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Characterization of the full length mRNA coding for *Lucina pectinata* HbIII revealed an alternative polyadenylation site

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Abstract

Lucina pectinata is a bivalve mollusk that lives in the Southwestern coast of Puerto Rico and houses intracellular symbiotic bacteria. This peculiar organism contains three types of hemoglobin, each characterized by distinct physico-chemical properties. Hemoglobin I (HbI) is a sulfide-reactive protein that reacts with H_2S to form ferric hemoglobin sulfide. In contrast, hemoglobin II and III are oxygen-reactive proteins that remain oxygenated in the presence of hydrogen sulfide. The partial coding region contained in the cDNA sequences we have cloned confirmed the *L. pectinata* HbIII amino sequence reported in the NCBI protein database) with a single amino acid difference (Asn72Asp; AsnE12Asp). The characterization of the full length mRNA coding for *L. pectinata* HbIII revealed an alternative polyadenylation site and an alternate transcription start site. The open reading frame (ORF) of the HbIII cDNA is composed of 459nts containing 153 codons. The initiation codon is preceded by 62 nts of untranslated region (5'UTR), whereas two 3'UTR regions of 640 nt and 455 nt long were identified, revealing the presence of alternative polyadenylation sites. Isoforms of the 3'UTR of HbIII only differed in the length of their sequences. It has been hypothesized that alternative polyadenylation acts through shortening of mRNA to regulate RNA localization, translation and stability. Interestingly, the HbIII mRNA is the only one of all the hemoglobin mRNAs from *L. pectinata* characterized so far with more than one 3'UTR. Primer extension products suggest two closely located start sites of HbIII mRNA transcription. We suggest that the *L. pectinata* hemoglobin genes may be under different cellular controls that direct them to exert their particular functions. These hypotheses need to be tested by functional studies and analysis of the regulatory elements of the cognate genes for *L. pectinata* hemoglobins.

Keywords: Alternative polyadenylation; Invertebrate hemoglobins; *Lucina pectinata*; mRNA isoform; HbIII

1. Introduction

The bivalve mollusk *Lucina pectinata* represents a peculiar organism belonging to the Lucinidae family. This bivalve mollusk lives in the Southwestern coast of Puerto Rico and houses intracellular symbiotic bacteria. The intracellular bacteria are considered a symbiotic sulfide-oxidizing chemoautotroph that needs to be supplied with both hydrogen sulfide and oxygen. *L. pectinata* contains three types of hemoglobin, each characterized by distinct physico-chemical properties. These hemoglobins transport oxygen and hydrogen sulfide from water to the bacterial endosymbiont, allowing the bacteria to sustain rapid hexose synthesis and supply the host animal with its entire carbohydrate nutrition (Arp, 1991; Kraus and Wittenberg,

Abbreviations: AMV, Avian Myeloblastosis Virus; ATP, adenosine 5'-triphosphate; bp, base pair; cDNA, complementary deoxyribonucleic acid; CO, Carbon dioxide; cpm, counts per min; Da, dalton; EDTA, ethylenediamine tetraacetic acid; FTIR, Fourier transform infrared spectroscopy; GSP, gene specific primers; HbI, Hemoglobin I; HbII, Hemoglobin II; HbIII, Hemoglobin III; MALDI-MS, Matrix-Assisted Laser Desorption/Ionization-Mass Spectrometry; Mb, myoglobin; mM, millimolar; mRNA, Messenger Ribonucleic Acid; NCBI, National Center for Biotechnology Information; nt, nucleotides; ORF, open reading frame; PCR, Polymerase Chain Reaction; RACE, Rapid Amplification of cDNA ends; RPM, Revolutions per minute; RT-PCR, reverse transcriptase-Polymerase Chain Reaction; SDS, Sodium dodecyl sulfate; 3' UTR, 3'-untranslated mRNA region; 5'-UTR, 5'-untranslated mRNA region; UV, Ultraviolet-visible.

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1990). Hemoglobin I (HbI) is a sulfide-reactive protein that reacts with hydrogen sulfide (H_2S) to form ferric hemoglobin sulfide (Kraus and Wittenberg, 1990). HbI is a monomeric hemoglobin of 142 amino acid residues. The *HbI* full-length cDNA sequence consists of 1322 nt (Antommattei et al., 1999). In contrast, Hb II and III are oxygen-reactive proteins that remain oxygenated in the presence of hydrogen sulfide (Kraus and Wittenberg, 1990). HbII is a dimeric hemoglobin of 151 amino acid residues. The *HbII* full-length cDNA sequence consists of 2114 nt (Torres et al., 2003). Until now, none of *L. pectinata* hemoglobin genes have been characterized.

HbII and HbIII have very similar amino acid compositions (percent identity = 64%) but differ significantly from HbI (percent identity = 32%). The similarities of these hemoproteins were further demonstrated by the kinetics of their reactions with ligands. The association rates of O_2 and CO with HbII and HbIII were found to be extremely slow in comparison with other monomeric hemoglobins. Likewise, dissociation rates of O_2 from HbII and HbIII were found to be 0.11 and 0.07 s^{-1}, respectively, which are very slow compared to the dissociation rate of this ligand from many other hemoglobins (Kraus and Wittenberg, 1990). The ligand binding kinetics of invertebrate hemoglobins are strongly influenced by the structure of the heme cavity, particularly the size and polarity of residues occupying the distal portion that exert steric and dielectric effects. In many invertebrate globins, the His E7 and Leu B10 residues are replaced by Gln and Tyr, resulting in a tight cage for O_2 and much higher O_2 binding affinities relative to vertebrate Myoglobin (Mb) (Peterson et al., 1997).

