Transient expression assays with the proximal promoter of a newly characterized actin gene from the oyster *Crassostrea gigas*[1]

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Abstract **We undertook the characterization of an actin gene and its proximal promoter in the oyster *Crassostrea gigas*. A complete actin cDNA was identified, sequenced and its amino acid sequence deduced. Comparative analysis showed a high homology with actin of other species and that this gene is closer to the cytoplasmic form of actins than to the muscle type. A probe derived from the 5′-untranslated region of the cDNA was then used to isolate the actin gene from a genomic library. The gene was sequenced and shown to contain a single 643 bp intron. A 1670 bp fragment upstream from the open reading frame was isolated and sequenced. This upstream region displays typical features of actins such as a serum response element (CarG box). This fragment was cloned into the promoterless vector pGL3-basic and the resulting construct was transfected into cells of dissociated oyster heart primary cultures. Its capacity to express the luciferase in this in vitro homologous system was monitored and showed high expression levels. This is the first complete actin sequence reported so far for the oyster *C. gigas* and its promoter is the first available among bivalves.**

Key words: Actin promoter; Actin gene; Gene transfer; Bivalve; Mollusk; Transient expression

1. Introduction

The possibility to generate transgenic organisms has opened the way to deepen the basic knowledge on the general biology of species. Transgenesis in animals offers sufficiently promising results to justify extensive investments in a variety of programs [1]. Genetic transformation aimed at the integration of new useful traits has been investigated in mammals [2,3] and in fish [4,5]. Invertebrates have been subjected to similar research but essentially in addressing fundamental biological studies, e.g. in insects [6], sea urchins [7] and nematodes [8]. Gene transfer has been attempted in marine invertebrates as a means to enhance growth or to develop resistance to various diseases [9,10].

Until recently, a serious drawback for molecular biological investigations in commercial mollusks has been the lack of reliable techniques for genetic transformation. This has been overcome using electroporation in the abalone *Haliotis rufescens* [11] and particle bombardment in the oyster *Crassostrea gigas* [12]. The first transgenic bivalves were produced recently using pantropic retroviral vectors [13].

Actin is a contractile protein found in eukaryotic cells. It is very abundant in muscle cells in the form of filaments involved in muscle contraction and in non-muscle cells as microfilaments involved in cytoskeletal morphology, cell division and organelle movements, as well as locomotion, phagocytosis, endocytosis and exocytosis. Encoded by a multigenic family, it displays a very high degree of conservation between species. The promoter for the actin gene has been chosen in several cases since the gene is very abundant and constitutively expressed [14–17]. The identification and isolation of an actin encoding gene in the oyster *C. gigas* will allow for comparative analysis in functional studies with other genes, studies on population variation and its use in transgenesis [12,18,19].

2. Materials and methods

2.1. Construction and screening of a complementary DNA (cDNA) library

A cDNA library was constructed from oyster *C. gigas* hemocytes by directional cloning into the λ ZAP Express vector (Stratagene, La Jolla, CA, USA). The library was screened with a ^{32}P-labelled probe obtained by polymerase chain reaction (PCR) amplification of DNA from the oyster *C. gigas* using the primers described for the oyster *Crassostrea virginica* [20] and renamed Avi1 (5′-TAA TCC ACA TCT GCT GGA AGG TGG-3′) and Avi2 (5′-TCA CCA ACT GGG ATG ACA TGG-3′).

Briefly, phage DNA was transferred to nylon membranes (Hybond N+, Amersham) and hybridized overnight at 48°C with the labelled probe according to standard techniques [21]. After several washes, the filters were exposed to X-ray films. A set of 10 randomly selected positive phages were isolated after two additional rounds of plaque screening and excised in vivo to release the phagemids carrying cloned DNA inserts from the λ ZAP Express vector.

