Primordial Germ Cell Development in the Chicken *Gallus gallus domesticus*

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Abstract

Germ cells are unique in that they are the only cells that undergo meiosis and pass their genetic information to the next generation. These cells develop from primordial germ cells (PGCs). While the physical aspects of PGC development are understood, there are currently gaps in our knowledge about the molecular processes that drive PGC development. This research focussed on investigating such molecular mechanisms in chicken. In particular its aims were to (1) identify chicken homologues of genes known to be involved in PGC development in other species and (2) develop an expression profile for each gene identified.

Gene homologues were identified by carrying out BLAST searches of the chicken genome and EST databases. Following identification, an expression profile of each gene was obtained using RT-PCR, Northern and *in situ* hybridisation analyses carried out on embryos at stages of embryonic development which PGC specification; PGC migration to the gonads and PGC development in the gonads occurred. Analyses focused on the genes *bruno*, *germ cell-less*, *oct4*, *mago nashi*, *nanog*, *nanos*, *piwi*, *pumilio* and *staufen* and aimed to determine (1) whether gene expression was comparable to the expression of homologues in other species, (2) whether the gene was expressed in areas known to contain PGCs, (3) whether the gene was expressed specifically in PGCs and (4) whether the gene could have a potential role in PGC development.

Of the genes chosen to be investigated, homologues of *bruno*, *germ cell-less*, *mago nashi*, *piwi*, *pumilio* and *staufen* were identified in the genome and in EST databases.

No *nanos* homologues were identified by this method. Homologues of *oct4* and *nanog* were identified by Dr B.Pain who provided sequence data for both genes. Analyses showed that the expression of all the genes were comparable to expression patterns in other species. *Nanog* and *oct4* were expressed in a pattern that indicated germ cell specific expression. This lead to additional *in situ* hybridisation, PGC transfer and qPCR analyses at later stages of development. These data showed how expression of *oct4* and *nanog* changed in relation to (1) the loss of PGC motility in males and females and (2) the onset of meiosis in females. Of the remaining genes, *germ cell-less* and *piwi* were detected specifically in adult testes, whereas the rest were detected in areas of the embryo known to contain PGCs. However, the gene expression was not PGC specific, which means that although the conserved expression patterns suggest possible roles in PGC development, further research would have to be carried out to confirm this.

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CHAPTER 1

INTRODUCTION

Reproduction is essential for the continuation of a species. The male sperm and female eggs are known as germ cells, which descend from primordial germ cells (PGCs). PGCs are the most primitive undifferentiated sex cells and in normal development will only produce germ cells. PGCs are larger than somatic cells with an approximate diameter of between 14 and 22 microns. They have a clearly defined nucleus that is surrounded by a definite membrane. In addition to being morphologically unique, PGCs have molecular components that are specifically found in germ cells. For example, *vasa* is a germ cell specific gene and stage specific embryonic antigen-1 (SSEA-1) is a lactoseries oligosaccharide antigen that is expressed on the surface of pluripotent cells. Detecting the presence of *vasa* mRNA, Vasa protein, or SSEA-1 are all well established methods for identifying germ cells during development and shall be referred to later.

In this section the mechanisms of PGC development and the genes involved I the mechanisms will be discussed. In section 1.1, the mechanics of the two model mechanisms of PGC development, epigenesis and preformation will be described. In section 1.2, these two mechanisms will be discussed in more detail with reference to *Drosophila melanogaster*, zebrafish, mice and chicken. In section 1.3, the genes involved in the mechanisms of PGC development will be briefly introduced, before describing the reasons for choosing particular genes for investigation in the chicken in section 1.4.

1.1 Epigenesis and preformation

In all species studied, germ cells develop by epigenesis or by preformation. Preformation is characterised by the formation of a substance in the early embryo known as germ plasm. Epigenesis is characterised by the absence of germ plasm.

1.1.1 Preformation

The main characteristic of preformation is that the oocyte inherits maternally supplied proteins and mRNAs, which are localised in the oocyte. The maternally supplied proteins and mRNAs that contribute to the germ line are collectively known as germ plasm. Germ plasm is not formed in the early embryo but in the germ cells (oocytes) of the previous generation. The number of species whose germline develops by preformation is considerable and includes *Drosophila*, *Caenorhabditus elegans*, zebrafish and *Xenopus*. Of these species, the most comprehensive data set on the molecular mechanisms of germ cell specification is that available for *Drosophila melanogaster* (Extavour and Akam, 2003). Whilst in the ovary, maternal proteins and RNAs are transported from the nurse cells of an egg into the oocyte. Some maternal factors are assembled at the posterior pole of the oocyte before fertilisation and form pole plasm, a germ cell specific substance (reviewed by Mahowald, 2001). Experiments that either transplant or force assembly of pole plasm in ectopic sites causing the induction of ectopic pole cells have demonstrated that pole plasm is a true germ cell determinant (Illmensee and Mahowald, 1974; Illmensee and Mahowald, 1976; Illmensee *et al.*, 1976; Ephrussi and Lehmann, 1992). After fertilisation, four to five pole cells are formed at the posterior pole,

which acquire PGC identity through the inheritance of pole plasm (Huettner, 1923; Mahowald, 2001).

Caenorhabditis elegans (*C. elegans*) provides a second example where germ cells are specified by preformation. In *C. elegans*, the embryos contain electron-dense granules called P granules. P granules are equivalent to pole plasm. Before and just after fertilisation, P granules are scattered evenly throughout the cytoplasm. During pronuclear fusion the P granules move to the posterior of the embryo (Hird *et al.*, 1996). During the next four cell cleavages, the P granules are asymmetrically segregated until all of them are found in the small P4 blastomere of the 16-cell embryo (Deppe *et al.*, 1978; Strome and Wood, 1982). The P4 blastomere is then recognised as a PGC. This sequence of events is the same in other nematode species studied, although there are differences in the timing of P4 formation relative to total embryonic developmental time (Extavour and Akam, 2003).

The first experimental evidence of preformation and the role of germ plasm in vertebrate germ cell specification was found in *Xenopus laevis* (Bounoure, 1939). During *Xenopus laevis* oogenesis, specialised cytoplasm characterised by an accumulation of mitochondria, is synthesized and localized to the vegetal subcortex and is known as the mitochondrial cloud. The mitochondrial cloud associates with electron-dense granules, specific proteins and RNAs (Heasman *et al.*, 1984; Houston and King, 2000; Kloc *et al.*, 2001; Kloc *et al.*, 2002; Zhou and King, 1996). Following fertilisation, the vegetal plasm, containing the mitochondrial cloud, forms patchy aggregates that segregate unequally into cleavage cells and finally accumulate

specifically in cells that become PGCs (Whitington and Dixon, 1975). Experiments that compromise the vegetal plasm have confirmed that the vegetal plasm contains germ cell determinants (Nieuwkoop and Suminski, 1959; Smith, 1966; Buehr and Blackler, 1970; Ikenishi *et al.*, 1974; Tanabe and Kotani, 1974; Züst and Dixon, 1975; Ikenishi *et al.*, 1986). Preformation is also the mechanism that is used for germ line specification by all other anuran amphibians that have been studied (Extavour and Akam, 2003).

These examples have demonstrated that the key characteristic of preformation is the localisation of molecular determinants in the oocyte. These factors are both necessary and sufficient for the formation of the germline, and cells that inherit these determinants will develop into germ cells.

1.1.2 Epigenesis

The time and site of origin of mammalian germ cells was a controversial issue for many years because germ plasm components could not be identified in the oocyte or early embryo. In embryos at 6.5 days post coitum (dpc), Lawson and Hage (1994) used lineage tracing studies and found that PGCs arise from the proximal part of the epiblast. In embryos between 7 and 7.5 dpc, alkaline phosphatase activity identified PGCs in the extraembryonic mesoderm (Ginsburg *et al.*, 1990). Even with the isolation of mouse *vasa* homologue, pre-PGCs could not be identified in earlier stages of mouse development. Although mouse *vasa* homologue protein is expressed in oocytes, it is not localised to a specific subcellular region, and no germ plasm is formed (Toyooka *et al.*, 2000). Instead, a true epigenetic mechanism for germ line

specification has been demonstrated by both descriptive and experimental evidence (Tsang *et al.*, 2001).

The only other evidence for inductive germ cell specification has arisen from studies on urodele amphibians. In many urodele species, germ cells are first identified in the lateral plate mesoderm (LPM; Humphrey, 1925; Humphrey, 1929; Ikenishi and Nieuwkoop, 1978). Explant and grafting experiments have shown that cells in the LPM are induced to form PGCs (Nieuwkoop, 1947). Recent studies in the axolotl *Ambystoma mexicanum* have confirmed that both a mitochondrial cloud and localised molecular determinants are absent in oocytes of this organism (Johnson *et al.*, 2001) The products of germ cell-specific genes, such as *Dazl* and *vasa,* are not localised in the oocytes or early embryos of this axolotl, and are not zygotically transcribed in PGCs until they approach the gonadal ridges (Johnson *et al.*, 2001; Johnson *et al.*, 2003). Although no data are available yet on the molecular nature of the endodermal signal that induces PGC and LPM differentiation in urodeles, BMP4 is known to induce ventral mesoderm in *X. laevis* (Dale *et al.*, 1992; Jones *et al.*, 1992), and it is therefore possible that this signal plays a role in axolotl PGC specification.

These examples have demonstrated that in epigenesis, instead of inheriting localised molecular determinants PGCs are induced from a population of germ cell competent cells. In comparison to PGCs that develop by preformation, during epigenesis PGCs are induced relatively late in development and prior to the point of induction there is no indication of PGC development.

1.1.3 Chicken primordial germ cells: preformation or epigenesis?

The origin of avian primordial germ cells is still a contentious issue. The main school of thought is that avian PGCs develop by preformation. In this section, the evidence for the preformation hypothesis will be discussed.

In 1880, Nussbaum first proposed the preformation thesis in birds (Callebaut, 2005). The characteristic of preformation is that the oocyte inherits maternally supplied proteins and mRNAs. Although the theory has been around for a long time, the first experimental evidence for preformation in the chicken came in the 1960's. Vakeat and Dubois concluded independently of each other that avian PGCs are derived from the deep layer of the blastodisc, known as the endophyll (Vakeat, 1962; Dubois, 1967 and 1969). Vakeat (1962) suggested that there are presumptive primordial germ cells (pPGCs) which are morphologically indistinguishable from the surrounding somatic cells in the peripheral deep rim of the area pellucidae. These pPGCs divide mitotically to produce one somatic cell and one PGC. Karagenç *et al* (1996), cultured disc fragments from stages VII-IX (EG & K) and found that the PGCs originate from the centre of the blastodisc. They found that PGCs could be derived from blastodiscs at stage VII (EG & K; Karagenç *et al*, 1996). They also found that the cultured disc fragments always generated a partial *area pellucidae* and suggested that the formation of the *area pellucidae* is a requirement for normal PGC development. As a consequence of this, they could not conclude that there is a population of cells committed to the germ cell lineage because PGCs could be

induced by the partial *area pellcidae*. To date the main evidence for suggesting that PGCs develop by preformation in avian species comes from Tsunekawa *et al* (2000). Tsunekawa *et al* (2000) identified and isolated a chicken *vasa* homolog (cvh). *Vasa* is germ cell specific mRNA and protein that has been identified in many species. In *Xenopus* oocytes, the cytoskeletal protein spectrin is co-distributed with the mitochondrial cloud, which has been implicated in the assembly and formation of germ plasm (Kloc *et al.*, 1998). Using antibodies to detect CVH protein and spectrin, Tsunekawa *et al* (2000) found that both CVH and spectrin proteins were co-localised in a characteristic globular shape in the mitochondrial cloud of oocytes. In pre-streak embryos, CVH positive cells were found in the centre of the blastodisc, supporting the data from Karagenç *et al.* (1996). Later in development, they found that CVH protein was germ cell specific as in other species. This data suggests the presence of germ plasm in chicken oocytes. Since the data on CVH protein was published, no further germ plasm components have been identified in the chicken. Therefore, although the data on CVH protein indicates that germ plasm is present in the chicken and that avian germ cells develop by preformation, the hypothesis is based mainly on the results from one paper and until further germ plasm components are identified the mechanism of PGC development in the chicken will remain controversial.

1.2 Primordial germ cell development in the model organisms *Drosophila melanogaster*, *Danio rerio*, *Mus musculus* and *Gallus gallus*

Observations of a wide range of animal groups allow us to define two classes of animals with regards to primordial germ cell (PGC) specification: animals whose PGCs develop via a preformation model and animals whose PGCs develop via an epigenetic model (see Section 1.1; Okada, 1998). In most organisms, PGCs are set aside from somatic cells early in development. It is hypothesised that this is to prevent possible modification to germinal DNA and cell differentiation (Rongo *et al.*, 1997). The germ cells migrate from their site(s) of specification, through the developing embryo to the mesodermal cell populations that contribute the somatic component of the gonad. In the gonad specific interactions between germ cells and soma regulate sex-specific development and differentiation into egg and sperm. Eventually germ cells undergo meiosis, a germ cell-specific cell cycle.

This section reviews what is known about the mechanism of PGC development in fruit flies (*Drosophila melanogaster*); zebrafish (*Danio rerio*); mice (*Mus musculus*) and chickens (*Gallus gallus*). First, section 1.2.1 discusses the mechanism of PGC development in *Drosophila*. The most comprehensive data set on the molecular mechanisms of germ cell specification is that available for *Drosophila melanogaster*, and orthologues of many of the *Drosaphila* genes have been found in other species, including vertebrates (Extavour and Akam, 2003). Second, section 1.2.2 outlines the mechanism of PGC development in zebrafish. Zebrafish are recognised as a lower vertebrate species, and so are closer relations to the chicken than *Drosophila* are.

Therefore, zebrafish act as a 'midpoint' reference species to this research. Third, section 1.2.3 reviews the mechanism of PGC development in mice. Mice are classed as higher vertebrates, and as such are evolutionarily more advanced than chickens. Finally, section 1.2.4 presents what is known about the mechanism of PGC development in chickens. From these sections the similarities between the mechanisms of PGC development in diverse species will become apparent, demonstrating how the results from other species can be used to inform investigations in the chicken.

1.2.1 Primordial germ cell development in fruit flies

In *Drosophila*, germ cells, which are known as pole cells, form via a preformation mechanism from a specialised, maternally supplied cytoplasm called pole plasm. Pole plasm is assembled at the posterior pole of the egg during oogenesis when maternal factors, supplied by nurse cells, are localised at the posterior pole of the growing oocyte (Mahowald, 1962). Piwi, Oskar, Vasa, Tudor, and Aubergine proteins are all essential for the assembly of the pole plasm (Hay *et al.*, 1988; Lasko and Ashburner, 1988; Golumbeski *et al.*, 1991; Ephrussi and Lehmann, 1992; Smith *et al.*, 1992; Kobayashi *et al.*, 1993; Harris and Macdonald, 2001; Megosh *et al.*, 2006). Pole plasm is incorporated into pole cells when they form at the syncytial blastoderm stage, adjacent to where the anlage of the posterior midgut form later in development (Bownes, 1975). As soon as the pole cells are formed, transcription is actively repressed (Zalokar and Erk, 1976; Schaner at al., 2003; Martinho *et al.*, 2004). Four localised RNAs, *germ-cell-less* (*gcl*), *nanos* (*nos*), *pumilio* (*pum*) and *polar granule component* (*pgc*) are all thought to be involved in transcriptional

repression (Wang and Lehmann, 1991; Barker *et al.*, 1992; Gavis and Lehmann, 1994; Wang *et al.*, 1994; Murata and Wharton, 1995; Kobayashi *et al.*, 1996; Nakamura *et al.*, 1996; Lin and Spradling, 1997; Asaoka *et al.*, 1998; Forbes and Lehmann, 1998; Asaoka-Taguchi *et al.*, 1999; Deshpande *et al.*, 1999; Sonoda and Wharton, 1999; Chagnovich and Lehmann, 2001; Forrest and Gavis, 2003; Deshpande *et al.*, 2004; Martinho *et al.*, 2004). Transcriptional silencing of the pole cells is maintained during migration and it is not until 3.5 hours after egg laying that the first germ cell transcripts are detected (Van Doran *et al.*, 1998a; Martinho *et al.*, 2004).

Proper gonadogenesis requires correct migration of germ cells from the posterior pole towards the somatic gonadal precursors, followed by association of germ cells with somatic gonadal mesoderm (Rongo *et al.*, 1997). Pole cells are closely associated with the midgut primordia at the posterior pole. During gastrulation, as the germ band extends and the midgut primordia move along the dorsal side of the embryo and invaginate into the embryo, the germ cells are carried along with them into the inside of the embryo and collect in the posterior midgut. This initial step of migration is passive. After entering the embryo, the migratory process and subsequent gonad assembly becomes active and can be broken down into discrete functional steps (see Figure 1.1): (1) Migration of germ cells through the posterior midgut; (2) migration of germ cells along the dorsal surface of the midgut; (3) migration of germ cells away from the midgut; (4) association of the germ cells with somatic precursors; (5) alignment of the germ cells with somatic gonadal precursors; and (6) gonad coalescence (Moore *et al.*, 1998).

First, to migrate across the posterior midgut the midgut rearranges its structure; apical junctions dissolve in the blind end of the midgut, and intercellular gaps form through which the pole cells migrate (Callaini *et al.*, 1995; Jaglarz and Howard, 1995). The formation of these junctions and gaps is a unique property of the posterior midgut, and therefore, mutations of genes such as *serpent* and *huckebein* that affect posterior midgut formation also prevent pole cell migration out of the gut (Reuter, 1994; Warrior, 1994; Jaglarz and Howard, 1995). Second, after leaving the gut, pole cells migrate towards the dorsal surface of the midgut. Wunen and Wunen-2 localised on the ventral most side of the midgut repel the pole cells towards the dorsal side (Zhang *et al.*, 1997; Starz-Gaiano *et al.*, 2001; Burnett and Howard, 2003). Third, from the midgut, the pole cells actively migrate towards 3-Hydroxy 3-Methylglutaryl Coenzyme A (HMGCoAR), an attractive signal that is expressed by the gonadal mesoderm (Van Doren, 1998b). Finally, having reached the mesoderm, the pole cells associate with the somatic gonadal precursors, and the gonads are assembled (Cumberledge *et al.*, 1992; Warrior, 1994; Boyle and DiNardo, 1995; Greig and Adam, 1995; Boyle *et al.*, 1997; Broihier *et al.*, 1998; Moore *et al.*, 1998).

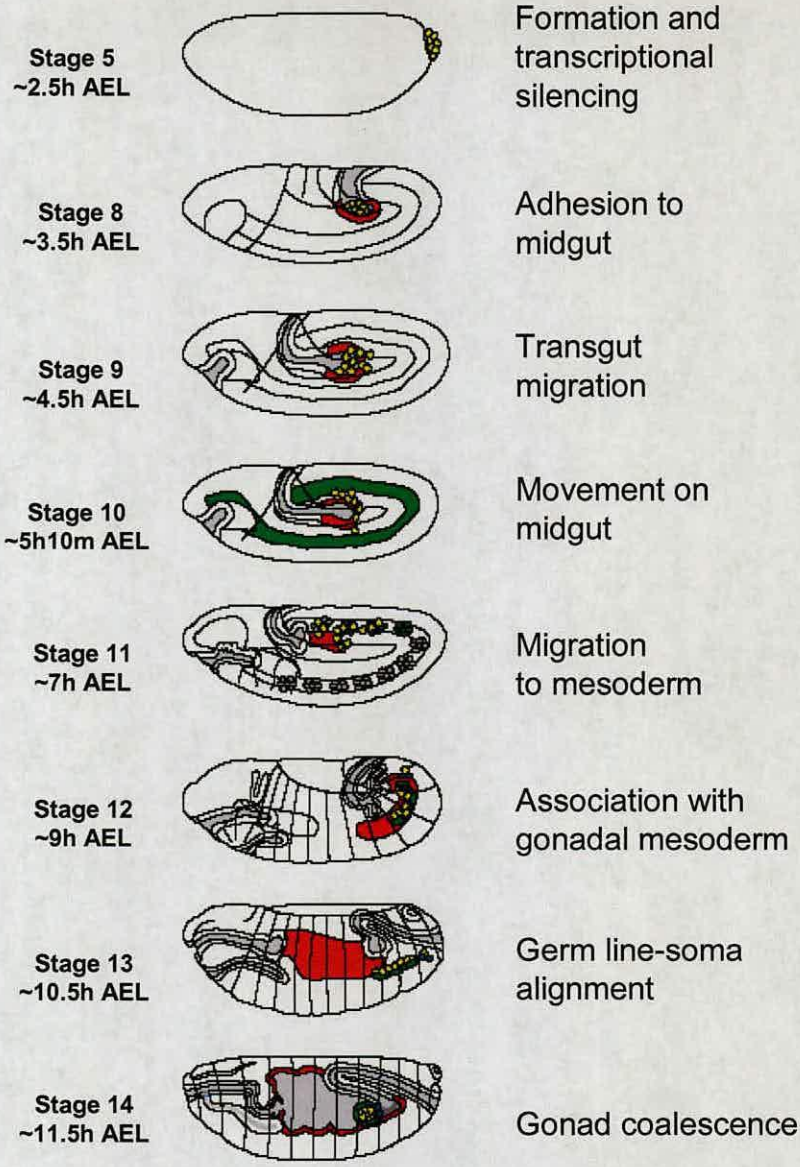


Figure 1.1: PGC specification and migration in *Drosophila melanogaster*. Schematic drawings of the *Drosophila* embryo with the anterior to the left and the dorsal to the top. Yellow: germ cells; red: midgut; green: mesoderm; blue: male specific gonadal precursors. AEL = after egg laying. Figure from Santos *et al.* (2004).

1.2.2 Primodial germ cell development in zebrafish

Zebrafish PGCs develop via a preformation mechanism. Germ cell determinants are found in the zebrafish oocyte. Molecular markers show localisation of maternal RNAs via the microtubule array to an electron dense region at the marginal position of the first cleavage plane (see Figure 1.2 A; Pelegri *et al.*, 1999; Knaut *et al.*, 2000). Transcripts that are known to be enriched in this region are *vasa*, *nanos 1*, *bruno-like*, *dazl* and *dead end* mRNAs, all known components of *Drosophila* pole plasm (Olsen *et al.*, 1997; Yoon *et al.*, 1997; Maegawa *et al.*, 1999; Pelegri *et al.*, 1999; Köprunner *et al.*, 2001; Weidinger *et al.*, 2003; Hashimoto *et al.*, 2004; Theusch *et al.*, 2006; Kosaka *et al.*, 2007). Although the germ cell specific mRNA *vasa* is localised to the germ plasm at the early stages of development, maternal vasa protein is found throughout the oocyte and does not become germ cell specific until later in development (Braat *et al.*, 2000; Knaut *et al.*, 2000). At the four-cell stage of development the germ plasm is found in four short strips along the first two cleavage planes (see Figure 1.2 B). The four strips of germ plasm become localised until each strip occupies one cell. The positions of the four germ plasm containing cells are determined by the orientation of the first two cleavages, which are random in relation to the future embryonic axes (Abdelilah *et al.*, 1994; Helde *et al.*, 1994). The germ plasm is maintained in four cells for the first four hours of development despite cell division occurring. This is achieved by asymmetrical inheritance of the germ plasm, where only one of the two dividing blastomeres inherits the germ plasm (Yoon *et al.*, 1997; Knaut *et al.*, 2000). The result of this is that the number of cells containing germ plasm does not increase for the first four hours of development (see Figure 1.2 A-D;). This changes at the sphere stage of development when the embryo consists of

approximately 4,000 cells. At this point the germ plasm spreads to the cytoplasm, so that when the cells divide both daughter cells inherit the germ plasm (Figure 1.2 E; Kimmel *et al.*, 1995; Knaut *et al.*, 2000). This transition in germ plasm distribution is the first time point at which PGCs can be phenotypically recognised (Raz, 2003). From the sphere stage the number of PGCs in each location increases from four to approximately 50. This results in the formation of four PGC clusters in an area away from where the gonads form and develop (Raz *et al.*, 2003). From their site of specification the PGCs make their way to the gonadal primordia, where they associate with somatic gonadal cells.

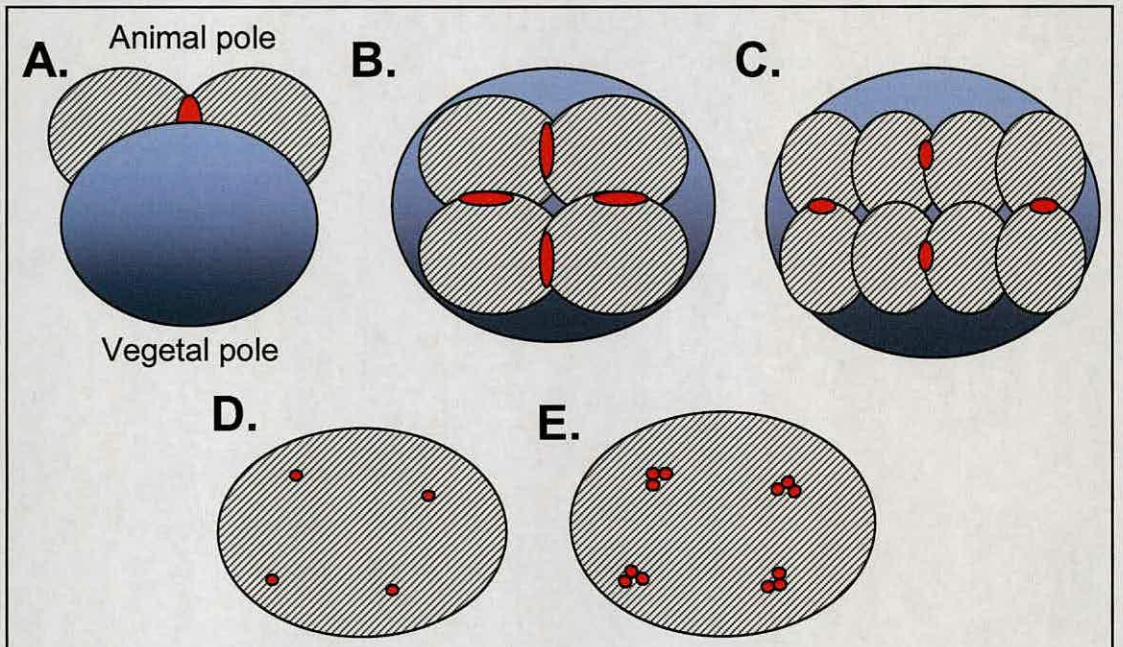


Figure 1.2: Schematic diagrams showing the localisation of maternal factors and formation of the germ plasm (red areas) in zebrafish. (A) two-cell stage, lateral view. (B) four-cell stage , animal view. (C) eight-cell stage, animal view. (D) 1K cell stage, animal view. (E) sphere stage, animal view. Modified from Raz (2003).

Shortly before beginning migration PGCs undergo a series of morphological alterations, following which they initiate motile behaviour (Raz *et al.*, 2006). To date, only *dead end*, a germ plasm component, is known to be essential to induce motile behaviour in zebrafish PGCs (Weidinger *et al.*, 2003). Unlike the migration of PGCs in the other species described here, zebrafish PGCs have no passive migratory step, and actively migrate from their site of origin to the developing gonadal primordia (Weidinger *et al.*, 1999 and 2003). Additionally, the PGCs are not all found in one location when migration starts, but in four clusters whose locations in the embryo are determined by the orientation of the first two cell cleavages, which are different in every embryo in relation to the embryonic axes. PGC migration starts at 60% epiboly as described by Weidinger *et al* (1999). The initial movement of the PGC clusters is towards the dorsal (see Figure 1.3 A). The clusters are then excluded from the midline of the embryo and migrate posteriorly down either side of the embryo, so that by 24hpf two clusters of PGCs level with the eighth somite are formed either side of the embryonic body (Figure 1.3 B and C).

Zebrafish PGC migration is driven by an attraction towards sources of the chemokine stromal-derived-factor-1a (SDF-1a; Doitsidou *et al.*, 2002). Only three factors are known to have a function for correct PGC migration in zebrafish, hydroxymethylglutaryl coenzyme A reductase (HMGCoAR), staufen-1 and staufen 2 (Thorpe *et al.*, 2004; Ramasamy *et al.*, 2006). When HMGCoAr activity is disrupted by atorvastatin, PGCs fail to align along the anterior and lateral borders of the trunk mesoderm and resulted in a delay in the PGCs populating the gaonads (Thorpe *et al.*, 2004). This suggests that inhibition of HMGCoAr activity leads to PGC migration

defects either by affecting the speed of migration or by impairing directional migration (Thorpe *et al.*, 2004). When the products of Staufen-1 and Staufen-2 are compromised *vasa* expression is lost, and although PGCs develop, they mis-migrate and eventually apoptose (Ramasamy *et al.*, 2006). Although these factors are known to have a function in PGC development, it is unknown what their specific functions are. As little is known about PGC development after migration is complete, it is currently not understood how the PGCs populate the gonads.

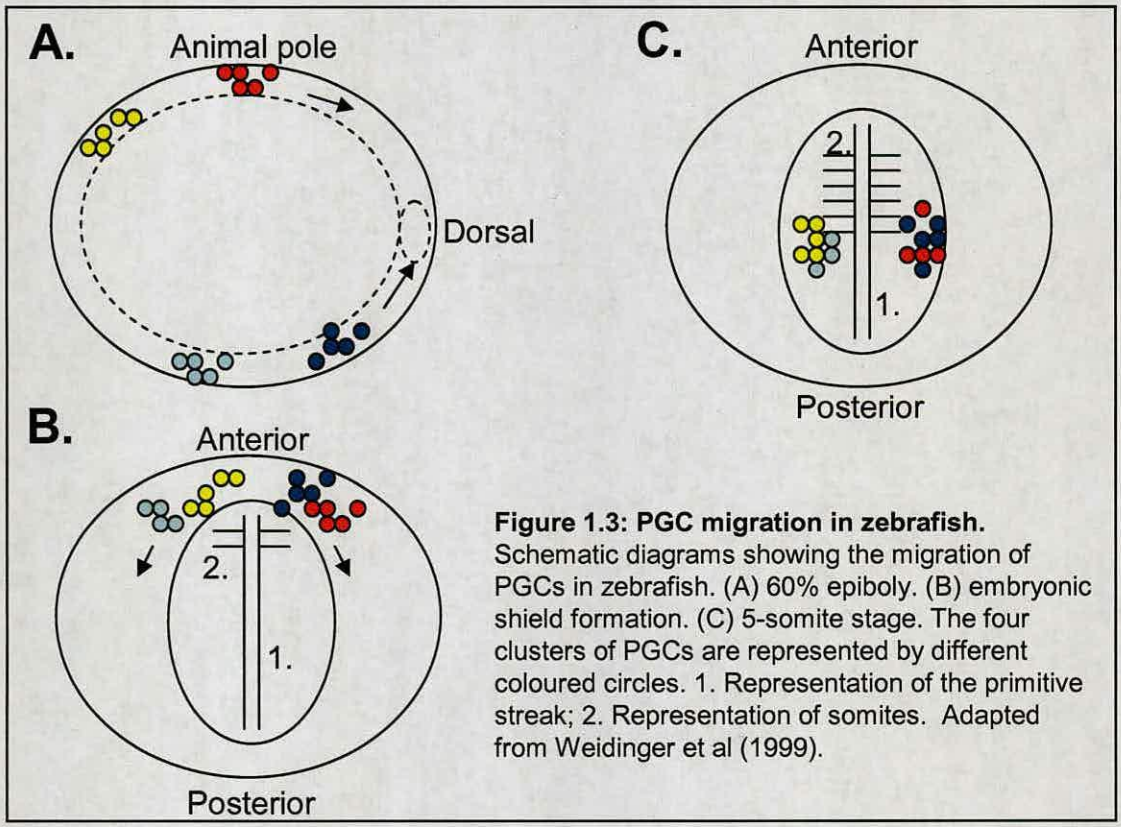


Figure 1.3: PGC migration in zebrafish. Schematic diagrams showing the migration of PGCs in zebrafish. (A) 60% epiboly. (B) embryonic shield formation. (C) 5-somite stage. The four clusters of PGCs are represented by different coloured circles. 1. Representation of the primitive streak; 2. Representation of somites. Adapted from Weidinger et al (1999).

1.2.3 Primordial germ cell development in mice

In mice, PGCs develop via an epigenetic mechanism. This is characterised by the absence of germ plasm in the early embryo: PGCs are induced later in development (Eddy and Hahnel, 1983; Gardner, 1977; Kelly, 1977). In mice, PGCs develop in the proximal region of the embryo. At 6.5dpc, when clumps of 5-20 cells of the distal epiblast, which would normally differentiate into ectoderm, were transplanted to the proximal region of a recipient embryo at the same stage, it was found that the grafted cells actually behaved like proximal cells and some differentiated into PGCs. This cell transplantation experiment indicated that environmental factors within the proximal region influence the fate of epiblast cells, and PGCs are induced by these factors (Tam and Zhou, 1996). It was found that prior to gastrulation, proximal epiblast cells are induced to form PGCs by three bone-morphogenetic-proteins (BMPs), members of the transforming growth factor β (TGFβ) family of secreted growth factors. Ectoderm derived BMP 4 and BMP 8b and endoderm derived BMP 2 combine to induce PGC specification (Lawson *et al.*, 1999; Ying *et al.*, 2000; Ying *et al.*, 2001; Ying and Zhao, 2001). The BMPs signal through the intracellular signal transducers SMAD1 and SMAD5, which by 6.0dpc are localised to the proximal region of the embryo (Matsui and Okamura, 2005). At 6.75-7.0dpc, the cell adhesion transmembrane protein E-cadherin is expressed in the proximal epiblast cells of the embryo. When E-cadherin is blocked, PGC specification is prevented, indicating that it has an important role during PGC specification (Okamura *et al.*, 2003). Clonal analysis was used to establish that germ cell fate is determined in a group of approximately 45 cells from 7.2 dpc, and PGCs are first detectable as alkaline phosphatase (ALP) positive cells at 7.25 dpc (Ginsburg *et al.*, 1990; Lawson

and Hage, 1994; McLaren, 2003). PGCs first emerge in the extraembryonic mesoderm at the posterior end of the primitive streak. At this stage PGCs are found in the centre of *fragilis* and *B lymphocyte-induced maturation protein-1* (*Blimp-1*) expression and they express the PGC specific gene *PGC7/stella* (Tunyaplin *et al.*, 2000; Saitou *et al.*, 2002; Ohinata *et al.*, 2005; Payer *et al.*, 2006). Within 24 hours of PGC specification, whilst *PGC7/stella* and *Blimp-1* expression is maintained in the PGCs, *fragilis* is downregulated and the cluster of PGCs starts to fragment (Saitou *et al.*, 2002). The fragmentation of the PGC cluster is important as in *Blimp-1* mutant embryos the PGC cluster fails to fragment, resulting in very few PGCs being able to start migration (Ohinata *et al.*, 2005).

At approximately 8.5dpc the hindgut forms in the endoderm and extends along the length of the embryo. PGCs are carried along with the endoderm cells, and once the hindgut is fully extended the PGCs lie along the ventral side of its entire length. PGCs move to the dorsal side of the hindgut, then into the body wall towards the notochord and the dorsal aorta, round the coelomic angle on each side of the embryo, and into the two forming genital ridges (McLaren, 2003; Molyneaux *et al.*, 2001). The active migration out of the gut is dependent upon the c-kit/Steel Factor signal transduction pathway and SDF-1 and its receptor CXCR4 (Mintz and Russell, 1957; Buehr *et al.*, 1993; Pesce *et al.*, 1993; Donovan, 1994; Molyneaux *et al.*, 2003). In *c-kit* mutant embryos, specification of the PGCs occurs normally, but at 8.5dpc PGCs form clumps along the length of the hindgut, and the majority fail to move to the dorsal side of the hindgut, suggesting an abnormality in PGC motility (Buehr *et al.*, 1993; Pesce *et al.*, 1993). PGCs in embryos carrying targeted mutations in CXCR4

are unaffected at earlier stages of development, and the PGCs migrate with the hindgut as normal, but the cells do not colonize the gonad normally. This indicates that the SDF-1/CXCR4 interaction is specifically required for PGC colonisation of the gonads, but not for earlier stages in germ cell migration. Germ cell counts at different stages suggest that both c-kit/Steel Factor and SDF-1/CXCR4 interactions also mediate germ cell survival (Pesce *et al.*, 1993; Molyneaux *et al.*, 2003). A number of other mutants interfere with correct PGC migration, including *β2 integrin* (Anderson *et al.*, 1999), *germ cell deficient* (Pellas *et al.*, 1991), *Fgf8* (Sun *et al.*, 1999), and *dead-end* (Youngren *et al.*, 2005; Bhattacharya *et al.*, 2007). However, it is not known when during the migratory process or how these genes function.

PGCs enter the genital ridges between 10 and 11dpc and are then known as gonocytes (McLaren, 2003). Once in the genital ridges the PGCs lose the ability to migrate and undergo a general reprogramming process (for a full list of transcriptional changes in PGCs after migration see Molyneaux *et al.*, 2004). They start to express new germ-cell specific genes including *mouse vasa homolog* (*MVH*), which is not expressed until this point, *germ cell nuclear antigen 1* (*Gcna1*) and *germ-cell-less* (*Gcl*) and downregulate genes such as *tissue non-specific alkaline phosphatase* (*TNAP*) and *Stage specific embryonic antigen-1* (*SSEA-1*; Enders and May, 1994; Kimura *et al.*, 1999; Toyooka *et al.*, 2000). The germ cells undergo two or three more rounds of mitosis, and at 12.5dpc both males and females enter a premeiotic stage and upregulate meiotic genes such as *Scp3* (DiCarlo *et al.*, 2000). In males, *Scp3* is then downregulated and the germ cells enter mitotic arrest as G0/G1 spermatogonia until after birth (McLaren, 1984). In females, germ cells

continue meiosis, before arresting in diplotene at about the time of birth (Upadhyay and Zamboni, 1982; McLaren, 1995).

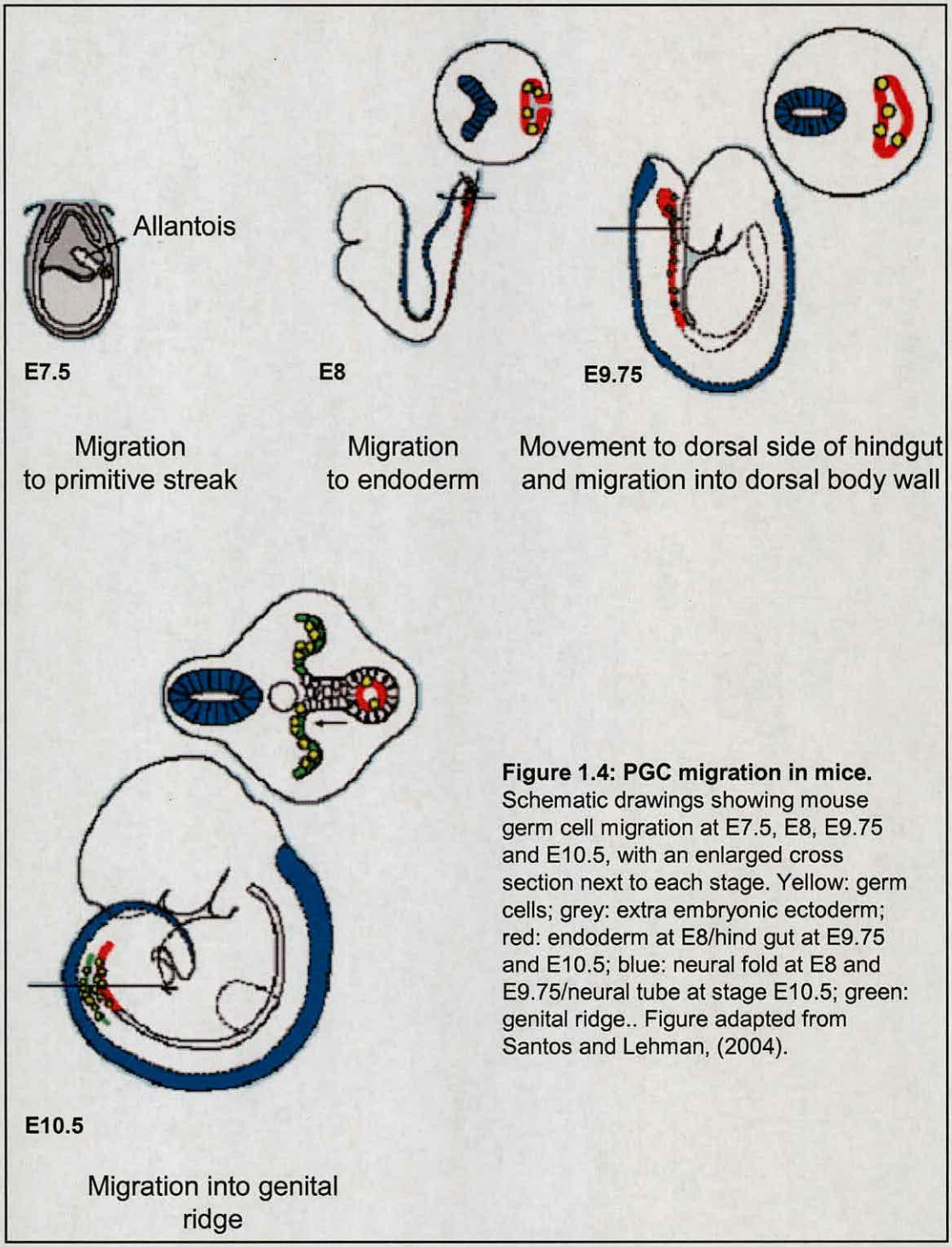


Figure 1.4: PGC migration in mice. Schematic drawings showing mouse germ cell migration at E7.5, E8, E9.75 and E10.5, with an enlarged cross section next to each stage. Yellow: germ cells; grey: extra embryonic ectoderm; red: endoderm at E8/hind gut at E9.75 and E10.5; blue: neural fold at E8 and E9.75/neural tube at stage E10.5; green: genital ridge.. Figure adapted from Santos and Lehman, (2004).

1.2.4 Primordial germ cell development in chickens

Although the chicken is a model organism for vertebrate development, remarkably little is known about the molecular mechanisms governing PGC development in comparison to the species described above. In chicken, PGCs develop via preformation. The evidence for the existence of germ plasm in the chicken has previously been discussed in section 1.1.3 where it was reported that only chicken vasa homologue (CVH) protein has been identified in the oocyte (see Figure 1.5 A; Tsunekawa *et al.*, 2000). The earliest stage of development that other germ cell determinants are known to exist is at stage VII (EG and K), when the blastodisc consists of approximately 60,000 cells. It was found that when a stage VII (EG and K) blastodisc is split into peripheral and central regions and each region is cultured separately, the majority of PGCs develop from the central region (Karagenç *et al*, 1996). Additionally, when the central region of a stage X blastodisc is removed and the embryos allowed to develop, the number of germ cells found is significantly reduced (Kagami *et al.*, 1997). These two experiments identify the presence of germ cell determinants in the centre of the blastodisc and cells containing these determinants are are known as PGC precursors (pPGCs).

At stage XI-XIV (EG and K) the embryo develops from a single layer into a two-layer blastula, consisting of an upper epiblast and a lower hypoblast separated by the blastocoel. The pPGCs translocate from the epiblast to the hypoblast (Eyal-Giladi *et al.*, 1981; Ginsburg and Eyal-Giladi, 1986; Urven *et al.*, 1988; Karagenç *et al.*, 1996). As the embryo develops from stage X-XIV (EG and K), the number of pPGCs increases from approximately twenty to seventy (Karagenç *et al.*, 1996). As

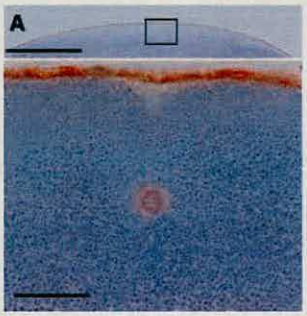
development continues the hypoblast containing the pPGCs is displaced anteriorly to the boundary between the area pellucida and the area opaca in a region known as the germinal crescent (Figure 1.5 B). At stage 4 (H and H), shortly after gastrulation, PGCs are identifiable in the germinal crescent lying free in the space between the ectoderm and endoderm (Romanoff, 1960).

From the germinal crescent the PGCs migrate via the embryonic blood system, which is formed as part of the mesoderm to the region where the gonadal primordia form (Kuwana, 1993). As the cells of the mesoderm grow forward between the ectoderm and the endoderm they surround the PGCs, blood islands begin to differentiate and the PGCs are incorporated into the newly formed blood vessels (Romanoff, 1960; Ukeshima and Fujimoto, 1984). At approximately 33 hours of incubation the embryonic and extraembryonic blood systems become continuous, PGCs begin to circulate in the blood system and enter the body of the embryo (Figure 1.5 C and D). Once in the body of the embryo, the PGCs pass through the walls of the capillaries on either side of the embryo body and migrate through the splanchnic mesoderm towards the epithelium and underlying stroma that will form the gonads (Figure 1.5 E; Romanoff *et al.*, 1960). The migration through the splanchnic mesoderm of the embryo is driven by an attraction to the chemokine stromal-derived-factor 1α (SDF 1α; Stebler *et al.*, 2004). Weidinger *et al* (2003) suggest that chicken *dead end* may have a similar function in chicken PGC migration as in zebrafish, but this has yet to be investigated. The PGCs migrate to the point where the gonadal primordia bud off from the surface of the embryonic kidneys, referred to as the mesonephroi or Wolffian bodies. At approximately 4.5 days of

incubation the gonadal primordia start to form on the surface of the mesonephroi and the PGCs are incorporated into the gonads (Figure 1.5 F). Despite initially colonising both gonads equally, the germ cells are found distributed unequally between the left and right gonads later in development, with a bias towards the left gonad (Zaccanti *et al.*, 1990). This is particularly apparent in females, and probably relates to the left ovary developing into a functional ovary, where as the right ovary degenerates. After the PGCs colonise the gonads migration continues between the right and left gonads (Swift, 1915).

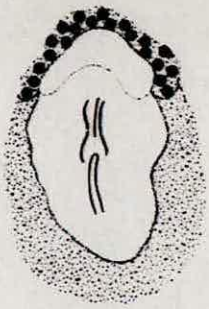
At around six days of incubation, the PGCs start to act differently depending on whether they are found in a male or a female environment. In females, the primary sex cords in the medulla of the left ovary degenerate (Ukeshima, 1996). At eight days of incubation PGCs in the medulla start to apoptose, whilst the PGCs in the outer cortical region multiply rapidly for four days (Swift, 1915; Ukeshima, 1996). After this period of division the PGCs are recognised as oögonia, and they enter meiosis between thirteen to fifteen days of incubation (Swift, 1915; Goldsmith, 1928). In males, after six days of incubation the primary sex cords, which eventually develop into the seminferous tubules, penetrate the inner medullary portion of the gonad (Romanoff, 1960). At thirteen days of incubation the PGCs multiply rapidly for four days, after which the PGCs are recognised as spermatogonia (Swift, 1916). Prior to hatching at approximately seventeen days of incubation the mitotic divisions slow down and the spermatogonia enter mitotic arrest (Van Krey, 1990).

A First cell cleavage



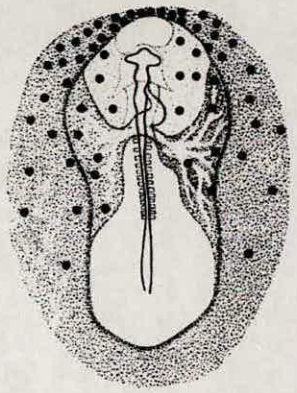
CVH protein is localised to the basal part of the first cell cleavage furrow.

B ~18 hours (stage 4)



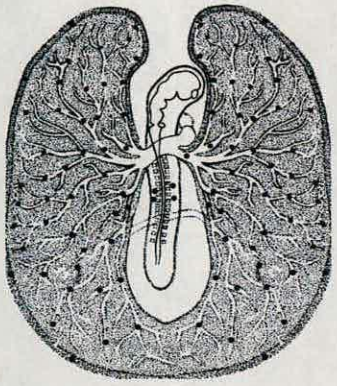
PGCs are first recognised in the germinal crescent.

C ~33 hours (stage 9)



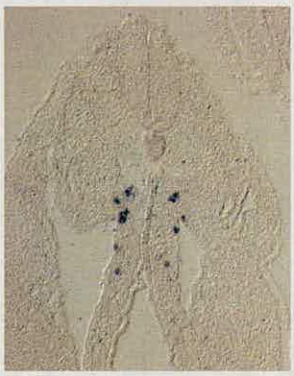
PGCs begin to migrate via the embryonic blood system.

D ~43 hours (stage 11)



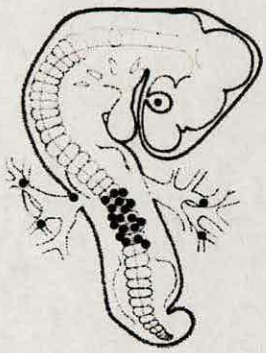
PGCs collect in the blood vessels of the splanchnic mesoderm and leave the blood stream.

E ~4.5 days (stage 18)



PGCs migrate through the splanchnic mesoderm towards the genital ridges.

F ~67 hours (stage 25)



PGCs populate the developing gonads, some are still migrating to their final destination.

Figure 1.5: PGC specification and migration in chicken. (A) Section through the blastoderm of a first cell cleavage embryo stained for CVH protein expression. (B-D and F) Schematic drawings of chicken embryos showing the positions of PGCs at different stages of development. (E) section through a stage 18 embryo stained for expression of *cPou5* mRNA. Pictures from Tsunekawa et al. (2000), Nieukoop and Satasurya (1979) and Chapter 8.

1.2.5 Similarities between diverse species

From the research described in these four sections it is possible to identify similarities in PGC development between all four species, despite their evolutionary divergence. In *Drosophila*, zebrafish and chicken in which the PGCs develop via preformation, germ plasm factors such as *nanos*, *vasa*, *bruno*, *dazl* and Piwi have been found in both *Drosophila* and zebrafish. However, parallel functions for these genes and proteins between the species have yet to be determined, and in chicken many of them still remain unidentified. Mammalian PGCs are specified later in development and so comparisons between mice and the other species described here cannot be drawn in relation to PGC specification. However, the mechanisms of migration and subsequent colonisation of the gonads can be compared between the four species discussed.

In the four species discussed the germ cells are set aside from the somatic cells, before migrating through the developing embryo to the mesodermal cell populations that contribute the somatic component of the gonad. In *Drosophila* and mice PGCs migrate into the gut and are initially carried by the gut as it enters the embryo and lengthens, before actively moving through the gut and migrating through the mesoderm to the developing gonadal primordia. In zebrafish, the whole migratory phase is active. In chicken, PGCs migrate into the developing blood system and are carried into the embryo where they migrate through the developing gut towards the gonadal primordial. In the three vertebrates, the SDF-1/CXCR4 interaction has a comparable function in attracting the germ cells towards their final destination, and *dead end* is also thought to have a comparable function in all three species.

HMGCoAR has been found to function similarly in zebrafish and *Drosophila*. However, it is the two most diverse species, *Drosophila* and mice, where the mechanism of PGC migration appears to be most similar. The description of PGC movement during gastrulation, the subsequent movement to the dorsal side of the gut and migration through the mesoderm is almost identical. Molecularly, G protein-coupled receptors in both species, Tre-1 in *Drosophila* and CXCR4 in mice, direct germ cell migration. Homologues of *wunen* and HMGCoAR have been identified in mice, but a function in PGC migration has not yet been investigated (Castellano *et al.*, 1994; Zhang *et al.*, 2000). Other similarities in the genes involved in PGC development will be discussed in the relevant chapters. What these reviews tell us is that the research on PGC development already carried out in other species can be used to direct research and support the results obtained in chicken.

1.3 Genes and germ cell development

The molecular mechanisms and the genes involved in germ cell development have been investigated in many species. Etavour and Akam (2003) produced a supplemental table that listed genes identified in germ cell development, the species the gene had been identified in as well as the function of the gene has in germ cell development if it is known. This table has been modified and reproduced here. The genes of interest to this research are highlighted in section 1.4 and will be covered in more detail in the relevant results chapter.

Table 1.1 (continued on next page): Genes known to be involved in PGC development (modified from Extavour and Akam, 2003)

KEY:

*Usually the name of the first gene in the family to be identified.

†Abbreviations for species names are as follows: A, *Ambystoma mexicanum* (axolotl); Aa, *Aurelia aurita* (moon jellyfish); Ad, *Acropora digitifera* (staghorn coral); B, *Bombyx mori* (silkworm); C, *Caenorhabditis elegans* (nematode); Ca, *Carassius auratus* (goldfish); Cb, *Cebus sp.* (capuchin monkey); Cc, *Cyprinus carpio* (carp); Ch, *Chironomous samoensis* (midge); Ci, *Ciona intestinalis* (ascidian); Cp, *Cynops pyrrhogaster* (newt); Cr, *Craspedacusta sowerbyi* (freshwater jellyfish); Cs, *Ciona savignyi* (ascidian); D, *Drosophila melanogaster* (fruit fly); Dd, *Dugesia dorotocephala* (flatworm); Dj, *Dugesia japonica* (flatworm); Dr, *Danio rerio* (zebrafish); Dv, *Drosophila virilis* (fruit fly); E, *Ephydatia fluviatilis* (sponge); Ec, *Equus caballus* (horse); G, *Gallus gallus* (chicken); Gd, *Gryllus domesticus* (cricket); H, *Hydra magnipapillata* (hydra); He, *Hydractinia echinata* (colonial hydroid); Hr, *Helobdella robusta* (leech); Hs, *Homo sapiens* (human); Hy, *Hyphessobrycon ecuadoriensis* (Columbian tetra); L, *Leucopsarion petersii* (ice goby); M, *Mus musculus* (mouse); Ma, *Macaca fascicularis* (crab-eating macaque); Md, *Musca domestica* (housefly); Mf, *Melanotaenia fluviatilis* (rainbowfish); Mm, *Macaca mulatta* (rhesus monkey); O, *Oryzias latipes* (medaka); Om, *Oncorhyncus mykiss* (rainbow trout); On, *Oreochromis niloticus* (Ukuobu); P, *Pantodon buchholzi* (butterfly fish); Pa, *Papio anubis* (baboon); Pt, *Pan troglodytes* (chimp); R, *Rattus norvegicus* (rat); S, *Schistocerca americana* (grasshopper); Sa, *Sanderia malayaensis* (Malaysian jellyfish); Sg, *Schistocerca gregaria* (locust); Sm, *Schmidtea mediterranea* (flatworm); Sp, *Sparus aurata* (gilthead bream); Sq, *Squalus acanthias* (spiny dogfish); Stp, *Strongylocentrotus purpuratus* (sea urchin); T, *Tetranychus urticae* (spider mite); Tf, *Tima formosa* (elegant jellyfish); X, *Xenopus laevis* (clawed frog).

‡Note that many homologues are not given new names, but may be called 'x-like gene', where x is the name of the first gene in the family to be identified.

§Species for which functional information is available are in parentheses.

Gene (common name)*	Species with homologues† (homologue names)‡						Gene product	Germ cell function§	References
	Fly (D)	Worm (C)	Frog (X)	Fish (Dr)	Mouse (M)	Other‡			
blimp-1				yes	yes	Stp	B lymphocyte induced maturation protein-1	Repression of a somatic programme (M)	(Chang *et al.*, 2002; Ohinata *et al.*, 2005; Wilm and Solnica-Krezel, 2005; Livi and Davidson, 2006; Wang *et al.*, 2007)
boule	yes		yes		yes	A (*Axdazl*), Cb, Hs (*DAZ*), Ma, Mm, Pt, Pa	RNP-type RNA binding protein with DAZ repeats	Meiosis; PGC differentiation (Hs, M, X)	(Eberhart *et al.*, 1996; Houston and King, 2000; Houston *et al.*, 1998; Johnson *et al.*, 2001; Ruggiu *et al.*, 1997; Venables *et al.*, 2001; Xu *et al.*, 2001)
aubergine	yes						Similar to eIFC2 (translation initiation factor)	Pole cell formation; translational regulation of *osk*	(Harris and Macdonald, 2001; Schüpbach and Wieschaus, 1991; Wilson *et al.*, 1996)
bruno	yes	yes	yes			Hs	RNP-type binding domains	Translational regulation of *osk* and *grk* (D)	(Castagnetti *et al.*, 2000; Filardo and Ephrussi, 2003; Knecht *et al.*, 1995; Timchenko *et al.*, 1996; Webster *et al.*, 1997)
capuccino	yes						Actin binding protein	*osk* and *stau* localisation in oocyte (D)	(Clark *et al.*, 1994; Emmons *et al.*, 1995)
dead end			yes	yes	yes	G	Novel protein	Required for germ cell motility	(Weidinger *et al.*, 2003; Horvay *et al.*, 2006 ; Aramaki *et al.*, 2007)
DEADSouth			yes				eIF4A-like helicase	Localised to germ granules (X)	(MacArthur *et al.*, 2000)
fragilis					yes		IFN inducible TM family member	Specific functions are unclear	(Saitou *et al.*, 2002; Lange *et al.*, 2003; Tanaka *et al.*, 2005)
germ-cell-less	yes	yes			yes		Nuclear pore associated protein	Transcriptional repression (D)	(Jongens *et al.*, 1992; Leatherman *et al.*, 2002; Robertson *et al.*, 1999)
gld-1		yes					KH motif RNA binding protein	Translational repression (C)	(Lee and Schedl, 2001; Schisa *et al.*, 2001)
gp130					yes		Cytokine receptor	Mutant has fewer PGCs (M)	(Koshimizu *et al.*, 1996)

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gurken	yes						EGFR ligand	Oocyte patterning and germ plasm assembly (D)	(Filardo and Ephrussi, 2003; Gonzalez-Reyes *et al.*, 1995; González-Reyes and St. Johnston, 1994; Neuman-Silberberg and Schupbach, 1993; Roth *et al.*, 1995; Styhler *et al.*, 1998; Tinker *et al.*, 1998; Tomancak *et al.*, 1998)
gustavus	yes						Novel protein	VAS localisation in oocyte	(Styhler *et al.*, 2002)
HMGCoA R	yes			yes	yes		Novel protein	An attractive signal expressed by gonadal mesoderm	(Van Doren, 1998b; Thorpe *et al.*, 2004).
homeless	yes						RNA-dependent ATPase	G plasm component localisation (D)	(Gillespie and Berg, 1995)
mago nashi	yes	yes	yes		yes	Hs	Novel protein	Germ plasm assembly (C, D)	(Li *et al.*, 2000; Mohr *et al.*, 2001; Newmark and Boswell, 1994; Newmark *et al.*, 1997; Zhao *et al.*, 1998)
mes-2		yes					Similar to *E(z)* (D polycomb gene)	Transcriptional repression (C)	(Capowski *et al.*, 1991; Garvin *et al.*, 1998; Holdeman *et al.*, 1998; Kelly and Fire, 1998)
mes-3		yes					Novel protein	MES-2 and MES-6 localisation (C)	(Garvin *et al.*, 1998; Holdeman *et al.*, 1998)
mes-4		yes					Novel protein	GC survival (C)	(Capowski *et al.*, 1991; Garvin *et al.*, 1998)
mes-6		yes					Novel protein	Transcriptional repression, MES-2 localisation (C)	(Capowski *et al.*, 1991; Garvin *et al.*, 1998; Holdeman *et al.*, 1998; Kelly and Fire, 1998)
mex-1		yes					Zinc finger protein	PIE-1 and P granule segregation (C)	(Guedes and Priess, 1997; Schisa *et al.*, 2001)
mex-3		yes					KN domain RNA binding protein	Blastomere identity; mutation leads to ectopic GCs (C)	(Draper *et al.*, 1996)
mtlrRNA	yes		yes				Mitochondrial ribosomal RNA	Localisation of mitochondrial ribosomes on P granules (D)	(Amikura *et al.*, 2001; Iida and Kobayashi, 1998; Kloc *et al.*, 2001; Kobayashi *et al.*, 1998; Kobayashi *et al.*, 1995; Kobayashi and Okada, 1989)
nanog					yes	Hs, G	Nanog homeoprotein	Maintains pluripotency	(Chambers *et al.*, 2003; Mitsui *et al.*, 2003; Clark *et al.*, 2004; Lavial *et al.*, 2007)

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nanos	yes	yes	yes	yes	yes	Ch, Dv, Gd, H (*Cnnos1*, *Cnnos2*), Hr (*Hrnos*), S, Md	CCHC Zn-finger protein	Translational and transcriptional repression (C, Ch, D, Dv, Md)	(Curtis *et al.*, 1995; Deshpande *et al.*, 1999; Forbes and Lehmann, 1998; Jaruzelska *et al.*, 2003; Kang *et al.*, 2002; Kobayashi *et al.*, 1996; Koprunner *et al.*, 2001; Lall *et al.*, 2003; Lehmann and Nusslein-Volhard, 1991; Mochizuki *et al.*, 2000; Mosquera *et al.*, 1993; Pilon and Weisblat, 1997; Sonoda and Wharton, 1999; Subramaniam and Seydoux, 1999; Tsuda *et al.*, 2002; Wang and Lehmann, 1991)
oct-4			yes		yes	Hs, G, Mm	DNA binding transcription factor	Maintains pluripotency	(Schöler *et al.*, 1990; Hinkley *et al.*, 1992; Mitalipov *et al.*, 2003; Lavial *et al.*, 2007)
orb	yes						RNA binding protein	*osk* localisation (D)	(Christerson and McKearin, 1994; Lantz *et al.*, 1992; Lantz *et al.*, 1994)
oskar	yes					Dv	Novel protein	Germ plasm assembly (D)	(Castagnetti *et al.*, 2000; Ephrussi and Lehmann, 1992; Kobayashi *et al.*, 1995; Lehmann and Nüsslein-Volhard, 1986; Markussen *et al.*, 1995; Webster *et al.*, 1994)
par-1	yes	yes			yes	Hs, R	Ser/Thr kinase	OSK phosphorylation, germ plasm assembly (C, D)	(Cox *et al.*, 2001; Doring *et al.*, 1993; Drewes *et al.*, 1997; Guo and Kemphues, 1995; Inglis *et al.*, 1993; Kemphues *et al.*, 1988; Riechmann *et al.*, 2002; Shulman *et al.*, 2000; Tomancak *et al.*, 2000)
pgc-1	yes						Non-coding RNA	PC migration (D)	(Nakamura *et al.*, 1996)
pie-1		yes					Zinc finger protein	Transcriptional repression (C)	(Mello *et al.*, 1996; Seydoux and Dunn, 1997; Seydoux *et al.*, 1996; Tenenhaus *et al.*, 2001)
pog					yes		Plant homeodomain motifs	PGC proliferation (M)	(Agoulnik *et al.*, 2002; Pellas *et al.*, 1991)
pumilio	yes	yes			yes	Hs (CUG-BP) S	Novel RNA binding domains	Translational repression (D, C)	(Barker *et al.*, 1992; Forbes and Lehmann, 1998; Jaruzelska *et al.*, 2003; Kraemer *et al.*, 1999; Lall *et al.*, 2003; Lin and Spradling, 1997; Moore *et al.*, 2003; Nakahata *et al.*, 2001; Sonoda and Wharton, 1999; Spassov and Jurecic, 2003; White *et al.*, 2001)
SDF-1				yes	yes	G	Chemokine	An attractive signal	(Doitsidou *et al.*, 2003; Ara *et al.*, 2003; Stebler *et al.*, 2004).
spire	yes						Novel protein	*osk* and *stau* localisation in oocyte (D)	(Clark *et al.*, 1994)
staufen	yes					Hs	dsRNA binding protein	Germ plasm assembly (D)	(DesGroseillers and Lemieux, 1996; St Johnston *et al.*, 1991; St Johnston *et al.*, 1992)

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stella					yes		Novel protein	Unknown	(Saitou *et al.*, 2002; Bortovin *et al.*, 2004)
tropomysin II	yes						Actin binding protein	*osk* and *stau* localisation in oocyte (D)	(Erdelyi *et al.*, 1995)
tudor	yes					Hs (*tudor domain protein*)	Novel 'tudor domain' repeats	Germ plasm assembly; *nos* localisation (D)	(Boswell and Mahowald, 1985; Callebaut and Mornon, 1997; Wang *et al.*, 1994)
valois	yes						Novel protein	Germ plasm assembly (D)	(Schüpbach and Wieschaus, 1989)
vasa	yes	yes	yes	yes	yes	Aa, Ad, B, Ca, Cc, Ci (*CiDEAD1b*), Cp, Cr, Cs (*CsDEAD1a, CsDEAD1b*), Dd (*Plvas1*), Dj (*Djvlga, Djvlgb*), Dv, E (*PoVAS1*), Ec, G (*Cvh*), H (*CnVAS1, CnVAS2*), He, Hs, Hy, L, Mf, O (*olvas*), Om, On, P, R (*RVLG*), Sa, Sg, Sm, Sp, Sq, T, Tf	DEAD-box RNA helicase; eIF4A (translation initiation factor) homology	Germ plasm assembly; translational regulation (D)	(Braat *et al.*, 2000; Castrillon *et al.*, 2000; Chang *et al.*, 2002; Dearden *et al.*, 2003; Fujiwara *et al.*, 1994; Gruidl *et al.*, 1996; Hay *et al.*, 1988a; Hay *et al.*, 1988b; Hay *et al.*, 1990; Ikenishi and Tanaka, 2000; Ikenishi *et al.*, 1996; Knaut *et al.*, 2002; Kobayashi *et al.*, 2000; Komiya *et al.*, 1994; Komiya and Tanigawa, 1995; Lasko and Ashburner, 1988; Miyake *et al.*, 2001; Mochizuki and Fujisawa, 2000; Mochizuki *et al.*, 2001; Nakao, 1999; Olsen *et al.*, 1997; Otani *et al.*, 2002; Sánchez Alvarado *et al.*, 2002; Sano *et al.*, 2002; Schüpbach and Wieschaus, 1989; Shibata *et al.*, 1999; Shinomiya *et al.*, 2000; Styhler *et al.*, 1998; Takamura *et al.*, 2002; Tsunekawa *et al.*, 2000; Tsunekawa *et al.*, 2002; Wang and Callard, 2001; Wang *et al.*, 1994; Woods *et al.*, 2002; Yoon *et al.*, 1997; Yoshizaki *et al.*, 2000)
Xlsirts			yes			Hs (*HumXist*)	Non-coding RNA	mRNA localisation to vegetal cortex (X)	(Kloc *et al.*, 2002; Kloc *et al.*, 1998; Kloc *et al.*, 1993)
Xpat			yes				Novel protein	Localised to germ plasm (X)	(Hudson and Woodland, 1998; Kloc *et al.*, 2002)

1.4 Selecting the genes to research in relation to PGC development in the chicken

Primordial germ cells (PGCs) go through three phases of development: determination, migration and colonisation. PGCs are determined via one of two model mechanisms: preformation, characterised by the assembly of germ plasm very early on in development; or epigenesis, involving induction signals from surrounding tissues to germ cell competent cells relatively late in development.

As described in Section 1.2.4, very little is known about genes that have a role in PGC development in the chicken. The aim of this investigation is to identify and investigate genes that could have a role in PGC development in the chicken. In this section the genes chosen for investigation will be introduced. Their roles in other species will be briefly outlined here and a more in depth review of each gene will be provided at the beginning of the relevant results chapter. In the final section, the reasons for choosing these genes will be outlined.

1.4.1 PGC determination

Eight known genes mediate the determination of PGC development in *Drosophila melanogaster*: *cappuccino*; *mago nashi*; *oskar*; *spire*; *staufen*; *tudor*; *valois* and *vasa* (Boswell and Mahowauld, 1985; Lehmann and Nüsslein-Volhard, 1986; Schüpbach and Wieschaus, 1986; Mahowald, 2001; Manseau and Schüpbach, 1989; Boswell *et al*, 1991; Newmark and Boswell, 1994). Of these eight genes *cappuccino*, *oskar*, *spire* and *valois* have not been found in species outside of the dipterans. A *tudor*

domain has been found in humans however, it is unknown whether the gene containing the *tudor* domain is a homologue of the *Drosophila tudor* gene or not. Mutations in these eight genes prevent germ cell determination. This phenotype can be attributed to defects, or the complete absence of polar granules in the germ plasm and leads to germ plasm not being assembled (Boswell and Mahowald, 1985; Lehmann and Nüsselein-Volhard, 1986; Schüpbach and Wieschaus, 1986; Newmark and Boswell, 1994).

1.4.2 Germ cell identity

The earliest gene to be expressed in the germ line is *vasa* (Sano *et al*, 2001). *Vasa* is expressed throughout germ cell development in a germ cell specific manner in many different species. *Vasa* has been shown to interact with several genes in PGC development and it has been proposed that Vasa protein is required for mRNA translation regulation, but their specific mechanism of action is still unknown (Salinas *et al.*, 2007). Due to the widespread conservation of the *vasa* gene and it's expression pattern, *vasa* is clearly an extremely important gene during germ cell development. In *Drosophila melanogaster* the two genes *nanos* and *pumilio* maintain *vasa* expression during the early stages of PGC development (Sano *et al*, 2001).

1.4.3 Maintaining the PGC population

Nanos, *pumilio* and components of the *decapentaplegic* pathway prevent germ cells from entering apoptosis. In other words they maintain the PGC population. This function is paralleled in GSCs in the gonad, suggesting a parallel mechanism exists

between PGCs and GSCs that is required for maintaining cell pluripotency (Gilboa and Lehmann, 2004; Wang and Lin, 2004). If apoptosis is suppressed, pole cells lacking maternal *nanos* or *pumilio* lose *vasa* expression and they then adopt a somatic cell fate (Gilboa and Lehmann, 2004; Hayashi *et al*, 2004). *Nanos* and *pumilio* have a further function in germ cell formation in *Drosophila*. They are needed to transcriptionally repress gene expression, and so prevent the premature activation of genes in PGCs. Embryos that lack either *nanos* or *pumilio* activity prematurely or inappropriately express genes and results in the failure to form functional germ cells (Leatherman *et al*, 2002). *Nanos* is also required during the migratory phase of PGC development for colonisation of the gonad (Kobayashi *et al*, 1996).

In more complex organisms such as zebrafish and mice there are more than one *nanos* gene. In zebrafish, *nanos1* is expressed in the germ plasm and its expression is restricted to the PGCs. When the levels of *nanos1* are reduced germ cell migration and survival are affected (Köprunner *et al*, 2001). Although *nanos1* is found in the germ plasm shortly after fertilisation, its function at this stage of development has not yet been investigated. Therefore, it is known that the migratory function of *nanos* is conserved between flies and vertebrates, but it is unknown whether *nanos* and *pumilio* maintain PGC identity in the zebrafish as found in *Drosophila*.

In mice there are three *nanos* genes. *Nanos1* is not involved in germ cell development but *nanos2* and *nanos3* are. Although *nanos2* and *nanos3* are not

expressed in a germ cell specific manner, they are differentially expressed in the PGCs. *Nanos3* is required for germ cell migration where as *nanos2* is a male specific gene required for spermatogenesis (Tsuda *et al*, 2003). The only part of the *nanos* gene that is conserved between species is the zinc-finger motif.

Two further genes, *oct-4* and *nanog*, are thought to maintain the PGC population in mammals. *Oct-4* and *nanog* are ES cell and germline specific genes that are expressed in totipotent and pluripotent cells during mammalian development. *Oct-4* has been knocked out conditionally in mouse germ cells and the germ cells apoptose indicating a requirement for germ cell survival (Kehler *et al*, 2004). However, *nanogs* function in germ cell development is currently unknown, but it is known that *nanog* maintains stem cell pluripotency and it is hypothesised that it could have a similar role in germ cell development.

1.4.4 Maintaining the GSC population in the gonad

Ziwi, the zebrafish homologue of *Drosophila piwi*, is first detected shortly after fertilisation, suggesting a maternal origin. Although it is detectable via RT-PCR at this early stage, the less sensitive technique of in-situ hybridisation cannot detect it. Later on in development *ziwi* co-localises with *vasa* to the embryonic genital ridge and it is expressed exclusively in the gonad of adults (Tan *et al*, 2002). In *Drosophila piwi* controls GSC maintenance and division (Cox *et al*, 2000). The early expression of *ziwi* in zebrafish is interesting and provides circumstantial evidence to support the theory of a parallel mechanism between PGCs and GSCs required for maintaining cell pluripotency (Gilboa and Lehmann, 2004).

1.4.5 Transcriptional repression

As previously mentioned *nanos* and *pumilio* prevent premature gene activation in *Drosophila*, and the failure to repress gene expression results in a failure to form PGCs (Leatherman *et al*, 2002). *Germ cell less* is a gene required for transcriptional quiescence and acts prior to pole cell formation (Leatherman *et al*, 2002). This places *germ cell less* as the earliest gene known to act in transcriptional repression of the germline (Leatherman *et al*, 2002). *Bruno* is another gene required for transcriptional quiescence. It is localised to the germ plasm in both *Drosophila* and zebrafish embryos (Webster *et al*, 1997; Hashimoto *et al*, 2005). In *Drosphila bruno* transcriptionally represses *oskar*, a gene important in establishing the anterior-posterior axis of the fly, and in the determination of PGCs (Lehmann and Nüsslein-Volhard, 1986; Castagnetti *et al*, 2000).

1.4.6 Choosing the genes for investigation

From researching the literature the following genes have been identified as key genes required for germ cell determination: *vasa*; *germ cell less*; *mago nashi* and *staufen*. Although other genes are required for germ cell determination in *Drosophila* the fact that gene homologues have not been identified outside of the dipterans suggests that they are not present in other species. *Vasa* has already been identified in the chicken (Tsunekawa *et al*., 2000), leaving *germ cell less*, *mago nashi* and *staufen* as novel targets for further research.

Nanos and *pumilio* have key roles throughout germ cell development, but it is their role that prevents gene activation in early germ cell development that is of particular interest to this research. Neither have been identified in the chicken, but homologues have been identified in diverse species including vertebrates and invertebrates. *Bruno* has a similar function to *Nanos* and *Pumilio* in the early development of germ cells making this gene another target of this research.

Piwi is of interest because of the suggestion of a parallel mechanism between PGCs and GSCs, which prevents cell differentiation. If this mechanism is paralleled in PGCs then it could be active during early development.

Recently, a homologue of *nanog* and a POU containing gene were isolated from chicken embryonic stem cell cultures (Lavial *et al.*, 2007). These genes are of interest in chicken PGC development because of their germ cell specificity in mammals.

The aims of this research are to: (1) identify chicken homologues of the genes mentioned above; (2) construct an expression profile for each gene to identify when and where each genes is expressed during chicken development; (3) use the expression profile to determine whether the genes are genuine homologues; (4) determine whether the expression pattern suggests a function during germ cell development.

CHAPTER 2

MATERIALS AND METHODS

2.1 Stock solutions

2.1.1 General stock solutions

CM-1

Dulbecco's Modified Eagles Medium (DMEM; Sigma cat#D5671), 450ml; L-Glutamine (Invitrogen), 6ml; Foetal Bovine Serum (Gibco), 50ml; Non Essential Amino Acids (Invitrogen), 5ml; 1x Penicillin/Streptomycin (Gibco).

100ml of 5x Creosol Red

60% sucrose, 60g; 1mM creosol red, 0.04g. The sucrose was dissolved in dH_2O on a hot plate before the creosol red dye was added.

1L of Dulbecco's solution

10 Phosphate Buffered Saline tablets (Oxoid) were dissolved in 800mls of dH_2O. The tablets contain: NaCl, 8g/L; KCl, 0.2g/L; KH_2PO_4, 1.15g/L; Na_2HPO_4, 0.24g/L. The pH was adjusted to pH7.4 with 1M HCl, and the solution made up to 1L with dH_2O.

Dulbecco's-Penicillin/Streptomycin solution

10µl of Penicillin/Streptomycin solution (Gibco) containing 10,000 units/ml Penicillin G sodium and 10mg/ml Streptomycin sulphate were added to 10ml of Dulbecco's.

Gel loading dye

Bromophenol blue 0.25% (w/v); Xylene cyanol FF 0.25% (w/v); Ficoll (Type 400; Pharmaicia) 15% (w/v) in H_2O.

Luria-Bertani Agar (LB Agar)

1% Bacto-tryptone, 0.5% Bacto-yeast extract, 0.125M NaCl and 1.5% agar (Difco).

1L Luria-Bertani Medium (LB Medium)

Tryptone, 10g; Yeast Extract, 5g; NaCl, 10g; dissolved in 950ml dH_2O. The pH was adjusted to pH7.0 with 2M NaOH and the solution made up to 1L with dH_2O.

4% Paraformaldehyde

An appropriate amount of Paraformaldehyde (PAF) was out weighed in a fume hood to give a final concentration of 4% (w/v). Half of the final volume of RNase free water was added. The solution was heated and stirred to 60°C in a fume hood. When the solution reached 60°C, drops of 1M sodium hydroxide were added until all solid PAF had dissolved. The solution was filtered through a 3mm Whattman filter paper. 10x PBS added to give a final concentration of 1x PBS and RNase free water

was added to give the final volume. 4% PAF solution was stored in 15ml aliquots at -20°C.

Phosphate buffered saline containing Tween-20 (PBT)

0.1% Tween-20 in Dulbecco's.

SOC-broth

2% Bacto-tryptone (w/v), 0.5% Bacto-yeast extract (w/v), 10mM NaCl, 2.5mM potassium chloride, 20mM Mg^{2+} stock (1M $MgCl_2$ $6H_2O$/1M $MgSO_4$ $7H_2O$) and 20mM glucose.

1L of 50x TAE electrophoresis buffer

Tris(hydroxymethyl)aminomethane hydrochloride (Tris) base, 242g; Glacial acetic acid, 57.1ml; 0.5M ethylenediaminetetraacetic acid (EDTA) solution pH8.0, 100ml; solution made up to 1L with dH_2O.

Tris/EDTA (TE)

10mM tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCL), 1mM ethylenediaminetetraacetic acid (EDTA).

2.1.2 Stock solutions for embedding tissue samples

0.24M Phosphate buffer pH7.2

For 3L: $NaH_2PO_4.H_2O$, 19.2g; Na_2HPO_4, 81g. The pH was adjusted to pH7.2 with 1M HCL and the solution made up to 3L with dH_2O.

0.12M Phosphate buffer-15% sucrose

0.24M phosphate buffer was diluted by half. Sucrose was added to give a final concentration of 15% (w/v). Solution stored at 4°C.

0.12M Phosphate buffer-15% sucrose-7.5% gelatine

0.24M phosphate buffer was diluted by half. Sucrose was added to give a final concentration of 15% (w/v). Gelatine was added to give a final concentration of 7.5% (w/v). Sucrose and gelatine were dissolved in a 37°C water bath. Solution stored in 50ml aliquots at -20°C.

2.1.3 Stock solutions for Northern analysis

10x MOPS

For 500ml: 20.9g MOPS was dissolved in 350ml dH_2O and the pH adjusted to pH7.0 with 2M NaOH. 10ml 1M sodium acetate and 10ml 0.5M EDTA pH8.0 were added. The solution was brought up to 500ml with dH_2O, and then filtered through a 0.45µm Millipore filter. The solution was autoclaved and stored at room temperature wrapped in tin foil. The solution was used within one week.

RNA sample buffer

2ml sample buffer was made up fresh prior to use. Molecular biology grade deionised formamide (Obiogene), 1ml; 10x MOPS, 200µl; formaldehyde, 360µl; dH_2O, 440µl.

Type III loading dye

0.25% bromophenol blue; 0.25% xylene cyanol FF; 30% glycerol in water.

10x SSC

20x SSC was made up and diluted down to 10x for use.

For 1L: 175.3g NaCl and 88.2g sodium citrate were dissolved in 800ml H_2O. pH adjusted to 7.2 with concentrated HCl and volume adjusted to 1L.

Church buffer

For 50ml: BSA (Sigma), 0.5g in 7.4ml dH_2O; 0.5M EDTA pH8.0, 100µl; 1M Sodium Phosphate pH 7.2, 25ml; 20% SDS, 17.5ml. For Northern analysis Church buffer was heated to 65°C prior to use. To prevent precipitation of BSA, all solutions were heated to 65°C before mixing.

1M Sodium phosphate pH7.2

For 1L: Na_2HPO_4, 142g dissolved in 850ml dH_2O. The pH was adjusted to 7.2 with orthophosphoric acid. Solution made up to 1L with dH_2O.

2.1.4 Stock solutions for whole mount *in situ* hybridisation analysis

Post-fix solution

Post-fix solution containing 4% formaldehyde final concentration (w/v) and 0.1% glutaraldehyde final concentration (w/v) in PBT.

Hybridisation mix

For 50ml: Molecular biology grade deionised formamide (Obiogene), 25ml; 20x SSC, 3.25ml; EDTA, 0.5ml; Yeast RNA (20 mg/ml), 125µl; 100% Tween-20, 0.1ml; CHAPS (10%), 2.5ml; Heparin (50mg/ml), 100µl; RNase free water, 16.4ml. Hybridisation mix was stored in aliquots of 50mls at -20°C until required.

Maleic Acid Buffer (MABT)

For 500ml: Maleic acid, 0.058g; sodium chloride, 0.04g; 100% Tween-20, 0.5ml (see *Storage note below); RNase free water to bring final volume to 500mls. The abbreviation MAB refers to Maleic acid buffer without Tween-20.

NTMT

For 50ml: 5M sodium chloride, 1ml; 2M Tris-HCL pH9.5, 2.5ml; 1M magnesium chloride, 2.5ml; 100% Tween-20, 0.5ml; double autoclaved water to bring final volume to 50ml. NTMT was discarded after two days.

TBST

For 500ml: 1M Tris pH7.5, 125ml; 5M Sodium chloride, 0.75ml; 100% Tween-20, 0.5ml (see *Storage note below); Potassium chloride, 0.1g; RNase free water to bring final volume to 500mls.

Blocking Reagent (BR)

10% blocking reagent (w/v; Roche) was added to MAB. The solution was heated to dissolve the solid and autoclaved. BR was stored in 15ml aliquots at -20°C.

Goat Serum

Goat serum (Sigma) was heat treated at 55°C for 30 minutes and stored in 15ml aliquots at -20°C.

In situ hybridisation blocking solution (also used on cryostat sections)

TBST/MABT containing 2% BR (w/v) and 20% goat serum (w/v).

DIG-AP antibody solution (also used on cryostat sections)

TBST/MABT containing 2% BR (w/v), 20% goat serum (w/v) and 1/2000 dilution of anti-DIG-AP antibody (Roche).

Staining solution (also used on cryostat sections)

NTMT containing 6.75µl/ml NBT (Promega) and 3.5µl/ml BCIP (Promega).

*Storage note for MABT and TBST: Each solution was made up without Tween-20, autoclaved and stored at room temperature. Tween-20 was added to give 0.1% final concentration prior to use. Solutions containing Tween-20 was discarded after two days.

2.1.5 Stock solutions for *in situ* hybridisation analysis of cryostat sections

10x Salt solution

For 100ml: NaCl, 11.4g; Tris HCl pH7.5, 1.404g; Tris base, 0.134g; $Na_2HPO_4.H_2O$, 0.78g; Na_2HPO_4, 0.71g; 0.5M EDTA 10ml; solution made up to 100ml with dH_2O.

Hybridisation mix

For 20ml: 10x salt, 2ml; molecular biology grade deionised formamide (Obiogene), 10ml; 50% dextran sulphate, 4ml; Yeast RNA (20mg/ml), 1ml; 50x Denhardt's (Sigma), 400ml; dH_2O 1.6ml. Solution stored in 15ml aliquots at -20°C.

Washing solution

For 10ml: 200µl 20 x SSC and 9.8ml formamide (Sigma).

2.1.6 Stock solutions for immunohistochemistry

Hoechst nuclear staining solution

BisBenzimide (Sigma) was dissolved in dH_2O to give a final concentration of 1mg/ml.

2.2 Centrifugation

Centrifugation of 1.5ml or 0.5ml microfuge tubes at room temperature was performed in a mini spin plus bench top centrifuge (Eppendorf).

Centrifugation of 1.5ml or 0.5ml microfuge tubes at a regulated temperature was performed in a Biofuge fresco centrifuge (Heraeus).

Centrifugation of PCR strip tubes was carried out in a mark IV refrigerated centrifuge (Baird and Tatlock).

Centrifugation of 15ml polypropylene tubes was performed in a GS-15R centrifuge (Beckman) using a Beckman S4180 rotor with bucket adapters.

Centrifugation of large volumes (>15ml) was performed in a Sorvall® RC-5B refrigerated superspeed centrifuge (Du Pont Instruments) using a Sorvall® SLA-1500 Super-Lite® rotor.

2.3 Agarose Gel Electrophoresis of DNA

Running buffer was made containing a final concentration of 1x TAE electrophoresis buffer and 12µl/L of 10mg/ml ethidium bromide (Sigma). Molecular biology grade agarose (BDH) was weighed to give a final concentration of 1.5% (w/v) when the band size predicted was less than 500bp, or 1% (w/v) when the band size predicted was greater than 500bp. The agarose was dissolved in running buffer by heating in a microwave, allowed to cool to hand hot before pouring. 6µl TrackIt™1kb plus DNA ladder (Invitrogen) was used as a size marker. For DNA quantification gels, 5µl HyperLadder I (Bioline) was used as a size marker. The Gel Logic 200 imaging system (Kodak) was used to visualise the gels.

2.4 Frozen sections

Sample pre-treatment

Embryonic tissues were fixed in 4% Paraformaldehyde for half an hour at room temperature. Adult tissues and tissues that had previously undergone *in situ*

hybridisation were fixed in 4% Paraformaldehyde overnight at 4°C. The tissues were rinsed three times in PBS. Tissues were transferred to stock 0.12M phosphate buffer-15% sucrose solution and incubated overnight at 4°C.

