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Hybridization Studies with Nucleic Acids from Murine Myelomas. Kinetics of the Reaction and Characterization of the Hybrid†

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ABSTRACT: The ability of pulse-labeled RNA from four different murine myelomas to hybridize with DNA isolated from homologous or heterologous tumors or mouse liver in the presence of formamide was examined. RNA from a given tumor reacted maximally with its homologous tumor DNA and to lesser extents with heterologous tumor or liver DNA. At low RNA/DNA ratios, a given tumor RNA reacted more rapidly with homologous DNA as compared to heterologous tumor or liver DNA. Homologous tumor RNA–DNA hybrids formed at low RNA/DNA ratios in the presence of 50% formamide have higher thermal stabilities than heterologous hybrids formed under the same conditions and are relatively resistant to RNase. At high RNA/DNA ratios, differences in the rates of formation and thermal stability of homologous and heterologous tumor hybrids were minimized, independent of the formamide concentration, and the hybrids were susceptible to RNase. These studies suggest differences among the different myeloma DNAs, possibly in their content of specific amplified genes.

It is thought that an individual is capable of synthesizing antibodies of 10^6 different specificities (Jerne, 1967). It has been proposed that the ability to generate antibody variability occurs during somatic development (Smithies, 1967; Brenner and Milstein, 1969; Gally and Edelman, 1970) or is the result of evolution (Dreyer *et al.*, 1967; Hood and Talmage, 1970). Basically, these theories differ as to whether each clone of antibody forming cells has one set of genes coding for the light and heavy chains of its specific antibody or whether all clones have the same information content in their DNAs for the synthesis of all possible antibody molecules but with specific derepression of one set of genes. For example, according to the latter hypothesis, each cell would have to devote approximately 0.2% of genetic material per haploid genome (2×10^4 variable genes) to immunoglobulin synthesis (Hood and Talmage, 1970). Storb (1972) has recently estimated that there are 6–14 $\times 10^3$ immunoglobulin genes the size of the variable region of the immunoglobulin peptide per mouse spleen haploid genome.

Murine myeloma represents a homogeneous, differentiated cell population committed to the synthesis of a large amount of homogeneous protein. These tumors apparently arise as a result of a malignant alternation in a plasma cell precursor committed to the synthesis of a specific immunoglobulin. It has been estimated that the myeloma globulin represents about 40% of the total cellular protein synthesized in a γG producing tumor (Askonas, 1961). Both somatic recombina-

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tion (Smithies, 1967; Brenner and Milstein, 1969; Gally and Edelman, 1970) and germ line theories (Dreyer *et al.*, 1967; Hood and Talmage, 1970) for generating antibody or immunoglobulin variation predict that only one or a small number of genes in the genome of an immunoglobulin producing cell specify the light and heavy chains of the immunoglobulin molecule. The intriging question is: how is the information encoded in a small number of genes amplified to produce a large amount of the total cellular protein as a single immunoglobulin species? At least four hypotheses have been advanced to explain amplification: (a) synthesis of a stable immunoglobulin mRNA species; and/or (b) a differential rate of transcription of the immunoglobulin genes to form a large pool of RNA; and/or (c) an increased rate of translation of the immunoglobulin mRNA; and/or (d) somatic duplication of the immunoglobulin gene. The mechanisms by which either of the above hypothesis could operate in immunoglobulin synthesis are speculative at this time. Evidence has been presented that the mRNA specifying myeloma globulin is relatively unstable with a half-life of 2–3 hr and would not in itself explain the high rate of myeloma globulin synthesis (Shutt and Krueger, 1972).

Greenberg and Uhr (1967), using the DNA–RNA hybridization technique, have reported on the genetic relationship of the MPC-11, RPC-20, and BJ myelomas. On the basis of hybridization reactions between homologous tumor DNAs and heterologous ribosomal RNAs labeled *in vivo*, they concluded that the DNAs of these tumors are different.

This article describes DNA–RNA hybridization studies with nucleic acids from four different murine myelomas that were carried out to assess the possibility that somatic gene duplication has occurred in murine myeloma. The kinetics and stoichiometry of reaction of pulse-labeled RNA with DNA indicate that the DNAs from these tumors are different and suggest that a given tumor DNA contains a multiplicity of DNA binding sites for its homologous RNA transcripts. At this time, however, it is not possible to establish that the differences in nucleic acids from different tumors result from genes or mRNA responsible for immunoglobulin synthesis. A preliminary report of these studies was presented earlier (Krueger and McCarthy, 1970).

Materials and Methods

The BALB/c myeloma tumors MOPC-104E, MOPC-46B, MOPC-173D, and ADJ-PC5 were supplied by Dr. Michael Potter. These tumors are maintained as solid tumors by serial *in vivo* transplantation in BALB/c mice of minced tumor tissue. These tumors synthesize antigenetically defined immunoglobulins: MOPC-104E synthesizes IgM[1] globulin; MOPC-173D, IgG_{2a}; ADJ-PC5, IgG_{2a}; and MOPC-46B synthesizes K light chains only.

Preparation of DNA. Nuclei were prepared from vigorously growing tumors and mouse livers and spleens by a modification of the method described by McCarthy and Hoyer (1964). The tumors were removed and minced into small fragments in Waymouths' medium. The fragments were collected by centrifugation, resuspenced in 2 vol of cold 0.32 M sucrose containing 0.03 M $MgCl_2$ and 0.03 M Tris, pH 7.4, and filtered through nylon gauze, and 5 vol of the sucrose solution was added. The fragments were rapidly homogenized by five strokes in a glass Teflon homogenizer and ten strokes of a tight fitting Dounce homogenizer. The homogenate was filtered through gauze and centrifuged at 1000*g* for 5 min at 4° and the supernatant was decanted and recentrifuged at 3000*g* for 10 min at 4°. The pellets from the 1000*g* and 3000*g* centrifugations were gently resuspended in a total of 10 vol of 2 × SSC, shaken by hand, and filtered through nylon gauze.

