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Molecular Analysis and Expression of Human IgE Transcripts

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Dedicated to Fabiola, Manuel, Mariana, Nestor, Ruth, and Soledad.

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List of abbreviations

BSA	bovine serum albumin
C	constant region of immunoglobulin
cDNA	complementary DNA
CDR	complementarity determining region
cpm	counts per minute
dATP	deoxy adenosine triphosphate
ddATP	dideoxy adenosine triphosphate
dCTP	deoxy cytosine triphosphate
D	region, diversity region of Ig
DMEM	Dulbecco's modified Eagle's medium
DNA	deoxyribonucleic acid
EBV	Epstein-Barr virus
EDTA	ethylene diamine tetra acetate disodium salt
ER	endoplasmatic reticulum
FCS	fetal calf serum
FW	framework
g	gram
h	hour
H-	immunoglobulin heavy chain
HPLC	high performance liquid chromatography
Ig	immunoglobulin
J	region, joining region of immunoglobulin
kb	kilobase
kDa	kilodalton
L-	immunoglobulin light chain
mRNA	messenger ribonucleic acid
MW	molecular weight
NIP	4-hydroxy-5-iodo-3-nitrophenacetyl
NP-40	Nonidet P-40
nt	nucleotide
PBL	peripheral blood lymphocyte
PBMC	peripheral blood mononuclear cells
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCR(s)	polymerase chain reaction(s)
poly (A)	polyadenylation
R/S	replacement/silent
RAST	Radio Allergoabsorbant Test
RNA	ribonucleic acid
RT	reverse transcription/transcribed
s	second
SCID	severe combined immunodeficient
SDS	sodium dodecyl sulphate
SSC	sodium saline citrate buffer
SSPE	sodium saline phosphate EDTA buffer
Tris	tris (hydroxymethyl)aminomethane
TBE	Tris borate EDTA buffer
TE	Tris EDTA buffer
UV	ultraviolet
V,	volt
V	region, variable region of Ig
vol	volumen
W	watt
WGA	wheat germ agglutinin

CHAPTER 1

INTRODUCTION

1.1 Immunoglobulin Structure and Function

The human immune system is capable of specifically recognizing and responding to an enormous number of antigens. The interaction of antigens with the cells involved in the immune response is mediated by two distinct antigen specific receptor molecules, the immunoglobulins and the T-cell receptor, expressed on the surface of B and T lymphocytes, respectively.

The immunoglobulin molecule is composed of two identical heavy (H) and two identical light (L) polypeptide chains linked together by disulfide bonds (Fig. 1.1). Both the H and the L-chains are organized in domains of approximately 110 aminoacids 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. In both the L and H-chains the amino-terminal ends constitute the so called variable (V) region domains. These V domains are not uniformly variable throughout their lengths: in particular, three small regions show greater variability; they are called hypervariable regions or complementarity determining regions 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 amino acid residues that make direct contact with the antigen [1, 2]. The three CDRs are separated by the so called frame-work regions (FW1, FW2, FW3 and FW4) that stabilize the structure of the β barrel [3].

The remaining domains constitute the constant regions (C) of the immunoglobulin and are identical for molecules of a given class. These regions determine the effector functions that are specific for a given class or subclass. The amino acid sequences within the constant

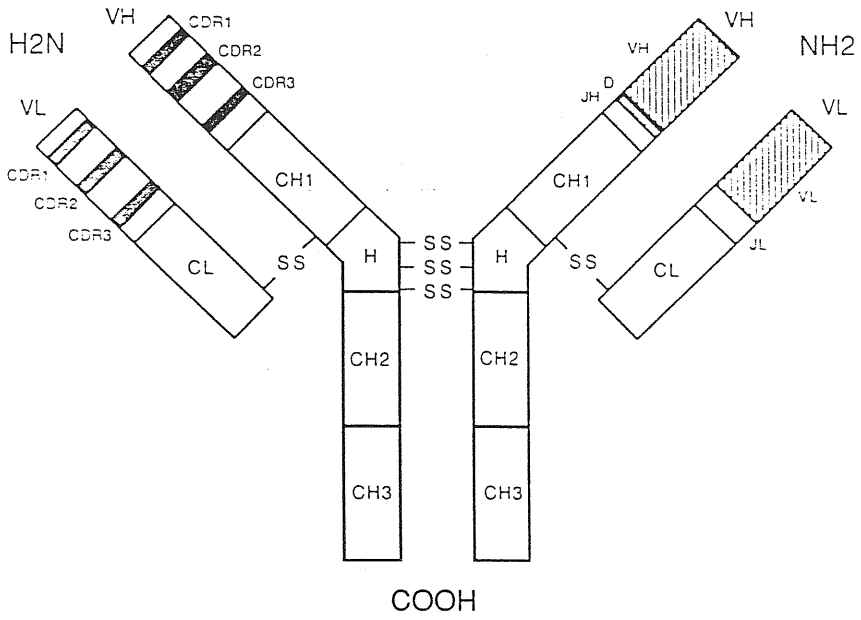


Figure 1.1: Structure of a IgG molecule. The V_H, C_H, V_L and C_L homology domains are shown as boxes and the hinge region is denoted H. Only the disulfide linkages H-H and H-L chains are shown. The aproximate boundaries of CDR regions and the sequences encoded by V_H, D, J_H, V_L and J_L are indicated by different shadings.

domains of the H and the L-chains are relatively conserved, although variations are found among the different isotypes that were originally classified according to their reactivity with specific antisera. In humans 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. Five types of H-chains exist which define the corresponding immunoglobulin classes IgM, IgD, IgG, IgA, and IgE, and which are designated by the Greek letters mu (μ), delta (δ), gamma (γ), alpha (α) and epsilon (ε), respectively. The IgG and IgA classes are further divided into subclasses defined by distinct CH region amino acid sequences. In humans the IgG class is divided into subclasses 1, 2, 3, and 4, whereas in the mouse these subclasses are defined as IgG1, IgG2a, IgG2b, and IgG3.

One of the most remarkable consequences of the amino acid variability between isotypes is the multimerization of immunoglobulins A and M. By contrast to the IgD, IgG, and IgE antibodies that generally consist of a single monomeric unit of two H and two L-chains (H2L2), circulating IgM antibodies are polymers consisting of five ((μ2L2)5-J) or six ((μ2L2)6) such units. The μ secreted (μs) tailpiece contains a cysteine residue at the penultimate position (cys 575) that can bridge to other μs chains or the glycoprotein J chain to form the pentameric secreted IgM molecule. This terminal cysteine residue has also been involved in the intracellular retention of unpolymerized intermediates [4]. IgM hexamers can be secreted by cells that lack the J chain [5] and by transfectoma cells expressing a μs gene construct with a mutated terminal cysteine [6, 7]. Like IgM, IgA also possesses a special C-terminal extension containing an extra cysteine that can cross-link monomer subunits together. As a late event in the synthesis, J chains initiate the polymerization reaction and become incorporated into the molecule. The major product from IgA secreting cells is dimeric IgA but small amounts of trimers, tetramers and pentamers are also secreted.

The antibodies of each class can be produced in two structurally different forms: membrane bound and secreted [8]. These two forms have the same rearranged VDJ segment,

but differ in their carboxyterminal aminoacids. The two forms are encoded by mRNA species that arise by alternative splicing of transcripts derived from a single H-chain gene [9-14]. It has been proposed that differential transcription termination and polyadenylation of the primary RNA transcripts determines which of the two mRNAs is expressed [12, 15]. Each immunoglobulin H-chain gene contains at least two polyadenylation sites. Usage of the promoter proximal poly A site, located immediately 3' to the stop codon of the last CH domain results in a mRNA encoding the secreted H-chain. If, however, transcriptional termination and polyadenylation occur at a site located several kilobases to the 3' of the first poly A site, a mRNA containing sequences from two additional exons (M1 and M2) is produced. These exons encode the hydrophobic transmembrane and hydrophilic intracytoplasmic carboxyterminal portions of the membrane H-chain. RNA processing events remove the first poly A site from the membrane mRNA by splicing from a conserved donor in the terminal CH exon (either the CH3 or CH4 exon depending on the H-chain isotype) to the M1 exon. Thus, it appears that in the case of immunoglobulin H-chain gene expression, transcriptional termination at alternative end sites determines the protein expression.

The constant regions of the different H-chain isotypes determine the effector functions of the immunoglobulins. The monomeric membrane form of IgM serves as an antigen-specific receptor for B cell activation which transmits an activation signal through the interaction of the μ constant region with accessory molecules [16-18]. The polyvalent nature of the soluble pentameric or hexameric IgM contributes to increase the avidity of these antibodies [6]. Secreted IgM is capable of initiating classical complement fixation. It can mediate phagocytosis indirectly by fixing C3b and can also be secreted at mucosal surfaces and in breast milk. The most well known function of IgG is complement activation via the classical cascade [19]. IgG1 is more efficient than IgG3 in complement-mediated hemolysis, while IgG2 and IgG4 are not able to mediate cytolysis. Another action of IgG entails its binding to antigen for destruction through phagocytosis by macrophages and granulocytes or by

antibody dependent cellular cytotoxicity (ADCC) mediated by lymphocytes and natural killer (NK) effector cells. These functions are mediated by Fcγ receptors (FcγR) present on the effector cells [20]. Signals transmitted by the FcγR not only stimulate antigen destruction but can also modulate immunity locally by regulating antigen presentation as well as lymphokine secretion and sensitivity of the effector cells. IgA in its dimeric form is the dominant immunoglobulin in secretions. Secreted IgA is the first line of defence against pathogenic invasion through mucosal surfaces since it coats all external tissues except skin. It may also play a role in neonatal immunity since it is present in calostrum milk. IgA posseses three effector properties: it fixes complement via the alternative pathway, it can serve as an opsonin for phagocytosis through a specific FcαR on macrophages, and it can also induce eosinophil degranulation through a specific receptor. Apart from its established role in mediating allergic reactions, IgE plays an important role in the antiparasitic response. The interaction of IgE with eosinophils and macrophages through specific receptors and the ability of these inflammatory cells to kill parasites in vitro indicate that IgE might have a function in the parasite attrition in vivo [21]. The function of IgD, either in its membrane-bound or soluble form, has not yet been established although its conservation in all mammals and the presence of the Cδ receptor suggests some role in immunity. Surface IgD, with accessory proteins like that of surface IgM, is a marker for mature B cells and its role as a receptor is generally accepted; however, the nature and purpose of the signal it transmits remains controversial [22].

1.2 Genetic of Immunoglobulin Genes

1.2a Human Heavy Chain Genes

The expression of a complete immunoglobulin molecule is the consequence of a DNA recombination event that takes place independently in each lymphocyte precursor and leads to the assembly of specific DNA segments in both the H and the L-chain loci.

The human H-chain locus is located on chromosome 14 at band 14q32.33 [23]. In this case the V region 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 diversity (D) and a joining (J_H) segment [24]. Each V_H gene segment contains two exons: the first one encodes a hydrophobic leader sequence that facilitates vectorial translocation of the molecule to the endoplasmic reticulum; while the second exon encodes the FW1, CDR1, FW2, CDR2 and FW3 amino acids of the V region H-chain. The D and J_H segments code for the third hypervariable region, CDR3, and the FW4 of the V domain (Fig. 1.2).

The number of different V_H gene segments belonging to this locus have originally been estimated by Berman et al [25] to be about 100-200 germline VH genes. Walter et al [26] identified 76 VH-genes by means of two dimensional DNA electrophoresis, while, Matsuda et al [27] counted 120 VH genes with a yeast artificial chromosome system. Seven of these genes are on chromosome 16 and therefore can not join to rearranged D-JH segments on chromosome 14. Recently, Tomlinson et al [28] determined the sequences of a large number of functional VH genes in a single individual: they found 83 segments with open reading frames, some of which may be duplicates. In the light of these results, it is likely that the human H-chains locus may contain as few as 60-70 functional VH genes. The VH segments have evolved by unequal crossing-over, conversion, duplication and deletion. They can be

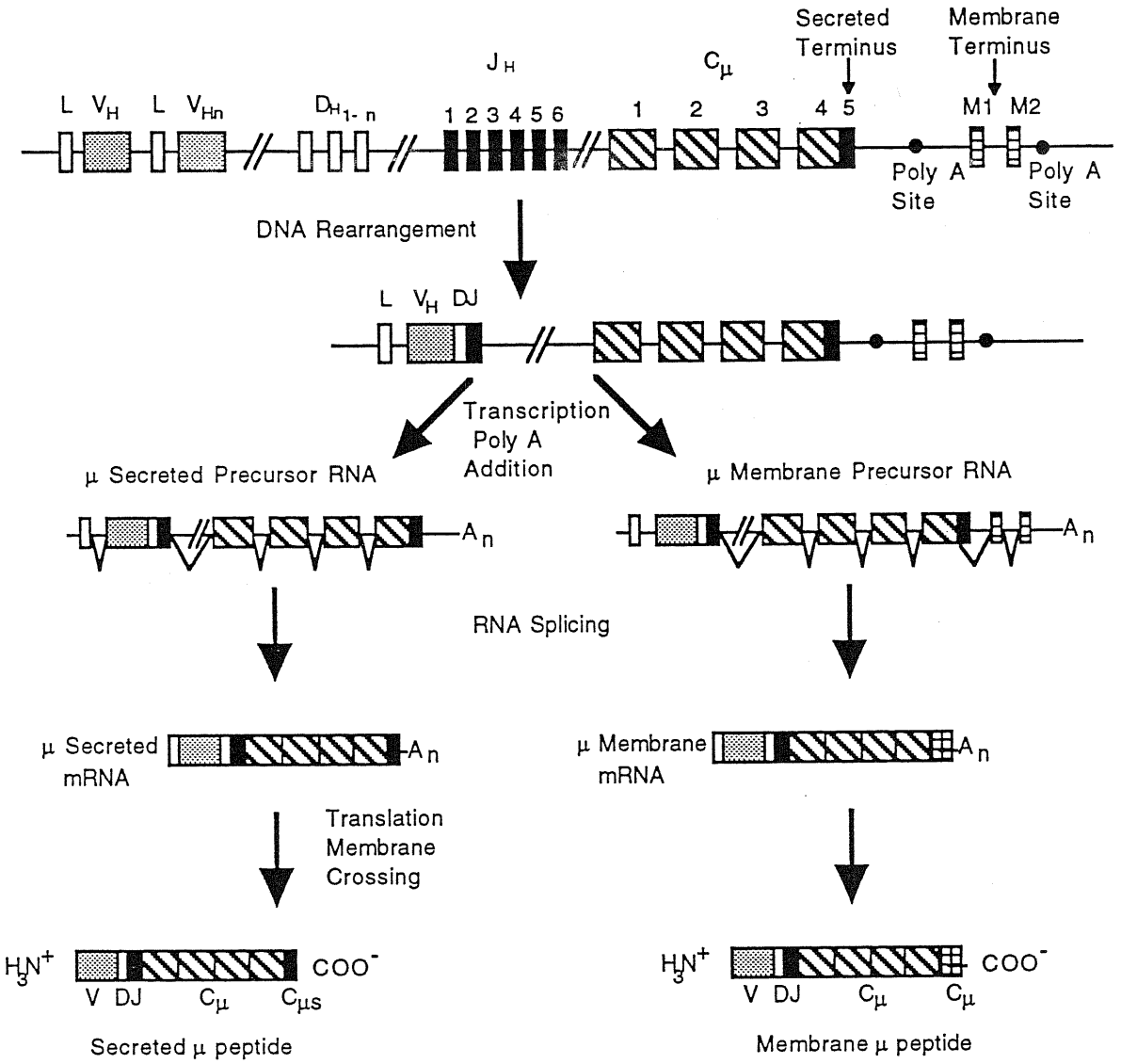


Figure 1.2: Schematic model of the organization of the human μ heavy chain gene. There are multiple V_H regions, each with a leader sequence, families of diversity (D) segments, six functional joining (J_H) and a single constant (Cμ) μ gene that is made up of a number of domains. Single V_H, D, and J_H 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.

divided into six families on the basis of nucleotide sequence homology of 80 % or more (25 VH1 segments, 5 VH2 segments, 28 VH3 segments, 14 VH4 segments, 3 VH5 segments, and 1 VH6) [28].

The number of D genes have been estimated by Southern blot analysis to be about 35. Most of these genes have been sequenced and based on sequence similarities they have been classified in 7 different families [29, 30].

In humans there are six active J_H gene segments which are located upstream of the CH genes. The H-chain C region genes are organized in 200 kb of DNA. Each C region is encoded by a separate exon cluster. The μ constant 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 results in the simultaneous expression of μ and δ H-chains that contain the same V region. During the maturation of the immune response, B lymphocytes can switch to the expression of different H-chain isotypes with 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).

The H-chain locus is the first to be rearranged. This process occurs in two steps. The first event is a D-to-J rearrangement, which almost invariably occurs on both H-chain alleles. Precursor B cells then undergo V-to-DJ rearrangement on one or both alleles, generating potentially functional VDJ H-chain genes. V-to-D rearrangement does not occur on alleles that have not first undergone D-to-J rearrangement [31, 32]. Since the V-to-DJ rearrangements are not precise, many of them generate "non productive" out-of-frame joined segments (VDJ^- alleles). When productive rearrangement of any one allele occurs, further changes in the second allele are inhibited (allelic exclusion), and the cells proceed to immunoglobulin L-chain gene rearrangement. That μm expression is essential for allelic exclusion has been established by elegant experiments with heterozygous mice carrying one disrupted μm allele which can encode μs but not μm and one wild-type allele [33]. The two

alleles are of different allotypes and their protein products can be distinguished by specific monoclonal antibodies. B cells develop in normal numbers in these heterozygous mice. However, a significant proportion (10%) of mature B cells in the bone marrow and periphery express membrane-bound IgM of the wild-type allotype as well as secretory IgM of the mutant allotype, showing that the mutant μm locus is incapable of signalling to prevent rearrangement of the wild-type allele.

In addition, recent results indicate that the organization of this locus, such as the relative localization and orientation of each segment, seems to affect the relative frequency of its usage and thus the diversity of the V_H repertoire. For instance, it has been shown that the V_H segments located proximaly to the J_H segments are more frequently used, especially during the early stages of ontogeny [34].

1.2b Human Light Chain Genes

In humans, kappa light (Lκ) chains comprise approximately 60 per cent of the human L-chain protein. The genes of this locus are located on chromosome 2 at band 2p13 [35]. The V protein domain is the consequence of a DNA rearrangement event which joins the Vκ and Jκ gene segments. The first 95 aminoacids of the Vκ region protein are encoded by one of the multiple Vκ gene segments (the total number is estimated to be around 75-80), divided in four families based on sequence homology. Similar to the V_H genes, 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 codes for the remaining portion of the leader sequence and most of the V 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 bp of non coding sequences (Fig 1.3)

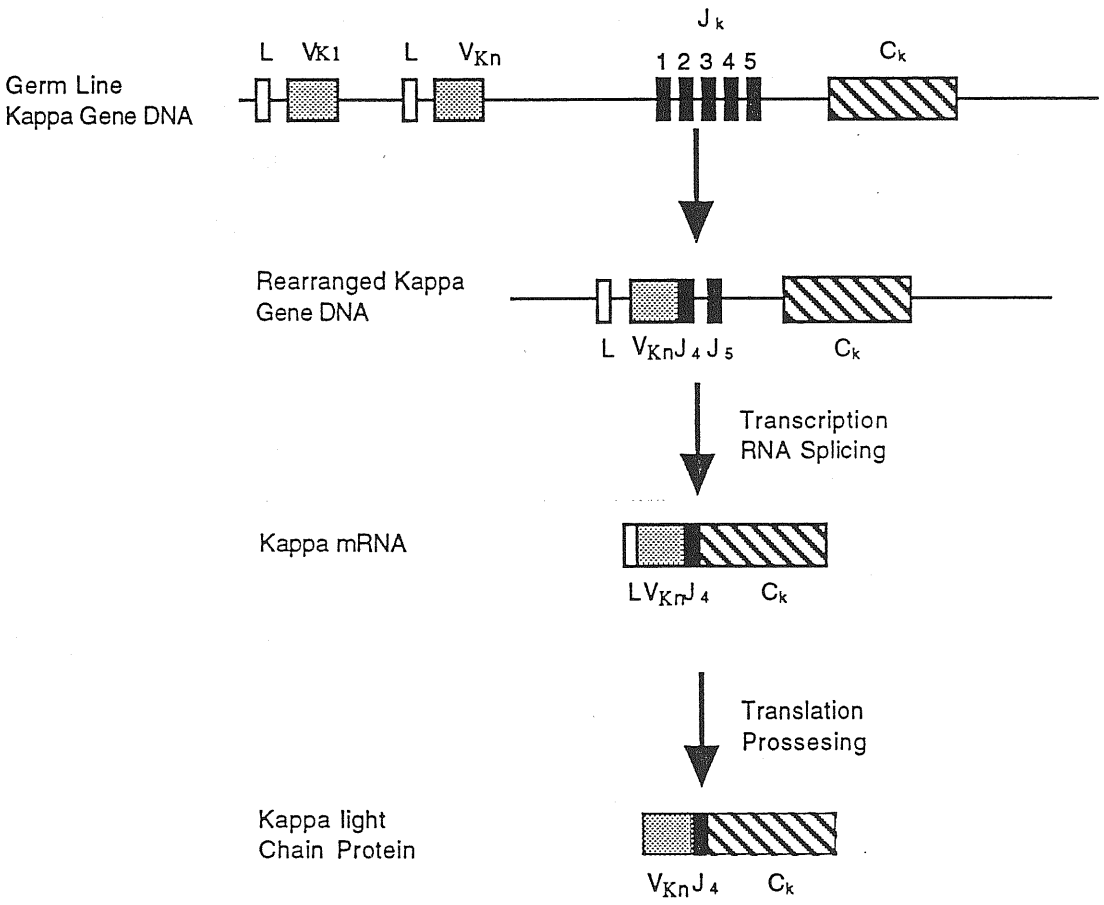


Figure 1.3: Arrangement of the human κ gene locus. Multiple variable (Vκ) regions each with an associated leader (L) sequence. There are five alternative joining (Jκ) segments encoding the remainding of the variable region. There is a single constant region per chromosome. DNA rearangements lead to the junction of a single Vκ with one of the five Jκ segments. When the gene is activated, RNA is transcribed and the intervining sequence are removed by RNA splicing.

Lambda light (Lλ) chains are present in approximately 40 per cent of human immunoglobulin molecules. The amino acid sequences of human lambda polypeptides indicate that humans also have multiple Vλ gene segments that can be divided into seven homology-based subgroups [36, 37]. The λ locus is present on the long arm of chromosome 22 at band 22q11 [35] and is organized in a slightly different fashion from the other immunoglobulin loci. It consists of a set of seven different Cλ genes, each one associated with its own upstream Jλ segment. Three of them are known to be pseudogenes, with either in-frame stop codons or frame-shifting deletions [38, 39]. They are clustered within a 33-Kb region of DNA that has been entirely sequenced [40]. In addition to the seven homologous Jλ-Cλ segments in the cluster, three additional homologous segments have been characterized [41].

The assembly of L-chain genes follows H-chain gene rearrangement, usually after production of a H-chain polypeptide. This DNA recombination event joins one of the many germline V 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 H-chain VDJ recombination, a μ,κ surface immunoglobulin B cell results. Frequently, however, both the maternal and paternal set of κ alleles rearrange aberrantly, or are deleted. If this occurs, λ gene rearrangements are initiated and, if effective, this results in a μ, λ bearing B cell. Cells that only have aberrantly rearranged H or L gene loci are later eliminated.

1.2c Recombination Mechanism Involved in Joining the Segments of the Variable Region

The mechanisms leading to the assembly of the gene segments encoding the V regions of the immunoglobulin molecules 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 [42, 43]. These segments are flanked by conserved recombination signal sequences which are composed of a palindromic heptamer and an AT-rich nonamer separated by a nonconserved spacer DNA segment that may be either 12±1 or 23±1 nucleotides long (Fig 1.4). These spacer lengths 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 [44, 45]. The heptanucleotide, which is an inverted repeat or palindrome of CACAGTG or CACTGTG, is found on the 3' side of each V segments, the 5' side of each J segment, and both sides of the D segments. Flanking each V_H, J_H, Jκ, and Vλ heptanucleotide is a spacer of 22 or 23 nucleotides and then the corresponding nonamer element. This 9-bp segment is complementary to a nonanucleotide that is 11 or 12 bases away from the heptanucleotide flanking the germline D, Vκ, and Jλ 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 with a 12±1 bp spacer always pair with a matched set of sequences containing the 23±1 bp spacer. According to this 12/23 rule, the joining of two D gene segments or joining of a V_H and J_H segment would not be possible. The possibilities for recombination appear to be defined by these spacers; joining between two segments is apparently not permitted if their respective recognition elements are separated by spacers of the same length. The fact that the heptanucleotides, nonanucleotides and their spacers flank all of the immunoglobulin gene 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 two segments is that the two complementary heptanucleotides and the two complementary nonanucleotides would base pair and create a stem-and-loop-like structure that would facilitate recombination between the strands. This stem-and-loop structure would be deleted by enzymatic action.

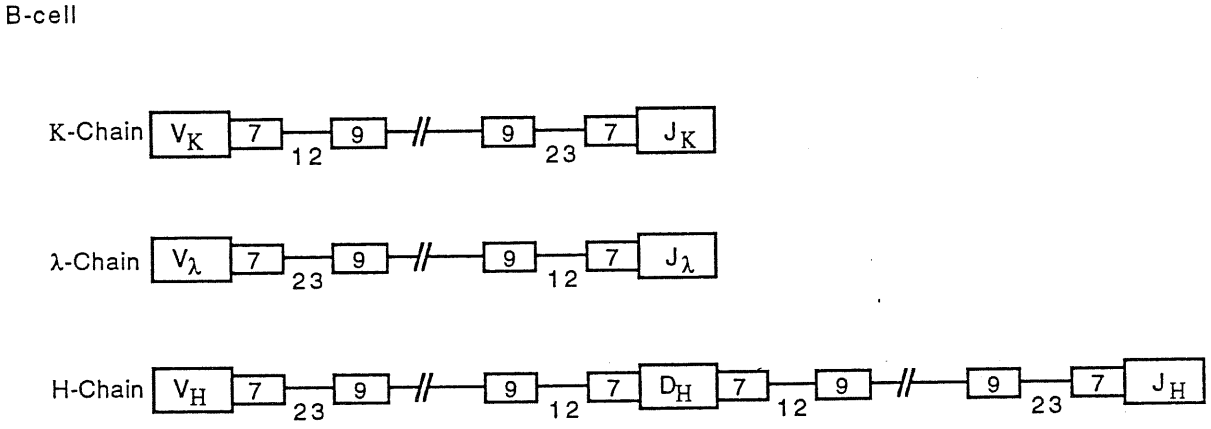


Figure 1.4: Immunoglobulin genes are flanked by conserved heptamer and nonamer sequences separated by nonconserved spacer of DNA that may be 12 or 23 nucleotides long. The heptamers and nonamers 3' to a segment are complementary to the nonamers and heptamers 5' of the segment. A heptanucleotide and nonanucleotide possessing an 11- or 12- spacer always pairs with a complementary set of secuences containing a 23±1 -bp spacer.

