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Evidence for Somatic Generation of Antibody Diversity

(RNA·DNA hybridization/κ-chain mRNA)

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ABSTRACT RNA preparations containing 70–80% mouse κ-chain mRNA have been prepared. The remainder consists of many RNA species, each of which represents a small fraction of the total RNA. The κ-chain mRNA preparation hybridizes with mouse liver DNA with biphasic kinetics, indicating that it consists of two fractions —"unique" and "reiterated." Competition hybridization experiments show that the homology among the unique fractions from different mRNAs is the same as the homology among the amino acid sequences of the corresponding κ-chains. Hence, in addition to the C-region (constant-region) sequences, (most of) the V-region (variable-region) sequences are also derived from unique germ line genes. The reiterated fractions from different κ-chain mRNAs show essentially complete homology with each other. This fraction seems to consist mostly of sequences which do not code for amino-acid sequences of the secreted polypeptide chain, i.e., the "external" section of the mRNA molecule. It is concluded that the number of germ line genes is too small to account for the observed diversity of antibody molecules.

One of the most fascinating problems of immunology is posed by the enormous diversity of the antibody molecules that one animal is able to synthesize. One inbred strain of mice has been shown to be capable of producing eight thousand different antibody molecules against the hapten NIP (4-hydroxy-5-iodo-3-nitrophenacetyl) (1). Another inbred mouse strain was shown to produce many different antibody molecules against DNP (dinitrophenyl), of which more than 500 cross-react strongly with TNP (trinitrophenyl) (2). The occurrence of species-specific residues in the V-regions of antibody polypeptide chains and the mendelian inheritance of rabbit *a* allotypes point against a germ line theory (3). Nevertheless, controversial ideas of evolutionary versus somatic generation of antibody diversity have continued to coexist (3, 4). The direct approach to solving this controversy is to count the number of antibody *V*-genes present in the DNA of one cell. To this end, we have analysed the kinetics of hybridization of a mouse κ-chain mRNA to mouse liver DNA. For the analysis to be meaningful, three points are essential:

1. The physical purity of the κ-chain mRNA preparation must be known; that is, the amount and nature of contaminants must be determined.

2. Since κ-chain mRNA contains both a unique and a reiterated fraction (5, 6), it is necessary to assign the V-region sequences to one of these fractions.

3. The proportion of all possible *V*-genes which would have cross-hybridized with V-region sequences of the particular κ-chain mRNA used, must be determined.

We have recently reported a preliminary account of our work along this line (5, 7), which strongly suggested that there are very limited numbers of genes coding for the variable region of κ-chains. Now, our work comes close to meeting all three of the above conditions. In this paper, we present cogent evidence that the number of germ line *V*-genes is so low that there must exist a mechanism for extensive somatic generation of antibody diversity.

MATERIALS AND METHODS

Myeloma Tumors. We are indebted to Dr. M. Potter for kindly supplying tumor lines. Tumors were maintained by subcutaneous injection of 1 to 2 × 10^7 cells into 8 to 20-week-old BALB/c female mice.

Purification of κ-Chain mRNA. The method has been modified from that in our previous report (8) and details will be published elsewhere. Briefly, RNA was extracted from membrane-bound polysomes isolated from solid tumors. From this, κ-chain mRNA was enriched by poly(dT)-cellulose chromatography followed by repeated sucrose gradient centrifugation. The 13S peak fraction was then subjected to preparative polyacrylamide gel electrophoresis in 98% formamide (9). A 2-mm thick gel slice was cut from the center of the major band and homogenized in 0.3 M sodium acetate. RNA was extracted from the crushed gel by repeated freezing and thawing, and soluble acrylamide was removed by passage through a small hydroxyapatite column.

Iodination of RNA. Purified κ-chain mRNA (or 18S rRNA) was iodinated according to Scherberg and Refetoff (10) at specific activities of 1 to 2 × 10^7 cpm/μg. No changes in hybridization properties were detected over 1 month.

Preparation of Mouse Liver DNA. DNA was prepared from isolated nuclei by the method of Bishop (11) and sonicated at a concentration of 0.5–1 mg/ml so that there was an average of 400 to 500 nucleotides per strand.

