Nature and Origins of Chromatin-Associated Ribonucleic Acid of Avian Reticulocytes†

Paul Tolstoshev and Julian R. E. Wells*

ABSTRACT: Chromatin from eukaryotic cells contains associated RNA which can be isolated as 3–4S chromosomal RNA. Similar material is readily prepared from avian erythroid chromatin by classical methods, the CsCl procedure or the deoxycholate procedure (see below). However, the status of such RNA as a possible regulator of transcription is seriously questioned by the finding that a number of steps involved in the purification of chromatin-associated RNA result in the degradation of higher molecular weight RNA to the 3–4S product. The CsCl procedure was investigated in the most detail; chromosomal RNA (cRNA) isolation involves centrifugation of chromatin through 4 M CsCl, Pronase digestion of the pellicle, phenol extraction, and DEAE-Sephadex chromatography. Degradation can occur as a result of (a) sonication of chromatin (initially used to disperse chromatin in 4 M CsCl), (b) endogenous RNase activity in the pellicle material, or (c) RNase activity in preincubated Pronase. The final point is very significant in considering the origins of cRNA for the following reasons. (1) When Pronase digestion is omitted, the majority of chromatin-associated RNA is recovered as ribosomal RNA (rRNA). (2) Covalent attachment of chromatin-associated RNA to protein is unlikely since as much or more RNA can be extracted from the pellicle in the absence of Pronase digestion. (3) For a given preparation of chromatin, there is insufficient chromatin-associated RNA to account for both the rRNA present and for the low molecular weight material obtainable by the CsCl procedure. (4) The RNase activity in preincubated Pronase has little effect on either reticulocyte or yeast tRNA (as judged by recovery of tRNA and of its amino acid acceptor activity), but reticulocyte rRNA is extensively degraded. This, together with the fact that dihydrouridine is found in tRNA (yeast, avian reticulocyte) but not in avian reticulocyte cRNA, suggests that cRNA is not a degradation product of tRNA in these cells. Isolation of cRNA by a deoxycholate procedure also yields a low molecular weight RNA which contains low levels of protein and no detectable saturated pyrimidine bases. About 70% of chromatin-associated RNA fractionates with the membrane component when chromatin is purified through a 1.7 M sucrose barrier, and high molecular weight RNA is found in both fractions. We suggest that membrane-bound ribosomes are the source of rRNA in avian erythroid chromatin, and that this is degraded to 3–4S fragments during preparative procedures for cRNA isolation.

Chromatin, the genetic material isolated from eukaryotic cells, contains three well-recognized components: DNA, protein, and RNA. The proteins (histones and nonhistone proteins) and chromatin-associated RNA have been suggested as possible regulators of transcription and as such to have relevance to this process in all eukaryotic cells. Here we report studies on the chromatin-associated RNA of avian erythroid cells as one aspect of a wider investigation of the processes which regulate specific transcription in these cells (Appels *et al.*, 1972; Appels and Wells, 1972; Harlow *et al.*, 1972).

Chromosomal RNA (cRNA[1]), a low molecular weight fraction first isolated from pea bud chromatin by Huang and Bonner (1965), has been studied extensively (Huang, 1967; Bonner and Widholm, 1967; Bekhor *et al.*, 1969; Shih and Bonner, 1969; Dahmus and McConnell, 1969; Jacobson and Bonner, 1971). The concept of RNA[2] molecules as regulators of eukaryotic transcription has been proposed (Britten and Davidson, 1969), and it is possible that cRNA preparations contain such molecules (Mayfield and Bonner, 1971, 1972). Properties attributed to cRNA in several tissues include: (a) covalent linkage between cRNA and chromosomal proteins (Huang, 1967; Jacobson and Bonner, 1971); (b) a high content (8–10 mol %) of a saturated pyrimidine base (Jacobson and Bonner, 1968); and (c) preferential hybridization to intermediate repetitious DNA (Sivolap and Bonner, 1971).

There is no doubt that application of the CsCl (see Dahmus and McConnell, 1969) or deoxycholate (Mayfield and Bonner, 1971) procedures to chromatin preparations from a variety of tissues will result in the isolation of low molecular weight cRNA. Avian erythroid chromatin is no exception. The central point, however, is whether such material can legitimately be considered as a separate class of RNA. Others (Heyden and Zachau, 1971; Artman and Roth, 1971) have questioned this proposal and studies reported here show that in general there is the distinct possibility of cRNA arising from degradation during preparative procedures and that, in particular, cRNA from avian erythroid chromatin is a degradation product of higher molecular weight RNA, chiefly rRNA.[3]

Materials and Methods

Cells. Reticulocytes, mainly mid- and late-polychromatic erythrocytes (Appels *et al.*, 1972), were obtained from the circulation of fowls made highly anemic with phenylhydra-

† From the Department of Biochemistry, University of Adelaide, Adelaide, South Australia 5001. *Received June 7, 1973.* This work was supported by the Australian Research Grants Commission D65/15758.

[1] Chromosomal RNA refers to the species of RNA defined by the preparative procedures of Dahmus and McConnell (1969) and of Mayfield and Bonner (1971) and is abbreviated to cRNA in the text.

[2] Protein molecules as regulatory elements were not excluded from this scheme.

[3] Abbreviations used are: HnRNA, heterogeneous nuclear ribonucleic acid; cRNA, chromosomal RNA; rRNA, ribosomal RNA.

zine. Induction of anemia, usually over a 7-day period, was carefully monitored by hematocrit measurements and a microgradient technique (Williams, 1972).

Preparation of Chromatin. Nuclei were isolated and washed essentially as described by Dingman and Sporn (1964). Chromatin was prepared from nuclei by homogenization in 0.2 mM EDTA (pH 7.2), in a Dounce homogenizer, followed by centrifugation. Purified chromatin refers to material which had been sedimented through a 1.7 M sucrose barrier. Details of these procedures have been described elsewhere (Harlow *et al.*, 1972).

Preparation of Chromosomal RNA. CsCl PROCEDURE. This was as described by Dahmus and McConnell (1969) except that, for initial experiments, sonication (Soniprobe, type 1130A, Dawe Instruments, U. K., 8 × 15 sec, setting 8, maximum output) was used to disperse the chromatin in 4 M CsCl. Subsequently, this was done using a Waring Blendor. The protein pellicle obtained after centrifugation was homogenized with a glass–Teflon homogenizer, in a small volume (usually 8 ml) of Pronase solution (Calbiochem, B grade, 4 mg/ml in 0.01 M Tris-Cl (pH 8.0), preincubated at 37° for 90 min) and incubated at 37° for 4 hr. The digest was then phenol extracted by the cold phenol method (see below) and ethanol precipitated.

DEOXYCHOLATE PROCEDURE. This was applied to crude reticulocyte chromatin exactly as described by Mayfield and Bonner (1971), except that only one cycle of chromatography on DEAE-Sephadex was performed.

