N° 144 Arch. Biol. Med. Exper. 11: 97-103, 1978

Polyadenylic acid-rich RNA in rat bone marrow cells

Presencia de RNA rico en ácidos poliadenílicos en las células de la médula ósea de la rata

ALFONSO VALENZUELA B., EUGENIO SPENCER O., MARCO PERRETTA P.

Instituto de Nutrición y Tecnología de los Alimentos (INTA) Universidad de Chile, Casilla 15138
Santiago 11, Chile

(Recibido para publicación el 8 de enero de 1976)

VALENZUELA, A.; SPENCER, E.; PERRETTA, M. Polyadenylic acid-rich RNA in rat bone marrow cells. (Presencia de RNA rico en ácidos poliadenílicos en las células de la médula ósea de la rata). Arch. Biol. Med. Exper. *11*:97-103, 1978.

The RNA isolated from rat bone marrow cells gives characteristic sedimentation profiles in a wide range from 85 S to 4 S. The cellulose chromatography elution profiles are characterized by a RNA fraction without poly A and by a RNA fraction with poly A sequences.

The polyadenylate-rich portion is present in 85 S, 72 S and 17 S, RNA, while in the 58 S 45 S, 28 S and 18 S RNA it is absent, 9 S and 4 S RNA species with and without poly A regions in their molecules have been detected.

The presence of the poly-A rich regions in high molecular weight RNA and in functional RNA, like the 9 S RNA, suggest a metabolic pathway that can explain the origin of messenger RNA in bone marrow cells.

HETEROGENOUS NUCLEAR RNA BONE MARROW POLY A RNA RNA MATURATION

The study of the high molecular wight precursors of functional RNA that participate in protein synthesis has become an increasingly important issue in the understanding of eukaryotic cells metabolism (1, 2). The precursors RNA have some common properties such as high molecular weight, short halflife, heterogeneous base composition and they are confined exclusively to the cell nucleus. They have been denominated under a variety of names, e.g. heterogeneous nuclear RNA (HnRNA) by Penman (3) and Perry (4), giant nascent RNA and nuclear nascent RNA by Scherrer (1), and giant D-RNA by Samarina *et al.* (5). We will refer to them as HnRNA.

It is believed that when HnRNA are processed by specific enzymes they can originate functional RNA, which are then able to reach the cytoplasm as such or under the form of higher precursors (1). In this mechanism hormones may be involved (6, 7).

The messenger RNA of eukaryotic cells, with the exception of histones mRNA (8), have short polyadenylic sequences of 100 to 150 nucleotides long, covalently bounded to the free 3'-hydroxyl group of the RNA chain. These poly A sequence are not present in ribosomal and transfer RNA, neither in their known precursors (9).

The presence of HnRNA has been reported in bone marrow by several authors. Scherrer (1) has described a giant RNA of $5\text{-}10 \times 10^6$ MW in duck erythroblasts that serves as a carrier of genetic information from DNA to the functional 9 S RNA, the globin messenger RNA; Gross and Goldwasser (10) have reported the synthesis of a 150 S RNA after the action of erythropoietin in rat bone marrow cells *in vitro,* while Perretta *et al.* (11) have suggested the appearance of precursor RNA with informational properties under the effect of erythropoietin in rat bone marrow cells *in vivo.*

The properties and characteristics of 9 S globin mRNA are well known, but the intermediary molecules formed from the giant RNA molecules until the apparition of the functional mRNA are not full identified yet.

The experiments described in this paper were undertaken to obtain information about the existence and relationships between the HnRNA with poly A sequences and the formation of 9 S RNA in rat bone marrow cells. Thus we have been able to show that in bone marrow cells there exist several RNA species with sedimentation coefficients higher than 45 S that contain poly A sequences in their structure. These RNA may be separated from the bulk RNA by chromatography in cellulose columns and by nitrocellulose filfration.

