

Characterization of the human immunoglobulin ϵ mRNAs and their polyadenylation sites

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ABSTRACT

Several IgE heavy (H) chain transcripts are produced by alternative splicing between constant region (CH3 and CH4) and membrane (M1 and M2) exons and by differential cleavage–polyadenylation at poly(A) sites downstream of the CH4 and M2 exons. We have now characterized the poly(A) signal of the ϵ transcripts that contain membrane exon sequences (ϵ CH4-M1'-M2, ϵ CH4-M1-M2, ϵ CH4-M2' and ϵ CH4-M2'') and have determined the complete sequence of the M2 exon and 1.4 kb of downstream genomic DNA. The membrane locus poly(A) site was identified by RACE-PCR analysis of ϵ transcripts obtained from IgE-producing myeloma cells and normal peripheral blood lymphocytes (PBL). All membrane exon transcripts were found to be polyadenylated following a CA dinucleotide located 1046 nt from the beginning of the M2 exon. An AGTAAA hexamer, located 13 nt upstream from the site of cleavage and polyadenylation, was the only poly(A) signal sequence present in the 1.4 kb of genomic DNA downstream of the M2 exon. A (G+T)-rich region, which is also conserved in most poly(A) signals, was present 50 nt downstream of the AGTAAA hexamer. Northern blot analysis confirmed that this poly(A) site is used by the membrane exon ϵ mRNAs expressed by the U266 myeloma. The four membrane exon transcripts were detected in different relative amounts in PBL and IgE-producing myeloma cells, which could reflect different ϵ mRNA splicing patterns during B-cell differentiation.

INTRODUCTION

During terminal B cell differentiation to plasma cells a switch from production of membrane-bound to secreted immunoglobulin (Ig) occurs (1–5). The production of these two types of Ig heavy chains (H chains) is regulated by differential splicing and polyadenylation of primary H chain transcripts (6–9). The membrane and secretory H chain isoforms differ only in their C-termini, where they contain a hydrophobic or hydrophilic sequence respectively.

The pattern of splicing appears to be more complex in the case of human IgE, since a number of alternatively spliced ϵ transcripts have been detected in IgE-producing myeloma cell lines and in unstimulated or stimulated peripheral blood lymphocytes (PBL) (10–14). These transcripts are generated by alternative splicing between constant region (CH3 and CH4) and membrane (M1 and M2) exons and by differential cleavage–polyadenylation at poly(A) sites downstream of the CH4 and M2 exons (Fig. 1a).

The different ϵ mRNAs encode two membrane (ϵ CH4-M1-M2 and ϵ CH4-M1'-M2) and five potentially secreted (ϵ CH4-S, ϵ CH4*, ϵ CH4'-I, ϵ CH4-M2' and ϵ CH4-M2'') ϵ heavy chains. The short membrane transcript (ϵ CH4-M1-M2) is homologous to the murine membrane transcript, but the longer variant (ϵ CH4-M1'-M2), which contains an extra 52 amino acid segment between the CH4 and M1 domains, has been detected at 100 times higher levels in IgE-producing myeloma cells and B cells treated with IL-4 plus CD40 (10,11). The secretory isoforms include the classical secretory IgE H chain (ϵ CH4-S) and four isoforms which either lack portions of the CH4 domain (ϵ CH4* and ϵ CH4'-I) or contain additional amino acids in their C-termini encoded by different parts and reading frames of the M2 exon (ϵ CH4-M2' and ϵ CH4-M2''). Among these isoforms only the ϵ CH4-M2'' transcript encodes a functional H chain which is efficiently secreted by both plasma cells and B lymphocytes (14,15). The other three alternatively spliced H chain transcripts appear to be only splicing by-products, which are eliminated by post-translational quality control mechanisms because of their inability to assemble into complete IgE molecules.

An important issue that requires further study is the investigation of expression of the two membrane and the two functional secretory IgE H chain isoforms. To facilitate such studies we have characterized the poly(A) signals used by these isoforms and investigated their expression in normal IgE-producing B lymphocytes and myeloma cells.

MATERIALS AND METHODS

RNA and DNA samples

The human IgE-secreting myeloma cell line U266BL (American Type Culture Collection, Rockville, MD) was maintained in RPMI 1640 medium supplemented with 10% (v/v) fetal calf serum, penicillin (100 U/ml) and streptomycin (100 μ g/ml).

