*Kpn*I fragment from this plasmid was used to replace the same fragment in the pCIG-C ϵ , thereby generating the pCIG-C ϵ CH4-S, which was used for the transfection.

ϵ CH4'-I. A PCR fragment obtained by amplifying cDNA with the E3/M2a primer pair (ϵ CH4'-I-M2') was cloned in the *Sma*I site of pUC18. The *Bbs*I-*Kpn*I fragment from this plasmid, which contained the CH4' and I exons and part of the M2' exon, was used to replace the corresponding fragment from pUC18 Δ C ϵ S. The *Bgl*II-*Kpn*I fragment from this new plasmid (pUC18 Δ C ϵ CH4'-I) was introduced between the *Bgl*II and *Kpn*I sites in the pCIG-C ϵ , thereby generating pCIG-C ϵ CH4'-I.

ϵ CH4-M2'. This form was obtained by PCR of independently amplified fragments containing the CH3-CH4 and M2' exons. The entire M2' exon was amplified from U266 genomic DNA having two primers located in its 5' end (M2l, 5'-AATCCCGGTGCGAGCGGTTCTCTCA-3') and 3'-untranslated region (M2d, 5'-TGGGTGCCGGGCCCTCCTTGGC-3'). The M2l primer contained a 5' overhang complementary to the 3' end of the CH4 exon (underlined). Another primer set, which consisted of E3 and a new oligonucleotide from the 3' end of the CH4 exon (E4l, 5'-ACCGCTGCACCGGGATTACAGACAC-3') having a 5' overhang complementary to the 5' end of the M2' exon (underlined), was used to amplify the 3' end of the CH3 exon joined to the complete CH4 exon. The two PCR fragments obtained from the two separate amplifications were mixed together and reamplified with the E3/M2d primer pair. The presence of the 5' overhangs allowed annealing between the two fragments after the initial denaturation and their extension and subsequent amplification, thereby resulting in a new fragment that contains the 3' end of the CH3 and the complete CH4 and M2' exons. This fragment was cloned in the *Sma*I site of pUC18, and the *Bbs*I-*Kpn*I fragment was subcloned in pUC18 Δ C ϵ S. Then, the pCIG-C ϵ CH4-M2' was generated by transferring the *Bgl*II-*Kpn*I fragment to the pCIG-C ϵ .

ϵ CH4*. A PCR fragment, which contained part of the V region, CH1, CH2, CH3, and the first 15 nt from the CH4* exon of an ϵ transcript was obtained by cDNA amplification with a CDR3-specific primer (9) and a primer from the CH3/CH4* boundary (E4*, 5'-CTCAGGCATGAAGT TCGCT-3'). This fragment was cloned in the *Sma*I site of pUC18. The *Hpa*I-*Kpn*I fragment, which contained the CH3 and the CH4* exons, was

subcloned in the corresponding sites of the pUC18CeS. Then, the *Bgl*II-*Kpn*I fragment was subcloned in pCIG-C ϵ to produce pCIG-C ϵ CH4*.
 ϵ CH4-M1'-M2. A PCR fragment obtained with oligonucleotides E3/M2d was cloned in the *Sma*I site of pUC18, and the *Bbs*I-*Kpn*I fragment was subcloned in pUC18 Δ CeS. Then, pCIG-C ϵ CH4-M1'-M2 was generated by transferring the *Bgl*II-*Kpn*I fragment to the pCIG-C ϵ .

To check for possible Taq polymerase errors, all constructs were sequenced and compared with published sequences (6, 8). The sequence of the M2 exon was found to be identical with that published in Reference 8.

Immunoprecipitations

Metabolic labeling and immunoprecipitation were performed as described (14). Briefly, 5×10^6 cells/ml were labeled with [^{35} S]methionine (Amersham International, Amersham, UK) at 100 to 250 μ Ci/ml (1 Ci = 37 GBq) for the indicated times. For the pulse-chase experiments, 50% of the cells from each cell line were lysed immediately after 1 h of labeling, and the remaining cells were incubated for an additional 4 h with an excess of cold methionine. Cell lysates and supernatants were immunoprecipitated with rabbit IgG to human IgE (ϵ -chains) (Dako, Glostrup, Denmark) and purified by protein A-Sepharose. The samples were analyzed by SDS-PAGE in the presence or absence of mercapto-ethanol, as indicated in the legends to Figures 3, 5, and 6. Treatment of labeled supernatants with recombinant PNGase F (*N*-glycosidase F) and recombinant Endo H_f (endoglycosidase H) was performed by following the protocol recommended by the manufacturer (New England Biolabs, Beverly, MA).

NIP-Sepharose affinity chromatography and Western blot analysis

Functional Ig molecules were isolated from cell lysates and supernatants of transfected cells by immunoadsorption on NIP-Sepharose. The purified IgE proteins were fractionated by SDS-PAGE under nonreducing or reducing conditions. Bands were electroblotted to nitrocellulose filters, reacted with rabbit IgG to human IgE (ϵ -chains) (Dako), and developed with alkaline-phosphatase-conjugated goat anti-rabbit polyclonal serum (Dako).

