Low Molecular Weight RNA Species from Chromatin†

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ABSTRACT: Several methods of preparing low molecular weight RNA from chick embryo chromatin have been examined. Traditional methods for dissociating chromatin utilizing high concentrations of salt (>2 *M*) followed by high-speed centrifugation resulted in very low yields of RNA. Increased yields of RNA were obtained by treating chromatin at lower salt concentration (0.2–0.5 *M*). By using low salt extraction and sodium dodecyl sulfate–phenol deproteinization, six to eight low molecular weight homogeneous RNA species were isolated from chick embryo chromatin and mouse myeloma chromatin. In the myeloma system, all these RNAs are metabolically stable. Each component is homogeneous as examined by gel electrophoresis and hybridizes with mouse DNA at a rate consistent with a single species. There are multiple gene copies for these RNA species in the mouse genome, varying from 100 to 2000 copies for the different species. One of these RNAs is identical with 5S rRNA. In addition, the redundancy of genes for 18S, 28S, and 5S rRNA and tRNA was determined. Approximately 300 copies for 18 and 28S rRNA and 500 copies for 5S rRNA were found. tRNAs were on an average 110-fold redundant with about 55 different species measured.

It has been known for many years that chromatin consists primarily of DNA and protein. However, small amounts of RNA are also reproducibly found in chromatin (Bonner et al., 1968). Portions of this RNA must be the nascent RNA chains associated with DNA template. The origin of the remaining RNA (cRNA) has been the subject of much recent controversy. Several workers have claimed that this RNA is an artifact of preparation arising from ribosomal RNA degradation or from contamination with transfer RNA (Artman and Roth, 1971; Heyden and Zachau, 1971; Tolstoshev and Wells, 1974). On the other hand, Bonner and coworkers (Holmes et al., 1974) and Monahan and Hall (1973a,b) have presented evidence that this RNA is a true chromatin component.

Several properties of low molecular weight cRNA have led to the proposal that it may have a regulatory function in chromatin (Bonner et al., 1968; Huang and Huang, 1969). These properties include the reported high sequence complexity and the restriction of cRNA to the intermediate repetitive fraction of DNA (Holmes et al., 1974). In addition, it has been claimed previously that this RNA is intimately associated with protein (Jacobsen and Bonner, 1971; Marzluff et al., 1972). Results of this paper indicate that RNA is not covalently linked to protein and that the bulk of the cRNA (80%) is composed of discrete species apparently identical with the low molecular weight nuclear RNAs described by Moriyama et al. (1969) and Weinberg and Penman (1968). They are complementary to the intermediate repetitive region of the genome and have sequence complexity varying from 100 to 2000 copies for the different cRNA species per mouse haploid genome.

A major difficulty in the earlier incomplete studies on this problem has been the lack of a quantitative procedure for the RNA isolation. Consequently, no detailed characterization of this RNA was possible. In this paper, by using chick embryos as a primary source for chromatin, we compare several methods of isolation of its low molecular weight RNA. These methods have been designed to allow recovery of RNA under relatively mild conditions at neutral pH allowing the isolated RNA and residual chromatin to be useful in functional studies. We have found that 0.35 *M* NaCl or 0.15 *M* deoxycholate, as originally reported by Mayfield and Bonner (1971), followed by phenol deproteinization gave the best yield of chromatin RNA. It consists of several discrete low molecular weight species ranging from 4 to 8 S. They are distinct from both ribosomal RNA and transfer RNA.

Using methods for rapid and quantitative recovery of low molecular weight RNA species, we have also examined these RNA species in mouse myeloma chromatin. $^{32}PO_4$-labeled RNA of high specific activity was prepared. Chemical complexity of these RNAs was examined by polyacrylamide gel electrophoresis; sequence complexity and genomic redundancy were determined by DNA:RNA hybridization. We find that the vast majority of low molecular weight RNA species is represented by six discrete, homogeneous RNA species ranging in size from 100 to 200 nucleotides. The genes for these RNA species are repetitive varying from 100 to 2000 copies per genome occupying only a very small portion of the total genome (<0.1%). Although they may not have the complexity required for specific gene regulators, the relative abundance of these RNAs in actively growing cells suggests that they may play a role in chromosomal structure and function.

Materials and Methods

Labeling of Cells. Mouse myeloma 66-2 cells were used in these experiments and grown as previously described (Marzluff et al., 1973). To label the RNA with $^{32}PO_4$ the cells were grown for 12 hr (to a concentration of 5–7 × 10^5/ml) in medium with reduced phosphate; the medium

† From the Department of Biology, The Johns Hopkins University, Baltimore, Maryland 21218. *Received November 26, 1974.* This work was supported in part by Grants CA 13953 and 1R01HD 08185 from the National Institutes of Health.

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did not contain any phosphate but undialyzed horse serum (10%) was used as the sole phosphate source. $^{32}PO_4$ (80–100 μCi/ml) was added. The cells were diluted with 0.5 vol of phosphate-free media every 6 hr. At the end of the 24 hr, the volume had tripled and the cell concentration was 3–5 $\times 10^5$/ml. The cells doubled during the labeling period. Approximately 2×10^8 cells were used for each preparation of $^{32}PO_4$-labeled RNA from chromatin.

Preparation of Chromatin from Chick Embryos. Chromatin was prepared from the purified nuclei of 11-day whole chick embryo or from chick embryo thighs as described by Huang and Huang (1969). The chromatin was dissolved in deionized water at a concentration of 10–15 A_{260} units/ml.

Preparation of Chromatin from Mouse Myeloma Culture Cells (66-2). The cells were harvested by centrifugation and washed twice with cold 0.14 *M* NaCl–0.01 *M* Tris (pH 7.5). They were then suspended in 25 m*M* KCl, 3 m*M* $CaCl_2$, 5 m*M* $Mg(OAc)_2$, 10 μg/ml of dextran sulfate, and 10 m*M* Tris (pH 8) (2×10^7 cells/ml) for 5 min, 2 *M* sucrose was added to 0.32 *M*, and the cells were broken by homogenization in a Dounce homogenizer (20 strokes, B pestle). Nuclei were pelleted by centrifugation at 1000*g* for 10 min. The crude nuclei were suspended in 0.88 *M* sucrose–5 m*M* $MgCl_2$–10 m*M* Tris (pH 8)–1% Triton-X-100 and homogenized 5 strokes. The nuclei were pelleted by centrifugation at 1000*g* for 10 min.

The nuclei were lysed by suspending them in 0.075 *M* NaCl–0.024 *M* EDTA (pH 8) (2×10^7 cells/ml) (Shaw and Huang, 1970) and centrifuging at 8000*g* for 10 min. Nuclear sap RNA was prepared from the supernatant (saline–EDTA extract). Chromatin was prepared as described by Huang and Huang (1969).

