

The Role of DNA Rearrangement and Alternative RNA Processing in the Expression of Immunoglobulin Delta Genes

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Summary

We have established the exon-intron structure of the gene coding for the constant (C) region of the mouse immunoglobulin δ heavy chain, using DNA clones isolated from BALB/c embryos and the δ mRNA extracted from two δ -producing hybridomas, B1-8. δ 1 and GCL2.8. At least three types of C δ gene structures are identified. A 2.7 kb δ mRNA reveals six exons. This δ mRNA may code for a membrane-bound δ chain. A second δ mRNA of 1.8 kb shares the first (5' side relative to direction of transcription) three exons with the 2.7 kb δ mRNA and in addition contains a fourth exon unique to this mRNA species. This δ mRNA most likely codes for a secreted δ chain. A third δ mRNA, also of 1.8 kb, shares the first four exons and a part of the fifth exon with the 2.7 kb mRNA. Its function, if any, remains unclear. We investigated the question of how a lymphocyte can produce the μ and δ heavy chains simultaneously, using the hybridoma GCL2.8, which makes both IgM and IgD. Results of Southern gel blot analysis and gene cloning experiments indicate that this cell utilizes the same rearranged V_H gene for the synthesis of the μ and δ chains, and yet maintains the embryonic configuration for the C μ and C δ genes and for the intervening region. Based on these results, we conclude that the V_H sequence is spliced alternatively to the C μ or C δ sequence during processing of the primary RNA transcript. An alternative mechanism for the expression of the δ gene is found in the hybridoma B1-8. δ 1, which actively secretes δ chains and synthesizes no μ chain. This mechanism involves deletion of the C μ gene, which brings the complete V_H gene closer to the C δ gene.

Introduction

Somatic rearrangement of DNA sequences plays a key role in the developmentally controlled synthesis of immunoglobulin heavy chains. The conventionally defined variable region is encoded in three types of independent germline DNA segments, referred to as

V_H, D_H and J_H, which are brought together by DNA recombination to create a complete V gene (Sakano et al., 1980, 1981; Early et al., 1980a; Kurosawa et al., 1981). The assembled V_H gene and the C μ gene located about 8 kb downstream (that is, on the 3' side) are cotranscribed in B cells that synthesize the free μ chain or complete IgM molecules. Activation of one of the γ , α or ϵ constant genes requires a second type of recombination (switch recombination), which replaces the C μ gene with the corresponding C γ , C α or C ϵ gene (Davis et al., 1980a; Kataoka et al., 1980; Maki et al., 1980). While recent studies have accumulated a considerable amount of information on the organization of heavy chain genes in "pre-B" and postswitch B cells, no analogous data are available for the third major state, namely the simultaneous synthesis of μ and δ chains in mature B lymphocytes. Recently, Liu et al. (1980) and Tucker et al. (1980) have mapped the C δ gene at 2.47 kb 3' to the C μ gene.

In this study we have analyzed the structure and expression of the δ gene using two hybridomas, B1-8. δ 1 and GCL2.8. The latter is a subclone of a hybridoma prepared by fusion of a Syrian hamster lymphoma (GD36A) with mouse spleen cells (BALB/c), and bears both IgM and IgD on the cell surface, which appear to share common idiotypic determinants (Raschke, 1978; Wabl et al., 1980). This hybridoma was recently shown by karyotype analysis to contain only one copy of mouse chromosome 12 (Wabl et al., 1980), on which the heavy chain genes reside (Hengartner et al., 1978; Meo et al., 1980). The other hybridoma, B1-8. δ 1, secretes IgD and has cell-surface IgD molecules but no IgM molecules (Neuberger and Rajewsky, 1981). This hybridoma is a derivative of a cell prepared by the fusion of a mouse myeloma cell (Ag8653) with a mouse spleen cell (C57BL/6); the original hybridoma produced IgM but no IgD. The loss of IgM has been linked to the deletion of the C μ gene (F. Sablitski, personal communication).

Results

Cloning of the Genomic DNA Segments Containing C μ and C δ in Mouse Embryo

Liu et al. (1980) and Tucker et al. (1980) mapped the C δ gene 2.5 kb 3' to the C μ gene, using cDNA prepared from the δ mRNA present in myeloma TEPC1017. Two types of recombinant DNA libraries, an Eco RI partial and an Eco RI* library, were utilized to cover the approximately 27 kb region containing the four J μ s, C μ and C δ sequences (Figure 1). Clone MEP203 (Figure 1c) was isolated from an Eco RI partial library with the 0.8 kb Xba I-Eco RI fragment (J probe) as a hybridization probe (Figure 1b). Previous characterization of this clone suggested that it was composed of three Eco RI fragments in the order 5' to 3' of 1.5 kb, 6.4 kb and 10.5 kb (Maki et al., 1980). Using the

4.5 kb Xba I-Xba I fragment indicated in Figure 1b as a hybridization probe, we isolated several clones from the Eco RI* library, one of which was ME501. This clone was shown to contain three Eco RI fragments: 3 kb, 10.5 kb and 2 kb. Heteroduplex analysis revealed that this 3 kb fragment was at the 5' end and was completely homologous to the 3' end of clone MEP203. Thus the clone ME501 extended 12.5 kb to the 3' side of clone MEP203. Additional heteroduplex and restriction enzyme mapping of various clones allowed us to order the fragments as shown in Figure 1c.

Other clones were isolated in this region with the 2 kb Eco RI fragment isolated from ME501 (Figure 1b). One of these clones, ME645 (Figure 1c), has an insert size of slightly more than 16.5 kb. It was composed of three Eco RI fragments, whose order of 10.5 kb, 2 kb and 4 kb was established by heteroduplex analysis and restriction enzyme mapping with clone ME501 and other clones not shown. These clones cover the genomic DNA segment for J_H , C_μ and C_δ .

Multiple Forms of δ mRNA in B1-8. δ 1

The poly(A)⁺ RNA extracted from B1-8. δ 1 was fractionated on a 10–40% sucrose gradient; and an aliquot of each fraction was electrophoretically separated on a 1% agarose gel containing methylmercury, transferred to diazonitrobenzoxymethyl (DBM) paper and hybridized with a 2.1 kb Xba-Xba probe (Figure 1) that contains the first two exons of the C_δ gene (Liu et al., 1980; Tucker et al., 1980). The results are shown in Figure 2A. In fractions 8 and 9 the predominant RNA species was 2.7 kb, while in fractions 11, 12 and 13 the predominant RNA was 1.8 kb, but was slightly heterogeneous. The 1.8 kb species appeared to be present at a level 3–4 times that

of the 2.7 kb species. To demonstrate that at least two RNA species code for δ chains, aliquots of RNA from fractions 8 and 12 were translated in a reticulocyte lysate system and the proteins synthesized were precipitated with an anti- δ antiserum. As shown in Figure 2B, RNA from fraction 8 coded for a product of about 48,500 daltons, while RNA from fraction 12 coded for a protein of about 47,000 daltons. Control precipitates with anti- μ or anti-light chain antisera gave results like those shown here for normal rabbit serum. Thus it would appear that the two RNA species observed by the RNA blotting experiment code for two immunoprecipitable forms of δ chain.

The exon-intron structure of the C_δ gene coding for the two δ mRNAs was studied by electron microscopy. RNAs from fraction 8 and fraction 12 were separately

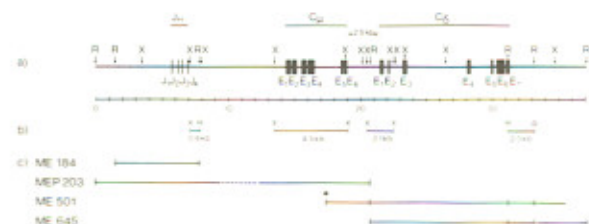


Figure 1. Physical Map of Mouse Embryo DNA

(a) Physical map of mouse embryo DNA segment encompassing J_H , C_μ and C_δ . The positions of the four J_H s were mapped according to the method of Sakano et al. (1980). The exons of C_μ (E₁'-E₄') were mapped according to the methods of Maki et al. (1980), Calame et al. (1980) and Gough et al. (1980). The exons of C_δ (E₁-E₇) were mapped with data presented here as well as by Liu et al. (1980) and Tucker et al. (1980). R: Eco RI. X: Xba I. Scale is in kb.

