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**DNA METHYLTRANSFERASE IN THE ZEBRAFISH *DANIO*
RERIO: 5-AZA-CYTIDINE TREATMENT OF EMBRYOS IN
VIVO CAUSES DISRUPTION IN THE DEVELOPMENT OF
THE AXIAL MESODERM**

BY

© C. CRISTOFRE MARTIN

**A Thesis
Submitted to the Faculty of Graduate Studies
in Partial Fulfillment of the Requirements
for the Degree of**

Doctor of Philosophy

**Department of Anatomy & Neurobiology
University of Ottawa
Ottawa, Ontario (c) July, 1997**



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Abstract

I have conducted sequence analysis of a zebrafish cDNA that encodes the protein DNA (5-cytosine) methyltransferase. I show that during development transcription of this zebrafish DNA methyltransferase gene is most high at blastula where it is ubiquitously expressed. At 24 hours of development, highest expression is observed in the brain, neural tube, eyes, and differentiating somites. To further assess the role of DNA methylation during development, I treated zebrafish embryos with 5-aza-cytidine (5-azaC), a nucleotide analog known to induce cellular differentiation and DNA hypomethylation in mammalian cell cultures. Treatments with 5-azaC during blastula and early gastrula caused a perturbation of the body axis resulting in loss of tail, and loss or abnormal development of the somites. Histological sections and *in situ* hybridization revealed whole or partial loss of a differentiated notochord and mid-line muscle in treated embryos. There was loss of expression of *no tail* in the notochord and *eng* in muscle-pioneer cells at 24 hours. When examined during gastrulation, 5-azaC treated embryos had a shortened and thickened axial mesoderm. DNA analysis on 5-azaC-treated embryos indicated an overall decrease in DNA methylation when compared to untreated controls. Embryos treated with 6-aza-cytidine, a cytidine analog not known to affect DNA methylation, showed neither abnormal development nor hypomethylation. Our findings suggest that normal DNA methylation is required for proper differentiation of dorsal mesoderm and pattern development of the dorsal-ventral body axis.

Résumé

J'ai analysé de la séquence d'un clone d'ADNc de danio (poisson-zèbre) codant pour l'enzyme ADN (5-cytosine) méthyltransférase. J'ai démontré qu'au cours du développement, la transcription de la méthyltransférase du danio est à son maximum lors du stade blastula alors que le gène est exprimé de manière ubiquitaire. A 24 heures de développement, la méthyltransférase est exprimée principalement dans le cerveau, le tube neural, les yeux et les somites en cours de différenciation. Afin de mieux comprendre le rôle de la méthylation de l'ADN au cours du développement, j'ai traité des embryons de danio avec la 5-aza-cytidine (5-azaC), un analogue nucléotidique reconnu pour induire la différenciation cellulaire et l'hypométhylation de l'ADN dans des cellules de mammifères en culture. Des traitements avec la 5-azaC au cours du stade blastula ou au début de la gastrulation causent des perturbations de l'axe embryonnaire résultant dans la perte de la queue et la perte ou le développement anormal des somites. Des sections histologiques et des expériences d'hybridation *in situ* montrent la perte partielle ou totale de notocorde différenciée et des muscles au niveau de la ligne médiane chez les embryons traités. On observe une perte de l'expression du gène *no tail* dans la notocorde et du gène *eng* dans les cellules musculaires pionnières au stade 24 heures. Lorsque les embryons sont examinés au cours de la gastrulation, le traitement à la 5-azaC produit un mésoderme axial raccourci et épaissi. L'analyse de l'ADN des embryons traités à la 5-azaC indique une diminution globale du niveau de méthylation lorsque comparé à l'ADN d'embryons contrôles. Les embryons traités avec la 6-aza-cytidine, un analogue de la cytidine n'affectant pas la méthylation de l'ADN, ne montrent aucun signe de développement anormal ou de diminution du niveau de méthylation. Nos résultats suggèrent qu'une méthylation normale de l'ADN est nécessaire pour la différenciation correcte du mésoderme dorsal et la morphogénèse de l'axe dorso-ventral de l'embryon.

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DNA Methyltransferase in the Zebrafish *Danio rerio*: 5-Aza-Cytidine Treatment Of Embryos In Vivo Causes Disruption In The Development Of The Axial Mesoderm

Introduction

DNA modification by methylation has been shown to occur in an evolutionary diverse group of organisms such as bacteria, plants, vertebrates and a number of invertebrates. In bacteria, methylation can occur on both adenine and cytosine residues and is primarily involved in the host 'immune' response against virus infection via the restriction modification system (reviewed in Hubacek, 1992). Methylation of adenine is also involved in regulating DNA replication and targeting the correction of replication errors (Noyer-Weidner and Trautner, 1993). At least in higher eukaryotes, methylation of cytosine is the primary modification and occurs most frequently at the dinucleotide CpG. In vertebrates approximately 60% of all CpG dinucleotides are methylated (Jaenisch *et al.*, 1995) and 5-methylcytosine represents approximately 1% of nucleotide bases (Bestor and Verdine, 1994). The methylation of DNA residues can be preserved through the replication process by the action of maintenance methylases which use the replicated hemimethylated DNA as a template (Razin and Riggs, 1980; Gruenbaum *et al.*, 1982). Demethylation of DNA can occur by passive means via DNA replication in the absence of maintenance methylation and is also thought to occur by an active mechanism in the absence of DNA replication (Kafri *et al.*, 1992; Kafri *et al.*, 1993; Brunk *et al.*, 1996). This type of DNA modification is important since typically methylated DNA is transcriptionally inactive (Cedar, 1988). While DNA methylation is not the only factor to

determine expression of a gene, at least for some genes there is a direct correlation between DNA hypomethylation and the establishment of a transcriptionally active chromatin state (Michalowsky and Jones, 1989). Methylation may also affect gene expression by direct influence on the binding of transcription factors (Ben-Hattar *et al.*, 1989), the association of repressor proteins such as MeCP1 and MeCP2 (methyl-cytosine binding proteins) (Boyes and Bird, 1991, 1992) or by influencing nucleosome positioning (Englander *et al.*, 1993). Housekeeping genes which are continuously active are frequently characterized by the presence, in their 5' region, of CpG islands which appear to be refractory to DNA methylation (Bird, 1986; Antequera and Bird, 1988). Furthermore, genes which are expressed in a tissue specific manner are highly methylated in non specific cell types (Cedar and Razin, 1990; Tate and Bird, 1993). The globin genes of chicken and human red blood cells are unmethylated, while the same genes are methylated in tissues that do not produce globin (Groudine and Weintraub, 1981). The chicken ovalbumin gene is unmethylated in oviduct cells but is methylated in other tissues (Mandel and Chambon, 1979) and demethylation is associated with immunoglobulin class switching (Rogers and Wall, 1981). The clonal propagation of DNA methylation patterns and its apparent repressive effect on transcription makes this epigenetic modification of DNA similar to the developmentally important homeotic repressors of *Drosophila* such as *polycomb* (Paro and Hogness, 1991) and the heterochromatin proteins (Wregget *et al.*, 1994). In mammals DNA methylation has been shown to participate in a number of important processes including the control of gene expression, developmental regulation (Monk *et al.*, 1987), genomic imprinting (Li *et al.*, 1993), cellular mosaicism (McGowan *et al.*, 1989),

X-chromosome inactivation (Riggs, 1975) and the development and progression of a number of human diseases (Hall, 1990) including cancers (Sapienza and Hall, 1995; Versteeg, 1997). Bestor (1990) and Bestor and Tycko (1996) have suggested that cytosine methylation in eukaryotes may also provide a host-defense system against viral infection similar to that found in prokaryotes. The methylation of cytosine also forms a mutational "hotspot" due to the hydrolytic deamination of m⁵C to thymine. The mutational rate of a methylated cytosine is 4X greater than an unmethylated DNA (Duncan, 1980). The result being that these C -> T transitional mutations can account for around one-third of all mutation in humans (Bestor and Coxon, 1993). We have recently proposed that gamete-of-origin specific methylation and imprinting of loci evolved in order to enhance the evolutionary potential of these loci (McGowan and Martin, 1997).

DNA Methyltransferase

The sequences of many bacterial and several eukaryotic DNA (cytosine-5) methyltransferase genes (human, mouse, chicken, *Xenopus*, sea urchin, and *Arabidopsis*) are known (Yen, *et al.*, 1992; Bestor *et al.*, 1988; Tajima *et al.*, 1995; Kimura *et al.*, 1996; Aniello *et al.*, 1996; Finnigan and Dennis, 1993). The most studied of the vertebrates MTases is that of the mouse. The mouse MTase contains 500 amino acids in the C-terminal catalytic domain (Lauster *et al.*, 1989; Postfai *et al.*, 1989) and an N-terminal regulatory domain of 1000 amino acids (Bestor, 1990). Comparison of the derived protein sequences between all these sequences has revealed the presence of at least 8 conserved regions located in the c-terminal portion of the protein (Adams, 1995). These conserved motifs,

numbered I, II, IV, VI, VII, VIII, IX, and X according to their prokaryotic counterparts, are involved in sequence target recognition and the catalytic/methyl transfer process. The methyltransfer process involves an initial disruption of the DNA causing unpairing and flipping of the cytosine 180° out from the duplex (Erlanson *et al.*, 1993; Klimasauskas *et al.*, 1994). This is followed by the activation of position 5 of cytosine which catalyzes the acceptance of a methyl group from S-adenosyl methionine (AdoMet). The N-terminal of the protein contains a region responsible for localization of the enzyme to the replication foci and, unique to eukaryotes, a nuclear localization signal which is required for the enzyme to locate to the nucleus (Leonhardt *et al.*, 1992). The C-terminal and the N-terminal of the protein is separated by a series of lysine-glycine repeats that have been proposed to be a junction site between a carboxy region similar to prokaryotic cytosine-5 methyltransferase and an amino terminal domain specific to eukaryotic DNA methyltransferases (Bestor *et al.*, 1988).

DNA Methylation During Development

The activity of DNA methyltransferase (MTase) during development has been studied in a number of animals. In both *Xenopus* and sea urchin, nuclear methyltransferase activity is highest in blastula stage embryos (Adams *et al.*, 1981; Tosi *et al.*, 1995) and a number of genes in sea urchin have shown developmental stage specific changes in methylation and correlated changes in transcription (Fronk *et al.*, 1992). In chick embryos, methylase activity is highest in the early embryo while DNA methylation remains relatively constant throughout development (Tanaka *et al.*, 1980).

The roles of DNA methyltransferase activity and DNA methylation during embryogenesis are best understood in mammals. Methyltransferase activity is highly regulated during spermatogenesis (Benoit and Trasler, 1994; Jue *et al.*, 1995) and differences in the methylation of many genes have been observed between sperm and eggs (Sanford *et al.*, 1987; Kafri *et al.*, 1992). Using antibodies for DNA methyltransferase, Carlson *et al.* (1992) demonstrated stage specific changes in the cellular localization of the enzyme during mouse development. Methyltransferase is localized to the cytoplasm during 1-4 cell stage and blastocyst stage. Between the 1-4 cell and blastocyst stages, the compacted 8-cell stage, the enzyme is localized in the nucleus. Later, methyltransferase is also observed in the nuclei of all tissues of post-implantation mouse embryos but is highest in the neural tissues (Goto *et al.*, 1994; Trasler *et al.*, 1996). Initially the sperm genome is relatively methylated compared to the genome of the oocyte. During development of the mouse, a period of overall demethylation of the genome has been observed post- 8 cell stage which lasts until approximately the blastocyst stage. A period of extensive de novo methylation begins at gastrulation and ultimately produces levels higher than those observed in either of the gametes (Monk *et al.*, 1987). These dynamic changes in DNA methylation and methyltransferase localization during early development and its relationship to gene expression suggest a role for methylation in development. In support, a number of tissue specific genes and genes expressed during mammalian development has been shown, at least in part, to be regulated by DNA methylation (Eden and Cedar, 1994). Some of the most well studied examples of these genes include myoD (Brunk *et al.*, 1996), *Xist* (Ariel *et al.*, 1995) and the imprinted genes: A 300 bp enhancer sequence located -20 kb from the myoD promoter undergoes

demethylation just prior to myogenesis in the mouse embryo. The promoters of most alleles on the inactive X-chromosome are methylated while the same sequences on the active X-chromosome are unmethylated (Rastan, 1994). The exception in this case being the *Xist* locus, a candidate for the X-inactivation center, which is unmethylated on the inactive X chromosome and methylated on the active X chromosome (Ariel *et al.*, 1995). Genomic imprinting is a process that differentially restricts the genetic availability of an allele depending on the sex of the parent contributing that allele. The alleles of a number of imprinted mouse transgenes (Sapienza *et al.*, 1987; Reik *et al.*, 1987) and endogenous imprinted genes such as *H19*, *Igf2*, and *Igf2r* are differentially methylated depending on parent-of-origin (Efstratiadis, 1994; Reik and Allen, 1994). Mice, homozygous for a targeted mutation of DNA methyltransferase die at 10-20 somite stage and display stunted growth, abnormal somites and evidence of cell death. However, early development, the time when changes in methylation are most pronounced, was virtually normal. This is thought to be due to the large amounts of maternally deposited methyltransferase (Li *et al.*, 1992). This fact also makes it difficult to determine when a lack of methylation has its greatest effect on mouse development. However, improper imprinting of several genes in these mice demonstrates the importance of DNA methylation for the establishment and maintenance of the 'imprint' (Li *et al.*, 1993). Regardless, in order to evaluate the role of DNA methylation during very early development it may be necessary to directly inactivate the methyltransferase enzyme present in the embryo.

While of considerable evolutionary distance from mammals, experiments with plants have also shown to implicate a role for methylation in

development. The genome of plants is even more methylated than in vertebrates. Mutants of the plant *Arabidopsis thaliana* showed 70% reduction in genomic 5-methylcytosine levels and important developmental defects such as abnormal number and shape of leaves, and a delayed onset of flowering (Kakutani *et al.*, 1995). Nearly identical results were observed in *Arabidopsis* which were transformed with an expression construct containing *Arabidopsis* MTase in the antisense orientation (Finnegan *et al.*, 1996; Ronemus *et al.*, 1996). Mutants of the fungi *Neurospora* that have reduced levels of MTase show abnormal chromosome segregation and stability (Foss *et al.*, 1993).

All of these examples suggest that DNA methylation plays an important epigenetic role in the development of a wide range of organisms.

5-azacytidine and DNA Hypomethylation

The nucleotide analog 5-aza-cytidine (5-azaC), through its incorporation into DNA (Jones and Taylor, 1980) or by interfering with the action of the enzyme methyltransferase (Santi *et al.*, 1983) is routinely used to produce demethylation of DNA sequences in a number of mammalian cell culture systems (reviewed in Cedar and Razin, 1990). While the primary result of 5-azaC treatment is DNA hypomethylation, some have suggested its effects on gene expression and cell differentiation are not directly linked to demethylation (Christman *et al.*, 1985; Jutterman *et al.*, 1994). However, cells treated with other cytidine analogs such as 6-azacytidine and cytosine arabinoside are ineffective at inducing cell differentiation or DNA demethylation (Jones and Taylor, 1980). Cells treated with 5-azaC show a number of effects. These treatments often resulted in cell differentiation (transformation) and activation of a number of previously silent genes

including genes located on the 'silent' X-chromosome (Grant and Worton, 1989). The most classic experiment showing 5-azaC induced cell differentiation was performed by Taylor and Jones (1985). Mouse embryonic 10T1/2 cells treated with 5-azaC differentiated into muscle cells and adipocytes. The DNA from 5-azaC-treated cells was hypomethylated relative to DNA from untreated cells. Similar phenotypic changes and cell differentiation effects are observed in 10T1/2 cells that are transfected with plasmid constructs which express DNA (cytosine-5) methyltransferase in the antisense orientation (Szyf *et al.*, 1992). This supports the hypothesis that the differentiation state of some cell types is controlled by DNA methylation and the effects of 5-azaC treatment are the result of DNA hypomethylation. In mammals, the centromeric heterochromatin has been found to be enriched in 5-methyl-cytosine (Miller *et al.*, 1974). Its role in the production of heterochromatin can be demonstrated by the use of 5-azaC and 5-azadC (5-aza-2'-deoxycytidine) which leads to the under condensation of the same centromeric heterochromatin (Schmid *et al.*, 1984) and the inhibited condensation of inactive euchromatin or facultative heterochromatin (Haaf and Schmid, 1989). Experiments exposing embryos *in vivo* to 5-azaC have suggested that methylation plays a role in early development. Sea urchin embryos treated prior to the 16 cell stage with 5-azaC showed arrested and abnormal development (Branno *et al.*, 1993). Analysis of the *in vitro* translation products of mRNA from 5-azaC-treated and untreated embryos showed that 5-azaC treatment did not impair transcription. In agreement with results from cell culture experiments, 5-azaC treated sea urchin embryos expressed an increased number of mRNA species. Chick embryos treated during blastoderm stage with 5-azaC developed a short, thickened primitive streak, an abnormal neural plate,

and similar to that in sea urchin expressed larger numbers of polypeptides than control embryos (Zagris and Podimatas, 1994). In mammals, rat embryos derived from hypomethylated sperm (via 5-azaC injection of donor males) developed abnormally or did not develop past 1 cell stage while sperm derived from 6-azaC injected donor males developed normally (Doerksen and Trasler, 1996). Post-implantation embryos, retrieved from pregnant mice and rats administered 5-azaC showed a number of malformations including limb and digit anomalies, exencephaly, micrognathia, and rib defects (Takeuchi and Takeuchi, 1985; Rosen and House, 1990; Cummings, 1994). The fact that 5-azacytidine and not other cytidine analogs prevented sporangia formation in the slime mold *Physarum polycephalum* lead Hildebrandt (1986) to propose that methylation was required to repress some genes during that part of the slime mold life cycle.

Early Zebrafish Development

As I will show here, treatment of developing zebrafish embryos with 5-azacytidine results in abnormal development of the axial mesoderm.

The adult zebrafish, like most fish, develops initially from a fertilized egg by a series of discoidal meroblastic cleavages (incomplete cleavage) (Gilbert, 1991). These incomplete cleavages produce a mound of blastomeres on top of a large vegetal yolk. This period occurs over approximately 2 hours and produces a blastodisc containing 64 cells (Kimmel *et al.*, 1995). Between 2 and 5 hours of the development the embryo is said to be in the blastula stage. During this period the embryo enters the mid blastula transition (or the beginning of RNA transcription), forms the yolk syncytial layer, and begins a period of coordinated rapid

cellular movements called epiboly. It is during this period of epiboly that different cell layers of the embryo are established. Investigators have been unable, due to extensive cell mixing, to produce a fate map of the zebrafish prior to epiboly stages. Analysis of cell fate by cell labeling and lineage tracing in epiboly stage zebrafish embryos suggest that the organization of the zebrafish fate map is similar to those constructed for *Xenopus* (Kimmel *et al.*, 1990). Epiboly is a process of cell movement where the cells of the blastodisc spread radially over the yolk cell, engulfing it. Along with the streaming of cells over the yolk, there are additional, complex cell movements of involution, convergence and extension which ultimately produce the primary germ layers (ectoderm, mesoderm, and endoderm) and the embryonic axis. The involution movements of gastrulation produce a thickened marginal region around the entire rim of the blastoderm called the germ ring. Mediolateral convergent and intercalation cell movements at one end of the embryo produce a localized thickening and accumulation of cells in the germ ring. This region is called the embryonic shield and is thought to be equivalent to the Spemann's Organizer of amphibians (i.e. having the ability to induce a secondary axis (Ho, 1992)) and can be identified by the expression of the gene *goosecoid* (Stachel *et al.*, 1993). The involuting cells of the embryonic shield and the germ ring (the hypoblast) ultimately form the prechordal plate and chorda mesoderm (the notochord and lateral mesoderm). These continued cell movements towards the dorsal side of the embryo lead to extensive elongation of the embryo along the anterior-posterior axis with the zebrafish head positioned at the animal pole and the tail bud positioned at the vegetal pole. Rapid cellular differentiation follows this formation of the body axis at approximately 10 hour post-

fertilization. The cells (epiblast) overlying the involuted hypoblast cells differentiate to form the neural keel, ultimately forming the central nervous system. The involuting cells of the hypoblast begin to differentiate to form the notochord, muscle begins the differentiation process in an anterior to posterior direction, and the primary organ rudiments begin to be observed. The final differentiation of the notochord and somatic muscle further elongates the zebrafish embryo producing a prominent tail and by 24 hours of development the embryo shows the appearance of a small fish. As stated previously, treatment of zebrafish embryos with the hypomethylating agent 5-azacytidine produced abnormal development of the axial and paraxial mesoderm and dorsal ventral patterning (notochord, somites and CNS). The role of the axial mesoderm in patterning the trunk of the zebrafish embryo has become apparent by the analysis of a number of zebrafish mutants, especially the *no tail* mutation. The *no tail* mutants possess a mutation in the gene homologous to the mouse gene *brachyury* (Schulte-Merker *et al.*, 1994) and fail to develop a differentiated notochord (Halpern *et al.*, 1993). Another zebrafish mutation *floating head*, whose mutant gene encodes the homologue of the *Xenopus* gene *Xnot*, also fails to form a differentiated notochord (Talbot *et al.*, 1995). Both of these mutations showed a shortened tail, abnormal development of the floor plate of the neural keel, and abnormal differentiation of the somites. Mosaic analysis using the *no tail* mutants (i.e. transplantation of wild-type cells into mutant embryos) has demonstrated the requirement for signals from the notochord for normal differentiation of the somites into chevron-shaped myotomes. *No tail* mutant embryos completely lack *eng* expression in the muscle pioneer cells of the somites revealing that expression of *eng* appears to be required for this final differentiation of the somites (Halpern *et al.*,

1993). The notochord has also been shown to be important in the differentiation of the ventral floorplate of the neural tube. In chick, grafting of notochord cells next to lateral portions of the neural tube results in the transformation of these lateral neural tube cells to cells showing floorplate morphology (van Straatum and Hekking, 1991; Smith and Schoenwolf, 1989). Mutants which lack a notochord such as the mouse T mutants and the zebrafish mutant *floating head* do not develop a floorplate (Herrmann *et al.*, 1990; Talbot *et al.*, 1995). The zebrafish mutant *no tail* does however develop a normal floorplate but this normal floorplate is located only in the anterior portions (trunk) of the embryo (Halpern *et al.*, 1993). The ability of the notochord to induce floorplate cells via cell contact suggest that a secretory protein might be involved in this process. Studies using the zebrafish mutant *cyclops* have revealed that the gene *sonic hedgehog* is a likely candidate for this floorplate inducing molecule (Krauss *et al.*, 1993). This gene is initially expressed in the germ ring and notochord of zebrafish embryos and is then later expressed in the floorplate suggesting that *sonic hedgehog* autoregulation is probably involved.

