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Functional role of high mobility group proteins 14 and 17 during early mouse development

by

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A thesis submitted to the Faculty of Graduate Studies and Research in a partial fulfillment of the requirements of the degree of Master of Science.

Dedication

To my Parents, my Family
and my beloved Country
Libya

إهداء

إلي والدي و أهلي

و وطني العزيز .... ليبيا

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Abbreviations

A.....adenine
BSA.....bovine serum albumin
C.....cytosine
CAT.....chloramphenicol acetyl transferase
cDNA.....complementary deoxyribonucleic acid
CMV.....cytomegalo virus
DIG.....digoxogenin
DNA.....deoxyribonucleic acid
EGA.....embryonic genome activation
eLF-4e.....eukaryotic initiation factor-4e
En-1&2.....engrailed 1&2
fg.....femtogram
G.....guanine
GFP.....green fluorescent protein
GV.....germinal vesicle
hCG.....human chorionic gonadotropin
HIV.....human immunodeficiency virus
HMG.....high mobility group
hsc.....heat shock complex
hsp.....heat shock protein
KD.....kilodalton
Kr.....Kruppel
mRNA.....messenger ribonucleic acid
PCR.....polymerase chain reaction
PMSG.....pregnant mares serum gonadotropin
RNA.....ribonucleic acid
rRNA.....ribosomal ribonucleic acid
RSV.....rous sarcoma virus

RT-PCR.....reverse transcription polymerase chain reaction

t-PA.....tissue plasminogen activator

T.....thymine

TK.....thymidine kinase

TRC.....transcription requiring complex

tRNA.....transfer ribonucleic acid

ZP.....zona pellucida

Abstract

Following fertilization, the embryo undergoes a sequence of precisely timed cleavage cycles to produce a blastocyst. The timing of these cycles likely depends in part on appropriate levels of gene activity. I have investigated whether high mobility group (HMG) proteins 14 and 17, which are associated with chromatin containing transcribed genes, are expressed in mouse embryos and are required to maintain normal early developmental timing. As assayed using RT-PCR, mRNAs encoding both HMG-14 and HMG-17 were present throughout preimplantation development to the blastocyst stage. By immunofluorescence, both proteins were detected in the nuclei of prophase I-arrested oocytes and embryos begining at the 2-cell stage. To investigate their function, antisense oligonucleotides targeting the 5' end of each mRNA species were injected into 1-cell stage embryos which were then cultured to develop to the blastocyst stage. At the 2- and 4-cell stages, only weak nuclear immunofluorescence was observed; however, by the 8-cell stage, the staining pattern of injected embryos was indistinguishable from controls. Thus, the injected antisense oligonucleotides transiently depleted the cellular supply of HMG-14 and HMG-17. Furthermore, the embryos in which both HMG-14 and HMG-17 had been depleted progressed significantly more slowly through successive stages of preimplantation development, as compared with embryos in which the proteins were individually depleted or injected with nonsense oligonucleotides. Therefore, it can be concluded that depletion of HMG-14 and HMG-17 from embryonic chromatin transiently delays preimplantation development, demonstrating a crucial role for these proteins in maintaining the normal temporal co-ordination of development.

Résumé

Suivant la fertilisation, l'embryon de 1-cellule entreprend une série de cycles de division. La durée de ces divisions est connue et dépend probablement en partie d'un niveau approprié d'activité de la part des gènes. Les protéines HMG-14 et HMG-17 (*High Mobility Group*) sont associées avec la chromatine contenant des gènes qui sont transcrits. Nous avons donc décidé d'étudier la possibilité que ces protéines soient exprimées dans les embryons de souris et qu'elles soient requises pour maintenir une durée normale des premiers stades de développement. En utilisant le RT-PCR, nous avons trouvé que les ARN messagers encodant les protéines HMG-14 et HMG-17 sont présents à travers les stades de pré-implantation jusqu'au stade de blastocyste. Par immunofluorescence, nous avons détecté ces deux protéines dans les noyaux d'ovocytes arrêtés en prophase I et dans les noyaux d'embryons au début du stade 2-cellules. En vue d'étudier la fonction de ces protéines, nous avons injecté des oligonucléotides antisens, ayant pour cible le bout 5' de chaque espèce d'ARN messager, dans des embryons au stade 1-cellule. Nous avons ensuite mis ces derniers en culture pour se développer jusqu'au stade de blastocyste. Un faible signal nucléaire d'immunofluorescence a été détecté aux stades 2 et 4-cellules; cependant, au stade 8-cellules, le signal était identique à celui des contrôles. Alors, les oligonucléotides antisens injectés ont épuisé, pour un certain temps, la quantité de protéine HMG-14 et HMG-17 présente dans les cellules. De plus, les embryons dont on a éliminé la quantité conjointe de HMG-14 et HMG-17 progressaient à travers les stades successifs de pré-implantation de manière significativement plus lente que les embryons dans lesquels une ou l'autre des protéines a été individuellement éliminée ou alors ceux qui ont été injectés avec des oligonucléotides non-sens. Nous pouvons donc conclure que l'élimination transitoire de HMG-14 et HMG-17 de la chromatine embryonnaire retarde le développement pré-implantatoire, démontrant ainsi un rôle crucial pour ces deux protéines dans le maintien normal de la coordination des premiers stades de développement.

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CHAPTER 1
INTRODUCTION

1.1 Oogenesis

The ovum, or the egg, is the bridge that connects one generation to the next. Life starts on one side with the formation of a mature egg and on the other side with the formation of a mature sperm. The sperm and the egg then meet to form the new embryo which will eventually give rise to the fully grown organism. It is the egg that is responsible for supplying most, if not all, the materials required by the embryo during its early stages of development (Howlett and Bolton, 1985). The process of development of the egg is termed oogenesis. In mammals, oogenesis begins early in fetal development, continues for up to months or years, in some species, and ends later in the sexually mature adult (Peters, 1969). Oogenesis begins with the formation of primordial germ cells which have an extragonadal origin (Chiquoine, 1954; Clark and Eddy; 1975; Eddy et al., 1981). In mouse, at day 8 of embryonic development there are as few as 15 and as many as 100 primordial germ cells found in the yolk-sac endoderm and the region arising from the primitive streak (Wassarman, 1988). By day 9 of embryonic development, these primordial germ cells have migrated into the endodermal epithelium of the hindgut, and by day 10 to 11 of embryonic development, these primordial germ cells have reached the dorsal mesentery of the genital ridges which is the site of the gonadal development (Wassarman, 1988).

As a result of continuous mitotic divisions, the number of primordial germ cells, which are the sole source of the germ cells, reaches 5,000 in day 11 to 12 and 20,000-25,000 in day 13 to 14 of mouse embryonic development (Wassarman, 1988). At day 13, migration of the primordial germ cells is complete and in the female all of the primordial germ cells have become actively dividing oogonia in the sex cords (Wassarman, 1988). Around this time, some of the oogonia transform to oocytes by stopping mitotic divisions and entering meiosis, where they arrest at the diplotene stage of the prophase of the first meiotic division (Bakken and McClanahan, 1978;

Bachvarova et al., 1982). By about day 17, the ovary contains only oocytes at various stages of the first meiotic prophase stage (Bakken and McClanahan, 1978; Bachvarova et al., 1982). This pool of small (12-15 μm in diameter), non growing oocytes is what gives rise to the differentiated unfertilized eggs in the sexually mature adult.

1.2 Oocyte growth

Shortly after birth, in mouse, some of these non growing oocytes, by a process apparently regulated within the ovary (Krarup et al., 1969), begin to grow, while remaining arrested in the first meiotic prophase stage. The size of the oocyte in this growth phase increases from 12 μm in diameter to a final diameter of 80 μm to become one of the largest cells of the body (Wassarman, 1988). During this growth phase, oocytes synthesize and accumulate large amounts of RNA (mRNA, rRNA and tRNA), proteins, lipids, and organelles (e.g., ribosomes, mitochondria, etc.), which constitute the maternal contribution towards early development. It has been estimated that fully grown oocyte contains about 200 times more RNA (Olds et al., 1973; Sternlicht and Schultz, 1981; Kaplan et al., 1982), 1,000 times more ribosomes and 50-60 times more proteins (Lowenstein and Cohen, 1964; Brinster, 1967; Schultz and Wassarman, 1977) than a typical mammalian somatic cell does (Wassarman, 1988). In mouse, an oocyte completes growth within 2 to 3 weeks (Wassarman, 1988) and during this oocyte growth phase, the nucleus, or the germinal vesicle (GV) increases in diameter from 9 μm in small oocyte to 22 μm in fully grown oocyte (Chouinard 1975; Wassarman and Josefowicz, 1978).

When the female is sexually mature, which is around 6 weeks in mouse, and under the influence of sexual hormones, some of these fully grown oocytes undergo a process termed meiotic maturation, which converts them to mature eggs ready for fertilization. During meiotic maturation, which occurs just prior to ovulation, the fully grown oocytes stop transcription, break the meiotic arrest and complete the first meiotic reductive division, by extruding the first polar body, and advance to the metaphase II stage of meiosis. Mature eggs arrested at the metaphase II of meiosis are transcriptionally inactive (Wassarman and Letourneau, 1976; Brower et al., 1981;

DeLeon et al., 1983) and it is in this state that they are finally released from the follicles and enter the oviduct until stimulated to complete meiosis after fertilization.

1.3 Fertilization

When mating occurs, approximately 60 million sperms are ejaculated into the female reproductive tract but only one sperm will be able to penetrate the surface of the egg. In order for the sperm to reach the surface of the egg it has to first penetrate the cumulus mass and then the zona pellucida of the egg. Binding of the sperm to the zona pellucida is highly species-specific, ensuring that only sperm from the same species can get its way inside the egg. Sperm head and part of the sperm tail then fuse into the cytoplasm of the egg resulting in what is called fertilization of the egg. Sperm binding to the zona pellucida initiates what is termed the zona reaction, which alters the structure of the zona pellucida in a way that inhibits the entrance of more sperm into the egg, thus preventing polyspermy.

Fertilization triggers the resumption of meiosis of the egg chromosomes, in which the egg completes meiosis II and extrudes the second polar body. In mouse, completion of meiosis and extrusion of the second polar body occurs between 2 to 5 hours after fertilization. The male and the female pronuclei are formed within 4 to 9 hours after fertilization, where they then start to move toward the center of the egg. DNA replication takes place 11 to 18 hours after fertilization, during the migration of male and female pronuclei towards the center of the egg. The two pronuclei migrate close to each other but do not fuse; instead, when the nuclear membranes break down at mitosis, the maternal and paternal chromosomes assemble together on the spindle. The first cleavage takes place between 17 to 20 hours after fertilization (Howlett and Bolton, 1985).

All these processes of pronuclear formation, DNA replication, and cytokinesis of the first cleavage division are sustained and directed by proteins and RNAs synthesized during oogenesis and stored in the egg (Howlett and Bolton, 1985), and it is only after mid-two-cell stage, 27 hours postfertilization, that the major transcriptional activation of

the embryonic genome takes place in the mouse (Flach et al., 1982; Sawicki et al., 1982; Bensuade et al., 1983; Latham et al., 1991; Manejwala et al., 1991; Schultz, 1993).

1.4 Embryonic Genome Activation (EGA)

Transcriptional activation of the embryonic genome (embryonic genome activation; EGA) is a critical step the embryo has to accomplish during its early development, in which a transition from maternal to embryonic control of development takes place. Degradation of the maternal RNA begins during the oocyte maturation and its essentially complete by the 2-cell stage in the mouse (Bachvarova and DeLeon, 1980; Bachvarova et al., 1985, 1989; Paynton et al., 1988); thus new embryonic transcripts must appear by this stage of development, in order to provide what will be needed for upcoming events of embryogenesis.

In mouse, several lines of evidence indicate that the major phase of EGA occurs at G2 phase of the 2-cell stage (Flach et al., 1982; Sawicki et al., 1982; Bensuade et al., 1983; Latham et al., 1991; Manejwala et al., 1991; Schultz, 1993). More recent experiments, however, have shown that minor embryonic transcription may start as early as G2 phase of the 1-cell embryos (Vernet et al., 1992; Ram and Schultz, 1993; Matsumoto et al., 1994; Christians et al., 1995; Bounil et al., 1995). Thus, embryonic genome activation has been described as a sequence of two phases of transcriptional activity, the minor EGA (Vernet, et al., 1992, Schultz, 1993) and the major EGA (Flach et al., 1982; Howlett, 1986; Vernet et al., 1992; Schultz, 1993) phase, which lead to the transition from maternal to embryonic control of development by the end of the 2-cell stage. The first phase of EGA is independent of the first round of DNA replication (Flach et al., 1982; Bolton et al., 1984; Howlett, 1986; Schultz, 1993), while the second phase is dependent on the first round of DNA replication (Howlett, 1986).

Interestingly, the existence of two phases of EGA suggests that these could be regulated by two different mechanisms. Since there is no transcription prior to the minor phase of EGA, its regulation presumably depends on post-transcriptional and post-translational modifications of maternally derived RNAs and proteins. For example, post-transcriptional mechanisms, such as mRNA processing (Cascio and Wassarman, 1982)

could give rise to novel proteins that may be involved in initiating the minor phase of EGA, and post-translational mechanisms such as phosphorylation (Van Blerkom, 1981; Howlett, 1986) might directly or indirectly regulate the activity of the RNA polymerase resulting in initiation of the minor phase of EGA (Poueymirou and Schultz, 1987, 1989; Latham et al., 1992; Schwartz and Schultz, 1992; Schultz, 1993). In the case of the major phase of EGA, regulatory events that control the selectiveness of the RNA polymerase seem to operate as indicated from experiments showed the requirement of enhancer sequences and the occurrence of stage-specific gene expression (Martinez-Salas et al., 1989; Wiekowski et al., 1991; Rothstein et al., 1992; Majumder et al., 1993). The mechanism of selectiveness of the RNA polymerase and how it is initiated, however, are still not understood.

The timing of EGA, as indicated by experiments done to study the synthesis of a complex of proteins called the transcription-requiring complex (TRC), which are only transiently expressed in the 2-cell stage of mouse embryo (Conover et al., 1991; Latham et al., 1991) appears to be more related to the time elapsed from fertilization rather than to cell cycle staging. In these experiments, when the cytokinesis in 1-cell embryos was inhibited using cytochalasin D or an activator of protein kinase C, the TRC appeared essentially at the same time in these cleavage-arrested 1-cell embryos as in normal 2-cell embryos (Poueymirou and Schultz, 1987). Similarly, when DNA synthesis was inhibited by aphidicolin in the 1-cell embryos, the TRC was synthesized at the same time in these arrested 1-cell embryos as in control 2-cell embryos (Howlett, 1986; Poueymirou and Schultz, 1987). Thus, minor phase of EGA seems to be regulated by an internal zygotic clock (Wiekowski et al., 1991) that monitors the time after fertilization rather than the developmental stage of the embryo such as cell number. However, the identity of this zygotic clock and its mechanism of controlling EGA are not understood.

In mouse, although, the first cleavage division is entirely driven by maternal materials stored in the egg and does not require transcription, subsequent cleavages do require transcription and embryonic gene activity is absolutely required for the embryo to develop to further stages (Golbus et. a., 1973; Bolton et al., 1984; Poueymirou and Schultz, 1989). Despite the fundamental importance of successfully passing this critical

step of development, very little information is available about the molecular events involved in the regulation of initiation and maintenance of EGA and the identity of factors that are involved.

1.5 Cleavage divisions

After initiation of the EGA, the embryo follows a series of cleavage divisions (Figure 1) in a temporally controlled manner, during which the embryo progresses from one stage to the next within a relatively defined time that is specific for each stage. The temporal control of the cleavage divisions depends on the transcriptional activity of the new embryo. When transcription is completely blocked by drugs, progression beyond the 2-cell stage is inhibited (Golbus et al., 1973; Bolton et al., 1984; Poueymirou and Schultz, 1989). In the same way that transcription, and thus embryonic genome activity, is necessary for the embryo to develop beyond the 2-cell stage, maintaining transcriptional activity is also necessary for the embryo to successfully progress through the subsequent cleavage divisions (Clarke et al., 1992). Failure to maintain the normal rate of transcription during these series of cleavage divisions may result in growth retardation of the embryo and disruption of the temporally controlled pattern of development.

The overall goal of this research project is to identify the factors and mechanisms that control and maintain the embryonic genome activity in the early mouse embryo. The particular focus of the research reported here was to analyze the function of a pair of proteins called the high mobility group (HMG) proteins 14 and 17. HMG-14 and HMG-17 proteins are non-histone proteins that are associated with the chromatin (Sandeen et al., 1980; Mardian et al., 1980; Albright et al., 1980) and are thought to enhance transcriptional activity (Crippa et al., 1993; Ding et al., 1994; Trieschmann et al., 1995; Paranjap et al., 1995). They are described further in the following sections.

1.6 Chromatin structure and regulation of transcription

Chromatin is the term that describes DNA and its associated proteins. Regulation of expression of the genetic information encoded in DNA is determined by

different and precise interactions between the DNA and the protein components of the genome (Cartwright et al., 1982; Igo-Kemenes, 1982). Proteins that contribute to chromatin structure and function can be categorized into three general classes of DNA-associated proteins. The first category represents the histones, which are the predominant class of chromosomal proteins. These proteins are highly abundant and conserved between different species and their role is to condense the DNA into a more compact structure. However, they do not have sufficient diversity to identify and specifically bind to selected DNA sequences in the genome. The second category of chromosomal proteins includes the so called tight-binding proteins which interact directly and remain complexed with DNA in high salt and urea conditions. This category of proteins includes the enzymes involved in processes such as replication, transcription, repair, and recombination, and structural proteins which are part of the nuclear matrix (Barrack and Coffey, 1982) and the chromosome scaffold (Earnshaw et al., 1983). The third class of DNA-associated proteins is represented by the HMG chromosomal proteins. Although they are the most abundant and ubiquitous non-histone chromosomal proteins found in the nuclei of all higher eukaryotes (Einck and Bustin, 1985), they are much less abundant than the histones, as there are approximately 30 histone molecules per HMG molecule (Einck and Bustin, 1985). Some HMG proteins display a certain preference for binding to certain DNA conformations but, like the histones, have no sufficient diversity to identify specific DNA sequences for binding and subsequent gene-specific regulation (Einck and Bustin, 1985).

Most of the studies on the structure and function of chromatin have focused on interactions occurring between histones and DNA. Complex processes such as transcription, replication , recombination and repair, however, are facilitated by different interactions between variety of proteins present in the chromatin and the DNA and it is clear now that chromatin fiber serves not only to package the DNA into the nucleus but also to control the accessibility of the regulatory factors to their specific sequences in the DNA and to potentiate interactions between distant regulatory elements (Grunstein, 1990; Wolffe, 1994).

1.6.1 The high mobility group proteins

Among all non-histone chromosomal proteins, the HMG proteins are the largest and best characterized group. High mobility group proteins are defined as a family of nuclear proteins that are extractable from nuclei and chromatin with 0.35 M NaCl (Goodwin et al., 1979). These proteins are also soluble in 2-5% perchloric acid and have a molecular mass less than 30 kD (Bustin et al., 1990). Currently, HMG proteins are classified into three families: the HMG-1/-2 family, the HMG-I(Y) family and the HMG-14/-17 family.

HMG-1/-2 proteins are non-specific DNA-binding proteins. They are the largest (~ 25 kD) and most abundant of the high mobility group proteins, and they are highly conserved between species. Although their cellular function remains elusive, data suggests that these proteins play a role in the regulation of chromatin structure, replication, transcription and DNA repair processes (Bustin and Reeves, 1996).

The HMG-I(Y) family in mammals consists of three members: HMG-I, HMG-Y and HMGI-C. These proteins are smaller than the HMG-1/-2, where HMG-I is ~11.9 kD, HMG-Y is ~10.6 kD and HMGI-C is ~12 kD. HMG-Y is identical to HMG-I except for an 11-amino acid deletion in HMG-Y, and these are products of alternative mRNA splicing (Johnson et al., 1988; Johnson et al., 1989) of the same gene (Friedmann et al., 1993). HMGI-C is product of separate gene and has ~50% homology to HMG-I and HMG-Y (Manfioletti et al., 1991; Friedmann et al., 1993; Patel et al., 1994). The HMG- I/-Y proteins have been implicated in numerous aspects of chromatin activity, including nucleosome phasing, metaphase chromosome condensation, DNA replication, and 3`-end processing of mRNA transcripts (Bustin and Reeves, 1996). Direct evidence has been obtained for a role of HMG-I(Y) proteins in transcriptional regulation in vivo, either positive regulation such as in the case of the human genes coding for β-interferon (Thanos and Maniatis, 1992, 1993) and for the α-subunit of the interleukin-2 receptor (Reeves et al., 1995) or negative regulation such as in the case of the genes coding for the human interleukin-4 (Chuvpilo et al., 1993) and the murine gene coding for the heavy chain embryonic ∈-immunoglobulin (Kim et al., 1995).

Recently, HMG-I(Y) proteins were found to be required for function of HIV-1 preintegration complexes in vitro (Farnet and Bushman, 1997).

HMG-14 and HMG-17 proteins represent the third member of the HMG proteins family. These closely related proteins have been found in nuclei of all higher eukaryotes (Giri et al., 1987). Avian erythrocytes contain two types of HMG-14 proteins - a major species, HMG-14a, and a minor species, HMG-14b (Srikantha et al., 1990). In contrast to the abundance of these two proteins in higher organisms, they have not been detected in yeast or other lower eukaryotes. The fact that HMG-14 and HMG-17 proteins are present in the tissues of all higher eukaryotes strongly suggest that this HMG family of proteins is necessary for proper cellular function. Additionally, since all cells contain both HMG-14 and HMG-17, it has been suggested that the two proteins may have distinguishable functions (Bustin and Reeves, 1996). Nevertheless, their exact cellular function and their mode of action are still not fully understood. Most of the data available, however, obtained from studies on their protein structure, their mode of interaction with the nucleosome cores, and their effect on the transcriptional potential of chromatin templates assembled under controlled conditions, suggest that HMG-14 and -17 help to regulate transcription (Crippa et al., 1993; Ding et al., 1994; Trieschmann et al., 1995; Paranjape et al., 1995).

