EVIDENCE FOR MULTIPLE IMMUNOGLOBULIN GENES[1]

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SUMMARY

RNA/DNA hybridization was used to quantitate immunoglobulin (Ig) genes. The RNA was prepared from microsomes of mouse myeloma cells and further purified by selecting poly A containing (poly A +) species. (^{125}I) labeled myeloma microsomal poly A + RNA (mainly Ig κ-chain mRNA) was hybridized in solution with an excess of mouse DNA. Biphasic hybridization kinetics showed that a significant portion of the hybrids were formed at low Cot indicating reaction with about 2,500 to 5,000 copies in the DNA. Krebs microsomal poly A + RNA, and myeloma postmicrosomal poly A + RNA, did not show a rapidly hybridizing phase. The data suggest the existence of a large number of immunoglobulin genes per haploid genome, enough probably to satisfy germ line theories of antibody synthesis.

INTRODUCTION

Since partially purified mRNA extracted from the microsomes of Ig L-chain producing myeloma tumors can be faithfully translated into L-chains in cell free systems (1,2), it is likely that structural genes exist for all Igs. Thus, many thousand different Ig genes would be required to code for the multitude of chemically different antibodies which an individual can synthesize (3). It is unknown whether every cell contains all Ig genes, as postulated by the germ line theories (4), or whether the variability is created somatically so that different cells would have different Ig genes, and a given cell would possess only a small number of them (5,6,7).

In filter hybridization experiments I had found evidence for approximately 10^4 Ig genes (8). I am reporting here a quantitation of Ig genes using hybridization in solution with DNA in vast excess. Poly A + RNA from myeloma cells was used as a source of Ig mRNA and its hybridization kinetics were compared with those of RNAs which are devoid of Ig mRNA.

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[1]Some of the results were presented at the 57th FASEB meeting, April, 1973.

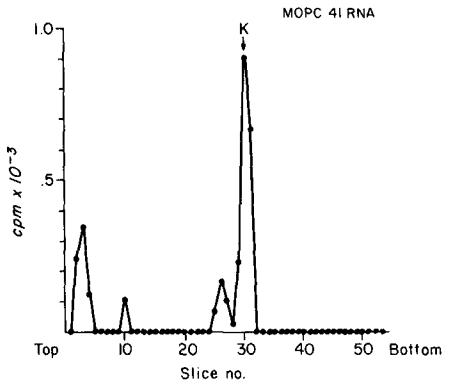

Figure 1. SDS-polyacrylamide gel electrophoresis of proteins synthesized by wheat germ cell free system with MOPC-41 microsomal poly A + RNA. 100 µl incubation mix contained: 60 µl wheat germ extract, 0.2 mM GTP, 1 mM ATP, 5 mM creatine phosphate, 0.015 mg creatine phosphokinase, 40 mM of 19 amino acids (minus serine), 5 µC (^{3}H)-serine (at 2 C/mM, NEN), 45 mM KCl, 3.8 mM Mg Ac, 13 µg MOPC-41 RNA. 100 µl incorporated 23 pmole (^{3}H)-serine into TCA precipitable radioactivity. cpm of endogenons background were subtracted.

METHODS

Tumors. MOPC-41 myeloma tumors were serially passaged in Balb/c mice as solid subcutaneous tumors. Krebs II ascites tumor was serially passaged in outbred Swiss mice.

Preparation of DNA and RNA. DNA was extracted from myeloma tumors or Krebs ascites cells (8). Microsomes were prepared from solid myeloma tumors (8,9) or from Krebs ascites cells (10). RNA was extracted by the hot phenol method in pH 5.2 Na acetate (8). Poly A + RNA was isolated from total microsomal and postmicrosomal RNA by chromatography on poly U Sepharose (11). Poly A + RNAs and E. coli RNA were iodinated with (^{125}I) (12).

Cell free protein synthesis. MOPC-41 microsomal poly A + RNAs were translated in a cell free system of a wheat germ cytoplasmic extract (13).

RNA/DNA hybridization in DNA excess. DNAs were sheared in a pressure cell at 12,000 psi. The sheared DNAs sedimented in alkaline sucrose gradients as a sharp, symmetrical peak of 9S as determined by comparison with a reference marker of linear polyoma DNA molecules included in the same gradient; the single stranded molecular weight was calculated from this to be 380,000 daltons (14). RNA/DNA hybridization was done essentially as described by Melli et al. (15). Sheared DNA was mixed with iodinated RNA at DNA/RNA ratios 1.1×10^4 to 2.4×10^5 in 0.24 M phosphate pH 6.8 (PB). The mixtures were heat denatured for 7 mins at 95°C and then immediately placed into a water bath at the hybridization temperature. After incubation for various times at 67°C the samples were rapidly cooled and frozen. To check for the % hybrid formation, samples were diluted 10 to 20 x in 0.24M PB and incubated for 25 mins at 37°C with or without 20 µg/ml of pancreatic ribonuclease, cooled and precipitated with 8% TCA without added carrier, and the precipitates were scintillation-counted. For the determination of background RNAase resistant radioactivity, hybridization mixtures were frozen immediately after heat denaturation.

