ABSTRACT

The two major zona pellucida proteins of the zebrafish chorion, Zp2 and Zp3, are encoded by multycopied genes arranged in tandem arrays on chromosomes 20 and 2, respectively. Expression of these zp genes in zebrafish is oocyte specific, and we report herein that their activity in developing oocytes is dependent on conserved CCAAT box sites in their promoters. A 140-bp region immediately upstream of the transcription initiation site (position 1) of the zp2 genes has been homogenized by gene conversion and contains a single CCAAT box located at –138 that is necessary for promoter activity in oocytes residing in stage I and early stage II ovarian follicles as determined by microinjection of promoter constructs linked to a luciferase reporter gene. The zp3 gene promoters have two inverted CCAAT boxes located in a region of shared homology within the initial 175 nucleotides. Serial deletion of these sites resulted in incremental decreases in luciferase activity. Double-stranded oligonucleotides containing CCAAT box sequences from both genes formed CCAAT box-specific complexes with ovariace extracts in an electrophoretic mobility shift assay. We also found that the expression of the separate zp3b gene, more closely related to two oocyte-expressed medaka zpc genes than to the tandemly arrayed zebrafish zp3 genes, is not CCAAT box dependent. The significance of these results have in furthering our understanding of the regulation of zebrafish zp gene evolution and regulation is discussed.

INTRODUCTION

Zebrafish eggs are surrounded by a thick glycoprotein envelope called the chorion which serves as an interface between the egg and sperm and later as a protective barrier between the developing embryo and its environment. The two major components of the zebrafish chorion are proteins Zp2 and Zp3. The first clones of CDNA encoding zebrafish Zp2 and Zp3 were characterized by Wang and Gong [1]. Subsequently, we cloned and sequenced a cluster of three zp2 genes arranged in the zebrafish genome as tandem repeats and matched their coding sequence to peptide sequences obtained from the major 44-kDa protein found in purified zebrafish chorion [2]. More recently, three tandemly repeated zp3 genes with high sequence identity have been isolated and characterized [3]. With these new data and with the July 13, 2007, release of the zebrafish genome project assembly version 7 (Zv7) (URL: http://ensembl.genomics.org.cn:8050/Danio_rerio/index.html), we are able to fully detail in this article the genomic structure, organization, and chromosome locations of the complete array of six zebrafish zp2 genes, as well as two additional members of the zp3 gene array of tandem repeats. These genomic sequence data also provided important clues that have allowed us to make significant progress toward elucidating the promoter elements responsible for regulating the coordinate expression of zp genes in the developing zebrafish oocyte.

The zebrafish chorion is absent from prefollicle phase (stage 1A) oocytes but begins to form as a unilaminar layer (<0.15 μm thick) during the primary growth phase within stage IB ovarian follicles [4]. The zebrafish zp genes are coordinately expressed in these developing oocytes [1, 3, 5, 6], and the synthesized Zp2 and Zp3 proteins are secreted and deposited as an extracellular matrix between the oocyte and the surrounding follicle cells. Expression of the zp genes is temporally constrained to the earliest phases of developing oocytes, and their regulation must therefore be tightly regulated in a developmental stage- and tissue-specific manner. Thus far, speculation about the mechanism of this regulation has centered on a zebrafish homologue of the mouse transcription factor Figla as a likely transactivator. In the mouse, regulation of oocyte expression of the three zona pellucida genes Zp1, Zp2, and Zp3 has been shown to be mediated by the binding of a heterodimer of the basic helix-loop-helix transcription factors Figla and TCF3 (previously known as E12) to E-box sequences located about 200 bp upstream of the initiation site of transcription [7]. Of additional significance was the finding that, in mice lacking Figla, primordial ovarian follicles never formed [8]. Although a zebrafish homologue of mouse Figla has been identified and its mRNA expression pattern is compatible with its possible role in zebrafish zp3 gene regulation [6], a direct connection between Figla and zebrafish zp gene regulation has not been established.

Transgenic zebrafish containing stably integrated copies of zebrafish zp3 promoters linked to the green fluorescent protein (GFP) have been generated independently by two laboratories. The first transgenic line used 412 bp of sequence upstream of the ATG codon of a single-copy zp3 homologue, zp3b (previously described and denoted as zpc [5] and zp3b [3]), which is a distant relative of mammalian Zp3 and is more closely related to medaka genes zpc4 and zpc5 than to the other zebrafish zp3 genes [6]. The second line contained 3.8 kb of the 5’ flanking region of zp3.2, one of the tandemly repeated zp3 genes [3]. Both transgenes expressed GFP in developing oocytes in a pattern resembling that of endogenous zp genes. Both groups of investigators cited E-box sequences, CAnnTG, as possible regulatory elements in the promoters that were used, but no promoter deletion or site mutation data were reported. Herein, we combine a rigorous examination of sequence data from zebrafish zp gene promoters and zp gene promoters from carp and medaka with promoter dissection experiments using microinjection of developing oocytes in ovarian follicles with luciferase reporter constructs to elucidate...
the zebrafish zp promoter elements responsible for coordinated stage- and tissue-specific expression.

**MATERIALS AND METHODS**

**Zebrafish and Maintenance**

Zebrafish (*Danio rerio*) were purchased from a local pet store and were maintained in a freshwater aquarium at room temperature. All experiments were performed using adult female fish and were performed in accord with the guidelines of the Johns Hopkins University Animal Care and Use Committee.

**Nucleotide Sequence Analysis**

Nucleotide sequence data and genome location information for zp genes of zebrafish (*D. rerio*) and medaka (*Oryzias latipes*) were obtained by direct and BLAST search of the Zv7 (July 13, 2007) and of the assembly for MEDAKA1 (October 2005) using Ensembl (URL: http://www.ensembl.org). Carp zp promoter sequences were obtained from the National Center for Biotechnology Information Entrez Nucleotide database (URL: http://www.ncbi.nlm.nih.gov/sites/entrez?db=nucl-nucleotide). Multiple sequence alignment was performed using ClustalW2 (URL: http://www.ebi.ac.uk/Tools/clustalw2/index.html). BLAST 2 (URL: http://blast.ncbi.nlm.nih.gov) was used for pairwise nucleotide sequence alignment.

