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Genetics

Variable and constant parts of the immunoglobulin light chain gene of a mouse myeloma cell are 1250 nontranslated bases apart

(gene cloning/R-loop mapping/RNA splicing)

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ABSTRACT **A DNA fragment carrying an immunoglobulin gene coding for both variable (V) and constant (C) regions of a mouse λ light chain was enriched about 15-fold from a total endonuclease *Eco*RI digest of a plasmacytoma (HOPC 2020) DNA by preparative agarose gel electrophoresis. The DNA fraction was used for cloning of a λ chain gene in the phage λgt_{WES} vector. After screening [Benton, W. D. & Davis, R. W. (1977) *Science* 196, 180–182] of about 70,000 plaques, each arising from an independent transfection event, we isolated one clone (Ig 303) that contained both a V_λ and a C_λ DNA sequence. Electron microscopy of R-loops formed between the cloned DNA and purified λ chain mRNA (HOPC 2020) revealed that the V_λ and C_λ DNA sequences are separated by a 1250-base DNA fragment.**

To study molecular mechanisms involved in organization and expression of immunoglobulin genes, we have been cloning DNA fragments containing these genes by *in vitro* DNA recombination. In a previous paper, we reported isolation of a mouse embryonic V_λ gene clone (1). Characterization of this clone by electron microscopy and nucleotide sequencing confirmed that immunoglobulin *V* and *C* genes (coding for the variable and constant regions, respectively, of immunoglobulin) occupy separate positions in embryo DNA (1, 2). On the basis of restriction enzyme analysis of total cellular DNAs, we previously concluded that the *V* and/or *C* genes are somatically rearranged during differentiation of lymphocytes (3–5). The rearrangement event was thought to join the two DNA segments, thereby permitting RNA polymerase to transcribe the *V* and the *C* genes in contiguity.

We here report isolation and partial characterization of another DNA clone from a λ chain-producing plasmacytoma. R-loop mapping by electron microscopy revealed that the cloned DNA contains both a V_λ and a C_λ gene, but that they are separated by 1250 nontranslated bases. The possibility is discussed that both DNA translocation and RNA splicing are involved in expression of a full immunoglobulin gene.

MATERIALS AND METHODS

Bacteria and Phages. *Escherichia coli* 803 (r_k^-, m_k^-, SuIII$^+$) originating from K. and N. E. Murray at the University of Edinburgh was obtained from W. Arber, Biocenter, Basel; *E. coli* DP50 and phage λgt_{WES}-λB (6) from P. Leder at the National Institutes of Health; *E. coli* χ1776; from R. Curtis and his coworkers at the University of Alabama; plasmid pCRI from J. Carbon, University of California, Santa Barbara.

Ligation and Transfection. Ligation was carried out as described (1) except that left and right arms of λgt_{WES} DNA were prepared from λgt_{WES}-λB instead of λgt_{WES}-λC. Details of transfection procedures were as described (1). A 7.5-μg sample of 7.0 kilobase (kb) mouse DNA fragments and 30 μg of the λgt_{WES} DNA arms were ligated in a volume of 0.75 ml and diluted 4-fold with 100 mM Tris·HCl, pH 7.1. The entire DNA solution was used in transfection of 6 ml of $CaCl_2$-treated *E. coli* 803 (~5×10^{10} bacteria) and was plated in 30 petri dishes (diameter, 8 cm).

Plaque Screening by *In Situ* Hybridization. Procedures described by Benton and Davis (7) were followed except that nick-translated, cloned λ chain cDNA (in pCRI) was used as the hybridization probe. Filters were coated with bovine serum albumin before hybridization (8).

Construction of a λ Chain cDNA Clone and Preparation of Hybridization Probe. A plasmic pCRI clone that carries in the *Eco*RI site a 970-base DNA sequence complementary to HOPC 2020 λ chain mRNA (1200 bases long) was constructed according to the procedures described (9) for globin cDNA clones. The details of the construction procedures are to be described elsewhere. The cloned DNA was nick-translated (10).

