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cDNA cloning and sequencing of tarantula hemocyanin subunits

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Summary. Tarantula heart cDNA libraries were screened with synthetic oligonucleotide probes deduced from the highly conserved amino acid sequences of the two copper-binding sites, copper A and copper B, found in chelicerate hemocyanins. Positive cDNA clones could be obtained and four different cDNA types were characterized.

Key words: Hemocyanin - cDNA - Oligonucleotide screening - Sequences - Copper-binding site

Introduction

Hemocyanins are oxygen-carrying binuclear copper proteins that are widely distributed in arthropod and molluscan phyla. The hemocyanin of the tarantula *Eurypelma californicum* is composed of 24 subunits which belong to seven different types (a-g) with molecular masses ranging over 68-74 kDa (Schneider et al. 1977). These seven polypeptide chains differ in amino acid composition, electrophoretic mobility, and in their immunological properties. The complete primary structures of subunits d and e have been determined by protein sequence analysis (Schneider et al. 1983; Schartau et al. 1983). In order to determine the amino acid sequence of all seven subunit types found in the tarantula hemocyanin, primary structure analysis is now being performed via the corresponding cDNAs that were isolated from tissue-specific tarantula cDNA libraries.

Sites of hemocyanin biosynthesis have been identified in different tissues depending on the species studied: in cyanoblasts accumulating behind the compound eye (*Limulus polyphemus*; Fahrenbach 1970), in the gizzard, and in the hepatopancreas of *Carcinus maenas* (Ghiretti-Magaldi et al. 1977), *Cancer pagurus, Astacus leptodactylus* (Préaux et al. 1986), and *Homarus americanus* (Senkbeil and Wriston 1981). In molluscs the branchial glands of cephalopodes (Préaux et al. 1986; Vandamme et al. 1987) and the pore cells of gastropodes (Wood et al. 1981; Sminia and Vlugt-van Daalen 1977) were found to be specialized in hemocyanin synthesis.

In the tarantula, hemocyanin is synthesized in hemocytes attached to the inner heart wall, as shown by immunohistochemistry. Extensive proliferation of hemocytes is observed subsequent to bleeding the animal (Kempter 1983). In previous studies the quantity of hemocyanin mRNA in tarantula heart tissue has been determined by using in vitro translation systems: about 7% of the in vitro translation products could be immunoprecipitated using antiserum against dissociated 37S *Eurypelma* hemocyanin (Voit and Schneider 1986). Knowing this, hemocyanin-enriched cDNA libraries were established.

Experimental procedures and results

Synthesis and cloning of cDNA

About 50 µg total RNA, obtained from one spider heart, were used for synthesis of double-stranded cDNA according to the method of Gubler and Hoffman (1983). The double stranded cDNA was either tailed homopolymer and inserted into the *Pst*I site of plasmid vectors pUC9/19 (Maniatis et al. 1982) or cloned into phage λgt10 DNA subsequent to *Eco*RI linker ligation (Huynh et al. 1985). Four independent plasmid cDNA libraries were established containing a total of about 40000 recombinants. Another two cDNA libraries were constructed in phage λgt10 resulting in 240000 and 300000 recombinant phages.

Screening of the cDNA libraries

Screening of the libraries was performed with two mixed 17-mer synthetic oligonucleotide probes. The probes were deduced from the well known amino acid sequences -His-His-Trp-His-Trp-His- and -His-Asn-