2.2. Rapid amplification of 5′-cDNA end (RACE-PCR)

A RACE-PCR was performed as specified by the manufacturer (Boehringer, Mannheim, Germany). Briefly, 2 μg of total RNA from hemocytes was subjected to reverse transcription using two antisense primers derived from the cDNA sequence: G1 (5′-CAA GAA AGT GAT GGT TTG AGT TCG-3′) and G2 (5′-ATT GTG AAG TTG TAA GAT TAA TAT-3′). After PCR amplification, the products were cloned in the cloning vector pCR-Script (Stratagene, La Jolla, CA, USA) and sequenced.

2.3. Construction and screening of the genomic library

For construction of *C. gigas*, genomic DNA was subjected to a

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Abbreviations: cDNA, complementary DNA; UTR, untranslated region; ORF, open reading frame

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partial digestion with *Sau*3AI to yield 12–20 kb fragments. *Sau*3AI overhangs were partially filled-in using Klenow and ligated to the dephosphorylated *Xho*I half-site arms of the cloning vector λGEM-12 (Promega, Southampton, UK). Ligation reactions were carried out at a vector to insert molar ratio of 2:1. Recombinant phages were encapsidated with Gigapack III Gold packaging extracts (Stratagene, La Jolla, CA, USA) and used to transform the *Escherichia coli* LE 392 strain, yielding a titer of 4.9×10^9 plaque-forming units per μg of ligated DNA. The genomic library was screened by in situ plaque hybridization and probed with a ^{32}P-labelled 110 bp *Bam*HI fragment comprising 52 bp of the 5′-untranslated region (UTR) of the cloned actin cDNA together with the initial 40 bp of the actin open reading frame (ORF) and 18 bp of the pBluescript SK+ phagemid. Hybridization conditions were as described in Section 2.1.

2.4. Cloning of the promoter and vector construction

PCR amplifications were performed with the high fidelity polymerase PFU (Stratagene, La Jolla, CA, USA), directly on the genomic clone, using the T7 primer present in the λGEM-12 vector left arm and a reverse primer called HUTR (5′-TAA GAT TAA TAT CCA GAC AA-3′) encompassing a 20 bp DNA tract of the 5′-UTR, 10 bp distal from the initiator codon of the actin gene. The PCR product was then ligated to the *Sma*I site of the promoterless expression vector pGL3-basic (Promega, Southampton, UK). Proper orientation of inserts was checked by restriction analysis. Nested 5′-deletions of the recombinant vector were performed with the Erase-a-Base system (Promega, Southampton, UK) following the manufacturer's instructions. Briefly, a 5′-protruding site upstream of the cloned sequence and a 3′-overhang downstream of the promoter were generated with *Kpn*I and *Nhe*I.

2.5. Transfection assays

The transfection assays were performed on oyster dissociated heart cells as previously described [18]. Briefly, heart tissue was enzymatically dissociated (overnight at 4°C) in Hank's buffered salt solution (HBSS) (Gibco BRL, 042-04185M) containing 0.012% pronase (7000 U/g, Boehringer, 1459643). After two washes in HBSS, cells were resuspended in medium without fetal bovine serum and distributed in four-well plates at a concentration of 3×10^6 cells/well and incubated at 25°C. Trypan blue exclusion was used to monitor cellular viability. After 8 h of incubation, a mixture of liposomes (Dotap, Boehringer, 1202375) and DNA from the recombinant plasmid Gia1-L3 at ratios 7 μg/1 μg, 20 μg/3 μg and 30 μg/5 μg were added. The cells were incubated further for 12 h at 25°C and then diluted with 1 ml of culture medium. Finally, 16 h later, the cells were lysed and the luciferase activity of each lot was monitored on a scintillation counter using the luciferase assay system (Promega, kit ref: E1500). Variability between experiments was averaged by measuring the total protein content of the samples using a protein quantitation assay [22]. Controls consisted of non-transfected cells and cells transfected with the expression vector pGL3-basic lacking promoter. Statistical significance of differences ($P<0.05$) between mean values was determined by a Mann-Whitney test.

3. Results and discussion

Screening of the ZAP Express cDNA library with the 850 bp fragment, generated upon amplification with the oyster *C. virginica* PCR primers, yielded several positive clones and the longest insert was sequenced in both strands (Fig. 1). To map the transcription initiation of the actin mRNA, a 5′-RACE-PCR assay was performed, yielding a stretch of 16 additional nucleotides containing a putative 5′-cap site.