Phenol Extractions. Samples were extracted with 90% aqueous phenol–0.1% 8-hydroxyquinoline, either at 4° using 0.01 M Tris-Cl (pH 8.0) as the extraction buffer (Dahmus and McConnell, 1969) or at 60°, using 0.01 M EDTA–0.01 M sodium acetate buffer (pH 5.1), as described by Artman and Roth (1971). These two procedures are referred to in the text as the cold phenol and hot phenol methods, respectively. After phenol extraction, the aqueous phase was sometimes extracted with diethyl ether to remove residual phenol, 0.1 vol of 20% potassium acetate (pH 6.5) and 2 vol of cold ethanol were added, the mixture was left at −15° overnight, and nucleic acids were recovered by centrifugation. The DNA contamination in hot phenol extracts of reticulocyte chromatin, or the pellicle derived from it, were low, ranging from 1 to 10% of the RNA content.

DEAE-Sephadex Chromatography. Samples were dissolved in 5 ml of 0.2 M NaCl–7 M urea–0.01 M Tris-Cl (pH 8.0) and loaded onto a 1 cm × 30 cm column of A-25 DEAE-Sephadex (Pharmacia, Uppsala) equilibrated with the same buffer. The column was eluted at 4° with a linear 300-ml gradient of NaCl from 0.2 to 1.0 M (in 7 M urea–0.01 M Tris-Cl (pH 8.0)). The appropriate fractions were pooled, dialyzed against 4 × 5 l. of distilled water at 4° for 10–16 hr, freeze-dried, dissolved in 0.01 M Tris-Cl (pH 8.0), and dialyzed further against this buffer (2 × 2 l., 6–8 hr, 4°).

Sucrose Gradient Centrifugation. Samples (40–200 μg) of RNA were loaded onto linear 5–20% sucrose gradients (sucrose gradient buffer, 5 mM magnesium acetate, 10 mM Tris-Cl, and 150 mM NaCl adjusted to pH 7.4) and centrifuged in the SW 41 rotor of a Beckman Model L_2 ultracentrifuge at 38,000 rpm (174,000*g*) at 3° for the times shown in the legends to figures. The gradients were fractionated through either an Optica or LKB spectrophotometer fitted with a flow cell, and the absorption at 260 mμ recorded. For the gradient of ^{32}P-labeled DNA, 20-drop fractions were collected, and high molecular weight DNA was precipitated with 5 ml of 5% Cl_3CCOOH–0.01 M sodium pyrophosphate using 500 μg of bovine serum albumin per fraction as carrier. Fractions were placed on ice for 10–30 min, filtered onto glass fiber disks, washed with 3 × 5 ml of 5% Cl_3CCOOH–0.01 M sodium pyrophosphate, and 2 × 1 ml of ether, dried, and counted using a scintillation spectrometer.

Preparation of Other RNA Samples. Yeast tRNA was extracted by the method of Holley *et al.* (1961). Reticulocyte tRNA was isolated by the cold phenol method from a high-speed supernatant of a reticulocyte lysate and was further purified by DEAE-cellulose chromatography as for yeast tRNA.

Reticulocyte rRNA was isolated as follows. Washed reticulocytes were lysed with 4 vol of ice-cold 2 mM $MgCl_2$, stirred for 10 min at 4°, and then 1 vol of 1.5 M sucrose–0.15 M KCl was added. The lysate was centrifuged at 15,000*g* for 10 min at 4° to remove nuclei and cell debris, and the supernatant was then centrifuged at 105,000*g* for 2 hr at 3°. The supernatant was used to isolate tRNA (see above) and the pellets were resuspended in 0.01 M EDTA–0.01 M sodium acetate buffer (pH 5.1), using a glass–Teflon homogenizer. Sodium dodecyl sulfate and Macaloid (a gift of Baroid Division, National Lead Co., Tex.) were added to 1%. The mixture was incubated at 37° for 10 min and then extracted by the hot phenol method. The RNA was ethanol precipitated as described, and, when analyzed on 5–20% sucrose gradients, contained predominantly 18S and 28S material, with a small 4S component (see Figure 3A).

Preparation of Reticulocyte Aminoacyl-tRNA Synthetase and Testing of Amino Acid Acceptor Activity. Charging enzymes were prepared as described for rabbit reticulocytes by Bhaduri *et al.* (1970). Before testing for acceptor activity, all RNA samples were deacylated by incubation in 0.5 M Tris-Cl (pH 8.9), for 1 hr at 37° followed by extensive dialysis.

The charging reaction (RajBhandary and Ghosh, 1969) contained, in a total volume of 0.5 ml, approximately 4 μg of tRNA or the RNA being tested, 0.33 mg of charging enzymes, and 0.2 μCi of ^{14}C-labeled algal hydrolysate (Schwarz-Mann, sp act. 249 μCi/μmol) in 0.025 M Tris-Cl–0.005 M $MgCl_2$–0.005 M NaATP (pH 7.5). The mixture was incubated for 30 min at 37° and the reaction stopped with 0.5 ml of cold 10% Cl_3CCOOH. The samples were placed on ice for 10–30 min and then filtered onto glass fiber disks, washed with 3 × 5 ml of 7% Cl_3CCOOH–0.1% casamino acids, and 2 × 1 ml of ether. The disks were dried and counted using a scintillation spectrometer. Corrections were made for the low levels of radioactivity incorporated in the absence of RNA. All of the radioactivity rendered Cl_3CCOOH insoluble in the above assay was sensitive to pancreatic RNase (1 μg/ml) or to 0.3 N KOH (37° for 10 min in both cases).

Assay for DNase Activity in Pronase. Pronase (preincubated in 0.01 M Tris-Cl (pH 8.0) for 90 min at 37°) was assayed for DNase activity by measuring the loss of Cl_3CCOOH-precipitable radioactivity from ^{32}P-labeled λ DNA (sp act. 6.91 × 10^3 cpm/μg). Bovine deoxyribonuclease 1 (Sigma Chemical Co.) was used in a control experiment. Reaction mixtures (0.55 ml) contained 0.05 ml of ^{32}P-labeled λ DNA (about 11 μg, 7.51 × 10^4 cpm) and 0.5 ml of either preincubated Pronase (4 or 8 mg/ml in 0.01 M Tris-Cl (pH 8.0), 0.01 M Tris-Cl (pH 8.0) alone, or DNase 1 (1 μg in 0.05 M Tris-Cl–0.025 M KCl–0.01 M $MgCl_2$ (pH 7.4)). Incubation was at 37° and 0.1-ml samples were taken at various times for measurement of Cl_3CCOOH-precipitable radioactivity.

For the sucrose gradient analysis of Pronase-digested ^{32}P-labeled DNA, the reaction mixture was extracted with cold

phenol and a portion of the aqueous phase layered directly onto the gradient.

Ribonuclease Digestions. Samples were digested, where indicated in the text, with 1–20 μg/ml of pancreatic RNase (bovine pancreas, Sigma Chemical Co.) in 0.01 M Tris-Cl (pH 8.0) at 37° for 10 min.

Estimation of Saturated Pyrimidine Bases in RNA Preparations. Dihydrouridine and dihydroribothymidine in RNA samples were measured by the production of their specific alkaline hydrolysis products (β-alanine and β-aminoisobutyric acid, respectively) as previously described (Tolstoshev and Wells, 1973).