**Semiquantitative RT-PCR and PCR**

Total RNA was isolated from the ovaries of adult female zebrafish using Trizol reagent (Invitrogen). The RT-PCR was executed in two steps. The initial RT step was catalyzed with Superscript II RT (Invitrogen) after priming 2.0 μg of total RNA using random hexamers. For the PCR amplification, one-tenth volume of the RT reaction was used in a standard PCR reaction with zebrafish zp gene repeat-specific primers (Table 1). From a 50-μl total reaction, samples were removed after 16, 20, and 24 cycles for analysis of products on ethidium bromide-stained agarose gel. Genomic DNA was isolated from zebrafish ovaries by protease K and SDS treatment, followed by phenol:chloroform extraction. The PCR amplification was performed on 2.5 μg of genomic DNA template under standard conditions using zp repeat-specific primers in a 50-μl reaction. Aliquots were removed after 16, 20, and 24 cycles for product analysis by agarose gel.

**Reporter Gene Constructs**

The highly conserved zebrafish zp2.3 and zp3.5 promoter sequences were generated by PCR amplification of genomic DNA and were cloned initially into pGEM-T Easy vector (Promega). After verifying the fidelity of the PCR amplification by nucleotide sequencing (Seqwright), the promoters were excised from the pGEM-T Easy vector using XbaI and SalI sites for zp2.3 promoters and into the BglII site for the zp3 promoters. The primers for generation of the zp2.3 promoter were forward primer 5' CAT AGA TCT AAC CAA TAA GGA TCA GCC GAG A 3' and reverse primer 5' CAT AAG ATT ATG CAG GCA GAG C 3' and the forward primer to generate the megaprimer, resulting in conversion of the CCAAT sequence to CCATT. The PCR amplification was performed on 1.0 mM Tris-HCl (pH 8.0) and 0.1 mM dithiothreitol (DTT), and 5% [v/v] Protease Inhibitor Cocktail (Sigma), followed by incubation on ice for 30 min. The homogenates were cleared by centrifugation, and protein concentration was determined using the Bio-Rad Protein Assay. The DNA templates used in the electrophoretic mobility shift assays were generated by annealing oligonucleotides 5' AAA GTG TCA ACA ACC AAT AAG GAT CCA GAG C 3' and 5' TGA TCC TGA GCC TCC TAA TGG GTT GTT AAG C 3' of the zp2.5 promoter, resulting in conversion of the CCAAT to AGCGA.

**Microinjection of Ovarian Follicles**

Ovarian follicles were isolated from ovaries of adult female zebrafish by mechanical disruption in ovarian follicle culture media consisting of 75% Liebovitz L-15 medium containing 20 mM HEPES [pH 7.5] and 0.2 mg/ml of bovine serum albumin. Ovarian follicles of different stages were identified by size [4] and were separated by sorting using fine forceps under a dissecting microscope (Bausch and Lomb). Microinjections were performed under the dissecting microscope using Leitz micromanipulators and glass microinjection needles prepared from borosilicate glass capillaries with filament (1.2-mm outer diameter and 0.69-mm inner diameter, Sutter Instrument Company) using a model p-87 micropipette puller (Sutter Instrument Company). Reporter plasmid DNA for microinjection was prepared in 1.0 mM Tris-HCl (pH 8.0) and 0.1 mM dithiothreitol (DTT) at 0.04 mg/ml. For normalization purposes, each microinjection solution contained a second reporter plasmid, pRL-CMV or pRL-SV40 (Promega), at 0.04 mg/ml. Microinjected oocytes were transferred to wells of a 96-well plate (10–15 follicles/well), cultured for 18 h at room temperature in 100 μl of ovarian follicle culture medium containing penicillin and streptomycin, and then assayed sequentially for firefly and Renilla luciferase activity using the Dual Glo Luciferase Assay System (Promega) according to the manufacturer’s protocol for cultured cells.

**Electrophoretic Mobility Shift Assays**

Extracts of stages I and II ovarian follicles were prepared by homogenization of one volume of follicles with an equal volume of 2× Extraction Buffer (40 mM HEPES [pH 7.9], 40% glycerol, 0.2 M KCl, 0.4 mM EDTA, 1 μM dithiothreitol (DTT), and 5% [v/v] Protease Inhibitor Cocktail [Sigma]), followed by incubation on ice for 30 min. The homogenates were cleared by centrifugation, and protein concentration was determined using the Bio-Rad Protein Assay. The DNA templates used in the electrophoretic mobility shift assays were generated by annealing oligonucleotides 5' AAA GTG TCA ACA ACC AAT AAG GAT CCA GAG C 3' and 5' TGA TCC TGA GCC TCC TAA TGG GTT AAG C 3' of the zp2.5 promoter, resulting in conversion of the CCAAT to AGCGA.

**TABLE 1.** zp2 gene specific primer pairs used for quantitative RT-PCR analysis.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Primer (5'–3')</th>
<th>Reverse Primer (5'–3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>zp2</td>
<td>GTCTTCATCCACTTGATAATACAG</td>
<td>ATTAAAAGAACATGAAACTTAAAGC</td>
</tr>
<tr>
<td>zp2.2</td>
<td>GTCTTCATCCACTTGATAATACAG</td>
<td>CATGAGATGGGAGCATCAAGT</td>
</tr>
<tr>
<td>zp2.3</td>
<td>GTCTTCATCCACTTGATAATACAG</td>
<td>CATGAGATGGGAGCATCAAGT</td>
</tr>
<tr>
<td>zp2.4</td>
<td>GTCTTCATCCACTTGATAATACAG</td>
<td>CATGAGATGGGAGCATCAAGT</td>
</tr>
<tr>
<td>zp2.5</td>
<td>GTCTTCATCCACTTGATAATACAG</td>
<td>CATGAGATGGGAGCATCAAGT</td>
</tr>
<tr>
<td>zp2.6</td>
<td>GTCTTCATCCACTTGATAATACAG</td>
<td>CATGAGATGGGAGCATCAAGT</td>
</tr>
</tbody>
</table>

A copy of the previously described zebrafish zp2 promoter was generated by PCR amplification of genomic DNA using forward primer 5' CAT CTT GAG GAC CTA CAA TAG GAG CTT GCA CAG CAT AGG 3' as the mutant primer, resulting in conversion of the CCAAT sequence to AGCGA. A luciferase reporter gene construct containing an additional 1701 bp of zp2.3 5' flanking sequence was generated by excising a 1735-bp XhoI-SpeI fragment from a previously isolated 4873-bp Xhol genomic fragment in pGEM-4Z [2] and by inserting it across the pGL3 NheI site and the zp2.3 SpeI site of the original 140-bp zp2 promoter-reporter construct.