MATERIALS AND METHODS

Chemicals

Cellulose Sigmacell type 19, DNase RNase — free and bovine pancreatic RNase type II were purchased from Sigma Chemical Co (^{14}C) formate (S.A. 20 μCi/mMol), PPO and liquifluor were purchased from New England Nuclear Millipore filters type HA 0,45 were obtained from Millipore Filters Corp. Sucrose (density gradient grade, RNase free) was from Schwartz Bio-research. E. coli 23 S rRNA was purchased from Miles Res., Labs.

Extraction and incubation of bone marrow cells

Ten male rats of the "Wistar" strain were used, with weights ranging from 250 to 260 g. Bone marrow cells were removed from pooled femurs and tibias and washed with ice-cold saline as previously described (12). The cellular pellet was suspended in 12 ml of 1 mM Tris-HCl buffer (pH 7.4) containing 0.25 M sucrose, 5 mM glucose, 1 mM $MgCl_2$ and 50 uC of (^{14}C) formate. The cell suspension was incubated for 30 min, at 37 °C with continuous agitation and then collected by centrifugation at 600 × g for 10 min. The cell pellet was washed three times with the same buffer (without the isotope) to eliminate the excess of (^{14}C) formate.

RNA extraction

The cellular pellet thus obtained was disrupted by suspension in 50 mM sodium acetate buffer (pH 5.2) containing 5% sodium dodecyl sulphate. A 0.5 ml volume of a 6% bentonite suspension in acetate buffer was added to the mixture. RNA was then extracted using Kirby's method (13) modified as follows: Both the interphase and aqueous phase obtained after phenol: chloroform extraction at 54 °C were reextracted once with an equal volume of phenol: chloroform (1:1 v/v) containing 1% (v/v) isoamyl alcohol and three times with chloroform containing 1% (v/v) isoamyl alcohol. In each of these reextractions a cycle of homogenization, heating (at 54 °C for 5 min), cooling and centrifugation (27,000 × g for 10 min at 2 °C) was worked out. The aqueous phase was treated with 200 μg of DNase for 30 min at 37°C and the extractions with phenol: chloroform were repeated in order to eliminate the enzyme. The final aqueous phase obtained was treated according to Kirby's method and the RNA thus extracted was stored at −20° C. As judged by Lowry's method, the final RNA preparation was free of protein.

RNA sucrose gradient centrifugation

Sucrose gradients (5-20% w/w) were prepared from ribonuclease-free sucrose solutions in 10 mM Tris-HCl buffer (pH 7.4) containing 1 mM EDTA and 10 mM NaCl, in nitrocellulose tubes using a Buchler density gradient system. After overnight equilibration, 300 μg of RNA were layered on the top of the gradient and the charged tubes were ultracentrifuged at 105,000 × g at 2° C. At the end of the run, the tubes were pierced at the bottom and 40 fractions (0.12 ml each) were collected. To each fraction 0.88 ml of the gradient buffer was added and after measuring absorbance at 260 nm, an aliquot was taken for radioactivity measurement.

The sedimentation coefficients were calculated with the Mc Ewen tables (14); the computed values agreed well with the sedimentation profiles obtained with the *E. coli* 23 S rRNA.

RNA fractionation by cellulose chromatography

RNA was fractionated in a microcrystalline cellulose column by using the methods of Schutz *et al.* (15) and Delarco *et al.* (17). 300 absorbance units (260 nM) of RNA dissolved in Tris-HCl-saline buffer (pH 7.4) containing 500 mM KCl and 0.2 mM $MgCl_2$ were applied to a column containing 2 cm^3 of cellulose and eluted with the same buffer until the absorbance at 260 nm fell to 0.02 units: at this point a volume of 15 ml was collected which was inmediatly precipitated with 2 vol of cold absolute ethanol. The pellet formed was separated by centrifugation and is named throughout the work as RNA fraction S.

