

1 Lack of evidence for the presence of an interferon in invertebrate

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3 Pei-Hui Wang

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5 Address correspondence and reprint request to Pei-Hui Wang, Department of

6 Biochemistry, The University of Hong Kong, 3/F Laboratory Block, Faculty of

7 Medicine Building, 21 Sassoon Road, Pokfulam, Hong Kong. E-mail address:

8 wph326@gmail.com (P.-H. Wang)

9 Abstract

10 In vertebrates, the interferon (IFN) response is the primary form of innate antiviral
 11 defense. Previously (2005), a partial cDNA which could encode an interferon-like
 12 protein (IntIP) is reported in shrimp, later Rosa et al. (2008) argue that this partial
 13 cDNA should encode a portion of insect mitochondrial ATP synthase (MAS) B-chain.
 14 Recently (2009), it is demonstrated IntIP also possess antibacterial activity beside
 15 antiviral activity reported before. Lacking of a consensus opinion to the question of
 16 whether this gene encodes IntIP or MAS, we try to provide more evidences to identify
 17 this gene exactly. Here we obtain the full length cDNAs of IntIP/ MAS in *Litopenaeus*
 18 *vannamei*, and perform the tissue distribution and induced expression analysis. Our
 19 results confirm that IntIP is coded by a mistaken ORF and the actual protein indeed is a
 20 *L. vannamei* mitochondrial ATP synthase (LvMAS) whose function is unknown in
 21 antiviral responses.

22 **Keywords:** shrimp interferon; mitochondrial ATP synthase; antiviral activity;
 23 antibacterial activity

24

25 **1. Introduction**

26 Interferons (IFNs) constitute a large group of cytokines that are best known for their
 27 ability to induce vertebrate cells into an antiviral state. It is also reported IFN system
 28 can defense against bacterial and protozoal infection. Binding of IFNs to their receptors
 29 initiates signaling that leads to a global shutdown in protein translation, cellular RNA
 30 degradation and deamination and often the death of virus-infected cells. However, until
 31 recently, there was no IFN cDNA of invertebrates found. In 2005 He et al. reported a
 32 partial cDNA encodes an interferon-like protein (IntIP) homologue to mammalian IFN-
 33 α which was expressed only in the WSSV-resistant shrimp *Penaeus japonicus* (but not
 34 in naïve shrimps) and showed non-specific antiviral activity to SGIV (grouper
 35 iridovirus). But later Rosa et al. (2008) argue that this partial cDNA actual encodes a
 36 portion of the mitochondrial ATP synthase (MAS) which shows high identity (60–73%)
 37 with insect MAS b-chain and was expressed not only in naïve and WSSV-infected *L.*
 38 *vannamei* but also in two wild Brazilian shrimp species. As well as He, Rosa didn't
 39 obtain the full length cDNA of shrimp ATP synthase. Thus, like IntIP, it is unclear
 40 whether this portion of the ATP synthase is in the right coding region of the full length
 41 cDNA. It is also unknown whether this gene has an induced expression by pathogen
 42 infection and where it localizes (secreted or not) for function. Recently, Mai et al. (2009)
 43 report IntIP also possesses significant antibacterial activity to the shrimp pathogens *V.*
 44 *alginolyticus* and *V. parahaemolyticus*. About this partial cDNA encoding IntIP or MFS
 45 has two different points of view. Maybe more empirical evidences are needed to
 46 confirm a substantive protein encoded by this gene. We obtain the full length cDNAs of

47 IntIP/MAS in *L. vannamei* by RACE-PCR approach, and then several cDNAs show
48 very high nucleotide identities (>60%) with *L. vannamei* MAS (LvMAS) in other
49 lobsters and crabs were retrieved in NCBI. All these cDNA sequences encode proteins
50 show high identities with insect MAS but very low with mammal IFNs. RT-PCR
51 reveals that LvMAS mRNA cannot be induced by immune challenge. Further
52 homology searching and sequence analysis indicate IFNs most probably origin from
53 cartilaginous fish and to date no correct invertebrate IFN cDNAs have been cloned.
54 This study also makes it clear that IFN system is limited in high vertebrates, while RNA
55 interference is used by nematode and insects as a main antiviral strategy. And this also
56 points an interesting question that which mechanism is used in the ancestor of high
57 vertebrate like sea urchins, amphioxus, hagfish, and lamprey and so on which lack IFN
58 system and no RNA interference pathway found.