The nuclei were lyzed by making the solution 1% with sodium dodecyl sulfate. The viscous mixture was shaken until homogeneous and an equal volume of redistilled water-saturated phenol (pH 7.8) added. The mixture was shaken by hand for 10 min and centrifuged at 1500*g* for 10 min at 4°. The viscous aqueous phase was removed and made 1 M with sodium perchlorate. An equal volume of chloroform–octanol (10:4) was added and the mixture shaken for 10 min and centrifuged at 1500*g* for 10 min at 4°. The viscous aqueous phase was removed and the DNA spooled following addition of 2 vol of cold 95% ethanol.

The spooled DNA was resolubilized in 0.1 × SSC, the solution was made 1 × SSC, and the DNA was respooled and resolubilized. The solution was brought to 1 × SSC and treated with 100 μg/ml of α-amylase for 1 hr at 37°. One volume of redistilled water-saturated phenol (pH 7.5) was added and the mixture was shaken and centrifuged for 10 min at 1500*g*. The aqueous phase was removed and the DNA spooled. The DNA was resolubilized in 0.1 × SSC and treated with 10 μg/ml of boiled RNase for 1 hr at 37°. The digestion was followed by two phenol extractions as before. After removal of the final aqueous phase, the remaining traces of phenol were removed by ether extraction. The DNA was spooled three times as before, taken up in 0.1 × SC and centrifuged at 10,000*g* for 2 hr after the addition of 0.01% acid-washed Norite to remove traces of protein and polysaccharide. The DNA was stored frozen in 0.1 × SSC until used. *Bacillus subtilis* and *Escherichia coli* DNA were isolated by the procedure described by Marmur (1961) and purified as above.

Preparation of Labeled Tumor DNA. BALB/c mice were transplanted subcutaneously with 2 × 10^6 viable tumor cells and when the tumors reached a diameter of 1 cm, 500 μCi of [^{14}C]thymidine was inoculated directly into the tumor mass. The animals were sacrificed 48 hr later, a cell suspension was prepared, and DNA isolated as described above.

The purity of the DNA was evaluated by the following criteria: special properties, absence of detectable protein, polysaccharide or RNA, and the ability to be retained on a nitrocellulose filter and to remain absorbed for the duration of the hybridization reaction.

Isotopic Labeling of RNA. Tumor-bearing BALB/c mice were sacrificed by cervical dislocation, and the tumors aseptically removed and placed in Waymouth's medium. The tumor tissue was minced into small fragments and the fragments were gently homogenized by a hand held mismatched glass homogenizer. The resulting cell suspension was filtered through nylon gauze to remove large fragments, filtered through 400 mesh stainless steel wire gauze, and centrifuged at 300*g* for 10 min. The cell pellet was resuspended in Waymouth's medium to a concentration of 2 × 10^6 viable cells/ml and incubated on a rocker platform for 1 hr at 37° in an environment of 15% CO_2–85% air. Cellular RNA was isotopically labeled by pulsing cultures with 2 μCi/ml of [^{3}H]uridine (4.24 Ci/mmol) for 20–30 min.

Preparation of RNA. RNA was isolated and purified by a

[1] Nomenclature and abbreviations for immunoglobulins correspond to those recommended by the World Health Organization (*Bull. W. H. O.*, 1964). Abbreviations used are: SSC, 0.15 M NaCl and 0.015 M sodium citrate; T_{m_i}, mean temperature of irreversible strand dissociation; sRNA, soluble ribonucleic acid.

modification of the procedure of Church and McCarthy (1968). The cells are removed from culture, centrifuged for 10 min at 1000*g*, and resuspended in 2 vol of cold 0.25 M sucrose containing 0.03 M $CaCl_2$–0.01 M Tris, pH 7.4. The cell suspension was homogenized at 4° by five–ten strokes of a tight fitting motor driven Teflon homogenizer and an equal volume of a solution containing 0.28 M LiCl–0.02 M sodium acetate–0.003 M $MgCl_2$, and 1% sodium dodecyl sulfate (pH 5.0) was added and the mixture rehomogenized. The homogenate was degraded by sonication in a Branson Sonifier LS for 1 min at full output to increase the hybridization efficiency of the RNA. An equal volume of freshly prepared redistilled water-saturated phenol (pH 7.0), preheated to 60°, was added and the mixture was incubated at 60° for 5 min with occasional stirring. The mixture was rapidly cooled, shaken hard by hand for 10 min, and centrifuged at 1500*g* for 10 min. The aqueous phase was removed and reextracted with an equal volume of water-saturated phenol (pH 6.3). After centrifugation, the aqueous phase was removed and made 0.2 M with potassium acetate and the RNA precipitated with 2 vol of cold ethanol for 16 hr at −20°. The precipitate was collected by centrifugation at 1500*g* for 10 min, dissolved in 0.1 × SSC, extracted three times with an equal volume of ether, and ethanol precipitated as before. The precipitate was dissolved in 0.01 M $MgCl_2$–0.01 M Tris, pH 7.4, and digested with 10 μg of RNase-free DNase/ml at 37° for 30 min. After three phenol extractions, the RNA was precipitated with ethanol as before. The precipitate was dissolved in 0.01 M potassium acetate, pH 5.2, and chromatographed on G-50 Sephadex buffered with 0.01 M potassium acetate. Finally, the RNA solution was centrifuged in the presence of 0.01% acid-washed Norite and ethanol precipitated, and the precipitated RNA was dissolved in 0.1 × SSC at a concentration of 5–10 μg/ml and stored at −20°.

