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.

The isolation and characterization of cytoplasmic messenger ribonucleoprotein particles (mRNP) containing specific mRNA's has so far been achieved in relatively few biological systems. The two best studied examples are globin mRNP and myosin mRNP (see reference 1 for review).

Insects remain relatively unexploited as model systems for the study of mRNP structure and function. A promising system for such studies is the posterior silk gland of *Bombyx mori*, in which silk fibroin mRNA is by far the major mRNA present in the cell.

The isolation of fibroin polyribosomes has so far proven difficult because of the lability of the very long mRNA molecule (2–4). In this paper I describe a method that utilizes a novel ribonuclease inhibitor, hydroxystilbamidine isethionate, to achieve the isolation of giant silk gland polyribosomes containing undegraded fibroin mRNA. I also present a preliminary characterization of fibroin-specific mRNP particles obtained from the giant polyribosome fraction. The fibroin mRNA present in these mRNP particles is biologically active.

MATERIALS AND METHODS

Reagents

Hydroxystilbamidine isethionate was a gift from May and Baker, Ltd., Degenham, Essex, England and from Merrell-National Laboratories, Cincinnati, Ohio. The yellow powder was dissolved in small aliquots in sterile H_2O (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.

Polysome Isolation

Silkwork larvae (Gunka × Hushun strain) were raised in the laboratory as described (5). Larvae on the fourth or fifth day of the fifth instar (~4.2 g body weight) were injected with 35 μl of a solution of 10 mg/ml cycloheximide in H_2O. After 5 min, the animals were immobilized in ice, and posterior silk glands were dissected and washed in ice-cold 0.15 M NaCl, 0.015 M Na citrate, 100 μg/ml cycloheximide. Washed glands from two larvae were placed in a Dounce homogenizer (Kontes Co., Vineland, N.J.) containing 4.7 ml of 40 mM triethanolamine-HCl, pH 7.5, 0.15 M sucrose, 0.1 M KCl, 3 mM $MgCl_2$, 2 mM reduced glutathione, 10 μg/ml cycloheximide, 750 μg/ml *Escherichia coli* tRNA, and an appropriate concentration of RNase inhibitor (sodium heparin, 1.5 mg/ml or hydroxystilbamidine isethionate, 1.5 mM). Homogenization was accomplished by seven slow strokes of a large-clearance Dounce plunger, followed by the addition of 0.3 ml of 2.5 M KCl, 16.7 mM $MgCl_2$ (to raise the KCl level to 0.25 M). Nonidet P-40 (NP-40) was added to a final concentration of 1%, and the material was stirred for 8–10 min with a glass rod to release membrane-bound polysomes. Nuclei were pelleted by centrifugation for 4 min at 1,000 g_{avg}, and the supernate was saved. About 1.5 ml of supernate was loaded on gradients (15–60% sucrose) made up in 40 mM triethanolamine-HCl, pH 7.5, 0.25 M KCl, 4 mM $MgCl_2$ reduced glutathione. Centrifugation was carried out for 3 h at 25,000 rpm (2.5°C) in the Beckman SW27 rotor (Beckman Instruments, Inc., Fullerton, Calif.). The polysome absorbance profiles were monitored at 254 nm and fractions of interest were pooled. Up to 16 ml of polysomal material were placed over a 5-ml cushion of 1.7 M sucrose (made up in the same buffer) and centrifuged for 4 h at 55,000 rpm (2.5°C) in a 60 Ti rotor (Beckman Instruments, Inc.). The clear polysome pellets were rinsed with cold sterile H_2O and stored at −80°C.

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 1 M formaldehyde was performed as described elsewhere (4).

Translation products were analyzed by electrophoresis in 3.2–10% polyacrylamide gels containing SDS in a Neville buffer system (7). Fluorography was performed as described by Bonner and Laskey (8).

RESULTS AND DISCUSSION

Treatment of postnuclear supernates of posterior silk glands of *B. mori* with nonionic detergents to release membrane-bound

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polyribosomes results in rapid nucleolytic degradation of 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 (~5.8×10^6 daltons; see references 3 and 4).