A luciferase reporter gene construct containing an additional 1701 bp of zp2.3 5' flanking sequence was generated by excising a 1735-bp XhoI-SpeI fragment from a previously isolated 4873-bp Xhol genomic fragment in pGEM-4Z [2] and by inserting it across the pGL3 NheI site and the zp2.3 SpeI site of the original 140-bp zp2 promoter-reporter construct. A luciferase reporter gene construct containing an additional 1701 bp of zp2.3 5' flanking sequence was generated by excising a 1735-bp XhoI-SpeI fragment from a previously isolated 4873-bp Xhol genomic fragment in pGEM-4Z [2] and by inserting it across the pGL3 NheI site and the zp2.3 SpeI site of the original 140-bp zp2 promoter-reporter construct.

A luciferase reporter gene construct containing an additional 1701 bp of zp2.3 5' flanking sequence was generated by excising a 1735-bp XhoI-SpeI fragment from a previously isolated 4873-bp Xhol genomic fragment in pGEM-4Z [2] and by inserting it across the pGL3 NheI site and the zp2.3 SpeI site of the original 140-bp zp2 promoter-reporter construct.

A luciferase reporter gene construct containing an additional 1701 bp of zp2.3 5' flanking sequence was generated by excising a 1735-bp XhoI-SpeI fragment from a previously isolated 4873-bp Xhol genomic fragment in pGEM-4Z [2] and by inserting it across the pGL3 NheI site and the zp2.3 SpeI site of the original 140-bp zp2 promoter-reporter construct.

A luciferase reporter gene construct containing an additional 1701 bp of zp2.3 5' flanking sequence was generated by excising a 1735-bp XhoI-SpeI fragment from a previously isolated 4873-bp Xhol genomic fragment in pGEM-4Z [2] and by inserting it across the pGL3 NheI site and the zp2.3 SpeI site of the original 140-bp zp2 promoter-reporter construct.

A luciferase reporter gene construct containing an additional 1701 bp of zp2.3 5' flanking sequence was generated by excising a 1735-bp XhoI-SpeI fragment from a previously isolated 4873-bp Xhol genomic fragment in pGEM-4Z [2] and by inserting it across the pGL3 NheI site and the zp2.3 SpeI site of the original 140-bp zp2 promoter-reporter construct.

A luciferase reporter gene construct containing an additional 1701 bp of zp2.3 5' flanking sequence was generated by excising a 1735-bp XhoI-SpeI fragment from a previously isolated 4873-bp Xhol genomic fragment in pGEM-4Z [2] and by inserting it across the pGL3 NheI site and the zp2.3 SpeI site of the original 140-bp zp2 promoter-reporter construct.
the binding reaction products were separated on a 5% nondenaturing polyacrylamide gel containing 2.5% glycerol in a 0.25M Tris-borate-EDTA buffer system. After electrophoresis, the gels were dried and exposed to x-ray film to visualize the protein-DNA complexes. Competition experiments were performed using 100-fold excess of unlabeled full-length DNA (filled in) templates and using the zp2.3 template carrying the mutated CCAAT binding site formed by annealing oligonucleotides 5'AAA GTC TTA AAG CGA AAG GAT CAG GCA GAG CAT AA 3' and 5'TTA TGC TCT GCC TGA TCC TTT CGC TTT GTT AAG ACT TT 3'.

RESULTS
Genomic Structure and Organization of Zebrafish zp2 and zp3 Genes

In a previous study [2], we described a cluster of three tandemly repeated zebrafish zp2 genes, namely, zp2a, zp2b, and zp2c, now designated as zp2.2, zp2.3, and zp2.4, respectively. Nucleotide sequence comparisons showed that they were 99% identical from 140 bp upstream of the initiation site to the end of exon 6 but that their sequences varied considerably (64%–71% identity) from intron 6 through exon 8 and the 3' flanking region [2]. We hypothesized that the genes were the result of duplication events and had subsequently been homogenized by gene conversion to maintain regions of functional importance. We also discovered that the sequence variation in exons 7 and 8 could be used to differentiate between different family members and that the nucleotide sequence of a previously published zp2 cDNA [1] indicated the existence of a fourth copy of the gene. Using nucleotide sequence data from the Zv7, two additional family members can be identified, bringing the total to six. To be consistent with the conventional nomenclature of genes in an array, the two new genes are designated as zp2.5 (ENSDARG00000038435 and ENSDART00000008439) and zp2.6 (ENSDARG00000038435 and ENSDART00000024598). These six genes are arranged in a tandem head-to-tail array on chromosome 20 extending from nucleotide positions 55,516,065 to 55,540,225 (Fig. 1A). From these data and from our Southern blot analysis using a full-length cDNA probe [2] and exon 7 and exon 8 probes specific for individual zp2 genes (data not shown), it seems certain that only six gene copies of the major zp2 gene are present in the zebrafish genome. The six-gene array also includes the previously described zp2 gene [1], and its arrangement from 5' to 3' is zp2.5, zp2.6, zp2.2, zp2.3, and zp2.4 (Fig. 1A).

The zebrafish zp3 gene family is also arranged as a cluster of tandemly repeated members. A recent study [3] reports the isolation and characterization of three tandemly repeated zp3 genes designated as zp3.1, zp3.2, and zp3.3. A search of the Zv7 revealed two additional family members at the 3' end of the same cluster. The five-gene array, zp3.1, zp3.2, zp3.3, zp3.5 (ENSDART00000078959), and zp3.4 (LOC100008088), is located on chromosome 2 and extends from nucleotides 52,117,532 to 52,143,450 (Fig. 1B). Sequence comparisons revealed a high degree of identity (94%–99%) for all eight exons of these genes and a region extending approximately 175 bp upstream of exon 1. Unlike the zp2 genes, whose introns are almost identical in size, introns 1, 2, 4, and 6 of individual zp3 family members have significant variation in their length (Fig. 1B).

