Isolation of Giant Silk Fibroin Polysomes and Fibroin mRNP Particles Using a Novel Ribonuclease Inhibitor, Hydroxystilbamidine

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ABSTRACT Hydroxystilbamidine isethionate, a dye capable of binding to both DNA and RNA, has been found to be a powerful inhibitor of cellular ribonucleases. A procedure has been developed that, with the aid of this compound, permits the preparative isolation of giant silk fibroin polyribosomes from the posterior silk gland of Bombyx mori. The polyribosomes contain ~45–112 ribosomal particles, as judged by electron microscopy.

Treatment of giant fibroin polyribosomes with EDTA releases a particle that sediments at 125S. This mRNP particle contains biologically active silk fibroin mRNA, as judged by cell-free translation in an mRNA-dependent reticulocyte cell-free system.

MATERIALS AND METHODS

Hydroxystilbamidine isethionate was a gift from May and Baker, Ltd., Dagenham, Essex, England and from Merrell-National Laboratories, Cincinnati, Ohio. The yellow powder was dissolved in small aliquots in sterile H2O (25–50 mM) and stored up to 4 mo at −20°C in the dark. Sodium heparin was obtained from Sigma Chemical Co., St. Louis, Mo.

Analytical Techniques

RNA was extracted from silk glands or polysomal pellets with proteinase K and sodium perchlorate (6). Electrophoresis of RNA in fully denaturing gels (1.6% acrylamide, 0.6% agarose) containing 52% formamide and 1M formaldehyde was performed as described elsewhere (4).

RESULTS AND DISCUSSION

Treatment of postnuclear supernates of posterior silk glands of B. mori with nonionic detergents releases membrane-bound
polysomal mRNA. This degradation is indicated by the absence of a band of fibroin messenger RNA after electrophoretic analysis in denaturing polyacrylamide-agarose gels. We have attempted polyribosome release in the presence of a number of commonly used ribonuclease inhibitors, such as heparin. Fig. 1, lane 2, shows that even in the presence of heparin it is not possible to recover intact fibroin mRNA from polysome pellets. The gel shows a faint band at ~32S, which probably represents nuclear ribosomal RNA precursor leakage, and a strong band near 18S consisting of cytoplasmic ribosomal RNA (ribosomal 28S RNA from B. mori contains a cryptic nick [3]). The nature of the RNA band below 18S has not been determined. The use of higher concentrations of heparin was found to be impractical because of an adverse effect on the integrity of nuclei during cell fractionation.

Using the gel electrophoresis assay shown in Fig. 1, I tested a number of other compounds and found that the trypanocidal dye hydroxystilbamidine isethionate (9, 10) permitted the recovery of mRNA after polysome release with NP-40 (Fig. 1, lane 3). Intact fibroin mRNA is in evidence as a faint, but very sharp, band at the expected position in the gel (~5.8 x 10^6 daltons; see references 3 and 4).

Sucrose gradient analysis of detergent-lysed postnuclear supernatants was used to analyze the size distribution of NP-40-released polysomes. Fig. 2 shows the results of one such analysis in which lysates were prepared in the presence of either hydroxystilbamidine isethionate (HSB) or heparin. The heparin gradient (B) shows some polyribosomes, whereas the HSB gradient (A) shows a remarkably large peak of very heavy polyribosomes. This peak is obtained reproducibly if HSB is present before the addition of NP-40. If the order of addition is reversed, no polyribosomes are obtained. In a set of similar experiments, I tested aurintricarboxylic acid (11) as a ribonuclease inhibitor, using concentrations in the range of 1–3 mM. The polysome profiles obtained with this compound (data not shown) were similar to those obtained with heparin.

To further characterize the large polyribosomes obtained with HSB, I examined the material present in the heavy peak (Fig. 2A, bracket 1) by electron microscopy with the spreading techniques of McKnight et al. (12).

Fig. 3 shows several electron micrographs of typical fields from spreadings on fine carbon films. Whereas the polysome size distributions have not been subjected to detailed statistical analysis, careful observation of >25 randomly chosen polysomal structures on the spreading yielded counts of 45–112 ribosomal particles per polysome. The mRNA strand seems to be in evidence in some of the more stretched polysomes, as seen in the lower part of Fig. 3. From the known size of silk fibroin (mol wt ~350,000–400,000; see references 4 and 13), one would expect a full-length fibroin polysome to contain ~100 ribosomal particles (12).

Polyribosomes prepared in the presence of HSB were dissociated with EDTA to release ribosomal subunits and messenger RNA. Fig. 4 shows a sucrose gradient analysis of EDTA-released fractions from polyribosome size cuts. The material released from giant polysomes (A) shows the expected ribosomal subunits and a very distinct peak sedimentsing at 12S. This peak is absent from material obtained from fractions of smaller polysomes (B) and is, therefore, a good candidate for a fibroin mRNP particle. Isopycnic banding of the 12S material in matrilmid gradients (14, 15) shows that it bands at a density different from that of ribosomal subunits. Gel electrophoresis in the presence of SDS shows two major protein components with molecular weights of 72,000 and 46,500, respectively (data not shown).