Other Analytical Procedures. Protein was estimated by the method of Lowry *et al.* (1951), DNA by the diphenylamine reaction (Burton, 1956), and RNA by the orcinol method (Dische, 1955) or, for tRNA, by the extinction coefficient of 26.2 for the OD_{260} of a 1 mg/ml solution (Stephenson and Zamecnik, 1963). For chromatin samples and samples contaminated with DNA, the orcinol reaction was performed on the perchloric acid supernatant after alkaline hydrolysis (Fleck and Monro, 1962).

Results

Properties of Avian Reticulocyte Chromosomal RNA Prepared by the CsCl Procedure. In this section, we examine the properties of cRNA prepared as described by Dahmus and McConnell (1969). Briefly, chromatin is dispersed in 4 M CsCl; the pellicle obtained after centrifugation is digested with Pronase, phenol-extracted, and RNA-chromatographed through DEAE-Sephadex.

RNA Content of Chromatin. Table I shows the RNA content of avian reticulocyte chromatin, expressed relative to the DNA content. Harlow *et al.* (1972) have found that when this chromatin is further purified by centrifugation through a 1.7 M sucrose barrier, only about 30% of the RNA remains associated with the chromatin, while the rest is found in the membrane material floating on the sucrose. The significance of this will be discussed later. The values obtained are higher than those of Artman and Roth (1971) for avian reticulocyte chromatin, but we have found, in agreement with the above authors, that endogenous ribonuclease activity greatly reduces the RNA content of chromatin, expecially after lengthy procedures such as dialysis. Therefore, the RNA content was always measured immediately after preparation of the chromatin. In animals not fully anemic, the RNA content of chromatin was lower, and mature erythrocytes yielded chromatin with very low levels of associated RNA (less than 1% of the DNA; P. Tolstoshev, unpublished observations).

TABLE I: RNA Content of Avian Reticulocyte Chromatin.[a]

	RNA	DNA
Chromatin	0.219 ± 0.021 (n = 6)	1.0
Chromatin purified through 1.7 M sucrose	0.075 ± 0.001 (n = 4)	1.0

[a] The RNA contents of reticulocyte chromatin, together with the standard error of the mean, were estimated as described under Materials and Methods and are expressed relative to the DNA content.

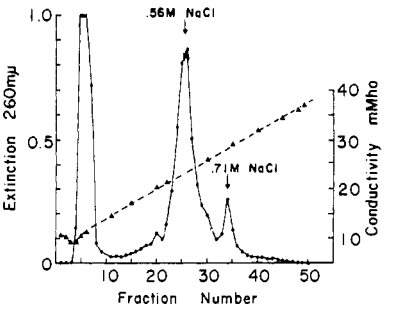

FIGURE 1: DEAE-Sephadex chromatography of chicken reticulocyte cRNA. The cold phenol extract of Pronase-digested CsCl pellicles derived from sonicated reticulocyte chromatin was chromatographed on a 1 × 30 cm column of DEAE-Sephadex, equilibrated in 0.2 M NaCl–7 M urea–0.01 M Tris-Cl (pH 8.0). The column was eluted with a 300-ml linear gradient of NaCl, from 0.2 to 1.0 M, in the presence of 7 M urea–0.01 M Tris-Cl (pH 8.0), at 25 ml/hr at 4°. The conductivity (▲) and the extinction at 260 mμ (2 mm) (●) of 5-ml fractions were measured.

Chromosomal RNA eluted as a sharp peak on DEAE-Sephadex chromatography, at an NaCl concentration between 0.54 and 0.56 M (Figure 1). The first peak in Figure 1 is phenol, while the third, smallest peak is contaminating DNA. When analyzed on 5–20% linear sucrose gradients, the cRNA showed a broad peak in the 3–4S region (Figure 2). The size distribution was considerably greater than that of yeast tRNA. Chromatin further purified by centrifugation through a 1.7 M sucrose barrier gave essentially the same results, although the amount of cRNA obtained was smaller.

The amount of RNA associated with the pellicles was highly variable, ranging from 40 to 80% of the total RNA in the chromatin. This is in agreement with the findings of Artman and Roth (1971). The final yield of cRNA was also variable, but averaged 25% of the total RNA of the chromatin.

Sonication and RNA Breakdown. Chromatin is difficult to disperse in 4 M CsCl and sonication overcame this difficulty. However, to check whether such treatment could contribute to the size heterogeneity of cRNA (Figure 2), yeast tRNA and chicken red cell rRNA were sonicated under the same conditions originally used to disperse chromatin (see Materials and Methods) and although no effect on tRNA was observed, rRNA was extensively degraded so that 18S and 28S RNA material now sedimented as a broad band in the 7–10S region of sucrose gradients (Figure 3B). Although precautions were taken, degradation may have resulted from local heating. When milder sonication conditions were used (setting 5 on the sonicator), degradation was not as extensive,

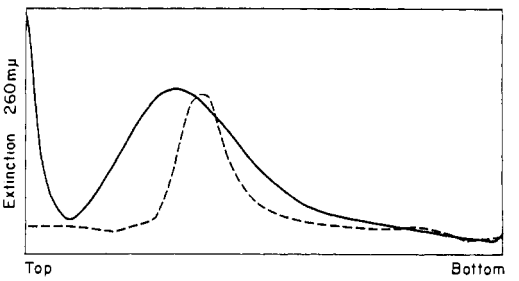

FIGURE 2: Sucrose gradient analysis of reticulocyte cRNA and yeast tRNA. Approximately 40 μg of yeast tRNA (- - -) and reticulocyte cRNA (–) in 0.01 M Tris-Cl (pH 8.0) were analyzed on linear 5–20% sucrose gradients as described under Materials and Methods. Centrifugation was at 174,000g for 16 hr at 3°.

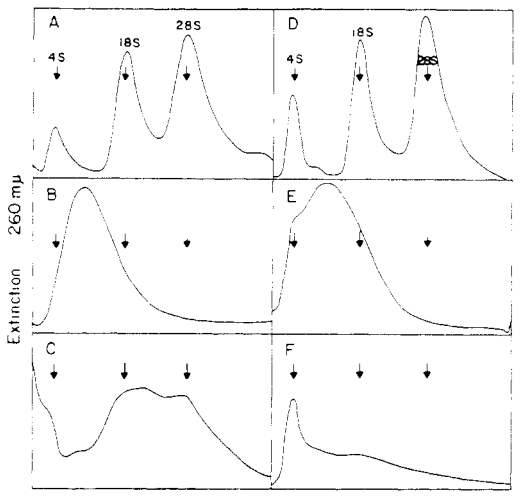

FIGURE 3: Sucrose gradient analysis of reticulocyte rRNA after sonication (B, C) or incubation with preincubated Pronase (E, F). For sonication experiments ~120 μg of RNA sample in 0.01 M Tris-Cl (pH 8.0) was analyzed on 5–20% sucrose gradients (174,000 *g*, 4 hr, 3°): (A) unsonicated rRNA; (B) rRNA sonicated 8 × 15 sec, setting "8"; (C) as for B, but at setting "5." For Pronase-digestion experiments, 2 mg of rRNA was incubated with 8.0 ml of 0.01 M Tris-Cl (pH 8.0) containing 4 mg/ml of preincubated Pronase for 4 hr, 37°: (D) incubation in buffer alone; (E) Pronase digested, hot phenol extracted; (F) Pronase digested, cold phenol extracted.

and some 18S and 28S material remained (Figure 3C). Therefore, sonication was abandoned as a method of dispersing chromatin in 4 M CsCl prior to centrifugation, and instead chromatin was sheared with a Waring Blendor. Chromosomal RNA prepared in this way still eluted at 0.55 M NaCl on DEAE-Sephadex chromatography, and sedimented in the 3–4S region of sucrose gradients. However, the amount of DNA associated with the protein pellicle floating on 4 M CsCl was considerably greater, sometimes amounting to 50% of the total DNA of the chromatin. Artman and Roth (1971) also reported that for unsonicated chromatin preparations from several chicken tissues large amounts of DNA remained associated with the protein pellicle after centrifugation through 4 M CsCl.