Expression of zp2 Family Members

Before embarking on a study of the zebrafish zp2 gene promoters, it was essential to determine if each of six individual zp2 family members is actively expressed in the zebrafish ovary. By taking advantage of the nucleotide sequence variability at the 3' end (intron 6 through exon 8) of the zp2 genes [2], we were able to design gene-specific PCR primers that could be used to measure mRNA amounts by semiquantitative RT-PCR (Table 1). Equal amounts of total RNA isolated from zebrafish ovary were subjected to RT-PCR using the gene-specific primer sets. After 16, 20, and 24 cycles, the products were fractionated by agarose gel electrophoresis.
and were visualized by ethidium bromide staining, allowing semiquantitative analysis of relative zp2 mRNA abundance. In each case, an RT-PCR product of the expected size was obtained, with the primer pair specific for zp2.4 producing the most intensely stained band, followed closely by zp2.2, zp2.3, and zp2.5, with zp2.6 having the least amount of product (Fig. 2). To ensure that any differences in mRNA abundance were not the result of bias introduced by the primers, we utilized the same gene-specific primer pairs in PCR reactions using equivalent amounts of genomic DNA as a template. These results showed almost equal amounts of product for each primer set, consistent with a single gene copy for each zp2 family member. Having established that all six zp2 genes are transcriptionally active, we turned our attention to determining the zp2 promoter sequences responsible for regulating this gene activity.

Nucleotide Sequence Analysis of Zebrafish zp2 and zp3 Promoters

Before undertaking deletion and mutation analysis of the zp2 gene promoter, we first compared the 5' flanking regions of the zp2 gene family members. The six nucleotide sequences extending from nucleotide positions 9 to –286 were aligned and were examined for nucleotide sequence identity and the presence of cis-regulatory elements. As previously reported [2], the promoters exhibit very high sequence identity (>99%) until nucleotide −140, where their sequences diverge. In the Zv7, this region of the zp2.4 promoter is not attenuated as had been previously reported [2]. In addition to a TATA box at position −30, we identified a consensus Sp1 binding site at −60 and a CCAAT box sequence at −138 just within the boundary of the highly conserved 5' upstream region of the six zp2 genes. No E-box sequences (CAnnTG) are present. Three E-box sequences are located within the highly conserved region, but neither has a counterpart in zebrafish.

Because the zebrafish zp2 and zp3 genes are coordinately expressed, they likely possess common regulatory sequences in their promoters. Alignment of the five zp3 promoters revealed a high degree of identity (approximately 95%) through nucleotide position −175 (except for a 20- to 21-bp deletion at position −113 for zp3.1, zp3.3, and zp3.4). The sequences further upstream diverge, with shared nucleotide identity ranging between 48% and 66%. Within the conserved region are a TATA box (position −31), a potential Sp1 binding site (position −58), and two inverted CCAAT box sequences (positions −142 and −171), a pattern not unlike that of the zp2 gene promoters. A search for E-box sequences in the highly conserved region of the tandemly arrayed zp3 gene promoters revealed only one, located in the zp3.2 promoter at nucleotide position −83. The promoter for the distantly related zp3b gene has also been shown to be expressed during the same developmental period as the tandemly arrayed zebrafish zp2

and zp3 genes [5, 6]. Examination of its nucleotide sequence also revealed the presence of two CCAAT boxes (not inverted) at −144 and −179. There is a TATA box sequence at −29 but no consensus Sp1 site. Three E-box sequences are located within the previously described promoter boundaries [6] at nucleotides −69, −135, and −321.

When predicting the identity of essential promoter elements, it is useful to examine the promoters of homologues from closely related species. Nucleotide sequence divergence is expected in nonessential regions; however, those elements that are required for promoter function should be conserved. The common carp (Cyprinus carpio) is a close relative of the zebrafish; they both belong to the fish family Cyprinidae. The two species split from a common ancestor 50 million years ago [10]. Like zebrafish, the carp zp genes are present in multiple copies; however, nucleotide sequence data exist for only one copy each of the carp zp2 and zp3 promoter regions [11, 12]. Pairwise alignment of the 5' upstream region of the carp zp2 gene with the zebrafish zp2 promoter confirmed the importance of the 140-bp region, which is uniquely conserved among all six zebrafish zp2 genes (data not shown). Zebrafish and carp shared 77% nucleotide sequence identity from positions 1 to −140. Sequence identity decreased dramatically to 27% in the region immediately upstream. Similar results were observed for comparison of the zebrafish zp3.4 and carp zp3 promoters, with the zebrafish homogenized region having 74% identity with the same region in carp and 34% decreased identity farther upstream. Notably, both carp promoters possess TATA boxes, Sp1 sites (5'GGGAGG3'), and CCAAT boxes in the same positions as their zebrafish counterparts. The carp sequences each contained a single E-box sequence in this conserved region, but neither has a counterpart in zebrafish.

Sequence analysis and comparison of the 5' upstream regions of zp2 and zp3 from individual zebrafish family members and the closely related fish species carp have allowed us to define the promoter boundaries and to identify several potential cis-regulatory elements of the zp genes in zebrafish. The promoters seem to occupy a region of almost homogenized sequence between nucleotides −69 and −135, and −321.

Deletion and Mutation Analysis of Zebrafish zp2 and zp3 Promoters

To test the activity of the zp2 promoter, a firefly luciferase reporter plasmid containing nucleotides 7 to −140 of the zebrafish zp2.3 promoter was generated and used to microinject developing zebrafish oocytes residing within stage IB and early

FIG. 2. Semiquantitative RT-PCR analysis of transcriptional activity of individual zp2 gene family members in zebrafish ovary. Top) Ethidium-stained RT-PCR products generated from total ovary RNA by gene-specific primer sets after 16, 20, and 24 cycles. Bottom) The PCR amplification of genomic DNA was performed as a control for primer efficiency. Ethidium-stained products are shown for each primer set after 16, 20, and 24 cycles.
stage II ovarian follicles. With our apparatus, we were able to successfully microinject follicles between 80 and 540 μm in diameter; however, the highest luciferase activities were obtained from follicles between 80 and 230 μm in diameter. After overnight incubation, luciferase activity from the zp2.3 promoter could be detected consistently in as few as 10–15 follicles, with values 700-fold higher than those produced by the promoterless luciferase vector (pGL3-Basic). The zp2.3 luciferase reporter constructs were co-injected with plasmid pRL-CMV containing the Renilla luciferase gene to provide an internal control for the efficiency of microinjection. The value of firefly luciferase expression from each zp2.3 reporter gene construct was adjusted accordingly to the level of Renilla luciferase expression from the pRL-CMV internal control plasmid. All reported values are the averages of at least three separate sets of microinjected oocytes.

