CHAPTER I

STUDIES ON CHROMOSOMAL RNA

CHAPTER II

EFFECT OF HYDROCORTISONE ON THE TEMPLATE ACTIVITY OF LIVER CHROMATIN

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ABSTRACT

CHAPTER I

Chapter I of this thesis is concerned with the isolation and characterization of ascites chromosomal RNA. The isolation of rat ascites chromosomal RNA as well as some of its physical and chemical properties are described in Section 1. Chromosomal RNA is characterized by its small size, lack of amino acid acceptor activity, and, relative to transfer RNA, low content of methylated bases. It has a base composition similar to that of ascites ribosomal RNA and is not labeled when the cells are exposed to a short pulse of ^{32}P. An RNA (3S cytoplasmic RNA), with properties similar to those of chromosomal RNA but contained in the cytoplasm, has also been isolated.

Section 2 is concerned with the hybridization properties of ascites chromosomal RNA to denatured ascites nuclear DNA. Chromosomal RNA hybridizes to about 4% of ascites nuclear DNA and has no sites in common with ascites messenger RNA. 3S cytoplasmic RNA hybridizes to about 2% of ascites nuclear DNA and contains no sequences not also contained in chromosomal RNA. The 3S RNA contained in the nuclear sap is homologous to 3S cytoplasmic RNA. Therefore, it appears that a fraction of chromosomal RNA is confined to the chromatin while the remainder is homologous to an RNA contained in both the cytoplasm and nuclear sap.

CHAPTER II

This chapter is concerned with the effect of hydrocortisone on the template activity of liver chromatin. Hydrocortisone administered to an adrenalectomized rat causes a two- to threefold increase in the rate of RNA synthesis in the liver. Chromatin isolated from the liver of hydrocortisone-treated rats possesses a 30% greater template activity for DNA-dependent RNA synthesis than does chromatin isolated from control rats. The difference in template activity is abolished by removal of the proteins associated with the DNA. Hydrocortisone, administered to isolated purified chromatin, does not alter its template activity.

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GENERAL INTRODUCTION

The genetic material as present in the interphase nucleus is collectively referred to as chromatin. The chromatin of higher organisms is composed of DNA complexed with proteins, both histone and nonhistone, and contains a small amount of RNA (for a review on the properties of isolated chromatin see Bonner et al., 1968). The amount of RNA present varies with the source of the chromatin, ranging from 26% (mass relative to DNA) for pea stem to 0.7% for calf thymus. Such RNA was first assumed to be a mixture of nascent messenger RNA, ribosomal RNA from the nucleolus together with other contaminating RNAs of the cell. Chromatin associated RNA took on added significance however when Huang and Bonner (1965) reported that a portion of it is covalently bound to a fraction of the chromosomal proteins. They demonstrated that salt extracted histones banded in CsCl contain RNA. It was later shown (Bonner and Huang, 1966) that the RNA is covalently bound to a small nonhistone protein, resulting in a complex having a buoyant density in CsCl of 1.57 g cm^{-3}.

This RNA, which has been named 'chromosomal RNA,' has many unusual properties which clearly distinguish it as a unique class of RNA. The chromosomal RNA of peas is small: $s_{20,w} = 3.2S$, and has a chain length of 40 nucleotides as determined by end group analysis. Its base composition differs from other classes of RNA in that it contains 27.5 mole per cent dihydrouridylic acid. (The complete base composition is presented and discussed in Section 1.) Bonner and Widholm (1967) have shown by hybridization experiments that chromosomal RNA is complementary to about

5% of pea nuclear DNA, and by hybridization competition experiments that it has no long sequences in common with transfer and ribosomal RNA. Total cytoplasmic RNA did not compete with chromosomal RNA in hybridization to denatured DNA. This was interpreted by Bonner and Widholm (1967) as evidence for its confinement to the nucleus and also its lack of homology to messenger RNA. Chromosomal RNA was also shown to be organ specific; i.e. pea cotyledon chromosomal RNA competes only partially with pea bud chromosomal RNA in hybridization to denatured pea DNA.

The chromosomal RNA of peas as it is present in chromatin is resistant to attack by RNase. It becomes RNase sensitive when the nucleohistone is heated to 60°C or when the DNA is destroyed by DNase treatment (Huang and Bonner, 1965).

These unique properties have led Huang and Bonner to suggest that chromosomal RNA may be involved in gene regulation. A large body of data has been accumulated which suggests that histone may be the agent of gene repression in higher organisms (Bonner et al., 1968). However, histones do not contain in themselves the molecular heterogeneity necessary to interact with and repress specific genes. It has therefore been suggested that chromosomal RNA by association with histone confers the required specificity.

The initial objective of the present work was to determine whether or not chromosomal RNA occurs in rat liver and the heptoma, Novikoff ascites tumor. At the time this investigation was started the existence of chromosomal RNA had been reported only for the various

organs of the pea plant. It was therefore of interest to investigate the chromatin of mammalian cells to determine if a similar RNA is present and if so, how its properties compare to those of pea chromosomal RNA. Chromosomal RNA was found in both rat liver and rat ascites cells. The ascites tumor system was chosen for detailed physical and chemical studies because of the ease of labeling ascites cells and the simplified method for the preparation of chromatin. For the hybridization studies, presented in Section 2, it was necessary to obtain uniformly labeled RNA. This would have been an impossible task in the rat liver system.

During the course of the present investigation the isolation of chromosomal RNA from chick embryo was reported by Huang et al. (1965) and from rat liver by Benjamin et al. (1966). The chromosomal RNA from chick embryo has the following properties in common with pea chromosomal RNA: a) high dihydrouridylic acid content (10 mole per cent), b) covalent linkage to a small nonhistone protein, the complex having a buoyant density in CsCl of 1.57 g cm^{-3}, c) small size, $s_{20,w}$ = 3.8S, and a chain length of 50 nucleotides, and d) complementarity to a large portion of the nuclear DNA. The base composition of chick embryo chromosomal RNA presented in Section 1 is, however, very different from that of pea. The properties of rat liver chromosomal RNA as reported by Benjamin et al. (1966) show little similarity to those of pea or chick chromosomal RNA. The liver RNA is size heterogeneous ranging from 4S to 28S and contains no dihydrouridylic acid. The properties and significance of the RNA reported by Benjamin et al. (1966) will be discussed in Section 1.

It is unlikely that this RNA is homologous to the class of chromosomal RNA previously investigated.

Evidence has also accumulated which sheds light on the biological function of this new class of RNA. This evidence supports the model initially proposed by Bonner and Huang (1966). The new data concerns the role of chromosomal RNA in the reconstitution of pea cotyledon chromatin (Bekhor, Kung and Bonner, 1968) and chick embryo chromatin (Huang and Huang, 1968). When pea cotyledon chromatin is dissociated into chromosomal proteins, chromosomal RNA, and DNA, by 2 M NaCl and reconstituted by gradient dialysis, non-native chromatin results, i.e. the chromatin does not support the synthesis of the same spectrum of RNA sequences as does native chromatin. Reconstitution of the chromatin by gradient dialysis from 2 M NaCl in 5 M urea, a hybridizing condition for chromosomal RNA, however, results in chromatin capable of synthesizing the same spectrum of RNA sequences synthesized by native chromatin. Destruction of the associated chromosomal RNA by RNase or by $Zn(NO_3)_2$ prevents the reconstitution of native chromatin. Similar experiments with chick embryo chromatin also show that the presence of chromosomal RNA is required for the reconstitution of native chromatin.

The name 'chromosomal RNA' for the new class of RNA will be used throughout this dissertation. It must be kept in mind that this designation does not refer to all of the RNA associated with chromatin but only to that small fraction which is associated with the chromosomal proteins and elutes appropriately on DEAE Sephadex. It also does not mean to imply that this class of RNA is confined to the chromatin.

Section 1 deals with the isolation of chromosomal RNA and a similar fraction of RNA from the cytoplasm as well as some of their chemical and physical properties. Section 2 is concerned with the hybridization properties of chromosomal RNA and the partially homologous cytoplasmic fraction of RNA to denatured rat ascites DNA.

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SECTION 1. ISOLATION AND CHARACTERIZATION OF CHROMOSOMAL RNA

INTRODUCTION

This section is concerned with the isolation and characterization of rat ascites tumor cell chromosomal RNA. The isolation of a similar RNA from the cytoplasmic fraction is also described. Various chemical and physical properties of chromosomal RNA are investigated to further characterize the RNA and hopefully to provide some insight into its biological function. Among the properties investigated are size, base composition, degree of methylation, and rate of synthesis.

MATERIALS AND METHODS

Maintenance of tumor line

The Novikoff ascites tumor line was used for the following investigation and was maintained by serial transplantation in male albino Sprague-Dawley rats purchased from Berkeley Pacific Laboratories. The tumor line, under the conditions employed, has a cycle of 6 to 7 days. During the first several days very little cell division takes place. This is followed by a period of rapid cell proliferation culminating in death of the animal. The tumor was transferred by innoculating 1-1.5 ml of the ascites fluid of an infected rat into the peritoneum of a healthy rat. Although the size of the rat has little effect on the length of the cycle, rats weighing between 150-250 gms were used. The transfer was carried out the sixth or seventh day of the cycle. Cells for experimental use were harvested on the sixth or seventh day.

Harvest and purification of tumor cells

The tumor was harvested from an infected animal by first lightly anesthetizing the rat with ether. The rat's abdomen was then swabbed with 70% ethanol and a two inch square section of skin just below the rib cage removed. The exposed muscle surface was then swabbed with ethanol and a small incision made. The ascites fluid was then drained from the peritoneum into a small beaker and stored on ice until all the rats had been sacrificed. Often clots appeared in the fluid. These were removed by filtration through several layers of cheesecloth. A 6 to 7 day infected rat contained 30 to 40 ml of ascites fluid which yielded

8 to 12 ml of washed packed cells.

The ascites fluid contains in addition to the tumor cells a large number of contaminating erythrocytes which can be separated by either of the following methods. The pellet was considered free of erythrocytes when no red color remained.

All procedures were carried out at 0-4°C.

a) The erythrocytes are significantly smaller than the tumor cells and can be completely separated 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 minutes at 700 g in the International Refrigerated Centrifuge. The supernatant was then removed and the pellet washed by repeated centrifugation. Three to four washes were required to obtain pure cells.

b) The erythrocytes are more sensitive to hypotonic solutions and can be preferentially lysed in the presence of tumor cells. The ascites fluid was diluted with an equal volume of TNKM and pelleted as previously described. The cells were then suspended in 3 vol. deionized water and immediately centrifuged at 700 g. Most of the contaminating cells are removed in this centrifugation. The remainder can be removed by an additional wash with TNKM.

c) An additional method, similar in theory to the first, employs a buffer containing 0.02 M tris, pH 8.0, 0.11 M NaCl, 0.01 M EDTA, 0.25 M sucrose (CWM). The cells are purified by differential centrifugation as previously described. This method is not as efficient as the previous two and more washes are required to obtain cells of equal purity.

The washed pellet of cells can be frozen in ethanol dry ice and stored at -80°C.

Preparation of chromatin

The initial investigations were carried out on chromatin isolated by the first of the following procedures (Nicolson, 1965). 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 to 80% of the DNA. The amount of chromosomal RNA associated with each was identical. Because of its simplicity, procedure (b) was routinely used. All extraction procedures were carried out at 0 to 4°C.

a) Isolation of chromatin in the presence of EDTA

Fresh ascites cells, or frozen cells thawed in a water bath at 24°C, were suspended by hand with the aid of a teflon homogenizer in 15 vol. cold deionized water. The cells were pelleted immediately by centrifugation at 3,000 rpm for 10 minutes in the No. 284 rotor of the International Centrifuge. The cells were then resuspended in 4 vol. of cold deionized water and homogenized with the power driven teflon, 20 strokes at full speed. One-half volume of 3X medium (0.09 M tris, pH 8.0, 0.075 M NaCl, 0.024 M Na_2EDTA, 0.75 M sucrose) and an equal volume of 1X medium in 2.2 M sucrose were added. The crude chromatin was then

pelleted by centrifugation at 3,100 rpm for 40 minutes in the International Centrifuge. The pellet was homogenized (by hand with a teflon homogenizer) in 1X medium and diluted with an equal volume of 2.2 M sucrose in 1X medium followed by centrifugation at 3,100 rpm for 40 minutes. The pellet was then suspended in 2 vol. of 0.03 M tris, pH 8.0, 0.16 M sucrose, and sheared in a Virtis at 30 volts for 60 seconds. Five ml aliquots were layered over 25 ml 1.6 M sucrose in 0.03 M tris, pH 8.0, the upper two-thirds of each tube stirred to form a two step gradient and centrifuged in the Spinco SW25 rotor for 2 hours at 22,000 rpm. Purified chromatin was recovered as a clear gelatinous pellet.

b) Isolation of chromatin in the absence of EDTA

The cells were first suspended by hand with the aid of a teflon homogenizer in 15 vol. of cold deionized water. Nuclei and unlysed cells were then pelleted by centrifugation at 1,500 g for 15 minutes. 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. Under these conditions the cells are lysed by the hypotonic solution, the nuclei, however, remain intact, probably because of the increased tonicity due to cell lysis. The crude nuclear pellet is then homogenized by hand with the aid of a teflon homogenizer in 0.01 M tris, pH 8.0, stirred slowly for 30 minutes on a magnetic stirrer, and centrifuged at 10,000 g for 15 minutes. To obtain complete lysis of the nuclei it is necessary to maintain a large volume of buffer, an amount equal to that added in the water lysis of the cells. The chromatin

was then washed 3 to 4 times by repeated suspension in 0.01 M tris 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, pH 8.0. Five ml aliquots were then layered onto 25 ml of 1.7 M sucrose containing 0.01 M tris, pH 8.0, the top two-thirds of the tube stirred to form a two step gradient, and centrifuged for 2 hours 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 M H_2SO_4 at 4°C and precipitated with 20% trichloroacetic acid (TCA). The amount of protein was determined following the method of Lowry, Rosebrough, Farr and Randall (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 V 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 starting material for the preparation of chromosomal RNA.

The isolation method involves dissociation of the chromatin by high salt, centrifugation to separate the DNA from the chromosomal protein followed by purification of the RNA from the protein fraction. The following procedure was carried out at 0 to 4°C with the exception of the steps indicated.

The chromatin pellets were suspended in equal volume of 0.01 M tris, pH 8.0 and diluted with 2 vol. of 6 M CsCl in 0.01 M tris, pH 8.0 (Industrial grade CsCl, American Potash and Chemical Corporation). The resulting solution was extremely viscous and was homogenized for 30 seconds at 20 volts in a Waring blender to facilitate solution of the chromatin. The solution was then centrifuged for 15 hours 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 to 4 mg/ml pronase (Pronase, B grade from Calbiochem, was preincubated for 90 minutes at 37°C to destroy any nuclease activity) in 0.01 M tris, pH 8.0 for 8 to 12 hours at 37°C.

The solution of digested chromosomal proteins and associated nucleic acids was then phenol extracted at 4°C. Sodium dodecyl sulfate (SDS) 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 minutes the phases were

separated by centrifugation and the phenol phase was extracted with 1/2 vol. of water. The combined aqueous phase was then re-extracted twice with half a volume of phenol. The nucleic acids were precipitated by the addition of 1/10 vol. of 20% potassium acetate and 2 vol. of 95% ethanol. After 2 hours at -20°C 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, pH 8.0.

The nucleic acids accompanying the chromosomal proteins contain some DNA in addition to chromosomal RNA. This DNA was separated from the chromosomal RNA by chromatography on A-25 DEAE Sephadex (Hall et al., 1965). Fractionation was routinely carried out on a 9 mm x 25 cm column; however, analytical columns as small as 1 mm x 28 cm and preparative columns as large as 1 cm x 50 cm have been used. The nucleic acids were eluted with a linear gradient of NaCl ranging from 0.2 M to 1.0 M in the presence of 7 M urea and 0.01 M tris, pH 8.0. Chromosomal RNA was recovered by precipitation with 2 vol. of 95% ethanol in the presence of 2% potassium acetate. The purified RNA was dissolved in and dialyzed against 2X SSC (SSC buffer contains 0.15 M NaCl and 0.015 M sodium citrate).

Preparation of 3S cytoplasmic RNA

The possible existence of a fraction of RNA with properties comparable to chromosomal RNA but localized in the cytoplasm was investigated by applying the isolation procedure for chromosomal RNA to

the cytoplasmic fraction. 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 and it should thus contain no chromosomal RNA. The supernatant was precipitated with 2 vol. of 95% ethanol in the presence of 2% potassium acetate, in order to concentrate the cytoplasmic proteins before CsCl centrifugation. The precipitate was next dissolved in 0.01 M tris, pH 8.0 and diluted with 2 vol. of 6 M CsCl, 0.01 M tris. Under these conditions the ribosomal proteins are dissociated and the ribosomal 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 purified RNA, isolated by this procedure, has a sedimentation constant of between 3 to 4S and will thus be referred to as 3S RNA.

Amino acid activation analysis

a) Preparation of aminoacyl-tRNA synthetase

Aminoacyl-tRNA synthetase was prepared from rat liver according to the method ("short procedure") of Holley et al. (1961). The final $(NH_4)_2SO_4$ precipitate was dissolved in 0.1 M sodium phosphate buffer, pH 6.8, dialyzed against the same buffer, and stored in liquid nitrogen.

b) Preparation of RNA

Rat liver transfer RNA was recovered from the DEAE Cellulose column used in the preceding preparation of aminoacyl-tRNA synthetase. Transfer RNA was eluted from the column with 1.0 M NaCl, 7 M urea, 0.01 M tris, pH 8.0, and precipitated with 2 vol. of 95% ethanol. The RNA was then phenol extracted, precipitated, and chromatographed on A-25 DEAE Sephadex. Transfer RNA elutes at a NaCl concentration of about 0.55 M. The RNA was then precipitated, dissolved, and uncharged in 0.5 M tris, pH 9.15, by heating at 37°C for 45 minutes (Yamane et al., 1963). After reprecipitation the transfer RNA was dissolved in and dialyzed against 0.04 M K maleate buffer, pH 6.9, containing 0.05 M dithiohectol, 0.025 M KCl, and 0.0075 M $Mg(Ac)_2$.

Transfer RNA from rat ascites cells was prepared from the 105,000 g supernatant by phenol extraction according to the method of Attardi et al. (1966) followed by chromatography on DEAE Sephadex. The RNA was uncharged as described above, and suspended in the maleate buffer used for liver transfer RNA.

Chromosomal RNA was prepared by the standard procedure, heated at 37°C for 45 minutes in 0.5 M tris, pH 9.15, precipitated and dissolved in maleate buffer.

c) Incubation and assay

Acceptor activity of the various RNA fractions was measured by determining the amount of radioactive amino acids incorporated into RNA in the presence of activating enzyme.

The complete incubation mixture contained in a volume of 0.25 ml: 0.1 μM ATP, (0.1 μM CTP when present), 1 μC ^{14}C amino acids (reconstituted ^{14}C protein hydrolysate from Schwarz Bioresearch Inc., average specific activity 170 μc/μmole), aminoacyl-tRNA synthetase (0.02 ml) and RNA. After a 20 minute incubation at 37°C, 50 μl aliquots were removed and absorbed onto Whatman 3 MM filter paper (2.3 cm diameter). The samples were then placed in a large volume of cold 10% TCA. After 10 minutes in TCA, the filters were removed and several (10 to 15) filters placed between two large filter papers in a Buchner funnel. The filters were then washed at 4°C with 1 liter of the following solutions: 66% ethanol containing 0.5 M NaCl, 10% TCA, 5% TCA, and 3:1 ethanol:ether. The filters were then dried and counted in the Beckman liquid scintillation spectrometer. Phenol extraction before TCA precipitation, thus removing the ^{14}C amino acids complexed with activating enzyme, had little effect on the result.

The background of the assay and the dependence of the reaction on ATP, activating enzyme, and transfer RNA are shown in Table 1.

Extraction of ^{32}P-labeled RNA

Carrier free ^{32}P phosphoric acid ($H_3{}^{32}PO_4$) was obtained from Volk Radiochemical Company. The solution was neutralized with 0.1 N NaOH and diluted to the required volume and salt concentration of 0.15 M NaCl.

a) Uniformly labeled RNA

Uniformly ^{32}P-labeled chromosomal RNA was prepared from cells

TABLE 1

Dependence of the Amino Acid Activation of Rat Liver Transfer RNA on the Presence of ATP, Activating Enzyme, and Transfer RNA

Incubation medium	Acceptor activity cpm ^{14}C amino acid incorporated per 50 µl
Complete	1573
- ATP	239
- enzyme	161
- transfer RNA	335
+ heated enzyme*	56

Each incubation, with the exception of those indicated, contained 10 µg rat liver transfer RNA.

*Enzyme heated at 100°C for 3 minutes before addition to the incubation medium.

which had been grown for 24 to 48 hours in the presence of ^{32}P. Rats, infected 4 to 5 days prior with tumor, were given an interperitoneal injection of carrier free ^{32}P in 0.5 ml physiological saline (0.15 M NaCl). The standard injection of ^{32}P was 3 mC per rat; dosages as low as 500 μC and as high as 5 mC per rat have been used.

b) Pulse labeled RNA

For the determination of the base composition of rapidly labeled RNA, 6 day infected rats were given an injection of 4 mC ^{32}P and sacrificed 60 to 80 minutes later. The cells were washed and the nucleic acids extracted with phenol following the method of Attardi et al. (1966). The final ethanol precipitate was dissolved in 0.01 M sodium acetate buffer, pH 5.1 and chromatographed on a Sephadex G-50 column (1.5 cm x 60 cm). The excluded nucleic acid was then precipitated with ethanol and dissolved in 2X SSC.