(b) Probes isolated from DNA clones.

(c) Clones of mouse DNA fragments in Charon 4A. Vertical bars: Eco RI sites. *, Eco RI site created by Eco RI* activity and ligation into the vector Charon 4A. Dashed line in clone MEP203: deletion of about 2.5 kb, which resulted during the cloning, probably due to recombination within a repeated sequence (Davis et al., 1980b; Sakano et al., 1980).

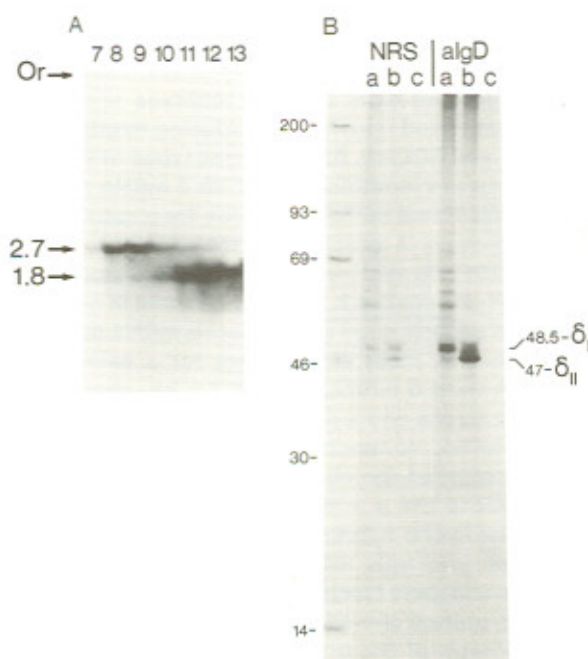


Figure 2. RNA Blotting and in Vitro Translation and Immunoprecipitation of δ mRNA

(A) RNA blot of B1-8. δ 1 mRNA. RNA was first fractionated on a 10–40% exponential sucrose gradient. An aliquot (0.5 μ g) of each fraction was electrophoretically separated on a 1% agarose gel containing methylmercury and processed as described in Experimental Procedures. The 2.1 kb Xba I-Xba I DNA fragment described in Figure 1 was used as a hybridization probe. Fractions are indicated at the top of the figure. Or: origin. Sizes of bands are indicated in kb. The rRNA extracted from B1-8. δ 1 was used as a standard; 28S = 5000 bp, 18S = 2100 bp. The filter was exposed for 20 hr before developing. (B) In vitro translation and immunoprecipitation of δ mRNAs. One microgram per lane of sucrose gradient fraction 8 (a) or fraction 12 (b) mRNA or nonspecific control mRNA (c) was translated in vitro with ³⁵S-methionine. The products were then immunoprecipitated with 2 μ l of nonimmune rabbit serum (NRS) or rabbit anti-mouse myeloma T1087 (algD), followed by Protein A-Sepharose 4B beads. The immunoprecipitates were eluted and run on SDS-PAGE under reducing conditions using a linear 8–18% 20 cm gradient gel. Autoradiograms of the dried gel, including the positions and sizes of marker proteins in kd, are shown.

hybridized with the clone ME645. The predominant form of the hybrid observed with the fraction 8 RNA is shown in Figure 3a. This RNA-DNA hybrid (type I) displayed six exons covering a distance of 9.8 kb (Table 1). The position of E1 places the 5' end of this exon about 2.5 kb from the 3' end of exon 6 of the C_μ gene (see Figure 1). This value is in good agreement with the previously determined 2.47 kb distance between the C_μ and C_δ genes (Liu et al., 1980). However, this gene structure differs from that of Liu et al. (1980), who observed only four exons.

RNA from fraction 12 gave a variety of different structures. The most prevalent structure consisted of four exons (Figure 3b; type II), three of which corresponded in size and position to E1, E2 and E3 exons

observed for the 2.7 kb species (Table 1). The fourth exon, E4, was estimated to be 300 bp in length and located 4.6 kb from the 3' end of E3 (Table 1). This form of the C_δ gene closely resembles that reported by Liu et al. (1980). RNA from fraction 12 gave another hybrid, which exhibited an exon-intron structure similar to but distinct from that of the 2.7 kb RNA species (Figure 3c; type III; Table 1). The exons E1, E2, E3 and E5 were all present, but only about 300 bp (5' portion) of the total 600 bp of exon 6 and no exon 7 hybridized. Several other hybrid structures were also observed with RNA from fraction 12, but each of them comprised a relatively minor fraction of the total hybrid molecules observed. They will not be considered further here.

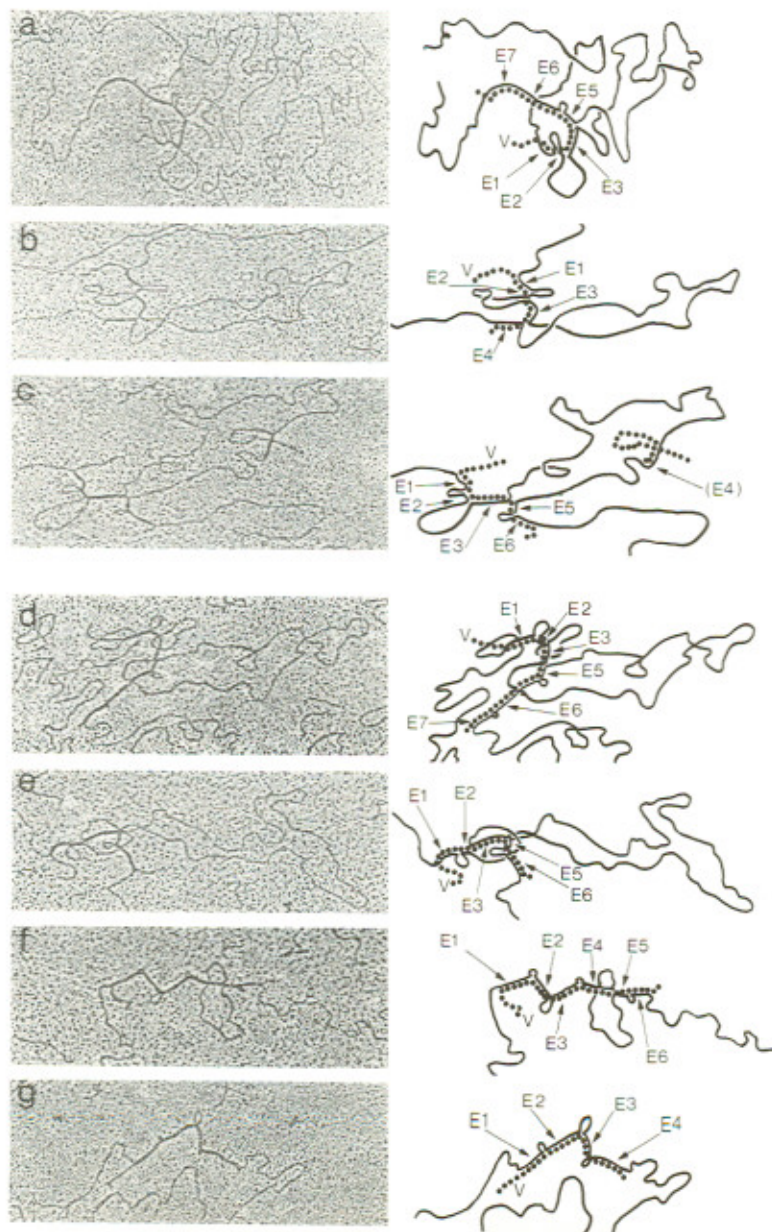


Figure 3. Electron Micrographs and Schematic Interpretation of mRNA-DNA Hybrids Observed with RNA from B1-8.81 or GCL2.8 and DNA from Clones in Figure 1

In the schematic drawings the solid line is DNA and the dotted line is mRNA. The measurements for each exon (E) are given in Table 1. V: RNA tail interpreted as coding for the V gene.

(a) Type I δ mRNA-DNA hybrid observed with B1-8.81 mRNA (fraction 8) and DNA from clone ME645. Six exons for the C_δ gene are visible: E1, E2, E3, E5, E6 and E7, as described in the text.

