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Organization of Immunoglobulin Genes: Reiteration Frequency of the Mouse κ Chain Constant Region Gene

(gene reiteration/hybridization/synthetic DNA/mRNA)

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ABSTRACT **Hybridization kinetic analyses with synthetic DNA indicate that there are only two to three copies of the κ constant region gene per haploid genome. This result lends weight to the argument that the immunoglobulin light chain is encoded by more than one continuous gene sequence.**

Several models for the organization of immunoglobulin genes have been proposed to account for the striking diversity of the variable (V) and the identity of the constant (C) regions of immunoglobulin molecules. The most straightforward of these, the stringent germ line hypothesis, states that all immunoglobulin molecules are encoded, intact, in the genome and that diversity has arisen during evolution (1, 2). Naturally, this hypothesis requires a large number of genes for both V-region and C-region sequences. The remaining major hypotheses, the recombinational germ line (3–5) and the somatic mutation (6–16), require only one or a few C-region genes. They differ from one another as regards the origin of the diversity in V-region sequences. The recombinational germ line hypothesis relies entirely upon evolutionary processes to generate diversity and requires a large number [at least 1000 (17)] of V-region genes, whereas the somatic mutation hypothesis envisages a smaller number (100 or less) of V-region genes which undergo somatic mutation. Therefore, to distinguish among them, we have set out to quantitate the immunoglobulin genes using hybridization kinetic analysis (18, 19) with radioactive probes derived from pure, immunoglobulin light chain mRNA (20).

Workers in several laboratories, including our own, have reported partial purification of immunoglobulin mRNAs from mouse myeloma tumors and translation of the mRNAs in cell-free or *Xenopus* oocyte systems (21–29). In the present study, we have further purified a κ chain mRNA from mouse myeloma tumor, MOPC-41, to an essentially homogeneous state, as judged by several independent criteria. Using synthetic DNA complementary to the purified mRNA (cDNA) as a hybridization probe, we have been able to quantitate the κ chain C-region gene.

MATERIALS AND METHODS

The sources of many of the reagents used in this study have been indicated (30–32). [^{3}H]dCTP (26 Ci/mmol) was obtained from Schwarz/Mann. Myeloma tumors were kindly supplied by Dr. M. Potter, being maintained and harvested as described (25).

Purification of κ Chain mRNA from MOPC-41 Myeloma. mRNA was extracted from membrane-bound polysomes and purified initially by oligo(dT)–cellulose column chromatography (dT1), as described by Swan *et al.* (25). A second oligo-(dT)–cellulose chromatographic step was carried out (dT2) in essentially the same way, except that the column was washed with 0.18 M KCl–0.01 M Tris·HCl (pH 7.5) before the elution of mRNA with 0.01 M Tris·HCl (pH 7.5). The mRNA preparation eluted with 0.01 M Tris·HCl (pH 7.5) was precipitated with ethanol and dissolved in H_2O. The mRNA solution was heated for 10 min at 75°, followed by rapid chilling. It was then layered on a 5–22% sucrose gradient containing 0.01 M Tris·HCl (pH 7.8)–0.1 mM EDTA in a Beckman SW 41 rotor and centrifuged at 40,500 rpm for 16 hr at 15° (SG1). Fractions (0.4 ml each) were collected, and their absorbance at 260 nm was determined. Aliquots of each fraction were assayed for their capacity to direct protein synthesis in a wheat germ cell-free system (33, 34) and by hybridization to [^{3}H]cDNA complementary to the purified MOPC-41 mRNA. The heavier half of the activity peak fractions was pooled and subjected to a second sucrose gradient centrifugation in an identical procedure (SG2).

Purification of Other mRNAs. Light chain mRNAs from other myeloma tumors were purified as described above. Krebs II ascites cell RNA was extracted from total polysomes and purified up to the dT1 step as described above. Mouse globin mRNA was purified as described (35).

