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LOW MOLECULAR WEIGHT SILK PROTEINS IN *GALLERIA MELLONELLA*

KRYSTYNA GRZELAK,[1] PIERRE COUBLE,[2] ANNIE GAREL,[2] BARBARA KLUDKIEWICZ[1] and HICHAM ALROUZ[2]

[1]Institute of Biochemistry and Biophysics, Polish Academy of Sciences, Rakowiecka 36, Warszawa, Poland

[2]Département de Biologie Générale et Appliquée, Unite Associée au CNRS n° 92, Université Claude Bernard—Lyon 1, 45 Boulevard du 11 Novembre 1918, Villeurbanne Cedex, France

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Abstract—*Galleria* cocoon proteins have been extracted by different solubilizing agents. Nine protein bands were observed by gel electrophoresis, with molecular weights ranging from 18 to 420 kD. Three silk proteins of 24, 29 and 30 kD were extracted only in the presence of β-mercaptoethanol, suggesting that they are covalently linked by disulfide bonds to the large fibroin. They are likely to be the products of the highly abundant mRNA of the posterior silk gland cells. *In vitro* translation analysis of this mRNA yielded 24, 29 and 30 kD proteins. Thus, as in *Bombyx*, the *Galleria* silk is composed of several subunits, including fibroin and low molecular weight polypeptides. However, the genes coding for fibroin or low molecular weight silk proteins in *Bombyx* and *Galleria* do not show nucleotide base homology.

Key Word Index: *Galleria mellonella*, silk gland, fibroin, cocoon

INTRODUCTION

The silk of *Galleria* is produced by a paired organ, the silk gland, with two morphologically distinct territories: the posterior silk gland (PSG) and the middle silk gland (MSG), both active in silk protein synthesis. The number of protein species in the cocoon and their molecular sizes has not yet been determined. The main silk component, fibroin, whose amino acid composition is unbalanced (22.6 glycine, 22.2 alanine, 16.5 serine) is produced by the PSG cells (Lucas *et al.*, 1960; Michalik *et al.*, 1984). Silk synthesis occurs all through the larval life and is associated with a continuous synthesis of mRNA. However, this mRNA production becomes massive during the last larval instar, at which stage the silk of the cocoon is elaborated (Grzelak *et al.*, 1982; Michalik *et al.*, 1984).

As a prelude to a study of the mechanism of regulation of silk production, we characterized the different protein components of *Galleria* cocoon and identified three polypeptides of low molecular weight that appear to be linked by disulfide bonds with fibroin. These proteins appear to be coded by 1100 nucleotide long mRNAs made in PSG cells. We hypothesize that these proteins are analogous to the low molecular weight silk proteins of *Bombyx mori*, but exhibit no nucleotide sequence homology.

MATERIALS AND METHODS

Insects

The wax moth, *G. mellonella* (Lepidoptera, Pyralidae), was reared on a standard diet at 30°C as reported by Sehnal (1966). The silk worm, *B. mori* (Lepidoptera), hybrids of the European strains 200 and 300, were reared in the laboratory as described previously (Couble *et al.*, 1981). Silk glands were dissected from the last instar larvae.

RNA extraction

Total silk gland RNA was extracted from fresh as well as from frozen PSG or MSG according to the LiCl–urea method of Auffray and Rougeon (1980). Poly A$^+$ RNA was purified either on oligo-dT-cellulose (Collaborative Research) (Aviv and Leder, 1972) or by using messenger affinity paper (Orgenics). Further fractionation of *Galleria* PSG mRNA was carried out on a linear 5–20% sucrose gradient according to the method of Couble *et al.* (1981).

Synthesis of complementary DNA

DNA complementary to PSG or MSG mRNAs was synthesized as described by Maniatis *et al.* (1982).

The reaction mixture (10 μl) contained: 50 mM Tris (pH 7.9); 8 mM $MgCl_2$; 80 mM NaCl; 5 mM dithiothreitol; 10 μg/ml oligo-dT; 200 μM each of dGTP, dATP and dTTP; 100 μCi [^{32}P]dCTP (3000 Ci/mmol, Amersham); 50 μg/ml poly A$^+$ RNA; and 100 U/ml of avian myeloblastosis virus reverse transcriptase. The reaction was carried out at 37°C for 30 min and the cDNA was purified according to Maniatis *et al.* (1982).