Many genes that are believed to be involved in axis formation and dorsal ventral patterning have been cloned from the zebrafish. I will use these genes as probes to analyze the developmental phenotype of experimentally hypomethylated zebrafish embryos.

DNA Methylation in Zebrafish

The zebrafish *Danio rerio* has become increasingly popular as a model organism for vertebrate development and genetics. These fish have external fertilization, high reproductive output, and optically clear

embryos giving them a number of advantages over much more established models of development such as mouse. The establishment of genetic methods, and large scale mutagenesis, has identified mutations that affect the development of this organism (Streisinger *et al.*, 1981; Kimmel, 1989; Rossant and Hopkins, 1992; Mullins and Nüsslein-Volhard, 1993; Solnica *et al.*, 1994). The production of a genetic linkage map of the zebrafish genome consisting of random amplified polymorphic DNAs (RAPDs), simple sequence repeats (SSRs), and cloned genes (Goff *et al.*, 1992; Postlethwait *et al.*, 1994), along with tools such as zebrafish X mouse somatic cell hybrids for example (Ekker *et al.*, 1996) has aided in the analysis of zebrafish mutations and genetics.

Despite the fact that we are making rapid strides in our understanding of the roles of different genes in zebrafish development, there are obvious gaps within the zebrafish research communities agenda. Possibly one of the most important examples is the study of epigenetic effects on development (which have been shown to be so important in *Drosophila*) such as chromatin modification, DNA modification, and the proteins and DNA sequences involved in directing these processes. The role of DNA methylation in zebrafish development can be included within this group. Furthermore, it appears that DNA methylation may at least in part be responsible for the poor expression of most zebrafish transgenes (Gibbs *et al.*, 1994; Martin and McGowan, 1995a; Martin and McGowan, 1995b), a fact that has hindered much progress in zebrafish transgenic work.

At present, except for my own work, there is virtually nothing known about DNA methylation in the zebrafish or its role in zebrafish embryonic development. I had previously studied a zebrafish transgene which showed an inverse correlation between the level of transgene DNA

methylation and the level of mosaic transgene expression in the skin epithelium of the fish (Martin and McGowan, 1995a). By breeding these transgenic zebrafish with various zebrafish strains, I demonstrated the presence of strain specific modifiers of DNA methylation. Furthermore, factors such as environmental temperature and the drug sodium butyrate which prevents histone-deacetylation were shown to affect the methylation level of this transgene. More interesting, this transgene also displayed parent-of-origin specific modification of DNA methylation (Martin and McGowan, 1995b) (i.e.. its methylation was affected by the sex of the parent contributing the transgene allele) that was remarkably similar to the genomic imprinting phenomenon as seen in mammals. While these studies did show differences in transgene methylation in the gametes and later in development, they tell us little about the direct role of DNA methylation in zebrafish development. These first experiments were important however, since they introduced the zebrafish as a model organism for the study of DNA methylation in vertebrates and furthermore suggested that we may have to reevaluate hypotheses concerning the evolutionary importance of DNA methylation and genomic imprinting in mammals (McGowan and Martin, 1997).

I have continued to study DNA methylation in the zebrafish. Here I report the sequence analysis of a partial cDNA encoding the gene for the zebrafish DNA (5-cytosine) methyltransferase and its spatial and temporal expression during zebrafish development. To complete the final tier of the methylation process, I have also analyzed the global level of DNA methylation in zebrafish sperm and throughout early embryonic development. In order to investigate possible roles for DNA methylation in the development of the zebrafish, I experimentally hypomethylated the

genomes of developing zebrafish embryos *in vivo* by treatment with 5-azacytidine. Using histological analysis and whole mount *in situ* hybridization with probes of known genes, I was able to define a specific phenotype for these hypomethylated embryos.

Materials and Methods

DNA Sequencing and Analysis

The 5-cytosine DNA methyltransferase cDNA insert within Bluescript SK- was sequenced by the dideoxy-nucleotide termination method using a Sequenase Version 2.0 Kit (Amersham) for manual sequencing and a Thermo Sequenase Fluorescent Labeled Primer Cycle Sequencing Kit (Amersham) for automated sequencing. Both the M13 forward primer (5'-CCCAG TCACG ACGTT GTAAA ACG-3') and the M13 reverse primer (5'-AGCGG ATAAC AATTT CACAC AGG-3') were used to sequence the complete 2.4 kb. cDNA insert. Sequencing reactions were subject to electrophoresis in a 6% Long Ranger Gel (Mandel) on a standard adjustable long plate sequencing apparatus (CBS Scientific Co.) or a LiCor 4000L automated sequencing apparatus.

The sequence was compared to all registered sequences stored at a number of data banks using the BLAST Network Service (Blast) (Altschul *et al.*, 1990) available via e-mail at the National Center for Biotechnology Information (NCBI) (e-mail address: blast@ncbi.nlm.nih.gov). This program aligns DNA or protein sequences and suggests registered sequences with the greatest degree of sequence identity.

DNA sequence and protein 'stackup' alignments of the zebrafish methyltransferase sequence and other known eukaryotic MTase DNA and amino acid sequences were performed using AlignSeq (W.R. Pearson, Univ. of Virginia) and Clustal IV software (Higgins *et al.*, 1991).

Animals

Embryos were obtained from zebrafish purchased from a local supplier. Fish were maintained in 20 gallon aquaria. Embryos were obtained by placing 3-4 male and female zebrafish in breeding cages the evening prior to the morning collection. The breeding cages were composed of two stacked plastic mouse cages where the floor of the top cage contained many holes. This allows fertilized eggs, upon being laid, to fall between the space of the two cages. The next morning, embryos are collected shortly after spawning which is induced by the onset of the photo period. Embryos were maintained at 28.5°C in 100 mm petri dishes and staged according to hours post fertilization (p.f.) (Westerfield, 1995).

Northern Analysis

Total RNA was extracted from zebrafish embryos and tissues using Trizol Reagent (Gibco BRL). Approximately 300 embryos were crushed in 1.5 ml of Trizol Reagent using a ground glass dounce. The resulting Trizol mixture was then processed according manufactures recommendations. 20 µg of total RNA from each sample were denatured in loading buffer solution (50% formamide, 0.12% bromophenol blue, 0.12% xylene cyanol - final conc.) by heating at 70°C for 5 minutes. The RNA was subject to electrophoresis in an agarose gel containing 1.2% agarose, 6.7% formaldehyde, and 1X borate buffer (0.12 M boric acid, 0.25 mM EDTA). The gel was run at 3-4 V/cm for approximately 4 hours in 1X borate buffer. A sample of a 0.24 - 9.5 kb RNA size markers (Gibco BRL) was included for each northern analysis. Following electrophoresis the size marker lane was removed and stained using ethidium bromide and photographed for later size determination. The gel containing the RNA

samples was soaked in distilled water 3X 15 minutes to remove formaldehyde. The RNA was then transferred to nylon membrane and hybridized to ^{32}P labeled probes (see methods under Southern blot).

Whole mount in situ hybridization

Fixation of embryos and *in situ* hybridization were performed as previously described (Akimenko *et al.*, 1994). The RNA probes used in this and the following chapter were made from: a 2.4 kb zebrafish DNA methyltransferase cDNA, a 1.1 kb α -tropomyosin cDNA (Weinberg *et al.*, 1996), 1.75 kb *axial* cDNA (Strähle *et al.*, 1993), 0.7 kb *floating head* cDNA (Talbot *et al.*, 1995), 2.2 kb *no tail* cDNA (Halpern *et al.*, 1993), 1.44 kb *shh* cDNA (Krauss *et al.*, 1993), 2.6 kb *engrailed 2* cDNA (Ekker *et al.*, 1992a), 2.0 kb *msxC* cDNA (Ekker *et al.*, 1992b), a 1.4 kb *Z-FGF4R* cDNA (Thisse *et al.*, 1995), 1.9 kb *krx20* cDNA (Oxtoby and Jowett, 1993).

The *in situ* hybridization used was based on the hybridization of a digoxigenin (DIG) labeled antisense RNA probe to corresponding native sense RNA of the study tissue or organism. The labeled probes are produced by *in vitro* RNA synthesis from a linearized DNA template in a reaction containing 1 μg linearized template DNA, NTPs (10 mM ATP, 10 mM CTP, 10 mM GTP, 6.5 mM UTP, 3.5 mM DIG -11-UTP (Boehringer Mannheim)), transcription buffer (40 mM Tris pH 8.0, 6 mM MgCl_2 , 10 mM DTT, 10 mM NaCl, 2 mM spermidine), 20 units RNasin (Promega), 20 units RNA polymerase (typically T7 RNA polymerase; BRL). This reaction was incubated at 37°C for 1 hour and another 20 units RNA polymerase was added to the mixture. After another hour of incubation, the synthesized probe was precipitated by the addition of LiCl

(0.4 M final concentration) and ethanol (75% final volume). The precipitated probe was resuspended in distilled water to a final concentration of approximately 100 ng/ μ l.

Embryos were prepared for *in situ* hybridization by fixation in 4% paraformaldehyde in PBS (0.07 M Na_2HPO_4 , 0.03 M NaH_2PO_4 , 0.5 M NaCl) at 4°C overnight. Embryos were washed 2X in PBS and placed at -20°C in methanol overnight. Embryos were then rehydrated in serial dilutions of methanol and PBS (75% MeOH/25% PBS; 50% MeOH/50% PBS; 25% MeOH/75% PBS) for 5 minutes each. The final rehydration of the embryos occurs in three, five minutes washes of PBST (1X PBS, 0.1% Tween-20). Following hydration, embryos were acetylated (believed to reduce background signal) by incubating in a solution containing 125 μ l triethanolamine and 27 μ l acetic anhydride in 10 ml distilled water for 10 minutes. After two, ten minute washes in PBST, embryos were prehybridized at 65°C for three hours in a solution containing 50% deionized formamide, 5X SSC (20X stock=3.0 M NaCl, 0.3 M citric acid), 0.1% Tween-20, 50 μ g/ml heparin, 10 mM citric acid and 100 μ g/ml yeast tRNA. The prehybridization solution was replaced by a fresh solution containing 1 ng/ μ l of DIG-labeled antisense RNA probe and the embryos were hybridized overnight. The hybridized embryos were then washed at 65°C for ten minutes in a serial dilution of hybridization mix and 2X SSC (75% hyb mix/25% 2X SSC; 50% hyb mix/50% 2X SSC; 25% hyb mix/75% 2X SSC; 100% 2X SSC), 2X 30 minute washes at 60°C in 0.2X SSC, and room temperature washes for 5 minutes in a serial dilution of 0.2X SSC and PBST (75% 0.2X SSC/25% PBST; 50% 0.2X SSC/50% PBST; 25% 0.2X SSC/75% PBST; 100% PBST). Detection of the digoxigenin RNA probe uses an Anti-DIG alkaline phosphatase conjugated antibody

(Boehringer Mannheim) and a chromogenic reaction. The embryos were preincubated for 1 hour at room temperature in PBS containing 2% calf serum and 2 mg/ml BSA. Embryos were then incubated at room temperature in PBST containing 0.2% calf serum, 0.2 mg/ml BSA, and preabsorbed Anti-DIG AP antibody (1:5000 dilution) for 4 hours. Embryos were washed overnight at 4 °C in PBST, followed by 6X 15 minutes washes in PBST. Prior to the chromogenic color reaction, the embryos were equilibrated for 5 minutes in reaction buffer (100 mM Tris pH 9.5; 50 mM MgCl₂; 100 mM NaCl; 0.1% Tween-20; 1 mM levamisole). The embryos were then stained at room temperature in darkness in reaction buffer (4 ml) containing 14 µl of BCIP (50 mg/ml in 70% dimethylformamide (DMF) and 30% water) and 27 µl NBT (50 mg/ml in DMF). The staining reaction was carried out for variable periods of time in order to obtain adequate staining for photography. For this reason, the level of staining can not be used as a quantifiable method of determining the absolute level of transcription. After completion of the color reaction, embryos were washed in PBST, post-fixed for 2 hours in 4% PFA in PBS, and stored in PBS containing 5 mM sodium azide.

Whole embryos were photographed using a Nikon HFX-DX stereo microscope camera system or mounted and photographed using a Zeiss AxioScope compound microscope.

DNA preparation and End-labeling.

Mature sperm was collected from 12 male fish. DNA were prepared from the offspring of random breedings containing approximately 1000 blastula stage embryos, and 500 embryos of gastrula stage (6 hour), and

similar numbers of embryos collected at 16 hour or 24 hour. DNA was extracted by placing zebrafish tissue or embryos in a 1.5 ml eppendorf tube containing 500 μ l. of digestion buffer (1% SDS, 100 mM NaCl, 100 mM EDTA, 50 mM Tris pH 8.0) and 0.5 μ g/ml proteinase K (Sigma). The samples were incubated for 3-4 hours at 56°C in a water bath. The DNA was extracted from the digested tissues with phenol (saturated with 0.5 M Tris-Cl pH 8.0), PCI (25 phenol:24 chloroform:1 iso-amyl alcohol) and CIA (24 chloroform:1 iso amyl alcohol). The extracted DNA was precipitated with sodium acetate (0.2M final conc.) and isopropanol (50% final conc.). The resulting DNA pellet was washed in 70% ethanol, 100% ethanol, and vacuum dried at room temperature. The DNA pellet was redissolved in TE (100 mM Tris pH 8.0, 1 mM EDTA). The DNA concentration was determined spectro-photometrically or using a fluorimeter. DNA preparations were digested 3 hours-overnight with the restriction enzymes *HpaII* and *MspI* according to the manufacturers instructions (Gibco/BRL, New England Biolabs). Complete digestion was tested by the addition of control plasmid DNA (typically pBluescript KS+) to an aliquot of the experimental digest. Digestion was considered complete when the internal control digest gave a digest pattern identical to the pattern obtained when the test plasmid was digested alone.

Digested DNA was end-labeled with [α -³²P]dCTP using the Klenow fragment of DNA polymerase I (Monk *et al.*, 1987). DNA fragments were separated by electrophoresis in a 0.8% agarose gel. Approximately 100,000 cpm of labeled fragments from each sample were used. *Hind* III cut lambda DNA was added to a single well to act as a DNA fragment size marker. Following electrophoresis, the DNA within the gel was visualized by ethidium bromide staining and a photograph was taken using

a transilluminator and Polaroid camera. DNA fragments were transferred in 20X SSC by capillary action onto Hybond nylon membrane (Amersham).

Southern Analysis

Southern blots containing 5 µg of *HpaII* and *MspI* digested DNA or uncut DNA were prepared and hybridized according to Sapienza *et al.* (1987). Uncut DNA and digested fragments were subjected to electrophoresis as previously described. Prior to Southern transfer of DNA to nylon membrane (Hybond-N, Amersham), the DNA fragments were denatured in 0.6 M NaCl and 0.2 M NaOH for 45 minutes. The DNA was immobilized on the nylon membrane by baking for 2 hours at 80°C. The membrane was washed in 0.1X SSC (20X stock=3.0 M NaCl, 0.3 M citric acid) and 0.1% SDS at 65°C, followed by overnight prehybridization at 43°C in a solution containing 6X SSC, 10X Denhardts solution (100X stock=2% ficoll, 2% BSA, 2% polyvinylpyrrolidone), 500 µg/ml denatured salmon sperm DNA and 0.5% SDS. ³²P-labeled probes were prepared by random primer labeling (Sambrook *et al.*, 1989). Blots were hybridized overnight at 43°C in a solution containing 50% deionized formamide, 4X SET (1X=0.15 M NaCl, 0.03 M Tris, 2.0 mM EDTA, pH 8.0), 1X Denhardts solution, 100 µg/ml denatured salmon sperm DNA, 0.5% SDS and 1 X 10⁶ cpm/ml of ³²P-labeled probe. Blots were then washed at 65°C in several changes of 0.1X SCC and 0.1% SDS. Blots were hybridized to a ³²P random primer labeled probe of the zebrafish interspersed repetitive element *Mermaid /DANA* (Izsvák *et al.*, 1996; Shimoda *et al.*, 1996a; Shimoda *et al.*, 1996b).

Blots were analyzed using a Bio-Rad GS-525 Molecular Imager System.

DNA Probe Preparation

E. coli (strain XL1-Blue) were made competent and transformed, with plasmids containing inserts used as probes for Southern and northern hybridization, using the hexaminecobalt chloride method (Sambrook *et al.*, 1989). Transformed bacteria were plated on LB media agar plates containing 100 µg/ml. ampicillin as a selective agent. A single bacterial colony from these plates was used to inoculate a 75 ml culture of L-broth liquid media containing 100 µg/ml ampicillin. This culture was grown overnight at 37°C with rapid agitation in a shaker incubator. The plasmid DNA was purified from the confluent bacterial culture using a Qiagen Midi Plasmid Prep Kit (Qiagen). The purified plasmid was digested with the appropriate restriction endonuclease to release the DNA fragment to be used as a probe. The resulting DNA fragments were separated by electrophoresis in a 0.8% agarose gel. The separated fragments were visualized using a UV transilluminator and ethidium bromide staining. A scalpel was used to cut a line in the gel just ahead and immediately behind the DNA fragment of interest. Pre-wetted NA45 DEAE ion exchange membrane (Schleicher & Schuell) was placed into the gel incisions. The gel was replaced into the electrophoresis box and the voltage re-applied for 10-30 minutes. The fragment migration was monitored periodically using a UV transilluminator. When the fragment of interest had completely migrated into the collection membrane, electrophoresis was discontinued. The collection membrane was removed and rinsed in Tris-acetate running buffer, and placed in a high salt elution buffer (1.0M NaCl, 50 mM arginine) and incubated at 68°C for 1-2 hours. The fluid from the tube containing the DEAE membrane was extracted with PCI and CIA, and

precipitated with 0.3 M sodium acetate and isopropanol. The resulting DNA pellet was washed in ethanol, dried, and re dissolved in TE and stored at -20°C until later use.

5-aza-cytidine and 6-aza-cytidine treatment

5-aza-cytidine and 6-aza-cytidine (Sigma) were dissolved in distilled water. Embryos were treated with a final concentration of 50 μ M 5-aza-cytidine in embryo medium (Westerfield, 1995). The concentration of 50 μ M was determined by producing a dose-response curve with treatment concentrations ranging from 0 μ M to 100 μ M. Embryos resulting from these treatments were scored as normal, showing the short-tailed phenotype, or dead. After the treatment period, embryos were washed extensively in embryo medium (0.137 M NaCl; 5.4 mM KCl; 0.25 M Na₂HPO₄; 0.44 mM KH₂PO₄; 1.3 mM CaCl₂; 1.0 mM MgSO₄; 4.2 mM NaHCO₃). Control embryos were raised in normal embryo medium. DNA and Southern analysis of 6-azacytidine, 5-azacytidine and untreated zebrafish embryos were prepared as described previously.

Preparation of embryos for cryostat sectioning and slide mounting

Embryos were cryosectioned according to Westerfield (1995) and photographed using a Zeiss Axioscope. Whole fixed embryos (post *in situ* hybridization) were embedded in a molten 40°C solution of 1.5% agar and 5% sucrose. After solidification of embedding media, a block containing the embryo to be sectioned was cut with a razor blade from the embedding media. These trimmed blocks were placed in a 30% sucrose solution and stored at 4°C overnight (or until the block has sunk in the sucrose solution). The embryo containing blocks were mounted on the cryostat

chuck by first preparing a raised platform by freezing a layer of OCT compound (Tissue Tek, Miles) with 2-methyl butane (-50° C) to the chuck. A drop of OCT compound was then applied to the platform followed by the agar block. The chuck and block were then frozen again in liquid nitrogen, and allowed to equilibrate to -25°C (sectioning temperature). The block was sectioned 16 µm thick using a LKB cryostat. Serial sections were adhered to glass slides and allowed to dry for 24 hours. Slides containing sectioned embryos were prepared for mounting by dehydrating for 5 minutes in a serial dilution of ethanol (20% EtOH; 40% EtOH; 60% EtOH; 80% EtOH; 90% EtOH; 95% EtOH; 2X 100% EtOH). Sections were cleared in two washes of xylene for 1 minute. Several drops of Cytoseal 60 mounting media (Stephens Scientific) were applied onto the tissue sections and a cover glass was laid over sections. Mounting media was allowed to dry overnight.

Results

Zebrafish Methyltransferase cDNA Sequence Analysis

During the process of conducting an expressed sequence tagging (EST) project on the zebrafish, Dr. Z. Gong (University of Singapore) retrieved a partial cDNA clone thought to encode the zebrafish DNA (cytosine-5) methyltransferase gene. This clone was generously donated to conduct this research.

The cDNA clone was sequenced in both directions using M13 forward (5'-CCCAG TCACG ACGTT GTAAA ACG-3') and reverse primers (5'-AGCGG ATAAC AATTT CACAC AGG-3') as well a primer created from known sequences within the cDNA clone (5'-TGTGA AGCAG GAGAA AATGG AGC-3'). The cDNA was found to be 2412 bp long and containing a 21 nucleotides of poly (A) tail (Figure 1). The nucleotide sequence contains an open reading frame (ORF) starting at nucleotide position 1 and continues to the stop codon (TAA) at position 2101 (Figure 1). This results in a 3' untranslated region of 308 bp.

A Blast (basic search alignment tool) search (Altschul *et al.*, 1990) performed with this sequence revealed very high sequence identity with all cloned eukaryotic DNA (5-cytosine) methyltransferases, the highest identity occurring with *Xenopus* (Genbank # D78638)(Figure 2). In comparing the sequence of the zebrafish MTase cDNA with the sequence of *Xenopus* we see a 75% identity between the zebrafish sequence and the frog counterpart. The higher sequence identity of 77% in the 3' portion of the cDNA probably reflects the fact that it is this region which contains the sequences encoding the functional portions of the MTase protein. The Blast search also indicated high, but slightly lesser, levels of sequence identity with DNA (cytosine-5) methyltransferases from other species:

mouse (Genbank # X14805), chicken (Genbank #D43920), human *H. sapiens* (GenBank # X63692), and sea urchin *P. lividus* (GenBank # Z50183) (Table 1). The high sequence identity of our cDNA sequence and that of other MTases suggests that this cDNA does represent the zebrafish DNA (cytosine-5) methyltransferase.

The deduced zebrafish DNA (cytosine-5) methyltransferase amino acid sequence was also aligned with those of the carboxyl-terminal region of the MTases previously aligned at the nucleotide level (Figure 3). When the zebrafish MTase amino acid sequence is aligned together with the amino acid sequences of human, mouse, chicken, *Xenopus*, and sea urchin MTases, I observed an overall 62% amino acid identity between all groups. Individual amino acid comparisons against the zebrafish MTase show the highest identity with *Xenopus* at 86% and the lowest identity with sea urchin at 70% (Table 1).

