Hybridization Studies with Nucleic Acids from Murine Myeloma. Comparison of Pulse-Labeled Ribonucleic Acid Prepared from Four Different Tumors†

K. Kosky and R. G. Krueger*

ABSTRACT: Pulse-labeled RNAs were prepared from four different murine myelomas and normal mouse and spleen tissue. Quantitative and qualitative differences in the RNA populations were compared by competitive hybridization of various RNA preparations with tumor and liver DNA. Tumor cells contain RNA populations not present in normal mouse liver or spleen. Differences in the RNA populations in the different tumors were maximized if the reaction of the labeled RNA and DNA was carried out at low RNA/DNA ratios under moderately stringent reaction conditions. In the competition experiments, the effectiveness of each unlabeled RNA preparation as competitor appeared to be related with the type of immunoglobulin produced by each tumor. A comparison of nuclear and cytoplasmic tumor RNAs suggests that the major portions of the RNA populations studied were mRNA species present in both the nucleus and cytoplasm of tumor cells.

Since the amino acid sequence of the light and heavy chains of a given immunoglobulin or antibody molecule is specified by genes in the genome, an immunoglobulin-producing cell responds to antigenic stimulus by synthesizing specific messenger RNAs. In principle, such RNA transcripts may be identified by RNA–DNA hybridization experiments assuming sufficient specificity of the reaction conditions. However, because of the incidence of partially related base sequences in mammalian DNA (Britten and Kohne, 1968), RNA–DNA hybridization reactions may not display locus specificity if the reaction conditions are not sufficiently specific.

RNA–DNA hybridization studies have revealed the presence of antigen-induced changes in mouse spleen RNA (Church *et al.*, 1968) and an increased diversity and antigen-directed specificity of RNA molecules synthesized in mouse spleen and peritoneal lymphocytes (Cohen, 1967; Raska and Cohen, 1968). Greenberg and Uhr (1967) presented evidence that the genomes of three murine myelomas were different. Recently Storb (1972) used this technique in an attempt to quantitate the number of immunoglobulin genes in immune spleen DNA.

The previous paper (Krueger and Kosky, 1973) described the stoichiometry and kinetics of the reaction of pulse-labeled myeloma RNA with homologous and heterologous tumor DNA and the nature of the hybrids. The data suggested that the DNAs of various tumors were different in those segments of the genome from which some of the pulse-labeled RNA was transcribed. The present report describes hybridization competition studies with nucleic acids from four different myelomas. The data indicate that the RNA transcripts from these tumors are distinguishably different.

Materials and Methods

The materials and methods used here have been described in the previous paper (Krueger and Kosky, 1973) with the exception of those described below.

Competition Experiments. The desired mixtures of pulse-labeled and unlabeled RNAs were prepared in 2 × SSC[1] and 50% formamide and heated to the reaction temperature (37°) before the addition of the DNA filter. The reaction mixtures were incubated at 37° for 16 hr. After annealing, the DNA filter was washed once with 2 × SSC containing 50% formamide at the incubation temperature, once with 2 × SSC at the incubation temperature, and twice with 1 × SSC before being dried and counted in a Packard Tri-Carb liquid scintillation counter to 2% error. The input amount of labeled RNA was calculated from 10% trichloroacetic acid precipitable counts of parallel RNA mixtures carried through the reaction conditions without DNA filters. The background hybridization for each reaction mixture was determined with *Bacillus subtilis* filters in a parallel series of reaction mixtures. Background hybridization was normally less than 0.4% of the input RNA.

Criteria used in setting up competitive hybridization reactions were: (1) the ratio of competing RNA to labeled RNA was high enough so that the labeled RNA represented a negligible fraction of the total. Competing RNA is not necessarily synonymous with total unlabeled RNA since a large part of the unlabeled RNA may be unrelated to the labeled RNA and not participate in the reaction. (2) The amount of competing RNA was sufficient to saturate all of the sites of mutual complementarity (as defined by the specificity of the reaction conditions) in the amount of DNA used (Shearer, 1969).