Sucrose gradient analysis of detergent-lysed postnuclear supernates 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. 2*A*, bracket 1) by electron microscopy with the spreading techniques of McKnight et al. (12).

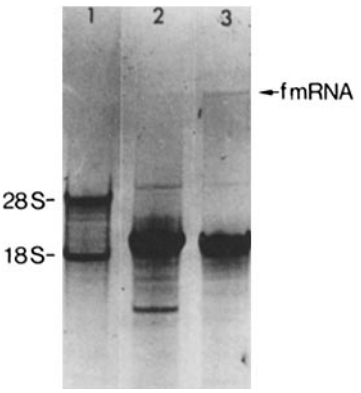

FIGURE 1 Electrophoresis of RNA from polysomal pellets of posterior silk glands. Postnuclear supernates were prepared as described in Materials and Methods, using ribonuclease inhibitors as indicated below. Each supernate was layered over a cushion of 1.8 M sucrose in 4 mM triethanolamine-HCl, 0.25 M KCl, 3 mM $MgCl_2$, and centrifuged at 50,000 rpm for 10 h at 3°C in a Beckman 50 Ti rotor. RNA was extracted from the polysome pellets and analyzed in denaturing polyacrylamide-agarose gels, as described in Materials and Methods. Lane *1*, marker ribosomal RNA from 3T6 cells; lane *2*, RNA from polysomes prepared in the presence of 1.5 mg/ml heparin; lane *3*, RNA from polysomes prepared in 1.5 mM HSB.

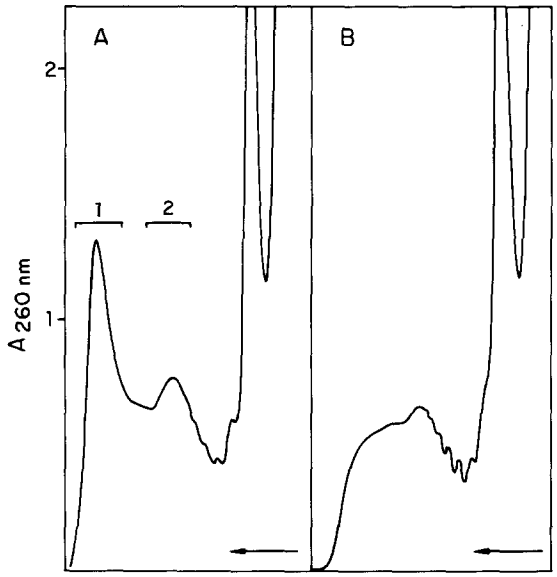

FIGURE 2 Sedimentation profiles of posterior silk gland polysomes prepared in the presence of different nuclease inhibitors. Postnuclear supernates were prepared in the presence of RNAase inhibitors and analyzed in 15–60% sucrose gradients, as described in Materials and Methods. The arrow indicates the direction of sedimentation. (*A*) Polysomes from cells lysed in the presence of HSB. (*B*) Polysomes from cells lysed in the presence of sodium heparin.

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 sedimenting at 125S. 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 125S material in matrizamide 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