Post-labeling of cloned DNA

DNA from clones carrying *Bombyx* silk encoding genes were nick translated (Rigby *et al.*, 1977), using [^{32}P]dCTP as radioactive tracer.

RNA blotting

"Northern" blots were performed according to Maniatis *et al.* (1982). Total or poly A$^+$ RNA were denatured in 50% formamide and 6% formaldehyde for 15 min at 60°C, then electrophoresed on horizontal 1.5% agarose gels prior to transfer onto nitrocellulose filters. ^{32}P-labeled cDNA or nick-translated cloned DNA were used as probes and hybridized to the filters in 50% formamide, 5 × SSC (0.15 M NaCl, 0.015 M sodium citrate), 1 × Denhardt's solution, 100 μg/ml denatured herring sperm DNA, for 18 h at 42°C. Filters were then washed stepwise with 2 × SSC and 0.1 × SSC containing 0.1% SDS, at 50 or 65°C with gentle agitation. Filters were exposed to Kodak X-ray films at −70°C in light-tight boxes with intensifying screens.

Analysis of cell-free translation products

Micrococcal nuclease-treated rabbit reticulocyte lysate (Amersham) was supplemented with 2.5 μCi/μl of tritiated leucine (147 Ci/mmol, Amersham) and used in a final volume of 13 μl. 0.5–2.4 μg of RNA were tested per assay and incubated at 30°C for 1 h. Wheat germ S-23 extract was prepared by the method of Morch *et al.* (1986). The reaction mixture (25 μl) containing: 17.5 μl of S-23 extract; 100 mM CH_3COOK; 0.5 mM Mg $(CH_3OO)_2$; 0.3 μCi/μl [^{14}C]leucine (211 mCi/mMol) or 0.3 μCi/μl [^{14}C]alanine (120 mCi/mMol) or 0.3 μCi/μl [^{14}C]glycine (113 mCi/mMol). 1.2 μg of RNA was tested per assay and incubated at 25°C for 1.5 h. The translation products were then brought to 5% SDS and 5% β-mercaptoethanol, heated at 100°C for 3 min and electrophoresed on a 15% acrylamide gel according to Laemmli (1970). ^{14}C-labeled protein markers (Amersham) were co-electrophoresed to calibrate the migration. Gels were treated for fluorography according to Bonner and Laskey (1974) and then autoradiographed.

Analysis of silk proteins

Silk proteins were extracted from *Galleria* or *Bombyx* cocoons by either 60% w/w neutral LiSCN under reducing conditions or by a mixture of 2% SDS and 8 M urea, with or without 5% β-mercaptoethanol.

Proteins were analyzed by electrophoresis on 15% acrylamide gels and on linear 3.5–12.5% acrylamide gradient gels according to Laemmli (1970).

RESULTS AND DISCUSSION

Analysis of Galleria cocoon proteins

In order to analyze the cocoon proteins of *Galleria*, we tried different methods of extraction resulting in either partial or complete solubilization of the silk components. The treatment of *Galleria* cocoons with 8 M urea and 2% SDS resulted in the solubilization of five distinct proteins of molecular weights of 170, 98, 71, 37 and 18 kD, respectively (Fig. 1a). Interestingly, when β-mercaptoethanol was added to the mixture, three additional bands (30, 29 and 24 kD) were distinguishable in the gel (Fig. 1b). The 30 kD band was faint, while the 29 and 24 kD bands were more intense. Complete solubilization of cocoon proteins was achieved by use of concentrated (60%) LiSCN under reducing conditions. It led to the extraction of the above proteins plus a highly abundant protein at the top of the 15% gel (Fig. 1c). Its molecular weight was estimated at about 420 kD on the basis of its migration relative to *Bombyx* sericin (380 kD) and fibroin (340 kD) proteins in 3.5–12.5% acrylamide gradient gels (Fig. 1e). We identified this high molecular weight silk protein as the *Galleria* fibroin by its solubility in concentrated LiSCN (Prudhomme *et al.*, 1985). However, becuase of the difficulty in electrophoretic separation of fibroin the relative abundance of the different silk proteins could not be estimated precisely. *B. mori* cocoons extracted with 60% LiSCN also yielded at least seven protein bands, including a 340 kD fibroin and a 25 kD small molecular weight protein (Shimura *et al.*, 1976).

