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Structure of the Large β-Chain Polyribosome of Rabbit Reticulocytes Rendered Isoleucine-Deficient by O-Methylthreonine

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SUMMARY

Incubation of rabbit reticulocytes with L-*O*-methylthreonine, an analogue of L-isoleucine, results in the formation of a unique, large β-chain polyribosome containing approximately 12 ribosomes. Two possible mechanisms for the formation of this polyribosome are considered: (a) the polyribosome may be formed by maximal packing of ribosomes on a β-chain mRNA strand proximal to the limiting isoleucine codon, or (b) there may be dimerization of lighter polyribosomes by an interaction of the blocked nascent β-chains. The nascent chains were removed from the ribosomes in intact cells by incubation with puromycin, and from the isolated ribosome-polyribosome component by incubation with Pronase. In both cases the large polyribosome remained after the nascent chains were removed, suggesting that it is formed by maximal packing of ribosomes on a β-chain mRNA strand.

INTRODUCTION

When rabbit reticulocytes are incubated with the L-isoleucine antagonist L-*O*-methylthreonine (1), the incorporation of isoleucine into the α- and β-polypeptide chains of hemoglobin is inhibited (2). Isoleucine is located only near the NH_2 terminus of the 141-amino acid α-chain, at positions 10, 17, and 55 (3, 4). In the 146-amino acid β-chain, it is located only at the COOH-terminus, at position 112 (5). Since protein synthesis proceeds from the NH_2-terminal amino acid to the COOH-terminal amino acid (6, 7), a deficiency of isoleucine results in retardation of ribosomal movement at the proximal portion of α-chain mRNA and at the distal portion of β-chain mRNA. It may be predicted, therefore, that inhibition of isoleucine incorporation into nascent α- and β-chains would result in smaller α-chain polyribosomes and larger β-chain polyribosomes than occur under normal conditions *in vitro*. Indeed, the unique and characteristic alterations in polyribosome profile seen after incubation with L-*O*-methylthreonine include an increase in the amount of dimers and trimers, a decrease in the normal components heavier than trimers, and the formation of a new, heavy polyribosome containing approximately 12 monomers (2, 8). The differential incorporation of valine and threonine into the nascent chains on the ribosomes suggested that the dimers and trimers are predominantly blocked α-chain polyribo-

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somes, and that the new, heavy peak represents blocked β-chain polyribosomes (2). This conclusion has been confirmed by peptide analysis of the nascent chains (8).

Up to six ribosomes are normally found on mRNA coding for either the α- or β-chain of rabbit hemoglobin (9). Since 12 ribosomes usually are not accommodated on mRNA of either chain, two possible mechanisms for the formation of a heavy β-chain polyribosome may be considered: (a) the polyribosome may be formed by maximal packing of ribosomes on a β-chain mRNA strand proximal to the limiting isoleucine codon, or (b) there may be a dimerization of lighter polyribosomes by an interaction of the blocked nascent β-chains. In this study, we describe experiments which differentiate between these two possibilities.

MATERIALS AND METHODS

Incubation of cells. Reticulocyte-rich blood was collected in heparin by cardiac puncture of phenylhydrazine-treated rabbits (10). The cells were centrifuged free of plasma and washed twice with low-magnesium saline (11). The cells were incubated in a gyrotory shaker water bath at 37°, with air as the gas phase. The washed reticulocytes were incubated in low-magnesium saline containing 5 mM Tris-HCl (pH 7.4), amino acids, glucose, and iron-transferrin, as previously described (2). The final mixture, in a 25-ml Ehrlenmeyer flask, contained 1.5 ml of packed cells in a total volume of 12 ml. The cells were incubated for 9 min in this fortified medium minus valine. L-Valine-1-^{14}C (34.2 mCi/mmole) was then added to give a final concentration of 0.1 mM, and the incubation was continued for 1 min. L-*O*-Methylthreonine (final concentration, 25 mM) was then added, and the incubation was continued for 5 min. In the experiments with puromycin, 2-ml aliquots of the incubation mixture were added either to flasks containing buffered saline (controls) or to flasks containing puromycin, and the incubation was continued for 5 min more. Metabolism was terminated by the addition of cold saline and shaking of the flask in ice. The cells were isolated by centrifugation at 600 × *g* at 4°. The cells were washed and lysed, and the course particulate fraction was removed as previously described (11).

