HYBRIDIZATION STUDIES WITH AN ANTIBODY HEAVY CHAIN mRNA

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1. Introduction

In a previous report we presented evidence which indicated that antibody diversity is generated in large part by somatic processes [1]. That evidence was based on DNA–RNA hybridization studies with a purified mouse κ-chain mRNA. The present report deals with similar studies using a preparation of mRNA coding for a mouse heavy chain. The results confirm and extend our previous finding, namely there are a very limited number – possibly a single copy per subgroup – of genes coding for the variable region of immunoglobulin polypeptides.

2. Methods

mRNA coding for a mouse γ2b chain was purified from the plasmacytoma MPC 11 by a modification of the method previously described for mouse κ chain mRNA's [2]; details will be reported elsewhere. The final step of the purification was polyacrylamide gel electrophoresis in 99% formamide as described by Gould and Hamlyn [3].

The methods for RNA iodination, DNA purification, and DNA–RNA hybridization were as described in our previous report [1].

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3. Results

3.1. *Preparation of γ2b-chain mRNA:*

Fig. 1 shows the acrylamide gel electrophoresis pattern of MPC 11 16S RNA which had been purified from membrane bound polysomes by repeated poly dT-cellulose chromatography and sucrose density gradient centrifugation. Approximately one-third of the input material migrated as a single major band (band H) with a mobility very similar to that of 18S ribosomal RNA (rRNA). The rest of the material was distributed on both sides of the major band, but no distinct banding was observed. The heavy chain mRNA activity was assayed in a cell-free translation system of M. Schreier and T. Staehelin. The activity was associated almost exclusively with band H. The molecular weight of band H RNA was estimated using MPC 11 and *E. coli* rRNA's as the migration standards. We obtained 6.5×10^5 daltons which corresponds to about 1910 nucleotides.

RNA was eluted from a 2 mm thick gel slice cut out of the center of band H, iodinated and used in the hybridization studies.

3.2. *Hybridization of γ2b-chain mRNA*

The hybridization of mouse liver DNA with band H RNA and with 18S rRNA is shown in Fig. 2. The Cot curve for the mRNA is composed of two components. The major component hybridizes with an apparent $Cot_{1/2}$ of 1259. Taking *E. coli* cRNA as a standard, we obtained a nominal reiteration frequency of 4 for the major component.

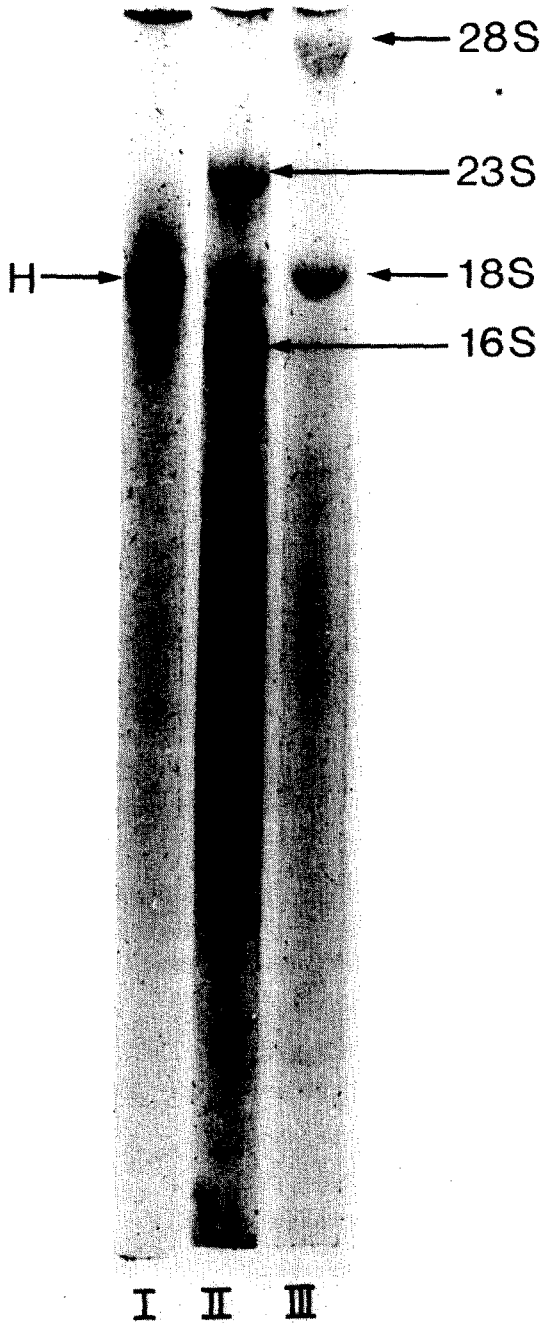

Fig. 1. Polyacrylamide gel electrophoresis of MPC 11 16S RNA in 99% formamide. Gels were stained by Pyronin Y. Gel I: 10 μg 16S MPC 11 RNA. Gel II: 17 μg 16S and 23S *E. coli* mRNA (Miles Laboratory). Gel III: 15 μg 18S and 28S MPC 11 rRNA.

