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Genetics

Reiteration frequency of immunoglobulin light chain genes: Further evidence for somatic generation of antibody diversity

(RNA·DNA hybridization/λ- and κ-chain mRNAs)

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ABSTRACT **Methods have been developed for preparing mouse immunoglobulin light chain mRNA of better than 90% purity. Hybridization of both λ and κ mRNAs to excess liver DNA yielded results compatible with gene reiteration frequencies of two to three. There was no evidence of hybridization of these highly purified mRNAs to reiterated DNA, and, in fact, the kinetics of hybridization were very similar to that of purified globin mRNA. Purified λ mRNA from tumors producing structurally different λ chains were used in competition hybridization experiments. An unlabeled λ mRNA competed with another, labeled λ mRNA to the same extent as homologous unlabeled λ mRNA. That is, base sequence homology among λ mRNAs is so high that any λ mRNA should cross-hybridize with all germ line variable (V_λ) genes at least for those V-regions which are represented among myelomas. From amino-acid sequence data, it is argued that there are probably more than 25 different λ V regions. Hence it is concluded that the number of germ line genes is too small to account for the diversity of λ chains. A similar conclusion is drawn for κ chains.**

The origin of antibody diversity is one of the central issues in immunology. Controversial ideas of evolutionary versus somatic generation of antibody diversity have coexisted until recently (1, 2).

In previous reports (3, 4) we presented evidence which strongly suggested the existence of a somatic mechanism for antibody diversification. The evidence was based on the observation that a number of germ line variable region (*V*) genes, as estimated by RNA·DNA hybridization, was too small to account for the observed amino-acid sequence diversity of mouse κ chains. In this communication I will report similar evidence for mouse λ chains. The results indicate that mouse λ chains, at least those represented among myelomas, are derived from no more than three germ line *V* genes, possibly from a single one. In addition I will describe hybridization experiments with κ chain mRNA of improved purity; these help to simplify the interpretation of our previous experimental results.

MATERIALS AND METHODS

Myeloma Tumors. The following Balb/c myeloma lines have been used and maintained as described previously (3): MOPC 104E, MOPC 321, and HOPC 2020. I am indebted to Drs. M. Potter and M. Cohn for kindly supplying these tumor lines.

Purification of λ- and κ-Chain mRNA. The method employed has been modified from that in our previous report (5). Tumors, about 1 cm or less in diameter, were dissected. After careful removal of connective and necrotic tissue, tumors were kept in ice-cold Dulbecco-modified Eagle's medium (GIBCO) containing 100 μg/ml of cycloheximide. After dissection, tumors were transferred to a mixture (30 ml/10 g tumor) composed of equal parts of buffer B (Tris·HCl, pH 7.5, 10 mM; $MgCl_2$, 5 mM; KCl, 50 mM; dithiothreitol, 1 mM; sucrose, 0.3 M) and rat liver supernatant prepared in the same buffer (6), and homogenized by 10 strokes in a motor-driven Teflon–glass homogenizer (1200 rpm, clearance = 0.3 mm). Nuclei, unbroken cells, mitochondria, and large membrane fragments were removed by centrifuging at 4000 × *g* for 10 min. The cloudy supernatant was centrifuged at 48,000 × *g* for 10 min in order to sediment membrane fragments which were then suspended in 10 ml of a mixture composed of equal parts of TKM buffer (Tris·HCl, pH 7.5, 20 mM; KCl, 100 mM; $MgCl_2$, 5 mM) and rat liver supernatant. The polysomes bound to the membrane fragments (MBP) were released by adding NP 40 (Shell Co.) to a final concentration of 1%. The solution was cleared by low-speed centrifugation (12,000 × *g*, 5 min), layered on 7 ml of 1.8 M sucrose in TKM buffer, and centrifuged at 26,000 rpm in a Spinco SW27 rotor for 24 hr at 4°. The pelleted polysomes were gently dissolved in ice-cold water and stored at −70° until further use. Each polysome preparation was checked for quality by subjecting an aliquot to sucrose gradient centrifugation. Only those preparations showing no or nearly no indication of degradation were used for further RNA purification. (The frequency of successful preparation depends largely on the myeloma lines and the condition of tumor passage. With MOPC 104E, HOPC 2020, and MOPC 321 tumors it was higher than 80%.)

