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Unique Chimeric Composition of the Trehalase Gene from Brine Shrimp, *Artemia franciscana*

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Abstract: To investigate the exon/intron structure of the *Artemia* trehalase gene, four overlapping clones were isolated from a genome library derived from an inbred strain of crustacean *Artemia franciscana*, and a 49 kb genetic area was re-constructed. The re-constructed area contained eight exons corresponding to the trehalase cDNA sequence that we had previously reported [1]. Comparative analysis of the *Artemia* trehalase gene with other animal trehalase genes revealed the existence of conserved exon/intron boundaries among different phyla. Comparison of the 5' UTR region of trehalase mRNA obtained by the 5' RACE method with the trehalase genes indicated the existence of a novel exon/intron boundary in the region designated "Exon I". Surprisingly, a part of a mitochondrial ribosomal protein gene (MRP-S33) was found to be inserted in the 5' UTR region of the trehalase gene. This sequence had the same polyadenylation signal that the *Artemia MRP-S33* cDNAs did. Using the 3' RACE method, it was demonstrated that the poly (A) additional signal is still functional and that the chimeric mRNAs composed of the 5' UTR of the trehalase mRNA and of the 3' end derived from the MRP-S33 gene are transcribed.

Key words: anhydrobiosis, *Artemia*, brine shrimp, alternative splicing, alternative polyadenylation.

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Introduction

Many organisms accumulate α,α -trehalose as a protectant against desiccation and freezing [2,3]. Dormant *Artemia* cysts also store trehalose and have been shown to be highly resistant to desiccation [4]. After breaking diapause, *Artemia* resume their development and the stock of trehalose is hydrolyzed by trehalase (EC 3.2.1.28) [5].

To date, literature on the genes of animal trehalase has been accumulated. The trehalases reported from mammals were allocated in the small intestine and kidney [6–9]. The trehalases reported from the small intestine are thought to work as a digestive enzyme to catalyze the hydrolysis of trehalose contained in food, such as mushrooms [6], whereas the function of trehalases in the kidney is still unclear. On the other hand, trehalases reported from insects are essential enzymes to utilize trehalose, the main blood sugar of insects. Two types of insect trehalase are known: membrane-bound trehalase located on the cell membrane, and soluble trehalase contained in the cell matrix [10–14]. In the silkworm, soluble and membrane-bound trehalases are each coded by independent genes, trehalase-1 [15] and trehalase-2 [16]. The two genes are quite different from each other, especially in their nucleotide sequence.

We reported that *Artemia* trehalase protein predicted from cDNA had a putative transmembrane region [1], whereas purified *Artemia* trehalase was a soluble type [5]. We supposed that the soluble trehalase was generated by the cleavage of the transmembrane region because the molecular mass of the soluble trehalase was almost the same as that of a protein encoded by cDNAs without a “transmembrane” region [1]. Although the assumption could be reinforced by analyzing a copy number of the trehalase gene, previous southern blot analyses using cDNA probe were not clear because the boundaries of exons were not known [17]. These reports also suggested alternative splicing or switching of transcriptional start site occurred [1,17]. However, details of mRNA variants or exon/intron structure of the *Artemia* trehalase gene have not been reported, either. We screened the genomic library of *Artemia* to obtain clones of the trehalase gene, and the *Artemia* trehalase gene was re-constructed using these clones. In the present paper, we report the unique structure of the *Artemia* trehalase gene. We also analysed a copy number of the trehalase gene using appropriate probes based on the exon/intron structure of the *Artemia* trehalase gene.

Materials and Methods

Animals

Cysts of *Artemia franciscana* from the Great Salt Lake (Utah, USA) were purchased from Japan Pet Drugs Co. Ltd. (Tokyo, Japan and Los Angeles, CA, USA). The cysts were hydrated and allowed to develop under appropriate conditions (2% sea salt water, 28°C under

constant light). Hatched nauplii were fed with Baby shrimp food (NT Laboratories Ltd., East Peckham, UK). Mature adults were mated in isolated beakers and their descendants were used to establish inbred strains as described by Nambu *et al.* [18]. Individuals at F22-F24 belonging to a single inbred strain were collected, starved for two days, and used to construct a genomic library.