(b) Type II δ mRNA-DNA hybrid observed with B1-8.81 mRNA (fraction 12) and DNA from clone ME645. Four exons for the C_δ gene are visible: E1, E2, E3 and E4, as described in the text.

(c) Type III δ mRNA-DNA hybrid observed with B1-8.81 mRNA (fraction 12) and DNA from clone ME645. Five exons for the C_δ gene are visible: E1, E2, E3, E5 and E6, as described in the text. (E4): a type II δ RNA that had only hybridized to E4 of the molecule.

(d) Type I δ mRNA-DNA hybrid observed with GCL2.8 mRNA (fraction 9) and DNA from clone ME645. Six exons for the C_δ gene are visible: E1, E2, E3, E5, E6 and E7, as described in the text.

(e) Type III δ mRNA-DNA hybrid observed with GCL2.8 mRNA (fraction 13) and DNA from clone ME645. Five exons for the C_δ gene are visible: E1, E2, E3, E5 and E6, as described in the text.

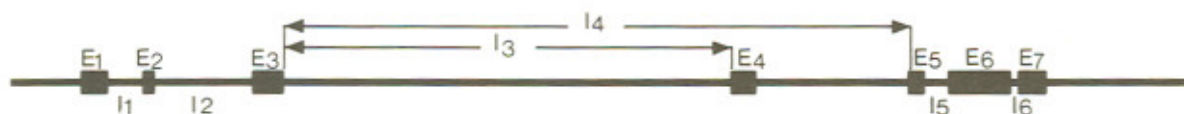
(f) Membrane μ mRNA-DNA hybrid observed with GCL2.8 mRNA (fraction 9) and DNA from clone MEP203. Six exons of the C_μ gene are visible: E1, E2, E3, E4, E5 and E6, as described in the text.

(g) Secreted μ mRNA-DNA hybrid observed with GCL2.8 mRNA (fraction 10) and DNA from clone MEP203. Four exons of the C_μ gene are visible: E1, E2, E3 and E4, as described in the text.

Table 1. Length Measurements of C δ Exons and Introns

Hybridoma	Type	N	E ₁	I ₁	E ₂	I ₂	E ₃	I ₃	E ₄	I ₄	E ₅	I ₅	E ₆	I ₆	E ₇
B1-8.δ1	I	10	0.26 ±0.03	0.42 ±0.05	0.11 ±0.02	1.0 ±0.03	0.29 ±0.02	—	—	6.42 ±0.20	0.15 ±0.02	0.28 ±0.02	0.95 ± 0.09		
	II	20	0.26 ±0.03	0.37 ±0.06	0.10 ±0.01	0.99 ±0.07	0.32 ±0.05	4.58 ±0.24	0.25 ±0.2	—	—	—	—		
	III	6	0.26 ±0.04	0.37 ±0.03	0.11 ±0.02	1.09 ±0.07	0.33 ±0.04	—	—	6.53 ±0.21	0.17 ±0.02	0.30 ±0.04	0.33 ±0.07	—	
GCL2.8	I	14	0.26 ±0.05	0.31 ±0.05	0.12 ±0.03	0.99 ±0.06	0.31 ±0.05	—	—	6.30 ±0.39	0.17 ±0.03	0.19 ±0.03	0.63 ±0.02	0.1	0.28 ±0.02
	III	5	0.26 ±0.01	0.34 ±0.04	0.11 ±0.01	0.98 ±0.09	0.30 ±0.02	—	—	6.31 ±0.35	0.15 ±0.02	0.24 ±0.02	0.36 ±0.01	—	

* Sizes are given in kilobase pairs ± S.D.; N = number of molecules measured.



Multiple Forms of δ and μ mRNA in GCL2.8

We analyzed the μ and δ mRNA of GCL2.8 in a manner similar to that used for the analysis of the δ mRNA from B1-8.δ1. As shown in Figure 4A, in sucrose gradient fractions 9 and 10 the predominant RNA species was 2.7 kb, while in fractions 12 and 13 a species of 1.8 kb was observed. The amount of 2.7 kb species observed here was about 1/10 of that in B1-8.δ1, while the 1.8 kb species was on the order of 1/100 of that in B1-8.δ1. Figure 3d depicts the predominant hybrid structure observed when RNA from fraction 9 was hybridized with the clone ME645. The six exons observed with this RNA were indistinguishable in size and position from the exons observed for type I δ RNA from B1-8.δ1 (Figure 3a; Table 1). RNA from fraction 12 gave hybrid structures indistinguishable from those observed with the type III δ mRNA from B1-8.δ1. No RNA was observed that used exon E4.

The same RNA blot was washed free of the ³²P-labeled C δ probe and was subsequently hybridized to the 4.5 kb Xba I-Xba I probe containing the C μ gene (Figure 1b). The results, shown in Figure 4B, indicate the presence of two RNA species of 2.7 and 2.4 kb. Figure 3f displays the hybrid structure that was observed with clone MEP203 and fraction 9 RNA containing predominantly the 2.7 kb species. This six-exon structure is characteristic of the C μ gene coding for the membrane-bound μ chain (Rogers et al., 1980; Early et al., 1980b). Fraction 11 RNA, containing the two species of μ mRNA in roughly equal proportion, gave two types of hybrid structure. One was indistinguishable from that observed with fraction 9 RNA and the other contained only the first four exons (Figure 3g). The latter structure is characteristic of the C μ gene coding for a secreted μ chain (Rogers et al., 1980; Early et al., 1980b). Thus, although GCL2.8 appears to synthesize only the membrane form of IgM,

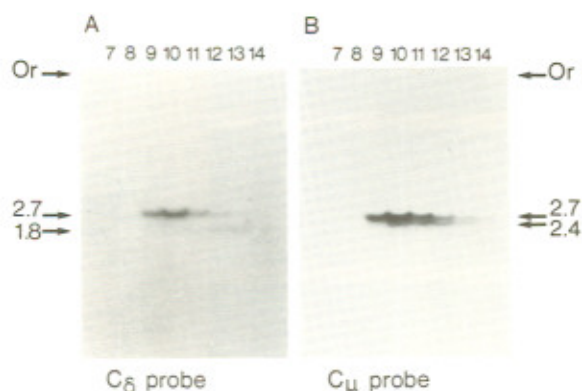


Figure 4. RNA Blot of GCL2.8 mRNA

RNA was first fractionated on a 10–40% exponential sucrose gradient. An aliquot (0.5 μ g) of each fraction was electrophoretically separated on a 1% agarose gel containing methylmercury and processed as described in Experimental Procedures. Fractions are indicated at the top of the figure. Or: origin. Sizes of bands are in kb. rRNA 28S (5000 bp) and 18S (2100 bp) extracted from GCL2.8 were used as size markers.

(A) C δ probe (2.1 kb Xba I-Xba I fragment; Figure 1) used in hybridization. The filter was exposed for 72 hr before developing.
(B) C μ probe (4.5 kb Xba I-Xba I fragment; Figure 1) used in hybridization. The filter was exposed for 10 hr before developing.

the RNAs for both the secreted and membrane forms of μ chains are present in this hybridoma.) This suggests, as pointed out by Sidman (1981), that translational or posttranslational controls may also play an important role in the expression of the various Ig genes.