Analysis for Saturated Pyrimidines in cRNA. Chromosomal RNA from several sources has been reported to contain 8–10 mol % of either dihydrouridine or dihydroribothymidine (Huang, 1967; Jacobson and Bonner, 1968; Getz and Saunders, 1970). The positive uriedo reaction (Ceriotti and Spandrio, 1963) of these two saturated pyrimidines has been used to measure their concentrations in cRNA. However, owing to the difficulty in removing the last traces of urea from cRNA after chromatography in 7 M urea (urea also gives a positive uriedo reaction) we used the alternative method of looking for the specific hydrolysis products of these saturated pyrimidines after alkaline hydrolysis (McGrath and Shaw, 1967). The hydrolysis products are β-alanine from dihydrouridine and β-aminoisobutyric acid from dihydroribothymidine and these amino acids can be resolved and quantitated by amino acid analysis. The details of the estimations for reticulocyte and mouse ascites cRNA have been reported elsewhere (Tolstoshev and Wells, 1973). We were not able to find either of the saturated pyrimidines, at levels of detection from 0.2 to 0.4 mol %. Dihydrouridine in both yeast and reticulocyte tRNA was readily detected.

Hill *et al.* (1971) also failed to find saturated pyrimidines in the RNA, of predominantly high molecular weight, associated with sea urchin embryo chromatin. Similarly, Arnold and Young (1972) were unable to detect uriedo-positive material in rat liver cRNA that had been carefully freed of contaminating urea, although a value of about 8 mol % saturated pyrimidine in rat liver cRNA has been reported by Mayfield and Bonner (1971).

Thus, the presence of high levels of saturated pyrimidine bases is not a universal property of cRNA from higher cells.

cRNA and Covalent Attachment to Protein. cRNA from several tissues is claimed to be covalently bound to protein (Huang and Bonner, 1965; Huang, 1967) and binding proteins for cRNA from rat ascites cells (Jacobson and Bonner, 1971) and chick embryo (Huang, 1967; Marzluff *et al.*, 1972) have been isolated. The existence of a covalent RNA–protein linkage has been questioned (Artman and Roth, 1971).

A number of factors influence the composition of the CsCl pellicle and the nucleic acids extractable from it and these are discussed further below. However, with appropriate conditions of hot phenol extraction, we find that as much, or more, RNA can be extracted from the pellicle if prior Pronase digestion is omitted (Table II). DNA is also found (in variable amounts) in the pellicle, but is not considered to be covalently bound to protein. It is likely that nucleic acids are nonspecifically trapped in the pellicle floating on 4 M CsCl and that no significant amount of RNA is covalently bound to protein in avian reticulocyte chromatin.

Amino Acid Acceptor Activity of Reticulocyte cRNA. Heyden and Zachau (1971) have suggested that calf thymus cRNA arises from degradation of tRNA during the Pronase digestion step. Ribonuclease activity in preincubated Pronase is of significance in the preparation of cRNA but our results suggest that cRNA is not derived from tRNA.

TABLE II: Extraction of RNA from CsCl Pellicles of Reticulocyte Chromatin before and after Pronase Treatment.[a]

Yield after Pronase Digestion	Yield after Direct Extraction
100	158.3
100	95.6

[a] The CsCl pellicles of avian reticulocyte chromatin, prepared as described under Materials and Methods, were extracted, either directly or after Pronase digestion by the hot phenol extraction procedure. The amount of RNA extracted is expressed as a percentage of the amount extracted after Pronase digestion.

The amino acid acceptor activities of various RNA preparations were tested in a standard assay system using an aminoacyl synthetase preparation from avian reticulocytes (Table III). Both reticulocyte tRNA and yeast tRNA had acceptor activity; rRNA and reticulocyte cRNA did not. Significantly, cRNA preparations obtained from CsCl pellicle material by direct cold phenol extraction without prior Pronase digestion (the RNA is still of low molecular weight) still did not show acceptor activity (Table III).

When reticulocyte and yeast tRNA were incubated with predigested Pronase (4 mg/ml in 0.01 M Tris-Cl (pH 8.0); 4 ml/mg of RNA) for 4 hr at 37°, and reextracted with cold phenol, the recovered material showed identical profiles on sucrose gradient analysis to those of undigested tRNA. The recoveries of tRNA and of its acceptor activity are shown in Table IV. Pronase digestion had little effect on the amino acid

TABLE III: Amino Acid Acceptor Activity of RNA Samples.[a]

Sample	Amino Acid Acceptor Act. (cpm/OD_{260} unit)
Reticulocyte tRNA	15,664
Reticulocyte tRNA + pancreatic RNase (2 μg/ml, 37°, 10 min)	0
Reticulocyte rRNA	125
Reticulocyte cRNA, extracted with Pronase	0
Reticulocyte cRNA, extracted without Pronase	0
Yeast tRNA	2,977

[a] The RNA samples, after deacylation, were assayed for amino acid acceptor activity as described under Materials and Methods. The values, expressed as cpm/OD_{260} unit of RNA, were corrected for control incubations without added RNA. A zero indicates that the acceptor activity was equal to or less than that of control incubations.

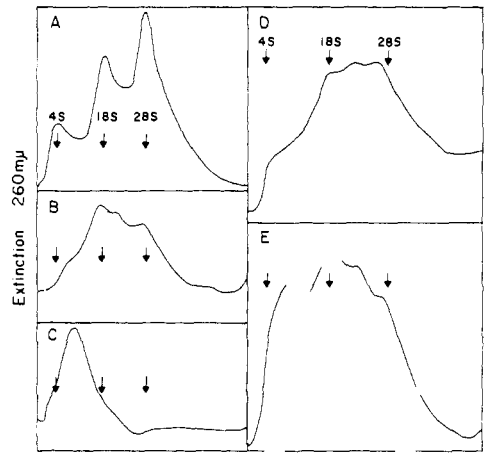

FIGURE 4: Sucrose gradient analysis of hot ph ol extracts from chromatin fractions: (A) crude chromatin; (B) 4 M CsCl pellicle material; (C) 4 M CsCl pellicle material prepared from sonicated chromatin; (D) chromatin purified through 1.7 M sucrose; (E) the membrane fraction from the 1.7 M sucrose barrier. RNA samples were analyzed as described in the legend to Figure 3.

acceptor activity of tRNA from both sources. These results agree with the observations of Holmes *et al.* (1972). Heyden and Zachau (1971) reported a slow loss of acceptor activity of yeast tRNA after Pronase digestion.

