*HYBRIDIZATION OF CHROMOSOMAL RNA TO NATIVE DNA**

By Isaac Bekhor, James Bonner, and Grace Kung Dahmus

DIVISION OF BIOLOGY, CALIFORNIA INSTITUTE OF TECHNOLOGY, PASADENA

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Abstract and Summary.—Chromosomal RNA, which is associated with chromosomal proteins in the chromosomes of higher organisms, possesses the ability to hybridize to homologous native DNA. The proportion of native DNA thus hybridized is similar to the proportion of denatured DNA which hybridizes with chromosomal RNA, and both are similar to the proportions of chromosomal RNA and DNA in native chromatin.

We have found that chromosomal RNA possesses the ability to hybridize to homologous native DNA. Chromosomal RNA is a particular class of RNA distinguished by short chain length (40–60 nucleotides in length in different organisms),[1, 2] high (8–10 mole %) content of dihydroprimidine (dihydrouridylic acid in pea, cow, and chick, dihydroribothymidylic acid in rat),[3] and by its association with DNA in the chromosomes of higher organisms. In such chromosomes, chromosomal RNA is on the one hand bound to DNA in RNase-resistant form,[4] and on the other is bound covalently to chromosomal protein.[5] We have previously shown that chromosomal RNA is sequence-heterogeneous and hybridizes to the extent of 3–5 per cent to homologous denatured DNA, the exact percentage depending upon the organism involved.[2, 6] We show below that chromosomal RNA hybridizes to this same extent with homologous native DNA.

Materials and Methods.—Preparation of chromatin: All of the experiments reported below were done with chromosomal RNA of rat Novikoff ascites tumor. The chromatin was prepared from tumor cells by the method of Dahmus and McConnell.[2] The ascites cells were removed from the rat on the fifth to sixth day after infection with the tumor and were immediately washed by pelleting for 6 min at 700 *g* in TNKM buffer (0.05 *M* tris, pH 6.7, 0.13 *M* NaCl, 0.025 *M* KCl, 0.0025 *M* $MgCl_2$), followed by a single pelleting from 4×-diluted TNKM buffer. The cells were then lysed in deionized water and the nuclei pelleted at 1500 *g* for 15 min. The nuclear pellet was homogenized by hand in a Teflon homogenizer and in 0.01 *M* tris, pH 8.0. The chromatin was pelleted and washed by repelleting four times from the same buffer. The resulting crude chromatin was then purified by layering it on 1.7 *M* sucrose and pelleting it for 2 hr at 22,000 rpm in the Spinco SW25 rotor.

Preparation of chromosomal RNA: The sucrose density gradient-purified chromatin, dialyzed against 0.01 *M* Tris to free it of sucrose, was dissolved in 4 *M* CsCl and centrifuged for 18 hr at 35,000 rpm in the Spinco no. 40 rotor. Under these conditions, DNA and messenger RNA pellet, while chromosomal proteins, together with chromosomal RNA, float as a skin or pellicle.[6] The chromosomal protein-RNA skin was next washed free of CsCl by pelleting it from 70% EtOH, suspended in 0.01 *M* tris pH 8.0, and protein-digested at 37°C with preincubated (90 min, 37°C) pronase, final concentration 1 mg/ml, for 4 hr at 37°C. Any undigested and aggregated protein was centrifuged off; the RNA phenol-extracted in the cold and then precipitated from 0.2 *N* KAc by addition of 2 vol of EtOH. The RNA, redissolved in 7 *M* urea containing 0.01 *M* Tris, pH 8.0, and 0.2 *N* in NaCl, was next applied to a DEAE Sephadex A-25 column. The column was then developed with a linear gradient of NaCl, 0.2–1.0 *M*, all in 7 *M* urea, and 0.01 *M* in Tris, pH 8.0. Chromosomal RNA elutes at a NaCl concentration of 0.55 *M*.[6] The peak fractions were pooled, precipitated with EtOH, and stored in liquid nitrogen until used.

For the preparation of P^{32}-labeled chromosomal RNA, the rats bearing the ascites tumor were supplied with P^{32}-labeled orthophosphate (4 mc/rat) 1 day before being killed. RNA so prepared was totally hydrolyzable with boiled pancreatic RNase or with KOH (0.3 *N*, 18 hr at 37°C) and exhibited a sedimentation constant of 3.4*S*. Chromosomal RNA was also isolated by the method of Dahmus and McConnell[2] from cytoplasmic and/or nuclear supernatants. Following lysis of the ascites cells in water, and pelleting of the nuclei, the supernatants from the water washes were made 0.2 *N* in KAc and 2 vol of EtOH then added to precipitate cytoplasmic RNA's. The Tris supernatants from homogenized nuclei were also pooled and precipitated for similar treatment. Chromosomal RNA (cRNA) from these fractions was purified on DEAE-Sephadex as described above.