Different Extraction Procedures for Low Molecular Weight RNAs from Chick Embryo Chromatin. 3 *M* NaCl. Solid NaCl was added to a final concentration of 3 *M* (Shaw and Huang, 1970) and centrifuged at 27,000 rpm for 36 hr to pellet the DNA, or alternatively the DNA was separated from the extracted protein and RNA by chromatography on Bio-Gel A-50 (Graziano and Huang, 1971).

4*M* Guanidine Hydrochloride. Solid guanidine hydrochloride was added to a final concentration of 4 *M* and the solution centrifuged at 36,000 rpm for 44 hr to pellet the DNA (Hill et al., 1971).

0.015 *M* Sodium Deoxycholate. Extraction was performed exactly as described by Mayfield and Bonner (1971).

0.35 and 0.2 *M* NaCl. The chromatin was adjusted to the appropriate salt concentration by dropwise addition of 5 *M* NaCl with stirring for 30 min. The precipitated chromatin was pelleted at 15K for 15 min in the SS-34 rotor of the Sorvall. The supernatant was recovered and used for RNA preparation.

0.5 *M* NaCl. NaCl (5 *M*) was added dropwise to the chromatin solution to a final concentration of 0.5 *M*. Solid sucrose was added to a concentration of 0.85 *M* and the NaCl concentration adjusted to 0.5 *M*. The residual chromatin was pelleted by centrifugation at 24K for 12 hr and the supernatant was used.

Isolation of RNA from Extracts of Chick Embryo Chromatin. The RNA was isolated from the chromatin extract by two different methods.

(a) By DEAE-Cellulose Chromatography Followed by CsCl–Guanidine Hydrochloride Centrifugation. DEAE-cellulose was equilibrated with 7 *M* urea, 0.25 *M* NaCl, 0.05 *M* Tris (pH 5.5), or, in the case of the deoxycholate-extracted RNA, 0.05 *M* Tris (pH 8). A column 2 × 10 cm was sufficient for 2000 A_{260} units of chromatin. The chromatin extract was adjusted to 7 *M* urea–0.25 *M* NaCl by dialysis and was applied to the column at a flow rate of 0.5–1 ml/min and the column washed with 7 *M* urea–0.3 *M* NaCl until the A_{230} was 0. The RNA was eluted with a gradient of 0.3–1.0 *M* NaCl, 7 *M* urea, or routinely eluted with 1 *M* NaCl in the presence of 7 *M* urea. Under these conditions 95% of the protein passed directly through the column.

The RNA was recovered by dialysis against H_2O followed by lyophilization. To further purify the RNA, the RNA was dissolved in 4 *M* guanidine hydrochloride and the solution adjusted to 2 *M* guanidine hydrochloride, 4 *M* CsCl, and 0.01 *M* Tris (pH 8), and centrifuged for 44 hr at 36K. The tubes were punctured and 0.5-ml fractions collected. RNA is concentrated to the bottom of the tube while some DNA fragments and residual protein are near the top of the gradient.

(b) By Sodium Dodecyl Sulfate–Phenol Extraction. When a large volume of extract was used, 1 drop of diethyl pyrocarbonate was added along with 2 vol of ethanol at −20° to completely precipitate the protein and RNA from the extracts. The pellet was redissolved in 1/10 the volume of 1% sodium dodecyl sulfate and 0.01 *M* sodium acetate (pH 5). The solution was extracted twice with water-saturated phenol and the solution adjusted to 0.2 *M* NaCl and the RNA precipitated with 70% ethanol at −20°. This procedure was used for the extracts at low ionic strength (≤0.5 *M* NaCl) and in 0.015 *M* deoxycholate. When a small volume of extract was used, the solution was made to 1% sodium dodecyl sulfate–0.01 *M* sodium acetate (pH 5) directly and extracted with phenol.

Preparation of rRNA, tRNA, and Nuclear Sap RNA from Myeloma Cells. The cytoplasm and 0.88 *M* sucrose wash from the crude nuclei were combined and centrifuged for 4 hr at 36K through a pad of 1 *M* sucrose–10 m*M* Mg^{2+}–10 m*M* Tris (pH 8.0) in the 65 rotor. The pelleted polyribosomes were suspended in 2% sodium dodecyl sulfate–10 m*M* EDTA–10 m*M* Tris (pH 7.5) and extracted with phenol–chloroform and the aqueous phase precipitated with ethanol. These were the source of ribosomal RNAs and 5S rRNA and 5.4S rRNA.

The supernatant (material above the sucrose cushion) was adjusted to 0.2% sodium dodecyl sulfate, 0.05 *M* NaOAc (pH 5), and extracted with phenol and then precipitated with 2 vol of ethanol. tRNA was further purified by polyacrylamide gel electrophoresis.

Polysomal RNA and nuclear sap RNA were first fractionated on 10–70% sucrose gradients as previously described (Marzluff et al., 1973) in the SW 27 rotor for 19 hr at 25K. The 4–8S RNAs of nuclear sap were collected from the gradient and further purified by gel electrophoresis as described for chromatin RNA. Pure 18S and 28S rRNA were collected after electrophoresis in 2% acrylamide–0.1% bisacrylamide–0.5% agarose gels in 0.04 *M* Tris-acetate (pH 7.2)–0.002 *M* EDTA–0.1% sodium dodecyl sulfate. The RNAs were isolated from the gels as described below. tRNA was prepared by electrophoresis of the cytoplasmic RNA on 10% acrylamide gels. RNA (5.4 S) associated with 28S RNA (Pene et al., 1968; Sy and McCarty, 1971) was prepared by heating 28S RNA to 60° for 10 min followed by electrophoresis in 10% acrylamide gels.

Purification of Individual Low Molecular Weight RNA

Species from Nuclear Sap and Chromatin of Myeloma Cells. RNA from nuclear sap (after saline-EDTA extraction of nuclei) and chromatin was further purified by preparative gel electrophoresis. The RNA was dissolved in 0.2% sodium dodecyl sulfate-10 m*M* EDTA-0.001% Bromophenol Blue-10% glycerol and layered onto 10% polyacrylamide-0.3% bisacrylamide gels prepared by the method of Loening (1968). The gels were 15 cm long and 1.1 cm diameter. They were pre-electrophoresed for 8 hr at 4° at 70 V, samples applied, and electrophoresis continued at 70 V until the dye just ran out of the gel (about 20 hr). The gels were sliced longitudinally and autoradiographed. The bands were cut from the gel, broken up, and placed in a plastic pipet plugged with filter paper. The RNA was recovered by eluting electrophoretically into a dialysis tubing attached to the pipet. This procedure yields RNA in excellent recovery free of polyacrylamide. The electrophoresis was at 150 V and the elution of the RNA of this size class was complete in 5-6 hr (monitored with a Geiger counter). The RNA was recovered by the ethanol precipitation. Bands 3-6 were sufficiently pure after this procedure. Bands 1 and 2 were generally eluted together and further separated by electrophoresis in the presence of formamide. Gels (0.8 × 12 cm) were polymerized (12% acrylamide-0.6% bisacrylamide) in 70% formamide-0.04 *M* Tris-acetate (pH 7.2)-0.002 *M* EDTA and preelectrophoresed for 2 hr at 70 V. The samples in 90% formamide were heated to 37° for 10 min and applied to the gels. Electrophoresis was for 18 hr at 70 V at 27°. Bromophenol Blue ran through the gel in 5-6 hr. The separated RNA bands were recovered as described above.