The results suggest that preincubated Pronase contains only low levels of RNase activity, but this activity can be detected when rRNA is incubated with Pronase (Figure 3E,F). Taken together with results showing the presence of dihydrouridine in reticulocyte tRNA and its absence in cRNA, the amino acid acceptor activity data suggest that for avian reticulocytes, cRNA is not derived from tRNA.

Investigation of Procedures Which Lead to Variability in cRNA Preparations. In the preceding section, we have shown that 3–4S RNA is readily obtained from avian reticulocyte chromatin by the CsCl procedure for cRNA isolation. Here we examine first some of the parameters which influence the nature of nucleic acids extracted from the pellicle and secondly the possible origins of chromatin-associated RNA of avian reticulocytes.

TABLE IV: Recovery of tRNA and Acceptor Activity after Pronase Digestion.[a]

Sample	% Recovered	% Original Acceptor Act.
Yeast tRNA	81.0, 89.3	77.4, 78.1
Reticulocyte tRNA	69.0, 65.2	92.0, 90.3

[a] Samples of yeast and reticulocyte tRNA were incubated with predigested Pronase as described in the text, and re-extracted with cold phenol. The amount of RNA recovered and the amino acid acceptor activity of tRNA before and after digestion were measured as described under Materials and Methods. The acceptor activity of tRNA samples after digestion is expressed as a percentage of the activity of the same amount of undigested material. Two separate digestions of each type of tRNA were performed.

Analysis of RNA Extracted Directly from Chromatin and the Pellicle. In the preparation of cRNA, incubation of the pellicle with Pronase digests associated protein. However, results in Table II indicate that incubation with Pronase is not necessary for the extraction of RNA in the pellicle. When either chromatin or the pellicle derived from it were extracted directly by the hot phenol procedure and the RNA analyzed on 5–20% sucrose gradients, it is apparent that the major component is rRNA with only a small amount of material in the 4S region (Figure 4A,B). The extract from the CsCl pellicle shows considerably more degradation, but 18S and 28S materials are still present. An extract of the pellicle derived from sonicated chromatin (Figure 4C) yields a profile identical with that of sonicated rRNA (Figure 3B). Artman and Roth (1971) also found rRNA as the major species of chromatin-associated RNA in several chicken tissues.

Since the majority of the RNA associated with avian reticulocyte chromatin is of high molecular weight, the low molecular weight RNA produced during isolation of cRNA is almost certainly a degradation product, and therefore the nuclease activity during Pronase digestion was investigated.

RNase Activity in Preincubated Pronase and the 4 M CsCl Pellicle. We have shown that preincubated Pronase does not degrade tRNA to any appreciable extent during incubation at 37° for 4 hr. However, when reticulocyte rRNA was incubated with Pronase under these conditions and mixtures reextracted by both the hot and cold phenol extraction methods, the material shown in Figure 3E,F was recovered. Two conclusions can be drawn from these results. Firstly, considerable degradation of the rRNA occurred, and material of low molecular weight was produced. Secondly, the cold phenol extraction procedure appears to preferentially extract low molecular weight RNA, since, after the Pronase digestion, the 4S component of the rRNA appears as the major product. This is reflected also in the recoveries of RNA after Pronase digestion: in the experiment described here, 68.5% with the hot phenol method compared to 45.9% for the cold phenol extraction. Any high molecular weight material remaining after Pronase digestion of the CsCl pellicle would presumably be extracted only in low yield by the cold phenol method.

If homogenized pellicle material which yielded the high molecular weight RNA shown in Figure 4B was incubated for

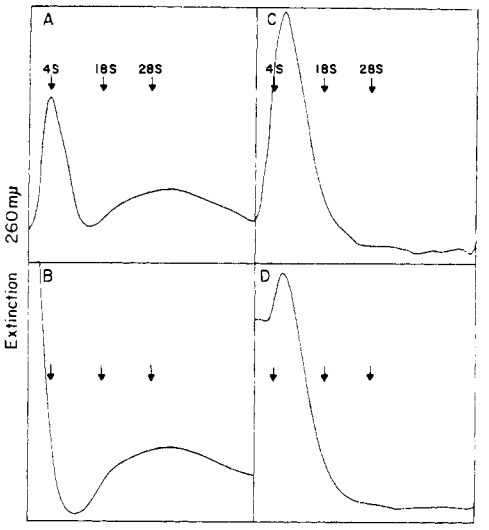

FIGURE 5: Sucrose gradient analysis of 4 M CsCl pellicle material from unsonicated chromatin after hot phenol extraction: (A) pellicle homogenized in 10 ml of 0.01 M Tris-Cl (pH 8.0) alone and incubated for 4 hr, 37°; (B) as for A, but also incubated with 10 μg of pancreatic RNase for 10 min, at 37° just prior to analysis; (C) pellicle incubated in predigested Pronase (4 mg/ml in 0.01 M Tris-Cl (pH 8.0), 4 hr, 37); (D) as for C, but also including pancreatic RNase digestion. Samples were analyzed as described in the legend to Figure 3.

4 hr at 37° in 0.01 M Tris-Cl (pH 8.0) and then extracted by the hot phenol method, only low molecular weight RNA was recovered (Figure 5A). The high molecular weight material in this gradient is DNA, as is shown by its insensitivity to RNase (Figure 5B).

Thus, endogenous RNase, as well as RNase present in Pronase, must be responsible for degradation of chromatin-associated RNA during the preparation of cRNA. The extent of degradation influences the proportion of RNA extracted by the hot and cold phenol procedures. The relative resistance of tRNA to ribonuclease attack is possibly due to its secondary structure.

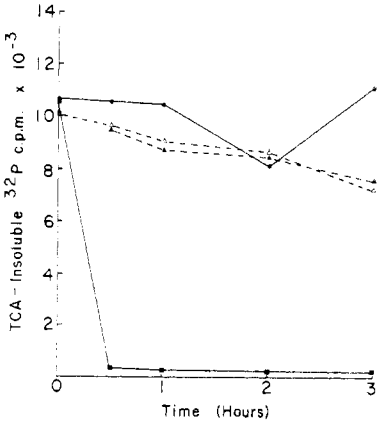

FIGURE 6: The DNase activity of preincubated Pronase solutions. Solutions (0.5 ml) of predigested Pronase (4 mg/ml, 8 mg/ml in 0.01 M Tris-Cl (pH 8.0) were assayed for their ability to produce Cl_3CCOOH-soluble fragments from 0.05 ml of ^{32}P-labeled λ DNA, as described under Materials and Methods: (Δ) Pronase, 4 mg/ml; (▲) Pronase, 8 mg/ml; (●) control incubation, containing 0.5 ml of 0.01 M Tris-Cl (pH 8.0) and 0.05 ml of ^{32}P-labeled λ DNA; (■) 0.05 ml of ^{32}P-labeled λ DNA incubated with 1 μg of pancreatic DNase I in 0.5 ml of 0.05 M Tris-Cl–0.025 M KCl–0.01 M $MgCl_2$ (pH 7.4).

