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Expression and characterisation of the maternal factor Zygote arrest 1 (Zar1) gene in chicken tissues and embryos

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Zygote arrest 1 (Zar1) is an oocyte-specific maternal effect gene that plays essential role during the oocyte-to-embryo transition in many species including human, mouse, rat, bovine, pig, frog and zebrafish. This study reports that this maternal-effect gene is also conserved in the avian species, namely the chicken *Gallus gallus domesticus. In silico* mining of the chicken genome shows that the chicken Zar1 (cZar1) gene consists of four exons and three introns, and the coding region of this gene is 903 bp. Using rapid amplification of cDNA ends analysis, the 3′-untranslated regions of the gene were isolated, where putative Pumilio (XPum) recognition sites as well as hexamer polyadenylation signals were identified. The gene is predicted to encode a 33 kDa protein, characterized by a conserved C-terminal FYVE/PHD zinc finger domain. In the present study the expression profile of this gene in chicken tissues and embryos was analyzed using reverse transcription-polymerase chain reaction (RT-PCR). The data presented in this study suggests that Zar1 is conserved in another vertebrate species, the chicken, and is preferentially expressed in the chicken reproductive tract, oocytes and embryos, suggesting a role in reproduction and embryonic development.

Key words: oocyte-specific gene, Zar1, zygote, ovary, testis, chicken embryos.

INTRODUCTION

Maternal mRNAs, which are expressed in the oocytes, play an important role in the success of early embryo development, as they allow the first cleavages to occur (Schultz, 2002; Hamatani *et al*., 2004; Minami *et al*., 2007). Recently, a growing number of maternal effect oocyte-specific genes such as Mater, Nalp5, Nalp9, Zar1, Stella, Hsf1 and Nmp2 have been identified in many vertebrates including human (Wu *et al*., 2003a, b; Uzbekova *et al*., 2006), mouse (Tong *et al*., 2000; Dean, 2002; Burns *et al*., 2003; Payer *et al*., 2003; Wu *et al*., 2003a, b), rat (Wu *et al*., 2003b), frog (Wu *et al*., 2003b), zebrafish (Wu *et al*., 2003b), pufferfish (Wu *et al*., 2003b), bovine (Brevini *et al*., 2004; Pennetier *et al*., 2004; Dalbiès-Tran *et al.*, 2005; Uzbekova *et al*., 2006) and pig (Uzbekova *et al*., 2006).

Zygote arrest 1 (Zar1) was the first identified oocyte-specific maternal-effect gene that functions at the oocyte-to-embryo transition in human and mouse (Wu *et al*., 2003a). *In silico* sequences studies revealed that Zar1 orthologues were found to be evolutionary conserved in six vertebrate species including human, mice, rat, frog, zebrafish and pufferfish (Wu *et al*., 2003b). Expression analysis studies have reported that Zar1 expression was restricted to ovary in mice (Wu *et al*., 2003a) and to ovary and testis in human (Wu *et al*., 2003a), while in frog expression was observed in ovary, lung and muscle, but not testis (Wu *et al*., 2003b). In pig and cattle, Zar1 expression was observed in ovary, oocyte, testis, hypothalamus and pituitary (Uzbekova *et al*., 2006).

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At present, limited information is available on oocyte-specific maternal effect genes in chicken. Chicken Vasa Homolog (CVH) was the first identified maternally inherited protein in the chicken embryo, and was found to be expressed in chicken oocytes and during first cleavage (Tsunekawa *et al*., 2000). More recently, another gene encoding for the Epidermal Growth Factor (EGF) protein, was found in the germinal disc of F2 oocytes, suggesting a role in chicken follicular development (Wang *et al*., 2007).

Although Zar1 has been cloned and studied in many vertebrates, there is little information concerning the chicken Zar1 (cZar1) homologue. Recently on a study based on candidate gene approach and on chicken Affymetrix microarray, several hundred chicken genes with potential maternal effect were identified (Elis *et al*., 2008). Among them, a chicken Zar1 transcript was detected. cZar1 was found to be expressed in the ovary and oocyte during follicular maturation and was expressed at higher levels after activation of the chicken embryonic genome (Elis *et al*., 2008). However, there is no information about the structure of the gene and its expression in the male reproductive tract and during embryonic development.

