Chromosomal Ribonucleic Acid of Rat Ascites Cells*

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ABSTRACT: When chromatin isolated from rat ascites cells is dissociated in the presence of high salt and the chromosomal proteins separated from the deoxyribonucleic acid by buoyant density centrifugation, a portion of the ribonucleic acid contained in the chromatin remains associated with the chromosomal proteins. This ribonucleic acid (chromosomal ribonucleic acid) is characterized by its small size ($s_{20,w}$ = 3.3 S), its high content of dihydroribothymidine, and its ability to form hybrid with about 4% of the nuclear deoxyribonucleic acid. It appears to have no sequences in common with ascites transfer, ribosomal, or messenger ribonucleic acid. A class of ribonucleic acid with similar properties but associated with the cytoplasmic proteins has also been isolated. This ribonucleic acid hybridizes to about 2% of the nuclear deoxyribonucleic acid and contains very few, if any, sequences not also contained in chromosomal ribonucleic acid. This fraction of ribonucleic acid is however unable to compete with about 50% of the sequences present in chromosomal ribonucleic acid indicating that a large portion of chromosomal ribonucleic acid is confined to the chromatin. A further class of ribonucleic acid associated with the nuclear sap proteins appears to be identical with the ribonucleic acid associated with cytoplasmic proteins.

It was shown earlier that the chromosomal proteins dissociated by salt from pea bud chromatin contain associated RNA of a special class (Huang and Bonner, 1965). This RNA has been assigned the name chromosomal RNA and can be distinguished from previously reported classes of RNA by its small size and high content of dihydrouridine. It has been suggested that chromosomal RNA may play a key role in gene regulation by conferring specificity on the DNA–chromosomal protein interaction (Bonner and Huang, 1966; Bonner and Widholm, 1967; Bekhor *et al.*, 1969; Huang and Huang, 1969).

The following work shows that such an RNA is also associated with the chromosomal proteins of rat ascites cells.

Methods

Growth and Labeling of Cells. The Novikoff ascites tumor line used in the following investigation was maintained by serial transplantation in male albino Sprague–Dawley rats purchased from Berkeley Pacific Laboratories. The transfer was carried out on days 6 or 7 of the cycle. Cells for experimental use were harvested on days 6 and 7.

Uniformly ^{32}P-labeled chromosomal RNA was prepared from cells which had been grown for 24–48 hr in the presence of ^{32}P. Rats, infected 4–5 days prior with tumor, were given an intraperitoneal injection of carrier-free [^{32}P]orthophosphate (Volk Radiochemical Co.) in 0.5 ml of physiological saline (0.15 M NaCl). The standard injection of ^{32}P was 3 mCi/rat; dosages as low as 500 μCi and as high as 5 mCi/rat have been used.

For the preparation of ^{3}H-labeled DNA, 4-day-old tumor-infected rats were each injected with 1 mCi of [^{3}H]thymidine (14,600 μCi/μmole; Nuclear-Chicago) and the cells were harvested 48 hr later.

The tumor cells were purified from the ascites fluid by preferential lysis of the blood cells followed by differential centrifugation. The ascites fluid was diluted with an equal volume of TNKM (0.05 M Tris buffer (pH 6.7)–0.13 M NaCl–0.025 M KCl–0.0025 M $MgCl_2$) and centrifuged for 6 min at 700g in the International refrigerated centrifuge. The cells were then suspended in three volumes of deionized water and immediately centrifuged at 700g. The supernatant was then removed and the pellet was washed by repeated centrifugation until no contaminating erythrocytes were apparent. The purified cells were then used immediately or frozen in ethanol–Dry Ice and stored at −80°.

Preparation of Chromatin. The initial investigations were carried out on chromatin isolated in the presence of EDTA following the procedure of M. Nicolson (personal communication). The second procedure is a direct modification of the method of Marushige and Bonner (1966) and was developed to study the effect of the absence of EDTA on the isolation of chromosomal RNA. The two methods of extraction resulted in purified chromatin of the same chemical composition and a final recovery of about 70–80% of the DNA. The amount of chromosomal RNA associated with each was identical. Because of its simplicity, the following procedure was routinely used. All extraction procedures were carried out at 0–4°.

Isolation of chromatin in the absence of EDTA. The cells were first suspended by hand with the aid of a Teflon homogenizer in 15 volumes of cold deionized water. Nuclei and unlysed cells were then pelleted by

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centrifugation at 1500*g* for 15 min. The pellet was then examined to determine the extent of cell lysis. If lysis was incomplete (*i.e.*, less than 70% of the cells lysed) the water wash was repeated. The crude nuclear pellet was then homogenized by hand with the aid of a Teflon homogenizer in 0.01 M Tris buffer (pH 8.0), stirred slowly for 30 min on a magentic stirrer, and centrifuged at 10,000*g* for 15 min. The chromatin was then washed three to four times by repeated suspension in 0.01 M Tris buffer (pH 8.0) and sedimentation at 10,000*g*. The chromatin at this stage is referred to as crude chromatin. If purified chromatin was to be prepared, the chromatin was suspended in an equal volume of 0.01 M Tris buffer (pH 8.0). Aliquots (5 ml) were then layered onto 25 ml of 1.7 M sucrose containing 0.01 M Tris buffer (pH 8.0) and the top two-thirds of the tube was stirred to form a two-step gradient and centrifuged for 2 hr at 22,000 rpm in the Spinco SW25 rotor. The purified chromatin was recovered as a clear gelatinous pellet.

Chemical Composition. DNA was determined by the diphenylamine reaction as described by Burton (1956) using rat liver DNA as a standard. RNA was determined by the orcinol reaction following the method of Dische and Schwarz (1937) using purified yeast RNA as a standard. Histone was extracted from chromatin with 0.2 N H_2SO_4 at 4° and precipitated with 20% trichloroacetic acid. The amount of protein was determined following the method of Lowry *et al.* (1951) using rat liver histone as a standard. The nonhistone protein content of the acid-insoluble, alkali-soluble material was determined by the same procedure, using bovine serum albumin fraction IV as a standard.

Preparation of Chromosomal RNA. Chromosomal RNA was prepared from both purified and crude chromatin. The purified RNAs isolated from the two sources are identical in size, base composition, and hybridization properties. Crude chromatin was, therefore, routinely used as a starting material for the preparation of chromosomal RNA. The following procedure was carried out at 0–4° with the exception of the steps indicated.