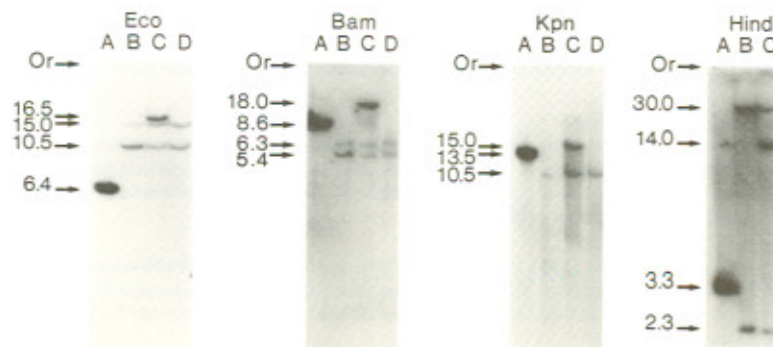
Heavy Chain J Rearrangement in GCL2.8

Previous karyotype and enzyme marker analysis revealed that GCL2.8 contains only one copy of the mouse chromosome 12 (Wabl et al., 1980). This suggests that only a haploid set of heavy chain V genes or their components are present in this cell. To verify

this conclusion by a more direct molecular means, Southern blot hybridization and genomic DNA cloning were used. The DNAs of embryos (BALB/c), GD36A (the Syrian hamster tumor used in cell fusion with mouse spleen cells), GCL2.8 and either 1M10 or 21M3 (subclones of GCL2.8 that have lost the one copy of mouse chromosome 12) were digested with the restriction endonucleases Eco RI, Bam HI, Kpn I or Hind III, fractionated by gel electrophoresis, transferred to nitrocellulose and hybridized with the 0.8 kb Xba I-Eco RI fragment (J probe) indicated in Figure 1b. This probe is the 3' end of the 6.4 kb embryonic Eco RI fragment containing the four J_H segments. A V-D-J joining that utilizes one of the four J_Hs brings in a new 5' Eco RI site and will therefore likely alter the size of this fragment. The results with this probe and various digests of the DNAs mentioned are shown in Figure 5. The Eco RI digest of embryo DNA gave one band at 6.4 kb, while the parent tumor GD36A of Syrian hamster origin had two bands, 15 kb and 10.5 kb. The hybridoma GCL2.8 gave three bands; two of the bands corresponded to the bands in GD36A—that is, 15 kb and 10.5 kb—and a third band of 16.5 kb was unique to this cell. The hybridoma 1M10, which has lost the single copy of mouse chromosome 12 present in GCL2.8, lacks this 16.5 kb band and displays only the two bands present in GD36A.

To confirm these results we repeated the experiment using Kpn I, Hind III and Bam HI digests of the same DNAs (Figure 5). In the Kpn I-digested DNA of GCL2.8 the unique band appeared at 15 kb and was absent from the DNA from 21M3. In the Hind III digests a unique band was identified at 14 kb; and finally, in the Bam HI-digested DNA a unique band in GCL2.8 appeared at 18 kb.

The Southern blot experiments allow us to conclude that: there is only one J-positive band in GCL2.8 that cannot be accounted for by DNA donated by the parent tumor GD36A; this J-positive DNA fragment is rearranged for all enzymes used when compared with embryo DNA; and the disappearance of this J-positive band in cells that have lost mouse chromosome 12 is correlated with the loss of IgM and IgD synthesis (Wabl et al., 1980).



Cloning and Characterization of the J-positive Fragment in GCL2.8

We extended the characterization of the J-positive rearranged sequence by cloning the 16.5 kb Eco RI fragment from Eco RI-digested GCL2.8, using the phage vector Charon 4A. Four identical clones were isolated, one of which, GCL-J5, is described here. As expected, the cloned fragment was 16.5 kb (Figure 6). The fragment was shown to be rearranged at J_{H4} by two criteria: first, heteroduplexes formed between the clone GCL-J5 and ME184 (the embryonic 6.4 kb J-positive Eco RI clone) indicated complete homology of 1.2 kb from the 3' Eco RI site to J_{H4}; second, restriction maps of both the embryo DNA clone and the hybridoma DNA clone revealed that cleavage sites 3' to J_{H4} were common while those 5' to J_{H4} were unique to each (Figure 6).

Since the 16.5 kb Eco RI fragment is the unique fragment containing a rearranged J_H sequence in GCL2.8, we conclude that this fragment contains the complete V_H gene (see below).

The Same V_H Gene Is Used for μ and δ Chains in GCL2.8

Prior serological studies indicated that the IgM and IgD molecules synthesized by GCL2.8 carry the same idiotype (W. Raschke, unpublished observations). This and the fact that only one complete V_H gene is detectable in GCL2.8 strongly suggest that the μ and δ chains employ the same V_H gene. To confirm this concept, we wished to demonstrate that the μ and δ

Figure 6. Restriction Enzyme Cleavage Maps of the DNA inserts from Clones ME184 and GCL-J5

The four J_Hs (J₁, J₂, J₃ and J₄) were mapped as previously described by Sakano et al. (1980). The V-J₄ indicated in GCL-J5 is interpreted as the rearranged V gene in GCL2.8 described in the text. E: Eco RI. P: Pst I. X: Xba I. H: Hpa II. H: Hind III. B: Bam HI. K: Kpn I.

Figure 5. Southern Blot Hybridization of Various DNAs with J Probe

DNA was from BALB/c mouse embryo (A), GD36A (B), GCL2.8 (C) or 1M10 (with Eco RI) or 21M3 (with Bam HI and Kpn I) (D). The J probe used for hybridization was the 0.8 kb Xba I-Eco RI fragment (see Figure 1). Eco: DNAs digested with Eco RI. Bam: DNAs digested with Bam HI. Kpn: DNAs digested with Kpn I. Hind: DNAs digested with Hind III. Or: origin. Sizes of bands are in kb.

mRNAs from GCL2.8 both contain a sequence complementary to the V_H gene presumably present on the clone GCL-J5. Since the mRNAs were too similar in size (Figure 4) to be resolved by electrophoresis, direct hybridization experiments with the V_H DNA probe did not provide convincing results. To circumvent this difficulty, we combined RNA-DNA hybridization with DNA-DNA heteroduplex formation and examined the hybrid molecules by electron microscopy (referred to as R hybrid).

In one experiment the phage DNA of two DNA clones, the V_H gene clone GCL-J5 and the C_μ gene clone MEP203, was mixed, denatured and annealed in the presence of the partially enriched GCL2.8 μ mRNA (fraction 9 of Figure 4B). Since the mouse DNA inserts of clones GCL-J5 and MEP203 are in an opposite orientation relative to the long and short phage DNA end fragments, the two DNA strands containing the noncoding sequences of the respective genes form a heteroduplex molecule by virtue of the homology in the phage DNA end fragments. A μ mRNA molecule in the partially enriched RNA preparation will hybridize with the C_μ exons on one DNA strand of the heteroduplex molecule and also with the V_H exon on the other DNA strand, if the mRNA is indeed encoded by this V_H gene. The hybrid structures shown in Figures 7A, 7B, 7G and 7H demonstrate that an RNA molecule in fraction 9 hybridized with both the V_H sequence on GCL-J5 and the C_μ sequence on MEP203. The DNA-RNA hybrids depicting the six C_μ exons for the membrane form of μ are clearly visible; and the 5' end of the mRNA hybridizes with the 300 bp V gene on clone GCL-J5. Note that the latter homology is 1.2 kb from the 3' Eco RI end of the insert, which is exactly the position where the V_H gene is expected to reside. We thus conclude that the V_H part of the mRNA coding for membrane μ in GCL2.8 is transcribed from the GCL-J5 V_H gene.

In a similar manner, the DNAs from phages ME645 (containing C_δ) and GCL-J5 and RNA from fraction 9 of the sucrose gradient (Figure 4A) were mixed and incubated. As shown in Figures 7C-7F, DNA-RNA hybrid structures similar to those previously demonstrated for the 2.7 kb δ RNA species are formed on

the ME645 strand of the heteroduplex. The 5' end of the same mRNA molecule hybridizes with the GCL-J5 strand. Again, 1.2 kb of single-stranded DNA separate the latter homology and the DNA-DNA hybrid formed by one end of the phage genome. These results demonstrate that the V_H gene on the GCL-J5 clone encodes the V part of the δ mRNA. Taken together, the results allow us to conclude that both μ and δ mRNA of GCL2.8 are transcribed from the same V_H gene.

There Is No DNA Rearrangement between C_μ and C_δ in GCL2.8

One possible mechanism that would allow the simultaneous expression of the same V_H gene in association with C_μ and C_δ genes is a duplication of the complete V_H gene and an insertion of the duplicated copy between the C_μ and C_δ genes. To test this and other models that include DNA rearrangement in the C_μ - C_δ gene region, we examined the DNA from GCL2.8 and embryos by Southern blot hybridization. Eight DNA fragments from the embryo DNA clones that encompass the genomic segment of DNA containing the C_μ and C_δ exons were used as hybridization probes to examine total DNA from embryos or GCL2.8 digested with the restriction endonucleases Eco RI, Bam HI or Xba I. Typical Southern blots are shown in Figure 8; the results have been summarized in Figure 9.

