Haemocyanin mRNA from arthropod and mollusc origin

Evidence for a multi-unit structure in mollusc haemocyanin mRNA

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mRNA from two molluscs, the snail *Levantina hierosolima* and the cuttlefish *Sepia officinalis*, and one arthropod, the scorpion *Leiurus quinquestratus*, were found to contain species that hybridize with an oligodeoxynucleotide sequence corresponding to the six-amino-acid sequence His-His-Trp-His-Trp-His postulated to carry binuclear copper in arthropod haemocyanins. The duplexes formed between the oligodeoxynucleotide and mRNA from *Leiurus* and *Levantina* had similar 'melting' temperatures, namely 46.5 and 44.5 °C respectively. The hybridizable mRNA species were identified with mRNA that codes for haemocyanin, Hc mRNA. Electrophoresis under denaturing conditions of *Leiurus* Hc mRNA gave a single band corresponding to 2.3 kb (kilobases), consistent with the value of 75000 reported for the M_r of the polypeptide chain of arthropod haemocyanin. Electrophoresis of mollusc Hc mRNA yielded RNA bands corresponding to 9.4, 6.7, 4.0 and 1.7 kb for *Levantina* and 9.5, 2.8 and 1.7 kb for *Sepia*. The 9.4 and 9.5 kb species represent authentic Hc mRNA and are consistent with an M_r of 350000 for molluscan haemocyanin polypeptide chain. The faster-moving RNA bands are attributed to Hc mRNA cleavage by nucleases during isolation of mRNA. Analysis of the electrophoretic band pattern indicates a multi-unit structure for mollusc Hc mRNA.

INTRODUCTION

Haemocyanins, a class of multisubunit respiratory proteins that contain copper, are of interest because of their occurrence in two very diverse phyla, the arthropods and the molluscs (Van Holde & Van Bruggen, 1971; Lontie & Witters, 1973; Wood, 1980; Van Holde & Miller, 1982; Ellerton *et al.*, 1983). Mollusc and arthropod haemocyanins differ entirely in their architecture and in the mass of protein per oxygen site, 50 kDa for molluscan and 75 kDa for arthropod haemocyanins. Another difference concerns the size of the constituent polypeptide chains. In the arthropods, the chain has M_r of about 75000 and carries a single site for binding oxygen. In contrast, the polypeptide chains of mollusc haemocyanins are very large, each carrying six to eight oxygen-binding sites. There is controversy regarding the exact M_r of the polypeptide chain in mollusc haemocyanins. For cephalopods, the values range from 330000 to 385000 (Van Holde, 1983). For gastropod haemocyanins, many values cluster around 350000 (Van Holde & Miller, 1982); however, values as low as 300000 (Quitter *et al.*, 1978) and as high as 474000 (Lontie, 1983) have been reported. The weight of the evidence for a relationship between molluscan and arthropod haemocyanins rests on a common stoichiometry for the oxygen-binding reaction, $2\text{Cu}:\text{O}_2$, and on indirect evidence, mainly spectroscopic (Himmelfright *et al.*, 1980), regarding a similarity in the environment of the oxygen site.

Sequence determinations of a number of haemocyanins, all of arthropod origin, have singled out a common stretch of six amino acid residues, His-His-Trp-His-Trp-His, as the most likely candidate to carry binuclear copper

responsible for the binding of oxygen (Schneider *et al.*, 1982, 1983; Schartau *et al.*, 1983; Nemoto & Takagi, 1983; Yokota & Riggs, 1984). Recent determination of the three-dimensional structure of haemocyanin from *Panulirus interruptus* provides direct evidence that two out of the four histidine residues in the six-amino-acid stretch serve as ligands for copper (Gaykema *et al.*, 1984). If the oxygen-binding site is the same in arthropod and molluscan haemocyanin, the latter should also carry this six-amino-acid stretch. Whether this is indeed so can evidently best be answered by direct comparison of haemocyanin amino acid sequences. The exceptionally large size of the polypeptide chain has so far, however, precluded the amino acid sequencing of haemocyanin from a molluscan species.

The relationship between mollusc and arthropod haemocyanins can also be examined at the mRNA level. A deoxynucleotide sequence corresponding to the six-amino-acid stretch thought to be involved in the binding of copper in haemocyanin was synthesized and used in hybridization experiments to probe mRNA from two mollusc and one arthropod species. Our findings show that the study of haemocyanin mRNA can be profitable not only in elucidating the relation between haemocyanin from mollusc and arthropod origin, but in shedding light on some open problems in haemocyanin research.