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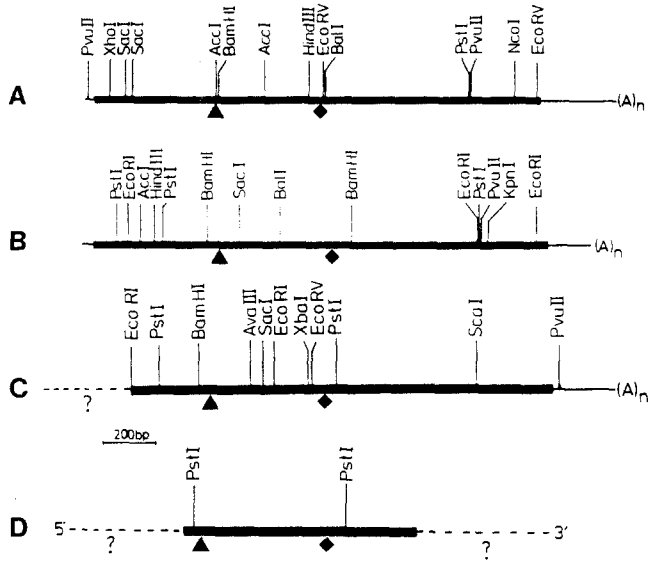

Fig. 1. Restriction maps of cDNAs encoding different tarantula hemocyanin subunits. (**A**) Full-length cDNA for subunit e; (**B**) full-length cDNA for subunit a; (**C**) partial cDNA from clone N22; (**D**) partial cDNA from clone pHC312. The positions of copper A (▲) and copper B (◆) binding sites are marked. Solid bars indicate the coding regions, thin lines indicate the flanking 5′ and 3′ untranslated regions

Trp-Gly-His-Val- which form part of the 'copper A' (CuA) and 'copper B' (CuB) oxygen-binding site in chelicerate hemocyanins (Linzen et al. 1985). From a total of 600000 original recombinants, about 150000 cDNA clones were probed with the oligonucleotide mixtures, 71 positive clones being obtained.

Characterization of hemocyanin cDNA clones

In the plasmid libraries three different clones (pHC4, pHC234, pHC312) hybridized with the radioactively labeled synthetic CuA oligonucleotide probe. The cDNA size of each clone was analyzed by *Pst*I digestion but none of those analyzed was long enough to include the coding regions with a predicted length of 1900 bp. Restriction mapping of the cDNAs revealed distinct patterns of restriction sites, demonstrating that each cDNA was coding for a particular hemocyanin subunit type (Fig. 1). In order to obtain full-length cDNAs, the phage cDNA libraries were screened using the cDNAs described above as subunit-type-specific hybridization probes. From the several clones hybridizing to pHC4 and pHC234 cDNA, the ones having the longest inserts were selected for further analysis. The nucleotide se-

