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Role of Maternal Oct-4 During Oogenesis and Early Embryogenesis in the Mouse

by

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ABSTRACT

Oct-4 is a transcription factor that belongs to the POU family. The expression of Oct-4 is found in growing oocytes and in totipotent or pluripotent cells of the early mouse embryo, and is down-regulated in embryos during differentiation events associated with blastocyst implantation and gastrulation. To study the correlation between cell pluripotency and Oct-4 expression, a polyclonal antibody was raised against a unique peptide sequence in the C-terminus of mouse Oct-4. It specifically recognized Oct-4 protein as tested by Western blot and gel mobility shift assays. This antibody was used to measure Oct-4 protein levels during retinoic acid induced differentiation of F9 cells. It was observed that Oct-4 protein was abundant in undifferentiated F9 cells but decreased to levels below detection as the cells differentiated, consistent with changes in levels of expression in early embryos. The role of maternal Oct-4 during oogenesis and early development in the mouse was investigated by a strategy to underexpress Oct-4 in transgenic mice by antisense inhibition. Antisense Oct-4 RNA was expressed under the control of the mouse zona pellucida ZP3 promoter. Oocyte-specific expression of antisense Oct-4 RNA was demonstrated by RT-PCR and by Southern blot analysis. Quantitative RT-PCR revealed that each unfertilized mouse oocyte contained about 800 copies of Oct-4 mRNA. The number was reduced by 69% to 310 in oocytes from transgenic animals. The level of Oct-4 protein in oocytes from transgenic mice was decreased by an average of 53% compared to the value in oocytes of normal mice when measured by immunoprecipitation. The reduced Oct-4 levels had no significant effect on reproductive capacity as measured by oocyte number and litter size in transgenic mice

compared to their normal counterparts. However, the transgenic strategy used in this study, with modifications, may be useful for functional analysis of other maternal factors.

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DEDICATION

To my husband, Dr. Maolong Lu

and

my daughter, Joanna Qi Lu

and

my son, Jonathan Xuan Lu

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ATP	adenosine triphosphate
BCIP	5-bromo-4-chloro-3-indolyl phosphate
bp	base pair
BSA	bovine serum albumin
dcAMP	dibutyryl cyclic AMP
CFA	Complete Freund's adjuvant
CIP	calf intestine alkaline phosphatase
dATP	deoxyadenosine triphosphate
dCTP	deoxycytidine triphosphate
dGTP	deoxyguanosine triphosphate
DIG	digoxigenin
DMEM	Dulbecco's Modified Eagle Medium
DMSO	dimethyl sulfoxide
dNTP	deoxynucleoside triphosphate
dTTP	deoxythymidine triphosphate
DTT	dithiothreitol
EC	embryonal carcinoma
ECL	enhanced chemiluminescence
EDTA	ethylenediamine-tetraacetate
EGTA	ethylene glycol-bis(β-aminoethyl ether)-tetraacetic acid
ES	embryonic stem

FCS	fetal calf serum
FITC	fluorescein iso-thiocyanate
FSH	follicle stimulating hormone
HCG	human chorionic gonadotrophin
HPLC	high pressure liquid chromatography
hr	hour
HRP	horse-radish peroxidase
ICM	inner cell mass
IFA	Incomplete Freund's adjuvant
kb	kilobase
kDa	kilodalton
KLH	keyhole limpet hemocyanin
LDH	lactate dehydrogenase
LH	lutenizing hormone
MBS	*m*-maleimidobenzoyl-*N*-hydroxysuccinimide
MOPS	3-(N-morpholino)propanesulfonic acid
NBT	nitroblue tetrazolium
nt	nucleotide
PAGE	polyacrylamide gel electrophoresis
pBS	pBluescript-SK
PBS	phosphate-buffered saline
p.c.	post coitum

PCR	polymerase chain reaction
PGC	primordial germ cell
PMS	pregnant mare serum
PMSF	phenylmethylsulfony fluoride
RA	retinoic acid
RT	reverse transcription
SDS	sodium dodecyl sulfate
SSC	saline sodium citrate
SSPE	saline sodium phosphate EDTA
TBE	tris-borate EDTA
TEMED	tetramethylethylenediamine
vol	volume

§1 INTRODUCTION

A central question in developmental biology concerns the role of maternal information during oogenesis, fertilization, and early embryogenesis. One of the maternal products of the mouse that is thought to have an important regulatory role in early development is Oct-4. Oct-4 is a transcription factor that belongs to the POU gene family. It has a unique expression pattern in that Oct-4 mRNA is restricted to totipotent and pluripotent embryonic stem cells before gastrulation and to the germ cell lines thereafter (Rosner et al., 1990; Schöler et al., 1990a; Yoem et al., 1991). This interesting expression led to the work described in this thesis. The study was designed to improve our understanding of the functional role of maternal (oocyte-derived) Oct-4 during oogenesis and early development in the mouse. Specially, a strategy to underexpress Oct-4 in the oocytes of transgenic mice was developed. To place the questions addressed in this study in context, the thesis begins with a brief review of relevant aspects of this field. The introductory chapter, therefore, includes a description of the landmarks of oogenesis and early embryogenesis in the mouse, including both morphological and molecular aspects of the processes, followed by an overview of recent studies on the POU gene family and especially, the Oct-4 gene.

§1.1 Overview of Mouse Oogenesis

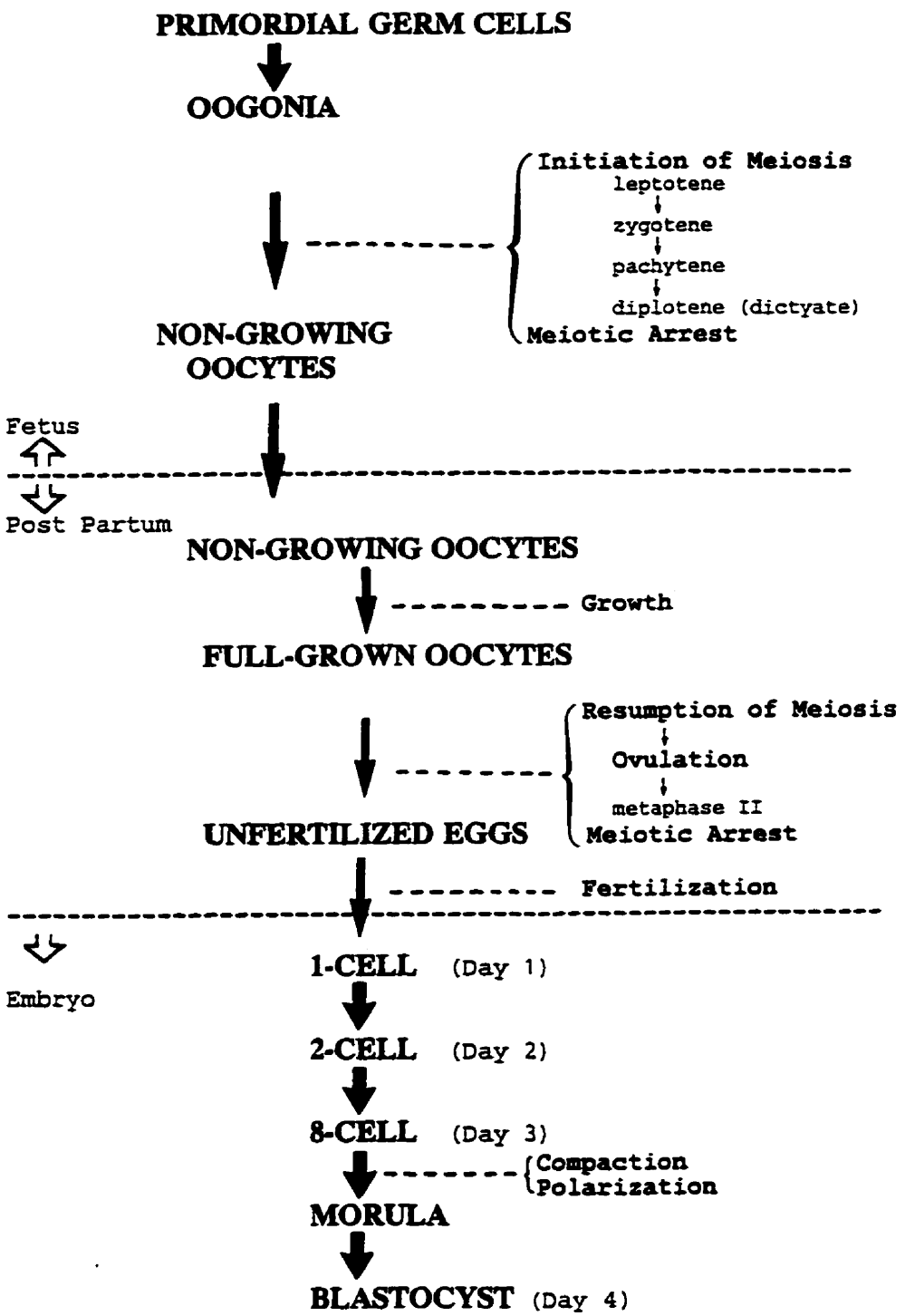
§1.1.1 Development of ovum

Oogenesis is a highly specialized and regulated biological process. The life cycle of oocytes begins with the appearance of primordial germ cells (PGCs) during fetal development and terminates with ovulation of unfertilized eggs in sexually mature adults. Oogenesis encompasses a series of cellular transformations (PGCs→ oogonia→ oocytes→ unfertilized eggs) and many important cellular biological events that include meiosis, oocyte growth, meiotic maturation and the establishment of a maternal store of macromolecules important for early development of the embryo (Fig.1).

Primordial germ cells (PGCs) are the earliest recognizable precursors of oocytes and have an extragonadal origin. They first become visible in 7- to 9- day mouse embryos in the yolk sac endoderm and the embryonic rudiment of the allantois. Either by active movement along tracts of extracellular matrix material or in response to chemotactic substances released by the cells of the genital ridges, PGCs migrate, first into the endodermal epithelium of the hind gut, then into the dorsal mesentery and finally into the genital ridges (for review, see Wassarman and Albertini, 1994). En route to their final destination, PGCs divide actively. It is estimated that there are between 15 to 100 PGCs in 8-day mouse embryos and this number increases to about 25,000 when the genital ridges are fully colonized in 13.5-day embryos (Tam and Snow, 1981).

By the 13th day of embryonic development, migration of PGCs is complete with virtually all of the cells converted to actively dividing oogonia in the female sex cord.

Fig. 1. Outline of oogenesis and preimplantation development in the mouse. Primordial germ cells are transformed into oogonia and then to oocytes. Oocytes are arrested at the dictyate stage of meiosis prophase I until hormonal signals trigger meiotic maturation and ovulation. Progression of oogenesis from primordial germ cells to non-growing oocytes takes place during fetal development, while progression from non-growing oocytes to unfertilized eggs takes place during post partum life. At fertilization, the second meiotic division is completed and male and female pronuclei are formed. The first cleavage occurs 20 - 26 hr post fertilization. Later cleavages occur at approximately 12 hr intervals. At the 8-cell stage, the embryo undergoes compaction and polarization and is transformed into morula. At the day 4 blastocyst stage, two distinct cell lineages, inner cell mass and trophectoderm, are established. Following implantation of the blastocyst, new primordial germ cells arise in the developing embryo and the cycle is repeated. (Modified from Fig. 1 in "The Mammalian Ovum" by Wassarman and Albertini, 1994).


PRIMORDIAL GERM CELLS
OOGONIA
Initiation of Meiosis
leptotene
zygotene
pachytene
diplotene (dictyate)
Meiotic Arrest
NON-GROWING OOCYTES
Fetus
Post Partum
NON-GROWING OOCYTES
Growth
FULL-GROWN OOCYTES
Resumption of Meiosis
Ovulation
metaphase II
Meiotic Arrest
UNFERTILIZED EGGS
Fertilization
Embryo
1-CELL (Day 1)
2-CELL (Day 2)
8-CELL (Day 3)
Compaction
Polarization
MORULA
BLASTOCYST (Day 4)

Oogonia, in turn, change into non-growing oocytes once meiotic prophase commences. By day 17 post coitum (p.c.), all of the oogonia are transformed into oocytes in various stages of the first meiotic prophase (Speed, 1982). When oocytes progress through the first meiotic prophase (leptotene, zygotene, pachytene, diplotene), homologous chromosomes pair in zygotene and crossing-over and recombination occur in the pachytene phase of meiosis. By parturition, most oocytes enter the late diplotene stage (dictyate stage) of first meiotic prophase. By day 5 post-partum, nearly all oocytes have reached the dictyate stage and remain arrested at this stage until preovulatory or atretic changes occur in the large follicle.

Throughout the juvenile period and each reproductive cycle of the female mouse, a small percentage of the pool of non-growing oocytes (~ 12 μm) is activated to begin growth. Oocytes undergo more than a 300-fold increase in volume through the 2-3 week growth period, resulting in fully grown oocytes of 85 μm in diameter that are still arrested at the dictyate stage (Wassarman and Albertini, 1994). The fate of all growing oocytes is to degenerate (atresia) in immature mice, whereas in sexually mature mice, growth ends in either ovulation of unfertilized oocytes or atresia (Greenwald and Roy, 1994). With the growth of the oocyte, the surrounding follicle cells proliferate and differentiate. Eventually, a Graafian follicle forms with an antrum containing follicular fluids (Greenwald and Roy, 1994). The Graafian follicle contains two morphologically distinct subpopulations of granulosa cells: mural granulosa cells that line the follicle wall, and cumulus granulosa cells that surround the oocyte.

As the oocyte increases in size, it gradually acquires the competence to undergo

meiotic reduction in response to follicle stimulating hormone (FSH) and luteinizing hormone (LH). Meiotic maturation involves nuclear (germinal vesicle) breakdown, chromosome condensation, spindle formation, emission of the first polar body and ovulation. The cumulus cells undergo a process of cumulus expansion and are ovulated together with the oocyte. A corpus luteum develops from the remnants of the ovarian follicle (Eppig, 1991). Ovulated oocytes are then arrested at the second meiotic metaphase until fertilization occurs in the ampullary region of the oviduct.

§1.1.2 Macromolecular stores of the oocyte

During the process of oogenesis, oocytes accumulate abundant stores of ribosomes, mitochondria, Golgi complex, cortical granules, RNA and proteins that are required to support fertilization and early embryonic development. Synthesis of RNA mainly occurs in growing oocytes. Experiments based on incorporation of radiolabeled RNA precursors indicate that the rate of RNA synthesis is very high during the oocyte growth phase and the RNA synthesized is very stable (DeLeon et al., 1983). Fully grown mouse oocytes contain about 500 to 600 pg of RNA, which is roughly 200 times the amount found in typical mammalian somatic cells (Olds et al., 1973; Sternlicht and Schultz, 1981). RNA continues to be synthesized in fully grown oocytes at a diminished rate. At the onset of meiotic maturation, RNA synthesis is barely detectable. There is a loss of about 20% of total RNA and 50% of polyadenylated RNA during this period (Bachvarova et al., 1985). The newly ovulated mouse oocyte contains about 350 to 500 pg of total RNA, of which about 6-8% is polyadenylated RNA (Olds et al., 1973; Piko

and Clegg, 1982; Bachvarova and DeLeon, 1980). The relative amount of maternal mRNA is unusually high when compared to the relative amount of poly(A)$^+$ mRNA (about 2%) in cells of somatic tissues (Reiners and Busch, 1980).

One of the most abundantly expressed genes during oogenesis in the mouse is zona pellucida ZP3 (mZP3) gene. The zona pellucida is an extracellular coat surrounding the oocyte and preimplantation embryo. It is composed of three glycoproteins, called mZP1, mZP2 and mZP3, that associate with one another through noncovalent interactions to form an insoluble network of crosslinked filaments (Wassarman, 1988). Besides its structural role, mZP3 also serves as a sperm receptor on the oocyte. Transcription of the mZP3 gene occurs exclusively in growing oocytes, not in non-growing oocytes or cells in any other tissues (Philpott et al., 1987; Roller et al., 1989). Use of RNase protection assays to measure the steady state level of the mZP3 mRNA during oogenesis indicates that there are approximately 300,000 copies of mZP3 mRNA in oocytes 60-70 μm in diameter and about 240,000 copies in fully grown oocytes (Roller et al., 1989). This is about 0.27% of the total oocyte poly(A)$^+$ RNA pool, making mZP3 one of the most abundant mRNAs in mouse oocytes. Expression of mZP3 is regulated by cis-acting sequences located in the 5'-flanking region of the gene (Lira et al., 1990). Because of its oocyte-specific expression and strong activity, the mZP3 promoter was chosen to drive expression of the transgene used in the study described in this thesis.

During the process of oocyte growth (as mRNA molecules are accumulated), translational activity also becomes more active. The absolute rate of protein synthesis increases 38-fold, from 1.1 pg/hr/oocyte in the non-growing oocyte to 41.8 pg/hr/oocyte

in the fully-grown oocyte (Schultz et al., 1979). The fully-grown mouse oocyte contains about 20-25 ng of protein exclusive of zona pellucida (3 ng), which is about 50-60 fold more protein than that contained in a typical mammalian somatic cell. Fully grown oocytes are particularly rich in structural proteins, such as actin and tubulin, and certain enzymes, such as LDH (lactate dehydrogenase) (Wassarman and Albertini, 1994). Protein synthesis is very active in both growing and fully-grown oocytes. However, unlike RNA synthesis (which is undetectable during meiotic maturation), protein synthesis continues in ovulated oocytes albeit at a slightly reduced rate of 33 pg/hr/oocyte (Schultz et al., 1979). This 23% decrease in overall protein synthetic rate is reflected in a number of specific changes in individual proteins. For example, the rate of actin and tubulin synthesis decreases by 40% (Wassarman, 1983) and zona pellucida proteins, which are abundantly synthesized in growing oocytes, are barely synthesized during the period of meiotic maturation (Shimuzu et al., 1983). The mRNA encoding the enzyme t-PA (tissue plasminogen activator), on the other hand, is first translated during oocyte maturation (Huarte et al., 1985). These types of changes in protein synthetic activity are reflected in the pool of maternally-inherited molecules that are contained in the ovulated oocyte and are used to support and regulate development of preimplantation mouse embryos.

§1.2 Overview of Mouse Preimplantation Embryogenesis

§1.2.1 Morphological events in early embryogenesis

Preimplantation development in the mouse spans the period between fertilization

and attachment of the blastocyst to the uterine wall. At the time of ovulation, the unfertilized mouse oocyte is arrested at the second meiotic metaphase (Fig. 1). Completion of meiosis and extrusion of the second polar body occurs within 1 to 3 hr post-fertilization. Nuclear membranes then form around the paternal and maternal chromosomes to generate male and female pronuclei, respectively. The pronuclei move toward the centre of the egg, DNA replication takes place, the pronuclear membrane breaks down, the chromosomes assemble on the spindle, and the first cleavage occurs soon after (Howlett and Bolton, 1985; reviewed by Schultz, 1986). Mouse embryos undergo their first cleavage within 20-26 hr after fertilization. Later cleavages occur at approximately 12 hr intervals (Schultz, 1986).

Up to the early 8-cell stage, each blastomere of the mouse embryo is totipotent and capable of giving rise to a complete mouse (Tarkowski and Wroblewska, 1967). The developmental potency of the blastomeres is gradually restricted from the late 8-cell stage onward. At this point, the blastomeres undergo the first morphogenetic event known as compaction, during which the blastomeres flatten against one another with obscured boundaries and merge into a single coherent mass called the morula. This first visible embryonic differentiation event involves cytoskeletal reorganization and changes at the membrane surface. The cytoplasmic and surface components become asymmetrically distributed (or polarized), allowing the first distinction to be made between the basolateral and apical domains of the blastomeres (reviewed by Gueth-Hallonet and Maro, 1992).

Polarity is maintained as cell division continues in the embryo. Between the fourth

and the sixth cleavages, individual blastomeres become partitioned into either of two cell lineages depending upon their outer or inner position in the compacted morula (Ziomek and Johnson, 1980). The outer blastomeres which are polar and have an epithelial organization contribute to trophectoderm, while the inner blastomeres which are apolar in nature contribute to the inner cell mass (ICM). By the 32- to 64- cell blastocyst stage, two distinct cell populations are established: the ICM and the trophectoderm (Hogan et al., 1986). At the same time, a blastocyst cavity is formed that contains fluid transported by the trophectoderm, and this leads to the expansion of the blastocyst. The trophectoderm differentiates into mural trophectoderm, which surrounds the blastocyst cavity, and polar trophectoderm, which overlies the inner cell mass (Pedersen and Burdsal, 1994). Shortly before implantation (4.0 days p.c.), differentiation in the ICM commences and results in the appearance of the primitive endoderm, an epithelial layer facing the blastocyst cavity. The trophectoderm and primitive endoderm give rise to extraembryonic structures and the embryonic contribution to the placenta. The remaining core of ICM cells known as the primitive ectoderm stays pluripotent and will mainly give rise to the embryo proper (Hogan et al., 1986).

§1.2.2 Utilization of maternal information

The first cleavage of the fertilized mouse oocyte can occur in the absence of transcription from the zygote genome. That is, it can proceed entirely under the control of maternally-inherited components and is not blocked when one-cell embryos are cultured in the presence of the transcriptional inhibitor α-amanitin (Flach et al., 1982;

Bolton et al., 1984). Activation of transcription from the zygote genome in the mouse embryo occurs in two phases. There is a "minor" round of RNA synthesis coupled to the appearance of a complex of 70 kDa polypeptide that occurs in the one-cell embryo (Flach et al., 1982; Bolton et al., 1984; Bensaude et al., 1983; Conover et al., 1991; Latham et al., 1991, 1992). This "minor" activation precedes a second phase of transcriptional activity at the 2-cell stage wherein this is a marked transition in types of mRNAs and polypeptides synthesized (Flach et al., 1982; Howlett, 1986; Schultz, 1993).

In the aspect of translation, a small increase in the rate of protein synthesis from 33 pg/hr/oocyte to 45 pg/hr/1-cell embryo occurs following fertilization (Schultz et al., 1979). High-resolution two-dimensional gel electrophoresis has revealed that most proteins synthesized during meiotic maturation continue to be produced in the 1-cell embryo, although quantitative variability is apparent (Schultz et al., 1979; Howlett and Bolton, 1985). On the other hand, fertilization triggers a number of stage-specific changes in the patterns of protein synthesis. Many of these changes are regulated by maternally-inherited (oocyte) factors since they occur in the absence of concurrent transcription from the genome in physically enucleated oocytes or 1-cell embryos cultured in the presence of α-amanitin (Petzoldt et al., 1980; Flach et al., 1982; Bolton et al., 1984). Pulse-chase experiments have demonstrated that post-translational modification of pre-existing proteins (maternal proteins) plays an important role in changes of protein synthetic patterns (Howlett and Bolton, 1985). Selective utilization of stored maternal mRNA also contributes to these changes as examined by use of *in vitro* translation of total RNA extracted from oocytes and embryos followed by examinations

of polypeptide synthetic profiles (Cascio and Wassarman, 1982; Howlett and Bolton, 1985). The involvement of both post-translational modification and selective utilization of maternal mRNA is reflected in changes of the polypeptide synthetic patterns of a protein complex with a molecular mass of 35 kDa during the first 24 hr after fertilization (Howlett and Bolton, 1985). To this end, it is intriguing to propose that developmentally regulated selective maternal mRNA activation also results from post-translational modification of regulatory proteins. In essence, whatever the underlying molecular mechanism is, development to the first cleavage stage in the mouse embryo is governed largely by maternally-derived macromolecules: proteins and RNAs.

§1.3 POU Gene Family

Based on studies of *Drosophila* embryogenesis, current models of development predict a sequential activation of a hierarchy of regulatory genes guiding the transformation of genetic information into embryonic structure. Much effort has been directed towards the investigation of regulatory processes in the early mouse embryos. Several multigene families of putative developmental control genes have been identified by sequence similarity to *Drosophila* regulatory genes. Examples of such multigene families are the Hox genes and the Pax genes (reviewed by Pedersen and Burdsal, 1994). The majority of these genes identified, however, exert their influence during morphogenetic processes at the late stages of mouse development, such as axis specification and pattern formation. Less is known about genes important for regulation

of preimplantation development. Identification of maternal regulatory proteins and regulatory proteins specific for early stages of embryogenesis might help to unravel the predicted developmental gene hierarchy of the mouse. By employing promoter and enhancer elements to screen for trans-activating factors playing a role in developmental processes, another class of genes, the POU family, have been isolated (reviewed by Schöler, 1991). Some genes in the POU family are expressed in oocytes as well as in early mouse embryogenesis. Oct-4, the gene product of interest in this thesis, falls in this category.

§1.3.1 Structure and function of the POU domain

The POU family was initially defined through the characterization of three mammalian transcription factors (Pit-1, Oct-1 and Oct-2) and one nematode regulatory protein (Unc-86). These four regulatory factors share a conserved DNA binding motif, referred to as the POU domain (Herr et al., 1988). Subsequently, several other members of this family have been identified and characterized. Their common DNA binding motif, the POU domain, is a conserved 160-amino acid bipartite structure that contains a 74 - 82 amino acid POU-specific domain and a 60 amino acid POU homeodomain, connected by a short variable linker region (reviewed by Ruvkun and Finney, 1991). The POU homeodomain is distantly related to the prototype *Antennapedia* homeodomain while the POU-specific domain is highly homologous among the POU factors. The regions outside these two domains are highly divergent and contain domains required for transcriptional activation.

The entire POU domain is involved in DNA binding. The POU homeodomain is similar to the helix-turn-helix DNA-binding motifs of several prokaryotic transcriptional regulators and itself alone can bind to DNA. Addition of the POU-specific domain increases specificity and strength of DNA binding by the POU homeodomain (Ingraham et al., 1990; Verrijzer et al., 1990a). Mutations in conserved regions of either the POU-specific domain or the POU homeodomain strongly affect binding of Oct-1 and Pit-1 to their sites (Ingraham et al., 1988; 1990; Verrijzer et al., 1990a).

In addition to their DNA binding function, both the POU-specific domain and the POU homeodomain can participate in protein-protein interactions with either POU proteins or other transcriptional factors. For example, the POU-specific domain is essential for Pit-1/Pit-1 homodimerization (Igraham et al., 1990); Pit-1 heterodimerizes with Oct-1 on the Pit-1 responsive element of the prolactin promoter through the POU homeodomain (Voss et al., 1991).

§1.3.2 Expression of octamer-binding proteins in different organisms

Members of the POU family recognize distinct high affinity DNA binding sites (reviewed by Ruvkun and Finney, 1991). The DNA sequence of one binding site can bear little resemblance to that of the other. A class of the POU proteins have been defined as the octamer-binding proteins (or the Oct family) due to their ability to bind to an octamer motif (ATGCAAAT) which is found in promoters and enhancers of a variety of genes and in viral origins of replication. The octamer-binding proteins have been named as Oct-1, Oct-2, Oct-3 and so on.

The octamer-binding proteins have been identified in different organisms ranging from mammals to arthropods. In the sea urchin, a POU gene, designated SpOct, has been isolated from *Strongylocentrotus purpuratus* (Char et al., 1993). SpOct mRNA is expressed in oogenesis and early embryogenesis and encodes the major octamer-binding protein in the sea urchin early embryo. The function of the SpOct gene in sea urchin development has been evaluated by the microinjection of antisense oligodeoxynucleotides into 1-cell zygotes (Char et al., 1994). It has been found that single-stranded antisense oligodeoxynucleotides specifically block the first embryonic cleavage and reduce protein accumulation in the zygotes. DNA replication is also partially inhibited in antisense-injected embryos as measured by ^{3}H-thymidine incorporation. Because the activation of protein synthesis prior to the first cleavage does not require zygotic transcription, the effect of antisense oligodeoxynucleotides on protein accumulation suggests that the SpOct protein may play a novel, non-transcriptional role during early cleavage of the sea urchin embryo.

Several *Drosophila* Oct family genes have also been characterized (Lloyd and Sakonju, 1991; Billin et al., 1991; reviewed by Verrijzer and van der Vliet, 1993). The *Drosophila* POU genes pdm-1 and pdm-2 encode proteins that resemble the mammalian Oct-1 and Oct-2 transcription factors. They are expressed in stripes in embryos during the blastoderm and germ band extended stages. In later stage embryos, both pdm-1 and pdm-2 are expressed in the developing nervous system (Lloyd and Sakonju, et al., 1991). Their expression profiles suggest that pdm-1 and pdm-2 may regulate *Drosophila* segmentation and cell fate specification during early embryogenesis and neurogenesis. A

definitive determination of these roles awaits a mutational analysis.

In *Xenopus*, multiple octamer-binding proteins have been observed by gel mobility shift assays using extracts from staged embryos and a consensus octamer motif (Hinkley et al., 1992). These octamer binding proteins are expressed in a sequential manner during embryogenesis. cDNAs encoding several octamer binding proteins have been isolated from *Xenopus* cDNA libraries (Verrijzer and van der Vliet, 1993). Three of them, namely Oct-25, Oct-60 and Oct-91, are related to mammalian Oct-4 according to the amino acid sequences of the POU domain (Hinkley et al., 1992; Whitfield et al., 1993). Oct-60 is primarily expressed in oocytes as a maternal transcript and is localized within the animal hemisphere in mature oocytes and remains localized to the animal cap in the developing embryo. The Oct-60 mRNA declines abruptly during gastrulation and it is succeeded sequentially during early development, first by Oct-25 transcripts and then by Oct-91 transcripts. The expression of all three genes decreased during late gastrulation and early neurulation (Hinkley et al., 1992; Whitfield et al., 1993). What roles these genes play are still under investigation.

Most studies on octamer-binding proteins have been carried out in mammalian systems. Mammalian Oct-1 and Oct-2 were the first two octamer-binding proteins that were cloned. Oct-1 is present in all cell types and activates the transcription of the ubiquitously expressed histone and snRNA genes (LaBella et al., 1988; Tanaka et al., 1988). Oct-2 stimulates the transcription of lymphoid-specific expression of immunoglobin light- and heavy- chain genes (Sen and Baltimore, 1986). To date, about 10 members of octamer binding proteins have been identified in nuclear extracts from

embryos at various developmental stages and in nuclear extracts from various tissues in the mouse by gel mobility shift assays with an oligonucleotide probe containing an octamer motif (Schöler et al., 1989). Except for Oct-1, most genes in the Oct family are differentially expressed throughout embryogenesis.

Among the ten octamer binding proteins identified in the mouse, only four of them (Oct-1, Oct-2, Oct-4 and Oct-6) have been defined by cloning the corresponding cDNAs. Oct-3 and Oct-4 were identified simultaneously by Rosner et al. (1990) and Schöler et al. (1990b), respectively. The two are identical but the Oct-4 label has been adopted in the current literature. Oct-4 is inherited as a maternal transcript and its expression is restricted to totipotent and pluripotent embryonal stem cells before gastrulation and to the germ cell lines (Rosner et al., 1990; Schöler et al., 1990a; for detail, see below). It is also expressed in undifferentiated embryonal carcinoma (EC) cells and embryonic stem (ES) cells. Thus, the presence of Oct-4 seems to correlate with a pluripotent, undifferentiated cell phenotype. Similar to Oct-4, Oct-6 is expressed in undifferentiated ES cells and F9 EC cells and its expression is down-regulated upon differentiation of these cells (Suzuki et al., 1990; Meijer et al., 1990). However, during mouse embryogenesis, the expression of Oct-6 is not detected at the blastocyst stage when Oct-4 expression is readily detectable. Oct-6 expression first becomes detectable in day 10 mouse embryos and increases significantly by day 12. The expression is restricted to the developing brain. Moreover, Oct-6 is also found in cells of the adult brain. Thus, Oct-6 might take part in brain development and maintenance of certain cellular functions of the brain (Suzuki et al., 1990). There is substantial evidence to

indicate that octamer-binding proteins such as Oct-1 and Oct-4 can function as transcription factors, but the exact molecular mechanism underlying their developmental role is still unclear.