EXPERIMENTAL

Materials

[γ - ^{32}P]ATP (3000 Ci/mmol) and phage- T_4 polynucleotide kinase were obtained from Amersham International.

Salmon sperm DNA was obtained from Sigma. *Hind*III-endonuclease-restricted λ -phage DNA marker and endonuclease *Eco*RI were from Biolabs. Benzoylnaphthoyl-DEAE-cellulose was obtained from Serva, Heidelberg, Germany.

Preparation of mRNA

Snails (*Levantina hierosolima*) were collected from Yaar Hanassi near Jerusalem. Scorpions (*Leiurus quinquestriatus*) were collected from Negev in the vicinity of Beer Sheva. Cuttlefish (*Sepia officinalis*) were fished in the eastern Mediterranean. Extraction of RNA was carried out from whole *Leiurus* and from the branchial gland of *Sepia* by the phenol/*m*-cresol method of Kirby (1968). Two alternative methods were applied for the extraction of RNA from whole (excluding the shell) *Levantina*, the urea/LiCl method (Auffray *et al.*, 1980) and the guanidinium thiocyanate/CsCl method (Glisin *et al.*, 1974). Poly(A)⁺ mRNA was isolated by oligo(dT)-cellulose chromatography (Aviv & Leder, 1972).

Preparation of oligodeoxynucleotide probe

Oligodeoxynucleotide probe was synthesized for us by Dr. J.J. Rossi by the phosphotriester method (Miyoshi *et al.*, 1980). Labelling of the 5'-end of the probe followed the protocol of Maxam & Gilbert (1980). A 44.8 pmol sample of the oligodeoxynucleotide probe was incubated with 100 μ Ci of [γ -³²P]ATP (30 pmol) and T4 polynucleotide kinase (3 units) in a 25 μ l reaction mixture containing 50 mM-Tris/HCl buffer, pH 7.6, 10 mM-MgCl₂, 0.1 mM-spermidine and 0.1 mM-EDTA. After 60 min at 37 °C, the reaction was stopped by addition of 5 μ l of 0.2 M-EDTA and the mixture was loaded on a benzoylnaphthoyl-DEAE-cellulose column in a plastic Gilson pipette tip equilibrated with 0.3 M-NaCl/1 mM-EDTA/0.01 M-Tris/HCl, pH 7.4. The column was extensively washed with equilibration buffer, and the 5'-³²P-labelled probe was eluted with 15% (v/v) ethanol/1 M-NaCl/1 mM-EDTA/0.01 M-Tris/HCl, pH 7.4, and used directly for hybridization. Specific radioactivities of about (3–4) $\times 10^5$ c.p.m./pmol were obtained.

Sucrose-gradient fractionation

Poly(A)⁺ mRNA was fractionated as described by Boedtker & Lehrach (1976). Preformed 11 ml 5–20%-(w/v)-sucrose gradients in 85% (v/v) formamide, containing 20 mM-Tris/HCl (pH 7.4)/1 mM-EDTA, were loaded with 50 μ g of poly(A)⁺ mRNA. The RNA was previously dissolved in 20 mM-Tris/HCl/1 mM-EDTA, pH 7.4, heated for 3 min at 70 °C and rapidly cooled. The gradient was centrifuged for 40 h at 284 000 *g* (*r*_{av}, 10.95 cm) in a SW 41.1 rotor at 20 °C. RNA was recovered by ethanol precipitation and dissolved in 20 μ l of 10 mM-Tris/HCl, pH 7.4.

Electrophoresis

Electrophoresis was carried out in 0.8% agarose gels (15 cm $\times$ 20 cm $\times$ 0.8 cm) in the presence of 6% (v/v) formaldehyde as described by Lehrach *et al.* (1977). A voltage of 170 V was applied for the time needed for the Bromophenol Blue marker to migrate 13 cm.

Hybridization

Hybridization of mRNA with ³²P-labelled oligodeoxynucleotide probe was carried out on nitrocellulose filters.