Recombination substrate experiments have been used to define the sequence requirements of the recombinase. These studies have shown that joining between immunoglobulin V region gene segments is not dependent upon chromosomal location and little, if any, flanking sequence beyond the recognition elements is required [46-49]. Whithin these substrates, segments that are flanked by distinctively different heptamers undergo rearrangement at approximately the same frequency, suggesting that the recombinase can probably recognize many different sequences in the genome efficiently and that sequences other than recognition elements might influence the frequency and specificity of the recombination [50].

Based on analysis of the products of many H and L-chain gene rearrangement events and of particularly unusual rearrangements of D and J_H segments, a multi-step model has been proposed for the recombination mechanism [51]. 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 the terminal deoxynucleotidyl transferase (TdT). This enzyme is present in the bone marrow and thymus and adds nucleotides to 3' ends, preferentially G residues [51], and 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 of the CDR3, and thus can generate different antibody specificities among immunoglobulin V regions encoded by the same V, D, and J segments (Fig. 1.5).

To unequivocally evaluate the role of TdT in V(D)J recombination and repertoire development, gene-targeting was used to generate chimeric mice in which all mature lymphocytes develop from precursors lacking TdT expression [52, 53]. The inability to express TdT had no gross effect on lymphocyte differentiation suggesting that this enzyme is

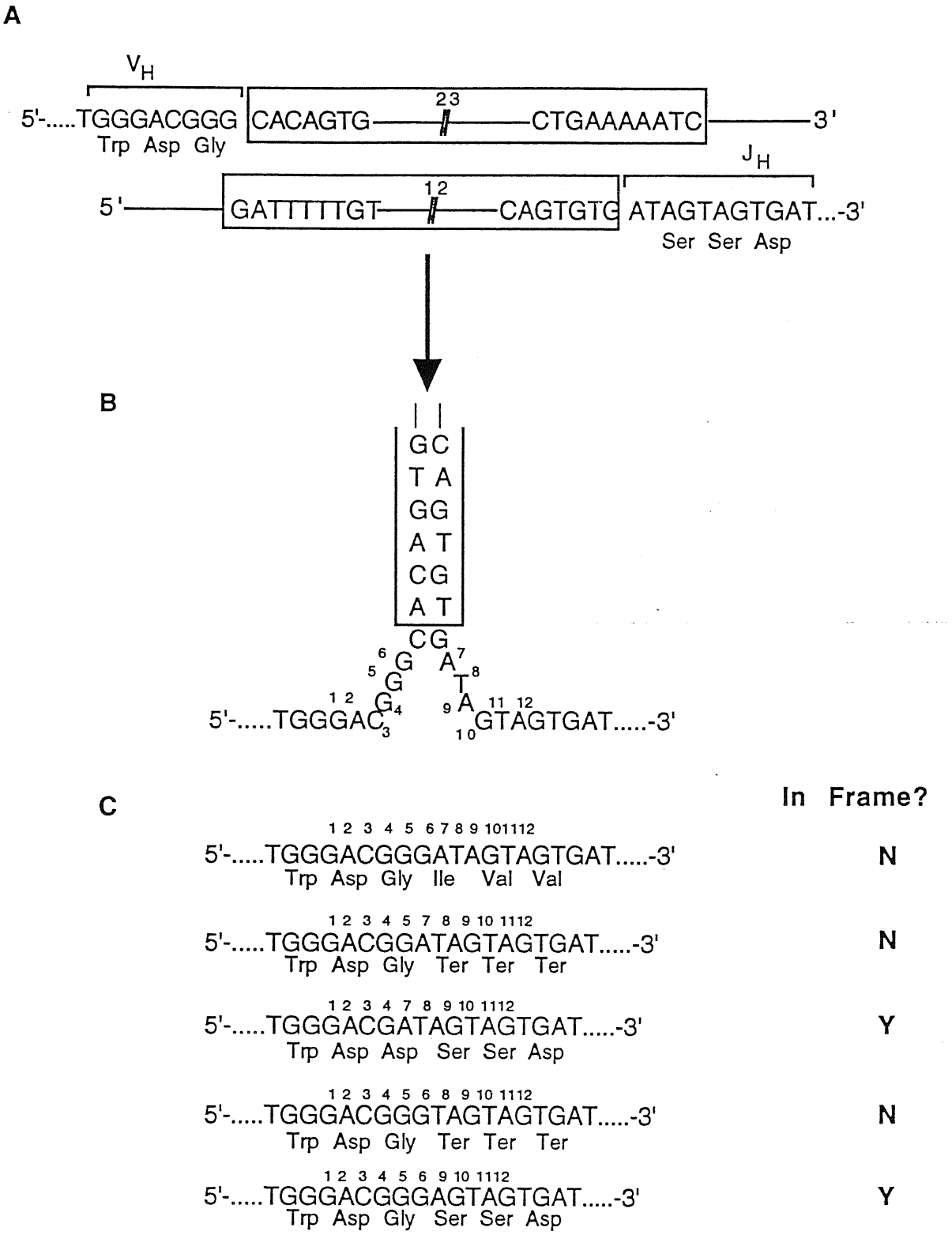


Figure 1.5: V(D)J recombination , signal sequences. The nucleotide sequences the 3' border of a V segment and 5' border of a J segment is shown. The signal heptamer and nonamer 3' are boxed. There is a "spacer" of 23 nucleotides (nt) separing heptamers from nonamers of a V coding segment. A 12 nt spacer is found 5' of a J segment. A signal sequence abuts the V coding segment but is separed from the J coding segment by 2 nt. The amino acid encoded at the border of these segments in their germilne state are shown. B, the coding segments are approximated by the V(D)J recombinase complex, possibly aided by potential hydrogen bonding between complementary nucleotides of the opposing signal sequences. A DNA break occurs at the border of the signal sequences, which are then ligated to one other. C, Variable nucleotide deletion occurs at the ends of the coding segment prior to ligation. Depending on the extent of deletion, the resolved contiguous V-J region can maintain a single reading frame and non-functional (examples are given). For in-frame structures additional variability can occur at the amino acid encoded at the V-J junction depending on the deletion.

not required for the rearrangement but if expressed it qualitatively modifies the out come by adding N regions to V(D)J junctions. Thus the presence or absence of TdT expression during V region assembly can affect the resulting V region repertoire in at least two ways. First, N region addition generates a substantial amount of diversity in the repertoire. In addition, absence of TdT leads to the appearance of particular V region genes at increased frequency, probably because N addition reduces the probability of homology-mediated joining of certain V region gene segments.

Until recently, the above model had been adequate to explain the known features of V(D)J recombination. However, it came under closer scrutiny with the discovery of an unexpected form of non random base pair addition first observed in the V(D)J joining of avian species [54]. These non random base pairs, now referred to as P nucleotides [55], are found only at the ends of the full-length coding segments and are always the reverse complement of the coding segment nucleotides next to the recombination sequences. Since the original description many other examples of P nucleotide addition have been described [33, 56].

The identification of P nucleotides led to the consideration of a new model of recombination sequence mediated recombination [57]. In this model, rearrangement is initiated by the binding of the recombinase, which introduces a single strand nick between the coding sequence and its recombination sequences. The base affected by this bond is activated and attacks its complementary base on the other strand, generating a hairpin intermediate and creating a double-stranded break between the coding region and the recombination sequences. Resolution of this hairpin could occur through the action of a single-stranded endonuclease, which can cut between any two bases in the hairpin structure. If the cut occurs at the base of the loop on the antisense strand, a sense strand with overhang is created containing a short inverted repeat (the P nucleotides). If this 3' end is then used to ligate the coding elements, the stretch of P nucleotides will be incorporated into the product. In

contrast, if the loop is opened at its base on the sense strand then the sense strand is shortened. If this shortened 3' end is used to ligate the coding element, a product containing a coding segment will be observed (Fig. 1.6) [58, 59].

By analogy to other site-specific recombination systems, the V(D)J reaction could be orchestrered 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 V(D)J recombination [60]. Recently, two genes have been identified that when expressed simultaneously are sufficient to generate VDJ recombinase activity in all cell types examined [57, 61]. These two genes were designated "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 V(D)J recombinase; it remains possible that one or both serve to regulate expression of the recombinase gene(s) or in some way activate the actual V(D)J recombinase.

The RAG-1 and RAG-2 genes were found to be coexpressed at substantial levels in the nucleus only in the primary lymphoid tissues and in cell lines that represent precursor lymphocytes [57, 61], consistent with their role either as transcriptional factors or as VDJ recombinase components. The RAG genes are conserved in vertebrates but no close homology has been found in lower organisms. While the RAG-2 gene product has no clear homology to any other gene, the RAG-1 protein does have limited homology to a yeast protein HPR-1 which has holomogy to topoisomerase I.

To evaluate the role of the RAG gene products in the recombination process, their function was eliminated by gene targeting of mice embryonic stem cells [62, 63]. The mice lacking either RAG-1 or RAG-2 gene function had identical and very specific phenotypes; both were viable, but show a complete severe combined immune deficiency, due to the apparent inability to initiate the VDJ recombination process in developing T and B cells. The specificity of the RAG-2 defect has been demostrated by transfection of RAG-2 expression

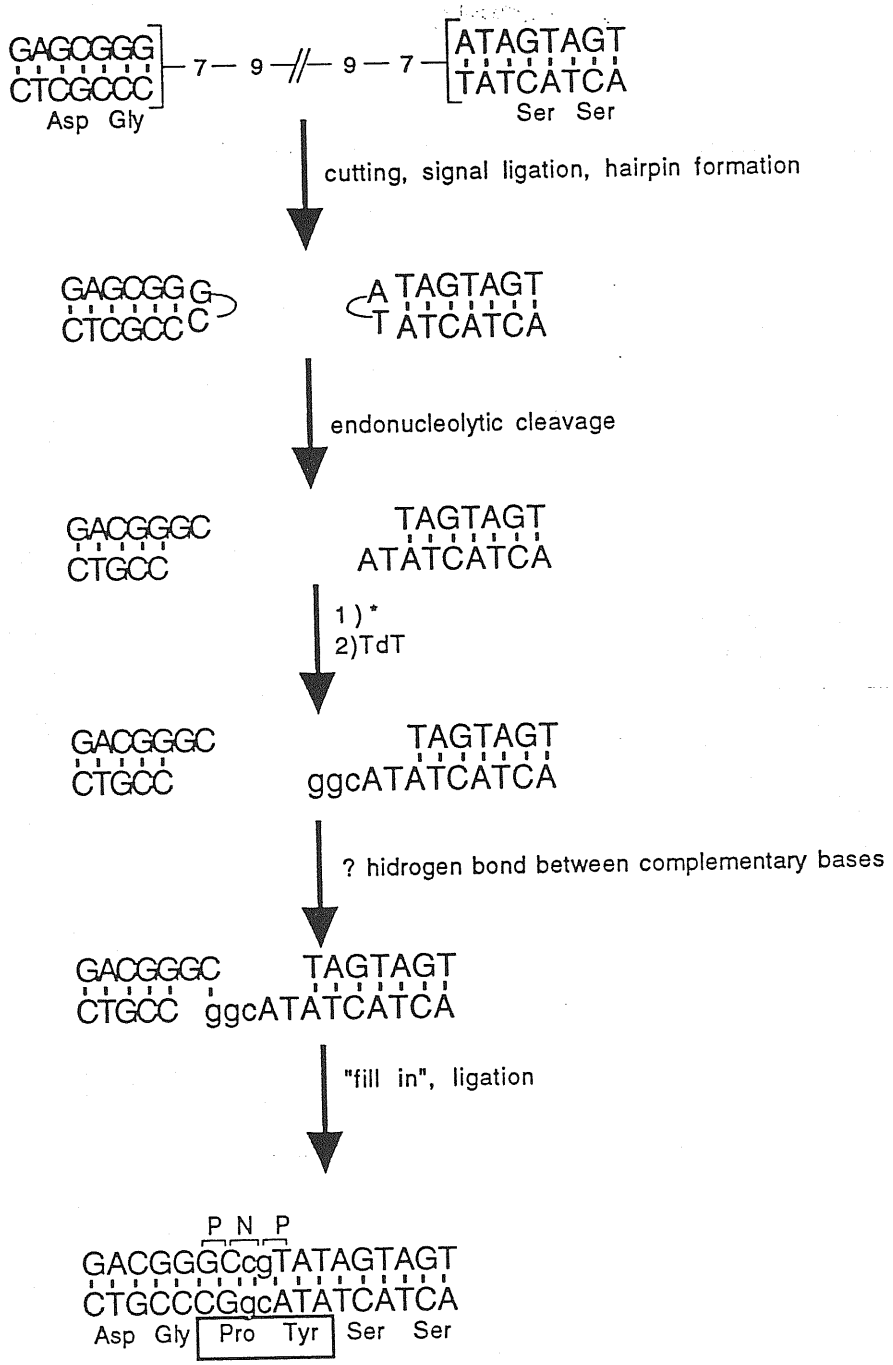


Figure 1.6: V(D)J recombination, nucleotide insertion, "P" and "N" nucleotides. An abbreviated and speculative model is show using a VDJ juxtaposition. Following approximation of the signal ends of the coding segments via a postulated hairpin intermediate. Terminal deoxinucleotyidyl transferase (TdT) adds non-templated nucleotides to the junctional ends. Complementary of terminal nucleotides aids in aligning the two coding ends, and after double stranded repair mechanisms and ligation a contiguous V-J segment is formed. The "P" nucleotides are the short, single-nucleotide, inverted repeats resulting from endonucleolytic cleavage and repair. They can be longer than single nucleotides, depending on the point of exonucleolytic cleavage. The "N" sequences shown in the lower cases, are result of TdT activity. In these example, these two processes have combined to created additional diversity at the V-J junction. Two additional amino acids, proline and tyrosine, will now be part of the H chain CDR3.

vectors into RAG-2$^-$ A-MuLV transformants; such cells acquire the ability to rearrange transfected VDJ recombination substrates [62]. Because RAG deficiency is manifested at the earliest lymphocyte precursor stages, the studies of RAG-deficient animals do not allow the investigation of the potential roles for these genes in events that occur in more mature cells of the immune system, such as immunoglobulin H-chain class switching or somatic mutation.

It seems likely that the specific components of the VDJ recombinase recruit additional, ubiquitously expressed cellular activities to perform certain aspects of the process. A spontaneuos mouse mutation has provided the first insight into such activities . The murine SCID mutation was defined as an autosomal recessive mutation that resulted in a general (but often not complete) absence of mature B and T cells due to an impairment in the VDJ recombination process [64]. Unlike the RAG mutant pre-B cells, SCID pre-B cells undergo the initial steps of the VDJ recombination relatively efficiently, including recombination sequence recognition and introduction of precise double strand DNA break repair. Similar defects in V(D)J recombination that can not be corrected by transfection with RAG-1 and RAG-2 expression vectors have been identified in several repair mutant cell lines [64].

1.3 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 the adult life. A series of stages in B cell development have been defined based on immunoglobulin gene rearrangement patterns [65, 66]. The first step of B cell maturation involves rearrangement of immunoglobulin H-chain genes and expression of cytoplasmatic μ H-chains. Late in the pre-B stage two new molecules named Vpre-B and λ5, are expressed. These molecules together function as a

"surrogate" for the conventional L-chain [67] associating with the μm to form a complex which is transported to the surface of the B cell precursor [68]. The λ5 molecule, which corresponds in sequence to the L-chain constant region, is covalently bound through disulfide bounds to μm chain, whereas the Vpre-B molecule, with sequence homology to the L-V region, associates non-covalently with the VH segment of the μm [67].

In mature B cells, membrane bound immunoglobulin molecules of all classes are expressed at the cell surface with at least two other proteins (the products of the mb-1 and B29 genes) which function as the signal-transducing components of the complex. The mb-1 and B29 genes are switched on very early in B cell development. In pre B cells protein products of these genes may associate with μm-surrogate L-chains and other molecules to signal success in IgH rearrangement. The stringency of the mechanism that screens for μm synthesis is revealed by targeted disruption of the exons encoding the μm transmembrane segment [54]. Mice homozygous for this mutation completely lack mature B cells in the periphery and also μm expressing pre-B cells in the bone marrow, suggesting that their B cell development is blocked at or during the stage of IgH rearrangement. This also implies that the μm has a functional role even in B cell development before the L-chain is expressed and supports the idea that μm, toghether with the surrogate L-chains, forms a membrane receptor through which pre-B cell differentiation is controlled.

The small pre-B cells subsequently acquire the capacity to synthesize L-chains, which interact with H-chains to form complete IgM molecules that are expressed on the cell membrane surface. The membrane IgM serves as an antigen receptor to trigger subsequent stages of differentiation 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 with identical V domains and hence identical antigen-binding specificities. Thus, each B cell displays antibodies with a single type of VH/VL regions sequence that function as antigen receptor. When a mature B lymphocyte binds antigen, in

the presence of appropriate 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 accompanied by the loss of surface immunoglobulin expression as a consequence of the conversion from the synthesis of membrane to secretory immunoglobulin molecules. The mature plasma cells are short-lived and produce large amounts of secreted immunoglobulins.

The process of early B cell maturation initially gives rise to a repertoire normally skewed towards autoreactive antibody production [68-71]. It has been reported that only a limited fraction of the total repertoire of H-chain V genes are represented in the fetal liver B cells. Two of the expressed genes found in this population encode H-chain frequently encountered in rheumatoid factor antibodies [72]. Moreover, it has been demonstrated that early human B cells express poly-reactive IgM antibodies that can bind to a variety of different autoantigens [70, 71].

The maturation of human B cell precursors into mature B cells proceeds through a highly regulated process involving the coordinated acquisition and loss of B lineage functional molecules. More than 20 distinct differentiation antigens have been identified on B lineage cells. The most immature B cell precursors (pro-B cells) express surface CD10 and CD34 and nuclear terminal deoxynucleotidyl transferase (TdT). These cells lack the B-lineage-specific antigens CD19 and CD22, are cytoplasmatic and surface immunoglobulin negative and have immunoglobulin genes in germline configuration. The next stage in B cell development, the pre-pre-B cell, is marked by the acquisition of the CD19 and CD22 antigens [73, 74]. The immunoglobulin H-chain genes undergo rearrangement, but are not yet expressed. Two other B cell antigens, the CD38 and CD40, are expressed later at this stage [66]. The hallmark of the next maturation stage, the pre-B cell, is the expression of Cμ H-chains and the appearance of CD73 and CD21 [66, 73, 74]. On the other hand, expression

of CD34 and CD10 ceases and nuclear TdT is no longer present [75]. Late at this stage there is expression of the μm-λ5-Vpre-B complex on the surface [75, 76] which is finally followed by rearrangement of the immunoglobulin L-chain genes and expression of surface IgM on immature B cells. As immature B cells develop into mature B cells, they start to express surface IgD and CD23 [65, 66]. At this point B-lineage development in the bone marrow is complete, and B cells exit into peripheral circulation and migrate to secondary lymphoid organs [77].

1.4 Somatic Mutation

The production of antibodies with progressively higher affinity for antigen is an important feature of B-cell clonotypes recruited by repeated antigenic challenge, and is referred to as affinity maturation. This phenomenon, which is restricted to T-cell dependent responses, requires that the antigen-binding sites of the antibody molecules in the matured response be structurally different from those present in the primary response [78]. The molecular basis of this process was first unveiled by the analysis of the binding affinity and primary structure of monoclonal antibodies generated at succesive stages of murine immune responses to different conjugated haptens and antigens. The first exposure to antigen results in recruitment of B cell clonotypes that bind the antigen by virtue of the combinatorial and junctional specificity of their unmutated receptors. Subsequent exposures to antigen lead to accumulation of somatic point mutations in the antibody V segment and antigen selection of the high-affinity mutated B cell clonotypes. Sequential rounds of mutation and selection eventually result in restriction of the response to those B cells with the best "fit" for antigen and this high affinity variants are then selected to enter to the pool of memory cells [79, 80]. Thus, the antibodies present in a memory response have a higher affinity than those of the

early primary response. Many of these antibodies are structuraly unrelated to those expressed in the primary response suggesting that there is a shift in the repertoire of germ line genes used in the secondary response [81].

Studies of murine anti-hapten responses have shown that the vast majority of B cells recovered in the first six to seven days after immunization express unmutated germ-line genes [82, 83]. In the second week, however, somatic mutations rapidly accumulate in the expressed H- and L- chains genes. Moreover, particular H- and L-chains gene combinations are found to carry the so-called "key mutations" that increase the affinity of the antibody for its antigen by a factor of 10 [84]. These key mutations are unique for each germline combination so that different key mutations are found in different responses [85, 86], and they are present in almost all of the responding B cells. Thus, not only does the repertoire diversifies through a hypermutation mechanism but, in addition, mechanisms must exist where rare B cells with higher affinity receptors can be selected and cells with low affinity receptors eliminated so that they do not contribute significantly to the pool of memory cells [81].

The intrinsic specificity of the hypermutation process has been investigated by analysis of silent mutations, of mutations that lie adjacent but outside the coding portions of rearranged V genes and of mutations in passenger transgenes [81-83, 87, 88]. These studies have shown that the mutations are targeted in and around the rearranged V gene segments of both the H and the L-chain loci; they do not extend significantly upstream beyond the promoter or dowstream into the constant region exons. The mutation rate has been estimated at approximately 0.4×10^{-3} per base pair per generation [89]. The mutations are usually base substitutions with a bias towards transitions and marked nucleotide substitution preferences. Although the mutations can be scattered over the rearranged V genes, the process is not random, in that intrisic hot spots can be discerned [80].

Little is known about the cis-acting DNA sequences responsible for the hypermutation mechanism. An approach for the systematic delineation of such sequences came from the observation that the rearranged V gene of an immunoglobulin Lκ chain transgene can constitute a target for the hypermutation process [90]. Using this approach it was demo strated that sequences downstream of Ck are necessary for somatic mutation of the linked V-transgene [91, 92]. Removal of either the 3' enhancer or the intron enhancer resulted in a dramatic fall of the rate of hypermutation. However the substitution of the V gene promoter and 5'-unstranslated region with that of the β-globin gene did not affect the mutation rate to any significant degree suggesting that the V gene promoter does not possess factors involved in recruiting the hypermutation mechanism [90].

The appearence of mutations in the V regions of the immunoglobulins coincides with the time when B cells are proliferating in follicles and particularly with the time when germinal centres are present in the follicles. The germinal center (GC) is a histologically defined area in peripheral lymphoid organs, such as lymph nodes and spleen, that develops following antigenic stimulation [93-95]. GCs contain proliferating lymphoblastoid B cells as well as some T cells, macrophages and a network of follicular dendritic cells. The latter have the capacity to take up antigen and store it on their surface in the form of antigen-antibody complexes [96]. The B cells that give rise to GCs seem to be recruited initially into the antibody response by antigen-driven activation outside follicles. In the first few days after primary immunization, proliferating B cells can be found in the T-cell rich areas of the periarteriolar lymphocyte sheath (PALS) of the spleen [80, 97]. Small numbers of these cells, about three for each follicle, colonize the follicular dendritic network and enter a phase of rapid exponential growth . Within three or four days they fill the network [98]. At this stage a marked change takes place within the follicle. The proliferating B cells move to one pole of the network, the dark zone of the GC, and cease to express immunoglobulin. These cells, now termed centroblasts, remain in the cell cycle but do not increase in number. Half of

their progeny become centrocytes, which come out of the cell cycle and re-express surface immunoglobulin. They pass back into the follicular dendritic cell network, which now fills the light zone of the GC. There is a high death rate among centrocytes, but some leave the germinal centre to become memory B cells or plasma cells [99]. Isolated centrocytes on culture spontaneously kill themselves by apoptosis, but this tendency can be delayed by one or two days if their antigen-binding receptors are cross-linked in vitro by using anti-immunoglobulin immobilized on solid surface [98]. It seems plausible that this reflects a process by which cells that have undergone somatic mutation in their immunoglobulin V-region genes are selected on the basis of their capacity to be activated by antigen held on follicular dendritic cells. GCs gradually decrease in size from the time of immunization, so that after three to four weeks the centroblasts and centrocytes are no longer apparent. When centroblasts and centrocytes are no longer present in the follicule, small numbers of B cells can still be found in the FDC network. As recirculating memory B cells can respond to antigen held on FDCs, it seems likely that there is an equilibrium between these follicular B cell and memory cells. It is also possible that these follicular B cell produce plasmablasts that migrate to the bone marrow or lamina propia of the gut. The estimated life span of these plasma cells of about four weeks is too short to maintain secondary antibody responses which may continue for more than a year. Antigen has been shown to be held on FCD for extented periods and presumably is involved in maintaining these late follicular B cell in cell cycle [100].

1.5 The Diversity of Antibodies

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-chain gene segments that create substantial diversity due to their combinatorial joining. In addition, diversity is introduced at the junction of V and J gene segments in the L-chain and at the VD and DJ junctions in the H-chain. 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 and also in the case of the H-chain, by the addition of the so called N and P elements at both sides of the D gene. Furthermore, a large number of antigen binding specificities are 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 further expanded by the occurence of somatic hypermutation that results in amino acid sequence changes in the V regions. This last process is the only antigen mediated mechanism that contributes to antibody diversity. Taking into account the number of different gene segments, the processes of recombination and generation of diversity, it can be estimated that more than 10^9 different antibody molecules can be generated from a few hundred different genetic elements found in the embryonic DNA.