The *C. gigas* actin cDNA clone (*Gia1*) contains 1439 bp and encodes a canonical protein of 376 amino acids flanked by leader and trailer tracts (Fig. 1).

The sequence is highly homologous to various known actins. A databank search using the Basic Local Alignment Search Tool [23] yielded highest homologies with the complete actin sequence from the scallop *Placopecten magellanicus* (86%) [24] and with another identified molluscan actin from the Californian sea hare *Aplysia californica* (84%). At the amino acid level, a Blitz search on the SwissProt database showed 97.7% identity with a sea-star and a nematode, the homology with the first gastropod mollusk actin being 95.7% (muscle actin of *A. californica*).

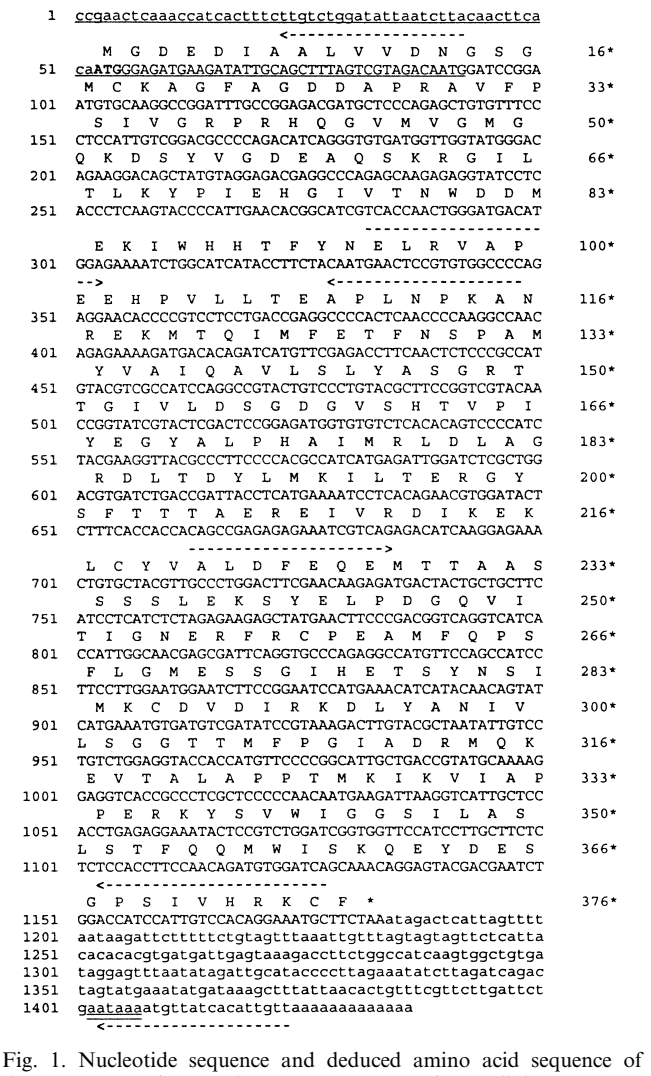

Fig. 1. Nucleotide sequence and deduced amino acid sequence of the *C. gigas* actin cDNA (*Gia1*). Sequences in small letters correspond to UTRs, *Bam*HI-excised probe used for genomic library screening is underlined, translation start codon in bold face letters, putative polyadenylation signals is double-underlined, arrows show primers for upstream region amplification and sequencing of the gene.

It is known that actin sequences from mammals, sea urchins, amoeba and yeast usually share over 87% homology. The initial 18 amino acids of the protein are considered as the most variable inter- and intra-species and are commonly used to discriminate between actin types: α for muscle and β for cytoplasmic actin in vertebrates [25]. This discrimination, however, remains difficult between the two types among invertebrates. Actin sequences have been reported and cloned from Echinodermata [26,27], Nematoda [28], insects [29] and crustaceans [30]. Among mollusks, actin sequences from three gastropods have been published [17,31,32]. In bivalves, a partial actin sequence of the oyster *C. virginica* [20] and the complete sequence of the scallop *P. magellanicus* [24] and the zebra mussel *Dreissena polymorpha* [33] are now available.