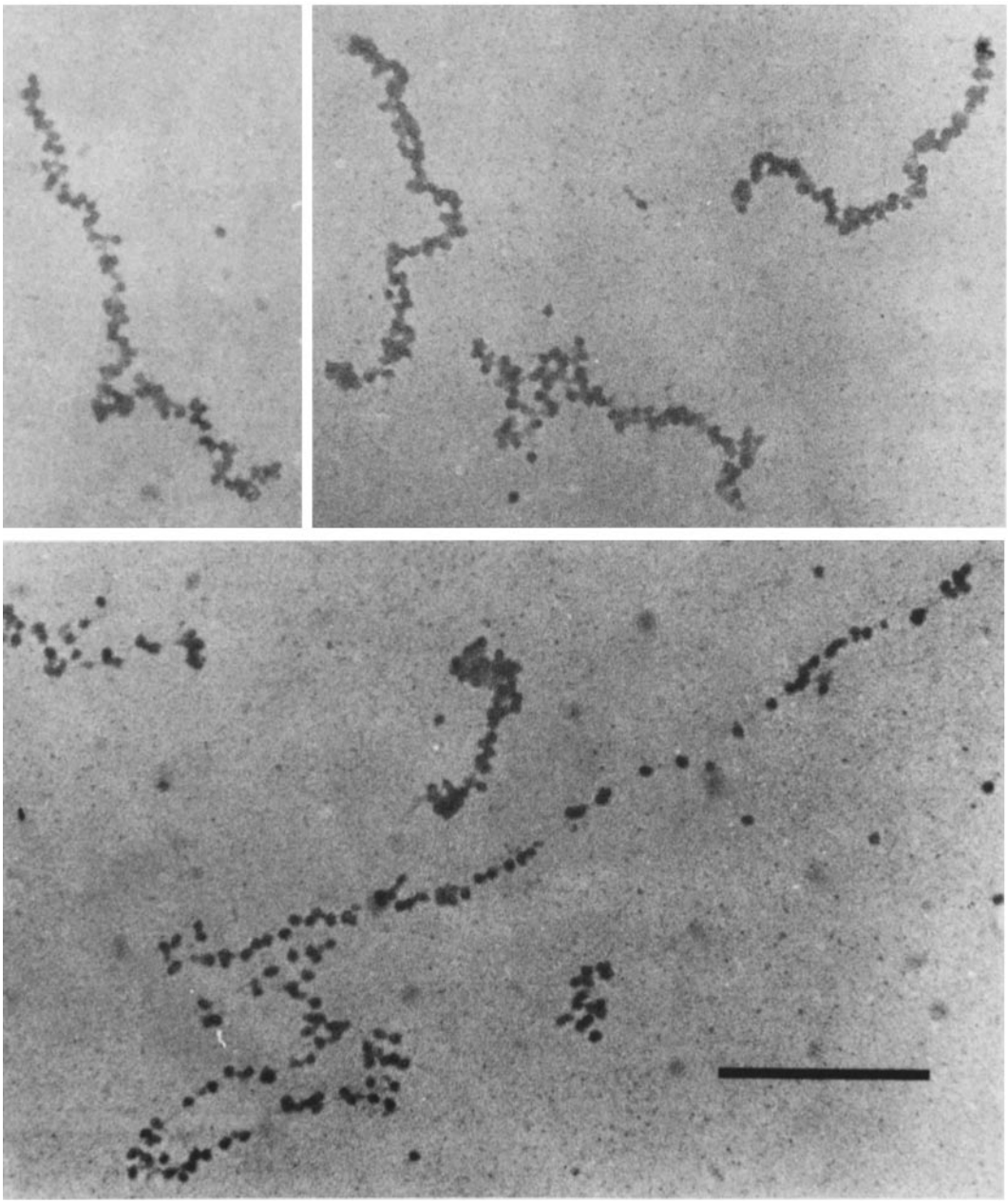

FIGURE 3 Electron micrographs of phosphotungstic acid-stained material from a fibroin polysome peak fraction. Samples from sucrose gradients (peak 1, Fig. 2 *A*) were processed for electron microscopy on carbon-coated grids, essentially as described by McKnight et al. (10), except that polysome spreading was performed simply by contact of sample droplets with coated grids, and the shadow-casting step was omitted. Bar, 0.5 μm. × 58,700.