59 **2. Materials and methods**

60 Based on the partial cDNA (accession no. **EU246975**) which could encode an
61 interferon-like protein (IntIP) in *L. vannamei*, specific primers (Table. 1) were designed
62 to obtain the 3' and 5' end cDNA sequences of LvMAS by rapid amplification of cDNA
63 ends (RACE) as described before(Wang et al., 2009). The genes of American Lobster,
64 Blue Crab, *Petrolisthes cinctipes* and Grass Shrimp show high nucleotide identities (>
65 70%) with LvMAS were obtained through the NCBI programs blastn and blastx, and
66 the ORFs were predicted through the NCBI program ORF Finder. RT-PCR was
67 performed with LvMAS-F and LvMAS-R. cDNA templates for RT-PCR were prepared
68 previously(Wang et al., 2009), and the conditions were the same as described before

except that the cycles were modified as indicated in Fig. 2B. Reported vertebrate IFN- α and IFN- γ were used as seed sequences to search IFN homologous of *C. elegans*, insects, sea urchin *S. purpuratus*, amphioxus, lamprey in UCSC Genome Browser and NCBI using “BLAST”. When search IFN homologous of pacific oyster *Crassostrea gigas*, shrimps and crabs, databases of Marine Genomics Project (<http://www.marinegenomics.org/>) are also referred using the provided search tool.

3. Results and discussion

The predicted ORFs of American Lobster, Blue Crab, *L. vannamei*, *Petrolisthes cinctipes* and Grass Shrimp all encode a protein (AlMAS, BcMAS, LvMAS, PcMAS and GsMAS) which possesses a mitochondrial ATP synthase domain; no alternative ORF which could encode a protein possesses an interferon domain is available (Fig. 1D). The ORF encodes the IntIP used by He et al which does not contain an interferon domain and shows very low identity with vertebrate IFN- α (Fig. 1B). As later Rosa described, the analysis of IntIP gene through the NCBI programs blastx resulted in a translated nucleotide sequence that strongly matched with the MASs of other species (Fig. 1C). In addition, IFNs are secreted proteins which possess a signal peptide in the N-terminus. But we can not find any signal peptide using all possible translation patterns of these five genes (Fig. 1D). The obtaining of the full length cDNA of LvMAS and further sequence analysis make us believe that IntIP most probable is encoded by a mistaken ORF. To confirm this conclusion, RT-PCR was performed to investigate the distribution and induced expression of LvMAS (Fig. 2B). We observe that LvMAS is widely distributed in healthy and immune challenged shrimp *L.*

91 *vannamei*, a result correlated with later Rosa. When challenged by saline, LPS (from *E.*
92 *coli*), Gram-negative *V. alginolyticus*, Gram-positive *S. aureus*, Yeast *S. cerevisiae*,
93 white spot syndrome virus (WSSV) or polyinosinic polycytidylic acid (poly I: C) as
94 described before, the expression of LvMAS has no obvious changing (Fig. 2B). The
95 constitutive expression of LvMAS is more like MAS rather than IFNs which could be
96 highly induced after immune challenge. As for the results that recombinant IntIP
97 possess non-specific antiviral and antibacterial activities described by He et al and Mai
98 et al, it remains unexplained. In the article (He et al., 2005), Fig. 3B displays the
99 antiviral activity of recombinant IntIP by a cytotoxicity experiment by inhibiting SGIV
100 on fish GP cell lines (grouper embryo cells). According to the authors, GP cells (Fig.
101 3A) were completely destroyed by SGIV (Fig. 3B) while parts of the cells remained
102 alive when incubated in the SGIV and IntIP protein mixture for 48 h, (Fig. 3C). But the
103 figures are not clear enough and no parallel experiments were declared. In the paper
104 (Mai et al., 2009), three different methods are mentioned in detecting IntIP antibacterial
105 experiments, but they were only done in duplicates and no significant differences were
106 calculated. And in these two papers, the antiviral and antibacterial assays both lack
107 positive control groups. In the later paper (Mai et al., 2009) in Table 1, a negative group
108 is also lacking. In addition, IntIP indeed shows some similarity with a portion of
109 mammalian IFNs although it is very low, so it can not be rule out that recombined IntIP
110 protein holds some functions like the IFNs.