1.6 Class Switching

As shown in figure 1.7 the H-chain genes are clustered in a 200 Kb region 3' to the JH gene segments. The order of the human CH genes is 5' Cμ-Cδ-Cγ3-Cγ1-Cψε-Cα1-Cψγ-Cγ2-Cγ4-Cε-Cα2 3'. The Cμ gene is the first to be expressed during B cell differentiation in combination with a V(D)J gene of the same allelic chromosome. The mature, resting B cells that coexpress mIgM and mIgD produce mature μ mRNA predominantly by termination of transcription immediately 3' to the Cμ membrane or secretory exon, while mature δ mRNA is

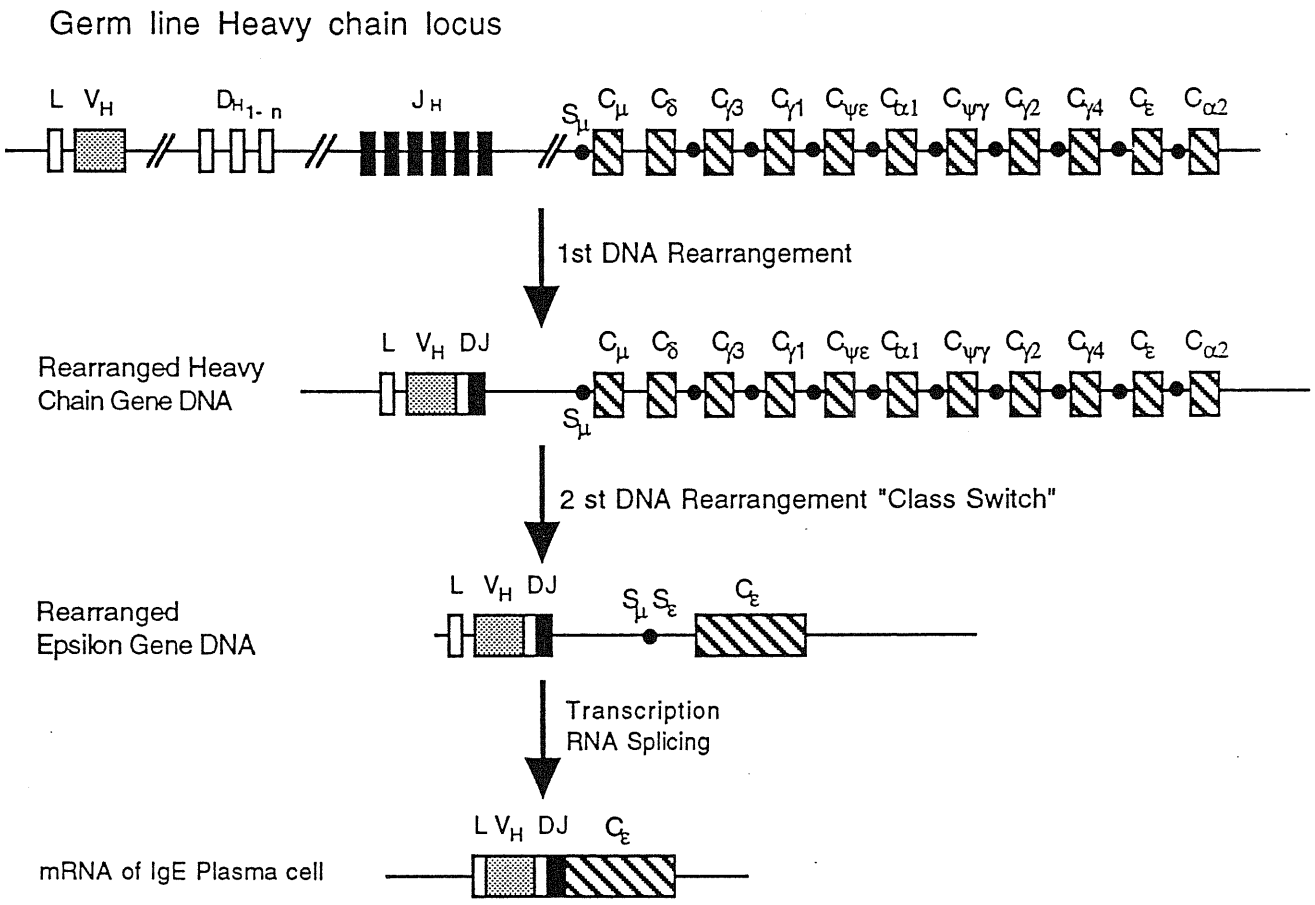


Figure 1.7. Schematic diagram of the human heavy chain gene locus showing the order of the constant region genes. Following the initial DNA rearrangement combining V_H , D, J_H , the B cell can alternate splice sites and produce IgM and IgD simultaneously . Alternatively, the B cell can switch to the 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 Sε switch regions are used, leading to the formation of an active ε transcript.

produced by transcription of a long RNA transcript that contains VDJ, Cμ and Cδ, followed by the splicing out of Cμ [101-104]. The isotype switch that occurs in the later stages of B cell differentiation is associated with deletion of all CH genes 5' to the one being expressed, with juxtaposition of the VHDJH gene, with its promoter and 3' enhancer, 5' to the expressed CH gene [105-107]. Both the non expressed, as well as the expressed H- loci can undergo class switching, with VH genes in both chromosomes in a given cell often switching to genes that encode the same CH isotype [108-111]. Deletional isotype switching is independent of membrane expression of productively rearranged immunoglobulin H and L-chains, as it can occur in pre-B cell lines in addition to B cells [112-114]. DNA sequencing studies have demonstrated the presence of switch (S) regions, which are located 5' to each CH gene, except Cδ [115-118]. All S regions contain repeats of the nucleotides GAGCT and TGGGG, which suggests that these sequences play a pivotal role in the class-switch process. In addition, switch regions preferentially contain the sequence (C or T)AGGTTG, which is typically located 5' to switch recombination sites and has been suggested to be involved, in association with the GAGCT and TGGGG tandem repeats, in mediating class switching [119-121].

Molecular analysis of stimulated B cells has revealed that induction of class switch recombination to a particular CH gene almost invariably correlates with the transcriptional activation of the same gene in its germinal configuration [122]. The germline transcripts that result from this process start a few kilobases upstream of the S region and proceed through one or more short exons (I exons) that are spliced to the first exon of the CH gene. The I exons generally display multiple stop codons in the three reading frames and therefore are unable to code for any peptide of significant length. Although there are no significant sequence conservations of the different germline CH transcripts, their overall structure is conserved. Together, these observations strongly suggest that germline transcription and/or germline transcripts function in the regulation of class switching recombination [122].

The relation between germline transcription units and regulation of class switching recombination has been confirmed by gene targeted mutation experiments. These studies showed that deletion of the Iγ1 or Iγ2b exons and their promoters resulted in inhibition of class switching to the corresponding genes [123, 124].

Considerable evidence exists that non-homologous recombination between two sites on the same DNA segment with looping out of the deleted DNA is the usual mechanism of deletional isotype switching and that this recombination is not directed to specific sites within the switch region. Analysis of circular excision products, which are produced during isotype switching of normal B cell and which contain the deleted 5' CH genes provided direct evidence that deletion occurs through formation and splicing out of a DNA loop [125-128]. A similar process has been described for VλJλ recombination in B cells and for the T cell receptor α and β genes [129-131]. The occasional occurrence of switch inversions, instead of deletions, in the genome of B cell tumor lines is consistent with the formation of a DNA loop [132, 133].

Although looping out and deletion as a mechanism for immunoglobulin isotype switching appears well established, recent studies indicate that switching to a given CH gene does not always occur directly from Cμ but may occur through sequential deletional events. This was first suggested by the demonstration that switch regions from some isotype-switched B cell tumors contained fragments of Sμ and the S region associated with the expressed CH gene, separated by a fragment of the S region associated with a third CH gene. This third CH gene was typically located between Cμ and the expressed CH gene [117, 134]. Additional support for the existence of sequential switching came from studies in which circular DNA, which was obtained from actively switching normal B cells and represented the reciprocal deletion products from class-switch events, was shown to contain switch region sequences of Sμ ligated to two different Sγ sequences: Sμ ligated sequentially to a Sγ sequence and to Sε, and Sμ ligated sequentially to an Sγ sequence and to Sα [108,

128]. Although it seems obvious that B cells that have switched to one immunoglobulin isotype may later be able to undergo a second switch event, evidence has also accumulated that sequential switching in some circumstances can be the usual mechanism for switching to a particular isotype. Restriction analysis of total, unselected circular DNA from LPS plus IL-4-stimulated cells indicated that the majority of Cγ1-containing circles, generated by an IgE class switch, had rearranged Cγ1 DNA [135]. In addition, DNA circles cloned from B cells stimulated to secrete IgE in vivo by infection with a nematode parasite that induces IL-4 production demonstrated sequences derived from Sγ1 as well as from Sμ and Sε [125]. Both of these observations suggest that IgE switching may typically occur by a sequential IgM to IgG1 to IgE switch. These observations are compatible with the following two possibilities (a) sequential switching in the generation of an IgE response simply reflects the greater likelihood of B cells that are activated by some stimuli, such as LPS plus IL-4, to switch to IgG1 rather than to IgE, so that most B cells that switch to IgE have already been induced to switch to IgG1; and (b) switching to IgE from IgG1 is more easily accomplished than is switching from IgM. Studies with B cells favor the first hypothesis by providing evidence that some stimuli appear to favor switch to IgE from IgM, while other stimuli appear to favor sequential switching to IgE through IgG. For example, human B cells induced to switch to IgE by EBV culture plus IL-4 can switch to IgE via IgG [136], while human B cells cultured with IL-2, IL-4, and PMA-activated mouse EL-4 thymoma cells [137] or with anti-CD40 antibody plus IL-4 [138] appear to switch directly to IgE.

In contrast to the situation with the IgG isotypes, there is considerable information about the control of switching to IgE in both mouse and human. This isotype appears to be regulated similarly in both species, with IL-4 being a critical stimulus. This cytokine stimulates IgE responses by purified mouse cell cultures with either LPS [139-141] or activated T cells [142-144], or by purified B cells cultured with EBV [145-147], Anti-CD40 antibody [148, 149], cortisol [150, 151], mouse EL-4 cells PMA [152] or activated T cells.

The IL-4 induced IgE production can be blocked with anti-IL-4 antibodies, further confirming the role for this cytokine in the IgE class switching.

In vivo studies in mouse support the view that IL-4 is absolutely required to generate an IgE response. Mice that lack a functional IL-4 gene fail to develop any IgE response to the appropiate stimuli. The only in vivo system in which an IgE response can be elicited that is not inhibited by IL-4 antagonists is the one in which the response is stimulated in mice by injection of a goat anti-IgE antibody [153]. It is believed that this response is produced by B lymphocytes that had switched to expression of mIgE prior to immunization, and that the resulting lack of a requirement for switching during the course of the response explains the lack of requirement for IL-4.

Although IL-4 stimulates switching of mouse B cells to both IgE and IgG1 and by human B cells to both IgE and IgG4, differences exist in the kinetics of switching to the different isotypes, in the concentrations of IL-4 that are required to induce the switching and in the requirement for costimuli. There is a 12 to 16 hours delay in the generation of most of the IgE response, as compared to the IgG1 response, by mouse B cells cultured with LPS plus IL-4 [135]. Similarly, human B cells stimulated with IL-4 plus anti-CD40 antibody secrete detectable IgG4 approximately 2 days earlier than they secrete detectable IgE [148]. Both observations are consistent with the interpretation that the switch from IgM to IgE typically involves an initial switch to an IgG isotype and then a second switch to IgE, as already discussed in the previous section. Also consistent with this interpretation is the observation that B cells must be exposed to IL-4 for a longer period of time to induce an IgE response than to an IgG response [139].

Three cytokines other than IL-4 (IL-2, IL-5 and IL-6) are required for, or contribute to, the induction of an IgE response in some systems [152, 154, 155]. All three cytokines have been implicated, however, in stimulating the differentiation of B cells into antibody-secreting cells, and it is unlikely that they are specifically involved in stimulating B cells to switch to

IgE expression. Another factor that may play a role in the induction of IgE responses is CD23, the low-affinity receptor for IgE, which is upregulated on B cells by IL-4 [156-158]. Some monoclonal antibodies to this receptor inhibit the IgE response of human peripheral blood mononuclear cells cultured with IL-4 [159, 160] and by human B cells cultured with IL-4 plus cortisol [151]. Soluble CD23 has been reported to upregulate IgE secretion in some systems [161, 162].

A number of different compounds have been identified that specifically inhibit IgE responses. The inhibitory effects of these compounds vary, however, depending on the costimulus that is employed to induce the IgE response. IFN-γ inhibits germline ε mRNA expression [163] and IgE secretion by human peripheral blood mononuclear cells cultured with IL-4 [160, 164]. IFN-γ has no effect, however, on IgE secretion by human B cells cultured with IL-4 plus anti-CD40 antibody [148]. IFN-α also inhibits IgE secretion by human peripheral blood mononuclear cells cultured with IL-4 [160, 164], and by human B cells cultured with IL-4 plus Epstein-Barr virus [165, 166]. Another cytokine, IL-12, has been reported to suppress IgE secretion by human B cells stimulated with IL-4 plus cortisol [167]. This cytokine suppresses IgE production indirectly, by inducing IFN-γ, as well as by an indirect effect on B cells. These observations suggest that IL-4 is an absolute requirement for induction of switching to IgE, but that other costimuli further modulate this response.

1.7 IgE and Allergy

Prausnitz and Küstener first demonstrated the presence of a factor in the blood of allergic subjects which when transferred to the skin of non-allergic individuals rendered them sensitive to allergens [21]. This substance was identified as a cytophilic antibody in the late 1960s by Ishizaka and Ishizaka [168], but the lack of human cells that produce it in sufficient

amounts was a serious drawback for its further investigation. It could, nevertheless, be separated from the known immunoglobulins IgM, IgG, IgA and was named IgE for the erythema that allergens provoke in sensitive skin [21]. The discovery of rat immunocytomas by Bazin and his colleagues [169], together with a rare human myeloma reported by Johansson and Bennich [170], was the major breakthrough that provided the first tangible evidence for the existence of IgE [171]. The protein produced by these cell lines afforded a standard for the measurement of IgE concentration in blood as well as material for the first structural studies. These cells were also the source of messenger RNA for the ε-chain cDNA cloning and the starting point for some of the later structural studies of IgE [21].

The word allergy comes from a Greek term that means altered reactivity and thus includes all mechanisms of hypersensitivity. The most common allergic diseases are asthma, allergic rhinitis, atopic dermatitis and food allergies, but the most dangerous is anaphylactic shock, usually provoked by insect stings or parenteral medication. Allergy in one form or another afflicts more than 20 per cent of the population, and is generally caused by the overproduction of IgE in response to common enviromental antigens, such as those present in pollen, food, house dust mites, animal danders, fungal spores, and insect venoms. IgE is produced only in mammals and is one of the five classes of human antibody. It constitutes a minuscule fraction of the total immunoglobulins in serum (50-300 ng/ml as compared with 10 mg/ml of IgG) and is therefore of little help in neutralizing antigens. However, its action is powerfully amplified by the receptors to which it binds.

The interaction between IgE and its high affinity receptor (FcεRI) on mast cells and basophils is a key step in the allergic response. When a multivalent allergen associates with FcεRI-bound IgE it crosslinks the receptor molecules on the cell membrane, triggers degranulation of the cell and cause a rapid release of stored mediators, notably histamine, and stimulates the synthesis and secretion of cytokines that attract and activate inflammatory cells. The FcεRI has recently been discovered also on Langerhans cells [172, 173], eosinophils

and activated monocytes, but its biological function in these cell types have not been completely elucidated [174].

Early studies by Nissim and colleagues [175] have indicated that the high affinity receptor binding site of the IgE molecule can be assigned to the ε H-chain constant region, since isolated Fcε fragments (Cε2-Cε4), obtained by limited proteolysis of IgE, were able to bind to mast cells with an affinity similar to that of the intact molecule. However, fragmentation of the Fcε into smaller pieces destroyed its receptor binding capacity [175]. In addition, it has been shown that maintenance of the native conformation of IgE is important in its binding to the FcεRI [176]. Studies in rodents demonstrated that a region in the cleft between the Cε2 and Cε3 is protected from proteolysis when bound to the FcεRI [177]. The precise site on the IgE molecule that interacts with the FcεRI was further elucidated using recombinant DNA technology. Cloned Cε gene segments of both human [178, 179] and mouse IgEs [180, 181] were expressed in bacteria [182-184] and mammalian cells [175, 185] as complete molecules, recombinant peptides or chimeras containing both rodent and human domains. Functional studies of these molecules provided direct evidence that the first twelve amino acids of Cε3 are required for binding to the FcεRI, but also showed that other residues in Cε3 or Cε4 influence this binding. Similar results were obtained from mapping studies with anti-IgE monoclonal antibodies [185-187]

Activation of inflammatory cells by the products of mast cell degranulation induces the expression of the "low affinity" IgE receptor, FcεRII, also known as CD23, on eosinophils, monocytes, and Langerhans cells [188]. This molecule is constitutively expressed on B lymphocytes and has been implicated in the regulation of the IgE response [21, 172, 189]. Like in the case of the FcεRI, studies with monoclonal anti-IgE antibodies and recombinant or chimeric IgE molecules pointed to Cε3 as the site of FcεRII interaction [190]. Studies with recombinant IgE confirmed that the Cε3 domain is involved in binding and also indicated that this domain must be dimeric since a single Cε3 did not bind to FcεRII. These studies also

localized the IgE binding region in the vicinity of the glycosylated residue Asn 371, and showed that the carbohydrate components of IgE are not required for this interaction.

CHAPTER 2

MATERIALS AND METHODS

2.1 Clinical samples and RNA preparation for ε fingerprint

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 [191].

2.2 Oligonucleotide primers

Oligonucleotides were synthesized by the solid phase triester methodology on a DNA synthesizer (380A; Applied Biosystems, Inc., Forster City, CA). Following synthesis, the oligonucleotides were removed from the solid support by an ammonia treatment (55°C) overnight, dried under vacuum, and purified by G-50 Sephadex molecular sieve

chromatography using TE elution buffer. These oligonucleotides were further used as PCR primers, oligonucleotide probes for hybridization, and as sequencing primers.

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 100 ml reaction mixtures containing each dNTP at 200mM, 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 [192] 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 (5' TAAGGTCATAGTTGTCCCGT 3') oligonucleotide followed by 30 cycles of PCR with the V1 (V1 5' GTGCAGCTGC(G)A(T)GG(C)AGTCG(T)GG 3') 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 (5' GGAAGACCGATGGGCCCTTG 3'). Two microliters of the first PCR reactions were reamplified in 50 μl PCR reactions with internal primers (^{32}P labeled V2 (5' CTGAGGACACGGCCGTGTATTACTG 3') and E1 (or E6, 5' GAGGTGGCATTGGAGGGAATGT 3') 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 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 CDR3/FW4 regions

A 30 min. autoradiography of the PCR fragments obtained from the second PCR reaction with the ^{32}P labeled V2 and E2 or E6 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" [192]. The amplified DNA fragments were precipitated, reamplified for 15 cycles with the same primers, and purified by electroelution from 1.2% agarose gels. The recovered DNA fragments were ligated in the Sma I site of pUC18 (Pharmacia LKB, Uppsala, Sw) and used to transform E. coli strain DH5α. Clones were picked randomly, and double-stranded DNA template was prepared and sequenced by the dideoxy chain termination method [193] using the T7 Sequencing Kit (Pharmacia LKB). Sequences were identified by comparison to reported germline D and JH gene segment sequences [29, 30, 34, 37].

2.5 Analysis of µ and γ4 transcripts

Total cellular RNA (1µg) from individual A3 was reverse transcribed with the oligonucleotide µ1 (5' GTCCTGTGCGAGGCAGCCAA 3') or γ4.1 (5'

TGATGGGCATGGGGGACCAT 3'). First PCR was performed on the total cDNA sample with the TB1 (5' GGTCATTACGATTTTTGG 3')/μ1 or the TB1/γ4.1 primer pair, followed by a second nested PCR reaction with the ^{32}P labeled oligonucleotide TB3 (5' TTTGGAGTGCATATTATAAG 3') and the corresponding μ2 (5' ATCCGACGGGGAATTCTCAT 3') or γ4.2 primer (5' GTTGCAGGTGTAGGTCTTC 3') 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 (5' CCGTCCTGGTTCGACC 3'). 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 minutes 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.

2.6 Cloning and sequencing of the polymorphic Cγ4b gene

DNA was isolated from white blood cells of individual A3 using standard methodology [192]. PCR amplification was performed with the 5'γ4 primer (5' CACACCGCGGTCACATGGCA 3', 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.

2.7 Analysis of membrane and secreted A3 b ε transcripts

Two 1 µg RNA samples from individual A3 were reverse transcribed with Oligo dT and amplified with the CDR3 specific oligonucleotide TB1 and the S1 (5' CCACTGCACAGCTGGATGGAG 3') or M2a (5' GGCTGGAGGACGTTGGTGTA 3') primers for the secreted and membrane forms, respectively. A 1 µl aliquot of each first PCR reaction was then reamplified with the TB3/S2 (5' GGAGGCAGGAGTACGTCATT 3') or TB3/M2b primer (5' TGCCGCGTGGCTGAGAGGAA 3') combination, respectively. The obtained fragment was electroeluted from a 1.2% agarose gel, cloned and sequenced as described above. Radioactive PCRs were performed on 1 µl aliquots of the first PCR reactions with ^{32}P 5' end labeled TB3 and E2 or E6. In reactions containing total cellular RNA from the IgE secreting myeloma cell line U266 (American Type Tissue Collection), 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.

2.8 Cell lines and transfection

U266BL cell line (ATCC, Rockville, USA) was maintained in RPMI 1640 medium supplemented with 10% (vol/vol) fetal calf serum, penicillin (100 units/ml) and streptomycin (100 µg/ml). The J558L and the Hu-pSVCε plasmacytoma cell lines were obtained from Dr. M. Neuberger and were grown in DMEM containing 10% FCS, penicillin (100 units/ml) and streptomycin (100 µg/ml). Transfections were performed by electroporation. Briefly, 2 x 10^6 cells were washed, resuspended in 1 ml cold PBS, mixed with linearized DNA (15 µg in

20 µl H_2O) and electroshocked using the Bio-Rad Gene Pulser at 450 volts and 250 µF. After 5 min. on ice, the cells were resuspended in 50 ml DMEM/10% FCS/gentamycin and transferred to five 96-well plates. Selective medium containing 400 µg/ml of G418 was applied 24 h later. Anti-NIP IgE antibody production was assayed by western blot analysis and the cells were cloned by limiting dilution. In the case of double expressors the screening was performed by RT/PCR. To discriminate between DNA and cDNA amplification the E7 (5' GGGTCGACAACAAAACCTCA 3') oligonucleotide located in CH2 was used together with the S2 oligonucleotide for the detection of the εCH4-S mRNA or with the M2X oligonucleotide (5' CTAGCAGCCACCCCTCCTCGATGA 3') for the detection of the εCH4-M2" mRNA.

2.9 RT/PCR analysis of alternativly spliced ε transcripts

Total cellular RNA was isolated from U266 cells and from PBMC obtained by fractionation of fresh whole blood on a Ficoll/Hypaque gradient, using the acid guanidinium thiocyanate procedure of Chomczynski and Sacchi [191]. Two to six micrograms of each RNA sample were reverse transcribed with the GeneAmp RNA PCR kit (Perkin Elmer Cetus, Norwalk, CT) and oligo-dT as primer. Thirty five cycles of PCR were next done on each RT sample with the E3/M2a, E3/S1, or E4M/M2W oligonucleotide primer pair (E3: 5' CATGCGGTCCACGACCAAGAC 3', E4M: 5' GACGCCCGGCACAGCAACGCAG 3', M2W: 5' GAATGGGAGTGACCCGGAGAC 3'). The locations of the different oligonucleotides used are given in Fig. 3.2.1 or 3.2.5. The RT and PCR analyses were performed according to the instructions of the manufacturer of the GeneAmp RNA-PCR kit. The PCR reactions were carried out with 1 min. of denaturation at 95°C, 1 min. of annealing at 62°C, and 2 min. of extension at 72°C in the first two PCR reactions while the annealing in

the case of E4M/M2W PCR was 65°C because of the higher melting point of the oligonucleotides. The specificity of the PCR products obtained with E3/M2a and E3/S1 was confirmed by Southern blot analysis with the E4 oligonucleotide (5' AAGTCTATGCGTTTGCGACGCCGG 3'). One third of the PCR reaction was subjected to electrophoresis on a 1.2 % agarose gel, transferred on a nylon membrane and hybridized with the ^{32}P labeled E4 oligonucleotide for 2 hours at 50° C in 5x SSPE + 5x Denhardts solution. Membranes were washed for 2 x 20 min. at room temperature with 2 x SSPE + 0.1% SDS, followed by a stringent wash at 65° C with 5 x SSPE and 0.5% SDS. Semi-nested PCR experiments with internal 5' ^{32}P end-labeled oligonucleotide primers (S2, M2b and E4Y) were performed on the first PCR samples to increase the specificity and sensitivity of the assay. Details of this procedure and the subsequent analysis on denaturing polyacrylamide gels have been given before.

2.10 Cloning and sequencing of alternativly spliced ε transcripts

The different bands seen on the autoradiograms of the Southern blot experiments were purified by electroelution from 1.2 % agarose gels. In the case of the semi-nested PCR experiments, a 30 min. autoradiography on non-denaturing, non-fixed, non-dried sequencing gels was performed to identify the location of the ^{32}P labeled PCR fragments. Slices containing these fragments were excised from the polyacrylamide gels, and the DNA was eluted by the "crush and soak procedure". The recovered DNA fragments were ligated in the Sma I site of pUC18 (Pharmacia LKB, Uppsala, Sw) and used to transform E. coli strain DH5α. Clones were picked randomly, and double-stranded DNA template was prepared and sequenced by the dideoxy chain termination method [193] using the T7 Sequencing Kit (Pharmacia LKB).

2.11 Construction of chimeric mouse/human ε chains

An Aat II/Kpn I fragment from Hu-pSVCε [194] (kindly provided by Dr. M. Neuberger), containing the mouse immunoglobulin H-chain enhancer and promoter, a mouse anti-NIP VH segment and the entire human genomic Cε gene, was subcloned in a plasmid derived from pRc/CMV (Invitrogen, San Diego, USA) in which the cytomegalovirus promoter and the fd portion had been deleted. This new plasmid (pCIG-Cε) contains a single Kpn I site downstream of the Cε gene and was used for the construction of the six alternatively spliced ε species (forms εCH4-S, εCH4*, εCH4'-I, εCH4-M2', εCH4-M1'-M2 and εCH4-M2" in Fig. 3.2.7). The polyadenylation site of the bovine growth hormone gene from pRc/CMV was retained in all constructs. Details of each construct are given below.

εCH4-S. A PCR fragment obtained by amplification of cDNA from PBL with the S2 primer and a CDR3 specific primer was cloned in the Sma I site of pUC18. The Sal I-Hpa I fragment from this plasmid, which contained part of the variable region, CH1, CH2, and part of CH3, was replaced with the 835 bp fragment between the Sal I site in exon 1 and the Hpa I site in exon 3 of the human genomic Cε gene from Hu-pSVCε. This plasmid, pUC18εCH4 -S, contains the 3' part of the ε CH1 exon, the 2nd intron, the CH2 exon, the 3rd intron and the CH3 and CH4 exons joined together. The Bgl II/Kpn I fragment from this plasmid was used to replace the same fragment in the pCIG-Cε, generating the pCIG-CεCH4-S which was used for the transfection.

εCH4'-I. A PCR fragment obtained by amplification of cDNA with the E3/M2a primer pair (εCH4'-I-M2') was cloned in the Sma I site of pUC18. The Bbs I/Kpn I fragment from

this plasmid, containing the CH4' and I exons and part of the M2' exon, was used to replace the corresponding fragment from pUC18εCH4 -S. The Bgl II/Kpn I fragment from this new plasmid (pUC18εCH4'-I) was introduced between the Bgl II and Kpn I sites in the pCIG-Cε, generating pCIG-CεCH4'-I.

εCH4-M2'. This form was obtained by PCR of independently amplified fragments containing the CH3-CH4 and M2' exons. The entire M2' exon was amplified from U266 genomic DNA with two primers located in its 5' end (M2L 5' AATCCCGGTGCAGCGGTTCCTCTCA 3') and 3' untranslated region (M2d 5' TGGGTGCCGGGCCCTCCTTGGC 3'). The M2L primer contained a 5' overhang complementary to the 3' end of the CH4 exon (underlined). Another primer set consisting of E3 and a new oligonucleotide from the 3' end of the CH4 exon (E4L 5' ACCGCTGCACCGGGATTTACAGACAC 3') with a 5' overhang complementary to the 5' end of the M2' exon (underlined) was used to amplify the 3' end of the CH3 exon joined to the complete CH4 exon. The two PCR fragments obtained from the two separate amplifications were mixed together and reamplified with the E3/M2d primer pair. The presence of the 5' overhangs allowed annealing between the two fragments after the initial denaturation and their extension and subsequent amplification, resulting in a new fragment containing the 3' end of the CH3 and the complete CH4 and M2' exons. This fragment was cloned in the Sma I site of pUC18, and the Bbs I/Kpn I fragment was subcloned in pUC18εCH4 -S. The pCIG-CεCH4-M2' was then generated by transfering the Bgl II/Kpn I fragment to the pCIG-Cε.

