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Molecular Analysis of IgE Heavy Chain Transcripts Expressed by Peripheral Blood Lymphocytes

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Candidate:
Facundo Damian Batista

Supervisor:
Prof. Oscar Burrone

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SISSA - SCUOLA INTERNAZIONALE SUPERIORE DI STUDI AVANZATI
TRIESTE
Strada Costiera 11

TRIESTE

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1. INTRODUCTION

1.1. Immunoglobulin Structure

The human immune system is capable of specifically recognizing and responding to an enormus number of antigens. The interaction of antigens with the cells involved in the immune response is mediated by two distinc antigen specific receptor molecules, the immunoglubulins (Ig) and the T-cell receptor (TRC), expressed on the surface of B and T lymphocyte repectevely.

The Ig molecule is composed of 2 identical heavy (H) and 2 identical light (L) polypeptide chains linked together by disulfide bonds (Fig. 1). Both the H and the L chain are organized into domains of aproximately 110 aa that form a conserved structure containing a loop of 65 amino acids stabilized by an intrachain disulfide bond. The L chains contain two while the H chains contain four or five such domains. Both in the L and H chains the N-terminal domain constitute the so called variable (V) region domain which are the elements responsable for the binding to the antigen. They are characterized by a large amino acid sequence variation between different Ig (1). On the other hand the remaining domains constitute the constant region domain (C) which are virtualy indentical for molecules of a given class. Within the variable light (V_L) and variable heavy (V_H) region that make contact with the antigen, three different subregion with higher amino acid. sequences differences have been identified and named CDR1, CDR2, and CDR3. These subregions form loops emerging from the same side of the β barrel structure that constitutes the V domain of the complete assembled molecule, and contain the aa. residues that directly make contact with the antigen (2, 3). The three CDRs are separated by less variable regions termed frame-work regions (FR1, FR2, FR3 and FR4) that stabilize the structure of the β barrel.

The amino acid sequences within the constant domains of the H and the L chain are relatively conserved, although variations are found among the different isotypes

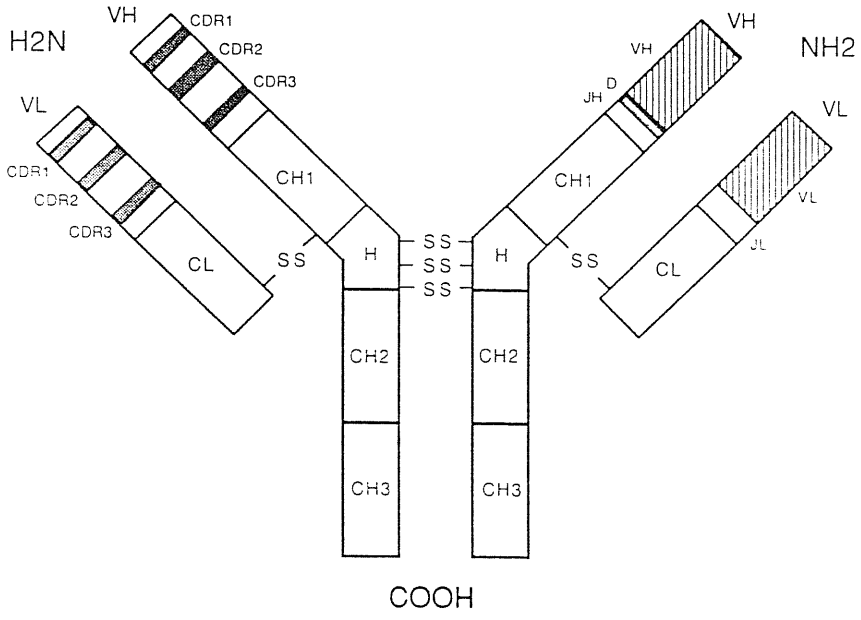

Figure 1. Structure of a IgG molecule. The V_H, C_H,V_L and C_L homology domains are shown as boxes and the hinge region is denoted H. Only the disulfide linkages H-H and H-L chains are shown. The aproximate boundaries of CDR regions and the sequences encoded by V_H, D, J_H, V_L and J_L are indicated by different shadings.

that were originally classified according to reactivity to specific antisera (4). There are 2 types of L chains kappa (κ) and lambda (λ); a single subtype of κ and at least 4 subtypes of λ have been identified with no functional differences between them. On the other hand, the constant region of the 5 differents H chain isotypes have specific functions such as complement fixation, placenta trasnfer capacity and binding to specific surface cell receptors (5). The H chain are designated by the Greek letters mu (μ), delta (δ), gamma (γ), alpha (α) and epsilon (ε). and define the corresponding immunogloulin classes IgM, IgD, IgG, IgA, and IgE, respectively. Some classes can be further divided into subclasses defined by distintc CH region amino acid sequences. In humans the γ class can be divided into subclasses 1, 2, 3, and 4; whereas in the mouse are identified as the four IgG subclasses 1, 2a, 2b, and 3.

IgD, IgG, and IgE antibodies generally consist of a single monomeric unit of two H and two L chains. Circulating IgM antibodies are polymers consisting of five such units. The secreted form of the μ chain has a penultimate cysteine residue that can bridge to other μ chains or the glycoprotein J chain to form the pentameric secreted IgM molecule. IgA antibodies may consist of one, two, or more units; the dimeric form seems to be the most commonly found.

The antibodies of each class can be produced in two structurally different forms: one membrane bound and the other secreted (6). The ability of immunoglobulins to appear as both secreted or membrane receptors is due to differences in the amino acids sequences of the carboxiterminal tail of these two forms of the molecule. This is achieved by means of the alternative splicing of specific exons coding for a transmembrane retention domain.

1.2. Differentiation of B Lymphocytes.

Antibody-producing cells are derived from hematopoietic stem cells first recognizable during embryonic life in the fetal liver. Shortly after birth the bone

marrow becomes the site of lymphopoiesis and remains so throughout adult life. In the early stages of differentiation along the B-cell pathway, progenitors cells committed to B-cell differentiation give rise to cells containing only cytoplasmatic μ H chain. The small pre-B cells subsequently acquire the capacity to synthesize L chains, which interact with H chains to form complete IgM molecules, expressed on the cell membrane surface, where they serve as antigen receptor to trigger subsequent stages of differenttiation and proliferation. Cells at this stage are called immature or virgin B cells. The immature B cells differentiate into Bμδ cells, which express on their surface both IgM and IgD molecules that have identical variable domains and hence identical antigen-binding specificities. Thus, each B cell displays on its membrane antibodies with a single type of V region sequence that function as antigen receptor. When a mature B lymphocyte binds antigen, in presence of the apropiate helper T cell, it is induced to proliferate and differentiate in either a memory or a plasma cell. The memory B-cells are relatively long lived and are more easily triggered than virgin B cells on subsequent encounters with the same antigen. The differentiation of the mature B cell to plasma cell is accopanied by the loss of surface immunoglobulin expression as a consequence of the conversion from the synthesis of membrane to secretory immunoglobulin molecules. These secreted Igs are produce in large amounts by the mature, short-lived plasma cell.

1.3 Genetic of Immunoglobulin Genes.

1.3.A. Human Heavy Chain Genes.

The expression of a complete Ig molecule is the consecuence of a complex mechanism that takes place independently on each lymphocyte and that involves a DNA recombination leads to the specific assembly of different DNA segments that both the H and the L chain loci.