111

112 The encodings of IFNs in animal genomes and EST sequences indicate that IFNs are

113 limited in bony vertebrates (teleost fish, amphibians, reptiles, birds, mammals) and all
 114 kinds of interferons are absent in pacific oyster, *C. elegans*, insects, sea urchin *S.*
 115 *purpuratus*, amphioxus, hagfish and lamprey, some of which are consistent with
 116 previous studies (Huang et al., 2008; Krause and Pestka, 2005; Savan et al., 2009) (Fig.
 117 3A). Although a human IFN-R1 homology was found in the sea squirt *Ciona*
 118 *intestinalis*, there is no significant IFN-R sequence found in amphioxus, hagfish or
 119 lamprey (Krause and Pestka, 2005). Until cartilaginous fish, a shark IFN γ -R is found
 120 (Savan et al., 2009). In evolution before cartilaginous fish, most of the vertebrate
 121 cytokines except for the tumor necrosis factor like gene are absent, including most
 122 interleukins, all interferons, chemokines, colony-stimulating factors, and their cognate
 123 receptors (Huang et al., 2008; Krause and Pestka, 2005). The same situation is also
 124 observed in other invertebrates including pacific oyster, nematode *caenorhabditis*
 125 *elegans*, crustacea and genome available insects (Rosa and Barracco, 2008). But from
 126 cartilaginous fish some cytokines begin to emerge (Krause and Pestka, 2005; Savan et
 127 al., 2009). We propose that vertebrate cytokines especial IFNs origin from cartilaginous
 128 fish (Fig. 3B). So we would not agree with the existence of an unknown cytokines to
 129 perform the antiviral protection in shrimps (Rosa and Barracco, 2008). In invertebrates
 130 such as nematode and insects, RNAi is critical for protecting from viral infections
 131 (Saleh et al., 2009; Schott et al., 2005). But RNAi is replaced by the IFNs system in
 132 high vertebrates as the primary antiviral responses (Cullen, 2006; Myles et al., 2008).
 133 RNAi now exists in vertebrates only as a mechanism of post-transcriptional regulation
 134 'programmed' by endogenously encoded miRNA. But RNAi is a sequence-specific

135 gene-silencing mechanism, which are different from the dsRNA-sequence independent
 136 unspecific antiviral responses of shrimps (Rosa and Barracco, 2008). We believe that
 137 some virus-induced immune proteins such as C-type lectins, hemocyanins and AMPs
 138 would play a very important role in this unspecific antiviral responses rather than
 139 unknown interferon-like proteins or cytokines similar to vertebrates proposed by Rosa
 140 et al (Lei et al., 2008; Zhao et al., 2009). To have a better understanding of shrimp
 141 antiviral response, further investigations should focus on these virus-induced immune
 142 proteins and a similar RNAi pathway in shrimps. As for the antiviral mechanism of low
 143 vertebrates, it is still a gap. Further investigation would be very interesting and
 144 contribute to the better understanding of the origin and evolution of animal antiviral
 145 system.

146 In conclusion, this current work demonstrates that IntlP is not a real interferon-like
 147 protein, but encoded by a mistaken ORF of MAS. To our knowledge, the reported IFNs
 148 are limited in bony vertebrates, and further homology searching and sequence analysis
 149 make us believe that most vertebrate cytokines especial IFNs origin from cartilaginous
 150 fish.

151

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157 Province. The nucleotide sequence of LvMAS has been submitted to NCBI database
158 and GenBank with an accession number of DQ923424.