εCH4*. A PCR fragment containing part of the variable region, CH1, CH2, CH3 and the first 15 nt from the CH4* exon of an ε transcript was obtained by cDNA amplification with a CDR3 specific primer and a primer from the CH3/CH4* boundary (E4 * 5'

CTCAGGCATGAAGTTCGCT 3'). This fragment was cloned in the Sma I site of pUC18. The Hpa I/Kpn I fragment containing the CH3 and the CH4* exons was subcloned in the corresponding sites of the pUC18εCH4-S. The Bgl II/Kpn I fragment was then subcloned in pCIG-Cε to produce pCIG-CεCH4*.

εCH4-M1'-M2. A PCR fragment obtained with oligonucleotides E3/M2d was cloned in the Sma I site of pUC18, and the Bbs I/Kpn I fragment was subcloned in pUC18εCH4 -S. The pCIG-CεCH4-M1'-M2 was then generated by transfering the Bgl II/Kpn I fragment to the pCIG-Cε.

εCH4-M2". A PCR fragment was obtained with oligonucleotides E3/M2"L (5' TGGGTACCTAGCAGCCACCCCTCCTCGATGACTCGGGATTTACAGACCACC 3'). The M2"L oligonucleotide contains 18 nucleotides complementary to the 3' of the CH4 (underlined) while the rest of the oligonucleotide encode the complement of the complete coding region of the M2" exon followed by Kpn I site (3' to the stop codon). The fragment obtained by PCR was cloned in the Sma I site of pUC18, and the Bbs I/Kpn I fragment was subcloned in pUC18εCH4-S. The pCIG-CεCH4-M2" was then generated by transfering the Bgl II/Kpn I fragment to the pCIG-Cε.

To check for possible Taq polymerase errors, all constructs were sequenced and compared with published sequences [195, 196] . The sequence of the M2 exon was found to be identical to that of Ref. [196]

2.12 Immunoprecipitations

Metabolic labeling and immunoprecipitation were performed as described [197]. Briefly, $5x10^6$ cells/ml were labeled with [^{35}S]methionine (Amersham, Amersham, UK) at 100-250 μCi/ml (1Ci=37 GBq) for the indicated times. The cells were washed with PBS and lysed by addition of cold cell lysis medium (50 mM Tris ph 8.0, 150 mM NaCl, 5 mM $MgCl_2$ 1% NP-40 and 1mM PMSF). For the pulse-chase experiments, 50% of the cells from each cell line were lysed immediately after one hour of labeling, while the remaining cells were incubated for additional 4 hours or 5 hours with an excess of cold methionine. Cells lysates and supernatants were immunoprecipitated with rabbit immunoglobulins to human IgE (ε chains) (DAKO, Glostrup, Denmark), and were purified by protein A-sepharose. The samples were analyzed by SDS-PAGE in the presence or absence of mercaptoethanol, as indicated in the figure legends. Treatment of labeled supernatants with recombinant PNGase F (N-glycosidase F) and recombinant Endo H_f (Endoglycosidase H) was done following the protocol recommended by the manufacturer (New England Biolabs, Beverly, USA).

2.13 NIP-Sepharose affinity chromatography and Western blot analysis

Functional immunoglobulin molecules were isolated from cell lysates and supernatants of transfected cells by immunoadsorption on NIP-Sepharose. The purified IgE proteins were fractionated on SDS-PAGE under non-reducing or reducing conditions. Bands were electroblotted to nitrocellulose filters and reacted with rabbit immunoglobulins to human IgE (ε chains) (DAKO) and developed with alkaline-phosphatase conjugated goat anti-rabbit polyclonal serum (DAKO).

2.14 Immunofluorescence

Cells were attached to poly-L-lysine-coated multi-well slides, fixed with ice cold methanol for 10 min, washed with PBS, and permeabilized for 10 min. with 0.3% Triton X-100 in PBS. Surface immunoglobulins were analyzed on non-permeabilized cells fixed for 20 min. with 3% paraformaldehyde at room temperature. The slides were incubated with rabbit immunoglobulins to human IgE (ε chains) (DAKO) and biotin labeled wheat germ aglutinin (Boehringer Mannheim Corp., Germany) (kindly provided by Dr. Claudio Schneider) for 1 hour. After three 5 min. washes with 1% BSA in PBS, the slides were stained with FITC-conjugated goat immunoglobulins to rabbit immunoglobulins (DAKO) and rhodamine conjugated streptavidin for 1 hour, washed, mounted in mounting medium (KPL, Gaithersburg, USA) and analyzed by either conventional immunofluorescence or by confocal microscopy on a Zeiss LS 410 microscope. Optical sections were collected for each label in 0.3 μm steps.

2.15 Northern blot Hybridizations

Twenty μg each of total RNA from the U266 cell line was separated by electrophoresis in 1% (w/v) agarose gel containing 6% formaldehyde and transferred to nylon membrane (Hybond-N, Amersham, UK) by overnight capillary blotting in 20x SSC and fixed with UV light. Probes were labelled with [α-^{32}P]dCTP (Amersham, UK) using the random priming labelling kit (Pharmacia LKB, Uppsala). Hybridizations were carried out at 42°C. Filters were washed in 2x SSC and 0.1% SDS at 65°C, and autoradiographed. The HεC, M2 and M2" probes were generated from the ε locus by the use of different restriction enzymes as it

is described in section 3.3.3. The M1' and 3'M2 probes were generated by PCR amplification of their respective subcloned DNA sequences. Paired primers were selected based on the sequence of the ε human region The primers for the M1' probe were M1B (5' TGTAGCTCACGCTCAGCAGG 3') and M1L (5' TCTGCCCACTCCGGACAGGCAG 3'). The primers for the 3'M2 fragment were M2V (5' CGTCTCGAGAGGGCTCTGT 3') and M2T (5' AGGGCCTGGCTTCCTGAGACT 3').

2.16 3'-End Amplification of ε cDNA

Three microgram of total U266 RNA in 16,5 µl of water was heated for 3 min, quenched on ice, and reverse transcribed with the GeneAmp RNA PCR kit (Perkin Elmer Cetus, Norwalk, CT) using oligo-(dT)17-adaptor (dT/Ad, 5' GACTAGAGTCGACATCGATTTTTTTTTTTTTTTT 3') as primer. Thirty five cycles of PCR were next done on the RT sample either with the M2N/Ad (M2N: 5' ACAGAGCCTCCTGCTGCTCT 3', Ad: 5' GACTAGAGTCGACATCGA 3') or E3/Ad oligonucleotide primer pair. The PCR reactions were carried out with 1 min. of denaturation at 95°C, 1 min. of annealing at 56°C, and 2 min. of extension at 72°C. The specificity of the obtained PCR products was confirmed by Southern blot analysis with the M2d or S2 oligonucleotide.

CHAPTER 3

RESULTS

Section 1

3.1.0 Molecular Analysis of the CDR3/FW4 regions of IgE Heavy Chain Transcripts Expressed in vivo

3.1.1 Introduction

The characterization and comparison of the IgE repertoires of normal and atopic individuals is an important step in the investigation of the phatophysiologic mechanisms underlaying allergy. Due to the extremely low serum levels and low numbers of IgE producing B cells, this investigation was only possible using a sensitive RT/PCR procedure which allowed the amplification and subsequent cloning and sequencing of the ε transcripts.

3.1.2 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-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 to allow their amplification. We found the second

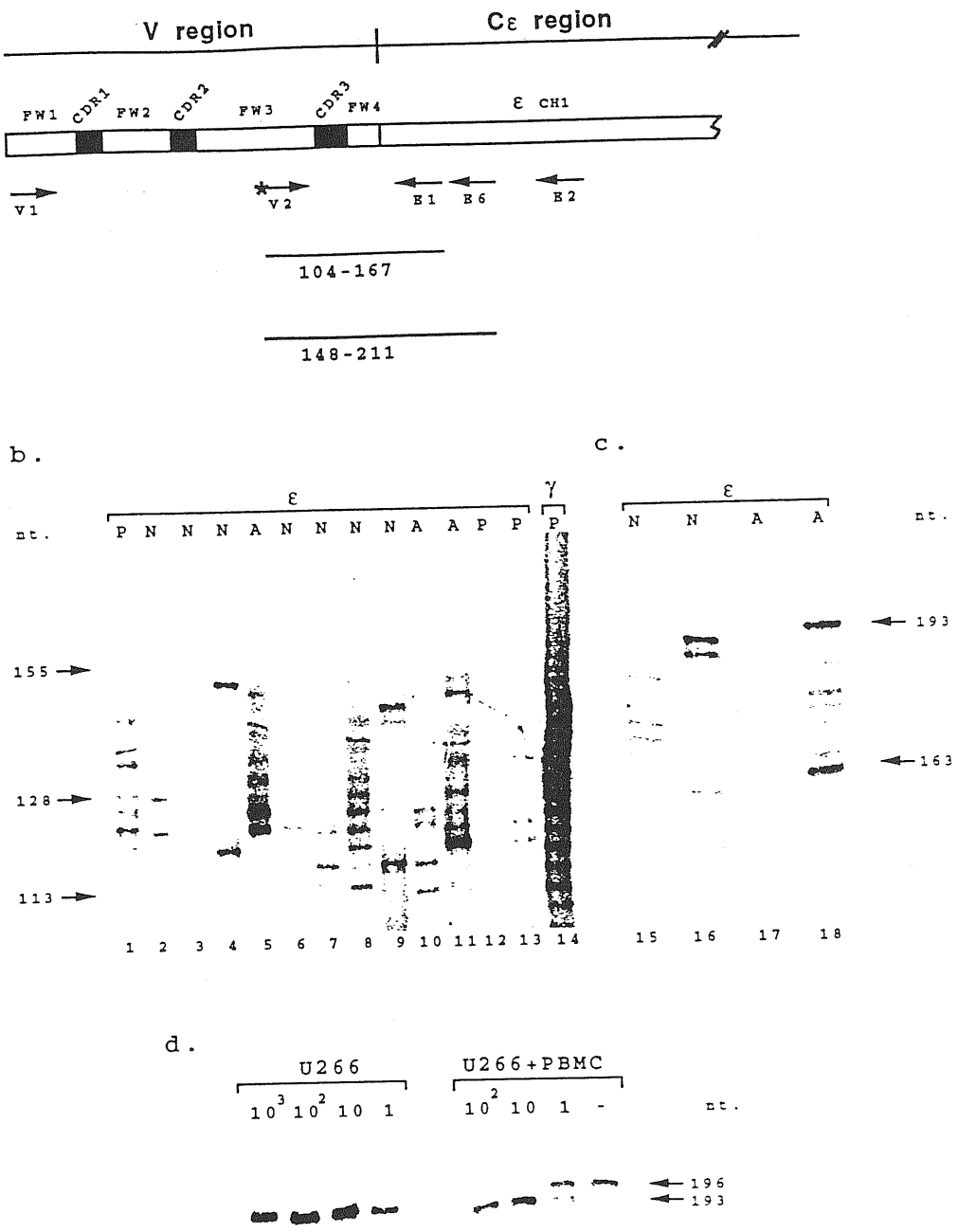


Figure 3.1.1: Analysis by RT/PCR of the ε CDR3/FW4 regions. a) Schematic representation of the positions of the different oligonucleotide primers. The size ranges of the expected fragments corresponding to the ε CDR3/FW4 sequences are indicated. b) Total PBMC cellular RNA from normal (N, lanes 2, 3, 4, 6, 7, 8, 9), and allergic (A, lanes 5, 10,11) individuals, and from 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-V2 and E1 in the second round of PCR (ε, lanes 1 to 13). Amplification of γ chain cDNAs was performed with primers V1 and G1, followed by a second PCR with primers ^{32}P-V2 and G1 (γ, lane 14). c) The first PCR samples from four individuals (corresponding to lanes 8, 9, 10 and 11 of fig 3.3.1b.) were reamplified with primers ^{32}P-V2 and E6 (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 V1/E2 and the second with ^{32}P-V2/E6. 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.

explanation unlikely, since sequence comparison of our degenerate 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 [28]. 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 [183] and which can be amplified even at low concentration (see below, Fig. 3.1.1d). 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. 3.1.1b). 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 (E6) which is located 44 nt downstream of the E1 oligonucleotide (Fig. 3.1.1c). 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 non atopic individual who showed a pattern consisting of a single band of 196 nt (Fig. 3.1.1d). 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.

3.1.3 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. 3.1.1b, 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. 3.1.2). 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).

N1

	_FW3_	CDR3 / NDN	JH	FW4	_ECH1_	JH Gene	Size nt.
(2)	tgcgaga	ggtccgtagtacggtattttgactggccgacatcct	tactactactactacggtatggacgtctggggccaagggaccacggtcaccgtctcctca		gcctcca	JH6	155
(12)	tgcaaaa	gacattggaggacgggtacggtgactac	gactactggggccagggaaccctggtcaccgtctcctca		gcctcca	JH4	128
(12)	tgcgaga	gcccgtggcagctactacgt	ctttgactactggggccaaggacccctggtcaccgtctcctca		gcctcca	JH4	122
(6)	tgcgaga	gtaccgcgctacagttac	ttcgacccctggggccagggaaccctggtcaccgtctcctcg		gcctcca	JH5	119

N6

	_FW3_	CDR3 / NDN	JH	FW4	_ECH1_	JH Gene	Size nt.
(1)	tgccctg	agctttttgcgtggatacagctatgttaccgagacggc	tactttgactactggggccagggaaccctggtcaccgtctcctca		gcctcca	JH4	143
(1)	tgcaaag	agcccctatagcagctcgttgc	ctgaagacttccgccactggggccagggcaccctggtcatcgtctcgtca		gcctcca	JH1	131
(1)	tagtaga	cataattcttatggtgactacgatcgtcct	tttgactactggggccagggcaccctggtcaccgtctcctca		gcctcca	JH4	
(1)	tgcgaga	actactatagcagtggaag	ggttcgacccctggggccagggaaccctggtcaccgtctcctca		gcctcca	JH5	122
(1)	tgccagg	ggggactgcagctggtgcc	actttgaatactggggccagggaaccctggtcatcgtctcctca		gcctcca	JH4	

Figure 3.1.2: Nucleotide sequences of PCR amplified ε CDR3/FW4 and adjacent regions obtained from two normal (N1 and N6) and two atopic (A1 and A3, 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. 3.1.1b. (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.

A3

	_FW3_	CDR3 / NDN	FW4 / JH	_ECH1_	JH Gene	Size nt.
		DHQ52-C1 DXP3				
(1)	tgcgaga	gatcataatccccaggattrctatgctagtagtgggtatttcccc	tactttgattcctggggccagggaacccsggtcaccgtctcctca	gcctcca	JH4	149
		DXP4				
(1)	tgcgaga	ggtcattacgatttttggagtgccattattataagggccccccgtc	ctggttcgacccctggggccagggaaccctggtcaccgtctcctcr	gcctcca	JH5	
(1)	--------	----------c----------a		-------		
(3)	--------	----------c---t----------a		-------		
(7)	--------	----------c----------t---------a		-------		
(1)	--------	----------c----------t-a-------a		-------		
(1)	--------	----------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	gttagtccagggagagtctggtgcctcgggtag	ttttgacatctggggccaagggacagtggtcaccgtctcttca	gcctcca	JH3	
		DXP4				
(2)	tgcaaac	ctaccacatgattacgatctttggagtggttattatatt	cagtggggccagggaarccsggtcaccgtctcctca	gcctcca	JH1	134
		DA4				
(2)	tgcgagg	gctgaggccacatacaataaccc	gctgaatacttccaacactggggccagggcaccctggtcaccgtctccsca	gcctcca	JH1	
		DN1				
(1)	tgcgaga	agtattacagcagcctgggcactgcc	tacttcgaccctggggccagggaaccctggtcaccgtctcctca	gcctcca	JH4	
(1)	-------	----------a--------	----------t----------	-------		128
		DL4 DM2				
(2)	tgcgagg	gacttagtcgtctatccgaaccat	gatatggacgtctggggccagggactacggtcaccgtctcctca	gcctcca	JH6	
		DA1				
(3)	tgcgaga	gcaatggaccgcagtgccgat	gcttttgatatctggggccaagggacaatggtcaccgtctcttca	gcctcca	JH3	125
		DXP'1				
(1)	tgcgaga	tctgggacaaccccccgggtactatgg	gatatctggggccaagggacaatggtcaccgtctcctca	gcctcca	JH3	
		DN1				
(1)	tgcgcga	gcaggggcagcagctgat	gcttatgatcctctggggccaagggacaatggtcaccgtctcttca	gcctcca	JH3	122
(7)	-------	----------	----------c---	-------		
(1)	----a--	----------	----------c---	-------		
		DM1*				
(5)	tgcgcga	acggtagaggaggacc	gttcgacccctggggccagggaaccctggtcaccgtctcctca	gcctcca	JH5	119
		DXP1**				
(1)	tgcgaga	gataacagtattgat	gcttttgatatctggggccaagggataatggtcaccgtctcttca	gcctcca	JH3	

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

A1

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

* DIR like sequence.

3.1.3a 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. 3.1.2). 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 [198-200]. It is interesting, however, that in these studies the JH1 gene was greatly underrepresented or absent, whereas we identified it in 4 out of the 26 different VDJ joinings sequenced (15 %).

3.1.3b 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 [201] 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) [198], 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. 3.1.2), 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 predicted value of 1.5 for FW mutations [202], 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.

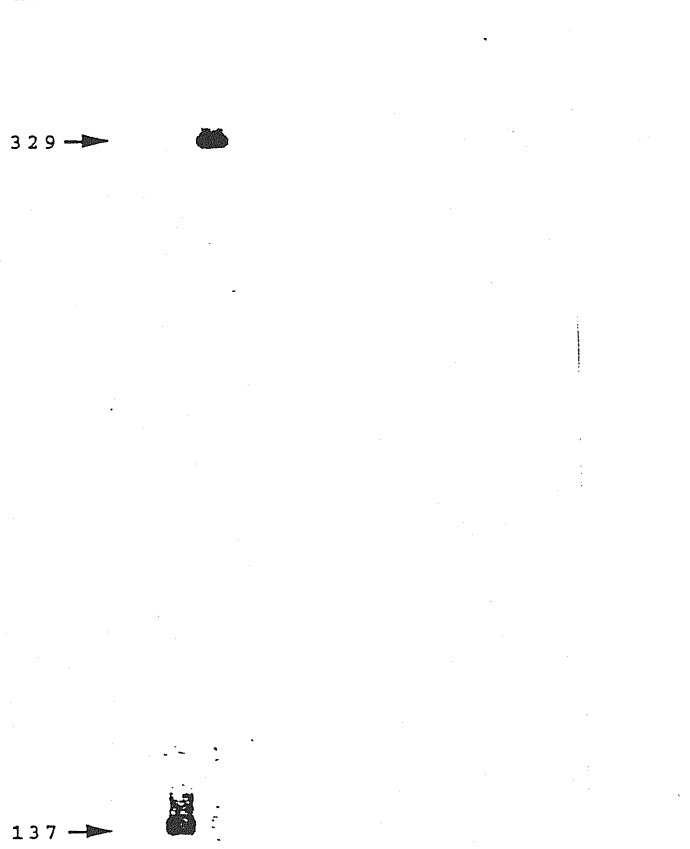
3.1.3c Clonally related ε transcripts with nucleotide differences in the FW4 sequence

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. 3.1.2), 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 (see below); 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).

3.1.4 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. 3.1.3a). 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. 3.1.3b). 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. 3.1.3c). 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-chain region was identical in all three γ4 transcripts, but contained three



b.

```
          _____ CDR3 _____/_____ FW4 _____/
(4)  tttggagtgcatattataagggcccccgtcctggttcgacccc tggggccagggcaccctggtcaccgtctcctca
(3)  ---------------------------------------- ----------------------t----------

     _____ μ CH1 _____.
     gggagtgcatccgccccaacccttttcccctcgtctcctatgagaattccccgtcggat.......
```

c.

```
          _____ CDR3 _____/_____ FW4 _____/
(2)  tttggagtgcatattataagggcccccgtcctggttcgacccc tggggccagggaaccctggtcaccgtctcctca
(1)  ----------------------------------------a- ----------------------t----------
(1)  ----------------------------------------ta- --------------------------------

     _____ γ4 CH1 _____.
     gccgtccaccaagggcccatcggtcttcccctggcgccctgctccaggagcacctccgagagcacagcggccctgggct
     _____.
     gcctggtcaaggactacttccccgaaccggtgacggtgtcgtggaactcaggcgccctgaccagcggcgtgcacacctt
     _____.
     cccggctgtcctacagtcctcaggactctactccctcagcagcgtggtgaccgtgccctccagcagcttgggcacgaag
     _____.
     acctacacctgcaac
```

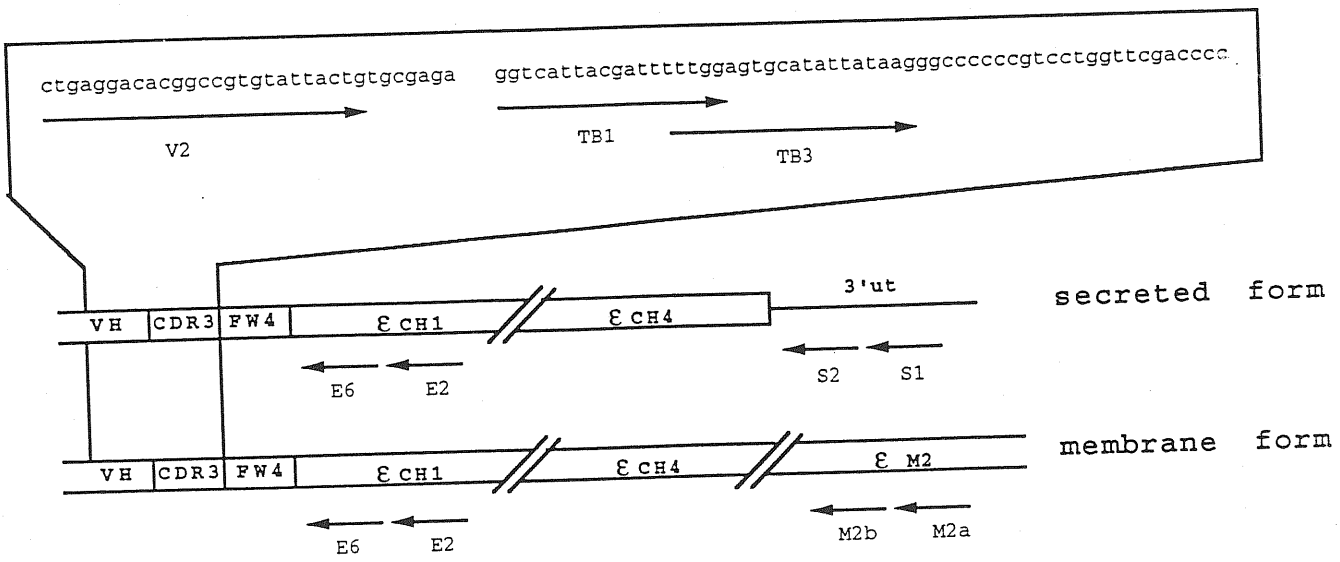
Figure 3.1.3: Detection of the A3/b CDR3/FW4 regions in μ (lane 1) and γ4 (lane 2) transcripts. a) The precise sizes of the radiolabelled PCR products obtained with primers ^{32}P-TB3/μ2 (lane 1) and ^{32}P-TB3/γ4.2 (lane 2) were determined with a sequencing ladder. b) and c) Nucleotide sequences corresponding 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 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 γ4b gene is presented with the three silent substitutions underlined. In parenthesis are indicated the number of clones sequenced.

substitutions with respect to the reported sequence of the γ4 constant region gene [37] 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. 3.1.3c, while the fourth one is absent since it corresponds to the 5' end nucleotide of the primer γ4.2.

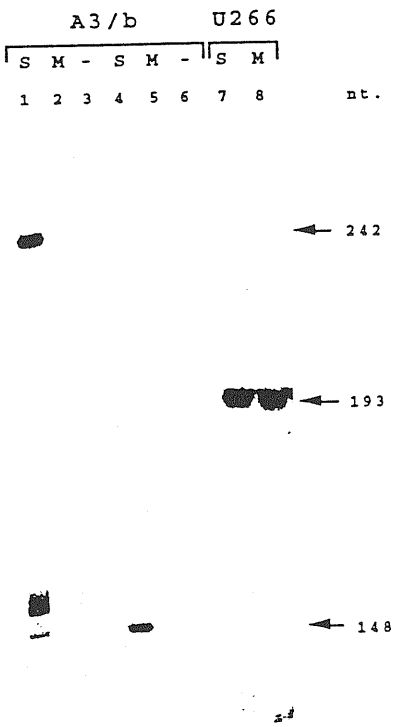
3.1.5 The clonally related A3/b transcripts are of the secreted form

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 εM2 exon [196] (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. 3.1.4a). A band of the expected size (app. 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 to the secreted ones, we performed additional PCR reactions with the internal ^{32}P labeled CDR3 specific primer TB2 and either the E2 or E6 primer, both from the first exon of the Cε 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. 3.1.4b). 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 which contain the M2 exon and that have been reported as being

a.


ctgaggacacggccgtgtattactgtgcgaga
ggtcattacgatttttggagtgcatattataagggccccccgtcctggttcgaccc
V2
TB1
TB3
VH
CDR3
FW4
ε CH1
ε CH4
3'ut
secreted form
E6
E2
S2
S1
VH
CDR3
FW4
ε CH1
ε CH4
ε M2
membrane form
E6
E2
M2b
M2a

b.


A3/b
U266
S M - S M - S M
1 2 3 4 5 6 7 8
nt.
242
193
148

c.

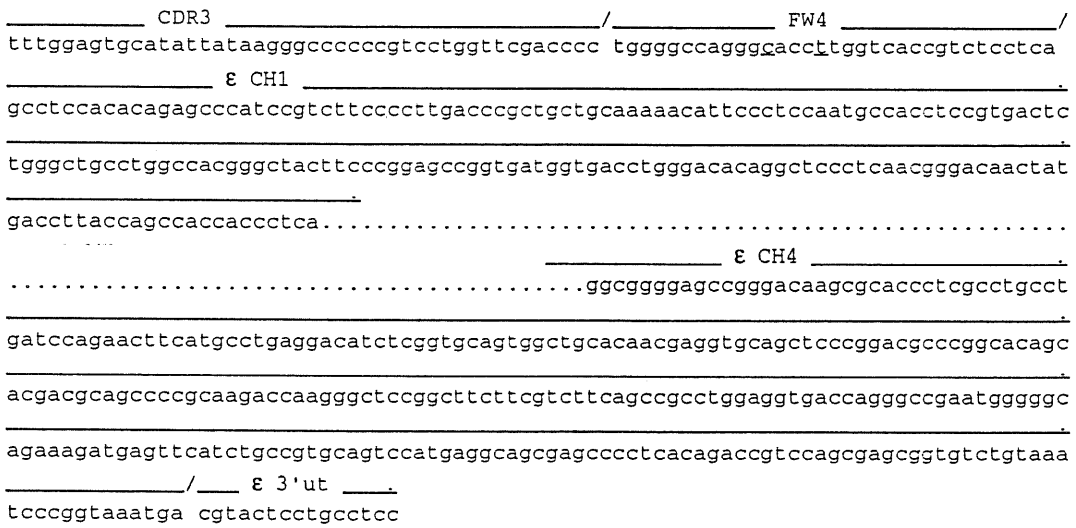


Figure 3.1.4: Analysis of the membrane (M) and secreted (S) forms of ε mRNAs containing the A3/b sequences. a) Schematic representation of the primers used for the PCR analysis. b) First PCR reactions were carried out with primers TB1/S1 (lanes 1, 4) and TB1/M2a (lanes 2, 5) on cDNA from individual A3 and primers V1/S1 (lane 7) and V1/M2a (lane 8) on cDNA from the U266 cell line. Lanes 3 and 6 represent negative controls with no cDNA (-). Radiolabelled products obtained after a nested PCR reaction with primers ^{32}P-TB3/E2 (lanes 1, 2, 3), ^{32}P-TB3/E6 (lanes 4, 5, 6) and ^{32}P-V2/E6 (lanes 7, 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' unstranslated region are shown. The two somatic mutations in FW4 are underlined.

putative secreted forms [196]. Positive control reactions were carried out with RNA from the cell line U266 which expresses the membrane and classical secreted form of ε mRNA. As seen in Fig. 3.1.4b, 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. 3.1.4c). 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 germline JH5 gene segment. The sequence of the 3' end confirmed that it belonged to the classical secreted form of ε mRNA.