2.4.1 Embedding tissue samples in gelatine

0.12M Phosphate buffer-15% sucrose-7.5% gelatine stock solution was thawed at 37°C for 2 hours. A 'gelatine bed' was made by adding 3ml 0.12M Phosphate buffer-15% sucrose-7.5% gelatine to a small plastic weighing tray and left to set at room temperature for 30 minutes. The tissue samples to be embedded were incubated in 1ml of 0.12M Phosphate buffer-15% sucrose-7.5% gelatine stock solution at 37°C for 30 minutes. The tissue samples were poured onto the gelatine bed and positioned under a microscope. 0.12M Phosphate buffer-15% sucrose-7.5% gelatine stock solution was added to cover the tissues and left to set at 4°C for 15 minutes. Samples were cut from the gelatine bed as blocks and mounted onto card with OCT (Bright Instruments Co. Ltd.). Isopentane was chilled in liquid N_2 to a temperature of -65°C. The gelatine blocks were frozen in the chilled isopentane and stored at –80 °C until sectioning.

2.4.2 Sectioning of frozen material

An OTF5000/HS-001 cryostat with solid knife block holder (Bright instruments) was used. The cryostat chamber was set at –20°C and the sample temperature was set at -23°C. Samples were transported and maintained on dry ice. Samples were mounted to the chuck using OCT. Sections were cut at a 15° angle and a thickness of 20μ

unless stated otherwise. Sections were collected on Super Frost ® Plus slides (VWR international) and stored at -20°C until use.

2.5 Animal methods

Chicken embryonic and adult material was obtained from the Roslin Institute flock of ISA Brown strain layer birds. Embryos for injection were incubated stationary in an Octagon 100 incubator (Brinsea) at 38°C. Post-injection, eggs were kept in a prototype hatcher (developed by Brinsea). All other embryos were incubated in an A.B. Multilife 290 GP setter (A.B. Incubators Ltd) at 38°C, with a turning cycle of thirty minutes.

2.5.1 Dissection of embryos at stages 1-3 and stages 4-10

Paper rings were made from Whatmann filter paper with an approximate diameter of 4cm, and an approximate diameter of the hole in the centre of 2cm. Each egg was broken into a small bowl (see Figure 2.1A) and the egg white and the capsule were poured off (see Figure 2.1B). The yolk was transferred to a 15mm petri dish lid with the embryo uppermost (see Figure 2.1C). A paper ring was placed onto the surface of the yolk with the embryo in the centre (see Figure 2.1D) and the yolk sac was cut around the paper ring (see Figure 2.1E). The embryo, attached to the paper ring, was gently slid away from the yolk (see Figure 2.1F), washed in Dulbecco's to remove excess yolk (see Figure 2.1G), and then removed from the vitelline membrane (see Figure 2.1H). For RNA extraction, embryos were transferred to a 1.5ml Cryovial (Nalgene). Excess Dulbecco's was removed using a 1ml fine-tipped pipette and the

tube was quick frozen in liquid nitrogen and stored at -80°C. For *in situ* hybridisation, the embryos were fixed in 4% PAF for 1-2 hours at room temperature.

2.5.2 Dissection of intact embryos from stages 18-20

Each egg was broken into a small bowl and the yolk sack was cut around the embryo. The embryo was transferred to a petri dish containing Dulbecco's and the extra-embryonic tissues and vitelline membrane were removed with tweezers. For RNA extraction, embryos were transferred to a 1.5ml Cryovial, quick frozen in liquid nitrogen and stored at -80°C. For *in situ* hybridisation, a small hole was put into the head of the embryos with the tip of the tweezers and they were fixed overnight at 4°C in 4% PAF.

2.5.3 Dissection of embryos and gonads at stages 26-28 and stages 33-35

An egg was cracked open and the embryo was transferred from the eggshell to a petri dish containing Dulbecco's. The head and vitelline membranes were removed and the embryo's abdominal region was opened and the gut removed. For removal of the gonads from the embryo proper, a 10A scalpel (Swann-Morton) was slid between the dorsal wall of the embryo and the mesonephros and moved the length of the embryo. The gonads were removed from the mesonephros using tweezers. For RNA extraction, embryos/mesonephros and gonads were transferred to a 1.5ml Cryovial, quick frozen in liquid nitrogen and stored at -80°C. For *in situ* hybridisation, embryos/ mesonephros and gonads were fixed overnight at 4°C in 4% PAF.

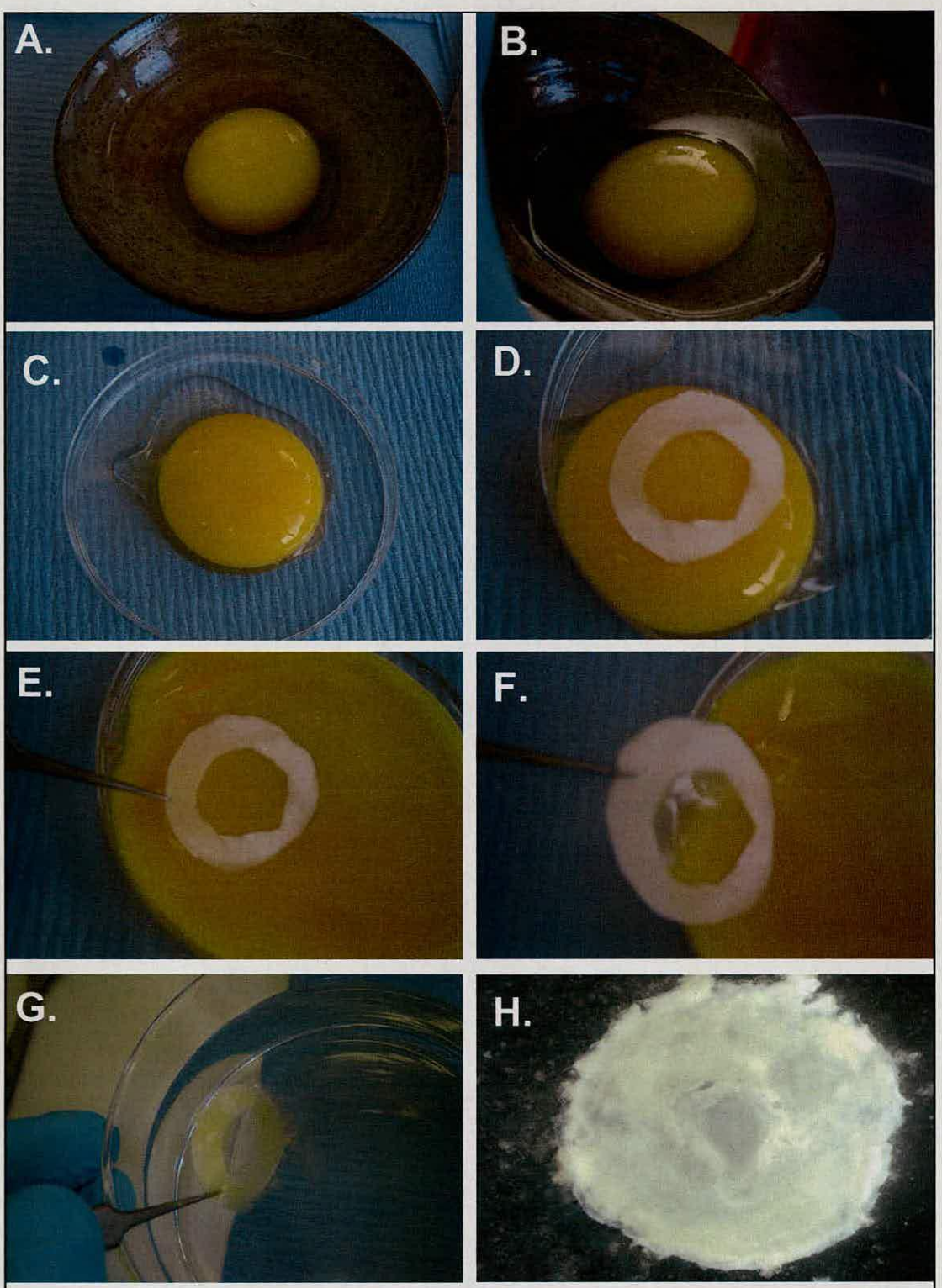


Figure 2.1: Dissection of an embryo at stage 5 (24hrs of development) using the paper disc method. (A) an egg was broken into a dish. (B) the egg white and capsule were poured. (C) the yolk was transferred to a petri dish. (D) a paper ring was laid over the embryo. (E) the yolk sac was cut around the paper ring. (F) the paper ring with the embryo attached was gently slid from the yolk. (G) the embryo was washed in Dulbeccos. (H) the embryo was dissected away from the vitelline membrane.

2.5.4 Dissection of the germinal crescent from embryos ubiquitously expressing green fluorescent protein (GFP) at stages 4-8

Each egg was broken into a small bowl and the embryo was checked for green fluorescent protein (GFP) expression using a UV light source passed through a FIT-C filter. When an embryo expressing GFP was found the germinal crescent region was removed using a tungsten wire that had been electrophoretically sharpened in 0.2M NaOH and UV sterilised. Approximately 0.5ml of Dulbecco's-Penicillin/Streptomycin solution was added using a disposable 1ml graduated pipette, and the crescent region was transferred to a sterile 0.5ml microfuge tube.

2.5.5 Dissection of adult and neonate tissues

Adult birds were killed following a schedule 1 method by a trained individual. 1-2ml of sodium pentabarbitone (Pentoject) was injected intravenously to kill the bird. To avoid contamination each adult tissue was removed to a separate petri dish and cut into small pieces. For RNA extraction, tissues were quick frozen in liquid nitrogen and stored at -80°C. For fixation, tissues were fixed in 4% PAF overnight at 4°C. Neonate birds (up to one week post hatch) were killed by intraperotoneal injection of 0.5ml sodium pentabarbitone. Adult birds were killed by intravenous injection of 10ml sodium pentabarbitone.

2.5.6 Injection of a single cell suspension into the embryonic cardio-vascular system

Preparation of cell suspension from blastodermal disc/germinal crescent region from embryos at stages 1-3 and stages 4-8

The isolated blastodermal disc/germinal crescent regions from staged embryos were broken up by pipetting vigorously with a 200µl Gilson pipette to form a single cell suspension.

Preparation of gonads from embryos older than stage 8

0.5ml of TrypLE Express (Invitrogen) was added to a minimum of five dissected gonads and the solution was pipetted vigorously with a 200µl Gilson pipette for several minutes to give a single cell suspension. 200µl of CM-1 (see 2.1.7) was added.

The resulting cell suspensions were pelleted at 3,000rpm for 2 minutes and the liquid was removed. The pellet was resuspended in 2µl of DMEM per blastodermal disc/germinal crescent region. 0.1µl of fast green dye (Sigma Aldrich) was added to facilitate visualisation of the cell suspension injection. 2µl of cells were added to 8µl dH_2O, and were counted on a haemocytometer.

Injection of cell suspensions into the vascular system

Wild type host embryos were incubated prior to injection for 2.5 days. Glass needles were made using a model 753 moving-coil microelectrode puller (Campden Instruments Ltd) from glass capillaries with an outer diameter of 1.5mm and an inner

diameter of 1.17mm (Harvard Apparatus). The needles were bevelled with a MB3-T turbo microbeveller (Research Instruments Ltd) to make an aperture of 35μm and they were UV sterilised before use. A small hole was made in the blunt end of a host egg with scissors and 2ml of egg white was removed using a 5ml syringe and a 21-gauge needle. The egg was windowed for injection. Approximately 1-2μl of single cell suspension was injected into the cardiovascular system of the embryo via the heart or the circumferal vein using a bevelled needle (see Figure 2.2). 1ml of Dulbecco's-pen/strep solution was added to the surface of the embryo to prevent drying out after sealing the window. The eggs were sealed using brown parcel tape and incubated at 38°C. After a minimum of four days of incubation post-injection, the gonads and other areas of the embryo were checked for GFP positive cells.

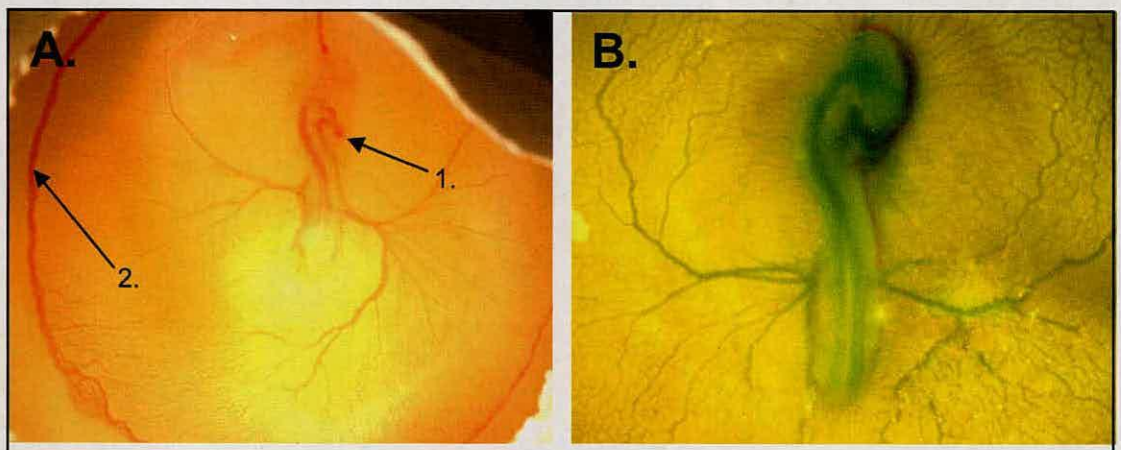


Figure 2.2: Vascular injection of single cell suspension into stage 15 embryos. (A) stage 15 embryo identifing the position of the heart (1.) and circumferal vein (2.) relative to the rest of the embryo. (B) a recently injected embryo.

2.6.1 Purification of DNA fragments from agarose gels

The GFX PCR DNA and Gel Band Purification Kit (Amersham Biosciences) was used. Bands were cut from the agarose gel and transferred to 1.5ml microfuge tubes. 10µl of capture buffer were added per 10mg of agaorse gel. The tubes were vortexed vigorously and incubated at 60°C for fifteen minutes and spun down. Samples were transferred to GFX columns, incubated for one minute at room temperature, and centrifuged at full speed for thirty seconds. The flow-through was discarded and 500µl of wash buffer was added. Tubes were centrifuged at full speed for thirty seconds. The GFX columns were transferred to 1.5ml microfuge tubes and dried at room temperature for five minutes. 50µl of dH_2O was added and the columns were incubated for one minute at room temperature, then centrifuged at full speed for one minute. A quantification gel was run to determine the amount of DNA recovered.

2.6.2 Cloning of Polymerase Chain Reaction products

The TOPO TA Cloning® kit (Invitrogen) was used. A 45µl PCR was run for 25 cycles. The PCR product was run on an agarose gel, purified and then quantified. 1µl salt solution and 1µl TOPO® vector were added to 4µl of fresh PCR product in a 0.5ml microfuge tube and incubated for five minutes on ice. One vial of One Shot® Mach1™ –T1^R Competent Cells per transformation was thawed on ice. 2µl of the TOPO® cloning reaction were added to one vial of cells, swirled gently and incubated on ice for thirty minutes. The cells were heat shocked for thirty seconds at 42°C and immediately transferred to ice. 250µl room temperature SOC medium

were added and the tubes incubated for one hour at 37°C with shaking at 200 rpm. 10-50µl of the transformation were spread onto a pre-warmed LB-agar plate containing 100µg/ml ampicillin, 1µl/ml Xgal (50mg/ml) and 1µl/µl IPTG (10mM) and then incubated overnight at 37°C. White or light blue colonies were picked for DNA purification.

2.6.3 Transformation of ultracompetent bacteria

Ultracompetent *E.coli* XL-10 cells (Stratagene) were used. 14ml BD Falcon polypropylene round-bottomed tubes were pre-chilled on ice and SOC broth was pre-heated to 42°C. Ultracompetent cells were thawed on ice. 100µl of cells and 4µl β-ME were aliquoted into each of the pre-chilled Falcon tubes. The tubes were swirled gently and incubated on ice for ten minutes with occasional swirling. 2µl of plasmid DNA were added to one tube and 1µl of 1/10 dilution pUC18 positive control DNA to the other. The tubes were swirled gently and incubated on ice for thirty minutes. The tubes were heat shocked for thirty seconds at 42°C and then incubate on ice for two minutes. 900µl pre-heated SOC were added, and the tubes were incubated at 37°C for one hour with shaking at 250 rpm. 5µl of the mix were spread onto a LB-agar plate containing 100µg/ml ampicillin and was incubated overnight at 37°C.

2.6.4 Small-scale preparation of plasmid DNA

The Wizard® Plus SV Minipreps DNA Purification System (Promega) was used. Cells containing the wanted plasmid were streaked onto LB-agar plates containing an appropriate antibiotic for selection and incubated overnight at 37°C. A single colony was picked and used to inoculate 5ml aliquots of LB medium containing the same

antibiotic as previously used. The LB medium was incubated at 37°C with shaking for a minimum of six hours. Each culture was centrifuged at 4800rpm for 5 minutes and the liquid discarded. The cell pellet was resuspended with 250µl cell resuspension solution. 250µl cell lysis solution were added and the tubes inverted four times to mix. 10µl alkaline protease solution were added, the tubes were inverted four times to mix and incubated for five minutes at room temperature. 350µl neutralization solution were added, the tubes inverted four times to mix and then centrifuged at 4800rpm for ten minutes at room temperature. The clear lysate was added to the spin column, centrifuged for one minute at 14500rpm at room temperature and the flowthrough discarded. 750µl wash solution were added, centrifuged for one minute at 14500rpm at room temperature and the flowthrough discarded. 250µl wash solution were added, centrifuged for two minutes at 14500rpm at room temperature and the flowthrough discarded. The spin column was transferred to a 1.5ml microfuge tube and 100µl nuclease-free water were added. The column was centrifuged for one minute at 14500rpm at room temperature. The amount of DNA recovered was quantified using the RNA/DNA calculator. DNA was stored at -20°C.

2.6.5 Large-scale preparation of plasmid DNA

The HiSpeed plasmid maxi kit (Qiagen) was used. Cells containing the wanted plasmid were streaked onto LB-agar plates containing an appropriate antibiotic for selection and incubated overnight at 37°C. A single colony was picked and used to inoculate 5ml LB medium containing 100µg/ml ampicillin. This was incubated at 37°C with shaking for a minimum of six hours. 1ml of the culture was added to

100ml fresh LB medium containing 100μg/ml ampicillin and incubated overnight at 37°C with shaking. The culture was centrifuged at 6000 x g for fifteen minutes at 4°C. The cell pellet was resuspended in 10ml buffer P1. 10ml buffer P2 were added and the solution was mixed by inverting several times. 10ml of chilled buffer P3 were added and the solution was mixed by inverting several times. The solution was added to a QIAfilter Maxi cartridge and incubated at room temperature for ten minutes. 10ml of buffer QBT were added to a HiSpeed Maxi Tip and allowed to enter the column. The cell lysate was added to the HiSpeed tip and allowed to enter the column by gravity flow. 60ml Buffer QC were added. 15ml Buffer QF were added and the elute was collected. 1.5ml isopopanol were added and the solution incubated at room temperature for five minutes. A QIAprecipitator Maxi Module was attached to a 30ml syringe and the solution added. The elute was filtered through the QIAprecipitator at constant pressure. The QIAprecipitator was removed, the plunger removed from the syringe and the QIAprecipitator reattached. 2ml 70% ethanol were added and filtered through the QIAprecipitator. The QIAprecipitator was removed, the plunger removed from the syringe and the QIAprecipitator reattached. The filter was dried by twice pressing air through the QIAprecipitator quickly. The QIAprecipitator was attached to a 5ml syringe. 1ml of buffer TE was added, passed through the QIAprecipitator and collected in a 1.5ml microcentrifuge tube. The QIAprecipitator was removed, the plunger removed from the syringe and the QIAprecipitator reattached. The collected filtrate was then passed back through the QIAprecipitator and re-collected in the same tube. The amount of DNA recovered was quantified using the RNA/DNA calculator. DNA was stored at -20°C.

2.6.6 Sequencing of plasmid DNA

Sequencing was carried out by the University of Dundee Sequencing Service. 200-300ng of DNA in 15µl dH_2O per reaction was sent to The Sequencing Service. The University of Dundee supplied the primers. DNA was sequenced both ways.

2.6.7 Isolation of RNA from tissues

RNA extraction using the NucleoSpin kit (BD Biosciences)

The NucleoSpin kit is designed to work on samples weighing between 10-30mg. All centrifugation steps were carried out at 4°C. 3.5µl of ß-Mercaptoethanol (ß-ME) were added to 350µl of RA1 in a fume hood. The RA1/ ß-ME solution was added to the tissue sample and the sample was homogenised using a PT-2100 Polytron homogeniser with a PT-DA 2107/EC attachment (Kinematica AG). The homogenate was transferred to a purple unit and centrifuged at 11,000 x g for one minute. 350µl of 70% ethanol were added to the lysate and the mixture was vortexed. The sample was loaded into a blue pre-assembled filter tube, centrifuged at 8,000 x g for thirty seconds and the lysate discarded. 350µl of MDB were added, the sample was centrifuged at 11,000 x g for one minute and the lysate discarded. 95µl of DNaseI reaction were added and the tubes were incubated at room temperature for fifteen minutes. 200µl of RA2 were added. The sample was centrifuged at 8,000 x g for thirty seconds and the lysate discarded. The filter top was transferred to a fresh 2ml collection tube and 600µl of RA3 containing ethanol were added. The sample was centrifuged at 8,000 x g for thirty seconds and the lysate discarded. 250µl RA3 containing ethanol were added. The tubes were centrifuged at 11,000 x g for two minutes and the lysate discarded. The filter top was transferred to a 1.5ml microfuge

tube, 50µl of nuclease free water were added and the sample was centrifuged at 11,000 x g for one minute. RNA was quantified using the GeneQuant RNA/DNA calculator (Pharmacia).

RNA extraction using RNA-Bee

The volumes given equate to one extraction containing 50mg of tissue. Samples were homogenised in 1ml of RNA-Bee using a PT-2100 Polytron homogeniser with a PT-DA 2107/EC attachment. 0.2ml of chloroform were added and samples were shaken vigorously for thirty seconds. The samples were incubated on ice for five minutes and then centrifuged at 12,000 x g for fifteen minutes at 4°C. Approximately 80% of the aqueous phase was transferred to a clean 1.5ml microfuge tube and 0.5ml of isopropanol were added. Samples were incubated for ten minutes at room temperature and then centrifuged at 12,000 x g for five minutes at room temperature. The supernatant was removed and the RNA pellet was washed with 1ml of 75% ethanol. Samples were spun for five minutes at 7,500 x g at room temperature and the supernatant removed. The RNA pellet was air-dried for 5-10 minutes on ice, and then dissolved in 50µl double autoclaved water. RNA was quantified using the GeneQuant RNA/DNA calculator.

2.6.8 Analysing RNA quality

The Agilent 2100 bioanalyzer was used to analyse RNA quality. Sample RNA (200ng) and RNA 6000 ladder (Ambion) were denatured at 70°C for two minutes and then stored on ice. The RNA Nano chip was loaded following the manufactures

instructions. The chip containing the RNA samples was loaded into the Agilent 2100 Bioanalyzer and the Eukaryote total RNA Nano program run.

Preparation of gel for use with Agilent 2100 bioanalyzer

Reagents were equilibrated to room temperature for thirty minutes before use. 550µl of RNA 6000 Nano gel matrix were added to a spin filter provided, and then centrifuged for ten minutes at 1500 x g. The gel was stored as 65µl aliquots at 4°C.

Preparation of gel-dye mix for use with Agilent 2100 bioanalyzer

Reagents were equilibrated to room temperature for thirty minutes before use. The RNA 6000 Nano dye was vortexed for ten seconds and spun down. 1µl of dye was added to a 65µl aliquot of filtered gel and the solution vortexed. The tube was centrifuged at 13000 x g for ten minutes at room temperature.

2.6.9 cDNA synthesis

The Promega Reverse Transcription System was used. 1µg of RNA was heat-treated at 70°C for ten minutes. The following 20µl reaction mix was made up: 25mM $MgCl_2$, 4µl; 10x Reverse Transcription buffer, 2µl; 10mM dNTP Mixture, 2µl; Recombinant Rnasin, 0.5µl; AMV Reverse Transcriptase, 0.75µl; Random primers, 0.5µl; total RNA, 1µg. The samples were incubated at room temperature for ten minutes; followed by 45°C for fifteen minutes; followed by 95°C for five minutes and followed by 4°C for five minutes. cDNA was stored at -20°C. For negative control samples the above reactions were carried out without AMV Reverse Transcriptase

2.6.10 RT-PCR

PCR primers were designed flanking an intron using primer3 software (http://frodo.wi.mit.edu/cgi-bin/primer3_www.cgi). β-actin primer sequences were supplied by Cheryl Hunter, and were used as a positive control. The primer sequences for Oct-4 and Nanog were supplied by Dr Bertrand Pain. All primer sequences used are shown in Appendix 1.

The following 15μl reaction mixture was made up: dH_2O, 6.0μl; 10x buffer (Roche), 1.5μl; 15mM $MgCl_2$ (Roche), 1.5μl; dNTP (Promega), 0.3μl; 3' primer (100pmol/ul), 0.3μl; 5' primer (100pmol/ul), 0.3μl; Fast Start Taq (Roche), 0.1μl; 5x Creosol Red, 3.0μl; sample cDNA, 2ul. The reaction mixture was vortexed and then spun down. Master mix without cDNA was made up for each set of primers and pippetted into PCR tube strips (Greiner Bio-One Ltd). After addition of the cDNA, the tubes were spun down and loaded into a JMBS 0.2G (Hybaid) PCR machine. The following PCR reaction was carried out: Stage 1 (1 cycle), 95°C for twenty minutes; Stage 2 (25-30 cycles) step 1, 95°C for thirty seconds; step 2, annealing temperature for thirty seconds; step 3, 72°C for one minute; Stage 3 (1 cycle), 72°C for thirty minutes. When completed, the samples were run on an agarose gel.

2.6.11 Quantitative PCR

The Platinum® SYBR® Green qPCR SuperMix-UDG w/ROX (Invitrogen) was used. Quantitative PCR (qPCR) primers were designed using Primer3 software (http://frodo.wi.mit.edu/cgi-bin/primer3_www.cgi) predicted to generate a product of

approximately 150bp. Primer stock was 20μM. cDNA was made from RNA extracted from gonads at 9.5, 11.5, 13.5, 15.5 and 17.5 days of incubation. cDNA was made from this RNA and qPCR was carried out. A minimum of three sets of gonads was used at each stage of development. cDNA was diluted 1/100. For one reaction: SYBR Green supermix 12.5μl; ROX 0.5μl; dH_2O 1μl; forward primer 0.5μl; reverse primer 0.5μl; cDNA 10μl. Note, when the qPCR primers to detect Nanog were used, 1μl of each primer was added and the water was removed. A Stratagene Mx3000P machine was used to run the qPCR. The pre-loaded program SYBR Green Invitrogen was used with the following settings: Well type- Unknown; Collect fluorescence data- ROX and SYBR; Reference dye- ROX; Normalising dye- none. The thermal profile of the program was: Stage 1 (1 cycle) 50°C for 2 minutes; 95°C for 2 minutes; Stage 2 (40 cycles) 95°C 15 seconds; 60°C 30 seconds; Stage 3 (1 cycle) 95°C 1 minute; 60°C 30 seconds.

Primer efficiency was calculated using a standard curve. A standard curve was generated for each primer set using cDNA made from RNA extracted from embryos at one day of incubation. cDNA was added at the following concentrations: 1/50; 1/500; 1/5000; 1/50000 and 1/500000. Each reaction was run four times for each primer set. A standard curve was drawn using the Mx3000P software and the equation for the line was given: $y = m*\log(x) + b$, where m is the slope of the line. The amplification efficiency, calculated from the slope, was also displayed.

In this work, the $2^{-\Delta\Delta Ct}$ method of relative quantification (described in detail in Livak and Schmittgen, 2001) was used to estimate copy numbers of *cPouV* and *nanog*

genes. This method allows an estimation of gene copy numbers in unknown samples. To be able to calculate the estimate, a house-keeping gene that permits normalization of the quantitative data is required. In this instance the gene LBR was used. The efficency of the primers was calculated prior to experimentation. Quantitative PCR was carried out six times for each sample. Following completion of the qPCR, the results were normalised for each sample. This involved subtracting the average Ct value for the LBR standard from each sample. In order to compare the results between the stages, the normalised Ct values for samples at 9 days of incubation were used as the baseline. To determine the difference in expression between stages, the Ct value for the baseline 9 day of incubation sample was subtracted from the normalised Ct value for each sample. This figure then replaced X in the following formulae to give a value for the increase/decrease in levels of mRNA in the sample compared to the baseline: $=2^{-X}$.

2.6.12 Restriction enzyme digests

Restriction enzymes (Roche; New England BioSciences) were added at a concentration of five units per μg of DNA. 10x Buffer was added to give a final concentration of 1x. Digests were incubated at 37°C for one hour. 4μl of the digest were run on an agarose gel to check the digest had been successful, and was complete.

2.7 Northern analysis

2.7.1 Preparation and running of 1% agarose formaldehyde gel for RNA analysis

To 1g molecular biology grade agarose was added to 10ml 10x MOPS and 73ml double autoclaved water. The agarose was melted in a microwave and allowed to cool to 60°C in a fume hood. 17ml 36% formaldehyde were added. A gel tank and comb were rinsed in 0.2M NaOH, double autoclaved water and RNaseZAP. The gel was poured.

Sample pre-treatment

Ethanol precipitate containing 10µg RNA was centrifuged at 10,000 x g for twenty-five minutes at 4°C and the supernatant removed. The pellet was washed with 500µl cold 85% ethanol, centrifuged at 10,000 x g for ten minutes at 4°C and the supernatant removed. The pellet was air-dried and resuspended in 15µl RNA sample buffer. The RNA was heated at 65°C for ten minutes and placed on ice. 4µl of 6x Type III loading dye and 1µl ethidium bromide (10mg/ml) were added.

Gel electrophoresis

10x MOPS was used as running buffer. The denatured RNA samples were loaded and the gel was run at 80volts for three hours. The gel was rinsed in dH_2O for thirty minutes and then rinsed in 10x SSC for thirty minutes. The RNA was transferred to a Hybond®-N membrane overnight by capillary transfer. The membrane was baked at 80°C for two hours between two pieces of Whatmann's filter paper.

2.7.2 Preparation of radiolabelled probes

ESTs obtained from Ark Genomics were used for the plasmid DNA source (see Appendices 2-9). 2μg of plasmid DNA were digested to isolate the insert and run on an agarose gel. The insert fragment was purified and a quantification gel run. The Rediprime™ II Random Prime Labelling System (Amersham Biosciences) was used to radiolabel the DNA. Approximately 20ng DNA were added to 1x TE to give a final volume of 45μl. The DNA was denatured at 99°C for five minutes, chilled on ice for five minutes and spun down. The denatured DNA was added to a Rediprime tube, and 5μl of P^{32} radiation from a dCTP source (Amersham Biosciences) were added and the solution mixed by pipetting. The tube was incubated at 37°C for thirty minutes, then at 99°C for five minutes, then chilled ice for five minutes and spun down. NICK™ columns (Amersham Biosciences) were used to separate the unincorporated nucleotides from the labelled DNA. The top of a NICK™ column was removed, the liquid poured off and the column was rinsed with 1x TE. The bottom cap was removed and 3ml TE buffer were passed through the column. The Rediprime reaction was added directly onto the filter and allowed to enter the column. 400μl 1x TE were added and discarded. A further 400μl 1x TE were added and collected. The column was washed out twice with 3ml 1x TE. 4μl of the collected probe were added to 1ml scintillation fluid and the activity checked using a Wallace 1410 Liquid Scintillation Counter (Pharmacia).

2.7.3 Northern hybridisation

Membranes were pre-wet in 50mM sodium phosphate pH7.2. 200µl of 100µg/ml Herring sperm DNA (Sigma) and 40µl 25µg/ml tRNA (Sigma) were denatured at 99°C for ten minutes and then chilled on ice for five minutes. The denatured Herring sperm and tRNA were added to 20ml Church buffer that had been pre-heated to 65°C. The membrane was pre-hybridised in the solution for a minimum of four hours at 65°C. A radiolabelled probe with a specific activity of $1\text{-}3x10^9$cpm/µl was added and the membrane was incubated at 65°C overnight. The hybridisation solution was discarded and the membrane rinsed two times for forty-five minutes each in 50mM sodium phosphate pH7.2/0.1% SDA at 65°C and then two times for forty-five minutes each in 25mM sodium phosphate pH7.2/0.1% SDA at 65°C. BioMax MS film (Kodak) was exposed to the membrane at -80°C for varying lengths of time. To remove the radiolabelled probe after exposure, the membranes were incubated for thirty minutes in boiling 0.1% SDS. Stripped membranes were kept at -20 °C until re-use.

2.7.4 Loading control

RT-PCR was carried out using primers to amplify 18S ribosomal DNA (rDNA; see Appendix 1). The product was purified. During Northern hybridisation, 50ng/µg of RNA on the blot of cold rDNA was added to the pre-hybridisation solution. The rest of the protocol was carried out as normal.

2.8 *in situ* hybridisations of whole-mounted tissues and frozen sections

2.8.1 Preparation of DNA template to produce RNA probes

A restriction digest was set up containing the following: DNA, 5µg; appropriate 10x buffer, 5µl; appropriate restriction enzyme, 3µl; and dH_2O to bring the final volume to 50µl. The reaction was briefly vortexed, spun down, and incubated in a water bath at 37°C for 1 hour. 1.5µl of the completed digest were set aside until the end of the procedure.

50µl of TE buffer and 95µl of phenol/chloroform were added to the remaining digest. The tubes were vortexed for 5 seconds and spun down for 2 minutes at 14500 x g at room temperature. The aqueous layer was transferred to an RNase-free microfuge tube. 0.1 times the volume of RNase-free 3M NaAc and 2.5 times the volume 100% ethanol were added. The DNA templates were precipitated at -20°C for a minimum of two hours.

The tubes were centrifuged for 15 minutes at 13000 x g at 4°C and the supernatant was removed. The pellet was air-dried and reconstituted in 30µl RNase-free water. To check the quality of the template, 2.5µl of template were run on a 1% agarose gel. The digest sample previously set aside was also run the 1% agarose gel to check the recovery efficiency. Templates were stored at -20°C.

2.8.2 Synthesis of a DIG-labelled RNA probe

The following 20µl reaction was made up in an RNase-free microfuge tube: linearised DNA template, 5µl; 5x transcription buffer (Promega), 4µl; DIG RNA labelling mix (Roche), 2µl; RNase-free water, 5µl; 10mM DTT (provided with RNA polymerase), 1µl; appropriate RNA polymerase (T3, T7 or SP6; Promega), 2µl; RNAsin (Promega), 1µl. The tubes were flicked and spun down. Tubes were incubated for three hours at either 37°C (T3 and T7) or 40°C (SP6).

2µl of RQ1 DNAse (Promega) and 1µl of RNAsin were added. The tubes were flicked to mix and spun down. 180µl of RNase-free water, 20µl of RNase-free 4M lithium chloride and 600µl of 100% ethanol were added. The RNA probes were precipitated at -80°C for a minimum of 12 hours.

The tubes were centrifuged at 1300 x g at 4°C for 30 minutes and the supernatent removed. The pellet was air-dried. To reconstitute the pellet, 100µl of RNase-free water was added and the pellet was left for one hour at room temperature. The solution was pipetted several times and left for a further hour at room temperature. 4µl of probe was run on a 1% agarose gel to verify the quality and quantity of the probe. Probes were stored at -80°C.

2.8.3 *In situ* hybridisation of whole mount embryos

Embryo pretreatment after dissection

Embryos were dissected in Dulbecco's and fixed in 4% PAF (see section 2.3). After fixing, the 4% PAF was discarded and the embryos were washed three times in PBT.

Embryos were stored in 100% methanol at -20°C and used within one month of fixation.

Rehydration and hybridisation

The embryos were rehydrated through 50% methanol/PBT, allowed to settle, and then washed three times in PBT, being allowed to settle each time. The embryos were treated with 10μg/ml proteinase K (Roche) in PBT, on a roller/shaker at room temperature. The time of proteinase K treatment in minutes was determined by using the Hamburger and Hamilton (1951) stage number of the embryos being treated. For example embryos at stage 15 were treated for 15 minutes. The proteinase K was removed and the embryos were rinsed twice in PBT. The embryos were post-fixed for twenty minutes in post-fix solution on a roller/shaker at room temperature. Embryos at stages 4-10 were post fixed for five minutes. The embryos were rinsed once with PBT and transferred to 2ml round bottomed microfuge tubes. Embryos at stages 4-10 were transferred to 35mm petri-dishes. The embryos were rinsed in 1ml of 1:1 PBT/hybridisation mix, followed by 1ml hybridisation mix. The embryos were incubated for one hour at 65°C in 1ml hybridisation mix. Hybridisation mix was pre-warmed at 65°C for future steps.

Fresh hybridisation mix was added and embryos incubated overnight at 65°C in 1ml pre-warmed hybridisation mix containing 10μl of DIG-labelled RNA probe. Hybridisation mix was pre-warmed at 65°C for future steps.

Post-hybridisation washes

Embryos were rinsed twice with pre-warmed hybridisation mix, and then washed twice for one hour each in 1ml pre-warmed hybridisation mix at 65°C. The embryos were washed twice for ten minutes each in 1ml pre-warmed 1:1 hybridisation mix:TBST/MABT at 65°C (MABT was used instead of TBST when the embryos were younger than three days of incubation). Embryos were rinsed three times with TBST/MABT, and then washed for thirty minutes at room temperature in TBST/MABT on a roller/shaker. Embryos were pre-incubated in 1ml *in situ* hybridisation blocking solution for two hours at room temperature, incubated at 4°C overnight on a roller/shaker in 1ml DIG-AP antibody solution.

Post-antibody washes and histochemistry

Embryos were rinsed three times with TBST and transferred to 15ml polypropelene tubes. Embryos at stages 4-10 were transferred to clean 35mm petri-dishes. Embryos were washed three times for one hour each in 10ml TBST/MABT on a roller/shaker at room temperature. Embryos were washed twice for ten minutes each in 5ml NTMT on a roller/shaker at room temperature. Embryos were transferred to 35mm petri dishes and incubated in 2ml staining solution in the dark. If embryos were to be left overnight the staining solution was replaced with NTMT, and embryos were stored at 4°C. When the colour had developed to the desired extent embryos were observed in PBS solution.

2.8.4 *In situ* hybridisation of tissue sections

Adult tissues were dissected, fixed in 4% PAF, embedded in gelatine-sucrose and sectioned. The sections were defrosted for a minimum of one hour at room temperature. 4µl DIG-labelled RNA probe were added to 0.5ml hybridisation buffer and the solution vortexed. The probe mix was denatured at 70°C for ten minutes. 0.5ml probe mix were added to a slide, and then covered with a RNase free Hybri-Slip (Sigma). Slides were incubated in a slide box containing a paper towl soaked in 1x salts/50% formamide and wrapped in parafilm at 65°C overnight. Glass Coplin jars and metal slide racks were baked at 120°C prior to use. Slides were transferred to a slide rack and washed in a pre-baked glass Coplin for thirty minutes in washing solution at 65°C. The cover slips were allowed to fall away. Two one hour washes in washing solution at 65°C, and then two one hour washes in MABT at room temperature were carried out. A paraffin pen was used to mark off the half of the slide that contained sections. The slides were blocked in 0.5 ml blocking solution made up with MABT for one hour at room temperature in a humidity chamber. 0.5ml DIG-AP antibody solution made up with MABT were added and the slides were incubated overnight at 4°C in a humidity chamber. Slides were washed three times one hour in 0.5ml MABT at room temperature. 0.5ml staining solution were added. Slides were stained at room temperature until the desired intensity was reached.

2.9 Protein detection on frozen sections

2.9.1 Protein detection of frozen sections

Frozen sections were incubated in Dulbecco's medium at 37°C for 30 minutes to remove the remains of the sucrose-gelatine block and then air-dried. The sections were incubated in 1ml 0.3% hydrogen peroxide for thirty minutes at room temperature to quench endogenous peroxidase activity. The sections were washed five times in 1ml Dulbecco's. 1ml antibody blocking serum added and the sections incubated for twenty minutes at room temperature. Primary antibody/antibodies were diluted in Dulbecco's and 1ml was added to the sections. From this point, all steps were carried out in a humidity chamber until mounting. The sections were incubated overnight at 4°C. The sections were rinsed three times in 1ml Dulbecco's and washed for one hour in 1ml Dulbecco's at room temperature. The sections were incubated in 1ml secondary antibody solution for one hour at room temperature. The sections were rinsed three times with 1ml Dulbecco's and washed for one hour in 1ml Dulbecco's at room temperature. Hoechst nuclear staining solution was added to Dulbecco's to give a 1/100 dilution and 1ml was added to the sections for one minute. The sections were rinsed three times with 1ml Dulbecco's and mounted using Hydromount. Sections were viewed under a microscope.

2.9.2 GFP and SSEA-1 co-stain of frozen sections

Primary antibody solution was made up in Dulbecco's containing 1/40 MC-480 SSEA-1 primary antibody (Developmental Studies Hybridoma Bank); 1/500 anti-green fluorescent protein; rabbit IgG fraction, Alexa Fluor® 488 conjugate primary

antibody (Invitrogen); and 15µl/ml of sheep serum. The sections were incubated overnight at 4°C in the primary antibody solution in a humidity chamber. Secondary antibody was applied as described above. Secondary antibody solution contained 1/200 anti-mouse IgM Texas red (Abcam) in Dulbecco's. SSEA-1 positive cells were visible when illuminated by UV light transmitted through a rhodamine filter and GFP positive cells were visible when illuminated by UV light transmitted through a FIT-C filter.

CHAPTER 3

IDENTIFICATION AND EXPRESSION ANALYSIS OF A CHICKEN HOMOLOGUE OF THE POST-TRANSCRIPTIONAL REGULATOR *BRUNO*

3.1 Introduction

In eukaryotic cells, gene expression is controlled by either transcriptional or post-transcriptional mechanisms. This chapter discusses the latter. Post-transcriptional regulation is often activated in response to environmental cues and involves the on/off regulation of particular gene products in a temporally and spatially regulated manner, allowing cells of different types or at different developmental stages to fine-tune their patterns of gene expression (Siomi and Dreyfuss, 1997). Many important events in development, such as pattern formation and terminal differentiation, are regulated by a range of post-transcriptional mechanisms that control mRNA stability, localisation and translation (Curtis *et al.*, 1995; St Johnston, 1995; Wickens *et al.*, 1996).

This chapter focuses on *bruno*, a gene which is a member of the Bruno family of RNA binding proteins (RBPs) that are known to regulate post-transcriptional gene expression. Members of the Bruno family contain an 80-90 amino acid conserved domain called the RNA recognition motif (RRM; Kenan *et al.*, 1991; Birney *et al.*, 1993; Burd and Dreyfuss, 1994). There are two signature sequences within the RRM called ribonucleoprotein 1 (RNP1) and ribonucleoprotein 2 (RNP2). These two RNPs are highly conserved sequences that have been shown to specifically interact

with RNA and to mediate protein-protein interactions (Merill *et al.*, 1988; Nagai *et al.*, 1995; Sakashita *et al.*, 1996; Wang *et al.*, 1997; Samuels *et al.*, 1998; Ding *et al.*, 1999; Draper, 1999).

3.1.1 *Bruno* genes in *Drosophila*

Bruno was first identified through UV cross-linking experiments in *Drosophila* as an ovarian protein that binds specific sequences in the 3'-untranslated region (3'UTR) of *oskar* mRNA (Kim-Ha *et al.*, 1995). A second *bruno* gene has since been found in *Drosophila*, but expression and functional data are still to be published (Good *et al.*, 2000).

Bruno mRNA is first detected throughout the cytoplasm of the nurse cells and the oocyte (Webster *et al.*, 1997). In the oocyte, Bruno protein is localised in a pattern that corresponds to the expression of two mRNAs: *gurken*, which is localised to the anterodorsal zone and *oskar*, which is found in the pole plasm at the posterior of the embryo (Neuman-Silberberg and Schüpbach, 1993; Webster *et al.*, 1997). Bruno protein localised to the anterodorsal zone acts to accumulate Gurken protein during dorsal-ventral patterning of the oocyte, and Bruno protein found at the posterior pole is involved in the localisation of Oskar protein (Lie and Macdonald, 1999; Yan and Macdonald, 2004; Chekulaeva *et al.*, 2006). During oogenesis, *bruno* mRNA is detected in all of the germ cells when the egg is in region 2A of the germarium (Webster *et al.*, 1997).

In *Drosophila*, Bruno protein plays a key role in the localisation of Oskar protein during early development. Oskar protein is required for pole cell formation and patterning of the posterior of the embryo (Lehmann and Nüsslein-Volhard, 1986). To ensure these two events happen correctly, both *oskar* mRNA and Oskar protein must be properly localised to the posterior of the embryo (Ephrussi and Lehmann, 1992; Kim-Ha *et al.*, 1999). Before it is localised to the posterior of the oocyte, *oskar* mRNA is translationally silenced, a process that is partly responsible for the localisation of Oskar protein. This repression is mediated by *cis*-acting sequences in the 3'UTR of *oskar* mRNA called Bruno response elements (BREs) and the corresponding *trans*-acting factor, the Bruno protein (Good *et al.*, 2000; Chekulaeva *et al.*, 2006). Deletion of the BREs in the *oskar* mRNA 3'UTR results in inappropriate translation of *oskar* mRNA at the anterior end of the oocyte, meaning that embryos develop two posterior poles (Lie and Macdonald, 1999). Repression of *oskar* translation by Bruno is stopped once *oskar* mRNA is localised to the posterior pole of the oocyte (Kim-Ha *et al.*, 1995; Markussen *et al.*, 1995; Rongo *et al.*, 1995; Wilson *et al.*, 1996). Although the mechanism of reactivation of *oskar* mRNA translation mechanism is currently unknown, it is thought that Vasa protein is involved because Vasa protein is known to be required for efficient activation of *oskar* translation and has been shown to physically interact with Bruno protein (Hay *et al.*, 1990; Lasko and Ashburner, 1990; Markusson *et al.*, 1995; Rongo *et al.*, 1995; Webster *et al.*, 1997). This regulation of localised expression of Oskar demonstrates that Bruno has a key role in germ cell formation and in early embryogenesis in *Drosophila*.

3.1.2 Conservation of *bruno* genes

Although *bruno* gene sequences have been identified in many vertebrate species, to date only data on zebrafish and human *bruno* genes have been published. In zebrafish, the number of *bruno-like* genes is still to be determined, but from looking at the zebrafish genome it is expected that there are between two and four *bruno-like* genes. Data has only been published on one of the zebrafish genes identified. Suzuki *et al.* (2000) identified a *bruno-like* gene with high homology to the following: human CUG-binding protein 1 also known as human Bruno-like2; mammalian and *Xenopus* Etr-3; *Xenopus* EDEN-BP also known as *Xenopus* Bruno-like2; and *Drosophila* Bruno. Zebrafish *bruno-like* mRNA is initially detected ubiquitously in the early oocyte, but is then localised to the vegetal pole (Good *et al.*, 2000; Suzuki *et al.*, 2000). This vegetal localisation of *bruno-like* is similar to the localisation of the germ plasm specific mRNA *dazl*. Between the 2-cell stage of development and 24 hours post-fertilisation (hpf), *bruno-like* mRNA is detected uniformly throughout the embryo (Suzuki *et al.*, 2000). Bruno-like protein is also detected ubiquitously at these times, but at the 4-cell stage of development there is evidence of protein accumulation at the ends of the cleavage furrows, which corresponds to the areas where zebrafish germ plasm is assembled (Hashimoto *et al.*, 2006). At 28hpf the levels of both *bruno-like* mRNA and protein detected increase throughout the embryo, but particularly in the lens fiber cells and somites (Suzuki *et al.*, 2000; Hashimoto *et al.*, 2006).

In humans, Good *et al.* (2000) identified the Bruno family of RNA-binding proteins consisting of six members. Of the six *bruno-like* genes in humans, data has only

been published on *bruno-like2*, previously identified as CUG-binding protein 1, and *bruno-like3*. The two genes are 80% identical over the entire length of the protein and 92% identical to each other in the RNA-binding domains, suggesting that they bind to the same targets (Good *et al.*, 2000). Northern analysis shows that the expression patterns of the two genes are quite different: *bruno-like2* is expressed ubiquitously, whereas *bruno-like3* is detected predominantly in the heart, muscles and nervous system (Good *et al.*, 2000). No data on any of the six human *bruno-like* genes has been published in relation to germ cells or germ cell development.

To summarise, it is known that Bruno protein is required during germ cell specification in *Drosophila*. The accumulation of Bruno-like protein at the cleavage furrows of the 4-cell stage zebrafish embryo suggests that the protein could have a role in pole cell specification in zebrafish, which would indicate that *bruno* has a conserved function between invertebrates and vertebrates. However, there is currently no published evidence in higher vertebrates that would suggest this is the case. The potential role of *bruno* in vertebrate PGC specification and the results from zebrafish that show an accumulation Bruno protein in the germ plasm make this gene interesting to this research. The following sections in this chapter will describe the identification and expression analysis of a chicken *bruno-like* homologue, and will discuss whether the gene could have a conserved role in chicken PGC specification.

3.2 Overview of methods

This section gives a brief overview of the methods and samples used to investigate *bruno* in PGC development. For a full account of the methods see chapter 2.

3.2.1 BLAST analysis of the chicken genome and EST databases

To identify putative *bruno* homologues in the chicken genome, BLAST searches of the genome sequence were carried out using as the query sequence the zebrafish *bruno-like* nucleotide sequence (acc.no. NM_131613). This sequence is the zebrafish *bruno* gene whose protein has been found at higher levels in the germ plasm (see Section 3.1.3) and is also known as CUG binding protein 1 (CUGbp-1).

3.2.2 Samples for RT-PCR and Northern analysis

RNA was extracted from chicken embryos at several stages of development and several adult tissues. The embryonic stages were: whole embryos at one and three days of incubation; gonads and mesonephros at five and eight days of incubation; the remaining embryo after the gonad and mesonephros had been removed at five and eight days of incubation. Adult tissues were taken from: brain; kidney; liver; ovary and testes.

3.2.3 RT-PCR analysis

RT-PCR primers to detect *bruno* were designed flanking an intron using primer 3 software (see Appendix 1; http://frodo.wi.mit.edu/). The primers hybridise to nucleotides located at 273-461bp of the predicted full-length sequence to give an

expected product size of 188bp. Amplification of β-actin using primers to give a product with an expected size of 428bp was used as a positive control. The annealing temperature was 59.5°C.

To make sure the PCR primers were amplifying the predicted *CUGbp-1* sequence, the RT-PCR product was cloned from testes cDNA and then sequenced. The sequence data were aligned with the expected product sequence resulting in a 100% match. This confirmed that the PCR primer set was amplifying the correct sequence.

3.2.4 Northern and *In situ* hybridisation probes

The EST ChEST696m9 from ARKGenomics was used to make a radiolabelled Northern probe (see Appendix 2).

The RT-PCR product cloned from testes cDNA was used to make sense and anti-sense digoxigenin *in situ* hybridisation probes. Whole mount *in situ* hybridisation was carried out on embryos at one, three and five days of incubation.

3.3 Results

3.3.1 Identification of a chicken *bruno* homologue

BLAST searches of the chicken genome identified one predicted chicken *bruno-like* homologue that is located on chromosome 5 at location 24988410-24992600. The predicted gene consists of 14 exons that are predicted to encode a transcript 4,487bp long and spans approximately 48kb of the genome. A further five Bruno family

members have since been identified using the 'Gene Tree' function in the Ensembl database (see Figure 3.1). These additional Bruno family members will not be investigated further here because of the six Bruno family members found in the genome, the one identified by the initial BLAST search of the chicken genome is the most similar to the zebrafish protein and as such is most likely to be involved in PGC development.

The naming of the predicted gene on chromosome 5 in reference to homologues in other species is quite complicated because of the naming of human Bruno family members by Good *et al.* (2000). The naming of the human family members does not take into account homologues of lower vertebrates or invertebrates, and as such the numbering of the *bruno-like* genes between species is inconsistent and somewhat confused in the literature. In the NCBI database and the Ensembl genome, the genes that are most closely related to the predicted chicken sequence are also known as *CUGbp-1*. Therefore, to avoid confusion the chicken Bruno family member identified on chromosome 5 shall be referred to as chicken *CUGbp-1* and it's protein as CUGbp-1 from this point forwards.

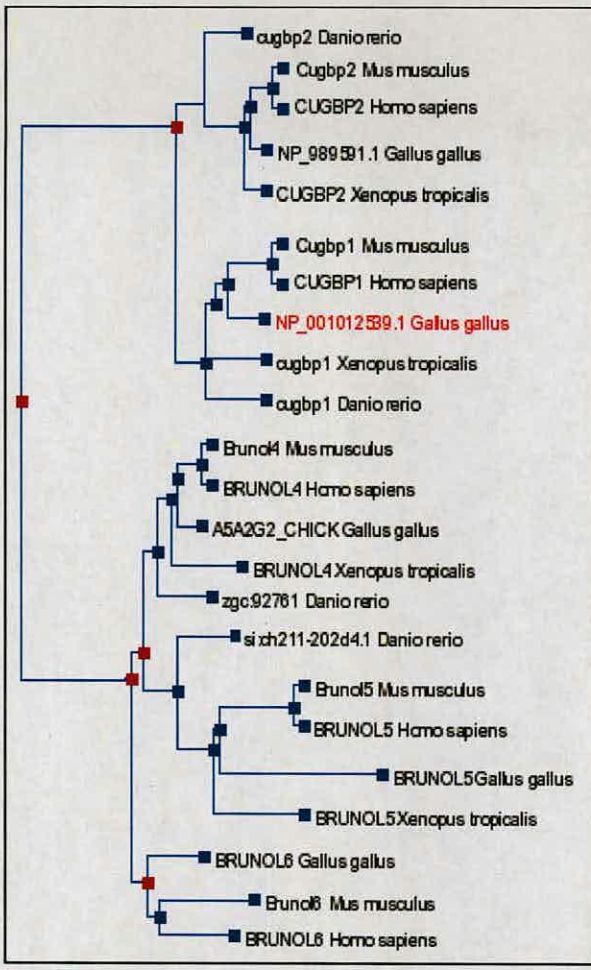


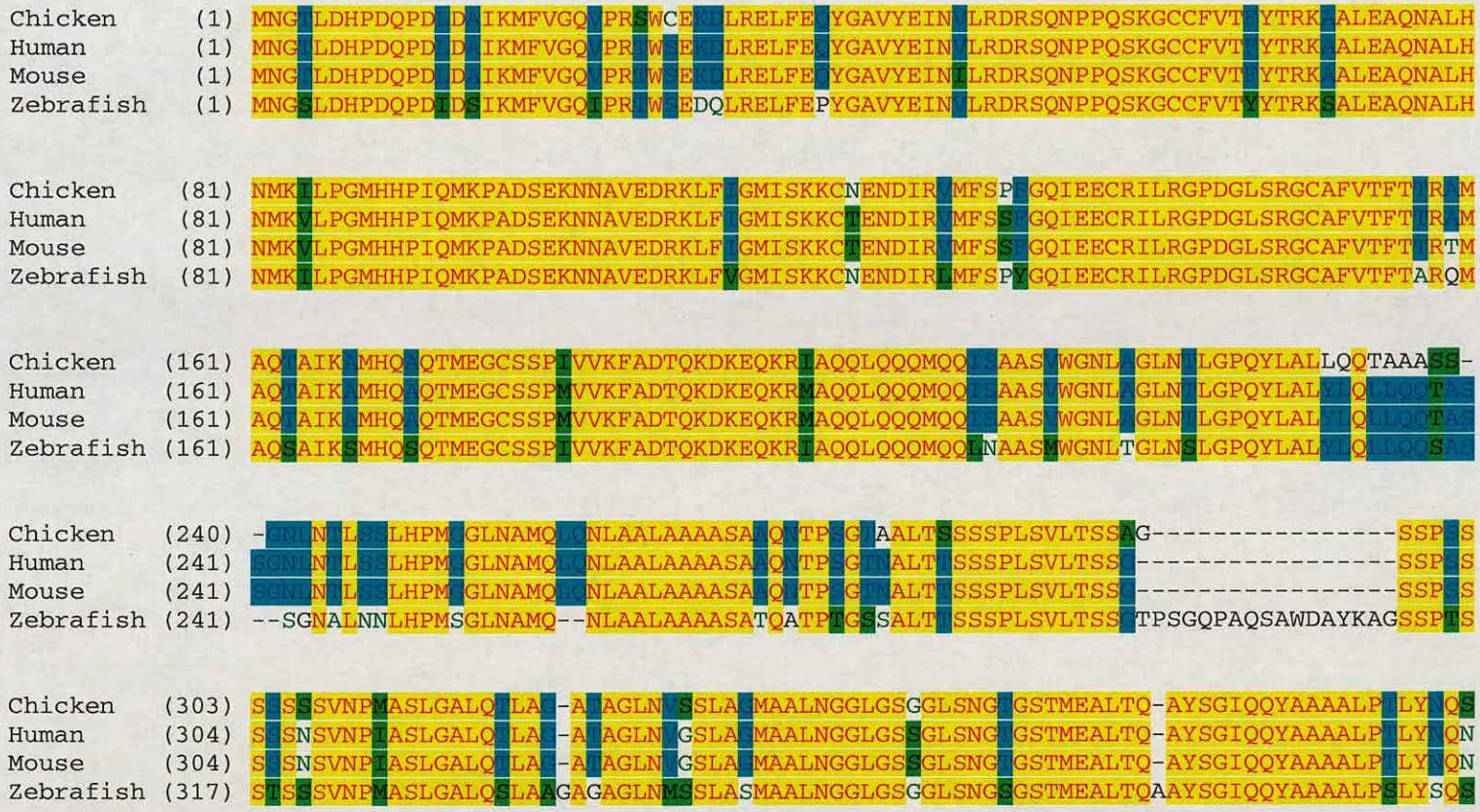
Figure 3.1: A gene tree of the Bruno family. The Gene Tree was generated in www.ensembl.com using PHYML displaying the maximum likelihood polygenetic gene tree (Guindon et al, 2005). The red squares represent gene duplication nodes, the blue squares represent speciation nodes and the branch length represents the number of substitutions that have occurred since the common ancestor. The gene highlighted in red is the gene identified by the BLAST searches of the genome as *chicken CUGbp-1*, or *chicken bruno-like 2*. The gene tree has been modified to show genes from the bruno family in humans, mice, zebrafish, *Xenopus* and zebrafish only and shows the complexity of the gene family.

In order to confirm that the sequence identified in the genome was accurate, BLAST searches of the chick UMIST database were carried out to identify previously isolated and cloned ESTs. The BLAST searches identified several ESTs with identical sequences to the chicken *CUGbp-1* sequence obtained from the chicken genome, indicating that the genome data is accurate (see Appendix 2). To confirm the identity of the sequence, the predicted chicken protein sequence was used as the query sequences to BLAST the NCBI database. The resulting hits with the highest similarity to the query sequence were CUGbp-1 proteins. Additionally, the BLAST search of the NCBI database identified three conserved RNA recognition motif domains within the protein sequence. To determine the level of conservation between species, the chicken CUGbp-1 protein sequence was aligned with known CUGbp-1 protein sequences from zebrafish, mice and humans (see Figure 3.2 and Table 3.1). The predicted chicken sequence is 95% identical to human CUGbp-1 protein, 94% identical to mouse CUGbp-1 protein, 91% identical to zebrafish CUGbp-1 protein and 37% identical to *Drosophila* Bruno protein. These percentage identities are high, indicating a high level of conservation between species.

Gene (species)	E value	Identity	Similarity
CUG bp1 *(Homo sapiens)*	0.0	95%	97%
CUG bp1*(Mus musculus)*	0.0	94%	96%
CUG bp1*(Danio rerio)*	0.0	91%	94%
Bruno *(D. melanogaster)*	8e-112	37%	49%

Table 3.1: E values, the percentage of identical residues (Identity) and the percentage of conservative substitutions (Similarity) are shown to identity of the predicted chicken Bruno protein.

Figure 3.2:



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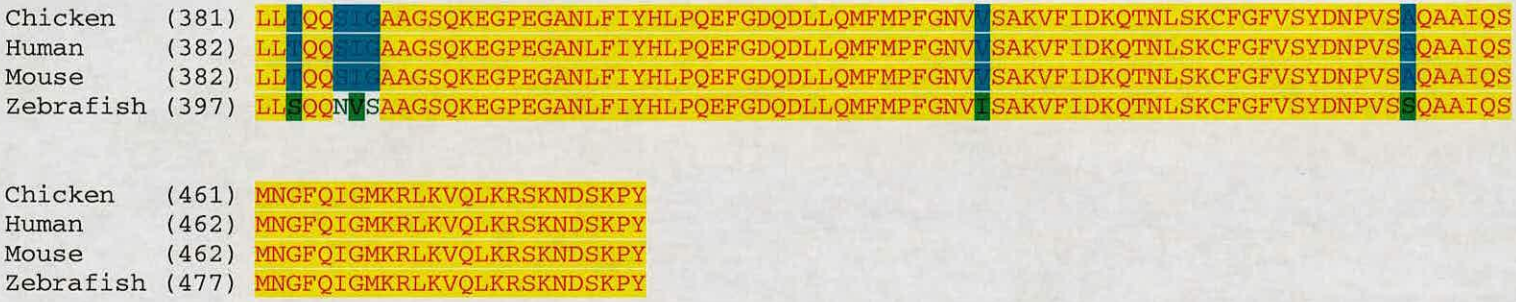


Figure 3.2 continued: Alignment of the identified chicken CUGbp-1 protein sequence with known CUGbp-1 protein sequences from human, mice and zebrafish. Identical residues are highlighted in yellow. Residues that are identical between two or three species are highlighted in blue. Residues that are different between species but where the different residues are predicted to act the same way are highlighted in green.

3.3.2 Detection of chicken *CUGbp-1* expression by RT-PCR and Northern analysis

RT-PCR was carried out on RNA from embryonic and adult tissues to determine when during development and which adult tissues express *chicken CUGbp1* (see Section 3.2.2). In embryonic tissues, *chicken CUGbp-1* mRNA was detected in all samples after 25 cycles of amplification (see Figure 3.3A). In adult tissues, *chicken CUBbp-1* was detected in all samples after 25 cycles of amplification (see Figure 3.3C). This expression is consistent with results from human (Good *et al.*, 2000) and zebrafish (Hashimoto *et al.*, 2006).

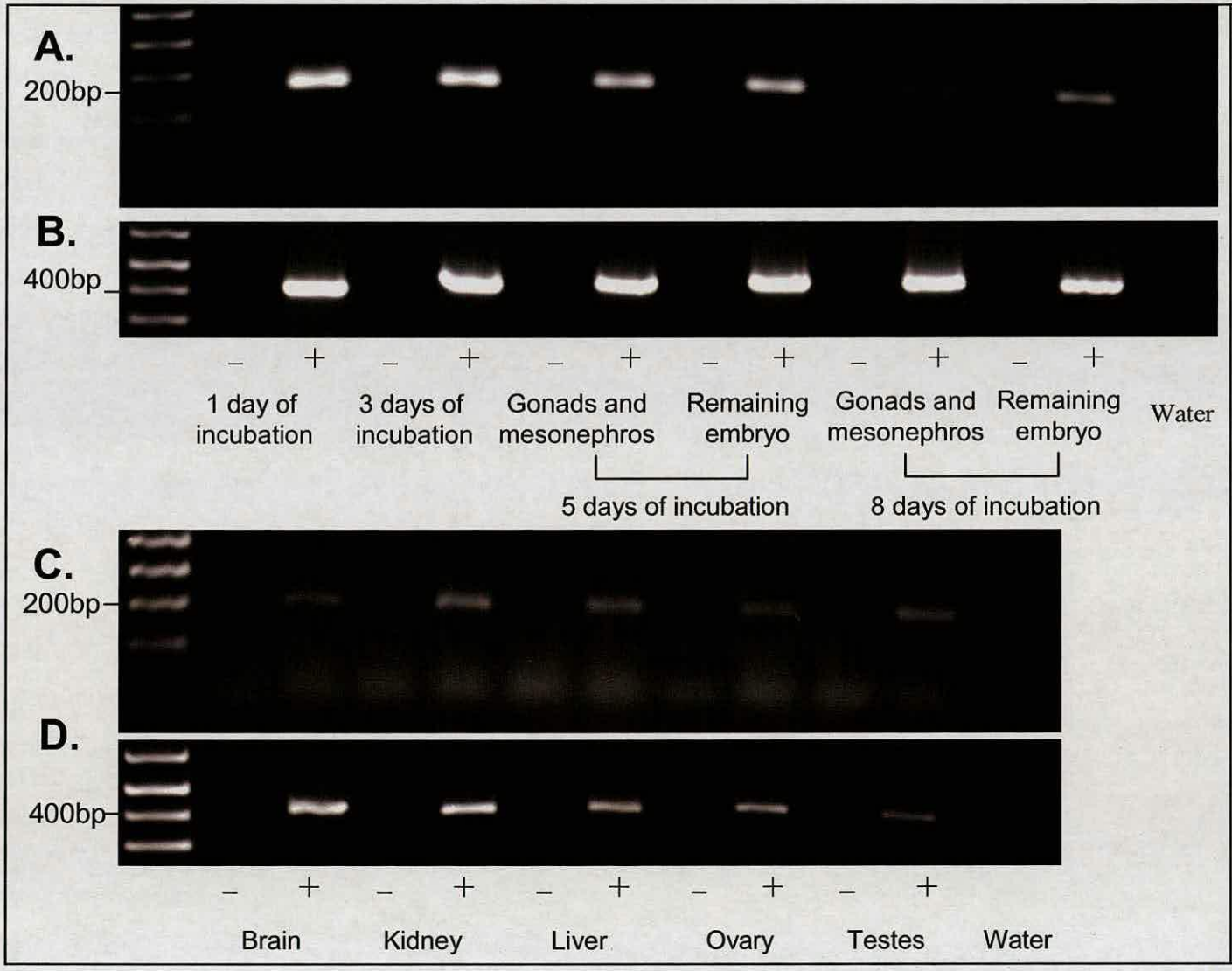


Figure 3.3: RT-PCR to detect the expression of *CUGbp-1* nRNA in embryonic and adult tissues. (A) *CUGbp-1* expression in embryonic tissues. (C) *CUGbp-1* expression in adult tissues. (B and D) ß-actin positive controls. +: indicates the presence of reverse transcriptase during cDNA synthesis. -: indicates the absence of reverse transcriptase during cDNA synthesis.

Northern analysis was carried out to extend the RT-PCR data. The radiolabelled probe did not hybridise to any of the embryonic or adult samples (see Figure 3.4). The probe synthesised was 87.5x103 cpm/µl, indicating that radiolabel had bound to the DNA in the reaction tube, but there is still the possibility that there was a technical error in the synthesis of the probe. In future experiments, use of poly A+ RNA instead of total RNA would increase the sensitivity of the analysis.

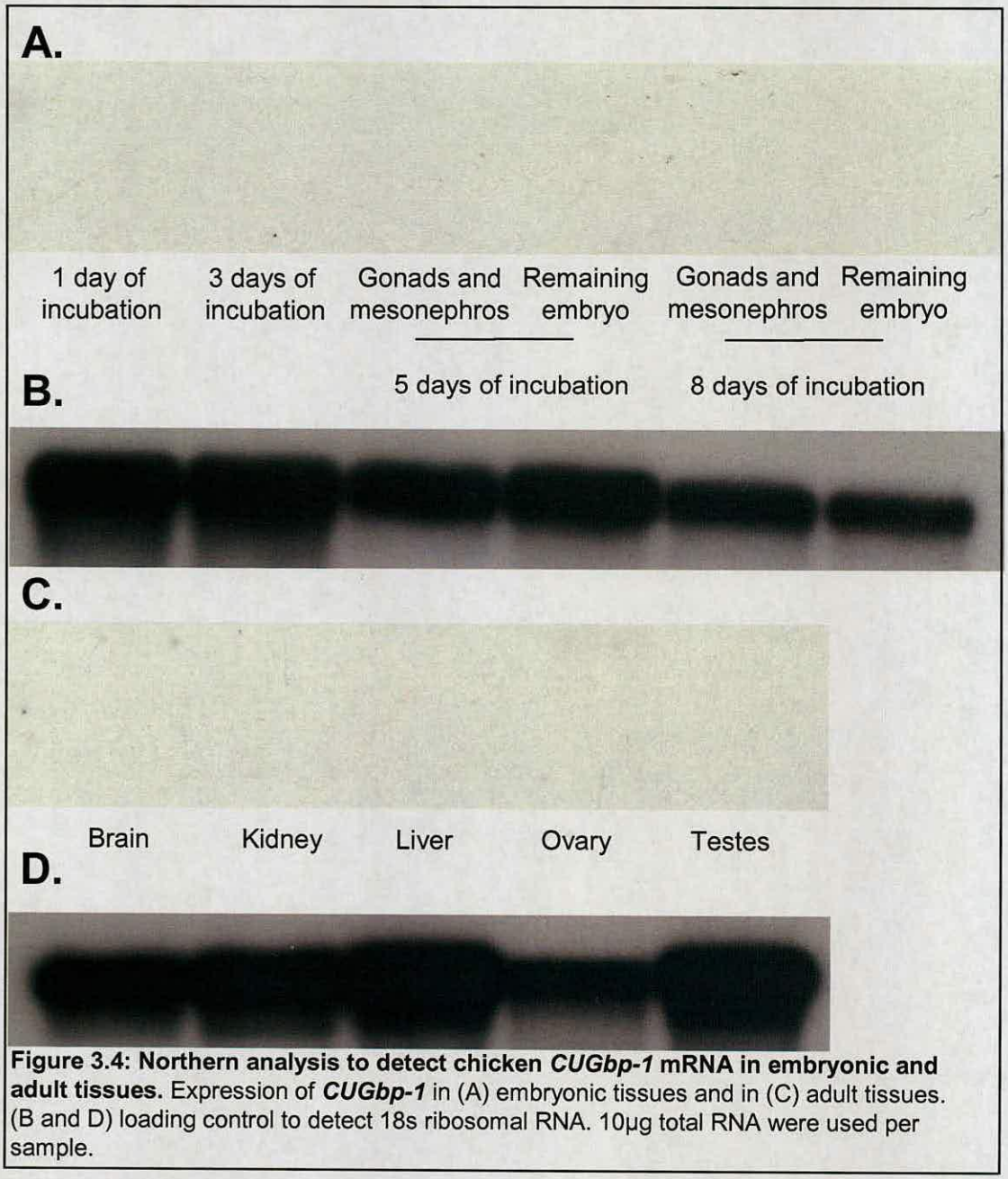


Figure 3.4: Northern analysis to detect chicken *CUGbp-1* mRNA in embryonic and adult tissues. Expression of ***CUGbp-1*** in (A) embryonic tissues and in (C) adult tissues. (B and D) loading control to detect 18s ribosomal RNA. 10µg total RNA were used per sample.

3.3.3 Expression of *chicken CUGbp-1*

The RT-PCR data indicates that *chicken CUGbp-1* mRNA is found in all embryonic stages tested and the Northern data was inconclusive. To further extend these results and identify which areas of the embryo express *chicken CUGbp-1*, a series of *in situ* hybridisations on whole-mount embryos at one, three and five days of incubation were carried out. Using the RT-PCR product cloned from testes cDNA (see Section 3.3.2), sense and anti-sense digoxigenin *in situ* hybridisation probes were made for *chicken CUGbp-1*.

At one day of incubation *chicken CUGbp-1* was faintly detected surrounding the primitive streak (1) and in a crescent of expression in a region that will develop into the head fold (2; see Figure 3.5A).

At three days of incubation, *chicken CUGbp-1* was detected in the eye and in the somites. The expression in the eye is strong in comparison to the expression ion the somites, which is weak (see Figure 3.5 C). The sense controls indicate that these are genuine signals and not background (see Figure 3.5D). This staining pattern is the same as that observed in zebrafish at 28hpf.

At five days of incubation staining is detected in the mesoderm (see Figure 3.5). Sectioning of the embryo revealed that *CUGbp-1* is expressed in deep mesoderm of the embryo (see Figure 3.5 F and G). Interestingly, *CUGbp-1* expression is excluded from the dorsal third of the neural tube, indicating a possible role for *CUGbp-1* in

neural tube patterning (1). This patterning of the neural tube is not observed in the posterior of the embryo (2).

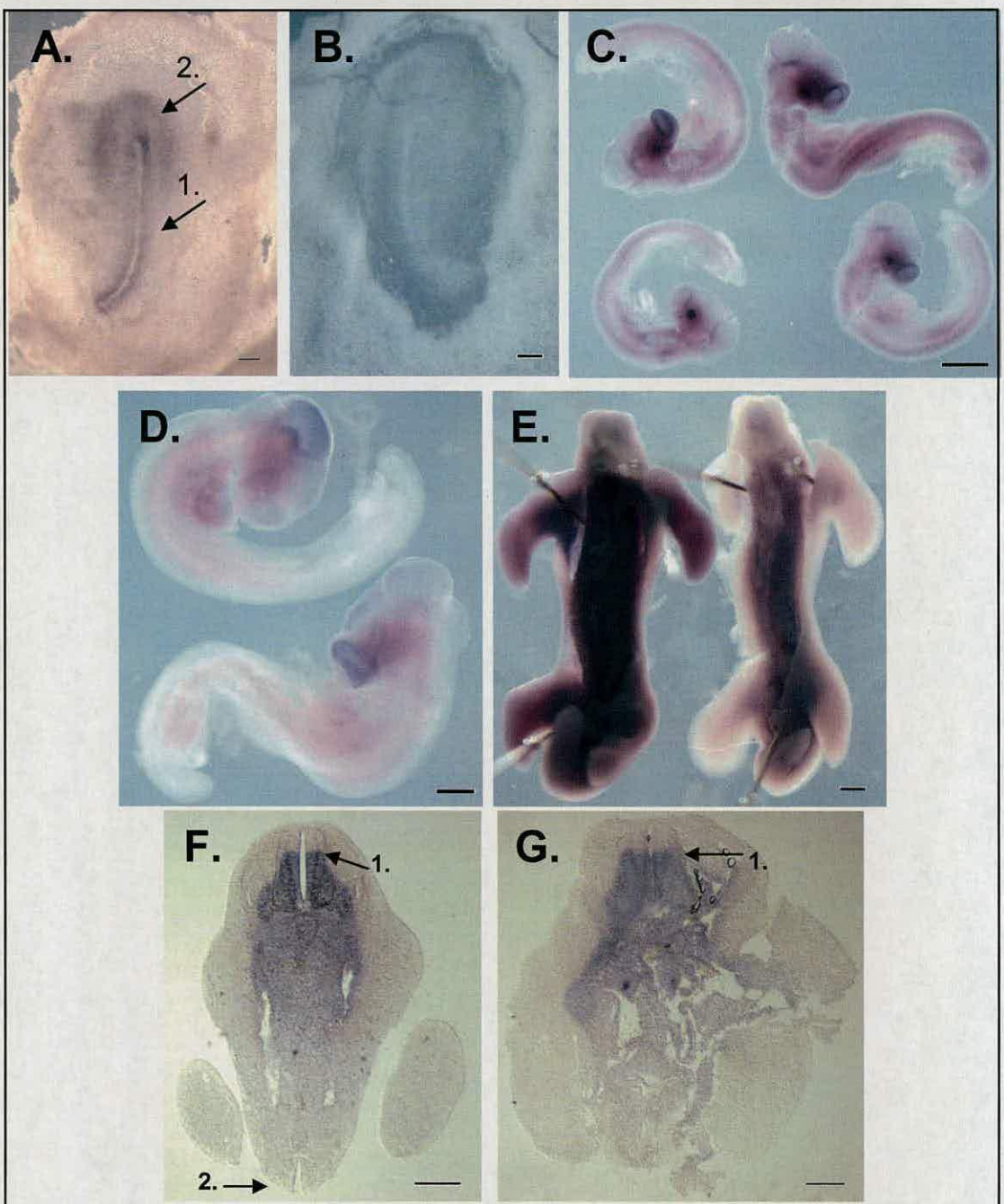


Figure 3.5: *In situ* hybridisation to detect *CUGbp-1* in embryos at 1 day, 3 days and 5 days of incubation. (A) 1 day of incubation anti-sense. (1) primitive streak, (2) head fold. (C) 3 days of incubation anti-sense. (E) 5 days of incubation anti-sense probe on left and sense probe on right. (F and G) 20μ sections through (E left). (B and D) sense controls. (1) neural tube pattering in the anterior. (2) absence of neural tube pattering in the posterior Scale bar represents 250μm.

neural tube patterning (1). This patterning of the neural tube is not observed in the posterior of the embryo (2).

Figure 3.5

3.4 Discussion

Chicken CUGbp-1, or *chicken bruno-like 2*, was identified in a BLAST search of the chicken genome and an expression profile developed for the gene. RT-PCR and *in situ* hybridisation analyses determined that *chicken CUGbp-1* mRNA is found throughout development, and the RT-PCR results indicate that the expression is maintained in a variety of adult tissues. The *in situ* hybridisation analyses have indicated that the *chicken CUGbp-1* is expressed in the deep mesoderm of embryos. The expression pattern of *chicken CUGbp-1* at five days of incubation indicates that the protein may function in patterning of the neural tube.

In relation to PGC development, these preliminary experiments do not indicate a function for *chicken CUGbp-1* during PGC development because the mRNA is not expressed in the germinal crescent at one day of incubation, the germinal ridge at three days of incubation or the developing gonad at five days of incubation. As previously described, *bruno* genes function during germ plasm assembly in *Drosophila* and zebrafish. In the stages of chicken development investigated here germ plasm has already been assembled and PGCs have formed. Therefore, *chicken CUGbp-1* could still have a role in PGC development in earlier stages of chicken development. It was decided not to pursue this gene any further based on observations in zebrafish. In zebrafish, Bruno protein is found at higher levels in the germ plasm of four-cell stage embryos, whereas the mRNA is ubiquitously expressed. The sequences of zebrafish *bruno* and *chicken CUGbp-1* are 91% identical, indicating good conservation between the two species. Additionally, the expression pattern of *chicken CUGbp-1* mRNA at three days of incubation is similar

to that observed in zebrafish embryos at 28 hours post fertilisation. Therefore, the next step in investigating a potential role for *chicken CUGbp-1* in PGC development is to look at protein expression in the early embryo. In order to do this an antibody would have to be raised against chicken CUGbp-1 protein because one is not commercially available. This process is very time consuming. Additionally, in order to investigate development prior to germ plasm formation, eggs would have to be removed from the oviducts of hens. This would mean killing multiple hens. Taking these two points into consideration it was decided to focus on other genes identified in the chicken genome rather than doing further work on *chicken CUGbp-1*.

CHAPTER 4

IDENTIFICATION AND EXPRESSION ANALYSIS OF A *GERM CELL-LESS* HOMOLOGUE IN THE CHICKEN

4.1 Introduction

4.1.1 Transcriptional repression

Transcriptional silencing is an important process during germ cell development. It is hypothesised that this prevents them from either differentiating into somatic cells or apoptosing (Lamb and Laird, 1976; Zalokar, 1976; Seydoux and Fire, 1994; Seydoux *et al.*, 1996). A gene that functions to transcriptionally silence germ cells in early *Drosophila* development is *germ cell-less* (see Section 4.1.2). The protein product of *germ cell-less*, Germ cell-less protein, contains a conserved BTB/POZ protein-protein interaction domain that is known to mediate transcriptional repression (Deweindt *et al.*, 1995; Huynh and Bardwell, 1998; Wong and Privalsky, 1998). The BTB domain, also known as the POZ (poxvirus and zinc finger) domain, is a protein-protein interaction module consisting of approximately 120 amino acids that is found in over 600 different proteins in organisms ranging from yeast to humans. The BTB domain is often found at the N-termini of zinc finger transcription factors as well as Shaw-type potassium channels. Experimental studies have implicated that the BTB domain serves to organise higher order macromolecular complexes involved in nuclear events such as chromatin folding (Albagli *et al.*, 1995).

This chapter reviews existing literature on the transcriptional repressor gene *germ cell-less* and its protein Germ cell-less. In particular, it will discuss the role of *germ cell-less* in PGC development in *Drosophila* and expression data from vertebrate species. The remainder of the chapter presents the identification of a partial chicken *germ cell-less* homologue in the genome and its expression pattern in chicken embryonic and adult tissues.

4.1.2 *Germ cell-less* in *Drosophila*

Germ cell-less was first identified in *Drosophila* in a genetic screen for grandchildless mutants. The mRNA is first detected in the nurse cells of stage 8 egg chambers, but is not detected in the oocyte until stage 10 (Jongens *et al.*, 1992). Between stage 11 of oogenesis and egg deposition the mRNA is localised to the posterior of the oocyte, which leads to the localisation of Germ cell-less protein at the posterior pole (Jongens *et al.*, 1992). At the posterior pole the protein induces the formation of pole cell pre-cursors called pole buds (Jongens *et al.*, 1994). After the pole buds develop into pole cells, both *germ cell-less* mRNA and Germ cell-less protein are detected specifically in the pole cells, with the protein localised to the nuclear envelope (Jongens *et al.*, 1992 and 1994; Robertson *et al.*, 1999). *Germ cell-less* mRNA remains detectable in the pole cells until early gastrulation, shortly before pole cells start to migrate (Jongens *et al.*, 1992). Later in development, the mRNA is no longer detected in the germ cells, but is found in a number of other tissues including the fore- and hindgut, muscle and the central nervous system (Jongens *et al.*, 1992).

The expression pattern described above suggests that Germ cell-less protein in *Drosophila* has a role during pole cell specification, but not in later pole cell development. This hypothesis was confirmed in *germ cell-less* mutants and overexpression experiments. Examination of mutant flies that have reduced levels of maternal *germ cell-less* mRNA (*germ cell-less* null embryos) do not form pole cells (Robertson *et al.*, 1999). In order to determine how Germ cell-less protein functions in pole cell development, expression of germ plasm components in *germ cell-less* null embryos was examined. These results suggested that the failure to form pole cells was due to a direct requirement of Germ cell-less protein and not due to a defect in the formation, maintenance or levels of germ plasm (Robertson *et al.*, 1999). Examination of embryos that overexpress Germ cell-less protein determined that extra pole cells are formed (Jongens *et al.*, 1994). However, when Germ cell-less protein is mislocalised to the anterior pole it is not sufficient to cause mislocalisation of other pole plasm factors or cause the formation of ectopic pole cells at the anterior (Jongens *et al.*, 1994). The results from the *germ cell-less* null embryos have shown that Germ cell-less is required for germ cells to form, but the results do not tell us how the gene functions.

The function of Germ cell-less protein was determined when wildtype embryos stained with an antibody against active transcription were compared with *germ cell-less* null embryos stained with the same antibody. This experiment found that prior to pole cell formation the pole bud nuclei of the wildtype embryos were transcriptionally silenced, whereas in the *germ cell-less* null embryos this did not happen (Leatherman *et al.*, 2002). Furthermore, it was found that the failure to

establish quiescence is strongly correlated with failure to form pole cells (Leatherman *et al.*, 2002).

These experiments in *Drosophila* have shown that Germ cell-less protein is required for germ cells to maintain their identity and the protein causes transcriptional repression. This demonstrates that transcriptional repression is essential for pole cell specification in *Drosophila*.

4.1.3 Conservation of *germ cell-less* function in vertebrates

Germ cell-less homologues have been identified in many vertebrate species, but to date research has only been published on *germ cell-less* homologues in zebrafish and mice. In zebrafish, *germ cell-less* mRNA is detected in all blastomeres from the cleavage stage to the blastula stage, which is different to the pattern observed for known germ plasm factors such as *vasa*, *nanos1* and *dead end* (Li *et al.*, 2006). During PGC migration, *germ cell-less* mRNA is found in two clusters of cells migrating towards the gonads, suggesting expression in the PGCs (Li *et al.*, 2006). This expression pattern suggests that *germ cell-less* functions during PGC migration. It is currently unknown whether *germ cell-less* has a role during PGC specification, although Li *et al.* (2006) argue that their results show that *germ cell-less* is required in zebrafish PGC specification. In adults, *germ cell-less* mRNA is detected in the ovary in developing oocytes of different stages and in the testes in germ cells (Li *et al.*, 2006).

In mice, two *germ cell-less* homologues have been identified, but research has only been published on one of these. Kimura *et al.* (1999) identified and cloned a mouse *germ cell-less* homologue with 34% identity to *Drosophila* Germ cell-less protein. Since its identification, a second *germ cell-less* homologue was identified in mice, leading to the renaming of the first gene to *mouse germ cell-less 1* (*mgcl-1*; Leatherman *et al.*, 2000). In embryonic stages, *mgcl-1* mRNA was detected in post-migratory PGCs, but not migratory or pre-migratory PGCs (Kimura *et al.*, 1999). In adult tissues, *mgcl-1* mRNA is highly expressed in the testes where it is expressed in pachytene stage spermatocytes (Kimura *et al.*, 1999). The expression pattern for *mgcl-1* described above are quite different to those observed in *Drosophila* and zebrafish and suggest that *mgcl-1* does not have a function during early PGC development, but does in later stages of male gametogenesis. Homozygous mutant mice for *mgcl-1* were produced to determine when during development *mgcl-1* functioned. In the mutant mice, it was found that pachytene sperm had abnormal nuclei, which ultimately resulted in a significant reduction in male fertility (Kimura *et al.*, 2003; Maekawa *et al.*, 2004). However, because germ cells had developed, it would suggest that *mgcl-1* does not have a function during PGC development.

To summarise, *germ cell-less* is a gene that has a vital role in early pole cell formation in *Drosophila* and expression patterns indicate that the zebrafish homologue has a role in migratory PGCs and possibly in pre-migratory PGCs. In mice, *mgcl-1* expression is not detected until after PGC migration is complete and the phenotype observed in *mgcl-1* null mice rule out a function in early germ cell

development. However, the second *mouse germ cell-less* gene could be required during early PGC specification.

4.2 Overview of methods

This section gives a brief overview of the methods and samples used to investigate *germ cell-less* in PGC development. For a full account of the methods see chapter 2.