Preparation of DNA: DNA was prepared from chromatin dissolved and pelleted from 4 *M* CsCl. The DNA pellet, redissolved in 1 *N* NaCl, was deproteinized by the Sevag method according to Marmur.[7] The thus purified DNA was then treated with ribonuclease, 100 μg/ml, 1 hr at room temperature; pronased with preincubated pronase, 1 mg/ml for 1 hr at 37°C, to remove any residual ribonuclease; and again phenol-extracted and ethanol-precipitated. The resulting DNA containing less than 0.2% protein was dialyzed against 0.01 *M* Tris, pH 8.0, to free it of phenol and stored in liquid nitrogen until use. DNA was also prepared by the Sevag method from rat liver chromatin purified according to the method of Marushige and Bonner.[8] DNA was prepared from *E. coli* by the method of Marmur.[7]

Hybridization: Hybridization of RNA to DNA was done according to the low-temperature method of Bonner, Kung, and Bekhor.[9] The hybridization reaction mixture consisted of 4 *M* urea (unless otherwise specified), 1 × SSC (standard saline citrate, 0.15 *M* NaCl–0.015 *M* Na citrate). Incubation was for 24 hr at a temperature of 1°C. Counting of hybridized labeled RNA was done with a Beckman liquid scintillation spectrometer.

Results.—Formation and isolation of the hybrid: The interaction of chromosomal RNA with DNA is exemplified by the data of Figure 1. In these experiments, chromosomal RNA was incubated with homologous (rat) or heterologous

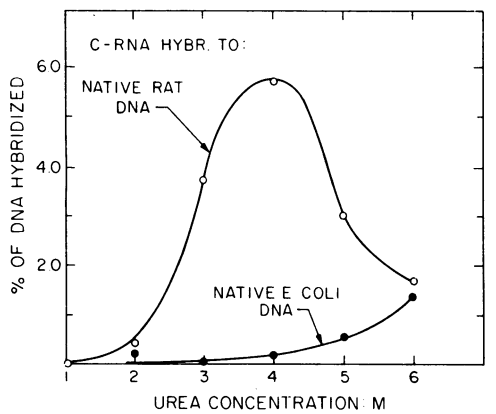


FIG. 1.—Fraction of rat liver native DNA hybridized by rat ascites tumor chromosomal RNA as a function of urea concentration in the hybridization reaction mixture. Reaction mixtures contained 1 × SSC and an RNA/DNA ratio of 5 throughout. Hybridization for 24 hr at 1°C. The RNase-resistant, DNA-associated RNA was then isolated as described in *Materials and Methods*.

(*E. coli*) native DNA at 1°C and in solutions containing increasing concentrations of urea. After incubation (24 hr), the DNA was pelleted by centrifugation, the pellet redissolved and treated with ribonuclease (50 μg/ml) for one hour at room temperature to remove RNA not specifically associated with DNA, and the complex precipitated with EtOH and redissolved in 0.01 *M* Tris, pH 8.0. The data of Figure 1 show that under optimum hybridization conditions, and in the presence of a large excess (saturating) amount of RNA, an amount of RNA equal to

approximately 4 per cent of DNA becomes associated with homologous DNA in ribonuclease-resistant form. Such association does not occur with heterologous DNA and is also lessened at urea concentrations lower or higher than the optimum, 4 *M*. That urea solutions in the range 4–5 *M*, and containing 1 × SSC, constitute a hybridizing condition for the association of RNA with homologous denatured DNA has been shown earlier.[10] It may be noted that in urea solution native DNA is reversibly altered in the direction of melting. Thus, as shown in Figure 2, the melting temperature of the DNA is lowered and its hyperchromicity (OD_{260} in urea solution when completely melted minus that in same solution at 20°C) diminished.

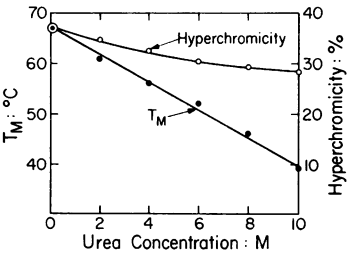


FIG. 2.—Melting temperature and hyperchromicity of rat liver native DNA as a function of urea concentration. DNA was dissolved in urea of stated concentration containing 1/100 SSC and melted in a Gilford recording spectrophotometer. Hyperchromicity refers to increase in optical density on melting above optical density in same solution before melting.