Before attempting a more thorough physicochemical characterization of the presumptive fibroin mRNP particles, it was important to determine whether isolation in the presence of HSB yielded biologically active ribonucleoproteins. To this effect, polyribosome and 125S mRNP fractions were tested as templates for in vivo translation in an mRNA-dependent reticulocyte cell-free system (16). Translation of pure fibroin mRNA in this system is known to give rise to a ladder of

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polypeptide products that represent nascent silk fibroin chains (17). These nascent chains accumulate transiently at specific positions on the mRNA template, which have been termed translation "pause" sites. Relatively little full-length fibroin is accumulated because of its extremely high molecular weight. The experiment in Fig. 5, lane 1, shows a typical pattern of translation products obtained with pure fibroin mRNA as template. All the radioactive bands in this fluorograph are known to represent growing silk fibroin polypeptides (17). Translation of material obtained from the fraction of giant silk fibroin polyribosomes (lane 2) demonstrates the synthesis of a product with the characteristic size of silk fibroin (see marker protein on lane 4). The relative proportion of smaller radioactive products is lower than in lane 1, which is to be expected because the polysomal material contains nascent chains at the beginning of the incubation. Because the time required for the synthesis of fibroin in the reticulocyte lysate is 85–90 min (5,000 amino acids polymerized at a rate of nearly one amino acid per second), loaded polyribosomes have an advantage relative to purified mRNA in a 100-min translation "race." The translation products of EDTA-released mRNP particles (lane 3) are similar to those of pure mRNA. Comparison of the electrophoretic mobilities of radioactive products in the range of 68,000–212,000 daltons shows that the "pause" positions are essentially the same for mRNP and for purified fibroin mRNA. The absence of the largest polypeptide chains could be caused by premature termination of translation inasmuch as other experiments showed that the mRNA recovered from the 125S mRNP particles was often slightly nicked. In any event, the predominance in lane 3 of authentic fibroin

**FIGURE 3** Electron micrographs of phosphotungstic acid-stained material from a fibroin polysome peak fraction. Samples from sucrose gradients (peak 1, Fig. 2 A) were processed for electron microscopy on carbon-coated grids, essentially as described by McKnight et al. (10), except that polysome spreading was performed simply by contact of sample droplets with coated grids, and the shadow-casting step was omitted. Bar, 0.5 μm. × 58,700.
Sucrose gradient analysis of EDTA-dissociated polyribosomes. Material from peaks 1 and 2 in Fig. 2A was collected and pelleted through a cushion of 1.7 M sucrose, as described in Materials and Methods. The pellets were briefly washed with sterile water to remove sucrose and dissolved in 0.6 ml of 30 mM triethanolamine-HCl, pH 7.4, 100 mM NaCl, 5 mM EDTA. After vortexing for 5 min at 4°C, NaCl was added to a final concentration of 200 mM, and the material was layered over a 10–29.6% wt/wt isokinetic sucrose gradient made up in 30 mM triethanolamine-HCl, pH 7.5, 200 mM NaCl, 2 mM EDTA. The gradient was run for 4 h at 26,000 rpm (3.5°C) in a Beckman SW 27.1 rotor. (A) Material from peak 1 in Fig. 2A. (B) Material from peak 2 in Fig. 2A. The sedimentation position of rabbit reticulocyte polysomes run on a separate gradient containing 4 mM MgCl₂.

polypeptides demonstrates that the mRNA in the 125S particles is biologically active fibroin messenger. Further studies on the structure and protein composition of the 125S mRNP particles are in progress.

In a different set of experiments (data not shown) it was found that the addition of 0.5 mM HSB to the reticulocyte cell-free system completely inhibits fibroin translation. This was not unexpected because HSB has been shown to bind to both DNA and RNA (9). The fact that polyribosomes isolated from cell lysates prepared in the presence of 1.5 mM HSB are active in in vitro translation (Fig. 5) suggests that most of the polypeptide-bound HSB is removed by the final polysome pelleting step. The recovery of biologically active structures in the silk gland system suggests that HSB could have wide application in eliminating nucleolytic activities during cell fractionation. In this respect, it is of interest to note reports that claim that this compound also has antiproteolytic and lysosome-stabilizing activities (18, 19). The exact mechanism of HSB inhibition of ribonuclease action remains to be elucidated.

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Note Added in Proof. Recent experiments using mammalian tissue culture cells have shown that the ratio of total cell RNA (and added E. coli tRNA) relative to HSB is critical for optimal polysome yield.

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