1.6.1.1 Structure of the HMG-14 and HMG-17 genes

The genes for the human HMG-14 (Landsman et al., 1989b) and HMG-17 (Landsman et al., 1989a) and for the chicken HMG-14b (Srikantha et al., 1990) and HMG-17 (Landsman et al., 1988a), have been isolated and sequenced. In man, HMG-14 maps to the region 21q22.3 (Pash et al., 1990) which is associated with the etiology of Down's syndrome, whereas HMG-17 maps to the region 1p36.1 (Popescu et al., 1990) which is associated with some types of malignancies and neoplasia. The human HMG-14 and HMG-17 and chicken HMG-17 genes each consist of six exons and five introns. The chicken HMG-14b has no intron 2, meaning that exon II and III are fused together. The first exon of all the genes encodes a 5`-untranslated region as well as the first four amino acids of each protein. The final exon, which represents ~ 70% of the transcript,

codes for a 3`-untranslated region. Analysis of the promoter regions has revealed that HMG-14 genes lack a TATA box whereas HMG-17 genes possess two TATA boxes suggesting that there could be a transcription regulation difference between the two genes (Bustin et al., 1990).

1.6.1.2 Structure of the HMG-14 and HMG-17 transcripts

cDNA clones of mouse (Landsman et al., 1988b), chicken (Landsman and Bustin, 1987; Srikantha et al., 1988; Dodgson et al., 1988) and human (Landsman et al., 1986) HMG-14 and HMG-17 have been isolated and sequenced. In chicken, HMG-14 protein is present in two types, the major species HMG-14a which has higher molecular weight and a minor species HMG-14b which is thought to be the homologue of the mammalian HMG-14 (Srikantha et al., 1988). Each HMG-14 and -17 gene produces only a single species of mRNA as revealed by Northern analysis of different tissues from various species (Bustin et al., 1990). Comparing the HMG-14 and -17 mRNAs sequence between various species has revealed common characteristics of the transcripts. The mRNAs are similar in size, ranging between 1.2 and 1.25 kb. The 5`-untranslated regions are relatively short, highly G-C rich, and have no sequence similarities, whereas the 3`-untranslated regions are extremely long, have a very high A-T content, and are remarkably conserved among species (Bustin et al., 1990). Quantitation analysis of HMG-14 and HMG-17 mRNAs in several human cell lines revealed that HMG-14 mRNA is less abundant than that of HMG-17 in most of these cells, and the ratio of HMG-14 mRNA to that of HMG-17 mRNA is relatively constant (Crippa et al., 1990).

1.6.1.3 Structure of the HMG-14 and HMG-17 proteins

Sequence analysis of 12 known HMG-14 and HMG-17 proteins reveals structural motifs that are characteristic of this family of proteins (Schneider and Stephens, 1990). These two proteins have over 40% of their sequence in common and possess four regions of particular note. The first region consists of the first four amino acids of the N-terminus of the proteins, which is encoded by exon I and is absolutely

conserved among the HMG proteins (Bustin and Reeves, 1996). The second region is a 30-amino acid sequence that represents the DNA-binding domain of the proteins (Cook et al., 1989; Crippa et al., 1992). This region is encoded by exons III and IV and is highly conserved between the two proteins. The third region is five amino acids encoded within exon V that are also highly conserved among the HMG proteins. The fourth region is a stretch of eight amino acids region encoded within exon V, which represent the C-terminus of these two proteins. The protein region encoded by exon II is conserved among the HMG-17 family of proteins, but not among the HMG-14 proteins.

HMG-14 and HMG-17 are positively charged proteins. Analysis of the distribution of charged amino acids along their polypeptide chains indicates a conservation pattern between both proteins. Based on this category of analysis, HMG-14 and HMG-17 proteins can be subdivided into three regions. The first region is the N-terminal region of the proteins which has a slightly net positive charge of +1 in the case of HMG-14 and a net positive charge of +2 in the case of HMG-17. The second region is the central region with a net positive charge of +15 in the case of HMG-14, and a net positive charge of +16 in the case of HMG-17. The third region is the C-terminal region of the proteins with a net negative charge of -9 in the case of HMG-14, and a net negative charge of -3 for HMG-17 (Bustin and Reeves, 1996).

Furthermore, there seem to be a correlation between the structure of the HMG-14 and HMG-17 genes and that of their proteins. Interestingly, many of the conserved protein domains are encoded by the 3` end of distinct exons. For example, domain A, which represent the first conserved region of the proteins, is encoded by the 3` end of the genes exon I, the 3 amino acids of the N-terminal region of domain B is encoded by the 3` end of exon II , domain D, which represent the fourth conserved region of the proteins, is encoded by the 3` end of exon V, the conserved sequence of 109-111 amino acids is encoded by the 3` end of exon VI (Bustin and Reeves, 1996).

However, despite the fact that HMG-14 and HMG-17 are similar in many aspects and have many features in common, the structural analysis of the two proteins reveals a clear distinction between them. For example, the two proteins have less than 50% in their amino acids sequence in common, there is a higher degree of sequence

conservation among the members of each individual subgroup than between the two subgroups. Specifically, the homology among the HMG-17 family is over 91%, whereas the sequence homology among the HMG-14 family ranges from 49 - 94%. Finally, the C-terminal of the nucleosomal binding domain of the two proteins, which is encoded by exon IV, shows a marked difference between the two proteins. This structural difference between the two proteins as well as their co-existence in every tissue raises the possibility that the two proteins participate in specific interactions, each of which is necessary for proper cellular function (Bustin and Reeves, 1996).

1.6.1.4 Cellular localization of HMG-14 and HMG-17 proteins and their interaction with chromatin

HMG-14/-17 proteins have been detected exclusively in the nucleus, mostly associated with nucleosomes (Sandeen et al., 1980; Mardian et al., 1980; Albright et al., 1980). Immunofluorescence studies have localized HMG-14 and HMG-17 to the regions of polytene chromosomes, which are transcriptionally active regions of chromosomes (Weisbrod et al., 1980; Westermann and Grossbach, 1984, Disney et al., 1989). Furthermore, HMG-14 and HMG-17 proteins have been found to specifically recognize the 146-bp nucleosomal core particle (Sandeen et al., 1980; Mardian et al., 1980). Both proteins bind to the nucleosome cores without any specificity for the underlying DNA sequence, suggesting that these two proteins recognize structural features specific to the chromatin subunit. The binding sites of HMG-14 and HMG-17 have been mapped to a distance of 25-125 nucleotides from the termini of nucleosomal particle DNA, which is close to the H2A-H2B dimer (Shich et al., 1985). HMG-14 and HMG-17 proteins bind to nucleosomes through their positively charged nucleosomal binding domain spanning residues 12 to 41 in the HMG-14 family and residues 17 to 47 in the HMG-17 family (Cook et al., 1989; Crippa et al., 1992). This region is highly conserved and studies with synthetic peptides indicated that a 30 amino-acid peptide, corresponding to the nucleosomal binding domain of HMG-17, binds specifically to the nucleosome cores (Postnikov et al., 1994). Additionally, point mutations in this region reduce its affinity to the nucleosome cores (Postnikov et al., 1994). Also, histone tails

were shown to be required for binding of both the peptide and the intact protein to the nucleosome particles (Crippa et al., 1992). Recent results indicate that the binding of HMG-14 and-17 to the nucleosome particles is not random and that this interaction produces complexes containing either two molecules of HMG-14 or two molecules of HMG-17 (Postnikov et al., 1995).

Post-translational modifications of the HMG-14 and HMG-17 proteins can affect their interaction with nucleosomes. For example, phosphorylation of ser-6 in HMG-14, which is one of the first molecular events associated with the induction of immediate-early genes in mitogenic stimulation (Barratt et al., 1994), reduces the affinity of HMG-14 for nucleosome core particles (Spaulding et al., 1991), therefore, this post-translational modification might result in structural changes in chromatin regions containing HMG-14 protein.

1.6.1.5 Expression of HMG-14 and HMG-17 proteins during cell cycle and differentiation

Since HMG-14/-17 proteins are considered to be a major component of chromatin and chromosomes structure, it was expected that their pattern of synthesis during the cell cycle would be similar to that of histones (Bustin et al., 1992). However, experiments done during liver regeneration to measure the incorporation of labeled amino acids into the HMG proteins indicated that the synthesis of these proteins occurs during late S phase or early G2, when most of histones and DNA synthesis have ceased (Kuehl, 1979; Smith, 1982). Measurement of the mRNA level of HMG-14 and HMG-17 in synchronized HeLa cells showed that the level of HMG-14 and HMG-17 mRNAs level reaches it highest in the late S or early G2 phase, when the level of histones mRNA is reduced to its lowest level (Bustin et al., 1987). Thus the amount of cellular mRNA level correlates well with the time of the protein synthesis.

A more detailed analysis of the mRNA level of HMG-14 and HMG-17 during cell cycle of these cells, however, showed that there is earlier increase in the level of mRNA of these proteins during the very early S-phase before significant amounts of histones mRNA are observed (Bustin et al., 1987). Interestingly, this earlier increase in

the level of the HMG-14 and HMG-17 mRNA before the full onset of DNA synthesis would create a pool of these proteins available to be incorporated into the early replicating DNA (Bustin et al., 1992) which correlates with the observation that HMG-14 and HMG-17 proteins are accumulated in the nucleus during DNA synthesis (Bonne et al., 1982), and also favours the suggestion that these proteins are associated with active genes, as early replicating DNA is enriched in transcriptionally active genes (Alberts et al., 1989). Nevertheless, measurement of the mRNA level of HMG-14 and HMG-17 in synchronized HeLa cells showed that HMG-14 and HMG-17 transcription occurs throughout the cell cycle (Bustin et al., 1987).

Changes in the expression of HMG-14 and HMG-17 have also been identified during differentiation. The levels of HMG-14 and HMG-17 mRNAs decrease during mouse myoblast differentiation, such that myotubes contain only 10-20% of the mRNA present in growing myoblasts (Begum et al., 1990). Analysis of HMG-14 and HMG-17 during erythropoiesis (Crippa et al., 1991) and the differentiation of several cell lines (Crippa et al., 1990) have also indicated that undifferentiated cells contain more HMG-14/-17 mRNA than those of differentiated ones. The biological significance of the down-regulation of HMG-14 and HMG-17 expression during differentiation is not clearly understood. Myoblasts cells transfected with plasmids expressing HMG-14 were inhibited from undergoing myogenesis (Pash et al., 1993), however, which suggests that myogenic differentiation may require regulated levels of HMG-14 protein.

1.6.1.6 Cellular function of the HMG-14 and HMG-17 proteins

The precise function of HMG-14/-17 has not been fully clarified. Several lines of experimental data, however, indicate that the function of these proteins is related to transcriptional regulation. It has been almost twenty years since the proteins were suggested to modulate the chromatin structure of active genes (Weisbrod and Weintraub, 1979). In vitro; reconstitution experiments with isolated nucleosomes showed that HMG-14 and HMG-17 proteins preferentially bind to nucleosomes enriched in sequences from transcribed genes (Sandeen et al., 1980; Brotherton and Ginder, 1986), incorporation of HMG-17 in chromatin during DNA replication

enhanced 5-fold the transcriptional potential of assembled chromatin template (Crippa et al., 1993), addition of recombinant human HMG-14/-17 proteins to *Xenopus laevis* egg extracts assembling the 5-S RNA gene of *Xenopus borealis* into minichromosomes, increase the transcriptional potential of the 5-S RNA gene when the proteins were added during but not after the minichromosome assembly (Crippa et al., 1993; Trieschmann et al., 1995). In vivo; microinjection of antibodies against HMG-17 into human fibroblasts showed to inhibit transcription (Einck and Bustin, 1983) and in-vivo-assembled chromatin with elevated amounts of HMG-14 experiments showed that HMG-14 stimulates transcription from chromatin template in a dose dependent fashion (Ding et al., 1994). Immunofluorescence studies showed that HMG-14 is more abundant in regions contain transcriptionally active genes (Weisbrod et al., 1980; Westermann and Grossbach, 1984; Disney et al., 1989). Immunoaffinity chromatography experiments revealed that chromatin regions containing transcribable genes contain approximately three-fold more of HMG-14 and HMG-17 compared to total nuclear DNA (Drobic and Wittig, 1986, 1987; Druckman et al., 1986).

Despite the many structural and functional similarities between both proteins, it is puzzling, however, that all cells contain both of the proteins. Recent findings that the nucleosome particle has two binding sites for either HMG-14 or HMG-17, however, suggest that both of the proteins may be involved in different functions or affect the transcription of different genes (Postnikov et al., 1995).

1.7 Objective

The objective of this study was to investigate the role of HMG-14 and HMG-17 during early mouse embryogenesis. The specific aims were to study their pattern of regulation and their function during the early cleavage stages of development. First, despite the evidence linking these proteins to transcriptional activity, their role in normal cells has not been identified. In the mouse embryo, transcriptional activity varies during development, being very low or absent until the late 2-cell stage, which offers the opportunity to study a potential correlation between HMG-14 and HMG-17 levels and transcription. Second, although cell cycle regulation of their expression has been studied

in cultured cells, such a study has not been performed in normal or embryonic cells. Third, almost all studies of these proteins have focused on structural aspects or employed in vitro systems. There are no reports of the effects of their depletion in mammalian cells, and such studies could provide more evidence of their biological role. Fourth, there has been no previous study done to directly discriminate between the functions of both proteins, and depletion of these proteins in an individual and combined manner could provide more direct evidence on their puzzling function.

In this study, the expression pattern of HMG-14 and HMG-17 mRNAs was studied using RT-PCR, and the cellular localization of the two proteins at different stages of mouse preimplantation was revealed using immunfluorescence. Antisense techniques were applied to deplete their supply in the cells so that their function during early mouse embryogenesis could be tested. It was anticipated that the new information obtained would increase our understanding of the function of these two proteins and of the regulation of early mouse development.

CHAPTER 2

MATERIALS AND METHODS

2.1 Collection of prophase I-arrested oocytes

To obtain immature oocytes arrested at prophase I of meiosis, 21- to 35-day old CD-1 female mice (Charles River Canada) were killed and their ovaries were removed and transferred to Hepes-buffered minimum essential medium (MEM-H), modified as described (Schroeder and Eppig, 1984; Clarke et al., 1988) and supplemented with 50-100 μg/ml dibutyryl cyclic AMP (dbcAMP). The ovarian follicles were punctured using a 30-gauge needle to release the enclosed oocytes, and immature oocytes were recognized by the presence of the germinal vesicle (GV). The dbcAMP prevents the immature oocytes from undergoing germinal vesicle breakdown (GVBD) in culture (Cho et al., 1974). Immature oocytes were incubated in 5-μl microdrops of bicarbonate-buffered minimal essential medium (MEM, GIBCO, Burlington, Ontario) containing 50-100 μg/ml dbcAMP under oil at 37°C in an atmosphere of 5% CO_2 in air until they were used. Prophase I-arrested oocytes to be microinjected were processed immediately. Those to be subjected to PCR analysis were either immediately processed for RNA extraction followed by cDNA synthesis and PCR amplification, or stored (usually 30 oocytes in each group) in 10 μl PBS at -70°C until processing.

2.2 Collection of embryos at different stages of preimplantation development

Embryos at different stages of preimplantation development were obtained as previously described (Clarke et al., 1992). Briefly, females that were eight weeks of age or older (Charles River Canada) were superovulated by an injection of 7.5 IU of pregnant mares' serum gonadotropin (PMSG, Sigma) followed 44-48 h later by 5 IU of human chorionic gonadotropin (hCG, Sigma) and caged individually with CD-1 males overnight. The next morning, the females were checked for the presence of a vaginal plug to confirm that mating had occurred. One-cell embryos were obtained from mice killed at day-0.5 (day-0 = day of plug). The oviducts were dissected and collected in a

35-mm tissue culture dish containing 2 ml of Hepes-buffered KSOM medium (KSOM-H, Biggers et al., 1993), and torn with forceps to release the egg-cumulus cell mass. This mass was then transferred to a dish containing 2 ml of KSOM-H supplemented with 1 mg/ml hyaluronidase (Boehringer, Montreal) for 30 min. at 37°C, to remove the cumulus cells. Cumulus-free eggs were collected and cultured in 5-µl drops of bicarbonate-buffered KSOM at 37°C in an atmosphere of 5% CO_2 in air. Two-cell embryos were collected by flushing the oviducts of pregnant females killed on day-1.5. When older embryos were required, the two-cell embryos were transferred to 5-ul drops of KSOM medium under paraffin oil, and incubated at 37°C in an atmosphere of 5% CO_2 in air until the desired stage. Embryos either were microinjected (1-cell stage only) or were processed for RNA extraction, cDNA synthesis and PCR as described for the oocytes.

2.3 Immunocytochemistry

Embryos at different stages were freed of the zona pellucida by a brief exposure to acidified (pH 2.5) Tyrode's medium. Then they were incubated for 1 hr at 37°C in KSOM-H medium, so they could recover from the Tyrode's treatment before fixation. Then they were fixed in freshly prepared 4% paraformaldehyde in phosphate-buffered saline (PBS) for 15 min. at room temperature, permeabilized in storage solution consisting of PBS, 3% bovine serum albumin. (BSA), 0.5% Triton X-100, and either stored at 4°C until the time of staining or processed immediately for immunostaining .

Before staining, embryos were incubated for 30 min. in blocking solution consisting of 900 µl storage solution, 100 µl horse serum (GIBCO), 20 µl fish gelatin (1 mg/ml, Sigma). They were then transferred to a solution containing affinity-purified rabbit antibody against HMG-14 or HMG-17 (Kindly supplied by Dr. M. Bustin, National Institute of Health, USA) as appropriate, in blocking solution. Following overnight incubation at 4°C with agitation, the embryos were washed in blocking solution twice for 15 min. each and then incubated for 1 hr at room temperature with agitation in the presence of a fluorescein isothiocyanate (FITC)-conjugated anti-rabbit

antibody (Jackson Immunoresearch) diluted 1:100 in blocking buffer. The embryos were then washed in blocking buffer twice for 15 min. each and mounted on a microscope slide in a 30-µl drop of Moviol (Hoechst, Montreal) containing 1,4-diazabi-cyclo[2.2.2]octane (25 mg/ml, Sigma) as an antifading agent and 1 µg/ml of DAPI to stain the DNA. Specimens were examined using a Leitz Laborlux S microscope, equipped for epifluorescence with FITC and UV filter sets, or using a laser confocal microscope.

2.4 Collection of embryos at different phases of the cell cycle

Embryos at different phases of the cell cycle were obtained as follows. To obtain 1-cell, G2 embryos, 1-cell stage embryos were collected as described above at 24-28 hr post-hCG injection. All other embryos at the different phases of the cell cycle were obtained by collecting 2-cell embryos at 32-36 hr post-hCG and culturing them to the desired stage. 2-cell, G1 phase were obtained at 32-36 hr post-hCG; 2-cell, S-phase at 40-44 hr post-hCG; 2-cell, G2 at 44-48 hr post-hCG; 4-cell, G1 at 50-54 hr post-hCG; 4-cell, S- phase at 58-62 hr post-hCG; 4-cell, G2 at 64-68 hr post-hCG. Embryos were stored at -70°C after collection and all groups were processed for RNA isolation, cDNA synthesis and PCR amplification together.

2.5 RNA isolation

To recover cellular RNA, oocytes or embryos (usually 30 embryos per tube) were transferred into a 0.5-ml microfuge tube containing 100 µl of lysing buffer [Trizol reagent (GIBCO)] and 10 µg of glycogen was added to each tube, which were mixed well and allowed to stand for 5 min. at room temperature. As a control in some of the experiments, 1.8 - 3.0 pg of *in vitro*-transcribed RNA corresponding to the vasa gene of *Drosophila* was added at this step. Twenty µl of chloroform was then added and the tubes were vigorously shaken for 15 sec, then allowed to stand for three min. at room temperature. The tubes were then centrifuged in an Eppendorf-type centrifuge for 15 min. at 4°C at 13,000 rpm. The upper aqueous phase was transferred to a clean tube, to

which was added 100 µl of isopropanol. After a 10 min. incubation at room temperature, the tubes were centrifuged for 15 min. at 4°C at 13,000 rpm and the supernatant was carefully withdrawn, leaving the precipitated RNA in the tube. The pellet was washed with 100 µl of 70% ethanol, the tube was centrifuged again for 15 min. at 4°C at 13,000 rpm, and the supernatant was removed. The pellet was allowed to dry for 10-15 min. and then dissolved in up to 10 µl of DEPC-treated water and incubated for 10 min. at 55°C. The RNA was then stored at -70°C or in most cases was immediately processed for cDNA synthesis.

2.6 cDNA synthesis

Synthesis of cDNA from the RNA was carried out using standard procedures. Briefly; each reaction mixture contained between 10 µl of RNA solution, prepared as described above, 27 units of RNase inhibitor (Pharmacia), 2 µl of a dNTP mix of 10 mM concentration, 4 µl of 5X reverse buffer (GIBCO), 2 µl of 10 mM DTT, 500 ng of oligo-d$(T)_{12\text{-}18}$ (Pharmacia) or 200 pmole of random hexamers (Pharmacia), and 200 units of MMLV reverse transcriptase (GIBCO). The cDNA synthesis was allowed to proceed for 2-3 hrs at 37°C, and the mixture was then heated at 85°C for 10 min., after which the product was used immediately for PCR amplification or was stored at -20°C.