All cloning experiments were carried out with an EK2 vector in a P3 facility, in accordance with the NIH guidelines issued in June 1976.

Other Procedures. Isolation of high molecular weight myeloma DNA, purification of light chain mRNA, isolation of the 3′-end half of mRNA, preparative agarose gel electrophoresis, and use of iodinated RNA in the detection of DNA fragments carrying immunoglobulin V and C sequences have all been described (3, 11).

R-Loop Formation and Electron Microscopy. The conditions for R-loop formation and spreading of the hybrid molecules were as described (1). Electron micrographs were taken with a Philips 300 at magnifications of 6800 and 16,000. Length measurements were made with a Numonics digitizer at final magnification of 160,000.

RESULTS

***Eco*RI Digestion and Fractionation of Myeloma DNA.** λ chain mRNA purified from HOPC 2020 myeloma (a λ chain producer) hybridized with three DNA fragments of 7.0, 4.7, and 3.2 kb, when *Eco*RI-digested total DNA from the same myeloma was fractionated by agarose gel electrophoresis (Fig. 1; also see ref. 4). The extent of hybridization with the 7.0-kb fragment was roughly 2 times higher than in the other two DNA fragments. Fig. 1 also shows that the 3′-end half of the same mRNA (*C* gene probe) hybridized with the 7.0-kb fragment but not with the 4.7- and 3.2-kb fragments. Thus, the latter two DNA fragments contain only *V* gene sequences. In the 7.0-kb fragment, the level of hybridization obtained with the whole mRNA was reproducibly higher than that obtained

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Abbreviations: kb, kilobase (1000 nucleotide bases or base pairs); SSC, standard saline citrate (0.15 M NaCl/0.015 M sodium citrate, pH 7).

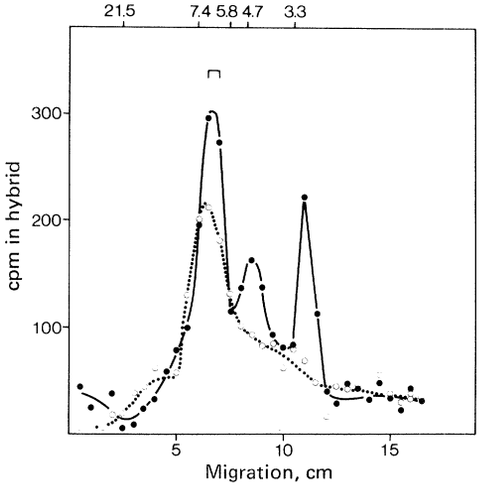

FIG. 1. Agarose gel electrophoresis profiles of an *Eco*RI digest of total HOPC 2020 myeloma DNA. DNA (4 mg) was fractionated in 0.9% agarose (1 cm thick, 20 cm wide). One-fifth aliquot each of DNA eluted from 5-mm gel slices was hybridized with ^{125}I-labeled (5×10^7 cpm/μg) whole λ chain mRNA from HOPC 2020 myeloma (●—●) or with its 3′-end half (O···O) to C_0t_e = 10,000 mol sec/liter (see ref. 4 for definition of C_0t_e). Inputs were 1300 cpm and 620 cpm for the whole and half RNA probes, respectively. Other procedures have been described (3). Numbers at the top indicate size (in kb) of duplex DNA markers. Fractions indicated by the brackets were pooled and used in ligation reaction.

