

# Structure and Expression of Germline $\epsilon$ Transcripts in Human B Cells Induced by Interleukin 4 to Switch to IgE Production

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## Summary

Interleukin 4 (IL-4)-induced IgE production coincides with the appearance of the 2.2-kb productive  $\epsilon$ -mRNA, but is preceded by synthesis of a 1.7-kb  $\epsilon$ -RNA. Analysis of cDNA copies of the 5' end of this RNA indicated that the 1.7-kb  $\epsilon$ -RNA is a germline  $\epsilon$  immunoglobulin heavy chain transcript with an exon mapping 5' to the switch region. Transcription through switch regions has been implicated in the control of class switching. However, IL-4 or cloned CD4<sup>+</sup> T cells were able to induce germline  $\epsilon$  transcripts without inducing IgE synthesis, for which both signals were required. These results indicate that induction of human germline  $\epsilon$ -RNA does not necessarily result in IgE synthesis, and that additional regulatory mechanisms are involved in class switching.

Cytokines play an important role in regulating Ig isotype production. The lymphokines IL-4 and IFN- $\gamma$ , which are produced by T cells (1), and TGF- $\beta$ , which is produced by many cell types (2-5), have been shown to affect the pattern of secreted isotype. Murine splenic B cells polyclonally activated by LPS produce predominantly IgM, IgG2b, and IgG3 (6). Addition of IL-4 to these cultures reduces IgG2b and IgG3 synthesis, whereas IgG1 (7), and particularly IgE synthesis, are enhanced (8, 9). IFN- $\gamma$  selectively enhances IgG2a synthesis (10). More recently, it has been shown that TGF- $\beta$  induces LPS-activated murine B cells to switch to IgA (11, 12). Human IL-4 induces human IgE synthesis in cultures of mononuclear cells (MNC)<sup>1</sup> from blood, tonsils, or spleen cells in the absence of polyclonal B cell activators (13-15). In addition to IL-4, a second signal transmitted by CD4<sup>+</sup> T cells was required (16; Gascan et al., manuscript submitted for publication). Limiting dilution analyses and studies with single B cells have indicated that IL-4 induces certain murine and human B cells to switch to IgE-producing cells at high frequencies (17; Gascan et al., manuscript submitted for publication).

Recent results suggest that class switching induced by lymphokines and polyclonal B cell activators in vitro is preceded by expression of the corresponding germline CH gene (12, 18-27). Murine,  $\mu$ ,  $\gamma$ 2b,  $\gamma$ 1,  $\gamma$ 3,  $\alpha$ , and germline  $\epsilon$  transcripts have been shown to initiate upstream of the corresponding S regions (19, 22-28). The structure of the switch

germline transcripts implies that transcription transverses these regions, and it has been proposed that isotype switching may occur through modulation of accessibility of the specific switch regions to a common recombination system, as has been suggested for VDJ rearrangements (18, 20, 22, 29, 30). On the other hand, a correlation between germline transcript expression and subsequent switching to the corresponding isotype could mean that such transcripts play a direct regulatory role, as has been suggested by Collier et al. (31).

In the present study, we investigated the relationship between C $\epsilon$  transcripts and switching to IgE in a culture system in which B cells of normal, nonallergic individuals are induced to switch to IgE-producing cells by IL-4 and CD4<sup>+</sup> T cells (13, 14). A germline  $\epsilon$  transcript exon was identified, cloned, and characterized. The requirements for germline  $\epsilon$  transcription and switching to IgE synthesis by normal human B cells and the relationship between these processes were investigated.

## Materials and Methods

**Cells and Cell Cultures.** Induction of IgE synthesis by IL-4 was carried out as described (13, 14). Briefly, PBMC of healthy donors were isolated by centrifugation over Ficoll-Hypaque and resuspended in Yssel's medium (32) supplemented with 10% FCS and incubated at a final density of 10<sup>6</sup> cells/ml in the presence of IL-4 (300 U/ml) overnight. The nonadherent cells were collected, washed twice, and resuspended in fresh medium in the presence or absence of IL-4 (300 U/ml) at 10<sup>6</sup> cells/ml in volumes of 1 ml in 2-ml microtiter plates. Supernatants and cells were collected for IgE determination and mRNA analysis, respectively, at various time points

<sup>1</sup> Abbreviations used in this paper: A-PCR, anchored PCR; MNC, mononuclear cells; SAC, *Staphylococcus aureus* Cowan.

of the cultures, as indicated. IgE synthesis induced in cultures of purified B cells and cells of the HLA-DR4-specific CD4<sup>+</sup> T cell clone A3 (33) were carried out as described by Gascan et al. (manuscript submitted for publication). Briefly, human spleen MNC were isolated by centrifugation over Ficoll-Hypaque. The B cells were labeled with an FITC-conjugated anti-CD20 mAb (Becton Dickinson & Co., Mountain View, CA) and isolated by FACS sorting. 10<sup>6</sup> sorted B cells (>99% pure as judged by staining with FITC anti-CD20) were cocultured with 10<sup>6</sup> A3 cells in the presence or absence of IL-4 for the time periods indicated. Cultures were carried out in Yssel's medium supplemented with 10% FCS in volumes of 1 ml in 2-ml microtiter wells. Supernatants and cells were harvested for measuring IgE synthesis and RNA isolation (10<sup>6</sup> B cells were used for each RNA isolation). The T cell clone A3, which fails to produce IL-4 (33), was maintained in Yssel's medium supplemented with 10% FCS, as described previously. The U266 IgE-producing myeloma cell line (34) (kindly provided by Dr. A. Saxon, UCLA School of Medicine, Los Angeles, CA) was also expanded in Yssel's medium supplemented with 10% FCS.

**Stimulation of PBMC and B Cells.** To determine the effects of lymphokines and polyclonal B cell activators on germline  $\epsilon$  expression and IgE synthesis, B cells or PBMC were incubated with the following reagents: rIL-4 (sp act, 10<sup>7</sup> U/mg; Schering Research, Bloomfield, NJ), used at 300 U/ml; rIL-2 (Cetus Corp., Norwalk, CT), used at 40 U/ml; and rIFN- $\gamma$  and rIFN- $\alpha$  (Schering Research) used at 250 U/ml. The polyclonal B cell activator Pansorbin and its non-B cell-activating counterpart Sansorbin (Calbiochem-Behring Corp., La Jolla, CA) were used at 0.5% (wt/vol); Immunobeads anti-human IgM (Bio-Rad Laboratories, Richmond, CA) at 5  $\mu$ g/ml; and PMA (Sigma Chemical Co., St. Louis, MO) at 1 ng/ml. Sansorbin and Pansorbin are *Staphylococcus aureus*-fixed cells. Sansorbin has no protein A, whereas Pansorbin contains protein A.