Preparation and Purification of cDNA. [^{3}H]cDNA was made with the MOPC-41 mRNA as a template by the avian myeloblastosis virus RNA-dependent DNA polymerase as described (31, 32, 36). [^{3}H]dCTP was used as a labeled precursor. The [^{3}H]cDNA synthesized was layered on a 5–20% alkaline sucrose gradient (pH 12.5) containing 0.9 M NaCl and 1 mM EDTA, and centrifuged in a Beckman SW 41 rotor at 40,5000 rpm for 30 hr at 5°. Fractions were collected, and an aliquot of each fraction was taken to determine its acid-insoluble radioactivity. The peak fractions of radioactivity were pooled. The cDNA preparation thus obtained was annealed to a 10-fold excess of the purified mRNA to a C_rt value of 10^{-2}, and the hybrid, which melted above 82.5°, was collected by elution from hydroxyapatite (37). The hybrid isolated was treated with alkali to destroy all the RNA. The

Abbreviations: V and C regions, variable and constant regions, respectively, of immunoglobulin molecules; cDNA, synthetic DNA complementary to mRNA; C_rt and C_0t values, product of concentration of nucleotide sequences of RNA and DNA, respectively, and time of incubation.

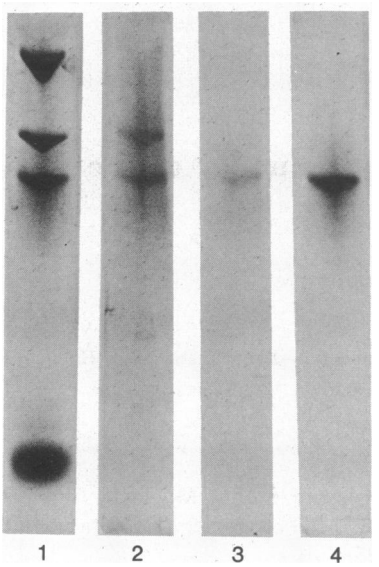

FIG. 1. Acrylamide gel electrophoresis of MOPC-41 mRNA in formamide. Acrylamide gel (5%) electrophoresis of RNA was carried out in 100% formamide by the procedure of Staynov *et al.* (62). Electrophoresis was at 100 V for 5 hr at room temperature. Gels were stained with Stains-all (Eastman Kodak Co.). *1*, Purified mRNA (SG2) with marker 18S and 28S rRNA. *2*, mRNA at dT2 stage. *3*, mRNA at SG1 stage. *4*, Purified mRNA (SG2).

final yield of cDNA was about 25% of the cDNA synthesized. The specific activity of [^{3}H]cDNA was estimated to be 10^7 cpm/μg, based on the assumption that cDNA contains 25% dCMP. [^{3}H]cDNA complementary to mouse globin mRNA was synthesized and purified as above, up to the alkaline sucrose gradient step (a generous gift of Dr. P. Venetianer).

RESULTS AND DISCUSSION

Characterization and Reverse Transcription of mRNA. MOPC-41 and other light chain mRNAs direct the cell-free synthesis of polypeptides that are somewhat larger than secreted light chain, but that yield appropriate tryptic peptides (21–29). In addition to using a wheat germ protein synthetic system to follow our mRNA through purification, we analyzed the mRNA at various steps using acrylamide gel electrophoresis in formamide (Fig. 1). Crude RNA preparations contain several components, including 28S and 18S ribosomal RNA, whereas the purified mRNA migrates as a single, homogeneous band. With 28S and 18S ribosomal RNA as standards, the molecular weight of this mRNA is calculated to be 450,000, corresponding to a chain length of about 1300 nucleotides. As shown previously (36) and described under *Materials and Methods*, when annealed to oligo(dT), this mRNA also serves as an efficient template-primer for the RNA-dependent DNA polymerase-catalyzed synthesis of [^{3}H]cDNA.

