ISOLATION AND MOLECULAR WEIGHT OF PURE FEATHER KERATIN mRNA

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SUMMARY: Biologically active feather keratin mRNA which directs the synthesis of most or all the keratin polypeptide chains, M.W. 10,000, was prepared either by cellulose chromatography or by dissociation of mRNP particles with Na dodecyl sulphate. Both preparations gave 1 RNA band of MW 250,000 on formamide-acrylamide gels indicating the presence of long untranslated segment(s) whose length has been conserved during the evolution of individual keratin genes.

INTRODUCTION

The embryonic chick feather represents a useful system for characterising the events involved in cytodifferentiation, and it has a number of advantages for investigating keratin synthesis when compared to other keratinizing tissues(1,2). The keratins of embryonic chick feather (3) consist of a family of at least 25 homologous polypeptide chains (4) all of MW about 10,000 (4,5). The rapid onset of keratin synthesis during embryonic development takes place at about 13 days and accounts for most of the protein synthetic activity of the embryonic feather by 14 days (6,7).

We have reported (1) the isolation of a 12S RNA species from 14-day feather polyribosomal RNA that directed the synthesis of keratin in a rabbit reticulocyte lysate system. Furthermore, this 12S RNA was shown to be selectively bound to cellulose. We now report procedures for isolation of this mRNA in an apparently homogeneous form, as judged by electrophoresis on polyacrylamide gels in 98% formamide. The molecular weight of the mRNA indicates that it contains long untranslated sequences, the length of which have been stringently conserved during the evolution of the separate keratin genes.

MATERIALS AND METHODS

The cellulose used was either Whatman Standard Grade (W.R. Balston, England) or Sigmacell Type 38 (Batch 1268-1831-9; Sigma Chemical Company). The fines were removed from both preparations, which were then treated with 0.1% diethylpyrocarbonate and stored at 2-4° in the presence of 0.1% sodium azide. [^{14}C]-serine (120 mC/mM) was obtained from ICN Pharmaceuticals Incorporated.

Rigorous precautions were observed to minimise RNAse contamination (1). The preparation of polysomes, phenol extraction of the polysomal RNA, preparation of rabbit reticulocyte cell-free system and identification of the products obtained were all as previously reported (1). For preparation of mRNA-protein complexes (mRNP) a procedure modified from those of Pemberton *et al.* (8) and Zehavi-Willner (9) was developed. Polysomes were taken up at 5-10 mg/ml in 0.0125 M KCl, 0.5 M Tris/KCl, pH 9.0 at 0°. The mixture was then made 0.015 M in EDTA by the addition of 0.3 M EDTA/KOH, pH 7.5, incubated at 37° for 2 min. and chilled on ice.

Electrophoresis was according to Staynov *et al.* (10), with the modifications suggested (11) except that Zeokarb 225 was used in place of Amberlite. Deionization was for 5 hours. Electrophoresis was at 1 ma/gel (8 cm x 6 mm diameter) After electrophoresis the gels were stained with 0.5% toluidine blue or with 'stains-all' (12).

RESULTS AND DISCUSSION

Purification of Keratin mRNA by Chromatography on Cellulose. Eukaryotic mRNAs with polyA segments bind to cellulose during chromatography on this material in high-salt buffers (13-15). Our binding experiments with RNA prepared by phenol extraction of feather polysomes demonstrated that at saturation, about 0.33 and 0.47 A_{260} units of polysomal RNA bound respectively per ml of packed Whatman cellulose and Sigmacell 38. Under non-saturating conditions, between 4% and 7% of feather polysomal RNA bound to cellulose compared to about 4% of rabbit reticulocyte polysomal RNA. A value of 1% was obtained for reticulocyte RNA by Schutz *et al.* (14), who used a much greater input ratio of RNA to cellulose.

The bound fraction of feather RNA was greatly enriched in 12S RNA, although not all of the 12S RNA bound (Fig. 1a,b). The bound fraction contained peaks of RNA sedimenting at 6, 9, 18, 20 and 28S, as did the bound fraction of rabbit reticulocyte RNA (not shown:cf. Ref. 16). The binding of ribosomal RNA to cellulose has been observed previously (17).