From 3000 to 6000 A_{260} units (an A_{260} unit is that amount of material giving an A_{260} of 1.0, through a 1 cm light path, when dissolved in 1 ml) of the frozen MBP where thawed at one time. Lithium dodecyl sulfate and proteinase K (Merck, chromatographically pure) were added to 0.2% and 200 μg/ml, respectively. The mixture was incubated at 0° for 10 min. The concentration of lithium dodecyl sulfate was raised to 1% and Tris·HCl, pH 9.0, and Na_2EDTA were added to 100 mM and 2 mM, respectively. The mixture was shaken at 4° with an equal volume of phenol–cresol–H_2O mixture (100:14:40) for 15 min. The phases were separated by centrifuging at 12,000 × *g*, 10 min. The aqueous phase was saved. The interphase was suspended in 20 ml of TE-I buffer (Tris·HCl, pH 9.0, 100 mM; Na_2EDTA, 2 mM) and shaken at 4° with an equal volume of chloroform–isoamyl alcohol mixture (25:1) for 15 min. The separated aqueous phase was combined with the first aqueous phase. The phenol–cresol extraction was repeated twice more and RNA was finally precipitated with 2.5 volumes of ethanol after adding Na-CH_3CO_2, pH 5, to 0.5 M. The RNA precipitate collected by centrifugation was dried *in vacuo*.

Abbreviations: C_0t, concentration of DNA in moles of nucleotide per liter times incubation time in sec; V_λ and V_κ, variable region of λ and κ chains, respectively; C, constant region.

The RNA was dissolved in TE-II buffer (Tris·HCl, pH 7.5, 10 mM; Na_2EDTA, 0.2 mM) at 100 A_{260} units/ml, heated at 70° for 7 min, and chilled quickly in ice. Prior to application to an oligo(dT)-cellulose column, the mixture was warmed to 25° and KCl was added to 0.5 M. Preparation and chromatography procedures of oligo(dT)-cellulose were according to Gilham (7) and Aviv *et al.* (8), respectively. The poly(A)-containing RNA eluted from the column by TE-II buffer was subjected to a second oligo(dT)-cellulose chromatography after heat treatment.

Poly(A)-containing RNA (20 A_{260} units) was dissolved in 0.5 ml of TLE buffer (Tris·HCl, pH 7.2, 5 mM; lithium dodecyl sulfate, 0.1%; Na_2EDTA, 0.2 mM), heated at 70° for 3 min, chilled to 0°, and layered on a 13 ml, 10–24%, convex sucrose gradient made in TLE buffer. The gradient was centrifuged at 40,000 rpm for 20–24 hr in an IEC SB283 rotor at 6°. After centrifugation fractions were collected from the bottom of the tube while A_{260} was monitored by a Gilford 2400 spectrophotometer fitted with an IEC 4 mm continuous flow cell. The light chain mRNA activity was monitored by adding an aliquot of each fraction to a cell-free translation system as previously described (5). The details of the analysis of translation products will be reported elsewhere. The 13S peak fraction, enriched in light chain mRNA, was subjected to a second sucrose gradient centrifugation.

The peak fraction of the second sucrose gradient was collected and RNA was precipitated by ethanol. It was dissolved in deionized 98% formamide (Merck) buffered with 0.02 M $NaPO_4$ and 0.1 M CH_3COOH (pH 6.8). After addition of sucrose and bromphenol blue (15% and 0.01%, respectively), the mixture was applied to a 3.5% acrylamide gel (0.6 × 8 cm) which had been polymerized in H_2O and equilibrated with the buffered formamide (9). Electrophoresis was carried out at 16° with continuous circulation of the buffered formamide. After electrophoresis, the gel was either stained by 0.005% Stain All (Eastman Kodak Co.) or scanned for A_{260} in a Gilford 2400 spectrophotometer fitted with a 2410-S linear transport device. The approximate position of the main band was estimated from the absorbance profile. In order to locate the band position more precisely, a small amount of 1% bromphenol blue solution was injected transversely into the gel with a gauge 26 syringe needle at both sides of the estimated band position, 5 mm apart, and the gel was rescanned at 260 nm. Injected bromphenol blue forms a thin line in the gel and produces two sharp absorption lines on the recording paper. A gel slice (1–2 mm thick) was cut from the center of the band, and RNA was eluted by homogenizing in 0.5 M $NaCH_3CO_2$ containing a few drops of phenol. Linear polyacrylate also elutes from the gel and coprecipitates with RNA on subsequent addition of 2.5 volumes of ethanol. It was removed by passage through a small hydroxyapatite column (Clarkson Chemical Co.).