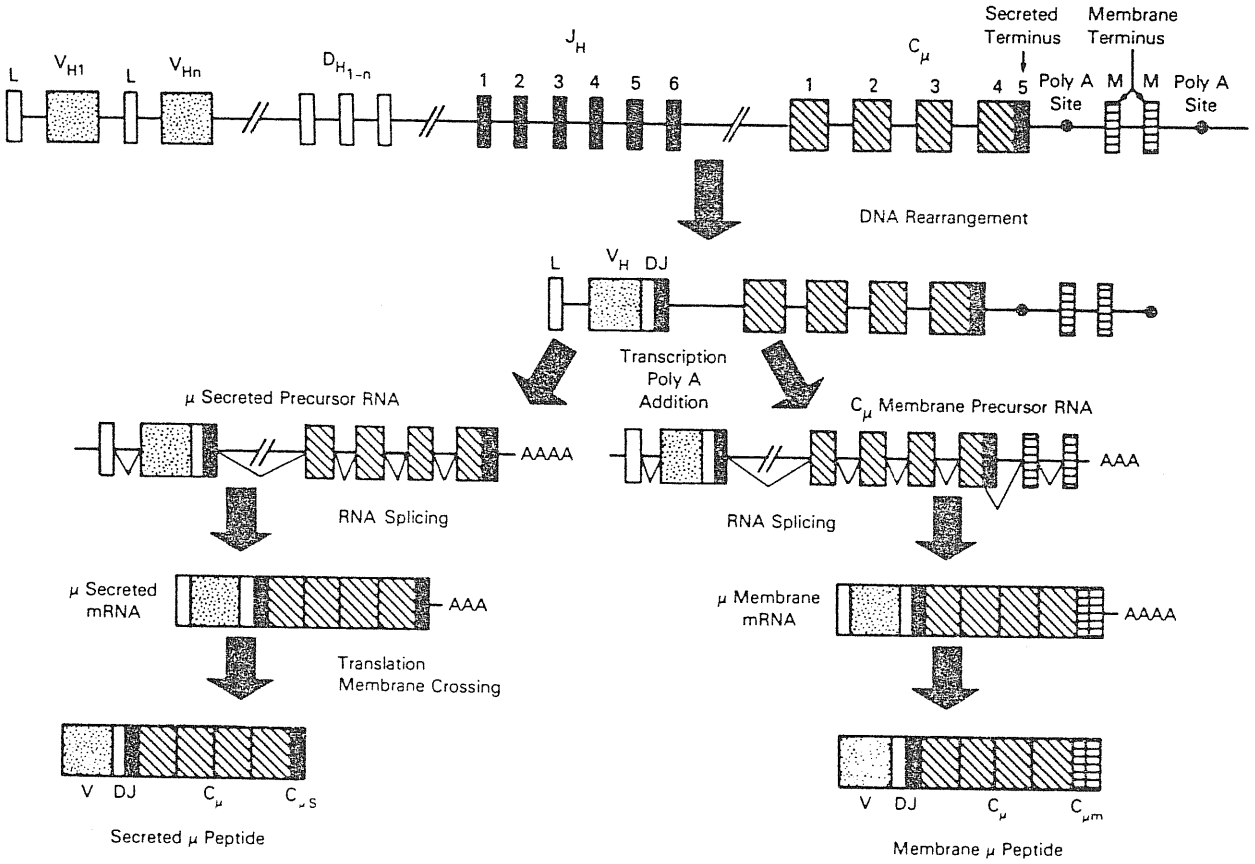

Figure 2. Schematic model of the organization of the human μ heavy chain gene. There are multiple V_H regions, each with a leader sequence, families of diversity (D) segments, six functional joining (J_H) and a single constant (Cμ) μ gene that is made up of a number of domains. Single V_H, D, and J_H are joined at the DNA level. Both secreted and membrane form of IgM are derived from a single constant μ region locus. Alternative sites of polyA addition and RNA splicing result in the different mRNAs containing either the secreted (Cμs) or the hydrophobic membrane (Cμm)terminus

The human H chain genes locus is located on chromosome 14 at band 14q32 (7). In this case the V region protein domain is encoded by a newly formed exon assembled upstream of the C region as a consequence of the recombination process that joins three different gene segments: a variable (V_H), a diversisty (D) and a joining (J_H) segments (8). Each V_H segment contains two exons encoding a 5' highly hydrophobic leader sequence that facilitates vectorial translocation of the molecule to the endoplasmatic reticulum and the first 98 aa of the variable region of the protein including the CDR1 and CDR2. The D_H and J_H segments contribute to the coding of the third hypervariable region, CDR3, and the FR4 of the V domain (Fig. 2).

In the human genome, the number of V_H gene segments has been estimated over 100. Based on sequence homology they can be grouped in 7 families . The human and mouse V_H elements were likely derived from three distinct progenitor V_H elements whose descendants populated three "clans" of V_H segments. These clans are defined based upon nucleotide sequence homology between the families (both between and across species) in the 6-24 codon interval in FR1 and the 67-85 interval of the FR3. Clan I includes the human families V_H1, V_H5, and V_H7. The primordial clan II was splited into 2 distinct subclans before divergence of human and mouse: the V_HII subclan represented by V_H2 family and the V_HIV subclan is represented by families V_H4 and V_H6. Finally, clan III consists of the human V_H3 family (9).

Based on sequence similarities the 17 human identified D_H genes can be also classified in 7 differents families (10) but the total number estimated is of around 35 differents D_H genes .

In man there are six active J_H gene segments located 5' to the C region genes. Additionaly, there are three J_H pseudogenes and also a region containig D_H genes, which lies between J_H1 and J_H2.

The H chain locus is the first to be rearranged. This process occur in two steps. The first event is a D-to-J rearrangement, which almost invariably occurs on both heavy-chain alleles. Precursor B cells then undergo V-to-DJ rearrangement on one or both DJ alleles, generating potentionally functional VDJ heavy-chain genes.

V-to-D rearrangement does not occurs on alleles that have not first undergone D-to-J rearrangement (11, 12). Since none of the V-to-DJ rearangements is precise, many of them generate "non productive" out-of-frame joined segments (VDJ^- alleles). In addition, recent results indicate that the organizatioin in this locus such as relative localization and orientation of each segment seems to affect the relative frecuency of its usage and thus the diversity of the V_H repertoire. For instance, it has been shown that the V_H segements located proximal to the J_H segments are more frecuently used especially during early stages of ontogeny (13).

The H chain C region genes are organized in a 200 kb of DNA. Each C region domain is encoded by a separate exon cluster. The μ C region (Cμ) is located closest to the J segments, and is followed by the Cδ gene. The differential processing of a long H chain transcript result in simultaneous expression of μ and δ H chains that contain the same V region. During the maturation of the immune response, B lymphocytes can express a different H chain isotype mantaining the same VDJ rearrangement and therefore the same antigen specificity, by replacing the Cμ coding sequences with those of a different C region through a different DNA recombination mechanism (class switching).

1.3.B. Human Kappa and Lambda Light Chains Genes.

In humans kappa light (Lκ) chains comprise approximatly 60 per cent of the human L chain protein. The genes of this locus are located on chromosome 2 at band 2p13 (14). In the case of the L chain the V protein domain is also the consequence of a DNA rearrangement joining two segments Vκ and Jκ. The first 95 aa. of the Vκ region protein are encoded by one of the multiple Vκ gene segments (the total number estimated is of around 75-80), divided in two families based on sequences homology. Each of these Vκ segments has two exons separated by an intron. The 5' exon codes for a major portion (residues -19 to -4) of the leader sequence, while the 3' Vκ exon