2.7 PCR amplification

Amplification of DNA using PCR was carried out using cDNA from 10-15 embryos or embryo-equivalents in a buffer consisting of 10 mM Tris (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 200 mM dNTPs, 60-100 pmoles of each primer and 2.5 units of *Taq* polymerase (various suppliers: Applied Biosystems, GIBCO, Qiagen) in a total volume of 50 µl. Amplification was performed using a PTC-100 (MJ Research) thermal cycler. Each cycle consisted of 1 min. at 94°C, 45 sec at a gene-specific temperature (58°C for HMG-14, 60°C for HMG-17, 58°C for vasa, 60°C for actin) and 45 sec at 72°C. Between 35 and 45 cycles were run for most experiments.

2.8 Agarose gel electrophoresis

To visualize the amplified products, 20 µl of each PCR reaction was electrophoresed through a 0.8-1.5% (w/v) agarose gel prepared in TBE buffer (45 mM Tris-borate, 1 mM EDTA, pH 8.0) containing 0.025% ethidium bromide, and using TBE as the running buffer. Gels were visualized and photographed under an UV source of light.

2.9 Primers used for PCR amplification and oligonucleotides used for microinjection

Primers for PCR amplification HMG-14, HMG-17, actin and vasa were designed from the corresponding GenBank sequences and are shown here:

HMG-14 primers

5'- A T G C C C A A G A G G A A G G T T A G

3'- G A G A C A G T C A C A G C C T C T C C

HMG-17 primers

5'- A C A C T T C A T T A C T G G G T G G G

3'- A G C C G A C C C T G A C T T T T A G G

vasa primers

5'- C T G G A G G T T A T C G A G G A G G A

3'- T C G G C A T C T T C G G C G A T A T T

Actin primers

5'- G T G G G C C G C T C T A G G C A C C A

3'- T G G C C T T A G G G T G C A G G G G G

Anti-sense, sense and nonsense oligonucleotides corresponding to HMG-14 and HMG-17 were designed to target the 5` end of the cDNA sequences; specifically, they were 24 nt. in length and began few bases before the protein synthesis initiation codon. The following oligonucleotides were used.

Non modified HMG-14 antisense oligonucleotides

T A A C C T T C C T C T T G G G C A T C G T G G

Non modified HMG-17 antisense oligonucleotides

C A G C C T T T C T T T T G G G C A T G G T G G

Non modified HMG-17 sense oligonucleotides

C C A C C A T G C C C A A A A G A A A G G C T G

Phosphorothioate-linked HMG-14 antisense oligonucleotides

C T A A C C T T C C T C T T G G G C A T C G T G

Phosphorothioate-linked HMG-14 nonsense oligonucleotides

A G C T T C T T C G C C C G T A G C C T A G T T

Phosphorothioate-linked HMG-17 antisense oligonucleotides

T C A G C C T T T C T T T T G G G C A T G G T G

All PCR primers and non modified oligonucleotides were synthesized at the Sheldon Biotechnology Center at McGill University, Montreal. Phosphorothioate-linked oligonucleotides were synthesized at the University Core DNA Services at The University of Calgary, Calgary.

2.10 Calculation of DNA, RNA, primer and oligonucleotide concentrations

When primers and oligonucleotides were received, they were resuspended in a volume of 200 µl autoclaved H_2O and kept as a stock solution at -20°C. Nucleotide concentrations were measured using a DU70 spectrophotometer as following. 5 µl of DNA, RNA, primer stock, or oligonucleotide stock solutions was diluted in 1 ml of water. The optical density (O.D.) at 260 nm and 280 nm was then measured. Provided the 260/280 ratio was greater than 1.5, l, the O.D.(260) was multiplied by 50 (for DNA), 40 (for RNA), or 30 (for primers and oligonucleotides) and the number was multiplied by 200 (dilution factor) to determine the concentration in ng/µl.

To determine the number of moles in each primer, Σ total was calculated as follows. The number of T, C, G and A nucleotides were counted in the primer or the oligonucleotide and Σ total was calculated using the following formula: Σ total = number of Ts X 8.8 + number of Cs X 7.3 + number of Gs X 11.7 + number of As X 15.4. The number of moles was then calculated using the following formula: number of

moles in μmole/ml = O.D / Σ total. The result was multiplied by 40 (dilution factor) to get the number of moles per 200 μl. The result was then divided by 200 to get the number of moles per μl. The result was multiplied by 10^6 to get the number of pmole/μl. The stock was then diluted accordingly to produce a final concentration of 60-100 pmoles per μl. Usually 1 μl of primers was used in each PCR reaction.

2.11 In vitro transcription

In vitro transcription was used to prepare unlabelled and digoxogenin (DIG)-labeled anti-sense RNAs corresponding to HMG-14 and HMG-17 and to prepare the vasa RNA that was used as a control for the efficiency of RNA purification, cDNA synthesis, and PCR amplification. Anti-sense RNAs for HMG-14 and HMG-17 were prepared by *in vitro* transcription of pM14c and pM17cbsk.1 plasmids, respectively (kindly supplied by Dr. M. Bustin, National Institutes of Health, USA). These plasmids contain the mouse HMG-14 and HMG-17 cDNAs with bacterial T7 RNA polymerase promoter at their 5` end and bacterial T3 RNA polymerase promoter at their 3` end. The vasa cDNA was kindly supplied by Dr. P. Lasko, Department of Biology, McGill University.

Unlabelled RNA was prepared and purified using the kit supplied by Promega, and RNA labeled with DIG was prepared and purified using the kit supplied by Boehringer, in both cases following the instructions supplied by the manufacturer. Briefly, to a microfuge tube at room temperature the following solutions were added; 20 μl of 5X transcription buffer (200 mM Tris-HCl pH 7.9, 30 mM MgCl2, 10 mM spermidine and 50 mM NaCl), 10 μl of 100 mM DTT, 100 U of RNAse inhibitor, 20 μl of dNTP mixture, 2.5-5 μg of plasmid DNA, 3 μl of the required RNA polymerase, and sufficient nuclease-free water to bring the volume to 100 μl. The reaction was allowed to proceed at 30°C or 37°C for 3 hrs. Following the transcription reaction, 5 μl of RNAse-free DNAse was added and the incubation was continued for 15 min. at 37°C to digest the plasmid DNA. After this, RNA was precipitated using the phenol-chloroform protocol as follows. 100 μl of TE-saturated phenol-chloroform (1:1) was added to the

mixture, which was vortexed for 1 min. and centrifuged for 2 min. The upper aqueous phase was transferred to a clean tube and 100 µl of chloroform:isoamyl alcohol (24:1) was added, the solution was vortexed for 1 min. and centrifuged for 2 min., and the upper aqueous phase was transferred to a clean tube. Fifty µl of 7.5 M ammonium acetate and 250 µl of pre-chilled 100 % ethanol were added and the solution was incubated at -70°C for 30 min. The tube was then centrifuged at 13,000 rpm for 5 min. at 4°C, the supernatant was removed carefully, and the pellet was washed with 70% ethanol, centrifuged for 5 min. at 13,000 rpm at 4°C, dried for 15 min. at room temperature, and redissolved in 50 µl of DEPC-treated water.

2.12 Microinjection of oocytes and embryos

Microinjection was carried out using a Leica inverted microscope equipped with Leica micromanipulators (Leica Canada, Montreal). A 10-µl drop of MEM-H containing 50 µg/ml dbcAMP in the case of oocytes, or of KSOM-H in the case of embryos, was placed in the center of the depression of a hanging drop microscope slide (Fisher, Montreal), and next to this was placed a 5-µl of the solution to be injected. The depression was then carefully overlaid with light mineral oil (Fisher). About 10 oocytes or embryos at a time were transferred into the drop of MEM-H or KSOM-H in preparation for microinjection.

The microinjection needles were prepared by pulling 1.0 mm (outside diameter) X 0.75 mm (inside diameter) capillary tubes (FHC, Brunswick, Maryland, USA) using a vertical pipette puller (David Kopf Instruments, California, model 720). They were filled with the solution to be injected, using the fill function of a PLI-100 microinjector (Medical Systems Corp., Greenvale, NY). The needle was pushed into the cytoplasm or nucleus, as required, of an embryo, and the solution was introduced by a pulse of positive pressure supplied by compressed N_2. In most cases it was possible to verify visually that an embryo had been successfully micoinjected by a dispersal of cytoplasmic or nuclear granules in the vicinity of the micropipette tip. Between injections, a constant positive pressure was maintained in the micoinjection needle, since preliminary

experiments showed that the needle otherwise tended to fill with culture medium. Based on displacement during injection of the meniscus marking the interface between solution and N_2 and on the inner diameter of the microinjection needle as determined using an eyepiece graticule, it is estimated that about 5-10 pl was injected into each cell.

Microinjected embryos were then transferred to MEM medium for oocytes or KSOM medium for embryos and incubated at 37°C in an atmosphere of 5% CO_2 in air. Embryos were inspected at regular intervals to determine the extent of embryonic development.

2.13 Plasmid construction

2.13.1 Restriction digestion

All restriction digestions used to construct the different plasmids were carried out following the standard protocol. Restriction enzymes from various suppliers were used and in each case the recommended buffer was used. Briefly, the protocol involves incubation of the DNA wanted to be digested in a 1X or 2X (depending on the restriction enzyme used) assay buffer, which typically contains [100 mM Tris-acetate (pH 7.5), 100 mM magnesium acetate and 500 mM potassium acetate]. 1 unit of the required restriction enzyme was usually added per 1 µg of DNA used and the volume was usually brought to 30 µl by H_2O. The mixture was then incubated for 1 hr at 37°C or at the temperature recommended for each specific restriction enzyme.

2.13.2 Purification of DNA fragments after restriction digestion

Following restriction digestion, usually the entire volume of the reaction mixture was run on 0.8 - 1.5 % (depending on the size of the desired fragment) agarose gel containing ethidium bromide and the gel was then visualized under an UV source of light. The desired fragment was then excised from the gel using a clean scalpel and transferred to a clean 1.5 ml microfuge tube. The amount of the agarose gel excised was then weighed and the DNA fragment was extracted from the agarose gel using Nucleotrap (MACHEREY - NAGEL) extraction kit following the supplier's instructions.

2.13.3 Cloning of the purified fragments

When the inserted fragment and the cloning vector had been cut with the same restriction enzyme(s), they were processed for ligation without prior treatment. Ligation was carried out using T4 DNA ligase (Pharmacia). The concentration of the inserted fragment was usually between 5 and 10 times higher than that of the cloning vector. 3 µl of ligation buffer (100 mM Tris-acetate, 100 mM magnesium acetate, and 500 mM potassium acetate), 3 µl of ATP, 3 µl of DTT and 1µl of the ligase enzyme were then added in sequence and the volume was brought to 30µl by H_2O. The reaction mixture was then incubated at 15°C overnight.

When the inserted fragment and the cloning vector had not been cut with the same restriction enzyme(s), both DNA fragments were blunt-ended using *Pfu* polymerase (Boehringer). Typically the blunt ending reaction involves incubation of the DNA fragment in an 1X reaction buffer (200 mM Tris-HCl pH 8.2, 100 mM KCl, 60 mM $(NH_4)_2S0_4$, 20 mM $MgCl_2$, 1% Triton X-100 and 100 µg/ml nuclease-free BSA), dNTPs. and 1 unit of polymerase enzyme. The volume was brought to 30 µl by H_2O. The reaction mixture was then kept for 30 min. at 72°C and the DNA was then phenol-chloroform extracted. The vector was then phosphorylated using polynucleotide kinase (Pharmacia) as follows. DNA was incubated in 1X reaction buffer (100 mM Tris-HCl, 100 mM magnesium acetate, and 500 mM potassium acetate), 48.5 pmol ATP, 20 units of polynucleotide kinase and the volume was then brought to 50 µl with H_2O, for 30 min. at 37°C, after which 5 µl of 0.25 M EDTA (pH 7.0) was added to stop the reaction. The insert was dephosphorylated using alkaline phosphatase (Pharmacia) as follows. DNA was incubated in 1X reaction buffer (100 mM Tris-HCl, 100 mM magnesium acetate and 500 mM potassium acetate), 0.1 unit of alkaline phosphatase and the volume was brought to 50 µl with H_2O, for 30 min. at 37°C after which the mixture was heated to 85°C for 10 min. DNA was then phenol-chloroform extracted and processed for ligation as described above.

2.13.4 Bacterial transformation

After ligation, the plasmids was used to transform bacteria. DH5α competent bacterial cells (GIBCO BRL) were used for transformation. Briefly, the transformation protocol involved mixing 1μl (1-10 ng) of ligated DNA with 100 μl competent cells in pre-chilled polypropylene tubes. The cells were then incubated on ice for 30 min. Cells were then heat-shocked for 45 seconds in a 42°C water bath and incubated on ice for 2 min. 900 μl of S.O.S. (97 ml H_2O, 2 g bactotryptone, 0.5 g yeast extract, 1 ml 1 M NaCl, 0.25 ml 1M KCl, autoclave, add 1 ml 2M Mg^{++}, 1 ml 2 M glucose, filter) medium was then added and cells were incubated for 1 hr at 37°C with shaking at 225 rpm. Cells were then diluted 1:100 or 1:150 with S.O.S. medium and 100 μl of diluted cells was then spread on TB plates containing 100 μg/ml Ampicillin or Kanomycin (depending on the kind of resistance gene carried by the cloning vector). Plates were then incubated at 37°C overnight. The next morning, plates were checked for colony formation and single colonies were picked up, inoculated into 3 ml TB medium containing antibiotic, and grown for 12-16 hr. Plasmid isolation was then carried out. TB plates were prepared by adding 1.5% Bacto-Agar to TB medium (1.2 g Bactotryptone, 2.4 g yeast extract, 0.4 ml glycerol, 0.231 g KH_2PO_4, 1.254 g K_2HPO_4, adjust volume to 100 ml with H_2O, autoclave, cool down and add 10 g ampicillin) and the mixture was then poured into plastic peter dishes.

2.13.5 Plasmid isolation (minipreps)

Plasmid isolation from transformed bacteria was carried out using Qiagen miniprep preparation kits following the supplier's instructions.

2.14 Detection of β-galactosidase activity

Embryos injected with plasmids carrying the *lacZ* gene were stained for β-galactosidase activity as follows. Embryos were collected at the stage desired for staining, washed once with PBS, and fixed in 2 ml of fixative (0.54 ml 3.7% formalin, 1.46 ml PBS, 4 μl 1M $MgCl_2$, 8 μl 50% glutaraldehyde, 0.1 % BSA) for 20 min. at 4°C.

Embryos were then washed twice in 2 ml of washing solution (PBS, 4µl 1M $MgCl_2$, 0.05% BSA and 0.1% Na deoxycholate) for 10 min. each. Embryos were then transferred to a dish containing 2 ml staining mixture (4 mM potassium ferricyanide, 4 mM potassium ferrocyanide, 2 mM $MgCl_2$, 0.05% BSA, 0.1% Na-deoxycholate, 1 mg/ml X-Gal) and incubated at 37°C overnight. Embryos were then scored either positive or negative for β-galactosidase activity by the presence or absence of blue colour inside the embryo cytoplasm under a light microscope.

2.15 Detection of DIG-labelled RNAs injected into mouse embryos

Embryos injected with DIG-labelled antisense RNAs were tested for DIG detection as follows. Embryos were collected at the time and stage desired for detection, washed once with PBS, and fixed in 10% formalin, 0.5 % Triton X-100 for 15 min. at room temperature and stored at 4°C. Embryos were then washed twice for 15 min.in 2 ml of blocking buffer (PBS, 3% BSA, 0.5% Triton X-100). Embryos were then transfered to primary antibody solution (anti-DIG antibody diluted 1:100 in blocking solution) and incubated in a humidified chamber overnight at 4°C with gentle agitation. Embryos were then washed twice for 15 min.in 2 ml of blocking buffer. Embryos were then washed twice for 15 min.in 2 ml of alkaline phosphatase (100 mM NaCl, 5 mM $MgCl_2$, 100 mM Tris, pH 9.5) (AP) buffer. Embryos were then transfered to detection solution [2 ml AP buffer, 13.2 µl NBT (Boehringer) solution, 6.6 µl DCIP (Boehringer) solution] and incubated in the dark and monitored regularly until the colour reached the appropriate intensity.

CHAPTER 3
RESULTS

3.1 Detection of HMG-14 and HMG-17 encoding mRNAs during early mouse developmental stages

As a first step to study whether the HMG-14 and HMG-17 proteins play a role during early mouse development, I investigated whether the corresponding mRNAs were detectable in fully grown oocytes arrested at prophase I of meiosis, ovulated eggs at metaphase II, and embryos at successive stages of preimplantation development from the 1-cell to the blastocyst stage. Total RNA was extracted from approximately 30 oocytes or embryos of each stage, as described in Materials and Methods, and reverse-transcribed to produce cDNA using hexamer primers. The cDNA was usually divided into two equal parts, one of which was used to detect HMG-14 mRNA, the second to detect HMG-17 mRNA along with vasa RNA, which worked as an exogenous control (explained below). This permitted HMG-14 and HMG-17 to be analyzed in the same sample. Each sample was subjected to PCR using the appropriate primers, which were expected to produce a 544-nt fragment in the case of HMG-14, and a 414-nt fragment in the case of HMG-17. Reaction products were analyzed in 1.5% agarose gels stained with ethidium bromide. In order to minimize any variations that may happen between different experiments, samples from all different stages were first collected and stored at -70°C and then subjected to simultaneous RNA extraction, cDNA synthesis and PCR amplification. Each oocyte and embryo stage was analyzed in at least three separate trials.

As shown in Figure 2, HMG-14 and HMG-17 mRNAs were detected in oocytes and in embryos throughout preimplantation development. As determined by the signal intensity of the PCR products, there was a gradual decline in the level of both mRNAs during early embryonic development, with PCR products of both mRNAs reaching their lowest level at the 2-cell stage. This result is in agreement with the previous observations that maternal RNA in the mouse becomes largely degraded by the 2-cell

stage (Bachvarova and DeLeon, 1980; Bachvarova et al., 1985; Paynton et al., 1988). After the 2-cell stage, both mRNAs increased in abundance, which likely reflects the expression of these genes following embryonic genome activation at the 2-cell stage.

To be certain that the observed changes in the relative amounts of the mRNAs between the different developmental stages were not due to technical artifacts, in these experiments a known amount of in vitro transcribed vasa RNA encoding the *Drosophila* vasa protein was added to the tube of embryos prior to RNA isolation. This RNA, which is not present in mouse embryos, served as an internal standard to correct for differences between samples in RNA recovery and efficiency of the RT-PCR. As shown in Figure 2A, when HMG-17 and vasa were coamplified in the same tube, there was no decline in the level of amplified vasa product during the stages when there relatively little HMG-17 mRNA was detected. This indicates that the observed decline in HMG-14 and HMG-17 mRNA abundance at the 2-cell stage and subsequent increase at the 4-cell stage were not due to inefficient RNA recovery or cDNA synthesis at these stages. More likely, the changes reflect an endogenous developmentally regulated process. The signals obtained, however, are not likely due to genomic DNA contamination, since when the reverse transcription step was bypassed and the RNA extract was directly amplified with PCR, there was no signal detected (not shown).

3.2 Detection of HMG-14 and HMG-17 proteins during early mouse developmental stages

Having demonstrated that the mRNAs encoding both HMG-14 and HMG-17 were present in oocytes and early embryos, I next examined whether the proteins were also detectable. Previous attempts to identify HMG-14 and HMG-17 by immunoblotting were unsuccessful (Clarke, H., unpublished data), likely owing to inability to collect sufficient number of cells. Therefore, oocytes and embryos were fixed and processed for immunofluorescence following procedures previously established in our laboratory (Clarke et al., 1992). Embryos from each stage were divided into two groups, one of which was used to detect HMG-14 protein and the other to detect HMG-17 protein. HMG-14 and HMG-17 proteins were revealed using affinity-purified antibodies

specifically recognizing each protein. These antibodies were provided by Dr. M. Bustin, National Institute of Health, USA, and their specificity has been previously established.

Figure 3 shows confocal microscopic images of embryos at the 2, 4 and 8-cell stages stained with anti-HMG-14 (Fig. 3A, B and C) or with anti-HMG-17 antibody (Fig. 3D, E and F). It may be seen that the nuclei of 2-cell embryos were moderately stained with both antibodies and that, at later stages, the nuclei were brightly stained. Similar nuclear staining using each antibody was also observed in prophase I-arrested oocytes (not shown). These results suggest that HMG-14 and HMG-17 are associated with the chromatin of transcriptionally active cells.

In contrast, the condensed chromosomes of metaphase II-arrested oocytes were not stained and the pronuclei of 1-cell embryos were only weakly stained (not shown). At these stages, however, the cytoplasm was stained. At this time, we can not distinguish whether this cytoplasmic staining is due to cytoplasmic presence of HMG-14 and HMG-17 or to binding of the antibodies to other cytoplasmic proteins. If the cytoplasmic immunoreactive species are in fact HMG-14 and HMG-17, this may indicate that they are displaced from the chromatin of these transcriptionally inactive cells.

3.3 Detection of HMG-14 and HMG-17 encoding mRNAs through the various phases of the cell cycle

Recently, we found out that mRNAs encoding the somatic histone subtypes, which are a major component of chromatin structure, are cell cycle regulated, in which thay are much more abundant during S-phase than during other phases, in mouse embryos (Clarke et al., 1998), which were previously shown in somatic cells (Marzluff and Pandey, 1988; Harris et al., 1991; Heintz, 1991), thus co-ordinating histone synthesis with DNA replication. As HMG-14 and HMG-17 are also abundant chromatin components, it was interesting to examine whether similar results would be obtained. Embryos at different stages of cell cycle were collected as described in Materials and Methods. Vasa RNA was added to each tube containing embryos (30 embryos per tube) prior to RNA extraction and total RNA was extracted and reverse-transcribed to

produce cDNA. The cDNA was divided into three equal parts, one of which was used to detect HMG-14 mRNA, the second to detect HMG-17 mRNA, and the third to detect vasa RNA.