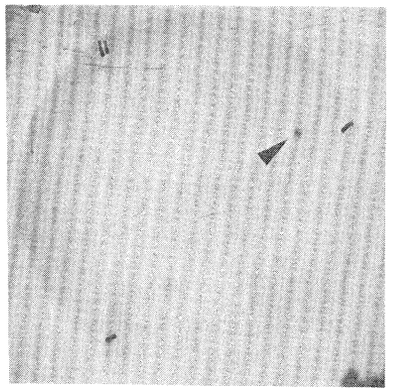

FIG. 2. Autoradiograph of an *in situ* hybridization. Filter paper (diameter 8 cm) to which the phages in the plaques had been transferred was alkali treated, neutralized, baked, precoated with albumin, and incubated at 65° for 12 hr in a solution (3 ml per filter) containing 4× SSC (1× SSC is 0.15 M NaCl, 0.015 M sodium citrate, pH 7), sonicated denatured calf thymus DNA (Sigma) at 100 μg/ml, *E. coli* DNA at 300 μg/ml, poly(A) (Miles) at 5 μg/ml, 0.5% sodium dodecyl sulfate Denhardt's mixture (8), and nick-translated, denatured DNA at 2×10^5 cpm/ml (specific activity ~5×10^7 cpm/μg) from λ chain cDNA clone (in pCRI). Filters were washed batchwise at 65° first in 4× SSC containing 0.5% sodium dodecyl sulfate and Denhardt's mixture (two changes, total time 3 hr), then 2× SSC containing 0.5% sodium dodecyl sulfate (one change, total time 2 hr), using 20 ml per filter per wash. Filters were air dried, marked, and exposed for 20 hr at −70° to a pre-fogged X-ray film (Kodak X Royal) with a tungsten intensifying screen (Ilford). The arrow points to an autoradiographic spot representing the Ig 303 phage plaque. Marks at the edge of the filter are for orientation.

with the 3′-end half, suggesting that this DNA fragment contains both *V* and *C* gene sequences. There may also be an additional fragment of 7.5 kb that contains only a *C* gene sequence but did not resolve from the 7.0-kb fragment (4). When pooled as indicated in Fig. 1, the hybridization-positive 7.0-kb DNA fragment was enriched approximately 15-fold over the total *Eco*RI digest. We used this DNA fraction for ligation with phage vector DNA.

Isolation of a *V* Plus *C* Gene Clone. We screened about 70,000 plaques, each arising from an independent transfection event, by the rapid *in situ* filter hybridization method and obtained one positive clone. Fig. 2 shows an autoradiograph of one filter that revealed the presence of this clone (clone Ig 303). The original petri dish contained about 2500 plaques.

R-Loops Formed Between Ig 303 DNA and HOPC 2020 mRNA. The cloned λgt_{WES}-Ig 303 DNA was incubated with HOPC 2020 λ chain mRNA under the conditions for R-loop formation. Upon examination in the electron microscope, more than 50% of the molecules displayed DNA·RNA hybrid regions. However, we did not observe the single R-loop of about 1000-nucleotide length that would have been expected if the mRNA had hybridized with a stretch of DNA corresponding to contiguous *V* and *C* gene sequences. Instead, at least three different types of hybrid could be observed (Fig. 3 *a–c*). Hybrids *a* and *b* were present in roughly equal amounts (50% each); hybrid *c* was much less frequent (less than 1%).

In the *a* class of hybrids (Fig. 3*a*) two small R-loops are separated by a double-stranded DNA loop. Similar triple-loop structures have recently been described for R-loops formed between *Drosophila melanogaster* 28S rRNA and rDNA (12). The interpretation of such a hybrid structure is that one RNA molecule is annealed to two stretches of DNA that are separated by a duplex DNA region. This interpretation is supported by the *b* class of hybrids (Fig. 3*b*), in which the mRNA was hybridized only to one strand of the DNA molecule, forming two RNA·DNA hybrid segments flanking a single-stranded DNA loop; the other strand had been displaced in a long single-stranded loop. In the *c* class of hybrids (Fig. 3*c*) the two small R-loops are independent, separated by a duplex region of exactly the same length as the double-stranded DNA loop in hybrids *a*, or the shorter single-stranded DNA loop in hybrids *b*. Hybrid *c* structures could have been formed by two separate RNA molecules each hybridizing to one of the two homology regions. In this case, however, both R-loops should have an RNA whisker corresponding to the nonhybridized moiety of the mRNA molecule. A more likely interpretation is that a triple-loop structure has been mechanically broken during the spreading and that each R-loop is formed by one half of the same mRNA molecule. The length of the cloned λgt_{WES}-Ig 303 DNA is 42.5 kb, and the two hybrid regions are located at 24.6 and 26.4 kb from the left end of the molecule.