We have reported (1) that the bound 12S RNA was more active in directing keratin synthesis in a rabbit reticulocyte lysate than the non-bound 12S RNA. In addition, keratin mRNA activity sedimenting at greater than 12S was also evident in the bound fraction (Expts. 1 and 2, Table 1). Bound RNA was therefore treated with formamide in order to dissociate any aggregated RNA and the 6S-28S peaks were each isolated by two successive cycles of sucrose gradient centrifugation. (Fig. 1b,c). The yield of 12S RNA on the gradients was increased by a factor of

TABLE I: TRANSLATION OF PURIFIED CELLULOSE-BOUND RNA FRACTIONS

	Experiment			
	1*	2	3	4
No RNA	nd	-55	86	19
6S	nd	nd	-23	138
9S	nd	nd	-26	-11
12S	894	1103	762	1041
18S	636	185	-68	104
20S	30	386	35	113
28S	78	172	20	93

In experiments 1 and 2, 12-28S RNA fractions were obtained by sucrose gradient centrifugation of the RNA as described in Fig. 1a. The 12S RNA (expts. 3 and 4) was isolated by two cycles of sucrose gradient centrifugation (Figs. 1b and c) and the other fractions (experiments 3 and 4) were isolated in a similar manner.

After ethanol precipitation and drying *in vacuo*, the individual final RNA preparations were taken up in 0.01 M Tris/HCl, pH 7.4. Samples (4 µg) were translated in 0.1 ml reticulocyte lysate systems and the lysates were assayed for radioactive keratin by immunoprecipitation as described (1). Results are expressed as immunoprecipitable cpm incorporated per 0.1 ml system, after subtraction of the non-immune serum control performed on each sample.

*In Experiment 1, the total preparations of 12-28S RNA fractions obtained after sucrose gradient centrifugation of 3.0 A_{260} units of cellulose-bound RNA, were added to a 0.2 ml system.

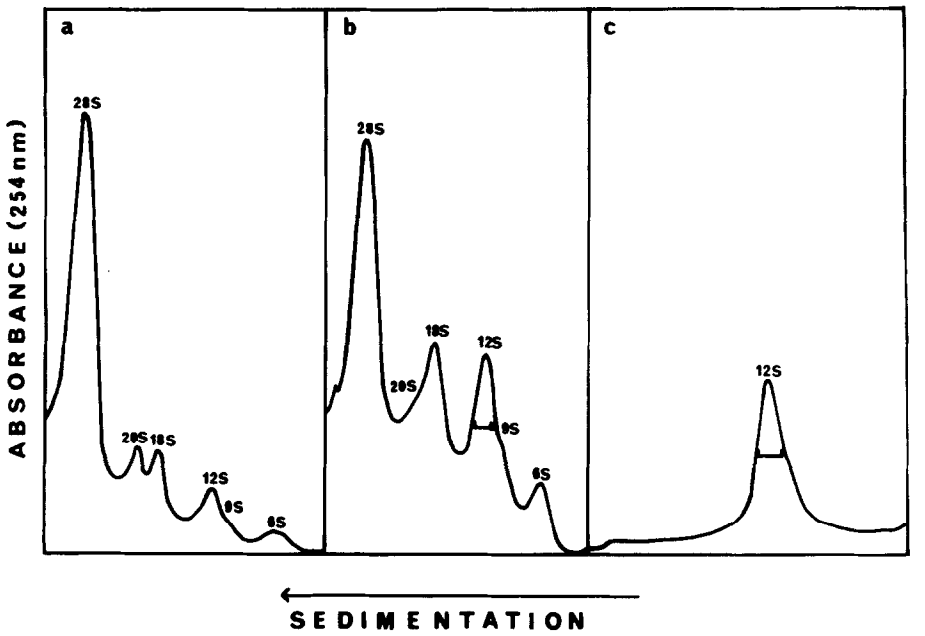

FIGURE 1. Sucrose gradient centrifugation of feather polysomal RNA fractions isolated by chromatography on cellulose. RNA (300 A_{260} units) was fractionated on a column (1.2 x 75 cm) of Whatman standard-grade cellulose as described (14). The RNA bound to cellulose but eluted with water and the non-bound RNA were separately collected by ethanol precipitation, dried *in vacuo* and dissolved in 1.0 ml of 0.1 M Tris-HCl, pH 9.0. Aliquots (0.5 ml) were centrifuged on 10-40% (w/v) sucrose gradients in buffer containing 10 mM KCl, 2 mM EDTA, 20 mM Tris-HCl, pH 7.6 for 16 hours at 41,000 rpm in the SW41 rotor. (a) Bound fraction. (b) As for (a) except that the RNA was taken up in 75 µl of 98% formamide (37°, 10 min), then chilled and diluted with 0.45 ml of buffer immediately before loading. (c) RNA isolated as shown by the bar in (b) was collected by ethanol precipitation, taken up in formamide and re-centrifuged as in (b).

about 2-4 by the treatment with formamide (cf. Figs. 1a,b). Treatment with formamide and re-centrifugation diminished the mRNA activity from RNA species sedimenting at greater than 12S (Expts. 3 and 4, Table 1), but did not affect the specific activity or S value (not shown) of 12S keratin mRNA (Expts. 3 and 4, Table 1). Other mRNAs have also been observed to aggregate (18-20).