As shown in Figure 4A and B, HMG-14 and HMG-17 were detected through out all cell cycle phases and their abundance, as determined by the PCR signal intensity, seem to be similar during the different phases. The constant amplification of vasa RNA (Figure 4C) indicate that the signals observed were not due to an experimental artifact. Previous experiments using synchronized HeLa cella (Bustin et al., 1987), showed that HMG-14 and HMG-17 mRNAs are present in the cells throughout the cell cycle. Therefore, HMG-14 and HMG-17 seem to have similar cell cycle regulation in embryos as in culture cells.

3.4 Strategies to deplete HMG-14 and HMG-17 proteins from embryonic cells

The experiments described above established that HMG-14 and HMG-17 were expressed during early mouse development. To investigate the role of HMG-14 and HMG-17 proteins during early mouse embryogenesis, I chose to deplete these two proteins in an individual and combined manner from mouse embryos and examine the effects on the development of these embryos.

3.4.1 Injection of plasmids expressing antisense constructs

The first strategy to deplete these proteins was to inject plasmids coding for the antisense RNA of each protein, individually (a plasmid encoding HMG-14 antisense RNA or a plasmid encoding HMG-17 antisense RNA) and together (both plasmids), along with a control plasmid coding for a reporter gene in each case, into the male pronucleus of 1-cell stage embryos. The embryos injected with the antisense plasmid would be cultured and their developmental progression to the blastocyst stage would be compared with embryos injected with only the control plasmid. This strategy was chosen for several reasons. First, in principle, there could be a continuous supply of antisense RNA to counter the continuing synthesis of the targeted mRNA during development; second, there might be less concern about the stability of the antisense

RNA, since antisense RNAs would be synthesized inside the embryo rather than in vitro; third, because both the reporter gene and the antisense RNA constructs were inserted in the same kind of plasmid (p610ZA; see Figure 5A and below) and would be coinjected together at the same concentration, expression of the reporter gene would give a strong indication that the antisense RNAs were also expressed.

3.4.1.1 Studies using lacZ as a reporter gene

Before injecting the antisense RNA-encoding plasmids, it was necessary to examine, first, whether injection of a plasmid coding for a reporter gene would lead to the expression of the reporter gene; second, what percentage of injected embryos would express the reporter gene; and third, for how long during development would the reporter gene be expressed.

The first reporter gene plasmid chosen was p610ZA (Figure 5A, received as a gift from Dr. Rashmi Kothary, Institut du Cancer, Montreal). The reporter gene in this plasmid is the *lacZ* gene, coding for the *E. coli* β-galactosidase protein, driven by the mammalian heat shock protein-68 (hsp-68) gene promoter. As shown in Figure 6, β-galactosidase activity could be detected in embryos that had been injected with the encoding plasmid. Data obtained from 11 experiments comprising 883 injected embryos showed that only about 40% of the injected embryos expressed detectable β-galactosidase activity (Table 1). Similar frequencies of expression have been reported by other laboratories using similar constructs (Bevilacqua and Mangia, 1993). This frequency of expression was judged too low to permit the planned antisense studies to be reliably interpreted. Therefore, alternative strategies were investigated.

3.4.1.2 Studies using green fluorescent protein as a reporter gene

The second strategy was to use the green fluorescent protein (GFP), derived from the jellyfish *Aequorea victoria*, as a reporter gene. An advantage of this strategy is that the expression of the reporter protein can be detected in living cells using brief exposure to UV irradiation. Thus, embryos that expressed GFP (and therefore

presumably also the antisense construct) could be separated and cultured to examine their further development. Thus, even if the expression rate were 40%, this would be sufficient to allow further studies following selection of the positive embryos. To this end, plasmid pEGFP-N1 containing GFP driven by the cytomegalovirus (CMV) promoter (Figure 5B) was obtained from Dr. Paul Lasko, Department of Biology, McGill University. This plasmid was injected into 1-cell embryos as in the previous experiment. As shown in Table 1, although GFP was expressed in some injected embryos, the percentage of injected embryos that expressed GFP (8.3%) was less than had been observed using the β-galactosidase as the reporter protein. Furthermore, of the embryos that developed to the 2-cell stage, none expressed detectable GFP.

I reasoned that the relatively low expression of GFP that was detected might indicate that, in mouse embryos, the activity of the CMV promoter was weaker than that of the hsp-68 promoter. Therefore, a plasmid containing the hsp-68 promoter driving GFP was constructed. To accomplish this, the hsp-68 promoter was removed from the p610ZA plasmid using *BamHI* and *SalI* restriction enzymes and cloned in the multiple cloning site of plasmid pEGFP-N1 in the *BamHI* and *SalI* restriction sites. This placed the hsp-68 promoter between the CMV promoter and the GFP protein-coding sequence. Figure 5C shows a schematic diagram of the hsp-GFP-N1 construct. Following injection into 1-cell embryos of this construct, which contained both the hsp-68 and CMV promoters, the expression frequency of GFP (17.8%) was about twice that observed using the CMV promoter alone. However, it remained less than half that of p610ZA construct that contained only the hsp68 promoter, and the expression frequency in 2-cell embryos was again very low.

These results suggested the possibility that the low expression frequency might be due an inhibitory activity associated with the CMV promoter. Therefore, a 400-nt *HindIII-NdeI* restriction fragment between position 200 of the CMV promoter and the multiple cloning site located 5' of the hsp68 promoter was excised. This construct, termed p68-GFP-N1 is shown in Figure 5D. However, none of the embryos injected with p68-GFP-N1 produced detectable GFP activity (Table 1). Next, to completely

remove all CMV promoter sequences, *EcoRI* and *AseI* restriction enzymes were used to remove the region between position 1 of the CMV promoter and the multiple cloning site located 5' of the hsp68 promoter. This generated plasmid pMO-GFP (Figure 5E) from which all of the CMV promoter sequences had been removed. As shown in Table 1, expression of GFP protein from pMO-GFP was detected in most injected cells that remained at the 1-cell stage, but in none of those that reached the 2-cell stage. This could mean that the procedure for detecting β-galactosidase activity is more sensitive than the fluorescent detection of GFP protein or that the steady-state level of β-galactosidase protein in 2-cell embryos is higher.

The low expression frequency of the reporter genes at the 2-cell stage embryos, as well as the fact that the expression frequency declined further beyond the 2-cell stage (not shown), led me to conclude that plasmid-based strategy was unlikely to be an efficient mechanism to deplete cellular HMG-14 and HMG-17.

3.4.2 Injection of in vitro transcribed HMG-14 and HMG-17 antisense RNAs

As a second approach, the possibility of transcribing antisense RNAs in vitro and injecting these into 1-cell embryos was explored. To confirm that in vitro-transcribed antisense RNA would be stable inside the embryos after injection, the following experiments was carried out. DIG-labeled HMG-14 and HMG-17 antisense RNAs were prepared by in vitro transcription and injected into 1-cell stage embryos. These embryos were then divided into three groups: group one was fixed immediately after injection, group two was fixed 7 hrs after injection, and group three was fixed at the 2-cell stage, 24 hrs after injection. The fixed embryos were exposed to an anti-DIG antibody conjugated to alkaline phosphatase and then stained for alkaline phosphatase activity. Figure 7 shows that the injected antisense RNAs could be detected at least up to the 2-cell stage. This result confirmed that the preparation and injection procedures preserved the integrity of antisense RNAs.

Unlabelled antisense RNAs complementary to HMG-14 and HMG-17 mRNAs were then transcribed in vitro and injected into 1-cell embryos. Approximately 10 pl of a

500 ng/µl solution (HMG-14 antisense RNA) or a 460 ng/µl (HMG-17 antisense RNA) was injected. Two experiments for each were carried out. Following microinjection, the embryos were cultured to the 2-cell stage. Total RNA was then extracted and used for RT-PCR analysis to examine whether injection of antisense RNA had led to degradation of its target mRNA. Figure 8 shows the result of two experiments done using HMG-14 antisense RNA. As judged by RT-PCR analysis, there was no difference in signal intensity between antisense-RNA-injected embryos (lanes, 1 and 3) and control non-injected embryos (lanes 2 and 4). This result suggested that antisense RNA injection did not lead to destruction of the target mRNA.

At least two possible explanations may account for this result. First, it may be that the antisense sequences failed to hybridize to the target mRNAs. For example, the RNAs may assume secondary structure that inhibits hybridization. Second, it may be that antisense-sense hybridization occurred but that the double-stranded RNA complexes were not digested. It is not likely, however, that the signal obtained was due to excess of non-hybridizing antisense RNAs remained undigested and served as a template for cDNA synthesis and subsequent PCR amplification because cDNA synthesis in these experiments was made using oligo-dT primers and not random hexamer primers and that in vitro transcribed antisense RNAs do not contain poly-A tails; therefore, no cDNA would be expected to be synthesized from these in vitro transcribed RNAs.

3.4.3 Injection of HMG-14 and HMG-17 antisense oligonucleotides

As a third strategy to deplete HMG-14 and HMG-17 mRNAs, I investigated the use of antisense DNA oligonuclnucleotides. Antisense oligonucleotides complementary to viral and cellular RNAs have been shown to inhibit gene expression when taken up by mammalian somatic cells (Zamecnik et al., 1978; Kawasaki, 1985; Minshull and Huut, 1986; Cazenave et al., 1987; Goodchild et al., 1988; Agrawal et al., 1989; Caceres et al., 1991; Sadler et al., 1995; Bavik et al., 1996 and Dorri et al., 1997), and have been used to deplete other mRNAs in mouse oocytes (O'Keefe et al., 1989; Paules et al., 1989; Tong et al., 1995). This strategy offers several advantages. First, the

oligonucleotides are short, typically comprising 20-30 nucleotides, and sequences can be chosen to reduce potential secondary structure such as by self-hybridization. Second, DNA may be less prone than RNA to degradation both in vitro and after microinjection. Third, modifications that increase the stability of oligonucleotides might extend their activity within the cell for a prolonged period of time.

3.4.3.1 Determining the concentration of nonmodified and modified antisense oligonucleotides that could be injected

The first experiments were designed to determine the maximal concentration of oligonucleotides that could be injected into embryos without causing non-specific effects on development. Such non-specific effects were evaluated in the following manner. The first embryonic cell cycle in mice proceeds independently of embryonic transcription (Golbus et al., 1973; Bolton et al., 1984). As HMG-14 and HMG-17 are thought to regulate transcriptional activity, this implies that depleting their supply in 1-cell embryos should not prevent cleavage to the 2-cell stage. Therefore, 1-cell embryos were injected with antisense, sense, or nonsense (containing the same nucleotides in scrambled sequence) oligonucleotides. If the injected embryos failed to cleave, this was inferred to reflect a non-specific action of the injected oligonucleotides.

Antisense oligonucleotides spanning the translation-initiation codon of the mRNAs encoding HMG-14 and HMG-17 were microinjected into 1-cell stage embryos. Controls were injected with sense-strand or nonsense oligonucleotides. Both oligonucleotides containing phosphorothioate linkages, which are thought to be relatively stable inside the cell (Agrawal et al., 1990), and those that had not been modified were tested. Eighteen experiments involving injection of 1398 embryos were performed. As shown in Table 2 and Table 3, embryos injected with phosphorothioate-modified oligonucleotides at a concentration of 0.1 μg/μl or less could divide to the 2-cell stage whereas those injected with higher concentrations could not. Using non-modified oligonucleotides, embryos could tolerate up to 0.5 μg/μl for HMG-17 and up to 2.5 μg/μl for HMG-14 as judged by this assay. These results established the suitable working concentrations for subsequent experiments.

3.4.3.2 Diminishing of target mRNA following injection of non modified HMG-14 & HMG-17 antisense oligonucleotides into mouse oocytes and embryos

I next investigated whether antisense oligonucleotides injected at these concentrations would lead to degradation of the target mRNA. First, these were injected into fully grown prophase I-arrested oocytes, a cell type in which such antisense-mediated degradation has previously been established (O'Keefe et al., 1989; Paules et al., 1989; Tong et al., 1995). Oocytes were injected, incubated for 18 hrs during which they underwent meiotic maturation, and subjected to RT-PCR analysis. As shown in Figure 9, lane 2, injection of non-modified antisense oligonucleotides corresponding to HMG-14 led to a significant decrease in the amount of HMG-14 mRNA in those oocytes as compared to nonsense injected controls (lane 1). Similar result was observed when the oligonucleotides were introduced using a liposomal-based technique (lane 3). Since RT-PCR technique is highly sensitive, a small amount of undegraded RNA, however, could be still detected.

In contrast, injection of the modified oligonucleotides did not lead to similar degree of degradation of the target mRNA (Figure 9, lane 4). One possible explanation is that, owing to the relatively low concentration of injected oligonucleotide, a portion of the target mRNA did not hybridize to antisense sequences. It has been reported, however, that double-stranded nucleic acid complexes consisting of RNA and phosphorothioate-modified oligonucleotides are not efficiently degraded by cellular RNAses (Agrawal et al., 1990). Therefore, an alternative explanation is that the phosphorothioate-modified nucleotides were able to associate with the target mRNA but that the DNA-RNA hybrid remained intact. Results described below are consistent with this latter possibility.

Next, I investigated whether injection of oligonucleotides into 1-cell embryos would also lead to the destruction of the target mRNA. One-cell stage embryos were injected in their cytoplasm as in the previous experiments, incubated overnight to the 2-cell stage, then processed for RT-PCR analysis. As shown in Figure 10, embryos injected with nonmodified oligonucleotides had relatively less HMG-14 mRNA than non-injected control embryos as judged by the intensity of the PCR products. Injection of

phosphorothioate-linked oligonucleotides, however, did not result in a similar difference between injected and non-injected groups (not shown). The difference in signal intensity between antisense injected and non-injected groups observed in embryos, however, was not as significant as that observed in oocytes.

At least three explanations could account for this observation. First, cytoplasmic conditions may differ between unfertilized oocytes and fertilized embryos such that hybridization of the oligonucleotide with its target mRNA occurred more efficiently in oocytes. Second, newly synthesized embryonic RNA at the 2-cell stage may have provided an excess of target mRNA over oligonucleotide. According to these explanations, target mRNA would remain in the embryo and so the encoded protein might not be depleted. A third explanation is that the oligonucleotide hybridized to the target mRNA as efficiently in the embryos as in the oocyte, but that the embryo did not possess as much activity that digested the double-stranded complex as did the oocyte. According to this view, the target protein might nonetheless become depleted if the RNA in the double-stranded complex could not serve as a template for efficient translation. To test this possibility, I examined the fate of the HMG-14 and HMG-17 proteins in microinjected embryos.

3.4.3.3 Transient depletion of HMG-14 and HMG-17 proteins from mouse embryos

To test whether HMG-14 and HMG-17 proteins were depleted following antisense oligonucleotides injection at the 1-cell stage, groups of injected embryos were fixed at the 2-cell, 4-cell, and 8-cell stage and processed for immunofluorescence using either anti-HMG-14 or anti-HMG-17. As shown in Figure 11 and 12, following injection of the antisense oligonucleotides, only weak fluorescent signals for both proteins were observed in 2- and 4-cell embryos. By the 8-cell stage, however, the signal strength had increased to a level close to that of controls. These observations established that injection of antisense oligonucleotides at the 1-cell stage led to a depletion of the target HMG protein by the 2- and 4-cell stages. This depletion was transient, however, and the protein quantity returned to near normal levels by about the

8-cell stage. Thus, injected oligonucleotides appear to provide an effective method to transiently deplete the cellular supply of HMG-14 and HMG-17.

3.5 Preimplantation development of embryos injected with antisense oligonucleotides

Having shown that HMG-14 and HMG-17 proteins could be transiently depleted form mouse embryos, I then investigated whether the absence of these proteins affected the development of the embryos. The results of the previous section demonstrated that, despite the transient depletion of the proteins, embryos could reach the 8-cell stage. Thus, a normal quantity of the proteins apparently is not absolutely required for progression through the early embryonic cell cycles. As HMG-14 and HMG-17 are thought to increase the rate of transcription, it may be hypothesized that they might be required to maintain the normal timing of development. Thus, embryos might develop relatively slowly in the absence of the proteins. To test this, 1-cell embryos were injected with antisense oligonucleotides, or with sense or nonsense oligonucleotides as controls, and the proteins were depleted in an individual and combined manner.

3.5.1 Development of embryos injected with either HMG-14 or HMG-17 antisense oligonucleotides

HMG-14 and HMG-17 proteins are closely related proteins and their function has been attributed to enhance transcription. There has been no experiments done, however, to directly discriminate between the functions of both proteins. Depletion of either protein from mouse embryos would directly indicate whether these two proteins perform a similar or different function. To establish such result, the following experiments were carried out; HMG-14 or HMG-17 antisense oligonucleotides were injected into 1-cell embryos. As a control, a group of embryos were left without injection in each case. Following microinjection and overnight culture, those that cleaved to the 2-cell stage from each group were selected. These were further cultured, examined at regular intervals, and the developmental stage of each embryo, as indicated by cell number, was recorded.

Table 4 and 5 show the results of experiments done using 0.1 μg/μl phosphorothioate-linked HMG-14 and HMG-17 antisense oligonucleotides, respectively. To facilitate interpretation of the results, these have also been represented in Figure 13 and 14, respectively. As can be interpreted from these figures, there is no significant difference between embryos injected with either HMG-14 or HMG-17 antisense oligonucleotides and control non-injected embryos. The small difference between injected and non-injected embryos in both cases may be attributed, as indicated below, to be a result of microinjection manipulation. These results demonstrate that depletion of either HMG-14 or HMG-17 proteins individually from mouse embryos has no effect on the rate of development of those embryos and suggest that HMG-14 and HMG-17 proteins may indeed perform a similar function, in which the absence of one protein is being compensated in the embryo by the presence of the other.

3.5.2 Growth retardation of embryos injected with a mixture of HMG-14 and HMG-17 antisense oligonucleotides

To test the effect of depletion of both HMG-14 and HMG-17 proteins on the rate of embryonic development, the following experiments were carried out. An equal mix of HMG-14 and HMG-17 antisense oligonucleotides was injected into 1-cell embryos. As a control, other groups of embryos either were injected with nonsense or sense oligonucleotides or left without injection. Following microinjection and overnight culture, those that cleaved to the 2-cell stage from each group were selected. As in the previous experiments, these were further cultured, examined at regular intervals, and the developmental stage of each embryo, as indicated by cell number, was recorded.

Table 6 shows the results of two experiments in which embryos were injected with 0.1 μg/μl phosphorothioate-modified oligonucleotides. To facilitate interpretation of the results, these have also been represented in Figure 15. At 34 h post-injection, 56% of non-injected embryos had reached the 4-cell stage. By comparison, about 50% of the embryos injected with a nonsense oligonucleotide had reached the 4-cell stage. At 48 h, as shown in Table 6, some of the non-injected embryos had reached the morula stage while none of the nonsense injected embryos had passed the 8 cell stage. At 56 h,

92% of the non-injected embryos had reached the morula stage, compared with 58% of the embryos receiving nonsense oligonucleotides. These results suggest that injection of oligonucleotides into embryos was associated with a slight delay in the progression of a population of embryos through preimplantation development.

Injection of HMG-14 and HMG-17 antisense oligonucleotides into embryos, however, was associated with an effect on development. At 34h, only 33% of the embryos had reached the 4-cell stages, which is less than in the other groups. At 48 h, the fraction of embryos that had reached the 8-cell (37%) was also less than in the other groups. At this time, most of the remaining embryos were at the 4-cell stage. This indicated that those embryos which had been at the 2-cell stage at 34 h were not developmentally arrested but continued to progress through the cleavage divisions. At 56 h, 33.3% of the embryos had reached the morula stage, which again was less than in the other groups. Of the remainder, about half had reached the 8-cell stage. Thus, at each time point examined, the fraction of embryos that had reached a particular stage of development was less in embryos injected with both antisense oligonucleotides than in control groups. However, these embryos were clearly not developmentally arrested, but continued to progress through the cleavage divisions. These results suggested that, when embryos were injected with antisense oligonucleotides targeting HMG-14 and HMG-17, they progressed more slowly through preimplantation development.

The retarded progression through development described above, although observed in each of the two experiments, was moderate. To verify the results, similar microinjection experiments were carried out using non-modified oligonucleotides. One-cell embryos were injected with a mix HMG-14 and HMG-17 antisense (0.8 μg/μl) or with a sense HMG-17 (0.8 μg/μl) oligonucleotides. Three experiments were carried out as described above.

At each time point examined (Table 7, Figure 16), the antisense-injected embryos were developmentally delayed as compared to the sense-injected embryos. Furthermore, by comparing Figures 15 and 16, it is evident that this delay was substantially more pronounced than when phosphorothioate-linked oligonucleotides were employed. These results confirm that microinjection of antisense oligonucleotides

directed against HMG-14 and HMG-17 mRNAs into 1-cell embryos retarded their subsequent progression through development. In addition, they indicated that non-modified oligonucleotides, which can be injected at a higher concentration without causing a non-specific developmental block, were more effective than phosphorothioate-modified oligonucleotides.