More than 50 DNA (5-cytosine) methyltransferase genes have been cloned and characterized from both prokaryotes and eukaryotes (reviewed in Kumar *et al.*, 1994). Comparative analysis of these genes has revealed the presence of a conserved set of 10 ordered motifs, 8 of which are conserved within the eukaryote MTases. Similar to other eukaryotic MTases, the zebrafish MTase also contains these 8 conserved motifs (termed I, II, IV, VI, VII, VIII, IX, X) which are arranged in a similar order and are located in the carboxyl-terminal end of the protein (Figure 3). Motif IV of the zebrafish MTase contains an invariant (G-PC---S) amino acid group that is conserved in all prokaryote and mammalian MTases. While high amino acid conservation is observed in the carboxyl-terminal between eukaryote MTases, we observed a large relatively non-conserved or variable region between amino acid motifs VIII and IX. It has also been shown that the

amino-terminal region (a region not present in this partial cDNA clone) of eukaryote MTases is not present in prokaryote MTases. In eukaryotes including the zebrafish, the conserved carboxyl-terminal domain is separated from the amino-terminal domain by a set of lysine-glycine repeats (KG - repeats). Regardless, the conservation of amino acid motifs within the different MTases proteins suggests that these domains provide important function to the activity of the enzyme. The partial zebrafish cDNA contains the complete carboxyl terminal region of the proteins, and therefore also contains the primary functional domains of the zebrafish protein.

To map the chromosomal location of the zebrafish MTase gene, the differences in nucleotide sequence between zebrafish and mouse MTase were used to design PCR primers located in the 3' untranslated region of the zebrafish MTase (#1 5' TGT GAA GCA GGA GAA AAT GGA GC 3' and #2 5' CAC ATG AAT GGC ACT GCA CAG AC 3'). These primers were used to specifically amplify the zebrafish MTase gene from template DNAs collected from a panel of zebrafish X mouse somatic cell hybrids (Egger *et al.*, 1996). While these primers were effective in specifically amplifying the zebrafish MTase gene from these templates, only 1 hybrid line (ZFB 212) which unfortunately contained several different zebrafish chromosome segments (linkage groups: I, III, IV, IX, XIV, XX, and XXII) was found to be positive for the zebrafish MTase gene (data not shown). At this time I am unable to map this gene to a specific zebrafish linkage group.

DNA (cytosine-5) methyltransferase Expression in Zebrafish

Using a zebrafish MTase probe, I performed northern blot analysis on RNA collected from blastula (3 hr), gastrula (6 hr), somite stage (16 hr) and tail stage (24 hr) embryos. A transcript of approximately 5.0 kb was observed from all samples, however, there was a significant drop in the transcription of MTase following the blastula stage (Figure 4a). These blots were subsequently stripped and re-probed with the zebrafish Max gene (Schreiber-Agus *et al.*, 1994) (Figure 4b). The Max gene has a relatively uniform level of expression throughout development and serves to confirm equal loading of RNA and RNA integrity. The total size of my zebrafish MTase cDNA is 2.4 kb. Since northern analysis indicates a transcript size of approximately 5.0 kb, my cDNA represents the 3' half of a complete zebrafish MTase cDNA.

Whole mount *in situ* hybridization using an antisense RNA MTase probe also confirms a drop in MTase expression following blastula stages. During blastula and gastrula stages, MTase is expressed ubiquitously in all cells of the embryo (Figure 5 a, b, c). During somatogenesis (12 hr pf.), the highest level of MTase expression were observed in the brain, neural keel and in the newly forming anterior somites (Figure 5 d, e). At 24 hours, MTase expression was strongest in the eyes, CNS and the newly forming somites of the tail bud (Figure 5 f, g). Expression in the CNS was strongest in the forebrain, mid-brain, and hind-brain. Observations of stained cells at high magnification show that within the somites, expression of this gene appears to be strongest in a vertical row of cells at the anterior boundary between somites (Figure 5g). The onset of expression of MTase in the zebrafish somites appears to approximate the time of initial *myoD* expression in the somites (Weinberg *et al.*, 1996).

MyoD is a gene that has been shown to be involved in the initial events of muscle differentiation. Close inspection of the expression patterns of both MTase and *myoD* show that MTase is expressed in the anterior portion of the somite, while *myoD* expression occurs in the posterior portion of the somites. The spatial and temporal expression of MTase in the zebrafish, especially in the tissues where it is strongest, suggest that MTase may be important for their development and differentiation.

Dynamics of DNA Methylation During Early Zebrafish Development.

To evaluate the level of DNA methylation during development, DNA samples from sperm and pooled embryos of different stages were digested with the restriction enzyme *HpaII* or its isoschizomer *MspI* both of which cut DNA at the sequence CCGG. These two enzymes are different in that *MspI* will cleave this sequence regardless of whether or not the cytosine residues are methylated, while *HpaII* will only cleave this sequence in the absence of methylation (Waalwijk and Flavell, 1978). The relative amount of DNA methylation can then be determined by comparing the relative proportion of high and low molecular weight DNA fragments. This method is sufficiently sensitive to assay the very small amounts of DNA obtained from very early stage embryos. Southern blots were prepared from these digested DNAs and hybridized with the zebrafish repetitive sequence *Mermaid* (Shimoda *et al.*, 1996a; Shimoda *et al.*, 1996b). This repetitive sequence is interspersed and represents 5-10% of the zebrafish genome. The results of this analysis, presented in Figure 6, show that mature sperm DNA is hypermethylated relative to blastula and gastrula stages. The methylation status of egg DNA was not tested due to the difficulty in obtaining sufficient quantities of DNA. From fertilization to

gastrulation, DNA methylation levels became markedly reduced. After gastrulation, DNA methylation in the embryo increased to levels similar to those observed in sperm. This was indicated by the relative changes in mid-molecular weight DNA fragments. Densitometric scans were performed across lanes to display relative differences in the quality of DNA fragments. DNA from both above experiments were also subjected to enzymatic digestion by *MspI*, an isoschizomer of *HpaII* whose activity is insensitive to methylation (Figure 6). The distribution of labeled DNA fragments in these digests was equivalent in all cases irrespective of the treatment. This last result, and the complete digestion of internal control plasmids (see Materials and Methods), suggest that the differences in DNA fragment sizes produced by the *HpaII* digestion of the various samples are attributable to differences in cytosine methylation. To ensure these sample DNAs do not contain degraded DNAs fragments which might skew my analysis, Southern blots containing uncut DNAs (Figure 6b) were hybridized with the repetitive sequence probe. These blots showed single bands of high molecular weight DNA (> 24 kb) and showed no evidence of low molecular weight DNA degradation products. While we do see an apparent difference in molecular weight between some samples (samples 2 and 3, for example), this does not appear to affect the outcome of my analysis since we see distinct methylation differences between these samples and the fact that *MspI* digests between all samples appear identical.

Nearly identical results of the analysis presented above were obtained using three different sets of zebrafish sperm and embryo DNAs representing three different collection times and source of zebrafish. Furthermore, a similar although less sensitive analysis was conducted using the protocol of Monk *et al.* (1987) (data not shown). This protocol was successfully used

to determine the overall level of DNA methylation in the gametes and embryos of the mouse and involves ^{32}P end-labeling and electrophoresis of restriction enzyme digested DNA fragments. Monk *et al.* (1987) also used repetitive sequence hybridization in their analysis. This analysis of zebrafish sperm and embryo DNA produced near identical results to the Southern blot analysis described above.

Treatment of Zebrafish Embryos With 5-azacytidine During Blastula Results in an Abnormal Body Axis.

To investigate a role for DNA methylation during vertebrate development, I treated developing zebrafish embryos with 50 μM 5-azacytidine (5-azaC) starting immediately after fertilization. The concentration used in these experiments was determined by producing a dose-response curve whereby fertilized zebrafish embryos were treated with concentrations ranging from 0 μM to 100 μM (Figure 7). When such embryos were examined 24 hours post fertilization, approximately 20-30% of treated embryos showed a shortened trunk and tail and loss or abnormal development of somites. Later at 48 hours, these embryos displayed a beating heart, normal head and eyes, loss of tail, block shaped somites and an enlarged yolk sac (Figure 8 A, B). The trunk muscles of these abnormal embryos show distinct muscle fibers, however the fibers are poorly organized and the somites failed to form chevron shaped myotomes (Figure 9). A mortality level of 10% or less, above untreated controls, was observed when treating at 50 μM concentration. The remaining embryos did not show any apparent phenotype. Embryos treated with 5 μM or 10 μM 5-azaC showed no obvious external phenotype at 24 hours, whereas treatments with concentrations of 100 μM

or greater caused severe lethality. A number of studies have used the cytidine analog 6-azacytidine (6-azaC) to control for possible cytotoxic effects (Jones and Taylor, 1980; Doerksen and Trasler, 1996). This molecule possesses an azide group on position 6 of cytidine and has been shown not to affect DNA methylation. We treated zebrafish embryos with concentration of 6-azaC ranging from 5 - 100 μ M and did not observe any effects on development (data not shown). This suggests that the abnormal development of 5-azaC treated embryos is the consequence of DNA hypomethylation.

The phenotype caused by 5-azaC treatment resembles that seen in the zebrafish *no tail* and *floating head* mutants which are known to completely lack a differentiated notochord (Halpern *et al.*, 1993; Talbot *et al.*, 1995). The region underlying the neural keel where the notochord normally resides appeared to be filled with undifferentiated mesoderm and cells that express α -tropomyosin, a marker of terminally differentiated muscle (Figure 8 C-H). While muscle fibers which express α -tropomyosin were present in 5-azaC phenotypic embryos, the muscle did not become properly organized into chevron-shaped myotomes. Transverse sections of 24 hour zebrafish showed a conspicuous notochord underlying the neural keel of wild-type embryos (Fig. 8 E, F) and the absence of a differentiated notochord in 5-azaC treated embryos (Figure 8 G, H). Notochord was observed in some embryos displaying the abnormal trunk phenotype ("phenotypic embryos"); however, in these cases, a normal vacuolated notochord was primarily restricted to anterior portions of the trunk. Development of the brain and neural keel did not appear to be affected in 5-azaC-treated embryos. To show the lack of effect of 5-azaC treatment on the differentiation of the CNS, I performed *in situ*

hybridization using probes for a number of genes expressed in this tissue. The expression patterns of genes such as *krx20* in the 3rd and 5th rhombomeres of the brain (Oxtoby and Jowett, 1993) (Figure 10 A, B) and Z-FGFR4 in the telencephalon, diencephalon, and the hindbrain (also expressed in the tail bud)(Thisse *et al.*, 1995) (Figure 10 C, D) are normal. The expression patterns of *eng2* in the mid/hindbrain boundary (Ekker *et al.*, 1992a) (see figure 13), and *msxC* in the neural keel (Ekker *et al.*, 1992b) (data not shown) were also normal. The phenotype of the embryos treated with 5-azaC suggest that this drug's developmental effect is to perturb the development of the axial mesoderm.

5-azacytidine Causes DNA Hypomethylation in vivo, and Acts Specifically During Blastula and Early Gastrula

In order to determine if 5-azaC was effective in inhibiting DNA methyltransferase in developing zebrafish embryos, I collected DNA from 24 hour control and 5-azaC-treated embryos and analyzed the overall methylation status of the genome with the method described above.

Southern blot analysis, like that used to determine DNA methylation vs. developmental stage, of the *HpaII* cleaved DNA probed with the repetitive sequence *Mermaid/DANA* (Izsvák *et al.*, 1996; Shimoda *et al.*, 1996a; Shimoda *et al.*, 1996b) were consistent with reduced DNA methylation in 5-azaC-treated embryos (Fig. 11A). The *Mermaid/DANA* sequence used as a probe in this experiment is a repetitive element which comprises approximately 10% of total zebrafish DNA and is thought to be uniformly distributed throughout the zebrafish genome (Izsvák *et al.*, 1996). Our Southern analysis with this probe is therefore indicative of global DNA methylation. Embryos treated with 5-azaC showed reduced DNA

methylation compared to untreated siblings (Figure 11A). The labeling of DNA in the untreated lane is skewed towards the higher molecular weight, while the 5-azaC-treated lanes had a much higher abundance of labeled fragments of low molecular weight (Figure 11A). This skewing towards the high molecular weights in the control DNA relative to the DNA collected from treated embryos is indicative that 5-azaC treatment resulted in decreased DNA methylation. Densitometric tracings of the lanes confirm this observation. In fact, if we compare these blots with the Southern blots (Figure 6) where I determined the relative DNA methylation of developing zebrafish embryos, the genome methylation of 24 hour 5-azaC treated embryos approximates the genome methylation of 6 hour gastrula embryos. The densitometric scans indicate that both 6 hr wildtype embryos and 24 hr 5-azaC treated embryos have relative lane densities, at the levels tested, of nearly 2X relative to that of 24 hr untreated embryos and other embryonic stages. The DNA collected from embryos treated with 6-azaC however, showed levels of DNA methylation similar to untreated embryos. This confirms previous reports that 6-azaC is unable to induce DNA hypomethylation.

DNA from both above experiments were also subjected to enzymatic digestion by *MspI*, an isoschizomer of *HpaII* whose activity is insensitive to methylation (Figure 11A). The distribution of labeled DNA fragments in these digests was equivalent in all cases irrespective of the treatment. This last result, and the complete digestion of internal control plasmids (see Materials and Methods), suggest that the differences in DNA fragment sizes produced by the *HpaII* digestion of the various samples are attributable to differences in cytosine methylation. Again, to ensure these sample DNA do not contain degraded DNA fragments which might skew my analysis,

Southern blots containing uncut DNA (Figure 11B) were hybridized with the repetitive sequence probe. These blots show single bands of high molecular weight DNA (> 24 kb) and show no evidence of low molecular weight DNA degradation products.

A proportion of embryos treated with 5-azacytidine showed no abnormal phenotype. These embryos, although to a lesser degree, did show evidence of DNA hypomethylation (data not shown). This indicates that there is a minimal level of DNA hypomethylation in the genome (or at least a minimal level of hypomethylation to shift the probability of hypomethylating important developmental genes) to produce the abnormal short tailed phenotype.

To determine the developmental time period during which 5-azaC is acting to produce the abnormal axial phenotype, we conducted a time-course experiment on developing embryos. Figure 12 shows the developmental periods for which embryos were treated with 5-azaC (solid lines) and the resulting percentage of embryos at 24 hours exhibiting the "short-tailed phenotype". Few embryos showed this phenotype when 5-azaC was administered for the first 3 hours after fertilization and then removed. Embryos that received 5-azaC starting at 3 hours were just as affected as those that received the drug immediately after fertilization. However, embryos that received 5-azaC starting at 6 hours did not show any phenotype. Assuming that the 5-azaC enters the embryo immediately upon treatment and is completely washed away following treatment, these results suggest a sensitive period during blastula and early gastrula, a time that corresponds to the stages of highest DNA methyltransferase expression (Figure 4).

5-azacytidine Causes Disruption in the Normal Differentiation of the Notochord

Analyses of the zebrafish *brachyury* mutant *no tail* (Halpern *et al.*, 1993) and the mouse *T* mutants (Herrmann *et al.*, 1990; Smith *et al.*, 1991) showed a requirement for the *brachyury* gene product in the differentiation of the notochord and subsequent development of the floorplate of the neural tube and the muscle pioneer cells. In order to better define the phenotype caused by 5-azaC treatment, I have examined the expression of the genes *no tail*, *eng*, and *sonic hedgehog*. In zebrafish, *no tail* is first expressed at 4 hours post fertilization at the margin, and continues to be expressed in the later involuting axial mesoderm (Schulte-Merker *et al.*, 1992). By 24 hours, *no tail* expression is limited to the differentiated notochord and the caudal mesoderm of the tail bud (Figure 13A). 5-azaC phenotypic embryos showed large gaps (Figure 13B) in *no tail* expression in the differentiated notochord or near complete loss of *no tail*-expressing cells (Figure 13C). However, *no tail* expression in the tail bud cells was completely normal in 5-azaC treated embryos.

Mosaic analysis using wild type and *no tail* mutant embryos has demonstrated a requirement for *no tail* signaling to induce *eng* protein expression in the muscle pioneer cells of the developing somites (Halpern *et al.*, 1993). In 5-azaC treated embryos, expression of *eng2* transcripts in the muscle pioneer cells was nearly completely absent along the trunk and tail (Figure 13 E, F) compared to that of controls (Figure 13D). While loss of expression of *eng2* was observed in 5-azaC treated embryos, these same embryos showed normal expression of *eng2* in the mid/hindbrain boundary. This result is consistent with a role for *no tail* in inducing *engrailed* expression in muscle pioneer cells. Loss of *eng*

expression in muscle pioneer cells was correlated with failure in proper myotome formation. Since I observed virtually no *eng2* expression in 5-azaC treated embryos, I wished to determine if the little expression I did observe was located in regions which possessed normal, *no tail* expressing notochord cells. I performed double *in situ* hybridization labeling on embryos with both *no tail* and *eng2* antisense probes. Along with control embryos, 5-azaC treated embryos which showed fragments of *no tail* expressing notochord cells were serial sectioned to correlate both *no tail* in the notochord and *eng2* expression in muscle pioneers. In control embryos, *no tail* and *eng2* expression could be directly correlated (Figure 13G). However, I observed no *eng2* expression in muscle pioneer cells even in sections which revealed *no tail* expressing notochord cells (Figure 13H). While mosaic analysis experiments imply a requirement for *no tail* expression from the notochord to induce *eng2* expression in muscle pioneers, this result suggests that either normal DNA methylation is required for the muscle pioneer cells to be receptive to signals from the notochord or that normal methylation of the somatic mesoderm is required for muscle differentiation to proceed to the point where these signals can be received.

In 24 hour wild-type zebrafish embryos the gene *sonic hedgehog* (Krauss *et al.*, 1993; Roelink *et al.*, 1994) is expressed in the ventral floorplate of the neural tube (Figure 14A). The ventral floorplate in 5-azaC-treated embryos was present; however, a number of abnormalities in the structure of the floorplate were observed as discontinuities (Figure 14B), kinking, and terminal forking. Similar abnormalities in the ventral floorplate have been observed in *floating head* mutant zebrafish, which also lack a differentiated notochord, (Talbot *et al.*, 1995) and the mouse T mutants

(Herrmann *et al.*, 1990). Studies on these notochord mutant animals have suggested a requirement for signals from notochordal precursor cells to induce floorplate in the overlaying neural tube. The phenotype observed in hypomethylated embryos using these molecular markers suggests a specific loss of differentiated notochord cells, and abnormal muscle development.

5-azacytidine Affects the Intercalation and Organization of the Involuting Axial Mesoderm

The results of the time course experiment indicate that 5-azacytidine has to be administered during blastula and early gastrula in order to produce the short-tail phenotype. The process of gastrulation defines the dorsal-ventral body axis and involves the involution and intercalation of the epi- and hypoblastic cells, followed by the differentiation of these cells to specific mesodermal fates, a process that involves a number of inductive events. We have looked at the expression of a number of genes during gastrulation that are thought to be involved in both these morphogenetic events.

During gastrulation the genes *no tail*, *floating head*, and *axial* are expressed in the axial mesoderm and are involved in the proper development of the axial mesoderm (Halpern *et al.*, 1993; Talbot *et al.*, 1995; Strähle *et al.*, 1993, respectively). At 10 hours post fertilization expression of these genes was observed as an elongated domain appearing on most of the length of the developing body axis, with a node of stronger expression occurring in the tail bud region (Figure 15 A, C, E). Expression of these genes in 5-azaC-treated embryos reflected a shortened and thickened axial mesoderm (Figure 15 B, D, F). The region of strong expression in the tail bud was

absent in 5-azaC-treated embryos. The intensity of the expression signal in individual cells did not appear to be affected by 5-azacytidine. The apparent decrease in the intensity of the *in situ* hybridization signal in treated embryos can be attributed to the fact that the cells expressing these genes are much less compacted in treated embryos than in controls, suggesting a disruption in the normal intercalation movements of the axial mesoderm cells. Twenty to thirty percent of 10 hour 5-azaC-treated embryos displayed these abnormal expression patterns, a percentage similar to the percentage of treated embryos showing the short-tailed phenotype after 24 hours of development.

Figure 1 - Partial cDNA sequence (3' end) and protein translation of the zebrafish DNA (5-cytosine) methyltransferase gene. This partial cDNA sequence contains 2412 nucleotides and a 3' untranslated region consisting of 309 nucleotides.