The chromatin pellets were suspended in an equal volume of 0.01 M Tris buffer (pH 8.0) and diluted with two volumes of 6 M CsCl in 0.01 M Tris (Industrial Grade CsCl, American Potash and Chemical Corp.). The resulting solution was extremely viscous and was homogenized for 30 sec at 20 V in a Waring blender to facilitate solution of the chromatin. The solution was then centrifuged for 15 hr at 36,000 rpm in the Spinco 40 rotor. Under these conditions the DNA pellets, while the chromosomal proteins, being buoyant, form a skin at the top of the tube. The skins were removed with a spatula and washed three times with 70% ethanol. The chromosomal proteins were then digested by treatment with 2–4 mg/ml of pronase (pronase, Grade B from Calbiochem, was preincubated for 90 min at 37°) in 0.01 M Tris buffer (pH 8.0) for 4–6 hr at 37°. Sodium dodecyl sulfate was added to a final concentration of 1% followed by the addition of an equal volume of water-saturated phenol containing 0.1% 8-hydroxyquinoline (Kirby, 1962). After shaking for 30 min the phases were separated by centrifugation and the phenol phase was extracted with one-half volume of water. The combined aqueous phase was then reextracted twice with one-half volume of phenol. The nucleic acids were precipitated by the addition of one-tenth volume of 20% potassium acetate and two volumes of 95% ethanol. After 2 hr at −20° the precipitate was recovered by centrifugation, washed once with 70% ethanol, and dissolved in several ml of 0.2 M NaCl–7 M urea–0.01 M Tris buffer (pH 8.0).

The nucleic acids accompanying the chromosomal proteins include some DNA in addition to chromosomal RNA. This DNA was separated from the chromosomal RNA by chromatography on A-25 DEAE-Sephadex. Fractionation was routinely carried out on a 9 mm × 25 cm column, eluted with a linear gradient of NaCl ranging from 0.2 to 1.0 M in the presence of 7 M urea and 0.01 M Tris buffer (pH 8.0). Chromosomal RNA was recovered by precipitation with two volumes of 95% ethanol in the presence of 2% potassium acetate at −20° for 2 hr. The purified RNA was dissolved in and dialyzed against two-times standard saline citrate (0.3 M NaCl and 0.03 M sodium citrate).

Preparation of Extranuclear Protein-Bound RNA. To avoid the possibility of contamination by chromosomal RNA, RNA was isolated from the supernatant from the first water lysis of the cells. This fraction contains no visible nuclei nor does it contain detectable DNA as measured by the diphenylamine method of Burton (1956). It is therefore unlikely that this supernatant is contaminated with a significant amount of chromatin. The supernatant was first precipitated with two volumes of 95% ethanol in the presence of 2% potassium acetate. The precipitate was then dissolved in 0.01 M Tris buffer (pH 8.0) and diluted with two volumes of 6 M CsCl–0.01 M Tris. Under these conditions the majority of ribosomal proteins are dissociated and the RNA pellets in the following centrifugation. Centrifugation, pronase treatment, phenol extraction, and DEAE-Sephadex chromatography were carried out exactly as in the preparation of chromosomal RNA.

The RNA associated with the cytoplasmic proteins and purified by the above procedure elutes from DEAE-Sephadex as a single sharp peak at a NaCl concentration of 0.55 M. It has a sedimentation constant of 3.2–3.5 S and will be referred to as 3S cytoplasmic RNA.

Preparation of t- and rRNA. Total cytoplasmic RNA was isolated from the cytoplasmic fraction by cold phenol extraction following the method of Attardi *et al.* (1966). The RNA was first fractionated on a Sephadex G-100 column (1.5 × 200 cm equilibrated and eluted with two-times standard saline citrate). The r- and tRNA fractions were diluted to a final salt concentration of less than 0.1 M NaCl in 0.05 M sodium phosphate buffer (pH 6.7) and separately chromatographed on methylated albumin kieselguhr as described by Mandell and Hershey (1960). The RNA was eluted with a linear gradient of NaCl, concentrated by pressure dialysis followed by ethanol precipitation, and rechromatographed on Sephadex

G-100. The finally purified RNA was precipitated with ethanol and dialyzed against two-times standard saline citrate.

Preparation of Pulse-Labeled RNA. Pulse-labeled RNA was prepared from *in vitro* labeled cells. The cells were pelleted directly from the ascites fluid, washed once with Eagle's Medium (Eagle, 1959) deficient in phosphate, containing 5% dialyzed calf serum, and suspended in ten volumes of the same medium preheated to 37°. After 5 min neutralized ^{32}P was added (4 mCi of ^{32}P was added for each rat sacrificed) and the incubation was continued at 37° for 10 min. The cells were then diluted with cold TNKM and washed free of contaminating erythrocytes by differential centrifugation at 4°. A portion of the cells was extracted with phenol at 66° according to the method of Scherrer and Darnell (1962). Nucleic acids associated with the chromosomal proteins were extracted from the remainder of the cells as previously described in this section. The final nucleic acid extracts were exhaustively dialyzed against 0.05 M sodium phosphate buffer (pH 6.7) and chromatographed on methylated albumin kieselguhr as described by Mandell and Hershey (1960). The radioactivity of each fraction was determined by evaporation of 0.2-ml aliquot on a planchet and counted in a Nuclear-Chicago D-47 gas-flow counting system.

In Vivo Methylation and Extraction of Labeled RNA. Each rat, infected 6 days previously with tumor, was given an intraperitoneal injection of 50 μCi of [^{14}C]-methylmethionine (12 μCi/μmole purchased from New England Nuclear Corp.). After 5 hr the rats were sacrificed and the tumor was harvested and washed as previously described. Chromosomal, 3S cytoplasmic, and tRNA were prepared from the same cells. Chromosomal and 3S cytoplasmic RNA were prepared as previously described in this section. tRNA was prepared from a portion of the cytoplasmic fraction used in the preparation of 3S cytoplasmic RNA by phenol extraction according to the method of Attardi *et al.* (1966). All nucleic acid fractions were purified by chromatography on A-25 DEAE-Sephadex and developed with a linear gradient of NaCl from 0.2 to 1.0 M containing 0.01 M Tris (pH 8.0) and 7 M urea. tRNA elutes at a salt concentration of 0.53 M NaCl, rRNA is irreversibly bound to the column. Each fraction (1-ml aliquots) was precipitated with cold 10% trichloroacetic acid in the presence of 1 mg of carrier yeast RNA and acid-insoluble material collected by filtration on trichloroacetic acid presoaked membrane filters (Schleicher & Schnell B-6). The filters were then washed with 10 ml of cold 10% trichloroacetic acid, dried, and counted in the Beckman liquid scintillation spectrometer.