§1.3.3 The POU domain stimulates adenovirus DNA replication

Besides transcriptional activity, another functional feature of octamer-binding proteins is their ability to stimulate adenovirus DNA replication (Verrijzer et al., 1992). The adenovirus origin of DNA replication consists of a core origin and an auxiliary region. The core origin is the minimal region required for initiation of replication at the basal level which is enhanced by the auxiliary region (De Pamphilis, 1988). Cellular transcription factors such as Oct-1 are able to bind to the auxiliary region to stimulate the initiation reaction (Rosenfeld et al., 1987). Deletion analysis of Oct-1 has demonstrated that the POU domain is sufficient for activation of DNA replication (Verrijzer et al., 1990b). In addition to Oct-1, divergent POU domain transcription factors, including Pit-1, Oct-2, Oct-4 and Oct-6 can stimulate adenovirus DNA replication (albeit at different levels) in a reconstituted *in vitro* system (Verrijzer et al., 1992). Moreover, two octamer motifs in the simian virus 40 (SV40) enhancer have also been implicated in SV40 viral DNA replication (Haas et al., 1991). The involvement of POU domain proteins in viral DNA replication suggests the possibility that they might also be involved in cellular DNA replication. In line with this notion, disruption of SpOct function in the sea urchin by antisense oligodeoxynucleotides, as described earlier, blocks the first embryonic cleavage and partially inhibits DNA replication (Char et al., 1994).

However, direct proof for a function of POU domain proteins in cellular DNA replication in mammals is still lacking.

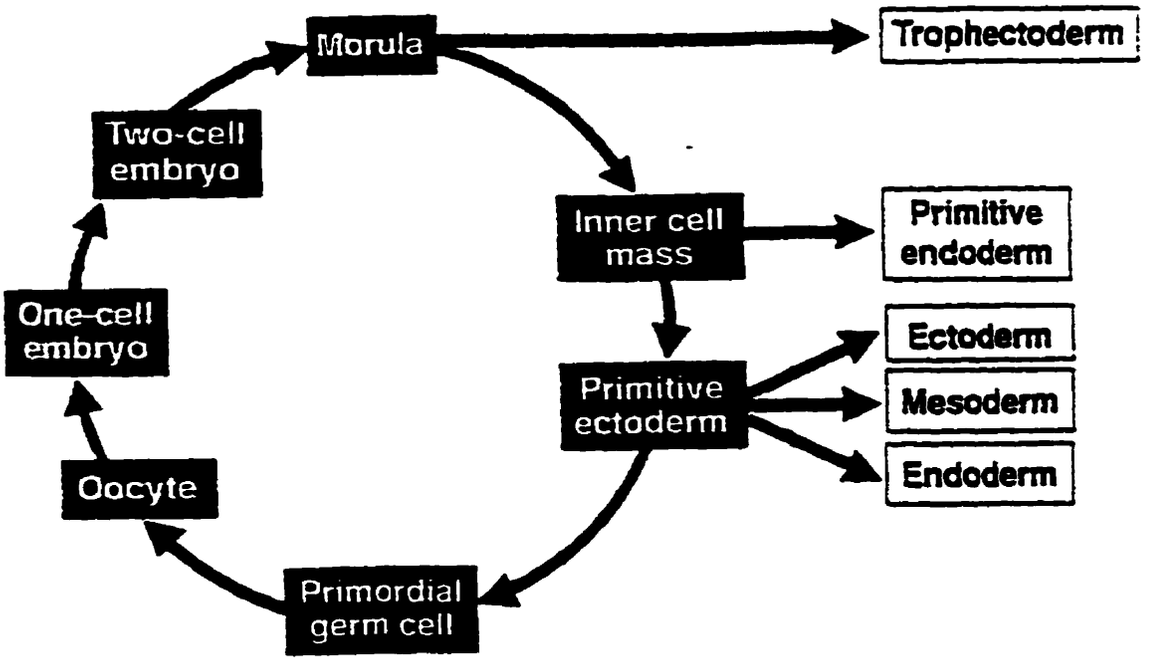
§1.4 Expression Patterns of Oct-4 Transcripts in the Mouse

Distinct from other octamer binding proteins, Oct-4 shows unique and interesting expression patterns in the mouse. Its expression is restricted to totipotent and pluripotent embryonal stem cells and to the germ cell lines (Schöler et al., 1990a; Rosner et al., 1990; Yoem et al., 1991). A diagram of Oct-4 expression during mouse development is shown in figure 2.

Oct-4 mRNA is maternally expressed and is found in unfertilized oocytes (Rosner et al., 1990; Schöler et al., 1990a; Yoem et al., 1991). Upon fertilization, maternal Oct-4 messenger RNA gradually decreases and is at background levels of detection at the 4-cell stage. By the 8-cell stage, the mRNA level is increased and is similar to that in growing oocytes, suggesting that zygotic Oct-4 gene expression is activated between the 4- and 8-cell stage (Yoem et al., 1991). Oct-4 transcripts are evenly distributed throughout all cells of embryo at these early stages of development (Yoem et al., 1991).

At the early and in the expanding blastocyst, Oct-4 is expressed uniformly in the embryo (Schöler et al., 1990a). Thereafter, expression increases in the ICM but decreases significantly in the trophectoderm and becomes undetectable when these cells differentiate into extraembryonic tissues (Schöler et al., 1990a). In ICM, the expression of Oct-4 remains high in the pluripotent primitive ectoderm but decreases in the more

Fig.2. Expression pattern of Oct-4 mRNA during mouse development. The black boxes at the left indicate those stages that express Oct-4. The white boxes at the right indicate those cell types that have little or no expression of Oct-4.


Morula
Trophectoderm
Two-cell embryo
Inner cell mass
Primitive endoderm
One-cell embryo
Ectoderm
Primitive ectoderm
Mesoderm
Endoderm
Oocyte
Primordial germ cell

committed primitive endoderm. With the differentiation of the primitive ectoderm, Oct-4 expression is also down-regulated. After 8.5-day p.c., Oct-4 expression is undetectable in somatic cells and is restricted to PGCs. PGCs express Oct-4 throughout their migration from the allantois to the genital ridges (Schöler et al., 1990a; Rosner et al., 1990). In the adult, Oct-4 is found in both the ovary and the testis (Rosner et al., 1990; Schöler et al., 1989). The identity of the cells that express Oct-4 is not clear in the testis. In the ovary, Oct-4 is confined to oocytes. Maturing oocytes express a higher amount of Oct-4 than resting oocytes (Rosner et al., 1990).

The expression of Oct-4 in PGCs and oocytes in the mouse suggests that Oct-4 might be involved in the regulation of the process of oogenesis, including aspects such as oocyte growth and oocyte maturation. It should be noted that Oct-4 is one of the first maternal transcription factors identified. Besides Oct-4 and the ubiquitously expressed Oct-1, another octamer binding protein, Oct-5, is also present in mouse oocytes as detected by gel mobility shift assays (Schöler et al., 1989). However, both Oct-4 and Oct-5 are encoded by the Oct-4 gene. The Oct-5 protein is 19 amino acids shorter than Oct-4 at the N-terminus (Schöler et al., 1990b). Unlike Oct-4, Oct-5 is not detectable in PGCs (Schöler et al., 1989).

The expression patterns of Oct-4 in mouse embryos seem to correlate with an undifferentiated cell phenotype. Consistent with this notion, Oct-4 is expressed in undifferentiated EC cells and ES cells, and its expression is down-regulated when the cells are induced to differentiate by retinoic acid (Okamoto et al., 1990). Further evidence for a correlation between the expression of Oct-4 and the cell pluripotency

comes from the study of hybrid cells (Shimazaki et al., 1993). When pluripotent EC cells (Oct-4$^+$) and fibroblasts (Oct-4$^-$) were fused to produce hybrid cells with a differentiated phenotype, expression of Oct-4 was extinguished at the transcriptional level. Re-introduction of Oct-4 into the hybrid cells by transfection, however, led to de-differentiation of these cells. These results suggest that Oct-4 may indeed play a role in maintaining cell pluripotency. In addition, Oct-4 is one of the few homeobox genes known to be expressed during the preimplantation stage of development in the mouse. Thus, it is a good candidate to act very early in the developmental control hierarchy and Oct-4 may play an important role in the activation of other developmental control genes. An initial report on the importance of Oct-4 in early development included results which indicated that antisense Oct-4 could block the first embryonic cleavage in the mouse (Rosner et al., 1991a). This work was subsequently retracted because results from appropriate controls had not been reported correctly by the first author of the paper. This prompts one to bring up the question again: what does Oct-4 do during early development in the mouse?

The study described in this thesis was conducted to test the hypothesis that maternal (oocyte-derived) Oct-4 is required for successful oocyte development and early embryogenesis in the mouse. In order to clarify the correlation between the expression levels of Oct-4 and the cell pluripotency, the F9 EC cell line was also used as a model system for studying early embryogenesis. A polyclonal antibody that can recognize a unique peptide sequence in the C-terminus of mouse Oct-4 was prepared. It specifically

recognizes Oct-4 protein as tested by western blots and bandshift assays. This antibody has been used to measure Oct-4 protein levels during retinoic acid induced differentiation of F9 cells. The expression patterns of Oct-4 protein and mRNA were compared. It was observed that Oct-4 protein was abundant in undifferentiated F9 cells but decreased to levels below detection as the cells differentiated, consistent with changes in levels of expression in early embryos. This study demonstrates a close correlation between Oct-4 expression levels and F9 cell differentiation status.

To analyze the role of maternal Oct-4 in oocytes and in early embryos especially during the first two embryonic cleavages (which occur before zygotic Oct-4 activation), transgenic mice were made to underexpress maternal Oct-4 by antisense inhibition. The mZP3 promoter was used to direct transgene expression. Tissue-specific expression of the antisense Oct-4 gene was obtained as examined by RT-PCR and further confirmed by southern blots. Levels of Oct-4 maternal mRNA from normal and transgenic mice were measured by quantitative RT-PCR. Levels of Oct-4 protein in oocytes from normal and transgenic mice were compared by radioactive labelling of oocytes followed by immunoprecipitation. The data showed that antisense inhibition resulted in reduced levels of both Oct-4 mRNA and protein in oocytes derived from transgenic animals. The effects of antisense inhibition of maternal Oct-4 were evaluated by histological examination of mouse ovaries and by assessment of oocyte and embryo numbers (reproductive performance) of normal and transgenic mice. The results indicate that reduced Oct-4 levels had no significant effect on early embryonic development or reproductive capacity of the transgenic mice. However, the transgenic strategy used in this thesis (with

modifications) may be useful for the study of the involvement of other maternal factors in oogenesis, early embryogenesis, and reproduction in mice.

§2 MATERIALS AND METHODS

§2.1 F9 Cell Culture

§2.1.1 Preparation of gelatin-coated tissue culture flasks

To facilitate attachment of F9 cells, the surface of each culture flask was covered with a sterile solution of 0.1% (w/v) gelatin in distilled water and placed at 4°C for 2 hr. This allowed the gelatin to form a thin film on the surface. The gelatin solution was then removed and the flask was washed 3 times with sterile distilled water (Gibco-BRL). Treated flasks could be used immediately or stored at room temperature for several months.

§2.1.2 Culturing of F9 cells

F9 cells were cultured at 37°C in Dulbecco's Modified Eagle's Medium (DMEM) with 4.5 g/L glucose (Gibco-BRL) supplemented with 10% heat-inactivated fetal bovine serum (FCS) in an atmosphere of 5% CO_2 in air and saturated humidity. For subculture, a solution of 0.25% trypsin (Gibco-BRL) in phosphate-buffered saline (PBS) was added to cover the cell monolayer with a minimal volume. The flask was incubated at 37°C for a few minutes to allow cells to detach and fresh medium with serum was then added to inhibit trypsin activity. Following recovery of cells by centrifugation and removal of supernatant by aspiration, the cells were resuspended in fresh medium and dispersed into new flasks for subsequent culture.

§2.1.3 Freezing F9 cells

After washing trypsinized F9 cells with fresh medium, the cells were resuspended in DMEM/10% FCS plus 10% DMSO (dimethyl Sulfoxide; Sigma). Aliquots of the cells were pipetted into 2-ml cryovials (Nalgene). The vials were placed within a styrofoam box and frozen at -70°C overnight. On the second day, the vials were transferred to liquid nitrogen where they were stored until use. To Culture F9 cells from the frozen stock, cells were thawn by rapid agitation in a 37°C waterbath. Fresh warm medium was added drop-wise to allow the cells to equilibrate. The medium was aspirated from the cells after centrifugation and replaced with fresh medium. The cells were then ready for seeding.

§2.1.4 Differentiation into parietal endoderm-like cells

The method used for inducing F9 cells into parietal endoderm-like cells was the same as described by Strickland et al. (1980). F9 cells were seeded at a low density, approximately 3 X 10^5 cells per gelatin-treated 75 cm^2 Falcon^R flask (Becton Dickinson) in DMEM/10% FCS containing 10^{-7}M retinoic acid (RA) and 10^{-3}M dibutyryl cyclic AMP (dcAMP; Sigma). Retinoic acid was prepared as a 10^{-4}M stock in ethanol and diluted in the medium while dcAMP was dissolved directly in the medium. The culture medium was replaced every 48 hr. The cell culture was protected from fluorescent light.

§2.1.5 Photography

Undifferentiated and differentiated F9 cells were photographed with a Zeiss IM35

photomicroscope with bright-field illumination using Kodak Gold Plus 100 print film.

§2.2 RNA Extraction From F9 Cells

The method used for total RNA extraction from F9 cells was modified from the protocol of Chomczynski and Sacchi (1987). Aliquots of undifferentiated or differentiated F9 cells were collected in a microfuge tube and washed 3 times with PBS. The cells were resuspended in GIT solution (4 M guanidine thiocyanate, 25 mM sodium citrate, pH 7.0, 0.5% sarcosyl, 1% 2-mercaptoethanol), using about 1 ml GIT per 100 mg of cell pellet. The following components were then added: 1/10 vol of 2 M sodium acetate, pH 4.0, an equal vol of H_2O-saturated phenol, an equal vol of Sevag's solution (chloroform : isoamyl alcohol = 24 : 1, v/v). The preparation was mixed after each addition, and vortexed for two bursts of 10 sec each after addition of the Sevag's solution. The mixture was incubated on ice for 15 min followed by centrifugation at 10,000 g for 10 min at 4°C. The upper aqueous phase was transferred to a fresh tube and an equal volume of isopropanol was added. RNA was allowed to precipitate at -20°C for at least 1 hr. After centrifugation at 10,000 g for 20 min at 4°C, the precipitate was dissolved in 1/2 vol GIT. RNA was re-precipitated at -20°C for at least 1 hr following addition of 2.5 vol of cold 95% ethanol and recovered by centrifugation in a microcentrifuge for 10 min at 4°C. The RNA pellet was washed in 70% cold ethanol, dried under vacuum and resuspended in sterile distilled water.

§2.3 Cloning of Oct-4 Partial cDNA From F9 Cells

§2.3.1 Reverse transcription- polymerase chain reaction (RT-PCR)

To obtain a cDNA fragment of Oct-4, first strand cDNA was synthesized by reverse transcription of F9 cell total RNA primed with oligo$(dT)_{12-18}$ (Pharmacia). The reverse transcription reaction was carried out in a volume of 20 μl, and 1 μg of RNA and 200 U (1 μl) of Superscript™II RT (Gibco-BRL) was used. The reaction was carried out in a microfuge tube to which was added the following:

1 μl 0.5 μg/μl oligo$(dT)_{12-18}$

1 μg RNA

dH_2O to 12 μl

The mixture was heated to 70°C for 10 min and quickly chilled on ice. Following brief centrifugation, the following components were added:

4 μl 5 X RT buffer (250mM Tris-Cl, pH 8.3, 375mM KCl, 15mM $MgCl_2$)

2 μl 0.1 M DTT (dithiothreitol)

1 μl 10 mM dNTPs (10 mM of each deoxynucleoside triphosphate: dATP, dGTP, dCTP and dTTP)

1 μl 200 U/μl Superscript™II RT

The tube was incubated at 42°C for 1 - 2 hr. The reaction was then heated at 95°C for 5 min to denature the RNA-cDNA hybrid and to inactivate the reverse transcriptase. After quenching on ice, distilled water was added to bring the reaction volume up to 50 μl. The sample was then used for PCR immediately or stored at -20°C.

The PCR primers for Oct-4 cDNA were designed from the sequence data of Rosner et al. (1990). The sequences for primer pairs were chosen in regions that were unique to the Oct-4 gene and distinct from other members of the Oct gene family. The upstream primer had the sequence 5'-gagatatcGTGGATCCTCGAACCTGGCTAA-3' and the downstream primer had the sequence 5'-cagatatcACCTTCTCCAACTTCACGGCAT-3'. Bases designated by lower case at the 5' extension of each primer were added to generate an *Eco*RV restriction site for the convenience of cloning. The primers were synthesized by the Regional DNA Synthesis Laboratory (the University of Calgary).

The PCR conditions utilized were the same as described by Arcellana-Panlilio and Schultz (1994). Typically, one tenth of the RT product was used as the template for PCR amplification. The PCR reaction contained the following components:

5.0 μl	10 X PCR buffer (100 mM Tris-Cl, pH 8.3, 500 mM KCl, 1 mg/ml BSA)
5.0 μl	15 mM $MgCl_2$
1.0 μl	10 mM dNTPs
1.0 μl	each primer (100 μM stock)
0.2 μl	*Taq* polymerase (5 U/μl; Pharmacia)

5.0 μl RT product

dH_2O to 50 μl

The mixed components were overlaid with light liquid paraffin (BDH) and placed in a thermocycler with the following program: (1) 4 min at 94°C; (2) denaturation for 1 min at 94°C, annealing for 2 min at 55°C and extension for 2 min at 72°C; (3) repeat of (2) for 35 cycles; (4) incubation for 7 min at 72°C and soaking at 4°C.

After amplification, the PCR products were resolved on 2% agarose gels to verify that bands migrated as expected for their predicted size. The expected 297 bp RT-PCR product (including the *Eco*RV restriction sites in the primers), corresponding to nucleotide from 127 to 407 in the cDNA sequence described by Rosner et al. (1990) was observed. In addition, the PCR fragment was cut with the restriction enzyme *Fok*I that cleaved the fragment at a known site to verify the identity of the PCR product.

§2.3.2 Cloning of PCR fragments into a pBluescript vector

To prepare the insert, the 297 bp Oct-4 PCR fragment was digested with *Eco*RV to yield a 287 bp insert. The 287 bp fragment was purified from 2% agarose gel by using GeneClean™ (Bio 101 Inc.) according to the instructions of the manufacturer.

To prepare the vector, pBluescript-SK (pBS) plasmid was digested with *Eco*RV. After confirming complete digestion, the DNA was extracted with phenol:chloroform and precipitated with ethanol. The blunt-ended DNA was redissolved in 90 μl of 10 mM Tris-Cl, pH 8.3 and dephosphorylated using calf intestine alkaline phosphatase (CIP) in a

reaction containing the following components:

90 μl blunt-ended vector DNA

10 μl 10 X CIP buffer (10 mM $ZnCl_2$, 10 mM $MgCl_2$, 100 mM Tris-Cl, pH 8.3)

One unit of CIP was added for every 2 pmol of blunt termini. The reaction was incubated at 37°C for 15 min after which another aliquot of CIP was added and incubation was continued for a further 45 min at 55°C. At the end of the reaction, the tube was heated at 90°C for 10 min to inactivate CIP. Finally, the vector was extracted by phenol : chloroform and precipitated in ethanol.

The prepared insert was mixed with the prepared vector in a molar ratio of 3:1 for ligation according to protocols provided by Gibco-BRL. The ligation reaction contained the following:

150 ng	vector (3 kb)
45 ng	insert (287 bp)
2 μl	5 x ligation buffer (250 mM Tris-Cl, pH 7.6, 50 mM $MgCl_2$, 5 mM ATP, 5 mM DTT and 25% PEG-8000)
1 μl	T4 DNA ligase (1 U/μl, Gibco-BRL)
H_2O to 10 μl	

The ligation reaction was incubated at 14°C overnight and was then used for

transformation.

Transformation was carried out according to the procedure described by Sambrook et al. (1989). Competent E.*coli* HB101 cells were prepared using calcium chloride. For transformation, 3 μl of ligation reaction was incubated with 200 μl of competent cells in a sterile tube on ice for 30 min. The tube was transferred to a 42°C waterbath for 90 seconds and then rapidly transferred to ice to allow chilling for 1-2 min. LB medium (800 μl) was added to the mixture which was incubated for 45 min in a 37°C waterbath with gentle shaking every 10 min. The cells were plated onto LB-agar plates containing 50 μg/ml Ampicillin incubated at 37°C overnight. At the same time, the following transformations were set up as negative or positive controls respectively: (i) self-ligation of pBS vector cut with *Eco*RV and dephosphorylated, (ii) supercoiled pBS plasmid.

To screen recombinant clones, colonies from the plates were cultured in LB-Amp medium and used for plasmid mini-preps by using the alkali lysis method (Sambrook et al., 1989). The plasmid DNA was digested with *Eco*RV to release the 287 bp insert, or cut with *Hind*III to test the orientation of the insert relative to the T3 polymerase promoter. The *Hind*III enzyme cuts the Oct-4 PCR product at nucleotide 29, producing fragments of 29 and 269 bp. Inserts with the sense orientation relative to the T3 promoter produce two bands of 3 kb and 269 bp whereas inserts with the antisense orientation relative to the T3 promoter produce fragments of 3.3 kb and 29 bp. Two recombinant clones were identified: pBS.Oct4 (#1) which was in the sense orientation to the T3 promoter, and pBS.Oct4 (#2) which was in the antisense orientation to the T3 promoter.

Both pBS.Oct4 (#1) and pBS.Oct4 (#2) were sequenced to verify the identity of the clones.

§2.4 Northern Blot Hybridization

Two methods were used to resolve RNA on gels.

§2.4.1 Methyl mercuric hydroxide-agarose gel

This method was modified from Bailey and Davidson's protocol (1976). Agarose (1%, w/v) was dissolved by boiling in 1x running buffer (50 mM boric acid, 5 mM sodium borate, 10 mM sodium sulfate, pH 8.2) and cooled to 60-65°C. Methyl mercuric hydroxide was added to a final concentration of 10 mM. A vertical gel was cast using a Bio-Rad Mini-protean™ gel apparatus which was preheated at 60°C before pouring.

For sample preparation, aliquots of F9 RNA (10 μg) were dissolved in 20 μl of 0.1X running buffer containing running dye mixture (5% glycerol, 0.1 mM EDTA, pH 8.0, 0.025% bromophenol blue, 0.025% Xylene cyanol FF). The samples were heated at 60°C for 5 min, quenched on ice, loaded on the gel, and subjected to electrophoresis at 30 mA for about 1 hr. At the end of the run, the gel was soaked in 0.5 M NH_4Ac for 30 min, stained with 0.5 M ammonium acetate containing 0.5 μg/ml ethidium bromide for 20 min, and destained for 20 min with 0.5 M ammonium acetate prior to photography. The gel was then blotted onto Hybond™-N^+ membrane by capillary transfer with 3 mM NaOH overnight. The membrane was neutralized in 2x SSC and baked for 1 hr at 80°C (SSC is standard saline citrate and contains 0.15 M sodium

chloride and 0.015 M sodium citrate).

§2.4.2 Formaldehyde gel

To make a 1% formaldehyde-agarose gel, 1 g agarose was dissolved in 74 ml H_2O by boiling. When the gel was cooled to 65°C, 10 ml of 10X MOPS (10X MOPS: 0.2 M 3-[N-Morpholino]propanesulfonic acid (Sigma), 50 mM sodium acetate, 10 mM EDTA, pH 7.0 with NaOH) and 16 ml of formaldehyde (supplied as 37% or 12.3 M solution in H_2O; BDH) were added, mixed and the gel was poured in a horizontal gel apparatus.

To prepare RNA samples, aliquots containing 10 μg F9 cell total RNA in a volume of 4.5 μl were mixed with 2 μl of 10 X MOPS, 3.5 μl of formaldehyde and 10 μl of formamide. The samples were preheated at 65°C for 5 min, combined with 2 μl of RNA loading buffer (25% glycerol, 1 mM EDTA, pH 8, 0.25% bromophenol blue and 0.25% xylene cyanol FF), and resolved on the prepared formaldehyde gel using 1 X MOPS as running buffer. After staining the gel with 0.5 μg/ml ethidium bromide and photography, RNAs were immobilized onto a Hybond^{TM}-N^+ membrane by capillary transfer with 10X SSC. The membrane was baked at 80°C for 1 hr after transfer.

§2.4.3 Probe preparation

The Oct-4 DNA fragment was retrieved from the plasmid pBS.Oct4 (#1) by *Eco*RV digestion and radiolabelled by the random priming method. The Oct-4 DNA fragment (50 - 200 ng) was mixed with 60 ng of random hexanucleotide primer in 12 μl

volume. The tube was boiled for 5 min and quenched on ice. To the denatured DNA and primers were added 2 μl of 10X buffer (0.5 M Tris-Cl, pH 6.9, 0.1 M $MgSO_4$, 1 mM DTT, 0.6 mM each of dATP, dGTP and dTTP), 5 μl α-^{32}P-dCTP (10 μCi/μl, 3000 Ci/mmol, Amersham) and 1 μl Klenow (2 - 3 units). The reaction mixture was incubated at room temperature for 1 hr. The probe was purified by a Sephadex G-50 spun-column. The specific activity of the probe was about $3-5 \times 10^8$ cpm/μg.

§2.4.4 Hybridization

Conditions for Northern hybridizations were modified from the protocol described by Arcellana-Panlilio and Schultz (1993). Briefly, membranes containing immobilized RNAs were incubated with pre-hybridization mix at 65°C for 2-4 hr. The prehybridization mix contains 5X SSPE (20X SSPE: 3.6 M NaCl, 0.2 M sodium phosphate, pH 7.4, 20mM EDTA), 5X Denhardt's (50X Denhardt's: 1% each of Ficoll [type 400, Sigma], polyvinylpyrrolidone [PVP-360, Sigma] and bovine serum albumin [fraction V, Sigma]), 1% SDS, 100 μg/ml yeast tRNA and 100 μg/ml salmon sperm DNA. After prehybridization, the Oct-4 probe was added to the prehybridization mix at 1×10^6 cpm/ml. The membrane was allowed to hybridize overnight at 65°C.

On the following day, the membrane was washed twice for 30 min each in 2 X SSPE, 0.1% SDS at 65°C and twice for 20 min each in 0.1X SSPE, 0.1% SDS at 65°C. The membrane was then placed against XAR-5 film (Eastman Kodak Co.) for exposure.

§2.5 Protein Extraction From F9 Cells

Two methods were used to prepare whole cell protein from F9 cells. Proteins extracted using Method 1 (see below) were useful for Western blot only. Proteins extracted using Method 2 (see below) were useful for both Western blot and bandshift assays.

§2.5.1 Method 1: Freeze-thaw method

F9 cells were collected in a microfuge tube and washed 3 times with PBS. The cells were resuspended in PBS (approximately 1 ml PBS per 100 mg cells) and proteins were released from the cells by freeze-thawing three times by transfer between dry ice and a 37°C water bath. After centrifugation in a microcentrifuge at 14,000 rpm at 4°C for 10 min, the supernatant was transferred to a fresh tube. The protein extract was quantified (see below) and stored in aliquots at -70°C.

§2.5.2 Method 2: Sonication method

This method was modified from the procedure of Schöler et al. (1989). F9 cells were collected and washed 3 times with PBS. The cells were then resuspended in chilled protein extraction buffer (1 ml buffer per 100 mg cell pellet) that contained 20 mM Hepes, pH 7.8, 450 mM NaCl, 0.2 mM EDTA, 0.5 mM DTT and a mixture of protease inhibitors (0.5 mM PMSF [phenylmethylsulfonyl fluoride], 1 μg/ml leupeptin, 1 μg/ml pepstatin and 2 μg/ml aprotinin). The protease inhibitors were made as stock solutions

(PMSF, 0.1 M in isopropanol; leupeptin, 1 mg/ml in H_2O; pepstatin, 1 mg/ml in methanol; aprotinin, 2 mg/ml in PBS) which could be stored at -70°C for several months. They were added to extraction buffer at the desired concentrations just before use. The cells were sonicated for 30 sec at 80W using a microprobe in Braun-Sonic 2000. After sonication, the extracts were cleared by centrifugation at 14,000 rpm for 10 min in a microfuge at 4°C. The proteins were quantified (see below), dispersed into small aliquots and stored at -70°C.

Nuclear extracts from P19 cells were kindly provided by Mr. D. Hewitt and Dr. T. Tamaoki, Department of Medical Biochemistry, The University of Calgary (Hewitt, 1996). The P19 cells were cultured and induced to differentiate along a neuronal pathway with RA according to the procedure of Rudnicki and McBurney (1987).

§2.5.3 Determination of protein concentrations

The protein concentrations were determined using the Bradford assay (Bradford, 1976). Protein samples (5 μl) were placed into polystyrene cuvets and brought to a final volume of 800 μl with dH_2O. Duplicates of BSA standards containing 0, 2.5, 5, 10, 15, 20 and 30 μg of protein, respectively, in 800 μl H_2O were also prepared. To all standards and samples, 200 μl of protein assay dye reagent (Bio-Rad) was added and mixed well. After incubation in the dark for 30 min, spectrophotometric measurements were taken at 595 nm. Using the standards, a standard curve (OD_{595} vs. μg/ml) was constructed. From the curve and the sample readings, the protein concentration of each

sample was determined.

§2.6 Preparation of Oct-4 Antibodies

§2.6.1 Synthetic peptide

A 21-amino acid peptide CEGEAFPSVPVTALGSPMHSN was synthesized that was identical to the C-terminal 20 amino acids in the sequence of Oct-4 protein (Rosner et al., 1990), with the addition of a cysteine residue to the N-terminus of the peptide for the convenience of conjugation. The peptide was synthesized by the Peptide Synthesis Laboratory in Queen's University and purified by high pressure liquid chromatography (HPLC). Purity was greater than 95% and further confirmed by amino acid analysis.

§2.6.2 Choice of carriers

Because of their size, peptides may not contain all the features of a good immunogen and, therefore, may not be immunogenic on their own. Thus, most peptides need to be coupled to carrier proteins before injection into rabbits to stimulate antibody production. The two most frequently used carriers are keyhole limpet hemocyanin (KLH) and bovine serum albumin (BSA). KLH is totally alien to the host animal (rabbits) and, therefore, is the most immunologically provocative in terms of production of antibodies. However, KLH tends to precipitate upon conjugation with peptides. BSA, on the other hand, is very soluble even when highly coupled with peptide, and often is a good immunogen in its own right. In the protocol used herein, both KLH and BSA were

employed as carriers to generate antibodies of high specificity.

§2.6.3 Coupling of the peptide to carrier proteins

The Oct-4 peptide was coupled to the carrier proteins KLH or BSA through the cysteine residue of the peptide in the presence of *m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester (MBS) as coupling reagent (Green et al., 1982). The detailed procedure is as follows:

A. Preparation of spun-column and solutions: Sephadex G-50 was soaked in water and washed sequentially with water, ethanol, glacial acetic acid, excess of water, and 50 mM phosphate buffer, pH 6.0. The treated sephadex was packed in a 5-ml syringe and equilibrated with 1 ml 50 mM phosphate buffer pH 6.0 for 3 times. The packed spun-columns could be wrapped and stored in a refrigerator in an upright position for several days.

Solutions prepared just prior to use included: (i) Cysteine: 1 mg/ml (8.25 mM) in H_2O, diluted from 10 mg/ml. (ii) Ellman's reagent (5,5'- dithio-bis(2-nitrobenzoic acid); DTNB, Sigma): 4 mg/ml in 0.1 M phosphate buffer, pH 8.0. (iii) MBS: 35 mg/ml in dimethyl-formamide. (iv) Oct-4 peptide: 10 mg/ml in phosphate-buffered saline (PBS), adjusted to pH 7.0 - 7.5 with NaOH.

