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Structural analysis of the murine IgG3 constant region gene

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We have determined the complete sequence of the γ3 heavy chain constant (Cγ3) region gene of the BALB/c mouse including the 5′-flanking region up to the switch site and the 3′-flanking region past the end of the membrane exons. The Cγ3 coding region, typical of other IgGs, is divided into six exons corresponding to the protein domains ($C_{\gamma 3}1$, hinge, $C_{\gamma 3}2$, and $C_{\gamma 3}3$) and to the membrane carboxyl terminus (M1 and M2). The predicted amino acid sequence of the γ3 chain has three potential N-linked carbohydrate addition sites (including one in the membrane spacer segment), as compared with a single occurrence in the other mouse IgGs. Between the switch recombination region and the body of the Cγ3 gene, there is a remarkable homology with a sequence between Cμ and Cδ which provides a rationale for an alternative, T cell-independent class-switch mechanism. We have used a computer to analyze the secondary structure of the γ3 mRNA precursor for the membrane form. We predict that this RNA precursor (~12 000 bp) folds into four leaf-like domains which correspond to the variable region, the large IVS, the body of the constant region, and the membrane exons. This organization may have a role to play in the function of the mRNA precursor.

Key words: DNA sequence/mouse immunoglobulin/RNA secondary structure

Introduction

The mouse produces eight classes of immunoglobulin (Ig) molecules which differ in the amino acid sequence of the heavy (H) chain constant (C) region. The genes coding for them have been cloned and mapped in the order Cμ-Cδ-Cγ3-Cγ1-Cγ2b-Cγ2a-Cε-Cα, corresponding to classes IgM, IgD, IgG3, IgG1, IgG2b, IgG2a, IgE and IgA (see Shimizu *et al.*, 1982; Marcu, 1982, for review) on chromosome 12 (D'Eustachio *et al.*, 1981). The various classes exhibit different effector functions and display differing tissue localization. Each Ig class can be expressed as a membrane (m) or a secreted (s) form which differ in sequence at the carboxyl terminus of the H chain (reviewed by Blattner and Tucker, 1984). Developmental control of membrane *versus* secreted expression is exerted at the RNA level by alternate processing of exons at the 3′ end of the mRNA.

Most antigens elicit prompt serum expression of IgM, followed later by a secondary, 'class switch' response in which products of downstream H chain genes, especially Cγ1 and Cγ2a predominate (Slack *et al.*, 1980; McKearn *et al.*, 1982). Regulation of Ig class expression is mediated through DNA recombination. The class switch is generally associated with the deletion of DNA (including Cμ) up to the expressed class (reviewed by Marcu, 1982). This deletion is thought to result from recombination between blocks of repetitive DNA ('switch sites') located on the 5′ side of each C_H gene except Cδ (Nikaido *et al.*, 1982; Marcu, 1982; Richards *et al.*, 1983).

Paradoxically, certain T cell-independent antigens, generally carbohydrate in nature, elicit a prompt response in which IgG3 is the predominant or, in some cases, the exclusive membrane and secreted Ig product (Perlmutter *et al.*, 1978; Fulton *et al.*, 1983). It is not known whether the same 'class switch' is involved in the T-independent response but this response never progresses beyond γ3 to the more downstream C_H genes in the absence of T cell help (Mongini *et al.*, 1982).

Despite its biological importance, γ3 is the only murine H chain gene which had not been sequenced, although fragments have been determined (Marcu, 1982; Komaromy *et al.*, 1983). To provide the structural information that could eventually lead to biological understanding, we have analyzed in detail this last remaining gene and its surrounding DNA. In addition to providing structural comparisons, the data show a flanking sequence homology that could participate in an unusual mechanism for a T-independent class switch. By fusing the Cγ3 sequence with appropriate upstream regions we have calculated a remarkable secondary structure for the 12-kb RNA precursor of the membrane γ3 chain.

