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Germ Line Basis for Antibody Diversity: Immunoglobulin V_H- and C_H-gene Frequencies Measured by DNA·RNA Hybridization

(mouse myeloma/H-chain mRNA/variable region/constant region)

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ABSTRACT **The reiteration frequency for mouse immunoglobulin V_H-genes and C_H-genes has been directly estimated by hybridization of purified MOPC 315 α-chain mRNA with a vast excess of mouse DNA. A biphasic C_0t curve resulted. The low C_0t transition ($C_0t_{1/2}$ about 1.5) was interpreted as hybridization to V_H-genes and the high C_0t transition ($C_0t_{1/2}$ about 10^3) as hybridization to C_H-genes. These values correspond to about 5000 V_H-genes and less than 8 C_H-genes. This germ-line content of V_H-genes is sufficient to account for antibody diversity given a comparable set of V_L-genes. Minimal-gene models are invalidated and there is no need to invoke somatic generators of diversity.**

Immunoglobulin heavy (H) or light (L) chain structure can be explained by assuming that each chain is encoded by two genes, one variable (V)- and one constant (C)-gene (1). Heavy chains of all classes share the sme V_H-gene pool and different C_H-genes can be linked to the same V_H-gene. One implication of this two gene-one polypeptide chain hypothesis is that there is no necessity for an equal number of V- and C-genes in the germ line. This satisfies the genetic evidence for a single C-gene for each constant region while permitting multiple V-genes as implied by amino-acid sequence data. That data points to a minimum essential number of V-genes which could account for the extent of antibody diversity only if there is a superimposed somatic generation of diversity (2–5). Alternatively a larger number of V-genes could be present in the germ-line and this could easily be a sufficient number to code for the total repetoire of antibodies without invoking diversification (1, 6, 7).

A direct estimate of the reiteration frequency of a given gene can be made by hybridization of a suitable radioactive probe, either RNA or DNA, in the presence of a vast excess of DNA (8). The probe, either mRNA or a reverse transcript thereof, must be labeled at very high specific activity so that the vast excess of DNA can be achieved.

We have used as a probe the mRNA for the α-chain of mouse myeloma protein 315. The mRNA was labeled in 315 cells grown in tissue culture and was isolated by virtue of its specific interaction with immunoglobulin (refs 9 and 10, Premkumar, Stevens, and Williamson, in preparation). Hybridization of this RNA with whole mouse embryo DNA, spleen DNA, or 315 cell DNA give closely similar biphasic C_0t curves. The proportions of hybridization in the two components supported the interpretation that the low C_0t transition represents hybridization to V_H-genes and the high C_0t transition represents hybridization to C_H-genes. From the $C_0t_{1/2}$ values for each transition the reiteration frequency for V_H-genes is about 5000 and for C_H-genes the reiteration frequency is about four copies.

Abbreviations: H, heavy; L, light; V, variable; C, constant; SDS, sodium dodecyl sulfate; cRNA, complementary RNA.

MATERIALS AND METHODS

Cells. Mouse myeloma cells 315 were maintained in exponential growth at 37° in RPMI medium supplemented with 2X amino acids and 20% fetal-calf serum.