Upon comparison with vertebrate α and β actins, the *C. gigas* actin appears more related to vertebrate non-muscular actin forms. Thus, a valine residue at position 11, as well as a methionine and a cysteine residue at positions 17 and 18, are characteristic of a cytoplasmic actin form [25]. Characteristic cytoplasmic residues are also found at positions 77, 104 and 163. In addition, residues at position 4, 77, 235, 279, 326 and 369 are clearly distinctive of cytoplasmic actins according to Mounier et al. [34]. In contrast to most other invertebrates, even to closely related species: the scallop *P. magellanicus* [24] and the mussel *D. polymorpha* [33], the second amino acid residue is not a cysteine but a glycine. This feature has been confirmed by the subsequent whole gene sequencing following amplification directly on the genomic DNA using PCR. In addition, similar replacements have also been found in another bivalve, the mussel *Mytilus edulis*, in which the second residue in one of the isolated isoforms is an aspartic acid instead of the usual cysteine (Cadoret, unpublished). Van Loon et al. [32], in their study on early expressed actin genes in the gastropod *Patella vulgata*, found glutamic and aspartic acids in second positions in three sequenced isoforms. The mentioned substitutions found in invertebrates reveal an intraspecific variability at the level of this second amino acid position. Finally, the canonical polyadenylation motif AATAAA is found at the end of the gene sequence (Fig. 1).

Subsequently, the genomic library was screened with an actin 5′-cDNA fragment. The inclusion of a 5′-untranslated sequence within the probe was made to favor preferential hybridization of translated actin isoforms, thus preventing the isolation of non-functional upstream regions. One positive clone was identified by plating at decreasing densities. A PCR experiment involving the universal primer T7 and a reverse oligonucleotide derived from the previously sequenced cDNA yielded a single amplification product of approximately 1670 bp. The sequence of this fragment shows the serum response element (SRE) characteristic of actins. This 10 bp element in the upstream region is typical of muscular and cytoplasmic actins in both vertebrates and invertebrates. It consists of a repeated motif displaying the consensus $CC(A/T)_6GG$ also termed CarG for CCA/TrichGG. This feature is of importance when examining the presence of putative enhancer elements, as reported in the silkworm *Bombyx mori* cytoplasmic actins [35]. These CarG boxes are found in varied positions, one of them close to the TATA box. Indeed, the oyster promoter displays a putative TATA box 38 bp downstream from the SRE and 25 bp upstream from the putative transcription start point (+1) (Fig. 2). Further characterization of the genomic actin cloned by PCR amplification permitted the subcloning of five overlapping and contiguous DNA fragments. Comparison of the DNA sequence of the subclones with that of the cDNA indicated that this gene harbored a single 643 bp intron starting at position 127 of the actin ORF (Fig. 2). This