4.2.1 BLAST analysis of the chicken genome and EST databases

To identify putative *germ cell-less* homologues in the chicken genome, BLAST searches of the genome sequence were carried out using the mouse *germ cell-less* cDNA nucleotide sequence (acc.no. NM_011818.2) as the query sequence.

4.2.2 Samples for RT-PCR and Northern analysis

RNA was extracted from chicken embryos at several stages of development and several adult tissues. The embryonic stages were: whole embryos at one and three days of incubation; gonads and mesonephros at five and eight days of incubation; the remaining embryo after the gonad and mesonephros had been removed at five and eight days of incubation. Adult tissues were taken from: brain; kidney; liver; ovary and testes.

4.2.3 RT-PCR analysis

RT-PCR primers to detect *germ cell-less* were designed flanking an intron using primer 3 software (see Appendix 1; http://frodo.wi.mit.edu/). The primers hybridise

to nucleotides located at 64-102bp of the predicted full-length sequence to give an expected product size of 42bp. Amplification of β-actin using primers to give a product with an expected size of 428bp was used as a positive control. The annealing temperature was 59.5°C.

To make sure the PCR primers were amplifying the predicted *germ cell-less* sequence, the RT-PCR product was cloned from testes cDNA and then sequenced by the Sequencing Service (University of Dundee). The sequence data were aligned with the expected product sequence resulting in a 100% match, confirming that the PCR primer set was amplifying the correct sequence.

4.2.4 Northern and *In situ* hybridisation probes

The EST ChEST703j13 from ARKGenomics was used to make a radiolabelled Northern probe (see Appendix 3).

The RT-PCR product cloned from testes cDNA was used to make sense and anti-sense digoxigenin *in situ* hybridisation probes. Whole mount *in situ* hybridisation was carried out on embryos at one, three and five days of incubation and 20μ frozen testes sections.

4.3 Results

4.3.1 Identification of a chicken *germ cell-less* homologue

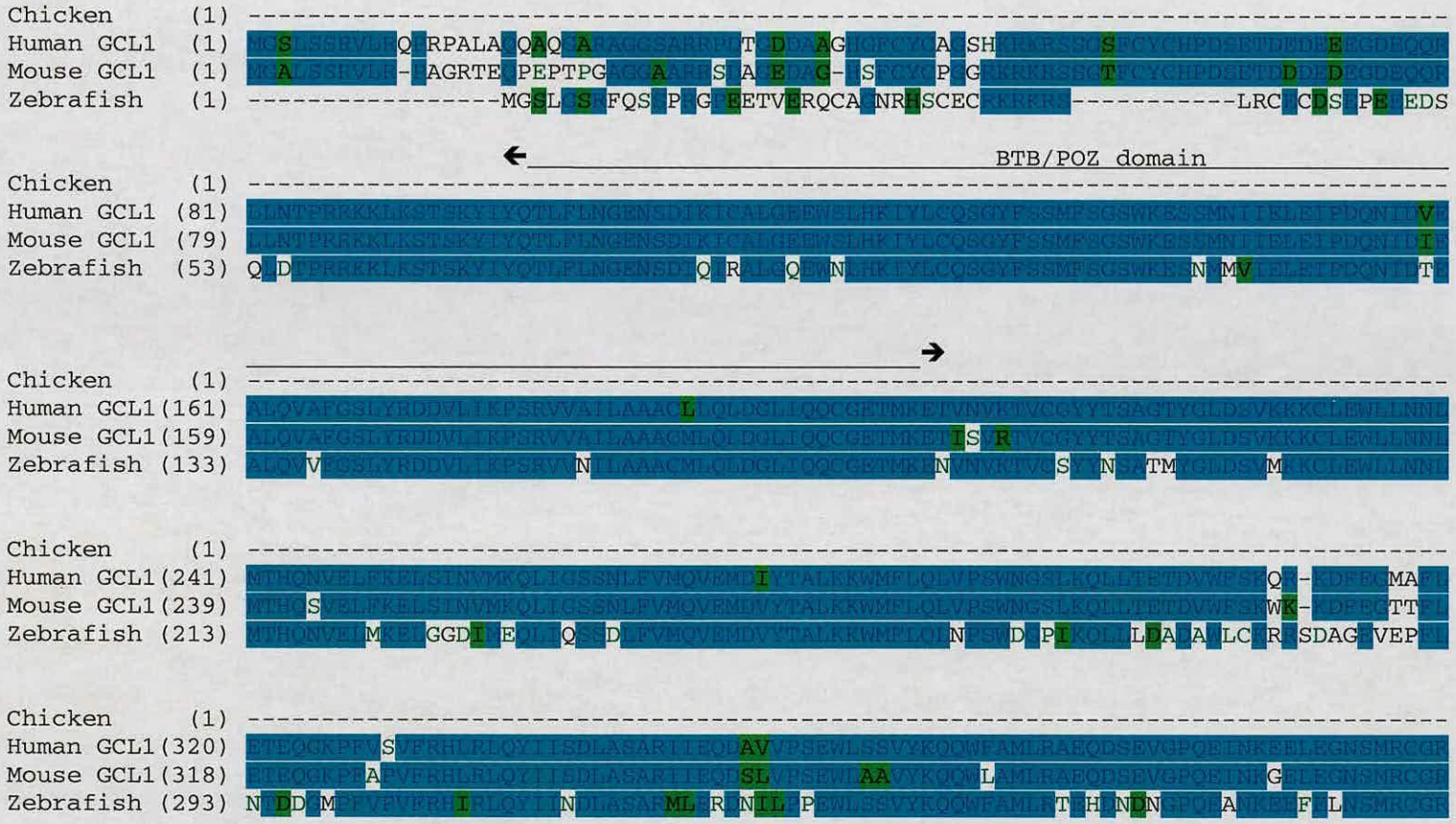
BLAST searches of the chicken genome identified one predicted chicken *germ cell-less* homologue that was still to be located on a chromosome. BLAST searches of

the chick UMIST database identified several ESTs with identical sequences to the sequence obtained from the chicken genome (see Appendix 3). This indicates that the genome data is accurate. To confirm that the sequence is a *germ cell-less* homologue, the predicted chicken protein sequence was used as the query sequences to BLAST the NCBI database. No conserved domains were detected and the genes with the highest similarity to the chicken sequence were *germ cell-less* genes. The E values obtained during the BLAST search of the NCBI database are shown in table 4.1 for various species. To investigate the genes identity further, the sequence was aligned with known Germ cell-less protein sequences from zebrafish, mice and humans (see Figure 4.1 Table 4.1). The proteins that the chicken sequence was being aligned with were truncated to the same length as the chicken sequence. The predicted chicken sequence shows the highest sequence similarity to truncated vertebrate Germ cell-less proteins (71-82% identical). The predicted chicken sequence is much shorter than the equivalent homologues in other species and the fact that the sequence has not yet been assigned to a chromosome suggest that the sequence identified is incomplete. The partial sequence identified corresponds to the 5' end of known germ cell-less proteins. Therefore, more of the sequence was looked for beyond the 3' end of the identified sequence. No sequences similar to Germ cell less were identified in this manner. This could mean that the sequence identified is an incomplete pseudogene.

Gene (species)	E value	Identity	Similarity
Germ cell-less 1 *(Homo sapiens)*	2e-40	80%	84%
Germ cell-less 1 *(Mus musculus)*	2e-40	82%	85%
Germ cell-less 2 *(Mus musculus)*	3e-12	34%	46%
Germ cell-less *(Danio rerio)*	6e-36	71%	83%
Germ cell-less *(Drosophila melanogaster)*	8e-39	16%	22%

Table 4.1: E values, the percentage of identical residues (Identity) and the percentage of conservative substitutions (Similarity) are shown to identify the putative chicken Germ cell less protein.

Figure 4.1:



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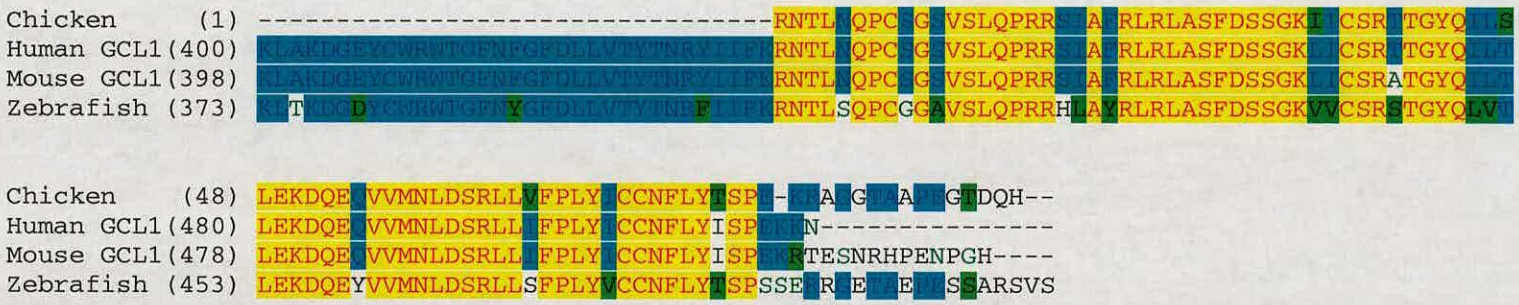


Figure 4.1: Alignment of the identified Germ cell-less protein sequence with known Germ cell-less protein sequences from human, mice and zebrafish. Identical residues are highlighted in tallow. Residues that are identical between two or three species are highlighted in blue. Residues that are different between species but where the different residues are predicted to act the same way are highlighted in green.

4.3.2 Detection of chicken *germ cell-less* expression by RT-PCR and Northern analysis

RT-PCR was carried out on RNA samples from different embryonic stages and adult tissues to determine when during development and which adult tissues express chicken *germ cell-less*. *Germ cell-less* mRNA was detected specifically in the adult testes after 25 cycles of amplification. *Germ cell-less* was not detected in any other tissue. The number of amplification cycles was increased to 30 to determine if low levels of *germ cell-less* mRNA were present in any other sample, but *germ cell-less* was only detected in the adult testes (see Figure 4.2).

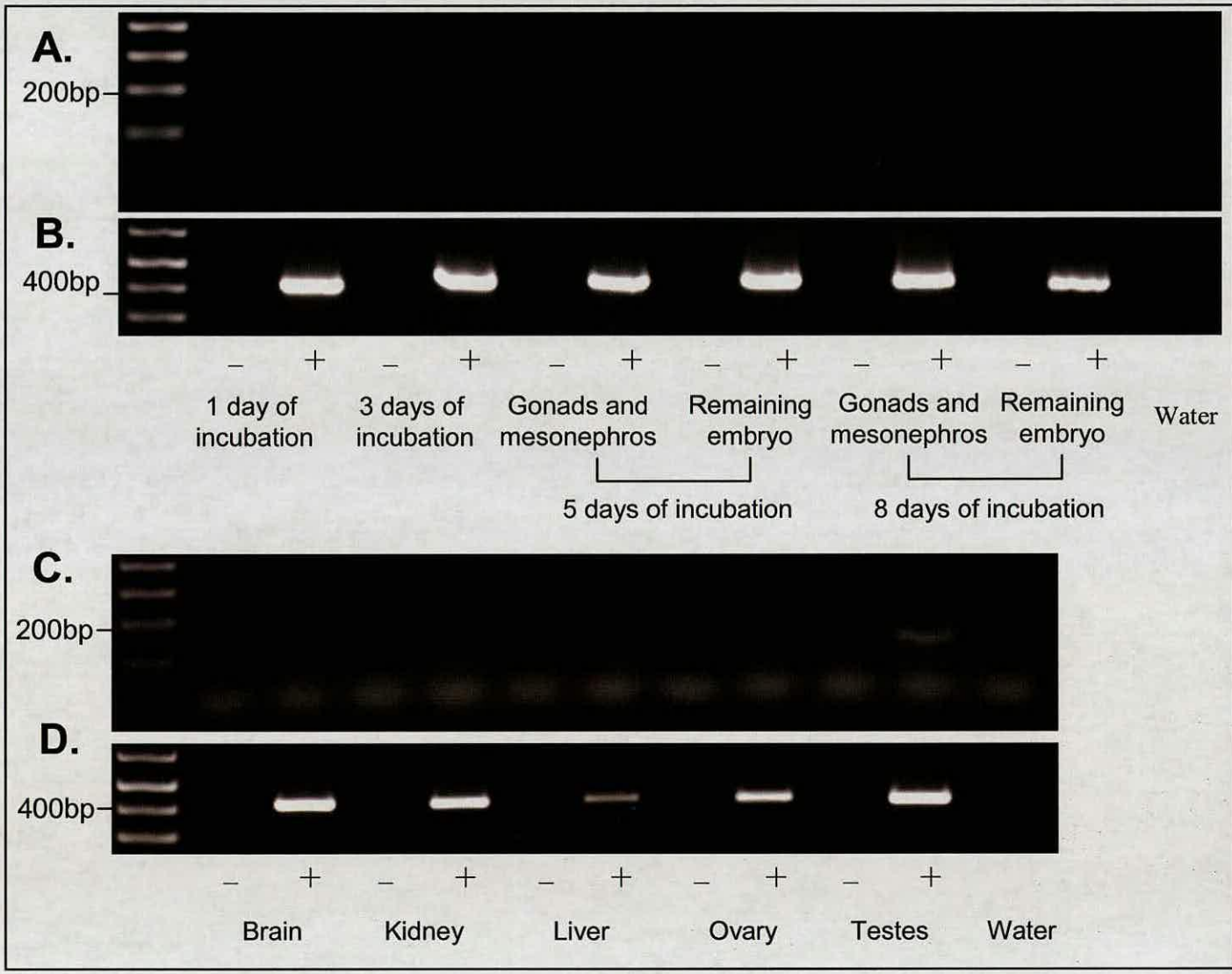


Figure 4.2: RT-PCR to detect the expression of *germ cell-less* mRNA in embryonic and adult tissues. (A) RT-PCR to detect *germ cell-less* mRNA in embryonic tissues. (C) RT-PCR to detect *germ cell-less* mRNA in adult tissues. (B and D) ß-actin positive controls. +: reverse transcriptase present during cDNA synthesis. –: no RT control.

Northern analysis was carried out to extend the RT-PCR data. A probe was made using DNA from a chicken EST previously identified, obtained from ARKGenomics (see Appendix 3). No hybridisation signal was detected in any embryonic or adult tissues (see Figure 4.3). The probe synthesised was 55x10-3 cpm/µl, indicating that radiolabel had bound to the DNA in the reaction tube, but there is still the possibility that there was a technical error in the synthesis of the probe. In future experiments, use of poly A+ RNA instead of total RNA would increase the sensitivity of the analysis.

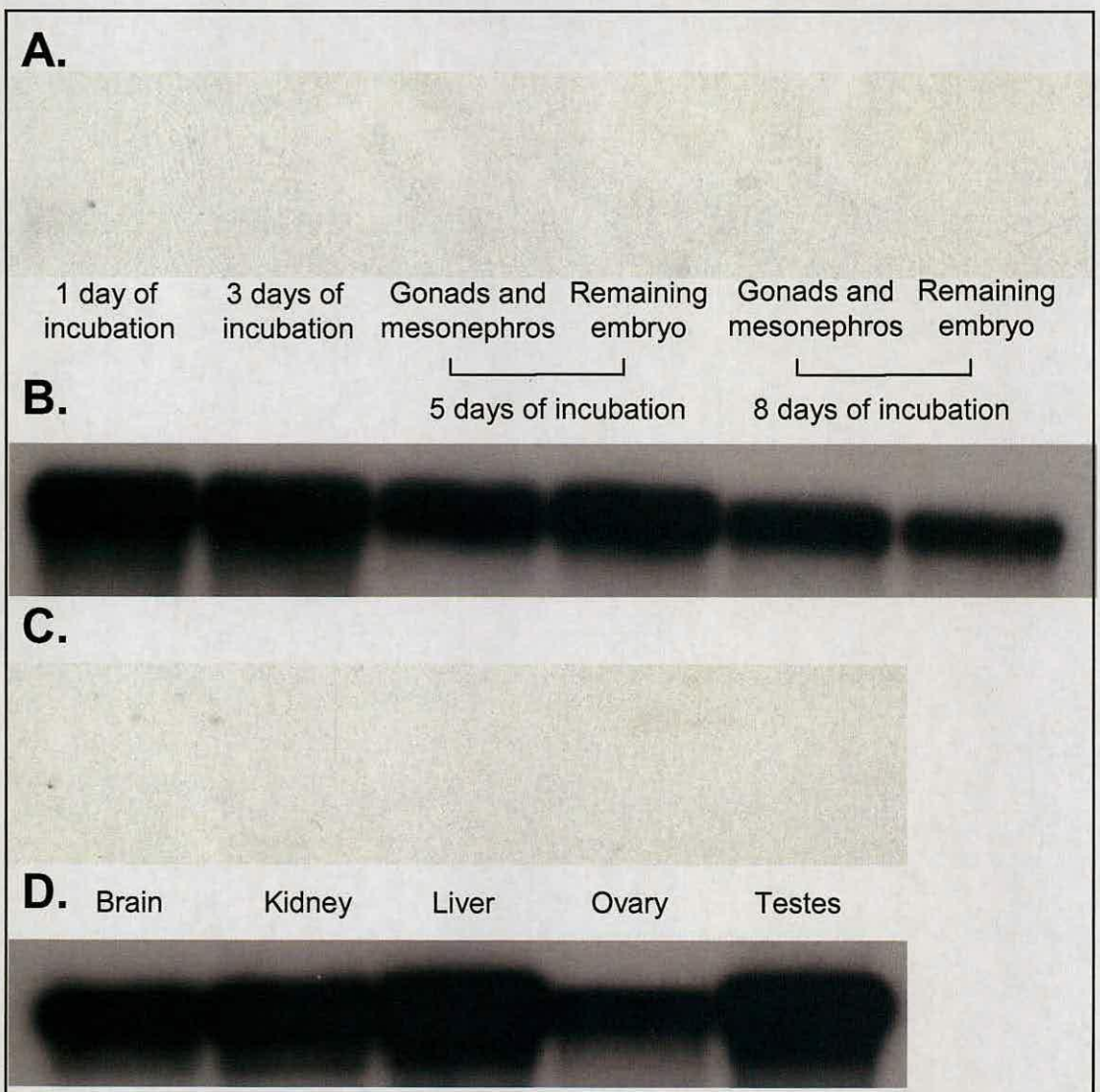


Figure 4.3: Northern analysis to detect *germ cell-less* mRNA in embryonic and adult tissues. (A) expression of *germ cell-less* in embryonic tissues. (C) expression of *germ cell-less* in adult tissues. (B and D) loading controls. 10µg total RNA were used per sample.

4.3.3 Expression of *germ cell-less*

The RT-PCR data indicates that *germ cell-less* mRNA is only found in adult testes, but the Northern data is inconclusive because no specific hybridisation was seen. To further extend these results and identify which areas of the testes express *germ cell-less*, *in situ* hybridisation was carried out. Using the RT-PCR product cloned from testes cDNA, sense and anti-sense digoxigenin *in situ* hybridisation probes were made for *germ cell-less*.

In situ hybridisations were carried out on embryos at one, three, and five days of incubation. Consistent with the results already obtained, *germ cell-less* mRNA was not detected at any stage of embryonic development (see Figures 4.4 A-D). RT-PCR detected *germ cell-less* mRNA in the adult testes, therefore *in situ* hybridisation was carried out on 20μ frozen adult testes sections. No specific staining was detected in the adult testes (see Figures 4.4 E). This suggests that either the *germ cell-less* transcript is rare and that neither the basic Northern analysis used here nor *in situ* hybridisation are sensitive enough to detect the transcript or there is the possibility of a technical error occurring during probe synthesis for both experiments.

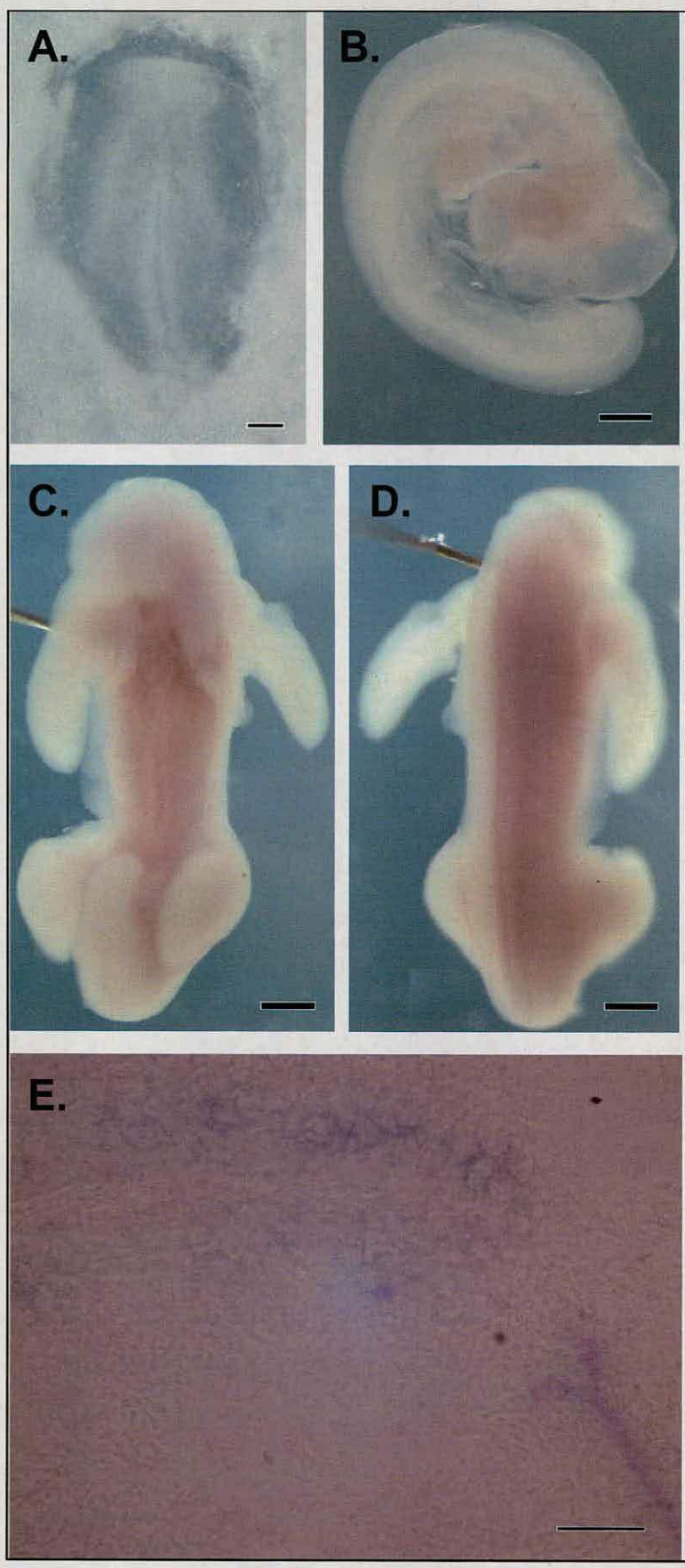


Figure 4.4: *In situ* hybridisation to detect *germ cell-less* in embryos at 1 day, 3 days and 5 days of incubation and adult testes section. (A) *in situ* hybridisation of embryos at 1 day of incubation using anti-sense probe. (B) *in situ* hybridisation of embryos at 3 days of incubation using anti-sense probe. (C) *in situ* hybridisation of embryos at 5 days of incubation using ant-sense probe ventral view (D) dorsal view. (E) *in situ* hybridisation of testes section. Sense controls not shown. Scale bar represents 250μm (A-D) 25μm (E).

4.4 Discussion

A single *germ cell-less* homologue was identified in the chicken genome, for which a cDNA sequence had already been cloned and logged in the UMIST EST database showing that the genome data is accurate. However, the identity of the gene was called into question when it was found that the transcribed protein did not contain the recognised BTB/POZ conserved domain of a Germ cell-less protein. Furthermore, the sequence is only a partial sequence that is still to be located on a chromosome. As the sequence would be the very 5' end of germ cell-less, the sequence 3' of this was examined for additional exons. None were found. This indicates that the partial sequence identified could be a non-transcribed pseudo-gene.

The alignment the protein sequence with known Germ cell-less protein sequences from other species showed that the protein had good conservation to the last 100 amino acids of vertebrate Germ cell-less 1 proteins. Additionally, when the identified protein sequence was used to query the NCBI database it showed highest homology to Germ cell-less proteins. These points suggest that the sequence identified in the genome is a *germ cell-less* gene, but that the sequence is incomplete.

RT-PCR analysis of the identified sequence determined that the mRNA is expressed exclusively in adult testes. However, Northern and *in situ* hybridisation analysis did not detect the transcript in any sample tested. This could be due to a technical error in probe synthesis. The RT-PCR results indicate that the gene identified does not have a function in PGC development because it is not expressed in any embryonic stage tested. This raised the hypothesis that there could be a second *germ cell-less*

gene that was not identified in this study. This is a possibility because more than one *germ cell-less* gene has been identified in mice and *germ cell-less* is known to be expressed in migrating PGCs in zebrafish. Although a second *germ cell-less* gene is known to exist in mice, research is yet to be published on its expression and function, which means that the gene might not have a role in PGC development. If this were the case and when taking into consideration the conservation of expression between chicken *germ cell-less* and mouse *germ cell-less 1*, it would indicate that chicken PGC development is more like mammalian PGC development than first thought. In order to test this theory, two lines of research would need to be taken up: first, investigate the expression of the second *germ cell-less* gene in mice; second, identify a second *germ cell-less* homologue in the chicken develop an expression profile for it.

CHAPTER 5

IDENTIFICATION AND EXPRESSION ANALYSIS OF CHICKEN HOMOLOGUES OF *MAGO NASHI*, *STAUFEN 1* AND *STAUFEN 2*

5.1 Introduction

5.1.1 Localisation of mRNAs

Intracellular localisation of messenger RNAs (mRNAs) by RNA binding proteins is a common process through which proteins are targeted to the regions of the cell where they are required (Palacios, 2002). The localisation of mRNAs to form germ plasm in early development is a characteristic of animals in which PGCs develop via a preformation mechanism. In *Drosophila*, the microtubule dependent localisation of *oskar* mRNA to the posterior pole of the oocyte is essential to the formation of pole plasm and the specification of pole cells. The localisation of maternal determinants, such as *oskar*, to the posterior pole of the oocyte is critical for anterior-posterior patterning, in particular abdominal segmentation, and germ cell formation. Both anterior-posterior patterning and germ cell formation are disrupted by maternal effect mutations of a number of genes called the posterior group genes. Two of the posterior group genes, *mago nashi* and *staufen* are required to localise maternal mRNAs to the posterior of the *Drosophila* oocyte (Newmark *et al.*, 1994 and 1997; Roegiers and Jan, 2000). It is not known whether vertebrate homologues of *mago nashi* and *staufen* have a comparable function in the early localisation of maternally supplied factors. However, in zebrafish, *mago nashi* maternal transcript is detected from the beginning of development. In other vertebrates, *mago nashi* is highly

conserved, has comparable functions in splicing mRNA and interacts with highly conserved RNA binding proteins. All of these points make *mago nashi* a potentially interesting subject for investigation in terms of PGC development in chicken. Both *staufen 1* and *staufen 2* are required for localisation of *Xenopus vasa homologue* mRNA to the vegetal side of the embryo, and are known to function in PGC migration in zebrafish. The known functions of *mago nashi* and *staufen* will now be discussed in more detail.

5.1.2 The *mago nashi* gene and Mago protein

Mago nashi was first identified in *Drosophila* in a genetic screen designed to identify genes essential for pattern formation and germ cell determination (Boswell *et al.*, 1991). Homologues of *Drosophila mago nashi* with high conservation at the nucleotide level have since been identified in *Caenorhabditis elegans* (80%; Newmark *et al.*, 1997; Li, 2000), *Xenopus* laevis (88%; Newmark *et al.*, 1997), zebrafish (42%; Pozzoli *et al.*, 2004), mice (88%; Newmark *et al.*, 1997) and humans (88%; Zhao *et al.*, 1998).

The *Drosophila mago nashi* gene codes for Mago protein, which co-localises to the posterior of the *Drosophila* oocyte with key germ plasm factors such as *oskar* mRNA and Staufen protein (Palacios, 2002). The localisation of Mago protein to the posterior pole is essential for both anterior-posterior patterning of the *Drosophila* embryo and germ plasm assembly, particularly for *oskar* mRNA and Staufen protein localisation (Boswell *et al.*, 1991; Newmark *et al.*, 1994 and 1997). In both vertebrates and invertebrates, Mago binds specifically to the RNA binding protein

Y14, also known as Tsunagi, to form a stable complex that localises to the nucleus (Zhao *et al.*, 1998; Hachet and Ephrussi, 2001; Mohr *et al.*, 2001). In *Drosophila*, Y14 accumulates at the posterior pole with *oskar* mRNA (Hachet and Ephrussi, 2001; Mohr *et al.*, 2001). In *y14* mutants, oskar mRNA does not localise to the posterior pole as normal, suggesting that the Y14/Mago complex is a component of the localisation machinery that transports *oskar* mRNA to the posterior pole of the oocyte (Hachet and Ephrussi, 2001; Mohr *et al.*, 2001). Additionally, in mutants in which the polarity of the oocyte is altered and *oskar* mRNA does not localise to the posterior pole, the Y14 protein co-localises to the same ectopic sites as *oskar* mRNA (Hachet and Ephrussi, 2001; Mohr *et al.*, 2001). The Y14/Mago complex is also involved in post-transcriptional events in the nucleus and cytoplasm and is thought to have a role during mRNA nuclear export and non-sense mediated mRNA decay (Hachet and Ephrussi, 2001; Le Hir *et al.*, 2001; Mohr *et al.*, 2001; Palacios, 2002). In *C.elegans*, when *mago nashi*, known as *mag-1*, is knocked down by RNA-mediated interference the germline is masculinised. In order to prevent masculinisation of the germline, *mag-1* inhibits the function of one or more of the masculanising genes *fog*, *fem* or *gld* (Li *et al.*, 2000). The idea that *mag-1* inhibits gene function coincides with data from humans and flies that have indicated a possible role for Mago in mRNA mediated decay (Zhao *et al.*, 1998; Hachet and Ephrussi, 2001; Le Hir *et al.*, 2001; Mohr *et al.*, 2001; Palacios, 2002). Of the *mago nashi* homologues found in vertebrates, expression analysis of zebrafish *mago nashi* has been undertaken (Pozzoli *et al.*, 2004), and the Y14/Mago complex has been investigated in human cell cultures (Zhao *et al.*, 1998 and 2000; Kataoka *et al.*,

2001). However, it is yet to be determined whether *mago nashi* has any role in vertebrate PGC development.

5.1.3 The *staufen* genes and proteins

Staufen codes for Staufen protein and was first identified in *Drosophila* in a genetic screen for maternal effect mutations (Schüpbach and Wieschaus, 1986). In vertebrate species, *staufen* homologues have been identified in *Xenopus* (Allison *et al.*, 2004), zebrafish (Bateman *et al.*, 2004; Ramasamy *et al.*, 2006) and mice (Saunders *et al.*, 2000). Additionally, *staufen* sequences have been added to the NCBI database from various other vertebrate species, including humans.

In *Drosophila* oocytes, Staufen protein is known to be essential for *bicoid* localisation to the anterior, *oskar* localisation to the posterior and *oskar* de-repression (Broadus *et al.*, 1998; Kim-Ha *et al.*, 1991; St Johnston *et al.*, 1991). De-repression is a process that reverses silencing and allows translation to take place. During localisation, mRNAs are transcriptionally silenced until correctly localised and de-repressed, resulting in protein localisation (Saunders *et al.*, 2000). In order to carry out these functions, Staufen protein contains five copies of a double-stranded RNA-binding domain (dsRBD), which are evolutionarily conserved (St Johnston *et al.*, 1991). Staufen also contains an evolutionarily conserved tubulin-binding domain (TBD). Of the five dsRBDs, only binding domains 1, 3 and 4 can bind double-stranded RNA *in vitro*. Domain 2 is required for microtubule-dependent localisation of *oskar* mRNA and domain 5 is involved in de-repression of *oskar* mRNA once it is localised to the posterior pole (Wickham *et al.*, 1999; Micklem *et al.*, 2000). In

Drosophila mutants in which the polarity of the oocyte is altered and *oskar* mRNA does not localise to the posterior pole, Staufen protein co-localises to the same ectopic sites as *oskar* mRNA, indicating that Staufen is not sufficient for correct localisation of *oskar* (Gonzalez-Reyes *et al.*, 1995). As with Mago, these results demonstrate that in *Drosophila* Staufen is essential for both anterior-posterior patterning and germ plasm assembly through the localisation of *oskar* mRNA. As well as localising factors early in development, later in development Staufen protein binds to *prospero* in maturing neuroblasts and aids in localising and anchoring *prospero* to the basal plasma membrane in the central nervous system (Broadus *et al.*, 1998; Schuldt *et al.*, 1998; Shen *et al.*, 1998).

Two *staufen* genes have been identified in *Xenopus*, zebrafish and mice (Saunders *et al.*, 2000; Allison *et al.*, 2004; Bateman *et al.*, 2004; Ramasamy *et al.*, 2006). In *Xenopus*, the protein products of both *xstau1* and *xstau2* are vegetally localised with *Xenopus vg1* (*Xenopus vasa* homologue) mRNA during oogenesis. If either Xstau protein is disrupted, *Vg1* mRNA localisation is blocked (Allison *et al.*, 2004; Yoon and Mowry, 2004). In mice, *staufen 1* and *staufen 2* are expressed in the germ cells during oogenesis and spermatogenesis and they have a function in the formation of RNA granules in neuronal dendrites (Saunders *et al.*, 2000; Miki *et al.*, 2005). However, it has not been investigated whether they have any role in PGC development. In zebrafish, both Staufen 1 and Staufen 2 proteins are required for the survival of neurons in the dorsal central nervous system and the survival and migration of PGCs (Ramasamy *et al.*, 2006). The fact that PGCs are formed but mismigrate in embryos where Stau1 or Stau2 are depleted indicates that neither

staufen gene has a function in germ plasm localisation in zebrafish. Although not required for germ plasm localisation, both Stau proteins have an evolutionarily conserved function in neuron development and have a function in germ cell development.

The aims of the experiments described here were to identify chicken homologues of *mago nashi* and *staufen* by carrying out BLAST searches of the chicken genome and EST databases. Following this, detection of mRNA using RT-PCR and Northern analysis were carried out to investigate expression of *mago nashi* and *staufen* homologues, and *in situ* hybridisation analysis was conducted. These experiments were undertaken to determine when and where during development the genes are expressed and from this try to predict whether they have a role during PGC development.

5.2 Overview of methods

This section gives a brief overview of the methods and samples used to investigate *germ cell-less* in PGC development. For a full account of the methods see chapter 2.

5.2.1 BLAST analysis of the chicken genome and EST databases

To identify putative *mago nashi* homologues in the chicken, BLAST searches of the chicken genome were carried out using the zebrafish *mago nashi* nucleotide sequence (acc.no. BC093273) as the query sequence.

To identify putative *staufen* homologues in the chicken, BLAST searches of the chicken genome were carried out using the zebrafish *staufen 1* (acc.no. NM_205561) and *staufen 2* nucleotide sequences (acc.no. NP_957219).

5.2.2 Samples for RT-PCR and Northern analysis

RNA was extracted from chicken embryos at several stages of development and several adult tissues. The embryonic stages were: whole embryos at one and three days of incubation; gonads and mesonephros at five and eight days of incubation; the remaining embryo after the gonad and mesonephros had been removed at five and eight days of incubation. Adult tissues were taken from: brain; kidney; liver; ovary and testes.

5.2.3 RT-PCR analysis

RT-PCR primers to detect *mago nashi* were designed flanking an intron using primer 3 software (see Appendix 1; http://frodo.wi.mit.edu/). The primers hybridise to nucleotides located at 158-317bp of the transcript to give an expected product size of 159bp. Amplification of β-actin using primers to give a product with an expected size of 428bp was used as a positive control. The annealing temperature was 59.5°C.

RT-PCR primers to detect *chicken staufen 1* and *chicken staufen 2* were designed flanking an intron using primer 3 software (see Appendix 1; http://frodo.wi.mit.edu/). The primers bind to nucleotides located at 1166-1371bp of the transcript to give an expected product size of 205bp for *chicken staufen 1*. The primers bind to nucleotides located at 505-740bp of the cDNA to give an expected product size of

235bp for *chicken staufen 2*. Amplification of β-actin using primers to give a product with an expected size of 428bp was used as a positive control. The annealing temperature was 59.5°C.

To ensure that the PCRs were amplifying the predicted *mago nashi*, *staufen* and *staufen 2* sequences, the RT-PCR products were cloned from testes cDNA and sequenced. The sequence data were aligned with the expected product sequences obtained from the chicken genome sequence. 100% match was observed in all three cases confirming that the PCR primer sets were amplifying the correct sequences.

5.2.4 Northern and *In situ* hybridisation probes

The ESTs ChEST710p7, ChEST622p16 and ChEST684h6 from ARKGenomics were used to make radiolabelled Northern probes to detect *mago nashi*, *staufen* and *staufen 2* respectively (see Appendices 4-6).

The RT-PCR products cloned from testes cDNA were used to make sense and anti-sense digoxigenin *in situ* hybridisation probes to detect *mago nashi*, *staufen* and *staufen 2*. Whole mount *in situ* hybridisation was carried out on embryos at one, three and five days of incubation.

5.3 Results

5.3.1 Identification of a chicken *mago nashi* homologue

BLAST searches of the chicken genome identified a single putative chicken *mago nashi* homologue was identified on chromosome 8 at location 25,398,748-25,400,444. The predicted transcript is 636bp long located over 1.68kb of the chromosome. The transcript is predicted to have 5 exons, which encode for a 145 amino acid protein. BLAST searches of the chicken UMIST database identified two ESTs with identical sequences to the sequence obtained from the chicken genome (see Appendix 4). This indicates that the genome data is of a genuine gene rather than of an artefact. To confirm that the sequence is a *mago nashi* homologue, the putative chicken Mago protein sequence was used as the query sequence to BLAST the NCBI database. The genes with the highest similarity to the chicken sequence were *mago nashi* genes. To investigate the gene's identity further, the sequence was aligned with known Mago protein sequences from zebrafish, mice and humans (see Figure 5.1 and Table 5.1). The chicken sequence shows the highest sequence similarity to Mago proteins from humans (99% identical), mice (99% identical), possums (99% identical) and *Xenopus* (99% identical). High sequence similarity to Mago proteins from zebrafish (98% identical) and *Drosophila* (89% identical) was also observed. These high sequence similarities and the BLAST search of the NCBI database confirm that the sequence found is chicken *mago nashi*.

Gene (species)	E value	Identity	Similarity
Mago nashi (*Homo sapiens*)	6e-80	99%	99%
Mago nashi *(Mus musculus)*	4e-80	99%	99%
Mago nashi (*Xenopus tropicalis*)	5e-80	99%	99%
Mago nashi (*Danio rerio*)	1e-79	98%	98%
Mago nashi (*D. melanogaster*)	1e-72	89%	91%

Table 5.1: E values, the percentage of identical residues (Identity) and the percentage of conservative substitutions (Similarity) are shown to identify the putative chicken Mago nashi protein.

Figure 5.1: Alignment of the identified Mago nashi protein sequence with known Mago nashi sequences from humans, mice, zebrafish and *Drosophila*. Identical residues are highlighted in yellow. Residues that are identical between two or more species are highlighted in blue. Residues that are different between species but where the different residues are predicted to act in the same way are highlighted in green. The conserved hydrophobic amino acids separated by heptad residues are marked with a plus. The conserved leucine residues that constitute a potential leucine zipper motif are marked with an asterisk.

5.3.2 Detection of chicken *mago nashi* by RT-PCR and Northern analysis

In order to determine when *mago nashi* is expressed during early embryonic development and which adult tissues express *mago nashi*, RT-PCR analysis was carried out. *Mago nashi* mRNA was detected in all embryonic stages and all adult tissues tested after 25 cycles of amplification (see Figure 5.2).

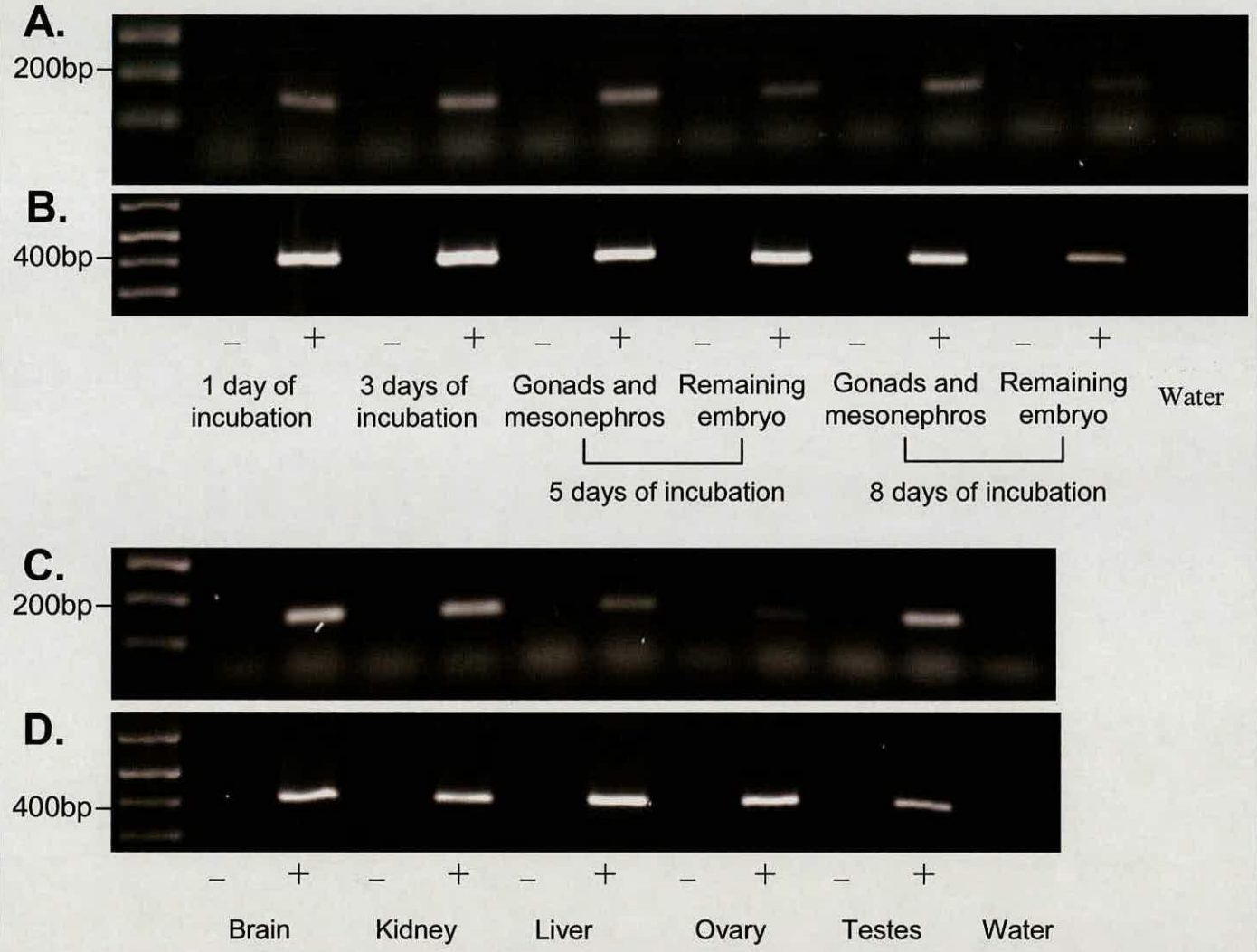


Figure 5.2: RT-PCR to detect the expression of *mago nashi* mRNA in embryonic and adult tissues. (A) RT-PCR to detect *mago nashi* mRNA in embryonic tissues. (C) RT-PCR to detect *mago nashi* in adult tissues. (B and D) *ß-actin* positive controls. +: reverse transcriptase present during cDNA synthesis. -: no RT control.

Northern analysis was carried out to extend the RT-PCR data. At embryonic stages, the probe hybridised strongly to RNA from one day of incubation; three days of incubation; gonads and mesonephros from embryos at five days of incubation; and embryos without gonads and mesonephros at five days of incubation. A faint hybridisation signal to embryos without gonads and mesonephros at eight days of incubation was detected, but no hybridisation signal was detected to gonadal and mesonephros RNA at eight days of incubation (see Figure 5.3 A). Hybridisation signals were detected in adult ovaries and testes, although it was not as strong as the signal detected during early embryonic stages. A faint hybridisation signal was seen in the adult brain and kidney, but not in the liver (sees Figure 5.3 B). In all cases, a single product of 600bp was generated, which is the size expected because the chicken *mago nashi* transcript was predicted to be 635bp.

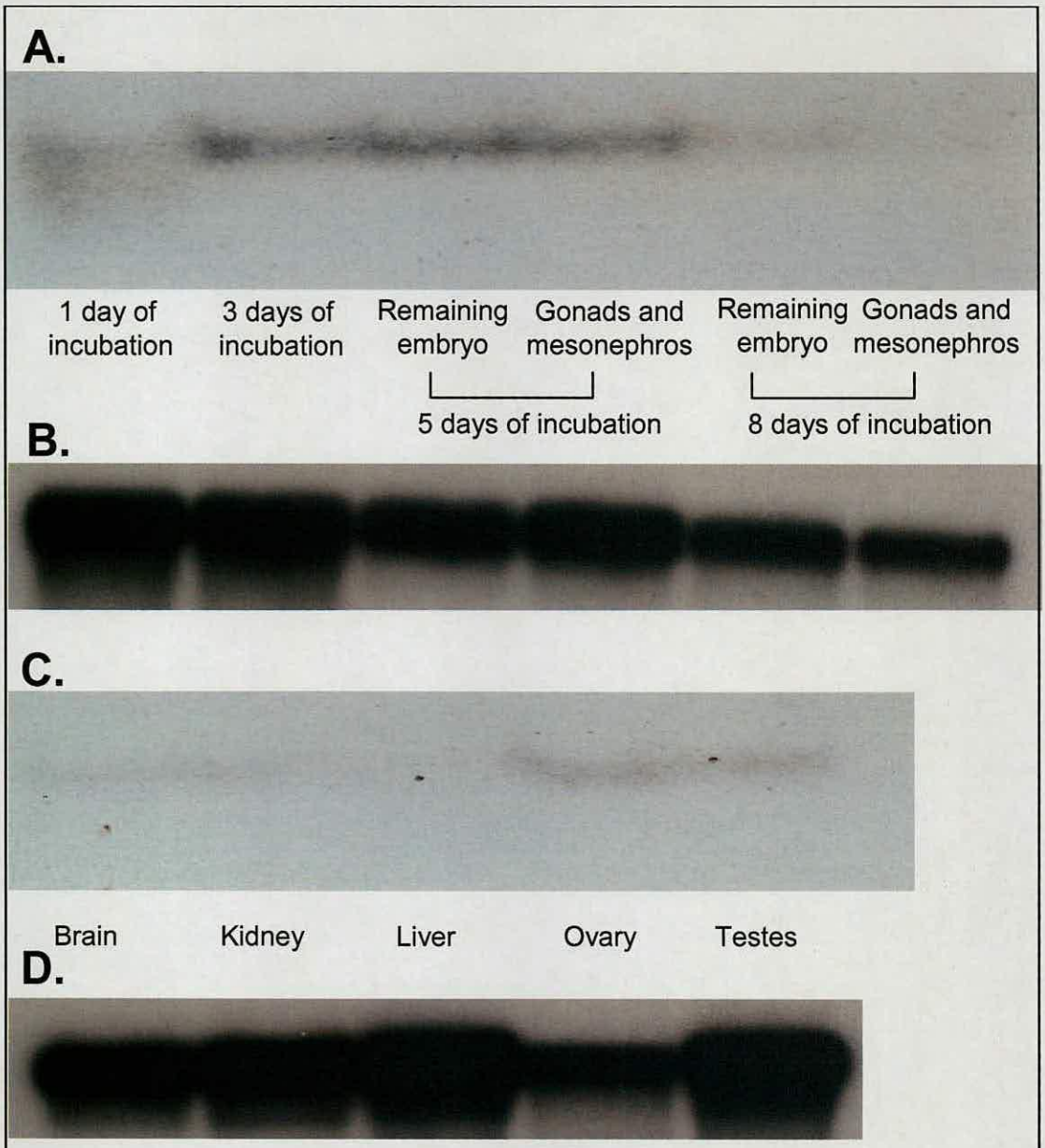
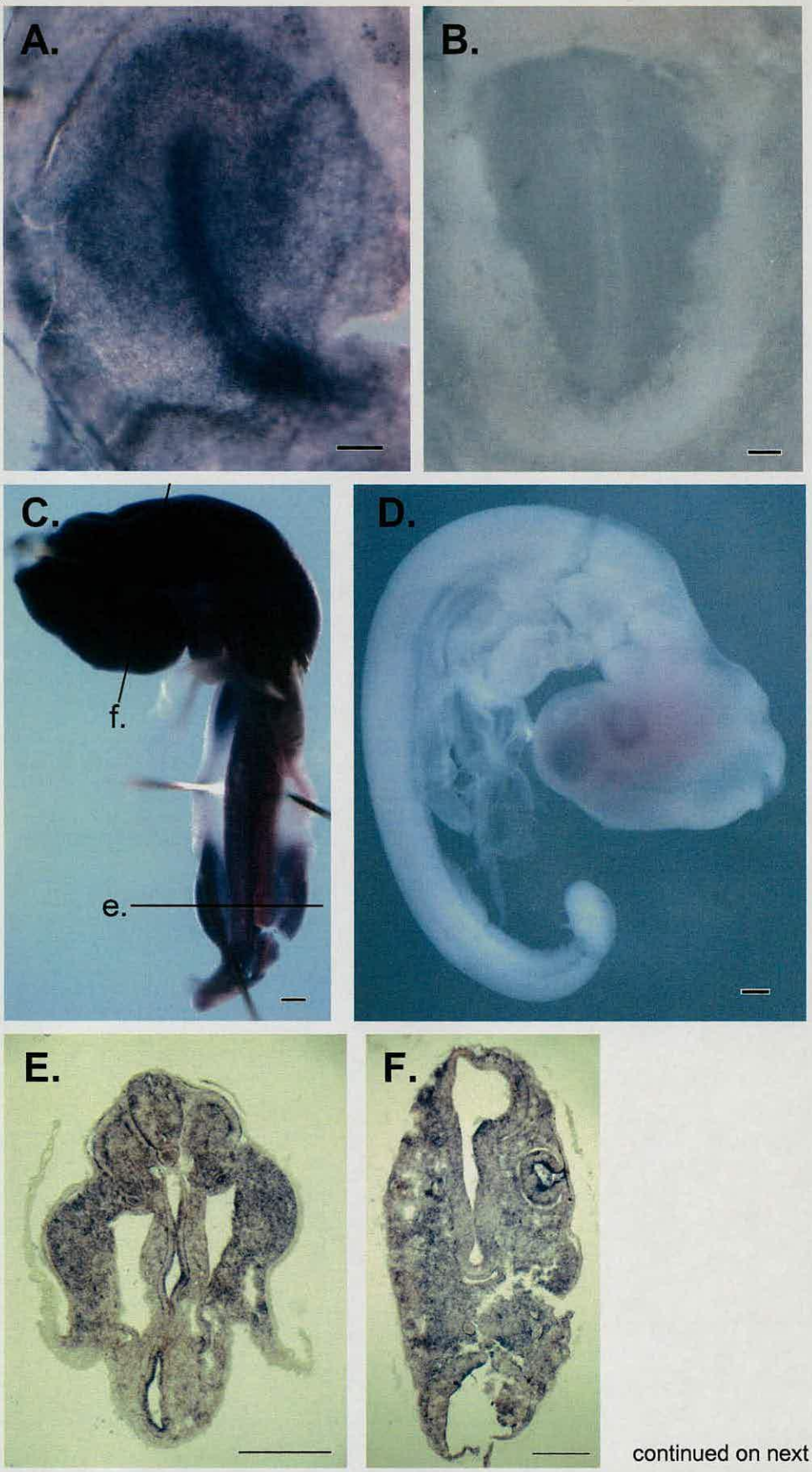


Figure 5.3: Northern analysis to detect *mago nashi* mRNA in embryonic and adult tissues. (A) Expression of *mago nashi* in embryonic tissues. (C) Expression of *mago nashi* in adult tissues. (B and D) loading controls. 10µg total RNA were used per sample.

5.3.3 Expression pattern of *mago nashi*

Whole mount *in situ* hybridisation was carried out to determine the pattern of expression of *mago nashi* in embryos at one, three, five and six days of incubation. Extensive staining was observed in all four embryonic stage of development (see Figure 5.4). At one day of incubation, *mago nashi* mRNA was detected surrounding the primitive steak, in the neural plate and in the extraembryonic membranes including the germinal crescent (see Figure 5.4 A). At three days of incubation, *mago nashi* mRNA was detected in the mesoderm of the main embryonic body and the developing limb buds, but was not detected in the endoderm (see Figures 5.4 C, E and F). At five and six days of incubation, *mago nashi* mRNA was detected ubiquitously throughout the embryo (see Figures 5.4 G, I and J).

Figure 5.4



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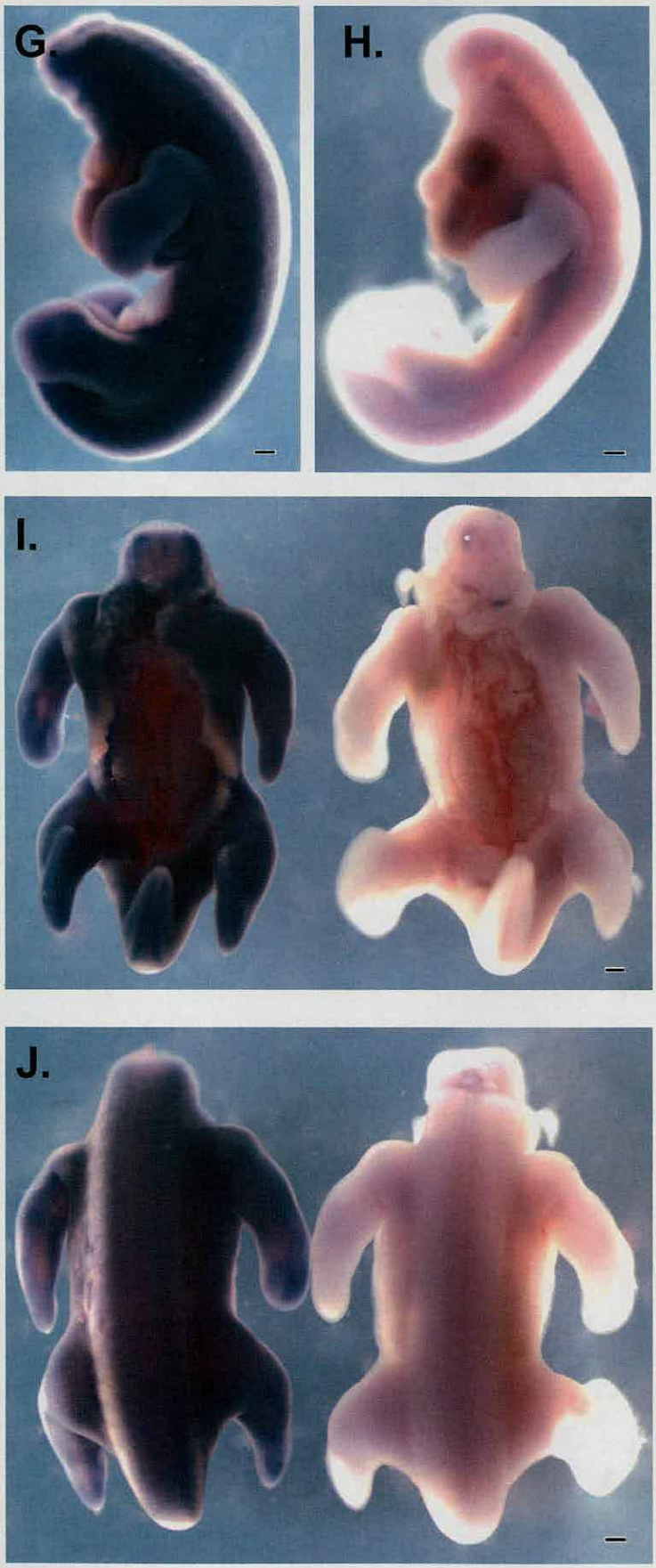

Figure 5.4 continued: *In situ* hybridisations to detect *mago nashi* mRNA in embryos at 1 day, 3 days, 5 days and 6 days of incubation. (A) *in situ* hybridisation of embryos at 1 day of incubation. (C) *in situ* hybridisation of embryos at 3 days of incubation. (E and F) 20μ sections through (C). (G) *in situ* hybridisation of embryos at 5 days of incubation. (I) *in situ* hybridisation of embryos at 6 days of incubation ventral view and (J) dorsal view. (B, D, F, I right and J right) sense controls. Scale bars represent 250μm.

5.3.4 Identification of chicken *staufen* homologues

BLAST searches of the chicken genome identified two putative chicken *staufen* homologues. The first sequence consists of a 15 exon, 2.8kb long transcript found on chromosome 20 at location 6,169,937-6,193,380. The transcript stretches over 17.3kb of the chromosome and encodes a 712 amino acid protein. The second sequence consists of a 12 exon, 2.2kb long transcript found on chromosome 2 at location 117,357,375-117,440,224. The transcript stretches over 82.83kb of the chromosome and encodes a 634 amino acid protein. Both genes contain five double-stranded RNA binding domains and one tubulin-binding domain (see Figures 5.5 and 5.6). BLAST searches of the chick UMIST database identified multiple ESTs with identical sequences to both sequences obtained from the chicken genome (see Appendices 5 and 6). This indicates that the genome data is genuine. To confirm that the sequences are *staufen* homologues, the putative chicken Staufen protein sequences were used as the query sequence to BLAST the NCBI database. The genes with the highest similarity to the chicken sequence were *staufen* genes. Additionally, double-stranded RNA binding domains were identified on both sequences. To investigate the genes' identity further, the sequences were aligned with known Staufen 1 and Staufen 2 protein sequences from zebrafish, *Xenopus*, possums, mice and humans and *Drosophila* Staufen (see Figure 5.5 and 5.6 and Tables 5.2 and 5.3). The chicken sequence on chromosome 20 shows the highest sequence similarity to Xstau1 protein from *Xenopus* (84% identical). High homologies were also observed between the chicken chromosome 20 sequence and other vertebrate Staufen 1 sequences (60-74% identical). Lower homologies were observed between the chicken chromosome 20 sequence and vertebrate Staufen 2

proteins (43-54% identical), confirming that the chromosome 20 is a *staufen* gene and is most closely related to *staufen 1* genes. Therefore, it shall be referred to as *chicken staufen 1*. The chicken sequence on chromosome 2 shows the highest sequence similarity to human Staufen 2 protein (69% identical), and similar homologies were observed between chicken and mice (67% identical), possums (66% identical) and *Xenopus* (65% identical). Homologies to zebrafish Staufen 2 and *Drosophila* Staufen were not as high (59% and 23% identical respectively). Lower homologies were observed between the chicken chromosome 2 sequence and vertebrate Staufen 1 proteins (38-51% identical). This confirms that the chromosome 2 is a *staufen* gene and is most closely related to *staufen 2* genes. Therefore, it shall be referred to as *chicken staufen 2*.

Gene (species)	E value	Identity	Similarity
Staufen 1 (Homo sapiens)	0.0	71%	75%
Staufen 2 (Homo sapiens)	3e-148	40%	48%
Staufen 1 (Mus musculus)	0.0	60%	62%
Staufen 2 (Mus musculus)	3e-134	40%	49%
Staufen 1 (Xenopus tropicalis)	0.0	84%	89%
Staufen 2 (Xenopus tropicalis)	5e-137	44%	54%
Staufen 1 (Danio rerio)	0.0	66%	73%
Staufen 2 (Danio rerio)	8e-143	36%	43%
Staufen (Drosophila melanogaster)	none	23%	30%

Table 5.2: E values, the percentage of identical residues (Identity) and the percentage of conservative substitutions (Similarity) are shown to identify the putative chicken Staufen protein on chromosome 20.

Figure 5.5

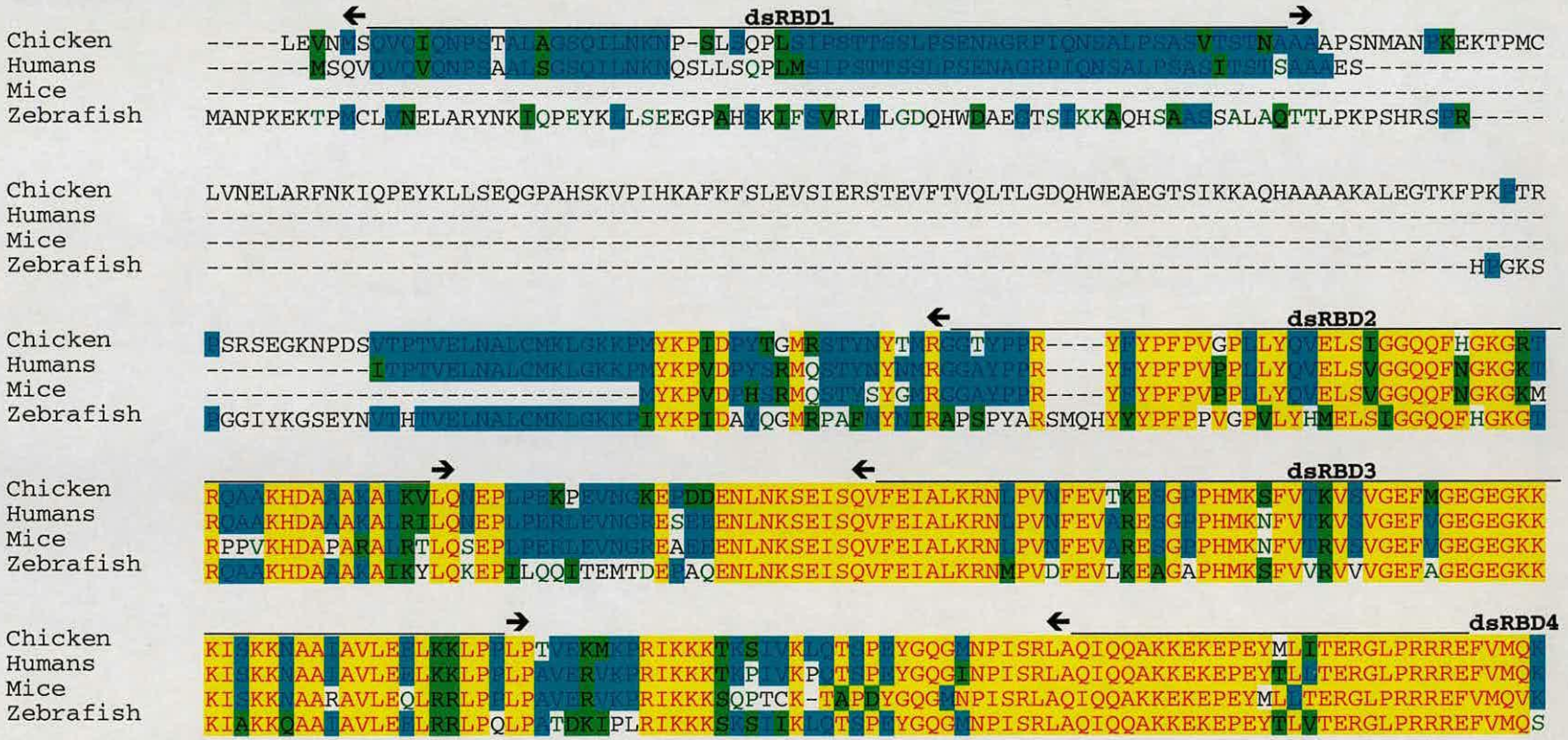

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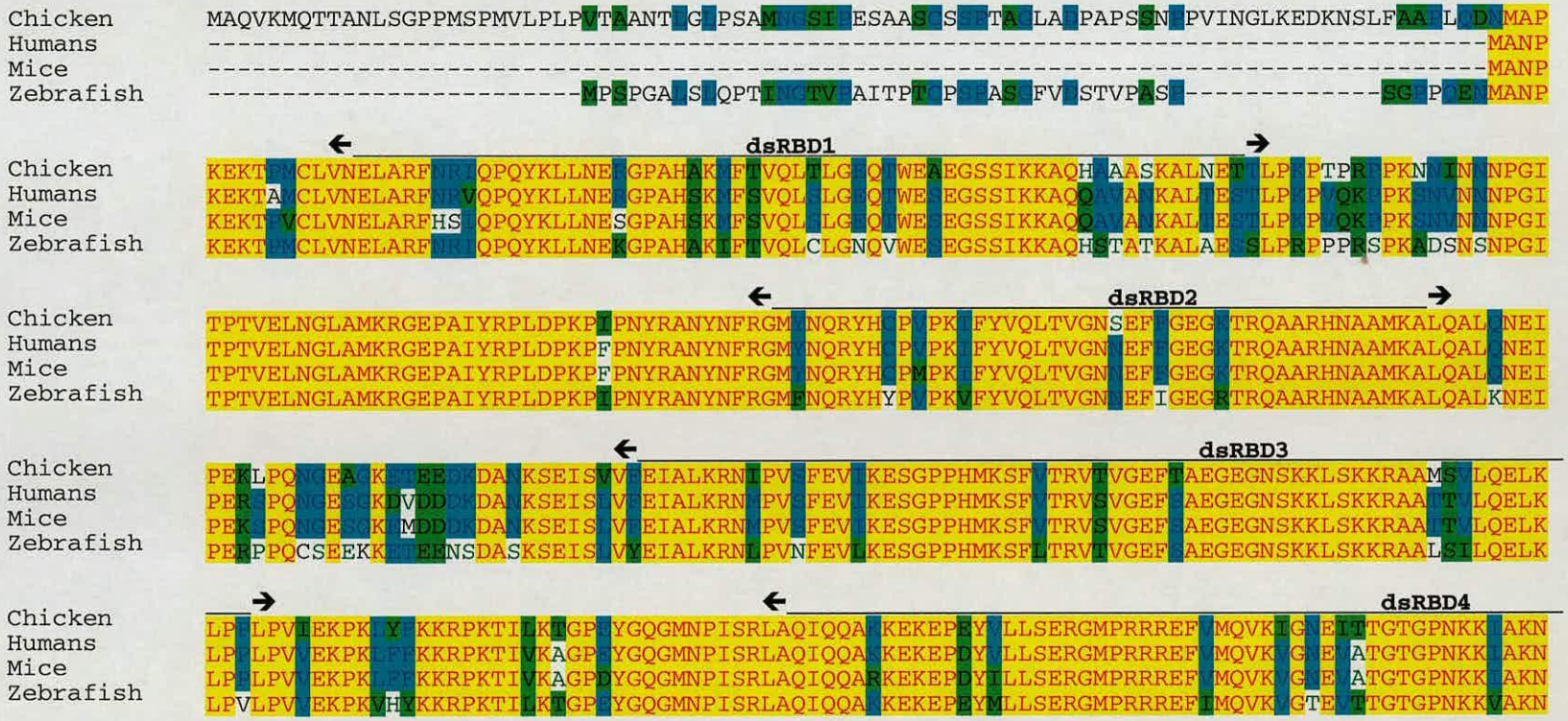
Figure 5.5: Alignment of the identified Staufen 1 protein sequence with known Staufen 1 sequences from humans, mice and zebrafish. Identical residues are highlighted in yellow. Residues that are identical between two or three species are highlighted in blue. Residues that are different between species but where the different residues are predicted to act in the same way are highlighted in green. dsRBD1-5 indicates the five conserved double-stranded RNA Binding Domains; TBD indicates the tubulin Binding Domain (positions obtained from Bateman *et al.*, 2004).

Gene (species)	E value	Identity	Similarity
Staufen 1 (Homo sapiens)	4e-96	37%	43%
Staufen 2 (Homo sapiens)	0.0	69%	74%
Staufen 1 (Mus musculus)	4e-99	31%	38%
Staufen 2 (Mus musculus)	0.0	67%	74%
Staufen 1 (Xenopus tropicalis)	5e-121	43%	51%
Staufen 2 (Xenopus tropicalis)	0.0	65%	72%
Staufen 1 (Danio rerio)	7e-96	38%	48%
Staufen 2 (Danio rerio)	0.0	59%	63%
Staufen (Drosophila melanogaster)	None	23%	30%

Table 5.3: E values, the percentage of identical residues (Identity) and the percentage of conservative substitutions (Similarity) are shown to aid in identifying the putative chicken Staufen protein on chromosome 2.

Figure 5.6



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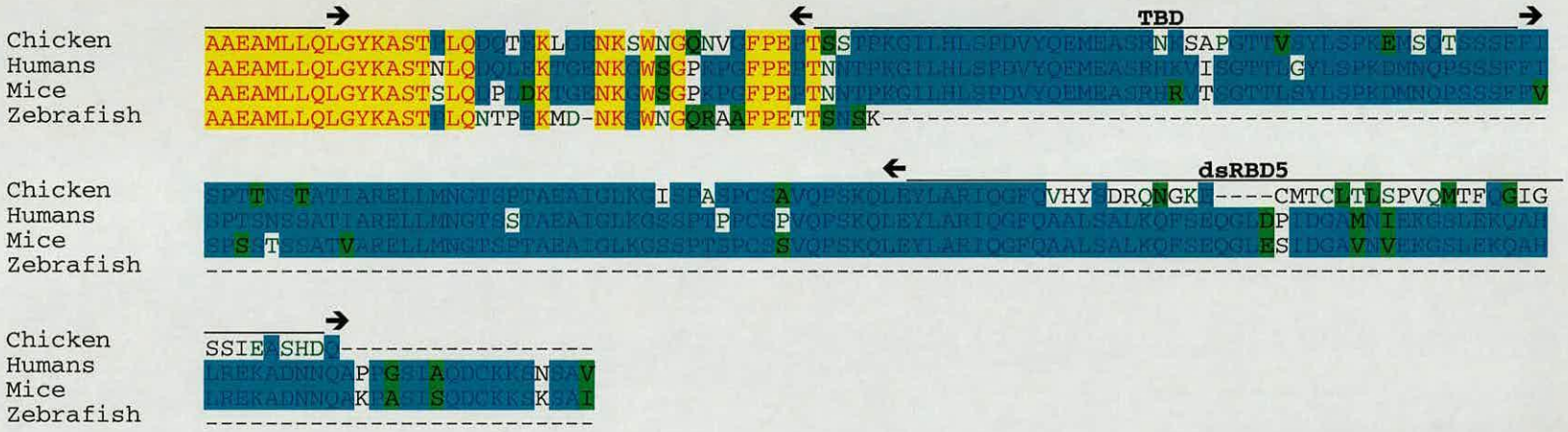


Figure 5.6: Alignment of the identified Staufen 2 protein sequence with known Staufen 2 sequences from humans, mice and zebrafish. Identical residues are highlighted in yellow. Residues that are identical between two or three species are highlighted in blue. Residues that are different between species but where the different residues are predicted to act in the same way are highlighted in green. dsRBD1-5 indicates the five conserved double-stranded RNA Binding Domains; TBD indicates the Tubulin Binding Domain (positions obtained from Bateman *et al.*, 2004).

5.3.5 Detection of chicken *staufen 1* and *chicken staufen 2* gene expression by RT-PCR and Northern analysis

RT-PCR was carried out in order to determine when *chicken staufen 1* and *chicken staufen 2* are expressed during early embryonic development and which adult tissues express them. *Chicken staufen 1* mRNA was detected in all embryonic stages after 30 cycles of amplification (see Figure 5.7A) and all adult tissues tested (see Figure 5.7C). *Chicken staufen 2* mRNA was detected in all embryonic stages (see Figure 5.8A) and all adult tissues tested except liver (see Figure 5.8C).

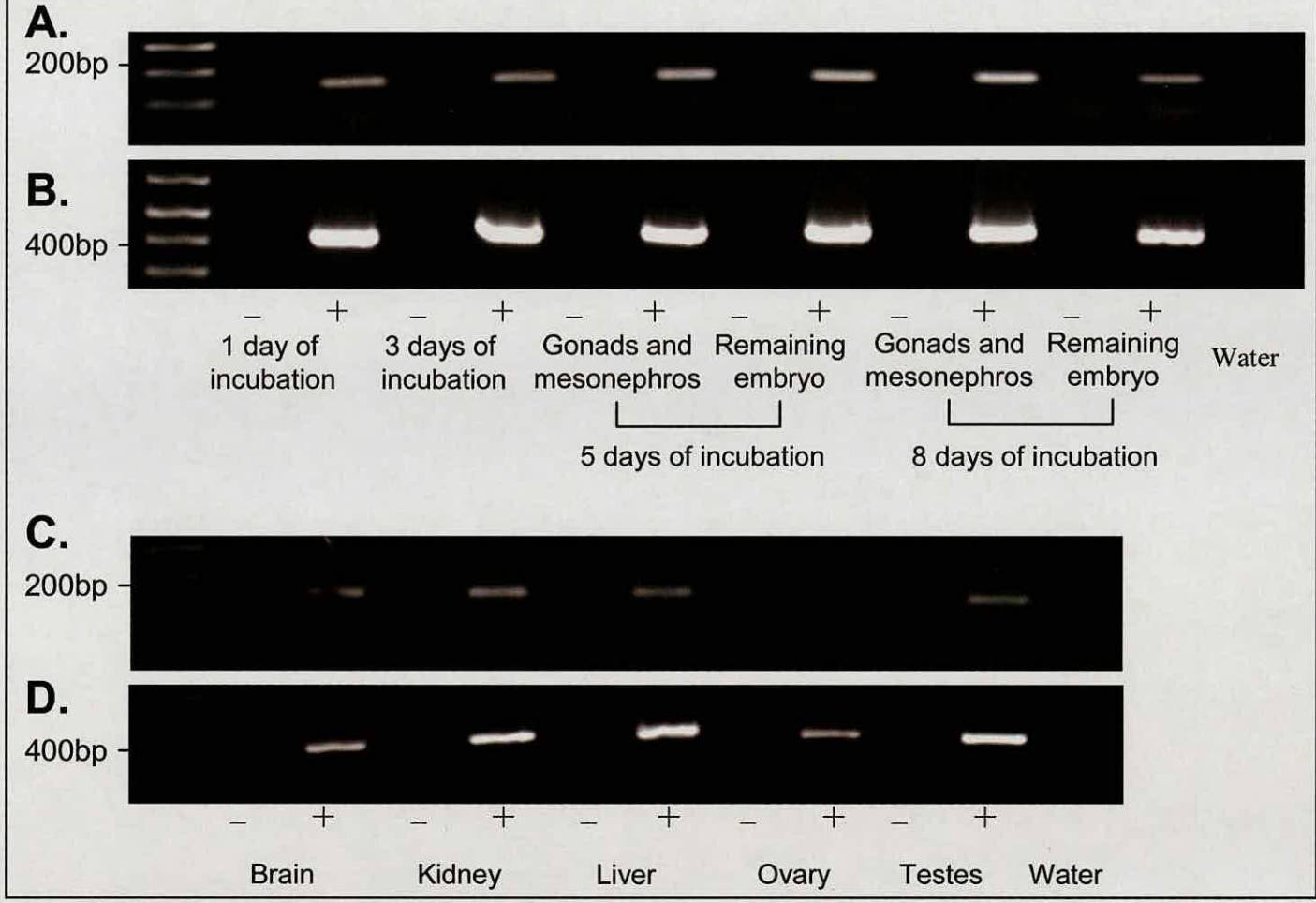


Figure 5.7: RT-PCR to detect the expression of *staufen* mRNA in embryonic and adult tissues. (A) RT-PCR to detect *staufen* mRNA in embryonic tissues. (C) RT-PCR to detect *staufen* in adult tissues. (B and D) *ß-actin* positive controls. +: reverse transcriptase present during cDNA synthesis. -: no RT control.

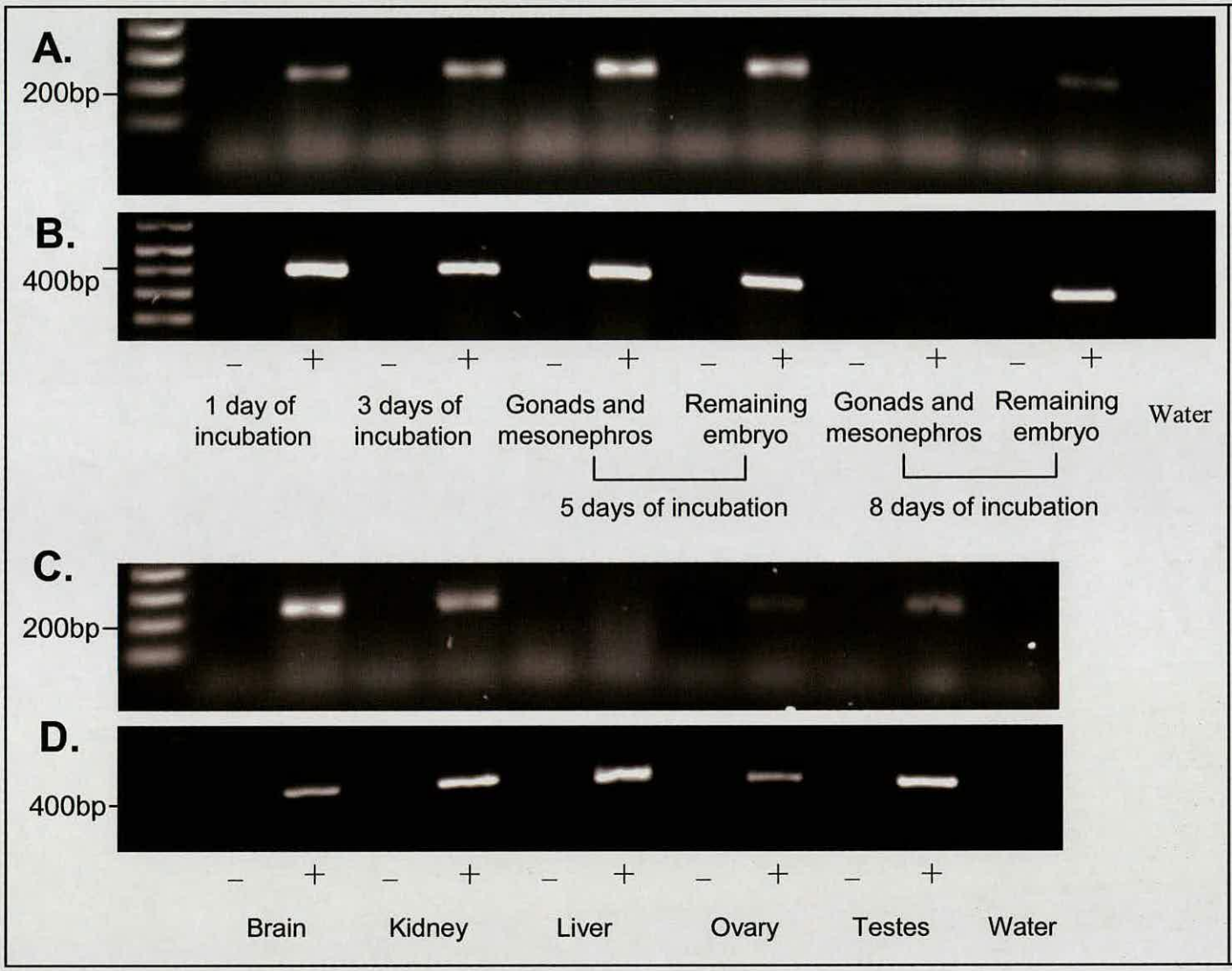


Figure 5.8: RT-PCR to detect the expression of *staufen 2* mRNA in embryonic and adult tissues. (A) RT-PCR to detect *staufen 2* mRNA in embryonic tissues. (C) RT-PCR to detect *staufen 2* in adult tissues. (B and D) *ß-actin* positive controls. +: reverse transcriptase present during cDNA synthesis. -: no RT control.

Northern analysis was carried out to extend the RT-PCR data. A probe was made using DNA from a chicken EST previously identified, obtained from ARKGenomics (see Section 5.2.1 and Appendices 5 and 6). Northern analysis to detect *staufen-1* identified specific hybridisation to all embryonic samples except the mesonephros and gonad sample at eight days of incubation. At one day of incubation, a faint smudge was detected, rather than a distinct band. At three, five and eight days of incubation, two different size products were generated. At three and five days of incubation a product of approximately 2600bp was detected. At eight days of incubation a product of approximately 2900bp was detected (see Figure 5.9A). No specific hybridisation was detected to any adult sample (see Figure 5.9D). Northern analysis to detect *staufen-2* did not result in specific hybridisation to any sample (see Figure 5.9B and E). This could be due to a technical error in the synthesis of the probe. In future experiments, use of poly A^+ RNA instead of total RNA would increase the sensitivity of the analysis.

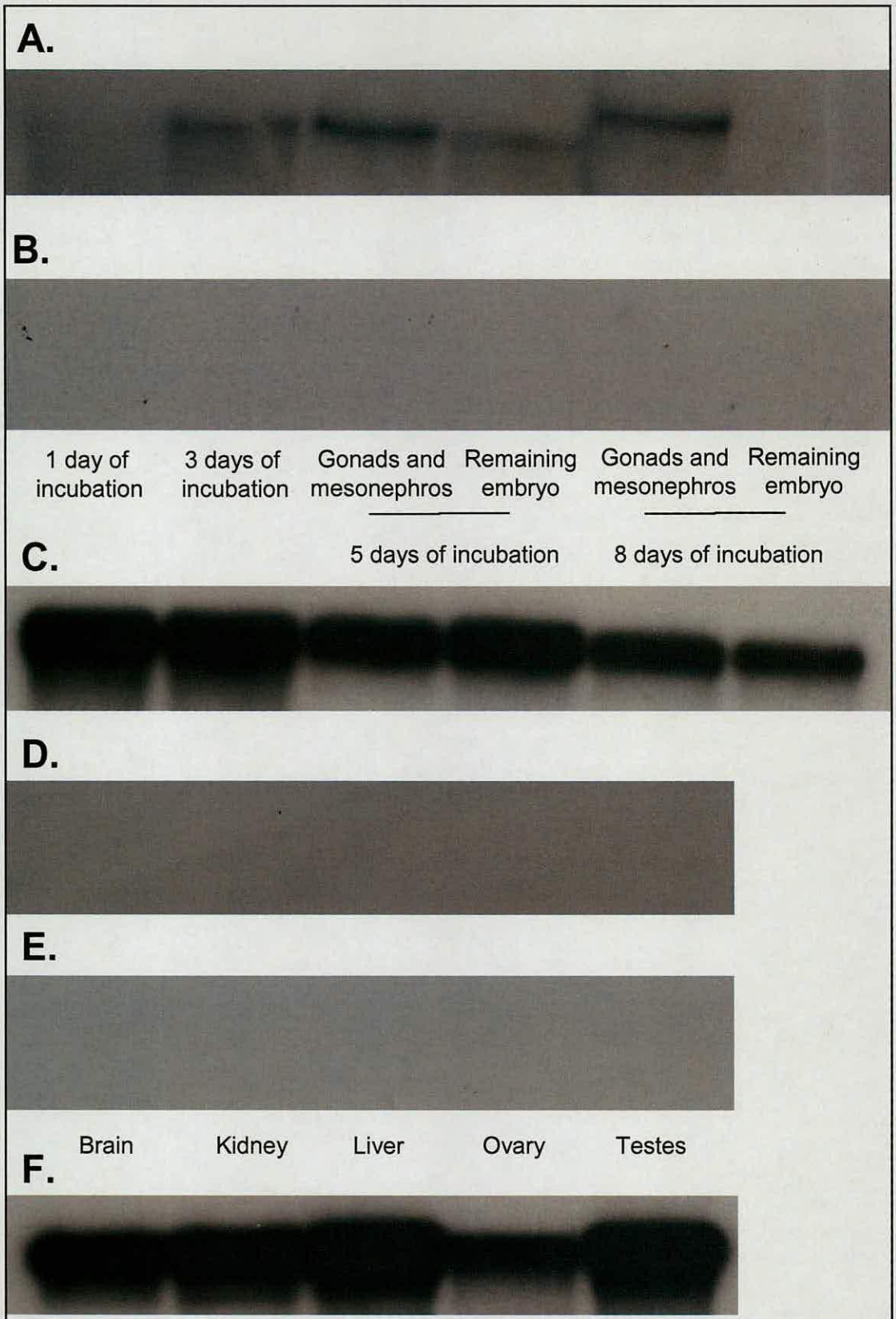


Figure 5.9: Northern analysis to detect chicken *staufen-1* and chicken *staufen-2* mRNAs in embryonic and adult tissues. Expression of *staufen-1* in (A) embryonic tissues and in (D) adult tissues. Expression of *staufen-2* in (B) embryonic tissues and in (E) adult tissues. (C and F) loading control to detect 18s ribosomal RNA. 10µg total RNA were used per sample.

5.3.6 Expression of *chicken staufen 1* and *chicken staufen 2*

Whole mount *in situ* hybridisations were carried out on embryos at one, three, and five days of incubation. Extensive staining was observed at one and five days of incubation for both *chicken staufen 1* and *chicken staufen 2* (see Figures 5.10 and 5.12). At one day of incubation, *chicken staufen 1* mRNA was detected primarily surrounding the primitive steak, and fainter expression was detected in the extraembryonic membranes (see Figure 5.10A). *Chicken staufen 2* mRNA was only detected surrounding the primitive streak (see Figure 5.11A).

At three days of incubation, no specific staining was observed using either *chicken staufen 1* or *chicken staufen 2* anti-sense probes, meaning that neither are detectable by *in situ* hybridisation at this stage of development (see Figures 5.10C and 5.11C).