Isolation of Half Fragments of κ mRNA Containing the 3′-End. MOPC 321 κ mRNA, purified by the procedures described above, was iodinated (see below) to about 3×10^7 cpm/μg. During iodination this RNA received about 1.2 cuts per molecule as estimated by sucrose gradient centrifugation of an aliquot. Intact molecules and those fragments containing poly(A) sequences at the 3′-terminus were separated from 5′-terminus-containing and internal fragments by two successive steps of chromatography in oligo(dT)-cellulose. They were then fractionated by two successive steps of sucrose density gradient centrifugation. The fraction sedimenting at 9 S was collected. The size distribution of the fragments was checked by acrylamide gel electrophoresis in formamide (see above). More than 90% of the mass (iodine radioactivity) migrated within the size range of 550 to 650 nucleotides.

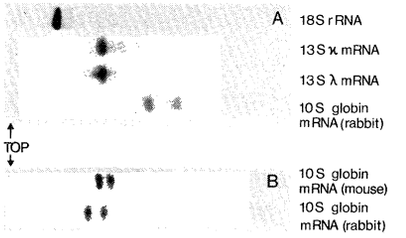

FIG. 1. Acrylamide gel electrophoresis of RNA in 98% formamide. 3–5 μg of indicated RNA were electrophoresed at 100 V for 12 hr (A), and 80 V for 10 hr (B) as described in *Materials and Methods*. Gels were stained with Stain All.

Isolation of Mouse Globin α- and β-Chain mRNA. Reticulocyte polysomes were isolated according to the procedures described by Williamson *et al.* (10). Mixed α- and β-chain mRNA was purified by oligo(dT)-cellulose chromatography and sucrose gradient centrifugation as described by Aviv and Leder (8). The acrylamide gel electrophoresis pattern is shown in Fig. 1B.

Other Methods. Procedures for RNA iodination, preparation of mouse liver or myeloma DNA, RNA·DNA hybridization and competition hybridization have been previously described (3).

RESULTS

Characteristics of light chain mRNA preparation

Fig. 1A shows the stained acrylamide gels of 13S MOPC 321κ and MOPC 104Eλ mRNA after electrophoresis. 18S rRNA and rabbit globin α- and β-chain mRNAs used as migration standards are also shown. In the light chain RNA gels, material lightly stained is noticeable on both sides of the major band. Our earlier preparation of 13S κ-chain RNA from MOPC 70E showed a higher degree of heterogeneity in a similar gel (see ref. 3). With such a preparation, the purity of the RNA eluted from the major band was estimated to be 70 to about 80% by "fingerprint" analysis and by competition hybridization (3). Considering the increased strength of the major band in the acrylamide gel and the higher content of 13S component in the total membrane-bound polysomal RNA, it is reasonable to conclude that the present κ- and λ-chain mRNA preparations as eluted from the gel are at least 90% pure.

Hybridization kinetics [concentration of nucleic acid × time (C_0t) curve] of λ mRNA

In contrast to the reported biphasic hybridization kinetics of κ mRNA (see below), the λ mRNA hybridized to mouse liver DNA with apparently monophasic kinetics (Fig. 2). Under the hybridization conditions employed the apparent $C_0t_{1/2}$ was 950 mol sec liter^{-1}. This corresponds to a nominal reiteration frequency of 6, based on *Escherichia coli* cRNA as a standard (11). The nominal reiteration frequency is an upper limit and corrections are required to account for a finite DNA/RNA ratio and for heat degradation of RNA. When corrections were made according to Bishop *et al.* (12), I obtained a corrected reiteration frequency of 3. Under similar conditions purified globin mRNA hybridized to liver DNA with $C_0t_{1/2}$ of 1050 mol sec liter^{-1} or a nominal reiteration frequency of 5 (Fig. 2). Based on hybridization ki-

