Regulatory elements and transcriptional control of chicken vasa homologue (CVH) promoter in chicken primordial germ cells

So Dam Jin¹, Bo Ram Lee¹, Young Sun Hwang¹, Hong Jo Lee¹, Jong Seop Rim¹ and Jae Yong Han¹,2*

Abstract

Background: Primordial germ cells (PGCs), the precursors of functional gametes, have distinct characteristics and exhibit several unique molecular mechanisms to maintain pluripotency and germness in comparison to somatic cells. They express germ cell-specific RNA binding proteins (RBPs) by modulating tissue-specific cis- and trans-regulatory elements. Studies on gene structures of chicken vasa homologue (CVH), a chicken RNA binding protein, involved in temporal and spatial regulation are thus important not only for understanding the molecular mechanisms that regulate germ cell fate, but also for practical applications of primordial germ cells. However, very limited studies are available on regulatory elements that control germ cell-specific expression in chicken. Therefore, we investigated the intricate regulatory mechanism(s) that governs transcriptional control of CVH.

Results: We constructed green fluorescence protein (GFP) or luciferase reporter vectors containing the various 5' flanking regions of CVH gene. From the 5' deletion and fragmented assays in chicken PGCs, we have identified a CVH promoter that locates at −316 to +275 base pair fragment with the highest luciferase activity. Additionally, we confirmed for the first time that the 5' untranslated region (UTR) containing intron 1 is required for promoter activity of the CVH gene in chicken PGCs. Furthermore, using a transcription factor binding prediction, transcriptome analysis and siRNA-mediated knockdown, we have identified that a set of transcription factors play a role in the PGC-specific CVH gene expression.

Conclusions: These results demonstrate that cis-elements and transcription factors localizing in the 5' flanking region including the 5' UTR and an intron are important for transcriptional regulation of the CVH gene in chicken PGCs. Finally, this information will contribute to research studies in areas of reproductive biology, constructing of germ cell-specific synthetic promoter for tracing primordial germ cells as well as understanding the transcriptional regulation for maintaining germness in PGCs.

Keywords: Chicken, Chicken vasa homologue, Primordial germ cell, Regulatory element, siRNA-mediated knockdown
continue to proliferate until they enter meiosis. This development of the PGC lineage is a highly complex process that is controlled by the coordinated action of many key factors, such as the expression and regulation of germline-specific genes [13, 14].

Evolutionarily conserved germ cell-specific *vasa* has been characterized in germ cells in several organisms, including chicken [15–17], zebrafish [18], mouse [19], and human [20]. Several studies have demonstrated that *vasa* plays critical roles in germ cell specification, supporting germ line development, translational control of transcribed genes, and RNA processes involving the biosynthesis of PIWI-interacting RNAs (piRNAs) in germ cells at the post-transcriptional level [21–26]. However, the intricate regulatory mechanism(s) that governs transcriptional control of *vasa* expression during chicken germ line development has yet to be investigated in detail.

Understanding the cellular and molecular mechanisms that regulate germ cell-specific gene expression during PGC development is critical for the practical use of genetic modifications and germ-cell biology. In the current study, to characterize the promoter of chicken *vasa* homologue (*CVH*) for inducing germ cell-specific gene expression, we conducted 5' deletion and fragment assays using both enhanced green fluorescent protein (eGFP) and NanoLuc luciferase expression vector. Furthermore, we investigated the predicted putative binding of transcription factors (TFs) on the promoter for *CVH*. Finally, we demonstrated that the transcriptional control of *CVH* expression through cis-elements and TFs is important for germ cell-specific gene expression in chicken PGCs.

**Methods**

**Experimental designs, animals and animal care**

This study was designed with the aim of investigating the cis- and trans-regulatory elements for modulating the transcription of *CVH* gene in chicken PGCs through dual luciferase assay and transcriptome analysis. The care and experimental use of chickens were approved by the Institute of Laboratory Animal Resources, Seoul National University (SNU-150827-1). The chickens were maintained in accordance with a standard management program at the University Animal Farm, Seoul National University, Korea. The procedures for animal management, reproduction, and embryo manipulation adhered to the standard operating protocols of our laboratory.

**Construction of eGFP and NanoLuc luciferase expression vectors controlled by *CVH* promoters of different sizes**

For construction of eGFP expression vectors, the 5' flanking regions of the *CVH* gene (NM_204708.2) were amplified using genomic DNA extracted from adult chicken blood, and subsequently inserted into the pGEM T easy vector (Promega, Madison, WI, USA). Primer sets were used to clone fragments of the *CVH* promoter of different sizes (Table 1). The eGFP coding sequence and polyadenylated (Poly-A) tail were inserted into the clone vectors including CVH promoter using the restriction enzymes *SpeI* and *Ndel*. For the construction of NanoLuc luciferase expression vectors, different lengths of the 5' upstream region of the *CVH* gene were inserted between the *KpnI* and *Xhol* sites of pNL1.2 vectors (Promega).

**Culture of chicken PGCs and DF-1**

Chicken PGCs were cultured in accordance with our standard procedure [2]. Briefly, PGCs from White Leghorn embryonic gonads at 6 days old (Hamburger-Hamilton stage 28) were maintained in knockout Dulbecco's Modified Eagle's Medium (DMEM) (Gibco, Grand Island, NY, USA) supplemented with 20% fetal bovine serum (FBS) (Hyclone, South Logan, UT, USA), 2% chicken serum (Sigma-Aldrich, St. Louis, MO, USA), 1× nucleosides (Millipore, Billerica, MA, USA), 2 mmol/L L-glutamine (Gibco), 1× nonessential amino acids (Gibco), β-mercaptoethanol (Gibco), 1 mmol/L sodium pyruvate (Gibco), and 1× antibiotic-antimycotic (Gibco). Human basic fibroblast growth factor (bFGF) (Koma Biotech, Seoul, Korea) at 10 ng/mL was used for PGC self-renewal. The cultured PGCs were subcultured onto mitomycin-inactivated mouse embryonic fibroblasts at 5- to 6-day intervals by gentle pipetting without any enzyme treatment. For DF-1, the cells were maintained in DMEM with high glucose (Hyclone), 10% FBS, and 1× antibiotic-antimycotic. Cultured cells were grown at 37 °C in a 5% CO₂ incubator.

