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Identification of storage-protein messenger RNA of the fleshfly *Sarcophaga peregrina*

Torakazu TAHARA, Yoichi MAEDA, Atsushi KUROIWA, Kohji UENO, Masuo OBINATA and Shunji NATORI*
Faculty of Pharmaceutical Sciences, University of Tokyo, Bunkyo-ku, Tokyo 113, Japan

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Storage-protein mRNA was found to be abundant in poly(A)-containing RNA extracted from the fat-body of third-instar larvae of *Sarcophaga peregrina* (fleshfly). This RNA sedimented at the position of 19S on sucrose-density-gradient centrifugation and the product of its translation *in vitro* was 75K protein (protein of mol.wt. 75000), which was precipitated specifically with antibody against storage protein. This product was suggested to contain a signal sequence that is missing in mature storage protein. The poly(A)-containing RNA was also found to contain much of another mRNA coding for 25K protein (protein of mol.wt. 25000), but the function of this protein is unknown.

Storage protein of holometabolous insects is synthesized in the fat-body during the larval feeding stage and is secreted into the haemolymph (Wyatt & Pan, 1978). It is used as a source of amino acids and energy for the synthesis of new proteins during metamorphosis (Thomson, 1975). This protein shows a characteristic behaviour on pupation (Martin *et al.*, 1971). In larvae of the fleshfly *Sarcophaga peregrina*, storage protein amounts to 70–80% of the total haemolymph protein at the end of the third instar. On puparium formation, it is selectively taken up again by the fat-body and incorporated into large granules in fat-body cells (Ueno & Natori, 1982). On histolysis of the fat-body during metamorphosis, most of the storage protein seems to be degraded *in situ* and the resulting amino acids are released into the haemolymph.

The reabsorption of storage protein by fat-body cells was found to be regulated by the moulting hormone 20-hydroxyecdysone (Ueno & Natori, 1982). So this system is a good model of uptake of serum protein by eukaryotic cells.

We are interested in the mechanism regulating the synthesis of storage protein, since the protein is so abundant in the haemolymph of third-instar larvae. Its synthesis may be regulated at the level of transcription or translation. It is also possible that the storage-protein gene is amplified manyfold during the larval stage, like the chorion gene of the fruitfly *Drosophila melanogaster* during oogenesis (Spradling & Mahowald, 1980). As a first step in studies on expression of the storage protein, we tried to identify storage-protein mRNA in poly(A)-containing RNA of the fat-body of *S. peregrina* larvae. We found that the main translation product of poly(A)-containing RNA *in vitro* is 75K protein, which is the same size as the monomer of the storage protein, indicating that storage-protein mRNA is abundant in poly(A)-containing RNA of fat-body cells. This product was specifically precipitated with antibody against storage protein.

* To whom reprint requests should be addressed.

Abbreviations used: SDS, sodium dodecyl sulphate; 75K protein, protein of mol.wt. 75000.

Materials and methods

Materials

S. peregrina was reared by the method of Ohtaki (1966). The fat-body was dissected out from freshly harvested third-instar larvae under a binocular microscope and stored at −80°C. Oligo(dT)–cellulose was prepared by the method of Gilham (1964). A cell-free protein-synthesizing system was prepared from rabbit reticulocytes by the method of Hunt *et al.* (1972). *Staphylococcus aureus* V-8 proteinase was purchased from Miles Laboratories. [^{35}S]Methionine (1030Ci/mmol) was purchased from The Radiochemical Centre (Amersham, Bucks., U.K.).

Preparation of total RNA

Fat-bodies from 40–60 larvae were homogenized in a mixture of 10ml of extraction buffer [0.1M-Tris/HCl, pH9.0, containing 10mM-EDTA, 0.1M-

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dithiothreitol and 2% (w/v) SDS] and 10ml of phenol/chloroform/3-methylbutan-1-ol (25:25:1, by vol.) saturated with extraction buffer. The organic phase was re-extracted with 10ml of extraction buffer and the two aqueous phases were combined. The combined aqueous phase was extracted three times with an equal volume of the phenol/chloroform/3-methylbutan-1-ol mixture described above, and nucleic acids were precipitated with ethanol in the presence of 0.3M-NaCl. The precipitate was dissolved in 1ml of 0.01M-Tris/HCl buffer, pH7.4, containing 10mM-EDTA, 25mM-NaCl and 0.2% SDS. RNA was selectively precipitated by adding LiCl at a concentration of 2M and the resulting precipitate was collected by centrifugation for 30min at 10000*g*.

