Vol. 60: 1–9, 2004 | DISEASES OF AQUATIC ORGANISMS
Dis Aquat Org | Published July 5

Identification of two novel interferon-stimulated genes from cultured CAB cells induced by UV-inactivated grass carp hemorrhage virus

Yi Bing Zhang, Jian Fang Gui*

State Key Laboratory of Freshwater Ecology and Biotechnology, Wuhan Center for Developmental Biology, Institute of Hydrobiology, Chinese Academy of Sciences, Wuhan 430072, PR China

ABSTRACT: Interferon (IFN) exerts its antiviral effect by inducing the expression of a number of IFN-stimulated genes (ISGs) to establish a host antiviral state. Earlier studies identified some important fish IFN system genes from IFN-induced CAB cells (crucian carp *Carassius auratus* L. embryonic blastulae cells) after treatment with UV-inactivated GCHV (grass carp hemorrhage virus). Herein, the cloning of 2 novel IFN-stimulated genes, termed Gig1 and Gig2, is described for the same cell system. The complete cDNA sequences of Gig1 and Gig2 contain 1244 bp encoding for a 194-amino-acid protein and 693 bp for a 158-amino-acid protein, respectively. A search of public databases revealed that these are 2 novel IFN-stimulated genes, since neither significant homologous genes nor conserved motifs were identified. Active GCHV, UV-inactivated GCHV and CAB IFN-containing supernatant (ICS) induced transcription of these genes and distinct kinetics were observed. An analysis of differences in expression between the 2 genes and the IFN signal factors CaSTAT1 and CaIRF7 indicated that GCHV infection activated different signal pathways for their up-regulation. Upon virus infection, the transcription of Gig1 but not of Gig2 is strongly suppressed by cycloheximide (CHX). In contrast, following treatment with CAB IFN-containing supernatant, CHX does not inhibit either gene transcription. The results suggest that GCHV infection can induce expression of both Gig1 and Gig2 via newly synthesized CAB IFN, most probably through the JAK-STAT signal pathway, and can also directly activate Gig2 transcription without ongoing protein synthesis.

KEY WORDS: Interferon · Interferon-stimulated gene · Antiviral-relevant gene · JAK-STAT signal pathway · Crucian carp embryonic blastulae cells · CAB · Grass carp hemorrhage virus

INTRODUCTION

The interferon (IFN) system is the first line of cellular defense against virus invasion: upon infection, host cells are stimulated to produce IFN, which in turn up-regulates a subset of downstream IFN-stimulated genes (ISGs) to develop a host antiviral state (Samuel 2001). In mammals, more than 300 ISGs have been identified so far by oligonucleotide arrays (Der et al. 1998). However, except for several ISG products, including double-stranded RNA-activated protein Kinasel (PKR), 2-5(A) synthetase/RNaseL, Mx, ADAR and IFI56, which have been known to affect virus replication, transcription or cell growth, the exact biochemical and cellular functions of most ISGs remain unknown (Samuel 2001). In addition to these genes, numerous reports strongly indicated that other yet-undefined ISGs possibly mediate the antiviral activity of IFN against virus infection (Zhou et al. 1999, Presti et al. 2001, Wieland et al. 2003).

Earlier studies have described IFN-like activity in different fish species and cultured fish cells (Zhang & Yu 2000), and several ISGs, such as the IFN regulatory factor (IRF) (Yabu et al. 1998, Zhang et al. 2003b), Mx (Trobridge & Leong 1995, Robertsen et al. 1997, Trobridge et al. 1997, Yap et al. 2003), Vig1 (Boudinot et al. 1999) and Vig2 (Boudinot et al. 2001) have been reported in independent studies, although their functions have not yet been fully elucidated. More recently, significant progress has been made in isolating virally induced genes (O'Farrell et al. 2002, Zhang et al.

*Corresponding author. Email: jfgui@ihb.ac.cn

2003a), identified as IFN-responsive genes (O'Farrell et al. 2002). A zebrafish IFN gene has also been identified (Altmann et al. 2003). In comparison to mammals, however, relatively few fish genes involved in IFN multiple effects have been characterized, especially from a single studying system (Zhang et al. 2003a).

Grass carp hemorrhage virus (GCHV) is a fish reovirus, and is the first fish virus to be isolated in China. It is responsible for high contagious hemorrhage disease in grass carp *Ctenopharyngodon idellus* leading to mortality of at least 60%, and to significant economic losses for grass carp farmers (Zhang 2002). Over the past 2 decades, an enormous amount of work has been focused on the morphology, physiochemical characterization of and vaccination against GCHV. Recently, the entire genome of GCHV was sequenced (Zhang 2002). Previous studies have shown that GCHV is able to induce IFN-like activity in grass carp and its cultured cells (Wang et al. 1999, Zhang & Yu 2000, Zhang et al. 2000), and that UV-inactivated GCHV is more effective in inducing IFN in the embryonic blastulae cells of crucian carp *Carassius auratus* L. (CAB) (Wang et al. 1999). Based on work concerning induction and characterization of fish IFN from CAB cells (Wang et al. 1999, Zhang et al. 2000), an experimental system for studying fish antiviral-relevant genes was established (Zhang et al. 2003a,b,c, Zhang & Gui 2004a,b). Suppression subtractive hybridization (SSH) has been employed to identify mRNAs that accumulate in higher concentrations in UV-inactivated GCHV-infected CAB cells than in mock-infected cells (Zhang et al. 2003c), and a number of differentially expressed sequence tags (ESTs), representing TLR3, STAT1, JAK1, IRF7, Mx, Viperin (Vig1), PKR (IFN-inducible eIF2α kinase), IFI56, IFI58, ISG15 and other important immune-relevant genes, have been identified (Zhang et al. 2003a). Also, 23 unidentified ESTs that show no similarity by Blast search have also been retrieved. Further studies revealed several unidentified ESTs, representing novel genes (Zhang et al. 2003a). Herein we describe the identification and cloning of 2 novel fish ISGs, termed Gig1 and Gig2 (GCHV-induced Gene Nos. 1 and 2, respectively), according to the sequences of 3 unidentified ESTs with relatively high screening frequency during screening of the established subtractive cDNA library. Expression analysis revealed that GCHV infection induces the expression of 2 ISGs, possibly through distinct signaling pathways.