The filters were prewashed with 20 $\times$ SSC (1 $\times$ SSC = 0.15 M-NaCl/0.015 M-trisodium citrate) and dried in air. mRNA (2–10 μ g) was spotted directly on nitrocellulose filter (dot hybridization) or transferred from agarose gel after electrophoresis. In the latter case, the gel was soaked for 30 min in 20 $\times$ SSC, and the RNA was blotted on to the nitrocellulose filter in 20 $\times$ SSC for 16 h at room temperature (Thomas, 1980). The filters with the RNA were air-dried for 1 h and baked for 3 h at 70 °C. The filters were then washed for 4 h at 37 °C with hybridization buffer: 6 $\times$ SSC, 10 $\times$ Denhardt's (1966) solution (1 $\times$ Denhardt's solution = 0.02% bovine serum albumin/0.02% Ficoll/0.02% polyvinylpyrrolidone), 50 μ g of sheared salmon sperm DNA/ml, 0.1% sodium pyrophosphate and 0.1% sodium dodecyl sulphate. RNA on the filter was then hybridized with the ³²P-labelled oligodeoxynucleotide probe (5 $\times 10^6$ c.p.m.) in hybridization buffer in sealed plastic bags at 37 °C for 24 h. The filters were removed from the hybridization solution, washed for 20 min at room temperature three times with 6 $\times$ SSC, dried, and counted for radioactivity or exposed to Kodak XR-1 film at –70 °C for 1–4 days.

Thermal denaturation of oligodeoxynucleotide-mRNA duplexes

Nitrocellulose filters containing mRNA hybridized with ³²P-labelled oligodeoxynucleotide and thoroughly washed at 21.5 °C with 6 $\times$ SSC (eight washes for 5 min each) were used for thermal-denaturation study (Wallace *et al.*, 1979). The filter was covered with 5 ml of 6 $\times$ SSC in a small glass container, which was then kept for 5 min in a fixed-temperature bath. The filter was removed, dried in air for 1 min, and the radioactivity was counted. The procedure was repeated at several increasing temperatures. From the radioactivity measured at each temperature, the fraction of duplex denatured was determined.

RESULTS

Synthesis of oligodeoxynucleotide probe

A set of octadecadeoxynucleotides complementary to the six-amino-acid sequence postulated to be involved in binding binuclear copper in haemocyanin was chemically synthesized. All 16 deoxynucleotide sequences consistent with code degeneracy (Fig. 1) were included in the mixture that served as the probe.

Hybridization of oligodeoxynucleotide probe with Hc mRNA from *Leiurus*

Poly(A)⁺ mRNA from whole *Leiurus* scorpion, an arthropod, was examined for its ability to hybridize with oligonucleotide probe on a nitrocellulose filter. The poly(A)⁺ mRNA was fractionated on a sucrose gradient, and samples from each fraction were spotted on

	His	His	Trp	His	Trp	His	
mRNA	5' – CA _C ^U	CA _C ^U	UGG	CA _C ^U	UGG	CA _C ^U	3'
cDNA	3' GT _G ^A	GT _G ^A	ACC	GT _G ^A	ACC	GT _G ^A	5'

Fig. 1. Putative amino acid sequence involved in binding of copper in haemocyanin and nucleotide sequences of the corresponding mRNA and cDNA

Fig. 2. Distribution of *Leiurus* Hc mRNA in sucrose-gradient centrifugation

Poly(A)⁺ mRNA from whole *Leiurus* was fractionated. Haemocyanin mRNA was detected by RNA dot hybridization with ³²P-labelled deoxynucleotide probe. Radioactivity retained on the nitrocellulose filter is plotted against fraction number.

nitrocellulose filters and hybridized with the 5'-³²P-labelled oligodeoxynucleotide probe. Fig. 2 shows a peak of hybridization of the probe with an RNA molecule migrating closely together with 18 S rRNA. The species of mRNA that hybridizes with the probe was identified with mRNA that codes for haemocyanin, Hc mRNA (see the Discussion section).

mRNA from whole *Leiurus* was electrophoresed in agarose gel under denaturing conditions, blotted, and Hc mRNA was detected by hybridization with ³²P-labelled oligodeoxynucleotide probe. A single band of Hc mRNA with mobility corresponding to 2.3 ± 0.1 kb was observed (Fig. 3).

Hybridization of oligodeoxynucleotide probe with Hc mRNA from *Levantina*

The question as to whether the deoxynucleotide probe can hybridize with Hc mRNA from a mollusc was examined next. Total poly(A)⁺ mRNA from the snail *Levantina* was electrophoresed on a denaturing agarose gel, transferred on to a nitrocellulose filter and hybridized with the ³²P-labelled probe. Four bands, of lengths 9.4 ± 0.4 , 6.7 ± 0.5 , 4.0 ± 0.4 and 1.7 ± 0.1 kb, were generally obtained (Fig. 3).