Pulse labeled chromosomal RNA was prepared from in vitro labeled cells according to the following procedure. The cells were pelleted directly from the ascites fluid, washed once with Eagles Medium (Eagle, 1959) deficient in phosphate, containing 5% dialyzed calf serum, and suspended in 10 volumes of the same medium preheated to 37°C. After 5 minutes neutralized ^{32}P was added (4 mC of ^{32}P was added for each rat sacrificed) and the incubation continued at 37°C for 10 minutes. The cells were then diluted with cold CMM and washed free of contaminating erythrocytes by differential centrifugation at 4°C. A portion of the cells were extracted with phenol at 66°C 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 (MAK) as described by Mandell and Hershey (1960). The radioactivity of each fraction was determined by evaporation of a 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 interperitoneal injection of 50 μC of ^{14}C-methyl-methionine (12 μC/μmole purchased from New England Nuclear Corporation). After 5 hours the rats were sacrificed and the tumor harvested and washed as previously described. Chromosomal, 3S cytoplasmic, and transfer RNA were prepared from the same cells. Chromosomal and 3S cytoplasmic RNA were prepared as previously described in this section. Transfer RNA 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. Transfer RNA elutes at a salt concentration of 0.55 M NaCl, ribosomal RNA is irreversibly bound to the column.

For the determination of radioactivity, 1 ml aliquots of each fraction were precipitated with cold 10% TCA in the presence of 1 mg carrier yeast RNA. Acid insoluble material was collected by filtration on TCA presoaked membrane filters (Schleicher and Schnell B-6), washed with 10 ml cold 10% TCA, dried, and counted in the Beckman liquid scintillation spectrometer.

Base composition analysis

The base composition of the various RNA fractions was determined by alkaline hydrolysis of the RNA in 0.3 N KOH at 37°C for 18 hours and separation of the nucleotides on a column of Dowex 1-X8, formate form (Cohn and Volkin, 1953). Base composition was calculated both from UV absorbance and from ^{32}P incorporation. The following molar extinction coefficients were used: CMP, $\epsilon_{280} = 13 \times 10^3$; AMP, $\epsilon_{260} = 14.7 \times 10^3$; UMP, $\epsilon_{260} = 10.0 \times 10^3$; GMP, $\epsilon_{260} = 11.7 \times 10^3$. The radioactivity was determined by evaporating aliquots of each fraction on a planchet and counting in a Nuclear Chicago gas flow counting system.

RESULTS

Tumor cell chromatin

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 2. The UV absorption spectrum of such chromatin is shown in Figure 1. The properties of ascites purified chromatin are in general very similar to those of rat liver chromatin (Marushige and Bonner, 1966). Ascites chromatin, however, contains slightly more protein and about three times the amount of RNA. The template activity of ascites DNA in the form of chromatin is restricted in its ability to act as template for RNA synthesis. Ascites purified chromatin coupled with E. coli RNA polymerase supports about 10 to 15% the amount of RNA synthesis as does an equal amount of deproteinized ascites DNA. This is similar to the template activity of rat liver chromatin which is 20% that of purified liver DNA.

Isolation of chromosomal RNA

When ascites 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 proteins. Purification of this nucleic acid by chromatography on DEAE Sephadex as described under Materials and Methods results in the two peak elution profile shown in Figure 2. Both peaks exhibit a characteristic nucleic acid UV absorption spectra with absorption maxima at 257 mμ. Figure 3 shows the UV absorption spectrum of

TABLE 2

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

Figure 1. Absorption spectrum of rat ascites purified chromatin in 0.01 M tris, pH 8.0.

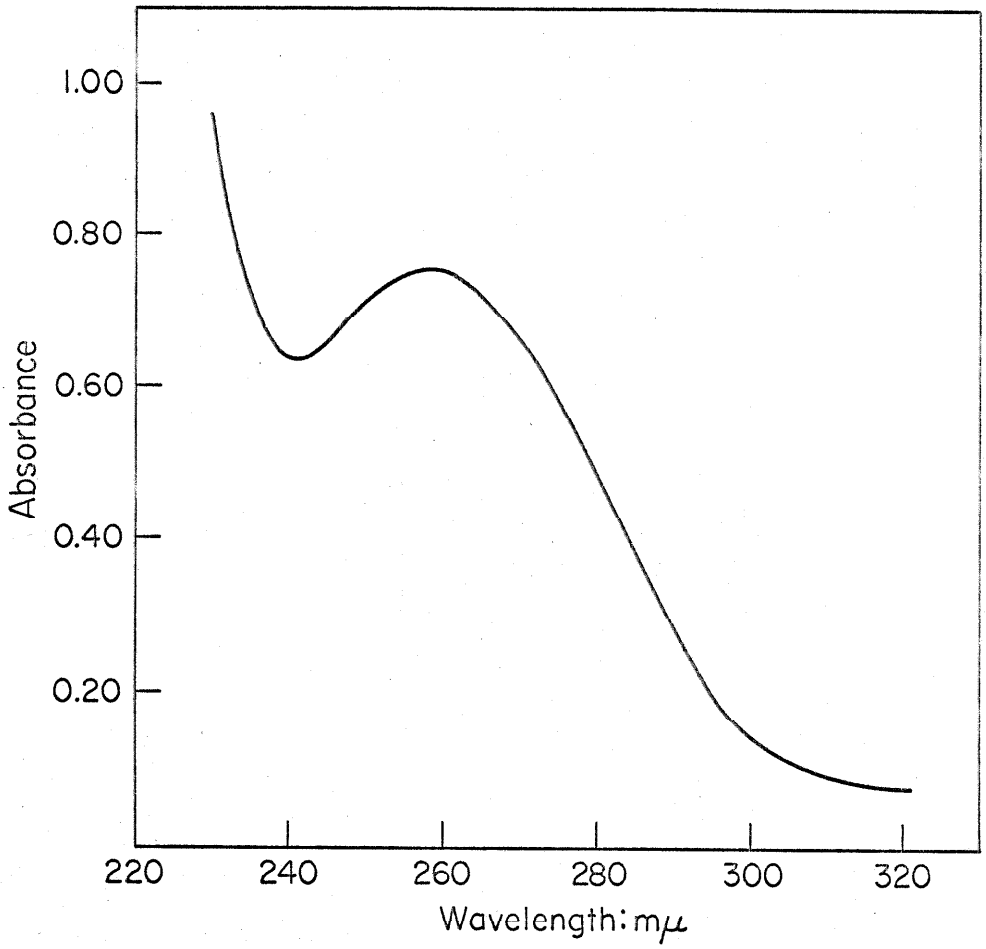

1.00
0.80
0.60
0.40
0.20
Absorbance
220
240
260
280
300
320
Wavelength: mμ

Figure 2. Elution profile of chromosomal protein associated nucleic acids from DEAE Sephadex. Nucleic acid eluted with a linear gradient of NaCl from 0.2 M to 1.0 M in the presence of 7 M urea and 0.01 M tris, pH 8.0.

—o—o—, OD_{260}; --Δ--Δ--, cpm.

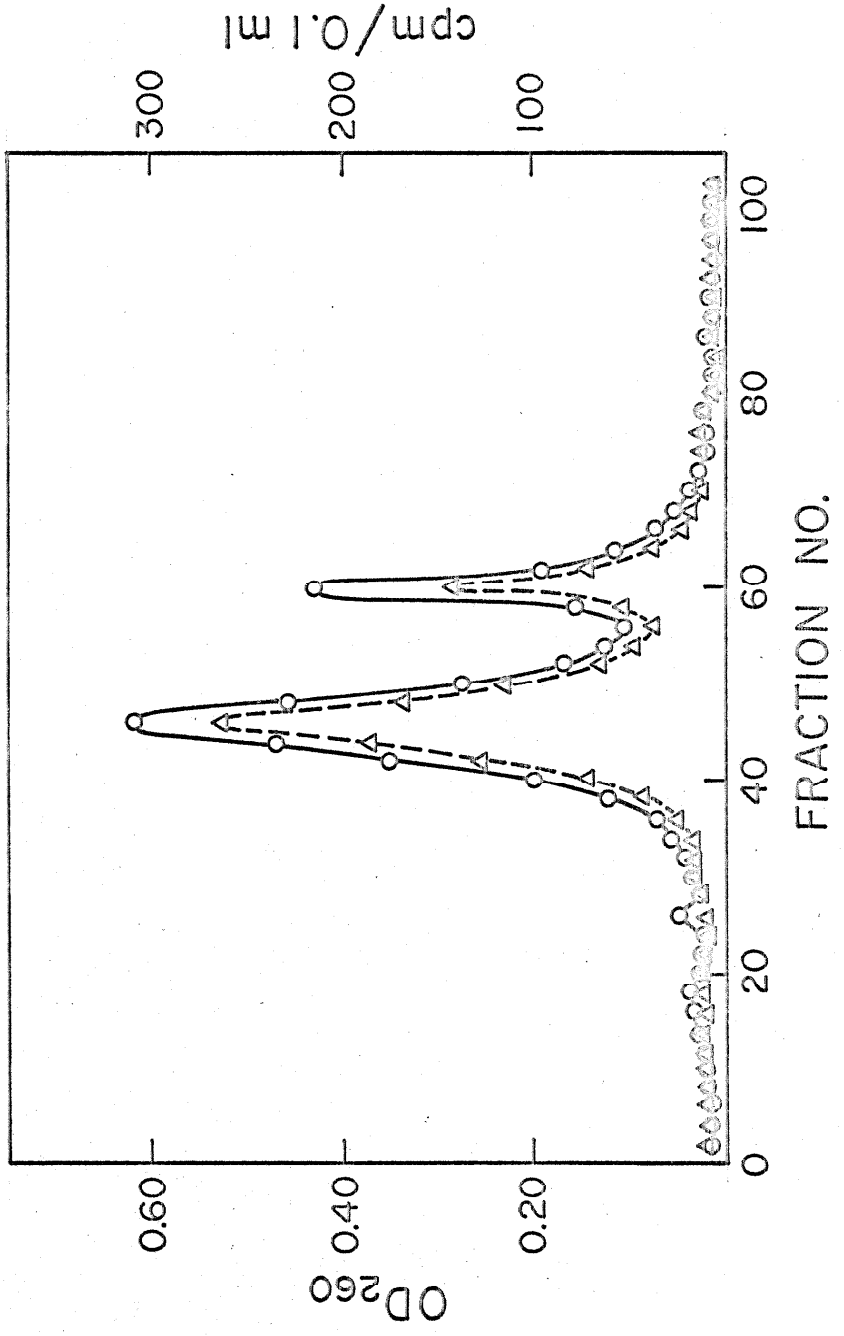
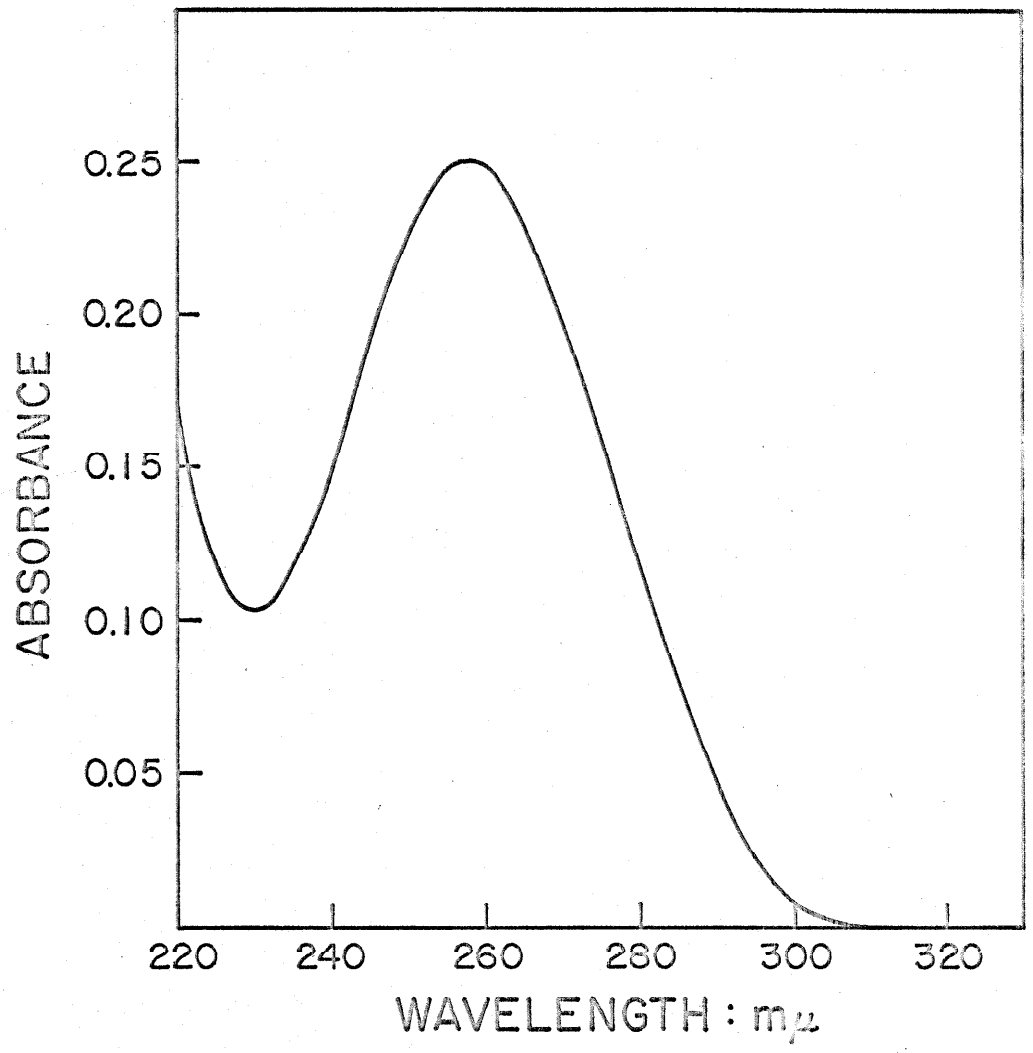

cpm/0.1 ml
300
200
100
OD260
0.60
0.40
0.20
0
20
40
60
80
100
FRACTION NO.

Figure 3. Absorption spectrum of DEAE Sephadex fraction I (chromosomal RNA) in 2X SSC.


ABSORBANCE
0.25
0.20
0.15
0.10
0.05
220
240
260
280
300
320
WAVELENGTH : mμ

the first peak, fraction I. As shown in Table 3, fraction I, which elutes at a NaCl concentration of 0.55 M, is totally base labile and is sensitive to RNase; the second peak (fraction II) which elutes at 0.65 M NaCl, is not hydrolyzed by base and is resistant to RNase. The thermal denaturation profile of fraction I is characteristic of RNA while that of fraction II is characteristic of DNA (Fig. 4). It is clear that fraction I is RNA and fraction II is DNA.

The RNA associated with chromatin can therefore be fractionated into a portion which accompanies the chromosomal proteins when they are separated from the DNA by buoyant density centrifugation, this fraction known as chromosomal RNA, and a remaining fraction which does not do so, this fraction referred to as "free" RNA. The data presented in Table 2 show that in crude chromatin, chromosomal RNA is present in an amount equal to about 4% of the DNA. This RNA constitutes 20 to 35% of the RNA contained in the chromatin. Even though different preparations of crude chromatin differ in their "free" RNA content, ranging from 10 to 25%, the amount of chromosomal RNA remains relatively constant at about 4%. The so called "free" RNA associated with ascites chromatin has not been investigated; it seems likely, however, that it is partially composed of nascent messenger, ribosomal, and nucleolar RNA. The "free" RNA from pea cotyledon has been shown to be partially composed of messenger RNA (Bekhor, Kung and Bonner, 1968). Purified chromatin contains less total RNA and proportionally less chromosomal RNA.

Very little is known about the DNA of fraction II. The amount varies a great deal from preparation to preparation ranging from an

TABLE 3

Base Hydrolysis and RNase Digestion of

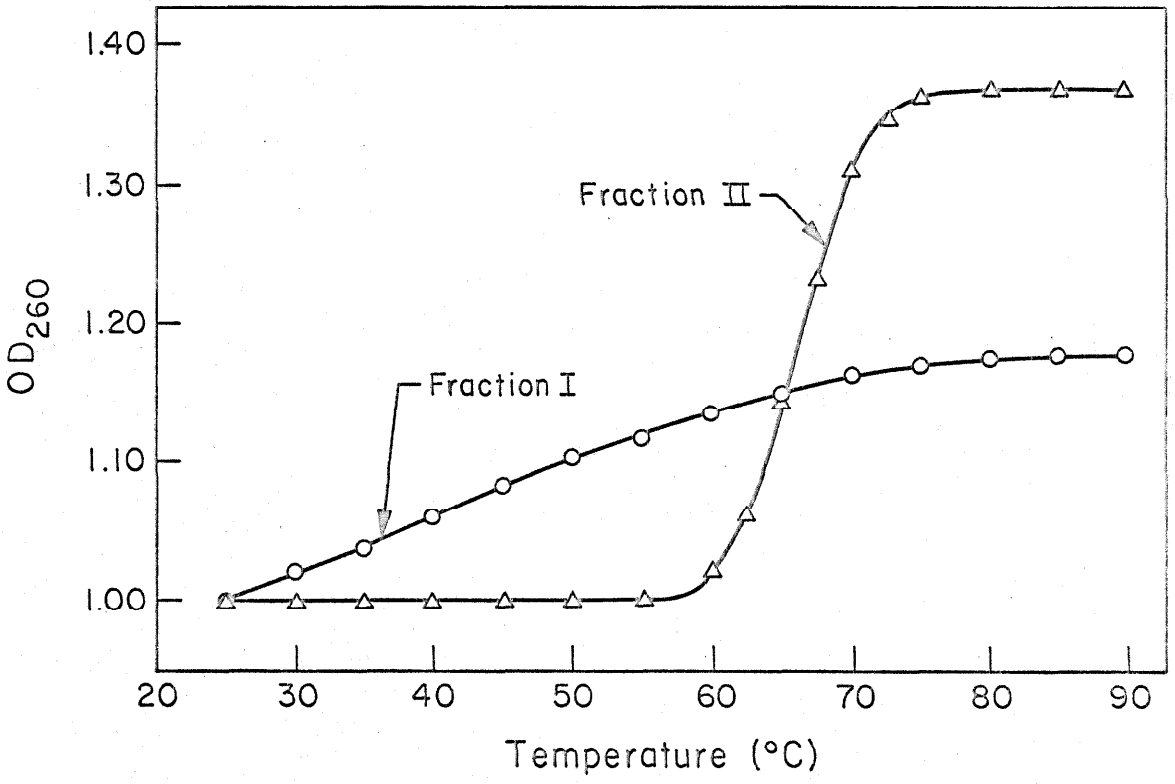
Fractions I and II

Treatment	Fraction	Input (cpm)	Acid soluble (cpm)	% Acid soluble
0.3 N KOH 18 hrs 37°C	I	1748	1742	99.7
	II	1981	56	2.8
RNase* 18 hrs 37°C	I	1648	1608	97.6
	II	9499	780	8.2

All samples were made 5% in $HClO_4$ at the end of the incubation and the radioactivity of the supernatant determined.

*RNase digestion was carried out in 2X SSC in the presence of 20 µg/ml pancreatic RNase.

Figure 4. Melting profiles of fractions I and II. Samples were melted in 0.01 M tris, pH 8.0 and monitored with a Gilford Model 2000 Multiple Sample Absorbance Recorder. Data has been normalized to an initial $OD_{260} = 1.0$.


1.40
1.30
1.20
1.10
1.00
OD_{260}
Fraction II
Fraction I
20
30
40
50
60
70
80
90
Temperature (°C)

undetectable amount to as much as 5% of the total DNA. The DNA is double stranded, exhibiting a sharp melting profile with a T_m of 66°C in 0.01 M tris, pH 8.0, and has a hyperchromicity of 38% (Fig. 4). It may be the product of enzymatic degradation during the preparation of chromatin or a result of shearing during salt extraction. The amount of DNA in fraction II is reduced when chromatin is prepared according to procedure (a) described in Materials and Methods. This may be a consequence of decreased DNase activity due to the presence of EDTA.

Chemical and physical properties of chromosomal RNA

Chromosomal RNA elutes from DEAE Sephadex as a single homogeneous peak. Since the strength of binding to DEAE Sephadex is dependent both on the charge of the molecule (which is in turn dependent on chain length) and on its secondary structure (Hall et al., 1965), a sharp peak could be composed of RNA molecules of different chain lengths and of different degrees of secondary structure, the larger molecules having more secondary structure. Thermal denaturation of chromosomal RNA in the presence of 0.2 M NaCl, 7 M urea show that it retains no secondary structure under the conditions of chromatography. The sharp peak therefore suggests that chromosomal RNA is size homogeneous.

Transfer RNA retains some secondary structure in the presence of 7 M urea as demonstrated by its chromatographic properties on DEAE Cellulose. When co-chromatographed with chromosomal RNA on DEAE Cellulose at room temperature in the presence of 7 M urea and 0.01 M tris, pH 8.0, chromosomal RNA elutes at about 0.48 M NaCl; transfer RNA elutes at between 0.35 to 0.4 M NaCl and can be completely resolved under these conditions. Chromosomal RNA also elutes as a sharp peak at a NaCl

concentration of 0.50 M when chromatographed on DEAE Cellulose at 55°C. Transfer RNA, when chromatographed under these conditions, elutes as a broad peak at a NaCl concentration of between 0.4 to 0.5 M (Jacobson, 1967). The fact that the binding of chromosomal RNA is not changed by chromatography at a higher temperature indicates that it contains very little secondary structure. The sharp elution profile is therefore most likely due to a small homogeneous chain length.