From this analysis it can be concluded that *Galleria* cocoon is composed of at least nine polypeptides. These proteins can be solubilized sequentially: five in urea and SDS; three in urea/SDS with 5% β-mercaptoethanol; and one, fibroin, in 60% LiSCN. *Bombyx* cocoon proteins are also extracted sequentially, using the same solubilizing agents (Couble *et al.*, 1983; Prudhomme *et al.*, 1985). By analogy with this system, the highly soluble proteins of *Galleria* would represent sericin-like proteins, which are surrounding the silk thread. As in *Bombyx* cocoon proteins, fibroin and the three other *Galleria* silk proteins probably constitute the major silk proteins (Shimura *et al.*, 1976, 1982).

The low molecular weight silk proteins of *Galleria* (24, 29 and 30 kD) are solubilized only in the presence of reducing agents, which could indicate that they are bound to larger silk protein(s) and freed upon the disruption of disulfide bonds. The fact that the size of the 5 proteins extracted by urea and SDS is not modified by subsequent treatment with β-mercaptoethanol, shows that the smaller 24, 29 and 30 kD silk proteins are not bound to them. These smaller silk proteins appear to be bound to fibroin; as indicated by recovery of larger amounts of these proteins following treatment with LiSCN, when fibroin is solubilized (Figs 1b and 1c). Thus, it can be assumed as is the case for *Bombyx* (Tokutake, 1980; Shimura *et al.*, 1976; Couble *et al.*, 1983), that *Galleria* silk proteins of small size are bound to fibroin.

Galleria PSG mRNA

In order to determine whether or not the posterior silk gland cells in *Galleria*, as in *B. mori*, produce such proteins, we analyzed the PSG mRNAs in *Galleria*. We identified the major species of mRNA from *Galleria* PSG by hybridizing blotted PSG mRNA with homologous cDNA. As shown in Fig. 2a, a large mRNA band can be seen as an intense radioactive signal. However, when total PSG RNA was blotted and probed with homologous cDNA, two bands were distinguishable (Fig. 2b). This observation suggests the presence of two mRNA species of similar size (about 1100 nucleotides, from 4.1–4.5 $\times$ 10^5 D).

In order to demonstrate that these major mRNAs are specific to the posterior silk gland cells, we hybridized blots of MSG mRNA with MSG cDNA (Fig. 2f) and PSG cDNA (Fig. 2g) separately. The MSG probe (Fig. 2f) revealed several mRNA species with molecular weight ranging from about 600 to 5000 nucleotides, but not a 1100 nucleotide long species. Furthermore, the PSG cDNA probe hybridized to MSG mRNA to a much lesser extent than the homologous probe, but no distinct 1100 nucleotide mRNA species was detected (Fig. 2g). Therefore, it can be concluded that PSG mRNAs contain a high proportion of mRNAs of 1100 nucleotides, whose respective genes are selectively expressed in the PSG cells.

The pattern of hybridization of *Galleria* mRNA with homologous cDNA (Fig. 2a) is very similar to the one obtained when blots of *Bombyx* PSG mRNA was annealed with *Bombyx* PSG cDNA (Fig. 2c). In both cases, 1100 nucleotide long mRNAs appeared as the most abundant species. This 1100 nucleotide mRNA in *Bombyx* has been identified as the message encoding the silk protein P25, which is bound to fibroin (Tokutake, 1980; Shimura *et al.*, 1976; Couble *et al.*, 1983).

