

Molecular Analysis of IgE H-Chain Transcripts Expressed in Vivo by Peripheral Blood Lymphocytes from Normal and Atopic Individuals

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ABSTRACT. The low levels of IgE produced by PBMC from normal individuals has so far prevented an analysis of their IgE H chain repertoire. Using a nested polymerase chain reaction approach, we were able to detect ϵ transcripts in all normal and allergic individuals we investigated. We further cloned ϵ CDR3/FW4 regions from two normal and two atopic individuals with low serum IgE levels. Sequence analysis of 104 clones identified 26 different ϵ CDR3/FW4 regions and an additional number of clonally related transcripts in the two atopic individuals. Preferential usage of DH genes from the DXP family (33%) and of the JH4b gene (35%) were observed, similar to reported findings for the IgM-producing peripheral blood B cell subset. Using CDR3 specific oligonucleotides, we detected the CDR3/FW4 regions of a particular set of clonally related ϵ transcripts in μ and $\gamma 4$ transcripts of the same individual. This finding demonstrates the in vivo production of IgE together with the two other Ig isotypes (IgM and IgG4) by the progeny of a common B cell precursor, and suggests a possible mechanism for regulating the allergic response. The clonally related ϵ transcripts were found to be only of the secreted form. We give also evidence that the IgE-producing B cells undergo somatic mutation because a number of identical mutations were observed in the FW4 regions of ϵ and μ clonally related transcripts. Some of these mutations were shared with other transcripts from the same and other individuals, supporting the existence of sequence-specific hot spots for the somatic hypermutation machinery in the JH gene segments. *Journal of Immunology*, 1993, 151: 2195.

The V regions of the IgH chains (IgH) are encoded by three separate genes: variable, diversity, and joining segments. Upon their assembly three CDR² are formed that code for the amino acid residues that directly contact the Ag. The most variable of them is the CDR3 which is encoded by a D segment and the VH-D and D-JH junctional areas, whereas CDR1 and CDR2 belong to a VH segment. During these joining steps enormous diversity is generated through somatic recombination of multiple VH, DH, and JH segments, imprecise joining with loss

of germ-line sequences, and the addition of N region nucleotides (1–3). Further diversity is introduced by somatic hypermutation, a process that occurs in the germinal centers of the follicles where activated Ag-specific cells proliferate, acquire mutations at random in their V regions, and become selected on the basis of their capacity for activation by the same Ag presented by follicular dendritic cells (4). This process allows the formation of B cells expressing high affinity antibodies that are able to further differentiate into plasma cells or memory B cells.

Diversity of Ig effector functions is accomplished through the Ig class switching mechanism where the C region (C μ) of the Ig is replaced through a deletional recombination event with another CH region (5). This process is T cell dependent and occurs in cells committed to differentiation into plasma cells. In the case of IgE, two signals are required for the switch to occur (6). The first one is provided by the cytokine IL-4, which initiates germ line transcription through the ϵ locus (7, 8). The deletional recombination switch takes place after a second signal is delivered from Th2 cells, EBV, or the anti-CD40 mAb (9–12).

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² Abbreviations used in this paper: CDR, complementarity determining region; FW, framework; PCR, polymerase chain reaction; R/S, replacement/silent ratio; RAST, radioallergoabsorbent test; SSPE, Saline phosphate EDTA solution; nt, nucleotide.

It has been shown that PBMC derived from atopic donors produce spontaneous IgE (13), whereas B cells from normal nonatopic donors produce IgE only if cultured in the presence of exogenously added IL-4 (14, 15). However, IgE-producing B cell clones from nonatopic individuals have also been obtained in the absence of IL-4 after 5 wk culture of EBV-transformed surface IgM/IgD-positive cells (16, 17). These cells co-expressed IgM and IgE, and contained nonrearranged C ϵ genes. Double isotype-producing B cells that express other Ig classes have also been identified (18–20). It has been suggested that these cells represent a stage of B cell differentiation before switch recombination (for review see References 21 and 22). In contrast to IgG- and IgA-producing clones, multiple isotype expression might be a frequent and stable characteristic of the IgE-producing peripheral blood B cell subset in nonatopic individuals (16).

Using a sensitive assay for the detection of ϵ transcripts expressed by fresh PBMC, we were able to analyze the ϵ CDR3/FW4 regions in normal and atopic individuals. The data obtained allowed us to investigate the membrane or secreted character of the expressed ϵ transcripts, as well as the presence of the same VDJ rearrangements in other Ig isotypes (IgM and IgG4).

Materials and Methods

Clinical samples and RNA preparation

PBMC were obtained from three atopic and seven normal white Caucasian adults and from a pool of white blood cells from five unclassified individuals by fractionation on a Ficoll/Hypaque gradient. The two atopic individuals that were analyzed by sequencing of their ϵ transcripts had a clinical history of mild allergic disease. Patient A1 suffered from occasional asthmatic attacks, had normal serum IgE levels (93 U/ml), and was positive to 11 out of 23 environmental allergens (grade 3 for *Lolium perenne*, *Phlaeum pratense*, and *Poa pratensis*) when tested using the Radio Allergoabsorbent Test (RAST). Patient A3 suffered from allergic rhinitis and atopic dermatitis, had slightly elevated serum IgE levels (166 U/ml), and her RAST showed serum IgE positivity to 14 environmental allergens with grade 4 response to *Lolium perenne*, *Phlaeum pratense*, and *Poa pratensis*, and grade 3 to cat epithelium. The normal individuals had serum IgE levels below 100 U/ml. Total cellular RNA was isolated according to the acid guanidinium thiocyanate procedure of Chomczynski and Sacchi (23).

Oligonucleotide primers and probes

Oligonucleotides were synthesized by the solid phase triester methodology on a DNA synthesizer (380A; Applied Biosystems, Foster City, CA). Unless otherwise indicated, the oligonucleotides were synthesized according to sequence data from Reference 24. Position and sequence of the oligonucleotides used for PCR amplification of the