159

160 *Abbreviations:* IFNs, interferons; MAS, mitochondrial ATP synthase; IntlP,
161 interferon-like protein; ORF, open reading frame; IPTG,
162 isopropyl-β-D-thiogalactopyranoside; LB, Luria broth; LPS, Lipopolysaccharide; ORF,
163 open reading frame; WSSV, white spot syndrome virus; SGIV, Singapore grouper
164 iridovirus; RACE, rapid amplification of cDNA end; RT-PCR, Reverse
165 Transcriptase–Polymerase Chain Reaction; S2, *Drosophila* Schneider 2; SDS-PAGE,
166 sodium dodecyl sulfate polyacrylamide gel; ISKNV, infectious spleen and kidney
167 necrosis virus; MFF, mandarin fish fry; poly I: C polyinosinic polycytidylic acid.

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208 **Figure Legends**

209 **Fig.1 cDNA cloning (A) and sequence analysis (D) of *LvMAS*.** The ORF encodes the
210 IntIP used by He et al shows very low identity with vertebrate IFN- α (B). The analysis
211 of IntIP gene through the NCBI programs blastx resulted in a translated nucleotide
212 sequence that strongly matched with the MASs of other species (Fig. 1C).

213 **Fig.2 Expression of *LvMAS* mRNA in healthy and immune-challenged shrimps..**

214 Tissue distribution of *LvMAS* mRNA in hemocyte (1), epithelium (2), hepatopancreas
215 (3), nerve (4), eyestalk (5), heart (6), pyloric caecum (7), intestine (8), gill (9) and
216 muscle (10) in healthy shrimps by RT-PCR analysis (left). Induced expression of
217 *LvMAS* mRNA in hemocyte by saline (12), LPS (from *E.coli*) (13), Gram-negative *V.*
218 *alginolyticus* (14), Gram-positive *S. aureus* (15), Yeast *S. cerevisiae* (16), white spot
219 syndrom virus (WSSV) (17) or polycytidylic acid (poly I: C) (18), and untreated
220 hemocyte (12) was used for control (right).

221 **Fig.3 Tree of life showing the emergence and evolution of IFNs.**

222

223

224 **Table 1. PCR primers used in this study**

225

| Primers | Primer sequences (5'-3') | Purpose/note |
|------------------|--------------------------|---|
| 5' RACE1 | TCGCATTGAGCAGAAGCAC | cDNA cloning; using nucleotides of <i>P. japonicus</i> to amplify 5' and 3' cDNA ends of <i>L. vannamei</i> MAS |
| 5' RACE2 | TGCCAAGCGAGAAAATGTTG | |
| 3' RACE1 | ATGCCAAGCGAGAAAATGTT | |
| 3' RACE2 | AACTCGCATTGAGCAGAAGC | |
| LvMAS-F | CTTTGTCATGGCTGTTTACGC | RT-PCR; the distribution and induced expression analysis of LvMAS |
| LvMAS-R | CATCTTGAACCCAATAAGTCCTAC | |
| β -actin-F | GAAGTAGCCGCCCTGGTTG | |
| β -actin-R | CGGTTAGCCTTGGGGTTGAG | |

226

227

[illegible]

AMDEKKRQVRAQGGIIMDAKKENVALQLEAAYRERAAV
 AMDEKRRQVETGCGQMFDAHRENVALQLEAAYRERLAV
 AMDEKRRQVETGCGRIIFDAHRENVALQLEAAYRERLAV
 AMDEKRRQVAGCGRIIFDAHRENVALQLEAAYRERLAV
 ---EKDAWFAEGCGQMFDAHRENVALQLEAAYRERLAV
 YREVKKRLDYQISGVNDRRTISKHNVWII *A. aegypti*
 HSEVKKRLDYQISGVNDRVTRISKHNVWII *F. paulensis*
 HSEVKKRLDYQISGVNDRVTRISKHNVWII *L. schmitti*
 HSEVKKRLDYQISGVNDRVTRISKHNVWII *L. vanna*
 HAEVKKRLDYQISGVNDRVTRISKHNVWII *M. japonicus*

(A)

(C)