Examination of the electrophoretic patterns of *Levantina* Hc mRNA preparations obtained by the urea/LiCl method or by the guanidinium thiocyanate/CsCl method showed that the relative intensities of the four bands are not conserved. In some preparations, some of the bands were altogether absent. The mobilities associated with the bands are, in contrast, constant.

Thermal stability of oligodeoxynucleotide-Hc mRNA duplexes

A comparison of the thermal stabilities of the duplexes of oligodeoxynucleotide probe with Hc mRNA from *Leiurus* and *Levantina* was performed. The results,

Fig. 3. Electrophoresis of *Leiurus* and *Levantina* Hc mRNA

Poly(A)⁺ mRNA from whole *Leiurus* (a) and poly(A)⁺ mRNA prepared by the urea/LiCl method from whole *Levantina* (b) were electrophoresed in formaldehyde agarose gel and blotted on to nitrocellulose filter. Hc mRNA was detected by hybridization with ³²P-labelled oligodeoxynucleotide probe. Indicated on the left are nucleotide lengths in kb determined from ³²P-labelled DNA markers electrophoresed in the same gel: λ Charon 4A DNA restricted with *Eco*RI; λ -phage DNA restricted with *Hind*III and *Eco*RI endonucleases; pBR322 DNA restricted with *Hinf*I and *Eco*RI endonucleases.

Fig. 4. Thermal denaturation of oligodeoxynucleotide-Hc mRNA duplexes

Poly(A)⁺ mRNA from *Leiurus* (a) and *Levantina* (b) were hybridized with ³²P-labelled oligodeoxynucleotide probe and the duplexes subjected to thermal denaturation.

Fig. 5. Electrophoresis of *Sepia* Hc mRNA

Left: poly(A)⁺ mRNA from two preparations of branchial glands of *Sepia* (a and b) were electrophoresed in formaldehyde agarose gel. Detection of Hc mRNA was as described in Fig. 3. Indicated on the right are nucleotide lengths in kb determined from ³²P-labelled DNA markers electrophoresed in the same gel: λ Charon 4A DNA restricted with *Eco*RI; pBR322 DNA restricted with *Hinf*I. Right: densitometric scans of lanes (a) and (b) (O is the origin).

expressed as plots of percentage of denatured duplex as a function of temperature, are presented in Fig. 4. From the plots, values for T_d , the temperature at which half the duplex is dissociated, can be determined. For the duplex of oligodeoxynucleotide and Hc mRNA from *Leiurus*, a value for T_d of 46 °C was obtained. A similar value, $T_d = 44.5$ °C, was determined for the duplex involving Hc mRNA from *Levantina*.

Hybridization of oligodeoxynucleotide probe with Hc mRNA from *Sepia*

The synthetic oligodeoxynucleotide was also used to probe the presence of Hc mRNA in a cephalopod mollusc, *Sepia*. Total poly(A)⁺ mRNA was isolated from the branchial gland of *Sepia*, electrophoresed on agarose gel, transferred on to nitrocellulose and hybridized with the probe. The electrophoretic pattern obtained is shown in Fig. 5. Bands corresponding to nucleotide lengths of 9.5 ± 0.3 , 2.8 ± 0.4 and 1.7 ± 0.1 kb were obtained.

DISCUSSION

A major finding of the present study is the detection in two molluscs, *Sepia* and *Levantina*, and one arthropod, *Leiurus*, of mRNA species that hybridize with the octadecadeoxynucleotide probe corresponding to the six-amino-acid sequence His-His-Trp-His-Trp-His. This amino acid sequence has been found in chains a, e (Schneider *et al.*, 1982, 1983) and d (Schartau *et al.*, 1983) of *Eurypelma californicum*, chains II (Yokota & Riggs, 1984) and IIIB (Schneider *et al.*, 1982) of *Limulus polyphemus* and in the α -chain of *Tachypleus*

tridentatus (Nemoto & Takagi, 1983) haemocyanins, all of arthropod origin. Further, the duplexes formed by the oligodeoxynucleotide probe with Hc mRNA from *Leiurus* and *Levantina* have similar thermal stabilities, showing that the nucleotide sequences involved in hybridization in the Hc mRNA species from the two phyla are similar. Our results constitute a first indication, although indirect, that the coding sequence for the six-amino-acid stretch is present in mollusc as well as in arthropod Hc mRNA. This finding is consistent with the identification of the six-amino-acid stretch as the site of attachment of binuclear copper involved in the oxygen-binding function.