Immunofluorescence

Cells were attached to poly-L-lysine-coated multi-well slides, fixed with ice cold methanol for 10 min, washed with PBS, and permeabilized for 10 min with 0.3% Triton X-100 in PBS. Surface IgG were analyzed on nonpermeabilized cells that had been fixed for 20 min with 3% paraformaldehyde at room temperature. The slides were incubated with rabbit IgG to human IgE (ϵ -chains) (Dako) and biotin-labeled WGA (Boehringer Mannheim Corp., Mannheim, Germany) (kindly provided by Dr. Claudio Schneider, Laboratorio Nazionale Consorzio Interuniversitario Biotecnologie, Trieste, Italy) for 1 h. After three 5-min washes with 1% BSA in PBS, the slides were stained with FITC-conjugated goat IgG to rabbit IgG (Dako) and rhodamine-conjugated streptavidin for 1 h, washed, mounted in mounting medium (KPL, Gaithersburg, MD), and analyzed by either conventional immunofluorescence or confocal microscopy on a Zeiss LS 410 microscope. Optical sections were collected for each label in 0.3- μ m steps.

Results

RT-PCR and sequence analysis of alternatively spliced ϵ transcripts

The ϵ H chain isoforms produced by unstimulated PBL were initially investigated by Southern blot analysis of RT-PCR-amplified RNA samples obtained from several blood donors. For comparison, RNA from the IgE-producing myeloma cell line U266 was analyzed in parallel. Five major bands and a few weak bands were obtained from the U266 analysis (Fig. 1*b*). The E3/S1 amplification of all

PBL samples yielded the same bands as those in U266, whereas no visible product could be detected in some of the reactions designed to amplify the membrane transcripts. To further increase the sensitivity of the assay and to avoid the formation of PCR heteroduplex artifacts, all samples were reamplified with ^{32} P-labeled internal oligonucleotides and analyzed on denaturing polyacrylamide gels (Fig. 1*c*). After reamplification, five major bands were present in almost all samples, four of which corresponded to bands from the Southern blots. The identity of these bands, together with those from the Southern blots, was determined by cloning and sequencing. Seven different species, which included the already reported ϵ CH4-S, ϵ CH4-M1'-M2, ϵ CH4-M2', ϵ CH4'-I, ϵ CH4'-I-M2 (5, 6, 8), and two novel alternatively spliced isoforms, were identified. Of the latter, the ϵ CH3-I3-CH4 form contained the complete intron 3, and the ϵ CH4* form was generated by splicing from the donor splice site in intron 3 to a new acceptor splice site located 90-nt downstream from the 5' end of the CH4 exon. The ϵ CH4* species was not observed on the Southern blots because it lacked the sequences complementary to the E4 oligonucleotide, which was used for the hybridization. At the protein level, the new forms would be rather similar, inasmuch as both of them would lack the complete CH4 domain. The new reading frames would create stop codons 12-nt downstream from the CH3 exon in the ϵ CH4* form and 66-nt downstream from the same exon in the ϵ CH3-I3-CH4 form. A diagram of the detected ϵ isoforms and the sequences of their carboxyl termini are shown in Figure 2.

Immunoprecipitation of U266 cellular extracts and supernatants

By RT-PCR, cloning, and sequencing we showed that the same alternatively spliced ϵ transcripts are present in RNA from both PBMC and the plasmacytoma cell line U266. To identify the proteins that correspond to these ϵ mRNA species, cellular extracts and supernatants from U266 were immunoprecipitated with a polyclonal anti-human IgE rabbit antiserum and analyzed on an 8% SDS-PAGE under reducing conditions (Fig. 3*a*). Two major bands, in the size range expected for the classical secreted form (76 to 79 kDa), appeared after immunoprecipitation of the cellular extract. The bands found in the supernatant were slightly higher in molecular mass (78 to 82 kDa), as was expected from terminal glycosylation of secreted proteins (Fig. 3, *a* and *b*). The possibility that the presence of two bands was caused by different degrees of glycosylation was investigated by treating the labeled U266 supernatants with Endo H_f (which cleaves high mannose and some hybrid oligosaccharides, but does not cleave complex oligosaccharides present in terminally glycosylated proteins) and with PNGase F (which cleaves high mannose, hybrid,