Results and Discussion

Organization and sequence of the Cγ3 gene

The physical map and complete nucleotide sequence of the Cγ3 gene, including 1961 bases of the 5′-flanking region and 738 bases beyond the 3′ end of membrane mRNA, are presented in Figures 1 and 2. The organization of the gene is typical of the other IgGs (Tucker *et al.*, 1979; Honjo *et al.*, 1979; Rogers *et al.*, 1981) in that each domain of the protein ($C_{\gamma 3}1$, $C_{\gamma 3}2$, $C_{\gamma 3}3$) and its hinge (H) as well as the spacer-transmembranal and cytoplasmic portions (M1 and M2, respectively) of the membrane carboxy terminus are coded on separate exons. Econ boundaries for the 3′ side of $C_{\gamma 3}1$, the hinge, and the 5′ side of $C_{\gamma 3}2$ were confirmed by comparison of the genomic sequence with the sequence of the truncated cDNA clone, p606γ3 (see Materials and methods). The other C_H domain boundaries were assigned by analogy with the other IgGs since excellent consensus RNA splice sites (Mount, 1982) were present in the sequence at the expected positions. However, the M1-M2 splicing pattern is not so clear since the M1 donor RNA splice site is very poor (see below).

In Figure 2 the predicted amino acid sequence of the γ3 heavy chain is presented beneath the coding sequence. There is no experimentally determined amino acid sequence available for this class but the genomic sequence agrees perfectly (where comparable) with the cDNA sequence of RNA from MOPC 606, a certified IgG3 myeloma (Potter,

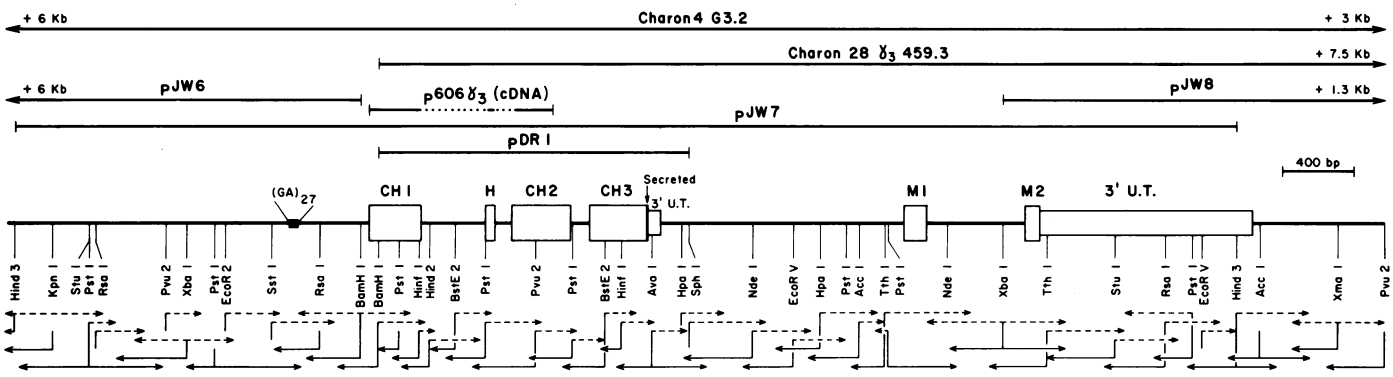

Fig. 1. Organization and sequence analysis of the Cγ3 gene. Shown on the top two lines are the genomic phage clones and on the next four lines the plasmid subclones positioned relative to the germline Cγ3 gene (diagrammed below). The cDNA clone, p606γ3, is 3′ truncated within the C_H2 region as indicated; dots represent intronic DNA not included in the cDNA. The domains and membrane exons are the larger open boxes and are appropriately labeled. The 3′-untranslated (UT) regions of the mRNA for both the secreted and membrane-bound forms are shown as smaller open boxes. The exon for the secreted form is a solid box to distinguish it from the contiguous C_H3 domain. The string of GAs which is located 5′ to the C_H1 domain is denoted as a small black box. The restriction endonucleases shown are those used for end-labeling. Sequencing reactions representing the coding strand are dashed horizontal lines and those representing the non-coding strand are solid horizontal lines. 5′ and 3′ end-labelings are implied by the direction sequenced (indicated by the arrow head on the end of the line).

1972). The protein sequence is typical of IgG class antibodies, including cysteine residues spaced ~60 amino acids apart within each domain.