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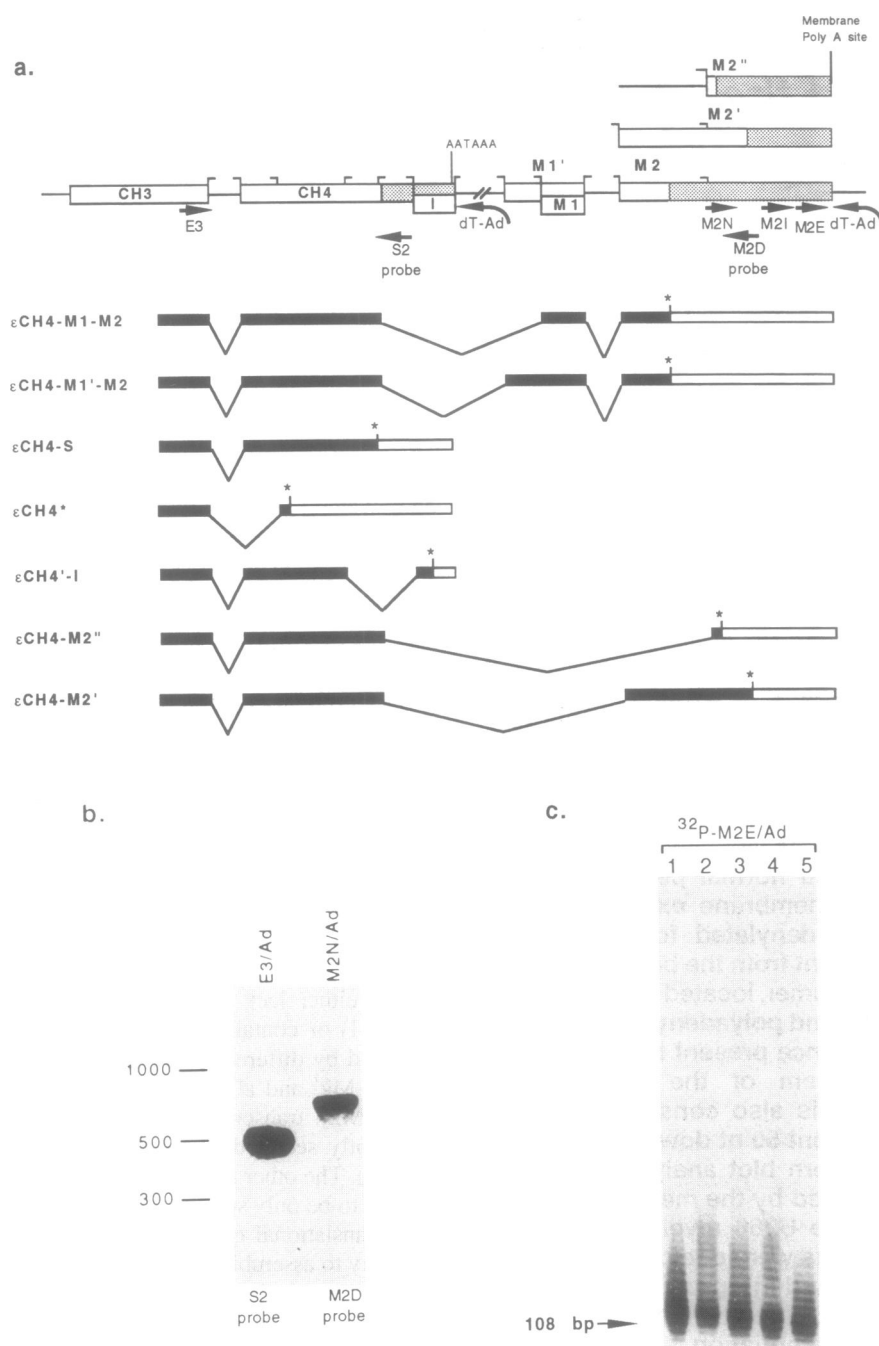


Figure 1. RACE-PCR analysis of poly(A) site usage in ϵ mRNAs. (a) Schematic representation of the 3'-end of the $C\epsilon$ gene. The locations of the RACE-PCR primers are indicated by arrows. Open boxes represent coding sequences, shadowed boxes represent 3' untranslated regions. (b) Autoradiogram of a Southern blot analysis of the RACE-PCR products. The S2 and M2D oligonucleotides were used to hybridize the fragments obtained with primers E3/Ad and M2N/Ad respectively. (c) RACE-PCR analysis of membrane exon ϵ transcripts expressed by unstimulated PBL. U266 mRNA (lane 1) was analyzed as a control. The distance between the membrane locus poly(A) addition site and the ^{32} P labeled M2E oligonucleotide (108 nt) is indicated.