CHAPTER 4

Discussion

4.1 Expression of HMG-14 and HMG-17 mRNAs and proteins in mouse oocytes and early embryos

The aim of my study was to investigate the pattern of expression and the function of the high mobility group proteins HMG-14 and HMG-17 during the early stages of development in mouse embryos. Here I report, based on RT-PCR analysis, that mRNAs encoding HMG-14 and HMG-17 are present in oocytes and throughout all preimplantation embryonic stages investigated. As judged by the RT-PCR analysis, the mRNAs encoding HMG-14 and HMG-17 both follow a similar pattern of regulation. They are relatively abundant in fully grown-prophase I arrested oocytes and then decline as the embryo develops to the 2-cell stage. After the 2-cell stage, HMG-14 and HMG-17 mRNAs increase in abundance as the embryo develops to further stages.

Using RT-PCR assay, differences in signal strength observed at different developmental stages might be due to variability in the recovery of RNA, reverse transcription, or PCR procedure. However, this is unlikely to be the case here, because of the following reasons. First; all stages were processed for RNA extraction, cDNA synthesis and PCR amplification simultaneously, therefore, any decrease or increase in the intensity of PCR product at one particular stage would not likely reflect an artifact that happened for that particular stage. Second, the same differences between stages were observed in several independent replicates. Third, as an additional test, an exogenous RNA species, encoding the *Drosophila* vasa protein, was added to embryos before RNA extraction and thus was subjected to the same conditions as the endogenous mRNAs. Using this procedure, I was able to coamplify HMG-17 mRNA with vasa RNA when ~ 60-100 fg vasa RNA was added per embryo. I had difficulty, however, in coamplifying HMG-14 mRNA with vasa RNA. Although the reason for this is unknown, it has previously been reported that, the relative efficiencies of co-amplification differ greatly between two primers pairs and others, in which the presence

of one pair, in some cases, inhibits the amplification of the other (Foley and Engle, 1992). Based on co-amplification of HMG-17 and vasa, it may be concluded that differences in HMG-14 and HMG-17 mRNA observed at different stages of embryogenesis were not artifacts due to differences in RNA recovery or RT-PCR efficiency.

The decline in mRNA abundance that was observed between the fully grown oocytes and the 2-cell stage and the re-accumulation beyond the 2-cell stage are consistent with previous observations. Fully grown prophase I-arrested oocytes are transcriptionally active, but transcription ceases during oocyte maturation and remains undetectable after fertilization (Wassarman and Letourneau, 1976; Brower et al., 1981; DeLeon et al., 1983). The mRNA synthesized by the oocyte directs early development, but the destruction of this maternal RNA is initiated by oocyte maturation and is essentially complete by the 2-cell stage (Bachvarova and DeLeon, 1980; Bachvarove et al., 1985; Paynton et al., 1988). Some embryonic genes are expressed as early as the late 1-cell stage, but the major transcriptional activation of the embryonic genome occurs at G2 phase of the 2-cell stage (Flach et al., 1982; Sawicki et al., 1982; Bensaude et al., 1983; Latham et al., 1991; Manejwala et al., 1991; Schultz, 1993). Thus, it appears that maternally encoded HMG-14 and HMG-17 mRNAs are present in oocytes and early embryos and become almost completely destroyed by the early-2-cell stage, and that embryonically encoded transcripts are produced beginning at the mid 2-cell stage.

Earlier we found out that mRNAs encoding the histones, which are major components of chromatin, are much more abundant during S-phase than during other phases in mouse embryos (Clarke et al., 1998), as previously shown in somatic cells (Marzluff and Pandey, 1988; Harris et al., 1991; Heintz, 1991), thus co-ordinating histone synthesis with DNA replication. As HMG-14 and HMG-17 are also abundant chromatin components, I examined whether the abundance of the encoding mRNAs was also cell cycle regulated. Analysis using RT-PCR showed that their mRNAs were present during all phases of the cell cycle (Figure 4). It has also been observed using RT-PCR analysis of synchronized HeLa cells that mRNAs encoding HMG-14 and HMG-17 are present throughout all phases of the cell cycle (Bustin et al., 1987).

Therefore, there do not appear to be major differences in the quantity of these transcripts present at different stages of the cell cycle. Interestingly, the observation that HMG-14 and HMG-17 are expressed as early as the S phase of the 2-cell stage makes them among the earliest genes to be expressed when embryonic genome activation takes place.

Both HMG-14 and HMG-17 proteins, as revealed by immunofluorescence, are present in the nuclei of fully grown-prophase I arrested oocytes and of embryos at the 2-cell stage and beyond. However, no nuclear staining of either HMG-14 or HMG-17 could be detected on the condensed chromosomes of metaphase II-arrested oocytes, and only a very weak nuclear staining of both proteins was observed in the 1-cell embryos.

These results indicate the following; first, HMG-14 and HMG-17 mRNAs are present in every stage of early mouse development; second, embryonic HMG-14 and HMG-17 transcripts are detectable by mid 2-cell stage and increase in their abundance as the embryo develops to further stages; third, HMG-14/-17 proteins are detectable in the nuclei of transcriptionally active cells and undetectable in the nuclei of transcriptionally inert cells and both proteins associate more with the nuclei of the cells as the embryo develops beyond the 2-cell stage.

This constitutive expression of HMG-14/-17 mRNAs throughout all developmental stages and their early expression at the 2-cell stage as well as the increase in their abundance as the embryo develops to further stages strongly suggest that these two proteins perhaps are essential for the embryo to successfully progress through the cleavage stages. The observation that HMG-14 and HMG-17 proteins are detectable in the nuclei of transcriptionally active cells and undetectable in the nuclei of transcriptionally inert cells, is in good support for the data indicating that the function of these two proteins is related to transcription (Crippa et al., 1993; Ding et al., 1994; Trieschmann et al., 1995). Furthermore, the increase of nuclear association of these proteins as the embryo develops beyond the 2-cell stage adds another indication that the function of these two proteins is necessary for the progression of the embryo to further stages.

4.2 Strategies to deplete cellular proteins from mouse oocytes and early embryos

As an approach to address the function of HMG-14 and HMG-17 proteins and their potential role during early mouse embryogenesis, I chose to deplete these two proteins from the mouse embryo in an individual and combined manner and study the effect of absence of either or both proteins on the development of mutated embryos.

My strategy to deplete these proteins was to target their encoding mRNAs using antisense techniques. Alteration of gene expression by antisense techniques has caught a large amount of attention during the past few years since this may provide important applications in the genetic analysis of complex organisms using a technique that is simpler than the time-consuming and difficult gene-knockout method.

The 1-cell embryo was chosen to target the mRNAs encoding these two proteins for several reasons. First, there is ~ 20 hours between fertilization and the first cleavage, which is enough time to manipulate the embryo in order to destroy the target mRNA. Second, 1-cell embryo is the stage prior to the stage in which embryonic genome activation occurs, so depletion of these two proteins before EGA would directly address their role in the initiation of EGA and, depending on for how long the proteins remained depleted, would address their role in maintaining embryonic gene activity. Third, because there is no transcription at early phases of 1-cell stage embryo, then destruction of the maternal mRNAs encoding HMG-14 and HMG-17 would, in principle, prevent new synthesis of these proteins. However, since previous studies showed that HMG-14 and HMG-17 proteins are relatively stable proteins (Bustin et al., 1992), destruction of their mRNAs at the 1-cell stage might not completely eliminate these two proteins by the 2-cell stage.

4.2.1 Plasmids expressing antisense constructs

The first strategy to destroy the mRNAs encoding for HMG-14 and HMG-17 was to inject plasmids encoding HMG-14 and HMG-17 antisense RNAs into the male pronuclei of 1-cell stage embryos. Several lines of experiments showed that plasmids encoding antisense genes were successful in altering the expression of both endogenous and exogenous genes when transferred to cultured cells. For example, cotransfecting of

3T6 mouse fibroblasts with plasmids encode for the sense and the antisense RNAs of *E.coli lacZ*, gave less β-galactosidase activity than those cells transfected with only sense *lacZ* plasmid (Rubenstein, et al., 1984), transfection and microinjection of plasmid encoding the antisense RNA of the actin gene in eukaryotic cells, suppressed the endogenous actin gene activity (Izant and Weintraub, 1985), transfection of *Drosophila* tissue culture cells with plasmid encoding the antisense RNA of the heat shock protein (hsp26), gave much less hsp26 after heat shock than did untransformed cells, and the expression of closely related heat shock proteins hsp22, hsp23, and hsp28 was not affected indicating that inhibition of expression of endogenous genes using antisense genes is very specific (McGarry and Lindquist,1986), introducing a vector expressing antisense RNA complementary to eLF-4E into rat embryo fibroblasts resulted in over 50% reduction of the normal level of eIF-4E (Rinker-Schaeffer et al., 1993), transfection of human hepatoblastoma cell lines with vectors carrying an antisense gene from the hepatitis B virus inhibited HBeAg expression by 71% (Ji W. and St C. W. 1997), and transfection of lung carcinoma cell lines with antisense cDNA constructs encompassing different regions of the c-erbB-2 gene, significantly reduced the c-erbB-2 RNA and protein levels (Casalini et al., 1997).

However, there are no previous studies show the alteration of gene expression using antisense genes in mouse embryos. Therefore, I attempted to carryout such study, but before going through injection of the antisense RNA-encoding plasmids, it was necessary, first, to examine the expression of injected plasmid coding for a reporter gene in terms of percentage of embryos express the reporter gene and how long during development the reporter gene would be expressed. The plasmid chosen to be examined was p610ZA encoding for the *lacZ* gene driven by the mammalian hsp68 promoter. This plasmid was chosen because the heat-shock genes, hsc70 and hsp68, are among the first to be expresses in mouse embryos (Bensaude et al., 1983; Howlett and Bolton, 1985; Manejwala et al., 1991), and plasmids encoding a *lacZ* gene driven by the hsp68 promoter are expressed following injection into pronuclei of 1-cell stage (Bevilacqua and Mangia, 1993). Thus, the antisense transcripts would be expected to be present

when the embryonic HMG-14 and HMG-17 genes became activated at the mid-2-cell stage.

Injection of plasmid p610ZA into the male pronuclei of 1-cell stage embryos resulted in an expression of the *lacZ* gene and detection of β-galactosidase activity in different developmental stages (Figure 6). However, I observed that the rate of expression of the reporter gene varied considerably among experiments. The average rate of expression in 11 experiments comprising 883 injected embryos was about 40%, which is close to values reported by other laboratories (Bevilacqua and Mangia, 1993). The reason why a large percentage of embryos do not express a detectable *LacZ* activity remains unclear. However, since it is not clearly understood whether injected plasmid integrates into the chromosomes or remains in the nucleus and assemble into chromatin, at least two explanations could be interpreted from these results. First, if the injected DNA remains in the nuclear matrix, it is possible, however, that it does not always assemble into chromatin, perhaps due to the limitation in resources. Second, if the injected DNA integrates into the chromosomes, its integration must be random due to the absence of any homologous regions between injected and endogenous DNA sequences. Therefore, it is possible that it occasionally integrates in silent regions in the chromosomes, such as heterochromatin regions, resulting in its silencing. This could explain why some embryos express injected DNA and some do not as well as the variation of expression observed between the different experiments. A further potential limitation of this construct is that transcription of the endogenous hsp68 gene is down-regulated at the 4-cell stage (Christians et al., 1995; Bevilacqua et al., 1995), and this may also occur in the *hsp68-lacZ* construct. Consistent with this, I observed that expression of the *lacZ* declined in embryos beyond the 2-cell stage (not shown). Based on insufficiently high frequency of expression and its loss beyond the 2-cell stage, this *hsp-lacz* construct was judged unsatisfactory to carry out the planned antisense studies.

I next tested a different reporter gene driven by different promoter, namely, pGFP-N1 carrying the GFP gene under the control of the CMV promoter. Using GFP as a reporter gene, expression can be detected in living cells under UV illumination, and

this would allow positive cells to be selected for culture, overcoming the limitation of a 40% expression rate. However, this plasmid was expressed at a much lower frequency than plasmid p610ZA (Table 1). This could reflect a lower activity of the CMV promoter in mouse embryos compared to the hsp68 promoter. Even when the GFP was placed under the control of the hsp68 promoter, however, expression frequency was restored only to about half the level of the *hsp-lacZ* construct. This could be due at least to two reasons. First, the staining procedure for β-galactosidase activity may detect a smaller quantity of protein than the fluorescent detection of GFP. Second, the stability of β-galactosidase mRNA or protein inside mouse embryos could be higher than that of GFP mRNA or protein. The latter explanation could account for the fact that GFP was detected only in 1-cell embryos. Due to the low and inconsistent expression of different reporter genes with different promoters, alternative strategies had to be considered.

4.2.2 In vitro-transcribed RNA

The next strategy to deplete HMG-14 and HMG-17 mRNAs from mouse embryos was to inject in vitro transcribed HMG-14 and HMG-17 antisense RNAs (RNAs complementary to HMG-14 and HMG-17 mRNAs). An advantage of this strategy is that it would not depend on activity of a promoter in the embryonic cells. Antisense RNAs have been used in a variety of cell types, including *Xenopus* oocytes, to inactivate an endogenous mRNA. For example, Harland and Weintraub (1985), showed that injection of thymidine kinase (TK) and chloramphenicol acetyl transferase (CAT) antisense RNAs into *Xenopus* oocytes cytoplasm inhibited expression of injected sense TK and CAT mRNAs. With respect to endogenous mRNAs, Melton (1985), showed that in vitro transcribed globin antisense RNA injected into *Xenopus* oocytes formed a hybrid with globin mRNA and selectively prevented its translation, and Wormington (1986), selectively and efficiently inhibited synthesis of an endogenous ribosomal protein, L1, by microinjection of antisense L1 RNA and demonstrated using an RNase-protection assay that endogenous L1 mRNA and injected antisense RNA formed RNA:RNA duplexes. In *Drosophila*, Rosenberg et al., (1985) showed that injection of

in vitro transcribed Kruppel (Kr) gene antisense RNA resulted in development of those antisense injected embryos into phenocopies of Kr mutant embryos. Antisense RNAs have also been used in mouse oocytes and embryos. Strickland et al., (1988), showed that injection of antisense RNA complementary to both coding and noncoding portions of tissue plasminogen activator (t-PA) into mouse oocytes resulted in a cleavage of the RNA:RNA hybrid region, yielding unstable mRNA that was not translated and Bevilacqua et al., (1988), showed that injection of 1-cell embryos with β-glucuronidase antisense RNA resulted in almost 50% inhibition of β-glucuronidase activity at later stages. Ao and Erickson (1992) showed that injection of antisense E-cadherin-RNA into each cell of two-cell embryos resulted in a delay in compaction of antisense injected embryos as compared to sense-RNA injected embryos.

To test whether HMG-14 and HMG-17 antisense RNAs are effective in depleting HMG-14 and HMG-17 endogenous mRNAs in mouse embryos, HMG-14 and HMG-17 antisense RNAs were in vitro transcribed and injected into the cytoplasm of 1-cell embryos. These cells were cultured usually for 20 hrs and total RNA was then extracted and RT-PCR was performed to examine whether HMG-14 and HMG-17 mRNAs were present. In each experiment either HMG-14 or HMG-17 antisense RNA was injected. Results from two experiments for HMG-14 and two experiments for HMG-17 revealed that PCR products corresponding to HMG-14 and HMG-17 mRNAs could be detected after injection of their antisense RNAs (Figure 8). Several possible explanations may account for the presence of PCR products in the antisense-injected embryos and these are discussed below.

First, the injected RNA may have been rapidly degraded. This could be due to its structure; for example it contained no poly(A) tail, which promotes mRNA stability (Brawerman, 1981) or together with massive degradation of maternal RNA that occurs after fertilization and before embryonic genome activation (Flach et al., 1982). To test the stability of injected HMG-14 and HMG-17 antisense RNAs, HMG-14 and HMG-17 antisense RNAs labeled with DIG were prepared and injected into the cytoplasm of 1-

cell embryos. Staining of 2-cell stages, after 24 hrs of injection, with anti-DIG antibody revealed that injected RNAs were detectable at least up to this stage (Figure 7).

Second, since a full length HMG-14 and HMG-17 antisense RNAs were used, the injected antisense RNAs may have taken a secondary structure that inhibited their binding to the target mRNAs. Antisense RNA complementary to large portion of target mRNA, however, was shown to inhibit the activity of target gene (Coleman et al., 1984).

Third, the injected antisense RNAs may have failed to stably hybridize to the target mRNAs. An activity that unwinds RNA:RNA duplexes exists at high levels in *Xenopus* eggs and early embryos, although it is absent in oocytes and late blastula embryos, and this has limited the use of antisense RNA to inactivate mRNAs in the early amphibian embryo (Rebagliati and Melton,1987, Bass and Weintraub,1987). In the mouse, Strickland et al., (1988) observed that injection of antisense RNA complementary to both coding and noncoding portions of tissue plasminogen activator into mouse oocytes resulted in a cleavage of the RNA:RNA hybrid region, implying that, unwinding activity was not present, and cleaving activity was present in mouse oocytes. The fact that antisense RNA also was effective in mouse embryos (Bevilacqua et al., 1988) implies that an unwinding activity is not present in embryos, although in this case RNA levels were not directly measured.

Fourth, there may not be an activity in mouse embryos that works to cleave the formed RNA:RNA duplex. If the RNA:RNA duplex is not being unwound in mouse embryos, it remains possible, however, that it is not being degraded due to the absence of cleaving activity that recognizes and destroys RNA:RNA hybrids. However, this cleavage activity has not, so far, been reported in mouse embryos and the persistence of HMG-14 and HMG-17 mRNAs after injection of their antisense RNAs could be indeed due to the absence of cleaving activity in mouse embryos.

Fifth, the signals detected for HMG-14 and HMG-17 might have been due to extensively newly synthesized mRNAs after the embryonic genome activation starts at the 2-cell stage. It is still possible, however, that mouse embryos possess cleaving activity as well, and the signals obtained for HMG-14 and HMG-17 are due to

extensively newly synthesized HMG-14 and HMG-17 mRNAs since RNA extraction was done around late G2 phase of 2cell embryos and as shown above HMG-14 and HMG-17 mRNAs were detected as early as the S phase. The signals obtained, however, were not likely due to excess of non-hybridizing antisense RNAs remained undigested and served as a template for cDNA synthesis and subsequent PCR amplification because cDNA was synthesized using oligo-dT and not random hexamer primers and that in vitro transcribed antisense RNAs do not contain poly-A tails, therefore, no cDNA could be synthesized from them.

Bevilacqua et al., (1988) showed that the RNA:RNA unwinding activity is not present in mouse embryos. However, they observed differences between capped and uncapped antisense RNAs, and between targeting different regions in the targeted mRNA in terms of inhibition. These observations were also observed by others, Leibhaber et al., (1984) showed that antisense hybridization to the 5' region of β-globin mRNA, including the ribosomal binding site, is important for inhibition of translation of β-globin mRNA in an in vitro system. On the other hand, Kim and Wold (1985) observed that antisense constructs complementary to either the 3' or 5' end of the herpes simplex thymidine kinase gene gave no difference in the degree of inhibition. Thus, the effectiveness of inhibition by antisense RNAs appears to be variable among the different target mRNAs and depends upon the particular gene selected for study, therefore, each mRNA must be tested experimentally.

4.2.3 Antisense oligonucleotides

The next strategy examined, was to inject antisense oligodeoxynucleotides complementary to the 5' end of HMG-14 and HMG-17 mRNAs, starting at few bases prior to the translation initiation codon. Injection of HMG-14 and HMG-17 antisense oligonucleotides into the 1-cell stage embryos resulted in transient depletion of HMG-14 and HMG-17 proteins during subsequent stages, in which both proteins were depleted at the 2- and 4-cell stages and were restored back near to their normal level at the 8- and subsequent-cell stages (Figures 11 and 12.). These results demonstrate that

HMG-14 and HMG-17 antisense oligonucleotides were effective in transiently depleting HMG-14 and HMG-17 proteins from mouse embryos. Furthermore, injection of unmodified HMG-14 antisense oligonucleotides into prophase I-arrested oocytes (GVs) resulted in diminishing of HMG-14 mRNA in those oocytes as judged by the low level of HMG-14 PCR product obtained after antisense but not nonsense oligonucleotides injection (Figure 9).

The use of antisense DNA fragments to block the translation or processing of specific mRNAs was first demonstrated around 20 years ago (Zamecnik,1978 and Stephenson, 1978). Since then, numerous studies have shown that antisense oligonucleotides are able to block the translation of selected mRNAs (Kawasaki, 1985; Minshull and Huut, 1986; Cazenave et al., 1987; Goodchild et al., 1988; Cazenave et al., 1989; Caceres et al., 1991; Sadler et al., 1995; Bavik et al., 1996; Tsark et al., 1997; and Dorri et al., 1997).

Antisense oligonucleotides have also been applied to deplete different proteins in mouse oocytes. For example, O'Keefe et al., (1989) and Paules et al., (1989), showed that injection of c-mos antisense oligonucleotides into mouse oocytes gave similar patterns to c-mos knockout, Tong et al., (1995) showed that injection of antisense oligonucleotides targeting the 5' ends of zona pellucida (ZP2 and ZP3) mRNAs abolished the synthesis of targeted zona pellucida proteins and that the targeted mRNAs were degraded as revealed by the sensitive RNase protection assay. Degradation of selected mRNAs was successfully done using other systems. Cazenave et al., (1986) showed that the use of antisense oligonucleotides leads to translation arrest of complementary mRNAs in the wheat germ translation system by causing a degradation of mRNA. Dash et al., (1987) showed that injection of antisense oligonucleotides into *Xenopus* oocytes resulted in a complete degradation of both injected and endogenous mRNAs by means of RNase H-like activity. Walder et al., (1988) showed that addition of α- and β-globin antisense oligonucleotides to freshly prepared rabbit reticultocyte lysates inhibited the translation of targeted mRNA and that nearly 100% of the targeted mRNAs were degraded at the site of hybridization. The authors also showed that

antisense oligonucleotides spanning the initiation codon were most effective. This site selection of inhibition was also observed by Goodchild et al., (1988), in which the sites most sensitive to translation inhibition of the rabbit beta-globin mRNA were found to be the beginning of the 5' noncoding region and a sequence including the initiation codon and several upstream bases.