Preparation of Nuclear and Cytoplasmic RNA. Nuclear and cytoplasmic cell fractions were prepared by a modification of the procedure of Shearer and McCarthy (1967). A suspension of *in vivo* tumor cells, prepared as described before (Krueger and Kosky, 1973), was centrifuged and the cell pellet was re-

† From the Department of Microbiology, University of Washington, School of Medicine, Seattle, Washington 98105. *Received June 22, 1972.* This research was supported by Grant No. E546 from the American Cancer Society and U. S. Public Health Service Grant No. CA-13333. We wish to thank Dr. B. J. McCarthy for many helpful discussions.

* Address correspondence to this author at: Department of Microbiology and Immunology, Mayo Medical School, Mayo Foundation, Rochester, Minn. 55901.

[1] Abbreviations used are: SSC, standard saline–citrate.

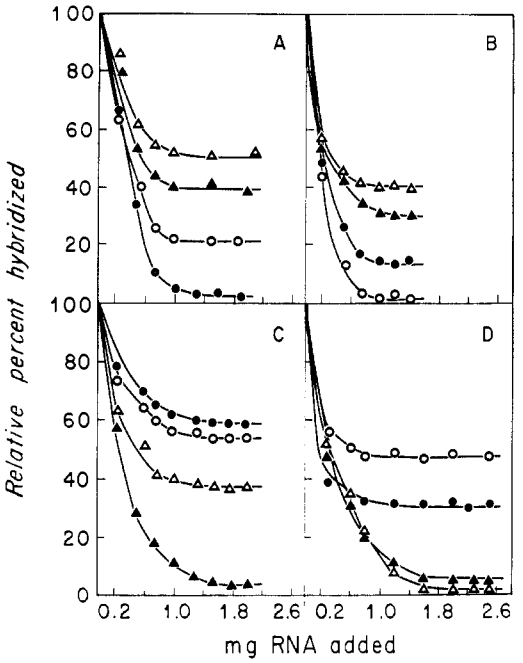

FIGURE 1: Competition by unlabeled RNA from different myelomas in the hybridization of (A) 5.8 μg of labeled MOPC-173D RNA (250 cpm/μg) and 11.6 μg of MOPC 173 DNA; (B) 7.0 μg of labeled ADJ-PC5 RNA (235 cpm/μg) and 14.0 μg of ADJ-PC5 DNA; (C) 4.1 μg of labeled MOPC-104E RNA (290 cpm/μg) and 8.2 μg of MOPC-104E DNA; (D) 6.4 μg of labeled MOPC-46B RNA (288 cpm/μg) and 12.8 μg of MOPC-46B DNA. Incubation conditions consisted of 0.25 ml of 2 × SSC and 50% formamide incubated at 37° from 20 hr in the presence of increasing amounts of unlabeled RNA: MOPC-173D (●), ADJ-PC5 (○), MOPC-104E (▲), MOPC-46B (△), Per cent reaction without competition was (A) 7.1, (B) 7.2, (C) 7.5, and (D) 7.6%.