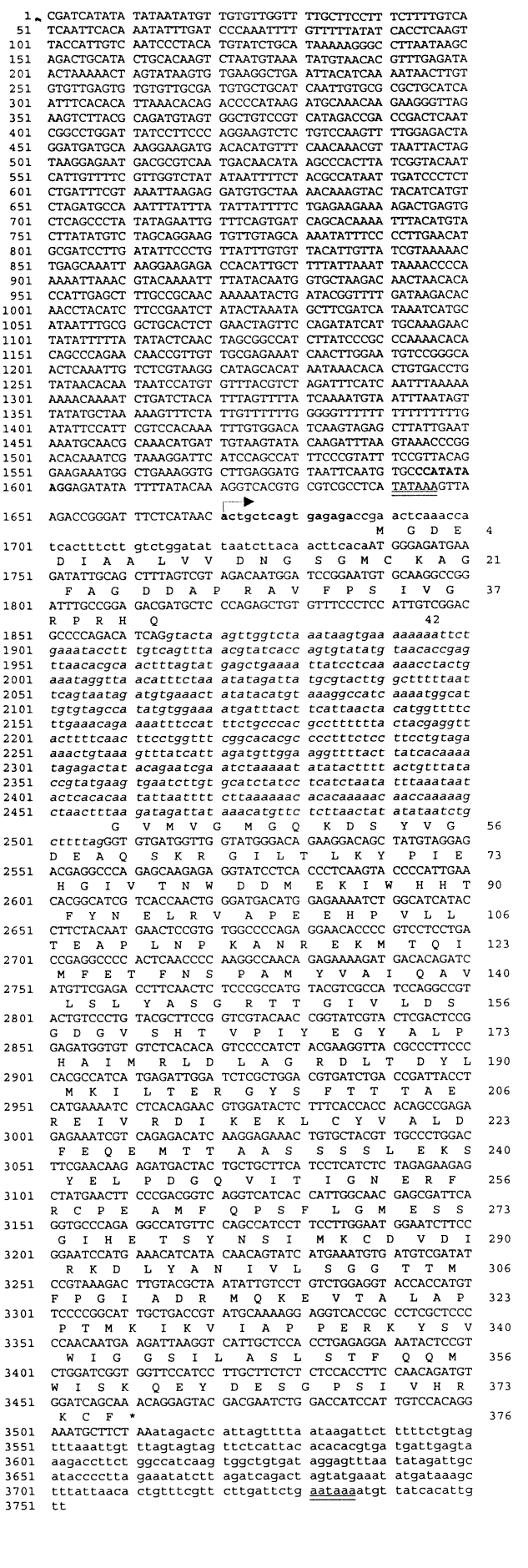

Fig. 2. Nucleotide sequence of the oyster actin gene *Gia1*. Intron, 5′-cap site upstream and 3′-untranslated sequences are shown in lower case letters. The sequences corresponding to the cDNA and the promoter region are in capital letters. The cap site is indicated by an arrow. The TATA-like box and polyadenylation signal are double-underlined. The deduced amino acid sequence of the actin sequence is above cDNA. An asterisk indicates the stop codon. In bold face, lower case letters: the additional 16 bp identified by the RACE-PCR. The CarG box (SRE) is in bold face capital letters.

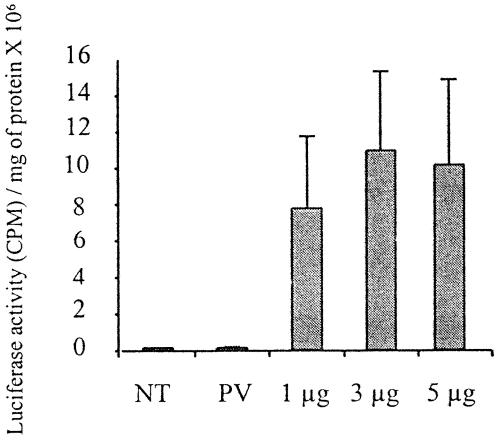

Fig. 3. Transfection of dissociated heart cells of the oyster *C. gigas* with the expression vector Gia1-L3. Non-transfected cells served as the control. A promoterless vector was transfected as an additional control. Each bar represents the mean of several transfections on 3×10^6 cells. Mean values are presented ± S.E.M. NT: Non-transfected, PV: promoterless vector.

position is conserved in vertebrate actins described so far and is consistent with previous descriptions concerning invertebrate actins [29]. Intron length polymorphisms provide neutral tools for the determination of genotype frequencies [36,37].

We undertook the cloning of this promoter region upstream of the luciferase gene in order to test its functionality and its potential use in gene transfer experiments. The luciferase system has proved to be a convenient reporter in earlier experiments [12]. The PCR fragment obtained with the primer T7 and the reverse oligonucleotide was directly inserted into the *Sma*I site of the pGL3-basic multicloning site. This specific expression construct was termed Gia1-L3. Fig. 3 shows the level of expression with this vector containing the actin promoter.