CDR3/FW4 and adjacent regions is as follows: hV1 5' GTGCAGCTGC(G)A(T)GG(C)AGTCG(T)GG 3' (codons 2 to 8) and hV2 5' CTGAGGACACGGCCGTGTATTACTG 3' (codons 84 to 92) are from the V regions of the H chain, genes, oligonucleotides E1 5' GAAGACGGATGGGC-TCTGTG 3' (codons 116 to 122), E2 5' TAAGGTCAT-AGTTGTCCCGT 3' (codons 167 to 173), and E3 5' GAG-GTGGCATTGGAGGGAATGT 3' (codons 130 to 137) belong to the first exon of the constant ϵ gene, and primer G1 5' GGAAGACCGATGGGCCCTTG 3' is from a consensus sequence in the first exon of the constant γ genes. The CDR3-specific oligonucleotides synthesized according to sequence data obtained in this paper are as follows: TB1 5' GGTCATTACGATTTTGG 3', TB2 5' CCGTCCTGGTTCGACC 3', and TB3 5' TTTGGAGTGCATATTATAAG 3'. Secreted form ϵ transcripts were detected with primers S1 5' CCACTGCACAGCTGGATGGAG 3' and S2 5' GGAGGCAGGAGTACGTCATT 3', whereas the membrane forms were amplified with oligonucleotides M2a 5' GGCTGGAGGACGTTGGTGTA 3' and M2b 5' TGC-CGCGTGGCTGAGAGGAA 3' (25). Constant H chain-specific oligonucleotides used for analysis of μ and γ 4 transcripts were: μ 1 5' GTCCTGTGCGAGGCAGCCAA 3' (codons 139 to 145) and μ 2 5' ATCCGACGGGGAAT-TCTCAT 3' (codons 127 to 133) from the first exon of the constant μ gene, γ 4.1 5' TGATGGGCATGGGGGACCAT 3' (codons 229 to 241) from the hinge region of the constant γ 4 gene and γ 4.2 5' GTTGCAAGGTGTAGGTCTTC 3' (codons 200 to 209) from the first exon of the γ 4 gene. The sequence of the 5' γ 4 primer located from nucleotide -35 to -15 upstream of the γ 4 CH1 exon was: 5' CACAC-CGCGGTCACATGGCA 3'.

Reverse transcription and PCR analysis of ϵ CDR3/FW4 regions

Reverse transcriptions were performed on total cellular RNA from PBMC (2 to 6 μ g) or from the U266 cell line (1 μ g and 10-fold dilutions) with the GeneAmp RNA PCR kit (Perkin Elmer Cetus, Norwalk, CT), following the procedure recommended by the manufacturer. All PCR reactions were performed on the total cDNA sample in 100- μ l reaction mixtures containing each dNTP at 200 μ M, 300 ng of each PCR primer, 2.5 U of AmpliTaq DNA polymerase (Perkin Elmer Cetus) and PCR reaction buffer provided by the same manufacturer. In some nested PCR experiments the second PCR reaction was performed with oligonucleotide primers 5' end labeled to high specific activity (26) with γ -[^{32} P]ATP (Amersham, Amersham, UK) and polynucleotide kinase (New England Biolabs, Beverly, MA). Amplifications of the ϵ CDR3/FW4 regions were performed after reverse transcription with the E2 oligonucleotide followed by 30 cycles of PCR with the V1 and E2 primers under the following conditions: 95°C for 1 min, 58°C for 1 min, and 72°C for 1 min and 30 s. In parallel

to these reactions γ CDR3/FW4 regions were amplified under the same conditions except that the E2 oligonucleotide was substituted with the G1 primer. Two microliters of the first PCR reactions were reamplified in 50- μ l PCR reactions with internal primers (^{32} P-labeled V2 and E1 or E3 for amplification of the ϵ transcripts, and ^{32} P-labeled V2 with G1 for γ transcript amplification) using the following thermal cycler (Perkin Elmer Cetus) conditions: 95°C 1 min, 64°C 1 min, and 72°C 1 min for 30 cycles. After one chloroform extraction to eliminate the mineral oil, 2 μ l of the second PCR reaction were analyzed on a denaturing 6M urea 6% polyacrylamide DNA-sequencing gel. The gels were fixed, dried, and exposed for 1 to 12 h. Negative control reactions containing no cDNA were always included. Sequencing reactions were used as size markers.

Cloning and sequencing of ϵ transcripts

A 30-min autoradiography of the PCR fragments obtained from the second PCR reaction with the ^{32} P-labeled V2 and E2 or E3 primers was performed on a nonfixed, nondried sequencing gel to identify the location of the specific bands. Slices containing the specific bands were excised from the polyacrylamide gel, and the bands were eluted by the "crush and soak procedure" (26). The amplified DNA fragments were precipitated, reamplified for 15 cycles with the same primers, and purified by electroelution from 1.2% agarose gels (26). The recovered DNA fragments were ligated in the *Sma*I site of pUC18 (Pharmacia LKB, Uppsala, Sweden) and used to transform *Escherichia coli* strain DH5a. Clones were picked randomly, and dsDNA template was prepared and sequenced by the dideoxy chain termination method (27) using the T7 Sequencing Kit (Pharmacia LKB). Sequences were identified by comparison with reported germ-line D and JH gene segment sequences (24, 28–30).

Analysis of membrane and secreted ϵ transcripts

Two 1- μ g RNA samples from individual A3 were reverse transcribed with oligo dT and amplified with the CDR3-specific oligonucleotide TB1 and the S1 or M2a primers for the secreted and membrane forms, respectively. A 1- μ l aliquot of each first PCR reaction was then reamplified with the TB3/S2 or TB3/M2b primer combination, respectively. The obtained fragment was electroeluted from a 1.2% agarose gel, cloned and sequenced as described above. Radioactive PCR were performed on 1- μ l aliquots of the first PCR reactions with [^{32} P]5' end-labeled TB3 and E2 or E3. In reactions containing total cellular RNA from the IgE-secreting myeloma cell line U266 (American Type Culture Collection, Bethesda, MD), the TB1 and TB3 primers were substituted with primers V1 and V2, respectively. The PCR products were analyzed on denaturing sequencing gels as described above.

Analysis of μ and γ 4 transcripts

Total cellular RNA (1 μ g) from individual A3 was reverse transcribed with the oligonucleotide μ 1 or γ 4.1. First PCR was performed on the total cDNA sample with the TB1/ μ 1 or the TB1/ γ 4.1 primer pair, followed by a second nested PCR reaction with the ^{32} P-labeled oligonucleotide TB3 and the corresponding μ 2 or γ 4.2 primer for amplification of μ or γ 4 transcripts, respectively. The resulting PCR fragments were analyzed on denaturing sequencing gels as already described. The PCR fragments from the second PCR reactions were purified and cloned. Transformants were identified by filter hybridization with the ^{32} P-labeled TB2 oligonucleotide. Hybridizations were performed overnight at 42°C in 5X SSPE/5X Denhardt's/0.5% SDS. The nylon filters were then washed once at room temperature for 20 min in 2X SSPE/0.1% SDS, and once at 54°C for 10 minutes in 5X SSPE and 0.5% SDS. Positive clones were picked up and sequenced.