Preparation of DNA. DNA was prepared from 66-2 tumor cells grown in Balb/c mice. Crude nuclei were prepared essentially as described for tissue culture cells. They were dissolved in 0.5% sodium dodecyl sulfate-10 μg/ml of Pronase at 37° for 3 hr, and then repeatedly extracted with phenol. The aqueous phase was dialyzed against 1 m*M* EDTA and then treated with pancreatic RNase (10 μg/ml) for 10 min at 37°, followed by Pronase in 0.5% sodium dodecyl sulfate (1 hr at 37°, 10 μg/ml), repeatedly extracted with phenol, and then dialyzed exhaustively against 1 m*M* EDTA.

DNA-RNA Hybridization. DNA was immobilized on 24 m*M* nitrocellulose filters (B-6, Schleider and Schuell) after denaturation with alkali in 6 × SSC.[1] Over 90% of the DNA prepared as described above bound to the filter. The filters were washed with 6 × SSC and baked in a vacuum desiccator at 80° for 2 hr. DNA (40 μg) was put on each filter.

Hybridization conditions were essentially as described by Birnstiel et al. (1972). The RNA samples from myeloma chromatin were dissolved in 50% formamide-6 × SSC-0.01 *M* Tris (pH 8) and the filters added to the solution. One milliliter of solution was used per 6 filters. At indicated times a blank filter and DNA filter were removed from the reaction and placed in a beaker of cold 4 × SSC. After all the filters had been removed from the RNA solution, the filters were washed in 4 × SSC (30 ml/filter) with occasional stirring, twice at 58° and twice at 25°, dried, and counted. Control filters either contained no DNA or 40 μg of *Escherichia coli* DNA. The temperature of the hybridization was 58° for all samples except for tRNA which was 50°.

[1] Abbreviations used are: SSC, 0.15 *M* NaCl-0.015 *M* Na_3-citrate (pH 8).

Table I: Extraction of RNA from Chick Embryo Chromatin.

Method	mg of RNA Recovered/mg of DNA	% RNA Recovered
3 *M* NaCl–DEAE	0.004	5
3 *M* NaCl–7 *M* urea	0.005	6
4 *M* Gdn·HCl	0.005	6
0.2 *M* NaCl–phenol	0.025	30
0.35 *M* NaCl–phenol	0.045	60
0.5 *M* NaCl–phenol	0.030	40
0.015 *M* Na deoxycholate–phenol	0.045	60
0.015 *M* Na deoxycholate–DEAE	0.030	40
0.35 *M* NaCl–DEAE	0.030	40

RNA concentration was determined by counting directly an aliquot of the reaction mixture. Specific activities of the rRNAs and tRNAs were determined spectrophotometrically (1 A_{260} = 40 μg of RNA). DNA on the filter was determined as described by Brown et al. (1971) (after hydrolysis in 1 *N* HCl) using 27 A_{260}/mg of DNA. In 24 hr approximately 20% of the DNA was lost from the filters at 58°, <5% at 50°. Results were corrected for DNA loss.

Melting of DNA-RNA Hybrids. For melting the hybrids, the filters were placed in 1 ml of 0.1 × SSC for 3 min at the indicated temperature. The solution was chilled, 50 μg of yeast RNA was added, and RNA was precipitated in 10% Cl_3CCOOH at 4° for 10 min. The precipitate was collected on glass-fiber filters, washed, and counted.

Polyacrylamide Gel Electrophoresis. Gel electrophoresis was performed according to Loening (1968) using 10 or 7.5% gels with an acrylamide:bis ratio of 33:1. Gels were stained with Stains-All (0.005% in 50% formamide).

Chemicals. All chemicals were of reagent grade. CsCl was purchased from Harshaw Chemical and guanidine hydrochloride from Heico. Stains-All (1-ethyl-2-[3,11-ethylnaphtho[1,2-*d*]-thiazolin-2-ylidene)-2-methylpropenyl]naphthol[1,2-*d*]thiazolium bromide) was purchased from Eastman.

Results

Comparison of Extraction Methods for Low Molecular Weight RNA Species from Chick Chromatin. Chromatin prepared from 11-day chick embryos has a protein:DNA ratio of 3:1 and contains 8-10% RNA. Several methods were employed for dissociation of chromatin and recovery of the dissociated RNA. All methods which involved dissociation of chromatin in solutions of high ionic strength, followed by high-speed centrifugation, commonly employed in dissociation of chromosomal proteins, resulted in extremely low recovery of RNA from chromatin (Table I). In contrast, a dissociation of chromosomal components under low salt conditions, while not as effective in removing protein, allowed recovery of large amounts of chromosomal RNA. Apparently the RNA was also extracted from chromatin at higher ionic strengths but much of this RNA then aggregated and pelleted with the DNA during high-speed centrifugation or eluted in the void volume of a Bio-Gel A-50 column with the DNA.

Originally, chromatography on DEAE-cellulose in the presence of 7 *M* urea was employed for separation of the RNA from dissociated chromosomal proteins. However, 20-30% of the dissociated RNA is of large enough molecular weight to be irreversibly absorbed to the column. Hence, for most complete recovery of RNA, direct deproteinization

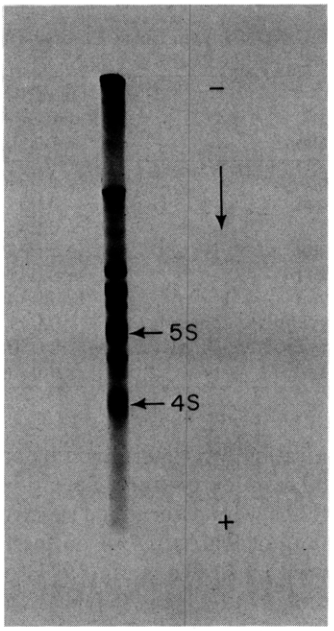

FIGURE 1: Gel electrophoresis pattern of low molecular weight RNAs from chromatin. RNA prepared from 11-day chick embryo thighs was analyzed by gel electrophoresis on 10% polyacrylamide gels. The gels were stained with Stains-All (Eastman). Chromatin RNA was prepared by extraction of chromatin with 0.35 *M* NaCl followed by phenol extraction. A similar pattern was found for total embryo chromatin RNA. Migration of 4S and 5S RNA in this system is indicated by an arrow.