RNA was extracted and purified from freshly excised tumors, mouse spleens, and livers as described above.

Hybridization of RNA with DNA. Purified, high molecular weight DNA, dissolved in 0.1 × SSC at 100 μg/ml, was heat denatured at 95° for 10 min and quickly cooled in a large volume of 4 × SSC. DNA was immobilized on membrane filters by slowly passing a DNA solution (10 μg/ml) through 145 mm diameter Schleicher and Schull type B6 filters. The filters were treated with the preincubation medium of Denhardt (1966), air dried at room temperature, and baked at 70° for 16 hr. The filters were cut to the required size (5 mm diameter) and their DNA content was checked by one or more of three ways: (1) decrease in A_{260} of the DNA solution during filtration, (2) A_{600} after the diphenylamine reaction (Burton, 1968), or (3) radioactivity when [^{14}C]DNA was used. The cut filters were stored dry at 4°.

The hybridization reaction between various amounts of pulse-labeled RNA and DNA immobilized on filters was carried out in 0.2 ml of 5 × SSC and 50% formamide (Eastman Kodak, Rochester, N. Y.) at 37–40° for 16–24 hr. At the end of the incubation period, the filters were removed from the reaction vials, washed once with 5 × SSC containing 50% formamide at the incubation temperature and four times with 1 × SSC before being dried, and counted in a Packard Tri-Carb scintillation counter to 2% error.

Nonspecific binding of RNA to DNA was determined by incubation of the labeled RNA with a filter containing *B. subtilis* DNA at an RNA/DNA ratio equal to that used in the experiment. Values obtained were between 0.01 and 0.4% of input counts and this background was subtracted from the values obtained with tumor DNA.

TABLE I: Specificity of the Hybridization Reaction.[a]

Source of DNA	Hybridization (%)
Myeloma tumor	3.8
Mouse spleen	2.0
Mouse liver	1.9
Rat liver	1.5
B. subtilis	0.45
E. coli	0.28

[a] ADJ-PC5 tumor RNA (11.6 μg, 1260 cpm/μg), labeled with [^{3}H]uridine, was hybridized with 23.2 μg of each DNA immobilized on filters in 0.2 ml of 5 × SSC and 50% formamide for 20 hr at 37°.

The stability of the RNA–DNA hybrids was assessed by incubating the hybrids at increasing temperatures. Filters were removed from the incubation vials and washed two times in 5 × SSC containing 50% formamide at the incubation temperature, and the hybrids were dissociated by heating in 2 ml of 1 × SSC. The temperature of the buffer was raised in 5° steps allowing 15 min for equilibration at each temperature. The amount of radioactivity removed from the filter at each temperature was determined by precipitating the wash with 10% Cl_3CCOOH after the addition of 10 μg of yeast RNA (Calbiochem, Los Angeles). The precipitates were collected on glass filters (Gelman type GSA), dried, and counted in a liquid scintillation counter to 2% error.

Loss of DNA from filters during RNA–DNA hybridization reactions and thermal stability experiments were monitored by measuring the DNA content of filters carried through reaction conditions without labeled RNA by the diphenylamine reaction (Burton, 1968) or determination of the amount of ^{14}C radioactivity lost from [^{14}C]DNA filters carried through mock reaction conditions or hybridized with ^{3}H-labeled tumor RNA. By all procedures, 93–95% of the DNA was retained on the filters during reassociation and thermal stability experiments in the presence of formamide, in good agreement with the results of Gillespie and Gillespie (1971).

The specificity of the hybridization reaction with one representative myeloma nucleic acid using these reaction conditions is shown in Table I. Using highly purified ADJ-PC5 DNA and RNA, the background level of pulse-labeled RNA complexed with DNA of *B. subtilis* or *E. coli* was less than 0.5% of input radioactivity. Reaction of pulse-labeled RNA with either rat liver or mouse liver and spleen was 1.0–1.5% less than that observed with homologous tumor DNA. While RNase will reduce the level of reaction even further, a reduction in the per cent hybridization of homologous reactions was also observed.

Specificity of Reaction with Tumor DNAs. The distribution of various classes of tumor RNA molecules was analyzed by examining the fraction of RNA hybridized with DNA as a function of the amount of input RNA present (Figure 1). Throughout the range of RNA/DNA ratios studied, tumor RNA displayed maximum hybridization efficiency with homologous tumor DNA at low RNA/DNA ratios. At the low RNA/DNA ratios (*i.e.*, 0.25 or 0.5), hybridization of ribosomal and sRNA would make up only a relatively small fraction of the total reaction whereas mRNA could hybridize with relatively high efficiency.

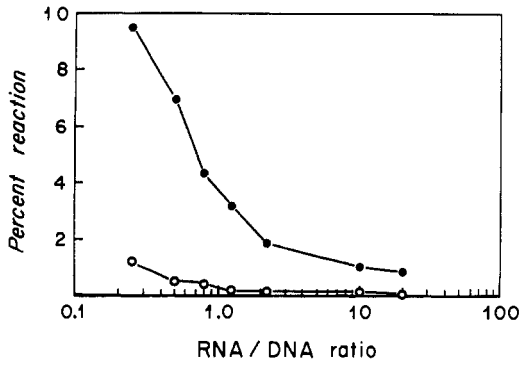

FIGURE 1: The efficiency of hybridization of pulse-labeled tumor RNA as a function of the ratio of RNA to DNA. Various amounts of filter-bound MOPC-104E DNA (●) or *B. subtilis* DNA (○) were incubated with 24.8 μg of MOPC-104E RNA (650 cpm/μg) in 0.2 ml of 5 × SSC and 50% formamide for 20 hr at 37°: MOPC-104E DNA, 11.2-μg filter; *B. subtilis*, 10.5-μg filter.