Assessment of mRNA Purity by Hybridization Kinetic Analysis. Further evidence of the purity of the mRNA is provided by hybridization kinetic analysis of the mRNA and its [^{3}H]-cDNA. Under conditions of excess mRNA concentration, the C_rt value at 50% hybridization ($C_rt_{1/2}$) can be used as a measure of the relative purity of the mRNA (37–39). This is so because the $C_rt_{1/2}$ value is proportional to the complexity of RNA in such a hybridization reaction. If the RNA

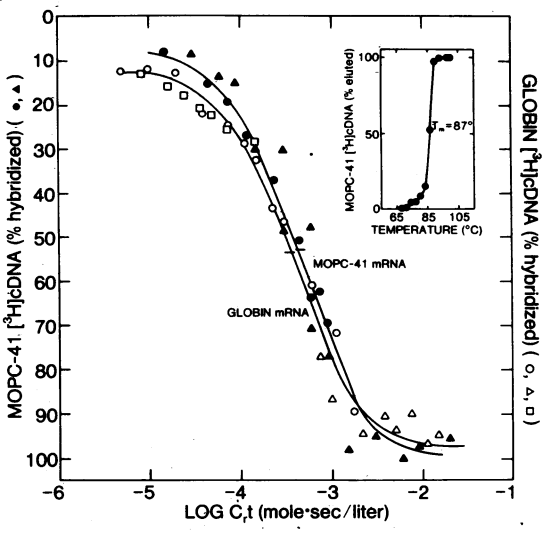

FIG. 2. Kinetics of annealing of [^{3}H]cDNA to mRNA and thermal denaturation of the hybrids. Hybridization reaction was performed in 0.6 M NaCl–0.2 mM EDTA–20 mM Tris·HCl (pH 7.2) at 75°. The amount of RNA was in at least 20-fold excess over cDNA. The reaction mixture was covered with mineral oil to prevent evaporation, and aliquots were taken at time intervals for the assay of the hybrid formed by S1 nuclease digestion (32, 63, 64). The MOPC-41 cDNA·mRNA hybrid formed at a C_rt value of 2×10^{-2} was diluted 10-fold in 0.014 M potassium phosphate (pH 6.8) and applied to a water-jacketed hydroxyapatite column. The column was washed with 0.14 M potassium phosphate (pH 6.8) at increasing temperatures, and acid-insoluble radioactivity, eluted at different temperatures, was determined (37). The filled symbols are for MOPC-41 [^{3}H]cDNA hybridized to MOPC-41 mRNA: ●, 90 ng/ml of RNA; ▲, various amounts of RNA incubated for 1 hr. The open symbols are for mouse globin [^{3}H]cDNA hybridized to mouse globin mRNA; ○, 228 ng/ml of RNA; △, 760 ng/ml of RNA; □, 50 ng/ml of RNA.

preparation were not pure, the $C_rt_{1/2}$ value would be greater than expected for an RNA of a given molecular weight. Mouse globin mRNA that has been purified from reticulocyte polysomes together with its cDNA (570 bases long), provides a reference mRNA preparation. As shown in Fig. 2, hybridization between globin mRNA and its [^{3}H]cDNA gives a $C_rt_{1/2}$ value of 3.7×10^{-4}. Assuming that globin mRNA is 1:1 mixture of α and β globin mRNAs, each of which is about 600 nucleotides long, the expected $C_rt_{1/2}$ value* for MOPC-41 mRNA (1300 nucleotides long) would be 4.0×10^{-4}. The experimental $C_rt_{1/2}$ value for the MOPC-41 mRNA is 4.2×10^{-4}, very close to the expected value. The thermal elution profile of the hybrid formed between the MOPC-41 mRNA and its [^{3}H]cDNA is also shown. The profile is sharp, with a T_m of 87.0°, slightly higher than that of an analogous globin mRNA·cDNA hybrid (37), suggesting that the hybrid is congruently base-paired with little or no mismatching.