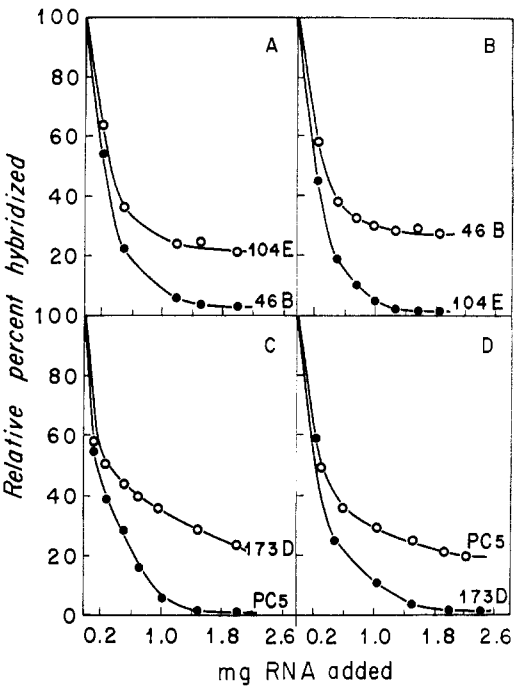

FIGURE 2: Competition by unlabeled RNA from different myelomas in the hybridization reaction of (A) 5.8 μg of labeled MOPC-104E RNA (290 cpm/μg) with 11.6 μg of MOPC-46B DNA; (B) 6.4 μg of labeled MOPC-46B RNA (288 cpm/μg) with 12.8 μg of MOPC-104E DNA; (C) 5.8 μg of labeled MOPC-173 RNA (250 cpm/μg) with 11.6 μg of ADJ-PC5 DNA; (D) 7.0 μg of ADJ-PC5 RNA (235 cpm/μg) with 14.0 μg of MOPC-173D DNA. Incubation conditions as described for Figure 1 except D where the reaction volume was 0.4 ml. Per cent reaction without competition was (A) 4.1, (B) 4.4, (C) 4.5, and (D) 4.7%.

suspended in 0.25 M sucrose, 0.15 M NaCl, and 0.1% Tween 80 and incubated for 5 min at 37°. The cells were ruptured by 10–15 strokes of a tight fitting Dounce homogenizer and nearly all nuclei were free of cytoplasmic tags when viewed by phase microscopy. The homogenate was layered over 2.2 M sucrose and centrifuged at 200*g* for 5 min, and the cytoplasm was removed and recentrifuged over 2.2 M sucrose. The nuclear pellicle was washed by slowly flowing 0.5 M sucrose down the side of the centrifuge tube to lift the remaining cytoplasm. The nuclei were resuspended in 0.25 M sucrose and recentrifuged over 2.2 M sucrose three times or until no trace of cytoplasm was seen when examined by phase microscopy. If the nuclei contained any trace of remaining cytoplasm they were treated with 0.05% Nonidet-P40 for 5 min at room temperature. The nuclei were layered over 2.2 M sucrose, centrifuged at 500*g* for 5 min, and washed with 0.5 M sucrose as before. The nuclear pellet was resuspended in distilled H_2O.

RNAs from the two cell fractions were isolated and purified as described previously (Krueger and Kosky, 1973).

Results

Existence of Unique RNA Species in Myeloma Tumors. Competitive hybridization reactions under stringent reaction conditions between labeled and unlabeled RNAs for binding sites in DNA can be used to compare various RNA preparations and the information content in the DNA. Figure 1 shows the data from competitive hybridization experiments with MOPC-173D, ADJ-PC5, MOPC-104E, and MOPC-46B DNA in the presence of 50% formamide and 2 × SSC. It can be seen that the addition of increasing amounts of homologous RNA in all experiments essentially competed the reaction to zero and achieved plateau values indicating saturation of the DNA binding sites. The addition of increasing amounts of heterologous tumor RNA, with one exception (Figure 1D), did not compete as efficiently as the homologous RNA. Thus, heterologous RNA was not as effective a competitor as homologous RNA for binding sites in a given DNA, indicating that the RNA transcripts from the four tumors are distinguishably different.

The one exception in the difference in effectiveness in competition between homogous and heterologous RNAs was observed in the reaction between labeled MOPC-46B RNA and its homologous DNA (Figure 1D). In this case, unlabeled heterologous MOPC-104E RNA was as efficient a competitor as the homologous RNA. These data would indicate that approximately 90% of the hybridizable molecules in these two tumors are similar and may suggest that they were transcribed from similar DNA base sequences.

To explore this possibility, experiments were conducted where the reaction between pulse-labeled MOPC-46B RNA and MOPC-104E DNA (Figure 2A) and pulse-labeled MOPC-104E RNA and MOPC-46B DNA (Figure 2B) was competed by the addition of either unlabeled MOPC-46B or MOPC-104E RNA. In these experiments RNA homologous to the pulse-labeled heterologous RNA was a moderately effective competitor but was not as effective as was the RNA homologous to the DNA immobilized on the filter. The results in-

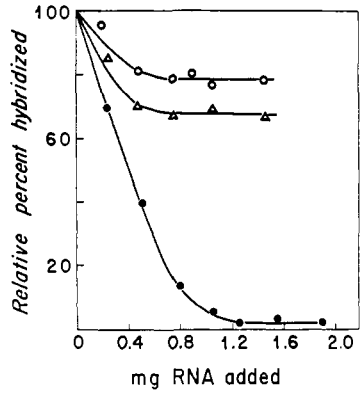

FIGURE 3: Competition by unlabeled RNA from ADJ-PC5 (●), mouse spleen (△), and mouse liver (○) in the hybridization of 7.0 μg of labeled ADJ-PC5 RNA (1092 cpm/μg) with 14.0 μg of ADJ-PC5 DNA. Incubation conditions as described for Figure 1. Per cent reaction without competition was 7.0%.