**Measurement of IgE Production.** The ELISA for IgE and its specificity have been described in detail elsewhere (13). Briefly, flat-bottomed microtiter plates (Nunc, Roskilde, Denmark) were coated with rabbit anti-IgE (Dako Corp., Glostrup, Denmark) diluted 1:2,000 in a bicarbonate buffer (pH 9.6). After incubation for 18 h at 4°C, the plates were washed with PBS containing 0.05% Tween 20 and incubated for 1 h at 37°C with RPMI 1640, 10% FCS to saturate protein-binding sites. Next, the culture supernatants were diluted to the appropriate concentration in PBS + 0.1% BSA, added to the plates, and incubated for 6 h at 20°C. The plates were washed, and the murine anti-IgE mAb I-27 (35) was added and incubated for 18 h at 20°C. After washing, the plates were finally incubated for 3 h at 37°C with goat anti-mouse Ig coupled to peroxidase (1:2,000) (Tago Inc., Burlingame, CA). After washing, 1 mg/ml of 2,2'-azinobis (3-ethylbenzthiazoline sulfonic acid) (Sigma Chemical Co.) in 0.1 M citrate-0.1 M phosphate buffer, pH 4.5, containing 0.003% H<sub>2</sub>O<sub>2</sub>, was added as enzyme substrate. ODs were measured using a VMAX ELISA reader (Molecular Devices, Palo Alto, CA). An IgE serum (Behring Diagnostics, Marburg, FRG) was used as a reference standard. The limit of sensitivity of the assay was 150 pg of IgE/ml.

**RNA Isolation and Northern Blot Assay.** Total RNA was isolated from PBMC or U266 cells by the guanidinium thiocyanate-CsCl procedure (36). For the experiment with purified B cells, RNA was purified by extraction with RNAzol B (CNNA/Biotec, Friendswood, TX), according to the instructions of the manufacturer, using *Escherichia coli* tRNA as carrier. Aliquots of RNA (2  $\mu$ g) were subjected to electrophoresis in 1% agarose, 6% formaldehyde gels (37), electrotransferred to Gene Screen nylon membranes (NEN Research Products), and fixed by UV irradiation (38).

Hybridization with cRNA probes and washing conditions were the same as described previously (39). Washes include a treatment with ribonuclease A, which prevents rehybridization of the RNA with a probe for actin or rRNA. Therefore, membranes were stained with methylene blue to localize and quantify rRNA before hybridization (40). A 0.24–9.5-kb RNA ladder (Bethesda Research Laboratories, Gaithersburg, MD) was used as marker.

To produce a cRNA probe specific for C $\epsilon$ , the 4-kb BamHI fragment of Ch4AHIGe12 (kindly provided by Dr. Honjo, Kyoto University, Japan) (41) containing C $\epsilon$  was recloned. A Ball-Ball subfragment (containing the CH4 domain) was inserted in pBS<sup>-</sup> (42) and used for in vitro transcription of a <sup>32</sup>P-labeled cRNA probe (43). Similarly, the SphI-HaeII fragment of the longest germline  $\epsilon$ -cDNA I-4 was recloned in pBS<sup>-</sup> and used to synthesize the corresponding cRNA probe. The cRNA probe used to detect actin was complementary to a BglII-SmaI fragment of pHF $\gamma$ A-1 (44).

**Preparation of cDNA and Cloning Procedures.** The germline transcript exon was cloned using anchored PCR (A-PCR) (45). Poly(A)<sup>+</sup> RNA was isolated from PBMC incubated for 6 d in the presence of IL-4 by oligo-dT chromatography (40). Integrity of the 1.7-kb  $\epsilon$ -RNA was assessed by Northern blot analysis. cDNA was synthesized using a mixture of four oligonucleotides derived from CH1 as primers (complementary to bases 368–388, 398–418, 428–448, and 458–478, according to Seno et al. [46]) and mouse Moloney leukemia virus reverse transcriptase (47). The cDNA was extracted with phenol/chloroform, precipitated twice with spermine-HCl, and tailed with dGTP (48). The tailed cDNA was phenol/chloroform extracted and ethanol precipitated. cDNA was amplified by A-PCR using recombinant TaqI DNA polymerase (AmpliTaq; Perkin-Elmer Cetus, Norwalk, CT) and the recommended buffer in five 50- $\mu$ l reactions. The primers were an oligonucleotide containing an XbaI site followed by a sequence complementary to bases 289–265 of C $\epsilon$  (5'ATTAGCTCTAGAACGGAGGTGGCATTGGAGGGAATGT 3') and, for the poly(dG) tail, a 9:1 mix of a primer containing the restriction sites for PstI, XbaI, and SphI, followed by a short poly(C) tail (5' GGCGCCCTGCAGCGGTCTAGAGCATGCC 3') and the same primer with a longer C tail (5' GGC-GCCCTGCAGCGGTCTAGAGCATGCCCCCCCCCCCCCCCC 3'). The first cycle was carried out with an annealing temperature of 42°C, in the absence of the C $\epsilon$  primer. This second-strand synthesis was followed by 40 cycles of PCR with an annealing temperature of 55°C. The A-PCR product was phenol extracted, ethanol precipitated, and subjected to PAGE (40). Amplified cDNA was analyzed by Southern blotting: DNA was electrotransferred from a fragment of the polyacrylamide gel to nylon membrane and fixed by UV irradiation (38). Hybridization with 5' <sup>32</sup>P-labeled oligonucleotide complementary to bases 216–240 of C $\epsilon$  was carried out at 55°C in 2 $\times$  SSC, 1% SDS, 10 $\times$  Denhardt's solution, 100  $\mu$ g/ml sonicated herring sperm DNA, 10  $\mu$ g/ml poly(A), and 10  $\mu$ g/ml poly(C), and was followed by three washes in 2 $\times$  SSC, 1% SDS at the same temperature. Amplified cDNA localized by Southern blot analysis was eluted, cut with XbaI and SphI, and ligated into pBS<sup>-</sup>. DNA was isolated from individual colonies (40), cut with XbaI and SphI, and analyzed by Southern blotting as described for the amplified cDNA, except that DNA was subjected to agarose gel electrophoresis. The inserts analyzed were all positive for C $\epsilon$  sequences as judged by hybridization with oligonucleotide complementary to bases 216–240 of C $\epsilon$ . Inserts of 15 clones were sequenced (both strands) by the dideoxy method (49) using modified T7 polymerase (Sequenase; United States Biochemical Corp., Cleveland, OH).