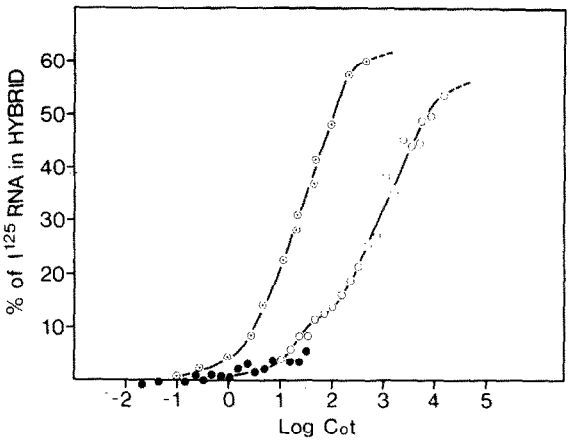

Fig. 2. Hybridization of MPC 11 band H RNA or 18S rRNA with mouse liver DNA. ○—○: MPC 11 band H RNA vs. mouse liver DNA; Co = 22.0 mg/ml; DNA/RNA ratio, 1.1×10^7. ●—●: MPC 11 Band H RNA vs. mouse liver DNA; Co = 0.223 mg/ml; DNA/RNA ratio, 5.6×10^4. ⊙—⊙—⊙: 18S rRNA vs. mouse liver DNA; Co = 2.2 mg/ml; DNA/RNA ratio 5.5×10^5. Intrinsic RNase resistancies subtracted are 3.9%, and 4.3% for band H RNA and 18S rRNA respectively.

As discussed previously [4], this is an extreme upper limit and can be interpreted to mean that the major component consists of unique sequences. Using the same standardization, we obtained a reiteration frequency of 250 for the 18S rRNA gene, which is in good agreement with reported estimates [4].

About 13% of the input RNA hybridized with a $Cot_{1/2}$ of approximately 18, indicating a gene reiteration frequency of 290. Since band H RNA migrates indistinguishably from 18S rRNA in the electrophoresis system used, the possibility that some of the low Cot component is due to contaminating rRNA was explored by competitive hybridization studies. In the presence of a large excess of unlabelled 18S rRNA, the fraction of the low Cot RNA was reduced to 10%. Thus, correcting for the maximal hybridization level of 18S rRNA (67% in fig. 2), band H RNA is contaminated by 4.5% of this RNA.

As shown in fig. 2, in the range of Cot values corresponding to a gene reiteration frequency of 1000 or more, we obtained no more than 3% of the input RNA as hybrid. Since even homogeneous

preparations have a breadth of about two decades, this is about the fraction expected from the transition at $Cot_{1/2}$ = 18. Essentially no RNA hybridized with Cot values corresponding to gene reiteration frequencies of 5000 and higher.

3.3. *Competition experiments*

I^{125}-labeled band H RNA was also hybridized in the presence of varying amounts of unlabeled homologous RNA (fig. 3). About 50% of the labeled RNA was prevented from hybridizing in the presence of the maximum amount of the competitor used. The amounts of competitor used were such that only unique or nearly unique sequences would be competed. The ratio of RNA to DNA at which a half maximal competition was reached is a measure of gene reiteration frequency independent of the one obtained from the Cot curve. An apparent reiteration frequency of 6.5 is obtained from fig. 3. This value is reduced to 2–1 after various corrections have been made [1]. Hence, the reiteration frequencies determined by this method agrees rather well with that determined from the Cot curve. Of the other 50% of I^{125}-labeled RNA which was not competed out, about 23% can be accounted for by the low Cot RNA component. The rest of the uncompeted RNA (about 27%) is attributed to many different species of mRNA which apparently contaminate the heavy chain mRNA preparation. These mRNA species hybridize with mouse DNA with a high Cot value and, therefore, are composed of unique sequences. They are, however, not competed significantly because the ratio of RNA to DNA employed here is too low for any of the individual RNA species.

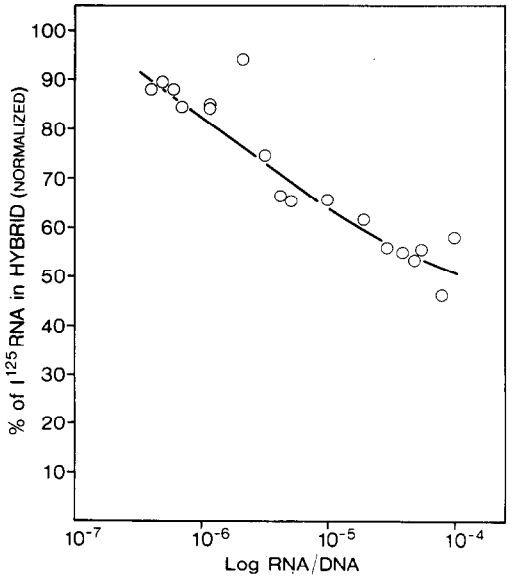

Fig. 3. Competition hybridization of I^{125}-labeled MPC 11 band H RNA with mouse liver DNA in the presence of unlabeled MPC 11 band H RNA. All samples were incubated to Cot = 11 200; intrinsic RNase resistancy 3.5%. The data are normalized against that hybridization level obtained without competitor (49.4%).