This effect could be accounted for by one of two explanations: the synthesis of either a large variety of different RNA molecules or synthesis of a few RNA species from multiple DNA sites. Likewise, if a large proportion of the [^{3}H]uridine were entering RNA species other than stable ribosomal components, an extensive fraction of the label would hybridize. It is possible that both of these contribute in view of the extensive synthesis of a single type of immunoglobulin protein by the neoplastic plasma cells. However, the role of the hybridized RNA molecules as templates for protein synthesis has not been demonstrated and it is conceivable that other types of RNA molecules, such as rapidly labeled nuclear RNA (Shearer and McCarthy, 1967) or RNA specific to the intracisternal A particles found in all myelomas (Kuff *et al.*, 1968; Volkman *et al.*, 1971), are present and actively synthesized in the tumor cells.

The extent of hybridization of pulse-labeled RNAs with homologous and heterologous tumor DNAs at an RNA/DNA ratio of 0.25 is shown in Table II. In all instances, pulse-labeled tumor RNA hybridized maximally with its homologous DNA and to lesser extents with heterologous tumor DNAs. At higher RNA/DNA ratios (1.0) the differences in

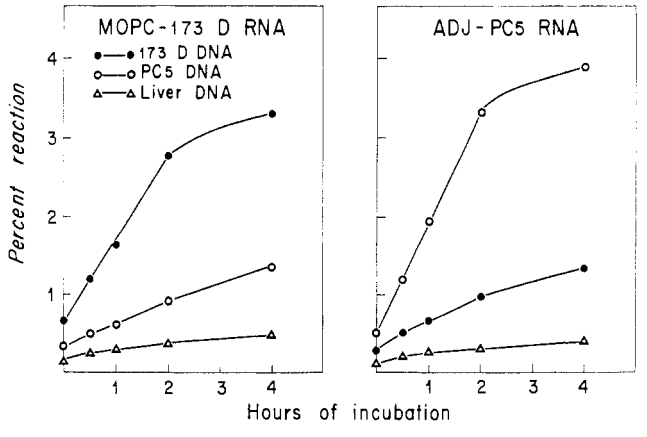

FIGURE 2: Hybridization of pulse-labeled ADJ-PC5 (1260 cpm/μg) or MOPC-173D RNA (800 cpm/μg) with ADJ-PC5, MOPC-173D, and mouse liver DNA at an RNA/DNA ratio of 0.5. The amount of DNA per reaction was 15.7 μg for ADJ-PC5, 17.34 μg for MOPC-173D, and 15.4 μg for mouse liver. Reactions were carried out in 0.2 ml of 5 × SSC and 50% formamide at 37°.

TABLE II: Percentage Hybridization of Tumor RNAs with Homologous and Heterologous Tumor DNAs.[a]

	DNA			
RNA	173D	PC-5	104E	46B
173D	7.20	5.40	4.90	5.40
PC-5	5.80	7.15	3.80	4.00
104E	4.90	3.40	8.80	6.10
46B	3.90	2.36	6.40	7.70

[a] Tumor RNA (5.8 μg) was incubated with tumor DNA filters (23.2 μg) at an RNA/DNA ratio of 0.25 in 0.2 ml of 5 × SSC and 50% formamide at 37° for 20 hr. The data are the average of three experiments and have been corrected for the background reaction to *B. subtilis* DNA: MOPC-173D RNA, 800 cpm/μg; MOPC-104E RNA, 649 cpm/μg; MOPC-46B, 680 cpm/μg; ADJ-PC5 RNA, 1260 cpm/μg.

the extent of reaction of all RNAs with homologous and heterologous tumor DNAs were minimal (unpublished).

Kinetics of RNA–DNA Hybrid Formation. The kinetics of the reaction of pulse-labeled MOPC-173D and ADJ-PC5 RNAs with MOPC-173D, ADJ-PC5, and mouse liver DNA at an RNA/DNA ratio of 0.50 are shown in Figure 2. An initial rapid reaction was observed with homologous DNA whereas the extent of reaction was appreciably less with the heterologous tumor and mouse liver DNAs. These data suggest a fraction of the pulse-labeled tumor RNA preparation reacts rapidly with a number of DNA binding sites in the homologous DNA and these binding sites occur less frequently in the heterologous tumor and mouse liver DNAs. Similar results were obtained with other pulse-labeled tumor RNAs reacting with homologous and heterologous DNAs.

Figure 3 shows the rate and extent of reaction of pulse-labeled MOPC-104E and ADJ-PC5 RNAs with homologous tumor DNAs as a function of the RNA/DNA ratio. Detection of the rapidly reacting component was dependent upon the RNA/DNA ratio at which the incubation was carried out. This suggests that with higher amounts of input RNA, the DNA binding sites for the rapidly reacting component are quickly saturated and the RNA reacts with partially related DNA sequences. Figure 4 shows the rate of reaction of ADJ-PC5 RNA with homologous, MOPC-104E, and mouse liver DNA at RNA/DNA ratios of 0.25 and 1.0. At the lower ratio (Figure 4A) detection of the rapid component and the ability of the labeled tumor RNA to discriminate between these DNAs was maximized whereas at the higher ratio (Figure 4B) it was minimized. At even higher ratios the extent of reaction with all DNAs was approximately equal (unpublished). Similar results were obtained with labeled RNAs from MOPC-173D and MOPC-46B reacting with homologous and heterologous DNAs.