Cross-Hybridization Between MOPC-41 cDNA and Other Light Chain mRNAs. The MOPC-41 and MPC-11 κ chains are identical in their C-regions, while MOPC-41 κ chain and RPC-20 λ chain C-regions are so different as to preclude

* Expected $C_rt_{1/2}$ for MOPC-41 mRNA

$$= C_rt_{1/2} \text{ for globin mRNA} \times \frac{\text{complexity of MOPC-41 mRNA}}{\text{complexity of globin mRNA}}$$

$$= 3.7 \times 10^{-4} \times \frac{1300}{600 + 600} = 4.0 \times 10^{-4}.$$

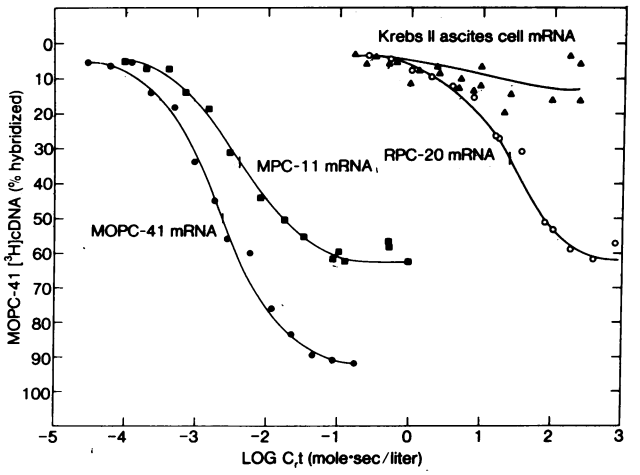

FIG. 3. Cross-hybridization of MOPC-41 [^{3}H]cDNA. Hybridization reaction was carried out as described above. All the mRNAs used were purified to the dT1 stage. ●, MOPC-41 mRNA; ■, MPC-11 mRNA; O, RPC-20 mRNA; ▲, Krebs II ascites cell mRNA.

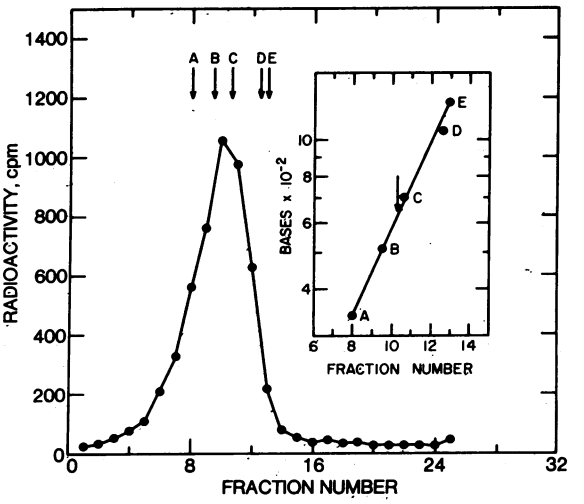

FIG. 4. Alkaline sucrose centrifugation of MOPC-41 cDNA. Purified MOPC-41 [^{3}H]cDNA was centrifuged in an alkaline sucrose gradient as described in *Materials and Methods*. ^{32}P-labeled λ phage DNA fragments, produced by *Hemophilus influenzae* restriction enzyme digestion (a generous gift of Dr. T. Maniatis of Harvard University), were added to the same tube; their peak locations are shown by letters. (Inset) Length (bases) of the fragments are: A, 340; B, 510; C, 700; D, 1050; E, 1250. Arrow indicates the peak location of [^{3}H]cDNA.

stable cross-hybridization (37, 40–42). There is, as expected, extensive cross-hybridization between MOPC-41 cDNA and mRNA derived from the κ chain-producing tumor, MPC-11 (Fig. 3). The extent of hybridization likely reflects that portion of the cDNA complementary to the κ C-region sequence (see below). The comparable $C_rt_{1/2}$ values, 2.4 and 4.0 × 10^{-3}, respectively, suggest a 1.7-fold greater relative concentration of κ chain mRNA in the MOPC-41 tumor. In contrast, hybridization to comparably prepared mRNA derived from a λ chain-producing tumor, RPC-20, has a 10,000-fold greater $C_rt_{1/2}$ (2.5×10^1), indicating hybridization to a complementary sequence present at 1/10,000th the concentration of κ chain mRNA in the MOPC-41 tumor.

