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Isolation of chicken *vasa* homolog gene and tracing the origin of primordial germ cells

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SUMMARY

To obtain a reliable molecular probe to trace the origin of germ cell lineages in birds, we isolated a chicken homolog *(Cvh)* to *vasa* gene *(vas)*, which plays an essential role in germline formation in *Drosophila*. We demonstrate the germline-specific expression of CVH protein throughout all stages of development. Immunohistochemical analyses using specific antibody raised against CVH protein indicated that CVH protein was localized in cytoplasm of germ cells ranging from presumptive primordial germ cells (PGCs) in uterine-stage embryos to spermatids and oocytes in adult gonads. During the early cleavages, CVH protein was restrictively localized in the basal portion of the cleavage furrow. About 30 CVH-expressing cells were scattered in the central zone of the area pellucida at stage X, later 45-60 cells were found in the hypoblast layer and subsequently 200-250 positive cells were found anteriorly in the germinal crescent due to morphogenetic movement. Furthermore, in the oocytes, CVH protein was predominantly localized in granulofibrillar structures surrounding the mitochondrial cloud and spectrin protein-enriched structure, indicating that the CVH-containing cytoplasmic structure is the precursory germ plasm in the chicken. These results strongly suggest that the chicken germline is determined by maternally inherited factors in the germ plasm.

Key words: Primordial germ cell, Germ plasm, Spectrin, Chick

INTRODUCTION

Understanding the molecular mechanisms underlying germline specification is important not only for fundamental research in embryology but also for the practical utilization of genetic resources. In birds, as in other vertebrates, many approaches have been used to investigate the origin of germlines; however, because of the lack of reliable molecular markers, it still remains unclear how primordial germ cells (PGCs) originate during early embryogenesis. Until recently, this was also true for fishes, but isolation of the zebrafish homolog of the *Drosophila vasa* gene has provided the first clues as to the origin of germ cells in fishes (Yoon et al., 1997; Olsen et al., 1997).

In *Drosophila*, the *vasa* gene is one of the genes responsible for maternal-effect mutations that cause a deficiency in the formation of germline precursor cells (pole cells; Hay et al., 1988; Lasko and Ashburner, 1988). Despite the uniform distribution of *vasa* mRNA in the oocyte cytoplasm, VASA protein is specifically localized in the germ plasm (pole granules) and is exclusively expressed in germline cells throughout subsequent stages of development. The VASA protein is a member of an ATP-dependent RNA helicase of the DEAD-box family protein. Structural conservation allows us to isolate the conserved sequences of *vasa* homolog genes using a PCR cloning technique. Many vertebrate *vasa* homolog genes have been reported (Komiya et al., 1994; Fujiwara et al., 1994; Komiya and Tanigawa, 1995).

The molecular function of proteins encoded by *Drosophila vasa* and its homologs is not yet fully understood. A possible function of the VASA protein is to bind target mRNAs involved in germline determination, such as *Oskar* and *Nanos*, and to control the onset of the translation (Hay et al., 1990; Lasko and Ashburner, 1990). In the case of *Caenorhabditis elegans vasa* homolog (*Glh*), an essential role for germline segregation has been demonstrated by the injection of antisense RNA (Gruidl et al., 1996). Similarly, in *Xenopus*, microinjection of antibodies against *Xenopus* VASA homolog protein (XVLG) into blastomeres at the 32-cell stage caused a reduction in the number of PGCs in the tadpole stage (Ikenishi and Tanaka, 1997). These findings indicate that the function of vasa family genes is indispensable for germ cell development and is essentially conserved in the evolution of animal species.

In the chicken, PGCs in the early embryos have been distinguished by their morphological characteristics, their high glycogen content stained with periodic acid-Schiff (PAS)

reaction and immunocytochemical staining of cell surface antigens with several monoclonal antibodies such as EMA-1 and SSEA-1. Previous studies have shown that chicken PGCs are recognized at the early somite stage as a population of about 200 dispersed cells located in an extraembryonic region anterior to the head fold, referred to as the germinal crescent (Rogulska et al., 1971). From the germinal crescent region, PGCs migrate into newly formed vascular veins and are passively transported by the blood stream to the vicinity of the embryonic gonads, where they actively invade the gonads. In contrast, little is known about PGC precursors prior to the germinal crescent stage. An excellent study conducted by Ginsburg (1994), in which microdissected fragments of blastodiscs at the intrauterine stage were cultured until the somite stage and the resulting PGCs examined, revealed that PGC precursors had emerged from the most central part of the blastodisc. However, to verify several questions, such as whether PGC precursors are determined at the earlier stage or whether the induction event of PGC differentiation takes place only in the central region, reliable germline-specific probes are required.

In this study, we have isolated a chicken *vasa* homolog *(Cvh)* gene and shown its germline-specific expression. Immunohistochemical analyses using specific antibodies against CVH protein demonstrated that CVH-expressing cells were detectable during early embryogenesis starting from the first cleavage of fertilized eggs, in which CVH protein was restrictively localized in the basal portion of the cleavage furrow. Furthermore, subcellular distribution of CVH protein in granulofilamentous structures in the cytoplasm of chicken oocytes appeared to be equivalent to the precursor material of the germ plasm found in *Xenopus* oocytes. These findings suggest that the chicken germ lineage is maternally predetermined, as is the cases for lower vertebrates such as fishes and frogs.

MATERIALS AND METHODS

Experimental animals

Freshly laid White Leghorn chicken (*Gallus gallus domesticus*) eggs were purchased from Livestock Industry Research Institute (Kanagawa, Japan). Eggs were incubated at 38°C and then the chicken embryos staged according to Hamburger and Hamilton (1951, in Arabic numerals). Fertilized eggs and intrauterine embryos were obtained from anesthetized hens (White Leghorn), which were raised at the National Institute of Animal Industry. The intrauterine embryos were staged according to the normal table of Eyal-Giladi and Kochav (1976, in Roman numerals).

Cloning of chicken *vasa* homolog (*Cvh*) gene

Single-strand cDNAs used for PCR templates were prepared from 10 μg of testis RNA (White Leghorn) using a reverse transcriptase reaction (Superscript preamplification kit; Gibco-BRL). For PCR cloning of chicken *vasa*-like genes, two sets of primers were used. The first set of primers (#1) was designed from two amino acid sequences (Fig. 1), which were common motifs in the DEAD-box protein: 5′-ATGGCNTG(G/T)GCNCA(A/G)ACNGG-3′ and 5′-CATNCG(A/G)TCNGC(C/T)CT(A/Q)TCNAGNAC-3′, respectively. The second set of primers (#2) was designed to sequences of mouse *vasa* homolog *(Mvh)* cDNA: 5′-GGTCCAAAAGTGACATATA-TACCC-3′ (nt: 718-741) and 5′-TTGGTTGATCAGTTCTCGAGT-3′ (nt:1137-1117), corresponding to GPKVTYIP and TRELINQ, respectively (Fujiwara et al., 1994). The PCR cycling conditions were as follows: 1 minute at 94°C, 1 minute at 56°C and 1 minute at 72°C (25 cycles). The PCR products were subcloned into the pGEM-T vector (Promega) and sequenced. A 0.4 kb cDNA fragment derived from PCR using Mvh primers was used as a probe to screen approximately 1×10^6 independent phage clones of a chicken testis (60 days old) cDNA library, which was kindly provided by Dr Nakabayashi of Tohoku University (Japan).