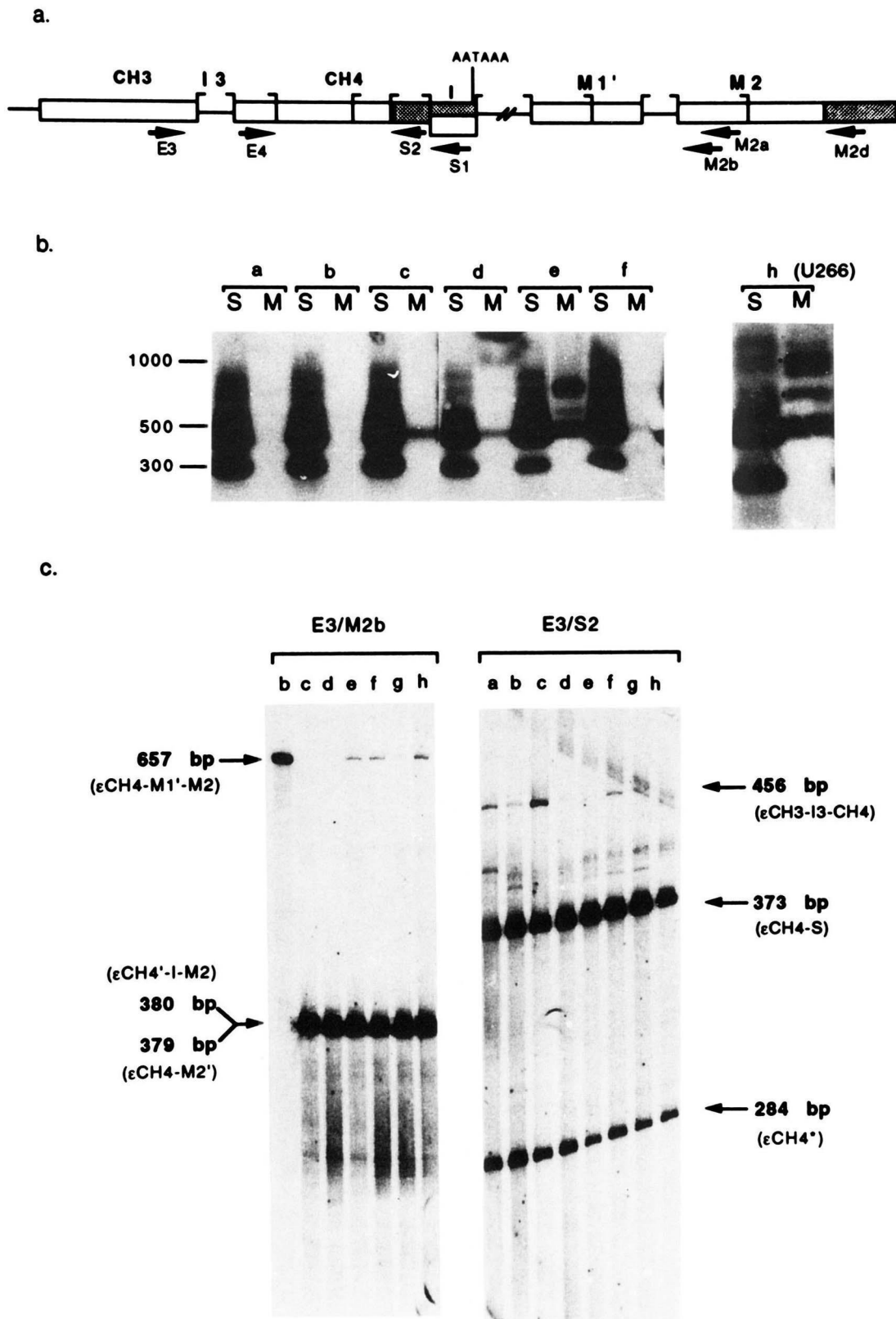


FIGURE 1. Analysis by RT-PCR of the alternatively spliced ϵ mRNAs. *Panel a*) schematic representation of the 3' end of the ϵ gene and the locations of the PCR primers (indicated by arrows). The identified donor and acceptor splice sites are shown. Open boxes represent coding sequences, shaded boxes represent 3' untranslated regions. *Panel b*) autoradiogram of a Southern blot hybridization performed on RT-PCR products of PBMC RNA from different individuals (a to f) and from U266 cells (h), amplified with primers E3 and S2 (lanes S) and E3 and M2a (lanes M). The E4 oligonucleotide was used as a probe. *Panel c*) autoradiogram of a denaturing PAGE of radioactive nested PCR products of samples S and M used in b, amplified with primers E3/ ^{32} P-S2 and E3/ ^{32} P-M2b, respectively. Sequencing reactions were included as size markers.