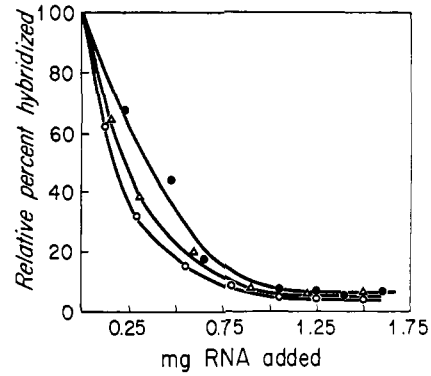

FIGURE 4: Competition by unlabeled RNA from MOPC-173D (△), mouse spleen (●) and mouse liver (○) in the hybridization of 5.8 μg of labeled MOPC-173D RNA (800 cpm/μg) with 11.8 μg of mouse liver DNA. Incubation conditions as described for Figure 1. Per cent reaction without competition was 2.4%.

dicate that the addition of increasing amounts of competitor RNA homologous to the DNA completely competed for the DNA binding sites at RNA/DNA ratios of 300, whereas RNA heterologous to the DNA resulted in a gradual approach to saturation and competes only to 80% at the same RNA/DNA ratios. Somewhat similar results were observed with heterologous competitive experiments between nucleic acids from ADJ-PC5 and MOPC-173D (Figures 2C and 2D). However, in these reactions the addition of increasing amounts of competitor RNA heterologous to the DNA resulted in a faster approach to complete competition. These results are what would be expected if there was a small number of DNA binding sites with which the heterologous RNA could specifically react, and an excess of unlabeled RNA was required to specifically saturate these sites.

Considering that (a) a minimum difference in the extent of reaction of homologous or heterologous RNA–DNA hybrids at RNA/DNA ratios above 12.5 (Krueger and Kosky, 1973, (b) that only labeled RNA species heterologous to the DNA were present, and (c) the large excess of competitor required to completely compete for the binding of labeled RNA, the difference between the RNA competitors homologous or heterologous to the DNA (Figure 2) could be due to different rates of reaction for hybrids of different degrees of base paring. Sutton and McCallum (1971) have observed that the rate of hybrid formation decreased as the degree in base pair mismatching increased. If there were a significant difference in the degree of base pair matching in homologous *vs.* heterologous tumor RNA–DNA hybrids, it could be predicted that RNA homologous to the DNA would be more efficient in competing the reaction of heterologous labeled RNA than would heterologous to the DNA which would react more slowly. To establish this, however, it would be necessary to compare the thermal stabilities of the hybrids after partial competition with homologous and heterologous RNA.

