Characterization of DNA Methylation in the Zebrafish, *Danio rerio*: Implications for Zebrafish Use as a Model System to Study the Role of DNA Methylation in Normal and Abnormal Development.

By

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A Thesis submitted to the Faculty of Graduate Studies of the University of Manitoba in partial fulfillment of the requirement for the degree of

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Abstract

DNA methylation at CpG dinucleotides plays an important role in the regulation of gene expression and is essential for normal vertebrate development. This DNA methylation has been well studied in mammals and is thought to play a crucial role in a number of important developmental processes including X-chromosome inactivation, genomic imprinting and gene silencing during lineage determination. The zebrafish has become a well established model system for the analysis of vertebrate development and a variety of disease phenotypes. The many favourable traits that make it such a popular model organism readily lend themselves to the study of DNA methylation in normal and abnormal vertebrate development. In this study, I have determined the full-length cDNA sequence corresponding to the zebrafish *DNA (cytosine-5) methyltransferase 1* gene, *dnmt1*, analyzed its expression patterns and elucidated the cycle of demethylation in germ-cells and during early embryogenesis of the zebrafish.

The *dnmt1* gene is 4907 base-pairs long and has an open reading frame predicted to encode a 1499 amino acid protein that is similar in size and sequence to a number of DNA (cytosine-5) methyltransferases identified in other organisms. Dynamic temporal and spatial patterns of expression of *dnmt1* were noted. During oogenesis, early oocytes contained significant amounts of *dnmt1* transcript while message abundance declined as the oocytes matured. Transcript levels remained low during early embryogenesis until the blastula stage. Methyltransferase enzyme assays revealed that the maternal *dnmt1* message accumulated during oogenesis was translated into protein as evidenced by the high enzyme activity in the mature oocyte and in the early cleavage and blastula embryos

presumably providing necessary dnmt1 stockpiles to support early embryonic development prior to zygotic gene activation.

Stage- and tissue-specific global demethylation and remethylation occurred in the germ cells and during early development of the zebrafish. The sperm genome was heavily methylated compared with that of the unfertilized oocyte. Following fertilization, the diploid genome of the early-cleavage zebrafish embryo underwent a wave of pronounced demethylation continuing through to the early blastula (2.2 hrs) stage embryos. By the late blastula (4.0 hrs) stage, the global methylation levels commenced to rise, approaching the sperm methylation levels by the gastrula (6.0 hrs) stage.

The conservation of all functional motifs of dnmt1, the developmentally regulated expression patterns and the presence of genome-wide DNA methylation changes in early development argue that this epigenetic control mechanism plays an important role in the early development of zebrafish. The conservation of dynamic changes in global genome demethylation among vertebrates implies functional significance of these changes in the early development of a complex vertebrate. My work provides evidence for the suitability of this attractive model system to study and better understand the role of DNA methylation in normal early development and the abnormal phenotype(s) ensuing from its disruption.

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List of Abbreviations

%- percentage

a.a.-amino acid

bp-base pair

Cat.-Catalogue

Ci-curie

cm-centimeter

CpG-cytosine connected to guanine by a 3`-5` phosphodiester bond

ddH_2O-distilled deionized water

dNTP-deoxynucleotide triphosphate

EDTA-ethylenediaminetetraacetic acid

EtBr-ethidium bromide

ds-double stranded

g-gram

HCl-hydrochloric acid

kb-kilobase

KDa-kiloDalton

ul-microlitre

ug-microgram

M-molar

ml-millilitre

mM-millimolar

$MgCl_2$-magnesium chloride

MTase-DNA cytosine-5 methyltransferase

NaCl-sodium chloride

ng-nanogram

nm-nanometer

No-number

MBP-methyl binding protein

MBD-methyl binding domain protein

OD-optical density

PAGE-polyacrylamide gel electrophoresis

PCR-polymerase chain reaction

SAM-S-adenosyl methionine

Taq-Thermus aquaticus DNA (polymerase)

UV-ultraviolet

V-volts

v/v-volume/volume

w/v-weight/volume

General Introduction

I. Introduction

Methylation of the cytosine residue within CpG dinucleotides is a major modification of DNA in higher eukaryotes (Adams and Burdon, 1982). This DNA methylation is introduced enzymatically by a group of DNA cytosine-5-methyltransferases (MTases) using *S*-adenosyl-L-methionine (SAM) as the donor of an activated methyl group. The methyl group is positioned in the major groove of the DNA where it is recognized by methyl-binding proteins (MBP) interacting with the DNA consequently adding new properties that are not encoded in the base pair sequence. The resulting DNA methylation constitutes an important mechanism of epigenetic control of genome function (reviewed in Razin and Riggs, 1980 and Razin and Cedar, 1991).

II. DNA Methyltransferases

II.A. Prokaryotic DNA methyltransferases

The DNA cytosine-5 methyltransferase enzymes are best understood in prokaryotes (Razin and Riggs, 1980). In general, bacterial MTases are two-domain proteins comprising one large and one small domain. The small domains of different MTases are dissimilar in amino acid sequence, size, and structure. The large domain contains a set of up to ten conserved amino acid motifs (I-X) (Wilson, 1992; Kumar *et al.,* 1993; Malone *et al.,* 1995) arranged in the same order within a protein of about 40 kDa (Kumar *et al.,* 1993). Motifs I (DXFXGXG), IV (GFPCQ) and VI (ENV) are the most conserved (Kumar *et al.,* 1994). The large domain of MTases forms the binding

site for S-adenosyl methionine (SAM) and the catalytic center of the enzyme. The high degree of conservation within the prokaryotic system of MTases allowed the identification of eukaryotic orthologues (Bestor & Ingram, 1983).

II.B. DNA Methyltransferases in Higher Eukaryotes

II.B. 1. Mammalian DNA cytosine-5 methyltransferases

Two distinct DNA methylation activities are found in mammalian cells, maintenance and *de novo* methylation activities. Maintenance methylation activity has a preference for hemimethylated DNA and it ensures the clonal propagation of DNA methylation patterns to daughter cells through mitosis. *De novo* methylation activity, on the other hand, establishes a new DNA methylation pattern on unmethylated DNA (reviewed in Bird, 2002).

To date, four mammalian DNA cytosine-5 methylatransferases (MTases) have been identified (reviewed in Colot and Rossignol, 1999), all of which contain a highly conserved C-terminal catalytic domain, but vary in their N-terminal extensions. With the exception of Dnmt2, MTases are active and are capable of methylating cytosines in CpG sequences (reviewed in Bestor, 2000). The properties of each of these enzymes are reviewed in the next sections.

II.B.1.i.a. DNA cytosine-5 methyltransferase 1 (Dnmt1)

Following the announcement of Bestor *et al.* (1988) of the purification, identification and cloning of the first eukaryotic DNA cytosine-5 methyltransferase from the mouse (Dnmt1; OMIM#126375), several other MTases were characterized from a variety of species. Dnmt1 is highly conserved among eukaryotes and has been found in every eukaryotic species with cytosine-5 DNA methylation that has been investigated. Dnmt1 is a large protein of 190 kDa relative molecular weight and is comprised of 1620 amino acid residues. Its 500-residue C-terminal part forms the catalytic domain and contains the amino acid sequence motifs characteristic for prokaryotic DNA cytosine-5 methyltransferases (Bestor *et al.,* 1988; Kumar *et al.,* 1994). The 1100-residue N-terminal domain serves a regulatory role with respect to substrate specificity and MTase targeting to different nuclear and cellular sites. It has a zinc-binding domain containing eight-conserved cysteine residues and binding to zinc (Chuang *et al.,* 1996), a polybromo-1 protein homologous region (reviewed in Bestor and Verdine, 1994) that is thought to be involved in protein-protein interactions and motifs for Dnmt1 enzyme nuclear localization (Leonhardt *et al.,* 1992). In addition, the N-terminal domain of Dnmt1 has been shown to interact with several other proteins like PCNA (Chuang *et al.,* 1997), the transcriptional co-repressor DMAP1 (Rountree *et al.,* 2000), the histone deacetylases HDAC1 (Fucks *et al.,* 2000) and HDAC2 (Rountree *et al.,* 2000) and the transcription factor E2F1 (Robertson *et al.,* 2000), as well as the Rb tumor-suppressor protein (Robertson *et al.,* 2000).

Dnmt1 was found to have 5 to 30-fold preference for hemimethylated DNA over unmethylated substrates (Yoder *et al.,* 1997; Tollefsbol and Hutchison, 1995) and hence

is thought to function as a maintenance methyltransferase (Yoder *et al.,* 1997). However, Dnmt1 does possess *de novo* methylation activity as well (Yoder *et al.,* 1997). Although Dnmt1 knockout embryonic stem cells and mice show strongly reduced levels of DNA methylation, cells continue to possess *de novo* MTase activity (Li *et al.,* 1992).

The *Dnmt1* gene expression is driven by sex specific promoters (reviewed in Bestor, 2000). Promoter 1s functions as the house keeping promoter (Yoder *et al.,* 1997). It drives translation from an ATG codon contained in exon 1s, initiating the full length Dnmt1 in somatic cells (Mertineit *et al.,* 1998). Just 3` of exon 1s lies a promoter in exon 1p that is active only in pachytene spermatocytes (Mertineit *et al.,* 1998). It is thought that the multiple short open reading frames contained in exon 1p interfere with the translation of the Dnmt1 open reading frame and hence the pachytene spermatocytes do not contain any detectable Dnmt1 protein (Mertineit *et al.,* 1998). An enzymatically active shorter form of Dnmt1 enzyme lacking the N-terminal 118 amino acids is found exclusively in oocytes (Dnmt1o) (Mertineit *et al.,* 1998). It results from the initiation of translation at an ATG codon in exon 4. Dnmt1o levels are found to be very high and the protein is cytoplasmically localized in mature oocytes. It has recently been implicated in the maintenance of the parental imprint during the first cleavage divisions, where a global demethylation occurs (Howell *et al.,* 2001).

II.B.1.i.b. Subcellular localization of DNA cytosine-5 methyltransferase 1

The Dnmt1 protein is subjected to a complex regulation of its subcellular localization during development and during the cell cycle. It is localized to the cytoplasm in oocytes and in preimplantation embryos (Carlson *et al.,* 1992; Cardoso and

Leonhardt, 1999) and is associated with replication foci during S-phase of the cell cycle in somatic cells (Leonhardt *et al.,* 1992). Sequences within the N-terminal domain are required for the localization of Dnmt1 to the nucleus and to replication foci during S-phase (Leonhardt *et al.,* 1992). The Dnmt1 protein is expressed in abundance in developing embryonic cells and is readily detected in nuclei of all cells of the early postimplantation embryo (Yoder *et al.,* 1997). In preimplantation embryos, only the ovarian form of Dnmt1, Dnmt1o, is detected (Mertineit *et al.,* 1998). Dnmt1o is cytoplasmically localized in mature oocytes and all preimplantation mouse embryos except at the eight-cell stage where it is found briefly in the nuclei of blastomeres in these embryos (Carlson *et al.,* 1992; Cardoso and Leonhardt, 1999; Howell *et al.,* 2001).

II.B.1.i.c. Expression patterns of DNA cytosine-5 methyltransferase 1 (Dnmt1)

Large amounts of Dnmt1o protein are present in MII oocytes and in all preimplantation cleavage-stage embryos. There are approximately 50,000-fold higher levels of Dnmt1o on per-cell basis in oocyte and early emryos as compared to Dnmt1 protein present in any somatic cell type (Carlson *et al.,* 1992). Monk *et al.,* (1991) detected very high methyltransferase activity in unfertilized eggs. Similarly, the enzyme activity was high in fertilized eggs, 2-cell and 8-cell embryos on a per-cell basis (Monk *et al.,* 1991). The fall in methylase activity, on a per-cell basis, between the one-cell embryo and the blastocyst stage was found to be equivalent to a 1000-fold decrease (Monk *et al.,* 1991).

II.B.1.ii. DNA cytosine-5 methyltransferase 2 (Dnmt2)

Dnmt2, a candidate methyltransferase identified by searching EST databases, belongs to a large family of proteins conserved from *S. pombe* to humans (Yoder *et al.,* 1998). Sequences with strong similarity to Dnmt2 were found in *X. laevis*, *A. thaliana* and the zebrafish *Danio rerio* EST databases (Dong *et al.,* 2001). The Dnmt2 enzyme is relatively small, consisting of 391 amino acid residues, and lacking the large N-terminal domain present in the Dnmt1 and Dnmt3 families (Yoder *et al.,* 1998). Although these proteins contain all sequence motifs characteristic for cytosine-MTases, catalytic activity has not yet been demonstrated for any protein belonging to this family (Wyngaert *et al.,* 1998; Dong *et al.,* 2001). Dnmt2 knock outs in embryonic stem cells show no obvious effects on genomic methylation patterns and these mutant embryonic stem-cells retain the capacity to methylate newly integrated retroviral DNA (Okano *et al.,* 1998). Further studies are needed to delineate any biological role(s) of Dnmt2.

II.B.1.iii. Dnmt3a and Dnmt3b

A recent addition to the vertebrate MTases is the Dnmt3 family which was identified by screening mouse and human databases with sequences corresponding to the catalytic domain of Dnmt1 (Okano *et al.,* 1998). The Dnmt3 MTase family was discovered in 1998 by Okano and colleagues (Okano *et al.,* 1998). Specifically, two closely related genes containing the ten conserved DNA cytosine-5 methyltransferase motifs have been identified in both mouse (Dnmt3a and Dnmt3b) and humans (DNMT3A and DNMT3B) (Okano *et al.,* 1998). They are abundantly expressed in embryonic tissues, whereas their expression is down regulated in differentiated cells (Okano *et al.,*

1998; Robertson *et al.,* 1999). Murine Dnmt3a and Dnmt3b are comprised of 908 and 859 amino acid residues respectively. Their C-terminal domains are over 80% identical with about 36% amino acid sequence overall identity with each other. The Dnmt3a and 3b enzymes contain a plant homeodomain (PHD)-like zinc finger cysteine-rich region that is similar to X-linked chromatin remodeling factor ATRX zinc-finger (α thalassemia/mental retardation syndrome X-linked). Moreover, they contain a proline-tryptophan-tryptophan-proline (PWWP) domain that occurs in proteins playing a role in cell growth and differentiation. A recently identified gene related to Dnmt3a and Dnmt3b, Dnmt3l, was found to lack the C-terminal domain and methylase catalytic activity (Aapola *et al.,* 2001).

Dnmt3a and Dnmt3b were found to play a role in *de novo* DNA methylation because baculoviruses expressing Dnmt3a and Dnmt3b proteins methylate CG dinucleotides without significant preference for hemimethylated DNA (Okano *et al.,* 1998; Gowher *et al.,* 2001). *De novo* methylation of DNA by Dnmt3a and 3b was also demonstrated *in vivo* after expression in human cell lines (Hsieh, 1999) and by expression of Dnmt3a in transgenic *D. melanogaster* (Lyko *et al.,* 1999).

The critical role of Dnmt3a and Dnmt3b in development was demonstrated in transgenic mice lacking Dnmt3a and/or Dnmt3b, which were hypomethylated and died in early embryogenesis ($Dnmt3a^{-/-}/Dnmt3b^{-/-}$, $Dnmt3b^{-/-}$) or shortly after birth ($Dnmt3a^{-/-}$) (Okano *et al.,* 1999). The phenotype of a double knockout was more severe than either of the single knockouts suggesting that Dnmt3a and 3b have partially overlapping functions. Mutations in human DNMT3b cause immune deficiency, centromeric instability and facial dysmorphism (ICF) syndrome, a severe autosomal recessive disease

(Xu *et al.,* 1999; Hansen *et al.,* 1999). In ICF patients, hypomethylation of the normally heavily methylated classical satellite regions 2 and 3 of chromosomes 1, 9 and, 16 is observed (Okano *et al.,* 1999; Xu *et al.,* 1999). Knockout experiments also demonstrated that Dnmt3a and Dnmt3b were critical in establishing the differential methylation of imprinted loci (Hata *et al.,* 2002). Dnmt3l was found to colocalize with Dnmt3a and Dnmt3b and was thought to be essential for the establishment of such imprints (Hata *et al.,* 2002).

II.B.2. DNA methyltransferases in plants

A higher number of DNA Mtases is known in plants than in animals. For example, *A. thaliana* has eleven genes that could encode DNA methyltransferases as compared with the four MTases found in mice (reviewed in Finnegan and Kovac, 2000 and Martienssen and Colot, 2001). In *A. thaliana*, four enzymes of the Dnmt1 family, one enzyme of the Dnmt3 family, one member of the Dnmt2 family, three members of the family of chromomethylases, and one member of the masc1 family are known. The chromomethylase class, Dnmt1 related, is unique to plants and consists of three related CMT genes in *A. thaliana*. There are two predicted genes in the domain rearranged methyltransferase (DRM) class, *DRM1* and *DRM2*. These are most related to *Dnmt3*, but their functions have not been fully characterized yet. Interestingly, a sequence resembling Dnmt2, a highly conserved putative MTase with an unknown function, has been found in *A. thaliana* (Dong *et al.*, 2001).

III. Dynamics of DNA methylation during development

Both gametogenesis and early embryogenesis of the mouse are accompanied by changes in the global levels, and locus-specific, DNA methylation changes. These variations are discussed in the next two sections.

III.A. Genome-wide DNA methylation changes during mouse preimplantation development

In mice, complex changes in DNA methylation levels are observed during development and differentiation (Monk *et al.,* 1987; Razin and Kafri, 1994). These changes involve a global demethylation during the cleavage stage, which is thought to reach its lowest level during the bastocyst stage (Monk *et al.,* 1987; Howlett and Reik, 1991; Kafri *et al* 1992). The earliest phase of this demethylation is confined to the paternal pronucleus, prior to DNA replication (Mayer *et al.,* 2000; Oswald *et al.,* 2000), and has been described as "active" demethylation. After the completion of the first cell cycle, DNA methylation continues to decline due to the absence of the maintenance methylase, Dnmt1 (Carlson *et al.,* 1992; Howlett and Reik, 1991; Monk *et al.,* 1991). This is referred to as the passive phase of demethylation. During this time, methylation declines in housekeeping genes and repeat sequences throughout the genome. Remarkably, imprinted genes are largely exempt from this process and maintain their methylation. Not long after implantation, DNA methylation is restored and maintained thereafter in somatic lineages (Monk *et al.,* 1987). This restoration of methylation is carried out by Dnmt 3a and 3b, the so-called *de novo* methylases (Okano *et al.,* 1999), but the precise time point and lineage specificity for *de novo* methylation are not known.

Perhaps the most intriguing feature of methylation reprogramming is the asymmetric loss of methylation by the male pronucleus after fertilization.

Recently a detailed analysis of the demethylation cycle during preimplantation mouse development was conducted by Santos *et al.* (2002). Using indirect immunofluoresence with an antibody to 5-methyl cytosine, they closely dissected DNA methylation reprogramming in mouse preimplantation embryos. Their results showed that demethylation of the male pronucleus is an active process that is completed within 4 hours after fertilization. Interestingly, paternal pronuclear demethylation in fertilized oocytes deficient for MBD2, the only candidate demethylase (Bhattacharya *et al.,* 1999), occurred normally (Santos *et al.,* 2002). Evaluation of fertilized oocytes homozygous for a MBD2 knockout allele gave an identical staining pattern of 5-methylcytosine to the wild type embryos (Santos *et al.,* 2002) suggesting the presence of a yet to be determined demethylase activity in the zygote.

The investigation by Santos *et al.* (2002) also revealed that passive loss of methylation occurred in a stepwise fashion up to the morula stage embryos. Thereafter, *de novo* methylation was observed specifically in the inner cell mass (ICM), but not in the trophectoderm of the blastocyst. It has been argued that these phases of methylation reprogramming in the developing mouse embryo involve erasing of the epigenetic modifications present in the zygote, followed by subsequent *de novo* methylation necessary for resetting the developmental patterns of gene expression in differentiating cell lineages (Razin and Kafri, 1994).

III.B. Tissue-specific methylation of single loci

Tisuue-specific genes are initially methylated in all cell types in the embryo and only expressed genes undergo demethylation during tissue-specific differentiation. In addition, many tissue-specific genes are methylated in non-expressing tissues (Jones, 1999). However, there are several tissue-specific genes that are never methylated, even in tissues where they are not expressed (Walsh and Bestor, 1999).

Despite the active and rapid demethylation of the paternal genome at fertilization, some genes are protected from demethylation including the imprinted genes *H19* (Olek and Walter, 1997) and *Ras Grff1* (Shibata *et al.,* 1998). Maintenance of the DNA methylation pattern of imprinted genes is carried out by Dnmt1 enzyme as evidenced by the loss of 50% of imprinted allele methylation in *Dnmt1o* knockout mouse embryos (Howell *et al.,* 2001). Although some tissue-specific genes are abnormally expressed in $Dnmt1^{-/-}$ embryos (reviewed in Jaenisch, 1997), these genes were not activated in these mutants (Walsh and Bestor, 1999). This implies that cytosine methylation in mammals has a role in specialized processes such as allele-specific gene expression, whereas it plays a minor role in the regulation of mammalian development.

IV. DNA methylation and chromatin

A complex integration occurs between the DNA methylation machinery and chromatin modifying pathways (reviewed in Ng and Bird, 1999; Burgers *et al.,* 2002; Okano and Li, 2002). The Dnmt1 N-terminal domain and Dnmt3a and Dnmt3b PHD like motif bind to HDACs and can consequently repress gene transcription through the histone deacetylase activity (Fucks *et al.,* 2000; Fuks *et al.,* 2001). Through the action of

methyl-binding domain proteins, which are either components of or recruit HDACs, MTases and histone deacetylase complexes operate to repress transcription. Burgers *et al.* (2002) proposed a two-step model of chromatin remodeling and gene silencing by DNA methylation. Initially, the MTase cooperates with the HDAC to bring about partial transcriptional repression. The generation of methylated chromatin by the MTase allows DNA binding of methyl binding domain (MBD) proteins. These in turn recruit further HDAC activities and thereby render chromatin more stably silenced. Currently a complex picture is beginning to emerge of how MTases cooperate with chromatin modifying enzymes and processes to orchestrate gene silencing networks in the cell.

V. The Roles of DNA Methylation

V.A. Role of DNA methylation in prokaryotes

In prokaryotes, one of the major biological roles of DNA methylation is the distinction of self and non-self DNA (Cheng, 1995). This is associated with restriction-modification systems that function as a defense mechanism against infection of bacteria by bacteriophages. The cellular DNA is protected against this attack by methylation within the recognition site of the restriction enzyme. In addition, in prokaryotes DNA methylation plays a role in DNA repair and control of DNA replication (reviewed in Barras and Marinus, 1989).

V.B. The functions of DNA methylation in higher eukaryotes

DNA methylation has been extensively studied in mammals and has been shown to play an important role in a number of developmental processes including X-chromosome inactivation (Panning and Jaenisch, 1996), gene silencing and lineage determination (Cedar, 1988; Riggs and Pfeiffer, 1992) as well as genomic imprinting (Li *et al.,* 1993; Efstratiadis A, 1994; Reik and Allen, 1994). Focal demethylation or hypermethylation at imprinted loci have been observed in several pathologic states including certain types of human cancer and developmental abnormalities. Ectopic *de novo* methylation of tumor suppressor genes may contribute to the development of specific types of tumors (Tycko, 2000). Although there has been a reasonable degree of progress in our ability to detect the presence of these abnormalities, very little is known about how and why such abnormalities occur. More over, our knowledge of the regulation of DNA methylation and of the control of DNA cytosine-5-methyltransferase enzyme biosynthesis is still scarce.

Several theories on the general function of DNA methylation have been proposed. Although all models agree that DNA methylation serves as a general mechanism of gene repression, they attribute different major roles to this process. In the noise-reduction model, general gene repression is the main function of DNA methylation. In the genome-protection model, silencing of selfish genetic elements by methylation is considered the main function of methylation. The development model proposes that gene-specific methylation and demethylation of the promoter regions is the cardinal function of DNA methylation.

V.B.1. Repression of gene transcription by DNA methylation

Methylation of CG sites is involved in gene regulation, with methylation of CG sites in the promoter regions of genes usually leading to a reduction of gene expression (reviewed in Kass *et al.,* 1997). The degree of transcriptional silencing caused by methylation of specific genes *in vivo* and *in vitro* may depend upon the location and density of the CpG islands relative to the promoter (Bird, 1992). The inhibition of gene expression can be controlled at different levels. First, several transcription factors, like AP-2, c-Myc/Myn, E2F, and NFB, are not able to bind to methylated target sites due to their inability to recognize their respective binding sites because the 5mC changes the recognition sequence (Bird, 1992). Secondly, DNA methylation may recruit 5-methylcytosine binding proteins (MeCPs) that act as repressors of gene transcription (Bird, 1992). Finally, DNA methylation may trigger histone deacetylation and thereby induce chromatin compaction leading to a stronger and more stable repression of gene expression (Kass *et al.,* 1997). Therefore, DNA methylation is a general tool for gene regulation that can be considered as an evolutionary device involved in many different biological functions (Colot, 1999).

V.B.2. DNA methylation and protection from selfish genetic elements

The human genome is challenged by different types of selfish genetic elements like transposons, retrotransposons, and viruses. Surprisingly >40 % of our genome is made of such selfish DNA (Smit, 1999). Since a random integration of transposable elements into the genome is an important source for mutations, prevention of transposon mobility by transcriptional silencing of these sequences is crucial for life. It has been

suggested that DNA methylation is involved in protection of the genome against genetic parasites (Yoder *et al.,* 1997). Transposons and other repetitive DNA sequences are usually relatively rich in CG sequences and are heavily methylated (Smit, 1999) and an increase in transcription of transposons is observed in Dnmt1 knockout embryonic stem cells (Walsh *et al.,* 1998) and cell lines (Jackson-Grusby *et al.,* 2001). These cells also show an elevated rate of mutations involving gene rearrangements (Chen *et al.,* 1998). Similar observations were made in plants where reduction of DNA methylation leads to the expression and mobilization of transposons (Miura *et al.,* 2001).

V.B.3. DNA methylation and the reduction of transcriptional noise

It has been proposed that DNA methylation serves to reduce transcriptional noise in organisms with complex genomes (Bird, 1995). This model is supported by the findings that in mammals most CG sequences are methylated and therefore the default expression status of most genes is off. In Dnmt1 (and p53) knockout cell lines the expression of 4-10 % of the detectable genes is induced while only 1-2 % are repressed (Jackson-Grusby *et al.,* 2001) lending further support to this model. This, of course, does not rule out a specific role of DNA methylation and demethylation during development. However the noise-reduction model is based on older estimates of the number of genes in different species, which recently have been revised considerably. According to the results of the genome-sequencing projects, the number of genes in *C. elegans* (19,000) and *Drosophila melanogaster* (13,000) is not dramatically lower than in *Arabidopsis thaliana* (20,000) and humans (35,000-40,000). Nevertheless, in humans and *A. thaliana* most of the CG sequences are methylated but *C. elegans* does not have DNA methylation

and *D. melanogaster* does not carry genome-wide DNA methylation, although some methylcytosine is present (Gowher *et al.,* 2000; Lyco *et al.,* 2000).

V.B.4. DNA methylation role in development

It was suggested more than two decades ago that DNA methylation as a heritable but flexible epigenetic mark is involved in the development of an organism (Holliday and Pugh, 1975; Riggs, 1975). There now is firm evidence that DNA methylation is essential for normal development in many vertebrates (Li *et al.,* 1992; Martin *et al.,* 1999; Stancheva and Meehan, 2000). Dnmt1 and Dnmt3b knockout mice die during early embryogenesis; Dnmt3a knockout mice are stunted and die shortly after birth (Li *et al.,* 1992; Okano *et al.,* 1999; Okano and Li, 2002). It is interesting that Dnmt1 knockout embryonic stem cells are viable despite a reduced level of methylation, but die after induction of differentiation (Li *et al.,* 1992; Jackson-Grusby *et al.,* 2001) a fact which also supports a role of DNA methylation in development. *Xenopus laevis* eggs depleted in Dnmt1 show disregulation of gene expression and premature activation of genes (Stancheva and Meehan, 2000). In zebrafish DNA methylation is required during gastrulation and somite patterning (Martin *et al.,* 1999).

V.B.5. DNA methylation and X-chromosome inactivation

In mammals, females carry two X chromosomes while males have only one. Therefore, dosage compensation is required for the genes encoded by the X chromosome. One X chromosome is inactivated in a process that involves specific expression of the *Xist* RNA from the inactivated chromosome, as well as significant methylation and

histone deacetylation of the inactivated chromosome (Avner and Headr, 2001). The importance of DNA methylation for X-chromosome inactivation is illustrated by the lack of stable maintenance of X-chromosome inactivation in Dnmt1 deficient mice (Sado *et al.,* 2000). The initial choice of which X chromosome is to be inactivated is made in early embryogenesis. In eutharian mammals' embryonic tissues, one chromosome is selected for inactivation in a random fashion, whereas in extraembryogenic tissues, the paternal chromosome is always chosen for inactivation. Therefore X-chromosome inactivation is also an example of parental imprinting. Interestingly, X-chromosome inactivation, like imprinting, depends on the CTCF repressor protein that detects the methylation state of the DNA (Bell and Felsenfeld, 2000; Hark *et al.,* 2000).

V.B.6. DNA methylation and genomic imprinting

In mammals a small number of genes carry an imprint that allows the paternal and maternal copies of these imprinted genes to be distinguished. Imprinted genes are only expressed from one allele. For example, only the maternal copy of *H19* and only the paternal copy of *Igf2* are active (Tilghman, 1999; Reik and Walter, 2001). DNA methylation represents a reasonable candidate responsible for the imprinting of specific loci (Razin and Cedar, 1991). Several lines of experimental evidence suggest that differential methylation of these loci is essential for maintenance of imprinting (Li *et al.*, 1993; Jaenisch, 1997). Imprints are established in the gonads during spermatogenesis and oogenesis. After fertilization, the imprint persists in the somatic cells for the whole life of the organism. In contrast, in the germ cells, the imprint must be erased and reset, because the new imprint depends on the sex of the individual. So, the paternal and

maternal copies of imprinted genes will obtain a female imprint in the oocytes and a male imprint in the sperm. Therefore, this process depends on both characteristic features of DNA methylation, stable and heritable silencing of genes, and the possibility to alter the encoded information, if required.

Differences in the pattern of methylation of imprinted genes are observed in many cases. *Dnmt1* knockout mice, which show a strongly reduced level of DNA methylation, have lost their imprint (Li *et al.,* 1993). Dnmt3a and Dnmt3b are found to be critical in establishing the differential methylation of imprinted loci (Hata *et al.,* 2002) while Dnmt1o has been implicated in the maintenance of the imprint during the first cleavage divisions, when a global demethylation of the embryo genome occurs (Howell *et al.,* 2001).

The mechanism of imprinting of the *H19/Igf2* pair has recently been determined. It involves one enhancer which has the potential to activate both genes but whose effect is only directed to the nearby H19 in the maternal genome. Its influence on the *Igf2* gene is blocked by binding of the CTCF protein, a chromatin boundary element, between the enhancer and the gene. In the paternal genome, the H19 promoter sequence and the CTCF binding site are methylated and thereby inactivated. Under these conditions, CTCF does not bind and the enhancer acts on the Ifg2 gene (Bell and Felsenfeld, 2000; Hark *et al.,* 2000).

Disruption of the normal imprinting process, and consequently the abnormal expression, of specific loci has been implicated in a number of human diseases and cancer. The best characterized syndromes related to defects in imprinting include Beckwith-Wiedemann syndrome (BWS) on chromosome 11p15 (OMIM#130650) and

the two clinically distinct syndromes of Prader-Willi (OMIM#176270) and Angelman (OMIM#105830) on chromosome 15q11-q13.

VI. DNA methylation and Human Disease

VI.A. Aberrant DNA methylation results in specific syndromes

Abnormal DNA methylation, aside from producing lethal results, may lead to a particular disease phenotype and has been implicated in many human diseases including some types of cancer. Recently, it has become increasingly clear that both correct levels and proper interpretation of methylation of specific loci are important factors for normal development and function of the developing human embryo. Diseases resulting from the abnormal regulation of genes that occurs with the loss of appropriate methylation controls during human development are best exemplified by the neurological disorders Fragile X (FRX) (OMIM#309550) and Rett syndromes (RTT) (OMIM #312750) and the disorder of the immune system, immunodeficiency, centromeric instability and facial dysmorphism (ICF) syndrome (OMIM# #242860).

There are several mechanisms by which pathology ensues when the normal methylation process goes awry. Inappropriate methylation and the consequent silencing of a normally active gene is one of these mechanisms. An example of this is fragile-X syndrome (Oberle *et al.,* 1991; Pieretti *et al.,* 1991), one of the commonest causes of mental retardation in humans. A normally inactive gene may become active due to loss of the normal transcription silencing process mediated by DNA methylation exemplified by Rett syndrome. A proportion of children with this disorder, characterized by

neurodevelopmental regression starting in late infancy, have mutations in the methyl CpG binding protein 2 (MeCP2) (Amir *et al.,* 1999). MeCP2 protein was identified by its ability to bind specifically to CpG-methylated DNA and is intricately involved in the methylation pathway (Meehan *et al.,* 1992). The abnormal MeCP2 protein fails to trigger the cascade of events that follows its binding to a methylated gene (Ballestar *et al.,* 2000) and therefore failure of transcription repression and consequently expression of a normally silenced gene or genes.

Recently, the molecular pathophysiology of "Immunodeficiency, centromere instability and facial anomalies" (ICF) syndrome has been linked to abnormal DNA methylation. ICF is a rare autosomal recessive disease characterized by variable immunodeficiency, dysmorphic features and centromeric decondensation and chromosome instability involving chromosomes 1, 9 and 16 (Tiepolo *et al.,* 1979). Homozygous or compound heterozygous mutations in the gene encoding DNA methyltransferase 3b (DNMT3b) have been shown to be the molecular pathology causing this syndrome (Scott-Hansen *et al.,* 1999; Xu *et al.,* 1999).

VI.B. Aberrant DNA methylation results in cancer

Alterations in the pattern of DNA methylation are frequently observed in cancerous tissues (Warnecke and Bestor, 2000; Baylin and Herman, 2000). Most often a general hypomethylation of the DNA is accompanied by a hypermethylation at specific loci. Both of these processes can have cancer-promoting effects. Hypomethylation leads to the genomic instability often observed in cancer cells and may lead to an activation of retrotransposons. In addition, the expression of oncogenes may become stimulated.

Hypermethylation is often found in the promoter regions of tumor-suppressor genes (TSG) and there it has the same effect as a mutation in the gene itself. Depending on the tumor type, epigenetic inactivation of tumor-suppressor genes can be the predominant method of functional gene loss in tumor cells. Hypermethylation of a TSG as a potential inactivating mechanism, and thus providing a 'second hit' of a TSG, was first described for the retinoblastoma gene, *RB* (Ohtani-Fujita *et al.,* 1993, Greger *et al.,* 1994). Another example of a tumor-suppressor gene inactivation by hypermethylation has been recently noted in several tumors of patients with Von Hippel-Lindau syndrome (Herman *et al.,* 1994; Prowse *et al.,* 1997). It should be noted that methylation defects in cancer cells, at least in principle, are reversible, which makes DNA methylation a promising target for a new generation of anticancer drugs.

VII. Zebrafish as a model system for developmental genetics

Vertebrates are not as amenable to genetic analysis as invertebrates such as *Drosophila* and *Caenorhabditis*. The mouse has been the classical vertebrate for genetic analysis. However, discovery of mutations that interfere with the earliest stages in development, using the mouse model system, has been impeded by the high cost of maintenance of animals and by the difficult accessibility due to the intrauterine mode of development (Driever *et al.*, 1996). George Streisinger (1981) of the University of Oregon recognized that the zebrafish, a small tropical teleost, has many of the advantages of *C. elegans* and *Drosophila*. Due to the favorable physical and genetic characteristics of this organism, there has been an exponential increase in its popularity as a model system for studying the genetic control of most developmental processes (reviewed in

Driever *et al.,* 1994). Since the fundamental develpmental genetic mechanisms have been well conserved among vertebrates, the zebrafish provides a useful model for understanding many aspects of human development. Over the past two decades, the zebrafish has emerged as one of the most tractable model systems for the study of vertebrate development. It has been exploited for elucidating the molecular pathophysiology of a wide range of human diseases.

VII.A. Life cycle

The zebrafish, *Danio rerio*, is a small tropical freshwater teleost native to streams in India. Development of zebrafish embryo consists of several cardinal stages including the zygote, cleavage, blastula, gastrula, segmentation and the hatching periods. The following brief description of zebrafish development is derived predominantly from Kimmel *et al.* (1995).

The zygote is formed of a mound of cytoplasm (the blastodisc) which sits on the large mass of yolk. The embryo proper is derived from the blastodisc and the remainder of the zygote becomes the yolk sac, which is later digested. This period lasts for the first 30 minutes. The newly fertilized egg is 0.7 mm in diameter. After the first cleavage stage, the cells divide at around 15 minute intervals. The cleavage stage runs from 30 minutes to 2.25 hours. After cleavage, the embryo enters the blastula stage as the blastodisc forms a sphere of cells sitting atop the yolk. There is no defined blastocoel but rather small irregular extracellular spaces are formed between the deep cells of the blastodisc. The blastula stage lasts from 2.25 to 5.25 hours.

The onset of gastrulation occurs at 50% epiboly. At this time, a thickened marginal region termed the germ ring appears around the blastoderm rim. The germ ring is formed by a folding of the blastoderm back upon itself (involution). Hence, within the germ ring there are two germ layers. The upper layer (the epiblast) continues to feed cells into the lower (the hypoblast) throughout gastrulation. The cells remaining in the epiblast when gastrulation ends correspond to the ectoderm and will give rise to such tissues as epidermis, the central nervous system, neural crest, and sensory placodes. The hypoblast gives rise to both the mesoderm and endoderm, although it is unclear how this layer subdivides into endoderm and mesoderm. The gastrula period runs from 5.25-10 hours. Primary organs will begin to appear visible. The tail becomes more prominent and the embryo elongates.

After epiboly, the somites and neural tube develop, the rudiments of the primary organs become visible, the tail bud becomes more prominent and the embryo elongates. The first somites form anteriorly (see also somite morphogenesis), and the posterior ones form last. Soon, the first cells differentiate morphologically, and the first body movements appear. Formation of the neural tube occurs by a process known as "secondary neurulation". The lumen of the neural tube, the neurocoele, forms by a process of cavitation, rather than by an uplifting and fusion of neural folds as in the amphibia, for example. The final stage is the hatching period, which runs from 48–72 hours. Individual eggs within a single developing clutch hatch sporadically during the whole third day of development and occasionally later. The embryo continues to grow at the same rate as it had earlier.

VII.B. The zebrafish model system attributes

The zebrafish as a model system for vertebrate development offers many distinct experimental advantages for the molecular analysis of most, if not all, vertebrate developmental processes. Some of the attributes that make the zebrafish such a versatile system for genetic studies include:

1. Due to its small adult size of approximately 3 centimeters length and a tolerance for high population densities, large populations can be easily and cheaply maintained.
2. Zebrafish have a short generation time of 3 months.
3. Zebrafish adult females have a large reproductive potential. Several hundred oocytes can be produced per mature zebrafish female per week.
4. Zebrafish oocytes are fertilized and develop externally to the female. This allows for easy accessibility to the embryos without sacrificing the mother. It also enables observation of all embryonic processes by several methods including time-lapse photography (Woo and Fraser, 1995). The external fertilization and development makes manipulations of the embryos such as transplantation of tissues, injection of antisense sequences or exposure to chemicals feasible. The ability to expose the embryo to various chemicals independent of the mother is particularly desirable in toxicology studies to test the teratogenetic effects of certain compounds. Such experiments are conducted simply by placing zebrafish embryos in water containing the chemical (Akimenko and Ekker; 1995).

5. Many embryos at a desired developmental stage can be easily collected due to the rapid development of zebrafish embryos and the fact that embryos obtained in a single breeding from one female develop relatively synchronously.
6. The zebrafish embryos are translucent allowing easy observation of all cells and internal organs during different stages of development.
7. Production of germline transgenic zebrafish is possible (Stuart *et al.,* 1995).
8. It is possible to manipulate fertilized zebrafish eggs so that they develop as haploids thus revealing the phenotype of recessive mutations.
9. It is anticipated that the zebrafish genome will be sequenced entirely soon.

VII.C. DNA methylation in the zebrafish

Despite the increasing interest in zebrafish as a model system and the crucial role DNA methylation plays in normal development and in disease etiology, our understanding of this important epigenetic control mechanism of gene expression in the zebrafish is very limited. Martin and McGowan (1995a) studied the effect of parental origin on the methylation of a transgene locus. Their work suggested the presence of a parent of origin effect on the methylation of a transgene in the zebrafish similar to genomic imprinting in mammals. This suggested that genomic imprinting might exist in the zebrafish (Martin and McGowan, 1995b). A role for DNA methylation in normal gastrulation and subsequent patterning of the dorsal mesoderm of zebrafish was suggested by Martin *et al.* (1999). Recently Macleod *et al.* (1999) noted the absence of genome-wide changes in DNA methylation during zebrafish development contrary to what has been shown by Martin *et al.* (1999). Collas, (1998) investigated the

methylation status of a transgene during zebrafish development and its correlation with the transgene expression. His data suggested that the transgene methylation and demethylation is developmentally regulated. In addition, a correlation between the extent of DNA methylation and transgene expression in zebrafish embryos was demonstrated.