Inasmuch as the extent of hybridization is similar to that observed with the κ chain MPC-11 mRNA and since the thermal elution profiles of these hybrids are identical (not shown), it appears that MOPC-41 cDNA is hybridizing to small amounts of κ chain mRNA present in mRNA derived from the λ chain-producing tumor. These κ sequences could arise from circulating lymphocytes present in tumor tissue or from endogenous κ chain mRNA present in the predominately λ chain-producing tumor. Myeloma tumors producing two classes of antibody have been observed (ref. 43; M. Weigert, M. Potter, and R. McIntire, personal communication). The possibility that this cross-hybridization reflects nonlight chain sequences is ruled out by the extensive hybridization of the cDNA probe together with the 10,000-fold difference in $C_rt_{1/2}$ values. The additional possibility that this cross-hybridization represents sequences (possibly untranslated) common to both κ- and λ-chain mRNAs is inconsistent with such large differences in $C_rt_{1/2}$, since amounts of λ-chain mRNA present in RPC-20 tumor are comparable to κ chain mRNA concentrations in MOPC-41 (T. Honjo and P. Leder, unpublished data). In further contrast to these results, there is little significant hybridization of MOPC-41 cDNA to Krebs II ascites tumor mRNA, even at very high C_rt values (2.8×10^2).

cDNA Covers the C-Region Sequence of mRNA. The average length of the cDNA probe is about 630 nucleotides, as determined by alkaline sucrose gradient centrifugation (Fig. 4). cDNA may be assumed to start at the middle of the poly(A) sequence located at the 3′ end of the 1300-nucleotide-long mRNA (32, 44) (Fig. 5). About 650 bases are required to encode the secreted form of the light chain (40); at least 900 are required to encode the putative precursor chains (30,000 daltons) synthesized in the wheat germ cell-free system (T. Honjo, M. Nau, and P. Leder, unpublished data). Further, about 200 adenylate residues comprise the 3′-terminus (44), leaving an untranslated sequence of about 200 bases. Although we do not know the exact location of the putative untranslated sequence in the mRNA, we can postulate two possible extremes in which all the untranslated sequence is either at the 3′ end or at the 5′ end of the translated sequence (Fig. 5). It is obvious that in the former case, the cDNA would cover at least 70% of the C-region sequence, whereas the V-region sequence would not be covered. In the latter case, the cDNA would correspond to the entire C-region and about one-third of the V-region. Therefore, as a minimum, the cDNA cor-

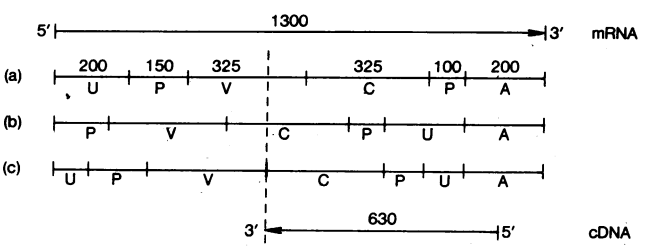

FIG. 5. Possible arrangements of sequences in MOPC-41 and its [^{3}H]cDNA. About 150 bases of the precursor sequence were put at the 5′ end of the V-region sequence because at least 50 extra amino-acid residues were found at the amino-terminal end of the precursor protein synthesized *in vitro* (D. McKean, T. Honjo, D. Swan, M. Nau, and P. Leder, unpublished data). A, poly(A) sequence; C, C-region sequence; P, precursor sequence; U, untranslated sequence; V, V-region sequence. (a) Untranslated sequence at the 5′ end; (b) untranslated sequence at the 3′ end of the translated sequence; (c) half the untranslated sequence at the 5′ end, half at the 3′ end of the translated sequence.