Thermal dissociation of RNA/DNA hybrids in solution. After incubation the DNA-RNA mixtures were diluted 30 x with ice cold 0.24 M PB. Each sample was divided into 20 equal aliquots. Duplicate tubes were heated for 10 mins at 5°C intervals between 65°C to 100°C and then immediately cooled in ice. All samples were RNAsed, precipitated with TCA and counted as above.

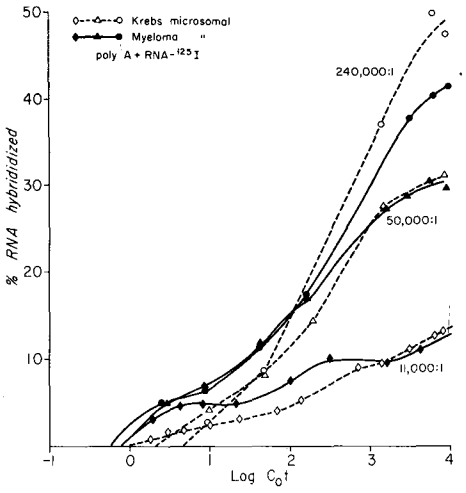

Figure 2. RNA/DNA hybridization in DNA excess. Myeloma or Krebs DNA was hybridized with (^{125}I) RNA at overall DNA/RNA ratios of 11,000 (◆ MOPC-41 microsomal poly A + RNA, ◇ Krebs microsomal poly A + RNA), 50,000 (▲ MOPC-41, △ Krebs), and 240,000 (● MOPC-41, ○ Krebs). Background RNAse resistant radioactivity was subtracted from all values.

RESULTS

Isolation of κ-chain mRNA. MOPC-41 microsomal poly A + RNA shows a major peak between 12S to 16S in sucrose gradients, and two minor peaks at 18S and 28S, probably contaminating rRNA (data not shown). In cell free protein synthesis the RNA stimulates the production of one major protein which tracks with MOPC-41 urinary κ-chains on SDS-gel electrophoresis and three minor peaks, which have not been identified (Fig. 1). 84% of the cell free product is specifically precipitated by anti κ-chain antibodies. The majority of the translatable RNA is therefore κ-chain mRNA.

Hybridization of myeloma microsomal mRNA with myeloma DNA in excess. Figure 2 shows the hybridization of iodinated myeloma microsomal poly A + RNA and of Krebs microsomal poly A + RNA with varying amounts of myeloma or Krebs DNA. Both RNAs hybridized increasingly well as the DNA/RNA ratio increased, indicating that iodination did not alter the base pairing capacity of the RNA. The myeloma RNA shows biphasic hybridization kinetics not seen with the Krebs RNA. Figure 3 compares the hybridization of myeloma microsomal poly A + RNA

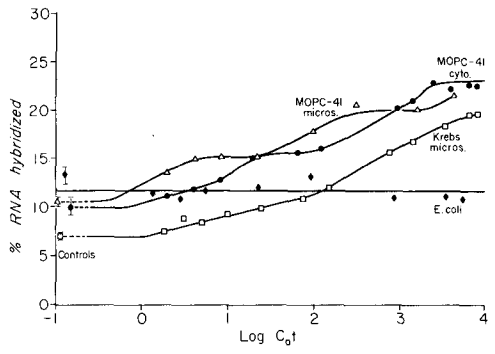

Figure 3. RNA/DNA hybridization in DNA excess. -△- MOPC-41 microsomal poly A +RNA (2.7×10^6 cpm/µg), a 500-fold excess of total Krebs RNA, and myeloma DNA; -●- MOPC-41 postmicrosomal cytoplasmic poly A + RNA (5×10^5 cpm/µg), a 500-fold excess of total Krebs RNA, and myeloma DNA; -□- Krebs microsomal poly A + RNA (10^7 cpm/µg), 200-fold excess of MOPC-104E myeloma RNA and Krebs DNA; -◆- E. coli RNA (2.1×10^7 cpm/µg) and myeloma DNA; DNA/RNA ratios of 11,300 in 0.24 M PB at 67°C. The "controls" indicate background RNAse resistant radioactivity obtained in hybridization mixtures which were cooled immediately after heat denaturation.