DNA·RNA Hybridization. Our procedure follows that of Melli *et al.* (12). Hybridization was carried out at 70° in 2 × SSC at a final concentration of 0.22, 2.2, or 22 mg of DNA

Abbreviations: C_0t, moles of deoxyribonucleotide/liter of incubation × sec; SSC, standard saline–citrate solution (0.15 M sodium chloride–0.015 M sodium citrate, pH 7); 2 × SSC means that the concentration of the solution used is two times that of the standard saline–citrate solution; V-region, variable region; C-region, constant region.

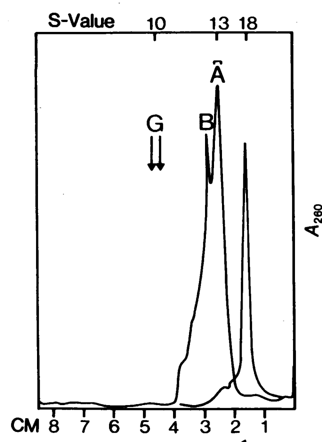

FIG. 1. Gel electrophoresis of MOPC 70E 13S RNA. Twenty micrograms of MOPC 70E 13S RNA from the second sucrose gradient centrifugation was loaded on a cylindrical 4.0% acrylamide gel in 98% formamide and electrophoresed at 8 V/cm at 15° for 12 hr with recirculation of 98% formamide containing 20 mM NaCl and 50 mM Tris·acetate at pH 8.5. The gel was scanned at A_{260} in a Gilford model 2400 spectrophotometer. MOPC 70E 18S rRNA was electrophoresed on a parallel gel. The positions of rabbit globin mRNAs are shown by the arrows labeled G. The bracket labeled A shows the fraction which was eluted and used in all subsequent experiments.

per ml. For the C_0t curves, DNA in 0.1 × SSC was denatured by heating at 100° for 5 min. in siliconized conical tubes under mineral oil. [^{125}I]RNA in 0.1 × SSC was added and the heating was continued for another 2 min. An aliquot was withdrawn for determination of the "intrinsic RNase-resistant fraction." The tube was then transferred to a 70° water bath. After 30 sec, one-ninth volume of preheated 20 × SSC was added and quickly mixed; this was taken as time 0. At intervals, 50- to 200-μl samples were withdrawn and quickly mixed with 10–20 ml of precooled 2 × SSC. The diluted hybridization mixture was divided into four equal parts. In two parts, the nucleic acid was directly precipitated with one-fifth volume of 50% trichloroacetic acid. When the DNA concentration for annealing was 0.22 or 2.2 mg/ml, 500 μg of yeast RNA (Worthington) was added as precipitation carrier. The other two parts were treated with 20 μg/ml of bovine pancreatic RNase and 2 units/ml of T-1 RNase at 37° for 20 min. (Both nucleases had been preheated at 80° for 10 min in 2 × SSC at pH 5.0 in order to inactivate contaminating DNase.) The RNase-treated samples were chilled in ice, (carrier added if necessary), and precipitated with one-fifth volume of 50% trichloroacetic acid. The ratio of radioactivity in the RNase treated samples to radioactivity in the untreated samples, corrected for the intrinsic RNase-resistant fraction, gave the fraction hybridized.

For competition hybridization experiments, DNA and ^{125}I-labeled RNA were mixed, and the salt concentration was adjusted to 4 × SSC. Equal aliquots (10–90 μl) of the mixture were distributed to siliconized conical tubes, appropriate amounts of unlabeled RNA (competitor) in 2–10 μl of water were added, and the final volume was adjusted with water to give 2 × SSC. The solution was covered with a thin layer of mineral oil and heated for 4 min at 100° before annealing at 70° was started. The intrinsic RNase-resistant fraction was determined separately.

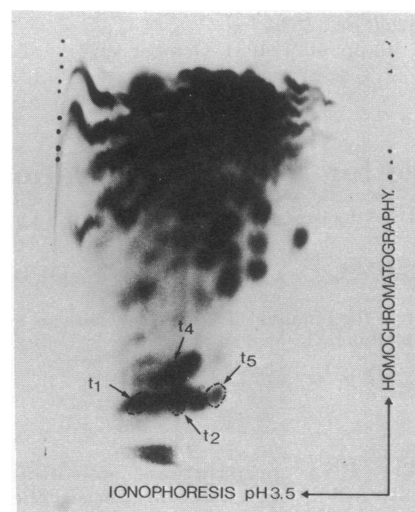

FIG. 2. Autoradiograph of a two-dimensional "fingerprint" of a T1-RNase digest of ^{125}I-labeled MOPC 70E band A RNA. The digest was fractionated in a two-dimensional system using high voltage electrophoresis on a cellulose acetate strip at pH 3.5 for the first dimension and thin-layer homochromatography on a DEAE cellulose plate for the second (30, 31). See *text* for explanation of labeled spots.