The above results suggest caution in the interpretation of experiments indicating the presence of high MW mRNA precursors if aggregation of mRNA has not been rigorously precluded (21).

Purification of Keratin mRNA from mRNP. Rabbit globin mRNA released from

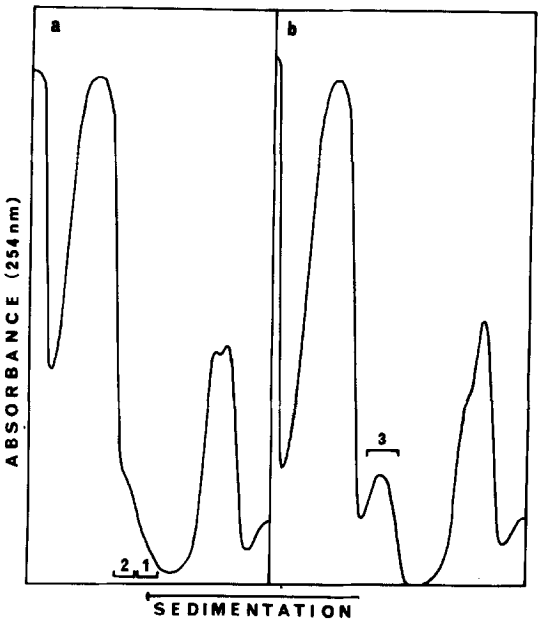

FIGURE 2. Sucrose gradient centrifugation of EDTA-treated polysomes. Polysomes from feathers or rabbit reticulocytes (dissociated with EDTA as described) were centrifuged for 14 hours at 3° on sucrose gradients (10 - 40% w/v, in buffer containing 0.015 M KCl, 0.01 M Tris/HCl, pH 7.5) at 41,000 rpm in an SW41 rotor. (a) EDTA-treated feather polysomes (44 A_{260} units); (b) EDTA-treated rabbit reticulocyte polysomes (55 A_{260} units).

polysomes as mRNP-complexes by treatment with EDTA (22, 23) was of greater purity than that obtained by dissociation of polysomes in Na dodecyl sulphate (24,25). Feather polysomes, when dissociated with EDTA and fractionated by sucrose gradient centrifugation yielded two partially resolved RNP peaks as shoulders on the trailing edge of the small ribosomal sub-unit (Fig. 2a). Under identical conditions rabbit globin mRNP was clearly resolved (Fig. 2b). The feather RNP peaks were collected (see Fig. 2a), ethanol precipitated and dissociated with 0.1% Na dodecyl sulphate. The released RNA was analysed by sucrose gradient centrifugation (Fig. 3a, b). The major RNA species from the faster sedimenting RNP fraction (Fig. 3a) sedimented at 12S, but species sedimenting at 5, 7, 9, 14 and 18S were also present. The slower sedimenting RNP fraction was enriched in 9S RNA (Fig. 3b). The predominant RNA species from rabbit reticulocyte mRNP (Fig. 3e) was globin mRNA sedimenting at 9S (8,22).

