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Germ-line human ε heavy chain gene RNA transcripts utilize the full range of alternative 3′ splicing seen in productive ε mRNA

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SUMMARY

An early step in immunoglobulin isotype switching involves the initiation of active transcription of downstream heavy chain loci while they are still in germ-line configuration. This results in the production of 'sterile' germ-line RNA transcripts that do not contain VDJ sequences, the role of which in isotype switching is under intense scrutiny. We investigated whether such human ε germ-line transcripts employ the full complement of complex alternative 3′ RNA splicing, splicing we have recently reported occurring with productive ε mRNA transcripts. Using a reverse transcriptase polymerase chain reaction (RT-PCR) strategy, in which the 5′ primer was located in the I region of the ε gene, a region expressed in germ-line but not productive (VDJ containing) ε transcripts, we showed that the full range of alternative 3′ ε splices occur with germ-line transcripts. These results demonstrate that ε 3′ splicing events are independent of 5′ isotype DNA switching. Additionally, we showed that, just as with mature ε mRNA, the relative production of the various ε germ-line mRNA isoforms was responsive to modulation by stimuli such as interleukin-10 (IL-10). Thus B cells, when stimulated to produce ε germ-line transcripts, generate a family of germ-line mRNA that differ not only in their initiation sites but, more importantly, also differ in their 3′ sequences, sequences that could be important in regulation of the parent gene itself. Furthermore, by discontinuous or trans-splicing, cells could utilize these various ε germ-line transcripts to produce the full range of mature IgE proteins prior to undergoing deletional recombination of isotype switching.

INTRODUCTION

An initial step in immunoglobulin isotype switching involves the initiation of active transcription of downstream unrearranged heavy chain loci.[1] Transcription of targeted loci prior to DNA rearrangement results in the production of germ-line RNA transcripts. Transcripts have been described for all isotypes, including ε, under specific conditions that may lead to subsequent rearrangement to the given isotypes. Transcriptionally active loci then may later undergo DNA rearrangement resulting in a productively rearranged VDJ region being placed immediately 5′ to a downstream heavy chain locus.[1] The specific role of the germ-line RNA transcripts in the ultimate switching remains highly debated.[2–6] For many heavy chain loci such as the human ε locus, germ-line transcripts do not themselves code for proteins as they contain stop codons in all three reading frames early in the sequence.[7] Thus the relative role of the act of transcriptional activation of locus vs. roles for germ-line RNA transcripts themselves is under intense scrutiny.[8]

The 5′ end of germ-line transcripts is defined by several start sites within the several hundred-base pair long I region located 5′ to the switch region sequences and located several thousand base pairs upstream of the first constant region exon. The production of germ-line transcripts with distinct 3′ ends generated by splicing to the membrane exons, as well as employing the secreted terminus, has been shown for the μ heavy chain gene.[9] RNA splicing has also been shown to generate alternative 5′ forms of germ-line α immunoglobulin transcripts.[10–12] Recently we and others have shown that productive human ε mRNA employs complex alternative splicing in producing the 3′ end of mature mRNA. This results in the generation of a series of nine distinct ε mRNA that encode two membrane and five secreted ε proteins.[13–16] Furthermore, we have shown that the relative production of these splice variants could be modulated by interleukin-10 (IL-10) as well as other stimuli.[17]

We have undertaken to determine if the full set of alternative splicing events we had observed at the 3′ end of the ε gene occurred independent of isotype switching and, if so, if it was responsive to modulation in a fashion analogous to

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that for mature ε RNA transcripts. Alternative RNA splicing of ε germ-line transcripts may provide a mechanism for the regulation of isotype switching, either directly via distinct mRNA species or indirectly via RNA trans-splicing or discontinuous transcription, to produce various mature ε mRNA and IgE proteins.[18–21]

MATERIALS AND METHODS

Cell line and cell cultures

The human IgE-secreting cell line AF-10 was maintained in complete RPMI-1640 medium, as described previously.[13] Fresh human B cells were isolated from tonsil and purified by two cycles of the Aminoethylisothiouronium bromide–sheep red blood cell (SRBC) rosette method and monocyte depletion, as described previously.[22–24] The resulting tonsil cell population contained 99% B cells ($CD19/CD20^+$); T cells ($CD3^+$) and monocytes ($CD14^+$) were less than 1%, as determined by flow cytometry. The purified B cells (1×10^6 cells/ml) were cultured for 1–4 days in the presence of 100 U/ml recombinant IL-4 (a kind gift of Drs E. S. Medlock and L. Souza, Amgen, Thousand Oaks, CA) for induction of ε germ-line transcripts. In some cultures of fresh human B cells, in addition to IL-4, either IL-10 (a kind gift from Dr K. Moore, DNAX, Palo Alto, CA) at concentrations of 1–100 ng/ml or IL-6 (a kind gift of Drs E. S. Medlock and L. Souza) at concentrations of 1–100 U/ml were added at the same time.