MATERIALS AND METHODS

Cell culture and virus propagation. We cultured 2 cyprinid fish cell lines, crucian carp *Carassius auratus* L. embryonic blastulae cells (CAB) and grass carp *Ctenopharyngodon idellus* kidney cells (CIK), in Medium 199 supplemented with 10% fetal calf serum (FCS) and antibiotics. Pathogenic Isolate 873 of GCHV was used. CIK cells were used for GCHV propagation and titer detection. When necessary, GCHV was inactivated completely by UV irradiation. Briefly, the harvested cell-culture fluid containing GCHV were centrifuged at $4 \times 10^3 \times g$ for 20 min to remove cell debris, and the supernatant was then ultracentrifuged at $1 \times 10^5 \times g$ for 1.5 h. The virus pellet thus obtained was resuspended in optimal Medium 199 without FCS, and centrifuged again at $1.5 \times 10^3 \times g$ at 4°C for 20 min. Purified GCHV was obtained from the supernatant and was used for UV-inactivation. Virus sample volumes of 3.5 to 4 ml in 40 mm petri dishes were placed on a shaker for constant and slow shaking during virus inactivation. GCHV could be completely inactivated by UV irradiation for 5 min with a 30 W General Electric germicidal lamp placed at a distance of 15 cm (Zhang et al. 2003b).

Induction and preparation of CAB IFN-containing supernatant (ICS). Briefly, after CAB cells had been grown in plastic flasks (25 cm^2) at 28°C for 4 d, the culture fluid was removed, and the cells were washed 3 times with non-FCS Medium 199. The washed cells were then treated with 0.5 ml UV-inactivated GCHV for 2 h at 28°C. After the treated medium had been removed, the cells were washed again 3 times with non-FCS medium 199. We added 5 ml of non-FCS Medium 199, and incubated the cells for 24 h at 28°C. The cell culture fluid was collected, and ultracentrifuged at $1 \times 10^5 \times g$, 4°C for 3 h to remove the remaining virus and cell debris. The upper one-third of supernatant was used as ICS to detect the IFN titer (Wang et al. 1999, Zhang et al. 2003b) and to characterize the expression of 2 novel ISGs.

Rapid amplification of cDNA ends (RACE)-PCR. In accordance with the instructions of Clontech PCR-Select™ cDNA subtractive kit and Clontech SMART (switch mechanism of the 5′ end of RNA template) PCR synthesis kit, a subtractive cDNA library was constructed with mRNA derived from UV-inactivated GCHV-infected and mock-infected CAB cells as described previously (Zhang et al. 2003a,c). Typically, CAB cells were firstly treated with UV-inactivated GCHV for 2 h, and then replaced by fresh non-FCS Medium 199 and cultured at 28°C for 14 h. Total RNA was extracted from UV-inactivated GCHV-infected and mock-infected CAB cells by CsTFA ultracentrifugation with an extraction kit (Pharmacia), and mRNA was isolated from total RNA with the PolyATtract mRNA isolation system III (Promega).

Differential screening and sequencing analysis revealed 23 unknown ESTs of putative genes. Of these, 3, ND3, ND5 and ND11, had relatively high abundant

frequencies (Zhang et al. 2003a). To clone their complete cDNAs, 3 pairs of primers, ND3-F and ND3-R, ND5-F and ND5-R, ND11-F and ND11-R, were designed against their cDNA sequences (Table 1). RACE-PCR was used to clone the 5′ and 3′ end of these genes using SMART cDNA as templates. Briefly, the upstream primer (ND3-F or ND5-F or ND11-F) and primer SMART-R were used in the 5′ RACE-PCR reaction. The downstream primer (ND3-R or ND5-R or ND11-R) and primer SMART-F were used in the 3′ RACE-PCR reaction. These reactions yielded 2 separate clones that overlapped in the region between upstream and downstream. Gig1 gene was generated from EST ND3 and ND11, and Gig2 gene from ND5.

Virtual Northern blot. The open reading frames (ORFs) of Gig1 and Gig2 were amplified with 2 pairs of primers, Gig1-F and Gig1-R, Gig2-F and Gig2-R (Table 1), designed against complete cDNA sequences of Gig1 and Gig2, and 1 µg of PCR products was then probed with the Dig High Prime system (Boehringer). SMART cDNA products (30 µl) derived from UV-inactivated GCHV-infected and mock-infected CAB cells, respectively, were electrophoresed on 1.0% agarose gel, and transferred to nylon membrane after denaturation and neutralization. Baking, prehybridization, hybridization, washing and color detection procedures were similar to those used in previous studies (Xie et al. 2001, Zhang & Gui 2004a).

