ANALYSES OF GLOBIN RELEASE FROM RETICULOCYTE RIBOSOMES*

Allan J. Morris

Department of Biochemistry, Michigan State University,
East Lansing, Michigan

Received January 25, 1966

Investigations of the biosynthetic processes which constitute the respective stages of protein synthesis indicate the presence of a specific mechanism for the release of completed protein from the biosynthetic template (Morris, 1964). It was demonstrated early in the studies of protein biosynthesis that cell-free systems prepared from rabbit reticulocytes synthesize hemoglobin molecules which are no longer attached to the ribosomal template and are chromatographically indistinguishable from carrier hemoglobin (Schweet et al, 1958). The details of the process by which completed protein chains are liberated to a nonparticulate state have been a subject of previous publications from this laboratory. It is the purpose of this communication to present the results of further studies which indicate the release of globin molecules from reticulocyte ribosomes involves cleavage of the globyl-sRNA bond at the ribosomal level and that the first soluble product of the release reaction is the protein molecule, free from soluble RNA. A mechanism for the termination and release of completed protein chains from the ribosomal template is proposed.

Experimental

Ribosomes and soluble enzymes were prepared from rabbit reticulocytes as described previously (Morris, 1964). Reticulocyte sRNA used as carrier in the density gradients was obtained from rabbit liver by the method of Harshaw et al (1962). Tritium labeled sRNA was prepared using RNA isolated by the procedure of Hoagland et al (1958). Labeled sRNA possessing a tritium labeled adenylate residue

* Michigan State Journal Number 3786

in the terminal position was then synthesized according to the method of Starr and Goldthwait (1963) using H^3-ATP (Schwarz BioResearch Inc., Orangeburg, N.Y.). H^3-sRNA was recovered from the labeling medium by phenol extraction and was washed twice by precipitation from 66% ethanol at -18°.

Globin was prepared from methemoglobin by extraction with 2-butanone at pH2 and dialyzed against 0.1 M LiCl, $5x10^{-3}$M Tris-Cl(pH 7.0) and 0.5% sodium dodecyl sulfate (SDS).

Ribosomes used for the gradient analyses were prelabeled as described previously (Morris, 1964) except using C^{14}-valine of specific activity 100 μC per μM. Labeled ribosomes used for those studies reported in Table 1 were prepared from washed ribosomes using either H^3-sRNA or C^{14}-valine (sp.act. 20 μC/μM) as the sole source of radioactivity. The introduction of H^3-sRNA into a ribosomal-bound state was accomplished by preincubation in the complete cell-free system (5 min. x 37°) with soluble enzymes from which endogenous sRNA had been largely removed by protamine precipitation. Preincubation conditions were identical in the C^{14}-valine counterpart except for the source of radioactivity. Following dilution with 20 volumes of cold 0.25 M sucrose, 17.5 mM $KHCO_3$ and 2 mM $MgCl_2$, the H^3-labeled ribosomes or C^{14}-labeled ribosomes were reisolated by centrifugation, resuspended in cold 0.25 M sucrose and added to the release assays as indicated. Ribosomes prepared in this manner possessed an estimated 1.5 H^3-sRNA molecules per ribosomal particle.

Sucrose density gradient analysis was carried out by a modification of the procedure of Traut and Monro (1964). Linear sucrose gradients containing 5 to 20 percent sucrose, 0.1 M LiCl, 0.5% SDS and $5x10^{-3}$M Tris-Cl buffer (pH 7.0) were prepared at room temperature. Materials to be analyzed were adjusted to 0.5% SDS, a total volume of 1.0 ml and layered onto a gradient of 58 ml. Centrifugation was carried out for 48 hours at 20° in a Beckman SW 25.2 rotor at 75,500 x g. Contents of each tube were then analyzed for materials absorbing at 260 mμ by pumping through a Gilford spectrophotometer equipped with a flow cell (0.5 cm path length). Results were plotted automatically by a Sargent Model SR recorder. Flow rate through the cell was maintained at 5 ml. per minute using a Buchler polystaltic pump. Effluent from the flow cell was collected in 1.0 ml portions with a Packard Model 231 fraction collector. To each fraction was added 1 mg of bovine serum albumin and the precipitate which formed in 10% TCA solutions was collected on nitro-

cellulose filters (Schleicher and Schull, Keene, N.H.). The filters were washed twice with 5 ml of 10% TCA and dried. Following the addition of 15 ml of toluene, PPO, POPOP counting fluid to each vial containing a dried filter, radioactivity was determined by a Packard Model 3003 liquid scintillation spectrometer.