Construction and screening of genomic library

After being washed with sea salt water, adult *Artemia* were homogenized in a DNA extraction buffer (10 mM Tris, 10 mM EDTA and 100 mM NaCl, pH 0.8) and incubated overnight with 0.5% SDS and 0.2 mg/ml proteinase K at 37°C. Genomic DNA was extracted three times with an equal volume of phenol and collected by ethanol precipitation. The DNA was dissolved in a solution containing 10 mM Tris (pH 8.0), 0.1 mM EDTA and 2 µg/ml RNase A (WAKO Pure Chemical Industries Ltd, Osaka, Japan). After RNase treatment (at 37°C for 1 hr), 0.5% SDS and 0.1 mg/ml proteinase K (SIGMA-Aldrich Corporation, St. Louis, USA) were added to the solution and further incubated for 3 hr. The DNA was deproteinated by phenol extraction followed by chloroform extraction, and was precipitated with ethanol.

Finally, the DNA was redissolved in TE buffer (1 mM EDTA and 10 mM Tris-HCl, pH 8.0). The genomic DNA was partially digested with *Sau3AI* and was ligated into the *Bam*HI site of an EMBL3 phage and packaged into a lambda phage particle using gigapack[®] III Gold (Stratagene Cloning System, La Jolla, CA, USA).

A trehalase cDNA clone, called *TreE2* [1], was labeled using a DIG DNA Labeling Kit (Roche Molecular Biochemical, Basel, Switzerland) and used as a probe. Approximately 5.0×10^5 plaque-forming units (pfu) of recombinant phages were plated with the *Escherichia coli* XL1-Blue MRA (P2) strain. The phage plaques were transferred to a nylon membrane, as described by Sambrook *et al.* [19]. Phage clones containing the *Artemia* trehalase gene were detected by a DIG-labeled probe according to a method described by Engler-Blum *et al.* [20]. Although four clones (λ gTre9, 11, 13 and 23) were obtained by the screening, these clones did not have regions corresponding to the 5' and 3' end sequences of the *TreE2*. Additional screenings were performed to obtain the missing genomic regions using DIG-labeled PCR probes. Probe I was synthesized using the primers TreEX1sen and TreEX1ant (see Table 1) to detect the upstream region of the *Artemia* trehalase. Probe VII was synthesized using the primers TreEX7sen and TreEX7ant (Table 1) to obtain the genetic region corresponding to the 3' end of the trehalase cDNA. As a result of additional screenings, λ gTre44, λ gTre68-1 and λ gTre70 were isolated. Four overlapping clones (λ gTre9, 11, 44 and 68-1) were selected and sequenced to re-construct the *Artemia* trehalase gene.

Sequencing of genomic clones

Specimens of lambda DNA extracted from each clone were digested by *Sal*I. *Sal*I frag-

Table 1. Primers used in this paper

primer	sequence(5' to 3')	position	direction
TreEX1sen	GTGAGAACACTGTATACAATAC	7908- 7929(a)	sense
TreEX1sen.new	GTGAGAACACTGTATACAATA	7908- 7928(a,b)	sense
TreEX1ant	CTAGCACAAACAGGCGGTAG	8256- 8275(a)	antisense
TreEX4SEN	GCAAAACATTACCTGTTGG	25584-25603(a)	sense
TreEX4ANT	TCTACAGGTAGTATGTCACG	25864-25883(a)	antisense
TreEX6SEN	TATGACGTAACGGTCGTTGG	32675-32694(a)	sense
TreEX6ANT	CCAATCCAAGCACCGAGG	32869-32876(a)	antisense
TreEX7sen	TCTTGCAAGTACTCGATTCTAT	42543-42564(a)	sense
TreEX7ant	TTTACTAAGAAAATATCACACTCG	47196-47219(a)	antisense
TreRACE.A2	TATGCAGGAATGTTCCATGG	17569-17588(a)	antisense
TreRACE.A1	GATTTTGATCGGTTGAGTTC	17689-17708(a)	antisense
TreRACE.S1	GGGTCAGAAATTTGAAGAATG	17759-17778(a,b)	sense
TreRACE.S2	GATTGGCATTCTAACCCAAG	17789-17808(a,b)	sense
TreRT-1	ATATATCCTTCCACCATTGG	22396-22415(a)*	antisense
TreRACE.A1-2	CCTCTTTGAAATCCTGATGTTC	8014- 8035(a,b)	antisense
TreRACE.S1-2	GGAGAAGGCAAACGTGCAAG	8083- 8102(a)	sense
TreRACE.S2-2	ATCTGATTCTCAGTGAAAGAGC	8227- 8248(a)	sense
TreRT-2	3CTAGCACAAACAGGCGGTAG	8256- 8275(a,b)*	antisense
TreUnkSEN	TCTTAAGCCTTCCAGCGAGT	(b)	sense
TreUnkANT	AACACCTTGTAGACCAATCTTG	(b)	antisense
MRP-S33sen	TTTCTAAGATCAGCATTATTTGG	(c)	sense
MRP-S33ant	AACCATAAAGTCTTAAATGCTTC	(c)	antisense
Oligo dT 3'-sites Adaptor	CTGATCTAGAGGTACCGGATCC(T) _n	(d)	
3'-sites Adaptor	CTGATCTAGAGGTACCGGATCC	(d)	