Preparation of in Vitro Synthesized RNA. RNA was synthesized *in vitro* by *Escherichia coli* RNA polymerase (f_4 of Chamberlin and Berg, 1962) using ascites-purified chromatin or ascites DNA as template. The complete incubation mixture for RNA synthesis contained in a final volume of 10 ml: 400 μmoles of Tris buffer (pH 8.0); 40 μmoles of $MgCl_2$; 10 μmoles of $MnCl_2$; 120 μmoles of β-mercaptoethanol; 8 μmoles each of GTP, UTP, and CTP; 8 μmoles of ATP-8-^{14}C (2 μCi/μmole); ascites DNA (100 μg) or chromatin (an amount containing 200 μg of DNA); and 1 mg of f_4. After 2-hr incubation at 30°, sodium dodecyl sulfate was added to a final concentration of 1% and the sample was extracted at 4° with an equal volume of water-saturated phenol containing 0.1% 8-hydroxyquinoline. Nucleic acids were precipitated from the aqueous phase with two volumes of ethanol in the presence of 2% potassium acetate at −20° for 2 hr. The precipitate was dissolved in 4 ml of TKM (0.05 M Tris buffer (pH 7.4)–0.025 M KCl–0.0025 M $MgCl_2$) and treated with 30 μg/ml of electrophoretically purified DNase (Worthington Biochemical Corp.) at 25° for 1 hr. The sample was again phenol extracted, the nucleic acid was precipitated, and dissolved in and dialyzed against two-times standard saline citrate.

Preparation of DNA. Rat ascites DNA was prepared from crude chromatin by the procedure of Marmur (1961) followed by an additional step including pronase digestion and phenol extraction. After RNase treatment (20 μg/ml) for 2 hr at 37° (RNase had been previously heated at 80° for 15 min), the DNA was incubated for 2 hr at 37° with 40 μg/ml of pronase (pronase, Grade B from Calbiochem, previously autodigested for 90 min at 37°). After digestion, the solution was made 1% in sodium dodecyl sulfate and extracted with an equal volume of water-saturated phenol. The extraction was repeated twice in the absence of sodium dodecyl sulfate with 0.5 volume of phenol. The DNA was then spooled from the aqueous phase with the addition of two volumes of 95% ethanol, dissolved in $\frac{1}{100}$ standard saline citrate, and reprecipitated with 0.54 volume isopropyl alcohol in the presence of 0.3 M sodium acetate plus 0.1 mM Na_2EDTA (pH 7.0) (Marmur, 1961). The purified DNA was then dissolved in and dialyzed against $\frac{1}{100}$ standard saline citrate. RNase activity was monitored by incubation of the DNA with ^{32}P-labeled RNA in two-times standard saline citrate at 37° for 20 hr. DNA preparations which resulted in any loss of trichloroacetic acid precipitable counts were subjected to additional phenol extractions until no detectable RNase activity remained.

DNA–RNA Hybridization. Denatured DNA was immobilized on nitrocellulose filters (Schleicher & Schuell B-6, 25 mm) as described by Gillespie and Spiegelman (1965). DNA filters were prepared in the presence of six-times standard saline citrate and contained 40 μg of denatured DNA. Trace amounts of ^{3}H-labeled ascites DNA were added to allow easy monitoring of the DNA in subsequent steps.

Hybridization was carried out at 66° or at 25° in the presence of 30 vol % formamide (Bonner *et al.*, 1967). The specificity of hybrid formation is identical under both conditions as measured by the specificity for ascites DNA and the total amount of hybrid formed. Before the addition of the filters the hybridization solution containing the RNA was heated at 95° for 10 min and cooled to 0–4°. Each vial contained two DNA filters and one blank filter in a volume of 1 ml and a final salt concentration of two-times standard saline citrate. At the end of the incubation the filters

TABLE I: Chemical Composition of Rat Ascites Chromatin.

Component	Mass Ratio	
	Crude Chromatin	Purified Chromatin
DNA	1.00	1.00
RNA		
Chromosomal	0.04	0.02
"Free"	0.17	0.11
Total	0.21	0.13
Histone		1.16
Nonhistone		1.00

TABLE II: Base Hydrolysis and RNase Digestion of Fractions I and II.[a]

Treatment	Fraction	Input (cpm)	Acid Soluble (cpm)	% Acid Soluble
0.3 N KOH, 18 hr, 37°	I	1748	1742	99.7
	II	1981	56	2.8
RNase,[b] 18 hr, 37°	I	1648	1608	97.6
	II	9499	780	8.2

[a] All samples were made 5% in $HClO_4$ at the end of the incubation and the radioactivity of the supernatant was determined. [b] RNase digestion was carried out in two-times standard saline citrate in the presence of 20 μg/ml of pancreatic RNase.

were removed, rinsed with two-times standard saline citrate in a large beaker, and washed on each side by filtration with 50 ml of two-times standard saline citrate. RNase digestion was carried out at 25° in a large volume of two-times standard saline citrate containing 20 μg/ml of preheated pancreatic RNase (about 24 filters/100 ml). After 1-hr incubation, the filters were rinsed with two-times standard saline citrate and again washed on each side with 50 ml of two-times standard saline citrate. The filters were dried in a vacuum oven and counted in a Beckman liquid scintillation spectrometer.

Results

General Properties of Ascites Chromosomal RNA. Purified chromatin of Novikoff ascites tumor cells is composed of DNA, chromosomal proteins, both histone and nonhistone, and RNA in the mass ratios shown in Table I. When chromatin is fractionated by separation of the DNA from the chromosomal proteins by salt dissociation and buoyant density centrifugation in CsCl, a portion of the nucleic acid remains associated with the chromosomal proteins. Purification of this nucleic acid as described under Methods and chromatography on DEAE-Sephadex results in the two peak elution profiles shown in Figure 1. Both peaks exhibit a characteristic nucleic acid ultraviolet absorption spectra with absorption maxima at 257 mμ. As shown in Table II the first peak which elutes at a NaCl concentration of 0.55 M, is totally base labile, and is sensitive to RNase; the second peak, which elutes at 0.65 M NaCl, is not hydrolyzed by base and is resistant to RNase. The RNA contained in the first peak is referred to as chromosomal RNA and represents only a fraction of the RNA contained in the chromatin. The ultraviolet absorption spectrum of chromosomal RNA is shown in Figure 2.

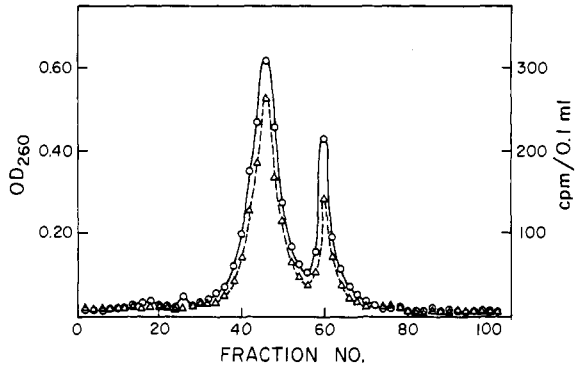

FIGURE 1: Elution profile of uniformly ^{32}P-labeled chromosomal RNA and DNA from DEAE-Sephadex. Nucleic acid eluted with a linear gradient of NaCl from 0.2 to 0.1 M in the presence of 7 M urea and 0.01 M Tris buffer (pH 8.0). ○—○, OD_{260}; △---△, ^{32}P counts per minute.