**Mapping of Germline  $\epsilon$  Exon on Genomic DNA.** DNA of the  $\lambda$  derivative containing unrearranged or rearranged DNA (U266)

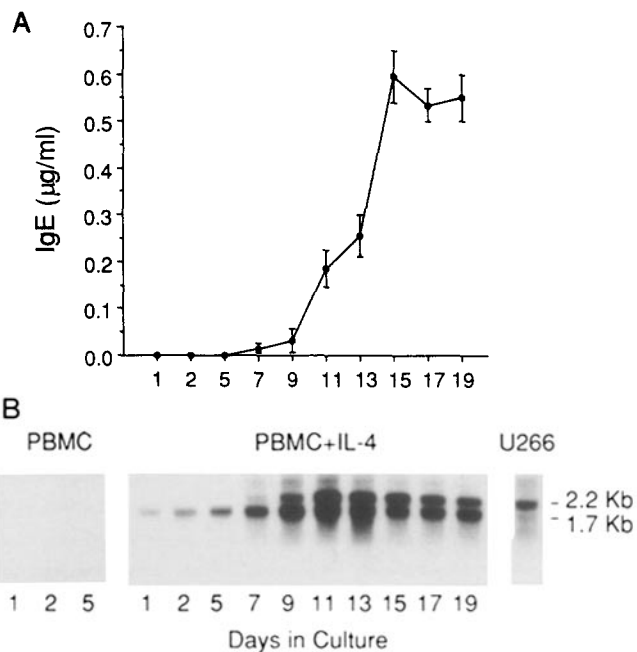
in front of C $\epsilon$  (Ch4AHlgE12 and Ch4AHlgE11, respectively [41]) were cut with BamHI, HindIII, or both restriction enzymes, subjected to electrophoresis in 0.7 and 1.5% agarose, denatured, and electrotransferred to Gene Screen membranes. Hybridization was performed with a cRNA probe obtained by *in vitro* transcription of cDNA I-3. Hybridization was performed as described for Northern blotting, but at 42°C, and was followed by three washes in 2 $\times$  SSC, 1% SDS at 65°C. Mapping was done according to published restriction maps (41, 50). The BamHI-HindIII Ch4AH-IgE12 fragment and its two SmaI subfragments were recloned in pBS, and the SmaI-HindIII subfragment was sequenced.

**S1 Nuclease Protection Assay.** The single-stranded DNA probes used for the S1 nuclease protection assay were derived from two constructs: the SmaI-HindIII Ch4AHlgE12 fragment and a chimeric construct obtained by ligating the SmaI-BglII subfragment of this genomic DNA with the BglII-XbaI fragment of cDNA I-4 in pBS<sup>-</sup>. Single-stranded DNA templates obtained from the pBS<sup>-</sup>-recombinant phagemids were used to synthesize uniformly <sup>32</sup>P-labeled probes (40). Probes were purified on 5% polyacrylamide-urea gels (40). S1 nuclease protection assays were carried out according to standard procedures (40). Hybridization was done in 80% formamide, 40 mM Pipes, pH 6.4, 1 mM EDTA, 400 mM NaCl at 55°C with 15  $\mu$ g of poly(A)<sup>+</sup> RNA, and 15  $\mu$ g of *E. coli* tRNA (51). Hybrids were incubated for 1 h at 37°C with 200 U S1 nuclease (Boehringer Mannheim Biochemicals, Indianapolis, IN) and subjected to electrophoresis on a 6% polyacrylamide-urea gel.

**Detection of Germline  $\epsilon$  Transcript and IL-4 mRNA by PCR.** To detect germline  $\epsilon$  transcripts by PCR, cDNA was synthesized using 1  $\mu$ g of total RNA as template, random sequence hexanucleotides as primers, and AMV reverse transcriptase (47). Amplification of the cDNA was performed using 30 cycles of PCR and a primer derived from C $\epsilon$  (bp 289–265; 5' ACGGAGGTGGCATTGGAGG-GAATGT 3'), and a primer located in the germline exon (5' AGGCTCCACTGCCCGGCACAGAAAT 3'). Amplified cDNA was analyzed by Southern blotting, as described for the cDNA clones using an oligonucleotide derived from sequence within the germline exon (5' AGCTGTCCAGGAACCCGACAGGGAG 3'). Hybridization and washes were performed at 42°C. For amplification of IL-4 cDNA primers, 5' ACTCTGTGCACCGAGTTGACCGTAA 3' and 5' TCTCATGATCGTCTTTAGCCTTTCC3' were used. Southern analysis was performed using an NheI-EcoRI cDNA fragment (52) labeled by random priming (53).

## Results

**IgE Synthesis and IgE mRNA Expression by B Cells Cultured with IL-4.** The addition of IL-4 (300 U/ml) to cultures of PBMC results in a relatively high rate of IgE synthesis (Fig. 1 A) (13, 14). Significant levels of IgE were detected at day 7 of culture, whereas maximal IgE levels were observed after  $\sim$ 13 d of culture. To determine IgE mRNA expression in these cultures, Northern blot analysis was carried out using a probe complementary to C $\epsilon$ . Two major cytoplasmic  $\epsilon$ -mRNA species could be detected (Fig. 1 B). The larger 2.2-kb mRNA was first detected after 7 d of culture, and maximal expression of this  $\epsilon$ -mRNA was observed on day 11 of culture. The observation that this 2.2-kb  $\epsilon$ -mRNA had exactly the same size as the  $\epsilon$ -mRNA from the IgE-secreting myeloma cell line U266 (Fig. 1 B), and that expression of the 2.2-kb  $\epsilon$ -mRNA occurred concomitantly with the appearance of IgE in the culture supernatants, supported the



**Figure 1.** Kinetics of IgE production (A) and  $\epsilon$  RNA (B) expression in PBMC stimulated with IL-4. For Northern blot analysis (B), RNA was isolated from PBMC incubated with or without IL-4 (300 U/ml) for the times indicated, or from cells of the IgE-producing myeloma U266. RNA was hybridized with a probe complementary to C $\epsilon$ . Autoradiography exposure times: PBMC  $\pm$  IL-4, 24 h; U266, 30 min.

notion that it represents a productive  $\epsilon$  transcript. A smaller 1.7-kb  $\epsilon$ -mRNA species was detected after 24 h of incubation with IL-4 (Fig. 1 B). Maximal expression of this transcript was also observed on day 11 of the culture. Since the truncated 1.7-kb  $\epsilon$ -mRNA was expressed well before (at least 6 d) IgE synthesis could be measured, we speculated that it was a germline  $\epsilon$  transcript analogous to those that precede the appearance of productive  $\epsilon$ -mRNA in the murine system (18, 21, 22, 25, 27).