Expression analysis. CAB cells were seeded at a cell density of 9×10^4 cells per 25 cm^2 flask and grown to confluence at 28°C for 1 to 2 d before the experiment began. Cells were first washed 3 times with non-FCS Medium 199, and then treated with active GCHV (1000 $TCID_{50}$ ml^{-1}), UV-inactivated GCHV (1×10^9 $TCID_{50}$ ml^{-1} exposed to UV irradiation), or CAB IFN-containing supernatant (2000 U ml^{-1}) (Wang et al. 1999, Zhang et al. 2000) for 1 h at 28°C, respectively. Inducers were then removed and the cells were washed 3 times followed by an addition of 5 ml of fresh non-FCS Medium 199. Cycloheximide (CHX) (Serva) was used 8 µg ml^{-1} to block protein synthesis. Cells were collected at 1, 3, 6, 8, 12, 24, 48, and 72 h post-treatment and total RNA was extracted using RNA-Solv Reagent (OMEGA BIOTEK). Control cells were treated with non-FCS Medium 199. First-strand cDNA was synthesized using random primers. The expression kinetics of Gig1 and Gig2 was analyzed by RT-PCR with 2 pairs of primers, ND3-F and ND3-R (expected size = 196 bp), ND5-F and ND5-R (expected size = 374 bp). PCR was performed with an initial denaturation step of 5 min at 94°C, and then 30 cycles were run as follows: 30 s denaturation at 94°C, 30 s annealing at 50°C for Gig1 or 52°C for Gig2, and 45 s extension at 72°C. The total amount of cDNA was calibrated on the basis of the amplification of crucian carp β-actin using primers β-actin-F and β-actin-R.

Table 1. Primers used for sequencing and expression studies

Name	Sequence (5′ to 3′)
ND3-F	GTAAGTGCTGTGAGGATGG
ND3-R	GGCTTCGATTCAACTG
ND11-F	GCTCCCAGATTATGTCACGG
ND11-R	CAGGTCGCTGTGTTCAGT
ND5-F	GCACATCAAGGGAGGCTGC
ND5-R	GCACATCAAGGGAGGCTGC
Gig1-F	TGAGACGGTGAACTTCACTC
Gig1-R	ACCAAATGAATCTTCGGGCGCT
Gig2-F	GAAGCAACATCAGAGGTTCAAAC
Gig2-R	CATACTCAGCAACAGGGTCAG
CaIRF7-F	GCGGTATGAGGGATTACGCAT
CaIRF7-R	TGGCTTTGTCGTTAGGGTGCT
CaSTAT1-F	AAGAAGCGGCAACAGATGGC
CaSTAT1-R	CATACACGGCTGTCTCTCCA
Smart-F	AACGCAGAGTACGCGGG
Smart-R	$CAGAGTACT_{16}$
β-actin-F	CACTGTGCCCATCTACGAG
β-actin-R	CCATCTCCTGCTCGAAGTC

RESULTS

Cloning of Gig1 and Gig2 on basis of 3 differentially expressed ESTs

In CAB cells treated with UV-inactivated GCHV, 3 unknown ESTs, ND3, ND5 and ND11, were differentially expressed and showed relatively high screening frequency being retrieved at 17, 11, 26 from 272 sequenced ESTs (Zhang et al. 2003a). This implies that the putative genes represented by these ESTs had relatively high abundant expressions and possibly played critical roles in defense against virus infection. Furthermore, their full-length cDNA sequences were obtained by RACE-PCR using SMART cDNA as templates. Sequence analysis found that 2 ESTs, ND3 and ND11, actually belonged to 1 common gene, named Gig1 (= GCHV-induced Gene No. 1), which was verified by PCR amplification with Primers ND11-F and ND3-R to generate a PCR product that was identical to the expected size of 716 bp according to the full-length cDNA of Gig1 (data not shown). ND5 represented another gene, termed Gig2. Sequence comparison revealed that ND156, another unknown EST (Zhang et al. 2003a), also belonged to the Gig1 gene, and was located at Nucleotide (nt) Position 21–182 (Fig. 1 top).

Gig1 cDNA is 1244 nt in length, starting 94 nt upstream from the first ATG codon, and contains a 585 bp open reading frame (ORF) encoding 194 amino-acid residues (Fig. 1 top). An AATAAA poly(A) consensus signal is present 515 nt downstream of the termination codon and 16 nt upstream of the poly(A) stretch.