Preparation of Cvh-GST fusion protein and its rabbit antibody

A full-length *Cvh* cDNA fragment containing *Bam*HI and *Sal*I sites at the 5′ and 3′ ends, respectively, was generated by PCR amplification (primers: 5′-GGATCCTTGGAGGAGGACTGGGACAC; 88-106 and 5′-GTCGACCCCATGACTTAAATGTTGT; 2070-2052). The cDNA fragment (2.2 kb) was subcloned in frame into the *Bam*HI-*Sal*I site of a GST-expression vector (pGEX-5X3, Pharmacia). GST-MVH fusion protein was purified using a GST gene fusion system (Pharmacia) according to the manufacturer's instruction. Approximately 300 μg of purified protein was immunized four times into a rabbit. The resulting antiserum was purified by affinity chromatography using GST-CVH-conjugated agarose beads and designated anti-CVH antibody.

Immunoblot analysis

Approximately 20 μg protein extracted from chicken tissues was analyzed by an immunoblot method as described previously (Fujiwara et al., 1994). Transferred filters were incubated overnight at 4°C with anti-CVH antibody (1:100,000 diluted), incubated with alkaline phosphatase (AP)-conjugated goat anti-rabbit IgG (1: 1000, BioRad) for 1 hour and stained using AP-detection solution containing 4-nitro-blue tetrazolium chloride (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) as the chromogen.

Immunohistochemistry

Tissues were fixed with Bouin's solution and embedded in paraffin or OTC compound (Tissue-Tek, Miles). Paraffin sections (7 μm) were dewaxed and dehydrated by passing through a xylene-ethanol series. To inactivate endogenous peroxidase activity, specimens were treated with methanol containing 0.3% H_2O_2 for 30 minutes. After three 20 minute washes in PBS containing 3% BSA, sections were incubated overnight at 4°C with primary antibodies; rabbit anti-CVH (1:10,000), rabbit anti-chicken spectrin (1:1000, Sigma S-1390) or mouse anti-human spectrin mAb (1:500, MAB2622, Chemicon). Goat HRP-conjugated anti-rabbit IgG (Chemicon), goat Oregon Green-conjugated anti-rabbit IgG (Molecular Probes) and goat Texas Red-conjugated anti-mouse IgG (Amersham) were used as secondary antibodies at 1:100-300 dilution in PBS. For HRP-conjugated antibody, sections were stained with 0.2 mg/ml 3,3-diaminobenzidine (DAB), 0.03% H_2O_2 in 0.1 M Tris-HCl (pH 7.5) and counterstained with Hematoxylin. MitoTracker-Red (Molecular Probes) was used according to the manufacturer's instructions to visualize the mitochondria cloud in sections.

For electron microscopic analysis, adult ovary was fixed with 4% paraformaldehyde (PFA), 0.1% glutaraldehyde in PBS. Paraffin sections of adult ovary were stained with anti-CVH as described above. The CVH-positive portions of the sections were dissected, fixed in 0.1% osmium tetroxide for 10 minutes, dehydrated in increasing concentrations of ethanol (70-99%) and embedded in Epon 812. The ultrathin sections were viewed in an electron microscope (JEM-1200EX, JEOL, Japan).

Whole-mount immunostaining

After fixation in 4% PFA in PBS and dehydration in 100% methanol, embryos were rehydrated overnight in PBS containing 0.1% Tween-20 (PBS-T) and incubated overnight with anti-CVH antibody (1:10,000). After three washes in PBS-T, embryos were incubated overnight in AP-conjugated anti-rabbit IgG (1:300). All of steps were

performed at 4°C with gentle shaking. AP-staining was developed with BCIP/NBT solution and the stained embryos were postfixed in 4% PFA-PBS, mounted on slide glasses and photographed.

RESULTS

Isolation of chicken *vasa* homolog cDNA

As for other vertebrates, such as mouse, frog and zebrafish, degenerated primers (#1 shown in Fig. 1B) to the conserved ATP-binding motifs were used to isolate the chicken homolog to *Drosophila vasa* gene. However, the DNA sequences of two different products (approximately 400 bp were amplified using chicken testis cDNA as a template, lane #1 in Fig. 1A) indicated that the deduced amino acid sequences were 95% and 76% identical to mouse PL10 (Leroy et al., 1989) and mouse p68 protein (Lemaire and Heinlein, 1993), respectively: chicken *PL10*, accession number, AB004874 and chicken *p68*, AB004875. Indeed, the latter matches to a partial sequence of a full-length cDNA that has been recently reported as a chicken homolog to mouse *p68* (Jost et al., 1999).

Next, we used another set of primers designed to amplify a different part of the putative ATP-binding domain (#2 shown in Fig. 1B), sequences of which were derived from the mouse *vasa* homolog cDNA. The resulting product with an expected size of 400 bp (Fig. 1A) showed a significant similarity to *Drosophila vasa*. The nucleotide sequence (2,985 bp) of the full-length cDNA isolated by screening a chicken testis cDNA library revealed an open reading frame for 663 amino acids (Fig. 1B) and the predicted amino acid sequence contains eight