The remainder of the RNA associated with chromatin pellets with the DNA and is referred to as "free" RNA. The nature of this "free" RNA is not known.

TABLE III: Nucleotide Composition of Different RNA Fractions of Rat Ascites Cells.[a]

Species of RNA	Mole Per Cent					
	A	C	Dihydro-ribothymidine	U	G	G + C
Chromosomal	17.4 ± 1.1	24.2 ± 0.8	8.1[b]	19.4 ± 1.8	30.9 ± 1.9	55.1
Ribosomal	18.1 ± 0.4	28.4 ± 0.6		18.2 ± 0.7	35.4 ± 0.5	63.8
Transfer	17.5	26.0		23.4	33.0	59.0

[a] The base composition of the various RNA fractions was determined by alkaline hydrolysis of the RNA in 0.3 N KOH at 37° for 18 hr and separation of the nucleotides on a column of Dowex 1-X8, formate form (Cohn and Volkin, 1953). [b] Taken from Jacobson and Bonner (1968).

TABLE IV: Degree of Methylation of Various RNA Fractions.

Species of Nucleic Acid	Methylation[a] (cpm/mg of RNA × 10^{-2})	% Methylation Rel to tRNA
tRNA	387.0	100
Chromosomal RNA	77.0	20
3S RNA	65.0	17
Fraction II (DNA)	6.5	1.7

[a] Average specific activities of the peak fractions. In the case of tRNA the value reported is the average of the fractions at the leading edge of the peak.

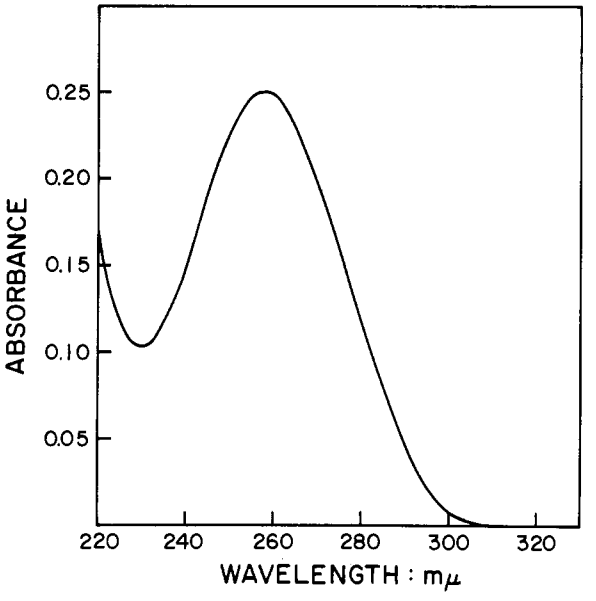

FIGURE 2: Absorption spectrum of purified rat ascites chromosomal RNA in 0.01 M Tris buffer (pH 8.0).

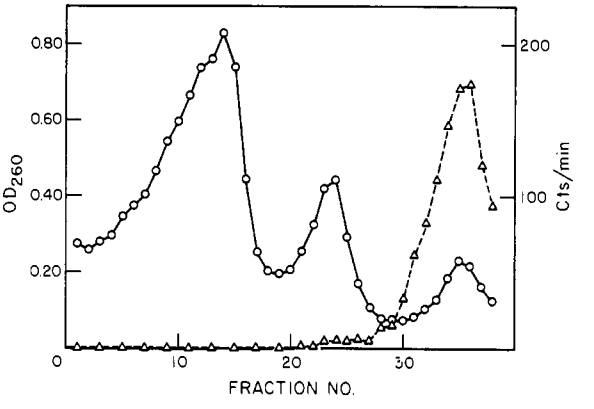

FIGURE 3: Sedimentation pattern of ^{32}P-labeled ascites chromosomal RNA in the presence of total ascites RNA. Sucrose (5–20%) gradient in the presence of 0.01 M sodium acetate buffer (pH 5.1)–0.1 M NaCl. Centrifugation at 39,000 rpm at 4° for 4.5 hr in the Spinco SW39 rotor. ○—○, OD $_{260}$; △--△, ^{32}P counts per minute.

The fact that chromosomal RNA elutes from DEAE-Sephadex as a single sharp peak indicates that it is composed of molecules of a relatively homogeneous chain length (Hall *et al.*, 1965). The pattern obtained from sucrose density gradient centrifugation of labeled chromosomal RNA in the presence of total ascites cytoplasmic RNA is shown in Figure 3. Chromosomal RNA moves as a sharp band and under these conditions is not distinguishable from tRNA. Analytical band velocity sedimentation gives an $s_{20,w}$ of 3.3 S. Equilibrium sedimentation in the Spinco Model E, according to the method of Van Holde and Baldwin (1958), yields a molecular weight of 10,140 daltons (D. Brutlag, personal communication).