Peripheral blood mononuclear cells were obtained from random blood donors by fractionation of fresh whole blood on a Ficoll-Hypaque gradient. Total cellular RNA was isolated using the acid guanidinium thiocyanate procedure of Chomczynski and Sacchi (16). High molecular weight genomic DNA was isolated from normal white blood cells and U266 cells by standard methodology (17).

Identification of poly(A) sites by RACE-PCR

The 3'-ends of the ϵ mRNAs were amplified using the RACE technique (18). Briefly, 3 μ g total cellular U266 RNA were reverse transcribed with the GeneAmp RNA PCR kit (Perkin-Elmer Cetus, Norwalk, CT) using an oligo(dT)₁₅ adaptor (dT-Ad, 5'-GACTCGAGTCGACATCGATTTTTTTTTTTTTTTT-3') as

primer. Thirty five cycles of PCR were next done on the RT sample with either the M2N/Ad (M2N, 5'-ACAGAGCCTC-CTGCTGCTCT-3'; Ad, 5'-GACTAGAGTCGACATCGA-3') or E3/Ad oligonucleotide primer pairs (E3, 5'-CATGCGGTCCAC-GACCAAGAC-3') (shown in Fig. 1a). The PCR reactions were carried out with 1 min denaturation at 95°C, 1 min annealing at 56°C and 2 min extension at 72°C. The specificity of the obtained PCR products was confirmed by Southern blot analysis with the M2D (5'-TGGGTGCCGGGCCCTCCTTGGC-3') or S2 (5'-GGAGGCAGGAGTACGTCATT-3') oligonucleotides. 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 oligonucleotides for 2 h at 50°C in 5× SSPE/5× Denhardt's solution. Membranes were washed for 2 × 20 min at room temperature with 2× SSPE/0.1% SDS, followed by a stringent wash at 56°C with 5× SSPE and 0.5% SDS. RACE-PCR analysis of ε mRNAs produced by PBL was similarly performed, with some modifications to increase the sensitivity. In this case the PCR was done with the M2I oligonucleotide (5'-CGTCCGACCTCGTCCCTATGA-3') and Ad. A second, semi-nested PCR was performed on 2 µl aliquots of the first PCR reactions using again Ad and an internal ³²P-labeled oligonucleotide (M2E, 5'-ATGACCTCGTCCAGC-CTCT-3'). The radioactively labeled PCR fragments were analyzed on denaturing polyacrylamide gels using sequencing reactions as size markers. Details of this procedure have been reported in a previous publication (19).

Cloning and sequencing

PCR fragments obtained by amplification of U266 and PBL ε cDNAs were purified from 1.2% agarose gels by electroelution. The recovered DNA fragments were ligated in the *Sma*I site of pUC18 (Pharmacia LKB, Uppsala, Sweden) and used to transform *Escherichia coli* strain DH5α. At least three clones from each transformation were sequenced using the T7 Sequencing kit (Pharmacia LKB).

PCR amplification of unknown 3' flanking DNA

The sequence of the 3' flanking region of the membrane locus poly(A) site was determined by analyzing 'misprimed' or 'jumping' PCR artefacts which were generated using primers complementary to sequences from the M2 exon and several non-specific primers, a modification of a method previously described (20). The first PCRs were performed with 1 µg genomic DNA, 50 pmol M2M primer (5'-TTCTACCCTGAGATC-CAAGTG-3', located in the M2 exon), 50 pmol non-specific primer (complementary to sequences of the human C8 β chain gene, the human γ- and β-globin genes and the α chain gene of human FcεRI), 200 µmol each dNTP, 0.5 U Taq polymerase and 10 µl 10× PCR buffer (both from Perkin-Elmer Cetus) in a total volume of 100 µl. Thirty five cycles of denaturation at 95°C for 1 min, annealing at 56°C for 1 min and extension at 72°C for 2 min were done in a Perkin-Elmer Cetus thermal cycler. The PCR artefacts containing the sequence of interest were selected in semi-nested PCRs in which 2.5 µl of the first PCRs were amplified with the internal M2I primer and the same non-specific primer from the first reaction. The specific PCR products were identified by Southern blot analysis with the internal M2G oligonucleotide (5'-GGGTTTCTTAGTAAAGATCCT-3') and were cloned and sequenced as described above.