Given the importance of Zar1 gene in reproduction, it was of interest to examine the chicken orthologue because of the wide use of the chick as a model system in developmental biology. In the present study we cloned the full length of cZar1 gene, including the 3′-untranslated regions (3′-UTR), and we examined the expression pattern of the cZar1 gene in various chicken tissues including the male and the female reproductive tract and embryos, during the chicken embryonic development.

MATERIALS AND METHODS

Collection of tissues and embryos

One year old birds (*Gallus gallus domesticus*, Gold Line) provisioned by a commercial supplier were sacrificed by cervical dislocation. Chicken ovary, oviduct, oocytes, testis, epididymis, brain, liver, kidney, spleen and heart were dissected, snap frozen in liquid nitrogen and stored at –80°C.

Freshly-laid chicken (Gold Line) eggs were obtained from a commercial supplier and incubated under humid conditions at 38°C. Eggs were kept for 3 to 7 days. Embryos were removed from eggs at days 3 to 7 of incubation, snap frozen in liquid nitrogen and stored at –80°C until analysed.

RNA Isolation and RT-PCR analysis

Total RNA was isolated from chicken tissues and embryos stored at –80°C. Initially, the tissues and embryos were ground to a fine powder, and the RNA was extracted using the Total RNA Isolation (TRI) Reagent (Ambion) according to the instructions provided by the manufacturer. To reduce degradation, RNAse inhibitor (Invitrogen) was added to each sample (1 Unit μg^{-1} of RNA) before storage at –80°C. All samples were pretreated, before reverse transcription (RT), with DNAse (Fermentas) at a concentration of 1 Unit μg^{-1} of RNA.

One μg of total RNA was reverse transcribed to cDNA using the SuperScript II Reverse Transcriptase kit (Invitrogen) according to the manufacturer instructions.

Two μl of the RT products were subjected to PCR amplification. A 249 bp fragment of the cZar1 cDNA was amplified using two specific primers, which were designed based on a chicken Zar1 EST deposited in Genbank (XM_001234451), primer cZar1F: 5′-TCTC CGGGAGTAACAAGGTG-3′ and primer cZar1R: 5′-GAGGGCACACAAGTCAGACA-3′. This primer pair was designed to cross intron sequences and resulted in the amplification of an 806 bp sequence if the samples were contaminated with genomic DNA. PCR amplification was performed using 0.1 μg genomic DNA as template, 200 nM of each primer, 1 mM Deoxynucleotide Triphosphates (dNTPs) and 1 unit of Taq DNA Polymerase Recombinant (Invitrogen) in 50 μl total volume reaction. PCR conditions were 94°C for 3 min, 35 cycles of 94°C for 30 sec, 56°C for 30 sec, 72°C for 30 sec and a final extension period at 72°C for 10 min.

RT-PCR products were resolved by electrophoresis using 1% Tris Borate EDTA (TBE) agarose gels, visualised with ethidium bromide and imaged under UV illumination.

Amplification of 18S ribosomal RNA was performed as a control to check the quality of the synthesized cDNAs, using the Classic 18S Internal Standard primer pair (Ambion) and an annealing temperature of 57°C.

In control samples, reverse transcriptase was omitted to demonstrate that PCR amplification was not due to contamination with genomic DNA.

The cDNA products were identified by cloning the PCR products into the pCR 2.1-TOPO vector using the TOPO TA cloning Kit (Invitrogen) according to the manufacturer protocol. DNA sequencing was performed by Macrogen (Korea).

3′ RACE PCR

The sequence of the 3′-untranslated regions (3′-UTR) including the PolyA tail of the cZar1 cDNA was obtained using the 3′ RACE PCR (Rapid Amplification of cDNA End by PCR) technique.