VIII. Project Description

DNA methylation is essential for the development of vertebrates (Li *et al.,* 1992; Okano *et al.,* 1999; Martin *et al.,* 1999; Stancheva and Meehan, 2000), but despite over two decades of intensive enquiry, researchers still do not know exactly why. Since the identification and cloning of the mouse Dnmt1 and subsequently Dnmt-3a and Dnmt-3b, the mouse model system continued to lend itself readily to furthering our understanding of the phenomenon of DNA methylation. However, due to the difficult accessibility to embryos and the lethal phenotype ensuing in Dnmt1-/- mice (Li *et al.,* 1992), this model system has admittedly been less favorable in dissecting the exact role of DNA methylation in early development. Furthermore, it is not yet clear whether the specific role of DNA methylation is conserved between vertebrates or whether methylation has been adapted to regulate different aspects of gene expression among mammals in particular.

The zebrafish has recently emerged as a very popular model system in the study of vertebrate development. This model system enjoys several traits that would make it a promising system to exploit for the enquiry into the exact role(s) of DNA methylation in early development.

VIII.A. Hypothesis

The attributes zebrafish enjoys would make it an ideal model system to examine the specific role(s) of DNA methylation in early vertebrate development.

VIII.B. Objectives

To test our hypothesis, we set forth to answer the following questions:

1. Is there a Dnmt1 homologue in the zebrafish? [Chapter-1].

2. What are its temporal and spatial expression patterns? [Chapter-2].

3. Are there genome-wide, and/or locus-specific, DNA methylation changes during zebrafish development? [Chapter-3].

Chapter One

Isolation, characterization and sequence analysis of the full-length *DNA (Cytosine-5)methyltransferase-1, dnm1* of the zebrafish, *Danio rerio.*

Abstract: Chapter One

The zebrafish has become a well-established animal model for the analysis of vertebrate development and of several disease phenotypes. Many of the favorable traits that make it a popular model organism would also be beneficial for the study of normal and abnormal vertebrate development in which DNA methylation may play a role. I have determined the full-length cDNA sequence corresponding to the zebrafish *DNA (cytosine-5) methyltransferase-1* gene, *dnmt1*. It is 4907 base pairs long and has an open reading frame predicted to encode a 1499 amino acid protein that is similar in size and sequence to a number of DNA (cytosine-5) methyltransferases identified in other organisms.*

*(Mhanni *et al.,* 2001).

Introduction: Chapter One

DNA methylation is a major epigenetic modification of the genome that regulates crucial aspects of its function. DNA methylation is remarkably dynamic during early mammalian development (Monk *et al.,* 1987) and alteration of the DNA methylation status of the entire genome (Monk *et al.,* 1987; Kafri *et al.,* 1992), individual chromosomes (Grant and Chapman, 1988) and specific genes (Kafri *et al.,* 1992; Li *et al.,* 1993; Norris *et al.,* 1994; Jones and Laird; 1999) are essential for the establishment and progression of normal mammalian development (Li *et al.,* 1992 and reviewed in Jones and Takai, 2001). Understanding how these DNA methylation dynamics are established, regulated and maintained requires the definition of the underlying enzymatic processes.

The DNA methylation process involves the addition of a methyl group, donated by S-adenosyl methionine (SAM), to the 5-position of the cytosine base in DNA. This reaction is catalyzed by a group of DNA (cytosine-5) methyltransferases (MTases). The mouse Dnmt1 was the first to be recognized and is the best characterized MTase to date. In mammals, Dnmt1 is ubiquitously expressed and is the major type of DNA methyltransferase present. The phenotype resulting from the targeted disruption of this gene showed that DNA methylation is crucial for mammalian development and is involved in the maintenance of genomic imprinting (Li *et al.,* 1992; Howell *et al.,* 2001) and X-chromosome inactivation (Li *et al.,* 1992; Panning and Jaenisch, 1996).

To commence characterization of DNA methylation in zebrafish, I set forth to look for the presence of a *DNA (cytosine-5) methyltransferase 1* (*dnmt1*) gene and to

characterize its entire coding sequence. Two approaches were used to accomplish this goal. The first employed screening cDNA libraries that were prepared from different developmental stages. The partial sequences obtained from the screening of cDNA libraries were used to design gene specific primers to preferentially amplify and isolate the entire length of the zebrafish *dnmt1* cDNA exploiting the rapid amplification of cDNA ends-polymerase chain reaction (RACE-PCR) technology. Analysis of the full-length of zebrafish DNA (cytosine-5) methyltransferase obtained revealed striking conservation of the overall nucleotide sequence and the functional motif-specific sequences in particular among vertebrates.

Materials and Methods: Chapter One

I. cDNA Libraries Screening

I.1: cDNA library screening strategy

cDNA libraries (adult and neurula stage)

⇓

Preparation of plating bacteria (*XL1-blue*)

⇓

Titration of lambda phage library and library plating

⇓

Immobilization of phage plaques on nylon membranes

⇓

Hybridization with zf-specific DNA methyltransferase probe

⇓

Selection of positive plaques

⇓

Secondary and tertiary screening of positive colonies

⇓

Selection of positive plaques

⇓

Excision of bluescript phagemid from the lambda ZAP vector

⇓

Amplification and purification of recombinant bluescript phagemid

⇓

Characterization of the insert:

Restriction digestion
Southern blotting
Direct sequencing

I.2: cDNA libraries

cDNA libraries derived from zebrafish poly A^+ RNA extracted from different stages of development were made by Robert Riggleman and Kathryn Helde in the laboratory of Dr. David Jonah Grunwald at the University of Utah. These cDNA libraries, graciously supplied by Dr. D.J. Grunwald, were cloned into lambda ZAP II, from which Bluescript plasmids may easily be excised.

Two different cDNA libraries were screened. A neurula (9-16 hour) stage library (Appendix 3) with a mean insert size 0f 1.1 kb (0.4-2.2 Kb) and an adult stage library with an average insert size of 2.0 Kb (0.8-3 Kb).

I.3: Preparation of plating bacteria

XL1-Blue E. coli strain bacteria (Stratagene, Cat. No.200268) were streaked on LB-agar plates and incubated in a 37 °C incubator overnight. An isolated colony was used to inoculate a sterile 250 ml flask containing 50 ml of sterile LB-broth supplemented with 0.2% maltose to increase the efficiency of lambda phage adsorption. The culture was grown overnight in a 37 °C shaker water bath. The cells were harvested by centrifugation at 4000g for 10 minutes at room temperature in a clinical centrifuge (International Equipment Co.; Mass, USA). The cell pellet was resuspended and diluted in 0.01 M MgSO4 to a density of about 1.6 X 10^9 (OD_{600} = 2). This cell suspension was stored at 4 °C and was used for up to a week.

I.4: Titration and plating of lambda phage libraries

Ten fold serial dilutions of libraries stocks were prepared in SM [5.8 g NaCl, 2 g $MgSo_4$, 50 ml 1 M TrisCl (pH 7.5), 5 ml 2% gelatin solution, H_2O to 1 litre]. One hundred microlitres of plating bacteria were added to a 100 ul of each phage dilution to be assayed in 15 ml sterile tubes. The contents of each tube were mixed by gentle shaking and then incubated at 37 °C for 20 minutes to allow adsorption of phage particles to the bacteria. Then three ml of molten (47 °C) agarose (0.7%) were added to each tube. The tube contents were vortexed and immediately poured onto an appropriately labeled 30-35 ml LB-agar plate. The plate was swirled gently to ensure even distribution of bacteria and top agarose. These steps were repeated for each tube. The closed plates were left to stand at room temperature for about 5 minutes to allow the top agarose to harden. The plates were inverted and incubated at 37 °C for 12-16 hours. Bacteriophage lambda plaques were counted in each plate and the appropriate phage dilution to be used was determined. Three hundred ul of 1:1000 dilution of each library was added to 1 ml of plating bacteria. After a 20-minute incubation period at 37 °C, 30 ml of molten (47 °C) agarose (0.7%) were added and the contents of each tube poured onto 23 cm LB-Tetracyclin (25 ug/ml) agar plates. The closed plates were allowed to stand at room temperature for 5 minutes to allow hardening of the top agarose. The plates were then incubated inverted in a 37 °C incubator for 12-16 hours. Approximately 150,000 plaques were obtained and screened.

I.5: Immobilization of bacteriophage lambda plaques on nylon membranes

The plates containing phage plaques were chilled at 4 °C for 1-2 hours to allow the top agarose to harden. Nylon membranes (Boehringer Mannheim, Cat. No. 1417240) of appropriate sizes were labeled with a soft-lead pencil. At room temperature, the nylon membranes were neatly placed onto the surface of the top agarose so that it was in direct contact with the plaques. The membrane was marked in four asymmetric locations by stabbing through it and into the agar beneath with an 18-gauge needle attached to a syringe containing waterproof black drawing ink (India Ink). After 30-60 seconds the membrane was peeled off using a blunt-ended forceps. The membrane was placed, DNA side up, in a shallow tray with a piece of 3MM paper soaked in denaturing solution (0.5 N NaOH, 1.5 M NaCl) for 5 minutes and then in neutralizing solution (1.5 M NaCl, 0.5 M Tris.Cl [pH 7.4]) for 5 minutes. The membrane was rinsed in 2X SSC and then baked for 2 hours at 80 °C. The membrane was then prehybridized, hybridized and washed as in section I.9.

I.6: Picking positive bacteriophage lambda plaques

Positive bacteriophage plaques were determined by matching the positive signals on the autoradiograph to the specific plate used. Potentially positive single plaques chosen were stabbed using a sterile Pasteur pipette equipped with a rubber bulb into the hard agar beneath. With mild suction the plaque, together with the underlying agar, was drawn into the pipette and placed in a 1.5 ml microfuge tube containing 1 ml of SM and a

drop of chloroform. The bacteriophage particles were allowed to diffuse out of the agar by incubating the tube for 1-2 hours at room temperature. The tube containing the infectious bacteriophage particles was then stored at 4 °C.

Secondary screening was carried out using phage particles obtained from positive plaques in the primary screen and this was followed by tertiary screening of phage obtained from positive secondary ones. The methods used to perform these screens are similar to the ones used to perform the primary screen.

I.7: Rapid analysis of bacteriophage lambda isolates from single positive plaques

I.7:A. In vivo excision using the ExAssist helper phage/*SOLR* strain system

The ExAssist interference-resistant helper phage in conjunction with *SOLR* strain of *E.coli* (Stratagene, Cat. No.200253) allows efficient excision of the bluescript phagemid from the lambda ZAP vectors. The helper phage contains a mutation that prevents replication of the phage genome in nonsuppressing *E. coli* strain cells. Therefore only the excised phagemid will replicate in the host.

Host cells were prepared by streaking splinters, scraped from frozen cells, with a sterile wire loop on a LB-agar plate containing the appropriate antibiotic. *E.coli* of the *XL1-Blue MRF`* (Stratagene, Cat. No.200230) strain were plated on LB-tetracyclin (12.5 ug/ml) and *SOLR* (Startagene, Cat. No.200298) strain of *E.coli* were plated on LB-kanamycin (50 ug/ml). Fifty millilitres of LB broth [supplemented with 0.2% (v/v) maltose and 10 mM MgSO4] were inoculated with a single colony of the *XL1-Blue MRF*

(Stratagene, Cat. No. 200230) cells. In addition, 50 ml of LB broth (without supplements) was inoculated with a single colony of the *SOLR* (Startagene, Cat. No. 200298) cells. Cultures were grown overnight with shaking at 30 °C to ensure that the cells did not overgrow. The next morning, the cells were harvested by centrifugation at 4000g for 10 minutes and the cells were resuspended in 15 ml of 10 mM MgSO4. The cells were then diluted to an OD_{600} of 1.0 in 10 mM MgSO4.

In a sterile 50 ml tube, 200 ul of *XL1-Blue MRF`* cells (OD_{600} of 1.0), 250 ul of the phage stock and 1 ul of ExAssist helper phage were combined and the mixture was then incubated at 37 °C for 15 minutes. Three millilitres of LB broth were added and the culture was further incubated for 2-2.5 hours at 37 °C with shaking. On occasion the culture was incubated overnight since this is a single clone excision reaction and clonal representation is not relevant. The culture tube was heated at 70 °C for 15 minutes and then spun at 4000g for 15 minutes. The supernatant containing the excised phagemid Bluescript was transferred to a sterile tube. The excised phagemid was plated by adding 200 ul of freshly grown *SOLR* cells (OD_{600} = 1.0) to two sterile 15 ml tubes. One hundred microlitres of the phage supernatant was added to one tube and 10 ul aliquot of the phage supernatant added to the other tube. The tubes were incubated at 37 °C for 15 minutes. Two hundred microlitres from each tube were plated on LB-ampicillin (100ug/ml) agar plates and incubated overnight at 37 °C. Single colony isolation was achieved by titration of the supernatant when necessary. At this stage colonies appearing on the plates contain the bluescript double-stranded phagemid with the cloned cDNA insert.

I.7:B. Plate lysate method

In a small sterile culture tube, 100 ul of the bacteriophage suspension obtained above (section 1.6) was mixed with 100 ul of plating bacteria (XL1-blue). The culture tube was incubated at 37 °C for 20 minutes and then 2.5 ml of molten (47 °C) top agarose (0.7%) was added. The bacterial suspension was spread on the surface of a freshly poured 90-mm LB-agarose plate. The plate was inverted and incubated at 37 °C overnight or until the plaques were almost confluent. Three millilitres of lambda diluent [10 mM Tris.Cl (pH 7.5), 10 mM MgSO4] were added directly onto the surface of the top agarose. The bacteriophage particles were allowed to elute for two hours at room temperature with constant gentle shaking. The lambda diluent was transferred to a centrifuge tube and the bacterial debris was removed by centrifugation at 4000g for 10 minutes. The supernatant was then transferred to a centrifuge tube and 1 ul of each RNase A (1 mg/ml) and DNAase I (1 mg/ml) were added. The solution was incubated at 37 °C for 15 minutes. An equal volume of a solution containing 20% w/v polyethylene glycol (PEG 8000) and 2 M NaCl in lambda diluent was added. The mixture was mixed by gentle vortexing and then incubated on ice for an hour. The precipitated bacteriophage particles were recovered by centrifugation at 10,000g for 10 minutes at 4 °C. The supernatant was discarded and the tube was inverted on a paper towel to drain. The bacteriophage particles were resuspended in 0.5 ml TE (pH 8.0).

Five microlitres of 10% SDS were added to the resuspended bacteriophage particles and the tube incubated for 5 minutes at 68 °C. Ten microlitres of 5 M NaCl were added and the bacteriophage DNA was purified by extracting once with

phenol:chloroform and once with chloroform:isoamyl alcohol alone. An equal volume of isopropanol was then added to the aqueous phase and the sample stored at -70 °C for 15 minutes . The DNA was finally recovered by centrifugation at 12,000g for 15 minutes at 4 °C in a microfuge. The DNA pellet was washed with 70% ethanol and the DNA pellet was allowed to dry at room temperature. The DNA was dissolved in an appropriate volume of TE (pH 8.0).

I.8: Probe used to screen libraries:

In collaboration with Dr. T. Bestor (Columbia University), a putative zebrafish methyltransferase sequence was generated using universal primers and RIP-PCR. The identity of this PCR generated fragment was established in our laboratory and was subsequently used to probe a zebrafish cDNA library to isolate a 2021 base-pair long sequence. This clone constituted less than a half of the total size of the transcribed sequence of *dnmt1* as predicted from the RNA hybridization analysis experiments (data presented in chapter two). Therefore this partial-length clone was used to further screen cDNA libraries in an attempt to obtain the full length of the transcribed sequence of *dnmt1*.

I.8:A. Preparation of the probe

Plasmids containing cDNA clones were transformed into *XL1-blue* competent cells (Stratagene, Cat. No.200268). Preparation of competent bacteria and transformation

was according to the method of Nishimura *et al.* (1990). Five hundred microlitres of an overnight culture of *XL1-blue* cells was inoculated into 50 ml of transformation LB [10 g Bactotryptone, 5 g Yeast Extract, 10 g NaCl, 10 ml 1M MgSO4.7H2O, 20% glucose, pH 7.0, dissolved in 1000 ml ddH2O]. The cells were grown in a 37 °C shaker water bath to mid logarithmic phase (OD_{600} = 0.3-0.4). The cells were then chilled on ice for 10 minutes and pelleted at 4000 rpm for 10 minutes at 4 °C. The cells were resuspended in 0.5 ml of transformation LB that had been precooled on ice and then 2.5 ml of precooled storage LB medium [1.0 g Tryptone, 0.5 g Yeast extract, 1.0 g NaCl, 36 ml glycerol, 12.0 g PEG (8000), 1.2 ml 1.0 M MgSO4.7H2O] were added. The cells were subsequently stored at -70 °C in a 100 ul aliquots. A 100 ul aliquot of frozen competent cells was thawed on ice and then 1 ul (50 ng/ul) of plamid DNA was added to the cells. After mixing the contents the tube was kept on ice for 30 minutes, heat shocked at 42 °C for 2 minutes and cold shocked on ice for 2 minutes. Three hundred microliters of room temperature LB broth [10 g Bactotryptone, 5 g Yeast Extract, 10 g NaCl, pH 7.0 with NaOH, dissolved in 1000 ml ddH2O] were added and the tube was then incubated at 37 °C for one hour. One hundred microlitres of the solution was spread on a LB-Ampicillin [100ug/ml] agar plate and placed in a 37 °C incubator overnight. As well, two dilutions [1:10 and 1:100] were made of the solution and one hunderd microlitres of each were plated on LB-Ampicillin agar plates and incubated in a 37 °C incubator overnight.

I.8:B. Purification of plasmid DNA

A single isolated colony selected from one of the plates was inoculated into 3 mls of LB broth and allowed to grow overnight in a 37 °C shaker water bath. Mini plasmid preparation was conducted using the "Rapid Pure Miniprep" (BIO 101 Cat. No.2070-200) from 3.0 ml sample of the culture. After pelleting by centrifugation at 1300 rpm for 30 seconds, the cells were resuspended thoroughly in 50 ul of prelysis buffer. One hundred microlitres of alkaline lysis buffer were then added to the resuspended cells and inverted 5 times to mix the contents of the tube. The suspension was left at room temperature for two minutes. One hundred microlitres of neutralizing solution were added and contents were vortexed producing a white precipitate. The precipitate was removed by centrifugation at 13000 rpm for two minutes and the supernatant was transferred to a glass milk (250 ul) containing filter in a "catch tube" (kit provided). The assembled catch tube was spun at 13000 rpm for 30 seconds and the filter was subsequently washed twice with 350 ul of wash buffer. The plasmid DNA was eluted with 50 ul of low TE [10 mM Tris, 0.1 mM EDTA pH 8.0]. The plasmid DNA was quantified by optical density measurement. Quality and size of recovered plasmid was checked on an agarose minigel (0.8% agarose).

Larger amounts of plasmid DNA were obtained using the "Big Prep DNA" kit (Temco 5'-3', Inc. Cat. No.1-891792) and the "Concert High Purity Plasmid Midiprep System" kit (Gibco-BRL, Cat. No.11451). In the Big prep 100 ml of LB broth was inoculated with a single bacterial colony from the LB-Ampicillin [100 ug/ml] agar plate. The culture was grown in a 37 °C water bath with vigorous shaking for 18 hours. The

bacteria were pelleted by centrifugation at 6000 g for 5 minutes. The cell pellet was resuspended in 4 ml of Solution I (Buffered RNase A solution). Solution II (Alkaline lysis buffer) (6 ml) was then added to the cell suspension. The contents of the tube were gently mixed by rolling the tube several times and then the mixture was neutralized by the addition of 3 ml of solution III (potassium acetate solution). The bacterial cell wall and other proteins forming the white precipitate were pelleted by centrifugation at 6000 g for 5 minutes. The supernatant was filtered using a Acrodisc Syringe Filter (0.2 um pore size filter; Gelman Cat. No.4192) and 10 ml syringe. Big Prep DNA Binding Matrix (11 ml) (Big prep DNA Binding Matrix suspension in guanidine-HCl) was added to the filtered supernatant and mixed by vigorous inversion. The solution was placed on wet ice for 5 minutes and then placed in a Big Prep Spin Column inserted into a sterile 50 ml centrifuge tube. The bound plasmid DNA was washed twice with Purification Solution (Buffered salt solution plus 95% ethanol). The bound plasmid DNA was then eluted from the matrix spin column with 65 °C prewarmed low TE [10mM Tris-Cl, 0.1 mM EDTA pH 8.0].

The "Concert High Purity Plasmid Midiprep System" (Gibco-BRL, Cat. No. 11451) purifies plasmid from 15-25 ml of overnight grown culture. The pelleted cells were homogeneously resuspended in 4 ml of Cell Suspension Buffer (E1) [50 mM Tris-HCl (pH 8.0), 10 mM EDTA] containing RNase A (20 mg/ml in water). The cells were then lysed by adding 4 ml of Cell Lysis Solution (E2) [200 mM NaOH, 1% SDS (w/v)]. After 5 minutes incubation at room temperature, 4 ml of Neutralizing Buffer (E3) [3.1 M potassium acetate (pH 5.5)] were added and the tube was immediately mixed by repeated inversion. Cell debris was precipitated by centrifugation at 13,000g for 10 minutes at

room temperature. The supernatant was loaded on a pre-equilibrated column using 10 ml of Equilibration Buffer (E4) [600 mM NaCl, 100 mM sodium acetate (pH 5.0), 0.15% Triton X-100 (v/v)]. The solution in the column is allowed to drain by gravity flow. The column was washed twice with 10 ml of Wash Buffer (E5) [800 mM NaCl, 100 mM sodium acetate (pH 5.0)]. Plasmid DNA was then eluted by adding 5 ml of Elution Buffer (E6) [1.25 M NaCl, 100 mM Tris-HCl (pH 8.5)]. The solution in the column was allowed to drain by gravity flow. The plasmid DNA was precipitated by adding 3.5 ml of isopropanol to the elute and centrifugation at 15,000g at 4 °C for 30 minutes. The plasmid DNA pellet was washed with 70% ethanol and centrifugation at 15,000g at 4 °C for 5 minutes. The pellet was air dried and then dissolved in 200 ul of low TE Buffer (TE) [10 mM Tris-HCl (pH 8.0), 0.1 mM EDTA].

I.8:C. Purification of insert DNA from plasmid vector

Restriction endonuclease digests to isolate the inserted clone were performed as follows: A one hundred microlitre reaction was performed. Twenty microlitres [0.5 ug/ul] of plasmid DNA, 65 ul sterile ddH2O, 10 ul of 10X React buffer 4 (Gibco-BRL,Cat. No.16304-016) and 5 ul of KpnI restriction enzyme (Gibco-BRL Cat. No.15232-010) were mixed in a 1.5 ul microfuge tube. After mixing the contents and briefly spinning them down the tube was incubated at 37 °C for one hour. A five microlitre aliquot of the digest was removed to be checked later on a minigel (0.8% agarose). Five microlitres of SmaI restriction enzyme (Gibco-BRL Cat. No.15228-018) were then added to the digest and the sample was incubated at 30 °C for one hour. The

double digest generated two distinct bands, ~2.958 Kb band (vector) and ~2 Kb band (insert).

The remaining volume of the digest was mixed with 1X loading buffer [20% Ficoll , 10mM Tris (pH 8.0), 50 mM EDTA, 0.25% Bromophenol blue, 0.25% Xylene cyanol] and loaded in a 0.8% agarose gel. TAE (1 X) [40 mM Tris, 10mM sodium Acetate, 1mM EDTA] was used as a running buffer and the gel was run for one hour at 100 volts. The DNA was visualized by ethidium bromide (1.0 ug/ml) staining and the appropriate size band was excised from the gel and placed in a 1.5 ml microfuge tube. The DNA fragment was then extracted from the overnight frozen (-20 °C) gel using the Sephaglas Band Prep kit (Pharmacia Biotech Cat. No.27-9285-01) as follows: based on the weight of the gel fragment a suitable amount of gel solublizer was added to the tube which was then placed in a 55 °C water bath for 5 minutes or until the gel was dissolved. An appropriate amount of glass milk solution was added and allowed to sit at room temperature for 5 minutes with mixing by inversion of the tube at one minute intervals. The tube contents were then centrifugated at 13000 rpm in a microfuge for one minute to pellet the Sephaglass milk. The pellet was washed twice with the provided wash solution. The DNA was then eluted from the glass milk with 50 ul of elution buffer.

The final concentration of the eluted cloned insert was determined spectrophotometrically by measuring the absorbance at 260 nm of a properly diluted sample. The final concentration was calculated based on the fact that an OD of 1 is equal to 50 ug/ml of DNA. Purity of the DNA was determined at the same time by measuring the absorbance at 280 nm and calculating the OD_{260}/OD_{280} ratio. In addition, the

concentration of the eluted DNA was estimated by spotting it against known standard plasmid DNA concentrations.

I.8:D. Labeling DNA probes

The purified 2021 bp DNA cytosine-5-methyltransferase clone was radioactively labeled using the Random Primer DNA Labeling System (Gibco-BRL Cat. No.18187-013). In a fifty microlitre reaction, one hundred nanograms of the insert were diluted in water and mixed with 2 ul of dATP, dGTP , dTTP [0.5 mM dNTP in 3 mM Tris-HCl (pH 7.0), 0.2 mM disodium EDTA], 10 ul random primer buffer mix [0.67 HEPES, 0.17 M Tris-HCl, 17 mM MgCl2, 33 mM 2-mercaptoethanol, 1.33 mg/ml BSA, 18 OD 260 units/ml], 5 ul α ^{32}PdCTP (NEN Life Science Products, Inc. Cat. No.BLU513H) [approximately 50 uCi] and 1 ul of Klenow enzyme (large fragment DNA polymerase I) [3 units/ul]. The reaction tube was incubated at 25 ^{o}C for one hour and the labeled clone was precipitated with 8 ul of 5 mg/ml calf thymus DNA (Sigma Cat. No.D-4522), 8 ul of 100 mM spermine and 134 ul of TE. The precipitation was allowed to proceed on ice for 30 minutes and then the DNA was pelleted by centrifugation at 13000 rpm for 10 minutes. The pellet was washed twice with 200 ul of a TE solution containing 1 mM spermine and then resuspended in 500 ul of a TE solution containing 0.5 M NaCl by boiling the tube for 5 minutes. Two microlitres of the reaction were used for scintillation counting to determine the incorporated radioactivity. This 2 ul aliquot was added to 0.5 ml of Bioflour (New England Nuclear Cat.No. NEF 961) and was counted using the Chicago Nuclear Scintillation Counter.

I.9: Pre-hybridization, Hybridization, washing, exposure and film developing

Once DNA was fixed to the membrane by baking, the membrane was placed in a plastic bag and an appropriate volume of prehybridization solution [6X SSC, 10X Denhardt solution [0.02 M Ficoll, 0.02 M Polyvinylpyrrolidone, 0.02 M Bovine Serum Albumin], 500 ug/ml salmon sperm DNA, 0.5% SDS and water] was added. The blot was allowed to prehybridize at 42 °C for 18 hours.

Four million counts per ml of radiolabeled DNA was added to the appropriate volume of hybridization solution [50% Formamide (Gibco-BRL Cat. No.15515-026), 4X SET [0.15M NaCl, 0.03M Tris, 2.0mM EDTA (pH 8.0)], 1X Denhardt's solution, 100 ul/ml boiled and sheared salmon sperm DNA (Sigma Cat. No.D-9156), 0.5% SDS and water]. Each hybridization was left for approximately 18 hrs in a 42 °C oven.

The blot was removed from the hybridization solution and agitated in 1X wash solution [0.1X SSC, 0.1% SDS] at room temperature for 20 minutes. The prewash solution was then replaced with 67-68 °C prewarmed 1X wash solution and was agitated for 20 minutes in a 67-68 °C water bath. The 67-68 °C wash was repeated four times for a total of 5 washes. The blot was then removed from the solution, dried and plastic wrap sealed. The blot was arranged in a film cassette with X-Ray film (Kodak) and an intensifying screen. The cassette was then placed at -70 °C for variable lengths of time ranging from two hours to overnight. The film was developed in Kodak Developer GBX (8124331) for 5 minutes and then fixed in Kodak Rapid Fix (KP 65351v) for five to ten minutes, rinsed in water and air-dried. The exposure intensity was then visually assessed and decisions regarding length of further exposure were made.

I.10: Restriction digestion of positive clones

The purified isolated plasmid DNA samples were quantified and 0.5 ug of each was digested with a number of appropriate restriction enzymes to either determine the size of the insert or to prepare the insert DNA for sequencing. Fifty microlitre restriction digests were performed in 500 ul microfuge tubes. One tenth of the total volume of the appropriate 10X REACT buffer and 2 ul of the appropriate restriction enzyme were added to the plasmid DNA and digestion was allowed to proceed at the appropriate temperature for one to two hours.

To an aliquot of the digestion product, 1 ul of loading buffer was added and the sample was loaded on a 1% agarose gel. The gel was run at 100 volts for one hour or until the Bromo-phenol blue dye had migrated approximately three quarters down the gel. The gel was then stained with ethidium bromide for 15-30 minutes and destained in distilled water for 30 minutes. An ultraviolet trans-illuminator allowed visualization of the ethidium bromide stained DNA restriction digestion products and the images were either captured using Polaroid 667 film or digitally using a Fluorochem digital setup.

I.11. Characterization of isolated positive clones

I.11:A. Sequencing of the isolated positive clones

The initial sequencing cycle was performed using the M13 forward and reverse primers supplied with the Gibco-BRL dsDNA Cycle Sequencing System (Cat. No.18196). The primers were end labeled by adding 1 ul of 5X kinase buffer [300 nm Tris HCl pH 7.8, 50 mM MgCl2, 1 M KCl] to 1 ul of T4 polynucleotide kinase [1 u/ul], 1 ul of γ-^{33}P dCTP [1000 Ci/mmol stabilized] (NEN Life Science Products, Inc., Cat. No.NEG302H) and 2 ul of primer suspended in water. The 5 ul reaction was incubated at 37 °C for 10 minutes then at 55 °C for 15 minutes and finally placed on ice.

To the 5 ul end-labeled primer 100 ng of appropriately digested recombinant plasmid, 4.5 ul 10X Taq sequencing buffer [300 mM Tris HCl pH 9.0, 50 mM $MgCl_2$, 0.5% w/v], 36 ul of water and 0.5 ul of Taq DNA polymerase [2.5 U/ul] were added. Eight microlitre samples of this prereaction mixture were added to four different tubes each with 2 ul of a specific termination mixture. Each termination mixture corresponds to one of the four DNA nucleotides. The 10 ul reaction mixture were mixed, briefly centrifuged and a drop of silicon oil was placed on top. The tubes were placed into the Thermolyne Temptronic Temperature Cycling Incubator and the following program was run: initial denaturing at 95 °C for 3 minutes followed by 30 cycles of 95 °C for 30 seconds, 55 °C for 30 seconds and 70 °C for 60 seconds and a final dwell cycle at 4 °C. Tubes were then removed from the thermal cycler, placed on ice and 5 ul of a stop

solution-loading buffer [95% (v/v) formamide, 10 mM EDTA (pH 8.0), 0.1% (w/v) bromophenol blue, 0.1% (w/v) xylene cyanol] was added.

I.11:B. Polyacrylamide gel electrophoresis and exposure to X-ray film

Five hundred millilitres of 5% polyacrylamide solution (23.75 grams of acrylamide, 1.25 grams of bisacrylamide, 210 grams of urea, 100 ml of 5X TBE [1.0 M Tris pH 8.0, 0.45 M Boric Acid, 0.5 M EDTA pH 8.0] diluted in 500 ml water and filtered through Whatman No. 1) were made and stored at 4 °C. To 65 ul of the polyacrylamide solution, 500 ul of 10% ammonium persulfate and 70 ul of TEMED (Gibco-BRL Cat. No.15524-010) were added and the solution was poured into the glass plate arrangement (0.4 mm spacers). After solidification of the gel, the glass plate arrangement was rinsed and placed in the Model S2 upright sequencing gel apparatus (Gibco-BRL Cat. No.21105-010). After assembly of the sequencing apparatus, one litre of 1X TBE was added as the running buffer and the gel was pre-run for about an hour. Five microlitres of each sequencing mix was denatured at 95 °C for three minutes and then loaded on the gel. The gel was run at approximately 55 amperes, 1800 volts, 80 watts for 2 hours or until the blue dye just ran off the bottom of the gel. The gel was loaded three separate times and allowed to run for appropriate lengths of time and distance. The sequencing apparatus was then disassembled and the glass plates were separated. The gel was transferred to a sheet of Watman No. 1 filter paper and placed in the Gel Dryer Model 583 on Cycle Two and allowed to dry at 80 °C for an hour. The dried gel was then arranged in the film cassette with Kodak BioMax MR film. The film

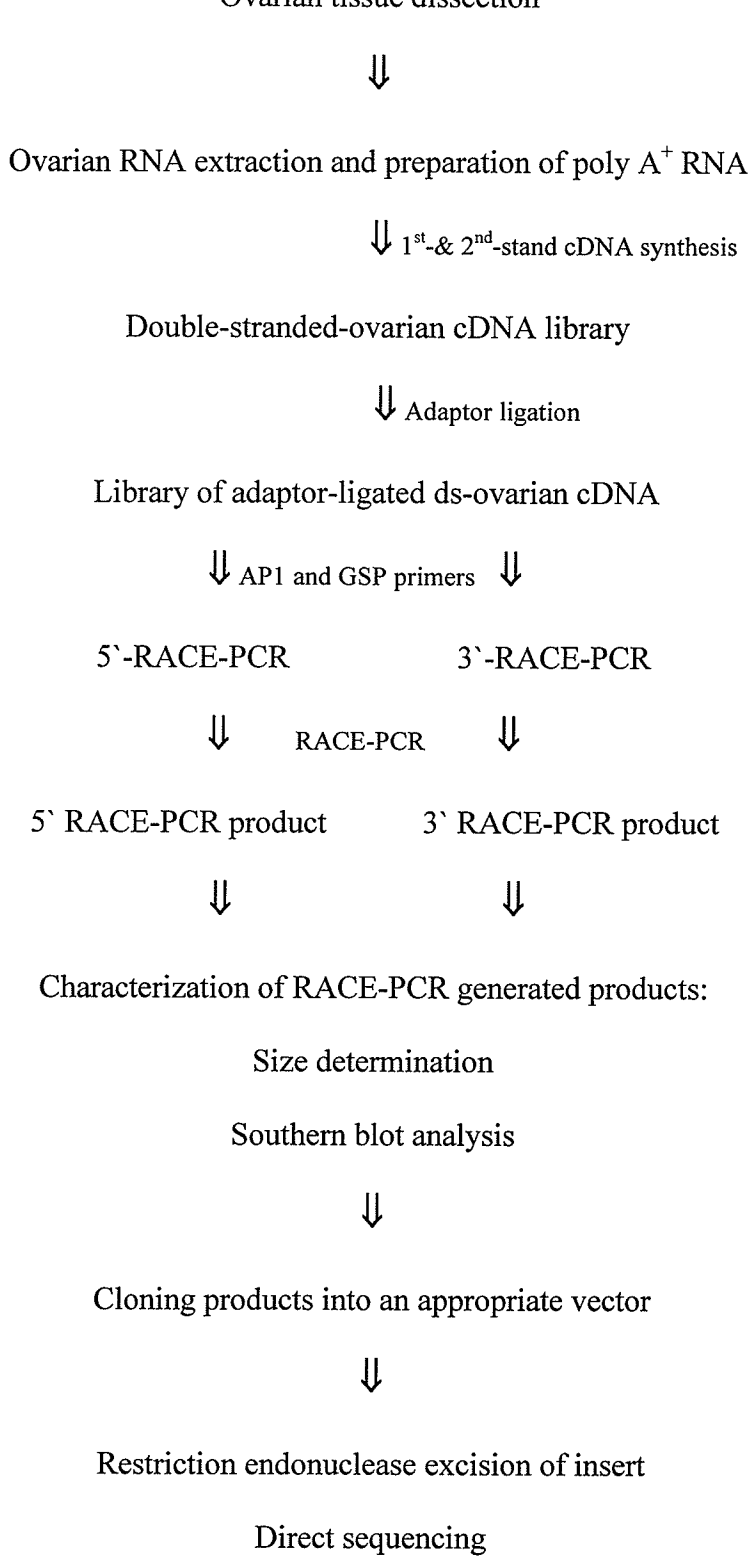
was developed after an overnight exposure at -70 °C. The film was developed in Kodak Developer GBX (8124331) and then fixed in Kodak Rapid Fix (KP 65351v) for five to ten minutes, rinsed in water and air-dried. The exposure intensity was then visually assessed and decisions regarding length of exposures were made. Analysis of the sequence was performed using the "Basic Local Alignment Search Tool" (BLAST) (Altchul *et al.,* 1997) program.

II. Preferential amplification of the zebrafish DNA (Cytosine-5) methyltransferase-1 cDNA using RACE-PCR

Rapid amplification of cDNA ends (RACE) is a polymerase chain reaction (PCR)-based technique designed to obtain full-length cDNA 5' and 3' ends after a partial cDNA sequence has been obtained by other methods (reviewed in Schaefer, 1995). Both 5` and 3`cDNA ends can rapidly be amplified from the same template. A prerequisite to designing RACE-PCR experiments is the knowledge of at least 23-28 nucleotides of sequence information in order to design the gene-specific primers for both the 5`- and 3`-RACE experiments. The minimal requirement for sequence information suits the characterization of partial clones obtained by cDNA library screening.

We employed this technique using dnmt1 gene-specific primers to obtain the full-length cDNA of dnmt1. We utilized sequence information obtained from the positive clones obtained by cDNA libraries screening to design gene-specific primers to preferentially amplify both the 5`- and the 3`-ends of the zebrafish DNA cytosine-5 methyltransferase cDNA. The Marathon cDNA amplification kit (Cat. No. K1802-1, Clonetech Laboratories, Inc.) was used in these experiments, which resulted in obtaining the full-length of dnmt1 cDNA.

II. 1: RACE-PCR strategy:



II. 2: General considerations for RACE-PCR

An overview of the experimental design of the preferential amplification of both the 3`- and 5`-regions of the zebrafish DNA cytosine-5 methyltransferase cDNA by the RACE-PCR approach is presented in figure 1. The general principles to be considered prior to starting the RACE-PCR set of experiments and the detailed experimental procedures are presented in the following sections.

II.2.A. Primer Design

After verification of the identity of the positive clones obtained by cDNA library screening, the sequence data obtained was used to design the gene-specific primers (GSPs). The GSPs (Appendix 1) were designed to have a length between 23-28 nucleotides, 50-70% GC content and a melting temperature ≥ 65 °C. GSP1 was designed to amplify the 5' region of *dnmt1* cDNA with an expected size of about 3.0 kb. To preferentially amplify the 3' region of *dnmt1* cDNA, a second gene specific primer was designed and was called GSP2 (Appendix 1). The 3` RACE-PCR product was expected to be about 2.0 kb.

The GSP1 and GSP2 were designed so that if used together, they would generate a 200 bp amplicon. This served as a control to indicate gene specific amplification. Another GSP1 primer was designed to be internal to GSP1 (nested GSP1) and was called GSP1b (Appendix 1). This nested primer was designed for use in nested RACE-PCR experiments if needed.

II.2.B. cDNA Synthesis

RACE-PCR experiments start with cDNA synthesis. Total and poly A^+ ovarian RNA were used to provide a template for first strand cDNA synthesis. A modified lock-docking oligo (dT) primer with two degenerate nucleotide positions at the 3' end was used to prime the first strand cDNA synthesis. Second strand synthesis was performed with a combination of *E. coli* DNA polymerase I, RNase H and *E. coli* DNA ligase. A radioactive tracer (^{32}P-dCTP) was incorporated in the first stand cDNA synthesis so that the efficiency and the quality of the generated double-stranded cDNA could be monitored prior to their use in the preferential amplification by RACE-PCR of the cDNA of interest.

II.2.C. Adaptor Ligation

Following creation of blunt ended cDNA ends with T4 DNA polymerase, the ds-cDNA was ligated to the cDNA adaptor (Appendix 1). The adaptor was partially double-stranded and was phosphorylated at the 5' end to facilitate blunt-end ligation of the adaptor to both ends of the ds-cDNA by T4 DNA Ligase. After adaptor ligation, an uncloned library of adaptor-ligated ds-cDNAs was obtained.

II.2.D. 5' and 3' RACE-PCR

Both 5' and 3' RACE-PCR reactions were primed with the internal, gene-specific primer (GSP), and the adaptor primer (AP1). The adaptor-ligated double stranded-cDNA

does not contain a binding site for AP1. However, during the first round of thermal cycling, an AP1 binding site was created by the extension of the GSP to the end of the adaptor. Subsequent PCR cycles would then exponentially amplify the cDNA of interest while minimizing nonspecific products.

II.2.E. Characterization of RACE Products

To confirm that the gene of interest had been amplified, the RACE products were characterized by several methods:

1. Determination of the size of the RACE-PCR product via fractionation on agarose gels.

2. Comparing PCR products obtained using GSP1 and AP1 to products generated with nested GSP1 (GSP1c).

3. Production of expected size amplicons that were generated using GSP1 and GSP2 primers.

4. Southern analysis by probing the membrane-bound RACE-PCR amplicons with a radioactive gene specific oligomer.

5. Direct sequencing of the PCR products.

II. 3: RACE-PCR amplification of 5' and 3' ends of zebrafish DNA cytosine-5-methyltransferase

II.3.A. Choice of cDNA synthesis template

II.3.A:i. Tissues used for total and poly A^+ RNA

To ensure the isolation of the cDNA of interest, zebrafish ovarian total and poly A^+ RNA was chosen to provide a template for the first-strand cDNA synthesis because RNA hybridization experiments have shown it to be rich in zf-*DNA (cytosine-5) methyltransferase-1* mRNA (chapter two). Zebrafish tissue dissection and total RNA extraction are described in chapter two.