The importance of the conserved CCAAT box sequence at nucleotide –138 to zp2.3 promoter activity in the zebrafish oocyte was initially assessed by microinjection of a reporter construct containing the zp2.3 promoter fragment deleted from nucleotides –140 to –103. Deletion of these nucleotides containing the CCAAT box resulted in a 9.2-fold reduction of luciferase activity, with 10.9% ± 7.4% activity relative to that of the full-length promoter (100.0% ± 48.2%; Fig. 3). That this difference in promoter activity was CCAAT box related was confirmed by comparison of luciferase activity generated by the full-length promoter with that from a promoter harboring a 5-bp replacement mutation of the CCAAT box sequence (CCAAT → AGCGA). The mutated promoter luciferase activity was reduced 18.9-fold, with an expression level that was 5.3% ± 2.2% of the wild-type full-length promoter (Fig. 3).

Dissection of the zp3 promoter was performed using a similar deletion strategy. The conserved zp3.5 promoter (nucleotides –155 to 5) was cloned upstream of the firefly luciferase gene in the plasmid pGL2. The promoter activity of this construct was compared with the activity of the luciferase reporter gene driven by zp3.5 promoter fragments deleted from nucleotides –155 to –134 and from nucleotides –155 to –106 after microinjection into the nucleus of oocytes in primary growth phase of zebrafish ovarian follicles. Deletion of nucleotides containing the CCAAT box at position –150 reduced the zp3.5 promoter activity to 25.0% ± 4.8% relative to full activity (100.0% ± 36.2%). Almost all of the promoter activity was lost after further deletion of the second CCAAT box at nucleotide –120 (3.3% ± 1.4%; Fig. 4). From these experiments, it is evident that the conserved CCAAT box sequences found in the zebrafish zp2 and zp3 promoters are essential for promoter activity.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Promoter sequencea</th>
<th>Orientation</th>
<th>Position</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zebrafish</td>
<td>zp2</td>
<td>CACCAATAAGAA</td>
<td>&gt;</td>
</tr>
<tr>
<td></td>
<td>zp2.2</td>
<td>CACCAATAAGAA</td>
<td>&gt;</td>
</tr>
<tr>
<td></td>
<td>zp2.3</td>
<td>CACCAATAAGGA</td>
<td>&gt;</td>
</tr>
<tr>
<td></td>
<td>zp2.4</td>
<td>CACCAATAAGGA</td>
<td>&gt;</td>
</tr>
<tr>
<td></td>
<td>zp2.5</td>
<td>CACCAATAAGGA</td>
<td>&gt;</td>
</tr>
<tr>
<td></td>
<td>zp2.6</td>
<td>CACCAATAAGGA</td>
<td>&gt;</td>
</tr>
<tr>
<td>Carp</td>
<td>zp2</td>
<td>GAATCAATGAGAA</td>
<td>&gt;</td>
</tr>
<tr>
<td>Zebrafish</td>
<td>zp3.1</td>
<td>TAGCCAATAAGGA</td>
<td>&lt;</td>
</tr>
<tr>
<td></td>
<td>zp3.2</td>
<td>TAGCCAATAAGGA</td>
<td>&lt;</td>
</tr>
<tr>
<td></td>
<td>zp3.3</td>
<td>TAGCCAATAAGAT</td>
<td>&lt;</td>
</tr>
<tr>
<td></td>
<td>zp3.4</td>
<td>TAGCCAATAAGAT</td>
<td>&lt;</td>
</tr>
<tr>
<td></td>
<td>zp3.5</td>
<td>TAGCCAATAGGA</td>
<td>&lt;</td>
</tr>
<tr>
<td>Carp</td>
<td>zp3</td>
<td>TACCAATAGGA</td>
<td>&lt;</td>
</tr>
<tr>
<td></td>
<td>zp3</td>
<td>TACCAATAGGA</td>
<td>&lt;</td>
</tr>
<tr>
<td>Consensus</td>
<td>C(A/G)(A/G) CCAAT (C/G) (A/G) G (A/C) G</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

a Sequence identities of the five core nucleotides are indicated in bold type and nucleotides matching the consensus flanking sequences are delineated by underlining.

FIG. 3. Deletion and mutation analysis of the zebrafish zp2.3 promoter. Luciferase reporter gene constructs containing 140, 103, and 0 bp of DNA upstream of the transcription initiation site of the zebrafish zp2.3 gene were microinjected into oocytes of stages IB and II ovarian follicles and were assayed after 18 h for firefly luciferase activity. The results were normalized to Renilla luciferase expression from the comicroinjection internal control plasmid pRL-CMV. The conserved CCAAT box is indicated by a shaded rectangle, and the putative Sp1 site is indicated by an oval. The percentage luciferase activity (mean ± SEM) was calculated relative to the activity of the 140-bp promoter, whose value was set at 100%. The effect of mutating the CCAAT box on the activity of the zebrafish zp2.3 promoter was also determined. Oocytes were microinjected with a luciferase reporter gene construct containing the 140-bp promoter, with the CCAAT box sequence replaced with the sequence AGCGA (shaded rectangle with an X).
CCAAT Box Binding Activity in Zebrafish Ovarian Follicle Extracts

Electrophoretic mobility shift assays were used to test ovarian follicle extracts for the presence of protein factors that bind specifically to the CCAAT box sequences of the *zp2* and *zp3* promoters that were shown to be essential for activity in developing oocytes. Protein-DNA complexes were formed when ovarian follicle extracts were incubated with radiolabeled double-stranded oligonucleotides spanning the CCAAT box sequences of the *zp2* and *zp3* promoters (Fig. 5A). Protein binding persisted in the presence of nonspecific poly dI-dC DNA and was ablated in the presence of excess unlabeled target DNA from either promoter. The CCAAT box specificity of the binding was further confirmed by the inability of an excess of oligonucleotide containing the CCAAT box replacement mutant (CCAAT → ACGCA) to compete for protein binding to the *zp2* target oligonucleotide (Fig. 5B). The trimeric transcription factor Nfy binds the CCAAT sequence with high affinity and specificity [13]. In supershift assays, the mobility of the protein-DNA complex was unaffected by antibody specific for the human NFYA subunit. Two *nfya* protein-coding transcripts have been identified in zebrafish (ENSDART00000061546 and ENSDART00000003493). The encoded proteins share 86% and 82% identity with human NFYA. Antibodies directed against zebrafish Nfy were unavailable for these experiments.