**Cloning of a cDNA Complementary to Germline  $\epsilon$ -mRNA.** To characterize the truncated 1.7-kb  $\epsilon$ -mRNA species, we cloned the cDNA complementary to the putative germline  $\epsilon$  exon using the previously described technique for amplification of cDNA with an unknown 5' end A-PCR (45). cDNA was synthesized by reverse transcription of poly(A)<sup>+</sup> RNA tailed with dGTP, and amplified by A-PCR using oligonucleotides complementary to C $\epsilon$  and to the dGTP tail as primers. Amplified cDNAs containing sequence from the first exon of C $\epsilon$  were digested with restriction enzymes that cut in the oligonucleotide primers and ligated to the vector. 15 clones were sequenced and found to contain the expected C $\epsilon$  nucleotide sequences followed by stretches of previously uncharacterized cDNA. These cDNA clones were composed of novel 5' sequences of 58 bp (for the shortest cDNA insert, clone I-3) to 134 bp (for the longest cDNA insert, clone I-4), directly spliced to the C $\epsilon$  gene region (Fig. 2). They did not contain an initiation codon in frame with the C $\epsilon$  region, and stop codons were observed in all three reading frames. Varia-

1 CTACCCAGGCTCCACTGCCCGGCACAGAAATAACAACCACGGTTACTGATCATCTGGGAGCTGTCCAGGA 70  
 BglI HaeII Cε  
 71 ACCCGAAGAGGAGCCGGACGGCCACACCATCCACAGGCACCAATGGAGACCCGGCGCTTCAAG 135  
 137 CCCTCCACACAGAGCCCATCCGCTCTCCGCTTGACCCGCTGCTGCAAAAAGATTCCTCCAAATGCC 201  
 A-PCR Primer  
 202 ACCTCCGT

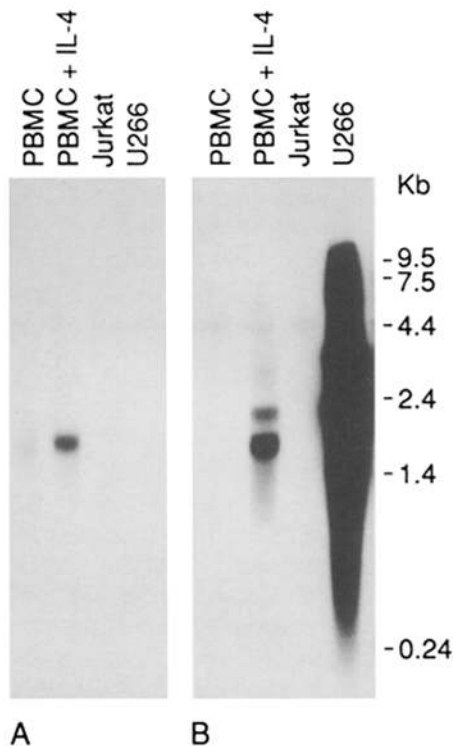
**Figure 2.** Nucleotide sequence of the cDNA clone I-4. The 5' ends of 14 other clones that were sequenced are indicated by dots. The Cε region is boxed. The Cε region contained in the primer used for A-PCR is underlined.

tions were observed in the poly(dC) tail length, indicating that the A-PCR products were derived from different cDNAs. To find the longest cDNA clone, inserts of 72 other cDNA clones hybridizing with the Cε probe were subjected to gel electrophoresis. None of them were longer than the insert of cDNA clone I-4, as judged by their migration (not shown). Although the cDNAs analyzed varied in size, their sequences were all homologous to cDNA I-4, with the exception that the sequences of some cDNA clones differed by one base (Fig. 2), determining the presence or absence of an HaeII restriction site. This single base difference may represent polymorphism because RNA was isolated from PBMC of four different donors. On the other hand, it cannot be ruled out that an

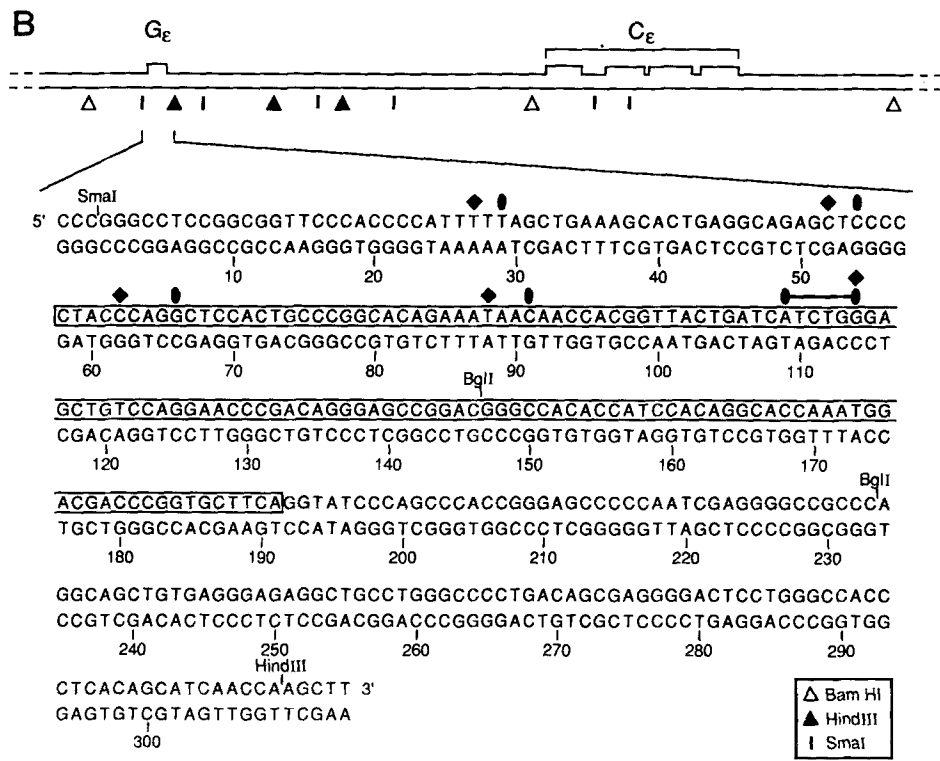
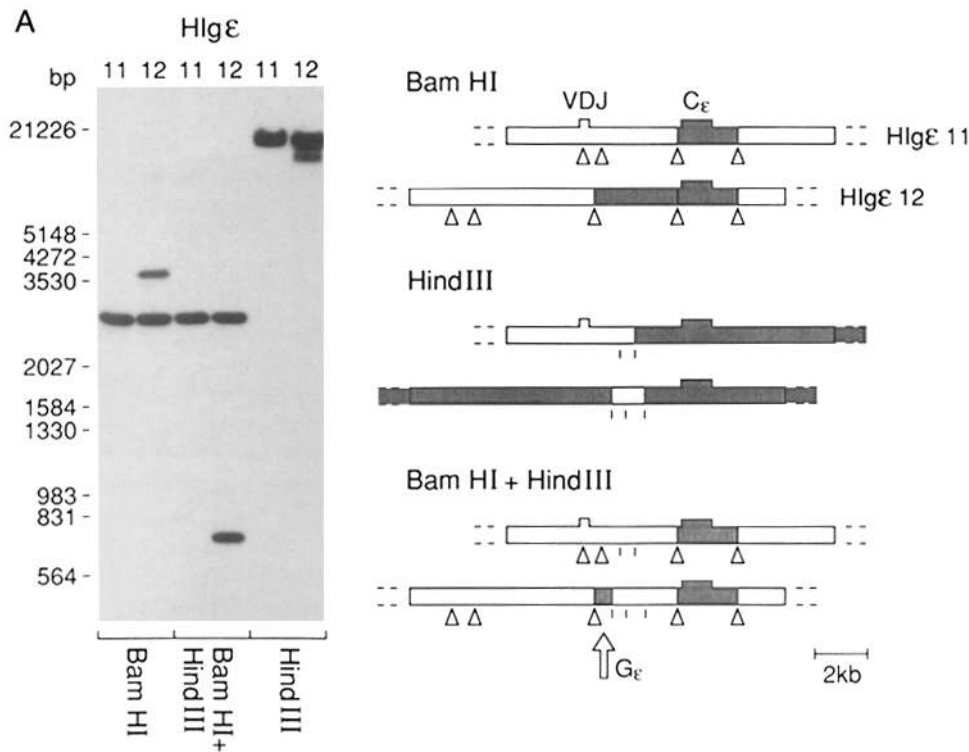
artifact was introduced by the TaqI polymerase during the cDNA amplification procedure. It can, however, be excluded that this variation reflects a Cε2 gene product, since the primer used for PCR was located in a region deleted in this pseudo gene (50, 54).