At five days of incubation, *chicken staufen 1* mRNA was detected in the mesoderm (see Figures 5.10E). Sectioning revealed that *chicken staufen-1* was expressed specifically in the deep mesoderm of the embryo body and limbs. *Chicken staufen 2* mRNA was detected ubiquitously throughout the embryo at this stage (see Figure 5.11E).

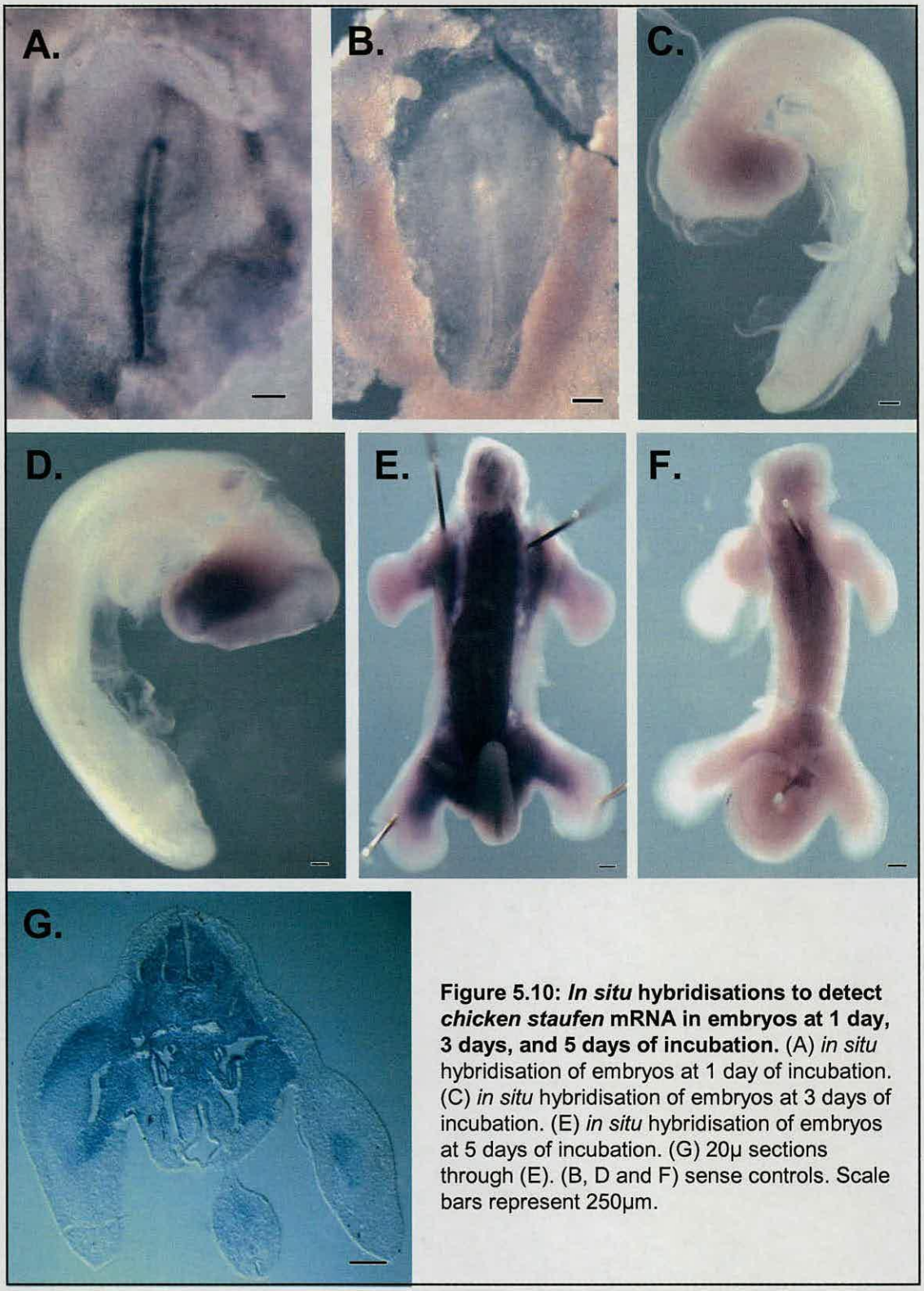


Figure 5.10: *In situ* hybridisations to detect *chicken staufen* mRNA in embryos at 1 day, 3 days, and 5 days of incubation. (A) *in situ* hybridisation of embryos at 1 day of incubation. (C) *in situ* hybridisation of embryos at 3 days of incubation. (E) *in situ* hybridisation of embryos at 5 days of incubation. (G) 20µ sections through (E). (B, D and F) sense controls. Scale bars represent 250µm.

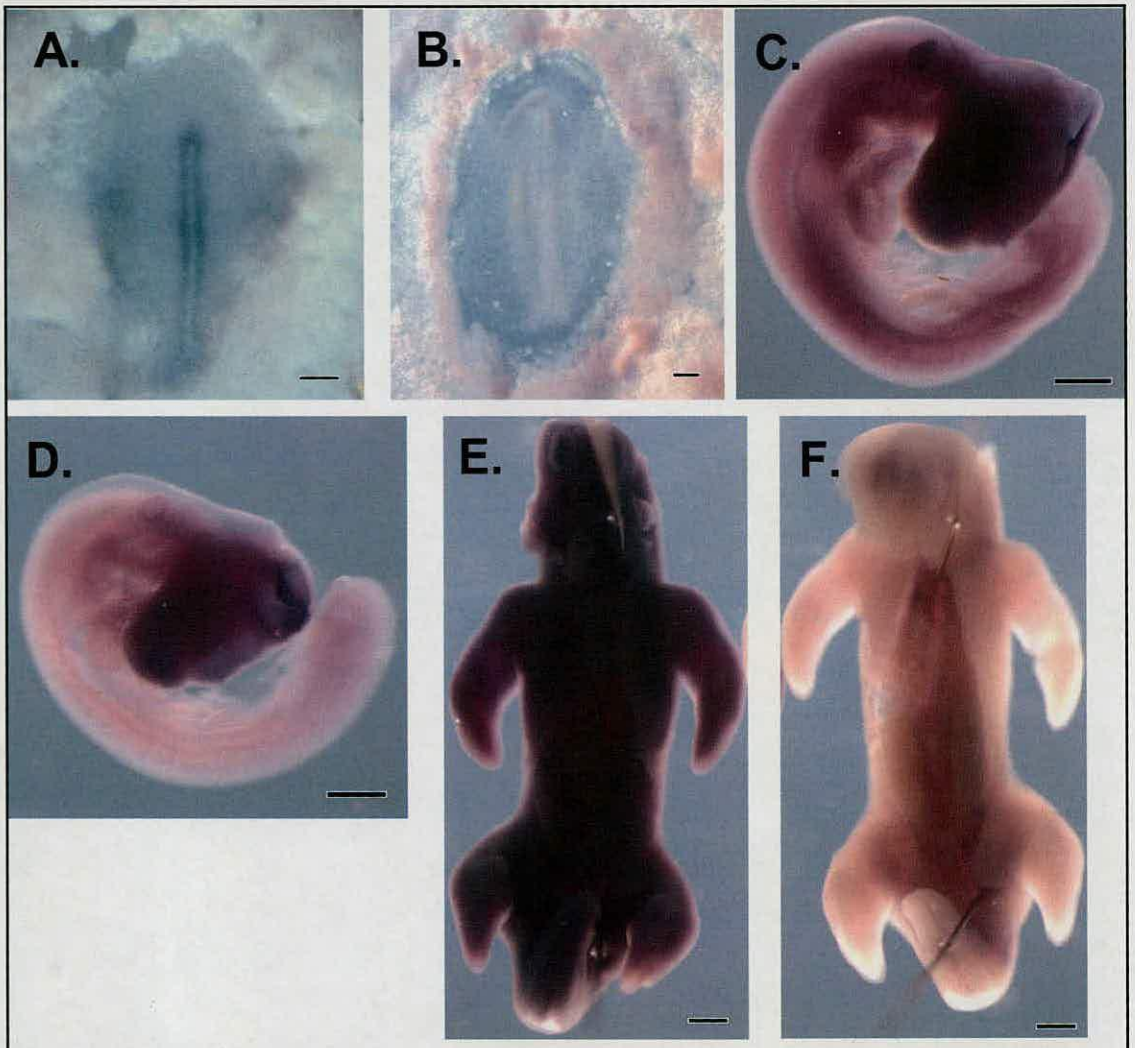


Figure 5.11: *In situ* hybridisations to detect *chicken staufen 2* mRNA in embryos at 1 day, 3 days, and 5 days of incubation. (A) *in situ* hybridisation of embryos at 1 day of incubation (C) *in situ* hybridisation of embryos at 3 days of incubation (E) *in situ* hybridisation of embryos at 5 days of incubation. (B, D and F) sense controls. Scale bars represent 250µm.

5.4 Discussion

Homologues of *mago nashi*, *staufen 1* and *staufen 2* were identified in the chicken genome and expression analysis carried out using RT-PCR, Northern analysis and *in situ* hybridisation. *Mago nashi* is extremely well conserved at the amino acid level, and although not so well conserved, the two *staufen* genes do contain the five conserved double-stranded RNA binding domains and the single tubulin-binding domain.

Mago nashi mRNA was detected by RT-PCR in all embryonic and adult tissues. The Northern analysis indicates that *mago nashi* mRNA is found during early embryonic development, but it is harder to detect by eight days of incubation by this method. In adult tissues, *mago nashi* mRNA was principally detected in the ovaries and testes. Low levels were also detected in the brain and kidney, but it was not detected in the liver. *In situ* hybridisation at embryonic stages showed *mago nashi* mRNA throughout the embryo. At one day of incubation *mago nashi* was detected in both embryonic and extraembryonic regions of the embryo, including the germinal crescent where the PGCs are located at this stage (see Figure 5.4A). By three days of incubation, *mago nashi* was detected in the mesoderm, and by five days of incubation expression was ubiquitous. This widespread expression of *mago nashi* during embryonic development was also observed in zebrafish, indicating the possibility of a conserved function between the two species (Pozzoli *et al.*, 2004). The expression of *mago nashi* mRNA at these three embryonic stages does not indicate expression in the germ cells. Overall from these preliminary experiments it is unknown whether *mago nashi* has a function in PGC or not.

Staufen and *staufen-2* mRNAs were detected by RT-PCR in all embryonic and adult tissues. *Staufen* transcript was detected by Northern in all embryonic tissues except the gonad and mesonephros at eight days of incubation. Interestingly, two different sized products were obtained between samples. This suggests the existence of a splice variant. *Staufen* was not detected in any adult tissues using Northern analysis. *Staufen-2* was not detected by Northern analysis either. Expression of both genes at one day of incubation and three days of incubation do not occur in a pattern to suggest expression in the PGCs. At five days of incubation, expression of *staufen* is found throughout the deep mesoderm, including the gonads (see Figure 5.10E). *Staufen-2* is ubiquitously expressed at this stage. Therefore, both genes could potentially have a role during PGC development at 5 days of incubation.

The expression data of all three genes does not confirm a role during PGC development in the three stages of development investigated, but does indicate that the three genes are genuine homologues. In *Drosophila*, all three genes function during germ cell determination. In chicken, PGC determination happens earlier than one day of incubation. Therefore, in order to investigate whether the genes have a possible function into PGC development, experiments would have to be carried out on embryos shortly after fertilisation. It was decided not to pursue these three genes any further because other experiments being carried out at the same time on two different genes were yielding more promising results (see Chapter 8).

CHAPTER 6

IDENTIFICATION AND EXPRESSION ANALYSIS OF TWO CHICKEN *PUMILIO* HOMOLOGUES. *NANOS* HOMOLOGUES ARE STILL TO BE IDENTIFIED IN THE CHICKEN GENOME

6.1 Introduction

In all of the organisms discussed in this thesis, the primordial germ cells migrate to the gonads and colonise them after they form. In *Drosophila*, the products of two genes, Pumilio and Nanos, are known to silence transcription and mitosis in PGCs during migration and both proteins are required for correct PGC migration. In addition to these functions in *Drosophila*, Nanos and Pumilio are required for dendrite morphogenesis, regulation of neuronal excitability and interact with *piwi* in germline stem cell self-renewal and differentiation (Schweers *et al.*, 2002; Edwards *et al.*, 2003; Ye *et al.*, 2004; Szakmary *et al.*, 2005). In all of the functions listed above, Pum protein acts as a cofactor of Nos protein (Barker *et al.*, 1992; Macdonald, 1992; Asaoka-Taguchi *et al.*, 1999).

This section discusses the multiple roles of *nanos* and *pumilio* in *Drosophila* germline development and outlines their conserved functions in other species. It then presents the identification of two chicken *pumilio* homologues and the corresponding expression data will be presented. The absence of any *nanos* homologues in the chicken genome will be discussed.

6.1.1 *pumilio* and *nanos* have multiple functions in *Drosophila* pole cell development

Pumilio and *nanos* were first identified as maternal proteins in *Drosophila*, where they were found to be essential for anterior-posterior patterning of the oocyte through repression of *hunchback* mRNA translation at the posterior of the oocyte (Tautz, 1988; Irish *et al.*, 1989; Barker *et al.*, 1992). *Pumilio* and *nanos* encode for Pumilio and Nos proteins respectively, both of which contain conserved domains. The Pumilio protein has a highly conserved C-terminal end consisting of N- and C-conserved regions and eight 36 amino acid PUF domains (see Figure 6.1 and 6.2). The Nanos protein has a highly conserved CCHC metal binding Nanos zinc finger motif (Curtis *et al.*, 1997; see Figure 6.9).

Maternally transcribed *nanos* mRNA is concentrated in the pole plasm at a late stage of oogenesis by the actions of *oskar* and *vasa* (Kobayashi *et al.*, 2005). After egg laying, *nanos* mRNA is translated and the protein diffuses away from the posterior to form a Nanos protein gradient with the highest concentration at the posterior pole (Barker *et al.*, 1992; Ephrussi and Lehmann, 1992; Smith *et al.*, 1992; Wang *et al.*, 1994; Thomson and Lasko, 2004). The Nanos gradient specifies the abdomen by repressing posterior translation of maternal *hunchback* mRNA, which would otherwise inhibit abdomen formation (Tautz, 1988; Hülskamp *et al.*, 1989; Irish *et al.*, 1989; Struhl, 1989; Tautz and Pfeifle, 1989; Barker *et al.*, 1992). Translational repression of *hunchback* is mediated by discrete target sites, known as *nanos* response elements (NREs) in its 3' UTR which Pumilio binds to directly in a sequence-specific manner (Wharton and Struhl, 1991; Wharton *et al.*, 1999).

Pumilio then interacts with Nanos protein to repress translation of *hunchback* mRNA (Murata and Wharton, 1995; Wharton *et al.*, 1998; Sonoda and Wharton, 1999). Although maternal *nanos* mRNA is localised to the germ plasm, maternal Nanos protein is initially found in the posterior half of the embryo until the cellular blastoderm stage when it is incorporated solely into the pole cells and remains detectable throughout pole cell migration (Lehmann and Nüsslein-Volhard, 1991; Wang and Lehman, 1991; Wang *et al.*, 1994). In comparison, maternal Pumilio protein is distributed ubiquitously in embryos (Barker *et al.*, 1992; Macdonald, 1992).

In *Drosophila*, pole cells cease mitosis at gastrulation and remain quiescent in the G2 phase of the cell cycle until they have populated the gonads. Asaoka-Taguchi *et al.* (1999) showed that when Cyclin B protein is induced in wildtype pre-migratory pole cells, the cells are driven from the G2 phase of the cell cycle and enter into mitosis before they migrate. *Cyclin B* is a maternally supplied mRNA that is localised to the pole plasm and is subsequently incorporated into the pole cells (Dalby and Glover, 1993; Asaoka-Taguchi *et al.*, 1999). Furthermore, *cyclin B* mRNA contains a NRE-like sequence in its 3' UTR that Nanos and Pumilio can bind to (Sonada and Wharton, 2001). In normal development, *cyclin B* mRNA is translationally repressed until the pole cells have populated the gonads and Nanos protein has degraded, but *pumilio* and *nanos* mutations cause premature expression of Cyclin B protein in pre-migratory pole cells (Asaoka-Taguchi *et al.*, 1999). These findings demonstrate that Nanos and Pumilio proteins inhibit the transition from G2 to mitosis in migrating pole cells by repressing Cyclin B protein production (Kobayashi *et al.*, 2005).

Although *nanos* is a germ plasm component, embryos that lack Nanos protein activity are still able to form pole cells called *nanos* pole cells. When *nanos* pole cells are transplanted into a wildtype embryo they migrate through the midgut epithelium into the hemocoel as normal, but they are never incorporated into the gonads of the host embryo and are eliminated by an apoptotic mechanism (Kobayashi *et al.*, 1996; Forbes and Lehmann, 1998; Hayashi *et al.*, 2004). The same series of events is also seen when pole cells taken from embryos mutant for *pumilio* are introduced into wildtype hosts. Pumilio and Nanos proteins have already been shown to repress Cyclin B production to prevent the pole cells entering mitosis, but repression of Cyclin B production is not required for pole cell migration because its induction does not affect pole cell migration. This suggests that *cyclin B* is not the only regulatory target of Nanos and Pumilio proteins in pole cells (Asaoka-Taguchi *et al.*, 1999; Kobayashi *et al.*, 2005). It was found that Nanos and Pumilio proteins also regulate *Head involution defective* (*hid*) mRNA, which contains a NRE in its 3' UTR and encodes a protein required for the induction of apoptosis (Grether *et al.*, 1995; Kobayashi *et al.*, 2005). Apoptosis is suppressed in flies that have a small deletion within the genomic region that includes the *hid* gene known as H99. In H99 embryos the pole cells are prevented from apoptosing and when they are transplanted into wildtype embryos they are able to migrate into the gonads as normal (Kobayashi *et al.*, 2005). This demonstrates that Nanos inhibits apoptotic responses in pole cells to permit their proper migration into the gonads.

The final known function of *nanos* in *Drosophila* pole cell development is repression of somatic differentiation. Hayashi *et al* (2004) created a mutant line that is both mutant for *nanos* and contains the H99 deletion to prevent apoptosis. When *nos*-H99 pole cells were transplanted into wildtype embryos, the pole cells were integrated within somatic tissues such as the midgut epithelium, tracheal epithelium and gastric caeca (Hayashi *et al.*, 2004). The integrated *nos*-H99 cells were not only morphological indistinguishable from surrounding somatic cells, but they ectopically expressed somatic genes of the host tissue they occupied and they no longer express the germline specific protein Vasa (Kobayashi *et al.*, 2005). Additionally, Sano *et al* (2001) found that Nanos and Pumilio proteins promote zygotic vasa expression.

From these results it can be seen that in *Drosophila* pole cells *nanos* and *pumilio* are important in preventing early activation of mitosis, are required for the colonisation of the gonads, prevent apoptosis of the pole cells and prevent differentiation into somatic cells. In the next section the conservation in other species of the roles discussed above will be outlined.

6.1.2 Conservation of *nanos* and *pumilio*

Multiple *pumilio* and *nanos* homologues containing the conserved domains have been identified in many species including *Caenorhabiditis elegans* (The *C.elegans* Sequencing Consortium), zebrafish (Pelegri, 2003), *Xenopus* (Nakahata *et al.*, 2001), mice (Haraguchi *et al.*, 2003; Tsuda *et al.*, 2003) and humans (Husi *et al.*, 2000; Spassov and Jurecic, 2002). In these species the interaction between Nanos and Pumilio proteins is conserved, as are some of their functions.

Three *nanos* homologues have been identified in *C.elegans*, the roles of which overlap during germ-line development. *Nanos-1* is expressed in PGCs after gastrulation, and is required for the efficient incorporation of PGCs into the somatic gonad. *Nanos-1* is also required redundantly with *nanos-2* to prevent PGCs from dividing in starved animals and to maintain germ cell viability during larval development. In the absence of *nanos-1* and *nanos-2*, germ cells cease proliferation at the end of the second larval stage, and die in a manner that is partially dependent on the apoptosis gene *ced-4* (Subramaniam and Seydoux, 1999). In *nanos-3* deficient animals, the hermaphrodite sperm-oocyte switch was defective, leading to the production of excess sperm and no oocytes (Kraemer *et al.*, 1999).

In zebrafish, a *nanos-like* homologue is expressed in the germ plasm and in PGCs and knock down of expression causes aberrant migration and PGC death (Köprunner *et al.*, 2001). Additionally, the 3' UTR of zebrafish *nanos* has been shown to interact with the micro RNA miR430, which is thought to contribute to the primordial germ cell-specific gene expression of *nanos* (Mishima *et al.*, 2006). Two *pumilio* genes have been identified in zebrafish, but expression and functional analysis are yet to be published.

In *Xenopus*, two Pumilio proteins have been identified, but investigative work has only been done on one protein. Pumilio interacts with Nanos, known as Xcat-2, and binds in a sequence specific manner to *cyclin B1* in a similar manner to *Drosophila*

(Nakahata *et al.*, 2001). However, whether this interaction functions during germ cell development is currently unknown.

In mice, three *nanos* homologues have been identified (Haraguchi *et al.*, 2003; Tsuda *et al.*, 2003). *Nanos-1* is expressed in the central nervous system and has no role in germ cell development (Haraguchi *et al.*, 2003). *Nanos-2* and *nanos-3* are expressed in embryonic germ cells (Tsuda *et al.*, 2003). *Nanos-2* expression is male-PGC specific and is only detectable in PGCs that have entered the male genital ridge (Suzuki *et al.*, 2007). Loss of *nanos-2* function results in a decrease in the number of male germ cells, irrespective of the presence of *nanos-3* (Suzuki *et al.*, 2007). In comparison, *nanos-3* is first detected in early PGCs at the base of the allantois shortly after specification (Suzuki *et al.*, 2007). Expression is maintained until E13.5 in the female, corresponding with the onset of meiosis, and E15.5 in the male (Yamaguchi *et al.*, 2005; Suzuki *et al.*, 2007). Loss of Nanos-3 protein in the mouse causes the gradual loss of germ cells during migration, which results in a germ cell-less phenotype in both sexes (Suzuki *et al.*, 2007). Nanos-2 can partially rescue a Nanos-3 null mutant phenotype (Suzuki *et al.*, 2007). As with zebrafish, two *pumilio* sequences have been identified in the mouse, but expression and functional analysis are yet to be published.

In humans, two *pumilio* genes have been identified containing the highly conserved C- and N-conserved regions and the eight PUF repeat motifs (Spassov and Jurecic, 2002). Pumilio-2 protein is expressed in embryonic stem cells and germ cells and has been shown to interact with the germ cell specific protein Dazl, suggesting a

function in germ cell development, but no direct evidence has been found to link the two (Moore *et al.*, 2003; Urano *et al.*, 2005). *Nanos* genes have been identified, as has a *pumilio-1* sequence, but further research is yet to be published.

Although homologues of both *nanos* and *pumilio* have been identified in diverse organisms, functions relating to primordial germ cell development are, on the whole, still to be confirmed. Some of the interaction and expression data described above suggests that some if not all of the functions described in *Drosophila* pole cell development are conserved between invertebrates and vertebrates.

6.2 Overview of methods

This section gives a brief overview of the methods and samples used to investigate *pumilio* and *nanos* in PGC development. For a full account of the methods see chapter 2.

6.2.1 BLAST analysis of the chicken genome and EST databases

To identify putative *pumilio* homologues in the chicken denome, BLAST searches of the genome sequence were carried out using the mouse *pumilio-1* and *pumilio-2* nucleotide sequences as the query sequences (acc.nos. BC050747 and BC041773).

To identify putative *nanos* homologues in the chicken, BLAST searches of the chicken genome were carried out using the mouse *nanos-1*, *nanos-2* and *nanos-3*

nucleotide sequences as the query sequences (acc.no. NM_178421; NM_194064; NM_194059 respectively).

6.2.2 Samples for RT-PCR and Northern analysis

RNA was extracted from chicken embryos at several stages of development and several adult tissues. The embryonic stages were: whole embryos at one and three days of incubation; gonads and mesonephros at five and eight days of incubation; the remaining embryo after the gonad and mesonephros had been removed at five and eight days of incubation. Adult tissues were taken from: brain; kidney; liver; ovary and testes.

6.2.3 RT-PCR analysis

RT-PCR primers to detect *pumilio-1* and *pumilio-2* were designed flanking an intron using primer 3 software (http://frodo.wi.mit.edu/; see Appendix 1). The *pumilio-1* primers hybridise to nucleotides located at 2767-2925bp of the predicted full length sequence to give an expected product size of 158bp. The *pumilio-2* primers bind to nucleotides located at 1685-1894bp of the predicted full length sequence to give an expected producted size of 209bp. Amplification of β-actin using primers to give a product with an expected size of 428bp was used as a positive control. The annealing temperature was 59.5°C.

To make sure the PCR primers were amplifying the predicted *pumilio-1* and *pumilio-2* sequences, the RT-PCR product was cloned from testes cDNA and then sequenced by the Sequencing Service (University of Dundee). The sequence data were aligned

with the predicted sequences resulting in a 100% match in both instances, confirming that the PCR primer sets were amplifying the correct sequences.

6.2.4 Northern and *In situ* hybridisation probes

The ESTs ChEST99k7 and ChEST588f13 from ARKGenomics were used to make a radiolabelled Northern probe to detect chicken *pumilio-1* and *pumilio-2* respectively (see Appendices 7 and 8).

The RT-PCR product cloned from testes cDNA was used to make sense and anti-sense digoxigenin *in situ* hybridisation probes. Whole mount *in situ* hybridisation was carried out on embryos at one, three and five days of incubation.

6.3 Results

6.3.1 Identification of two chicken *pumilio* homologues

These BLAST searches identified two predicted chicken *pumilio* homologues containing the N- and C-conserved domains and eight PUF repeats each. The first predicted sequence is on chromosome 23 at location 438,326-508,237, consisting of 22 exons, is 3240bp long and covers 66.91Kb of the genome (see Appendix 7). The second predicted sequence is on chromosome 3 at location 104,495,277-104,538,316, consisting of 20 exons, is 3685bp long and is located over 43.04Kb of the chromosome (see Appendix 8). BLAST searches of the chick UMIST database identified several ESTs with identical sequences to each sequence obtained from the chicken genome (see Appendices 7 and 8). This indicates that the genome data is

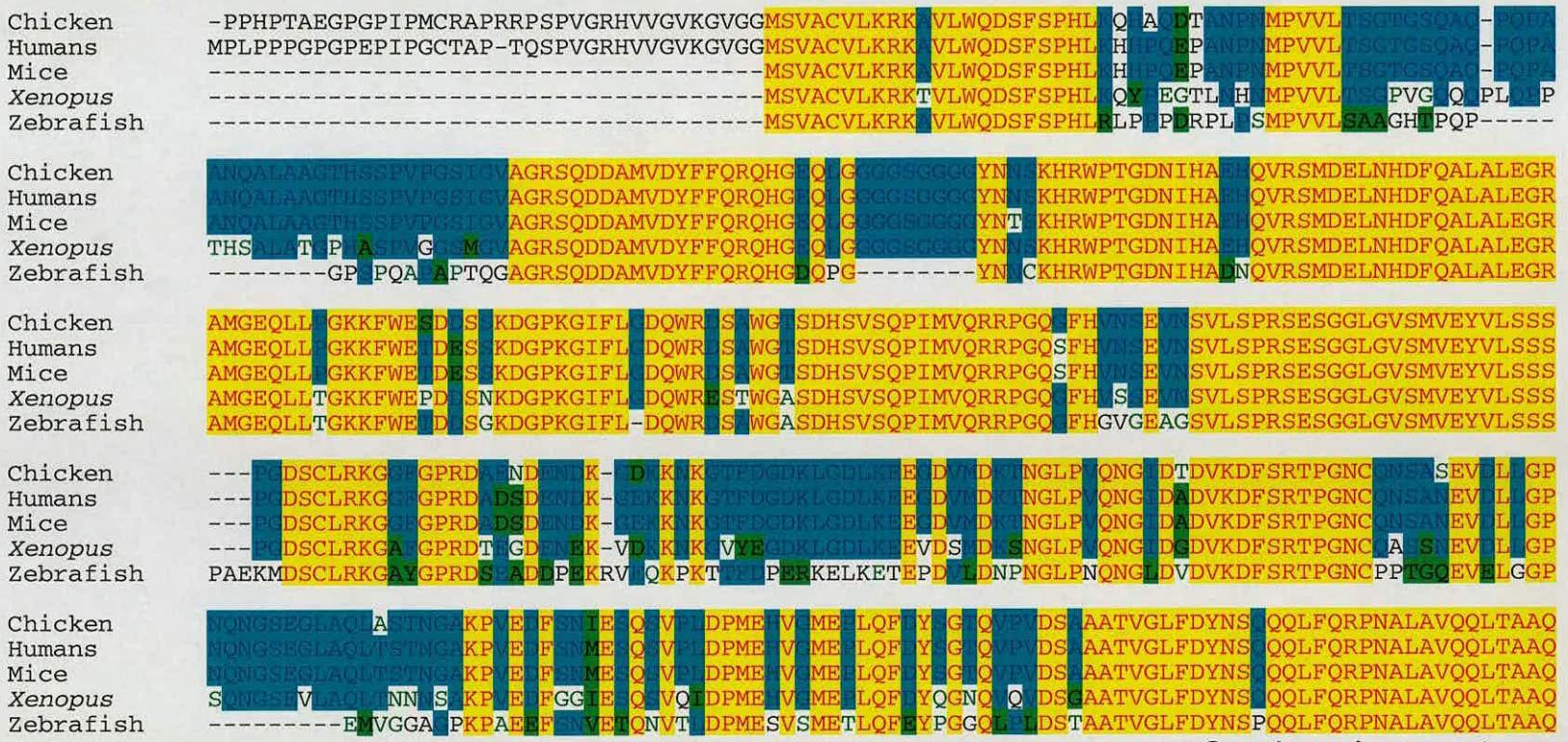
accurate. To confirm that the sequences are *pumilio* homologues, the predicted chicken protein sequences were used as the query sequences to BLAST the NCBI database. The genes with the highest similarity to the chicken sequences were *pumilio* genes. To investigate the genes' identity further, the sequences were aligned with known Pumilio protein sequences from *Xenopus*, zebrafish, mice and humans (see Figures 6.1 and 6.2 and Tables 6.1 and 6.2). The predicted chicken sequence on chromosome 23 shows the highest sequence similarity to Pumilio 1 proteins, with the highest similarity to human Pumilio-1 (97% identical; see Table 6.1). Additionally, high sequence similarity to mouse Pumilio-1 (94% identical), and *Xenopus* Pumilio-1 (86% identical) was observed. Lower sequence similarity to zebrafish Pumilio-2 (54% identical) and *Drosophila* Pumilio (27% identical) was observed. Overall the chicken Pumilio protein on chromosome 23 shows highest sequence similarity to other Pumilio-1 proteins in comparison to Pumilio-2 proteins. Therefore, the gene shall be referred to as chicken *pumilio-1*, which encodes chicken Pumilio-1 protein.

The predicted chicken sequence on chromosome 3 shows the highest sequence similarity to Pumilio 2 proteins, with the highest similarity to human Pumilio-2 (94% identical; see Table 6.2). Additionally, high sequence similarity to mouse Pumilio-2 (93% identical) was observed. Again, these percentage identities are exceptionally high. Lower sequence similarity to *Xenopus* Pumilio-2 (76% identical), zebrafish Pumilio-2 (59% identical) and *Drosophila* Pumilio (26% identical) were observed. The chicken Pumilio protein on chromosome 3 shows highest sequence similarity to Pumilio-2 proteins, therefore the gene shall be referred to as chicken *pumilio-2*, which encodes chicken Pumilio-2 protein.

Gene (species)	E value	Identity	Similarity
Pum-1 *(Homo sapiens)*	0.0	97%	98%
Pum-2 *(Homo sapiens)*	Not shown	66%	71%
Pum-1 *(Mus musculus)*	0.0	94%	95%
Pum-2 *(Mus musculus)*	Not shown	65%	71%
Pum-1 *(Xenopus tropicalis)*	0.0	86%	89%
Pum-2 *(Xenopus tropicalis)*	Not shown	66%	74%
Pum-1 *(Danio rerio)*	0.0	26%	28%
Pum-2 *(Danio rerio)*	Not shown	51%	58%
Pum *(Drosophila melanogaster)*	0.0	27%	36%

Table 6.1: E values, the percentage of identical residues (Identity) and the percentage of conservative substitutions (Similarity) are shown to identity of the predicted chicken Pumilio protein on chromosome 23.

Figure 6.1:



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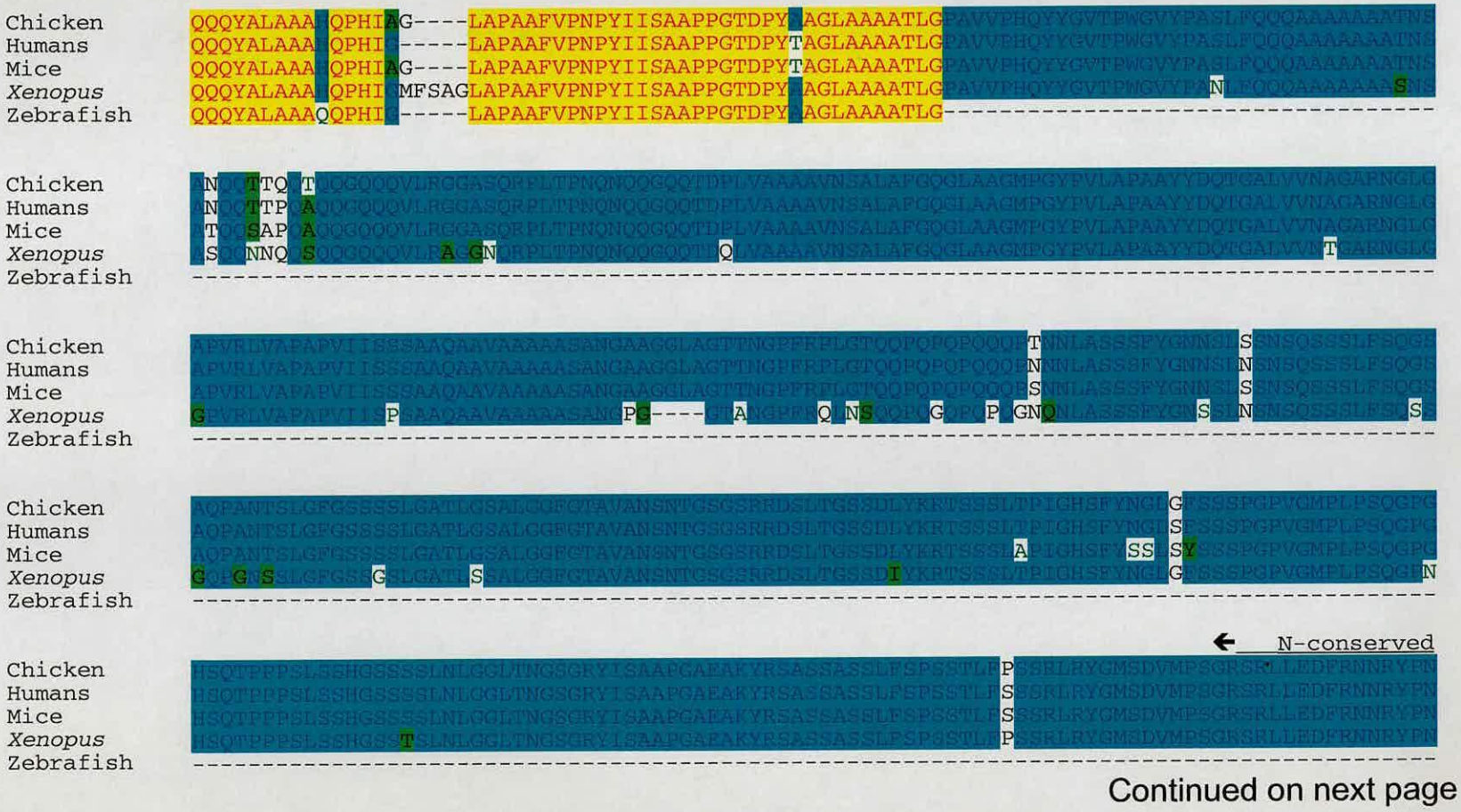

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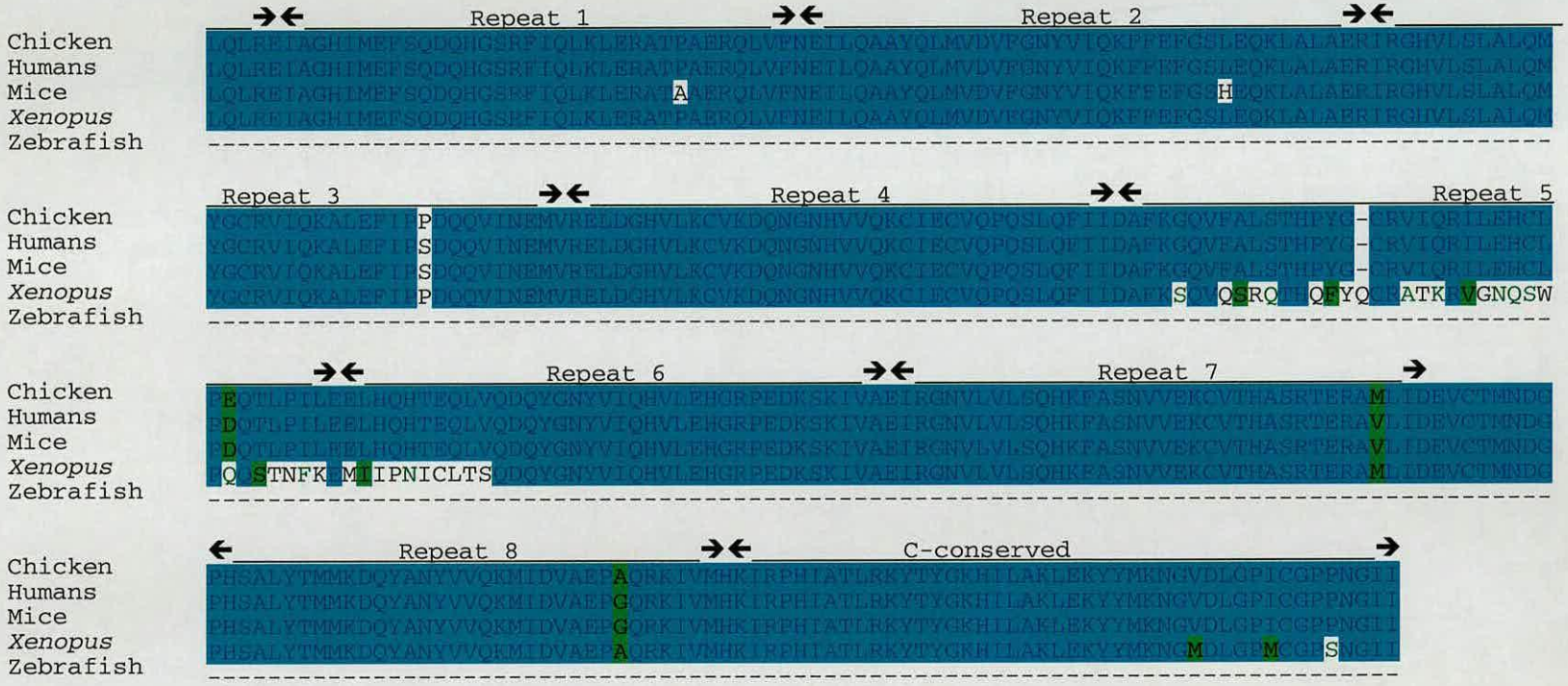
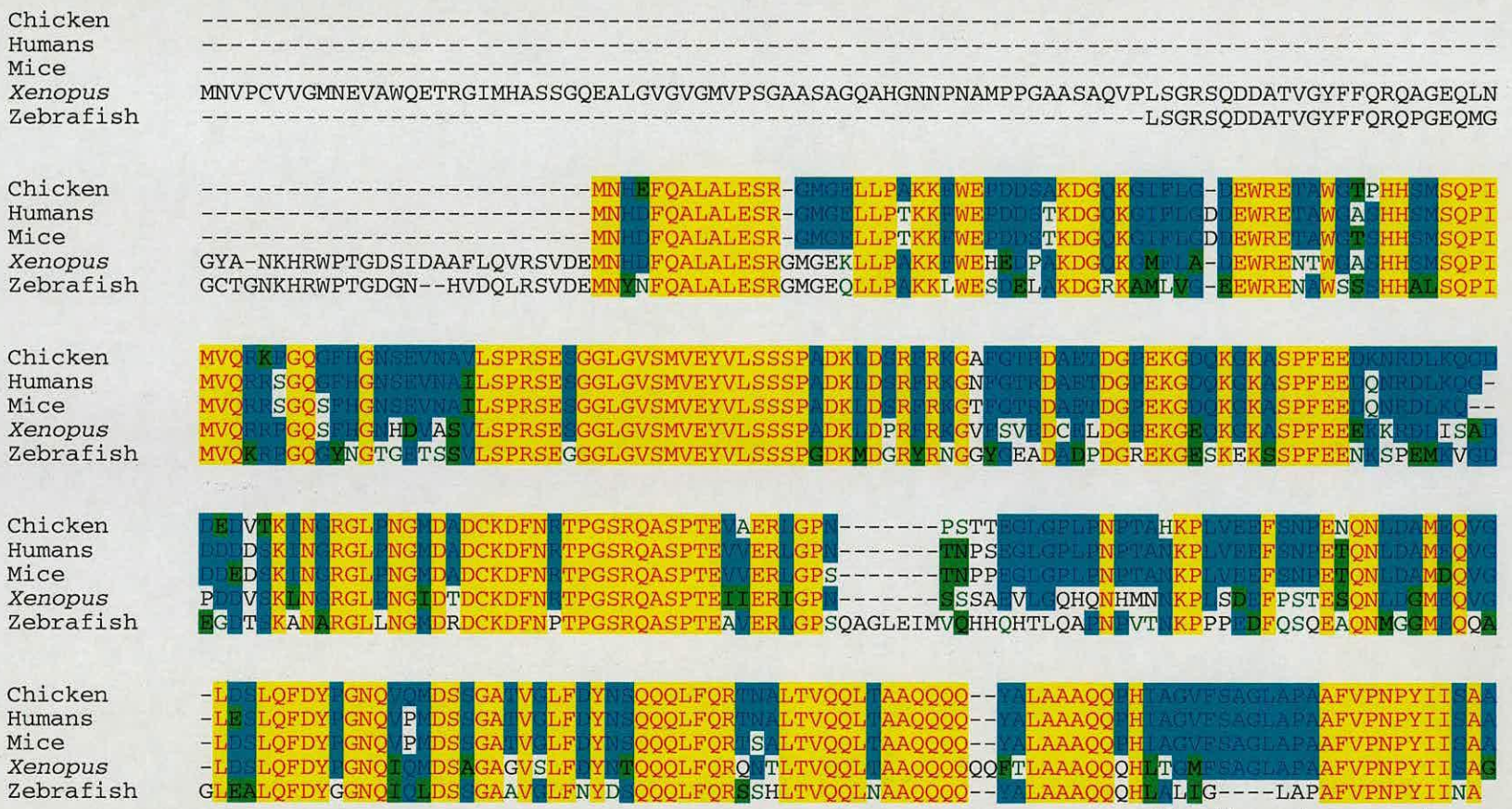


Figure 6.1: Alignment of the identified Pumilio protein sequence found on chromosome 23 with known Pumilio 1 sequences from humans, mice, *Xenopus* and zebrafish. Identical residues are highlighted in yellow. Residues that are identical between two or three species are highlighted in blue. Residues that are different between species but where the different residues are predicted to act in the same way are highlighted in green. The N- and C-conserved regions are shown, as are the eight PUF repeats of 36 amino acids each.

Gene (species)	E value	Identity	Similarity
Pum-1 *(Homo sapiens)*	Not shown	65%	71%
Pum-2 *(Homo sapiens)*	0.0	94%	95%
Pum-1 *(Mus musculus)*	Not shown	67%	74%
Pum-2 *(Mus musculus)*	0.0	93%	95%
Pum-1 *(Xenopus tropicalis)*	Not shown	64%	70%
Pum-2 *(Xenopus tropicalis)*	0.0	76%	80%
Pum-1 *(Danio rerio)*	Not shown	18%	21%
Pum-2 *(Danio rerio)*	0.0	59%	66%
Pum *(Drosophila melanogaster)*	0.0	26%	34%

Table 6.2: E values, the percentage of identical residues (Identity) and the percentage of conservative substitutions (Similarity) are shown to identity of the predicted chicken Pumilio protein on chromosome 3.

Figure 6.2:



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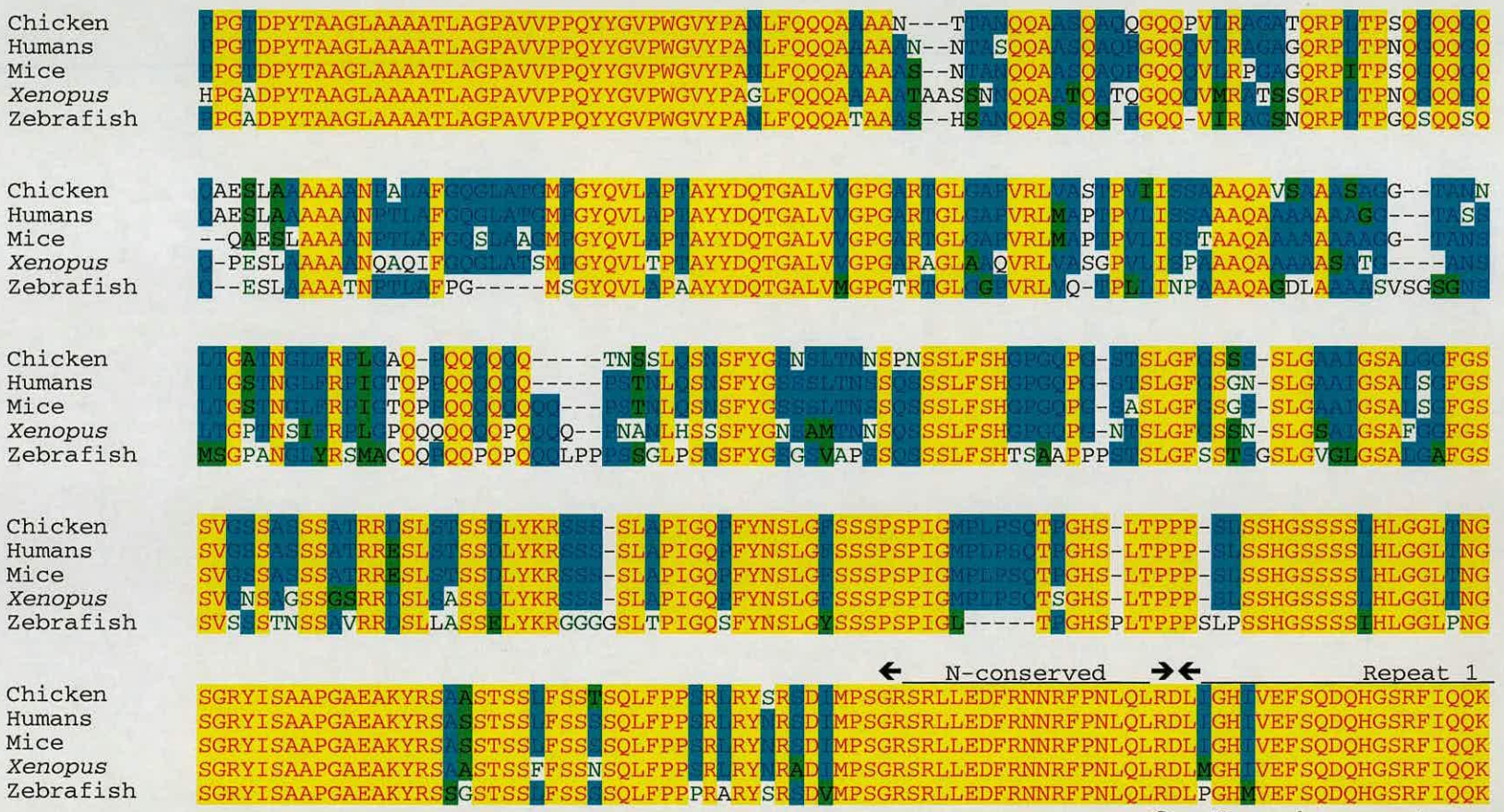

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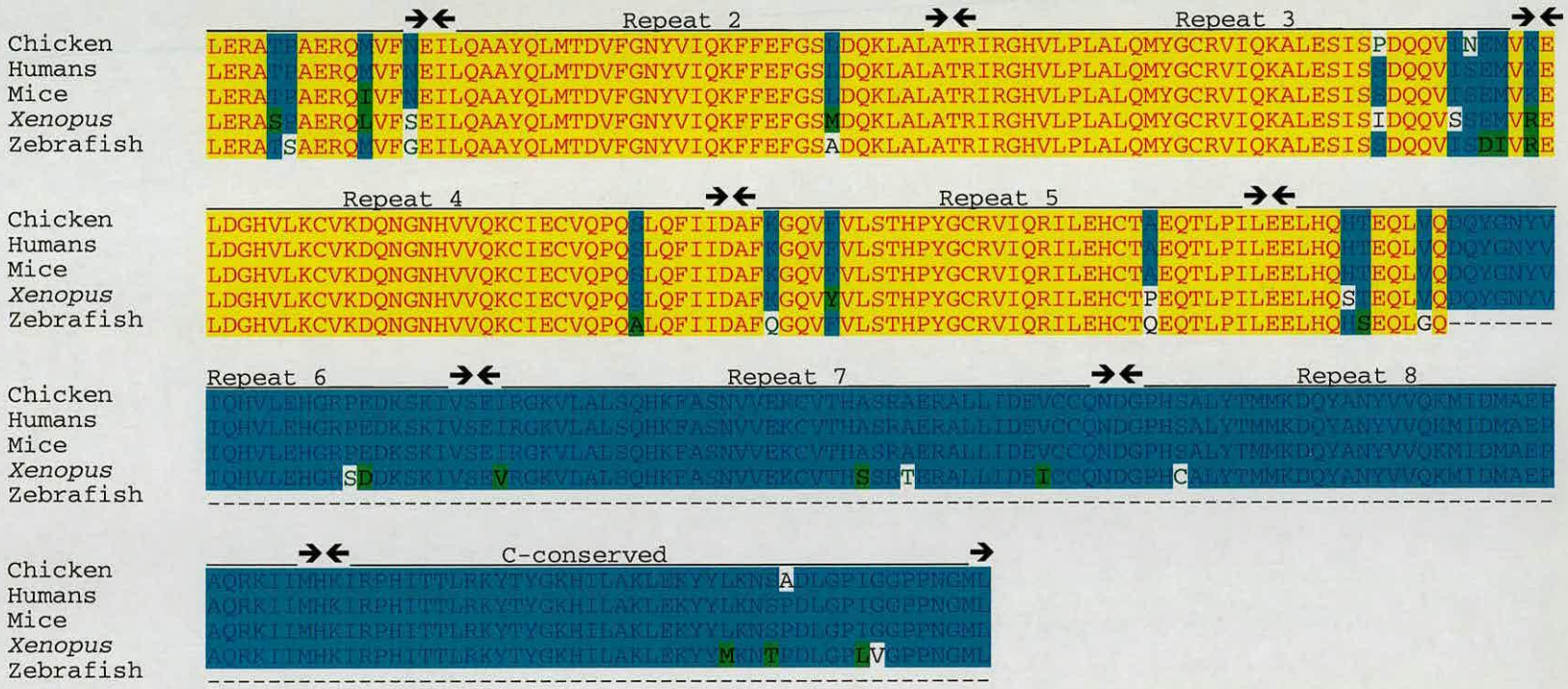


Figure 6.2 continued: Alignment of the identified Pumilio protein sequence found on chromosome 3 with known Pumilio 2 sequences from humans, mice, *Xenopus* and zebrafish. Identical residues are highlighted in yellow. Residues that are identical between two or three species are highlighted in blue. Residues that are different between species but where the different residues are predicted to act in the same way are highlighted in green. The N- and C-conserved regions are shown, as are the eight PUF repeats of 36 amino acids each.

6.3.2 Detection of chicken *pumili-1* and *pumilo-2* by RT-PCR and Northern analysis

In order to determine when *pumilio-1* and *pumilio-2* are expressed during early embryonic development and which adult tissues express them, RT-PCR and Northern analyses were carried out using RNA extracted from embryos at several stages of development and a range of adult tissues.

Looking at the RT-PCR results, after 25 cycles of amplification, both *pumilio-1* and *pumilio-2* mRNAs were detected at all embryonic stages and in all adult tissues (see Figures 6.3 and 6.4).

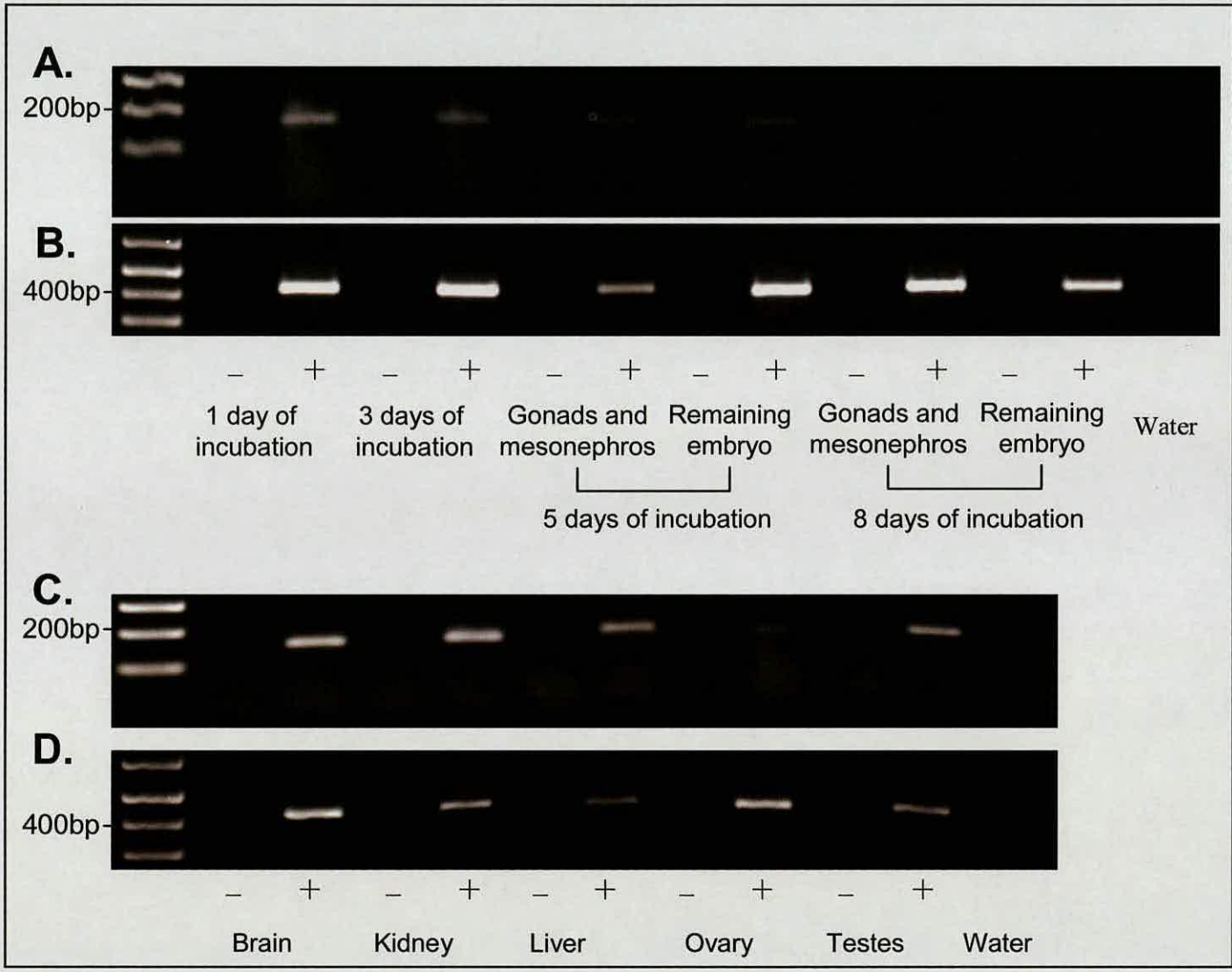


Figure 6.3: RT-PCR to detect the expression of *pumilio-1* mRNA in embryonic and adult tissues. (A) RT-PCR to detect *pumilio-1* mRNA in embryonic tissues. (B) RT-PCR to detect *pumilio-1* mRNA in adult tissues. (C and D) *ß-actin positive controls.* +: reverse transcriptase present during cDNA synthesis. -: no reverse transcriptase control.

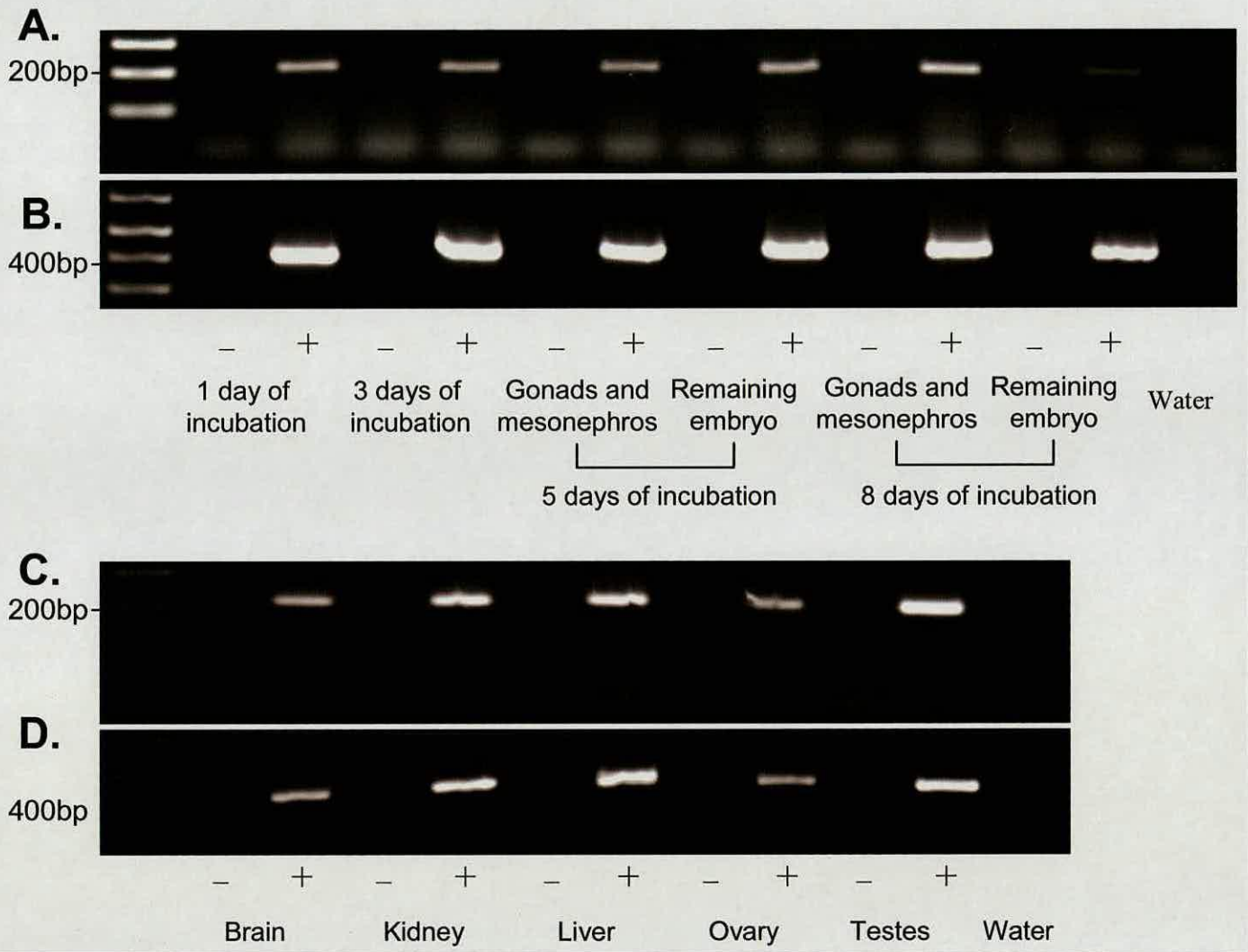


Figure 6.4: RT-PCR to detect the expression of *pumilio-2* mRNA in embryonic and adult tissues. (A) RT-PCR to detect *pumilio-2* mRNA in embryonic tissues. (B) RT-PCR to detect *pumilio-2* mRNA in adult tissues. (C and D) *ß-actin positive controls.* +: reverse transcriptase present during cDNA synthesis. -: no reverse transcriptase control.

Northern analysis was carried out to extend the RT-PCR data. Probes were made using DNA from previously identified chicken ESTs obtained from ARKGenomics (see Appendices 7 and 8). Neither of the radiolabelled probes hybridised to any of the embryonic or adult samples (see Figure 6.5). The probe synthesised to detect - *pumilio-1* was $22.5x10^3$ cpm/µl, and the probe synthesised to detect *pumilio-2* was $17.5x10^3$ cpm/µl indicating that radiolabel had bound to the DNA in both reactions, but there is still the possibility that there was a technical error in the synthesis of the probe. In future experiments, use of poly A^+ RNA instead of total RNA would increase the sensitivity of the analysis.

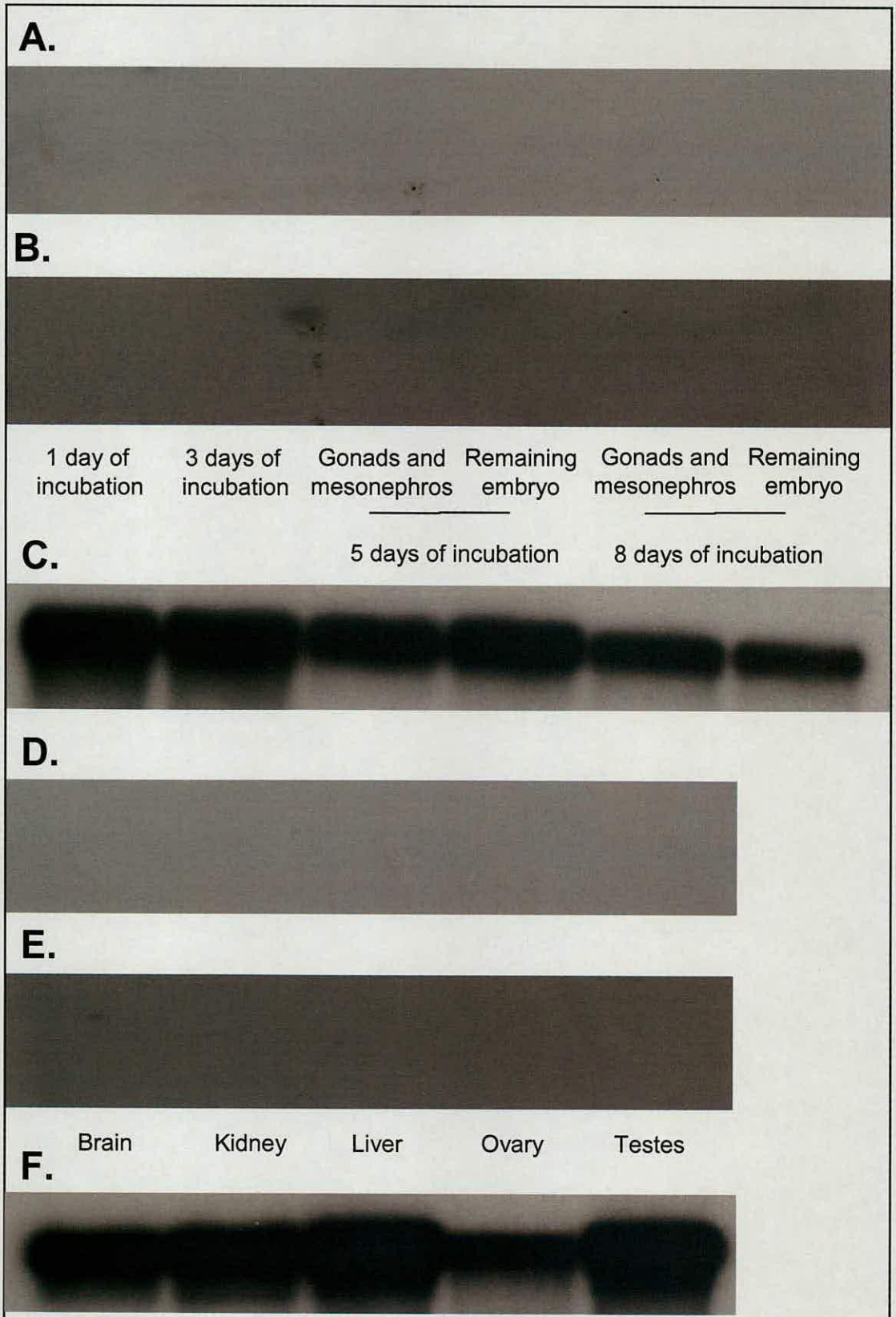


Figure 6.5: Northern analysis to detect chicken *pumilio-1 and chicken* pumilio-2 mRNAs in embryonic and adult tissues. Expression of *pumilio-1* in (A) embryonic tissues and in (D) adult tissues. Expression of *pumilio-2* in (B) embryonic tissues and in (E) adult tissues. (C and F) loading control to detect 18s ribosomal RNA. 10µg total RNA were used per sample.

6.3.3 Expression of *pumilio-1* and *pumilio-2*

Whole mount *in situ* hybridisation was carried out on embryos at one, three, and five days of incubation to detect the expression of *pumilio-1* and *pumilio-2* mRNA and determine the likely areas where the protein functions during development.

First, looking at *pumilio-1*, at one day of incubation, *pumilio-1* mRNA was detected very faintly around the anterior of the primitive streak (see Figure 6.6 A). The mRNA was not detected anywhere else in the embryos at this stage.

At three days of incubation, *pumilio-1* expression was detected in the eye and the forming blood vessels positioned between the somites (see Figures 6.6 C and E). The sense control indicates that these are genuine signals and not background staining or trapping (see Figure 6.6D).

At 5 days of incubation, *pumilio-1* was detected in the mesoderm of the embryo body and limbs (see Figure 6.6 F). The mRNA is absent from the outer ectoderm layers in a similar pattern to chicken *bruno* and *staufen-1*.

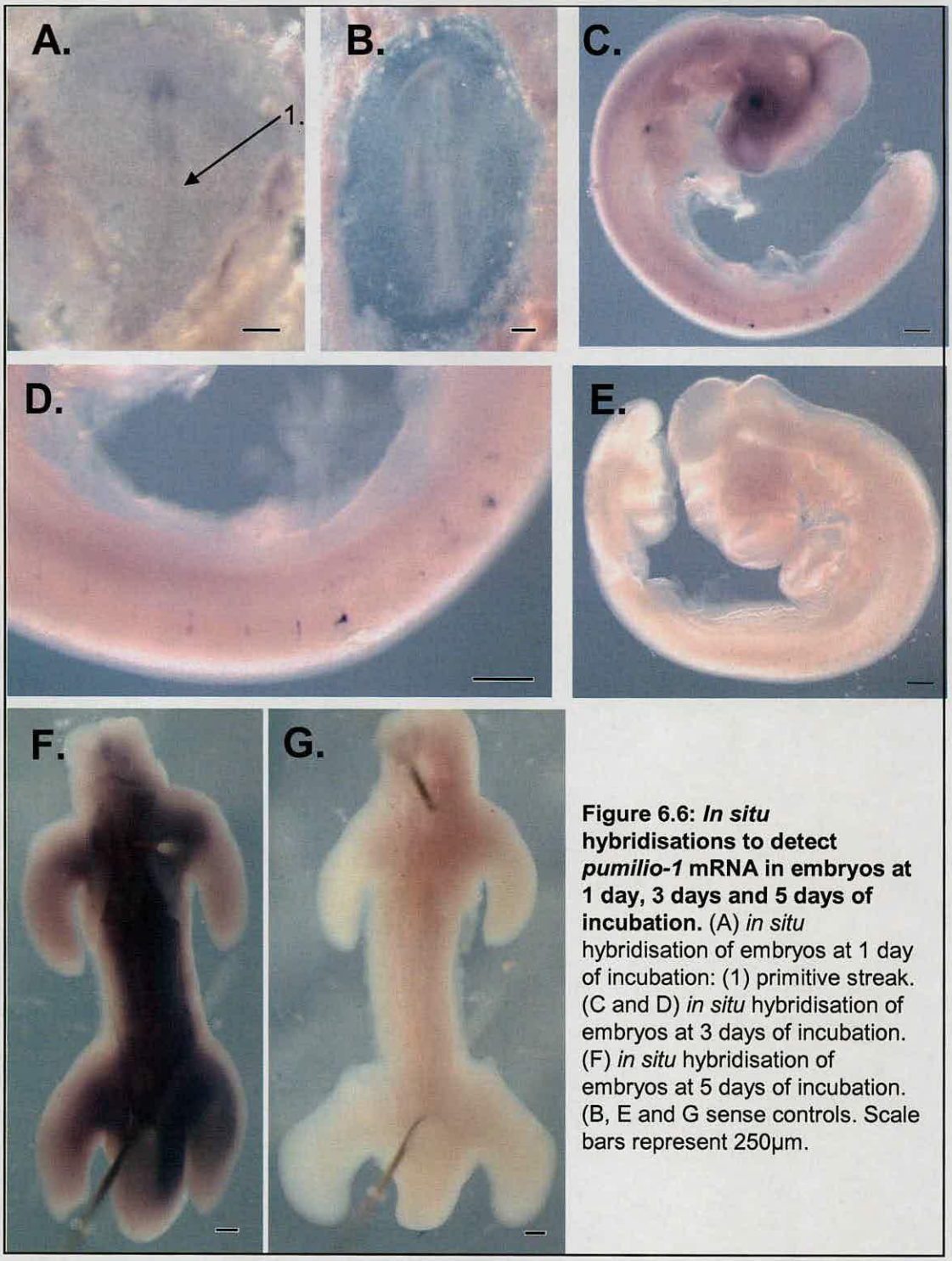


Figure 6.6: *In situ* hybridisations to detect *pumilio-1* mRNA in embryos at 1 day, 3 days and 5 days of incubation. (A) *in situ* hybridisation of embryos at 1 day of incubation: (1) primitive streak. (C and D) *in situ* hybridisation of embryos at 3 days of incubation. (F) *in situ* hybridisation of embryos at 5 days of incubation. (B, E and G sense controls. Scale bars represent 250μm.

Looking at the expression of *pumilio-2*, at one day of incubation, *pumilio-2* mRNA was detected surrounding the primitive steak and in the surrounding neural plate. Expression is restricted to the area pellucida. Two bands of darker expression were detected either side of the primitive streak, approximately half way down the trunk of the embryo (see Figure 6.7A). Slightly later in development, the expression pattern of *pumilio-2* mRNA changes. Expression appears to be down-regulated in the neural plate during the process of primitive streak closure when the node travels down the streak. A line of expression was detected along the newly closed primitive streak. The remaining unclosed streak was still surrounded by expression. The two lines of expression detected either side of the trunk, adjacent to the area pellucidae/area opaca boundary noted earlier are still present (see Figure 6.7 B).

At three days of incubation, *pumilio-2* mRNA was only detected in the head (see Figure 6.7 D). However, the sense control indicates that this staining is likely to be trapping rather than genuine signal (see Figure 6.7 D).

At five days of incubation, *pumilio-2* mRNA was detected ubiquitously throughout the embryo (see Figures 6.7 F).

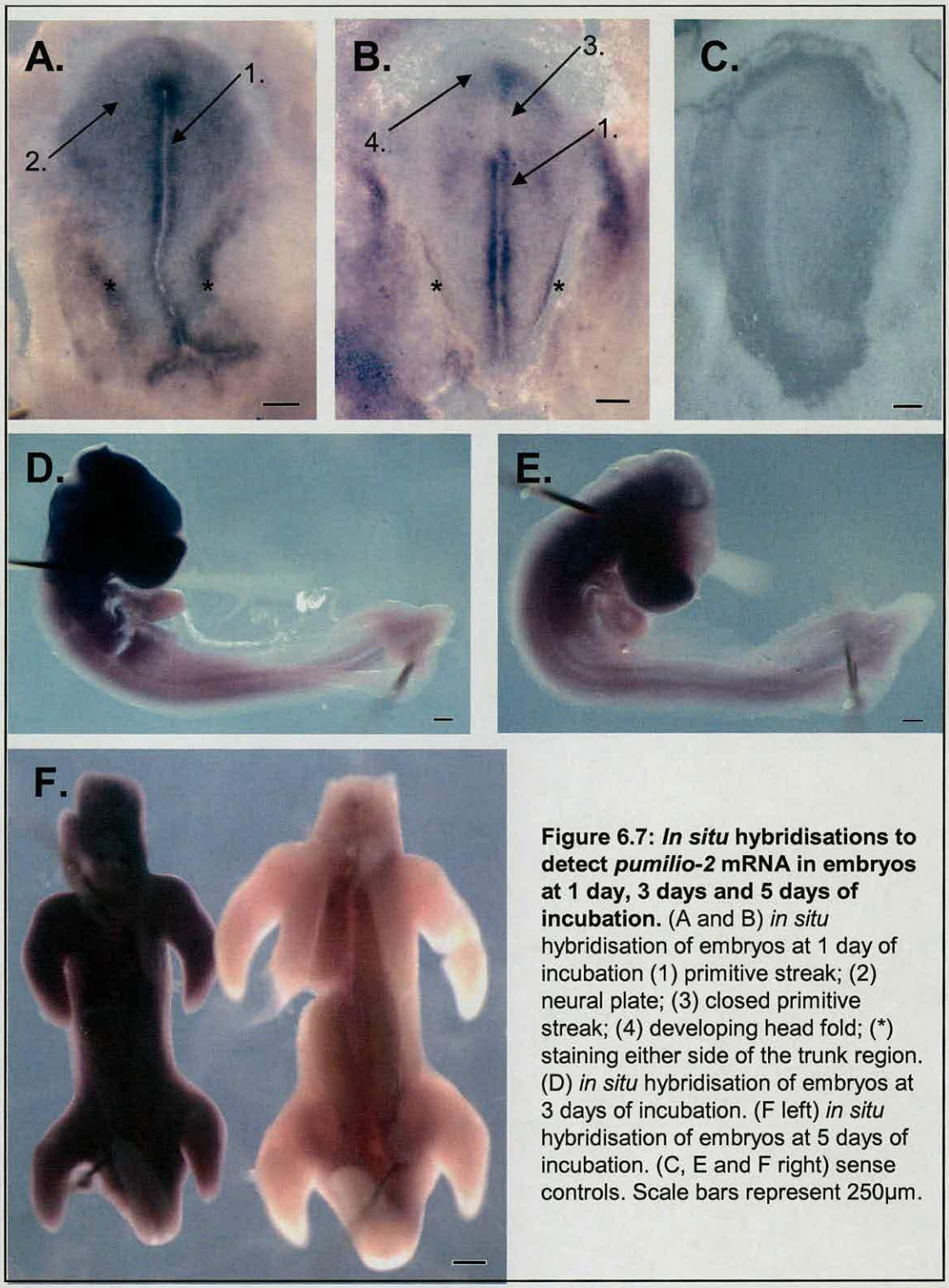


Figure 6.7: *In situ* hybridisations to detect *pumilio-2* mRNA in embryos at 1 day, 3 days and 5 days of incubation. (A and B) *in situ* hybridisation of embryos at 1 day of incubation (1) primitive streak; (2) neural plate; (3) closed primitive streak; (4) developing head fold; (*) staining either side of the trunk region. (D) *in situ* hybridisation of embryos at 3 days of incubation. (F left) *in situ* hybridisation of embryos at 5 days of incubation. (C, E and F right) sense controls. Scale bars represent 250µm.

In all of the species discussed here, *pumilio* genes work together with *nanos* genes. The remainder of this chapter discusses the methods used to try and identify *nanos* homologues in the chicken.

6.3.4 *nanos* homologues are not found in the chicken genome

BLAST searches of the chicken genome using mouse *nanos-1*, *-2* and *-3* were carried out. The only query sequence that identified a possible *nanos* homologue was the mouse *nanos-1* sequence. When the BLAST search was carried out using mouse *nanos-1* one predicted chicken *nanos* homologue was identified on chromosome 6. However, a CCHC *nanos* zinc-finger domain was not present on the predicted sequence. The CCHC *nanos* zinc-finger domain is the only region of the protein that is highly conserved between species and all *nanos* sequences have a conserved zinc-finger which is characterised by the precise location of six cysteine amino acids and two histidine amino acids in the sequence. The exception to this is the *C.elegans* Nanos-2 and Nanos-3 sequences which do not contain all of the eight conserved amino acids. However, taking this into account there is still no region of the predicted sequence that resembles a *nanos* zinc-finger domain. Therefore, in order to identify putative *nanos* zinc-fingers in the chicken genome, the amino acid sequences of zinc-finger domains of Nanos proteins from various species were used as query sequences to BLAST the chicken genome with. This resulted in no hits with a good match to the original query. The zinc finger domains were then used to BLAST the ARKGenomics and the University of Deleware EST databases. This also resulted in no hits. A keyword search of the EST databases using nanos as the keyword yielded several hits, but none of the hits where of a region of a gene containing a *nanos* zinc-

finger. The EST sequences identified during the keyword search were used as the query sequence to BLAST the chicken genome, but no hits with significant similarity to the original query sequence were obtained. The fact that no *nanos* homologues are to found in the chicken genome at the present time is probably due to the genome being incomplete, particularly because recently a predicted chicken *nanos-3* sequence containing a *nanos* zinc-finger domain was published in the NCBI database (acc. No. XM_001236366).

6.4 Discussion

Two homologues of *Drosophila pumilio* have been identified in the chicken, a gene with closest homology to vertebrate *pumilio-1* genes on chromosome 23 and a gene with closest homology to vertebrate *pumilio-2* genes on chromosome 3. Located at the C-terminus end of both genes eight highly conserved PUF binding domains and conserved C- and N-regions were found, which are characteristic of *pumilio* genes (see Figures 6.1 and 6.2). The sequence conservation between the chicken sequences identified and the mammalian sequences previously identified is very high; the chicken *pumilio-1* sequence is 97% identical to human *pumilio-1* and 94% identical to mouse *pumilio-1*. The chicken *pumilio-2* sequence is 94% identical to human *pumilio-2* and 93% identical to mouse *pumilio-2*. The high sequence conservation of chicken *pumilio-1* and *pumilio-2* suggests that their function may also be conserved. The expression of the two chicken *pumilio* genes also suggests conservation of function. The mRNA of both genes is detected by RT-PCR in all adult and embryonic tissues tested, a result that was confirmed in a recent publication by Lee *et al.* (2007). The authors showed the identification of *pumilio-1* and *pumlio-2* in the

chicken and their expression in various tissues by RT-PCR, with the exception of the expression of *pumilio-1* in the liver, which they did not detect. This widespread expression is also seen in murine tissues (Spassov and Jurecic, 2003; White *et al.*, 2001) and human tissues (Spassov and Jurecic, 2002). Interestingly, *pumilio-1* is detected in the liver of the mouse, but not in the liver of humans.

This chapter has presented the first spatial analysis by *in situ* hybridisation of both *pumilio-1* and *pumilio-2* mRNAs in a vertebrate species. In this preliminary expression analysis, expression of *pumilio-1* mRNA is faintly detected around the anterior of the primitive streak, but is hard to detect until three days of incubation. At this stage in development it is found in the blood vessels forming between the somites, and in the developing eye. At five days of incubation expression is found throughout the mesodermal tissues.

Pumilio-2 mRNA was detected extensively at one day of incubation in the neural plate and surrounding the primitive streak. As development progresses the detection of *pumilio-2* mRNA in the neural plate begins to lessen. At three days of incubation *pumilio-2* mRNA was detected exclusively in the head. However, the sense control also showed staining in the head, which indicates that the staining detected is likely to be background staining rather than genuine signal. By five days of incubation *pumilio-2* mRNA was detected in a ubiquitous manner. These patterns of mRNA expression at different time points of development suggest that Pumilio-1 and Pumilio-2 proteins are synthesised and function in the same tissues in the embryo. However, any conclusions that the function of these two proteins is conserved

between species remain speculative without carrying out protein expression or functional analyses.

With regards to PGC development, neither gene was expressed in a pattern to suggest expression in germ cells. From the work carried out in *Drosophila*, *pumilio* is required for pole cell migration. The expression in the chicken of either *pumilio* gene at three days of incubation when the PGCs are migrating through the splanchnic mesoderm indicates that neither gene in involved during PGC migration through the gut. At five days of incubation, both genes are expressed in the gonads, so they could have a function during later PGC development, but expression of both genes is widespread so this hypothesis cannot be confirmed from these analyses alone.

The failure to identify any *nanos* genes in the chicken was surprising because *nanos* gene(s) have such an important function in different areas of development which are known to be conserved between species and *nanos* genes are functionally linked with *pumilio* genes, it seem unlikely that chickens do not contain these genes. The main reasons that *nanos* homologues were not identified in the chicken are likely to be two-fold. Firstly, the chicken genome is incomplete, and secondly the sequence coding for a *nanos* zinc-finger was used as the query sequence when carrying out BLAST searches of EST databases. The zinc finger is the only highly conserved region of *nanos* genes and because it is a relatively small sequence the BLAST searches of the EST databases were narrowed down in comparison to the BLAST searches carried out for *pumilio-1* and *pumilio-2*, which used the full-length sequences identified in the chicken genome. In order to carry this research forward

and to try and identify additional putative chicken *nanos* sequences beyond the one recently published in the NCBI database degenerate RT-PCR primers designed around known zinc-finger sequences should be utilised.

CHAPTER 7

IDENTIFICATION AND EXPRESSION ANALYSIS OF A CHICKEN HOMOLOGUE OF *PIWI*

7.1 Introduction

7.1.1 The Argonaute protein family

Piwi is a member of the highly conserved Argonaute protein family (for a review see Carmell *et al.*, 2002). Argonaute proteins are at the core of a RNA-silencing machinery that uses small RNA molecules as guides to identify homologous sequences in RNA or DNA. The effects that are induced by Argonaute proteins include the induction of histone and DNA methylation, deletion of DNA sequences, mRNA breakdown, and inhibition of translation (Zamore and Haley, 2005; Houwing *et al.*, 2007). Proteins that belong to the Argonaute family have been found in diverse species including Ciliates (Mochizuki *et al.*, 2002; Obara *et al.*, 2000), yeast (Volpe *et al.*, 2002) fungi (Cogoni and Macino, 1997), *Arabidopsis* (Moussian *et al.*, 1998), *Drosophila* (Cox *et al.*, 1998, 2000; Megosh *et al.*, 2006), *Caenorhabditis elegans* (Tabara *et al.*, 1999), planarians (Reddien *et al.*, 2005), fish (Houwing *et al.*, 2007; Tan *et al.*, 2002) mice (Grivna *et al.*, 2006; Kotaja *et al.*, 2006; Kuramochi-Miyagawa *et al.*, 2001), and humans (Sharma *et al.*, 2001). Argonaute proteins are ~100kD highly basic proteins that contain two conserved protein domains, PAZ and PIWI (Cerutti *et al.*, 2000). The 130 amino acid PAZ domain has no defined function, but is thought to be a protein–protein interaction domain (Cerutti *et al.*, 2000). The C-terminal 300-amino acid PIWI domain has no known function, but is

highly conserved between species. Argonaute proteins can be separated by sequence comparisons into two subclasses: those that more closely resemble *Arabidopsis* AGO1, and those that resemble *Drosophila piwi* (Lin *et al.*, 1997; Bohmert *et al.*, 1998; Carmell *et al.*, 2002). Here the main interest is the latter subclass; sequences that more closely resemble *Drosophila piwi.*

7.1.2 The subclass Piwi

Piwi was first identified in *Drosophila* germ-line stem cells in a screen of P element mutant lines (Lin *et al.*, 1997). When mutated it was found that germline stem cell division was prevented (Cox *et al.*, 1998, 2000). As well as having a crucial role in germline stem cell division, *piwi* has also been found to have a role in germ cell development (Megosh *et al.* 2006). Since first being identified in *Drosophila*, *piwi* homologues and orthologues have been identified in a number of diverse species including *Arabidopsis* (Moussian *et al.*, 1998), ciliates (Obara *et al.*, 2000), annelids (Tadokoro *et al.*, 2006), planarians (Reddien *et al.*, 2005; Rossi *et al.*, 2006), sea urchins (Rodriguez *et al.*, 2005), zebrafish (Tan *et al.*, 2002), mice (Kuramochi-Miyagawa *et al.*, 2001), and humans (Sharma *et al.*, 2001). In contrast to members of the AGO1 subclass, generally Piwi proteins do not associate with small-interfering RNAs (siRNAs) and micro-RNAs (miRNAs), but they do associate with a separate class of germ-cell specific small RNA molecules called Piwi-interacting RNAs (piRNAs; Aravin *et al.*, 2006; Girard *et al.*, 2006; Lau *et al.*, 2006). In species other than *Drosophila*, *piwi* genes have been found to be crucial to spermatogenesis, through associations with piRNAs, stem cell self-renewal and germ cell development (Sharma *et al.*, 2000; Deng and Lin, 2002; Kuramochi-Miyagawa *et al.*, 2004;

Reddien *et al.*, 2005; Grivna *et al.*, 2006; Jin and Xie, 2006; Kim, 2006; Kotaja *et al.*, 2006). Of these roles, the one of interest in relation to this research is the function of *piwi* genes during germ cell development.