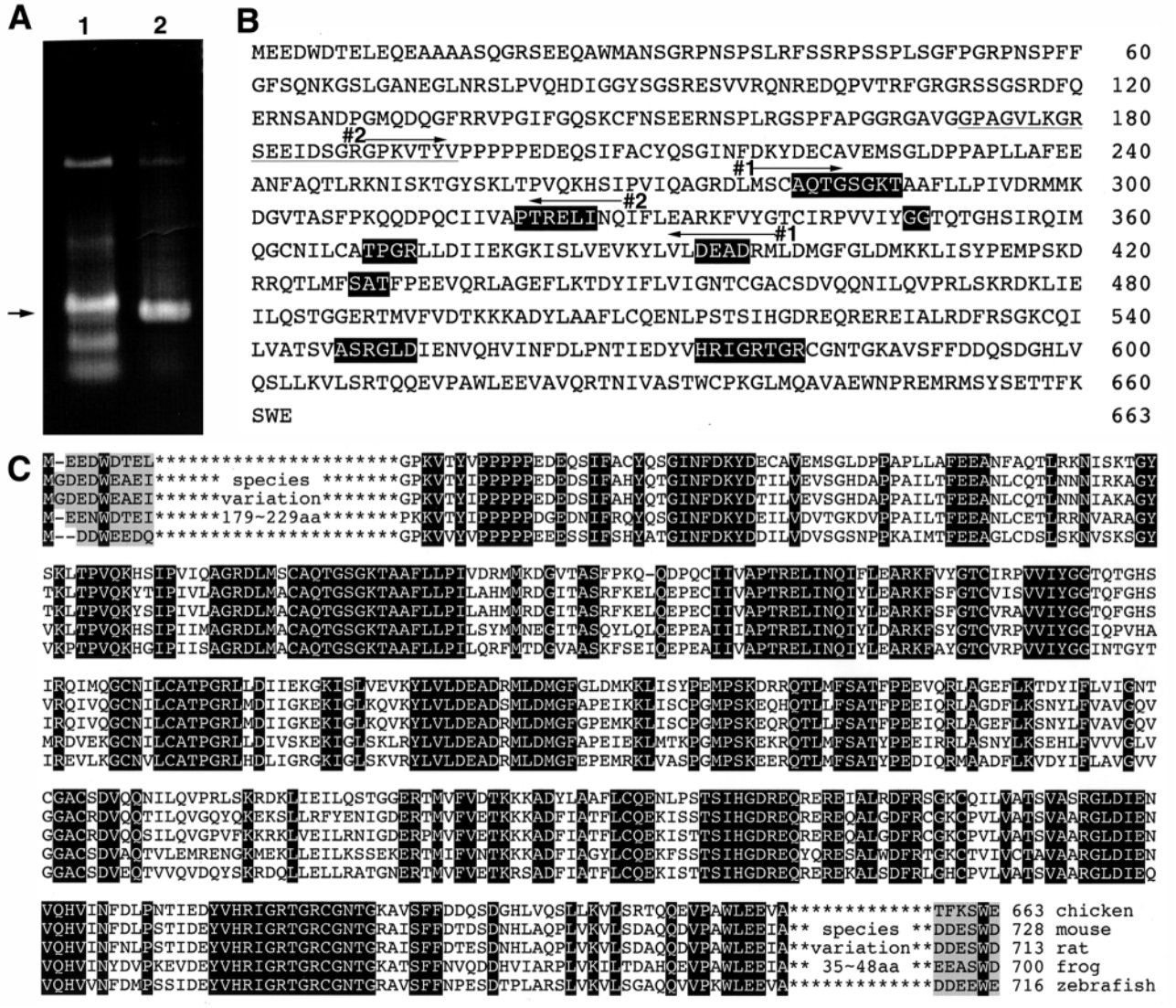

Fig. 1. PCR cloning of Cvh gene and its deduced amino acid sequence. (A) PCR amplification was performed using two sets of primers: DEAD-family-specific primers (#1) and primers generated from *Mvh* cDNA (#2). The PCR products were loaded for each lane on 1.2% agarose gel. (B) Deduced amino acid sequence. Eight conserved motifs are indicated as black boxes. Arrows are placed above the amino acids for which each degenerated primer used for PCR cloning is based. (C) Comparison of the entire sequence of CVH with other vertebrate vasa homolog protein, except for the variation regions of 220 (N-terminal) and 50 (C-terminal) amino acids. Amino acids identical among five vasa homolog proteins are indicated as black boxes and N- and C-terminal regions containing the conserved E, D, W residues are indicated as shaded boxes. GenBank accession number: chicken (Cvh; this work; AB004836), mouse (*Mvh;* Fujiwara et al., 1994; D14859), rat (*Rvlg;* Komoya and Tanigawa, 1995; S75275), Xenopus (*Xvlg;* Komiya et al., 1994; S69534) and zebrafish (*Vas;* Yoon et al., 1997; AB005147).

motifs absolutely conserved among DEAD-box helicase proteins. Comparison with other vertebrate vasa homolog proteins over the entire alignment indicated significant identities: 52% to rat, 50% to mouse, 49% to zebrafish and 45% to *Xenopus*. Furthermore, alignment of the conserved domain of vasa homolog proteins of these five species revealed numerous identical stretches among them: approximately 55% identical in 435 amino acids (Fig. 1C). In addition, as described below, similarities in the germline-specific expression and cytoplasmic localization lead us to conclude that the gene is a chicken homolog to *Drosophila vasa.* We therefore designated it *Cvh* (chicken *vasa* homolog).

Identification of Cvh product and its tissue-specific expression

The *Cvh* transcript (a single band of approximately 3.5 kb) was specifically detectable in adult testis (Fig. 2A). In situ hybridization analyses of adult testis sections revealed that specific signals were observed in cells at stages from spermatogonia to mature spermatocytes (data not shown).

To examine the cellular localization of CVH protein during chicken germ cell development, three kinds of antibodies were prepared against CVH protein. First, rabbit mono-specific antibody was raised against a synthetic 24-mer oligopeptide (the sequence is underlined in Fig. 1B). Subsequently, rabbit polyclonal antibody against the full-length CVH protein was prepared using GST-CVH fusion protein as the antigen, which was specifically recognized with the anti-CVH peptide antibody (data not shown). Using the same GST-CVH fusion protein, a mouse myeloma cell line (clone #234) producing a monoclonal antibody against CVH protein was established. Immunoblot analyses using these three kinds of antibodies showed the same result (Fig. 2B,D), i.e. a single band of 80 kDa protein was specifically detected in adult testis (Fig. 2B) and CVH protein having the same size was detected in immature testis and ovary of newly hatched chickens (Fig. 2C), in which germ cells were at the spermatogonium stage in males and the primary oocyte stage in females. Among these antibodies, anti-GST-CVH fusion protein, designated as anti-CVH, was used for the following analysis because it demonstrated the highest specific reactivity in the immunohistochemical detection.

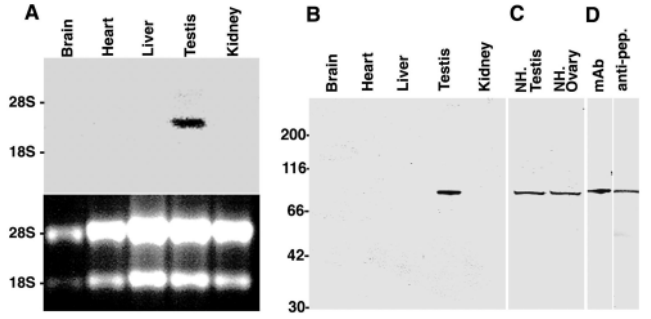

Fig. 2. Tissue-specific expression of Cvh. (A) Northern blot analysis of total RNA (15 μg) from the indicated chicken adult tissues. Positions of 28S and 18S rRNAs are shown. *(Lower)* 28S and 18S rRNA bands stained with ethidium bromide. A *Pst*I fragment from CVP0.8 was used as the probe. (B) Immunoblot detection of CVH protein. Proteins (20 μg) from the indicated adult tissues were used for each lane and immunostained with anti-CVH antibody. Signals were detected with enzymatic colorization by AP-conjugated anti-rabbit IgG. (C) Proteins from testis and ovary of newly hatched chicken were analyzed as in A. (D) Proteins from adult testis were immunostained with a monoclonal antibody against CVH protein or with antibody against CVH-oligopeptide.

CVH expression during germ cell development

Anti-CVH staining of adult testis sections revealed that CVH protein was exclusively localized in the cytoplasm of spermatogenic germ cells but not in somatic cells such as Leydig and Sertoli cells (Fig. 3A). Strong staining was detected in cell stages from the spermatogonia to round spermatids, but was absent by the elongated spermatid stage as the spermiogenesis proceeded (Fig. 3a). Interestingly, CVH protein in spermatocytes appeared to be distributed in a granulofibrillar manner, suggesting an association with some cytoplasmic structures. Section staining of adult ovaries revealed CVH distribution in the cytoplasm of the immature oocytes. Primary oocytes located in the periphery of the ovary were stained the most strongly, with the staining becoming progressively weaker as the follicles grew (Fig. 3B). CVH protein appeared to be localized underneath the plasma membrane and in spherical fibriform structures (Fig. 3b). Staining of gonadal sections prepared from newly hatched male chickens and from female embryos at 9 day after incubation also revealed the germ-cell-specific expression of CVH protein in the cytoplasm of spermatogonia and oogonia (Fig. 3C,D).