**Table 1 List of primer sequences used for cloning of the CVH promoter**

| Primer sets | Primer sequence (5'→3') |
|-------------|-------------------------|
| CVH_−1,575 bp_F | GACACAGCTTCCCCACGTGAG |
| CVH_−1,231 bp_F | TGGCCACGTCATCATATTAGT |
| CVH_−625 bp_F | CTCTGATCATGCTCAGCC |
| CVH_−316 bp_F | CAGGCAGCAAGCTAGGCACAGA |
| CVH_−227 bp_F | AGCTAAGACGAGGAAAGG |
| CVH_−135 bp_F | GCCGCCACCTTTCACCC |
| CVH+25 bp_F | GCTATTTGGACGGAGAATGAAAA |
| CVH promoter_R | AGGCAAATGGACGAGCAGC |
| CVH_−135/+222_R | CCCTCCGAGGACCAT |
| CVH_−135/+162_R | AGCACGACTGCCCCCTG |
| eGFP poly A _F | ACTAGTCCGCGATGGTGAGCAAG |
| eGFP poly A_R | CATAGGACGCTTCCCCCACGATGCC |
**In vitro transfection**

In vitro transfection was performed using Lipofectamine 2000 in accordance with the manufacturer’s instructions (Invitrogen, Carlsbad, CA, USA). For expression analysis of eGFP, the constructed CVH promoter vector (1 μg) and 2 μL of Lipofectamine 2000 were separately diluted with 50 μL of Opti-MEM 1 reduced serum medium (Invitrogen) and incubated at room temperature for 5 min. Liposome-DNA solutions were then mixed and incubated at room temperature for 20 min to form the lipid-DNA complex. Liposome-DNA complex solution was added to 2.5 × 10^5 cultured PGCs in 500 μL of PGC culture medium. Transfected cells were incubated for 24 h without feeders. After incubation, cells were analyzed using a fluorescence microscope.

**Luciferase reporter assay**

Nano-Glo Dual-Luciferase Reporter Assay System (Promega) was used to measure the CVH promoter activities. The prepared cells were seeded in a 96-well plate and co-transfected with pGL4.53 firefly luciferase (Fluc) and pNL1.2 (Nluc/P/CVH RE) NanoLuc luciferase (Nluc) plasmid using Lipofectamine 2000 (Invitrogen). The transfected cells were then lysed with lysis buffer with Fluc substrate and incubated on an orbital shaker for 3 min. Fluc signals were then quenched, followed by reaction with Nluc substrate. The signals in arbitrary unit (AU) from both Nluc and Fluc were measured using a luminometer (Glormax-Multi-Detection System; Promega). The promoter activities were calculated by the ratio of the respective AU values of Nluc/Fluc. pNL1.2, an empty vector, was used as a negative control.

**Prediction of putative transcriptional binding elements by in silico sequence analysis**

The 591-base pair (bp) fragment (~316+/275) of the CVH promoter that had the highest activity was analyzed for TF binding sites. Such sites were predicted by MatInspector, a Genomatix program (http://www.genomatix.de) using TRANSFAC matrices (vertebrate matrix; core similarity 1.0 and matrix similarity 0.8) PROMO, which uses version 8.3 TRANSFAC (http://alggen.lsi.upc.es/cgi-bin/promo_v3/promo/promoinit.cgi?dirDB=TF_8.3) and TFBIND, which uses weight matrix in the database TRANSFAC R.3.4 (http://tfbind.hgc.jp).

**Small interfering RNA (siRNA) transfection in chicken PGCs**

Chicken PGCs were seeded at a density of 2.5 × 10^5 per well of a 12-well plate in 1 mL of medium. Then, the cells were transfected with each siRNA (50 pmol/L) using RNAiMAX (Invitrogen). Negative control siRNA with no complementary sequence in the chicken genome was used as a control. The sequence of each siRNA is listed in Table 2. After transfection for 48 h, total RNA was extracted using TRIzol reagent (Invitrogen). The knockdown efficiency of predicted TFs and their effects on the expression of germ cell-related genes including CVH, cDAZL, CIWI, and CDH were measured using quantitative reverse transcription-polymerase chain reaction (RT-PCR).