7.1.3 *piwi* genes in germ cell development

Although the majority of research into *piwi* function has been restricted to its function in the stem cell cycle and spermatogenesis, some research has looked at its potential role during germ cell development. As previously discussed, *Drosophila* pole cells contain germ plasm which is formed early in development when maternal mRNAs and proteins are localised to the posterior of the embryo. During early embryogenesis, Piwi protein is localised with Vasa protein by Oskar protein to the polar granules, a germ plasm specific organelle, in a crescent shape at the posterior pole, before becoming incorporated into the pole cells (Megosh *et al.*, 2006). When maternal Piwi protein is depleted, germ cells fail to form and the localisation of Oskar and Vasa proteins is severely affected, demonstrating that this early localisation of Piwi protein is essential for germ cell formation (Megosh *et al.*, 2006). Furthermore, when the number of copies of the *piwi* gene were doubled or tripled, the levels of Oskar and Vasa protein were doubled or tripled correspondingly, as were the number of PGCs within the embryos (Megosh *et al.*, 2006). These experiments identify a key role for Piwi in pole cell development. As well as interacting with Oskar and Vasa proteins, Piwi protein forms a complex with DICER-1, a key component of miRNA pathways (Megosh *et al.*, 2006). Depletion of DICER-1 results in a reduction in the number of PGCs developing and causes severe germ plasm defects, highlighting a possible role for a Piwi-mediated miRNA

pathway in pole cell determination in *Drosophila*. Although the mechanism by which the *piwi* gene and protein work through is currently unknown, it clearly has a major function during the specification of pole cells and their subsequent development.

In zebrafish, two Piwi homologs have been found, *ziwi* and *zili* (Houwing *et al.*, 2007; Tan *et al.*, 2002). Neither *zili* expression nor function has been investigated to date. However, both *ziwi* transcript and Ziwi protein synthesis have been investigated. *Ziwi* transcripts are detected via RT-PCR shortly after fertilisation, suggesting a maternal origin, but they cannot be detected by *in situ* hybridisation (Tan *et al.*, 2002). Ziwi protein is found localised to the germ plasm at the cleavage planes of the two and four cell embryo in a pattern similar to *vasa* RNA localisation, indicating that Ziwi protein is part of the germ plasm (Houwing *et al.*, 2007). *Ziwi* transcripts are detectable from approximately 16 hours of development, but they are not expressed in a germ cell specific manner (Tan *et al.*, 2002). From 24hpf *ziwi* mRNA co-localises with *vasa* to the embryonic genital ridge and Ziwi protein is found to be PGC specific (Tan *et al.*, 2002; Houwing *et al.*, 2007). As in *Drosophila*, if Ziwi protein function is lost the germ cells are also lost (Houwing *et al.*, 2007). In adults, *ziwi* is found exclusively in both the ovaries and the testes, but at a higher level in the testes in comparison to the ovaries (Tan *et al.*, 2002). piRNAs are also found exclusively in the ovaries and the testes in a pattern that mirrors *ziwi* expression, and have a similar bias in the level of expression between the ovaries and the testes (Houwing *et al.*, 2007). It is strongly suggested, although not yet confirmed, that piRNAs do interact with Ziwi in both the adult ovaries and testes (Houwing *et al.*, 2007). The transcript and protein expression patterns indicate that

ziwi has a role in zebrafish germline development, and the Ziwi knockdown data confirms this. However, as with *Drosophila* the mechanism by which the *piwi* gene and protein work through during PGC development is currently unknown.

In mice, there are three *piwi* homologues *mili*, *miwi*, and *miwi2*, which are all known to be essential for spermatogenesis (Kuramochi-Miyagawa *et al.*, 2001; Deng and Lin, 2002; Kuramochi-Miyagawa *et al.*, 2004; Grivna *et al.*, 2006; Carmell *et al.*, 2007). In adult mice, Mili and Miwi proteins are found exclusively in the testes where they interact with piRNAs (Kuramochi-Miyagawa *et al.*, 2001; Aravin *et al*, 2006; Girard *et al.*, 2006). The specificity of the *miwi2* gene or protein has not yet been investigated, and it is unknown whether it interacts with piRNAs in a similar manner to other *piwi* homologues. Of the three *piwi* homologues, only *mili* is known to be expressed during the embryonic stages of development (Kuramochi-Miyagawa *et al.*, 2001). *Mili* is detected by RT-PCR from E12.5 until adult in males, and in females, *mili* is detected from E12.5, but expression is lost shortly after birth (Kuramochi-Miyagawa *et al.*, 2001). To determine whether *mili* is expressed in the PGCs or the supporting cells during the embryonic stages of development, Kuramochi-Miyagawa *et al.* (2001) carried out *in situ* hybridisations with a *mili* anti-sense probe and immunohistochemistry with anti-mouse vasa homologue antibody on serial sections. The results from this experiment show that *mili* expression is restricted to the PGCs in both males and females (Kuramochi-Miyagawa *et al.*, 2001). This suggets that *mili* has a function during germ cell development, although the relatively late expression of *mili* indicates that any role will not start until shortly before the PGCs differentiate.

The aims of the experiments described here were to identify one or more homologues of *piwi* by carrying out BLAST searches of the chicken genome and EST databases. Following this, detection of mRNA using RT-PCR and Northern analysis were carried out to indicate expression of *piwi* homologues, and *in situ* hybridisation analysis was done to determine when and where during development the gene(s) are expressed and from this try to ascertain whether they have a role during PGC development.

7.2 Overview of methods

This section gives a brief overview of the methods and samples used to investigate *chiwi* in PGC development. For a full account of the methods see chapter 2.

7.2.1 BLAST searches

To identify putative *piwi* homologues in the chicken, BLAST searches of the chicken genome were carried out using the zebrafish *ziwi* nucleotide sequence (acc.no. NM183338), and the mouse *mili* nucleotide sequence (acc.no. NM021308) as the query sequences.

7.2.2 Samples RT-PCR and Northern analysis

RNA was extracted from chicken embryos at several stages of development and several adult tissues. The embryonic stages were: whole embryos at one and three days of incubation; gonads and mesonephros at five and eight days of incubation; the

remaining embryo after the gonad and mesonephros had been removed at five and eight days of incubation. Adult tissues were taken from: brain; kidney; liver; ovary and testes.

7.2.3 RT-PCR analysis

RT-PCR primers to detect *chiwi* were designed flanking an intron using primer 3 software (see Appendix 1; http://frodo.wi.mit.edu/). The primers hybridise to nucleotides located at 2426-2586bp of the predicted full-length sequence to give an expected product size of 160bp. Amplification of β-actin using primers to give a product with an expected size of 428bp was used as a positive control. The annealing temperature was 59.5°C.

To ensure the PCR primers were amplifying the expected product, RT-PCR was carried out using cDNA from adult testes. 25 cycles of amplification were carried out. The product of the *chiwi* PCR primers was cloned, purified and sent for sequencing. The sequence data of the cloned PCR product was aligned with the expected product sequence. This resulted in a 100% match, confirming that the PCR primers were amplifying the correct sequence.

7.2.3 Northern and *In situ* hybridisation probes

The EST ChEST709n12 obtained from ARKGenomics was used to make the radiolabelled Northern probe (see Appendix 9).

The RT-PCR product cloned from testes cDNA was used to make sense and anti-sense digoxigenin *in situ* hybridisation probes. Whole mount *in situ* hybridisations were carried out on embryos at one, three and five days of incubation and on frozen 15μ adult testes sections.

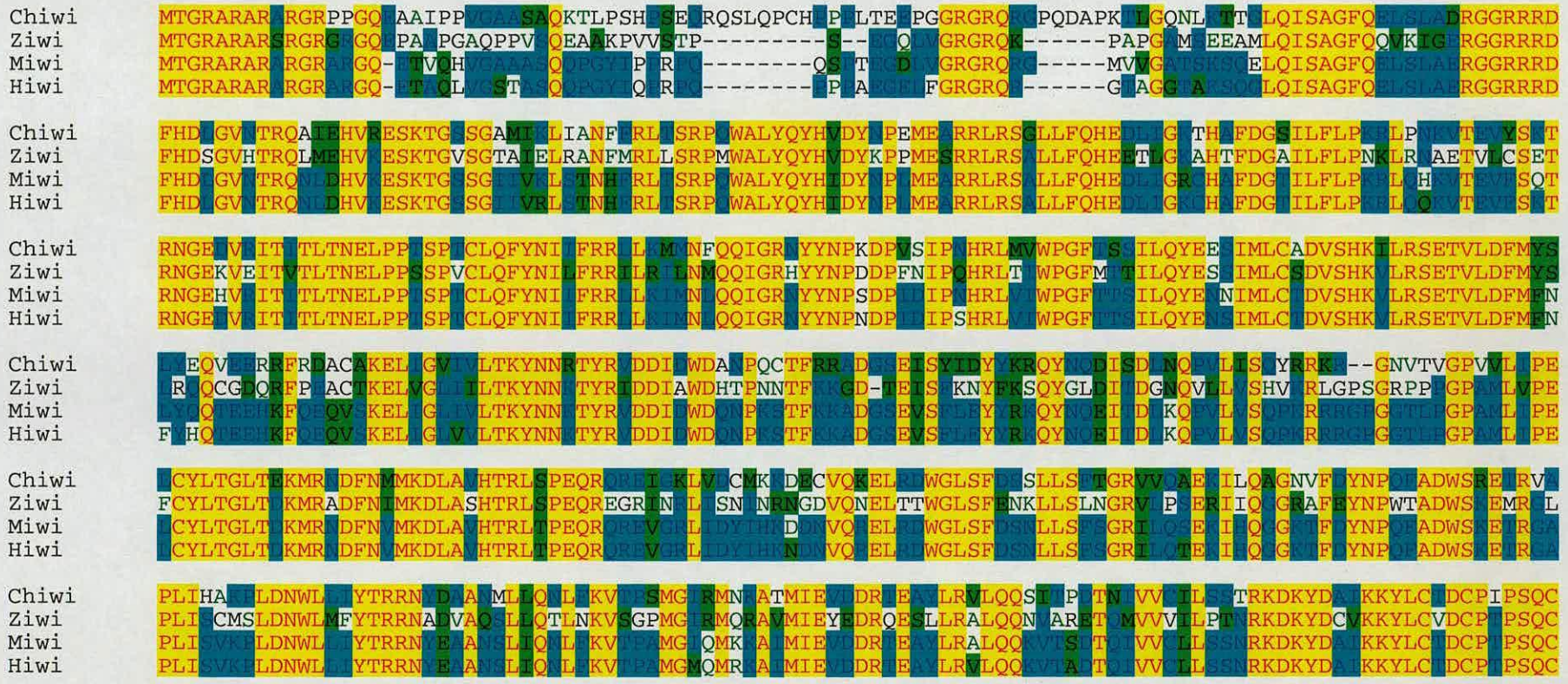
7.3 Results

7.3.1 Identification of a chicken *piwi-like* homologue

BLAST searches of the chicken genome identified a single putative chicken *piwi-like* homologue containing both a PAZ and Piwi protein domain. The putative sequence (see Appendix 9) consisted of a 20 exon, 2.6kb long cDNA spanning a region of 16.68Kb on chromosome 15 at location 3,306,381-3,323,061. The gene encodes an 874 amino acid protein. BLAST searches of the chick UMIST database identified several ESTs with identical sequences to the sequence obtained from the chicken genome (see Appendix 9). This indicates that the genome data is of a genuine gene and it is not an artefact. To confirm that the sequence is a *piwi* homologue, the putative chicken Piwi-like protein sequence was used as the query sequence to BLAST the NCBI database. The genes with the highest similarity to the chicken sequence were *piwi* genes. To investigate the gene's identity further, the sequence was aligned with known Piwi protein sequences from zebrafish (Ziwi and Zili), mice (Miwi and Mili) and humans (Hiwi and Piwi-like 2; see Figure 7.1). The chicken sequence shows the highest sequence similarity to Piwi-like 1 proteins, with the highest similarity to Miwi and Hiwi (76% identical). High sequence similarity to Ziwi (64% identical) was also observed. These high sequence similarities and the

BLAST search of the NCBI database confirm that the sequence found is a *piwi* gene. Furthermore, when the chicken Piwi-like protein sequence was aligned with Piwi-like 2 proteins from zebrafish (Zili), mice (Mili) and humans; with Piwi-like 3 protein from humans and Piwi-like 4 proteins from mice (Miwi2) and humans low sequence similarity was observed in all cases. From this it can be concluded that the sequence identified is chicken *piwi-like 1* and not *piwi-like 2, 3* or *4*. Subsequently the sequence was named *chiwi*.

Figure 7.1



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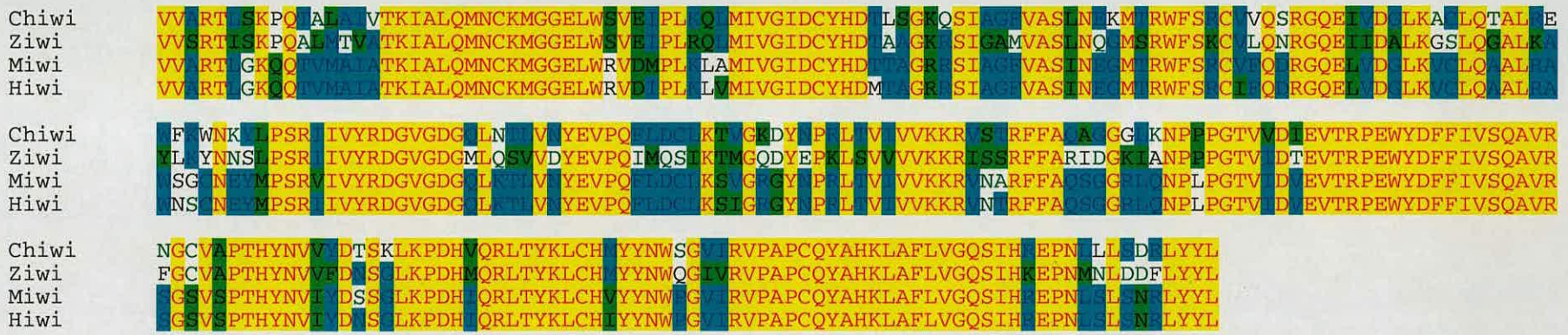


Figure 7.1: Alignment of the identified Chiwi protein sequence with known Piwi sequences from zebrafish (Ziwi), mice (Miwi) and humans (Hiwi). Identical residues are highlighted in yellow. Residues that are identical between two or three species are highlighted in blue. Residues that are different between species but the different residues are predicted to act in the same way are highlighted in green. Chiwi shows the highest sequence similarity to Miwi and Hiwi (76% identical). High sequence similarity is also observed between Ciwi and Ziwi (64% identical). Chiwi is only 36% identical to Mili, which is expressed in mouse PGCs (alignment not shown; Kuramochi-Miyagawa et al, 2001).

7.3.2 Detection of chicken *chiwi* by RT-PCR and Northern analysis

RT-PCR analysis was carried out to determine when *chiwi* mRNA is expressed during early chicken development and which adult tissues express *chiwi*. *Chiwi* mRNA was detected specifically in the adult kidney and testes after 25 cycles of amplification (see Figure 7.2). *Chiwi* mRNA was not detected in any other sample and remained undetected when the number of amplification cycles was increased to 30.

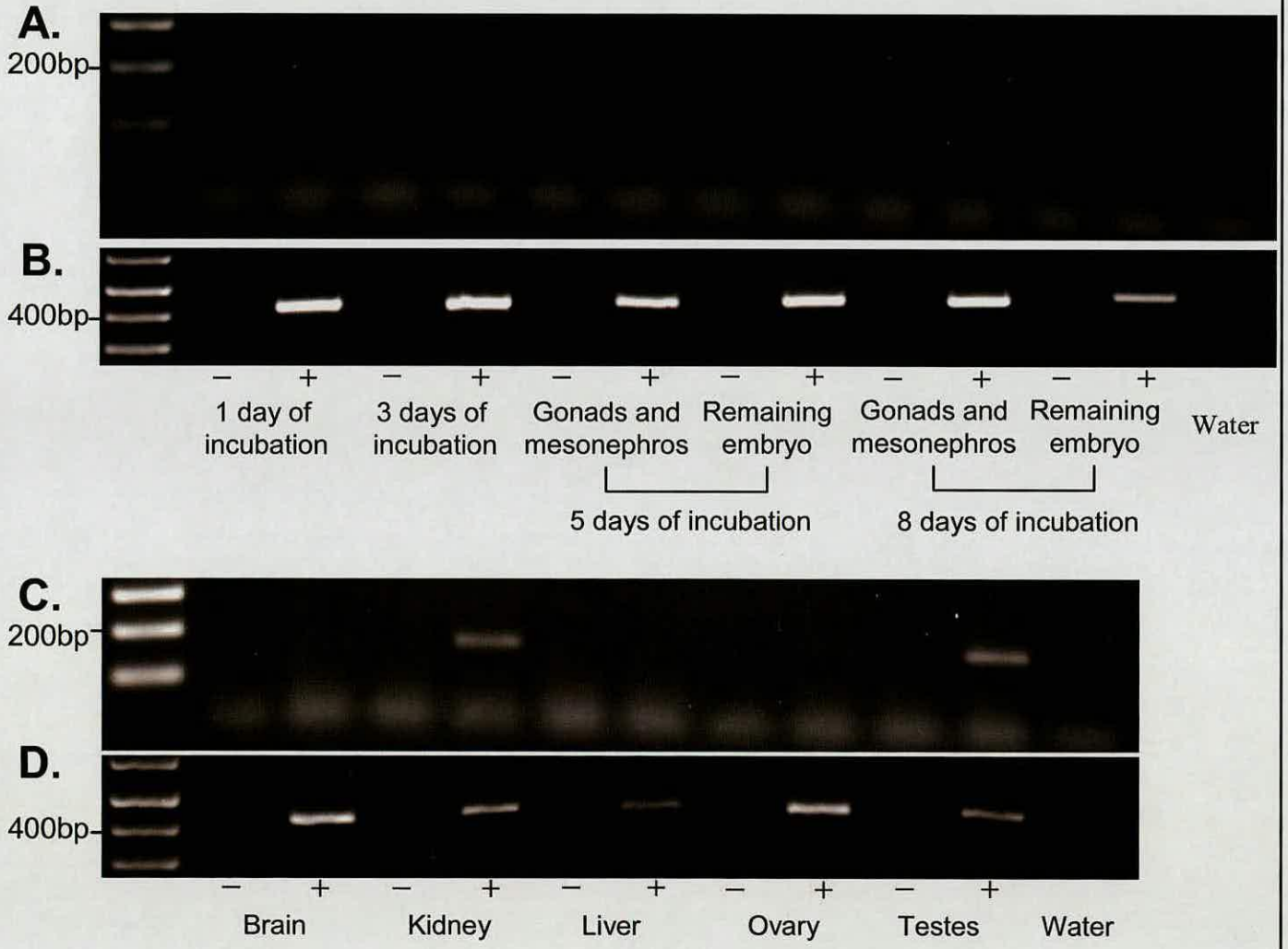


Figure 7.2: RT-PCR to detect the expression of *chiwi* nRNA in embryonic and adult tissues. (A) *chiwi* expression in embryonic tissues. (C) *chiwi* expression in adult tissues. (B and D) ß-actin positive controls. +: indicates the presence of reverse transcriptase during cDNA synthesis. -: indicates the absence of reverse transcriptase during cDNA synthesis.

Northern analysis was carried out to extend the RT-PCR data. A probe was made using DNA from a chicken EST previously identified, obtained from ARKGenomics (see Section 7.2.1 and Appendix 9). A strong hybridisation signal of *chiwi* mRNA was detected in the adult testes at approximately 1.8kb (see Figure 7.3). No hybridisation signal was detected in any other adult or embryonic tissues.

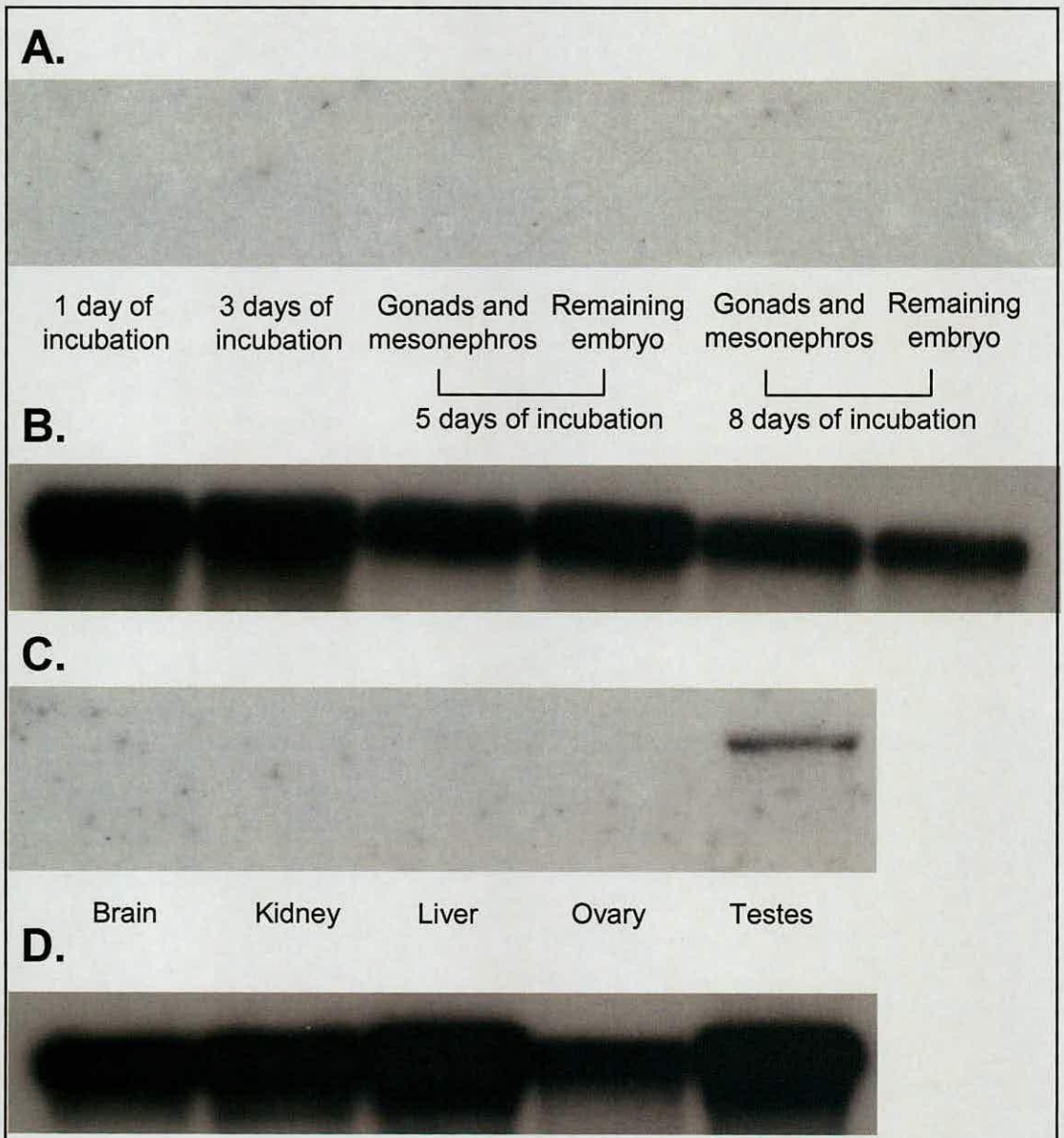


Figure 7.3: Northern analysis to detect *chiwi* mRNA in embryonic and adult tissues. Expression of *chiwi* in (A) embryonic tissues and in (C) adult tissues. (B and D) loading control to detect 18s ribosomal RNA. 10µg total RNA were used per sample.

7.3.3 Expression of chicken *chiwi*

At one, three and five days of incubation, *in situ* hybridisation analysis did not detect *chiwi* mRNA (see Figure 7.4). This is consistent with the RT-PCR and Northern data previously obtained.

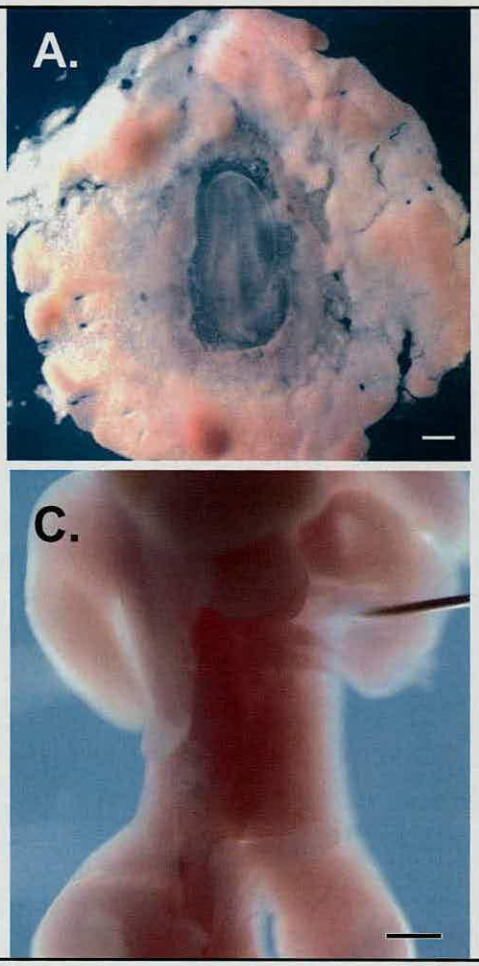


Figure 7.4: *In situ* hybridisations to detect *chiwi*. (A) *In situ* hybridisation on an embryo at 1 day of incubation. (B) *In situ* hybridisation on an embryo at 3 days of incubation. (C) *In situ* hybridisation carried out on an embryo at 5 days of incubation. Sense embryos not shown. Bar represents 250μm.

To confirm that the anti-sense *in situ* hybridisation probe made worked, an *in situ* hybridisation was carried out on frozen 15μm testes sections. *Chiwi* mRNA was detected in cells near the basement membrane of the testes section (see Figure 7.5A). This area of the seminiferous tubules is known to contain the spermatogonium and primary spermatocytes (see Figure 7.5C). The location of the cells in the seminiferous tubules staining positively for the presence of *chiwi* mRNA is consistent with the expression pattern of *miwi* in mice testes, indicating that this is a genuine *piwi* homologue.

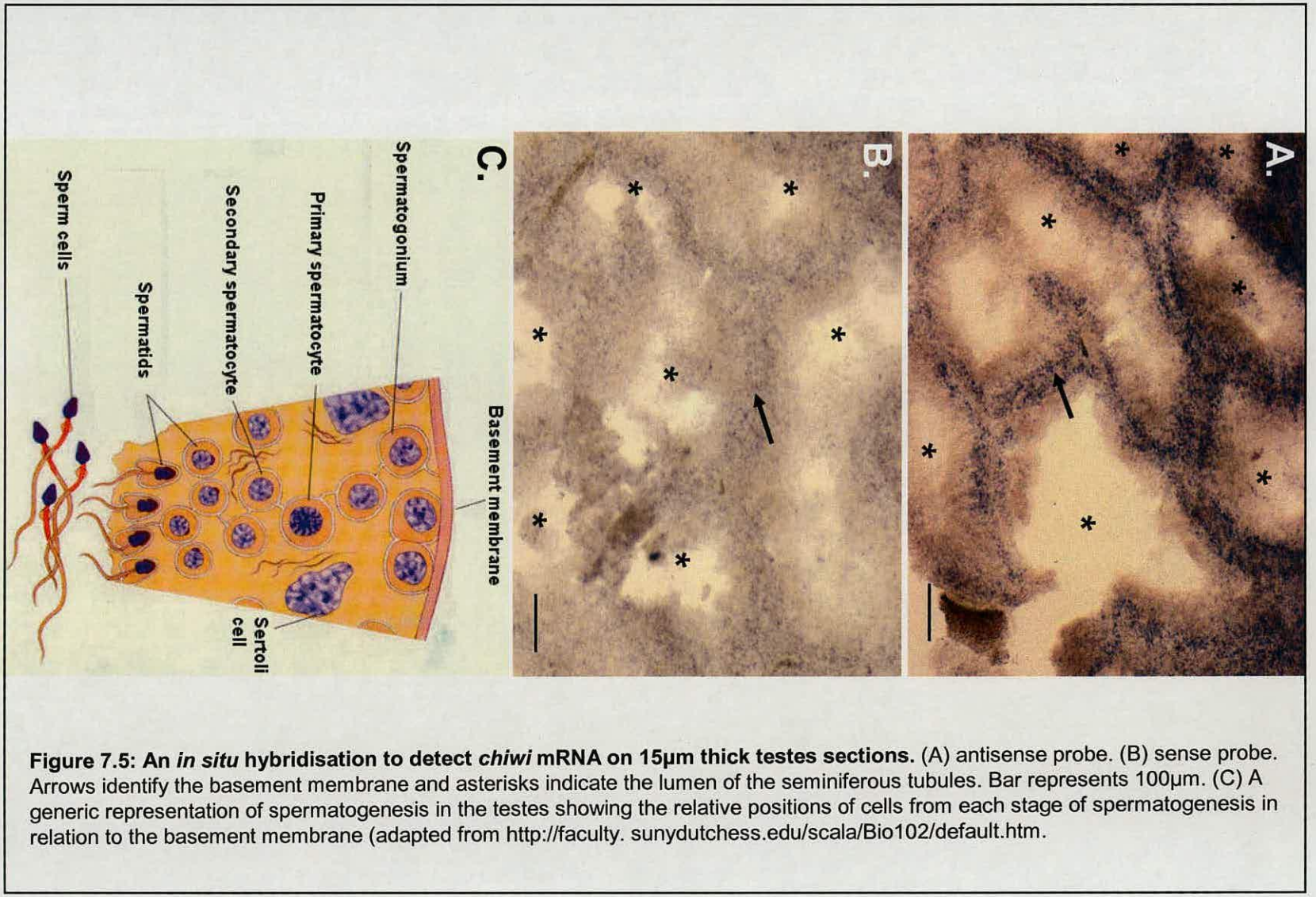


Figure 7.5: An *in situ* hybridisation to detect *chiwi* mRNA on 15µm thick testes sections. (A) antisense probe. (B) sense probe. Arrows identify the basement membrane and asterisks indicate the lumen of the seminiferous tubules. Bar represents 100µm. (C) A generic representation of spermatogenesis in the testes showing the relative positions of cells from each stage of spermatogenesis in relation to the basement membrane (adapted from http://faculty. sunydutchess.edu/scala/Bio102/default.htm.

7.4 Discussion

A single *Piwi* homologue was identified in the chicken, with the highest sequence similarity to *piwi-like 1* genes from zebrafish (*ziwi*), mice (*miwi*) and humans (*hiwi*). The homologue has low sequence similarity to *piwi-like 2* genes, indicating that the sequence identified is chicken *piwi-like 1* and was consequently named *chiwi*.

In mice, *miwi* is expressed exclusively in the testes from two weeks after birth and expression continues in adulthood (Kuramochi *et al.*, 2001). The RT-PCR data presented here shows that chicken *chiwi* is expressed in the testes and kidney, and the Northern data suggests that the gene is testes specific. However, because of the difficulties experienced with Northern experiments with other genes, it would suggest that the RT-PCR data is more reliable. The expression data from all of the methods used to detect *chiwi* do all show that *chiwi* is not expressed during embryonic development. Therefore, *chiwi* cannot be involved in PGC development, but it could have a role in spermatogenesis. This is of particular interest because zebrafish *ziwi* is expressed during embryonic stages and is known to play an important role during early zebrafish PGC development. The lack of expression of *chiwi* in the chicken embryo indicates that its function is more likely to be homologous to mouse *miwi* rather than zebrafish *ziwi*. However, mice still have a *piwi-like* gene expressed in the PGCs in the embryo, which would suggest that at least one more *piwi-like* gene will be found in the chicken. A gene tree that displays the maximum likelihood phylogenetic gene tree representing the evolutionary history of the Piwi gene family constructed in www.ensembl.com using PHYML supports

this hypothesis (See Figure 7.5; Guindon *et al.*, 2005). A gene duplication event (red square) is shown between the ancestral Piwi family member found in *Drosphila melanogaster* and vertebrates. At this point, *piwi-like 1* and *piwi-like 2* have diverged. The gene tree shows that most vertebrate species have a *piwi-like 1* and *piwi-like 2* gene, and because *piwi-like 1* and *piwi-like 2* genes have been identified in species that are recognised to be evolutionarily more primitive and more sophisticated than chickens, it would indicate that a *piwi-like 2* homologue should be found in the chicken.

BLAST searches using the *mili* sequence (acc.no. NM021308) from mice of the chicken genome and of the University of Delaware and the UMIST chick EST databases did not generate any matches with high homology to the query sequence. However, neither the chicken genome nor the EST databases contain every chicken gene, and so it is still possible that a *piwi-like 2* gene will be found. Because a number of closely related vertebrate species have a *piwi-like 1* and a *piwi-like 2* gene and that *piwi* genes are known to have functions during PGC development in *Drosophila*, zebrafish and mice, the suggestion that chickens have evolved a system of germ cell development which does not require a *piwi-like* gene seems highly unlikely. In future, to identify a putative *piwi-like 2* sequence in the chicken, I would design degenerate primers around the known *piwi-like 2* sequences from zebrafish, mice and humans.

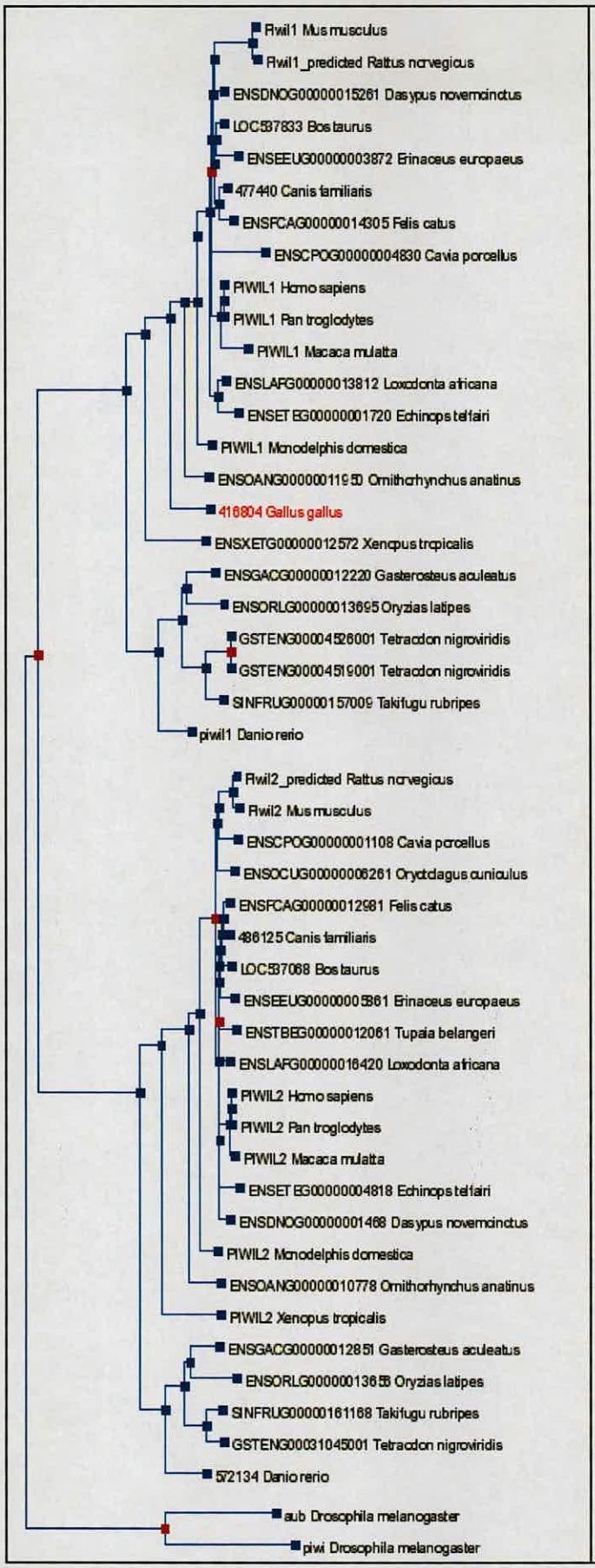


Figure 7.6: A gene tree representing the evolutionary history of the *piwi* gene family. The Gene Tree was generated in www.ensembl.com using PHYML displaying the maximum likelihood polygenetic gene tree (Guindon et al, 2005). The Gene Tree has been modified to show the evolutionary relationship between *piwi-like 1* genes and *piwi-like 2* genes. The red squares represent gene duplication nodes, the blue squares represent speciation nodes and the branch length represents the number of substitutions that have occurred since the common ancestor.

CHAPTER 8

EXPRESSION OF THE CHICKEN HOMOLOGUES OF MAMMALIAN *POU5F1* AND *NANOG* DURING PGC DEVELOPMENT

8.1 Introduction

Mature germ cells are totipotent cells, which means that despite being differentiated and highly specialised, they maintain the ability to generate extraembryonic tissues or cells from any of the three germ layers (Saffman and Lasko, 1999). In comparison, pluripotent cells have the potential to differentiate into cells from any of the three germ layers, but are unable to generate extraembryonic tissues. This means that pluripotent cells cannot develop into an organism on their own (Pan and Thomson, 2007). The mechanism that maintains pluripotency in embryonic stem (ES) cells has been investigated extensively, while less is known about the mechanism that maintains totipotency in germ cells. Recent work suggests that at least two of the genes required for maintaining ES cell pluripotency are also involved in maintaining germ cell totipotency in mice. These two genes are *oct-4*, also known as *pou5f1*, and *nanog*. As part of this study, a chicken *nanog* homologue and a gene containing a POU domain with high similarity to mammalian *oct-4* were investigated. The first part of this chapter reviews the background literature, highlighting the domains, regulation and expression of each gene. The remainder of the chapter then briefly outlines the experimental approach, before presenting and discussing the results of an extensive expression profile of the two chicken genes.

8.1.1 Oct-4 in vertebrates

Oct proteins are part of the Pit-Oct-Unc (POU) transcription factor family, whose members are characterised by the POU domain (Ryan and Rosenfeld, 1997). The shared POU domain is a bipartite DNA binding domain, which contains the POU homeodomain (POU_H) and the POU specific domain (POU_S), which are connected by a variable length, flexible linker. The POU_H and POU_S binding domains bind to the two halves of the consensus octamer sequence, AAAT and ATGC (Klemm *et al.*, 1994). The POU protein family is divided into five classes of POU protein according to the sequence of the linker region. Oct-4 belongs to class V (Ryan and Rosenfeld, 1997). Class V POU domain proteins have been identified in a number of vertebrate species including mice (Okamoto *et al.*, 1990; Rosner *et al.*, 1990; Schöler *et al.*, 1990), *Xenopus* (Frank and Harland, 1992; Hinkley *et al.*, 1992), zebrafish (Takeda *et al.*, 1994) and axolotl (Bachvorova *et al.*, 2004). In all of these species the protein has been identified in early embryonic development. However, a function in PGC development has only been investigated in mice.

Oct-4 was first identified in mice as an ES cell and germline specific transcription factor that has a dynamic expression pattern during early development (Okamoto *et al.*, 1990; Rosner *et al.*, 1990; Schöler *et al.*, 1990; Palmieri *et al.*, 1994; Yeom *et al.*, 1996; Nichols *et al.*, 1996; Yoshimizu *et al.*, 1999). In the eight-cell embryo and subsequent morula stage, Oct-4 protein is present in the nuclei of all cells. At the blastocyst stage, the trophetoderm is formed and *oct-4* expression is down-regulated in the trophectodermal cells. *Oct-4* expression is restricted to the inner cell mass

(ICM). After implantation *oct-4* is specifically expressed in the epiblast. During gastrulation, *oct-4* expression in the epiblast is down-regulated in an anterior to posterior manner so that by 1.5 days after gastrulation expression is completely lost in all tissues except the PGCs (Yeom *et al.*, 1996; Yoshimizu *et al.*, 1999). After *oct-4* becomes germ line specific, its expression is maintained in PGCs of both sexes until 13.5 days post coitum (dpc), when expression begins to differ between the sexes. In females, *oct-4* expression is down-regulated at 13.5dpc, when female germ cells enter meiosis. In adult females, expression is up-regulated in oocytes from the diplotene/dictyate stage. In males, *oct-4* expression is maintained throughout development, even when the male germ cells are mitotically arrested (Yoshimizu *et al.*, 1999). In adult males, *oct-4* is expressed in type A spermatogonia, but down-regulated in type B spermatogonia and in spermatocytes (Pesce *et al.*, 1998; Yoshimizu *et al.*, 1999). The expression patterns described above are diagrammatically represented in Figure 8.1 below.

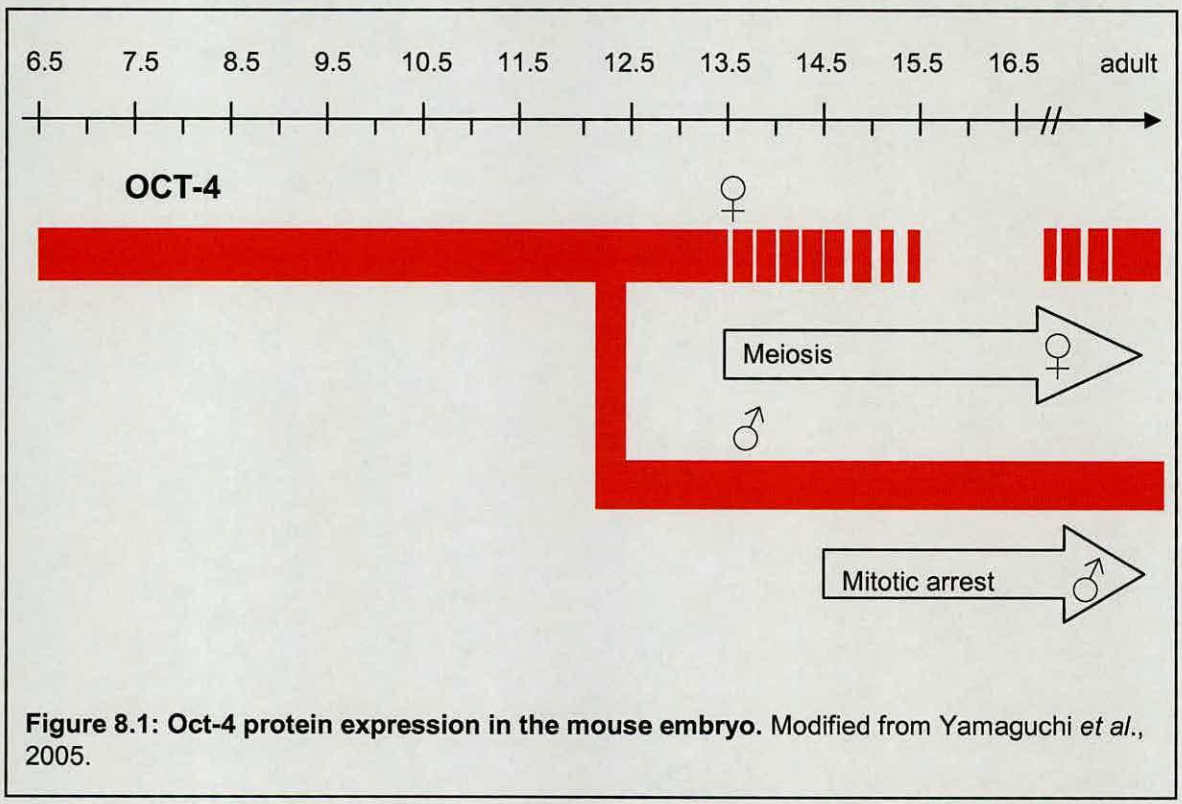


Figure 8.1: Oct-4 protein expression in the mouse embryo. Modified from Yamaguchi *et al.*, 2005.

Four factors are currently known to regulate *oct-4* expression in mammals. The first factor is Oct-4 protein, which regulates *oct-4* through two elements called the proximal element (PE) and the distal element (DE) in the 5' regulatory sequence (Yeom *et al.*, 1996). The two elements allow separate regulation of germline and epiblast expression. The PE is stage and tissue specific and activates *oct-4* expression in the epiblast of mouse embryos, whereas the DE is lineage specific and activates *oct-4* expression in the germ cells (Yeom *et al.*, 1996). The second factor, Sox-2, acts synergistically with Oct-4 to activate Oct-Sox enhancers. Oct-Sox enhancers have been identified on several genes and regulate the expression of pluripotent stem cell specific genes including *nanog*, *oct-4* and *sox-2* (Masui *et al.*, 2007). The third factor is germ cell nuclear factor (GCNF), which regulates *oct-4* expression by preventing expression of *oct-4* as pluripotent stem cells differentiate (Fuhrmann *et al.*, 2001). The fourth factor, transiently RA-induced factor (TRIF) has also been implicated in the regulation of *oct-4* expression through an interaction with GCNF. However, this is still to be confirmed (Fuhrmann *et al.*, 2001; Gu *et al.*, 2005).

8.1.2 Nanog in mammals

Nanog was first described as an early embryo specific NK (ENK) gene that contained a homeodomain and was specifically expressed in ES cells (Wang *et al.*, 2003). The homeodomain was later determined to be unique and the gene was re-named *nanog* by two independent groups (Chambers *et al.*, 2003; Mitsui *et al.*, 2003). In mice, *nanog* expression is first detected in morula-stage embryos. In E6.5-E7.5 embryos, *nanog* expression is maintained in the ICM and the epiblast, but

nanog expression is not detected in PGCs found in the proximal region of the allantois at this stage of development (Hatano *et al.*, 2005). At E7.75, Nanog protein is first detected in some PGCs. At E8.5, Nanog protein is germline specific and expressed in all PGCs. At E13.5 expression patterns begin to differ between sexes. In females, Nanog expression is down-regulated at E13.5 and from E15.5 Nanog is no longer detected (Yamaguchi *et al.*, 2005). This coincides with the onset of meiosis. In males, Nanog expression is down-regulated at E14.5 and from E16.5 onwards Nanog is no longer detected (Yamaguchi *et al.*, 2005). This coincides with the onset of mitotic arrest. In adults of both sexes Nanog remains down-regulated. The expression patterns described above are diagrammatically represented in Figure 8.2 below.

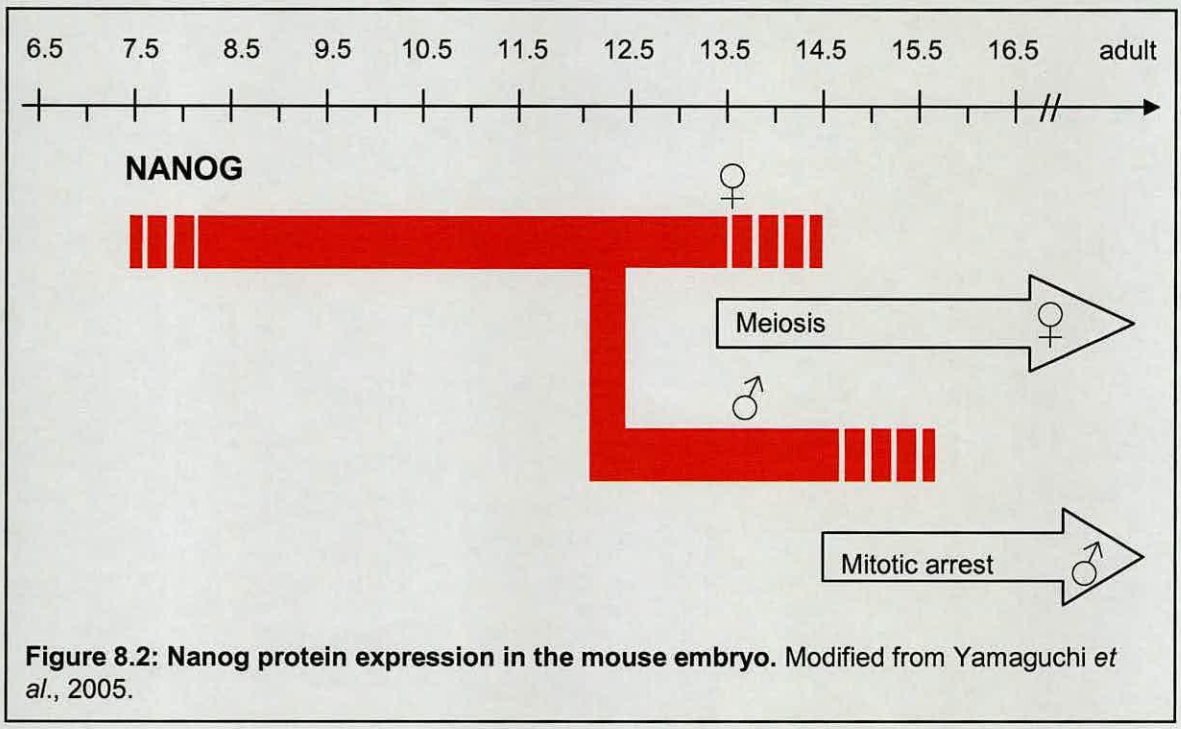


Figure 8.2: Nanog protein expression in the mouse embryo. Modified from Yamaguchi *et al.*, 2005.

Five elements are currently known to regulate *nanog* expression. Three factors, Oct-4, Sox-2 and FoxD3, promote *nanog* expression and two factors, p53 and Tcf3, inhibit *nanog* expression (see Figure 8.3). To promote Nanog expression, Oct-4 and Sox-2 bind to a composite Oct-4/Sox-2 motif located upstream of the Nanog transcription start site (Kuroda *et al.*, 2005; Rodda *et al.*, 2005). Mutagenesis of the Oct-4/Sox-2 binding motif showed that the motif is required for the activity of the Nanog promoter, suggesting that Oct-4 and Sox-2 act together to promote Nanog transcription (Rodda *et al.*, 2005). FoxD3 is a forkhead family transcription factor that is highly expressed in mouse ES cells and in pluripotent cells of the early embryo (Sutton *et al.*, 1996). Reporter assays show that FoxD3 can activate the Nanog promoter through a pluripotent cell specific enhancer located upstream of the Nanog transcription start site (Pan *et al.*, 2006).

Nanog is negatively regulated by p53 and Tcf3. During ES cell differentiation, the rapid down-regulation of Nanog correlates with the induction of transcription activity and Ser315 phosphorylation of p53 (Lin *et al.*, 2005). When the induction of p53 by knock-in of $p53^{S315A}$ is impaired, Nanog is inefficiently suppressed during ES cell differentiation, suggesting that p53 is a negative regulator of Nanog (Lin *et al.*, 2005). Tcf3 is a transcription factor that functions downstream of the Wnt pathway and that is highly expressed in undifferentiated mouse ES cells. Ablation of Tcf3 in mouse ES cells causes elevated levels of Nanog protein and a delay in differentiation in favour of self-renewal with (Pereira *et al.*, 2006).

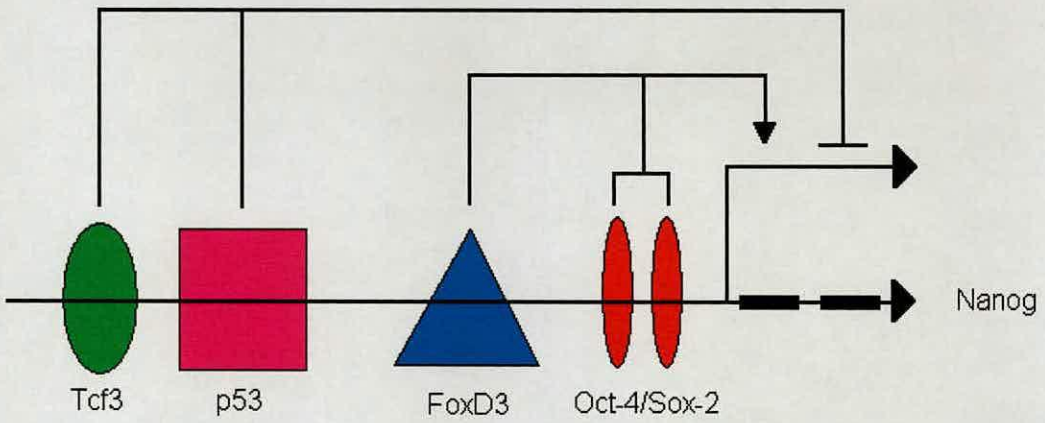


Figure 8.3: Nanog regulation in mouse ES cells (modified from Rodda *et al.*, 2005).

8.1.3 Chicken *nanog* and *cPouV*

Recently, *nanog* and a member of the *PouV* gene subfamily have been identified in the chicken. Chicken *nanog* was predicted in the chicken genome at reference *IDENSGALG00000014319* in chromosome 1 and identified independently by two groups (Canon *et al.*, 2006; Lavial *et al.*, 2007). Lavial *et al.* (2007) found that *nanog* is down-regulated in chicken embryonic stem cells (cESCs) at the point of differentiation. Furthermore, when chicken *nanog* is overexpressed in mouse ESCs, the cells are able to proliferate in the absence of LIF. This is similar to the effect observed when mouse *nanog* is overexpressed in ESCs, which suggests that the chicken *nanog* gene is a functional orthologue of mouse *nanog*.

A chicken gene containing a POU domain was identified by subtractive hybridisation of cDNAs from cESCs and chicken embryoid bodies (Lavial *et al.*, 2007). The POU domain is statistically more closely related to class V POU proteins than any other class of POU gene and was named chicken *PouV* (*cPouV*). Like chicken nanog, *cPouV* is down-regulated in cESCs at the point of differentiation. Furthermore,

cPouV is able to partially rescue *oct-4* deficient mouse ESCs. It is therefore thought that *cPouV* may be an orthologue of mammalian *oct-4* (Lavial *et al.*, 2007).

The literature reviewed above shows that in mammals, *nanog* and *oct-4* are expressed in pluripotent cells in early development and become germline specific later in development. *Nanog* and *oct-4* have key roles in maintaining pluripotency in stem cells and may have a similar role in maintaining totipotency in germ cells. In lower vertebrates, it is know that *oct-4* homologues are expressed in the early embryo, however, both the role of *nanog* and the expression of *oct-4* in the germline remains to be investigated. In chicken, *nanog* and a *PouV* subfamily member are expressed in the early embryo, in cESCs and in late stage PGCs. In this research study, a full expression analysis is carried out in relation to expression of chicken *nanog* and *cPouV* during PGC development. The remainder of the chapter briefly outlines the methods used, before presenting expression data for *cPouV* and *nanog* and discussing how expression changes in relation to germ cell differentiation.

8.2 Experimental methods overview

This section gives a brief overview of the methods and samples used to investigate *nanog* and *cPouV* in PGC development. For a full account of the methods see chapter 2. The cDNAs and PCR primer sequences for chicken *nanog* and *cPouV* used for RT-PCR, Northern and *in situ* hybridisation analyses were provided by Dr Pain for work to be carried out on these two genes in relation to PGC development.

8.2.1 Samples for RT-PCR and Northern analysis

RNA was extracted from chicken embryos at several stages of development and several adult tissues. The embryonic stages were: whole embryos at one and three days of incubation; gonads and mesonephros at five and eight days of incubation; the remaining embryo after the gonad and mesonephros had been removed at five and eight days of incubation. Adult tissues were taken from: brain; kidney; liver; ovary and testes.

8.2.2 RT-PCR analysis

The primer sequences for *cPouV* and *nanog* give an expected product size of 279bp and 180bp respectively (see Appendix 1). Primers that amplify β-actin to give an expected size of 428bp were used as a positive control (see Appendix 1). PCRs were run with an annealing temperature of 59.5°C and 25 cycles of PCR were carried out. To ensure the PCR primers were amplifying the expected product, RT-PCR was carried out using cDNA from adult testes and the product was cloned. The *nanog* PCR primers did not generate a product using testes cDNA, so the RT-PCR was repeated using cDNA isolated from embryos at three days of incubation and the resulting product was cloned. The cloned PCR products were then sequenced. The sequence data were aligned with the expected product sequences. This resulted in a 100% match for both *cPouV* and *nanog* and confirmed that the PCR primer sets were amplifying the correct sequences.

8.2.3 Northern and *in situ* hybridisation probes

Radiolabelled RNA probes for Northern and digoxigenin RNA probes for *in situ* hybridisation were made using the full-length cDNA sequences of *cPouV* and *nanog* supplied by Dr Pain.

8.2.4 *In situ* hybridisation analyses

In situ hybridisation analysis was carried out on whole-mount embryos at one, three and five days of incubation. Following the preliminary experiments, *in situ* hybridisation analysis was carried out on gonads and mesonephros dissected from embryos at 7.5 days of incubation; male and female gonads and mesonephros at 9.5, 11.5, 13.5, 15.5 and 17.5 days of incubation; and gonads from male and female hatchlings.

8.2.5 Sexing embryos

Embryos were sexed by eye from 9 days of incubation. The following morphological features distinguish the sexes: In females, the right ovary is significantly smaller in comparison to the left ovary. The left ovary is no longer tubular in shape, but has flattened out. In males, both gonads remain similar in size. The gonads are tubular shaped.

8.2.6 Consecutive sections

Consecutive 20μ frozen sections of gonads at five days of incubation were taken. These sections contained at least one common PGC, because PGCs are large cells, measuring between 15-20μ in diameter. Each set of consecutive sections was stained

for the presence of the carbohydrate epitope SSEA-1 and either *oct-4* or *nanog* mRNA. MC-480 monoclonal antibody to detect SSEA-1 was developed by Solter and Knowles (antibody obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by The University of Iowa, Department of Biological Sciences, Iowa City, IA, 52242).

8.2.7 Detecting proteins in frozen sections

SSEA-1 was detected using antibody MC480 (DSHB) at 1/40 concentration. Secondary used was anti-mouse IgM Texas red (Abcam) at 1/200 concentration.

Meiotic cells were detected using an antibody against SCP3 protein (ab15092; Abcam). The antibody was used at a 1/100 concentration. The secondary antibody used was Alexa Fluor $F(ab')_2$ fragment of goat anti-mouse IgG (Molecular Probes) at 1/200 concentration.

8.2.8 Quantitative PCR (qPCR)

RNA was extracted from male and female gonads at 9.5, 11.5, 13.5, 15.5 and 17.5 days of incubation. cDNA was made from this RNA and qPCR was carried out. A minimum of three sets of gonads was used at each stage of development. In this work, the $2^{-\Delta\Delta Ct}$ method of relative quantification (described in detail in Livak and Schmittgen, 2001) was used to estimate copy numbers of *cPouV* and *nanog* genes. This method allows an estimation of gene copy numbers in unknown samples. To be able to calculate the estimate, a house-keeping gene that permits normalization of the quantitative data is required. In this instance the gene LBR was used. The efficiency

of the primers was calculated prior to experimentation. Quantitative PCR was carried out six times for each sample. Following completion of the qPCR, the results were normalised for each sample. This involved subtracting the average Ct value for the LBR standard from each sample. In order to compare the results between the stages, the normalised Ct values for samples at 9 days of incubation were used as the baseline. To determine the difference in expression between stages, the Ct value for the baseline 9 day of incubation sample was subtracted from the normalised Ct value for each sample. This figure then replaced X in the following formulae to give a value for the increase/decrease in levels of mRNA in the sample compared to the baseline: $=2^{-X}$.

8.3 Results

8.3.1 Initial expression analysis of *cPouV* and *nanog*

RT-PCR, Northern and *in situ* hybridisation analyses were used to develop a preliminary expression profile for *cPouV* and *nanog*. RT-PCR and Northern analyses determined whether each gene was expressed at four stages of embryonic development and in various adult tissues. *In situ* hybridisation analysis was then used to determine the pattern of expression of each gene at one day of incubation, three days of incubation and five days of incubation.

RT-PCR analysis was carried out to investigate when *cPouV* and *nanog* were expressed during early chicken development and which adult tissues express *cPouV* and *nanog*. In embryonic tissues, both *cPouV* and *nanog* were detected at one and

three days of incubation; at five days of incubation in the gonad and mesnephrous sample and the remaining embryo sample; and at eight days of incubation in the gonad and mesonephros sample (see Figures 8.4A and 8.5A). In adult tissues, *cPouV* was detected specifically in the ovary and testes (see Figure 8.4C). *Nanog* was not detected in any adult tissues (see Figure 8.5C).

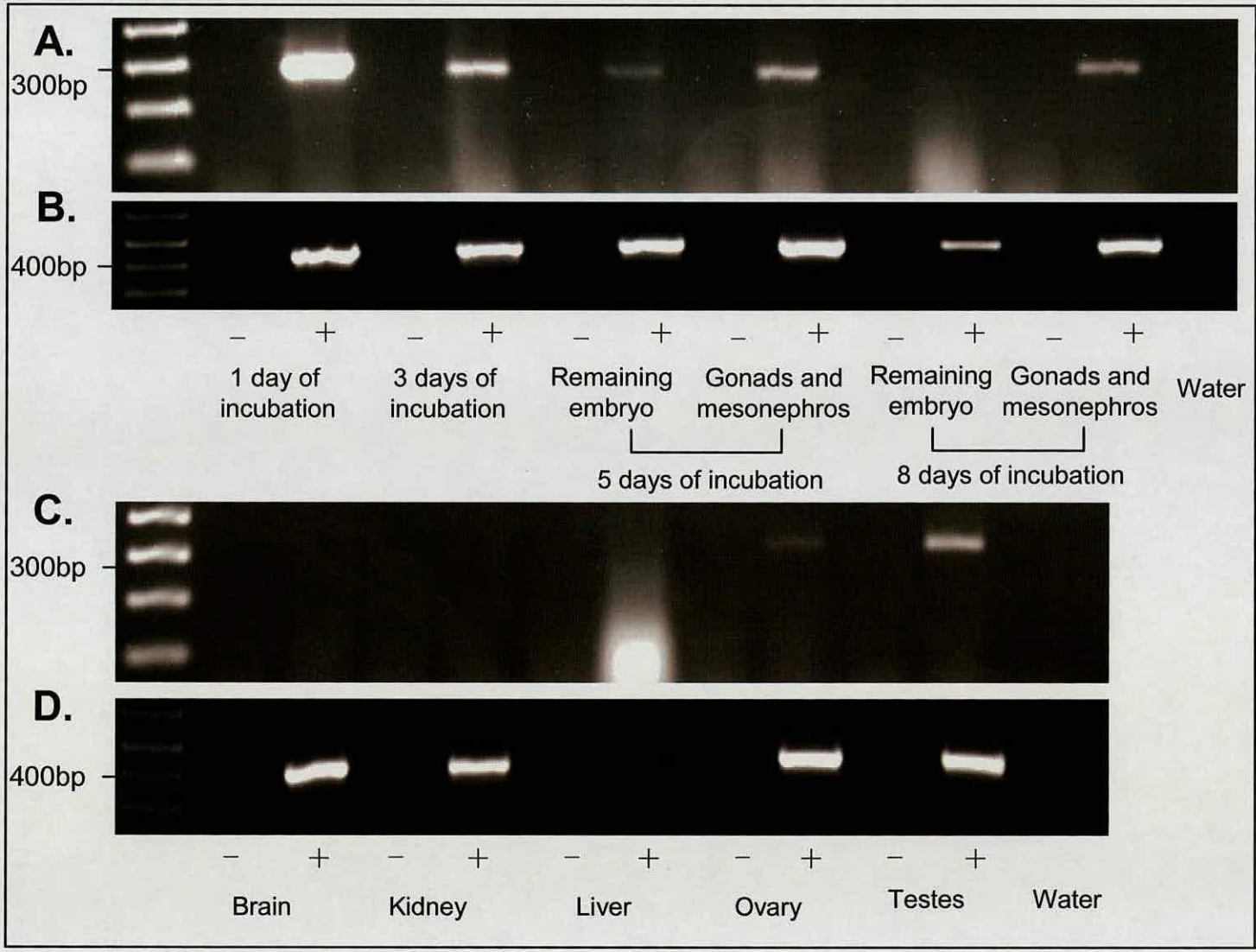


Figure 8.4: RT-PCR to detect the expression of *oct-4* mRNA in embryonic and adult tissues. (A) RT-PCR to detect *oct-4* mRNA in embryonic tissues. (C) RT-PCR to detect *oct-4* mRNA in adult tissues. (B and D) *ß-actin* controls. +: reverse transcriptase present during cDNA synthesis. –: no reverse transcriptase control.

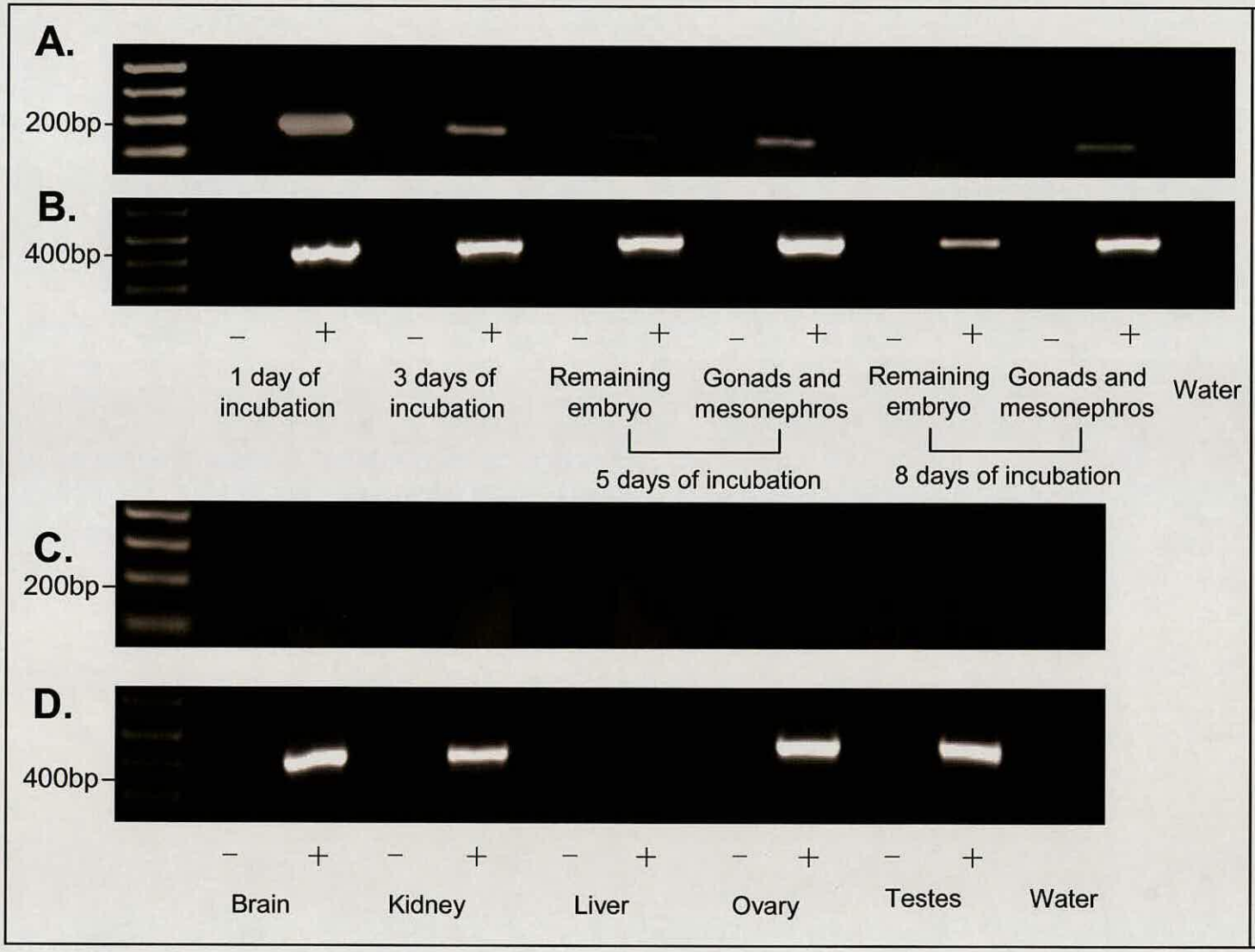


Figure 8.5: RT-PCR to detect the expression of *nanog* mRNA in embryonic and adult tissues. (A) RT-PCR to detect *nanog* mRNA embryonic tissues. (C) RT-PCR to detect *nanog* mRNA in adult tissues. (B and D) *ß-actin* controls. +: reverse transcriptase present during cDNA synthesis. –: no reverse transcriptase control.

Northern analyses were carried out to extend the RT-PCR data. The *cPouV* radiolabelled probe hybridised specifically to RNA extracted from embryos at one day of incubation. This produced a single product of approximately 900bp (see Figure 8.6A). This size of product is consitent with the predicted length of the transcript, which is 888bp. The *cPouV* radiolabelled probe did not hybridise to any other RNA sample (see Figures 8.6A and C).

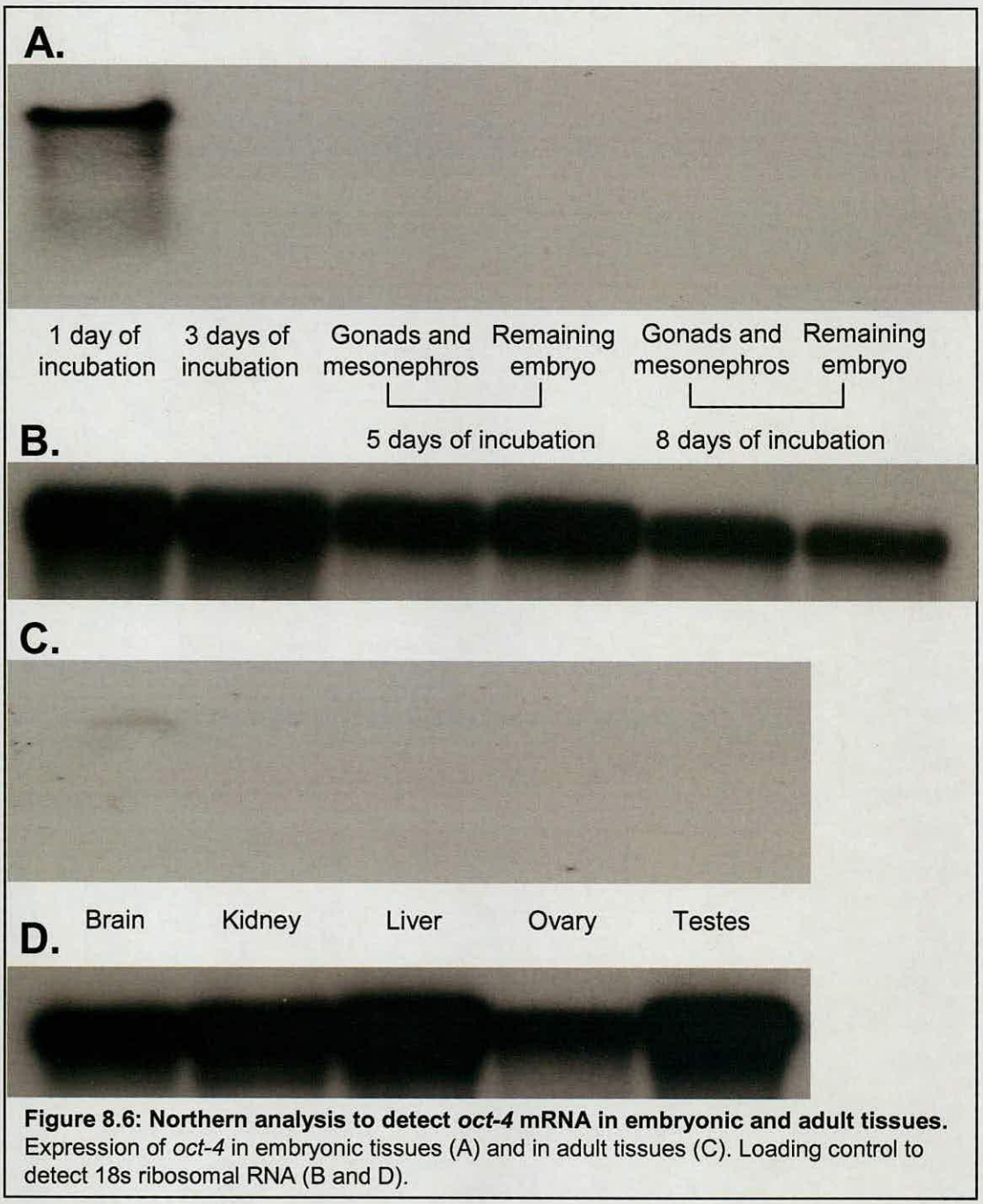


Figure 8.6: Northern analysis to detect *oct-4* mRNA in embryonic and adult tissues. Expression of *oct-4* in embryonic tissues (A) and in adult tissues (C). Loading control to detect 18s ribosomal RNA (B and D).

The *nanog* radiolabelled probe hybridised specifically to RNA extracted from embryos at one day of incubation, producing a single product of approximately 1000bp (see Figure 8.7A). This size of product is consistent with the predicted size of the *nanog* transcript, which is 930bp. The *nanog* radiolabelled probe did not hybridise to any other RNA sample (see Figures 8.7A and C).

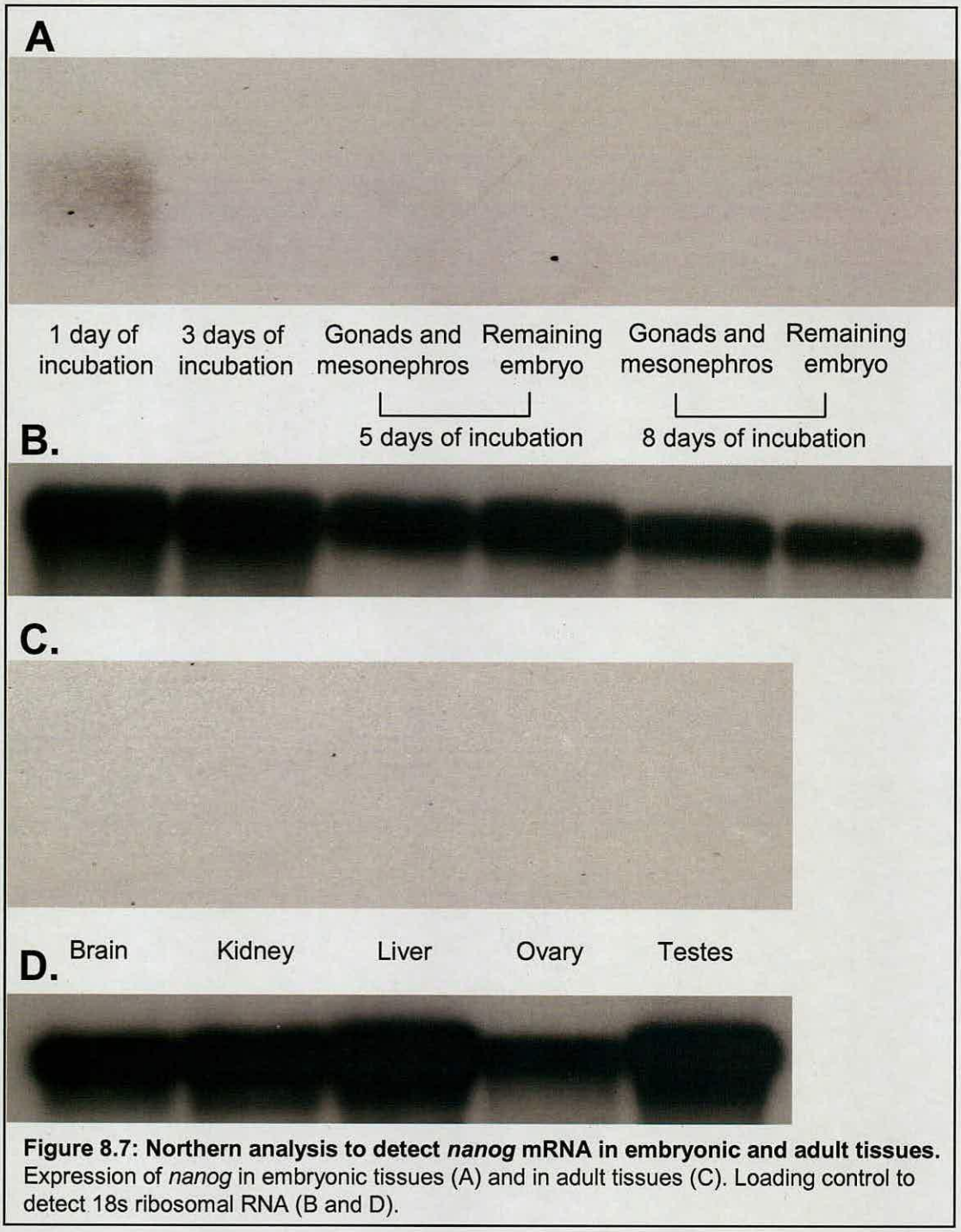


Figure 8.7: Northern analysis to detect *nanog* mRNA in embryonic and adult tissues. Expression of *nanog* in embryonic tissues (A) and in adult tissues (C). Loading control to detect 18s ribosomal RNA (B and D).

8.3.2 Expression of *cPouV* at one, three and five days of incubation

To determine where *cPouV* is expressed during development, *in situ* hybridisation was carried out on embryos at one, three and five days of incubation. At stage four (H and H), *cPouV* mRNA was detected throughout the neural plate (1 in Figure 8.8A). *cPouV* positive cells were not detected in the germinal crescent (2 in Figure 8.8A) at this stage of development. At three days of incubation, *cPouV* mRNA was detected in the genital ridges located between the fore and hind limb buds (3 in Figure 8.8C and E). The expression occurred in a salt and pepper pattern, suggesting that *cPouV* is expressed in the PGCs. The anti-sense control indicated that any staining observed in the head and otic vesicle (4 in Figure 8.8D) was trapping and not a genuine signal (see Figure 8.8D). Analysis of sections of the embryo revealed that *cPouV* positive cells were found either side of the midline in the splanchnic mesoderm (6 in Figure 8.8e') and in the genital ridges (3 in Figure 8.8e') slightly below the notochord (5 in Figure 8.8e'). At five days of incubation, when gonads (8 in Figure 8.8F) are budding away from the mesonephros (9 in Figure 8.8F), *cPouV* positive cells were detected specifically in and around both the left and right gonad (see Figure 8.8F). Analysis of sections of a whole-mount embryo confirmed this location of *cPouV* positive cells (see Figures 8.8H).

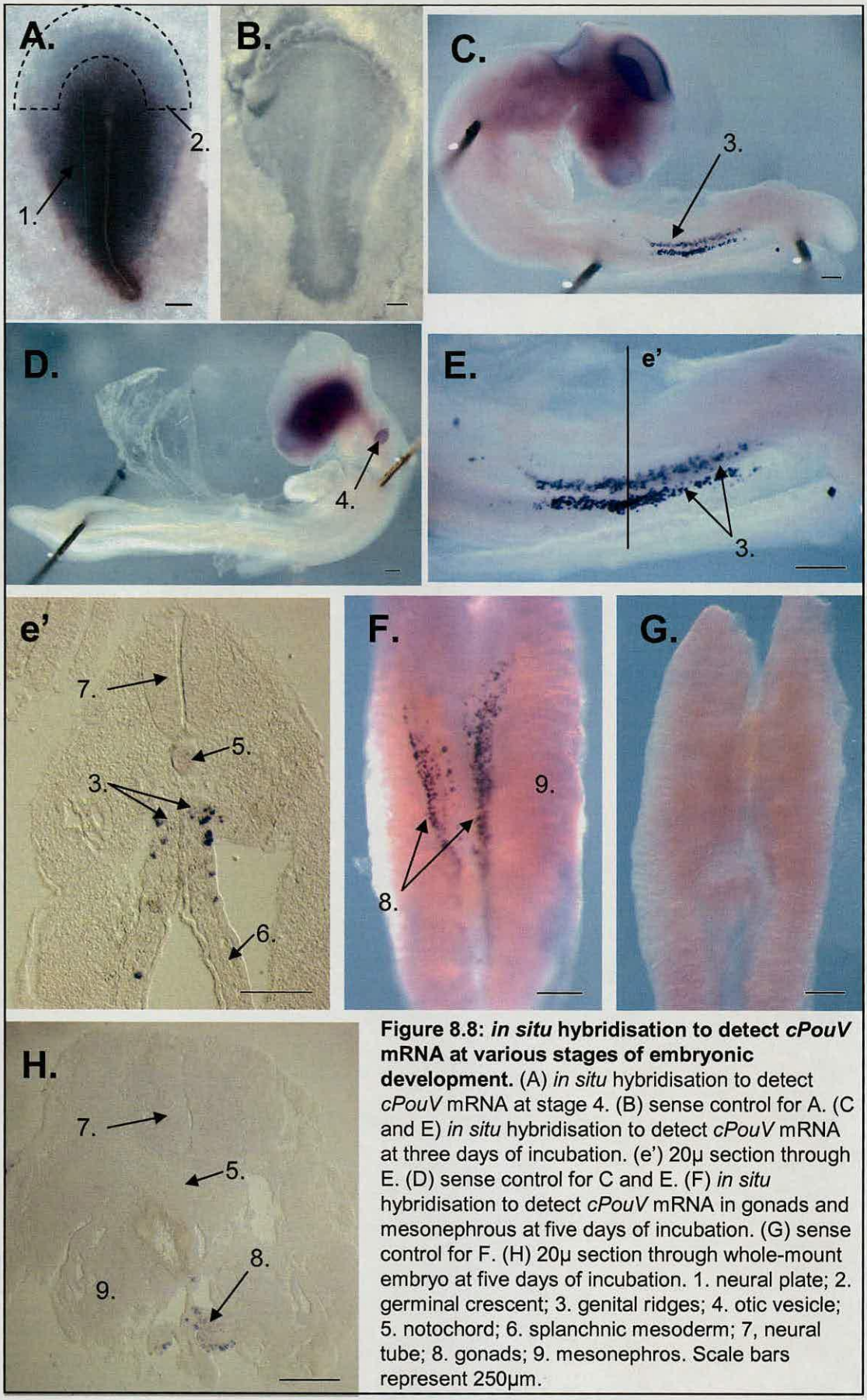


Figure 8.8: *in situ* hybridisation to detect *cPouV* mRNA at various stages of embryonic development. (A) *in situ* hybridisation to detect *cPouV* mRNA at stage 4. (B) sense control for A. (C and E) *in situ* hybridisation to detect *cPouV* mRNA at three days of incubation. (e') 20µ section through E. (D) sense control for C and E. (F) *in situ* hybridisation to detect *cPouV* mRNA in gonads and mesonephrous at five days of incubation. (G) sense control for F. (H) 20µ section through whole-mount embryo at five days of incubation. 1. neural plate; 2. germinal crescent; 3. genital ridges; 4. otic vesicle; 5. notochord; 6. splanchnic mesoderm; 7, neural tube; 8. gonads; 9. mesonephros. Scale bars represent 250µm.

8.3.3 Expression of *nanog* at one, three and five days of incubation

To determine where *nanog* is expressed during development, *in situ* hybridisation was carried out on embryos at one, three and five days of incubation. At early stage four (H and H), *nanog* mRNA was detected in the neural plate (1 in Figure 8.9A), surrounding the primitive streak. Expression was located further away from the primitive streak at the anterior than at the posterior. The pattern of expression was punctated, particularly at the posterior of the embryo (see Figure 8.9A). At late stage 4 (H and H), *nanog* expression was detected in neural plate cells located around the anterior and posterior ends of the primitive streak. *Nanog* positive cells were detected in the germinal crescent (2 in Figure 8.9B) surrounding the anterior neural plate. This pattern suggests staining of PGCs (see Figure 8.9B). At three days of incubation, *nanog* positive cells were detected in the genital ridges either side of the embryonic mid-line (3 in Figures 8.9D and F). Some staining was also detected in the head, heart and otic vesicle (4 in Figure 8.9D), however, the sense control indicated that this occurred due to trapping (see Figure 8.9E). Sectioning of the embryo revealed *nanog* positive cells clustered below the notochord (5 in Figure 8.9f'), either side of the midline, in the genital ridges (3 in Figure 8.9f'). At five days of incubation, *nanog* positive cells were mainly detected in and around the gonads (7 in Figure 8.9G left). Some staining was also detected in the mesonephros (8 in figure 8.9G), however, the sense control indicates that this was endogenous β-galactasidase activity (see Figure 8.9G right). Sectioning of one of these embryos revealed specific staining in and around the gonads (7 in Figure 8.9H).

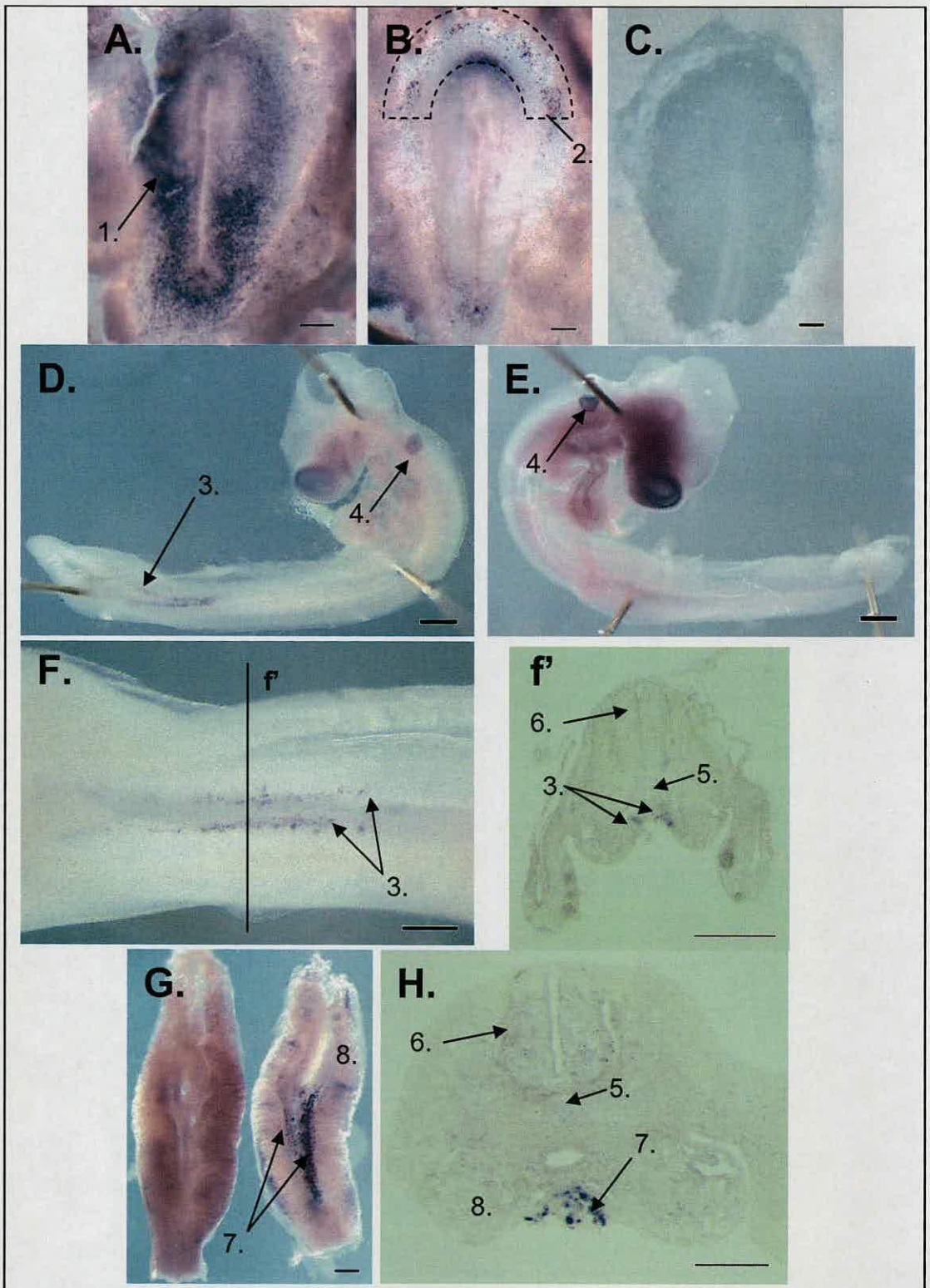


Figure 8.9: *in situ* hybridisation to detect *nanog* mRNA at various stages of embryonic development. (A) *in situ* hybridisation to detect *nanog* mRNA at stage 4 and (B) late stage 4 embryo. (C) sense control for A and B. (D and F) *in situ* hybridisation to detect *nanog* mRNA at three days of incubation. (f') 20µ section through F. (E) sense control for D and F. (G right) *in situ* hybridisation to detect *nanog* mRNA in gonads and mesonephrous at five days of incubation. (G left) sense control for G right. (H) 20µ section through whole-mount embryo at five days of incubation. 1. neurral plate; 2. germinal crescent; 3. genital ridges; 4. otic vesicle; 5. notochord; 6. neural tube; 7. gonads; 8. mesonephros. Scale bars represent 250µm.