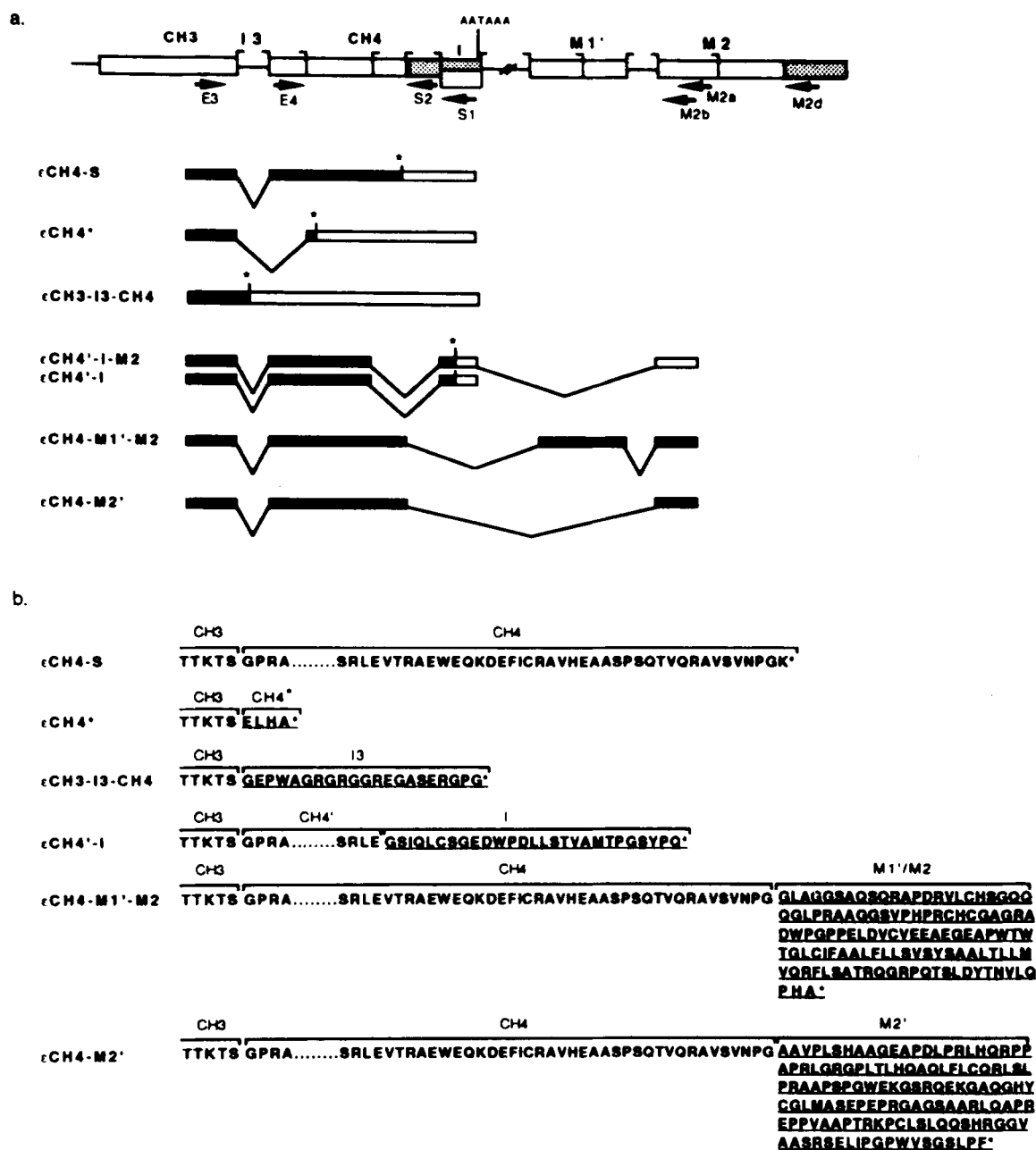


FIGURE 2. ϵ mRNA isoforms. *Panel a*) diagram of the identified alternatively spliced ϵ transcripts. Filled and open boxes correspond to translated and untranslated regions, respectively. Stop codons are indicated by asterisks. *Panel b*) amino acid sequences of the carboxyl termini of the ϵ isoforms shown from position 434 in the CH3 domain. The sequences that differ from the ϵ CH4-S form are underlined, and their exon origins are indicated above them.

and complex oligosaccharides). Figure 3*b* shows the analysis of these samples on a gradient 7 to 12% SDS-PAGE under reducing conditions. Treatment with Endo H_f led to a slight reduction in the size of the bands from the doublet, which suggests that a few nonterminally glycosylated oligosaccharides are present in the secreted ϵ -chains. The persistence of the doublet after the treatment excluded the possibility that the two bands

represent ϵ -chains that differ only in the content of high mannose and hybrid oligosaccharides. On the other hand, treatment with PNGase F completely eliminated the doublet and yielded a single band of approximately 62 kDa, thereby demonstrating that the two bands represent ϵ -chains that differ in the content of complex oligosaccharides. Although the size of this band corresponded to that of the ϵ CH4-S species, it cannot be

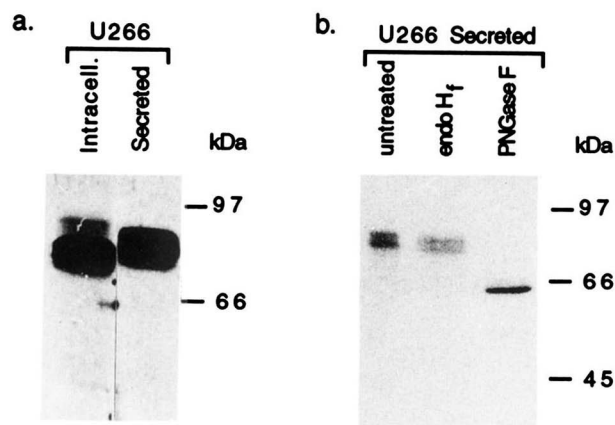


FIGURE 3. Immunoprecipitation of ϵ -chains produced by U266 cells. Cells were labeled with [^{35} S]methionine for 5 h, and the total cell lysate (Intracell.) and culture supernatant (Secreted) were immunoprecipitated with polyclonal rabbit anti-human IgE. *Panel a*) samples of intracellular and secreted material were separated on a reducing 8% SDS-polyacrylamide gel. Apparent molecular masses are expressed in kDa. *Panel b*) treatment of material immunoprecipitated from U266 supernatant with Endo H_f and PNGase F. Samples were analyzed on gradient 7 to 12% SDS-polyacrylamide gel under reducing conditions. The untreated sample corresponds to the secreted sample in Figure 3a.

excluded that it also contained other ϵ isoforms, such as the ϵ CH4'-I, with similar predicted m.w.s.