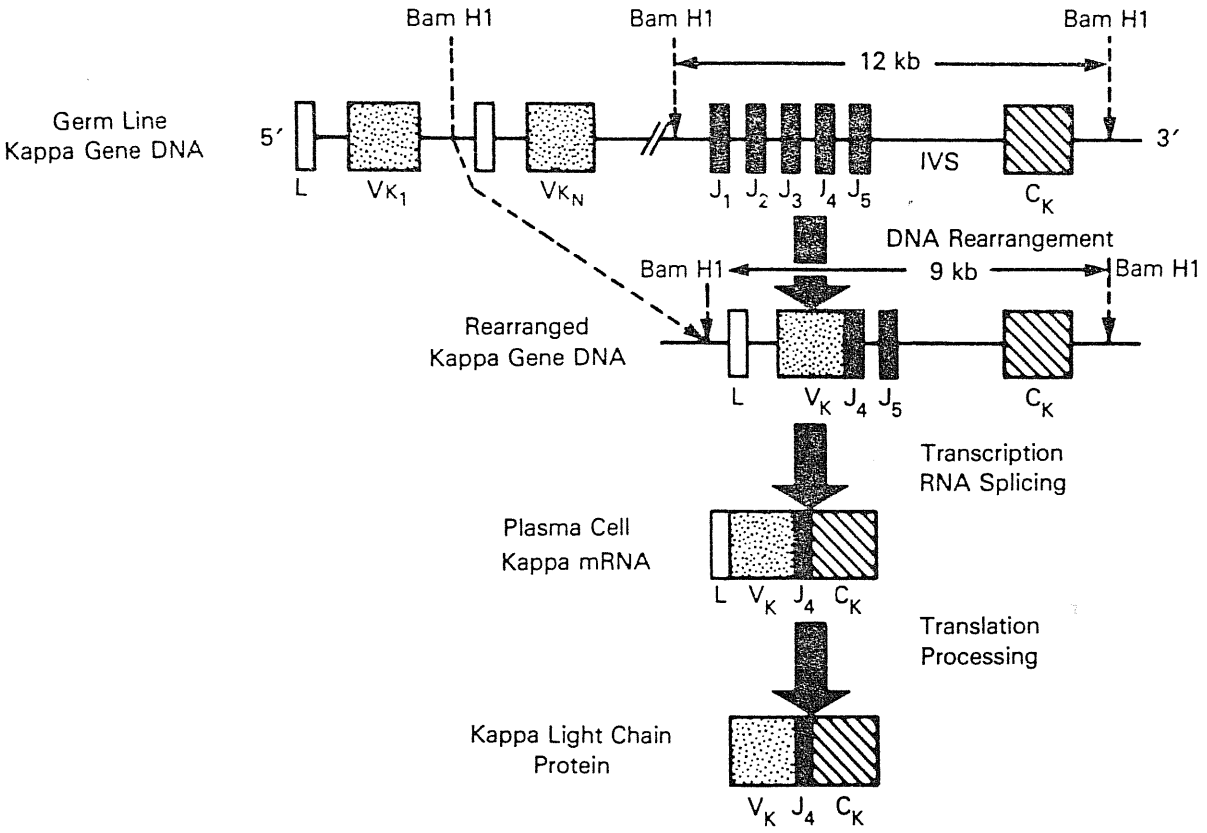

Figure 3. Arrangement of the human κ gene locus. Multiple variable (Vκ) regions each with an associated leader (L) sequence. There are five alternative joining (Jκ) segments encoding the remainding of the variable region. There is a single constant region per chromosome. DNA rearangements lead to the junction of a single Vκ with one of the five Jκ segments. When the gene is activated, RNA is transcribed and the intervining sequence (IVS) are removed by RNA splicing. The rearrangement that juxtapose single Vκ and Jκ segments results in a change in the location of a Bam HI restriction endonuclease site. This event alters the size of the restriction fragment that bears the Cκ gene segment and allows this allele to be distinguished from the germ-line configuration of the κ gene.

codes for the reamining portion of the leader sequence and most of the variable region (residues -3 to 95). The rest of the Vκ region chain is encoded by one of the five functional Jκ gene segments which are separated by 250 to 300 pb of non coding sequences (Fig 3)

Lambda light (Lλ) chains are presents in approximately 40 per cent of humans immunoglobulins molecules. The amino acid sequences of human lambda polipeptides indicate that humans also have multiple Vλ gene segemnts that can be divided into homology-based subgroups (3). The λ locus is present at the long arm of chromosome 22 at band 22q11 (14) and is organized in a slightly different fashion from the other immunoglobulin loci. It consist of a set of different Cλ genes, each one associated with its own upstream Jλ segment (16-18).

The assembly of L chain genes follows H chain gene rearrange, usually after production of a H chain polypeptide. This DNA recombination event joins one of the many germline variable regions with a particular joining (Jκ) region. If a Vκ and a Jκ gene segment are effectively joined in a cell already possessing an effective VDJ recombination a μ,κ surface immunoglobulin B cell results. Frecuently, however, both the maternal and paternal set of κ alleles aberrantly rearrange or are even deleted. If this occurs, λ gene rearrengements are initiated and, when effective, it results in a μ,λ bearing B cell. Cells that only have aberrant rearranged H or L gene loci are later eliminated.

1.3.C. Recombination Mechanism Involved in Joining the Segments of the Variable Region.

The mechanisms that leads to the joining of the variable region of the gene segments have not been completely elucidated. However, DNA sequences that appear to serve as signals for the joining of the V, D, and J segments have been identified (19, 20). These segments are flanked by conserved, usually palindromic heptamer and by

an AT-rich nonamer sequence, which are separated by nonconserved spacer DNA that may be either 12±1 or 23±1 nucleotides long (Fig 4). These spacer lenghts are approximately equivalent to either one or two turns of the DNA helix, which could suggest that the helical groves within recognition elements are aligned on the DNA molecule in the same rotational orientation (21, 22), although the spacer lenghts can vary and the alignment may not be exact. This heptanucleotide which is an inverted repeat or palindromic of CACAGTG or CACTGTG is found on the 3' side of each V segments, on the 5' side of each J segment, and on both sides of the Dh segment. Flanking each heptanucleotide of a V_H, J_H, $J\kappa$, and $V\lambda$ is a spacer of 22 or 23 nucleotides and then the corresponding to the nonamer element. This 9-bp segement is complementary to a nonanucleotide that is separated by 11 or 12 bases from the heptanucleotide flanking the germline D_H, $V\kappa$, and $J\lambda$ segment. The heptameric and nonameric sequences following a gene segment are complementary to those preceding the particular gene segment with which they combine. Furthermore, it appears that a heptanucleotide and a nonanucleotide possessing 12±1 bp spacer always pairs with a matched set of sequences containing the 23±1 bp spacer. According to this 12/23 rule, the joining of two D heavy chains or joining of a Vh and Jh would not be possible. The possibilties for recombination appear to be defined by these spacers; joining between two segments is apparently not permitted if their respective recognition elements are separed by spacers of the same length. The fact that the heptanucleotides, nonanucleotides and their spacers flank all of these immunoglobulin genes segments and are found in all species examined suggests that they are active participants in bringing V_L/J_L and $V_H/D/J_H$ regions together. One possible model for the mechanism of joining of two segments is that the two complementary heptanucleotides and the two complementary nonanucleotides would base pair, creating a stem and loop like structure that would facilitates recombination between the strands. The stem and loop structure would be deleted by enzimatic action.

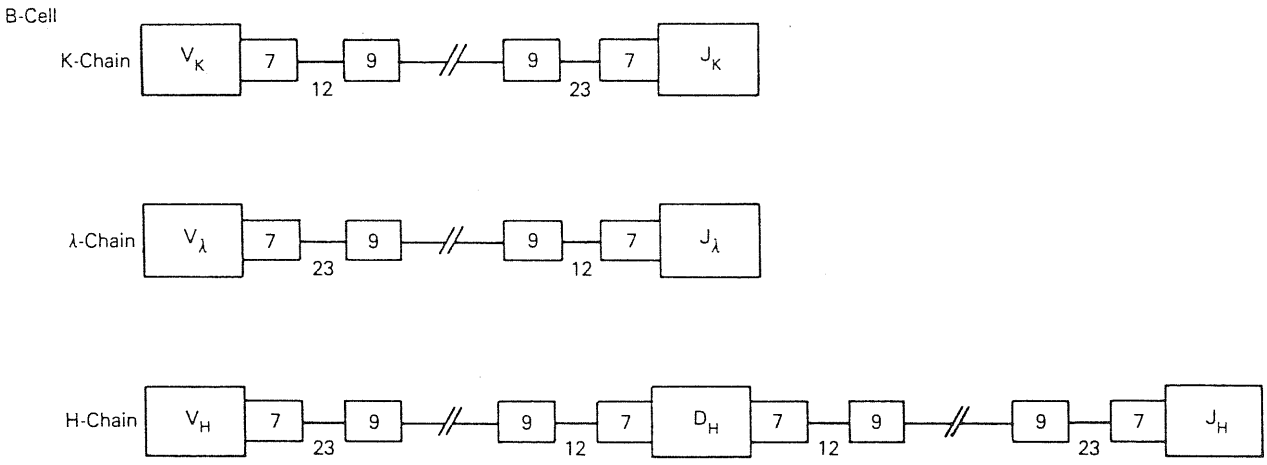

Figure 4. Immunoglobulin genes are flanked by conserved heptamer and nonamer sequences separated by nonconserved spacer of DNA that may be 12 or 23 nucleotides long. The heptamers and nonamers 3' to a segment are complementary to the nonamers and heptamers 5' of the segment. A heptanucleotide and nonanucleotide possessing an 11- or 12- spacer always pairs with a complementary set of secuences containing a 23±1 -bp spacer.