mRNA Labeling and Isolation. Myeloma cells growing exponentially at a density of 6×10^5 cells per ml were incubated with 500 μCi/ml of [^{3}H]uridine (27 Ci/mmol) and 500 μCi/ml of [^{3}H]cytidine (26 Ci/mmol) for 8 hr at 37°. The cells were then sedimented, washed, and separated into nuclei and cytoplasm (11), and the total RNA was isolated from the cytoplasmic fraction by the phenol–chloroform method of Singer and Penman (12). After phenol extraction, the RNA was precipitated with 2 volumes of ethanol overnight at −20°. The RNA precipitates were collected by centrifugation, dissolved in water, and fractionated into poly(A) containing RNA and nonpoly(A) RNA by the method of Schutz, Beato, and Feigelson (13). The poly(A) containing RNA fraction was precipitated with 3 volumes of ethanol overnight at −20°. The precipitate was collected by centrifugation, dissolved in 0.5 ml of solution A [0.5 mM $MgCl_2$–75 mM NaCl–20 mM phosphate buffer (pH 7.6)], 50 μg of 315 protein, purified by affinity chromatography on DNP-Sepharose column (14), in Solution A was added and the mixture incubated on ice for 3 min. Anti-315 antibody, purified on DEAE-Sephadex, was added at equivalence and the mixture was incubated on ice for a further 5 min (9). The precipitates formed were collected by centrifugation (3000 × *g* for 10 min) and washed three times with ice cold solution A. The precipitates were dissolved in 0.5 ml of 0.5% sodium dodecyl sulfate (SDS) solution and then diluted to 5 ml with RNA extraction buffer (0.1 M sodium acetate–0.2 M NaCl–1 mM EDTA–0.2% SDS), pH brought to 5.0 with acetic acid, and shaken with an equal volume of phenol–chloroform (3:1) mixture at 45° for 3 min. The mixture was then chilled, centrifuged, and the aqueous extract precipitated with 3 volumes of ethanol overnight at −20°. The H-chain mRNA thus obtained had a specific activity of 1.2×10^6 cpm/μg.

Sucrose Gradient Sedimentation. The RNA sample in 0.1 ml of 0.1 M phosphate buffer (pH 7.6) was layered on 5 ml of 15–30% (w/w) sucrose gradient containing 0.01 M Tris·HCl (pH 7.4)–0.1 M NaCl–0.01 M EDTA–0.1% SDS in a Beckman SW 50.1 rotor and centrifuged at 50,000 rpm for 2.5 hr.

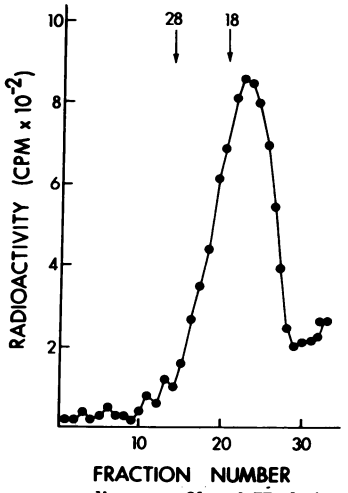

FIG. 1. Sucrose gradient profile of H-chain mRNA isolated from 315 cell cytoplasm. Isolation of the RNA and sucrose gradient analysis was done as described in *Materials and Methods*. The positions of ^{32}P-labeled 28S and 18S rRNA markers are indicated by *arrows*. The numbers on the ordinate have been multiplied by 10^{-2} in Figs. 1 and 2.

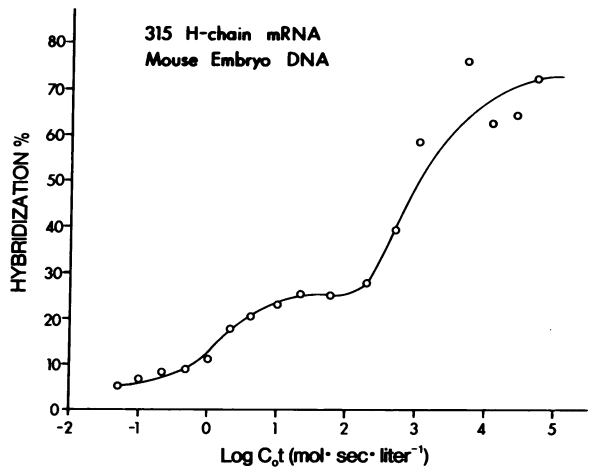

FIG. 3. Kinetics of hybridization of mouse-embryo DNA with 315 H-chain mRNA. Hybridization of mRNA with excess of mouse DNA was carried out as described in *Materials and Methods*.

Fractions were collected from the bottom of the tube and their radioactivity measured.