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1/1
AAT TCG GCA CGA GGG TTG GTG AAC TAT AAG GGG GAG CAG TAC AAA GTA GGG GAC AGT GTC
asn ser ala arg gly leu val asn tyr lys gly glu gln tyr lys val gly asp ser val
61/21
TAC CTA CCA CCT GAG GCC TTT AAC TTT GTG GTA AAG GCT GCA AGC CCG GTG AAG CGT TCC
tyr leu pro pro glu ala phe asn phe val val lys ala ala ser pro val lys arg ser
121/41
CAC AGA AAA GAT GAC GTT GAT GAA GAT CTT TAC CCT GAA TAC TAC AGG AAG TCC TCT GAT
his arg lys asp asp val asp glu asp leu tyr pro glu tyr tyr arg lys ser ser asp
181/61
TAC ATC AAA GGC TCA AAT CTG GAT GCT CCT CAG CCT TTT CGC ATT GGG CGC ATC AAA GAA
tyr ile lys gly ser asn leu asp ala pro gln pro phe arg ile gly arg ile lys glu
241/81
ATC TTC TGC AAC AAG CGC AGC AAT GGA AAG CCA GAC ACC ACA GAG ATC AAA CTG CGC CTC
ile phe cys asn lys arg ser asn gly lys pro asp thr thr glu ile lys leu arg leu
301/101
TAT AAA TTC TAC AGA CCT GAA AAC ACT CAC AAG GGA CCA AAG GGA GCA TAT CAT TCT GAC
tyr lys phe tyr arg pro glu asn thr his lys gly pro lys gly ala tyr his ser asp
361/121
ATA AAC CAA CTC TAC TGG AGT GAC GAA GAG GCG ACC GTC AGC ATG ACT GAG GTG CTG ACA
ile asn gln leu tyr trp ser asp glu glu ala thr val ser met thr glu val leu thr
421/141
CGC TGT CGT GTT GAG TAT GCA GAG GAT CTG GTT GAA AGT GTG CAG GAC TAT TCA AAC AAA
arg cys arg val glu tyr ala glu asp leu val glu ser val gln asp tyr ser asn lys
481/161
GGA CCC GAT CGC TTC TAT TTC CTT GAG GCA TAC AAT GCC AAA ACC AAG AGC TTT GAG GAT
gly pro asp arg phe tyr phe leu glu ala tyr asn ala lys thr lys ser phe glu asp
541/181
CCT CCC AAT CAT GCT AGA TCT GCT GTT AAT AAG GGC AAA GGG AAA GGA AAG GGC AAG GGA
pro pro asn his ala arg ser ala val asn lys gly lys gly lys gly lys gly lys gly
601/201
AAG GGA AAG GGA AAG CAG CGC TCA AGA ACC ACA GGA TCA GGA GCA CAA GAG CCA GTG GTT
lys gly lys gly lys gln arg ser arg thr thr gly ser gly ala gln glu pro val val
661/221
CCC AAA CTG CGC ACT CTG GAT GTA TTC TCT GGC TGT GGT GGT CTG TCT GAG GGC TTC CAT
pro lys leu arg thr leu asp val phe ser gly cys gly gly leu ser glu gly phe his
721/241
CAA GCA GGC ATC TCA GAG ACT CAC TGG GCA ATA GAG ATG TGG GAC CCT GCC GCT CAG GCT
gln ala gly ile ser glu thr his trp ala ile glu met trp asp pro ala ala gln ala
781/261
TTC AGG TTG AAC AAT CCA GGC ACG ACC GTT TTC ACA GAG GAC TGT AAC GTC CTG CTG AAG
phe arg leu asn asn pro gly thr thr val phe thr glu asp cys asn val leu leu lys
841/281
CTG GTA ATG TCT GGA GAG AAG ACT AAC TCT CTT GGA CAG AAG CTG CCA CAG AAG GGT GAC
leu val met ser gly glu lys thr asn ser leu gly gln lys leu pro gln lys gly asp
901/301
GTG GAG ATG CTC TGC GGT GGG CCC CCT TGC CAA GGC TTC AGT GGA ATG AAC CGC TTC AAC
val glu met leu cys gly gly pro pro cys gln gly phe ser gly met asn arg phe asn
961/321
TCT CGC ACA TAC TCA AAG TTC AAG AAC TCT CTG GTT GTT TCT TAT CTG AGT TAT TGT GAC
ser arg thr tyr ser lys phe lys asn ser leu val val ser tyr leu ser tyr cys asp
1021/341
TAC TAC AGA CCC AGA TTC TTC CTG CTG GAG AAC GTG AGG AAC TTT GTG TCA TTT AAG CGC
tyr tyr arg pro arg phe phe leu leu glu asn val arg asn phe val ser phe lys arg
1081/361
TCC ATG GTC CTG AAG CTC ACA CTG CGC TGT CTA GTG CGC ATG GGC TAC CAG TGC ACC TTT
ser met val leu lys leu thr leu arg cys leu val arg met gly tyr gln cys thr phe
1141/381
GGT GTG CTG CAG GCT GGA CAG TAT GGG GTG GCC CAG ACT CGA CGC AGA GCT ATC ATC CTG
gly val leu gln ala gly gln tyr gly val ala gln thr arg arg arg ala ile ile leu
1201/401
GCT GCA GCC CCT GGT GAA AAG CTG CCC CGC TAC CCT GAA CCC TTG CAT GTG TTT GCC CCG
ala ala ala pro gly glu lys leu pro arg tyr pro glu pro leu his val phe ala pro

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1261/421	1291/431
CGA GCA TGT TCT CTT AGT GTG GCA GTT GAT	GAG AAG AAA TAT GTC AGC AAT GTT ACT CGT
arg ala cys ser leu ser val ala val asp	glu lys lys tyr val ser asn val thr arg
1321/441	1351/451
GGA AAT GGG GGC ATT TAT CGC ACC ATC ACA	GTA CGG GAC ACC ATG TCT GAC CTC CCA GAG
gly asn gly gly ile tyr arg thr ile thr	val arg asp thr met ser asp leu pro glu
1381/461	1411/471
ATC CGC AAT GGA GCT GCT GCA CTA GAG ATT	TCC TAC AAT GGC GAG CCA CAG TCC TGG TTT
ile arg asn gly ala ala ala leu glu ile	ser tyr asn gly glu pro gln ser trp phe
1441/481	1471/491
CAG AGG CAA ATT CGG GGC TCT CAG TAT CAG	CCC ATC CTC AGG GAC CAC ATC TGC AAG GAC
gln arg gln ile arg gly ser gln tyr gln	pro ile leu arg asp his ile cys lys asp
1501/501	1531/511
ATG AGT GCC CTG GTT GCT GCC CGC ATG CGT	CAC ATT CCT CTG GCT CCT GGT TCA GAC TGG
met ser ala leu val ala ala arg met arg	his ile pro leu ala pro gly ser asp trp
1561/521	1591/531
AGG GAT CTA CCT AAT ATT GAG GTG CGG TTG	CGG GAT GGC ACC ACC ACA AAA AAG CTT CGC
arg asp leu pro asn ile glu val arg leu	arg asp gly thr thr thr lys lys leu arg
1621/541	1651/551
TAC ACA CAC TCT GAC AAA AAG AAT GGC CGC	AGT GGC ACC GGT GCC CTG AGA GGA GTG TGT
tyr thr his ser asp lys lys asn gly arg	ser gly thr gly ala leu arg gly val cys
1681/561	1711/571
TCA TGT TCT GAA GGA AAA CAG TGT GAC CCT	GCA GAC AGG CAG TTC AAC ACC CTG ATT CCC
ser cys ser glu gly lys gln cys asp pro	ala asp arg gln phe asn thr leu ile pro
1741/581	1771/591
TGG TGT CTG CCT CAT ACG GGT AAC CGC CAT	AAT CAC TGG GCT GGT CTG TAT GGC CGC CTG
trp cys leu pro his thr gly asn arg his	asn his trp ala gly leu tyr gly arg leu
1801/601	1831/611
GAA TGG GAC GGA TTC TTC AGC ACT ACA GTT	ACC AAT CCT GAG CCA ATG GGA AAG CAG GGA
glu trp asp gly phe phe ser thr thr val	thr asn pro glu pro met gly lys gln gly
1861/621	1891/631
CGT GTC CTT CAC CCT GAG CAG CAC CGT GTG	GTG AGT GTC AGG GAA TGT GCA CGC TCT CAG
arg val leu his pro glu gln his arg val	val ser val arg glu cys ala arg ser gln
1921/641	1951/651
GGC TTC CCA GAC ACC TAC CGC TTC TTT GGC	AAT GTC CTA GAC AAA CAC AGA CAG GTT GGC
gly phe pro asp thr tyr arg phe phe gly	asn val leu asp lys his arg gln val gly
1981/661	2011/671
AAT GCT GTT CCT CCA CCC CTC TCC GAA ACC	ATT GGC CTG GAG GTC AAG AAA TGT GTG CTG
asn ala val pro pro pro leu ser glu thr	ile gly leu glu val lys lys cys val leu
2041/681	2071/691
GAG AAA ATG AGG GAG AAT GCT ACA GAG CCT	GTG AAG CAG GAG AAA ATG GAG CTC TCT GAC
glu lys met arg glu asn ala thr glu pro	val lys gln glu lys met glu leu ser asp
2101/701	2131/711
TAA AGC CAT CAC TCT GTA AAA TTG AAG CAC	ATT TTT CAT TGT GTT TGG ATC CAT CTG AAG
OCH	
2161/721	2191/731
CAT CAC TCC ATT TTA AAC ATT TTG TTA TTC	ATA AGC TGG TGT AGA GCT TGC CAC TTC TGC
2221/741	2251/751
GGC CTT TCT ATG GAC AGT CTG TGC AGT GCC	ATT CAT GTG ATG TTT TAT CAA TTT CTA AAT
2281/761	2311/771
GTG ATT TTA ACC ATG TAT TTG CAA TTG TCC	ACT CTA AGA CAG TGG TAT AAA TTC TAT ATA
2341/781	2371/791
GGT TTT TAT ATG TTG TAA TAT TTC AAA TAA	AGC TCT TAT TAA ATG TTA AAA AAA AAA AAA
2401/801	
AAA AAA AAC TGC	

Figure 2: DNA sequence alignment between the partial cDNA of the zebrafish DNA (5-cytosine) methyltransferase gene (I) and the corresponding region of the *Xenopus* DNA (5-cytosine) methyltransferase cDNA (GenBank #D78638) (II).

alignment of zebrafish (2412 NT) and xenopus (5033 NT)

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      10      20      30      40      50
I  AATTCGGCACGAGGGTTGGTGAACATAAGGGGAGCAGTACAAAGTAGGGGACAGTGT-
   : : : : : : : : : : : : : : : : : : : : : : : : : : : :
II AATTTGCTAC-AGTACAGCT-ACAAAGAATGATGTACATTATAAGGTTGGAGATGGAGTG
   2640      2650      2660      2670      2680      2690

      60      70      80      90     100     110
I  CTAC-CTACCACCTGAGGCC-TTTAACTTTGTGGTAAAGGCT-GCAAGCCCGGTGAAGCG
   : : : : : : : : : : : : : : : : : : : : : : : : : : : :
II CTACACTTCC--CTGATGCCTTTTCATTTAGT-GTAAA-ACTGGGTAGCCCTATGAAGCG
   2700      2710      2720      2730      2740      2750

     120     130     140     150     160     170
I  TTCCACAGAAAAGATGACGTTGATGAAGATCTTTACCCTGAATACTACAGGAAGTCCTC
   : : : : : : : : : : : : : : : : : : : : : : : : : : : :
II ACCCCAGAGGAAGGATGATGTGGATGAAGATCTATACCCAGAATACTACCGCAAATCATC
   2760      2770      2780      2790      2800      2810

     180     190     200     210     220     230
I  TGATTACATCAAAGGCTCAAATCTGGATGCTCCTCAGCCTTTTCGCATTGGG-CGCATCA
   : : : : : : : : : : : : : : : : : : : : : : : : : : : :
II AGACTACATTAAGGGTAGCAACCTGGATGCTCCAGAACCGTACCG-ATTGGGTGCGATTA
   2820      2830      2840      2850      2860      2870

     240     250     260     270     280     290
I  AAGAAATCTTCTGCAACAAGCGCAGCAATGGAAAGCCAGACACCACAGAGATCAAACCTGC
   : : : : : : : : : : : : : : : : : : : : : : : : : : : :
II AGGAGATCTTCTGCAATAAGAGAAGTAATGGAAAGCCTAATGAGGCAGATATAAAGCTGC
   2880      2890      2900      2910      2920      2930

     300     310     320     330     340     350
I  GCCTCTATAAATTCTACAGACCTGAAAACACTCAC-AAGGGACCAAAGGGAGCATATCAT
   : : : : : : : : : : : : : : : : : : : : : : : : : : : :
II GGATTTACAAGTTTTACAGACCTGAAAATACTCACAAGGTATGAAAGCAAG-TTACCAT
   2940      2950      2960      2970      2980

     360     370     380     390     400     410
I  TCTGACATAAACCAACTCTACTGGAGTGACGAAGAGGCGACCGT-CAGCATGACTGAGGT
   : : : : : : : : : : : : : : : : : : : : : : : : : : : :
II TCAGACATAAACATGGTTTACTGGAGCGATGAAGAGGCTGTAGTGGAG-TTTAAGGCGGT
   2990      3000      3010      3020      3030      3040

     420     430     440     450     460     470
I  GCTGACACGCTGTCTGTTGAGTATGCAGAGGATCTGGTTGAAAGTGTGCAGGACTATTC
   : : : : : : : : : : : : : : : : : : : : : : : : : : : :
II GCAAGGACATTGTACAGTAGAATATGGTGAAGATCTAACCGAATCCATTTCAGGAGTATTC
   3050      3060      3070      3080      3090      3100

     480     490     500     510     520     530
I  AAACAAAGGACCCGATCGCTTCTATTTTCCTTGAGGCATACAATGCCAAAACCAAGAGCTT
   : : : : : : : : : : : : : : : : : : : : : : : : : : : :
II TGCTGGAGGATCAGATCGCTTTTACTTCCCTTGAGGCCTATAATGCAAAGACCAAAAGCTT
   3110      3120      3130      3140      3150      3160

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	540	550	560	570	580	590
I	TGAGGATCCTCCCAATCATGCTAGATCTGCTGTTAATAAGGGCAAAGGAAAGGGAAGGG :: :::::::::: : :::::: : :::::::::: :::::::::: :::::::::: :::::::::: II TGAAGATCCTCCAAACCATGCTCGCGGTGCTGTT---AA---CAAAGGCAAAGGAAAGGG 3170 3180 3190 3200 3210 3220					
	600	610	620	630	640	650
I	C-AAGGGAAAAGGGAAAAGGGAAGCAGCGCTCAAGAACCACAGGATCAGGAGCACAAAGAGC : :::: :::::::: ::::: : : : : ::::: : : : : : : : : : : : : : : : : : II CAAAGGAAAAGGCCAAGGC AAAAC-TCCTTCAA-AGTCAGAGAATGAACAGCTGAA-CTC 3230 3240 3250 3260 3270					
	660	670	680	690	700	
I	CAGTGGT----T-CCC AA ACTGCGCACTCTGGATGTATTCTCTGGCTGTGGTGGTCTGTCTC ::: : :.: ::::::::::: ::::::::::: ::::::::::: ::::::::::: ::::::::::: ::: II TGGTGATAAACTGCCTAAACTGCGCACCTTGGATGTATTTTTCTGGTTGTGGAGGTCTTTTC 3280 3290 3300 3310 3320 3330					
	710	720	730	740	750	760
I	TGAGGGCTTCCATCAAGCAGGCATCTCAGAGACTCACTGGGCAATAGAGATGTGGGACCC ::: : : : : : ::::::::::: : ::::: ::::::::::: ::::::::::: ::::::::::: : : II TGAAGGTTTTTCACCAAGCAGGCATATCTGAGACAAACTGGGCAATAGAGATGTGGGAGCC 3340 3350 3360 3370 3380 3390					
	770	780	790	800	810	820
I	TGCCGCTCAGGCCTTTCAGGTTGAACAATCCAGGCACGACCGTTTTTCACAGAGGACTGTAA ::: : : : : : : : ::::::::::: II TGCAGCCCAGGCCTTCAGACTGAACAATCCTGGTACTACAGTGTTTACTGAAGACTGCAA 3400 3410 3420 3430 3440 3450					
	830	840	850	860	870	880
I	CGTCCTGCTGAAGCTGGTAATGTCTGGAGAGAAGACTAActCTCTTGGACAGAAGCTGCC : II CAT'TCTCCTGAACTAGTCATGTCTGGGGAAAAAACAAATTCCTAGGGCAGCGGTTGCC 3460 3470 3480 3490 3500 3510					
	890	900	910	920	930	940
I	ACAGAAGGGTGACGTGGAGATGCTCTGCGGTGGGCCCCCTTGCCAAGGCTTCAGTGG AAT : II GCAGAAAGGCGACGTGGAAATGCTCTGCGGAGGGCCCCCTTGCCAAGGTTTTTAGTGGCAT 3520 3530 3540 3550 3560 3570					
	950	960	970	980	990	1000
I	GAACCGCTTCAACTCTCGCACATACTCAAAGTTCAAGAACTCTCTGGTTGTTTCTTATCT : II GAACCGGTTTAACTCAAGAACCTATTCAAAGTTCAAGAACTCACTGGTGGTCTCGTATCT 3580 3590 3600 3610 3620 3630					
	1010	1020	1030	1040	1050	1060
I	GAGTTATTGTGACTACTACAGACCCAGATTCTTCCTGCTGGAGAACGTGAGGAACCTTTGT : II CAGCTACTGTGACTATTACAGACCTAAATACTTTCCTCTTGGA AACGTCCGGAACCTTTGT 3640 3650 3660 3670 3680 3690					

47


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2140      2150      2160      2170      2180      2190
I  TTGTGTTTGGATC-CATCTGAAGCATCACTCCATTTTAAACATTTTGTATTTCATAAGCT
   :. :  : :  : :  : :  : :  : :  : :  : :  : :  : :  : :  : :
II GAAT-TAAAGAGCAGATATTAA--ATC-GGACATTGT-CTCTTCTGGTTGATCA-AATTT
   4780      4790      4800      4810      4820

2200      2210      2220      2230      2240      2250
I  GGTGTAGAGCTTGCCACTTCTGCGGCCTTTCTATGGAC--AGTCTGTGCAG-TGCCATTC
   : :  : : : :  : :  : :  : :  : :  : :  : :  : :  : :
II TCTCTTCTGGTTG-ATCAAATGTTGACATTCCAAGCTCTTAATTTTATCTGCTGAAACGG
   4830      4840      4850      4860      4870      4880

2260      2270      2280      2290      2300
I  ATGTGATGTTTTATCA--ATTTCTAAATGTGATTTT--AACCA-TGTATTTGCAATTGTC
   : :  : :  : : : : : : : : : : : : : : : : : : : : : :
II ATGAGCTG-CGCAACAGCACTTCT-AA-GAAATTTTAAAAACAGTGGATTT--TATTAT-
   4890      4900      4910      4920      4930

2310      2320      2330      2340      2350      2360
I  CACTCTAAGACAGTGGTATAAATTCTATATAGGTTTTTATATGTTGTAATATTTCAAATA
   : :  : :  : :  : :  : :  : :  : :  : :  : :  : :  : :
II -ACGTTGTTACGTTGGCGTCTTACCTCTCCA-CTTTGTATACGTGCGAATATGTAGTTTT
   4940      4950      4960      4970      4980      4990