To examine the expression of CVH protein in PGCs in the migrating and gonadal phases, sections of early embryos after 1-6 days of incubation were stained with anti-CVH. During these periods, PGCs were carried by the circulation from the germinal crescent to the vicinity of the gonads before they migrated with active cell movement into the gonads (Meyer, 1964). In 6-day embryos, PGCs localized both in the embryonic gonads and in the dorsal mesentery near the gonads were clearly recognized as CVH-positive cells (Fig. 3E). Similarly, in 3-day embryos, CVH protein was detected in the cytoplasm of PGCs migrating in the dorsal mesentery (Fig. 3F). In 2-day embryos, PGCs circulating in the blood stream were recognizable as CVH-positive cells (Fig. 3G). Before the circulating stage, PGCs localized in the extraembryonic germinal crescent region anterior to the head fold of 1-day embryos (stage 11) were morphologically distinguishable from other types of cells by their larger size. CVH protein was specifically detectable in the cytoplasm of PGCs located in the germinal crescent (Fig. 3H,h), whereas no significant staining was observed in any type of cell other than germ cells throughout development.

Expression of CVH protein during formation of the primitive streak

Based on the germline specificity of anti-CVH staining, we further investigated the developmental kinetics of chicken PGC precursors during the early blastodisc stages, stage X (EG&K) to stage 4 (H&H). Anti-CVH staining revealed that cells expressing CVH protein already existed in pregastrulation embryos at stage IX-X (CVH expression at the stage X was also detectable with immunoblot analysis, data not shown). In whole-mount staining, CVH-positive cells were found scattered in the central region of the area pellucida

(Fig. 4A). Section staining of the corresponding stage revealed CVH-positive cells in the ventral region of the epiblast layer (Fig. 5A). The positive cells appeared to be relatively larger than the neighboring negative cells and, interestingly, subcellular localization of CVH protein was detected as a crescent-shaped region in the ventral part of the cytoplasm. The population of CVH-positive cells was estimated at 0.11% (average) by immunostaining the cells dissociated from the stage X blastodiscs (Fig. 5D). Assuming that stage X blastodiscs consist of about 3×10^4 cells (Stepinska and Olszanska, 1983), the number of PGC precursors is calculated to be approximately 33 cells per embryo. In a whole-mount staining of the stage 3 (H&H) embryos, CVH-positive cells were detected with a 'C'-shaped distribution in the anterior region of a primitive streak (Fig. 4B). In sections of the equivalent stage, the positive cells were predominantly located on dorsal surface of the newly developed hypoblast layer in the central part of blastodiscs (Fig. 5B). The CVH-positive cells in the hypoblast appeared be morphologically similar to PGCs in the germinal crescent. Staining of serial sections of whole blastodiscs indicated about 40-60 CVH-positive cells per stage 2-3 (H&H) embryo and showed a few CVH-positive cells adjacent to each other. At the stage 4, CVH-positive cells were found in a crescent-shaped distribution around the anterior edge of the primitive streak (Fig. 4C). In sections, the positive cells were mainly localized in the anterior region of the presumptive amniocardiac vesicle (Fig. 5C) and a few positive cells were observed in the endodermal and ectodermal layers. The number of CVH-positive PGCs per embryo at this stage was estimated as approximately 200-250 by analyzing the serial sections. These observations suggest that translocation of PGCs from the central region of the area pellucida (stage X) to the germinal crescent in the extraembryonic region (stage 4) is due to passive movement caused by formation of the primitive streak.

When a blastodisc of a stage X embryo was dissociated and cultured on mouse STO cells as a feeder layer, blastodermal cells proliferated for at least the first 24 hours. After 20 hours of culture, some CVH-positive cells were found to be adjacent to each other and the number, an average of about 150 cells, and morphological characteristics resembled those of stage 4 embryos (Fig. 5D,E), indicating that CVH-positive cells in stage X blastoderms have already acquired the developmental potency to give rise to PGCs, which localize in the germinal crescent of stage 4 embryos.