On the contrary, injection of phosphorothioate-linked HMG-14 antisense oligonucleotides into mouse oocytes did not lead to noticable loss of HMG-14 mRNA (Figure 9). This could be explained by the fact that modified oligonucleotides are more resistant to degradation by RNase-H activity. Indeed, Agrawal et al., (1990), in a comparison study between unmodified and differently modified oligonucleotides with respect to their ability to be acted on by ribonuclease H activities present in the nuclear extracts of HeLa cells after hybridization with complementary sequences in RNA, clearly demonstrated that phosphorothiaoate-linked oligonucleotides were much more resistant to cleavage with RNase H than unmodified oligonucleotides were.

The use of modified antisense oligonucleotides have caught a great deal of interest among researchers where the objective was to find modifications that permit hybridization with RNA on one hand but that are more metabolically stable than unmodified oligonucleotides on the other hand. Among the various modifications done for oligonucleotides, phosphorothioate-linked oligonucleotides, in which the backbone phosphodiester groups were replaced with phosphorothioate groups, were found to be most effective. Phosphorothioate-linked antisense oligonucleotides have been previously used to inhibit the translation of various proteins. Agrawal et al., (1989) showed that phosphorothiaoate antisense oligomers were up to 100 times more potent in their inhibition efficiency of replication and expression of the HIV virus than unmodified oligomers of the same concentration. Lisziewicz et al., (1992) showed that five phosphorothioate oligonucleotides complementary to different regions of HIV-1 RNA, blocked replication of the virus in a sequence-specific manner. Augustine et al., (1995) showed that injection of En-1 and En-2 phosphorothioate antisense oligonucleotides into early mouse somite embryos resulted in inhibition of En-1 and En-2 proteins. Quaggin et al., (1997) showed that incubation of embryonic mouse kidney cells with

Cux-1 phosphorothioate antisense oligonucleotides caused increased apoptosis in antisense but not sense treated cells.

Injection of unmodified HMG-14 into the 1-cell stage, also resulted in diminishing of HMG-14 mRNA as compared to non-injected embryos (Figure 10). Injection of modified HMG-14 and HMG-17 antisense oligonucleotides into the 1-cell stage embryos, however, did not result in any loss of their target mRNAs (not shown). The diminishing degree of HMG-14 mRNA observed after injection of antisense oligonucleotides into embryos, however, was not as pronounced as that observed in oocytes. This suggests that mouse embryos may not possess as high RNase H activity as oocytes do. No previous experiments, however, demonstrated that mouse embryos possess such activity. Therefore, the observation that HMG-14 and HMG-17 proteins were depleted at the 2- and 4-cell stages indicates that although injection of HMG-14 and HMG-17 antisense oligonucleotides into 1-cell mouse embryos did not result in complete degradation of the mRNAs, the corresponding proteins, nonetheless, were depleted.

4.3 Effect of HMG-14 and HMG-17 transient depletion on the development of mouse embryos

The precise function of HMG-14 and HMG-17 proteins is not fully understood. Several lines of experiments, however, indicated that these two proteins associate with regions of active genes in chromosomes and work to enhance both the initiation and elongation of transcription. Immunofractionation experiments, for example, revealed that these proteins preferentially associate with transcriptionally active regions (Druckmann et al., 1986). Antibodies against HMG-14 bind specifically to transcriptionally active genes (Westermann and Grossbach, 1984). Addition of HMG-14/-17 proteins to in vitro assembled *Xenopus* chromatin increased the transcriptional potential of a polymerase III transcribed gene 5-S RNA when the proteins were added during but not after the chromatin assembly (Crippa et al., 1993; Trieschmann et al., 1995). Addition of HMG-17 to assembled *Drosophila* chromatin stimulated transcription initiation by RNA polymerase II (Paranjape et al., 1995). Increased level of

HMG-14 protein stimulated the rate of RNA polymerase II elongation but not the level of initiation of transcription (Ding et al., 1994), and microinjection of anti-HMG-17 antibodies into human fibroblast inhibited transcription (Einck and Bustin, 1983).

4.3.1 Effect of HMG-14 and HMG-17 depletion on initiation of Embryonic Genome Activation (EGA)

Previous studies showed that injection of HMG-17 antibodies into human somatic cells inhibited transcription (Einck and Bustin,1983). It was interesting, therefore, to test if depletion of both HMG-14 and HMG-17 proteins prior to the embryonic genome activation would result in inhibition of transcription and arrest of mutated embryos at the 2-cell stage, since transcription was shown to be required for subsequent cleavages (Golbus et al.,1973, Bolton et al.,1984, Poueymirou and Schultz, 1989).

Depletion of HMG-14 and HMG-17 proteins at the 2-cell stage by antisense oligonucleotides, however, did not result in arrest of those mutated embryos. Development of antisense injected embryos up to the 8-cell stage despite low levels of HMG-14 or HMG-17 proteins suggests that normal quantity of the proteins perhaps is not absolutely required for initiation of the embryonic genome activation or for progression through the early embryonic cell cycles. These results, however, do not exclude the role of HMG-14 and HMG-17 in transcription regulation and the observation that these proteins may not be required for the initiation of EGA suggests that this event is a highly complex process that probably involves a large number of regulators. A developmental study to examine the effect of depletion of either protein on the rate of embryonic development was performed. In these experiments mutated embryos were cultured to further stages and their rate of development was recorded and compared to controls.

4.3.2 Effect of depletion of either HMG-14 or HMG-17 on the rate of embryonic development

HMG-14 and HMG-17 proteins are closely related proteins and most of the previous studies suggest that both proteins perform a similar function. Some other experiments, however, suggest that both proteins may perform different functions. For example, Postinkov et al., (1995) showed that each nucleosome has two binding sites for either HMG-14 or HMG-17 suggesting that both proteins may indeed perform different functions or affect the transcription of different sets of genes. Barratt et al., (1994) found that immediate-early gene transcription after mitogenic stimulation is associated with rapid and extensive phosphorylation of HMG-14 but not HMG-17. There are no previous studies, however, that have directly discriminated between the function of both proteins.

Depleting of either HMG-14 or HMG-17 individually from mouse embryos would be a good system to test whether these two proteins perform a similar or different function. Injection of either HMG-14 or HMG-17 antisense oligonucleotides individually at the 1-cell stage of mouse embryos, however, did not result in a significant difference, in respect to their rate of development, as compared to control non injected embryos (Tables 4&5, Figures 13&14). These results suggest two possible interpretations. First, HMG-14 or HMG-17 may play no role on the rate of embryonic development and that embryos do not need either of them to develop normally. Second, HMG-14 and HMG-17 proteins may perform a similar function, at least with respect to embryonic development, and the absence of one protein is compensated by the presence of the other. It would be interesting to test whether depletion of HMG-14 results in upregulation of HMG-17 protein and vice versa.

4.3.3 Effect of depletion of both HMG-14 and HMG-17 on the rate of embryonic development

As the previous experiments showed that depletion of either HMG-14 or HMG-17 proteins did not result in a growth retardation of mutated embryos, next I examined whether depleting both proteins would result in growth retardation. A series of

developmental experiments were conducted, in which embryos were injected with a mixture of HMG-14 and HMG-17 antisense oligonucleotides at the one cell stage and their development to the blastocyst stage was monitored regularly along with control injected and non-injected embryos. Developmental pattern of embryos injected with phosphorothioate-linked HMG-14 and HMG-17 antisense oligonucleotides to the blastocyst stage as compared to embryos injected with phosphorothioate-linked nonsense oligonucleotides as well as non-injected embryos revealed that, antisense injected embryos developed more slowly than nonsense injected and non-injected embryos (Table 6 and Figure 15). At each time point examined, fewer embryos that had been injected with HMG-14/-17 antisense oligonucleotides reached a certain stage as compared to nonsense and non-injected control embryos. These results clearly demonstrate that transient depletion of both HMG-14 and HMG-17 from mouse embryos results in growth retardation of these embryos as compared to control embryos.

To test whether injection of higher concentrations of antisense oligonucleotides would result in a more dramatic affect, a series of developmental experiments using unmodified HMG-14 and HMG-17 antisense oligonucleotides was conducted. Injection of unmodified HMG-14 and HMG-17 antisense oligonucleotides at a concentration of 0.8 μg/μl into 1-cell embryos resulted in slow rate of development of those embryos as compared to sense injected embryos (Table 7 and Figure 16). The slow rate of development using unmodified HMG-14 and HMG-17 antisense oligonucleotides was more dramatic than that obtained using phosphorothioate-linked HMG-14 and HMG-17 antisense oligonucleotides, in which at each time point examined there was less than 50% of antisense injected embryos reached a certain stage as compared to sense injected embryos.

This retarded development implies that embryos progress through cell division cycles more slowly than normal embryos. In other words, embryos lacking HMG-14/-17 proteins remain longer in each cell cycle. This increase in the cell cycle time could reflect an increase in the time of all phases of the cell cycle, or could reflect a selective lengthening of one phase. At this time we can not discriminate whether depletion of

HMG-14/-17 proteins increases the duration of each phase of the cell cycle or specifically increases the time of a certain phases.

Based on previous results showing that HMG-14/-17 proteins positively enhance transcription, we propose that the increase in cell cycle time likely to be general and include every phase. Since both proteins were shown to enhance transcription, their depletion would probably down regulate transcription. Down regulation of transcription will result in less RNA and presumably less protein; therefore, each phase, in principle, could be affected. One way to test whether the effect of HMG-14/-17 depletion is specific or general for the cell cycle phases, is by monitoring the timing of DNA synthesis and the time of cleavage in HMG-14/-17 depleted and normal embryos. If DNA synthesis begins later in depleted embryos than in normal embryos, this will indicate that depleted embryos had longer G1 phase. The duration of DNA synthesis will indicate whether the length of the S phase is affected. If there is no difference in the G1 and S phases between both groups, then the difference in the length of the cell cycle observed must be in the G2 or M phase. Monitoring the time of cleavage will discriminate between these possibilities.

The findings of this study clearly demonstrate that injection of HMG-14 and HMG-17 antisense oligonucleotides results in transient depletion of HMG-14 and HMG-17 proteins from mouse embryos and that these proteins play an important role in keeping the normal progression of the embryo through the cleavage stages. Depleting either protein individually, however, did not result in a significant developmental delay, suggesting that these proteins perform a similar function. Our interpretation, based on these results, is that depletion of HMG-14 and HMG-17 proteins from mouse embryos results in down regulation of either the transcriptional process of certain genes that are responsible for driving the embryo through cleavage stages or the general transcriptional process in the embryo resulting in general slowing of development. Therefore, it can be concluded that HMG-14 and HMG-17 proteins are required for the embryo to develop normally and absence of these two protein results in growth retardation of the embryo.

4.4 Future studies

Based on previous studies showed that HMG-14 and HMG-17 proteins work to enhance transcription and since the developmental retardation of antisense injected embryos was interpreted as a result of down regulation of the transcription process in those embryos, I am carrying out another series of experiments to measure the transcriptional activity of embryos injected with HMG-14 and HMG-17 antisense oligonucleotides. Measuring the transcription level will be carried out as follows. One group of 1-cell embryos will be injected with HMG-14 and HMG-17 antisense oligonucleotides and another group will be injected with sense oligonucleotides. Both groups will then be cultured up to the late 2-cell stage or the early 4-cell stage. Embryos will then be permeablized using detergents and incubated in medium containing radio-labeled UTPs. Unincorporated UTPs will then be washed away and embryos will be harvested and the radio activity will be counted. Alternatively, BrUTP may be used. Recently, Aoki et al., (1997), showed a procedure to monitor and measure the transcription level of endogenous genes in preimplantation stages of mouse embryos. Applying either method will show whether depletion of HMG-14 and HMG-17 from mouse embryos is correlated with a decrease in the transcription level in these embryos as anticipated.

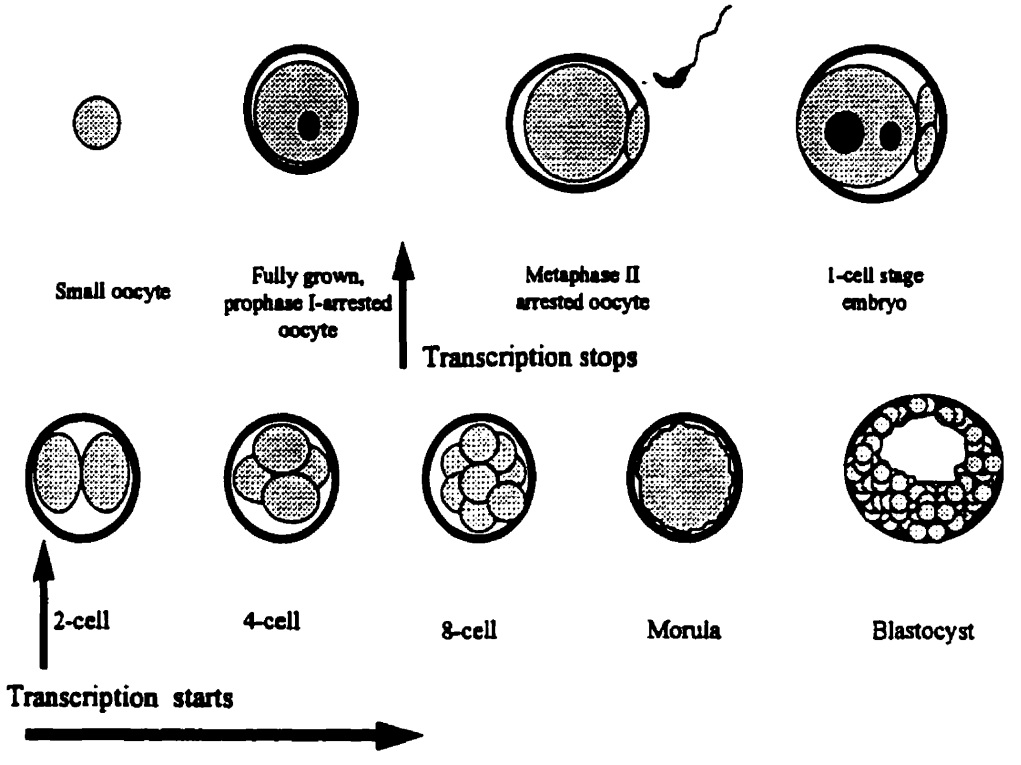
5

Figures and Tables

5.1 Figures

5.1.1 Figure 1

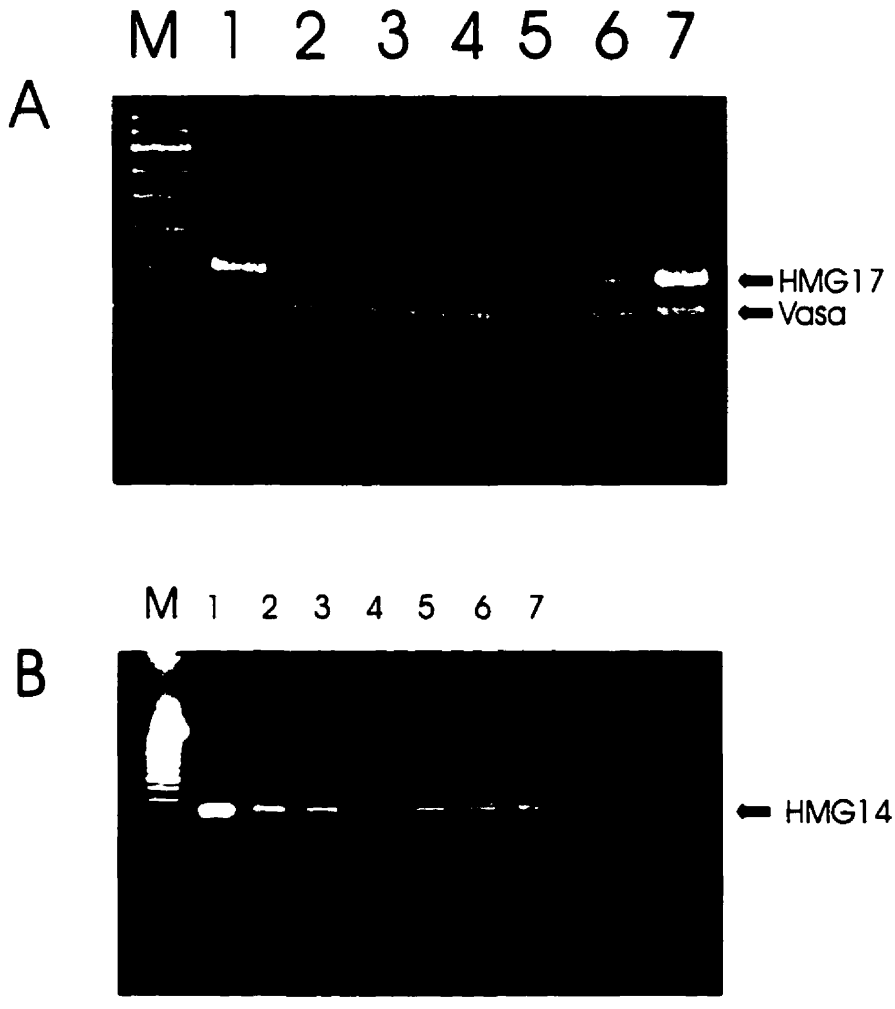
Schematic diagram represents the different developmental stages of mouse embryogenesis.


Small oocyte
Fully grown, prophase I-arrested oocyte
Metaphase II arrested oocyte
1-cell stage embryo
Transcription stops
2-cell
4-cell
8-cell
Morula
Blastocyst
Transcription starts

5.1.2 Figure 2

RT-PCR analysis of HMG-14 and HMG-17 mRNAs during preimplantation stages of mouse development.

30 embryos were collected from each stage, vasa RNA was added prior to RNA extraction, RNA was then extracted and cDNA was made from all stages simultaneously. cDNA was divided into two equal parts. One part equivalent to 15 embryos was used to detect HMG-17 mRNA and the other part to detect HMG-14 mRNA by PCR. PCR amplification of all stages was carried out simultaneously. **(A)** Co-amplification of HMG-17 mRNA with vasa RNA. **(B)** Amplification of HMG-14 mRNA. (M) 100 bp ladder; (1) Prophase I-arrested oocytes (GV); (2) Metaphase II-arrested oocytes; (3) 1-cell stage embryos; (4) 2-cell stage embryos; (5) 4-cell stage embryos; (6) 8-cell stage embryos; (7) Blastocyst stage embryos.


M 1 2 3 4 5 6 7
A
HMG17
Vasa
M 1 2 3 4 5 6 7
B
HMG14

5.1.3 Figure 3

Detection of HMG-14 and HMG-17 proteins during preimplantation stages of mouse development.

Confocal microscopy image of embryos stained with anti-HMG-14 and anti-HMG-17 antibodies. Embryos were fixed at different stages of development and processed for immunostaining using either HMG-14 or HMG-17 antibody. **(A)** 2-cell stage embryo stained with anti-HMG-14 antibody. **(B)** 4-cell stage embryo stained with anti-HMG-14 antibody. **(C)** 8-cell embryo stained with anti-HMG-14 antibody. **(D)** 2-cell embryo stained with anti-HMG-17 antibody. **(E)** 4-cell stage embryo stained with anti-HMG-17 antibody. **(F)** 8-cell stage embryo stained with anti-HMG-17 antibody. Magnification is about 60X.

5.1.4 Figure 4

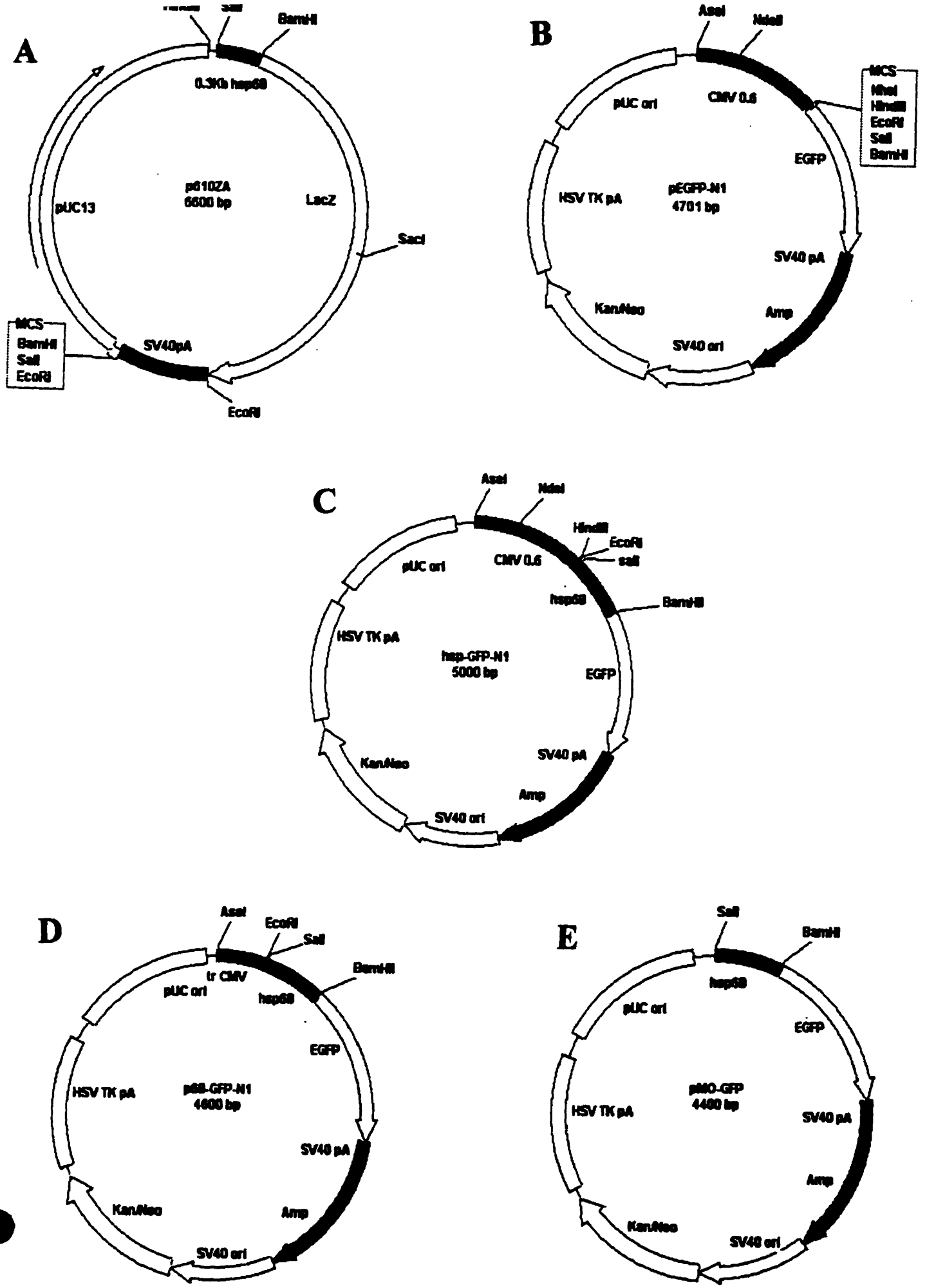
RT-PCR analysis of HMG-14 and HMG-17 mRNAs during cell cycle stages.