First strand cDNA synthesis was performed using the OligodT Adaptor primer 5′-GGCCACGCGTC GACTAGTAC$(dT)_{17}$-3′. PCR was performed in ovarian cDNA using the cZar1F and the Abridged Universal Anchor Primer (AUAP: 5′-GGCCACGCGT CGACTAGTAC-3′) primer pair. PCR amplification, cloning and DNA sequencing of the PCR product were performed as detailed above.

Protein sequence comparisons and phylogenetic analysis

The deduced amino acid sequences of the known vertebrate Zar1 genes together with cZar1 were aligned using the multiple sequence alignment program T-COFFEE ver. 5.72 (Notredame *et al*., 2000).

Phylogenetic relationships of ZAR1 proteins were performed using the Neighbor-Joining Method with Poisson correction (Saitu & Nei, 1987). Bootstrap values were derived from 1000 pseudoreplicates. A phylogenetic tree was constructed using the MEGA 4.0 software (Tamura *et al*., 2007). The GenBank accession numbers of the sequences were: AY191415 (*Mus musculus*), AY191416 (*Homo sapiens*), AY283175 (*Rattus norvegicus*), AY283176 (*Xenopus laevis*), AY283178 (*Danio rerio*), AY283177 (*Takifugu rubripes*), DQ231456 (*Bos taurus*), DQ231444 (*Sus scrofa*) and XM_001234451 (*Gallus gallus*).

RESULTS

Isolation and structure of cZAR1 gene

Using PCR and *in silico* analysis we isolated the full length of the cDNA encoding the cZar1 gene. This sequence was deposited in GenBank under accession number FJ914588. Alignment between the cZar1 cDNA and the chicken genome revealed the structure of the gene. As illustrated in Fig. 1, the gene consists of four exons (591 bp, 93 bp, 75 bp and 144 bp in size, respectively) and three introns (176 bp, 154 bp and 403 bp in size, respectively); the coding region of this gene is 903 bp and encodes a 33 kDa protein.

We also performed 3′ RACE PCR, in order to determine the full-length of the cZar1 transcript, including the 3′-UTR. Sequencing of the resulting clone revealed the full-length of the 3′-UTR of the cZar1 cDNA (Fig. 2). *In silico* analysis revealed the presence of XPum (Pumilio) recognition sites (TGTA), approximate to hexamer polyadenylation signals (HPS) AATAAA within the 3′-UTR of the cZar1 cDNA. The PolyA tail was found 177 bp after the stop codon.

Phylogenetic analysis

To determine the phylogenetic relation of the cZar1 protein with the known vertebrate Zar1 proteins, we performed an alignment based on the known sequences of various Zar1 proteins from different vertebrates reported in genome databases. Nine amino acid sequences, including mouse, rat, human, frog, zebrafish, pufferfish, bovine, pig and chicken Zar1 proteins, were aligned using the T-COFFEE ver. 5.72

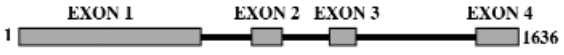

FIG. 1. Structure of chicken Zar1 gene. Grey boxes represent exons. Black lines represent introns.

TGACTTGTGTGCCCTCCAATTTGTGCTCTGCACTTTGTCAGTCTTTTGTGATGTTTTGACCTTAGGCTTT 70

TGACCTGGCACTGGATAATGGATGAAGACTTCTGTGTTATTTATAAAATATTTAACTTTATTGTATATAT 140

GCTATAATTAAAGTTCAATAAAAGTTTGCACTGATTTGTTAAAAAAAAAAAAAAAAAAAAAAAAAAAAA 210

AAAAAAAAAAAAAAAAAA 229

FIG. 2. Sequence of the 3′ UTR of the chicken Zar1 cDNA. The TGA stop codon is bold-typed. Hexamer polyadenylation signal is boxed in grey. Putative Pumilio-binding site is underlined.