(a): Nucleotide number is based on reconstructed trehalase gene illustrated in Fig. 1,(b): position of the primer is illustrated in Fig. 3, (c): position of the primer is illustrated in Fig. 4A, (d): involved in 3' RACE core kit, *: phosphorylated at 5' end

ments of the trehalase gene were inserted into a pBluescript SK(-). Subclones were constructed using restriction endonucleases, and overlapping clones were generated from each subclone using a Kilo-Sequence Deletion Kit (Takara bio, Otsu, Japan). DNA sequencing was carried out using a BigDye[®] Terminator v3.1 Cycle Sequencing Kit and ABI PRISM[®] 377 (Applied Biosystems).

RNA extraction

Fully washed and hydrated cysts or 12 h-cultured embryos of wild type *A. franciscana* were homogenized in ten volumes of ISOGEN (NIPPON GENE, Tokyo, Japan). Total RNA was isolated according to the manufacturer's protocol.

Rapid amplification of the 5' and 3' cDNA ends (RACE)

We analyzed the 5' end of *Artemia* trehalase mRNA according to the cRACE method, a

modified 5' RACE method described by Maruyama *et al* [21]; it was performed according to the manufacturer's protocol of the 5' Full RACE Core Set (Takara bio, Otsu, Japan). The samples of total RNA were reverse-transcribed into single stranded cDNAs using a *TreE2* specific primer (TreRT-1, Table 1) and AMV Reverse Transcriptase XL (Takara bio, Otsu, Japan). The cDNA chains were self-ligated by T4 RNA ligase and were made into circular forms. Nested PCR reactions were performed using trehalase-specific primers as follows: TreRACEA1 and TreRACES1 were used in the first amplification, and TreRACEA2 and TreRACES2 were used in the following amplifications (Table 1).

The results of the 5' RACE strongly suggested that alternative splicing occurred in the trehalase 5' UTR region called "Exon I". To detect the 5' end of the novel sequence found upstream of the new splicing site, an additional 5' RACE was performed using the following primers (Table 1 and Fig. 3): TreRT-2, a primer for reverse transcription; TreS1-2 and TreA1-2, primers for the first amplification; TreS2-2 and TreA2-2, primers for the second amplification.

To detect the products of the alternative splicing, 3' RACE was performed according to the manufacturer's protocol of the 3' -RACE Core Kit (Takara bio, Otsu, Japan). Primers corresponding to different 5' ends of trehalase mRNA (TreEX1sen.new or TreUnkSEN, Table 1) were used with a poly(dT) primer in the 3' RACE reaction. The PCR products were subcloned into the *EcoRV* site of the pBluescript SK (-) and sequenced as described above.

Isolation and phylogenetic analyses of a novel cDNA corresponding to a sequence inserted into trehalase gene structure

To obtain variants of trehalase cDNA, the region corresponding to Exon I was DIG-labeled by PCR reaction using TreEX1sen and TreEX1ant primers (Table 1). The λ gt10 library of 12 hr cultured embryos [1] was plated with the *Escherichia coli* NM514 strain. Plaque lifting to nylon membranes and detection of positive clones were performed as described by Engler-Blum *et al* [20], followed by washing at 65°C. *NotI* digested samples of lambda DNA extracted from each clone were inserted into the pBluescript SK (-) and sequenced as described in "Sequencing of genomic clones". An amino acid sequence coded by one of the clones was unexpectedly shown to have a high similarity to a known mitochondrial ribosomal protein gene, as described in the "Results" section. GENETYX ver.13 (GENETYX, Tokyo, Japan) was used to draw a phylogenetic tree of homologous proteins.

Northern blot analysis

In order to detect the mRNA of the mitochondrial ribosomal protein gene, a DIG-labeled PCR probe was synthesized from the subcloned cDNA (Fig.4A), using sequence-specific primers (MRP-S33sen and MRP-S33ant in Fig.4A). Samples of total RNA were extracted from re-hydrated dried cysts and 12hr developed embryos. Each specimen (20 μ g) was electrophoresed in a 1.0% agarose gel containing formaldehyde [19], and blotted onto Hy-

bondN+ membrane (Amersham). Hybridization was performed at 68°C, as described by Engler-Blum *et al* [20], followed by washing at 65°C.