B. Free cysteine assays: For the peptide to be coupled efficiently, free cysteines must be available on the peptide. The content of free cysteine for Oct-4 peptide was

determined by Ellman's test (Ellman, 1959). In this method, the following were mixed in a polystyrene cuvette: 200 μl of 0.1 M phosphate buffer, pH 8.0, 10 μl of 1 mg/ml cysteine (standard, 8.25 mM) or 10 μl of 10 mg/ml Oct-4 peptide (sample), 100 μl of Ellman's reagent and 690 μl H_2O. The Ellman's reagent reacts with thiol groups and develops a color rapidly (2 min) which has absorbency at 412 nm. According to the absorbances of the cysteine standard (8.25 mM) and the Oct-4 peptide, the free cysteine molar concentration in Oct-4 peptide was calculated. The free cysteine content was also calculated according to the molar concentration of free cysteine in Oct-4 peptide and the molar concentration of the Oct-4 peptide (the molar concentration of 10 mg/ml Oct-4 peptide is 4.70 nM). It ranged from 50% to 80%.

C. Reaction of KLH or BSA with MBS: Samples (10 mg) of KLH or BSA were dissolved in 10 mM phosphate buffer, pH 7.0 to a final volume of 500 μl in a 1.5-ml microfuge tube. To this was added 1.75 mg (50 μl of 35 mg/ml) MBS and the reaction was shaken for 30 min at room temperature. To prevent an increase of the local concentration of formamide which might result in precipitation of KLH, the MBS was added in 10 μl aliquots every 5 min for the first 20 minutes of the reaction. At the end of the reaction, free MBS was removed by passage through the prepared Sephadex G-50 spun-column. The eluates containing the reaction product KLH-MBS or BSA-MBS were collected.

D. Conjugation of Oct-4 peptide with KLH-MBS or BSA-MBS: For a good

conjugation reaction, the ratio of the peptide to KLH-MBS (or BSA-MBS) should be at least 1 mg peptide for each mg of carrier protein. The above KLH-MBS (or BSA-MBS) eluates were mixed with 10 mg Oct-4 peptide which was dissolved in 1 ml PBS. The mixture was adjusted to pH 7.0-7.5 with NaOH and allowed to react for at least 3 hr at room temperature with shaking. The conjugate was purified by dialysis (dialysis tubing cut off 12,000 - 14,000 M.W.) of the coupling mixture against PBS overnight in a cold room.

§2.6.4 Production of Oct-4 polyclonal antibodies

New Zealand White rabbits, 3 months of age, were used for immunization. A bleed to obtain preimmune serum was carried out prior to the initiation of the immunization protocol. On day 0, 0.5 ml of 2% (w/v) Evans Blue Dye/PBS was injected between two toes of each hind foot of each rabbit 1 - 2 hr before inoculation (the dye became concentrated in the lymph nodes and made them easier to find). Thereafter, 200 μg (in 50 μl) of KLH-Oct4 was emulsified in 50 μl of Complete Freund's Adjuvant (CFA) and injected into the popliteal lymph node of each hind leg. On day 14, 0.5 ml of emulsion containing 100 μg KLH-Oct4 and an equal volume of Incomplete Freund's Adjuvant (IFA) was injected subcutaneously into each side of the rabbits. Subsequently, the rabbits were boosted with 100 μg KLH-Oct4/IFA at biweekly intervals. On day 56, 50 μg of BSA-Oct4 in IFA was injected subcutaneously. To maintain a high antibody titre, 50 μg booster injections were continued with IFA and BSA-Oct4 at monthly intervals. Bleedings were taken weekly after day 70. Finally, cardiac puncture was taken

to collect large amount of antisera. Three animals were used to develop antibodies to the Oct-4 peptides. Antiserum from rabbit #3 had the highest titre and the least amount of cross-reactivity with peptides other than Oct-4 and was used for all experiments described in this thesis. Whole sera were used in all the experiments.

§2.7 Immuno-dot Blot

Because the concentration of the antibodies in the Oct-4 antisera was unknown, a dot blot procedure was used as a quick and effective method to determine the optimal antibody dilution for use in western blot experiments. To do this, Oct-4 peptide was spotted onto a strip of Hybond™-ECL nitrocellulose membrane (Amersham) in amount of 10 pg, 1 ng and 1 μg. One blot was prepared for each antiserum dilution to be tested. The membranes were blocked in 5% BSA/PBS with 0.2% Tween-20 (PBS-T) for 1 hr at room temperature and then incubated for 1 hr at room temperature in Oct-4 anti-serum with dilutions of 1:500, 1:2,000 and 1:10,000, respectively. After several washes in PBS-T, the membranes were incubated in horseradish peroxidase (HRP)-conjugated anti-rabbit secondary antibody (Amersham) with 1:2,000 dilution for 1 hr at room temperature and washed as before. Signals were detected using enhanced chemiluminescence (ECL) detection reagent (Amersham) in the same manner as described in the next section (Western Blot). The optimal Oct-4 anti-serum dilution was chosen by comparison of the signals obtained with the background.

§2.8 Western Blot Analysis of Oct-4 Expression in F9 Cells

§2.8.1 Electrophoresis and blotting

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was employed to resolve polypeptides. Typically for western blots, 10 μg of F9 cell protein extract was loaded in each lane of protein mini-gels containing 10% separating gel and 4% stacking gel. The gel was subjected to electrophoresis at 200V for about 45 minutes.

After the run, the gel was equilibrated in protein transfer buffer (25 mM Tris, 192 mM glycine, 20% v/v methanol, pH 8.3) for 30 min. Hybond^{TM}-ECL membrane cut to the size of the gel was pre-wetted in distilled water followed by equilibration in transfer buffer for at least 15 min. Proteins were transferred in a mini Transfer-Blot^{R} electrophoretic transfer cell (Bio-Rad) in a cold room at 30V overnight or at 100V for 2 hr. After the transfer, the blot was used immediately or air-dried and stored in a refrigerator for up to 3 months.

§2.8.2 ECL immunodetection

Western blot detection of Oct-4 proteins from F9 cell extracts or oocyte extracts was modified from Amersham ECL Western blotting protocols. The membrane was blocked in PBS-T (80 mM Na_2HPO_4, 20 mM NaH_2PO_4, 100 mM NaCl and 0.3% (v/v) of Tween-20) containing 5% BSA for 1 hr at room temperature with shaking. The membrane was washed with PBS-T 1X for 15 min and 2X for 5 min. Oct-4 antiserum was preincubated in 5% BSA/PBS-T at a dilution of 1:2,000 for 30 min at room

temperature before use. The membrane was then soaked in the diluted antiserum for 1 hr at room temperature with gentle shaking, followed by vigorous washes with PBS-T 2X for 15 min and 2X for 5 min. The membrane was then incubated with HRP-linked anti-rabbit secondary antibody diluted at 1:3,000 for 1 hr at room temperature. The membrane was washed with PBS-T at least 3X for 15 min and 3X for 5 min prior to use for detection. The detection procedure followed the manufacturer's instruction (Amersham) in which the detection reagents (solution 1 and solution 2) were mixed in an equal volume (final 0.125 ml/cm^2) and added to the membrane on the surface carrying the protein for precisely 1 min at room temperature. The membrane was placed against Kodak XAR-5 film for exposure for 10 sec to 10 min.

For the control experiments, the same membrane was stripped of bound antibodies and reprobed with rabbit preimmune serum (1:2,000 dilution) or Oct-4 antiserum pre-incubated with excess Oct-4 peptide. To strip the membrane, the membrane was submerged in stripping buffer (100 mM 2-mercaptoethanol, 2% SDS, 62.5 mM Tris-Cl pH 6.7) and incubated at 65°C for 1 hr (with occasional agitation), followed by two washes with PBS-T for 10 min each at room temperature. For the peptide absorption control, 1 μl of Oct-4 antiserum was incubated with 1 μg Oct-4 peptide overnight. The antibody-peptide complexes were precipitated by centrifugation at the top speed in a microcentrifuge for 10 min before use.

§2.9 Bandshift And Supershift Assays

§2.9.1 Oligonucleotides

Two complementary single-stranded 22-mer oligonucleotides were synthesized: 5'-GATCGGTAATTTGCATTTCTAA-3' (oligo #1) and 5'-GATCTTAGAAATGCAAATTACC-3' (oligo #2). Upon annealing, these oligonucleotides produced an octamer binding motif ATTTGCAT with surrounding sequences identical to the mouse IgH enhancer sequence region 536-584 nt (Ephrussi et al., 1985). They each also contained a *Bam*HI overhang at the 5' end for the purpose of labelling. These oligonucleotides were synthesized by the Regional DNA Synthesis Laboratory (the University of Calgary).

§2.9.2 Oligonucleotide annealing and labelling

Synthesized oligonucleotides were made up as 20 μM (20 pmol/μl) stocks. The annealing reaction was set up as follows:

9 μl oligo #1 (20 pmol/μl)

9 μl oligo #2 (20 pmol/μl)

2 μl 10 X annealing buffer (1.5 M NaCl, 100 mM Tris-Cl, pH 8, 10 mM EDTA)

After the mixture was centrifuged briefly in a microcentrifuge, the tube was incubated

at 90°C for 10 min in a heat block. At the end of the incubation, the heater was turned off and allowed to cool down slowly at room temperature for 1-2 hrs until the temperature dropped under 30°C.

The annealed oligonucleotides were labelled using the Klenow fragment of DNA polymerase I to fill in the ends. The following components were added to a 1.5-ml microfuge tube on ice:

2 μl	annealed oligonucleotides
2.5 μl	10 X nick-translation buffer (0.5 M Tris-Cl, pH 7.5, 0.1 M $MgSO_4$, 1 mM DTT, 0.5 mg/ml BSA)
5 μl	α-^{32}P-dCTP (10 μCi/μl, 3000 Ci/mmol, Amersham)
1 μl	2 mM d(AGT)TPs
13.5 μl	H_2O
1 μl	Klenow (6 U/μl)
total volume 25 μl	

The reaction was mixed and incubated at room temperature for 30 min. The volume of the reaction was then increased to 100 μl with TE (10 mM Tris-Cl, pH 8, 1 mM EDTA). The labelled oligonucleotides were purified with a Sephadex G-50 spun-column.

§2.9.3 Bandshift reactions

DNA-protein binding reactions were carried out as described previously by Wang

and Schultz (1996). The reactions contained the following:

4 μl 5X EMSA buffer (50 mM Hepes, pH 7.8, 5 mM spermidine, 25 mM $MgCl_2$, 250 mM KCl, 2.5 mM DTT, 45% glycerol)

2 μl labelled oligonucleotides ($\sim 10^4$ cpm)

1 μl 1 mg/ml poly (dI-dC)·poly (dI-dC)

1 μl 10 mg/ml BSA

12 μl F9 protein extract (+ H_2O)

The final volume was 20 μl.

In this reaction, the components were mixed on ice. The protein extract was added last. After incubation for 30 min at room temperature, 10 μl of the binding reaction was applied to a non-denaturing polyacrylamide gel (see below). As a gel running indication, a mixture containing 4 μl 5X EMSA buffer, 2 μl labelled oligonucleotides, 2 μl DNA loading dye (containing bromophenol-blue) and 12 μl H_2O were mixed and loaded onto the same gel.

The amount of the protein added in the above reaction was initially tested using 1 μg, 2 μg, 6 μg, 12 μg, 16 μg and 22 μg of protein, respectively. The optimal protein amount (6 μg) for the assays was chosen on the basis of signal:background ratio.

§2.9.4 Competition assays

To verify that the protein-DNA complexes in the above reactions were a result

of specific binding, competition assays were carried out. For specific competition, a 100-fold excess of unlabelled double-stranded oligonucleotides was added in the above reaction mixture and incubated for 30 min at room temperature before loading onto the gel. For non-specific competition, a 100-fold excess of sheared salmon sperm DNA was used.

§2.9.5 Supershift assays

In order to examine whether Oct-4 antibodies could supershift the DNA-protein complexes, the bandshift reactions were carried out initially in the manner described in §2.9.3. After a 30 min incubation, an aliquot (1 μl) of Oct-4 antiserum was added to the reaction and the incubation was continued for a further 1 hr at room temperature before loading the gel. For control experiments, 1 μl of preimmune serum or new-born goat serum instead of Oct-4 antiserum were added to the bandshift reactions.

§2.9.6 Non-denaturing PAGE

The bandshift and supershift reactions were analyzed by non-denaturing polyacrylamide gel electrophoresis (non-denaturing PAGE). Typically, 5% polyacrylamide gels were used. To make a 50 ml gel, 8.33 ml of 30% acrylamide stock, 2.5 ml of 10X TBE buffer, 38.82 ml of water, 0.35 ml of 10% ammonium persulfate and 25 μl of TEMED were combined and poured into a vertical slab gel unit (Hoffer Scientific Instrument) with 15 cm X 15 cm plates and 1.5 mm spacers. The gel was allowed to polymerize for about 40 min. After loading samples, the gel was run at 125V

for about 2 hr at room temperature using 0.5 X TBE as running buffer until the bromophenol blue dye ran about 8 cm. The gel was then transferred to Whatman paper and dried for 45 min in a gel dryer. The dried gel was placed against XAR-5 film for exposure.

§2.10 Immunofluorescent Staining of F9 Cells

Cultured F9 cells were stained with Oct-4 antiserum followed by FITC-conjugated secondary antibody to localize Oct-4 protein expression. The detailed procedure is outlined below:

F9 cells were collected, washed 3 times with PBS, and resuspended in PBS to a desired density. Poly-L-Lysine coated adhesion slides (Avestin, Inc.) were rinsed under tap water to remove the protective green coating. To each well of the slide, 10 μl of F9 cell suspension were spotted and allowed to adhere for about 15 min at room temperature. Free cells were removed by rinsing gently with PBS. The attached cells were fixed for 10 min by placing the slide into freshly prepared 4% paraformaldehyde/PBS, and then washed in PBS 3X for 2 min. The slide was incubated in 2% goat serum for 30 min at room temperature to block non-specific binding sites on the cells. After a rinse in PBS, the cells were permeabilized with 0.05% Triton X-100/PBS for 4 min. The slide was washed with PBS 2X for 2 min. To each well on the slide, either 10 μl of diluted Oct-4 antiserum or preimmune serum were applied. The

antisera were diluted 1:10 in 5% BSA/PBS and the dilution was incubated for 30 min at room temperature before use. For the preimmune serum control, preimmune serum was also diluted 1:10. For a "no antibody" control, 10 μl of 5%BSA/PBS was applied to the well. The reactions were incubated for 1 hr at 37°C in a humidified chamber to prevent evaporation. After the primary antibody reaction, the slide was washed in PBS 2X for 2 min and 10 μl of FITC-conjugated anti-rabbit goat IgG (Zymed Laboratories, Inc.) was added to each well in a dilution of 1:100. The reaction was incubated for 45 min at 37°C in a humidified chamber. After washing as before, the slide was mounted with a drop of anti-fade medium (Citifluor), observed and photographed under a Zeiss photomicroscope II using UV illumination.

§2.11 Animals And Oocytes Recovery

Seven to eight weeks old randomly bred CD1 Swiss albino mice (Charles River Breeding Laboratories) were used throughout the second part of the study. For superovulation, the female mice were stimulated by intraperitoneal injection of 7.5 I.U. pregnant mare serum (PMS) gonadotrophin (Folligon, Intervet, Cambridge, U.K.) followed 44-48 hr later by the intraperitoneal injection of 7.5 I.U. human chorionic gonadotrophin (HCG; Sigma).

Approximately 16 to 18 hr after HCG administration, oocytes, surrounded by the cumulus cells, were recovered from the ampullary end of mouse oviducts. The oocytes were freed from cumulus cells by incubation in 300 U/ml of hyaluronidase (Sigma) in

M2 medium (Hogan et al., 1986) containing 0.4% BSA (w/v) for a few minutes. The cumulus cells were subsequently removed following passage of dissociated cumulus masses over a Nitex-screening fabric with a mesh size which permits passage of follicular cells while retaining eggs. After several washes in M2, oocytes were collected and pooled for RNA or protein analyses.

§2.12 *In Situ* Hybridization

§2.12.1 Fixation and embedding

Mouse ovaries or the ampulla end of oviducts containing newly ovulated oocytes in a cumulus mass were recovered by dissection. Ovaries and oviduct segments were fixed overnight at 4°C in freshly made 4% paraformaldehyde in a buffer containing 85 mM Pipes, 25 mM Hepes and 10 mM EGTA, pH 6.5. The tissues were then rinsed in 70% ethanol 3 times and stored in 70% ethanol at 4°C until ready for subsequent treatment.

Before embedding, the tissues were dehydrated through an alcohol series with 30 min in 80% ethanol, 30 min in 95% ethanol and 2 X 1hr in 100% ethanol. The tissues were then cleared in chloroform overnight.

On the following day, the ovaries or oviduct segments were infiltrated with paraplast (Oxford® Labware) at 60°C for 1 X 30 min and 2 X 1 hr. The last half hour of the paraffin incubation was under -20 in.Hg vacuum. Individual ovaries were embedded in Tissue-Tek base molds (15 mm X 15 mm X 5 mm; Miles) in conjunction

with embedding rings (VWR). Sections of 6 μm thickness were cut on a microtome and spread on glass slides treated with 3-aminopropyltriethoxysilane (TESPA; Sigma).

§2.12.2 Riboprobe synthesis

Digoxigenin-11-UTP (Boehringer-Mannheim) labelled RNA probes were used for *in situ* hybridization. The antisense Oct-4 probe was transcribed off the T7 polymerase promoter of plasmid pBS.Oct4(#1) linearized with *Eco*RI. The sense Oct-4 probe was produced via the T3 polymerase promoter from plasmid pBS.Oct4(#1) linearized with *Cla*I. To label the probes, the following components were added to a microfuge tube in the given order:

1 μg template DNA

2 μl DIG NTP labelling mix (10 mM of each ATP, CTP and GTP, 6.5 mM UTP and 3.5 mM DIG-UTP)

2 μl 10 X TB (400 mM Tris-Cl, pH 8, 60 mM $MgCl_2$, 100 mM DTT, 20 mM spermidine, 100 mM NaCl)

1 μl T7 or T3 RNA polymerase (60 U)

H_2O to 20 μl

The reactions were incubated at 37°C for 2 hr. To remove the DNA templates, 20 U of RNase-free DNase I were added to the reactions and incubated for a further 30 min. The volume of the reactions were increased to 200 μl with TE. The riboprobes were purified

using G-50 spun-column and precipitated with 50 μg yeast tRNA (carrier), 1/10 vol of 4 M LiCl and 3 vol of cold 95% ethanol overnight at -20°C. The transcripts were analyzed by agarose gel electrophoresis and ethidium bromide staining. The yields of the transcripts were estimated by comparing a DNA sample of known amount run side by side.

§2.12.3 *In situ* hybridization

Before hybridization, slides mounted with mouse ovary or oviduct sections were dewaxed, rehydrated, proteinase K-treated to open up the tissue and acetic anhydride-treated to neutralize positive charges on proteins in the tissue, which in turn prevents charge interactions with probes. The pretreatment procedures were applied to groups of slides by transfer in Wheaton jars as follows:

Toluene	2 X 2 min
Absolute ethanol	2 min
95% ethanol	2 min
80% ethanol	2 min
70% ethanol	2 min
2 X SSC	2 X 2 min
Proteinase K at 20 μg/ml in 20 mM Tris-Cl pH7.5 and 2 mM $CaCl_2$	
	7.5 min at room temperature
2 X SSC	2 X 5 min

1.25 ml acetic anhydride in 250 ml triethanolamine	
	10 min
2 X SSC	2 X 5 min

After these pretreatments, slides were incubated in prehybridization mix (50% formamide, 5 X SSPE, 20 mM DTT, 1 X Denhardt's solution) for 2-4 hr at 50°C. Slides were taken out of prehybridization mix one by one and excess solution on the slides was removed with Kimwipes. An aliquot (20 μl) of prehybridization mix containing 100 ng of DIG-labelled Oct-4 riboprobe (sense or antisense) and 400 ng of yeast tRNA was spotted onto the sections of each slide. The slides were sealed with coverslips and rubber cement and transferred to a sealed container in an air oven at 50°C. The hybridization reaction was allowed to proceed for 12-36 hr.

On the following day, the rubber cement was gently peeled off and coverslips were removed by soaking in 2 X SSC. The slides were washed as outlined below:

2 X SSC	30 min at 37°C
20 μg/ml RNase A in 2 X SSC	30 min at 37°C
2 X SSC	30 min at 37°C
2 X SSC	30 min at 50°C
0.5 X SSC	30 min at 50°C

After the final wash, the slides were soaked for 5 min in buffer 1 (100 mM Tris-

Cl, pH 7.5, 150 mM NaCl), followed by the DIG probe detection procedure as described below:

Blocking buffer (buffer 1 plus 0.3% Triton X-100, 2% normal sheep serum)	1 hr at room temperature
1:500 dilution goat anti-DIG antibody in blocking buffer (100 μl per slide)	4 hr at room temperature
Buffer 1	3 X 10 min
Buffer 2 (100 mM Tris-Cl, pH 9.5, 100 mM NaCl, 50 mM $MgCl_2$)	5 min

At this point, the slides were ready for color reactions. The color reaction mix was made by adding 35 μl of 100 mg/ml NBT (nitroblue tetrazolium, Boehringer-Mannheim), 35 μl of 50 mg/ml BCIP (5-bromo-4-chloro-3-indolyl phosphate, Boehringer-Mannheim), 25 μl 1 M levamisole (Sigma) to 10 ml buffer 2 immediately before use. Each slide was wiped dry with Kimwipes and spotted with 450 μl of the color reaction mix. The colour reaction was incubated at room temperature in a dark humidified chamber (paper towels moisted with buffer 2) for 12 to 36 hrs.

When a desired color is achieved, the color reaction is stopped by soaking the slides in buffer 3 (10 mM Tris-Cl, 1 mM EDTA, pH 8.0). Before mounting, the slides were stained in 0.1 mg/ml nuclear fast red for 2 min and dehydrated in increasing concentrations of ethanol (10 dips each), followed by 10 dips in xylene. The slides were

mounted with Permount® (Fisher), examined and photographed under a Zeiss photomicroscope II.

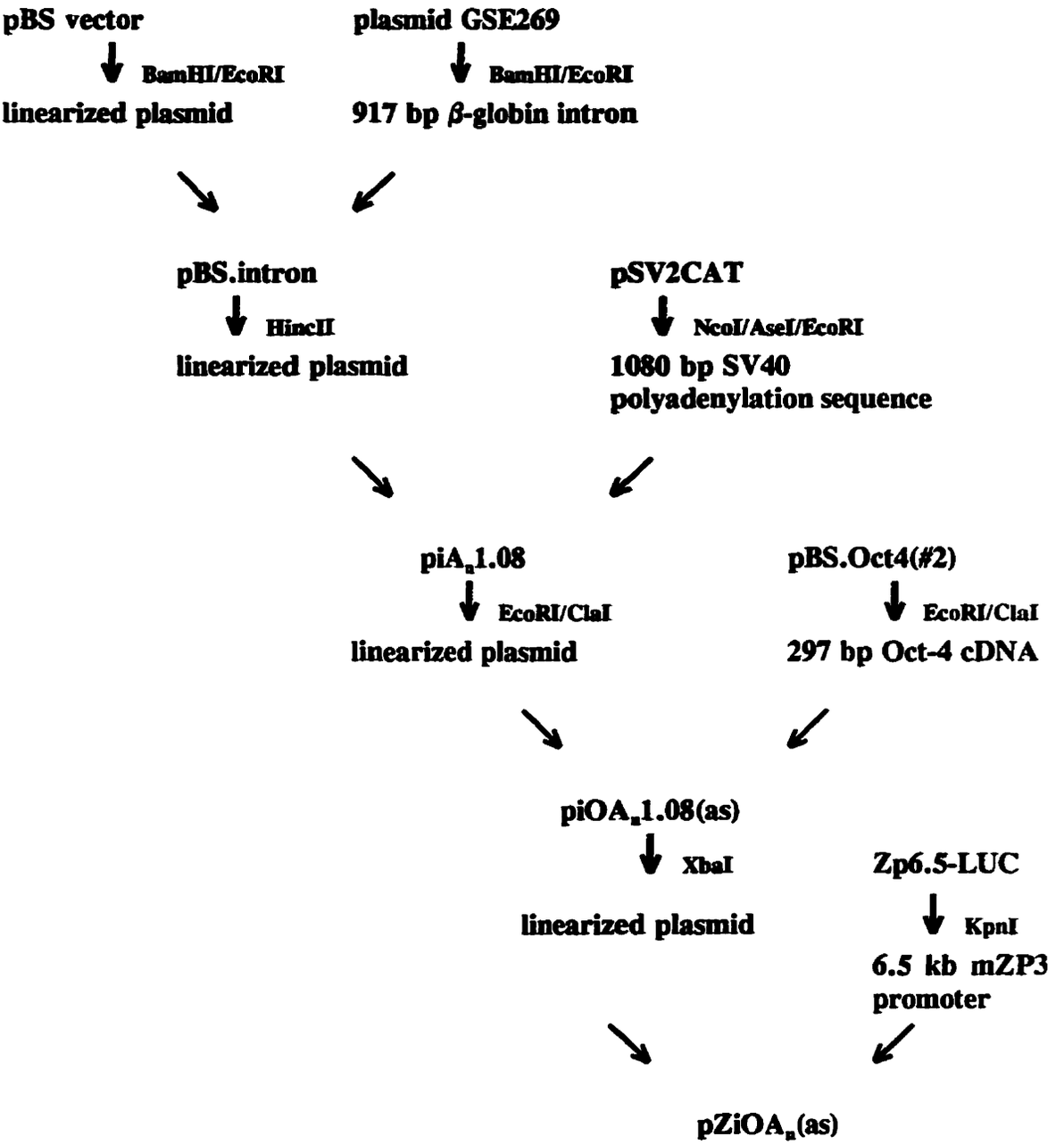
§2.13 Preparation of Transgenic construct

To make the transgenic construct, a pBluescript.SK (pBS) vector was used. The construct contained four DNA components in the following order: mouse ZP3 promoter, human β-globin second intron, Oct-4 cDNA fragment in antisense orientation and SV40 small t-antigen polyadenylation signal sequence. Figure 3 shows the flow chart of the procedure. The detailed scheme of the whole procedure is described in the following sections.

§2.13.1 Cloning of the intron

Plasmid GSE269 was obtained as a gift from Dr. Gordan Chan in Department of Medical Biochemistry, The University of Calgary. GSE269 contains a 5.5 kb human β-globin genomic fragment which encompasses the second intron. The 917 bp intron was retrieved from the plasmid by *Bam*HI and *Eco*RI double digestions. The pBS vector was also digested with *Bam*HI and *Eco*RI. The 917 bp intron and the digested vector were ligated directionally in a reaction as described in Section §2.3.2. The resulting plasmid was named pBS.intron.

Fig.3. A flow chart of making the transgenic construct. The detailed scheme of the procedure is described in section §2.13.


pBS vector
BamHI/EcoRI
linearized plasmid
plasmid GSE269
BamHI/EcoRI
917 bp β-globin intron
pBS.intron
HincII
linearized plasmid
pSV2CAT
NcoI/AseI/EcoRI
1080 bp SV40
polyadenylation sequence
piA$_n$1.08
EcoRI/ClaI
linearized plasmid
pBS.Oct4(#2)
EcoRI/ClaI
297 bp Oct-4 cDNA
piOA$_n$1.08(as)
XbaI
linearized plasmid
Zp6.5-LUC
KpnI
6.5 kb mZP3
promoter
pZiOA$_n$(as)

§2.13.2 Cloning of the polyadenylation sequence

Plasmid pSV2CAT (Gorman et al., 1982) was digested with *NcoI*/ *AseI*/*Eco*RI to produce six fragments: 301 bp, 359 bp, 752 bp, 822 bp, 1080 bp and 1689 bp, respectively. The 1080 bp fragment corresponds to the position 2618-3698 in pSV2CAT and contains the SV40 t-antigen polyadenylation signal sequence. The fragment was purified and end-blunted by a Klenow fill-in reaction essentially as described in Section §2.9.2 except that no radioactivity was used. The blunt-ended fragment was ligated to the *Hinc*II site of plasmid pBS.intron. The orientation of the insert was examined by *Pst*I/ *Hind*III digestions which produced two bands on gel electrophoresis, the larger band being mostly vector. The other band was indicative of orientation: a 870 bp species meant that the insert was correctly oriented, i.e., the polyadenylation sequence had the same orientation as the intron; a 206 bp band meant incorrect orientation. The plasmid with the correct orientation was designated as piA$_n$1.08.

§2.13.3 Cloning of the antisense Oct-4 cDNA

To insert the Oct-4 fragment into piA$_n$1.08 in an antisense orientation, an Oct-4 mini-cDNA clone pBS.Oct4 (#2) which is in antisense orientation relative to the T3 promoter (see Section §2.3.2) was cut with *Eco*RI and *Cla*I restriction enzymes. The Oct-4 fragment with *Eco*RI and *Cla*I overhangs was purified from a 2% agarose gel using GeneClean™ (Bio 101 Inc.). Plasmid piA$_n$1.08 was also digested with *Eco*RI and *Cla*I. The prepared insert and vector were ligated directionally to produce a plasmid piOA$_n$1.08(as).

§2.13.4 Cloning of the promoter

As a final step, the 6.5 kb mZP3 promoter (Lira et al., 1990) was retrieved from plasmid Zp6.5-LUC (kindly provided by Dr. Paul M. Wassarman, Department of Cell Biology and Anatomy, Mount Sinai School of Medicine, New York, N.Y.) by *Kpn*I digestion. To produce blunted ends, the 6.5 kb *Kpn*I fragment was subjected to 3' exonucleolytic digestion to remove 3' termini using the 3'→ 5' exonuclease activity of T4 DNA polymerase according to the following procedure:

10 μl 6.5 kb DNA fragment in TE, pH 8 (~3 μg)

1 μl 2mM dNTPs

2 μl 10X any restriction digestion buffer

6 U T4 DNA polymerase

water to 20 μl

The reaction was incubated for 15 min at 12°C. T4 DNA polymerase was inactivated at 75°C for 10 min. The reaction was phenol:chloroform extracted and the blunt-ended DNA fragment was precipitated with ethanol.

Plasmid piOA$_n$1.08(as) was linearized with *Xba*I and the 5' protruding ends were filled-in using Klenow fragment. The blunt-ended 6.5 kb mZP3 promoter and the vector piOA$_n$1.08/*Xba*I were ligated. The orientation of the mZP3 promoter was confirmed by *Cla*I or *Bam*HI digestion which gave different patterns of bands between the two orientations of the promoter. The resulting plasmid with the correct orientation of the

promoter was designated as pZiOA$_a$(as).

§2.13.5 Preparation of the transgenic fragment

The transgenic construct, pZiOA$_a$(as), was cut with restriction enzymes *Sma*I and *Kpn*I to release the 8.8 kb DNA fragment (which contained promoter, intron, antisense Oct-4 and polyadenylation site) from the plasmid. To purify this fragment, the digestion reaction was resolved in a 0.8% agarose gel. A strip of DEAE-cellulose membrane was inserted into a slot made in the gel in front of the 8.8 kb band. Before use, the DEAE membrane was activated by soaking in 10 mM EDTA, pH 8 for 5 min and then soaking in 0.5 N NaOH for a further 5 min, followed by washing six times in sterile water. When the 8.8 kb DNA band had migrated from the gel and was trapped on the membrane, the membrane was removed and washed at room temperature with low-salt wash buffer (20 mM Tris-Cl, pH 8, 0.15 M NaCl, 0.1 mM EDTA, pH 8) to remove residual agarose. DNA was then eluted by incubating the membrane in high-salt elution buffer (20 mM Tris-Cl, pH8, 2.5 M NaCl, 0.1 mM EDTA) at 65°C for 10-45 min. After extraction with phenol:chloroform and precipitation twice with ethanol, the purified DNA was finally dissolved in filter-sterilized injection buffer (5 mM Tris-Cl, pH 7.4, 0.1 mM EDTA) to a concentration of 4 ng/μl.