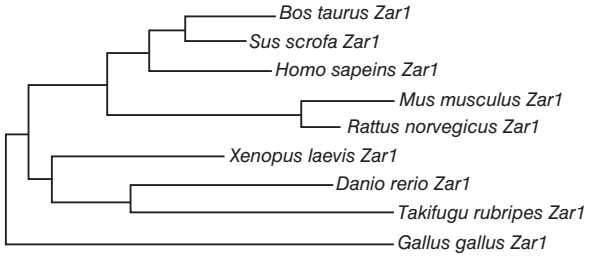

FIG. 3. Phylogenetic relationships of vertebrate Zar1 proteins.

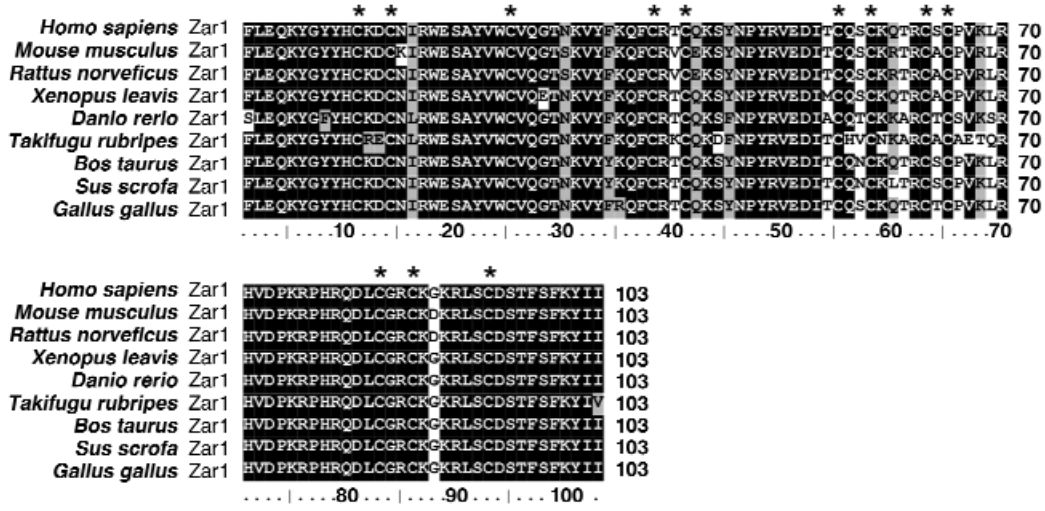

FIG. 4. Alignment of the Zar1 C-terminal regions from different vertebrates. Black and grey areas indicate identical amino acids and conservative substitutions, respectively. Asterisks indicate the 12 conserved cysteines.

software (Notredame *et al*., 2000) and a tree was subsequently constructed using the neighbor-joining tree building algorithm (Saitu & Nei, 1987). As shown in Fig. 3, the unrooted tree generated has three main branches. One of them consists of only the chicken Zar1 protein. Mouse and rat, as well as pig and bovine, are the most closely related, whereas bovine and chicken are the least. Frog, zebrafish and pufferfish Zar1 proteins appear closely related, while the human Zar1 homologue appears more closely related to big and bovine. However, as observed from the amino acid alignment (Fig. 4), the C-termini of the proteins are highly conserved. Comparisons of the C-terminal 103 amino acids of these proteins revealed more than 80% identity between the chicken and the other Zar1 proteins. Similar to other species, an atypical Plant Homeo Domain (PHD) motif was also identified in the C-termini of the chicken Zar1 protein as was evident by the presence of the 12 conserved cysteines.