Southern blot hybridization

Three DIG-labeled PCR probes corresponding to Exon I, Exon IV and Exon VI of the *Artemia* trehalase were synthesized and used in the following analysis. The primers used in the probe synthesis are described in Table 1: TreEX1sen and TreEX1ant for probe I; TreEX4SEN and TreEX4ANT for probe IV; TreEX6SEN and TreEX6ANT for probe VI.

Genomic DNA was prepared from 20 hr-cultured and fully washed wild type larvae (nauplii) as described in "Construction and screening of genomic library", and digested by four kinds of restriction enzyme (*Eco*RI, *Hind*III, *Bam*HI and *Pst*I). The digested specimens were separated by electrophoresis through a 0.6% agarose gel and transferred to Nylon membranes. Fragments of the trehalase gene were detected as described in "Northern blot analysis".

Results

Re-construction of the Artemia trehalase gene

Four genomic clones (λ gTre9, 11, 13 and 23) were isolated by the first screening using the whole trehalase cDNA probe. Though the clones contained five exons corresponding to known trehalase cDNA, 5' and 3' ends of the cDNA were not found in these clones. In additional screenings, λ gTre44, 68-1 and 70 were obtained. Four clones (λ gTre9, 11, 44 and 68-1) were selected to re-construct the *Artemia* trehalase gene (Fig. 1). The identities be-

Fig. 1. Trehalase gene structure of *Artemia franciscana*. Eight exons corresponding to trehalase cDNAs [1] are designated as Exon I-Exon VIII.

tween the genomic clones were higher than 99% in their overlapping areas. The reconstructed genetic area spanned about 49 kb (accession number of Genbank/EMBL/DDBJ: AB458252) and contained eight exons (Exon I-Exon VIII, see Fig. 1) corresponding to known trehalase cDNA, *TreE2* and *E3* [1]. The protein encoded by the trehalase gene was aligned with animal trehalases, and the positions of the exon/intron boundary were compared. Boundaries corresponding to the position S38 and K526/Y527 were widely conserved among the animal trehalases (Fig. 2).

A new exon/intron boundary found in "Exon I" and alternative splicing of the Artemia trehalase gene

The 5' ends of some transcripts obtained by 5' RACE were different from those of reported cDNA clones, suggesting the existence of an unknown transcriptional start site. We verified a new exon/intron boundary in the exon designated "Exon I" (Fig. 3). It is probable that splicing at this site might occur according to the "GT-AG" rule, as with other splicing sites found in the *Artemia* trehalase gene. In order to detect the sequence of the 5' end of the new transcript, an additional 5' RACE was performed. The maximum length of the new 5' end sequence was 114 bp from the new exon/intron boundary found in Exon I ("5' RACE TreA6", Fig. 3).

A short sequence inserted in the trehalase gene: the sequence derived from a mitochondrial ribosomal protein gene, ArMRP-S33

The results of the 5' RACE suggested that *Artemia* trehalase mRNA had at least two variants in its 5' end. To confirm the existence of these variants, we re-screened the cDNA library of 12 hr-developed nauplii [1] using the Exon I as a probe. Only two clones were isolated by the screening of 200,000 clones. One of them was a fragmented 5' end of trehalase mRNA containing the new 5' end found by 5' RACE (data not shown). The other was 430 bp long and had an open reading frame (375 bp). The cDNA was composed of a novel sequence of 296 nucleotides and 134 nucleotides that were identical to the sequence downstream of the new exon/intron boundary found in the Exon I described above (Fig. 4A).

The protein predicted by the 375 bp ORF comprised 116 amino acids (Fig. 4A). The protein showed a high degree of similarity to a small subunit of mitochondrial ribosomal protein, MRP-S33 [22] reported from higher eukaryotes: 65% to fruit fly (AE014297), 62% to silkworm (DQ311287), malaria mosquito (AAAB01008859) and rat (DQ480753), 60% to clawed frog (BC084770) and yellow fever mosquito (CH477286), 59% to mouse (BC092135) and human (BC105793), 58% to bovine (BC109522), 56% to rainbow trout (AB161470), 53% to zebrafish (BC095219), and 48% to *Caenorhabditis elegans* (Z54271) and *C. briggsae* (CAAC02000533). In phylogenetic analyses, *Artemia* MRP-S33 (ArMRP-S33, Genbank/EMBL/DDBJ accession no. AB456740) was allocated into the group of arthropod MRP-S33 proteins (Fig. 4B). The location of ArMRP-S33, on the branch next to that

Fig. 2. Intron-inserted sites of animal trehalases. Trehalase proteins of *Artemia* (Af), silkworm (Bm-1 and Bm-2 [15, 16], fruit fly (Dm; CG9364 in database), European honeybee (Am; [25]), human (Hs; [9]) and *Caenorhabditis elegans* (Ce-1, Ce-2, Ce-3, Ce-4 and Ce-5; [23]) are aligned. Exon /intron boundaries are indicated by small arrows. Conserved boundary corresponding to S38 in *Artemia* trehalase is indicated by large arrowhead. Conserved boundary corresponding to K526/Y527 in *Artemia* trehalase is indicated by gray arrowhead. Conserved amino acids are shaded in gray.