Centrifugation studies also demonstrate that chromosomal RNA is of small and homogeneous size. The pattern obtained from sucrose density gradient centrifugation of labeled chromosomal RNA in the presence of total ascites cytoplasmic RNA is shown in Figure 5. Chromosomal RNA moves as a sharp band and under these conditions is not distinguishable from transfer RNA. Analytical band velocity sedimentation gives an $s_{20,w}$ of 3.3S. 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 (Brutlag, 1967).

Base composition

The base composition of ascites chromosomal, pulse labeled, transfer and ribosomal RNA was determined by alkaline hydrolysis followed by chromatography of the nucleotides on Dowex 1-X8. A typical fractionation of hydrolyzed chromosomal RNA is shown in Figure 6.

The base composition of the various RNA fractions studied appear in Table 4. The nucleotide composition of chromosomal RNA calculated from UV absorbance is identical to that based on ^{32}P incorporation. The base composition is in general very similar to ascites transfer and ribosomal RNA but differs markedly from pulse labeled RNA. The slight differences in the contents of U and G between chromosomal and ribosomal

Figure 5. Sedimentation pattern of ^{32}P-labeled ascites chromosomal RNA (7 μg) in the presence of total ascites RNA (0.5 mg). 5 to 20% sucrose 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°C for 4 1/2 hours.

—o—o—, OD_{260}; --Δ--Δ--, ^{32}P cpm.

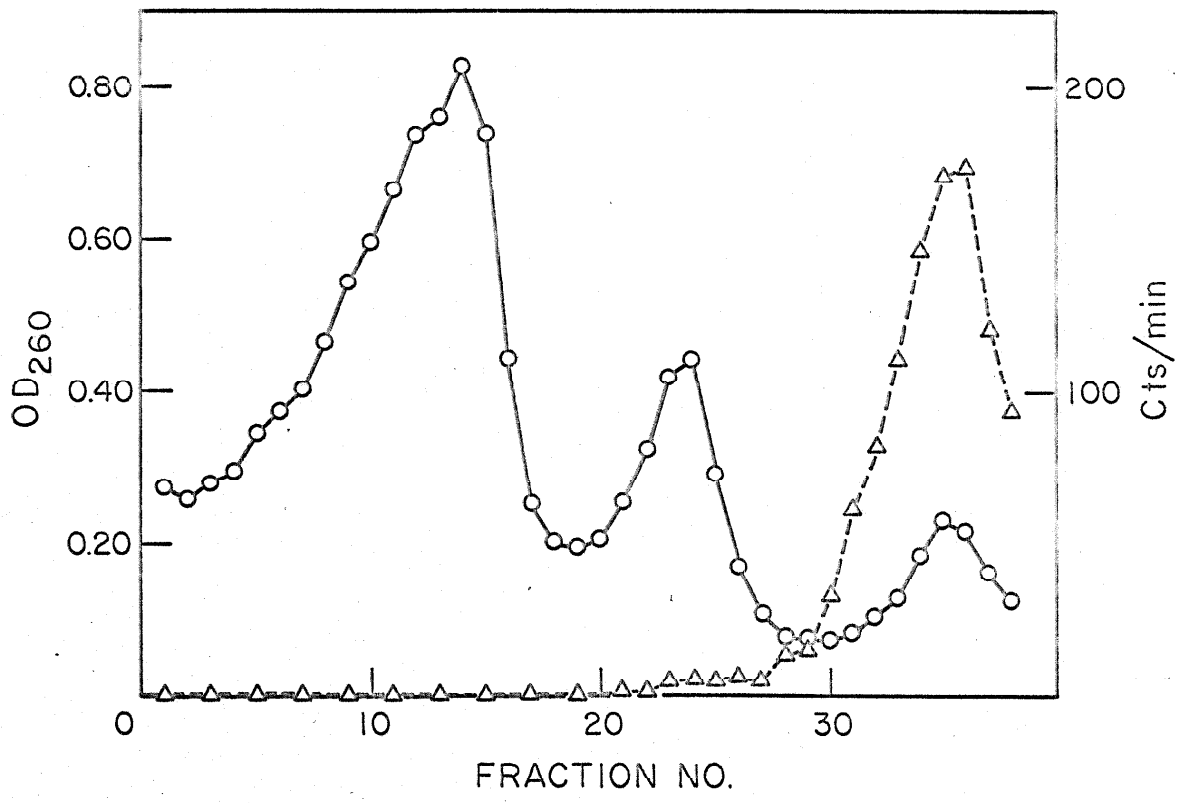

0.80
0.60
0.40
0.20
OD260
200
100
Cts/min
0
10
20
30
FRACTION NO.

Figure 6. Typical fractionation on Dowex 1-8X of the products of alkaline hydrolysis of ^{32}P-labeled ascites chromosomal RNA. Nucleosides were eluted with 0.005 N HCOOH; cytidylic acid with (b) 0.025 N HCOOH; adenylic acid with (c) 0.2 N HCOOH; uridylic acid with (d) 0.01 N HCOOH + 0.05 N $HCOONH_4$; guanylic acid with (e) 0.1 N HCOOH + 0.2 N $HCOONH_4$; and the remaining material with (f) 2.5 N $HCOONH_4$. 5 ml fractions were collected at a flow rate of about 0.7 ml/min.

—O—O—, OD_{260}; --Δ--Δ--, ^{32}P cpm/ml.

1,500
1,000
500
cpm/ml
0.50
0.40
0.30
0.20
0.10
OD260
10
20
30
40
50
60
70
Fraction no.
b
c
d
e
f

TABLE 4

Nucleotide Composition of Different RNA Fractions of Rat Ascites Cells

Fraction	Method of analysis	Mole per cent				G + C
		A	C	U	G	
Chromosomal RNA						
	UV	18.4	27.4	20.7	33.5	60.9
	^{32}P	18.9	26.7	20.6	33.8	60.5
	Average*	18.9 ± 1.2	26.3 ± 0.9	21.1 ± 2.0	33.6 ± 2.1	59.9
Ribosomal RNA						
	UV	18.1 ± 0.4	28.4 ± 0.6	18.2 ± 0.7	35.4 ± 0.5	63.8
Transfer‡ RNA						
	UV	17.5	26.0	23.4	33.0	59.0
Pulse labeled RNA						
	^{32}P	18.9	27.0	24.4	29.7	56.7

*Average of seven independent determinations.

‡Average of two determinations.

RNA are reproducible but of unknown significance.

The chromosomal RNA from pea cotyledon and chick embryo is characterized by a relatively high content of dihydrouridine; 27.5 mole per cent for pea cotyledon (Huang and Bonner, 1965) and 9.6 mole per cent for chick embryo (Huang, Smith and Alexander, 1968). Dihydrouridine was detected as a major component of pea chromosomal RNA by chromatography of the nucleotides on Dowex 1-X8. Under these conditions dihydrouridylic acid elutes just prior to U and is characterized by its high 230 mμ, low 260 mμ absorbance. It is apparent from Figure 6 that ascites chromosomal RNA does not contain an appreciable amount of dihydrouridylic acid eluting in this region.

The dihydrouridine content of chick embryo chromosomal RNA was determined by reaction of the nucleotides with paradimethylaminobenzaldehyde according to Fink, Cline, McCaughey and Fink (1956). The ureido group formed by alkali treatment of dihydrouridylic acid reacts to produce a yellow color which when compared to the proper standards can be used as an estimate of the dihydrouridine content. The total alkaline digest of ascites chromosomal RNA was, therefore, compared to yeast transfer RNA in its ability to react with paradimethylamino benzaldehyde. Ascites chromosomal RNA contained about 2 to 2 1/2 times as much ureido positive material as did yeast transfer RNA, which is known to contain about 4 mole per cent dihydrouridine (Jacobson, 1967).

Treatment of 5,6-dihydrouracil at 100°C for 3 hours in the presence of 0.2 N NaOH results in its conversion to β-ureidopropionic

acid which decomposes to form β-alanine (Magrath and Shaw, 1967). This reaction is nearly quantitative (78% conversion when in the form of 5,6-dihydrouridine) and can be used to estimate the content of dihydrouridine in RNA (Magrath and Shaw, 1967). When ascites chromosomal RNA was digested under these conditions and the products analyzed on a Spinco amino acid analyzer, no β-alanine was detected (Jacobson, 1967).

It, therefore, appears that 5,6-dihydrouridine is not a component of ascites chromosomal RNA. It does, however, contain a high level of ureido positive material the nature of which is still under investigation. It is possible that this material is a derivative of 5,6-dihydrouridylic acid.

Amino acid activation

Early in the course of these investigations, chromosomal RNA appeared to have many properties in common with transfer RNA, i.e. chromatographic behavior on DEAE Sephadex, sedimentation properties in a sucrose gradient, and base composition. It was, therefore, necessary to determine whether or not chromosomal RNA contained any amino acid acceptor activity. Activating enzymes (aminoacyl-tRNA synthetases) were prepared from rat liver and transfer RNA was prepared from both rat liver and ascites tumor cells. As shown in Table 5 both rat liver and ascites transfer RNA have a high acceptor activity. Chromosomal RNA is, however, completely devoid of any amino acid acceptor activity. In an effort to eliminate the possibility that transfer RNA had been inactivated by the prolonged preparative procedure of chromosomal RNA, purified rat liver

TABLE 5

Absence of Amino Acid Acceptor Activity

in Chromosomal RNA

Rat liver transfer RNA (μg)	Ascites transfer RNA (μg)	Ascites chromosomal RNA (μg)	Acceptor activity‡ cpm ^{14}C amino acid incorporated per 50 μl
10			1027
20			2284
30			3244
10*			1249
20*			2165
30*			3033
	10		757
	20		1248
	30		2274
		10	0
		20	27
		30	0
20		20	2273
	20	20	1025

*Transfer RNA treated with pronase (2 mg/ml) at 37°C for 7 hours followed by phenol extraction. The RNA precipitated and dissolved in maleate buffer as described in Materials and Methods.

‡Incorporation by enzyme alone (335 cpm/50 μl) subtracted.

transfer RNA was pronased and chromatographed on DEAE Sephadex following the procedure used for the purification of chromosomal RNA. As shown in the second row of data in Table 5, its activity was not affected by this treatment. Incubation of chromosomal RNA in the presence of CTP, a condition suitable for the addition of the very labile CCA end of transfer RNA, had no effect on its activity. The last two rows of Table 5 show there is no factor present in chromosomal RNA which inhibits the charging of active transfer RNA. It is, therefore, clear that chromosomal RNA does not contain a detectable amount of transfer RNA nor is it likely that chromosomal RNA results from degradation of transfer RNA during the preparation. (These results do not rule out the possibility that degradation occurred before pronase treatment and DEAE Sephadex chromatography. This possibility is, however, ruled out in Section 2 by hybridization competition experiment with transfer RNA.)

Isolation of 3S cytoplasmic RNA

The cytoplasmic proteins, banded in 4 M CsCl, also contain RNA. This RNA elutes from DEAE Sephadex as a sharp peak at the same molarity of NaCl as does chromosomal RNA and is, therefore, small in size. (The peak material exhibits a characteristic nucleic acid UV absorption spectrum, is totally base labile, and is sensitive to RNase.) This RNA constitutes about 6% of the total cytoplasmic RNA. The hybridization data presented in Section 2 indicate that this RNA is not a homogeneous fraction. The RNA has a sedimentation constant of about 3.2S as determined by analytical band velocity sedimentation in the Spinco Model E. For

convenience it will be referred to throughout this dissertation as 3S cytoplasmic RNA.

Methylation of chromosomal RNA in vivo

The different classes of RNA can be distinguished by their different content of methylated bases. The relative degree of methylation of E. coli RNA is as follows: transfer RNA = 1.00, 16S ribosomal RNA = 0.25, and 23S ribosomal RNA = 0.20 (Starr and Fefferman, 1964). About 6% of the nucleotides contained in E. coli transfer RNA are methylated. The degree of methylation of HeLa cell RNA is similar to that of E. coli (the mole per cent of methylated bases as follows: transfer RNA = 8.3, 18S ribosomal = 2.1, and 28S ribosomal RNA = 1.4 [Brown and Attardi, 1965]).

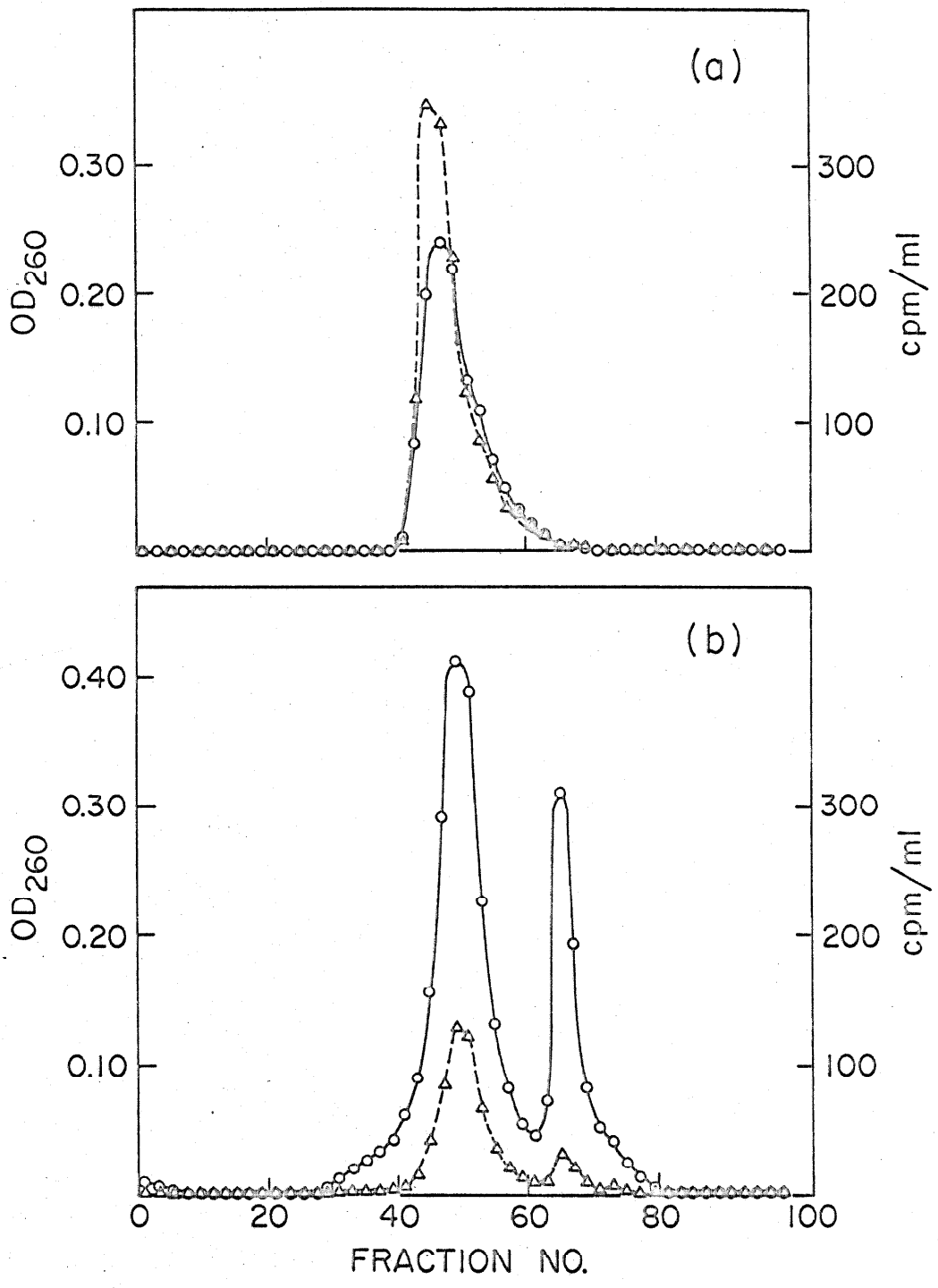
It has been shown by Mandel and Borek (1963) for the case of E. coli and by Biswas, Edmonds and Abrams (1961) for the case of Ehrlich ascites that the methyl donor for the methylation reaction is methionine. The extent of methylation of chromosomal RNA was, therefore, measured by exposing the cells to ^{14}C methylmethionine and measuring the extent of incorporation of the label into RNA. Incorporation into chromosomal RNA was compared with that into transfer and 3S cytoplasmic RNA. The various fractions of RNA were prepared as described in Materials and Methods and purified by chromatography on DEAE Sephadex. The optical density and radioactivity were determined and the specific activities of each fraction of RNA calculated.

The elution profiles of transfer RNA and chromosomal RNA are shown in Figure 7. The results of DEAE Sephadex chromatography of 3S

Figure 7(a). Elution profile of <u>in vivo</u> ^{14}C methylated ascites transfer RNA from DEAE Sephadex.

(b). Elution profile of <u>in vivo</u> ^{14}C methylated ascites chromosomal RNA from DEAE Sephadex.

—o—o—, OD_{260}; --△--△--, ^{14}C cpm.


(a)
0.30
0.20
0.10
OD260
300
200
100
cpm/ml
(b)
0.40
0.30
0.20
0.10
OD260
300
200
100
cpm/ml
0
20
40
60
80
100
FRACTION NO.

cytoplasmic RNA are shown in Figure 8. In the case of chromosomal and 3S cytoplasmic RNA the specific activity throughout the peak is constant, indicating that the fractions are pure. In the case of transfer RNA, 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 transfer RNA. The specific activities of the various fractions of RNA are listed in Table 6. The leading edge of the transfer RNA peak contains 5X 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 to ribosomal RNA but clearly much less than transfer RNA.

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

Rate of synthesis of chromosomal RNA

Among the RNAs contained in chromatin are messenger RNA (Bekhor, Kung and Bonner, 1968), probably in the act of being synthesized, and ribosomal RNA precursor associated with the nucleoli. Both of these classes of RNA may be closely associated with 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.

Figure 8. Elution profile of *in vivo* ^{14}C methylated ascites 3S RNA from DEAE Sephadex.

—O—O—, OD_{260}; --Δ--Δ--, ^{14}C cpm.

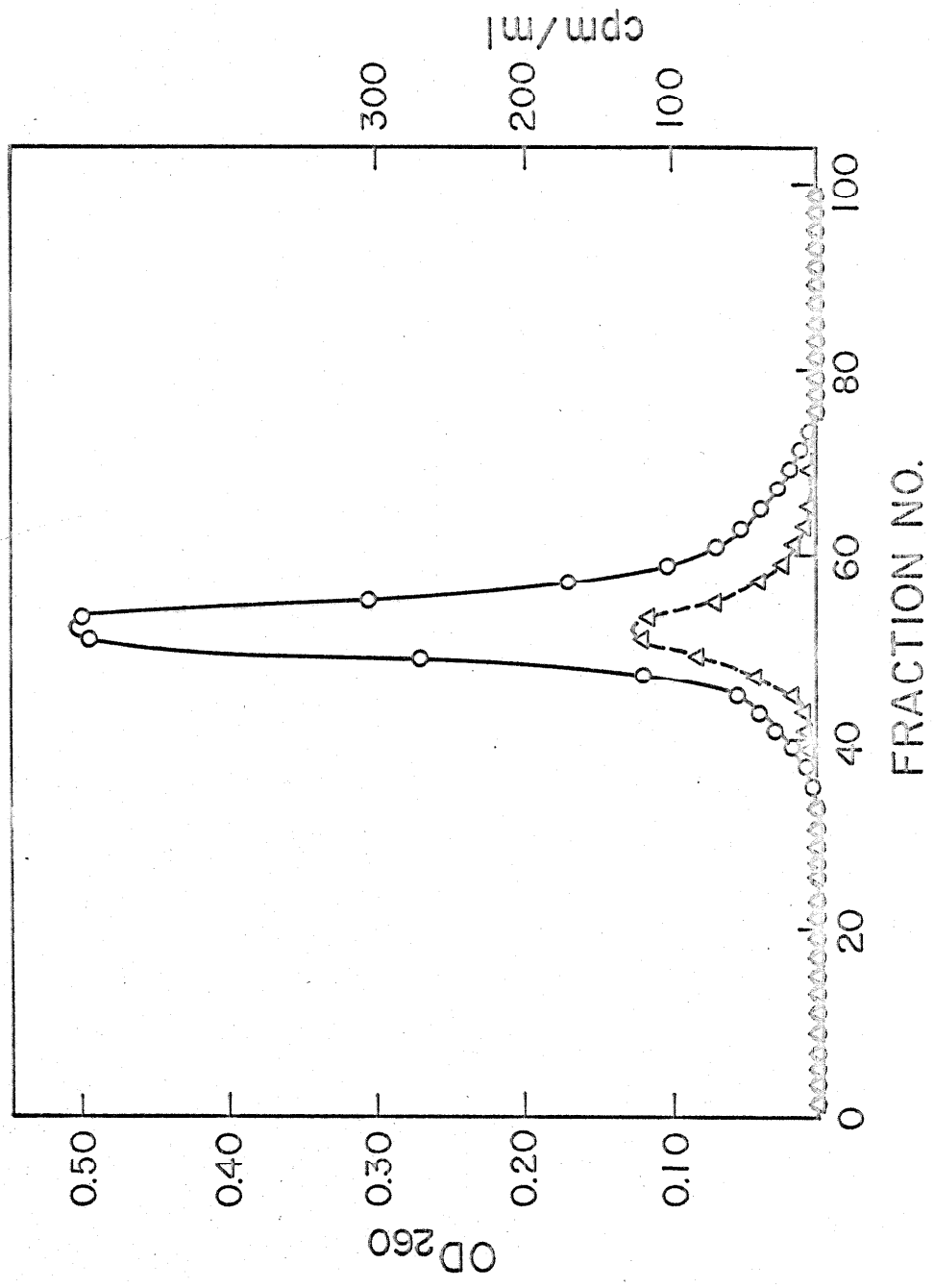

cpm/ml
300
200
100
OD260
0.50
0.40
0.30
0.20
0.10
0
20
40
60
80
100
FRACTION NO.