§2.14 Generation of Transgenic Mice

Transgenic mice were kindly produced by Dr. Saul Zackson, Department of

Medical Biochemistry, The University of Calgary. In brief, the prepared transgenic fragment was injected into the male pronuclei of fertilized mouse eggs that were subsequently transferred into oviducts of pseudopregnant CD1 female mice.

§2.15 Identification of Transgenic Mice

§2.15.1 Tail DNA extraction

Transgenic founder mice and offspring were identified by PCR using mouse tail DNAs as templates. Mouse tails were snipped when the mice were about 4-weeks old. Two methods were used for mouse tail DNA extraction. Both methods yielded DNA that could be digested with most restriction enzymes and that could be used successfully for PCR.

A. Method 1: About 1 cm of mouse tail was cut and placed in a 1.5-ml microfuge tube containing 0.5 ml of lysis buffer (50 mM Tris-Cl pH 8, 100 mM EDTA, 0.5% SDS, 0.5 mg/ml proteinase K) and incubated in a 55°C waterbath. The reaction was agitated after 1-3 hr incubation and left overnight. On the second day, proteinase K was inactivated by heating to 95°C for 15 min. The debris was pelleted by centrifugation in a microcentrifuge at top speed for 5 min. The supernatant was transferred to a fresh tube and extracted once with phenol and once with phenol:chloroform. The aqueous phase was transferred to a new tube, 1/10 vol of 3M sodium acetate, pH 6 was added and the DNA was precipitated by addition of 2.5 vol of 95% ethanol. After recovery by centrifugation,

the DNA was washed thoroughly with 70% ethanol to remove traces of salt and SDS. The pellet was resuspended in 100 μl TE and allowed to dissolve overnight at room temperature.

B. Method 2: Mouse tail of 1-2 cm length was incubated in 0.5 ml of lysis buffer (10 mM Tris-Cl pH 8.2, 400 mM NaCl, 2 mM EDTA, 0.5% SDS, 0.2 mg/ml proteinase K) at 55°C overnight with shaking. The debris was pelleted by centrifugation in a microcentrifuge at the top speed for 5 min. To the supernatant, 125 μl of H_2O-saturated NaCl (~6 M) was added and mixed for 15 sec to salt out proteins. After centrifugation at 10,000 rpm for 15 min, the supernatant was transferred to a fresh tube and mixed with 2 vol of absolute ethanol. The thread-like DNA was transferred using a pipet tip to a tube containing 100 μl of TE, pH 8. DNA was allowed to dissolve completely by heating the tube to 55°C for several hours.

§2.15.2 PCR analysis

Two pairs of primers were used in each PCR reaction. Primer pair 5'-ACCTTCGCCTGGACTGCCGC-3' and 5'-GCTCATGGGATTCGCGCCCG-3' were used as a positive control which amplified a 231 bp fragment of the endogenous mouse Thy-1 gene. Primer pair, Zioprime.A and Zioprime.B, had sequences 5'-ATGAGGTTTGAGGCCACAGGT-3' and 5'-CCTGAGACTTCCACACTGATGC-3', respectively, which amplified a 309 bp fragment spanning the mZP3 promoter and the β-globin intron of the transgene. This fragment could only be present if the transgenic

construct had been stably integrated into the mouse genome.

Before setting up PCR reactions, the mouse tail DNA was denatured at 95°C for 10 min and quenched on ice. The conditions for PCR were the same as described in Section §2.3.1, except that 0.5 μl of each of the primer stocks (100 μM of Thy 5', Thy 3', Zioprime.A and Zioprime.B primers) were used. The temperature programs in the thermocycler were optimized to the following: (i) initial denaturation 6 min; (ii) denaturing at 94°C for 1 min, annealing at 60°C for 1 min, extension at 72°C for 1 min; (iii) repeat (ii) for 35 cycles; (iv) final extension at 72°C for 7 min; and (v) soaking at 4°C. After the PCR, 10 μl of PCR products were analyzed on a 2% agarose gel.

§2.15.3 Identification of homozygous transgenic mice

Homozygous transgenic mice were produced by setting up heterozygous intercrosses. As a first step to identify the homozygous mice, partial quantitative PCR method was employed. Mouse tail DNAs from each litter were quantified carefully by spectrophotometry. In each reaction, 4 ng of mouse DNA was used as template. Conditions for PCR were the same as above (Section §2.15.2) except that 25 to 30 cycles were used. A small aliquot (10 μl) of PCR reaction products was analyzed on a 2% gel. The mice with a stronger 309 bp band than the corresponding bands in other lanes were chosen for further analysis.

The chosen mice were then mated (back-cross) with normal (non-transgenic) CD1 mice. Tail DNAs from the offspring were analyzed with PCR to determine whether they contained the transgenic construct. Mice in which all of the offspring generated in the

back-cross were transgenic were recognized as homozygous transgenic mice. Initially, 5 homozygous females and 5 homozygous males were identified within the transgenic line. The line was maintained by homozygous intercrosses.

§2.16 Marking Transgenic Lines

Each potential transgenic mouse was assigned an identification number and marked with that number. The scheme for marking a mouse using a continuous number series was the same as described by Hogan et al. (1986). The scheme involved ear punching and used numbers from 1 to 99 only. Ear punching was performed at the time of tail biopsy when the mice were about 4-weeks old.

§2.17 Analysis of Transgene Expression

§2.17.1 RNA extraction

Ovary, brain, heart, kidney, liver, muscle, spleen and testis were collected from transgenic mice. Ovaries were also collected from normal CD1 mice. Tissues were homogenized on ice in a 1.5-ml microfuge tube with a Deltaware™ Pellet Pestle disposable mixer (VWR). GIT solution (4 M guanidinium thiocyanate, 25 mM sodium citrate pH7, 0.5% sarcosyl, 1% 2-mercaptoethanol) was added to each tube at 1 ml solution per 100 mg tissue. The remainder of the RNA extraction procedure was identical to that used for F9 cell RNA extraction (Section §2.2).

§2.17.2 Radioactive RT-PCR analysis

All RNA samples were treated with RNase-free DNase I before reverse transcription to remove any contaminating genomic DNA in the RNA samples. To do this, 1 μg of RNA was incubated with 10 U of DNase I (Pharmacia) in a volume of 11 μl at 37°C for 15 min. The tube was then heated to 95°C for 10 min to inactivate DNase I and quenched on ice. The DNase I-treated RNA samples were used directly for reverse transcription following the procedures described in Section §2.3.1.

For PCR, primer pairs, Zioprime G and Zioprime H, or, Zioprime I and Zioprime J, were used. The nucleotide sequences of these primers were: 5'-TCCAACCTGAGGTCCACAGTAT-3' (Zioprime G); 5'-CCTCTACAAATGTGGTATGGCT-3' (Zioprime H); 5'-TTGCTTTCCACTCGTGCTCC-3' (Zioprime I) and 5'-ATGATCTCTAGTCAAGGCAC-3' (Ziopime J). The target sequences for Zioprime G and Zioprime I were located within the antisense Oct-4 cDNA fragment. The target sequences for Zioprime H and Zioprime J were located within the SV40 polyadenylation sequence but upstream of the AATAAA polyadenylation signal. The PCR products of these two primer pairs were 291 bp and 339 bp, respectively. In each PCR reaction, 1 μCi of α-^{32}P-dCTP was added. The conditions for PCR were similar to those described in Section §2.15.2, except that 1 μl of 100 μM each primer stock was used and the cDNA were amplified for 40 cycles.

As a control for DNase I treatment, 20 ng of transgenic mouse tail DNA was digested with DNase I in the same way as RNA samples, followed by PCR analysis.

To analyze the radioactive PCR products, 10 μl of the reactions were resolved on a 2% agarose gel. At the end of the run, the gel was soaked in transfer buffer (0.4 N NaOH, 0.6 M NaCl) for 15 min and blotted onto Hybond™-N$^+$ membrane by capillary transfer overnight. After the transfer, the membrane was rinsed briefly in 5X SSC and air dried. A Kodak XAR-5 film was placed against the blot for exposure.

§2.17.3 Southern blot analysis

To confirm the identity of the transgene transcripts, Southern blot analysis was employed. The DNase I-treated RNA samples were reverse transcribed and amplified by PCR using Zioprime G and Zioprime H or Zioprime I and Zioprime J as described above except that no radioactivity was added in the reactions. After running a 2% agarose gel, the PCR products were immobilized onto Hybond™-N$^+$ membrane using 0.4 N NaOH and 0.6 M NaCl as transfer buffer. The membrane was baked at 80°C for 1 hr and prehybridized at 65°C for 2 hrs in a solution containing 5X SSPE, 5X Denhardt's, 1% SDS, 100 μg/ml yeast tRNA and 100 μg/ml salmon sperm DNA. The Oct-4 DNA fragment retrieved from pBS.Oct4(#1) was radioactively labelled by random priming as described in Section §2.4.3. The probe (specific activity of 4 X 10^8 cpm/μg) was added to the prehybridization solution at 5 X 10^5 cpm/ml and allowed to hybridize overnight.

On the second day, the membrane was washed twice for 30 min each time in 2X SSC, 0.1% SDS and twice for 20 min each time in 0.1X SSC, 0.1% SDS at 65°C. A Kodak XAR-5 film was placed against the membrane for exposure.

§2.18 Quantitative RT-PCR

§2.18.1 Cloning of the template DNA into a Poly A vector

To prepare the insert, a 255 bp Oct-4 cDNA fragment corresponding to nucleotide sequence 680-934 (Rosner et al., 1990) was amplified from F9 RT products using an Oct-4 specific primer pair (Oct4.ds). The positions of these primers are downstream from the Oct-4 primers described previously in Section §2.3.1. This primer pair had sequences 5'-TATGCAAATCGGAGACCCTG-3' (Oct4.ds 5' primer) and 5'-AAGGTGTCCCTGTAGCCTCA-3' (Oct4.ds 3' primer), respectively. Several PCR reactions were pooled and resolved on a 2% agarose gel. To purify the 255 bp fragment, the gel slice containing the 255 bp fragment was cut and placed in dialysis tubing filled with 1X TAE buffer. An electrophoretic current was applied for about 30 min with the tubing submerged in an electrophoretic chamber until the DNA was trapped on the side of the tubing. The current was then reversed for 30 sec to loosen the DNA on the tubing wall. The liquid in the tubing was recovered and extracted with phenol:chloroform. DNA was precipitated with 1/10 vol of 3 M sodium acetate, pH 5.2 and 2.5 vol of 95% ethanol.

The purified PCR fragment was "polished" with Klenow in a fill-in reaction to create blunt ends using the same procedure as described in Section §2.13.2. Since the PCR primers used lacked 5' terminal phosphorylation, phosphates were added using T4 polynucleotide kinase (Gibco-BRL) in the following reaction:

5 pmol	255 bp DNA fragment, blunt-ended
5 μl	5 X forward reaction buffer (350 mM Tris-Cl, pH 7.6, 50 mM $MgCl_2$, 500 mM KCl, 5 mM 2-mercaptoethanol)
10 U	T4 polynucleotide kinase
2.5 μl	10 mM ATP
H_2O to 25 μl	

The components were mixed thoroughly and incubated at 37°C for 10 min. The reaction was heat-inactivated at 65°C for 10 min. DNA was extracted with phenol:chloroform and precipitated with ethanol.

To prepare the vector, plasmid pBS.Poly A (Arcellana-Panlilio and Schultz, 1994) was linearized with *Sma*I and dephosphorylated as described in Section §2.3.2. The prepared insert and vector were ligated to produce a plasmid designated as pOct4ds.Poly A (Fig. 18A). The sequence of the Oct-4 fragment in pOct4ds.Poly A was confirmed to be in a correct orientation by *Bgl*I and *Pst*I double digestions and by sequencing using the T7 primer, i.e., transcription off the T7 RNA polymerase promoter would produce a sense strand RNA with the poly A region at the 3' end.

§2.18.2 Preparation of RNA standards

After the Oct-4 downstream cDNA fragment was cloned into the pBS.Poly A transcription vector, the plasmid was linearized with the restriction enzyme *Hind*III. The reaction was extracted with phenol:chloroform and precipitated with ethanol. The DNA

pellet was dissolved in H_2O to a final concentration of 0.2 μg/μl.

Standard RNA synthesis reactions were carried out by *in vitro* transcription using the above linearized DNA as a template. At room temperature, the following components were combined in the given order:

10 μl	5 X RPB (200 mM Tris-Cl, pH 8, 40 mM $MgCl_2$, 125 mM NaCl, 10 mM spermidine, 25 mM DTT)
1.5 μl	40 U/μl RNasin (Pharmacia)
10 μl	2.5 mM NTPs
5 μl	0.2 μg/μl pOct4ds.Poly A/*Hind*III
22 μl	H_2O
1.5 μl	T7 RNA polymerase (60 U/μl)

The synthetic reaction was continued for 1-2 hr at 38-39°C. The volume of the reaction was increased to 100 μl with water. The RNA was then extracted with phenol:chloroform and precipitated in ethanol overnight at -20°C.

On the following day, the Oct4ds.PolyA RNA was purified using oligo(dT)-cellulose as described by Jacobson (1987). All steps were carried out at room temperature. Oligo(dT)-cellulose (Collaborative Research, Inc.) was saturated with water and then equilibrated with oligo(dT) binding buffer (10 mM Tris-Cl, pH 7.5, 0.5 M NaCl, 1 mM EDTA, 0.5% SDS) in a microcentrifuge. *In vitro* transcribed Oct4ds.Poly A RNA was resuspended in 1 ml oligo(dT) binding buffer and added to the above

oligo(dT)-cellulose. After shaking gently for 15 min to allow binding, the suspension was centrifuged at 1500g for 5 min. The cellulose was washed 3 times with 1 ml of oligo(dT) wash buffer (10 mM Tris-Cl, pH 7.5, 0.1 mM NaCl, 1 mM EDTA). After the final wash, the Oct4ds.Poly A RNA was eluted 3 times with 400 μl of oligo(dT) elution buffer (10 mM Tris-Cl, pH 7.5, 1 mM EDTA). The eluates were pooled and 120 μl of 3 M sodium acetate (pH 5.2) and 3.6 ml of 95% ethanol were added to allow precipitation of RNA overnight at -20°C. The RNA pellet was dissolved in 50 μl water.

The purified RNA standards were accurately quantitated by spectrophotometric measurement at 260 nm. A dilution series ranging from 10^7 copies of RNA (650 nt long) per μl to 10^4 copies per μl were precisely prepared and stored at -70°C in 10 μl aliquots.

§2.18.3 Preparation of RNA samples

Oocytes were collected from superovulated normal and homozygous transgenic mice as described in Section §2.11. Total RNA was isolated by phenol:chloroform extraction (Arcellana-Panlilio and Schultz, 1994). Briefly, pools of oocytes were collected in a 500 μl microfuge tube with a minimum volume of M2 medium. In a separate tube, the following were combined: 100 μl RNA extraction buffer (0.2 M NaCl, 25 mM Tris-Cl, pH7.4, 1 mM EDTA), 100 μl H_2O-saturated phenol, and 100 μl Sevag's solution (chloroform:isoamyl alcohol=24:1). To the oocytes, 10 μg of E. coli rRNA and the above mix were added. The tube was vortexed vigorously for two bursts of 10 sec each and centrifuged at 13,000 rpm for 10 min in a microcentrifuge. The aqueous phase was transferred to a fresh tube and re-extracted with 200 μl of Sevag's solution. Total

RNA was precipitated with ethanol at -20°C. Typically, the recovery of RNA was 69%.

When the RNA was needed for reverse transcription, it was recovered by centrifugation at 20,000 g for 30 min at 4°C. The pellet was washed in 200 μl of cold 70% ethanol and re-centrifuged for 30 min. The final pellet was dried under speed-vacuum and dissolved in 5 μl of cold water.

§2.18.4 Quantitative RT-PCR using external standards

Before reverse transcription, the RNA samples were treated with RNase-free DNase I to remove any contaminating DNA. At the same time, 5 μl RNA standards of different dilutions were combined with 2.5 μl of 4 μg/μl E.*coli* rRNA and were also treated with DNase I. The RNA samples and the standards (ranging from 5X 10^6 RNA copies per tube to 5X 10^4 copies per tube) were reverse transcribed as described in Section §2.3.1. To minimize tube-to-tube variability, a master mix was made for the RT reactions.

To set up PCR reactions, one tenth of the RT products were used as templates. Reactions for the standards and for the samples were set up in parallel. The Oct4.ds primer pair were used (see Section §2.18.1). Radiolabelled precursor (1 μCi of α-^{32}P-dCTP) was included in each reaction. Conditions and the temperature profile were the same as for radioactive PCR (Section §2.17.2) except that 25 cycles were run. Again, a master mix was needed to minimize tube-to-tube variability.

The PCR products were resolved on a 2% agarose gel and transferred to Hybond™-N$^+$ membrane (see Section §2.17.2). Autoradiograms were analyzed by

densitometry on a Ultroscan XL Laser Densitometer (LKB). The copy number of each sample was determined by comparisons of the area under each scan with those of the standards.

§2.19 Measurement of Relative Levels of Oct-4 Protein

§2.19.1 Labelling and lysing cells

Oocytes were cultured in M16 medium (Hogan et al., 1986) containing 1 mCi/ml ^{35}S-methionine (>1000 Ci/mmol, Amersham). DMEM with 0.4% BSA containing 1 mCi/ml ^{35}S-methionine was used to label F9 cells (suspension culture). The media were made 1-2 hr before use and kept warm in a 37°C incubator. The remainder of the procedures were the same for F9 cells and oocytes.

Oocytes were collected in M2 medium from superovulated homozygous or normal CD1 mice as described in Section §2.11. The oocytes were transferred in a minimum volume (about 10 μl) to the warm M16 labelling medium in a 4-well Nunclon^{R} multidish and cultured at 37°C for 5 hr. At the end of labelling period, oocytes were recovered from the labelling medium by centrifugation at 3000 rpm for 2 min in a microcentrifuge. The oocytes were washed three times with 1 ml of PBS. To lyse the oocytes, 150 μl of lysis buffer (150 mM NaCl, 1% Nonidet P40, 50 mM Tris-Cl pH 8) was used to resuspend the oocytes. The mixture was incubated on ice for 40 min with occasional mixing. The protein lysate was separated from the cell debris by centrifugation at 10,000

g for 10 min at 4°C. A small aliquot (1 μl) of the protein lysate was spotted onto a filter for TCA precipitation and for measurement and calculation of ^{35}S incorporation efficiency. The remainder of the protein lysate was used for immunoprecipitation.

§2.19.2 Immunoprecipitation

To precipitate the Oct-4 proteins, 1.7 X 10^7 cpm of the protein lysate was mixed with 5 μl of Oct-4 antiserum in a 1.5-ml microfuge tube. The same amount of radioactively-labelled protein (cpm) from normal mouse oocytes and homozygous transgenic mouse oocytes was used in each experiment. The mixture was incubated on ice for 2 hr to allow protein-antibody complexes to form. To each reaction, 15 μl of protein A-agarose beads (Upstate Biotechnology Inc.) were then added. The reactions were incubated for 3 hr at 4°C with gentle rocking. The beads were collected by quick centrifugation in a microcentrifuge and washed 3 times with 1 ml of lysis buffer. Wash buffers were removed using a 25-gauge needle. Care was taken to remove as much of the final wash as possible. To the beads, 15 μl of Laemmli sample buffer (2% SDS, 10% glycerol, 100 mM DTT, 60 mM Tris-Cl pH 6.8, 0.001% bromophenol-blue) were added. The samples were then heated at 85°C for 10 min. After centrifugation, the supernatants were loaded onto an SDS mini-protein gel. At the end of the run, the gel was soaked in 5% glycerol for 1 hr and then dried in a gel drier. The gel was placed against an XAR-5 film for exposure. Autoradiograms were analyzed by densitometry to measure the relative amount of radiolabelled Oct-4 protein synthesized by normal and transgenic mouse oocytes.

§2.20 Histological Examination of Mouse Ovaries

The stages of estrus were determined by analysis of vaginal smears (Rugh, 1968). A few drops of 0.9% sodium chloride solution were drawn into a pipette, introduced into the vagina and then retracted into the pipette. The fluid was transferred onto a slide and mounted under a coverslip with a trace of methylene blue to add contrast and bring out the nuclei. Examination for cell types was carried out under low and then high power magnification using a Zeiss photomicroscope II.

Ovaries in determined phases of estrus were fixed and embedded as described in section §2.12.1. Sections taken from the mid region of ovaries were spread onto slides. After dewaxing in xylene and rehydrated in decreasing concentrations of ethanol, the slides were stained in haematoxylin for 20 min, then in 1% eosin for 20 sec. The slides were then rinsed in water, dehydrated in ethanol series and cleared in xylene. The slides were mounted with coverslips, observed and photographed under a Zeiss photomicroscope II.

§2.21 Statistical Analysis

Statistical comparisons of the relative amount of Oct-4 protein in oocytes derived from normal or transgenic animals were made using one-way analysis of variance (ANOVA) through the Number Cruncher Statistical System (version 4.1) program. Statistical analysis of oocyte numbers or embryo numbers from normal and transgenic

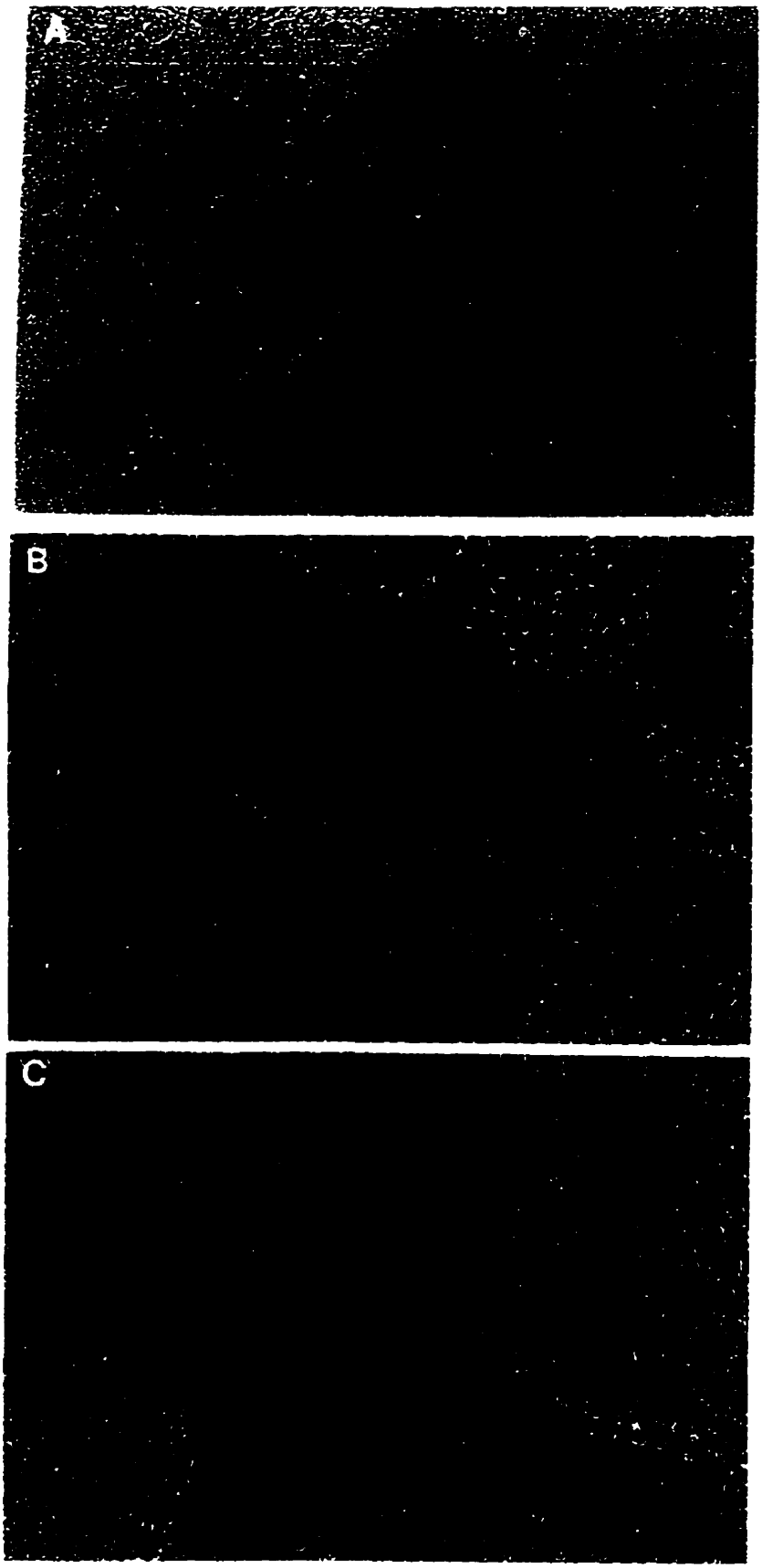
mice was made using ANOVA or the student t-test using the same program.

§3 RESULTS

§3.1 Morphology of F9 Cells During Differentiation

F9 cells have been used by many investigators as an in vitro model system to study differentiation events related to early embryogenesis in the mouse. These embryonal carcinoma cells, which resemble inner cell mass (ICM) cells of mouse blastocysts and undergo very limited differentiation under normal culture conditions, can be induced into either parietal endoderm or visceral endoderm-like cells depending on the culture conditions. The F9 cell line is routinely propagated on gelatin-coated surface so that the cells can adhere better to the culture flask. In the undifferentiated state, F9 cells grow as tightly packed colonies (Fig. 4A). The cell population appears predominantly homogenous. When cultured in the presence of 10^{-7} M retinoic acid (RA) and 10^{-3} M dibutyryl cyclic AMP (dcAMP), the cells become more rounded and begin to form aggregates (Fig. 4B). The phenotype of these cells is characterized by active secretion of large amounts of extracellular matrix, including type-IV collagen and laminin, and by low levels of alkaline phosphatase and lactate dehydrogenase (Strickland and Mahdavi, 1978; Strickland et al., 1980). By day 6 or 7, most cells form aggregates (Fig. 4C) containing differentiated cells that resemble parietal endoderm, an extraembryonic cell that is generated early in mouse embryogenesis. The differentiation pattern shown in Figure 4 closely mimics that published previously by Strickland et al.(1980) and Hogan et al. (1986). Aliquots of cells from the undifferentiated state to day

Fig.4. Differentiation of F9 cells in the presence of 10^{-7}M RA and 10^{-3}M dcAMP. (A) Monolayer culture of undifferentiated F9 cells; (B) Cells 4 days after addition of RA and dcAMP; (C) Cells and aggregates 6 days after addition of RA and dcAMP. Magnification X100.


A
B
C

7 of treatment with RA and dcAMP were used for RNA and protein preparations for analysis of Oct-4 expression during this program of differentiation.

§3.2 Northern Blot Analysis of Oct-4 RNA Expression in F9 Cells

An Oct-4 mini-cDNA fragment was amplified from F9 cells by RT-PCR. The 297 bp RT-PCR product including the *Eco*RV restriction sites in the primers corresponds to nucleotide 127 to 407 in the Oct-4 cDNA sequence described by Rosner et al. (1990). The sequence in this region is unique to the Oct-4 gene and not shared by other members of the gene family. The identity of the amplified DNA was verified by *Fok*I restriction digestion which produced 123 and 174 bp fragments (data not shown). The RT-PCR products were cloned into the *Eco*RV site of the plasmid pBluescript.SK (pBS). The resulting recombinant clones, pBS.Oct4 (#1) and pBS.Oct4 (#2), were confirmed by sequencing (data not shown).

As a probe for northern blots, the partial Oct-4 cDNA fragment was isolated following *Eco*RV digestion of plasmid pBS.Oct4 (#1) and was radiolabelled by random priming. After hybridization, a single band of about 1.5 kb was detected as the Oct-4 transcript (Fig. 5A). Pluripotent undifferentiated F9 cells expressed a large amount of Oct-4 mRNA (Fig. 5A, lane 1). A marked decrease in abundance of Oct-4 mRNA was observed in RNA samples obtained from F9 cells after 2 to 7 days of treatment with 10^{-7} M RA and 10^{-3} M dcAMP (Fig. 5A, lanes 2 to 5). Ethidium bromide staining of 28S and 18S rRNA was used to verify that the amount of total RNA loaded in each lane was

Fig.5. Relative amount of Oct-4 mRNA in differentiating F9 cells. (A) Autoradiograph of Northern blot probed with ^{32}P-labelled Oct-4 cDNA. A 1.5 kb Oct-4 transcript was detected. Lane 1, 10 μg of undifferentiated F9 cell total RNA; lane 2 to 5, 10 μg of total RNA from F9 cells after 2, 4, 6, and 7 days of treatment with 10^{-7}M RA and 10^{-3}M dcAMP, respectively. The probe specific activity was 3.3 X 10^{8}cpm/μg and the autoradiograph was exposed for 3 days. (B) Ethidium bromide stained pattern of 28S and 18S rRNA on the agarose gel before transfer for northern blot in Fig.5A (RNA loading control).

A

-28S

-18S

-1.5kb

1 2 3 4 5

B

-28S

-18S

1 2 3 4 5

more-or-less equal (Fig. 5B). The slight increase of Oct-4 signal on day 7 (Fig. 5A, lane 5) may result from overloading of total RNA in the same lane (Fig. 5B, lane 5). Although Oct-4 mRNA levels are remarkedly reduced in F9 cells treated with RA and dcAMP, the detection of low levels of Oct-4 mRNA suggests that a few undifferentiated F9 cells are still present and express the Oct-4 gene. The down-regulation of Oct-4 mRNA observed in differentiating F9 cells is similar to that reported previously (Schöler et al., 1989; Schoorlemer and Kruijer, 1991).

§3.3 Oct-4 Protein Expression in F9 Cells

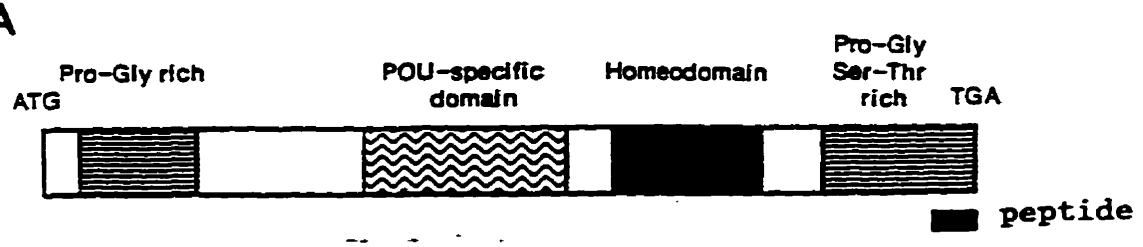
§3.3.1 Antibody

A 21-amino acid peptide was synthesized for the purpose of Oct-4 antibody production. The peptide has an amino acid sequence that is unique to Oct-4 protein as assessed by protein sequence data that were available. The position of the peptide (Fig. 6A) corresponds to the C-terminal 20 amino acids of Oct-4 (Rosner et al., 1990) which is located in the putative transactivation domain and should be located at the surface of the native protein.