Fig. 3. A novel exon/intron boundary found by 5' RACE. 5' RACE products and partial sequence of *Artemia* trehalase cDNA, *TreE2* [1] are aligned. A novel exon/intron boundary is indicated by vertical bold line. The new sequences found in four 5' RACE products (TreA2, TreA6, TreC9, TreC10) are indicated in bold letters. The 151 bp sequence which is identical to *ArMRP-S33* transcripts is indicated in red letters. Positions and direction of primers used in additional 5' RACE (TreRT-2, TreRACES1-1, TreRACES1-2, TreRACEA1-2 and TreUnkANT) and 3' RACE (TreEX1sen.new and TreUnkSEN) are indicated by boxes and arrows, respectively.

Fig. 4. Characterization of *Artemia* MRP-S33. **A:** Nucleotide and predicted amino acid sequences of *ArMRP-S33*. The nucleotide and amino acid sequences are numbered from the first nucleotide and the first methionine. Asterisks indicate stop codons. The 134 nucleotides that were identical to the sequence downstream of the new exon/intron boundary found in the Exon I are indicated in bold letters. A truncated polyadenylation signal is heavily underlined. **B:** Phylogenetic analysis of animal MRP-S33 protein. Non-rooted neighbor-joining tree was drawn by GENETYX ver. 13 software. Scale bar indicates 0.1 expected amino-acid substitution per site. Values on each branching represent percent of bootstrap probability. **C:** Northern blotting of *Artemia* MRP-S33. Total RNA samples were extracted from *Artemia* nauplii at 0 hr and 12 hr of post-dormant development, and 20 µg of each sample was electrophoresed, transferred to nylon membrane, and detected with DIG-labeled PCR probe. Positions of molecular weight markers are indicated. Position of MRP-S33 mRNA is indicated by an arrowhead.

of insect proteins, showed a good agreement with the taxonomic position of *Artemia*.

Southern blotting to determine the copy number of the Artemia trehalase gene

To investigate the copy numbers of the *Artemia* trehalase gene, Southern blotting was performed using three kinds of DIG-labeled PCR probes, corresponding to parts of Exon I, IV and VI (probe I, IV and VI in Fig. 5). Only the probe I showed double signals in the sample digested by *EcoRI*. In the cases of probe IV and VI, the trehalase gene was detected as a single band.

Detection of chimeric composition of trehalase transcripts

In the 3'RACE analysis, we used three types of 5' primer: "TreEX1sen.new" corresponded to the 5' UTR of known trehalase cDNA (Fig. 3 and Table 1), "TreUnkSEN" corresponded to the novel 5' UTR found by 5'RACE (Fig. 3 and Table 1), and "MRP-S33sen" that is specific to *ArMRP-S33* cDNA (Fig. 4A and Table 1). In the analysis using TreEX1sen.new primer, subcloned PCR products were approximately 2.5 kb and had an open reading frame (Fig. 6), coding 703 amino acids. In the case of TreUnkSEN, we found two types of cDNA (Fig. 6 and 7). One was about 2.5 kb long and had an open reading frame coding the trehalase protein (Fig. 6, TreUnkSEN1~TreUnkSEN7, Fig. 7), as in the case of TreEX1sen.new. Except for the 5' end, the 2.5 kb cDNAs derived from the different primers had essentially the same exon composition, and the predicted amino acid sequences were 96-99% identical to each other. Another type of PCR product obtained by TreUnkSEN primer was 0.3 kb (TreUnkSEN8~TreUnkSEN11, in Fig. 7). They had poly(A) tails at 15-17 base

Fig. 5. Southern blot analysis of *Artemia* trehalase gene. DNA from *Artemia* nauplii digested with *EcoRI*, *HindIII*, *BamHI* or *PstI* was hybridized with probe I, probe IV and probe VI labeled with digoxigenin. Digoxigenin-labeled molecular markers were used on the gel.