TABLE 6

Degree of Methylation of Various RNA Fractions

Species of nucleic acid	Methylation* cpm/mg RNA X10^{-2}	Per cent methylation relative to transfer RNA
Transfer RNA	387.0	100
Chromosomal RNA	77.0	20
3S RNA	65.0	17
Fraction II (DNA)	6.5	1.7

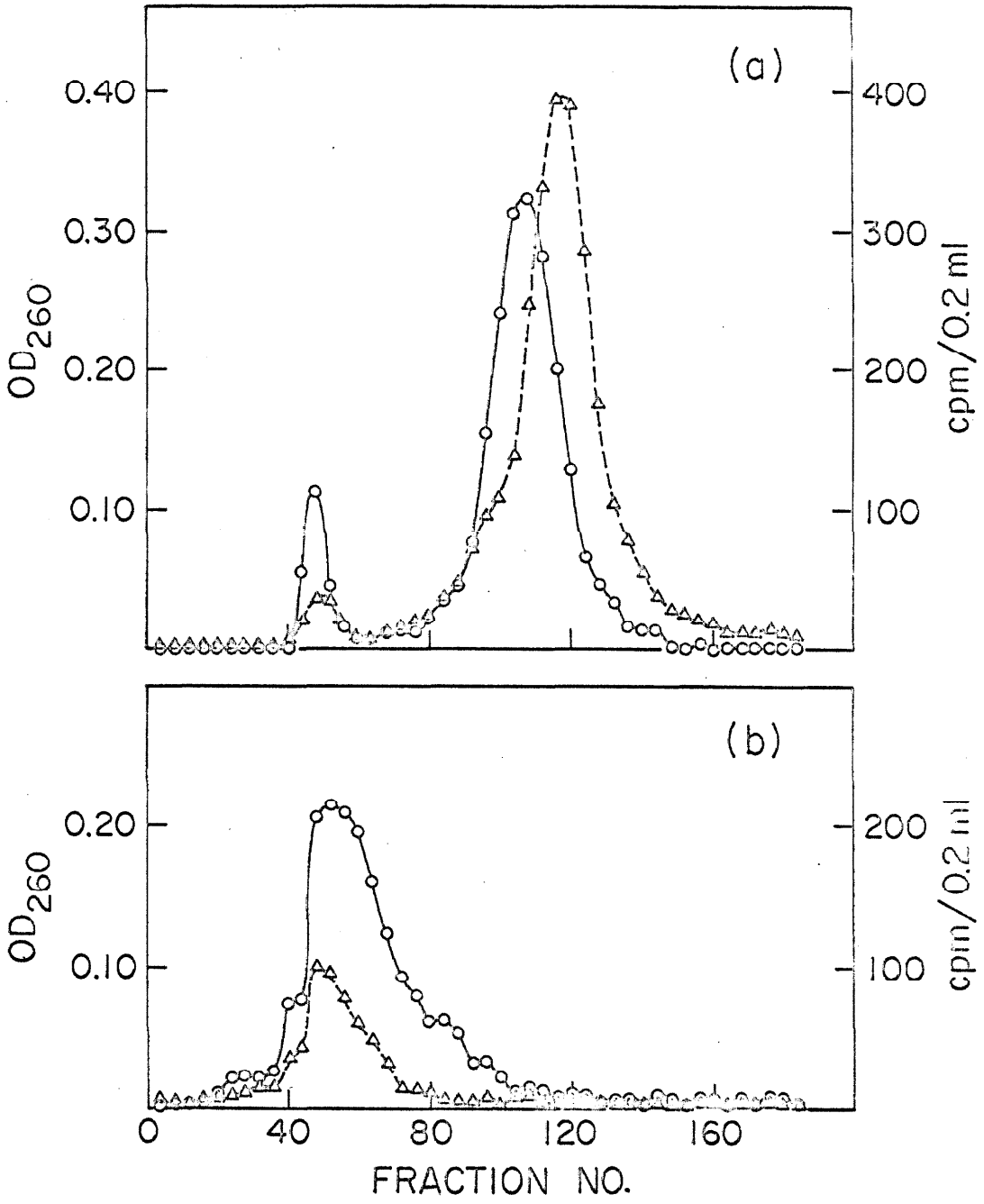
*Average specific activities of the peak fractions. In the case of transfer RNA the value reported is the average of the fractions at the leading edge of the peak.

Detailed studies on the turnover of ascites chromosomal RNA were prevented by the difficulties incurred in the preparation of many samples of chromosomal RNA by the present procedure, and by the inherent difficulties in studying RNA metabolism in a rapidly dividing system.

Ascites cells were subjected to a 10 minute pulse of ^{32}P and chromosomal and total RNA extracted as described in Materials and Methods. The RNA fractions were chromatographed on MAK and the specific activities of the different RNA species determined. (Independent experiments in which chromosomal RNA was co-chromatographed with total nucleic acid extracts, have shown chromosomal RNA to elute in the region of transfer RNA.) The elution profiles are shown in Figure 9. In part (a), total RNA, the first UV absorbing peak is transfer RNA, the second ribosomal RNA. Rapidly labeled messenger RNA elutes at the tail end of the ribosomal RNA peak as is apparent from the high specific activity in that region. Comparing this profile to that of chromosomal RNA (Fig. 9b) it is clear that chromosomal RNA is not rapidly labeled as is messenger RNA but is synthesized at a rate comparable to that of transfer RNA. The presence of DNA incompletely resolved from chromosomal RNA accounts for the decreased specific activity in fractions 70-100).

Figure 9. Elution profile of ^{32}P pulse labeled ascites total RNA (a) and chromosomal RNA (b) from MAK.

—o—o—, OD_{260}; --Δ--Δ--, ^{32}P cpm/0.2 ml.


(a)
0.40
0.30
0.20
0.10
OD260
400
300
200
100
cpm/0.2 ml
(b)
0.20
0.10
OD260
200
100
cpm/0.2 ml
0
40
80
120
160
FRACTION NO.

DISCUSSION

In the case of pea and chick chromosomal RNAs, the base compositions are very different from any previously reported RNA. It is, therefore, unlikely in these systems that the RNA in question results from degradation of another class of RNA. The base composition of rat ascites chromosomal RNA is not unique and consequently the question is more difficult to resolve. The most sensitive assay for similarity or difference of two kinds of RNA is, of course, to compare their base sequences. This has been done by hybridization competition experiments as described in the following section. However, many of the chemical and physical properties investigated already suggest that chromosomal RNA is not a degradation product.

a) The amounts of chromosomal RNA isolated from the chromatins of different creatures appears to be relatively constant. In addition different methods of isolation applied to the same kind of cells result in the same amount of chromosomal RNA. (Ascites chromatin isolated in the presence of divalent metal ions contained the same amount of chromosomal RNA as chromatin isolated in the presence of EDTA.) If chromosomal RNA were the result of degradation we would expect its amount to vary with the organism and conditions used for its isolation. We are, of course, at this stage dealing with limited data from tissues of similar nature.

b) Chromosomal RNA is very homogeneous with respect to size. If it resulted from degradation of other RNAs we would expect a broader size distribution. It is also unlikely that in the purification of chromosomal or 3S cytoplasmic RNA we have selected for degradation products of a specific size. Degradation was not apparent in the remainder of the total nucleic acid.

c) The low degree of methylation of chromosomal and 3S cytoplasmic RNA makes it unlikely that they are the result of degradation of transfer RNA. The slow rate of incorporation of ^{32}P into chromosomal RNA also makes it unlikely that chromosomal RNA is the result of degradation of messenger or ribosomal RNA precursor.

The chromosomal RNA isolated from rat ascites cells has many properties similar to those previously reported for pea bud and chick embryo chromosomal RNA (Huang and Bonner, 1965; Bonner and Huang, 1966; Bonner and Widholm, 1967; Huang et al., 1968). The amount of chromosomal RNA (relative to DNA) contained in the purified chromatin from rat ascites, pea bud, and chick embryo is between 2 to 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 preparation. Nucleohistone prepared from rat ascites cells by the method of Zubay and Doty (1959) contains less chromosomal RNA than does the starting chromatin. The mere fact that nucleohistone can be prepared so as to contain smaller amounts of RNA than chromatin, is no indication that this represents

the more native state. Purified chromatin, however, is known to possess the same template active and template inactive regions of the genome as native chromatin (Paul and Gilmour, 1966; Smith, Church and McCarthy, 1968). This information is not available for nucleohistone. The chromosomal RNA from rat liver reported by Benjamin et al. (1966) was 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 one would expect very little incorporation into chromosomal RNA, but a large amount into nascent messenger. In fact the base analysis and sedimentation profile reported by Benjamin et al. (1966) are most representative of messenger RNA. 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 3.3S for ascites chromosomal RNA is in good agreement with the sedimentation constants reported for pea (3.2S) and chick embryo (3.8S).

The base compositions of ascites, pea bud and chick embryo chromosomal RNA are presented in Table 7. The base compositions of pea bud and chick embryo are similar only in the sense that both RNAs contain a high per cent of dihydrouridylic acid. No dihydrouridylic acid was detected in ascites chromosomal RNA; it does, however, contain

TABLE 7

Nucleotide Composition of Chromosomal RNA from Various Sources

Source of RNA	Mole per cent					% GC	Pu/Pyr
	A	C	U	diHU	G		
Rat ascites	18.9	26.3	21.1	--	33.6	59.9	1.11
Pea bud*	31.6	10.4	15.2	27.5	15.3	25.7	0.88
Chick embryo‡	27.6	25.6	12.8	9.6	24.6	50.2	1.09

*Taken from Huang, Smith and Alexander (1968).

‡Taken from Huang and Bonner (1965).

a large amount of urcido positive material. If as has been suggested, chromosomal RNA binds, by base pairing to a region of the gene, and thus controls the activity of that gene, the base composition of chromosomal RNA should be complementary to this locus. Such large differences in base compositions would not have been predicted for a species of RNA with such similar physical properties and presumably the same biological function. The significance of these differences is unclear at the present time.

The properties of ascites chromosomal RNA are, therefore, generally similar to those of pea bud and chick embryo chromosomal RNAs previously studied. It seems likely then, that ascites chromosomal RNA is analogous to the chromosomal RNA of peas and chicks.

No attempts have been made, in the other systems in which chromosomal RNA has been investigated, to isolate a comparable fraction of RNA from the cytoplasm. This RNA fraction will be discussed in detail in Section 2.

Bonner and Huang (1966) have suggested that the possible function of chromosomal RNA is to detect the gene which is to be repressed. This would be accomplished by chromosomal RNA base pairing with a site analogous to the operator locus in bacteria. Once the chromosomal RNA had detected the proper gene, histones would complex with the DNA of the adjacent structural gene thereby completing the act of repression. Most of the properties of ascites chromosomal RNA investigated were responsible for the formulation of the present model. However, the degree of methylation and the rate of synthesis of chromosomal RNA had not been previously studied. We can, therefore, discuss these

findings in light of the proposed model. According to the present model, the synthesis of chromosomal RNA would be required only when new genes are to be repressed. The majority of genes in rat ascites chromatin are repressed (85% of the genome as indicated by the template activity of ascites purified chromatin relative to deproteinized ascites DNA) and are probably not active at any time in the life of an ascites cell. We would, therefore, expect the majority of chromosomal RNAs to be synthesized when DNA synthesis occurs so that they are available for repression in the new cell. Its rate of labeling would, therefore, be comparable to that of transfer and ribosomal RNA in which cases there are also pre-existing pools. If chromosomal RNA recognizes a specific site in the DNA by base pairing, we would not expect it to contain a significant number of altered bases, such as methylated bases, which would interfere with such recognition. Therefore, these properties investigated, although they do not confirm the model, are consistent with it.

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SECTION 2. HYBRIDIZATION PROPERTIES OF CHROMOSOMAL RNA

INTRODUCTION

This section is concerned with the hybridization properties of ascites chromosomal RNA. Its level of hybridization to homologous nuclear DNA was measured and compared to the levels obtained with other classes of RNA. In addition the homology between chromosomal RNA and 3S, transfer, and ribosomal RNA was investigated by hybridization competition. The base composition of the RNA hybrid formed was analyzed.

MATERIALS AND METHODS

Preparation of RNA

a) Chromosomal and 3S cytoplasmic RNA

Ascites chromosomal and 3S cytoplasmic RNA were prepared as described in Section 1. All samples were dissolved in and dialyzed against 2X SSC (0.3 M NaCl, 0.030 M sodium citrate). In these and all following preparations of RNA, RNase activity was monitored by incubation of the RNA for 20 hours at 37°C (when unlabeled RNA preparations were assayed ^{32}P-labeled RNA served as substrate). At the end of the incubation, RNA was precipitated in the presence of 1 mg carrier RNA by the addition of an equal volume of cold 20% trichloroacetic acid (TCA). Acid-insoluble material was collected by filtration on TCA presoaked membrane filters (Schleicher and Schuell B-6), washed with cold 10% TCA, and counted in a Nuclear Chicago D-47 gas flow counting system. Any samples containing RNase activity were subjected to additional phenol extractions until no detectable activity remained.

b) Total cytoplasmic, transfer and ribosomal RNA

The following extraction procedure allows the purification of transfer, ribosomal, chromosomal and 3S cytoplasmic RNA from the same lot of cells and was, therefore, routinely used.

The supernatant from the first centrifugation in the preparation of chromatin (see Materials and Methods of Section 1 for procedure) was precipitated with 1/10 vol. of 20% potassium acetate and 2 vol. of 95% ethanol (this step was necessary to reduce the volume for the following

extractions). A portion of the precipitate was used for the preparation of 3S cytoplasmic RNA as described in Section 1; the remainder was dissolved in 10 vol. of TKM (0.05 M tris, pH 6.7, 0.025 M KCl, 0.0025 M $MgCl_2$) containing 1% sodium dodecyl sulfate (SDS), 5 mg/ml sodium naphthalene disulfonate (Kirby, 1962), and 1 mg/ml bentonite (Fraenkel-Conrat, Singer and Tsugita, 1961). The latter was then shaken for 30 minutes with an equal volume of water saturated phenol containing 0.1% hydroxyquinoline (Kirby, 1962). The aqueous phase was separated by centrifugation and re-extracted twice with one-half volume of phenol. The nucleic acids were then precipitated in the presence of 2% potassium acetate and 2 vol. of 95% ethanol for 2 hours at -20°C. The precipitate was dissolved in TKM and treated with 20 μg/ml DNase (RNase free electrophoretically purified Worthington DNase I) at 25°C for 1 hour. The solution was made 1% in SDS and extracted twice with one-half volume of phenol. RNA was reprecipitated with ethanol and dissolved in a small volume of 2X SSC. In the preparation of total cytoplasmic RNA the sample was chromatographed on a 1 cm x 50 cm column of Sephadex G-50 in the presence of 2X SSC.

Transfer and ribosomal RNA were fractionated by a series of gel filtration and methylated albumin keiselguhr (MAK) chromatography. The sample was first fractionated on a Sephadex G-100 column (1.5 cm x 200 cm equilibrated and eluted with 2X SSC). Ribosomal, 5S, and transfer RNA were clearly separated in this fractionation. The ribosomal and transfer RNA 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 MAK as described by Mandell and Hershey (1960). Because of the low capacity of MAK columns, it was necessary to scale up the procedure by as much as 20 times. The RNA was eluted with a linear gradient of NaCl, concentrated by pressure dialysis followed by ethanol precipitation, and rechromatographed on Sephadex G-100. No contamination of the purified RNA fractions with other species of RNA could be detected in this final chromatographic procedure. The finally purified RNA was precipitated with ethanol and dialyzed against 2X SSC.

c) Partially degraded ribosomal RNA

Ascites ribosomal RNA was degraded and fractionated by the following procedure to obtain RNA fragments of about 3S. Purified ribosomal RNA (20 mg) was incubated with 40 μg pancreatic RNase at 4°C in SSC in a final volume of 4.5 ml. After 60 minutes SDS was added to a final concentration of 1% and the digest extracted twice with an equal volume of phenol. The RNA was precipitated with ethanol, dissolved in 0.2 M NaCl, 7 M urea, 0.01 M tris, pH 8.0 and chromatographed on DEAE Sephadex (chromatography procedure was identical to that used in the preparation of chromosomal RNA described in Materials and Methods of Section 1). The nucleic acid eluting at 0.55 M NaCl was precipitated with ethanol and dialyzed against 2X SSC. This material had an average sedimentation coefficient of about 3.3S as determined by band velocity sedimentation.

d) In vitro synthesized RNA

RNA was synthesized in vitro by E. coli RNA polymerase (f_4 of Chamberlin and Berg, 1962) using ascites purified chromatin or ascites DNA as template. (The preparation of ascites purified chromatin was described in Section 1.) The complete incubation mixture for RNA synthesis contained in a final volume of 10 ml: 400 μmoles tris buffer, pH 8.0; 40 μmoles $MgCl_2$; 10 μmoles $MnCl_2$; 120 μmoles mercaptoethanol; 8 μmoles each of GTP, UTP, CTP; 8 μmoles of ATP-8-^{14}C (2 μc/μmole); ascites DNA (100 μg) or chromatin (an amount containing 200 μg of DNA) and 1 mg of f_4. After 2 hours incubation at 30°C, SDS was added to a final concentration of 1% and the sample extracted with an equal volume of water saturated phenol containing 0.1% 8-hydroxyquinoline. Nucleic acids were precipitated from the aqueous phase with ethanol in the presence of 2% potassium acetate at -20°C for 2 hours. The precipitate was dissolved in 4 ml of TKM and treated with 20 μg/ml electrophoretically purified DNase at 25°C for 1 hour. The sample was again phenol extracted, the nucleic acid precipitated, and dissolved in and dialyzed against 2X SSC. When purified chromatin served as template, 3 mg of RNA were generated; when DNA served as template 5 mg of RNA were generated.

Preparation of DNA

Rat ascites DNA was prepared from crude chromatin (see Materials and Methods of Section 1) 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 hours at 37°C, RNase had been

previously heated at 80°C for 15 minutes to destroy any DNase activity) the DNA was incubated for 2 hours at 37°C with 40 μg/ml pronase (pronase, B grade from Calbiochem, had been previously autodigested for 90 minutes at 37°C). After digestion, the solution was made 1% in SDS and an equal volume of water saturated phenol added. The extraction was repeated twice in the absence of SDS with 1/2 vol. of phenol. The DNA was then spooled from the aqueous phase with the addition of 2 vol. of 95% ethanol, dissolved in 1/100 SSC and reprecipitated with isopropanol (Marmur, 1961). The purified DNA was then dissolved in and dialyzed against 1/100 SSC. RNase activity was monitored by incubation of the DNA with ^{32}P-labeled RNA in 2X SSC at 37°C for 20 hours followed by TCA precipitation as previously described. Phenol extraction was found to be more effective than extraction with chloroform-isoamyl alcohol for the removal of RNase activity. The DNA was stored at 4°C over a few drops of chloroform. For the preparation of ^{3}H-labeled DNA, 4 day tumor infected rats were each injected with 1 mc ^{3}H thymidine (14,600 μC/μmole obtained from Nuclear Chicago), the cells harvested 48 hours later and DNA extracted as described previously.

Preparation of DNA filters

DNA was denatured in 1/100 SSC either by alkali treatment, at pH 13 for 10 minutes, or by heating in a 100°C water bath for 15 minutes. Denaturation was monitored by following the increase in OD_{260}. Trace amounts of ^{3}H-labeled ascites DNA were added before denaturation of the unlabeled DNA to allow easy monitoring of the DNA in subsequent steps.

The denatured DNA was neutralized or, in the case of heat denaturation, quickly cooled, and diluted to 8 μg/ml and a final salt concentration of 6X SSC. Five ml aliquots were applied to 25 mm nitrocellulose filters (type B-6 Schleicher and Schuell) as described by Gillespie and Spiegelman (1965). Each DNA filter, therefore, contained 40 μg of denatured DNA (including 2 μg of ^{3}H-labeled ascites DNA, 7,200 cpm). Filters were washed with 6X SSC as prescribed and dried at room temperature for at least 4 hours followed by 2 hours at 80°C in a vacuum oven.

Hybridization

Hybridization was carried out at 66°C or at 25°C in the presence of 27 to 30 vol.% formamide (Bonner, Kung and Bekhor, 1967). Before the addition of the filters the hybridization solution containing the RNA was heated at 95°C for 10 minutes and cooled to 0 to 4°C. With the exception of the base analysis and kinetic studies, each vial contained two DNA filters and one blank filter in a volume of 1 ml and a final salt concentration of 2X SSC. Blank filters contained no DNA but received the same washing and drying procedures as did the DNA filters. At the end of the incubation the filters were removed, rinsed with 2X SSC in a large beaker and washed on each side with 50 ml of 2X SSC. RNase digestion was carried out at 25°C in a large volume of 2X SSC containing 20 μg/ml preheated pancreatic RNase (about 24 filters per 100 ml). After 1 hour incubation, the filters were rinsed with 2X SSC and again washed on each side with 50 ml of 2X SSC. The filters were dried in the vacuum oven and counted in a Beckman liquid scintillation spectrometer.