The domains can now be delineated, using the Kabat numbering system (Kabat *et al.*, 1983) as: $C_{\gamma 3}1$, positions 115–223 (96 total residues); Hinge, 226–240 (16 residues); $C_{\gamma 3}2$, 245–360 (110 residues); $C_{\gamma 3}3$, 361–478 (107 residues including the secreted terminal dipeptide). Although not confirmed at the protein level, we anticipate that the secreted carboxyl-terminal lysine residue, conserved among all the IgG subclasses, is likewise proteolytically removed from the nascent γ3 chain (Tucker *et al.*, 1979).

The prototypic sequence for attachment of N-linked sugars, Asn-X-Ser or Thr, where X is any amino acid (Marshall, 1972) is present in $C_{\gamma 3}2$, $C_{\gamma 3}3$ (beginning at base numbers 3008 and 3649, respectively) and in the amino-terminal portion (base 5131) of the membrane carboxyl terminus. This segment, which we have termed the 'spacer' (Cheng *et al.*, 1982), is postulated to reside immediately outside the membrane. The first of these glycosylation sites is conserved in all Igs of mouse. The second and third are unique to γ3. The finding of a potential carbohydrate attachment to the membrane spacer is quite unusual since it would imply a carbohydrate moiety specific to the membrane form of the immunoglobulin. It is interesting to note that the three isotypes (γ, δ, and γ3) involved in the primary, T-independent response are glycosylated significantly more than those (other γs and α) involved in the secondary response (see Kabat *et al.*, 1983).

Homology with other IgG classes

With the determination of the $C_{\gamma 3}$ gene nucleotide sequence presented here, all of the mouse C_γ gene sequences are available to be compared with each other to detect nucleotide and amino acid homologies. Consideration of such homologous regions permits one to propose a mode of evolution in which domains or domain-intron segments are translocated between Ig classes as unitary evolutionary events (Miyata *et al.*, 1980). The detailed presentation of this scheme is the subject of the accompanying paper by Hayashida *et al.*

The membrane coding exons

The membrane carboxy-terminal coding region of the Cγ3 gene is very similar to the other γ classes (Figure 2), with strong homology among the M1 and M2 exons that code, respectively, for the spacer-transmembranal and cytoplasmic portions of these proteins. There is also substantial intervening sequence homology. However, in γ3 the donor splice site on the 3′ side of M1 (G/GTCAT) that is responsible for splicing M1 to M2 has a very poor match to the usual donor consensus, G/GTRRR (where R = purine) (Mount, 1982). No case of a splicing at this sequence has been reported and in only a few instances has splicing at a donor sequence with only one of the three purines been observed (see Mount, 1982 for review).

We, therefore, performed experiments to determine whether the M1 to M2 splice occurs in γ3 mRNA. Poly(A)$^+$ RNA isolated from two γ3 hybridomas and lipopolysaccharide (LPS) activated splenic B cells was sized on Northern blots (Figure 3A). The major band at 1.8 kb is in good agreement for the size (1732 bp) of the secreted form of the γ3 chain as estimated from the DNA sequence [adding 450 bases of 5′-untranslated-variable region and 200 bases of poly (A)]. However, the presumed membrane form band at 3.6 kb migrated considerably larger than the calculated value of 3064. Confirmation that the 3.6-kb band was indeed the major membrane RNA was achieved by its hybridization to the cloned *XbaI-Hind*III fragment (Figure 1) that spans the M2 exon (data not shown). We then subjected these mRNAs to S1 nuclease protection analysis using strand-separated DNA probes. These experiments were hampered by the very minute levels of membrane form γ3 mRNA present in the hybridomas as judged by Northern blot analysis (Figure 3A). The most critical test employed a DNA fragment labeled at its 3′ end at an *AsuI* site within the M1 exon and extending into the putative intervening sequence between M1 and M2 (Figures 1 and 3C). The expected result if the splice occurred would be a shortening of this fragment from 422 to 102 bases in a DNA-mRNA hybrid by S1 nuclease digestion. As shown in the experiment depicted in Figure 3B, we observed quantitative cleavage to the 102 base fragment showing that, at least in the particular hybridoma used in this experiment (IIID311), splicing at GTCAT occurred. However in some experiments using splenic mRNA or other hybridomas, unspliced mRNA in the poly(A)$^+$ fraction was observed (data not