Fig. 6. Schematic diagram of *Artemia* trehalase gene and detected transcripts. Length of introns is deformed. Eight exons that corresponded to trehalase cDNA [1] are designated as Exon I-Exon VIII. Exon I is divided into Exon Ia and Exon Ib at a novel exon/intron boundary. A new sequence found in 5' RACE products is indicated as a black box. Polyadenylation signal sequences are indicated by small arrows and vertical gray lines. Dotted areas indicates coding region of *Artemia* trehalase and MRP-S33, respectively. Coding region of the trehalase is indicated by black arrowhead (translational start codon) and white arrowhead (stop codon). Positions of probes used in Southern blotting are indicated on trehalase gene with horizontal lines.

polyadenylation signal		
MRP-S33-1	TTGAAAAGAATAAAAGATATTTTATCAGCAAAAAAAAAAAAAA-----	442
MRP-S33-2	TTGAAAAGAATAAAAGATATTTTATCACTAAAAAAAAAAAAA-----	440
MRP-S33-3	TTGAAAAGAATAAAAGATATTTTATCAGCAAAAAAAAAAAAAA-----	443
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TreEX1.sen.new1	TTGAAAAGAATAAAAGATATTTTATCACTAAACATGTGCGCATTTTGTAGTTTTTCGTCGTTTTTGGATTGCTACAAAC	302
TreEX1.sen.new2	TTGAAAAGAATAAAAGATATTTTATCACTAAACATGTGCGCATTTTGTAGTTTTTCATCGTTTTTGGATTGCTACAGC	302
TreEX1.sen.new3	TTGAAAAGAATAAAAGATATTTTATCACTAAACATGTGCGCATTTTGTAGTTTTTCGTCGTTTTTGGATTGCTACAAAC	302
TreEX1.sen.new4	TTGAAAAGAATAAAAGATATTTTATCACTAAACATGTGCGCATTTTGTAGTTTTTCGTCGTTTTTGGATTGCTACAAAC	307
TreEX1.sen.new5	TTGAAAAGAATAAAAGATATTTTATCACTAAACATGTGCGCATTTTGTAGTTTTTCGTCGTTTTTGGATTGCTACAAAC	302
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TreUnkSEN1	TTGAAAAGAATAAAAGATATTTTATCACTAAACATGTGCGCATTTTGTAGTTTTTCATCGTTTTTGGATTGCTACAGC	298
TreUnkSEN2	TTGAAAAGAATAAAAGATATTTTATCACTAAACATGTGCGCATTTTGTAGTTTTTCATCGTTTTTGGATTGCTACAGC	297
TreUnkSEN3	TTGAAAAGAATAAAAGATATTTTATCACTAAACATGTGCGCATTTTGTAGTTTTTCATCGTTTTTGGATTGCTACAGC	297
TreUnkSEN4	TTGAAAAGAATAAAAGATATTTTATCACTAAACATGTGCGCATTTTGTAGTTTTTCATCGTTTTTGGATTGCTACAGC	297
TreUnkSEN5	TTGAAAAGAATAAAAGATATTTTATCACTAAACATGTGCGCATTTTGTAGTTTTTCATCGTTTTTGGATTGCTACAGC	297
TreUnkSEN6	TTGAAAAGAATAAAAGATATTTTATCACTAAACATGTGCGCATTTTGTAGTTTTTCATCGTTTTTGGATTGCTACAGC	297
TreUnkSEN7	TTGAAAAGAATAAAAGATATTTTATCACTAAACATGTGCGCATTTTGTAGTTTTTCATCGTTTTTGGATTGCTACAGC	298
TreUnkSEN8	TTGAAAAGAATAAAAGATATTTTATCAGCAAAAAAAAAAAAAA-----	260
TreUnkSEN9	TTGAAAAGAATAAAAGATATTTTATCACTGAAAAAAAAAAAAA-----	261
TreUnkSEN10	TTGAAAAGAATAAAAGATATTTTATCACTAAA	298
TreUnkSEN11	TTGAAAAGAATAAAAGATATTTTATCACTAAAAAAAAAAAAA-----	269

Fig. 7. Polyadenylation by signal sequence derived from *MRP-S33* gene. Four types of 3' RACE products amplified by three independent primers (MRP-S33sen, TreEX1sen.new and TreUnkSEN) were aligned. Sequences are numbered from the first nucleotide.

downstream from the typical poly(A) additional signal, AATAAA, in the same way as *MRP-S33* cDNAs amplified by *MRP-S33sen* primer (*MRP-S33-1* ~ *MRP-S33-3*, Fig. 7).