A minor band of 88 kDa also was obtained after immunoprecipitation of the U266 cellular extract and corresponded in size to the classical membrane form ϵ CH4-M1'-M2.

Expression of five alternatively spliced ϵ mRNA species

The immunoprecipitation experiments with the U266 cell line did not confirm the existence of proteins corresponding to all of the alternatively spliced ϵ mRNAs. This could have been because of the lower mRNA levels, less efficient translation, or more rapid turnover of the protein products of some of these species. To investigate the properties of each of these isoforms in the absence of competition for protein-processing factors, we generated chimeric mouse/human ϵ -chain gene constructs, which contained a mouse V_H segment from an Ab having anti-NIP specificity and human ϵ H chains with carboxyl termini corresponding to the ϵ CH4*, ϵ CH4-M2', ϵ CH4-S, ϵ CH4'-I, and ϵ CH4-M1'-M2 forms. The ϵ CH3-I3-CH4 form was not included because this species most likely represented incompletely processed ϵ mRNA.

A schematic diagram of the five chimeric human/mouse gene constructs is depicted in Figure 4. These chimeric genes were independently transfected in the J558L mouse

myeloma cell line, which produces only the light chain of the anti-NIP Ab. Transfectants that express the different proteins were identified by Western blot analysis of the anti-NIP affinity-purified cellular extracts by using anti-IgE polyclonal rabbit antiserum (data not shown).

Pulse-chase experiments were performed on each cell line, and the immunoprecipitated proteins were analyzed under reducing conditions in a 10% SDS-PAGE. As shown in Figure 5, a band of the expected size was immunoprecipitated in all cases from the cellular extracts obtained after 1-h labeling with [^{35} S]methionine. A protein of 80 kDa appeared in the supernatant of the ϵ CH4-S cell line after the 4-h chase, and, as expected from terminal glycosylation, it was a few kDa larger than the intracellular form. On the other hand, no protein could be detected in the supernatants of the ϵ CH4*, ϵ CH4-M2', and ϵ CH4'-I cell lines, which suggested that these forms are not secreted. These forms also seemed to be rapidly degraded, because the amount of intracellular protein was substantially decreased after the 4-h chase period, but it remained constant in the cell line expressing the classical membrane form (Fig. 5). Identical results were obtained by NIP-Sepharose affinity purification and Western blot analysis of proteins from supernatants of long-term cultures, except for the presence of reactive material having a molecular mass of less than 50 kDa, which was obtained from the ϵ CH4*, ϵ CH4-M2', and ϵ CH4'-I cell lines (data not shown). Because this material was much smaller than the corresponding intracellular protein, it may have represented degradation products released from dead cells or partially degraded IgE molecules in which the removal of particular sequences allowed their secretion.

To investigate whether the Ab molecules were correctly assembled, unreduced samples from immunoprecipitated, labeled cellular extracts of the ϵ CH4*, ϵ CH4-M2', ϵ CH4-S, and ϵ CH4'-I cell lines and the supernatant of the ϵ CH4-S cell line were analyzed on a nonreducing 7% SDS-PAGE (Fig. 6a). Dimerization to the complete H₂L₂ was observed for the ϵ CH4-S form both intracellularly and secreted, and the other forms were present as intracellular HL monomers. Most of the secreted ϵ CH4-S was dimerized, but a small quantity of terminally glycosylated ϵ HL monomer also was present in the supernatant, thus suggesting that some ϵ HL monomers or noncovalently linked dimers also can be secreted. These data were confirmed by Western blot analysis of NIP affinity-purified IgE (Fig. 6b).

The cellular localization of these forms was investigated by performing immunolocalization experiments with use of a confocal microscopy. As shown in Figure 7, immunofluorescence analysis showed a clearly different intracellular compartmentalization for the ϵ CH4-S and ϵ CH4-M1'-M2 forms, consistent with accumulation in the Golgi, whereas the other three forms were scattered throughout the cytoplasm, suggesting that they are retained in the ER. Costaining with WGA, a marker that is specific for the