MLSRLALRSVQEARPFLVLPVRPTSTAVNKATE--RDEVNERRRPVPGKVRMGFLPEEWEQFFYSKGTGVTGPYLFGVG
 MLSRLANRLQEARPFLVLPVRPTSTAVNKREE--RDEVNERRRPVPGKVRMGFLPEEWEKFFYAKGTGVTGPYLFGVG
 MLSRLVTRSLQEVRFALVIRPTSTAVSRAAERDEVNERRRPVPGKVRMGFLPEEWEQFFYSKGTGVTGPYLFGVG
 MLSRLAARTVQEVRF--LVIRPTSTASVQRAEQ--RDEVNERRRPVPGKVRMGFLPEEWEQFFYSKGTGVTGPYLFGVG
 MLSRLATSLQEARF--VVIVRATSTAVRAAEE--RDEVNERRRPVPGKVRMGFLPEEWEQFFYSKGTGVTGPYLFGVG
 MLSRLVALRSQASKQACTIVARGASDVSA--TH--DQKTRPVRP--PGKVRMGFLPEEWEQFFYSKGTGVTGPYLFGVG
 MLSRLVALRSAAAKQS--TRIVARGASADVSGVR--DEKNERRRPVPGKVRMGFLPEEWEQFFYSKGTGVTGPYLFGVG
 MLSRLALILAAKPPA--AVILARGASATD-----GNRPVRADPGKVRMGFLPEEWEQFFYSKGTGVTGPYLFGVG
 MLSRLPALIAAARPPA--GLILARGASATD-----GNRPVRADPGKVRMGFLPEEWEQFFYSKGTGVTGPYLFGVG

LDTFLCISKEIYVMEHEFYVGLSLIFMVAIVAIRKIGECIFNYLDKEIEDIEGSSWVKYRQESVEGIEQATBIDERRAQMFAEG
 VGTFFIEISKEIYVMEHEFYVGLTILMLTVVGVKFGEGCLAYLIDKEIEDEGSSWVNYRGRSSNNIKESIBAEKKAQNEAF
 LDTFLCISKEIYVMEHEFYVGLSIFIMVYVAKKIGFLCQSLDKIEDEIGSSWVYTRDGSITDILKRSVBEKAAQLSAG
 LDTFLCISKEIYVMEHEFYVGLSILIMVYALKRKGIVIGETLDRIEILBASWNYREGSVIVQVQNTBFEKALISAG
 LDTFLCISKEIYVMEHEFYVGLSLIIMVAFVAKRFGESLQSDKEIDIEHLWNYRDESTQGLDKDSVCEKAAQASAG
 LDTYLCSKEIYVMEHEFYVGLSLIMVYVHVKGFELAKWIDKEVATEENNEGKNTVIALEDAIBGETEIQFRAAG
 LDTYIFSKEIYVMEHEFYVGLSLLIMVAVAKKFGSLAAWLDKEVQATBNIDNSSRVESKALEDAVBAEKKAQMFAGG
 MLTYLCSKEIYVMEHEFYVGLSLAIMVIAVVRKEGFAVAYCDREIDRIGEWGQRENTIAQLINQATBIEKFEQFRAAG
 LDTYLCSKEIYVMEHEFYVGLSLAIMVIAVKKFGFAVAYCDREIDRIGEWKALRENNQOTACAMFETFEKFEQFRAAG

GATLFDKAKRNVSLQLEAAAYRRLATVHSEVKKRLDYQLBTANVKTIEQKHMVNWTVSNVFKASTTFAQAAATKCCFAD
 GSVLFDKAKRNVSLQLEAAAYRRLATVHSEVKKRLDYQLBTANVKTIEQKHMV-----LVNGVRS-----
 EAMLFDKAKRNVSLQLEASRYRRLAMVHPEVKKRLDYQLBTANVKTIMQKHMV-----HDCPKT-----
 CMLFDKAKRNVSLQLEAAAYRRLAKVHSEVKKRLDYQLBVAHVKTILQKHMVDWTVSNVHSSITFAQAAATKCCMAAD
 BSMILFDKAKRNVSLQLEAAAYRRLATVHSEVKKRLDYQLBTANVKTILQKHMVDWTVSNVFKASTTFAQAAATKCCFAD
 CELLIQAKKNVLLQLEAAAYRRLIMYAEVKKRLDYQLBKSNVKTLQKHMVDWTVSNVTKATHTDQKQADRCIAD
 CELLIQAKKNVLLQLEAAAYRRLPMYAEVKKRLDYQLBKSNDLERLAQKHMVDWTVSNVTKATHTDQKQADRCIAD
 GTLIMAKKNVLLQLEAAAYRRLATYAEVKKRLDYQVBRQNVDRISQKHMVDWTVSNVTKATHTDQKQADRCIAD
 GTLIMAKKNVLLQLEAAAYRRLATYAEVKKRLDYQVBRQNVDRISQKHMVDWTVSNVTKATHTDQKQADRCIAD