The column was then washed with 15 ml of distilled water (pH 7.4) and the eluted treated as above. The pellet thus obtained is named throughout the work as RNA fraction A. All procedures were carried out at 4° C.

The RNA fraction S and A were ultracentrifuged in sucrose density gradients according to the conditions described above and the RNA species thus separated were used for identification and determination of its poly A content.

(a) *Identification of poly A sequences*

Poly A sequences in "RNA fraction S and A" were identified using the method described by Mendecki *et al.* (18), but employing thin layer chromatography for adenine identification instead of paper chromatography. Thin layer chromatography was performed using microcrystalline cellulose as support and K_2HPO_4 (pH 5.2) as running solvent.

(b) *Determination of poly A content*

The fractions obtained after ultracentrifugation were diluted to 2 ml with Tris-saline buffer and incubated for 30 min at 37° C with 0.2 ml of a solution containing pancreatic

bovine ribonuclease (100 μg/ml) and T_1 ribonuclease (5 unit/ml) in Tris-saline buffer. After incubation, aliquots (0.1 ml) were taken for radioactivity determination and the rest of the incubate (2.1 ml) was passed through nitrocellulose filtres as described by Brawerman (16). The filters were washed twice with Tris-saline buffer and counted. An aliquot (0.5 ml) of each filtrate was counted for radioactiviy.

Miscellaneous procedures

Absorbance was determined in a Beckman-Gilford spectrophotometer at 260 nm using 1 ml quartz cuvettes.

Radioactivity was measured in a Nuclear Chicago spectrometer model Mark I using a scintillator mixture of 4 gr of PPO, 400 ml of ethanol and 600 ml of toluene for liquid samples and a scintillator mixture of Liquifluor-Toluene for Millipore filters.

The sucrose gradient centrifugations were performed in a Beckman L2-65B ultracentrifuge using a SW-39 rotor.

RESULTS

RNA sucrose gradient centrifugation

Total RNA extracted from bone marrow cells was subjected to ultracentrifugation through sucrose gradients to identify the different RNA species. The radioactivity and absorbance profiles are shown in figure 1. It can be seen that the preparation sedimentates in a wide range. In the high molecular weight range of the gradient three species can be detected showing sedimentation coefficients of 85 S, 72 S and 58 S. In the center of the gradient RNA species with sedimentation coefficient ranging from 45 S to 18 S can be detected that correspond to the precursor ribosomal RNA (45 S and 32 S) and to the typical ribosomal RNA components (28 S and 18 S). In the low molecular weight