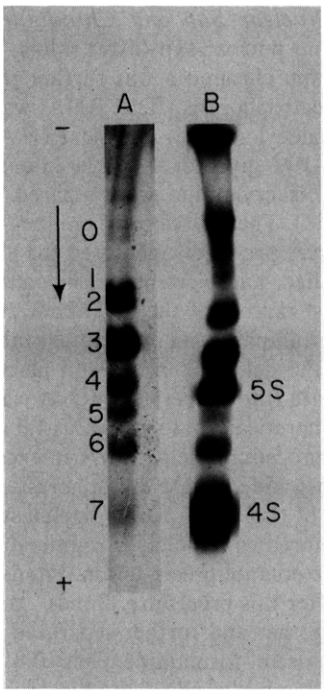

FIGURE 2: Gel electrophoresis patterns of low molecular weight RNAs. $^{32}PO_4$-labeled myeloma chromatin RNA (extracted with 0.35 *M* NaCl followed by phenol) and 4–8S RNA from the saline–EDTA wash of the myeloma nuclei was fractionated by gel electrophoresis on 10% polyacrylamide gels as described under Materials and Methods. The gels were sliced longitudinally and autoradiographed. Exposure time was 1 hr: (A) RNA from chromatin; (B) RNA from saline–EDTA wash. Eight bands (0–7) were observed.

of the extract by extraction with phenol–sodium dodecyl sulfate followed by ethanol precipitation of the RNA was employed. Over 90% of the RNA extracted from chromatin was recovered by this procedure. Extraction procedures which removed large amounts of nonhistone chromosomal proteins (0.015 *M* deoxycholate or 0.35 *M* NaCl) were most effective in removal of RNA from chromatin. A certain proportion (30–40%) of the RNA, however, was not extracted by the technique described here.

Some polypeptide material copurified with the RNA on DEAE-cellulose and a smaller amount in the guanidine hydrochloride–CsCl gradients. Polypeptide material was separated from most of the cRNA after banding the RNA in mixed Cs_2SO_4–CsCl gradients (Szybalski, 1968) and the RNA banded in a single broad band at an identical density with tRNA.

Electrophoretic Analysis of Low Molecular Weight RNA Species from Chromatin. Since myeloma chromatin and chick embryo chromatin contain approximately the same amount of RNA, it would, therefore, be interesting to know whether they are in a similar molecular weight range. The RNAs were prepared by extracting chromatin with low ionic strength buffer. They were analyzed by gel electrophoresis (Figure 1). At least 8 major RNA species of chick thigh chromatin ranging in size from 4 to 8 S are resolved by this procedure. No evidence of any RNA significantly smaller than 4 S was found in these experiments after any of the procedures tested. One of the major components had a mobility identical with 5S ribosomal RNA. These species were strikingly similar in their electrophoretic mobilities to those found in rat hepatoma nuclei described by Moriyama et al. (1969) and in HeLa cells by Weinberg and Penman (1968). The electrophoretic patterns of low molecular weight RNA species from chick embryo chromatin and myeloma chromatin were similar although not identical (Figures 1 and 2).

The low molecular weight RNAs are released into solution upon extraction of myeloma nuclei and chromatin with moderate salt. One fraction resulted from extraction of the nuclei by saline–EDTA (0.075 *M* NaCl–0.024 *M* EDTA (pH 7.9)) and another when isolated chromatin was extracted with 0.35 *M* NaCl. Figure 2 shows the gel electrophoresis patterns of RNAs in these two fractions. A total of 8 discrete species are present in both fractions, numbered 0–7 in order of increasing mobility (C_0–C_7). All but two of these (4 and 7) are restricted to the nucleus. Band 4 is identical with 5S rRNA and band 7 with tRNA (see below). The components are present in the two fractions in different proportions. The obvious differences appeared in bands C_1, C_3, and C_5; C_1 is missing in the saline–EDTA washing and C_3 and C_5 are also present in chromatin in much higher proportions. This pattern was not altered if the extraction with saline–EDTA was repeated. C_4 is 5S rRNA (see below), and it is found in both fractions. In the saline–EDTA wash, a portion may be derived from nuclear ribosomes as 90% of the RNA in the saline–EDTA wash is ribosomal 18S and 28S RNAs (not shown) as is the tRNA.

These low molecular weight chromatin RNA species were very stable. During a chase period of 8 hr, there was essentially no turnover of these RNA species (Figure 3). The relative labeling of the RNA species also remained the same, indicating that there was no preferential turnover of a single species of RNA. Less than 10% of the radioactivity was lost from each fraction during the chase. Hence, these RNAs are metabolically stable and not transiently associ-

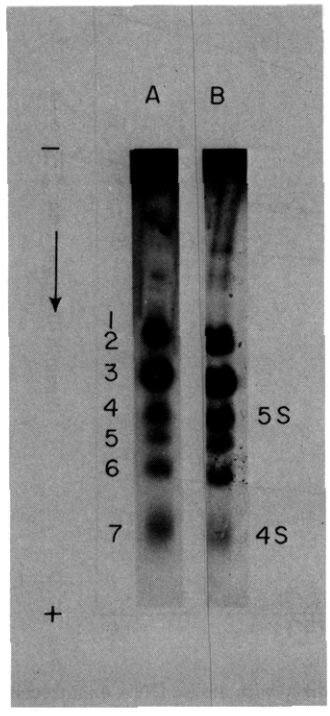

FIGURE 3: Stability of chromatin RNAs. Cells were labeled with $^{32}PO_4$ as described under Materials and Methods for 20 hr. The cells (10%) were then centrifuged and resuspended in fresh media (3×10^5 cells/ml) for 8 hr containing unlabeled phosphate and no $^{32}PO_4$. Chromatin RNA was prepared from each sample: (A) RNA from labeled cell chromatin (90% of the preparation); exposure time, 30 min; (B) RNA from labeled cell chromatin after chase (10% of the preparation); exposure time, 4.5 hr. Less than 10% of the radioactivity in chromatin RNA was lost during the chase period.

Table II: Molecular Weights of Myeloma Chromatin RNA as Examined by Polyacrylamide Gel Electrophoresis.[a]

Bands	Aqueous Gels	Formamide Gels
C_1	62,000	65,000
C_2	60,000	58,000
C_3	56,000	56,000
C_4	40,000	40,000
C_5	37,000	38,000
C_6	33,000	35,000
5.4S rRNA	43,000	48,000

[a] The molecular weights of the various species were determined by polyacrylamide gel electrophoresis both in aqueous and formamide gels. As standards 5S (mol wt 40,000) and 4S (mol wt 25,000) RNA were electrophoresed on the same gels and a semi-log plot of mobility vs. molecular weight was constructed.

ated with chromatin, i.e. chromatin 5S RNA is not eventually found in ribosomes.