RNA isolation and reverse transcription (RT)

Total cytoplasmic RNA was isolated from both AF-10 cells and induced/uninduced B cells as described previously.[13] 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 (BRL, Gaithersburg, MD). The cDNA mixture was heated to 95° for 5 min and then immediately used as a polymerase chain reaction (PCR) substrate.

Amplification and analysis of ε cDNA

Pairs of PCR primers were designed for detecting different forms of ε mRNA containing membrane exon sequences. Table 1 shows the oligonucleotide primers and probes used in this study. For the specific detection of ε germ-line transcripts containing membrane exon region sequences, the 5′ primer was located within the I exon (primer Gε1; Table 1) and the 3′ primer was in the 3′ untranslated region of the M2′ exon (primer 8; Table 1). For the specific detection of productive ε transcripts (those containing the VDJ–CHε sequence), a conserved sequence derived from all the human J regions was chosen as the 5′ primer (primer JH; Table 1) and the 3′ primer was the same as that for ε germ-line transcripts. PCR amplifications were performed as described previously.[13]

Southern blot analysis of PCR products

PCR products were extracted with phenol:chloroform and then 20 μl of each reaction mixture was electrophoresed on 1·5% agarose gel (NuSieve and Seakem agarose 1:1 mixture; FME Bioproducts, Rockland, ME) in Tris–borate–EDTA (TBE) buffer. DNA was transferred to nylon membranes (Nytran; Schleicher & Schuell Inc., Keene, NH) in 0·4 M NaOH, and blots were analysed by probing with end-labelled oligonucleotides corresponding to an internal region of each putative ε membrane exon. Blots were prehybridized for 2 hr at 60° in 5 × sodium chloride–sodium phosphate–EDTA buffer (SSPE) Denhardt's solution, 0·5% sodium dodecyl sulphate (SDS) and 250 ng/ml of salmon sperm DNA. Hybridization with kinased oligonucleotide probes was performed overnight at 60°. The blots were washed for 20 min at room temperature with 2 × sodium chloride–sodium citrate (SSC) plus 0·1% SDS and then twice more at 60° with 0·2 × SSC plus 0·1% SDS for 20 min.

Table 1. Oligonucleotide sequences of primers and probes

Name	Sequence (5′ to 3′)	Position and reference[13,14]
Gε1	AGGCTCCACTGCCCGGCACAGAAAT	5′ end of Iε exon[7]
Gε3	AGCTGTCCAGGAACCCGACAGGGAG	Internal Iε exon[7]
JH	ACCCTGGTCACCGTCTCCTCA	3′ end of conserved J chain[31]
1	ACGGAGGTGGCATTGGAGGGAATGT	Cε1 domain[32]
2	TCATGCGGTCCACGACCAAGA	3′ end of Cε3 domain[32]
3	AGGCAGCGAGCCCCTTCACAGACCG	274–297 of fig. 2a in ref. 14
4	GAGGACTGGCCAGACCTTCTG	383–403 of fig. 2a in ref. 14
5	GTCATTGCAACAGTGGACAG	420–401 of fig. 2a in ref. 14
6	TGTAGCTCACGCTCAGCAGG	281–262 of fig. 1b in ref. 13
7	GAATGGGAGTGACCCGGAGAC	495–475 of fig. 2b in ref. 14
8	GACGGGGATGCCAGGCAGTTGGATC	648–624 of fig. 2b in ref. 14
9	GTGGGGCGCCCCAGGCACCA	β-actin gene
10	CTCCTTAATGTCACGCACGATTTC	β-actin gene
11	CGCGAGAAGATGACCCAGATC	5′ end of third exon of β-actin gene
12	ATCACGATGCCAGTGGTACGG	3′ end of fourth exon of β-actin gene

Quantitative analysis of ε mRNA

The procedure for quantifying and comparing the levels for different ε mRNA isoforms by employing RT and PCR amplification has been detailed elsewhere.[24] Briefly, total cellular RNA was isolated from B cells ($1–5 \times 10^6$ cells/sample) that had been cultured with IL-4, CD40 and/or different concentrations of IL-10, as described above. The RNA was reverse transcribed as described elsewhere.[14]