II.3.A.ii. Poly A^+ preparation

Oligo dT columns (GIBCO-BRL; Cat.No. 15939-010) were flushed with 5 ml NaOH twice and then five times with 5 ml of 0.1% diethylpyrocarbonate (DEPC-Sigma)-treated H_2O (depc-H_2O). Columns were then equilibrated by flushing five times with 5 ml binding buffer [0.5M NaCl, 0.5% SDS depc treated and autoclaved]. Total RNA sample was prepared by the addition of Tris to a final concentration of 0.01M and SDS to a final concentration of 0.5% in 5 ml total volume. The sample was heated at 65 ^{o}C for 5 minutes and NaCl was added to a final concentration of 0.5M. After applying and passing the RNA sample through the column twice, the column was flushed with 10 ml of binding buffer followed by 10 ml of 0.5M NaCl. Finally, mRNA was eluted by flushing the column with depc-H_2O slowly and then precipitated with 1/10 volume 3.0M

Na Acetate and 2.5 volumes ethanol.

II. 3.B: First-strand cDNA Synthesis

Ten micrograms of total, or one microgram of poly A^+, ovarian RNA in five microlitres of depc-treated H_2O and one microlitre of cDNA synthesis primer (10 uM) were combined in a 0.5 ml microfuge tube and incubated at 70 °C for 2 minutes. The tube was then placed on ice and 2 ul 5X first-strand buffer, 1 ul dNTP mix (10 mM), 1 ul [α-^{32}P]dCTP (1 uCi/ul) and 1 ul of AMV reverse transcriptase (20 units/ul) were added. After mixing the contents, the tube was incubated at 42 °C for an hour in an air incubator (Isotemp Incubator, Fisher Scientific). Termination of first-strand synthesis was achieved by placing the tube on ice. A positive control cDNA synthesis using Human Placental Poly A^+ RNA was simultaneously performed. This first-strand cDNA made was used as a positive control in all subsequent steps.

II. 3.C: Second-strand cDNA synthesis

Second-strand cDNA synthesis was performed by adding 48.4 ul of sterile water, 16 ul of 5X Second-Strand buffer, 1.6 ul dNTP Mix and 4 ul 20X Second-Strand Enzyme cocktail to the 10 ul First-Strand reaction. The reaction mixture was then incubated at 16 °C for 1.5 hours. Two microlitres (10 units) of T4 DNA Polymerase were then added and the reaction was incubated for 45 minutes at 16 °C. The Second-Strand synthesis

reaction was terminated by adding 4 ul of EDTA/Glycogen Mix. Phenol/chloroform extraction and ethanol precipitation were performed as described in earlier sections. The yield and size of cDNA was assessed by running a 2 ul aliquot on a 1% agarose gel and ethidium bromide staining. The gel was then dried using a vacuum gel drying system and exposed to an X-Ray film for several hours at –70 °C.

II. 3.D: Adaptor ligation

Ligation of the Marathon cDNA Adaptor to the ds cDNA was performed by combining half of the ds cDNA made in the previous step (5 ul) with 2 ul of Marathon cDNA Adaptor (10uM), 2 ul of 5X DNA ligation buffer and 1 ul T4 DNA ligase (1 unit/ul). The reaction was allowed to proceed at room temperature for 3-4 hours. Subsequently the ligase was inactivated by heating at 70 °C for 5 minutes.

II.3.E: Dilution of adaptor-ligated ds-cDNA

The adaptor-ligated ds cDNA was diluted to a concentration suitable for subsequent RACE-PCR procedures (0.1 ug/ml). As the experimental cDNA yield was comparable to that obtained with the positive control RNA, 1/250 and 1/500 dilutions of the experimental and control cDNA were made in Tricine-EDTA Buffer. The diluted samples were then heated at 94 °C for 5 minutes to denature the double stranded cDNA and were placed on ice for 5 minutes. All subsequent PCR reactions were performed using these diluted samples of ds-cDNA.

II.3.F: Control PCR experiment

A positive control RACE-PCR experiment was performed to ensure that the Marathon RACE protocol works with our thermal cycler and other variables. Master mix for all PCR reactions was prepared by mixing the following for each 50 ul PCR reaction: 36 ul of water, 5 ul of 10X cDNA PCR reaction buffer, 1 ul dNTP Mix (10 mM) and 1 ul Advantage cDNA Polymerase Mix (50X). PCR reactions were prepared as shown in table 1.

Table 1. Control RACE-PCR experiment:

Component	5`-RACE control	3`-RACE control	Internal control
PCR ctrl. CDNA	5 ul	5 ul	5 ul
5`-RACE TFR primer (10 uM) (Appendix 1)	1 ul	0.0	1 ul
3`-RACE TFR primer (10 uM) (Appendix 1)	0.0	1 ul	1 ul
AP1 primer (10 uM) (Appendix 1)	1 ul	1 ul	0.0
Master mix	43 ul	43 ul	43 ul
Total volume	50 ul	50 ul	50 ul
Expected size	2.6 Kb	2.9 Kb	0.3 Kb

The contents of each tube were overlaid with 2 drops of mineral oil, capped firmly and placed in the thermal cycler [MiniCycler, MJ Research].

Thermal cycling was commenced with a denaturing cycle at 94 °C for 1 minute followed by 5 cycles of 94 °C for 30 seconds and 72 °C for 4 minutes. This was followed by 5 cycles of 94 °C for 30 seconds and 70 °C for 4 minutes and then 20-25 cycles of 94 °C for 20 seconds and 68 °C for 4 minutes. The PCR products were electrophoresed on a 1% agarose gel along with a size marker. The gel was ethidium bromide stained and sizes of the different bands were determined.

II:3.G: Rapid Amplification of 5` and 3` cDNA ends (RACE)

The 5`- and 3`-RACE PCR reactions along with positive controls for both the 5` and 3` ends of the zf-MTase cDNA were performed as follows:

II.3.G.i. 5`-RACE reactions

The 5`-RACE PCR reactions were conducted as shown in table 2.

Table 2. 5`-RACE reactions set up.

Component	Exp. sample	TFR pos. ctrl.	GSP1 + 2 pos. ctrl.	AP1 only neg. ctrl.	GSP1 only neg. ctrl.
Exp. CDNA	5 ul	0.0	5 ul	5 ul	5 ul
Pos. ctrl. cDNA	0.0	5 ul	0.0	0.0	0.0
AP1 primer (10 uM)	1 ul	1 ul	0.0	1 ul	0.0
GSP1 primer (10 uM)	1 ul	0.0	1 ul	0.0	1 ul
GSP2 primer (10 uM)	0.0	0.0	1 ul	0.0	0.0
Ctrl. 5'-RACE TFR primer (10 uM)	0.0	1 ul	0.0	0.0	0.0
H_2O	0.0	0.0	0.0	1 ul	1 ul
Master mix*	43 ul	43 ul	43 ul	43 ul	43 ul
Final volume	50 ul	50 ul	50 ul	50 ul	50 ul
Expected product size	3.0 Kb	2.6 Kb	0.2 Kb	None	None

*PCR master mix was prepared as described in the previous section.

II.3.G.ii. 3`-RACE reactions

The 3`-RACE PCR reactions were conducted as shown in table 2.

Table 3. 3`-RACE reactions set up.

Component	Exp. sample	TFR pos. ctrl.	GSP1 + 2 pos. ctrl.	AP1 only neg. ctrl.	GSP2 only neg. ctrl.
Exp. cDNA	5 ul	0.0	5 ul	5 ul	5 ul
Pos. ctrl. cDNA	0.0	5 ul	0.0	0.0	0.0
AP1 primer (10 uM)	1 ul	1 ul	0.0	1 ul	0.0
GSP2 primer (10 uM)	1 ul	0.0	1 ul	0.0	1 ul
GSP1 primer (10 uM)	0.0	0.0	1 ul	0.0	0.0
Ctrl. 3'-RACE TFR primer (10 uM)	0.0	1 ul	0.0	0.0	0.0
H2O	0.0	0.0	0.0	1 ul	1 ul
Master mix	43 ul	43 ul	43 ul	43 ul	43 ul
Final volume	50 ul	50 ul	50 ul	50 ul	50 ul
Expected product size	2.0 Kb	2.9 Kb	0.2 Kb	None	None

The contents of each tube were overlaid with 2 drops of mineral oil, capped firmly and placed in the thermal cycler [MiniCycler, MJ Research]. Thermal cycling was

commenced at 94 °C for 1 minute followed by 5 cycles of 94 °C for 30 seconds and 72 °C for 4 minutes, 5 cycles of 94 °C for 30 seconds and 70 °C for 4 minutes and 20-25 cycles of 94 °C for 20 seconds and 68 °C for 4 minutes.

Five microlitres of each PCR product were electrophoresed on a 1% agarose gel along with appropriate DNA size markers. The gel was ethidium bromide stained and sizes of the different bands were determined. The DNA was then transferred to a nylon membrane and hybridized using a gene specific cDNA probe (Southern analysis, see section I). The remainder of the 5' and 3'-RACE PCR products were run on a 0.8% agarose gel. The respective bands were cut out of the gel and the cDNA was purified as described in the previous section.

II.3.H: Subcloning of RACE-PCR products

The purified amplified 5' and 3' RACE fragments were inserted into pCR2.1 plasmid using the Original TA Cloning Kit (Invitrogen; Cat. No.K2000-J10,K2000-10 and K2000-40). This method is based on the fact that *Taq* Polymerase has a nontemplate-dependent activity which adds a single deoxyadenosine (A) to the 3' ends of PCR products. The linearized vector pCR2.1 [Invitrogen] has single 3' deoxythymidine (T) residues. This allows PCR inserts to ligate efficiently with the vector. The concept of TA cloning method is shown in figure 2 (results section).

II.3.I: Cloning into pCR2.1 vector

The amount of PCR product needed to ligate with 50 ng of pCR2.1 vector was estimated by the formula:

X ng PCR product = (Y bp PCR product)(50 ng pCR2.1 vector)/(Size in bp of pCR2.1 vector: 3900 bp). Where X ng is the amount of PCR product of Y base pairs to be ligated for a 1:1 (vector:insert) molar ratio.

A 10 ul ligation reaction was set up using 2 ul of PCR product, 1 ul of 10X ligation buffer, 2 ul (25 ng) pCR2.1 vector, 4 ul of sterile water and 1 ul of T4 DNA ligase enzyme (4.0 Weiss units). The reaction was allowed to proceed by incubation at 14 °C overnight. The ligation product was then transformed into competent INVαF' cells (Invitrogen; Original TA Cloning Kit, Cat. Nos.K2000-J10, K2000-01 and K2000-40). LB plates containing 50 ug/ml of ampicillin were prepared the night before the transformation. The plates were equilibrated at 37 °C prior to use. After temperature equilibration of the plates, 40 ul of 40 mg/ml X-Gal (GIBCO BRL, Cat.No.15520-034) were spread on each plate and the liquid was allowed to soak into the plates for each ligation/transformation one 50 ul vial of One Shot competent cells was used. Two microlitres of 0.5 M β-mercaptoethanol were pipetted into the vial of competent cells and mixed by gentle stirring. Two microlitres of the ligation reaction product were then added to the cells and the contents of the tube were gentle stirred. The cells were heat-shocked for 30 seconds at 42 °C and then placed on ice for 2 minutes. Two hundred and fifty microlitres of SOC medium (2% Tryptone, 0.5% Yeast Extract, 10.0 mM NaCl, 2.5 mM KCl, 10.0 mM MgCl2-6H2O, 20.0 mM glucose) were added and cells were

incubated at 37 °C for one hour in a shaking incubator. Fifty microlitres and 200 ul from each transformation vial were spread on separate previously prepared LB agar plates. Once the liquid was absorbed, the plates were inverted and incubated in a 37 °C incubator overnight. The plates were examined for the presence of white colonies and ten of these were picked for plasmid isolation and restriction analysis. Each isolated colony was grown overnight in 5 ml of LB broth containing 50 ug/ml ampicillin in a 37 °C water bath with vigorous shaking. Plasmid preparation was performed the following morning as described in the previous sections. The recombinant plasmids were characterized by restriction digestion and sequencing.

II.3.J: Sequencing of RACE-PCR products

The cloned RACE-PCR products were initially sequenced by dideoxy chain termination using the M13-reverse and forward primers (Appendix 1). Double stranded DNA sequencing and sequence analysis were performed as described in the previous section. Subsequent sequencing using internal primers (Appendix 1) that were designed based on the obtained initial sequence was continued using an ABI PRISM Gene Analyzer (Model 310, Version 3.0) (Institute of Cell Biology, University of Manitoba, MB Canada).

II.3.K: Sequence analysis

The identity of the assembled nucleotide sequences was determined using the "Basic Local Alignment Search Tool" (BLAST 2.0) (Altschul *et al.,* 1997). The National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/) nucleotide and protein sequence databases, "Nucleotide Entrez" and "Protein Entrez", were used to retrieve the nucleotide and amino acid sequences of DNA cytosine-5 methyltransferase-1 from several organisms as indicated in the results section. Multiple alignments were performed using Clustal V (Higgins *et al.,* 1991) program.

Results: Chapter One

I. Zebrafish DNA cytosine-5 methyltransferase clones obtained from cDNA library screening

Several positive clones resulted from the cDNA library screening experiments. Figure 3 is an illustrative example of one of these screens. The identity of all positive clones was confirmed to be *dnmt1* by sequencing. However the size of the largest positive clone obtained was 2.8 Kb. All sequenced positive clones represented the 3`-region of *dnmt1* and no clones containing the 5`-region of dnmt1 were obtained.

II. RACE-PCR Amplification of 5` and 3` Regions of *dnmt1*

To obtain the 5` end of dnmt1 cDNA, we employed RACE-PCR technology using sequence data from the positive clones obtained by the cDNA library screens. Along with the 5`-RACE-PCR cloning, we designed experiments to preferentially amplify the 3`-region of *dnmt1* for comparison with the *dnmt1* 3` region obtained by cDNA library screening as a control for amplification efficiency. Using 5` RACE-PCR, a 3.0 Kb fragment was amplified and the 3` RACE-PCR generated a 2.0 Kb fragment as expected (Figure 4). The amplicon sizes obtained were as expected based on our knowledge of *dnmt1* message size (~5.0 kb) and our experimental design. Both the 5`- and 3`-TFR positive controls gave the expected amplicon sizes of 2.6 kb and 2.9 kb respectively (figure 4).

III. Cloning of the full length *dnmt1* cDNA using RACE-PCR

The 5`- and 3`-RACE-PCR fragments obtained were subcloned into pCR 2.1 plasmid using the TA Cloning Kit (Invitrogen) and restriction digests that release the insert from the cloning vector are shown in figure 5. The inserts were subsequently sequenced on both strands, the forward and the reverse. The sequence of the 3` fragment obtained by RACE-PCR was identical to the one obtained by library screening suggesting efficient PCR amplification. The entire cDNA sequence obtained was 4907 bp (Figure 6) (GenBank Accession No.AF483203; Mhanni *et al.,* 2001).

IV. Sequence analysis of *dnmt1*

The assembled nucleotide sequence is capable of encoding an open reading frame of 1499 amino acids (figure 6) comparable in length to the human enzyme (1495 amino acids). The inferred amino acid sequences of zebrafish and human methyltransferases are 82% similar overall (figure 7 and 8). The overall amino acid sequence similarity with the mouse and *Xenopus* MTases is 70% and 81% respectively (figure 8-B). However the similarity was more pronounced for all the conserved functional motifs found in other vertebrates. A comparison of the order and degree of similarity of all the known functional domains of the human and zebrafish MTase enzymes is shown in figure 8-B.

Discussion: Chapter One

The assembled nucleotide sequence contains an open reading frame encoding 1,499 amino acids (figure 6), which is similar in size to methltransferase proteins found in other organisms. In-frame termination codons are found up-stream of the ATG further supporting the assumption that the entire coding region is found within the present clone. The zebrafish sequence described above was found to be very similar to a large number of MTases isolated from other organisms. The results of an examination of the presence, order, and relative positions of the known functional motifs of the mammalian Dnmt1 (Bestor *et al.,* 1988; Leonhardt *et al.,* 1992; Cardoso and Leonhardt, 1999) in the zebrafish dnmt1 enzyme are shown in figure 7 and 8-A. Overall, the zebrafish *dnmt1* cDNA had the highest degree of similarity to the *Xiphophorus* XDNMT-1 (87%). This was followed closely by the human DNMT1 (84%), *Xenopus* XDnmt1 (83%), chicken (82%), and mouse Dnmt1 (72%) (figure 8-B). Moreover, as illustrated in figure 8, all conserved functional motifs of the enzyme in these organisms are well conserved in the zebrafish.

At least three independently functional nuclear localizing regions (NLRs) have been identified in the mouse Dnmt1 (Cardoso and Leonhardt, 1999). The putative zebrafish dmnt1 protein has motifs that correspond in position (figure 7 and 8) and sequence (figure 7) to these regions and therefore may represent functional NLRs. A number of RNA splicing proteins were found to bear some resemblance to the mouse Dnmt1 N-terminal region between amino acids 120 and 210 (Leonhardt *et al.,* 1992). The corresponding region in *Xiphophorus*, chicken, *Xenopus*, human and also the zebrafish Dmnt1 proteins show limited similarity based on amino acid identity and

conservative changes (figure 8). The functional significance, if any, of the sequence similarities between Dmnt1 and the splicing factors remains to be determined (Leonhardt *et al.,* 1992).

Cardoso and Leonhardt (1999) mapped a Dnmt1 cytoplasmic retention sequence and they noted that fusion constructs containing the broad region from amino acids 308–854 of the oocyte-specific Dnmt1 (426–972 of the somatic form of Dnmt1) are efficiently retained in the cytoplasm of early mouse embryos. Interestingly, the corresponding region in zebrafish dmnt1 shows 76% similarity (figure 8-B). This suggests that there may well be subcellular localization of the zebrafish dnmt1 protein as well. Investigating this possibility awaits the availability of an antibody against zebrafish dmnt1.

Conclusions: Chapter One

In conclusion, the degree of similarity between the zebrafish cDNA described in this report and other vertebrate DNA MTases, both at the nucleotide and the amino acid levels, strongly suggests that our sequence represents zebrafish *dnmt*1. Furthermore, its high degree of similarity to other vertebrate methyltransferases and the significant conservation of all functional motifs, including the cytoplasmic retention sequence, argues that it may play a role in early development of zebrafish. Further work to delineate the expression patterns of dmnt1 as well as investigating its possible subcellular localization will help further clarify the role of this enzyme in zebrafish early development. Such work will provide necessary data to investigate the potential use of

this attractive model system to better understand the role of DNA methylation in normal and abnormal early development.

Figure 1. An overview of the experimental design of dnmt1 RACE-PCR. The template and primers used for the preferential amplification of dnmt1 are shown as is the expected size of the amplification products. AP = adaptor primer. GSP = gene specific primer (see Appendix 1 for primer and cDNA adaptor sequences).

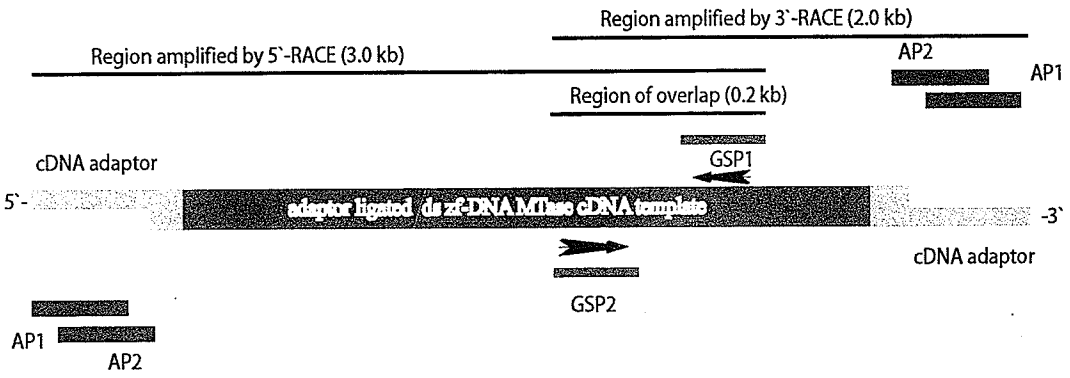

Region amplified by 3`-RACE (2.0 kb)
Region amplified by 5`-RACE (3.0 kb)
AP2
AP1
Region of overlap (0.2 kb)
GSP1
cDNA adaptor
5`-
adaptor ligated ds zf-DNA MMuso cDNA template
-3`
cDNA adaptor
GSP2
AP1
AP2

Figure 2. TA cloning strategy. The Taq polymerase generated deoxyadenosine (A) residues at the 3` ends of PCR product are able to ligate efficiently with the linearized vector 3` deoxythymidine (T) residues.


3`
5`
pCR2.1 Vector
T
5`
3`
A
5` or 3` RACE-PCR product
A
3`
5`
5`
pCR2.1 Vector
T
3`

Figure 3. An illustrative example of cDNA library screening results. (A) Primary library screen. (B) Secondary library screen. (C) Tertiary library screen.

Immobilized plaques on nylon membrane were hybridized with zebrafish dnmt1 specific probes. After hybridization and washing, the membranes were exposed to X-ray film. The positive plaque detected in the primary screen (arrow) was identified, isolated and replated in the secondary screen. One positive plaque was isolated from the secondary screen and similarly used in the tertiary screening process. The dramatic increase in the number of positive colonies (arrows) noted in the tertiary screen indicated that these colonies were positive ones.

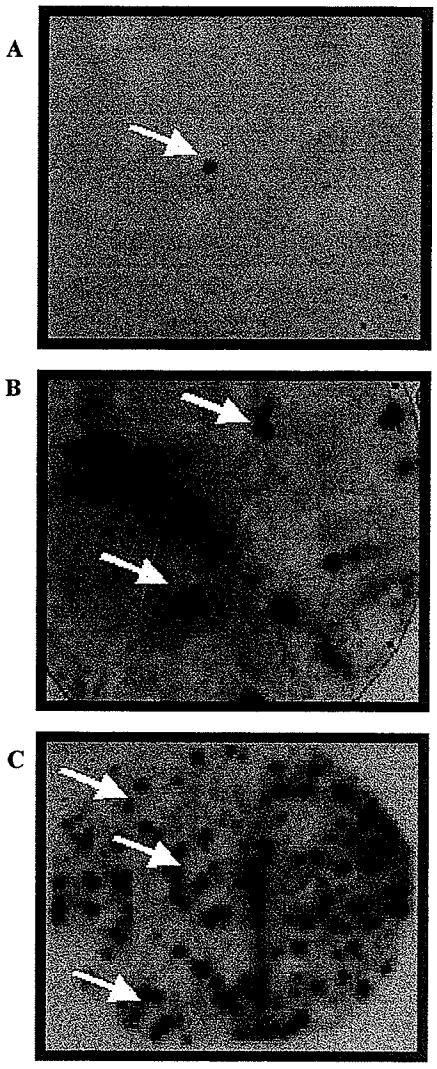

A
B
C

Figure 4. A representative example of the results of the 5`- and the 3` RACE-PCR experiments.

Lane 1. 1.0 kb plus ladder

Lane 2. Experimental 5`-RACE-PCR product (3.0 kb).

Lane 3. Control 5`-RACE-PCR product (2.6 kb).

Lane 4. Experimental 3`-RACE-PCR product (2.0 kb).

Lane 5. Control 3`-RACE-PCR product (2.9 kb).

Lane 6. Positive control (0.2 kb). PCR product generated from the use of GSP1 and GSP2 together.

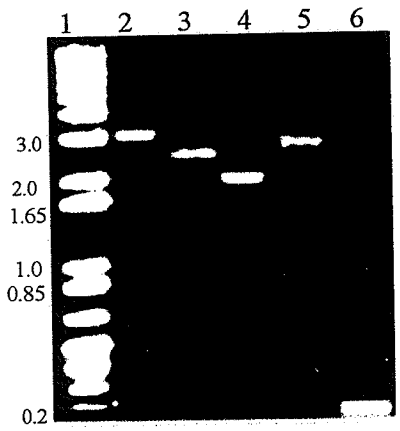

1
2
3
4
5
6
3.0
2.0
1.65
1.0
0.85
0.2

Figure 5. EcoRI digestion of 5`- and 3`- RACE recombinant pCR2.1 plasmid.

Lane 1. 1.0 kb plus ladder.

Lane 2. 5`-RACE-PCR product (3.0 kb).

Lane 3. 3`-RACE-PCR product (2.0 kb).