Thermal Stabilities of RNA–DNA Hybrids. The T_{m_i}[2] values and extent of reaction of homologous myeloma RNA–DNA hybrids formed at a low RNA/DNA ratio (0.5) were different from that of heterologous hybrids formed at the same ratio (Figure 5 and Table III) or homologous hybrids formed at a high RNA/DNA ratio (Figure 6). Homologous hybrids formed at low RNA/DNA ratios (Figure 7) were affected to a lesser extent by RNase treatment than were heterologous

TABLE III: Thermal Stability of Myeloma RNA–DNA Hybrids.[a]

RNA	DNA	RNA/DNA Ratio	RNase Treatment	% Reaction	T_{m_i} (°C)
173D	173D	0.25	−	4.60	81
173D	173D	0.25	+	4.60	81
173D	PC-5	0.25	−	3.90	80
173D	PC-5	0.25	+	3.85	80.5
173D	173D	0.50	−	3.80	78
173D	173D	0.50	+	3.76	78
173D	PC-5	0.50	−	2.50	73.5
173D	PC-5	0.50	+	1.90	77
173D	173D	12.5	−	1.47	71
173D	173D	12.5	+	0.71	73.5
173D	PC-5	12.5	−	1.46	66
173D	PC-5	12.5	+	0.65	70
PC-5	PC-5	0.25	−	5.76	81
PC-5	PC-5	0.25	+	5.75	81
PC-5	173D	0.25	−	3.80	80
PC-5	173D	0.25	+	3.78	80.5
PC-5	PC-5	0.50	−	3.67	78
PC-5	PC-5	0.50	+	3.63	78
PC-5	173D	0.50	−	2.75	74.5
PC-5	173D	0.50	+	1.95	77
PC-5	PC-5	15.0	−	1.20	70
PC-5	PC-5	15.0	+	0.62	74
PC-5	173D	15.0	−	1.18	67
PC-5	173D	15.0	+	0.50	70

[a] Tumor RNA was incubated with 34.8–35-μg DNA immobilized filters at the indicated RNA/DNA ratios in 0.2 ml of 5 × SSC and 50% formamide at 37° for 20 hr. The T_{m_i} of each hybrid was determined as described in Materials and Methods. The data are the average of three experiments with two separate RNA and DNA preparations. The specific activities of the RNA preparations are given in the footnotes to Table II.

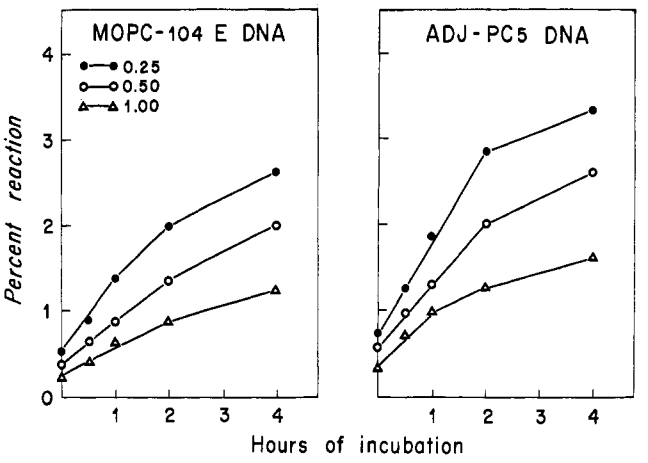

FIGURE 3: Hybridization of pulse-labeled MOPC-104E (650 cpm/μg) or ADJ-PC5 RNA (625 cpm/μ) with homologous tumor DNA as a function of the RNA/DNA ratio. The amount of DNA per reaction was 11.57 μg for MOPC-104E and 15.3 μg for ADJ-PC5. Incubation conditions are as in Figure 2.

hybrids formed at a similar ratio or homologous or heterologous hybrids formed at a higher RNA/DNA ratio (Figure 6). The thermal dissociation profiles and extent of reaction of homologous hybrids formed at the lower ratio with or without RNase treatment to remove grossly mispaired structures were nearly identical (Figure 7A), but detectably different in the case of high ratio homologous or heterologous hybrids or low ratio heterologous hybrids (Figures 6A and 7B, respectively). This result is not surprising since RNase removes more of the bound RNA from homologous hybrids formed at high RNA/DNA ratios or heterologous hybrids formed at low ratios. The fact that the T_{m_i} of homologous myeloma hybrids was decreased when large amounts of RNA were used to form more hybrid indicates that the type of structure that formed was dependent upon the input ratio of RNA to DNA. At high input RNA levels, the RNA is able to form mismatched complexes with DNA sequences other than those from which it was presumably transcribed, because under these conditions such cross-reactions are maximized. Thus, the degree of matching achieved by hybrids formed after many DNA sites are occupied is lower than when a large number of such sites are available. Similar results were obtained with nucleic acids from all the tumors.

Figure 8 depicts the thermal dissociation profiles of MOPC-173 RNA–DNA hybrids formed at an RNA/DNA ratio of 0.5 allowed to react for 2, 16, and 24 hr. The T_{m_i} values of these structures were a continuous function of the duration of reaction. For example, hybrids formed at 2 hr have a T_{m_i} of approximately 80°, whereas complexes formed at 24 hr have a T_{m_i} of 74°. A similar result was obtained for ADJ-PC5 RNA–DNA hybrid formation. These results appear to be consistent with the view that certain DNA sequences exist in high frequency and react with certain RNA species with high specificity. With time, however, the predominant reaction products represent associations between RNA molecules and partially related DNA sequences.