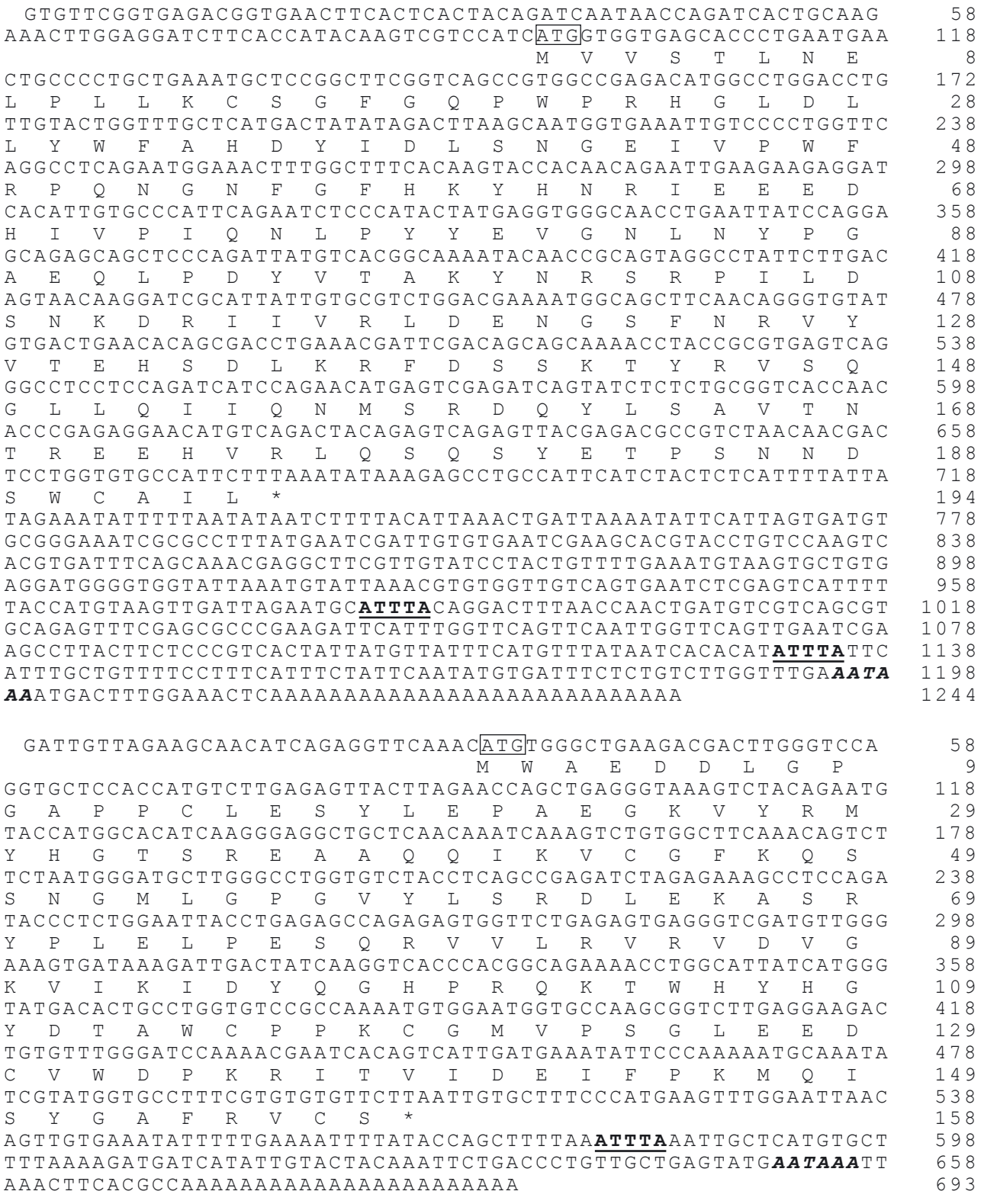

Fig. 1. Nucleotide (nt) and putative amino acid sequences of cDNAs of (top) GCVH-induced gene 1, Gig1, and (bottom) GCHV-induced gene 2, Gig2. Box indicates start (ATG) codon, asterisk indicates stop (TAA) codon. Italics and boldface indicate polyadenylation signals, underlining and boldface indicates motifs associated with mRNA instability (ATTTA). Top: 3 EST clones, ND3, ND11 and ND156, corresponding to nt 21–182, nt 826–1244, nt 273–825 of Gig1 cDNA sequence, respectively; bottom: EST ND5 sequence, corresponding to nt 120–619 of Gig2 gene cDNA sequence

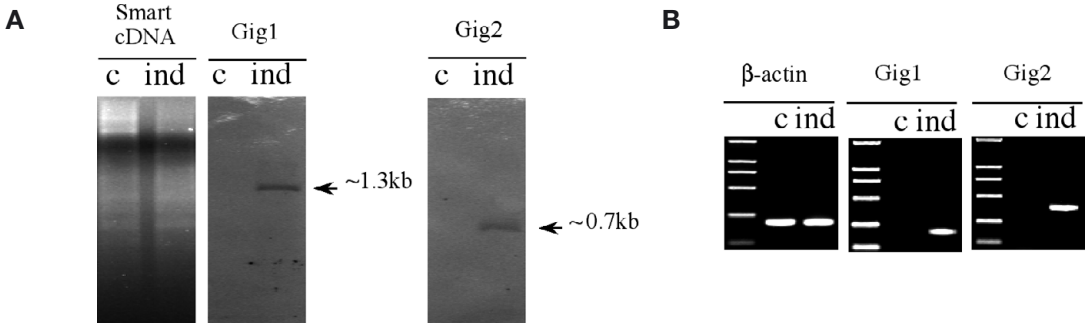

Fig. 2. Expressions of Gig1 and Gig2 induced by (A) UV-inactivated GCHV and (B) CAB interferon (IFN)-containing supernatant, ICS. (A) Virtual Northern blot detection of gene mRNA in CAB cells (*Carassius auratus* embryonic blastulae cells) treated with UV-inactivated GCHV (ind) and mock-infected cells (c); SMART cDNA products (30 µl) generated from UV-inactivated GCHV-infected and mock-infected CAB cells were used to hybridize with ORF (open reading frame) fragments of Gig1 and Gig2 labeled by Dig prime, respectively. (B) RT-PCR detection of gene cDNA from CAB cells induced by CAB IFN-containing supernatant (ind) for 72 h and from control cells (c). Samples were normalized on basis of β-actin expression

The 3′ untranslated region (UTR) has 2 ATTTA sequences, a characteristic motif possibly involved in rapid message degradation (Shaw & Kamen 1986). Gig2 cDNA is 693 nt in length, and is made up of 31 nt of 5′UTR, a single ORF of 477 nt, and 185 nt of 3′ UTR, with the poly(A) consensus signal 14 nt upstream of poly(A) tail (Fig. 1, bottom). The ORF encodes a protein of 156 amino acid residues, and there is also 1 ATTTA motif in its 3′UTR.