2370      2380      2390      2400
I  AAGCTCTTATTAAATGTTAAAAAAAAAAAAAAAAAAAA
   : :  : :  : :  : :  : :  : :  : :
II TATATGTTGTAATATTTGAAATAAACTTCTCTTGT
   5000      5010      5020      5030

```

Table 1: DNA and deduced amino acid sequence identity between the zebrafish DNA (cytosine-5) methyltransferase and MTases from *Xenopus*, chicken, human, mouse, and sea urchin. DNA sequence comparisons (average identity) were conducted using Blast and amino acid sequence comparisons were conducted using ClustalV.

<u>Species</u>	<u>DNA (BLAST)</u>	<u>Amino Acid</u>
Xenopus	75.5 %	86.0 %
chicken	75.5 %	83.5 %
human	75.0 %	82.0 %
mouse	75.5 %	79.5 %
sea urchin	71.5 %	70.0 %

Figure 3: Multiple alignment of deduced amino acid sequence for 5-cytosine DNA methyltransferase of zebrafish, human, mouse, chicken, *Xenopus*, and sea urchin. Alignment was determined using Clustal IV software (Higgins *et al.*, 1991). The symbol "*" indicates an amino acid that was identical in all six sequences, while the symbol "." indicates an amino acid that was similar in all six sequences. The conserved sequences of the KG-repeat, motifs I, II, IV, VI, VII, VIII, IX, and X are indicated and bolded.

zebrafish	-----NSARGLVNYKGEQYKVGDSVYLPPEAFNFVVKAAASPV
human	-----QLEDLSRVLYYSATKNGILYRVGDGVYLPPEAFTFNILKSSPV
mouse	-----GRVYCSSITKNGVCYRLGDSVYLPPEAFTFNIVKASPV
chicken	-----QYRVGDSVYLLPEAFSFSMKPASPA
xenopus	-----LDSKICYSTATKNDVHYKVGDLHFPDAFSFSVVKLGSPM
sea_urchin	RPGAKLEDQDDSSKVLVSSWHYKGNFQIGDGVYLLPEVFSFNIQKVVT
	... * * * . * * *
zebrafish	KRSHRKDDVDEDLYP-EYYRKSSDYIKGSNLDAQPFRIGRIKEIFCNKR
human	KRP-RKEPVDEDLYP-EHYRKYSYDIKGSNLDAPEPYRIGRIKEIFCPKK
mouse	KRP-KKDPVKENPVPRDTYRKYSYDIKGRNLDAPEPYRIGRIKEIHCGKK
chicken	KRP-KKEAVDEDLYP-EHYRKSEYIKGSNLDAPDYRVGRIKEIFCHIR
xenopus	KRPQRKDDVDEDLYP-EYYRKSSDYIKGSNLDAPEPYRLGRIKEIFCNKR
sea_urchin	KKPVSKDDVDEDLYP-ENYRKSSDYIKGSNLECEPFRIGKIISIYTTKS
	* . * * * * . * * * * * . * * * * * . * * * * *
zebrafish	SNGKPDTEIKLRLYKFYRPENTHKGPKGAYHSDINQLYWSDEEATVSMT
human	SNGRPNETDIKIRVNKFYRPENTHKSTPASVHADINLLYWSDEEAVVDFK
mouse	-KGKVNEADIKLRLYKFYRPENTHRSYNGSYHTDINMLCWSDEEAVVNF
chicken	TNGKPNADIKLRWKFYRPENTHKSMAKYHADINLLYWSDEETTVDFC
xenopus	SNGKPNADIKLRWKFYRPENTHKGMAKYHADINMVYWSDEEAVVEFK
sea_urchin	-----NSTVRLRVNKMYPEDTHKGRTAAYQADLNVLYWSEEEAVTELE
	 * . * * * * * . * * * * * . * * * * *
zebrafish	EVLTRCRVEYAEDLVESVQDYSNKGPDYFLEAYNAKTSFEDPPNHAR
human	AVQGRCTVEYGEDLPECVQVYSMGGPNRFYFLEAYNAKSKSFEDPPNHAR
mouse	DVQGRCTVEYREDLLESIQDYSQGGPDRFYFLEAYNSKTNFEDPPNHAR
chicken	AVQGRCTVYGEDLTESIQDYSAGGLDRFYFLEAYNAKTSFEDPPNHAR
xenopus	AVQGHCTVEYGEDLTESIQEYSAGGSDRFYFLEAYNAKTSFEDPPNHAR
sea_urchin	VVQGKCSVCAEDLNVSTDEYSAGGPHKFYFREAYDSERKCFEDPPSKSR
	* . * * * * . * * * * * . * * * * * . * * * * *
	<u>KG-repeats</u> <u>I</u>
zebrafish	SAVNKGKGKGKGKGKGKGK--KQRSRTTGSGAQEPVVPKLR TL DVFSGCGG
human	SPGNKGKGKGKGKGKGKPK---SQACEPSEPE-IEIKLPKLRTLDVFSGCGG
mouse	SPGNKGKGKGKGKGKGKGK--HQVSEPKEPE-AAIKLPKLRTLDVFSGCGG
chicken	SSGNKGKGKGKGKGKGKGSSTTCEQSEPEPTTELKLPKLRTLDVFSGCGG
xenopus	GAVNKGKGKGKGKGKGKGT--PSKSENEQLNSGD-KLPKLRTLDVFSGCGG
sea_urchin	STRMKGKGKGKGKGKAG-KIAVEKEEKEESTETPFNKLKCLDVFAGCGG
	***** * * * * . * * * * * . * * * * *
	<u>II</u>
zebrafish	LSEGFHQAGISETHWAIEMWDPAAQAFRLNNPGTTVFTEDCNVLLKLVMS
human	LSEGFHQAGISDTLWAIEMWDPAAQAFRLNNPGSTVFTEDCNILLKLVMA
mouse	LSEGFHQAGISETLWAIEMWDPAAQAFRLNNPGTTVFTEDCNVLLKLVMA
chicken	LSEGFHQAGVSETLWAIEMWEPAAQAFRLNNPGTTVFTEDCNVLLKLVMS
xenopus	LSEGFHQAGISETNWAIEMWEPAAQAFRLNNPGTTVFTEDCNILLKLVMS
sea_urchin	LSEGFHQAGICESSWAIEKEEPAAQAYRLNNPGSTVFSDDCNELLRLVMQ
	***** * * * * . * * * * * . * * * * *
	<u>IV</u>
zebrafish	GEKTNSLGQKLPQKGDVEMLCGGPPCQGFSGMNRFNRSRTYSKFKNLSLVVS
human	GETTNSRGQRLPQKGDVEMLCGGPPCQGFSGMNRFNRSRTYSKFKNLSLVVS
mouse	GEVTNSLGQRLPQKGDVEMLCGGPPCQGFSGMNRFNRSRTYSKFKNLSLVVS
chicken	GEKTNSLGQKLPQKGDVEMLCGGPPCQGFSGMNRFNRSRTYSKFKNLSLVVS
xenopus	GEKTNSLGQRLPQKGDVEMLCGGPPCQGFSGMNRFNRSRTYSKFKNLSLVVS
sea_urchin	GEKTSRTGQKLPQKGDVELLCGGPPCQGFSGMNRFNRSREYSKFKNLSISS
	** * * * * . * * * * * . * * * * * . * * * * *

	<u>VI</u>	<u>VII</u>
zebrafish	YLSYCDYYRPRFFLLENVRNFVSFKRSMVLKLTLRCLVRMGYQCTFGVLQ	
human	FLSYCDYYRPRFFLLENVRNFVSFKRSMVLKLTLRCLVRMGYQCTFGVLQ	
mouse	FLSYCDYYRPRFFLLENVRNFVSFKRSMVLKLTLRCLVRMGYQCTFGVLQ	
chicken	FLSYCDYYRPRFFLLENVRNFVSFKRSMVLKLTLRCLVRMGYQCTFGVLQ	
xenopus	YLSYCDYYRPRFFLLENVRNFVSFKRSMVLKLTLRCLVRMGYQCTFGVLQ	
sea_urchin	YLSYCDYYRPRFFLLENVRNFVSFKKSMVLKLTLRCLVRMGYQCTFGVLQ	
	.*****..*****... *****.*****.*****..**	
	<u>VIII</u>	
zebrafish	AGQYGVAQTRRRRAIILAAAPGEKLPRIYPEPLHVFAPRACSLSVAVDEKKY	
human	AGQYGVAQTRRRRAIILAAAPGEKLPRIYPEPLHVFAPRACQLSVVVDDKKF	
mouse	AGQYGVAQTRRRRAIILAAAPGEKLPRIYPEPLHVFAPRACQLSVVVDDKKF	
chicken	AGQYGVAQTRRRRAIILAAAPGEKLPRIYPEPLHVFAPRACQLSVVVDDKKF	
xenopus	AGQYGVAQTRRRRAIIVLAAAPGEKLPRIYPEPLHVFAPRACQLSVVVDDKKF	
sea_urchin	AAPGEKLPRIYPEPLHVFSSRACSLSVMIGEKKI	
	***** *****.*****.*****.*** ** *	
zebrafish	VSNVTRGNGGIYRTITVRDMSDLPEIRNGAAALEISYNGEPQSWFQRQI	
human	VSNITRLSSGPFRTITVRDMSDLPEVRNGASALEISYNGEPQSWFQRQL	
mouse	VSNITRLSSGPFRTITVRDMSDLPEIQNGASNSEIPYNGEPLSWFQRQL	
chicken	VSNITRTYSGPFRTITVRDMSDLPEIRNGASALEISYNGEPQSWFQRQI	
xenopus	VSNITRTNSSLFRTITVRDMSDLPEIRNGASALEISYNGEPQSWFQRQI	
sea_urchin	ESNNQWCLSAFYRTITVRDMSDLPTINNGAQKLEISYDGEQSDQFKKI	
	** *****.*** ** *	
zebrafish	RGSQYQPILRDHICKDMSALVAARMRHIPLAPGSDWRDLPNIEVRLRDGT	
human	RGAQYQPILRDHICKDMSALVAARMRHIPLAPGSDWRDLPNIEVRLSDGT	
mouse	RGSQYQPILRDHICKDMSALVAARMRHIPLAPGSDWRDLPNIEVRLSDGT	
chicken	RGSQYQPILRDHICKDMSALVAARMRHIPLAPGSDWRDLPNIEVRLSDGT	
xenopus	RGSQYQPILRDHICKDMSALVAARMRHIPLAPGSDWRDLPNIEVRLSDGT	
sea_urchin	RGNQYQPILRDHICKDMSALVAARMRHIPLAPGSDWRDLPNIEVRLSDGT	
	** *****.***** ** *	
zebrafish	TTKKLPHTHTLTKKNGRSGTGALRGVCSCEG-KQCDPADRQFNTLIPWCL	
human	MARKLRYTHHDRKNGRSSSGALRGVCSCEAG-KACDPESRQFNTLIPWCL	
mouse	IADKLQYTFHDVKNGYSSSTGALRGVCSCEAG-KACDPESRQFNTLIPWCL	
chicken	STRKLRYTHHEKNGRSGTGALRGVCSCEAG-KPCDPADRQFNTLIPWCL	
xenopus	TSRKLRYTHHDKKNGRSGTGALRGVCCCEG-KQCDPADRQFNTLIPWCL	
sea_urchin	TCRKLRYTHKDKKNGSSSTGALRGVCSCEAG-DACDPSDRQFNTLIPWCL	
	. ** *****.*** * *****.*** *****	
zebrafish	PHTGNRHNHWAGLYGRLEWDGFFSTTVTNPEPMGKQGRVLHPEQHRVSV	
human	PHTGNRHNHWAGLYGRLEWDGFFSTTVTNPEPMGKQGRVLHPEQHRVSV	
mouse	PHTGNRHNHWAGLYGRLEWDGFFSTTVTNPEPMGKQGRVLHPEQHRVSV	
chicken	PHTGNRHNHWAGLYGRLEWDGFFSTTVTNPEPMGKQGRVLHPEQHRVSV	
xenopus	PHTGNRHNHWAGLYGRLEWDGFFSTTVTNPEPMGKQGRVLHPEQHRVSV	
sea_urchin	PHTGNRHNHWAGLYGRLEWDGFFSTTVTNPEPMGKQGRVLHPEQHRVSV	
	*****.***** *****	
	<u>IX</u>	<u>X</u>
zebrafish	RECARSQGFPDITYRFFGNVLDKHRQVGNVPPPLSETIGLEVKKCVLEKM	
human	RECARSQGFPDITYRFLFGNILDKHRQVGNVPPPLAKAIGLEIKLCMLAKA	
mouse	RECARSQGFPDSYRFFGNILDRHRQVGNVPPPLAKAIGLEIKLCMLAKA	
chicken	RECARSQGFPDITYRFLFGNILDKHRQVGNVPPPLAKAIGLEIRACVGMARM	
xenopus	RECARSQGFPDITYRFLFGNILDKHRQVGNVPPPLAKAIGLEIKSCVLTRM	
sea_urchin	RECARSQGFPDITYRFFGSILDKHRQIGNVPPPMAAAIGMEIKVCLQTKT	
	*****.*** ** *.***.***.*****..***... *	

zebrafish	R-ENATEPVK-----QEKMELSD
human	R-ESASAKIK-----EEEEAAK---
mouse	R-ESASAARK-----AKEEAATKD
chicken	R-EESGAARVAPPAPKMEMTAAAD
xenopus	K-ENGTEPVK-----AEKMETD-
sea_urchin	KRDQERAALEP---VKEETESMD
	 *

Figure 4: Northern blot of blastula (3 hr), gastrula (6 hr), somite stage (16 hr) and tail stage (24 hr) zebrafish embryo total RNA (20 μ g) hybridized to the zebrafish DNA (cytosine-5) methyltransferase (MTase) cDNA (a) and the zebrafish 'Max' cDNA (b). MTase message is strongest in blastula stage embryos. The zebrafish 'Max' gene expression is relatively uniform throughout early development.

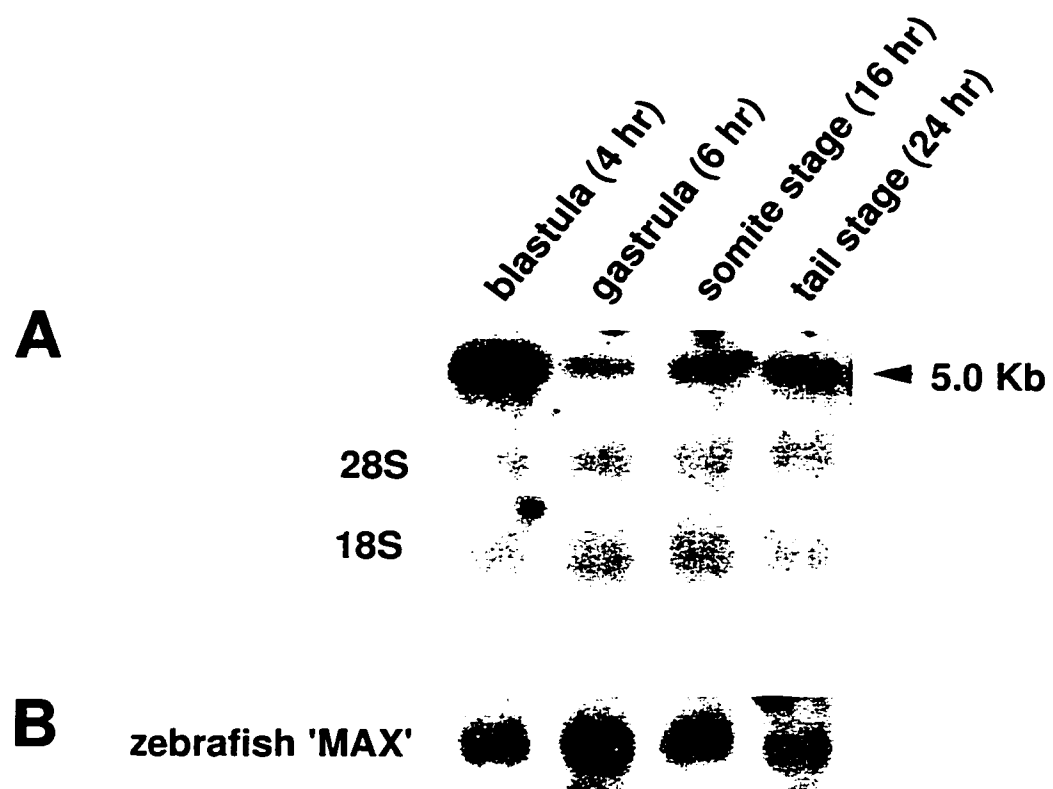


Figure 5: Expression of DNA (cytosine-5) methyltransferase in zebrafish embryos. Whole mount *in situ* hybridization of blastula (3 hr) (a), 4 hour (b), gastrula (6 hr) (c), somite stage (13 hr) (d) and tail stage (24 hr) (e & f: head and tail) zebrafish embryos with a zebrafish MTase antisense RNA probe. MTase is ubiquitously expressed in all cells of blastula and gastrula embryos. At 13 hours, MTase expression is highest in the CNS and the anterior differentiating somites (arrow; D , E). At 24 hours MTase expression is highest in the eyes, and brain (F) and the posterior differentiating somites (arrow; G). Scale bar: (A-D=135 μm ; E=95 μm ; F=200 μm ; G=50 μm).

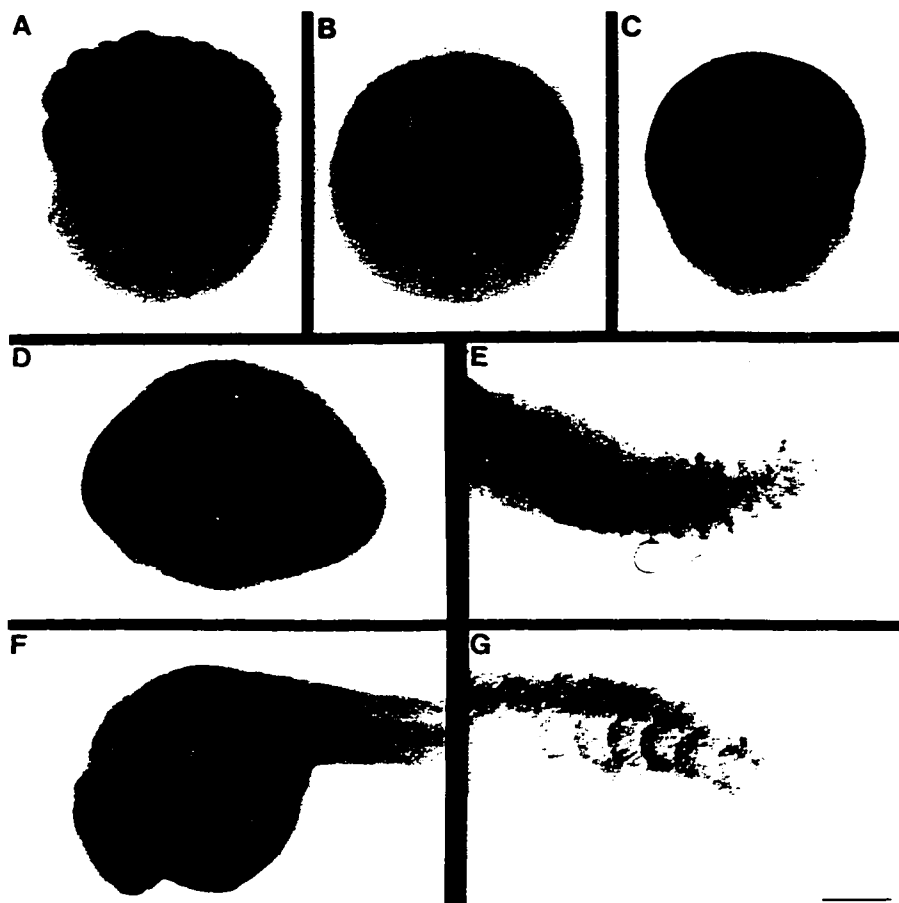


Figure 6: Global changes in DNA methylation during early zebrafish development. DNAs collected from sperm, blastula stage embryos (4 hr pf.), gastrula stage embryos (6 hr pf.), somite stage embryos (12 hr pf.), and 24 hour embryos were digested with *HpaII*, *MspI* (a) and hybridized to the zebrafish interspersed repetitive sequence *Mermaid*. Sperm DNA is hypermethylated relative to blastula and gastrula stages. Global DNA methylation levels decrease following fertilization until approximately gastrulation. After gastrulation, the level of DNA methylation in the embryos increases to levels approximating those found in sperm DNA. To confirm the integrity of the DNA samples used, uncut DNAs were subjected to electrophoresis and stained with ethidium bromide(b). This gel was Southern transferred and hybridized to the same zebrafish repetitive sequence. No evidence of DNA degradation or low molecular weight DNA fragments were observed in these uncut samples.

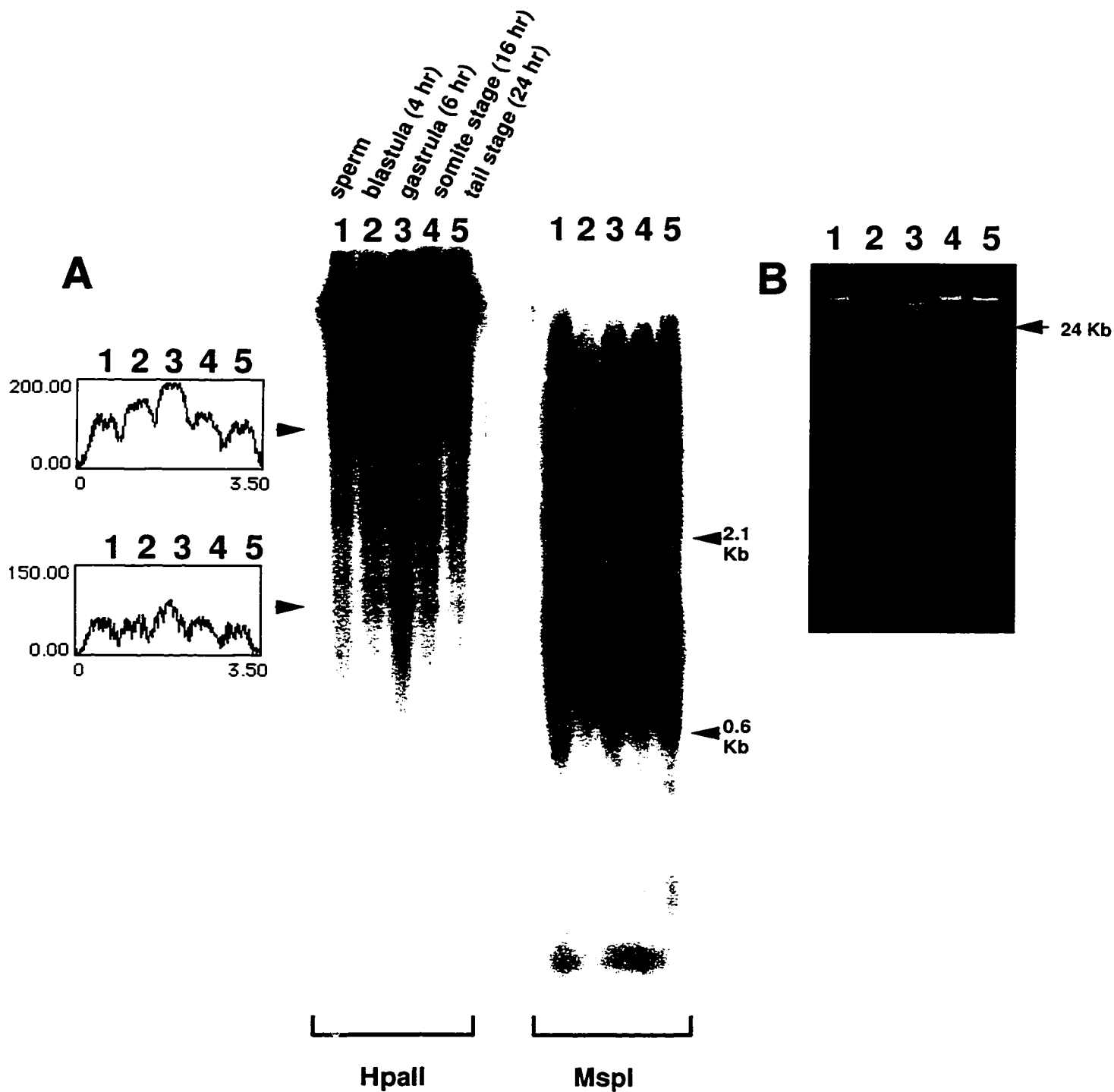


Figure 7: Dose-response curve for fertilized zebrafish embryos treated with 5-azacytidine. Zebrafish embryos were treated following fertilization with 0 μ M, 5 μ M, 10 μ M, 25 μ M, 50 μ M, 75 μ M and 100 μ M 5-azacytidine. Embryos were allowed to develop for 24 hours post-fertilization when they were scored as phenotypically normal, abnormal showing the short-tailed phenotype, or dead. 150-200 embryos were treated at each dose concentration.

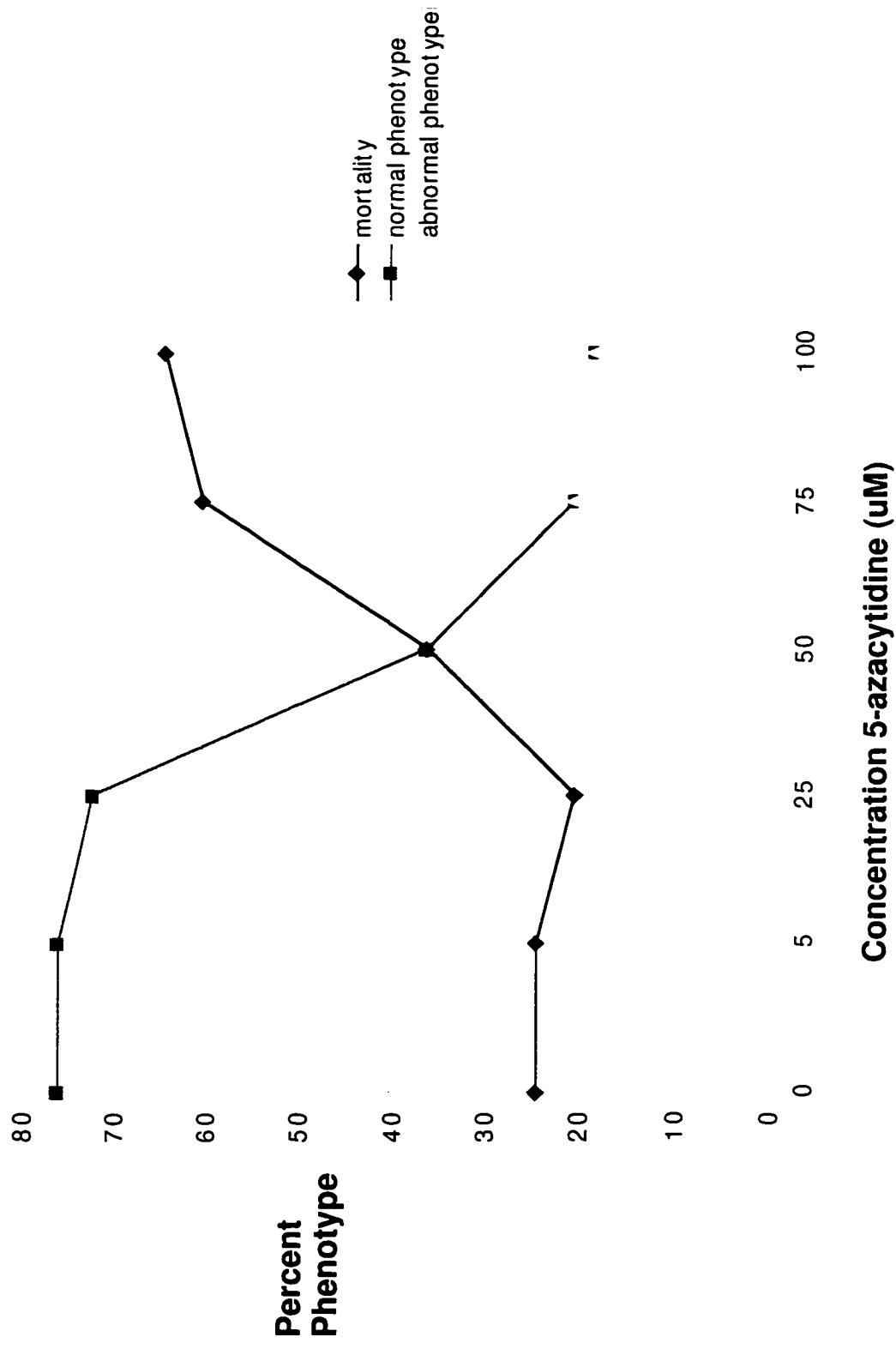


Figure 8 (A , B, C, D, E, F, G, H): Zebrafish embryos treated with 5-azacytidine lack tails and a differentiated notochord. (A) Wild-type embryos at 48 hours of development have an elongated tail and possess a fully differentiated notochord in the trunk and tail. (B) Approximately, 20-30% of embryos treated with 50 μ M 5-azacytidine show reductions in the elongation of the tail, and loss or abnormal development of posterior somites. Structures anterior to the trunk appeared to develop normally. (C and D) *In situ* hybridization with a probe for the muscle specific transcript α -tropomyosin shows that somatic mesoderm was restricted to lateral bands along the body axis in 24 hour wild-type embryos (dorsal view) (C). However, in 5-azaC-treated embryos α -tropomyosin transcripts were detected in lateral bands and in the tissues that underlay the neural tube (arrow) (D). (E ,F , G and H) Transverse sections of a 24 hours wild-type (E , F) and 5-azaC-treated embryo (G , H). Wild-type embryos show a large vacuolated notochord (n) directly beneath the neural keel (nk) and well developed lateral somites (s), while sibling 5-azaC treated embryos completely lack tissues resembling a notochord (arrow). (E , G - transverse sections posterior to hindbrain; F , H - transverse sections within posterior trunk/tail). Scale bar: (A=300 μ m; B=250 μ m; C=280 μ m; D=150 μ m; E-H=110 μ m).

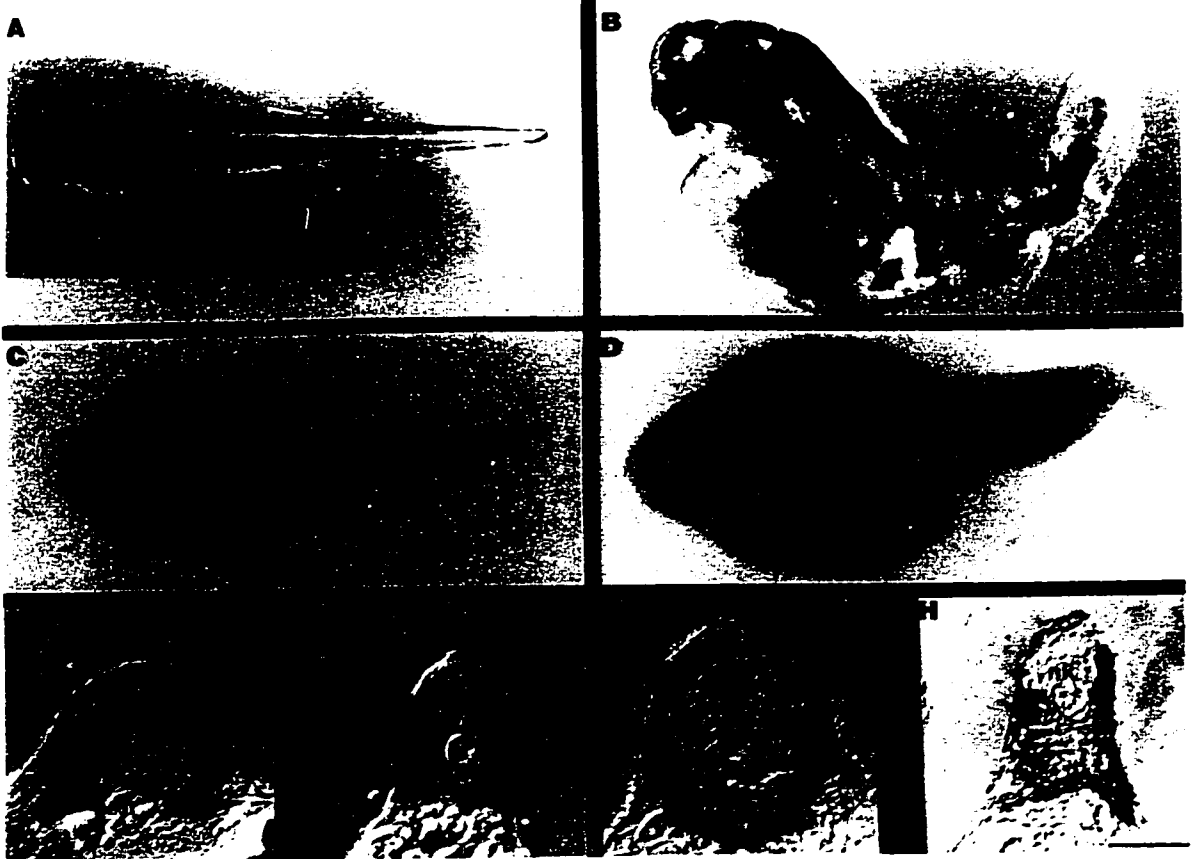


Figure 9 (A , B): The somites of zebrafish embryos treated with 50 μ M 5-azacytidine failed to develop into chevron-shaped myotomes. A) By 48 hours, the somites of the zebrafish embryo develop into chevron-shaped myotome with directionally organized muscle fibers. B) The somites of embryos treated with 5-azacytidine are block shaped and show disorganized muscle fibers. Scale bar = 40 μ m.



Figure 10 (A, B, C, D): Zebrafish embryos treated with 5-azacytidine show normal expression of *krx-20* and *ZFGFR4*. *In situ* hybridization to 24 hour zebrafish embryos with a *krx-20* and *ZFGFR4* antisense RNA probe. At 24 hours p.f. *krx-20* is expressed in the 3rd and 5th rhombomeres (r3, r5) of the brain in both untreated (A) and 5-azacytidine treated zebrafish embryos (B). *ZFGFR4* is expressed in the telencephalon (t), Diencephalon (d), the midbrain/hindbrain border (arrow) and the caudal mesoderm of the tail (cm). (m=midbrain). Scale bar: (A=165 μ m; B=200 μ m; C=280 μ m; D=200 μ m).

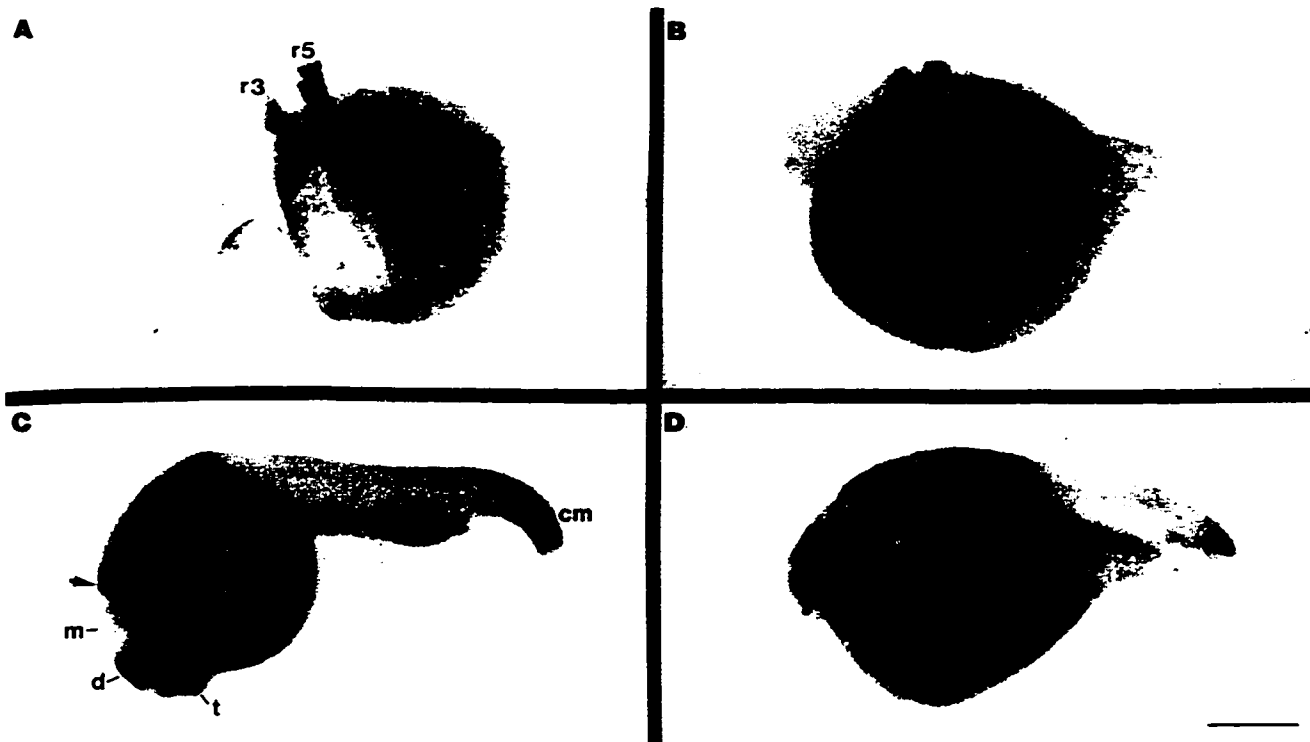


Figure 11 (A, B): Treatment of zebrafish embryos with 5-azacytidine *in vivo* results in reduced DNA methylation. DNA collected from 24 hour wild-type embryos, and from 5-azaC-treated embryos were digested with *HpaII* or *MspI*, Southern blotted and probed with the *mermaid/DANA* interspersed repetitive DNA sequence (A) as described in Materials and Methods. This revealed a much higher relative abundance of low molecular weight fragments in *HpaII* digested DNA from 5-azaC-treated embryos compared to untreated embryos indicating DNA hypomethylation in treated embryos. DNA collected from embryos treated with 6-azaC show a methylation level similar to that observed from untreated embryo DNA. Digestion of the same DNA with *MspI* shows a similar distribution of DNA fragments in all samples. This confirms that cleavage differences observed in *HpaII* digested DNA are the result of differences in the degree of DNA methylation. Quantitative differences in the relative abundance of DNA fragments are also indicated in densitometric tracings of these blots. To confirm the integrity of the DNA samples used, uncut DNA was subjected to electrophoresis and stained with ethidium bromide(B). No evidence of DNA degradation or low molecular weight DNA fragments were observed in these uncut samples. Each experiment was carried out in triplicate.

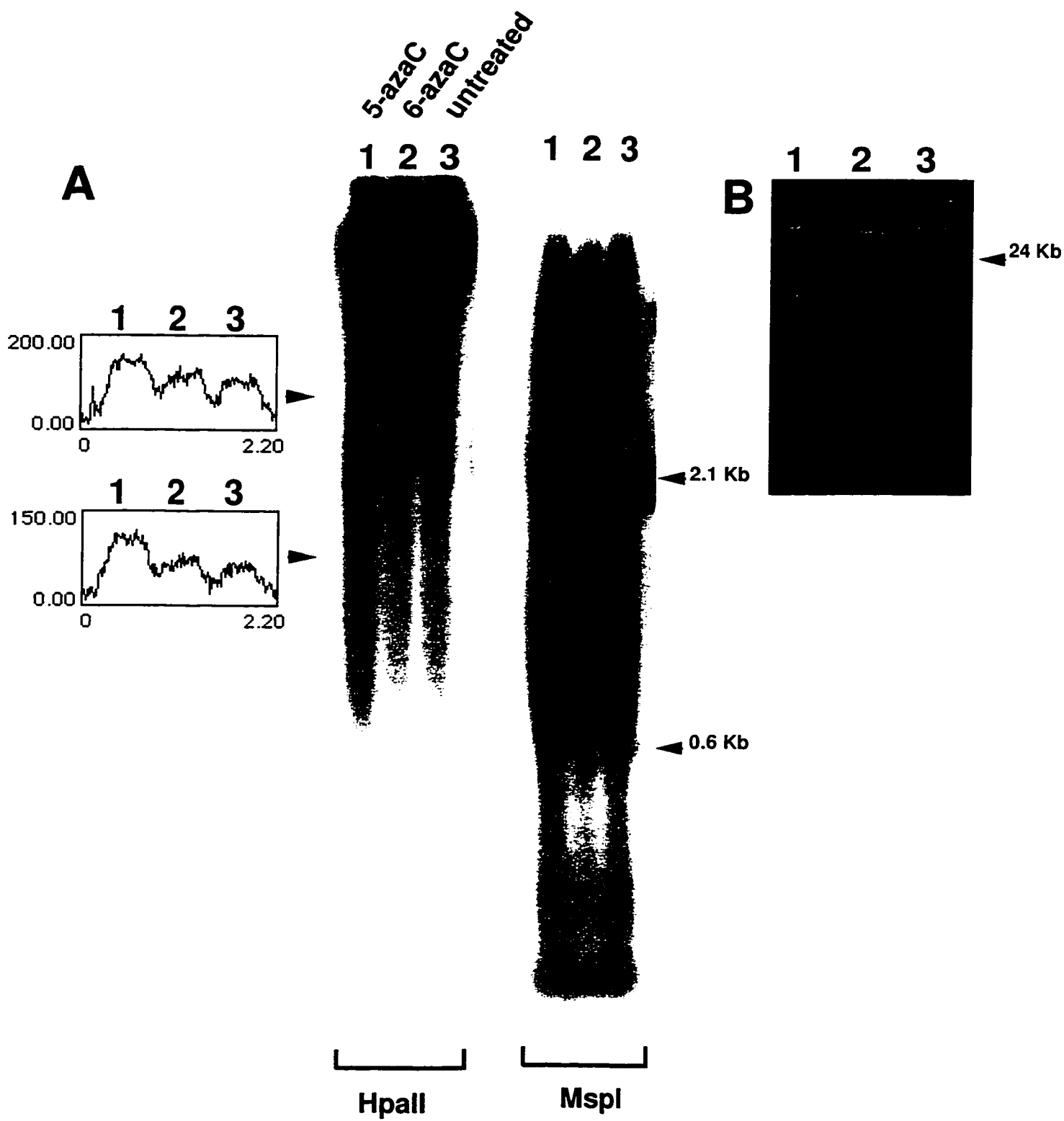


Figure 12: Time-course of the effect of 5-azaC on the development of axial structures. Embryos were treated with 50 μ M 5-azaC for the periods represented by the solid lines. The percentage of embryos showing the short-tail phenotype is indicated.

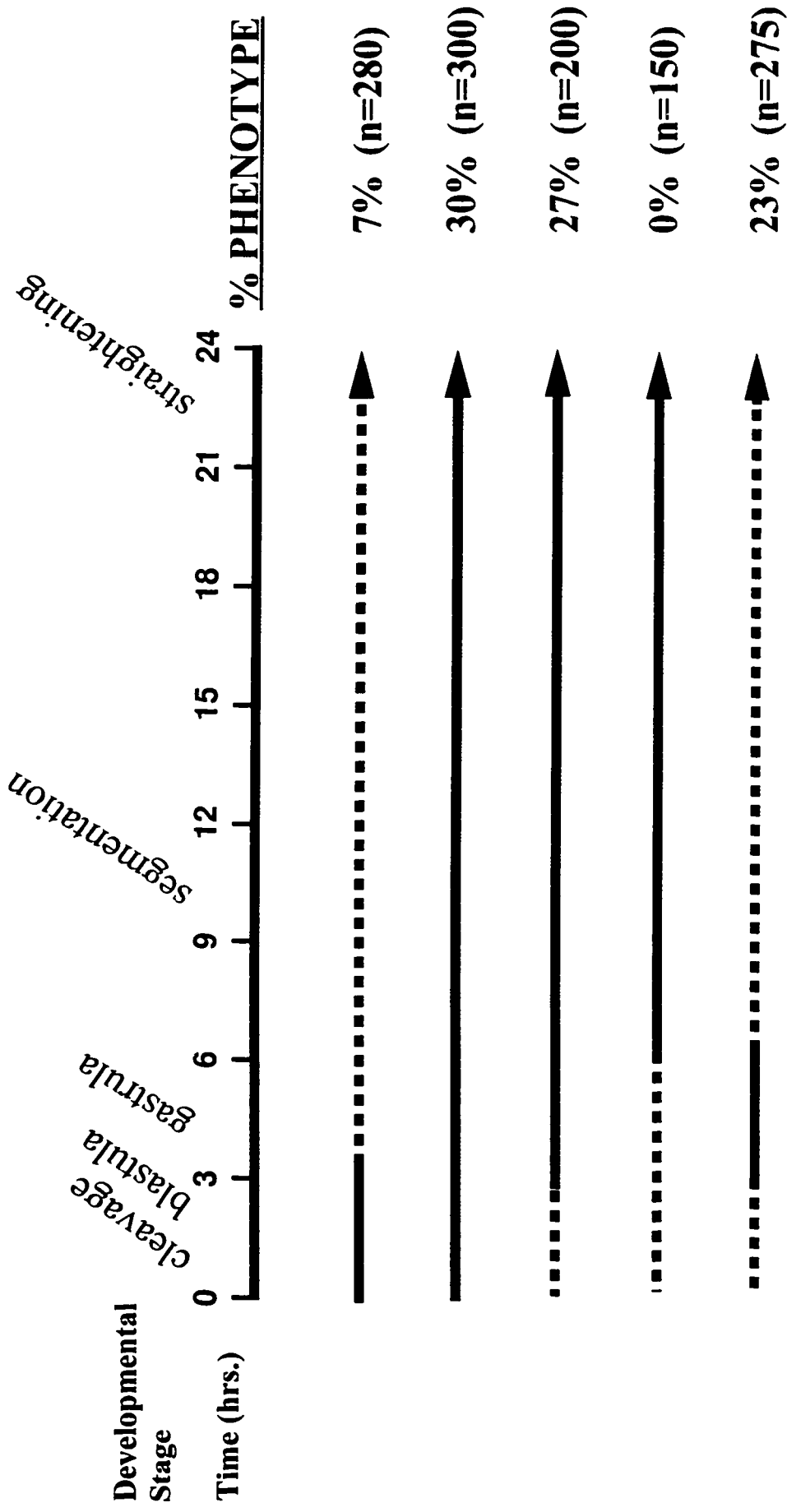
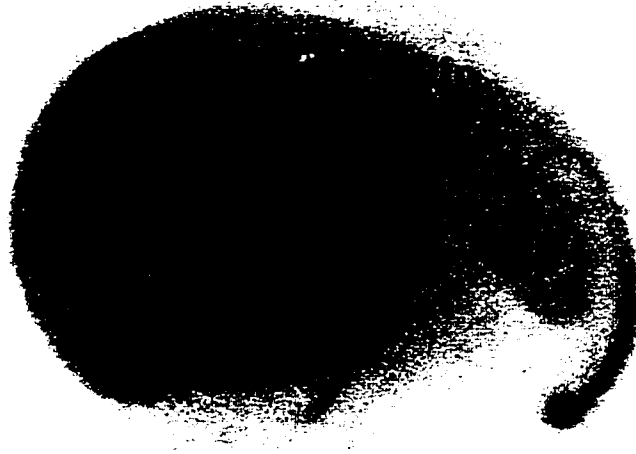


Figure 13: 5-azacytidine treatment of zebrafish embryos causes loss of *no tail* in the notochord and *eng2* expression in muscle pioneer cells. (A) In 19 hour wild-type zebrafish embryos, *no tail* is expressed in continuous pattern in cells of the differentiated notochord (n) and the caudal mesoderm of the tailbud (tb). (B, C) *no tail* expression in 5-azaC-treated embryos is discontinuous ranging from short gaps of absent expression along the body axis or (B) to near complete loss of expressing cells (C). Expression of *no tail* in the tailbud mesoderm is normal in 5-azaC treated embryos. (D) Wild-type embryos express *eng2* in the muscle pioneer cells (mp) located along the developing somites and in the mid/hindbrain boundary (mh). (E, F) 5-azacytidine-treated embryos show loss of expression of *eng2* in the muscle pioneer cells, but maintain normal *eng2* expression in the midbrain/hindbrain boundary. (G - wild-type, H - 5-azaC treated) Sectioning of double labeled embryos with antisense probes for *no tail* and *eng2* shows that the loss of expression of *eng2* in the muscle pioneer cells (arrow) of 5-azaC treated embryos occurs irrelevant of the presence of *no tail* expressing notochord cells (n). Scale bar: (A-F=200 μ m; G-H=110 μ m).



Figure 14 (A, B): 5-azacytidine treatment of zebrafish embryos causes partial loss of the ventral floor plate of the neural tube. *In situ* hybridization of 20 hour p.f. zebrafish embryos with a *shh* antisense RNA probe. At 20 hours of development *shh* (sonic hedgehog) is expressed in the ventral floor plate of the neural tube (dark staining) (A). Treatment of zebrafish embryos with 5-azacytidine causes partial loss of floorplate (B; arrow). Scale bar = 200 μ m.

A



B

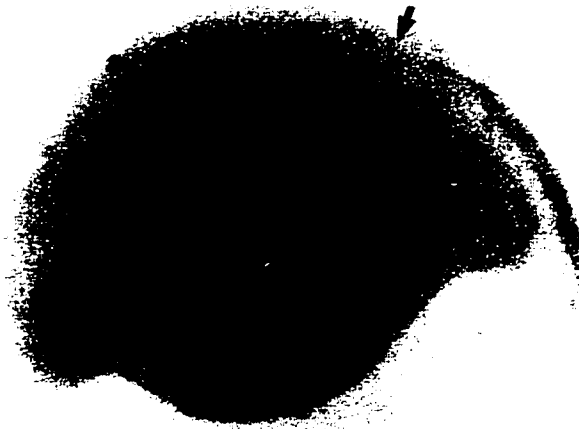
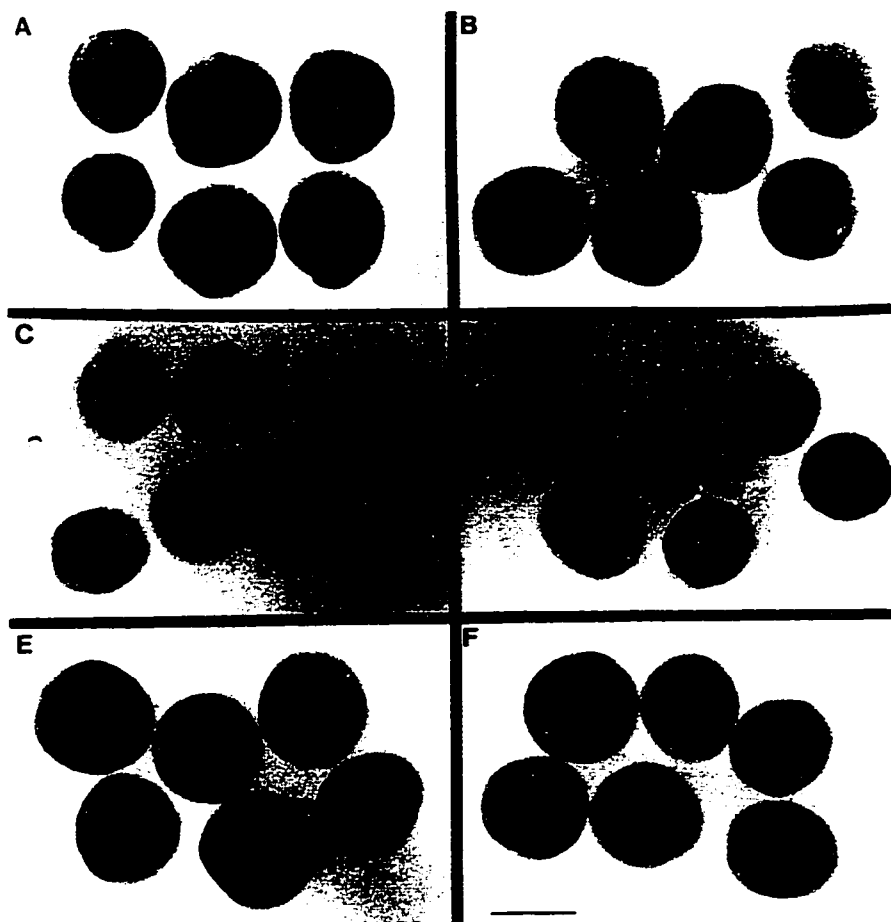


Figure 15: Zebrafish embryos treated with 5-azacytidine have a shortened and thickened axial mesoderm. The expression of the genes *no tail* (A, B), *floating head* (C, D), and *axial* (E, F) occurs in the axial mesoderm of 10 hours post-fertilization zebrafish. (A, C, E) wild-type untreated embryos. (B, D, F) embryos treated with 50 μ M 5-azacytidine from fertilization. Wild-type embryos show an elongated band of gene expression and a node of strong gene expression in the tail bud. In 5-azaC treated embryos, the stripe of cells expressing these genes are more dispersed and there is a poor expression in the tail bud. Scale bar: (A, B, E, F = 500 μ m; C, D = 580 μ m).



Discussion