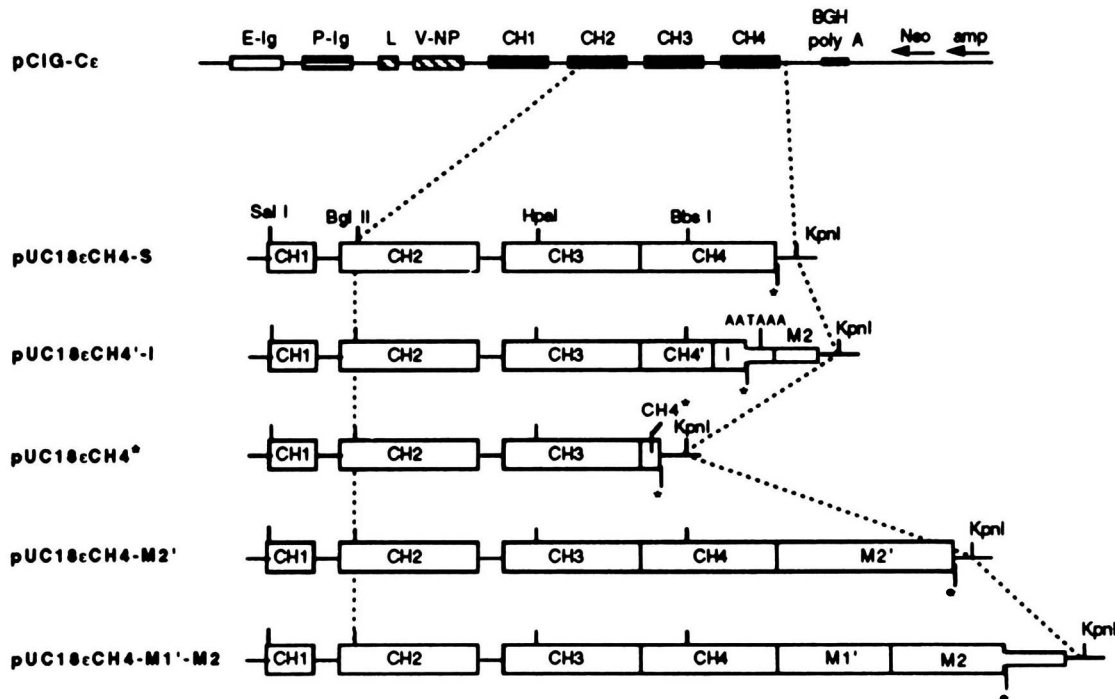


FIGURE 4. Diagram of the chimeric mouse V-Nip/human C ϵ gene constructs. The 3' ends of the different ϵ isoforms were cloned (in their cDNA form from exon CH3) into pCIG-C ϵ , as described in *Materials and Methods*. pCIG-C ϵ contains the mouse Ig enhancer (E-Ig) and promoter (P-Ig) located upstream of the mouse leader (L) and variable (V-NP) exons, the four exons of the human C ϵ gene, and the bovine growth hormone gene poly(A) site (BGH-poly(A)). The relative positions of the selectable marker genes, neomycin (Neo) and ampicillin (Amp), are shown by arrows. Stop codons are indicated by asterisks.

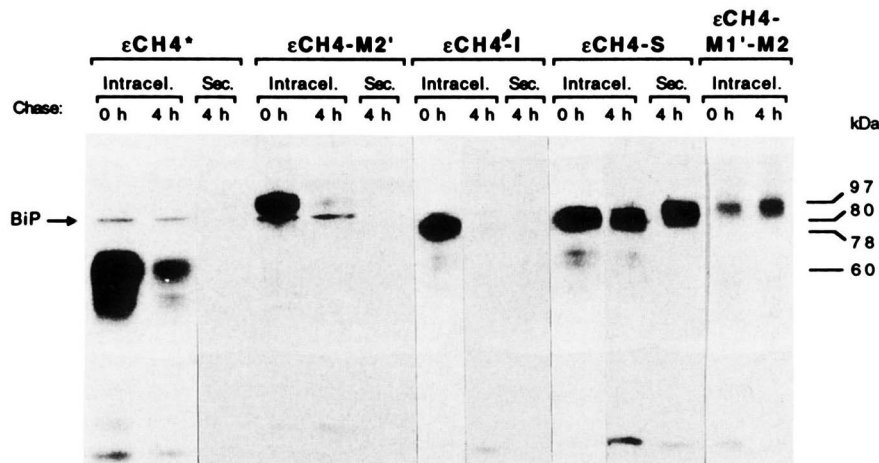


FIGURE 5. Immunoprecipitations of the chimeric IgE. Transfectoma cell lines were pulse labeled with [^{35} S]methionine for 1 h and chased in the presence of cold methionine for 4 h. Cell lysates (Intracel.) and supernatants (Sec.) were immunoprecipitated with an anti-human IgE rabbit antiserum and analyzed on a reducing 10% SDS-polyacrylamide gel. The estimated sizes of the different ϵ -chains are: ϵ CH4*, 60 kDa; ϵ CH4-M2', 97 kDa; ϵ CH4'-I, 78 kDa; intracellular ϵ CH4-S, 78 kDa; secreted ϵ CH4-S, 80 kDa; and ϵ CH4-M1'-M2, 88 kDa. A band corresponding in size to BiP is indicated with an arrow on the left.

Golgi apparatus (15) and that also stains the membrane glycoproteins, confirmed the subcellular localization of the ϵ CH4-S and ϵ CH4-M1'-M2 chains. Immunofluorescence analysis of nonpermeabilized cells showed that only the classical membrane form is expressed as surface Ig (Fig. 7 and data not shown).

Discussion

The classical secreted and membrane forms of all Igs are produced by alternative splicing of the sequences between the last C region exon and the first membrane exon of the H chain gene (16–22). More recently, a number of alternatively

FIGURE 6. Assembly of the IgE molecules. *Panel a*) Western blot analysis of NIP-Sepharose purified Ab molecules from cell lysates (Intrac.) and supernatants (Sec.) of the transfectomas. Electrophoresis was performed under nonreducing conditions in a 7% SDS-polyacrylamide gel. Rabbit polyclonal anti-human IgE was used and visualized with alkaline phosphatase-conjugated goat anti-rabbit Igs. *b*) Autoradiogram of immunoprecipitations with a rabbit polyclonal anti-human IgE antiserum of [^{35} S]methionine labeled (4 h). cellular extracts or supernatants. Samples were analyzed on a 7% nonreducing SDS-polyacrylamide gel. The localizations of the H_2L_2 and HL molecules are indicated by brackets. The position of BiP is indicated with an arrow. Apparent molecular masses are shown on the right in kDa.