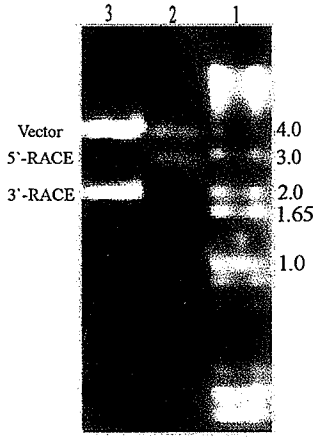

3
2
1
Vector
5`-RACE
3`-RACE
4.0
3.0
2.0
1.65
1.0

Figure 6. Sequence of zebrafish *dnmt1* (GenBank Accession No.AF483203). The sequence of *dnmt1* cDNA is shown and underneath is the deduced amino acid sequence in standard one letter code (Appendix 2).

```
   1 GCCCGGGCAG GTGCGCGCGC TGCAGTTGGC ACTGTCACAT ATTAATACCC GCTTTTATCT
  61 GTTGTTGTTT TAAATCGTGT TTTTAAGTAG TCAATCCCTT TGATCAAAAT CGAACTTGAA
       M  P  T    K  T  S  L    S  L  P    E  D  V    K  E  R  L    Q  V  L
 121 ATGCCTACCA AGACCTCATT GTCTCTGCCA GAGGATGTCA AAGAACGGCT TCAGGTGTTG
       D  E  G    G  D  S  L    S  D  E    E  C  V    K  E  K  L    R  L  L
 181 GATGAAGGTG GAGACAGTTT GTCAGATGAG GAGTGTGTAA AAGAAAAGCT CAGGTTACTA
       Q  E  F    L  L  A  D    T  Q  D    Q  L  K    N  L  E  D    K  L  K
 241 CAGGAGTTCC TGCTCGCTGA TACTCAGGAC CAGCTCAAAA ACCTTGAGGA CAAGTTAAAG
       S  S  E    L  S  T  E    V  Y  M    S  E  V    K  A  V  L    K  K  A
 301 AGCTCTGAGC TCTCCACGGA GGTTTACATG TCAGAGGTGA AGGCTGTACT GAAGAAAGCT
       L  G  V    G  K  E  G    D  G  V    E  Q  N    G  H  S  N    G  F  S
 361 CTGGGAGTTG GCAAGGAGGG TGATGGAGTG GAGCAGAATG GACATTCAAA TGGCTTCTCT
       E  N  G    S  H  K  D    N  G  E    Q  E  G    A  M  D  T    Q  D  E
 421 GAAAATGGAT CTCACAAAGA CAATGGCGAG CAGGAGGGAG CCATGGACAC TCAGGACGAA
       G  D  A    I  K  S  P    S  A  P    K  G  R    G  G  R  R    S  K  A
 481 GGAGACGCTA TAAAGTCTCC CAGTGCCCCA AAGGGAAGGG GCGGACGTCG CAGTAAAGCA
       D  S  E    P  K  K  S    P  A  S    S  R  V    T  R  N  T    G  K  Q
 541 GACTCTGAGC CCAAGAAATC TCCAGCCAGT TCCAGGGTTA CACGCAACAC TGGAAAGCAA
       Q  T  I    V  S  M  F    S  R  V    P  K  R    K  S  D  E    L  N  G
 601 CAAACTATTG TGTCAATGTT TTCTAGAGTC CCAAAACGAA AGTCTGATGA GCTGAATGGA
       E  P  A    N  G  D  T    E  I  K    T  E  E    T  I  T  E    E  V  R
 661 GAACCAGCAA ATGGTGACAC AGAAATTAAG ACTGAGGAAA CTATCACTGA GGAGGTCCGT
       E  E  K    R  L  K  T    E  D  E    K  P  E    A  E  N  A    A  N  L
 721 GAAGAGAAAC GCCTTAAAAC AGAAGATGAG AAGCCTGAAG CTGAGAATGC TGCTAACCTC
       K  P  V    S  T  A  K    T  P  P    P  K  C    P  D  C  R    Q  Y  L
 781 AAACCTGTGT CTACAGCAAA GACTCCTCCG CCGAAGTGTC CTGACTGCAG GCAGTATCTG
       D  D  S    D  L  K  F    F  Q  G    D  P  D    D  A  L  D    E  P  E
 841 GATGATTCAG ACCTAAAGTT CTTTCAAGGG GATCCTGATG ATGCTTTGGA TGAACCAGAG
       M  L  T    D  E  R  L    S  L  F    D  S  N    E  D  G  F    E  S  Y
 901 ATGTTAACCG ATGAGCGTCT CTCCCTCTTT GATTCAAATG AGGATGGTTT TGAAAGCTAT
       E  D  L    P  Q  H  K    I  T  N    F  S  V    Y  D  K  R    G  H  L
 961 GAAGATCTTC CTCAGCACAA GATCACGAAC TTCAGTGTGT ATGACAAGCG TGGTCATCTG
       C  P  F    D  S  G  L    I  E  K    N  V  E    L  Y  F  S    C  A  V
1021 TGTCCATTTG ATTCTGGCCT CATTGAGAAA AATGTGGAAC TGTACTTTAG TTGTGCTGTA
       K  P  I    Y  D  D  N    P  C  M    D  G  G    V  P  A  K    K  L  G
1081 AAGCCCATCT ATGATGACAA CCCATGCATG GATGGTGGGG TTCCTGCCAA AAAGCTTGGT
       P  I  N    A  W  W  I    T  G  F    D  G  G    E  K  A  L    I  G  F
1141 CCCATCAATG CTTGGTGGAT CACTGGTTTT GATGGTGGGG AGAAGGCTTT GATTGGCTTC
       T  T  A    F  A  D  Y    I  L  M    D  P  S    E  E  Y  S    A  I  F
1201 ACTACAGCCT TTGCTGACTA TATCCTAATG GACCCTAGTG AGGAGTACTC TGCTATTTTT
       A  L  M    Q  E  K  I    C  M  S    K  I  V    V  E  F  L    Q  K  N
1261 GCTCTGATGC AGGAGAAGAT CTGCATGAGC AAAATTGTAG TTGAATTTCT TCAGAAAAAC
       Q  D  A    T  Y  E  D    L  L  N    K  I  E    T  T  V  P    P  A  G
1321 CAGGATGCCA CATATGAGGA CCTGCTGAAC AAAATAGAGA CTACTGTCCC ACCTGCGGGG
       L  N  F    N  R  F  T    E  D  T    L  L  R    H  A  Q  F    V  V  E
1381 CTCAACTTCA ACCGTTTCAC AGAAGACACA CTCTTGCGCC ATGCCCAGTT TGTGGTAGAG
       Q  V  E    S  Y  D  E    A  G  D    S  D  E    Q  P  I  I    I  T  P
1441 CAGGTCGAGA GCTACGATGA GGCTGGAGAC TCTGATGAAC AACCCATTAT AATCACTCCA
       C  M  R    D  L  I  K    L  A  G    V  T  L    G  K  R  R    A  A  R
1501 TGTATGCGTG ACTTGATCAA GCTGGCTGGT GTTACTTTGG GCAAGAGGAG AGCAGCCAGA
       R  Q  A    V  R  H  P    T  K  I    E  K  D    N  K  G  P    T  K  A
1561 AGACAAGCCG TCCGCCATCC CACTAAGATT GAAAAGGACA ACAAGGGCCC GACTAAAGCC
       T  T  T    K  L  V  H    L  I  F    D  T  F    F  S  D  Q    I  D  Q
1621 ACCACCACTA AACTGGTCCA CCTGATCTTT GACACATTCT TCTCTGATCA GATTGACCAG
       N  N  K    D  G  G  V    K  R  Q    R  C  G    V  C  E  V    C  Q  A
1681 AACAATAAGG ATGGAGGTGT GAAGAGACAA CGGTGTGGCG TCTGTGAGGT GTGTCAAGCT
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      P  D  C   G  K  C  S   A  C  K   D  M  I   K  F  G  G   S  G  R
1741 CCAGACTGCG GCAAGTGTTC AGCTTGTAAG GATATGATCA AGTTTGGAGG CAGTGGCAGA
      S  K  Q   A  C  Q  K   R  R  C   P  N  L   A  V  K  E   A  E  D
1801 AGTAAACAAG CTTGTCAGAA GAGGAGGTGT CCCAACCTGG CAGTGAAAGA AGCTGAGGAT
      D  E  N   M  D  E  E   D  V  L   P  V  K   D  T  K  K   M  S  Q
1861 GATGAGAACA TGGATGAGGA GGATGTGTTG CCAGTTAAGG ATACAAAGAA AATGTCTCAG
      T  K  K   K  K  Q  T   K  N  K   I  S  W   V  G  E  P   L  K  T
1921 ACCAAGAAGA AGAAACAAAC CAAGAATAAA ATCAGCTGGG TTGGTGAGCC ATTAAAGACT
      E  G  K   K  E  Y  Y   M  K  V   R  V  E   N  E  V  L   E  V  G
1981 GAGGGAAAGA AGGAATATTA CATGAAAGTG CGTGTGGAAA ATGAGGTGTT GGAGGTGGGA
      D  C  V   S  V  S  P   D  D  P   S  H  P   L  Y  L  A   R  I  T
2041 GATTGTGTAT CTGTAAGCCC TGATGACCCA TCACATCCTC TCTACCTGGC CAGGATTACA
      A  L  W   D  D  G  E   K  M  F   H  A  H   W  F  C  R   G  T  D
2101 GCTTTGTGGG ATGATGGTGA GAAGATGTTT CATGCCCATT GGTTCTGCCG TGGCACTGAT
      T  V  L   G  E  S  S   D  P  L   E  L  F   L  V  D  E   C  E  D
2161 ACGGTCCTTG GAGAGTCATC AGACCCTCTT GAGCTCTTCC TAGTGGATGA GTGTGAAGAC
      M  Q  L   S  F  I  H   G  K  V   N  V  F   Y  K  A  P   S  E  N
2221 ATGCAGCTAA GTTTTATCCA TGGCAAGGTC AACGTCTTTT ACAAGGCACC ATCTGAAAAC
      W  Y  M   E  G  G  M   D  E  D   I  K  V   I  D  D  D   G  E  S
2281 TGGTATATGG AGGGTGGAAT GGATGAAGAC ATCAAAGTGA TTGACGATGA TGGTGAAAGC
      F  F  Y   Q  L  H  Y   E  G  E   C  A  R   F  E  T  P   P  K  V
2341 TTCTTTTATC AGCTCCATTA TGAAGGTGAA TGTGCTCGCT TTGAGACTCC TCCCAAGGTC
      T  P  S   E  D  C  K   Y  K  F   C  A  S   C  T  R  N   K  E  R
2401 ACACCCTCTG AGGACTGCAA GTATAAGTTT TGTGCCAGCT GCACTAGAAA CAAGGAGCGA
      E  A  E   S  V  P  H   A  Y  E   P  L  E   D  E  E  S   D  S  K
2461 GAGGCTGAGT CGGTTCCCCA TGCCTATGAG CCCTTAGAGG ATGAGGAGAG TGACTCTAAA
      V  F  Y   G  L  V  N   Y  K  G   E  Q  Y   K  V  G  D   S  V  Y
2521 GTCTTCTATG GGTTGGTGAA CTATAAGGGG GAGCAGTACA AAGTAGGGGA CAGTGTCTAC
      L  P  P   E  A  F  N   F  V  V   K  A  A   S  P  V  K   R  S  H
2581 CTACCACCTG AGGCCTTTAA CTTTGTGGTA AAGGCTGCAA GCCCGGTGAA GCGTTCCCAC
      R  K  D   D  V  D  E   D  L  Y   P  E  Y   Y  R  K  S   S  D  Y
2641 AGAAAAGATG ACGTTGATGA AGATCTTTAC CCTGAATACT ACAGGAAGTC CTCTGATTAC
      I  K  G   S  N  L  D   A  P  Q   P  F  R   I  G  R  I   K  E  I
2701 ATCAAAGGCT CAAATCTGGA TGCTCCTCAG CCTTTTCGCA TTGGGCGCAT CAAAGAAATC
      F  C  N   K  R  S  N   G  K  P   D  T  T   E  I  K  L   R  L  Y
2761 TTCTGCAACA AGCGCAGCAA TGGAAAGCCA GACACCACAG AGATCAAACT GCGCCTCTAT
      K  F  Y   R  P  E  N   T  H  K   G  P  K   G  A  Y  H   S  D  I
2821 AAATTCTACA GACCTGAAAA CACTCACAAG GGACCAAAGG GAGCATATCA TTCTGACATA
      N  Q  L   Y  W  S  D   E  E  A   T  V  S   M  T  E  V   L  T  R
2881 AACCAACTCT ACTGGAGTGA CGAAGAGGCG ACCGTCAGCA TGACTGAGGT GCTGACACGC
      C  R  V   E  Y  A  E   D  L  V   E  S  V   Q  D  Y  S   N  K  G
2941 TGTCGTGTTG AGTATGCAGA GGATCTGGTT GAAAGTGTGC AGGACTATTC AAACAAAGGA
      P  D  R   F  Y  F  L   E  A  Y   N  A  K   T  K  S  F   E  D  P
3001 CCCGATCGCT TCTATTTCCT TGAGGCATAC AATGCCAAAA CCAAGAGCTT TGAGGATCCT
      P  N  H   A  R  S  A   V  N  K   G  K  G   K  G  K  G   K  G  K
3061 CCCAATCATG CTAGATCTGC TGTGAATAAG GGCAAAGGGA AAGGAAAGGG CAAGGGAAAG
      G  K  G   K  Q  R  S   R  T  T   G  S  G   A  Q  E  P   V  V  P
3121 GGAAAGGGAA AGCAGCGCTC AAGAACCACA GGATCAGGAG CACAAGAGCC AGTGGTTCCC
      K  L  R   T  L  D  V   F  S  G   C  G  G   L  S  E  G   F  H  Q
3181 AAACTGCGCA CTCTGGATGT ATTCTCTGGC TGTGGTGGTC TGTCTGAGGG CTTCCATCAA
      A  G  I   S  E  T  H   W  A  I   E  M  W   D  P  A  A   Q  A  F
3241 GCAGGCATCT CAGAGACTCA CTGGGCAATA GAGATGTGGG ACCCTGCTGC TCAGGCATTC
      R  L  N   N  P  G  T   T  V  F   T  E  D   C  N  V  L   L  K  L
3301 AGGTTGAACA ATCCAGGCAC GACCGTTTTC ACAGAGGACT GTAACGTCCT GCTGAAGCTG
      V  M  S   G  E  K  T   N  S  L   G  Q  K   L  P  Q  K   G  D  V
3361 GTAATGTCTG GAGAGAAGAC TAACTCTCTT GGACAGAAGC TGCCACAGAA GGGTGACGTG
      E  M  L   C  G  G  P   P  C  Q   G  F  S   G  M  N  R   F  N  S
```

```
3421 GAGATGCTCT GCGGTGGGCC GCCTTGCCAA GGCTTCAGTG GAATGAATCG CTTCAACTCG
      R  T  Y   S  K  F  K   N  S  L   V  V  S   Y  L  S  Y   C  D  Y
3481 CGCACATACT CAAAGTTCAA GAACTCTCTG GTTGTTTCTT ATCTGAGTTA TTGTGACTAC
      Y  R  P   K  F  F  L   L  E  N   V  R  N   F  V  S  F   K  R  S
3541 TACAGACCCA AGTTCTTCCT GCTGGAGAAC GTGAGGAACT TTGTGTCATT TAAGCGCTCC
      M  V  L   K  L  T  L   R  C  L   V  R  M   G  Y  Q  C   T  F  G
3601 ATGGTCCTGA AGCTCACACT GCGCTGTCTA GTGCGCATGG GCTACCAGTG CACCTTTGGT
      V  L  Q   A  G  Q  Y   G  V  A   Q  T  R   R  R  A  I   I  L  A
3661 GTGCTGCAGG CTGGACAGTA TGGGGTGGCC CAGACTCGAC GCAGAGCTAT CATCCTGGCT
      A  A  P   G  E  K  L   P  R  Y   P  E  P   L  H  V  F   A  P  R
3721 GCAGCCCCTG GTGAAAAGCT GCCCCGCTAC CCTGAACCCT TGCATGTGTT TGCCCCGCGA
      A  C  S   L  S  V  A   V  D  E   K  K  Y   V  S  N  V   T  R  G
3781 GCATGTTCTC TTAGTGTGGC AGTTGATGAG AAGAAATATG TCAGCAATGT TACTCGTGGA
      N  G  G   I  Y  R  T   I  T  V   R  D  T   M  S  D  L   P  E  I
3841 AATGGGGGCA TTTATCGCAC CATCACAGTA CGGGACACCA TGTCTGACCT CCCAGAGATC
      R  N  G   A  A  A  L   E  I  S   Y  N  G   E  P  Q  S   W  F  Q
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      R  Q  I   R  G  S  Q   Y  Q  P   I  L  R   D  H  I  C   K  D  M
3961 AGGCAAATTC GGGGCTCTCA GTATCAGCCC ATCCTCAGGG ACCACATCTG CAAGGACATG
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      D  L  P   N  I  E  V   R  L  R   D  G  T   T  T  K  K   L  R  Y
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      T  H  S   D  K  K  N   G  R  S   G  T  G   A  L  R  G   V  C  S
4141 ACACACTCTG ACAAAAAGAA TGGCCGCAGT GGCACCGGTG CCCTGAGAGG AGTGTGTTCA
      C  S  E   G  K  Q  C   D  P  A   D  R  Q   F  N  T  L   I  P  W
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      C  L  P   H  T  G  N   R  H  N   H  W  A   G  L  Y  G   R  L  E
4261 TGTCTGCCTC ATACGGGTAA CCGCCATAAT CACTGGGCTG GTCTGTATGG CCGCCTGGAA
      W  D  G   F  F  S  T   T  V  T   N  P  E   P  M  G  K   Q  G  R
4321 TGGGACGGAT TCTTCAGCAC TACAGTTACC AATCCTGAGC CAATGGGAAA GCAGGGACGT
      V  L  H   P  E  Q  H   R  V  V   S  V  R   E  C  A  R   S  Q  G
4381 GTCCTTCACC CTGAGCAGCA CCGTGTGGTG AGTGTCAGGG AATGTGCACG CTCTCAGGGC
      F  P  D   T  Y  R  F   F  G  N   V  L  D   K  H  R  Q   V  G  N
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      K  M  R   E  N  A  T   E  P  V   K  Q  E   K  M  E  L   S  D
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4621 AGCCATCACT CTGTAAAATT GAAGCACATT TTTCATTGTG TTTGGATCCA TCTGAAGCAT
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4861 TTTTATATGT TGTAATATTT CAAATAAAGC TCTTATTAAA TGTTAAA
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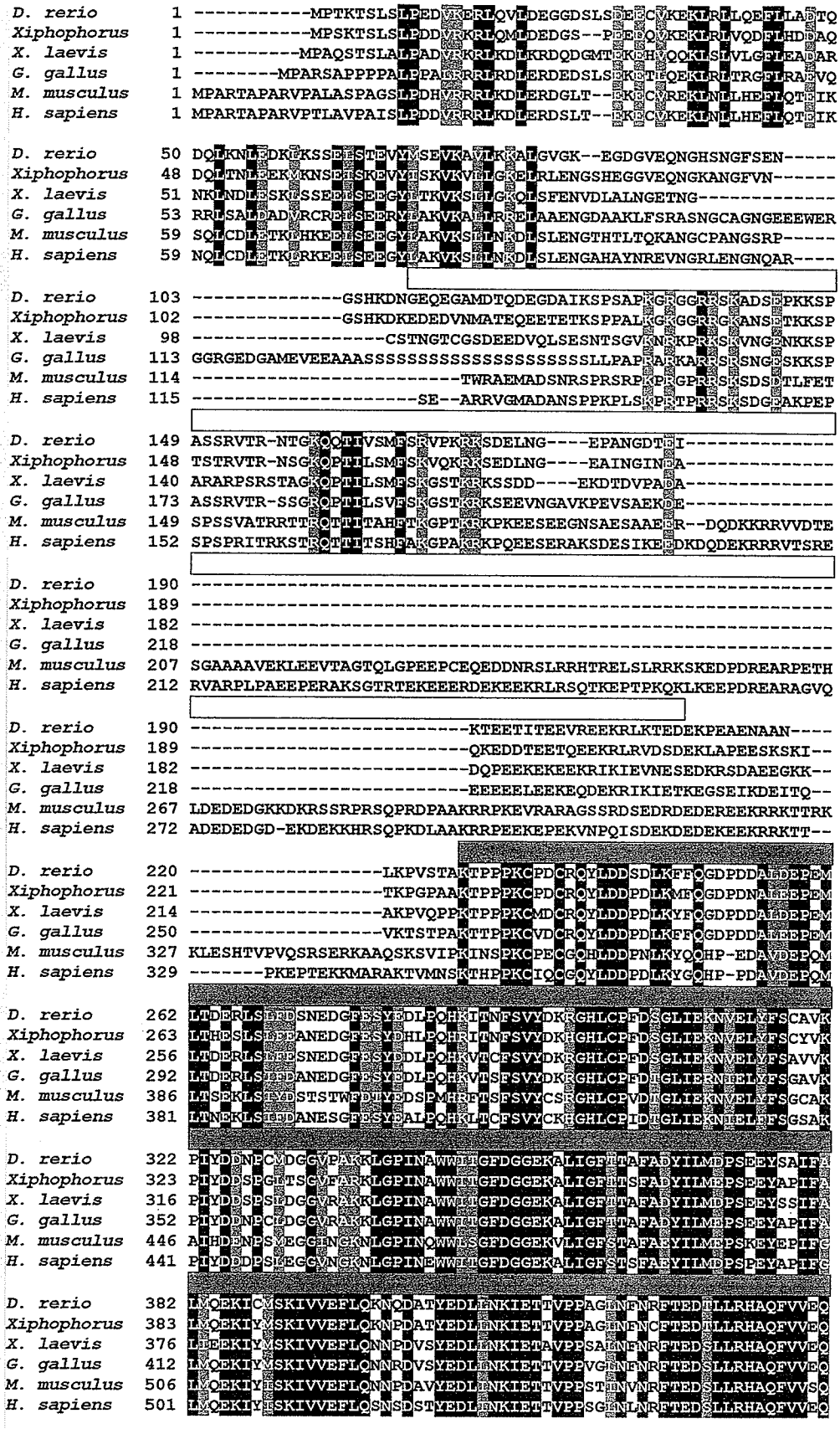
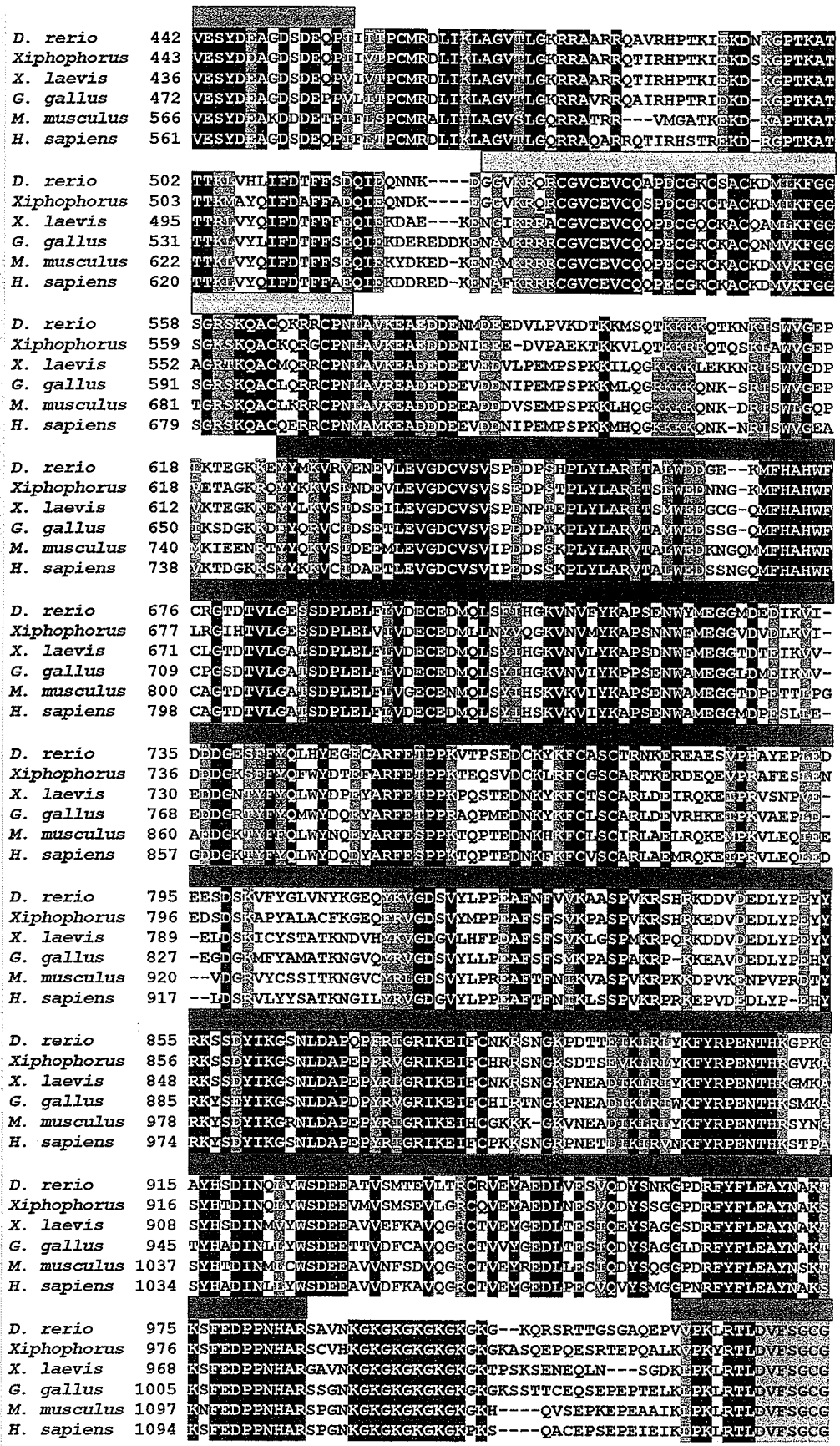
Figure 7. Conservation of MTase functional domains in vertebrates. Approximate amino acid alignment of the predicted zebrafish dnmt1 functional domains (*D. rerio*) with other vertebrate Dnmt1 proteins. (Xiphophorus, GenBank #AF152342; X. laevis, GenBank #D78638; G. gallus, GenBank #D43920; M. musculus, GenBank #X14805; H. sapiens, GenBank #X63692). The colored bars represent domains corresponding with the coloring in figure 8-A.


D. rerio
Xiphophorus
X. laevis
G. gallus
M. musculus
H. sapiens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G. gallus 472
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G. gallus 531
M. musculus 622
H. sapiens 620
D. rerio 558
Xiphophorus 559
X. laevis 552
G. gallus 591
M. musculus 681
H. sapiens 679
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Xiphophorus 618
X. laevis 612
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Xiphophorus 677
X. laevis 671
G. gallus 709
M. musculus 800
H. sapiens 798
D. rerio 735
Xiphophorus 736
X. laevis 730
G. gallus 768
M. musculus 860
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Xiphophorus 796
X. laevis 789
G. gallus 827
M. musculus 920
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X. laevis 848
G. gallus 885
M. musculus 978
H. sapiens 974
D. rerio 915
Xiphophorus 916
X. laevis 908
G. gallus 945
M. musculus 1037
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G. gallus 1005
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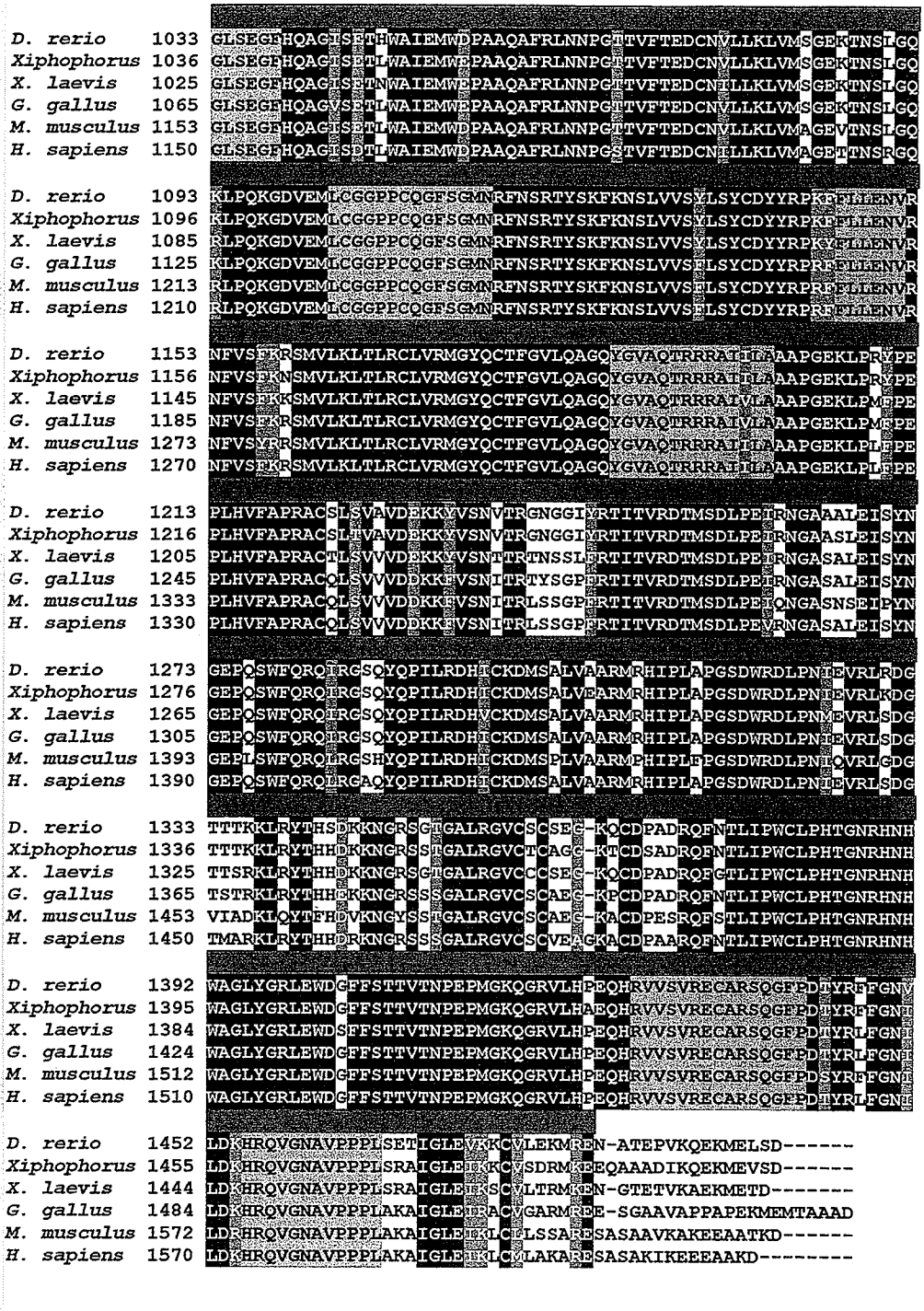
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X. laevis 1025 GLSEGFHQAGISETNWAIEMWEPAAQAFRLNNPGTTVFTEDCNTLLKLVMSGEKTNSLGQ
G. gallus 1065 GLSEGFHQAGVSETLWAIEMWEPAAQAFRLNNPGTTVFTEDCNVLLKLVMSGEKTNSLGQ
M. musculus 1153 GLSEGFHQAGISETLWAIEMWDPAAQAFRLNNPGTTVFTEDCNVLLKLVMAGEVTNSLGQ
H. sapiens 1150 GLSEGFHQAGISPTLWAIEMWDPAAQAFRLNNPGSTVFTEDCNILLKLVMAGETTNSRGQ
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Xiphophorus 1096 KLPQKGDVEMLCGGPPCQGFSGMNRFNSRTYSKFKNSLVVSYLSYCDYYRPKFFLLENVR
X. laevis 1085 RLPQKGDVEMLCGGPPCQGFSGMNRFNSRTYSKFKNSLVVSYLSYCDYYRPKYFLLENVR
G. gallus 1125 KLPQKGDVEMLCGGPPCQGFSGMNRFNSRTYSKFKNSLVVSFLSYCDYYRPRFFLLENVR
M. musculus 1213 RLPQKGDVEMLCGGPPCQGFSGMNRFNSRTYSKFKNSLVVSFLSYCDYYRPRFFLLENVR
H. sapiens 1210 RLPQKGDVEMLCGGPPCQGFSGMNRFNSRTYSKFKNSLVVSFLSYCDYYRPRFTLLENVR
D. rerio 1153 NFVSFKRSMVLKLTLRCLVRMGYQCTFGVLQAGQYGVAQTRRRAIILAAAPGEKLPRYPE
Xiphophorus 1156 NFVSFKNSMVLKLTLRCLVRMGYQCTFGVLQAGQYGVAQTRRRAIILAAAPGEKLPRYPE
X. laevis 1145 NFVSFKKSMVLKLTLRCLVRMGYQCTFGVLQAGQYGVAQTRRRAIVLAAAPGEKLPMFPE
G. gallus 1185 NFVSFKRSMVLKLTLRCLVRMGYQCTFGVLQAGQYGVAQTRRRAIVLAAAPGEKLPMFPE
M. musculus 1273 NFVSYRRSMVLKLTLRCLVRMGYQCTFGVLQAGQYGVAQTRRRAIILAAAPGEKLPLFPE
H. sapiens 1270 NFVSFKRSMVLKLTLRCLVRMGYQCTFGVLQAGQYGVAQTRRRAIILAAAPGEKLPLFPE
D. rerio 1213 PLHVFAPRACSLSVAVDEKKYVSNVTRGNGGIYRTITVRDTMSDLPEIRNGAAALEISYN
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X. laevis 1205 PLHVFAPRACTLSVVVDEKKYVSNTTRTNSSLFRTITVRDTMSDLPEIRNGASALEISYN
G. gallus 1245 PLHVFAPRACQLSVVVDDKKTVSNITRTYSGPFRTITVRDTMSDLPEIRNGASALEISYN
M. musculus 1333 PLHVFAPRACQLSVVVDDKKTVSNITRLSSGPFRTITVRDTMSDLPEICNGASNSEIPYN
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Xiphophorus 1276 GEPQSWFQRQIRGSQYQPILRDHICKDMSALVEARMRHIPLAPGSDWRDLPNIEVRLKDG
X. laevis 1265 GEPQSWFQRQIRGSQYQPILRDHVCKDMSALVAARMRHIPLAPGSDWRDLPNMEVRLSDG
G. gallus 1305 GEPQSWFQRQIRGSQYQPILRDHICKDMSALVAARMRHIPLAPGSDWRDLPNIEVRLSDG
M. musculus 1393 GEPLSWFQRQLRGSHYQPILRDHICKDMSPLVAARMPHIPLFPGSDWRDLPNIQVRLGDG
H. sapiens 1390 GEPQSWFQRQLRGAQYQPILRDHICKDMSALVAARMRHIPLAPGSDWRDLPNIEVRLSDG
D. rerio 1333 TTTKKLRYTHSDKKNGRSGTGALRGVCSCSEG-KQCDPADRQFNTLIPWCLPHTGNRHNH
Xiphophorus 1336 TTTKKLRYTHHDKKNGRSSTGALRGVCTCAGG-KTCDSADRQFNTLIPWCLPHTGNRHNH
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G. gallus 1365 TSTRKLRYTHHDKKNGRSSSGALRGVCSCAEG-KPCDPADRQFNTLIPWCLPHTGNRHNH
M. musculus 1453 VIADKLQYTFHDVKNGYSSTGALRGVCSCAEG-KACDPESRQFSTLIPWCLPHTGNRHNH
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D. rerio 1392 WAGLYGRLEWDGFFSTTVTNPEPMGKQGRVLHPEQHRVVSVRECARSQGFPDTYRFFGNV
Xiphophorus 1395 WAGLYGRLEWDGFFSTTVTNPEPMGKQGRVLHAEQHRVVSVRECARSQGFPDTYRFFGNT
X. laevis 1384 WAGLYGRLEWDSFFSTTVTNPEPMGKQGRVLHPEQHRVVSVRECARSQGFPDTYRLFGNT
G. gallus 1424 WAGLYGRLEWDGFFSTTVTNPEPMGKQGRVLHPEQHRVVSVRECARSQGFPDTYRLFGNT
M. musculus 1512 WAGLYGRLEWDGFFSTTVTNPEPMGKQGRVLHPEQHRVVSVRECARSQGFPDSYRFFGNT
H. sapiens 1510 WAGLYGRLEWDGFFSTTVTNPEPMGKQGRVLHPEQHRVVSVRECARSQGFPDTYRLFGNT
D. rerio 1452 LDKHRQVGNAVPPPLSETIGLEVKKCVLEKMREN-ATEPVKQEKMELSD------
Xiphophorus 1455 LDKHRQVGNAVPPPLSRAIGLEIKKCVSDRMKEEQAAADIKQEKMEVSD------
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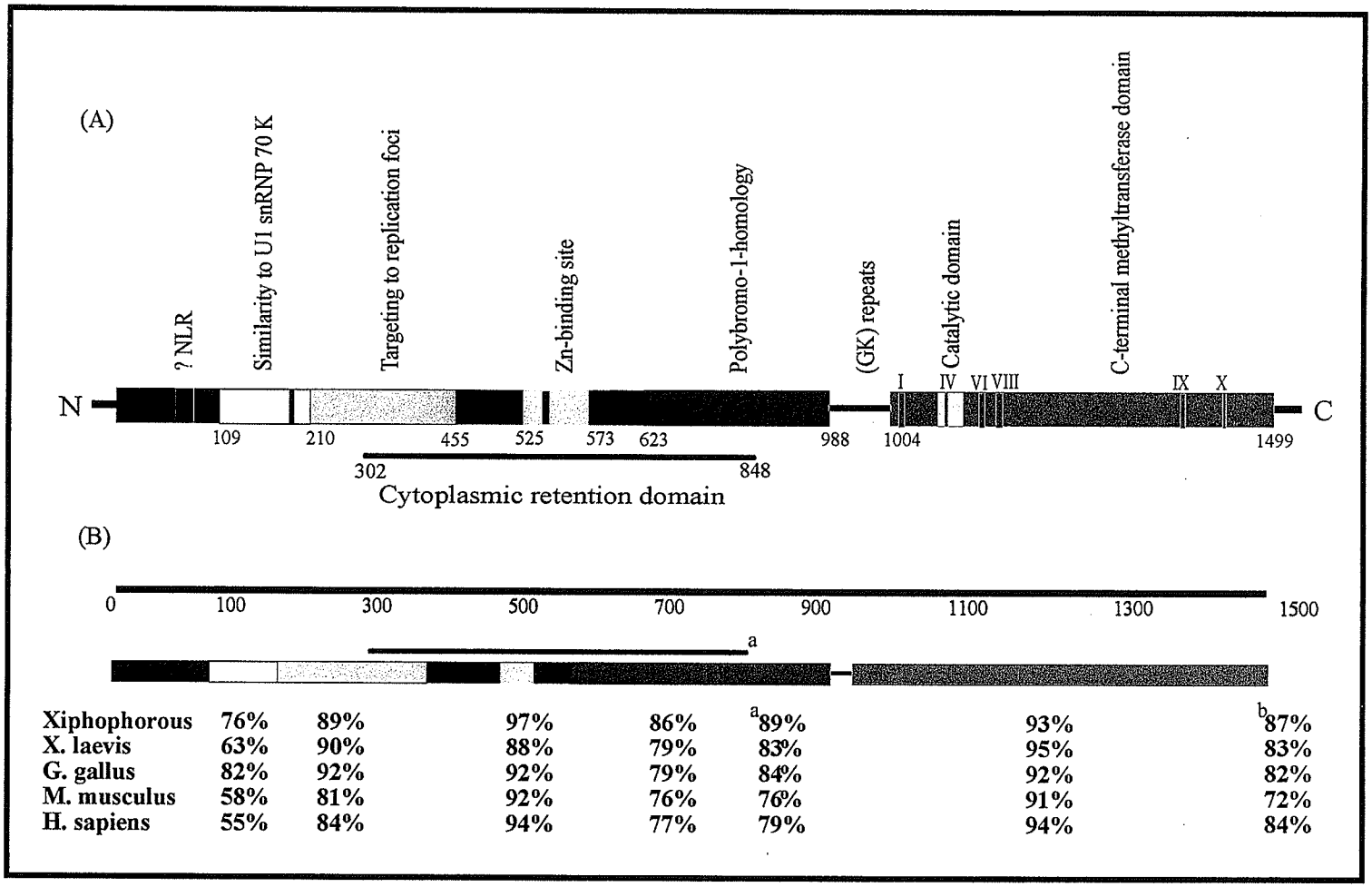
M. musculus 1572 LDRHRQVGNAVPPPLAKAIGLEIKLCVLSSAREESASAAVKAKEEAATKD------
H. sapiens 1570 LDKHRQVGNAVPPPLAKAIGLEIKLCVLAKAREESASAKIKEEEAAKD--------

Figure 8. Diagram of sequence elements and functional domains of zebrafish dnmt1. The conservation of the order and relative positions of the known functional motifs of the mammalian Dnmt1 in the zebrafish dnmt1 enzyme is shown in (A). The deduced amino acid sequence was compared to the mouse Dnmt1 and the conserved motifs and features are shown.

(B) Conservation of MTase functional domains in vertebrates. Approximate amino acid alignment of the predicted zebrafish dnmt1 functional domains with other vertebrate Dnmt1 proteins (Xiphophorus, GenBank AF152342; X. laevis, GenBank D78638; G. gallus, GenBank D43920; M. musculus, GenBank X14805; H. sapiens, GenBank X63692). The colored bars represent domains corresponding to the coloring in part (A). The column and solid black bar denoted (a) represent the approximate position and homology of the cytoplasmic retention domain. The column denoted (b) represents the overall similarity of Dnmt1 in the organisms indicated. (%) = similarity of functional domains to DNMT1. NLR = sequences representing possible nuclear localization regions; GK repeats = repeats of glycine and lysine residues that join the N- and the C-terminal domains. Roman numericals denote the six most conserved motifs in nearly all DNA methyltransferases.



Xiphophorous	**76%**	**89%**	**97%**	**86%**	**[a]89%**	**93%**	**[b]87%**
X. laevis	**63%**	**90%**	**88%**	**79%**	**83%**	**95%**	**83%**
G. gallus	**82%**	**92%**	**92%**	**79%**	**84%**	**92%**	**82%**
M. musculus	**58%**	**81%**	**92%**	**76%**	**76%**	**91%**	**72%**
H. sapiens	**55%**	**84%**	**94%**	**77%**	**79%**	**94%**	**84%**

Chapter two

Variations in *DNA (cytosine-5) methyltransferase 1* (*dnmt1*) expression during oogenesis and early development of the zebrafish

Abstract: Chapter Two

The zebrafish *DNA (cytosine-5-) methyltransferase* (*dnmt1*) temporal and spatial patterns of expression in gonadal tissues and during early development were determined. Only a single sized *dnmt1* message of ~5 Kb was observed in all tissues examined and its levels were highest in gonadal tissues. During the course of oogenesis, early oocytes contained significant amounts of *dnmt1* transcript while message abundance declined as oocytes matured. During early embryogenesis, message levels remained low until the blastula stage. Methyltransferase enzyme assays revealed that the maternal *dnmt1* message accumulated during oogenesis had been translated into protein presumably providing necessary dnmt1 stockpiles to support early embryonic development prior to zygotic gene activation. Such spatial and temporal regulation of dnmt1 expression suggests specific functions for the enzyme during oogenesis and early development of zebrafish.*

*(Mhanni and McGowan, 2002)

Introduction: Chapter Two

DNA methylation has been proposed as a mechanism that may account for much of the epigenetic control of gene regulation occurring during early development and normal embryogenesis. In mammals therefore, it is expected that DNA methyltransferase message would be expressed in the developing embryo. If DNA methylation plays an equally important role in zebrafish, one may expect dnmt1 to be expressed during development, to exhibit stage- and tissue-specific expression and to manifest developmentally regulated changes in abundance, activity and localization. If such regulation exists, one would further expect that disruption of normal progression of developmentally regulated changes in DNA methylation to result in developmental abnormalities.

I carried out a series of RNA hybridization experiments and employed quantitative RT-PCR to elucidate the temporal and tissue specific expression patterns of zebrafish *DNA (cytosine-5)methyltransferase 1 (dnmt1)* transcript during early development, in gonads and in somatic tissues (section one). To examine the levels of *dnmt1* mRNA during the different stages of oogenesis, I conducted a series of *in situ* hybridization experiments to ovarian whole-mounts and tissue sections (section two). After the characterization of *dnmt1* mRNA abundance during oogenesis, early development and in somatic tissues, I set forth to test whether the changes in *dnmt1* mRNA levels translate into protein levels and enzyme activity or not (section three).

Section one: Developmental profiling of the temporal dynamics of the zebrafish *DNA (cytosine-5) methyltransferase 1* mRNA

Materials and methods: Section One

I. Zebrafish maintenance and general care

Adult zebrafish, *Danio rerio*, maintenance, breeding and embryo collection were according to the Zebrafish Book (Westerfield, 1995).

I.A. Zebrafish general care

The zebrafish lab stocks were supplemented with locally purchased wild-type zebrafish, *Danio rerio*. Adult zebrafish stocks were maintained in the lab in 5-gallon aquaria on a constantly circulating system at 28 °C on a 14-hr light/10-hr dark cycle. The day-night cycle was controlled by an automatic-timer. Fish were kept well fed with two feeds a day consisting of flaked food and Brine shrimp. One third of the tank water was replaced each day and the tank was cleaned at least once a week.

I.B. Fish breeding

Zebrafish are photoperiodic in their breeding and produce embryos in the morning shortly after the start of the light cycle. A few hours before the end of the light period, 6-8 females and 4-5 males were placed in a breeding tank. Embryos were harvested a half hour after the initiation of the light cycle by siphoning the bottom of the tank. Embryos

were then immediately cleaned by rinsing them several times with fresh dechlorinated water.

I.C. Raising embryos

Embryos were kept in patches of 25-50 embryos in 100 ml water in a 250 ml beaker each. The water temperature was kept constant at 28 °C by placing the covered beakers in a constant temperature water bath.

I.D. Embryo staging and pooling

Embryo staging is determined by examining for the presence of stage-specific morphological landmarks and the hours post fertilization (Kimmel *et al.,* 1995). Approximate embryonic staging was estimated based on the time lapse from the onset of the first morning light which is considered as zero developmental time. In combination, a dissecting microscope with transmitted illumination and high magnification of 25X was used to establish the approximate developmental stage. Embryos at desired developmental stages were procured, pooled and flash frozen in either liquid nitrogen or dry ice/ethanol and placed at –80 °C till ready to use.

I.E. Sperm and oocytes collection

Fish were chosen for sperm and egg collection based on appearance. Females should look large and fat in the belly while males should look yellow and spry. Selected male and female fish were separated during the night in holding tanks. In the morning, fish were anaesthetized using 2-phenoxyethanol in water, rinsed in water and placed

upside down in a dish with the fish under the objective lens of the dissecting microscope. The sides by the anal fins were pushed gently and the sperm or eggs collected. Collected samples were immediately frozen in liquid nitrogen and stored at – 80 °C till ready to use.

I.F. Tissue dissection

Selection of the adult fish for tissue dissection depended on its size, breeding capacity, sex and general health. Chosen fish were deeply anaesthetized by adding 2-phenoxyethanol to the water then the fish were placed on the dissecting plate under the dissecting microscope. After decapitation, the fish were immediately dissected for the desired tissue. Collected tissues were instantly frozen in liquid nitrogen or dry ice/ethanol and stored at –80 °C till ready to be used.

II. Preparation of total RNA

II.A. Isolation of RNA from zebrafish embryos, gonads and somatic tissues

Isolation of total RNA from zebrafish embryos and tissues was according to Chomczynski and Sacchi (1987). The method was modified for smaller volumes of tissue. For approximately 500 ul of embryos (Appendix 3), 1 ml of solution D [4 M guanidium thiosulfate, 25 mM sodium citrate pH 7.0, 0.5% sarcosyl, 0.1 M 2-mercaptoethanol] was added and the tissue was homogenized. One hundred microlitres of sodium acetate [2 M, pH 4.0], 1 ml of water saturated phenol (pH 5) and 500 ul of CIA (24 parts Chloroform: 1 part Isoamyl Alcohol) were added and mixed with the solution

D-homogenate. The mixture was incubated on ice for 15 minutes and then centrifugated at 10,000 rpm for 10 minutes at 4 °C. The upper aqueous layer was placed in a clean microfuge tube. The aqueous layer was then precipitated with an equal volume of isopropanol [100%] and stored at -20 °C for one hour. The RNA was pelleted by centrifugation at 13000 rpm for 10 minutes. The pellet was resuspended in 700 ul of solution D and vortexed after adding 700 ul of CIA. After 5 minutes of centrifugation, the aqueous layer was transferred to a clean microfuge tube. Seventy microlitres of sodium acetate [0.5 M] and 700 ul isopropanol [100%] were added and the tube was stored at -70 °C for one hour. RNA was then pelleted by centrifugation at 13000 rpm.

The RNA pellet was washed with 70% ethanol, air-dried and resuspended in an appropriate volume of depc-H_2O. The concentration of total RNA was determined by measuring the absorbance at 260 and 280 on a spectronic 601 (Milton Roy; Fisher Scientific). The RNA concentration was calculated in ug/ul according to the OD_{260} and an estimation of protein contamination was obtained by the 260/280 ratio. An OD_{260} of 1 is equal to 40ug/ml of RNA. Any sample with an $OD_{260/280}$ ratio of less than 1.8 was considered contaminated and was re-extracted with phenol/chloroform and ethanol precipitated. If this ratio was 1.8 or greater, the RNA was considered free from contamination and the sample was run on a formaldehyde gel (see next section) for examination.

II.B. Formaldehyde RNA denaturation and electrophoresis

Aliquots of the newly made RNA were run on a formaldehyde gel for staining with ethidium bromide to examine the integrity of RNA and confirm the concentrations

obtained by optical density determination. Formaldehyde was chosen to denature RNA as it stains more efficiently with ethidium bromide than other denaturation methods.

Three volumes of fresh formaldehyde denaturation buffer [50% deionized formamide, 18% fomaldehyde, 10 mM NaPO4 in depc-H2O] were added to 3-5 ug of RNA in a 3-4 ul volume. The RNA was incubated at 50 °C for 30 minutes and then removed to ice. Two microlitres of RNA loading buffer [50% sucrose, 0.1% bromo phenol blue] were added and samples were loaded into a 1% agarose-formaldehyde gel [1% agarose, 10 mM NaPO4, 18% formaldehyde]. The gel was run at 100 V until the dye was ¾ of the way down the gel. The gel was removed from the box and soaked in a large volume of water for 30 minutes then in water containing ethidium bromide (1 ul/ml) for a further 30 minutes. The gel was destained in water for few hours and the gel was photographed on a UV transilluminator.

III. RNA hybridization experiments

III.1. RNA hybridization strategy

Extraction of total RNA from desired stage embryos, gonads and somatic tissues

⇩

Fractionation of RNA on glyoxal gels

⇩

Transfer of fractionated RNA to nylon membranes

⇩

Hybridize with *dnmt1* cDNA probe and a control (*β-actin/max/smn*) probe

⇩

Expose blot to X-ray film (autoradiography)

⇩

Develop autoradiograph

⇩

Scan autoradiograph

⇩

Perform densitometric analysis

III.2. RNA hybridization (Northern) analysis

III.2.i. Fractionation of RNA on Glyoxal RNA gels

Twenty micrograms of total RNA in a 10-ul volume were used in each of the RNA hybridization experiments. In some instances RNA had to be concentrated by ethanol precipitation to obtain the desired RNA concentration in a ten-microlitre volume. Twenty microlitres of RNA sample buffer [10 mM sodium phosphate pH 6.8, 50% DMSO, 4.5% deionized glyoxal, 10% SDS] were added and the RNA samples were placed in a 56 °C water bath for 25 minutes. The tubes were immediately placed on ice for 5 minutes and then 2-5 ul of bromophenol blue in 50% sucrose were added to each tube as a loading buffer. The samples were loaded on a 1% agarose gel immersed in 10 mM sodium phosphate running buffer and a current of 80 volts for approximately 3 hours. The running buffer was circulated to maintain the pH of the buffer during the electrophoresis period.

III.2.ii. Transfer of RNA to nylon membranes

RNA gels that were to be transferred were not stained. After the gel was run as long as desired, the gel was taken out of the electrophoresis apparatus and placed upside down in the transfer apparatus containing sponges immersed in 20X SSC solution. The sponges were covered with Whatman filter paper (No.1). An appropriate size nylon transfer membrane (Micron Separations Inc.) was soaked in water for 10 minutes and was then placed on top of the gel after edging the gel with plastic sheets. Two pieces of Whatman filter paper were placed on top of the membrane and then a stack of paper

towels was placed on top. A balanced weight was placed on top and the assembled apparatus was left to transfer overnight.

III.2.iii. Prehybridization, hybridization and autoradiography of RNA blots

Following RNA transfer, the membrane was removed from the apparatus and baked in an 80 °C oven for two hours to permanently immobilize the RNA. The dried membrane was then sealed in a decosonic bag with an appropriate volume of RNA prehybridization solution [4X SSC, 1X Denhardt's (0.2 M Ficoll, 0.2 M Polyvinylpyrolidone, 0.2 M Bovine serum albumin), 0.05 M Sodium Phosphate, 500 ug/ml of denatured salmon sperm DNA (Sigma Cat. No.D-9156), 0.5% SDS] and placed in a 42 °C oven overnight.

An appropriate volume of RNA hybridization solution (5-10 ml) [50% deionized formamide (GIBCO-BRL Cat. No.15515-026), 4X SSC, 1X Denhardt's (0.2 M Ficoll, 0.2 M Polyvinylpyrolidone, 0.2 M Bovine serum albumin), 0.05 M Sodium Phosphate, 500 ug/ml of denatured salmon sperm DNA (Sigma Cat. No.D-9156)] containing four million counts per ml of desired labeled probe (see next section) was added to the membrane. Hybridization was allowed to proceed overnight at 42 °C in an air incubator (Isotemp Incubator, Fisher Scientific). In the morning, the membrane was washed with agitation in a 0.1X Wash Solution [0.1X SSC, 0.1% SDS] five 30-minute washes at 67 °C. The blot was then removed from the wash solution, patted dry with paper towels, wrapped in plastic wrap and arranged in a cassette with an X-ray film (Kodak) and an intensifying screen. The cassette was placed at –70 °C from a few hours to overnight. Film development was performed as described in section I.9, Chapter 1.

III.3. Probes used for RNA hybridization

Membranes were hybridized with a ^{32}P-labeled zebrafish-*DNA (cytosine-5) methyltransferase 1* (GenBank accession no. AF483203; Mhanni *et al.*, 2001) cDNA fragment, a zebrafish *β-actin* (GenBank accession no. AF057040) clone (gift of G. Kelly, University of Western Ontario), a zebrafish-*max* (GenBank accession no. AAA02483) clone (gift of G. Kelly, University of Western Ontario) and a zebrafish-*smn* (McGowan *et al.*, 1996) clone at 42 °C overnight. *β-actin*, *max* and *smn* were used as controls to normalize for RNA loading differences. These probes were labeled in the same fashion as previously described in chapter 1, section I.8.D.

III.4. Methylene blue staining of RNA after transfer to nylon membranes

Immobilized RNA on nylon membranes was stained with methylene blue to examine the integrity, quality and quantity of each RNA sample. The staining procedure started by soaking the dried membrane in 5% acetic acid for 15 minutes at room temperature and then transferring it to a solution of 0.5 M sodium acetate (pH 5.2) and 0.04% methylene blue for 10 minutes at room temperature. The membrane was rinsed in water for 30 minutes to several hours to remove the excess stain. The membrane was then dried, examined for the presence of 18S and 28S rRNA bands and scanned. An illustrative example is shown in figure 9.

III.5. Densitometric analysis and estimation of levels of expression of *dnmt1*

Evaluation of the approximate differences in levels of expression of zebrafish *dnmt1* in different developmental stages, gonads and somatic tissues was conducted using

comparisons between the density of the *dnmt1* band and the corresponding *B-actin*, the *max* and the *smn* bands as controls. These comparisons were conducted to control for RNA loading differences between different samples.

The autoradiographs were scanned on a "Plustek" scanner, processed using "Picture Publisher" software and analyzed using "Scion image" software (Scion Corporation).

IV. Quantitation of *dnmt1* mRNA using coamplification RT-PCR with an endogenous control

IV.1. Coamplification RT-PCR with an endogenous control

The reverse transcription polymerase chain reaction (RT-PCR) has been extensively used for analyzing low abundance messenger RNA (Foley *et al.,* 1993; Zimmermann and Mannhalter, 1996). Chelly *et al.* (1988) described an approach to quantitate RNA levels by PCR. Their method uses an endogenously expressed RNA as an internal control that is coamplified along with the RNA of interest. This endogenous control serves as a control for both sample to sample variations in RT and PCR reaction conditions as well as the extent of degradation and recovery of RNA.

The ubiquitously expressed zebrafish *max* gene (Schreiber-Agus *et al.,* 1993) was used as an internal control. Samples were assayed within the exponential phase of the PCR reaction. Target RNA levels in different samples were directly compared after normalization with respect to the endogenous control.

Strategy of coamplification RT-PCR with an endogenous control

RNA extraction from desired embryonic stages, gonadal and somatic tissues

⇩

First-strand cDNA synthesis

⇩

Determine the exponential phases of amplification for each set of primers

⇩ ⇩

RT-PCR (test) PCR-no RT (control)

⇩

Use two sets of primers:

dnmt1 (test)

max (endogenous control)

⇩

Fractionate amplicons

⇩

Quantify:

Densitometric analysis of ethidium bromide stained gels

Measurement of radioactivity by scintillation counting or densitometric analysis of autoradiograms of the transferred amplicons.

IV.2. Coamplification RT-PCR with an endogenous control

IV.2.A. Extraction of RNA

RNA extraction, examination of integrity and quantitation was performed as described in section II.

IV.2.B. Reverse transcription

First-strand cDNA was made using RNA extracted from different developmental stages of interest, gonads and somatic tissues. One microgram of total RNA in 5 ul of depc-H_2O and 1 ul of cDNA synthesis primer (10 uM) were combined in a 0.5 ml microfuge tube and incubated at 70 °C for 2 minutes. The tube was then placed on ice and 2 ul 5X first-strand buffer, 1 ul dNTP mix (10 mM), and 1 ul of AMV reverse transcriptase (20 units/ul) were added. After mixing the contents the tube was incubated at 42 °C for an hour in an air incubator (Isotemp Incubator, Fisher Scientific). Termination of first-strand synthesis was achieved by placing the tube on ice. A similar reaction was set up with no AMV-RT enzyme added. This control was used to role out possible genomic DNA contamination.

IV.2.C. RT-PCR assay to determine the exponential phases of amplification

To ensure that *dnmt1* and *max* amplicons generated were in the exponential phase of amplification, six similar 50-ul reaction-mixtures for each set of primers, each containing 0.2 uCi of [α^{32}P] dCTP were subjected to 15, 20, 25, 30, 35, 40 and 45 cycles of amplification respectively. First strand cDNA generated from either ovarian or

blastula-stage RNA was used as the template in this experiment. A master mix for all PCR reactions was prepared by mixing the following for each 50 ul PCR reaction: 36 ul of water, 5 ul of 10X cDNA PCR reaction buffer (CLONTECH, Cat.No.K1910-y), 1 ul dNTP Mix (10 mM), 0.2 uCi of [α^{32}P] dCTP (NEN Life Science Products, Inc. Cat. No.BLU513H) and 1 ul Advantage Taq Polymerase Mix (50X) (CLONTECH, Cat.No.K1910-y). A separate reaction was set up for each set of primers. One microlitre of each set of primers [10 uM] was added to 1 ul of cDNA diluted in 4 ul of ddH2O. Forty-three microlitres of master mix was added to each reaction and the sample was overlaid with two drops of mineral oil. The tubes were placed in the thermal cycler [MiniCycler, MJ Research] and thermal cycling was commenced using the following program: one cycle of denaturation at 94 °C for 3 minutes; 45 cycles of denaturation at 94 °C for 30 seconds, annealing at 55 °C for 30 seconds, and extension at 72 °C for 1 minute.