In the above mode of preparation, pelleting serves to separate the DNA-RNA complex from the bulk of the nonhybridized RNA, and RNase treatment serves to remove from the complex any RNA not specifically associated with DNA in ribonuclease-resistant form. Both of these functions can be accomplished by chromatography on hydroxyapatite, which serves to retain double-stranded nucleic acid of high molecular weight while permitting single-stranded material to pass through. Thus chromosomal RNA applied to a hydroxyapatite column according to the procedures of Britten *et al.*[11] is completely eluted by 0.12 *M* potassium phosphate buffer; that is, it is not retained by the hydroxyapatite. Native DNA, on the contrary, is eluted only by 0.4 *M* phosphate buffer. Alternatively, selective elution from hydroxyapatite may be achieved at constant buffer concentration (0.12 *M*) by use of an increasing temperature gradient. RNA elutes at 25–40°C, native DNA only at its melting temperature, 80°C, under the present conditions. For this type of experiment the RNA-DNA complex, after incubation under hybridizing conditions and pelleting of the DNA-RNA complex, was applied to a jacketed column of hydroxyapatite and developed with 0.12 *M* phosphate buffer. The temperature of the column was then gradually raised. The data of Figure 3 show that under these conditions free RNA not associated with DNA is eluted at a temperature of 40°C or lower. A further portion of the RNA, however, is retained by the hydroxyapatite and is liberated only at the temperature at which the DNA itself is melted, approximately 80°C. A further portion of the bound RNA is eluted only at a still higher temperature. Under these conditions again approximately 4 per cent as much RNA as DNA is associated in the complex that is retained by hydroxyapatite. As may be seen in Figure 3*b*, no RNA is retained in this way by heterologous *E. coli* DNA, even though the chromosomal RNA and heterologous DNA have been subjected to hybridizing conditions for 24 hours. Figure 3 also

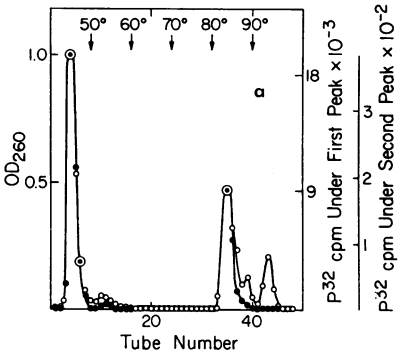
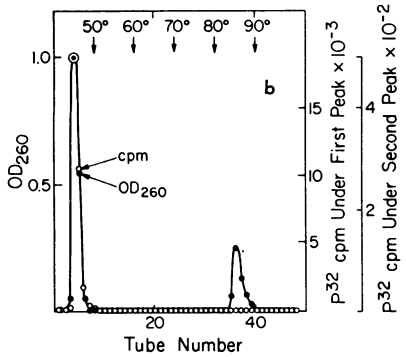
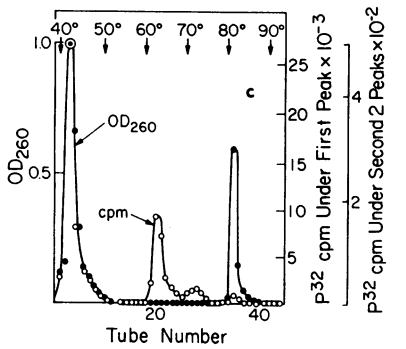


FIG. 3.—Temperature elution profiles from hydroxyapatite column of varied DNA-rat ascites tumor chromosomal RNA mixtures. The RNA/DNA mixtures were first incubated for 24 hr under hybridizing conditions. The reaction mixtures were then applied to the hydroxyapatite column (0.9 × 15 cm) and eluted with 0.12 *M* potassium phosphate buffer, pH 6.5. 5-ml fractions were collected and temperature was increased stepwise in 10° increments as indicated by arrows. Unhybridized RNA elutes from column at or below 40°.

(*a*) Homologous (rat) DNA retains 3.2% as much chromosomal RNA as DNA. The two elute together at temperatures of 80–90°.



(*b*) Heterologous (*E. coli*) DNA retains 0.0% as much rat chromosomal RNA as DNA. Chromosomal RNA is P^{32}-labeled, DNA unlabeled throughout.