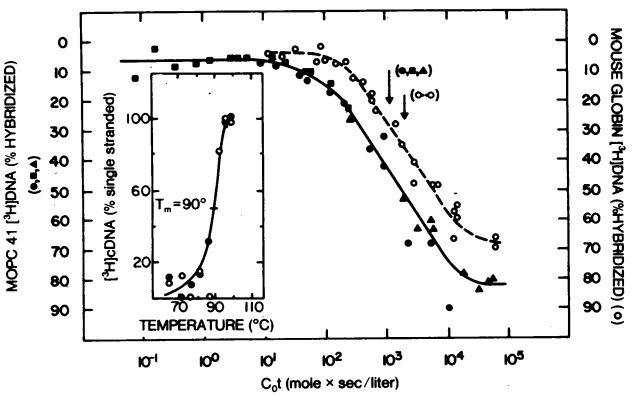

FIG. 6. Hybridization kinetic analysis of [³H]cDNA in the presence of total cellular DNA and thermal denaturation of the hybrid. Hybridization reaction was carried out in the presence of up to a 1.2×10^7-fold excess of unlabeled cellular DNA and assayed by S1 nuclease digestion as described (53). Purification of DNA from various tissues was also described (53). The C_0t values are those that would obtain at 0.18 M Na^+ (18). $C_0t_{1/2}$ for MOPC-41 cDNA hybridized to MOPC-41 DNA equals 1130. $C_0t_{1/2}$ for globin cDNA hybridized to MOPC-41 DNA equals 2100. MOPC-41 cDNA hybridized to MOPC-41 DNA: ■, 0.99 mg/ml of DNA, 0.18 M Na^+; ●, 9.9 mg/ml of DNA, 0.54 M Na^+; ▲, 9.9 mg/ml of DNA, 1.06 M Na^+; O, Results for globin cDNA hybridized to MOPC-41 DNA. (Inset) The hybrids formed at a C_0t value of 5×10^5 were analyzed by thermal denaturation. The cDNA·DNA hybrid was diluted to 0.25 M Na^+ and heated at each temperature for 8–10 min. S1 nuclease-resistant radioactivity was determined as a function of increasing temperature (20). ●, MOPC-41 cDNA hybridized to MOPC-41 DNA; O, MOPC-41 cDNA hybridized to mouse spleen DNA.

responds to a major portion (70%) of the C-region sequence. As a maximum, it might represent a portion of the untranslated and/or a small portion of the V-region as well. Consistent with this expectation, about 66% of the hybridizable MOPC-41 [³H]cDNA forms a stable hybrid with mRNA derived from MPC-11, a myeloma tumor producing a κ chain with the same C-region sequence, but a different V-region sequence (Fig. 3). This hybrid has a thermal elution profile identical to that of the homologous hybrid, suggesting closely congruent base-pairing (not shown). The residual, nonhybridizing portion, about 34%, could correspond either to a short V-region sequence or to an untranslated portion of the mRNA.

Two Genes Encoding One Polypeptide Chain? A Test of the Stringent Germ Line Hypothesis. We have already indicated that the most stringent germ line hypothesis requires hundreds, if not thousands, of adjacent V–C sequences. However, several genetic analyses with regard to linkage, allotype, and crossover of heavy and light chain C-region genes (45–52) have strengthened hypotheses requiring only one or few copies of C-region genes that must join with one of many V-region sequences (3, 11). Implicit in these hypotheses is the notion that (at least) two genes encode one polypeptide chain.

A direct test of the stringent germ line hypothesis is possible using the MOPC-41 cDNA as a hybridization probe to assess the reiteration frequency of the constant region gene sequence. The relevant experiment is shown in Fig. 6, in which MOPC-41 [³H]cDNA is hybridized to a vast excess of unlabeled, total DNA derived from MOPC-41 tumor. A similar analysis using mouse globin[³H]cDNA is shown for comparison. The results are quite clear. The $C_0t_{1/2}$ value for MOPC-41 cDNA is 1130, as compared to a $C_0t_{1/2}$ value of 3000 for "unique copy" mouse DNA (37, 65). This is equivalent to a reiteration frequency of 2.7 copies per haploid genome. A comparable reiteration frequency, 2.3, can be calculated by a method that is dependent upon the final extent rather than the rate of hybridization† (53, 65). The comparable globin gene frequency is 1.4 copies per haploid genome, in agreement with a previous report (53, 65). Similar analyses have been carried out with MOPC-41 [³H]cDNA and total DNA from a λ chain-producing tumor, RPC-20, and from normal mouse liver (not shown). These give reiteration frequencies at 3.0 and 3.2 per haploid genome, respectively, and indicate that only two to three copies of the C-region gene are present per haploid mouse genome regardless of whether the cells are actively producing κ chains. These results are not compatible with the stringent germ line hypothesis.