As shown in Figure 8A, the 1.5 kb Xba I-Xba I fragment (probe B in Figure 9) located between C_μ and C_δ hybridized to a 12 kb fragment in Bam HI digests of embryo and GCL2.8 DNAs. The 2.1 kb Xba I-Xba I fragment (probe C in Figure 9), containing the first two exons of C_δ , hybridized to a 10.5 kb Eco RI fragment in both embryo (lane a) and GCL2.8 (lane b) DNAs (Figure 8B) or a 12 kb Bam HI fragment in both (Figure 8C). The smear in the background of both embryo and GCL2.8 DNAs is due to crosshybridization of the probe sequence to a large number of DNA fragments. In Figure 8D the DNA blot of the 3' end of the C_δ gene is shown with a 2.5 kb Bam HI-Bam HI fragment (probe F in Figure 9). When this probe was hybridized to Eco RI-digested embryo and GCL2.8 DNAs, it gave the expected result of a common frag-

Figure 7. Electron Micrographs and Schematic Interpretation of R Hybrids with RNA from GCL2.8 and DNA from Clones Described in Figures 1 and 6

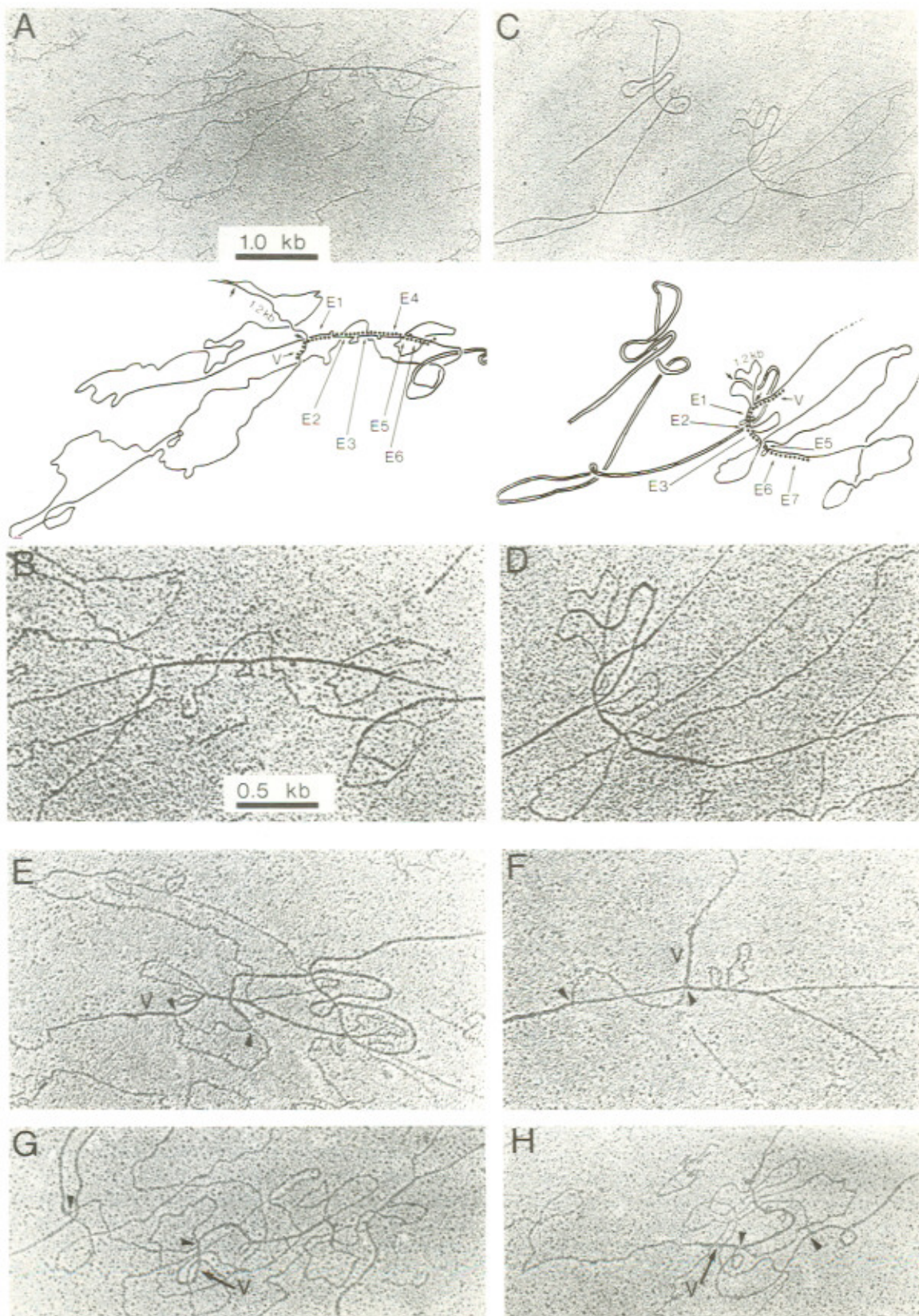
In the schematic drawings the solid lines are DNA and the dotted line is mRNA.

(A, B, G, H) R hybrid structures observed with GCL2.8 mRNA (fraction 9) and DNA from clones MEP203 and GCL-J5. The double-stranded DNA structures are the complementary strands to the phage DNA that have hybridized. The single-stranded DNA inserts are derived from clones MEP203 and GCL-J5. mRNA has hybridized to the six exons of C_μ on MEP203 and the V gene of GCL-J5. Short arrows in schematic drawings: 1.2 kb length between the end of the insert in GCL-J5 and the V gene discussed in the text.

(B) Enlargement of the V-C junction shown in A.

(C, D, E, F) R hybrid structures observed with GCL2.8 mRNA (fraction 9) and DNA from clones ME645 and GCL-J5. The double-stranded DNA structures are the complementary strands to the phage DNA that have hybridized. The single-stranded DNA inserts are derived from clones ME645 and GCL-J5. mRNA has hybridized to six exons of C_δ (type I) on ME645 and the V gene of GCL-J5. Arrowheads (E-H): 1.2 kb length between the end of the insert in GCL-J5 and the V gene discussed in the text.

(D) Enlargement of the V-C junction shown in C.



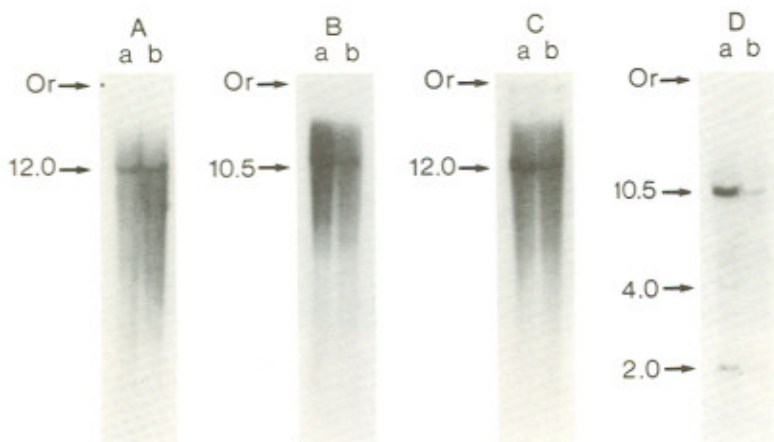


Figure 8. Southern Blot Hybridization of Two DNAs with Probes Shown in Figure 9

(Lane a) BALB/c mouse embryo DNA; (lane b) GCL2.8 DNA. Or: origin. Sizes of bands are in kb.

(A) DNAs digested with Bam HI and hybridized with probe B in Figure 9 (1.5 kb Xba I-Xba I fragment).

(B) DNAs digested with Eco RI and hybridized with probe C in Figure 9 (2.1 kb Xba I-Xba I fragment).

(C) DNAs digested with Bam HI and hybridized with probe C in Figure 9 (2.1 kb Xba I-Xba I fragment).