**A RNA Probe Derived from the Germline ε Transcript cDNA Hybridizes with the 1.7-kb ε mRNA Species.** To confirm that the cloned cDNA was specific for the germline ε exon, a probe containing no Cε sequences was made by taking advantage of the SphI restriction site in the anchor primer and the HaeII restriction site present in the cDNA 5' to Cε to reclone a fragment of clone I-4. An RNA probe prepared from this cDNA subfragment hybridized to the 1.7-kb RNA in Fig. 3, but not to the 2.2-kb band. However, both bands hybridized to a Cε probe, demonstrating that the 1.7-kb ε-mRNA is a germline transcript, whereas the 2.2-kb band consists of productive ε transcripts. No germline ε-mRNA transcripts could be detected in the IgE-producing myeloma cell line U266 in the absence of IL-4 (Fig. 3) or after incubation with IL-4 (300 U/ml) for 1, 3, 5, or 7 d (data not shown). In U266, the Sε region is deleted in only one of the alleles (55). Therefore, the failure to detect germline ε transcripts in this myeloma cell line suggests that germline ε transcripts are not present in cells that have undergone switching to IgE (Fig. 3).

**Mapping of the Germline ε Exon.** To localize the germline ε exon, we attempted to map it on genomic ε DNA. Phages containing either the germline (HIgE12) or the rearranged ε (HIgE11) DNA 5' to Cε isolated from U266 (41) were redigested by BamHI, HindIII, or combinations of these restriction enzymes. Hybridization of the restriction fragments with a cRNA probe produced by transcription of one of the germline cDNA clones (clone I-3) demonstrated that the germline ε transcript was present in a BamHI-HindIII fragment 3.5 kb upstream from Cε (Fig. 4 A). This fragment is part of DNA deleted by switch recombination in the expressed allele of the U266 myeloma (41). Sequencing of a SmaI-HindIII subfragment (50) of the BamHI-HindIII fragment showed a region of full homology with the cDNA sequences, except for the cDNAs containing a HaeII restriction site, where a single mismatch is observed and enabled us to precisely localize the germline ε exon (Fig. 4 B). The sequence of the first 54 nucleotides of the SmaI-HindIII fragment has been published (50). Interestingly, the SmaI-HindIII fragment containing the germline exon, as well as the 113 bp upstream of this fragment, were found to be retained in Cε2 the ψε gene located 5' from α1 (50, 55).



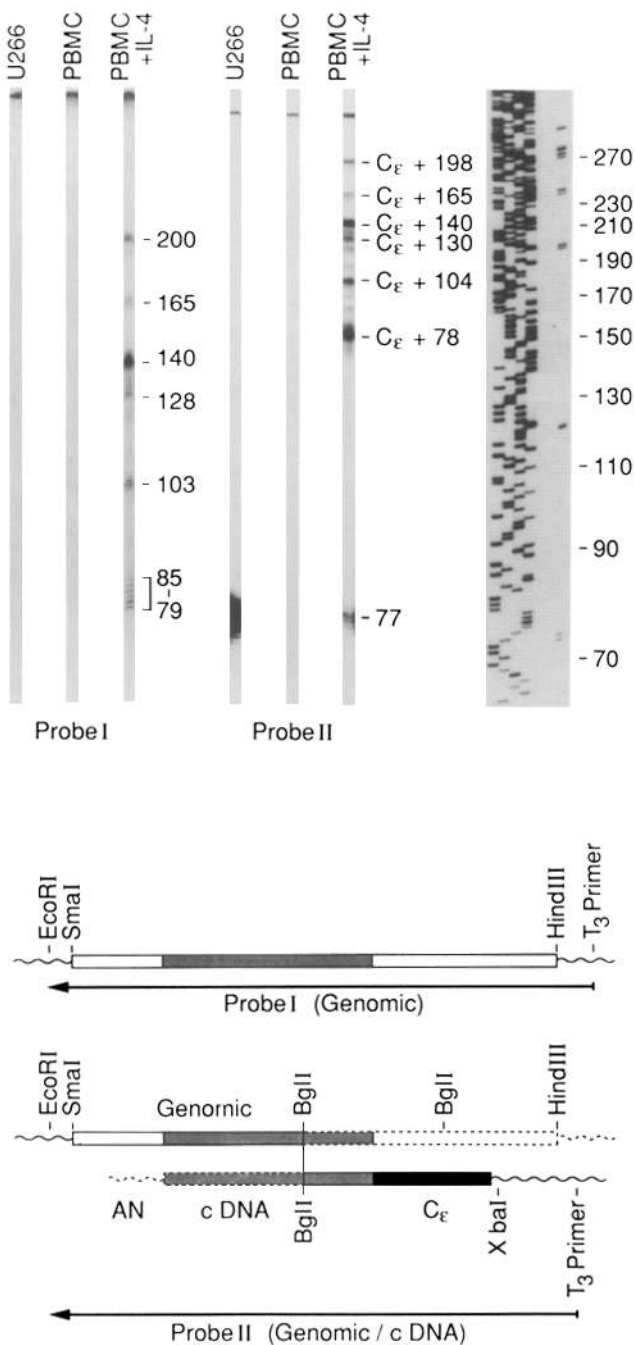
**Figure 3.** Expression of germline and productive ε transcripts as detected by Northern blot assays using probes complementary to germline ε exon (A) and Cε (B). RNA was isolated from PBMC and incubated for 9 days with or without IL-4, cells from the T leukemia cell line Jurkat, or the IgE-producing myeloma U266. Autoradiography exposure time was 24 h. Migration of the six bands of the RNA ladder is indicated.