The study of DNA methylation at least in vertebrates has been primarily limited to mammalian model systems. However, the consistent similarities in the MTase sequence, MTase expression and DNA methylation between the zebrafish and mouse lead us to suggest that methylation may play similar roles in both mammalian and non-mammalian vertebrates. Coupled with my previous work which indicated a genome-imprinting like phenomenon (parent-of-origin specific modification of DNA methylation) in the zebrafish, it may be that methylation plays several roles in zebrafish that were once thought to be exclusive to mammals. This work establishes the zebrafish as a potential model for studying the role of DNA methylation in early development. Furthermore, given some of the attributes of zebrafish development and genetics, the zebrafish may in some instances provide a better system for studying DNA methylation in development than mammalian model systems.

I have sequenced a partial cDNA isolated from a zebrafish embryonic cDNA library. The DNA and deduced amino acid sequences of this cDNA showed high identity to the sequences of DNA (5-cytosine) methyltransferase genes from a number of eukaryotes. This suggests that the cDNA clone represents the zebrafish MTase coding sequence. The 2.4 kb zebrafish cDNA is a partial cDNA whose deduced protein sequence I have shown contains eight conserved amino acid motifs (a KG-repeat, and domains: I, II, IV, VI, VII, VIII, IX, and X) in the carboxyl-terminal region of the protein. These conserved motifs have been used to imply a common functional architecture between all MTases whose activities involve the recognition of a specific amino acid sequence and the catalysis of methyl group transfer to the cytosine base (Kumar *et al.*, 1994). As

with most proteins, the regions containing the sequences related to these distinct functions are segregated into domains. Firstly, the amino terminal region of MTases contains a nuclear localization signal (NLS) and a region believed to be responsible for the enzymes localization and protein-protein interaction at the replication foci during DNA synthesis. Experiments disrupting parts of these sequences resulted in failure of the enzyme to localize to the nucleus (Leonhardt *et al.*, 1992; Hozak and Cook, 1994). My zebrafish MTase cDNA contains sequence encoding only what I estimate to be the carboxyl-terminal half of the MTase proteins and therefore I am unable at present, to make a comparison of this region from zebrafish to MTases of other species. However, I am able to make comparisons between the carboxyl-terminal regions between these proteins. Separating the amino- and carboxyl- terminal regions of eukaryote MTases is a set of lysine-glycine repeats (KG- repeats). There is no equivalent to the amino-terminal regions of eukaryotic MTases in the MTase enzyme of prokaryotes. It has previously been proposed that during evolution of MTases in eukaryotes, this amino-terminal region was added by gene fusion and that the KG-repeat is a junction site between the putative gene fusion event (Bestor *et al.*, 1988). The indicated conserved motifs found in the carboxyl-terminal of the protein are probable sites for providing important functional properties to the protein. At present, only two of these motifs have been assigned specific roles related to the common biochemical activity of these enzymes. Motif I of the MTases shows sequence similarity to other AdoMet-dependent MTases and is thought to be the binding site for the AdoMet (methyl group donor) cofactor (Klimasauskas *et al.*, 1989; Ingrosso *et al.*, 1989). Motif IV is known to be part of the catalytic site, which together with the other motifs (I-V and

X) form a pocket which holds the reactive cytosine during the methylation process (Adams, 1995). Between domains VIII and IX of the conserved carboxyl-terminal of the zebrafish MTase is a region of non-conservation. This variable region was thought to be a natural candidate for providing sequence (target) specificity. Swapping of this region with the variable regions of other MTases demonstrated that it provides both sequence specificity and the choice of which base becomes methylated within the target sequence (reviewed in Noyer-Weidner and Trautner, 1993). It is known that in mammals, DNA methylation plays very important roles in such things as gene silencing during embryonic development, X-inactivation and phenomenon such as genomic imprinting. While such involvement of MTase in the zebrafish remains to be conclusively demonstrated (cf. Martin and McGowan, 1995a; 1995b), the structural conservation of the MTase protein between zebrafish and mammalian MTases suggests the zebrafish MTase is functionally very similar and thus may be involved in similarly important epigenetic processes.

The expression of 5-cytosine DNA methyltransferase in the zebrafish is highest during the blastula stage where levels of the gene transcripts are at least 10X higher than later stages of development. While we see an abrupt decrease in MTase expression following blastula, studies from the MTase 'knockout' mice have shown that active maternally deposited MTase can persist in the embryo until at least 9.5 days post-fertilization (Carlson *et al.*, 1992). At present we know nothing about the stability of MTase in the zebrafish. If it is as long lived as in the mouse, the products of the high MTase expression in zebrafish blastula could persist throughout the entire embryonic developmental period of the zebrafish which occurs over a couple days. This very high MTase expression occurs in zebrafish just

prior and during major tissue differentiation events of zebrafish embryogenesis. The tissue specific expression of MTase in the zebrafish at later stages of development would seem to argue against a long lived MTase protein and possibly suggests that MTase is rapidly degraded following gastrulation. Post-somatogenesis, we see highest expression of MTase in the neural tissues and within developing somites. The observation of high MTase expression in neural tissues has been reported in mice (Trasler *et al.*, 1996). However, expression in the developing somites as we see in zebrafish has not previously been reported. At least in the adult brain, high levels of MTase activity is in part thought to function in remethylating newly incorporated cytosines required due to high levels DNA mismatch repair occurring in these cells (Brooks *et al.*, 1996). Brunk *et al.* (1996) observed active demethylation of the mouse *myoD* enhancer prior to somite differentiation (Brunk *et al.*, 1996). In zebrafish, MTase is expressed in the anterior half of the somite whereas *myoD* is expressed in the posterior portion of the somite. The complimentary expression of these two genes in the somites might suggest that MTase expression occurs to repress *myoD* expression in the anterior portion of the somite. These observations conclude that while a process of gene specific demethylation is required for allowing muscle differentiation, methylation may also be required to stabilize this terminally differentiated state by methylating genes no longer required for that cell lineage. Alternatively, proper patterning of the somite may require that MTase spatially regulate *myoD* expression. The tissue specificity of the zebrafish MTase during organogenesis in the CNS and cells of the somites suggest that it is important for the development of these tissues. The correlation between MTase in the somite cells and

myogenesis also suggest that methylation may be specifically important in muscle differentiation, by transcriptionally repressing genes which were expressed in these cells prior to myogenesis.

The high MTase expression during blastula and gastrula stages has been observed in sea urchin, chick and mouse. An interesting aspect of the relatively high MTase expression observed in blastula and gastrula of at least mouse and zebrafish is that the expression does not translate into a relative increase in levels of genome DNA methylation. Following fertilization, the diploid genome of the early zebrafish embryo undergoes a pronounced overall demethylation until approximately gastrula with later stages showing global methylation levels similar to the high methylation observed in sperm. Monk *et al.* (1987) observed similar genome wide demethylation in early mouse embryos, even in the presence of large amounts of the methyltransferase enzyme (Howett and Reik, 1991; Monk *et al.*, 1991). It has been suggested that this discrepancy results from the absence, in pre-gastrula, of regulatory factors that are required for methyltransferase activity and the localization of methyltransferase in the peripheral cytoplasm of early embryonic cells (Carlson *et al.*, 1992). Unfortunately we do not have, at present, an antibody specific for zebrafish MTase or one that will cross-react to zebrafish MTase to address the possible changes in cellular localization of MTase in zebrafish. This increase in DNA methylation following gastrulation occurs during a time of rapid cellular differentiation. Monk (1986; 1995) proposed that these methylation events occur as part of an epigenetic program to stabilize or permanently repress genes no longer required for a specific cell lineage. The increases in DNA methylation following gastrula in zebrafish are nearly identical to the pronounced

changes observed during mouse development. This might suggest that DNA methylation plays a similar role in cementing cell differentiation in the development of non-mammalian vertebrates and may also be involved in important events such as sex chromosome inactivation and establishing a genomic imprinting-like phenomenon in the zebrafish. I have also shown that zebrafish sperm DNA is more methylated compared to early developmental stages. Contrary to this, we previously observed a zebrafish transgene locus that was hypomethylated in sperm compared to eggs and somatic tissues (Martin and McGowan, 1995b). This dichotomy between the methylation of a specific locus and the overall genomic methylation levels in sperm is not unique to zebrafish. It also occurs in mice at some imprinted loci such as *Xist*, *H19*, *Igf2r* and an imprinted mouse transgene (Ariel *et al.*, 1995; Ferguson-Smith *et al.*, 1993; Ströger *et al.*, 1993; Chaillet *et al.*, 1991). This zebrafish transgene also showed characteristics of parent-of-origin specific methylation. These last results suggest that important epigenetic programming by DNA methylation occurs during gametogenesis in the zebrafish.

The dynamic changes in DNA methylation during early development and the association of methylation with transcriptional silencing and the phenomenon of genomic imprinting have long suggested that methylation plays an important role in early development. Holliday (1990) has discussed two points which have brought skepticism to this proposal: 1) At present, no developmentally important genes have been identified that are directly regulated by DNA methylation; and 2) Other eukaryotes such as yeast as well as some organisms such as *Drosophila*, and *C. elegans* that have very complex developmental processes don't methylate their DNA (Urieli-Shoval *et al.*, 1982; Simpson *et al.*, 1986; Proffitt *et al.*, 1984).

Despite the fact that DNA methylation does not occur in fruitflies, it appears that *Drosophila* may at one time have used cytosine methylation throughout its genome (Vanyushin and Poirier, 1996). However, we and others have recently proposed that methylation is functionally and biochemically similar to the developmentally important mechanisms of homeotic repression used by these organisms, such as chromatin structural modification and the binding of Polycomb and HP1 proteins (Wolffe, 1995; Singh and James, 1995; Bestor, 1995; Martin and McGowan, 1995a). While, these types of regulation are used in vertebrates, methylation is thought to be required to stabilize these epigenetic modifications in organisms which have more cell divisions, longer cell cycle times and larger genomes (Holliday, 1987; Martienssen and Baron, 1994; Bestor and Verdine, 1994).

In order to inactivate the MTase enzyme in zebrafish and observe its effects on embryo development, I treated zebrafish embryos in vivo with 5-azaC. Treatments of zebrafish embryos with 5-azacytidine were able to induce DNA hypomethylation in vivo which resulted in pronounced developmental consequences. A consistent proportion of zebrafish embryos treated with 5-azacytidine displayed developmental characteristics similar to notochordless mutant animals such as the *no tail* and *floating head* zebrafish mutants and the mouse *T* mutants (Halpern *et al.*, 1993; Talbot *et al.*, 1995; Herrmann *et al.*, 1990). My investigations show that 5-azacytidine treated embryos, in whole or partially, lack a notochord and that the mesoderm occurring in the position of the notochord is differentiated muscle. The somites are poorly developed particularly the most posterior tail somites. The fact that no other tissues appeared to be affected in these embryos suggests that methylation is

specifically important in the differentiation of the axial mesoderm. This effect was observed in approximately 30% of treated embryos. The remaining embryos were morphologically normal and their DNA methylation was less affected than phenotypic embryos. This minimal level of DNA hypomethylation required to produce a developmental effect is similar to the methylation threshold required to produce transformation of mammalian cell cultures (Paroush *et al.*, 1990). This effect is supported by observations of graded expression of a reporter episomal system in cell culture where repression was exponentially proportional to the construct's CpG methylation density (Hsieh, 1994). Treatments of zebrafish embryos with 5-azacytidine at different stages of development indicate that the effective interval at which the drug must be administered, in order to produce the axial phenotype occurs during blastula and early gastrula. It is during this time, that expression of MTase in the zebrafish embryo is highest. This correlation might indicate that 5-azacytidine treatment inactivates the high levels of MTase enzyme present at these early embryonic stages. The relative level of hypomethylation in these embryos appears to approximate methylation levels observed in gastrula stage embryos. This suggests that 5-azaC is effectively inhibiting the MTase enzyme and preventing the relative increase of DNA methylation observed in post-gastrula zebrafish embryos. These experiments clearly demonstrate that DNA methylation provides important epigenetic influences (other than those occurring in the gametes), very early in development, that are required for proper differentiation of the notochord and associated structures later in development.

My observations of MTase expression in early development and the ability to induce DNA hypomethylation in developing embryos with 5-azaC

treatment may allow us to develop techniques to improve the expression of zebrafish transgenes. It is believed that the poor expression of zebrafish transgenes is the result of DNA hypermethylation of the transgene locus (Gibbs *et al.*, 1994; Martin and McGowan, 1995a; Martin and McGowan, 1995b). This makes sense since microinjected transgene constructs would be considered by the embryo as invading DNA, and that these embryos which develop externally would probably develop a strong methylation host defense system (likely to a greater extent than that in mouse whose embryos develop internally) (Bestor, 1990). Since I have shown DNA hypomethylation by 5-azaC treatment, it may be possible to treat embryos with 5-azaC following DNA microinjection and thus increase the level of transgene expression in later development. If the transgene is able to escape methylation in early development (methylation which may occur prior to integration) then the transgene may remain hypomethylated since a hemi-methylated substrate would be absent for maintenance methylase to act.

Initially, I was concerned that the effects I observed were the result of 5-azacytidine cytotoxicity. However, the action of 5-azaC appears to specifically affect the differentiation of the notochord and that of structures such as the floor plate and muscle, whose development depends upon proper notochord function. As far as I could tell, initial differentiation of ectoderm and other mesodermal derivatives was unaffected. Time-course treatments have also shown that 5-azaC was only effective in producing the abnormal phenotype when given at the beginning of gastrulation. The sensitive period corresponds to the period of highest MTase expression in the embryo and just prior to dramatic increases in global DNA methylation. This further suggesting that the