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

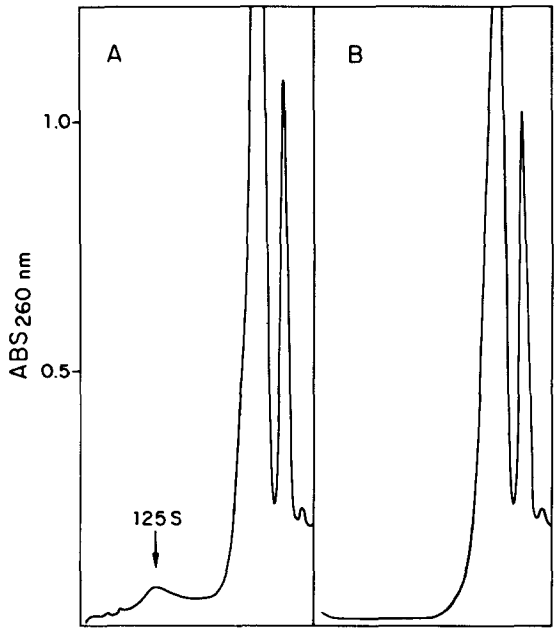

FIGURE 4 Sucrose gradient analysis of EDTA-dissociated polyribosomes. Material from peaks 1 and 2 in Fig. 2 *A* was collected and pelleted through a cushion of 1.7 M sucrose, as described in Materials and Methods. The pellets were briefly washed with sterile water to remove sucrose and dissolved in 0.6 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. 2 *A*. (*B*) Material from peak 2 in. Fig. 2 *A*. The sedimentation value for the mRNP peak (125S) was estimated from the sedimentation position of rabbit reticulocyte polysomes run on a separate gradient containing 4 mM $MgCl_2$.

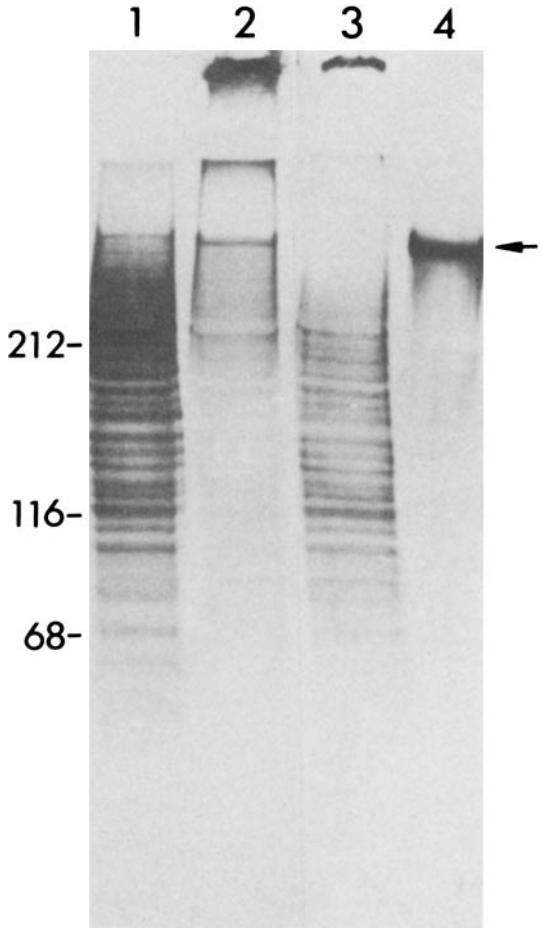

FIGURE 5 Fluorography of products of in vitro translation analyzed by SDS polyacrylamide gel electrophoresis. An mRNA-dependent rabbit reticulocyte cell-free system supplemented with posterior silk gland tRNA was used, as described elsewhere (14, 15). The labeled amino acid was [^{14}C]alanine. Lane *1*, standard translation (2 h) in the presence of 70 μg/ml fibroin mRNA. Lane *2*, translation in the presence of 40 A_{260}/ml of fibroin polysomes (estimated fibroin mRNA content, 30 μg/ml). Lane *3*, translation in the presence of 0.75 A_{260}/ml fibroin mRNP (estimated fibroin mRNA content, 30 μg/ml). Lane *4*, authentic fibroin marker, labeled in vivo with [^{14}C]-alanine. The dark bands at the top in lanes *2* and *3* represent labeled material that did not penetrate the gel. The arrow denotes the full-size fibroin marker (mol wt ~400,000). Stained markers (not shown) were muscle myosin (212,000 daltons), β-galactosidase (116,000 daltons), and serum albumin (68,000 daltons).

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 polysome-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* pRNA) relative to HSB is critical for optimal polysome yield.

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