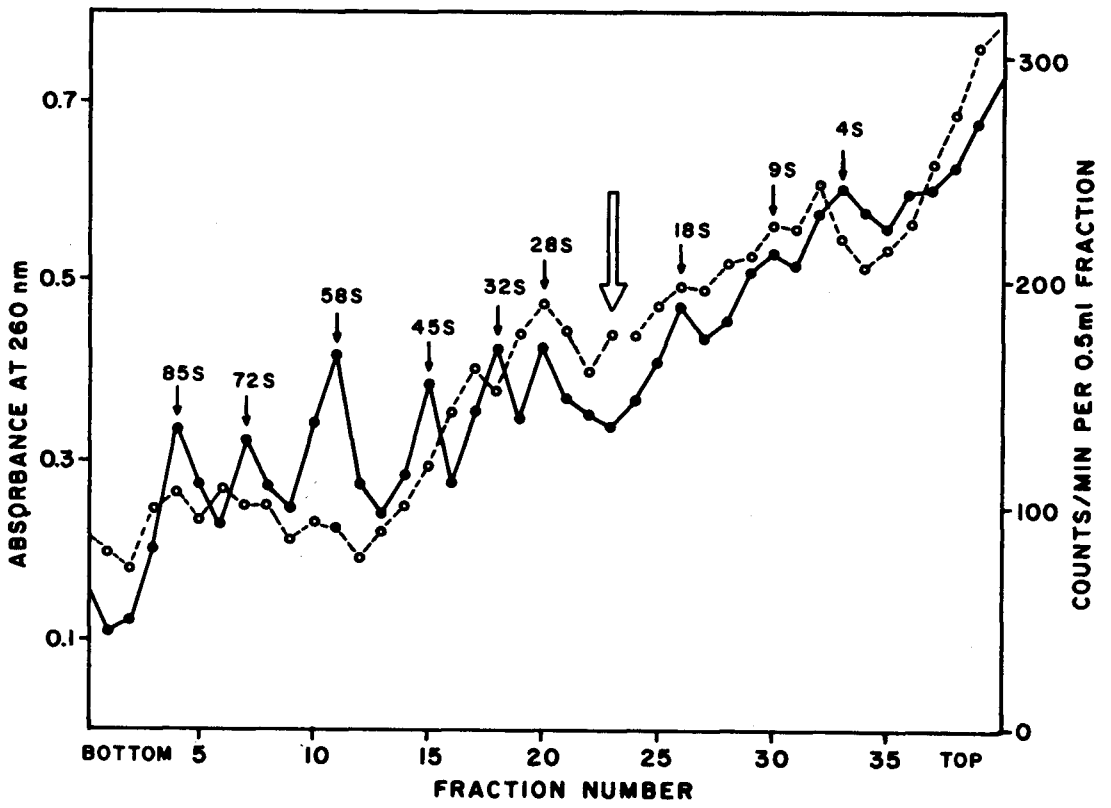

Figura 1. Sucrose gradient centrifugation of rat bone marrow RNA. The arrow shows the position of 23 S *E. coli* rRNA, used as sedimentation marker. Conditions were as described in Materials and Methods.

o____ o Absorbance measured at 260 nm.

• ____ • ^{14}C-radioactivity.

range, both 9 S and 4 S RNA are highly noticiable, in spite of the great heterogeneity observed in this part of the gradient.

RNA fractionation by cellulose Chromatography

After it characterization by sedimentation analysis, the bone marrow RNA was subjected to a fractionation on cellulose columns in an attempt to identify the presence of species containing poly A in their structure. The elution profile of an RNA sample is shown in figure 2. Both, the absorbance and the radioactivity profiles show the presence of two RNA fractions: one that elutes at high ionic strength (RNA fraction S) and other that elutes at very low ionic strength (RNA fraction A). According to Schutz *et al.* (15) it is assumed that RNA fraction S contains RNA species without poly A residues, while RNA fraction A is rich in species with poly A sequences in its structure.

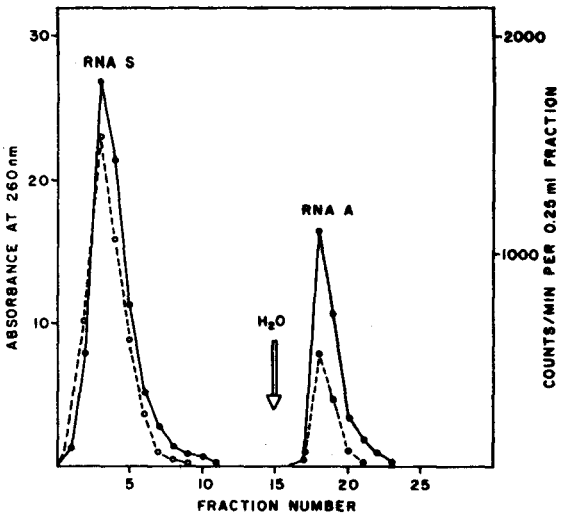

Fig. 2. Cellulose chromatography of rat bone marrow RNA. Conditions were as described in Materials and Methods.
o ____ o Absorbance measured at 260 mm.
• ____ • ^{14}C-radioactivity.