Isolation of DNA. Whole mouse (Swiss-Webster strain) embryo or Balb/c spleen or a pellet of 315 myeloma cells were homogenized for 30 sec in NTE buffer (0.1 M NaCl–0.02 M Tris–1 mM EDTA and 1% mercaptoethanol; pH 8.5) and concentrated SDS solution added to make it 1% with respect to SDS. The homogenate was warmed to 60° and shaken at this temperature for 10 min. It was then cooled to 25°, an equal volume of chloroform-isoamyl alcohol mixture (24:1) was added and the mixture shaken at room temperature for 15 min. It was then centrifuged in cold for 30 min at 3000 × *g*; the aqueous extract was collected, mixed with an equal volume of water-saturated phenol, and shaken again at room temperature for 15 min. The mixture was centrifuged again in cold for 30 min, and the aqueous extract precipitated with 2 volumes of cold ethanol and the DNA strands spooled on a glass rod. The DNA was then dissolved in 0.3 N KOH solution and incubated at 37° for 18 hr. The DNA solution was dialyzed in cold extensively against 1 mM EDTA solution. The DNA was fragmented to a size of 6.4S by using a Precision cell insonator. It was then precipitated with 2 volumes of ethanol, pelleted by centrifugation, and dissolved in a minimum volume of 0.01 M phosphate buffer (pH 6.8).

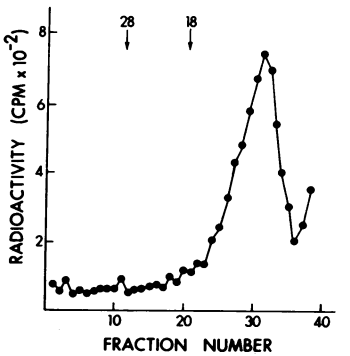

FIG. 2. Sucrose gradient profile of 315 H-chain mRNA that has been sheared by sonication prior to use for DNA·RNA hybridization studies. The positions of ^{32}P-labeled 28S and 18S rRNA markers are indicated by *arrows*.

Preparation of Escherichia coli cRNA to Homologous DNA. Highly purified *Escherichia coli* DNA dependent RNA polymerase was purchased from Miles Laboratories, Indiana. This enzyme (40 μg) and 10 μg of *E. coli* DNA were incubated together for 20 hr at 37° in 0.5 ml of reaction mixture containing 0.04 M Tris (pH 8.0), 0.01 M $MgCl_2$, 0.25 mM dithiothreitol, 0.15 M KCl, 0.25 mg of bovine-serum albumin, 0.15 mM GTP, ATP, CTP, and 0.15 mM [^{3}H]UTP (16.6 Ci/mmol). DNA was then digested with DNase (20 μg/ml for 20 min at 37°), 1 mg of yeast RNA and SDS (0.5%) was added, and the mixture was passed through a Sephadex G-50 column (1.8 × 32 cm) in TE buffer [0.02 M Tris (pH 7.4) and 1 mM EDTA] containing 0.1% SDS. The first peak was collected, extracted twice with water saturated phenol and precipitated with 2 volumes of ethanol overnight at −20°. This RNA had a specific activity of 1.9×10^7 dpm/μg.

Hybridization of ^{3}H-Labeled RNA with Vast Excess of DNA. The hybridization mixture, containing 10 mg/ml of DNA and 6000 cpm/ml of 315 H-chain mRNA (sonicated to 6–7S size) in 0.4 M phosphate buffer (pH 6.8) was heated to 100° for 5 min. in water-ethylene glycol bath and quickly transferred to a bath at 65°. The hybridization was carried out at 65° in silicone stoppered tubes. To achieve lower C_0t values in a workable time period, lower DNA and lower salt concentrations were used. Aliquots were taken out at different time intervals and cooled rapidly in an ice-water bath. The reaction mixture was diluted to a DNA concentration of 50 μg/ml with 2 × SSC (SSC = 0.15 M NaCl, 0.015 M Na citrate) and halved. One half was incubated with pancreatic ribonuclease A (15 μg/ml) and with ribonuclease T_1 (15 units/ml) for 1 hr at 37°. The other half of the sample was incubated similarly but in the absence of ribonuclease. Ice-cold trichloroacetic acid (50%) was added to give a final concentration of 5% and after incubating at 0–4° for 30 min., the precipitate was col-