effects of 5-azaC are the results of impaired methylation. Thus, the tissue specificity and temporal specificity of 5-azacytidine action are inconsistent with effects of a purely cytotoxic nature. Treatment of zebrafish embryos with the cytidine analog 6-azaC resulted in no abnormal development at concentrations tested. This drug which does not affect DNA methylation has been used in a number of studies to control for 5-azaC cytotoxic effects (Jones and Taylor, 1980; Doerksen and Trasler, 1996). Therefore, the effects we observe on zebrafish development are likely due to abnormal DNA hypomethylation.

Expression of the genes *no tail*, *floating head*, and *axial* in 10 hour 5-azaC treated embryos reveals that the axial mesoderm in these embryos is much shorter and wider relative to untreated embryos. These observations are consistent with experiments where chick embryos were treated with 5-azaC. While these experiments in chickens did not include specific gene expression analyses, 5-azaC-treated chick embryos displayed a shortened and thickened primitive streak (Zagris and Podimatas, 1994). These observations are consistent with a disruption in proper axis elongation and intercalation of axial mesoderm cells during epiboly like I report here for the zebrafish. It is formally possible that there are simply more cells expressing these markers along the medio-lateral axis and less expressing cells along the anterior-posterior axis. However, visual inspection of the body axis suggests a lower cell density of marker expressing tissues which is more consistent with the first proposition. Interestingly, this similar expression pattern of markers for axial mesoderm also occurs at this stage of development in *floating head* mutant zebrafish (Halpern *et al.*, 1995), and similarly, these tissues also form muscle, a non-axial tissue.

Analysis of mouse *T* mutants and the zebrafish mutants *no tail* and *floating head*, and the cloning of the mutant genes has revealed the requirement for these gene products for complete notochord differentiation (Halpern *et al.*, 1993; Talbot *et al.*, 1995). These mutations also demonstrate the requirement of a signal from the notochord for proper development of somites and the floorplate of the neural tube. While the phenotype of zebrafish embryos treated with 5-azaC is consistent with the developmental conclusion made by studying both these mutations, these fish most closely resemble the phenotype of homozygous *floating head* mutant embryos. Both embryos show replacement of notochord by fusion of somites underlying the neural tube and disruption in the posterior ventral floorplate. The phenotype of 5-azaC-treated zebrafish is also reminiscent of the mouse *T* mutants and zebrafish *floating head* mutants which show disruption in the floorplate. Our results suggest that proper methylation is required at one step of the pathway leading to differentiation of the axial mesoderm. The change in fate for the axial mesoderm from notochord to muscle suggests that methylation is required to stabilize that fate of the axial cells. That is, genes in axial cells are actively repressed by DNA methylation (likely via local changes in chromatin) preventing them from receiving muscle differentiation signals or transcriptional queuing. They then can progress to a notochord fate. We cannot however, preclude that the loss of differentiated notochord cells is not the result of apoptosis occurring specifically in these cells and 'filling in' of the notochord region by muscle cells. Preliminary studies have shown that apoptosis has been observed to occur in a number of cell types in the embryos of MTase knockout mice (Trasler *et al.*, 1996). However, the similarity in the phenotype of 5-azaC treated embryos and

the zebrafish mutants which lack a notochord suggests that the phenotype we observe is the result of improper cell differentiation, an assumption made by the analysis of the zebrafish mutants. On the other hand, I have observed a very distinct and important difference between the phenotype of 5-azaC treated embryos and the phenotype of the zebrafish mutants *no tail* and *floating head*. Experiments using these mutant embryos have shown the requirement for signals from the notochord to induce *eng* expression in the muscle pioneer cells which is subsequently involved in final muscle differentiation and morphological changes in the muscle. I observed no *eng* expression in muscle pioneer cells irrespective of the presence of *no tail* expressing notochord cells. This is very significant since it implies that the muscle pioneer cells in these embryos are not responsive to *no tail* signaling from the notochord cells. I have observed specific MTase expression in the cells of wild-type zebrafish embryos that have initiated the process of myogenesis. At present we do not know the exact identity nor the developmental function of the cells within the somites expressing high levels of MTase. We do know that improper methylation results in loss of *eng* expression in the muscle pioneer cells which have been shown to be important in defining somite morphology. While clearly, these cells in 5-azaC treated embryos do proceed through the initial stages of myogenesis, as evident by expression of α -tropomyosin, proper DNA methylation may be required for these cells to proceed to the final stage of muscle patterning.

Given our present state of knowledge of specific gene methylation events during development, it is difficult to explain in the context of the actual genes involved our observations of the loss of a differentiated notochord and muscle abnormalities in the hypomethylated zebrafish embryos. I can

make some suggestions that could serve to provide a framework for future studies. Firstly, treatment of 10T1/2 cell with 5-azaC causes DNA hypomethylation and expression of the gene *myoD* resulting in transformation of these cells to myoblasts (Cedar and Razin, 1990). It was recently shown that regulated demethylation of the *myoD* distal enhancer in cells of the myogenic lineage precedes *myoD* transcription and myogenesis in mouse embryos (Brunk *et al.*, 1996). Therefore, treatment of zebrafish embryos with 5-azaC during gastrulation may result in demethylation of the *myoD* distal enhancer in the axial mesoderm altering its fate to muscle instead of notochord. Furthermore, improper *myoD* expression in the somites may also be responsible for the failure of the muscle to pattern properly. The existence of this enhancer has yet to be demonstrated for the zebrafish *myoD* gene. A second possibility is that some genes that are regulated directly by DNA methylation modify *no tail* expression in the notochord as was previously shown for its likely mouse ortholog, *brachyury*. In mice, expression of *brachyury* shows incomplete genomic imprinting where transcripts originate primarily from the paternal allele (Foreijt *et al.*, 1995). This results in parental effects on the phenotype of offspring produced from *T* mutant reciprocal crosses (Johnson, 1974; Winking and Silver, 1984). The *T-associated maternal effect* (Tme) locus, which is believed to be the gene *Igf2r*, was proposed to be the modifier gene responsible for the observed imprint of *brachyury* (Barlow *et al.*, 1991). Both the genes for *Igf2r* and its ligand *Igf2* are imprinted in mice and show transcriptional modification by DNA methylation. Hypomethylation of the developing zebrafish genome, therefore, could indirectly disrupt *brachyury* expression and notochord

differentiation by altering the normal expression patterns of *Igf2r* or *Igf2*, two genes that have yet to be identified in this species.

By taking advantage of the external development of the zebrafish, we have been able to demonstrate a role for DNA methylation in early development by inducing DNA hypomethylation with 5-azacytidine. This data does not implicate methylation directly in differentiation nor do they suggest wide involvement in early development. However, they do suggest that expression of one or a few genes during early embryogenesis is influenced directly by DNA methylation, and hypomethylation of these genes and their perturbed expression is responsible for the abnormal phenotype I have observed in these experiments. Therefore, control of gene expression by DNA methylation is important in zebrafish and probably other vertebrates for the proper differentiation of the mesoderm, specifically, the notochord.

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Appendices - Introduction

During the tenure of my Ph.D. in the laboratory of Dr. Marc Ekker, I was funded in part from a grant to Marc Ekker and Mario Chevrete (McGill University) by the Canadian Genome Analysis and Technology program (CGAT). My involvement in this project included the analysis of a panel of zebrafish X mouse somatic cell hybrids and the cloning and analysis of a number of zebrafish repetitive DNA sequences. The work on this project has resulted in two publications (one published and one paper that is in preparation). I have included these two papers in Appendix 1 and 2. Further to my academic achievements in the past and as a result of work at the University of Ottawa, I have included as reference a complete curriculum vitae (Appendix 3).

Appendix 1: Stable Transfer of Zebrafish Chromosome Segments into Mouse Cells

Stable Transfer of Zebrafish Chromosome Segments into Mouse Cells

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Whole-cell fusion between zebrafish fibroblast-like ZF4 cells and mouse B78 melanoma cells resulted in hybrids containing one or a few zebrafish chromosome segments in a murine chromosomal background. Fluorescence *in situ* hybridization to hybrid cell metaphases with a zebrafish genomic DNA probe revealed that many hybrids contained zebrafish chromosome segments that were either inserted or translocated to a mouse chromosome, whereas other hybrids contained zebrafish chromosomes with no evidence of insertion or translocation. We have assigned hybrids to 17 linkage groups of the genetic map of the zebrafish genome. Our results demonstrate the feasibility of producing somatic cell hybrids between distantly related species. Zebrafish/mouse cell hybrids will provide a useful tool for the physical mapping of the zebrafish genome and for the cloning of genes affected in zebrafish mutants.

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INTRODUCTION

The establishment of genetic methods in the zebrafish, *Danio rerio*, allows a systematic mutational analysis of vertebrate development (Streisinger *et al.*, 1981; Kimmel, 1989; Rossant and Hopkins, 1992; Mullins and Nüsslein-Volhard, 1993; Solnica-Krezel *et al.*, 1994). Although the identification and analysis of mutations affecting development are facilitated by the biology of the zebrafish, including the production of haploid and parthenogenetic diploid individuals, the molecular characterization of mutations in zebrafish is still a challenging task. To date, only three genes detected by mutation have been cloned (Halpern *et al.*, 1993; Schulte-Merker *et al.*, 1994; Sepich and West-

erfield, 1993; Talbot *et al.*, 1995). The availability of tools such as a genetic linkage map and a physical map of overlapping YAC, P1, or cosmid clones will facilitate the cloning of genes that are affected in zebrafish mutants. Recently, a genetic linkage map of the zebrafish genome has been reported (Postlethwait *et al.*, 1994). The map consists of more than 400 random amplified polymorphic DNAs (RAPDs) (Williams *et al.*, 1990; Welsh and McClelland, 1990). It was originally organized into 29 linkage groups but has now been consolidated into 25 linkage groups (S. L. Johnson and J. Postlethwait, unpublished data), corresponding to the number of zebrafish chromosomes identified by karyotype (Schreeb *et al.*, 1993). The map has been complemented by the integration of anonymous DNA markers based upon simple sequence repeats (SSRs) (Goff *et al.*, 1992) and of cloned genes (Postlethwait *et al.*, 1994). The linkage map of the zebrafish genome will contribute to the molecular analysis of zebrafish mutations affecting development.

Panels of human/mouse and mouse/hamster somatic cell hybrids have greatly helped the mapping of human and mouse genes, respectively (Ringertz and Savage, 1976; Ruddle, 1981). Somatic cell hybrids containing zebrafish chromosomes would likewise allow rapid assignments of cloned genes or anonymous DNA markers to specific chromosomes or chromosome regions. However, stable somatic cell fusion has not been reported for species as distantly related as mouse and fish. In the present report, we describe a panel of zebrafish/mouse somatic cell hybrids that contain one or a few zebrafish chromosomes or chromosome fragments.

MATERIALS AND METHODS

Whole-cell fusion. Calcium phosphate precipitation (Maniatis *et al.*, 1989) was used to transfer the pCMV neo plasmid into ZF4 cells. The CMV promoter/enhancer has previously been shown to have high activity in ZF4 cells (Driever and Rangini, 1993). Stable transfectants were selected in the presence of 600 µg/ml of G418. A pool of more than 400 clones was produced to ensure that each of the 25 zebrafish chromosomes (Schreeb *et al.*, 1993) was represented. For whole-cell fusion, 500,000 cells of each of the zebrafish ZF4 and mouse B78 parental cell lines were plated together into a 25-cm²

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flask 24 h before fusion. The cell monolayer was fused with 1 ml of 50% (wt:wt) polyethylene glycol (1540; NBS Biologicals) solution in Dulbecco's modified Eagle's/F12 medium for 90 s. After three washings with serum-free medium, the cells were incubated at 37°C for a period of 24 h, after which they were split and transferred to two 162-cm² flasks. The selection drugs G418 (800 µg/ml) and ouabain (6 µM) were added to both flasks to kill parental cell lines. One flask was then incubated at 37°C and the other at 28°C until colonies of cells resistant to both drugs developed.

All somatic hybrids reported here can be obtained from the authors upon request.

Fluorescence in situ hybridization. Metaphase chromosomes were prepared from ZF4, B78, and zebrafish/mouse hybrid cells according to standard trypsin harvest techniques under the following conditions: 90 min in Colcemid (0.05 µg/ml); 20 min in hypotonic solution (ZF4 cells, 1% sodium citrate; B78 and hybrid cells, 0.075 M KCl); brief prefix of approximately 2 drops Carnoy's (3:1 methanol:acetic acid) per milliliter of hypotonic cell suspension, added just prior to pelleting; standard fixation and cell preparation (Lawce and Brown, 1991). Fluorescence *in situ* hybridization (FISH) was performed as described (Pinkel *et al.*, 1988). Probes consisted of the zebrafish satellite type I (SRI) monomeric unit (Ekker *et al.*, 1992a) or of total zebrafish genomic DNA that had been previously digested with a mixture of the restriction enzymes *EcoRV*, *PstI*, and *PvuII*. The hybridization conditions were as follows: Hybridization mixture: 100 ng of biotinylated probe, 1 µg salmon sperm DNA in 10 µl of 70% formamide/2× SSC; Posthybridization washes: 10 min, 37°C, 50% formamide/2× SSC followed by 10 min, 37°C, 2× SSC.

PCR analysis. Genomic DNA was prepared (Westerfield, 1993) from four nearly confluent 100-mm dishes. PCR using zebrafish-specific primers (MapPairs, Research Genetics, Huntsville, AL) was performed on genomic DNA in a final volume of 50 µl according to the manufacturer's instructions. Primers for zebrafish genes were either generously provided to us by J. Postlethwait and A. Fritz or were synthesized by us from published sequences. These sequences can be obtained upon request. The following genes were tested on hybrids: *HoxC5* (Ericson *et al.*, 1993), *Hox C6* (Njølstad *et al.*, 1990), *PouC* (Johansen *et al.*, 1993), *Wnt1* (Molven *et al.*, 1991), *sonic hedgehog* (Krauss *et al.*, 1993), *Eng3* (Ekker *et al.*, 1992b), *msxD* (Ekker *et al.*, 1992c), *axial* (Strähle *et al.*, 1993), *Cyclin E* (GenBank Accession No. X83596), and *sup 44* (Fjose *et al.*, 1995). The PCR conditions were as follows: denaturing step, 50 s at 92°C; annealing step, 1 min and 30 s at the temperature indicated below; extension step, 2 min at 72°C. Annealing temperatures were 52°C for *axial*; 55°C for *Hox C5*, *PouC*, *sonic hedgehog*, and *cyclin E*; 57°C for *Hox C6* and *msxD*; 60°C for *Eng3* and *sup44*; and 61°C for *Wnt1*. All PCR analyses were carried out for a total of 40 cycles except for that for *sonic hedgehog*, which was carried out for 30 cycles.