RESULTS

Section 2

3.2.0 Characterization and Expression of Alternatively Spliced IgE Heavy Chain Transcripts

3.2.1 Introduction

The ε H-chain gene consists of four constant region exons (CH1-CH4) and two membrane exons (M1 and M2); the latter encode the transmembrane and intracytoplasmic parts of the membrane bound IgE [180, 203, 204] *The sequence of the human ε membrane exons was recently reported and allowed identification of two types of membrane transcripts which differ in the length of the first membrane exon (M1 and M1')* [196, 205, 206]. *These two ε mRNA species arise by alternative splicing between a donor splice site at the 3' end of the CH4 exon and two acceptor splice sites separated by 150 bp and located several hundred nucleotides downstream of the CH4 exon. The RNA species containing the longer variant of the first membrane exon (εCH4-M1'-M2) was found to be at least 100 times more abundant than the species containing the short membrane exon (εCH4-M1-M2) in IgE producing myeloma (SKO-007) and transfectoma cell lines* [206]. *Furthermore, the εCH4-M1'-M2 form was the only membrane protein that could be detected by immunoprecipitation in these cell lines* [206]. *An ε mRNA species that lacks both the M1' or M1 exon but contains the second membrane exon (M2) in a different reading frame has also been described* [196, 206]. *This RNA species appears to be rather abundant since it can be detected, together with the classical secreted (εCH4-S) and membrane (εCH4-M1'-M2) transcripts, by Northern blot analysis in the U266 cell line and its subclones SKO-007 and AF-10* [195, 196, 206, 207]. *A secreted ε protein of the corresponding size has been described to be expressed (by Western blotting) in the AF-10 cell line and also in the serum of a patient with IgE myeloma, and it has been suggested that this εCH4-M2' form might play an important and distinct role in allergic reactions* [196]. *Several other alternatively spliced forms, which lack different parts of the CH4 exon or contain additional sequences 3' to it, have also been identified by*

RT/PCR analysis of the U266 and AF-10 cell lines and in PBL stimulated with IL-4 and an anti-CD40 mAb [195, 196] *The presence of their putative protein products has not been yet investigated and it is unclear whether these ε transcripts have any functional significance or are just by-products of alternative splicing in the Cε locus. Furthermore, this ε mRNA splicing pattern could be a characteristic of transformed plasma-cell lines or a consequence of in vitro culture conditions and might not be representative of the splicing pattern that occurs in vivo.*

We have addressed these issues by analyzing the ε mRNAs produced by unstimulated PBL. Using RT/PCR and sequence analysis we identified eight alternatively spliced ε transcripts which were also present in the U266 cell line. The properties of the protein products of the different ε transcripts were investigated by construction of IgE H-chain expression vectors and examination of their expression in transfected cell lines.

3.2.2 RT/PCR analysis of alternatively spliced ε transcripts

To investigate the nature of the 3' end termini of the IgE H-chain transcripts we performed RT/PCR analysis on total cellular RNA obtained from PBMC of several individuals and from the U266 cell line. Each sample was separately amplified with an oligonucleotide complementary to a sequence in the εCH3 exon (E3) and either with an oligonucleotide 3' to the εCH4 exon (S1) or with an oligonucleotide in the M2 exon (M2a). Southern blot analysis of the PCR products was done with an oligonucleotide probe complementary to the 5' end of the εCH4 exon (E4). This probe hybridized to three major bands of approximatelly 270 bp, 410 bp, and 490 bp in all samples amplified with the E3/S1 primer pair (Fig. 3.2.1b). In one of the PBMC samples and in the U266 sample two bands of about 450 bp and 700 bp were detected after the E3/M2a PCR. The 450 bp band was also present in some of the other PBMC samples, while no hybridizing product could be detected in the rest. A few weak bands were also obtained in the amplifications with either pair of

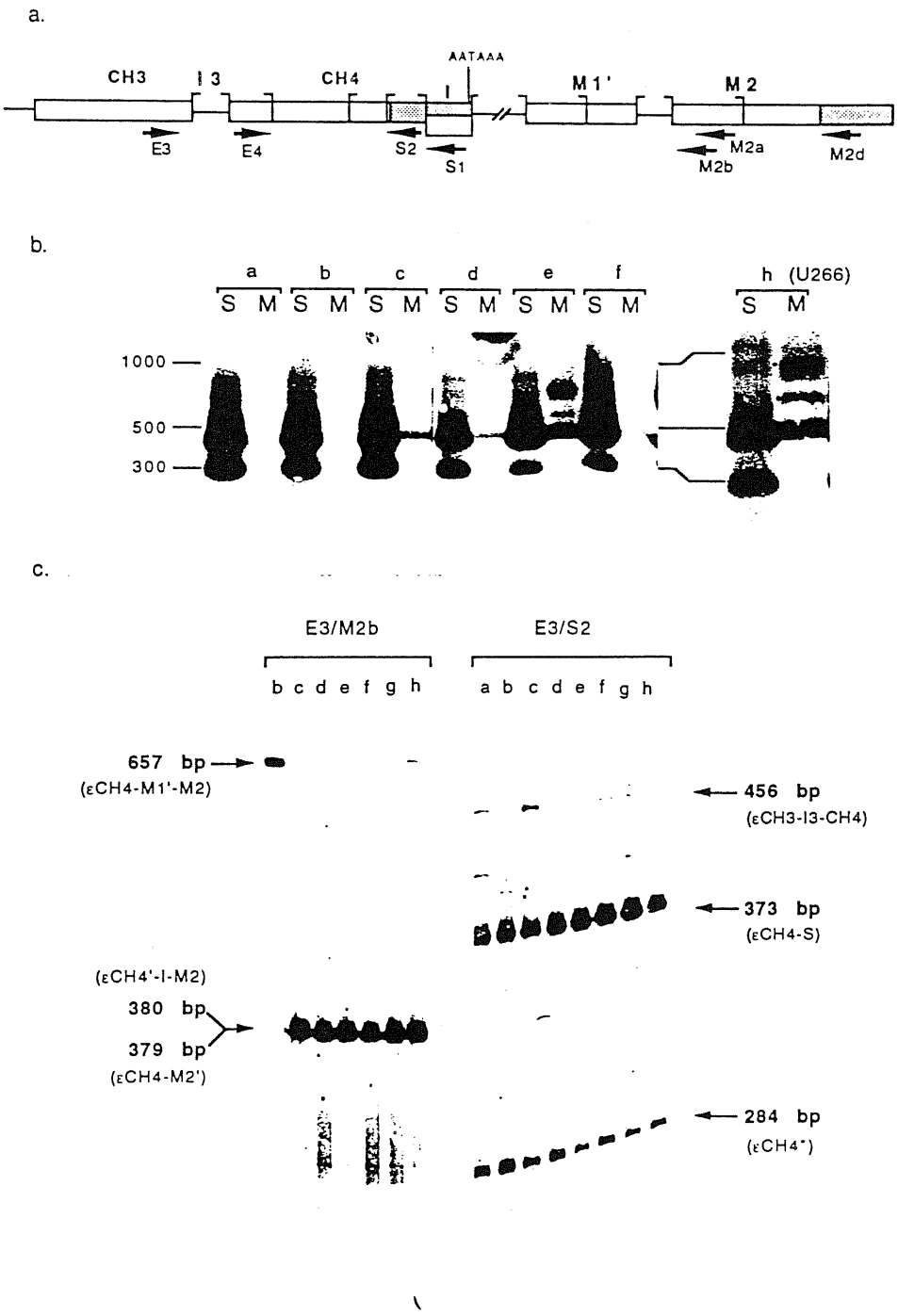


FIGURE 3.2.1: Analysis by RT/PCR of the alternatively spliced ε mRNAs. a) Schematic representation of the 3' end of the ε gene and the locations of the PCR primers (indicated by arrows). The identified donor and acceptor splice sites are shown. Open boxes represent coding sequences, shadowed boxes represent 3' untranslated regions. b) Autoradiogram of a Southern blot hybridization performed on RT/PCR products of PBMC RNA from different individuals (a to f) and from U266 cells (h), amplified with primers E3 and S1 (lanes S) and E3 and M2a (lanes M). The E4 oligonucleotide was used as a probe. c) Autoradiogram of a denaturing polyacrylamide gel electrophoresis of radioactive nested PCR products of samples S and M used in b, amplified with primers E3/^{32}P-S2 and E3/^{32}P-M2b, respectively. Sequencing reactions were included as size markers.

primers which could have been a consequence of heteroduplex formation. To investigate this possibility and to further increase the sensitivity of the assay, the E3/S1 and E3/M2a PCR samples were reamplified with the ^{32}P labeled internal oligonucleotides S2 and M2b, respectively. The radioactive PCR products were then separated on a denaturing polyacrilamide gel which prevented heteroduplex formation (Fig. 3.2.1c). Two bands of about 380 nt and 650 nt were now present in almost all samples amplified with the E3/M2b primer pair. Their sizes corresponded to the two major bands observed in the Southern blot analysis. The longer band of 650 bp was even detected, although in different relative amounts, in some of the samples that did not show it in the Southern blot. On the other hand, three major bands of about 280, 370 and 450 nt were obtained from the E3/S2 amplifications. The sizes of the two top bands corresponded to the two upper bands seen on the Southern blots, while the bottom band was approximatelly 50 nt longer than expected. These data suggested the existence of at least 6 alternatively spliced ε transcripts in the RNA from both PBMC and U266 cells.

3.2.3 Sequence analysis of the different ε mRNA species

The major bands observed on the autoradiograms from both the Southern blot and radioactive PCR analysis were cloned and sequenced. Figure 3.2.2 shows the 7 ε mRNA species that were identified with the assays described. Four of them, including the reported εCH4-S, εCH4-M1'-M2 and εCH4-M2' RNA species [195, 196, 206] and a new one containing the complete intron 3 (εCH3-I3-CH4) were cloned from both the first and the nested PCR reactions. These species corresponded to the bands of 410 bp, 700 bp, 430 bp and 490 bp from the Southern blots and the bands of 370 nt, 650 nt, 380 nt and 450 nt from the radioactive PCR analysis, respectively. The εCH4'-I species had also been previously

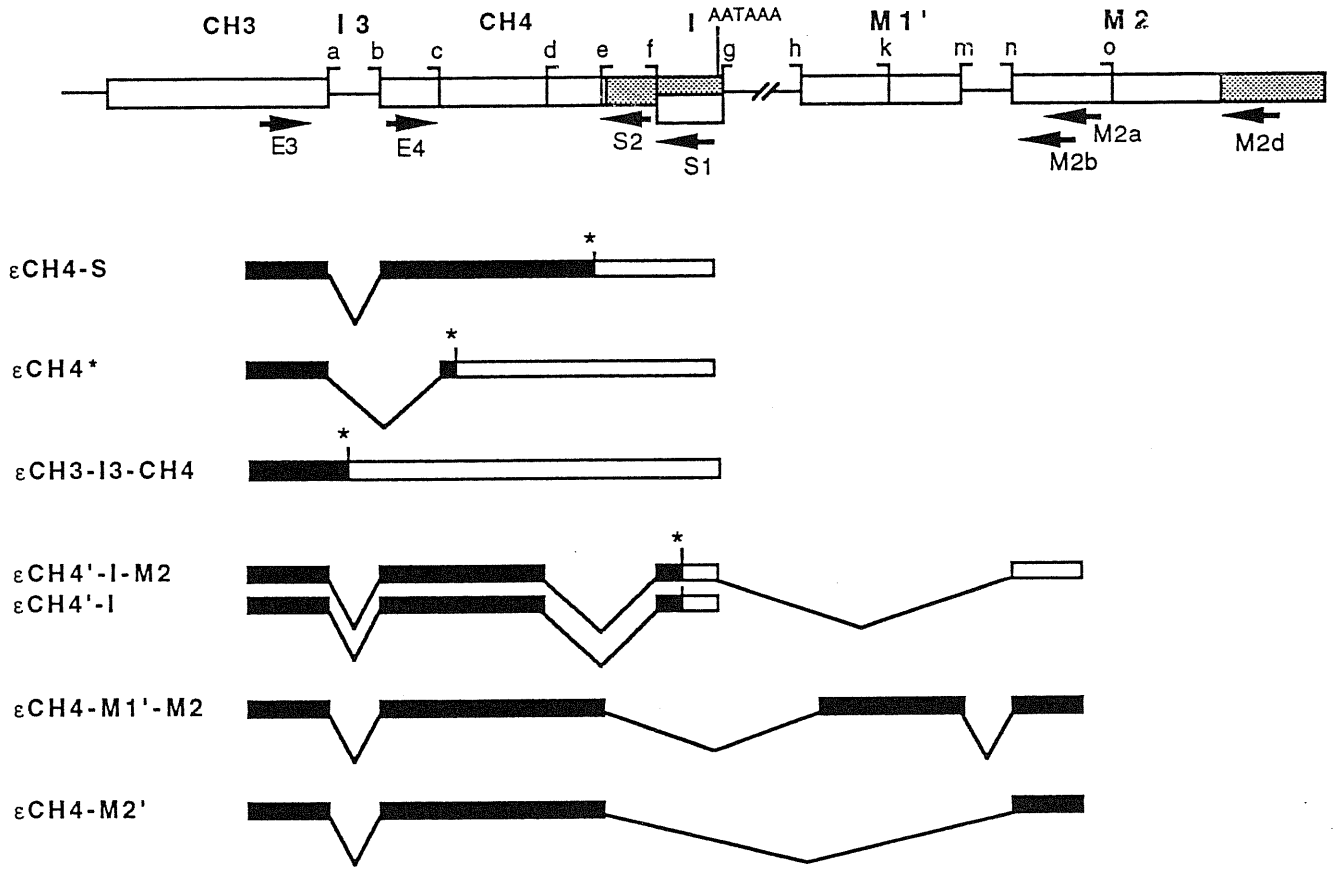


FIGURE 3.2.2: ε mRNA isoforms. Diagram of the identified alternatively spliced ε transcripts. Filled and open boxes correspond to translated and untranslated regions, respectively. Stop codons are indicated by asterisks. The donor and acceptor sites are indicated and named by letters.

reported and was cloned from the 1st PCR reaction. This species was seen as a band of 270 bp only in the Southern blot analysis because it did not contain the sequence complementary to the S2 oligonucleotide which was used in the nested PCR (Fig 3.2.3a). A variant of this species which contained the M2 exon (εCH4'-I-M2') was obtained from the E3/M2a PCR reaction. It could not be discriminated from the εCH4-M2' species in the Southern blots and the radioactive PCRs because of almost identical sizes (379 bp versus 380 bp), but appeared to be less abundant since it was identified in only one clone as opposed to 9 clones for the εCH4-M2'. The putative protein products of the εCH4'-I-M2 and εCH4'-I isoforms should be identical because of the presence of a stop codon in the I exon.

Another new species was identified and cloned from the 284 nt band of the nested PCR (Fig. 3.2.1c). This form, named εCH4*, was spliced from the donor splice site in the intron 3 to a new acceptor splice site 90 nt from the 5' end of the ε CH4 exon. The new reading frame created a stop codon 12 nt downstream of the new acceptor splice site in exon 4. This species was not observed on the Southern blots since it lacked the sequences complementary to the E4 oligonucleotide probe which was used for the hybridization.

3.2.4 Identification of a new ε mRNA species in PBL

During the course of this study a novel ε mRNA isoform was reported [208]. This form was generated by splicing from the usual donor site in the CH4 exon to a novel acceptor site in the terminal portion of the M2 exon. This CH4-M2" species was not detected with our initial RT/PCR assay due to the position of the primers used. The εCH4-M2" species was identified in the IgE producing myeloma cell line AF-10 and was also detected in EBV-transformed cells and lymphocytes from patients with high IgE levels after stimulation with IL-4 and anti-CD40 antibody. The εCH4-M2" mRNA has a one-nucleotide shift in the

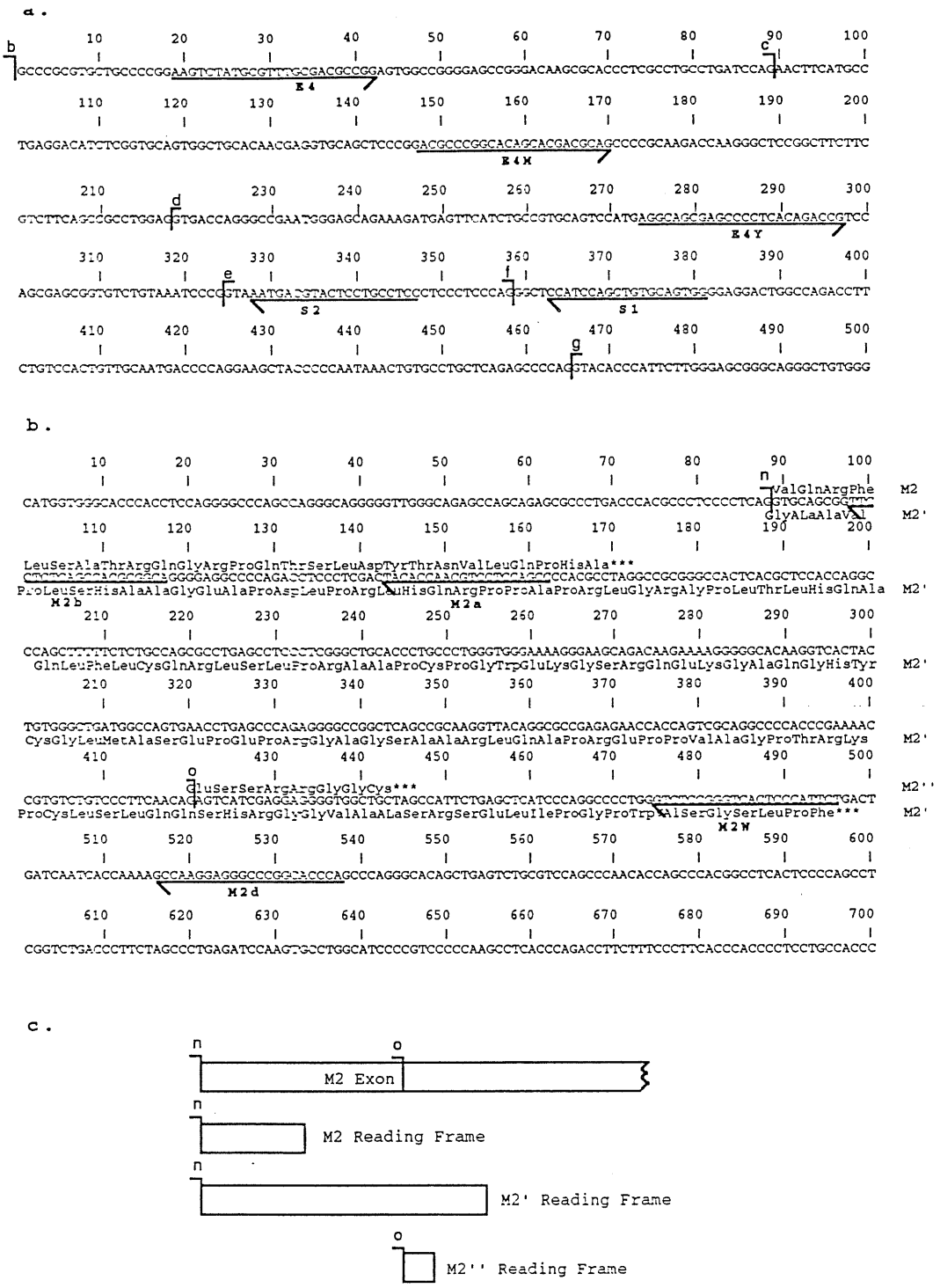


FIGURE 3.2.3: ε gene nucleotide sequences related to various alternative spliced mRNA. The donor and acceptor splice site are shown with the same nomenclature used in figure 3.2.2. a) The genomic sequence of the human CH4 (nucleotides 1-324) and its 3'-flanking sequence is presented. The oligonucleotides used for the PCRs analysis are also shown. The half arrow under the sequence indicates the orientation of the oligonucleotides. b) The sequence of the human ε membrane M2 exon (nucleotide 89-498) and part of its 3'-unstranslated region is shown. The amino acid sequences generated from the various alternative splicing products in the different reading frames are also given. The amino acids shown above the nucleotide sequence 89-169 represent the translation of the M2 exon in the M2 reading frame, whereas those below nucleotides 89-495 represent the translation in the reading frame named M2'. The amino acids shown above nucleotides 420-443 represent the translation of the third reading frame (M2"). c) Schematic representation of the three different reading frames in which the M2 exon can be stranslated to generated of the CH4-M1'-M2, CH4-M2', and CH4-M2" mRNAs.

reading frame of the M2 exon compared with the εCH4-M2' form (Fig 3.2.3b). As a result, the translation of the εCH4-M2" mRNA should terminate at the TAG codon at position 444 of the M2 exon. The εCH4-M2" polipeptide is six amino acids longer than the classical secreted ε H-chain and differs from it in the last 8 amino acids which are of considerable interest because of the presence of a cysteine in the terminal position. The protein sequences of the above described isoforms are shown in Figure 3.2.4.

To investigate the expression of this isoform in unstimulated PBL we used a semi-nested strategy with a new set of primers complementary to sequences in the CH4 and M2 exons. The E4M primer, which was used in the first PCR reaction, was located in the middle of the CH4 exon and could therefore prime all of the alternatively spliced ε mRNA species (Fig. 3.2.3a). This oligonucleotide was used together with the M2W primer which was located in the M2" exon, 230 bp downstream of the M2a primer used in the previous assay. To eliminate the species containing the I exon, a second PCR reaction was performed with the E4Y primer located at the 3' end of the CH4 exon. Samples from thirthy-two donors and also from the U266 cell line were analyzed with this assay. The analysis of ten donors and the U266 cell line is shown in Figure 3.2.5. Two bands of about 460 nt and 738 nt were present in all samples and were of the sizes expected for the εCH4-M2' and εCH4-M1'-M2 RNA isoforms, respectively. Their identities were confirmed by sequence analysis. A band of the expected size for the εCH4-M2" form was present in only two out of the thirthy-two individuals tested and also in the U266 cell line. These data suggested that the εCH4-M2" is not constitutively expressed in PBL and that its expression might be regulated.

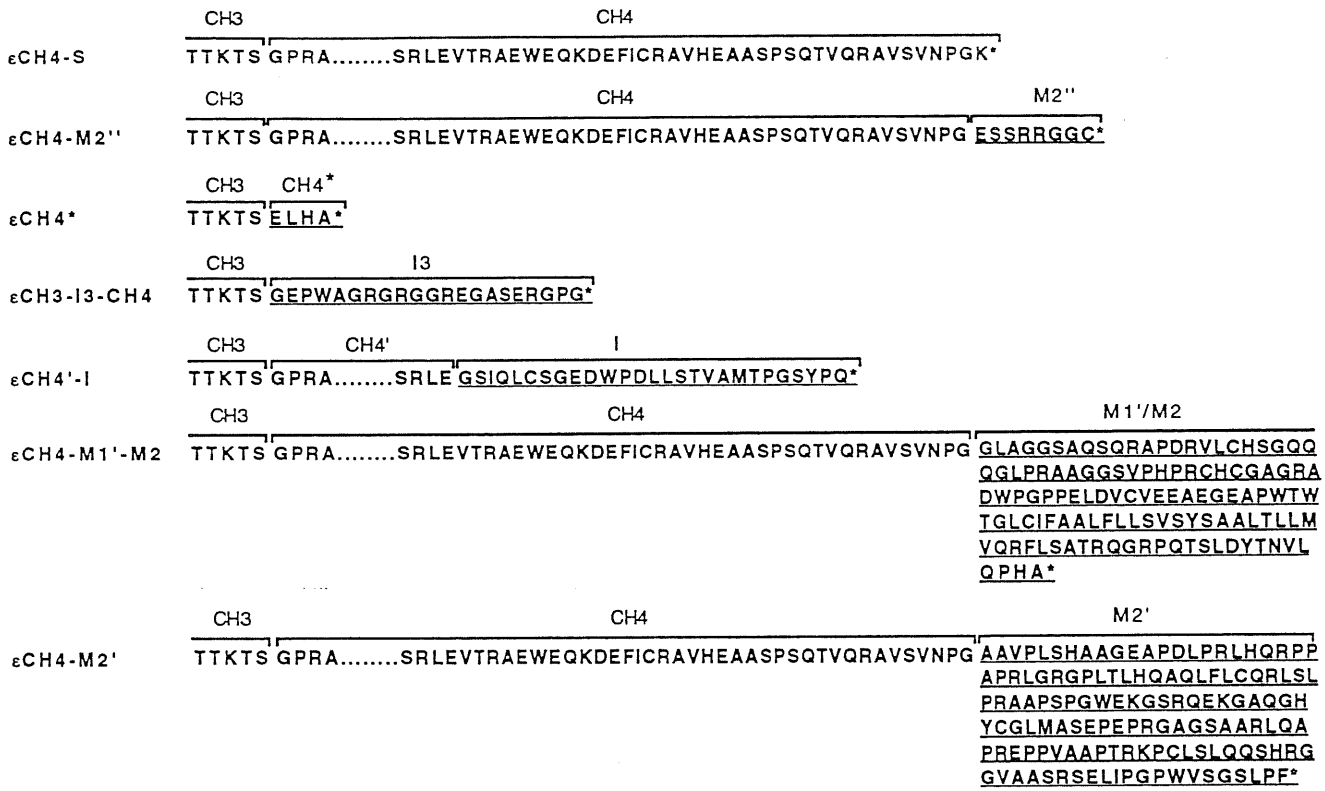


Figure 3.2.4: Amino acid sequences of the carboxyl termini of the ε isoforms shown from position 434 in the CH3 domain. The sequences that differ from the εCH4-S form are underlined and their exon origins are indicated above them.