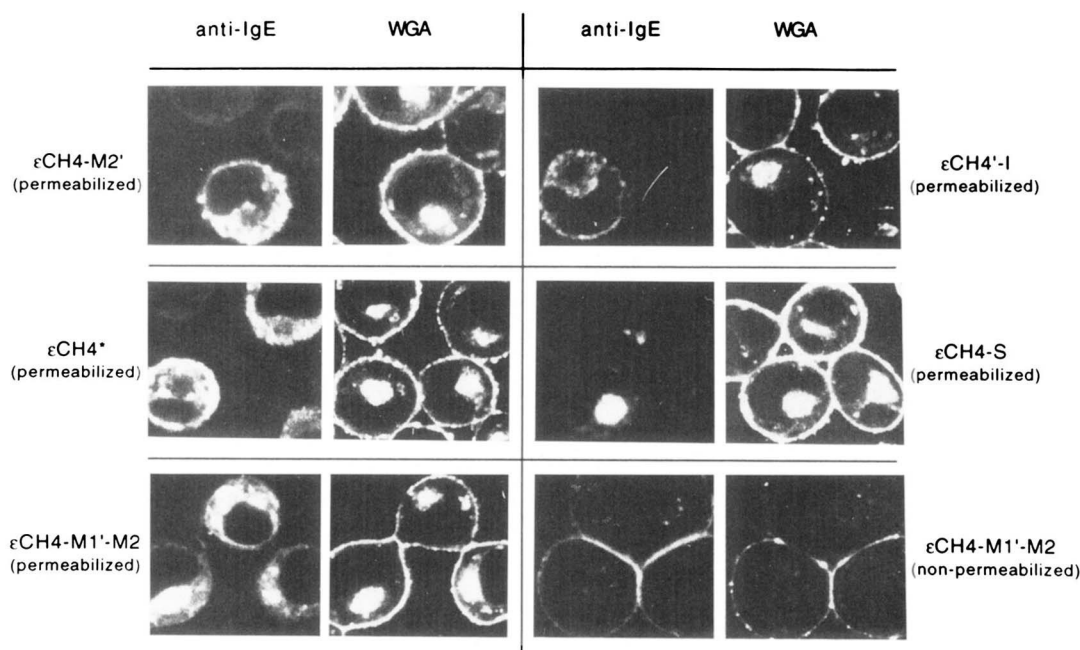
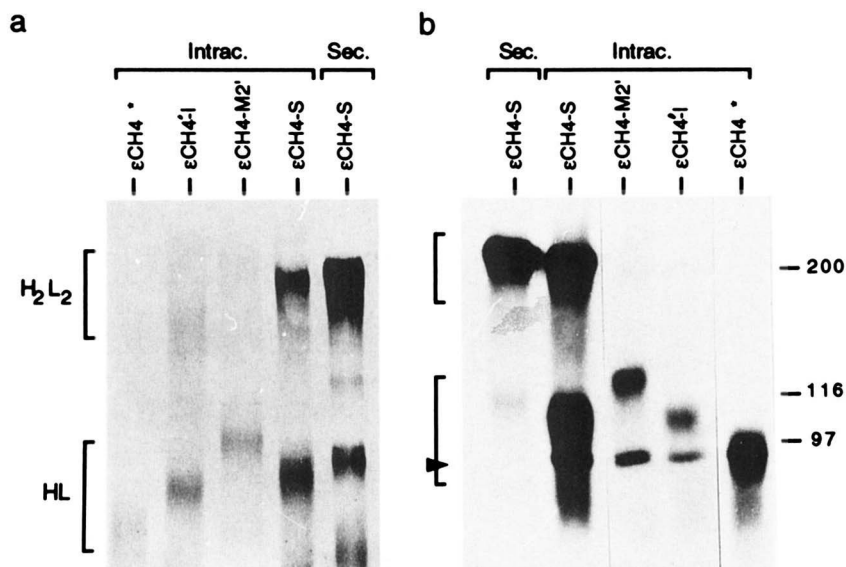


FIGURE 7. Double indirect immunofluorescence analysis (fluorescein for anti-IgE and rhodamine for WGA) by confocal microscopy of the transfectoma cell lines ϵ CH4-M2', ϵ CH4'-I, ϵ CH4*, ϵ CH4-S, and ϵ CH4-M1'-M2. The analysis was performed on permeabilized or nonpermeabilized cells, as indicated. The same section is shown for anti-IgE and WGA in each cell line.