### Table 2 List of small interfering RNA sequences used for knockdown analysis

| Target genes | Sense | Antisense |
|--------------|-------|-----------|
| ZNF143       | GACGCCUUAUAGUACAGUUAGUGCUAAGAACCGUC | GACGCCUUAUAGUACAGUUAGUGCUAAGAACCGUC |
| ZNF143       | GACGCCUUAUAGUACAGUUAGUGCUAAGAACCGUC | GACGCCUUAUAGUACAGUUAGUGCUAAGAACCGUC |
| ZNF143       | GACGCCUUAUAGUACAGUUAGUGCUAAGAACCGUC | GACGCCUUAUAGUACAGUUAGUGCUAAGAACCGUC |
| ZNF143       | GACGCCUUAUAGUACAGUUAGUGCUAAGAACCGUC | GACGCCUUAUAGUACAGUUAGUGCUAAGAACCGUC |
| ZNF143       | GACGCCUUAUAGUACAGUUAGUGCUAAGAACCGUC | GACGCCUUAUAGUACAGUUAGUGCUAAGAACCGUC |
| ZNF143       | GACGCCUUAUAGUACAGUUAGUGCUAAGAACCGUC | GACGCCUUAUAGUACAGUUAGUGCUAAGAACCGUC |
| ZNF143       | GACGCCUUAUAGUACAGUUAGUGCUAAGAACCGUC | GACGCCUUAUAGUACAGUUAGUGCUAAGAACCGUC |
| ZNF143       | GACGCCUUAUAGUACAGUUAGUGCUAAGAACCGUC | GACGCCUUAUAGUACAGUUAGUGCUAAGAACCGUC |
| ZNF143       | GACGCCUUAUAGUACAGUUAGUGCUAAGAACCGUC | GACGCCUUAUAGUACAGUUAGUGCUAAGAACCGUC |
| ZNF143       | GACGCCUUAUAGUACAGUUAGUGCUAAGAACCGUC | GACGCCUUAUAGUACAGUUAGUGCUAAGAACCGUC |
| ZNF143       | GACGCCUUAUAGUACAGUUAGUGCUAAGAACCGUC | GACGCCUUAUAGUACAGUUAGUGCUAAGAACCGUC |
| ZNF143       | GACGCCUUAUAGUACAGUUAGUGCUAAGAACCGUC | GACGCCUUAUAGUACAGUUAGUGCUAAGAACCGUC |
| ZNF143       | GACGCCUUAUAGUACAGUUAGUGCUAAGAACCGUC | GACGCCUUAUAGUACAGUUAGUGCUAAGAACCGUC |
| ZNF143       | GACGCCUUAUAGUACAGUUAGUGCUAAGAACCGUC | GACGCCUUAUAGUACAGUUAGUGCUAAGAACCGUC |
| ZNF143       | GACGCCUUAUAGUACAGUUAGUGCUAAGAACCGUC | GACGCCUUAUAGUACAGUUAGUGCUAAGAACCGUC |
| ZNF143       | GACGCCUUAUAGUACAGUUAGUGCUAAGAACCGUC | GACGCCUUAUAGUACAGUUAGUGCUAAGAACCGUC |
| ZNF143       | GACGCCUUAUAGUACAGUUAGUGCUAAGAACCGUC | GACGCCUUAUAGUACAGUUAGUGCUAAGAACCGUC |
| ZNF143       | GACGCCUUAUAGUACAGUUAGUGCUAAGAACCGUC | GACGCCUUAUAGUACAGUUAGUGCUAAGAACCGUC |
| ZNF143       | GACGCCUUAUAGUACAGUUAGUGCUAAGAACCGUC | GACGCCUUAUAGUACAGUUAGUGCUAAGAACCGUC |
| ZNF143       | GACGCCUUAUAGUACAGUUAGUGCUAAGAACCGUC | GACGCCUUAUAGUACAGUUAGUGCUAAGAACCGUC |
| ZNF143       | GACGCCUUAUAGUACAGUUAGUGCUAAGAACCGUC | GACGCCUUAUAGUACAGUUAGUGCUAAGAACCGUC |
| ZNF143       | GACGCCUUAUAGUACAGUUAGUGCUAAGAACCGUC | GACGCCUUAUAGUACAGUUAGUGCUAAGAACCGUC |
| ZNF143       | GACGCCUUAUAGUACAGUUAGUGCUAAGAACCGUC | GACGCCUUAUAGUACAGUUAGUGCUAAGAACCGUC |
| ZNF143       | GACGCCUUAUAGUACAGUUAGUGCUAAGAACCGUC | GACGCCUUAUAGUACAGUUAGUGCUAAGAACCGUC |
| ZNF143       | GACGCCUUAUAGUACAGUUAGUGCUAAGAACCGUC | GACGCCUUAUAGUACAGUUAGUGCUAAGAACCGUC |
Results

Identification of the minimal promoter region for transcription of the CVH gene

To investigate the gene promoter for the expression of CVH mRNA, we constructed eGFP expression vectors containing different sizes of the CVH promoter by 5′ deletion, spanning a 1,850-bp region from the 5′ flanking region to the 5′ untranslated region (UTR) (Fig. 1a). Subsequently, we tested whether the differently sized CVH promoters can induce the expression of eGFP in cultured chicken PGCs. As shown in Fig. 1b, the following eGFP reporters were associated with the expression of green fluorescence in chicken PGCs: 1,850-bp fragment (−1,575/+275), 1,506-bp fragment (−1,231/+275), 900-bp fragment (−625/+275), 591-bp fragment (−410/+275), and 250-bp fragment (−120/+275).

Table 3

| No. | Gene Symbol | Description | Accession No. | Forward | Reverse | Product Size, bp |
|-----|-------------|-------------|---------------|---------|---------|-----------------|
| 1   | EP300       | E1A binding protein p300 | XM_004937710.1 | AGCTGCAGATGGAGGAGAAATC | ACCTGAAATGCCTACCCAGTG | 242 |
| 2   | GABPA       | GA binding protein transcription factor, alpha subunit 60 kDa | NM_001007858.1 | TGAACAGATGACACGATGGG | GGGACCTGCTGAAGAAGTC | 225 |
| 3   | HSF2        | heat shock transcription factor 2 | NM_00116764.1 | CCAGTTATACCTGGAGGC | CCAAACAGTCCTCGACCC | 242 |
| 4   | NFYA        | nuclear transcription factor Y, alpha | NM_001006325.1 | TCGCCACCTGTGAAGACAC | TCGAAGTTCCCTCGACCC | 231 |
| 5   | SP3         | Sp3 transcription factor | NM_204603.1 | GGCACAAAGTGTCACTCGAC | GTGTGTTCTCCACCAAGTA | 229 |
| 6   | ZNF143      | zinc finger protein 143 | XM_004941377.1 | GAAGCGGCCCATTTACCT | CCCGACCTCCACACAGAATT | 216 |