Figure 9A illustrates the thermal dissociation profiles of hybrids formed by MOPC-173D RNA and DNA in theoretically increasingly stringent reaction conditions. Incubation at 37° in the presence of 32, 40, 50, and 60% formamide is

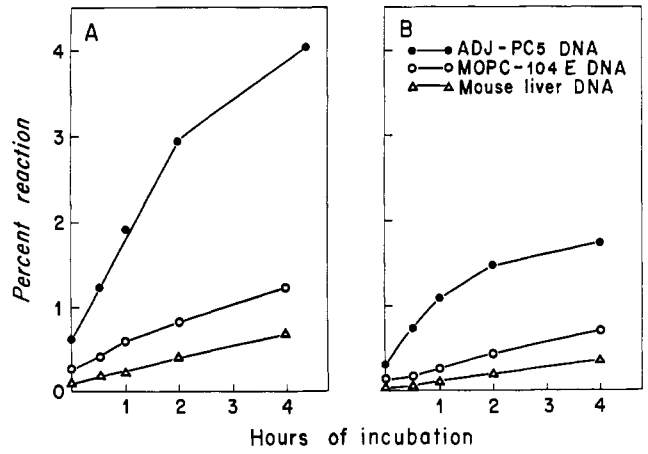

FIGURE 4: Hybridization of pulse-labeled ADJ-PC5 RNA (625 cpm/μg) with ADJ-PC5, MOPC-104E, and mouse liver DNA at an RNA/DNA ratio of 0.25 (A) and 1.0 (B). The amount of DNA per reaction was 14.0 μg for ADJ-PC5, 16.28 μg for MOPC-104E, and 15.4 μg for mouse liver. Incubation conditions are as in Figure 2.

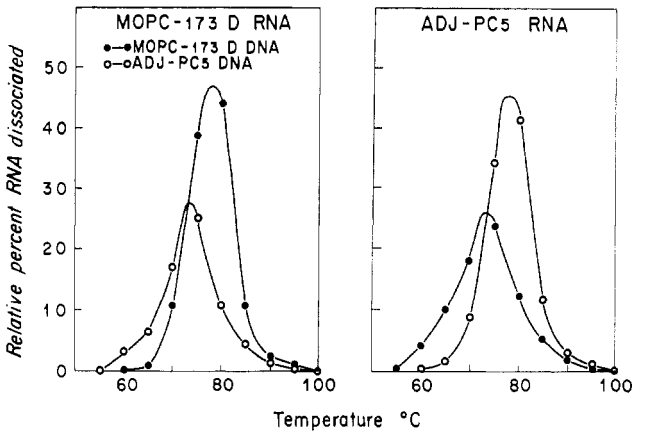

FIGURE 5: Thermal dissociation profiles on hybrids formed by incubating MOPC-173D (800 cpm/μg) or ADJ-PC5 RNA (625 cpm/μg) with MOPC-173D or ADJ-PC5 DNA at an RNA/DNA ratio of 0.50. The amount of DNA per reaction was 44.8 μg for MOPC-173D and 31.6 μg for ADJ-PC5. The T_{m_i} values of the hybrids are given in Table III. Hybrids were formed in 0.4 ml of 5 × SSC and 50% formamide at 37° for 20 hr. The amount of RNA dissociated is relative to the total RNA in homologous hybrids.

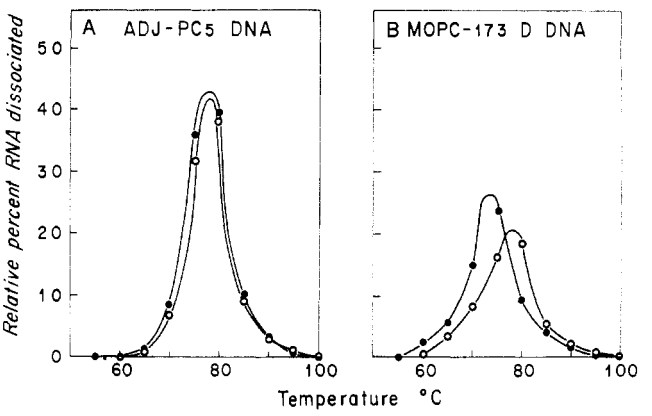

FIGURE 7: Thermal dissociation profiles of total and RNase-resistant hybrids formed by incubating 15.8 μg of pulse-labeled ADJ-PC5 RNA (1250 cpm/μg) with 31.6 μg of ADJ-PC5 DNA and 33.6 μg of MOPC-173D DNA. The total hybrid (●) and the RNase-resistant hybrid (○) were determined as described in Figure 6. The T_{m_i} values of the hybrids are given in Table III. The amount of RNA dissociation is relative to the total RNA in ADJ-PC5 RNA–ADJ-PC5 DNA hybrids.

theoretically equivalent to 60, 67, 74, and 82° in 1 × SSC, respectively (McConaughy *et al.*, 1969). It is apparent that the T_{m_i} values of the hybrids and the extent of reaction were a continuous function of the formamide concentration. The T_{m_i} values varied from 68 to 81.5° when formed in the range of 32–60% formamide. A fraction of the products formed under conditions of low stringency (32 and 40% formamide) overlap with those formed under conditions of high stringency (50 and 60% formamide). Even under the most stringent conditions used, however, it was apparent that a significant proportion of the hybrid was formed between molecules representing different genetic sites.