Results and Discussion

Analyses of carrier sRNA preparations alone (Fig. 1A) indicate soluble RNA is located approximately mid-point in the centrifuge tube after centrifugation in the manner employed here. Sedimentation of globin molecules, shown here in comparison to sRNA (Fig. 1B), is markedly slower than sRNA and permits the distinction of globin from sRNA molecules on the basis of sedimentation velocity.

In order to determine the stability of the peptidyl-sRNA bond during the centrifugation procedure, ribosomes containing C^{14}-labeled intermediates were dissociated with detergent by the method of Gilbert (1963) and the sedimentation pattern of the nascent protein analyzed. Over 90% of the radioactive ribosomal bound intermediates applied to the gradient were recovered from the region of the tube coincident with the carrier sRNA (Fig. 1C). Such recovery, plus the absence of free labeled peptides in the gradient indicate that no significant hydrolysis of the peptidyl-sRNA bond occurs during the prolonged time of centrifugation. In addition, these data suggest that most, if not all, of the ribosomal bound nascent protein is linked to molecules with the sedimentation properties of sRNA.

Having thus established the sedimentation properties of globin, of sRNA and of peptidyl-sRNA compounds, analyses were carried out to determine the state of soluble protein immediately after release from the ribosomal template. Supernatant solutions obtained from both the isolated release assay and the complete cell-free system (Morris, 1964), at two time periods of incubation in each type of assay, were analyzed. Active, energy dependent release of protein occurred during all periods studied (Fig. 1, E, through H). While the experimental conditions of the isolated release assay have been shown previously to prevent incorporation of amino acids into polypeptide structure, a GTP dependent release of completed globin chains has been shown to proceed for approximately 40 minutes at 37°. Figures 1E and 1F indicate that no globyl-sRNA could be detected in the sucrose gradients at times which are early (10 min. x 37°) or mid-point (20 min. x 37°) in the time course of the release process.