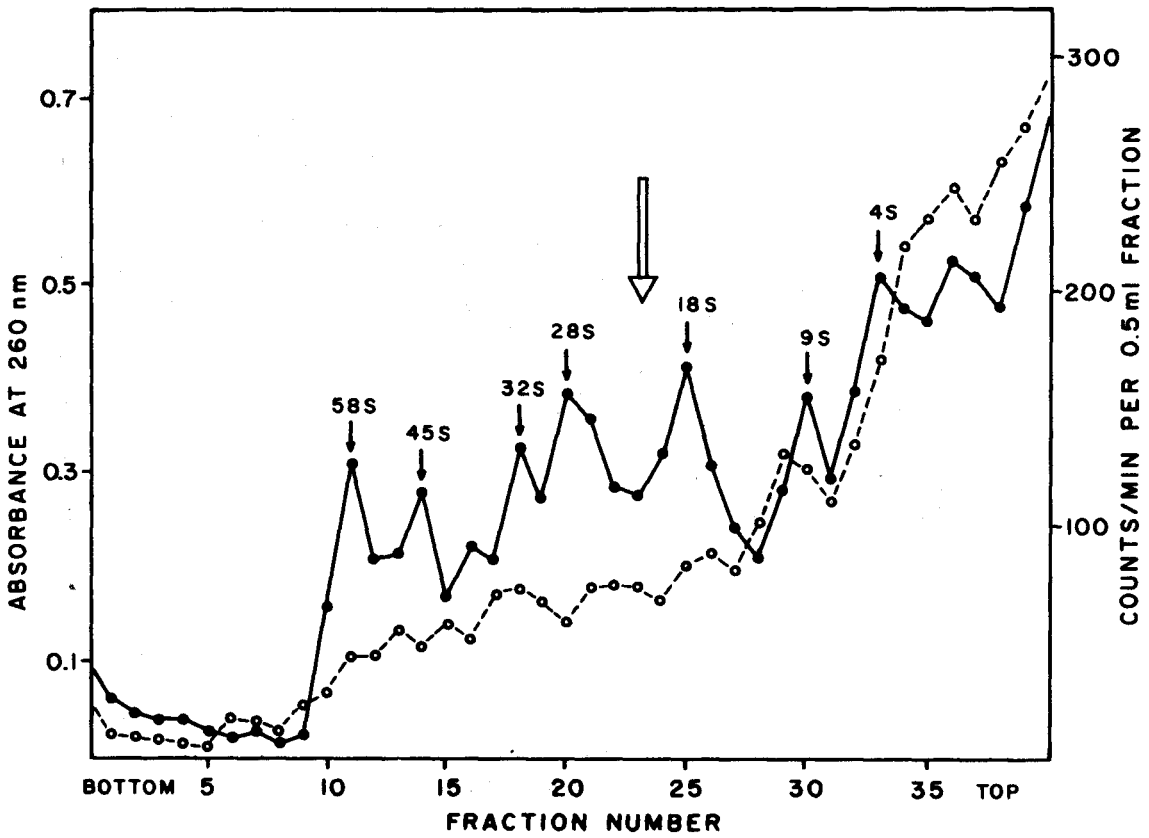

Fig. 3. Sucrose gradient centrifugation of RNA fraction S. The arrow snows the position of 23 S *E. coli* rRNA, used as sedimentation marker. Conditions were as described in Materials and Methods.
o ___ o Absorbance measured at 260 nm.
• ____ • ^{14}C-radioactivity.

Sucrose gradient centrifugation of RNA fraction S and A

In figure 3, the sedimentation profile obtained after sucrose gradient centrifugation of RNA fraction S is shown. If, this sedimentation profile is compared with the one obtained from the bulk RNA (fig. 1), the absence of RNA species with sedimentation coefficients of 85 S and 72 S is highly remarkable. However, RNA species with sedimentation coefficients lower than 72 S are still present in this fraction.

Similar analysis was performed with RNA fraction A and results presented in figure 4, demonstrate that this RNA fraction, as compared with the bulk RNA, lacks RNA species with sedimentation coefficients of 58 S, 45 S, 28 S and 18 S.