Northern blot analysis of ε transcripts

Twenty microgram samples of U266 RNA were separated by electrophoresis in 1% (w/v) agarose gels containing 6% formaldehyde, transferred to nylon membranes (Hybond-N, Amersham, UK) by overnight capillary blotting in 20× SSC and fixed with UV light. The filters were hybridized with four different probes generated by restriction enzyme digestion or PCR amplification of cloned ε gene fragments as described (11). The hεC probe was a 812 bp *Bgl*II-*Bbs*I cDNA fragment containing part of CH2 and the complete CH3 and CH4 exons. The M2'' probe was a 735 bp *Nhe*I-*Kpn*I cDNA fragment containing the last coding part of the M2 exon and its 3' untranslated region. The M2 probe was a 271 bp *Sac*II-*Nhe*I fragment containing the region of M2 upstream of the M2'' acceptor splice site. The M1' probe (207 bp), which contained almost the complete M1' exon, was generated by PCR with the M1B (5'-TGTAGCTCAGCT-CAGCAGG-3') and M1L (5'-TCTGCCCCACTCCGACAGG-CAG-3') primers. The probes were labeled with [α-³²P]dCTP (Amersham, UK) using the Oligolabelling kit (Pharmacia LKB). Hybridizations were carried out overnight at 42°C in 5× SSPE/0.5% SDS/5× Denhardt's/50% formamide/100 µg/ml salmon sperm DNA. Filters were washed in 2× SSC/0.1% SDS at 42°C and autoradiographed.

RT-PCR analysis of ε transcripts

Two micrograms of PBL or U266 RNA were reverse transcribed and amplified with the GeneAmp RNA PCR kit according to the instructions of the manufacturer. Thirty five cycles of PCR were done on each RT sample with the E4Y (5'-AGGCAGCGAGC-CCCTCACA-3') and M2W (5'-GAATGGGAGTGACCCGGA-GAC-3') oligonucleotides under the following conditions: 1 min denaturation at 95°C, 1 min annealing at 62°C and 1 min extension at 72°C. Two microliter aliquots of the first PCR were re-amplified under the same conditions in a semi-nested PCR with the internal 5'-³²P-end-labeled oligonucleotide E4Z (5'-CAGCGAGCGGTGTCTGTGA-3') and primer M2W and analyzed on denaturing polyacrylamide gels.

RESULTS

Characterization of the ε mRNA polyadenylation sites

Poly(A) site usage of the ε H chain transcripts was initially investigated on total cellular RNA from the IgE-secreting myeloma cell line U266 using the RACE-PCR procedure. In order to identify the poly(A) sites of both the secreted and membrane loci the cDNA resulting from reverse transcription was amplified with the Ad primer and the E3 or M2N oligonucleotide, which were located in the CH3 and M2 exons respectively (Fig. 1a). The PCR products were analysed by Southern blot using as probes internal radiolabeled oligonucleotides (Fig. 1b). In the E3/Ad amplification a single positive band of ~500 nt appeared after hybridization with the S2 oligonucleotide. This band was of the size expected for the classical secreted form if the AATAAA sequence located 105 nt downstream of the stop codon served as the polyadenylation signal. Cloning and sequencing of this band confirmed that this AATAAA sequence is the actual polyadenylation signal of the classical secreted form.

In the case of the M2N/Ad PCR products a single band of ~700 bp was observed after hybridization with the internal M2D oligonucleotide, suggesting that the transcripts containing the M2