Cloning and sequencing of the polymorphic C γ 4b gene

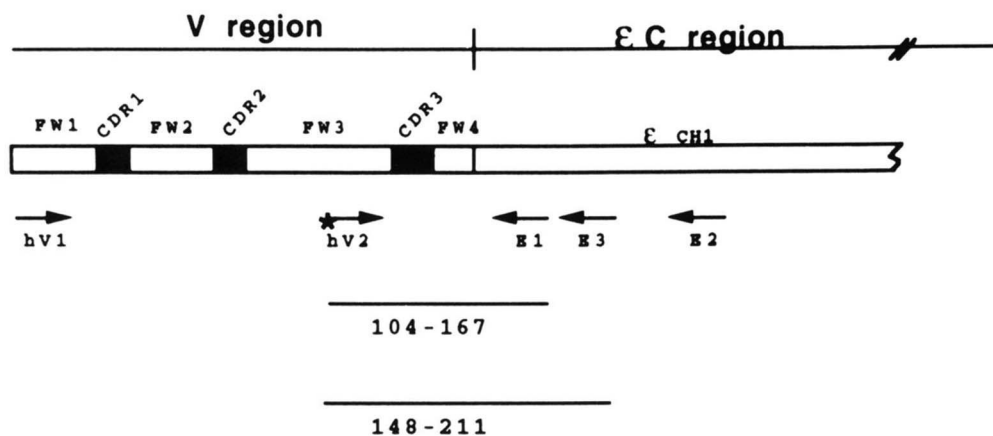
DNA was isolated from white blood cells of individual A3 using standard methodology (26). PCR amplification was performed with the 5' γ 4 primer (located 35 nt upstream of the γ 4 CH1 exon) and the γ 4.1 primer (from the hinge region) on 1 μ g of A3 DNA with 32 cycles of denaturation at 95°C for 1 min, annealing at 64°C for 1 min, and extension at 72°C for 1 min and 30 s. The obtained PCR fragment was eluted, cloned, and sequenced as already described. A total of six clones was sequenced using the 5' γ 4 and the γ 4.2 primers. The nucleotide sequence of the polymorphic γ 4 CH1 exon has been submitted to GenBank and assigned the accession no. L13487.

Results

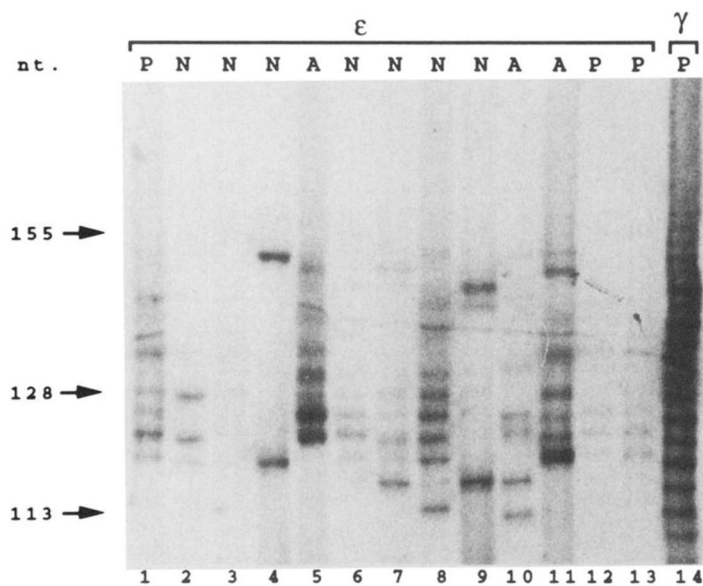
Amplification of the CDR3/Fw4 regions results in an ϵ fingerprint

We initially attempted the amplification of complete ϵ VH regions on RNA samples obtained from seven normal individuals, three atopic individuals, a pool of PBMC from five different individuals, and the IgE-secreting myeloma cell line U266 using a FW1 primer (V1) and a primer from the first exon of the C ϵ gene (E2). Bands of the expected size were visible on EtBr-stained agarose gels (data not shown) only in the amplifications of U266 RNA. This suggested that the PBMC IgE H chain transcripts, if present, were either below the limit of detection, or that there was insufficient homology of the V1 primer with the FW1 regions of the PBMC ϵ transcripts that did not allow their amplification. We found the second explanation unlikely, because sequence comparison of our degenerate FW1 primer with a number of published variable gene segments showed either perfect homology or one to three mismatches

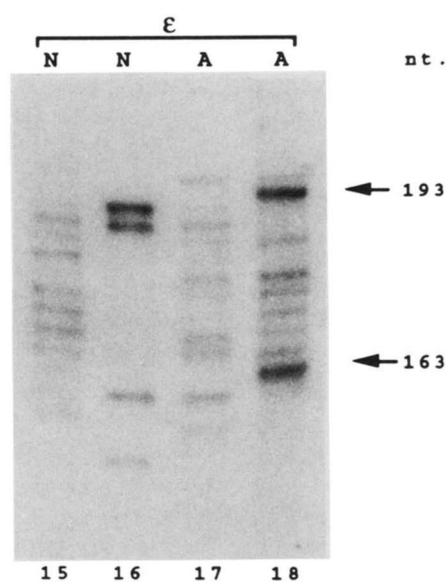
a .



b .



c .



d .

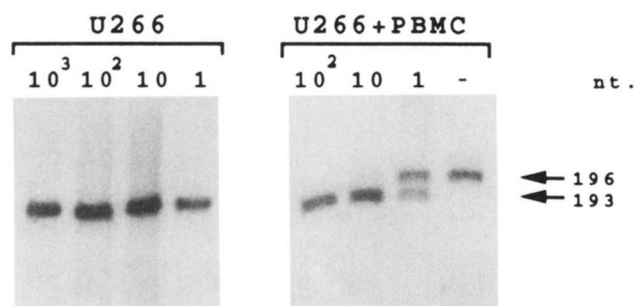


FIGURE 1. Analysis by RT/PCR of the ϵ CDR3/FW4 regions. *a*, Schematic representation of the positions of the different oligonucleotide primers. The size ranges of the expected fragments corresponding to the ϵ CDR3/FW4 sequences are indicated. *b*, Total PBMC cellular RNA from normal (N, lanes 2, 3, 4, 6, 7, 8, 9), and allergic (A, lanes 5, 10, 11) individuals, and from

with members of all VH families except the VH2 (31). The observed differences were at positions that should not affect primer annealing and extension, as observed for the U266 ϵ transcript, which contains three mutations with respect to the sequence of the V1 primer (32) and which can be amplified even at low concentration (see below, Fig. 1*d*). Also, the same primer had previously successfully amplified the complete V regions of μ transcripts in 12 cases with chronic lymphocytic leukemia (D. Efremov and O. Burrone, unpublished observations). Therefore, to further increase the sensitivity of the assay we included a second nested PCR reaction with the internal primers E1 (in the first C ϵ exon) and ^{32} P-labeled V2 (in FW3) to amplify the CDR3/FW4 regions and adjacent sequences. A parallel reaction was done with the G1 primer (in the first exon of the C γ genes). The radiolabeled PCR fragments were separated on a polyacrylamide sequencing gel, and a codon ladder of bands differing in size by three nucleotides that corresponded to the lengths of the different ϵ CDR3/FW4 segments was obtained. Only a few bands were obtained from amplification of the ϵ transcripts, whereas bands of much stronger intensity were present at every possible position in the sample amplified with the γ -specific primer (Fig. 1*b*). Although each individual had a particular pattern of bands, there were no major differences in the number or intensities of the bands between the normal and atopic individuals. To confirm that we were truly amplifying IgE H chain mRNA, the first PCR samples were reamplified with another ϵ -specific primer (E3), which is located 44 nt downstream of the E1 oligonucleotide (Fig. 1*c*). By obtaining the same ϵ fingerprint, only extended in length by 44 nucleotides, we demonstrated that the bands representing the CDR3/FW4 sequences were indeed amplified from the ϵ mRNA population.