Expression analysis of cZar1 mRNA

In order to determine the expression pattern of the cZar1 mRNA, we analyzed by RT-PCR chicken adult tissues including ovary, oviduct, oocytes, testis, epididymis, brain, liver, kidney, spleen, heart and embryos at various developmental stages. Expression a-

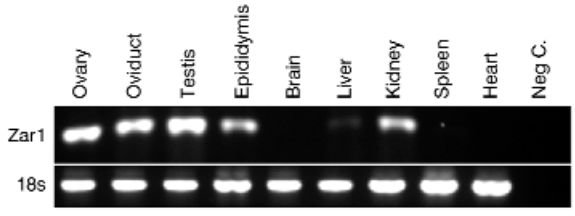

FIG. 5. Expression of chicken Zar1 gene in various chicken organs. cDNA samples from chicken Ovary, Oviduct, Testis, Epididymis, Brain, Liver, Kidney, Spleen and Heart were amplified by PCR with a primer pair designed to detect a 249 bp fragment of the cZar1 gene. Amplification of 18S rRNA was used as a control. Neg C.: no-template control.

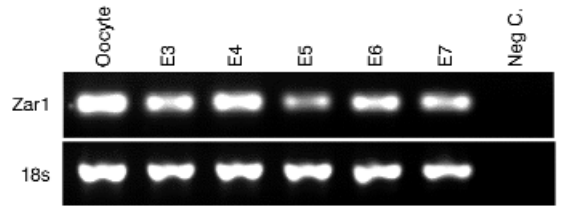

FIG. 6. Expression of chicken Zar1 gene in chicken oocyte and embryos during embryonic development. cDNA samples from chicken oocyte and embryos at day 3 (E3) to day 7 (E7) of embryonic development were amplified by PCR with a primer pair designed to detect a 249 bp fragment of the cZar1 gene. Amplification of 18S rRNA was used as a control. Neg C.: no-template control.

nalysis revealed that the cZar1 mRNA was expressed at high levels in both male (testis) and female (ovary) gonads and oocytes (Figs 5 and 6). cZar1 transcripts were also observed in the chicken epididymis and oviduct. Apart from the reproductive tract, kidney was the only tissue with detectable cZar1 mRNA.

Expression analysis in chicken embryos revealed that cZar1 mRNA transcripts were detected in every stage of embryonic development examined, from embryonic day 3 (E3) to day 7 (E7).

DISCUSSION

By means of 3′ RACE PCR, conventional PCR and *in silico* cloning we have isolated the full length of the cDNA encoding for the chicken Zar1 gene. Zar1 gene was originally identified in human and mouse (Wu *et al*., 2003a). This gene is evolutionarily conserved in vertebrate ovaries since orthologues were also cloned in rat (Wu *et al*., 2003b), frog (Wu *et al*., 2003b), zebrafish (Wu *et al*., 2003b), pufferfish (Wu *et al*., 2003b), cattle (Brevini *et al*., 2004), and pig (Uzbekova *et al*., 2006). Our data extend these findings by identifying Zar1 also in chicken.

In silico analysis revealed that the structure of the gene is composed of 4 exons and 3 introns. This finding revealed that the structure of the cZar1 gene is very similar to the other vertebrate orthologues reported to date, including pig (Uzbekova *et al*., 2006), cattle (Uzbekova *et al*., 2006), human (Wu *et al*., 2003a) and mice (Wu *et al*., 2003a), all of them consisting of 4 exons and 3 introns. This suggests that the Zar1 gene organization is very similar among vertebrates.

Analysis of the gene revealed the presence of a FYVE/PHD zinc finger domain in the C-terminus of the deduced cZar1 protein sharing highly homology with the zing finger domain of other vertebrate Zar1 proteins. Alignment of the C-terminus of the cZar1 protein with the orthologues of the other known Zar1 proteins from various vertebrates, revealed the conserved atypical PHD motif of the conserved 12 cysteine pattern. Although the PHD motif has been detected in many eukaryotes, little is known about its function. It has been suggested that this motif is involved in protein-protein interactions related to a possible role in chromatin-mediated transcriptional regulation (Aasland *et al*., 1995; Jacobson & Pillus, 1999). The homology of the nine Zar1 proteins was more than 80% and the spacing of the twelve cysteines (C-X2-C-X10-C-X12-C-X2-C-X13-C-X2-C-X4-C-X1-C-X17-C-X2-C-X6-C) was also conserved. The highly conserved, atypical PHD motif in these divergent vertebrate species suggests that this region is involved in transcriptional regulation events (Aasland *et al*., 1995; Bienz, 2006).