Figure 3 illustrates a competitive hybridization experiment between labeled ADJ-PC5 RNA and increasing amounts of unlabeled tumor, mouse spleen, and mouse liver RNA for binding sites in ADJ-PC5 DNA. It is evident that homologous tumor RNA was the most effective competitor whereas spleen and liver RNA could compete for the binding of only a fraction of the tumor RNA (30 and 20%, respectively). Spleen RNA contained more RNA species in common with tumor than did liver, as would be expected since spleen and myeloma tissues are of lymphoid origin. As a control, a competitive hybridization experiment between labeled ADJ-PC5 RNA and unlabeled tumor, liver, and spleen RNAs for binding sites in liver DNA was carried out (Figure 4). It can be seen that all RNAs were able to effectively compete the reaction as would be expected if there were a minimum of DNA binding sites in liver DNA with which the different populations of RNA transcripts could hybridize. Presumably these few sites represent the binding of rRNA, tRNA, and other mRNAs in common among these three tissues. It seems unlikely that only those tumor RNA species would bind to liver DNA which are also transcribed in liver (Figure 4). For example, genes concerned with the mechanisms of cell division are not transcribed in the bulk of normal liver cells, but they can be turned on after partial hepatectomy (Church and McCarthy, 1967). On the other hand, myeloma cells *in vivo* are rapidly dividing and a large proportion of their pulse-labeled RNA species would presumably be concerned with cell division. Most of these RNA species would bind to liver DNA, since the mechanisms of cell division are basically the same in tumor cells and nonmalignant cells. Therefore, RNA from normal, nondividing liver cells should not compete with these myeloma RNAs. The data in Figure 4 may be explained by the fact that myeloma cells in primary culture do not divide and thus a large proportion of their pulse-labeled RNA species may not be concerned with cell division. These data could also be interpreted according to the rate of reaction of competitor RNA homologous to the DNA being more rapid than the reaction of heterologous, labeled tumor RNA because of the difference in base pair matching in the hybrids. That is, liver DNA does not contain a sufficient number of DNA binding sites that tumor RNA could specifically hybridize with.

As pointed out in a previous report (Krueger and Kosky, 1973), the ability of pulse-labeled tumor RNAs to discriminate among tumor DNAs was dependent upon the RNA/DNA ratio at which the reaction was carried out. Figure 5 shows the data of a competitive hybridization experiment between labeled ADJ-PC5 RNA and unlabeled ADJ-PC5, MOPC-173D, MOPC-104E, and MOPC-46B RNA for binding sites in ADJ-PC5 DNA when the ratio of labeled RNA to DNA was 1.5. It is evident that under these conditions, the heterologous RNAs were able to compete the reaction almost as effectively as the homologous RNA, even though saturation with competitor was not reached in this experiment. Thus, the

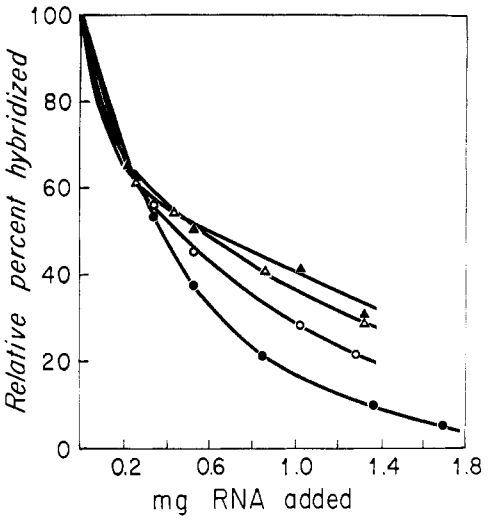

FIGURE 5: Competition by unlabeled RNA from ADJ-PC5 (●), MOPC-173D (○), MOPC-104E (△), and MOPC-46B (▲) in the hybridization of 21.0 μg of labeled ADJ-PC5 RNA (235 cpm/μg) with 14.0 μg of ADJ-PC5 DNA. Incubation conditions as described for Figure 1. Per cent reaction without competition was 2.8%.

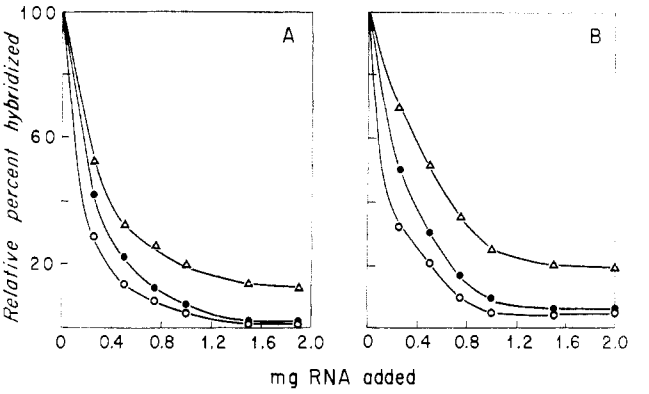

FIGURE 6: Competition by unlabeled nuclear (○), cytoplasmic (△), and total (●) tumor RNA in the hybridization reactions: (A) 7.0 μg of labeled ADJ-PC5 RNA (1092 cpm/μg) with 14.0 μg of ADJ-PC5 DNA; (B) 4.1 μg of labeled MOPC-104E RNA (975 cpm μg) with 8.2 μg of MOPC-104E DNA. Incubation conditions as described for Figure 1 except the reaction volume was 0.4 ml. Per cent reaction without competition was (A) 7.25% and (B) 8.3%.