The amount of DNA retained on the filter at the end of the hybridization was dependent on the age of the DNA filters and the conditions under which the hybridization was carried out. DNA retention of freshly prepared filters after a 10 hour incubation at 66°C, or at 24°C in the presence of 30% formamide, was between 95 and 100%. The age of the filter had little effect on the retention of DNA when the hybridization was carried out in 30% formamide. However, filters dried several days prior to hybridization lost as much as 30% of their DNA upon a 10 hour incubation at 66°C. A significant amount of the DNA lost was bound to the blank filter, thus making an accurate determination of the background very difficult. For this reason filters were prepared just prior to use. (We have on occasion received certain lots of nitrocellulose filters which exhibited poor DNA retention even when used immediately after their preparation. The poor retention was most apparent when the hybridization was carried out at 66°C.) The background at 66°C (i.e. counts absorbed to the filter containing no DNA) was normally less than 0.04% of the input counts; in the presence of 30% formamide the background was decreased to about 0.01% of the input counts.

Extraction and base analysis of hybridized RNA

The hybridized RNA was extracted by heating the DNA filters in deionized water at 100°C for 15 minutes. The extraction was repeated 4 times. The extracts were pooled and precipitated in the presence of 1 mg carrier ascites ribosomal RNA by the addition of 1/10

vol.20% potassium acetate and 2 vol.95% ethanol. This extraction and precipitation result in 100% recovery of the hybridized RNA. The ethanol precipitate was hydrolyzed in 0.3 N KOH at 37°C for 18 hours. A portion of the DNA was also extracted from the filters and was precipitated at the end of the hydrolysis by the addition of 60% perchloric acid to a final concentration of 0.5 N. The supernatant was then adjusted to pH 8.0 with KOH, diluted with 10 vol. of deionized water and the nucleotides separated on a 1-X8 Dowex column as described in Section 1. The ribosomal RNA added as carrier permits easy monitoring of the column and serves as a control for the fractionation.

RESULTS

Properties of chromosomal RNA hybridization

The dependence of chromosomal RNA hybridization on the amount of denatured ascites DNA is shown in Figure 1. Over the range of DNA amounts investigated, the amount of RNA hybridized is directly proportional to the amount of DNA on the filter. In all following experiments, with the exception of those concerned with the base analysis of hybrid RNA, DNA filters contained 40 μg of denatured DNA.

The amount of chromosomal RNA hybridized as a function of time is shown in Figure 2. The saturation value obtained was dependent on the input ratio of RNA to DNA. Saturation of the DNA, at various input ratios of RNA to DNA, was always complete by 10 hours. When the hybridization was carried out at 66°C the amount of RNA hybridized reached a maximum at 10 hours and then, due to loss of DNA from the filters, slowly decreased. All subsequent hybridizations were carried out for 10 hours.

The data contained in Table 1 show that the hybridization of ascites chromosomal RNA is specific for rat ascites DNA. About 1/6 the amount of hybrid is formed with calf thymus DNA and between 1/10 to 1/20 the amount with pea DNA. The specificity is slightly greater when the hybridization is carried out at 66°C than it is at 25°C in the presence of 27% formamide. Higher concentrations of formamide, which would result in more stringent hybridization conditions, are probably required to obtain specificity equal to that observed at 66°C. The

Figure 1. Hybridization of rat ascites chromosomal RNA to filters containing various amounts of denatured ascites nuclear DNA. Three DNA filters and one blank filter were incubated in 1 ml 2X SSC containing 27 μg ascites chromosomal RNA (813 cpm/μg) at 66°C for 16 hours.

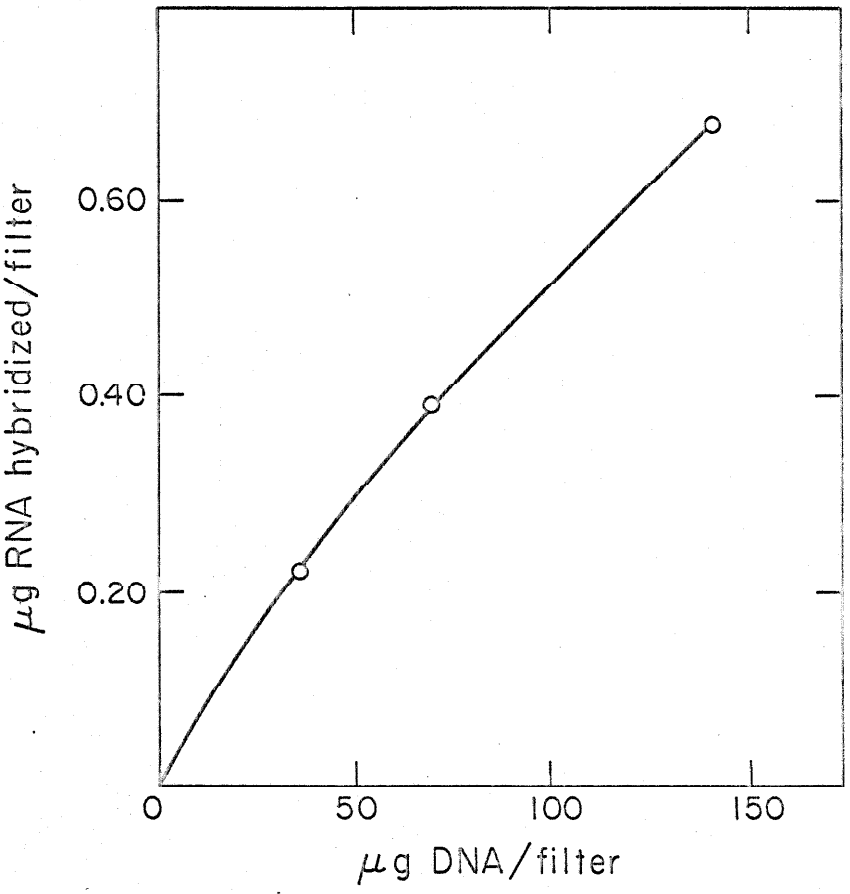

μg RNA hybridized/filter
0.60
0.40
0.20
0
50
100
150
μg DNA/filter

Figure 2. Hybridization of ascites chromosomal RNA to ascites DNA as a function of time. Eight DNA filters, each containing 40 μg DNA, and four blank filters were hybridized at 25°C in 3 ml 2X SSC containing 30% formamide and 585 μg chromosomal RNA (1419 cpm/μg). At various times two DNA filters and one blank filter were removed and the amount of hybrid assayed as described in Materials and Methods. The μg RNA hybridized per filter is plotted as a function of time.

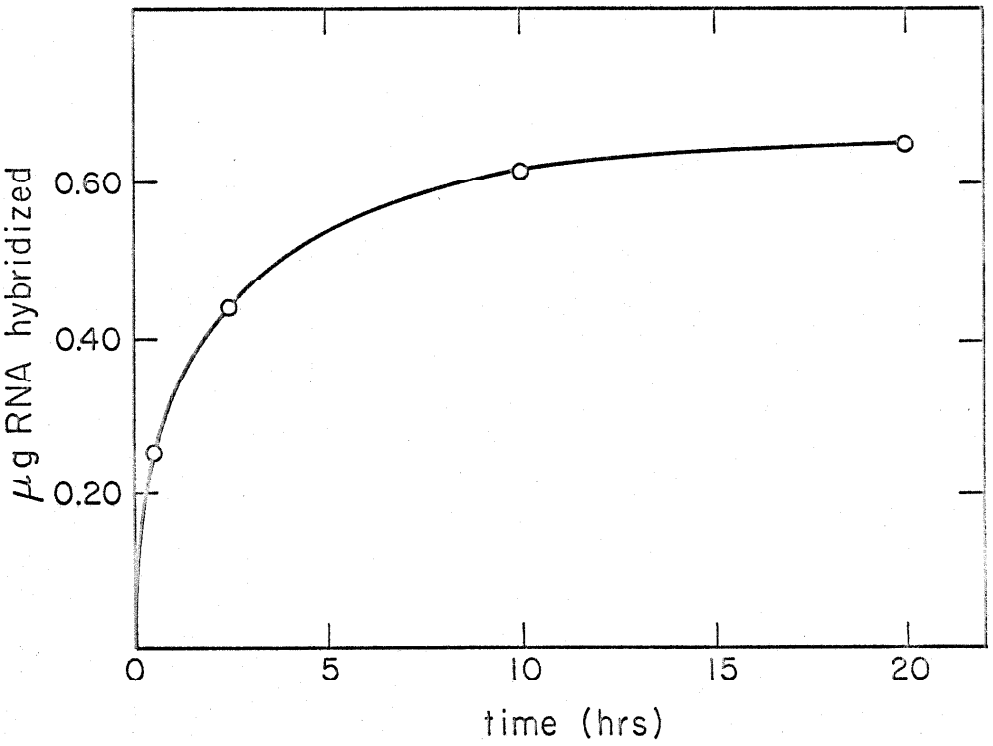

μg RNA hybridized
0.60
0.40
0.20
0
5
10
15
20
time (hrs)

TABLE 1

Hybridization of Rat Ascites Chromosomal RNA

to Various DNAs

Expt. no.	Hybridization condition	DNA source	Ascites chromosomal RNA		μg RNA hybridized/μg DNA x10²
			Input (μg)	Hybridized* (μg RNA/filter)	
1	66°C	Ascites	60	0.521	1.28
		Pea embryo	60	0.026	0.06
		Sea urchin	60	0.011	0.03
2	66°C	Ascites	27	0.254	0.72
		Calf thymus	27	0.052	0.13
		Pea embryo	27	0.025	0.08
3	27% formamide	Ascites	120	0.821	2.06
		Sea urchin	120	0.094	0.23
		Lambda	120	0.035	0.08

*Heterologous DNA filters of experiments no. 1 and 3 contained 2 μg ^{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 experiment 2 contained no labeled DNA and no correction has been made.

The values reported in experiment 3 are the average of four DNA filters (two DNA filters per vial). Specific activity of the RNA used in the various experiments was as follows: no. 1, 1155 cpm/μg; no. 2, 813 cpm/μg; no. 3, 1073 cpm/μg.

decreased specificity is reflected in the amount of hybrid formed under the different conditions. A 10 to 15% increase is observed, in the total amount of hybrid formed, when 27% formamide is substituted for 66°C. The amount of hybrid formed at 30% formamide is, however, equal to the amount formed at 66°C. With the exception of a few experiments, which will be clearly noted, all hybridization was carried out at 66°C. Many experiments were carried out under both hybridizing conditions; in these cases the results were identical.

The per cent of DNA hybridized in the presence of increasing amounts of chromosomal RNA is shown in Figure 3. The double reciprocal plot presented in Figure 4 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°C in the presence of 30% formamide. Hybridization in the presence of 27% formamide, however, resulted in a 10 to 15% increase in the amount of hybrid formed. Additional purification of chromosomal RNA by chromatography on methylated albumin keiselguhr or Sephadex G-50 did not alter its level of hybridization.

Hybridization of transfer, ribosomal, and 3S cytoplasmic RNA

Attardi *et al.* (1965) have reported HeLa 28S ribosomal RNA to be complementary to 3-5 x 10^{-3}% of HeLa DNA, 18S ribosomal RNA on the order of 2-3 x 10^{-3}%. These values are, however, minimum estimates, for

Figure 3. Hybridization of ascites chromosomal RNA to ascites nuclear DNA in the presence of increasing amounts of RNA. Hybridization at 66°C in 2X SSC.

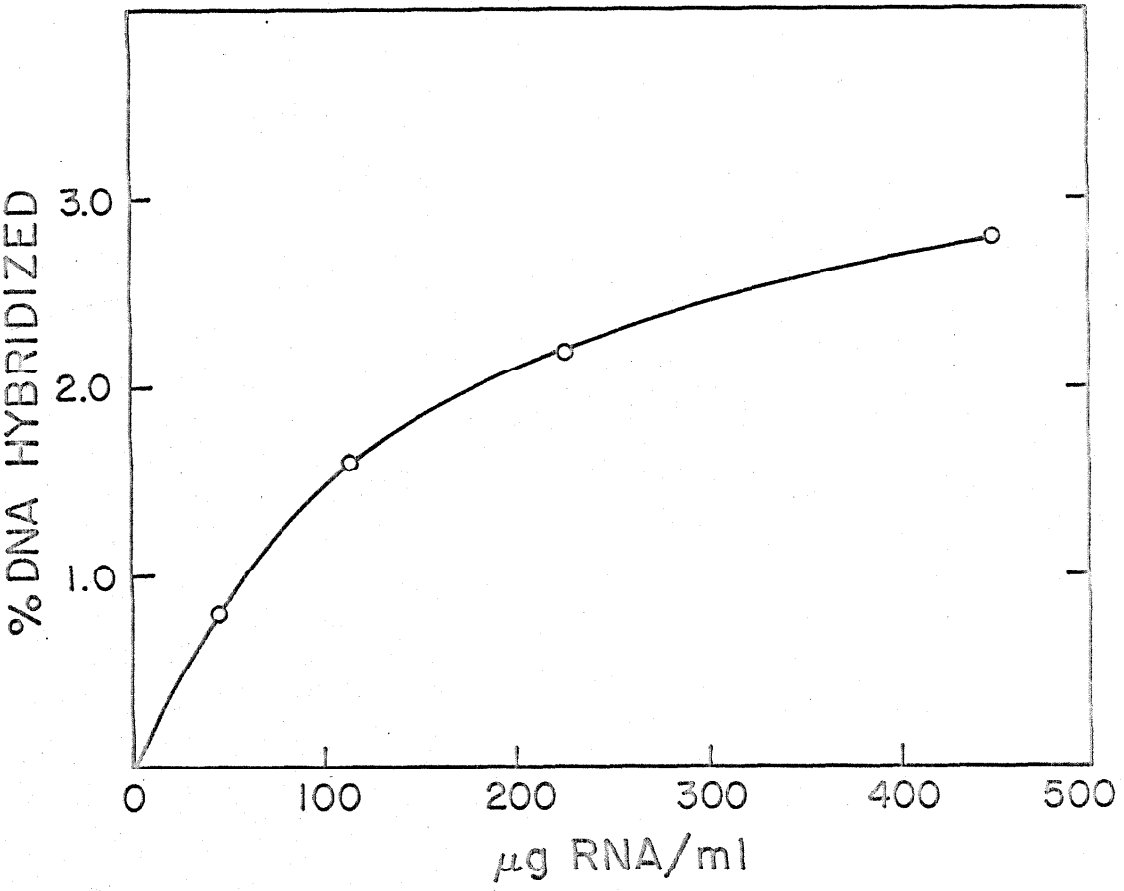
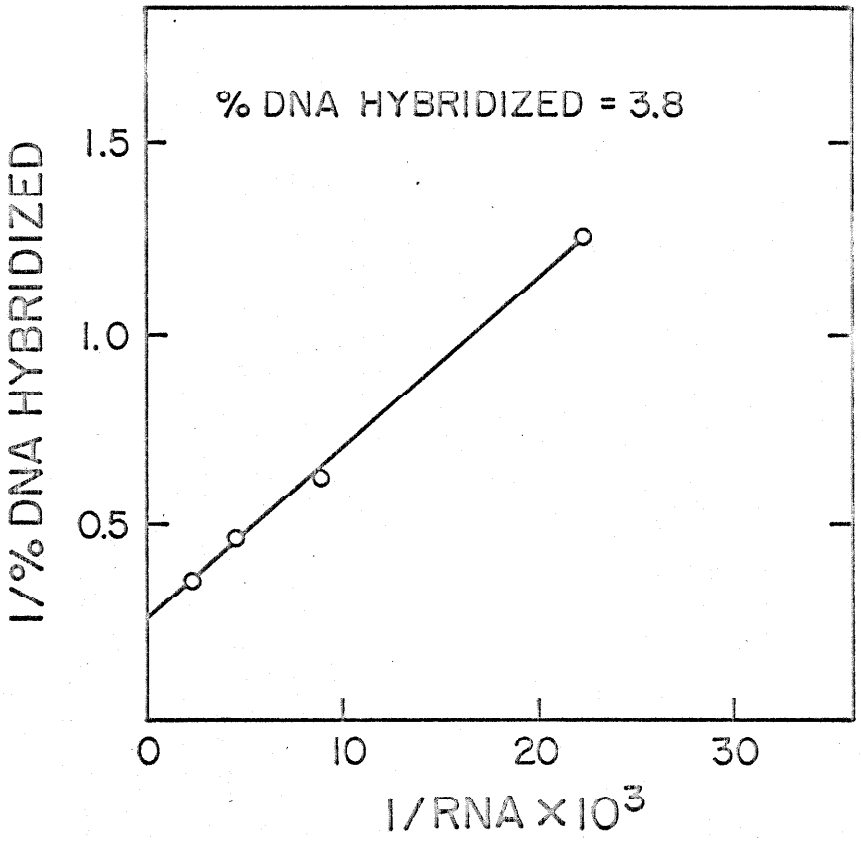

% DNA HYBRIDIZED
3.0
2.0
1.0
0
100
200
300
400
500
μg RNA/ml

Figure 4. Double reciprocal plot of saturation curve presented in Figure 3.


% DNA HYBRIDIZED = 3.8
1/% DNA HYBRIDIZED
1.5
1.0
0.5
0
10
20
30
1/RNA X 10³

only large molecules with a base composition similar to the input ribosomal RNA were measured. The total amount of hybrid formed involved 0.02% of the DNA. Transfer RNA of HeLa cells is homologous to about 5×10^{-3}% of HeLa DNA (Hatlen, 1968). This measurement is a measure of total hybrid with no selection with respect to size or base composition. The specific activities of ascites transfer and ribosomal RNA (1,000 to 2,000 cpm/μg) are not high enough to allow accurate measurements of such low levels of hybridization. It was necessary, however, to obtain a saturation value under the experimental conditions used in the hybridization of ascites chromosomal RNA.

The data of Table 2 show the level of hybridization obtained with ascites transfer and ribosomal RNA. For comparison, the table also includes data on the hybridization of chromosomal RNA. Concentrations of transfer and ribosomal RNA up to 600 μg/ml do not completely saturate the DNA (Figure 5). It seems unlikely, therefore, that this interaction represents the specific hybridization of transfer or ribosomal RNA but rather the background of the system. (The hybridization may be due to the presence of trace amounts of messenger RNA.) The amount of transfer or ribosomal RNA bound to the DNA filters is on the order of 0.02% of the input RNA. Similar results were obtained when the hybridization was carried out at 66°C; the background, however, was increased.

The level of hybridization of transfer and ribosomal RNA was greatly influenced by the purity of the samples. By repeated chromatography on Sephadex G-100 and methylated albumin keiselguhr both the

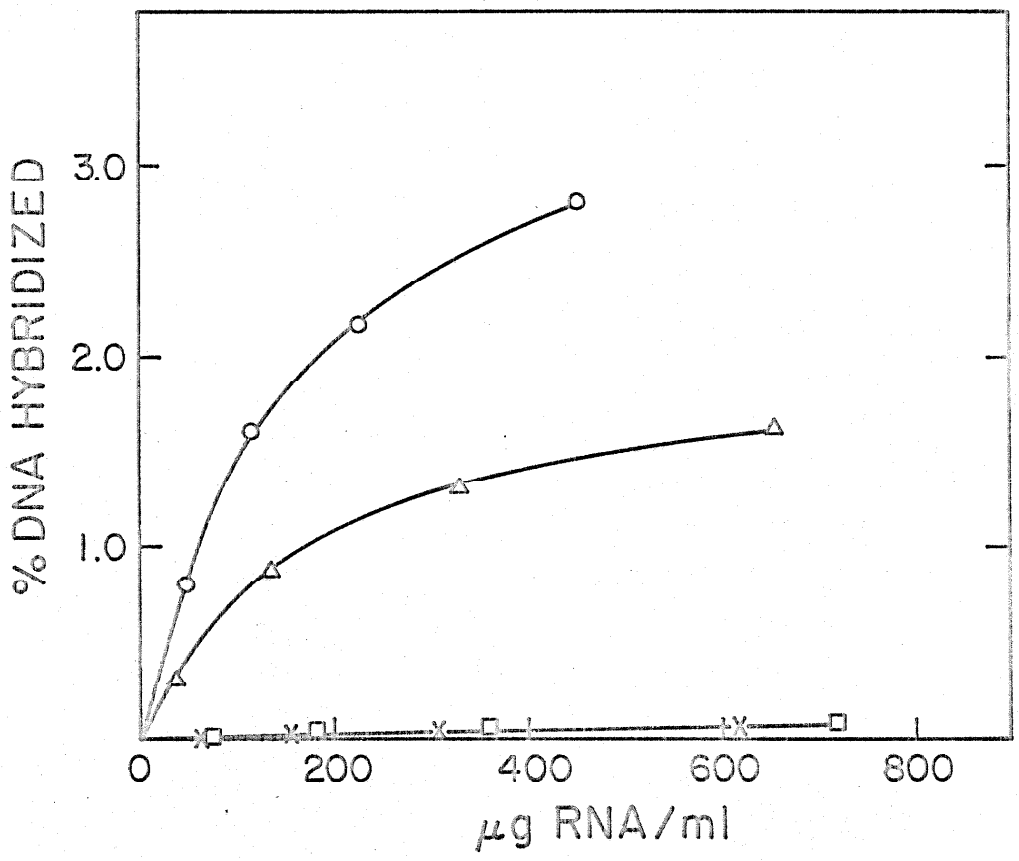
TABLE 2

Hybridization of Rat Ascites Chromosomal, Transfer, and Ribosomal RNA to Ascites Nuclear DNA

Species of ascites RNA	Specific activity cpm/μg	Input RNA (μg)	cpm hybridized/filter	Hybridized RNA (μg/filter)	μg RNA hybridized/μg DNA x10^2
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

Incubation at 25°C for 10 hours in the presence of 30% formamide.