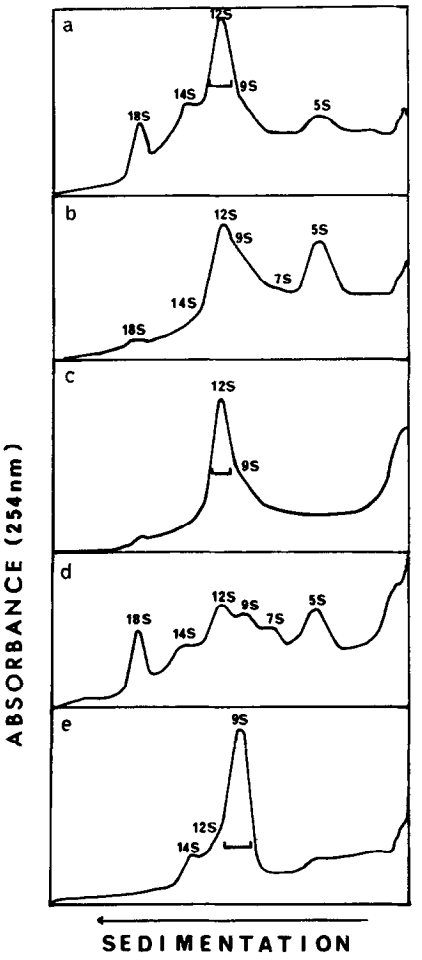

FIGURE 3. Sucrose gradient centrifugation of RNA fractions obtained from mRNP's. All samples, after ethanol precipitation and drying, were dissolved in 0.1 M Tris/HCl, pH 9.0, containing 0.1% Na dodecyl sulphate, and centrifuged for 16 hours at 3° on sucrose gradients (10 - 40%, w/v, in buffer containing 0.1 M NaCl, 0.01 M Tris/HCl, 0.001 M EDTA, pH 7.4) at 41,000 rpm in the SW41 rotor. The samples were, (a) Feather mRNP fraction 2 isolated as shown in Fig. 2a. (b) Feather mRNP fraction 1 isolated as shown in Fig. 2a. (c) Cellulose-bound fraction of RNA from mRNP; RNA was isolated by phenol extraction of mRNP from pooled fractions 1 and 2 as in Fig. 2a. The RNA obtained (2.15 A_{260} units) was fractionated on a column (1 x 15 cm) of Whatman cellulose. (d) As for (c), but the non-bound fraction. (e) mRNP from rabbit reticulocyte polysomes, isolated as shown in Fig. 2b.

Alternatively, RNA was prepared from the total feather mRNP by phenol extraction and then fractionated on cellulose. The 12S and 9S RNA species bound selectively to the cellulose (Fig. 3c and d). 12S RNA obtained as shown

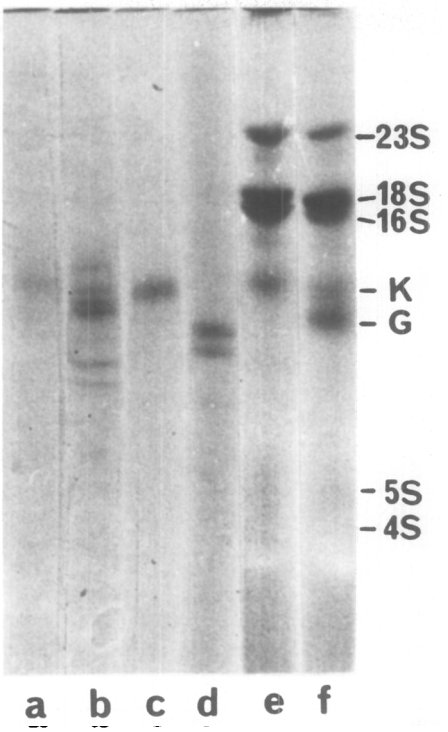

FIGURE 4. Polyacrylamide gel electrophoresis of RNA in the presence of 98% formamide. Conditions were as described in the text. (a) Cellulose-bound 12S feather RNA, prepared as shown in Fig. 1c. (b) As for (a), but the non-bound 12S fraction. (c) 12S feather RNA from dissociation of RNP with Na dodecyl sulphate, prepared as shown in Fig. 3a. (d) Globin mRNA, prepared as shown in Fig. 3e. (e) As for (c), but with the addition of *E. coli* 16S and 23S rRNA, and rabbit reticulocyte 18S rRNA and 4-5S RNA. (f) As for (e), but with the addition of globin mRNA as in (d). The gels shown in (e) and (f) were run in a separate experiment on RNA preparations different to those shown in (a) - (d). It should be noted that the globin mRNA preparation shown in (f) contained only one band, while that in (d) contained two bands. K, keratin mRNA; G, globin mRNA.

(Fig. 3a) also bound to cellulose. The 12S RNA from the preparation as shown in Fig. 3a was shown to be active in directing synthesis of keratin chains β2 - β5 by translating it (26) using the wheat embryo cell-free system of Efron and Marcus (27).

Determination of the Molecular Weight of Keratin mRNA. Electrophoresis of RNA on polyacrylamide gels in the presence of 98% formamide is a technique of high resolving power uncomplicated by conformational effects (10,11,25) for the estimation of the molecular weight of RNA.

The 12S keratin mRNA obtained from mRNP (Fig. 3a,c) or by cellulose chromatography (Fig.1c) reproducibly yielded only one discrete band when electrophoresed on formamide gels (Fig. 4a and c). In contrast the non-bound 12S fraction contained several bands (Fig. 4b). We have prepared rabbit globin mRNA from mRNP under the above conditions and have observed both double (Fig.4d) and single (Fig. 4f) bands in independent preparations respectively as reported by other workers(11,25).