(D) DNAs digested with Eco RI and hybridized with probe F in Figure 9 (2.5 kb Bam HI-Bam HI fragment).

ment at 10.5 kb in each as well as a 2 kb fragment in each, but also a crosshybridizing band in embryo DNA (lane a) at 4 kb, which is apparently absent in GCL2.8 DNA (lane b). The deletion of the 4 kb Eco RI fragment in GCL2.8 with probe F was verified by recent cloning and mapping results, which place this deletion 3' to the C_{δ} gene (R. Maki and W. Roeder, unpublished observations). These results, as well as the summary of various probes and digests in Figure 9, suggest that no large DNA rearrangement has occurred in the region of C_{μ} and C_{δ} in GCL2.8. While rearrangements of relatively short sequences may have been overlooked, it is unlikely that an insertion of a complete V_{H} gene somewhere between the C_{μ} and C_{δ} genes could have escaped detection.

Deletion of C_{μ} in B1-8.81

We were interested in determining whether the appearance of IgD and loss of IgM in B1-8.81 may be explained by a mechanism similar to that observed for class switch from IgM to IgG or IgA. In these cases the C_{μ} gene is deleted (Colecough et al., 1980; Cory and Adams, 1980; Rabbitts et al., 1980). To answer this, Southern blot hybridization of Eco RI-digested DNA from Ag8653 (the mouse tumor used in the cell fusion), B1-8.9 (IgM producer) and B1-8.81 was performed with the 4.5 kb Xba I-Xba I fragment (Figure 1b), which contains most of the C_{μ} gene. These results are shown in Figure 10. The Eco RI digest of B1-8.9 (lane B) shows a band at 15 kb, which corresponds to the Eco RI fragment containing the C_{μ} gene in C57BL/6 (Marcu et al., 1980). Two small bands were also observed, but these are due to crosshybridization of the sequences flanking the C_{μ} gene and are not considered further here. The DNA from Ag8653 (lane A) shows no C_{μ} gene-containing fragment; it was derived from a cell that previously synthesized IgG and had deleted the C_{μ} gene. The Eco RI-digested DNA from B1-8.81 (lane C) also did not show a band at 15 kb. Thus the appearance of IgD expression and loss of IgM expression in B1-8.81 is accompanied by the deletion of the C_{μ} gene.

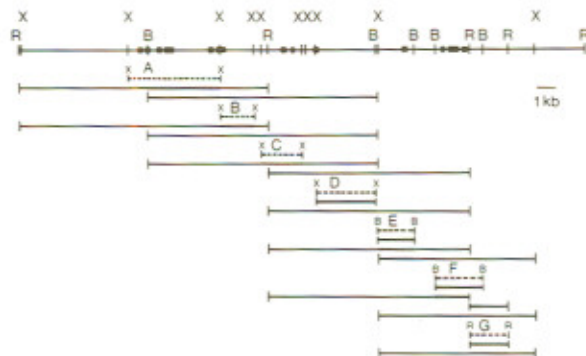


Figure 9. Summary of Southern Blot Hybridization Experiments on Mouse Embryo DNA and GCL2.8 DNA

(Top line) restriction endonuclease map derived from cloned DNA in Figure 1. Positions of exons for C_{μ} and C_{δ} (small black boxes) are as described in Figure 1. R: Eco RI. X: Xba I. B: Bam HI. The probes used are indicated by a dashed line; their homology with specific DNA fragments by the stippled area; and the hybridizable band common in mouse embryo and GCL2.8 by the solid lines.

Probe A: 4.5 kb Xba I-Xba I fragment. Probe B: 1.5 kb Xba I-Xba I fragment. Probe C: 2.1 kb Xba I-Xba I fragment. Probe D: 3.0 kb Xba I-Xba I fragment. Probe E: 1.9 kb Bam HI-Bam HI fragment. Probe F: 2.5 kb Bam HI-Bam HI fragment. Probe G: 2.0 kb Eco RI-Eco RI fragment.

The sizes of the fragments common to embryo and GCL2.8 DNAs that will hybridize with specific probes are as follows. Probe A: 13 kb Eco RI, 12 kb Bam HI. Probe B: 13 kb Eco RI, 12 kb Bam HI. Probe C: 12 kb Bam HI, 10.5 kb Eco RI. Probe D: 3 kb Xba I, 10.5 kb Eco RI. Probe E: 1.9 kb Bam HI, 10.5 kb Eco RI, 8.3 kb Xba I. Probe F: 2.5 kb Bam HI, 10.5 kb Eco RI, 2 kb Eco RI, 8.3 kb Xba I. Probe G: 2 kb Eco RI, 8.3 kb Xba I.

Discussion

The Multiple Forms of the C_{δ} Gene and δ mRNA

This report describes the exon-intron structure of the mouse C_{δ} genes. A total of seven exons encompassing about 10 kb was mapped by electron microscopy. At least three distinct types of δ mRNA were identified that differ from each other by their use of the exons. The largest mRNA (type I), observed in both GCL2.8 and B1-8.81, was 2.7 kb and hybridized to six of the seven exons (all except E4). This was the predominant

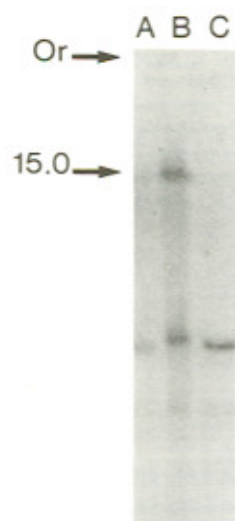


Figure 10. Southern Blot Hybridization of Eco RI-digested DNAs with C_δ Probe

DNAs were from Ag8653 (lane A), B1-8.9 (lane B) or B1-8.δ1 (lane C). Probe was C_δ gene (4.5 kb Xba I-Xba I fragment; Figure 1b). Or: origin. Sizes of bands are in kb.

species in GCL2.8, and since GCL2.8 has only membrane δ and does not secrete a detectable amount of IgD, we tentatively conclude that it codes for the membrane form of δ .

Of particular interest was the type II species of δ mRNA, identified in B1-8.δ1, which hybridized to exons E1, E2, E3 and E4. This δ mRNA was predominant in this hybridoma, as judged both by the gel blot analysis and by frequency of the characteristic hybrid structure observed by electron microscopy. Recently, in work published by Liu et al. (1980) and Tucker et al. (1980), a cDNA from TEPC1017 that contains the C_δ sequence was characterized in detail and was shown to hybridize to four exons in a pattern very similar to the type II RNA of B1-8.δ1. Both TEPC1017 and B1-8.δ1 secrete IgD, while GCL2.8, which lacks this type of δ mRNA, apparently does not. Based on this correlation we conclude that the type II mRNA codes for a secreted form of δ . This conclusion is supported by the finding that the amino acid sequence predicted from the nucleotide sequence of the TEPC1017 cDNA lacks a hydrophobic peptide at the carboxy-terminal end characteristic of the membrane-bound μ chain (Tucker et al., 1980). It would be interesting to see if the type I δ mRNA has a sequence coding for such a hydrophobic peptide.

An additional form of δ mRNA, which we have referred to as type III, was observed in both B1-8.δ1 and GCL2.8. This RNA has an exon-intron structure similar to that of the type I δ RNA, but is somewhat shorter at its 3' end and contains only a portion of exon 6 and none of exon 7 (Figures 3c and 3e; Table 1). Two possibilities can be considered for its presence: that this RNA, having a 3' end different from either type I

or type II δ RNAs, codes for yet a third type of δ protein; or that this RNA also codes for the same membrane form of δ as type I RNA. This latter alternative has become more reasonable based on the finding that dihydrofolate reductase mRNA has a large degree of heterogeneity at its 3' end but each RNA can be translated in vitro to produce the same protein (Setzer et al., 1980). Possibly the only difference between the type I and type III RNAs is the length of the 3' untranslated region.

If the above interpretation of the multiple forms of δ mRNA and C_δ genes turns out to be correct, it has a direct parallel with the multiple forms of μ mRNA and the C_μ gene. Two forms of μ mRNA have been identified; one is thought to code for secreted μ and the other for membrane μ . The critical difference between these two mRNAs is their use of different 3' exons (Alt et al., 1980; Rogers et al., 1980; Early et al., 1980b; Singer et al., 1980; Tonegawa et al., 1980).