Recombination substrate experiments have begun to define the recombination sequence requirements of the recombinase. Joining between Ig V region gene segments is not dependent upon chromosomal location and little, if any, flanking sequence beyond the recognition elements is required (23-26). Whithin these substrates, segments that are flanked by distinctively different heptamers undergo rearrengement at aproximately the same frecuency, suggesting that the recombinase can probably recognize many differents sequences in the genome efficiently and that sequences other than recognition elements might influence the frecuency and specificity of the recombination (27).

Based on analysis of the products of many H and L chain gene rearrangement events and of particulary unusual rearrangement of D and J_H segments, a multi-step model was proposed for the recombination mechanism (28). In the first step, a double-stranded break occurs on both recombining partners, usually at the junction of the heptamer and coding sequences. The heptamers are then ligated precisely back-to-back, but nucleotides can be removed from the exposed ends of one or both coding regions. At this stage during assembly of H chain genes extra bases (referred to as "N regions") may be added "de novo" to the ends of the coding region by enzyme terminal deoxynucleotidyl transferase (TdT). This enzyme is present in the bone marrow and thymus and adds nucleotides to 3' ends, with preference for adding G residues (28) and qualitatively modifies the VDJ junction. At the next stage, the added bases are filled in and the two coding regions are joined. The heterogeneity in joining between the coding sequences further diversifies the amino acid sequence on the CDR3, and thus can generate different antibody specificities among Ig V regions encoded by the same V, (D), and J segments.

By analogy to other site-specific recombination systems, the VDJ reaction could be orchestrated by one or two specific gene products, which confer specificity on the reaction, while other events (for example ligation) might be effected by ubiquitous cellular activities which carry out their functions in the context of VDJ recombination (29). Recently, two genes have been identified that when expressed

simultaneuosly, are sufficient to generate VDJ recombinase activity in all cell types examined. (30, 31) These two genes were designed "recombinase activation genes" RAG-1 and RAG 2. It has not yet been proven unequivocally that these two genes encode the tissue-specific components of VDJ recombinase; it remains possible that one or both serve to regulate expression of the recombinase gene(s) or in some way activate the actual VDJ recombinase.

The Rag-1 and Rag-2 genes were found to be coexpressed at substantial levels in the nucleus only in primary lymphoid tissue and in cell lines that represent precursor lymphocytes (30, 31), consistent with the role either of trascriptional factors or as VDJ recombinase components. The Rag genes are conserved in vertebrates but no close homologous have been found in lower organisms. While the RAG-2 gene product has no clear homology to any other gene product, the Rag-1 protein does have limited homology to a yeast protein HPR-1 which has holomogy to topoisomerase I.

1.4. Somatic Mutation.

The antibodies present in a memory response have, on average, a higher affinity than those of the early primary response. This phenomenon, which is restricted to T-cell dependent responses, is referred to as the maturation of the immune response. This maturation requires that the antigen-binding sites of the antibody molecules in the matured response be structurally different from those present in the primary response. This alteration is brought about in two quite different ways. Firstly, there is a shift in the repertoire of germ line genes which are used in the primary response and the memory response. Many of the antibodies found in the secondary response are structuraly unrelated to those expressed at early stages (32). Secondly, it is now clear that the primary repertoire is also diversified by the process of V region hypermutation. The examination of the sequences of

monoclonal antibodies expressing a well-characterized germ-line V region gene suggets that the base changes that appear during the immune response are generated by a hypermutation mechanism. This hypothesis is supported by many studies in which sets of monoclonal antibodies are analyzed by organizing them into a genealogical tree (33, 34). The construction of such genealogical trees may be complicated by the fact that similar mutations occur repeatedly even in antibodies from different animals, an indication of "hot spots" for the hypermutation mechanism and selection (32-34) Nevertheless, the apparence of additional base changes in successive branches of a genealogical tree indicates that single base changes, or small groups of changes, occur sequentially during successive cell divisions.

Apparently random changes mainly in the coding regions are introduced into the H and L chain genes and occasionally one such change will lead to an increase in the affinity of the antibodies. These mutations also extend into the immediate 5' and 3' flanking regions but not into the C region genes (35, 36). It is these high-affinity variants which are then selected to enter in the pool of memory cells. In a T cell dependent response, activated B cells may develop either along a pathway leading to the formation of plasma cells, or along one which leads to memory cells. Although somatic mutation is observed most frecuently in B lymphocyte that have undergone H chain class-switching (37, 38) the mutations are sometimes present in cells that produce IgM antibodies (39). Various assays such as the analysis of the point mutation in many families of related monoclonal antibodies led to the calculations of mutation rates of about 10^{-3}-10^{-4} bp/cell/generation, that is far greater than the background rate, suggesting that they are generated by a specific hypermutation mechanism. A number of different mechanisms have been proposed. These include an error-prone repair process utilizing either a special enzymatic system, or perhaps an unusual modification or combination of the usual repair systems (40).

1.5. The Mechanisms of Antibody Diversity

Combined effects of different somatic and germline mechanisms contribute to the generation of an individual's complete repertoire of antibody specificities. First, there are multiple H and L chains gene segments that create substantial diversity due to their combinatorial joining. In addition, diversity is introduce at the junction of the variable VJ region gene in the L chain and at the VD and DJ segments in the H chain. This is referred to as junctional site diversity. This junctional site diversity arises in part because of the flexibility in the frame of recombination at the sites of junction of the different H and L chain segments. In addition, in the case of the H chain more diversity is introduce by the insertion of one to several (up to 12) nucleotides at both sides of the D gene in a template-independent fashion. Furthermore, a large number of antigen binding specificities is obtained by the combinatorial association of the different L and H chains to produce the complete antibody molecules. Finally, this repertoire of antibody molecules is expanded still further by the occurence of somatic hypermutation that results in changes of the amino acid sequences of the variable region. This last process is the only one of all the mechanisms that contribute to antibodies diversity that is driven by antigen. Taken into account the number of different gene segments and the processes of recombination and generation of diversity, it can be calculated that more than 10^9 different antibody molecules can be generated from a few hundred different genetic elements found in the embrionic DNA.

1.6. Class Switching.

As shown in figure 5 the heavy chain genes are in a cluster in a 200 Kb region 3' to the J sequences. The order of the human Ch genes is 5' Cμ–Cδ–Cγ3–Cγ1–