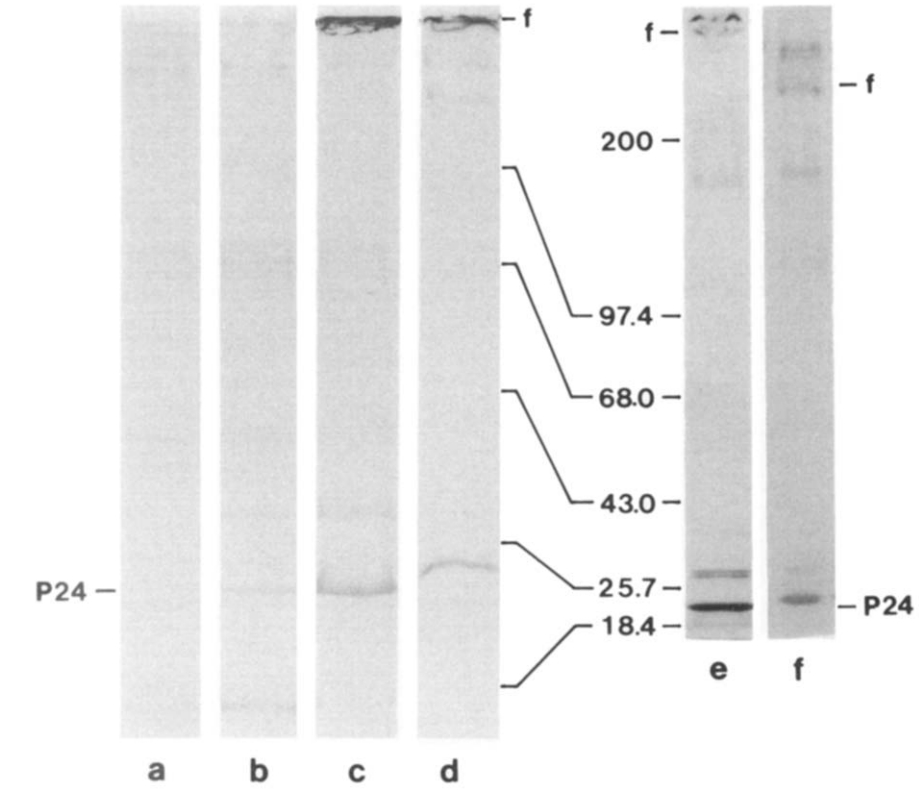

Fig. 1. Separation of *Galleria* cocoon proteins extracted by SDS–urea (a), SDS–urea–β-mercaptoethanol (b) and neutral LiSCN (c, e). LiSCN extracts of *Bombyx* cocoon proteins are shown in d and f. Proteins in a–d were analyzed on 15% acrylamide gel and in e and f, on 3.5–12.5% linear gradient of acrylamide. Size of marker proteins (β-lactoglobulin, α-chymotrypsinogen, ovalbumin, bovine serum albumin, phosphorylase *b* and myosin, respectively) are indicated in kD; f stands for fibroin.