4. Discussion

Before considering some of the complexities of hybridization experiments with mRNA molecules coding for immunoglobulin chains, we wish to call attention to one major point which is obvious from a glance at the curves. The results obtained here with heavy chain mRNA are essentially similar to those obtained previously with light chain mRNA. Both results indicate that there are a quite limited number of genes which code for immunoglobulin chains.

The kinetics of hybridization (fig. 2) demonstrates that about 77% of the RNA is complementary to unique DNA sequences, while the remainder (23%) seems to be derived from genes which are reiterated about 300 times. These figures, however, refer to the entire band H RNA preparation. Before we can draw conclusions about the heavy chain messenger, we must correct for the presence of impurities. The homologous competition experiment (fig. 3) gives us an estimate of purity. This experiment suggests that about 50% of the RNA consists of a single species of unique sequences. The remainder (27%) of the unique sequences is attributable to contamination of many different species of mRNA, each of which constitutes a very small fraction of the preparation. It is more difficult to estimate the purity of the reiterated fraction with confidence. Although our purification procedure strongly selects against RNA species which lack poly A (i.e. non mRNA), an obvious possible impurity is 18S ribosomal RNA (fig. 1), and indeed we could show that the reiterated

fraction includes about 5% rRNA. It is possible, of course, that the contaminating mRNA species also contribute to the reiterated fraction. Even though Rabbitts et al. [5] have shown that this is generally the case for mRNA molecules, we have made no attempt to allow for other impurities in the reiterated fraction since we are uncertain how large the correction should be. Note, however, that because of this our final estimate of 23% for the reiterated fraction of the heavy chain mRNA molecule itself is an upper limit rather than an unbiased estimate.

In the above discussion we have tacitly assumed that the major component is the heavy chain mRNA molecule. While the kinetic experiments demonstrate that there exists such a major component, they cannot, of course, identify the component. The purification procedure, involving repeated poly-dT cellulose chromatography ensures that the major component is an mRNA molecule. Yet the only mRNA actively detected in the band H RNA is that for immunoglobulin heavy chain. Hence it can be concluded with some confidence that the major component is indeed the heavy chain mRNA molecule.

The heavy chain mRNA molecule is composed of four sections: the poly-A section which is unlabeled in our experiments, the two sections which code for the variable and constant regions of the heavy chain, and the remainder of the molecule which we call the 'external' section. We can only speculate about the role of the external section but note that it need not consist of one contiguous stretch of nucleotides. The nucleotide sequences of the heavy chain messenger may also be partitioned into two fractions: a majority fraction which hybridizes with unique (or nearly so) DNA sequences, and a minority fraction which hybridizes with DNA sequences that are reiterated about 300 times per genome. These two ways of characterizing the heavy chain mRNA molecule are diagramatically illustrated in fig. 4. Unfortunately, the present experiments do not permit an unambiguous assignment of each of the three labeled sections to the unique or reiterated fractions. However, the striking similarity of these results with those of the light chain mRNA leads us to suggest a similar interpretation. That is, the section coding for the C-region and most of that encoding the V-region are included in the unique fraction, while

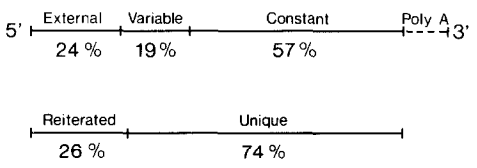

Fig. 4. Diagramatical representations of heavy chain mRNA. The bottom line represents the putative major component of band H RNA, divided into two sections according to the reiteration frequency of corresponding DNA sequences (see text). The top line represents the partitioning of the heavy chain mRNA molecule into four functional sections based upon a molecular weight determined in polyacrylamide gel electrophoresis (see text). According to this determination the molecule is composed of about 1.90 nucleotides. The poly A stretch at the 3′ terminus was assumed to contain 200 nucleotides. The sizes of the two sections coding for variable and constant regions were calculated from approximate numbers of amino acids in these regions of heavy chains. The remaining nucleotides were assigned to the external region. The nucleotides content of each of the latter three sections are expressed as per cent of the labeled sections, i.e. not including the poly A stretch.

the reiterated fraction consists mostly of the external section.

Although there exists a large body of genetic evidence to the contrary, there has until now been no direct proof that entire immunoglobulin chain genes (i.e. C- and V-regions) are not highly reiterated in the genome. Such a scheme would obviate the necessity for any mechanism ('translocation') for joining V- and C-region genes. Our results, however, clearly demonstrate that the C-region gene is not highly reiterated.

More important, however, is the fact that our DNA–RNA hybridization experiments provide no evidence whatsoever for the thousands of V-region genes which would be necessary if the entire repertoire of antibody specificities were present in all cells. This is true for the light [1] as well as for the heavy chain. This emphasizes the importance of searching for somatic generators of antibody diversity [6].

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