30 embryos were collected from each stage, vasa RNA was added prior to RNA extraction, RNA was then extracted and cDNA was made from all stages simultaneously. cDNA was divided into three equal parts. One part equivalent to 10 embryos was used to detect HMG-14 mRNA, the second part to detect HMG-14 mRNA and the third part to detect vasa RNA by PCR. PCR amplification of all stages was carried out simultaneously. **(A)** Amplification of HMG-14 mRNA at different stages of the cell cycle. (M) 100 bp ladder; (1) prophase I-arrested oocytes, (2) 1-cell stage embryos collected at G2 phase, (3) 2-cell stage embryos collected at G1 phase, (4) 2-cell stage embryos collected at S phase, (5) 2-cell stage embryos collected at G2 phase, (6) 4-cell stage embryos collected at G1 phase, (7) 4-cell stage embryos collected at S phase, (8) 4-cell stage embryos collected at G2 phase. **(B)** Amplification of HMG-17 mRNA at different stages of the cell cycle. (M) 100 bp ladder; (1) 2-cell stage embryos collected at S phase, (2) 2-cell stage embryos collected at G2 phase, (3) 4-cell stage embryos collected at G1 phase, (4) 4-cell stage embryos collected at S phase, (5) 4-cell stage embryos collected at G2 phase. **(C)** Amplification of vasa RNA as a control for RNA extraction, cDNA synthesis and PCR amplification. (M) 100 bp ladder; (1) 2-cell stage embryos collected at S phase, (2) 2-cell stage embryos collected at G2 phase, (3) 4-cell stage embryos collected at G1 phase, (4) 4-cell stage embryos collected at S phase, (5) 4-cell stage embryos collected at G2 phase.

5.1.5 Figure 5

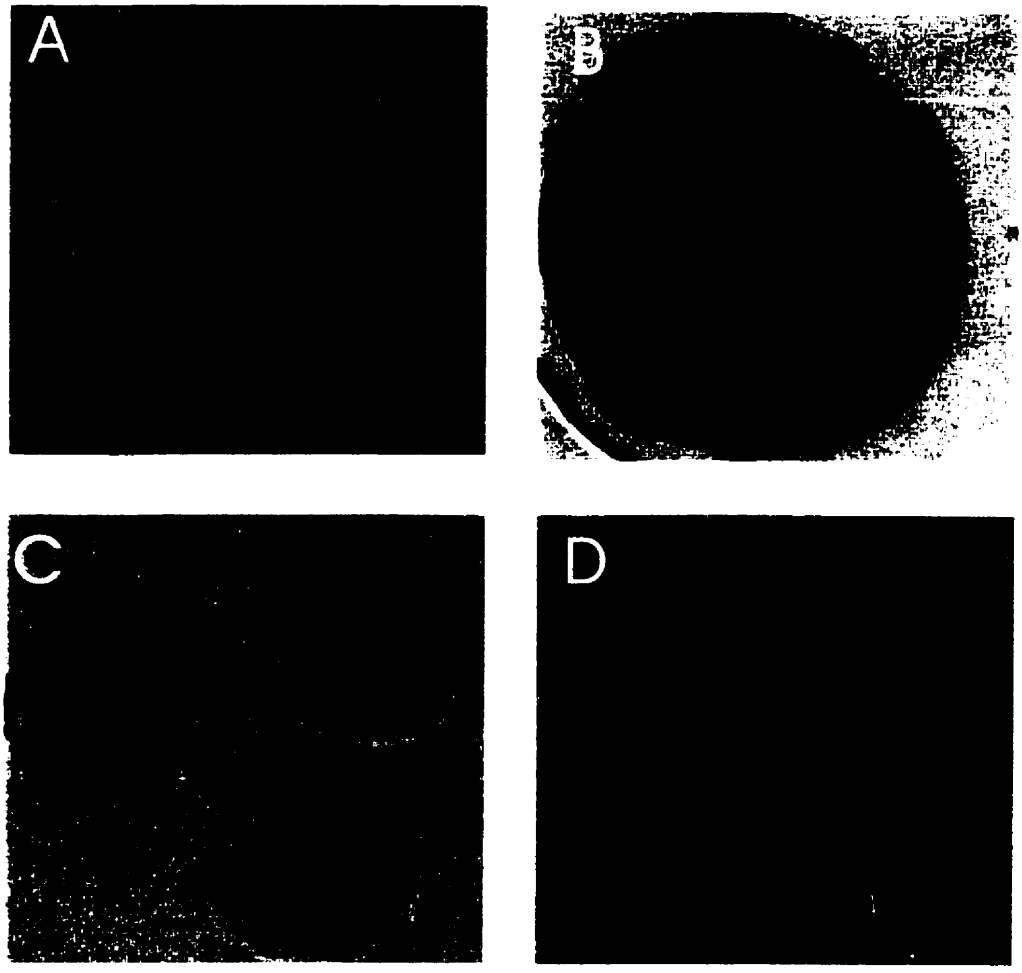
Schematic diagram of plasmids constructed and used for microinjection.

(A) Plasmid p610ZA encoding for the *lacz* gene driven by hsp68 promoter. **(B)** Plasmid pEGFP-N1 encoding for the GFP gene driven by CMV promoter. **(C)** Plasmid hsp-GFP-N1 encoding for the GFP gene driven by CMV and hsp68 promoters. **(D)** Plasmid p68-GFP-N1 encoding for the GFP gene driven by hsp68 promoter plus a 200 bp fragment from CMV promoter. **(E)** Plasmid pMO-GFP encoding for the GFP gene driven by hsp68 promoter.


A
BamHI
0.3Kb hsp68
pUC13
p610ZA
6600 bp
LacZ
SacI
MCS
BamHI
SalI
EcoRI
SV40pA
EcoRI
B
AseI
NdeI
MCS
NheI
HindIII
EcoRI
SalI
BamHI
pUC ori
CMV 0.6
EGFP
HSV TK pA
pEGFP-N1
4701 bp
SV40 pA
KanNeo
Amp
SV40 ori
C
AseI
NdeI
HindIII
EcoRI
sall
pUC ori
CMV 0.6
hsp68
BamHI
HSV TK pA
hsp-GFP-N1
5000 bp
EGFP
SV40 pA
KanNeo
Amp
SV40 ori
D
AseI
EcoRI
SalI
BamHI
tr CMV
pUC ori
hsp68
EGFP
HSV TK pA
p68-GFP-N1
4600 bp
SV40 pA
KanNeo
Amp
SV40 ori
E
SalI
BamHI
hsp68
pUC ori
EGFP
pMO-GFP
4400 bp
HSV TK pA
SV40 pA
Amp
KanNeo
SV40 ori

5.1.6 Figure 6

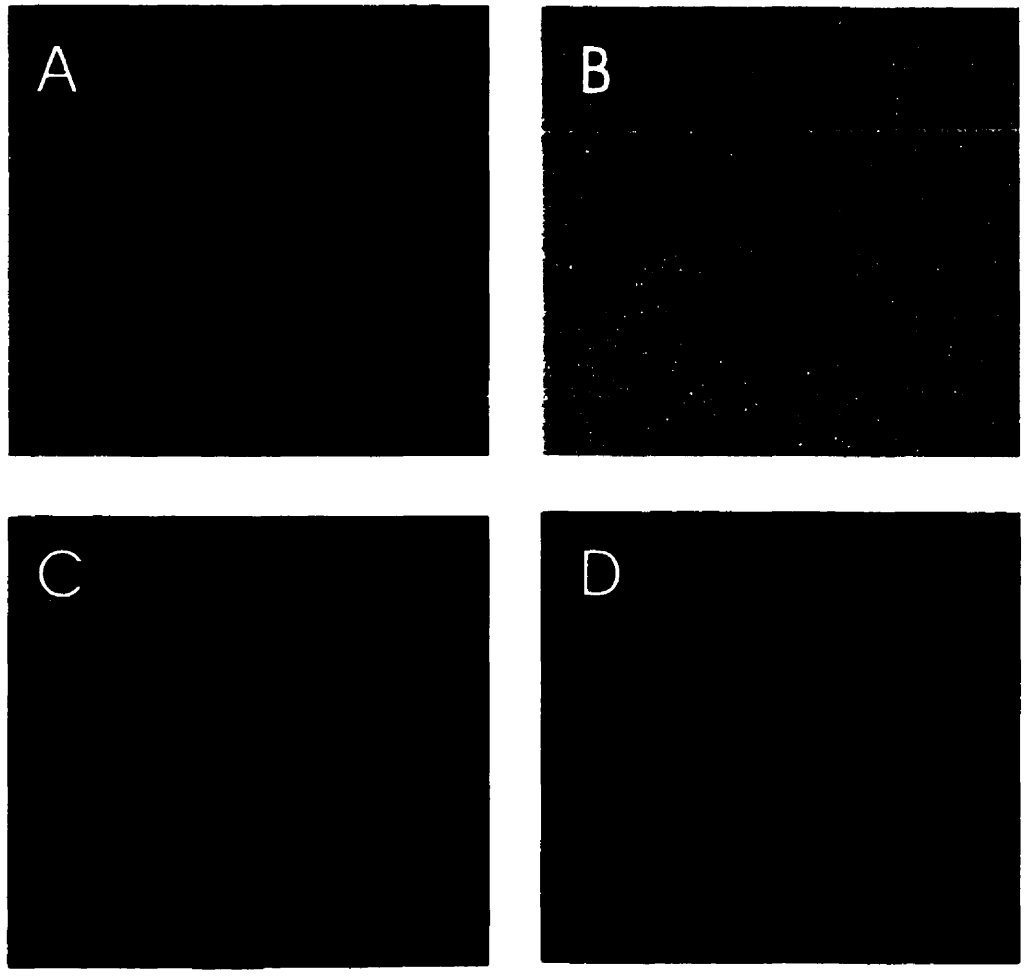
Expression of *lacz* gene during different stages of mouse preimplantation development. Embryos were injected with plasmid p610ZA into the male pronuclei of 1-cell stage, fixed 24 or 40 hrs after injection and processed for β-galactosidase activity. **(A)** 2-cell stage embryos that did not express the *lacz* gene after being injected at the 1-cell stage and fixed 24 hrs after injection. **(B)** Uncleaved 1-cell stage embryo that expressed the *lacz* gene after being injected 24 hrs earlier. **(C)** 2-cell stage embryos that expressed the *lacz* gene after being injected at the 1-cell stage and fixed 24 hrs after injection. **(D)** 4-cell stage embryo that expressed the *lacz* gene after being injected at the 1-cell stage and fixed 40 hrs after injection.


A
B
C
D

5.1.7 Figure 7

Detection of DIG-labeled RNAs injected into mouse embryos.

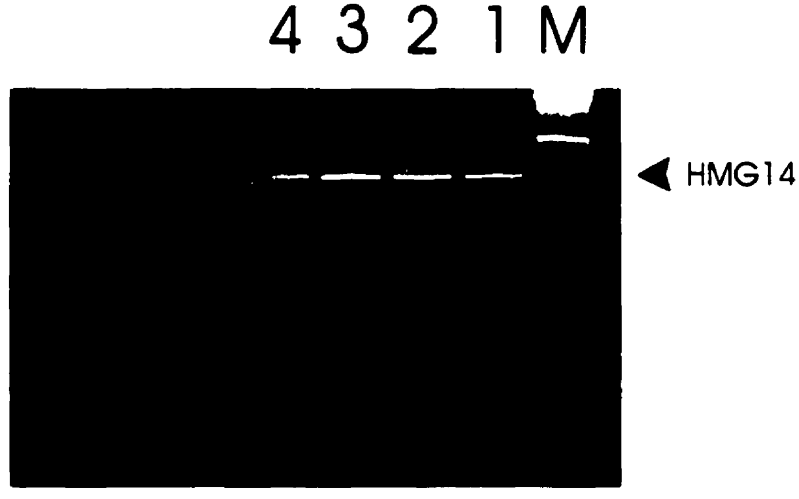
DIG-labeled anti-sense HMG-14 was prepared by in vitro transcription and injected into the cytoplasm of 1-cell stage embryos. Embryos then were fixed immediately, 7 hrs, or 24 hrs after injection and processes for DIG detection using anti-DIG antibody. Embryos from all groups were processed for DIG detection simultaneously. **(A)** Non-injected embryos as a control. **(B)** Embryos fixed immediately after injection. **(C)** Embryos fixed 7 hrs after injection. **(D)** Embryos fixed 24 hrs after injection.


A
B
C
D

5.1.8 Figure 8

Detection of HMG-14 mRNA after injection of HMG-14 antisense RNA.

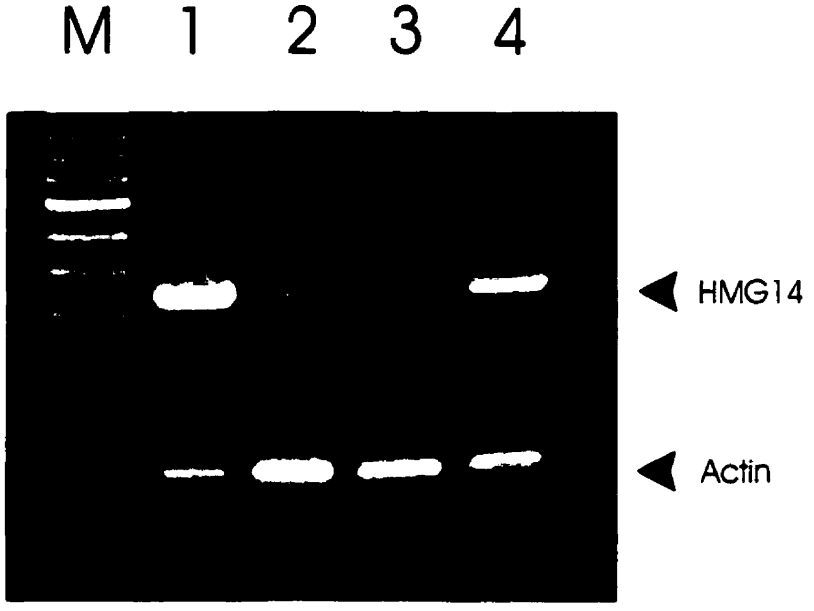
Two experiments were done separately in which 1-cell stage embryos were injected with HMG-14 antisense RNA, cultured to the 2-cell stage and then stored at -70°C. Controls were left without injection in each case. Both groups as well as the controls, which are ~20 embryos each, were subjected to simultaneous RNA extraction, cDNA synthesis using olig-dT primers and PCR amplification with the same reaction mixture containing HMG-14 primers. (M) 100 bp ladder, (1) Equivalent of 20 2-cell stage embryos injected at the 1-cell stage with HMG-14 antisense RNA in experiment 1, (2) Equivalent of 20 non-injected 2-cell stage embryos, (3) Equivalent of 20 2-cell stage embryos injected at the 1-cell stage with HMG-14 antisense RNA in experiment 2, (4) Equivalent of 20 non-injected 2-cell stage embryos.


4 3 2 1 M
HMG14

5.1.9 Figure 9

Diminishing of HMG-14 mRNA after introducing of unmodified HMG-14 antisense oligonucleotides into mouse oocytes

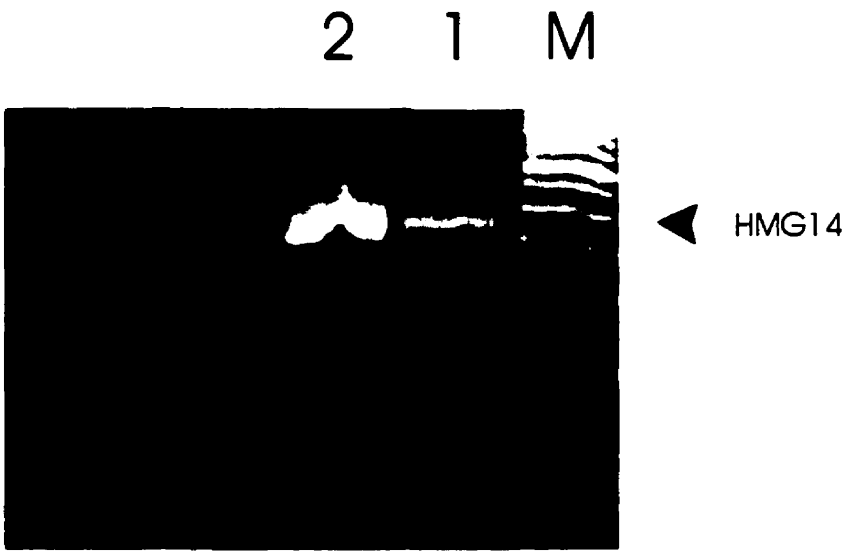
Prophase I-arrested oocytes (GVs) were collected and oligonucleotides were introduced. Oocytes were then incubated for ~ 18 hr in which they under went meiotic maturation. RNA was then extracted and cDNA was made and samples were subjected to PCR amplification with HMG-14 and actin primers. (M) 100 bp ladder, (1) Equivalent of 15 oocytes injected with nonsense oligonucleotides, (2) Equivalent of 15 oocytes injected with unmodified HMG-14 antisense oligonucleotides, (3) Equivalent of 15 oocytes treated with liposomal infection reagent containing unmodified HMG-14 antisense oligonucleotides, (4) Equivalent of 15 oocytes injected with phosphorothioate modified HMG-14 antisense oligonucleotides.


M 1 2 3 4
HMG14
Actin

5.1.10 Figure 10

Diminishing of HMG-14 mRNA after injection of unmodified HMG-14 antisense oligonucleotides into mouse embryos

One-cell stage embryos were collected and divided into two groups. One group was injected with unmodified HMG-14 antisense oligonucleotides and the other group was left with out injection. Embryos from both groups were then incubated and left to develop to the 2-cell stage. Both groups were then subjected to RNA extraction, cDNA synthesis and PCR amplification with HMG-14 primers. (M) 100 bp ladder, (1) Equivalent of 30 embryos injected with unmodified HMG-14 antisense oligonucleotides, (2) Equivalent of 30 non-injected embryos.


2 1 M
HMG14

5.1.11 Figure 11

Transient depletion of HMG-14 protein after injection of HMG-14 antisense oligonucleotides

One-cell stage embryos were collected and either injected with HMG-14 antisense oligonucleotides or left without injection. Embryos were then incubated and left to develop to further stages. A group of these were fixed at different stages of development and stored at 4°C in blocking buffer. All stages were then treated simultaneously with affinity purified anti-HMG-14 antibody and examined under confocal microscope. (A) Non-injected 2-cell stage embryo stained with anti-HMG-14 antibody, (B) Non-injected 4-cell stage embryo stained with anti-HMG-14 antibody, (C) Non-injected 8-cell stage embryo stained with anti-HMG-14 antibody, (D) 2-cell stage embryo injected with HMG-14 antisense oligonucleotides and stained with anti-HMG-14 antibody, (E) 4-cell stage embryo injected with HMG-14 antisense oligonucleotides and stained with anti-HMG-14 antibody, (F) 8-cell stage embryo injected with HMG-14 antisense oligonucleotides and stained with anti-HMG-14 antibody. Magnification is about 60X.