with various control RNAs at DNA/RNA ratios of 11,000. In order to prevent the reaction of any remaining rRNAs and tRNAs in the (^{125}I)-poly A + RNA, the hybridizations were carried out in the presence of a 200- to 500-fold excess of unlabeled RNA; unlabeled Krebs RNA was added to labeled myeloma RNA, and vice versa. The background RNAse resistant radioactivity in the hybridization mixtures was between 7 and 13%. DNA renaturing in the course of a hybridization experiment did not mechanically trap and protect (^{125}I)-RNA from RNAse action since the RNAase resistance of iodinated E. coli RNA in the presence of myeloma DNA did not increase during extended incubation times (Fig. 3). Myeloma microsomal poly A + RNA shows a rapidly hybridizing phase with a Cot[2] 1/2 of about 1 to 2. No such rapid phase was seen with Krebs microsomal poly A + RNA or poly A + RNA of myeloma postmicrosomal cytoplasm.

The thermal stabilities of the hybrids with myeloma microsomal poly A + RNA are shown in Fig. 4. Hybrids formed up to a Cot of 100 had a T_m of 79°C, hybrids formed up to a Cot of 4,700 had a T_m of 87°C.

[2]Cot = product of DNA concentration x incubation time expressed as mole nucleotide per litre x sec.

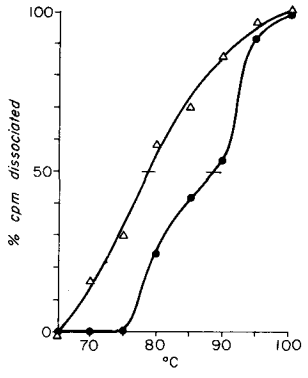

Figure 4. Thermal dissociation of RNA/DNA hybrids formed in DNA excess. Labeled myeloma microsomal poly A + RNA and unlabeled Krebs RNA were hybridized with DNA to Cot 100 (-△-) or Cot 4,700 (-●-). The hybrids were melted as described under "Methods".

DISCUSSION

Poly A + microsomal RNA from MOPC-41 myeloma cells was used in the present study as a source of κ-mRNA, since the main product of its cell free translation is κ((2) and this study). However, the RNA does contain some other species besides κ-mRNA ((16) and Fig. 1) and although the wheat germ system seems to translate all mRNAs (B. Gallis, pers. comm.), the presence of untranslatable mRNAs can not entirely be ruled out.

I have not studied the hybridization of mRNA transcribed from a unique gene, however, from the data of others (17,18) one can infer, that a unique sequence of the mouse genome would hybridize with RNA at a Cot 1/2 of about 5,000. In light of this, the data in Figs. 2 and 3 indicate that a portion of the myeloma microsomal RNA hybridized with DNA sequences which have about 2,500 to 5,000 copies (Cot 1/2 = 1-2) in the haploid genome. I wish to consider that these hybrids may consist of DNA and mRNA for sequences within V_{κ}-regions. Poly A could not have caused hybrids at low Cot (or at any Cot), since adenine is not iodinated (12), the samples were RNAsed, and the other poly A + RNAs did not show a rapid phase. It is also unlikely that the low Cot hybrids were due to RNA species common to membrane bound ribosomes in general, or to myeloma RNA species which might contaminate the microsomes, or to

short sequences common to all genes (19), since Krebs microsomal RNA and myeloma postmicrosomal RNA did not hybridize rapidly. rRNAs and tRNAs contaminating the (^{125}I)-κ-mRNA would not have hybridized in the experiments of Fig. 3, because of the excess of competitor RNAs. Furthermore, it has been generally found that, with the exception of histone mRNAs, the majority of other cellular mRNAs are transcribed from rare DNA sequences (20). On the other hand, I have always found this rapid phase when myeloma microsomal poly A + RNA (MOPC-41; P_3K ($\gamma_1\kappa$) and MPC-11 ($\gamma_2\kappa$) (data not shown)) was hybridized. Assuming that the rapidly forming hybrids are due to v_κ-sequences, they can not represent the whole v-region, because only a low percentage of RNA binds at low Cot. However, the data are compatible with the known amino acid sequences of a large number of v_κ-chains (21,22), which have regions of similar amino acid sequences interspersed with variable stretches. Since a sequence of at least 50 nucleotides (23) with at least 80% homology (24) is the lower limit for stable hybrid formation at 67°C, nearly identical stretches of at least 17 amino acids are required to allow the hybridization of a given κ-mRNA with sequences in most κ-genes. Such stretches exist in v_κ-chains. The partial mismatch of the low Cot hybrids is demonstrated in Fig.4 by a relatively low T_m (79°C) as compared with the T_m of native mouse DNA (90°C in 0.24 M PB), i.e. the low Cot hybrids must have been mismatched at about 7 to 10% of the base positions (25). Most RNA stretches with partial homology to most κ-genes would have hybridized independently of other κ-mRNA stretches, since by iodination and thermal fission the RNA had been degraded to pieces smaller than v-region mRNA (data not shown). This would explain the hybrids at higher Cots as due to κ-DNA sequences present in fewer and fewer v_κ-genes (Cot 20-1,000) or only in the gene homologous to $\kappa_{MOPC-41}$ (Cot >1,000). Genetic studies suggest that each constant region (c) gene exists only as a single copy per haploid genome. The data seem to support this theory, because if c_κ-genes were present in a large number of copies, low Cot hybrids of a perfect match should be formed. Part of the hybrids formed