Figure 5. Hybridization of different species of ascites RNA to ascites nuclear DNA as a function of RNA concentration. Chromosomal RNA, 1275 cpm/μg (—O—O—); 3S cytoplasmic RNA, 1025 cpm/μg (—△—△—); transfer RNA, 1575 cpm/μg (—x—x—); ribosomal RNA, 1265 cpm/μg (—□—□—). The hybridization of chromosomal RNA was performed at 66°C, all other samples were hybridized at 25°C in the presence of 30% formamide.


% DNA HYBRIDIZED
3.0
2.0
1.0
0
200
400
600
800
μg RNA/ml

background and hybridization levels were greatly reduced. (As mentioned previously, additional purification of chromosomal RNA did not alter its hybridization properties.)

Ascites 3S cytoplasmic RNA hybridizes at saturation with about 1.8% of denatured ascites DNA. The results of this experiment are presented in Figure 5 along with the RNA saturation curves for chromosomal, transfer and ribosomal RNA.

Hybridization competition

a) General properties of hybridization competition

The relationship between ascites chromosomal RNA and various other RNA species was investigated by hybridization competition. Competition reactions can be carried out simultaneously by allowing the mixture of labeled and unlabeled RNA to react together, or sequentially by preincubating the unlabeled RNA with DNA prior to the addition of labeled RNA. The results obtained by sequential hybridization are dependent on the stability of the hybrid formed in the first incubation. Clear results can be obtained only when the hybrid formed is stable during the subsequent incubation. A comparison of these two methods using ascites chromosomal RNA as both labeled, and unlabeled competing RNA is shown in Table 3. It is apparent that the DNA-RNA hybrid formed under these conditions is not completely stable but is reduced by 31% by a 10 hour additional incubation in the presence of 2X SSC. When both labeled and unlabeled competing RNA are added simultaneously and incubated for 10 hours the amount of labeled hybrid formed is reduced by 50%. However,

TABLE 3

Hybridization Competition by Presaturation and Simultaneous Addition of RNA

Time (hr)			μg ^{32}P RNA hybridized	% ^{32}P hybrid remaining
0-10	10	10-20		
Hot			0.279	100
Hot	Wash	2X SSC	0.191	69
Hot + Cold			0.143	51
Cold	Wash	Hot	0.205	74
Cold		Hot	0.114	41

Hybridization competition between ^{32}P-labeled chromosomal RNA and unlabeled chromosomal RNA.

Explanation of symbols: hot, 53 μg labeled chromosomal RNA (1007 cpm/μg); cold, 210 μg unlabeled chromosomal RNA; wash, filters removed from the hybridization medium, washed with 50 ml 2X SSC on each side and added to fresh medium. All incubations at 24°C in the presence of 30% formamide.

if the DNA is preincubated for 10 hours with unlabeled chromosomal RNA, washed, and allowed to hybridize with labeled RNA, the amount of labeled hybrid formed is reduced by only 28%. This difference is most likely due to the decomposition of the unlabeled RNA-DNA complex during the second 10 hours incubation, thus making more sites available for the hybridization of labeled RNA. Preincubation of the DNA for 10 hours with unlabeled RNA followed by the addition of labeled RNA resulted in a 60% reduction in the amount of labeled hybrid formed. In this case, even though the hybrid is unstable, the unlabeled RNA is still present to compete with the labeled RNA.

Therefore, because of the complications involved in sequential hybridization experiments, the following competition studies were carried out by the simultaneous addition of both RNA species.

b) Competition between labeled chromosomal and unlabeled transfer and ribosomal RNA

Transfer and ribosomal RNA, purified in the same manner as the transfer and ribosomal RNA used in the previous hybridization studies, does not compete with chromosomal RNA in the hybridization to ascites DNA (Table 4). This result is not surprising since both transfer and ribosomal RNA are capable of forming hybrids with less than 0.06% of the DNA (chromosomal RNA hybridizes with 4% of the DNA). Ribosomal RNA, however, when present in large amounts relative to chromosomal RNA, does interfere with the hybridization of chromosomal RNA. This point will be discussed in detail later in this section.

TABLE 4

Hybridization Competition Between Labeled

Chromosomal RNA and Unlabeled Ascites

Transfer and Ribosomal RNA

μg ^{32}P ascites chromosomal RNA	Competing RNA		Total RNA/^{32}P chromosomal RNA	μg ^{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

c) Competition between labeled chromosomal and unlabeled *in vitro* synthesized messenger RNA

The interference of ribosomal RNA in the hybridization of chromosomal RNA made it impossible to carry out competition experiments with pulse labeled RNA. A more suitable comparison of the messenger RNA fraction was made by the use of ascites purified chromatin. It has been shown by Paul and Gilmour (1966) and by Smith, Church and McCarthy (1968) that the RNA, generated by chromatin *in vitro*, possesses complete sequence homology with the messenger RNA of the tissue from which the chromatin was isolated. We can thus compare chromosomal RNA to 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 5. The addition of 10 times the amount of either ascites or pea messenger RNA 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.

TABLE 5

Hybridization Competition Between Labeled Chromosomal RNA and *in vitro* Generated RNA

μg ^{32}P ascites chromosomal RNA	Competing RNA		Total RNA/^{32}P chromosomal RNA	μg ^{32}P RNA hybridized/filter	% labeled hybrid remaining
	Species	Amount (μg)			
60				.96	100
60	Ascites mRNA	540	10	.95	98.9
60	Pea cotyledon mRNA	540	10	.99	103.0
60	Total RNA*	540	10	.87	91.0
60	Ascites chromosomal RNA	540	10	.26	27.5

*RNA synthesized *in vitro* by *E. coli* RNA polymerase with ascites DNA as template.

d) Competition between chromosomal RNA and 3S RNA

The competition curve obtained when ^{32}P-labeled chromosomal RNA is hybridized in the presence of increasing amounts of unlabeled homologous RNA is shown in Figure 6. The observed competition curve is in good agreement with the theoretical curve calculated from the chromosomal RNA hybridization saturation curve (Fig. 3). The data of Figure 6 also show that unlabeled 3S cytoplasmic RNA competes with ^{32}P-labeled chromosomal RNA. It is effective in competing with chromosomal RNA 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 not interference of the type observed with ribosomal RNA, was shown by sequential hybridization experiments in which the DNA was first hybridized with 3S cytoplasmic RNA followed by hybridization with labeled chromosomal RNA.

The data for Figure 7 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 probably no 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 shows that the

Figure 6. 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°C with 60 μg of labeled ascites chromosomal RNA throughout.

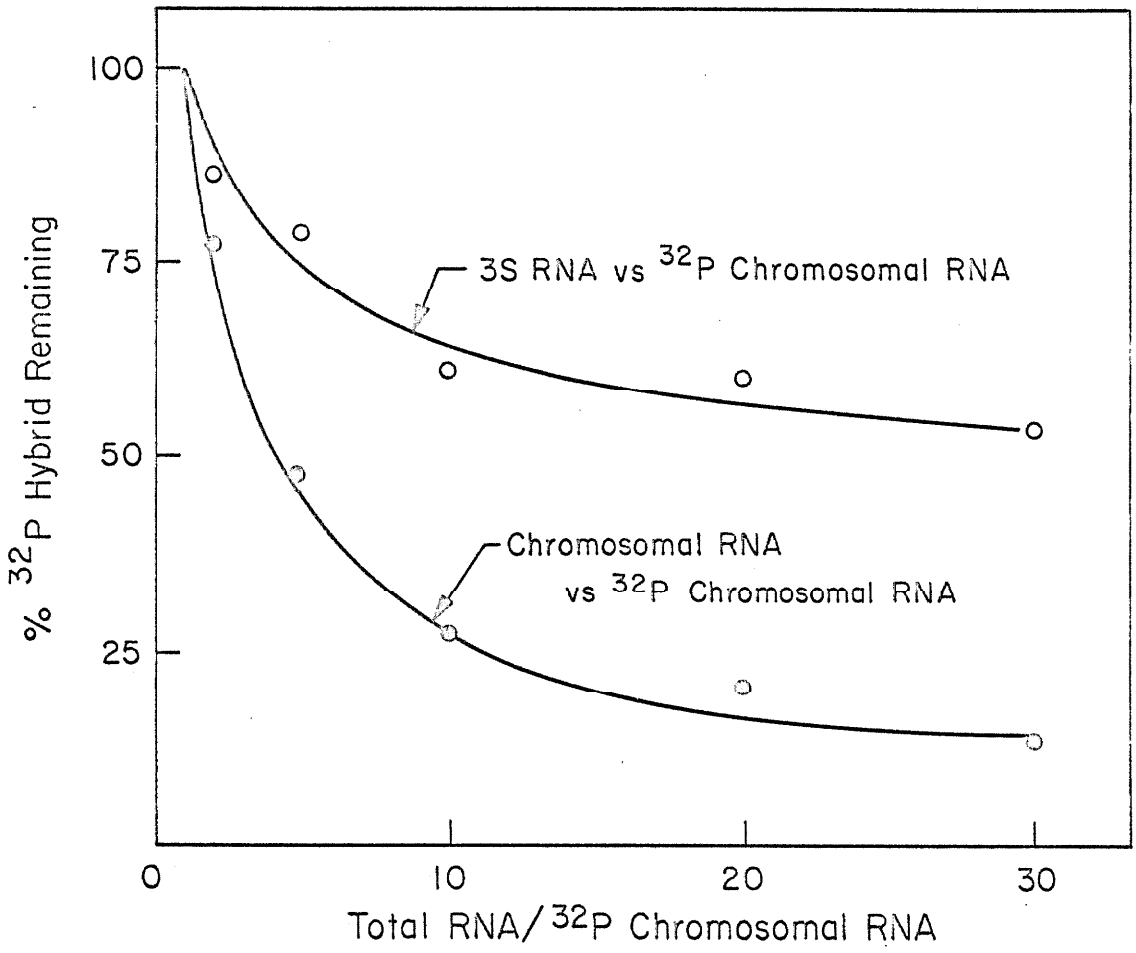
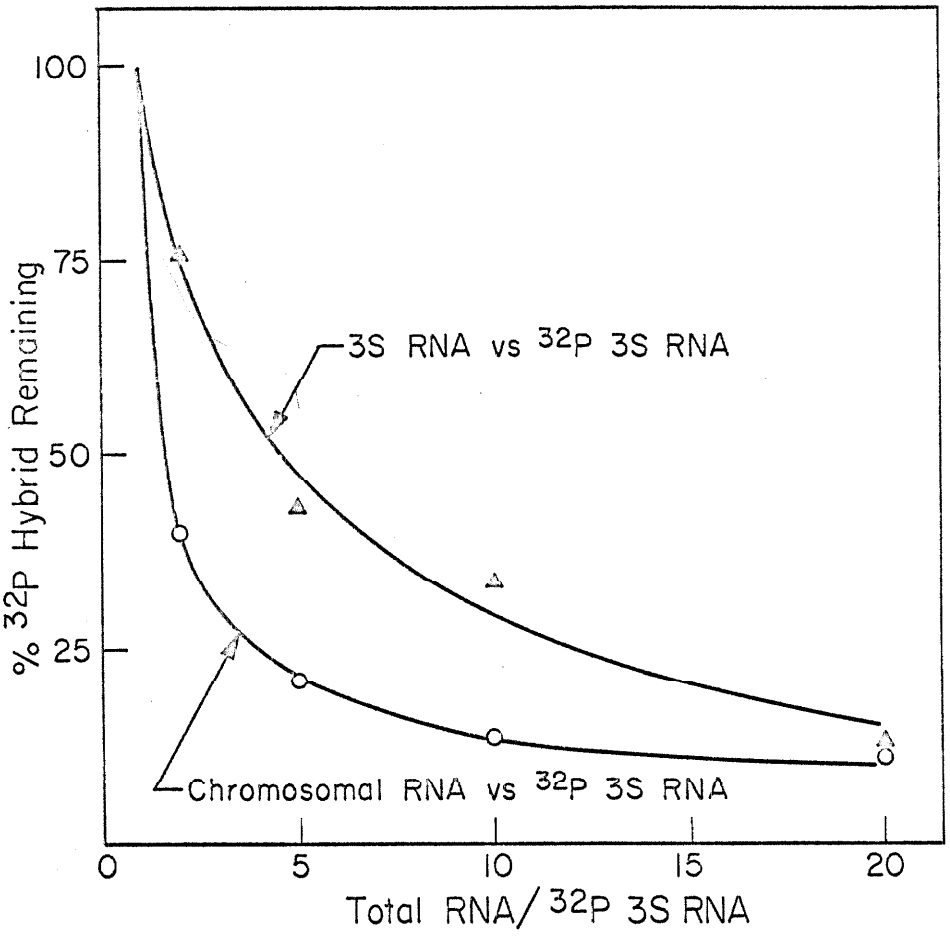

100
75
50
25
0
10
20
30
% 32P Hybrid Remaining
Total RNA/32P Chromosomal RNA
3S RNA vs 32P Chromosomal RNA
Chromosomal RNA
vs 32P Chromosomal RNA

Figure 7. 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 at 24°C in the presence of 27% formamide.


100
75
50
25
% 32P Hybrid Remaining
3S RNA vs 32P 3S RNA
Chromosomal RNA vs 32P 3S RNA
0
5
10
15
20
Total RNA/ 32P 3S RNA

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 (see Materials and Methods of Section 1). This fraction of RNA has hybridization properties identical to those of 3S cytoplasmic RNA (McConnell, 1967). It is capable of competing for only 50% of the chromosomal RNA binding sites and contains no sequences not also contained in chromosomal RNA.

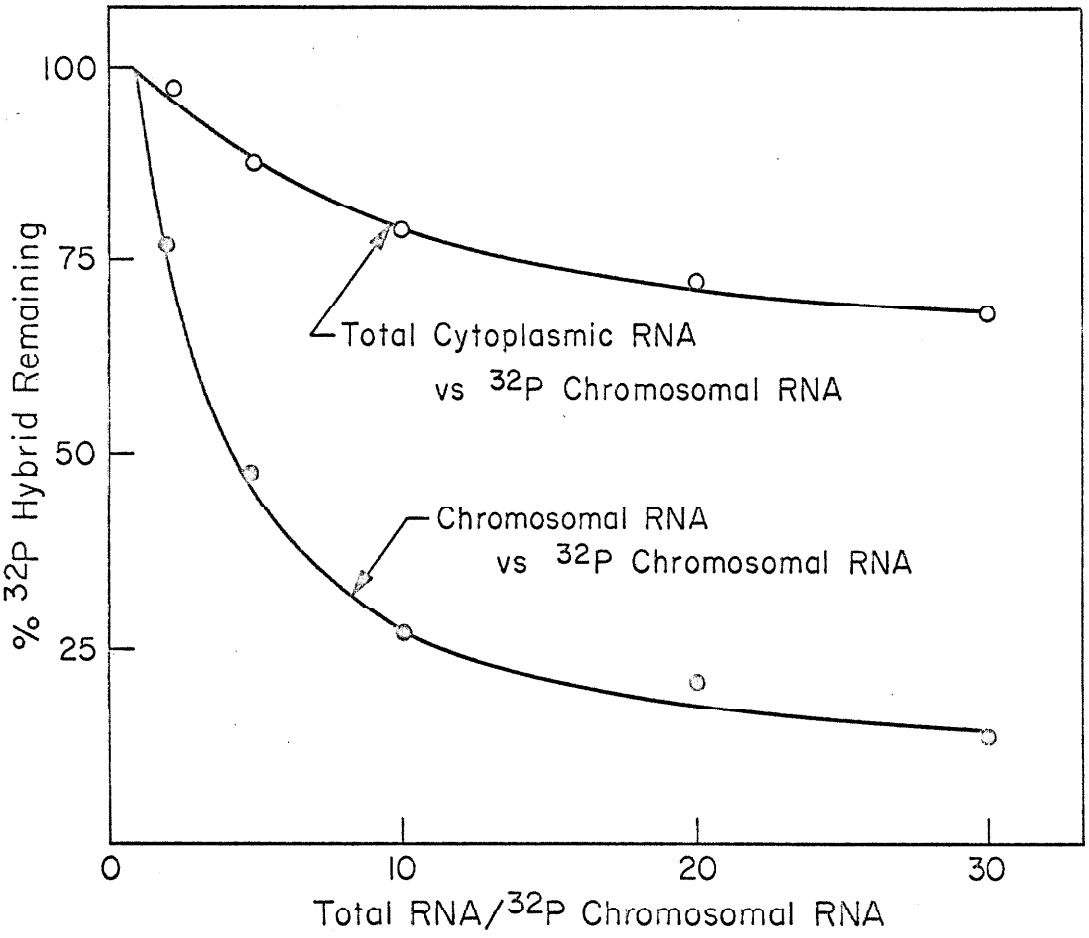
A portion of the chromosomal RNA, about 50% of the sequences present, therefore, appears to be confined to the chromatin. The remainder of the chromosomal RNA is homologous to a fraction of RNA present in the nuclear sap and cytoplasm.

Ribosomal RNA interference in chromosomal RNA-DNA hybridization

It is apparent from Figure 8 that cytoplasmic RNA is a relatively effective competitor in the hybridization of chromosomal RNA. To determine the specific RNA species contained in cytoplasmic RNA responsible for this competition, various fractions of cytoplasmic RNA were prepared and their ability to compete with chromosomal RNA, in the hybridization to DNA, determined.

Since transfer or ribosomal RNA are capable of forming hybrids with only 0.06% of the DNA, complete blockage of their sites, with cold transfer or ribosomal RNA, should not cause a significant change in the hybridization of chromosomal RNA which can form hybrids with 4% of the

Figure 8. Ability of unlabeled total cytoplasmic RNA to compete with ^{32}P-labeled chromosomal RNA in the hybridization to ascites DNA. All hybridization at 66°C with 60 μg of labeled ascites chromosomal RNA throughout.


100
75
50
25
% 32P Hybrid Remaining
Total Cytoplasmic RNA
vs 32P Chromosomal RNA
Chromosomal RNA
vs 32P Chromosomal RNA
0
10
20
30
Total RNA/32P Chromosomal RNA

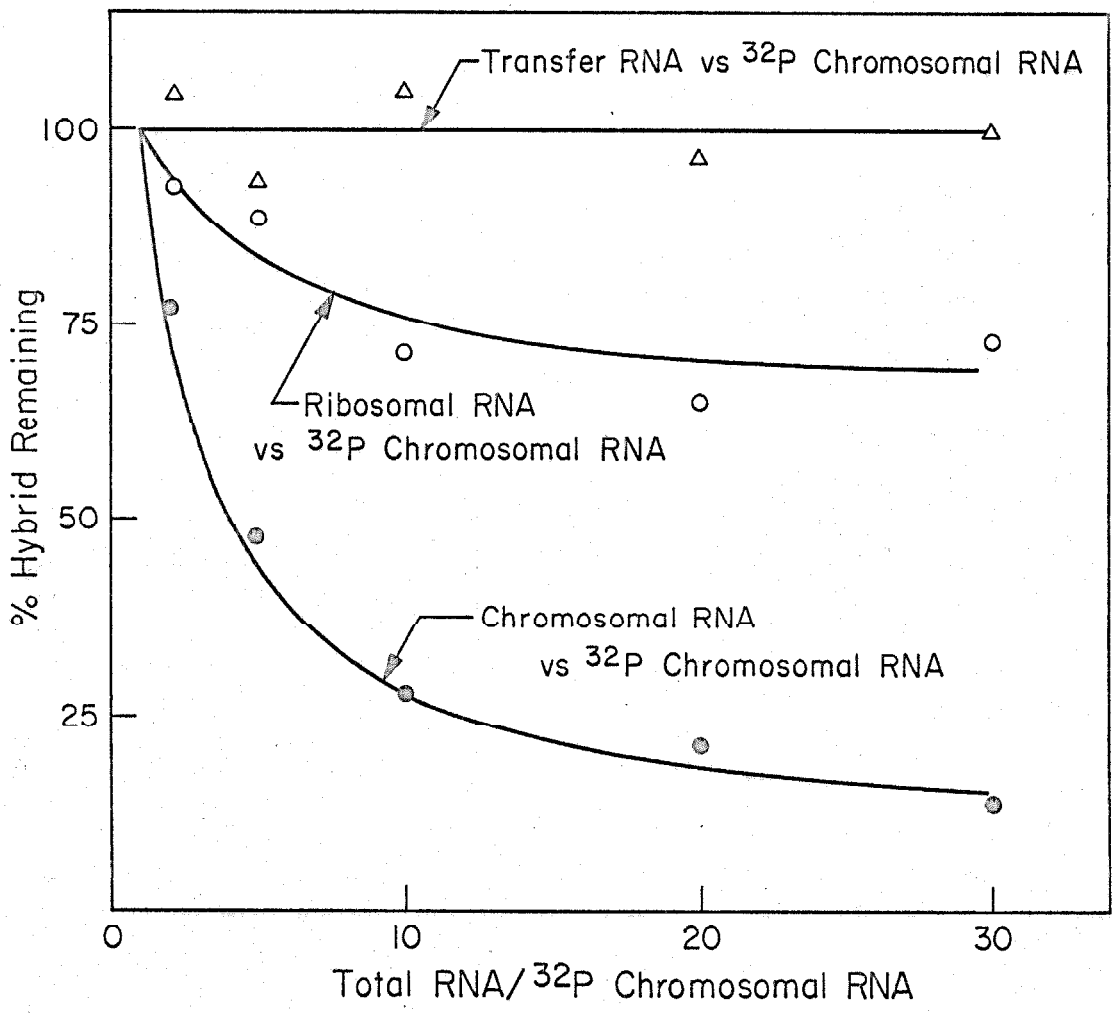
DNA. (If the transfer and ribosomal RNA binding sites are contained in the chromosomal RNA sites the reduction in chromosomal RNA hybridization should not be greater than $\frac{0.06}{3.8} = 0.016$ or 1.6%.) It was, therefore, anticipated that transfer and ribosomal RNA would behave as completely heterologous RNA in hybridization competition experiments with chromosomal RNA. The results of such an experiment are presented in Figure 9. As expected transfer RNA gives no measurable competition. Ribosomal RNA on the other hand is an effective inhibitor in the hybridization of chromosomal RNA. Similar results were obtained when the hybridization was performed at 66°C. For the reasons stated previously it is very unlikely that this interference caused by ribosomal RNA is due to competition for specific DNA sites. This was verified by hybridization competition experiments in which the DNA was first hybridized with ribosomal RNA, the unhybridized RNA removed and the DNA hybridized with labeled chromosomal RNA. No presaturation of the chromosomal RNA binding sites could be demonstrated under these conditions.