Quatitation of PCR products was performed by measuring the amount of incorporated radioactive tracer. This was achieved by differential precipitation of the DNA products with trichloroacetic acid (TCA). Whatman GF/C glass-fiber filters (W&R Balston, England) were labeled using a soft-lead pencil. Two microlitres of each PCR product to be assayed were spotted on the center of each of two labeled filters. One of the filters was used to measure the total amount of radioactivity in the reaction, the other measures only the acid-precipitable radioactivity. The filters were stored at room temperature until all the fluid had evaporated. One of each pair of filters was washed twice in ice-cold 5% TCA and 20 mM sodium pyrophosphate for two minutes. During these washes, the unincorporated nucleotides are eluted from the filters while the radioactive nucleic acids are fixed to them. Each filter was washed in 70% ethanol then

allowed to dry at room temperature. All washed and unwashed filters were placed in scintillation vials and radioactivity was counted using the Chicago Nuclear Scintillation Counter. The number of counts per minute in washed filters was plotted against the number of cycles.

Along with the above method of quatitation, the PCR products were analyzed by electrophoresis on 1.6% agarose gels and visualized by ethidium bromide staining. The DNA was then transferred to a nylon membrane (Micron Separations Inc.) and exposed to X-ray film at –70 °C for two hours. The autoradiogram was then analyzed using the same methods as section IV.4.

IV.2.D. Coamplification RT-PCR with an endogenous control

Master mix was prepared for all PCR reactions. For each 50-ul PCR reaction, the following reagents were mixed: 36 ul water, 5 ul 10X cDNA PCR Reaction Buffer (CLONTECH, Cat.No.K1910-y), 1 ul dNTP Mix [10 mM] and Advantage 2 Polymease Mix [50X] (CLONTECH, Cat.No.K1910-y). The contents were mixed well and then the tube was briefly spun in a microfuge.

The *dnmt1* primer-set 5MTR6 and 5MTF2 (Appendix 1) was used, which generates a 533 bp *dnmt1* amplicon. Primers, maxF and maxR (Appendix 1), to the constitutively expressed zebrafish *max* gene (Schreiber-Agus *et al*, 1993) were designed to generate a 313 bp amplicon. One microlitre [10 uM], per each 50 ul PCR reaction, of each of these three primer sets was added to the master mix. Then 49 ul of the mix were added to 1 ul of each of the first-strand cDNAs made. The contents of each tube were overlaid with 2 drops of mineral oil, capped firmly and placed in the thermal cycler

[MiniCycler, MJ Research]. The control samples prepared adding no AMV-RT were also treated similarly.

Thermal cycling was commenced using the following program:

- 94 °C for 5 minutes
- 30 cycles of
    - ➢94 °C for 30 seconds
    - ➢55 °C for 30 seconds
    - ➢72 °C for 60 seconds

Results: Section One

I. Expression analysis of *dnmt1* during early development, in gonads and in somatic tissues

In order to determine the developmental profile of *dnmt1* mRNA expression, RNA hybridization analysis experiments were carried out on a variety of developmental stages, the gonads and somatic tissues (figure 10). Three bands measuring approximately 5 kb, 2.7 kb and 1.65 kb corresponding to zebrafish *dnmt1*, *β-actin* and *smn* respectively were observed. *β-actin* and *smn* were used as controls to normalize for RNA loading differences. Another control (*max*) (1.1 kb) (Schreiber-Agus *et al.*, 1994) was also used for embryonic stages to confirm the values determined using *β-actin* and *smn*. Densitometric analysis (figure 11) of the *dnmt1* and corresponding *β-actin* (table 4), *smn* (table 5) or *max* (table 6) bands was conducted on two independent blots in order to determine the relative levels of *dnmt1* mRNA in the different developmental stages and tissues tested.

Table 4. Densitometric analysis of *dnmt1* and *β-actin*.

Dev. Stage/tissue	Dnmt1 density	β-actin density	dnmt1/β-actin ratio
1-2 cell stage (1)	103	667	0.15
1-2 cell stage (2)	70	437	0.16
Blastula (3.5-4.0 h) (1)	115	248	0.46
Blastula (3.5-4.0 h) (2)	112	255	0.44
Gastrula (6.0 h) (1)	445	1290	0.34
Gastrula (6.0 h) (2)	306	1015	0.30
Ovary (1)	621	833	0.75
Ovary (2)	687	909	0.76
Testes (1)	96	60	1.60
Testes (2)	159	99	1.61
Muscle (1)	149	228	0.65
Muscle (2)	203	318	0.64
Whole male	533	846	0.63
Whole female	356	584	0.61

Table 5. Densitometric analysis of *dnmt1* and *smn*.

Dev. Stage/tissue	Dnmt1 density	smn density	dnmt1/SMN ratio
1-2 cell stage	72	243	0.296
1-2 cell stage	176	607	0.289
Blastula (3.5-4.0 h) (1)	159	233	0.682
Blastula (3.5-4.0 h) (2)	1502	2113	0.710
Testes (1)	1028	710	1.450
Testes (2)	1917	1335	1.440
Ovaries (1)	226	188	1.200
Ovaries (2)	541	468	1.160
Muscle (1)	256	344	0.744
Muscle (2)	241	321	0.750

Table 6. Densitometric analysis of *dnmt1* and *max*.

Dev. Stage/tissue	Dnmt1 density	max density	dnmt1/max ratio
1-2 cell stage	121	480	0.252
1-2 cell stage	154	621	0.248
Blastula (3.5-4.0 h) (1)	176	310	0.570
Blastula (3.5-4.0 h) (2)	409	703	0.580
Gastrula (6.0 h) (1)	175	462	0.378
Gastrula (6.0 h) (2)	111	284	0.390
Ovary (1)	604	520	1.162
Ovary (2)	556	483	1.151
Testes (1)	572	416	1.380
Testes (2)	799	582	1.373
Muscle (1)	234	162	1.440
Muscle (2)	175	126	1.388

Overall, *dnmt1* transcripts were found in all of the tissues examined. However, mRNA levels seem to vary among early developmental stages, gonads and somatic tissues (figure 10 and 14). The dnmt1 mRNA levels were highest in the gonads. Although message abundance in ovaries was consistently high, it was more variable than in other tissues. RNA extracted from whole adult fish (both male and female) showed higher message levels likely reflecting the higher levels of *dnmt1* mRNA present in the gonads. In the 1-2 cell stage, *dnmt1* mRNA was relatively lower in abundance than all other stages tested (figure 10). During blastula stage (3-3.5 hrs) embryos *dnmt1* mRNA levels climbed to considerably higher levels than those noted during early cleavage. During the gastrula stage (6.0 hrs), although the transcript levels showed slight reduction, it remained significantly higher than in early cleavage (figure 10).

II. RT-PCR assays to determine the exponential phases of amplification of the primer-sets

To determine that the amplicons generated were in the exponential phase of amplification, separate experiments were undertaken whereby radioactivity was incorporated and samples were subjected to increasing numbers of cycles. This was undertaken for each set of primers used independently to avoid possible existing differences in exponential phase of amplification among them.

The counts per minute radioactivity obtained for each washed glass-fiber filter, reflecting the incorporated radioactivity, were plotted against the number of PCR cycles (Table 7 & Figure 12).

Table 7. The counts per minute (CPM) of incorporated radioactivity at specific numbers of PCR cycles for each of the two sets of primers used.

# of cycles	10	15	20	25	30	35	40	45
CPM								
Max	55	83	321	2284	8025	7888	7743	5413
Dnmt1	-	-	64	296	741	1472	1104	786

From this analysis we concluded that sampling at thirty cycles was within the exponential range of amplification. The exponential phases for the different sets of primers overlapped enabling us to perform multiplex PCR in the expression experiments.

III. Expression pattern of *dnmt1* using RT-PCR

RT-PCR was used to evaluate the temporal and tissue specific differences in abundance of *dnmt1* transcripts. Figures 13 and 14-D illustrate the expression pattern during development and in gonads. No bands were detected in the samples where the reverse transcriptase was omitted. This implies that the amplified product was derived from first strand cDNA and not the result of genomic DNA contamination. Amplicons of the expected sizes were noted in all samples. Table 8 indicates the ratios of *dnmt1/max* in gonads and early developmental stages. These ratios support the observations made on RNA hybridization analyses.

Table 8. Scintillation counts of each *dnmt1* and *max* amplicon.

Dev. stage/tissue	Dnmt1 (CPM)	max (CPM)	dnmt1/max ratio
1-2 cell stage	2953	10738	0.275
1-2 cell stage	2875	12075	0.238
Blastula (3.5-4.0 h)	8431	13620	0.620
Blastula (3.5-4.0 h)	8662	13210	0.650
Gastrula (6.0 h)	7579	18891	0.400
Gastrula (6.0 h)	7084	16144	0.439
Ovaries (1)	35093	51699	0.680
Ovaries (2)	32084	41156	0.780
Testes (1)	46451	40873	1.140
Testes (2)	41220	32580	1.258

Section two: Determination of the spatial and temporal patterns of *dnmt1* message expression during oogenesis and early-cleavage embryos:

Materials and methods: Section Two

I. Strategy of *in situ* hybridization analysis

An overview of the whole mount and tissue sections *in situ* Hybridization using DIG-labeled probes is outlined below and illustrated in figure 15.

DIG RNA probe labeling

⇩

Immobilization of target mRNA (whole mount, tissue sections)

⇩

Hybridization of DIG-labeled probes

⇩

Binding of anti-DIG AP antibody conjugates

⇩

Colorimetric detection of *in situ* with NBT/X Phosphate

II.Non-radioactive labeling using DIG-11-UTP

II.A. RNA labeling:

A pBluescript SK (+) (Stratagene) plasmid with a 2 Kb zebrafish specific cytosine-5 methyltransferase cDNA insert was used to make the RNA hybridization probes. The plasmid DNAs were linearized with SmaI, followed by *in vitro* transcription reactions with T7 RNA polymerase for the antisense RNA probe. Controls with sense strand probes were prepared by linearizing the plasmid with KpnI and *in vitro* transcription with T3 RNA polymerase.

To a sterile microfuge tube on ice, the following reagents were added in order: 1 ug of appropriately cut and purified DNA template, 2 ul of 10X NTP labeling mixture [10 mM ATP, 10 mM CTP, 10 mM GTP, 6.5 mM UTP, 3.5 mM DIG-UTP], 2 ul of 10X transcription buffer [400 mM Tris-HCl (pH 8.0), 60 mM $MgCl_2$, 100 mM dithioerythritol (DTT), 20 mM spermidine, 100 mM NaCl, 1 unit/ml RNase inhibitor], depc-H_2O to a total volume of 18 ul and 2 ul RNA polymerase (Boehringer Mannheim) (T7 or T3). This RNA synthesis reaction was incubated at 37 °C for 2 hours . Two microliters of 0.2 M EDTA were then added to terminate the transcription reaction.

The probes were hydrolyzed to an average length of 150-300 nucleotides by alkaline hydrolysis to allow diffusion of the probe into and out of the tissue (Cox *et al.*, 1984). RNA was hydrolyzed by adding an equal volume of depc-treated H_2O and two volumes of carbonate buffer [60 mM Na_2CO_3, 40 mM $NaHCO_3$ (pH 10.2)] and incubating the reaction for 60 minutes at +60 °C. Hydrolysis was stopped by the addition of an equal volume of hydrolysis neutralization buffer [200 mM sodium acetate, 1%

(V/V) acetic acid (pH 6.0)] and the RNA precipitated by adding three volumes of ETOH and incubation at -70 °C for 30 minutes. The RNA was pelleted by centrifugation at 13000 rpm for 15 minutes at +4 °C. The resulting probe length was determined by electrophoresis on a 1% agarose gel stained with ethidium bromide.

Estimation of DIG-labeled RNA yield was performed by preparing 10-fold serial dilutions of the labeled RNA in depc-H_2O. Serial dilutions were also done for DIG-labeled control RNA [DIG-labeled "antisense"-RNA]. One microliter of each dilution of the experimental and control RNA was spotted onto a nylon membrane (Micron Separations Inc.). The RNA was cross-linked to the membrane by exposure to UV light for 3 minutes. The membrane was rinsed briefly in washing buffer [100 mM maleic acid, 150 mM NaCl, 0.3% Tween 20 (pH 7.5)] and then placed in blocking solution [1% W/V blocking reagent (Boehringer Mannheim), 100 mM maleic acid, 150 mM NaCl (pH 7.5)] for 30 minutes at room temperature with agitation. After removal of the blocking solution, the membrane was incubated in diluted Anti-DIG-alkaline phosphatase (1:5000) in blocking solution for 30 minutes at room temperature. The membrane was washed twice in washing buffer, 15 minutes per wash. The membrane was then incubated in detection buffer [100 mM Tris-HCl (pH 9.5), 100 mM NaCl, 50 mM $MgCl_2$] for 2 minutes to activate the alkaline phosphatase conjugated to the antibody. The detection buffer was poured off and replaced with fresh color substrate solution [45 ul NBT, 35 ul BCIP in 10 ml of detection buffer]. Color development was allowed to take place in the dark. After color developed to a sufficient intensity, the reaction was stopped by washing the membrane with sterile H_2O for 5 minutes. Spot intensities of the control and

experimental dilutions were compared to estimate the concentration of the experimental probe.

II.B. DNA labeling:

DNA was labeled with DIG-11-dUTP using the Genius Nonradioactive Nucleic Acid Labeling and Detection System (Boehringer Mannheim). Using the random primer labeling method, 500 ng of DNA were denatured by boiling for 10 minutes and immediately chilled on ice. Two microlitres of 10X hexanucleotide mix [156 ug/ul random hexanucleotides in 500 mM Tris-HCl (pH 7.2), 100 mM $MgCl_2$, 1 mM DTE, 2 ug/ul BSA], 2 ul 10X dNTP labeling mix [1 mM dATP, 1 mM dCTP, 1 mM dGTP, 100 mM dTTP, 0.35 mM alkali-labeled DIG-dUTP (pH 6.5)] and 2 ul of large fragment DNA polymease I were added to the denatured DNA. The sample was incubated for an hour at 37 °C. The DIG-labeled DNA was precipitated with 3 volumes of isopropyl alcohol for 30 minutes at - 70 °C and centrifugation at 13000 rpm for 15 minutes. The pellet was washed with 70% ETOH, dried at 65 °C for 10 minutes and then resuspended in TE [10 mM Tris-HCl (pH 7.5) and 1 mM EDTA].

Estimation of DIG-labeled DNA yield was performed by preparing 10-fold serial dilutions of the labeled DNA in dilution buffer [50 ng/ul herring sperm DNA, 10 mM Tris-HCl (pH 8.0), 1 mM EDTA]. Serial dilutions were also done for DIG-labeled control DNA [5 ng/ul DIG-labeled pBR328 DNA]. The estimation procedure is as described above.

III.A. Whole mount *in situ* hybridization

III.A.1. Fixation of Embryos and Ovarian Tissues

Zebrafish maintenance, breeding, embryo collection and tissue dissection is described in chapter two-section one-I. Immediately after collection, the embryos and the gonads were fixed with 4% paraformaldehyde in 1X PBS [130 mM NaCl; 7 mM Na2HPO4; 3 mM NaH2PO4] at 4 °C overnight. Embryos and ovarian tissue were washed twice in PBS, 5 minutes each, at room temperature. Embryonic chorions were then removed mechanically. Embryos and ovarian tissue were placed in 100% methanol and cooled to –20 °C for at least 30 min to facilitate permeabilization. Embryos and ovaries were brought back to room temperature and rehydrated in 5-minute changes of 50% and 30% methanol in PBST [PBS and 1% polyoxyethylenesorbitan monolaurate (Tween 20) (Sigma Cat. No. P-1379)], rinsed twice in PBST for 5 min each, fixed for 20 min in 4% paraformaldehyde in PBS at room temperature and then rinsed twice in PBST for 5 min each.

III.A.2. Proteinase K digestion and postfixation

Embryos and ovarian tissue were digested with proteinase K (10 μg/ml in PBST) at room temperature for 5 to 10 minutes respectively, rinsed twice in PBST for 5 min each, fixed as above (4% paraformaldehyde in PBS, 20 min) and then washed twice in PBST for 5 minutes each.

III.A.3. Acetic anhydride treatment

PBST was replaced with dH2O as quantitatively as possible. As quickly as possible, dH2O was replaced with a fresh mixture of 2.5 µl acetic anhydride in 1 ml of 0.1M triethanolamine (pH 7.0) and incubated for 60 min at room temperature. The embryos and ovarian tissue were then washed 2 times for 10 min each in PBST. This acetic anhydride treatment helps reduce background due to endogenous phosphatases.

III.A.4. Prehybridization

Embryos and ovarian tissue were transferred into eppendorf tubes in approximately 300 µl of HYB* [50% formamide, 5xSSC, 0.1% Tween-20] and incubated for 5 min at 55°C; afterwards, HYB* was replaced with an equal volume of HYB+ [HYB* with 5mg/ml torula (yeast) RNA]. Prehybridization was carried out at 55°C overnight in HYB+.

III.A.5. Hybridization

As much of the HYB+ as possible was removed without letting the embryos touch air and 20 to 40 µl of fresh HYB+ containing 20-100 ng of RNA probe (about 0.5-5.0 ng/µl) were added so that all embryos were covered by the solution. The probe was heat denatured in HYB+ for 5 min at 68°C before adding to the embryos. Hybridization was allowed to proceed overnight at 55 C.

III.A.6. Probe removal, RNase treatment and washing

Probe was pipetted out and washing was commenced by soaking the embryos and ovarian tissue for 20 min at 55°C in 50% formamide in 2xSSCT, rinsing 3 times for 10 min each at 37°C in 2xSSCT [SSC plus 0.1% Tween] and then rinsing for 5 min at 37°C in PBST. RNase digestion (RNAse A, 20 µl/ml plus RNAse T1, 100 U/ml in PBST) was allowed to proceed for 30 min at 37°C and then embryos and ovarian tissue were rinsed for 10 min at 37°C in 2xSSCT. Embryos and ovarian tissue were soaked 60 min at 55°C in 50% formamide in 2xSSCT, rinsed 15 min at 55°C in 2xSSCT, rinsed twice for 15 min each at 50°C in 0.2xSSCT and finally rinsed 5 min in PBST at room temperature.

III.A.7. Colorimetric detection

Detection began with blocking for 1 hour at room temperature with PBST plus blocking reagent (Boehringer Mannheim). Fab-AP fragment (Boehringer Mannheim) was added at a 1:4000 dilution in PBST plus blocking reagent and the samples incubated for 4 hours at room temperature with agitation. Embryos and ovarian tissue were washed 4 times for 25 min each with PBST plus blocking reagent, washed 3 times for 5 min each in staining buffer [100 mM Tris pH 9.5, 50 mM MgCl2, 100 mM NaCl, 0.1% Tween-20, 1 mM Levamisol (added fresh)] and then incubated in staining buffer with 4.5 µl NBT and 3.5 µl X-Phosphate [NBT, 75 mg/ml in 70% dimethylformamide; X-Phosphate, 50 mg/ml in dimethylformamide] per ml added. Staining was allowed to proceed for 30 min to two hours. Once colour developed, the embryos and ovarian tissue were washed in PBS, dehydrated with 100% MeOH twice (10 min each), fixed in 4% paraformaldehyde/PBS for 30 minutes at room temperature and mounted in glycerol.

Whole mounts were visualized and photographed with a Nikon camera on a dissecting microscope using day light balanced Kodak Ektachrome 100 ISO slide film.

III.B. *in situ* hybridization of ovarian tissue sections

Ovarian tissue was embedded in paraffin and serially sectioned for *in situ* hybridization with a DIG-labeled probe. Frozen ovarian sections were also prepared for hybridization.

III.B.1. Paraffin Sections of Zebrafish Ovaries

Female zebrafish were anesthetized with 2-phenoxyethanol and subsequently ovarian tissues were dissected. The tissue was immediately placed in freshly prepared Ammerman's fixative [30 g chromium potassium sulfate, 30.0 ml formalin, 2.0 ml glacial acetic acid, 238.0 ml distilled water; Humason, (1979)] for two hours. The tissue was washed overnight under running tap water. Tissue dehydration in an ethanol series was then commenced. Dehydration in 75% and 95% ethanol was allowed to proceed for one hour. The sections were then placed in two changes of 100% ethanol for 30 minutes each. The tissue was then infiltrated with 2 changes of molten (56 °C) paraffin for 30 minutes each. Once infiltration was complete the tissue was embedded in paraffin. The paraffin block was then sectioned on a Leitz Wetzlar microtome to obtain 0.5-0.7 um-thick sections of the ovary. The sections were placed in a 37 °C water bath to allow the paraffin to expand and even out the sections. Sections were lifted from the water bath on a 0.1% poly-L-lysine- or albumin-coated slides and were placed on a slide warmer to dry. The slides were then used for *in situ* hybridization with the DIG-labeled probes.

III.B.2. Frozen Sections of Zebrafish Ovaries

Zebrafish ovaries were sectioned using a cryostat (CRYO-CUT II Microtome; American Optical). Female breeding zebrafish were anesthetized with 2-phenoxyethanol and subsequently ovarian tissues were dissected. The dissected tissues were placed immediately in 2-N-methylbutane cooled in a dry ice/ethanol bath. The frozen tissues were mounted in O.C.T. compound (Tissue-Tekll), and 10 um sections were cut at -20 °C. Each section was removed from the knife by placing precoated (0.1% poly-L-lysine) slides on top of the sections. The sections were allowed to dry at room temperature for 20 minutes and then fixed for 20 minutes in freshly prepared 4% paraformaldehyde/PBS [130 mM NaCl; 7 mM Na2HPO4; 3 mM NaH2PO4]. The sections were washed in 3X PBS for 5 minutes and then washed twice in 1X PBS for 5 minutes each. The sections were dehydrated in 5 minute changes of 30%, 60%, 80%, 95% and 100% ethanol and were then air dried and either used immediately or stored at -20 °C overnight.

III.B.3. *in situ* Hybridization of Paraffin and Frozen Sections

Paraffin embedded sections were treated for three minutes with slide clearing solution (Fisher Scientific; Cat. No.15-182-507) to remove paraffin from the sections. Sections were rehydrated in an ethanol series of 100%, 95% and 70% ethanol for three minutes each and then treated with 0.2M HCl for 20 minutes. The sections were rinsed in distilled water for 5 minutes before and after a 15 minutes wash in 2X SSC. Pronase treatment [0.25 ug/ul pronase in 50 mM Tris-HCl (pH 7.5) and 5 mM EDTA] was allowed to proceed at 37 °C for 5 minutes. The pronase solution was then removed and the slides were washed in 1X PBS and 2 ug/ml glycine for 45 seconds with agitation.

Sections were rinsed in 1X PBS twice for 45 seconds each and fixed for 20 minutes in 4% paraformaldehyde. Post fixation, the sections were washed once in 3X PBS for 5 minutes and then twice in 1X PBS for 5 minutes each. Acetic anhydride treatment [0.25% acetic anhydride in 100 mM triethanolamine] was allowed to proceed for 10 minutes followed with three 1X PBS washes.

Prehybridization [50% deionized formamide, 5X SSC, 0.1% N-lauroylsarcosine, 0.02% SDS and 2% blocking reagent (Boehringer Mannheim)] solution was added to each slide and a coverslip was placed on the sections to ensure even and constant distribution of solution on the tissue sections. The slides were placed in a humid chamber and prehybridization proceeded for 1 hour at 55 °C for DIG-labeled RNA probes and at 45 °C for DIG-labeled DNA probes. DIG-labeled probes were heat-denatured prior to hybridization. Hybridization solution [probe mixed with prehybridization solution] was added to each slide and coverslips were placed on top to ensure the solution remains restricted to the sections. The slides were then submerged in preheated mineral oil and hybridization was carried out overnight at 55 °C for RNA probes and at 45 °C for DNA probes.

The following morning, the slides were removed from mineral oil and rinsed three times, 5 minutes each, in chloroform to remove residual mineral oil. The slides were air dried and then rinsed in 2X SSC where cover slips, if remaining in place, were removed. The sections were washed twice in 2X SSC for 15 minutes each and once in 0.1X SSC for 20 minutes at either 55 °C, for RNA probes, or at 45 °C, for DNA probes. After a final wash in 0.1X SSC at room temperature for 10 minutes, the slides were ready for colorimetric detection of DIG-labeled probes.

III.B.4. Colorimetric Detection of DIG-Labeled Probe

Posthybridization washed slides were equilibrated for 1 minute in wash buffer [100 mM maleic acid, 150 mM NaCl (pH 7.5)] and then blocked in 1% blocking solution [1% (w/v) blocking reagent dissolved in wash buffer] for 30 minutes at room temperature with agitation. After draining and placing the slides in a humid chamber, 1:2000 dilution in 1% blocking solution of anti-DIG-alkaline phosphatase (Boehringer Mannheim) was added and the sections were incubated for 30 minutes. After incubation in the antibody, sections were washed twice in washing buffer [100 mM maleic acid, 150 mM NaCl (pH 7.5)] for 15 minutes each. The sections were incubated in detection buffer [100 mM Tris-HCl (pH 9.5), 100 mM NaCl, 50 mM $MgCl_2$] for 2 minutes to activate the alkaline phosphatase. Colour substrate solution was prepared by adding 4.5 ul of NBT [75 mg/ml nitroblue tetrazolium salt, 70% dimethylformamide] and 3.5 ul of X-phosphate [50 mg/ml 5-bromo-4-chloro-3-indolyl phosphate toluidinium salt in 100% dimethylformamide] to 1 ml of detection buffer. The colour substrate solution was added to drained slides in a humid chamber and coverslips were placed on top to ensure the substrate solution remained restricted to the sections. Colour was allowed to develop in the dark for a few hours to overnight. Once colour had developed, the sections were rinsed in TE-buffer [10 mM Tris, 1 mM EDTA (pH 8.0)] and then fixed for 20 minutes in 4% paraformaldehyde/PBS. The sections were rinsed in 1X PBS for 5 minutes, dehydrated in an ethanol series and mounted in glycerol for visualization. Sections were examined for localized color development using light microscopy and photographs were taken at various magnifications using Ektachrome Tungsten balanced 64 slide film (Kodak) and a Nikon camera on a Nikon SMZUII microscope.

IV. Hematoxylin and Eosin Staining of Paraffin Sections of Zebrafish Ovaries

Ovarian tissue was stained with hematoxylin to delineate normal histology of prepared sections. Paraffin was removed from sections by clearing twice in xylene, 3 minutes each. Sections were then rehydrated in 3 minute changes of 100%, 95% and 70% ethanol. Sections were placed in Lugol's solution [1.0 gm iodine crystals, 2.0 gm potassium iodide, 12.0 ml distilled water] for 3 minutes and then rinsed for 3 minutes under running tap water. Sections were placed in 5% sodium thiosulfate for 3 minutes and then rinsed for 3 minutes under running tap water and placed in hematoxylin for 3 minutes. The sections were rinsed in running tap water for 3 minutes and then placed in Scott's solution [2.0 gm sodium bicarbonate, 20.0 gm magnesium, 100.0 ml distilled water] for 3 minutes. The sections were rinsed under running tap water and then counterstained with eosin for 2 minutes. Sections were dehydrated in 3-minute periods of 70%, 95% and 100% ethanol and mounted in glycerol. Sections were examined under light microscopy and photographs at various magnifications were taken using Ektachrome Tungsten balanced 64 slide film (Kodak).

Results: Section Two

in situ hybridization to ovarian and two-cell stage embryo whole mounts and tissue sections

Whole mount and tissue section *in situ* hybridization of zebrafish ovaries and early cleavage embryos using antisense zebrafish-*DNA (cytosine-5) methyltransferase 1* riboprobe demonstrated abundant expression of *dnmt1* mRNA in ovarian follicles of different developmental stages (figure 16 and 17). Intense staining was noted in the earlier stages of oogenesis. Reduction in message abundance was noted in the more mature stages of ovarian follicles with no detectable message in the fully mature oocytes by this method. The levels of *dnmt1* message were also not detectable in the one- and two- cell stage embryos (figure 16).

Section three: Elucidation of developmental dynamics of DNA (cytosine-5) methyltransferase 1, dnmt1, enzyme activity.

Materials and methods: Section Three

I. Determination of DNA methyltransferase enzyme activity strategy

Methylation capacity was measured as described by Issa *et al.*, (1993).

Cell lysates from different developmental stages and tissues

+

poly(dI-dC) [substrate]

+

^{3}H-SAM [methyl group donor]

⇩

tritium incorporation measurement

II. Zebrafish care and embryo collection

As described in section one.

III. Preparation of protein lysates

Tissues and pooled embryos from desired developmental stages were used. Embryos/tissues were lysed in 3 volumes of lysis buffer (LB) (w/v) [50 mM Tris (GiBCO BRL, Cat.No.15504-038) pH 7.8, 1.0 mM EDTA (Invitrogen Life Technologies,

Cat.No.15576-028), 1.0 mM dithioerythritol (DTT) (Sigma, Cat.No.D-8255), 0.1% sodium azide (Fisher Scientific Cat. No. S227-78263), 6 mg/ml phenylmethylsulfonyl fluoride (PMSF) (Sigma, Cat.No.P-7626), 10% glycerol (Fisher Scientific, Cat.No.G-32) and 1% Tween 20. The suspension was then passed through a 20-gauge needle followed by a 25-gauge needle twice each and lysed by freeze–thaw three times. The samples were then stored at –70 °C till ready to quantitate.

IV. Quantitation of protein lysates

The protein concentrations were determined using the Bio-Rad protein assay reagent (Bio-Rad Laboratories, Cat.No.500-0006). Proteins were quantitated in 15 ml aliquotes in duplicates using bovine serum albumin (BSA) as standard. Dye reagent was prepared by diluting 1 part dye reagent concentrate with 4 parts lysis buffer (LB). Six protein standards ranging from 0.2 to 1.0 mg/ml were prepared in LB. An aliquot of 100 ul of each standard and sample solution was pipetted into a clean, dry test tube and 5.0 ml of diluted dye reagent was added to each. After 5 minutes incubation at room temperature, absorbance at 595 nm (OD_{595}) was measured. A relative measurement of concentrations of the unknowns were determined by comparison to the standard curve generated for the BSA (figure 18).

V. Methyltransferase assay

For the DNA methyltransferase assay, 20 μg of cellular protein from each desired developmental stage or tissue (Table 9) was incubated for two hours at 37 °C with a deoxyionosine-deoxycytosine [poly(dI-dC)-poly(dI-dC)] (Amersham-Pharmacia Biotech

Inc Cat.No.27-7880-01) template (0.5 ug) and 2 μCi of 3H-labeled SAM (S-adenosyl-L-[methyl-^{3}H] methionine) (Amersham-Pharmacia) in a total volume of 20 μl. Poly(dI-dC)-poly(dI-dC) was used since human and murine DNA methyltransferases have very high affinity for this substrate (Pedrali-Noy and Weissbach, 1986) and they respond to it as though it were hemimethylated (Bestor, 1992). Reactions were stopped by adding 300 ul stop solution (1.0 % SDS, 2.0 mM EDTA, 3% 4-amino-salicylate [Sigma, Cat.No.A-3505], 5% butanol [Fisher Scientific, Cat.No.A-399], 0.25 mg/ml Salmon sperm DNA, 1.0 mg/ml proteinase K [Sigma, Cat.No.P-2308]) and incubation at 37°C for 30 min. Protein was extracted twice with phenol-chloroform. The reacted DNA template was recovered from the aqueous phase by ethanol precipitation. The pellet was then resuspended in 0.3 M NaOH (Amachem Scientific & Chemical, Cat.No.SS-350) and incubated at 37 °C for an hour to remove RNA and avoid over-estimation of methyltransfrease activity due to methylation of RNA (Liu and Santi, 2000). The solution was then spotted on a Whatman GF/C filters (Whatman), dried, washed with 5% trichloroacetic acid (Fisher Scientific, Cat.No.SA433-500) followed by 70% ethanol then placed in scintillation vials (Fisher Scientific, Cat.No.3-337-11B) containing 5 ml of biofluor (New England Nuclear, Cat.No.NEF-961) and counted using a Beckman liquid scintillation counter (Beckman, LS6000TA). All assays were performed in duplicates and simultaneously. Background levels were determined in assays lacking poly(dI-dC)-poly(dI-dC) and the same sample was used as a positive control with poly(dI-dC)-poly(dI-dC) but no SAM.

Results: Section Three

Quantitation of dnmt1 enzyme activity in mature oocytes, in early embryonic stages and in somatic tissues

Results of duplicate experiments indicated that dnmt1 enzyme levels during early embryogenesis are higher relative to the enzyme levels in somatic tissues (table 9 and figure 19). In mature unfertilized oocytes, dnmt1 enzyme activity is 4-5 fold higher than in mature oocyte-depleted ovary. The dnmt1 enzyme levels were equally as high in mature unfertilized oocyte and in early cleavage (1-2 cell stage) embryos. At early blastula (2.2 hr) there was a slight reduction in enzyme levels relative to those observed in unfertilized mature oocytes and during early cleavage, but by late blastula (3.5 hrs) stage, significantly higher levels of dnmt1 enzyme activity were observed.

Table 9. dnmt1 enzyme assay of different developmental stages, oocytes and somatic tissues. The disintegrations per minute (DPM) were automatically calculated by an Auto DPM program* as a measure of the absolute activity within the sample.

Developmental stage/tissue	CPM	Auto DPM
Mature oocyte-depleted ovary (1)	295	694
Mature oocyte-depleted ovary (2)	318	752
Mature unfertilized oocytes (1)	1485	3498
Mature unfertilized oocytes (2)	1522	3463
1-2 cell stage (1)	1462	3369
1-2 cell stage (2)	1537	3543
Blastula (2.2 h) (1)	1255	2896
Blastula (2.2 h) (2)	1323	3023
Blastula (3.5 h) (1)	1911	4394
Blastula (3.5 h) (2)	1823	4223
Gastrula (6.0 h) (1)	1927	4486
Gastrula (6.0 h) (2)	1777	4138
Muscle (1)	76	202
Muscle (2)	79	195
Positive control (no SAM)	9	34
Negative control (no Poly dI-dC)	11	42

* The use of DPM makes the results independent of factors that affect counting efficiency such as choice of liquid scintillation cocktail, amount of cocktail and size and type of vial.

Discussion: Chapter Two

We have examined the spatial and temporal patterns of expression of *dnmt1* during oogenesis and early development of zebrafish (figures 10, 13 and 14) and found that the levels are developmentally regulated. RNA hybridization analyses of early embryonic stages and gonads (figure 10) revealed a single sized message of ~5 Kb in all tissues examined. The highest levels of *dnmt1* mRNA were observed in gonadal tissues of breeding adult zebrafish consistent with results previously found in mice (Monk *et al.*, 1991; Carlson *et al.*, 1992; Mertineit *et al.*, 1998). Message abundance in ovaries was consistently high, but it was more variable than in other tissues probably due to differences in breeding status of the female fish from which ovaries were obtained for RNA extraction.

Zebrafish *dnmt1* mRNA was also identified in 1-2 cell embryos *albeit* at relatively lower abundance. By blastula stage (3-3.5 hrs), *dnmt1* mRNA levels climbed to considerably higher levels than those noted during early cleavage. In the gastrula stage (6 hours), the transcript levels remained appreciably higher than those seen in early cleavage but had declined from the blastula stage levels in keeping with the investigation of Martin *et al.*, (1999) who observed that *dnmt1* mRNA abundance is greatest in blastula-stage with reduction in message level during gastrulation.

Whole mount and tissue-section *in situ* hybridization to zebrafish ovaries using antisense *dnmt1* mRNA probes demonstrated that there were variations in the abundance of *dnmt1* transcript levels among ovarian follicles of different developmental stages (figure 16 and 17). A noticeable degree of reduction in message abundance was evident

in the more mature stages of ovarian follicles with no detectable message in the fully mature oocyte.

Dnmt-1 enzyme assays were performed to determine whether the changes in *dnmt1* mRNA levels translate into similar changes in enzyme activity. Results of duplicate experiments (figure 19) indicated that dnmt1 enzyme levels during early embryogenesis were higher relative to the enzyme levels in somatic tissues, reminiscent of the high levels of Dnmt1 throughout mouse preimplantation development relative to somatic tissues (Carlson *et al.,* 1992). In mature unfertilized oocytes, dnmt1 enzyme activity is 4-5 fold higher than in mature oocyte-depleted ovary.

The relative changes in *dnmt1* mRNA and enzyme activity during oogenesis and early development are compiled in figure 20. Zebrafish dnmt1 enzyme was present at very high levels in mature oocytes and were equally as high in the early cleavage embryos while mRNA levels were low at these stages. Such a relationship closely mimics the mouse Dnmt1 protein and mRNA changes during oogenesis and pre-implantation development (Monk *et al.,* 1991; Bestor, 2000). This implies that the high levels of *dnmt1* mRNA noted during early stages of oogenesis are translated into protein during the latter stages of oogenesis in a similar fashion to what was described in *Xenopus* (Kimura *et al.,* 1999). This would presumably provide stockpiles of maternal dnmt1 to support early embryonic development prior to zygotic gene activation.

At early blastula (2.2 hr), there was a slight reduction in enzyme levels relative to those observed in unfertilized mature oocytes and during early-cleavage. This is prior to zebrafish midblastula transition (MBT) and zygotic gene activation (ZGA) which begins at cycle 10 (~ 2.75 hrs) (Kane and Kimmel, 1993) and may reflect maternal dnmt1

protein turnover. Late blastula (3.5 hrs) stage embryos have higher levels of dnmt1 enzyme activity than those noted prior to ZGA. This corresponds with the increase in *dnmt1* mRNA observed during this stage and likely represents embryonic dnmt1 expression.

Conclusions: Chapter Two

The developmentally regulated and stage specific expression changes in the levels of *dnmt1* mRNA and enzyme activity, particularly during the process of oogenesis suggest that DNA methylation may play a role in oogenesis and/or in early development of the zebrafish.

Figure 9. A representative example of a methylene blue-stained blotted and immobilized RNA on nylon membranes. Note intact 18S (~2.0 kb) and 28S (~5.0 kb) RNA. Poly A^+ RNA appears as a smear.

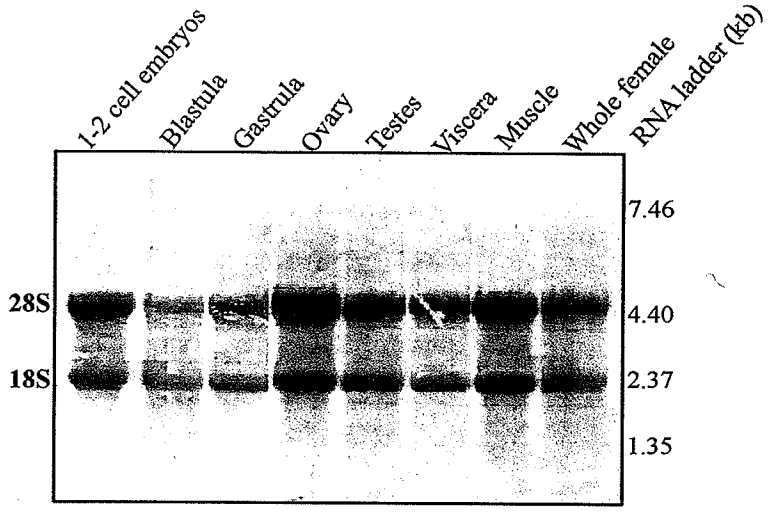
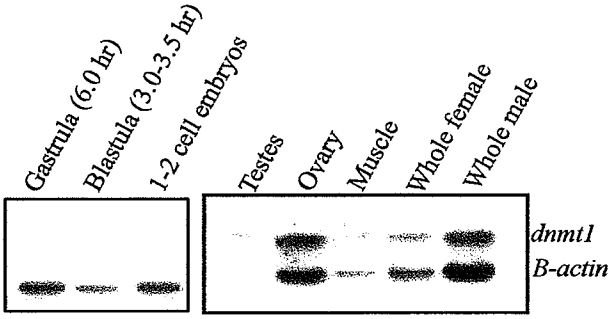
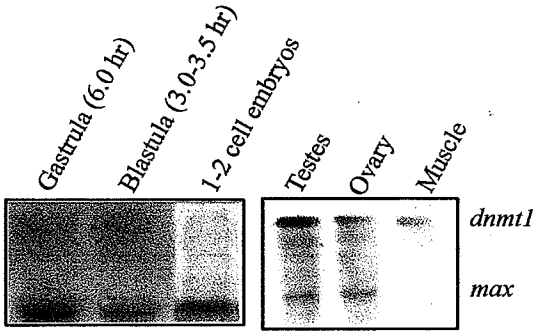

1-2 cell embryos
Blastula