8.3.4 Expression of *cPouV* in the germinal crescent at stage 10 (H and H)

The initial RT-PCR and *in situ* hybridisation analyses identified *cPouV* and *nanog* transcripts at one, three and five days of incubation. The Northern analysis identified *cPouV* and *nanog* transcripts at one day of incubation only. Looking at the *in situ* hybridisation analysis results, at one day of incubation a large proportion of cells in the embryo express the two genes, whereas at three and five days of incubation relatively few cells express *cPouV* and *nanog*. *CPouV* was detected in embryos at three days of incubation in a pattern that suggests expression in PGCs. In earlier development, *cPouV* was detected throughout the neural plate but not in cells in the germinal crescent, indicating that *cPouV* is not expressed in PGCs at this stage of development. *In situ* hybridisation analysis was carried out to identify when during development *cPouV* is first expressed in a pattern that suggests expression in PGCs.

At early stage 8 (H and H), *cPouV* mRNA was detected in the head fold (1 in Figure 8.10) and in the neural plate (2 in Figure 8.10) surrounding the primitive streak. The staining surrounding the primitive streak extended across the area pellucida (3 in Figure 8.10). The pattern of expression appeared punctated further away from the primitive streak. In the area opaca, the developing blood islands were also positive for *cPouV* (4 in Figure 8.10). Staining was not detected in the germinal crescent indicating that *cPouV* was not expressed in PGCs at this stage of development (see Figure 8.10A).

At late stage 8 (H and H), *cPouV* mRNA was detected in the neural tube (5 in Figure 8.10), the posterior neural plate and in the blood islands. Staining was not detected anterior of the head in the germinal crescent, indicating that *cPouV* is not expressed in the PGCs at this stage (see Figure 8.10B).

At stage 10, *cPouV* mRNA was detected in the head (7 in Figure 8.10), the developing eyes, the neural tube and the posterior neural plate. In the extraembryonic membranes, *cPouV* was detected in the blood islands and in cells in the germinal crescent (see Figures 8.10D and E). The staining of the cells in the germinal crescent suggests (6 in Figure 8.10) expression in the PGCs (8 in Figure 8.10). Analysis of sections of the embryo revealed: staining throughout the neural tube, neural plate and the blood islands at the posterior (see Figure 8.10I); staining throughout the neural tube and in the blood islands at mid-body level (see Figure 8.10H); and staining in individual cells attached to the surface of the mesoderm at the anterior (see Figure 8.10G). These results indicate that *cPouV* is first detectable in PGCs at stage 10.

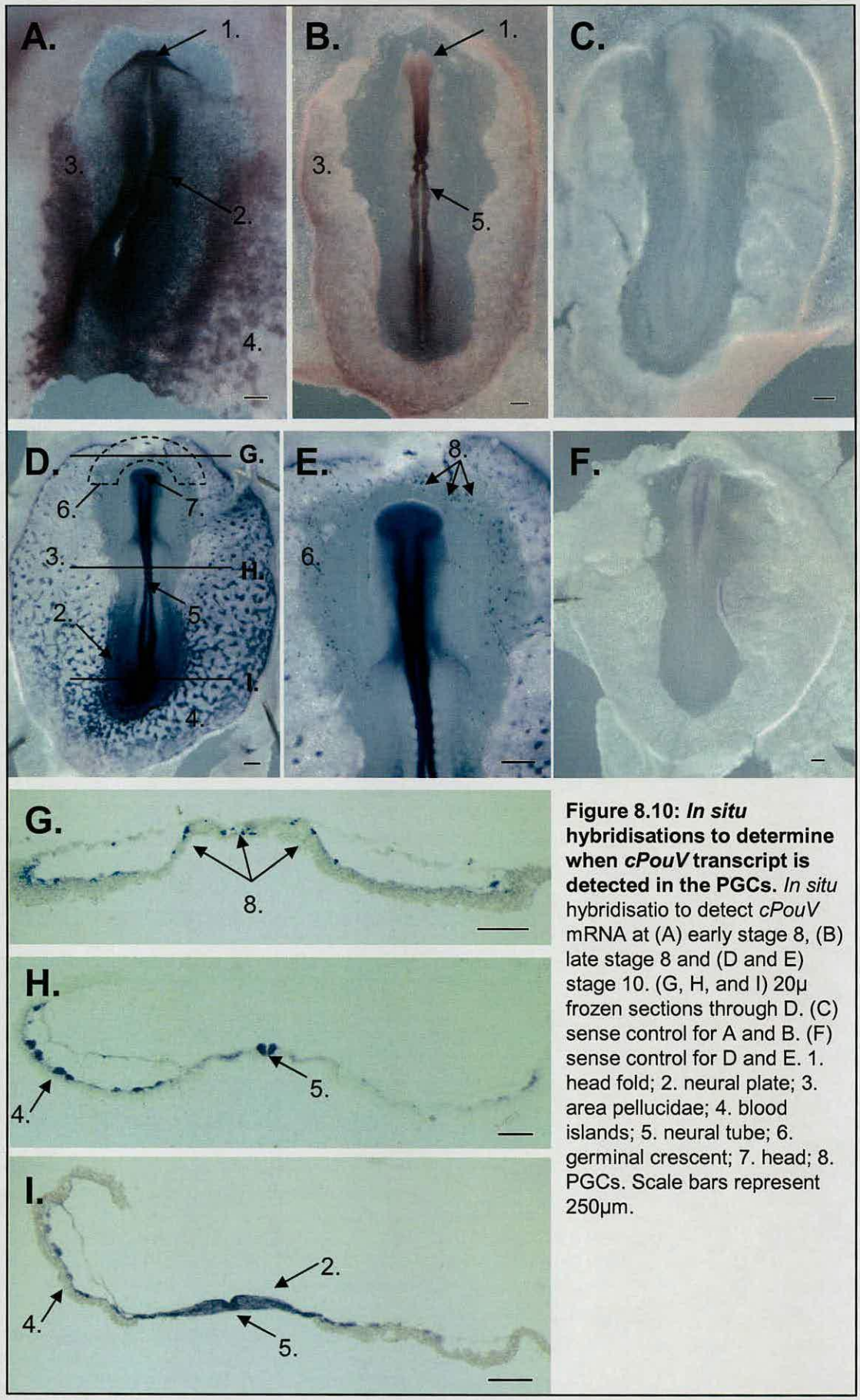


Figure 8.10: *In situ* hybridisations to determine when *cPouV* transcript is detected in the PGCs. *In situ* hybridisatio to detect *cPouV* mRNA at (A) early stage 8, (B) late stage 8 and (D and E) stage 10. (G, H, and I) 20µ frozen sections through D. (C) sense control for A and B. (F) sense control for D and E. 1. head fold; 2. neural plate; 3. area pellucidae; 4. blood islands; 5. neural tube; 6. germinal crescent; 7. head; 8. PGCs. Scale bars represent 250µm.

8.3.5 Staining consecutive sections for *cPouV/nanog* expression and SSEA-1

SSEA-1 is a pluripotent cell specific protein that can be used to detect germ cells. Consecutive 20μ sections of gonads from an embryo at five days of incubation were collected on two slides and stained for the presence of SSEA-1 and either *cPouV* or *nanog* mRNA to determine whether the cells expressing *cPouV* and *nanog* are germ cells. Cells containing *cPouV* mRNA were detected in the gonad (see Figure 8.11C). In the second section stained for SSEA-1 activity, the locations of several of the SSEA-1 positive cells were found in similar locations of the gonad to the *cPouV* positive cells (see Figures 8.11D arrows). Cells containing *nanog* mRNA were detected in the gonad (see Figure 8.11E). In the second section stained for SSEA-1 activity, the locations of several of the SSEA-1 positive cells were found in similar locations of the gonad to the *nanog* positive cells (see Figures 8.11F arrows). This was observed in several sections from different embryos and indicates that *cPouV* and *nanog* are expressed in PGCs and not in supporting cells see Figure 8.12).

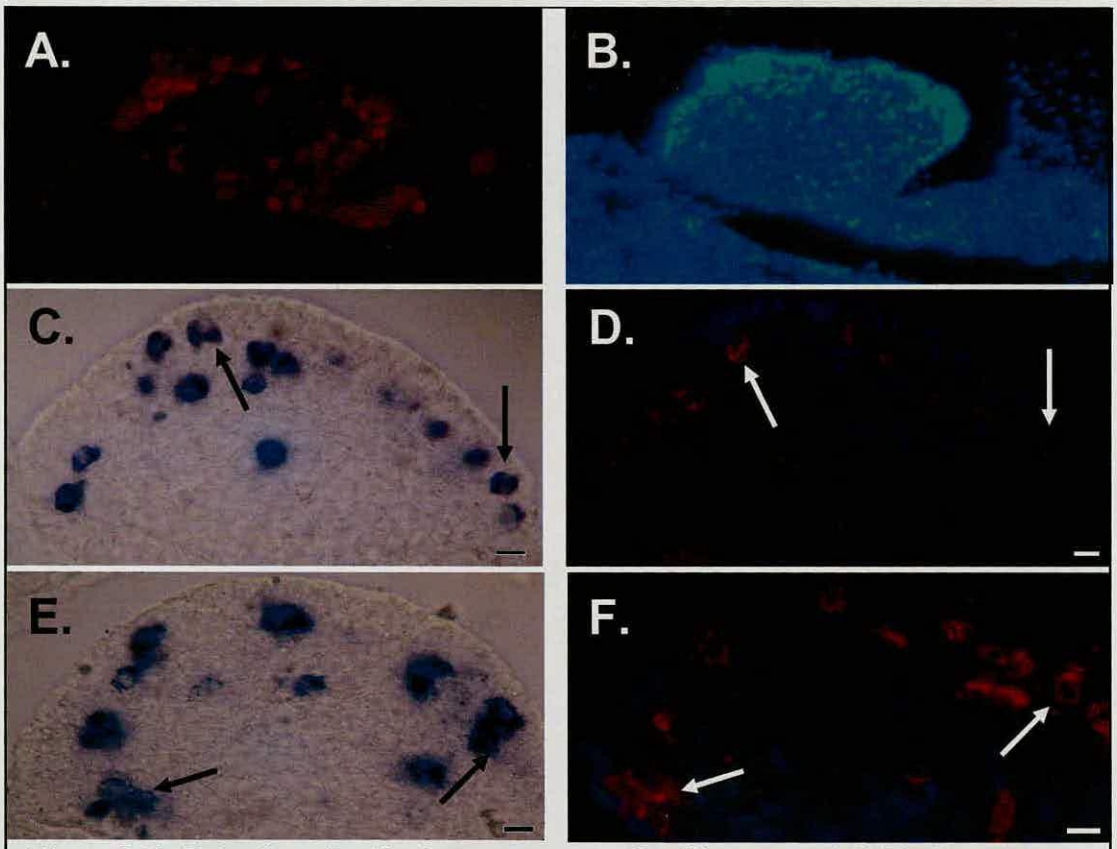
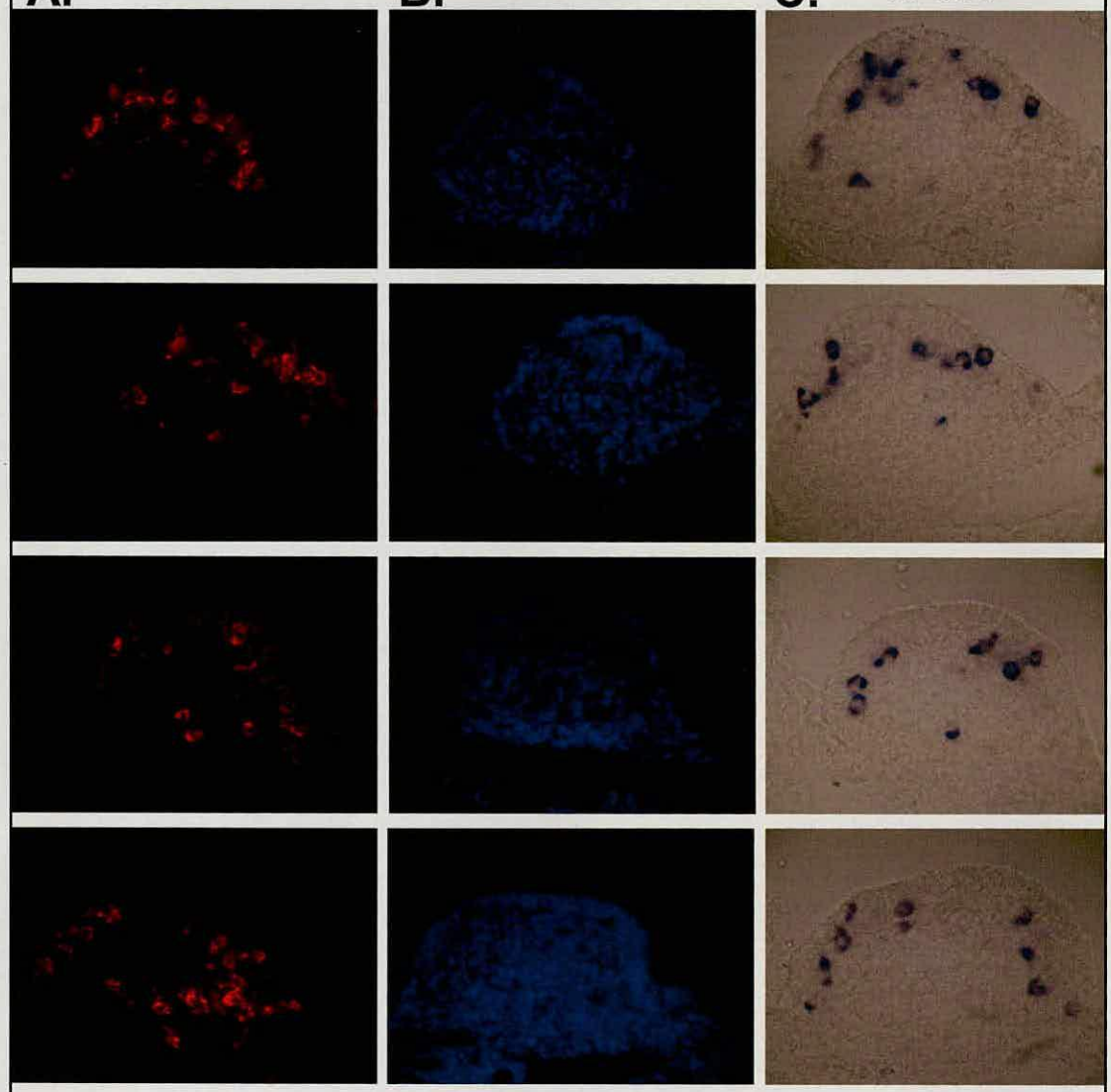


Figure 8.11: Detection of cells that co-express *cPouV*/*nanog* and SSEA-1 carried out on consecutive sections. (A) detection of PGCs in five day gonad 20µ frozen section using anti-SSEA-1 antibody. (B) Hoechst staining of A. (C) *in situ* hybridisation on five day gonad 20µ frozen section to detect *cPouV* mRNA. (D) detection of SSEA-1 on consecutive 20µ frozen section from C. (E) *in situ* hybridisation on five day gonad 20µ frozen section to detect *nanog* mRNA. (F) detection of SSEA-1 on consecutive 20µ frozen section from E. Scale bar represents 10µ.

Figure 8.12:



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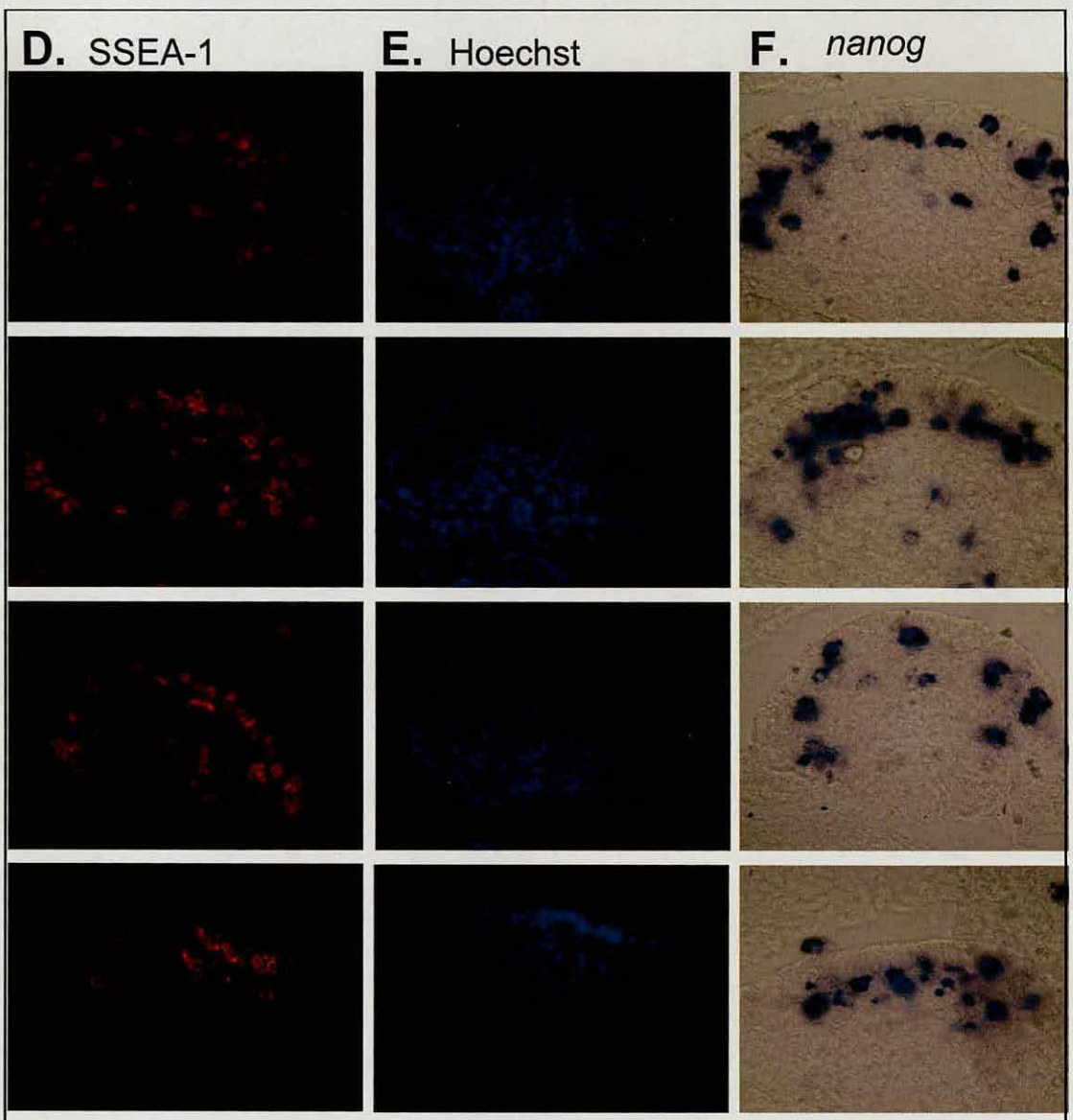


Figure 8.12: Detection of cells that co-express *cPouV*/*nanog* and SSEA-1 carried out on consecutive sections from different embryos. (A) SSEA-1 detection in PGCs in five day gonad 20µ frozen section using anti-SSEA-1 antibody. (B) Hoechst staining of A. (C) *in situ* hybridisation to detect *cPouV* on consecutive 20µ frozen section from A. (D) SSEA-1 detection in PGCs in five day gonad 20µ frozen section using anti-SSEA-1 antibody. (E) Hoechst staining of A. (F) *in situ* hybridisation to detect *nanog* on consecutive 20µ frozen section from D.

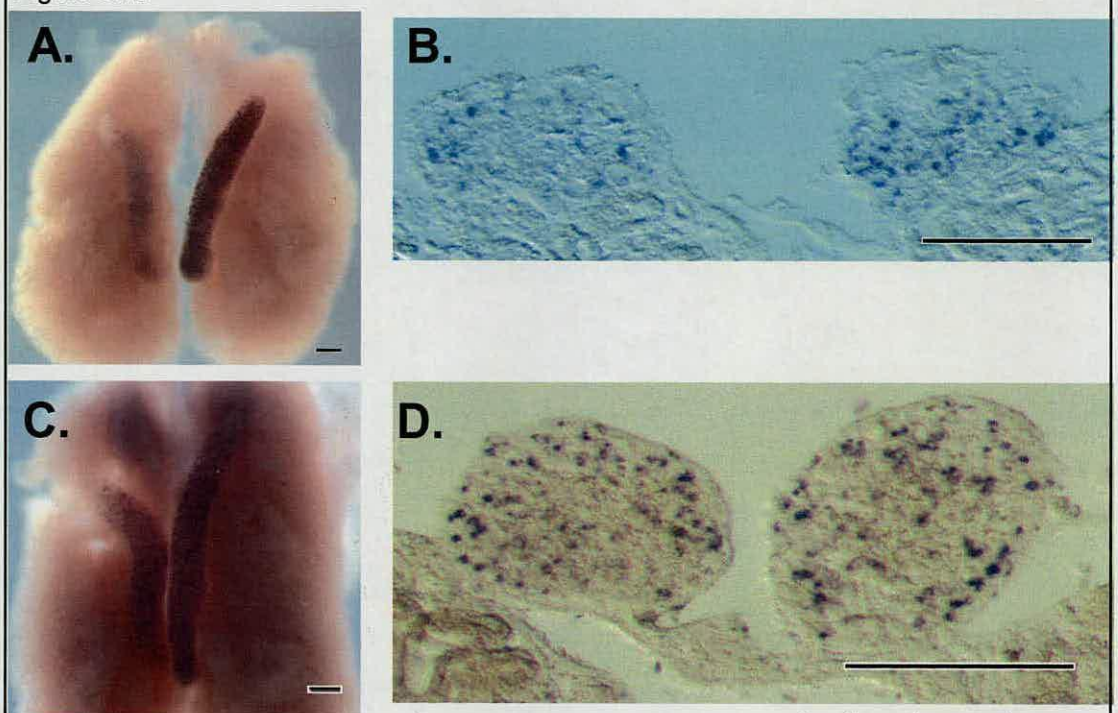
8.3.6 Expression of chicken *cPouV* in male and female gonads from embryos at late stages of development

Further *in situ* hybridisation analysis was conducted to determine the expression patterns of *cPouV* in gonads later in development. At 9 days of incubation, embryonic gonads can be sexed accurately by eye (see section 8.2.5) and the expression of *cPouV* was investigated in each sex separately. The results from each sex are presented separately.

In males at 9.5 days of incubation, *cPouV* was detected in a salt and pepper pattern in cells in the gonads, suggesting expression is maintained in the PGCs (see Figure 8.13A). Analysis of sections of the gonads revealed *cPouV* positive cells throughout the gonad (see Figure 8.13B). In males at 11.5 days of incubation, *cPouV* was detected in a salt and pepper pattern in cells in the gonads, indicating expression in the PGCs. The distribution of the *cPouV* positive cells appeared more uniform than at 9.5 days of incubation (see Figure 8.13C). Analysis of sections of the gonads revealed *cPouV* positive cells throughout the gonads (see Figure 8.13D). In males at 13.5 days of incubation, *cPouV* was detected in cells throughout the gonads. The overall number of *cPouV* positive cells in the gonads had increased. This observation is consistent with observations made by Swift (1916), who indicated that male PGCs begin dividing at approximately 13 days of incubation (see Figure 8.13E). Analysis of sections of the gonads revealed an increase in the number of *cPouV* cells per section in comparison to earlier stages (see Figure 8.13F). In males at 15.5 days of incubation, *cPouV* positive cells were detected throughout the gonads. Both gonads appeared black (see Figure 8.13G). Analysis of sections revealed

cPouV positive cells close to the surface of the gonad in a salt and pepper pattern, indicating expression of *cPouV* in germ cells. No cells at the centre of the gonads stained positively for *cPouV* (see Figure 8.13H). This suggests that either *cPouV* is being down-regulated, or that the tissues are too dense and less permeable at this stage of development to allow effective penetration of the *in situ* hybridisation probe. In males at 17.5 days of incubation, *cPouV* positive cells were detected in both gonads in a dispersed salt and pepper pattern. The number of *cPouV* positive cells detected was significantly lower than at 13.5 and 15.5 days of incubation (see Figure 8.13I). In hatchlings, no *cPouV* positive cells were detected (see Figure 8.13J). The decrease in *cPouV* positive cells in the gonads of 17.5 days of incubation embryos followed by complete absence in hatchlings may be because *cPouV* is down-regulated, or because the tissue is at a more advanced stage of development and the RNA probe was unable to penetrate it effectively.

Figure 8.13



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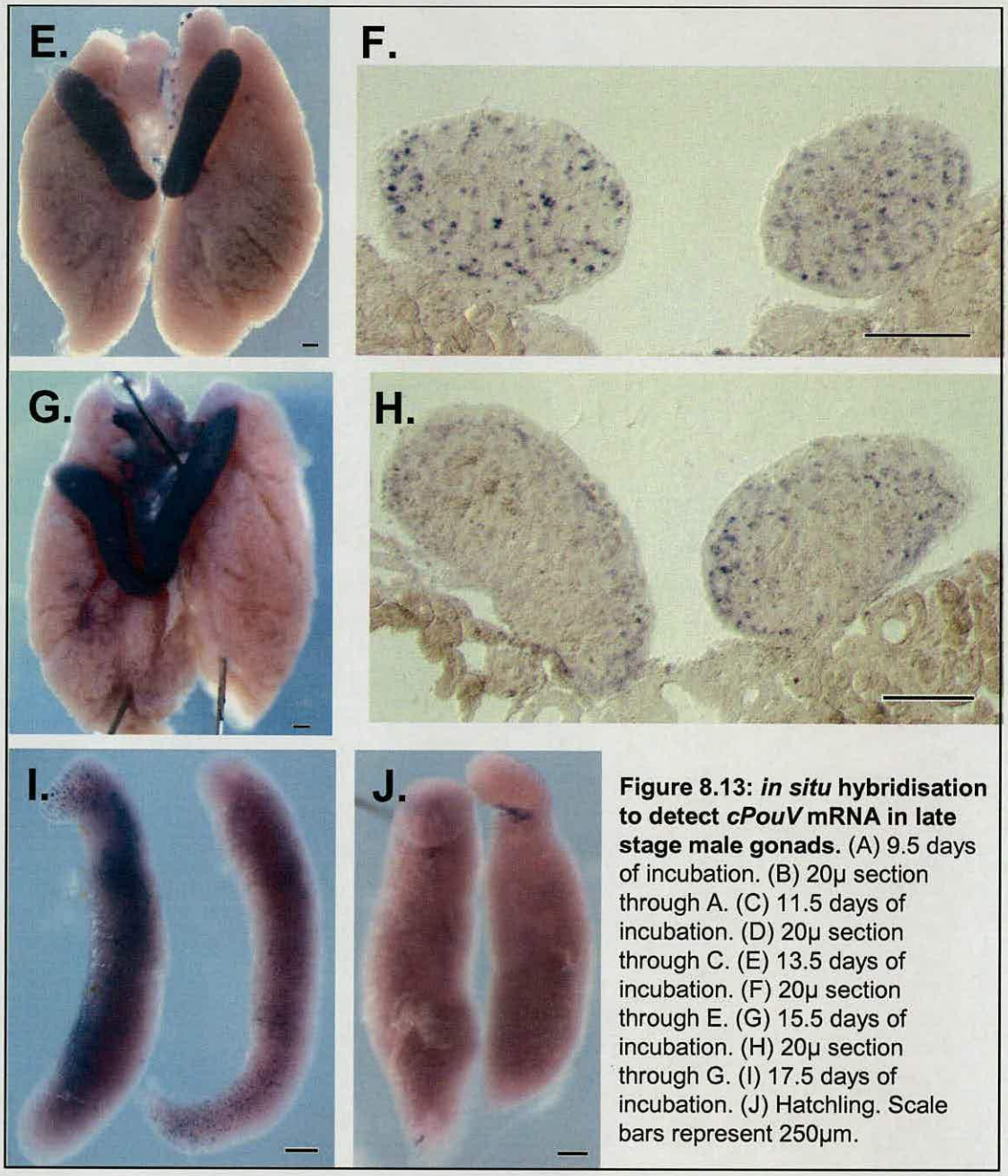


Figure 8.13: *in situ* hybridisation to detect *cPouV* mRNA in late stage male gonads. (A) 9.5 days of incubation. (B) 20µ section through A. (C) 11.5 days of incubation. (D) 20µ section through C. (E) 13.5 days of incubation. (F) 20µ section through E. (G) 15.5 days of incubation. (H) 20µ section through G. (I) 17.5 days of incubation. (J) Hatchling. Scale bars represent 250µm.

In females at 9.5 days of incubation, *cPouV* positive cells were detected specifically in the gonads. When the left ovary was removed from the mesonephros and viewed, the majority of the *cPouV* positive cells appeared to be located on the dorsal side of the ovary (see Figures 8.14 A-C). However, analysis of sections revealed that the *cPouV* positive cells were located in the cortex of the ovary on the ventral side (see Figure 8.14D). In females at 11.5 and 13.5 days of incubation, *cPouv* positive cells were detected in the right ovary and on the dorsal side of the left ovary (see Figure 8.14E and G-I). Analysis of sections of the gonad from 11.5 days of incubation confirmed that the *cPouV* positive cells were located in the dorsal side of the ovary (see Figure 8.14F). This observation was unexpected because the germ cells are known to be located in the cortex region of the ovary, which is found on the ventral side of the ovary. It suggests that either the germ cells located in the cortex no longer express *cPouV* or the *in situ* hybridisation probe was unable to penetrate the cortex layer. In females at 15.5 days of incubation, *cPouV* positive cells were detected at the lateral edges of the left ovary. *cPouV* positive cells were not detected in the right ovary (see Figures 8.14J-M). Analysis of sections revealed faint shading on one side of the left ovary, which is a pattern that does not suggest *cPouV* expression in the PGCs (see Figure 8.14N). In females at 17.5 days of incubation and in gonads taken from a hatchling, *cPouV* positive cells were not detected (see Figures 8.14O and P). The faint staining seen at the lateral edges of the ovary at 17.5 days of incubation is background staining from the endogenous alkaline phosphatase activity detected in the mesonephros.

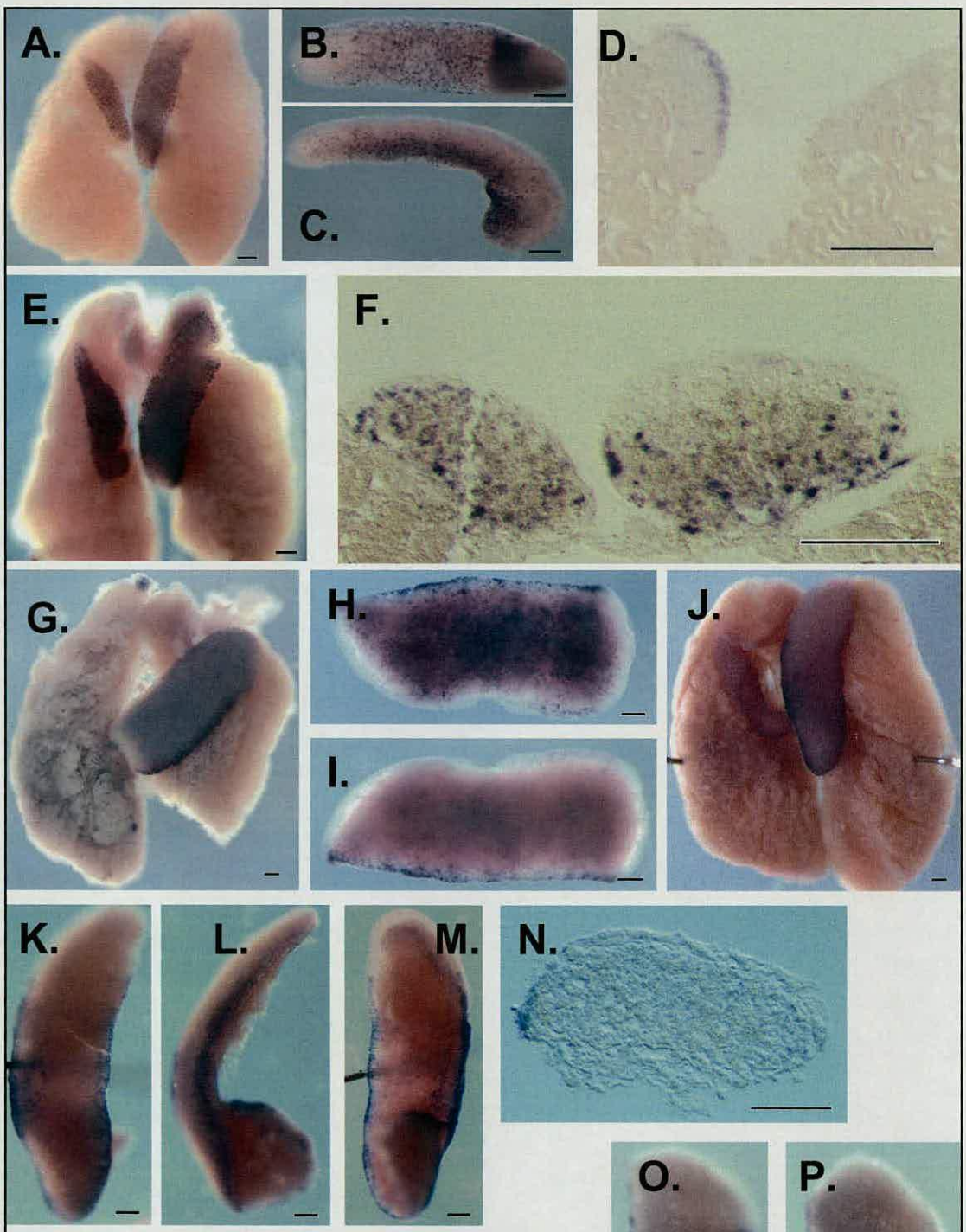


Figure 8.14: *in situ* hybridisation to detect *cPouV* mRNA in late stage female gonads. (A) 9.5 days of incubation (B) dorsal view and (C) side view. (D) 20µ section through A. (E) 11.5 days of incubation. (F) 20µ section through E. (G) 13.5 days of incubation (H) dorsal view and (I) ventral view. (J) 15.5 days of incubation (K) ventral view (L) side view and (M) dorsal view. (N) 20µ section through J. (O) 17.5 days of incubation. (P) Hatchling. Scale bars represent 250µm.

8.3.7 Expression of chicken *nanog* in male and female gonads from embryos at late stages of development

Further *in situ* hybridisation analysis was conducted to determine the expression patterns of *nanog* in gonads later in development. The expression of *nanog* was investigated in each sex separately from 9.5 days of incubation to hatchlings. The results from each sex are presented separately.

In males at 9.5 days of incubation, *nanog* positive cells were detected in the gonads (see Figure 8.15A). Analysis of sections of the gonads revealed *nanog* positive cells throughout the left and right testes (see Figure 8.15B). In males at 11.5 days of incubation, some *nanog* positive cells were detected in the gonads (see Figure 8.15C). The staining of endogenous alkaline phosphatase in the mesonephros was very high and the gonads had to be dissected away from the mesonephros in order to see the staining of *nanog* positive cells. Analysis of sections of the gonads revealed faint staining of cells in a salt and pepper pattern, suggesting germ cell expression of *nanog* (see Figure 8.15D). In males at 13.5 days of incubation, *nanog* positive cells were detected in the gonads. The staining was very faint at this stage (see Figure 8.15E). It was decided that the staining was too faint for *nanog* positive cells to remain visible after sectioning; therefore the gonads were not sectioned. In males at 15.5, 17.5 and in gonads taken from a hatchling, *nanog* positive cells were not visible (see Figures 8.15F-H).

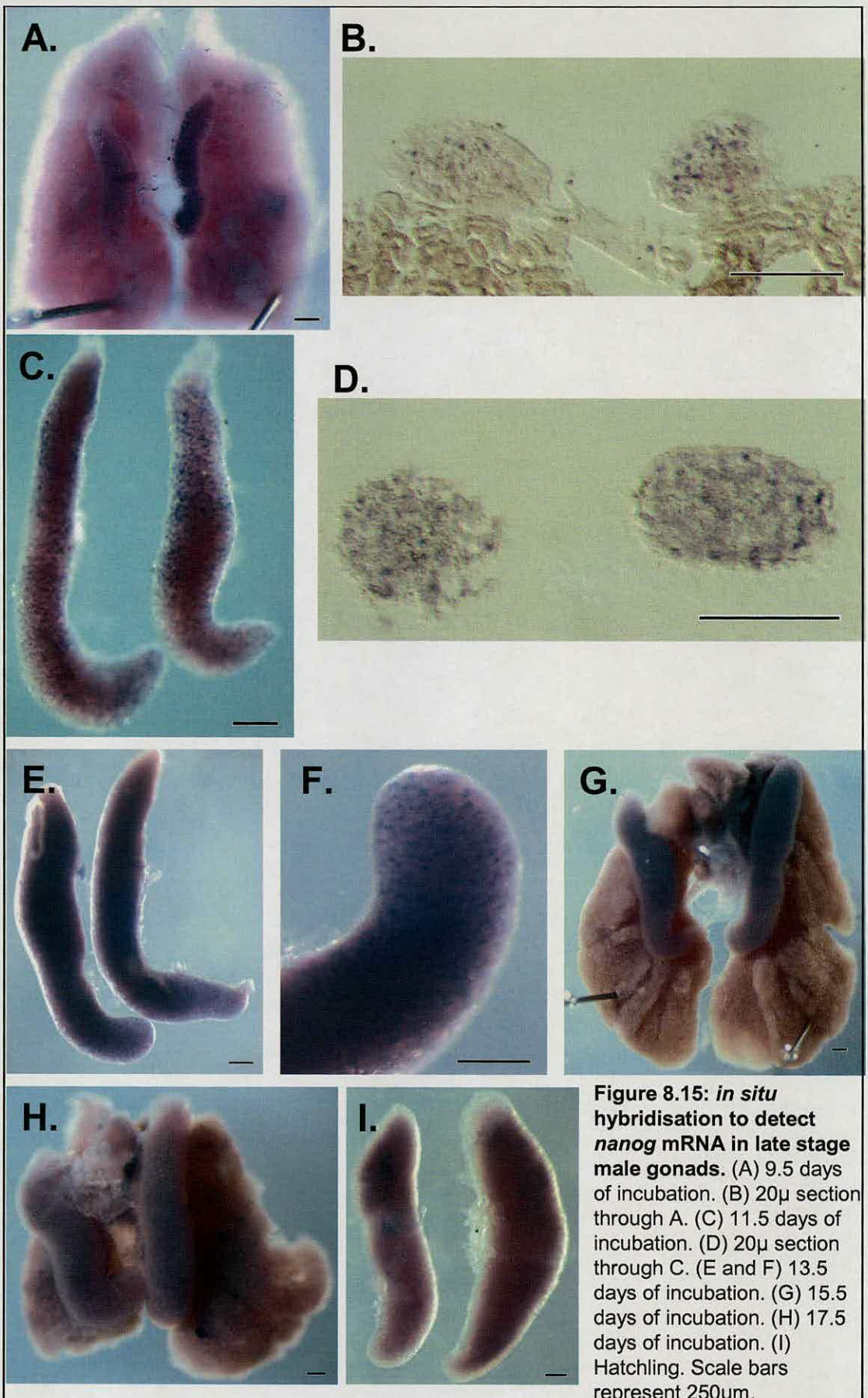


Figure 8.15: *in situ* hybridisation to detect *nanog* mRNA in late stage male gonads. (A) 9.5 days of incubation. (B) 20µ section through A. (C) 11.5 days of incubation. (D) 20µ section through C. (E and F) 13.5 days of incubation. (G) 15.5 days of incubation. (H) 17.5 days of incubation. (I) Hatchling. Scale bars represent 250µm.

In females at 9.5 days of incubation, *nanog* positive cells were detected on the ventral surface of the left ovary (see Figure 8.16A-C). Analysis of sections of the gonads revealed *nanog* positive cells in the cortex of the left ovary (see Figure 8.16D). In females at 11.5 days of incubation, *nanog* positive cells were detected on the dorsal side of the left ovary (see Figure 8.16E and F). Analysis of sections of the gonads revealed scattered *nanog* positive cells in the medulla of the ovary, but not the cortex (see Figure 8.16G). In females at 13.5 days of incubation, *nanog* positive cells were detected at the lateral edges of the ovary (see Figure 8.16H). *Nanog* positive cells were not visible on the ventral side of the ovary (see Figure 8.16I). A few *nanog* positive cells were seen on the dorsal side of the left ovary (see Figure 8.16J). This distribution of *nanog* positive cells was unexpected because germ cells are located in the cortex on the ventral surface of the ovary. In females at 15.5 and 17.5 days of incubation and in gonads taken from a hatchling, *nanog* positive cells were not detected (see Figures 8.16K-N). This could be because *nanog* is being down-regulated, or it could be because the probe was unable to penetrate the tissue.

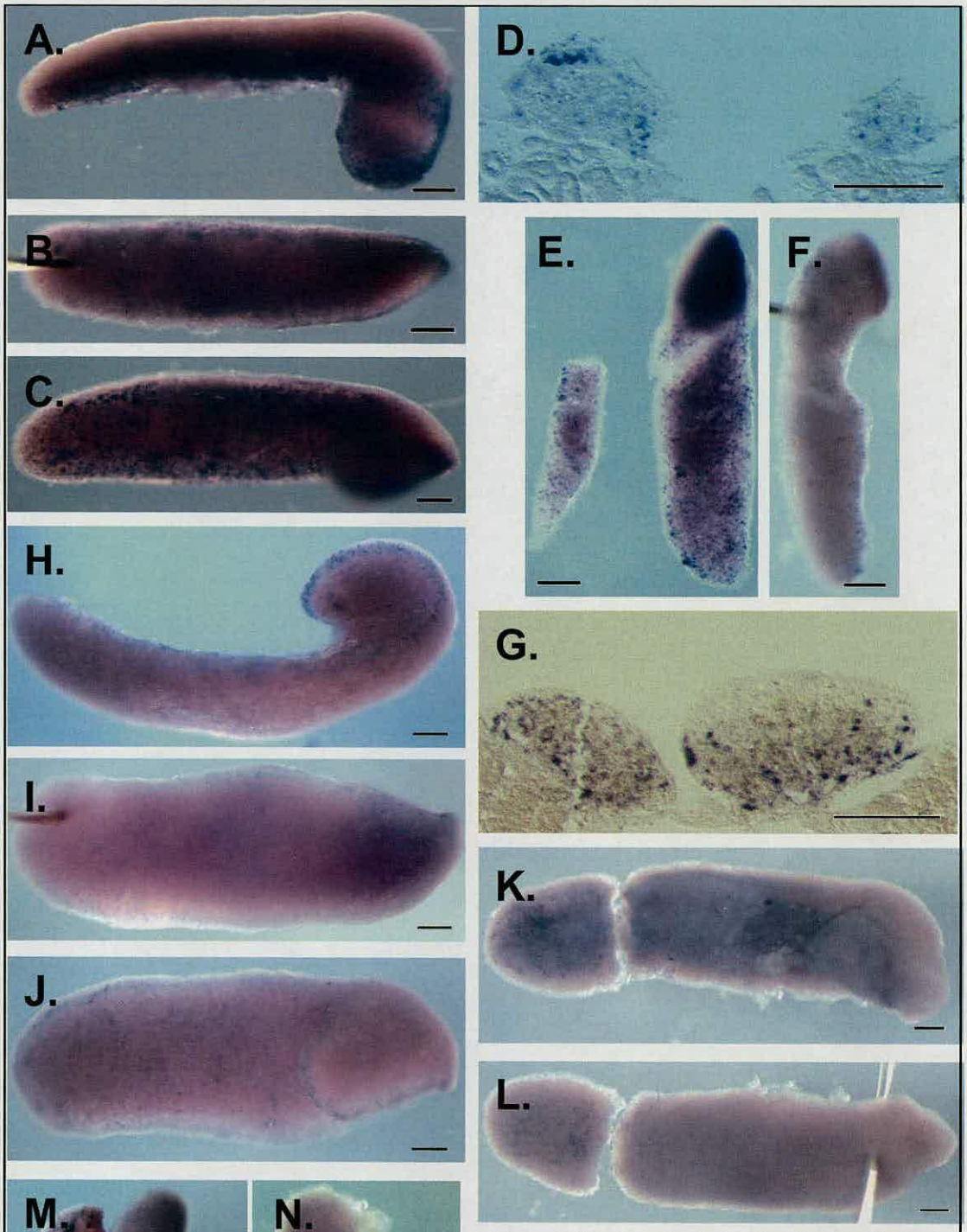


Figure 8.16: *in situ* hybridisation to detect *nanog* mRNA in late stage female gonads. (A) 9.5 days of incubation side view (B) ventral view and (C) dorsal view. (D) 20µ section through A. (E) 11.5 days of incubation dorsal view and (F) ventral view. (G) 20µ section through E. (H) 13.5 days of incubation side view, (I) ventral view and (J) dorsal view. (K) 15.5 days of incubation dorsal view and (L) ventral view. (M) 17.5 days of incubation. (N) Hatchling. Scale bars represent 250µm.

8.3.8 Quantitative PCR

To expand the *in situ* hybridisation results further the left gonads were removed from females and both gonads from male embryos at 9, 11, 13, 15 and 17 days of incubation. RNA was then extracted from them to carry out quantitative PCR (qPCR). The numbers of surrounding somatic cells increase as do the numbers of germ cells during these stages of development. Therefore, the ratio of germ cells to somatic cells will be different at each stage. However, changes in the levels of the genes could still provide useful data, especially if there are large changes in levels.

In this work, the $2^{-\Delta\Delta Ct}$ method of relative quantification (described in detail in Livak and Schmittgen, 2001) was used to estimate copy numbers of *cPouV* and *nanog* genes following normalisation of the quantitative data to LBR levels in the samples. A Student's t-test testing pairwise with a confidence interval of 95% was used to determine the significance of the figures. The baseline value was taken from the Ct values obtained at 9 days of incubation. In order for a change to be significant the level needs to change by at least a factor of two.

Looking at *cPouV*, the levels of *cPouV* mRNA significantly rose between males at 9 days of incubation and males at 13 days of incubation ($p<0.01$). A significant increase was also detected between males at 9 days of incubation and males at 15 days of incubation ($p<0.01$; see Table 8.1A and Figure 8.17B). These increases in *cPouV* levels coincide with the increase in the numbers of PGCs in the gonads. The changes detected between 9 days of incubation and either 11 days of incubation or 17 days of incubation are not statistically significant. In females, *cPouV* levels rose

significantly between gonads at 9 days of incubation and gonads at 15 days of incubation ($p<0.01$; see Table 8.1A and Figure 8.17A). All changes detected between other stages are statistically insignificant.

Looking at *nanog*, in males the levels of *nanog* mRNA significantly rose between gonads at 9 days of incubation and gonads at 13 days of incubation ($p<0.05$). A significant increase was also detected between males at 9 days of incubation and males at 15 days of incubation ($p<0.01$; see Table 8.1B and Figure 8.17D). This rise in *nanog* levels coincides with the increase in the numbers of PGCs in the gonads. All other changes detected are statistically insignificant. In females, the levels of *nanog* mRNA significantly rose between gonads at 9 days of incubation and gonads at 13 days of incubation ($p<0.05$). A significant increase was also detected between gonads at 9 days of incubation and gonads at 15 days of incubation ($p<0.01$; see Table 8.1D and Figure 8.18B). Although this rise in *nanog* levels coincides with the increase in the numbers of PGCs in the gonads, the levels detected at 15 days of incubation are far greater than the *cPouV* levels detected at the same stage. All other changes detected are statistically insignificant.

A.

Sample Name	Mean	Variance	Standard Deviation	Mean *LBR*	Mean *cPouV*- Mean *LBR*	ΔΔCt	Relative gene Expression
9 female cPouV	28.76	0.07	0.26	24.72	4.04	0.00	1.00 (0.76-1.32)
11 female cPouV	28.75	0.38	0.62	24.13	4.62	0.57	0.67 (0.37-1.23)
13 female cPouV	28.29	0.41	0.64	24.93	3.36	-0.68	1.60 (0.84-3.06)
15 female cPouV	27.74	0.27	0.52	24.82	2.92	-1.13	2.18 (1.40-3.40)
17 female cPouV	29.27	0.18	0.43	24.49	4.78	0.73	0.60 (0.42-0.87)
9 male cPou V	28.59	0.07	0.27	24.14	4.45	0.00	1.00 (0.83-1.21)
11 male cPouV	28.99	0.13	0.36	24.32	4.67	0.22	0.86 (0.67-1.10)
13 male cPouV	28.50	0.12	0.35	25.51	2.99	-1.46	2.75 (2.13-3.55)
15 male cPouV	28.12	0.03	0.17	24.88	3.24	-1.21	2.31 (1.92-2.77)
17 male cPouV	29.23	0.57	0.75	24.64	4.59	0.14	0.91 (0.39-2.09)

B.

Sample Name	Mean	Variance	Standard Deviation	Mean *LBR*	Mean *nanog*- Mean *LBR*	ΔΔCt	Relative gene Expression
9 female nanog	29.38	0.25	0.50	24.72	4.66	0.00	1.00 (0.66-1.51)
11 female nanog	28.91	1.39	1.18	24.13	4.78	0.12	0.92 (0.37-2.30)
13 female nanog	29.01	0.03	0.19	24.93	4.08	-0.58	1.49 (0.92-2.43)
15 female nanog	26.77	0.24	0.49	24.82	1.95	-2.71	6.55 (4.28-10.03)
17 female nanog	30.58	0.21	0.46	24.49	6.09	1.44	0.37 (0.25-0.54)
9 male nanog	28.65	0.23	0.48	24.14	4.51	0.00	1.00 (0.71-1.40)
11 male nanog	29.22	0.51	0.71	24.32	4.90	0.38	0.77 (0.47-1.26)
13 male nanog	29.46	0.17	0.41	25.51	3.95	-0.56	1.47 (1.10-1.98)
15 male nanog	28.52	0.31	0.56	24.88	3.64	-0.87	1.83 (1.21-2.77)
17 male nanog	31.94	0.04	0.20	24.64	7.31	2.80	0.14 (0.07-0.28)

Table 8.1: Mean Ct values for cPouV and nanog in male and female gonads and the 2-ΔΔCt method of relative quantification. (A) cPouV in females and in males. (B) nanog in females and in males. The change in Ct levels were calculated using values from 9 days of incubation as a baseline value. LBR was used as an internal control to normalise for the amount of RNA in each reaction. Standard deviations for each stage are shown. The values in brackets in the relative gene expression column are the range of standard deviation.

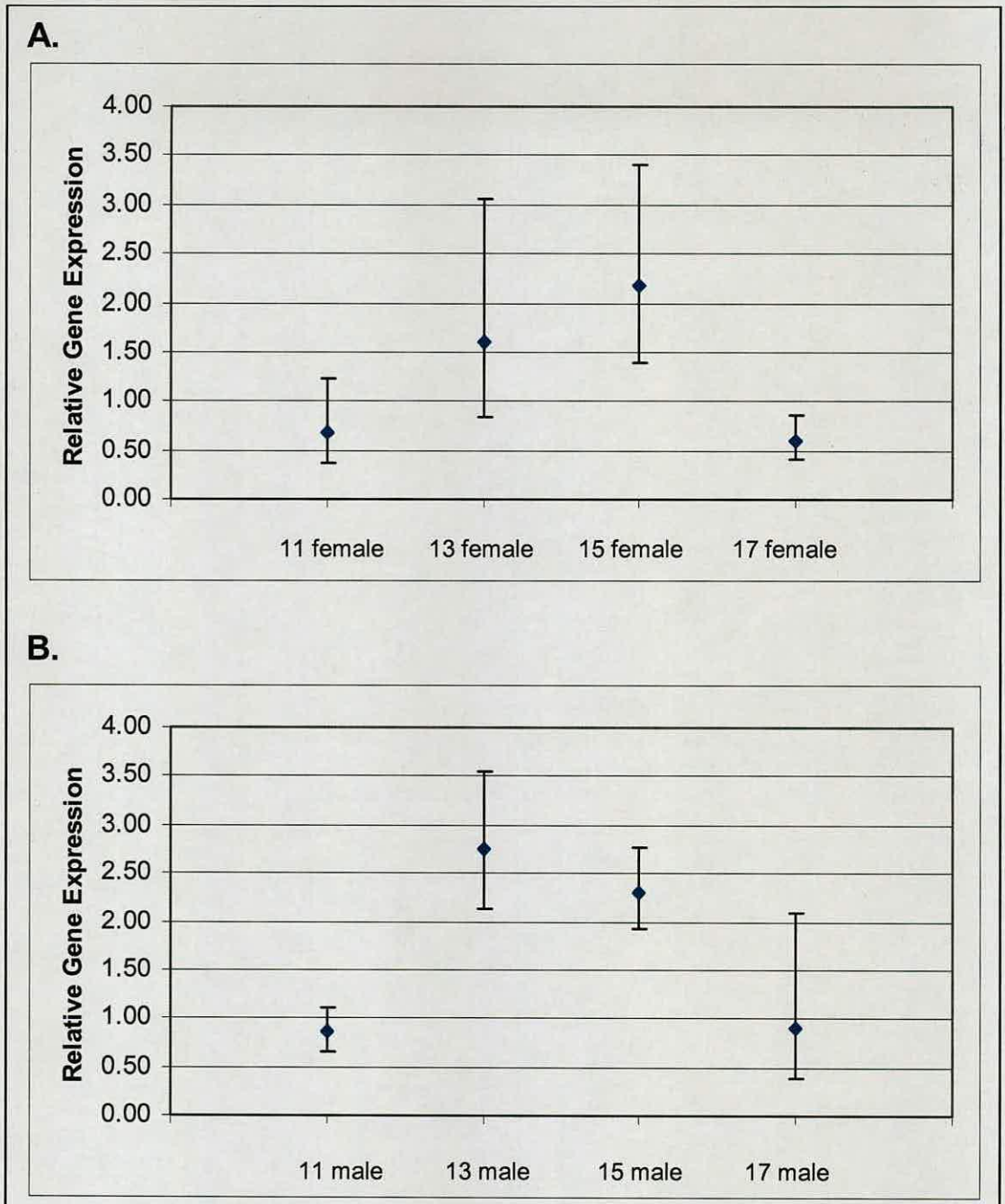


Figure 8.17: graphical representation of quantitative PCR data showing the levels of *cPouV* mRNA in comparison to levels at nine days of incubation. (A) data from female gonads at 11, 13, 15 and 17 days of incubation. (B) data from male gonads at 11, 13, 15 and 17 days of incubation. Error bars represent standard deviation.

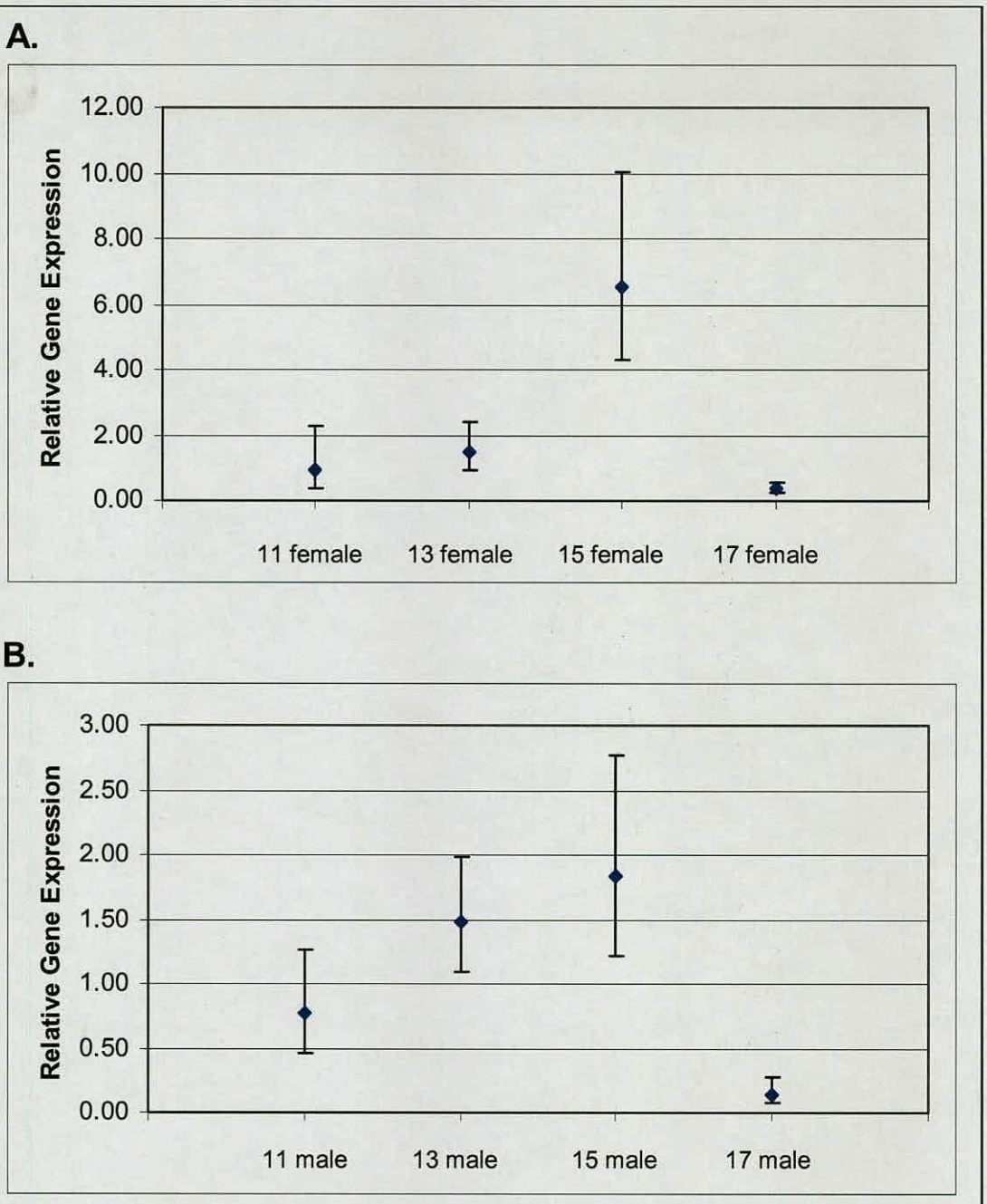


Figure 8.18: graphical representation of quantitative PCR data showing the levels of *nanog* mRNA in comparison to levels at nine days of incubation. (A) data from female gonads at 11, 13, 15 and 17 days of incubation. (B) data from male gonads at 11, 13, 15 and 17 days of incubation. Error bars represent standard deviation.

8.4 Discussion

The chicken homologues of *oct-4* and *nanog* were investigated to determine whether they are expressed in PGCs. The initial expression analysis of *cPouV* and *nanog* by RT-PCR analysis identified the mRNAs at several stages of embryonic development, but the Northern analysis only detected them at one stage of development, one day incubation. Taking into account results from *in situ* hybridisation, it was concluded that the type of Northern analysis carried out can detect genes when they are expressed in a large proportion of cells in the embryo, but that the method is not sensitive enough to detect germ cell specific transcripts in non-purified starting material.

The *in situ* hybridisation results suggested that both *cPouV* and *nanog* were expressed in PGCs. In late stage four (H and H) embryos, *nanog* was detected in cells in the germinal crescent. At this stage of development PGCs reside in the germinal crescent, suggesting expression of *nanog* in the PGCs. At three days of incubation, *cPouV* positive cells were detected in the splanchnic mesoderm and around the site of the future gonads, suggesting expression of *cPouV* in PGCs at this stage of development. As a result of these findings, it was decided to investigate *cPouV* and *nanog* further. The staining of consecutive sections for SSEA-1 activity and the mRNAs of either *cPouV* or *nanog* strengthened the hypothesis that the two mRNAs are expressed in PGCs.

In the initial expression profile, *cPouV* was expressed in the blood islands. The blood islands develop as part of the mesoderm, which is unusual because *oct-4* homologues have never been identified in the mesoderm layer, which suggests that *cPouV* is not a genuine *oct-4* homologue (Morrison and Brickman, 2006). However, the neural expression and the subsequent restriction of gene to the germ lineage, the overlapping expression patterns of *cPouV* and *nanog* shown here as well as the overlapping expression of *cPouV* and *sox-2* indicate that the gene is an *oct-4* homologue (Rex *et al.*, 1997; Matsushit *et al.*, 2002).

In the initial expression profile, it was found that *nanog* was expressed in a pattern that suggested expression in the PGCs earlier in development than *cPouV*. This was an unexpected result because in mice *oct-4* is expressed in germ cells before *nanog* and because of the expression of transcription factors that are known to regulate *nanog* expression. Three factors are known to positively regulate *nanog* expression in mammals: Oct-4, Sox-2 and FoxD3. From the work carried out here it is known that *cPouV* is not expressed in cells in the germinal crescent before *nanog*, which is confirmed by results from Lavial *et al.* (2007). The expression pattern of chicken *sox-2* has been published and no *sox-2* positive cells were observed in the germinal crescent (Rex *et al.*, 1997). This leaves FoxD3, which is still to be identified in chicken.

In the experiments carried out to detect the expression of *cPouV* and *nanog* in late stage gonads of males and females, it was found that expression differs between the sexes. In males, *in situ* hybridisation showed that in embryos at 13.5 days of

incubation the number of *cPouV* positive cells is higher than in earlier stages resulting in the gonads staining intensely. Analysis of sections of the gonads revealed that the same salt and pepper pattern observed in earlier stages is still seen, but the density of the cells is greater. This coincides with the stage when the PGCs multiply rapidly (Swift, 1916). In gonads at 15.5 days of incubation, the *cPouV* positive cells are restricted to cells close to the surface of the gonad and in gonads at 17.5 days of incubation the number of *cPouV* positive cells is greatly reduced.

In males, the change in the level of *cPouV* mRNA indicated by the qPCR is not as dramatic as that suggested by the *in situ* hybridisation. A likely explanation for this difference is that from approximately 15 days of incubation in males, the *in situ* hybridisation probe does not penetrate the tissue efficiently and that the *in situ* hybridisation should be carried out on frozen sections rather than whole-mounted tissues.

In females, *in situ* hybridisation analysis showed that *cPouV* is expressed in cells in the cortex at 9.5 days of incubation, but that after this stage the only *cPouV* positive cells were detected in the medulla of the ovaries. This was unexpected, because it is known that the germ cells reside in the cortex region of the ovary. Beyond 11.5 days of expression, *cPouV* expression appears to be down-regulated until shortly after 15.5 days of incubation, when *cPouV* mRNA is no longer detectable.

In females, the qPCR data shows that the levels of *cPouV* consistently increase between gonads at 9 days of incubation and gonads at 15 days of incubation, but by

17 days of incubation the change in the level of mRNA is insignificant, indicating that the level has dropped. At approximately 15 days of incubation the female germ cells begin to differentiate. The qPCR analysis indicates that at the onset of meiosis, the levels of *cPouV* mRNA begin to decrease. The levels of *cPouV* mRNA detected in female chickens is consistent with the levels of Oct-4 protein detected in female mice. However, the readings in male chickens may differ to mice. In mice the levels of Oct-4 protein increase, plateau and remain present. In the chicken, it has been shown that *cPouV* mRNA increase, but the difference between 9 days of incubation and 17 days of incubation is insignificant. Therefore, it cannot be concluded that *cPouV* mRNA remains. In order to determine whether *cPouv* is still expressed, a baseline from a younger embryo could be used because fewer germ cells will be present and therefore it should be expected that there will be a lower level of *cPouV*.

The findings from *nanog in situ* hybridisation experiments showed that in males, *nanog* mRNA was detected in gonads at 9.5, 11.5 and faintly at 13.5 days of incubation, indicating a possible down-regulation of *nanog* at the latter stage of development. This coincides with the onset of PGC multiplication followed by the start of mitotic arrest. The *in situ* hybridisation results do not agree with the qPCR results, which indicate that *nanog* mRNA was present at high levels at 15.5 days of incubation and that *nanog* levels drop after this stage. A possible explanation for this difference is that the *in situ* hybridisation probe could not penetrate the tissue effectively.

In females, *nanog* mRNA was detected in the cortex of the ovaries at 9.5 days of incubation. After this stage, *nanog* mRNA was detected in cells in the medulla of the ovary, but not the cortex. It is known that the germ cells are located in the cortex, which suggests that either the *in situ* hybridisation probe cannot effectively penetrate the cortex tissue after 9.5 days of incubation.

The qPCR analysis for *nanog* shows the same increases and decreases as those observed for *cPouV*, coinciding with the onset of meiosis in females and the start of the mitotic block in males (Van Krey, 1990). Of particular note is the dramatic increase in the levels of *nanog* at the onset of meiosis. At 15 days of incubation the levels of *nanog* increase to six and a half times the levels detected at 9 days of incubation. Observations by Smith (1916) and Goldsmith (1928) indicate that meiosis starts between 13.5 and 15.5 days of incubation. This could suggest a role for Nanog in triggering the start of meiosis in chickens.

CHAPTER 9

BEHAVIOUR OF GFP POSITIVE MALE AND FEMALE CHICKEN PRIMORDIAL GERM CELLS INJECTED INTO THE BLOODSTREAM OF WILDTYPE HOSTS

9.1 Introduction

In avian embryos, primordial germ cells (PGCs) display a unique migration pathway during early development (Kuwana *et al.*, 1993). In chicken, from the time that the blood vessels form at stage 10 (H and H) PGCs located in the germinal crescent enter the circulatory system and by stages 20-24 (H and H) they have migrated to the gonadal primordia (Swift, 1941; Fujimoto *et al.*, 1976).

PGCs have been transferred from turkeys to chickens, chickens to quails and quails to chickens (Reynaud, 1976; Nakamura *et al.*, 1992; Yasuda *et al.*, 1992). In these experiments, blood samples were taken from donor embryos at a stage of development when PGCs were migrating and transferred into the blood stream of host embryos at the same stage of development. The transferred PGCs migrated into the gonadal tissues of the host animal. Furthermore, both male and female PGCs migrated to the gonads, regardless of the sex of the host (Ono *et al.*, 1996; Tagami *et al.*, 2006).

9.1.1 Injecting PGCs into the bloodstream of embryos at different stages of development

Nakamura *et al.* (1991) carried out a series of experiments in which chicken PGCs were removed as part of a blood sample from host embryos at stages 13-14 (H and H) and were injected into host quail embryos ranging from stage 15-20. Quail PGCs are normally found migrating in the blood system at stages 15-16. At this time of development, the majority (90.6%) of the injected chicken PGCs were detected in the gonads of the quail host. When PGCs were injected into host quails at stage 17, 84.7% were observed in the gonads; at stage 18, 41.4% were observed in the gonads; at stage 19, 15.9% were observed in the gonads; and at stage 20, 6.2% were observed in the gonads.

In this chapter, the purpose of the injection experiments carried out here had three aims: First, to determine whether there are any cells in the embryo that can recognise the migratory signals prior to PGCs forming; second, to determine when PGCs are first able to respond to migratory signals and; third, to identify when changes in post-migratory PGCs occur.

9.2 Overview of methods

This is a brief overview of the methods used in this chapter. For a full account see chapter 2.

9.2.1 Production of GFP embryos

To carry out these experiments, embryos that ubiquitously express GFP were used. The GFP expressing birds were developed using lentiviruses to infect cells in the blastodisc of freshly laid eggs. Of the infected cells, some went onto develop into germ cells. When the first generation of birds was bred, a small proportion of the offspring expressed GFP ubiquitously in all tissues. The male birds of this generation were reared to a breeding age and used to produce a ready supply of GFP positive embryos.

9.2.2 Single cell suspension sources

Single cell suspensions were created from: the centre of the blastodisc of freshly laid eggs; the germinal crescent from embryos at one day of incubation; gonads taken from embryos at five days of incubation; gonads taken from male embryos at 9.5 days of incubation; gonads taken from female embryos at 9.5 days of incubation; and gonads taken from male embryos at 11.5 days of incubation.

9.2.3 Injection of single cell suspension

Host embryos were incubated for two and a half days at 38°C until they reached between stage 15 and 16. 1-2µl of prepared single cell suspension was injected into the vascular system of the host embryos via the heart or circumferal vein using a glass capillary needle with an aperture of 35µm. Post-injection, host embryos were incubated at 38°C for two days.

9.3 Results

Donor cells from the centre of the blastodisc of freshly laid eggs were able to migrate to the gonads. However, instead of the salt and pepper pattern expected, a large clump of cells was seen next to the gonads in three embryos and in the gut in one embryo (see Figure 9.1A-C). This suggests that there are cells in the centre of the early blastodisc that are capable of responding to the migratory signals that direct PGCs to the gonads. However, the cells are unable to populate the gonads and in one instance the cells were unable to successfully migrate through the splanchnic mesoderm, which will go onto develop into the gut.

Donor cells from the germinal crescent of embryos at 1 day of incubation were able to migrate to the gonads, populate them and were seen in the expected salt and pepper pattern (see Figure 9.1D-F). This result indicated that the green cells were PGCs, but too confirm this assumption the embryos were sectioned and a double antibody stain to detect GFP and SSEA-1 positive cells was carried out (see Figures 9.1J and K). This determined that the GFP positive cells arriving at the gonads were also SSEA-1 positive confirming their identity as PGCs. However, some cells in the mesonephros that were GFP positive were not SSEA-1 positive, suggesting that they were somatic cells.

Donor cells from embryonic gonads at 5 days of incubation were able to re-migrate and populate the gonads (see Figure 9.1G-I). The GFP positive cells in the gonads appeared in a salt and pepper pattern, indicating that the cells are PGCs.

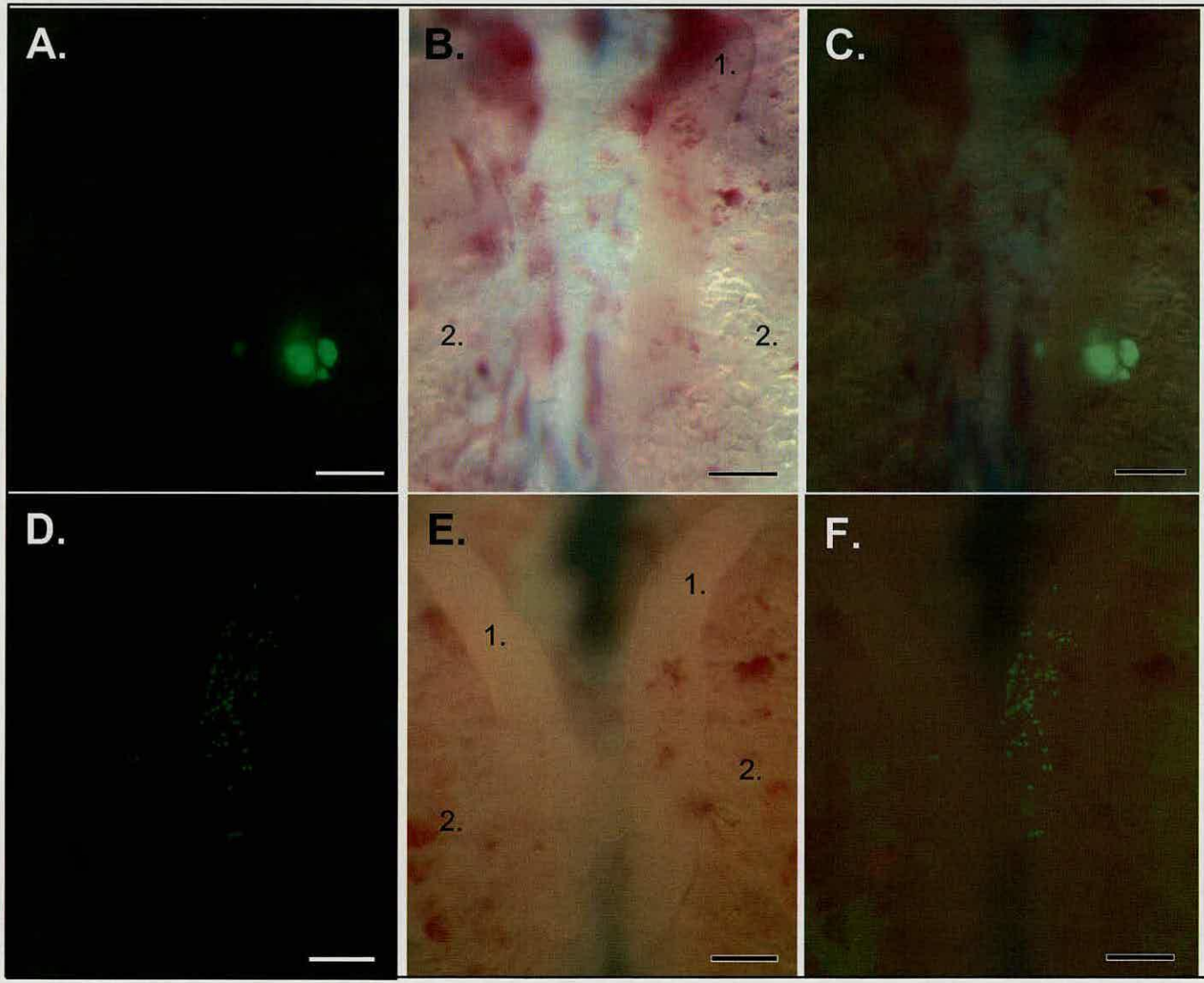


Figure 9.1: Cardiac injection of PGCs taken from ubiquitous GFP embyos at different stages. (A-C) Stage X blastodisc donor. (A) UV (B) brightfield (C) combined. (D-F) 1 day of incubation germinal crescent donor. (D) UV (E) brightfield (F) combined. (G-I) 5 days of incubation gonad donor. (G) UV (H) brightfield (I) combined. (J and K) Co-staining for SSEA-1 and GFP of 20µ frozen sections from an injected embryo. Arrows indicate cells staining positively for both proteins. Scale bars represent 250µm. 1. Gonads; 2. Mesonephros; 3. Gut. Continued on next page.

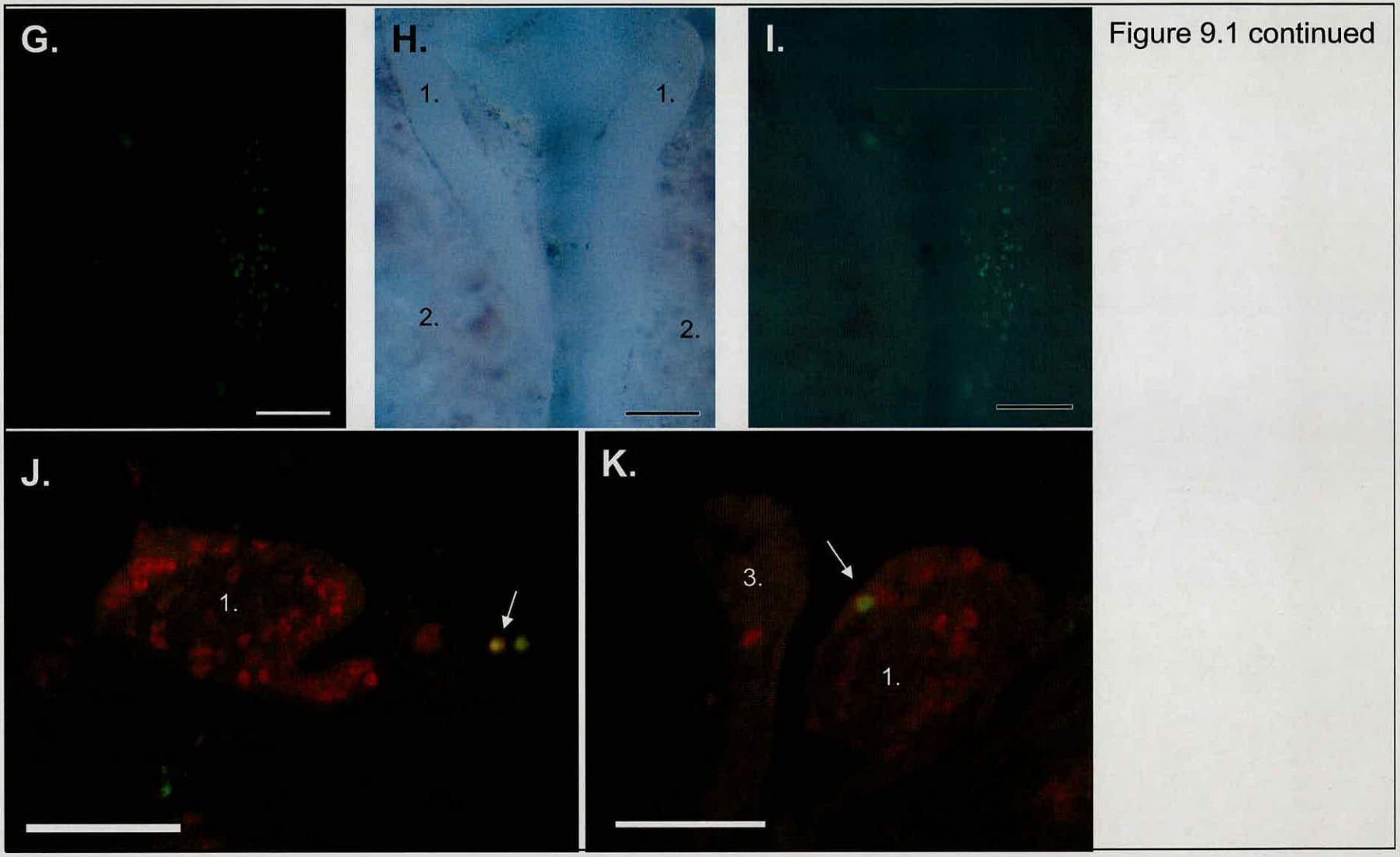


Figure 9.1 continued

The final two stages injected, 9.5 and 11.5 days of incubation, were injected as male donor cells and female donor cells. Donor cells from females at 9.5 days of incubation did not migrate back to the gonads in ten different embryos, indicating that they are no longer able to migrate (see Figures 9.2A-C). Donor cells from males at 9.5 days of incubation were able to re-migrate and populate the gonads (see Figures 9.2D-F). Donor cells from male embryonic gonads at 11.5 days of incubation were no longer able to migrate back to the gonads in ten different embryos, indicating that they are no longer able to migrate (see Figures 9.2G-I).

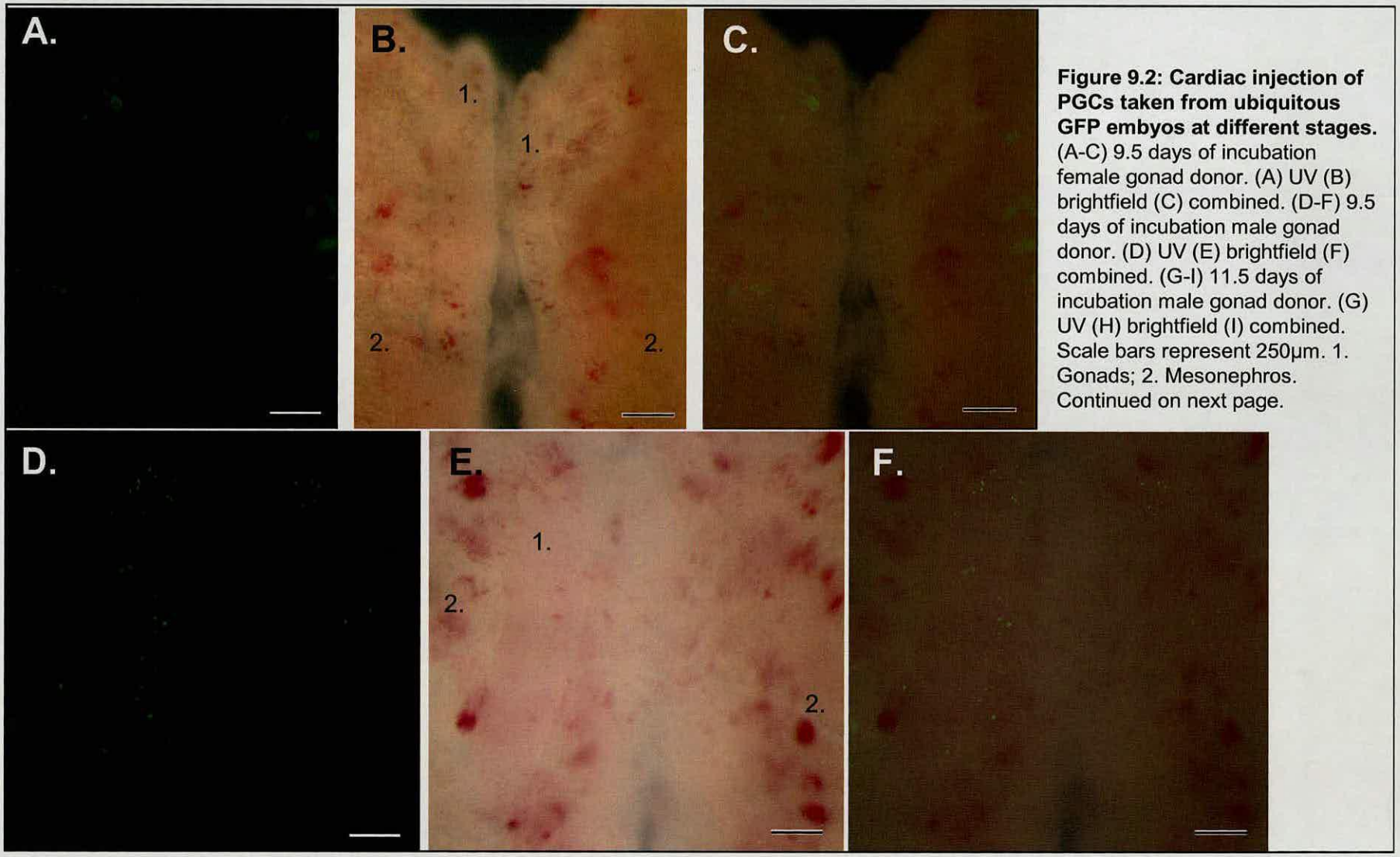
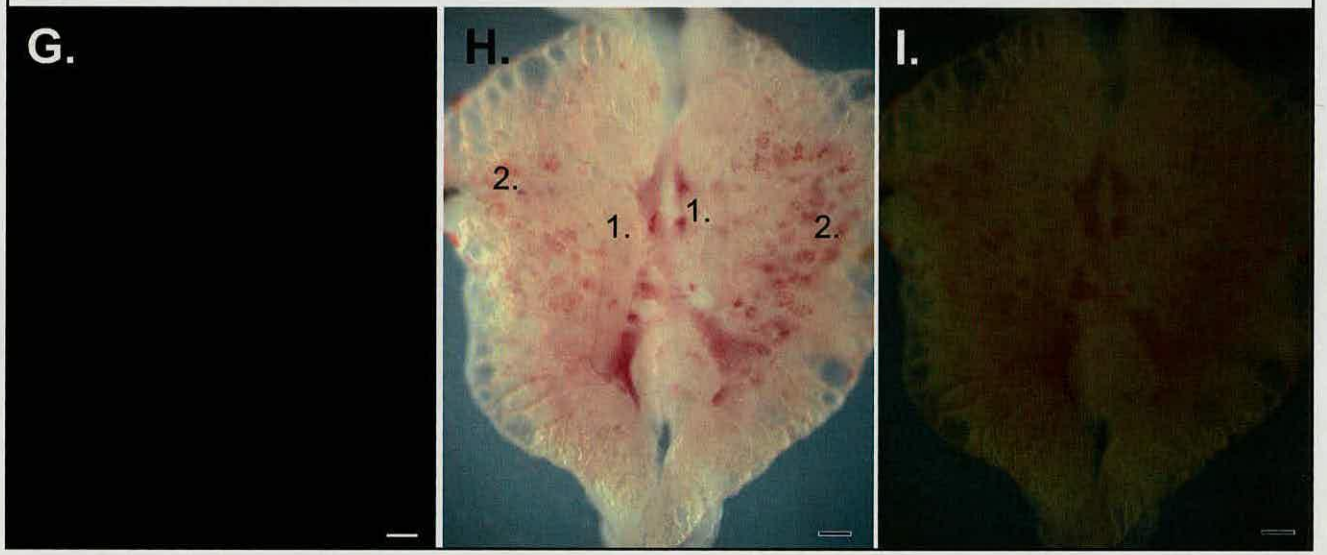


Figure 9.2: Cardiac injection of PGCs taken from ubiquitous GFP embyos at different stages. (A-C) 9.5 days of incubation female gonad donor. (A) UV (B) brightfield (C) combined. (D-F) 9.5 days of incubation male gonad donor. (D) UV (E) brightfield (F) combined. (G-I) 11.5 days of incubation male gonad donor. (G) UV (H) brightfield (I) combined. Scale bars represent 250µm. 1. Gonads; 2. Mesonephros. Continued on next page.