Fig. 1

Identification of promoter region for inducing germ cell-specific gene expression in the chicken vasa homologue (CVH) promoter through 5′ deletion assays. a Schematic diagram of the constructed enhanced green fluorescent protein (eGFP) expression vectors with CVH promoters of different sizes. By 5′ deletion assays, six constructs including differently sized 5′ flanking sequences containing the 5′ untranslated region (UTR) were randomly designed. eGFP expression vector of a 250-bp fragment of the CVH promoter containing only the 5′ UTR. b Twenty-four hours after transfection, the expression of eGFP under the control of the differently sized promoters in cultured chicken primordial germ cells (PGCs) was monitored by microscopy. Each fragment used for driving eGFP expression was ligated into the NanoLuc luciferase expression vector (pNL1.2-Basic) to measure promoter activity. Dual luciferase assay of CVH promoter activity in PGCs (c) and DF-1 (d). NanoLuc luciferase expression levels were normalized to the luciferase activity of internal firefly control and are expressed as relative luciferase units. Scale bar = 100 μm. Different letters (a–e) indicate significant differences (P < 0.05)
fragment (−625/+275), 591-bp fragment (−316/+275), and 410-bp fragment (−135/+275); however, the smallest fragment (+26/+275) did not induce this expression. To evaluate the promoter activity further, we performed the dual luciferase reporter assay using the same fragments of the CVH promoter in chicken PGCs and DF-1. Consistent with the findings for eGFP expression, the luciferase reporters containing the promoter region of CVH presented strong enzyme activity, but we could not detect enzyme activity from the smallest 250-bp fragment and pNL1.2-basic, an empty vector (Fig. 1c). Notably, compared with DF-1 fibroblast cells, chicken PGCs generally presented at least 10 times higher luciferase activities (Fig. 1c and d). Collectively, these results suggest that the minimal promoter region of the CVH gene is located at −135 to +275 bp, which includes the 5′ UTR, and plays an important role in the transcription of this gene in chicken PGCs.

**Investigation of the cis-regulatory elements of the CVH gene**

For further investigation of the potential transcriptional cis-elements in the CVH promoter, we performed 5′ and 3′ fragmentation assays using the 591-bp fragment (−316/+275) that presented the highest luciferase reporter activity, as well as a 410-bp fragment (−135/+275) (Fig. 2a). First, we confirmed the eGFP expression with the designed fragments of the CVH promoter in chicken PGCs. Among six fragment constructs, the 591-bp fragment (−316/+275), 502-bp fragment (−227/+275), and 410-bp fragment (−135/+275) were associated with the strong expression of green fluorescence in chicken PGCs compared with the 357-bp fragment (−135/+222) and the 297-bp fragment (−135/+162). These latter two fragments (357-bp and 297-bp fragments) still showed minimal promoter activity, while the 250-bp fragment (+26/+275) showed none (Fig. 2b). We also conducted a dual luciferase reporter assay using NanoLuc luciferase expression vectors to compare the CVH promoter activity in chicken PGCs and DF-1. As shown in Fig. 2c, deletion of the 92-bp fragment between −227/+275 bp and −135/+275 bp resulted in a dramatic decrease in luciferase activity. These results suggest that a positive transcriptional cis-element is located in this region. Furthermore, partial deletion of the 5′ UTR including intron 1 (−135/+222 bp and −135/+162 bp) also produced a dramatic change in promoter activity (Fig. 2c). Interestingly, all
tested fragments showed higher luciferase activity in PGCs than in DF-1 fibroblasts (Fig. 2d). Collectively, these results indicate that the PGC-specific gene expression requires at least a 410-bp sequence of the 5’ upstream region of the CVH gene along with the 5’ UTR including intron 1.

**Prediction and selection of the TFs involved in transcriptional control of the CVH promoter in chicken PGCs**

Based on the findings of the CVH promoter activity mentioned above, we predicted TFs that have binding sites in the 591-bp fragment (−316/+275) of the CVH promoter using three software programs (PROMO, TFBIND, and MatInspector). Additionally, we attempted to clarify the TFs that were more highly expressed in chicken PGCs than in other cell types, such as Stage X blastodermal cells, gonadal stromal cells (GSCs), and chicken embryonic fibroblasts (CEFs) using previously obtained transcriptome data (Fig. 3a) [3, 4, 13]. From these analyses, we identified six TFs (EP300, GABPA, HSF2, NFYA, SP3, and ZNF143) that were expressed at significantly higher levels in PGCs and have putative binding sites in the 591-bp fragment (−316/+275) of the CVH promoter. To summarize our findings, we marked the consensus sequences and positions of the predicted TFs in sequences of the CVH promoter including TATA-box sequence and transcription start codon in Fig. 3b and c.

**Predicted TFs affecting the transcriptional activity of germ cell-specific RBPs**

To confirm the expression of the selected TFs in chicken PGCs, we conducted quantitative RT-PCR using the RNA samples prepared from various cells/tissues (PGCs, Stage X, CEFs, DF-1, and GSCs). The results showed that the expression of five TFs is highly PGC-specific, with the exception being GABPA, which is expressed in both PGCs and Stage X equally (Fig. 4). These results indicate that these TFs may be involved in transcriptional control of the CVH promoter by directly interacting with it in chicken PGCs. We further examined whether these TFs affect the transcription of germ cell-specific RBPs.

![Fig. 3](image_url)
(CVH, cDAZL, CIWI, and CDH) in chicken PGCs using a siRNA-mediated knockdown assay. As shown in Fig. 5, in the samples with the highest knockdown efficiency of HSF2, NFYA, SP3, and ZNF143 mRNA expression in chicken PGCs, the expression of RBP mRNA was significantly reduced, suggesting that these TFs function in regulating transcriptional control of the CVH promoter, and other PGC-specific RBPs, such as cDAZL, CIWI, and CDH, while EP300 and GABPA remain unaffected. Taken together, these results suggest that these TFs (HSF2, NFYA, SP3, and ZNF143) play a role in the transcription of PGC-specific RBPs through direct binding to 5′ upstream promoter regions.