Effect of Additional Upstream Sequences on *zp2.3* Promoter Activity

Using nucleotide sequence analysis of the 5' flanking regions of the tandemly arrayed *zp2* zebrafish and carp genes, we tentatively identified a short highly conserved 140-bp sequence directly upstream of the transcription site of each gene copy as the tissue- and stage-specific *zp2* gene promoter. This promoter fragment actively promotes luciferase gene activity in microinjected developing zebrafish oocytes and contains a CCAAT box regulatory element whose ablation results in an almost complete loss of promoter activity. Because the so-defined promoter is short, lacks E-box sites known to regulate mammalian *Zp* genes, and is regulated by the ubiquitous transcription factor Nfy, additional upstream sequences were tested for the existence of other regulatory elements by their ability to enhance *zp2.3* promoter activity. The *zp2.2* and *zp2.3* gene copies are separated by 3068 bp of intervening sequence. Within this sequence are two repetitive elements of approximately 665 bp and 278 bp located at nucleotides 509 to 1174 and nucleotides 1761 to 1998, respectively. Using our previously reported [2] genomic clone of this region, we generated a luciferase reporter plasmid with
an additional 1701 bp of upstream sequence extending to nucleotide −1841. This fragment begins near the end of the most distal repetitive element and contains the entire unique zp2.3 upstream DNA except for about 1000 bp immediately downstream of the 3′ end of the zp2.2 gene copy. Compared with the 140-bp zp2.3 promoter in ovarian follicle microinjection experiments, this longer promoter sequence increased the mean ± SEM amount of luciferase activity produced 18 h after microinjection by 2.3-fold (100% ± 29.2% vs. 43.9% ± 12.1%) (Fig. 6).

Stage-Specific Activity of the Zebrafish zp2.3 Promoter

The highly conserved 140-bp region immediately upstream of each of the tandemly arrayed zp2 gene copies seems to contain most of the sequence information required for full promoter activity in zebrafish oocytes. To determine if this region also confers developmental stage specificity, we tested its activity in oocytes at different stages of development. It has been shown that the zebrafish zp3 genes are most highly expressed in oocytes residing in stage I primary growth phase follicles [3]. Levels of zp3 mRNA decrease as the oocytes develop. In parallel to the increase in the thickness of the oocytes’ zona pellucida layer, expression of zp3 genes was found to be high in early stage II oocytes but gradually decreased and was barely detectable in early stage III ovarian follicles.

As the oocyte develops and increases in size, the nuclear:cytoplasmic ratio decreases, and microinjection into the nucleus becomes increasingly difficult. In our experiments, microinjection of stage IB oocytes always produced dramatically higher luciferase expression from the zp2.3 promoter than microinjection of late stage II or early stage III follicles. To make certain that this difference in activity was the result of stage-specific expression and not a decrease in microinjection efficiency, it was necessary to demonstrate expression from a microinjected internal control plasmid in the later stage oocytes. The cytomegalovirus immediate-early promoter of the pRL-CMV plasmid contains multiple CCAAT sequences and might share the stage-specific properties of the zp2.3 promoter. For this reason, we used the pRL-SV40 plasmid, whose Renilla luciferase reporter gene is driven by the SV40 enhancer and early promoter, which is devoid of CCAAT sequences. By normalizing the firefly luciferase activity produced by the zp2.3 promoter with the amount of Renilla luciferase activity generated by the SV40 promoter, we were able to determine the relative zp2.3 promoter activity at different stages of oocyte development. Similar to the pattern of endogenous zp2 and zp3 gene expression, the microinjected 140-bp zp2.3 promoter had

### Ovarian Follicles

<table>
<thead>
<tr>
<th>Size</th>
<th>Stage</th>
<th>% LUC Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>80-230 μm</td>
<td>Ib – early II</td>
<td>100.0 ± 31.8</td>
</tr>
<tr>
<td>230-310 μm</td>
<td>late II</td>
<td>34.1 ± 16.6</td>
</tr>
<tr>
<td>310-540 μm</td>
<td>early III</td>
<td>25.0 ± 14.5</td>
</tr>
</tbody>
</table>

FIG. 6. Effect of additional upstream sequences on zebrafish zp2.3 promoter activity. Firefly luciferase activity from stages IB and II ovarian follicles microinjected with a luciferase reporter gene construct containing an additional 1701 bp of zp2.3` flanking sequence was compared with activity from similar stage follicles microinjected with the 140-bp zp2.3 promoter or the promoterless pGL3-Basic parent plasmid. The results were normalized to Renilla luciferase expression from the microinjected internal control plasmid pRL-CMV. Repetitive DNA elements in the extended promoter sequence are indicated by a black rectangle. The percentage luciferase activity (mean ± SEM) was calculated relative to the activity of the 1841-bp promoter, whose value was set at 100%.

FIG. 7. Analysis of zp2.3 promoter activity in zebrafish ovarian follicles of different developmental stages. The 140-bp zp2.3 promoter linked to the firefly luciferase reporter gene was microinjected into oocytes of stage IB to early stage II (80–230 μm), stage II (230–310 μm), or late stage II to stage III (310–540 μm) follicles and was assayed after 18 h for luciferase activity. The results were normalized to Renilla luciferase expression from the microinjected internal control plasmid pRL-SV40. The percentage luciferase activity (mean ± SEM) was calculated relative to the promoter activity in the oocytes residing in follicles of the earliest developmental group.
the highest relative activity in oocytes residing in stage IB and early stage II ovarian follicles. Promoter activity decreased to 34.1% ± 16.6% in oocytes in late stage II follicles and to 25.0% ± 14.5% in oocytes in early stage III follicles (Fig. 7).

Role of CCAAT Sequences in zp3b Promoter Function

A 366-bp sequence immediately upstream of the transcription initiation site of the zebrafish zp3b gene (a zp3 family member more closely related to the zpc4 and zpc5 genes of medaka than to the tandemly arrayed zebrafish zp3 genes) has been shown to have promoter activity in transgenic zebrafish that mimics the tissue- and stage-specific pattern of expression observed for endogenous zp genes promoters [6]. Nucleotide sequence comparison of this promoter with the highly conserved promoter region we identified in the repeated zp3 genes revealed no significant homology. Two similarly positioned CCAAT box sequences in the zp3b promoter (nucleotide positions -180 and -145) were identified, but no Sp1 sites were located. To determine if the two CCAAT box sequences have any role in regulating zp3b promoter activity in oocytes of stage IB follicles, we constructed reporter plasmids containing the 366-bp promoter sequence and tested the effect of mutating the CCAAT box sites on promoter activity in microinjection experiments.