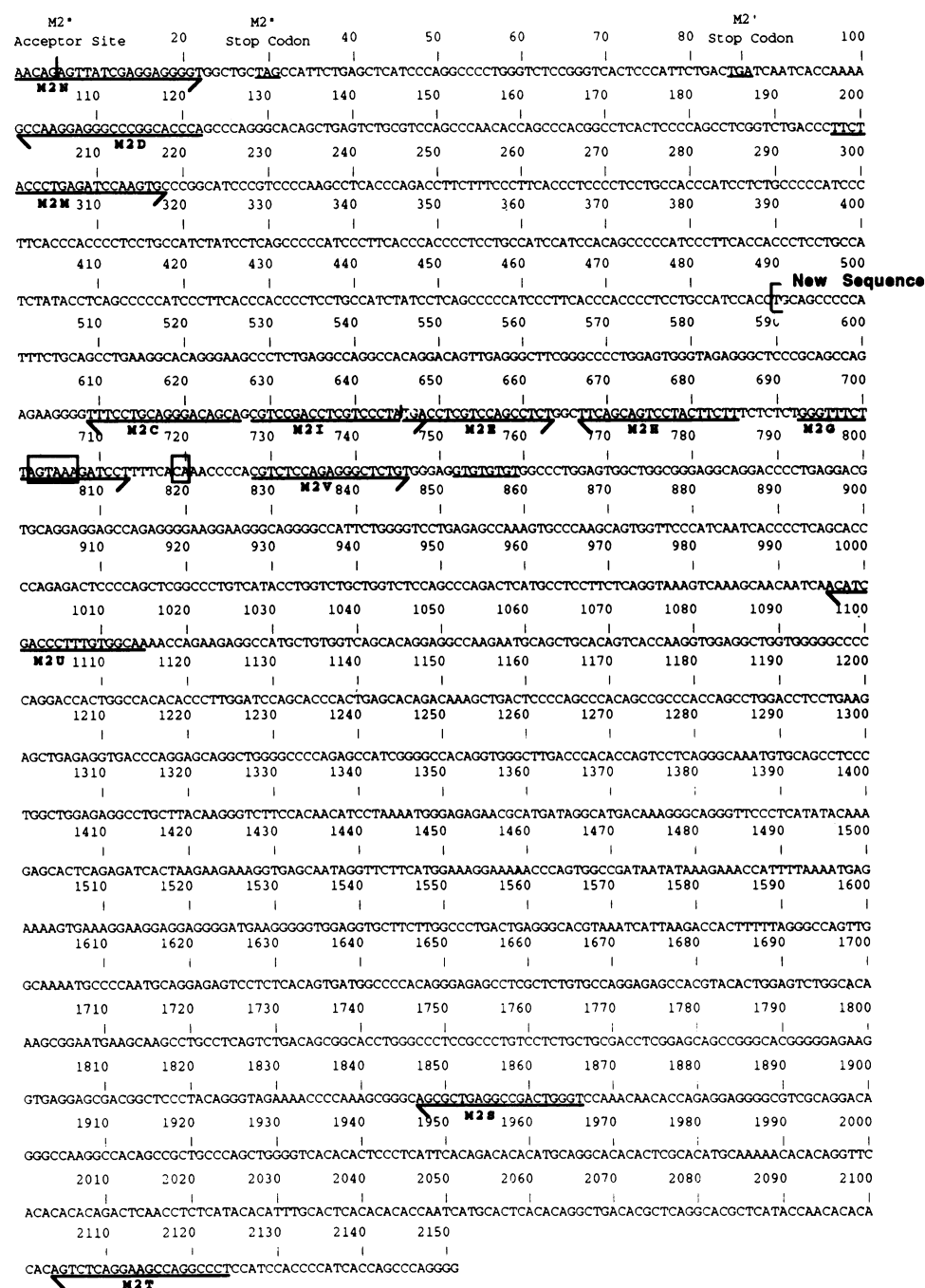


Figure 2. Genomic sequence of the 3' part of the human ε M2 exon and its 3' flanking region. The acceptor splice site of the M2'' exon and the stop codons of the M2'' (nt 29–32) and M2' (nt 87–90) exons are indicated. The AGTAAA polyadenylation signal and the site of cleavage and poly(A) addition (the CA dinucleotide at position 720) are boxed. The (G+T)-rich element (positions 752–759) is underlined. The 6× tandem repeat sequence is located between positions 290 and 490. The oligonucleotides used for the PCR analysis and their orientations are indicated by half arrows. New sequence is from position 490 (indicated by a bracket).

exon use a unique polyadenylation site (Fig. 1b). Cloning and sequencing of this fragment provided >200 bp of new sequence from the M2 exon, which finished with a CA dinucleotide (shown as part of Fig. 2). Thirteen nucleotides upstream of this poly(A) addition site an AGTAAA hexanucleotide was present, which presumably served as the polyadenylation signal. A 6× tandem repeat of a 42 nt sequence was present in the 3' untranslated region

of the M2 exon ~200 nt upstream of the AGTAAA hexanucleotide.