In order to determine the sensitivity of our assay, total cellular RNA was extracted from 2×10^6 U266 cells. RNA aliquots corresponding to 1000, 100, 10, and 1 U266 cells were then analyzed using the above described RT/PCR procedure either separately or mixed together with total cellular RNA corresponding to 3×10^5 PBMC of one non-atopic individual who showed a pattern consisting of a single band of 196 nt (Fig. 1*d*). A band of 193 nt corresponding to the U266 ϵ CDR3/FW4 region was present in all four dilutions when U266 RNA was reverse transcribed

and amplified alone or together with PBMC RNA. The 196 nt band of the nonatopic individual was present in the U266 RNA dilution corresponding to 1 cell, and was almost completely competed out by the RNA of 10 U266 cells.

Sequence analysis of ϵ CDR3/FW4 regions

We then cloned all four visible bands from one normal individual (N1), and a number of bands each from another normal (N6) and two atopic individuals (A1 and A3) (Fig. 1*b*, lanes 2, 8, 5, and 11, respectively). The total numbers of clones sequenced were 32 from N1, 5 from N6, 16 from A1, and 51 from A3 (Fig. 2). The obtained sequences were of the same sizes as the bands from which they were cloned. Twenty-six different VDJ joinings were observed in the 17 cloned bands from all four individuals, ranging from 4 in individual N1 to 12 in A3. An additional number of clonally related transcripts was identified in the atopic individuals (see below).

Biased use of the JH4 gene and D genes from the DXP family

In most cases, significant homology of the D regions with published D sequences was found allowing assignment to particular D gene families (Fig. 2). However, it is possible that some of the assignments are imprecise because of the presence of somatic mutation, the possibility that all D gene members have not yet been identified, and the unknown level of polymorphism within them. Nevertheless, our analysis shows preferential usage of members of the DXP family (33%), and the JH4b gene (35%), similar to reported findings for the IgM-producing B cell subset in adult peripheral blood (33–35). It is interesting, however, that in these studies the JH 1 gene was greatly underrepresented or absent, whereas we identified it in 4 out of the 26 different VDJ joinings sequenced (15%).

The ϵ Fw4 regions show evidence of somatic mutation

Fifty-four substitutions with respect to germ-line JH sequences were identified in the 43 different transcripts, corresponding to an exchange frequency of 1 in 35 sequenced nucleotides, which is much higher than the observed error

a pool of white blood cells from five different individuals (P, lanes 1, 12, 13, 14) was reverse transcribed with primer E2 (ϵ , lanes 1 to 13) or primer G1 (γ , lane 14). The CDR3/FW4 regions of ϵ -chain cDNA were amplified with primers hV1 and E2 in the first PCR, and with primers [^{32} P]hV2 and E1 in the second round of PCR (ϵ , lanes 1 to 13). Amplification of γ -chain cDNA was performed with primers hV1 and G1, followed by a second PCR with primers [^{32} P]hV2 and G1 (γ , lane 14). c, The first PCR samples from four individuals (corresponding to lanes 8, 9, 10 and 11 of b) were reamplified with primers [^{32} P]hV2 and E3 (lanes 15 to 18). d, Total cellular RNA from different number of U266 cells, as indicated above each lane, was used either alone or mixed with RNA from 3×10^5 PBMC and subjected to two rounds of PCR. The first reaction was carried out with primer pair hV1/E2 and the second with [^{32} P]hV2/E3. 193 and 196 correspond to the length in nucleotides of the ϵ CDR3/FW4 regions of the U266 and PBMC transcripts, respectively. b, c, and d, Autoradiograms of denaturing polyacrylamide gels in which sequencing reactions were included as size markers.

A1

	FW3	CDR3	FW4	εCH1	JH Gene	Size nt.	Name
		NDN	JH				
		DK5 DK4					
(1)	tgcgaga	gatcaggtctctgctatgacagcagtggt	ctgaatacttccagactggggccaggggcacgtggtaagctctctcc	gcctcca	JH1	140	A1/a
(1)	-----	-----	-----	-----			
(1)	-----	-----	-----	-----			
		DA1					
(2)	tgcgaga	gatcaggactacagcgaa	actactacggtatggaagctctggggccaaagggaacaggtcacgtctctcca	gcctcca	JH6	131	A1/b
(1)	-----	-----	-----	-----			
		DIR*					
(4)	tgcgaga	tctgggacaacccccgggtactatgg	gatatctggggccaaagggaacatgggtcacgtctctcca	gcctcca	JH3	125	A1/c
(1)	-----	-----	-----	-----			
(1)	-----	-----	-----	-----			
		DN1					
(2)	tgcgaga	aacacctatgacgcagcttcaaa	tgactactggggccagggaacccctgggtcacgtctctcca	gcctcca	JH4	122	A1/d
(1)	-----	-----	-----	-----			
(2)	tgcgagt	ggtctggtattttgaactggcc	ttgactactggggccagggaacccctgggtcacgtctctcca	gcctcca	JH4		A1/e

* DIR like sequence.