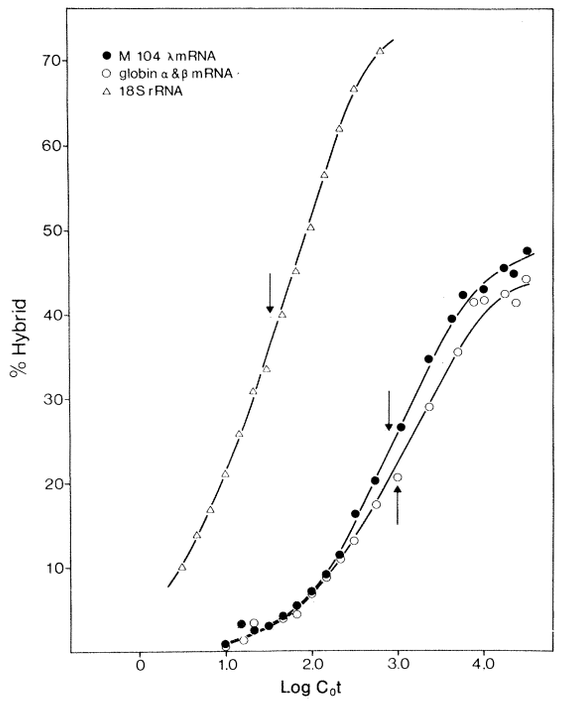

FIG. 2. Hybridization kinetics of MOPC 104E λ mRNA with mouse liver DNA (C_0t curve). DNA concentration, 22.2 mg/ml for λ mRNA and mouse globin mRNAs, 8.8 mg/ml for 18S rRNA; ratio of DNA to RNA, 1.5×10^7 for λ mRNA, 3×10^7 for globin mRNA, and 4.5×10^6 for 18S rRNA. Intrinsic RNase-resistant fraction (2.0%) has been subtracted. The arrows indicate the positions of $C_0t_{1/2}$.

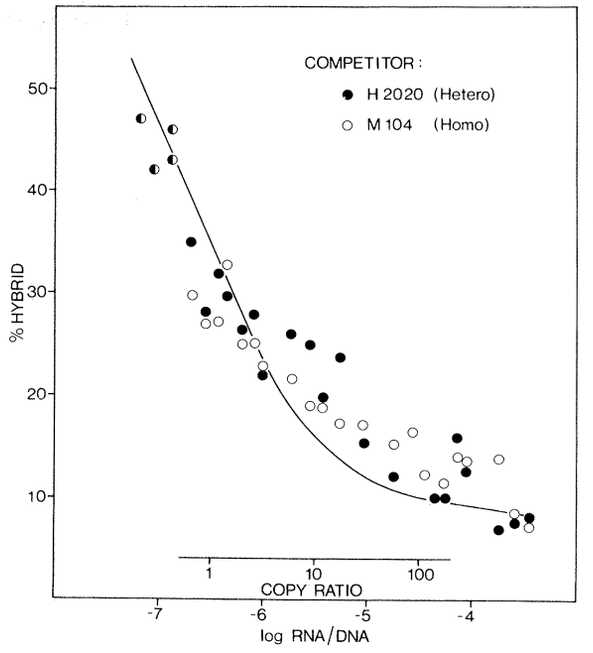

FIG. 3. Competition hybridization of λ mRNAs. Mouse liver DNA (22 mg/ml) was annealed with ^{125}I-labeled MOPC 104E λ mRNA (purified from gel) to $C_0t = 10^4$ in the presence of various amounts of unlabeled mRNA from HOPC 2020 or MOPC 104E tumors. The hybridization levels obtained were plotted against the overall ratio of RNA to DNA. The competitor RNAs were purified to the stage of second sucrose gradient centrifugation and the purity was estimated to be about 70%. This was taken into account in positioning the copy ratio (RNA/DNA) scale. Points represented by ◐ are from the hybridization mixtures without unlabeled RNA. The theoretical curve was derived by a computer program and represents homologous competition of the hypothetical RNA mixture described in the *text*. For computation it was assumed that $k_r/k_d = 0.30$ and $C_0t/C_0t^d{}_{1/2} = 25$, where k represents a rate constant and r and d represent RNA·DNA hybridization and DNA renaturation, respectively.