The PCR strategies were based on that reported by Go *et al.*,[25] in which a linear relationship is established for each PCR product. Two different PCR reactions were performed. The oligonucleotide sequences of the primers used are shown in Table 1. The first PCR, termed PCR1′, used the primer pairs 3 + 8 and 9 + 10 and was used to amplify the three ε mRNA isoforms CH4–M1′–M2, CH4–M2′ and CH4–M2″[14] and the house-keeping gene β-actin. The second PCR, termed PCR2′, used the primer pairs 2 + 5 and 11 + 12 and was used to amplify two other ε mRNA splices, the CH4–S form (the classic secreted isoform) and the alternative spliced CH4′–CH5 form, along with β-actin. The reaction mixtures and cycle times were chosen following extensive titration of each reagent and timing to ensure equal amplification of all products. The characters of the ε mRNA isoforms generated by alternative 3′ splicing are shown in Fig. 1. The location of the primers used for PCR1′ to measure the amounts of CH4–M1′–M2,

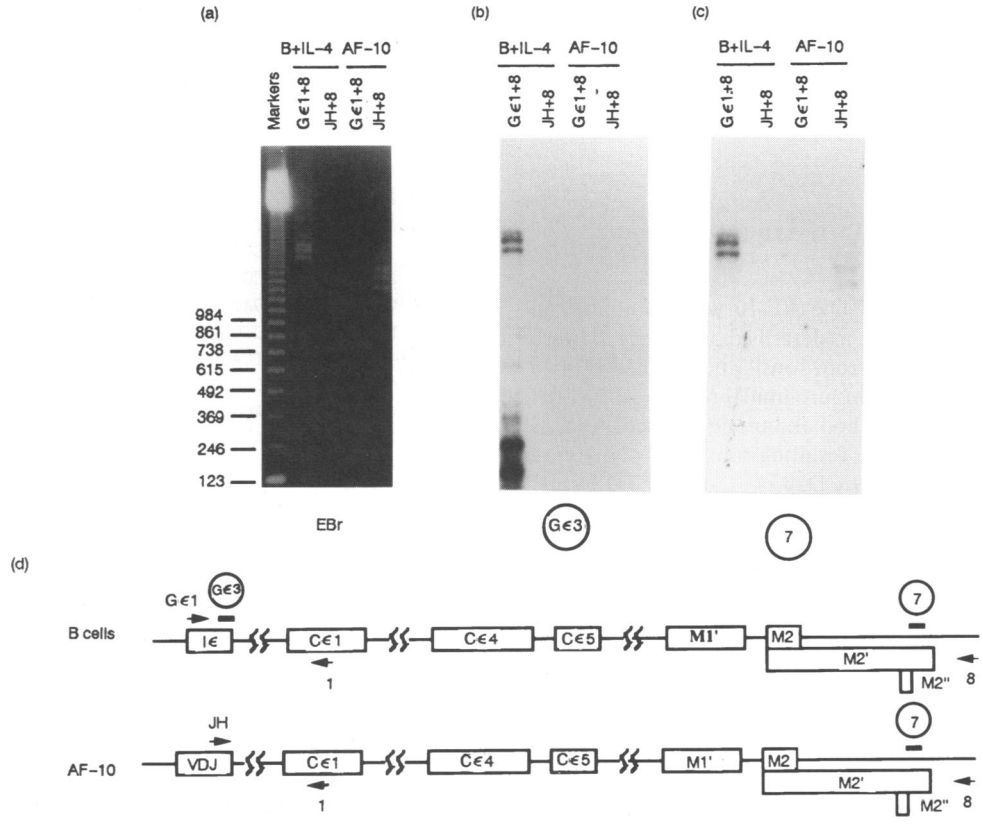

Figure 1. Demonstration that multiple forms of ε germ-line transcripts contain membrane exon region sequences. (a) RT-PCR products, amplified with primer sets shown from purified B cells, stimulated with IL-4 and AF-10 cells were electrophoresed and visualized by ethidium bromide (EB) staining. Primer set Gε1 + 8 amplifies products containing the I region sequence, i.e. germ-line transcripts, while primer set JH + 8 amplifies products containing the J region sequence, i.e. productive transcripts from VDJ rearranged ε genes. The markers lane represents base pairs (bp). (b) The blot in (a) was hybridized with oligo probe Gε3, an internal probe for the ε I exon. Multiple bands from B cells stimulated with IL-4 hybridized, showing that the various forms of ε germ-line transcripts were present in these cells while being absent from the IgE-secreting AF-10 cells. (c) The blot in (a) and (b) was again stripped and rehybridized, this time to a probe derived from the membrane exon 2′ region sequences (oligo probe 7). Multiple bands were observed with the PCR products from B cells stimulated with IL-4 and AF-10 cells. This result indicated that the multiple forms of ε germ-line transcripts (Gε1 + 8 products in B cells + IL-4), as well as productive ε transcripts (JH + 8 products in AF-10 cells), contained membrane region exon sequences. (d) Diagrammatic map of the relevant human ε heavy chain exons showing their relationships and the oligonucleotides used as PCR primers and hybridization probes in this study.