5.1.12 Figure 12

Transient depletion of HMG-17 protein after injection of HMG-17 antisense oligonucleotides

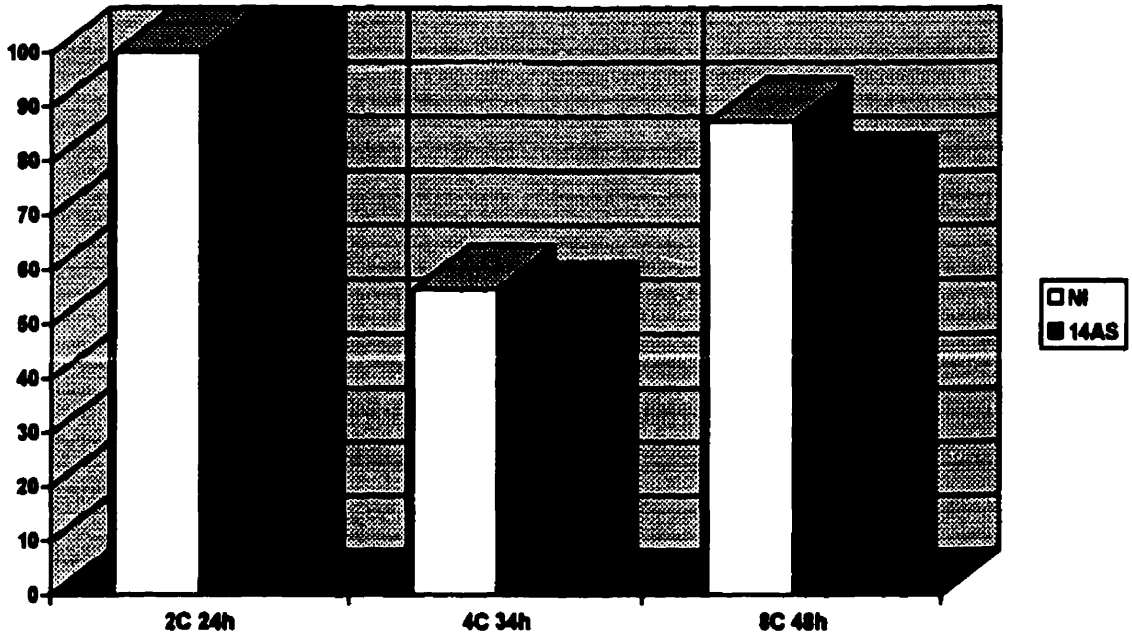
One-cell stage embryos were collected and either injected with HMG-17 antisense oligonucleotides or left without injection. Embryos were then incubated and left to develop to further stages. A group of these were fixed at different stages of development and stored at 4°C in blocking buffer. All stages were then treated simultaneously with affinity purified anti-HMG-17 antibody and examined under confocal microscope. (A) Non-injected 2-cell stage embryo stained with anti-HMG-17 antibody, (B) Non-injected 4-cell stage embryo stained with anti-HMG-17 antibody, (C) Non-injected 8-cell stage embryo stained with anti-HMG-17 antibody, (D) 2-cell stage embryo injected with HMG-17 antisense oligonucleotides and stained with anti-HMG-17 antibody, (E) 4-cell stage embryo injected with HMG-17 antisense oligonucleotides and stained with anti-HMG-17 antibody, (F) 8-cell stage embryo injected with HMG-17 antisense oligonucleotides and stained with anti-HMG-17 antibody. Magnification is about 60X.

5.1.13 Figure 13

Development of embryos injected with HMG-14 antisense oligonucleotides

Histogram represents the rate of development of embryos injected with HMG-14 antisense oligonucleotides as compared with controls non-injected embryos. Embryos were collected at the 1-cell stage and divided into two groups; one group was injected with 0.1 μg/μl phosphorothioate-modified HMG-14 antisense oligonucleotides and the other group was left without injection. 24 hrs following microinjection and overnight culture, those that cleaved to the 2-cell stage from each group were selected and represented as 100%. These were further cultured, examined at regular intervals, and the developmental stage of each embryo, as indicated by cell number, was recorded in each group. The number of embryos from each group reaching a certain stage at a certain time was represented as percentage of embryos from that group reached that stage at that time (see table 4 for actual numbers).

-NI (non-injected), 14AS (embryos injected with HMG-14 antisense oligonucleotides).

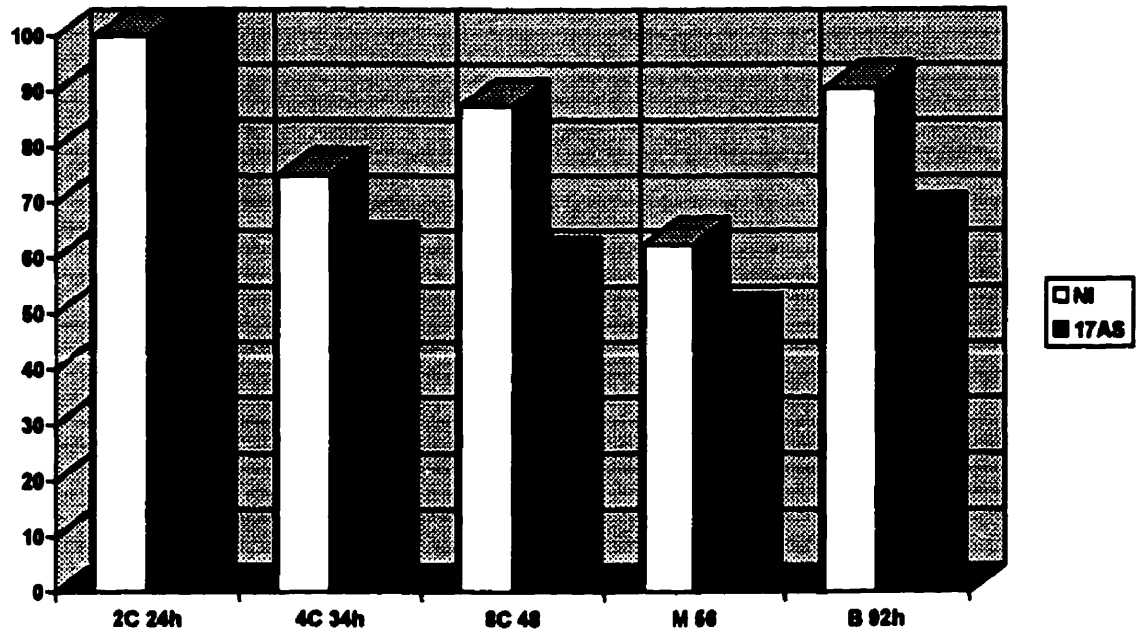

100
90
80
70
60
50
40
30
20
10
0
2C 24h
4C 34h
8C 48h
NI
14AS

5.1.14 Figure 14

Development of embryos injected with HMG-17 antisense oligonucleotides

Histogram represents the rate of development of embryos injected with HMG-17 antisense oligonucleotides as compared with controls non-injected embryos. Embryos were collected at the 1-cell stage and divided into two groups; one group was injected with 0.1 μg/μl phosphorothioate-modified HMG-17 antisense oligonucleotides and the other group was left with out injection. 24 hrs following microinjection and overnight culture, those that cleaved to the 2-cell stage from each group were selected and represented as 100%. These were further cultured, examined at regular intervals, and the developmental stage of each embryo, as indicated by cell number, was recorded in each group. Number of embryos from each group reached certain stage at certain time was represented as percentage of embryos from that group reached that stage at that time (see table 5 for actual numbers).

NI (non-injected), 17AS (embryos injected with HMG-17 antisense oligonucleotides).

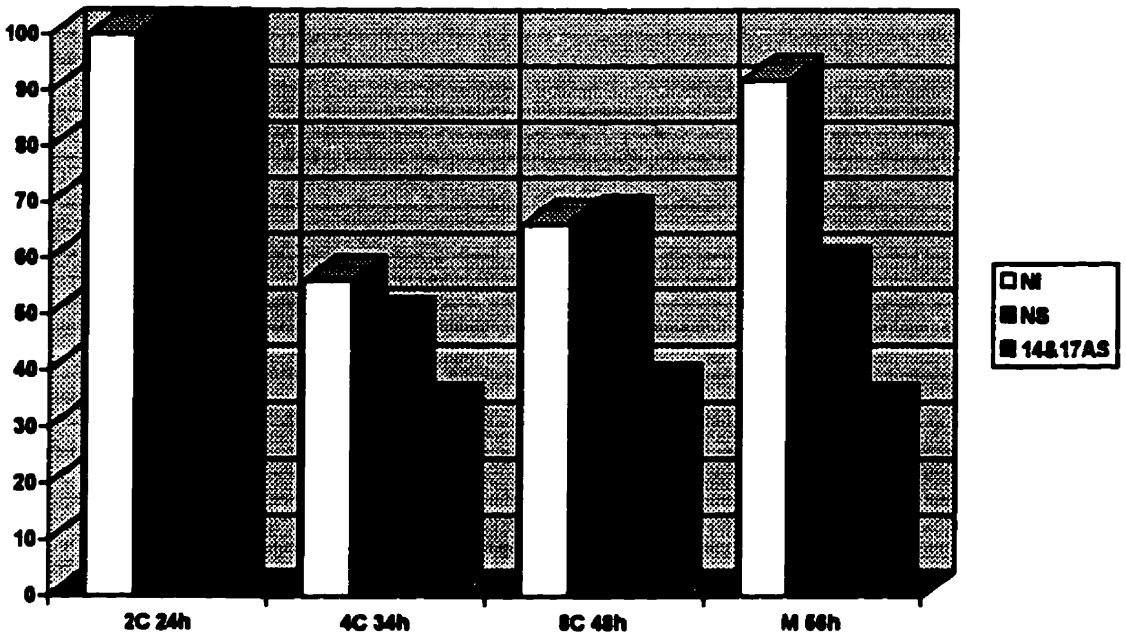

100
90
80
70
60
50
40
30
20
10
0
2C 24h
4C 34h
8C 48
M 66
B 92h
Ni
17AS

5.1.15 Figure 15

Development of embryos injected with a mixture of phosphorothioate-modified HMG-14 and HMG-17 antisense oligonucleotides

Histogram represents the rate of development of embryos injected with modified HMG-14 and HMG-17 antisense oligonucleotides as compared with controls injected with modified nonsense oligonucleotides and non-injected embryos. Embryos were collected at the 1-cell stage and divided into three groups; one group was injected with 0.1 μg/μl phosphorothioate-modified HMG-14 & HMG-17 antisense oligonucleotides, the second group was injected with 0.2 μg/μl modified nonsense oligonucleotides and the third group was left with out injection. 24 hrs following microinjection and overnight culture, those that cleaved to the 2-cell stage from each group were selected and represented as 100%. These were further cultured, examined at regular intervals, and the developmental stage of each embryo, as indicated by cell number, was recorded in each group. Number of embryos from each group reached certain stage at certain time was represented as percentage of embryos from that group reached that stage at that time (see table 6 for actual numbers).

- NI (non-injected), NS (embryos injected with HMG-14 nonsense oligonucleotides), 14&17AS (embryos injected with a mixure of HMG-14 and HMG-17 antisense oligonucleotides).

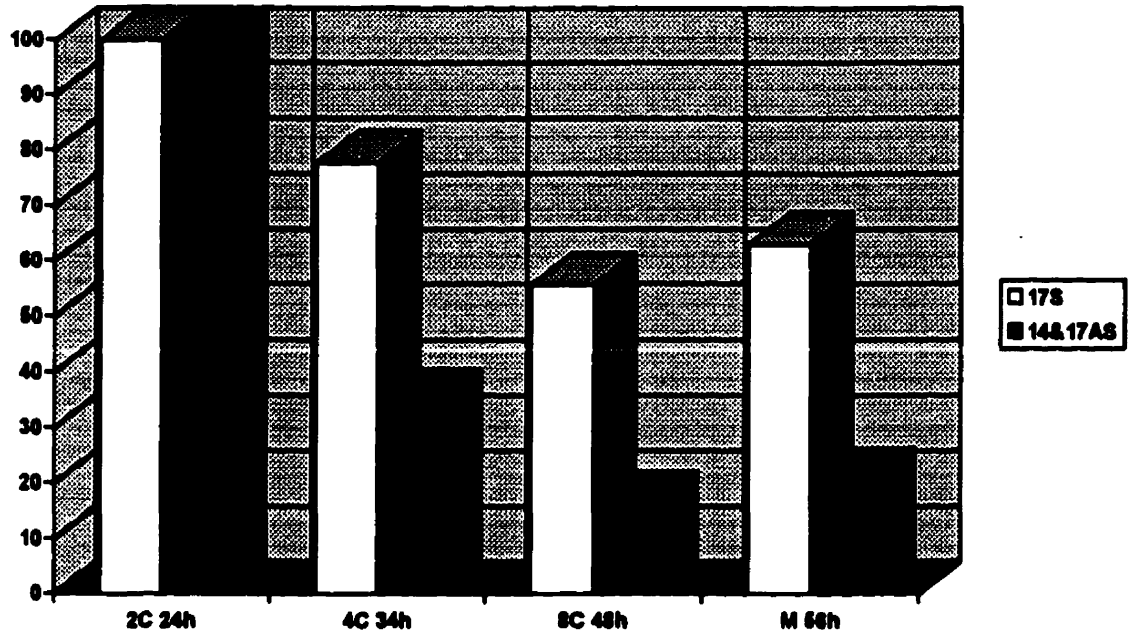

100
90
80
70
60
50
40
30
20
10
0
2C 24h
4C 34h
8C 48h
M 56h
NI
NS
14&17AS

5.1.16 Figure 16

Development of embryos injected with a mixture of unmodified HMG-14 and HMG-17 antisense oligonucleotides

Histogram represents the rate of development of embryos injected with unmodified HMG-14 and HMG-17 antisense oligonucleotides as compared with controls injected with unmodified HMG-17 sense oligonucleotides. Embryos were collected at the 1-cell stage and divided into two groups; one group was injected with 0.8 μg/μl unmodified HMG-14 & HMG-17 antisense oligonucleotides and the other group was injected with 0.8 μg/μl unmodified HMG-17 sense oligonucleotides. 24 hrs following microinjection and overnight culture, those that cleaved to the 2-cell stage from each group were selected and represented as 100%. These were further cultured, examined at regular intervals, and the developmental stage of each embryo, as indicated by cell number, was recorded in each group. Number of embryos from each group reached certain stage at certain time was represented as percentage of embryos from that group reached that stage at that time (see table 7 for actual numbers).

- 17S (embryos injected with HMG-17 sense oligonucleotides), 14&17AS (embryos injected with HMG-14 and HMG-17 antisense oligonucleotides).


100
90
80
70
60
50
40
30
20
10
0
2C 24h
4C 34h
8C 48h
M 66h
17S
14&17AS

5.2 Tables

5.2.1 Table 1

Reporter gene expression after injection of encoding plasmid into 1-cell stage embryos

Plasmid DNA encoding for different reporter genes driven by different promoters were prepared (see Results) and microinjected into the male pronuclei of 1-cell stage embryos usually at a concentration of 0.4 μg/μl. Injected embryos were cultured for 24 hrs and examined for the activity of the reporter gene injected (see Results and Material and Methods).

Plasmid	p610ZA	pEGFP-N1	hsp-PEGFP	p68EGFP	pMOEGFP
Promoter	hsp-68	CMV	CMV-hsp	ΔCMV-hsp	hsp
Reporter Gene	Lac-Z	GFP	GFP	GFP	GFP
No. of embryos injected	883	88	78	142	94
No. that survived (% of injected)	475 (53.8%)	36 (40.9%)	29 (37.1%)	67 (47.2%)	45 (47.8%)
No. that remained at the 1-cell stage (% of survivors)	76 (16.0%)	12 (33.3%)	7 (24.1%)	18 (26.9%)	11 (24.4%)
No. that reached the 2-cell stage (% of survivors)	399 (84.0%)	24 (66.6%)	22 (75.8%)	49 (73.1%)	34 (75.5%)
No. of positive 1-cell embryos (% of 1-cells)	43 (56.5%)	3 (25.0%)	4 (57.1%)	0 (0%)	9 (81%)
No. of positive 2-cell embryos (% of 2-cells)	140 (35.1%)	0 (0%)	1 (4.5%)	0 (0%)	0 (0%)
Total no. of positive embryos (% of survivors)	183 (38.5%)	3 (8.3%)	5 (17.2%)	0 (0%)	9 (20%)

5.2.2 Table 2

Determination of modified oligonucleotides concentrations used for microinjection

Phosphorothioate-modified oligonucleotides were prepared at different concentration and injected into the cytoplasm of 1-cell stage embryos. Embryos were then cultured for 24 hrs and the number of cleaved and uncleaved embryos were counted.

5.2.2 Table 2

Determination of modified oligonucleotides concentrations used for microinjection

Phosphorothioate-modified oligonucleotides were prepared at different concentration and injected into the cytoplasm of 1-cell stage embryos. Embryos were then cultured for 24 hrs and the number of cleaved and uncleaved embryos were counted.

Oligonucleotide injected	Concentration (μg/μl)	No. embryos injected	No. embryos that survived (% of injected)	No. of embryos that reached the 2-cell stage (% of survivors)
14-5AS	2.4	241	119 (49.4%)	11 (9.2%)
14-5AS	1	68	24 (35.3%)	3 (12.5%)
14-5AS	0.2	200	64 (32%)	16 (25.0%)
14-5AS	0.1	60	22 (36.6%)	20 (91.0%)
14-5AS	0.02	43	18 (41.8%)	17 (94.4%)
14-5NS	2.5	52	38 (73%)	2 (5.3%)
14-5NS	1	74	28 (37.8%)	2 (7.1%)
14-5NS	0.2	60	13 (21.6%)	0 (0%)
14-5NS	0.1	60	21 (35.0%)	18 (85.7%)

5.2.3 Table 3

Determination of unmodified oligonucleotides concentrations used for microinjection

Unmodified oligonucleotides were prepared at different concentration and injected into the cytoplasm of 1-cell stage embryos. Embryos were then cultured for 24 hrs and the number of cleaved and uncleaved embryos were counted.

Oligonucleotide injected	Concentration (μg/μl)	No. embryos injected	No. embryos that survived (% of injected)	No. of embryos that reached the 2-cell stage (% of survivors)
none	-	18	18 (100%)	18 (100%)
14-AS	2.5	96	50 (52%)	44 (88%)
14-AS	0.2	112	63 (56%)	56 (88.9%)
17-AS	7	64	31 (48.4%)	15 (48.4%)
17-AS	3.6	78	41 (52.5%)	19 (46.3%)
17-AS	1	82	37 (45%)	15 (40.5%)
17-AS	0.5	30	14 (46.6%)	12 (85.7%)
17-S	1	30	11 (36.6%)	6 (54.5%)
17-S	0.5	30	10 (33.3%)	9 (90%)

5.2.4 Table 4

Development of embryos injected with 0.1 μg/μl phosphorothioate-modified HMG-14 antisense oligonucleotides.

Embryos were collected at the 1-cell stage and divided into two groups; one group was injected with 0.1 μg/μl phosphorothioate-modified HMG-14 antisense oligonucleotides and the other group was left with out injection. 24 hrs following microinjection and overnight culture, those that cleaved to the 2-cell stage from each group were selected and counted in each group. These were further cultured, examined at regular intervals, and the developmental stage of each embryo, as indicated by cell number, was recorded in each group.

Oligonucleotide injected	No. of embryos injected	Time observed (h after injection)	No. of 2-cells	No. of 4-cells	No. of 8-cells
None injected	40	24	39		
		34	17	22	
		48		5	34
14-AS	60	24	30		
		34	14	16	
		48		7	23

5.2.5 Table 5

Development of embryos injected with 0.1 μg/μl phosphorothioate-modified HMG-17 antisense oligonucleotides.

Embryos were collected at the 1-cell stage and divided into two groups; one group was injected with 0.1 μg/μl phosphorothioate-modified HMG-17 antisense oligonucleotides and the other group was left with out injection. 24 hrs following microinjection and overnight culture, those that cleaved to the 2-cell stage from each group were selected and counted in each group. These were further cultured, examined at regular intervals, and the developmental stage of each embryo, as indicated by cell number, was recorded in each group.

Oligonucleo-tide injected	No. of embryos injected	Time observed (h after injection)	No. of 2-cells	No. of 4-cells	No. of 8-cells	No. of morulae	No. of Blastocyst
None	38	24	32				
		34	8	24			
		48		4	22	6	
		56			12	20	
		92				3	29
17-AS	60	24	39				
		34	15	24			
		48	1	15	23		
		56		2	18	19	
		92				13	26

5.2.6 Table 6

Development of embryos injected with a mixture of phosphorothioate-modified HMG-14 and HMG-17 antisense oligonucleotides

Embryos were collected at the 1-cell stage and divided into three groups; one group was injected with 0.1 μg/μl phosphorothioate-modified HMG-14 & HMG-17 antisense oligonucleotides, the second group was injected with 0.2 μg/μl modified nonsense oligonucleotides and the third group was left with out injection. 24 hrs following microinjection and overnight culture, those that cleaved to the 2-cell stage from each group were selected and counted. These were further cultured, examined at regular intervals, and the developmental stage of each embryo, as indicated by cell number, was recorded in each group.

Oligonucleotide injected	No. of embryos injected	Time observed (h after injection)	No. of 2-cells	No. of 4-cells	No. of 8-cells	No. of morulae
None	60	24	50			
		34	22	28		
		48		17	25	8
		56		1	3	46
NS	90	24	66			
		34	34	32		
		48		22	44	
		56		4	24	38
14-AS & 17-AS	96	24	57			
		34	38	19		
		48	3	33	21	
		56	2	18	18	19

5.2.7 Table 7

Development of embryos injected with a mixture of unmodified HMG-14 and HMG-17 antisense oligonucleotides

Embryos were collected at the 1-cell stage and divided into two groups; the first group was injected with 0.8 μg/μl unmodified HMG-14 & HMG-17 antisense oligonucleotides and the second group was injected with 0.8 μg/μl unmodified sense HMG-17 oligonucleotides as a control. 24 hrs following microinjection and overnight culture, those that cleaved to the 2-cell stage from each group were selected and counted. These were further cultured, examined at regular intervals, and the developmental stage of each embryo, as indicated by cell number, was recorded in each group.

Oligonucleotide injected	No. of embryos injected	Time observed (h after injection)	No. of 2-cells	No. of 4-cells	No. of 8-cells	No. of morulas
17-S	55	24	24	3		
		34	6	20	1	
		48	2	10	8	7
		56	2	4	4	17
14-AS & 17-AS	79	24	48	1		
		34	32	17		
		48	17	24	5	3
		56	16	16	7	10

6

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