Preparation of poly(A)-containing RNA

Poly(A)-containing RNA was prepared from total RNA by chromatography on oligo(dT)–cellulose. All preparations were done at 37°C with glassware and reagents that had been autoclaved. About 180 A_{260} units of total RNA was applied to a column (0.9cm × 3.0cm) of oligo(dT)–cellulose which had been equilibrated with washing buffer (0.01M-Tris/HCl, pH7.5, 0.5M-NaCl, 1mM-EDTA and 0.1% SDS). Adsorbed material was eluted stepwise with elution buffer I (0.01M-Tris/HCl, pH7.5, 1mM-EDTA, 0.1M-NaCl and 0.1% SDS) and elution buffer II (elution buffer I, but without NaCl). The RNA eluted with elution buffer II was precipitated with ethanol and used as poly(A)-containing RNA.

Translation of poly(A)-containing RNA in vitro

The incubation mixture (final volume 10μl) contained 6μl of nuclease-treated reticulocyte lysate (Pelham & Jackson, 1976), 20μg of haemin/ml, 10mM-phosphocreatine, 1mg of creatine kinase (P–L Biochemicals)/ml, 1mM-ATP, 0.2mM-GTP, 0.1M-KCl, 2mM-dithiothreitol, 1mM-magnesium acetate, 20mM-Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid], pH7.0, a mixture of 19 amino acids excluding methionine (50μM each), 1μCi of [^{35}S]methionine and 100ng of poly(A)-containing RNA. Incubation was done at 27°C for 120min.

Immunoprecipitation studies

Product analysis of immunoprecipitation was performed by the method of Kessler (1975) by using the Cowan I strain of *Staph. aureus* as an adsorbent of the immunocomplex. To 20μl of translation reaction mixture, 80μl of phosphate-buffered saline (40mM-sodium phosphate, pH7.2, 150mM-NaCl) containing 0.02% NaN_3, 0.1% gelatin and 1% Triton X-100, and 20μl of rabbit antiserum raised against purified storage protein or normal serum, were added. After incubation for 60min at 4°C, 300μl of *Staph. aureus* Cowan I cell suspension was added and incubation was continued for 20min. The cells were collected by centrifugation for 15min at 10000*g* and washed with 3 × 1ml of washing buffer (0.05M-Tris/HCl, pH7.4, 0.15M-NaCl, 5mM-EDTA, 0.02% NaN_3 and 1% Triton X-100). Then the pellet was suspended in 1ml of elution buffer (65mM-Tris/HCl, pH6.8, 2% SDS, 10% glycerol and 5% β-mercaptoethanol) and heated at 100°C for 4min. The mixture was centrifuged at 10000*g* for 15min, and the resulting supernatant was analysed by SDS/polyacrylamide-gel electrophoresis followed by fluorography.

Peptide mapping analysis by SDS/polyacrylamide-gel electrophoresis

This was done by the method of Cleveland *et al.* (1977). Protein samples were first subjected to electrophoresis by the method of Laemmli (1970) and stained with Coomassie Brilliant Blue. Regions corresponding to 75K storage protein were cut out and equilibrated in 0.125M-Tris/HCl buffer, pH6.8, containing 1mM-EDTA, 1mM-β-mercaptoethanol and 10% glycerol. Then each gel slice was placed in a well of another SDS/polyacrylamide slab gel consisting of stacking gel (3% polyacrylamide) and separating gel (15% polyacrylamide). Various amounts of *Staph. aureus* V-8 proteinase were added to the wells, and electrophoresis was carried out at 20°C. Protein was partially digested during electrophoresis, and the resulting peptides were separated on the gel.

Results and discussion

Previously we reported that the haemolymph of third-instar larvae of *S. peregrina* contains numerous particles consisting of storage protein, which has mol.wt. 75000 (Ueno & Natori, 1982). This protein was not detected in the haemolymph of newly hatched larvae, but was found to increase markedly 3 days after hatching, that is early in the third instar, as shown in Fig. 1. Judging from the intensity of protein bands on SDS/polyacrylamide gel, it amounts to more than 70% of the total haemolymph protein at this stage.