Constants. For computations, the following constants were used: Genome size of mouse liver DNA = 1.8 × 10^{12} daltons (13); $C_0t_{1/2}$ of *Escherichia coli* DNA in 2 × SSC at 70° = 8.1 (12); molecular weight of 18S rRNA = 6.5 × 10^5 (14); extinction coefficient of RNA = 25 A_{260}/mg per ml, of DNA = 20 A_{260}/mg per ml.

RESULTS

Characterization of κ-chain mRNA preparations

The 13S RNA component exhibits two major bands (bands A and B) and two or three minor bands after polyacrylamide gel electrophoresis in formamide (Fig. 1). The overwhelming majority of the κ-chain mRNA activity is associated with band A (8). The molecular weight of MOPC 70E band A RNA was determined by using, as migration standards, rRNA from *E. coli* and from the tumor as well as rabbit globin mRNA. The molecular weights obtained in three measurements were 4.0 ± 0.2 × 10^5 which corresponds to 1180 ± 60 nucleotides. The ratio of RNA in band A to that in band B was estimated from A_{260} tracings of the gel. The ratio varied somewhat in different preparations (from 3:1 to 5:1). The RNA was eluted from band A and iodinated. A part was used for "fingerprinting" while the rest was used in hybridization studies.

The assay of κ-chain mRNA activity in cell-free translation systems is convenient and sensitive, but, it does not by itself permit us to estimate the purity of the mRNA preparation. Homogeneity in electrophoresis is also not sufficient because contamination by an RNA of similar size can neither be excluded nor its extent estimated. We have, therefore, digested our κ-chain mRNA with T-1 RNase and prepared a two-dimensional "fingerprint" (Fig. 2) which shows that: (*1*) the preparation contains a major component which is about 70% pure, judging from the background and yields of characteristic polynucleotides; (*2*) the contaminants consist of many different species; (*3*) the major component contains all

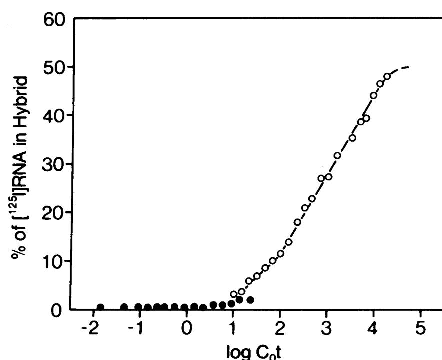

FIG. 3. Hybridization kinetics of MOPC 70E band A RNA with mouse liver DNA (C_0t curve). Closed circles: DNA concentration, 0.22 mg/ml; ratio of DNA to RNA, 5.5×10^4. Open circles: DNA concentration 22.4 mg/ml; ratio of DNA to RNA 1.1×10^7. Intrinsic RNase-resistant fraction was 2.1%.

three oligonucleotides (t_2, t_4, and t_5) of 16 to 20 bases which have previously been assigned to the C-region of MOPC 21 κ-chain mRNA (15); (*4*) the major component shares with MOPC 21 κ-chain mRNA, one oligonucleotide (t_1) of about 20 bases which could not be assigned to the translated sections of κ-chain mRNA (15).

We conclude that the major component is indeed the MOPC 70E κ-chain mRNA. The high molecular weight as well as the existence of an apparently untranslated oligonucleotide show that the κ-chain mRNA contains sequences that do not code for the V- and C-region of the secreted κ-chain. We call these apparently untranslated sequences the "external section" without implying that they constitute a contiguous stretch of nucleotides. There is a poly(A) sequence of 200 nucleotides at the 3′ end of the polynucleotide chain (15) which is not labeled by iodine and which will not concern us further. The sections coding for the V- and C-regions each consist of 327 nucleotides (33.5% of the labeled portion of the molecule). This leaves about 320 nucleotides (33%) for the "external section."