To investigate if the same poly(A) site is used by normal PBL we performed semi-nested RACE-PCR analysis on RNA from four different samples. U266 RNA was analyzed in parallel as a control. As shown in Figure 1c, a ladder of fragments was obtained due to priming of the dT-Ad oligonucleotide at different

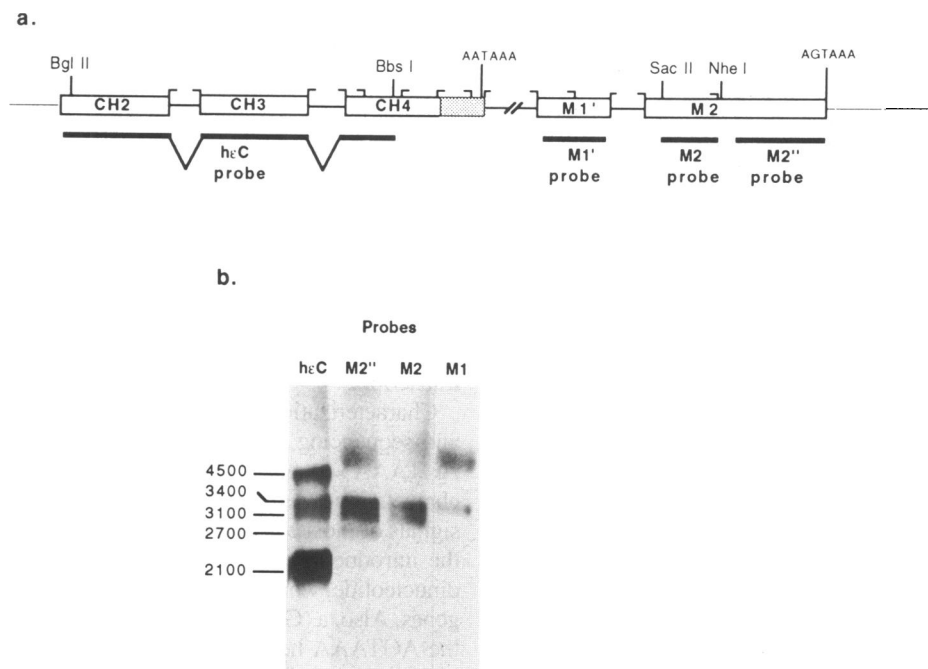


Figure 3. Northern blot analysis of the alternatively spliced ϵ mRNAs. (a) Schematic representation of the 3'-end of the ϵ chain locus. The four probes and the restriction enzyme sites used in their construction are indicated. (b) Four identical lanes containing 20 μ g total cellular RNA from U266 were independently hybridized with each of the four different probes. The sizes of the detected RNA species are indicated.

positions in the poly(A) tail. The bulk of the fragments were ~ 108 nt long, which is the distance between the above-described poly(A) addition site and the ^{32}P -labeled M2E primer used in the second PCR. Cloning and sequencing of these fragments confirmed that the membrane exon ϵ transcripts are cleaved following the CA dinucleotide located 13 nt downstream of the AGTAAA signal.

To further characterize the polyadenylation site of the membrane locus and to investigate the presence of other potential polyadenylation signals we determined the sequence of the genomic region downstream of the AGTAAA hexanucleotide. This was accomplished using a chromosome walking PCR technique (a modification of 20), which provided 1.4 kb of novel sequence from the C ϵ locus (Fig. 2). Analysis of this sequence showed the presence of a (G+T)-rich element 33 nt downstream of the site of cleavage and polyadenylation. No other consensus polyadenylation sequence was found in the 1.4 kb genomic DNA region downstream of the AGTAAA hexamer. The specificity of the new sequence was confirmed by PCR analysis of human genomic DNA with the primers M2N, M2M and M2I, located in the M2 exon, and primers M2V, M2S, M2T and M2U, derived from the new sequence (shown in Fig. 2). In all cases bands with the expected length and sequence were obtained (data not shown).

The newly identified poly(A) signal is used by the membrane exon ϵ transcripts

The ϵ mRNA species produced by U266 were investigated by Northern blot analysis using four different probes corresponding to distinct parts of the C ϵ gene (Fig. 3a). The h ϵ C probe contained CH2, CH3 and CH4 exon sequences and was used to detect all ϵ mRNA isoforms. The forms that use the membrane locus poly(A) site were identified with the M2'' probe, which contained the last coding part of the M2 exon and its 3' untranslated region.

Differentiation between the forms containing the complete M2 exon and those containing the M2'' exon was achieved with the M2 probe, which contained the region of M2 upstream of the M2'' acceptor splice site. The M1' probe, which contained almost the complete M1' exon, was used to identify the membrane form.