Since this protein is very abundant in the haemolymph of third-instar larvae, and since it is synthesized in the fat-body and eventually secreted into haemolymph, we expected that fat-body cells should contain a significant amount of mRNA for this protein. So we extracted poly(A)-containing RNA from the fat-body of third-instar larvae and examined its mRNA activity. In the reticulocyte lysate system, poly(A)-containing RNA isolated from the fat-body stimulated incorporation of amino acids into trichloroacetic acid-precipitable protein by

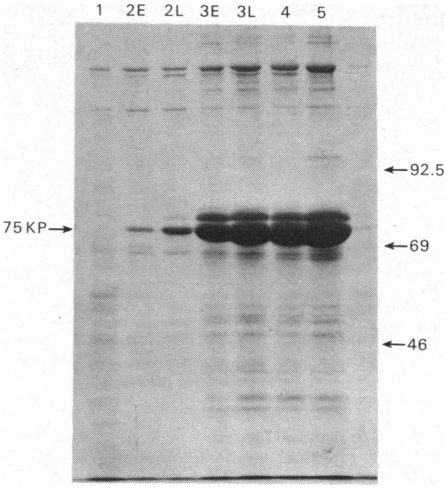

Fig. 1. *Analysis of haemolymph protein of Sarcophaga peregrina larvae*

Haemolymph was prepared from larvae at various times after hatching and 0.1 μl of each sample was subjected to SDS/polyacrylamide-gel electrophoresis. The gel was stained with Coomassie Brilliant Blue to locate proteins. Numbers represent days after hatching; E and L indicate the early and late part of the day respectively. The molecular weight of 75K protein (75KP) was determined by calibrating the gel with phosphorylase *b* (mol.wt. 92500), bovine serum albumin (69000) and ovalbumin (46000).

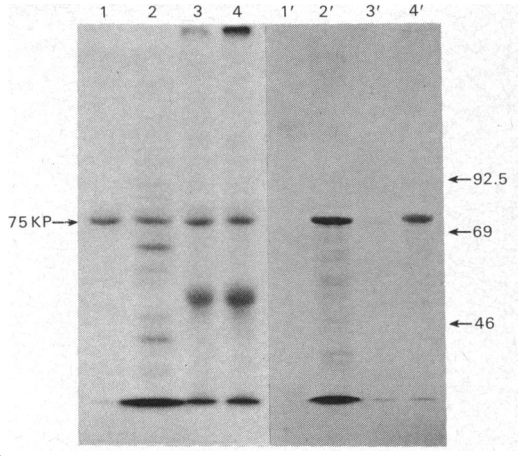

Fig. 2. *SDS/polyacrylamide-gel electrophoresis of the proteins formed in vitro directed by poly(A)-containing RNA*

Poly(A)-containing RNA prepared from fat-body was translated *in vitro* in a reticulocyte lysate. The resulting product labelled with [^{35}S]methionine was mixed with a small amount of haemolymph and subjected to SDS/polyacrylamide-gel electrophoresis. The gel was stained with Coomassie Brilliant Blue to locate proteins (lanes 1–4) and then fluorographed (lanes 1'–4'). Lanes 1 and 1', 0.02 μl of haemolymph; lanes 2 and 2', product of translation *in vitro*; lanes 3 and 3', immunoprecipitate of lane 2 with normal rabbit serum; lanes 4 and 4', immunoprecipitate of lane 2 with antiserum raised against storage protein. The gel was calibrated as described in the legend to Fig. 1.

20–40-fold compared with the value without added mRNA.

The translation product was analysed by SDS/polyacrylamide-gel electrophoresis. From Fig. 2, lane 2', it is obvious that there are two main translation products, and the mobility in polyacrylamide gel of the larger product coincides with that of 75K storage protein. Moreover, when antiserum raised against storage protein was added to the reaction mixture, the larger product was specifically precipitated, as shown in lane 4', whereas when normal serum was added, no significant precipitation was detected, as shown in lane 3'. Thus the larger product was 75K storage protein. From this experiment it is clear that the native storage protein has the same mobility as the product of translation *in vitro*. However, the product *in vitro* should contain the signal sequence that is essential for the protein to be secreted into the haemolymph (Blobel & Dobberstein, 1975). We examined this point by partially digesting both the native storage protein and the product of translation *in vitro* with *Staph. aureus* V-8 proteinase and analysing the resulting peptides by SDS/polyacrylamide-gel electrophoresis. As shown in Figs. 3(*a*) and 3(*b*), the distributions of peptides derived from the native storage protein and those from the product *in vitro*, located by fluorography, were quite similar, indicating that the product is storage protein. However, the peptides indicated by arrows in Fig. 3(*b*) were only detected in proteolytic peptides of the product *in vitro* and were slightly larger than the corresponding peptides of the native storage protein. It seems likely that these peptides are derived from the *N*-terminal of the product *in vitro* and contain a signal sequence that is missing from the native protein. This signal sequence may be small enough not to affect the mobility of the mature protein in the gel.