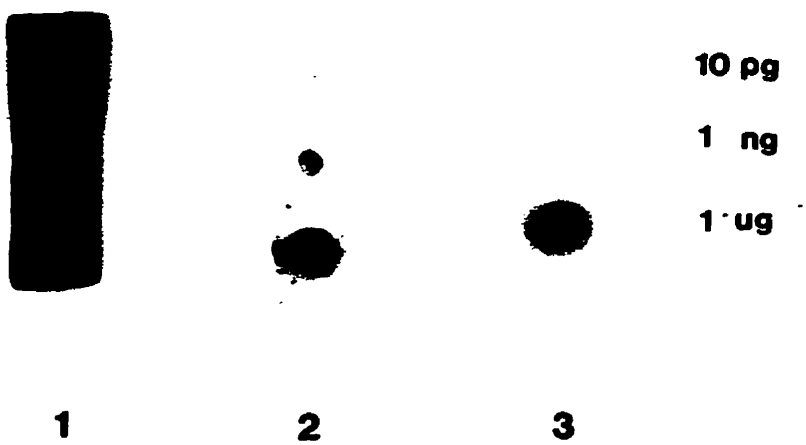
A polyclonal antibody was raised in rabbits against the synthetic peptide following its conjugation to carrier proteins. Upon production of an Oct-4 polyclonal antibody, immuno-dot blots were carried out to determine the optimal antibody dilution for later use. Antiserum dilutions of 1:500, 1:2,000 and 1:10,000 were tested on blots spotted with 10 pg, 1 ng and 1 μg of Oct-4 peptide (Fig. 6B). According to signal:noise ratios,

Fig.6. Immuno-dot blot analysis of Oct-4 antibody. (A) Schematic diagram of Oct-4 protein, showing the POU-specific domain, POU homeodomain and two putative transactivation domains located at the N-terminus and C-terminus, respectively. The amino acid sequence of the peptide used for Oct-4 antibody production is located at the C-terminus. (B) Immuno-dot blots to determine the optimal antibody dilution. Each blot contains 10 pg, 1 ng and 1 μg of Oct-4 peptide. Blots 1, 2 and 3 were reacted with Oct-4 antiserum of 1:500, 1:2,000 and 1:10,000 dilutions, respectively.

A


Pro-Gly rich
ATG
POU-specific domain
Homeodomain
Pro-Gly Ser-Thr rich
TGA
peptide

B


10 pg
1 ng
1 ug
1
2
3

a dilution of 1:2,000 was recognized to be optimal. The 500-fold dilution produced too much background and the 10,000-fold dilution resulted in reduced sensitivity.

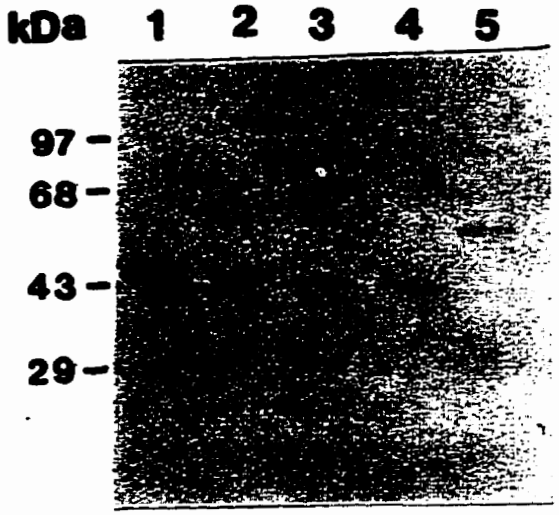
§3.3.2 Western blot analysis

To examine the Oct-4 protein expression patterns during F9 cell differentiation, the Oct-4 antiserum was used to probe a Western blot of proteins derived from undifferentiated and differentiated F9 cells using the ECL Western blotting system. A strong signal in undifferentiated cells was observed for a protein with a molecular weight of about 45,000 (Fig. 7A, lane 1). When cells were induced to differentiate for 2, 4, and 6 days, the level of Oct-4 protein was observed to progressively decrease (lane 2 to 4). By day 7, the level of Oct-4 protein was below detection (Fig. 7A, lane 5). As control experiments, when the same membrane was stripped of bound antibody and reprobed with rabbit preimmune serum or the antiserum pre-absorbed with excess Oct-4 peptide, the 45 kDa protein was not detected (Fig. 7B). These control experiments verified the specificity of the Oct-4 antiserum.

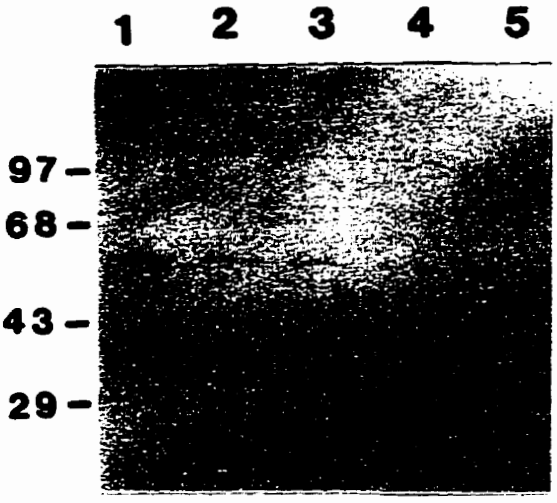
Besides F9 cells, another murine EC cell line, P19, was also examined for Oct-4 expression. P19 cells are also pluripotent and resemble the ICM cells of mouse blastocysts. Exposure of P19 cells to RA results in differentiation into neuroectodermal, endodermal and mesodermal derivatives (reviewed by McBurney, 1993). Previously work by Okamoto et al. (1990) has shown that Oct-4 mRNA is abundant in undifferentiated P19 cells but virtually absent in RA-induced P19 cells. Thus, Western blot analysis was carried out to examine Oct-4 protein abundance in undifferentiated and RA-induced P19

Fig.7. Western blot analysis of Oct-4 protein from undifferentiated and differentiated EC cell lines. (A) Detection of a 45 kDa Oct-4 protein in differentiating F9 cells. Lane 1, 10 μg of undifferentiated F9 cell protein extract; lane 2 to 5, 10 μg of protein extracts from differentiating F9 cells after 2, 4, 6, and 7 days of treatment with 10^{-7}M RA and 10^{-3}M dcAMP, respectively. (B) Peptide absorption control. The same blot as (A) was stripped of bound Oct-4 antibody and reprobed with the antiserum pre-absorbed with excess Oct-4 peptide. (C) Expression of Oct-4 protein in P19 cells. Lane 1, 10 μg protein extract from undifferentiated F9 cells; lane 2, 10 μg protein extract from day 7 differentiated F9 cells; lane 3, 5 μg undifferentiated P19 nuclear protein extract; lane 4, 5 μg differentiated P19 nuclear protein extract.

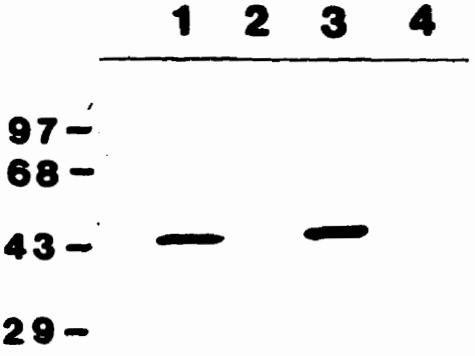
A



B



C



cells (Fig. 7C). Exactly as observed for F9 cells, Oct-4 protein was found to be present in undifferentiated P19 cells but undetectable in differentiated EC cells, consistent with the decline in mRNA expression pattern (Okamoto et al., 1990).

§3.4 DNA-protein Binding Assays

§3.4.1 Bandshift assays

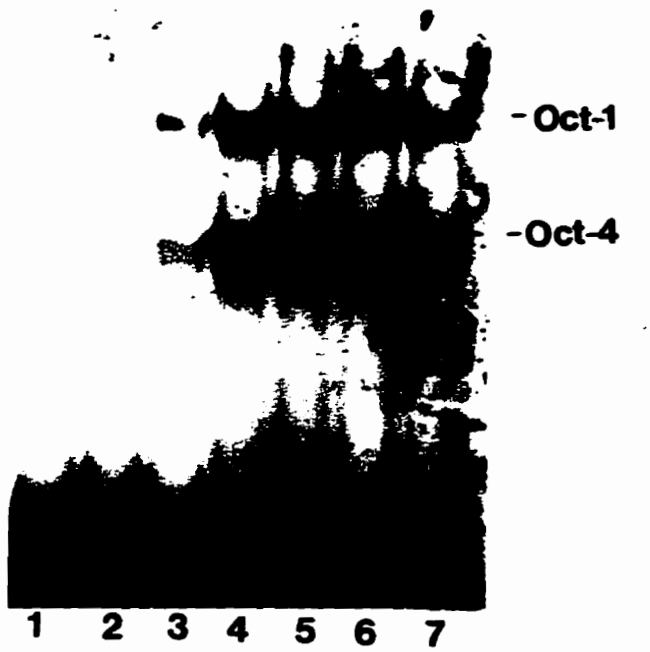
The presence of Oct-4 proteins in F9 cells was also analyzed by bandshift assays using a radioactively labelled oligonucleotide containing an octamer motif (ATTTGCAT) of mouse immunoglobin heavy chain gene enhancer (Fig. 8A). To test the optimal amount of F9 proteins for bandshift assays, proteins of different amount (1, 2, 6, 12, 16, and 22 μg, respectively) were used in the reactions (Fig. 8B). Complexes between F9 cell proteins and the DNA probe were allowed to form and were resolved on a non-denaturing polyacrylamide gel. Two DNA-protein complexes were detected from most lanes. According to previous reports (Schöler et al., 1989), two major octamer factors, Oct-1 and Oct-4, are present in undifferentiated F9 cells. The ubiquitously expressed Oct-1 factor has a molecular weight of 90 - 100 kDa (O'Neill et al., 1988; Sturm et al., 1987; Fletcher et al., 1987), while Oct-4 has a molecular weight of 45 kDa (see section §3.3.2). Thus, the larger protein-DNA complex observed was likely from Oct-1, while the more rapidly migrating complex was a result of interaction of Oct-4 with the DNA probe. Comparison of the signals from each lane showed that 6 μg of F9 protein extracts were sufficient for each reaction. (Fig. 8B, lane 4).

Fig.8. An octamer DNA-binding site interacts with Oct factors from F9 cells. (A) Sequence of complimentary oligonucleotides used to generate a DNA probe containing an ATTTGCAT octamer binding site. (B) The ^{32}P-labelled probe was incubated with different amount of protein extract from F9 cells and the resulting complexes were resolved by polyacrylamide gel electrophoresis under non-denaturing conditions. Lane 1, free probe; lane 2, probe plus 1 μg of F9 cell extract; lane 3, probe plus 2 μg of F9 cell extract; lane 4, probe plus 6 μg of F9 cell extract; lane 5, probe plus 12 μg of F9 cell extract; lane 6, probe plus 16 μg of F9 cell extract; lane 7, probe plus 22 μg of F9 cell extract. (C) Competition assays. Lane 1, free probe; lane 2, probe plus 6 μg of F9 cell extract; lane 3, complex incubated as lane 2 plus 100-fold excess of unlabelled probe DNA; lane 4, complex incubated as lane 2 plus 100-fold excess of non-specific (salmon sperm) DNA. (D) Supershift assays with anti-Oct-4 antibody. Lane 1, free probe; lane 2, probe plus 6 μg of F9 cell extract; lane 3, complex formed as in lane 2 and then incubated at room temperature for 1 hr with rabbit Oct-4 antiserum; lane 4, experiment as in lane 3 but with preimmune rabbit serum; lane 5, experiment as in lane 3 but with non-specific (new-born goat) serum.

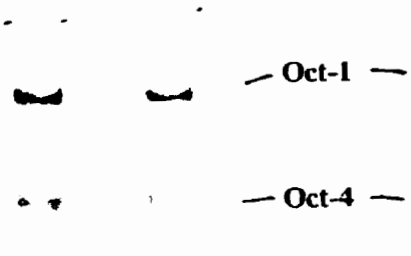
A

5'-GATCGGTAATTTGCATTTCTAA-3'
3'-CCATTAAACGTAAAGATTCTAG-5'
Octamer binding site

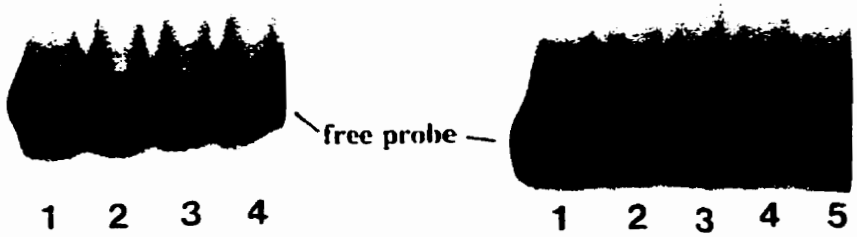
B



C



D



The specificity of the DNA-protein complexes formed was examined by competition assays (Fig. 8C). Both bands were competed away by 100-fold excess of the cold oligonucleotides, but not by 100-fold excess of non-specific DNA (salmon sperm DNA) (Fig. 8C, lanes 3 and 4). This result confirmed that the DNA-protein complexes specifically resulted from the octamer-binding factors and the octamer-binding motif.

§3.4.2 Supershift assays

To test whether the Oct-4 antiserum was able to recognize natural Oct-4 protein, gel mobility supershift assays were performed. When the DNA-protein complexes were incubated with the Oct-4 antiserum, the lower band was supershifted while the upper band was unaffected (Fig. 8D, lane 3). Overexposure of the X-ray film showed that the supershifted Oct-4-DNA complex was located at the sample well of the gel. Control experiments indicated that preimmune serum or new-born goat serum did not supershift the Oct-1 or Oct-4 complexes (Fig. 8D, lanes 4 and 5). The assays confirmed that the lower band was from the interaction of Oct-4 with the DNA probe and demonstrated that the Oct-4 antiserum contained antibodies that could recognize natural Oct-4 protein.

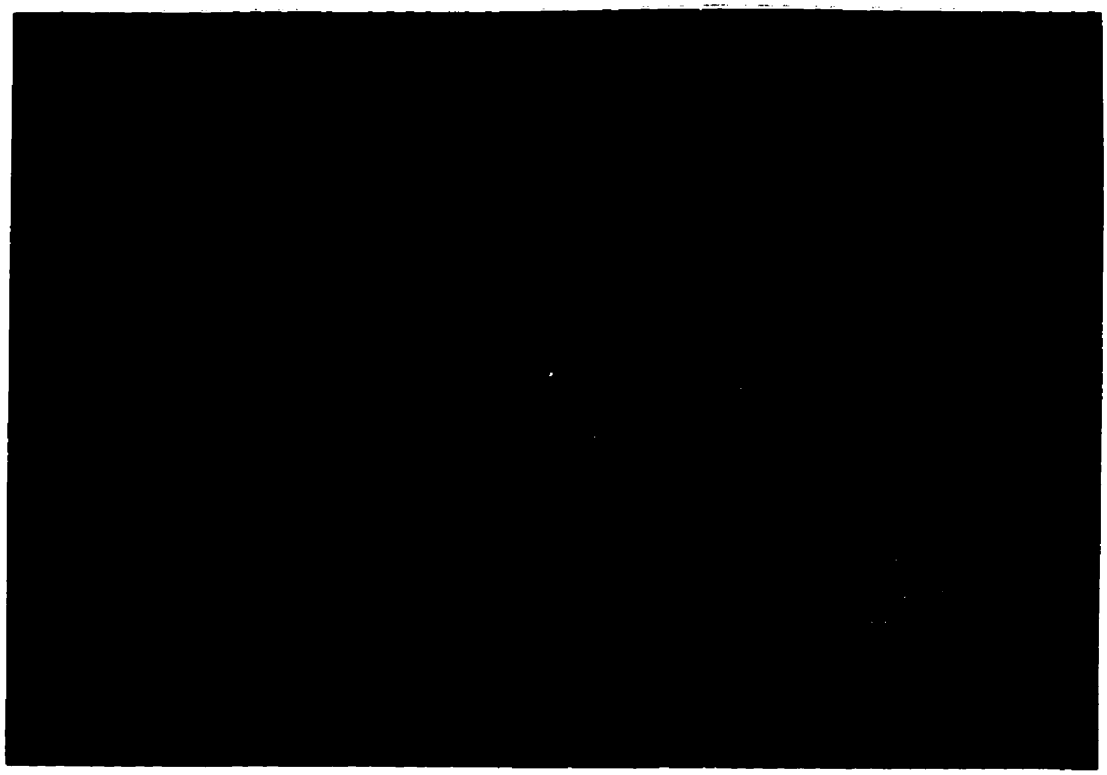
§3.5 Immunofluorescent Staining of F9 Cells

The Oct-4 antiserum was also used to localize the Oct-4 protein expression in F9 cells. Undifferentiated F9 cells were treated with the Oct-4 antiserum, followed by FITC-conjugated secondary antibody staining. Fluorescent staining was observed in both nuclei and cytoplasm of the cells, with stronger signals in the nuclei (Fig. 9A). In the control

Fig.9. Immunofluorescent staining of F9 cells. (A) Undifferentiated F9 cells were treated with the Oct-4 antiserum followed by FITC-conjugated secondary antibody staining. (B) Undifferentiated F9 cells were treated with preimmune rabbit serum followed by FITC-conjugated secondary antibody staining.

A

B

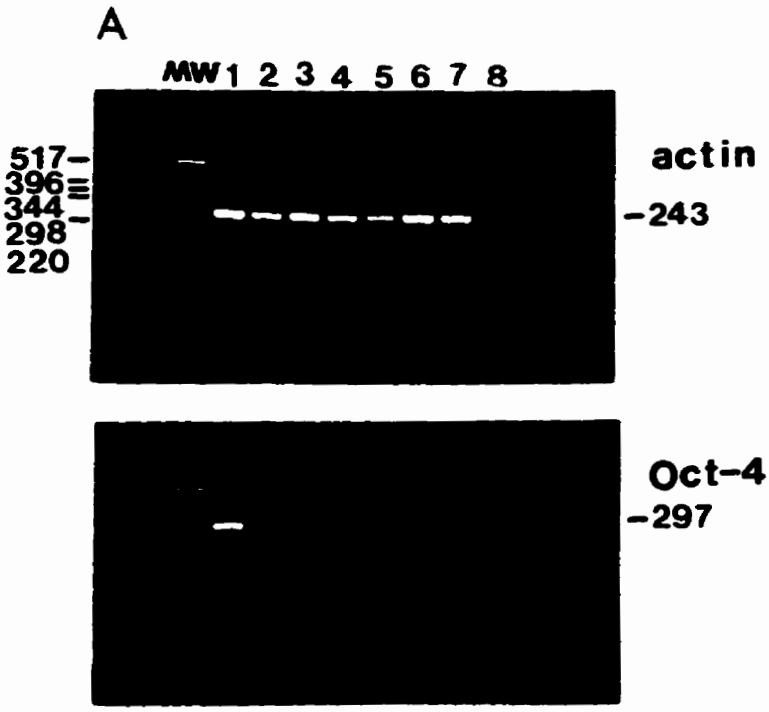


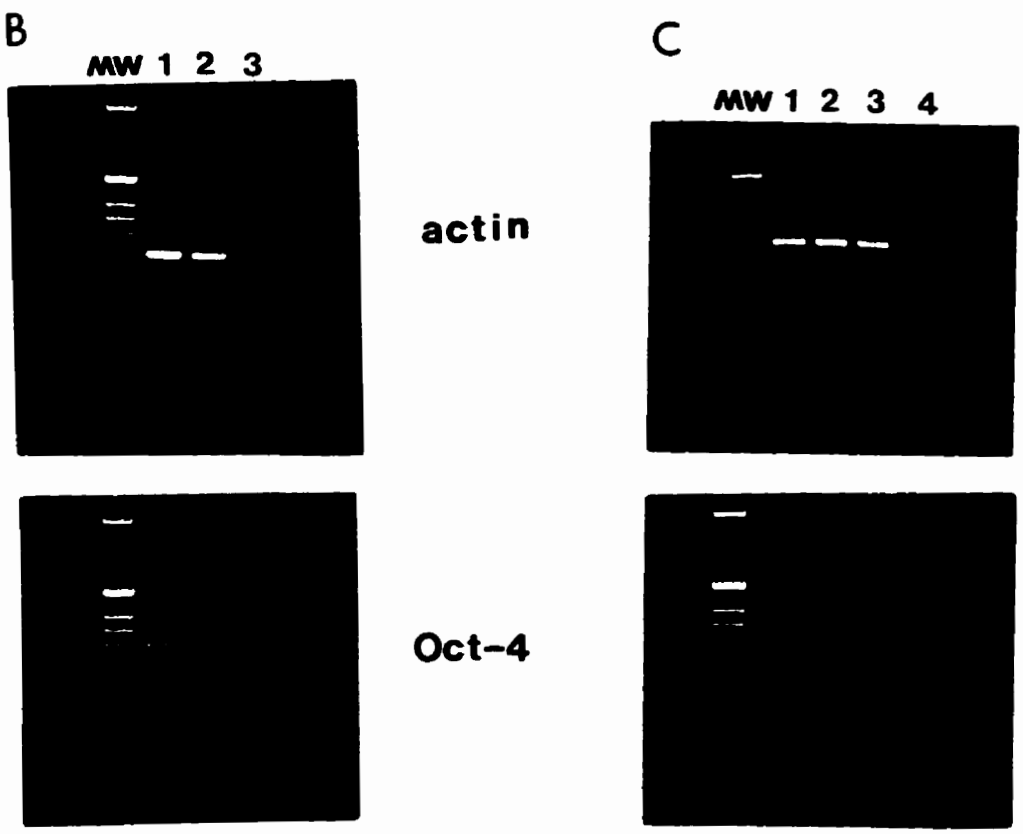
experiment with preimmune serum, much weaker signals were observed, especially in the nuclei (Fig. 9B). The uneven and reversed distributions of the signals in nuclei and cytoplasm for the Oct-4 antibody labelling and the preimmune serum control suggest that Oct-4 protein is mainly located in nuclei, consistent with its role as a transcription factor. The cytoplasmic staining of F9 cells with the Oct-4 antiserum may be due to non-specific interactions since cytoplasmic staining also occurs with the preimmune serum. The possibility that the Oct-4 protein is present in the cytoplasm is not excluded, however, considering that the cytoplasm is the location of protein synthesis.

§3.6 Oct-4 RNA Expression in Adult Mouse Tissues

RT-PCR was employed as a rapid and sensitive method to screen RNA samples for the presence of Oct-4 mRNA in various mouse tissues. Total RNA was extracted from adult mouse ovary, brain, heart, kidney, liver, muscle and spleen. To exclude any possible contaminating genomic DNA, the RNA samples were treated with RNase-free DNase I before reverse transcription. Each sample was amplified for both β-actin and Oct-4 RT-PCR products. Actin primers were designed to amplify a 243 bp cDNA PCR product near the 5'-terminus of the mRNA sequence and a 330 bp PCR product if genomic DNA was present (Telford et al., 1990). Actin primers were, therefore, used as a positive control for the quality of the RT-PCR and for the presence of contaminating genomic DNA. All the samples yielded a single strong 243 bp band in the absence of the 330 bp genomic DNA PCR product (Fig. 10, upper panels). However, when Oct-4

Fig.10. RT-PCR analysis of Oct-4 mRNA expression in adult mouse tissues. RNA extracted from adult mouse tissues were treated with RNase-free DNase I before reverse transcription. PCR was programmed for 40 cycles as follows: denaturation for 1 min at 94°C, annealing for 2 min at 55°C, and extension for 2 min at 72°C. Both actin primers and Oct-4 primers were used for PCR. Actin PCR products are shown in the upper panels and Oct-4 PCR products are shown in the lower panels in each case. (A) Expression of Oct-4 in various tissues. Lane 1, ovary; lane 2, brain; lane 3, heart; lane 4, kidney; lane 5, liver; lane 6, muscle; lane 7, spleen; lane 8, negative control. (B) Comparison of Oct-4 expression in female and male gonads. Lane 1, ovary; lane 2, testis; lane 3, negative control. (C) Analysis of Oct-4 expression in female reproductive tracts. Lane 1, ovary; lane 2, oviduct; lane 3, uterus; lane 4, negative control.


A
MW 1 2 3 4 5 6 7 8
517
396
344
298
220
actin
243
Oct-4
297


B
MW 1 2 3
actin
Oct-4
C
MW 1 2 3 4

primers were used, only the mouse ovary sample showed a 297 bp Oct-4 cDNA PCR product (Fig. 10A, lower panel). The distribution patterns of Oct-4 RNA in adult mouse tissues are identical to those observed for Oct-4 protein by gel mobility shift assays using nuclear extracts of different mouse tissues (Schöler et al., 1989).

The expression of Oct-4 in mouse testis was also examined by RT-PCR. As shown in Figure 10B, a 297 bp band of low intensity was observed from testis after 40 cycles of amplification (Fig. 10B, lane 2). Although the same amount of RNA from ovary or testis was used for RT-PCR, the PCR signal from testis was much lower than that from ovary (Fig. 10B, lane 1 and 2). The result suggests that Oct-4 mRNA appears more abundant in ovary than in testis. The observation that Oct-4 mRNA is detectable in the testes is consistent with the previous reports by Schöler et al. (1989) in which Oct-4 protein was found to be present in the male gonad by gel mobility shift assays. The identity of the cells expressing Oct-4 in the testis was not defined.

Heikinheimo et al. (1995) have recently reported that expression of Oct-4 mRNA in the cynomolgous monkey was detected, in addition to oocytes, in ovarian granulosa cells, fallopian tube, uterine tube, testis, liver and several other tissues. This predominant expression of Oct-4 in several tissues of the reproductive tract of the primate prompted us to re-investigate Oct-4 expression in the reproductive tract of the female mouse. Detection of RT-PCR products for Oct-4 mRNA was restricted to the ovary in the mouse (Fig. 10C, lane 1). No signals were detected in the oviduct or uterus (Fig. 10C, lane 2 and 3). These results along with those of others (Rosner et al., 1990; Schöler et al., 1990a) support the conclusion that, in the mouse, Oct-4 expression appears to be

confined to germ cells of the gonad and to pluripotent cells of the early embryo.

§3.7 In Situ Hybridization to Detect Oct-4 mRNA in the Oocytes

The expression of Oct-4 transcripts was also examined by *in situ* hybridization. Strand-specific RNA probes were labelled with digoxigenin (DIG) and the hybridized DIG probe was detected by anti-DIG alkaline phosphatase conjugate followed by the addition of enzyme substrates. The signals generated yield purple-blue staining. The sense or antisense Oct-4 DIG riboprobes were transcribed from linearized pBS.Oct4 (#1) using either T3 or T7 polymerases. Initially, experiments to examine the specificity of the riboprobes were carried out on F9 cell RNA. Samples of 5, 2 and 1 μg were dotted on a membrane and hybridized with Oct-4 sense or antisense DIG riboprobes. Since F9 cells express Oct-4 transcripts abundantly, it was expected that RNA-RNA hybrids should form between Oct-4 transcripts and its antisense riboprobe. The antisense Oct-4 riboprobe produced purple-blue signals on the membrane upon hybridization while the sense control showed no apparent signals (data not shown). Having demonstrated that the riboprobes behaved as expected, they were then applied to sections of the ampullary end of the oviduct of female mice stimulated to ovulate following administration of PMS and HCG. The pieces of oviduct with the residing ovulated cumulus masses were collected carefully, embedded and sectioned for in situ hybridization. Examples of two consecutive sections that were hybridized with either the antisense or sense riboprobes are shown in Fig.11A and Fig. 11B, respectively. Weak purple-blue punctate staining was observed

Fig.11. ***In situ*** **hybridization analysis of Oct-4 expression in mouse oocytes. Mice were superovulated and the oviduct segments with the residing cumulus masses were embedded and sectioned for in situ hybridization experiments using DIG labelled Oct-4 riboprobes. (A)** ***In situ*** **hybridization using Oct-4 antisense riboprobe. The section passes through 3 oocytes. The most prominently stained oocyte is identified by an arrow. (B) in situ hybridization using Oct-4 sense riboprobe. The section is consecutive to that used in panel A.**

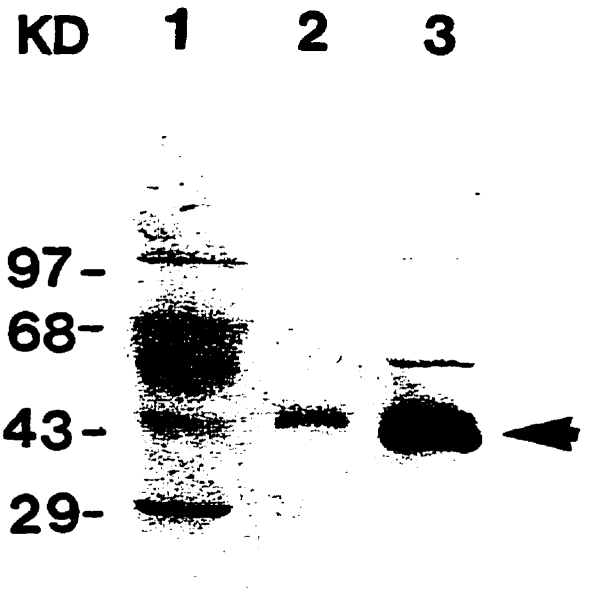

A
B

within some oocytes when the antisense riboprobe was used. No signals were observed in oocytes when the sense riboprobe was used. The oviduct wall did not stain with either probe. Overall, the *in situ* hybridization signals for Oct-4 mRNA were very weak. Several experiments on oviduct sections containing ovulated oocytes or sections of whole ovaries containing oocytes at various stages of growth were carried out. In every case, the antisense probe showed some staining of oocytes relative to the sense control, but the signals were always very weak. This observation suggests that Oct-4 mRNA levels are quite low in oocytes.

§3.8 Oct-4 Protein Levels in Mouse Oocytes

To examine whether the low level of Oct-4 mRNA in oocytes is translated into detectable amounts of Oct-4 protein, pools of mouse oocytes were used to make protein extracts for analysis on Western blot in comparison to protein extracts from F9 cells. In the example shown in Figure 12, protein extract prepared from 751 oocytes (lane 1) was analyzed along with 1 μg (lane 2) and 10 μg (lane 3) of protein extract from undifferentiated F9 cells. Ovulated oocytes in the mouse each contain, on average, 23 ng of protein (Hogan et al., 1986). Assuming 100% recovery, the 751 oocyte sample is estimated to contain about 17 μg of protein. A very weak signal for the band of the expected size for Oct-4 was detected (Fig. 12, lane 1). Several non-specific bands were also observed from the oocyte protein sample. These non-specific bands are also detectable when larger amounts of F9 cell protein are used (lane 3). In terms of relative

Fig.12. Western blot analysis of Oct-4 protein expression in mouse oocytes. Arrow indicates the band for Oct-4 protein. Lane 1, protein extract from 751 mouse oocytes; lane 2, 1 μg of F9 cell extract; lane 3, 10 μg of F9 cell extract. The migration position of molecular weight markers is shown on the left.