Discussion

The results of the current study suggest that the promoter region of the CVH gene, which extends from −316 to +275 bp and contains the 5′ UTR and intron 1, can control the transcription of the CVH gene in chicken PGCs. They also suggest that significantly up-regulated TFs such as HSF2, NFYA, SP3, and ZNF143 in chicken PGCs play a role in expression of the CVH gene by directly interacting with putative binding sites of the CVH gene promoter.

VASA, an evolutionarily conserved RBP that promotes translational control of germ cell-specific genes, is expressed specifically in germ cells during germline development [28]. Several reports have shown that vasa play a critical role in the formation of the germplasm and gametogenesis in invertebrates such as Caenorhabditis elegans and Drosophila melanogaster [29, 30]. In addition, VASA expression in germ cells is essential for their survival and proliferation [31, 32]. In transgenic animals, 5.1-kb, 4.7-kb, 2.4-kb, 5.6-kb, 8-kb and 4.3-kb of vasa promoter have been used for germ cell specific expression of reporter genes in medaka [33], rainbow trout [34], zebrafish [35], mice [36], cows [37] and pig [38], respectively. Moreover, in Drosophila melanogaster, it is reported that germline specific vasa gene expression in oogenesis is required for a 40-bp genomic region of the vasa gene though interacting specifically with certain ovarian protein [39]. In addition, in the malaria mosquito, vasa-like gene is specifically expressed in both the male and female gonads in adult mosquitoes and is characterized the regulatory regions that are the entire 5′UTR and only 380-bp of upstream sequence for the specific germline expression in the GSCs of both sexes [40]. Although studies on the transcriptional control of CVH for temporal and spatial regulation hold great promise for practical applications, regarding using a germ cell-specific promoter for tracing germ cells as well as understanding the molecular network of transcriptional regulation behind their unique characteristics, very limited information is available on the regulatory elements involved in transcriptional control of the CVH gene in chicken.

Previous study showed that the CVH gene requires a 5′ flanking region of 1,555-bp for higher induction of specific expression in germ cells at the transcriptional level [16]. However, as shown in Fig. 1, we described that the highest promoter activity region of the CVH gene, which is a 591-bp fragment (−316/+275) containing the 5′ UTR, is sufficient for the induction of specific expression in chicken PGCs, as determined by a 5′ deletion
assay. Additionally, our findings demonstrate for the first time that the 5′ UTR containing intron 1 is required for promoter activity of the CVH gene in chicken PGCs, as determined through 5′ and 3′ fragmentation assays (Fig. 2). With regard to the roles of introns in transcriptional control in diverse organisms, several reports have shown that introns play a pivotal role in controlling transcription, including that of germline-specific genes, and act as enhancers to control gene expression [41–47]. In search for regulatory elements of vasa promoter in medaka, Li et al. demonstrated that the first intron plays an important role in the VAS activity from total of 11 regions identified within the 5.1-kb vasa promoter [47], and subsequently found that the first 35-bp of exon 1 of vasa gene is sufficient to increase transcriptional activity as an enhancer [48]. Therefore, it seems likely that the 5′ UTR containing intron 1 of the CVH gene would be valuable for constructing a germ cell-specific CVH promoter vector for the practical utilization of genetic resources.

Transcriptional control is required for regulatory elements such as specialized promoter sequences and...
promoter recognition trans-acting factors [49]. But, there are few reports on transcriptional regulators of vasa gene. A previous study revealed that Mitf acts as transcriptional activator of germ cell-specific genes encoded RNA-binding proteins such as vasa, dazl and dnd in medaka spermatogonial cell line [50]. Therefore, we investigated whether predicted TFs that have putative binding sites in the 591-bp fragment (−316/+275) of the CVH promoter can directly regulate the expression of chicken CVH. Using our previous transcriptome analysis, we identified TFs that were more highly expressed in chicken PGCs than in other cell types. The integrated approaches used in this study were complementary for finding novel TFs with putative binding sites in the CVH promoter. However, we could not find Mitf in the analyzed CVH promoter above mentioned. Finally, we selected six TFs (EP300, GABPA, HSF2, NFYA, SP3, and ZNF143) that have putative binding sites in the 591-bp fragment (−316/+275) of the CVH promoter through a series of experiments. In Fig. 3, all TFs are marked for consensus sequences and positions in the sequence of the CVH promoter, including the TATA-box sequence for transcriptional initiation and the start codon. We also validated their expression levels using quantitative RT-PCR. Based on the results, five TFs (EP300, HSF2, NFYA, SP3, and ZNF143) were significantly expressed in PGCs compared with their levels in other samples, while GABPA were significantly expressed in PGCs compared with CEF, DF-1, and GSC, but showed no significant difference in expression compared with that at Stage X (Fig. 4). We further examined whether these TFs affect the transcription of germ cell-specific RBPs (CVH, cDAZL, CIWI, and CDH) in chicken PGCs through siRNA-mediated knockdown.