Figure 9.2 continued



9.4 Discussion

These experiments have demonstrated that there are cells in the embryo that can respond to signals directing them to populate and colonise the gonads. This research has identified cells from stages of development ranging from freshly laid eggs until 9.5 days of incubation that are capable of responding to the migratory signals. At all of these stages except the freshly laid egg, the donor cells were seen in a salt and pepper pattern in and around the gonads, suggesting that the cells were PGCs. SSEA-1 staining has demonstrated that the cells that migrate to the gonads are SSEA-1 positive, indicating that they are PGCs (see Figures 9.1J and K). However, there are some green cells that do not stain positively for SSEA-1. This suggests that not all of the cells migrating towards the gonads are PGCs. The cells taken from the centre of the blastodisc of freshly laid eggs clumped in a sphere close to the gonad, suggesting that there are cells that are capable of responding to the migratory signals, but they are not fully able to act like PGCs and colonise the gonads.

These experiments show that cells from female donors lose the ability to migrate to the gonads a few days earlier than males. This staggering is similar to the difference in the onset of germ cell proliferation in the gonads and indicates that the step is probably a regulated step in PGC development. The timing of the loss in migratory ability occurs several days before the PGCs differentiate, indicating that the cells are being reprogrammed several days prior to differentiation. This suggests that that the PGCs have changed sufficiently for them to no longer be recognised as PGCs.

CHAPTER 10

DISCUSSION

10.1 The identification of eight new chicken genes

For a considerable time it has been recognised that chicken germ cells develop via pre-formation. This assumption was based upon work carried out in other species, but the main evidence to support the assumption in chicken has only come about relatively recently showing the localisation of the germline specific protein Vasa in the oocyte. To date, no other proteins or mRNAs have been shown in avian oocytes in a pattern that would suggest expression in germ plasm. To definitively show that chicken germ cells develop via pre-formation, maternally supplied proteins and mRNAs that contribute to the germline need to be shown in the oocyte.

One of the aims of this research was to identify genes in the chicken that are known to have a function in the specification of germ cells in other species. Therefore, the chicken genome was used to try and positively identify homologues of the seven genes *bruno*, *germ cell less*, *mago nashi*, *nanos*, *piwi*, *pumilio*, and *staufen*. After successful identification, the expression of the mRNAs was to be determined at various stages of embryonic development and in several adult tissues and these results were compared to the results from other species. It was decided to look at mRNA expression of each gene in embryos starting at 48 hours post fertilisation because the stages of development are easy to obtain and the embryo is developing *in vitro*. Although many of the genes investigated do not have a known function in

germ cell development beyond specification, it was decided to investigate their expression at these stages before considering whether to look at expression in the oocyte and early cleavage stages. This is because an avian embryo goes through approximately 24 hours of development inside the mother hen and therefore, in order to obtain oocytes and early cleavage embryos adult hens have to killed and the resources were not available for doing this work.

10.1.1 Chicken *bruno*

The third chapter of this thesis presents the results for a chicken homologue of *bruno*. Initially, one *bruno* gene was identified in the chicken genome, but subsequent BLAST searches identified a further five *bruno* family members in the chicken. Of the six chicken *bruno* genes identified in the genome, the *bruno* gene with the highest similarity to the zebrafish *bruno* homologue known to be involved in germ cell development (Suzuki *et al.*, 2000) was investigated further.

The results of the RT-PCR analysis showed that chicken *bruno* mRNA is expressed in all stages of embryonic development and in all adult tissues, while the *in situ* hybridisation analyses showed mesodermal expression of *bruno* mRNA at five days of incubation. Earlier in development, at three days of incubation *bruno* mRNA is detected in the eye and somites and at one day of incubation *bruno* mRNA is detected surrounding the primitive streak. This expression pattern is similar to that reported in zebrafish (Suzuki *et al.*, 2000; Hashimoto *et al.*, 2006). Therefore, the next step would be to try and determine whether *bruno* is involved in germ cell specification. Initial experiments should look at the expression of both *bruno* mRNA

and Bruno protein in the oocyte to see if it is similar to the expression of CVH protein.

10.1.2 Chicken *germ cell less*

The fourth chapter of this thesis presents the results for a chicken *germ cell less* homologue. The BLAST searches identified a partial *germ cell less* sequence that shows strong sequence similarity to the 3' end of germ-cell less proteins from other species. However, the partial sequence did not include the conserved BTB/POZ domain. Furthermore, no additional sequence could be identified 3' of the identified sequence and the sequence is still to be located to a chromosome, suggesting that this could be an incomplete gene fragment. Additional BLAST searches at later dates failed to identify a more complete sequence.

The partial sequence was used to generate an expression profile for *germ cell less*, which showed that the mRNA is testes specific. This expression pattern was determined via RT-PCR alone because none of the other analyses carried out were able to detect the sequence and therefore, very few conclusions can be drawn from these results because the data from the different methods do not corroborate with each other. The inconsistencies in the results could have been due to a technical error during *in situ* and Northern probe synthesis, or it could be that the mRNA is very rare. What can be said is that this expression profile is different from *germ-cell less* genes in other species.

In order to carry this research forward, the rest of the gene sequence containing a BTB/POZ domain needs to be identified. EST libraries were searched for additional *germ cell less* sequence, but with no success. Therefore, degenerate primers could be used experimentally to try and identify more of the sequence. Once a more complete sequence has been identified, the expression analysis experiments should be repeated to confirm the data from the RT-PCR experiments. Following this, the presence or absence of the gene in the oocyte can be investigated.

10.1.3 Chicken *mago nashi* and *staufen*

The fifth chapter in this thesis presents the results for the chicken homologues of *mago nashi* and *staufen*. The BLAST searches of the chicken genome identified a single *mago nashi* homologue and the results of the RT-PCR analysis showed that *mago nashi* mRNA is expressed at all four of the embryonic time points and in all adult tissues that were investigated. It was shown by *in situ* hybridisation that the pattern of expression during embryonic development was widespread in the three time points looked at. This pattern is comparable to that observed in zebrafish.

For *staufen*, the BLAST searches identified two *staufen* homologues in the chicken genome, one related to vertebrate Staufen-1 proteins and the other related to vertebrate Staufen-2 proteins. The results of the expression analysis showed that both genes are expressed in multiple adult tissues and have a widespread expression pattern in the embryonic time points looked at.

The expression patterns of *mago nashi*, *staufen-1* and *staufen-2* do not confirm that these genes have a role in germline development at the stages of development investigated, nor do the results rule one out. However, a role was not expected at these time pointes. The reason for looking at *mago nashi* and *staufen* was to try and identify further germ plasm components. The results of the expression analysis show that the three genes have comparable expression patterns to known homologues in other species. Therefore, it would be advantageous to look at the expression of *mago nashi* and *staufen-1/-2* mRNA and protein in the oocyte and early cleavage stages to determine whether the genes are germ plasm components.

10.1.4 Chicken *puilio* and *nanos*

The sixth chapter of this thesis presents the results for the chicken homologues of *pumilio* and *nanos*. The BLAST searches of the chicken genome identified a two *pumilio* homologues. The results of the PCR and *in situ* hybridisation analyses show that both *pumilio-1* and *pumilio-2* are expressed in a widespread pattern. *Pumilio* is known to function during PGC migration, however, at three days of incubation neither *pumilio* gene was detected in a pattern that would suggest expression in PGCs. This would indicate that neither gene has a role in PGC migration, but to confirm this protein expression would have to be investigated, followed by functional analyses in relation to PGC migration.

The BLAST searches of the chicken genome were unable to identify any *nanos* homologues. BLAST searches of EST libraries identified several chicken ESTs in EST libraries that have been called *nanos-1*, but none contain any sequence relating

to the conserved *nanos* zinc-finger domain. These results were unexpected because *pumilio* and *nanos* interact with each other in several different developmental processes in multiple species. Therefore, the idea that *pumilio* genes are present in the chicken and *nanos* genes are not seems unlikely. Rather than wait for the chicken genome to be completed in order to identify potential *nanos* homologues, a different method needs to be employed. One option would be to use degenerate primers designed to identify *nanos* zinc finger sequences. Presuming this works, expression and functional analyses can then be carried out.

10.1.5 Chicken *piwi*

The seventh chapter of this thesis presents data for a chicken *piwi* homologue. The BLAST searches of the chicken genome identified a single *piwi* homologue. The results of the RT-PCR and Northern analyses showed that the chicken homologue of *piwi*, named *chiwi*, is not expressed during the stages of embryonic development investigated, but it is expressed in adult kidney and testes. An *in-situ* hybridisation analysis carried out on frozen adult testes sections determined that *chiwi* is expressed in cells close to the basement membrane of the seminiferous tubules. The location of the cells staining positively for *chiwi* mRNA relate to the location of spermatogonium and primary spermatocytes. This suggests that *chiwi* has a conserved function during spermatogenesis in chickens.

Based upon results in mice and zebrafish, the inability to detect *chiwi* in embryonic tissues was an unexpected result and indicates that the gene does not function in germ cell development during these stages of development. However, the result does

not rule out a possible function in germ cell specification. Assuming that chicken PGCs develop via preformation, chiwi could well function in the assembly of germ plasm. As with the genes previously described, in order to investigate this possibility expression of the mRNA and the protein should be looked at in the oocyte and early cleavage embryos.

10.1.6 Functional analyses

In order to confirm whether a particular gene has a function in germ cell development experiments should be carried out that show a requirement for the gene in germ cell development. The first experiment to attempt would be to knockdown the gene, with the ultimate aim of generating a sterile chicken. Although this would not tell us very much about what the gene actually does, it would show a requirement for germ cell development or survival. Of course, the initial mutagenesis might not generate a reduction in germ cell phenotype if germ cell development only requires maternally supplied mRNAs or proteins. If this is the case then effects will not be seen until the second generation. This leads onto another problem. A lot of the genes identified here have a widespread expression pattern and therefore, knockdowns could well prove to be embryonic lethal. Therefore, partial knockdowns would be the best way to proceed.

10.2 Oct 4 and nanog

The eight results chapter of this thesis presents results for chicken homologues of *nanog* and *oct*-4. Of all of the genes looked at in this project, the two of most

interest in terms of PGC development were *cPouV* and *nanog*. Results presented here showed that both of these genes are expressed in the PGCs from an early stage of development. During these early stages of development, of particular interest was the fact that *nanog* was detected in the PGCs before *cPouV*. This led to the question: what is promoting *nanog* expression? Known promoters of *nanog* in mouse stem cell work include Oct-4, Sox-2 and p53. This research has shown that the mRNA of the chicken homologue of *oct-4*, *cPouV*, is not detected in the PGCs until later in development. The expression of *sox-2* mRNA at this time in development mirrors *cPouV* expression (Rex *et al.* 1997), indicating that it is not this gene that is promoting *nanog* expression in the PGCs. The next factor to look at with regards *nanog* expression would be p53, but this is still to be identified in the chicken.

The levels of *nanog* and *cPouV* were investigated in late development via qPCR. Although most of the results obtained from this analysis were not significant because the increase/decrease compared to the baseline was below a factor of two, a general trend was observed for both genes, which mirrors expression in mice. Overall, the levels of both genes initially increase before dropping off later in development. This is likely to be because of the increase in the numbers of germ cells, but the numbers of supporting cells will also have increased. So is the increase in the levels of each gene caused solely by an increase in the number of germ cells or do the levels in each cell also increase? Certainly with *nanog* in females, it would appear to be the latter. This is because the seven fold increase in *nanog* levels at 15 days of incubation are not paralleled by *cPouV* which has only increased by a factor of two. If the increase in the levels were to be solely explained by an increase in the number of germ cells

then it would be expected that the levels of *cPouV* and *nanog* would be similar. Therefore, the dramatic increase in *nanog* levels at 15 days of incubation indicates that the levels of *nanog* in the germ cells increases.

The timing of the increase in *nanog* levels described above corresponds to the time at which meiosis is initiated in females, leading to the hypothesis that an increase in the levels of *nanog* triggers the onset of meiosis. Two experiments could be carried out to investigate this hypothesis: first, ovaries taken from various time points at around 15 days of incubation should be taken and each sample split into two. One half of each sample should be sectioned and used to detect when meiosis starts in females. The second half should be used in a qPCR analysis to accurately determine when the onset of meiosis occurs in relation to the peak in *nanog* levels. Second, an experiment could be carried out to determine if meiosis would be initiated early if the levels of *nanog* were artificially increased at an early time point?

10.3 Investigating PGC migration

The ninth results chapter of this thesis presents the results of a series of injection experiments designed to look at PGC migration. In avian species, the PGCs initially migrate via the embryonic blood system. This step does not require any known cellular interactions, unlike in systems such as mice and *Drosophila*, making avian species ideal for investigating PGC migration. The purpose of the injection experiments had three aims: First, to determine whether there are any cells in the embryo that can recognise the migratory signals prior to PGCs forming; second, to

determine when PGCs are first able to respond to migratory signals and; third, to identify when changes in post-migratory PGCs occur.

The results of the injections using cells taken from the centre of the stage X blastodisc showed cells migrating from the embryonic blood system to the vicinity of the gonads. This indicates that there are cells in the blastodisc that can respond to the PGC migratory signals. To try and prove this, SDF-1α can be ectopically expressed (Stebler *et al.*, 2004) and the cells from the centre of the blastodisc can then be injected as normal. If injected cells are found at the ectopic source of SDF1α, it would indicate that there are cells capable of not only recognising but also following the migratory signals that direct PGC migration.

The injection of cells taken from the germinal crescent at one day of incubation showed that PGCs are capable of successfully migrating to the gonads shortly after the cells have formed. The next thing investigated aimed to determine when PGCs lose the ability to migrate. The results showed that in females, PGCs are no longer able to respond to the migratory signals by 9.5 days of incubation. In males the PGCs are no longer able to respond to the signals by 11.5 days of incubation. This means that the PGCs have changed so it appears that they can no longer respond to the migratory cues. The most likely reason for this would be because they no longer have functional receptors that detect the ligand SDF-1. The SDF-1 receptors are known as CXCR-4, and a PCR to detect these receptors using cDNA from ovaries and testes would determine whether the receptors are lost at the time points when male and female PGCs lose the ability to migrate.

The injection method could be useful in future experiments for introducing modified PGCs into a host system. The recent development of a chicken PGC culture system by van de Lavoir (2006) gives a ready supply of PGCs that could potentially be manipulated. Following the manipulation the introduction of them into a living embryo could prove to be a useful tool to investigate genes involved in PGC migration and subsequent germ cell development. The injection experiments carried out here have highlighted a potential problem with this idea because the PGCs do eventually change and are no longer able to respond to migratory cues. Identifying what changes in the PGCs will be important in determining whether the method will work, especially when investigating genes involved in PGC migration. Additionally, it would be worth exploring reducing the endogenous PGC population. This could make changes in the manipulated PGCs more noticeable, but it would be particuarly impotant if the aim is to generate offspring from the manipulated PGCs. Methods to reduce the endogenous PGC population were investigated a little during this research (results not shown). The first method involved γ irradiating embryos at one day of incubation. This method did reduce the endogenous PGC population, but when trying to take the embryos to hatch the fatality rate was 100%. Therefore, a compromise between the dose of radiation and number of PGCs destroyed needed to be investigated. The second method involved the injection of busulphan developed by Song *et al.* (2005). Following their method the embryos generated did not develop properly because the mutagen remained in the egg yolk, but the endogenous PGC population was reduced. However, of the two methods investigated the

irradiation method results in a uniform dose to all of the embryos and generally is a more user friendly procedure.

10.4 Epigenesis or preformation in the chicken

Manipulating embryos when they are only a few cells big would be an alternative method to determine whether germline specific factors are found in the early embryo. The first manipulation method that could be employed involves taking an early cleavage, GFP ubiquitous embryo and transplanting each cell into a wildtype embryo at the same stage of development. If all of the GFP positive cells contribute to the germline it would indicate that there has been no segregation of germline determinants by this stage of development. This experiment can be carried out for the first few cellular divisions to try and determine the timing of segregation of germline determinants and the loss of germline potential in other cells. If germ cells develop via an epigenetic mechanism in the chicken then all of the early cells should be able to contribute to the germline in transplant experiments. This experiment would help to understand the timing of events and help focus the efforts of the next two methods. A second method that could be used would be to ablate a single cell at the eight cell stage and look later in development to see if there is a reduction in the number of germ cells. This method has been used most successfully in the arthropod *Parhyale hawaiensis* to investigate cell lineages (Gerberding *et al.*, 2002). A third method that could potentially be used would be to try and remove or at least disrupt the cytoplasm from the base of the first cell cleavage. This is the region of the embryo that CVH protein is known to localise to (Tsunekawa *et al.*, 2000). Removal

of all, or part of the cytoplasm should have an effect on the numbers of PGCs that develop late in development and this would demonstrate that there are factors in the early embryo that contribute to the germline.

An alternative mechanism to preformation is epigenesis. As discussed in section 1.1.2, the characteristic of epigenesis is the lack of germ plasm in the oocyte. Germ cells are induced from germ cell competent cells. In mice it has been shown that some of the key factors involved in inducing the germline are bone morphogenetic proteins (BMPs). To investigate whether chicken germ cells could develop via epigenesis the expression of three BMPs, BMP2, BMP4 and BMP8b, could be investigated. Chicken PGCs are first distinguishable from the surrounding somatic cells at approximately stage 3-4 (H and H), so the expression of the three BMPs could be investigated at this stage in development. If BMPs function to specify development of chicken PGCs in a similar manner to that observed in mice, then the expression pattern of BMP2, BMP4 and BMP8b would be similar to Figure 10.1A. If the patterns of the BMPs were found in this pattern, the next step would be to prove that there are germ cell competent cells elsewhere in the embryo and that a combination of the three BMP signals causes the induction of ectopic germ cells. This could be achieved by introducing beads covered in BMP2 protein at the posterior of an embryo at stage 3-4 (H and H) and then looking for ectopic germ cells in this location of the embryo (see Figure 10.1B).

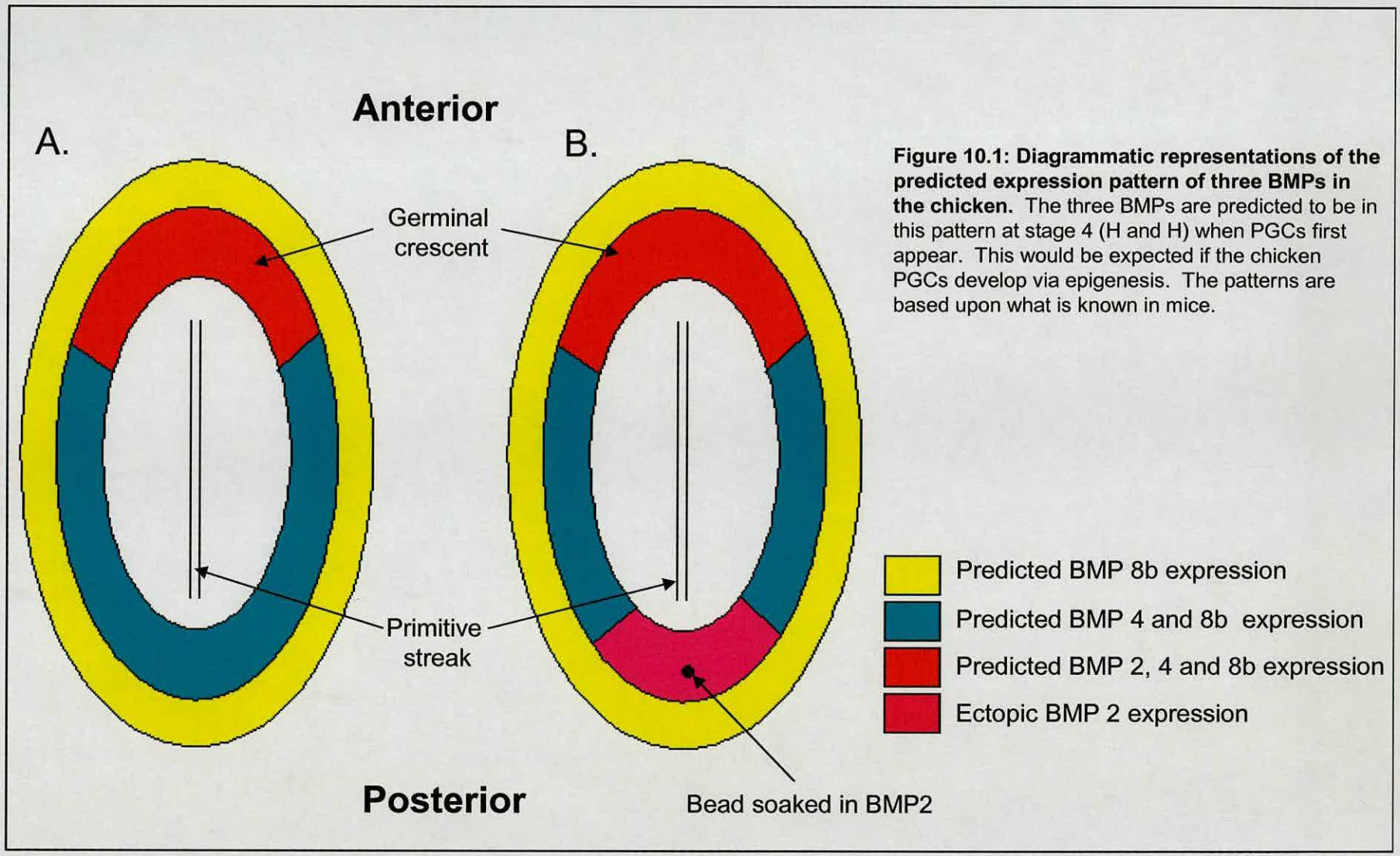


Figure 10.1: Diagrammatic representations of the predicted expression pattern of three BMPs in the chicken. The three BMPs are predicted to be in this pattern at stage 4 (H and H) when PGCs first appear. This would be expected if the chicken PGCs develop via epigenesis. The patterns are based upon what is known in mice.

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Appendix 1

RT-PCR primer sequences

Primer name	Primer sequence (5'-3')	Product size
18S rDNA forward	AGC TCT TTC TCG ATT CCG TG	
18S rDNA reverse	GGG TAG ACA CAA GCT GAG CC	1850bp
β-actin left	ATG GCT ACA TAC ATG GCT GG	
β-actin right	GGA TGA TGA TAT TGC TGC CG	428bp
Bruno left	CAG ACA CGC AGA AGG ACA AA	
Bruno right	ACG TGT TCA GGT TCC CAG AC	188bp
GCL left	GTT TGC TGA GCG AAG GAG AG	
GCL right	CAG ATG CCA GGT CAC TGA TG	42bp
Mago nashi left	ACA AGA GCGTGA TGG AGG AG	
Mago nashi right	GAC CCA ATT TTT GAC GTG GT	159bp
Nanog left	AGC TGC ACG TCG AAG TTT TT	
Nanog right	TTC CTT GTC CCA CTC TCA CC	180bp
cPouV left	TCC CTT TGC ATC CAC ACT ATC	
cPouV right	GCT TCA CCA CCA TTT TTG AGA	279bp
Piwi left	TGA AAC CAG ATC ATG TAC AAC G	
Piwi right	TCT GAG AGC AAC AGG TTT GG	160bp
Pumilio 1 left	TTG ATG GTG GAT GTA TTT GGA A	
Pumilio 1 right	AAC TCA AGG GCC TCC TGG AT	158bp
Pumilio 2 left	GCT CTG CAC TTG GTG GAT TT	
Pumilio 2 right	ATG TCC TGG AGT TTG GCT TG	209bp
Staufen 1 left	GAT ACA AGC AGC CAA GAA GG	
Staufen 1 right	AGG CTG AGG TTG AGG GAT TT	205bp
Staufen 2 left	ACA GTG GAG CTG AAC GGT CT	
Staufen 2 right	GGC CTT CAT TGC AGC ATT AT	235bp

Quantitative PCR primer sequences

Primer name	Primer sequence
LBR forward	GGT GTG GGT TCC ATT TGT CTA CA
LBR reverse	CTG CAA CCG GCC AAG AAA
Nanog 5'	TTG GAA AAG GTG GAA CAA GC
Nanog 3'	GGT GCT CTG GAA GCT GTA GG
cPouV 5'	TCA ATG AGG CAG AGA ACA CG
cPouV 3'	TCA CAC ATT TGC GGA AGA AG

Appendix 2

Chicken CUGbp-1 full length sequence. Exons are marked in different colours.

```
  1 ACCAGCTGATAGTGAAAAGAATAATGCAGTAGAAGATAGGAAGTTGTTTATTGGAATGAT
 61 ATCAAAGAAGTGCAATGAAAATGATATCCGAGTGATGTTCTCACCCTTTGGGCAGATTGA
121 AGAATGCAGGATATTACGGGGCCCAGATGGGCTGAGCCGAGGTTGTGCATTTGTGACTTT
181 TACAACAAGAGCCATGGCGCAAACAGCAATCAAAGCAATGCACCAAGCACAAACCATGGA
241 GGGTTGCTCTTCTCCCATTGTGGTAAAATTTGCAGACACGCAGAAGGACAAAGAGCAGAA
301 ACGAATTGCTCAGCAACTCCAGCAACAAATGCAACAGATCAGTGCTGCCTCTGTATGGGG
361 AAACCTGGCTGGTCTCAACACGCTTGGACCGCAATACTTAGCACTTTATTTGCAGCTCCT
421 TCAGCAAACAGCAGCAGCCTCGTCTGGGAACCTGAACACGTTGAGCAGCCTCCACCCAAT
481 GGGAGGACTGAATGCGATGCAGTTACAGAACCTAGCTGCGTTAGCAGCTGCAGCCAGTGC
541 AGCTCAGAATACACCAAGTGGCACCGCTGCACTCACCTCCTCCAGCAGTCCCCTCAGTGT
601 GCTCACCAGTTCAGCAGGTTCCTCACCTAGCTCCAGTAGCAGCTCTTCTGTTAATCCCAT
661 GGCTTCTCTTGGAGCATTGCAGACACTGGCAGGGGCTACAGCAGGCCTGAATGTTAGCTC
721 TCTAGCAGGCATGGCAGCTTTAAATGGAGGACTTGGCAGTGGTGGTCTTTCAAATGGGAC
781 AGGTAGCACAATGGAAGCTCTCACGCAGGCTTATTCTGGAATCCAGCAATACGCTGCTGC
841 TGCATTGCCCACACTCTATAACCAGAGTCTCTTAACACAGCAGAGTATTGGTGCAGCAGG
901 AAGTCAAAAAGAAGGTCCAGAGGGAGCCAATCTGTTTATCTACCATCTCCCCCAGGAGTT
961 TGGGGATCAAGATCTGCTGCAGATG
```

EST ID numbers identified in the UMIST database that correspond to chicken *CUGbp-1*.

ChEST696m9	ChEST398h10	ChEST233k22
ChEST559o11	ChEST324h19	ChEST322c1
ChEST761n24	ChEST184c17	ChEST900j12
ChEST571o17	ChEST490b23	ChEST772i4
ChEST999c11	ChEST599b2	ChEST549j11'

EST sequence used to make radiolabelled probe for Northern analysis

ChEST696m9

CACAGAAGATAGGAAGTTGTGGTATTGGAATGATATCAAAGAAGTGCAA
TGAAAATGATATCCGAGTGATGTTCTCACCCTTTGGGCAGATTGAAGAAT
GCAGGATATTACGGGGCCCAGATGGGCTGAGCCGAGGTTGTGCATTTGT
GACTTTTACAACAAGAGCCATGGCGCAAACAGCAATCAAAGCAATGCAC
CAAGCACAAACCATGGAGGGTTGCTCTTCTCCCATTGTGGTAAAATTTGC
AGACACGCAGAAGGACAAAGAGCAGAAACGAATTGCTCAGCAACTCCA
GCAACAAATGCAACAGATCAGTGCTGCCTCTATATGGGGAAACCTGGCT
GGTCTCAACACGCTTGGACCGCAATACTTAGCACTTTATTTGCAGCTCCTT
CAGCAAACAGCAGCAGCCTCGTCTGGGAACCTGAACACGTTGAGCAGCC
TCCACCCAATGGGAGTCTGTGTATATAGTCGTTGATCTCATGGTTAGGAG
TTGCCACTTAACCTTGTCTCACAGTTGACTGCTGCTTCTTGGCTGCCAAGG
AGGCCAGACAAAGCAAGTTGATGTTCAGCTGATGACAGGTTAAAGGACT
GAATGCGATGCAGTTACAGAACCTAGCTGCGTTAGCAGCTGCAGCCAGT
GCAGCTCAGAATACACCAAGTGGCACCGCTGCACTCACCTCCTCCAGCAG
TCCCCTCAGTGTGCTCCACCAGTTCAGGTTCCTCACCTAGCTCCAGTAGCC
AGCTCTTCTGTTAATCCCATGGCTTCTCTTGGAGCATTGCAGAACACTG

Appendix 3

Germ cell-less identified sequence.

AGCTGGAGGGCGATGCTGCATTCCTGGACATGGAGCAGGGCGCTGCCTT
CCGGCCCGTGTTCGCACACCTTCGCCTGCAGTACATCATCAGTGACCTGG
CATCTGCTCGGATCGTGGAGAGGGACGCCCTGATCCCCCCAG

EST ID numbers identified in the UMIST database that correspond to chicken *germ cell-less*.

ChEST701n13	ChEST57b4	ChEST703j13
ChEST293h8	ChEST712c6'	

EST sequence used to make radiolabelled probe for Northern analysis

ChEST703j13

CACATCGAGCTGGAGATCCCCGACCAGAACATCGACATAGAGGGTGAGT
GGAGCCCGCGGCGCTGCCACGGAGGGTCCCATCAGCTCCGCGCTGAGCT
CTGCGCTGCTGTTCCCCTCCCAGCCCTGCAGGTGGCATTCGGCTCTCTGTA
CCGGGATGATGTGCTGATAAAGCCCGGCCGAGTGGTTGCGCTGCTGGCG
GCCGCCTGCATGCTGCAGCTGGACGGTCTGATCCTGCAGTGCGGGGAAA
CGATGAAGGAAACCATCAATGCCAAAACTGTGTGCAGTTATTACAACTC
GGCGGGGACGTACGGGTTGGACTCGGTGAAGAAGAGGTGCCTCGAGTGG
CTGCTGAACAACCTGATGACTCACCAGAGCGTTGAACTCTTCAAAGAGCT
CAGCATAAACCTCATGAAGCAGCTGATCAGCTCTTCTAATCTCTTCGTGA
TGCAAGTGGAGATGGATCTGTACACTGCTCTCAAGAAGTGGATGTTCCTG
CAGCTCGTGCCTTCCTGGAACGGGCCTCTGAAACAGCTCTTAGCACAAGC
TGATGCCTGGTTTGCTGAGCGAAGGAGAGAGCTGGAGGGCGATGCTGCA
TTCCTGGACATGGAGCAGGGCGCTGCCTTCCGGCCCGTGTTCGCACACCT
TCGCCTGCAGTACATCATCAGTGACCTGGCATCTGCTCGGATCGTGGAGA
GGGGACGCCCTGATCCCCCC

Appendix 4

Chicken *mago nashi* full length sequence. Exons are marked in different colours.

```
  1 GGTGCGGCGCGGCGGGACCATGGCGAGCGACTTCTACCTGCGGTACTACGTGGGGCACAA
 61 GGGGAAATTCGGGCACGAGTTCCTGGAGTTCGAGTTCCGGCCCGACGGGAAGCTGCGCTA
121 CGCCAACAACAGCAACTACAAGAACGACGTCATGATCCGCAAAGAGGCCTACGTGCACAA
181 GAGCGTGATGGAGGAGCTGAAGCGGATCATCGACGACAGCGAGATCACCAAAGAGGACGA
241 CGCGCTGTGGCCGCCACCAGACAGGGTGGGCCGCCAGGAGCTTGAAATAGTAATTGGTGA
301 CGAGCACATCTCCTTTACCACGTCAAAAATTGGGTCGCTCATCGATGTAAATCAATCCAA
361 GGATCCAGAAGGTTTGAGAGTGTTCTACTACCTGGTCCAGGACCTTAAATGTCTAGTCTT
421 CAGTCTTATTGGACTACACTTCAAGATTAAGCCAATCTAAATCAAACAAACTGAAGTTTG
481 TACTGCAGTGTCTGTACATGAGGGGGGGGATGCTTTTTCAATTCCTTGCTTCTTCAGACC
541 CGAAACTTTTATGTATGTTAGAATTCTTTTTACAAACTGTAAGTGACTGTCTTAATAAAA
601 TGTTGACATGGGTATTTTTAACTT
```

EST ID numbers identified in the UMIST database that correspond to chicken *mago nashi*.

ChEST20k6	ChEST678c23	ChEST150f8
ChEST504h16	ChEST580h13	ChEST168o5
ChEST269a1	ChEST566d9	ChEST479j23
ChEST710p7	ChEST91h16	ChEST623e8
ChEST167m21	ChEST830k24	ChEST375b3
ChEST454i12	ChEST973c19	ChEST517a5
ChEST446a2	ChEST829p15	ChEST368a10
ChEST342c2	ChEST895j2	ChEST422o5
ChEST448e5	ChEST258o9	ChEST727m18
ChEST506a1	ChEST910i23	ChEST806m18
ChEST797i16	ChEST115k13	ChEST856e11
ChEST794g12	ChEST395e6	ChEST729o16
ChEST814a24	ChEST696f23	ChEST692m17
ChEST273a23	ChEST354k22	ChEST403a5

EST sequence used to make radiolabelled probe for Northern analysis

ChEST710p7

CGACTTCTACCTGCGGTACTACGTGGGGCACAAGGGGAAATTCGGGCAC
GAGTTCCTGGAGTTCGAGTTCCGGCCCGACGGGAAGCTGCGCTACGCCA
ACAACAGCAACTACAAGAACGACGTCATGATCCGCAAAGAGGCCTACGT
GCACAAGAGCGTGATGGAGGAGCTGAAGCGGATCATCGACGACAGCGA
GATCACCAAAGAGGACGACGCGCTGTGGCCGCCACCAGACAGGGTGGGC
CGCCAGGAGCTTGAAATAGTAATTGGTGACGAGCACATCTCCTTTACCAC
GTCAAAAATTGGGTCGCTCATCGATGTAAATCAATCCAAGGATCCAGAA
GGTTTGAGAGTGTTCTACTACCTGGTCCAGGACCTTAAATGTCTAGTCTTC
AGTCTTATTGGACTACACTTCAAGATTAAGCCAATCTAAATCAAACAAAC
TGAAGTTTGTACTGCAGTGTCTGTACATGAGGGGGGGGATGCTTTTTCAA
TTCCTTGCTTCTTCAGACCCGAAACTTTTATGTATGTTAGAATTCTTTTTA
CAAACTGTAAGTGACTGTCTTAATAAAATGTTGACATGGGTATTTCTAAA
AA

Appendix 5

Chicken *staufen 1* full length sequence. Exons are marked in different colours.

```
   1 CAAAACTGTTTACCTACAGTAACTTGCTCTGGTTGGAAGTGAACATGTCTCAAGTTCAAA
  61 TTCAGAATCCTTCTACTGCCCTCGCAGGGAGCCAAATATTGAATAAGAACCCGTCTCTTT
 121 CACAGCCACTGAGTATTCCTTCTACTACCAGTTCTTTGCCATCTGAAAATGCAGGTAGAC
 181 CTATCCAAAATTCTGCTTTACCCTCTGCGTCTGTTACATCTACCAATGCAGCTGCAGCTC
 241 CTTCTAACATGGCAAACCCCAAAGAGAAAACCCCAATGTGTCTTGTGAATGAGTTAGCCC
 301 GTTTCAACAAGATTCAGCCTGAATATAAGCTTCTGAGTGAGCAAGGTCCAGCTCATTCAA
 361 AGGTGTTTACAGTGCAGCTGACTCTTGGGGACCAGCACTGGGAAGCTGAAGGAACTAGTA
 421 TTAAAAAAGCGCAACATGCAGCAGCTGCCAAAGCCTTGGAAGGGACAAAGTTCCCTAAGC
 481 CTACAGCTCGTCCATCTCGTAGTGAAGGCAAGAATCCAGACAGTGTAACCCCCACAGTGG
 541 AGTTGAATGCACTTTGCATGAAGCTGGGAAAGAAACCTATGTATAAACCTATTGATCCTT
 601 ATACAGGGATGAGATCCACTTACAACTATACAATGAGAGGTGGTACTTATCCTCCACGGT
 661 ACTTTTACCCATTTCCTGTTGGGCCTTTACTTTATCAAGTTGAGCTTTCAATTGGGGGGC
 721 AACAGTTTCATGGGAAAGGAAGAACAAGACAAGCTGCTAAGCACGATGCAGCTGCTAAAG
 781 CACTGAAAGTTCTGCAGAATGAGCCCTTGCCTGAGAAACCAGAGGTTAACGGAAAAGAAC
 841 CAGATGATGAAAATCTCAATAAATCTGAAATAAGCCAAGTTTTTGAGATTGCACTTAAAA
 901 GGAACTTGCCTGTGAATTTTGAGGTGACCAAGGAAAGTGGTCCTCCCCATATGAAGAGCT
 961 TTGTAACCAAGGTGTCAGTTGGAGAATTCATGGGTGAAGGTGAAGGAAAGAGCAAGAAGA
1021 TCTCAAAGAAAAATGCTGCAATAGCAGTCTTAGAAGAACTGAAAAAATTGCCACCCCTTC
1081 CTACGGTTGAGAAAATGAAGCCACGAATCAAAAAGAAAACGAAATCAATAGTGAAGCTGC
1141 AGACAAGTCCAGAATATGGTCAAGGCATGAATCCCATTAGCAGACTTGCCCAGATACAGC
1201 AGGCCAAGAAGGAGAAGGAACCAGAGTATATGCTCATCACAGAACGTGGTCTTCCAAGAC
1261 GCAGGGAGTTTGTTATGCAGGTGAAAGTTGGTGTACACACAGCTGAAGGAATGGGCACAA
1321 ACAAAAAGGTTGCTAAACGCAATGCAGCTGAAAACATGTTGGAAATTTTAGGTTTCAAAA
1381 TCCCTCAACCTCAGCCTCCAAAACCAGCATTAAAGACAGAAGAGAAGACACCAGTGAAGA
1441 AACCAGGTGATGGAAGAAAAGTAACTTTCTTTGAGCCGGGCTCTGAAGAGACTTCAACTA
1501 GTAATAAAGAAGATGAGTTTAGGATGCCTTATCTCAGTCATCAGCAGCTTCCTGCTGGAA
1561 TTCTTCCCATGGTCCCTGAGGTTGCACAAGCTGTAGGAGCCAGTCAAGGACACCACACCA
1621 AAGAATTCAATAGGGCAGCCCCAAATCCTGCCAAGGCTACTGTAACAGCAATGATTGCTA
1681 GAGAGCTATTGTATGGTGGTACTTCTCCTACTGCTGAAACCATATTAAAAAATAACAACT
1741 CATCAGGCCATGTGCCCCACGGACCACTTACCAGGCCCTCTGAACAGCTGGACTATCTTT
1801 CCAATGTTCAAGGAATCCAGGTTGAATATAAAGACTTTCCAAAAAATAACAAGAATGAGT
1861 TTGTGTCTCTTATAAACTGTTCCTCTCAGCCACCACTGATCAGCCATGGAATTGGAAAGG
1921 ATGTAGAATCTTGTCACGATATGGCTGCATTGAACATTTTGAAGTTGCTGTCTGAGCTGG
1981 ACCAACAAACCACAGAGATGCCAAGAACAGGAAATGGACCAATGTCTGTATGTGTGAAAC
2041 AAGAAATGGAAAGTGATCCTCTTCTCAAACCGGCTAACTCAAACACTTTGGGACAAACAC
2101 TGGACAGCACTGCCTAAAAAGGCTTTTGACTGGACCCAAACATGAAAGCACCAGAGAAAA
2161 TCAAATGCTTCCTATTAATGTAACCTGTTTTAGAGTGCTACAGTCTTTACAACCTACTGT
2221 AGTGTCTAAATCATAACTGTTGCTTTTTCTTCAAACAGTGATAAATTTTTAGTTTCATTA
2281 TGTTGTTTTGATTGAAATGACACTATAAATTTTTTCATTTAAAAGTTTCTTAATTGTATC
2341 TAGAACAATAGCACAGTTTAGAAACTTTGTCTTCTGAGACTGACATGTTATCTGTGAACT
2401 AACTTGGGAAGATCATATCCATGTATGTGGTTATTTTGTTTTTATTGACATAGCGAGTTT
2461 CTCTGCAAACAAATTGTGTGCCCACCATTTTTAAGAAGTCCAGTATTTTCGCAAACCTGG
2521 TGTAATTATCCAACGGTTGAATGAATTATAACACTTCAGGAATTTTAAACTTTGATCATA
2581 TTTGGTTAATTTCTAGTTTTGTTGAGTAGGGGTATGGGAACTTGAATGCAACGTGACTTT
2641 AAGTGATCTCTGATCTGTGTTTTAAGGATTGTGTGTATAGATGGCACACAGCTCACTACA
2701 TTACAGGATATGATCTCAATGTATAAAACCGCAGATTGATTTTCCTTGAGTGCTTTATAC
2761 TGTTTAATTACATCTCCATGTAGGGCTGAAAAAAATTACCTATGTTTA
```

EST ID numbers identified in the UMIST database that correspond to chicken *staufen-1*.

ChEST333n20	ChEST86g12	ChEST946k9
ChEST748e23	ChEST845e23	ChEST301g11

ChEST599m6	ChEST126m10	ChEST856m3
ChEST384a23	ChEST302l23	ChEST668a22
ChEST622p16	ChEST1035k16	ChEST69a6
ChEST467j14	ChEST962b20	ChEST435k15
ChEST776a12	ChEST69e1	ChEST1012p22
ChEST235m14	ChEST407h14	ChEST447p21
ChEST383p21	ChEST452h14	ChEST176k16
ChEST200n24	ChEST695g13	ChEST6i18
ChEST110a13	ChEST394l13	ChEST813d18
ChEST738i22	ChEST423n20	ChEST684h6
ChEST233j9		

EST sequence used to make radiolabelled probe for Northern analysis

ChEST622p16

GAAGACACCAGTGAAGAAACCAGGTGATGGAAGAAAAGTAACTTTCTTT
GAGCCGGGCTCTGAAGAGACTTCAACTAGTAATAAAGAAGATGAGTTTA
GGATGCCTTATCTCAGTCATCAGCAGCTTCCTGCTGGAATTCTTCCCATGG
TCCCTGAGGTTGCACAAGCTGTAGGAGCCAGTCAAGGACACCACACCAA
AGAATTCAATAGGGCAGCCCCAAATCCTGCCAAGGCTACTGTAACAGCA
ATGATTGCTAGAGAGCTATTGTATGGTGGTACTTCTCCTACTGCTGAAAC
CATATTAAAAAATAACAACTCATCAGGCCTTGTTGCCCCAACGGACCACT
TACCAGGCCCCTCGTGAACAGCTGGACTATCTTTCCAATGTTCAAGGAAT
CCAGGTTGAATATAAAGACTTTCCAAAAAATAACAAGAATGAGTTTGTGT
CTCTTATAAACTGTTCCTCTCAGCCACCACTGATCAGCCATGGAATTGGA
AAGGATGTACGAATCTTGTCACGATATGGCTGCATTGAACATTTTGAAGT
TGCTGTCTGAGCTGGACCAACAAACCACAGAGATGCCAAGAACAGGAAA
TGGACCAATGTCTGTATGTGTGAAACAAGAAATGGACAGTGATCCT

Appendix 6

Chicken *staufen 2* full length sequence. Exons are marked in different colours.

```
   1 GTAGTGATAAACAATGGCACAAGTTAAGATGCAGACGACAGCAAACCTGTCCGGCCCTCC
  61 CATGTCCCCCATGGTGCTACCCCTGCCAGTTACAGCCGCAAACACGCTGGGCCTGCCTTC
 121 CGCCATGAACGGATCCATTCCGGAGTCGGCTGCCAGCTGCAGCAGCCCCACTGCTGGCCT
 181 TGCAGACCCCGCACCATCCAGCAACCCACCAGCTCCTCTCCAAGATAACATGGCAAACCC
 241 CAAAGAGAAAACTCCAATGTGTCTGGTAAATGAGTTAGCCCGTTTCAATAGAATTCAACC
 301 CCAGTATAAGCTTCTGAATGAAAGAGGGCCTGCTCATGCCAAGATGTTCACAGTGCAGCT
 361 GACACTTGGAGAACAGACATGGGAAGCTGAAGGAAGCAGTATTAAAAAGGCCCAGCATGC
 421 TGCTGCTAGCAAAGCTTTGAATGAAACTACCCTTCCCAAACCAACTCCTAGACCGCCCAA
 481 AAATAACATTAACAATAATCCAGGCAGTATAACTCCGACAGTGGAGCTGAACGGTCTGGC
 541 TATGAAAAGAGGAGAGCCCGCCATCTACAGGCCATTAGATCCAAAGCCAATCCCCAATTA
 601 TAGAGCAAATTATAATTTCCGGGGCATGTACAATCAGAGGTATCACTGCCCAGTGCCTAA
 661 AATTTTCTATGTCCAGTTAACTGTTGGCAACAGTGAGTTTTTTGGTGAAGGAAAGACTCG
 721 CCAAGCTGCTAGACATAATGCTGCAATGAAGGCCCTACAAGCTCTCCAGAATGAGCCTAT
 781 TCCAGAAAAATTACCTCAGAATGGGGAAGCAGGAAAAGAAACAGAAGAAGATAAAGATGC
 841 AAACAAGTCTGAGATCAGCGTTGTGTTTGAAATTGCTTTGAAGCGCAATATACCCGTCAG
 901 TTTCGAGGTGATAAAAGAAAGTGGACCTCCTCACATGAAGAGCTTTGTCACACGAGTTAC
 961 GGTAGGAGAATTCACCGCAGAAGGAGAAGGGAACAGTAAGAAGCTCTCAAAGAAACGTGC
1021 TGCAATGTCTGTCCTACAAGAGCTGAAGAAGCTTCCTCCTCTTCCTGTGATTGAAAAGCC
1081 AAAACTGTACTTCAAAAAACGTCCAAAAACAATATTGAAGACTGGACCTGAATATGGTCA
1141 AGGAATGAATCCTATCAGTCGTCTGGCTCAGATCCAGCAGGCCAAAAAGGAGAAGGAGCC
1201 GGAGTATGTTCTTCTTTCAGAGAGAGGGATGCCTCGCCGTCGAGAGTTTGTTATGCAGGT
1261 AAAAATAGGTAATGAGATTACTACTGGAACAGGCCCAAACAAGAAAATAGCTAAAAGAAA
1321 TGCAGCAGAAGCAATGTTGCTACAGCTGGGTTACAAAGCCTCTACTCCTCTTCAAGACCA
1381 GACAGAAAAGGTGAGAACCAATTGTGATGCCCGAATTTTACCTTTGTCTTGATTTCTTAG
1441 CTGTGTCAGCTAATCACTTGATATGAACAGAACCTTAAACTTGTGCACTGCATTGCTGGG
1501 TTGCATGTCAGATCAGCCTTCATTTCAGGGCAGCAGGCAATAGTAAGTGCACTAAAAGCA
1561 ATAGAGAACAGAAATGTAGTTGTAGAAGACAGTGTGTACACAAAAGCGTACATGTAATGT
1621 GTGGTTGTATTTCTGCTTAAAAATATGCCTTTTCTATGCTGCTGTAGGTACAATGAGAGG
1681 ACACCTCTTCTGGAATAGGAAAGAATGTTGTCCTTAATTTTCATACATAAAGTACTCATT
1741 GCTATTTGTCTTCCCATTGAATATGTTCTGGAATAATTCTAGAAAAATAATGGCCTTTCC
1801 AGCCTTTTAGTGACTGAAAAAGAAAGTGTGTGTCAATATTTTCTGGAGAAGAAATGATAT
1861 TTTAAATTCAAGTGTGTTATATTATGTATTTCCTCGTTAGCGATGTGCAGCTTAGCACTC
1921 CTGGCCTGTCTTGACTTCATTGATTAAAGGTTTTGCAATCAAAATAGGTGTTAAAGTTTT
1981 ACTTTTTATGTCCTGACAAGATCAGAACAAGGCATTTGGCTAAGGTGTGGACTCTGGACG
2041 GCTTCTGTCATACGCACACACAGGGTCCCTGCATGTGTGTATTTTGATCACATCTTAAGT
2101 CTTTGCTGTGGCTGCATGTGCCAACTGTTGACTCAGGTCAGCAGTATGTTCTACTGGGCT
2161 TTTATAGGGACCACATGATAAATCTGTATATAGTGCTGTTGAGTGGTTGGGGAAAGGGTG
2221 ATGCTGCTTTATTTCCTTGCATTTGTTTATCTATTGCTTACAAGTGCATTGTCC
```

EST ID numbers identified in the UMIST database that correspond to chicken *staufen-2*.

ChEST738i22	ChEST813d18	ChEST401m4
ChEST423n20	ChEST467e2	ChEST1032b3
ChEST455k24	ChEST394l13	ChEST914e10
ChEST822f13	ChEST233j9	ChEST350d7
ChEST249i23	ChEST582d21	ChEST333n20
ChEST461n21	ChEST684h6	ChEST435k15
ChEST99a3	ChEST6i18	ChEST69a6
ChEST249a23	ChEST360j7	ChEST632j3
ChEST156h21	ChEST555f15	ChEST479e12

ChEST103i16 ChEST110a13

Sequence of EST used to make Northern Radiolabelled probe

ChEST684h6

CACGGCTATGAAAAGAGGAGAGCCCGCCATCTACAGGCCATTAGATCCA
AAGCCAATCCCCAATTATAGAGCAAATTATAATTTCCGGGGCATGTACAA
TCAGAGGTATCACTGCCCAGTGCCTAAAATTTTCTATGTCCAGTTAACTG
TTGGCAACAGTGAGTTTTTTGGTGAAGGAAAGACTCGCCAAGCTGCTAGA
CATAATGCTGCAATGAAGGCCCTACAAGCTCTCCAGAATGAGCCTATTCC
AGAAAAATTACCTCAGAATGGGGAAGCAGGAAAAGAAACAGAAGAAGA
TAAAGATGCAAACAAGTCTGAGATCAGCGTTGTGTTTGAAATTGCTTTGA
AGCGCAATATACCCGTCAGTTTTGAGGTGATAAAAGAAAGTGGACCTCCT
CACATGAAGAGCTTTGTCACACGAGTTACGGTAGGAGAATTCACCGCAG
AAGGAGAAGGGAACAGTAAGAAGCTCTCAAAGAAACGTGCTGCAATGTC
TGTCCTACAAGAGCTGAAGAAGCTTCCTCCTCTTCCTGTGATTGAAAAGC
CAAAACTGTACTTCAAAAAACGCCCAAAAA

Appendix 7

Pumilio-1 full length sequence. Exons are marked in different colours.

```
   1 GGGGGCCGAGATCCAGCTTCATCCTACCGCTCCGCCCGTGTTGGTGGAATGAGCGTTGCG
  61 TGTGTGTTGAAGAGAAAAGCAGTGCTCTGGCAGGACTCGTTCAGCCCCCACCTGAAACAG
 121 CACGCTCAAGATACAGCTAATCCCAACATGCCTGTTGTATTGACATCTGGAACAGGGTCC
 181 CAGGCTCAGCCACAGCCAGCTGCAAACCAGGCTCTTGCAGCAGGGACACACTCCAGTCCT
 241 GTTCCTGGATCCATAGGAGTTGCAGGCCGCTCCCAGGACGACGCTATGGTGGATTACTTC
 301 TTTCAGAGGCAGCATGGTGAGCAGCTTGGGGGAGGAGGAAGTGGTGGAGGCGGCTATAAT
 361 AACAGCAAACATCGCTGGCCTACTGGGGATAACATTCATGCAGAACATCAGGTGCGTTCT
 421 ATGGATGAGCTGAACCATGATTTTCAAGCGCTTGCTCTGGAAGGACGGGCTATGGGAGAG
 481 CAGCTGTTGCCGGGTAAAAAGTTTTGGGAATCTGATGATTCCAGCAAAGATGGACCAAAA
 541 GGGATATTTCTGGGAGATCAGTGGAGAGACAGTGCTTGGGGAACATCAGATCACTCTGTT
 601 TCCCAACCAATTATGGTTCAGAGAAGACCTGGTCAGGGCTTTCATGTGAATAGTGAAGTC
 661 AACTCAGTGCTTTCACCACGGTCAGAGAGTGGAGGACTTGGAGTTAGCATGGTGGAATAT
 721 GTGTTGAGCTCATCTCCCGGAGATTCCTGCCTAAGGAAAGGAGGATTTGGGCCAAGGGAT
 781 GCAGAGAATGATGAGAATGACAAAGGGGATAAGAAAAATAAGGGCACATTTGATGGCGAT
 841 AAATTAGGAGATCTGAAGGAGGAGGGGGATGTGATGGATAAAACAAATGGTTTGCCTGTG
 901 CAGAATGGAATCGACACAGATGTCAAAGACTTCAGCCGTACACCTGGTAATTGCCAGAAC
 961 TCTGCTAGTGAAGTTGATCTTCTGGGTCCAAACCAGAATGGATCTGAGGGCTTAGCCCAG
1021 CTGGCGAGTACTAATGGTGCCAAGCCCGTGGAGGATTTTTCCAACATAGAGTCACAGAGT
1081 GTTCCCCTGGATCCCATGGAGCACGTTGGCATGGAGCCTCTGCAGTTCGATTATTCTGGC
1141 ACCCAGGTACCTGTGGACTCAGCCGCAGCCACCGTGGGGCTCTTCGATTACAATTCCCAA
1201 CAGCAGTTGTTCCAAAGACCGAATGCACTTGCTGTTCAGCAGCTAACAGCAGCCCAGCAG
1261 CAGCAGTATGCATTGGCAGCTGCCCATCAGCCTCACATAGCAGGTTTAGCTCCTGCTGCC
1321 TTTGTCCCCAATCCGTACATCATCAGCGCTGCTCCACCAGGAACGGATCCGTACGCAGCC
1381 GGGCTCGCAGCAGCTGCCACATTAGGCCCTGCAGTTGTCCCTCACCAGTACTACGGAGTT
1441 ACACCCTGGGGAGTTTATCCTGCCAGCCTCTTCCAGCAGCAAGCTGCAGCAGCTGCCGCT
1501 GCCACTAACTCAGCAAATCAGCAGACCACTCAGCAAACCCAGCAGGGCCAGCAACAGGTT
1561 CTACGTGGCGGAGCCAGTCAGCGTCCTTTGACCCCAAACCAGAACCAGCAGGGACAGCAG
1621 ACTGATCCGCTGGTGGCTGCCGCCGCCGTCAACTCTGCCCTTGCCTTTGGACAAGGGCTG
1681 GCAGCAGGGATGCCAGGGTACCCTGTGCTGGCTCCTGCTGCTTACTATGACCAAACTGGT
1741 GCTCTTGTGGTGAATGCGGGGGCCAGGAATGGTCTGGGGGCACCCGTGCGTCTGGTGGCC
1801 CCTGCACCAGTTATCATCAGCTCCTCTGCAGCACAGGCAGCAGTTGCAGCAGCGGCAGCT
1861 TCAGCCAACGGTGCTGCGGGGGGCCTGGCAGGAACCACCAACGGCCCCTTCCGCCCCCTG
1921 GGAACGCAGCAGCCCCAGCCTCAGCCGCAGCAGCAGCCCACCAACAACTTGGCCTCCAGC
1981 TCGTTTTATGGCAACAACTCACTCAGCAGCAATTCCCAGAGCAGCTCCCTCTTCTCTCAG
2041 GGCTCTGCCCAGCCTGCCAACACCTCCCTGGGCTTTGGGAGCAGCAGCTCCCTTGGTGCC
2101 ACTCTGGGGTCTGCACTGGGAGGCTTTGGGACAGCAGTTGCTAACTCCAACACTGGCAGC
2161 GGCTCTCGCCGAGATTCCCTGACAGGGAGCAGTGACCTGTACAAGAGGACATCCAGCAGT
2221 TTGACACCTATAGGACACAGTTTTTACAATGGCCTTGGGTTTTCCTCCTCTCCTGGACCT
2281 GTGGGTATGCCTCTGCCCAGCCAAGGACCTGGCCACTCTCAGACTCCACCACCTTCCTTA
2341 TCTTCACATGGATCTTCTTCCAGTCTAAACCTGGGAGGGCTCACAAATGGGAGCGGCCGT
2401 TACATCTCTGCTGCTCCTGGAGCTGAAGCCAAGTACCGCAGTGCAAGCAGTGCCTCCAGC
2461 CTCTTCAGCCCCAGCAGCACACTGTTCCCTTCGTCTCGCCTGCGCTATGGCATGTCTGAT
2521 GTTATGCCCTCCGGCCGAAGCAGGCTGCTCGAAGACTTCCGCAATAACCGGTACCCCAAT
2581 TTACAGCTGAGGGAGATTGCTGGGCACATCATGGAGTTCTCCCAGGACCAGCATGGATCC
2641 AGATTTATTCAGCTGAAACTGGAGCGTGCTACCCCAGCAGAACGTCAGCTTGTGTTCAAC
2701 GAGATCCTCCAGGCGGCTTATCAGTTGATGGTGGATGTATTTGGAAATTATGTCATCCAG
2761 AAGTTCTTTGAATTTGGCAGCCTGGAGCAAAAGTTAGCCTTGGCAGAACGTATCCGTGGG
2821 CATGTTCTGTCCCTGGCTTTGCAGATGTATGGATGTAGGGTGATCCAGAAGGCCCTTGAG
2881 TTTATTCCCCCAGACCAGCAGGTAATTAACGAGATGGTACGGGAGTTGGATGGCCATGTC
2941 CTGAAGTGTGTAAAAGACCAGAACGGTAATCATGTGGTGCAGAAGTGTATTGAGTGTGTG
3001 CAGCCTCAGTCCCTGCAGTTTATCATTGATGCATTTAAGGGACAGGTTTTTGCGTTGTCT
3061 ACACATCCATATGGCTGTCGTGTGATTCAGAGGATCCTCGAGCACTGTCTTCCTGAGCAG
3121 ACTCTTCCCATCTTGGAGGAGCTTCACCAGCACACTGAGCAGCTTGTGCAGGATCAGTAT
3181 GGGAACTATGTTATCCAGCATGTACTAGAGCACGGTCGGCCTGAGGACAAGAGCAAGATT
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3241 GTAGCAGAAATTAGAGGCAACGTGCTCGTCTTGAGTCAACATAAATTTGCTAGCAACGTT
3301 GTGGAGAAGTGTGTGACCCACGCCTCCCGTACGGAGCGTGCCATGTTGATCGATGAGGTG
3361 TGCACTATGAACGATGGCCCTCACAGTGCCTTATACACCATGATGAAGGATCAGTATGCC
3421 AACTACGTGGTACAGAAGATGATCGATGTGGCAGAACCAGCTCAGCGGAAGATTGTCATG
3481 CACAAGATCCGGCCCCACATCGCCACCCTGCGCAAGTACACCTACGGCAAGCACATCTTG
3541 GCCAAGCTGGAGAAGTACTACATGAAGAACGGCGTTGACCTCGGGCCCATCTGTGGACCT
3601 CCAAATGGCATCATCTGAGGCACGGAGCTGCCCCGGCCATCCGGCATCCACAACCAGCAA
3661 CCAGCCCTATTCCTATATACAGTTAGAGAGCGCGTTACGGTTGCTAAACTACTAAAAGAA
3721 GAAGAAGAAAAAAAGCCTTTGTAAATTTCTTTAATTTTATTATGCATAACATGTACTAAT
3781 TATTTTTTTTTAATTAACTGATTGCCCTGCTGTTTTACTGGTGTATAGAATACTTGTACAT
3841 AGGTATCACATGTACATGGAAGGCCACATTTTTGTTCACTGTTGTATCTATATTCCAAAT
3901 GTGGAAACTTTCAGGGTGGTTGGTTTGAAGTACAAAGAAAAAACACACGACGTTTTTAAT
3961 CCATCTGCCTTGCAGGCAACTCTGTGCAGATACCCGTTGTTCTCCTCTGACTCAGAAGTC
4021 AGCAGCAGGTTTGGATTTTAGTTGAACGGCGCGAGAGACGCATTTAATGCTGTTTATAAA
4081 TATTAAAAAA
```

EST ID numbers identified in the UMIST database that correspond to chicken *pumilio-1*.

ChEST651c3	ChEST99k7	ChEST865n18
ChEST500g19	ChEST730n20	ChEST183j14
ChEST335g7	ChEST511i14	ChEST993m8
ChEST488o8	ChEST387c20	ChEST359a12
ChEST739p19	ChEST618c14	ChEST387j7
ChEST505g20	ChEST634h19	ChEST618h14
ChEST486p9	ChEST996l22	ChEST1031b5
ChEST69i5	ChEST100n7	ChEST733f20
ChEST448b13	ChEST58e8	ChEST666l20
ChEST100i16	ChEST392p3	ChEST973l16
ChEST852g18	ChEST789f23	ChEST293b10
ChEST278h13	ChEST792e6	ChEST839d1
ChEST189p5	ChEST697a12	ChEST816i4
ChEST94c7	ChEST609a10	ChEST609j18
ChEST505h9	ChEST973n17	ChEST345i13
ChEST736e14	ChEST969b19	ChEST649p6
ChEST17i19	ChEST465m21	ChEST726p23
ChEST588f13	ChEST1035g21	ChEST47b2
ChEST42n4	ChEST204p10	ChEST347f17
ChEST280i6	ChEST853d17	ChEST1031b24
ChEST726j18	ChEST726h18	

EST sequence used to make radiolabelled probe for Northern analysis

ChEST99K7

GTGTAGCTTATCAGTTGATGGTGGATGTATTTGGAAATTATGTCATCCAG
AAGTTCTTTGAATTTGGCAGCCTGGAGCAAAAGTTAGCCTTGGCAGAACG
TATCCGTGGGCATGTTCTGTCCCTGGCTTTGCAGATGTATGGATGTAGGG
TGATCCAGAAGGCCCTTGAGTTTATTCCCCCAGACCAGCAGGTAATTAAC
GAGATGGTACGGGAGTTGGATGGCCATGTCCTGAAGTGTGTAAAAGACC
AGAACGGTAATCATGTGGTGCAGAAGTGTATTGAGTGTGTGCAGCCTCA
GTCCCTGCAGTTTATCATTGATGCATTTAAGGGACAGGTTTTTGCGTTGTC

TACACATCCATATGGCTGTCGTGTGATTCAGAGGATCCTCGAGCACTGTC
TTCCTGAGCAGACTCTTCCCATCTTGGAGGAGCTTCACCAGCACACTGAG
CAGCTTGTGCAGGATCAGTATGGGAACTATGTTATCCAGCATGTACTAGA
GCACGGTCGGCCTGAGGACAAGAGCAAGATTGTAGCAGAAATTAGAGGC
AACGTGCTCGTCTTGAGTCAACATAAATTTGCTAGCAACGTTGTGGAGAA
GTGTGTGACCCACGCCTCCCGTACGGAGCGTGCCATGTTGATCGATGAGG
TGTGCACTATGAACGATGGCCCTCACAGTGCCTTATACACCATGATGAAG
GATCAGTATGCCAACTACGTGGTACAGAAGATGATCGATGTGGCAGAAC
CAGCTCAGCGGAAGATTGTCATGCACAAGATCCATTTCACCCGTTGGGCC
AAATTTCTCAGAATAGAAGATGTGGGAACATCCTGGTTTCCCCCAAAAGA
GAGACAC

Appendix 8

Chicken *pumilio-2* full length sequence. Exons are marked in different colours.

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   1 GGGAGGGGAGGCCGGAAGGAGAGGCGCCGAGAGGAGCAGGCGGCGGGGGGCGGCGGGCGG
  61 GCGGCGGTGTCCCAGGGCCCGCTCCGGCTCCGTCTCGGCGAGCGGCGCCGCCAACCAGCC
 121 CGTTCGATCTGTGGATGAGATGAATCATGAGTTTCAAGCTCTTGCCCTAGAATCTCGGGG
 181 AATGGGAGAGCTTTTACCTGCCAAAAAGTTTTGGGAACCTGATGATTCAGCAAAAGATGG
 241 ACAAAAAGGGATATTCCTTGGTGATGAGTGGAGAGAGACTGCATGGGGAACTCCTCACCA
 301 TTCTATGTCCCAGCCTATTATGGTACAGAGAAAGCCTGGACAGGGTTTTCATGGAAACAG
 361 TGAAGTAAATGCTGTATTGTCTCCACGATCAGAAAGTGGTGGCCTTGGCGTGAGCATGGT
 421 AGAATATGTGTTAAGTTCCTCTCCAGCTGATAAATTGGATTCCCGGTTTAGGAAAGGAGC
 481 TTTTGGCACTAGAGATGCTGAAACAGATGGACCTGAGAAAGGAGATCAGAAAGGCAAGGC
 541 TTCTCCATTTGAGGAGGACAAAAACAGAGATCTTAAACAAGGAGATGATGAGGATGTTAC
 601 TAAAATAAATGGCAGAGGTTTGCCAAATGGAATGGATGCCGATTGCAAAGATTTTAATCG
 661 TACCCCTGGAAGTCGACAAGCCTCCCCAACAGAAGTAGCTGAACGCTTGGGTCCCAATCC
 721 CAGCACCACAGAAGGATTGGGCCCACTTCCCAATCCTACAGCCCACAAGCCCTTGGTAGA
 781 AGAATTTTCCAATCCAGAAAATCAGAATCTAGATGCCATGGAACAAGTTGGTCTTGATTC
 841 TCTACAATTTGATTATCCTGGCAACCAGGTACAGATGGACTCTTCGGGAGCTACTGTAGG
 901 ACTTTTTGACTACAATTCTCAGCAGCAGCTTTTCCAGAGGACTAATGCTCTGACTGTGCA
 961 GCAGTTAACAGCAGCCCAGCAGCAGCAGTACGCCCTGGCTGCGGCTCAGCAGCCTCACAT
1021 AGCAGGTGTATTCTCAGCAGGCTTGGCTCCAGCTGCTTTTGTGCCAAACCCATACATTAT
1081 CAGTGCTGCTCCTCCAGGTACTGACCCGTACACTGCAGCTGGATTAGCTGCAGCAGCCAC
1141 CCTAGCAGGTCCTGCTGTGGTTCCGCCTCAGTATTATGGTGTTCCATGGGGGGTGTATCC
1201 AGCCAATTTATTTCAGCAGCAAGCTGCAGCAGCAAATACCACAGCAAATCAACAAGCAGC
1261 CTCCCAGGCACAACAGGGACAGCAGCCGGTTCTGCGTGCTGGAGCAACTCAGCGCCCGCT
1321 TACTCCCAGTCAGGGTCAGCAAGGGCAGCAAGCAGAGTCACTTGCAGCAGCTGCAGCAGC
1381 AAATCCAGCTTTGGCTTTTGGTCAGGGTCTTGCTACAGGCATGCCAGGCTATCAAGTACT
1441 AGCTCCCACTGCCTATTATGATCAGACTGGTGCCTTAGTAGTAGGACCTGGAGCAAGAAC
1501 TGGCCTTGGAGCACCAGTCAGATTGGTGGCCTCAACTCCTGTTATAATTAGTTCTGCAGC
1561 AGCACAAGCAGCTGCAGCTGCTTCAGCTGGAGGAACAGCAAACAATCTCACGGGAGCCAC
1621 GAACGGTCTATTTCGGCCACTTGGTGCCCAGCCACAACAACAGCAACAGCAAACAAATAG
1681 TAGCCTACAATCCAATTCATTTTATGGCAGTAACTCTTTGACCAATAACTCCCCAAACAG
1741 TTCCCTTTTTTCACATGGTCCTGGCCAACCAGGAAGTACATCTCTTGGCTTTGGAAGTAG
1801 CAGCTCCTTAGGAGCAGCTATCGGCTCTGCACTTGGTGGATTTGGCTCATCAGTTGGCAG
1861 TTCTGCAAGTAGTAGTGCCACAAGGAGAGATTCTCTATCTACTAGCTCTGACTTGTACAA
1921 AAGATCTAGTAGCAGCCTAGCACCCATAGGGCAGCCATTTTACAATAGTCTGGGATTTTC
1981 CTCCTCTCCAAGTCCAATAGGCATGCCTCTGCCAAGCCAAACTCCAGGACATTCACTTAC
2041 GCCACCGCCATCACTTTCATCACATGGATCCTCATCCAGTTTGCATTTAGGAGGACTCAC
2101 AAATGGTAGTGGTCGCTATATTTCTGCGGCACCTGGAGCAGAAGCAAAGTATCGCAGTGC
2161 AGCAAGTACCTCCAGTCTTTTTAGCTCCACCAGTCAGCTCTTCCCTCCTTCACGCCTTCG
2221 TTACAGTAGGTCTGACATTATGCCTTCTGGACGTAGTCGATTGCTGGAAGATTTCCGAAA
2281 TAATCGTTTCCCCAATCTGCAACTAAGAGACCTTATTGGACATATTGTTGAGTTTTCTCA
2341 AGATCAGCATGGTTCTAGATTTATACAGCAAAAGCTGGAGCGAGCTACTCCAGCTGAGCG
2401 CCAGATGGTGTTTAACGAGATCTTGCAAGCAGCATATCAATTGATGACTGATGTGTTTGG
2461 AAACTATGTAATACAGAAGTTCTTTGAGTTTGGAAGCCTGGATCAAAAGTTAGCCCTGGC
2521 AACACGCATACGTGGTCATGTTCTGCCATTAGCCCTACAGATGTATGGTTGTCGTGTTAT
2581 TCAGAAAGCACTTGAGTCTATCTCACCTGACCAGCAGAACGAAATGGTGAAAGAGCTGGA
2641 TGGTCACGTTCTGAAATGTGTGAAAGATCAAAATGGGAATCATGTTGTGCAAAAGTGTAT
2701 CGAGTGTGTTCAGCCGCAGTCGCTCCAGTTCATCATCGATGCATTCAAAGGACAGGTATT
2761 TGTGCTTTCAACTCATCCGTATGGCTGTAGAGTAATTCAGCGTATTCTGGAACACTGCAC
2821 TGCTGAGCAGACTTTGCCAATCTTAGAAGAACTACATCAGCACACAGAACAACTAGTGCA
2881 GGATCAATATGGAAATTACGTTATTCAACACGTGCTGGAGCATGGTCGTCCTGAAGACAA
2941 GAGTAAAATAGTTTCAGAAATAAGAGGAAAAGTTCTAGCTCTGAGTCAGCACAAATTTGC
3001 CAGCAACGTGGTAGAAAAATGTGTAACTCATGCTTCTCGTGCTGAAAGAGCTTTACTTAT
3061 TGATGAGGTCTGCTGCCAGAATGATGGTCCTCACAGTGCCTTATACACCATGATGAAGGA
3121 CCAGTATGCCAACTATGTTGTTCAGAAGATGATTGATATGGCTGAACCTGCTCAGCGGAA
3181 GATAATAATGCACAAGATTCGACCCCACATTACAACTCTGCGTAAATACACCTACGGCAA
3241 ACACATTCTGGCGAAGCTGGAAAAGTATTACCTGAAGAACAGTGCTGATCTGGGGCCAAT
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3301 AGGTGGACCACCAAATGGGATGCTGTAAAAGGCAAAAAAAAAAAAAAGGAAGTGGAAGAAA
3361 AATTTTATTGTGAATGATCAAAACATACAACTTAACTATAAATGTTCTGATTTTTTTAAA
3421 TCTATTTATTGACTTTGTTCATCCATTTGTAAAATTTTTATTCTTTTGTATATTTTTGGG
3481 GAGTGAATTATAAAAAAAAAAATAAAAAAAAAAATCTCCAGCCCTGATCAGGAGACCTAT
3541 CAGATTGGATGGCTGGCAAAGCACAGAACGCCTGTATATGATGTAATTGTATCAAAATAG
3601 CTGTCACATATTTTGTAAATTTTTACCTTGTAAAGTCACTGAAATAGTTTTTAAAGGGAA
3661 AAAGTACAGTATTCTTTTAATACAC
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EST ID numbers identified in the UMIST database that correspond to chicken *pumilio-2*.

ChEST588f13	ChEST505h9	ChEST609j18
ChEST726p23	ChEST816i4	ChEST345i13
ChEST465m21	ChEST1031b24	ChEST973n17
ChEST94c7	ChEST768j9	ChEST347f17
ChEST280i6	ChEST853d17	ChEST1035g21
ChEST47b2	ChEST42n4	ChEST609a10
ChEST809j16	ChEST651f13	ChEST62o19
ChEST656f2	ChEST726j18	ChEST726h18
ChEST730n20	ChEST228a7	ChEST335g7
ChEST387j7	ChEST387c20	ChEST996l22
ChEST634h19	ChEST511i14	ChEST505g20
ChEST500g19	ChEST488o8	ChEST1031b5
ChEST392p3	ChEST993m8	ChEST618h14
ChEST486p9	ChEST618c14	ChEST733f20
ChEST789f23	ChEST204p10	ChEST839d1
ChEST739p19	ChEST359a12	ChEST651c3
ChEST852g18	ChEST499k13	ChEST183j14
ChEST58e8	ChEST672n6	ChEST865n18
ChEST100n7	ChEST293b10	ChEST448b13
ChEST792e6	ChEST69i5	

EST sequence used to make radiolabelled probe for Northern analysis

ChEST588f13

GCGAGCTACTCCAGCTGAGCGCCAGATGGTGTTTAACGAGATCTTGCAAG
CAGCATATCAATTGATGACTGATGTGTTTGGAAACTATGTAATACAGAAG
TTCTTTGAGTTTGGAAGCCTGGATCAAAAGTTAGCCCTGGCAACACGCAT
ACGTGGTCATGTTCTGCCATTAGCCCTACAGATGTATGGTTGTCGTGTTAT
TCAGAAAGCACTTGAGTCTATCTCACCTGACCAGCAGAACGAAATGGTG
AAAGAGCTGGATGGTCACGTTCTGAAATGTGTGAAAGATCAAAATGGGA
ATCATGTTGTGCAAAAGTGTATCGAGTGTGTTCAGCCGCAGTCGCTCCAG
TTCATCATCGATGCATTCAAAGGACAGGTATTTGTGCTTTCAACTCATCC
GTATGGCTGTAGAGTAATTCAGCGTATTCTGGAACACTGCACTGCTGAGC
AGACTTTGCCAATCTTAGAAGAACTACATCAGCACACAGAACAACTAGT
GCAGGATCAATATGGAAATTACGTTATTCAACACGTGCTGGAGCATGGTC
GTCCTGAAGACAAGAGTAAAATAGTTTCAGAAATAAGAGGAAAAGTTCT
AGCTCTGAGTCAGCACAAATTTGCCAGCAACGTGGTAGAAAAATGTGTA
ACTCATGCTTCTCGTGCTGAAAGAGCTTTACTTATTGATGAGGTCTGCTGC

CAGAATGATGGTCCTCACAGTGCCTTATACACCATGATGAAGGACCAGTA
TGCCAACTATGTGGTCAGAAGATGATTGATATGG

Appendix 9

Chiwi full length sequence. Exons are marked in different colours.

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   1 TGTGGCGTTGTGGAGACGGCGGTCTCCCGTTTGTCGCTGGAGCGGCGTCGCTTTGTGACT
  61 GAGGGAACATGACAGGAAGAGCTAGAGCCAGAGCGAGAGGGAGACCTCCAGGACAGGAGG
 121 CTGCCATTCCTCCTGTGGGAGCTGCATCTGCTCAAAAGACTTTGCCAAGTCATCCATCTG
 181 AACAGCGGCAATCTCTGCAGCCATGTCATCCTCCACCACTGACAGAAGAACCTGGTGGCC
 241 GTGGGCGACAGAGAGGCCCTCAGGATGCTCCAAAGACACTAGGTCAGAATCTCAAAACAA
 301 CAGGATTACAGATTTCAGCAGGGTTTCAGGAACTGTCTTTAGCAGATAGGGGCGGACGTC
 361 GTCGGGATTTCCATGACCTCGGGGTAAATACTCGACAAGCCATAGAACACGTTAGAGAAT
 421 CAAAAACTGGCTCTTCAGGTGCTATGATAAAATTAATTGCAAATTTTTTTCGTCTCACAT
 481 CTCGACCCCAATGGGCTTTATATCAATACCATGTAGACTATAATCCTGAGATGGAAGCAC
 541 GCCGCCTTCGATCAGGTTTGCTCTTTCAGCATGAAGACCTAATTGGAAAAACGCATGCAT
 601 TTGATGGATCAATATTATTCTTGCCAAAAAGACTGCCAAATAAGGTTACTGAAGTATATT
 661 CTAAGACCCGAAATGGAGAAGATGTGAGGATCACGATCACATTGACTAATGAATTACCAC
 721 CTACTTCACCTACATGTCTGCAGTTTTACAACATCATTTTTAGAAGGCTTCTGAAGATGA
 781 TGAATTTTCAGCAAATTGGACGTAACTATTACAACCCTAAGGACCCAGTCAGCATCCCTA
 841 ATCACAGGTTGATGGTTTGGCCAGGCTTCACAAGTTCTATTCTCCAGTATGAGGAGAGCA
 901 TTATGTTATGTGCAGATGTGAGCCATAAGATTCTTCGTAGTGAAACAGTTTTGGATTTTA
 961 TGTACAGTCTCTATGAACAGGTTGAAGAGAGAAGATTTAGAGATGCCTGTGCAAAGGAGC
1021 TGATAGGTGTAATTGTTCTTACGAAGTACAATAACAGAACATACAGAGTTGATGACATCG
1081 ACTGGGATGCCAATCCACAGTGTACTTTTAGACGAGCAGATGGCTCTGAAATCAGCTATA
1141 TAGACTACTACAAAAGGCAATATAACCAAGATATCAGTGACTTGAACCAGCCTGTCTTGA
1201 TCAGTCAGTATCGGAGGAAGAGAGGAAATGTGACGGTAGGACCTGTGGTTCTAATCCCAG
1261 AGCTGTGCTACCTAACAGGATTAACTGAGAAGATGAGGAATGATTTTAACATGATGAAAG
1321 ACTTGGCTGTTCATACACGACTTTCACCTGAGCAAAGACAACGTGAAATTGGAAAGCTTG
1381 TTGACTGCATGAAAAAAGATGAATGTGTTCAGAAGGAACTCCGGGACTGGGGTTTAAGCT
1441 TTGATTCTAGCTTACTGTCCTTTACGGGAAGAGTTGTTCAAGCAGAAAAGATCCTTCAAG
1501 CAGGAAATGTGTTTGATTACAATCCTCAGTTTGCTGATTGGTCACGGGAAACCAGGGTAG
1561 CTCCCTTAATCCATGCAAAGCCTTTGGACAACTGGTTACTGATATACACACGGCGCAACT
1621 ATGATGCTGCTAATATGTTACTTCAGAATCTGTTTAAAGTCACACCATCTATGGGAATCA
1681 GAATGAACAAGGCAACCATGATTGAAGTGGATGATAGAACAGAAGCTTATTTAAGGGTTT
1741 TGCAACAAAGTATTACTCCGGACACAAACATAGTAGTTTGTATTTTGTCTAGTACCCGAA
1801 AGGATAAGTATGATGCTATCAAGAAATACCTATGTACGGATTGTCCCATTCCAAGTCAGT
1861 GCGTGGTTGCTCGTACTTTAAGCAAGCCTCAGACTGCTCTGGCCATAGTGACAAAAATTG
1921 CCTTGCAGATGAACTGTAAAATGGGTGGAGAACTCTGGAGTGTTGAGATCCCTCTGAAGC
1981 AGTTAATGATTGTGGGCATTGATTGTTACCATGATACTTTATCTGGAAAGCAGTCAATTG
2041 CTGGATTTGTGGCTAGCCTGAATGAAAAAATGACACGGTGGTTTTCACGCTGCGTTGTTC
2101 AAAGCCGTGGGCAGGAAATTGTGGATGGGCTCAAAGCCTGCTTGCAAACTGCTCTAAGGG
2161 AATGGTTCAAGTGGAATAAGTATTTGCCCTCTCGTATTATTGTGTATCGTGATGGTGTAG
2221 GAGATGGACAGCTCAATACTTTAGTGAACTATGAAGTGCCTCAGTTTCTGGATTGCTTGA
2281 AGACTGTTGGTAAAGACTACAATCCAAGACTGACTGTGATCGTTGTGAAGAAACGAGTGA
2341 GTACCAGATTCTTTGCGCAGGCTGGTGGAGGACTTAAAAACCCACCCCCTGGTACTGTCG
2401 TTGATATAGAGGTGACCAGACCAGAATGGTATGATTTCTTTATTGTGAGTCAGGCAGTGA
2461 GAAATGGTTGTGTCGCACCCACTCATTATAACGTAGTGTATGACACTAGCAAACTGAAAC
2521 CAGATCATGTACAACGTTTAACCTACAAACTTTGCCACATGTACTATAACTGGTCGGGTG
2581 TTATCAGAGTACCTGCTCCTTGCCAGTATGCCCATAAACTGGCTTTCCTTGTGGGTCAGA
2641 GCATTCACAGAGAACCAAACCTGTTGCTCTCAGACAGACTTTACTATCTCTAATTGGTT
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EST ID numbers identified in the UMIST database that correspond to chicken *chiwi*.

ChEST329k18	ChEST757n8	ChEST764f14
ChEST489j21	ChEST432j12	ChEST295l24
ChEST709n12	ChEST810p1	ChEST422o10
ChEST1025n3	ChEST797p12	ChEST143d16

EST sequence used to make radiolabelled probe for Northern analysis

ChEST709n12

TGTATGACACTAGCAAACTGAAACCAGATCATGTACAACGTTTAACCTAC
AAACTTTGCCACATGTACTATAACTGGTCGGGTGTTATCAGAGTACCTGC
TCCTTGCCAGTATGCCCATAAACTGGCTTTCCTTGTGGGTCAGAGCATTC
ACAGAGAACCAAACCTGTTGCTCTCAGACAGACTTTACTATCTCTAATTG
GTTTAAAAAAAAAAAAAAGTTCCTTTCTGGGGAAGGTGGAAGTGTGGTT
GGGGTTGGGTGGGTTGGTTTTCATGAGACACAAGCAGGGGTCCATGCAG
TTGTGGAACTTTTATTTTCACTGTAGCGGGAAAAAGATTGAAGATAGCAG
ATCTGTGTTTGGAATAAGACTTTATTCAACTGCATAGAGGGAAACATTGT
TAGAGACTGCTACAGATTCCATAGGTGAATGAACTCAGTTTGGGGGTTGA
CATTGGTCCGTTACTTTTCTCATTTAATAAGCATTCTCTGGATTCCCTGTA
AGGAACATCAGTATCTACGTCAGCACTGCCTTGAGAGGGGGGGAACAAA
ACAAACACAAAAAAAGGAGGTGGGCCATATTTAACCGGGGCCTTAGGGG
GTTTTAACAGGCCCCAGATAATTGGGAAGGGGCTAAGGTTGACACGTCC
GAGATTGCGAAAATCGGAAGAGGGTCCCTAGGGCAGGGACACCTTGGAC
CCAGGGAGTGCGCTAAACTTGCGGAAAGGGGGACAACACACCAGGAGG
CTCCTCACAGTAGGTCTGTGGCTCATTACCCGCAGTGTAATCACGCGTTG
AACACTTAGGGATTGGGACAACACTTAAAGGGTGGTGGGGGACAAAACA
GGACCAGTTTATTTGGGACACAACAACGTGGGGTACACCTTGGGCGCAC
ATTTCACCCGTTGGGCCAAATTTCTCAGAATAGAAGATGTGGGAACATCC
TGGTTTCCCCCAAAAGAGAGACAC

Appendix 10

Publications:

Lavial F, Acloque H, Bertocchini F, Macleod DJ, Boast S, Bachelard E, Montillet G, Thenot S, Sang HM, Stern CD, Samarut J, Pain B. (2007) The Oct4 homologue PouV and Nanog regulate pluripotency in chicken embryonic stem cells. *Development*. 134:3549-63

Development 134, 3549-3563 (2007) doi:10.1242/dev.006569

The Oct4 homologue PouV and Nanog regulate pluripotency in chicken embryonic stem cells

Fabrice Lavial[1], Hervé Acloque[1,*], Federica Bertocchini[2], David J. MacLeod[3], Sharon Boast[2], Elodie Bachelard[1], Guillaume Montillet[1], Sandrine Thenot[1], Helen M. Sang[3], Claudio D. Stern[2], Jacques Samarut[1] and Bertrand Pain[4,5,†]

Embryonic stem cells (ESC) have been isolated from pregastrulation mammalian embryos. The maintenance of their pluripotency and ability to self-renew has been shown to be governed by the transcription factors Oct4 (Pou5f1) and Nanog. *Oct4* appears to control cell-fate decisions of ESC in vitro and the choice between embryonic and trophectoderm cell fates in vivo. In non-mammalian vertebrates, the existence and functions of these factors are still under debate, although the identification of the zebrafish *pou2* (*spg*; *pou5f1*) and *Xenopus Pou91* (*XlPou91*) genes, which have important roles in maintaining uncommitted putative stem cell populations during early development, has suggested that these factors have common functions in all vertebrates. Using chicken ESC (cESC), which display similar properties of pluripotency and long-term self-renewal to mammalian ESC, we demonstrated the existence of an avian homologue of *Oct4* that we call chicken *PouV* (*cPouV*). We established that *cPouV* and the chicken *Nanog* gene are required for the maintenance of pluripotency and self-renewal of cESC. These findings show that the mechanisms by which *Oct4* and *Nanog* regulate pluripotency and self-renewal are not exclusive to mammals.