*Eco*RI digestion of the λgt_{WES}-Ig 303 DNA and subsequent fractionation by agarose gel electrophoresis led to identification of three DNA fragments. These are the left and right arms of the λgt_{WES} vector and a 7.4-kb mouse DNA fragment (data not shown). The 7.4-kb fragment was purified and hybridized with HOPC 2020 λ chain mRNA to determine the position and the lengths of the homology regions more accurately. Under the conditions of R-loop formation employed (57°, 0.56 M Na^+, 70% vol/vol formamide) the 7.4-kb mouse DNA fragment denatured completely, and the mRNA hybridized with the complementary DNA strand (noncoding strand). This generated a structure in which a single-stranded DNA loop was flanked by two hybrid segments from which single-stranded DNA arms extended (Fig. 4). R-loop formation could then be observed to occur in two steps. Upon further incubation at 25°, the coding DNA strand annealed to the left and right arms of the structure (Fig. 5*a*) and finally also to the loop between the two hybrid regions to form the triple-loop structure similar to the one shown in Fig. 3*a* (Fig. 5*b*).

The results obtained by measurement of the various hybrid structures are summarized in Table 1. The table shows that one homology region (460 ± 40 nucleotides) is slightly longer than the other (380 ± 30 nucleotides). Orientation of the mouse DNA