Discussion

We re-constructed the *Artemia* trehalase gene using genomic clones isolated from a library derived from an inbred strain. The gene had 8 exons corresponding to the trehalase cDNAs reported in our previous study [1]. The eight exons ranged in size from 209 to 609 bp (Table 2). Seven introns ranged from 1.4 kb to 9.7 kb (Table 2). All the introns were in kilobase order, whereas those of mammalian trehalase genes were relatively small, except for the first one [9]. The rate of introns inserted in the coding area of *Artemia* trehalase was 94.7%. The rates of introns found in the coding area of other trehalase genes are smaller than those found in the *Artemia* trehalase gene [9, 16, 23–25]. An extreme compaction of introns can be found in the silkworm trehalase-1 gene [15]. In this gene, there is no intron in the protein coding area. It has been reported that *Artemia* has a considerably large genome (1.6×10^9 bp) for an arthropod [26]. The redundancy in size of introns, such as those in the trehalase gene, may explain the large genome size of *Artemia*, to some extent. In order to obtain a 5' end of trehalase transcripts, 5' RACE was performed. The 5' end of the longest RACE product corresponded to position 7882 of the re-constructed trehalase gene. No typical TATA box sequence was found around this area.

The trehalase genes identified by the insect genome projects were seemingly different from those of mammals in their exon/intron conformation. In many cases, the genes of insect trehalase were composed of a small number of exons (2-7 exons), whereas the trehalase genes of the human and mouse contained small 15 exons [9]. However, a new trehalase gene found in the silkworm, trehalase-2, had 14 exons [16]. The trehalase gene reported from *Spodoptera exigua* also had 13 exons [27]. Contrary to these reports, the trehalase gene previously reported from the silkworm (trehalase-1) has only two exons [15]. Furthermore, five treha-

Table 2. Structure of the *Artemia* trehalase gene

Exon name	Exon size(bp)	Sequence at exon-intron junction				Codon interrupted	Intron size (kb)
		5' splice		3' splice			
		↓		↓			
I	>390	TGCTAG	GTAAGT	TTTCAG	CGAAAT	S38	9.281
II	326	GGTTAG	GTTGGT	TTTCAG	GTCGAA	—	4.297
III	319	TTTAAA	GTAAGT	TCTTAG	GCAAAA	K253	3.086
IV	609	CTTGAA	GTAAGT	TTTAAG	GTTTCC	K455	4.831
V	214	GAAAAG	GTATTT	TTTTAG	TATGAC	—	1.437
VI	212	GATTGG	GTAAGG	TTTTAG	TCTTGC	G598	9.656
VII	217	TTACCC	GTAAGT	TTCTAG	GGCTTC	—	4.343
VIII	209						

lase genes of *Caenorhabditis elegans* were composed of 4-12 exons and have a large diversity in their patterns of exon/intron structure [23, Wormbase: <http://www.wormbase.org/>]. These findings suggested that trehalase genes could have a large diversity in their exon/intron structure, even in one species. The positions of the exon/intron boundary in the *Artemia* trehalase gene were compared with those in the trehalase genes of many species (Fig. 2). Boundaries corresponding to the position S38 of *Artemia* trehalase were found in insect, mammalian and nematoda trehalases (Fig. 2). Another boundary at K526/Y527 of the *Artemia* trehalase is conserved among most arthropods and mammals. Furthermore, several common exon/intron boundaries were also found among different phyla (Fig. 2). These common boundaries may reflect the evolutionary relationship of each trehalase gene.

We found a new exon/intron boundary in the 5' UTR region of the *Artemia* trehalase gene. A novel sequence was found at the 5' end of the cDNAs, using the 5' RACE method. This finding suggests another transcriptional start site of the trehalase gene. The novel 5' end sequence of the RACE products was not found in the reconstructed trehalase gene. This sequence seemed to exist in a much upper area of the reconstructed region. Unfortunately, this sequence was too short to synthesize useful screening probes, and genomic clones corresponding to this sequence have not been obtained yet. Gene walking or other efficient methods and laborious investigation will be needed to explore this region.

The 151 bp sequence found in Exon I and trehalase cDNA (Fig. 3) was identical to the transcripts of *ArMRP-S33*. Although the short sequence could be found in the *Artemia* trehalase mRNA reported in our previous study [1], it was too short to be recognized at that time as a part of an ORF derived from another gene. There has been no report about the *MRP-S33* sequence in trehalase genes hitherto reported. The 151 bp sequence is 100% identical to the *ArMRP-S33* transcripts and this strongly suggests that it was not so long ago when this sequence was inserted in the trehalase gene. A slight signal of *ArMRP-S33* mRNA was detected in dormant cysts. The signal became undetectable in 12 hr-cultured embryos (Fig. 4C). Although the amount of *ArMRP-33* transcript was much smaller than that of trehalase, its expression pattern was similar to the trehalase mRNA [1]. These results suggest that the *ArMRP-S33* is one of the genes required in the early post-dormant development, as the trehalase is [1]. Human MRP-S33 is a product of a nuclear gene and one of the five MRPs suggested to be related to non-syndromic hearing loss [28]. The precise roles of *ArMRP-S33* in *Artemia* dormant cysts and developing nauplii are not clear and need to be elucidated.