Molecular Weight Determination of Low Molecular Weight RNA Species from Myeloma Chromatin. The bands were eluted from the gels separately. Bands C_{3-7} were homogeneous on electrophoresis in gels containing formamide (Figure 4). Bands C_1 and C_2 were not adequately separated in the aqueous gels but separated much better on electrophoresis in formamide (Figures 1 and 5). The bands from the saline–EDTA wash had identical mobilities with the corresponding chromatin RNA bands in formamide.

They range from 25,000 to 67,000 or in chain lengths from 80 to 200 nucleotides. RNA band 4 has been identified as 5S RNA not only judged by its size, but also by its ability to hybridize with 5S DNA (Table III). Band 5 does not contain 5S RNA sequences. Also the T_1 fingerprint of band 4 is identical with that of 5S RNA (R. C. Huang, unpublished results). This does not completely rule out small differences in sequence in the two 5S RNAs (of the *Xenopus* oocyte and somatic cell 5S; Ford and Southern, 1972). Band C_7 is heterogenous; a portion of it may be tRNA.

The molecular weights were estimated on the basis of migration of the RNAs in both types of gels (Table II). As no standards other than 5S rRNA and 4S tRNA were available, these molecular weights are at best estimates. The U1 RNA recently sequenced by Reddy et al. (1974) is probably identical with our C_3 and has been included as a standard in deriving the estimates. C_3 showed the greatest relative differences in the different gels, indicating that may have unusual conformations compared to the other RNA species.

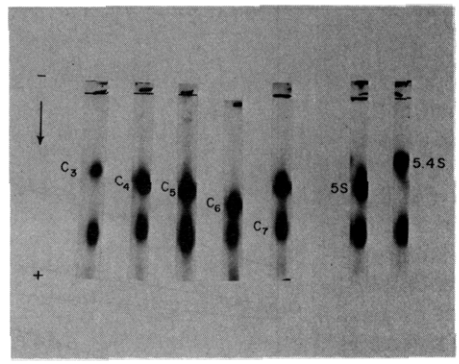

FIGURE 4: Electrophoresis of myeloma chromatin RNAs on formamide gels. Purified RNAs were eluted from the gels in Figure 1. The RNAs were precipitated with ethanol and electrophoresed in gels equilibrated in 70% formamide. To each sample was added 10,000 cpm of 4S tRNA, except for band C_7 to which was added 10,000 cpm of 5S rRNA. Left to right: chromatin RNA bands C_3, C_4, C_5, C_6, C_7, 5S rRNA, 5.4S rRNA.

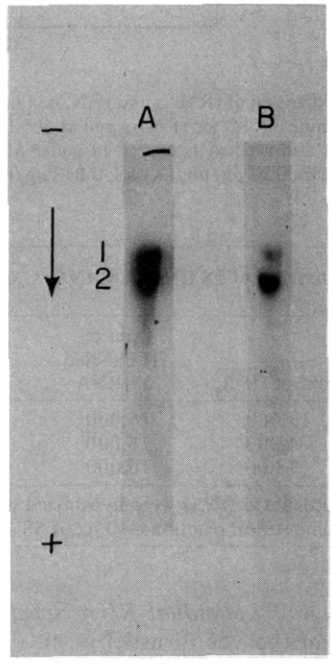

FIGURE 5: Purification of chromatin RNA bands C_1 and C_2. C_1 and C_2 were eluted from acrylamide gels as described in Figure 2 and further fractionated on gels equilibrated in 70% formamide: (A) bands C_1 and C_2 from chromatin; (B) bands 1 and 2 from saline–EDTA wash.

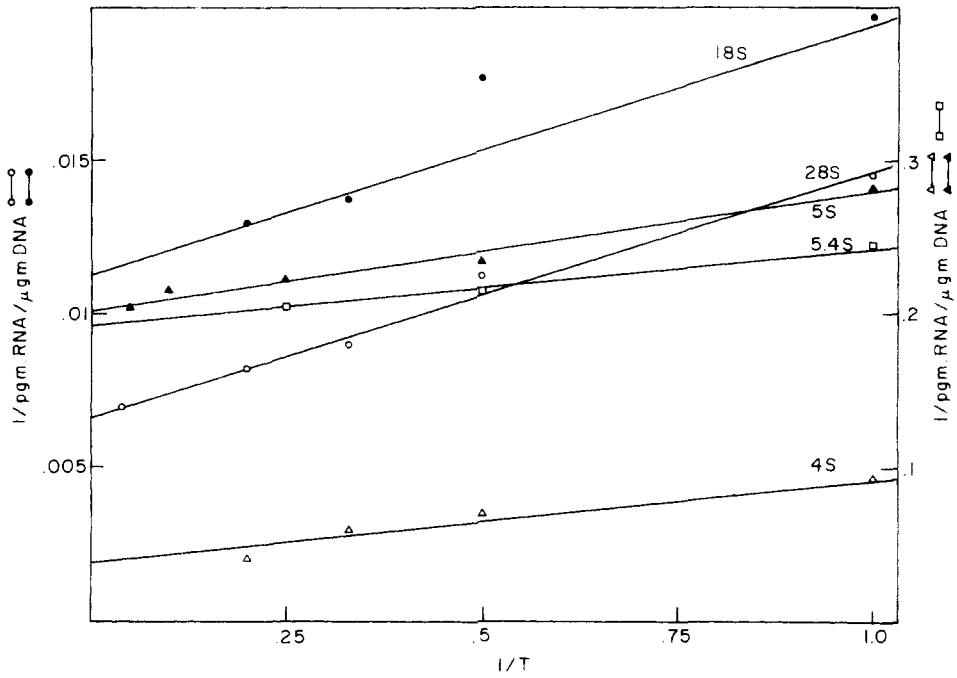

FIGURE 6: Hybridization of rRNAs and tRNAs. Purified myeloma RNA samples were hybridized with mouse DNA on nitrocellulose filters in 6 × SSC–50% formamide at 58° for rRNAs and at 50° for tRNA. The data were analyzed as described by Birnstiel et al. (1972). Filters were removed at indicated times and washed as described under Materials and Methods. $1/T$ is given in hours. Specific activity was 550,000 cpm/μg: (●) 18S, 0.64 μg/ml; (○) 28S, 0.97 μg/ml; (▲) 5S, 0.042 μg/ml; (□) 5.4S, 0.050 μg/ml; (△) 4S, 0.84 μg/ml.