populations of RNA transcripts from the different tumors do not appear to be distinguishably different when the competitive hybridization experiment is carried out at high RNA/DNA ratios, when the more slowly reacting RNA species in the labeled preparation dominate the reaction (Krueger and Kosky, 1973).

Competitive Hybridization with Nuclear and Cytoplasmic RNA, Samples of pulse-labeled ADJ-PC5 (Figure 6A) or MOPC-104E RNA (Figure 6B) were incubated with homologous tumor DNA in the presence of increasing amounts of unlabeled homologous nuclear, cytoplasmic, and total tumor RNA. Both nuclear and cytoplasmic RNA were efficient competitors when compared to total RNA; however, nuclear RNA was more efficient than cytoplasmic RNA as indicated by the slope of the initial part of the curves and makes up a large proportion of the total competitor RNA species. The cytoplasmic RNA preparation was unable to compete against a fraction of the pulse-labeled RNA as shown by the plateau at high ratios of competitor to labeled RNA. This fraction of the hybridized RNA molecules probably represents a population of RNA molecules in the mammalian cell nucleus which is not found in the cytoplasm (Shearer and McCarthy, 1967).

The fact that cytoplasmic RNA was capable of effectively competing the reaction may be attributed to a higher content of RNA hybridizable at low RNA/DNA ratios (nonribosomal RNA) in the cytoplasm as compared to the nucleus. A 500-fold dilution of pulse-labeled RNA by cytoplasmic RNA was required to reduce the hybridization level by 95%. These results indicate that a relatively large fraction of the unlabeled cytoplasmic RNA is identical with mRNA, which is presumably labeled by the short pulse with isotope (approximately $^1/_{45}$th of a cell generation), suggesting that the cytoplasm of these tumor cells is relatively enriched for these RNA species in accord with the dedication of these tumor cells in immunoglobulin synthesis. The fact that a similar dilution was required for unlabeled nuclear RNA to maximally reduce the hybridization level suggests that this RNA population may also be enriched for mRNA species. Similar results were obtained for the MOPC-173D tumor line. *In toto*, these data lend support to the idea that a significant proportion of the competitive hybridization data obtained with total RNAs from the four different tumors was contributed by metabolically unstable RNA species (mRNA ?) and suggests that some of the differences observed with the tumor nucleic acids could be related to immunoglobulin synthesis.

Discussion

The data from the competitive hybridization experiments indicate that the RNA transcripts coded for by a given myeloma DNA are distinguishably different from RNA transcripts from other myelomas. This is of interest since RNA populations of essentially different cell lines of the same type of lymphoid tissue would not be expected to show such extensive differences even under very stringent reaction conditions. Furthermore, the extent of the differences between the various myeloma RNA populations is not a maximum estimate. The RNA transcripts present in the various tumors will, even under moderately stringent reaction conditions, be able to compete for DNA binding sites which are either strictly complementary or sufficiently similar in base sequence but which may not be concerned with their synthesis.

The assumption in these studies was that we were employing moderately stringent reaction conditions through the use of 50% formamide and 2 × SSC. According to the observations of Schmuckpepper and Smith (1972) our reaction conditions were not as stringent as we originally assumed (68° in 2 × SSC). Therefore, the extensive differences as observed in the RNA transcripts from different myelomas were a minimal estimate; the differences would be even more extensive by the use of more stringent conditions (72° and 2 × SSC).