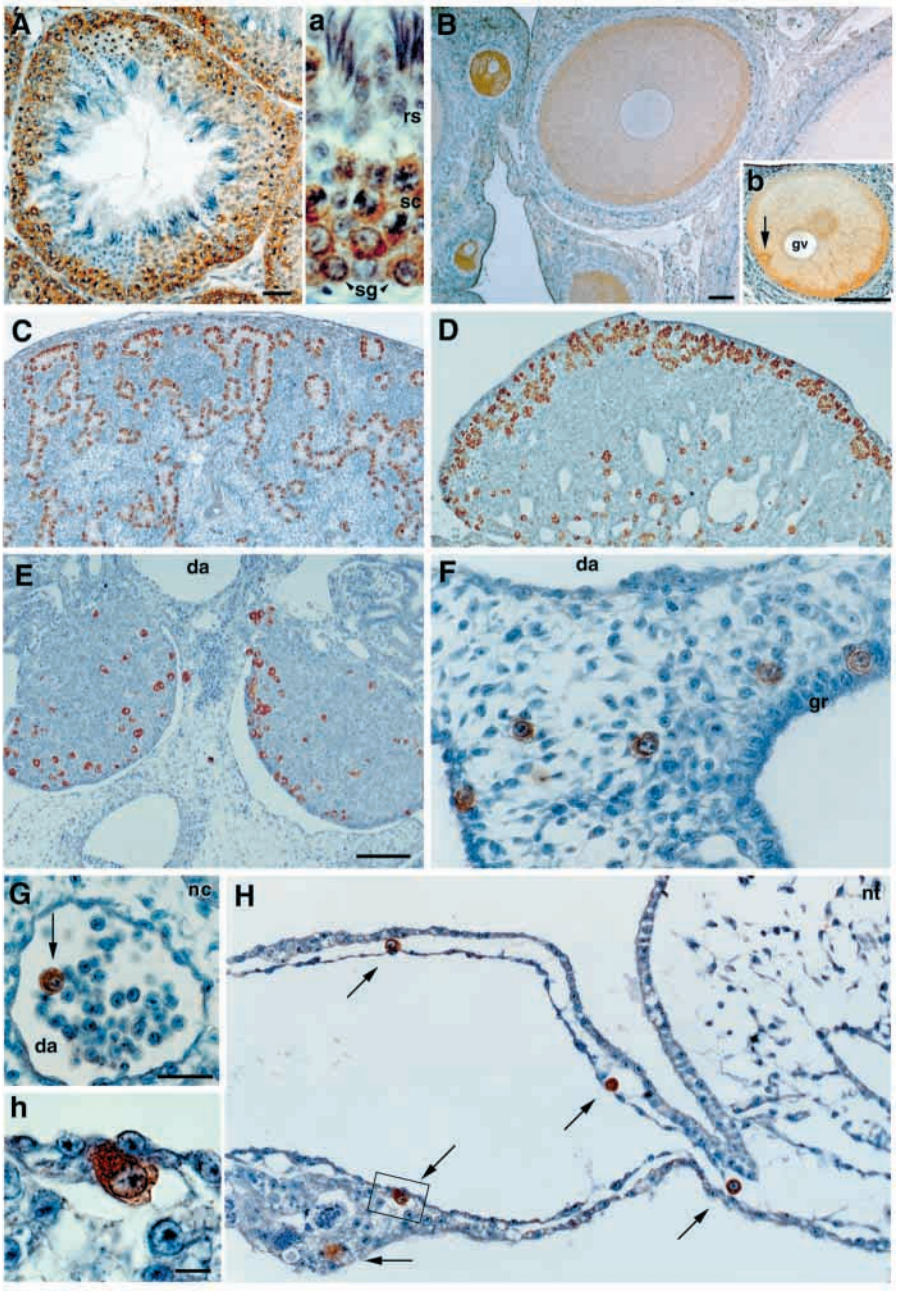

Fig. 3. Immunohistochemical staining of gonads and embryos exhibits germline-specific expression of CVH protein. Sections of the adult testis (A,a), the adult ovary (B,b), the embryonic testis of 21 days after incubation (C) and the embryonic ovary of 9 days after incubation (D) were immunostained with anti-CVH, using enzymatic colorization by HRP-conjugated secondary antibody. (a) A high magnification view of the seminiferous epithelium in A. (b) A high magnification view of an immature oocyte stained with anti-CVH. Similarly, transverse sections of embryos at 6 days (E), 3 days (F) and 2 days (G) after incubation, and a section in the germinal crescent region of the stage 11 (H&H) embryo of 1 day after incubation (H,h) were stained. (h) A high magnification view of the boxed zone in H. Abbreviations: da, dorsal aorta; gr, genital ridge; gv, germinal vesicle; nt, neural tube; rs, round spermatid; sc, spermatocyte; sg, spermatogonium. Bar in A, B and b, 50 μm; in E, C and D, 100 μm; in G and F, 25 μm; in h, 10 μm.

CVH protein is co-localized with the mitochondrial cloud in oocytes

In other animals, including several vertebrates, vasa homolog proteins are known to be localized in the germ plasm, for example, polar granules in *Drosophila* and germinal granules in *Xenopus*. In *Xenopus* oocytes, the cytoskeletal protein spectrin has been shown to be co-distributed with the mitochondrial cloud, which has been implicated in the assembly and formation of germ plasm (Kloc et al., 1998). Therefore, we carried out immunocytochemical staining of serial sections of chicken oocytes using both anti-CVH and anti-spectrin antibody. As shown in Fig. 6, double staining with anti-CVH and anti-spectrin revealed that both CVH and spectrin were co-localized in a characteristic globular shape structure in oocytes. Interestingly, spectrin was located in the center of the structure, whereas CVH localization was detected in the outer layer surrounding the spectrin-containing region (Fig. 6A-E). The mitochondria-specific dye MitoTracker-Red was used for the section staining of oocytes to examine the localization of the mitochondrial cloud. The fluorescent-labeled mitochondria-rich structure appeared to be identical to the spectrin-containing structure and the CVH-enriched region appeared to surround the mitochondria cloud (Fig. 6F-J). Electron microscopic analyses of the CVH-enriched globular shape structures also demonstrated the presence of mitochondria cloud within the structure (Fig. 7). By analogy to the findings in *Xenopus* oocytes, these data suggest the presence of germ plasm in chicken oocytes.

Distribution of CVH protein in cleavage-stage embryos

If chicken germ cells were determined by maternal heritage factors, a germ plasm-like structure expressing CVH protein must be detectable in some blastomeres during successive cleavages. Sections of the uterine-stage embryos were stained with anti-CVH to define the localization of CVH protein and distinguish the germ cell progenitors in the initial step of the development. At the first cleavage (stage I), a CVH-positive structure was found in the basal part of the cleavage furrow (Fig. 8A). Serial sections revealed that this structure was a sphere-shaped structure (approximately 20 μm in diameter) located in the middle portion of the furrow. At the second meroblastic cleavage, the CVH-positive structure changed to a 'V' shape at the basement of the first cleavage furrow, in which CVH staining was detected in tightly aggregated granules just beneath the furrow (Fig. 8B). Subsequently, similar localization of CVH protein was observed until the fourth asymmetric cleavage. At the 15-blastomere stage, corresponding to the intermediate stage between II and III

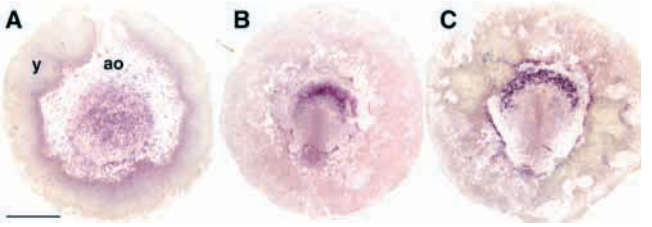

Fig. 4 Whole-mount immunostaining of the embryos from stage X (EG&K) to stage 4 (H&H). Embryos at stage X (A), stage 3 (B) and stage 4 (C) were immunostained with anti-CVH, using enzymatic colorization by AP-conjugated secondary antibody. Some staining observed in the boundary between the area opaca (ao) and the yolk (y) is non-specific because staining was also found in the control specimen stained without anti-CVH (data not shown). Bar in A for B and C, 1 mm.