With regard to significant expression and functions in germ cells, it has been reported that heat shock factor 2 (HSF2) plays a role during embryonic development and under stress conditions, prevents the formation of damaged gametes, and ensures the integrity of the reproductive process [51]. In addition, knockout mouse models have shown that HSF2 is involved in oogenesis and spermatogenesis and strongly expressed in PGCs [52, 53]. As a general transcription activator, NF-Y binds strongly at CCAAT motifs and consists of NF-YA, −YB, and −YC subunits [54]. In C. elegans, mutations in nfya-1 affect the development of germ cells and also reduce the number of sperm [55]. Moreover, NF-Y is greatly affected by the CCAAT motif in terms of its transcriptional activity regarding Miwi and CIWI gene [56, 57]. Additionally, SP3 is one of the Sp family of TFs, which is characterized by three conserved zinc fingers [58], and positively or negatively controls the transcriptional activity of numerous genes through binding to the GC box in cis-regulatory elements [59]. Importantly, it has been reported that regulation of Nanog gene expression is required for Sp1 and Sp3 expression, besides Oct4 and Sox2, in mouse [60]. Zinc finger protein 143 (ZNF143) was first identified in Xenopus [61], and most ZNF143 binding sites are disturbed in promoters associated with CpG islands near the transcription start site in the mammalian genome [62]. Znf143 is particularly expressed in the mouse ICM [63] and its expression has been implicated in the regulation of mammalian embryonic stem cell survival and renewal [64]. It was also proven that ZNF143 interacts with Oct4, governs Nanog expression through direct binding to the Nanog proximal promoter [65]. In addition, ZNF143 has recently been identified as a new factor connecting promoters and distal regulatory elements as an insulator function for lineage-specific gene expression [66]. With regard to transcriptional regulator of Mouse Vasa Homologue (MVH), Znf143 preferred histone H3 lysine 27 acetylation (H3K27ac)-marked regions associated with the early genes including Kit, Prdm1, and Sox2 rather than the late genes such as Mvh, Piwi1, Piwi2, Tdar7, and Tdar9 in germ cells [67]. However, our results revealed that HSF2, NFYA, SP3, and ZNF143 would be expected to function as transcriptional regulators in chicken PGCs. Collectively, our results demonstrate for the first time that these TFs are involved in promoter activity of germ cell-specific RBPs in chicken PGCs; however, it remains to be determined whether these TFs directly act on each gene promoter during chicken PGC development.

**Conclusion**

In conclusion, we have identified the promoter region of the CVH gene for PGC-specific gene expression and found TFs such as HSF2, NFYA, SP3, and ZNF143 associated with transcriptional control of the CVH gene in chicken PGCs. This information should aid a wide range of studies in constructing germ cell-specific synthetic promoters for tracing germ cells using transgenesis, as well as our understanding of the transcriptional regulation that maintains germness in PGCs.

**Abbreviations**

ACAT: Beta-actin; bFGF: Basic fibroblast growth factor; cDAZL: Chicken DAZL; CDH: Chicken dead end homologue; CEF: Chicken embryonic fibroblast; CIWI: Chicken PIWI-like protein 1; CVH: Chicken vasa homolog; DAZL: Deleted in azoospermia-like; DMEM: Dulbecco’s modified eagle medium; eGFP: Enhanced green fluorescent protein; EP300: E1A binding protein p300; FBS: Fetal bovine serum; GABPA: GA binding protein transcription factor, alpha; GADPH: Glyceraldehyde 3-phosphate dehydrogenase; GSC: Gonadal stromal cell; H3K27ac: Histone H3 lysine 27 acetylation; HSF2: Heat shock transcription factor 2; ICM: Inner cell mass; NFYA: Nuclear transcription factor Y, alpha; PGC: Primordial germ cell; piRNAs: PIWI-interacting RNAs; PIWI: P-element induced wimpy testis; qRT-PCR: Quantitative reverse transcription-polymerase chain reaction; RBP: RNA binding protein; siRNA: Small interfering RNA; SP3: Stimulating protein 3; TF: Transcription factor; UTR: Untranslated region; ZNF143: Zinc finger protein 143
Acknowledgements
Not applicable.

Funding
This work was supported by a National Research Foundation of Korea (NRF) grant funded by the Korea government (MSIP) (No. 2015R1A3A2033826).

Availability of data and materials
The datasets during and/or analyzed during the current study available from the corresponding authors on reasonable request.

Authors’ contributions
HJY participated in study design and coordination. JSD participated in the design of the study, carried out the experiments, statistical analysis and wrote the first draft of the manuscript. LBR participated in overview manuscript preparation. JSD, HYS and LHJ were involved in data interpretation. RJS participated in writing the final versions of the manuscript. All authors have read and approved the final manuscript.

Competing interests
The authors declare that they have no competing interests.

Consent for publication
Not applicable.

Ethics approval
The care and experimental use of chickens were approved by the Institute of Laboratory Animal Resources, Seoul National University.

Received: 5 August 2016 Accepted: 7 December 2016
Published online: 13 January 2017