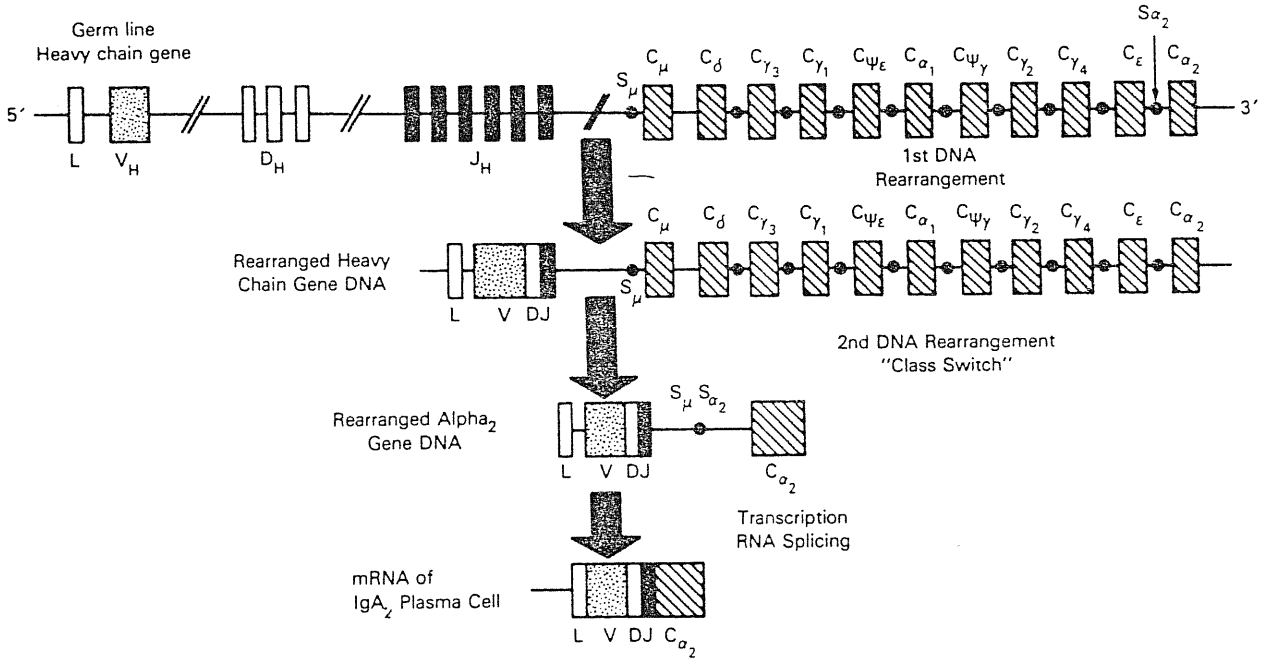

Figure 5. Schematic diagram of the human heavy chain gene locus showing the order of the constant region genes. Following the initial DNA rearrangement combining V_H , D, J_H , the B cell can alternate splice sites and produce IgM and IgD simultaneously . Alternatively, the B cell can switch to the production of other heavy chain isotype. For the class switch, a second DNA recombination occurs that involves highly homologous switch secuences (•) that are found 5' to each constant region with the exception of δ. In the example Sμ and Sα2 switch regions are used, leading to the formation of an active α2 transcript.

$C\psi\varepsilon$–$C\alpha1$–$C\psi\gamma$–$C\gamma2$–$C\gamma4$–$C\varepsilon$–$C\alpha2$. Unlike $C\kappa$, C_H genes are composed of multiple exons that correspon to the structural domains in the antibody molecule. During dofferentiation of a single B lymphocyte, a given V_H gene is first expressed in combination with the $C\mu$ gene of the same allelic chromosome, and later $C\mu$ and $C\delta$ are co-expressed. This phenomenon is called heavy chain class switch. The close proximity of the $C\mu$ and $C\delta$ regions allows for the simultaneus production of IgM and IgD, which bear the same assembled variable region (41). Nuclear RNA transcripts initiating from the recombinaned VDJ region undergo differential processing by alternative splicing, thus connecting either the $C\mu$ or the $C\delta$ constant region (42). Subsequently, following maturation of the immune response the same VDJ is found also expressed in combination with any of the other C_H isotype genes. Bud cells can differentiate and begin to synthesize other classes of antibodies. Such class switches occur by genetic recombination between switch specific DNA sequences [termed switch (S) regions] that lie 2 to 3 Kb 5' from each C_H gene with the exception of the $C\delta$ gene. These switch regions are large segments composed of multiple copies of short repeated elements. The repetitive nature of these switch regions may promote homologous recombination between the μ switch region and any of the further 3' switch regions. Such recombinations results in a DNA rearrangement that is accompained by deletion of the DNA between the u switch region ($S\mu$) gene and the switch region immediately 5' from the constant region to be used. This process of switching allows for creation of a new transcription unit containig the same original VDJ sequence joined to the new C_H recombined gene. During the immune response, the extent to which class switching to a particular H chain isotypes occurs can be influenced by the action of T lymphocytes. Class switching can be induced in vitro by mitogens or by stimulation with certain antigens which seems to mimic the apparent regulatory effect of T lymphocytes. A directed model for switching is supported by studies which have shown that many cell lines and normal B lymphocyte that have undergone S region recombination on both productive and non-productive alleles have switched to the same isotype on each chromosome (43-45). In the case of IgE

two signals are required for the switch to occur (46). The first one is provided by the cytokine IL-4, secreted by Th2 cells, which initiates germline transcription through the e locus (47, 48, 53, 54). The deletional recombination switch takes place after a second signal is delivered by TH_2 cells, or in vitro by EBV or the anti-CD40 monoclonal antibody (49-51). Interleukin 4 and TH_2 cells can also induce surface IgM positive human B cells to switch to IgG4 synthesis (52). B-cells that coexpress two different isotypes, such as IgM and IgE have also been identified (56, 57). It has been suggested that these cells represent a stage of B cell differentiation prior to switch recombination (59, 60). 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 (55).

1.7. Objectives

The low levels of IgE produced by peripheral blood mononuclear cells (PBMC) from normal individuals has so far prevented an analysis of their ε repertoire, while only recently, a few IgE antibodies from atopic individuals have been characterized. Investigation and characterization of the IgE repertoires of normal and atopic individuals could eventually lead to a better understanding of the pathophysiologic mechanisms underlying allergy. To that purpose in this work the following objectives were followed:

- to develop a sensitive procedure for analyzing ε transcripts from both normal and atopic individuals,
- to obtain sequence data which would allow determination of V_H, D and J_H gene segment usage and eventually the determination of the level of somatic mutation,
- to investigate the possible usage of the ε repertoire in other heavy chain (h.c.) isotypes.

1.8. Summary

We have investigate ε transcripts expressed in vivo by PBMC to obtain further insight in the mechanism regulating IgE production. Using a nested PCR 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 PB 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 γ4 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. We give also evidence that the IgE producing B cells undergo somatic mutation since 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 existance of sequence specific hot spots for the somatic hypermutation machinery in the JH gene segments.

2. MATERIALS AND METHODS

2.1. Clinical samples and RNA preparation

PBMC were obtained from 3 atopic and 7 normal white Caucasian adults and from a pool of white blood cells from 5 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 enviromental allergens (grade 3 for Lollum 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 enviromental allergens with grade 4 response to Lollum 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 (61).

2.2. Oligonucleotide primers and probes

Oligonucleotides were synthesized by the solid phase triester methodology on a DNA synthesizer (380A; Applied Biosystems, Inc., Forster City, CA). Unless otherwise indicated, the oligonucleotides were synthesized according to sequence data from ref. 4. 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 variable regions of the h.c. genes, oligonucleotides E1 5' GAAGACGGATGGGCTCTGTG 3' (codons 116 to 122), E2 5' TAAGGTCATAGTTGTCCCGT 3' (codons 167 to 173) and E3 5' GAGGTGGCATTGGAGGGAATGT 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' GGTCATTACGATTTTTGG 3', TB2 5' CCGTCCTGGTTCGACC 3', and TB3 5' TTTGGAGTGCATATTATAAG 3'. Constant h.c. specific oligonucleotides used for analysis of μ and γ4 transcripts were: μ1 5' GTCCTGTGCGAGGCAGCCAA 3' (codons 139 to 145) and μ2 5' ATCCGACGGGGAATTCTCAT 3' (codons 127 to 133) from the first exon of the constant μ gene, γ4.1 5' TGATGGGCATGGGGGACCAT 3' (codons 229 to 241) from the hinge region of the constant γ4 gene and γ4.2 5' GTTGCAGGTGTAGGTCTTC 3' (codons 200 to 209) from the first exon of the γ4 gene. The sequence of the 5'γ4 primer located from nucleotide -35 to -15 upstream of the γ4 CH1 exon was: 5' CACACCGCGGTCACATGGCA 3'.