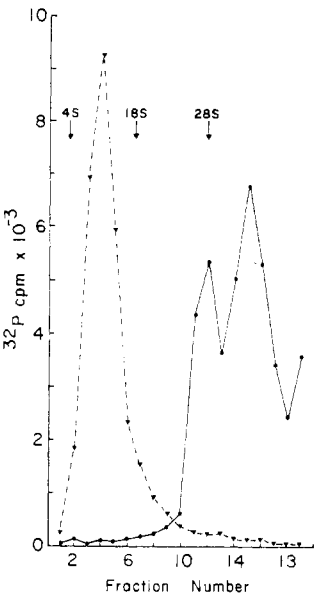

FIGURE 7: Sucrose gradient analysis of ^{32}P-labeled λ DNA after Pronase digestion. ^{32}P-Labeled λ DNA was incubated in predigested Pronase (4 mg/ml, in 0.01 M Tris-Cl (pH 8.0), reextracted with cold phenol, and analyzed on linear 5–20% sucrose gradients as described under Materials and Methods. Undigested ^{32}P-labeled λ DNA was also analyzed on a separate gradient. After centrifugation at 174,000*g* for 4 hr at 3°, 20-drop fractions of the gradients were collected, and the Cl_3CCOOH-insoluble radioactivity in each fraction was determined: (▼) Pronase-digested ^{32}P-labeled λ DNA; (●) undigested ^{32}P-labeled λ DNA.

DNase Activity in Preincubated Pronase. When chromatin or CsCl pellicles are extracted by the hot phenol method, only minimal amounts of DNA are extracted (1–10% of the total nucleic acid in the extract) and the DNA is of high molecular weight (see Figure 5A,B). However, when the CsCl pellicle is digested with Pronase and then extracted by the hot phenol method, considerable amounts of DNA are found in the extract, and all of it sediments as a well-defined peak at about the 10S region of the gradient (Figure 5C). Only about 20% of this extract is RNA, as is shown by the sucrose gradient profile (Figure 5D) after RNase digestion. This result indicates the presence of a DNase activity in Pronase, since extractions done on material which was not subjected to Pronase digestion yielded DNA of much greater size.

The DNase activity of preincubated Pronase was assayed using ^{32}P-labeled λ DNA as substrate (Figure 6). There was little release of Cl_3CCOOH-soluble radioactivity, even with Pronase concentrations of 8 mg/ml, whereas 1 μg of DNase 1 rendered the ^{32}P-labeled λ DNA completely Cl_3CCOOH-soluble in less than 30 min at 37°. When the ^{32}P-labeled λ DNA was incubated with 0.5 ml of preincubated Pronase (4 mg/ml, for 3 hr at 37°) and the mixture was reextracted with cold phenol and run on 5–20% linear sucrose gradients, all of the Cl_3CCOOH-insoluble radioactivity sedimented as a sharp peak in the 10S region of the gradient (Figure 7). The two peaks of labeled DNA not incubated with Pronase probably correspond to linear molecules and closed circles, with oligomers at the bottom of the gradient (Hershey *et al.*, 1963). The results are consistent with a restriction-type DNase activity of low specificity which degrades DNA into fragments sedimenting at about 10 S on sucrose gradients. Similar enzymes have been isolated from other bacterial sources (see Meselson *et al.*, 1972, for a review).

Content of rRNA and cRNA in Chromatin. Since the efficiency of extracting RNA from chromatin by the hot phenol method rarely exceeded 70% and since the cRNA extracted with cold phenol from the CsCl pellicle averaged only 25% of the total chromatin-associated RNA, we tried to eliminate the possibility that cRNA represented a separate class of RNA distinct from the high molecular weight RNA extractable from chromatin or the CsCl pellicle. This was done in the following way. Chromatin was prepared and was centrifuged through 4 M CsCl (no sonication was used). The pellicle was homogenized and divided into two. One-half was extracted directly with hot phenol (yielding high molecular weight rRNA) and the other half digested with Pronase before cold phenol extraction as for the preparation of cRNA. The recoveries of RNA were measured for all the extractions (Table V). When the recovery from direct extraction of the pellicle and the recovery after Pronase digestion are added and expressed as a percentage of the total RNA of each pellicle homogenate, the value is well in excess of 100%. Thus, the low molecular weight RNA extracted after Pronase digestion cannot be a separate class, but must be derived from the high molecular weight RNA which can be extracted directly from the pellicle.

TABLE V: Recoveries of RNA from CsCl Pellicles.[a]

	Expt 1	Expt 2
Yield after direct extraction of CsCl pellicle (%)	97.7	66.9
Yield after Pronase digestion of CsCl pellicle (%)	54.4	64.4
Total	151.1	131.3

[a] CsCl pellicles were prepared from unsonicated reticulocyte chromatin. The RNA content of the pellicle homogenate was measured. One-half of the homogenate was extracted directly with hot phenol, and the other half was digested with Pronase and then extracted with cold phenol. The yield of RNA from both extracts was measured and expressed as a percentage of the total RNA content of one-half of the CsCl pellicle.

Membrane Origin of RNA in Chromatin. The chromatin preparations used for the above experiments were not purified by centrifugation through 1.7 M sucrose. This treatment removes membranous material associated with chromatin (Marushige and Bonner, 1966). We have previously reported (Harlow *et al.*, 1972) that when avian erythroid chromatin is purified in this way, about 70% of the RNA in the chromatin is recovered in the membrane fraction, while the rest is found in the purified chromatin (see Table I). When purified chromatin and the membrane fraction are subjected to hot phenol extraction, both yield high molecular weight RNA containing 18S and 28S material (Figure 4D,E), indicating that ribosomes associated with membrane are the major source of chromatin associated RNA in avain reticulocytes. A specific class of membrane associated RNA, termed "membron" RNA, has also been reported (Shapot and Davidova, 1971), but it is difficult to eliminate the possibility that this RNA arises from degradation of rRNA. It is worth noting that cRNA isolated from rat ascites cells by Jacobson and Bonner (1968, 1971) was obtained from the membranous material separated from chromatin by centrifugation through 1.7 M sucrose.

Nature of cRNA Isolated by a Sodium Deoxycholate Procedure. The CsCl procedure for cRNA isolation can yield artefactually produced RNA fragments (Artman and Roth, 1971; Szeszak and Pihl, 1972; this work). Holmes *et al.* (1972) suggest that other cRNA isolation methods may overcome difficulties associated with the CsCl procedure. With the exception of the deoxycholate procedure (Mayfield and Bonner, 1971) the other methods cited involve long periods of centrifugation (Huang and Huang, 1969) or Pronase digestion (Jacobson and Bonner, 1971), thus increasing the chances of RNase action.