netics of cDNA, which require less correction, Harrison *et al.* (13) reported that mouse globin α- and β-chain genes are reiterated about twice. This gives me an additional justification for employing the above correction factor in obtaining the true reiteration frequency. The small deviation of the two C_0t curves, particularly at high C_0t values, is partly due to different DNA/RNA ratios; it is difficult to determine exact specific activity of iodinated RNA. For comparison a C_0t curve of purified iodinated 18S rRNA is also shown in Fig. 2. The nominal reiteration frequency of 18S rRNA gene was 210.

Competition hybridization of λ mRNAs

A mixture of highly purified, iodinated MOPC 104E λ mRNA and denatured liver DNA in excess over the labeled RNA was annealed to $C_0t = 10{,}000$ in the presence of various amounts of unlabeled homologous (MOPC 104E) or heterologous (HOPC 2020) λ mRNA. The specificity of such a competition reaction has been established (3). In Fig. 3 it can be seen that both homologous and heterologous competition take place over a wider range of ratios (of RNA to DNA) than expected for a single component. This is due to impurities in the labeled mRNA preparation. I expect that these impurities consist of a large number of different species (3). In order to effectively compete these species, sufficient unlabeled RNA must be added so that the sequences in RNA are in excess over those in DNA for each species. Also shown in Fig. 3 is a theoretical curve for a hypothetical RNA preparation containing 85% unique, major component and 15% impurities, assumed to consist of 1500 different equimolar species. The fit is as good as might be expected considering simplicity of the assumptions.

The competition experiments were carried out to test the hypothesis that the nucleotide sequences of mRNAs coding for various mouse λ chains are highly homologous as suggested by the amino-acid sequence data (see *Discussion*). If this is so, a particular λ mRNA would cross-hybridize with germ line genes for other λ chains if they existed. Although the points are somewhat scattered, it is clear from Fig. 3 that the two competition curves, homologous and heterologous, are indistinguishable. This indicates that the nucleotide sequences in the two mRNAs and consequently in the two genes are highly homologous.

Hybridization kinetics of κ mRNA

In our previous report we showed that a 70–80% pure κ mRNA preparation (MOPC 70E) consisted of two fractions: a major fraction (*ca* 85%) "unique" or nearly unique sequences and a minor fraction (*ca* 15%) "reiterated" (*ca* 230 times) sequences (Fig. 4A) (3). Based on the results of a series of competition hybridization experiments we concluded that both *V*- and *C*-region sequences are represented by the unique or nearly unique sequences (3). When κ mRNA (MOPC 321) was purified to a higher homogeneity it hybridized to liver DNA with apparently monophasic kinetics (Fig. 4A). The corrected reiteration frequency was 3, a value very similar to that of mouse globin α- and β-chain genes.