Fig. 2. The complete germline nucleotide sequence of the Cγ3 gene. Amino acid sequences predicted for the coding regions are shown direclty below the first base of the corresponding codon in single letter code: Phe,F; Le,L; Ile,I; Met,M; Val,V; Ser,S; Pro,P; Thr,T; Ala,A; Tyr,Y; His,H; Gln,Q; Asn,N; Lys,K; Asp,D; Glu,E; Cys,C; Trp,W; Arg,R; and Gly,G. The TGA translation terminator is a dot. Five potential end sites for mRNA (AATAAA or AATTAAA) are indicated in lower case type, only the two at nucleotide positions 3644 and 6961 are apparently used to terminate the secreted and membrane mRNAs, respectively. A string of 27 GA dinucleotides 5′ to C_H1 is underlined. The secreted carboxy-terminal exon which is contiguous with C_H3 has its protein sequence enclosed in brackets. The 5′-flanking sequence disagrees with that reported by Stanton and Marcu (1982) at positions 40 and 44−46 in this figure. None of the remaining sequence had been previously published 5′ to base pair position 52 (Komaromy *et al.*, 1983).

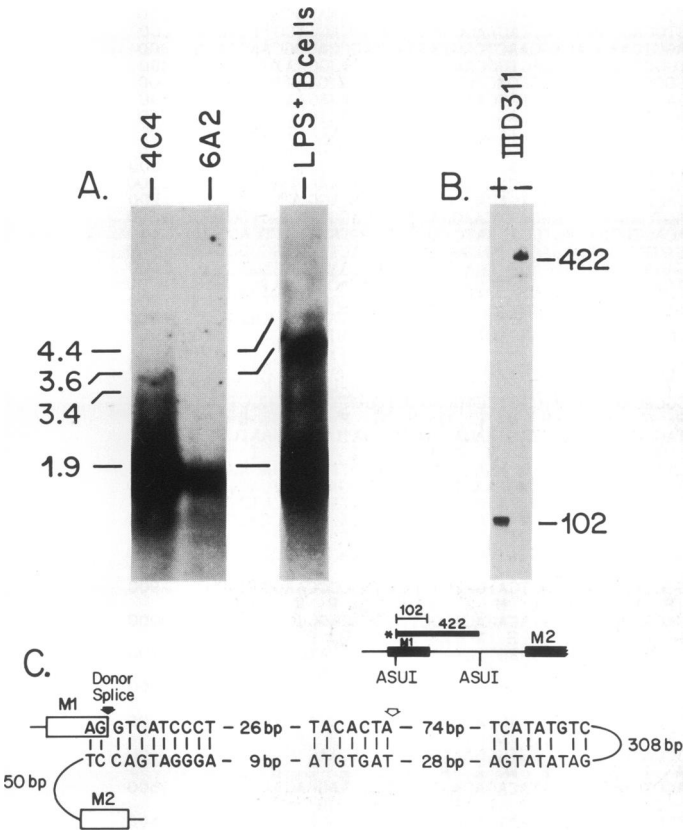

Fig. 3. Analyses of γ_3 mRNA by Northern blots and S1 nuclease protection. Cytoplasmic RNA was isolated from NP40 lysates of 5 day LPS-stimulated BALB/c splenic B cells and from three SP2/0 X murine B cell hybridomas by phenol extraction of LiCl precipitation (Auffray and Rougeon, 1980). **(A)** Aliquots of poly(A)$^+$ mRNA were denatured, electrophoresed on 2% agarose-6% formaldehyde gels and transferred to nitrocellulose filters (Schleicher and Schuell) in 20 x SSC (Southern, 1975). The *Bste*II-*Sph*I fragment from pDR1 containing the third constant region and 3′-untranslated regions of mouse γ3-specific DNA (Figure 1) was labeled with ^{32}P by nick translation (Rigby *et al.*, 1977). Hybridization of the probe to the filter was according to Wahl *et al.* (1979). The filters were washed with 0.1 x SSC, 0.1% SDS at 42°C for 1 h and subjected to autoradiography. **(B)** A 422-bp *Asu*I fragment (solid bar) from pJW7 which spans the putative donor site in the M1 exon was 3′ end-labelled as previously described (Word *et al.*, 1983) and the non-coding single-strand fragment was isolated from a 6.5% polyacrylamide gel (Maxam and Gilbert, 1980). Labelled DNA and poly(A)$^+$ mRNA from IIID311 cells were hybridized and S1 nuclease (Miles) digested as previously described (Word *et al.*, 1983). The protected S1 nuclease digested DNA fragment (+) and the untreated 422 base fragment (−) were electrophoresed on a 6% polyacrylamide/8.3 M urea gel and autoradiographed. **(C)** Possible homologous base pairings in γ3 mRNA involving the M1 exon and the IVS between the M1 and M2 exons are shown. The solid arrow marks the putative donor splice site at the 3′ end of M1 and the open arrow marks the position of a strong S1 nuclease protected fragment seen in one experiment (see text).