Table III: Hybridization of 5S RNA and RNAs C_4 and C_5 from Myeloma to *Xenopus* 5S DNA.[a]

Sample $^{32}PO_4$	cpm of $^{32}PO_4$	cpm of ^3H-Labeled 5S RNA	Bound		Competed		% Efficiency		
			$^{32}PO_4$	^3H	$^{32}PO_4$	^3H	^3H	^{32}P	% 5S
5S	13,500	16,000	138	192	13	9	1.2	1.1	95
C_4	4,000	16,000	42	188	2	20	1.2	1.1	95
C_5	7,000	16,000	5	252	0	0	1.6	<0.1	<10

[a] Purified $^{32}PO_4$ labeled RNAs were hybridized with *Xenopus* 5S DNAs. As an internal standard ^3H-labeled 5S RNA was added to each sample. In the competition reactions, 10 μg of 5S RNA was added to each vial.

Hybridization of Individual RNA Species of Myeloma to DNA. The method of Birnstiel et al. (1972) was used which allows both an evaluation of the sequence complexity of the RNA sample and the saturation value to be determined in a single experiment. Thus, hybridization of the major species can be distinguished from hybridization of minor species on the basis of hybridization rate. The rate is dependent on molar RNA concentration of a given sequence at the optimum temperature. The method was standardized with RNAs of known sequence complexity, 5S rRNA, 5.4S rRNA, 18S rRNA, and 28S rRNA (Figure 6). The second piece of information obtained from the saturation value is the number of copies of each RNA species in the genome. The ribosomal and tRNA genes are reiterated (Brown and Weber, 1968; Hatlen and Attardi, 1971) while the genes coding for most proteins apparently are not (Harrison et al., 1972; Bishop et al., 1974). Thus, it was of interest to determine whether the genes for these low molecular weight RNA species were reiterated or not.

The results of Figure 6 indicate that the cytoplasmic RNAs tested all hybridized as single components, at a rate consistent with their molecular weights (Birnstiel et al., 1972). As expected, the saturation values of 18 and 28 S gave identical copies, about 300 per genome, while 5 S was present in approximately 500 per genome (Table IV). The $C_r t_{1/2}$ values found were similar to those found by Birnstiel et al. (1972) under these conditions, 0.37 for 5 S, 5.3 for 18 S, and 11.5 for 28 S. tRNA hybridized at a much slower rate, indicative of the sequence complexity of this class of RNA. There were 4000–5000 copies of tRNA in the genome with about 55 different sequences being present. RNA (5.4 S) hybridized at a rate consistent with a single species which was present in 500–600 copies per genome.

The optimum temperature for hybridization of 5S and rRNAs was 58–60°, while for tRNA it was 50°, presumably due to the poorer hybrids with tRNA due to many modified bases present.

The low molecular weight species RNA were assayed in the same manner (Figure 7). Each hybridized at a rate consistent with a homogeneous species, varying dramatically in their reiteration frequency in the genome. From the hybridization kinetics the complexity of each species was calculated (Table V) and for each species it is consistent with species being a single sequence, certainly no more than 2 or 3.

However the number of copies varied from about 100 for C_1 and C_2 to about 2000 for C_6. The low molecular weight RNA species in the saline–EDTA washing (2, 3, and 6)

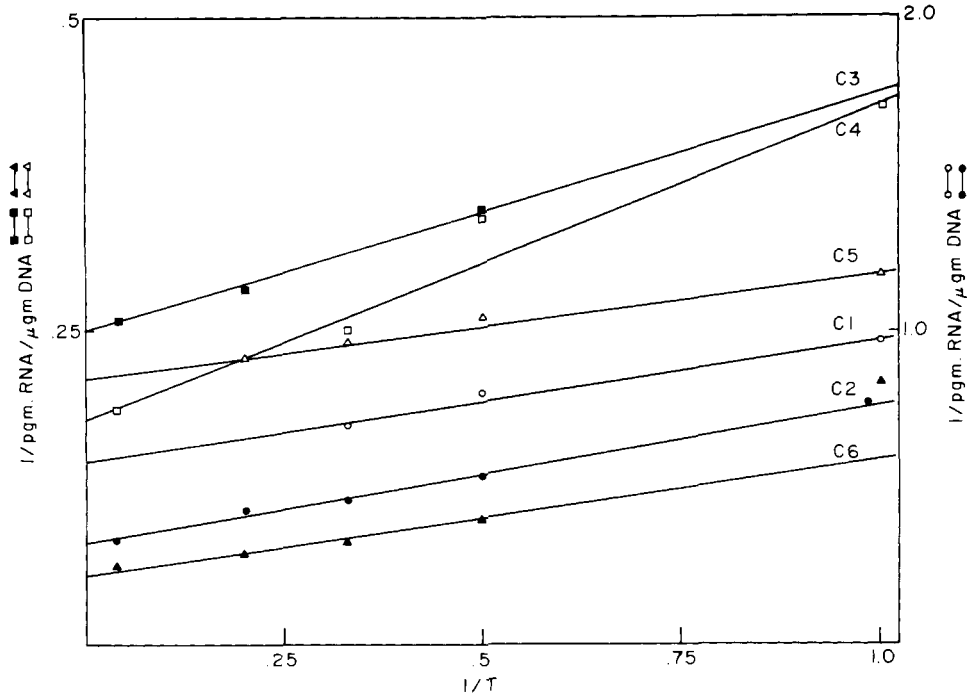

FIGURE 7: Hybridization of chromatin RNAs. Purified myeloma RNA fractions were hybridized to mouse DNA on nitrocellulose filters in 6 × SSC–50% formamide at 58° as described in Figure 6. The data were analyzed as described in Figure 6. $1/T$ is expressed in hours. Specific activity was 650,000 cpm/μg. Calculations for the specific activity were described in the text: (○) C_1, 0.040 μg/ml; (●) C_2, 0.045 μg/ml; (■) C_3, 0.02 μg/ml; (□) C_4, 0.016 μg/ml; (△) C_5, 0.042 μg/ml; (▲) C_6, 0.007 μg/ml.

Table IV: Gene Copies for Cytoplasmic RNAs of Myelomas as Determined by DNA–RNA Hybridization.[a]

RNA	Copies				
	Expt 1	Expt 2	Expt 3	Av	$C_r t_{1/2} \times 10^3$
4S	7500	5400		6450	11
5S	700	520	470	560	0.27
18S	290	490	200	[330]	4.7
28S	200	460			12.6
5.4S	500	570		535	0.31

[a] Purified $^{32}PO_4$ RNAs (specific activity 250,000–800,000 cpm/μg) were hybridized with mouse DNA as described under Materials and Methods. $t_{1/2}$ was determined from plots similar to Figure 6. Saturation values were extrapolated from the intercepts in Figure 6. The mouse haploid genome was taken as 3 pg of DNA. In all experiments at least 80% saturation was reached. The molecular weights used were 4S = 25,000, 5S = 40,000, 5.4S = 45,000, 18S = 6×10^5, 28S = 1.2×10^6. $C_r t_{1/2}$ is as defined by Birnstiel et al. (1972), where C_r is the nucleotide concentration in moles/liter and $t_{1/2}$ is the time for half-saturation to be reached in seconds.