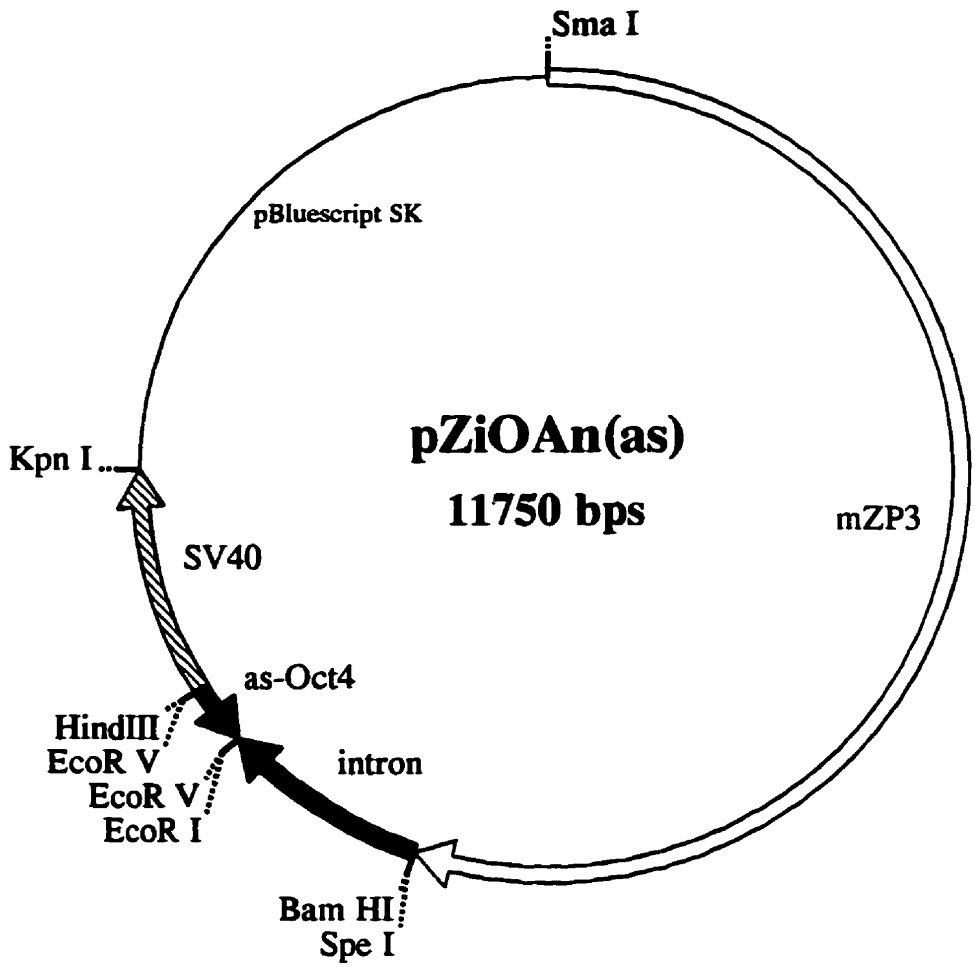

KD
1
2
3
97-
68-
43-
29-

intensity, Oct-4 protein band in the 1 μg F9 sample (Fig. 12, lane 2) is roughly twice as strong as that of 17 μg protein sample derived from the oocytes. This suggests that Oct-4 protein levels are about 35 times lower in oocytes than in undifferentiated F9 cells.

§3.9 Transgenic Construct

To study the role of the maternal Oct-4 in early mouse development, I attempted to generate transgenic mice in which expression of Oct-4 in oocytes was suppressed due to antisense inhibition. The mouse zona pellucida ZP3 (mZP3) promoter was chosen to direct antisense Oct-4 RNA expression due to its oocyte-specificity and strong promoter activity (high levels of endogenous mRNA) in oocytes. A 6.5 kb mZP3 promoter was retrieved by *Kpn*I digestion from plasmid pZP3/6.5-LUC (Lira et al., 1990) which contains the 6.5 kb mZP3 promoter and the coding region of firefly luciferase. The 6.5 kb mZP3 promoter was shown to be enough for oocyte-specific expression of the luciferase reporter (Lira et al., 1990). A human β-globin second intron was placed between the mZP3 promoter and the antisense Oct-4 cDNA in the transgenic construct because it has been shown that inclusion of heterologous introns often increases transgene expression (Palmiter et al., 1991). The β-globin second intron was previously successfully used in transgenic mice that expressed antisense RNA of myelin basic protein (Katsuki et al., 1988). In the 3'end of the transgene, an SV40 polyadenylation signal sequence was included to effect proper processing of the nascent RNA molecule. The resulting transgenic construct, pZiOAn(as) is shown in Figure 13. The entire

Fig.13. Schematic diagram of the transgene construct. The construct contains the 6.5 kb mouse zona pellucida ZP3 promoter, 917 bp human β-globin second intron, 297 bp Oct-4 cDNA in antisense orientation and 1.08 kb SV40 polyadenylation signal sequence in a pBluescript.SK vector. The transgene fragment can be retrieved by *Kpn*I and *Sma*I digestions of the recombinant plasmid DNA.


Sma I
pBluescript SK
pZiOAn(as)
11750 bps
mZP3
Kpn I
SV40
as-Oct4
HindIII
EcoR V
EcoR V
EcoR I
intron
Bam HI
Spe I

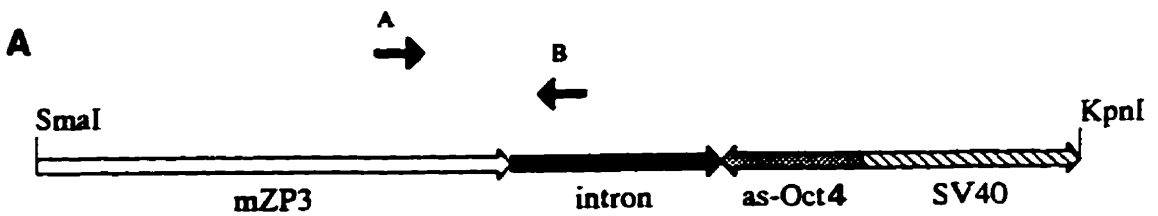
transgene, including the 6.5 kb mZP3 promoter, 917 bp β-globin intron, 297 bp antisense Oct-4 cDNA and 1.08 kb SV40 polyadenylation signal sequence, was retrieved by SmaI/KpnI digestions. In collaboration with Dr. Saul Zackson, Department of Medical Biochemistry, The University of Calgary, the transgenic fragment was injected into the pronuclei of 1-cell mouse embryos to produce transgenic mice.

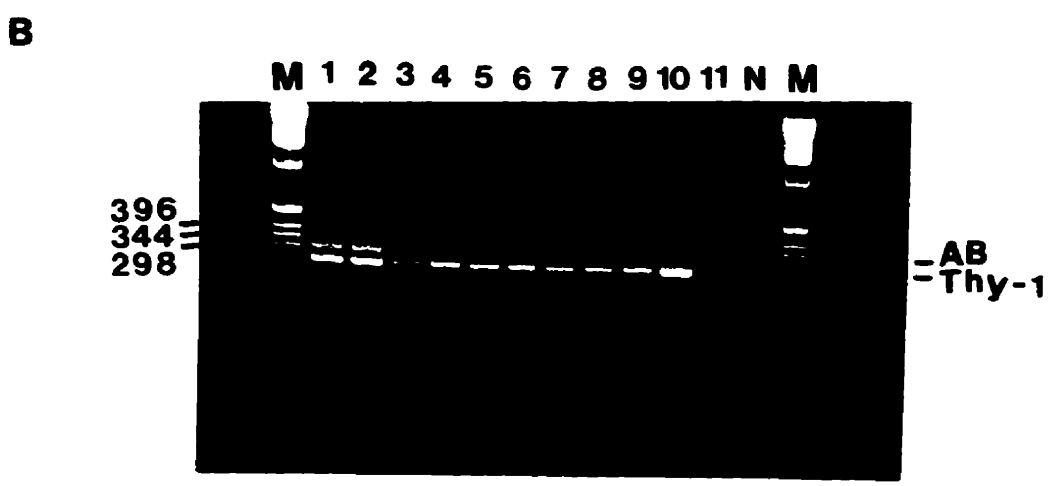
§3.10 Screening Transgenic Mice

Transgenic mice were identified by PCR using mouse tail DNA as template. Two pairs of primers were used in each reaction. Primer pair Thy-1 amplifies a 231 bp fragment of the endogenous mouse Thy-1 gene as a positive control for PCR. Primer pair Zioprime A and Zioprime B (Fig. 14A) are transgene-specific and amplify a 309 bp fragment spanning the ZP3 promoter and the intron of the transgene. This fragment could only be present if the transgene had been stably integrated into the mouse genome. Figure 14B is an example of PCR results. Transgenic mice showed two PCR products (Fig. 14B, lanes 1, 2, 7, and 9), while normal mice had only the 231 bp Thy-1 PCR product.

Using the above method, 128 potential transgenic founder mice were screened. Only one of them, named mouse #6, was transgenic. PCR data of the screens have not been shown to avoid repetition. The transgenic founder mouse was a female and mated with normal CD1 male mice. Heterozygous transgenic progeny were identified as above. Homozygous transgenic mice were produced from heterozygote intercrosses. Putative

Fig.14. Identification of transgenic mice. (A) Location of transgene-specific primers, Zioprime A and Zioprime B within the transgene construct. (B) An example of PCR reactions to identify transgenic mice using mouse tail DNA. Lane 1 to 11, DNA samples from 11 littermates; lane N, negative control. Each PCR reaction was amplified by the endogenous mouse Thy-1 gene primers and the transgene-specific primers, Zioprime A and Zioprime B.


A
A
B
SmaI
KpnI
mZP3
intron
as-Oct4
SV40


B
M 1 2 3 4 5 6 7 8 9 10 11 N M
396
344
298
AB
Thy-1

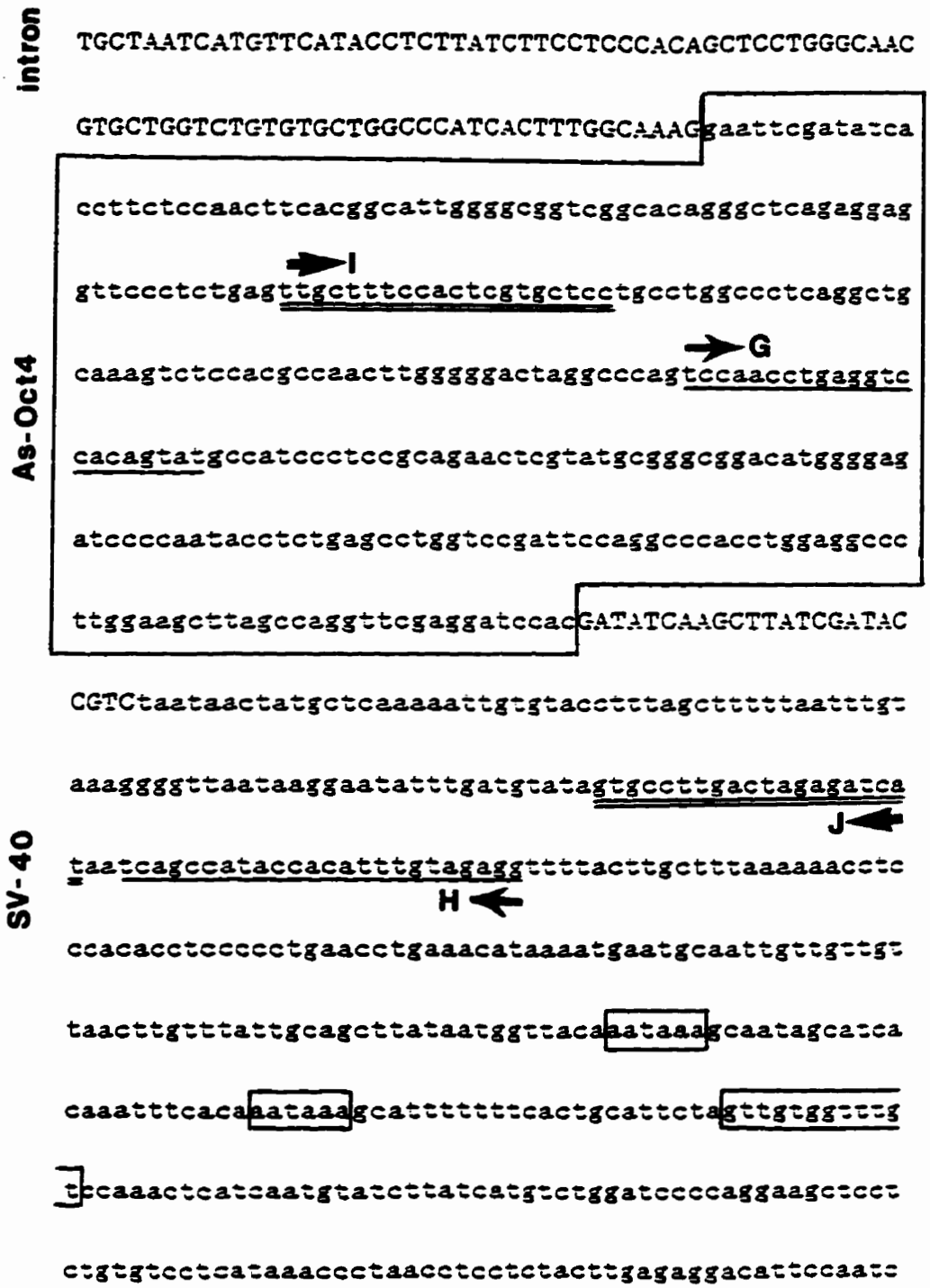
homozygotes were identified on the basis of the "double" strength of the PCR signal for the AB PCR fragment. The genotype of these animals was verified by back-crosses to non-transgenic animals. Animals in which all F1 progeny of the backcross inherited a copy of the transgene were used to breed the line to homozygosity.

§3.11 Transgene Expression

§3.11.1 Antisense Oct-4 RNA-specific primers

The expression of the transgene in transgenic mice was analyzed by RT-PCR using antisense Oct-4 RNA-specific primers. To design antisense RNA-specific primers, one of the two primers must target a region outside of antisense Oct-4 cDNA in the transgene so that the endogenous Oct-4 cDNA sequence is not amplified. Theoretically, transcription starts in the mZP3 promoter region of the transgene but most of the mZP3 promoter sequence is not expected to be present in the nascent antisense RNA. Upon transcription, the intron and most of the SV40 polyadenylation sequence should also be cleaved from the primary transcript of the transgene. Careful examination of the SV40 polyadenylation sequence revealed two AATAAA RNA termination signals followed by a GT-rich region (Fig. 15). The mature antisense RNA is expected to terminate somewhere between the second AATAAA signal and the GT-rich region. The SV40 sequence upstream of the AATAAA signal is, however, expected to be transcribed together with the antisense Oct-4. The primer pairs, Zioprime G and Zioprime H, or Zioprime I and Zioprime J, were thus chosen from within these sites for use as antisense

Fig.15. Design of antisense Oct-4 RNA-specific primers. Only part of nucleotide sequence of the transgene is shown. The AATAAA RNA termination signals and the GT-rich stretch of the SV40 polyadenylation signal sequence are marked as blocks. Zioprime G and Zioprime H are underlined. Zioprime I and Zioprime J are double underlined.


intron
As-Oct4
SV-40
TGCTAATCATGTTCATACCTCTTATCTTCCTCCCACAGCTCCTGGGCAAC
GTGCTGGTCTGTGTGCTGGCCCATCACTTTGGCAAAGgaattcgatatca
ccttctccaacttcacggcattggggcggtcggcacagggctcagaggag
I
gttccctctgagttgctttccactcgtgctcctgcctggccctcaggctg
G
caaagtctccacgccaacttgggggactaggcccagtccaacctgaggtc
cacagtatgccatccctccgcagaactcgtatgcgggcggacatggggag
atccccaatacctctgagcctggtccgattccaggcccacctggaggccc
ttggaagcttagccaggttcgaggatccacGATATCAAGCTTATCGATAC
CGTCtaataactatgctcaaaaattgtgtacctttagctttttaatttgt
aaaggggttaataaggaatatttgatgtatagtgccttgactagagatca
J
taatcagccataccacatttgtagaggttttacttgctttaaaaaacctc
H
ccacacctcccctgaacctgaaacataaaatgaatgcaattgttgttgt
taacttgtttattgcagcttataatggttacaaataaagcaatagcatca
caaatttcacaaataaagcatttttttcactgcattctagttgtggtttg
tccaaactcatcaatgtatcttatcatgtctggatccccaggaagctcct
ctgtgtcctcataaaccctaacctcctctacttgagaggacattccaatc

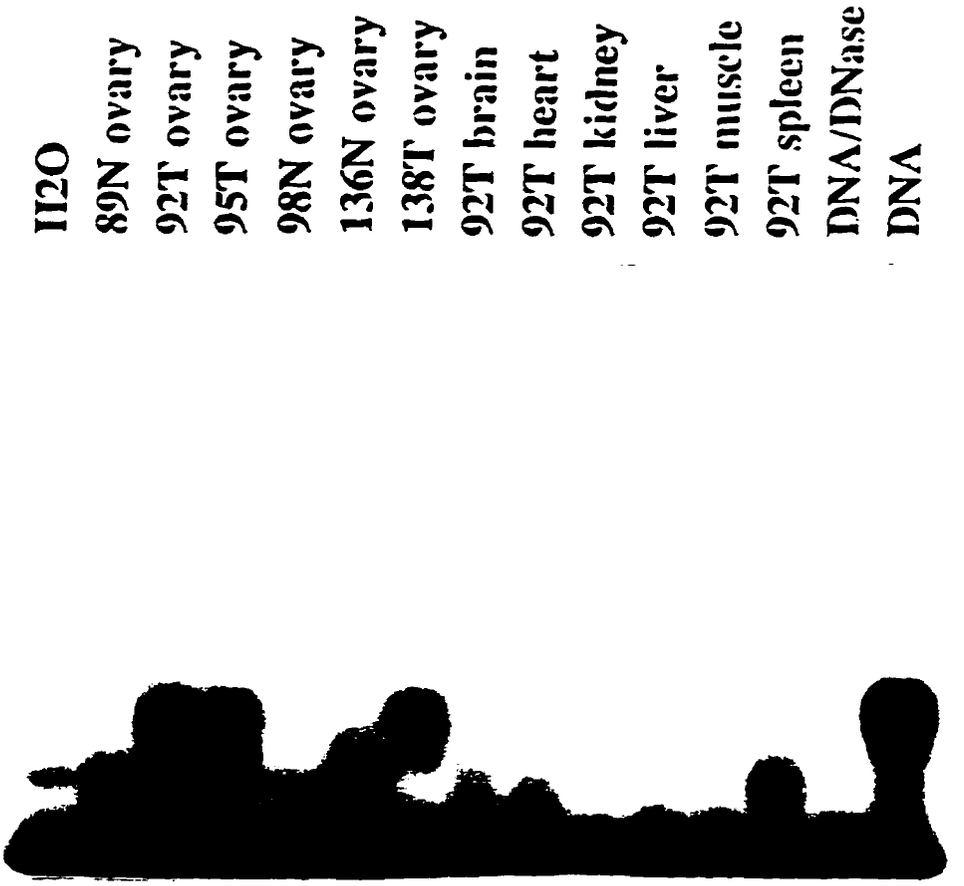
Oct-4 RNA-specific primers. The Zioprime G and Zioprime H primer pair amplify a 291 bp PCR product while the Zioprime I and Zioprime J primer pair generate a 339 bp PCR product.

§3.11.2 Radioactive RT-PCR analysis

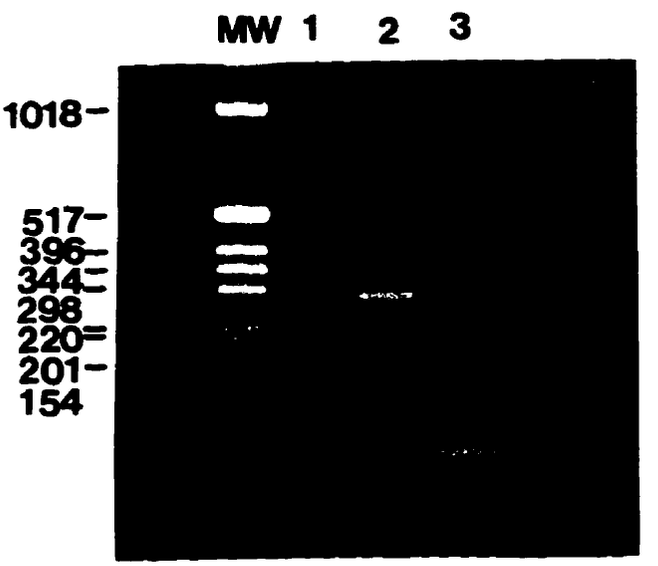
To examine the expression of the transgene, RNAs were extracted from tissues obtained from transgenic mice including ovary, brain, heart, kidney, liver, muscle, spleen and normal mouse ovary. The RNA samples were pretreated with RNase-free DNase I to prevent any possible genomic DNA contamination. Initially, ethidium bromide staining of RT-PCR products was used to identify the presence of RNA transcripts derived from the transgene using Zioprime G and Zioprime H as PCR primers. Unexpectedly, signals obtained when the RT-PCR products were resolved on an agarose gel were very weak or undetectable (data not shown). Thus RT-PCR, in which radioactive precursor was added to the PCR reaction, was carried out to increase sensitivity of detection. The RT-PCR products were resolved on a 2% agarose gel and blotted onto a membrane. Figure 16A shows the results of autoradiogram. A band with the expected size (291 bp) was detected from RNA extracted from transgenic mouse ovaries, but not from brain, heart, kidney, liver, muscle and spleen of transgenic mice or normal mouse ovary. As a control for DNase I treatment, 20 ng of transgenic mouse tail DNA was also treated with DNase I in the same way as the RNA samples followed by PCR amplification. No signals were detected although untreated DNA sample produced a 291 bp PCR product, indicating that the DNase I treatment was complete

Fig. 16. Expression patterns of the transgene in transgenic mice. (A) Radioactive RT-PCR analysis of transgene expression. RNAs from ovaries, brain, heart, kidney, liver, muscle and spleen of transgenic mice and ovaries of normal mice were treated with DNase I and then analyzed for transgene expression by RT-PCR using Zioprime G and Zioprime H with radioactivity added to the PCR reactions. PCR products were resolved on a 2% agarose gel and blotted onto a Hybond™-N⁺ membrane for autoradiography. Transgenic mouse genomic DNA treated with DNase I or the genomic DNA alone were also amplified using Zioprime G and Zioprime H. 89N, 98N and 136N represent normal mice. 92T, 95T and 138T are transgenic mice. (B) RT-PCR analysis of transgene expression in transgenic mouse oocytes using Zioprime G and Zioprime H. Lane 1, equivalent of 4 oocytes of normal mice; lane 2, equivalent of 4 oocytes of transgenic mice; lane 3, negative control. PCR conditions are as follow: denaturing at 94°C for 1 min, annealing at 60°C for 1 min, and extension at 72°C for 1 min. A 40 cycle program was used.

A


H2O
89N ovary
92T ovary
95T ovary
98N ovary
136N ovary
138T ovary
92T brain
92T heart
92T kidney
92T liver
92T muscle
92T spleen
DNA/DNase
DNA
291bp
250bp

B


MW 1 2 3
1018
517
396
344
298
220
201
154

(Fig. 16A) Thus the signals obtained from transgenic mouse ovary RNA were not derived from contaminating genomic DNA. Besides the 291 bp band, a 250 bp RT-PCR product was also observed in transgenic mouse ovary RNA samples and the DNA sample. This suggests that Zioprime G and Zioprime H also target somewhere else in the template sequence.

The expression of the transgene was further examined in transgenic mouse oocytes. Pools of 200 oocytes from superovulated normal mice or homozygous transgenic mice were collected for RNA extraction and reverse transcription. One tenth of the RT products were used for PCR with Zioprime G and Zioprime H. One fifth of these PCR products were subject to electrophoresis. A 291 bp product was readily detectable in the transgenic mouse oocyte sample by ethidium bromide staining, but not in the normal mouse oocyte sample, showing the expression of the transgene in the oocytes (Fig. 16B). The weak signal of the transgene in RT-PCR assays of whole ovary RNA (Fig. 16A), therefore, appears to reflect the fact that oocytes contribute only a very small component to the total cellular mass of the ovary.

§3.11.3 Southern blot analysis

To confirm the identity of the 291 bp fragment, Southern blot analysis was carried out. The RT-PCR products from various tissues of transgenic mice or normal mice were resolved on a gel, blotted onto a membrane and hybridized with an Oct-4 DNA probe corresponding to the nucleotide sequence of antisense Oct-4 cDNA in the transgene. Consistent with the radioactive RT-PCR results, signals were only observed from

transgenic mouse ovary samples (Fig. 17A). Both the 291 bp and 250 bp fragments of the RT-PCR products were hybridized, suggesting that each of them came from the antisense Oct-4 transcripts.

Because of the difficulty in generating high levels of RT-PCR products from the antisense Oct-4 transgene RNA with the primer pair Zioprime G and Zioprime H, an experiment was carried out to assess their efficiency in PCR reactions compared to primers used to detect endogenous Oct-4 transcripts. In brief, 20 ng of purified genomic DNA from a transgenic mouse was used as a template for PCR with either Oct-4 primers or the Zioprime G and Zioprime H primers. After 30 cycles of amplification, the amount of PCR product generated from the Zioprime pair was more than 10 times lower than that for the Oct-4 primers (data not shown). Thus low levels of RT-PCR products derived from antisense Oct-4 RNA templates is, at least in part, due to the inefficiency of the primer pair used in the PCR assay.

Another primer pair, Zioprime I and Zioprime J (see Fig. 15) were used to repeat the experiments carried out with Zioprime G and H. Unfortunately, this primer pair also exhibited poor efficiency in PCR reactions and signals were only detected easily when radioactive precursors were included in the reaction. Consistent with earlier results, a 339 bp band, which was the expected size for PCR product of Zioprime I and Zioprime J primers, was detected from transgenic mouse ovary samples, but not from any other tissue samples (Fig. 17B). Thus, the expression of the transgene was again shown to be restricted to transgenic mouse ovaries, which represents the critical feature of this transgenic approach.

Fig. 17. Southern blot analysis of transgene expression in transgenic mice. RNA samples were treated with DNase I, reverse transcribed and PCR amplified using Zioprime G and Zioprime H (A) or Zioprime I and Zioprime J (B). PCR products were resolved on a 2% agarose gel, transferred to a Hybond™-N^+ membrane and hybridized with Oct-4 DNA randomly labelled probe. The specific activity of the probe was about 4 X 10^8cpm/μg. The X-ray films were exposed overnight.

A
H2O
89N ovary
92T ovary
95T ovary
98N ovary
136N ovary
138T ovary
92T brain
92T heart
92T kidney
92T liver
92T muscle
92T spleen
DNA/DNase
DNA
-291bp
-250bp
B
H2O
89N ovary
92T ovary
95T ovary
98N ovary
136N ovary
138T ovary
92T brain
92T heart
92T kidney
92T liver
92T muscle
92T spleen
DNA/DNase
DNA
-339bp

§3.12 Quantitation of Oct-4 Messages in Oocytes

§3.12.1 The Oct-4 external standards

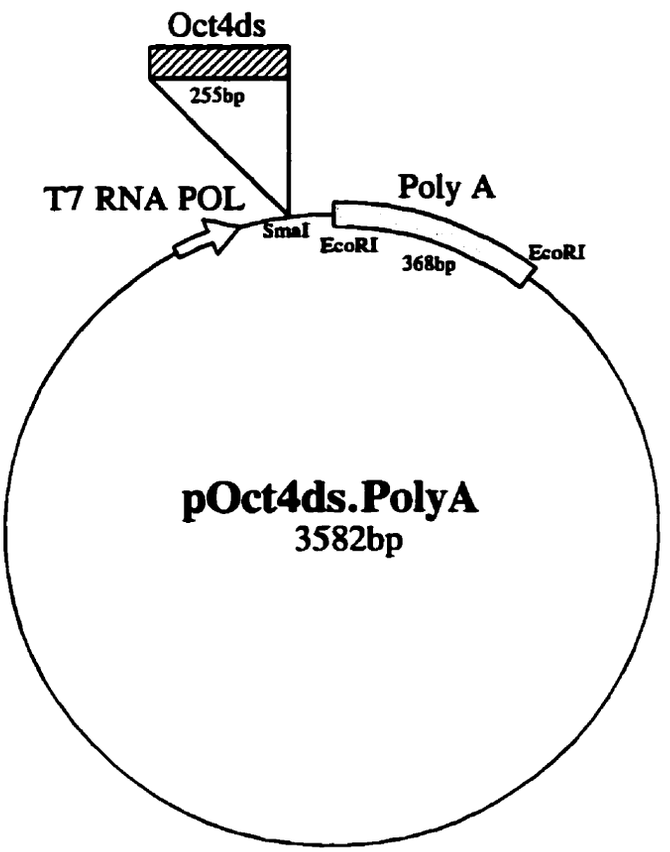
The copy numbers of Oct-4 transcripts in normal mouse oocytes and transgenic mouse oocytes were compared through quantitative RT-PCR using external standards. To ensure that RT-PCR does not amplify the antisense Oct-4 transcripts from the transgene in transgenic mouse oocytes, PCR primers (Oct.ds) were designed to target Oct-4 cDNA sequence (nt. 680 - 934, Rosner et al., 1990) downstream of the cDNA sequence (nt. 127 - 407, Rosner et al., 1990) used for the transgene construct. Thus, the Oct4.ds primers only amplify the endogenous Oct-4 RT products. A 255 bp DNA fragment amplified by using Oct.ds primers was cloned into the pBS.PolyA vector (Arcellana-Panlilio and Schultz, 1994) upstream of the poly A tract in a sense orientation (Fig. 18A). The resulting plasmid, pOct4ds.polyA, was linearized with *Hind*III restriction enzyme and used as a template for in vitro transcription of Oct-4 polyA RNA standards (Fig. 18B).

§3.12.2 Quantitative RT-PCR

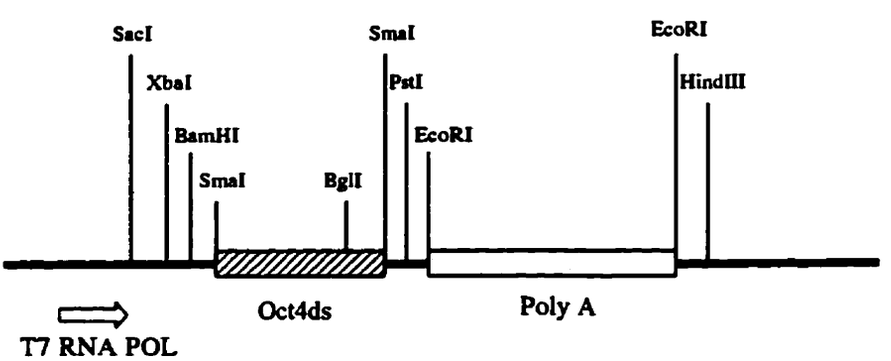
Oocytes were collected from superovulated normal or homozygous transgenic mice and were used for RNA extraction. Dilutions of standard RNA were reverse-transcribed in parallel with oocyte RNA preparations. Aliquots of 1/10 of standard RT and sample RT products were amplified with Oct4.ds primers in the presence of α-^{32}P-dCTP using a 25 cycle PCR program. The 25 cycles were initially chosen because of our

Fig.18. Plasmid maps of pOct4ds.polyA. (A) Circular map. Oct4.ds PCR fragment was cloned into *Sma*I site of the pBS.polyA vector in a sense orientation. (B) Linear map. The pOct4ds.poly A was linearized with *Hind*III. Oct-4 poly A RNA was synthesized by *in vitro* transcription using T7 RNA polymerase.