In experiments with Pronase, the incubation with fortified medium minus valine, L-valine-1-^{14}C, and L-*O*-methylthreonine was performed as described above. The ribosome-polyribosome component, however, was isolated by centrifuging 4.8 ml of cell lysate over a cushion of 7.2 ml of 30% sucrose for 3 hr at 100,000 × *g*. The pellet was gently resuspended in 4 ml of standard buffer (0.01 M Tris, pH 7.4; 0.01 M KCl; and 0.0015 M $MgCl_2$) and cleared by centrifugation for 5 min at 600 × *g*. The ribosome concentration was estimated using a value of 11.2 absorbance units/mg/ml at 260 mμ (12). The ribosomal suspension was divided into two equal portions and incubated in buffer alone or in buffer containing Pronase, 0.23 mg/1.14 mg of ribosomes per milliliter, for 1 hr at 4°.

Analysis of ribosome-polyribosome component. Aliquots (1 ml) of the cleared cell lysate (puromycin experiments) or the resuspended ribosome-polyribosome component (Pronase experiments) were placed on 27 ml of 15–30% linear sucrose gradients with standard buffer as the solvent. The gradients were centrifuged at 25,000 rpm for 2¾ hr at 4° in a Spinco SW 25.1 rotor. After centrifugation, the gradient was pumped through a flow-through cell attached to a Beckman DB spectrophotometer, and absorbance was measured at 260 mμ (10). Incorporation of the polyribosome into protein was determined by collecting approximately 40 fractions, adding carrier serum albumin (11), precipitating the protein in 5% trichloracetic acid, extracting at 90° for 10 min, and plating on 0.45-μ Millipore filters. Radioactivity was determined in a gas flow counter with Micromil windows (efficiency, 30%).

Materials. Pronase, grade B, was purchased from Calbiochem; L-valine-1-^{14}C, from New England Nuclear Corporation; and RNase-free, crystalline, ultrapure, density-gradient sucrose, from Mann Research Laboratories

RESULTS AND DISCUSSION

The two alternative structures of the heavy polyribosome formed in the presence

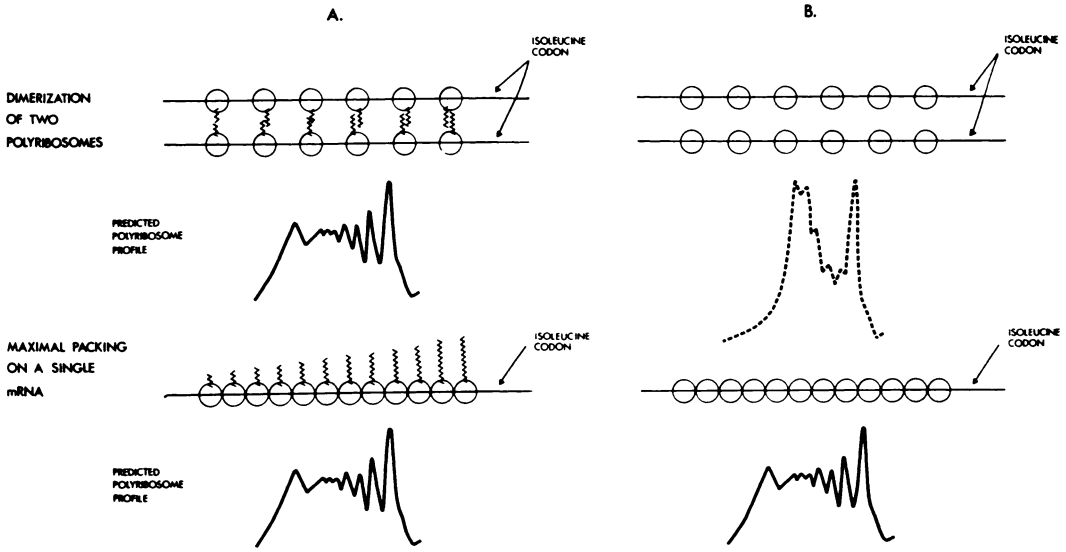

FIG. 1. *Possible mechanisms for formation of heavy polyribosome from cells incubated with* L-*O-methylthreonine*

A. Schematic representations of the possible mechanisms for the formation of heavy polyribosomes. B. Theoretical changes after the nascent chains have been removed.

of L-*O*-methylthreonine are represented in Fig. 1. If interaction of nascent β-chains serves to hold two lighter polyribosomes together, as indicated in the top of Fig. 1, removal of the nascent chains would result in an explosive shift of the polyribosome pattern from the heavy duodecamer peak to smaller units. If 12 ribosomes are attached to a single mRNA, there should be little change in the polyribosome profile when nascent chains are removed.