The apparent competition observed previously with total cytoplasmic RNA, therefore, may be almost entirely due to the interference of ribosomal RNA. (This fraction undoubtedly contains small amounts of 3S cytoplasmic RNA which, as previously shown, is partially analogous to chromosomal RNA.)

Because of the difficulty in preparing large amounts of labeled chromosomal RNA, below saturating amounts were used in most experiments. It was, therefore, necessary to add large amounts of competing RNA. In experiments performed at higher concentrations of labeled chromosomal

Figure 9. Ability of ascites transfer (—△—△—), or ribosomal (—○—○—), RNA to compete with ^{32}P-labeled chromosomal RNA in the hybridization to ascites DNA. 60 μg of labeled ascites chromosomal RNA throughout. With the exception of the competition by unlabeled chromosomal RNA, all hybridization was carried out at 25°C in the presence of 27% formamide. The same competition curve is obtained between labeled and unlabeled chromosomal RNA when the hybridization is carried out at 25°C in the presence of 27 to 30% formamide.

The degradation of ribosomal RNA to fragments of about 3S did not change its ability to interfere in the hybridization of labeled chromosomal RNA.


Transfer RNA vs 32P Chromosomal RNA
Ribosomal RNA
vs 32P Chromosomal RNA
Chromosomal RNA
vs 32P Chromosomal RNA
% Hybrid Remaining
100
75
50
25
0
10
20
30
Total RNA/32P Chromosomal RNA

RNA, which required the addition of much smaller amounts of competing RNA, little or no competition was observed in the presence of ribosomal RNA (Table 4). This result, in addition to the previous findings, suggest that RNA-RNA interactions are responsible for the decreased hybridization of chromosomal RNA.

The ability of ribosomal RNA to form complexes with pulse-labeled RNA has been previously reported in both bacteria (Hayes, Hayes and Guérin, 1966) and in rat liver (Staehelin, Wettstein, Oura and Noll, 1964). Chromosomal RNA is like messenger in that it has a low degree of secondary structure and is complementary to a large portion of the DNA, i.e. sequence heterogeneous. The existence of such complexes between ascites chromosomal RNA and ribosomal RNA can be demonstrated by sedimentation of the RNA contained in an aliquot of the hybridization solution at the end of an incubation. As is apparent from Figure 10(a), incubation of labeled chromosomal RNA in the presence of 9 times the amount of unlabeled chromosomal RNA does not significantly change its sedimentation properties (see Figure 5 of Section 1 for the sedimentation profile of input chromosomal RNA). However, incubation in the presence of ribosomal RNA causes a fraction of the labeled chromosomal RNA to sediment in the region of ribosomal RNA [Fig. 10(b)]. (The sedimentation profile of ribosomal RNA shows that considerable degradation has occurred during the incubation.)

The fact that ribosomal RNA readily interacts to form complexes with chromosomal RNA but does not interact with DNA (less than 0.02% of the input ribosomal RNA was found associated with the DNA at the end of

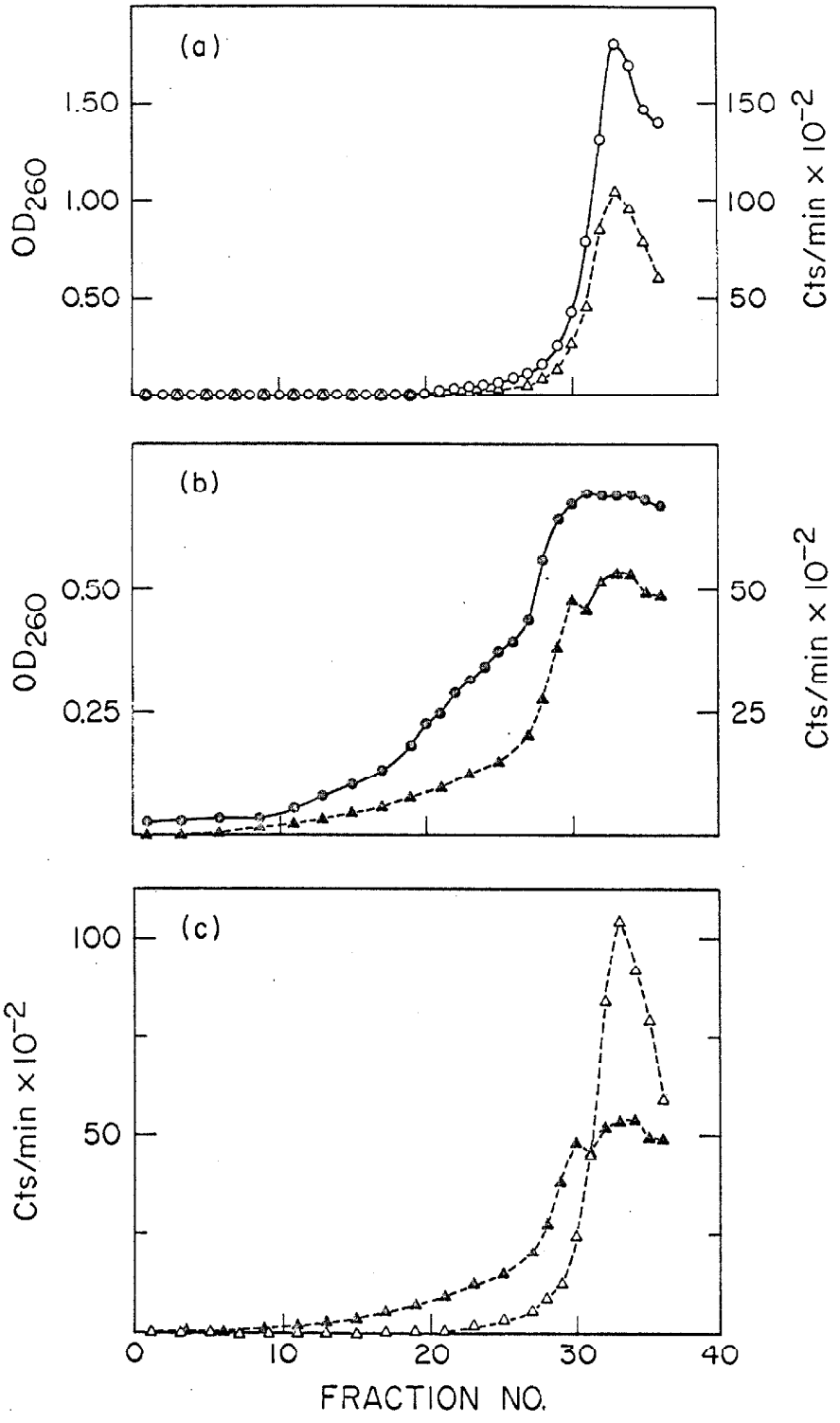
Figure 10. Sedimentation profile of non-hybridized RNA.

At the end of the hybridization reaction 0.5 ml of the hybridizing RNA solution was layered on a 5 to 20% sucrose gradient and centrifuged at 24,000 rpm for 13 hours in the Spinco SW25 rotor (sucrose gradient prepared in 2X SSC). At the end of the centrifugation fractions were collected, optical density at 260 mμ measured, and the TCA precipitable radioactivity of each fraction determined.

(a) Hybridization reaction contained 57 μg ^{32}P-labeled chromosomal RNA and 513 μg unlabeled chromosomal RNA in 1 ml 2X SSC, —O—O—, OD_{260}; --Δ--Δ--, cts/min.

(b) Hybridization reaction contained 57 μg ^{32}P-labeled chromosomal RNA and 513 μg unlabeled ribosomal RNA in 1 ml 2X SSC, —●—●—, OD_{260}; --▲--▲--, cts/min.

Hybridization at 66°C for 10 hours.


(a)
1.50
1.00
0.50
OD260
150
100
50
Cts/min x 10-2
(b)
0.50
0.25
OD260
50
25
Cts/min x 10-2
(c)
100
50
Cts/min x 10-2
0
10
20
30
40
FRACTION NO.

the hybridization) suggest that the interaction is not due to impurities in the ribosomal RNA preparation. It is more likely analogous to the interaction between rapidly labeled and ribosomal RNA previously described (Hayes, Hayes and Guérin, 1966).

Base composition of hybridized RNA

It is apparent from Table 6 that the hybridized RNA has a base composition very different from that of the input chromosomal RNA. The base composition differs from that of the input RNA in the manner expected from partial hydrolysis of the hybrid by RNase, i.e. an increase in the purine to pyrimidine ratio. (In the previous hybridization experiments the DNA-RNA complex was treated with RNase to remove any incomplete hybrids.) The poor agreement may, therefore, be due to a partial hydrolysis of the chromosomal RNA by RNase. It is also possible that the base composition of the hybrid differs from that of the input RNA because of a selective hybridization of a specific fraction of chromosomal RNA. The base composition of the hybrid formed in the presence of 30% formamide and not treated with RNase also differs markedly from that of the input RNA. (Omission of RNase treatment results in a 20 to 30% increase in the amount of hybrid RNA retained on the filters.) It is apparent from the increased purine to pyrimidine ratio in the RNased hybrid, relative to the non-RNased hybrid, that some hydrolysis does occur. The base composition of the hybrid formed at 66°C, in the absence of RNase digestion, more closely resembles that of the input RNA.

TABLE 6

Nucleotide Composition of Hybridized Ascites Chromosomal RNA

RNA	Expt. no.	Hybridization		Moles per cent					Pu/Pyr
		Input RNA/DNA	Conditions	A	C	U	G	% GC	
Chromosomal RNA (input)				18.9	26.3	21.1	33.6	59.9	1.11
Hybridized RNA (after RNase digestion)	1	0.03	30% formamide	26.1	14.3	19.0	40.5	54.8	2.00
	2	0.20	30% formamide	24.2	16.4	18.2	41.2	57.6	1.89
	3	0.30	30% formamide	28.4	14.0	16.2	41.4	55.4	2.31
Hybridized RNA (without RNase digestion)	2	0.20	30% formamide	25.3	20.4	19.3	35.1	55.5	1.53
	3	0.30	30% formamide	26.5	20.1	20.8	32.7	52.8	1.45
	4	0.5	30% formamide	20.9	21.6	20.7	36.8	58.4	1.36
	5	6.0	30% formamide	24.2	20.5	19.0	36.3	56.8	1.53
	4	0.5	66°C	20.5	24.2	21.8	33.5	57.7	1.17
	5	6.0	66°C	22.1	22.6	20.9	34.4	57.0	1.30

The hybrids formed at 25°C in 30% formamide generally have a higher content of A and G and a slightly lower content of C than the hybrids formed at 66°C. Because of the limited data and the variation within identical treatments it is difficult to say whether or not these differences are real. It is not clear why the different hybridization conditions should favor the hybridization of RNA molecules with different base compositions.

There are several possible explanations for the poor agreement between the base composition of the non-RNased hybrid and the input chromosomal RNA. The overall base composition of chromosomal RNA is clearly a weighted average of the base composition of all the molecules present. The base analysis of the hybrid, on the other hand, is not a weighted average because only one RNA molecule can hybridize to its complementary site, regardless of the number of identical molecules present. Such a distribution could account for the shape of the RNA hybridization saturation curve, i.e. at low concentrations of RNA the rate of hybridization is high because of the hybridization to DNA sites complementary to RNA molecules present in high concentration. The remaining sites are hybridized at a decreased rate and are saturated only at high input RNA concentrations. Such a distribution could also account for the fact that different concentrations of RNA hybridize at saturation to different levels of DNA regardless of the time of incubation.

Another possibility is that chromosomal RNA, like 3S cytoplasmic RNA, is not a pure fraction. The base composition of the hybrid may, therefore, represent the true base composition of chromosomal RNA.

DISCUSSION

The hybridization properties of ascites chromosomal RNA clearly distinguish it from the various other RNA species studied. It is immediately distinguished from transfer and ribosomal RNA by its high level of hybridization, being complementary to about 4% of ascites nuclear DNA. The lack of competition observed with in vivo generated messenger RNA, in addition to the incorporation studies described in Section 1, suggest that chromosomal RNA is also different from messenger RNA.

The hybridization properties of rat ascites chromosomal RNA are in good agreement with those reported for pea chromosomal RNA by Widholm and Bonner (1967). Pea chromosomal RNA hybridizes to about 5% of pea nuclear DNA and has no sites in common with either transfer or ribosomal RNA. Widholm and Bonner (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 (Huang, 1967). It has no sites in common with chick transfer or ribosomal RNA. 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 does not possess any sequence homology to ascites messenger RNA.

As clearly demonstrated by the recent work of Church and McCarthy (1968), the amount of hybrid formed is dependent on the stringency of the hybridizing conditions. They also point out the difficulties in forming specific hybrids in mammalian systems because of the large amount of redundancy in the DNA sequences. For these reasons, it is very difficult to determine the percentage of DNA complementary to a given RNA fraction. The change in base composition of the hybrid following RNase treatment indicates that many of the hybrids formed were incomplete hybrids and may represent hybridization to irrelevant DNA segments. Clearly the value reported for the per cent of ascites DNA homologous to ascites chromosomal RNA is a maximum value.

It is clear, however, that chromosomal RNA is complementary to a large portion of the ascites genome. The high level of hybridization may be the result of sequence heterogeneity of the chromosomal RNA, resulting in its hybridization to many unique sites, or the result of a sequence homogeneous population of RNA hybridizing to redundant sections of DNA. In ascites cells, we have no direct evidence to

distinguish between these possibilities at this time. In the case of pea chromosomal RNA, however, the high level of hybridization appears to be due to sequence heterogeneity of the chromosomal RNA. This follows directly from the fact that pea bud chromosomal RNA hybridizes equally well with the rapid, moderately fast, or slow reannealing fractions of pea nuclear DNA (Ho, 1968).

Bekhor, Kung and Bonner (1968) and Huang and Huang (1968) 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 is 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. Chromosomal RNA, therefore, appears to provide the required specificity for gene repression. 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.

If the binding of chromosomal RNA is necessary for the regulation of a specific gene, as the recent results of Bekhor *et al.* (1968) and Huang *et al.* (1968) suggest, the act of repression or derepression may involve a rearrangement of the chromosomal RNA. We would then expect to find a certain fraction of the chromosomal RNA molecules dissociated

from the chromatin at any time. The 3S RNA observed in the nuclear sap and cytoplasm might, therefore, represent chromosomal RNA in the free state.

The hybridization 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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CHAPTER II

EFFECT OF HYDROCORTISONE ON THE TEMPLATE ACTIVITY OF LIVER CHROMATIN

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*INCREASED TEMPLATE ACTIVITY OF LIVER CHROMATIN, A RESULT OF HYDROCORTISONE ADMINISTRATION**

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Communicated September 24, 1965

We have found that the administration of hydrocortisone to adrenalectomized rats increases the template activity of their liver chromatin for RNA synthesis. Such administration is known to cause a two- to threefold increase in rate of nuclear RNA synthesis in the liver.[1, 2] This increase is followed by an increase in the activities of a series of liver enzymes.[3–5] Since the induction of these enzymes by hydrocortisone is abolished by simultaneous treatment with actinomycin D, it is clear that new RNA synthesis is required to support their formation.[4, 5] The increased rate of liver RNA synthesis caused by administration of hydrocortisone might in principle be due to changes in the template activity of the liver genetic material such as would accompany derepression of genes previously repressed. We shall show below that the administration of hydrocortisone does result in an increased availability of the genetic material for transcription.

Materials and Methods.—Treatment of rats: Male albino Sprague-Dawley rats weighing 150–250 gm each were obtained in the bilaterally adrenalectomized condition from Berkeley Pacific Laboratories. Hydrocortisone A grade was obtained from Calbiochem.

Rats were fasted 18 hr prior to the beginning of the experiment. Hydrocortisone-treated rats were given an intraperitoneal injection of hydrocortisone, 5 mg/100 gm body weight, suspended in physiological saline. Control rats were injected with an equal volume of saline. Four hours after treatment the rats were sacrificed, their livers immediately removed and washed with cold saline. The livers were then frozen in dry ice and chipped into small pieces. Tissue from identical treatments (24 rats per treatment) was pooled and stored at −80°C.

Preparation of purified chromatin: Crude chromatin was prepared from 10-gm samples of frozen tissue by the procedures of Marushige and Bonner[6] with the modification that the tissue was homogenized in 0.05 *M* NaCl plus 0.016 *M* Na_2 EDTA (pH 8.0). The chromatin was purified by centrifugation through 1.7 *M* sucrose and dialyzed against two changes of 0.01 *M* tris, pH 8.0.

Preparation of deproteinized DNA: Protein was removed from the purified chromatin by centrifugation in 4 *M* CsCl according to the method of Huang and Bonner.[7] Samples were centrifuged at 35,000 rpm for 22 hr in a Spinco SW-39 rotor. The gelatinous DNA pellet was dissolved in 0.01 *M* tris, pH 8.0.

Preparation of RNA polymerase: RNA polymerase was prepared from early log phase cells of *E. coli* strain B (General Biochemicals) by the methods of Chamberlin and Berg[3] to the stage of their fraction 3, hereafter referred to as F_3.

Assay of template activity: The complete incubation mixture for RNA synthesis contained in a final volume of 0.25 ml: 10 μmoles tris buffer (pH 8.0), 1 μmole $MgCl_2$, 0.25 μmole $MnCl_2$, 3 μmoles β-mercaptoethanol, 0.05 μmole spermidine phosphate, 0.10 μmole each of CTP, UTP, and GTP, 0.10 μmole 8-C^{14}-ATP (spec. act. 1 μc/μmole), DNA or chromatin, and F_3. Samples were incubated at 37°C for 10 min. The reaction was then stopped by the addition of cold 10%

TCA. Acid-insoluble material was collected by filtration on TCA-presoaked membrane filters (Schleicher and Schuell B-6) and washed with four 5-ml portions of cold 10% TCA. The filters were then glued to planchets, dried, and counted in a Nuclear-Chicago D-47 gas flow counting system. That the 8-C^{14}-ATP is in fact incorporated into RNA has been shown by Marushige and Bonner.[6]

Enzyme assays: Tyrosine transaminase was determined following the method of Canellakis.[9] An aliquot (10 ml) of the first homogenate in the preparation of chromatin was frozen and stored at −80°C. This freezing and storage for several days at −80°C resulted in no loss of enzyme activity. The samples were thawed, further homogenized with 0.05 ml 2-octanol in an Omni Mixer (75 v-75 sec), and filtered through one layer of Miracloth. Aliquots (0.1 ml) were used for enzyme assays, and 1-ml aliquots were used in the determination of protein.

The loss of TCA-precipitable material from C^{14}-labeled RNA brought about by incubation of C^{14} RNA with purified chromatin was taken as a measure of RNase activity.

C^{14}-labeled RNA was prepared by incubation of liver DNA in the medium for RNA synthesis as outlined above. The incubation mixture was then diluted with 0.01 *M* NaOAc, pH 5.5, containing 5% butanol, 1% sodium lauryl sulfate, 10^{-3} *M* $MgCl_2$, and treated with an equal volume of water-saturated phenol (60°C) for 3 min. Carrier RNA was added and RNA twice precipitated with ethanol. The final C^{14}-RNA pellet was dissolved in 0.01 *M* tris, pH 8.0 (spec. act. 95,000 cpm/mg RNA). C^{14}-labeled RNA was incubated with purified chromatin in the standard mixture for RNA synthesis minus F_3 and nucleoside triphosphates. After 10 min incubation at 37°C, C^{14}-labeled RNA was assayed as described above under assay of template activity.

Chemical composition: DNA was determined by the diphenylamine method of Dische[10] using rat liver DNA as a standard. RNA was determined by the orcinol reaction following the method of Dische and Schwarz[11] using purified yeast RNA as a standard. Histone was extracted from chromatin with 0.2 *N* H_2SO_4 at 4°C and precipitated with 20% TCA. Its amount was determined following the method of Lowry *et al.*[12] using rat liver histone as a standard. Nonhistone protein was determined on the acid-insoluble material by the same procedure, using bovine serum albumin fraction V as a standard.