CH4–M2′ and CH4–M2″, and PCR2′ to measure the amounts of CH4–S and CH4′–CH5 are also denoted.

For quantification, aliquots of 20 μl of each PCR product were run on a 8% non-denaturing polyacrylamide gel at 100 V for 5 hr, dried on a Biorad 543 gel drier (Biorad, Hercules, CA) for 45 min at 65° and then exposed to Kodak XAR-5 film for 8–12 hr. In place of a densitometer, the autoradiograph was scanned on an Apple One 24-bit Scanner at 300 d.p.i. and converted into a TIFF uncompressed format and the resultant pattern analysed using the Scan Analysis program from Biosoft (Cambridge, UK), which delineates peaks and integrates the area under each peak.

The ability to quantify the PCR products was established and validated by employing plasmid cDNA representing the different ε mRNA products, and showed that there was a log–linear correlation between the number of starting copies of the plasmid and the PCR yield, as quantified by densitometry for each cDNA alone and in combination.[24] PCR titration of individual plasmids showed that, for each, this PCR strategy was sensitive to 10^2 copies of template. The individual isoform amplification rates were normalized to that of β-actin mRNA.

RESULTS

Multiple forms of human ε germ-line transcripts contain membrane exon region sequences

To define the possible existence of ε germ-line transcripts that contain various membrane exon sequences, we stimulated highly purified human B cells with IL-4, conditions under which ε germ-line[6,7] as well as multiple γ germ-lines, including γ4,[26] but not mature productive ε transcripts, are induced.[6,7] Using RT-PCR with a pair of primers designed for detection of ε germ-line transcripts from B cells (oligoGε1 and 1; Table 1 and Fig. 1) and a set of primers for productive ε transcripts from the VDJ rearranged, ε isotype-switched plasmacytoma cells from AF-10 (oligoJH and 1), we observed that RNA from