The chromosomal RNA from pea cotyledon and

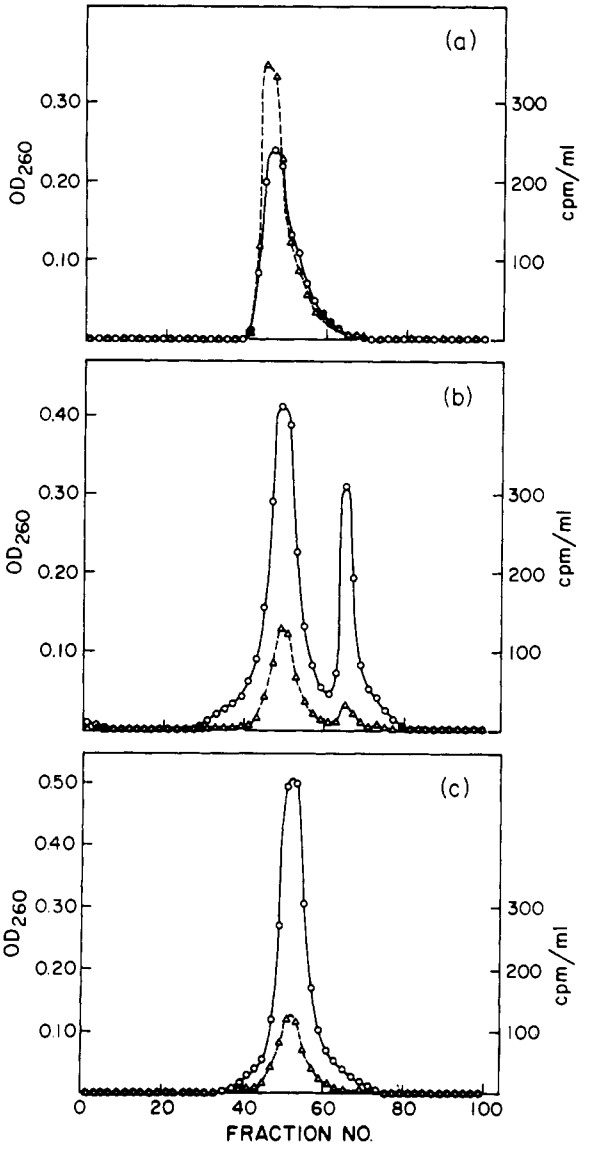

FIGURE 4: Elution profile of *in vivo* ^{14}C-methylated ascites tRNA (a), chromosomal RNA (b), and 3S cytoplasmic RNA (c) from DEAE-Sephadex. ○—○, OD_{260}; △--△, ^{14}C counts per minute.

chick embryo is characterized by a relatively high content of dihydropyrimidine; 8.5 mole % for pea cotyledon (Jacobson and Bonner, 1968) and 9.6 mole % for chick embryo (Huang, 1969). Dihydrouridine has been identified in pea bud and dihydroribothymidine in rat ascites cells. The base composition of ascites chromosomal RNA is presented in Table III along with the compositions of ascites t- and rRNA.

The different classes of RNA can be distinguished by their different content of methylated bases. The mole per cent of methylated bases contained in the various classes of HeLa cell RNA is as follows: tRNA = 8.3, 18S rRNA = 2.1, and 28S rRNA = 1.4 (Brown and Attardi, 1965). The extent of methylation of chromosomal RNA was measured by exposing the cells to [^{14}C]methylmethionine and measuring the extent of incorporation of the label into RNA. Incorporation into chromosomal RNA was compared with that into t- and 3S cytoplasmic RNA. The various fractions of RNA were prepared as described under Methods and purified by chromatography on DEAE-Sephadex. The elution profiles of transfer, chromosomal, and 3S cytoplasmic RNA are shown in Figure 4. In the case of chromosomal and 3S cytoplasmic RNA (4b,c) the specific activity throughout the peak is constant. In the case of tRNA (4a), however, the leading edge of the peak clearly has a higher specific activity than the remainder. This may be due to the presence of small amounts of 3S cytoplasmic RNA which elute slightly behind tRNA. The specific activities of the various fractions of RNA are listed in Table IV. The leading edge of the tRNA peak contains five times the [^{14}C]methyl group activity of the chromosomal or 3S cytoplasmic RNA fractions. The degree of methylation of 3S cytoplasmic RNA is probably not significantly different from that of chromosomal RNA. Chromosomal RNA is therefore methylated to a degree comparable with rRNA but clearly much less than tRNA.

These results make it unlikely that chromosomal RNA is a degradation product of tRNA, for if this were the case, we would expect the former to be methylated to the same degree as the latter.

Nascent RNA may be closely associated with the chromosomal proteins and may be isolated by the procedure used for the preparation of chromosomal RNA. The possible relationship between these classes of RNA and chromosomal RNA was investigated by comparing the amount of incorporation of ^{32}P into various classes of RNA following a short pulse of label. Ascites cells were therefore subjected to a 10-min pulse of ^{32}P and chromosomal and total RNA were extracted as described under Methods. The RNA fractions were chromatographed on methylated albumin kieselguhr and the specific activities of the different RNA species was determined. (Independent experiments in which chromosomal RNA was cochromatographed with total nucleic acid extracts have shown chromosomal RNA to elute in the region of tRNA.) The elution profiles are shown in Figure 5. In Figure 5a, total RNA, the first ultraviolet-absorbing peak is tRNA, the second rRNA. Rapidly labeled mRNA elutes at the tail end of the ribosomal peak as is apparent from the high specific activity in that region. Comparing this profile with that of chromosomal RNA (Figure 5b), it is clear that chromosomal RNA is not rapidly labeled as is mRNA but is synthesized at a rate comparable with that of tRNA. (The presence of DNA incompletely resolved from chromosomal RNA accounts for the decreased specific activity in fractions 70–100.)

Hybridization of Ascites Chromosomal RNA to Nuclear DNA. The data contained in Table V show that the hybridization of ascites chromosomal RNA is specific for rat ascites DNA. About $\frac{1}{6}$ the amount of hybrid is formed with calf thymus DNA and between $\frac{1}{10}$ and $\frac{1}{20}$ the amount with pea DNA.

The per cent of DNA hybridized in the presence of increasing amounts of chromosomal RNA is shown in

TABLE V: Hybridization of Rat Ascites Chromosomal RNA to Various DNAs.

Expt	DNA Source	Ascites Chromosomal RNA Input (μg)	Hybridized (μg of RNA/filter)[a]	μg of RNA Hybridized/μg of DNA $\times 10^2$
1	Ascites	60	0.521	1.28
	Pea embryo	60	0.026	0.06
	Sea urchin	60	0.011	0.03
2	Ascites	27	0.254	0.72
	Calf thymus	27	0.052	0.13
	Pea embryo	27	0.025	0.08

[a] Heterologous DNA filters of expt 1 contained 2 μg of ^{3}H-labeled ascites DNA. The amount of RNA expected to be hybridized to this amount of DNA has been subtracted (0.08 μg). The DNA filters of expt 2 contained no labeled DNA and no correction has been made. Incubation at 66° for 10 hr.

Figure 6a. The double-reciprocal plot presented in Figure 6b shows that, at an infinite RNA concentration, 3.8% of the DNA would be expected to be hybridized. The percentage of DNA hybridized at saturation, by different preparations of chromosomal RNA, ranged from 3 to 4%. The kinetics of hybrid formation and the final saturation level were not changed when the hybridization was performed at 25° in the presence of 30% formamide. Additional purification of chromosomal RNA by chromatography on methylated albumin kieselguhr or Sephadex G-50 did not alter its level of hybridization.

The level of hybridization obtained with ascites t- and rRNA is shown by the data contained in Table VI. For comparison, the table also includes data on the hybridization of chromosomal RNA. Since concentrations of t- and rRNA up to 600 μg/ml do not completely saturate the DNA, it seems unlikely that this interaction represents the specific hybridization of t- or rRNA. (The hybridization may be due to the presence of trace amounts of mRNA.) The specific activities of the t- and rRNA (1000–2000 cpm/μg) are not sufficiently high to allow an accurate measure of such low levels of hybridization. It is clear however that chromosomal RNA has very different hybridization properties than either t- or rRNA.