A3

	FW3	CDR3	FW4	εCH1	JH Gene	Size nt.	Name
		NDN	JH				
		DK052-D1 DXP3					
(1)	tgcgaga	gatcataatccccagattctctatgtagtagtggtattccccc	tactttgattctggggccagggaacccctgggtcacgtctctcca	gcctcca	JH4	149	A3/a
(1)	tgcgaga	ggctattacgatttttgagtgcaattataaaggccccccgtc	ctgggtcgacccctggggccagggaacccctgggtcacgtctctcca	gcctcca	JH5		A3/b
(1)	-----	-----	-----	-----			
(3)	-----	-----	-----	-----			
(7)	-----	-----	-----	-----			
(1)	-----	-----	-----	-----			
(1)	-----	-----	-----	-----			
(1)	-----	-----	-----	-----			
(1)	-----	-----	-----	-----			
(4)	-----	-----	-----	-----			
(1)	-----	-----	-----	-----			
		DXP1 DXP1					
(2)	tgcgaga	ggtatgtagggagctctggtccctccgggtag	tttttgatctggggccagggaacaggtcacgtctctcca	gcctcca	JH3		A3/c
(2)	tgcaaac	ctccacatgattacgactcttgagtggttattatatt	caatggggccagggaacccctgggtcacgtctctcca	gcctcca	JH1	134	A3/d
(2)	tgcgagg	gctgagccacatatacaaacccc	gctgaatacttccagactggggccagggaacccctgggtcacgtctctcca	gcctcca	JH1		A3/e
		DN1					
(1)	tgcgaga	agttatgagcaactgggactgcc	tacttgagcctctggggccagggaacccctgggtcacgtctctcca	gcctcca	JH4		A3/f
(1)	-----	-----	-----	-----			
		DL4 DM2					
(2)	tgcgagg	gagttatgctctatcagaacat	ggtatggaagctctggggccagggaacccctgggtcacgtctctcca	gcctcca	JH6	128	A3/g
		DA1					
(3)	tgcgaga	gcattggagcagtgagat	gcttttgatattggggccagggaacatgggtcacgtctctcca	gcctcca	JH3		A3/h
(1)	tgcgaga	tctgggacaacccccgggtactatgg	gatatctggggccaaagggaacatgggtcacgtctctcca	gcctcca	JH3	125	A3/i
		DN1					
(1)	tgcgaga	gcaggggcagcagctgat	gcttatgagctctggggccagggaacatgggtcacgtctctcca	gcctcca	JH3	122	A3/j
(7)	-----	-----	-----	-----			
(1)	-----	-----	-----	-----			
		DN1*					
(5)	tgcgaga	accggtgagggagagc	gttgacccctggggccagggaacccctgggtcacgtctctcca	gcctcca	JH5		A3/k
		DXP1**					
(1)	tgcgaga	gatagcagattgat	gcttttgatattctggggccagggaacatgggtcacgtctctcca	gcctcca	JH3	119	A3/l

* Other possible DH genes: DM2, DN1

** Other possible DH genes: DA4, DLR5, DK4

N1

	FW3	CDR3	FW4	εCH1	JH Gene	Size nt.	Name
		NDN	JH				
		DXP1					
(2)	tgcgaga	ggctcgtatgactgattttgactggccagacacct	tactactactactacggtatggaagctctggggccaaagggaacaggtcacgtctctcca	gcctcca	JH6	155	N1/a
		DXP1 DA1					
(12)	tgcaaaa	gacattggaggaggggtacgtgactac	gactactggggccagggaacccctgggtcacgtctctcca	gcctcca	JH4	128	N1/b
		DLR2					
(12)	tgcgaga	gcccgtggagctctactcgt	ctttgactactggggccaaagggaacccctgggtcacgtctctcca	gcctcca	JH4	122	N1/c
		DK1					
(6)	tgcgaga	gtaccgcgtacagttac	ttgacccctggggccagggaacccctgggtcacgtctctcca	gcctcca	JH5	119	N1/d

N6

	FW3	CDR3	FW4	εCH1	JH Gene	Size nt.	Name
		NDN	JH				
		DK4					
(1)	tgccctg	agctttttcgggtgatacagctatggtcacagagaagcgc	tactttgactactggggccagggaacccctgggtcacgtctctcca	gcctcca	JH4	143	N6/a
		DN4					
(1)	tgcaag	agccctctatagcagctcgttgc	ctgaagacttccgactggggccagggaacccctgggtcacgtctctcca	gcctcca	JH1	131	N6/b
		DK1					
(3)	tagtaga	cataattcttatgggtgactacagctcct	tttgactactggggccagggaacccctgggtcacgtctctcca	gcctcca	JH4		N6/c
		DN4					
(1)	tgcgaga	actactatagcagtggaag	ggttgacccctggggccagggaacccctgggtcacgtctctcca	gcctcca	JH5	122	N6/d
		DN1					
(1)	tgccagg	ggggagtagcagctggcgc	actttgactactggggccagggaacccctgggtcacgtctctcca	gcctcca	JH4		N6/e

rate of the Taq polymerase (36) and the error rate that we encountered in amplification of V regions from hybridoma cells under the same conditions (less than 1 mutation in 400 sequenced nucleotides). Because polymorphic JH genes have been reported (JH3 a and b, JH4 a and b, JH5 a and b, and JH6 a, b and c) (33), we cannot exclude that some of the sequence variation is caused by as yet unidentified polymorphic JH genes. However, several variants of a particular JH gene segment were present within a single individual, which supports the role of somatic mutation in the generation of the observed nucleotide differences. Evidence for this process comes also from the presence of nucleotide differences in the FW4 regions between the clonally related transcripts (see Fig. 2), which for reasons discussed below cannot be attributed to Taq polymerase errors. Out of the 54 JH substitutions, 36 belong to the FW4 regions, with 19 replacement and 17 silent nucleotide changes, resulting in an R/S ratio of 1.1. This value is in agreement with the predicted value of 1.5 for FW mutations (37), because the expected R/S ratio of 2.9 for a randomly mutated sequence would mean introduction of deleterious mutations that will result in the loss of the clone of antibody-producing cells. Even though there are considerable differences in the nucleotide sequence of some of the CDR3 regions with respect to the assigned D and JH gene segments, it is difficult to evaluate the incidence of somatic mutations in these regions because part of the substitutions could be accounted for by D gene polymorphism, usage of yet undescribed D genes, and imprecision in defining the boundary of the D/N junction.

Clonally related ϵ transcripts with nucleotide differences in the FW4 sequence

In the two atopic individuals, clonally related transcripts containing the same VDJ rearrangement but differing mainly in the FW4 regions were identified. In the case of the 149 nt long transcripts from individual A3 (A3/b in Fig. 2), 10 sequences differed among themselves by 1 to 6 nt substitutions. Five of these mutations were replacement, and eight were silent, resulting in an R/S ratio of 0.62. We exclude the possibility that these nucleotide differences were caused by Taq polymerase error for several reasons: first, amplified transcripts from two different bands of individual N1 were sequenced 12 times each and no nucleotide differences were present among the clones; second, there were no nucleotide differences (with one exception at the N/J boundary) in the CDR3 regions among the 21 se-