KEY WORDS: Nanog, Oct4, Avian homologue, cPouV, Stem cells

INTRODUCTION

Embryonic stem cells (ESC) are self-renewing pluripotent cells that can be maintained in culture for an indefinite period. In mammals, pluripotency is under the control of key transcription factors, including Oct4 (also known as Pou5f1 – Mouse Genome Informatics) (Nichols et al., 1998), Nanog (Mitsui et al., 2003; Chambers et al., 2003), Sox2 (Avilion et al., 2003) and FoxD3 (Hanna et al., 2002). Oct4 is found in oocytes and is expressed in cleavage stage cells up to the morula stage (Kirchhof et al., 2000), and subsequently in the epiblast of the pre-primitive streak stage embryos. *Oct4* expression is downregulated in trophectodermal cells but maintained in the inner cell mass, becoming restricted to primordial germ cells and oocytes (Kehler et al., 2004; Boiani et al., 2002). In vitro, *Oct4* is expressed in proliferating murine and primate (including human) ESC, as well as in tumourigenic cells such as embryonal carcinoma (EC) (Ben-Shushan et al., 1995) and germ cell tumour (GCT) cells (Looijenga et al., 2003).

Oct4 appears to control cell-fate decisions of ESC in vitro. Inhibition of *Oct4* expression in mouse ESC (mESC) causes a loss of proliferation and the induction of trophectodermal and endodermal markers (Velkey and O'Shea, 2003; Hay et al., 2004). By contrast, overexpression of *Oct4* leads to primitive endoderm differentiation (Niwa et al., 2000) and it appears that a fine balance between *Oct4* and *Cdx2* expression controls the choice between embryonic and trophectoderm cell fates (Niwa et al., 2005; Tolkunova et al., 2006).

Oct4 contains a POU-specific domain and a POU homeodomain and belongs to the class V POU homeodomain family of transcription factors. A complex of proteins including Oct4 and Sox2 has been found to regulate expression of the growth factor Fgf4 (Dailey et al., 1994; Ambrosetti et al., 1997) and of the transcription factors Utf1 (Nishimoto et al., 1999), Zfp42 (Rex1) (Ben-Shushan et al., 1998), Fbx15 (also known as Fbxo15 – Mouse Genome Informatics) (Tokuzawa et al., 2003), Nanog (Kuroda et al., 2005; Rodda et al., 2005) and Sox2 itself (Tomioka et al., 2002). Different nuclear receptors participate in the regulation of *Oct4* expression including Sf1 (Barnea and Bergman, 2000), Lrh-1 (Nr5a2) (Gu et al., 2005a), Gcnf (Nr6a1) (Fuhrmann et al., 2001; Gu et al., 2005b), CoupTF (Nr2f2) (Ben-Shushan et al., 1995) and Rar/Rxr heterodimers, the latter being responsible for the downregulation of *Oct4* expression by retinoic acid (Schoorlemmer et al., 1994; Pikarsky et al., 1994). *Oct4* expression is under the control of its own protein (Okumura-Nakanishi et al., 2005; Chew et al., 2005) through specific response elements located in its own promoter (Yeom et al., 1996; Nordhoff et al., 2001; Gu et al., 2005a; Gu et al., 2005b).

Nanog expression is also confined to pluripotent tissues and cell lines and its overexpression is able to maintain mESC in an undifferentiated state, even in the absence of Lifr/gp130 stimulation. Inhibition of *Nanog* expression in mESC results in their differentiation into primitive endoderm (Chambers et al., 2003; Mitsui et al., 2003).

To date, this relationship between *Oct4* and/or *Nanog* and stem cell pluripotency has only been demonstrated in mammals. Indeed, in zebrafish, it was reported that the *pou2* gene (also known as *spg* and *pou5f1* – ZFIN), initially identified by a mutation that caused neural and endoderm defects, is the fish homologue of the mammalian *Oct4* gene based on protein similarities, chromosomal syntenic relationship and developmental expression pattern, but not in terms of function (Burgess et al., 2002). No evaluation of a

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putative role in fish ESC pluripotency was described in an assessment of murine *Oct4* activity in medaka ESC (Hong et al., 1998). The *X. laevis Pou91* (*XlPou91*) gene product, encoded by one of three *X. laevis PouV* genes, has been demonstrated to have a similar activity to the mouse *Oct4* gene in mESC and to participate in the maintenance of putative stem cell populations during early development (Morrison and Brickman, 2006).

Given that *Oct4* appears to be so important in the maintenance of pluripotency, a report suggesting that the chicken genome lacks a homologue of *Oct4* (Soodeen-Karamath and Gibbins, 2001) was very surprising. Indeed, no corresponding sequence was identified in the chicken genome annotation, even in the latest release (Ensembl 42, December 2006).

Here we report the isolation of chicken *PouV* (*cPouV*) and *Nanog* (*cNanog*), homologues of mammalian *Oct4* and *Nanog*. Both genes are expressed in early embryos before gastrulation and thereafter in germ cells. Taking advantage of chick ESC (cESC) (Pain et al., 1996; Petitte et al., 2004), we demonstrate that chicken *PouV* and *Nanog* are required for the maintenance of cESC pluripotency and for continued proliferation. Together, these findings show that the mechanisms by which these two genes regulate pluripotency and self-renewal are not exclusive to mammals.

MATERIALS AND METHODS

Oligonucleotides and cDNA sequences

Oligonucleotides (Proligo) were designed using Primer 3 software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) and are listed in Table 1. The coding sequences of the different genes were identified using the chicken genome assembly (http://www.ensembl.org/Gallus_gallus/), GenBank (http://www.ncbi.nlm.nih.gov) or sequenced directly from newly isolated clones.

Subtractive liquid hybridisation

Total RNA from cESC and from 2-day-old chicken embryoid bodies (cEB) obtained as previously described (Pain et al., 1996) were reverse transcribed. The cDNAs were subject to a subtractive liquid hybridisation procedure (http://www.genome-express.com/). The transcripts enriched in cESC were subcloned, sequenced and filtered sequences assembled using PHRAP software. Target sequences delivered by the assembly process were subject to BLAST analysis.

Library screening and cloning of *cPouV*

The cDNA library from chicken embryonic stem cell mRNA (Acloque et al., 2001) was screened using T7 or T3 vector primers and internal sequences P06(381)S (5′-GTTGTCCGGGTCTGGTTCT-3′) or P06(382)AS (5′-GTGGAAAGGTGGCATGTAGAC-3′) derived from the 1P06g01 initial clone. A 5′-RACE strategy was developed with the P06RAAS2 (5′-TGAGTGAAGCCCAGCATGAT-3′) primer followed by a second amplification with P06RAAS1 (5′-AACATCTTCCCATA-GAGCGTGC-3′) and AnchPS (5′-GACCACGCGTATCGATGTC-GACTTTTTTTTTTTTTTT-3′) primers. A second round of amplification using P06(pL7-2)AS (5′-TGCTTGAGGTCCTTGGCAAA-3′) and PCRprimseq primers led to the isolation of 300 bp upstream of the 1P06g01 clone, including an in-frame ATG. A full-length cDNA was cloned into pGEM-T-easy (Promega) using primers P06EcoRIS (5′-AT-GAATTCATGCATGTAAAAGCCAAA-3′) and P06EcoRIAS (5′-AT-GAATTCTCAGTGGCTGCTGTTGTT-3′).

RNA extraction and RT-PCR

Real-time RT-PCR was performed using the MXP-3000P PCR-system (Stratagene) using Mix-Quantitect SYBR Green (Qiagen). Samples were run in duplicate and gene expression levels were calculated using the ΔΔCt method (http://www.gene-quantification.info) with the chicken ribosomal gene *RS17* (X07257) as reference. The number of independent experiments performed is indicated in each figure legend.

Table 1. Oligonucleotides used for gene expression analysis

Gene	Gene identification	Oligonucleotide (5′ to 3′) Sense	Oligonucleotide (5′ to 3′) Antisense
Chicken			
AP	U19108	CCTGACATCGAGGTGATCCT	CAAAGAGACCCAGCAGGAAG
Cdx2	NM_204311	TCAAAACCAGGACGAAGGAC	CCAGATTTTCACCTGCCTCT
Gata4	XM_420041	TGAGAAAAGAGGGCATTCAGA	GCAGGATGAATTGAAGATCCA
Gata6	NM_205420	CCGACCACTTGCTATGAAAAA	CAGCCCATCTTGACCTGAATA
Gcnf	ENSGALT00000001607	GTTTGCCAGGACTTCACAGAG	CGGGACATTCACCATCTTTC
Nanog	DQ867025	CAGCAGACCTCTCCTTGACC	TTCCTTGTCCCACTCTCACC
PouV	DQ867024	GTTGTCCGGGTCTGGTTCT	GTGGAAAGGTGGCATGTAGAC
Rarg	X73973	TCTACAAACCGTGCTTCGTCT	TCCTCCTTCACCTCCTTCTTC
RS17	X07257	ACACCCGTCTGGGCAACGACT	CCCGCTGGATGCGCTTCATCA
Sox2	U12532	GCAGAGAAAAGGGAAAAAGGA	TTTCCTAGGGAGGGGTATGAA
Sox3	U12467	TGTTCGCTTCCGAGTCTTAAA	CCTTTCCGTAGGAACAAAACC
Tert	AY502592	CCCAATAGAAGGGGCATAGAG	CTTGGTAACTGCGGGAATACA
Mouse			
brachyury	NM_009309	CCGGTGCTGAAGGTAAATGT	CCTCCATTGAGCTTGTTGGT
Cdx2	NM_007673	TCTCCGAGAGGCAGGTTAAA	GCAAGGAGGTCACAGGACTC
Fgf4	NM_010202	CGAGGGACAGTCTTCTGGAG	GTACGCGTAGGCTTCGTAGG
Gata4	AF179424	GCAGCAGCAGTGAAGAGATG	GCGATGTCTGAGTGACAGGA
Gata6	AF179425	GCCAACTGTCACACCACAAC	TGTTACCGGAGCAAGCTTTT
Hnf1 (*Tcf1*)	M57966	GATGTCAGGAGTGCGCTACA	CTGAGATTGCTGGGGATTGT
laminin B1	M15525	GTTCGAGGGAACTGCTTCTG	GTTCAGGCCTTTGGTGTTGT
Nanog	AY278951	AAGTACCTCAGCCTCCAGCA	GTGCTGAGCCCTTCTGAATC
Oct4 (*Pou5f1*)	NM_013633	CACGAGTGGAAAGCAACTCA	AGATGGTGGTCTGGCTGAAC
Rex1 (*Zfp42*)	NM_009556	GGCCAGTCCAGAATACCAGA	GAACTCGCTTCCAGAACCTG
Rs17 (*Rsp17*)	BC086901	ATGACTTCCACACCAACAAGC	GCCAACTGTAGGCTGAGTGAC
Sox17	NM_011441	CTCGGGGATGTAAAGGTGAA	GCTTCTCTGCCAAGGTCAAC
Sox2	U31967	CACAACTCGGAGATCAGCAA	CTCCGGGAAGCGTGTACTTA
Tert	AF051911	ACTCAGCAACCTCCAGCCTA	CATATTGGCACTCTGCATGG
Utf1	D31647	TTACGAGCACCGACACTCTG	GGCCCAGAACTGTTGAGATG

In situ hybridisation

Hens' eggs were incubated for 0-36 hours and embryos staged according to Eyal-Giladi and Kochav (Eyal-Giladi and Kochav, 1976) for pre-primitive streak stages and according to Hamburger and Hamilton (Hamburger and Hamilton, 1951) for later stages. Embryos were subjected to whole-mount in situ hybridisation (Streit and Stern, 2001). Fluorescent *Vasa* and *cPouV* probes were labelled with digoxigenin and fluorescein, respectively, and successively revealed using an HRP-coupled anti-digoxigenin and an HRP-coupled anti-fluorescein antibody and the TSA-Plus Cyanine3/Fluorescein system (Perkin Elmer). SSEA-1 labelling (DSHB, Iowa) was performed on frozen sections (15 μm) and revealed with an anti-mouse IgM conjugated to Texas Red (Abcam). Adjacent sections were processed for in situ hybridisation as previously described (Strähle et al., 1994).

Expression constructs

Reverse-transcribed chicken embryonic stem cell mRNA was used with P06GFPEcoRIS (5′-GGGAATTCGCATGTAAAAGCCAAA-3′) and P06GFPKpnIAS (5′-ATGGTACCTCAGTGGCTGCTGTTGT-3′) primers to amplify the *cPouV* coding region. The product was subcloned into pEGFP-C1 (Clontech) to produce the pGFP-cPouV expression vector. *cPouV* cDNA was amplified with P06EcoRIS (5′-ATGAATTCATG-CATGTAAAAGCCAAA-3′) and P06EcoRIAS (5′-ATGAATTCTCAG-TGGCTGCTGTTGTT-3′) primers and cloned into pCAGIP (Niwa et al., 2000). The 1.8 kb *pou2* zebrafish coding sequence was amplified from a pCSL2-Pou2 template using Pou2EcoRIS (5′-ATAGAATTCTATGAC-GGAGAGAGCGCAG-3′) and Pou2EcoRIAS (5′-GTAGAATTCTTAG-CTGGTGAGATGACCC-3′) primers and cloned into pCAGIP. Murine *Oct4* and *Nanog* coding sequences were reverse transcribed from mESC total RNA with primer pairs mOct4EcoRIS (5′-ATGAATTCTGCTGGAC-ACCTGGCTTC-3′) with mOct4EcorIAS (5′-ATGAATTCTTAACCC-CAAAGCTCCAG-3′) and mNanogXhoIS (5′-GTCTCGAGATGAGT-GTGGGTCTTCC-3′) with mNanogNotIAS (5′-ATGCGGCCGCTCAT-ATTTCACCTGGT-3′), respectively, then inserted into pCAGIP. The *cNanog* coding sequence was obtained from reverse-transcribed cESC total RNA using cNanogEcoRIS (5′-ATGAATTCATGAGCGCTCACCTG-GCC-3′) and cNanogEcoRIAS (5′-ATGAATTCCTAAGTCTCATAAC-CATT-3′) primers and cloned into pCAGIP.

Transactivation test

The p(ATGCAAAT)×3-luc reporter gene was constructed by inserting double-stranded oligonucleotides Oct4BS (5′-CTAGCATGCAAATAA-CAGCGCGCATGCAAATAACAGCGCATGCAAATAACAGCGCCCC-3′) and Oct4BAS (5′-GGGGCGCTGTTATTTGCATGCGCTGTTATT-TGCATGCGCGCTGTTATTTGCATG-3′) into the pGL3 vector (Promega). To construct the pΔPE-luc reporter gene, a 1.4 kb fragment from the *mOct4* distal enhancer was amplified from pGOF18ΔPE-GFP using ODES (5′-GTACGCGTGAATTCAGACAGGACTGCTGGGC-3′) and SVAS (5′-AGCATCACAAATTTCACAAATAAAGAATTCACG-GCTTT-3′) primers (Hong et al., 2004) and subcloned into pGL3. For luciferase assays, ZHBTc4 cells were plated at 1×10^5 cells per well with 2 μg/ml tetracyclin. Twenty-four hours later, 75 ng of reporter plasmid, 150 ng of the test plasmid and 10 ng of the *Renilla* reporter plasmid were co-transfected using 600 ng FuGENE 6 (Roche) and incubated overnight before fresh medium was added with 2 μg/ml tetracyclin. Cell lysates were analysed 48 hours after transfection as described by the manufacturer (Promega).

RNA interference (RNAi) vector construction

pFLΔNeo was obtained by inserting into pBSK the 2 kb PCR-amplified product mU6ΔNeoΔ, derived from the mU6ΔNeo-ΔApaIDXhoI template (Coumoul et al., 2004) using mU6SmaIS (5′-ATCCCGGGGTATATCC-GACGCCGCCAT-3′) and mU6HindIIIAS (5′-ATAAGCTTAACAAG-GCTTTTCTCC-3′) primers. Double-stranded short hairpin (sh) RNA was cloned into pFLΔNeo, generating pFLΔNeo-XshRNA vectors for each gene to be targeted. The oligonucleotides containing the *Hin*dIII and *Xho*I sites used for generating the 21 bp shRNA sequence were: cPouV-shRNA-2S (5′-AGCTTAAGATGTTCAGCCAGACCACCTTCAAGAGAGGTG-GTCTGGCTGAACATCTTTTTTTTC-3′) and cPouV-shRNA-2AS (5′-TCGAGAAAAAAAAGATGTTCAGCCAGACCACCTCTCTTGAAGG-TGGTCTGGCTGAACATCTTA-3′) against *cPouV*; cNanog-shRNA-1S (5′-AGCTTAACAGAAACCTTCAGGCTGTGTTCAAGAGACACAGC-CTGAAGGTTTCTGTTTTTTTTC-3′) and cNanog-shRNA-1AS (5′-TCGAGAAAAAAAACAGAAACCTTCAGGCTGTGTCTCTTGAACA-CAGCCTGAAGGTTTCTGTTA-3′) against *cNanog*; as well as cNanog-shRNA-3S (5′-AGCTTAAGGCCAAGAGCCGCACAGCTTTCAAGA-GAAGCTGTGCGCTCTTGGCCTTTTTTTTC-3′) and cNanog-shRNA-3AS (5′-TCGAGAAAAAAAAGGCCAAGAGCCGCACAGCTTCTCTT-GAAAGCTGTGCGGCTCTTGGCCTTA-3′) and cOct6-shRNA-3S (5′-AGCTTAAGCAGCGGCGGATCAAGCTGTTCAAGAGACAGCTTGA-TCCGCCGCTGCTTTTTTTTC-3′) and cOct6-shRNA-3AS (5′-TCGA-GAAAAAAAAGCAGCGGCGGATCAAGCTGTCTCTTGAACAGCT-TGATCCGCCGCTGCTTA-3′) against *cOct6*. The Cre-ERT2 coding sequence (Feil et al., 1997) was cloned into the pCIFL-Hygro vector, derived from pCINeo (Promega), by replacing the neomycin cassette with a hygromycin cassette to produce pCre-ERT2-Hygro.

Cell maintenance and transfection

cESC were maintained and transfected as previously described (Pain et al., 1996; Pain et al., 1999). For kinetic experiments, formation of cEB was achieved by allowing dissociated proliferating cESC to float in bacterial dishes. When used, retinoic acid was added at 10^{-7} M 24 hours after plating and considered as T=0. Cycloheximide and actinomycin D were added to the culture medium at 10 μg/ml for various times as indicated.

ZHBTc4 cells were maintained as described (Niwa et al., 2000). Expression of the endogenous murine *Oct4* can be downregulated by addition of 1 μM doxycyclin (Sigma). For transfection, 5×10^6 cells were electroporated (BioPulser, BioRad) at 575 μF with 25 μg of the various linearised vectors. From twenty-four hours after electroporation, doxycyclin was added at daily intervals, and puromycin was added 72 hours after electroporation at 1 μg/ml and administered daily for 6 days.

RNAi induction and proliferation assay

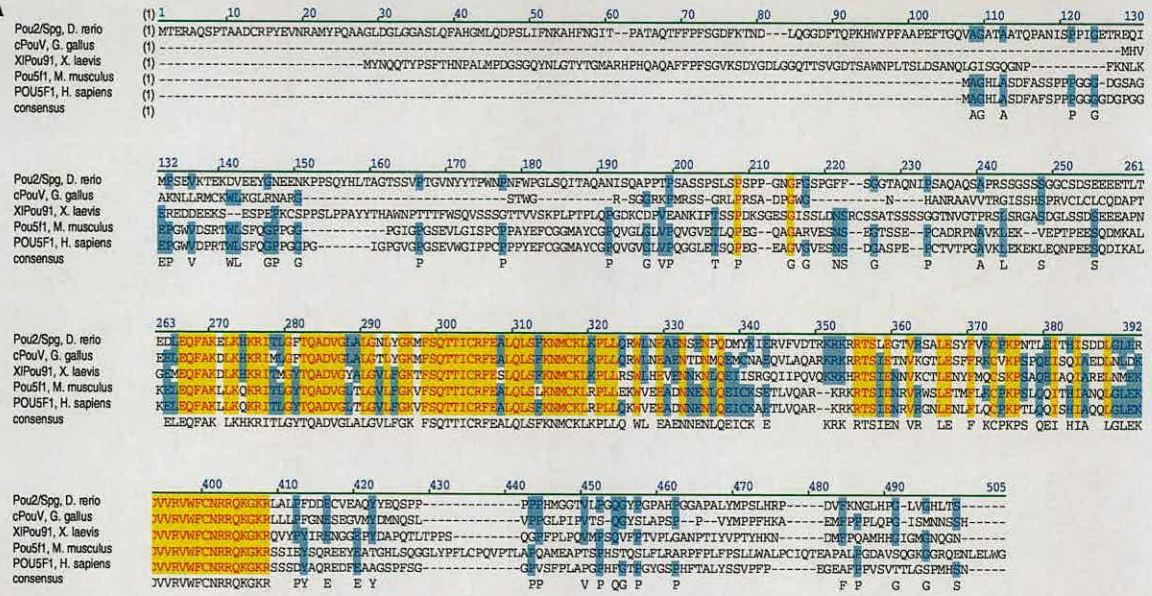
Once transfected and selected with 200 μg/ml neomycin for 7 days, resistant clones of cESC were pooled, transfected with the pCre-ERT2-Hygro vector and selected for 7 days with 0.75 μg/ml hygromycin. Clones were numbered, picked and individually observed during the induction of shRNA expression by adding 1 μM 4-hydroxytamoxifen to the medium. Morphology was assessed by direct microscopic observation and Wright Giemsa staining. For proliferation kinetic assays, clones were picked individually, the cells dispersed and plated in six wells in 250 μl medium. After 24 hours, 4-hydroxytamoxifen was added and proliferation was assessed at different times using two wells per time point using Cell Proliferation Kit II (XTT) (Roche).

RESULTS

Cloning of the chicken homologue of the mammalian *Oct4* gene

Subtractive hybridisation of cDNAs from cESC and chicken embryoid bodies (cEB) resulted in identification of a 228 bp cDNA fragment encoding a partial POU domain. A combination of the results of screening a cDNA library and 5′-RACE using mRNA from cESC, allowed us to define an open reading frame (ORF) of 888 bp coding for a 295 amino acid (aa) protein. Comparative analysis and phylogenetic tree construction using maximal parsimony and neighbour-joining methods revealed that this sequence is statistically more closely related to the other PouV proteins than to the other Pou factors (Fig. 1A) (Felsenstein, 1978; Saitou and Nei, 1987). This novel predicted protein is part of the PouV protein subfamily, which contains XlPou91, the *D. rerio* Pou2 and mammalian Pou5f1 proteins and exhibits high similarity with the other members of the family (Fig. 1B,C).

A



B

Species	Genbank nb	Bt	Ss	Hs	Pt	Mm	Tv	Dr	Gg	Xl		
										Pou25	Pou91	Pou60
B. taurus	NP 777005	100	97	93	93	74	58	32	51	33	34	33
S. scrofa	Q9TSV5		100	95	95	75	58	32	51	32	32	32
H. sapiens	NP 002692			100	100	76	57	31	51	33	35	34
P. troglodytes	Q7YR49				100	76	57	30	51	32	33	32
M. musculus	NP 038661					100	47	26	46	29	31	29
T. vulpecula	AY 345973						100	22	58	27	29	31
D. rerio	NP 571187							100	35	33	32	30
G. gallus	DQ 867024								100	37	37	38
X. laevis (Pou25)	AAA 49996									100	52	40
X. laevis (Pou91)	AAA 49999										100	38
X. laevis (Pou60)	AAA 49997											100

C

Species	Genbank nb	Homeodomain			Bt	Ss	Hs	Pt	Mm	Tv	Od	Dr	Gg	Xl		
		start	end	size										Pou25	Pou91	Pou60
B. taurus	NP 777005	142	288	146	100	100	97	98	92	69	48	67	67	63	65	55
S. scrofa	Q9TSV5	142	288	146		100	97	98	92	69	48	67	67	63	65	55
H. sapiens	NP 002692	142	289	147			100	99	93	69	48	65	67	63	66	56
P. troglodytes	Q7YR49	142	288	146				100	92	69	49	65	67	63	66	56
M. musculus	NP 038661	135	282	147					100	65	49	65	65	63	65	56
T. vulpecula	AY 345973	1	120	119						100	37	50	56	51	51	46
O. dioica	AAT 47873	224	372	148							100	44	48	42	48	46
D. rerio	NP 571187	253	402	149								100	76	66	63	60
G. gallus	DQ 867024	80	229	149									100	76	71	65
X. laevis (Pou25)	AAA 49996	231	380	149										100	76	68
X. laevis (Pou91)	AAA 49999	222	371	149											100	64
X. laevis (Pou60)	AAA 49997	206	355	149												100

D

	Gene	Nkx2.1	Nkx2.4	Nkx2.3		Nkx2.5				Nkx2.6	Nanog					Nkx2.2	
	Species	Gg	Mm	Gg	Mm	G. g	Xl	Hs	Mm	Mm	Mm	Rr	Hs	Hs-2	Gg	Hs	Mm
	Genbank Nb																
G. gallus, Nkx2.1	NP_989947	100	60	36	34	40	39	38	38	39	27	24	27	28	28	45	45
M. musculus, Nkx2.4	NP_075993		100	35	29	35	34	36	36	35	22	20	19	19	21	43	43
G. gallus, Nkx2.3	CAA66257			100	61	56	54	55	55	46	28	26	26	28	28	45	46
M. musculus, Nkx2.3	NP_032725				100	51	50	51	52	39	24	22	24	24	24	39	39
G. gallus, Nkx2.5	NP_990495					100	82	74	73	52	33	31	33	36	31	47	48
X. laevis, Nkx2.5	AAA19861						100	71	70	50	34	30	33	36	32	47	47
H. sapiens, NKX2.5	NP_004378							100	90	47	31	29	29	30	31	46	46
M. musculus, Nkx2.5	NP_032726								100	48	30	28	30	30	31	46	46
M. musculus, Nkx2.6	NP_035050									100	39	37	37	49	40	49	49
M. musculus, Nanog	BAC76998										100	80	66	59	44	33	33
R. rattus, Nanog	XP_575662											100	62	53	41	30	30
H. sapiens, NANOG	NP_079141												100	81	44	32	33
H. sapiens, NANOG2	AAS57555													100	41	33	33
G. gallus, Nanog	DQ 867025														100	33	33
H. sapiens, NKX2.2	NP_002500															100	99
M.musculus Nkx2.2	NP_035049																100

Fig. 1. See next page for legend.

Fig. 1. The *cPouV* gene encodes a chicken PouV protein. (**A**) Alignment of *D. rerio* Pou2 (NP_571187), *X. laevis* Pou91 (AAA49999.1), *M. musculus* Oct4 (Pou5f1, NP_038661) and *H. sapiens* OCT4 (POU5F1, NP_002692) PouV proteins with the chicken PouV coding sequence (DQ867024) using NTi Clustal software (Invitrogen). (**B,C**) Similarity table analysis of the full-length proteins (B) or of their homeodomains (C) of Oct4 homologues was performed with sequences of 360 aa for *B. taurus* (Bt) Oct4 (NP_777005), of 360 aa for *Sus scrofa* (Ss) Oct4 (Q9TSV5), of 360 aa for *H. sapiens* (Hs) OCT4 (NP_002692), of 360 aa for *P. troglodytes* (Pt) Oct4 (Q7YR49), of 352 aa for *M. musculus* (Mm) Oct4 (NP_038661), of 189 aa for *T. vulpecula* (Tv) Oct4 (AAQ24229), of 472 aa for *D. rerio* (Dr) Pou2 (NP_571187), of 448 aa for *X. laevis* (Xl) Pou25 (AAA49996), of 445 aa for *X. laevis* Pou91 (AAA49999) and of 426 aa for *X. laevis* Pou60 (AAA49997). The 295 aa were used for the *G. gallus* (Gg) PouV protein (DQ867024). (**D**) Similarity table analysis of Nkx and Nanog families performed with sequences of 344 aa for *G. gallus* (Gg) Nkx2.1 (NP_989947), of 354 aa for *M. musculus* (Mm) Nkx2.4 (NP_075993), of 323 aa for *G. gallus* Nkx2.3 (CAA66257), of 362 aa for *M. musculus* Nkx2.3 (NP_032725), of 294 aa for *G. gallus* Nkx2.5 (NP_990495), of 299 aa for *X. laevis* (Xl) Nkx2.5 (AAA19861), of 324 aa for *H. sapiens* (Hs) NKX2.5 (NP_004378), of 318 aa for *M. musculus* Nkx2.5 (NP_032726), of 216 aa for *M. musculus* Nkx2.6 (NP_035050), of 305 aa for *M. musculus* Nanog (BAC76998), of 312 aa for *R. rattus* (Rr) Nanog (XP_575662), of 305 aa for *H. sapiens* NANOG (NP_079141), of 232 aa for *H. sapiens* (Hs-2) NANOG2 (AAS57555), of 273 aa for *H. sapiens* NKX2.2 (NP_002500) and of 273 aa for *M. musculus* Nkx2.2 (NP_035049). The 310 aa were used for the chicken Nanog protein (DQ 867025). Red text and yellow highlights indicate a complete aa conservation between tested species; blue text and blue highlights indicate partial conservation between tested species.

At the genome level, the novel gene was mapped to chicken chromosome GGA17, specifically between primers SEQ0256 and SEQ0257 described in the ChickRH6 whole-genome radiation hybrid (WGRH) panel (http://chickrh.toulouse.inra.fr/). Syntenic comparison identified a relationship between this chicken gene, *XlPou91* and zebrafish *pou2*. This relationship appears to be absent, either deleted or displaced, in mammalian species, despite the presence of adjacent syntenic loci on mouse chromosome 2 (data not shown).

In conclusion, our data reveal the existence in the chicken genome of a gene belonging to the *PouV* gene subfamily. We will therefore henceforth refer to this new chicken gene as chicken *PouV* (*cPouV*) (GenBank accession DQ867024).

Cloning of chicken *Nanog* cDNA

A chicken *Nanog* gene was predicted in the chicken genome annotation at reference ID ENSGALG00000014319 on chicken chromosome 1 (GGA1). Primers designed using this sequence were used to isolate a clone from the chicken embryonic stem cell library with a 930 bp ORF (GenBank accession DQ867025). Comparative analysis and phylogenetic tree construction revealed that this sequence is closely related to mammalian *Nanog* genes and that the predicted protein exhibits high similarity with the other Nanog proteins (Fig. 1D). This sequence contains a homeodomain of 57 aa, located between aa 98 and 155, but does not have the WWW repeat in the C-terminus that is characteristic of the mammalian Nanog subfamily (Pan and Pei, 2005). In contrast to the recently reported chicken Nanog sequence identified in silico (Canon et al., 2006), our cloned protein does not indicate the existence of a 112 aa segment after aa 50 that could correspond to a putative alternatively spliced form.

cPouV and *cNanog* are highly expressed in proliferating cESC and downregulated during differentiation of cESC

To determine the expression profiles of *cNanog* and *cPouV*, we performed real-time RT-PCR experiments showing that proliferating cESC express high levels of *cPouV* and *cNanog* (Fig. 2A,D, time 0). cESC can be induced to differentiate either as cEB, by preventing cell attachment, or following treatment with chemical inducers such as DMSO or retinoic acid (RA) (Pain et al., 1996). During a 5-day RA treatment, *cPouV* expression was almost completely abolished in parallel to similar reductions in expression of the markers alkaline phosphatase (*AP*) and telomerase reverse transcriptase (*Tert*) (Fig. 2A). Chicken *Gcnf* expression was also strongly downregulated, as was expression of *Sox2* and *Nanog*, although with a more complex profile. By contrast, *Rarγ* expression was upregulated following RA treatment (Fig. 2A). Treatment with cycloheximide, known to block de novo protein synthesis, did not affect the downregulation of *cPouV* and *Gcnf* transcription, whereas *Nanog* transcription was no longer responsive to RA, suggesting that downregulation of *cPouV* and *Gcnf* transcription are direct transcriptional events following RA treatment (Fig. 2B). Moreover, following actinomycin D treatment, which blocks transcription, a 50% decrease in the expression of *cPouV* and *Gcnf* was observed 8 to 12 hours after addition of the drug. A decrease of greater than 50% in *Nanog* mRNA levels was seen as early as 30 minutes after treatment, suggesting that it has a very short half-life (Fig. 2C).

Expression of *cPouV* and *Nanog* was also strongly downregulated during formation of cEB (Fig. 2D), as was expression of *Sox2* and *AP*. By contrast, *Sox3*, *Cdx2* and *Gata6* were induced, suggesting a complex differentiation process during formation of these three-dimensional bodies (Fig. 2E).

cPouV and *cNanog* are expressed dynamically in early chick embryos

To determine the likely sites at which *cPouV* and *cNanog* function during normal development, transcripts were identified in embryos during progressive stages of development using whole-mount in situ hybridisation. *cPouV* mRNA was found to be ubiquitously expressed in the epiblast of pre-primitive streak stage embryos and in a salt-and-pepper fashion in the forming hypoblast (Fig. 3A,B,B′). As the primitive streak started to form, transcripts were strongly localised in the epiblast of the streak itself (Fig. 3C,C′) and in the mesoderm emerging from it, whereas expression in the lower layer tended to decrease (Fig. 3D-F,D′,F′). Expression in the area opaca was lost by stage 3^+ (Fig. 3F). At later stages, *cPouV* continued to be expressed in the mesoderm, but was undetectable in the endoderm (Fig. 3G-I). At stage 8 and subsequently, *cPouV* was strongly expressed in the neural plate and neural tube with particularly strong expression in the anterior hindbrain/posterior midbrain (Fig. 3I). Later, at stage 9 and subsequently, *cPouV* was still expressed in neural tissue and expression appeared in primordial germ cells (Fig. 3J).

cNanog showed a different pattern. In pre-streak embryos, transcripts were detected in the whole epiblast but not in the forming hypoblast (Fig. 4A,B,B′). As the primitive streak started to form, transcripts disappeared from the primitive streak epiblast but were still expressed throughout the area pellucida epiblast (Fig. 4C-E,C′,D′). At the end of gastrulation (stage 4^-4^+), *cNanog* mRNA was quickly downregulated in the epiblast and persisted in a crescent anterior to the emerging head process (Fig. 4F-H). As the neural plate formed (stages 6-8), expression in the epiblast was restricted to the anterior neural plate (Fig. 4H-J,H′).

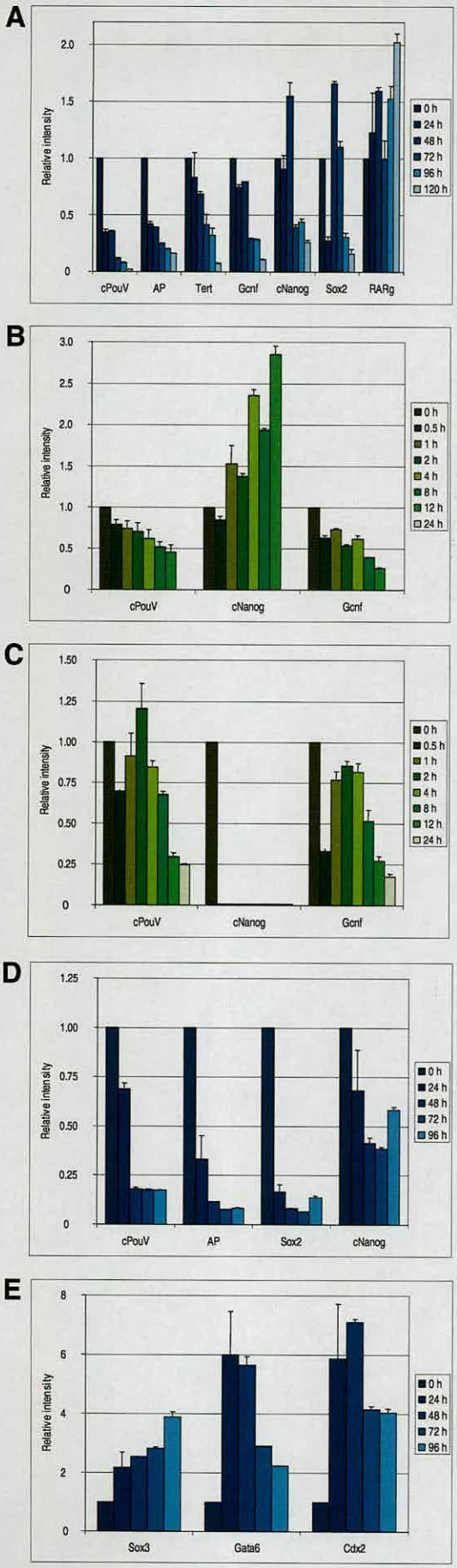


Fig. 2. Kinetics of expression of pluripotency genes during differentiation of cESC. Proliferating cESC were induced to differentiate (**A**) by retinoic acid treatment at 10^{-7} M for 5 days after plating or (**D**,**E**) by embryoid body formation for 4 days. Five independent experiments provided similar results. (**B**,**C**) As in A except that cycloheximide (B) or actinomycin D (C) was added to the culture medium at 10 μg/ml at T=0; two independent experiments provided similar results. Expression of some of the genes analysed, as measured by real-time RT-PCR, was downregulated (D) or upregulated (E). A value of 1 was assigned to expression levels at T=0, i.e. at the start of the induction of differentiation.

In conclusion, following initial, high levels of expression in early pluripotent epiblast cells, *cPouV* and *cNanog* present a very restricted in vivo pattern of expression during early embryonic development.

cPouV and *cNanog* are expressed in the germ cells during late embryonic development

In order to determine the expression profile of *cPouV* and *cNanog* during late embryonic development, quantitative (Q) RT-PCR analysis was performed on chicken embryo tissues at day 16 to 17, including intestine, muscle, kidney, spleen, lung, brain, liver, heart and gonads. Expression was detected in gonads (male and female, data not shown) but at a level 270-fold lower than in proliferative cESC, and also in spleen and brain but 530-fold and 1100-fold lower, respectively, than in cESC (data not shown). In situ hybridisations confirmed that *cPouV* is expressed in gonads, with expression restricted to germ cells. At stage 33 (7 days of incubation), *cPouV* expression was detected in a salt-and-pepper fashion in the forming gonads (Fig. 5A,B). The *cPouV*-positive cells were found to also express the germ-cell-specific markers *Cvh* (by mRNA detection, Fig. 5C-F) and SSEA-1 (by immunostaining, Fig. 5G,H). *Sox2* and *Cvh*, the expression of which is high in embryonic brain and gonads, respectively, were used as control gene markers for tissue specificity (data not shown).

cNanog expression was also detected by QRT-PCR in heart, brain, kidney and gonads, but at levels 20-, 25-, 90- and 100-fold lower, respectively, than in cESC (data not shown). In early embryos, *Nanog* was also expressed in scattered cells in the germinal crescent: these cells are likely to correspond to the future germ cells (Fig. 4I and arrowhead in H′). Later in development, *cNanog* was still expressed in germ cells at stage 33 (Fig. 5I-K) identified as SSEA-1–positive, in a similar manner to the *cPouV*-expressing cells (Fig. 5L,M). However, *cNanog* expression became weaker at this stage compared to the previous stages. This expression profile was observed in both male and female embryos (data not shown).

In conclusion, expression of *cPouV* and *Nanog* becomes restricted to germ cells at later stages of embryonic development.

Overexpression of *Oct4*-related genes in cESC and mESC

In order to compare *cPouV* function with its orthologues, coding sequences of *cPouV*, murine *Oct4* (*mOct4*), *XlPou91* and zebrafish *pou2* were transfected into cESC and mESC. In cESC, overexpression of *cPouV* using the pCAGIP vector impaired the isolation of proliferating clones. Using pCMV-based vectors, gene expression analysis revealed a 4-fold induction of *cPouV*, but a strong decrease in expression of *Nanog* and *Tert*. By contrast,

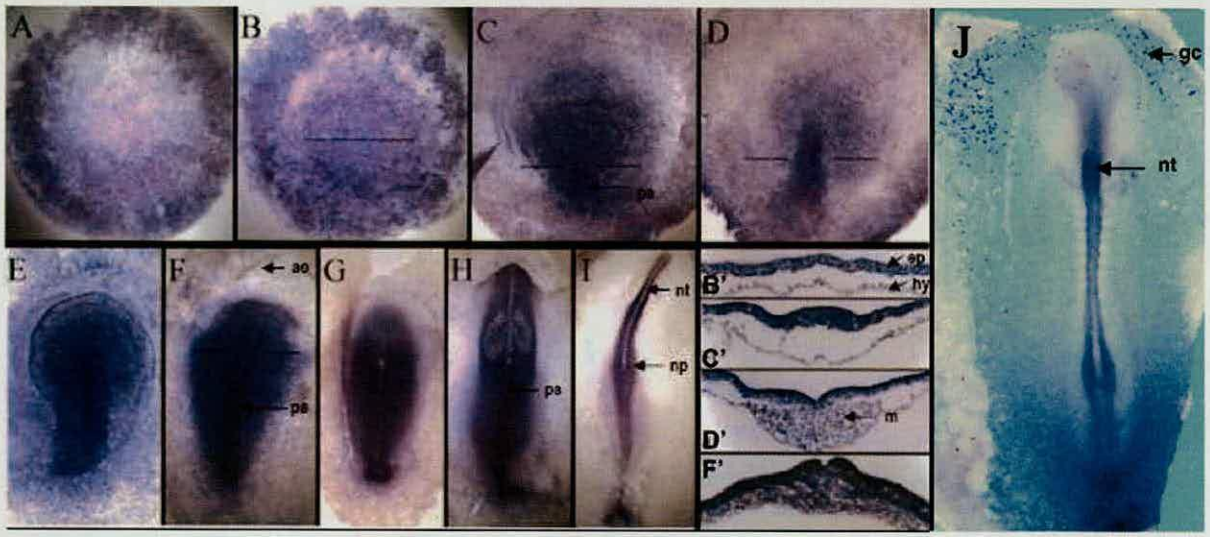


Fig. 3. *cPouV* expression during chick embryo development. (**A-J**) Whole-mount in situ hybridisation to *cPouV* transcripts. Transcripts are detected in the area pellucida and area opaca of the epiblast in pre-streak embryos (A, stage XI; B, stage XIII), and in the hypoblast in a salt-and-pepper manner (B'). At stage XIV (C), the expression is very strong in the area pellucida of the epiblast, especially where the streak is forming (C'). Transcripts are expressed in the ingressing mesoderm at stages 2-3 (D,D'). As the primitive streak elongates and the embryo grows, expression is still detected in the ectoderm and mesoderm (E, stage 3+; F,F', stage 4+; G, stage 5). At stage 7 (H) and 8 (I), *cPouV* mRNA is detected in the forming neural tube and in the underlying mesoderm, but is absent from the endoderm (data not shown). At stage 9 (J), *cPouV* mRNA is expressed in neural tissue (nt) and presumptive migrating germ cells (gc). B',C',D',F' are transverse sections of the embryos in B,C,D,F, respectively. ao, area opaca; ep, epiblast; gc, germ cells; hy, hypoblast; m, mesoderm; np, neural plate; nt, neural tube; ps, primitive streak.

strong upregulation of *Gata4*, *Gata6* and *Cdx2*, associated with differentiation, was observed (Fig. 6A). Ectopic expression of *XlPou91* induced a similar expression profile, with an increase in endogenous *cPouV* and of differentiation markers *Gata4*, *Gata6* and *Cdx2*, and a strong decrease in *Nanog* and *Tert* expression (Fig. 6B). By contrast, overexpression of *mOct4* did not modify *cPouV* or *Nanog* endogenous expression levels and induced only a slight increase in endogenous chicken *Cdx2* gene expression (Fig. 6B).

As previously described (Niwa et al., 2002), it was not possible to isolate clones of cells overexpressing *mOct4* after transfection of mESC with the neomycin resistance overexpression plasmid. The same pCAGIP vector was used to overexpress *cPouV* and *XlPou91* in mESC, but clones could only be isolated of mESC expressing *XlPou91*. Endogenous expression of *Oct4* was maintained and expression of *Gata4*, *Gata6* and *Cdx2*, as well as of mesendodermal markers including *Hnf1*, brachyury, *Sox17* and laminin B1, was observed (Fig. 6C). Using a pCMV-based expression vector, *cPouV* expression enabled identification of clones presenting a similar expression profile, i.e. with a maintenance of endogenous *Oct4* expression but only a slight increase in *Gata4*, *Gata6*, *Cdx2*, *Hnf1*, brachyury, *Sox17* and laminin B1 expression (Fig. 6D).

In conclusion, high ectopic expression of *cPouV* impairs the proliferation of both cESC and mESC, but a moderate level of expression of exogenous *cPouV* is tolerated by cESC and mESC with an associated modification of the observed gene expression profile.

cPouV is able to rescue partially *Oct4*-deficient ZHBTc4 mESC

A good test of whether *cPouV* is functionally equivalent to its murine counterpart *Oct4*, is to assay the ability of the chick gene to rescue the ZHBTc4-inducible cells in which endogenous *Oct4* expression is downregulated by addition of doxycyclin. Transfection of ZHBTc4 cells with expression vectors for *mOct4* or *XlPou91* allowed isolation of proliferating clones in the presence or absence of doxycyclin, (Fig. 7A,B) as predicted (Niwa et al., 2000; Morrison and Brickman, 2006). In the presence of doxycyclin, expression of *cPouV* was able to support the growth of slowly proliferating AP-positive colonies (Fig. 7C) with a rescue index (the ratio between the number of clones in the presence versus the absence of doxycyclin) of 0.5 (Fig. 7D), as compared with 1.0 for expression of *mOct4* and 3.5 for *XlPou91*. However, the colonies recovered after *cPouV* expression were limited in their capacity to be passaged or amplified and exhibited a differentiated morphology. No clones were obtained after expression of zebrafish *pou2* in the presence of doxycyclin.

Real-time RT-PCR analysis performed on RNA from the clones generated by *cPouV* complementation revealed a complete loss of endogenous *mOct4* mRNA, but high expression of the exogenous *cPouV* mRNA (data not shown). Expression of pluripotency-associated markers such as *Nanog*, *Sox2*, *Utf1* and *Zfp42* (*Rex1*) was maintained at the same level in cells complemented by *XlPou91* as in cells complemented by *mOct4* (and expression was even higher for *Tert* and *Fgf4*). Expression of these markers was reduced, but detectable, in cells complemented by *cPouV*, with the exception of *Sox2* and *Fgf4* for which no expression could be detected in the presence of *cPouV* (Fig. 7E).

To test the ability of this gene to transactivate specific *Oct4*-responsive elements, promoters containing either the *mOct4* consensus binding site (ATGCAAAT), or the 1.4 kb ΔPE fragment from the *mOct4* promoter (Yeom et al., 1996; Hong et al., 2004), linked to a luciferase reporter, were transfected into ZHBTc4 cells. These promoters were activated in ZHBTc4 cells treated with doxycyclin in the presence of the expression vectors coding for *mOct4*, *XlPou91* or *cPouV*, as measured by luciferase activity (Fig. 7F). This interesting result suggests that the cPouV protein is able to recognise *mOct4*-response elements and activate transcription.

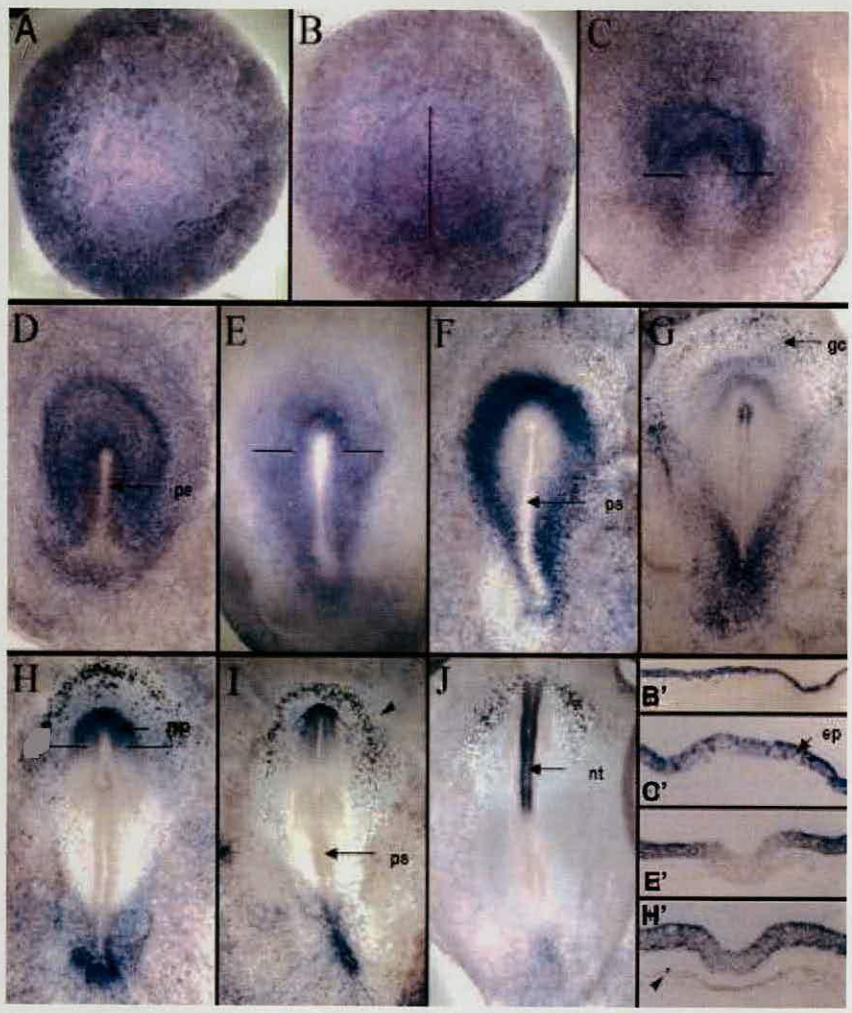


Fig. 4. *cNanog* expression during chick embryo development. (**A-J**) Whole-mount in situ hybridisation to *cNanog* transcripts. *Nanog* transcripts are localised in the epiblast of the area pellucida and area opaca of the pre-streak embryo (A, stage XI; B, stage XII), but not in the hypoblast (B′). From stage XIV, *cNanog* mRNA disappears from the posterior area pellucida (C,C′) and from the growing primitive streak (D, stage 3; E,E′, stage 3+; F, stage 4+). *cNanog* transcripts are downregulated in the epiblast from stage 4+ (F), and are confined anteriorly in a crescent region in the epiblast (G, stage 5+). At stage 6 (H), expression is restricted to the neural plate and the neural tube (I, stage 7; J, stage 8). *cNanog* is also expressed in scattered cells in the germinal crescent from stage 4 (arrowhead in I and H′). B′ is a longitudinal section of the embryo in B, anterior at the right; C′,E′,H′ are transverse sections of embryos in C,E,H, respectively. ep, epiblast; gd, gonad; ms, mesonephros; np, neural plate; nt, neural tube; ps, primitive streak.

In conclusion, these experiments suggest that the c*PouV* gene is able to partially rescue the loss of *mOct4* function in mESC, and does interact with and activate *mOct4*-dependent regulatory elements.

cNanog function in ES cells

Overexpression of mouse Nanog protein in mESC results in growth factor-independent maintenance of the pluripotent cell phenotype. To test whether overexpression of *cNanog* can confer the same growth-factor independence on mESC, proliferation of mESC was assessed in the absence of LIF, after transfection of a *cNanog* expression plasmid. Colonies did form in the absence of LIF, indicating that *cNanog* is able to confer growth factor independence (Fig. 8A). In the absence of LIF, the transfected cells were undistinguishable from the parental cells, on the basis of morphology, AP staining and growth rate (Fig. 8B-G). Real-time RT-PCR analysis of these proliferating clones indicated that expression of pluripotent factors, including *mOct4* and *Sox2*, was maintained, but with the notable exception of *Fgf4*, the expression of which was almost completely abolished (Fig. 8H).

In contrast to mESC, cESC are not dependent on a single cytokine for their proliferation and survival (Pain et al., 1996) (our unpublished results). Overexpression of *cNanog* conferred the ability of the cESC to grow in a low-serum medium in the absence of growth factors and cytokines that are usually required for proliferation (Fig. 8I). The clones obtained proliferated actively and were easily passaged and amplified (data not shown). It was particularly surprising to obtain proliferative avian primary stem cells in the presence of only 1% foetal bovine serum. Interestingly, under these drastic conditions, *mNanog* expression did not have any pronounced effect on the chicken cells. Real-time RT-PCR analysis confirmed overexpression of *cNanog*, the maintenance of *Tert* and reduced, but detectable, expression of *cPouV* and *AP* (Fig. 8J-K).

In conclusion, we have shown that *cNanog* functions in a very similar way to *mNanog* in mESC and has a more dramatic effect in cESC, where overexpression permits maintenance of the stem cell phenotype in the absence of growth factors and in low serum.

Inactivation of *cPouV* or *cNanog* inhibits ES cell proliferation and induces differentiation

To assess *cPouV* and *cNanog* function, constructs expressing shRNAs were designed to knockdown transcripts of these genes, using a tamoxifen-inducible Cre system to activate the expression of the shRNAs. Following induction of Cre recombinase activity by tamoxifen addition, a rapid and dramatic morphological change was observed, involving changes associated with differentiation (Fig. 9A-D). These changes were seen in ~60% of the clones when specific shRNAs were used against *cPouV* and *cNanog* (Fig. 9G) This morphological change was observed even in the presence of growth factors and was accompanied by a loss of AP activity and of SSEA-1 antibody staining (Fig. 9H), plus a growth rate alteration 48 and 96 hours after Cre induction (Fig. 9I). Comparison of the gene

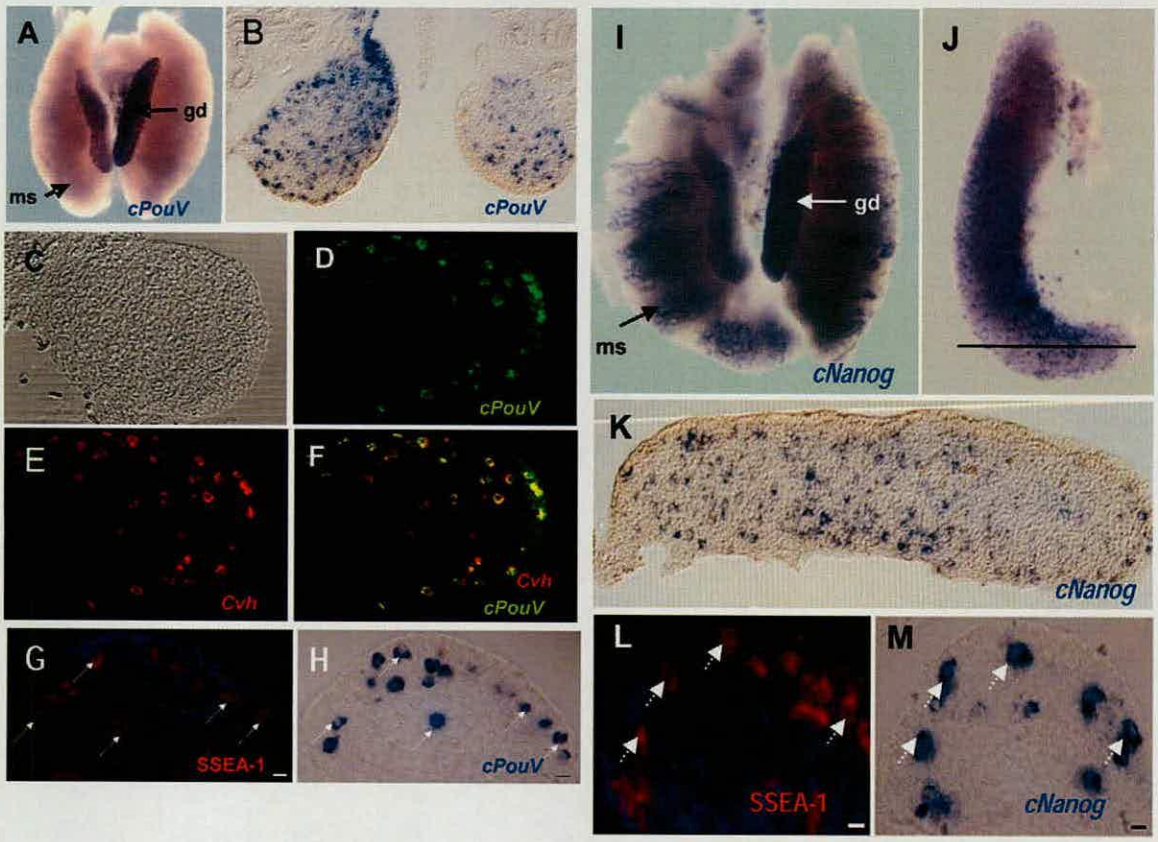


Fig. 5. *cPouV* and *cNanog* are expressed in germ cells during later embryonic development. (**A-H**) At stage 33, *cPouV* mRNA is detected in the developing gonad, which is attached to the mesonephros (A,B), in the germ cells (D), as detected by co-localisation (F) of *cPouV* (D) both with *Cvh* (chicken *Vasa*, E) expression and with SSEA-1-positive cells (G) revealed on adjacent sections counterstained by Hoechst and *cPouV* probe (H, arrows). (B) Section of gonad shown in A. (C) Bright field of the stage 33 gonad used for in situ hybridisation (D,E,F). (**I-M**) At stage 33, *cNanog* is highly expressed in gonads and in mesonophros tubules (I) and gonad (I,J), especially in germ cells (K), as revealed by SSEA-1 staining (L) on the same cells that express cNanog in adjacent sections (M, arrow) counterstained by Hoechst (L). (K) Section of dissected gonad from the urogenital tract (J). ms, mesonephros; gd, gonads. Scale bars: 15 μm.

expression profiles between differentiated clones and clones that continued to proliferate revealed strong inhibition of endogenous *cPouV* expression as well as of *cNanog* and *Gata4* and strong induction of *Gata6* (Fig. 9J). No upregulation of *Cdx2* was detected, nor of other mesendodermal markers such brachyury or *Hnf3β*. Similar experiments involving inhibition of another POU-domain gene, *Oct6* (Levavasseur et al., 1998), did not change the endogenous level of *cPouV* and proliferating clones were obtained (Fig. 9G,J).

When a similar analysis was performed using shRNA directed against *cNanog* mRNA, a similar process of differentiation occurred, with thin cytoplasmic protrusions (Fig. 9E,F), a loss of AP activity and SSEA-1 staining (Fig. 9G) and reduced proliferation (Fig. 9H). This phenomenon was observed with two distinct sequences, shRNA-1 and shRNA-3. Real-time RT-PCR expression analysis showed a drastic decrease in the expression of *cPouV*, *Gcnf* and *Gata4* (Fig. 9I) and an induction of *Gata6*.

In conclusion, inhibition of either *cPouV* or *cNanog* leads to a loss of proliferation of cESC and to the induction of differentiation.

DISCUSSION

Oct4 is established as one of the key factors controlling pluripotency and the unique self-renewing property of mammalian ESC (Chambers and Smith, 2004). Both overexpression and disruption of *Oct4* in mESC leads to a loss of pluripotency and induces the cells to differentiate into primitive endoderm, characterised by high *Gata6* expression (Li et al., 2004), and into trophectoderm expressing *Cdx2* (Niwa et al., 2000; Strumpf et al., 2005; Niwa et al., 2005; Tolkunova et al., 2006). In vivo, it is now thought that complex regulatory mechanisms lead to restricted expression in early pregastrulation embryos (Gu et al., 2005a; Boiani et al., 2002) and in the germ line (Kehler et al., 2004; Yeom et al., 1996).

Nanog, a homeodomain transcription factor, was identified as another key factor maintaining the pluripotency of mammalian ESC (Chambers et al., 2003; Mitsui et al., 2003; Hart et al., 2004). In mESC, *Nanog* overexpression has been shown to substitute for the requirement for growth factors in the maintenance of self-renewal. Disruption of *Nanog* leads to a loss of pluripotency and to induction of differentiation towards an endoderm-like state (Mitsui et al., 2003).

The existence and equivalent functions of homologues of these genes in non-mammalian vertebrates are still debated. Functional assays were used to identify the zebrafish *pou2* gene as the *Oct4* homologue (Burgess et al., 2002), but this gene appears to be mainly involved in the endoderm-specification cascade (Reim et al., 2004; Lunde et al., 2004). In *Xenopus*, *XlPou91*, a *PouV* gene, plays a significant role in the maintenance of pluripotent cells during early development and was shown to rescue *Oct4* depletion in mESC.

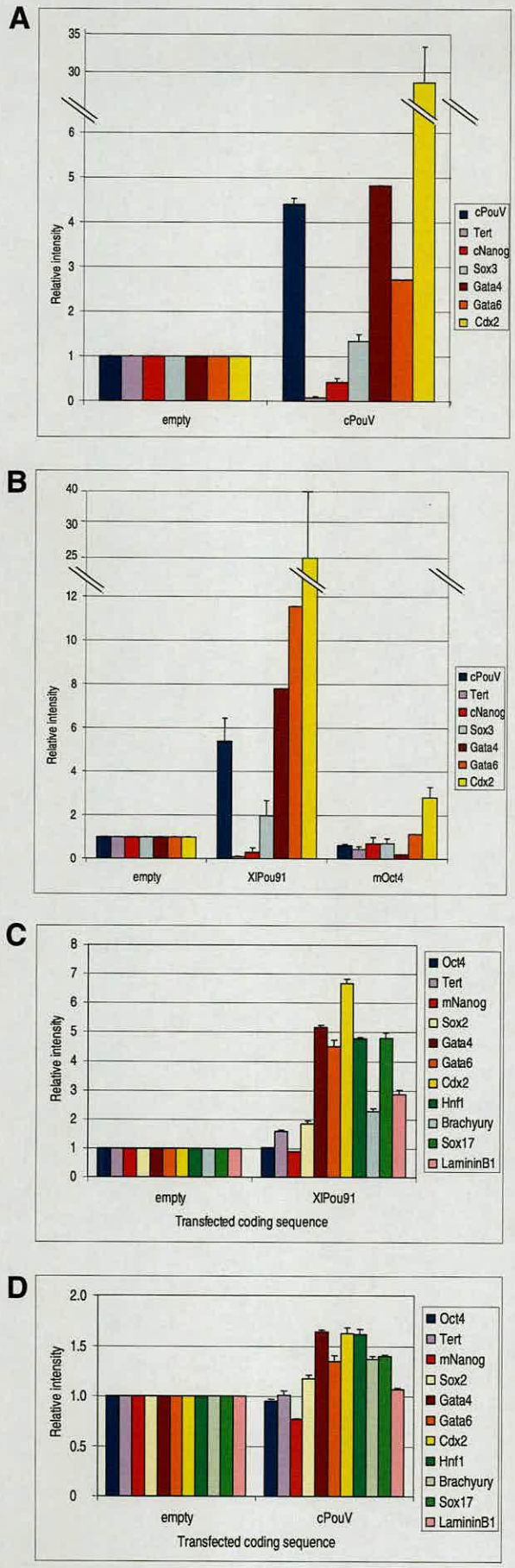


Fig. 6. Overexpression of *cPouV* in cESC and mESC. (**A**) Following transfection of the different *PouV* coding sequences, overexpression of *cPouV* in cESC revealed loss of endogenous *cNanog* expression and an increase in *Gata4*, *Gata6* and *Cdx2* expression. (**B**) A similar profile was observed for *XlPou91*, but only a slight increase in *Cdx2* when *mOct4* is transfected in cESC. (**C**,**D**) Upregulation of *Gata4*, *Gata6* and *Cdx2* is strong (C) when *XlPou91* is overexpressed in mESC with an induction of other markers (*Hnf1*, brachyury, *Sox17* and laminin B1), in contrast to a moderate induction when *cPouV* is overexpressed in mESC (D). A value of 1 was given to the gene expression level obtained in clones transfected with the empty vector (empty). Two independent experiments provided similar results.

XlPou91 knockdown in vivo using morpholinos induces expression of *Xcad3*, which is considered to be the *Xenopus* homologue of *Cdx2* (Morrison and Brickman, 2006). These data are consistent with the idea that *PouV* family members, including murine *Oct4*, could act to prevent premature commitment of pluripotent cells present in vertebrate embryos prior to and during gastrulation.

cESC have been isolated and maintained in culture for long periods (Pain et al., 1996; Petitte et al., 2004; Van de Lavoir et al., 2006). These cells were derived from the culture of pre-primitive streak blastodermal cells and are characterised by the presence of typical ESC markers such as AP, Tert activity and reactivity with particular antibodies including ECMA-7, SSEA-1, SSEA-3 and EMA-1 (Pain et al., 1996; Petitte et al., 2004).

In a differential screen from proliferative cESC and cEB, we identified a new coding sequence containing a POU domain. Several strands of evidence support the view that this gene is the chicken homologue of mammalian *Oct4*. First, comparative analysis and phylogenetic tree construction reveal that this sequence belongs, with high probability, to the *PouV* subfamily. Genomic analysis also demonstrates a clear syntenic conservation of the different loci between the non-mammalian species. We therefore refer to this gene as chicken *PouV* (*cPouV*). Second, this gene is expressed in vitro only in proliferating ESC. Its expression is rapidly downregulated once differentiation is induced by RA or during formation of cEB. This downregulation is maintained in the presence of cycloheximide, suggesting a direct effect of RA on transcription.

Third, *cPouV* is expressed in a complex pattern in the embryo, being expressed widely in the early epiblast and later becoming restricted to specific regions, including the mesoderm and nervous system. This initial expression in multipotent epiblast cells, which then becomes restricted once the cells start to be committed, is also shared by the zebrafish, *Xenopus* and mouse homologues, which have been implicated in regulation of early neural development and patterning (Ramos-Mejia et al., 2005; Burgess et al., 2002; Reim and Brand, 2002; Morrison and Brickman, 2006). During late development, *cPouV* expression becomes more restricted to migrating and proliferating germ cells, as demonstrated by co-localisation with *Cvh*-positive cells in the developing gonads. This germ-line-restricted expression is a feature shared with its murine counterpart, in contrast to the zebrafish and *Xenopus* homologues. We conclude that *cPouV* plays a similar role to its mammalian and non-mammalian homologues in pregastrulating embryos, but functions more like the mammalian homologue in germ cells.

Another feature of this chicken gene is its ability to induce differentiation when overexpressed in cESC. Expression of *cPouV* in cESC and mESC alters the morphology and reduces the growth

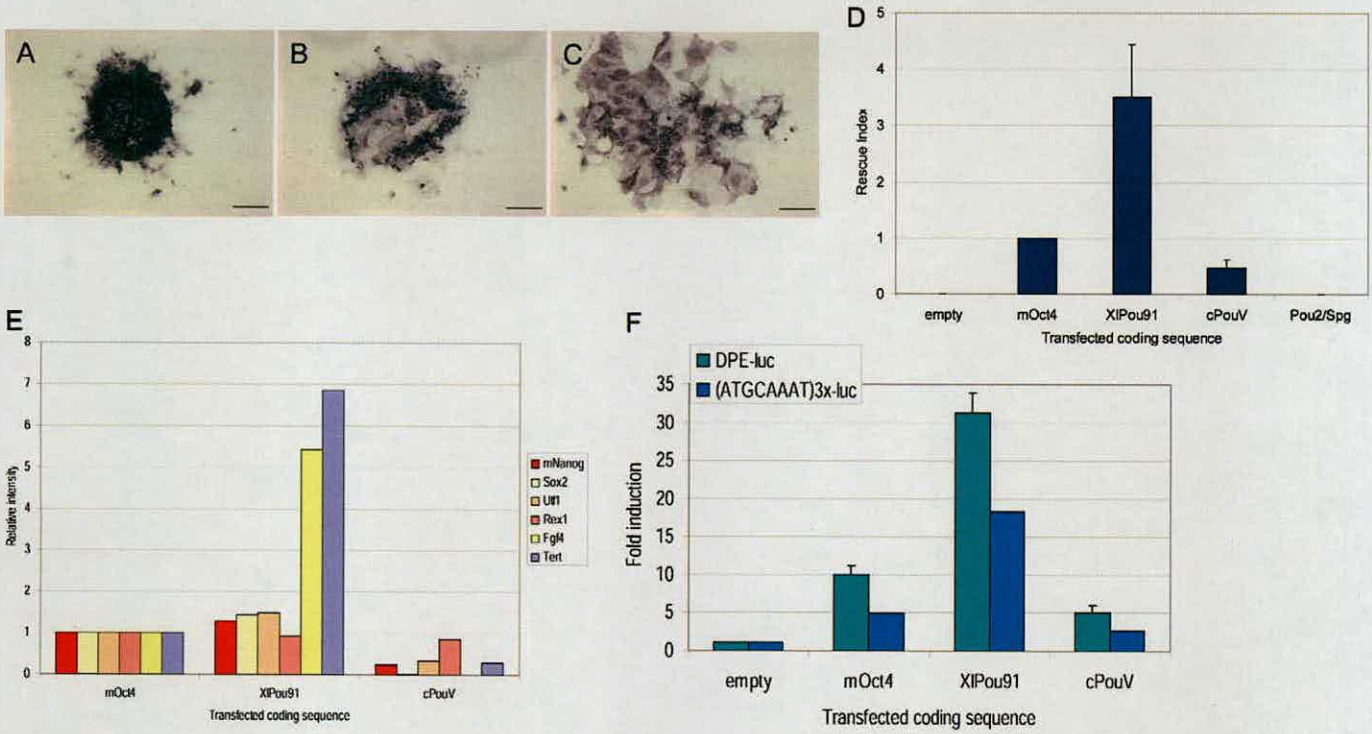


Fig. 7. *Oct4*-deficient ZHBTc4 mESC are only partially rescued by *cPouV* expression. ZHBTc4 cells transfected with *mOct4*, *XlPou91*, *cPouV* or *pou2* expression vectors were treated with doxycyclin after selection of stable clones. AP-positive clones were obtained with *mOct4* (**A**), *XlPou91* (**B**) and *cPouV* (**C**), but no clones were isolated with empty control vector (empty) or *pou2*. A rescue index (RI, the ratio between the number of clones in the presence versus the absence of doxycyclin) of 1 is given in the presence of *mOct4* (**D**). This RI is the result of two independent experiments with a total number of clones of 114/0, 82/19, 123/95, 74/8 and 82/0, respectively, in the absence/presence of doxycylin for the empty vector, the *mOct4*, *XlPou91*, *cPouV* and *pou2* expression vectors. (**E**) Expression of pluripotency-associated genes in ZHBTc4 complemented clones was analysed by real-time RT-PCR. A value of 1 was given to the level detected in the *mOct4*-complemented clones. *Nanog*, *Utf1*, *Zfp42* (*Rex1*) and *Tert* expression was lower in clones complemented by *cPouV* than in those complemented with *XlPou91*. (**F**) ZHBTc4 cells were co-transfected in the presence of luciferase reporter gene driven either by the ΔPE promoter or the Oct4 consensus sequence (ATGCAAAT). A value of 1 was given to the empty vector. Each result is the average of four wells per condition, and two independent experiments provided similar results. *cPouV* expression activated both promoters.

rate of the ESC, and inhibits isolation of clones from cells in which *cPouV* is expressed from a very strong promoter. Expression using a moderate CMV promoter induces expression of differentiation markers such as *Gata4*, *Gata6* and *Cdx2*. In mouse, these markers are associated with endodermal and trophectodermal lineages, but their function during early chicken development is still unknown. A similar profile of gene expression is obtained in parallel experiments with *XlPou91*, suggesting common target genes. Overexpression of *cPouV* in mESC led to a more moderate phenotype, with a slight induction of differentiation markers such as *Gata6* and *Cdx2*, and also of *Hnf1*, brachyury and *Sox17*, which are known in mouse to be strongly expressed in mesendoderm structures (Tada et al., 2005; Yasunaga et al., 2006). *cPouV* is able to trigger a differentiation programme when overexpressed in both mESC and cESC.

cPouV is only able to partially rescue the phenotype of *mOct4*-deficient cells. In ZHBTc4 ES cells, *cPouV* expression can restore limited proliferation, in contrast to the zebrafish *pou2* gene but in agreement with recent findings regarding *XlPou91* function (Morrison and Brickman, 2006). In the presence of *cPouV*, endogenous mouse *Nanog* expression in mESC is maintained at a low but detectable level. Expression of *Utf1*, *Zfp42* (*Rex1*) and *Tert* is maintained, but expression of *Sox2* and *Fgf4* is completely abolished. These observations could explain the limited ability of these clones to be passaged and amplified. However, under these same conditions, the various factors are able to transactivate specific promoters containing either the *Oct4* consensus binding site or the mouse endogenous *Oct4* promoter, suggesting that *cPouV* is able to substitute functionally for mouse *Oct4*. The N-terminal domains of the mouse and chicken genes are highly diverged. It is probable that stringent interprotein interactions are required for full activity of the chicken protein. A first attempt to test this hypothesis, by constructing molecular chimaeras between the mouse *Oct4* N-terminus and the *cPouV* homeodomain and C-terminus, proved unable to restore complete ZHBTc4 cell proliferation (data not shown), suggesting that other mechanisms and/or partners are likely to be required.

We also report the isolation of the chicken functional orthologue of *Nanog* (*cNanog*) from proliferating cESC and demonstrate that *cNanog* also plays a role in the maintenance of self-renewal and pluripotency of cESC. The sequence we cloned is shorter than the one recently reported by Canon et al. (Canon et al., 2006). The *cNanog* expression profile differs both in vitro and in vivo from that of *cPouV*. Specifically, *cNanog* expression is downregulated by differentiation but with a different time-course than *cPouV* in both RA-induced differentiation and cEB formation. In contrast to *cPouV*, *cNanog* transcription is maintained and possibly increased in the presence of cycloheximide and the mRNA half-life appears to be reduced in the presence of actinomycin, suggesting a short half-life

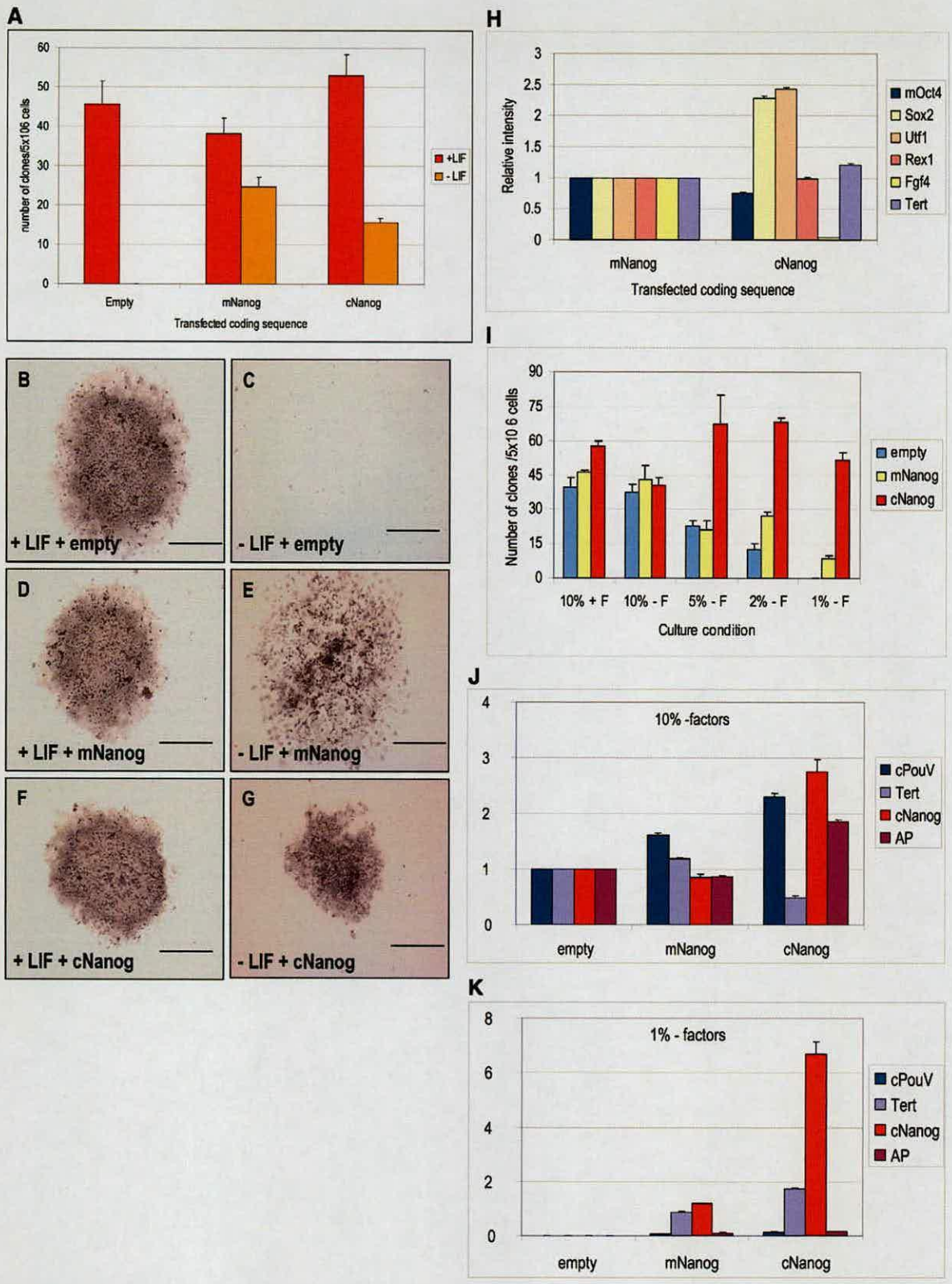


Fig. 8. Overexpression of *cNanog* induces growth factor-independent proliferation in both mESC and cESC. mESC or cESC were transfected with either *mNanog*, *cNanog* or empty expression vector. For mESC, LIF was removed, clones scored (**A**), stained for alkaline phosphatase activity and observed (**B-G**). Similar results were obtained in two independent experiments. Scale bars: 400 μm. (**H**) Gene expression analysis revealed expression of *mOct4* as well as of *Sox2*, *Utf1*, *Rex1* and *Tert*, but with the notable exception of *Fgf4*. A value of 1 was given to the level of expression in the clones obtained without LIF in the presence of *mNanog*. (**I**) For cESC, growth factors and cytokines (bFgf, Scf, Igf1, Il6, Il6R) (–F) were removed, serum concentration reduced as indicated (10% to 1%) and clones scored. (**J**,**K**) Gene expression analysis revealed maintenance of *cPouV* expression in the presence of 10% serum without factors (J), but a complete loss of expression when serum was reduced to 1% without factors (K). Under these conditions, the expression of *cNanog* is upregulated and the cells continue to proliferate.

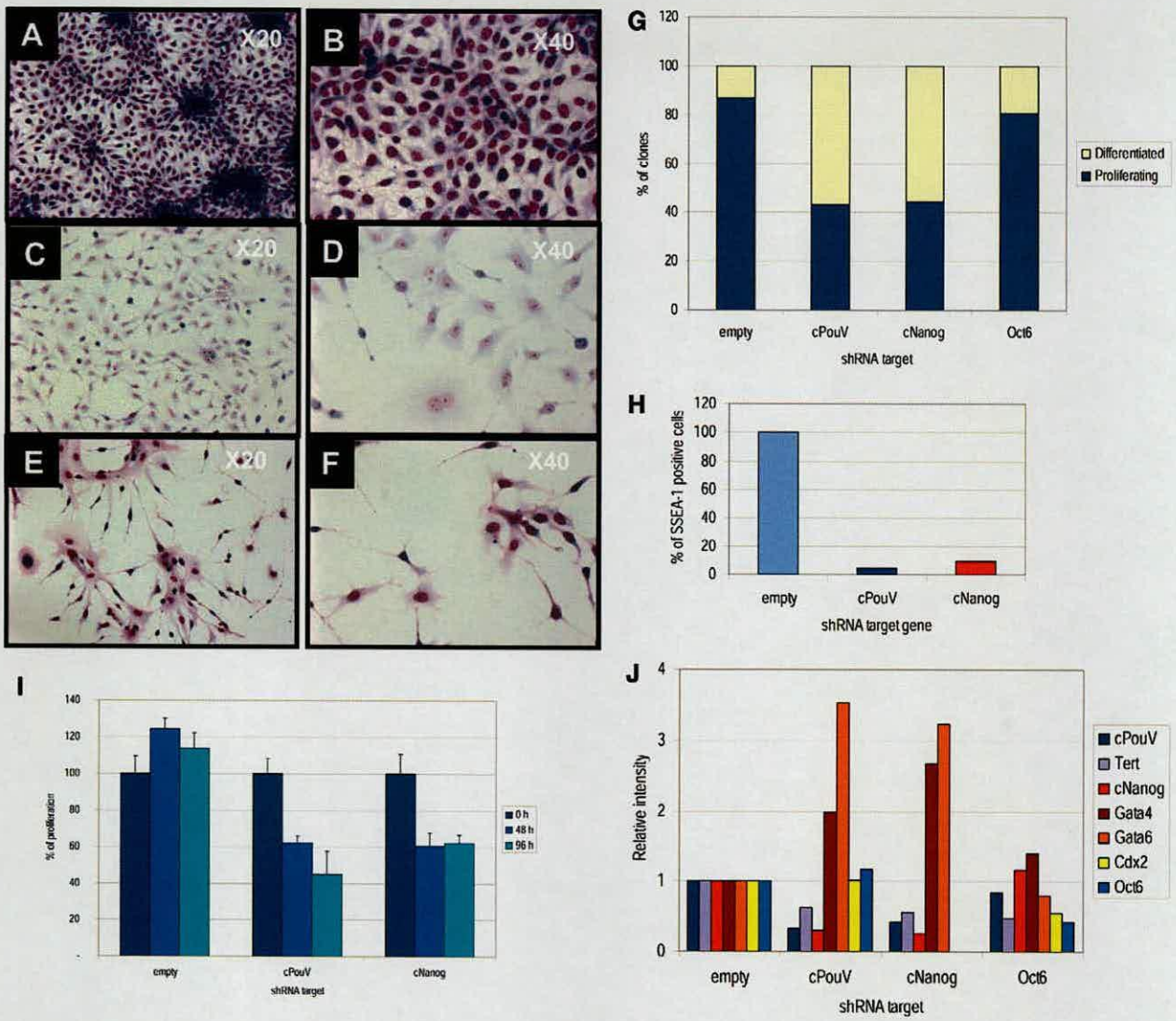


Fig. 9. Inhibition of *cPouV* and *cNanog* expression stops proliferation and induces cESC to differentiate. cESC were transfected with vectors allowing conditional expression of shRNA-2 and shRNA-1 against *cPouV* and *Nanog*, respectively, and of Cre-ERT2 recombinase, the activity of which was induced by 4-hydroxytamoxifen. Similar results were obtained with shRNA-3 against *cNanog*. The morphology of the cells targeted for *cPouV* (**C**,**D**) and *cNanog* (**E**,**F**) was strongly altered, compared with control cells (**A**,**B**) and with targeted cells for cOct6 (data not shown), in 60% of the clones (**G**). In these differentiated cells, the percentage of SSEA-1-positive cells was drastically reduced (**H**). Proliferation was also rapidly reduced as assessed by XTT proliferation on 12 independent clones followed for 4 days after tamoxifen addition (T=0 hours) (**I**). Gene expression analysis (**J**) revealed a strong decrease in expression of target genes *cPouV* and *cNanog* in contrast to a strong upregulation of *Gata4*, *Gata6* and *Cdx2* in cells expressing shRNA against *cPouV*, and the upregulation of *Gata4* and *Gata6* for those cells expressing shRNA against *cNanog*. In the latter case, no *Cdx2* expression could be detected. A shRNA-3 against another Pou family member, *Oct6*, does not alter *cPouV* and *cNanog* expression by comparison with the control vector (empty). *Oct6* expression cannot be detected in the cells targeted by shRNA against *cNanog* (J). Three independent experiments provided similar results.

of the mRNA. In vivo, the expression pattern of *cNanog* is also different from that of *cPouV*, with a more rapid disappearance from the epiblast and a subsequent restriction to the anterior neural plate. *cNanog* expression is detected in migrating germ cells, as was also observed for *Nanog* in the mouse (Yamaguchi et al., 2005), but also in germ cells of developing gonads.

Following overexpression of either mouse or chicken *Nanog* in mESC, the resulting proliferating clones are able to grow in the absence of the cytokine LIF, suggesting a functional complementation between the mouse and chicken genes. A notable exception is an almost complete loss of *Fgf4* expression in clones overexpressing *cNanog*. In cESC, a drastic reduction in serum in the culture medium revealed an important action of *cNanog* in maintaining proliferation of undifferentiated cells, suggesting that *cNanog* is able to stimulate proliferation and cell-cycle machinery in the absence of exogenous growth factors by acting directly on downstream targets.

Finally, inhibition of expression of *cPouV* and *cNanog*, using an inducible knockdown approach, promotes rapid growth arrest within 48 hours of shRNA induction. This inhibition of proliferation is accompanied by an induction of differentiation as detected by altered morphology, loss of SSEA-1 labelling and expression of *Gata6* and *Cdx2*. This suggests that these two genes play a key role in the maintenance of the pluripotent character of cESC.

In conclusion, the identification of chicken *PouV* elucidates some aspects of epiblast proliferation and maintenance of pluripotency in vitro and in vivo, and points the way for a better understanding of germ cell development and proliferation in the chicken embryo. The chicken *Nanog* gene also plays a role in this process and the functional relationship between these two key genes requires further investigation.

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