References
1. Han JY. Germ cells and transgenesis in chickens. Comp Immunol Microbiol Infect Dis. 2009;32:61–80.
2. Park TS, Han JY. Piggycb transposition into primordial germ cells is an efficient tool for transgenesis in chickens. Proc Natl Acad Sci U S A. 2012;109:9337–41.
3. Kim H, Park TS, Lee WK, Moon S, Kim JN, Shin JH, et al. MPPS profiling of embryonic gonad and primordial germ cells in chicken. Physiol Genomics. 2007;29:253–9.
4. Han JY, Park TS, Kim JN, Kim MA, Lim D, Lim JM, et al. Gene expression profiling of chicken primordial germ cell ESTs. BMC Genomics. 2006;7:220.
5. Donovan PJ. The germ cell–the mother of all stem cells. Int J Dev Biol. 1998;42:1043–50.
6. Zheng YH, Rengaraj D, Choi JW, Park KJ, Lee SI, Han JY. Expression pattern of meiosis associated SYCP family members during germline development in chickens. Reproduction. 2009;138:483–92.
7. Rengaraj D, Zheng YH, Kang KS, Park KJ, Lee BR, Lee SI, et al. Conserved expression pattern of chicken DAZL in primordial germ cells and germ-line cells. Theriogenology. 2010;74:765–76.
8. Kim TH, Yun TW, Rengaraj D, Lee SI, Lim SM, Seo HW, et al. Conserved functional characteristics of the PIWI family members in chicken germ cell lineage. Theriogenology. 2012;78:1948–59.
9. Reinke V. Germline genomics. WormBook. 2006. doi:10.1895/wormbook.1.74.1.
10. Seydoux G, Braun RE. Pathway to totipotency: lessons from germ cells. Mol Cell. 2000;5:181–8.
11. Han JY. Germ cells and transgenesis in chickens. Comp Immunol Microbiol Infect Dis. 2009;32:61–80.
12. Hansburger J, Hamilton HL. A series of normal stages in the development of the chick embryo. J Morphol. 1935;88:49–92.
13. Ginsburg M, Eyal-Giladi H. Temporal and spatial aspects of the gradual migration of primordial germ cells from the epiblast to the germinal crescent in the avian embryo. Development. 1986;95:53–71.
14. Lee SI, Lee BR, Hwang YS, Lee HC, Rengaraj D, Song G, et al. MicroRNA-mediated posttranscriptional regulation is required for maintaining undifferentiated properties of blastoderm and primordial germ cells in chickens. Proc Natl Acad Sci U S A. 2011;108:10426–31.
15. Tsunekawa N, Naito M, Sakai Y, Nishida T, Noce T. Isolation of chicken vasa homolog gene and tracing the origin of primordial germ cells. Development. 2000;127:2741–50.
16. Minematsu T, Harumi T, Naito M. Germ cell-specific expression of GFP gene induced by chicken vasa homologue (Cvh) promoter in early chicken embryos. Mol Reprod Dev. 2008;75:1515–22.
17. Livi K, Acloque H, Bachelard E, Nieto MA, Samarut J, Pain B. Ectopic expression of Cvh (Chicken Vasa homologue) mediates the reprogramming of chicken embryonic stem cells to a germ cell fate. Dev Biol. 2009;330:73–82.
18. Yoon C, Kawakami K, Hopkins N. Zebrafish vasa homologue RNA is localized to the cleavage planes of 2- and 4-cell-stage embryos and is expressed in the primordial germ cell development. 1997;124:157–65.
19. Fujisawa Y, Komiya T, Kawabata H, Sato M, Fujimoto H, Furusawa M, et al. Isolation of a DEAD-family protein gene that encodes a murine homolog of Drosophila vasa and its specific expression in germ cell lineage. Proc Natl Acad Sci U S A. 1994;91:12258–62.
20. Castillnon DH, Quade BJ, Wang T, Quigley C, Crum CP. The human VASA gene is specifically expressed in the germ cell lineage. Proc Natl Acad Sci U S A. 2000;97:9585–90.
21. Styrler S, Nakamura A, Swan A, Suter B, Lasko P. Vasa is required for GURKEN accumulation in the oocyte, and is involved in oocyte differentiation and germline cyst development. Development. 1998;125:1599–78.
22. Carrera P, Johnston O, Nakamura A, Casanova J, Jäckle H, Lasko P. VASA mediates translation through interaction with a Drosophila yif-2 homolog. Mol Cell. 2000;5:181–7.
23. Raz E. The function and regulation of vasa-like genes in germ-cell development. Genome Biol. 2001;1:1017.
24. Noce T, Okamoto-ito S, Tsunekawa N. Vasa homolog genes in mammalian germ cell development. Cell Struct Funct. 2001;26:131–6.
25. Liu N, Han H, Lasko P. Vasa promotes Drosophila germ stem cell differentiation by activating mei-P26 translation by directly interacting with a (U)-rich motif in its 3’ UTR. Genes Dev. 2009;23:2742–52.
26. Xiong J, Spinelli P, Laussmann MA, Holomola D, Yang Z, Cosa E, et al. RNA clamping by Vasa assembles a pRNA amplifier complex on transposon transcripts. Cell. 2014;157:1698–711.
27. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2−ΔΔCT method. Methods. 2001;25:402–8.
28. Gustafson EA, Wessel GM. Vasa genes emerging roles in the germ line and in multipotent cells. Bioessays. 2010;32:626–37.
29. Splice C, Meyer N, Racen E, Orsborn A, Kirchner J, Kuznikz K, et al. Genetic analysis of the Caenorhabditis elegans GLH family of P-granule proteins. Genetics. 2008;178:1973–81.
30. Illmensee K, Mahowald AP. Transplantation of posterior polar plasm in Drosophila, Induction of germ cells at the anterior pole of the egg. Proc Natl Acad Sci U S A. 1974;71:1016–20.
31. Parvinen M. The chromatic body in spermatogenesis. Int J Androl. 2005;28:189–201.
32. Medrano JV, Ramathal C, Nguyen HN, Simon C, Reijo Pera RA. Divergent RNA-binding proteins, DAZL and VASA, induce meiotic progression in human germ cells derived in vitro. Stem Cells. 2012;30:441–51.
33. Tanaka M, Kinoshita M, Kobayashi D, Nagahama Y. Establishment of medaka (Oryzias latipes) transgenic lines with the expression of green fluorescent protein fluorescence exclusively in germ cells: a useful model to monitor germ cell lineages in a live vertebrate. Proc Natl Acad Sci U S A. 2001;98:2544–9.
34. Yoshizaki G, Takeuchi T, Tomimaga H, Kobayashi T, Takeuchi T. Visualization of primordial germ cells in transgenic rainbow trout carrying green fluorescent protein gene driven by vasa promoter. Fish Sci. 2002;68:1067–70.
35. Kravel AV, Olsen LC. Expression of a vasa: EGFP transgene in primordial germ cells of the zebrafish. Mech Dev. 2002;116:141–50.
36. Gallardo T, Shirley L, John GB, Castrillon DH. Generation of a germ-cell-specific mouse transgenic Cre line. Vasa-Cre Genesis. 2007;45:413–7.
37. Luo H, Zhou Y, Li Y, Li Q. Splice variants and promoter methylation status of the Bovine Vasa Homolog (BVh) gene may be involved in bull spermatogenesis. BMC Genet. 2013;14:1.
38. Song Y, Lai L, Li L, Huang Y, Wang A, Tang X, et al. Germ cell-specific expression of Cre recombinase using the VASA promoter in the pig. FEBS Open Bio. 2016;6:50–5.
39. Sano H, Nakamura A, Kobayashi S. Identification of a transcriptional regulatory region for germline-specific expression of vasa gene in Drosophila melanogaster. Mech Dev. 2002;112:219–39.