Although, 18 S RNA is not present in this fraction it is important to mention the appearance of a new specie (17 S) not observed in the bulk RNA and whose significance should be commented later.

Determination of poly A content in RNA fraction S and A

In figure 5, the poly A content in the RNA species separated by sucrose gradient centrifugation from RNA fraction S and A is shown. The high poly A content in RNA species from RNA fraction A is notorious, while the poly A content in RNA species from RNA fraction S is scarce and represents inespecific binding to Millipore filters (19).

The fact that there is a good correlation on the poly A content and on the radioactivity profile of RNA species in fraction A (see figure 4 and 5), strongly suggests that RNA fraction

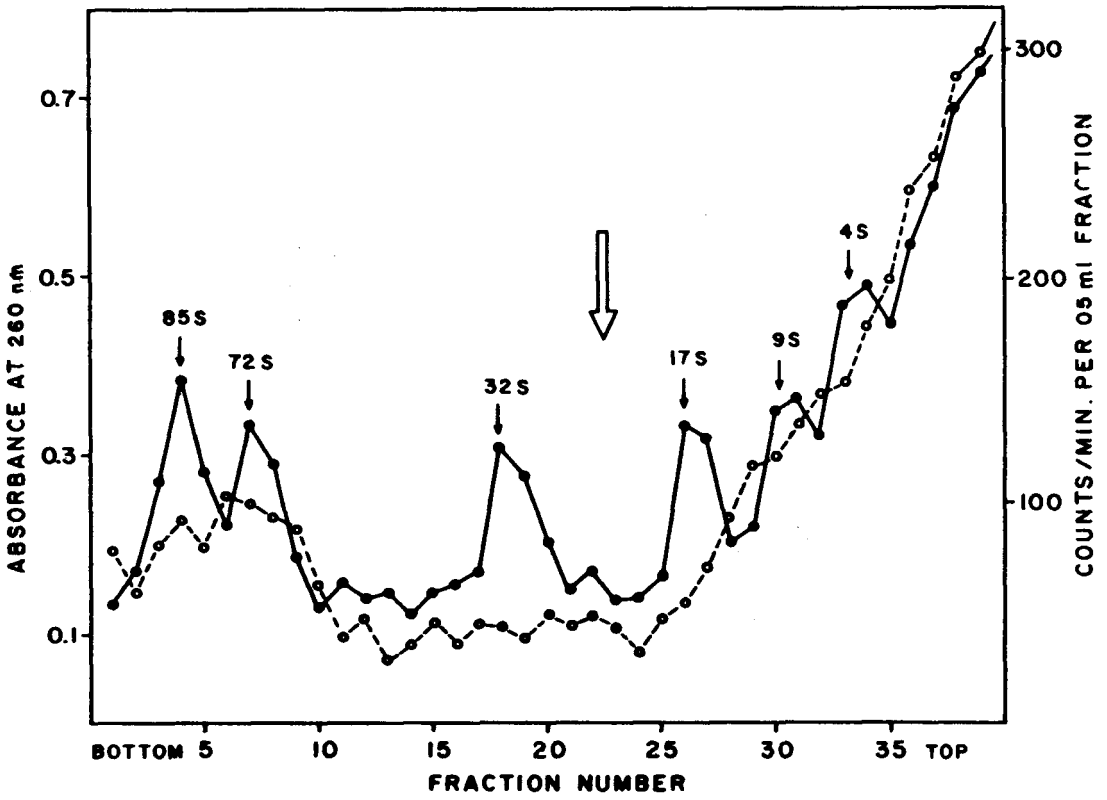

Fig. 4. Sucrose gradient centrifugation of RNA fraction A. The arrow shows the position of 23 S *E. coli* rRNA, used as sedimentation marker. Conditions were described in Materials and Methods.
o _ _ _ o Absorbance measured at 260 mm.
• ____ • ^{14}C-radioactivity.