Each bar represents the mean of three experiments. A Mann-Whitney test gave statistical significance to all lipofections carried out. This figure clearly demonstrates the capacity of the 1670 bp upstream region of the cloned oyster actin gene *Gia1* to be functional when transfected into oyster heart cells. Additional experiments involved nested 5′- deletions of the

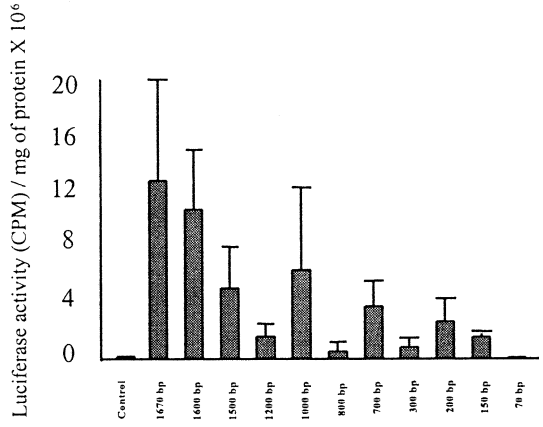

Fig. 4. Transfection of dissociated heart cells of the oyster *C. gigas* with the 5′-deletion mutants of the expression vector Gial-L3. Mean values are presented ± S.E.M.

recombinant vector resulting in a set of 10 plasmids with inserts approximately 70, 150, 200, 300, 700, 800, 1000, 1200, 1500 and 1600 bp, respectively, the intact insert stretching 1670 bp. The first four longest fragments including the intact fragment show a general decreasing promoter activity. The sixth and eighth display a clear drop ($P<0.05$) in the promoter response, which justifies further experimentation (Fig. 4). A minimal response similar to the control is obtained with the last deletion insert starting 70 bp upstream of the putative transcription start point. Sequencing of all the deletion fragments is underway and will allow for screening for putative transcription factors, enhancers and repressors responsible for the differences in the level of expression.

4. Conclusion

We have reported here the isolation of a full-length cDNA from a cDNA library of the oyster *C. gigas* encoding a non-muscular actin and the subsequent characterization of the corresponding actin gene containing a single intron. A functional actin promoter has also been cloned from the oyster genome, which has proved to be functional upon transfection into cells of primary cultures of dissociated oyster heart. The gene features reported could be useful as genetic markers in oyster population studies and the transient expression results may be exploited in future transgenesis experiments, as a means to study gene regulation in bivalves.

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References

[1] Houdebine, L.M. (1997) Transgenic Animals: Generation and Use (Houdebine, L.M., Ed.), p. 576, Harwood academic publishers, Amsterdam.
[2] Wall, R.J., Hawk, H.W. and Nel, N. (1992) J. Cell Biochem. 49, 113–120.
[3] Brem, G. and Muller, M. (1994) in: Animals with Novel Genes (Maclean, N., Ed.), pp. 179–244, Press Syndicate of the University of Cambridge, Cambridge.
[4] Gong, Z. and Hew, C.L. (1995) in: Current Topics in Developmental Biology, Vol. 30, Academic Press.
[5] Maclean, N. and Rahman, A. (1994) in: Animals with Novel Genes (Maclean, N., Ed.), pp. 63–105, Press Syndicate of the University of Cambridge, Cambridge.
[6] Crampton, J.M. and Eggleston, P. (1994) in: Animals with Novel Genes (Maclean, N., Ed.), pp. 21–62, Press Syndicate of the University of Cambridge, Cambridge.
[7] Flytsanis, C.N., Mc Mahon, A.P., Hough-Evans, B.R., Katula, K.S., Britten, R.J. and Davidson, E.H. (1984) in: Molecular Biology of Development (Liss, A.R., Ed.), pp. 621–632, New York.
[8] Fire, A. and Waterston, R.H. (1989) EMBO J. 8, 3419–3428.
[9] Mialhe, E., Bachère, E., Boulo, V. and Cadoret, J.P. (1995) Aquaculture 132, 33–41.
[10] Mialhe, E., Bachere, E., Boulo, V., Cadoret, J.-P., Saraiva, E., Carrera, L., Calderon, J. and Colwell, R. (1995) Mol. Mar. Biol. Biotechnol. 4, 275–283.
[11] Powers, D.A., Kirby, V.L., Cole, T. and Hereford, L. (1995) Mol. Mar. Biol. Biotechnol. 4, 369–375.
[12] Cadoret, J.P., Boulo, V., Gendreau, S. and Mialhe, E. (1997) J. Biotechnol. 56, 183–189.
[13] Lu, J.K., Chen, T.T., Allen, S.K., Matsubara, T. and Burns, J.C. (1996) Proc. Natl. Acad. Sci. USA 93, 3482–3486.
[14] Gomez-Chiarri, M., Hereford, L. and Powers, D. (1994) 3rd