A


Oct4ds
255bp
T7 RNA POL
Poly A
SmaI
EcoRI
368bp
EcoRI
pOct4ds.PolyA
3582bp

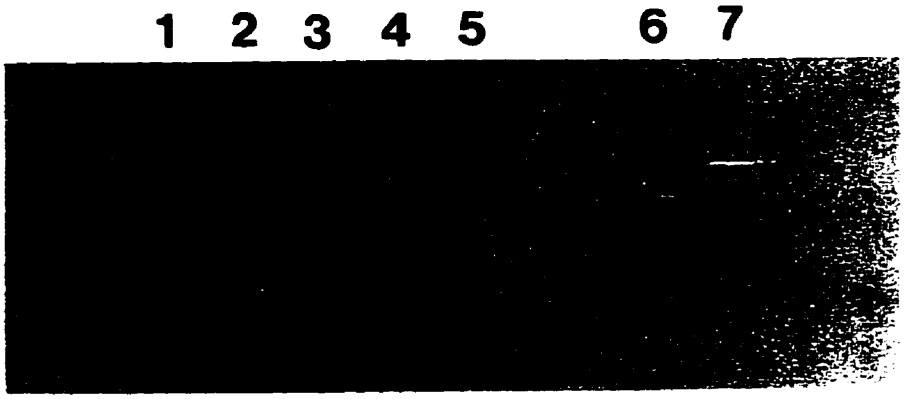
B


SacI
XbaI
BamHI
SmaI
BglI
SmaI
PstI
EcoRI
EcoRI
HindIII
Oct4ds
Poly A
T7 RNA POL

earlier experience that demonstrated rare messages such as those encoding most growth factors and/or transcription factors in early mouse embryos are usually maintained within the exponential phase of amplification in the PCR reactions with this cycle number (Schultz et al., 1993). Several trials using separate sets of material showed that, indeed, 25 cycles allowed the amplification of Oct-4 cDNA within the exponential phase. An example of the results of one quantitative PCR experiment is shown in Figure 19. RNA standards of $1x\ 10^6$, $5x\ 10^5$, $1x\ 10^5$, $5x\ 10^4$ copies and RNA samples from 200 normal mouse oocytes or 200 transgenic mouse oocytes were reverse-transcribed. One tenth of the RT reaction products were used for PCR and 1/5 of the PCR products were subjected to electrophoresis. After gel electrophoresis, the products were transferred onto a hybond™-N^+ membrane and subjected to autoradiography (Fig. 19). The bands appearing across the gel represent the 255 bp Oct-4 PCR products. The first four lanes represent PCR products derived from $2x\ 10^4$, $1x\ 10^4$, $2x\ 10^3$, and $1x\ 10^3$ copies of standard RNA, respectively. Lane 5 is the negative control. The last two lanes contain PCR products derived from the equivalent of 4 normal mouse oocytes and 4 transgenic mouse oocytes, respectively. The autoradiogram was analyzed by densitometry. The Oct-4 PCR signal for RNA derived from 4 normal oocytes (lane 6) was very similar to that of the standard sample containing 2,000 copies of Oct-4 mRNA (lane 3). This was equal to 500 copies per oocyte. When corrected for recovery of RNA during extraction (69%), the copy number was calculated to be 724. The level of Oct-4 mRNA in the transgenic mouse oocyte sample was about 20% of the normal in this experiment.

Five separate sets of oocytes and RNA preparations (each set contained the same

Fig.19. Quantitation of Oct-4 mRNA in oocytes. RNA standards and samples were reverse transcribed and PCR amplified in parallel in the presence of α-^{32}P-dCTP. PCR products were resolved on a 2% agarose gel and transferred to nylon membrane. The autoradiograph was exposed for 7 days. Lane 1 to 4, PCR products derived from 2X 10^4, 1X 10^4, 2X 10^3 and 1X 10^3 copies of RNA standard. Lane 5, negative control. Lane 6, equivalent of 4 oocytes of normal mice; lane 7, equivalent of 4 oocytes of transgenic mice.


1 2 3 4 5 6 7

number of oocytes from normal and homozygous transgenic mice) were carried out to quantitate Oct-4 mRNA. There were some variations from one set of samples to the next, but on average, the copy number of Oct-4 mRNA per oocyte derived from normal mice was approximately 800. The Oct-4 mRNA level in transgenic oocytes was 39% of the normal and equivalent to about 310 copies per oocyte.

§3.13 Measurement of Relative Levels of Oct-4 Protein in Oocytes

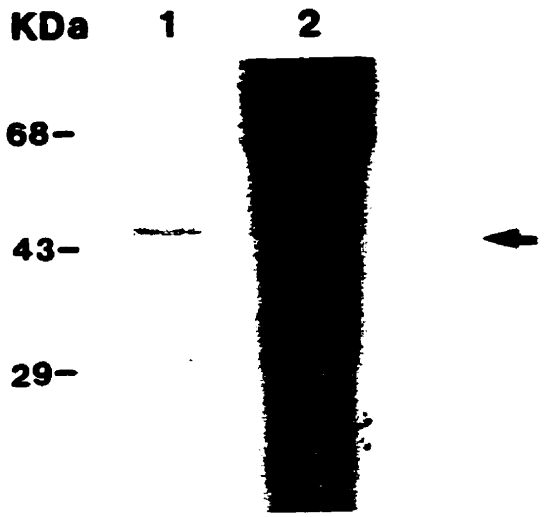
Previous experiments using Western blot analysis on protein extracts from 751 mouse oocytes showed that the relative level of Oct-4 protein was very low compared to that in F9 cells (Fig. 12). An alternative approach was taken to try to compare the relative levels of Oct-4 protein in oocytes derived from normal or transgenic mice. This approach involves metabolic labelling of the oocytes with ^{35}S-methionine, cell lysis, immunoprecipitation of ^{35}S-labelled Oct-4 protein with anti-Oct-4 antiserum, purification of the immune complexes with protein A agarose beads, SDS-polyacrylamide gel electrophoresis and autoradiography.

Initially, an experiment was carried out to test the immunoprecipitation approach on ^{35}S-labelled protein extracts from undifferentiated F9 cells. The immunoprecipitate did contain a band of the expected size for Oct-4 protein along with a few non-specific bands (Fig. 20A). This band was greatly enriched in the immunoprecipitate relative to the total protein extract (compare lane 2 and lane 1, Fig. 20A).

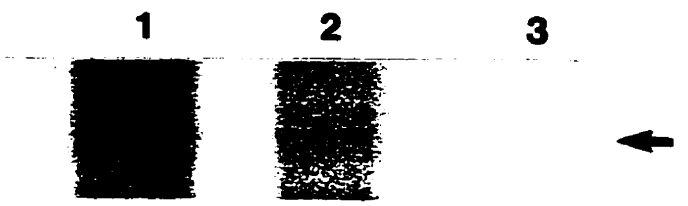
The next step in applying the immunoprecipitation method to analysis of Oct-4

Fig.20. Measurement of relative levels of Oct-4 protein in oocytes. (A) Immunoprecipitation of Oct-4 protein from F9 cells. F9 cells were metabolically labelled with ^{35}S-methionine and lysed. Lane 1, the immunoprecipitate from 3.5X 10^6 cpm of F9 cell lysate using Oct-4 antiserum and analyzed on an SDS-polyacrylamide gel. Lane 2, total proteins from F9 cell lysate of 2.4X 10^4 cpm. The autoradigraph was exposed for 6 days. The arrow indicates the band for Oct-4 protein. (B) Results of immunoprecipitation of Oct-4 protein from mouse oocytes and F9 cells. The same amount of radioactively labelled total protein (1.73X 10^7 cpm) from normal (lane 1) and homozygous transgenic mouse (lane 2) oocytes was used. The autoradiograph was obtained by exposure an X-ray film against the gel for 4 days. The arrow indicates the band for Oct-4 protein. The immunoprecipitate from 3.6X 10^6 cpm of F9 cells is shown in lane 3 for comparison. (C)Relative Oct-4 protein levels in normal mouse oocytes and transgenic mouse oocytes from three immunoprecipitation experiments. Autoradiographes were scanned by a densitometer. Densitometric readings of normal mouse oocytes were arbitrarily set to be 1 in each experiment. Column 1 represents the result from (B). Column 2 and 3 represent results from other two separate experiments. In column 2, the immunoprecipitates from 2.9X 10^7 cpm of normal and transgenic mouse oocytes were used. In column 3, the immunoprecipitates from 1.70X 10^7 cpm of normal and transgenic mouse oocytes were used.

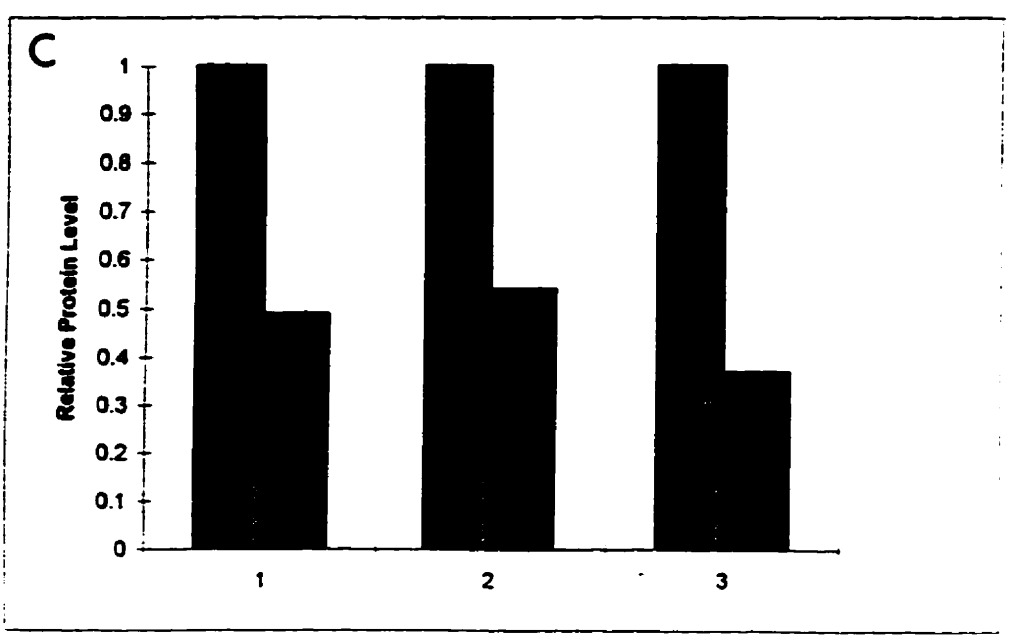
A


KDa
1
2
68–
43–
29–

B


1
2
3

C


Relative Protein Level
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
1
2
3

levels in oocytes was to optimize the level of Oct-4 antiserum needed for an experiment. Oocytes were collected, following superovulation, from 25 normal CD1 mice and cultured in the presence of ^{35}S-methionine for labelling. After the oocytes were lysed, 2 μl or 5 μl Oct-4 antiserum was added to the lysate of approximately 1.7X 10^7 cpm each to precipitate the Oct-4 protein. The resulting autoradiograph showed that 2 μl of antiserum was not sufficient to produce a visible signal of the Oct-4 protein but that 5 μl antiserum precipitated the Oct-4 protein to yield a band of moderate intensity albeit accompanied by several non-specific bands (data not shown). Because background would be increased if more antiserum was used, 5 μl of Oct-4 antiserum per aliquot of cell lysate (1.7X 10^7 cpm) was chosen as the optimal amount for these immunoprecipitation experiments.

To compare the relative levels of Oct-4 protein, the same amount of radioactively labelled total protein (cpm) from normal mouse oocytes and homozygous transgenic mouse oocytes was used in each immunoprecipitation experiment. Three separate experiments were conducted. An interesting observation was that oocytes from normal mice were always labelled better than those from transgenic mice in the three experiments. Fewer numbers of oocytes from normal mice were thus used in each experiment than from transgenic mice when normalized to the same count of protein lysate. In the three experiments, numbers of oocytes used were 563 (normal) versus 710 (transgenic) (equivalent to 1.73 X 10^7 cpm), 1288 (normal) versus 1501 (transgenic) (equivalent to 2.9 X 10^7 cpm), and 582 (normal) versus 690 (transgenic) (equivalent to 1.70 X 10^7 cpm), respectively. The result of one of the experiments is presented in

Figure 20B. The immunoprecipitated ^{35}S-labelled 45 kD Oct-4 protein band from normal mouse oocytes is shown in lane 1, from transgenic oocytes in lane 2, and from undifferentiated F9 cells in lane 3 (Fig.20B). Densitometric analysis of the intensity of the signal in the Oct-4 protein band showed that there was a 51% decrease in transgenic mouse oocytes (Fig. 20B, lane 2) when compared to normal mouse oocytes (Fig. 20B, lane 1). The relative levels of Oct-4 proteins from the three analyses have been summarized in Figure 20C. When the Oct-4 protein level in normal mouse oocytes was arbitrarily set to be 1, the Oct-4 protein levels in transgenic mouse oocytes from the three experiments were 0.49, 0.54, and 0.37, respectively. Taking the average of the three separate experiments, the Oct-4 protein level in transgenic mouse oocytes was 0.47 ± 0.09 (mean$\pm$SD, n=3), a 53% decrease when compared to that in normal mouse oocytes.

§3.14 Assessment of Reproductive Capacity of the Transgenic Mice

The transgenic mice, heterozygous or homozygous, did not show any visible physical phenotypes. Compared to normal CD1 mice, the transgenic mice had normal body weight, normal size, and no overt developmental abnormalities. To investigate the impact of antisense inhibition of Oct-4 in oocytes on reproductive capacity more thoroughly, the transgenic mice were scored with respect to numbers of oocytes produced and embryo numbers (litter sizes). In addition, general histological examination of the ovaries of normal and transgenic mice was carried out.

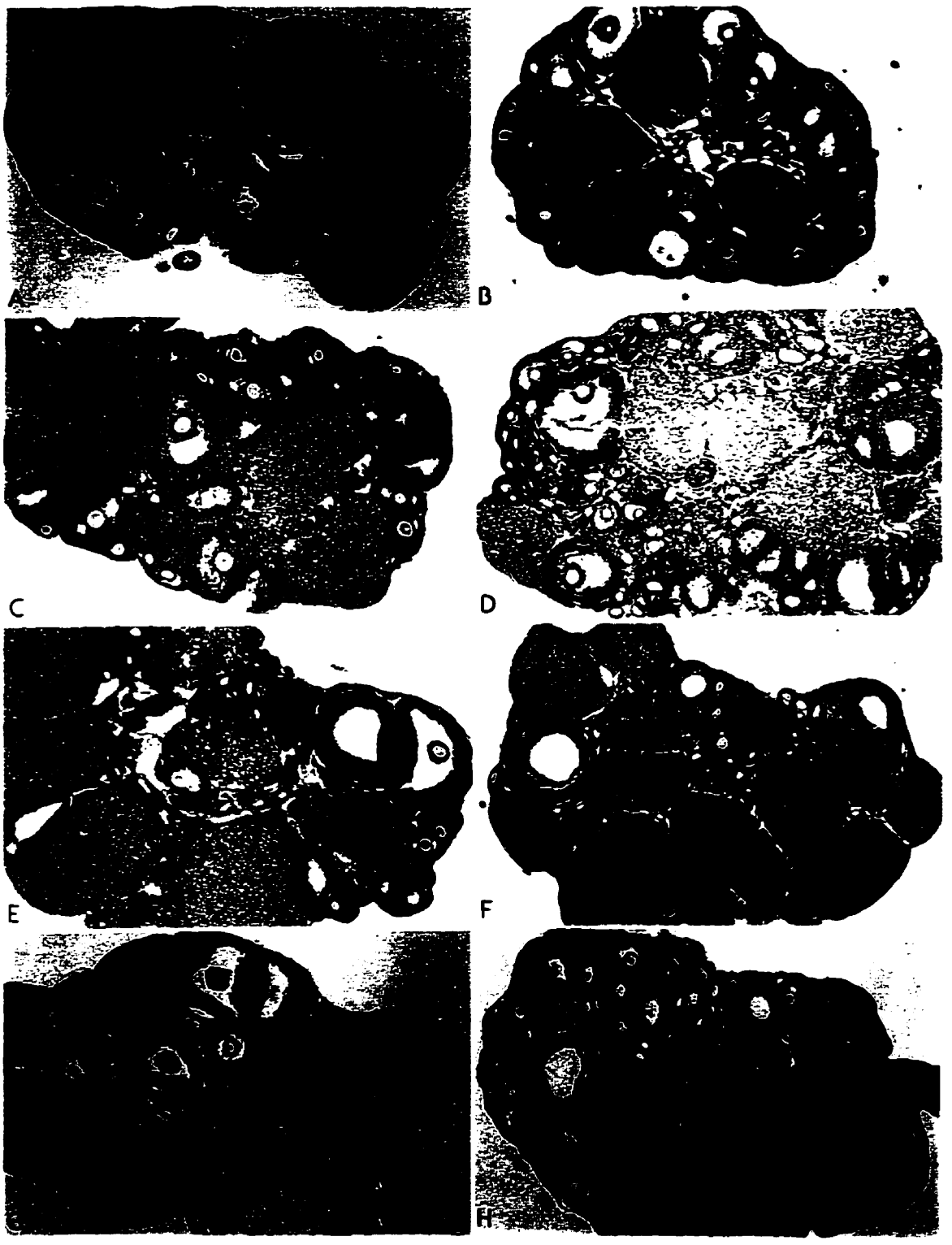
§3.14.1 Histological examination of ovaries

Ovaries from normal and homozygous transgenic mice collected at various phases of the estrus cycle were compared histologically. The phases of estrus cycle were determined by vaginal smears (Rugh, 1968). Mid-saggital sections from ovaries in each stage were stained with haematoxylin and eosin, which stained nuclei and cytoplasm, respectively. Overall, the sections from the ovaries of normal and transgenic mice at the same estrus phase appeared very similar, suggesting the antisense Oct-4 RNA expression did not significantly influence ovarian follicle number or size (Fig. 21). Occasionally, ovaries from some transgenic mice showed more small follicles than normal ones (compare for example, normal and transgenic ovaries at the proestrus stage in panels C and D, respectively, or at the metestrus stage in panels G and H, respectively). The significance of the observation is not clear since reproductive capacity appears unaffected in transgenic animals (see below). The sections may simply reveal individual variation in mice. Ovaries from only 4 mice were prepared for histological sections at each stage (total 16 mice). While three out of eight of the transgenic mice sections had a structure like those in panels D and H (Fig. 21) with many small follicles, the other sets were indistinguishable from their normal counterparts. The sample size is too small to draw firm conclusions and the observations made herein must be interpreted with extreme caution.

§3.14.2 Ability of superovulation

The effect of underexpression of maternal Oct-4 on oogenesis was further

Fig.21. Histological comparison of ovary sections from normal mice and homozygous transgenic mice in each stage of estrous cycle. Phases of estrus were determined by vaginal smears. Ovary sections were stained with hematoxylin and eosin. (A) and (B), diestrus; (C) and (D), proestrus; (E) and (F), estus; (G) and (H), metestrus. (A), (C), (E) and (G) are normal mouse ovary sections. (B), (D), (F) and (H) are homozygous transgenic mouse ovary sections. magnification X80.


A
B
C
D
E
F
G
H

evaluated by scoring oocyte yields in mice that were hormonally stimulated. Batches of normal CD1 mice and transgenic mice were injected with PMS and HCG for superovulation. The numbers of oocytes recovered were counted and results are summarized in Table 1. From 13 observations, the average egg yields of homozygous transgenic mice were 23.35±5.82 (mean±SD, n=13). In the other 13 observations, superovulation of normal CD1 mouse led to egg yields of 26.44±3.45 (mean±SD, n=13). The overall average egg yields were lower from transgenic mice than from normal mice. However, these values were not statistically different when analyzed by one-way analysis of variance (ANOVA) or the student t-test. The oocyte numbers per mouse were more variable in transgenic mice than normal mice (Table 1). One possible reason is that the ages of homozygous transgenic mice used in experiments were more heterogenous than the normal CD1 mice. For the most part, the mice were all 7 to 8 weeks old when used in the experiment except for groups 11, 12 and 13 in the transgenic groups that were 12 weeks of age (table 1).

§3.14.3 Reproductive performance

The only transgenic founder mouse obtained, #6, was fertile and, therefore, enabled us to develop this transgenic line. To assess reproductive performance of these transgenic mice, we bred heterozygous transgenic mice of either gender with heterozygous or normal mates. Homozygous transgenic mice were also intercrossed. The average number of viable offspring per breeding pair and the sex of offspring was determined for each type of cross (Table 2). Both homozygous and heterozygous

Table 1. Numbers of oocytes derived from superovulated normal and transgenic mice.

Exp. No.	mouse type	mouse number	oocyte number	oocyte number per mouse	mean oocyte number per mouse ± standard deviation[a]
1	homozygous	50	1501	30.02	23.60 ± 5.98 (n=13)
2		50	1060	21.20	
3		50	800	16.00	
4		35	1205	34.43	
5		25	692	27.68	
6		26	710	27.31	
7		26	803	30.88	
8		26	523	20.12	
9		26	394	15.15	
10		21	423	20.14	
11		20	450	22.50	
12		40	735	18.38	
13		25	575	23.00	
14	normal	50	1288	27.76	26.44 ± 3.45 (n=13)
15		50	1210	24.20	
16		50	1200	24.00	
17		40	881	22.03	
18		50	1340	26.80	
19		50	1035	20.70	
20		26	888	34.15	
21		26	740	28.46	
22		26	710	27.30	
23		26	703	27.04	
24		25	639	25.56	
25		26	670	25.77	
26		25	748	29.92	

[a]Enclosed in parentheses is the number of observations.

Table 2. Litter sizes and sex ratios in transgenic mice.

breeding pairs	heterozygous X normal	heterozygous X heterozygous	homozygous X homozygous
number of litters (n)	13	9	115
total mouse number in F1	135	103	1252
total female number in F1	66	52	592
total male number in F1	69	51	660
average litter size[a]	10.38 ± 1.85	11.44 ± 3.24	10.89 ± 3.67
average female number per litter[a]	5.08 ± 1.38	5.78 ± 1.99	5.15 ± 2.52
average male number per litter[a]	5.30 ± 2.50	5.67 ± 2.12	5.74 ± 2.26

[a] mean value ± standard deviation from the mean (Sd)

transgenic female mice became pregnant at a frequency similar to normal CD1 mice, suggesting that these transgenic mice underwent normal estrus cycles, ovulation and mating. The gestation period for female transgenic mice (homozygous or heterozygous) was 19 to 21 days, which is also the gestation time for normal CD1 mice. The average litter sizes for homozygote intercrosses, heterozygote intercrosses and heterozygote/normal mice intercrosses were 10.89 ± 3.67 (mean$\pm$SD, n=115), 11.44 ± 3.24 (mean$\pm$SD, n=9), and 10.38 ± 1.85 (mean$\pm$SD, n=13) littermates, respectively. These numbers fall within the range of those reported for CD1 mice which have 10 to 14 offspring per female (Hill, 1983). The average sex ratios of the littermates per breeding pair were also determined. There were similar numbers of female littermates and male littermates in each mating type, showing a normal mendelian distribution close to the expected ratio of 1:1 (Table 2).

In summary, antisense inhibition of maternal Oct-4 resulted in 69% decrease in Oct-4 mRNA level and 53% decrease in Oct-4 protein level in oocytes derived from transgenic mice. However, the overall reproductive capacity of the transgenic mice was not affected.

§4 DISCUSSION

A polyclonal antibody was raised against a unique peptide sequence of Oct-4. It specifically recognized Oct-4 protein in supershift assays. This antibody has been used to measure Oct-4 protein levels during retinoic acid induced differentiation of F9 embryonic carcinoma (EC) cells. Consistent with expression levels of Oct-4 mRNA in differentiating F9 cells as analyzed by northern blot analysis, Oct-4 protein levels were found to decline to undetectable levels as F9 cells differentiated and cell pluripotency was lost. To study the role of maternal (oocyte-derived) Oct-4 in the mouse, an attempt was made to underexpress maternal Oct-4 in transgenic mice by antisense inhibition. Antisense Oct-4 RNA was expressed under the control of mouse zona pellucida ZP3 (mZP3) promoter. Tissue-specific expression of the antisense gene was demonstrated by radioactive RT-PCR and Southern blot analysis. The level of Oct-4 mRNA in transgenic mouse oocytes was quantitated and shown to be 39% of that in normal mouse oocytes. The Oct-4 protein level was also reduced to 47% of the normal. The reduction in Oct-4 levels in oocytes of transgenic mice did not affect the number of oocytes or number of embryos produced.

§4.1 Analysis of Oct-4 Expression in F9 Cells and in the Mouse

Mouse blastocysts contain two cell types: the inner cell mass (ICM), which gives rise to the embryo proper, and the trophectoderm, which gives rise to extraembryonic

tissues and the embryonic portion of placenta. As development continues, the outer cell layer of the ICM differentiates and forms primitive endoderm which further differentiates into two morphologically and biochemically distinguishable subpopulations: the visceral endoderm, which remains associated with the developing ICM, and the parietal endoderm, which migrates onto the inner surface of the trophectoderm. The formation of primitive endoderm is readily mimicked in tissue culture by employing EC cells such as F9 cells. F9 cells are pluripotent and can be induced by retinoic acid (RA) to develop into primitive endoderm-like cells, which give rise to parietal endoderm when cultured in monolayers or to visceral endoderm when cultured in suspension (Hogan et al., 1986). This suggests that F9 EC cells share some of the properties of embryonic ICM cells. F9 cells have, therefore, been widely studied as a model system to study developmental events at these early stages of mouse embryogenesis.

In the present study, both Oct-4 mRNA and protein levels were measured in differentiating F9 cells cultured in monolayers. It was observed that both Oct-4 mRNA and protein were down-regulated upon RA-induced differentiation of F9 cells. In the case of Oct-4 mRNA, the levels were markedly reduced, but still detectable, when F9 cells were treated with RA and dcAMP for 7 days (Fig. 5). The most likely reason for the presence of residual Oct-4 mRNA is that a few undifferentiated F9 cells were still present and expressed the Oct-4 gene. At the protein level, no product was detected when F9 cells were treated for the same period of time (Fig. 7A). This may reflect differences in the level of sensitivity of detection of low levels of RNA versus protein. In line with its expression pattern in differentiating F9 cells, Oct-4 protein expression was also observed

to be down-regulated in differentiating P19 EC cells which also resemble mouse ICM cells and can differentiate along a neural pathway. The expression patterns of Oct-4 in these differentiating EC cells suggest that Oct-4 may indeed play a role in maintaining cell pluripotency.

The mechanism of Oct-4 gene repression in RA-treated EC cells has been studied in several laboratories (Schoorlemmer et al., 1994; Pikarsky et al., 1994; Ben-Shushan et al., 1995). The action of RA on gene expression is usually mediated by two families of the nuclear hormone receptor superfamily referred to as RARs (isoforms α, β and γ) and RXRs (isoforms α, β and γ) (Mangelsdorf et al., 1992; Petkovich et al., 1987). Besides RARs and RXRs, the nuclear hormone receptor superfamily also comprises a variety of orphan receptors such as COUP-TFI and COUP-TFII (O'Malley and Conneely, 1992). These nuclear hormone receptors bind to their corresponding hormone response element in the form of homodimers or heterodimers (Leid et al., 1992). Dissection of the promoter of the Oct-4 gene has revealed that the Oct-4 promoter harbours an RA-responsive element (RAREoct) (Schoorlemmer et al., 1994; Pikarsky et al., 1994). Transfection assays indicate that orphan receptors COUP-TFI and COUP-TFII repress Oct-4 promoter activity while three different RAR:RXR heterodimers, RARα:RXRα, RARβ:RXRα and RARβ:RXRβ, stimulate Oct-4 promoter activity through the RAREoct site. The orphan receptors bind the RAREoct site with a much higher affinity than the RAR:RXR heterodimers (Ben-Shushan et al., 1995). Moreover, RA treatment of EC cells strongly activates expression of COUP-TFI and COUP-TFII (Ben-Shushan et al., 1995). These observations suggest that expression of Oct-4 in EC

cells depends, at least in part, on the concentration and affinity of different nuclear hormone receptors for the RAREoct site. Although EC cell lines resemble particular cell types at a certain developmental stage of the mouse embryo, the correspondence between cell lines and *in vivo* cell types is never complete. Thus, whether the regulatory mechanism of Oct-4 gene expression in EC cells reflects the situation in normal embryogenesis in the mouse remains to be demonstrated.

Despite the caveat above, the expression pattern of Oct-4 in differentiating F9 cells is consistent with changes in levels of expression in early mouse embryos (Rosner et al., 1990; Schöler et al., 1990a; Yoem et al., 1991). The feature common to all of the cells expressing Oct-4 in the early embryo is that they retain the capacity for differentiation along multiple lineages. With the differentiation of the three primary germ cell layers, endoderm, mesoderm and ectoderm, Oct-4 expression is eventually confined to primordial germ cells (PGCs) during embryogenesis (Rosner et al., 1990; Schöler et al., 1990a). In this thesis, sensitive RT-PCR technology was used to examine expression patterns of Oct-4 in adult mouse tissues. Among the tissues examined, Oct-4 transcripts were detected only in the gonads: ovary and testis (Fig. 10). Expression of Oct-4 in the testis was previously observed by Schöler et al. (1989) and Rosner et al. (1990). The exact identity of the cells in the testis expressing Oct-4 has not been established. Neither mature sperm nor Sertoli cells have detectable Oct-4 (Schöler et al., 1989; Rosner et al., 1991b). Hence, expression may be restricted to spermatocytes. The location of the expression of Oct-4 in the ovary has been defined and is definitely restricted to oocytes (Fig. 11 in this study; Rosner et al., 1990; Schöler et al., 1990a). In conclusion, Oct-4

expression appears to be confined to pluripotent cells of mouse embryo and to germ cells.

The expression of Oct-4 in PGCs and oocytes may be accounted for by its putative role in maintenance of cell potency. Indeed, the developmental potency of PGCs and oocytes is unlimited. On the other hand, mouse ovarian oocytes were found to be able to develop parthenogenetically in single- and many-layer follicles forming "ovarian embryos" composed of many types of well differentiated tissues of embryonic and extraembryonic origin (Stevens and Varnum, 1974). Besides a role in maintaining cell pluripotency, in PGCs and oocytes, Oct-4 may also be involved in the processes of germ cell specification, oocyte growth and meiotic maturation. Direct proof for such functions is still lacking.

The correlation between Oct-4 expression and cell pluripotency is challenged by the studies of Oct-4 expression in other mammals. In human, the Oct-4 gene is expressed at low levels in diverse adult tissues such as kidney, heart, liver, spleen and pancreatic islets in addition to ovary and testis (Takeda et al., 1992). In cynomolgus monkey, expression is not as widespread as in the human but is still more diverse than in the mouse and is detectable in several reproductive tissues including oocytes, ovarian granulosa cells, fallopian tube, uterine tube, myometrium and cervix (Heikinheimo et al., 1995). The variations of Oct-4 expression patterns in these different mammals suggest that a role for Oct-4 in cell pluripotency might, therefore, be organism-specific (i.e. unique to the mouse). Nonetheless, a common feature of Oct-4 expression is that Oct-4 transcripts are found in oocytes and unfertilized eggs whether in mouse, human or

monkey (Rosner et al., 1990; Abdel-Rahman et al., 1995; Heikinheimo et al., 1995). This commonality does indeed support a possible role of maternal Oct-4 during oogenesis and early postfertilization development. Moreover, antisense inhibition of a maternal Oct factor, spOct, in the sea urchin *Strongylocentrotus purpuratus* results in developmental arrest of the embryo prior to the first cell division (Char et al., 1994). This suggests an important role for maternal octamer factors in early postfertilization development. On this basis, the role of maternal Oct-4 in the mouse was investigated.

§4.2 Evaluation of the Transgenic Strategy

Subsequent to the initial discoveries of antisense RNA molecules as natural repressors of prokaryotic gene expression (Mizuno et al., 1984; Takayama and Inouye, 1990), various investigators have taken advantage of antisense approaches to try to inhibit gene expression in eukaryotic systems. Generally, these approaches fall into three categories: (i) direct microinjection of antisense RNA or DNA molecules into cells, (ii) addition of antisense molecules to culture medium of cells for up-take and inhibition, and (iii) introduction of antisense genes for stable integration and expression of antisense RNA molecules in cells. All approaches have been used successfully in one system or another but each approach also has some disadvantages.

Direct microinjection of *in vitro* synthesized antisense RNA or antisense oligonucleotides has been used by several investigators to introduce large quantities of antisense RNA for target inhibition (Salles et al., 1993). This approach has worked

particularly well on large cells like *Xenopus* oocytes (see Melton, 1985). In studies of early mouse embryos, it has also been applied to inhibition of t-PA mRNA translation in mouse oocytes (Strickland et al., 1988), to interference with meiotic maturation by microinjection of antisense c-mos oligonucleotides (Paules et al., 1989; O'Keefe et al., 1989), to blockade of connexin mRNA translation with a resultant failure in gap-junction formation in early cleavage stage embryos (Bevilacqua et al., 1989). Although these examples demonstrate successful application of this method for studies of early development, the approach has limitations because the time course of antisense inhibition is restricted to a short-period related to the half-life (stability) of the injected antisense molecules and, in the case of embryos, it is simply impractical to microinject every cell of an embryo except at the very early cleavage stages.