Identification of Gig1 and Gig2 as 2 novel IFN-inducible genes

A search of 2 genes in the public database revealed no significant homologue for Gig2; however, a viral hemorrhagic septicemia virus (VHSV)-induced gene from rainbow trout (AF483531) encoding for 171 amino acids (O'Farrell et al. 2002) had a low-level homology to Gig1, with 27% overall identity. To further confirm whether they were homologues or belonged to a gene family, the cDNA sequences of Gig1 and the VHSV-induced gene were compared with the Fugu genome database (see: www.jgj.doe.gov/fug) to reveal their orthologues. Unexpectedly, no Fugu DNA sequence was found with a putative common gene, indicating that both Gig1 and Gig2 were 2 novel genes. In addition, no conserved motif was found in the putative protein sequences of Gig1 and Gig2 (signal polypeptide, putative N-glycosylation site, etc.).

The viral inducible characteristics of the 2 novel genes were further verified by a virtual Northern blot assay. As shown in Fig. 2A, only a single transcript was detectable for Gig1 (1.3kb), and for Gig2 (0.7 kb) in the UV-inactivated GCHV-infected CAB cell mRNA, but not in the control CAB cell mRNA, indicating that UV-inactivated GCHV was able to induce the transcription of these genes. Since CAB IFN activity could be produced after treatment of CAB cells with UV-inactivated GCHV (Wang et al. 1999, Zhang et al. 2000), it is suggested that Gig1 and Gig2 were likely to be induced directly by CAB IFN. To test this, CAB cells were treated with CAB IFN-containing supernatant at 2000 U ml^{-1} for 72 h. As shown in Fig. 2B, a strong inducible expression of the 2 genes was observed in the IFN-stimulated CAB cells compared with control cells.

Differences in expression between Gig1 and Gig2 in response to 3 inducers

To determine differences in expression between Gig1 and Gig2 in response to different inducers, 3 groups of CAB cells were treated in parallel with the 3 inducers, active GCHV, UV-inactivated GCHV and CAB IFN-containing supernatant, and Gig1 and Gig2 mRNAs were monitored by RT-PCR using the primers ND3-F and ND3-R, ND5-F and ND5-R, respectively. As shown in Fig. 3, a very weak constitutive expression was detected in mock-treated cells. In response to the 3 inducers, Gig1 induction exhibited diverse expression patterns. Its transcriptional activation occurred in CAB cells treated with CAB IFN-containing supernatant for 1 h, but the same level of expression occurred 12 h after treatment with UV-inactivated GCHV, and 24 h later with active GCHV. In contrast to Gig1, a similar expression pattern was observed for Gig2 after treatment with active GCHV, UV-inactivated GCHV and CAB IFN-containing supernatant, respectively. The Gig2 expression was initiated 1 h after induction in response to all 3 inducers, although some differences existed at the transcriptional level (Fig. 3). Therefore, the induction kinetics of Gig2 differed from that of Gig1, implying that the signal pathway regulating Gig2 expression may be different from that of Gig1 in GCHV-infected CAB cells.

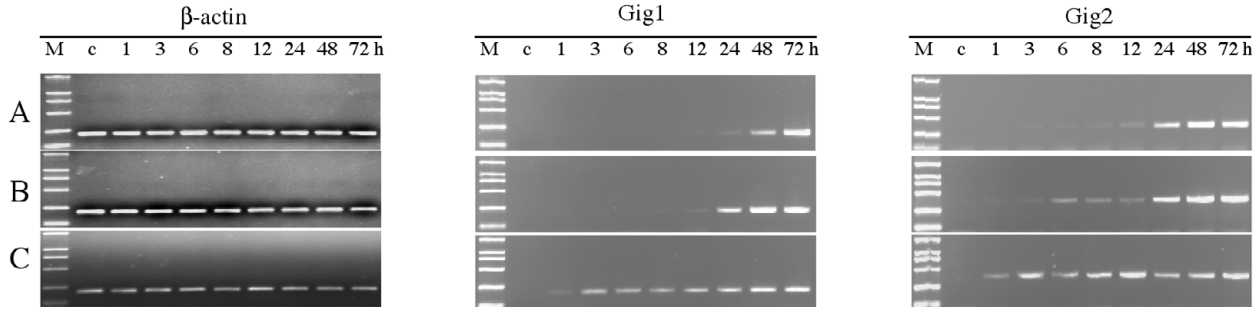

Fig. 3. Expression kinetics of Gig1 and Gig2 in (A) CAB cells induced by active GCHV, (B) UV-inactivated GCHV and (C) CAB IFN-containing supernatant, ICS. Three groups of CAB cells were treated with active GCHV, UV-inactivated GCHV and CAB IFN-containing supernatant for 1, 3, 6, 8, 12, 24, 48, and 72 h, respectively, and gene mRNA was then extracted and detected by RT-PCR. c: mock-treated CAB cells. β-actin was amplified using same conditions as for a positive control in each experimental group. M: DNA marker DL2000

Evidence of induction pathways of Gig1 and Gig2 after virus infection and IFN treatment

To further reveal the diverse induction pathways of Gig1 and Gig2, their expression kinetics after UV-inactivated GCHV infection was compared with CAB IFN signal molecules, CaSTAT1 and CaIRF7. As shown in Fig. 4, 4-genes were all up-regulated strongly in the virally infected CAB cells, but different expression patterns were observed. Compared to the base expressions, CaSTAT1 transcriptional activation occurred at 3 h post-virus-infection, at 12 h for CaIRF7 and Gig1, but at only 1 h for Gig2; i.e. the Gig1 gene was induced later than CaSTAT1, whereas Gig2 induction was nearly simultaneous to that of CaSTAT1 but clearly prior to CaIRF7. It is well known that the JAK-STAT signal pathway is activated by mammalian IFNs to establish an innate antiviral response in host cells and that IRF7 is responsible for expression of the delayed IFN subtypes and ISGs (Sato et al. 2000). Therefore, in UV-inactivated GCHV-infected CAB cells, Gig1 expression is possibly induced via an intermediate (such as IFN) but, unlike Gig1, both the CAB IFN and JAK-STAT pathways are possibly dispensable for expression of Gig2.