Nucleotide sequencing of our PCR-generated zp3b promoter revealed a high degree of identity with the published sequence (98.4%), yet there were several important nucleotide differences, most notably a T to A change in the CCAAT box sequence at position -180. This sequence difference seems to be owing to variation among individual zebrafish and not the result of polymerase infidelity during the PCR reaction, as a similar result was obtained from different genomic DNA samples and PCR reactions. Before testing the promoter activity, we regenerated the CCAAT box by site-directed mutagenesis and then generated a construct with two mutant CCAAT box sequences by mutating the second site at position -145 in the original promoter fragment. These two promoters were then linked to the firefly luciferase reporter, and their activity was assayed after microinjection into oocytes residing in stage IB ovarian follicles. The results of these experiments showed that these CCAAT box sequences have little if any role in regulating zp3b gene expression in oocytes. Mutation of the two CCAAT sequences resulted in a reduction to 70.6% ± 24.4% relative to the wild-type promoter (100.0% ± 45.2%) (Fig. 8).

**DISCUSSION**

In this study, we demonstrated that conserved CCAAT sequence elements in the promoters of the tandemly arrayed zebrafish zp2 and zp3 genes are necessary for reporter gene expression in developing oocytes. Confirmation of a determinate role for these sequences in conferring tissue specificity to the zebrafish zp genes will require the generation of transgenic fish carrying these short highly conserved promoter sequences linked to a histological marker. We have shown by microinjection into oocytes of different developmental stages that the 140-bp zp2.3 promoter is only active during the earliest stages of oocyte development. Combined with the nucleotide sequence analysis of multiple zp genes in multiple fish species, our microinjection results make it reasonably certain that the short promoter sequences we have described represent the minimal length required for oocyte-specific promoter activity of these genes in zebrafish and that the conserved CCAAT box sequences have a determinate role in regulating this activity.

The reproductive strategy of zebrafish is dependent on the frequent production of a large number of eggs. In response to this need for increased egg production, the zebrafish zp gene copy number has expanded through an evolutionary process called concerted evolution [14], whereby multiple highly conserved copies of a gene are generated and maintained by the process of unequal crossing over and/or gene conversion. Other examples of concerted evolution driven by selection for increased gene dosage include the genes encoding for rRNA [15], the highly repetitive sea anemone neurotoxin gene Nvi [16], and the M96 genes encoding a low-complexity extracellular protein component of the oospore walls of the oomycete Phytophthora [17]. In each case, the process has homogenized the genes so that their products remain uniformly functional.

It is reasonable to presume that the promoter sequences of tandemly repeated genes can also be conserved by concerted evolution. This seems to be the case for the zebrafish zp promoters, as the very high degree of sequence identity shared by the zebrafish zp2 genes extends 140 bp upstream of the transcription initiation site. It is almost certainly no coincidence that the percentage sequence identity decreases dramatically immediately upstream of the critical CCAAT box sequence. In addition, the conserved sequence boundary of the zp3 promoters is immediately upstream of the second inverted CCAAT sequence, and the highest sequence identity within the same promoters of carp, a related fish species, is precisely limited to these same evolutionarily homogenized regions.

**FIG. 8.** Mutation analysis of the zebrafish zp3b promoter. Firefly luciferase activity from stages IB and II ovarian follicles microinjected with a luciferase reporter gene construct linked to a PCR-generated copy of the stage-specific zp3b promoter was compared with activity from follicles microinjected with the same promoter containing two mutated CCAAT box sequences (CCAAA and AGCGA, respectively), indicated by shaded rectangles marked with an X. The firefly luciferase activity was normalized to Renilla luciferase expression from the comicroinjected internal control plasmid pRL-CMV. The percentage luciferase activity (mean ± SEM) was calculated relative to the activity of the wild-type promoter, whose value was set at 100%.
The CCAAT box is one of the most widespread promoter elements in the eukaryotic genome. It has been estimated that it is present in as many as 64% of human promoters [18]. Nuclear transcription factor Y, also known as CCAAT binding factor, is the activator of the CCAAT box; it is composed of three subunits, NFYA, NFYB, and NFYC, all required for specific binding to the consensus sequence G/A G/A CCAAT C/G A/G [13, 19]. It has generally been considered a constitutive ubiquitous transcription factor and not a candidate for mediating tissue specificity. However, there is now strong evidence for its differential expression in oocytes.

A recent study [20] of the expression patterns of 36,182 genes in 61 mouse tissues found Nfyα and Nfyγ (Nfyβ was not included in the targets) to be highly upregulated in mouse oocytes with 40-fold and 12-fold increases relative to their median expression in 60 other tissues. In adult fertile oocytes, transcription factor Y, also known as CCAAT binding factor, is included in the targets) to be highly upregulated in mouse oocytes. The genes

\[ nfyb \]

and

\[ nfya \]

are also highly expressed in the gonads, but their transcripts are present in a few other tissues as well [21]. Although the expression pattern of \( nfy \) in zebrafish tissues has not been reported (to our knowledge), the data from mouse and \( C. \ elegans \) suggest that the coordinated expression of \( nfy \) genes in developing zebrafish oocytes might be regulated by oocyte-specific overexpression of the \( nfy \) subunit genes. Nfy regulation of oocyte-specific gene expression might also be evident in transgenic zebrafish harboring the LTR of the ERV-9 human endogenous retrovirus coupled to the GFP gene. The LTR, containing 14 tandem repeats of 37–41 bp with recurrent CCAAT motifs, was found to be active in zebrafish oocytes and stem cell regions of adult tissues but not in spermatozoa [22].

In this study, we have not addressed whether the conserved Sp1 sites in the zebrafish \( zp \) promoters are required for activity. Although mutation of the CCAAT box sequence of the \( zp2 \) promoter reduced promoter activity to 5.3% of that of the 140-bp wild-type promoter, it is still possible that an interaction between Sp1 and Nfy is required and that both binding sites must be present for full activity. There are many examples where a functional synergism between Sp1 and Nfy has been demonstrated, including the genes for human \( EDF1 \), mouse \( Hdac1 \), human transforming growth factor beta receptor type II, rat pyruvate kinase muscle, and rat fatty acid synthase [23–27]. In some cases, a physical interaction between the two transcription factors has been shown [28]. Determining the role of Sp1 in the regulation of zebrafish \( zp \) gene expression will require additional microinjection experiments using luciferase reporter constructs containing \( zp \) promoters with deleted or mutated Sp1 binding sites. While the CCAAT box sequence and possibly the Sp1 binding site are necessary, they may not be sufficient for regulating zebrafish \( zp \) promoter. Because we showed that the addition of 1701 bp to the 140-bp \( zp2.3 \) promoter resulted in a 2.3-fold increase in activity, there may be still other enhancer binding sequences upstream of the homogenized region that affect the level of \( zp2 \) gene expression.