Table V: Gene Copies for Myeloma Chromatin RNAs as Determined by DNA–RNA Hybridization.[a]

Bands	Expt 1	Expt 2	Av	$C_r t_{1/2} \times 10^3$
C_1	110	100	105	0.37
C_2	125	165	145	0.30
C_3	295	270	282	0.22
C_4	550	550	550	0.32
C_5	560	475	517	0.28
C_6	3370	1820	2600	0.17

[a] $^{32}PO_4$ chromatin RNAs were purified as described in the text and hybridized to mouse DNA as described in Table III. The copies of band C_4 (5 S) were defined as 550 in each experiment and the specific activity of the RNAs calculated assuming this value from the saturation value (cpm/μg of DNA) of C_4 in each experiment (see text). The molecular weights used were an average of the two values from Table I. $C_r t_{1/2}$ is as defined in Table IV. Saturation values were determined from the intercepts in Figure 7 and at $t_{1/2}$ from plots similar to those of Figure 7. At least 80% saturation was reached in all determinations.

gave identical results with the same species found in chromatin. A mixture of band C_2 from the chromatin and saline-EDTA wash hybridized as a single species indicating these were identical (not shown).

The major problem in analyzing the data is accurate determination of specific activity of the RNA. This could be determined directly for 18, 28, 4, and 5S RNA and in all cases the specific activities of these were identical with one another in a given experiment. Insufficient amounts of chromatin RNAs were obtained to determine the specific activity directly.

The following assumptions were made: (1) all the individual RNA species have the same specific activity; (2) the specific activities of C_4 can be determined by comparison with that of 5S rRNA from the same preparation. The first assumption is supported by the following facts: (a) the RNAs are all very stable, not turning over detectably in an 8-hr period; (b) the cells grew for one generation, suggesting that all stable cellular RNAs should become labeled equally; (c) the relative amounts of different chromatin RNA species found in vivo reflect the relative amounts of radioactivity in the separate species, implying they all have about the same specific activity (Figure 8). The second assumption was derived by comparing the specific activity deduced from the hybridization properties of band 4 to those of 5S rRNA of known specific activity. In three different preparations analyzed the specific activity of band 4 relative to 5S rRNA varied from 0.5 to 2. In all cases, within one experiment the relative amounts of hybridization of the different species relative to band 4 were the same; only the magnitude (saturation counts per minute relative to 5 S)

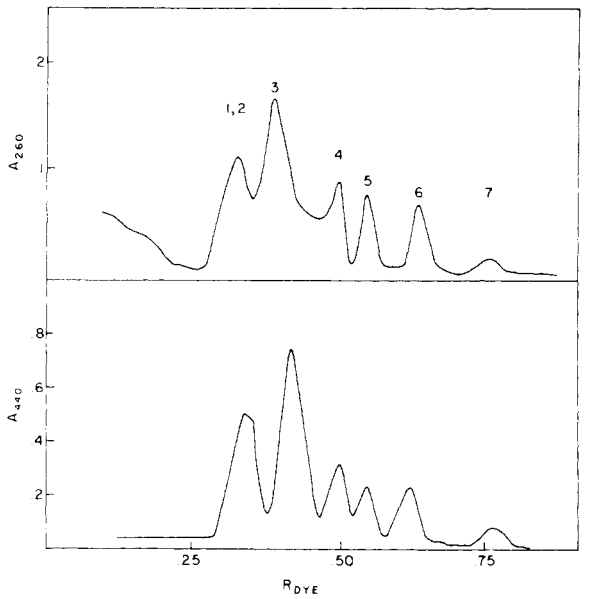

FIGURE 8: Specific activity of myeloma chromatin RNA as examined by gel electrophoresis. Top: Unlabeled chromosomal RNA was prepared from 10^8 cells exactly as described for the labeled RNA. The RNA was analyzed on 0.7 × 12 cm 10% polyacrylamide gels. The gel was scanned with a Gifford Linear Transport device. Bottom: An autoradiograph (from a gel in Figure 2) of chromatin RNA was scanned in a densitometer. The correspondence in relative magnitude in the two samples indicates the chromatin RNAs all have a similar specific activity.

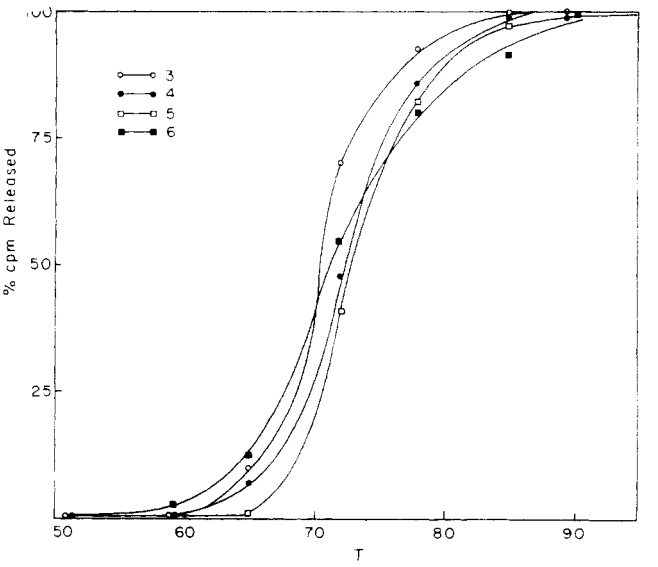

FIGURE 9: Melting of myeloma chromatin RNA–DNA hybrids. Purified chromatin RNA fractions were hybridized to mouse DNA for 24 hr as described in Figure 6. The filters were incubated for 3 min in 1 ml of 0.1 × SSC at the indicated temperature and the counts released determined as described under Materials and Methods: (○) C_3; (●) C_4; (□) C_5; (■) C_6.

was different in different species. Hence this assumption appears to be valid.

All the hybrids between individual RNA and DNA had sharp melting profiles (Figure 9) with no low melting material, indicating that excellent hybrids were formed.

C_7 RNA species hybridized at a slow rate and the hybridization was completed by cytoplasm tRNA (not shown). It was the least abundant low molecular weight species of chromatin.

Discussion

The most widely used methods of dissociating chromatin employ high ionic strength (Shaw and Huang, 1970; Levy et al., 1972; Elgin and Bonner, 1972; Hill et al., 1971). These methods result in a very low yield of RNA in the extract. In contrast, extraction of chromatin at lower ionic strength, while not removing as much protein, allows recovery of much of the RNA in chromatin. These same procedures release much of the nonhistone protein (Graziano, 1972) and little histone (except at 0.5 *M* salt where histone I is removed). Hence, it is possible the RNAs exist in chromatin in a complex with nonhistone protein.