The nuclear–cytoplasmic RNA competition experiments indicated that unstable RNA transcripts constituted the bulk of the pulse-labeled RNA species. Shearer and McCarthy (1967) observed that in mouse L-cells, while nuclear RNA species make up approximately 66% of the species labeled in a pulse of 25 min, the bulk of these transcripts were precursors to cytoplasmic RNA species. A similar observation was described here, in that unlabeled cytoplasmic RNA was nearly as effective a competitor as nuclear RNA in the reaction of pulse-labeled RNA and DNA. There was no indication that the difference observed in the various myeloma RNA popula-

tions could be attributed to the function of nuclear RNA restricted to the nucleus. This is what would be expected if the RNA molecules restricted to the nucleus function as mediators of the regulation of gene transcription and these might be expected to be conserved in base sequence relative to the total DNA (Shearer and McCarthy, 1967).

Competition experiments between the reaction of pulse-labeled RNA and its homologous RNA by unlabeled homologous nuclear and cytoplasmic RNA suggests that a significant proportion of the difference in tumor RNA populations resided in cytoplasmic RNA. This result would be expected if a large fraction of the cytoplasmic RNA was identical with mRNA which should be preferentially labeled by the 20-min radioactive pulse used. These data suggest, therefore, that mRNA species constitute a relatively significant fraction of the myeloma cytoplasmic RNA species and indirectly indicate that the differences demonstrated for the various myeloma RNA populations were due to different mRNA species, since the base sequence of the other cytoplasmic RNA species such as rRNA has been demonstrated to be conservative (Moore and McCarthy, 1968). The suggestion that cytoplasm of myeloma cells is relatively enriched for mRNA species, and in particular myeloma globulin mRNA, may not be as speculative as it might seem at first inasmuch as the characteristic immunoglobulin synthesized by these tumor cells can constitute 10–20% of the total cellular protein (unpublished).

It should be stressed, however, that at this time it is not possible to state how much if any of the differences between the tumor nucleic acids are caused by genes coding for immunoglobulin. If each clone of antibody-forming cells has a different DNA base sequence coding for the variable portion of each immunoglobulin chain, then each monoclonal myeloma that produces intact immunoglobulin molecules should produce one species of mRNA for the heavy and light chains of the immunoglobulin peptide. It is difficult to predict the differences in extent of hybridizability of such mRNAs with homologous as compared to heterologous DNA under the reaction conditions used here because of the existence of a high frequency of partially related base sequences in the mammalian genome (Britten and Kohne, 1968). Furthermore, the nucleotide sequences of the same variable region (V) subclass, different subclasses within the same class, and even different V classes would be closely related. On the other hand, such immunoglobulin mRNAs could hybridize to a much greater extent with homologous DNA than heterologous tumor DNA under the moderately stringent reaction conditions used here, particularly if the genes coding for immunoglobulin are replicated (duplicated) in antibody-forming cells following their stimulation by antigen.

References

Britten, R. J., and Kohne, D. E. (1968), *Science 161*, 529.

Church, R. B., and McCarthy, B. J. (1967), *Proc. Nat. Acad. Sci. U. S. 58*, 1548.

Church, R. B., Storb, U., McCarthy, B. J., and Weiser, R. S. (1968), *J. Immunol. 101*, 399.

Cohen, E. P. (1967), *Proc. Nat. Acad. Sci. U. S. 57*, 673.

Greenberg, L. J., and Uhr, J. W. (1967), *Proc. Nat. Acad. Sci. U. S. 57*, 1878.

Hood, L., and Talmage, D. W. (1970), *Science 168*, 325.

Krueger, R. G., and Kosky, K. (1973), *Biochemistry 12*, 1299.

Moore, R. L., and McCarthy, B. J. (1968), *Biochem. Genet. 2*, 75.

Raska, K., and Cohen, E. P. (1968), *Nature (London) 217*, 720.

Schmeckpepper, B. J., and Smith, K. D. (1972), *Biochemistry 11*, 1319.

Shearer, R. E. W. (1969), Ph.D. Dissertation, University of Washington, Seattle.

Shearer, R., and McCarthy, B. J. (1967), *Biochemistry 6*, 283.

Storb, U. (1972), *J. Immunol. 108*, 755.

Sutton, W. D., and McCallum, M. (1971), *Nature (London), New Biol. 232*, 83.