In a κ mRNA molecule about 350 nucleotides are un-

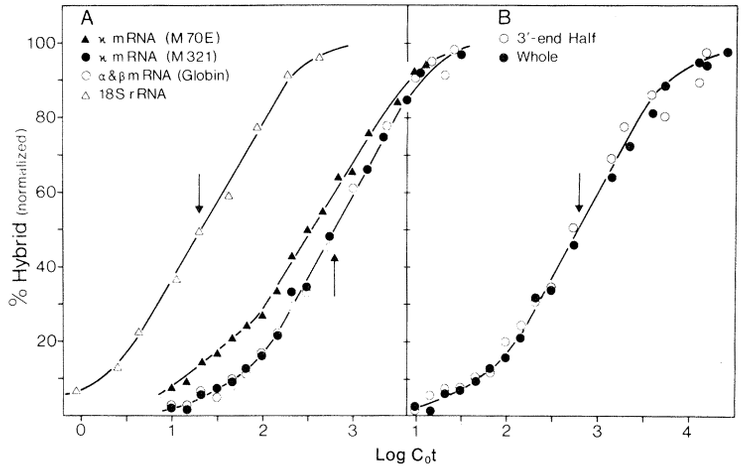

FIG. 4. (A) Hybridization kinetics of κ mRNAs with mouse liver DNA. DNA concentration, 22.2 mg/ml for κ mRNAs and globin mRNA, 2.2 mg/ml for 18S rRNA; ratio of DNA to RNA, 1.1×10^7 (MOPC 70E κ mRNA), 2.0×10^7 (MOPC 321 κ mRNA), 2.1×10^7 (globin mRNA), and 5.5×10^5 (18S rRNA). The curves are normalized, after subtraction of intrinsic RNase-resistant radioactive material (2.0%), taking the maximal hybridization levels as 100%. These levels were 51% (MOPC 70E), 50% (MOPC 321), 40% (globin), and 67% (18S rRNA).

(B) Hybridization kinetics of 3'-end half fragment and whole MOPC 321 κ mRNA with mouse liver DNA. DNA concentration, 22.2 mg/ml; ratio of DNA to RNA, 7×10^7 for half-fragment, 2×10^7 for whole. The maximal hybridization levels were 50% for both curves.

translated (4, 14); of these about 200 nucleotides lie between the section coding for the *C*-region (*ca* 330 bases) and the poly(A) (*ca* 200 bases) at the 3'-end (15). The κ mRNA fragment (see *Materials and Methods*) which is about 600 bases long and contains at least a part of the poly(A) sequence should be largely devoid of the section coding for the *V*-region and the untranslated section at the 5'-end. In Fig. 4B, the kinetics of hybridization of such a fragment was carefully compared with that of the whole molecular under equivalent conditions. The two C_0t curves were indistinguishable within the limit of experimental error.

DISCUSSION

Fig. 5 summarizes the amino-acid sequence data of the V-region of Balb/c mouse myeloma λ chains. It was modified from Fig. 1 of ref. 16. The following features of the sequence data are relevant for the present discussion. (*1*) There is an extensive sequence homology among all λ chains. Thus the homology in the corresponding nucleotide sequences is likely to be extensive. (2) The λ chains sequenced are not selected by any means other than the method of tumor induction. (3) Roughly two thirds of the λ chains synthesized by the different tumors have the M104E type sequence. The remaining λ chains have sequences very similar to but different from this.

The data shown in Fig. 5 permit certain conclusions about the diversity of mouse λ chains. So far Weigert and colleagues have sequenced six λ chains besides those of the M104E type and found that all six are different from one another. This suggests that there exist many other different V_λ sequences. If there are only seven different mouse V_λ sequences (M104 type and six others), the probability of picking six out of six non-104E type myeloma proteins, each with a different sequence, is: $6!/6^6 = 0.015$, a highly unlikely event. Assuming that all λ chains except for the M104E type occur at equal frequencies among tumors, 25 different λ chains are required in order to increase that probability to 0.5. The estimated number of different sequences further increases if the frequency of various tumors is unequal. I

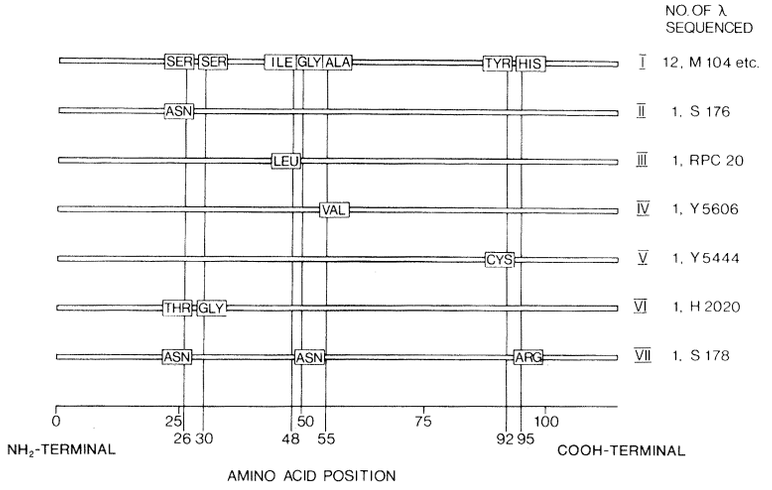

FIG. 5. Amino-acid sequences of Balb/c mouse myeloma λ chains (16). Only V-region sequences are shown. The thick bars represent amino acids common in all λ chains. The minimum number of base changes which are required to generate each chain from M104 type chain (I) are 1 for type II to type V, 2 for type VI, and 4 for type VII.