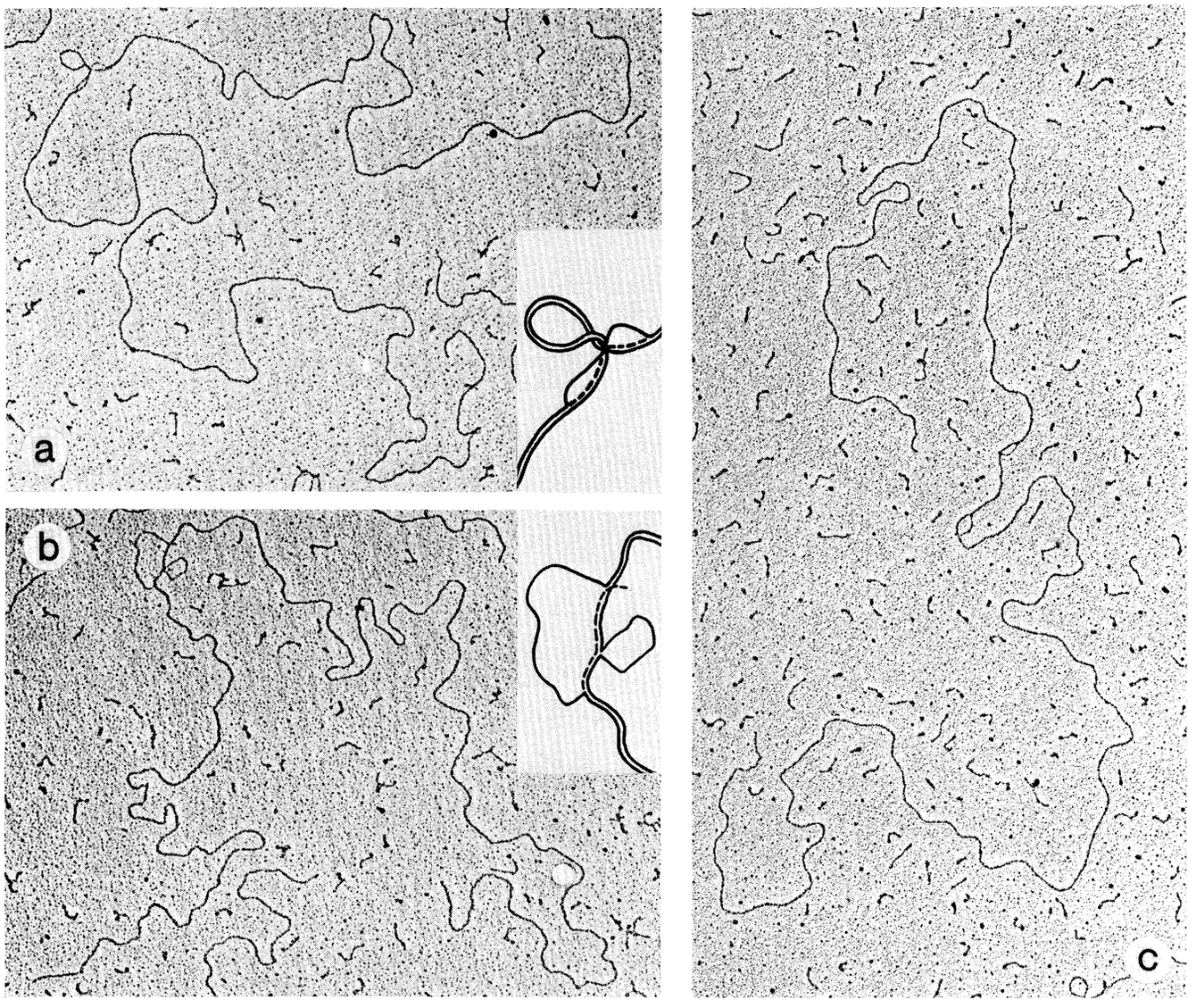

FIG. 3. R-loops formed between λgt_{WES}-Ig 303 DNA and HOPC 2020 mRNA. The three types of hybrids are described in the *text*. The magnified *insets* show schematic interpretations of the hybrid region, with the broken line representing the RNA.

insert in the phage genome was deduced from the position of the two homology regions on the whole λgt_{WES}-Ig 303 DNA. Thus, the longer homology lies to the left of the shorter one as shown in Fig. 7. In many cases the hybrid segment or R-loop generated by the longer homology had a short whisker (50–100 nucleotides) at its left end. The whisker is probably the poly(A) at the 3′ end of the mRNA. This suggests that the longer homology is composed of the *C* gene sequence and that the shorter one is composed of the *V* gene sequence.

Correspondence of the *V* Gene Sequence to the Shorter Homology Region. We previously reported isolation and partial characterization of an embryonic V_λ gene clone Ig 13 (1). Further characterization by restriction enzyme digestion and by nucleotide sequencing unequivocally confirmed that the cloned DNA fragment carries a V_λ gene and no C_λ gene (unpublished observation). One of the restriction enzyme fragments generated by digesting the Ig 13 DNA with endonuclease *Hae* III (Ig 13 *Hae* III B fragment) is 1.5 kb long and contains most of the V_λ gene sequence at one end. Hybrids formed between the 7.4-kb Ig 303 fragment and the Ig 13 *Hae*

Table 1. Length measurements on hybrid structures with Ig 303 DNA

Type of hybrid structure	Left arm	Right arm	"*C* gene"	"*V* gene"	Insert
RNA·DNA hybrids Ig 303 × HOPC 2020 mRNA (n = 23)	3.75 ± 0.37	1.49 ± 0.16	0.480 ± 0.027	0.388 ± 0.027	1.30 ± 0.13
R-loops Ig 303 × HOPC 2020 mRNA (n = 53)	3.75 ± 0.20	1.66 ± 0.08	0.440 ± 0.040	0.380 ± 0.030	1.20 ± 0.09
Hybrid Ig 303 × Ig 13 *Hae* III B fragment (n = 25)	5.44* ± 0.50	1.58 ± 0.12	—	0.360 ± 0.023	—

The different segments of Ig 303 DNA were measured on electron micrographs taken from the hybrid structures illustrated in Figs. 4, 5, and 6. The mean length of each segment and the standard error are given in kb units. n = number of hybrid molecules measured.
* Includes also "*C* gene" and insert.