Hybridization kinetics

The κ-chain mRNA preparation hybridizes to mouse liver DNA with biphasic kinetics, thus indicating that it consists of two fractions in terms of the reiteration frequency of the corresponding genes (Fig. 3). We call these two fractions "unique" and "reiterated." Most of the sequences (84%) are in the unique fraction which hybridizes with an apparent $C_0t_{1/2}$ of 1320. This corresponds to a nominal reiteration frequency of 4 (16). The nominal reiteration frequency is an upper limit. If corrections are made to account for a finite ratio of DNA to RNA and for heat degradation of RNA according to Bishop *et al.* (16), we obtain a corrected reiteration frequency of 1 to 2. The reiterated fraction consists of about 16% of the hybridizable RNA and hybridizes with a $C_0t_{1/2}$ of approximately 23, thus indicating a gene reiteration frequency of 230. We also obtained a reiteration frequency of 250 for the 18S rRNA gene (5). This is in good agreement with reported values (12). Competition experiments show that despite the similar reiteration frequencies, the reiterated fraction of the κ-messenger RNA is not ribosomal RNA (data not shown). In the experiments illustrated in Fig. 3, only about 50% of the RNA hybridized at the maximum C_0t value employed. In a separate experiment in which the dependency

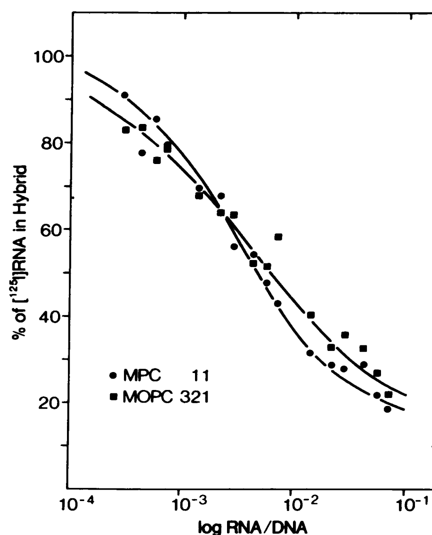

FIG. 4. Competition hybridization of the reiterated RNA fraction. Mouse liver DNA (2.07 mg/ml) was annealed with ^{125}I-labeled MOPC 70E band A RNA to C_0t = 198 at a ratio of DNA to [^{125}I]RNA of 5.75×10^4 in the presence of varying amounts of unlabeled mRNA from MOPC 321 or MPC 11 tumors. The competitor RNA was purified only to the stage of the second sucrose density gradient centrifugation. The data are normalized, taking the hybridization level obtained without competitor as 100%. This was 9.8% (average of four measurements) after subtraction of the intrinsic RNase-resistant fraction (2.0%).

of the final hybridization level on the ratio of DNA to RNA was studied, as high as 60% was hybridized (DNA/RNA = 2.7×10^7).

The competition hybridization experiment

DNA·RNA hybridization is intrinsically a competitive reaction. The codon strand of the DNA competes with the labeled RNA for the anti-codon strand. That is why it is necessary to use a "vast excess" of DNA to achieve maximal hybridization (12). As the ratio of DNA to RNA is reduced, the final, i.e., at infinite time, level of hybridization will be reduced. Assume that the competition occurs on an equal basis—that is, the rate constants for DNA·RNA hybridization and DNA·DNA renaturation are the same. In this case, half-maximal hybridization will occur when for each RNA molecule, there is exactly one homologous copy in the DNA. From this, the nominal gene reiteration frequency, N_κ, can be calculated by

$$N_\kappa = R_{1/2}(M_m/M_\kappa)$$

where $R_{1/2}$ = ratio of RNA to DNA that gives half-maximal hybridization; M_m and M_κ are the molecular weights of the mouse haploid genome and the κ-chain mRNA molecule, respectively.