Competition between Labeled Chromosomal and Unlabeled t-, r-, and in Vitro Generated mRNA. Neither transfer nor rRNA compete with chromosomal RNA in the hybridization to ascites DNA (Table VII). This result is not surprising since both t- and rRNA are capable of forming hybrids with less than 0.06% of the DNA. rRNA, however, when present in large amounts relative to chromosomal RNA, does intefere with the hybridization of chromosomal RNA. This appears to be due to interaction between ribosomal and chromosomal RNA.

The interference of rRNA in the hybridization of chromosomal RNA made it impossible to carry out competition experiments with pulse-labeled RNA. It has been snown by Paul and Gilmour (1966) and by

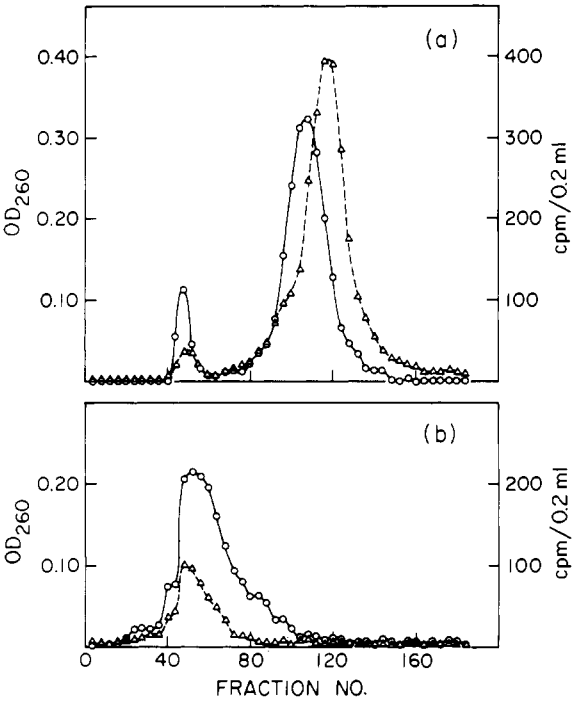

FIGURE 5: Elution profile of ^{32}P pulse-labeled total ascites RNA (a) and chromosomal RNA (b) from methylated albumin kieselguhr. ○—○, OD_{260}; △--△, ^{32}P cpm/0.2 ml.

Smith *et al.* (1969) that the RNA, generated by chromatin *in vitro*, possesses complete sequence homology with the mRNA of the tissue from which the chromatin was isolated. We have thus compared chromosomal RNA with the RNA generated by *E. coli* RNA polymerase with ascites purified chromatin as template. The RNAs generated from pea embryo chromatin and ascites DNA were also assayed for their ability to compete with chromosomal RNA in hybridization to denatured DNA. The results of such an experiment are presented in Table VIII. The addition of ten times the amount of RNA generated from either ascites or

TABLE VI: Hybridization of Rat Ascites Chromosomal, t- and rRNA to Ascites Nuclear DNA.[a]

Species of Ascites RNA	Sp Act. (cpm/μg)	Input RNA (μg)	cpm Hybridized/Filter	Hybridized RNA (μg/filter)	μg of RNA Hybridized/μg of of DNA × 10^2
Chromosomal	1007	53	367	0.365	0.91
	1615	225	1246	0.740	2.16
Transfer	1727	307	40	0.024	0.058
Ribosomal	1409	246	34	0.024	0.058

[a] Incubation at 25° for 10 hr in the presence of 30% formamide.

TABLE VII: Hybridization Competition between Labeled Chromosomal RNA and Unlabeled Ascites t- and rRNA.[a]

μg of ^{32}P Ascites Chromosomal RNA	Competing RNA		Total RNA/^{32}P Chromosomal RNA	μg of ^{32}P RNA Hybridized/Filter	% Labeled Hybrid Remaining
	Species	Amount (μg)			
390			1	0.767	100
390	Transfer	1108	3.8	0.795	103
390	Ribosomal	1108	3.8	0.792	103
390	Chromosomal	593	2.5	0.580	76
390	Chromosomal	1108	3.8	0.382	50

[a] Incubation at 66° for 10 hr.

TABLE VIII: Hybridization Competition between Labeled Chromosomal RNA and *in Vitro* Generated RNA.[a]

μg of ^{32}P Ascites Chromosomal RNA	Competing RNA		Total RNA/^{32}P Chromosomal RNA	μg of ^{32}P RNA Hybridized/Filter	% Labeled Hybrid Remaining
	Species (Template)	Amt (μg)			
60				0.384	100
60	*In vitro* RNA (ascites chromatin)	540	10	0.380	98.9
60	*In vitro* RNA (pea cotyledon chromatin)	540	10	0.396	103.0
60	*In vitro* RNA (ascites DNA)	540	10	0.348	91.0
60	Ascites chromosomal RNA	540	10	0.104	27

[a] Incubation at 25° for 10 hr in the presence of 30% formamide.

pea embryo chromatin had no effect on the level of hybridization of chromosomal RNA while the same amount of added homologous chromosomal RNA caused a 73% reduction in the amount of hybrid formed. RNA generated from ascites DNA slightly reduced the level of hybrid formation. From these data it is clear that ascites chromosomal RNA and ascites messenger RNA, synthesized *in vitro*, have no sequences in common. The low level of competition observed with RNA transcribed from DNA is probably due to the synthesis of small amounts of chromosomal RNA.

Sequence Homology between Chromosomal RNA and 3S RNA. The data of Figure 7 show that 3S cytoplasmic RNA hybridizes at saturation with about 1.8% of denatured ascites nuclear DNA.