FIGURE I

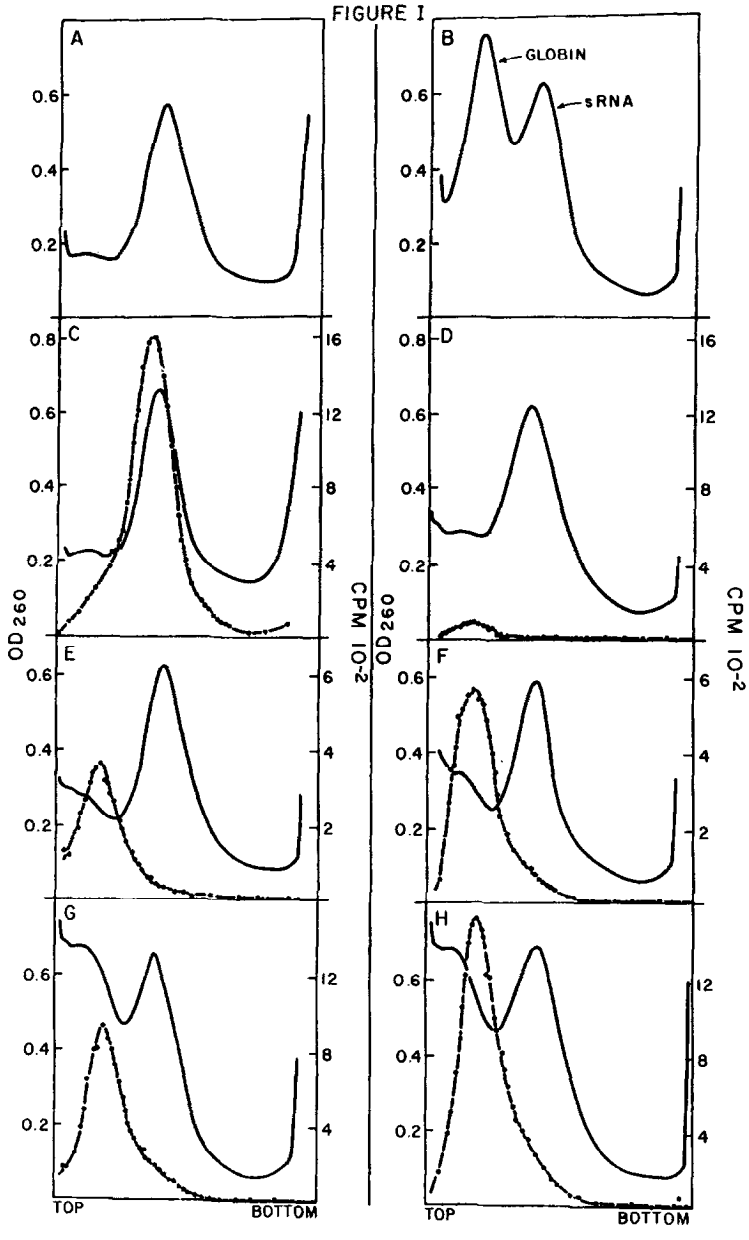

Fig. 1. Carrier sRNA, 20 OD units (260 mμ), was added to each sample analyzed. In addition the samples contained the following: (A) carrier sRNA only, (B) rabbit globin, (C) dissociated labeled ribosomes, (D) supernatant fraction following incubation of labeled ribosomes (3'x37°) in the complete cell-free system less nucleoside triphosphates and the ATP generating system, (E) supernatant fraction following incubation of labeled ribosomes (10'x37°) in the isolated release assay, (F) same as E except 40'x37°, (G) supernatant fraction following incubation of labeled ribosomes in the complete cell-free system (3'x37°), (H) same as G except 10'x37°. Optical density is depicted with solid lines, radioactivity with broken lines.

Similarly, analysis of the soluble fractions obtained following incubation of ribosomes containing labeled nascent protein in the complete cell-free system (containing unlabeled free amino acids) revealed no globyl-sRNA (Fig. 1, G and H). These "chase" conditions are known to lead to the formation of soluble labeled hemoglobin (Morris and Schweet, 1961). In the absence of the nucleoside triphosphates and ATP generating system the complete cell-free system contains only trace amounts of soluble protein (Fig. 1D). Supernatants from the isolated release assay analyzed in the same manner also showed only trace amounts of soluble labeled proteins in the absence of GTP, the amount of soluble protein being similar to that found in nonincubated assays.

While these data indicated the absence of detectible amounts of globyl-RNA in the supernatant fraction, it was of interest to examine the possibility that breakdown of soluble globyl-sRNA occurred so rapidly that none could be detected. One means of studying such a possibility would be to follow the distribution of ribosomal bound sRNA molecules during active release of protein. Table 1 presents the results of a parallel study of the release of C^{14}-labeled protein from prelabeled ribosomes, on one hand, and the release of H^3-sRNA from a ribosomal-bound state in the other analyses. In the isolated release assay, conditions which led to the release of 15% of the ribosomal bound C^{14}-labeled protein produced only trace amounts of soluble H^3-sRNA. In contrast, in the complete cell-free system where the polymerization of amino acids is proceeding, an energy dependent solubilization of the H^3-sRNA molecules occurred to a large extent. The latter result would be expected in view of the proposed role of sRNA in the polymerization reaction. With polypeptide synthesis the ribosomal bound H^3-sRNA would be replaced by unlabeled sRNA (added to the complete cell-free system) as the latter carried amino acids to the biosynthetic template.

A recent publication by Takanami and Yan (1965) has provided evidence that termination codon may program the end of the protein chain with a trinucleotide containing UA. Similarly Brenner and coworkers (1965) have conducted experiments with suppressor mutants which suggest the termination codon may be the "nonsense" trinucleotide sequences UAA and UAG, which do not code for amino acid incorporation. However, the mechanism of release must involve more than recognition of a specific codon since cleavage of the finished molecule from an sRNA-like molecule occurs during the process. Similarly, the existence

of polycistronic messenger RNA molecules presupposes that release of the completed protein is not due to having reached the end of the messenger RNA strand.