International Marine Biotechnology Conference, August 7–12, Tromso.
[15] Chan, K.M. and Chow, J.F.C. (1994) 3rd International Marine Biotechnology Conference, August 7–12, Tromso.
[16] Horard, B., Mangé, A., Pélissier, B. and Couble, P. (1994) Insect Mol. Biol. 3, 261–265.
[17] Lardans, V., Ringaut, V., Cadoret, J.P. and Dissous, C. (1997) DNA Seq. 7, 353–356.
[18] Boulo, V., Cadoret, J.P., Le Marrec, F., Dorange, G. and Mialhe, E. (1996) Mol. Mar. Biol. Biotechnol. 5, 167–174.
[19] Cadoret, J.-P., Gendreau, S., Delecheneau, J.-M., Rousseau, C. and Mialhe, E. (1997) Mol. Mar. Biol. Biotechnol. 6, 72–77.
[20] Unger, M.E. and Roesijadi, G. (1993) Mol. Mar. Biol. Biotechnol. 2, 319–324.
[21] Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) Cold Spring Harbor Laboratory Press, New York.
[22] Bradford, M. (1976) Anal. Biochem. 72, 248–254.
[23] Altshul, S.F., Gish, W., Miller, W., Myers, E.W. and Lipman, D.J. (1990) J. Mol. Biol. 215, 403–410.
[24] Patwary, M.U., Reith, M. and Kenchington, E.L. (1996) J. Shellfish Res. 15, 265–270.
[25] Wesseling, J.G., de Ree, J.M., Ponnudurai, T., Smits, M.A. and Schoenmakers, J.G.G. (1988) Mol. Biochem. Parasitol. 27, 313–320.
[26] Schuler, M.A., McOsker, O. and Keller, E.B. (1983) Mol. Cell. Biol. 3, 448–456.
[27] Crain, W.R., Boshar, M.F., Cooper, A.D., Durica, D.S., Nagy, A. and Steffen, D. (1987) J. Mol. Evol. 25, 37–45.
[28] Krause, M., Wild, M., Rosenzweig, B. and Hirsh, D. (1989) J. Mol. Biol. 208, 381–392.
[29] Mounier, N. and Prudhomme, J.C. (1986) Biochimie 68, 1053–1061.
[30] Macias, M.T. and Sastre, L. (1990) Nucleic Acids Res. 18, 5219–5225.
[31] DesGroseillers, L., Auclair, D. and Wickham, L. (1990) Nucleic Acids Res. 18, 3654.
[32] Van Loon, A.E., Goedemans, H.J., Daemen, A.J.J.M., Van de Kamp, A.J. and Van de Biggelaar, J.A.M. (1993) Roux's Arch. Dev. Biol. 202, 77–84.
[33] Lamers, A.E., Heiney, J.P. and Ram, J.L. (1998) J. Shellfish Res. 17, 1215–1217.
[34] Mounier, M., Gouy, M., Mouchiroud, D. and Prudhomme, C. (1992) J. Mol. Evol. 34, 406–415.
[35] Mangé, A., Couble, P. and Prudhomme, J.C. (1996) Gene 183, 191–199.
[36] Lessa, E.P. (1992) Mol. Biol. Evol. 9, 323–330.
[37] Corte-Real, H.B.S.M., Dixon, D.R. and Holland, P.W.H. (1994) J. Mar. Biol. 120, 407–413.