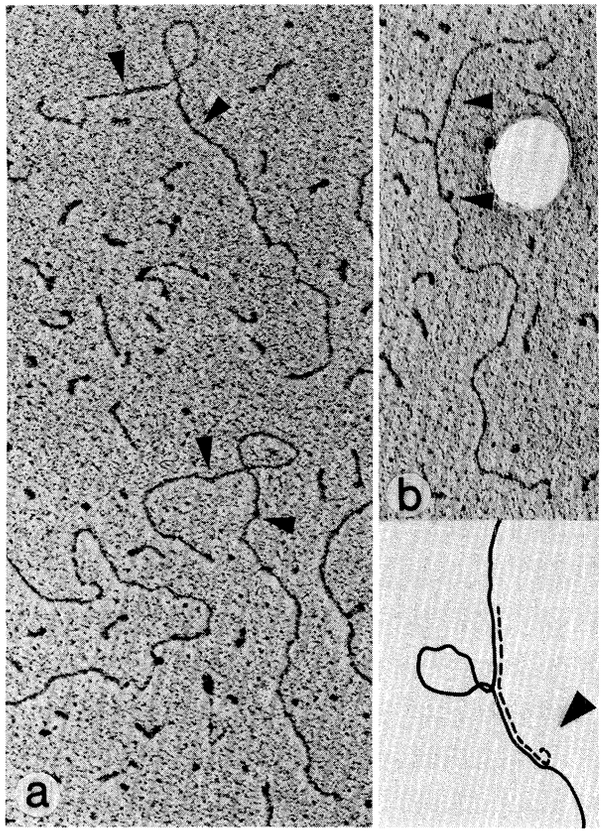

FIG. 4. Hybrids formed by annealing HOPC 2020 mRNA to denatured Ig 303 DNA. Arrows point to the ends of the RNA·DNA hybrid segments. Note the short tail on the end of the longer hybrid segment in *b*.

III B fragment are shown in Fig. 6. The Ig 303 DNA was either single-stranded with a short duplex region resulting from the annealed part of the Ig 13 fragment (Fig. 6*a*) or double-stranded, displaying the hybrid region as a D-loop structure (Fig. 6*b*). Measurements made on these structures support the conjecture that the shorter homology is the *V* gene sequence (Table 1). The data also allow us to deduce the direction of the

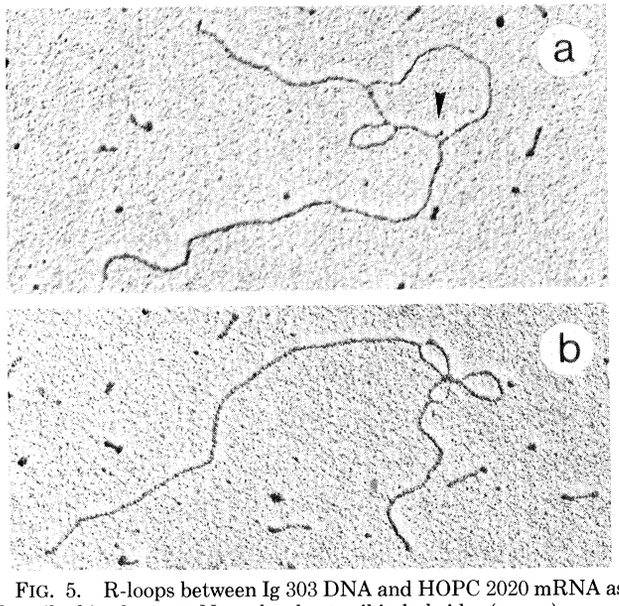

FIG. 5. R-loops between Ig 303 DNA and HOPC 2020 mRNA as described in the *text*. Note the short tail in hybrid *a* (arrow).