A subsequent experiment was designed to determine whether de novo protein synthesis was required for expression of the 2 genes by monitoring their mRNA levels in UV-inactivated GCHV-infected, IFN-treated and untreated cells in the presence or absence of cycloheximide (CHX), a potent inhibitor of protein synthesis. As shown in Fig. 5, a low dose of CHX

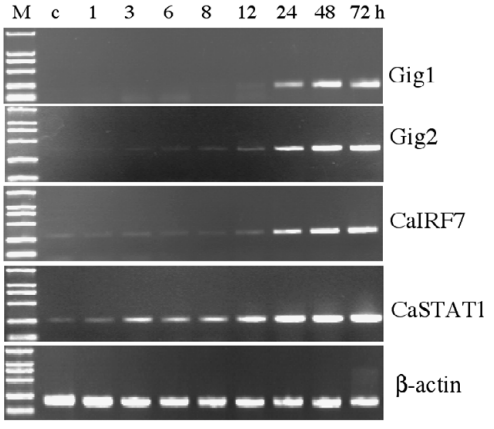

Fig. 4. RT-PCR detection of diverse expression kinetics among Gig1, Gig2, CaSTAT1 and CaIRF7 in CAB cells responsive to UV-inactivated GCHV. CAB cells were treated with UV-inactivated GCHV for 1, 3, 6, 8, 12, 24, 48, and 72 h, and cell mRNA was then extracted. RT-PCR was used to detect expression of these 4 genes. c: mock-treated CAB cells. β-actin was amplified using the same conditions as for a positive control. M: DNA marker DL2000

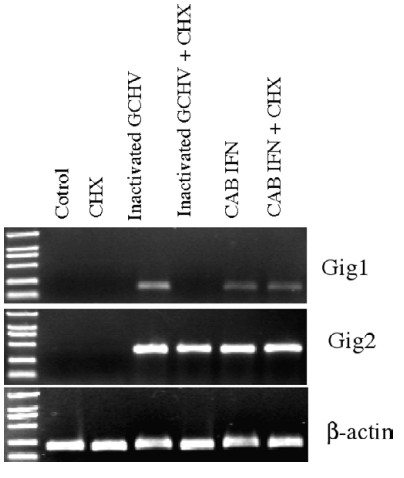

Fig. 5. Requirement for ongoing protein synthesis to induce Gig1 and Gig2 expression by UV-inactivated GCHV and CAB IFN-containing supernatant, ICS. CAB cells were treated with UV-inactivated and CAB IFN-containing supernatant in presence or absence of 8 μg ml^{-1} cloheximide (CHX) for 30 h, and total mRNA was then extracted. RT-PCR was employed to detect expression of Gig1 and Gig2. CAB cells cultured without inducers in presence or absence of CHX were used as controls, and samples were normalized on basis of β-actin expression

(0.8 µg ml^{-1}) had almost no cytotoxic effect on CAB cells (data not shown) and could not induce the expression of either gene. However, addition of CHX 30 h post-infection strongly suppressed the expression of Gig1 but had almost no inhibitory effect on Gig2. In the case of CAB IFN-containing supernatant treatment, as expectedly, 2 gene transcripts were detected irrespective of the presence of CHX. This result further showed that in the UV-inactivated GCHV-infected CAB cells, the expression of Gig1 and Gig2 was activated through distinct induction pathways with CAB IFN as the probable intermediate. That is, UV-inactivated GCHV first induces CAB IFN expression, and the newly secreted IFN in turn activates the expression of Gig1 and Gig2. On the other hand, GCHV infection may directly activate Gig2 expression without the need of cellular protein synthesis.

DISCUSSION

In mammals, although some IFN-inducible proteins, such as PKR, 2-5(A) synthetase/RNase L, and Mx, have been well characterized in IFN antiviral effects (Samuel 2001), experiments with mice (Zhou et al. 1999) and cell cultures (Presti et al. 2001) that lack these proteins suggest that additional pathways may also contribute to the antiviral activity of IFN. In support of this, an increasing number of IFN-inducible proteins have been identified, including known proteins (Der et al. 1998) and novel gene products (Chin & Crasswell 2001). Compared with mammals, relatively few fish IFN system genes have been isolated and characterized (but see O'Farrell et al. 2002, Zhang et al. 2003a, and Altmann et al. 2003). Recently, several studies isolating fish IFN-responsive genes have revealed a relatively low degree of similarity with mammalian homologues (Liu et al. 2002, Zhang et al. 2003c, Zhang & Gui 2004b). In addition, since fish have undefined unique characteristics, it is also plausible that there are other pathways involving novel ISGs that mediate the antiviral activity of fish IFN. In the present study, 2 novel fish ISGs were identified on the basis of a variety of evidence, including their isolation from an IFN-producing cell system, the lack of homologues in other species including mammals, and their up-regulation in response to GCHV infection and IFN treatment.