Eppig and Schroeder (1989) have developed a system to collect mouse oocytes from preantral follicles in large numbers, and to allow them to grow, mature and be fertilized *in vitro*. Theoretically, primary oocytes obtained from ovarian follicle by using this system could provide optional material to study the role of a maternal factor during oogenesis. Methodological conditions for microinjecting plasmid constructs into dictyate oocyte nuclei at different stages of follicle growth for transient expression have been developed. Exogenous genes such as the E. *coli* lacZ gene under the control of different promoters were able to be expressed when the circular DNA constructs were injected into small- and middle- sized growing oocytes (Bevilacqua et al., 1992). However, these studies only showed transient expression of exogenous genes (i.e., 17 to 40 hr post injection). Moreover, the ability to maintain proper balance between nuclear and

cytoplasmic maturation events in oocytes in culture is very difficult and except in the most experienced laboratories (like Eppig's) it is virtually impossible to maintain an *in vitro* system that reflects the *in vivo* situation.

There is also a large body of literature that contains reports of successful down-regulation of gene expression by addition of antisense oligonucleotides directly to the medium of cultured cells or embryos (van der Krol et al., 1988; Marcus-Sekura, 1988). The stability of these exogenously supplied antisense oligonucleotides can be increased through various modifications that include production of methyl-phosphonate or phosphorothioate derivatives and various end modifications to the terminal phosphodiester linkage (van der Krol et al., 1988; Marcus-Sekura, 1988; Koga et al., 1991; Shaw et al., 1991). These modifications also improve penetration of antisense oligonucleotides across the cell membrane. The advantage of this approach for the study of early mouse embryo development is that embryos can be cultured in relatively small droplets of medium (20-50 μl), thereby avoiding the expense of addition of large amounts of synthetic oligonucleotides in the medium and the method is non-invasive compared to microinjection. Antisense inhibition of IGF-II expression in mouse embryos has been partially achieved by culturing 2-cell mouse embryos in the presence of antisense oligonucleotides (Rappolee et al., 1992). The method did not, however, lead to complete quenching of IGF-II expression and only 65 to 80% of embryos were affected by the treatment as visualized by a delay in cavitation and blastocyst formation (Rappolee et al., 1992). Thus, this approach can be used where cell numbers are small and where embryos can be maintained in culture, but like microinjection procedures the time course of

experiment is limited by the stability of the antisense oligomers.

The preferred route for production of antisense RNA is the introduction of antisense genes into cultured cells or germ lines for stable integration into the host genome (Takayama and Inouye, 1990). Although most of the studies have been carried out in cultured cells, the introduction of antisense genes into the germ lines of mice, particularly into male pronuclei of fertilized eggs, has also been documented. The first successful whole-animal model of antisense inhibition was demonstrated by Katsuki et al. (1988). Plasmid construct containing antisense myelin basic protein (MBP) gene was used to inhibit MBP mRNA levels as much as 80% in the transgenic mice. Some of these mice were converted from the normal to mutant shiverer phenotype (Katsuki et al., 1988). For another example, expression of type II glucocorticoid receptor antisense RNA in transgenic mice resulted in up to a 70% decrease of endogenous type II glucocorticoid receptor mRNA (Pepin et al., 1992). These transgenic mice exhibited greatly increased fat deposition and thus displayed larger body weight and size. The examples indicate that introduction of antisense genes into the germline of mice can help to define the biological and developmental functions of specific genes *in vivo*.

The principle of the antisense RNA approach is presumably to inactivate the endogenous mRNA function by formation of an RNA-RNA duplex between the antisense RNA and its complementary target mRNA (Colman, 1990). The mechanism of action of antisense RNA remains obscure. An easily explained model is that duplex formation between antisense and sense RNAs interferes with either the binding or translocation of ribosomes on the mRNA and thus inhibits translation of the target mRNA (Colman,

1990). A more likely mechanism of antisense-mediated translational inhibition appears to be a degradation of the RNA in the duplexed region by a double-strand-specific RNase III-like nuclease activity as observed in E. *coli* (Krinke and Wulff, 1987). Strickland et al. (1988) reported that injection of t-PA antisense RNA into mouse oocytes resulted in cleavage of the RNA-RNA hybrid region. Thus, double-strand-specific nuclease activity appears to be present in mouse oocytes and the RNA "unwindase" activity found in *Xenopus* embryos appears to be absent (Bass and Weintraub, 1988; Bevilacqua et al., 1988). When antisense DNA is stably integrated into the host genome, the antisense RNA is transcribed in the nucleus. Thus, the antisense RNA may also function by sequestering the target mRNA in the nucleus, preventing its transport to the cytoplasm and by interfering with mRNA processing (Takayama and Inouye, 1990; van der Krol et al., 1988).

The temporal and spatial specificity of gene inactivation by an antisense strategy using transgenic mice can be manipulated by choosing an appropriate promoter. In the present study, the mZP3 promoter was used to direct antisense Oct-4 gene expression specifically to oocytes. As outlined in the introductory chapter of the thesis, mZP3 is expressed only in growing oocytes (Philpott et al., 1987; Roller et al., 1989). The expression level of mZP3 transcripts in oocytes is unusually high. It is estimated that there are approximately 300,000 copies of mZP3 mRNA in 60- to 70- μm diameter-stage oocytes and 250,000 copies in fully grown oocytes (Roller et al., 1989). The mZP3 mRNA constitutes as much as 0.27% of total oocyte poly(A)$^+$ RNA, making mZP3 one of the most abundant mRNAs in mouse oocytes (Philpott et al., 1987). Due to its strong

activity and oocyte-specificity, the mZP3 promoter permits expression of antisense transgenes specifically in growing oocytes of transgenic mice, thus creating maternal effect mutations in mice. We have used a 6.5 kb mZP3 promoter sequence to direct antisense Oct-4 RNA expression. The 6.5 kb mZP3 promoter was previously shown to express the reporter gene, firefly luciferase, in the same temporal and spatial patterns as endogenous mZP3 during mouse development (Lira et al., 1990). By using this promoter, it was anticipated that antisense Oct-4 RNA should only be produced in growing oocytes of transgenic mice and thereby, provide a means to suppress the synthesis of maternally derived Oct-4 proteins. Ultimately, the goal was to gain an insight into the role of maternal (oocyte-derived) Oct-4 mRNA in early mouse development.

One concern in the above approach was that of the efficiency of the mZP3 promoter. Although the 6.5 kb of the mZP3 5'-flanking region was shown by Lira et al. (1990) to correctly direct spatial and temporal expression of mZP3, the firefly luciferase protein level was very low in these transgenic mice at about 2.2 pg per fully grown oocyte (Lira et al., 1990). For comparison, it has been estimated that each fully grown mouse oocyte contains >100 pg of actin, >250 pg of tubulin, >200 pg of lactate dehydrogenase (LDH), and >1 ng of endogenous mZP3 for assembly into the zona pellucida (Wassarman, 1983). Although the steady level of luciferase mRNA in transgenic mice was not measured, it is possible that the rate of transcription of the transgenic construct was very low. In this context, it has been suggested that genomic constructs, or constructs containing introns, are expressed up to 10- to 100- fold more

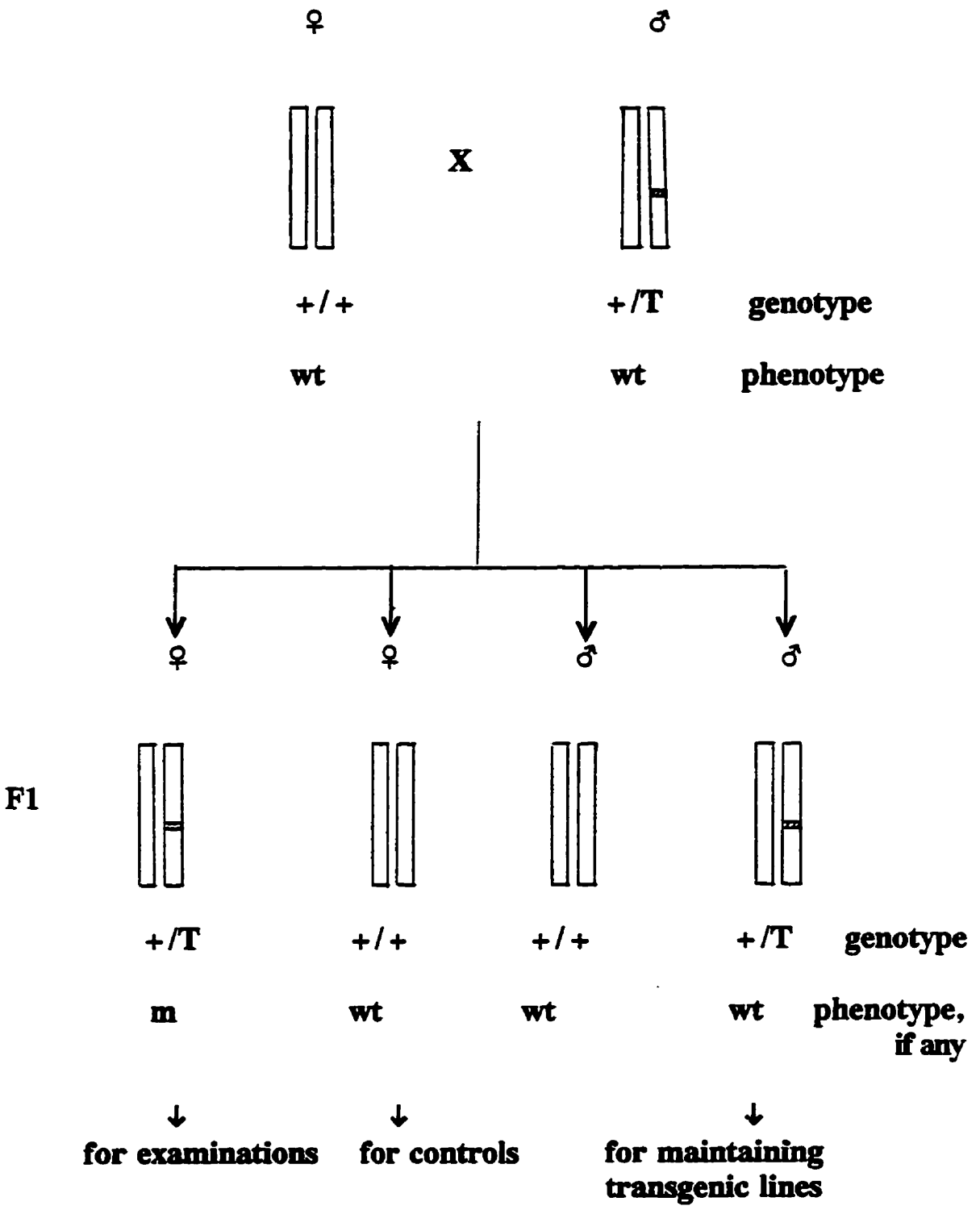
efficiently than identical constructs lacking introns (Brinster et al., 1988). Explanations for such improvement include the ideas that introns contain transcriptional enhancers or that introns contain sequences that facilitate opening of chromosomal domains during transcription. Heterologous introns have also been demonstrated to enhance expression of transgenes in mice (Palmiter et al., 1991). Katsuki et al., (1988) used the rabbit β-globin second intron to enhance myelin basic protein antisense cDNA expression in transgenic mice and successfully converted the normal to a shiverer phenotype. In a model system using the mouse metallothionein promoter-rat growth hormone gene construct (mMT-rGH), it was shown that insertion of heterologous introns such as the human β-globin second intron between the mMT promoter and the rGH gene was able to improve the expression of the rGH in transgenic mice (Palmiter et al., 1991). Thus, to try to achieve high levels of antisense Oct-4 RNA expression so as to inhibit the function of Oct-4 mRNA efficiently, I also inserted the human β-globin second intron between the mZP3 promoter and the antisense Oct-4 cDNA in the transgenic construct (Fig. 13).

In addition to transgenesis by pronuclear injection, a widely applicable approach for identification of gene function is gene targeting and screening for the products of homologous recombination (gene "knock-out"). This offers the most precise and effective method for extinction of gene activity. By abolishing its function, the role of a gene during mouse development can sometimes be established. However, when this project was initiated, we did not have the technical capability to create null mutations through homologous recombination using embryonic stem cells. Moreover, the approach we

chose has the advantage that any transgenic mice generated should express the antisense gene sequences solely in oocytes. Knocking out critical developmental control genes may result in embryonic lethality and thus result in failure to obtain transgenic lines. Similarly, by knocking out critical genes controlling gamete production, the transgenic mice may be sterile. In either situation, it would be difficult to generate transgenic lines and to be able to assess the developmental role of such genes. In contrast, expression of transgenes by using the mZP3 promoter circumvents this problem (Fig. 22). Even if the gene is very important for reproduction, infertility would only be observed in female transgenic mice because the mZP3 promoter is only expressed in oocytes. Male transgenic mice, on the other hand, should always have a wild type phenotype. These male transgenic mice can, therefore, be used to preserve the transgenic lines by mating with normal female mice. In such crosses, half of the offspring should carry the transgene as a heterozygote and could potentially be used for studies in assessing the role of the maternal factor, while the normal littermates would serve as controls. This approach, therefore, was applicable for functional studies of any genes expressed during oogenesis that are critical for reproductivity and early development.

When the work described in this thesis was still in progress, Richards et al. (1993) used exactly this approach with the mZP3 promoter to express antisense RNA directed against maternal mRNA encoding t-PA in transgenic mice. The t-PA mRNA was decreased up to 84% in the primary oocytes and up to 60% in mature eggs of homozygous transgenic mice. The t-PA enzyme activity was decreased by 60% in mature eggs of homozygous transgenic mice. Their experiments indicated that the strategy was

Fig.22. A strategy for transgene expression using the mZP3 promoter in transgenic mice. Male transgenic mice should have a wild type phenotype. When a male transgenic mouse is intercrossed with a wild type female mouse, the progeny will include transgenic female mice, transgenic male mice and normal (wild-type) littermates. Transgenic female mice should be the only individuals affected by transgene expression (mZP3 promoter; oocyte-specific). Transgenic male mice, although carrying the mutation, should not express the transgene and should be fertile for the purpose of maintaining the transgenic line. wt, wild type; m, mutant phenotype; +, wild type genotype; T, transgenic genotype.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potentially applicable to studies of murine Oct-4 mRNA during oogenesis and early development. The results obtained in this study showed that transgenic mice expressing antisense Oct-4 were not sterile and did not result in embryos that died early in development. Thus, the strategy to maintain the mutation via male mice was not really required. On the positive side, lack of a lethal phenotype allowed breeding of the line to homozygosity to facilitate experimentation.

§4.3 Expression of Oct-4 Antisense mRNA in Transgenic Mice

The expression of antisense Oct-4 transgene was investigated by RT-PCR. Antisense RNA-specific PCR primers were designed to amplify a sequence spanning the antisense Oct-4 cDNA and SV40 polyadenylation sequence upstream of the AATAAA signals (Fig. 15). Thus, any cDNA reverse transcribed from the endogenous Oct-4 mRNA could not be amplified. RT-PCR was carried out using RNAs extracted from ovaries and various other tissues of transgenic mice. As expected, antisense Oct-4 RNA was restricted to the transgenic mouse ovaries (Fig. 16A and Fig. 17) and within ovaries, the antisense RNA was localized in the oocytes (Fig. 16B). These results are consistent with the oocyte-specific activity of the mZP3 promoter. The oocyte-specific expression of the transgene represents a critical feature of this approach.

The level of antisense Oct-4 RNA within total ovarian RNA was very low. RT-PCR products of antisense Oct-4 RNA were not detected by ethidium bromide staining of the transgenic mouse ovary samples. The expression was only detected with

radioactive RT-PCR or RT-PCR coupled with southern blot hybridization. Perhaps this is not unexpected since expression of antisense Oct-4 is restricted to oocytes and oocytes make up only a very small component of the total cellular mass of the ovary. When RNA was extracted from oocytes of superovulated homozygous transgenic mice and used for RT-PCR, the intensity of the signal was markedly increased (Fig. 16B). Nevertheless, the level of Oct-4 antisense RNA was still low when compared to that of the endogenous Oct-4 mRNA in mouse ovary RNA samples (Fig. 10).

The low level of antisense Oct-4 RNA observed can be attributed to two factors. First, the efficiency of the antisense Oct-4 RNA-specific PCR primers was low. This was suggested by the results of radioactive RT-PCR or southern blot analysis. Zioprime G and Zioprime H did not only amplify the 291 bp target sequence, but also produced a 250 bp non-specific band (Fig. 16A and Fig. 17A). Similarly, Zioprime I and Zioprime J also amplified several minor non-specific products in addition to the major 339 bp specific band (Fig. 17B). The low efficiency of the antisense Oct-4 RNA-specific primers was directly demonstrated by PCR using genomic DNA of transgenic mice as a template. The amount of PCR products generated from either Zioprime G and H or Zioprime I and J were at least 10 times lower than from the Oct-4 primers when the same amount of DNA template was used (data not shown). The second factor, and probably the major factor, for the low level of the Oct-4 antisense RNA is the nuclease-mediated degradation of the antisense RNA. Double-stranded-specific nuclease activity is present in mouse oocytes (Strickland et al., 1988). Thus, when antisense Oct-4 RNA forms a duplex with the endogenous Oct-4 mRNA, it is expected that it will be rapidly degraded by the

double-strand-specific nuclease. Because of this, it has been commonly observed by several investigators that antisense transcripts are often extremely difficult to detect (Colman, 1990). In many cases, the levels of antisense transcripts are barely detectable even when a profound antisense gene effect was observed (Kim and Wood, 1985; Nishikura and Murray, 1987; Mercola et al., 1989). Thus, the low steady-state level of antisense RNA indicates, in a sense, that the antisense RNA is functioning.

The important test in an antisense experiment is to demonstrate that the target gene product is reduced in abundance. Therefore, the level of endogenous Oct-4 mRNA was quantitated to assess the degree of antisense inhibition. By using quantitative RT-PCR, the numbers of the copies of Oct-4 mRNA in oocytes derived from superovulated normal or transgenic mice were determined (Fig. 19). In five separate experiments, the average copy numbers of Oct-4 mRNA in normal and transgenic mouse oocytes were found to be approximately 800 and 310 per oocyte, respectively. Thus, antisense RNA expression resulted in 61% reduction in the Oct-4 mRNA level. Some variability was observed during quantitation, which is perhaps due to tube-to-tube variations in the RT-PCR procedures when the external standard method is used. The results could also be influenced by biological variations from one animal to the next.

The average copy number of 800 per oocyte of normal CD1 mice places Oct-4 in the moderately abundant class of transcripts (10 to 1,000 copies per cell). Lewin (1987) defined the abundant class of mRNA to be present at $> 1{,}000$ copies per cell and the low abundance class of mRNA to be present at < 10 copies per cell. Other molecules that fall into the same class as Oct-4 transcripts include interleukin-7 mRNA, which has

544 copies per oocyte (Rothstein et al., 1992), and MHC class I H-2K^q mRNA, which has 71 copies per oocyte (Arcellana-Panililio and Schultz, 1994). For comparison, each mouse unfertilized oocyte contains approximately 6-8 copies of IGF-II ligand and IGF-I receptor mRNA (Schultz et al., 1993), 4.1 X 10^3 copies of t-PA mRNA (Rothstein et al., 1992), 2.1 X 10^4 copies of β-actin mRNA (Taylor and Piko, 1990), 1.67 X 10^5 copies of histone RNA (Graves et al., 1985), and 9.6 X 10^5 copies of U1 SnRNA (Lobo et al., 1988). Overall, the level of Oct-4 transcripts in oocytes is relatively low, although it is higher than that of some growth factor transcripts such as those encoding IGF-II ligand and IGF-I receptor.

The 69% reduction in the Oct-4 mRNA level achieved in my transgenic mice compares favorably to reductions in t-PA mRNA observed by Richards et al. (1993) when a similar antisense transgenic approach employing the mZP3 promoter was used. In the study by Richards et al. (1993), two independent (founder) transgenic lines were established, one of which exhibited a 40% reduction of oocyte t-PA mRNA, the other a 60% reduction in tPA mRNA level. However, because the unfertilized mouse oocyte contains more t-PA mRNA than Oct-4 mRNA (Rothstein et al., 1992; this study), a higher level of antisense t-PA gene expression was required for a 60% or 40% reduction of t-PA mRNA than that of antisense Oct-4 gene expression needed for a 69% disruption of Oct-4 mRNA. It is very common that the levels of antisense inhibition vary from one experiment to another. A survey of 17 reports for which quantitative data were available indicated a degree of inhibition by antisense gene expression ranging from 0 to 99% (van der Krol et al., 1988).

The variabilities in the expression levels of antisense genes observed in these experiments may be due to chromosomal position of insertion or to variable numbers of transgene copies inserted into the host genome. Chromosomal position can influence expression due to location of heterochromatic domains or accessibility of transcription factors to the gene of interest (Palmiter and Brinster, 1986; van der Krol et al., 1988). Because of these variabilities, it is desirable to carry out studies on transgenic mice of more than one line. In my case, the first transgenic founder was identified within the first 20 mice born following pronuclear microinjection of the construct and embryo transfer. Unfortunately, in several other sets of microinjections, no further founder mice (out of 128) were generated. The same construct, purified in the same way, was used throughout and the reason for the low success rate in achieving transgenics (technical or biological consequences of the construct) is not clear. Because of the time and expense involved, a decision was made to proceed with work on the one transgenic line available. Clearly, the antisense gene is expressed, as expected, in an oocyte-specific manner and Oct-4 mRNA and protein levels were reduced. In the absence of other lines we will not, however, be able to rule out whether other putative lines might have led to higher levels of expression and/or altered phenotype.

The reduced level of Oct-4 mRNA was directly reflected in the Oct-4 protein level. Immunoprecipitation indicated that a 53% reduction in the synthesis of Oct-4 protein in oocytes expressing antisense Oct-4 RNA (Fig. 20). An interesting aspect observed in the experiments of measuring the relative protein levels in oocytes was that oocytes from normal mice always incorporated more radiolabelled precursor (about 20%

higher in radioactivity) than those from transgenic mice when the same number of oocytes were used in each experiment. It seems that the normal oocytes were metabolically more active than transgenic oocytes. In another set of experiments, fibroblasts cultured from pieces of skin from the transgenic mice also grew more slowly than typical fibroblasts (unpublished observations). These transgenic fibroblasts were also morphologically different, i.e. flatter than typical fibroblast cells. There were fewer cells per unit area (D. Nickel, personal communication). Because the mZP3 promoter is not active in fibroblasts, these phenotypes may result from insertional mutations. At the current time, we have not investigated this further.

§4.4 Role of Maternal Oct-4

As a first step to evaluate the role of maternal Oct-4 during oogenesis and early preimplantation development, histological examinations were carried out on ovaries from wild type and transgenic mice. The transgenic mouse ovaries were, in general, very similar to normal ovaries, except some of transgenic mouse ovaries contained many more small follicles than normal ovaries of the same estrous phase (Fig. 21). This occurred in 3 out of 8 transgenic animals examined. No wild-type ovaries contained this histologic characteristic. If the increased number of small follicles was in some way related to underexpression of the Oct-4 gene, these findings imply some role for Oct-4 during oocyte growth. Interestingly, Oct-4 mRNA is expressed in growing, but not resting oocytes in wild type mice (Rosner et al., 1990), consistent with its putative role in oocyte

growth. However, the increased number of small follicles was observed in only some of the transgenic mouse ovaries and may be reflective of individual variations in mice. A larger sample size is required before any conclusion can be drawn.

Another means to examine whether reduced Oct-4 levels had an effect on oocyte numbers was to simply count the number of oocytes produced following administration of PMS (FSH-like activity) and HCG (LH-like activity) to normal or transgenic females. The average oocyte yields were lower from homozygous transgenic mice (23.35 ± 5.83, mean$\pm$SD, $n=13$) than from wild type mice (26.44 ± 3.45, mean$\pm$SD, $n=13$), but statistically not significant. This slight decrease indicates that underexpression of maternal Oct-4 does not markedly affect oocyte numbers stimulated to grow and mature by the hormonal regime used for superovulation. In addition, these findings suggest that the histological preparations that indicated that there may be larger numbers of small follicles in transgenic mice are not reflected in altered numbers of mature ovulated oocytes following superovulation. Additional measurements of reproductive performance have revealed that transgenic female mice have normal fertility, normal gestation time, normal litter size and a 1:1 sex ratio. That is, under laboratory and vivarium conditions, they are indistinguishable from normal mice.

What do these results tell us? The simplest interpretation is that the reduced levels of Oct-4 mRNA and protein remaining in oocytes of transgenic mice are sufficient to allow normal function and development to proceed. The only real way to prove this is to obtain other transgenic lines that result in greater suppression of Oct-4 or to generate null mutants via targeted mutation. Unfortunately, we were not able to obtain more than

one founder transgenic mouse from the transgenic mouse facility (The University of Calgary) in the course of this experimental work. In addition, disruption of the Oct-4 gene by gene targeting has recently been carried out in a collaborative study between the laboratories of Dr. Austin Smith (University of Edinburgh) and Dr. Hans R. Schöler (EMBL). The work has not been published, but they have observed that embryos homozygous for null mutations for Oct-4 proceed through early cleavage and blastocyst formation but fail at the time of implantation due to defects in development of the inner cell mass and primitive ectoderm (A. Smith, personal communication). These results suggest a crucial role for zygotic Oct-4 gene expression in maintaining functional stem cells in the ICM and primitive ectoderm. However, because the mice were derived from crosses of heterozygous animals and the mutation is lethal, it is not possible to determine if maternal (oocyte) Oct-4 is also crucial for early developmental events. At the time the Oct-4 gene is transcribed during oogenesis, growing oocytes are 4N in chromosome complement and are in the dictyate stage of first meiotic prophase. Animals that are heterozygous would express Oct-4 from the normal copy of the gene.

Another possible interpretation for the lack of phenotype in the antisense transgenic mice is that other gene products can replace the functional role of Oct-4. That is, there could be redundancy. Redundancy is a prevalent phenomenon in mammals. For example, disruption of a tyrosine kinase gene, c-*src*, in mice did not reveal any defects in platelets and the nervous system where it was predominantly expressed (Soriano et al., 1991). In another example, disruption of the *tenascin* gene which encodes an extracellular matrix protein with a highly restricted expression pattern during mouse embryogenesis

did not lead to any abnormalities in mice and no compensatory changes were observed in other extracellular matrix proteins (Saga et al., 1992). Redundancy includes superfluous gene expression and duplicated function of related proteins (Erickson, 1993). Superfluous gene expression means that genes are expressed where they have no function at all. A possible explanation for superfluous gene expression is that gene expression (either transcription or translation) is very cheap from the perspective of cellular metabolism and it may be more economical for a cell to tolerate unnecessary gene expression rather to generate an additional control mechanism to turn it off (Erickson, 1993).

On the other hand, as a usual concept of redundancy, functional duplication of related genes can account for minimal phenotype when a supposedly important gene is disrupted (Joyner et al., 1991). In this context, two octamer binding proteins, Oct-4 and Oct-5, are expressed in mouse oocytes in addition to the ubiquitously expressed Oct-1 (Schöler et al., 1990a). However, the Oct-5 transcript is just a truncated form of Oct-4 mRNA (Schöler et al., 1990b). The antisense Oct-4 RNA generated from the transgene was designed to target both Oct-4 and Oct-5, and Oct-5 mRNA should be subjected to the same fate as Oct-4 when antisense Oct-4 RNA is expressed. No other octamer factors have been identified to be expressed in oocytes. However, it is possible that underexpression of the Oct-4 gene leads to up-regulation of a "back-up" gene by feedback mechanisms. An example of this is the *Myo-D* gene "knock-out" in which inactivation of *Myo-D* in mice leads to up-regulation of the myogenic gene *Myf-5* (Rudnicki et al., 1992). A large family of octamer-binding proteins have been identified

in the mouse (Schöler et al., 1989). One of them might possibly compensate for a reduction of Oct-4 in mouse oocytes. Studies to examine whether levels of other octamer binding proteins were altered in the antisense Oct-4 transgenic mice were not carried out.

If maternal Oct-4 has a function during oogenesis and early embryogenesis, which genes does it regulate? Studies from several laboratories have identified some genes encoding growth factors or peptide hormones as the putative Oct-4 downstream genes. Expression of these genes can either be stimulated (such as kFGF and PDGF-α receptor) or suppressed (such as HCG β subunit) by Oct-4 in cultured EC cells or choriocarcinoma cells (Yuan et al., 1995; Kraft et al., 1996; Liu and Roberts, 1996). Among these putative Oct-4 downstream genes, regulation of the kFGF gene by Oct-4 has been best illustrated (Schoorlemmer and Kruijer, 1991; Dailey et al., 1994; Yuan et al., 1995). kFGF (also called FGF-4) encodes a member of the fibroblast growth factor (FGF) family and was originally identified as an oncogene (Delli Bovi et al., 1987). It is expressed in preimplantation embryos and later in distinct embryonic tissues (Niswander and Martin, 1992; Rappolee et al., 1994). kFGF is also expressed in undifferentiated embryonic stem (ES) cells and EC cells, but not in their differentiated counterparts (Velcich et al., 1989). Activation of kFGF gene transcription in F9 cells depends on the synergistic action of Oct-4 and a member of Sry-related Sox family, Sox2, through an enhancer within the 3' noncoding region of the gene (Yuan et al., 1995). It remains unclear whether Oct-4 regulates kFGF gene expression in mice.

In summary, our results indicate that reduced levels of Oct-4 mRNA and protein in oocytes do not significantly affect the numbers of oocytes or embryos produced, nor

the number that go on to implant and yield live new-born pups. This implies that the depressed levels of Oct-4 protein present in the transgenic mice may be sufficient to promote early cleavage events, at least in the intact animal. Perhaps there are other factors within oocytes that can partially replace the functional role of Oct-4 in this way. If a major role of Oct-4 is to regulate the kFGF gene expression, then one might expect major disturbances in development just after implantation within Oct-4 "knock-out" mice (as observed by A. Smith and H. Schöler). This is the time when kFGF appears to exert important effects on mouse early embryo development (Rappolee et al., 1994). Dominant-negative mutations for FGF receptors that inhibit FGF signalling slow the growth of primitive ectoderm-like cells but not parietal endoderm-like cells in the implanting blastocysts (Rappolee et al., 1994). kFGF null mutants die shortly after implantation, apparently due to failure of proliferation of embryonic ectoderm (Feldman et al., 1995). One might speculate that Oct-4 expression (and in this case, Oct-4 expressed from the zygote genome of totipotent embryonic cells) is important for regulation of kFGF expression and that FGF signalling, in turn, is required for primitive ectoderm growth. Perhaps, when the work of Smith and Schöler is published, it will shed some light on this issue. Because kFGF is found as a maternal transcript and the sequential expression of the Oct-4 and kFGF genes coincides in the preimplantation stage of mouse development (Rappolee et al., 1994), one could also speculate that maternal Oct-4 may also play a role in the regulation of kFGF gene expression, particularly in kFGF zygotic gene activation. Because the Oct-4 null mutation is lethal, this latter question can only be answered by methods of the type used in this thesis that lead to

inhibition of Oct-4 expression selectively in oocytes. Unfortunately, the degree of inhibition achieved in this study was insufficient to lead to a firm conclusion. The problem with antisense methods is that it is difficult, as shown in the studies herein, to completely extinguish expression of a gene through this approach. If there is still a small amount of the protein end-product of the gene produced, it may be sufficient to mask an expected phenotype. Thus, new approaches to blockade of expression in oocytes will likely need to be developed to answer questions of the kind proposed in this thesis.

Clearly, the exact role of Oct-4 in oogenesis and early development still remains to be established. Nonetheless, we have acquired additional information on levels of expression of the Oct-4 during oogenesis and early development and have established that reducing the level of Oct-4 protein in mature oocytes by 53% through antisense techniques does not have serious consequences for early development. Additional molecular genetic approaches should help to identify the functional role of Oct-4 in the early developmental process in the years to come.

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