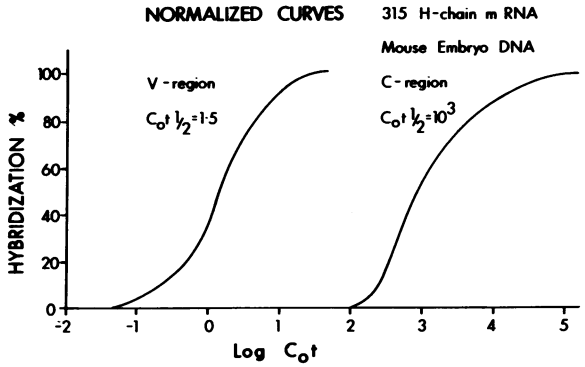

FIG. 4. Normalized kinetics of hybridization of 315 H-chain mRNA. The curves in this figure were drawn from data in Fig. 2 as described by Bishop (8) after making allowance for the fraction of RNA which did not react at very high C_0t and also the fraction of RNA which became RNase resistant in heated and quenched sample. The biphasic curve was broken into its two different components and each curve was normalized as described above.

lected on Millipore cellulose-nitrate filters, washed with 100 ml of 5% cold trichloroacetic acid, dried and counted.

Thermal Denaturation of DNA·RNA Hybrids. DNA·RNA samples hybridized to a C_0t of 100 mol·sec·liter^{-1} representing the fast hybridizing sequences and another set which hybridized between a C_0t of 200 and 10^4 mol·sec·liter^{-1} were quenched and diluted with 2 × SSC. Aliquots were taken and heated for 10 min at different temperatures followed by quenching in ice-water. Samples were further diluted with 2 × SSC to make DNA concentrations of 50 μg/ml and treated with RNase A and RNase T_1, as described earlier. They were then precipitated with trichloroacetic acid, filtered, and counted.

RESULTS

H-Chain mRNA. The method of isolation of H-chain mRNA depends on the specific interaction between this mRNA and immunoglobulin originally demonstrated for the 5563 mouse myeloma system (9, 10). We have since shown that this interaction occurs between other immunoglobulins and H-chain mRNA; α-chain mRNA isolated in this way from cytoplasm of 315 cells gave a single peak on acrylamide-gel electrophoresis and could be translated in *Xenopus laevis* oocytes yielding α-chains in the absence of detectable λ-chains (Premkumar, Stevens, and Williamson, in preparation).

For the present experiments high specific activity ^{3}H-labeled α-chain mRNA was prepared as described in *Materials and Methods.* On a sucrose density gradient this mRNA gave a single peak at 16–17S (Fig. 1). After sonication this RNA gave a symmetrical peak at 6–7S (Fig. 2), indicating that the mRNA has been sheared to approximately four fragments per molecule.

RNA·DNA Hybridization. This was performed as described in *Materials and Methods.* The necessary vast excess of DNA (approximately 10^7-fold) was achieved because of the high specific activity of the mRNA. DNA was obtained from three different sources. Whole Swiss-Webster mouse embryo was taken as a general source of DNA. Adult Balb/c mouse spleen gave us a source rich in lymphoid cells. The other source was the highly differentiated 315 plasmacytoma cells in which to check for amplification of C_H-genes or restriction of V_H-gene reiteration.