Figure 9B illustrates the thermal dissociation profiles of heterologous hybrids formed by MOPC-173D RNA and MOPC-104E DNA under the same conditions as Figure 9A. The T_{m_i} values of the hybrids were dependent upon the conditions of the reaction varying from 64 to 75° when formed in the range of 32–60% formamide. Furthermore, the T_{m_i} values were lower than those for homologous tumor hybrids (Figure 9A) and the proportion of hybrid that melted at high temperatures, when the hybrids were formed in the presence of high formamide concentrations, was reduced. These results indicate that the overall specificity of base pairing and the proportion of well-matched structures in the heterologous RNA–DNA hybrids were lower than those for the homologous hybrid. These data are suggestive that myeloma DNAs differ in the frequency of base sequences capable of binding homologous or heterologous tumor RNA transcripts.

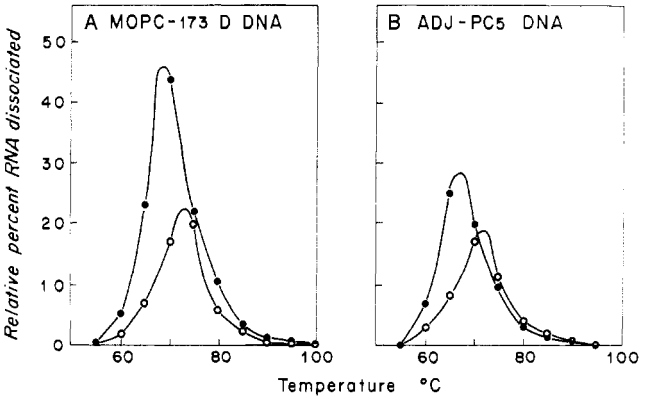

FIGURE 6: Thermal dissociation profiles of total and RNase resistant hybrids formed by incubating pulse-labeled MOPC-173D RNA (525 cpm/μg) with 44.8 μg of MOPC-173D DNA and 31.6 μg of ADJ-PC5 DNA at a RNA/DNA ratio of 1.0. The total hybrid (●) was determined by measuring the RNA–DNA complex stable to two washes of 5 × SSC at the incubation temperature. The RNase-resistant hybrid (○) that remained after treating washed filters with 10 μg/ml of RNase was described by Gillespie and Spiegelman (1965). The T_{m_i} values of the hybrids are given in Table III. Incubation conditions are the same as in Figure 5. The amount of RNA dissociated is relative to the total RNA in nonRNase-treated MOPC-173D RNA–MOPC-173D DNA hybrids.

Discussion

The data reported here suggest that a species of RNA transcript, present in high concentration in murine myeloma, specifically reacts with complementary or partially complementary binding sites in homologous tumor DNA. Hybridization of RNA to homologous tumor DNA was most readily observed when reactions were carried out for short periods of time at low RNA/DNA ratios under theoretically stringent reaction conditions. This suggests that there are multiple DNA binding sites in the genome which exist in greater frequency in homologous tumor DNA than in heterologous tumor or liver DNA. The high thermal stability of the homologous tumor RNA–DNA hybrids indicates that the binding sites are very similar in base sequence. In general, filter RNA–DNA hybridization reactions measure only RNA synthesized from the redundant sequences in mammalian DNA (Britten and Kohne, 1968) and have T_{m_i} values of 70–75° depending upon the stringency of the reaction conditions (Church and McCarthy, 1968). The homologous myeloma RNA–DNA hybrids formed at low ratios in the presence of formamide had high T_{m_i} values (80°). When reactions were carried out at high RNA/DNA ratios, slowly reacting RNA species dominated the reaction and the resulting hybrids had T_{m_i} values expected for redundant sequences.

The data in Tables II and III are different in the extent of

reaction of a given tumor RNA and DNA at low RNA/DNA ratios. This is apparently due to the amount of DNA used in the reaction, 11.6 μg in Table II and 34.8 μg in Table III. We have consistently observed that the extent of reaction of labeled tumor RNA with a given tumor DNA at a constant RNA/DNA ratio is higher when a small amount of DNA is used. For example, reaction of 6 μg of RNA with 12 μg of DNA gives a higher per cent reaction than the reaction of 12 μg of RNA with 24 μg of DNA. The T_{m_i} values of the respective hybrids are nearly identical. The explanation of this observation may be trivial, but could indicate that the increased availability of DNA binding sites enhances the reaction of RNA transcripts with related DNA base sequences.

Schmeckpepper and Smith (1972) studied the effect of formamide concentration on RNA–DNA hybridization reactions in 2 and 4 × SSC at 25 and 33° and concluded that there was no simple linear relationship between the mean thermal stability of hybrids and formamide concentration below 45° or the formamide concentration required to produce hydrids of specified thermal stability. According to their data, our reaction conditions allowed the same degree of base mispairing as occurs at 68° in 2 × SSC, not as stringent as we originally anticipated. However, myeloma RNA–DNA hybrids formed in the presence of 50% formamide and 5 × SSC at 37° have T_{m_i} values nearly identical with hybrids formed at 72° in 2 × SSC (unpublished data). Further, Gillespie and Gillespie (1971) demonstrated that RNA–DNA hybridization reactions with *E. coli* nucleic acids in 6 × SSC and 50% formamide at 35° were generally superior to reactions in 6 × SSC at 66°. Thus, there is further need to investigate the effect of formamide and low-temperature reactions on reassociation specificity.