Gastrula
Ovary
Testes
Viscera
Muscle
Whole female
RNA ladder (kb)
28S
18S
7.46
4.40
2.37
1.35

Figure 10. RNA hybridization analysis of *dnmt1*. RNA was isolated from zebrafish muscle, viscera, adult male and female, testes, ovaries and early developmental stages as shown. Fractionated and immobilized RNAs were hybridized with radiolabeled *dnmt1* and either *B-actin* (A), *max* (B) or *smn* (C) cDNA probes.

A


Gastrula (6.0 hr)
Blastula (3.0-3.5 hr)
1-2 cell embryos
Testes
Ovary
Muscle
Whole female
Whole male
dnmt1
B-actin

B


Gastrula (6.0 hr)
Blastula (3.0-3.5 hr)
1-2 cell embryos
Testes
Ovary
Muscle
dnmt1
max

C

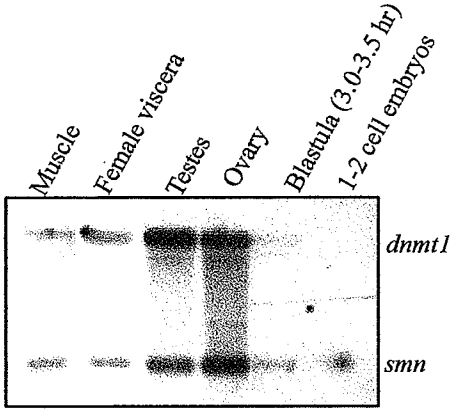
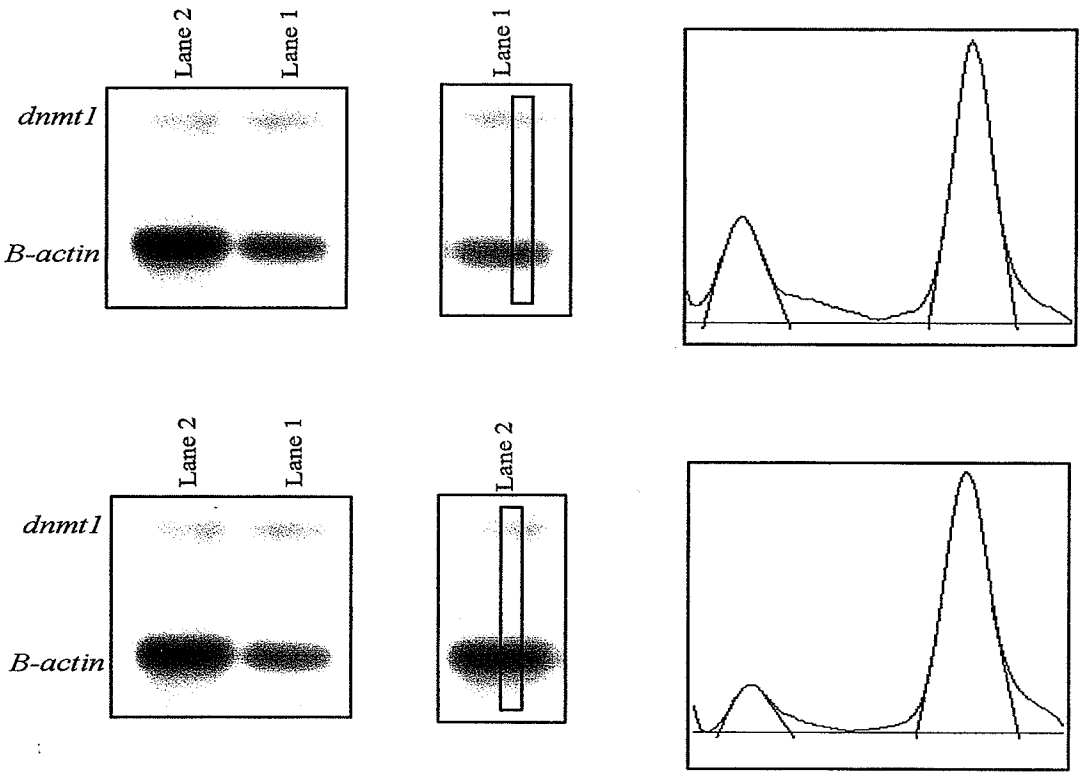

Muscle
Female viscera
Testes
Ovary
Blastula (3.0-3.5 hr)
1-2 cell embryos
dnmt1
smn

Figure 11. A representative example of densitometric analysis. After scanning the image and importing it into the software Scion Image, the desired lane is marked and plotted. The area under each curve is then estimated by the program.

A) Scan blot **B) Mark lane** **C) Plot lane**



D) Results

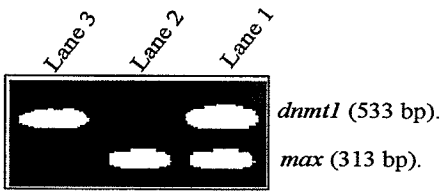
	dnmt1	*B-actin*	*dnmt1/B-actin*
Lane 1	103	235	0.44
Lane 2	63	244	0.26

Figure 12. Coamplification RT-PCR with an endogenous control.

A) Two sets of primers were used to generate a 533 bp *dnmt1* and a 313 bp *max* amplicons. Lane 1 shows a multiplex PCR using the two sets of primers combined. Lanes 2 and 3 show the *max* and *dnmt1* amplicons respectively.

B) Determination of the exponential phase of amplification of each primer set used in the coamplification RT-PCR analysis Two separate experiments were performed wherein each set of primers was subjected to an increasing number of cycles. The exponential range was determined to be between 27 and 35 cycles.

A


Lane 3
Lane 2
Lane 1
dnmt1 (533 bp).
max (313 bp).

B

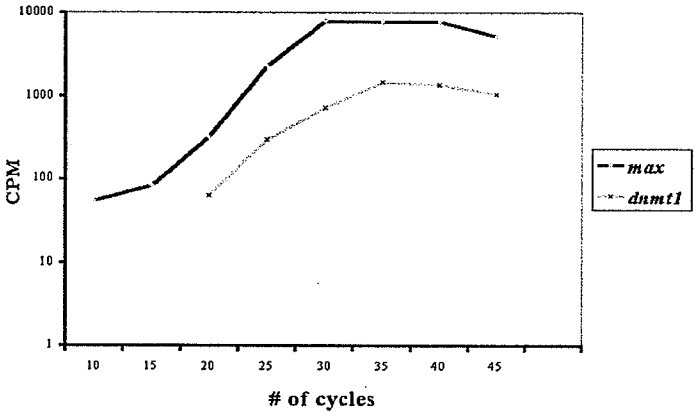

10000
1000
100
10
1
CPM
10
15
20
25
30
35
40
45
# of cycles
max
dnmt1

Figure 13. Coamplification RT-PCR analysis of embryonic and gonadal tissues. RNA from different developmental stages and gonadal tissues was used to make first strand cDNA. Lanes designated + or - refer to the presence or absence of RT in the first strand synthesis, respectively. The cDNAs were then PCR amplified using two sets of primers, one specific for *dnmt1* and one for *max* as an endogenous control. The relative amounts of amplification product were determined by comparing the *dnmt1* amplicon to the corresponding *max* band (*dnmt1*/*max* ratios are indicated below figure).

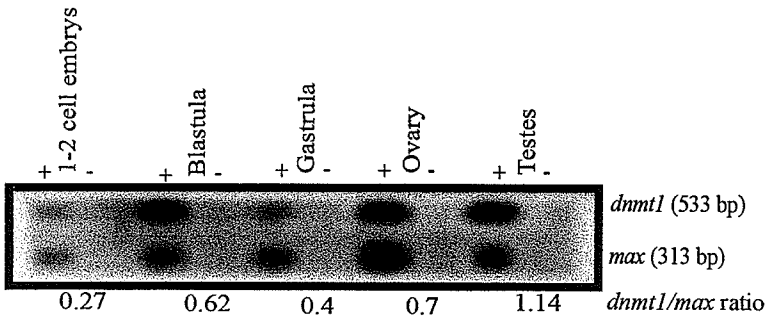
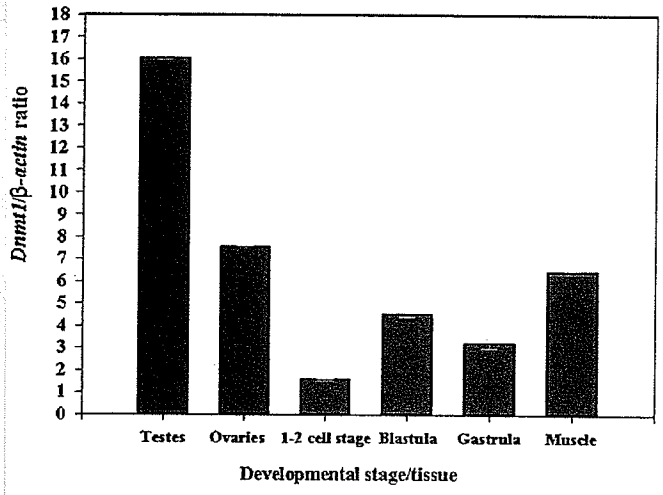
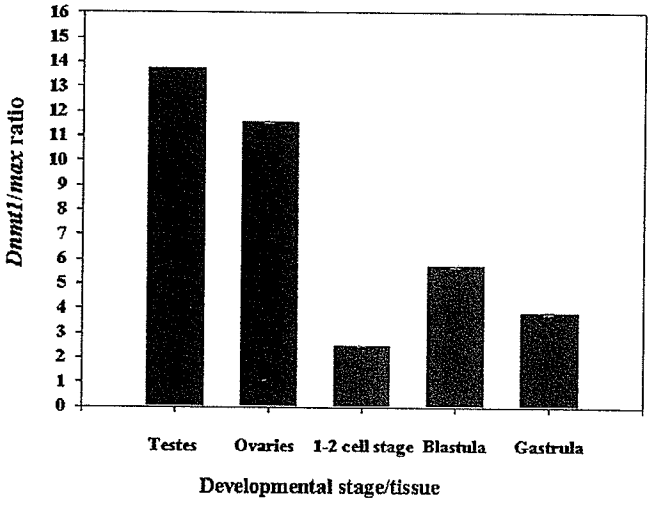

+ 1-2 cell embrys -
+ Blastula -
+ Gastrula -
+ Ovary -
+ Testes -
dnmt1 (533 bp)
max (313 bp)
0.27
0.62
0.4
0.7
1.14
dnmt1/max ratio

Figure 14. The relative abundance of *dnmt1* mRNA in different developmental stages, gonads and somatic tissues as compared to *β-actin* (A) , *smn* (B) and *max* (C) probes used in Northern blot analysis. (D) The relative abundance of *dnmt1* to *max* as obtained by coamplification RT-PCR analysis.

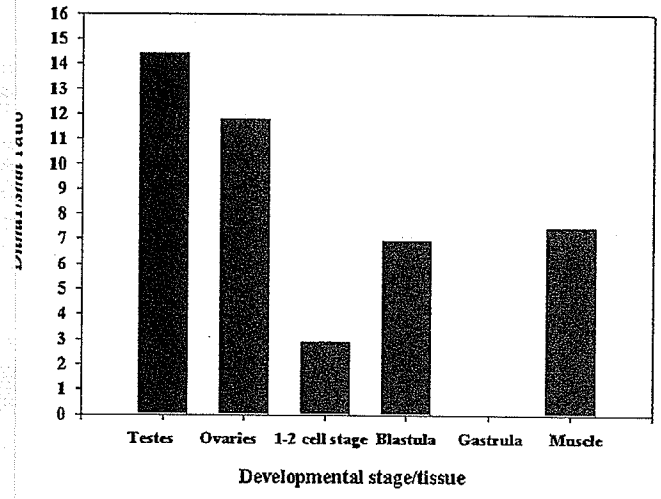
A


Dnmt1/β-actin ratio
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
Testes
Ovaries
1-2 cell stage
Blastula
Gastrula
Muscle
Developmental stage/tissue

C


Dnmt1/max ratio
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
Testes
Ovaries
1-2 cell stage
Blastula
Gastrula
Developmental stage/tissue

B


0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
Testes
Ovaries
1-2 cell stage
Blastula
Gastrula
Muscle
Developmental stage/tissue

D

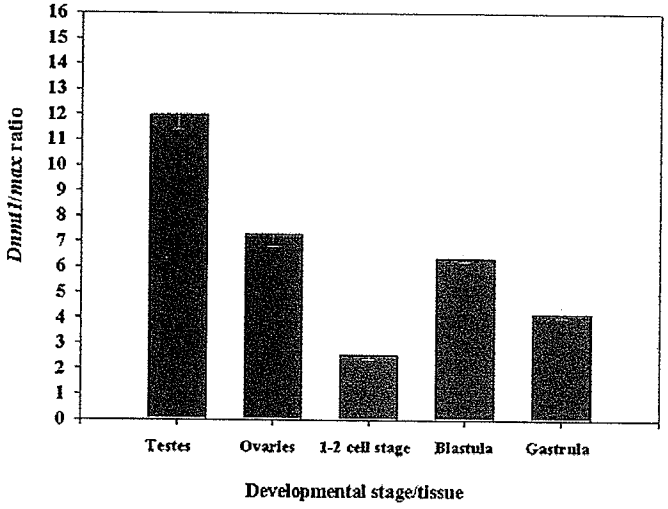
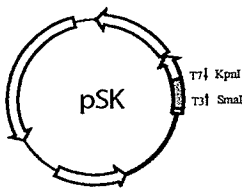

Dnmt1/max ratio
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
Testes
Ovaries
1-2 cell stage
Blastula
Gastrula
Developmental stage/tissue

Figure 15. An overview of *in situ* hybridization analysis strategy.

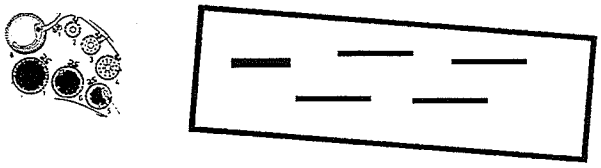


Linearization of plasmid

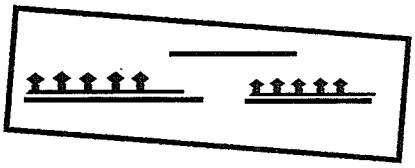
Sense and antisense MTase RNA synthesis

DIG-UTP
+ T7 or T3 RNA polymerase

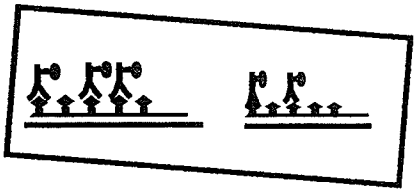
DIG-labeled MTase probe



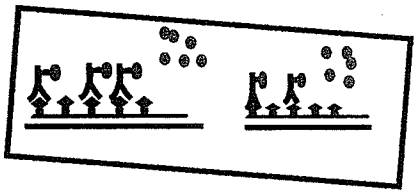
Immobilized target nucleic acid
(whole mounts and tissue sections)



Hybridization of DIG-labeled probe

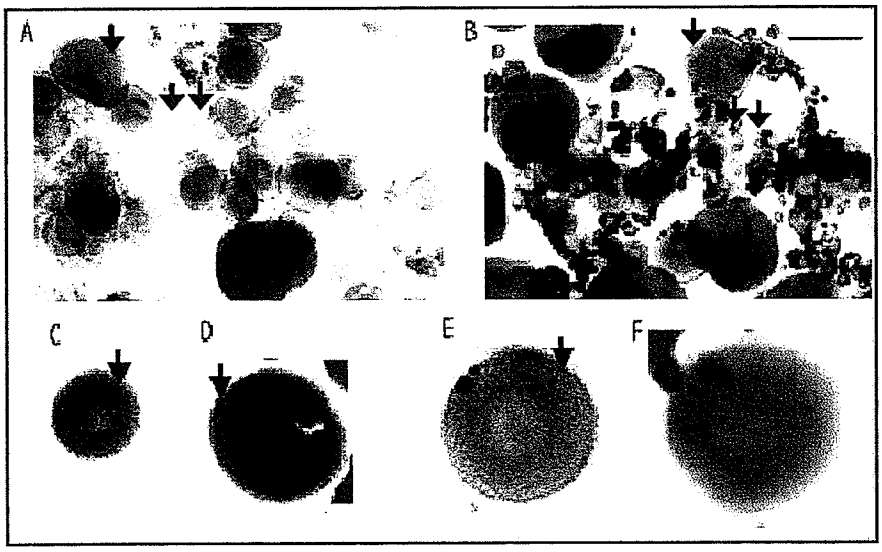


Binding of anti-DIG-AP antibody conjugates



Colorimetric detection by addition of substrates
NBT/X-phosphate

Figure 16. Expression of *dnmt-1* in zebrafish ovaries and 2-cell stage embryos. Zebrafish *dnmt-1* sense (A and G) and antisense (B-F and H) RNA probes were used in whole-mount in situ hybridizations to ovarian follicles at different stages of maturity and 2-cell stage embryos. The hybridized product was visualized using alkaline phosphatase-conjugated antibodies and the appropriate substrates. (B) An ovarian whole-mount with clear purple precipitate primarily in the early stages of oogenesis (*double arrows*) and no detectable precipitate visible in the mature ones (*single arrow*); the control (A) is devoid of purple precipitate. C-F Oocytes at increasing stages of maturity at higher magnification. The resultant purple precipitate is only evident in the early stages of oogenesis (C-E; *arrows*) while there is no detectable message in the mature oocyte (F). *Scale bar* in B represents 500 μm. (H) two-cell stage embryos not showing any purple precipitate.


A
B
C
D
E
F

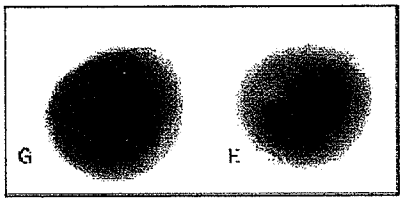
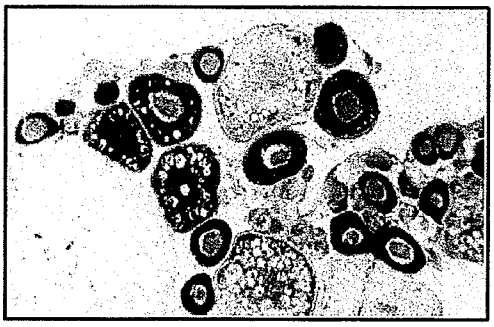

G

Figure 17. Expression of *dnmt-1* in zebrafish ovarian sections. Zebrafish *dnmt-1* antisense (**A**) and sense (**B**) RNA probes were used in ovarian tissue section *in situ* hybridization. The hybridized product was visualized using alkaline phosphatase-conjugated antibodies and the appropriate substrates. **(A)** An ovarian section with clear purple precipitate primarily in the early stages of oogenesis

A



B

Figure 18. A representative example of the standard curve for the Bio-Rad Protein Assay, bovine serum albumin (BSA).

Protein conc.	Absorption
0.0	0.0
0.2	0.277
0.4	0.478
0.6	0.65
0.8	0.768
1.0	1.023

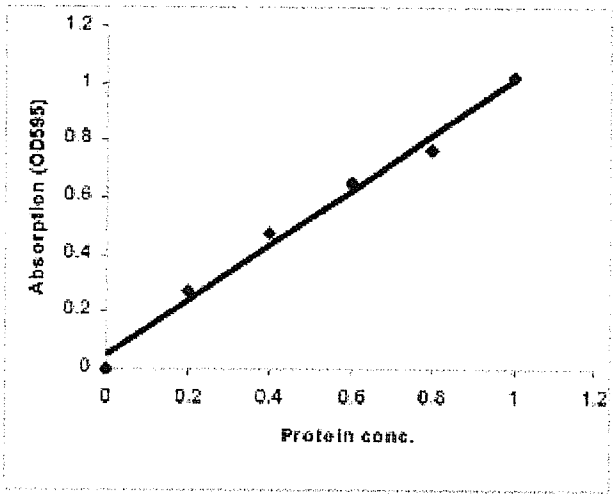


Figure 19. Developmental changes in DNA methyltransferase activity in mature oocyte-depleted ovaries, mature unfertilized oocytes (UO), pooled 1-2 cell-stage embryos (0.5 h), early (2.2 h) and late (3.5 h) blastula, and gastrula (6 h) stages and somatic tissue (muscle). Cellular proteins (20 ug) were incubated with poly(dI-dC)-poly(dI-dC) substrate and ^{3}H-SAM in duplicate reactions and then counted in a scintillation counter. Incubation of cell lysates in the absence of DNA substrate served as a background control and was subtracted from each point. Enzyme activity is expressed in terms of tritium incorporation (*DPM*)/20 μg cellular protein. DPMs were automatically measured by a specific DPM Program as a measure of the absolute activity within the sample. Error bars shown are calculated standard error of the mean.

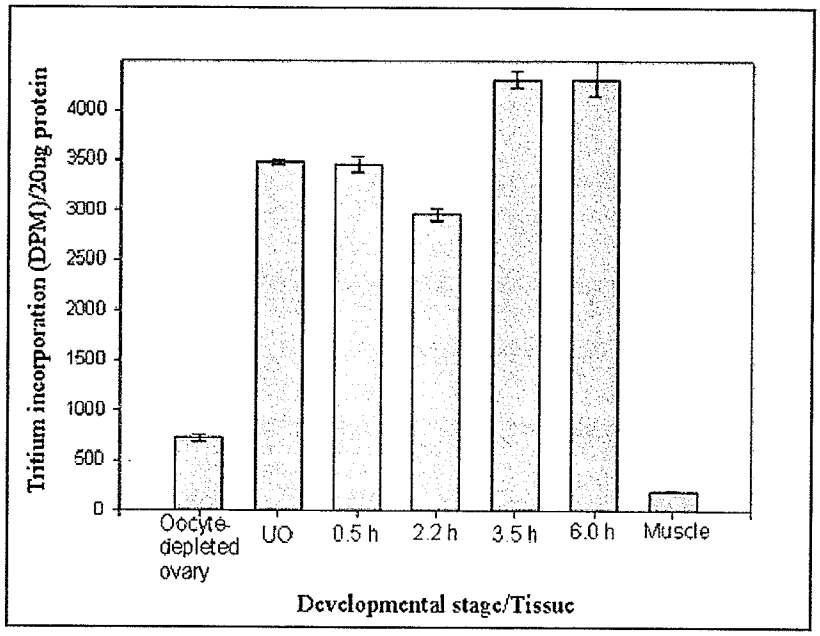
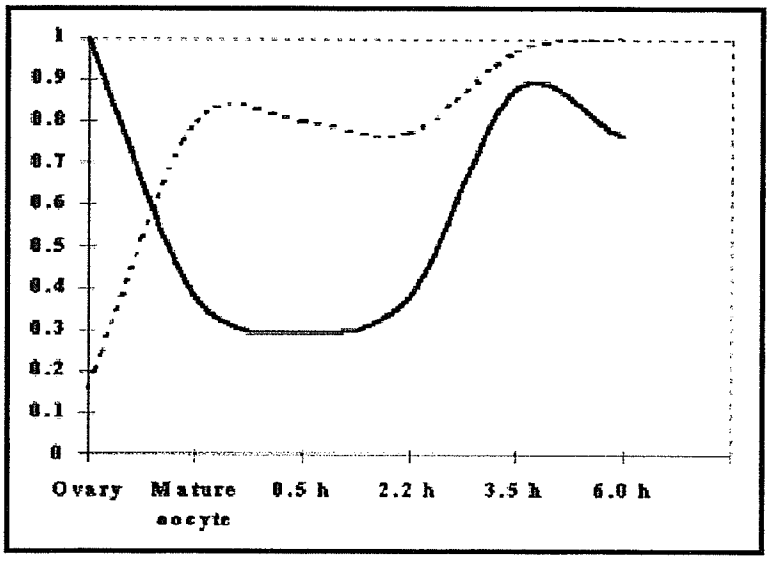

Tritium incorporation (DPM)/20ug protein
4000
3500
3000
2500
2000
1500
1000
500
0
Oocyte-depleted ovary
UO
0.5 h
2.2 h
3.5 h
6.0 h
Muscle
Developmental stage/Tissue

Figure 20. A composite graphic representation of the correlation between the relative changes in *dnmt-1* mRNA and dnmt-1 enzyme activity during oogenesis and early development. The highest mRNA and activity levels were considered unity. Levels of mRNA and activity for all other stages were represented relative to this value. The *solid line* represents mRNA levels while the *dotted one* represents the enzyme activity.


1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Ovary
Mature oocyte
0.5 h
2.2 h
3.5 h
6.0 h

Chapter Three

Elucidation of the Dynamics of Genome-Wide DNA Methylation Changes in Germ Cells, in Somatic Tissues and During Early Development

Abstract

A cycle of stage- and tissue-specific global demethylation and remethylation occurs in the germ cells and during early development of the zebrafish. The sperm genome is heavily methylated as compared with that of the unfertilized oocyte. Following fertilization, the diploid genome of the early zebrafish embryo undergoes a wave of pronounced demethylation continuing through to the early blastula (2.2 hrs) stage embryos. By late blastula (4.0 hrs) stage, the global methylation levels commence to rise approaching the sperm methylation levels by the gastrula (6.0 hrs) stage. These changes closely mimic those found during the mouse preimplantation development (Monk *et al.*, 1987; Howlett and Reik, 1991; Kafri *et al.*, 1992; Santos *et al.*, 2002). The conservation of these dynamic changes in global genome methylation among vertebrates implies functional significance of these changes in the early development of a complex vertebrate. However the exact role of these temporal and quantitative changes of methylation remain unclear.

Introduction: Chapter Three

Cytosine residues in the CpG islands of the vertebrate genome are specifically targeted and methylated by a group of DNA cytosine-5 methyltransferases (Bestor, 2000). This methylation leads to transcriptional repression and has been shown to be essential for normal development of the mouse (Li *et al.,* 1992). Both gametogenesis and early development of the mouse were found to be associated with global DNA methylation changes (Monk *et al.*, 1987; Howlett and Reik, 1991). Genome-wide loss of DNA methylation was observed during early mouse development reaching a low level during the blastocyst stage but by the time of implantation, DNA methylation was restored (Monk *et al.*, 1987). Recently, using a sensitive immunofluorescence assay with a well characterized antibody to 5-methyl cytosine, Santos *et al.* (2002) presented the first detailed description of the cycle of methylation reprogramming during preimplantation mouse development.

The earliest phase of methylation reprogramming occurs during gametogenesis. Methylation was found to be confined to the paternal pronucleus after fertilization and was noted to undergo rapid active demethylation (Mayer *et al.*, 2000; Santos *et al.*, 2002). While the oocyte genome undergoes passive demethylation, the paternal genome was selectively and actively demethylated shortly after sperm decondensation. Very low genomic DNA methylation remained until the morula stage. In contrast to the earlier report by Monk *et al.* (1987) that examined DNA extracted from whole blastocysts, Santos *et al.* (2002) found that *de novo* methylation occurred by the blastocyst stage but was confined only to the inner cell mass, while the trophectoderm significantly lacked methylation. This early *de novo* methylation of the inner cell mass is critical for early development as demonstrated by dnmt3a and dnmt3b deficient mice in which there was abnormal development of all embryonic lineages, but normal development of extraembryonic

tissues (Okano *et al.*, 1999). During the period of hypomethylation, several single-copy genes, repetitive elements and transgenes have been found to lose their methylation (Razin and Shemer, 1995). Remarkably, some imprinted regions remain highly methylated during this stage of global demethylation (Tremblay *et al.*, 1997).

The functional significance of the hypomethylation occurring prior to implantation is unclear. However it has been suggested that it plays a role in resetting the gametic methylation patterns to reprogram the genome for the next round of somatic development (Monk *et al*, 1987; Razin and Shemer, 1995) as well as playing a role in imprinting (Reik and Walter, 2001). If this hypomethylation wave is essential for gametic methylation resetting in the early embryo, one might expect conservation of this phenomenon among vertebrates. At the present time, there is no conclusive evidence for the presence or absence of this wave of demethylation in non-mammalian vertebrates.

In the zebrafish, one of the few other vertebrates in which DNA methylation has been examined, the occurrence of global DNA methylation changes, or the lack thereof, is still a matter of debate (Macleod *et al.*, 1999; Martin *et al.*, 1999). If DNA methylation contributes to the control of normal embryogenesis of the zebrafish, then developmentally regulated changes in DNA methylation, genome-wide and/or locus specific, should occur. I set forth to identify the presence or absence of genome-wide and locus-specific DNA methylation changes during early development, in the gonads and in somatic tissues in the zebrafish.

Materials and Methods: Chapter Three

I. Zebrafish care and maintenance

Adult zebrafish maintenance and breeding is carried out as described in Chapter two.

II. Embryos, tissues and germ-cells collection

Collection of desired embryonic stages, tissues and germ-cells was conducted as described in chapter two.

III. Choice of developmental stages and tissues

Embryos at various developmental stages, based on developmental time and morphological categorization (Appendix 3), were used. Embryos were pooled to obtain sufficient volumes for DNA extraction. Different developmental stages were chosen for the analysis of genome-wide and locus specific methylation changes based on our current understanding of the cycle of DNA methylation in mice, keeping in mind some of the cardinal differences between the zebrafish and the mouse model system such as the difference in speed of development.

The embryonic stages tested included 1-2 cell stage embryos, early blastula (2.2 hrs), late blastula (4.5 hrs) and gastrula stage (6.0 hrs) embryos. When possible, developmental stages were tested at half hourly developmental time increments starting at 1-2 cell stage up to 6.0 hrs stage.

Mouse germ cells are characterized by genome-wide DNA methylation changes (Monk *et al.*, 1987). To look for similar changes in zebrafish, sperm and eggs were obtained from breeding zebrafish. Due to the limited number of breeding males and females and the technical difficulty in getting pure sperm and unfertilized oocytes, whole ovaries and whole testes of breeding females and males respectively were tested at times as an approximate reflection of the egg and sperm genome methylation status. DNA from somatic tissues and from whole male and whole female fish were also dissected and included in the analyses.

IV. Isolation and preparation of high molecular weight genomic DNA

Embryos, tissues and germ cells were ground into powder using mortar and pestle. The powder was added to 15 ml tubes containing genomic DNA extraction buffer (10 volumes per volume of ground material) [10 mM Tris pH8; 100 mM EDTA pH8; 0.5% SDS; 200 ug/ml Proteinase K]. After 3 hours to overnight incubation at 50 °C, the solution was cooled to room temperature and extracted three times with an equal volume of Tris-saturated phenol. After gentle mixing and emulsion formation, the phases were separated by centrifugation at 3000-5000g for 10 minutes. The aqueous phase was then removed carefully using a wide bore pipette. To the aqueous phase removed after the third phenol extraction, an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) was added. After centrifugation, the aqueous phase was transferred into fresh tubes and the DNA was precipitated with NaCl (final concentration 200 mM) and two volumes of ethanol. The DNA was noted to precipitate immediately following gentle swirling of the

tube. The precipitated DNA was then washed twice with 70% and 95% ethanol respectively and allowed to air dry for 5-10 minutes.

Once dry, the DNA pellet was resuspended in an appropriate volume of 10 mM Tris pH8, 5 mM EDTA and 100 ug/ml (DNAse free) RNAse A and incubated at 37 °C for 30 minutes. The DNA solution was extracted once with phenol:chloroform:isoamyl alcohol as described above and the aqueous phase was transferred to a fresh tube. The DNA was reprecipitated by the addition of an equal volume of isopropyl alcohol and 0.5 M ammonium acetate. The precipitated DNA was washed with 70% and 95% ethanol and allowed to air dry. The air-dried DNA pellet was resuspended in low TE buffer [10 mM Tris pH8, 0.1 mM EDTA]. Appropriately diluted samples were used for absorbance readings on a Spectronic 601 (Milton Roy) spectrophotometer at 260 and 280 nm. The concentration was calculated in ug/ul and an estimation of DNA purity was determined by the 260/280 ratio.

All mixing and resuspension steps involved in DNA extraction were carried out very gently. Vortexing was avoided throughout the entire procedure. DNA was stored at 4 °C to avoid DNA degradation due to repeated freezing and thawing. Quality of DNA was determined by agarose gel electrophoresis and ethidium bromide staining.

V. Restriction endonuclease hydrolysis of DNA using methylation sensitive enzymes

V.A. *MspI/HpaII* isoschizomeric endonucleases

The differential sensitivity of *MspI* and *HpaII* restriction endonucleases to methylated cytosines was exploited to identify tissue- or temporal-specific differences in methylation patterns (Waalwijk and Flavell, 1978; Fuchs and Blakesley, 1983). To study

the methylation status of a particular genome, the respective DNA sample is digested with the isoschizomeric enzymes *MspI* or *HpaII*, which cut the DNA at the four-base sequence 5`-CCGG-3`. *MspI* cleaves this sequence whether the internal cytosine is methylated or not, whereas *HpaII* will only cut the unmethylated sequence (Waalwijk and Flavell, 1978). An indication of the extent of methylation can therefore be obtained by comparing fragment sizes resulting from *MspI* and *HpaII* restriction digests. Direct examination of fragment size by ethidium bromide staining allows detection of relative differences in degrees of methylation (section V.A). However, observation of such differences can be achieved with much higher sensitivity by end-labeling the resulting DNA fragments (section V.B). In addition, the methylation status of certain repetitive DNA elements may be a reflection of the overall genome-wide methylation changes. The methylation status of repeated sequences can be tested with the use of repetitive elements as probes of blots of *MspI* and *HpaII* digested DNAs (section V.C).

V.B. Restriction endonuclease digestion of DNA samples

DNA samples were prepared as described in section IV from different developmental stages and tissues including germ cells. DNA sample quality was examined by agarose gel electrophoresis, prior to restriction digestion, and subsequent ethidium bromide staining. DNA was cleaved for 2-4 hrs with either *HpaII* or *MspI* restriction endonucleases. One microgram and ten micrograms of DNA were digested for use in end labeling and Southern blot analysis respectively.

All DNA samples were hydrolyzed in a total final reaction volume of 300 ul. The desired DNA concentration was prepared by the addition of water to a final volume of 150 ul.

The appropriate restriction enzyme buffer (1X final concentration) (GIBCO-BRL), water and enzyme mix, either *MspI* (GIBCO-BRL,Cat. No.15419-021) or *HpaII* (GIBCO-BRL, Cat. No.15209-018) was made up separately in a large volume at 2x concentration and then 150 ul added to the diluted DNA mixture.

To test for completion of digestion, a control plasmid (pBluescript KS) DNA was added to a sample of the experimental digest. After setting up digests as above, a volume from each digest corresponding to 0.5 ug of sample DNA was added to 0.5 ug of control plasmid DNA. One control for each of the digest reactions was set up. In a separate tube, another restriction digestion was set up for the control plasmid alone to obtain the expected restriction digestion pattern of the control plasmid. Also separately, the same reaction was set up with no enzyme added to indicate the pattern of the uncut plasmid.

All the reactions mentioned above were incubated at 37 °C for 2-4 hours. After the incubation was complete, the digests were removed from the incubator and placed on ice. Control digests were loaded on agarose gels and the restriction digestion was examined after staining the gel with ethidium bromide. Restriction digestion was considered to be complete when the internal control digest gave a digest pattern identical to the pattern obtained with control plasmid and the same enzyme alone. If the control plasmid was cut to completion, the original digested DNA was precipitated by adding ammonium acetate (0.3 M) and an equal volume of isopropanol. Samples were placed at –20 °C for at least 30 minutes after which they were precipitated at 13000x g. The DNA pellet was then washed with 70% and 95% ethanol respectively and air dried. The dry DNA pellet was redissolved in an appropriate volume of low TE buffer (10 mM Tris pH8.0, 0.1 mM EDTA).

All experiments were repeated several times on independently extracted DNA samples from batches of independently procured embryos, germ cells and somatic tissues and the densitometric data were calculated from several independent gels/blots.

VI. Detection of genome-wide DNA methylation status of zebrafish germ cells, early embryogenesis and somatic tissues

V.A. *MspI/HpaII* ethidium bromide assay

V.A.i. Strategy

DNA from desired developmental stages, germ cells, gonads and somatic tissues

⇩

HpaII or *MspI* endonuclease digestion

⇩

Fractionate DNA on agarose gels

⇩

Ethidium bromide stain the DNA

⇩

Visualize under UV light

⇩

Capture image digitally

⇩

Densitometric analysis

V.A.ii. The assay

MspI or *HpaII* hydrolysed DNA samples (section V.B) were subjected to fractionation on 0.8% agarose gels. Ten micrograms of each sample were loaded and the gel was run overnight at

30 V. Once fractionation was complete, the gel was placed in 1 litre of H_2O in a plastic tray with 100 ul of ethidium bromide solution (10 mg/ml). After staining for one hour, the gel was destained in water for one to two hours. The DNA was visualized on either a UV transilluminator box or using a Flurochem system. Images were captured digitally and were analyzed using Scion Image software as described in chapter two.

V. B. *MspI/HpaII* end labeling assay

V.B.i. Strategy

DNA from different developmental stages, germ-cells, gonads and somatic tissues

⇩

HpaII or *MspI* endonuclease digestion

⇩

End-labeling of digested DNA

⇩

Fractionation of end-labeled DNA on agarose gels

⇩

Transfer of DNA to nylon membranes

⇩

Autoradiography

⇩

Densitometric analysis

V.B.ii. End labeling of digested DNA samples

MspI or *HpaII* digested DNA samples (~1 ug) were end labeled using 2 U of Klenow fragment of DNA polymerase I (Invitrogen, Cat. No. 18012-021) and 5 uCi of [α-^{32}P]dCTP (NEN Life Sciences Products, Inc. Cat. No.BLU513H) in 10 mM Tris-HCl (pH 8.0), 10 mM $MgCl_2$ for 1 hr at room temperature (Sambrook *et al.*, 1989). Unincorporated isotope was removed by ethanol precipitation of the DNA with ammonium acetate and sodium acetate. Uncut (mock digested) DNA was end-labeled in each experiment to ensure the high molecular weight of the starting DNA preparations and preclude the presence of DNA degradation. A 1 kb plus DNA ladder (GIBCO-BRL, Cat. No.10787-018) was also end labeled to serve as a marker.

V.B.iii. Fractionation of end labeled DNA and DNA blotting

The end labeled DNA was subjected to fractionation by electrophoresis for 16 hrs at 30 V on 0.8% agarose gels. After completion of fractionation, the gels were placed in denaturation solution (2.4 M NaCl, 0.8 M NaOH) for 40 minutes to assist in complete transfer of the high molecular weight DNA species. Once denaturation was complete, the gel was placed in the transfer apparatus and DNA transfer to nylon membranes (Boehringer Mannheim) was allowed to proceed overnight as described in chapter one. Completeness of the transfer was assured since only background counts were detected in the gels following blotting as well as the absence of any radioactive signal resulting from the exposure of the transferred gel to an X-ray film.

V.B.iv. Autoradiography and densitometric analysis

The DNA fragment size distribution was detected by processing the nylon membranes in a phosphoimager or by exposure to X-ray films. Densitometric analysis was then performed on the digitally acquired images using Scion Image software written by Wayne Rasband (National Institute of Health, Bethesda, MD, USA) as described in Chapter 2. Densitometric scans were performed in regions corresponding to mid- and low-molecular weight DNA restriction fragments. The regions of high and very low molecular weight DNA restriction fragments were avoided due to the saturation of the signals in these regions. Mean density values of several independent blots were used to produce a comparative density ratio between the corresponding *HpaII* and *MspI* lanes. A high ratio indicates low levels of DNA methylation due to the similarity between the densities of *HpaII* and *MspI* lanes. A low ratio indicates higher levels of DNA methylation. Mean ratios are indicated as the average of four independent gels ± SE.

V.C. *MspI/HpaII* Southern blot analysis

V.C.i. Strategy

DNA from different developmental stages, germ-cells, gonads and somatic tissues

⇩

HpaII or *MspI* endonuclease restriction digestion

⇩

Fractionation of the DNA on agarose gels

⇩

Transfer of the DNA to nylon membranes

⇩

Use a repetitive element as a hybridization probe

⇩

Autoradiography

⇩

Densitometric analysis

V.C.ii. Southern blotting

Genomic DNA extracted from desired developmental stages, gonads and somatic tissues was subjected to restriction endonuclease digestion by either *MspI* or *HpaII* as described above. Ten micrograms of each DNA sample were loaded and fractionated by electrophoresis on 0.8%

agarose gels overnight at 30 V. Once fractionation was complete, the gels were stained in a solution of ethidium bromide (1 ug/ml) for one hour, destained for one hour in H_2O and then visualized on a UV transilluminator and photographed. The gels were subjected to denaturation for 40 minutes (2.4 M NaCl, 0.8 M NaOH) with gentle shaking. The denatured gel was placed in the transfer apparatus filled with 20X SSC transfer buffer and then framed with plastic film. Prewetted nylon membrane was placed directly on top of the gel and covered with two pieces of filter paper followed by 4-6 inches of paper toweling. DNA transfer was allowed to take place overnight. In the morning, the membrane was removed from the transfer apparatus, labeled and dated, rinsed in ddH_2O and then baked in an 80 °C oven for two hours.

V.C.iii. Probes used for hybridization

Three different repetitive elements were used as hybridization probes to detect any global change in DNA methylation. The DANA interspersed repetitive sequence, which represents about 10% of the zebrafish genome and is found dispersed throughout the genome (Izsvak *et al.*, 1996), was used as a probe to detect any changes in the overall size of the *HpaII* digested genomic DNA. This would reflect the overall methylation status of the genome of each tested developmental stage or tissue. Two [A+T]-rich satellite DNA elements were also used as probes, type Ia (sr1) and Ib (sr14) elements, comprising approximately 8% and 0.2% of the zebrafish genome (Ekker *et al.*, 1992; He *et al.*, 1992). The zebrafish type I elements have been localized to centromeric regions of zebrafish chromosomes by fluorescence in situ hybridization (Ekker *et al.*, 1996).

Probe preparation

The DANA repetitive element was prepared by amplification of genomic DNA using primer sequences DANA-1 and DANA-2 (Appendix 1). In a 0.5 ml microfuge tube, 5 ul of 10X PCR buffer, 1 ul of 10 mM dNTP mix, 1.5 ul of 50 mM $MgCl_2$, 5 ul of each DANA-1 and DANA-2 primers, 2 ul of whole zebrafish template DNA, 0.5 ul of *Taq* DNA polymerase [5 U/ul] was added to 30 ul of ddH_2O. The 50 ul reaction was placed in a Minicycler [MJ Research] for an initial denaturation at 94 °C for five minutes followed by 30 cycles of 94 °C for 30 seconds, 60 °C for 30 seconds, 72 °C for 60 seconds. Once the cycles were complete a 4 °C dwell cycle was added to prevent degradation of the product. The PCR product was fractionated on a 1.5% agarose gel and visualized by ethidium bromide staining.

The repetitive elements sr1 and sr14 (a generous gift of Dr. M. Ekker; Loeb Institute Ottawa Civic Hospital) were prepared as described in Chapter one, Section I.8

V.C.iv. Labeling of probes, prehybridization, hybridization, washing and autoradiography

Labeling of the probes, prehybridization, hybridization, washing and autoradiography were performed as described in Chapter one, Section I.9.

VI. Locus-specific DNA methylation analysis

To investigate the presence of single-copy gene methylation changes and its potential correlation with the genome-wide methylation changes, *MspI/HpaII* digested DNA from different developmental stages, germ-cells and somatic tissues were surveyed using locus specific probes to determine their methylation status. The zebrafish *ck* (Harder and McGowan, 2001) gene cloned in our lab shows a developmentally regulated expression pattern. It becomes expressed at significant levels with the elaboration of somites, but it is not expressed prior to that stage. Hence along with the early developmental stages, early (12 hrs) and late (30 hrs) (Appendix 3) somite stage embryos were included in the analysis to look for any changes in the methylation status of this gene during these stages.

VI.A. *MspI/HpaII* Southern blot analysis: Strategy

DNA from different developmental stages, germ-cells, gonads and somatic tissues

⇩

HpaII or *MspI* endonuclease restriction digestion

⇩

Fractionation of the DNA on agarose gels

⇩

Transfer the DNA to nylon membranes

⇩

Use *ck* as a hybridization probe

⇩

Autoradiography

IV.B. *MspI/HpaII* Southern blot analysis

Southern blotting, probe preparation, prehybridization, hybridization, washing and autoradiography were conducted as presented in the previous section.

Results: Chapter Three

I. High molecular weight genomic DNA extraction and restriction enzyme digestion

The integrity of each high molecular weight DNA sample extracted was tested by fractionation on 0.8% agarose gels and staining with ethidium bromide (figure 21). The single high molecular-weight band present in each sample is indicative of intact DNA.

For each DNA sample subjected to restriction endonuclease digestion, completion of restriction digestion was determined as described in materials and methods. A representative example of a control restriction digest is presented in figure 22.

II. Temporal and Quantitative Genome-Wide DNA Methylation Dynamics During Early Development and in the Germ Cells

II. A. *MspI/HpaII* ethidium bromide assay

DNA samples from sperm, eggs, pooled embryos of different stages and skeletal muscle were digested with either the restriction endonuclease enzyme *HpaII* or its isoschizomer *MspI*. By digesting samples with both enzymes, the relative degree of DNA methylation can be compared between samples by obtaining a ratio between the densities of *HpaII*-and the respective *MspI*-digested DNAs lanes.

Representative examples of *MspI/HpaII* ethidium bromide assays are shown in figure 23. All gels were scanned between 7.0 kb and 2.0 kb for densitometric analysis to avoid any bias due to the over saturation of the high and low molecular weight DNA species. The relative degrees of DNA methylation was compared between DNA samples

by estimating the ratio between the lane density of the *HpaII*- and the corresponding *MspI*-digested DNAs (Figure 23 and Table 10).

Table 10. Quantified intensity of *HpaII* and *MspI* ethidium bromide stained lanes between 7 and 2 kb. Data were calculated from two independent gels.

Stage/tissue	Intensity of HpaII lanes	Intensity of MspI lanes	HpaII/MspI ratio	Relative levels of methylation (1-ratio)
1-2 cell stage	106	117	0.91	0.09
1-2 cell stage	100	113	0.885	0.115
2.2 hrs	78	95	0.821	0.179
2.2 hrs	76	95	0.80	0.2
4.0 hrs	105	224	0.469	0.531
4.0 hrs	111	245	0.453	0.547
6.0 hrs	93	245	0.380	0.62
6.0 hrs	100	247	0.40	0.6
Unfertilized oocytes	82	107	0.766	0.234
Unfertilized oocytes	90	103	0.874	0.126
Sperm	95	280	0.339	0.661
Sperm	101	279	0.362	0.638
Muscle	82	187	0.438	0.562
Muscle	81	182	0.445	0.555

Mature sperm DNA is hypermethylated relative to all other developmental stages examined while oocyte DNA is relatively hypomethylated as were the methylation levels of the early cleavage embryonic genome. The embryonic genome regained high methylation levels approaching those of somatic tissues by the gastrula (6.0 hrs) stage (table 10 and figure 29-A).

II.B. *MspI/HpaII* end labeling assay

DNA samples isolated from mature sperm, unfertilized oocytes, 1-2 cell-stage, early and late blastula-stage (2.2-5.0 hrs), gastrula-stage (6.0 hrs) (Appendix 3) embryos and skeletal muscle were subjected to restriction endonuclease digestion with the isoschizomeric enzymes *MspI* and *HpaII* exploiting their differential methylation sensitivity.

Figure 24 shows a representative example of an autoradiograph of end labeled-mock digested DNA preparations that were used in these experiments. The autoradiograph shows the integrity of the high molecular weight of the DNA samples used. Very minimal degradation indicated by the very low molecular weight fragments was noted for some of the samples at the bottom of the lanes.

Figure 25 shows a representative example of an experiment with end labeled embryonic, germ cell and somatic DNA following *MspI* or *HpaII* digestion. The degree of methylation of the total DNA is observed by comparison of *MspI* and *HpaII* digests. A densitometric ratio between the respective *MspI* and *HpaII* lanes gives an indication of the relative degree of methylation of the DNA of the relevant tissue or developmental stage examined. Skewing towards the higher molecular weight of the label in the *HpaII* lanes indicates heavier DNA methylation status. The intense signal at the bottom of all lanes results from the labeling of the high proportion of fragments of the clustered CpG islands which are mostly unmethylated (Bird *et al.*, 1985).

A measure of comparison of fragment size distribution in the different DNA samples using densitometric tracings was obtained from the ratio of fragments between 7 and 2 kb. Using this analysis the sperm DNA was found to be heavily methylated relative to the unfertilized oocyte and other developmental stages examined (figure 25 and 29-B and tables 11

and 12). Globally the embryonic genome is hypomethylated at early cleavage compared to testicular DNA. The methylation levels increased considerably by the gastrula stage (6 hrs) (figures 25 and 29-B and table 11 and 12). These results were reproducible and were conducted on several independent DNA samples.

Table 11. Quantified intensity of *HpaII* and *MspI* end labeled lanes between 7 and 2 kb of embryonic stages. Data were calculated from four independent blots.

Stage/tissue	Intensity of HpaII lanes	Intensity of MspI lanes	HpaII/MspI ratio	Relative levels of methylation (1-ratio)
1-2 cell stage	66	70	0.943	0.057
1-2 cell stage	81	82	0.988	0.012
1-2 cell stage	183	190	0.963	0.037
1-2 cell stage	94	99	0.949	0.051
2.2 hrs	1973	2653	0.744	0.256
2.2 hrs	824	1137	0.725	0.275
2.2 hrs	134	187	0.720	0.28
2.2 hrs	1310	1815	0.722	0.278
3.0 hrs	2021	3506	0.577	0.423
3.0 hrs	72	126	0.571	0.429
3.0 hrs	129	201	0.642	0.358
3.0 hrs	374	783	0.478	0.522
4.0 hrs	2805	5713	0.491	0.509
4.0 hrs	843	1827	0.461	0.539
4.0 hrs	110	222	0.491	0.509
4.0 hrs	98	213	0.460	0.54
4.5 hrs	92	278	0.331	0.669
4.5 hrs	2222	5467	0.41	0.59
4.5 hrs	98	206	0.476	0.524
4.5 hrs	1513	4066	0.372	0.628
5.0 hrs	1970	4440	0.444	0.556
5.0 hrs	2839	6152	0.461	0.539
5.0 hrs	2010	4790	0.42	0.58
5.0 hrs	116	260	0.446	0.554
6.0 hrs	315	1024	0.31	0.69
6.0 hrs	99	283	0.349	0.651
6.0 hrs	94	265	0.355	0.645
6.0 hrs	88	258	0.341	0.659

Table 12. Quantified intensity of *HpaII* and *MspI* end labeled lanes between 7 and 2 kb of germ-cells and somatic tissues. Data were calculated from three independent blots for the unfertilized oocyte and four independent ones for the sperm and muscle.

Stage/tissue	Intensity of HpaII lanes	Intensity of MspI lanes	HpaII/MspI ratio	Relative levels of methylation (1-ratio)
Unfertilized oocyte	90	113	0.796	0.204
Unfertilized oocyte	629	830	0.758	0.242
Unfertilized oocyte	609	800	0.761	0.239
Sperm	1416	7909	0.179	0.821
Sperm	918	3367	0.273	0.727
Sperm	68	226	0.30	0.70
Sperm	69	335	0.21	0.79
Muscle	104	295	0.353	0.647
Muscle	3038	9111	0.333	0.667
Muscle	1210	3780	0.320	0.68
Muscle	2647	7630	0.35	0.65

II.C. *MspI/HpaII* Southern blot analysis

To analyze the methylation status of different repeated sequences, Southern blots of *HpaII*- and *MspI*-digested DNA samples collected from pooled embryos of different stages, sperm, unfertilized egg or whole ovarian DNA and DNA from skeletal muscle were hybridized with three different probes.