have shown that the gene coding for H2020 λ mRNA would completely cross-hybridize with M104E mRNA if it existed as a separate germ line gene. Although many combinations of different λ chains have not been tested for cross-hybridizability, this is very likely to be the case, considering the extensive amino-acid sequence homology (Fig. 5). If the germ line theory of antibody diversification is correct we expect that the genome of a Balb/c cell would carry *at least* 20 to 30 different germ line V_λ genes (see above). These genes should all cross-hybridize with M104E mRNA efficiently. Yet I have shown that total number of λ genes which can cross-hybridize with M104E λ mRNA is no more than 3. Hence the results presented here indicate that the number of germ line V_λ genes is too small to account for the observed amino-acid sequence diversity.

Base mismatching causes a reduction in the rate of hybrid formation. This, of course, leads one to underestimate gene reiteration frequency. Bonner *et al.* (17) estimated the effect of base mismatching on the rate of reassociation of DNA. Their estimate was a rate reduction of a factor of two for each 10° reduction in melting temperature resulting from about 10% base mismatching. Aside from possible "neutral" base differences, there should be 1.1% base mismatching in the hybrid formed between M104 λ mRNA and a hypothetical germ line V_λ gene for S178 (four unpaired bases in 345 base long hybrid)—a value too small to affect significantly the rate of hybrid formation, if the above estimate of DNA reassociation applies also to hybrid formation. For other hypothetical germ line V_λ genes, base mismatching would be even less (Fig. 5). Base mismatching due to "neutral" base differences should not be substantial either, for the melting profile of the hybrid formed between a λ mRNA (MOPC 104E) and homologous DNA exhibited a sharp transition at the melting temperature of 87° in 2 × SSC (0.3 M NaCl, 0.03 M sodium citrate, pH 7) (my unpublished observation). In conclusion, assuming that the effect of mismatching is similar for DNA reassociation and RNA·DNA hybridization, I should not have substantially underestimated the reiteration frequency due to base mismatching in the hybrid.

Contrary to earlier reports from several laboratories including our own (3, 4, 18–21), the κ mRNA preparation (MOPC 321) used in the present work gave a monophasic C_0t curve of unique or nearly unique sequences. That the discrepancy results from differences in the purity rather than in the source (i.e., different tumor lines) of κ mRNA is suggested by the observation that the 13S fraction of MOPC 321 κ mRNA from the second gradient contained a small amount of reiterated sequences (my unpublished observation). The C_0t curve of the present κ mRNA preparation is very similar to that of mouse globin mRNA (Fig. 2). The corrected reiteration frequencies were 3.0 and 2.5, respectively. Furthermore, the two C_0t curves obtained with whole κ mRNA molecules and the 3′-end half fragments were indistinguishable (Fig. 4B). The latter should mainly represent sequences corresponding to the constant region (*C*) gene and the untranslated section at the 3′-end. There is abundant genetic and hybridization evidence which suggests that the *C* gene is unique (3, 22–26). These results confirm our previous conclusion: not only the nucleotide sequences for the *C* region but also those for the *V* region are derived from unique or nearly unique genes.

The present results, on the other hand, are incompatible with the hypothesis that the reiterated fraction observed in earlier κ mRNA preparations originates from the untranslated, 5′-end section of the RNA molecule (5, 27). Since sequences in this fraction were common among all κ mRNAs tested (3), it was hoped that careful characterization of this fraction would lead to an estimate of the total number of germ line V_κ genes. The present results make this an unlikely prospect. However, the absolute number of germ line *V* genes is a separate issue from the relative numbers of germ line genes and protein chains. The germ line theory demands that for each chain there exists a germ line gene. Based on a series of competition experiments and a statistical consideration of known κ chain sequences, we have previously concluded that the number of germ line V_κ genes is too small to account for the sequence diversity (3). The present results with λ and κ chains reinforce and extend this conclusion.

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