2.3. Reverse transcription and PCR analysis of ε CDR3/FW4 regions

Reverse transcriptions were performed on total cellular RNA from PBMC (2 to 6 μg) or from the U266 cell line (1 μg 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 100μl reaction mixtures containing each dNTP at 200μM, 300 ng of each PCR primer, 2.5 units 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 (62) with ^{32}P γATP (Amersham) and Polynucleotide Kinase (New England Biolabs). 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 sec. 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 µl 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^{o}C 1 min, 64^{o}C 1 min, and 72^{o}C 1 min, for 30 cycles. After one chloroform extraction to eliminate the mineral oil, 2 µl 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 hours. Negative control reactions containing no cDNA were always included. Sequencing reactions were used as size markers.

2.4. 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 polyacrilamide gel, and the bands were eluted by the "crush and soak procedure" (62). The amplified DNA fragments were precipitated, reamplified for 15 cycles with the same primers, and purified by electroelution from 1.2% agarose gels (62). The recovered DNA fragments were ligated in the Sma I site of pUC18 (Pharmacia LKB, Uppsala, Sw) and used to transform E. coli strain DH5a. Clones were picked randomly, and double-stranded DNA template was prepared and sequenced by the dideoxy chain termination method (63) using the T7 Sequencing Kit (Pharmacia LKB). Sequences were identified by comparison to reported germline D and JH gene segment sequences (10,13, 4,64).

2.5. Analysis of μ and γ4 transcripts

Total cellular RNA (1μg) from individual A3 was reverse transcribed with the oligonucleotide μ1 or γ4.1. First PCR was performed on the total cDNA sample with the TB1/μ1 or the TB1/γ4.1 primer pair, followed by a second nested PCR reaction with the ^{32}P labeled oligonucleotide TB3 and the corresponding μ2 or γ4.2 primer for amplification of μ or γ4 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 5xSSPE/5xDenhardt's/0.5% SDS. The nylon filters were then washed once at room temperature for 20 minutes in 2xSSPE/0.1% SDS, and once at 54°C for 10 minutes in 5xSSPE and 0.5% SDS. Positive clones were picked up and sequenced.

2.6. Cloning and sequencing of the polymorphic Cγ4b gene

DNA was isolated from white blood cells of individual A3 using standard methodology (62). PCR amplification was performed with the 5'γ4 primer (located 35 nt upstream of the γ4 CH1 exon) and the γ4.1 primer (from the hinge region) on 1 μg 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 sec. The obtained PCR fragment was eluted, cloned and sequenced as already described. A total of 6 clones were sequenced using the 5'γ4 and the γ4.2 primers. The nucleotide sequence of the polymorphic γ4 CH1 exon has been submitted to GenBank and assigned the accession number L13487.

3. RESULTS

3.1. 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 7 normal individuals, 3 atopic individuals, a pool of PBMC from 5 different individuals, and from the IgE secreting myeloma cell line U266 using a FW1 primer (V1) and a primer from the first exon of the Cε 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.c. 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, since sequence comparison of our degenarate FW1 primer with a number of published variable gene segments showed either perfect homology or one to three mismatches with members of all VH families except the VH2 (65). 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 (66) and which can be amplified even at low concentration (see below, fig. 6d). Also, the same primer had previously succesfully amplified the complete variable regions of μ transcripts in 12 cases with chronic lymphocytic leukemia (D.E. and O.B., 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 Cε 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 Cγ genes). The radiolabeled PCR fragments were separated on a polyacrylamide sequencing gel and a codon ladder of bands differing in size by 3 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, while bands of much stronger intensity were present at every possible position in the sample amplified with the γ specific primer (fig.6b). Altough each individual had a particular pattern of

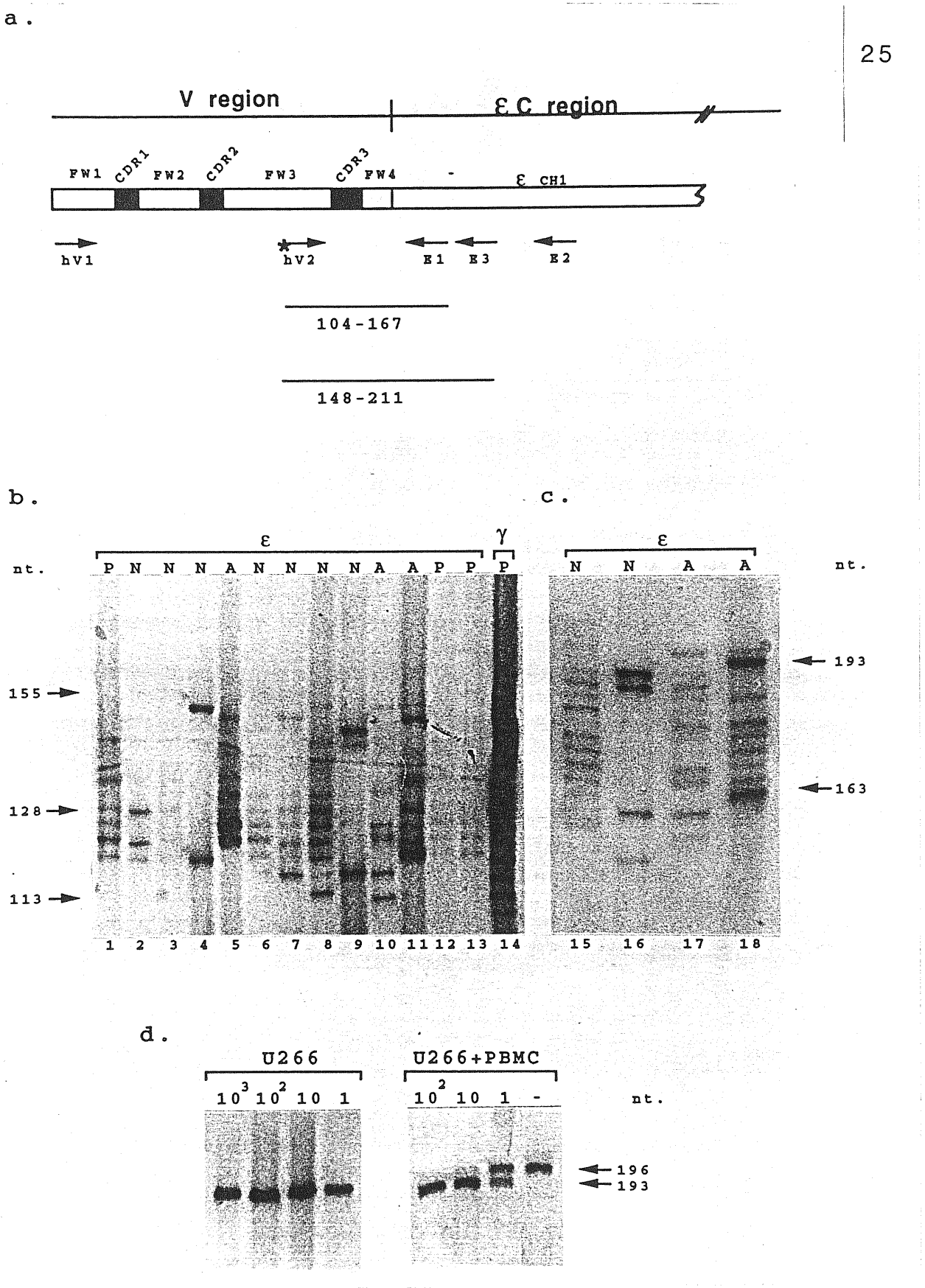

Figure 6: Analysis by RT/PCR of the ε CDR3/FW4 regions. a) Schematic representation of the positions of the different oligonucleotide primers. The size ranges of the expected fragments corresponding to the ε CDR3/FW4 sequences are indicated. b) Total PBMC cellular RNA from normal (N, lanes 2, 3, 4, 6, 7, 8, 9), and allergic (A, lanes 5, 10,11) individuals, and from a pool of white blood cells from five different individuals (P, lanes 1, 12, 13,14) was reverse transcribed with primer E2 (ε, lanes 1-13) or primer G1 (γ, lane 14). The CDR3/FW4 regions of ε chain cDNAs 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 cDNAs 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 fig 1b.) were reamplified with primers ^{32}P-hV2 and E3 (lanes 15, 16, 17 and 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 3x10^5 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 transcrits, respectively. b), c), and d) are autoradiograms of denaturing polyacrylamide gels in which sequencing reactions were included as size markers.