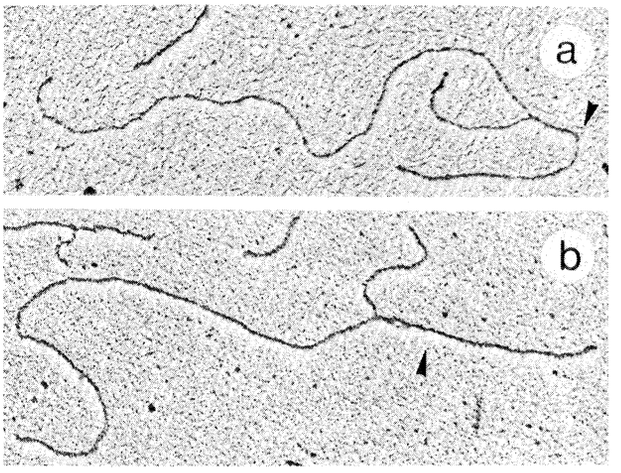

FIG. 6. Hybrids formed between Ig 303 DNA and Ig 13 *Hae*III B fragment. One end of the Ig 13 fragment anneals to the corresponding *V* region in Ig 303 DNA. Arrows delimit hybrid regions.

two homology regions. Fig. 7 illustrates an anatomy of the cloned mouse DNA fragment. It contains one copy each of V_λ and C_λ gene sequences, separated by a 1250-base fragment.

DISCUSSION

We previously presented evidence for a somatic rearrangement of immunoglobin light chain *V* and *C* genes during differentiation of lymphocytes (1, 5). The evidence was that very different patterns of hybridization are obtained when κ light chain mRNA from MOPC 321 (a κ chain producer) is hybridized to embryonic DNA or the homologous myeloma DNA, after digestion of the DNA with a restriction endonuclease, *Bam*HI, and subsequent fractionation by agarose gel electrophoresis (1). A hint that λ chain genes also underwent rearrangement was obtained when DNAs from embryo and λ chain-producing myeloma (HOPC 2020) were compared (4). Thus, digestion of embryonic DNA with *Eco*RI generated two V_λ gene-carrying fragments (4.7 and 3.2 kb) and one C_λ gene-carrying fragment (7.5 kb), whereas digestion of HOPC 2020 myeloma DNA generated, in addition to the three DNA fragments, a new fragment (7.0 kb) that probably carried both V_λ and C_λ genes (see *Results* and ref. 4). Because amino acid sequence studies (13, 14) ruled out the existence of an *Eco*RI site in the translated part of a λ chain gene, these results indicated that *V* and *C* genes are some distance away from each other in embryonic DNA. This conclusion was directly confirmed by our recent nucleotide sequencing studies of a $V_{\lambda II}$ gene clone that was isolated from the 4.7-kb embryonic fragment (ref. 2, and unpublished observation). How does the genetic information encoded in two separate DNA segments, *V* and *C*, become integrated in order to generate mRNA coding for a continuous polypeptide chain? Appearance of a new DNA fragment in myeloma DNA, which apparently carries both *V* and *C* gene sequences (7.0-kb fragment), suggested that the integration takes place directly at the DNA level by somatic recombination (1). The simplest model was that the DNA rearrangement event

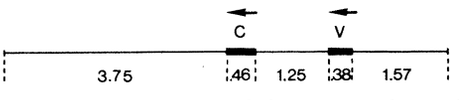

FIG. 7. Organization of *V* and *C* genes on the 7.4-kb *Eco*RI fragment isolated from Ig 303 DNA. The lengths of the different segments are given in kb units and are mean lengths obtained from all measurements summarized in Table 1. Arrows indicate the direction of transcription.

brings previously distant *V* and *C* genes in contiguity to create a continuous, full immunoglobulin gene.