The competition curve obtained when ^{32}P-labeled chromosomal RNA is hybridized in the presence of increasing amounts of unlabeled homologous or 3S cytoplasmic RNA is shown in Figure 8. The data of Figure 8 show that unlabeled 3S cytoplasmic RNA is an effective competitor for about 50% of the chromosomal RNA binding sites. This result is in agreement with the hybridization saturation curve obtained for 3S cytoplasmic RNA in which it saturated about one-half the amount of DNA as did chromosomal RNA. That this competition is in fact specific site competition, and

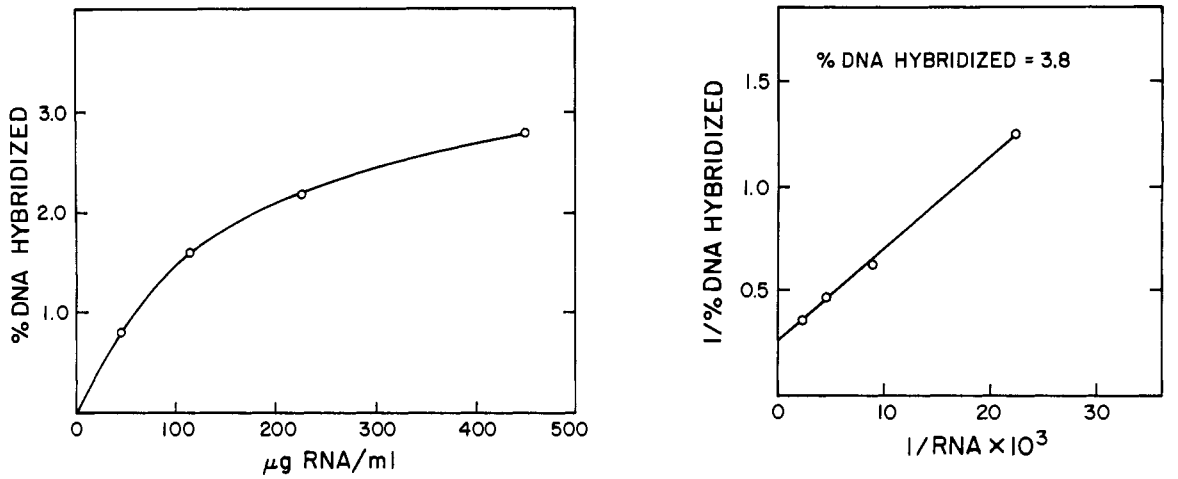

FIGURE 6: Hybridization of ascites chromosomal RNA to ascites nuclear DNA. Incubation at 66° for 10 hr. (a) Hybridization as a function of input RNA concentration. (b) Double-reciprocal plot of DNA saturation curve.

TABLE IX: Nucleotide Composition of Chromosomal RNA from Various Sources.

Source of RNA	Mole Per Cent						
	A	C	Dihydro-pyrimidine	U	G	% G + C	Pu/Pyr
Rat ascites[a]	17.4	24.2	8.1	19.4	30.9	53.9	0.93
Pea bud[b]	39.8	13.1	8.5	19.2	19.3	32.4	1.45
Chick embryo[c]	27.6	25.6	9.6	12.8	24.6	50.2	1.09

[a] Taken from Jacobson and Bonner (1968). [b] Huang and Bonner (1965) report a slightly different base composition. The present values are based on the more accurate analytical methods now available. [c] Taken from Huang (1969).

not interference of the type observed with rRNA, was shown by sequential hybridization experiments in which the DNA was first hybridized with 3S cytoplasmic or chromosomal RNA followed by hybridization with labeled chromosomal RNA.

The data for Figure 9 show the competition observed when ^{32}P-labeled 3S cytoplasmic RNA is hybridized in the presence of increasing amounts of unlabeled 3S cytoplasmic or chromosomal RNA. It is apparent that chromosomal RNA is a more effective competitor than 3S cytoplasmic RNA and is capable of competing for at least 90% of the 3S RNA binding sites. That is to say there are very few, if any, sequences contained in 3S cytoplasmic RNA that are not also contained in chromosomal RNA. The fact that a given concentration of chromosomal RNA produces more competition than the same concentration of 3S cytoplasmic RNA indicates that the 3S cytoplasmic RNA fraction contains less of the high hybridizing component than does chromosomal RNA and therefore is not a pure fraction.

RNA has also been prepared from the nuclear lysate, after pelleting of the chromatin, by the same procedure used for the preparation of 3S cytoplasmic RNA. This fraction of RNA has hybridization properties identical with those of 3S cytoplasmic RNA. It is capable of competing for only 50% of the chromosomal RNA binding sites and contains no sequences not also contained in chromosomal RNA. It competes on an equal basis with 3S cytoplasmic RNA in hybridization competition experiments with ^{32}P-labeled 3S cytoplasmic RNA. A portion of the chromosomal RNA, about 50% of the sequences present, therefore, appears to be confined to the chromatin. The remainder of the chromosomal RNA is homologous to a fraction of RNA present in the nuclear sap and cytoplasm.

Discussion

Chromosomal RNA isolated from rat ascites cells has many properties like pea bud and chick embryo chromosomal RNA (Huang and Bonner, 1965; Bonner and Huang, 1966; Bonner and Widholm, 1967; Huang and Huang, 1969; Huang, 1969). The amount of chromosomal RNA (relative to DNA) contained in purified chromatin from rat ascites, pea bud, and chick embryo is between 2 and 4% even though the "free" RNA content of these different chromatins varies from 4 to 17%. Commerford and Dehlias (1966) have reported extremely small amounts of RNA associated with the chromosomal proteins of mouse liver and intestine. This result may be the consequence of their method of nucleohistone preparation. The chromosomal RNA from rat liver reported by Benjamin *et al.* (1966) was

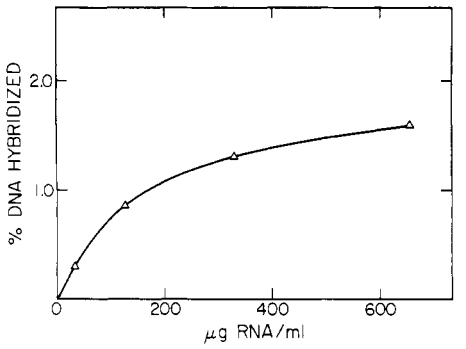

FIGURE 7: Hybridization of 3S cytoplasmic RNA to ascites nuclear DNA in the presence of increasing amounts of RNA. Hybridization at 66° for 10 hr.

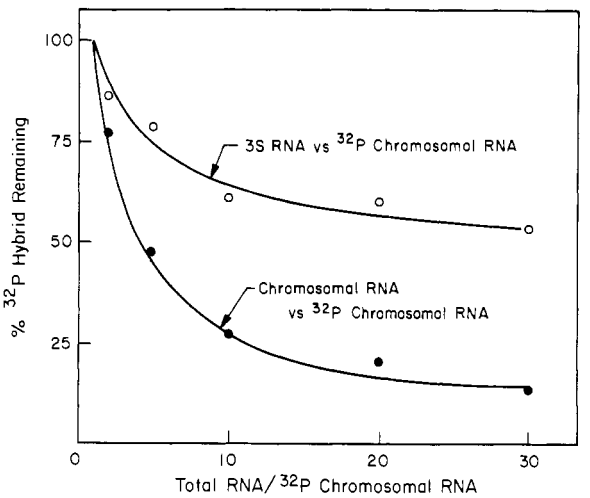

FIGURE 8: Ability of ascites 3S cytoplasmic or chromosomal RNA to compete with ^{32}P-labeled chromosomal RNA in hybridization to ascites DNA. All hybridization at 66° for 10 hr with 60 μg of labeled ascites chromosomal RNA throughout.