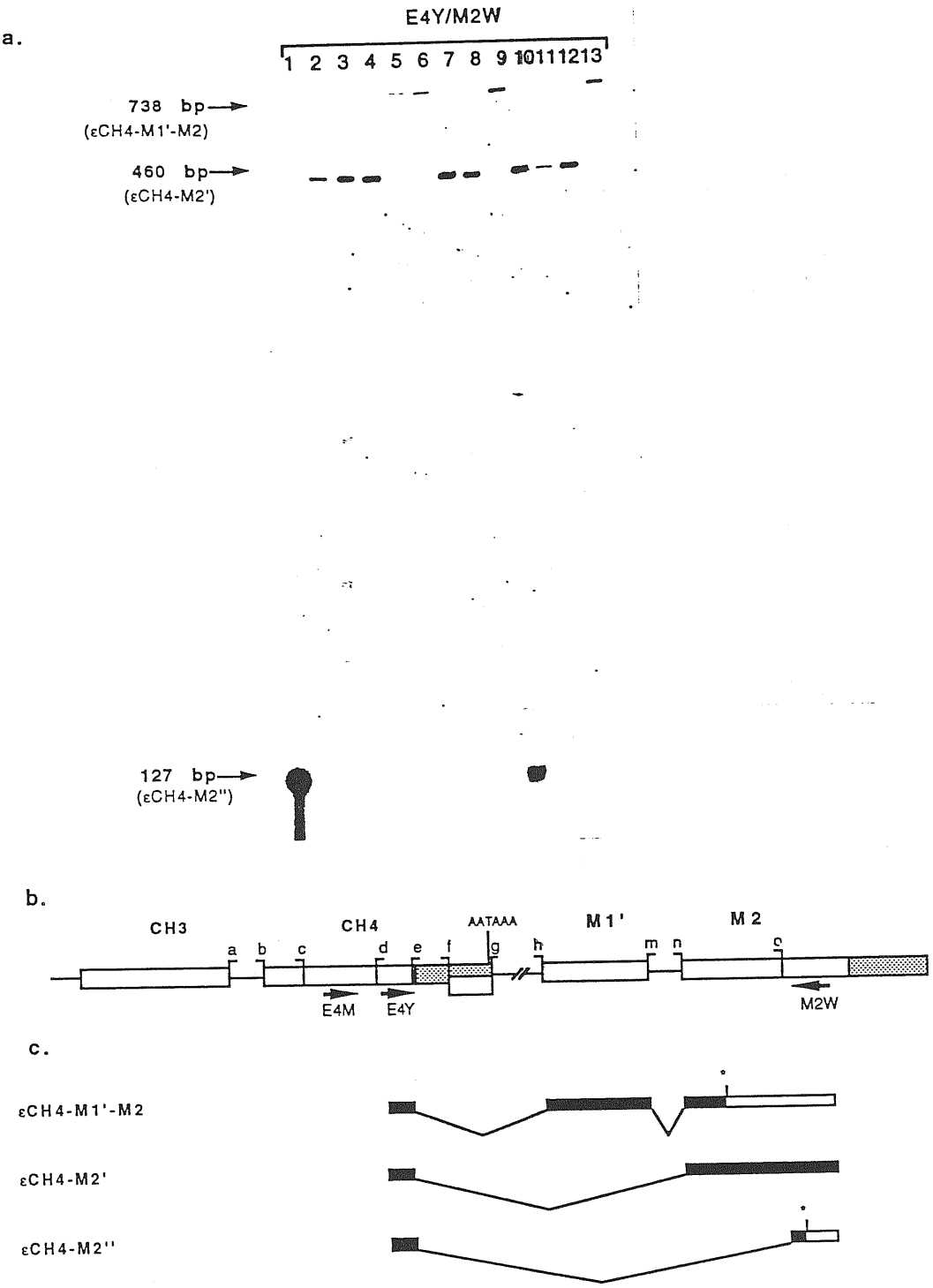


FIGURE 3.2.5: a) Autoradiogram of a denaturing polyacrylamide gel electrophoresis of radioactive nested PCR. A first round of amplification was performed with primers E4M and M2W and the products re-amplification with ^{32}P-E4M/M2W. Lane 1, RNA from U266, lanes 2-13 obtained from different healthy donors. b) Schematic representation of the 3' end of the ε gene and the locations of the PCR primers (indicated by arrows) used in the analysis showed in a). The identified donor and acceptor splice sites are shown. Open boxes represent coding sequences and shadowed boxes 3' untranslated regions. c) Diagram of the identified alternatively spliced ε transcripts. Filled and open boxes correspond to translated and untranslated regions, respectively. Stop codons are indicated by asterisks.

3.2.5 Immunoprecipitation of U266 cellular extracts and supernatants

By RT/PCR, cloning and sequencing we showed that the same alternatively spliced ε transcripts are present in RNA from both PBMC and the plasmacytoma cell line U266. To identify the proteins corresponding to these ε mRNA species, cellular extracts and supernatants from U266 were immunoprecipitated with a polyclonal anti-human IgE rabbit antiserum and analyzed on an 8% SDS-PAGE under reducing conditions (Fig. 3.2.6a). Two major bands, in the size range expected for the classical secreted form (76-79 kDa), appeared after immunoprecipitation of the cellular extract. The bands found in the culture supernatant were slightly higher in molecular weight (78-82 kDa), as expected from terminal glycosylation of secreted proteins (Fig. 3.2.6a and b). The possibility that the presence of two bands was due to different degrees of glycosylation was investigated by treating the labeled U266 supernatants with Endo Hf (which cleaves high mannose and some hybrid oligosaccharides, but does not cleave complex oligosaccharides present in terminally glycosylated proteins) and with PNGase F (which cleaves high mannose, hybrid and complex oligosaccharides). Figure 3.2.6b shows the analysis of these samples on a gradient 7-12% SDS-PAGE under reducing conditions. Treatment with Endo Hf led to a slight reduction in the size of the bands from the doublet, thus suggesting that a few non-terminally glycosylated oligosaccharides are present in the secreted ε chains. The persistence of the doublet after the treatment excluded the possibility that the two bands represent ε chains which differ only in the content of high mannose and hybrid oligosaccharides. On the other hand, treatment with PNGase F completely eliminated the doublet and yielded a single band of about 62 kDa, demonstrating that the two bands represent ε chains that differ in the content of complex oligosaccharides. Although the size of this band corresponded to the εCH4-S species, it could not be excluded that it also contained other ε isoforms, such as the εCH4'-I and/or the εCH4-M2", with similar predicted molecular weights.

FIGURE 3.2.6: Immunoprecipitation of ε chains produced by U266 cells. Cells were [^{35}S]methionine labeled for 5 hours and the total cell lysate (Intracell.) and culture supernatant (Secreted) were immunoprecipitated with polyclonal rabbit anti-human IgE. a) Samples of intracellular and secreted material were separated on a reducing 8% SDS-PAGE. Apparent molecular masses are expressed in kDa. b) Treatment of material immunoprecipitated from U266 supernatant with Endo H_f and PNGase F. Samples were analyzed on a gradient 7-12% SDS-PAGE under reducing conditions. The untreated sample corresponds to the secreted sample in Figure 3.2.6a.

A minor band of 88 kDa was also obtained after immunoprecipitation of the U266 cellular extract and corresponded in size to the classical membrane form εCH4-M1'-M2.

3.2.6 Expression of 6 alternatively spliced ε mRNA species

The immunoprecipitation experiments with the U266 cell line did not confirm the existence of proteins corresponding to all of the alternatively spliced ε mRNAs. This could have been due to lower mRNA levels, less efficient translation, or more rapid turnover of the protein products of some of these species. In order to investigate the properties of each of these isoforms in the absence of competition for protein processing factors, we generated chimeric mouse/human ε chain gene constructs which contained a mouse VH segment from an antibody with anti-NIP specificity and human ε H-chains with carboxy termini corresponding to the εCH4*, εCH4-M2', εCH4-S, εCH4'-I, εCH4-M2" and εCH4-M1'-M2 forms. The εCH3-I3-CH4 form was not included since this species most likely represented incompletely processed ε mRNA.

A schematic diagram of the 6 chimeric human/mouse gene constructs is depicted in Figure 3.2.7. These chimeric genes were independently transfected in the J558L mouse myeloma cell line which produces only the light chain of the anti-NIP antibody. Transfectants expressing the different proteins were identified by Western blot analysis of the anti-NIP affinity purified cellular extracts using the anti-IgE polyclonal rabbit antiserum (data not shown).

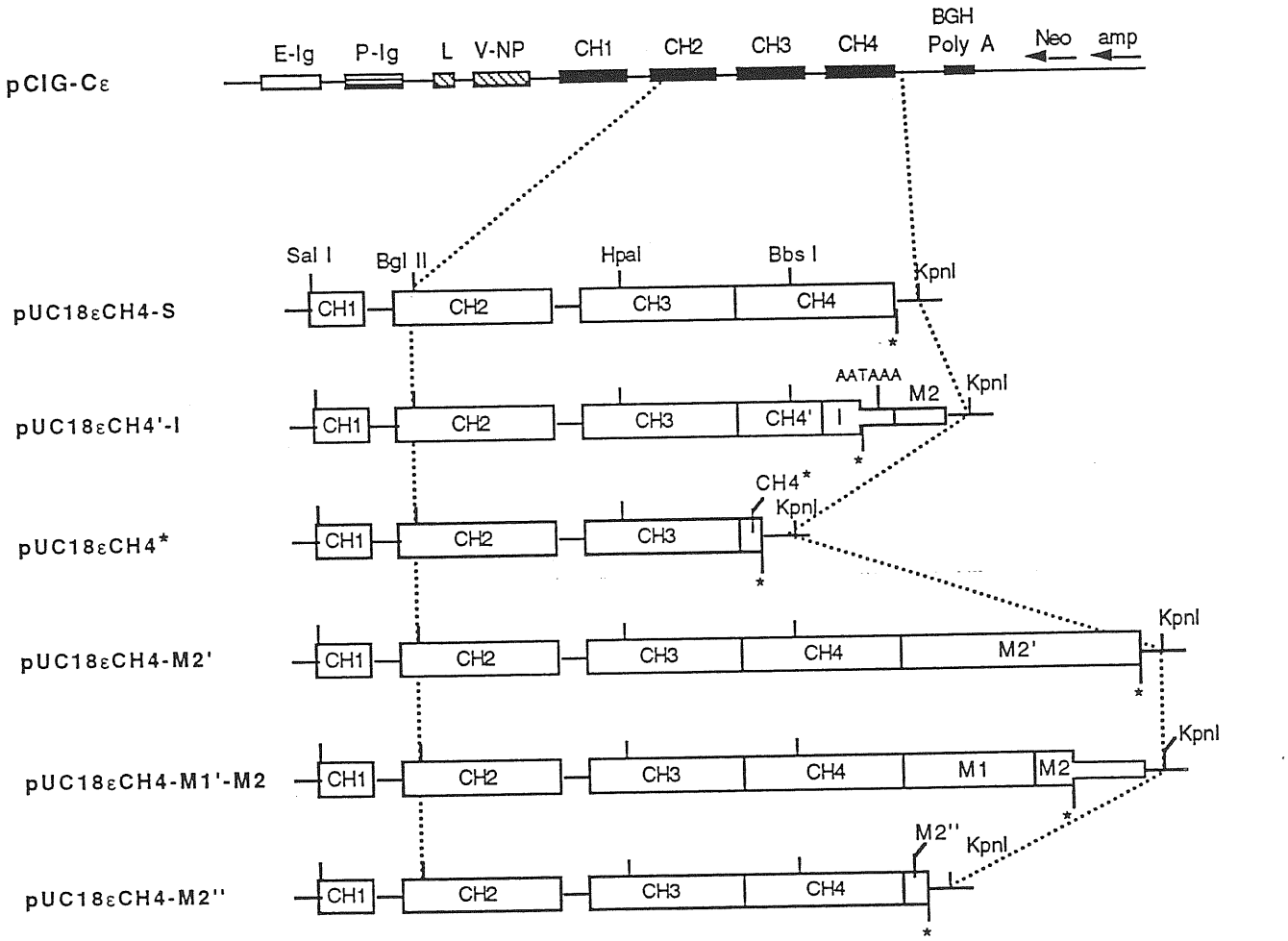


FIGURE 3.2.7: Diagram of the chimeric mouse V-Nip/ human Cε gene constructs. The 3' ends of the different ε isoforms were cloned (in their cDNA form from exon CH3) into pCIG-Cε, as described in Materials and Methods. pCIG-Cε contains the mouse immunoglobulin enhancer (E-Ig) and promoter (P-Ig) located upstream of the mouse leader (L) and variable (V-NP) exons, the four exons of the human Cε gene and the bovine growth hormone gene poly A site (BGH-poly A). The relative positions of the selectable marker genes, neomycin (Neo) and ampicillin (Amp), are shown by arrows. Stop codons are indicated by asterisks.

3.2.6a Analysis of the expression of εCH4*, εCH4-M2', εCH4-S, εCH4'-I and εCH4-M1'-M2 isoforms

Pulse-chase experiments were done in each of the cell lines and the immunoprecipitated proteins were analyzed under reducing conditions in a 10% SDS-PAGE. As shown in Figure 3.2.8, a band of the expected size was immunoprecipitated in all cases from the cellular extracts obtained after one hour labelling with [^{35}S]methionine. A protein of 80 kDa appeared in the supernatant of the εCH4-S cell line following the 4 hour chase and, as expected from terminal glycosylation, it was a few kDa larger than the intracellular form. On the other hand, no protein could be detected in the supernatants of the εCH4*, εCH4-M2' and εCH4'-I cell lines, suggesting that these forms are not secreted. These forms also appeared to be rapidly degraded since the amount of intracellular protein was substantially decreased after the 4 hour chase period, while it remained constant in the cell line expressing the classical membrane form (Fig. 3.2.8). Identical results were obtained by NIP-Sepharose affinity purification and Western blot analysis of proteins from supernatans of long term cultures, except for the presence of reactive material with a molecular weight of less than 50 kDa which was obtained from the εCH4*, εCH4-M2' and εCH4'-I cell lines (data not shown). Since this material was much smaller than the corresponding intracellular protein, it possibly represented degradation products released from dead cells or partially degraded IgE molecules in which the removal of particular sequences allowed their secretion.

The immunoprecipitation experiments of the cellular extracts and supernatants from the εCH4-M2', εCH4* and εCH4'-I transfectomas suggested that these forms are intracellularly retained and degraded. To investigate whether these forms can form hybrid molecules with the εCH4-S H-chains which could eventually be secreted we coexpressed the εCH4-M2' and εCH4-S proteins. This was done by transfecting the εCH4-M2' gene construct in a clone

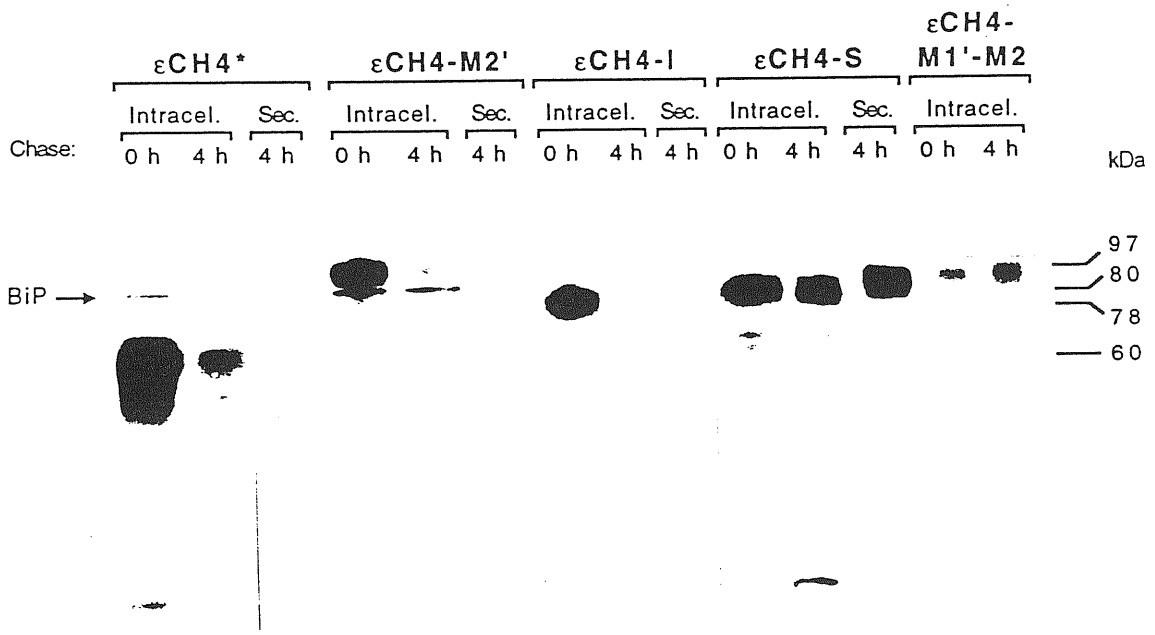


FIGURE 3.2.8: Immunoprecipitations of the chimeric IgE. Transfectoma cell lines were pulse labeled with [^{35}S]methionine for 1h. and chased in the presence of cold methionine for 4 hours. Cell lysates (Intracel.) and supernatants (Sec.) were immunoprecipitated with an anti-human IgE rabbit antiserum and analyzed on a reducing 10% SDS-PAGE. The estimated sizes of the different ε chains are: εCH4* 60 kDa, εCH4-M2' 94 kDa, εCH4'-I 78 kDa, intracellular εCH4-S 78 kDa, secreted εCH4-S 80 kDa, and εCH4-M1'-M2 88 kDa. A band corresponding in size to BiP is indicated with an arrow on the left.

derived from the J558L cell line which had previously been transfected with the Hu-pSVCε gene construct and produced the classical secreted form [194]. The presence of different drug resistance markers in the two constructs allowed the selection of a cell line expressing both isoforms. Due to the difference in size of the two polypeptides, double expressors producing both the εCH4-S and εCH4-M2' forms were identified by Western blot analysis of the anti-NIP affinity purified cellular extracts using the anti-IgE polyclonal rabbit antiserum. Pulse-chase experiments were done on two indepent clones expressing the εCH4-M2' and Hu-pSVCε constructs. The Hu-pSVCε transfectoma was included as a control of the classical secreted form. The immunoprecipitated proteins were analyzed under reducing conditions in a 10% SDS-PAGE. As shown in Figure 3.2.9, after immunoprecipitation of the 1 hour [^{35}S]methionine labeled cellular extracts, two bands of 78 kDa and of 94 kDa corresponding to the εCH4-S and the εCH4-M2' polipeptides were present in the double expressors, while only the first one was observed in the control. After the 5 hour chase, only a band of 80 kDa representing the εCH4-S polypeptide was immunoprecipited from the supernatants in all three cases. On the other hand, no protein of the expected size of εCH4-M2' could be detected suggesting that this form is not secreted even in the presence of the εCH4-S polypeptides. The amount of εCH4-M2' intracellular protein labeled during the pulse completely disappeared after the 5 hour chase period confirming the observed intracellular decay of this form in the εCH4-M2' transfectoma.

To investigate whether the antibody molecules were correctly assembled, unreduced samples from immunoprecipitated labelled cellular extracts of the εCH4*, εCH4-M2', εCH4-S, and εCH4'-I cell lines and the supernatant of the εCH4-S cell line were analyzed on a non reducing 7% SDS-PAGE (Fig. 3.2.10b). Dimerization to the complete H_2L_2 was observed for the εCH4-S form both intracellularly and secreted, while the other forms were present as intracellular HL monomers. Most of the secreted εCH4-S was dimerized, but a small quantity of terminally glycosylated HL monomer was also present in the supernatant,

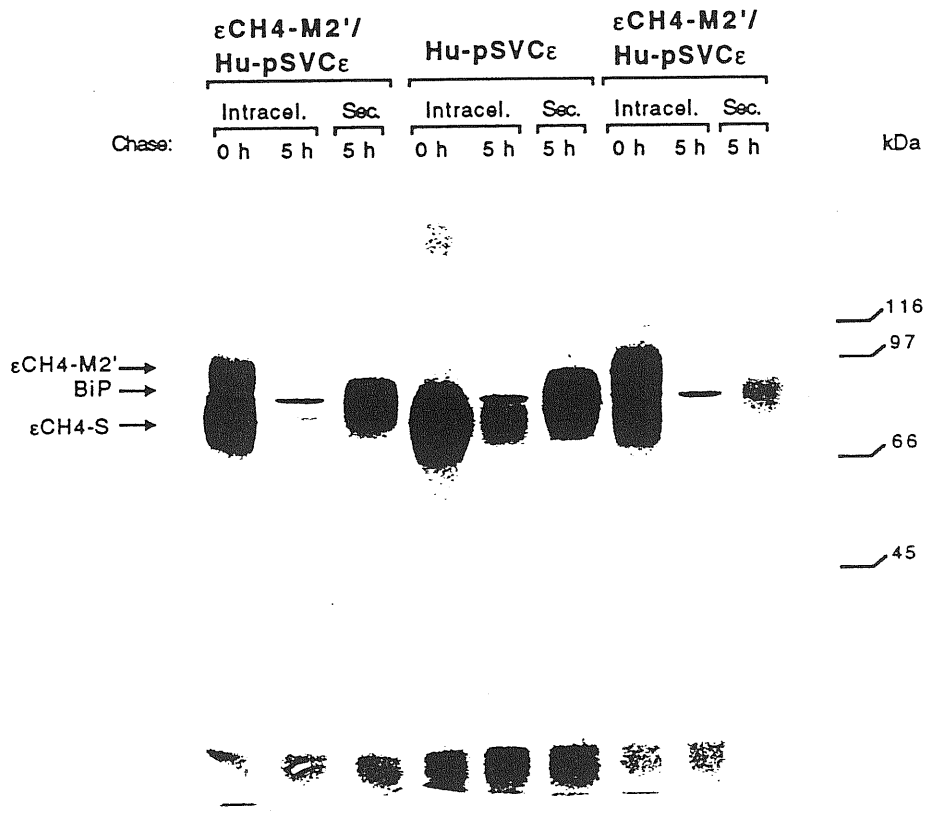


FIGURE 3.2.9: Immunoprecipitations of the chimeric IgE from two double expressor clones (εCH4-M2'/Hu-pSVCε) and a control, Hu-pSVCε, cell line. Transfectoma cell lines were pulse labeled with [35S]methionine for 1h. and chased in the presence of cold methionine for 5 hours. Cell lysates (Intracel.) and supernatants (Sec.) were immunoprecipitated with an anti-human IgE rabbit antiserum and analyzed on a reducing 10% SDS-PAGE. The estimated sizes of the intracellular εCH4-M2', εCH4-S, and of the secreted εCH4-S are 94 kDa, 78 kDa, and 80 kDa respectively. A band corresponding in size to BiP is indicated with an arrow on the left.

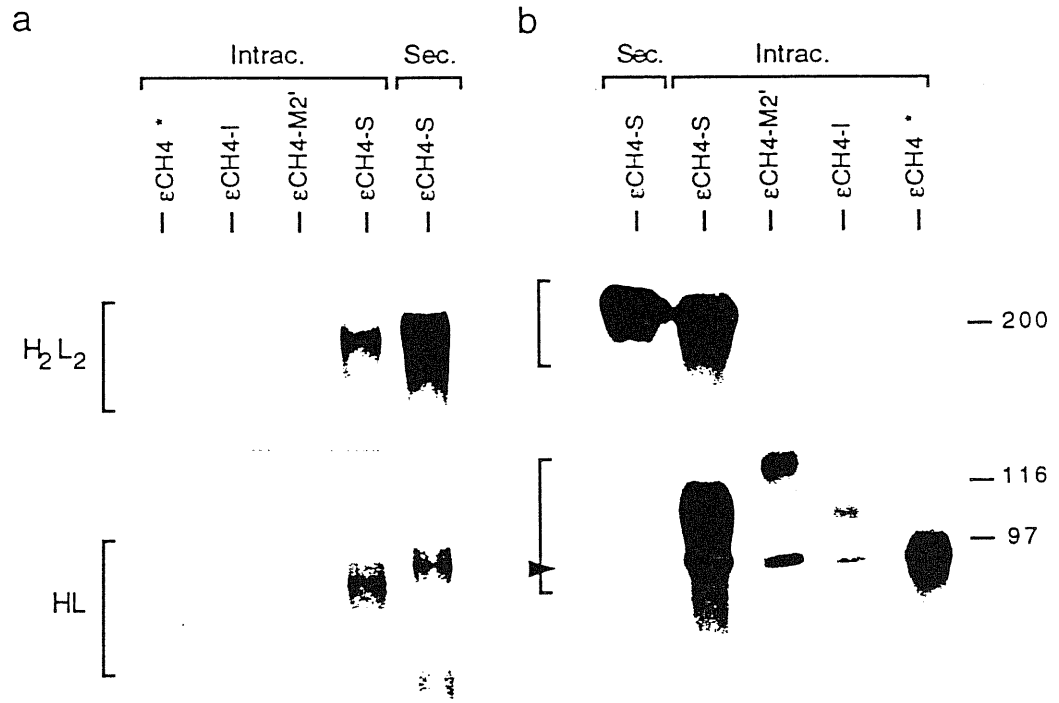


FIGURE 3.2.10: Assembly of the IgE molecules. a) Western blot analysis of NIP-Sepharose purified antibody molecules from cell lysates (Intrac.) and supernatants (Sec.) of the transfectomas. Electrophoresis was performed under non-reducing conditions in a 7 % SDS-PAGE. Rabbit polyclonal anti-human IgE was used and visualized with alkaline phosphatase-conjugated goat anti-rabbit immunoglobulins. b) Autoradiogram of immunoprecipitations with a rabbit polyclonal anti-human IgE antiserum of [^{35}S]methionine labeled (4h.) cellular extracts or supernatants. Samples were analyzed in a 7% non-reducing SDS-PAGE. The localizations of the H_2L_2 and HL molecules are indicated by brackets. The position of BiP is indicated

suggesting that some monomers or non-covalently linked HL dimers can also be secreted. These data were confirmed by Western blot analysis of NIP-affinity purified IgE (Fig. 3.2.10a).

The cellular localization of εCH4*, εCH4-M2', εCH4-S, εCH4'-I, and εCH4-M1'-M2 forms was investigated by immunostaining experiments using confocal microscopy. As shown in Figure 3.2.11, immunofluorescence analysis showed a clear different intracellular compartmentalization for the εCH4-S and εCH4-M1'-M2 forms consistent with accumulation in the Golgi, while the other three forms were scattered throughout the cytoplasm, suggesting that they are retained in the endoplasmic reticulum. Co-staining with wheat germ aglutinin, a marker specific for the Golgi apparatus [209] that also stains the membrane glycoproteins, confirmed the subcellular localization of the εCH4-S and εCH4-M1'-M2 chains. Immunofluorescence analysis of non-permeabilized cells showed that only the classical membrane form is expressed as surface immunoglobulin (Fig. 3.2.11 and data not shown).

3.2.6b Analysis of the expression of the εCH4-M2'' isoform

In order to investigate the properties of the protein encoded by the εCH4-M2" isoform, its corresponding gene construct was transfected in the J558L cell line. This construct was also used to transfect the Hu-pSVCε clone derived from the J558L cell line which had been described in the preceding section. Transfectants expressing only the εCH4-M2" form were identified by Western blot analysis of the anti-NIP affinity purified cellular extracts using the anti-IgE polyclonal rabbit antiserum. Double expressors producing both the εCH4-S and εCH4-M2" forms were identified by RT/PCR analysis of clones from the Hu-pSVCε transfectoma.