Puromycin promotes the premature release of the entire growing peptide chain in reticulocytes (13, 14). The antibiotic was added during incubation of intact reticulocytes, at a concentration that does not alter the normal polyribosome pattern but removes more than 70% of the radioactivity incorporated into the nascent chains (15). The incorporation of L-valine-1-^{14}C into the peptide chains of the ribosome-polyribosome component of hemoglobin synthesis that has been inhibited by L-*O*-methylthreonine is shown in Fig. 2A. When the cells were incubated under identical conditions, but with puromycin (Fig. 2B), approximately 70% of the radioactivity, representing prematurely released nascent chains, was removed from each fraction. The corresponding ultraviolet absorption profiles (Fig. 2) in this experiment reveal that puromycin caused only a small decrease in the heavy polyribosome region, with a corresponding rise in single ribosomes. There was no increase in the level of smaller polyribosomes. The slight shift toward single ribosomes might be explained by substitution of the antibiotic for isoleucyl-tRNA in a manner similar to its possible replacement of tryptophanyl-tRNA in tryptophan-deficient reticulocytes (15). The persistence of the heavy polyribosome peak, in the presence of puromycin, is suggestive evidence that this unique structure does not result from an interaction of large β-chains. It is possible, however, that the remaining radioactivity in the heavy polyribosome fractions represents a selective inability of puromycin to remove interacting chains. This seems unlikely, as radioactivity was removed uniformly from all fractions, suggesting that the nascent chains were released at random. To obtain further evidence against this possibility, experiments were performed in which nascent chains were removed with Pronase.

Pronase has been shown to hydrolyze that portion of the nascent chain which is not shielded by the ribosome (16). Approxi-

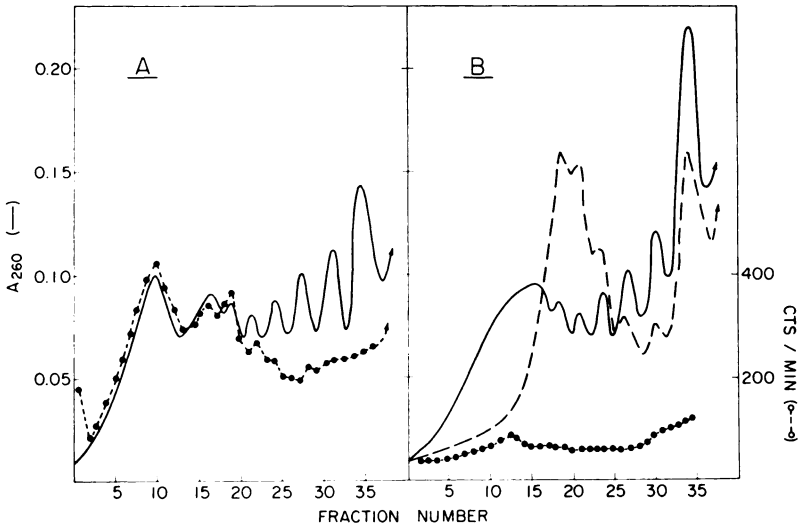

FIG. 2. *Effect of puromycin on ultraviolet absorption profile and incorporation of* L-*valine-1-*^{14}*C into peptide chains of ribosome-polyribosome component of rabbit reticulocytes with hemoglobin synthesis inhibited by* L-*O-methylthreonine*

Incubation of cells with L-*O*-methylthreonine and puromycin, sucrose density gradient analysis of the cleared cell lysate, and determination of the radioactivity incorporated into peptide chains were performed as described in MATERIALS AND METHODS. ——, ultraviolet absorption at 260 mμ; ●- - -●, incorporation of L-valine-1-^{14}C without puromycin (A) and with puromycin at a final concentration of 19 μM (B). The dashed line in panel B represents the ultraviolet absorption profile of the ribosome-polyribosome component from cells which had not been incubated with L-*O*-methylthreonine or puromycin.

mately 30–35 amino acid residues are buried within the ribosomal structure and thus are protected from proteolytic dissociation. The heavy polyribosome is formed only when ribosomal movement toward the 3′-nucleotide end of mRNA is retarded. When there is retardation toward the 5′-nucleotide end, as with the α-chain mRNA in this system, or with tryptophan deficiency (2), smaller polyribosomes result. The large polyribosome therefore is not a result of retardation of ribosomal movement. We assumed, on this basis, that if dimerization occurred, it would result from the long length of the nascent β-chains unique to this system, and the interaction would take place between the portions of the β-chain which are not buried within the ribosomal structure. Pronase, by digestion of this region, should eliminate any interaction. However, as shown in Fig. 3, the integrity of the large polyribosome was maintained in the presence of Pronase. The amount of radioactivity removed in this experiment was similar to that reported by Malkin and Rich (16); approximately 50% of the radioactivity in the nascent chains of the heavy polyribosomal region was removed.