Storb (1972) has recently reported that she was unable to observe any difference between the capacities of liver, hyperimmune mouse spleen, or MOPC-104E DNA to hybridize whole cell or microsomal RNA of hyperimmune mouse spleen or MOPC-104E. Three explanations could be advanced to explain the discrepancy between these results (Storb, 1972) and the experiments reported here. (1) Her hybridization conditions were not as stringent as ours (*i.e.*, 2 × SSC and 67°) and/or high RNA/DNA ratios. (2) We routinely carry out reactions at low temperatures (37°) in the presence of formamide, whereas Storb apparently did not use this reagent and incubates at considerably higher temperatures (67°). It is possible that a significant proportion of the DNA may fall off the filter under the latter reaction conditions and/or formamide may advantageously favor the reaction of certain types of RNA transcripts. (3) The pulse-labeled RNAs used here were whole cell preparations prepared from tumor cells incubated in primary culture. In addition, the RNAs were sonicated early in the extraction procedure as described by Church and McCarthy (1968) to increase their hybridization efficiency. Sonication produces a homogeneous, low molecular weight RNA preparation (unpublished). Storb utilized apparently high molecular weight *in vivo* labeled RNA preparations. Other experiments (Kosky and Krueger, manuscript in preparation) indicated that sonicated, pulse-labeled RNAs extracted from tumor cells in primary culture were superior to pulse-labeled nonsonicated RNA extracted from tumor cells in primary or established cell culture in their capacity to hybridize with homologous tumor DNA as compared to heterologous tumor or liver DNA. The difference in the capacities of these various RNA preparations to hybridize with the different DNAs could be attributed to a molecular weight effect similar to that observed for DNA–RNA hybridization reactions (McCarthy and Church, 1970) and/or RNA synthesis in primary cultures is partially repressed such that only a limited number of RNA species are synthesized.

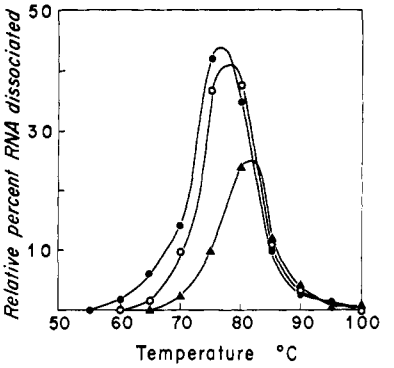

FIGURE 8: Thermal dissociation profiles of hybrids formed by incubating 11.6 μg of pulse-labeled MOPC-173D RNA (800 cpm/μg) with 22.4 μg of MOPC-173D DNA for 2 hr (▲), 16 hr (○), and 24 hr (●) in 5 × SSC and 50% formamide at 37°. Specific activity of MOPC-173D RNA was 800 cpm/μg. The amount of RNA dissociated is relative to the total RNA in 24-hr hybrids.

It must be cautioned that the experiments reported here were carried out with sonicated, total pulse-labeled RNA and we are therefore unable to ascribe the apparent increased hybridization of tumor RNA transcripts to any particular group of genes. Nevertheless, it is possible that the observed differences in tumor nucleic acids are associated with the production of immunoglobulins and that the specialization required of cells of the lymphoid series to produce immunoglobulins involves somatic amplification of the immunoglobulin genes. Little and Donahue (1970) found that RNA from antigen-stimulated rabbit lymph nodes hybridized to a greater extent with lymph node DNA than liver DNA whereas nonimmune lymph node RNA was able to discriminate between the two DNAs. Gene duplication could occur during the lymphoid cell proliferative response that results from antigenic stimulation. It has been reported that specific antigenic

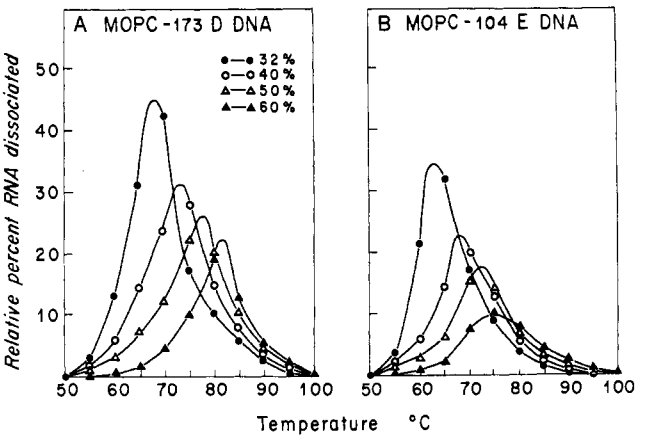

FIGURE 9: Thermal dissociation profiles of hybrids formed by incubating 22.4 μg of pulse-labeled MOPC-173D RNA (800 cpm/μg) with 44.8 μg of MOPC-173 DNA or 44.5 μg of MOPC-104E DNA in the presence of 5 × SSC and 32, 40, 50, or 60% formamide at 37° for 20 hr. The amount of RNA dissociated is relative to the total RNA in MOPC-173 RNA–MOPC-173D DNA hybrids formed in 32% formamide.

stimulation modified the pattern of DNA replication in antigenically stimulated guinea pig lymph node cells (Souleil and Panijel, 1970; Gottlieb *et al.*, 1970). Souleil and Panijel (1970) interpreted their results as the preferential replication of a large number of genes concerned with the process of antibody formation. On the other hand, Gottlieb *et al.* (1970) suggest that the appearance of the "new" DNA population is newly replicated DNA attached to a portion of the nuclear membrane, the newly replicated DNA being related to the early events occurring after antigen challenge but not necessarily associated with the selective replication of specific genes.

Further experimentation is obviously necessary to determine whether gene amplification is involved in immunoglobulin synthesis and the mechanism by which it occurs during the differentiation process associated with the synthesis of immunoglobulin.

Acknowledgment

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