**Figure 4.** Mapping of the germline  $\epsilon$  exon on genomic  $\epsilon$  DNA. (A) Localization by Southern blot assays. A probe complementary to cDNA was hybridized with DNA of  $\lambda$  derivative containing the region surrounding C $\epsilon$  in the germline (HlgE12) or rearranged configuration (HlgE11). HlgE12 and HlgE11 DNA was digested with the restriction enzymes indicated. Labeled fragments, which included vector sequences in case of the digestion with HindIII, are shaded on the restriction map (41). The arrow (G $\epsilon$ ) indicates the deduced position of germline  $\epsilon$  exon. (B) Schematic representation of the germline  $\epsilon$  exon and the C $\epsilon$  locus and nucleotide sequence of the SmaI-HindIII HlgE12 restriction fragment containing the germline  $\epsilon$  exon. The nucleotide sequence of cDNA clone I-4 is boxed. The main transcription initiation sites, as mapped with probes I and II, are indicated by black squares and ovals (see Fig. 5).

**Initiation Sites of the Germline  $\epsilon$  Transcripts.** The sizes of the 15 sequenced and the 72 nonsequenced inserts varied considerably, suggesting that transcription of germline  $\epsilon$  transcripts initiates at multiple sites. These initiation sites were further defined by using S1 nuclease protection analysis. For

this analysis, two probes were used. One probe was derived from the genomic SmaI-HindIII fragment (probe I), the other from a hybrid construct containing the genomic SmaI-BglII fragment linked to the BglII site of cDNA I-4 (probe II; Fig. 5). Fig. 5 shows that probe I, which only contained the germ-



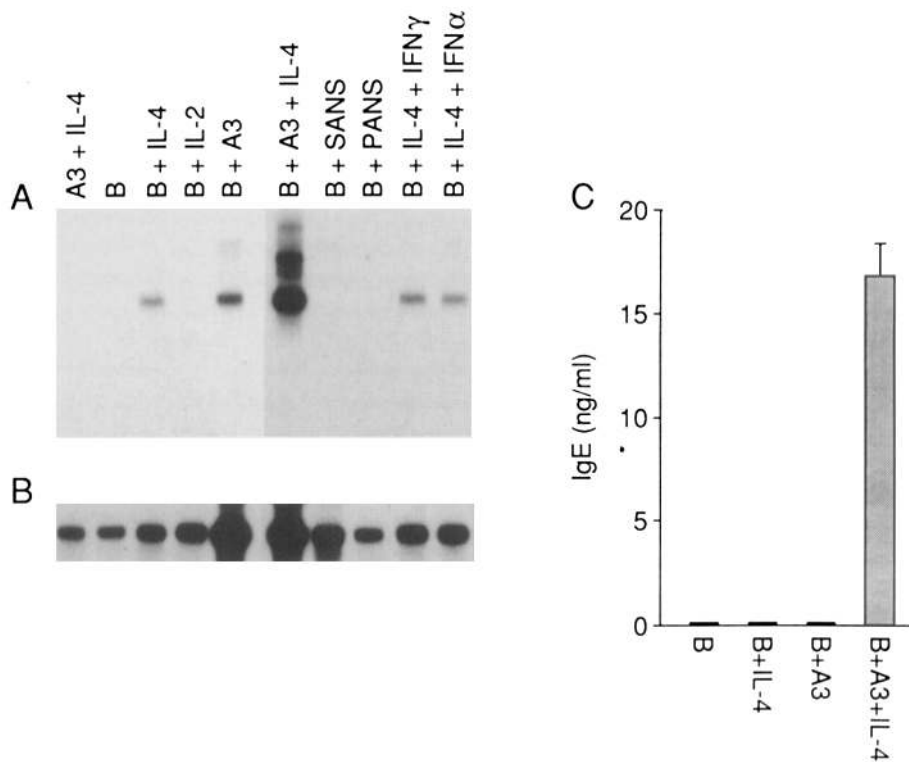
**Figure 5.** Mapping of germline  $\epsilon$  RNA transcription initiation sites, by S1 nuclease protection. Poly(A)<sup>+</sup> RNA isolated from U266 cells, PBMC, or PBMC incubated with IL-4 for 9 d was hybridized with the single-stranded probes I and II, which are shown at the bottom of the figure. The sizes of the fragments were determined by using a M13 sequencing ladder.

line  $\epsilon$  exon and no C $\epsilon$  cDNA sequences, was not protected by poly(A)<sup>+</sup> RNA from U266. However, U266 poly(A)<sup>+</sup> RNA was able to protect a fragment of the second probe with a length corresponding to that of the constant  $\epsilon$  region plus three bases common to the 3' end of the J6 segment, which is spliced to C $\epsilon$  in the U266 IgE-mRNA (46, 56).

In contrast, PBMC cultured with IL-4 produced transcripts that protected multiple-sized fragments of both probes, whereas poly(A)<sup>+</sup> RNA isolated from PBMC cultured in the absence of IL-4 was unable to protect any fragment of the probe (Fig. 5). The lengths of the protected fragments suggested that the germline  $\epsilon$  transcript initiates at various sites within a stretch of 90 bp (Figs. 4 B and 5). Judged by the intensity of the bands, there are four predominant initiation regions located  $\sim$ 80,  $\sim$ 100,  $\sim$ 130, and  $\sim$ 140 bp upstream from the C $\epsilon$  splice point (Figs. 4 B and 5). The detection of a protected fragment, with the size one would expect when only vector sequences were digested by S1 nuclease, indicated that germline  $\epsilon$  transcripts can even initiate 5' to the SmaI site. Similar to what has been described for the mouse  $\mu$ ,  $\gamma$ 2b, and  $\epsilon$  germline transcript promoters (19, 27, 28) the region upstream from the initiation sites lacks well-defined TATAA motifs (Fig. 4 B) (50).

*Transcription of Germline  $\epsilon$ -mRNA Does not Correlate Absolutely with IgE Secretion.* In murine models of isotype switching, the accumulation of a particular germline CH transcript precedes switching to corresponding CH chains, suggesting a correlation between germline CH transcript expression and subsequent production of the corresponding isotype. IL-4 is the only identified lymphokine inducing IgE synthesis, and polyclonal B cell activators consistently failed to do so. We found that IL-4 induces both germline  $\epsilon$  transcripts and IgE synthesis by PBMC, whereas IFN- $\gamma$ , PMA, and immobilized anti-IgM antibodies were ineffective. In contrast, IL-2 and *S. Aureus* Cowan (SAC) (Pansorbin) induced expression of germline  $\epsilon$ -mRNA, but no IgE synthesis. Collectively, these data (not shown) indicate that germline  $\epsilon$  transcripts can be induced by activators that fail to induce switch to and synthesis of IgE by PBMC.