spliced H chain transcripts have been identified in IgA- and IgE-producing B cell lines (5, 6, 8, 19). In this paper, we have shown, by RT-PCR, cloning, and sequencing analysis, that alternatively spliced ϵ mRNA species also exist in unstimulated PBL. Apart from the classical membrane (ϵ CH4-M1'-M2) and secreted (ϵ CH4-S) forms, five alternatively spliced ϵ transcripts were identified. The ϵ CH4'-I, ϵ CH4'-I-M2, and ϵ CH4-M2' forms have been previously reported and arise as a result of the alternate use of two different donor splice sites

in the CH4 exon and two acceptor splice sites in the CH4 3'-untranslated region of the secreted form or in the M2 exon (5, 6, 8). In the two new forms reported here (ϵ CH3-I3-CH4 and ϵ CH4*), the acceptor splice site at the end of intron 3 is not used, and, consequently, these forms retain either the complete intron 3 or are spliced to a new donor splice site in exon 4. At the protein level, both forms would lack the complete CH4 domain as a result of stop codons created by the new reading frames. Except for the ϵ CH4-M1'-M2 form, the

protein products of all of these transcripts should be secreted because they do not contain any hydrophobic segments that could act as transmembrane lipid anchors.

The above-described ϵ mRNA species also were identified in the RNA from the U266 cell line, thereby suggesting a similar pattern of ϵ mRNA splicing in plasmacytoma cells and PBL. However, with the exception of the classical secreted and membrane forms, analysis of the U266 cellular extracts and supernatants did not confirm the existence of proteins that corresponded to the alternatively spliced ϵ mRNA species and suggested that these isoforms are either inefficiently translated and/or rapidly degraded. To investigate the expression of these isoforms in the absence of competition for translational and other protein-processing factors, we independently introduced five suitably modified human ϵ H chain genes, corresponding to the ϵ CH4*, ϵ CH4-M2', ϵ CH4-S, ϵ CH4'-I, and ϵ CH4-M1'-M2 forms, in the mouse plasmacytoma cell line J558L, which is commonly used as an analogue to Ab producing cells (13, 23, 24). All five constructs were intracellularly expressed, but, with the exception of the classical secreted form (ϵ CH4-S), their protein products were absent in the supernatants. Pulse-chase experiments showed that the ϵ CH4*, ϵ CH4-M2', and ϵ CH4'-I forms are intracellularly retained and degraded. Immunofluorescence analysis suggested that the site of the retention is the ER, because none of them were found to be compartmentalized within the Golgi. Altogether, these data suggest that the ϵ CH4*, ϵ CH4-M2', and ϵ CH4'-I forms are only byproducts of alternative splicing in the C ϵ locus and, most likely, are without functional significance. The alternative possibility, that these proteins are membrane isoforms, also was excluded because only the classical membrane form was expressed as surface Ig, and it was shown not to be degraded during the chase period.

Unlike other Igs, the requirements for IgE secretion have not been thoroughly studied. Secretion of IgG requires assembly of two H chains with two light chains, whereas secretion of IgM requires additional assembly into pentamers (23). The secretion pattern of IgA is more complex and seems to contain monomeric, dimeric, and polymeric structures (25). In all cases, the incorrectly folded or improperly assembled proteins are retained and degraded in the ER (26). The ϵ CH4*, ϵ CH4'-I, and ϵ CH4-M2' forms assemble into HL monomers that retain the anti-NIP specificity, but fail to assemble into HL dimers. The absence of the complete CH4 domain in ϵ CH4* or the carboxyl-terminal part of it in ϵ CH4'-I (amino acids 449 to 510, including the Cys at 464) could explain this inability to form HL dimers, because this domain has been shown to be necessary for ϵ H chain dimerization (27). In the case of the ϵ CH4-M2' form, which contains the complete ϵ CH4 domain (except for the last two amino acids), the presence of the large M2' domain could prevent dimerization of the two H chains. However, the presence of a small amount of ϵ CH4-S HL monomers in the supernatant of the trans-

fected J558L plasmacytoma may indicate that the absence of dimerization is not the only reason why these forms are not secreted. Alternatively, the new carboxyl termini created by the alternative splicings could lead to incorrect folding of the C region domains or could contain retention and/or degradation signals.

Regarding the ϵ CH4-M2' form, our results are contradictory with those reported by Zhang et al. (6) who found that it is secreted in a patient having myeloma and in AF-10, a subclone of the U266 cell line. We were unable to detect it in U266 and found that it is produced in the J558L transfectoma but degraded within the secretory pathway. It is unlikely that this discrepancy is a result of the differentiation state of the cells, as observed for membrane and secreted forms of IgM (24), because all cells are of myeloma origin, although there may be differences in the subclones used by both groups. A possibility that was not investigated in our study is that this variant could form hybrid molecules with the ϵ CH4-S H chains, which eventually could be secreted by the AF-10 cells. However, we find this explanation unlikely, because this form also would be expected to be secreted by the U266 cells, which produce significant amounts of both ϵ CH4-M2' and ϵ CH4-S mRNAs (Ref. 8 and our unpublished observations).

More recently, a novel ϵ mRNA species, which is generated by splicing the CH4 exon to a novel distal acceptor splice site in the M2 exon (28), has been identified. This ϵ CH4-M2' species should encode a secreted ϵ protein, which is only 6 amino acids larger than the classical secreted ϵ H chain. This species was not detected in our study because of the position of our primers and could not be discriminated from the classical secreted form in the U266 immunoprecipitation because of identical expected sizes. Therefore, expression studies must be undertaken to determine if this form is a new functional isoform or just another byproduct of alternative splicing in the C ϵ locus.

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