Electrophoresis of *Leiurus* Hc mRNA yields a single band corresponding to 2.3 kb. In a previous study (Avissar *et al.*, 1983), we identified Hc mRNA from *Leiurus* by translation *in vitro* and immunoprecipitation of the translation products, and determined its size as approx. 2 kb by sucrose-gradient centrifugation. The size of *Leiurus* Hc mRNA determined in the present study is therefore in agreement with our previous determination. The constituent polypeptide chains of *Leiurus* haemocyanin, like those of other arthropod haemocyanins, have M_r values of about 75000. This value corresponds to about 700 amino acid residues, taking 100 as the average M_r of an amino acid residue. To code a polypeptide chain of this length, a message of about 2100 nucleotides would be needed. The observed size of the mRNA coding for haemocyanin is thus consistent with expectation.

The electrophoretic patterns of Hc mRNA from *Levantina* and from *Sepia* reveal a number of bands. Considering the observed size homogeneity of the mollusc haemocyanin polypeptide chains, it is plausible

that only one of the RNA bands corresponds to authentic Hc mRNA. The range of M_r values reported for mollusc haemocyanins, namely 300000–474000, leads one to expect that the length of authentic Hc mRNA would fall in the range 8.5–13 kb. The slowest-moving band in the electrophoresis of *Levantina* Hc mRNA (band a in Fig. 3b) corresponds to 9.4 kb and represents therefore authentic Hc mRNA. Similarly, a value of 9.5 kb is determined for *Sepia* authentic Hc mRNA (band a in Fig. 5). The sizes of the authentic Hc mRNA in *Levantina* and *Sepia* are consistent with a polypeptide chain of M_r 350000. The faster-moving bands (b, c, d in *Levantina*, Fig. 3b, and b, c in *Sepia*, Fig. 5) probably represent products of nuclease cleavage of authentic Hc mRNA during isolation. This conclusion is supported by the variable relative intensities of the bands in the different mRNA preparations.

A remarkable feature of the electrophoresis of *Levantina* and *Sepia* Hc mRNA is the discreteness of the band pattern. Examination of our data shows that the fastest-moving band in *Levantina* and *Sepia* Hc mRNA corresponds to 1.7 kb. This nucleotide segment seems to be relatively resistant to further nuclease action. We assume that the protection against nucleolytic cleavage is the result of folding and that the folded 1.7 kb nucleotide chain segment constitutes a structural unit of Hc mRNA. If the structural unit were to contain a message of 1.5 kb and a poly(A) chain of 0.2 kb, the slower-moving bands can be attributed to combinations of two, four and six structural units. Under this assumption, we calculate for the Hc mRNA electrophoretic band components expected values of 1.7, 3.2, 6.2 and 9.2 kb, in reasonable agreement with the observed values. Analysis of our data thus leads to a six-unit structure for authentic mollusc Hc mRNA.

The structure of Hc mRNA that emerges from the present study has its counterpart in the structure of the polypeptide chain of molluscan haemocyanin. A number of studies [for a review, see Van Holde & Miller (1982)] have indicated that a polypeptide chain from mollusc haemocyanin is folded into six to eight functional units. Each unit has an M_r of about 50000 and carries one oxygen-binding site. To code a polypeptide sequence of this size, a message of about 1.5 kb would be needed. This value is consistent with the length of the message carried in a structural unit of Hc mRNA determined in the present study. We thus have a correspondence between the structural unit of Hc mRNA and the functional unit of haemocyanin on the one hand and, on the other, between the multi-unit structure of the nucleotide chain of the Hc mRNA and the multi-unit structure of the polypeptide chain of haemocyanin.

In this work, the usefulness of the oligodeoxynucleotide probe corresponding to His-His-Trp-His-Trp-His in studying haemocyanin mRNA has been demonstrated. Such a synthetic oligodeoxynucleotide probe can also be a powerful tool in screening clones of DNA complementary to haemocyanin mRNA, as has recently been reported by Wood & Dixon (1985), opening the way toward the study of the haemocyanin gene.

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