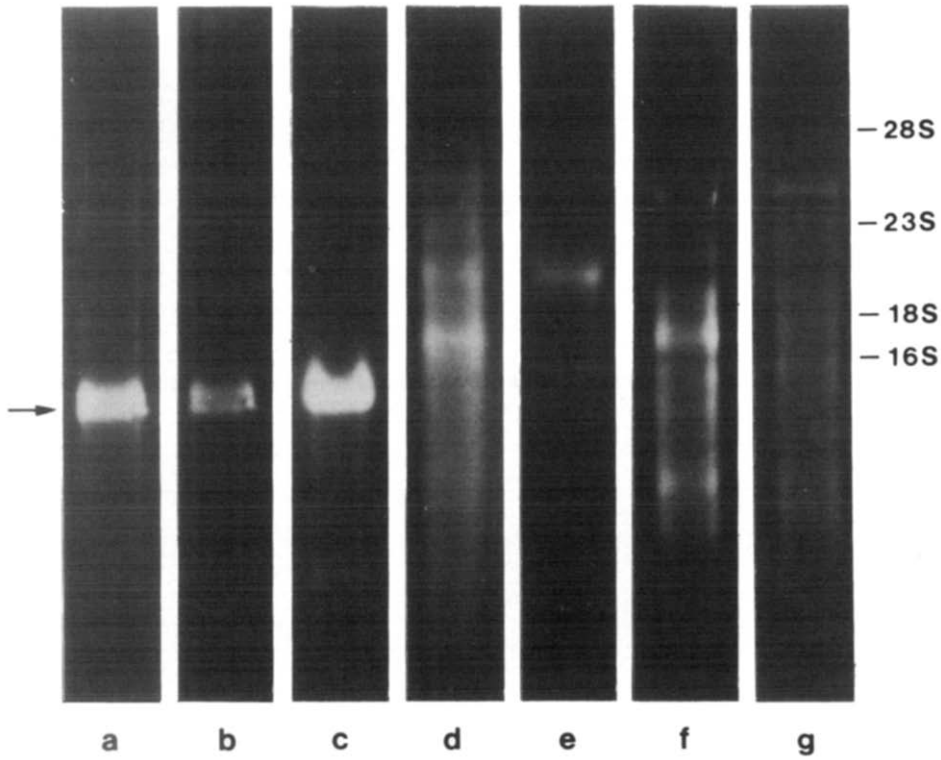

Fig. 2. Analysis of *Galleria* PSG and MSG mRNA. *Galleria* PSG mRNA 5 μg (a, d), PSG total RNA 20 μg, (b, e), MSG mRNA (f, g) and *Bombyx* PSG mRNA (c) were blotted on nitrocellulose and hybridized with *Galleria* [^{32}P]PSG cDNA (a, b, g), *Galleria* [^{32}P]MSG cDNA (f) and *Bombyx* [^{32}P]PSG cDNA (c–e), respectively. Size of markers (HeLa cell 28S and 18S rRNA and *E. coli* 23S and 16S rRNA) are indicated. Note the intense hybridization signal (arrow) over 1100 nucleotides mRNA species in a–c.

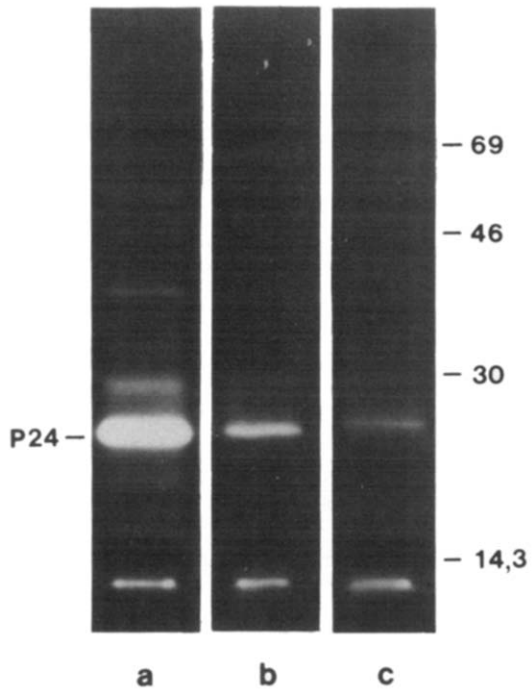

Fig. 3. Characterization of the protein encoded by *Galleria* PSG mRNA in a rabbit reticulocyte lysate system. Autoradiograms correspond to the cell-free [^{3}H]leucine-labeled proteins translated with *Galleria* RSG mRNA (a), 10S–18S fraction of *Galleria* PSG mRNA (b) and *Bombyx* PSG mRNA (c). Proteins were denatured in SDS and β-mercaptoethanol prior to electrophoresis in 15% acrylamide gel. Size of marker proteins (lysozyme, anhydrase carbonic, ovalbumin and bovine serum albumin, respectively) are indicated in kD.

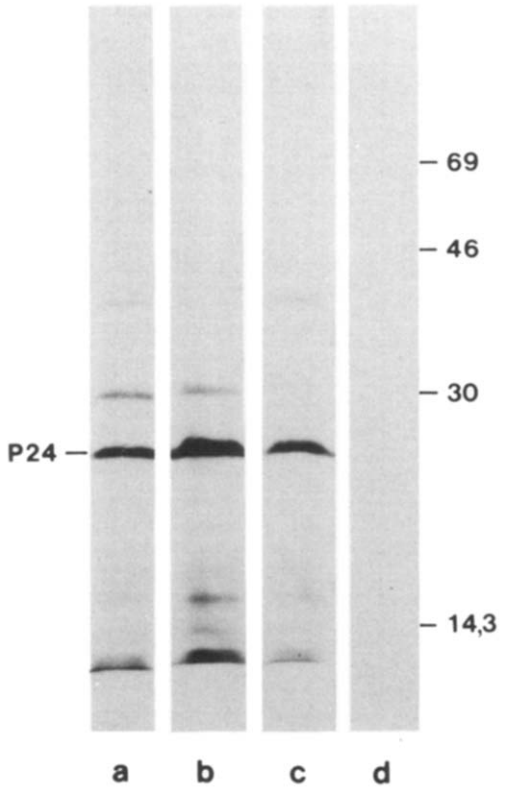

Fig. 4. Translation of *Galleria* PSG mRNA in a wheat germ cell-free system. The translation products were labeled with [^{14}C]leucine (a), [^{14}C]alanine (b), [^{14}C]glycine (c) and with [^{14}C]glycine without PSG mRNA added (d). Equal amounts of radioactive material (7000 cpm) were loaded into each slot of the gel. Proteins were denatured and separated on the gel as described in Fig. 3. Size of marker proteins as in Fig. 3.