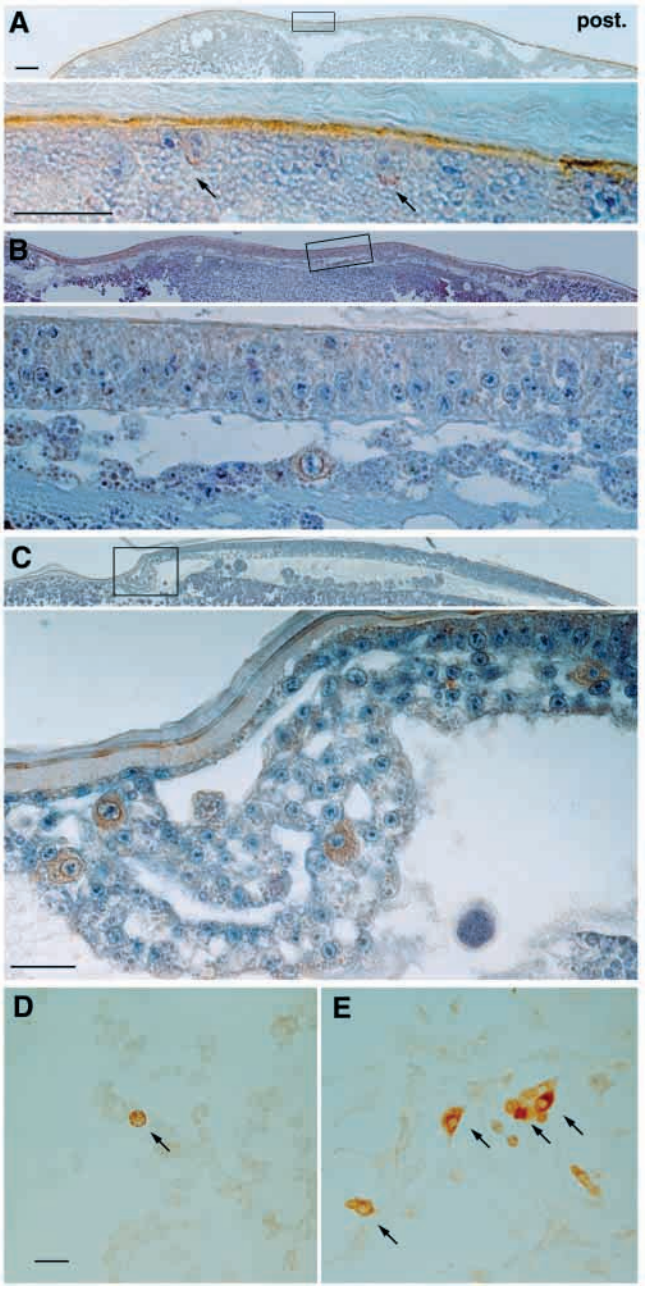

Fig. 5. Immunohistochemical identification of CVH-positive cells in intrauterine and early incubation embryos. Sections of embryos at stage IX-X (A), stage 3 (B) and stage 4 (C) were stained with anti-CVH, using enzymatic colorization by HRP-conjugated secondary antibody. Lower photographs are high magnification views of the boxed zones indicated in upper ones, which show the entire blastodisc of each embryo. (D,E) Blastodermal cells from stage X were seeded onto feeder layers of STO cells. They were immunostained with anti-CVH after 2 hours (D) and 20 hours (E) of culture. Arrows indicate CVH-positive cells. Bars, 30 μm.

(EG&K) embryos, CVH protein was localized in patch-like structures close to the ventral cleavage furrows (Fig. 8C). In stage III embryos, CVH-containing structures were observed in the constricted part of a specific blastomere or in a specific cell undergoing horizontal cleavage at the center of the blastodisc (Fig. 8D). Interestingly, MitoTracker-Red staining of the same section revealed mitochondrial localization in the CVH-containing structure (Fig. 8E). In stage IV and V embryos, which consist of about 300 and 600 cells, respectively, granular spherical structures containing CVH protein were detected in the ventral cytoplasm of 6 to 8 cells, which resided in the center of the blastodisc (Fig. 8F,G), as observed in stage IX-X embryos.

Anti-CVH shows cross-reactivity on testis sections of other vertebrates

To examine the cross-reactivity of anti-CVH against other birds and vertebrates, adult testis sections prepared from quail *(Coturnix coturnix japonica)*, turtle *(Pelidiscuc sinensis)* and snake *(Trimeresurus flavoviridis*) were stained with anti-CVH. As shown in Fig. 9, testicular germ cells in these animals were specifically stained with subcellular distributions similar to that of chicken testis. A protein of approximately 80 kDa was detected in testis of quail and turtle by immunoblot analyses (data not shown). In addition, similar results were obtained in testes sections of lizard *(Takydromus takydromus)* and frog (*Xenopus laevis)*, suggesting the presence of vasa homolog proteins with common sequences to the CVH protein in other birds and reptiles.

DISCUSSION

The objective of the present study was to isolate a chicken *vasa*

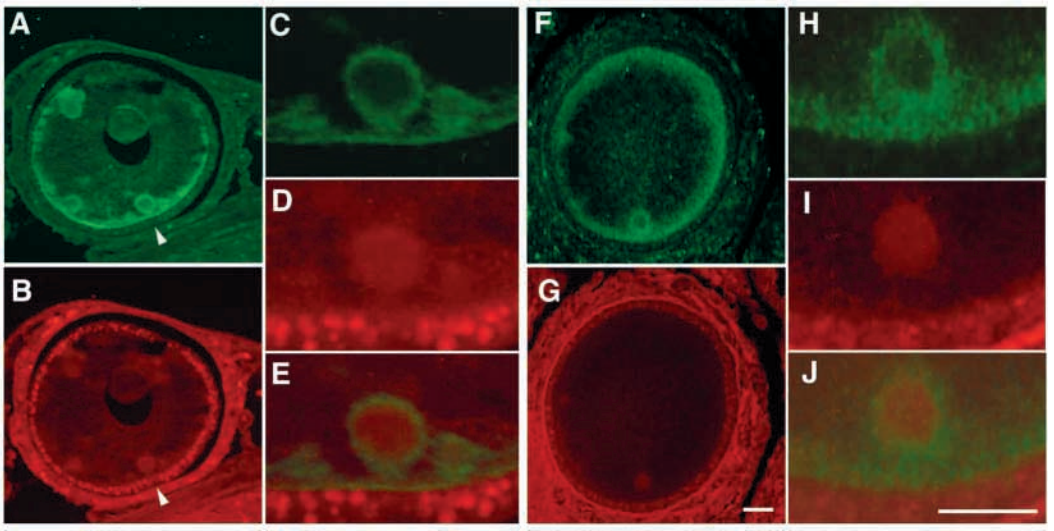

Fig. 6. Immunocytochemical analyses of chicken oocytes. Sections of oocytes were double-stained with anti-CVH (A,C) and anti-spectrin (B,D) or double-stained with anti-CVH (F,H) and MitoTracker-Red (G,I). C,D,H,I are higher magnification views of A,B,F,G, respectively. E and J are merged images of C plus D and H plus I, respectively. Arrowheads in A and B indicate the stained structures magnified in C and D. Bars, 50 μm.

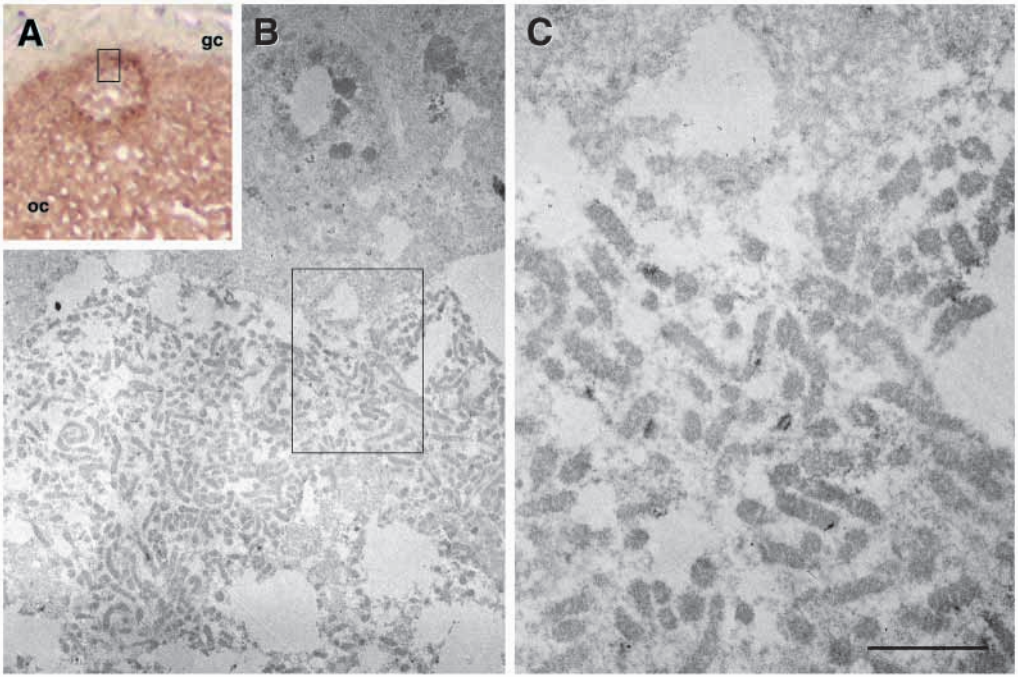

Fig. 7. Electron microscopic analysis. Oocyte sections were immunostained with anti-CVH antibody (A) and a portion of the positive region (boxed) was analyzed with electron microscope (B). (C) A high magnification view of the boxed zone in B. Abbreviations: oc, oocyte; gc, granulosa cell layer. Bar in C, 2 μm.