We discovered that regulation of the tandemly arrayed \( zp2 \) and \( zp3 \) genes in zebrafish is mediated differently from that of the mouse \( Zp \) genes, with the Nfy transcription factors having a key role instead of Figla and Tcf3. As we demonstrated herein, zebrafish \( zp3b \) gene expression is not dependent on CCAAT box binding of Nfy, and it may eventually be found to require Figla and Tcf3 for its oocyte-specific activity. This would not be entirely unexpected, as fish have evolved reproductive strategies that are different from those of other vertebrates and vary widely among different fish species. While the expression

<table>
<thead>
<tr>
<th>Gene</th>
<th>Position</th>
<th>Sequence</th>
<th>Position</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oryzias latipes</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>zpc1</td>
<td>−30</td>
<td>ATATAAAA</td>
<td>−74</td>
<td>CAGTTG</td>
</tr>
<tr>
<td>zpc2</td>
<td>−30</td>
<td>TATAAGA</td>
<td>−62</td>
<td>CAGTTG</td>
</tr>
<tr>
<td>zpc3</td>
<td>−30</td>
<td>TTTTAAT</td>
<td>−66</td>
<td>CAGTTG</td>
</tr>
<tr>
<td>zpc4</td>
<td>−30</td>
<td>TAAAAAA</td>
<td>−68</td>
<td>CAGTTG</td>
</tr>
<tr>
<td>zpc5</td>
<td>−30</td>
<td>TTTTTAA</td>
<td>−105</td>
<td>CAGTTG</td>
</tr>
<tr>
<td>Danio rerio</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>zp3b</td>
<td>−30</td>
<td>TAAAGC</td>
<td>−69</td>
<td>CAGTTG</td>
</tr>
</tbody>
</table>

of \( zp \) genes in zebrafish and carp occurs exclusively in the ovary (similar to the mouse), in other teleost fish (such as Atlantic salmon, rainbow trout, and winter flounder) the \( zp \) genes are expressed in the liver and are regulated by estrogen [29–32]. Medaka and the gilthead seabream have both liver-specific and ovary-specific \( zp \) genes [33, 34].

The major constituents of the medaka egg envelope are choriogenin H and choriogenin L, which are synthesized in the liver in response to estrogen and are encoded by genes of the \( zp2 \) and \( zp3 \) families, respectively [33]. The proteins encoded by the major \( zp2 \) and \( zp3 \) genes of zebrafish are most closely related in amino acid sequence to medaka choriogenins H and L. There are six oocyte-specific \( zp \) genes in the medaka genome as well [33]. Many of these are linked, a characteristic attributed to tandem gene duplication. In medaka, \( zpc1, zpc2, \) and \( zpc4 \) are located on linkage group 17, and \( zpc3 \) and \( zpc5 \) are on linkage group eight [33]. The nucleotide sequences are available for five of six oocyte-expressed medaka \( zpc \) genes. The protein encoded by the zebrafish \( zp3b \) gene is most closely related in amino acid sequence to the proteins encoded by medaka \( zpc4 \) and \( zpc5 \) [6].

Unlike the zebrafish, there is considerable sequence variation among the nucleotide sequences of the tandemly duplicated medaka oocyte-specific \( zpc \) genes throughout the gene body and 5’ flanking regions. Notably, on examination of the putative promoter sequences, we found no evidence of a zebrafish-like pattern of CCAAT boxes and Sp1 sites. Instead, as has been reported [33], the medaka oocyte-specific \( zpc \) gene promoters contain a number of E-box sequences. A thorough inspection of these \( zpc \) box sites reveals the presence of a conserved CAGGTG E-box sequence located between nucleotides −62 and −74 in four of five promoters (\( zpc1 \) through \( zpc4 \)) at nucleotide −105 in \( zpc5 \). Similarly, there is an E-box sequence located at nucleotide −69 in the zebrafish \( zp3b \) promoter (Table 3). Whether the oocyte-specific expression of these genes is regulated by Figla [35] remains to be determined.

The relatedness of the major zebrafish \( Zp2 \) and \( Zp3 \) proteins to the liver-expressed chorionins of medaka may be the key to understanding the evolution of the unique mechanism of oocyte-specific regulation of the major zebrafish \( zp2 \) and \( zp3 \) genes described herein. In most vertebrates, the genes for zona pellucida proteins are transcribed exclusively in the ovary. Teleosts are one of the exceptions to this rule [33, 34]. It has been suggested that ancient \( zp2 \) and \( zp3 \) genes were clustered and that (after duplication of this cluster) one group may have gained liver-specific regulation [33]. It is possible that one cluster of \( zp \) genes in ancestral zebrafish developed liver specificity. Sometime later, driven by selective pressure to produce more eggs, this \( zp \) gene group underwent concerted evolution to increase gene dosage and in the process converted from estrogen responsiveness to oocyte-specific expression by tapping into an alternative oocyte-specific regulation pathway.
using preexisting CCAAT box sequences in their gene conversion-homogenized immediate upstream promoter regions. The duplicate gene cluster that did not gain liver specificity may have given rise to the zp3b gene in zebrafish, which retained the standard Figla-type oocyte-specific regulation.

It is apparent from our results that different mechanisms of oocyte-specific zp gene regulation have evolved for different fish species and probably within individual species such as the zebrafish. It is tempting to speculate that these factors may be in part the result of the strategies used in expanding the zp gene dosage in fish. The elucidation of these factors may have important implications in unraveling the regulatory pathways that control the development and maturation of the egg. Certainly, one would not have predicted that Figla was one of the master switches of oogenesis when Liang and colleagues [7] first described its role in Zp gene regulation. Our goal is to determine how the transcription factor NfY fits into the bigger picture of egg development. We hypothesize that it could be downstream of Figla or another master oocyte-specific transcription factor such as a zebrafish homologue of NOBOX oogenesis homeobox [36, 37]. If so, it or one of its subunits may represent a candidate gene for reproductive disorders such as premature ovarian failure [38].

ACKNOWLEDGMENTS

The authors would like to thank Dr. Van Mouwmanakis for the use of his Leitz micromanipulator apparatus for our microinjections, Dr. Edward Hedgcock for the use of his microinjection needle puller, and undergraduate students Florence Barkats and Mehdri Draoua for their technical assistance.

REFERENCES