Alternative RNA Processing of a Common Primary Transcript Probably Generates μ and δ Chains in a Single Cell

The finding of only one copy of chromosome 12 in GCL2.8 by Wabl et al. (1980) and work presented here would seem to eliminate models for double expression that utilize two copies of chromosome 12, one for the synthesis of a V- C_μ product and the other for a V- C_δ product.

The possibility that the V_H gene was duplicated and inserted between the C_μ and C_δ genes—that is, to the 5' side of exon E1 of the C_δ gene—was considered but was found to be inconsistent with our Southern blot results. The conclusion reached by this analysis is that the organization of the C_δ gene and its 5' flanking sequences in GCL2.8 DNA is essentially unchanged from that of embryo DNA. The DNA rearrangement to the 3' side of the C_δ gene is curious. Although this deletion does not involve the seven δ exons described here, we consider several explanations possible: first, that this rearrangement somehow influences the expression of the δ gene; second, that this deletion is a preliminary step in switch recombination and may suffice to bring the C_μ genes closer to the rearranged V gene, while still allowing the expression of μ and δ ; or third, that this observation is simply an artifact in GCL2.8.

Given the results presented here, the most likely mechanism for the simultaneous synthesis of μ and δ chains is alternative processing of a common primary RNA transcript containing one copy each of the V_H , C_μ and C_δ sequences. A model describing this mechanism, which borrows heavily from the information accumulated for the late genes of adenovirus (reviewed in Ziff, 1980), is outlined in Figure 11. We have shown that the only functional DNA sequence rearrangement occurring in the double producer GCL2.8 is for the assembly of the V gene components,

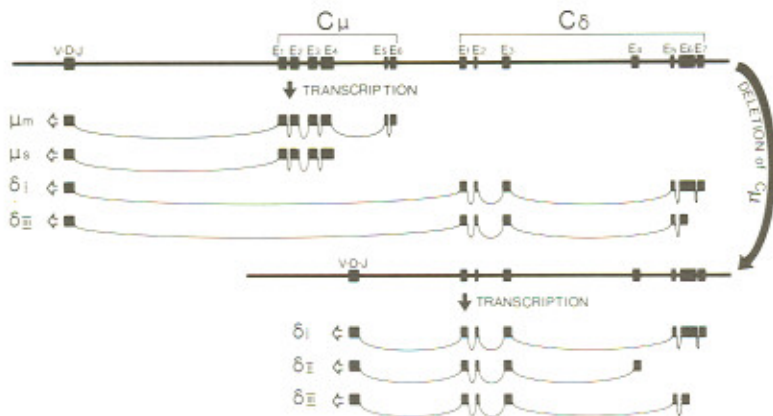


Figure 11. Two Ways to Express δ
Models are presented for expression of δ as observed in GCL2.8 (top) and B1-8. δ 1 (bottom). Straight lines: arrangement of exons (blocks) in the DNA for the V gene (V-D-J), C $_{\mu}$ gene (E1-E6) and C $_{\delta}$ gene (E1-E7). After transcription the exons are spliced together to code for membrane μ (μ_m), secreted μ (μ_s), δ_I , δ_{II} and δ_{III} .

namely V_H, D_H and J_H. We thus assume that the entire 25 or so kilobase region containing the complete V_H gene, C_μ gene and C_δ gene can be transcribed into a single RNA molecule. We propose that this primary transcript contains at least five potential poly(A) attachment sites and that polyadenylation at one of these sites generates as many RNA species. For the production of membrane and secreted μ chains polyadenylation would occur just 3' to μ exon 6 or μ exon 4, respectively. This has been supported by the recent DNA-sequencing studies of Rogers et al. (1980) and Early et al. (1980b), who found potential polyadenylation sites (Proudfoot and Brownlee, 1976) in these areas. Once poly(A) has been attached, the appropriate RNA splices as diagrammed in Figure 11 would occur to create a 2.7 kb mRNA for membrane μ chain or a 2.4 kb mRNA for secreted μ chain.

The δ_I (membrane δ), δ_{II} (secreted δ) and δ_{III} (δ of unknown nature) mRNAs are postulated to derive from precursor RNAs that are polyadenylated at sites just 3' to δ exon 7, 3' to δ exon 4 or in the middle of δ exon 6, respectively. However, GCL2.8, which secretes no IgD, does not accumulate a detectable amount of δ_{II} mRNA. This may indicate that polyadenylation sites are under developmental control (see below).

Production of viable δ_I and δ_{III} mRNAs from the corresponding precursor RNA requires the donor splice site at the 3' end of the V_H exon to choose the acceptor site at the 5' end of the δ exon 1, rather than the acceptor site at the 5' end of μ exon 1. This skipping of internal exon sequences has not been observed as a legitimate splicing process in the eucaryotic genes studied to date, but precedents are available in animal viruses (reviewed in Darnell, 1979; Ziff, 1980). For instance, in the expression of some of the late adenovirus genes a common (tripartite) leader must be attached to the distal exon sequences rather than to the proximal sequences (Chow and Broker, 1978; Nevins and Darnell, 1978; Berget and Sharp, 1979). In this case it has been proposed that polyadenylation at one of several alternative sites deter-

mines which exon sequences will be spliced to the common leader sequences. Those sequences immediately preceding poly(A) are the most preferred. There may be a direct recognition of poly(A) by the splicing apparatus; or the effect of polyadenylation may be indirect. For instance, the secondary structure of an RNA molecule may influence which pair of splice sites are preferred (Khoury et al., 1979). There may also be a gradient of "strength" among the alternative acceptor sites so that those located distal to the common donor site are preferentially used (Khoury et al., 1980). This idea of differential strength of alternative acceptor sites may also apply to the splicing of the complete V gene sequence to the C_μ gene sequences in GCL2.8, assuming that the 5' side of δ exon 1 is a "stronger" acceptor site than the 5' side of μ exon 1. However, it should be noted that there is no information presently available as to the relative frequency of splicing of the V_H sequence to the C_μ versus C_δ sequences in the RNA molecules on which all three sequences are present. A similar problem of selecting one of two acceptor sites arises when the δ exon 3 sequence splices to the δ exon 5 sequence for types I and III δ RNAs, since the δ exon 4 used for type II δ mRNA also carries a potential acceptor site for the δ exon 3 donor site.

In summary, there are two principal features in the proposed mechanism for simultaneous expression of μ and δ genes: selection of an appropriate polyadenylation site on a common primary RNA transcript, and selection of the correct pair of donor and acceptor splice sites.

IgD Expression after Deletion of C_μ

Recently, the expression of the γ and α heavy chain genes has been shown to be associated with a DNA rearrangement that occurs between the 5' flanking sequence of the C_μ gene and the expressed C_γ, C_α or C_ε gene. This rearrangement, referred to as a switch recombination, brings the V_H gene and C gene into close proximity (Maki et al., 1980; Davis et al., 1980a, 1980b) and involves the deletion of all DNA, including

the C_{μ} gene, between V_H and the expressed C gene (Honjo and Kataoka, 1978; Colecough et al., 1980; Cory and Adams, 1980; Rabbitts et al., 1980). Based on the history of the B1-8. δ 1 cell (see Introduction) and the demonstration that the C_{μ} gene is absent in this hybridoma (Figure 10), we suggest that a similar DNA recombination as described for the γ and α genes may have occurred for the δ gene in B1-8. δ 1. Thus, as shown in the bottom panel of Figure 11, a second way to express the δ gene is by deleting the C_{μ} DNA segment.

B1-8. δ 1 produces three major species of δ mRNA, two of which are indistinguishable from δ_I and δ_{III} present in GCL2.8; the third, δ_{II} , is unique to B1-8. δ 1. There are a number of possible steps, where synthesis of δ_{II} mRNA can be controlled. For instance, poly(A) attachment to the 3' side of δ exon 4 may be under a developmental control. Alternatively, the splice acceptor site at the 5' end of δ exon 4, which shares the δ exon 3 donor site with the δ exon 5 acceptor site may be functionally controlled.

Is secretion of IgD a natural phenomenon? In mice, serum IgD is extremely low and almost undetectable (Finkelman et al., 1979), the major portion being present on the membrane of lymphocytes. The most compelling argument for the natural occurrence of IgD secretion is our finding of the unique exon (δ exon 4) used to encode the δ chain secreted by B1-8. δ 1. We therefore conclude that IgD secretion and probably IgM-IgD switch are natural events, although the proportion of this form of δ gene expression varies among species.