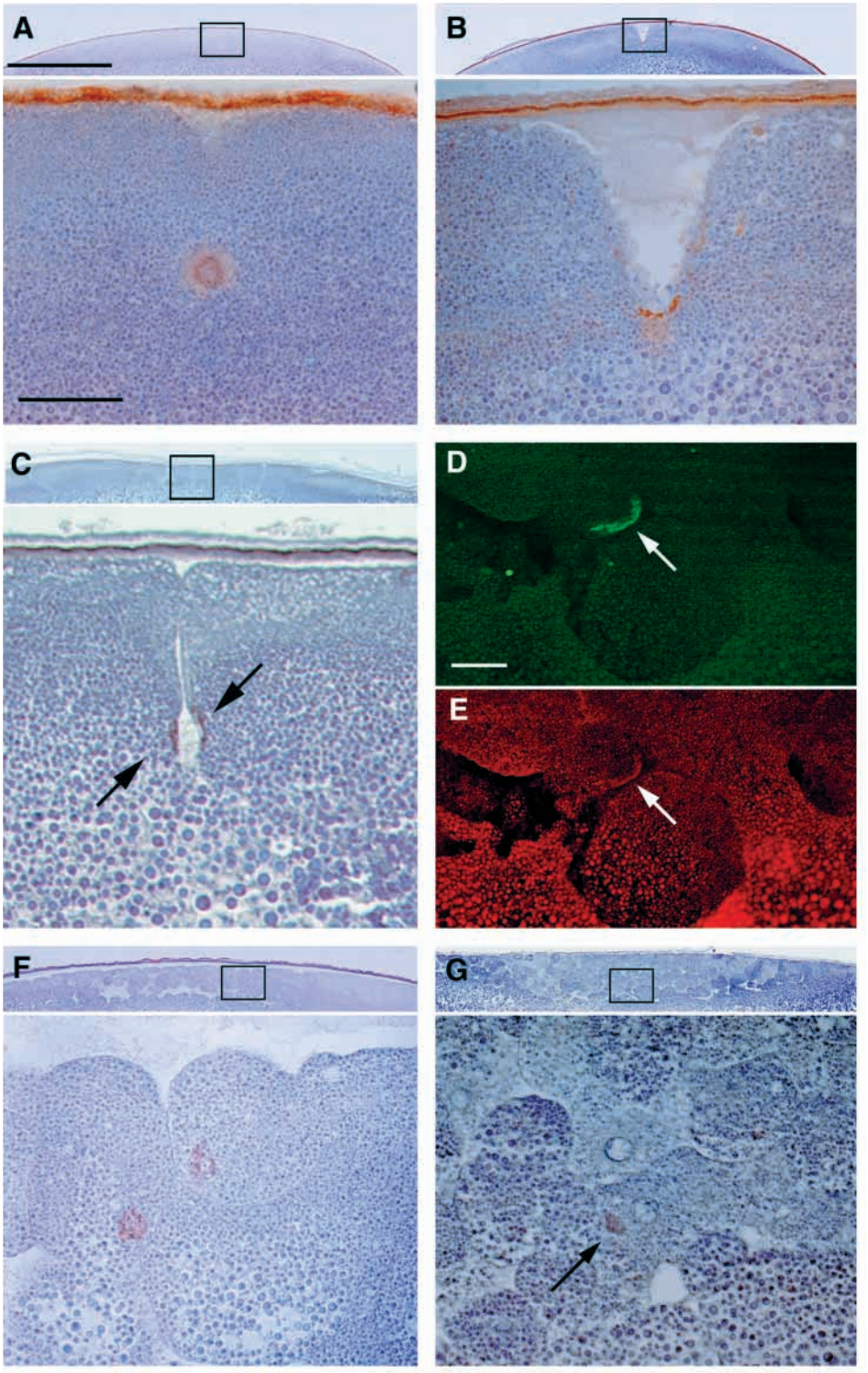

Fig. 8. Immunohistochemical identification of CVH-positive cells in early cleavage blastoderms. Sections of embryos were stained with anti-CVH, using enzymatic colorization by HRP-conjugated secondary antibody: the first cleavage stage (A), the second cleavage stage (B), the stage II-III consisting of 15 open and closed cells (C), the stage IV consisting of approximately 300 cells (F) and the stage V consisting of approximately 600 cells (G). Lower photographs in A-C, F and G are high magnification views of the boxed zones indicated in upper ones. A section of the stage III embryo was stained with anti-CVH using Oregon Green-conjugated secondary antibody (D), and simultaneously stained with MitoTracker-Red (E). The view showed the localization of CVH-protein in an asymmetrically dividing cell undergoing horizontal cell division. Bars, 50 μm.

homolog gene which could be used as a reliable molecular marker for investigating avian germ cell lineages. The *Cvh* gene, one of three DEAD-box family genes identified in this study, showed the highest identity to *vasa* homolog genes in vertebrates, the deduced amino acid sequences of which contain eight conserved motifs essential for ATP binding, ATP hydrolysis, RNA binding and RNA unwinding (Schmid and Linder, 1991; Pause and Sonenberg, 1992; Pause et al., 1993). Moreover, CVH protein shares several common characteristics with vasa family proteins, namely Trp (W), Glu (E) and Asp (D) residues near the start and stop codons, and multiple repeats of the RGG (Arg-Gly-Gly)-box, which is a putative RNA-binding motif (Kiledijian et al., 1992), in the N-terminal domain, while most of the sequences are RGR, RGA and RGP.

In the chicken, until recently the earliest identification of the presumptive PGC population has been in stage X embryos just prior to primitive streak formation, which are recognized with EMA-1 and SSEA-1 monoclonal antibodies (Karagenç et al., 1996). Karagenç et al. (1996) detected about 20 SSEA-1 (EMA-1)-positive cells at stage X on the ventral surface of the epiblast in association with other polyingressing cells, and later on the dorsal surface of the hypoblast. In the present study, we detected approximately 30 CVH-positive cells scattered in the central zone of the 1-cell-thick area pellucida at stage X, about 45-60 positive cells later at stage 2-3 on the hypoblast layer, and 200-250 positive cells at stage 4 located finally anteriorly in the germinal crescent as a consequence of morphogenetic movement. These developmental kinetics of CVH-positive cells are totally consistent with those of SSEA-1-positive cells and PAS-positive cells observed in cultures of microdissected blastodisc fragments (Ginsburg and Eyal-Giladi, 1987, 1989) and also consistent with the results from generating germline chimeras by transplantation of blastodermal cells (Kagami et al., 1997). Moreover, it is known that in vitro culture of dispersed stage X blastodermal cells gave rise to PGCs which were similar in number and cytological features to in vivo PGCs

localized in the germinal crescent. A similar developmental change in a culture was observed in a population of CVH-expressing cells (Fig. 5D,E). Taken together, these findings indicate that cells expressing CVH protein are indeed PGCs or their precursors.