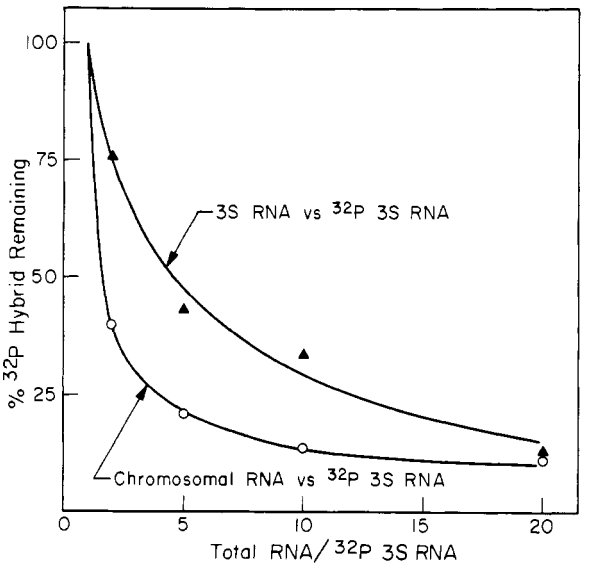

FIGURE 9: Ability of ascites chromosomal or 3S cytoplasmic RNA to compete with ^{32}P-labeled 3S cytoplasmic RNA in hybridization to ascites DNA; 60 μg of labeled ascites 3S cytoplasmic RNA throughout. Hybridization for 10 hr at 25° in the presence of 27% formamide.

followed by radioactivity only; we thus have no indication of the actual amount of RNA present. An additional complication of their work results from the short labeling time which they used; a time during which we would expect very little incorporation into chromosomal RNA of the nonreplicating liver chromosomes, but a large amount into nascent messenger. This is indicated by the fact that a 10-min pulse of ^{32}P given to rat ascites cells results in only slight incorporation into chromosomal RNA. In fact the base analysis and sedimentation profile reported by Benjamin *et al.* (1966) are most representative of mRNA. A more detailed investigation is required to determine if this RNA is really analogous to the chromosomal RNA reported in other systems.

Pea bud, chick embryo, and ascites chromosomal RNA are all small and size homogeneous. This is indicated by the fact that all three species of chromosomal RNA elute from DEAE-Sephadex as a sharp peak at the same molarity of NaCl. The sedimentation constant of 3.3 S for ascites chromosomal RNA is in good agreement with the sedimentation constants of pea (3.2 S) and chick embryo (3.8 S).

The base compositions of ascites, pea bud, and chick embryo chromosomal RNA are presented in Table IX. The base compositions are similar only in the sense that each contains about 9 mole % saturated pyrimidine. The significance of these differences is unclear at the present time.

The hybridization properties of rat ascites chromosomal RNA are in good agreement with those reported for pea chromosomal RNA by Bonner and Widholm (1967). Pea chromosomal RNA hybridizes to about 5% of pea nuclear DNA and has no sites in common with either t- or rRNA. Bonner and Widholm (1967) presented no evidence concerning a partially homologous cytoplasmic fraction. However, no competition was observed in the hybridization of pea chromosomal RNA in the presence of 32 times the amount of total cytoplasmic RNA. The chromosomal RNA of chick embryo is also complementary to a large portion of chick nuclear DNA, hybridizing at saturation to about 4% of the DNA (R. C. Huang, 1967, personal communication). It has no sites in common with chick t- or rRNA.

The hybridization properties of chromosomal and 3S cytoplasmic RNA are very similar to those reported by Shearer and McCarthy (1967) for pulse-labeled nuclear and cytoplasmic RNA from mouse L cells. They have reported the presence of a nuclear RNA that hybridizes to about 4.4% of the nuclear DNA. An additional fraction isolated from the cytoplasm hybridizes to about 1% of the nuclear DNA and contains no sequences not contained in the nuclear RNA. Thus, a fraction of the RNA isolated from the nucleus is confined to the nucleus. An important distinction between this work and that of Shearer and McCarthy (1967) is that their RNA is rapidly labeled while chromosomal RNA does not appear to be. In addition, ascites chromosomal RNA appears to possess no sequence homology to ascites mRNA.

Chromosomal RNA constitutes about 1–2 % of the

total cellular RNA. From the present experiments it is not possible to estimate the amount of 3S cytoplasmic and 3S nuclear RNA. We can however estimate the total amount of nonchromatin-bound 3S RNA to be about 5% of the total cellular RNA. The fact that these RNAs have not been observed previously may be due to their association with proteins which have prevented their extraction with phenol.

In establishing chromosomal RNA as a new class of RNA it is necessary to very carefully consider the possibility that it has arisen from the degradation of other classes of RNA. The experiments reported here, although they do not prove the point, strongly suggest that it does indeed represent a new class of RNA. The hybridization properties of ascites chromosomal RNA distinguish it from the various other RNA species studied. It is immediately distinguished from t- and rRNA by its high level of hybridization, being complementary to about 4% of ascites nuclear DNA. Neither t- nor rRNA are effective competitors for chromosomal RNA binding sites and in unreported data we have found that degradation of rRNA into 3S fragments does not increase its efficiency as a competitor. The lack of competition observed with *in vivo* generated mRNA suggests that chromosomal RNA is also distinct from mRNA. This is further supported by the fact that it is not pulse labeled. Because of the low degree of methylation of chromosomal RNA it is unlikely the result of degradation of tRNA. These results indicate that chromosomal RNA is not a degradation product of any one of these RNA fractions. The high content of dihydroribothymidine (8 mole %) is evidence that it is not a general composite degradation product.

Bekhor *et al.* (1969) and Huang and Huang (1969) have demonstrated that chromatin, completely dissociated in the presence of 2 M NaCl and then reconstituted under the proper conditions, supports the synthesis of the same spectrum of RNA sequences as does native chromatin. However, chromosomal RNA appears to be required for this sequence-specific reconstitution, *i.e.*, destruction of the chromosomal RNA by RNase or $Zn(NO_3)_2$ before reconstitution prevents sequence-specific reconstitution. In ascites cells only a fraction of the chromosomal RNA is confined to the chromatin. RNA homologous to the remaining fraction is found both in the nuclear sap and cytoplasm. What can be the role of this class of RNA? The close homology between 3S RNA and chromosomal RNA suggests that it also may be involved in gene regulation.

The properties of ascites chromosomal RNA clearly show that it is homologous to the chromosomal RNA isolated from pea bud and chick embryo. The presence of chromosomal RNA in rat, pea, and chick suggests that chromosomal RNA may be of general occurrence in higher organisms.

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