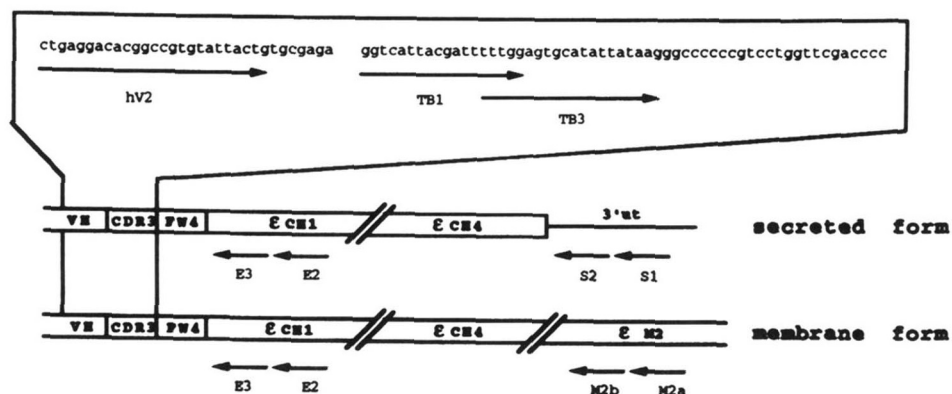
quenced clones; third, the same nucleotide differences were encountered when we amplified the clonally related transcripts in a separate set of PCR reactions for detection of the secreted form (see below); fourth, in these same long amplified fragments, no substitutions with respect to the germ-line sequence of the C region were present; and fifth, clonally related transcripts containing the same CDR3/FW4 sequences were also identified in the μ H chain mRNA (see below).

The clonally related A3/b transcripts are of the secreted form

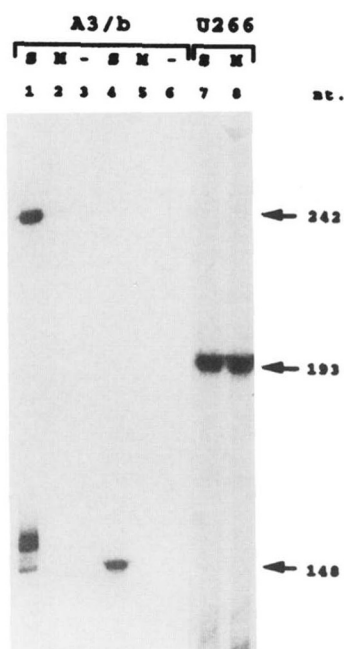
To further characterize the A3/b clonally related transcripts, we investigated whether they corresponded to membrane or secreted forms of ϵ -chains. For this purpose oligonucleotides specific for both the membrane ϵ M2 exon (25) (primers M2a and M2b) and the 3' untranslated region (primers S1 and S2) of the secreted ϵ mRNA were used together with two CDR3-specific oligonucleotides (TB1 and TB3) in a two-step nested PCR reaction (Fig. 3a). A band of the expected size (approximately 1400 nt) was observed only for the secreted form (not shown). To investigate whether membrane form transcripts were present but at much lower levels compared with the secreted ones, we performed additional PCR reactions with the internal ^{32} P-labeled CDR3-specific primer TB2 and either the E2 or E3 primer, both from the first exon of the C ϵ gene. Bands of expected lengths (242 nt and 148 nt) were present in the reamplifications of the first PCR reactions carried out with primers that amplify the classical secreted form (Fig. 3b). No signal was obtained from the reactions performed with the M2b-specific primer, which amplifies, apart from the classical membrane form, other alternative spliced products that contain the M2 exon and that have been reported as being putative secreted forms (25). Positive control reactions were carried out with RNA from the cell line U266, which expresses the membrane and secreted forms of ϵ mRNA (Reference 25 and our unpublished observations). As seen in Figure 3b, a band of 193 nt was observed for both the secreted and membrane U266 ϵ transcripts. The 1.4-kb band obtained in the second PCR amplification with primers TB3/S2 was cloned and partially sequenced from both ends (Fig. 3c). On the 5' end the sequence contained the same CDR3/FW4 region as one of the clonally related transcripts with two substitutions with respect to the germ-line JH5 gene segment. The sequence of the 3' end confirmed that it belonged to the secreted form of ϵ mRNA.

FIGURE 2. Nucleotide sequences of PCR-amplified ϵ CDR3/FW4 and adjacent regions obtained from two normal (N1 and N6) and two atopic (A1 and A3) individuals. Sequences are presented divided into VH, NDN, JH, and CH. The CDR3 and FW4 regions are also indicated. The sequences of the primers used are not included. Numbers in parentheses indicate the number of clones of each particular transcript sequenced. The names of the germ-line D and JH genes with maximum homology to the reported sequences are indicated. Sizes correspond to the length in nucleotides of the bands in Figure 1B (which include the amplification primers). Nucleotide substitutions with respect to germ-line sequences are *underlined* and those introducing amino acid replacements are shown in *italic letters*. Identical nucleotides are indicated with dashed lines.

a.



b.



c.

_____ CDR3 _____/_____ FW4 _____/

tttgagtgcatattataagggcccccgcctcgtggttcgacccc tggggccaggggacccttggtcaccgtctcctca

_____ ϵ CH1 _____

gcctccacacagagcccatcctcctcccttgaccgctgctgcaaaaacattccctccaatgccacctcgtgactc

tggtgctgctggccacgggctacttcccgagccgggtgatgggtgacctgggacacaggctccctcaacgggacaactat

gacctaccagccaccacctca.....