40. Papanotinos PA, Windlbichler N, Menichelli M, Burt A, Crisanti A. The vasa regulatory region mediates germline expression and maternal transmission of proteins in the malaria mosquito Anopheles gambiae: a versatile tool for genetic control strategies. BMC Mol Biol. 2009;10:65.

41. Kawamoto T, Makino K, Niwa H, Sugiyama H, Kimura S, Amemura M, et al. Identification of the human beta-actin enhancer and its binding factor. Mol Cell Biol. 1988;8:267–72.

42. Liu Z, Moav B, Faras A, Guise K, Kapucinski A, Hackett P. Functional analysis of elements affecting expression of the beta-actin gene of carp. Mol Cell Biol. 1990;10:3432–40.

43. Tomaras GD, Foster DA, Burren CM, Taffet SM. ETS transcription factors regulate an enhancer activity in the third intron of TNF-alpha. J Leukoc Biol. 1999;66:183–93.

44. Henkel G, Weiss DL, McCoy R, Deloughery T, Tara D, Brown MA. A DNase I-hypersensitive site in the second intron of the murine IL-4 gene defines a mast cell-specific enhancer. J Immunol. 1992;149:3239–46.

45. Wong TT, Tesfamichael A, Collodi P. Identification of promoter elements responsible for gonad-specific expression of zebrafish Deadend and its application to ovarian germ cell derivation. Int J Dev Biol. 2013;57:767–72.

46. Mohapatra C, Barman HK. Identification of promoter within the first intron of Plzf gene expressed in carp spermatogonial stem cells. Mol Biol Rep. 2014;41:6433–40.

47. Li M, Guan G, Hong N, Hong Y. Multiple regulatory regions control the transcription of medaka germ gene vasa. Biochimie. 2013;95:850–7.

48. Li M, Zhao H, Wei J, Zhang J, Hong Y. Medaka vasa gene has an exonic enhancer for germline expression. Gene. 2015;555:403–8.

49. DeJong J. Basic mechanisms for the control of germ cell gene expression. Gene. 2006;366:39–50.

50. Zhao H, Li M, Purwanti YJ, Liu R, Chen T, Li Z, et al. Mtif is a transcriptional activator of medaka germ genes in culture. Biochimie. 2012;94:759–67.

51. Abane R, Mezger V. Roles of heat shock factors in gametogenesis and development. FEBS J. 2010;277:4150–72.

52. Sarge KD, Park-Sarge OK, Kirby JD, Morimoto RI. Expression of heat shock factor 2 in mouse testis potential role as a regulator of heat-shock protein gene expression during spermatogenesis. Biol Reprod. 1994;50:1334–43.

53. Kallio M, Chang Y, Manuel M, Alastalo TP, Rallu M, Gitton Y, et al. Brain abnormalities, defective meiotic chromosome synapsis and female subfertility in HSF2 null mice. EMBO J. 2002;21:2591–601.

54. Mantovani R. The molecular biology of the CCAAT-binding factor NF-Y. Gene. 1999;1:15–27.

55. Deng H, Sun Y, Zhang Y, Lu X, Hou W, Yan L, et al. Transcription factor NF-Y globally represses the expression of the C. elegans Hox gene Abdominal-B homolog egl-5. Dev Biol. 2007;308:583–92.

56. Hou Y, Yuan J, Zhou X, Fu X, Cheng H, Zhou R. DNA Demethylation and USF Regulate the Meiosis-Specific Expression of the Mouse Miwi. PLoS Genet. 2012;8:e1002716.

57. Sohn YA, Lee SI, Choi HJ, Kim HJ, Kim KH, Park TS, et al. The CCAAT element in the CMI promoter regulates transcriptional initiation in chicken primordial germ cells. Mol Reprod Dev. 2014;81:871–82.

58. Philipsen S, Suske G. A tale of three fingers: the family of mammalian Sp/XLF transcription factors. Nucleic Acids Res. 1999;27:2991–3000.

59. Suske G. The Sp-family of transcription factors. Gene. 1999;238:291–300.

60. Wu DY, Yao Z. Functional analysis of two Sp1/Sp3 binding sites in murine Nanog gene promoter. Cell Res. 2006;16:319–22.

61. Mylinski E, Krol A, Carbon P. ZNF76 and ZNF143 are two human homologs of the transcriptional activator Stat. J Biol Chem. 1998;273:21998–2006.

62. Mylinski E, Gerard MA, Krol A, Carbon P. A genome scale location analysis of human Stat/ZNF143-binding sites suggests a widespread role for human Stat/ZNF143 in mammalian promoters. J Biol Chem. 2006;281:39953–62.

63. Yoshikawa T, Piao Y, Zhong J, Matoba R, Carter MG, Wang Y, et al. Highthroughput screen for genes predominantly expressed in the IC of mouse blastocysts by whole mount in situ hybridization. Gene Expr Patterns. 2006:6:213–24.

64. Chia NY, Chan YS, Feng B, Lu X, Orlow YL, Moreau D, et al. A genome-wide RNAi screen reveals determinants of human embryonic stem cell identity. Nature. 2010;468:316–20.

65. Chen X, Fang F, Liou YC, Ng HH. Zfp143 regulates Nanog through modulation of Oct4 binding. Stem Cells. 2008;26:2759–67.

66. Bailey SD, Zhang X, Desai K, Aid M, Corradin O, Cowper-Sal R, et al. ZNF143 provides sequence specificity to secure chromatin interactions at gene promoters. Nat Commun. 2015;2:6186.

67. Ng J-H, Kumar V, Muratani M, Kraus P, Yeo J-C, Yaw L-P, et al. In vivo epigenomic profiling of germ cells reveals germ cell molecular signatures. Dev Cell. 2013;24:324–33.