Cross hybridization of Bombyx and Galleria PSG RNAs

Since both *Galleria* and *Bombyx* PSG mRNAs contain 1100 nucleotide long mRNAs we assayed by heterologous hybridization of northern blots of *Galleria* mRNA with *Bombyx* PSG cDNA probes. The data presented in Figs 2d and 2e show that *Bombyx* PSG cDNA hybridized to several RNA species from *Galleria* PSG mRNA. Only one of these bands was detectable when total RNA was probed (Fig. 2e). However there was no homology between the 1100 nucleotide RNAs from *Bombyx* and *Galleria* even under conditions of low stringency (50°C; 1 × SSC). The same conclusion can be drawn from dot blot analysis of *Galleria* PSG RNA with pBmP25–11 clones (Couble *et al.*, 1985) coding for *Bombyx* P-25 proteins (data not shown).

Characterization of PSG mRNA translation products

In order to characterize further the PSG mRNAs they were translated in a rabbit reticulocyte lysate cell-free translation system using [^{14}C]leucine as marker. The *in vitro* translated proteins were analyzed by electrophoresis on 15% acrylamide gel under denaturing conditions.

Cell-free translation of *Galleria* PSG mRNA yielded many different proteins. Of these, a 24 kD protein is the most abundant; an unidentified 13 kD protein is the next most abundant, followed by a 30 kD band (Fig. 3a). As noted earlier, 24, 29 and 30 kD proteins are present in *Galleria* cocoons (Fig. 1c). Furthermore, the proportion of the *in vitro* translated 24 kD protein to the 30 kD protein is similar to that of the corresponding silk proteins in the cocoon (Fig. 1e). *B. mori* PSG mRNA on translation produced proteins which in SDS gels exhibited a pattern nearly identical to that of *Galleria* PSG mRNA except that it lacked the 30 kD band. In addition, cell-free translation of the 10S–18S *Galleria* mRNA fraction yielded 24 and 30 kD polypeptides (Fig. 3b). The 24 kD band was very prominent while the 30 kD band was very faint. This size class proteins were not observed when MSG mRNA was translated *in vitro* under the same conditions (data not shown).

Similar results were obtained when *Galleria* PSG mRNA was translated in a wheat germ cell-free system using [^{14}C]leucine (Fig. 4a), [^{14}C]alanine (Fig. 4b) or [^{14}C]glycine (Fig. 4c) as the label. In all these preparations the 24 kD protein was the main translation product. However, the 30 kD protein was detectable only when [^{14}C]leucine or [^{14}C]alanine were used but not with [^{14}C]glycine (Fig. 4c). In controls lacking PSG mRNA no labeled proteins were observed (Fig. 4d). These results indicate that depending on the radioactive precursor used there are significant differences in the efficiency of labeling proteins in cell-free translation system. These observations also suggest that the 24 and 30 kD proteins differ significantly in their amino acid composition.

The presence of 24, 29 and 30 kD proteins in the cocoon of *Galleria*, together with the abundance of PSG specific 1100 nucleotides long mRNAs that produce on translation proteins of similar size, strongly suggest that the silk proteins in question are encoded by these mRNAs.

In conclusion, the proteins of the cocoon of *Galleria* consist of three low molecular weight silk proteins which are probably bound to fibroin. They are encoded by abundant PSG specific mRNAs as in *Bombyx*. However, *Bombyx* and *Galleria* 1100 nucleotide mRNA do not bear any significant nucleotide sequence homology. These observations suggest that the incidence of such small proteins in silk-producing Lepidoptera is common although reported earlier (Shimura *et al.*, 1976; Couble *et al.*, 1983). The role of such proteins in stabilizing silk fibers in a cocoon remains to be elucidated. However, the fact that the RNAs coding for these low molecular weight proteins do not exhibit sequence homology suggests that even though their function is conserved these genes might have diverged in the course of evolution.

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