DANA sequences digested with *MspI* produced heterogeneous small fragments and a prominent band at 180 bp (figure 26). Sensitivity of DANA sequences to *HpaII* digestion varied indicating different levels of methylation of the different genomes tested. DANA sequences were resistant to *HpaII* digestion in the sperm DNA implying a high degree of methylation of the sperm genome (table 13). The oocyte genome on the other hand was relatively hypomethylated. The embryonic genome was noted to be hypomethylated at the 1-2 cell stage and the early blastula (2.2 hrs) embryos. The genome remains relatively hypomethylated till the late blastula (4.0 hrs) stage where methylation levels were noticeably higher and by the gastrula stage (6.0 hrs) it regained higher methylation levels approaching those of the sperm genome. These changes are presented in tables 13 and 14 and in figure 29-C.

Table 13. Quantified intensity of *HpaII* and *MspI* DANA probed lanes between 7 and 2 kb of embryonic stages. Data were calculated from three independent blots.

Stage/tissue	Intensity of HpaII lanes	Intensity of MspI lanes	HpaII/MspI ratio	Relative levels of methylation (1-ratio)
1-2 cell stage	99	107	0.93	0.07
1-2 cell stage	1136	124	0.935	0.065
1-2 cell stage	95	88	1.07	0.0
2.2 hrs	71	103	0.689	0.311
2.2 hrs	60	92	0.652	0.348
2.2 hrs	65	93	0.699	0.301
3.0 hrs	35	98	0.357	0.643
3.0 hrs	94	256	0.367	0.633
3.0 hrs	52	120	0.433	0.567
4.0 hrs	37	167	0.22	0.78
4.0 hrs	63	495	0.127	0.873
4.0 hrs	72	441	0.16	0.84
5.0 hrs	43	846	0.0508	0.9492
5.0 hrs	34	669	0.051	0.949
5.0 hrs	271	5365	0.0505	0.9495
6.0 hrs	26	714	0.0364	0.9636
6.0 hrs	35	981	0.038	0.962
6.0 hrs	35	1000	0.035	0.965

Table 14. Quantified intensity of *HpaII* and *MspI* DANA probed lanes between 7 and 2 kb of germ-cells and somatic tissues. Data were calculated from three independent blots.

Stage/tissue	Intensity of HpaII lanes	Intensity of MspI lanes	HpaII/MspI ratio	Relative levels of methylation (1-ratio)
Unfertilized oocyte	166	190	0.874	0.126
Unfertilized oocyte	155	179	0.866	0.134
Unfertilized oocyte	48	55	0.873	0.127
Sperm	43	1256	0.034	0.966
Sperm	27	1072	0.0255	0.9745
Sperm	35	1100	0.0318	0.9682
Skeletal muscle	513	7429	0.069	0.931
Skeletal muscle	106	1449	0.073	0.927
Skeletal muscle	170	2383	0.071	0.929

The two other repetitive elements used as probes in the *MspI/HpaII* Southern blot analysis were the [A+T]-rich pericentromeric satellite DNA elements, sr1 (type Ia) and sr14 (type Ib). The methylation status of these elements was analyzed by DNA hybridization analyses of *HpaII*- and *MspI*-digested DNA extracted from breeding adult testes and ovaries, 1-2 cell stage, 2.2-hr, 3.5-hr and 6.0-hr embryos. Hybridization with the sr1 repetitive sequence produced a large number of heterogeneous fragments giving similar smear patterns (figure 27) for both the *HpaII* and *MspI* lanes. Densitometric analysis of the resulting blots revealed that the smear pattern for each sample of *HpaII*-

digested DNA was not significantly different from its corresponding *MspI*-digested DNA (figure 27). The hybridization pattern obtained using the sr14 sequence as a probe contained a single 5 kb band. This band was similarly observed in the *HpaII* and *MspI* lanes of all the different DNA samples used (figure 28). Both the sr1 and the sr14 elements are pericentromeric (Ekker *et al.*, 1996) [A+T]-rich satellite DNA elements (Ekker *et al.*, 1992; He *et al.*, 1992). The lack of discernable differences between the *HpaII*- and *MspI*-digested DNA samples could be explained by the lack of *HpaII* and *MspI* restriction sites as expected from their [A-T]-rich sequences. In addition, these satellite sequence may reside in a hypomethylated area of the genome and would consequently show no difference in the restriction digestion capability of *HpaII* and *MspI*.

III. Locus specific methylation changes

Methylation-sensitive restriction endonucleases and DNA hybridization analyses of 1-2 cell stage, early (2.2 hrs) blastula, 3.0 hrs, 4.0 hrs, 5.0 hrs, gastrula (6.0 hrs), early somite (12 hrs), late somite (30 hrs) stage embryos, sperm and skeletal muscle genomic DNA were used to look for any differences in the methylation status of the ck gene (Genbank accession number AF259079).

One major band in all the *MspI* digested DNA-lanes of all stages tested (figure 30) was observed. In the *HpaII* lanes there was a single distinct high-molecular weight band in the 4-, 5- and 6-hr stage embryos and the sperm implying heavy methylation of the gene. The HpaII digested DNA in the 12- and 30-hr somite stage embryo lanes appears as a smear at the top of the lane. In the 30-hr stage a second distinct band at

about 12 kb was noted. These findings are suggestive of a lower degree methylation of the gene during these stages. The signal in the 1-2 cell and early blastula (2.2 hrs) embryo lanes was very weak due to lower DNA content in these lanes.

Discussion: Chapter Three

In this chapter, I provide the first detailed dissection of the cycle of methylation reprogramming in the zebrafish gametes and early embryogenesis. To fully characterize the overall genomic DNA methylation changes, I used several independent methods including a very sensitive assay for the quantitation of DNA methylation, the end-labeling assay (Monk *et al.*, 1987). In addition to its sensitivity, the end-labeling assay allows the analysis of DNA methylation in tissues where biological material is limiting. Changes in DNA methylation patterns have also been observed at various repetitive sequences in the mouse (Yoder *et al.*, 1997). In this investigation, I used a probe derived from the short interspersed repeat element (SINE), DANA, which makes up approximately 10% of the zebrafish genome.

Analyses of genome-wide DNA methylation changes revealed that the sperm genome was hypermethylated relative to the unfertilized oocyte. Monk *et al.* (1987) observed similar DNA methylation changes in the mouse sperm and oocyte genomes. Martin *et al.* (1999) and Macleod *et al.* (1999) have noted that zebrafish sperm genome is hypermethylated. Neither study however examined the oocyte genome methylation status.

The one- to two-cell and early blastula (2.2 hrs) stage zebrafish embryo genomes were found to be hypomethylated relative to the late blastula- (4.0-4.5 hrs) and the gastrula-stage (6.0 hrs) embryos. Macleod *et al.* (1999) reported the absence of genome wide DNA methylation changes in zebrafish early embryogenesis. However, in their investigation, Macleod *et al.* (1999) did not assay the methylation status of early cleavage embryos. Although Macleod *et al.* (1999) included early blastula stage (2.2 hrs)

embryos, their methods of detection of genome-wide DNA methylation differed from ours.

Santos *et al.* (2002) reported that the mouse paternal genome was selectively and actively demethylated immediately after sperm decondensation. The nature of this *in vivo* demethylating activity is not yet known however. Interestingly, Collas, (1998) investigated the methylation and demethylation of a transgene during zebrafish early development. This work suggested the presence of a demethylating activity in the zebrafish oocyte because methylated transgenes microinjected into the zebrafish oocytes were found to undergo active demethylation shortly after microinjection. The mechanism producing rapid active demethylation of exogenous sequences may also lead to the active demethylation of the zebrafish sperm genome shortly after fertilization producing the observed degree of hypomethylation in 1-2 cell and early (2.2 hrs) blastula embryo genome similar to the mouse.

The developmental survey of Martin *et al.* (1999) included late blastula (4.0 hrs), gastrula (6.0 hrs), somite stage (16 hrs) and tail (24 hrs) stage embryo genomes. Their investigation reported the presence of genome-wide DNA methylation changes during zebrafish development. I have confirmed their results and extended the investigation to included the germ-cells and all the critical stages of zebrafish early development and the results presented in this report demonstrate that in the zebrafish there is global genomic undermethylation of the oocyte, relative undermethylation of the early-cleavage and early blastula (2.2 hrs) embryo genomes and the presence of significant *de novo* methylation by the gastrula (6.0 hrs) stage embryos.

The significant degree of methylation observed in zebrafish late blastula (4.0 hrs) contrasts with the early finding that the mouse genome underwent pronounced overall demethylation reaching its lowest levels in the blastocyst stage (Monk *et al.*, 1987). However Santos *et al.* (2002), using a sensitive immunofluorescence assay with a well characterized antibody to 5-methyl cytosine, undertook a detailed dissection of the cycle of demethylation of the mouse preimplantation embryo and revealed that significant *de novo* methylation occurs by the blastocyst stage but is restricted to the inner cell mass, while the trophectoderm has virtually no methylation. Thus, our finding of significant methylation during the zebrafish late blastula (4.0 hrs) stage can be explained by lineage-based methylation differences. Since the zebrafish embryo lacks extraembryonic lineage cells, the dilution effect of the extraembryonic lineage seen in the mouse would not be observed in these fish.

Here we have shown significant differences in genome-wide DNA methylation of the zebrafish early embryos and germ cells. The sperm genome is heavily methylated as compared with that of the unfertilized oocyte. Following fertilization, the diploid genome of the early zebrafish embryo undergoes a wave of pronounced demethylation continuing through to the early blastula (2.2 hrs) stage. By late blastula (4.0 hrs) stage, the global methylation levels commence to rise approaching the sperm methylation levels by the gastrula (6.0 hrs) stage. These changes closely mimic those found during the mouse preimplantation development recently described by Santos *et al.* (2002).

Our investigation shows the first clear evidence for the presence of the cycle of demethylation in a non-mammalian vertebrate. The conservation of this wave implies that it is a necessary part of normal development of a complex vertebrate. The functional

importance of the loss of methylation prior to mouse implantation and zebrafish gastrulation is at present unclear. A role in imprinting was suggested by Reik and Walter, (2001) while Monk *et al.* (1990) proposed a role for this wave of demethylation in germ-line reprogramming. Monk (1995) also suggested that these methylation events play a role as an epigenetic program to repress genes that are no longer required by a specific cell lineage. The occurrence of significant hypermethylation in zebrafish late blastula and gastrula stages suggests that DNA methylation may play a similar role in zebrafish in commencing cell differentiation.

Our investigation in addition suggested the presence of differences in the DNA methylation status of specific single copy genes during zebrafish development related to the expression of the gene. However I was unable to examine the correlation between the methylation status of this single locus and the wave of embryonic global DNA demethylation.

Conclusions: Chapter Three

Using several independent and sensitive methods on a complete developmental survey of the zebrafish early embryonic stages and germ cells, I show that the cycle of demethylation in the zebrafish closely resembles that of the mouse (Monk *et al.*, 1987; Howlett and Reik, 1991; Kafri *et al.*, 1992; Santos *et al.*, 2002). The overall genome-wide DNA methylation in zebrafish germ cells and during early development are compiled in figure 31. The conservation of these dynamic changes in global genome methylation status among vertebrates implies functional significance of these changes in the early development of a complex vertebrate. However, the exact role of these temporal and quantitative changes of methylation remain unclear.

Figure 21. Ethidium bromide stained undigested high molecular weight genomic DNA extracted from different developmental stages, germ cells and somatic tissues as indicated above the figure. This figure shows intact high-molecular weight genomic DNA.

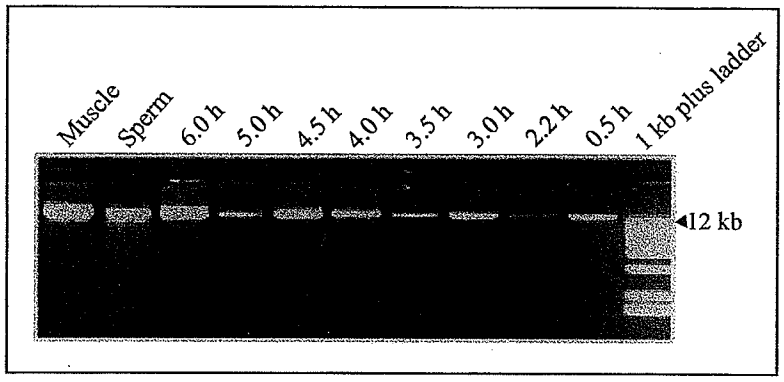

Muscle
Sperm
6.0 h
5.0 h
4.5 h
4.0 h
3.5 h
3.0 h
2.2 h
0.5 h
1 kb plus ladder
12 kb

Figure 22. An ethidium bromide stained product of *MspI* digestion of genomic DNA from various developmental stages. Experimental samples have the same digestion pattern as the control plasmid digested alone indicating complete digestion of the experimental DNA.

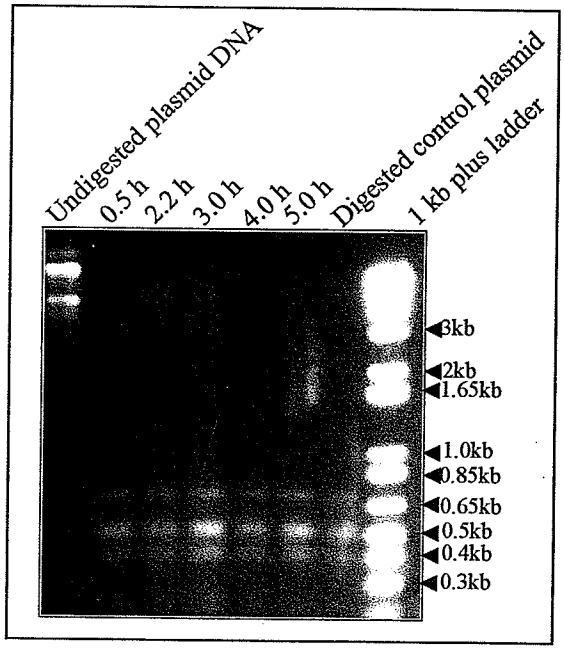
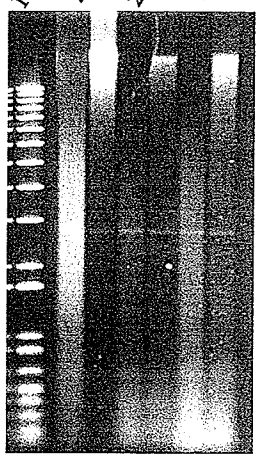
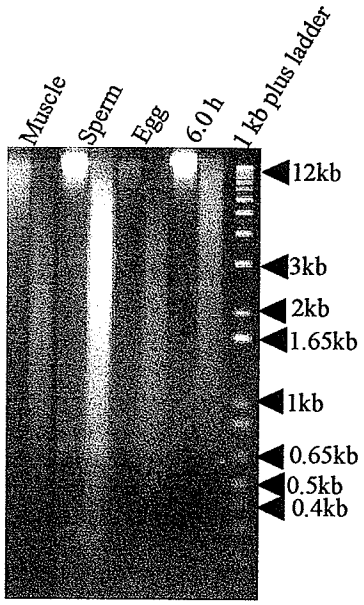
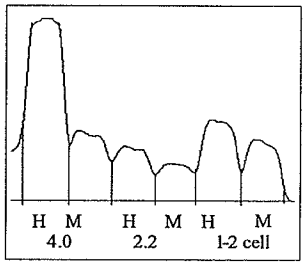
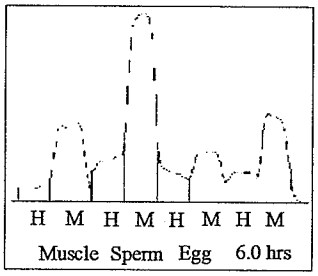

Undigested plasmid DNA
0.5 h
2.2 h
3.0 h
4.0 h
5.0 h
Digested control plasmid
1 kb plus ladder
3kb
2kb
1.65kb
1.0kb
0.85kb
0.65kb
0.5kb
0.4kb
0.3kb

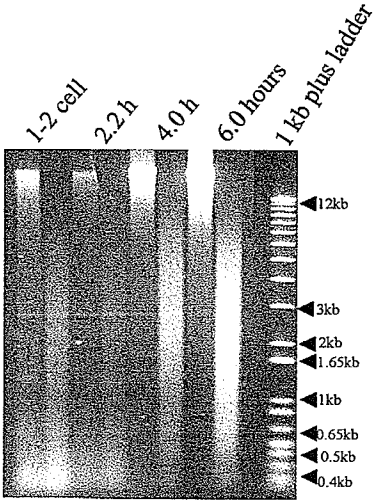
Figure 23. Ethidium bromide stained *HpaII* or *MspI* digested DNA from different developmental stages. Densitometric analysis was done on scanned lanes between 7 and 2 kb. The relative degrees of DNA methylation was compared between DNA samples by estimating the ratio between the lane density of the *HpaII*- and the corresponding *MspI*-digested DNAs.


1 kb plus ladder
4.0 h
2.2 h
0.5 h


Muscle
Sperm
Egg
6.0 h
1 kb plus ladder
12kb
3kb
2kb
1.65kb
1kb
0.65kb
0.5kb
0.4kb


H M H M H M
4.0 2.2 1-2 cell


H M H M H M H M
Muscle Sperm Egg 6.0 hrs


1-2 cell
2.2 h
4.0 h
6.0 hours
1 kb plus ladder
12kb
3kb
2kb
1.65kb
1kb
0.65kb
0.5kb
0.4kb

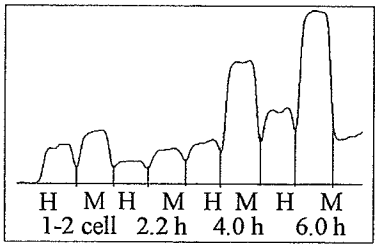

H M H M H M H M
1-2 cell 2.2 h 4.0 h 6.0 h

Figure 24. A representative example of an autoradiograph of mock digested-end labeled high molecular genomic DNA samples extracted from different developmental stages and gonads. This autoradiograph shows that the starting experimental DNA is of high molecular weight and not degraded. The labeling noted at the very bottom of some of the lanes indicates minimal degradation.

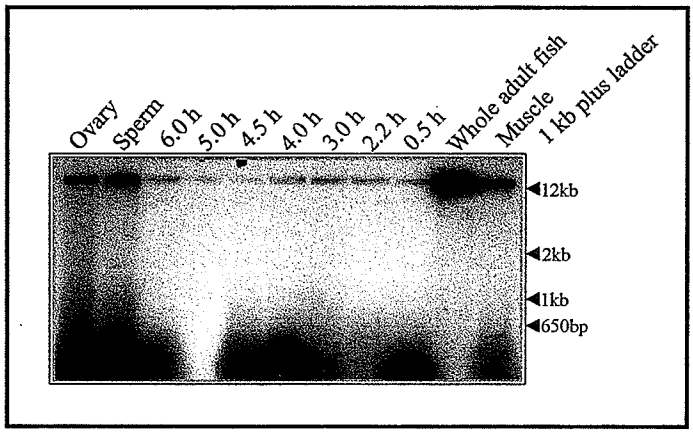
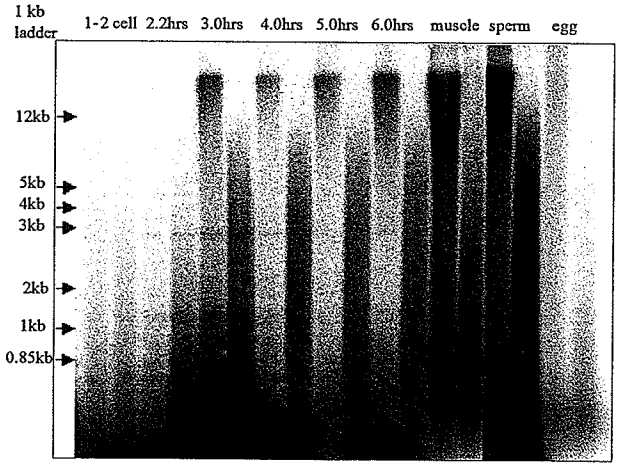
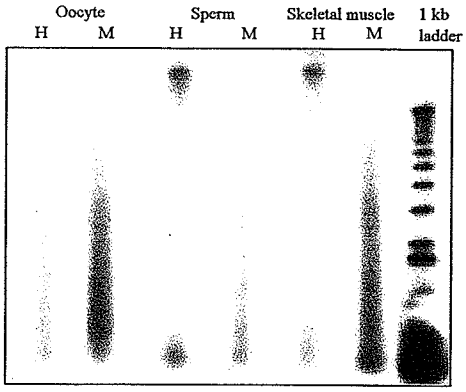

Ovary
Sperm
6.0 h
5.0 h
4.5 h
4.0 h
3.0 h
2.2 h
0.5 h
Whole adult fish
Muscle
1 kb plus ladder
12kb
2kb
1kb
650bp

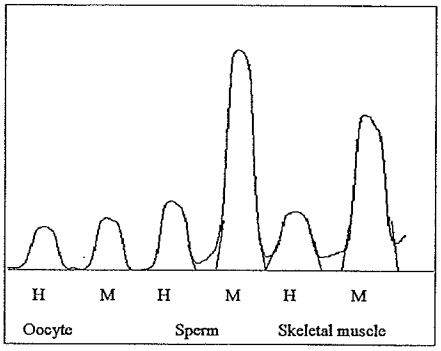
Figure 25. Representative examples of an *HpaII/MspI* end labeling assay of embryonic, germ cell and somatic DNA. The degree of methylation of the total DNA is observed by comparison of *MspI* and *HpaII* digests. A densitometric ratio between the respective *MspI* and *HpaII* lanes (shown in B and C underneath the autoradiogram) gives an indication of the relative degree of methylation of the DNA of the relevant tissue or developmental stage examined. The densitometric scans were performed between 7 and 2 kb.

A

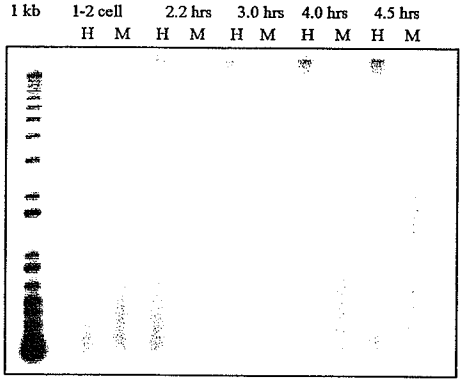

1 kb ladder
1-2 cell
2.2hrs
3.0hrs
4.0hrs
5.0hrs
6.0hrs
muscle
sperm
egg
12kb
5kb
4kb
3kb
2kb
1kb
0.85kb

B


Oocyte
Sperm
Skeletal muscle
1 kb ladder
H M H M H M


H M H M H M
Oocyte
Sperm
Skeletal muscle

C


1 kb
1-2 cell
2.2 hrs
3.0 hrs
4.0 hrs
4.5 hrs
H M H M H M H M H M

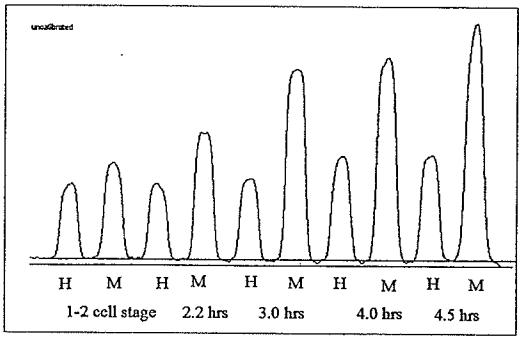
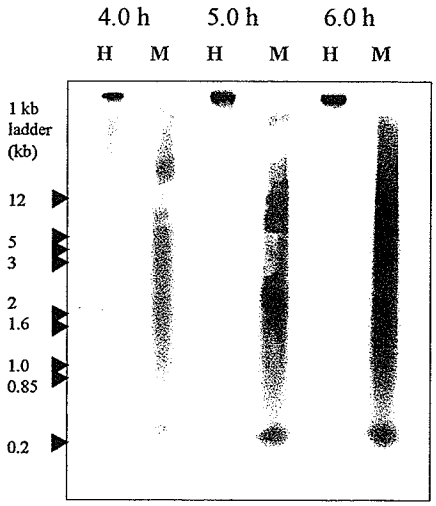
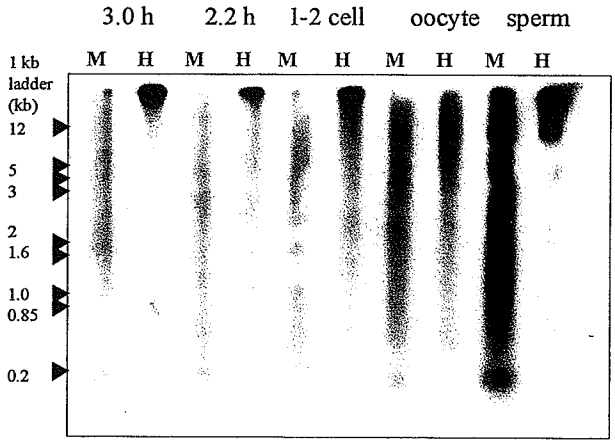
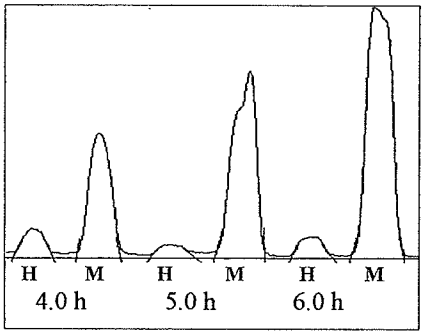

H M H M H M H M H M
1-2 cell stage
2.2 hrs
3.0 hrs
4.0 hrs
4.5 hrs

Figure 26. Representative examples of Southern blots of *HpaII*- and *MspI*-digested DNA samples collected from pooled embryos of different stages, sperm and unfertilized oocytes probed with the DANA repetitive sequence. The autoradiograms were scanned between 7 and 2 kb for densitometric analysis. The densitometric scans are shown underneath the respective autoradiograms.


4.0 h
5.0 h
6.0 h
H M H M H M
1 kb ladder (kb)
12
5
3
2
1.6
1.0
0.85
0.2


3.0 h
2.2 h
1-2 cell
oocyte
sperm
1 kb ladder (kb)
M H M H M H M H M H
12
5
3
2
1.6
1.0
0.85
0.2


H M H M H M
4.0 h 5.0 h 6.0 h

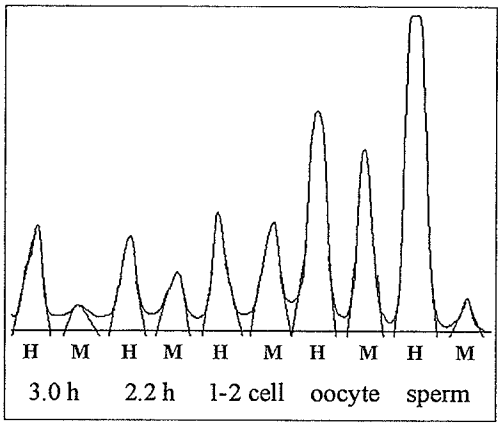
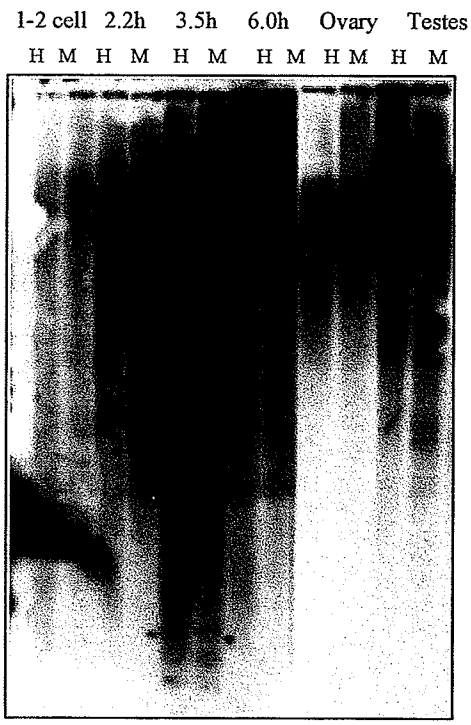
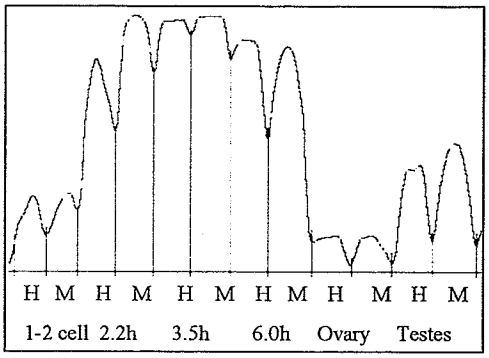

H M H M H M H M H M
3.0 h 2.2 h 1-2 cell oocyte sperm

Figure 27. A representative Southern blot of *MspI* and *HpaII* digested DNA extracted from different embryonic stages and gonadal tissues hybridized with the sr1 repetitive sequence. Note the similarity of the smears given by the large number of heterogeneous fragments for both the *HpaII* and *MspI* lanes. Densitometric analysis of the resulting blots revealed that the smear pattern for each sample of *HpaII*-digested DNA was not significantly different from its corresponding *MspI*-digested DNA.





stage/tissue	Intensity of HpaII lanes	Intensity of MspI lanes	HpaII/MspI ratio
1-2 cell stage	94	96	0.98
2.2 hrs	232	238	0.975
3.5 hrs	274	280	0.98
6.0 hrs	253	249	1.016
Ovary	68	65	1.046
Testes	133	142	0.936

Figure 28. The hybridization pattern of the probe sr14 to *HpaII* and *MspI* digested DNA of different developmental stages and gonads. A single 5 kb band was similarly observed in the *HpaII* and *MspI* lanes of all the different DNA samples tested.

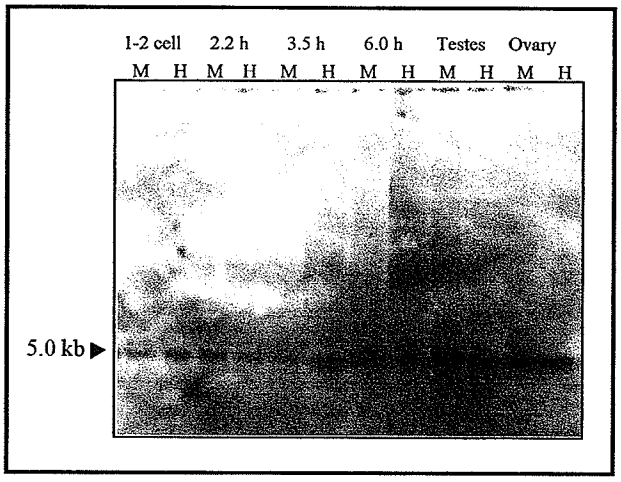
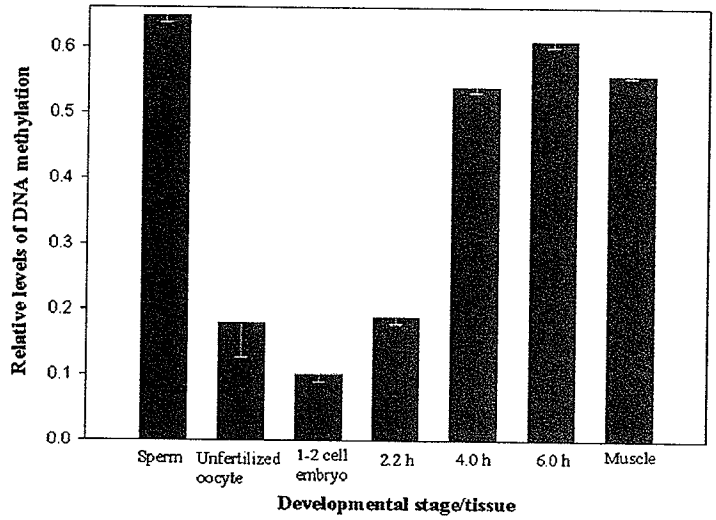
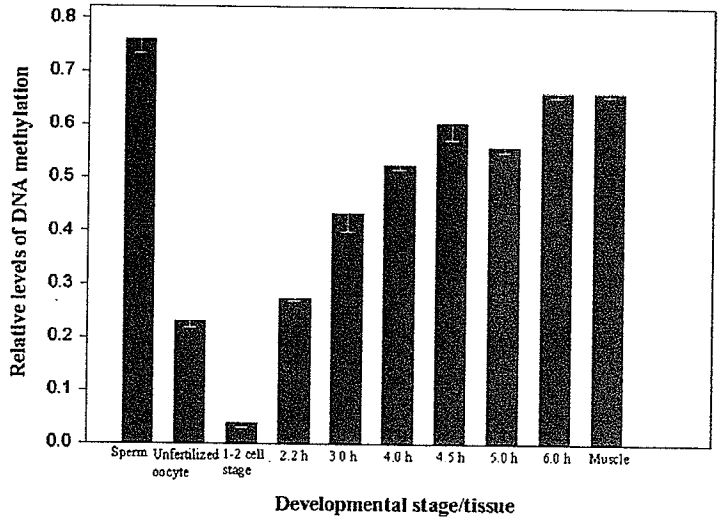

1-2 cell
2.2 h
3.5 h
6.0 h
Testes
Ovary
M H M H M H M H M H M H
5.0 kb ▶

Figure 29. A graphic representation of the genome-wide DNA methylation changes observed during gametogenesis and early development of the zebrafish. These changes were elucidated using the *MspI/HpaII* ethidium bromide assay (A), the *MspI/HpaII* end labeling assay (B) and the Southern analysis assay using DANA probe (C). The graphs represent the relative levels of DNA methylation. Error bars shown are calculated standard error of the mean. Data were quantified from independent duplicate gels (A), four independent blots (B) and three independent blots (C).

A


Relative levels of DNA methylation
0.0
0.1
0.2
0.3
0.4
0.5
0.6
Sperm
Unfertilized oocyte
1-2 cell embryo
2.2 h
4.0 h
6.0 h
Muscle
Developmental stage/tissue

B


Relative levels of DNA methylation
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
Sperm
Unfertilized oocyte
1-2 cell stage
2.2 h
3.0 h
4.0 h
4.5 h
5.0 h
6.0 h
Muscle
Developmental stage/tissue

C

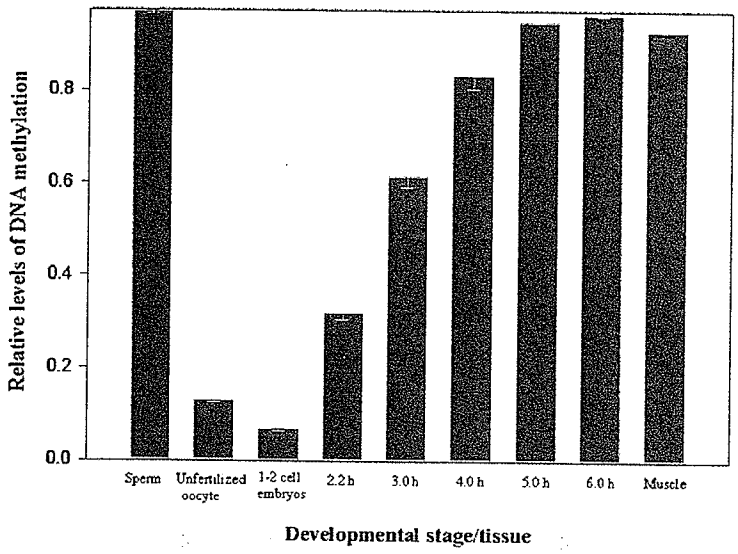

Relative levels of DNA methylation
0.0
0.2
0.4
0.6
0.8
Sperm
Unfertilized oocyte
1-2 cell embryos
2.2 h
3.0 h
4.0 h
5.0 h
6.0 h
Muscle
Developmental stage/tissue

Figure 30. Southern blot of *MspI* and *HpaII* digested DNA extracted from different developmental stages and probed with zebrafish *ck* gene. A single sized band was noted in all *MspI* digested samples. The *HpaII* digested samples of sperm, 4-hr, 5-hr and gastrula stage (6.0 hrs) embryos showed one band at the top of the lanes while that of 12-hr and 30-hr embryos show some smearing at the top of the lane and two distinct bands of high molecular weight were seen clearly in the 30-hr stage embryos.

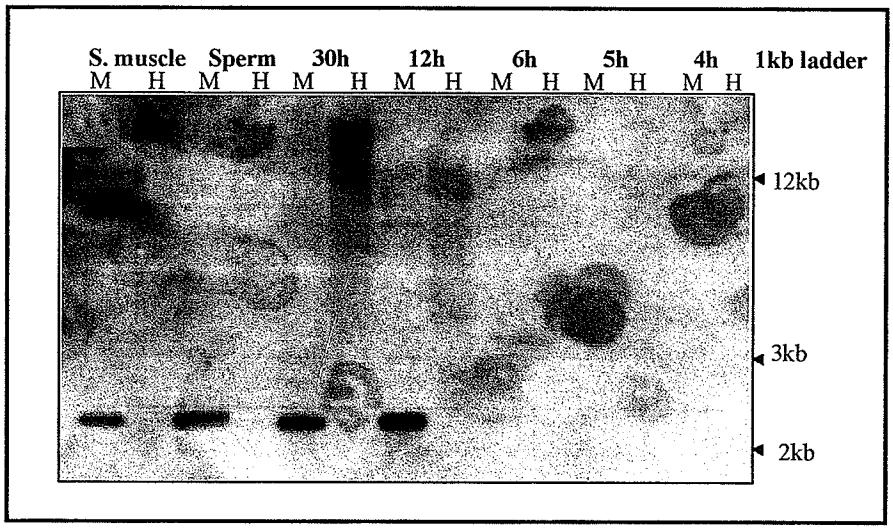
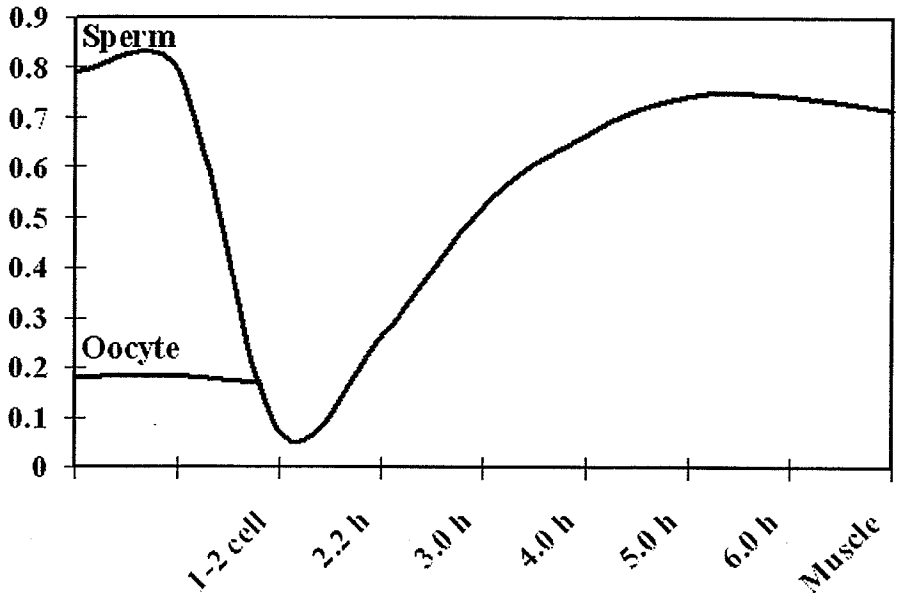

S. muscle
Sperm
30h
12h
6h
5h
4h
1kb ladder
M H M H M H M H M H M H M H
12kb
3kb
2kb

Figure 31. A diagram showing the dynamics of the global DNA methylation changes in germ cells and during early embryogenesis of zebrafish. The ratios of the relative levels of methylation derived from densitometric analyses were used to generate this graph.


0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Sperm
Oocyte
1-2 cell
2.2 h
3.0 h
4.0 h
5.0 h
6.0 h
Muscle

General Conclusions

The understanding of the epigenetic modification of DNA by methylation of the cytosine residues in the CpG dinucleotides is important, particularly as it relates to normal development and to human disease. The availability of a versatile animal model system to dissect such relationships is therefore of paramount importance. We have characterized some aspects of DNA methylation in the zebrafish in an attempt to test its suitability for such studies.

Our investigation showed a high degree of similarity of dnmt1 to other vertebrate methyltransferases and significant conservation of all its functional motifs. In addition, dnmt1 undergoes developmentally regulated and stage specific expression changes in mRNA levels and enzyme activity, particularly during the process of oogenesis (figure 32). The conservation of dnmt1 and its dynamic temporal and spatial expression profile argue that DNA methylation may play a role in the early development and/or in oogenesis of the zebrafish.

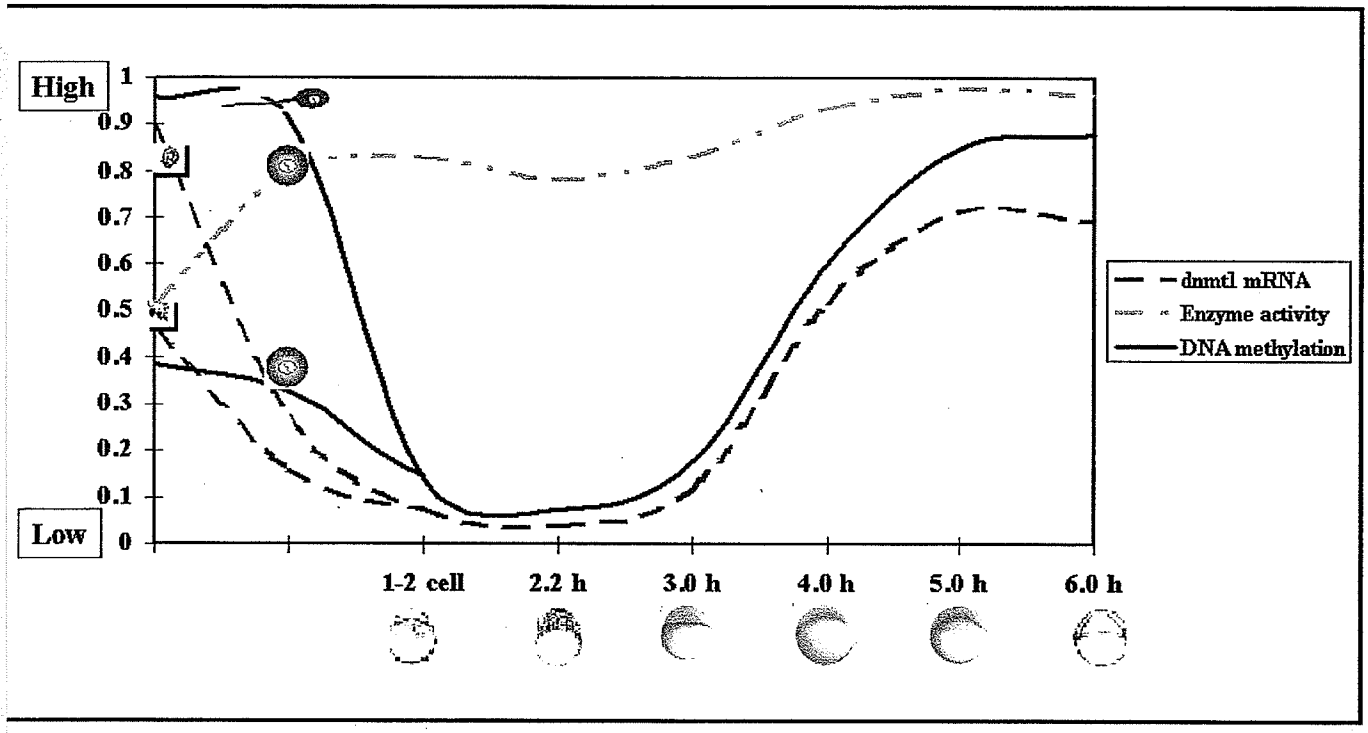
During the course of oogenesis in the zebrafish, early oocytes contained significant amounts of *dnmt1* transcript while message abundance declined as oocytes matured. The transcript levels remained low during early embryogenesis until the blastula stage. Methyltransferase enzyme assays revealed that the maternal *dnmt1* message accumulated during oogenesis is translated into protein as evidenced by the high enzyme activity found in mature unfertilized oocytes, 1-2 cell stage and early blastula (2.2 h) stage embryos (Mhanni *et al.*, 2002). Despite the high enzyme activity in the unfertilized oocyte, during the early cleavage and blastula stage embryos, their genomes are demethylated (figure 32). A similar paradoxical relationship has been observed in the

preimplantation mouse embryo which may result from the subcellular localization of Dnm1o to the cytoplasm conferred by the cytoplasmic retention domain of Dnmt1 (Carlson *et al.,* 1992; Howell *et al.,* 2001). The zebrafish dnmt1 has a similar cytoplasmic retention domain (Mhanni *et al.,* 2001), hence it may undergo similar subcellular localization. The lack of an antibody specific for, or one that will cross-react with, dnmt1 precludes confirmation of this premise.

The cycle of demethylation in the zebrafish (figure 32) closely resembles that of the mouse. The identification of a similar phenomenon of demethylation operating in a non-mammalian vertebrate suggests that this process is evolutionarily conserved and therefore probably is important in the development of a complex non-mammalian vertebrate. However the exact role of these temporal and quantitative changes of methylation remain unclear.

Overall, our work suggests that there is considerable potential for exploiting the zebrafish model system to understand the exact role of DNA methylation in the early development of vertebrates and to dissect the disease phenotype(s) ensuing from its disruption.

Figure 32. A composite graphic representation summarizing the developmental profile of the relative changes in *dnmt1* mRNA, dnmt1 enzyme activity and the cycle of demethylation in zebrafish germ-cells and during early embryogenesis.


High
Low
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
1-2 cell
2.2 h
3.0 h
4.0 h
5.0 h
6.0 h
dnmt1 mRNA
Enzyme activity
DNA methylation

Contribution to Ongoing Work and Future Prospects

I. Cloning of zebrafish *dnmt3* and characterization of the *de novo* methylation activity.

Introduction

The Dnmt3 family of methyltransferases, consisting of two related genes *Dnmt3a* and *Dnmt3b*, are expressed at low levels in adult tissues, but are abundant in mouse embryonic stem cells (Okano *et al.*, 1998). Both are highly expressed in ES cells but their expression is down regulated upon differentiation and remains low in adult somatic tissues. Recently, several lines of evidence suggested that they act as *de novo* methyltransferases (Okano *et al.*, 1998; Okano *et al.*, 1999; Lyko *et al.*, 1999).

Interestingly, Xie *et al.* (1999) identified a related zebrafish gene (*Zmt3*) (GenBank number AF135438) to the DNMT3 genes by searching the EST database. The catalytic domain of Zmt3 is highly conserved, indicating that it belongs to the DNMT3 family, whereas the N-terminal domain bears no significant homology to either DNMT3a or DNMT3b (Xie *et al.*, 1999).

Cloning the full length of zebrafish dnmt3 is part of our effort to characterize the role of DNA methylation in zebrafish early development. Obtaining the full-length dnmt3 will allow us to perform detailed homology studies, analyze its functional domains and investigate whether there are two active isoforms of dnmt3 in zebrafish as there are in the mouse.

Objectives:

1. Cloning of the full-length *dnmt3* cDNA and investigating whether there are two isoforms of this enzyme in the zebrafish.

2. Determine the *de novo* enzyme activity in the oocytes, during early development and in somatic tissues.

Approach and progress:

1. dnmt3 cloning:

RACE-PCR, as described in chapter one, was used to clone the full-length of *dnmt3*. Gene specific primers were designed using sequence data from the published expressed sequence tag (EST) (GenBank number AF135438) sequence (Appendix 4). Using these primers we were able to clone 1600 bp of the 3` sequence of the gene. Efforts are ongoing in our laboratory to clone and characterize the 5` end of the gene.

2. The *de novo* MTase enzyme activity in oocytes, during early development and in somatic tissues

Determination of the *de novo* enzyme activity was carried out essentially as described in Chapter two-Section three. The substrate used to monitor the *de novo* MTase activity consisted of a non-methylated double stranded oligonucleotide (Robertson *et al.*, 2000) [oligo-1 (top) and oligo-2 (bottom)-Appendix 4] (EPOCH Biosciences, California). To create a hemimethylated substrate, to monitor maintenance MTase activity as a control, the bottom strand oligonucleotide was synthesized with 5-

methylcytosine at all potential CpG sites (EPOCH Biosciences, California) and annealed to the same unmethylated top strand oligonucleotide.

Methyltransferase enzyme assays were carried out using these two substrates. The assay performed using the hemimethylated substrate did not work. After discussions with "EPOCH Biosciences company" technical support, the reason was determined to be the inappropriate synthesis of the hemimethylated oligonucleotide (oligo 3).
The *de novo* enzyme assay using the nonmethylated substrate was done in duplicate and it revealed a high level of enzyme activity during early embryogenesis and in mature oocytes (table 1 and figure 1-appendix 4). The levels were significantly lower at gastrulation as they were in somatic tissues. These results are in keeping with what has been observed in mice (Okano *et al.*, 1998).

II. Is there similar subcellular localization of zebrafish DNA (cytosine-5) methyltransferase 1 enzyme in gonads and during early development to the mouse Dnmt1?

Introduction

In the mouse, preimplantation development is characterized by a decrease in DNA methylation (Monk *et al.*, 1987; Monk, 1990; Santos *et al.*, 2002) paradoxically occurring in the presence very high levels of Dnmt1 (Carlson *et al*, 1992). This might be explained on the basis of Dnmt1 subcellular localization. In preimplantation mouse embryos, the oocyte-specific Dnmt1, Dnmt1o, is cytoplasmically localized in mature oocytes and in all preimplantation mouse embryos except at the eight-cell stage where it is detected briefly

in the nuclei of blastomeres in these embryos (Carlson *et al.,* 1992; Cardosos and Leonhardt, 1999; Howell *et al.,* 2001).

Cardoso and Leonhardt (1999) mapped a *Dnmt1* cytoplasmic retention sequence. They noted that fusion constructs containing the broad region from amino acids 308-854 of Dnm1o (426-972 of the somatic form of Dnmt1) to be efficiently retained in the cytoplasm of early mouse embryos. Interestingly, the corresponding region in zebrafish dnmt1 shows 73% similarity based on amino acid identity and conservative changes. This suggests that there may well be subcellular localization of the zebrafish dnmt1 protein and that it is controlled in the same way as in the mouse.

Approach

We are currently examining the intracellular localization of dnmt1 enzyme during different stages of oogenesis and early development of the zebrafish. There is no commercially available antibody to the zebrafish dnmt1, we therefore are using two different commercially available antibodies to mouse Dnmt1. One of these antibodies, Dnmt1 (K-18):sc-10221 (Santa Cruz Biotechnonology, Inc.), was raised against an epitope that is identical in both zebrafish dnmt1 and mouse Dnmt1.

Our attempts to use these antibodies for Western blot analysis generated too many nonspecific bands. We continue to attempt to optimize the experimental conditions to be able to use these antibodies. We are also conducting immunohistochemistry experiments using these antibodies to investigate the localization of the dnmt1 protein in oocytes and during early development of the zebrafish.

III. Is there a somatic form of zebrafish *dnmt1*?

Introduction

The mouse Dnmt1 gene expression is driven by sex-specific promoters (Bestor, 2000). The 5`-most promoter introduces an oocyte-specific 5` exon which causes translation to initiate at an ATG codon in exon 4. This results in a protein that is shorter than the somatic form by 118 N-terminal amino acids (reviewed in Bestor, 2000). In somatic cells, an active promoter located ~ 7 kb 3` of exon 1o, 1s, functions as the house keeping promoter and initiates translation in an ATG codon in exon 1s that initiates the full-length Dnmt1 in somatic cells (reviewed in Bestor, 2000).

To further study the possible mechanisms regulating DNA methylation during zebrafish early development, it is necessary to determine whether the two isoforms are expressed in the zebrafish.

Approach

RACE-PCR is being performed essentially as in chapter one. Gene specific primers (GSP3 and GSP4) (Appendix 4) were designed to clone the 5` end of *dnmt1* (figure 2). Somatic RNA (muscle) is being used as template for cDNA synthesis and ovarian RNA is being used as a control.

Ribonuclease protection and primer extension assays are the other approaches being used to map the 5` end of *dnmt1* mRNA that is extracted from several somatic tissues along with oocyte mRNA.

IV. Characterization of the phenotypes resulting from stage-specific disruption of *dnmt1* and *dnmt3* using Morpholino antisense oligos

To determine the phenotypic effects of loss of methylation in early development, we intend to conduct a series of experiments using antisense gene knockout. *dnmt1* and *dnmt3* antisense oligonucleotides (morpholino antisense oligos) will be obtained from "GENE TOOLS, LLC." Demethylation will be induced at different developmental stages to determine the phenotypic consequences of demethylation at each developmental stage. This experiment will be conducted to examine the role of *dnmt1* and *dnmt3* in early development. This will help answer questions regarding the specific role of DNA methylation in cell differentiation at different developmental stages.

Appendices

Appendix 1

Primer Sequences

I. Primers used for dnmt1 RACE-PCR

AP1 (CLONTECH Cat. No. K1802-1)

5' --CCA TCC TAA TAC GAC TCA CTA TAG GGC-- 3'

AP2 (CLONTECH Cat. No. K1802-1)

5' --ACT CAC TAT AGG GCT CGA GCG GC-- 3'

Marathon cDNA synthesis primer (CLONTECH Cat. No. K1802-1)

5' --TTC TAG AAT TCA GCG GCC $GC(T)_{30}\underline{N_{-1}N}$-- 3'

N_{-1}=G,A or C; N=G,A,C or T

Degenerate nucleotides anchor

primer at base of poly-A tail

5' RACE-TFR Primer (CLONTECH Cat. No. K1802-1)

5' --GTC AAT GTC CCA AAC GTC ACC AGA-- 3'

3' RACE-TFR Primer (CLONTECH Cat. No. K1802-1)

5' --ATT TCG GGA ATG CTG AGA AAA CAG ACA GA-- 3'

II. dnmt1 Gene Specific Primers (GSPs) (Gibco-BRL)

Name	Sequence	Tm	%GC
GSP1	5' --CCC TTT CCC TTG CCC TTT CCT TTC CC-- 3'	79	57
GSP2	5' --CTG TCT GAG GGC TTC CAT CAA GCA GGC-- 3'	81	59

III. Primers used for sequencing

M13 Reverse (Gibco-BRL Cat. No. 18424-010)

5' --AGC GGA TAA CAA TTT CAC ACA GC-- 3'

M13 Forward (Gibco-BRL Cat. No. 18257-014)

5' --CCC AGT CAC GAC GTT GTA AAA CG-- 3;

IV. Internal primers for 3` RACE-PCR clone and 5` RACE-PCR dnmt1 clone # 1

Name	Sequence	Tm	%GC content
5MTF1	5' -GCA CCT CAG TCA TGC TGA CGG- 3'	75	61
5MTF2	5' -CGC TTC ACC GGG CTT GCA GCC- 3'	79	71
5MTF3	5' -GAT GTC TTC ATC CAT TCC AC- 3'	66	45
5MTF4	5' -GAT ACA CAA TCT CCC ACC- 3'	65	50
5MTF5	5' -TAG TCG GCC CTT GTT GTC C- 3'	70	57
5MTF6	5' -TCA CTA GGG TCC ATT AGG- 3'	65	50
5MTF7	5' -GCT GTA GAC ACA GGT TTG- 3'	65	50
5MTR1	5' -ACT CAC TAT AGG GCT CGA GC- 3'	70	55
5MTR2	5' -TAA AGT CTC CCA GTG CCC- 3'	67	55
5MTR3	5' -GAG ATG TTA ACC GAT GAG CG- 3'	68	50
5MTR4	5' -AGG AGA AGA TCT GCA TGA G- 3'	65	47
5MTR5	5' -AGA CAA CGG TGT GGC GTC- 3'	69	61
5MTR6	5' -CAG CTT TGT GGG ATG ATG- 3'	65	50
3MTR1	5' -CAT CAC ATG AAT GGC ACT GCA C- 3'	71	50
3MTR2	5' -CAG GAT TGG TAA CTG TAG TGC- 3'	69	47
3MTR3	5' -GTC CTT GCA GAT GTG GTC C- 3'	70	57
3MTR4	5' -CAC ATG CAA GGG TTC AGG	67	55
3MTF1	5' -GGG CCG CCT TGC CAA GGC TTC- 3'	79	71

Note: All internal primers were made by Gibco-BRL

V. Primers used to generate the DANA repetitive element

DANA-1: 5`-GGCGACRCAGTGGCGCAGTRGG-3`

DANA-2: 5`-TTTTCTTTTTGGCTTAGTCCC-3`

Primers were made by GIBCO-BRL.

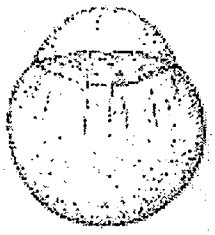
Appendix 2

Single and Three Lettered Amino Acid Abbreviations

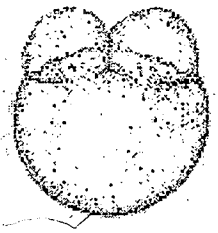
A	Alanine	Ala
C	Cysteine	Cys
D	Aspartic acid	Asp
E	Glutamic acid	Glu
F	Phenylalanine	Phe
G	Glycine	Gly
H	Histidine	His
I	Isoleucine	Ile
K	Lysine	Lys
L	Leucine	Leu
M	Methionine	Met
N	Asparagine	Asn
P	Proline	Pro
Q	Glutamine	Gln
R	Arginine	Arg
S	Serine	Ser
T	Threonine	The
V	Valine	Val
W	Tryptophan	Trp
Y	Tyrosine	Tyr

Appendix 3

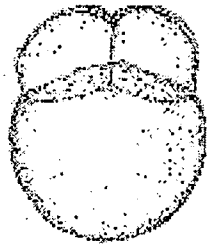
Developmental Stages
(After The Zebrafish Science Monitor, 1999)



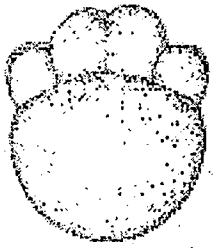
1-cell
0.2 h



2-cell
0.75 h

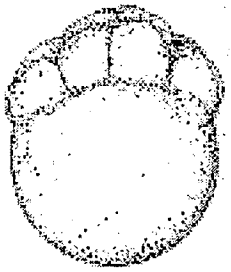


4-cell
1 h

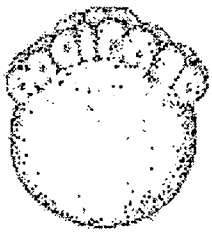


8-cell
1.25 h

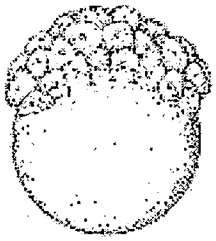
Cleavage



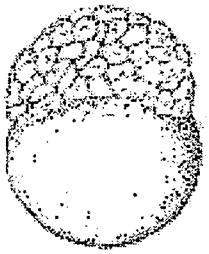
16-cell
1.5



32-cell
1.75

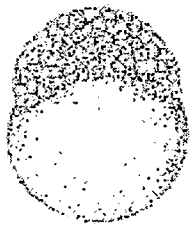


64-cell
2 h

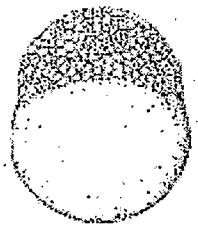


128-cell
2.25 h

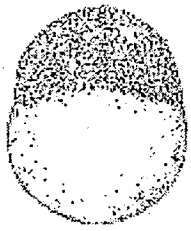
Blastula



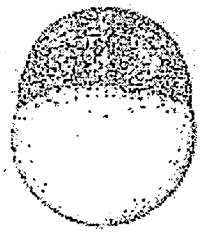
456-cell
2.5 h



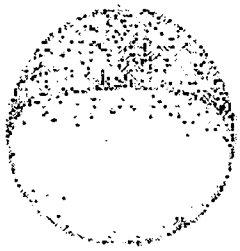
512-cell
2.75 h



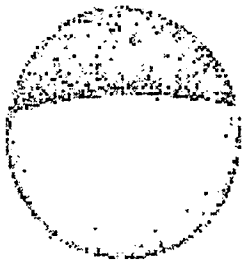
1k-cell
3 h



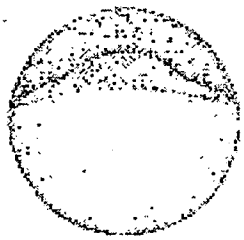
high
3.3 h



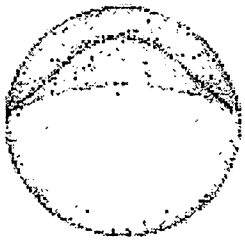
oblong
3.7 h



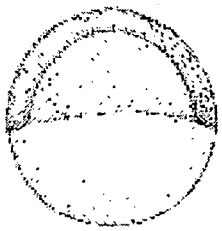
sphere
4 h



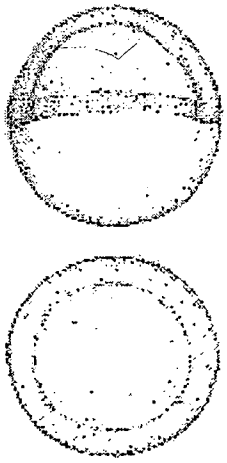
dome
4.3 h



30% epiboly
4.7 h



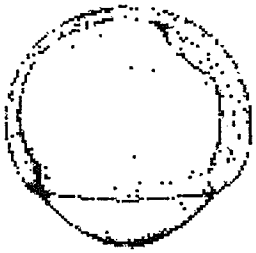
50% epiboly
5.3 h



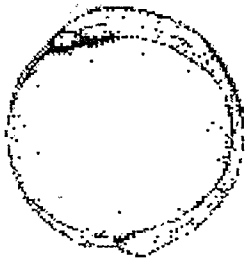
germ ring
5.7 h

Gastrula

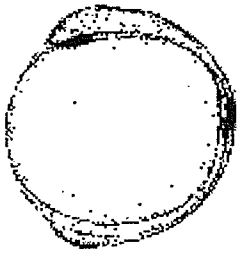
shield
6 h



75% epiboly
8 h



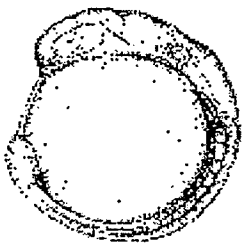
90% epiboly
9 h



bud
10 h

3-somite
11 h

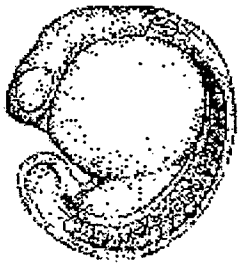
6-somite
12 h



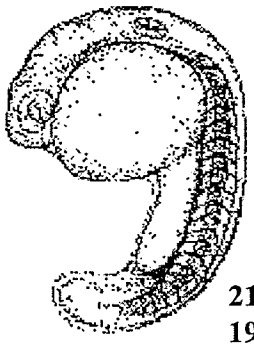
10-somite
14 h

14-somite
16 h

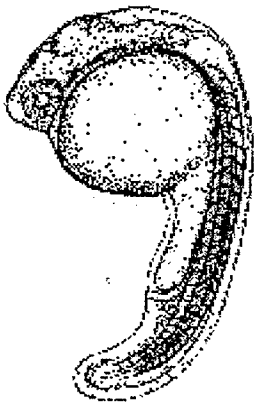
Segmented



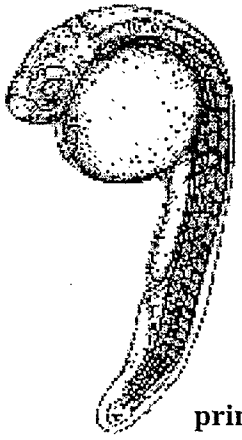
18-somite
18 h



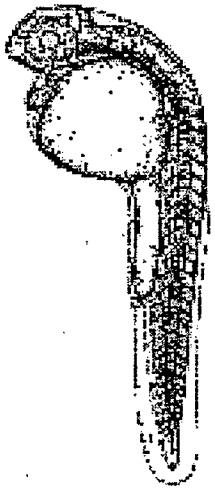
21-somite
19.5 h



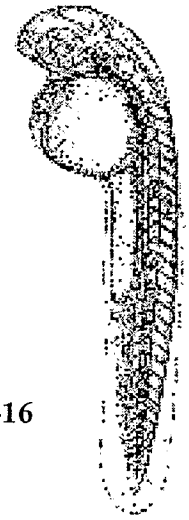
26-somite
22 h



prim-6
25 h



prim-16
31 h



prim-22
35 h

high pec
42 h

long pec
48 h

pec fin
60 h

protruding mouth
72 h

Appendix 4

I.

Sequences of the substrate used in the de novo MTase enzyme assay:

Oligo 1.

5`GGGGGCCAAGCGCGCGCCTGGCGCCCGGGCCGGCTCAAGCGCGCGCCTGG

CGCC-3`

OLIGO 2.

5`GATCCGGGCGCCAGGCGCGCGCTTGAGCCGGCCGGGCGCCAGGCGCG

CGCTTG-3

OLIGO 3.

5`GATC*GGG*CGCCAGG*G*G*GCTTGAGC*GGCC*GGG*GCCAGG*G*G*

GCTTGG-3`

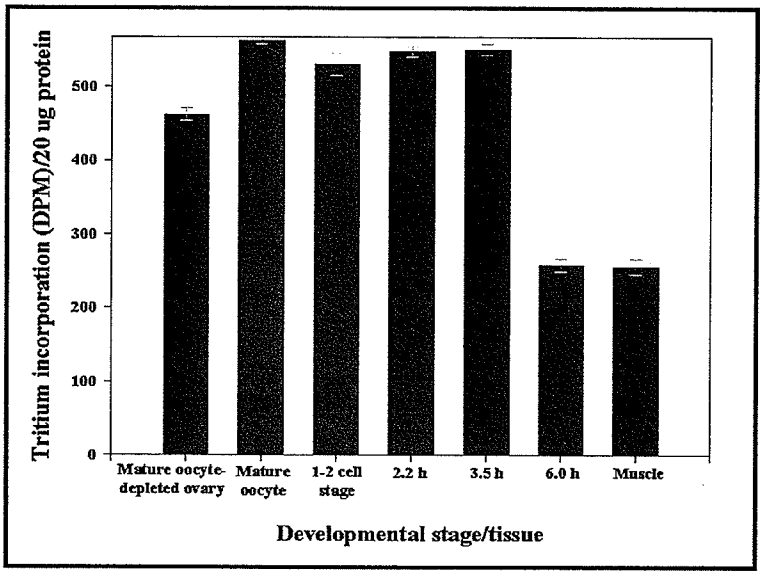
* = methylated cytosine

Oligonucleotides were made by EPOCH Biosciences, California.

Table 1. The *de novo* MTase enzyme acivity in the mature oocyte, during early development and in somatic tissues of the zebrafish. The disintegrations per minute (DPM) were automatically calculated by an Auto DPM program as a measure of the absolute activity within the sample.

Developmental stage/tissue	CPM	Auto DPM
Mature oocyte-depleted ovary	199	471
Mature oocyte-depleted ovary	199.7	454
Mature unfertilized oocytes	245	567
Mature unfertilized oocytes	242	558
1-2 cell stage	233	546
1-2 cell stage	227	515
Blastula (2.2 h)	235	555
Blastula (2.2 h)	229	541
Blastula (3.5 h)	237	558
Blastula (3.5 h)	234	543
Gastrula (6.0 h)	101	249
Gastrula (6.0 h)	111	267
Muscle	107	246
Muscle	110	267
Positive control (no SAM)	10.6	33.3
Negative control (no Poly dI-dC)	9.2	33.4

Figure 1. The *de novo* MTase enzyme activity in mature oocytes, in early development and in somatic tissues of the zebrafish.


Tritium incorporation (DPM)/20 ug protein
0
100
200
300
400
500
Mature oocyte-depleted ovary
Mature oocyte
1-2 cell stage
2.2 h
3.5 h
6.0 h
Muscle
Developmental stage/tissue

III.

Gene Specific Primers (GSPs) (Gibco-BRL) used for *dnmt1*-5`-RACE-PCR

Name	Sequence	Tm	%GC
GSP4	5' –CTG CTC CAC TCC ATC ACC CTC CTT GCC-- 3'	82	62
GSP5	5' –CCA TGG CTC CCT CCT GCT CGC CAT TGT C-- 3'	84	64