The UV-inactivated GCHV-infected CAB cell system established in our laboratory is very effective for identification of antiviral-relevant genes or immune-related genes (Zhang et al. 2003a,b). Consistent with the finding that CAB IFN activity occurs and an antiviral state arises in response to UV-inactivated GCHV infection (Wang et al. 1999, Zhang et al. 2000), some important crucian carp IFN system genes, including IFN (AY452069), Type I IFN signaling factors JAK1 and STAT1 (AY242386), IRF7 (AY177629), innate immune receptor TLR3, potential antiviral factors Mx1 (AY303813), Mx2 (AY303812), viperin (AY303809), PKR (AY293929), ISG15-1 (AY303810), ISG15-2 (AY303811), IFI56 (AY267212) and IFI58 (AY267211), have been successfully retrieved from this experimental system (Zhang et al. 2003a,b,c, Zhang & Gui 2004a,b). These results suggest that fishes can combat virus infection, possibly by the JAK-STAT signal pathway, and employ well-described and well-conserved antiviral pathways similar to those in mammals, e.g. the PKR pathway, Mx pathway, Viperin pathway and the IFI56 pathway.

Recent studies revealed that fishes have a mechanism similar to Type I IFN signal transduction and cascade (Collet & Secombe 2002). Oates et al. (1999) demonstrated that zebrafish STAT1 could rescue IFN-signaling functions in a STAT1-deficient human cell line. The identification and characterization of crucian carp CaSTAT1 (Zhang & Gui 2004a) and CaIRF7 (Zhang et al. 2003b) also indicated that the JAK-STAT signal pathway is involved in fish IFN antiviral function. Moreover, like most mammalian ISGs, the characteristic sequence of ISRE (IFN-stimulated response element) was verified to position in the promoters of some fish ISGs, including the pufferfish Mx (Yap et al. 2003), the ISG15 (Liu et al. 2002), and the rainbow trout Mx1 (Collet & Secombes 2001), and Vig2 gene (Boudinot et al. 2001). This was also supported by the expression analysis in the current study. Firstly, the delay in Gig1 induction by active and inactivated GCHV compared to CAB IFN indicated that its induction by GCHV possibly occurrs via induction of IFN. For the expression of Gig 2, an IFN dose-dependent trend was observed in response to 3 stimuli (Fig. 3). Secondly, expression comparison showed that the expression of Gig1 occurred much later than that of CaSTAT1 and CaIRF7 (Fig. 4). Finally, expression analysis using CHX further demonstrated that CAB IFN is the intermediate for the 2 gene inductions in virally infected cells (Fig. 5). Collectively, transcriptional activation of both Gig1 and Gig2 following treatment with IFN should be mediated by the JAK-STAT signal pathway.

In mammals, Type I IFN-stimulated genes can be subdivided into 2 groups: (1) immediate–early (IE) genes activated in response to viral infection by a protein synthesis-independent pathway; (2) delayed-type genes whose expression is dependent on *de novo* protein synthesis (Sato et al. 2000). IRF3 is involved in regulating IE gene expression (Sato et al. 2000, Grandvaux et al. 2002), but delayed-type gene expression is mainly mediated by the JAK-STAT signal pathway, whereby IRF7 is significantly up-regulated by newly

synthesized IFN and participates in the regulation of gene transcriptional induction by a positive feedback mechanism (Marié et al. 1998, Sato et al. 2000). Thus, on the basis of the expression characterization of the 2 genes, Gig1 appears to be a delayed-type gene induced by CAB IFN and Gig2 an IE gene activated directly by GCHV infection.

Although some putative antiviral genes, including PKR, Mx, Viperin and IFI56, were up-regulated significantly in response to GCHV infection, screening of a subtractive cDNA library found that these gene products were not the most abundant (Zhang et al. 2003a, Zhang & Gui 2004b). In fact, Gig1 was likely to be the highest abundant gene in those identified from the subtractive cDNA library (Zhang et al. 2003a). This observation indicates strongly that the Gig1 gene product plays an essential role in the host-defense response, although its exact biochemical and cellular function remains unknown. In mammals, IFI56 is known to be the most abundant ISG in response to virus infection (Der et al. 1998); however, crucian carp CaIFI56 is unlikely to be the most abundant gene, since only 2 cDNA ESTs were retrieved (Zhang et al. 2003a, Zhang & Gui 2004b). Therefore, it seems that the fish IFN system is not identical to that of mammals but possesses unique characteristics.

Acknowledgements. This work was supported by grants from the National 863 High Technology Research Program (2002AA626010), the National Natural Science Foundation of China (30200207, 30130240), the Innovation Project of Chinese Academy of Sciences (KSCX2-SW-303), and the Wuhan Dawn Plan (20015005055).

LITERATURE CITED

Altmann SM, Mellon MT, Distel DL, Kim CH (2003) Molecular and functional analysis of an interferon gene from the zebrafish, *Danio rerio*. J Virol 77:1992–2002

Boudinot P, Salhi S, Blanco M, Riffault S, Benmansour A (1999) Vig-1, a new fish gene induced by the rhabdovirus glucoprotein, has a virus-induced homologue in humans and shares conserve motifs with the MoaA family. J Virol 73:1846–1852

Boudinot P, Salhi S, Blanco M, Benmansour A (2001) Viral haemorrhagic septicaemia virus induces vig-2, a new interferon-responsive gene in rainbow trout. Fish Shellfish Immunol 11:383–397

Chin KC, Cresswell P (2001) Viperin(cig5), an IFN-inducible antiviral protein directly induced by human cytomegalovirus. Proc Natl Acad Sci USA 98:15125–15130

Collet B, Secombes CJ (2001) The rainbow trout (*Oncorhynchus mykiss*) Mx1 promoter: structural and functional characterization. Eur J Biochem 268:1577–1584

Collet B, Secombe CJ (2002) Type I interferon signaling in fishing. Fish Shellfish Immunol 12:389–397

Der SD, Zhou A, Williams BR, Silverman RH (1998) Identification of genes differentially regulated by interferon alpha, beta, or gamma using oligonucleotide arrays. Proc Natl Acad Sci USA 95:15623–15628