A has several species, containing polyadenylic sequences in their structures.

The above assumption was proved to be correct by the thin layer chromatography analysis of the poly A moiety from RNA fractions S and A. The results, not presented here, shown that RNA fraction A contains approximately 95% more adenine than RNA fraction S.

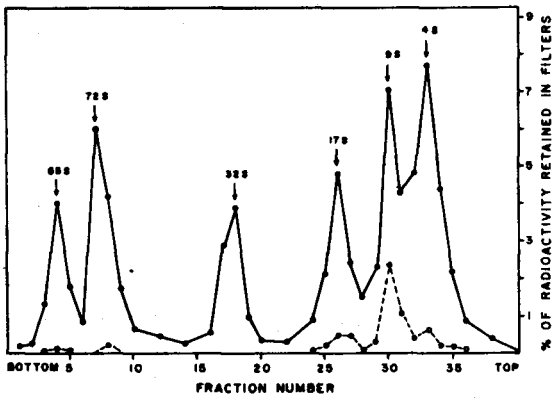

Fig. 5. Poly A content of RNA species derived from RNA fractions S and A after sucrose gradient centrifugation. Conditions were as described in Materials and Methods.
o___o Poly A percentage in RNA fraction S.
°____• Poly A percentage in RNA fraction A.
The results are expressed as percentage of radioactivity retained by filter $\frac{\text{counts/min in Filter}}{\text{counts/min in sample before Filtration}}$

DISCUSSION

The results presented in this paper draw attention to the following general conclusions: *(1)* RNA from bone marrow cells gives characteristics sedimentation and cellulose chromatography elution patterns which permit the identification of the several RNA species present in this tissue. *(2)* The poly A content, detected in RNA species, may serve as an indicator of the presence of mRNA or some of its precursors. *(3).* Cellulose chromatography proves to be a valuable tool for studing some aspects of RNA processing in eukaryotic cells.

Considering that all the steps involved in RNA maturation are open to speculation, it is conceivable to give some explanations for the data obtained including: *(1)* The 85 S RNA may correspond to the HnRNA described by Scherrer (1) in duck erythroblasts and might occur in bone marrow cells during the first stages of the differentiation process. *(2)* The 72 S RNA may represent the first or one of the earliest RNA products in the maturation process. *(3)* Both 85 S and 72 S RNA contain poly A sequences in their structure. *(4)* The 58 S RNA does not contain poly A and its presence can be explained assuming that it could be the RNA precursor for histone mRNA, also it could be a HnRNA in which the poly A region has not been incorporated yet or it could be a RNA with regulatory functions not related with protein synthesis (26). *(5)* The presence of 85 S, 72 S, 32 S, 17 S and 9 S RNA, all containing poly A residues in their molecules, suggest a role for these molecules as intermediate in the maturation process related with the origin of mRNA; while the existence of 45 S, 32 S, 28 S and 18 S RNA species without poly A in their structure, may characterize the genesis of ribosomal RNA. *(6)* The 9 S RNA detected in RNA fraction S could be the globin messenger that has lost most of its poly A residues. This explanation is based on the idea of Sheines and Darnell (20) that part of the poly A portion is cleaved off from mRNA during translation. Another support to this argument is the finding of a cytoplasmic ribonuclease which cleaves the poly A chain of RNA (21). *(7)* The existence of poly A in high percentages in 4 S RNA, isolated from RNA fraction A, is explained by the fact that it may be synthetized by a nuclear or a cytoplasmic poly A synthetase (22, 23, 24, 25) thus forming a poly A pool whose size could be increased by residues derived from the enzymatic splitting of poly A containing RNA. *(8)* The 4 S RNA isolated from RNA fraction S may correspond to tRNA.

Experiments are now in progress to know whether the different stage on the maturation of HnRNA, are induced by erythropoietin, the factor that triggers and makes possible differentiation in bone marrow cells.