Clearly storage-protein mRNA is abundant in poly(A)-containing RNA if all mRNA species are translated with the same efficiency. About 23% of the acid-precipitable radioactivity was specifically precipitated with antiserum against storage protein. Thus transcription of the storage-protein gene is probably selectively activated in fat-body cells of

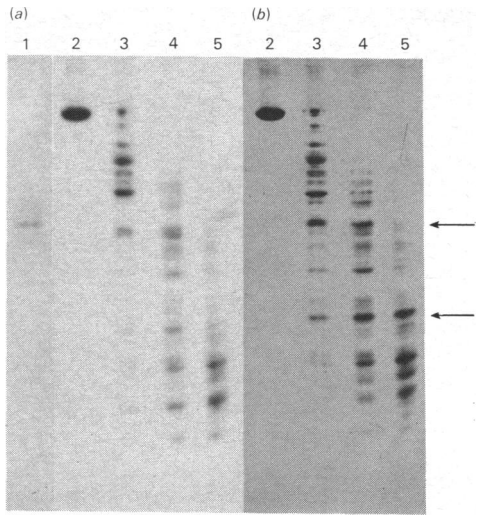

Fig. 3. *Partial digestion of storage protein and the product of translation in vitro*

The product of translation *in vitro* was mixed with 0.04 μl of haemolymph and subjected to SDS/polyacrylamide-gel electrophoresis. The band corresponding to storage protein was cut out from the gel and partially digested in another SDS/polyacrylamide-gel with various amounts of *Staph. aureus* V-8 proteinase. After electrophoresis, the gel was stained with Coomassie Brilliant Blue to locate peptides from unlabelled storage protein (*a*) and then fluorographed (*b*). Lane 2, no proteinase; lanes 3, 4 and 5, peptides obtained by digestion with 5, 50 and 500 ng of V-8 proteinase respectively; lane 1, 500 ng of proteinase alone. Arrows indicate peptides specific to the product of translation *in vitro*.

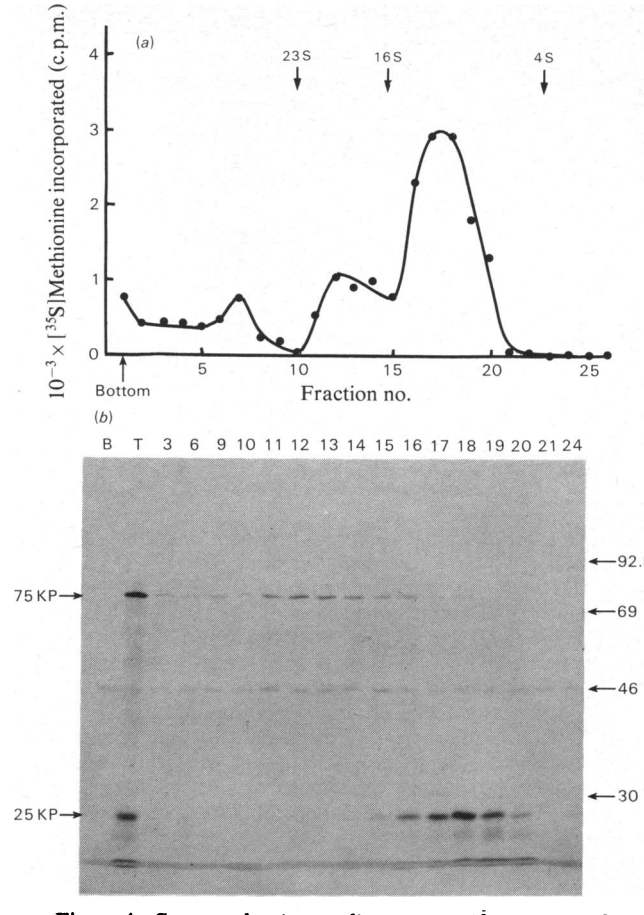

Fig. 4. *Sucrose-density-gradient centrifugation of poly(A)-containing RNA and analysis of the product of translation in vitro*