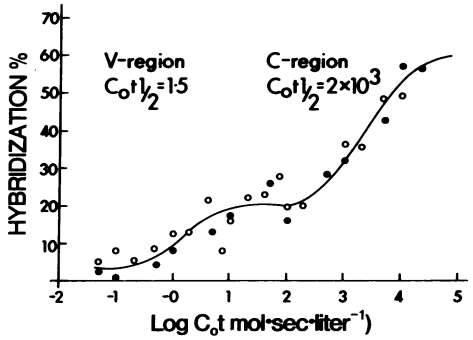

FIG. 5. Kinetics of hybridization of 315 H-chain mRNA with Balb/c spleen DNA and 315 cell DNA. The details are given in the text. Balb/c spleen DNA, O——O; 315 cell DNA, ●——●.

The C_0t curve for embryo DNA is shown in Fig. 3. It is clearly a biphasic transition curve with a maximum hybridization of about 70% at a C_0t of 50,000. The extent of hybridization in the low C_0t transition is about 20% of the input RNA or about 29% of the total hybridization achieved. This agrees well with the expected figure of about 25% of the mRNA coding for the V-region of the H-chain. We interpret this transition at low C_0t as hybridization to V_H-genes and the second transition at high C_0t as hybridization to C_H-genes. The two transitions have been normalized (Fig. 4) and the C_0t $^1/_2$ values are 1.5 and 10^3 mol·sec·liter^{-1} respectively. The C_0t $^1/_2$ value determined for *E. coli* cRNA hybridization was 20 mol·sec·liter^{-1} (curve not shown). Reiteration frequencies were calculated taking the analytical complexities for *E. coli* DNA to be 2.7 × 10^9 daltons and for "unique" sequences of mouse DNA to be 1.08 × 10^{12} daltons. This gives a value of 5300 for V_H-genes and 8 for C_H-genes.

The combined data for hybridization of α-chain mRNA with either Balb/c spleen DNA or 315 cell DNA is plotted as a single C_0t curve (Fig. 5). Both sets of points are fitted to a single biphasic-transition curve similar to Fig. 3. The definition of the low C_0t transition is less clear than in Fig. 3 where most points in that region are averages of duplicates. In the high C_0t transition there is striking agreement between the data obtained using splenic or 315 cell DNA. The two transi-

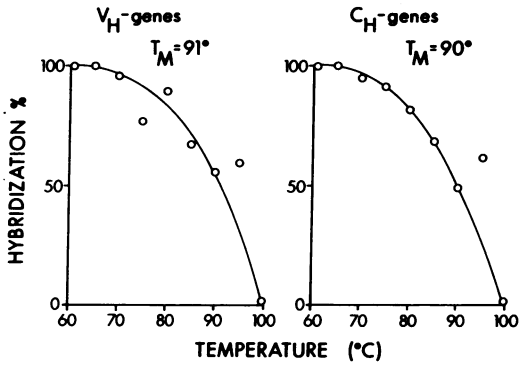

FIG. 6. Thermal denaturation profiles of DNA·RNA hybrids. Hybridization and melting of the hybrid was carried out as described in the *text.*

tions were normalized and C_0t $^1/_2$ values of 1.5 and 2×10^3 mol·sec·liter^{-1} measured. These values correspond to reiteration frequencies of 5300 for V_H-genes and 4 for C_H-genes.

Melting Temperatures (T_m) of RNA·DNA Hybrids. The thermal stability of an RNA·DNA hybrid gives an indication of the quality of sequence matching. The T_m values of the hybrids formed at low C_0t and high C_0t were measured separately (see *Materials and Methods*). The thermal stability curves shown in Fig. 6 give T_m values of 91° and 90° for the low C_0t and high C_0t hybrids respectively. This indicates equally good matching of base pairs in the two hybrids. The extent of homology between the reiterated V-genes is apparently high.