We have used light chain mRNA preparations from a series of myelomas as competitor RNAs to inhibit the hybridization of MOPC 70E ^{125}I-labeled band A RNA to mouse liver DNA. The unique and reiterated fractions are studied separately. To study the reiterated fraction, we tried to hybridize an amount of DNA which was in excess of labeled RNA for the reiterated sequences, but which was limiting for unique sequences; the results are shown in Fig. 4. To study the unique fraction, we used excess DNA and annealing was carried out

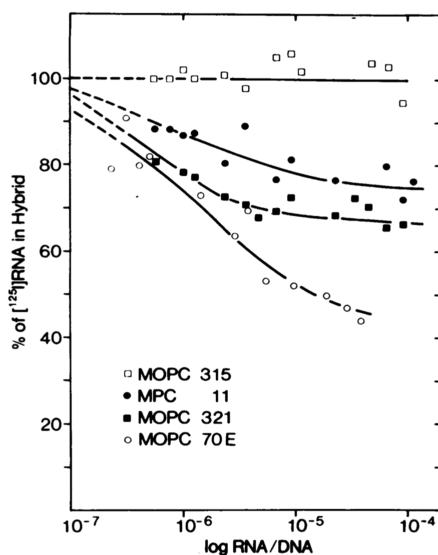

FIG. 5. Competition hybridization of the unique RNA fraction. Mouse liver DNA (22.0 mg/ml) was annealed with MOPC 70E band A [^{125}I]RNA to C_0t = 7920 at a ratio of DNA to [^{125}I]RNA of 1.1×10^7 in the presence of varying amounts of band A RNA from the myeloma lines indicated. The hybridization level without competitor was 45.5%. Intrinsic RNase-resistant fraction was 2.0%.

to C_0t = 7920. Under these conditions, both fractions hybridize, but the maximum amount of competitor used was such that only sequences of the unique fraction would be competed; the results are shown in Fig. 5. The conclusions which can be extracted from this experiment are considered separately.

Which of the two RNA fractions, unique or reiterated, contain the V-region sequence?

The amino acid sequence data (17, 18) show that nucleotide sequence homology within the V-region must be very limited between MOPC 70E and MPC 11. Thus, if either the unique or reiterated fractions contain MOPC 70E V-region sequences which are not shared by MPC 11, these sequences should be visualized as a difference in the extent of homologous (MOPC 70E) and heterologous (MPC 11) competitions. Furthermore, competing MOPC 321, which shows extensive homology with 70E (19), should inhibit hybridization to an intermediate degree. The result shown in Figs. 4 and 5 seem clear on this point. Since about 80% of the reiterated fraction was competed-for regardless of the kinds of competitor used, this fraction cannot contain most of the V-region sequences. (Only those rare sequences common to MOPC 70E and MPC 11 might be derived from reiterated genes.) In contrast, there is a good correlation between the estimated V-region sequence homology and the extent of competition in the unique fraction. MPC 11 RNA was an effective competitor for only 30% of the labeled 70E RNA in the range of RNA to DNA ratios where only (nearly) unique sequences are expected to compete. In the same range of RNA to DNA ratios, 70E RNA was an effective competitor for 55% of the homologous labeled RNA. Hence, a little over half of the sequences in the unique fraction of MOPC 70E are shared with MPC 11. Note that the λ-chain MOPC 315, which is very different from MOPC 70E in the C- as well as in the V-region (20), does not compete at all in this range of RNA to DNA ratios. We conclude that the unique fraction contains both C- and V-region sequences. This conclusion could be strengthened by performing competition experiments with a wider assortment of mRNAs.

Would we have counted multiple *V*-genes if they existed?

More precisely, would MOPC 70E κ-chain mRNA have cross-hybridized with DNA in other *V*-genes if they existed? MOPC 321 κ-chains share the same amino acids with MOPC 70E at 81% of positions within the V-region (17, 19). Thus, assuming that the minimum base sequence homology which hybridizes under our conditions is 15 contiguous bases (21), up to 65% of the base sequences in the V-region of MOPC 70E κ-chain are expected to be competed for by MOPC 321 κ-chain mRNA. Fig. 5 shows that about 40% of the sequences uncommon between the two κ-chain mRNAs of MOPC 70E and MPC 11 are inhibited by MOPC 321 in the range of RNA to DNA ratios where only (nearly) unique sequences can be expected to compete. The results indicate that if there were two separate *V*-genes, one each for MOPC 70E and MOPC 321, we would have counted both genes with the methods employed here. But MOPC 70E and MOPC 321 are representatives of a large group of mouse κ-chains which have a high degree of homology with each other. Six out of 42 myeloma κ-chains—about 14%—appear to belong to this group (19, 22). Our results strongly suggest that MOPC 70E mRNA would have hybridized with all *V*-genes coding for this group. Yet we found that the group-specific V-region sequences hybridized with unique or nearly unique DNA. We conclude that at most, a few germ line *V*-genes code for some 14% of mouse κ-chain V-regions.