(*c*) Heterologous higher-organism DNA (calf) retains 0.13% as much rat chromosomal RNA as DNA. An additional amount of RNA equal to 1.2% of the DNA elutes at temperatures higher than required to elute free RNA but lower than required to melt the DNA.

includes data on the hybridization of ascites tumor chromosomal RNA to DNA less heterologous to rat than *E. coli*, namely to cow DNA. It is noteworthy that cow DNA does retain rat chromosomal RNA to an appreciable extent, although the putative hybrid melts at a substantially lower temperature than does the hybrid with homologous DNA. The data of Figure 4 also show that if chromosomal RNA and homologous native DNA are merely mixed and applied to the column, all of the RNA separates from the DNA. The specific association of chromosomal RNA with native DNA thus requires pre-exposure to hybridizing conditions.

Ability to bind to native DNA is not a general property of RNA molecules. Messenger RNA, for example, does not bind to DNA under conditions suitable for the formation of the chromosomal RNA-DNA complex. To study this matter, RNA was transcribed from rat ascites tumor chromatin with *E. coli* RNA polymerase. Such RNA was then subjected to exclusion chromatography on Biogel P-30 and the retained fractions of mean sedimentation coefficient 3.5*S* pooled and used. RNA thus transcribed hybridizes at saturation with approximately 6 per cent of denatured ascites tumor DNA.[12] When it is incubated under hybridizing conditions with native rat DNA and then subjected to chromatography on hydroxyapatite, all of the RNA elutes as free RNA, and none is retained with the DNA, as is shown in Figure 5.

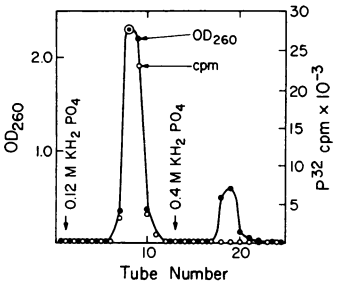


FIG. 4.—The elution of chromosomal RNA and rat native DNA from hydroxyapatite column with stepwise increase in ionic strength and at a constant temperature of 40°C. Chromosomal RNA and rat native DNA were merely mixed prior to layering on the column. RNA is P^{32}-labeled.

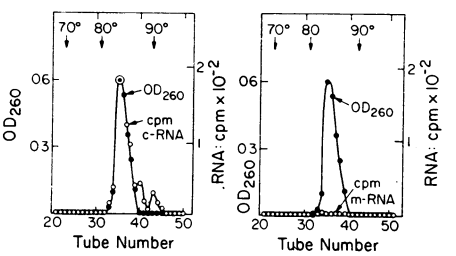


FIG. 5.—Elution of homologous mRNA-DNA hybrid as compared to chromosomal RNA–DNA hybrid from hydroxyapatite column. Procedure as described in legend to Fig. 3 was used. Chromosomal RNA had an $s_{20,w} = 3.45$, and *in vitro* synthesized mRNA had an $s_{20,w} = 3.50$. 0.07% of DNA is associated with mRNA; 3.60% of DNA is associated with chromosomal RNA.

Properties of the hybrid: Chromosomal RNA when hybridized to homologous native DNA remains associated with the DNA, both upon sucrose density gradient centrifugation and on banding in CsCl. For these experiments, the ribonuclease-resistant hybrid was separated by centrifugation as described above. Aliquots of the complex were then subjected to further centrifugation. The data of Figure 6 show that the chromosomal RNA sediments with DNA in a sucrose gradient and bands with it in CsCl (Fig. 7). Control experiments, not shown, verify that chromosomal RNA merely mixed with, but not hybridized to, either homologous or heterologous DNA is separated from the DNA by both of these techniques.

Since chromosomal RNA hybridizes readily to denatured DNA, one may ask whether its association with native DNA may be in part or whole with single-stranded regions of the otherwise double-stranded material. That this is not the case is indicated by the fact that the melting profiles for release of bound chromosomal RNA are different for the two cases. Determinations of the melting profile of chromosomal RNA hybridized to denatured or to native DNA were done on complexes prepared by hybridization in solution (4 *M* urea, 1 × SSC), which were then pelleted, RNased, and reprecipitated as described above. Aliquots were then treated at various temperatures in 1 × SSC, retreated with