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.c. mRNA, the first PCR samples were reamplified with another ε specific primer (E3) which is located 44 nt downstream of the E1 oligonucleotide (fig.6c). 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 $2x10^6$ 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 $3x10^5$ PBMC of one nonatopic individual who showed a pattern consisting of a single band of 196 nt (fig.6d). 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.

3.2. 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.6b, 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.7). 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).

N1

	_FW3_	CDR3 / NDN		CDR3 / JH	FW4	_εCH1_	JH Gene	Size nt.	Name
(2)	tgcgaga	DXP1	ggtccgtactacggtattttgactggccgacatcct	tactactactactacggtatggacgtctggggccaagggaccacggtcaccgtctcctca		gcctcca	JH6	155	N1/a
(12)	tgcaaaa	DXP'1 / DA1	gacattggaggacggggtacggtgactac	gactactggggccagggaaccctggtcaccgtctcctca		gcctcca	JH4	128	N1/b
(12)	tgcgaga	DLR2	gcccgtgggagctgctacgt	ctttgactactggggccaaggagccctggtcaccgtctcctca		gcctcca	JH4	122	N1/c
(6)	tgcgaga	DK1	gtaccgcgctacagttac	ttcgacccctggggccagggaaccctggtcaccgtctcctcc		gcctcca	JH5	119	N1/d

N6

	_FW3_	CDR3 / NDN		CDR3 / JH	FW4	_εCH1_	JH Gene	Size nt.	Name
(1)	tgccctg	DK4	agctttttgcgtggatacagctatgrttaccgagacggc	tactttgactactggggccagggaaccctggtcaccgtctcctca		gcctcca	JH4	143	N6/a
(1)	tgcaaag	DN4	agcccctatagcaactcgttgc	ctgaaaacttccgccactggggccagggcaccctggtcarcgtctcgtca		gcctcca	JH1	131	N6/b
(1)	tagtaga	DK1	cataattcttatggtgactacgatcgtcct	tttgactactggggccagggcacccttgtcaccgtctcctca		gcctcca	JH4		N6/c
(1)	tgcgaga	DN4	actactatagcagtggaag	ggttcgacccctggggccagggaaccctggtcaccgtctcctca		gcctcca	JH5	122	N6/d
(1)	tgccagg	DN1	ggggacrgcagctggtgcc	actttgaatactggggccagggaaccctggtcarcgtctcctca		gcctcca	JH4		N6/e

Figure 7: Nucleotide sequences of PCR amplified ε CDR3/FW4 and adjacent regions obtained from two normal (N1 and N6) and two atopic (A1 and A3, in the next page) 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 parenthesis indicate the number of clones of each partricular transcript sequenced. The names of the germline D and JH genes with maximum homology to the reported sequences are indicated. Sizes correspond to the length in nucleotides of the bands in fig.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.

A1

	_FW3_	CDR3 (NDN)	CDR3 (JH)	FW4	_ECH1_	JH Gene	Size nt.	Name
			DK5 / DK4					
(1)	tgcgagg	gatcggagtctatgtgtatgacagtagtggtt	ctcaatacttccaacactggggccagggcaccgtggtaatcgtctcctcc	gcctcca	JH1	140	A1/
(1)	-------	------------------------------	--g-----	-------			
(1)	-------	------------------------------	----------------a----------------------------	-------			
			DA1					
(2)	tgcgaga	gatccaggactacggcgaa	actactacggtatggacgtctggggccaagggaccacggtcaccgtctcctca	gcctcca	JH6	131	A1/
(1)	-------	-------------------	--------------------a----------------------------	-------			
		DIR*					
(4)	tgcgaga	tctgggacaacccccgggtactatgg	gatatctggggccaagggacaatggtcaccgtctcttca	gcctcca	JH3	125	A1/
(1)	-c-----	--------------------------	-----t----------------------------------	-------			
(1)	-------	----------a----------	--	-------			
		DN1					
(2)	tgcgaga	aacacctatgccggcagcttcaa	tgactactggggccagggaaccctggtcaccgtctcctca	gcctcca	JH4		A1/
		DXP1				122	
(2)	tgcgcgt	ggatctggtattttgactggcc	ttgactactggggccagggaaccctggtcaccgtctcctca	gcctcca	JH4		A1/

* DIR like sequence.

A3

	_FW3_	CDR3 (NDN)	CDR3 (JH)	FW4	_ECH1_	JH Gene	Size nt.	Nam
		DHQ52-C1 / DXP3						
(1)	tgcgaga	gatcataatccccagcattctatgctagtagtgggtatttcccc	tactttgattgctggggccagggaaccccggtcaccgtctcctca	gcctcca	JH4			A3,
		DXP4					149	
(1)	tgcgaga	ggtcattacgatttttggagtgccatattataagggccccccgtc	ctggttcgacccctggggccagggaaccctggtcaccgtctcctct	gcctcca	JH5			A3,
(1)	-------	----------	----------c----------a	-------				
(3)	-------	----------	----------c---t----------a	-------				
(7)	-------	----------	----------c---------t---------a	-------				
(1)	-------	----------	----------c---------t-a-------a	-------				
(1)	-------	----------t	----------c---------t---------a	-------				
(1)	-------	----------	----------t-----c---------t---------a	-------				
(1)	-------	----------	----------c----------g---a	-------				
(4)	-------	----------	----------c---------t-----g---a	-------				
(1)	-------	----------	----------a--g--aa----------t--a	-------				
		DXP'1 / DXP'1.						
(2)	tgcgaga	gttagtcgagggagctctggtgcgtcgggtag	ttttgacatctggggccaagggacagtggtcaccgtctcttca	gcctcca	JH3			A3
		DXP4						
(2)	tgcaaac	ctaccacatgattacgatctttggagtggttattatatt	cagtggggccagggaatccccggtcaccgtctcctca	gcctcca	JH1	134		A3,
		DA4						
(2)	tgcgagg	gctgaggccacatacaataaccc	gctgaatacttccaacactggggccagggcaccctggtcaccgtctccgca	gcctcca	JH1			A3
		DN1						
(1)	tgcgaga	agtattacagcacctggcactgcc	tacttcgaccctggggccagggaaccctggtcaccgtctcctca	gcctcca	JH4			A3
(1)	-------	----------a--------	----------------------------------t------------	-------				
		DL4 / DM2					128	
(2)	tgcgagg	gagttagtcgtctatccgaaccat	gatatggacgtctggggccagggactacggtcaccgtctcctca	gcctcca	JH6			A3
		DA1						
(3)	tgcgaga	gcaatggacggcagtgccgat	gcttttgatatctggggccaagggacaatggtcaccgtctcttca	gcctcca	JH3			A3
		DXP'1					125	
(1)	tgcgaga	tctgggacaacccccgggtactatgg	gatatctggggccaagggacaatggtcaccgtctcctca	gcctcca	JH3			A3
		DN1						
(1)	tgcgcga	gcaggggcagcagctgat	gcttatgatctctggggccaagggacaatggtcaccgtctcttca	gcctcca	JH3	122		A3
(7)	-------	----------	--c---	-------				
(1)	----a--	----------	--c---	-------				
		DM1*						
(5)	tgcgcga	accggtagagcaggacc	gttcgacccctggggccagggaaccctggtcaccgtctcctca	gcctcca	JH5			A3
		DXP1**					119	
(1)	tgcgaga	gataacagtattgat	gcttttgatatctggggccaagggataatggtcaccgtctcttca	gcctcca	JH3			A3

* Other possible DH genes: DM2, DIR1
** Other possible DH genes: DA4, DLR5, DK4

3.3. 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. 7). However, it is possible that some of the assignments are imprecise due to 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 (67,68,69). 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 %).