Direct phenol extraction of avian reticulocyte chromatin yields predominantly high molecular weight RNA. If the deoxycholate procedure does indeed maximize the chances of isolating cRNA as opposed to degradation products, then only a low percentage (see Figure 4A) of total chromatin-associated RNA should be isolated as "deoxycholate cRNA." However, in two separate preparations, 36.0 and 57.8% of the total RNA of chromatin was recovered as low molecular weight fragments eluting at 0.55 M NaCl on DEAE-Sephadex chromatography. When analyzed on sucrose gradients, the RNA showed an identical profile with that of cRNA obtained by the CsCl procedure. The RNA extracts contained less than 10% by weight protein, suggesting that most, if not all, of the RNA was *not* covalently bound to protein. Neither dihydrouridine nor dihydroribothymidine were found at levels of detection of 0.33 mol %.

It is thus clear that this procedure for cRNA isolation also results in the recovery of small RNA pieces which must be derived from the higher molecular weight RNA present in avian reticulocyte chromatin.

Discussion

Low molecular weight RNA is readily isolated from the chromatin of avian reticulocytes by two published procedures for the preparation of cRNA. However, the RNA is clearly heterogeneous and is not characterized by a high content of saturated pyrimidine, or by covalent attachment to proteins, as reported for cRNA from other tissues (Bonner and Widholm 1967; Huang, 1967; Jacobson and Bonner, 1968, 1971; Dahmus and McConnell, 1969; Shih and Bonner, 1969; Dahmus and Bonner, 1970; Getz and Saunders, 1970; Mayfield and Bonner, 1971, 1972). The major part of the RNA associated with avian reticulocytes is high molecular weight material, predominantly rRNA, as has also been reported by Artman and Roth (1971). We have previously reported that both RNA and nonhistone proteins of avian erythroid chromatin behave as membrane-associated entities, rather than DNA-associated moieties *in vitro* (Harlow, *et al.*, 1972). It is therefore likely that rRNA present in chromatin preparations comes from ribosomes bound to membranes. Ribosome attachment to membranes *in vitro* and *in vivo* is well documented (Schreml and Burka, 1968; Burka and Schickling, 1970; Attardi *et al.*, 1969; Rosbash and Penman, 1971a,b). It is shown here (Figure 4D,E) that high molecular weight RNA exists in membrane derived from avian erythroid chromatin and other data suggest that sufficient membrane is also present in purified chromatin to account for levels of RNA found in preparations pelleted through 1.7 M sucrose (Harlow *et al.*, 1972). It is significant that Jacobson and Bonner (1971) have also prepared cRNA from material remaining at the interface when crude chromatin from rat ascites cells is centrifuged into a sucrose barrier.

Investigation of the CsCl procedure for cRNA isolation shows that RNA (and also DNA) is nonspecifically trapped among the proteins floating on 4 M CsCl. Similar observations

have been reported by others (Artman and Roth, 1971; Szeszak and Pihl, 1972; Holmes *et al.*, 1972). Degradation of RNA can then occur due to RNase activity in Pronase, or in the 4 M CsCl pellicle material. Sonication of chromatin prior to centrifugation through 4 M CsCl can also degrade high molecular weight RNA. Use of the deoxycholate procedure for cRNA isolation results in the recovery of a large part of the chromatin-associated RNA as 3–4S material. The deoxycholate treatment presumably releases ribosomes bound to membranes present in the chromatin, and rRNA is then degraded by endogenous RNase activity during the subsequent isolation procedures.

During the preparation of chromatin, there is ample opportunity for cellular RNA (and other components) to associate with the DNA–protein complex, and the predominant species of RNA associated may vary depending on the tissue and the method used for chromatin preparation. The cRNA isolated from chromatin is defined by a series of experimental steps rather than by a functional assay. Without wishing to minimize the difficulties of such an assay it is nevertheless important to ask whether cRNA can be considered to be of general occurrence in eukaryotic cells and to have properties which distinguish it as a distinct class of RNA. In view of our findings and those of others (Artman and Roth, 1971), it is likely that 3–4S cRNA is predominantly an *in vitro* degradation product of other RNA species. If this is so, the precise nature of the original chromatin-associated RNA is not of great significance. The report of Heyden and Zachau (1971) is therefore significant in pointing to RNase activity in Pronase (used in the isolation of cRNA) rather than for the claim that cRNA of calf thymus is derived from tRNA. Since these authors used a selective tritium-labeling technique (NaB^3H_4), only RNA containing unusual bases (dihydrouridine) would be detected and the possibility of cRNA arising from other types of chromatin-associated RNA still exists. Our results of unusual base analyses and amino acid acceptor activities of RNA fractions argue against the notion of cRNA as a degradation product of tRNA in avian red cells.

Certain unique properties attributed to cRNA are incompatible with the suggestion that this RNA is an *in vitro* degradation product. First, a covalent linkage to chromosomal protein has been claimed for rat ascites cRNA (Jacobson and Bonner, 1971) and for chick embryo cRNA (Huang, 1967; Marzluff *et al.*, 1972), but cRNA isolated from rat liver and kidney (Mayfield and Bonner, 1971) has only low levels of protein associated with it. Other results argue against the proposed covalent linkage: those of Artman and Roth (1971) in which inclusion of urea in CsCl solutions was effective in release of RNA from the pellicle and results presented here (Table II) in which as much or more RNA was obtained from the pellicle in the absence of prior Pronase digestion. Secondly, reports of high levels of saturated pyrimidines in cRNA preparations must be treated with some caution, as urea contamination can lead to false estimates of uriedo-positive material in RNA samples. It is not clear from the report of Jacobson and Bonner (1968) whether estimation of uriedo-positive material was used to estimate the saturated pyrimidine content of rat ascites, pea bud, and calf thymus cRNA. However, this method was used for rat liver and kidney cRNA (Mayfield and Bonner, 1971) while for chick embryo cRNA (Huang, 1967; Marzluff *et al.*, 1972) the method of estimation was not stated. Estimation of specific hydrolysis products of saturated pyrimidines seems necessary to measure the levels of these bases in cRNA preparations (Tolstoshev and Wells, 1973). We find no evidence for these unusual bases in cRNA from nondividing reticulocytes (CsCl or deoxycholate methods) or from rapidly dividing mouse ascites cells. Thirdly, the hybridization behavior of cRNA from several sources (Bonner and Widholm, 1967; Bekhor *et al.*, 1969; Dahmus and McConnell, 1969; Mayfield and Bonner, 1971; Sivolap and Bonner, 1971) could be explained by fragments of heterogeneous nuclear RNA present in cRNA preparations. In fact, a precursor–product relationship between HnRNA and cRNA in regenerating rat liver has been proposed (Mayfield and Bonner, 1972) but the possibility of artefactual degradation of HnRNA is difficult to eliminate. Montecuccoli *et al.* (1972) have reported that synthesis of cRNA from rat liver is inhibited by α-amanitin. The short labeling period (20 min) used by these authors presumably allows preferential labeling of rapidly synthesized species of RNA. As previously mentioned, the nature of RNA associated with chromatin may vary from one tissue to another.