The cause of the minor shift of the heavy polyribosome peak to lighter components (approximately 12 monomeric units to 10 monomeric units) is not clear at present. However, when the ribosome-polyribosome component from control cells was treated with Pronase, the slight absorbance observed in the region corresponding to the heavy polyribosome was shifted similarly (data not shown). It should be noted that a portion of the single ribosomes was dissociated into ribosomal 60 S and 40 S subunits, as previously described (16). It has been shown recently that Pronase selectively dissociates ribosomes not attached to mRNA (17). Therefore, the ribosomes dissociated by Pronase treatment are those that have not initiated hemoglobin synthesis.

Our failure in these experiments to disrupt the large polyribosome by removing nascent

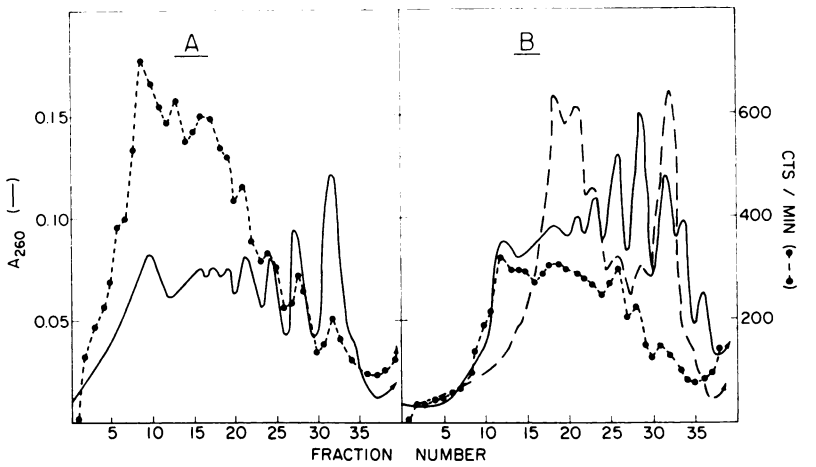

FIG. 3. *Effect of Pronase on labeled ribosome-polyribosome component isolated from rabbit reticulocytes with hemoglobin synthesis inhibited by* L-*O-methylthreonine*

Incubation of cells with L-*O*-methylthreonine, isolation of the ribosome-polyribosome component, incubation of this component with Pronase, sucrose density gradient analysis, and determination of radioactivity were performed as described in MATERIALS AND METHODS. ——, ultraviolet absorption at 260 mμ; ●- - -●, incorporation of L-valine-1-^{14}C without Pronase (A) and with Pronase at a concentration of 0.23 mg/1.14 mg of ribosomes per milliliter (B). The dashed line in panel B represents the ultraviolet absorption profile of the isolated ribosome-polyribosome component not exposed to Pronase and from cells which had not been incubated with L-*O*-methylthreonine.

chains is suggestive evidence that this structure does not result from blocked nascent β-chains causing dimerization of smaller polyribosomes. The hypothesis that the heavy polyribosome results from maximal packing of ribosomes on a β-chain mRNA strand proximal to the limiting isoleucine codon is a plausible alternative. The question remains, however, whether 12 ribosomes can fit on a single β-chain mRNA. Slayter *et al.* (9) have indicated that the expected length of mRNA coding for the α- or β-chain, of approximately 150 amino acids, would be 1500 A, provided that the nucleotides are stacked with a translation of 3.4 A. These authors suggested that the length could double when the nucleotides are not stacked, and lengths up to 2800 A were seen in electron micrographs (9). Eisenberg and Felsenfeld (18), measuring the coil dimensions in stacked and unstacked single-stranded polyribonucleotides, reported an interphosphate separation of about 6.5 A in both cases. Using this measurement, the β-chain mRNA would always be 2800 A, even if the nucleotides were stacked. In either case, the 333-nucleotide segment of β-chain mRNA preceding the isoleucine codon at position 112 could be approximately 2200 A. Such a length could conceivably accommodate 10 closely packed ribosomes with a diameter of approximately 220 A (19). Even without postulating distortion of ribosomes, a β-chain polyribosome containing 12 ribosomes on a single mRNA strand seems possible. If ribosomes are packed on mRNA with any distortion in their structure, even greater numbers could be accommodated.

Reports of a heavy class of "repressed polyribosomes" in unfertilized sea urchin eggs (20) and *Ascaris* eggs (21) indicate that terminal block and resulting enlargement may have a physiological counterpart. Such a block has been suggested by Spirin (22) as a possible mode of storing mRNA in a stable configuration.

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