shown). Furthermore, in one experiment we observed an *Asu*I-protected fragment of 147 bp. This corresponds to a cleavage product generated downstream of the M1 donor, at the position denoted by an open arrow in Figure 3C. Perhaps secondary RNA structure, such as depicted in Figure 3C (also see discussion below), could be responsible for the variations observed in the S1 pattern.

We feel that these experiments can be conservatively interpreted as showing that at least some and perhaps all membrane form γ3 has the protein sequence presented in Figure 2. More sensitive experiments are needed at the protein or mRNA level to establish whether any of the membrane protein is translated from an unspliced form of γ3 mRNA. Such a protein would have a completely different sequence of 100 amino acids on the interior of the membrane, although the exterior (spacer portion) would be identical. The corresponding cytoplasmic segment of IgA is expressed exactly in this way i.e., by-passing a cryptic M1-M2 donor splice site (Word *et al.*, 1983). Since the interior portion of the heavy chain may be involved in delivery of an external signal to the cytoplasm of the cell upon antigenic stimulation, the question of any possible variation in its sequence deserves careful scrutiny.

Most eukaryotic mRNAs contain the sequence AAUAAA (or in some cases, AAUUAAA) ~20 bases upstream of the 3′ poly(A) tail (Brownlee, 1982). This sequence was found downstream of M2 at position 6961. An S1 nuclease protection experiment was performed to determine if the 3′ end of the mRNA for the membrane form is terminated at that site. A fragment generated by 3′ end-labeling a *Tth*I site in the M1-M2 interval and recutting at a *Pvu*II site beyond the putative 3′ end of the message (Figure 1) was shortened from 2134 to 1415 bp (data not shown). This confirmed that the 3′ end position was correctly assigned.

Novel repeat sequence between the class switch region and Cγ3 gene

The ability of T-independent antigens to stimulate a rapid switch to an otherwise minor Ig class suggests that a special mechanism, perhaps involving a second type of switch site, may be operative in the γ3 response. If so, we reasoned that a second type of switch site might occur in the 5′-flanking region of Cγ3.

We therefore determined the sequence of 1961 bases from the 'conventional' Cγ3 switch site to the body of the gene. Indeed, we did find an interesting repeat, $(GA)_{27}$ (underlined in Figure 2), ~500 bp 5′ of the $C_{\gamma 3}1$ exon. This sequence is essentially identical to the $(GA)_{28}$ repeat 5′ to the first exon of Cδ (Richards *et al.*, 1983). Although this sequence bears no relationship to the pentamer repeats (GAGCT, GGGGT) normally associated with the class switch (Nikaido *et al.*, 1981, 1981) it could serve a similar function in T-independent responses. The result of such a recombination event would be to translocate Cγ3 to the region normally occupied by Cδ just downstream of Cμ. In that position, membrane IgG3 could be expressed in conjunction with IgM in a similar way to IgM-IgD double expression characteristic of virgin B cells (Blattner and Tucker, 1984).

Secondary structure model for the membrane γ mRNA precursor

The biological significance of RNA precursor folding is unclear. However in cases where processing decisions must be made (e.g., membrane *versus* secreted Ig expression), overall secondary structure of the RNA might provide a critical component of specificity.