3.4. The ε Fw4 regions show evidence of somatic mutation

Fifty four substitutions with respect to germline 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 rate of the Taq polymerase (70) 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). Since polymorphic JH genes have been reported (JH3 a and b, JH4 a and b, JH5 a and b, and JH6 a, b and c)(67), we cannot exclude that some of the sequence variation is due to 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.7), which for reasons discussed below cannot be atributed 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

a.

b.

```
          _________ CDR3 _____________________________/____________ FW4 _________________/
(4)  tttggagtgcatattataagggccccccgtcctggttcgacccc tggggccagggcaccctggtcaccgtctcctca
(3)  ---------------------------------------- ----------------------t----------

          ___________________ μ CH1 ____________________________________________.
     gggagtgcatccgccccaaccctttttccccctcgtctcctatgagaattccccgtcggat.......
```

c.

```
          _________ CDR3 _____________________________/____________ FW4 _________________/
(2)  tttggagtgcatattataagggccccccgtcctggttcgacccc tggggccagggaaccctggtcaccgtctcctca
(1)  ----------------------------------------a- ----------------------t-----------
(1)  ----------------------------------------ta- --------------------------------

          ___________________ γ4 CH1 ____________________________________________.
     gcctccaccaagggcccatcggtcttcccccctggcgccctgctccaggagcacctccgagagcacagcggccctgggct
     ____________________________________________________________________________.
     gcctggtcaaggactacttccccgaaccggtgacggtgtcgtggaactcaggcgccctgaccagcggcgtgcacacctt
     ____________________________________________________________________________.
     cccggctgtcctacagtcctcaggactctactccctcagcagcgtggtgaccgtgccctccagcagcttgggcacgaag
     _____________.
     acctacacctgcaac
```

Figure 8: Detection of the A3/b CDR3/FW4 regions in μ (lane 1) and γ4 (lane 2) transcripts. a) The precise sizes of th radiolabelled PCR products obtained with primers ^{32}P-TB3/μ2 (lane 1) and ^{32}P-TB3/γ4.2 (lane 2) were determined with sequencing ladder. b) and c) Nucleotide sequences corresponding to the A3/b μ (b) and A3/b γ4 (c) transcripts. The CDR3 FW4, and the μ and γ4b CH1 exons are indicated above the sequences. Underlined nucleotides in the CDR3 and FW correspond to differences with the JH5b sequence. Dashed lines below the sequence represent identical nucleotides. Th sequence of the CH1 exon of the polymorphic γ4b gene is presented with the three silent substitutions underlined. I parenthesis are indicated the number of clones sequenced.

predicted value of 1.5 for FW mutations (71), since the expected R/S ratio of 2.9 for a randomly mutated sequence would mean introduction of deleterious mutations which 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 since 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.

3.5. 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.7), ten 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 due to 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 sequenced 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 (data not shown); fourth, in these same long amplified fragments no substitutions with respect to the germline sequence of the constant region were present; and fifth, clonally related transcripts containing the same CDR3/FW4 sequences were also identified in the μ heavy chain mRNA (see below).

3.6. The A3/b CDR3/FW4 regions are present in μ and γ4 mRNAs

With the aim of studying whether the same CDR3/FW4 sequences of the IgE heavy chains were also present in other Ig isotypes we performed radioactive PCRs with A3/b specific oligonucleotides coupled with primers specific for μ and γ4 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.8a). To confirm these results we constructed from individual A3 μ specific and γ4 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 μ2 and γ4.2, respectively. Sequence analysis identified two clonally related μ heavy chain transcripts that contained identical CDR3/FW4 regions as the A3/b ε transcripts (Fig.8b). In the case of the γ4 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 germline configuration, while the other two contained two nucleotide substitutions each, which were all different from the ones present in the ε and μ transcripts (Fig.8c). These γ4 transcripts also differed among themselves by 1 to 3 nucleotides in the JH segment. The sequence of the first exon of the constant h.c. region was identical in all three γ4 transcripts, but contained three substitutions with respect to the reported sequence of the γ4 constant region gene (4). To investigate the origin of these substitutions we cloned and sequenced the CH1 exons of the γ4 genes of individual A3. Besides the already published Cγ4 sequence we also identified a polymorphic Cγ4 gene (further referred as Cγ4b) containing 4 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 γ4 transcripts shown in fig. 8c, while the fourth one is absent since it corresponds to the 5' end nucleotide of the primer γ4.2.

4. DISCUSSION:

Despite a significant biological effect of IgE as a trigger of allergic reactions, its serum levels are 3 to 4 orders of magnitude lower than the other Ig families. Immunoglobulin E is produced by a correspondingly low number of peripheral blood B lymphocytes, estimated over a wide range from 1/1000-1/100000 lymphocytes in normal individuals (72,73,74,75). Elevated levels are frequently encountered in atopic individuals and during certain parasitic infections. The low levels of immunoglobulin E production cause considerable difficulties in analyzing the ε chain variable 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 (76). 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, since the RNA content from 10 U266 cells almost completely competed out amplification of ε transcripts produced by $3x10^5$ PBMC. However, biased amplification of the U266 or PBMC transcripts due to 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 polyacrilamide 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 (67,68,69).

We give evidence that the IgE producing B cell clones undergo somatic mutation, since most of the ε transcripts contained nucleotide substitutions with respect to the germline 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 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.7, N1/b and N1/c). While it is possible that this finding is purely coincidential, it might also be a consequence of frequent antigen stimulation resulting in repeated rounds of somatic mutation in antigen 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 (52). A significant percentage of these cells produced combinations of two or all three immunoglobulin 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 Sμ-Sγ-Sε deletional recombination (51). However, other investigators have reported germ-line configuration of the Cε genes in IgM/IgE double isotype producing clones established by EBV transformation of surface IgM/IgD positive -surface IgE negative clones, cultivated in the abscence of exogeneously added IL-4 (55). These results suggest that other mechanisms such as trans-splicing between germ-line CH transcripts and VDJ-encoding RNA (77) or alternative splicing of a long RNA transcript encoding one VDJ and multiple CH genes in germ-line configuration (58) 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 PB B lymphocytes. To that purpose we searched for the presence of the A3/b ε CDR3/FW4 regions in μ and γ4 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 γ4 transcripts. While the obtained A3/b μ sequences were completely identical with the ε transcripts, the 3 identified γ4 transcripts contained different

mutations in the JH segment, and also 3 mutations in the CH1 exon as compared to the published Cγ4 gene sequence (fig.8,b and c). Cloning and sequencing of genomic DNA from this individual revealed a novel Cγ4 gene (Cγ4b) containing 4 silent polymorphisms in the CH1 exon.

The above described results could best fit in a model where 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, while the second would be composed of IgM/IgE double isotype producing cells. Since we could only identify secreted form A3/b ε transcripts (data not shown) , 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.B.,D.E., and O.B., unpublished observations). The observed presence of clonally related ε and γ4 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 (78). 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 likelyhood for an allergic reaction upon subsequent contact with allergen. Our inability to detect γ4 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 germline 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.7). Comparison with other

published sequences revealed that the same mutation was present in two of 19 ε VH5 transcripts (76) and also among the clonally related transcripts of two follicular lymphoma cases (79,80). Further, the substitutions at positions -1, -6, and -22 were also found in 4, 2, and 3 of the other analyzed transcripts, respectively. These mutations can not be accounted for by Taq polymerase error since 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 existance of "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 (76). Further evidence for the existance of sequence specific hot spots comes from a very recent study of passenger κ transgenes in which the somatic mutations were not selected by antigen and still preferentially occured in particular positions of the κ chain V regions (81).

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 antigen specificity, affinity and expression of functional immunoglobulin. However, while negative selection against deleterious mutations could acount for the low R/S ratio (0.62) observed in the FW4 regions, the almost complete abscence of mutations, including silent, in the CDR3 regions (only 1 of 13) which were almost twice the size of the FW4s, requires additional considerations. Studies of a large number of malignant antibody forming cells and hybridomas (82) have suggested that the somatic mutation process is actively turned off in the V regions during B cell differentiation, while continues to introduce changes, altough at a much lower rate, in the constant 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 FW4s. Decreased accesibility of the CDR3 regions to the somatic hypermutation mechanism could prevent introduction of mutations that could alter the antigen 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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