Grandvaux N, Servant MJ, ten Oever B, Sen GC, Balachandran S, Barber GN, Lin R, Hiscott J (2002) Transcriptional profiling of interferon regulatory factor 3 target genes: direct involvement in the regulation of interferon-stimulated genes. J Virol 76:5532–5539

Liu M, Reimschuessel R, Hassel BA (2002) Molecular cloning of the fish interferon stimulated gene, 15 kDa (ISG15) orthologue: a ubiquitin-like gene induced by nephrotoxic damage. Gene 298:129–139

Marié I, Durbin JE, Levy DE (1998) Differential viral induction of distinct interferon-alpha genes by positive feedback through interferon regulator fanctor-7. EMBO (Eur Mol Biol Organ) J 17:6660–6669

Oates AC, Wollberg P, Pratt SJ, Paw BH and 5 others (1999) Zebrafish stat3 is expressed in restricted tissues during embryogenesis and stat1 rescues cytokine signaling in a STAT1-deficient human cell line. Dev Dyn 215:352–370

O'Farrell C, Vaghefi N, Cantonnet M, Buteau B, Boudinot P, Benmansour A (2002) Survey of transcript expression in rainbow trout leukocytes reveals a major contribution of IFN-responsive genes in the early response to a rhabdovirus infection. J Virol 76:8040–8049

Presti RM, Popkin DL, Connick m, Paetzold S, Virgin HWT (2001) Novel cell type-specific antiviral mechanism of interferon gamma action in macrophages. J Exp Med 193: 483–496

Robertsen B, Trobridge GD, Leong JAC (1997) Molecular cloning double-stranded RNA inducible Mx genes from Atlantic salmon (*Salmo salar* L.). Dev Comp Immunol 21: 397–412

Samuel CE (2001) Antiviral action of interferons. Clin Microbiol Rev 14:778–809

Sato M, Suemori H, Hata N, Asagiri M and 6 others (2000) Distinct and essential roles of transcription factors IRF-3 and IRF-7 in response to viruses for IFN-alpha/beta gene induction. Immunity 13:539–548

Shaw G, Kamen R (1986) A conserved AU sequence from the 3′ untranslated region of GM-CSF mRNA mediates selective mRNA degradation. Cell 46:659–667

Trobridge GD, Leong JAC (1995) Characterization of a rainbow trout Mx gene. J Interferon Cytokine Res 15:691–702

Trobridge GD, Chiou PP, Leong JAC (1997) Cloning of the rainbow trout (*Oncorhynchus mykiss*) Mx2 and Mx3 cDNAs and characterization of trout Mx protein expression in salmon cells. J Virol 71:5304–5311

Wang TH, Zhang YB, Li GQ, Yi YL, Liu HQ, Zhu ZY (1999) Interferon induction in cultured fish cells. Chin J Virol 15: 43–49 (in Chinese)

Wieland SF, Vega RG, Müller VR, Evans CF and 5 others (2003) Searching for interferon-induced genes that inhibit hepatitis B virus replication in transgenic mouse hepatocytes. J Virol 77:1227–1236

Xie J, Wen JJ, Chen B, Gui JF (2001) Differential gene expression in fully-grown oocytes between gynogenetic and gonochoristic crucian carps. Gene 71:109–116

Yabu T, Hirose H, Hirono I, Katagiri T, Aoki T, Yamamoto E (1998) Molecular cloning of a novel interferon regulatory factor in Japanese flounder, *Paralichthys olivaceus*. Mol Mar Biol Biotechnol 7:138–144

Yap WH, Tay A, Brenner S, Venkatesh B (2003) Molecular cloning of the pufferfish (*Takifugu rubrips*) Mx gene and functional characterization of its promoter. Immunogenetics 53:705–713

Zhang QY (2002) A review of viral diseases of aquatic anima in China. Acata Hydrobiol Sin 26:93–101 (in Chinese)

Zhang YB, Gui JF (2004a) Molecular characterization and IFN signal pathway analysis of *Carassius auratus* CaSTAT1 identified from the cultured cells in response to virus infection. Dev Comp Immunol 28:211–227

Zhang YB, Gui JF (2004b) Identification and expression analysis of two IFN-inducible genes in crucian carp (*Carassius auratus* L.). Gene 325:43–51

Zhang YB, Yu XM (2000) Progresses in studies in fish interferon. J Fish Sci China 7:97–101 (in Chinese)

Zhang YB, Wang TH, Li G Q, Jia FJ, Yu XM (2000) Induction and partial characterization of CAB cell (blastulae embryonic cell line of crucian carp) IFN. Virol Sin 15:163–169 (in Chinese)

Zhang YB, Zhang QY, Xu DQ, Hu CY, Gui JF (2003a) Identification of antiviral-relevant genes in the cultured fish cells induced by inactivated virus. Chin Sci Bull 48: 581–588

Zhang YB, Hu CY, Zhang J, Huang GP, Wei LH, Zhang QY, Gui JF (2003b) Molecular cloning and characterization of crucian carp (*Carassius auratus* L.) interferon regulator factor 7. Fish Shellfish Immunol 15:453–466

Zhang YB, Shi YH, Gui JF (2003c) Construction of antiviral subtractive cDNA library of cultured fish cells. Acta Hydrobiol Sin 27:113–118 (in Chinese)

Zhou A, Paranjape JM, Der SD, Williams BR, Silverman RH (1999) Interferon action in triply deficient mice reveals the existence of alternative antiviral pathway. Virology 258: 435–440

Editorial responsibility: Jo-Ann Leong, Kaneohe, Hawaii, USA

Submitted: June 20, 2003; Accepted: December 23, 2003
Proofs received from author(s): June 23, 2004