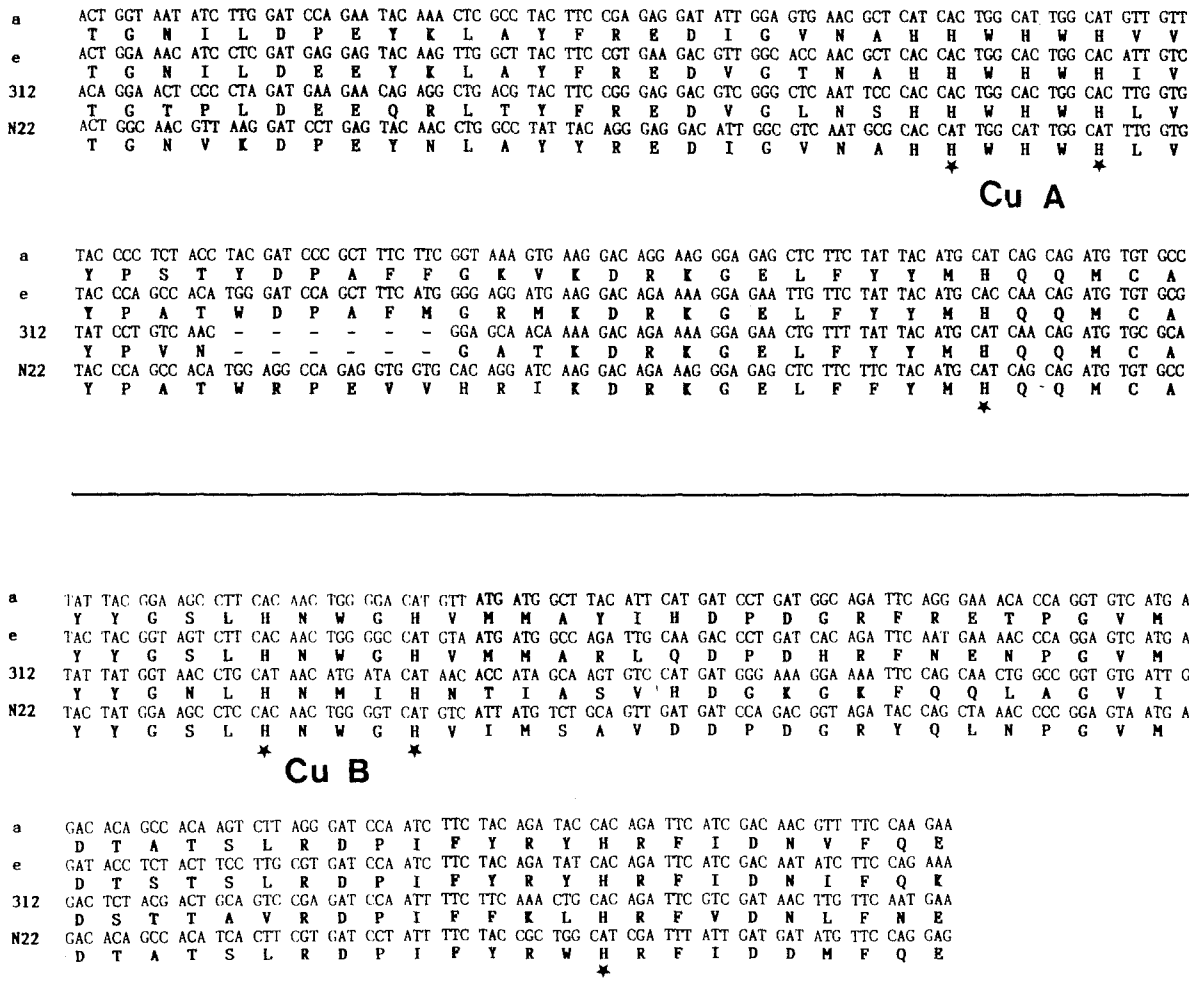

Fig. 2. Nucleotide and deduced amino acid sequences of the highly conserved copper A and copper B regions. The copper-binding histidine residues are marked by asterisks

quences of the 2.2-kb cDNA of clone M1 (homologous with pHC4) and of the 2.1-kb cDNA of clone K1 (homologous with pHC234) were determined by the dideoxy-chain-termination technique (Sanger et al. 1977) after construction of overlapping subclones. Sequence analysis showed that both phage clones contained the entire coding region for hemocyanin subunit a (K1) and subunit e (M1). They were assigned by comparing the amino acid sequences deduced from cDNA and data available from protein sequence analysis (Linzen et al. 1985). Clone pHC312 represents only a partial cDNA. So far, no full-length cDNA encoding the same subunit type could be identified in the plasmid and phage libraries by using cDNA pHC312 as a hybridization probe. Subsequent cDNA sequence analysis revealed that the cDNA exhibits a different copper B binding sequence, -His-Asn-Met-Ile-His-Asn- (Fig. 2).

Out of all CuA-positive cDNA clones selected from the phage libraries one particular clone, called N22, has been characterized in detail (Fig. 1). At present it is still unclear which hemocyanin subunit types are encoded by cDNA pHC312 and N22. In vitro transcription of these cDNAs and translation analysis followed by characterization with specific antibodies against the different hemocyanin subunits should solve this problem. However, it is evident from the pronounced sequence similarity of these two unidentified types to the sequences of subunits a and e that both cDNA types code for another two tarantula hemocyanin subunit types. Again, the highest degree of sequence similarity is found near the two copper-binding sites (Fig. 2). All three copper-binding histidine residues are invariant and conserved in copper A and copper B binding sites throughout all sequences.

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