Results.—A single intraperitoneal injection of hydrocortisone brings about an increase in the activity of liver tyrosine transaminase, as is presented in Table 1. This induction represents a five- to sixfold increase, indicating that our system is responding as previously reported.[3–5, 13]

Purified chromatin was prepared from livers of rats 4 hr after treatment either with hydrocortisone or with saline. The chromatin isolated from the livers of hydrocortisone-treated animals will be referred to as "induced" chromatin, while

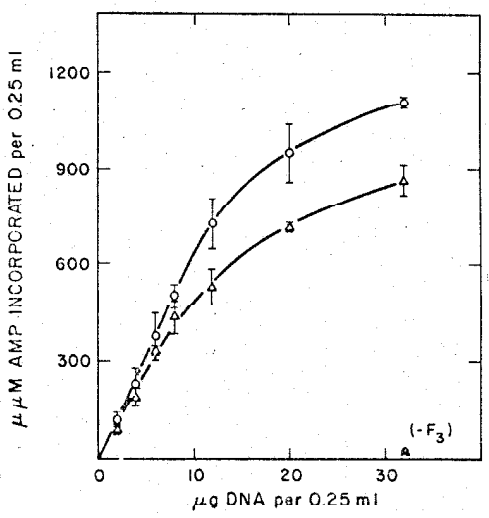

FIG. 1.—Template activity of rat liver chromatin isolated 4 hr after treatment with hydrocortisone (—○—○—) or saline (—△—△—). The incubation mixture (0.25 ml) contained various concentrations of DNA in the form of chromatin and 58 μg F_3. Incorporation by F_3 alone (100 μμM AMP/0.25 ml) has been subtracted. Each point is an average of three incubations. The brackets represent 90% confidence limits.

Samples incubated without added exogenous RNA polymerase are shown: (−F_3), ● = hydrocortisone-treated; ▲ = saline-treated.

TABLE 1

INDUCTION OF LIVER TYROSINE TRANSAMINASE BY HYDROCORTISONE TREATMENT OF ADRENALECTOMIZED RATS

Liver from:	Tyrosine transaminase activity* (μg product/30 min)
Control animals	13
Hydrocortisone-treated animals	72

* Activity is expressed as μg of p-hydroxy-phenyl-pyruvate formed per 30 min per mg protein at 38°C. Tyrosine transaminase was assayed 4 hr after treatment (see *Materials and Methods*).

TABLE 2

RNASE ACTIVITY OF PURIFIED LIVER CHROMATIN

Chromatin from livers of:	RNase activity*
Control animals	0.09
Hydrocortisone-treated animals	0.12

* The incubation mixture contained, in a final volume of 0.25 ml, 32 μg of purified chromatin and 25 μg C^{14}-labeled RNA in the standard mixture for RNA synthesis minus F_3 and nucleoside triphosphates. RNase activity is expressed as the fraction of initial C^{14}-labeled RNA (2,400 cpm) rendered TCA-soluble after a 10-min incubation with chromatin at 37°C. The values reported are the averages of two samples.

that isolated from saline-treated animals will be referred to as "noninduced."

The template activity for RNA synthesis of the two types of liver chromatin in the presence of added exogenous RNA polymerase is shown in Figure 1. It is clear that any given amount of DNA supplied as induced chromatin supports a greater rate of RNA synthesis than does an equal amount of DNA supplied as noninduced chromatin. The data of Figure 1 indicate that in the presence of added RNA polymerase at high template concentrations the template activity of induced liver chromatin is approximately 30 per cent greater than that of noninduced chromatin.

Chromatin purified according to the present procedure possesses essentially no endogenous RNA polymerase activity, as is apparent in Figure 1 ($-F_3$). No difference in the rate of RNA synthesis supported by induced chromatin or noninduced chromatin could be detected when such synthesis was catalyzed solely by endogenous polymerase.

The increase in template activity indicated in Figure 1 has been shown to be statistically significant by two methods. An analysis of variance shows that the increase of template activity induced by hydrocortisone is significant at the 99 per cent level. In addition, a regression analysis of the slopes of the linear portion of the template saturation curves shows that they are significantly different at the 95 per cent level. The increased template activity of induced chromatin is reproducible from experiment to experiment, ranging from 10 to 35 per cent.

The difference in template activity between induced and noninduced chromatin is maintained over the range of RNA polymerase concentrations 30–120 μg per 0.25 ml, and also over the range of nucleoside triphosphate concentrations 0.05–0.20 μmole each per 0.25 ml. The RNase activity of rat liver chromatin is small and not significantly different between induced and noninduced chromatin (Table 2). It seems, therefore, that the difference in rate of RNA synthesis observed is not due to the presence of degradative enzymes but rather to a real difference in template activity.

The difference in template activity between induced and noninduced chromatin is abolished by removal of DNA-bound protein. Purified liver chromatin was suspended in 4 *M* CsCl and centrifuged as described under *Methods* for the preparation of deproteinized liver chromatin DNA. The DNA thus isolated has an increased template activity of about 14-fold as compared to rat liver chromatin. The data of Figure 2 show that there is no difference in template activity between in-

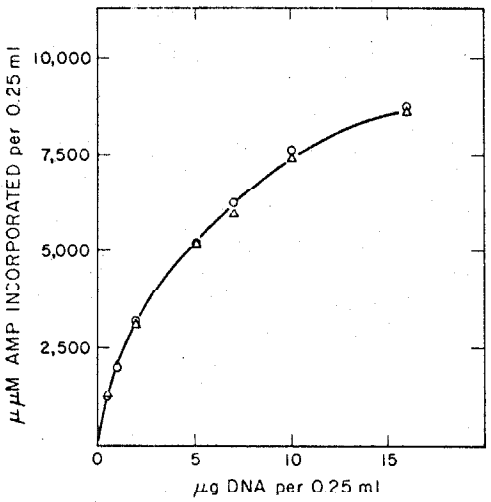

FIG. 2.—Template activity of CsCl deproteinized DNA. The incubation mixture contained various concentrations of deproteinized DNA and 58 μg F_3. Incorporation by F_3 alone (100 μμM AMP/0.25 ml) has been subtracted. —○—○—, Deproteinized DNA from induced chromatin of Fig. 1; —△—△—, deproteinized DNA from noninduced chromatin of Fig. 1.

duced and noninduced chromatin when the proteins associated with the DNA are removed. The DNA samples prepared from induced and noninduced chromatin are of similar molecular weight. Sedimentation coefficients were determined by boundary velocity sedimentation in the Spinco model E centrifuge and molecular weights calculated using the relation of Studier.[14]

The chemical compositions of induced and noninduced liver chromatin are given in Table 3. There is no detectable analytical difference in the amounts of the several components measured.

Addition of hydrocortisone to noninduced liver chromatin *in vitro* causes no increase in template activity for RNA synthesis. Preincubation of chromatin with hydrocortisone at 4° or at 37°C for 10 min prior to the addition of polymerase also elicits no effect upon liver chromatin template activity (Table 4).

Discussion.—The experiments reported above clearly demonstrate that the administration of hydrocortisone to adrenalectomized rats causes an increased template activity of liver chromatin for RNA synthesis. Lang and Sekerkis[15] and Barnabei *et al.*[16] have previously reported the isolation of template-polymerase omplexes from the liver of hydrocortisone-treated rats, and have shown that such

TABLE 3

CHEMICAL COMPOSITION OF RAT LIVER CHROMATIN

Component	Mass Ratios* Induced chromatin	Noninduced chromatin
DNA	1	1
RNA	0.059	0.056
Histone	0.82	0.82
Nonhistone protein	1.01	1.06

* Average values of four preparations.

TABLE 4

EFFECT OF HYDROCORTISONE ON TEMPLATE ACTIVITY OF PURIFIED CHROMATIN *in vitro*

μg Hydrocortisone/ 0.25 ml	μμM ATP incorporated/0.25 ml
0	561
10^{-10}	587
10^{-8}	587
10^{-6}	576
10^{-4}	575
10^{-2}	559
1	591
10	587

Each 0.25-ml incubation contained 26 μg DNA in the form of noninduced chromatin and 78 μg F_3. Hormone was preincubated 10 min at 4°C before the addition of F_3. Incorporation by F_3 alone (208 μμM AMP) has been subtracted.

complexes possess greater activity for RNA synthesis than do similar complexes from rats not treated with hydrocortisone. Their experiments do not, however, distinguish between increased rate of RNA synthesis resulting from increased template activity of chromatin and increased rate of RNA synthesis resulting from increased activity or amounts of RNA polymerase contained in the complex. In the present experiments the addition to the chromatins of equal and large amounts of exogenous RNA polymerase makes possible a clear measure of template activity.

The increase in template activity which results from hydrocortisone action would appear to be in some way associated with the proteins which are complexed with the chromosomal DNA. This follows from the fact that removal of chromosomal protein by treatment with high salt concentrations yields DNA of equal template activity from induced and noninduced liver chromatin. Differences in protein composition of chromatin induced by hydrocortisone treatment, if any, are apparently too small to be detected by present analytical methods.

The increase in rate of RNA synthesis by rat liver nuclei in response to hydrocortisone treatment is of the order of two- to threefold. The increases in template activity for RNA synthesis of rat liver chromatin caused by hydrocortisone treatment are of the order of 30 per cent. It is clear, therefore, that a portion of the increase in rate of RNA synthesis elicited by hydrocortisone administration is due to effects not preserved in isolated chromatin. A further portion may be due to an increased concentration or activity of RNA polymerase itself.

Finally, although the administration of hydrocortisone *in vivo* causes increased template activity to be developed *in vivo*, the administration of hydrocortisone directly to isolated, noninduced chromatin has no such effect. Therefore, there is some intermediary, not preserved in isolated chromatin, between the hormone and its ultimate effect upon the state of repression of the genetic material.

Summary.—Chromatin isolated from the liver of hydrocortisone-treated adrenalectomized rats possesses a greater template activity for DNA-dependent RNA synthesis than does chromatin isolated from the livers of rats not treated with hydrocortisone. This difference in template activity is abolished by the removal of proteins associated with the DNA.

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ADDITIONAL RESULTS

In the previous experiments the template activity of rat liver induced and noninduced chromatin was compared by measuring the amount of RNA synthesis primed by each template in 10 minutes. The amount of RNA synthesized by induced and noninduced chromatin as a function of time is shown in Figure 1. It is apparent that at any time the amount of RNA synthesis supported by induced chromatin is greater than that supported by noninduced chromatin. Both samples plateau at about 10 minutes.

The dependence of the rate of synthesis on nucleoside triphosphate concentration is shown in Figure 2. In these experiments the concentrations of all four nucleoside triphosphates were changed. There is only a slight change in the rate of RNA synthesis over the concentrations examined. At each concentration the difference in activity between induced and noninduced chromatin is maintained. The difference in activity is, therefore, not due to a difference in the availability of substrate.

The dependence of the rate of synthesis on the concentration of RNA polymerase (F_3) is also shown in Figure 2. An increase in the amount of RNA polymerase brings about a proportional increase in the rate of RNA synthesis. The increased rate of synthesis supported by induced chromatin is maintained over the range of RNA polymerase concentrations investigated.

Figure 1. Time course of RNA synthesis primed by rat liver induced (—O—O—) and noninduced (—Δ—Δ—) chromatin. The incubation mixture (4.25 ml) contained 544 μg of DNA in the form of chromatin, 1.3 mg F_3 and other ingredients as described in Materials and Methods of the preceding paper. At various times aliquots of 0.2 ml were removed from each tube, induced and noninduced, and assayed. Each point is an average of two determinations. Incorporation by F_3 alone has been subtracted.

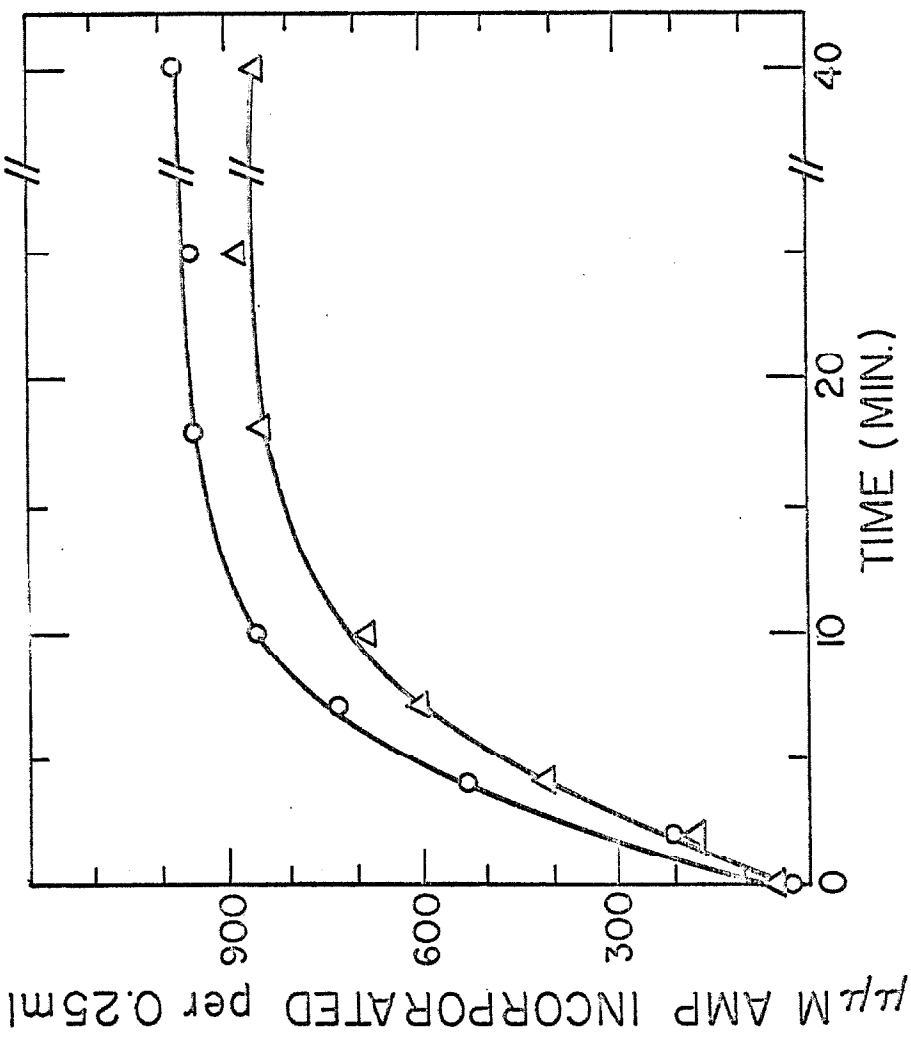
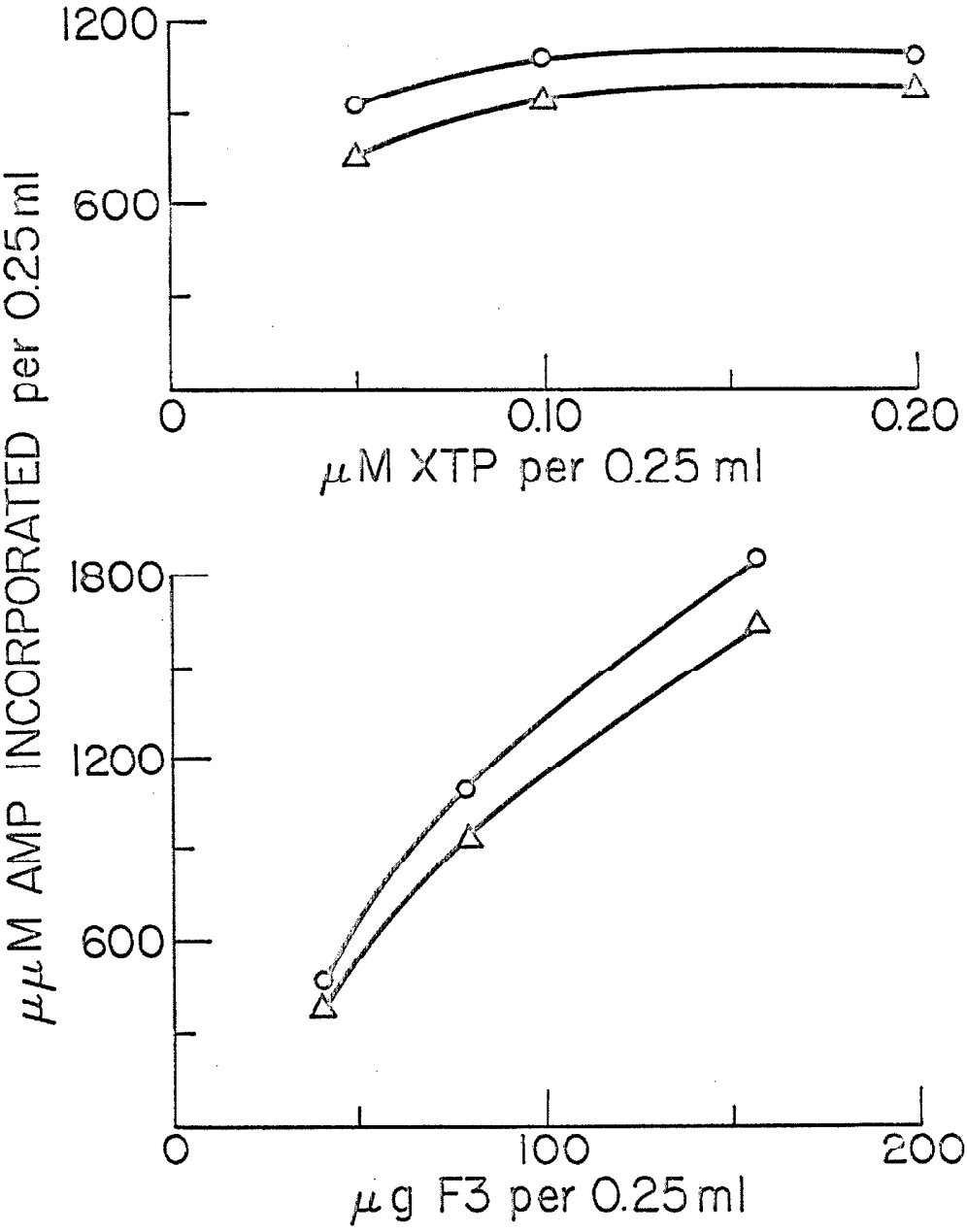

μμM AMP INCORPORATED per 0.25ml
900
600
300
0
10
20
40
TIME (MIN.)

Figure 2. Upper: Effect of nucleoside triphosphate concentration on the rate of RNA synthesis primed by induced and noninduced rat liver chromatin. The incubation mixture (0.25 ml) contained 26 μg of DNA in the form of chromatin, 78 μg of F_3, various concentrations of nucleoside triphosphates and other ingredients described in Materials and Methods of the preceding paper. Incorporation by F_3 alone (50, 130 and 150 μμM AMP/0.25 ml) has been subtracted.

Lower: Effect of RNA polymerase concentration on the rate of RNA synthesis primed by induced and noninduced rat liver chromatin. The incubation mixture (0.25 ml) contained 26 μg of DNA in the form of chromatin, various concentrations of F_3 and other ingredients previously described. Incorporation by F_3 alone (65, 130 and 260 μμM AMP/0.25 ml) has been subtracted.


1200
600
0
0.10
0.20
μM XTP per 0.25 ml
μμM AMP INCORPORATED per 0.25 ml
1800
1200
600
0
100
200
μg F3 per 0.25 ml

DISCUSSION

Several papers dealing with the nature of hormone stimulated RNA synthesis have appeared since the publication of the preceding paper. This discussion is not intended as a review of the recent work on the mechanism of hormone action, but merely a summary of how this particular approach has brought about insights into the mechanism of action of various hormones.

Barker and Warren (1966) have investigated the effect of estradiol on the template activity of uterine chromatin. They have reported a 70 to 80% increase in the activity of isolated chromatin as template for RNA synthesis as a result of hormone treatment. They could detect no difference in the histone, nonhistone or RNA content of the chromatin. Recently Hamilton (1968) has also reported an increase in the template activity of uterine chromatin following the administration of estradiol.

The administration of testosterone propionate, growth hormone, or both hormones cause an increase in the activity of aggregate RNA polymerase from femoral muscles of hormone deficient rats (Breuer and Florini, 1966). The chromatin isolated from testosterone treated rats, when coupled with E. coli polymerase, supports RNA synthesis at about twice the rate of chromatin isolated from control rats. The chromatin isolated from growth hormone treated rats has a slightly decreased template activity when compared to the chromatin from control rats. They have, therefore, concluded that testosterone stimulates RNA synthesis

by increasing the priming efficiency of the template while growth hormone probably acts by directly affecting the level of RNA polymerase.

Experiments have also been reported by Kim and Cohen (1966) concerning the effect of thyroxine on tadpole chromatin. The administration of thyroxine to tadpoles induces the synthesis of RNA which leads to the production of various enzymes. They have shown that chromatin isolated from the livers of thyroxine treated tadpoles has a template activity 20 to 50% higher than that of chromatin prepared from control animals. This difference is abolished by the removal of the chromosomal proteins. The hormone had no effect on the template activity of noninduced chromatin in vitro.

We, therefore, have several cases in which hormones stimulate RNA synthesis by modifying the chromatin in such a way as to make it a more effective template for RNA synthesis. This is not to say, however, that the hormone interacts directly with the chromatin. None of the hormones which cause an increase in the template activity of chromatin when administered in vivo have clearly been shown to have an effect on the template activity in vitro. [It has recently been reported that cortisol, administered to isolated liver chromatin, can cause an increase in the template activity of the chromatin (Stackhouse, Chetsanga and Tan, 1968). However, the increase reported was very small and was observed only at very high hormone concentrations. The results were further complicated by the high protein content of the chromatin. Since the experiments do not differ significantly from those described in the previous paper, it seems likely that the effect would be lost by the

additional purification of the chromatin.] The administration of cortisone, at low concentrations, to isolated rat liver nuclei has been reported, however, to cause an increase in the rate of RNA synthesis (Schmid, Gallwitz and Sekeris, 1967). It appears, therefore, that a substance present in the nucleus is required to mediate the effect of the hormone. The cytoplasm of endometrial cells contain a soluble protein which specifically binds β-estradiol (Jensen et al., 1968). This protein hormone complex appears then to break down into a smaller complex, still containing the hormone, which is found only in the nucleus. As suggested by the experiments of Maurer and Chalkley (1967), this complex may then become associated with the chromatin.

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