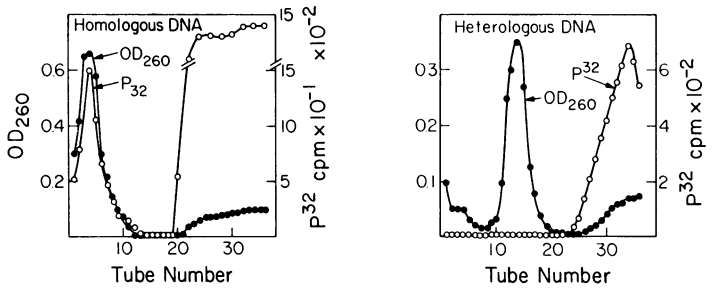
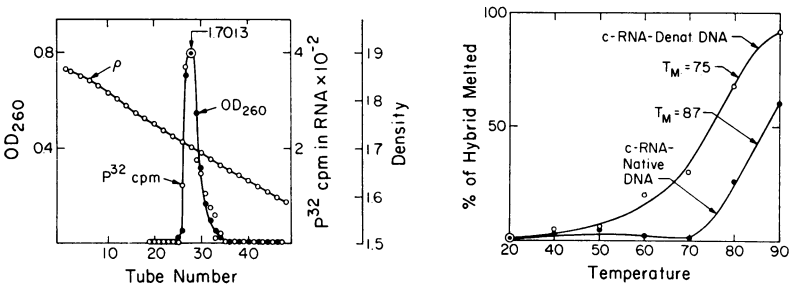


FIG. 6.—Sucrose density gradient centrifugation of rat chromosomal RNA–rat native DNA hybrid, and *E. coli* DNA–rat chromosomal RNA hybrid. Samples of hybridization reaction mixtures of 0.1 ml, of DNA concentration 20 OD in 0.01 *M* Tris, pH 8.0, were layered on 4.5 ml of 5–20% linear sucrose gradient in 0.01 *M* Tris, pH 8.0, and then centrifuged for 20 hr in SW39 rotor at 5°C and at 36,000 rpm. 10-drop fractions were collected from the bottom of the tube and diluted to 0.5 ml with deionized water for OD reading and P^{32} counting. $s_{20,w(\text{rat DNA})} = 20.36S$. $s_{20,w(\text{sheared } E.\ coli \text{ DNA})} = 17.14S$. 1.58% of rat DNA is associated with chromosomal RNA, none with *E. coli* DNA. Chromosomal RNA is P^{32}-labeled.



(*Left*) FIG. 7.—Density gradient equilibrium centrifugation (CsCl) of homologous chromosomal RNA–native DNA complex. The sample containing 20 OD_{260} of previously hybridized DNA was mixed in 4.0 ml of 5.680 *M* optical grade CsCl (ρ = 1.7008 at 25°C). and topped with 1.0 ml of paraffin oil. Centrifugation was for 72 hr at 5°C in SW39 rotor. 7-drop fractions were collected from the bottom of the tube, diluted to 0.5 ml with deionized water for absorbence and P^{32} counting. The unbound chromosomal RNA sediments towards the bottom of the tube under these conditions. A blank tube of CsCl alone was run at the same time for index of refraction readings. 1.0% of DNA is associated with chromosomal RNA, which is P^{32}-labeled.

(*Right*) FIG. 8.—Melting of chromosomal RNA-DNA complex in solution. The hybridization was carried out as described in text. The melting was done in 1 × SSC, and samples were heated at the indicated temperatures for 5 min. Following precipitation and washing of the DNA-RNA complex as described in text, the RNase-resistant precipitate was then counted.

RNase, and the RNase-resistant RNA precipitated with TCA and counted. The melting profiles of the two types of hybrids, shown in Figure 8, are different from each other. The T_M of the hybrid of chromosomal RNA with denatured DNA is, in fact, lower than that for the hybrid with native DNA.

Discussion.—Chromosomal RNA, unlike messenger RNA, possesses the ability to interact and bind to native DNA. The evidence available suggests that this interaction is in part at least by base pairing. Thus the interaction is specific for homologous DNA, implying sequence specificity. The complex is

formed only under hybridizing (as judged by formation of RNA-denatured DNA hybrids) conditions, implying the involvement of base pairing by hydrogen bonds. And finally, the complex once formed can be destroyed by melting as can duplex nucleic acid structures. How can it be that chromosomal RNA can interact with native DNA in a way in which messenger RNA cannot? The most evident clue would appear to be the uniformly high content of dihydropyrimidine in the chromosomal RNA's of all creatures and of all organs of all creatures yet studied.

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