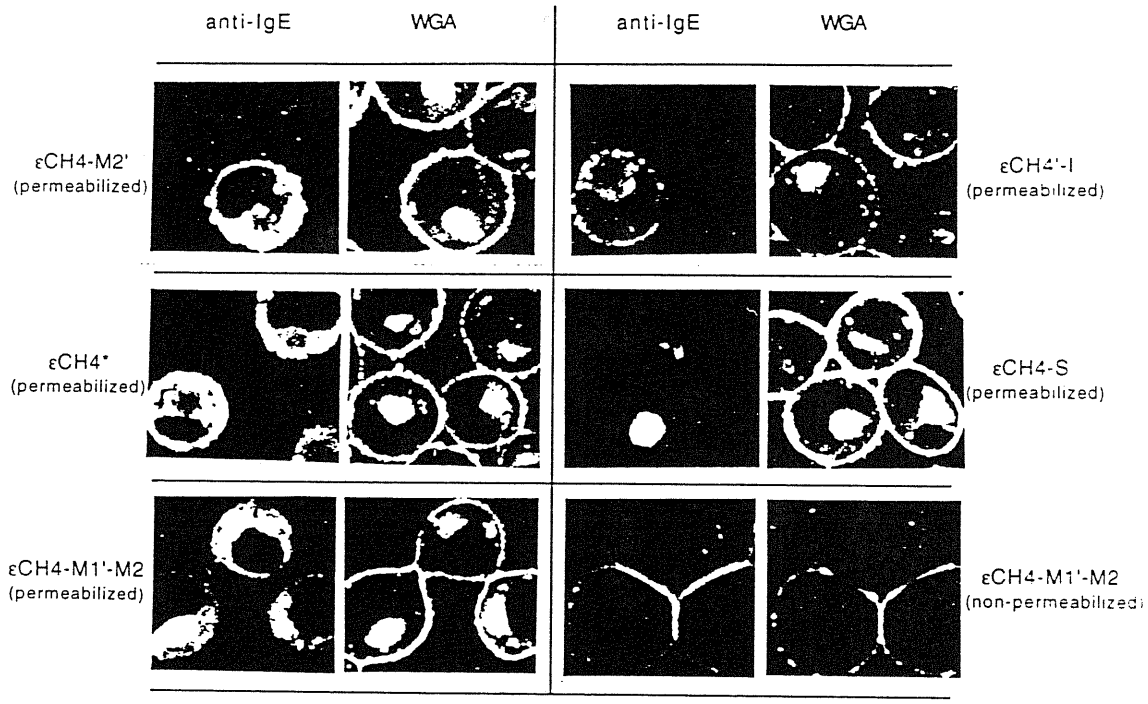


FIGURE 3.2.11: Double indirect immunofluorescence analysis (fluorescein for anti-IgE, rhodamine for WGA) by confocal microscopy of the transfectoma cell lines εCH4-M2', εCH4'-I, εCH4*, εCH4-S and εCH4-M1'-M2. The analysis was done on permeabilized or non permeabilized cells, as indicated. The same section is shown for anti-IgE and WGA in each cell line.

Pulse-chase experiments were done on the εCH4-M2" cell line and the immunoprecipitated proteins were analyzed under reducing conditions in a 10% SDS-PAGE. In this experiment the product of the Hu-pSVCε transfectoma was used as control of the classical secreted form. As shown in Figure 3.2.12, a band of 80 kDa was obtained from both cell lines after immunoprecipitation of the cellular extracts (1 hour labeled with [^{35}S]methionine) and supernatants (after the 5 hour chase). The presence of IgE molecules in the supernatant of the εCH4-M2" cell line clearly showed that this form is secreted in a plasmocytoma cell line. The quantity of protein that was immunoprecipitated from the supernatant was identical to the amount of protein obtained after the pulse, indicating that there was no intracellular retention or degradation of the εCH4-M2" isoform. Thus these experiments demonstrated the existence of two IgE secreted isoforms, the εCH4-S and the εCH4-M2" forms, which were both found to be correctly assembled and efficiently secreted by plasma cells.

An interesting aspect that was also investigated was the role of the terminal cysteine present in the εCH4-M2" protein. The possibility that it is involved in the polymerization of H2L2 dimers, as in the case of IgA and IgM, was addressed by analyzing the secreted material from the εCH4-M2" transfectoma on a non-reducing 6% SDS-PAGE. As shown in Figure 3.2.13, no band of higher molecular weight than that expected for the εCH4-M2" H2L2 dimers was present, demonstrating that these molecules do not associate as homo-dimers or -polymers. Moreover, the absence of HL monomers, which were present in the supernatant of the Hu-pSVCε transfectoma (Fig. 3.2.13), suggested that the extra cysteine is involved in an interchain disulfide bond between the two H-chains that stabilizes the H2L2 dimers or increases the rate of their association.

The experiments described above did not completely exclude the possibility that the terminal cysteine is involved in dimerization of H2L2 molecules, since dimers could also be formed by putative heterohybrids IgE antibodies containing one εCH4-S and one εCH4-M2" H-chain. These molecules would contain a terminal cysteine on only one chain which would

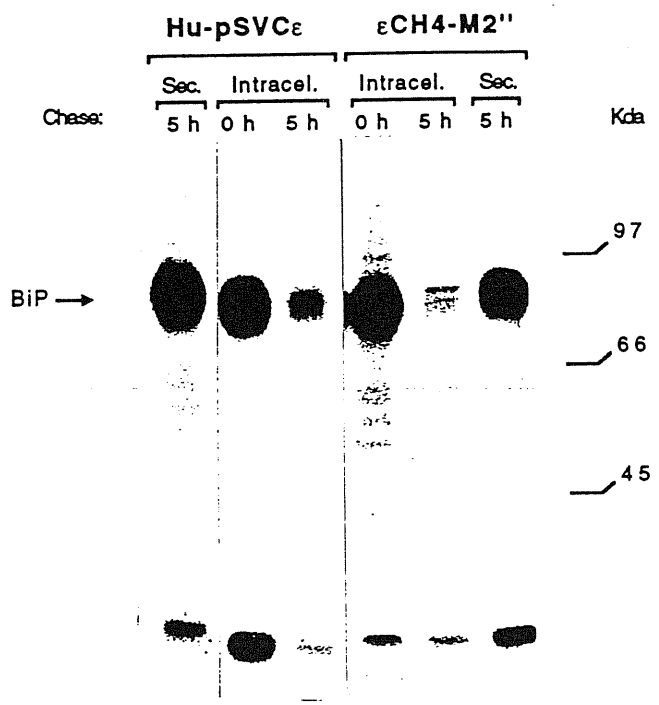


FIGURE 3.2.12: Immunoprecipitations of the εCH4-S from Hu-pSVCε and εCH4-M2" chimeric IgE. Transfectoma cell lines were pulse labeled with [35S]methionine for 1h. and chased in the presence of cold methionine for 5 hours. Cell lysates (Intracel.) and supernatants (Sec.) were immunoprecipitated with an anti-human IgE rabbit antiserum and analyzed on a reducing 10% SDS-PAGE. The estimated sizes of both ε chains are 78 kDa for the intracellular material and 80 kDa for the secreted one. A band corresponding in size to BiP is indicated with an arrow on the left.

FIGURE 3.2.13: Assembly of the εCH4-M2" secreted molecules. Autoradiogram of immunoprecipitations with a rabbit polyclonal anti-human IgE antiserum of the secreted material from the indicated cell lines. Cells were labelled with [^{35}S]methionine for 3 hours. Samples were analyzed on a 6% non-reducing SDS-PAGE. The localizations of the H2L2 and HL molecules are indicated by brackets.

be free to interact with a cysteine from an analogous molecule. This issue was addressed by analysing under non-reducing conditions the immunoprecipitated material from the double expressor cell line εCH4-M2"/Hu-pSVCε (Fig. 3.2.13). Like in the previous case, only a band of the size expected for the H2L2 form was present, demonstrating that the terminal cysteine in the εCH4-M2" form does not lead to polymerization of IgE antibodies.

RESULTS

Section 3

3.3.0 Identification and Characterization of the Second Poly (A) Site in the Human Constant ε Locus

3.3.1 Introduction

The mRNAs encoding the membrane-bound (m) and secreted (s) forms of immunoglobulin H-chains are produced from identical primary transcripts which are differently processed at their 3' ends. The relative abundance of these two types of mRNA is regulated during B-cell maturation, the membrane type being predominant in early stages and the secreted type being heavily favored in mature plasma cells. This regulation depends on competition between two mutually exclusive processing reactions, cleavage-polyadenylation at the secretory poly(A) site and removal of the intron that contains this site [210-213].

Studies of μm and μs production [212] *have indicated that the efficiencies of these alternative processing reactions must be delicately balanced in order to achieve developmental regulation. The work described in this section investigated the alternative processing of the most abundant ε isoforms in plasma cells and led to the identification and characterization of the poly (A) site of the membrane ε locus.*

3.3.2 The polyadenylation sites of the ε transcripts

The characterization of the 3' unstranslated region of the membrane ε H-chain mRNA was accomplished using the "RACE-PCR" procedure. Total RNA from the IgE secreting myeloma cell line U266 was reverse-transcribed using a 35-base oligonucleotide which

contained 17 dT residues and an adaptor sequence with three endonuclease recognition sites that are found infrequently in genomic DNA (dT/Ad). In order to identify the poly (A) sites of both the secreted and membrane loci, the resulting cDNA(-) was then amplified with an oligonucleotide that contained only the adaptor sequence (Ad) and the E3 or M2N oligonucleotide, located in CH4 and M2 exons, respectively (Fig. 3.3.1a). The PCR products were analysed by Southern blot analysis using as probe radiolabelled oligonucleotides complementary to sequences predicted to be present in the RACE products. In the E3/Ad amplification only one positive band of about 500 nt appeared after hybridization with the S2 oligonucleotide (Fig. 3.3.1b). This band was of the size expected for the classical secreted form if the AATAAA sequence located 110 nt dowstream of the stop codon in the ε CH4 exon served as the polyadenylation signal. Cloning and sequencing of this band confirmed that this AATAAA sequence is the actual polyadenylation signal of the classical secreted form (Fig. 3.3.2a).

In the case of the M2N/Ad PCR products, a single band of about 700 bp was observed after hybridization with the internal M2d oligonucleotide, suggesting that the transcripts containing the M2 exon also use a unique poly (A) site. Cloning and sequencing of this fragment provided more than 200 bp of new sequence from the membrane ε locus and also revealed an AGTAAA sequence located 13 nucleotides upstream from the poly (A) which presumably served as the polyadenylation signal (Fig. 3.3.2b). A 5 times tandem repeat sequence of about 41 nucleotides was also present (from nt 290 to 490 in Figure 3.3.2b) 200 nucleotides downstream of the M2' stop codon.

To further characterize the polyadenylation signal of the transcripts from the membrane ε locus and to investigate the presence of other potential polyadenylation signals we analyzed the genomic composition of the region downstream of the poly (A) site. The DNA sequence of the genomic flanking the poly (A) site in the 3' direction was obtained by a newly developed PCR procedure. This procedure took advantage of the PCR artifacts that are

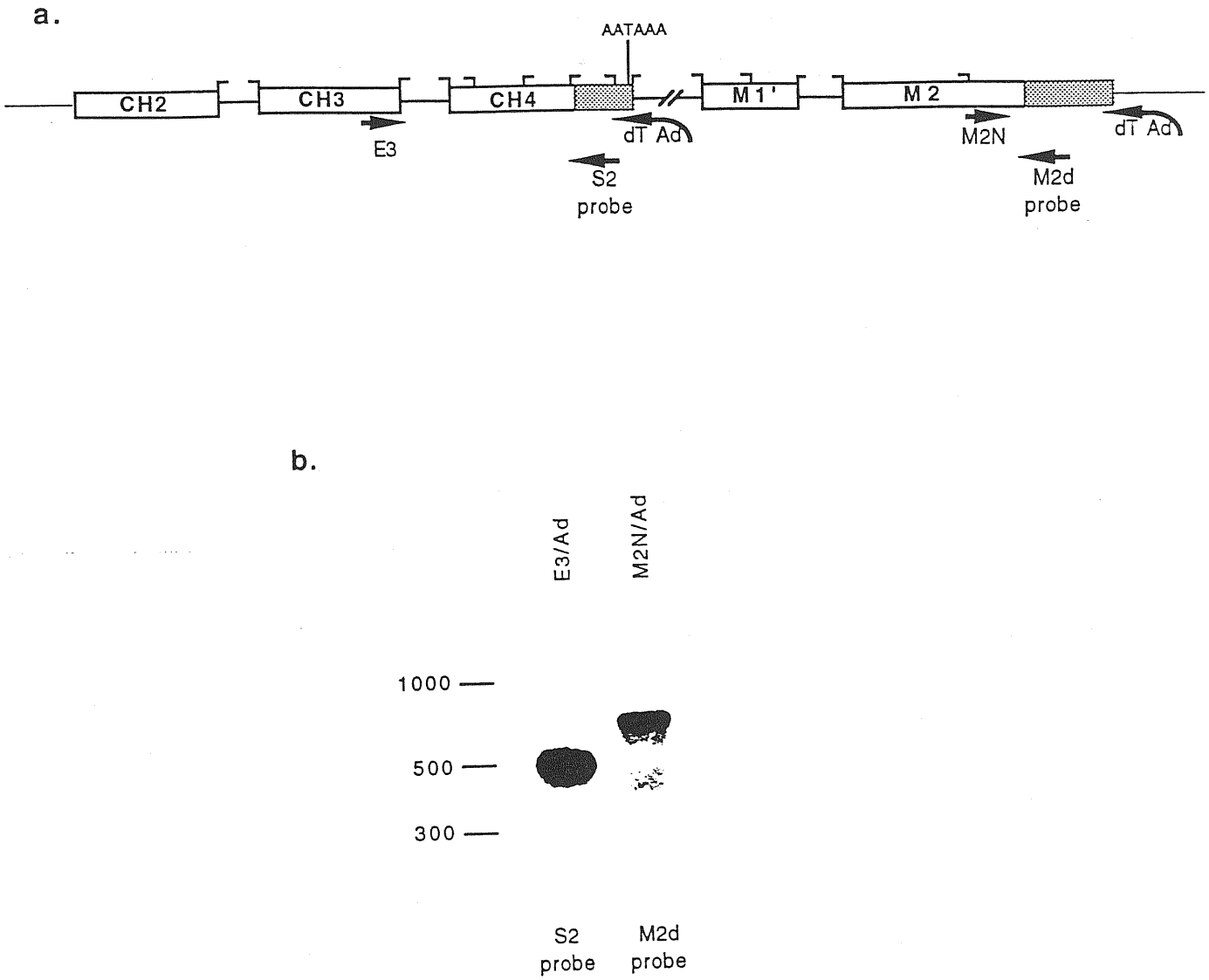


FIGURE 3.3.1: Analysis by RACE of the polyadenylation site usage in ε mRNAs. a) Schematic representation of the 3' end of the ε gene and the locations of the RACE/PCR primers (indicated by arrows). Open boxes represent coding sequences, shadowed boxes represent 3' untranslated regions. b) Autoradiogram of a Southern blot hybridization performed on RACE products with primers E3/Ad and M2N/Ad. The S2 and M2M oligonucleotides were used as probes.

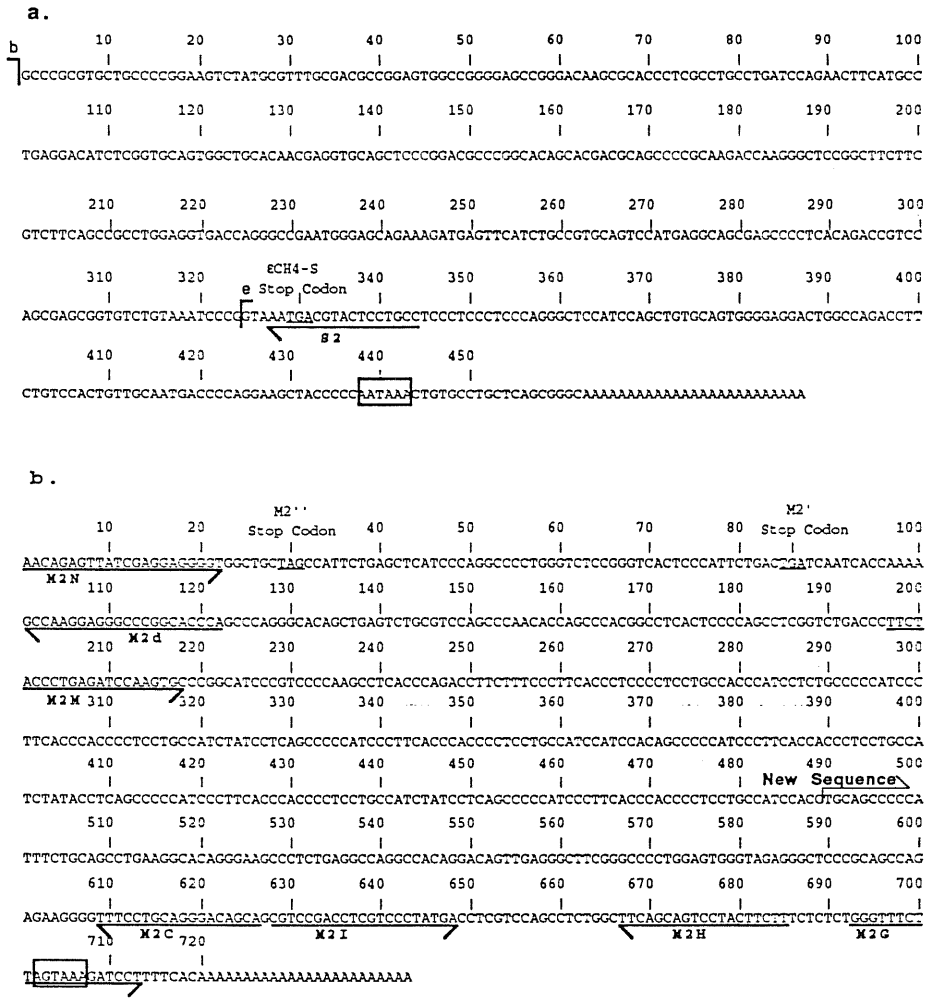


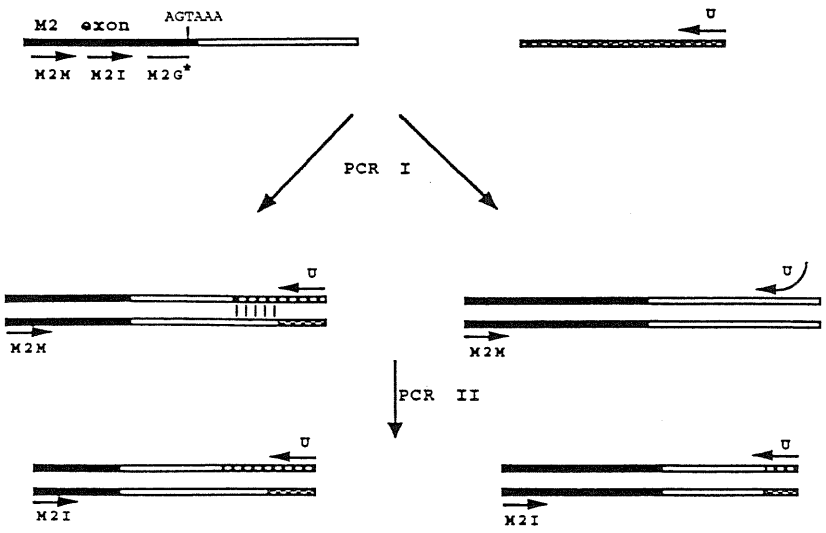
FIGURE 3.3.2: a) The genomic sequence of the human CH4 (nucleotides 1-324) and its 3'-flanking sequence is presented. The oligonucleotide S2 used for the hybridization is also shown. The AATAAA polyadenylation signal of the secreted ε locus is boxed. b) Genomic sequence of the 3' part of the human ε M2 exon (nucleotides 1-720). The M2' and M2" stop codon are underlined (nucleotides 29-32 and 87-90 respectively). The AGTAAA polyadenylation signal is boxed while a tract of A indicates the polyadenylation cleavage and addition site (position 720). The 5 times tandem repeat sequence is located between position 290-490. The oligonucleotides used for the PCR analysis and sequencing are also shown, the half arrow indicates the orientation of the oligonucleotides.

generated by primer misannealing and "jumping PCR" when the reaction is performed in the absence of correct template (Fig. 3.3.3a). The artifacts containing the sequence of interest were selected and further amplified in a seminested PCR to obtain the sequence of the genomic DNA region which lied outside the boundary of the known sequence of the Cε locus.

To create the artifacts, the primer M2M, located 5' to the unknown region, was used in a set of PCR reactions with unrelated primers, including primers from the α- and β-globin genes, the human immunoglobulin H-chain variable gene segments, the human immunoglobulin light chain (kappa) variable gene segments and primers from the IgE locus located upstream and oriented in the opposite direction of M2M. The artifacts created in these PCR reactions were reamplified in a second set of PCRs with the M2I oligonucleotide which is internal to M2M. As shown in Figure 3.3.3b, a number of bands appeared on the ethidium bromide stained agarose gels some of which were up to 3 kb long. The specific bands were identified by Southern blot analysis using as a probe the internal M2G oligonucleotide (Fig. 3.3.3c). Three of these bands were cloned and sequenced from both directions . Although they differed in size, they all contained the same sequence downstream of the poly (A) site. The longest one allowed the determination of more than 1.4 kb of new sequence (Fig. 3.3.4). Analysis of this sequence showed the presence of a conserved GT rich element located 33 nucleotides downstream of the CA dinucleotide where the cleavage/polyadenylation had occurred. The absence of any other consensus polyadenylation sequence in the 1.4 kb region downstream of this poly (A) site suggested that this is the only poly (A) site in the membrane ε locus.

The specificity of the obtained sequence was confirmed by PCR amplifications of genomic DNA with the primers M2N, M2M and M2I, located in the known region upstream of the poly (A) site, and primers M2S, M2T, M2V and M2U derived from the new

a.



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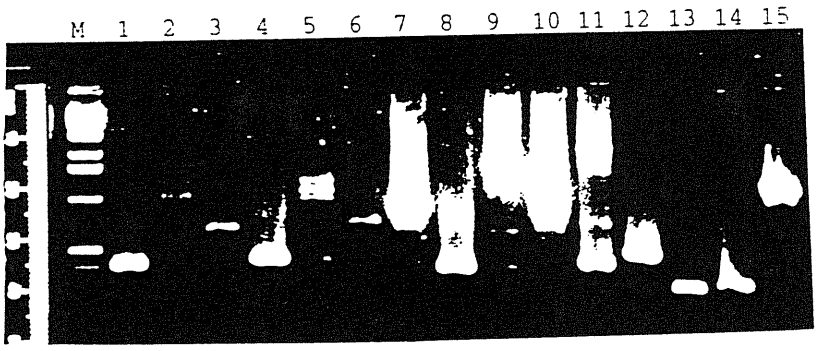

c.

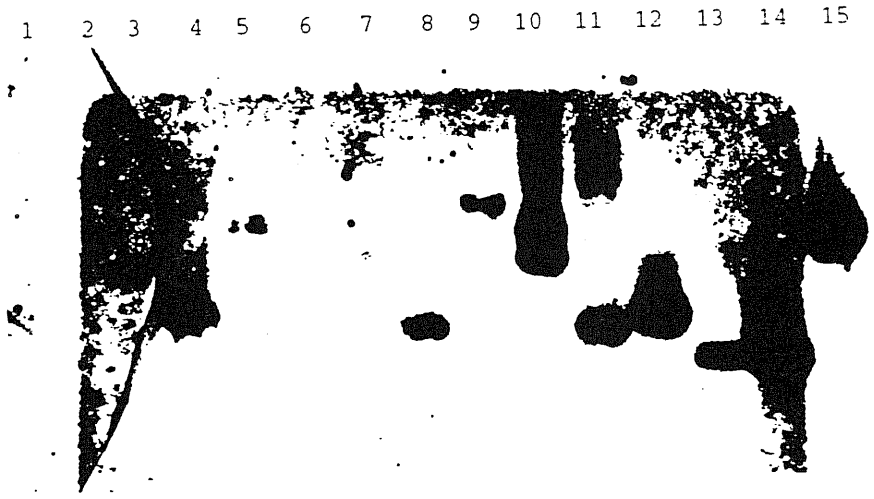

Figure 3.3.3: a) Schematic representation of the semi-nested PCR strategy used for chromosome walking. The genomic DNA region of known sequence is indicated by a solid line, the flanking unknown sequence by an open line and the unrelated sequence involved in the jumping PCR artifact by a hatched line. Vertical lines indicate the region of sequence homology between the unknown sequence and the unrelated sequence. Oligonucleotide primers are depicted by arrows and are defined in the text. b) Amplification of the membrane human ε locus and its 3' flanking region. (a) Agarose gel electrophoresis of the nested PCR products amplified with primers from the M2 exon (M2B and M2I) and the following unrelated primers: lane 1-β globin gene primer, lane 2-γ globin gene primer, lanes 3 to 7- human immunoglobulin heavy chain variable region primers, lanes 8 and 9- human immunoglobulin light chain (kappa) variable region primers, lane 10 to 15- E1, E2, E6, S1 and S2 primers from ε locus but oriented oposite to the M2B and M2I primers. 1kb DNA Ladder (BRL) was used as size marker (M). (b) Southern blot analysis of the same gel probe with the internal M2G oligonucleotide (from the M2 exon). Positive bands from lanes 10, 14 and 15 were cloned and sequenced.

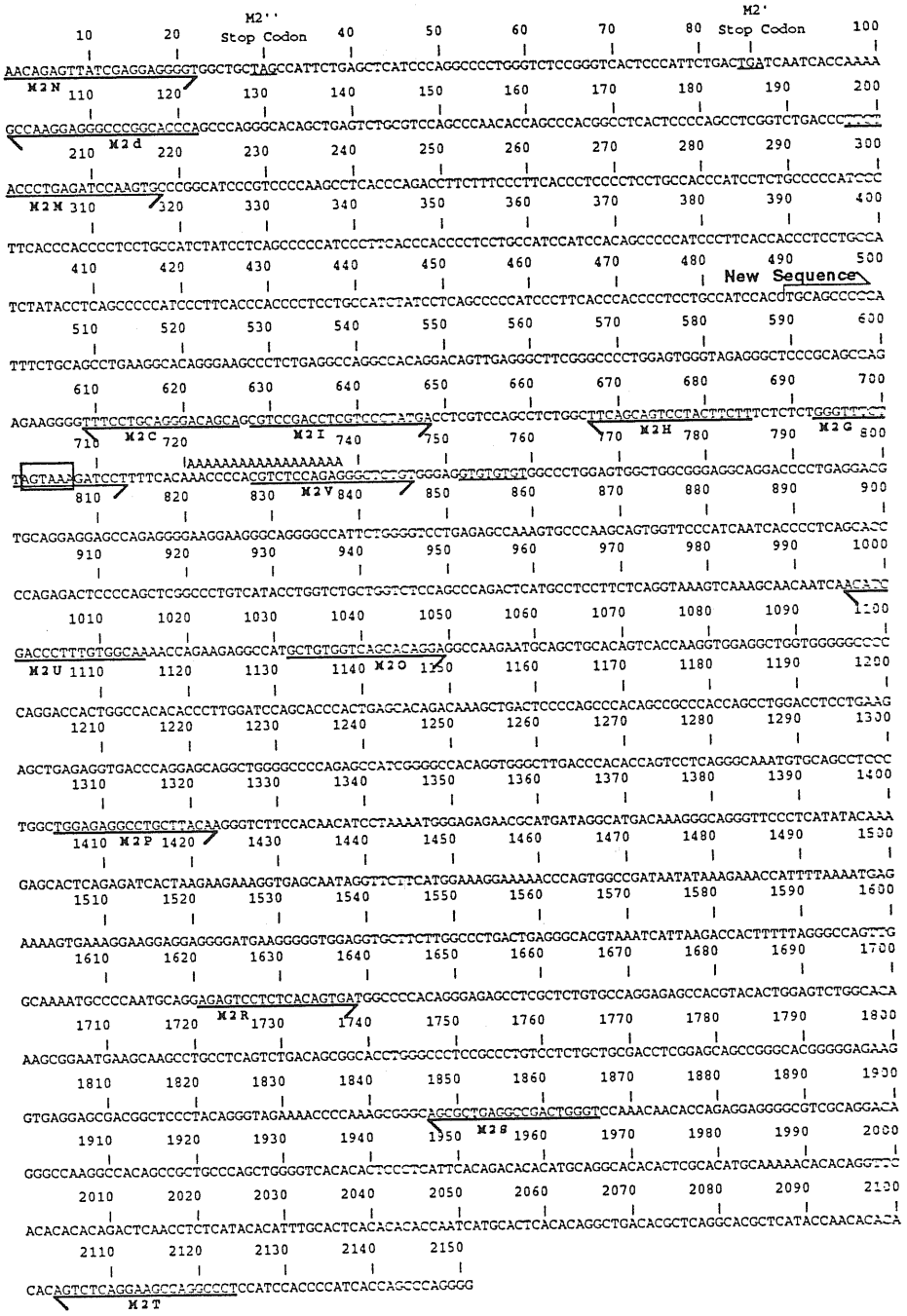

FIGURE 3.3.4: Genomic sequence of the 3' part of the human ε M2 exon (nucleotides 1-720) and its 3' flanking region. The M2' and M2" stop codon are underlined (nucleotides 29-32 and 87-90 respectively). The AGTAAA polyadenylation signal is boxed while a tract of A indicates the polyadenylation cleavage and addition site (position 720). The GT rich element (position 752-759) is underlined. The 5 times tandem repeat sequence is located between position 290-490. The oligonucleotides used for the PCR analysis and sequencing are also shown, the half arrow indicates the orientation of the oligonucleotides.

sequence. As shown in Figure 3.3.5, fragments of the expected size were obtained after each amplification with the different primer combinations.