Using the 3′ RACE PCR technique, we also obtained the 3′-UTR of the cZar1 cDNA. *In silico* analysis revealed the presence of a XPum (Pumilio) recognition site (TGTA), as well as a hexamer polyadenylation signal (HPS) AATAAA within the 3′-UTR of the cZar1 cDNA. Similar sequences have also been found in the 3′-UTR of the Zar1 cDNAs of other vertebrates, including human, pig and cattle (Uzbekova *et al*., 2006). Pumilio proteins are highly conserved, bearing around 90% amino acid identity in vertebrates and have been shown to act as translational repressor of cyclin B1 by binding to this sequence during oocyte maturation, in addition to cytoplasmic polyadenylation element mediated repression (Nakahata *et al*., 2003).

Expression analysis revealed that the cZar1 is preferentially expressed in reproductive organs of both male and female birds with high levels of expression observed in chicken ovaries and testis, showing a similar expression pattern to that observed in humans. In addition, cZar1 expression was also observed in the oviduct and epididymis, indicating the specificity of expression in the reproductive tract. It has recently been reported that cZar1 cDNA is expressed in the chicken ovary (Elis *et al*., 2008). Our data extend this finding, by identifying cZar1 also in the oviduct, as well as in the male reproductive tract (testis, epididymis). cZar1 mRNA transcripts were also observed at lower levels in kidney, but not in the other organs examined. Previous studies have reported that although in mouse Zar1 expression was exclusively localized in ovaries, human Zar1 transcripts have been detected in both ovaries and testis (Wu *et al*., 2003a). In addi-

tion, the *Xenopus laevis* orthologue is expressed in ovaries, muscle and lung but absent in the testis (Wu *et al*., 2003b). Bovine Zar1 was found in a range of tissues including ovary, testis, skeletal muscle, brain, pituitary and myocardium (Brevini *et al*., 2004; Uzbekova *et al*., 2006), while pig Zar1 transcripts were detected in ovary, testis, brain and pituitary (Uzbekova *et al*., 2006). Therefore, the exclusive ovarian localization of Zar1 seems to be limited to mouse in the species examined to date (Wu *et al*., 2003a).

cZar1 expression was also found at every stage of chicken embryo development investigated from day 3 to day 7 of embryonic development. It has been reported that cZar1 is expressed during early chicken embryogenesis from fertilization until 48 hrs post ovulation (Elis *et al*., 2008). Our data extend these findings by identifying cZar1 expression in chicken embryos until E7. In frog, Zar1 mRNA expression during embryogenesis decreases gradually after the midblastula transition and disappears in the tadpole-stage embryos (Wu *et al*., 2003b). Pig Zar1 mRNA has also been shown to decrease throughout pig embryo development from zygote to 8-cell stages, and very low levels of expression could be detected in morula and blastocyst stages (Uzbekova *et al*., 2006). In addition, human Zar1 mRNA was detected in one-cell and two-cell embryos, but was absent from four-cell, eight-cell embryos and blastocyst (Wu *et al*., 2003a). In mouse, Zar1 protein disappears after the two-cell embryo stage (Wu *et al*., 2003a). In contrast, bovine Zar1 expression was detected at every stage of embryo development from oocyte to blastocyst (Brevini *et al*., 2004; Sangiorgio *et al*., 2008). Thus, our data suggests an embryonic and not only maternal origin of the chicken Zar1 transcript, similar to that observed for the bovine orthologue.

The data presented in this study reveals that the maternal-effect Zygote Arrest 1 gene is conserved in another vertebrate species, the chicken, showing a tissue expression pattern similar to that observed in humans (cZar1 was expressed in chicken male and female gonads) and presented a similar embryo expression pattern with the bovine. Although the mechanisms by which Zar1 protein affects the oocyte-to-embryo transition are poorly understood, our data suggest that chicken Zar1 is an essential maternal factor for reproduction and embryonic development.

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