As shown in Figure 3b, the h ϵ C probe hybridized to four major U266 mRNA species, represented by bands of ~ 2.0 , ~ 2.7 , ~ 3.1 and ~ 3.4 kb. The 2.0 kb band did not hybridize to any of the probes derived from the membrane locus and should therefore include mRNA encoding the classical secretory ϵ heavy chain and the aberrantly spliced ϵCH4^* and $\epsilon\text{CH4}'\text{-I}$ transcripts. The 2.7 kb band corresponded to the $\epsilon\text{CH4-M2''}$ transcript, since it hybridized to the M2'' probe but to neither the M2 nor the M1' probes. The band of 3.1 kb hybridized to the M2'' and M2 probes, but did not hybridize to the M1' probe, indicating that this band represents the $\epsilon\text{CH4-M2'}$ species. The 3.4 kb band was the only one hybridizing with the M1' probe, as well as with the other two probes derived from the membrane locus (namely M2'' and M2), and corresponded in size to the $\epsilon\text{CH4-M1'-M2}$ transcript. The difference in size between the $\epsilon\text{CH4-M1'-M2}$, $\epsilon\text{CH4-M2'}$ and $\epsilon\text{CH4-M2''}$ mRNA species was in accordance with their usage of the newly identified poly(A) signal of the membrane locus. Higher molecular weight species of >4.5 kb were detected with all probes.

Different splicing patterns of the membrane exon transcripts can be detected in unstimulated PBL

In two recent studies we have shown that unstimulated PBL from normal individuals express low levels of ϵ mRNA (14,19). Most of the ϵ mRNA was of the $\epsilon\text{CH4-S}$ species, however, the other isoforms could also be detected following nested PCR experiments. The only exception was the $\epsilon\text{CH4-M2''}$ transcript, which could not be detected because of the position of the primers used

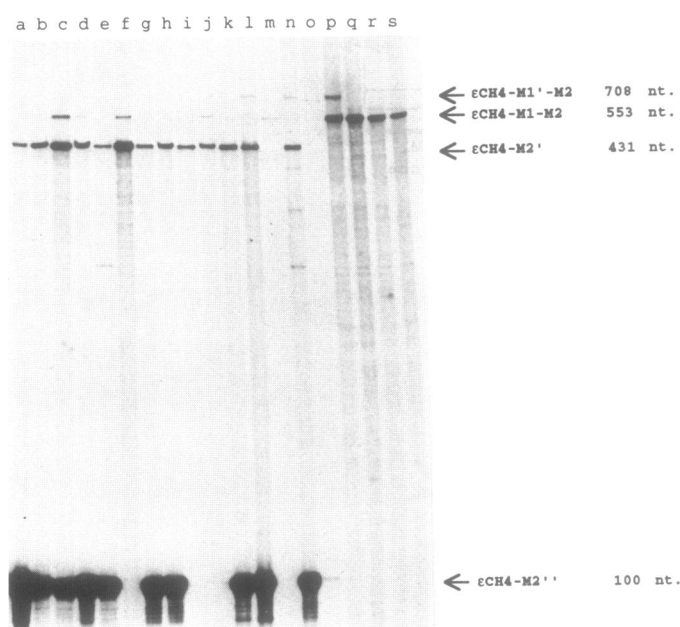


Figure 4. RT-PCR analysis of membrane exon ϵ transcripts expressed by PBL. The samples were amplified in a semi-nested radioactive PCR and analyzed by denaturing polyacrylamide gel electrophoresis.

for the PCR. Since this transcript has been detected only in IgE-producing cell lines and in PBL from patients with high serum IgE levels, we now investigated its expression in unstimulated PBL obtained from 20 random blood donors. As shown in Figure 4, this transcript was present in approximately half of the investigated samples, all of which also contained ϵ CH4-M2' mRNA. Most interestingly, we also detected the short membrane isoform (ϵ CH4-M1-M2) in about one third of the PBL samples and in some cases it was the only ϵ mRNA species present. The long membrane isoform was detected in only three samples.

DISCUSSION

Recent studies on the switch in the production of membrane to secretory IgM have shown that competition between splice and cleavage-polyadenylation reactions determines the pattern of alternative RNA processing (21,22). The expression of the different ϵ mRNA species also appears to be a regulated event (23,24). However, analysis of C ϵ gene expression has been hampered by a lack of information regarding the poly(A) signal of the membrane exon transcripts and their 3' untranslated regions. We now show that these transcripts are polyadenylated following a CA dinucleotide located 1046 nt from the beginning of the M2 exon and confirm that the poly(A) site used by the classical secreted ϵ isoform is the AATAAA sequence located 105 nt downstream of the stop codon in the CH4 exon.