```
1   GCCCGGGCAG GTGCGCGCGC TGCAGTTGGC ACTGTCACAT ATTAATACCC GCTTTTATCT
61  GTTGTTGTTT TAAATCGTGT TTTTAAGTAG TCAATCCCTT TGATCAAAAT CGAACTTGAA
121 ATGCCTACCA AGACCTCATT GTCTCTGCCA GAGGATGTCA AAGAACGGCT TCAGGTGTTG
181 GATGAAGGTG GAGACAGTTT GTCAGATGAG GAGTGTGTAA AAGAAAAGCT CAGGTTACTA
241 CAGGAGTTCC TGCTCGCTGA TACTCAGGAC CAGCTCAAAA ACCTTGAGGA CAAGTTAAAG
301 AGCTCTGAGC TCTCCACGGA GGTTTACATG TCAGAGGTGA AGGCTGTACT GAAGAAAGCT
361 CTGGGAGTTG GCAAGGAGGG TGATGGAGTG GAGCAGAATG GACATTCAAA TGGCTTCTCT
421 GAAAATGGAT CTCACAAAGA CAATGGCGAG CAGGAGGGAG CCATGGACAC TCAGGACGAA
```

Figure 2. Nucleotide sequence of 5` end of ovarian *dnmt-1*. The highlighted sequence in bold blue represents complementary sequence of GSP4 primer. The highlighted sequence in bold brown represents complementary sequence of GSP5 primer.

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Electronic-Database Information

The URLs for data presented herein are as follows:

www.gene-tools.com: GENE TOOLS,LLC.

http://www.ncbi.nlm.nih.gov/Omim/: Online Mendelian Inheritance in Man (OMIM).

http://www.ncbi.nlm.nih.gov/Entrez/: used to access sequence data bases for existing nucleatide and protein sequences.

http://www.ncbi.nlm.nih.gov/BLAST/: for standard nucleotide-nucleotide BLAST,protein-protein BLAST and pairwise BLAST.

http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi: for searching the conserved domain database.

http://www.ncbi.nlm.nih.gov/Genbank/index.html: for submitting sequence data to GenBank.

http://www.ncbi.nlm.nih.gov/genome/seq/DrBlast.html: To blast a sequence against zebrafish specific expressed sequence tags.

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