| | Signal peptide | Identity with hIFN- α /AaMAS |
|-----------------|----------------|-------------------------------------|
| IKGLAPQSA : 248 | LvMAS No | 14.4% / 59.3% |
| ----- : 218 | GsMAS No | 18.6% / 49.6% |
| ----- : 221 | AlMAS No | 18.5% / 47.8% |
| IKSLPFA- : 246 | BcMAS No | 15.6% / 57.8% |
| IKGLAPKVA : 247 | PcMAS No | 17.0% / 57.5% |
| IASLARK-- : 243 | BmMAS No | 14.7% / 63.0% |
| IAALPARK- : 244 | SeMAS No | 16.1% / 64.1% |
| IGALPARKS : 238 | CqMAS No | 16.9% / 89.5% |
| IGALPARAK : 238 | AaMAS No | 14.3% / 100% |

(D)

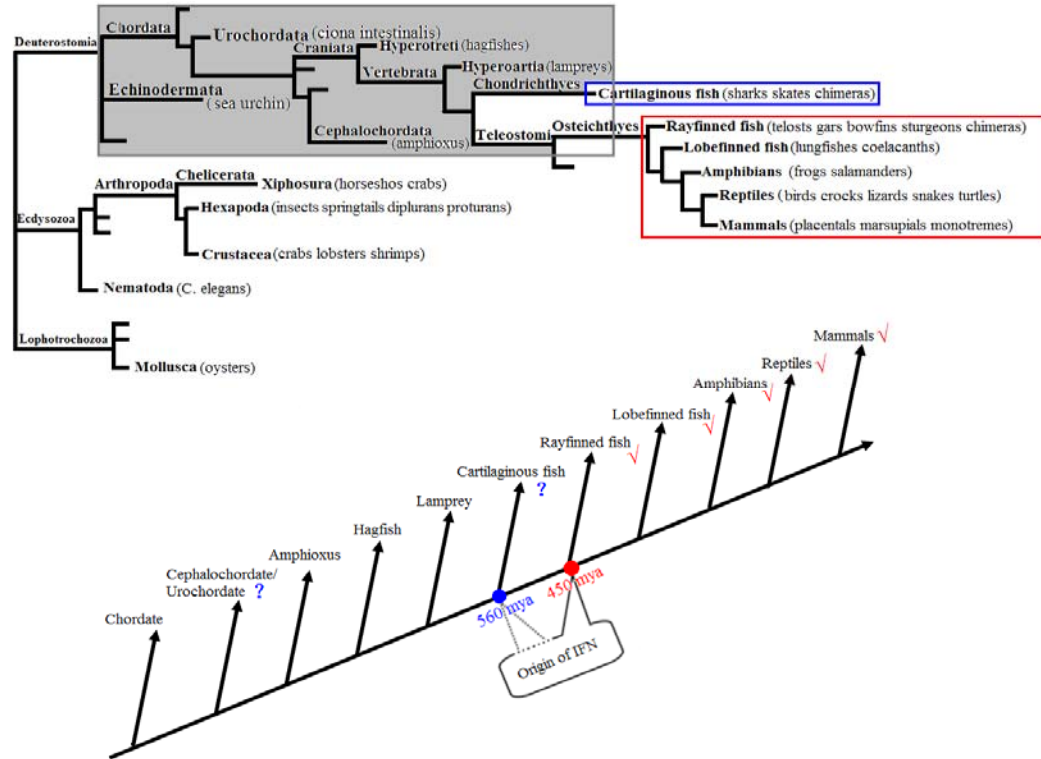
231 Fig.2



232

233

234 Fig.3



235