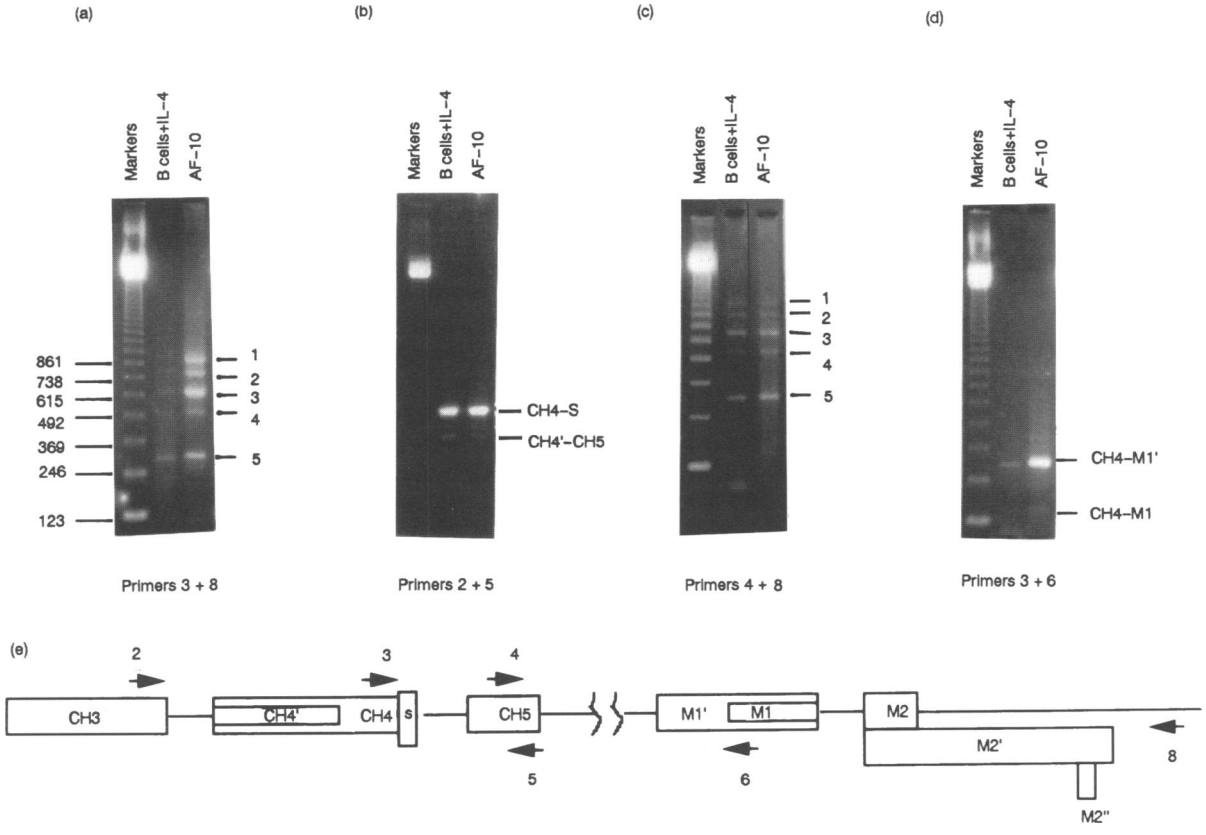

Figure 2. RT-PCR analysis of the alternative 3′ splicing of ε germ-line transcripts in B cells stimulated with IL-4. The RT-PCR products from AF-10 cells were also analysed as known splicing variants for comparison.[13,14] The PCR products were electrophoresed and visualized by ethidium bromide (EB) staining. The markers lanes represent base pairs (bp). (a) RT-PCR products from IL-4-stimulated B cells were amplified with primers 3 and 8. Five discrete bands representing three splice variants (see Results) were observed with RNA from both cell sources. (b) RT-PCR products from both B cells and AF-10 cells were amplified with primers 2 and 5. Both showed two products representing the CH4–S transcript and the CH4′–CH5 splicing product. (c) RT-PCR products showing splicing from CH5 to the downstream membrane exon region were amplified with primers 4 and 8. Five discrete bands representing three splice variants (see Results) were observed with RNA from both cell sources. (d) RT-PCR analysis of mRNA containing the CH4–M1′ and CH4–M1 splicing products. Amplification from IL-4-induced B cells, as well as AF-10 cells, was performed the primer set 3 + 6, which is known to amplify splicing representing the two membrane-coding alternative forms for ε mRNA. Products representing the CH4–M1′ and CH4–M1 products were apparent with both cell sources. (e) Diagrammatic map of the exons at the 3′ end of the human ε heavy chain, showing their relationships and the oligonucleotides used as PCR primers in this study.

the induced B cells produced ε germ-line transcripts while ε productive transcripts were absent (data not shown). As a control, AF-10 cells showed no signal for germ-line transcripts and a strong positive signal for productive transcripts (data not shown.)[6]

When the RNA from the IL-4-stimulated tonsil B cells was subjected to RT-PCR with primer set Gε1–8, which amplifies germ-line transcripts containing any ε membrane exon sequence, we detected several bands (Fig. 1a). These hybridized with both the internal Iε exon probe (oligoGε3, Fig. 1b), showing that they were of germ-line origin, and the internal membrane exon probe (oligo7; Fig. 1b), showing that the membrane exon sequence was also included. As a control, AF-10 cells only showed the productive ε transcripts containing membrane exon region sequences (Fig. 1a, c). These results identified that different forms of ε germ-line transcripts containing membrane exon(s) region sequences were present in the IL-4-stimulated B cells.

Membrane exon-containing ε germ-line transcripts use identical alternative splicing patterns to those of productive ε transcripts

To determine whether the ε germ-line transcripts detected used the same alternative splicing pattern as the productive ε mRNA, we employed our previously described sets of PCR primers for analysing the alternative splicing of ε mRNA. We examined the mRNA splicing pattern of ε germ-line transcripts and compared the results with those from ε productive mRNA. As shown in Fig. 2, when several pairs of primers were used for detection of different forms of mRNA generated by the alternative splicing, the ε germ-line transcripts from purified B cells stimulated with IL-4 gave rise to an identical pattern of ε mRNA PCR products as that of productive transcripts from the switched IgE-secreting AF-10 line cells.

In Fig. 2a, five PCR products can be seen. The bands 1, 3 and 5 correspond to the CH4–M1′–M2, CH4–M2′ and CH4–M2″ splicing products, respectively, as determined by previous work.[14] Bands 2 and 4 resulted from heteroduplex

formation between different alternative spliced products formed under the electrophoresis condition used.[14] Hybridization of these PCR products with the internal M2′ probe (oligo7) indicated that these PCR products were not the result of non-specific amplification (data not shown).