Estimation of gene reiteration frequency and purity by homologous competition

In Fig. 5 it can be seen that only about 55% of the labeled RNA was prevented from hybridizing. The 16% of the RNA which is in the reiterated fraction will not be competed for. Since the hybridization mixture was incubated only to C_0t = 7,920, where only 90% of the maximal hybridization level is reached (Fig. 3), the RNA which hybridized in the absence of competitor is enriched from about 16% to 18% in reiterated RNA. The rest of the uncompeted RNA can be explained by impurities. Since the impurities consist of many different species, the ratio of RNA to DNA for any individual species of contaminant is too low to allow effective competition with the amount of competing RNA used. The estimate of purity made in this way is in excellent agreement with the estimate from the fingerprint studies. Half maximal competition was reached at an RNA to DNA ratio of 1.2×10^{-6}, thus giving a nominal reiteration frequency of 5.4. This is an overestimate‡. A corrected reiteration frequency would be even closer to that estimated by time kinetics.

‡ Our nominal reiteration frequency is subject to at least three sources of error: (*a*) The rate constant for RNA·DNA hybridization may be different from that for DNA renaturation. (*b*) The purity of the *competing* RNA preparation is not 100%. (*c*) Even in the absence of competitor, the hybridization level is not maximal—in part due to limiting C_0t, in part due to lack of a large excess of DNA—so that the experimental 50% inhibition point is incorrect. Calculations to correct for these factors would be too long to give here and would not be very accurate because of the uncertainties in all of the parameters. Rough estimates indicate that the nominal reiteration frequency is about 3-fold too high.

DISCUSSION

We are aware that our results and conclusions are at variance with several published reports. Premkumar *et al.* (23) reported that a mouse heavy chain mRNA preparation contained a fraction with a reiteration frequency of 5000. The discrepancy is not due to a difference between heavy and light chains (7). While the paucity of mouse myeloma heavy chains with sequenced V-regions has prevented us from carrying out the type of detailed analysis presented above, we find that heavy chains, like light chains, contain, within the limits of experimental error, no sequences deriving from genes which are reiterated more than 1000 times. It is not clear whether the disagreement lies in the mRNA preparation or in the hybridization techniques. Be that as it may, Premkumar *et al.* simply assume that the reiterated RNA component represents the V-region sequences. The results presented in this paper make this assumption rather unlikely.

Using a mouse κ-chain mRNA preparation of unknown purity, Delovitch and Baglioni (24) observed a biphasic C_0t curve which, to a certain extent, is similar to ours. They also ignored the existence of the external section of the mRNA and assumed that the reiterated fraction represented "subclass" specific sequences. By further assuming that there are 30 "subclasses," they estimated that there are some 6000 germ line κ-chain *V*-genes. But our results show that the reiterated sequences are not, in fact, "subclass"-specific. Rather, they seem to be the same for all κ-chain mRNAs. They presumably represent, in large part, the external section (5).

The function of the external section (about 320 nucleotides) is unknown except that at least 30 to 60 nucleotides in this section must code for the extra peptide which is cleaved off before the κ-chain is secreted (25–28). A number of possibilities suggest themselves for the remaining external sequences. They might be involved in the "translocation," i.e., the possible joining of *V*- and *C*-genes. It should be noted, however, that the existence of reiterated external sections may not be peculiar to mRNAs coding for immunoglobulins (6, 29). Part of the external section sequences may play a role in transcription, messenger processing, and/or translocation, i.e., functions which would be common to most, if not all, mRNAs. This would imply that there are classes of genes which are regulated in common.

In conclusion, let us return to the question with which we started. How many κ-chain *V*-genes are there? At least part of the external section seems to be common to all κ-chain mRNAs. Since these common sequences appear to be reiterated about 200-fold, it would seem that there cannot be more than about 200 *V*-genes. On the other hand, all of our results are compatible with even a single *V*-gene. At present, our estimate must be between 1 and 200. We hope to be able to narrow this range in the near future. Whatever the outcome, however, the number of germ line genes seems to be too small to account for the observed variability of antibody V-regions.

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