The *L. pectinata* HbII structure has been elucidated by X-ray crystallography (Gavira et al., 2006). Computer model predictions of HbIII structure based on its similarity with HbII, suggest that both have Gln E7 and Tyr B10 in the hemo distal pocket. Native HbII and native HbIII are similar in amino acid sequence and the kinetics of their reactions with oxygen. Nevertheless, these similarities may not extend to the mechanisms of oxygen binding. Mechanisms of oxygen binding for HbII have been proposed but the HbIII mechanisms remain to be determined.

In *L. pectinata*, HbIII tends to form dimers, and when it is in an equimolar mixture with HbII, tretramers are formed at high concentrations (>4 mM) which suggests that the tetramer $(HbII)_2(HbIII)_2$ may exist in tissues (Kraus and Wittenberg, 1990). Several questions may be raised in the *L. pectinata* model system. What is the specific function of each *L. pectinata* hemoglobins in its symbiotic relationship with sulfide-oxidizing chemoautotrophic intracellular bacteria? Why are two oxygen-reactive binding hemoglobins necessary? What are the peculiarities of HbII and HbIII?

It is widely known that similarities at the protein level do not imply similarity at the genomic level. In order to clarify some of these questions, we decided to characterize the full-length mRNA coding for *L. pectinata* HbIII including determination of the length and the nucleotide sequences of the 5' and 3' untranslated regions (UTR) of the processed $mRNA^{HbIII}$ using RACE Methods. Surprisingly, some 3' UTR segments can be more conserved than coding exons reflecting an unexpected selective pressure in this region (Hughes, 2006). Phylogenetic studies of conserved poly (A) sites reported 500 genes with tandem conserved poly (A) sites (Moucadel et al., 2007). Moucadel et al. (2007) hypothesized that conservation of specific alternative 3' ends together with specific 3'UTR elements might reflect novel regulatory mechanisms. We hypothesize that the hemoglobin genes from *L. pectinata* may be under different cellular controls that direct them to exert their particular functions.

2. Materials and methods

2.1. Synthesis and cloning of cDNA

Total RNA from *L. pectinata* was extracted from ctenidias using Trizol reagent in a modification of the RNA isolation method developed by Chomczynski and Sacchi (Chomczynski and Sacchi, 1987). After designing degenerate oligonucleotides using the HbIII amino acid sequence reported in the NCBI protein database (GeneBank accession no. **P41262**), 1 μg of *L. pectinata* total RNA was reverse transcribed and amplified using the Thermostable rTth Reverse Transcriptase RNA PCR kit (Perkin Elmer). Total RNA was incubated with 1 μL of 15 μM HbIIIrev2 primer (Fig. 2C) and 10.4 μL of RNase free water for 10 min at 70 °C. Then, a PCR mix containing: 2 μL of 10× enzyme buffer, 2 μL of 10 mM $MnCl_2$ solution, 0.4 μL of each 10 mM dNTP and 2 μL RTth DNA Polymerase (2.5 U/μL) was added and incubated at 55 °C for 90 min. Polymerase Chain Reaction (PCR) was performed using the retrotranscriptase reaction products as template as recommended by manufacturer. The sequence of the primer HbIIIfow1 corresponds to amino acid residues NGTNFYM (positions 26–32). The sequence for the primer HbIIIrev2 corresponds to amino acid residues WEDFIAY (positions 136–139). The amplified product of 342 nt in size was cloned in the pCRII Topo vector (Invitrogen, Carlsbad, CA). The presence of the cloned insert was verified by colony PCRs. Plasmids were isolated using QIAprep Spin Miniprep Kits (Qiagen) and sequenced in both strands in an ABI 310 automated DNA sequencer using dye terminator chemistry (Big Dye V3 Dye Terminator Sequencing kit, Applied Biosystems). Analysis of the cDNA sequence was performed using the Basic Local Alignment Sequence Tool (BLAST http://www.ncbi.nlm.nih.gov/BLAST/).

2.2. Synthesis and cloning of cDNA ends

Gene specific primers (GSP) derived from the HbIII partial cDNA sequence were designed using the Primer3 software (http://fokker.wi.mit.edu/primer3/input.htm) to obtain the 5' and 3' end of the cDNA by the RACE method. The HbIIIRACER2 (GSP1) was used to obtain the 5' end and HbIIIRACEF (GSP2) to obtain the 3' end (Fig. 2C). Rapid Amplification of cDNA ends (RACE) reactions were carried out using the Marathon cDNA Amplification Kit (Clontech, Palo Alto, CA) as recommended by the manufacturing. The cDNAs synthesized by RACE methods were cloned into the pST Blue-1 vector (Novagen) and sequenced as described in Section 2.1.