One approach to this analysis is to attempt to predict the secondary structure of the RNA with a computer. It is currently not feasible to analyze such a large molecule by minimizing global free energy. The programs in common use which use variants of this method would require prohibitive amounts of computer time. The kinetically based approach (Martinez, 1984) was therefore used. This computer program employs the notion that nearby points on an RNA chain will have an opportunity to pair first. The pairing of nearby regions then brings more distant segments closer together and these pair next. If at any point a conflict develops for pairing of a region, a choice is made at random, weighted on the

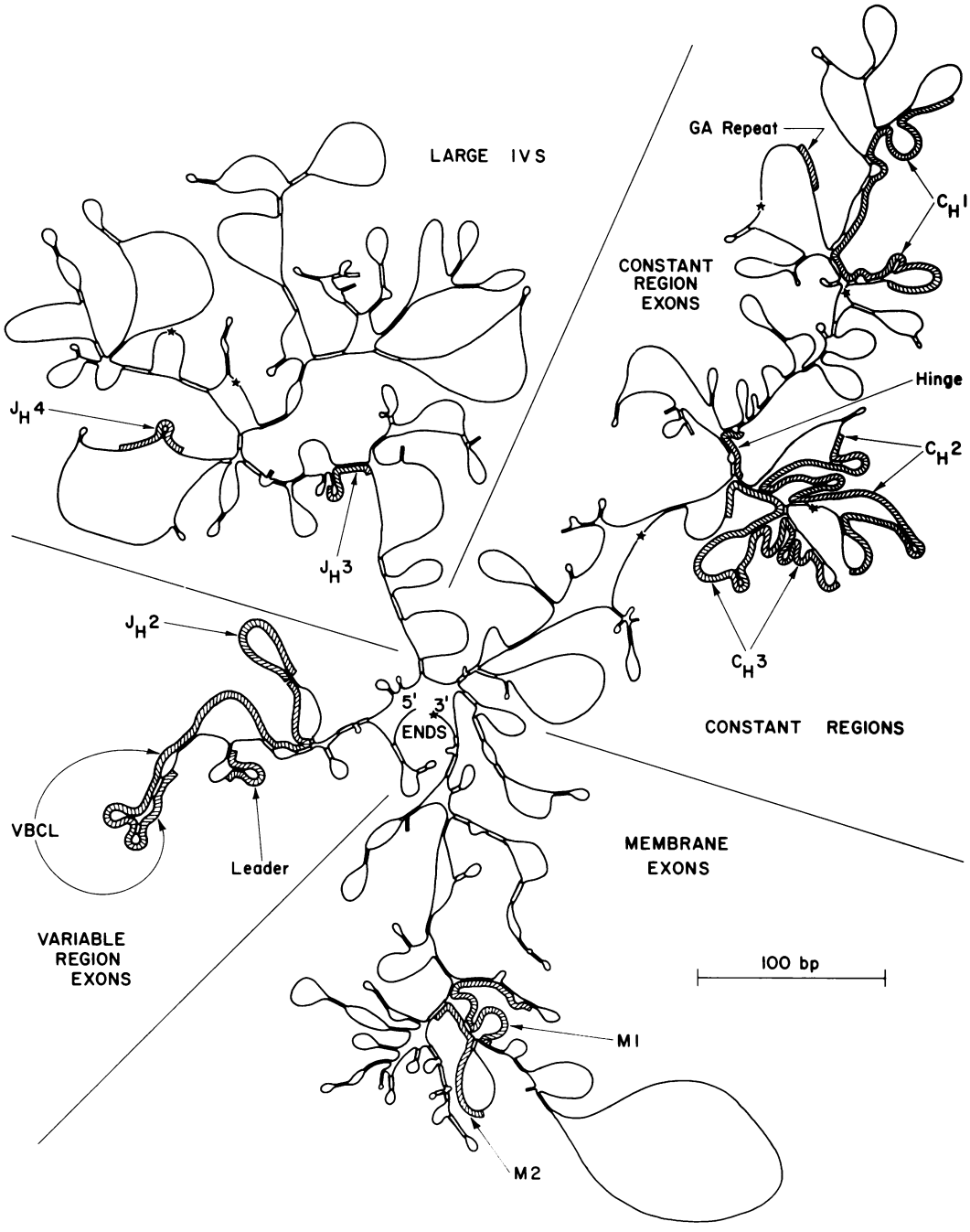

Fig. 4. Hypothetical folding of the membrane γ3 mRNA precursor. The 11 936 base sequence was assembled as follows: bases 1–944 , from Ch28 289.1 (Knapp *et al.*, 1982); 995–4685, from pNN12 (Newell *et al.*, 1980 and unpublished, Blattner and Tucker, in preparation); 4686–11 936, from sequence of Figure 2. The point used for fusion of the μ and γ3 switch regions was arbitrarily chosen according to the available rearrangement data (see Stanton and Marcu, 1982) on IgG3 producing myelomas. Exons are denoted by the hatched ropes. The variable region used (VBCL) is productively rearranged to J_H2. Thin lines represent intervening sequencing, and stars along these lines represent potntial poly(A) addition sites (AAUAAA or AAUUAAA). Boxes between separate strands denote complementary stem-folding sequences of proportional length. Solid boxes represent stems which formed in at least seven out of the 10 independent Monte Carlo foldings, while open boxes represent stems formed in fewer than seven foldings. Computational time was ~10 h on a VAX 11/750 computer.

basis of relative free energy of the two options. The entire process can be repeated several times to gauge the degree to which the random choices made affected the overall result. This strategy greatly limits the number of possible secondary structures examined. Although it does not guarantee that the minimal free energy structure will be found, it is likely to simulate actual biological folding kinetics. The algorithm used here previously predicted a secondary structure for the self-splicing rRNA intron in which 5′ and 3′ ends were brought to within 17 bases of each other (Martinez, 1984).

To carry out this analysis, we have assembled the sequence of the entire 11 936 base membrane form of the γ3 precursor mRNA (pre-$\gamma3_m$) from three segments. The 5′ end (containing the cap, untranslated region and rearranged heavy chain variable region) was derived from a B cell lymphoma sequence (Knapp *et al.*, 1982). The contiguous intronic sequence from the J_H2 to the μ switch site and 5′ portion of the μ switch region) was derived from Newell *et al.* (1980),