Regulation of Immunoglobulin Gene Expression

The accumulating evidence on the expression of heavy chain immunoglobulin genes is beginning to reveal the dynamic nature of the eucaryotic genome. To fulfill the demands placed on it, the immune system has utilized both DNA rearrangements and complex RNA processing events to create new gene products with different functions. We (Sakano et al., 1980, 1981; Kurosawa et al., 1981) as well as others (Early et al., 1980a) have described the DNA rearrangements that bring together V, D and J DNA segments for a complete V gene. By an entirely different mechanism of DNA rearrangements, this one complete V gene can be subsequently expressed with one of at least six other heavy chain C genes (Davis et al., 1980a, 1980b; Kataoka et al., 1980; Maki et al., 1980; Sakano et al., 1980). Thus the underlying principle in DNA rearrangements for the heavy chains is twofold: first, to have a large degree of flexibility in the V region for antigen binding by assembling a V gene from a vast array of different V, D and J_H segments; and second, to express the same V region with one of many C genes in order to alter the effector functions of the complete immunoglobulin chain.

The heavy chain gene system has also utilized

intricate RNA processing schemes to further enhance the flexibility of gene expression. This was first illustrated for membrane μ and secreted μ (Rogers et al., 1980; Early et al., 1980b). In this paper we have concluded that RNA processing is involved in the simultaneous synthesis of two immunoglobulins, namely IgM and IgD. These are the first cases in which RNA processing has been shown to play an essential role in regulating gene expression in eucaryotic development.

Experimental Procedures

Cells

The hybridoma B1-8. δ 1 was supplied by M. S. Neuberger and K. Rajewsky (1981). The cells were cultured in RPMI1640, 15% fetal calf serum, 4 mM glutamine, 5×10^{-5} M 2-mercaptoethanol and 100 U/ml penicillin-streptomycin at 37°C, 5% CO₂. The cell line GD36A was supplied by T. Meo. This cell and hybridomas GCL2.8, 1M10 and 21M3 were grown in Dulbecco's modified Eagle's medium containing 15% fetal calf serum, 4 mM glutamine, 5×10^{-5} M 2-mercaptoethanol and 100 U/ml penicillin-streptomycin at 37°C, 5% CO₂.

Bacteria and Phages

Phage Charon 4A and E. coli DP50 (Su II⁻, Su III⁺) were obtained from F. Blattner (Blattner et al., 1977). The λ gtWES- λ B was obtained from P. Leder (Leder et al., 1977). E. coli 803 (*r_K*⁻, *m_K*⁻, Su III⁺) was originally from K. and N.E. Murray. Lysogens used for preparation of packaging mixtures, BHB 2688 (N205 *recA*⁻ [*limm434 b2 red3 eam4 sam7*]/ λ) and BHB 2690 (N205 *recA*⁻ [*limm434 cts b2 red3 dam15 sam7*]/ λ) were obtained from B. Hohn (Collins and Hohn, 1978).

Preparation of DNA

DNA was extracted from the tissue culture cells using a combination of proteinase K treatment and phenol extraction. Cells were collected by centrifugation at 1500 rpm for 10 min. The cell pellet was washed once in 10 mM Tris-HCl (pH 8.0), 0.15 M NaCl. The cells were then resuspended at a concentration of about 2×10^7 cells/ml in a buffer containing 10 mM Tris-HCl (pH 8.0), 150 mM NaCl and 10 mM EDTA, to which was added 20% SDS to a final concentration of 0.2% and proteinase K to a final concentration of 100 μ g/ml. This mixture was incubated for 10 hr at 37°C. An equal volume of phenol saturated with 0.1 M Tris-HCl (pH 8) was added and slowly mixed with the aqueous solution. After centrifugation the aqueous phase was extracted again with phenol. This was followed by extracting the aqueous phase one time with an equal volume of chloroform:isoamylalcohol (25:1). The DNA was immediately precipitated from the aqueous layer with two volumes of ethanol, spooled on a glass rod and redissolved in a small volume of 10 mM Tris-HCl (pH 8), 5 mM EDTA. Usually it was necessary to dialyze the DNA against the same buffer overnight at 4°C. The following day RNAase A (50 μ g/ml final concentration) and RNAase T1 (2 U/ml final concentration) were added to the DNA solution and the mixture was incubated at 37°C for 5 hr. The SDS and proteinase K treatment were repeated and the DNA was extracted two times with phenol as described above. The final aqueous phase was dialyzed extensively against a buffer containing 10 mM Tris-HCl (pH 8.0), 10 mM NaCl and 1 mM EDTA. Usually DNA prepared in this manner was easily digested with restriction endonucleases.

DNA Blots

Southern blot hybridization was carried out essentially as modified by Wahl et al. (1979). For the cellular Southern blots 10 μ g of DNA digested with the appropriate restriction endonuclease were used per lane. In the hybridization of the nitrocellulose filters to which DNA had been blotted 1×10^7 cpm of the appropriate nick-translated probe were used.

Preparation of Libraries and Isolation of Clones

The preparations of the mouse embryo RI partial library (Maki et al., 1980) and RI* library (Roeder et al., 1981) have been described. The clone ME184 was isolated with the preparative agarose gel technique described previously (Sakano et al., 1979) and the J probe (0.8 kb Xba I-Eco RI fragment in Figure 1b). The procedure to prepare nick-translated probes (Brack et al., 1978) as well as screening of the recombinant phage carrying mouse DNA (Benton and Davis, 1977) have also been described.

Preparation of RNA

RNA was prepared from the cultured cells by a method of R. Wall (personal communication). Cells were collected by centrifugation at 1500 rpm for 10 min and the cell pellet was washed once in 10 mM Tris-HCl (pH 7.4), 0.15 M NaCl. The cells were resuspended to a concentration of 2×10^7 cells/ml in ice-cold buffer containing 0.32 M sucrose, 3 mM MgCl₂, 10 mM Tris-HCl (pH 9), 250 µg/ml heparin and 250 µg/ml spermidine. Nonidet-P40 was added to a final concentration of 0.5% and the cell suspension was gently mixed on ice for 5 min. The mixture was centrifuged at 3000 rpm for 2 min and the supernatant was recovered and saved. The extraction process was repeated on the pellet and the second supernatant was added to the first. To the collected supernatants was added 20% SDS to a final concentration of 0.1% and 0.2 M EDTA to a final concentration of 0.01 M. Extraction of the RNA from this solution was achieved by adding 3/4 volume of phenol saturated with 0.1 M Tris-HCl (pH 9.0) and 1/4 volume chloroform:isoamylalcohol (20:1). The mixture was placed at 65°C for 5–6 min followed by centrifugation at 6000 rpm for 10 min. The aqueous layer was reextracted as above and the RNA was precipitated from the aqueous layer by addition of 2 volumes of ethanol and 1/5 volume 3 M sodium acetate. The mRNA was prepared by two passages over oligo(dT) and was fractionated on a 10–40% sucrose gradient (Tonogawa, 1976).

RNA Blots

Messenger RNA, 10 µg of total poly(A)⁺ RNA or 0.5 µg from sucrose fractions, was subjected to electrophoresis on a 1% agarose gel containing 10 mM methylmercury hydroxide in buffer E (Alwine et al., 1977). Following electrophoresis the gel was stained with ethidium bromide and processed for transfer to DBM paper as described by Alwine et al. (1977). DBM paper was prepared as described by Levy et al. (1980).

In Vitro Translation and Immunoprecipitation

Sucrose gradient fraction or nonspecific control mRNAs were translated for 90 min at 37°C with the reticulocyte lysate system (Amersham) supplemented with ³⁵S-methionine. The entire translation mixture was then cooled to 4°C, solubilized with nonionic detergent, immunoprecipitated with the indicated antisera and Protein A-Sepharose beads and finally displayed on SDS-PAGE. A more detailed procedure is described by Sidman (1981).

Electron Microscopy

Procedures used for formation of single-stranded DNA-mRNA hybrids and for formation of R hybrids have been described by Brack (1981). Photographs were taken with a Philips EM 300. Measurements were made with a Numonics digitizer and the data were analyzed with a Hewlett-Packard HP 9825 calculator.

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