By co-electrophoresis of the keratin mRNA with RNA species of known (10) MW (Fig. 4e and f), a value of 250,000 was obtained for the MW of keratin mRNA. The MW of feather keratin proteins demands about 300 nucleotides in the coding sequence of mRNA. Available evidence indicates that the mRNA is not polycistronic and that keratin is not synthesised as a larger polypeptide precursor (28). Therefore, about 500 nucleotides of the mRNA must be non-coding sequence(s). Since keratin mRNA binds selectively to cellulose the presence of a polyA sequence (13-17) is indicated. The untranslated region is longer than any polyA sequence reported and may contain sequence(s) required for translational control.

Embryonic feather keratin is comprised of at least 25 homologous polypeptide chains (4) and the mRNA for at least 4 and probably all of these is present in 12S RNA. The mRNAs coding for these chains must be of almost identical MWs, as they migrate as one band on formamide gels(Fig. 4). This result suggests that the length of the non-coding sequence(s) has also been stringently conserved during the evolution of the individual keratin genes.

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REFERENCES

1. Partington, G.A. Kemp, D.J. and Rogers, G.E. (1973). Nature New Biology, *246*, 33-36.
2. Freedberg, I.M. (1972). J. Invest. Dermatol. *59*, 56-65.
3. Kemp, D.J. and Rogers. G.E. (1972). Biochem. *11*, 969-975.

4. Walker, I.D. (1974). Ph.D. Thesis, University of Adelaide.
5. O'Donnell, I.J. (1973). Aust. J. Biol. Sci. *26*, 415-437.
6. Kemp, D.J., Dyer, P.Y. and Rogers, G.E. (1974). J. Cell. Biol. In press.
7. Fraser, R.D.B., McRae, T.P. and Rogers, G.E. (1972). Keratins. Springfield, Illinois. Thomas.
8. Pemberton, R.E., Housman, D., Lodish, H.F. and Baglioni, C. (1972). Nature New Biology, *235*, 99-102.
9. Zehavi-Willner, T. (1970). Biochem. Biophys. Res. Commun. *39*, 161-169.
10. Staynov, D.Z., Pinder, J.C. and Gratzner, W.B. (1972). Nature New Biology, *235*, 108-110.
11. Gould, M.J. and Hamlyn, P.H. (1973). FEBS Letters, *30*, 301-304.
12. Dahlberg, A.E., Dingman, C.W. and Peacock, A.C. (1969). J. Mol. Biol. *39*, 139-147.
13. Kitos, P.A., Saxon, G. and Amos, H. (1972). Biochem. Biophys. Res. Commun. *47*, 1426-1437.
14. Schutz, G., Beato, M. and Feigelson, P. (1972). Biochem. Biophys. Res. Commun. *49*, 680-689.
15. DeLarco, J. and Guroff, G. (1973). Biochem. Biophys. Res. Commun. *50*, 486-492.
16. Brawerman, G., Mendecki, J. and Lee, S.Y. (1972). Biochemistry, *11*, 637-641.
17. Sullivan, N. and Roberts, W.K. (1973). Biochemistry, *12*, 2395-2403.
18. Schechter, I. (1973). Proc. Natl. Acad. Sci. U.S.A. *70*, 2256-2260.
19. Skoultchi, A. and Gross, P.R. (1973). Proc. Natl. Acad. Sci. U.S.A. *70*, 2840-2844.
20. Palmiter, R.D. (1973). J. Biol. Chem. *248*, 2095-2106.
21. Williamson, R., Drewienkiewicz, C.E. and Paul, J. (1973). Nature New Biology, *241*, 66-68.
22. Chantrenne, H., Burny, A. and Marbaix, G. (1967) in Prog. in Nucleic Acid Res. (Ed. Davidson and Cohn) New York, Academic Press, *7*, 173-194.
23. Lebleu, B., Marbaix, G., Huez, G., Temmerman, J., Burny, A., and Chantrenne, H. (1971). Eur. J. Biochem. *31*, 38-93.
24. Kazazian, H., Moore, P.A. and Snyder, P.G. (1973). Biochem. Biophys. Res. Commun. *51*, 564-571.
25. Lanyon, W.G. and Paul, J. (1972). Eur. J. Biochem. *31*, 38-43.
26. Kemp, D.J., Schwinghamer, M. and Rogers, G.E. Unpublished observations.
27. Efron, D., and Marcus, A. (1973). FEBS Letters, *33*, 23-27.
28. Kemp, D.J., Partington, G.A. and Rogers. G.E. Unpublished observation