at high Cot values are probably due to c_κ mRNA and part to contaminating mRNAs for other proteins.

Delovitch and Baglioni (26) in similar experiments had not seen a rapid hybridization phase. This was probably due to contamination of their RNA by other cytoplasmic RNAs which were transcribed from rare or unique DNA sequences. They have now recently also found a rapid phase of hybridization with the use of myeloma microsomal poly A + RNA. (Delovitch, pers. comm.).

In conclusion, the evidence presented here suggests the presence in mouse cells of 2,500 to 5,000 v_κ-genes related to but not identical with $v_{MOPC-41}$, i.e. probably enough to satisfy germ line theories (27). The same results were obtained with Krebs DNA, making somatic gene amplification unlikely (Storb, unpublished). More definitive experiments with hybridization-competition of RNAs from myelomas which produce immunoglobulins of known amino acid sequences are in progress.

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REFERENCES

1. Stavnezer, J. and Huang, R.C., Nature New Biol. 230, 172, (1971).
2. Swan, D., Aviv, H. & Leder, P., Proc. Nat. Acad. Sci. 69, 1967, (1972).
3. Jerne, N.K., C.S.H. Symp. Quant. Biol. 32, 591, (1967).
4. Dreyer, W.J., Gray, W.R. & Hood, L., C.S.H. Symp. Quant. Biol. 32, 353, (1967).
5. Smithies, O., Science 157, 267, (1967).
6. Brenner, S. & Milstein, C., Nature 211, 242, (1969).
7. Gally, J.A. & Edelman, G.M., Nature 227, 341, (1970).
8. Storb, U., J. Immunol. 108, 755, (1972).
9. Storb, U. & Martin, T.E., Biochim. Biophys. Acta 281, 406, (1972).
10. Storb, U., Biochem. Biophys. Res. Comm. 52, 1483, (1973).
11. Firtel, R.A., Jacobson, A., and Lodish, H.F., Nature New Biol. 239, 225, (1972).
12. Tereba, A. & McCarthy, B.J., Biochem. (1973) (in press).
13. Shih, D.S. and Kaesberg, P., Proc. Nat. Acad. Sci. 70, 1799, (1973).
14. Studier, F.W., J. Mol. Biol. 11, 373, (1965).
15. Melli, M., Whitfield, C., Rao, K.V., Richardson, M. & Bishop, J.O. Nature New Biol. 231, 8, (1971).
16. Aviv, H., Packman, S., Swan, S., Ross, J. and Leder, P., Nature New Biol. 241, 174, (1973).

17. Bishop, J.O., Pemberton, R. and Baglioni, C., Nature New Biol. 235, 231, (1972).
18. Harrison, P.R., Hill, A., Birnie, G.D. and Paul, J., Nature 239, 219, (1972).
19. Dina, D., Crippa, M. and Beccari, E. (1973) Nature New Biol. 242, 101, (1973).
20. Greenberg, J.R. and Perry, R.P. (1971) J. Cell Biol. 50, 774, (1971).
21. McKean, D., Potter, M. and Hood, L., Biochem. 12, 760, (1973).
22. Wu, T. and Kabat, E.A. J. Exp. Med. 132, 211, (1970).
23. Gillespie, D. and Spiegelman, S., Bacteriol. Proc. G 112, (1966).
24. McCarthy, B.J. and Church, R.B. Ann. Rev. Biochem. 39, 131 (1970).
25. Ullman, J.S. and McCarthy, B.J., Biochim. Biophys. Acta 294, 416, (1973).
26. Delovitch, T.L. and Baglioni, C., Proc. Nat. Acad. Sci. 70, 173, (1973).
27. Hood, L. and Talmage, D.W. Science 168, 325, (1970).