Similarly, when a primer set (oligo2 and 5) was used to assay for mRNA forms of the novel CH4′–CH5 transcripts in ε germ-line transcripts, an identical pattern from the both cell types was observed (Fig. 2b). The larger band represents the transcripts of the classic secreted form (CH4–S) and the smaller band represents the CH4′–CH5 transcripts. Hybridization of the bands on a Southern blot to the internal probe (oligo4) confirmed that the PCR products amplified were ε-specific products (data not shown). Figure 2c shows that amplification with the primer set 4 + 8 gave the same PCR pattern as that of the primer set 3 + 8, demonstrating that the CH4′–CH5 germ-line transcripts from the IL-4-induced B cells spliced from CH5 to all the common splicing acceptor sites in the membrane exon regions, as previously defined in productive transcripts from AF-10 cells, i.e., splicing from CH5 to M1′, M2′ and M2″ formed the CH4′–CH5–M1′–M2 (band 1), CH4′–CH5–M2′ (band 3) and CH4′–CH5–M2″ (band 5) products. As with the PCR products from primer set 3 + 8, bands 2 and 4 in Fig. 2c are the PCR artifacts generated by heteroduplex formation.[1]

We next determined whether ε germ-line transcripts contained both splice variants of mRNA that code for membrane ε normally found in IgE-producing cells, the relatively abundant CH4–M1′–M2 transcripts and the CH4–M1–M2 splice variant, a minor form of ε mRNA.[14–16] This was accomplished by using RT-PCR analysis of germ-line ε mRNA employing primer pair 3 + 6. As shown in Fig. 2d, pairs of identical PCR products were amplified with the ε germ-line transcripts of IL-4-induced B cells, as well as with the ε productive transcripts from AF-10 cells. The larger band represents the CH4–M1′ splicing product and the smaller band represents the CH4–M1 spliced transcripts. The exact nature of the CH4–M1 splicing product was confirmed by cloning and sequencing (results not shown).[13] These results demonstrate that ε germ-line transcripts employ both splices normally used to generate the two types of productive ε mRNA coding for the membrane-bound form of the ε chain.

The pattern of alternative spliced germ-line ε mRNAs is modulated by IL-10 in a fashion analogous to productive ε mRNA transcripts

We have shown that IL-10 can both increase and alter the production of different forms of productive ε mRNA *in vitro*.[17] Using a modified PCR with radiolabelled primers, the relative ratios of three of the ε isoforms, CH4–M1′–M2, CH4–M2′ and CH4–M2″, could be quantified and compared to a β-actin control in a single reaction. Since this method is very sensitive and is performed under very stringent conditions, we were able to use fewer cycles than those used in the experiments shown in Fig. 2a, thereby eliminating heteroduplex formation and resulting in the presence of only three PCR product bands. A second PCR detected the presence of both CH4–S and CH4′–CH5 transcripts and compared their production to that of a β-actin control.

ε germline transcripts from IL-4-stimulated B cells resulted in the production of a pattern of ε mRNA isoforms that was similar and reproducible for each individual donor (Fig. 3), with the most abundant forms being the secreted CH4–M2″ form and the classic secreted form. Cultures of IL-4 plus IL-10 did not show evidence of productive transcripts or IgE protein production (data not shown), so the changes in ε mRNA isoforms necessarily involved germ-line transcripts. The addition of IL-10 to IL-4-stimulated B cells induced a differential change in relative amounts of specific ε mRNA and at the same time increased total ε mRNA by over 10-fold. This change consisted of a large increase in the presence of the CH4–M2′ form and a smaller relative increase in the amounts of the CH4–M1–M2, CH4–M2″ and CH4–S splice variants. In contrast, the CH4′–CH5 mRNA, which uses both unique splice acceptor and donor sites, increased considerably less. Thus the overall pattern of germ-line ε mRNA produced was clearly altered by IL-10, e.g. the ratio of CH4–S to CH4′–CH5 increased from 9 : 1 to 40 : 1. This is similar to the change that is induced with productive ε mRNA by IL-10.[17]

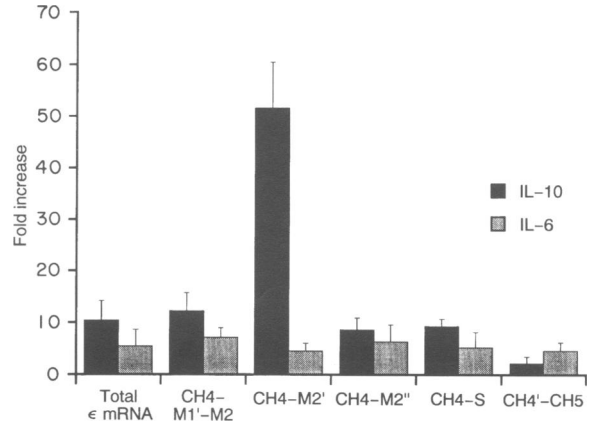

Figure 3. Quantitative RT-PCR analysis of the pattern of alternative 3′ splicing of ε germ-line transcripts in B cells and the modulation of this pattern by IL-10. The relative enhancement (as fold increase) for each of the ε mRNA variants noted, as induced by addition of IL-10 (50 ng/ml) or IL-6 (500 U/ml) to purified tonsil B cells stimulated with IL-4 (200 U/ml), compared to levels obtained when no cytokine was added. The amount of mRNA detected for each ε variant from cells recovered after 3 days of culture with the relevant cytokine was measured and standardized relative to the amount of β-actin present. The results shown are the mean and standard deviation of three experiments.