Presence of both V_λ and C_λ gene sequences in the 7.0-kb myeloma DNA fragment was verified by isolation of Ig 303 DNA, which was cloned from the gel fraction enriched for this fragment. (The slight discrepancy in the sizes of the two DNA fragments, the 7.0-kb agarose gel fragment and the 7.4-kb cloned DNA fragment, can be attributed to inaccuracy in size determination of the former.) However, electron microscopy of the R-loops formed between the cloned DNA and purified, homologous λ chain mRNA revealed that the V_λ and C_λ gene sequences are separated by 1250 bases. Because the clone was isolated from the DNA component present in a λ_I chain-producing myeloma (but not in the embryo genome), it seems that the *V* and *C* genes in the Ig 303 fragment are those that have gone through the rearrangement process. This conjecture is supported by our following observations. We have more recently cloned the 7.5-kb embryonic C_λ gene fragment and the 3.2-kb embryonic V_λ gene fragment. Electron microscopic examination of the heteroduplexes formed between either of these cloned DNA fragments and the Ig 303 DNA demonstrated that the 1250 nontranslated bases are also contained in the embryonic C_λ gene clone (data not shown). These results also make the following possibility remote, namely, that the 1250-base fragment is an artifact of cloning procedures. It is thus likely that the *V* and *C* gene sequences contained in the Ig 303 clone are those that are utilized in this myeloma for actual production of the λ_I light chain. We thus envisage the following scheme of events. In the embryo, immunoglobulin *V* and *C* genes are separate and are relatively distant from each other. During differentiation of B lymphocytes, a particular *V* gene (and/or the corresponding *C* gene) is translocated. The mechanism of the translocation is such that the two DNA segments come closer, but not in contiguity. How the structural gene containing a DNA gap leads to production of a continuous mRNA (15) coding for the polypeptide chain is a matter of speculation. One possibility is that RNA polymerase jumps across the gap from one DNA segment to the other. Another more attractive possibility is that the entire DNA region—including the *V* segment, the non-translated segment, and the *C* segment—is transcribed into a single precursor RNA molecule and that the RNA sequences corresponding to the *V* and *C* regions are subsequently spliced to yield a mature mRNA molecule. RNA splicing has been recently proposed to account for the presence of the short segments that form the 5′ region of adenovirus late mRNAs, and which are coded in DNA by short fragments that are separate from the larger fragment coding for the rest of the mRNAs (16, 17).

One may ask why the lymphocytes take the trouble of moving immunoglobulin DNA segments around if they are equipped with an RNA splicing apparatus. A key for answering this question may be that RNA splicing is an intramolecular event. Both amino acid sequence studies (18) and nucleic acid hybridization studies (4) suggest that there exist more different germ line *V* genes than *C* genes for a given type of immunoglobulin chain. (In this sense, mouse λ type chains may be exceptional.) Thus, in the mouse there might be as many as one hundred different V_κ genes per haploid genome, whereas there is probably only one C_κ gene (19). The assumption that RNA splicing operates only intramolecularly imposes a certain restriction as to how the immunoglobulin gene complex is transcribed; a given *V* gene must be cotranscribed with the *C* gene for its subsequent expression. An inevitable consequence of transcription without DNA translocation would be the synthesis of long RNA molecules containing multiple *V* gene sequences and a single *C* gene sequence. It is difficult to envisage a simple mechanism by which a lymphocyte picks for splicing one and the same *V* gene sequence in *all* such long RNA molecules that it synthesizes. This would be necessary because there is abundant evidence which indicates that all B lymphcytes of one clone express the same light chain *V* gene (20). Translocation at the DNA level would be a solution to the problem.

The finding of a noncoding DNA segment within a structural gene is not without precedents. It was discovered in 28S rRNA genes of *Drosophila melanogaster* (21). Genes coding for late mRNAs in adenovirus provide another example (16, 17). Our recent nucleotide sequencing studies of an embryonic $V_{\lambda II}$ gene clone (clone Ig 13) also revealed the presence of 93 nontranslated nucleotides in the region coding for the amino terminus (O. Bernard, W. Gilbert, A. Maxam, and S. Tonegawa, unpublished results). Nontranslated intragenic DNA seems to occur frequently in the genomes of eukaryotes and their viruses.

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