The complete sequence of the M2 exon with its polyadenylation signal was determined using the RACE-PCR procedure. An AGTAAA hexamer was found located 13 nt upstream from the poly(A) addition site. Although this sequence does not fit completely with the consensus AATAAA, a survey of the literature and a search in databanks showed that AGTAAA is also used by other eucaryotic genes, such as the human θ -globin gene

and the H2b-VIII avian histone gene (25,26). Analysis of the activity of different signals generated by site-directed mutagenesis has attributed to the AGTAAA sequence ~30% of the activity of the classical AATAAA (27). It is interesting that the AGTAAA poly(A) signal is conserved in the mouse membrane C ϵ locus (S.Anand, F.D.Batista, D.G.Efremov and O.R.Burrone, manuscript in preparation), whereas all other Ig H chain genes (both mouse and human) contain AATAAA or ATTAAA, which are the strongest poly(A) signal sequences (9,28,29). The presence of an inefficient poly(A) signal in the membrane C ϵ locus could constitute an additional aspect in the regulation of IgE production, as it could prevent high level expression of membrane transcripts.

Characterization of the poly(A) site was completed by cloning and sequencing >1.4 kb of genomic DNA downstream of the AGTAAA sequence. Analysis of this region identified sequence characteristics which are conserved in the polyadenylation signals of most eucaryotic genes. The point of RNA cleavage for the introduction of the poly(A) tract occurred following a CA dinucleotide, which is the most common site found in eucaryotic genes. Also, a (G+T)-rich region was found 50 nt downstream of the AGTAAA hexanucleotide. The absence of other AATAAA consensus sequences in the 1.4 kb of downstream genomic DNA suggests that the AGTAAA is the only poly(A) signal within the membrane ϵ locus.

The utilization of this poly(A) signal by membrane exon ϵ transcripts was confirmed by RACE-PCR analysis of ϵ transcripts from unstimulated PBL and U266 and also by Northern blot analysis of U266 mRNA with probes complementary to the C ϵ gene, the M1' exon, the M2 exon and the M2'' exon. However, higher molecular weight mRNA species were also detected, which could suggest the existence of another polyadenylation site in the C ϵ locus. We find this possibility unlikely, since we have detected species of similar size by Northern blot analysis of cell lines transfected with an IgE expression vector (described in 14) that contains only the bovine growth hormone poly(A) site. Furthermore, no other poly(A) site was detected by RACE-PCR analysis in PBL or U266. A more likely explanation is that these species represent unprocessed RNA precursors, such as those found in β -globin and β -spectrin I RNA (30,31). This possibility is also in agreement with the study of Hellman (12), who did not detect species of this size by Northern blot analysis of poly(A)⁺ mRNA.

By Northern blot analysis we also investigated expression of the different ϵ mRNAs in U266 cells. This analysis confirmed that the ϵ CH4-S, ϵ CH4-M1'-M2 and ϵ CH4-M2' species are the most abundant ϵ mRNA isoforms in this cell line (11,12). The high mRNA levels of the ϵ CH4-M2' isoform are intriguing, since its H chain cannot assemble into complete H₂L₂ molecules in either B lymphocytes or plasma cells and is retained and degraded in both cases (14). Since this mRNA has been shown to follow the expression of the classical secretory ϵ transcripts (23) and was absent in some of the PBL samples that contained the short membrane variant, we speculate that this species represents an aberrantly spliced transcript produced during down-regulation of membrane IgE expression. We also detected significant amounts of the ϵ CH4-M2'' species in U266, although it was expressed at a lower level than any of the other three isoforms. This species is of considerable interest, since it encodes a second secretory IgE isoform and our initial studies have already shown that it has some properties different from the classical secretory ϵ H chain (15).

Interestingly, this transcript was detected in significant quantities in a portion of the PBL samples but was completely absent in the remaining samples, which contained ϵ CH4-M1-M2 and/or ϵ CH4-M2' mRNA. Finally, we could not detect the ϵ CH4-M1-M2 isoform in U266 either by Northern blot or by PCR analysis, but found it in about one third of the PBL samples, in half of them as a unique species. Interestingly, this short membrane isoform was not observed when PBL were induced to secrete IgE by stimulation with IL-4 and anti-CD40 Ab (13). On the other hand, although we detected significant amounts of the long membrane variant in U266, we did not detect it in most of the PBL samples. These data suggest that the short membrane transcript is the predominant membrane species in B lymphocytes and that expression of the membrane exon ϵ transcripts changes during B cell differentiation.

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