ACKNOWLEDGEMENTS

This work was supported in part by "Comisión Nacional de Investigación Científica y Tecnológica de Chile" (Grant N° 204) and "Oficina Técnica de Desarrollo Científico y Creación Artística de la Universidad de Chile" (Grant N° 174). The authors gratefully acknowledge the stimulant interest and helpful discussion of Dr. José Minguell and the excellent technical assistance of Mr. Jorge Merino.

REFERENCES

1. SCHERRER, K., Karolinska Symposium on Research Methods in Reproductive Endocrinology. Stockholm, 1973, E. Diczfalusy (Ed.). Karolinska Institutet, 1973, p. 95.
2. WEINBERG, R.A., Ann. Rev. Biochem. *42*:329, 1973.
3. PENMAN, S., SCHERRER, K., BECKER, Y., DARNELL, J. E., Proc. Natl. Acad. Sci. USA. *49*:654, 1963.
4. PERRY, R., GREEMBERG, J., KELLEY, D., LATORRE, J., A. Hollaender (Ed). Gene Expression and its Regulations, vol. 1, Plenum Press USA., 1973, p. 199.
5. SAMARINA, O., LIKAMIDIN, E., MOLNAR, J., GEORGIEV, P., J. Mol. Biol. *33*:251, 1968.
6. SIERRALTA, W., MINGUELL, J., Biochem. Biophys. Res. Comm. *41*:50, 1970.
7. SIERRALTA, W., GONZÁLEZ, M.C., MINGUELL, J., J. Steroid Biochem, *5*:645, 1974.
8. ADESNIK, M., DARNELL, J.E., J. Mol. *Biol. 67*:397, 1972.
9. BURDON, R.H., SHENKIN, A., FEBS Letts *24*:11, 1972.
10. GROSS, M., GOLDWASSER, E., Biochemistry *8*:1795, 1969.
11. PERRETTA, M., VALEN. UELA, A., VALLADARES, L., A. Hollaender (ed.) Gene Expression and its Regulation, vol. 1, Plenum Press, USA. p. 199, 1973.
12. PERRETTA, M., VALENZUELA, A., SAGE, N., OYARGUREN, C., Arch. Biol. Med. Exper. *8*:30, 1971.
13. KIRBY, K.S., Biochem. J. *96*:266, 1965.
14. MC, EWEN, G.R., Analyt. Biochem. *20*:114, 1967.
15. SCHUTZ, G., BEATO, M., FEIGELSON, P., Biochem. Biophys. Res. Comm. *49*:680, 1972.
16. BRAWERMAN, G., MENDECKI, J., LEE, S.Y., Biochemistry *11*:637, 1972.
17. DELARGO, J., GUROFF, G., Biochem. Biophys. Res. Comm. *50*:486, 1972.
18. MENDECKI, J., LEE, S.Y., BRAWERMAN, G., Biochemistry *11*:792, 1972.
19. ROSENFELD, M.G., COMSTOCK, M., O'MALLEY, B., Biochem. Biophys. Res. Comm. *47*:387, 1972.
20. SHEINESS, D., DARNELL, J., Nature (New Biology) *241*:265, 1973.
21. ROSENFELD, M.G., ABRASS, J.B., PERKINS, L.A., Biochem. Biophys. Res. Comm. *49*:230, 1972.
22. EDMONDS, M., ABRAMS, R., J. Biol. Chem. *238*: 1186, 1963.
23. WINTERS, M.A., EDMONDS, M., J. Biol. Chem. *248*: 4756, 1973.
24. WINTERS, M.A., EDMONDS, M., J. Biol. Chem. *248*: 4763, 1973.
25. NIESSING, J., SEMERIS, E., Nature (New Biology) *243*:9, 1973.
26. DARNELL, J.E., JELINEK, W.R., MOLLOY, G.R., Science *181*:1215, 1973.