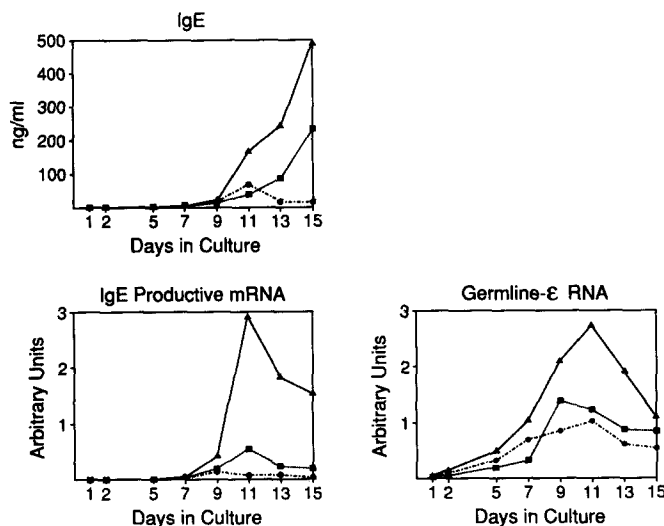
To exclude the possibility that germline  $\epsilon$ -mRNA expression in cultures of PBMC was induced indirectly via IL-4 produced by other cells (T cells, NK cells) present in the cultures, germline  $\epsilon$  transcript induction was also measured in highly purified B cells (>99.8% pure). IL-4 alone was sufficient to trigger germline  $\epsilon$  transcript synthesis, measured by Northern analysis or PCR (Fig. 6, and data not shown). Identical results were obtained using highly purified B cells (>98% pure) that were negatively selected (not shown). Interestingly, although IL-2 and SAC were capable of inducing germline  $\epsilon$  transcripts in cultures of PBMC, they failed to do so in cultures of highly purified B cells. In addition, even though IL-4 induced germline  $\epsilon$ -mRNA in cultures of purified B cells, these cells did not develop into IgE-secreting cells. Germline  $\epsilon$  transcripts were also induced when the purified B cells were cocultured with the CD4<sup>+</sup> T cell clone A3 (Fig. 6), which lacks the capacity to produce IL-4 upon activation (33). Also, no IL-4 mRNA, as measured by PCR followed by Southern blot assay, could be detected in the cultures containing both purified B cells and A3 cells. The germline  $\epsilon$ -inducing effect of A3 could not be attributed to the production of human lymphokines, since IL-1 $\alpha$ , IL-2, IL-3, IL-5, IL-6, murine IL-9, granulocyte-CSF, granulocyte/macrophage-CSF, IFN- $\gamma$ , IFN- $\alpha$ , TNF- $\alpha$ , and TGF- $\beta$  were all ineffective, as judged by Northern blot analysis (not shown).



**Figure 6.** Effect of various stimuli on germline  $\epsilon$  RNA levels in purified B cells incubated in the absence or presence of the T cell clone A3. RNA was isolated after 5 d of incubation, and aliquots corresponding to one-third of the culture were subjected to Northern blot analysis using probes complementary to  $\epsilon$  (A) or actin (B). Autoradiography exposure time: A, 72 h; B, 10 h. (B) IgE production by an aliquot of  $5 \times 10^3$  B cells in the presence or absence of  $5 \times 10^3$  A3 T cells is shown after 9 d of culture.

Although both IL-4 and A3 induced germline  $\epsilon$ -mRNA, each of these signals was insufficient to induce IgE synthesis (Fig. 6).

**Effects of IFN- $\gamma$  and IFN- $\alpha$  on IL-4-induced Germline  $\epsilon$ -mRNA Expression in PBMC and Purified B Cells.** We have previously demonstrated that both IFN- $\gamma$  and IFN- $\alpha$  strongly inhibit IL-4-induced IgE synthesis (16), and that these antagonists acted most efficiently in the first 48 h of the incubation period



**Figure 7.** Effects of IFN- $\gamma$  and IFN- $\alpha$  on IL-4-induced IgE synthesis, productive  $\epsilon$ -mRNA, and germline  $\epsilon$ -mRNA expression in PBMC. RNA levels were determined by densitometric scanning of Northern blot autoradiographs. ▲, PBMC + IL-4; ■, PBMC + IL-4 and IFN- $\gamma$ ; ●, PBMC + IL-4 and IFN- $\alpha$ .

(57). In Fig. 7, it is shown that IFN- $\gamma$  and IFN- $\alpha$  reduce the steady-state level of IL-4-induced germline  $\epsilon$ -mRNA by 57 and 64%, respectively. Furthermore, IFN- $\gamma$  and IFN- $\alpha$  have an even more potent effect on the steady-state level of productive  $\epsilon$ -mRNA, causing an 83 and 98% reduction, respectively. The strong reduction in productive  $\epsilon$ -mRNA expression reflected the strong reduction in IgE synthesis measured in these cultures (Fig. 7). In contrast, IFN- $\gamma$  and IFN- $\alpha$  had no effect on IL-4-induced germline  $\epsilon$ -transcript expression in highly purified B cells in the presence or absence of A3 T cells (Fig. 6, and data not shown), indicating that the suppressive effect on germline  $\epsilon$  transcript expression in PBMC is probably mediated indirectly via other cells and/or factors produced by these cells upon incubation with IFN- $\gamma$  and IFN- $\alpha$ .

## Discussion

In the present study, we demonstrate that B cells induced to switch to IgE-producing cells by IL-4 and T cells express two species of  $\epsilon$ -mRNA, with sizes of 1.7 and 2.2 kb, respectively. Kinetic studies indicate that the 2.2-kb  $\epsilon$ -mRNA appeared after 7 d of culture concomitantly with the first detectable levels of IgE in the cultures. From this observation, and the finding that the IgE-producing U266 myeloma cells also expressed a 2.2-kb  $\epsilon$ -mRNA (Fig. 1) (46, 54, 56), we concluded that the 2.2-kb  $\epsilon$ -mRNA induced by IL-4 represented productive  $\epsilon$ -mRNA encoding the secreted form of IgE. The 1.7-kb mRNA species could be detected as early as 2 h after incubation with IL-4 (not shown), which is  $\sim 6$  d before any significant production of IgE was observed.

To determine the nature of this 1.7-kb  $\epsilon$ -mRNA species, the sequences located in this 5' to C $\epsilon$  RNA were cloned using A-PCR (45). Analysis of the amplified cDNA resulted in the identification of a new exon spliced to C $\epsilon$  at the same site as VDJ in the productive  $\epsilon$  transcript. This human germline  $\epsilon$  exon was mapped in a 0.3-kb SmaI-HindIII fragment 3.5 kb 5' from C $\epsilon$ . Interestingly, the germline  $\epsilon$  exon, as well as the region immediately 5' to this exon, are retained in the  $\psi\epsilon$  gene located 5' to  $\alpha 1$  (50, 55). Although the  $\psi\epsilon$  gene also contains a switch region (50), it remains to be determined whether the  $\psi\epsilon$  germline exon is transcribed and if switching to  $\psi\epsilon$  truncated C region occurs.