DISCUSSION

The reiteration frequencies reported here of about 5000 V_H-genes which cross hybridize with the 315 V-region sequence and about 4 C_H-genes which are homologous to C_α-region sequence formally confirms the two genes—one polypeptide chain hypothesis (1). The number of V_H-genes defined by these experiments is a minimum estimate to the extent that 315 V-region sequence fails to cross hybridize with all V_H-genes (discussed below). However a germ-line content of 5000 V_H-genes is clearly sufficient to supply the total repetoire of antibody combining sites given a comparable number of V_L-genes. If we assume that V_L and V_H regions can associate at random, then 5000 V_L-genes and 5000 V_H-genes in the germ-line will generate 2.5×10^7 antibody combining sites. In addition, each of these combining sites probably have multiple shared specificities (7, 17). Thus, the germ-line information can fully account for the extent of antibody diversity. The data also show that there is neither significant restriction in the number of V_H-genes or significant amplification of C_H-genes in the differentiated, homogeneous population of 315 cells.

Our interpretation of the two transitions in the C_0t curve in terms of V_H- and C_H-gene hybridization depends on the purity of the 315 α-chain mRNA. The specificity of the isolation procedure (9, 10) assures a high degree of purity of the mRNA and this is attested by the single peak on a sucrose density gradient (Fig. 1), a single peak on polyacrylamide gels, and translation in oocytes yielding α-chains and no λ chains. (Premkumar, Stevens, and Williamson, in preparation). The proportion of mRNA hybridized in the low C_0t transition is slightly in excess of the value predicted from the sequence of H-chains; this could be due in part to the hybridization of an additional stretch of bases at the 5′-end of the mRNA preceding the initiation codon.

The reiteration frequency for V_H-genes measured in these experiments represents only that fraction of the total V_H-gene pool which has extensive cross-homology with the 315 V_H-sequence. The stringent ribonuclease treatment after hybridization should select for hybrids having close homology. The quality of the base pairing is shown by the high T_m (91°) measured for the low C_0t hybrids. Current knowledge of amino-acid sequences of V_H-regions is compatible with the extensive cross-homology indicated by our hybridization data. All (27/27) mouse V_H-regions so far checked have an unblocked amino terminal group. These proteins seem to fall into three major subgroups (Hood, personal communication). In other species there is remarkable constancy of aminoacid sequence among a range of heavy chains belonging to the same subgroup (15,16). Further hybridization experiments should demonstrate whether mRNA coding for H-chains of one subgroup can cross-hybridize with DNA sequences coding for the H-chains of other subgroups and help us in defining the true size of the V_H-gene pool.

The question remains as to whether we may reasonably assume a pool size for V_κ-genes similar to that for V_H-genes. A recent attempt to measure V_κ-gene reiteration by hybridization gave inconclusive results (18); this was in part due to the relatively low specific activity of the mRNA used which would not afford conditions of vast DNA excess. There is, however, an inherent problem in measuring the total V_κ-gene pool size and that is the uncertainty concerning the extent of homology between V_κ regions of different subclasses.

Recent experiments measuring the number of nitro-iodo-hydroxy-phenacetyl-binding antibodies which can be made in CBA/H mice indicated a minimum number of 70 V_H-genes and 70 V_L-genes are required if all of the information is carried in the germ-line (19). This number would be comfortably contained in a V_H-pool of 5000 genes. The stable inheritance of specific V-genes can be shown using the phenotypic markers, isoelectric focusing spectrum and idiotype (7, 20–23). The overall stability of the V-gene pools remains to be established.

The current estimate of the number of C_H-genes is approximate. The important point to be made here is the very low number of C_H-genes. The doubt is mainly due to the errors of measuring hybridization at very high C_0t. Speculation at present on the existance of undefined α-chain subclasses or on cross-homology between α-chain and other classes of H chain would be premature. The extent of internal homology between C_H-domains is not sufficient to give cross hybridization (24).

The present data obviates the necessity to search for somatic generators of diversity. Questions concerning the maintainence and control of a large V-gene pool in the germ-line and the expression of V-genes by integration with C-genes can now be brought more clearly into focus. It should also now be possible to approach the problem of the evolution of the multigenic system controlling antibody diversity.

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