_____ ϵ CH4 _____

.....ggcggggagccgggacaagcgacacctcgctgct

gatccagaactcatgctgaggacatctcggtgcagtggtgcacacagaggtgcagctcccgagcggcgacagc

acgacgcagccccgcaagaccaagggtccggctctctcgtcttcagccgctggaggtgaccagggccgaatgggggc

agaaagatgagttcatctgcccgtgcagtcctgagggcagcagccctcacagaccgtccagcagcggtgtctgtaaa

_____ ϵ 3'ut _____

tcccggtaatga cgtactcctgcctcc

The A3/b CDR3/FW4 regions are present in μ and $\gamma 4$ mRNA

With the aim of studying whether the same CDR3/FW4 sequences of the IgE H chains were also present in other Ig isotypes we performed radioactive PCR with A3/b-specific oligonucleotides coupled with primers specific for μ and $\gamma 4$ sequences. Only one radioactive band of the exact size was obtained in each case, thus indicating that the same VDJ rearrangement was present in IgE, IgM, and IgG4 isotypes (Fig. 4a). To confirm these results we constructed from individual A3 μ -specific and $\gamma 4$ -specific cDNA libraries containing CDR3/FW4 sequences. The cloned fragments were obtained by a two-step nested PCR using the A3/b-specific primer TB3 and the corresponding primers $\mu 2$ and $\gamma 4.2$, respectively. Sequence analysis identified two clonally related μ H chain transcripts that contained identical CDR3/FW4 regions as the A3/b ϵ transcripts (Fig. 4b). In the case of the $\gamma 4$ transcripts, we obtained three clonally related sequences that contained the same 3' part of the NDN region and used the same JH5 gene as the A3/b ϵ and μ transcripts. However, in one of these sequences the JH5 gene segment was in germ-line configuration, whereas the other two contained two nucleotide substitutions each, which were all different from the ones present in the ϵ and μ transcripts (Fig. 4c). These $\gamma 4$ transcripts also differed among themselves by 1 to 3 nucleotides in the JH segment. The sequence of the first exon of the C H chain region was identical in all three $\gamma 4$ transcripts, but contained three substitutions with respect to the reported sequence of the $\gamma 4$ C region gene (24). To investigate the origin of these substitutions we cloned and sequenced the CH1 exons of the $\gamma 4$ genes of individual A3. Besides the already published C $\gamma 4$ sequence we also identified a polymorphic C $\gamma 4$ gene (further referred as C $\gamma 4$ b) containing four silent substitutions in the CH1 exon: a T to C substitution at position 3, C to G at position 21, C to G at position 69, and C to T at position 252. The first three substitutions are present in the sequence of the $\gamma 4$ transcripts shown in Figure 4c, whereas the fourth one is absent because it corresponds to the 5' end nucleotide of the primer $\gamma 4.2$.

Discussion

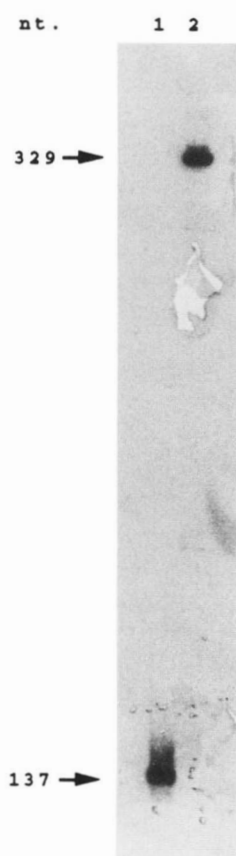
Despite a significant biologic effect of IgE as a trigger of allergic reactions, its serum levels are three to four orders of magnitude lower than the other Ig families. IgE is pro-

duced by a correspondingly low number of peripheral blood B lymphocytes, estimated over a wide range from 1/1,000 to 1/100,000 lymphocytes in normal individuals (38–41). Elevated levels are frequently encountered in atopic individuals and during certain parasitic infections. The low levels of IgE production cause considerable difficulties in analyzing the ϵ -chain V region repertoire, especially in normal individuals. Recently, van der Stoep et al. showed that ϵ transcripts from atopic individuals with high serum IgE levels can be analyzed after one round of PCR amplification using family-specific FW1 primers, but were unable to amplify any ϵ VH transcripts from PBMC of nonatopic individuals (42). Using a nested PCR approach we were able to amplify, and subsequently clone and sequence, the CDR3/FW4 regions of ϵ transcripts from both normal and atopic individuals with low serum IgE levels. The extreme sensitivity of this procedure allowed the analysis of ϵ mRNA produced by a single plasma cell, as shown for the myeloma cell line U266. The dilution experiment with U266 RNA also suggested that the amount of ϵ transcripts produced by PBMC from normal individuals is very low, in the order of 1 to 10 U266 cell equivalents, because the RNA content from 10 U266 cells almost completely competed out amplification of ϵ transcripts produced by 3×10^5 PBMC. However, biased amplification of the U266 or PBMC transcripts because of preferential annealing of the FW primers to the different ϵ VH regions could have also influenced this result.

Cloning of individual bands separated on denaturing polyacrylamide gels allowed us to analyze in greater detail particular CDR3/FW4 regions, and limited the possibility of repeatedly cloning only the most abundant or preferentially amplified ϵ transcripts. The sequence analysis of the ϵ CDR3/FW4 regions showed preferential usage of the JH4 gene segment and D genes from the DXP family, which were present in 35% and 33%, respectively, of the ϵ transcripts. A similar bias in the use of the same JH and D genes has been reported for the IgM-producing B cell population (33–35).

We give evidence that the IgE-producing B cell clones undergo somatic mutation, because most of the ϵ transcripts contained nucleotide substitutions with respect to the germ-line JH gene segments. A number of these mutations were identified in clonally related ϵ and μ transcripts, and therefore could not be accounted for by polymorphism or Taq

FIGURE 3. Analysis of the membrane (M) and secreted (S) forms of ϵ mRNA containing the A3/b sequences. *a*, Schematic representation of the primers used for the PCR analysis. *b*, First PCR reactions were carried out with primers TB1/S1 (lanes 1 and 4) and TB1/M2a (lanes 2 and 5) on cDNA from individual A3 and primers hV1/S1 (lane 7) and hV1/M2a (lane 8) on cDNA from the U266 cell line. Lanes 3 and 6 represent negative controls with no cDNA (–). Radiolabeled products obtained after a nested PCR reaction with primers [^{32} P]TB3/E2 (lanes 1 to 3), [^{32} P]TB3/E3 (lanes 4 to 6), and [^{32} P]hV2/E3 (lanes 7 and 8) were analyzed on a sequencing gel. Sizes in nucleotides are shown. *c*, Partial nucleotide sequence of one A3/b transcript belonging to the secreted form of the ϵ -chain. The CDR3, FW4, most of the ϵ CH1 and CH4 exons, and part of the 3' untranslated region are shown. The two somatic mutations in FW4 are underlined.

a .**b .**

_____ CDR3 _____ / _____ FW4 _____ /

(4) tttggagtgcattataagggtccccctgctggttcgacccc tggggccagggaccctggtcacctgtctctca

(3) -----t-----

_____ μ CH1 _____

gggagtgcattccgcccaacccttttccccctggtctctctatgagaattccccgtcggat.....

c .

_____ CDR3 _____ / _____ FW4 _____ /

(2) tttggagtgcattataagggtccccctgctggttcgacccc tggggccagggaccctggtcacctgtctctca

(1) -----a-----t-----

(1) -----ta-----

_____ γ 4 CH1 _____

gcgtccaccaagggtccatcgtcttccccctggcgccctgtccaggagcacctccgagagcacagcggccctgggct

gcctggtcaaggactacttccccgaaccggtgacggtgtcgtggaactcaggcgccctgaccagcggcgtgcacacctt

cccggctgtcctacgtcctcaggactctactccctcagcagcgtggtgaccgtgccctccagcagcttgggcacgaag

acctacacctgcaac

polymerase error. It is interesting that sets of clonally related transcripts were found only in the allergic individuals, and none were obtained when 12 clones each from two different bands of individual N1 were sequenced (Fig. 2, N1/b and N1/c). Although it is possible that this finding is purely coincidental, it might also be a consequence of frequent Ag stimulation resulting in repeated rounds of somatic mutation in Ag-specific clones of the two atopic individuals.