The greatest changes occurred at 50 ng/ml of IL-10. At other concentrations the results were less marked but still significant (Table 2). In contrast, altered relative production of the germ-line ε mRNA forms was not observed with IL-6 (Fig. 3). There was no apparent shift in the observed ε isoform pattern at the highest concentration of IL-6 (500 U/ml), even though the total amount of ε mRNA increased by 540%. Table 2 shows that this failure of IL-6 to alter the pattern of germ-line ε mRNA held throughout the range of concentrations of IL-6 tested.

DISCUSSION

We and others have previously shown that the single human functional ε heavy chain gene is able to generate an unexpected complexity of mRNA and resulting proteins

Table 2. IL-10 increases germ-line ε mRNA levels and changes the levels of germ-line ε mRNA containing differing 3′ ends*

	CH4–M1′–M2	CH4–M2′	CH4–M2″	CH4–S	CH4′–CH5
IL-10 (0·5 ng/ml)	0·1	0·9	0·1	0·3	0
IL-10 (5 ng/ml)	1·5	6·6	1·8	2·1	0·3
IL-10 (10 ng/ml)	5·1	20·2	5·7	8·2	0·5
IL-10 (50 ng/ml)	12·3	51·8	8·7	9·3	2·2
IL-10 (100 ng/ml)	8·9	38·6	7·7	9·0	1·1
IL-6 (0·5 U/ml)	0	0	0	0	0
IL-6 (5 U/ml)	1·8	0·9	1·2	0·7	1·3
IL-6 (50 U/ml)	5·2	4·3	5·3	3·8	5·5
IL-6 (500 U/ml)	7·2	4·6	6·4	5·3	4·7

* The amount of mRNA detected for each ε isoform from B cells was measured and standardized relative to the amount of β-actin present. The results shown are the mean and the standard deviation of five replicates. The relative enhancement (as fold increase) for each of the ε mRNA above that obtained when B cells were cultured with IL-4 alone is shown for different concentrations of IL-10 or IL-6.

through alternate mRNA splicing at the 3′ end of the primary ε RNA transcript.[13–16] It is now clear that for the ε gene, as for all immunoglobulin heavy chain genes, there is production of germ-line transcripts prior to actual DNA rearrangement and subsequent production of productive or mature VDJ-containing transcripts.[1] We undertook to determine whether these germ-line transcripts make use of the full set of 3′ splices that occurs with productive ε mRNA transcripts. if so, then germ-line transcripts of multiple ε mRNA species would exist, and this may have relevance towards the regulation of the ε gene itself, as discussed below.

Utilizing a RT-PCR strategy that we had developed originally to identify the various mature ε mRNA, we analysed RNA from B cells stimulated in the presence of IL-4 alone. IL-4 alone did not induce DNA rearrangement/isotype switching to the ε locus, but rather generated ε germ-line transcripts. These transcripts were initiated at several sites within the I exon, which is located just upstream of the ε switch region and is approximately 3 kb upstream of the first ε constant region exon. These germ-line transcripts are then spliced from the Iε exon to the first constant region exon. The presence of these germ-line transcripts was detected using a PCR-based strategy which employed an upstream primer within the I exon. We were able to demonstrate, as expected, that the IL-4-driven purified B cells did not contain mature transcripts (containing the J region sequence) but contained only transcripts containing the Iε region sequence upstream of the first ε constant region sequence. This germ-line RNA pool was then analysed for the alternate spliced mRNA. Indeed, we found an identical pattern of 3′ splices among this germ-line mRNA, as we had found among the productive ε mRNA. These experiments show that the 3′ events of alternate splicing of the ε transcripts are not linked or related to the events at the 5′ end of the ε gene or the resulting RNA transcripts, i.e. similar splicing occurs with both mature and germ-line transcripts.

The ability of the IL-4-stimulated B cells to produce multiple forms of ε germ-line transcripts with the different 3′ ends makes it likely that ε germ-line transcripts on Northern blot should occur as multiple species in addition to the one 1·7 kb species generally referred to. Indeed, we observed such bands in our blots, as well as in other published work,[6,7,13] although often the higher kb region is not shown in figures. Such multiple species of ε germ-line transcripts may be indistinguishable from the productive ε mRNA for both classic secreted and membrane-bound forms on Northern blot analysis. So species of RNA larger than 1·7 kg that hybridize to ε constant or even membrane region probes do not necessarily represent the productive forms of ε mRNA and need to be interpreted cautiously. To distinguish accurately productive from germ-line transcripts on Northern blot, it is necessary for the blot to be differentially hybridized with both a Cε and an Iε probe.