Table 1 A comparison of the release of protein and sRNA from reticulocyte ribosomes.

assay	Time at 37°	energy dependent release of C^{14} protein	percent of total C^{14} protein	energy dependent release of H^3-sRNA	percent of total H^3-sRNA
	min.	cpm		cpm	
isolated	10	560	6.1	-3	0
release	20	1,057	11.5	36	1.2
system	40	1,303	14.3	29	1.0
	60	1,360	14.9	33	1.1
complete system	40	3,394	37.1	766*	48*

*The supernatant enzyme (present in the complete system) converted 47 percent of the ribosomal-bound H^3-adenylate label of sRNA to a TCA soluble form during 40 min. at 37°. Percent calculations are corrected accordingly. The H^3-sRNA radioactivity was recovered quantitatively from the isolated release assays, following incubation, by TCA precipitation. C^{14}-labeled ribosomes (2 mg) contained 9,156 cpm total labeled protein. H^3-sRNA labeled ribosomes (2mg) contained 3,003 cpm. All values have been corrected for labeled protein or labeled sRNA present in the supernatant fraction when energy was omitted. Energy in the isolated release assay was 10^{-4}M GTP. Energy in the complete system was 10^{-4}M GTP, 10^{-3}M ATP, 5×10^{-3}M PEP and 40 µg pyruvate kinase. For details of the assay procedures see Morris (1964) reference.

On the basis of information available at this time one can propose a mechanism for the release of completed protein. Such a mechanism must take into account the observations that:(1) the release process requires GTP, (2) C-terminal amino acid residues of proteins contain more than a few kinds of amino acids i.e., the last AA-sRNA to react in polymerization is probably not the terminating RNA, (3) the release process can be demonstrated to proceed in the absence of amino acid polymerization (Morris, 1964), (4) the first soluble product is the free protein molecule.

It is proposed that a unique sRNA-like molecule is present which can accept the completed protein molecule and retain it in a ribosomal-bound form. This process would be energy-requiring in that GTP would be necessary to move the ribosome onto the termination

codon in a manner analogous to ribosomal motion along the messenger RNA strand (from codon to codon) during amino acid polymerization. (While the mechanism of action of GTP in protein synthesis had not been firmly established, studies with a model peptide bond forming system employing the single peptide bond formed between peptidyl-sRNA and puromycin found no requirement for GTP in that reaction (Casjens and Morris, 1965). This would tend to exclude a direct participation of GTP in peptide bond formation. The participation of GTP in ribosomal movement along the messenger RNA strand remains a reasonable possibility). In addition to the termination anticodon, the terminating sRNA-like molecule would possess a recognition site for the cleaving enzyme in a manner analogous to the sites on sRNA molecules which permit specific recognition by the activating enzymes. The cleaving reaction would separate the completed protein molecule from the terminating RNA--the product of the reaction being soluble protein and ribosomal-bound RNA. Studies of the mechanism of protein release are being continued in this laboratory.

Data, mentioned here in preliminary form, will be reported in detail elsewhere.

ACKNOWLEDGEMENTS

The author wishes to thank Miss Ann P. Stevens for excellent technical assistance. This investigation was supported by U.S. Public Health Service Grant AM-08755 and funds allocated from the Experimental Station, Michigan State University.

REFERENCES

Casjens, S. and Morris, A., Biochem. Biophys. Acta 108 677 (1965).
Gilbert, W., J. Mol. Biol. 6 389 (1963).
Harshaw, J.P., Brown, R.A. and Graham, A.F., Anal. Biochem. 4 182(1962).
Hoagland, M.B., Stephenson, M.L., Scott, J.F., Hecht, L.I., and Zamecnik, P.C., J. Biol. Chem. 231 241 (1958).
Morris, A.J. and Schweet, R.S., Biochem. Biophys. Acta 47 415 (1961).
Morris, A.J., Biochem. J. 91 611 (1964).
Schweet, R.S., Lamfrom, H. and Allen, E., Proc. Natl. Acad. Sci. 44 1029 (1958).
Starr, J.L. and Goldthwait, D.A., J. Biol. Chem. 238 682 (1963).
Stretton, A.O.W. and Brenner, S., J. Mol. Biol. 12 456 (1965).
Takanami, M. and Yan, Y., Proc. Natl. Acad. Sci. 54 1450 (1965).
Traut, R.R. and Monro, R.E., J. Mol. Biol. 10 63 (1964).