Earlier reports indicated that low molecular weight chromosomal RNA exhibited great sequence diversity and hybridized almost exclusively with intermediate repetitive DNA (Holmes et al., 1974). Isolating RNA by the same methods as used by others (Mayfield and Bonner, 1972) we found that much of the RNA had minimal sequence complexity. However, preliminary experiments with total cRNA preparations at high C_rt values indicated a much higher complexity than indicated by the isolated species. Whether this minor proportion of the RNA represents a true low molecular weight cRNA or contaminating heterogeneous nuclear RNA (HnRNA) is difficult to determine. However, it is certainly true that RNA species present in small numbers in the nucleus would not have been detected in these studies (see below). The report that much of the cRNA is complementary to intermediate repetitive DNA (Holmes et al., 1974) is not in conflict with these studies, but does not indicate the sequence complexity of the bulk of the complementary DNA, which we found to be minimal.

The major portion of low molecular weight RNA in chromatin is a series of defined RNA species which are sequence homogeneous as analyzed by RNA–DNA hybridization. The six species described here represent at least 75 to 80% of the RNA released from chromatin by these extraction procedures. All of these low molecular weight RNAs were metabolically stable, essentially not turning over during an 8-hr chase period. These RNAs are similar in all these respects to the low molecular weight nuclear RNAs first reported by Weinberg and Penman (1968) and Moriyama et al. (1969) and since found in many eucaryotic systems (Frederiksen et al., 1974). These RNAs are loosely associated with chromatin and within the nucleus may be in equilibrium with the chromatin. Certain species, C_1 and C_5, were more restricted to chromatin while others were found in equal abundance in both fractions of the nucleus.

There are large numbers of these RNAs in each nucleus, on the order of 200,000 to 1,000,000 molecules/nucleus. This implies that we have studied only major species of RNA and any molecules present at less than 40,000 molecules per nucleus certainly would not have been detected in these experiments.

One of the RNAs, band 4, is identical with 5S rRNA. This component represents about 10% of the total low molecular weight RNA associated with chromatin and 5% of the total chromatin RNA. This amount is much too large to represent contamination with large ribosomal subunits. No 28S RNA is detectable in the preparation and the conditions used (0.35 *M* NaCl) will not remove 5S RNA from the ribosomal subunit. In the saline–EDTA wash of the nuclei, all the rRNA associated with the nuclei was removed. In addition, during a chase period, the chromatin 5S RNA remains associated with chromatin and certainly does not

represent a biosynthetic intermediate on the pathway of ribosome biosynthesis.

Several years ago, Knight and Darnell (1967) showed that there existed a molar excess of 5S RNA in the nucleus and that this "pool" of 5S RNA was stable. Leibowitz et al. (1973) have shown that the rate of synthesis of 5S RNA is higher than that required for ribosome biosynthesis. Whether the 5S species in chromatin has an identical nucleotide sequence with 5S ribosomal RNA is not known, but the two are indistinguishable by their hybridization properties of 5S DNA, and T_1 fingerprints are similar if not identical.

The finding that these species are transcribed from reiterated genes extends the list of known gene products of the reiterated portion of the genome. The hybridization technique developed by Birnstiel and associates is ideal for these studies, since it requires a small amount of material and inherent in the experiment is a determination of purity of the RNA sequence being tested. Thus, it is different from saturation experiments where one might measure only a small proportion of the RNA which is complementary to a large fraction of the DNA.

The saturation values for the rRNAs and tRNAs found in the mouse by these methods are consistent with those found in other systems (Clarkson et al., 1973; Hatlen and Attardi, 1971). The 5S genes are present in slight excess over the ribosomal genes and the data indicate about 50 different tRNA sequences present at an average of around 100 per genome or slightly less than the number of ribosomal genes on a molar basis. Unlike the case of *Xenopus laevis*, where there are 9000–20,000 5S genes, a great molar excess over 28S and 18S rRNA (Brown and Weber, 1968; Birnstiel et al., 1966), there are only a few hundred 5S genes in mouse, of the same order of magnitude as the genes for 18 and 28S rRNA. A similar result has been reported for *Drosophila* (Ritossa et al., 1968; Wimber and Steffensen, 1970; Tartof and Perry, 1970). The lower number of 5S genes is consistent with only slightly higher rates of equimolar synthesis of 5S than that of rRNA in these cells. In contrast, in *Xenopus* during oogenesis the 28S and 18S rRNA genes are grossly amplified and the 5S genes are not. There is evidence that the bulk of the 5S genes do not code for the 5S rRNA found in somatic cells suggesting that the bulk of these genes is normally repressed and may only function during oogenesis (Ford and Southern, 1972). There is presumably no similar demand for 5S RNA at any stage in the mouse.

A higher value of gene copies for 5S RNA has been reported by Hatlen and Attardi in HeLa cells (1971). They have found 2000 5S genes in the HeLa cell using saturation hybridization, and a similar number of rRNA genes as reported here. The reason for the discrepancy is not clear, although very slight impurities in the preparation in principle can raise the saturation value, and hence raise the number of gene copies.

The genes for each RNA species of chromatin are present in widely different numbers. They account for an extremely small percentage of the total mouse DNA however. All the genes for structural RNAs so far described are repetitive, while most of the genes coded for proteins are found in unique DNA (Bishop et al., 1974). It is likely that the reason for this is that the number of RNA molecules a gene can produce is limited by its transcription rate, while the number of protein molecules a given gene can direct is affected at two stages, the production of many RNAs from a single DNA and then of many proteins from this RNA. An interesting exception is the histones (Kedes and Birnstiel, 1971). Extremely large numbers of these molecules have to be made in a short time during each cell cycle and it is possible that the maximum rate of synthesis is not sufficient without many copies of DNA for these proteins.

Engberg et al. (1974) have studied the redundancy of various low molecular weight nuclear RNAs from hamster kidney and have reached a similar conclusion that they are transcribed from reiterated sequences. Their values are somewhat higher than ours and similar for each species. Their experiments were performed at much higher RNA concentrations and hence for larger values of C_rt and this might contribute to the differences. Alternatively, they simply may be species-specific differences in gene numbers.

The function of the RNA species, C_1 to C_6, is totally unknown. They are restricted to the nucleus, but are not quantitatively associated with chromatin. The subnuclear localization is determined arbitrarily by the isolation conditions chosen. However, under the moderate salt conditions employed in this study (0.125 M Na^+) much of the low molecular weight RNA was specifically associated with the chromatin.

It seems likely that these RNAs may be complexed with nonhistone proteins in chromatin. RNA (5S) is known to form a complex with a specific ribosomal protein (Blobel, 1971) and it would be interesting to determine whether this protein is associated with the RNA in chromatin. Although these RNAs may not function as specific gene regulators, a correlation between the amount of RNA present in chromatin and the activity of the chromatin seems to exist (Bonner et al., 1968).

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