The structure of the human germline  $\epsilon$ -mRNA is similar to the structures of the murine  $\mu$ ,  $\gamma 2b$ ,  $\epsilon$ , and  $\alpha$  transcripts (19, 25–28). The described murine germline transcripts consist of a 5' exon originating from sequences 5' to the corresponding S region, which spliced to the appropriate downstream CH region. The human germline  $\epsilon$  transcript did not contain initiation sites in frame with C $\epsilon$ , suggesting that it probably is not capable of encoding large proteins, similar to what has been proposed for the murine  $\mu$ ,  $\gamma 2b$ ,  $\epsilon$ , and  $\alpha$  transcripts. S1 nuclease protection analysis of the germline  $\epsilon$  5' end suggested that transcription initiates from multiple sites within a region of 90 bases. More accurate mapping of the transcription initiation sites by primer extension is currently in progress. As with promoter region sequences of various other genes that have heterogeneous transcription initiation sites, this region lacks canonical TATAA sequences. Multiple transcription initiation sites has also been demonstrated for murine  $\mu$ ,  $\gamma 2b$ , and  $\epsilon$  germline transcripts (20, 27, 28). Collectively, these data indicate that because of their similarities in structure, human germline  $\epsilon$  and murine germline  $\gamma 2b$ ,  $\alpha$ , and  $\epsilon$  transcripts may have similar functions, despite the fact that no significant sequence homologies were found. Human germline  $\epsilon$  expression preceded switching to  $\epsilon$  in cultures of PBMC, or combinations of cloned CD4<sup>+</sup> T cells and purified B cells in the presence of IL-4. These data seem to be compatible with results obtained in murine models, which indicated that isotype production by B cells in response to LPS and various lymphokines was always preceded by induction of the corresponding germline transcripts (12, 18–27).

It has been proposed that synthesis of germline transcripts act by opening the chromatin structure of specific switch regions, making them accessible to a putative common switch recombination system (18, 20, 22, 30). In addition to this "accessibility" model, other models in which the germline transcripts themselves play an active role in the process of directed class switching cannot be ruled out to explain correlations between germline CH chain transcription and class switching (31). It is conceivable that germline transcripts in B cells induced by exogenous agents could function as substrates for *trans* RNA splicing, before switching at the DNA level, which has been proposed to lead to an intermediate stage of class switching (58, 59).

Induction of germline  $\epsilon$  transcripts in PBMC is not a specific property of IL-4. Both IL-2 and the polyclonal activator SAC induced germline  $\epsilon$  transcripts, although IFN- $\gamma$ , TPA, and

insolubilized anti-IgM antibodies did not. Despite the fact that IL-2 and SAC induced germline  $\epsilon$  transcription, they failed to induce productive  $\epsilon$  transcripts and IgE synthesis. These results indicate that germline  $\epsilon$  transcription, as such, is insufficient for switching to  $\epsilon$ , and that additional regulatory mechanisms are involved. Interestingly, both IL-4 and cloned CD4<sup>+</sup> T cells with nonrelevant specificities, derived from allogeneic donors, were able to induce germline  $\epsilon$  transcription but not IgE synthesis by purified B cells. These results are compatible with previous data that indicated that purified B cells in the absence of autologous T cells failed to produce IgE in response to IL-4 (13, 14, 16). However, addition of both IL-4 and cloned allogeneic CD4<sup>+</sup> T cells to purified B cells resulted in synergistic effects on germline  $\epsilon$  transcription in the purified B cells and subsequent high rates of IgE synthesis, indicating that in addition to IL-4, a second, yet to be defined, signal or set of signals provided by cloned CD4<sup>+</sup> T cells is required for induction of productive  $\epsilon$  transcripts and switching to  $\epsilon$ . That IL-4 and the signal provided by CD4<sup>+</sup> T cells indeed direct switching to  $\epsilon$  was confirmed by limiting dilution studies that indicated that the frequencies of precursor B cells that under these conditions could be induced to switch to  $\epsilon$  varied between 1:10 and 1:20 (Gascan et al., manuscript submitted for publication). Our data are consistent with studies conducted in murine systems (17). Although IL-4 can induce purified murine B cells stimulated with LPS to secrete IgG1 and IgE, in the absence of LPS, IL-4 induces germline  $\gamma 1$  transcription but not IgG1 secretion (23). In contrast, murine germline  $\epsilon$  transcripts cannot be induced in purified B cells by IL-4 alone, but require both LPS and IL-4 for expression (21, 22). If the accessibility model is correct, it would predict that modulation of germline  $\epsilon$  expression would reflect subsequent modulation of  $\epsilon$  switching, or alternatively, that agents that block  $\epsilon$  switching may act through inhibition of germline  $\epsilon$  expression. Recently, we demonstrated that both IFN- $\gamma$  and IFN- $\alpha$  strongly blocked IgE synthesis induced by IL-4 in vitro (16).

In agreement with the accessibility model, it was found that IFN- $\gamma$  and IFN- $\alpha$  inhibition of IgE synthesis by PBMC was associated with a decrease in transcription of  $\epsilon$ -mRNA. Considerable and sometimes complete inhibition of productive  $\epsilon$ -mRNA synthesis was observed. In general, IFN- $\alpha$  was found to be more effective than IFN- $\gamma$ . Inhibition of the productive  $\epsilon$ -mRNA was preceded by inhibition of the germline transcript, which, however, was always partial and never exceeded 70%. Similarly, partial inhibition of IL-4-induced germline  $\gamma 1$  transcripts by IFN- $\gamma$  was observed in murine models (23). Interestingly, IFN- $\gamma$  and IFN- $\alpha$  failed to reduce germline  $\epsilon$  transcription induced by IL-4 in purified B cells, suggesting that these factors do not directly act on the purified B cells, but mediate their suppressive effects via other cells present in the PBMC cultures.

Our results indicate that in the human system, in contrast to murine models, germline  $\epsilon$  expression can be induced by various signals, but that this does not necessarily result in switching to  $\epsilon$ . Only germline  $\epsilon$  expression induced by both IL-4 and CD4<sup>+</sup> T cells resulted in subsequent production of IgE, indicating that a special set of signals provided by IL-4



and cloned CD4<sup>+</sup> T cells are required for directing B cells to switch to IgE-producing cells. Nonetheless, induction of IgE synthesis was always preceded by the induction of germline  $\epsilon$  transcripts. However, the long time lag between germline  $\epsilon$  transcription and  $\epsilon$  switching suggests that different regulatory mechanisms are involved in which T cells and/or their

products play an important role. Further characterization of the relative contributions of IL-4 and the CD4<sup>+</sup> T cells to the mechanisms controlling germline and productive  $\epsilon$ -mRNA transcription will help us to define how class-switch recombination events are directed by exogenous signals.

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