Workers in several laboratories (54–60) have reported interesting experiments regarding the reiteration frequency of immunoglobulin genes, using ^{125}I-labeled mRNA as a hybridization probe. All observed different proportions of unique and reiterated components by hybridization kinetic analyses. However, the precise interpretation of these data was necessarily limited by the use of partially purified probes and/or the fact that the unique and reiterated portions of the curves could not be assigned specifically to the C- or V-region genes. The availability of homogeneous mRNA and its cDNA probe overcomes these difficulties. Nevertheless, our methods do not allow us to discriminate between only one or a few copies of the C-region sequence. If the latter is the case, redundant C-region copies must be very similar, since the thermal elution profile of the hybrid formed between MOPC-41 [³H]-cDNA and genomic DNA (Fig. 6) is quite sharp (T_m = 90°), suggesting closely matched complementary base-pairing.

Joining of V- and C-Region Sequences. Given considerable data on the differences among mouse κ chain V-region amino-acid sequences, it seems clear that a minimum of 25 V-region sequences (and possibly many more) must be encoded in the germ line genome (15). How, then, can these many V-region sequences be joined to the few C-region sequences we observe? Obviously this joining step could occur at the level of the C- and V-region genes, their mRNAs, or polypeptide chains. Since light chain mRNA contains both V- and C-region sequences (21–29), the last possibility is ruled out. While it is possible that C- and V-region mRNA sequences are joined after transcription, it is simpler to envisage a single recombinational event occurring at the level of DNA during immunodifferentiation rather than the thousands of joining events that must occur were mRNA fragments to be linked. Several interesting models by which V–C linkage events could occur at the DNA level have been proposed (3, 13, 61).

† Reiteration frequency = $R/E_r \times f$ (refs. 53 and 65), where E_r = unlabeled cellular DNA (mg/ml)/[³H]cDNA (mg/ml);
f = fraction of haploid mouse cell genome corresponding to one copy of the cDNA sequence = number of bases in [³H]cDNA/number of bases in haploid mouse genome;
and R = number of unlabeled cellular globin genes in reaction mixture/number of [³H]cDNA sequences in reaction mixture.
For the reaction in Fig. 6: % hybridization at completion = 76, corresponding to R = 3.2 (refs. 53 and 65).
E_r = (9.9 mg/ml)/(8.5×10^{-7} mg/ml) = 1.16×10^7.
f = $630/5.8 \times 10^9$ (ref. 66) = 1.09×10^{-7}.
Therefore, reiteration frequency = 2.5.

Somatic Mutation or Evolution as a Source of Diversity? Our present experiments focus on the genetic representation of the C-region sequence and, therefore, do not address the question of the origin of diversity in the V-region sequences. Nonetheless, initial experiments with purified, light chain ^{125}I-labeled mRNA have been done (20). These data indicate that a major portion, at least 70%, of the entire κ-chain mRNA is represented as a relatively unique sequence. The balance of the ^{125}I-labeled mRNA hybridizes with a reiteration frequency of 30–50 copies per haploid genome. These results are consistent with both somatic mutation and recombinational germ line hypotheses, taking into account the likelihood that distantly related V-region gene sequences would not form stable hybrids with the probe. Our ability to distinguish unambiguously between these hypotheses requires us to focus on the V-region sequences either by the use of appropriate, competing RNA sequences or especially fashioned V-region probes.

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