Recent studies have shown that cloned surface IgM-positive human B cells can be induced to switch with high frequencies to IgG4 and IgE production after a contact-mediated signal provided by T cell clones and IL-4 (12). A significant percentage of these cells produced combinations of two or all three Ig isotypes simultaneously. Analysis of switch junctions in established IgE producing human B cell lines and PBL cultivated with IL-4 have shown that at least part of these cells switch through sequential $S\mu$ - $S\gamma$ - $S\epsilon$ deletional recombination (11). However, other investigators have reported germ-line configuration of the $C\epsilon$ genes in IgM/IgE double isotype-producing clones established by EBV transformation of surface IgM/IgD-positive surface IgE-negative clones, cultivated in the absence of exogenously added IL-4 (16). These results suggest that other mechanisms such as *trans*-splicing between germ-line CH transcripts and VDJ-encoding RNA (43) or alternative splicing of a long RNA transcript encoding one VDJ and multiple CH genes in germ-line configuration (20), could also be responsible for IgE production. We therefore investigated whether production of IgM, IgE, and IgG4 antibodies by cells descending from a single B cell clone is only an in vitro phenomenon, or also a characteristic of the in vivo IgE-producing peripheral blood B lymphocytes. To that purpose we searched for the presence of the A3/b ϵ CDR3/FW4 regions in μ and $\gamma 4$ transcripts from the same individual using CDR3 specific oligonucleotides. Clonally related transcripts containing the same D segment, N region, and J segment were identified both among the μ and $\gamma 4$ transcripts. Although the obtained A3/b μ sequences were completely identical with the ϵ transcripts, the three identified $\gamma 4$ transcripts contained different mutations in the JH segment, and also three mutations in the CH1 exon as compared with the published C $\gamma 4$ gene sequence (Fig. 4, *b* and *c*). Cloning and sequencing of genomic DNA from this individual revealed a novel C $\gamma 4$ gene (C $\gamma 4b$) containing four silent polymorphisms in the CH1 exon.

The above described results could best fit in a model in

which a common progenitor gives rise to two cell populations that undergo somatic mutation independently. In this model one B cell population would switch to IgG4 production, whereas the second would be composed of IgM/IgE double isotype-producing cells. Because we could only identify secreted form A3/b ϵ transcripts, this second B cell subset would produce secreted IgE molecules. This could, however, represent only a stage of differentiation for this clone, and we do not exclude that it would ultimately express surface IgE, especially since we have identified membrane form ϵ transcripts in fresh PBMC from several normal and atopic individuals (F. Batista, D. Efremov, and O. Burrone, unpublished observations). The observed presence of clonally related ϵ and $\gamma 4$ transcripts could also point out to a mechanism of modulating the allergic response, as antibodies of the IgG4 subclass have recently been shown to represent the blocking antibodies identified in patients with chronic helminthic infections, which despite high levels of parasite-specific serum IgE, do not manifest allergic reactions (44). It is possible that preferential expansion of the IgE-producing clones could eventually create a disequilibrium in the levels of the IgE and the corresponding IgG4-blocking antibodies, increasing the likelihood for an allergic reaction upon subsequent contact with allergen. Our inability to detect $\gamma 4$ A3/a, A3/c, and A3/d transcripts in the allergic individual A3 using the same approach as described for the A3/b transcripts is in line with this possibility (data not shown).

The A3/b clonally related transcripts that were identified in the three different Ig isotypes were created by 15 different nucleotide substitutions in the germ-line JH5b gene, and one substitution at the N/J junction. A number of these mutations were found to be shared with transcripts from the other individuals. For example, the C for T substitution at position -11 (upstream of the CH1 exon) was also found on different JH gene segments (JH1 and JH4) in three other transcripts (N6/b, N6/c, and A1/a) (Fig. 2). Comparison with other published sequences revealed that the same mutation was present in 2 of 19 ϵ VH5 transcripts (42) and also among the clonally related transcripts of two follicular lymphoma cases (45, 46). Furthermore, the substitutions at positions -1, -6, and -22 were also found in 4, 2, and 3 of the other analyzed transcripts, respectively. These mutations cannot be accounted for by Taq polymerase error because they were present in sequences obtained from two separate PCR amplifications of ϵ transcripts and also in the A3/b μ transcripts, and therefore suggest the existence of

FIGURE 4. Detection of the A3/b CDR3/FW4 regions in μ (lane 1) and $\gamma 4$ (lane 2) transcripts. *a*, The precise sizes of the radiolabeled PCR products obtained with primers [^{32} P]TB3/ μ 2 (lane 1) and [^{32} P]TB3/ $\gamma 4$.2 (lane 2) were determined with a sequencing ladder. *b* and *c*, Nucleotide sequences corresponding to the A3/b μ (*b*) and A3/b $\gamma 4$ (*c*) transcripts. The CDR3, FW4, and the μ and $\gamma 4b$ CH1 exons are indicated above the sequences. Underlined nucleotides in the CDR3 and FW4 correspond to differences with the JH5b sequence. Dashed lines below the sequence represent identical nucleotides. The sequence of the CH1 exon of the polymorphic $\gamma 4b$ gene is presented with the three silent substitutions underlined. In parentheses are indicated the numbers of clones sequenced.

"hot spots" for the somatic hypermutation machinery in the JH gene segments. Van der Stoep et al. also observed a high number of shared mutations among the VH5 segments of the 19 ϵ transcripts they analyzed, and suggested an intrinsic sequence specificity of the somatic hypermutation mechanism (42). Further evidence for the existence of sequence specific hot spots comes from a very recent study of passenger κ transgenes in which the somatic mutations were not selected by Ag and still preferentially occurred in particular positions of the κ -chain V regions (47).

Another mechanism that can also be taken into consideration to explain the confinement of the somatic mutation in the FW4 regions of the A3/b ϵ transcripts is negative selection against mutations that could affect Ag specificity, affinity, and expression of functional Ig. However, although negative selection against deleterious mutations could account for the low R/S ratio (0.62) observed in the FW4 regions, the almost complete absence of mutations, including silent, in the CDR3 regions (only 1 of 13), which were almost twice the size of the FW4, requires additional considerations. Studies of a large number of malignant antibody-forming cells and hybridomas (reviewed in Reference 48) have suggested that the somatic mutation process is actively turned off in the V regions during B cell differentiation, while it continues to introduce changes, although at a much lower rate, in the C region genes. It is therefore possible that this subset of cells represents a stage in B cell differentiation in which the somatic mutation was being switched off in the CDR3 regions, while still operative in the FW4. Decreased accessibility of the CDR3 regions to the somatic hypermutation mechanism could prevent introduction of mutations that could alter the Ag specificity or affinity of clones that have already been selected for high binding affinity. Further studies will be required to determine whether such an event represents a more frequent feature of the somatic hypermutation mechanism.

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