Having established the ability of germ-line transcripts to undergo alternative splicing, we investigated whether this splicing was susceptible to modulation in a fashion analogous to what we have also shown occurs with the productive transcripts.[17,24] We employed IL-10 as a model modulating stimulus. IL-10 did induce both up-regulation of total ε germ-line mRNA and a clear change in the pattern of germ-line ε mRNA splicing in a fashion analogous to that which we have seen with IgE-producing cells. Thus not only are germ-line transcripts produced as various ε mRNA isoforms, but the splicing mechanism in germ-line ε-producing cells is modulated in a fashion analogous to that in IgE-producing cells. It is likely, but remains to be proven, that other external stimuli, such as CD23 ligation on B cells as well as aromatic hydrocarbons from diesel exhaust particles, can also alter the ε germ-line splicing pattern in their own individual ways.[17,24] We have shown previously that these two stimuli are capable of changing the mature splicing pattern of ε-producing cells. This change in splicing patterns showed specificity in that not all stimuli would up-regulate the ε transcription which induced the change in splicing pattern, as evidenced by the failure of IL-2 and IL-6 to affect a change in the ε mRNA isoform pattern.

The role of germ-line transcription in subsequent isotype switching that involves DNA rearrangement remains vigorously debated. None of the ε germ-line transcripts that we have identified codes for proteins, as there are multiple stop codons in the I exon. Enhanced transcription through the full Iε–Cε

locus is associated with increased isotype switching, as shown by Xu *et al.*[27] who put a constitutive promoter in the place of the IL-4 response element. On the other hand, replacing the entire upstream regulatory region and simply inducing transcription through this region does not lead to enhanced isotype switching, as shown by the experiments of Bottaro *et al.*,[8] indicating that more than simply enhancing transcription through a region 5′ to the ε switch region is required for isotype switching to occur.

Our studies gain increasing potential importance in view of recent studies by Rastinejad & Blau,[28] which investigated potential roles of the 3′ ends of different mRNA in muscle proteins. They demonstrated that distinct mRNA isoforms had the ability to regulate the expression of their own gene and alter cell differentiation. Thus, one can imagine that the production of different ε mRNA isoforms in germ-line configuration may enhance or inhibit the likelihood of a cell going on to undergo isotype switching and subsequent differentiation into a high rate IgE-secreting plasma cell. This becomes particularly germane in view of our evidence that the production of the different ε germ-line mRNA is susceptible to modulation by specific external stimuli such as IL-10. Should this be true, then manipulating potential or nascent IgE-secreting cells may be possible by either altering the absolute levels of germ-line mRNA containing different 3′ ends or by changing the relative amounts of the different germ-line RNA.

Production of heavy chain immunoglobulin gene germ-line transcripts is thought to be a necessary step preceding the deletional recombination mechanism that leads to isotype switching.[1,2] Additionally, germ-line transcripts themselves have also been demonstrated to be involved in another form of isotype 'switching', the production of downstream isotype proteins by RNA trans-splicing.[18–20] This trans-splicing process joins the downstream heavy chain constant region sequences from the corresponding germ-line RNA transcripts to the VDJ region from RNA transcripts associated with the μ chain, and thereby generates a productive immunoglobulin mRNA encoding a mature downstream immunoglobulin heavy chain protein. Such a trans-splicing mechanism has been shown to be involved in the isotype production for IgG1 and IgE from unswitched B cells, as evidenced by studies *in vitro* and in transgenic mice.[18–20] This same outcome could result from discontinuous transcription, which has been suggested to occur in murine BCL_1 cells.[21] These processes may also be involved in IgE formation from those human Epstein–Barr virus (EBV)-transformed B-cell lines that produce both IgM and IgE (or IgG) simultaneously without heavy chain gene rearrangement to the ε locus (or γ locus).[29] Data suggesting the production of multiple immunoglobulin proteins by trans-splicing or discontinuous transcription could provide evidence for the occurrence of IgE forms in IgH chain unrearranged B cells without the need to invoke the postulated existence of large primary RNA.[30]

Additionally, the multiple ε germ-line transcripts described in this paper, with various 3′ ends generated via alternative splicing, provide a substrate with which the aforementioned mechanisms could produce the full array of ε mRNA and resulting proteins in the absence of DNA isotype switching. Such trans-splicing- or discontinuous transcription-derived membrane IgE proteins may then play a role in the subsequent isotype switching of B cells via signalling through membrane IgE. Also, trans-splicing-derived antigen-specific secreted IgE may function to regulate IgE switching via Fc receptors I and II of IgE stimulation of various cells that express these receptors and their soluble products.

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