In many cases, the trehalase gene was detected as a single band in the Southern blotting. These results strongly suggest that this gene is a single copy, as we previously described [17]. Only probe I showed double signals in the sample digested by *EcoRI* (Fig. 5). As described above, Exon I had a sequence derived from *ArMRP-33*. Therefore, the double signals detected by probe I may be the original *ArMRP-33* and its partial copy inserted in the trehalase gene. However, this does not mean that the gene described in the present report is the only trehalase gene in the *Artemia* genome. In screening of the trehalase gene in the

silkworm, the gene of the membrane-penetrating trehalase was not detected by a probe derived from the soluble trehalase cDNA [15]. In a previous report, we pointed out that the molecular mass of the *Artemia* membrane-bound trehalase without the membrane-binding region was almost equal to the size of the soluble trehalase that we had purified [1, 5]. Therefore, we speculated that the soluble-type trehalase might be generated by the cleavage of the membrane-bound trehalase. However, the presence of other genes coding the soluble trehalase is still possible. Mammals appear to have a single trehalase gene in their genomes, whereas the silkworm, fruit fly and honeybee have at least two trehalase genes [9, 16, 23–25]. Pellerone *et al* [23] reported five trehalase genes of *Caenorhabditis elegans*. These findings owed a large part of themselves to genome projects or systematic cDNA databases. A comprehensive EST research or genome project in *Artemia* may bring rapid progress in this field.

We revealed that an alternative polyadenylation site has been introduced into the 5' UTR of the *Artemia* trehalase gene. As a result of the polyadenylation at this site, chimeric transcripts that had the 5' UTR region of trehalase and 3' end of *MRP-S33* with poly(A) tail could be transcribed (Fig. 6, 7). Such a composition of mRNA is quite interesting because the short transcript has no coding region of trehalase. If all of the transcripts were truncated by the poly(A) signal in the *ArMRP-S33* sequence, no trehalase protein would be synthesized. These findings strongly suggest the presence of a control mechanism of polyadenylation in the 5' UTR of trehalase mRNA. Recent progress in genome researches revealed that alternative polyadenylation could be found in various organisms [29–31]. The alternative polyadenylation signal found in the Exon I of the *Artemia* trehalase gene is unique because it was derived from another gene and was newly introduced during the evolution of the trehalase gene. Winter *et al.* reported that alternative polyadenylation signals are related to the tissue-specific expression of the Opitz Syndrome gene *MIDI* [32]. It is quite an attractive question whether the novel alternative polyadenylation site in the trehalase gene regulates the trehalase activity and/or tissue-specific expression.

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アルテミアトレハラーゼ遺伝子の持つ特異な構造について

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要 旨: 小型甲殻類アルテミアのトレハラーゼ遺伝子の構造解析を行うため、近交系アルテミア由来のゲノムライブラリーから得られた4つのクローンに基づいてアトレハラーゼ遺伝子を含む49 kb の領域を再構築した。この領域内には以前我々が報告したトレハラーゼ cDNA に対応する8つのエキソンが存在していた。他の動物で報告されているトレハラーゼ遺伝子と構造を比較した結果、動物界を超えて保存されているエキソン/イントロン境界が存在していることが判明した。5' RACE 法によりトレハラーゼ mRNA とトレハラーゼ遺伝子の関係を解析し、エキソン I の領域内にこれまで知られていなかったエキソン/イントロン境界が存在することを明らかにした。また、ミトコンドリアのリボソームタンパク質 (MRP-S33) 遺伝子の一部のコピーがトレハラーゼ遺伝子の5' 側の領域に組み込まれていることが判明した。組み込まれた配列は本来の MRP-S33 が持っていたポリA付加シグナルを保持していた。3' RACE 法により、このポリ A 付加シグナルが依然として機能しており、その結果としてトレハラーゼ mRNA の5' UTR と MRP-S33 遺伝子の3' 末端をもった短いキメラ mRNA が転写され得ることが示された。

キーワード: アンハイドロビオシス, アルテミア, ブラインシュリンプ, 選択的スプライシング, 選択的ポリA付加。

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