(*a*) Poly(A)-containing RNA (72 μg), which had been heated at 80°C for 10 min and then rapidly cooled, was layered on top of a 5 ml linear gradient of 5–20% (w/v) sucrose in 0.01 M-Tris/HCl buffer, pH 7.4, containing 0.01 M-EDTA, 0.025 M-NaCl and 0.2% SDS, and centrifuged for 2 h at 60000 rev./min in a Hitachi RPS-65T rotor at 22°C. After centrifugation, the mRNA activity was determined in a rabbit reticulocyte lysate by using 0.7 μl portions of each fraction. As positional markers, 4S, 16S and 23S RNA of *Escherichia coli* were centrifuged under the same conditions. (*b*) Analysis of product of translation *in vitro*. After SDS/polyacrylamide-gel electrophoresis, proteins were located by fluorography. Numbers at the top correspond to fraction numbers in (*a*). The gel was calibrated with various molecular-weight markers as described in the legend to Fig. 1, except that carbonic anhydrase (30000) was added. B, background, i.e. without mRNA; T, total poly(A)-containing RNA used as mRNA.

early third-instar larvae. However, the mechanism regulating expression of the storage-protein gene is totally unknown. Since storage protein is of mol.wt. 75000, we examined the size of storage-protein mRNA by sucrose-density-gradient centrifugation. After centrifugation, each fraction from the gradient was assayed for mRNA activity and the resulting product was analysed by SDS/polyacrylamide-gel electrophoresis followed by fluorography. As shown in Fig. 4(*a*), two main peaks with sedimentation coefficients of about 19S and 11S were found to have mRNA activity. The translation product of 19S-peak material was mainly storage protein and that of 11S-peak material was an unidentified fat-body protein of mol.wt. 25000, as shown in Fig. 4(*b*). Therefore two major mRNA species were separated by this procedure. Storage-protein mRNA was calculated to be 19S and its mol.wt. was estimated as 7.5×10^5, which corresponds to about 2300 nucleotides. This size is sufficient for coding 75K storage-protein.

On acid/urea/agarose-gel electrophoresis, 19S RNA gave a discrete band, and the same band was detected in total poly(A)-containing RNA. It was found that the translation product of RNA extracted from this band was exclusively 75K protein (results

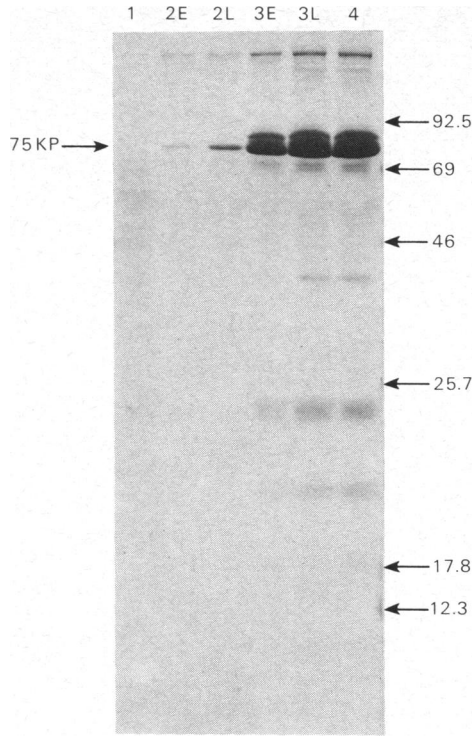

Fig. 5. *Analysis of haemolymph protein by SDS/polyacrylamide-gel electrophoresis*

The experimental procedures and abbreviations were the same as those in the legend to Fig. 1, except that 12.5% polyacrylamide-gel was used. In addition to the usual three marker proteins, α-chymotrypsinogen (mol.wt. 25700), myoglobin (17800) and cytochrome *c* (12300) were used to calibrate the gel.

not shown). Assuming that RNA in this band is entirely storage-protein mRNA, the purity of this mRNA is 19S RNA recovered from sucrose-density-gradient centrifugation should be very high, although it was difficult to quantify the purity.

In this work we found two major mRNA species in fat-body cells of third-instar larvae of *S. peregrina*. One of them was identified as storage-protein mRNA. The other mRNA, coding for 25K protein, was not characterized further, since the nature of that protein is unknown. This protein is probably not a storage protein, because a thick band comparable with that of 75K protein was not detected in the 25000-mol.wt. region on polacrylamide gel when haemolymph was analysed under conditions that ensure that proteins larger than 12300 mol.wt. remain in the gel, as shown in Fig. 5. But it should be pointed out that this mRNA was also very abundant in the fat-body.

Little is known about the gene of the storage protein, although it is known that a tremendous amount of protein is synthesized in a short period at the specific stage of insect development. This kind of protein is specific for holometabolous insects, and the present results should be useful in studies on the mechanism regulating expression of the storage-protein gene.

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