The SSEA-1 staining does not recognize any cells in intrauterine embryos prior to stage X. The epitope is a carbohydrate (galactose-N-acetylglucosamine-fucose; Gooi et al., 1981) and is expressed in inner cell mass cells, epiblastic cells and migratory PGCs in mice, indicating that the antigen is not germline-specific. The absence of SSEA-1-positive cells in intrauterine chicken embryos, therefore, cannot preclude the presence of cells committed to germ cell lineage. In fact, we found a small population of cells containing CVH protein in early cleavage embryos. At the first cleavage, CVH protein was found in spherical structures underneath the cleavage furrow. This subcellular localization is strikingly similar to that of vasa homolog (*vas*) transcripts in 2- and 4-cell-stage zebrafish embryos (Yoon et al., 1997; Olsen et al., 1997) and that of the germ plasm detected by immunostaining of spectrin in early cleavage *Xenopus* embryos (Kloc et al., 1998). These may imply that the structure containing vasa family gene products is involved in the organization of microtubule tracks regulating proper distribution of maternal RNAs to germline cells.

CVH-containing structures were found in only 6 cells among approximately 300 blastomeres at stage IV. Therefore there are presumably less than 6 founder cells of germ precursors, although we cannot specify the number because meroblastic cleavage prior to stage IV occurs in an incomplete and asynchronous manner. Thereafter CVH-containing cells increased to about 30 cells in stage X embryos, which consisted of approximately 3×10^4 cells, indicating that they divided approximately twice during 6-7 divisions of meroblastic cleavage. At present, we do not know whether this apparently three times longer period of doubling is solely due to the low proliferation activity of CVH-containing cells or partially due to the asymmetric distribution of CVH-containing structures.

It has been shown that in vitro cultures of dispersed blastodermal cells of stage IX embryos yield no SSEA-1-positive PGCs while stage X blastodermal cells yield 20-40 PGCs under the same culture conditions (Karagenç et al., 1996), suggesting a requirement for an epigenetic process for PGC differentiation. Taken together with our results, it is conceivable that cellular organization during formation of the area pellucida is required for the survival and/or differentiation of CVH-positive presumptive PGCs prior to stage X, and that PGCs after emerging from the area pellucida acquire an independent developmental potency as described by Karagenç et al. (1996). In this connection, it has been reported that chicken pluripotential blastodermal cells can be long-term cultured in an LIF-dependent manner (Pain et al., 1996). As some of the pluripotential blastodermal cells exhibit a germline contribution in chimeras when they are grafted into recipient stage X embryos, they seem to originate from PGCs. However, the fact that long-term culture of the blastoderm-derived cells causes a remarkable loss of the germline contribution indicates that PGCs gradually lose their developmental potency under immortalizing conditions. In mice, embryonic germ (EG) cell lines, which exhibit developmental pluripotency similar to ES cells, are established from migratory and gonadal PGCs (Matsui et al., 1992; Labosky et al., 1994), but most of them lose the ability to express mouse vasa homolog (MVH) protein (Fujiwara et al., 1994). Therefore, to establish a chicken ES (EG) system for molecular genetic approaches, further improvement is required to maintain or recover the ability of PGCs. CVH expression will provide the best reliable marker to develop such a novel system.

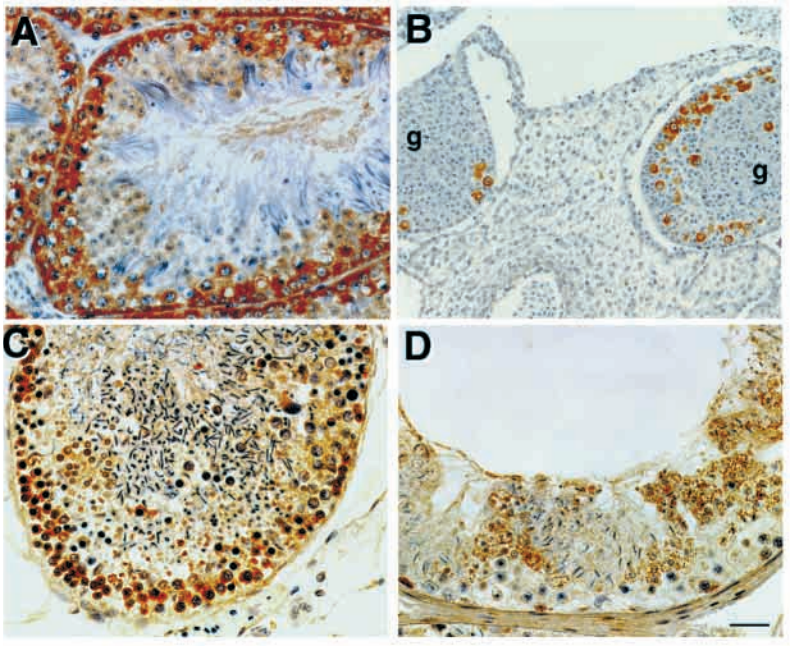

Fig. 9. Anti-CVH staining on testes sections of other animals. Sections of quail (*Coturnix coturnix japonica*) adult testis (A), quail embryonic gonad at 6 days after incubation (B), turtle (*Pelidiscuc sinensis*) adult testis (C) and snake (*Trimeresurus flavoviridis*) adult testis (D) were immunostained with anti-CVH as in Fig. 3. g, embryonic gonad. Bar in D for A-C, 50 μm.

In the present study, we have shown that CVH protein is localized in globular structures in chicken oocytes, which contain the mitochondrial cloud and spectrin-rich materials. Similar characteristic structures are immunostained with anti-CVH in the germinal disc at the first cleavage stage. In *Xenopus*, it has been found that spectrin is localized in the mitochondrial cloud during oogenesis and in the germ plasm during early embryogenesis, indicating that spectrin is involved in the organization of germ plasm (Kloc et al., 1998). As an analogy to frog germ plasm, it is most likely that CVH-containing structures in early embryos and oocytes are chicken germ plasm and its precursor materials. However, CVH localization in fertilized eggs still remains to be resolved. Anti-CVH staining of serial sections of the germinal disc region of fertilized eggs showed no distinct structure; therefore, it is unclear whether CVH protein is uniformly distributed in the germinal disc or is localized outside of the germinal disc, such as within Pander nuclei or beneath the plasma membrane. Moreover, in order to demonstrate that the CVH-containing materials are required for the formation of chicken germ cells as for the germ plasm in other animals, examination of the effects of removal or transplantation of the specialized

cytoplasm and/or molecular genetic approaches to disrupt the function of the *Cvh* gene will be needed; however, at present, various technical difficulties impede these studies.

From an evolutionary point of view, it is of great interest that anti-CVH can recognize germline cells in other avian species and reptiles. Previous studies have found that PGCs in snakes and in some lizards are found in the anterior germinal crescent and later migrate toward the gonads by a vascular transfer pathway, as in the case of chicken. In contrast, in turtles and in other species of lizards such as some *Lacerta*, PGCs locate at the posterior part of the primitive streak and their interstitial migration is similar to that in mammals (Hubert, 1969; Fujimoto, 1979). Interestingly, PGCs in *Sphenodon* locate and migrate in both manners (Tribe and Brambell, 1932). It seems possible that the difference in localization and subsequent migration of PGCs may be due to the timing of PGC allocation. Thus, further comprehensive study of *vasa*-expressing cells in each animal species will provide new insights into evolutionary changes in germ cell development during the diversification of reptiles and birds and, in turn, of all other vertebrates.

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