It is still an open question whether eukaryotic chromosomes contain structural RNA elements (analogous to that suggested for the *Escherichia coli* folded chromosomes; Stonington and Pettijohn, 1971; Worcel and Burgi, 1972) or "activator RNA" (Britten and Davidson, 1969). Avian reticulocyte chromatin *in vitro* contains predominantly rRNA and it is shown that there is insufficient total chromatin-associated RNA to account for both rRNA obtainable by direct extraction and cRNA obtainable from the same chromatin preparation by the CsCl procedure.

Although the results presented here do not eliminate the possibility of RNA molecules with regulatory functions in chromatin, it is clear that avian erythroid cRNA as defined by "CsCl" or "deoxycholate" preparative procedures is predominantly a degradation product and, as such, does not possess unique characteristics attributed to some other cRNA preparations. Although our interests are primarily concerned with the avian erythroid cell series, the fact that chromatin-associated RNA degradation occurs as a result of the experimental procedures used in the isolation of cRNA suggests that the findings reported here have relevance to studies on cRNA from other tissues.

Acknowledgments

We wish to thank Mr. R. Harlow, Dr. R. Appels, and Dr. R. H. Symons for valuable discussons and suggestions. We also thank Mr. M. Smith for the gift of ^{32}P-labeled λ DNA. The technical assistance of Mrs. J. Dinan and Miss A. Heylen is gratefully acknowledged.

References

Appels, R., Wells, J. R. E., and Williams, A. F. (1972), *J. Cell Sci. 10*, 47.

Arnold, E. A., and Young, K. E. (1972), *Biochim. Biophys. Acta 269*, 252.

Artman, M., and Roth, J. S. (1971), *J. Mol. Biol. 60*, 291.

Attardi, B., Cravioto, B., and Attardi, G. (1969), *J. Mol. Biol. 44*, 47.

Bekhor, I., Bonner, J., and Dahmus, G. K. (1969), *Proc. Nat. Acad. Sci. U. S. 62*, 271.

Bhaduri, S., Chatterjee, N. K., Bose, K. K., and Gupta, N. K. (1970), *Biochem. Biophys. Res. Commun. 40*, 402.

Bonner, J., and Widholm, J. (1967), *Proc. Nat. Acad. Sci. U. S. 57*, 1379.

Britten, R. J., and Davidson, E. H. (1969), *Science 165*, 349.

Burka, E. R., and Schickling, L. F. (1970), *Biochemistry 9*, 459.

Burton, K. (1956), *Biochem. J. 62*, 315.
Ceriotti, G., and Spandrio, L. (1963), *Clin. Chim. Acta 8*, 295.
Dahmus, M. E., and Bonner, J. (1970), *Fed. Proc., Fed. Amer. Soc. Exp. Biol. 29*, 1255.
Dahmus, M. E., and McConnell, D. J. (1969), *Biochemistry 8*, 1524.
Dingman, C. W., and Sporn, M. B. (1964), *J. Biol. Chem. 239*, 3483.
Dische, Z. (1955), *in* The Nucleic Acids, Chargaff, E., and Davidson, J. N., Ed., Vol. 1, New York, N. Y., Academic Press, pp 285–305.
Fleck, A., and Monro, H. N. (1962), *Biochim. Biophys. Acta 55*, 571.
Getz, M. J., and Saunders, G. F. (1970), *Fed. Proc., Fed. Amer. Soc. Exp. Biol. 29*, 671.
Harlow, R., Tolstoshev, P., and Wells, J. R. E. (1972), *Cell Differentiation 1*, 341.
Hershey, A. D., Burgi, E., and Ingram, L. (1963), *Proc. Nat. Acad. Sci. U. S. 49*, 748.
Heyden, H. W., and Zachau, H. G. (1971), *Biochim. Biophys. Acta 232*, 651.
Hill, R. J., Poccia, D. L., and Doty, P. (1971), *J. Mol. Biol. 61*, 445.
Holley, R. W., Apgar, J., Doctor, B. P., Farrow, J., Marini, M. A., and Merrill, S. H. (1961), *J. Biol. Chem. 236*, 200.
Holmes, D. S., Mayfield, J. E., Sander, G., and Bonner, J. (1972), *Science 177*, 72.
Huang, R. C. C. (1967), *Fed. Proc., Fed. Amer. Soc. Exp. Biol. 26*, 603.
Huang, R. C. C., and Bonner, J. (1965), *Proc. Nat. Acad. Sci. U. S. 54*, 960.
Huang, R. C. C., and Huang, P. C. (1969), *J. Mol. Biol. 39*, 365.
Jacobson, R. A., and Bonner, J. (1968), *Biochem. Biophys. Res. Commun. 33*, 716.
Jacobson, R. A., and Bonner, J. (1971), *Arch. Biochem. Biophys. 146*, 557.
Lowry, O., Rosebrough, N. J., Farr, A. L., and Randall, R. T. (1951), *J. Biol. Chem. 193*, 265.
Marushige, K., and Bonner, J. (1966), *J. Mol. Biol. 15*, 160.
Marzluff, W., Smith, M. M., and Huang, R. C. C. (1972), *Fed. Proc., Fed. Amer. Soc. Exp. Biol. 31*, 496.
Mayfield, J. E., and Bonner, J. (1971), *Proc. Nat. Acad. Sci. U. S. 68*, 2652.
Mayfield, J. E., and Bonner, J. (1972), *Proc. Nat. Acad. Sci. U. S. 69*, 7.
McGrath, D. I., and Shaw, D. C. (1967), *Biochem. Biophys. Res. Commun. 26*, 32.
Meselson, M., Yuan, R., and Heywood, J. (1972), *Annu. Rev. Biochem. 41*, 447.
Montecuccoli, G., Novello, F., and Stirpe, F. (1972), *FEBS (Fed. Eur. Biochem. Soc.) Lett. 25*, 305.
RajBhandary, U. L., and Ghosh, H. P. (1969), *J. Biol. Chem. 244*, 1104.
Rosbash, M., and Penman, S. (1971a), *J. Mol. Biol. 59*, 227.
Rosbash, M., and Penman, S. (1971b), *J. Mol. Biol. 59*, 243.
Schreml, W., and Burka, E. R. (1968), *J. Biol. Chem. 243*, 3573.
Shapot, V. S., and Davidova, S. Y. (1971), *Progr. Nucl. Acid Res. Mol. Biol. 11*, 81.
Shih, T. Y., and Bonner, J. (1969), *Biochim. Biophys. Acta 182*, 30.
Sivolap, Y. M., and Bonner, J. (1971), *Proc. Nat. Acad. Sci. U. S. 68*, 387.
Stevenson, C. T., and Zamecnik, P. C. (1963), *Proc. Nat. Acad. Sci. U. S. 47*, 1627.
Stonington, O. G., and Pettijohn, D. E. (1971), *Proc. Nat. Acad. Sci. U. S. 68*, 6.
Szeszák, F., and Pihl, A. (1972), *FEBS (Fed. Eur. Biochem. Soc.) Lett. 20*, 177.
Tolstoshev, P., and Wells, J. R. E. (1973), *Biochem. Biophys. Res. Commun. 51*, 223.
Williams, A. F. (1972), *J. Cell Sci. 10*, 27.
Worcel, A., and Burgi, E. (1972), *J. Mol. Biol. 71*, 127.