Sakano *et al.* (1980) and Richards *et al.* (in preparation). This was fused (see legend to Figure 4 for details) to the sequence of Figure 2 which includes the remainder of the transcription unit from γ3 switch to the 3′ end of M2.

For pre-$\gamma 3_m$, a total of 181 stems were chosen from among 31 851 that were considered in which the free energies of formation were ≤ -20 kc. The entire folding was repeated in 10 independent Monte Carlo trials. Most of the stems chosen were chosen in all 10 trials (see Figure 4).

Figure 4 shows one of the 10 computed structures for pre-$\gamma 3_m$. As suggested in other systems (Dumas and Ninio, 1982; Stucker and Stieger, 1981) short-range interactions appear to dominate the folding, so that the mRNA seems to be organized in domains. However, these do not correspond to the protein domains. Instead, four leaf-like segments can be delineated: the variable region exons, the large V_H-C_H intervening sequence (IVS), the Cγ3 constant region exons and the intronic-exonic region flanking the membrane carboxyl terminus. This is an interesting aspect of the structure that may be correlated to function. However, it does not provide any obvious rationale for the RNA splicing pattern observed; i.e., juxtaposition of 5′ and 3′ intronic ends. Subsequent binding to components of the splicing complex such as U1 small ribonuclear proteins might be required to accomplish this task.

It will be possible to confirm features of the predicted structure of the γ precursor RNA by nuclease cleavage experiments. Although our data are extremely limited, it appears that the stems suggested by our S1 analysis (Figure 3B and C) were also predicted at high criterion on the computer.

Materials and methods

The sequence was determined by the chemical degradation technique (Maxam and Gilbert, 1980) using subclones (Figure 1 and legend) isolated from BALB/c mouse liver DNA genomic clone, Charon 28γ3 459.3, constructed in our laboratory (Liu *et al.*, 1980) as well as a genomic clone, Charon 4 G3.2, provided by Dr. Jerry Adams (Adams *et al.*, 1980). The probe used for our genomic cloning was cDNA clone p606γ3 constructed by Drs. Phil Early and Leroy Hood (unpublished results). This truncated cDNA, isolated from an IgG3 secreting myeloma, MOPC 606, was also sequenced. Figure 1 illustrates the span of each of the clones, as well as the strategy used in the sequence determination. RNA extraction, Northern blotting, and S1 nuclease protection experiments were carried out as described in detail elsewhere (Word *et al.*, 1983). Routine sequence analysis was on a DNASTAR microcomputer system (Blattner and Schroeder, 1984). Secondary structure calculations were performed as described (Martinez, 1984) on a Vax 11/750 computer.

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