3.3.3 Indentification of the predominant ε-mRNA species in U266 by Northern blot analysis

Three major species have been identified by Northern blot analysis in the U266 cell line and its subclones AF10 and SKO-007, and have been assigned to the εCH4-S, εCH4-M2' and εCH4-M1'-M2 forms [195, 196, 206, 207]. The characterization of the poly (A) site of the membrane locus and its 3' flanking region allowed us to identify the most abundant ε mRNA species in the U266 cell line, to determine the poly (A) site usage of these isoforms and to define their exact length. Five isoform-specific probes corresponding to distinct parts of the Cε gene were used in the Northern blot analysis (Fig. 3.3.6a). An 812 bp Bgl II/ Bbs I cDNA fragment containing part of CH2 and the complete CH3 and CH4 exons was used to detect all ε-mRNA isoforms (hεC probe). The M2" probe was a 735 bp Nhe I/ KpnI cDNA fragment that contained the last part of the M2 exon and its 3' untranslated region and was used to identify all the forms that use the membrane locus poly (A) site. Differentiation between the forms containing the complete M2 exon and those containing the M2" exon was achieved with the M2 probe, a 271 bp Sac II/Nhe I fragment containing only the region of the M2 exon upsteam of the M2" acceptor splice site. The M1' probe, which contained almost the complete M1' exon of 207 bp and was generated by PCR, permitted identification of the membrane form. Finally, a 1.4 kb fragment 3' to the poly (A) site, the 3'M2 probe, was also used to investigate whether any sequences downstream of the identified membrane poly (A) site is present in ε transcripts.

As shown in Figure 3.3.6b, the hεC probe hybridized to five major U266 mRNA species, represented by bands of approximately 2.1 Kb, 2.7 Kb, 3.1 Kb, 3.4 Kb and 4.5

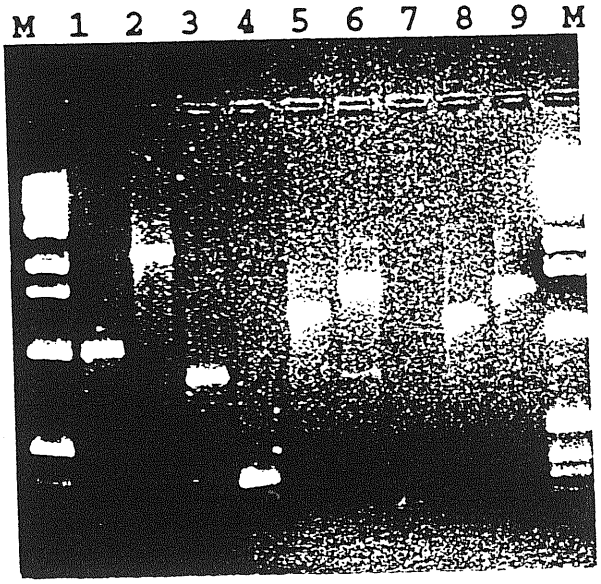

Figure 3.3.5: Amplification of the membrane human ε locus and its 3' flanking region. Agarose gel electrophoresis of the PCR products obtained with primers from the M2 exon (M2N, M2M and M2I) and primers from the 3' end flanking sequence (M2S, M2U, M2V and M2T): lane 1- M2N/M2U, lane 2- M2N/M2T, lane 3- M2M/M2U, lane 4- M2I/M2U, lane 5- M2I/M2S, lane 6- M2I/M2T, lane 7- M2V/M2U, lane 8- M2V/M2S, lane 9- M2V/M2T. 1kb DNA Ladder (BRL) was used as size marker (M).

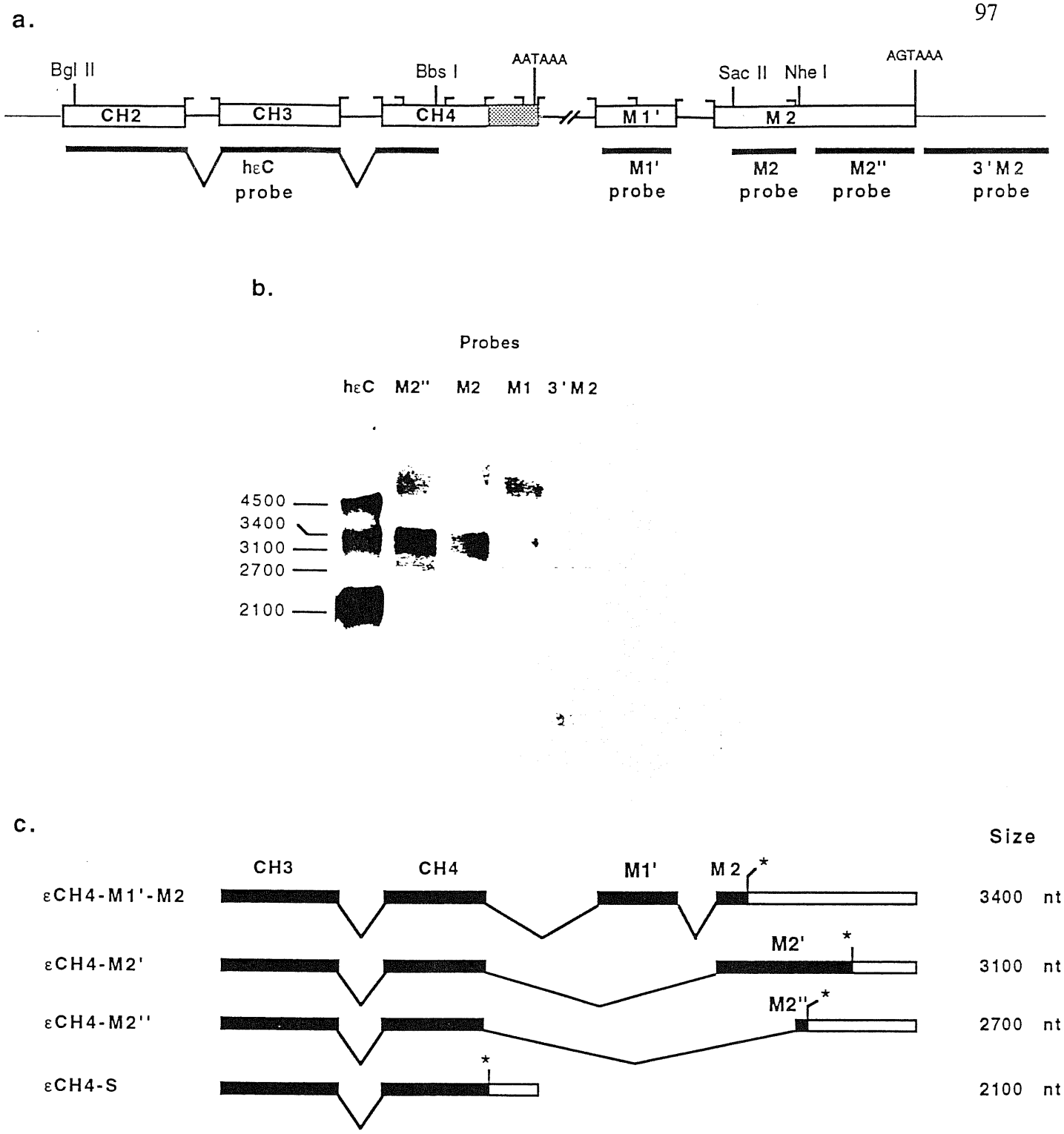

FIGURE 3.2.6: Northern blot analysis of the alternatively spliced ε mRNAs. a) Schematic representation of the 3' end of the ε chain locus with the position of the different probes indicated bellow the corresponding region of the gene. The position of the different restriction enzymes sites used in their construction are shown. b) Total RNA from the U266 cell line was separated on a formaldehyde-containig agarose gel. Five identical lanes were loaded on the same gel with 20 μg of RNA in each line. Each lane was hybridized with one of the five different probes from the various regions. c) Diagram of the identified ε transcripts. Filled and open boxes correspond to translated and untranslated regions, respectively. Stop codons are indicated by asterisks.

Kb. The 2.1 Kb band did not hybridize to any probe derived from the membrane locus and therefore should represent the mRNA encoding the classical secreted ε H-chain. The 2.7 kd band corresponded to the εCH4-M2" form since it hybridized to the M2" probe but neither to the M2' nor the M1' probe. The band of 3.1 Kb hybridized to both the M2" and the M2 probes, but not with the M1' probe, suggesting that this band represented the εCH4-M2' species. Finally, the 3.4 Kb band was the only one hybridizing with the M1' probe, as well as the other two probes derived from the membrane locus, suggesting that this band corresponded to the εCH4-M1'-M2 form. Additional transcripts, more than 4.5 kb long, were obtained with all probes and presumably represent mRNA precursors. A schematic representation of the different isoforms and their corresponding length is shown in fig 3.3.6c

CHAPTER 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 [214-217]. 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 [218]. Using the nested PCR approach described in chapter 3.1 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 [198-200].

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. 3.1.2, 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 [219]. 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 [136]. 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 [220]. These results suggest that other mechanisms such as trans-splicing between germ-line CH transcripts and VDJ-encoding RNA [221] or alternative splicing of a long RNA transcript encoding one VDJ and multiple CH genes in

germ-line configuration [222] 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. 3.1.4b 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, 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. 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 [223]. 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.3.1.2). Comparison with other published sequences revealed that the same mutation was present in two of 19 ε VH5 transcripts [218] and also among the clonally related transcripts of two follicular lymphoma cases [224, 225]. 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 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 [218]. Further evidence for the existance of sequence specific hot spots comes from a very recent study of passenger k transgenes in which the somatic mutations were not selected by antigen and still preferentially occured in particular positions of the k chain V regions [87].

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 [226] 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.

The classical secreted and membrane forms of all immunoglobulins are produced by alternative splicing of the sequences between the last constant region exon and the first membrane exon of the H-chain gene [9-14, 227]. More recently, a number of alternatively spliced H-chain transcripts have been identified in IgA and IgE producing B-cell lines [12, 195, 196, 206, 208]. We have now shown by RT/PCR, cloning and sequencing analysis that alternatively spliced ε mRNA species also exist in unstimulated PBL. Apart from the classical membrane (εCH4-M1'-M2) and secreted (εCH4-S) forms, six alternatively spliced ε transcripts were identified. The εCH4'-I, εCH4'-I-M2, εCH4-M2', and εCH4-M2" forms have been previously reported and arise by alternative usage of two different donor splice sites in the CH4 exon and different acceptor splice sites in the CH4 3'-untranslated region of the secreted form or in the M2 exon [195, 196, 206, 208]. In the two new forms reported here (εCH3-I3-CH4 and εCH4*), the acceptor splice site at the end of intron 3 is not used

and consequently these forms either retain the complete intron 3 (εCH3-I3-CH4)or are spliced to a new donor splice site in exon 4 (εCH4*). At the protein level both forms would lack the complete CH4 domain due to stop codons created by the new reading frames. Except for the εCH4-M1'-M2 form, the protein products of all of these transcripts should be secreted since they do not contain a hydrophobic segment which could act as transmembrane lipid anchor.

The above described ε mRNA species were also identified in the RNA from the U266 cell line, suggesting a similar pattern of ε mRNA splicing in plasmacytoma cells and PBL. However, with the exception of the classical secreted and membrane forms, analysis of the U266 cellular extracts and supernatans did not confirm the existence of proteins corresponding to the alternatively spliced ε mRNA species, and suggested that these isoforms are either inefficiently translated and/or rapidly degraded. To investigate the expression of these isoforms in the absence of competition for translational and other protein processing factors we introduced independently six suitably modified human ε H-chain genes, corresponding to the εCH4*, εCH4-M2', εCH4-S, εCH4'-I, εCH4-M2" and εCH4-M1'-M2 forms in the mouse plasmacytoma cell line J558L which is commonly used as an analogue to antibody producing cells [4, 194, 228]. All six constructs were intracellularly expressed but, with the exception of the εCH4-S and the εCH4-M2" forms, their protein products were absent in the supernatants. Pulse-chase experiments showed that the εCH4*, εCH4-M2' and εCH4'-I forms are intracellularly retained and degraded. Immunofluorescence analysis suggested that the site of the retention is the ER, since none of them were found to be compartmentalized within the Golgi. Altogether, these data suggest that the εCH4*, εCH4-M2', and εCH4'-I forms are only byproducts of alternative splicing in the Cε locus and, most likely, without functional significance. The alternative possibility that these proteins are membrane isoforms was also excluded since only the classical membrane form was expressed as surface immunoglobulin.

Unlike other Igs, the requirements for IgE secretion have not been thoroughly studied. Secretion of IgG requires assembly of two H-chains with two light chains, while secretion of IgM requires further assembly into pentamers [4, 229]. The secretion pattern of IgA is more complex and appears to contain monomeric, dimeric and polymeric structures [229, 230]. In all cases, the incorrectly folded or improperly assembled proteins are retained and degraded in the ER [231]. The εCH4*, εCH4'-I and εCH4-M2' forms assemble into HL monomers that retain the anti-NIP specificity, but fail to assemble into HL dimers. The absence of the complete CH4 domain in εCH4* or the carboxyl terminal part of it in εCH4'-I (a.a. 510 to 547, including the Cys at 524) could explain the inability to form HL dimers since this domain has been shown to be necessary for ε H-chain dimerization [232]. In the case of the εCH4-M2' form, which contains the complete ε CH4 domain (except for the last two amino acids), the presence of the large M2' domain could prevent dimerization of the two H-chains. However, the presence of a small amount of εCH4-S HL monomers in the supernatant of the transfected J558L plasmacytoma suggests that the absence of dimerization is not the only reason why these forms are not secreted and may indicat that the new carboxyl termini created by the alternative splicings could lead to incorrect folding of the constant region domains or contain retention and/or degradation signals.

Regarding the εCH4-M2' form, our results were contradictory with those reported by Zhang, et al., who found that it is secreted in a patient with myeloma and in AF-10, a subclone of the U266 cell line [196].We were unable to detect it in U266 and found that it is produced in the J558L transfectoma but degraded within the secretory pathway. It is unlikely that this discrepancy was due to the differentiation state of the cells, as observed for membrane and secreted forms of IgM [228], since all cells were of myeloma origin, although there could be differences in the subclones used by both groups. To investigate the possibility that this variant forms hybrid molecules with the εCH4-S H-chains which could eventually be secreted, we produced a cell line in which both forms were expressed at almost

equimolar amounts. As in the case of the single transfectant, the εCH4-M2' form is retained and degraded in plasma cells also when coexpressed with the classical secreted form.

The C-terminal tailpieces of μ and α chains display a remarkable sequence conservation in having a cysteine in the penultimate position. Early studies of the μ H-chain demonstrated that the Cys 575 in the Cμ tailpiece plays a role in polymerization [4]. The complete μs tailpiece has also been involved in the retention of secretory μ chains in the ER of B lymphoid cells. Furthermore, the addition of the eighteen C-terminal residues of the secretory μ chains to the IgG3 H-chain gene was sufficient to induce the formation of polymers of IgG3, which activated complement more efficiently than the IgG3 monomers [233]. Since the εCH4-M2" has a cysteine in the terminal position (though not penultimate), an atractive hypothesis was that this form could be assembled and secreted like polymeric IgE and therefore with different biological function. This hypothesis could not be tested using the IgE producing myeloma cell line U266 due to the low levels of expression of this form and its almost identical molecular weight to the classical secreted form. To overcome these problems, we expressed this form either in the presence or in the absence of the CH4-S polypeptide and investigated the possible polymerizations of both, the εCH4-M2" homodimers and the εCH4-M2"/εCH4-S heterodimers.

Analysis of the secreted material from the εCH4-M2" transfectoma showed that the terminal cysteine in this IgE isoform is not involved in polymerization of H_2L_2 molecules in myeloma cells, contrary to IgM and IgA. Moreover, the absence of HL monomers in the secreted material of the εCH4-M2" cell line indicated that the terminal cysteine may be involved in an interchain bridge between monomers, stabilizing the H_2L_2 molecules. Recent mutagenesis experiments have shown that the presence of charged residues upstream of the terminal cysteine in IgM and IgA decrease the secretion of polymeric molecules from myeloma cells [229]. For instance, insertion of acidic or basic residues in the vicinity of the Cys 575 results in the secretion of IgM monomers. Conversely, changing the Asp 572 of the

α tailpiece inserted in a μ chain, to a neutral residue reduces the percentage of IgM monomers secreted. In the case of the εCH4-M2", a possible explanation for the lack of dimerization or polymerization could be the presence of two arginines (Arg 550 and 551), located 3 and 4 residues upstream of the terminal cysteine which could prevent association of the H_2L_2 molecules.

The possible assembly of heterohybrids containing one εCH4-S chain and one εCH4-M2" chain and the eventual polymerization of these heterohybrids were investigated by producing a transfectoma that expressed similar quantities of both mRNAs. PAGE analysis under non-reducing conditions excluded the presence of heterohybrid dimers $(H_2L_2)_2$ or polymers $(H_2L_2)_n$, but did not provide an answer regarding the existence of the heterohybrids themselves which could not be discriminated by size from the εCH4-S and εCH4-M2" homodimers. Although other techniques such as HPLC ion exchange chromatography and isoelectric focusing were also used, the differences in amino acid content between the homodimers were too small to allow their separation and thus not useful to investigated the existence of heterohybrids molecules. Formal proof for the existence of εCH4-S/εCH4-M2" heterohybrids will have to await the production of antibodies specific for the carboxyl terminal tail piece of the εCH4-M2" form. Such antibodies are currently being developed and should also allow investigation of the functional significance of this second secreted IgE isoform.

The results discussed in the previous sections showed a complex pattern of alternative splicing in the Cε locus which lead to the generation of several ε mRNAs isoforms. Three of these isoforms encoded proteins which were found to be secreted or expressed as surface immunoglobulin, while the other isoforms encoded proteins that were retained and degraded in the plasma cells. The control of the alternative splicing of these mRNA isoforms is an important question which should provide further insight into the regulation of their expression. Recent studies of the switch in the production of membrane and secreted IgM

have suggested that the polyadenylation of the unprocessed transcripts could be the regulated event which determines the pattern of splicing [15, 213, 234]. While both poly (A) sites are known for the other immunoglobulin H-chain loci, only the poly (A) site used by the classical secreted isoform had been defined in the Cε locus. The results presented in chapter 3 section 3, described the identification of the second poly (A) site of the Cε locus and the definition of the most abundant εmRNA isoforms produced by the U266 cell line.

Cloning an sequencing of PCR products obtained with the RACE technique confirmed that the AATAAA sequence located 115 nt downstream of the stop codon in the ε CH4 exon served as the polyadenylation signal for the classical secreted form. This analysis also provided 200 bp of new sequence from the 3' unstranslated region of the M2 exon which contained an AGTAAA sequence located 13 nt upstream from the poly (A) addition site. Although this sequence does not fit completely with the consensus, analysis of mutated signals generated by site direct mutagenesis have attributed to this sequence about 30 % the activity of the classical AATAAA [235-238] A survey of the literature and search in data banks suggested that, besides the predominant AATAAA, the AGTAAA is also found in nature. Its location at an appropriate distance from the poly (A) addition site indicates that it may be a functional hexamer [239]. Its relative efficiency perhaps constitutes an additional aspect in the regulation of the ε gene.

The definition of the poly (A) signal was completed by cloning and sequencing more than 1.4 Kb of genomic DNA dowstream of the AGTAAA sequence and by comparing with sequences involved in the regulation of eukaryotic mRNA polyadenylation. Several studies have shown that the (G+T)-rich region found within 50 nt dowstream of the hexanucleotide is required for proper 3' end processing [237, 238]. These downstream sequence probably participates in forming secondary structures necessary for recognition and or activity of the cleavage enzymes [240, 241]. This signal is not as well conserved as the AATAAA and has been only vaguely defined as a T-rich or a (G+T) rich element. Analysis of our sequence

revealed a GT rich tract present 50 nt dowstream of the AGTAAA signal. The point of RNA cleavage for the introduction of the poly (A) tract also occured in CA dinucleotide conserved in many eukaryotic genes. This CA dinucleotide precedes the poly (A) tract in 70 % of the eukaryotic RNAs analyzed [242-244]. The absence of other AATAAA consensus sequences in the 1.4 Kb genomic DNA downstream of the AGTAAA described suggested that this hexanucleotide is the unique poly (A) signal in the human ε membrane locus.

The newly obtained sequences also allowed investigation by Northern blot analysis of the relative amounts of the ε mRNA species that were previously detected by RT/PCR in U266. The use of specific probes complementary to the Cε gene, the M1' exon, the M2 exon, the M2" exon and the 3' flanking region downstream the poly (A) site, confirmed that the εCH4-S, εCH4-M1'-M2 and εCH4-M2' species are the most abundant ε mRNA isoforms in U266 [195, 196]. A band corresponding in size to the εCH4-M2" species was detected with both the hεC and the M2" probe, suggesting a significant level of expression of this second secreted IgE isoform in U266. The differences in size between this band and the bands corresponding to the CH4-M1'-M2 and εCH4-M2' species are in agreement with their usage of the poly (A) signal of the membrane locus. Higher molecular weight mRNA species were detected with all probes. These species were not detected by Northern blot analysis of poly $(A)^+$ mRNA as reported by Hellman [195], which suggests that they represent ε mRNA precursors that are not polyadenylated.

Recent studies have suggested that the alternative splicing of the ε isoforms is not a stochastic but a regulated event. Saxon et al. reported that crosslinking of the CD23 molecule on IgE myeloma cells leads to a decrease in the levels of εCH4-S mRNA and to inhibition of the IgE secretion [207]. On the other hand, RT/PCR analysis of the ε mRNA isoform pattern in nasal lavage cells have shown an increase in the levels of the εCH4-S and the εCH4-M2" mRNA species upon challenge with diesel exhaust particles, suggesting that the expression of both isoforms can be induced [245]. The isoform specific probes designed in this study

should prove useful in future investigations of the IgE expression by allowing accurate quantification of the levels of the ε mRNA species and the effects of possible regulatory factors.

SUMMARY

The main objectives of the studies presented in this thesis were: 1) Analysis of the role of somatic mutation of D and JH gene segment usage and isotype switching in the generation of the IgE repertoire in normal and atopic individuals, 2) Characterization and expression of the alternatively spliced ε isoforms produced by peripheral blood lymphocytes and plasma cells and 3) Characterization of the poly (A) site of the human ε membrane locus.

A review of the literature is presented in Chapter 1. The structure and function of the antibody molecules are described, as well as the genomic organization of the genes encoding them. The recombination mechanisms involved in the joining of variable region segments are also presented, followed by a description of the gene expression during B cell differentiation. The role of somatic mutation in generating the diversity of immunoglobulin repertoire is discussed in a separate section. Special emphasis is given to the mechanisms involved in immunoglobulin class switching with a detailed description of current knowledge regarding to IgE production. The role of IgE in mediating the allergic response is discussed in the last section together with a short historical perspective of some of the more important accomplishments in the IgE research. A description of the methodology used is presented in Chapter 2.

Chapter 3.1 provides a detailed analysis of the IgE repertoire expressed in vivo by peripheral blood lymphocytes from normal and atopic individuals.The ε transcripts from several normal and allergic individuals were investigated by RT/PCR, cloning and sequencing. 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 demonstrated the in vivo production of IgE together with two other immunoglobulin isotypes (IgM and IgG4) by the progeny of a common B cell precursor, and suggested a mechanism for the production of blocking antibodies which play an important role in the regulation of the allergic response. This study also provided 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 existence of sequence specific hot spots for the somatic hypermutation machinery in the JH gene segments.

The investigation of the IgE H-chain isoforms produced in vivo is presented in Chapter 3.2. The ε mRNA species present in unstimulated PBL were characterized and expressed individually in a myeloma cell line. Eight ε mRNA species were identified using RT/PCR, cloning and sequencing analysis. These included the classical secreted (εCH4-S) and membrane-bound (εCH4-M1'-M2) IgE and six alternatively spliced ε transcripts. At the protein level, the six alternatively spliced ε transcripts (εCH4*, εCH4-M2', εCH4-M2", εCH4'-I, εCH4'-I-M2 and εCH3-I3-CH4) corresponded to five ε H-chain isoforms in which various parts of the CH4 domain were replaced by new stretches of amino acids at the carboxyl termini. The same ε mRNA species were also present in the IgE producing myeloma cell line U266. However, except for the classical membrane and secreted IgE, the existance of their corresponding proteins could not be confirmed. To further characterize the εCH4-S, εCH4*, εCH4-M2', εCH4'-I, εCH4-M2" and εCH4-M1'-M2 species, we expressed them as chimeric mouse/human anti-NIP antibodies in a mouse myeloma cell

line. Besides the classical secreted and membrane IgE forms which are properly transported, a novel ε chain from was identified. This correspond to the εCH4-M2" polypeptide which contains 6 extra amino acids sequence ESSRRGGC with a distinct carboxyterminal with respect to the classical εCH4-S. The role of the terminal cysteine is discussed. All the other ε polypeptides were found to be intracellularly degraded in plasma cells.

Chapter 3.3 deals with the identification of the poly (A) site of the human membrane ε locus. Both cDNA cloning of the 3' end of mRNA and chromosome walking allowed to complete sequence of the M2 exon and the identification of the poly (A) site of all ε transcripts containing this exon. Also the relative abundance of the ε mRNA isoforms were analaysed by Northern blot in the U266 myeloma cell using specific probes. The different band usually observed were assigned to the described mRNAs and the most relevant isoforms detected.

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