RESULTS

Production of Zebrafish/Mouse Somatic Cell Hybrids

We produced somatic cell hybrids containing zebrafish chromosomes by fusing the zebrafish fibroblast-like cell line ZF4 (Driever and Rangini, 1993) to mouse melanoma B78 cells (McGowan-Jordan *et al.*, 1994). Zebrafish chromosomes were tagged with a selectable marker, the aminoglycoside phosphotransferase gene conferring resistance to G418 (neo). Hybrids were selected in media containing G418, to kill

mouse parental cells, and ouabain, to kill zebrafish parental cells. Rodent cells are naturally more resistant to ouabain than are zebrafish (M. Ekker and M. Chevrette, unpublished data) or human cells, as determined empirically (Lugo and Fournier, 1986). After a 3- to 4-week period of culture at 37 or 28°C (the normal temperature for the culture of ZF4 cells), we observed clones resistant to both G418 and ouabain only in the flasks grown at 37°C.

Morphological examination of zebrafish/mouse hybrids revealed that the majority had a rounded epithelial-like appearance, resembling mouse B78 parental cells (not shown). Only a few hybrids, such as ZFB-34, had a flattened fibroblast-like appearance similar to that of the ZF4 parent (not shown). Together with the fact that all hybrids were isolated at 37°C, a nonpermissive temperature for the ZF4 parental cell line, these results suggested that most hybrids had retained more mouse chromosomes than zebrafish chromosomes.

FISH Analysis of Zebrafish Chromosomes in Hybrids

To detect zebrafish chromosomes in the hybrids, we performed FISH using two zebrafish-derived probes. The first probe (SRI) corresponded to a highly repetitive satellite-like DNA sequence from the zebrafish (Ekker *et al.*, 1992a), and the second consisted of total zebrafish genomic DNA (TZD probe). The SRI probe hybridized to the centromere region of all zebrafish chromosomes (Fig. 1B) of ZF4 cells, whereas the TZD probe labeled zebrafish chromosomes in their entirety (Fig. 1A). The latter result suggests the presence of one or several interspersed repetitive DNA sequence(s) in the zebrafish genome (Deininger and Daniels, 1986). Neither probe hybridized to mouse chromosomes (Figs. 1E and 1F).

When the same probes were used on the zebrafish/mouse hybrids, they identified only one zebrafish chromosomal element in 31 of 77 hybrids analyzed; the other hybrids contained two or three zebrafish chromosomal elements. Furthermore, as can be seen in Fig. 1C, the zebrafish chromosome fragment present in the hybrids was often either inserted or translocated into a mouse chromosome, as shown by the distal chromosome fragments that did not label with the TZD probe. We observed an inserted or translocated chromosomal element in 75% of hybrids examined (58/77; Table 1). However, in some hybrids, the zebrafish chromosomes were entirely labeled with the TZD probe (not shown), suggesting the absence of a translocation event. However, we cannot rule out the presence, at the telomeres of such chromosomes, of mouse elements that are too small to be visible. Similarly, very small zebrafish chro-

FIG. 1. Fluorescence *in situ* hybridization of zebrafish probes to chromosome spreads from zebrafish, mouse, and hybrid cells. (A, C, and E) Hybridization of a total zebrafish genomic DNA probe to (A) zebrafish ZF4; (C) hybrid ZFB8; and (E) mouse B78. (B, D, and F) Hybridization of a zebrafish type I satellite DNA probe (Ekker *et al.*, 1992a) to (B) zebrafish ZF4; (D) hybrid ZFB11; and (F) mouse B78. Microphotographs were taken using a 100X objective.

TABLE 1

Characteristics of the Zebrafish/Mouse Hybrid Panel

Number of hybrids	77 (100%)
Number of hybrids with one zebrafish chromosomal element	31 (40%)
Number of hybrids containing a translocated chromosomal element	19
Number of hybrids containing an untranslocated chromosomal element	12
Number of hybrids with multiple zebrafish chromosomal elements	46 (60%)
Number of hybrids containing only translocated chromosomal elements	14
Number of hybrids containing only untranslocated chromosomal elements	7
Number of hybrids containing both types of chromosomal element	25

mosome fragments present in a hybrid will be missed by such FISH analyses.

Assignment of Markers and Cloned Genes to Hybrids

To characterize further the zebrafish/mouse hybrid panel, we prepared DNA from each hybrid and analyzed it for the presence of specific zebrafish sequences. We performed PCR analysis on hybrid cell DNA with oligonucleotides corresponding to sequences from cloned genes or flanking specific SSRs from the zebrafish (Goff *et al.*, 1992). Some of these SSR markers were included in the genetic linkage map of the zebrafish (Postlethwait *et al.*, 1994). However, we were not able to use adequately most RAPD markers described by Postlethwait and collaborators (1994), presumably because interstrain differences at the sites of such short PCR primers (10 nucleotides long) seriously impair the amplification reaction. The ZF4 cell line that we have used was derived from the zebrafish mutant *tupfel* (also referred to as leopard danios) (Driever and Rangini, 1993; Kirschbaum, 1975), while the RAPD markers used for building the genetic linkage map originate from the AB and Darjeeling strains. It was nevertheless possible to assign a RAPD marker to the hybrid ZFB14, which contained a chromosome fragment encompassing the *floating head* mutation on linkage group XIII (Table 2; Talbot *et al.*, 1995).

Hybrids ZFB8, 10, 11, 43, 48, 54, and 201 contained SSR marker 13, which has been mapped to the linkage group VIII (Postlethwait *et al.*, 1994). The same hybrids

were also positive for SSR markers 9 and 25, which map to the same linkage group, thus confirming that markers linked on the genetic map have been transferred together in our hybrids (Table 2). At present, we have assigned hybrids to 17 of 25 linkage groups. Some hybrids contained more than one zebrafish chromosomal element and were positive for SSR markers or genes identifying different linkage groups (Table 2). We were able to isolate subclones from such hybrids, which retained only one zebrafish chromosomal element, as well as markers from a single linkage group (ZFB43-7; Table 2). It should be noted that 8 linkage groups on the zebrafish genetic map did not contain markers (SSRs and cloned genes) that we could assign to the hybrid panel. This, and the fact that, in some cases, only portions of chromosomes were transferred during the fusion, might explain why many hybrids have not yet been assigned to linkage groups. We and others are producing additional SSR markers that should help establish the correspondence between zebrafish/mouse hybrids and the genetic linkage map.

Stability of Zebrafish Chromosome Segments in Hybrids

To determine the stability of zebrafish chromosomes in the hybrids and to assess whether chromosomal translocation influences this stability, we grew hybrids ZFB10 and ZFB37 for over 100 generations (34 passages) in either the presence or the absence of G418. Hybrid ZFB10 contains a zebrafish chromosomal element that has been inserted into a mouse chromosome, whereas translocation was not apparent for the chromosomal elements present in ZFB37. The retention of zebrafish chromosomal elements in the hybrids was assayed by FISH analysis of 100 interphase nuclei. In the presence of G418, zebrafish chromosomal elements were detected in at least 95% of nuclei examined for both ZFB10 and ZFB37 (Table 3). Furthermore, the SSR markers originally present on hybrid ZFB10 were all retained (data not shown). However, in the absence of selection, zebrafish chromosomal elements were unstable in both hybrids, and as many as 54% of nuclei were no longer stained with the TZD probe after 100 generations (Table 3). Thus, it appears that, although zebrafish/mouse hybrids can be maintained for many generations in the presence of G418, continual selection is required to prevent the loss of zebrafish chromosomal elements from the mouse background, as pre-

TABLE 2. Note. FISH examination of at least 100 nuclei using the total zebrafish genomic DNA probe was performed to determine the number of zebrafish chromosomes present in at least 5% of interphase nuclei (in parentheses). Additional hybrids with characteristics identical to hybrids already present in the table were omitted. SSR markers (Goff *et al.*, 1992) and cloned zebrafish genes were detected by PCR on hybrid cell DNA. SSR markers were previously assigned to linkage groups (LG) by Postlethwait and collaborators (1994). The following genes were used as probes to assign hybrids to linkage groups: *HoxC5* (Ericson *et al.*, 1993), *HoxC6* (Njølstad *et al.*, 1990), *PouC* (Johansen *et al.*, 1993), *Wnt1* (Molven *et al.*, 1991), *sonic hedgehog* (Krauss *et al.*, 1993), *Eng3* (Ekker *et al.*, 1992b), *msxD* (Ekker *et al.*, 1992c), *axial* (Strähle *et al.*, 1993), *Cyclin E* (GenBank Accession No. X83596), and *sup 44* (Fjose *et al.*, 1995). Their assignment to linkage groups is a personal communication from J. Postlethwait (J. Postlethwait, Eugene, OR, pers. comm., 1995). A RAPD marker mapping to LG XIII was detected in hybrid ZFB14 (W. Talbot, Eugene, OR, pers. comm., 1995).

Assignment of Zebrafish/Mouse Hybrids to Linkage Groups

[illegible]

TABLE 3
Stability of Zebrafish Chromosomal Elements
in Hybrids

Hybrids and culture conditions	Zebrafish element ^a		
	0	1	>1
ZFB10			
Passage 4 + G418	5	94	1
Passage 34 + G418	5	91	4
Passage 34 no G418	32	68	0
ZFB37			
Passage 4 + G418	3	60	37
Passage 34 + G418	5	82	13
Passage 34 no G418	54	44	2

^a The number of zebrafish chromosomal elements was determined by FISH using the TZD probe on interphase nuclei.

viously observed for other hybrid cell lines after a high number of passages (Ruddle, 1981). Furthermore, insertion or translocation to a mouse chromosome does not seem to prevent the loss of the zebrafish chromosomal element from hybrids when selection is removed.

DISCUSSION

We have produced a panel of zebrafish/mouse hybrids containing between one and three zebrafish chromosomal elements. About half of the hybrids were monochromosomal as determined by FISH analysis. This contrasts with whole-cell fusions between mouse and rat cells, where resulting hybrids often contain a large subset of chromosomes from both parents (Peterson *et al.*, 1985; Gourdeau and Walker, 1994a). Thus, as suggested by our experiments on the stability of zebrafish chromosomal elements in hybrids (Table 3), in the absence of selective pressure, zebrafish chromosomes are probably unstable and gradually lost. This phenomenon is often observed in hybrids but usually occurs only after many cell divisions. The use of microcells (Fournier, 1981) is known to increase the proportion of monochromosomal hybrids obtained after fusion but the procedure results in fewer hybrids. However, our panel, which was produced by whole-cell fusion, already has a high proportion of monochromosomal hybrids, which will facilitate the assignment of genes and markers to specific chromosomes and will render the production of microcell hybrids unnecessary.

One additional advantage of our hybrid panel is that at least half of the stably retained zebrafish chromosomal elements were either inserted or translocated into a mouse chromosome. It is therefore likely that many of the hybrids have zebrafish subchromosomal fragments. These will allow the isolation of markers and the mapping of genes to specific chromosome regions without the need to generate radiation hybrids.

The 77 zebrafish/mouse hybrids were obtained from fusions involving a total of 2×10^7 cells from each

parental cell line. Thus, the yield of whole-cell fusion experiments between zebrafish and mouse cells (3.8×10^{-6}) was considerably lower than that obtained in fusions involving two mammalian cells (10^{-4} to 10^{-3}) (Gourdeau and Walker, 1994b). This could be due to the apparent intrinsic instability of the zebrafish chromosomes in a mouse cell line.

This is the first report in which single chromosomes or chromosomal elements have been transferred, in a stable manner, between animal species as distantly related as zebrafish and mouse. Somatic cell hybrids have been made by fusion of chicken erythrocytes with either rat or hamster cells (Schwartz *et al.*, 1971; Kao, 1973; Trisler and Coon, 1973; Leung *et al.*, 1975). Such hybrids contained a complete set of chromosomes from the mammalian parent and between one and three chick microchromosomes, a number comparable to the number of zebrafish chromosomal elements that we observed in our hybrid panel. There was, however, no characterization of the chicken/mammal hybrids using FISH or molecular markers, making the comparison with the zebrafish/mouse hybrids presented here difficult. Karyotype analysis of the chicken/hamster hybrids indicated that the chicken chromosomes did not translocate to Chinese hamster chromosomes (Kao, 1973). Moreover, chicken chromosomes were stable in the absence of selection for over 100 generations in the chicken/hamster hybrids (Kao, 1973). This contrasts with our observations that zebrafish chromosomes are often inserted or translocated to mouse elements (Fig. 1) and tend to be lost from the hybrids when cells are grown in the absence of selection (Table 3). These differences could be attributed to the recipient cells, although both hamster and mouse cells are known to segregate exogenous chromosomes such as the human chromosomes in human/rodent hybrids. Alternatively, zebrafish chromosomes could be less stable in a mammalian cell background, when selection is removed, due to the larger evolutionary distance that separates fish and mammalian lineages (400 million years) than that separating bird and mammalian lineages (300 million years). Finally, the difference in optimal temperatures for the growth of fish cells and for the growth of cells from birds or mammals may contribute to the lower stability of fish chromosomes in a mammalian cell background.

The presence of interspersed repetitive sequences in the zebrafish genome is suggested by the FISH results using whole zebrafish genomic DNA as a probe. These sequences were absent from the mouse genome as assessed by FISH. Therefore, characterization of zebrafish interspersed repetitive sequence(s) will allow the isolation of YAC or P1 clones containing large portions of specific zebrafish chromosomes from a library made with our zebrafish/mouse hybrids.

In conclusion, we have produced a panel of zebrafish/mouse hybrids containing a large proportion of monochromosomal hybrids. In many hybrids, the zebrafish chromosomal elements are inserted or translocated

to a mouse chromosome. The zebrafish chromosomes are stably maintained in a mouse background for a large number of generations without detectable loss provided the hybrids are grown in the presence of the selective agent (G418). These hybrids will not only allow rapid assignment of known genes and anonymous markers to individual zebrafish chromosomes or chromosome regions, but also will constitute a useful tool for the cloning of genes identified by mutation. The ability to generate somatic cell hybrids between distantly related species will likewise facilitate the mapping of genomes from other organisms.

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Appendix 2: Analysis Of Repetitive DNA Sequences From The Zebrafish *Danio rerio* Using A Panel Of Zebrafish X Mouse Somatic Cell Hybrids

ANALYSIS OF REPETITIVE DNA SEQUENCES FROM THE ZEBRAFISH *DANIO RERIO* USING ZEBRAFISH-MOUSE SOMATIC CELL HYBRIDS

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Subject Category: Chromosome structure

ABSTRACT

We have isolated two new tandem repetitive DNA sequences from the zebrafish *Danio rerio* and show chromosomal localization of three previously cloned repetitive sequences. Southern analysis of restriction endonuclease digested genomic DNA with some of these repetitive sequences as probes resulted in a ladder pattern. This suggests that they are mainly composed of tandem arrays of short monomeric sequences. Fluorescence *in situ* hybridization with two of these repetitive DNA sequences show small amounts of hybridization to all chromosomes at or near the centromere. One of these probes also completely paints a single chromosome from zebrafish ZF4 cells. Hybridization of this sequence to chromosomes and DNAs of a collection of zebrafish-mouse somatic cell hybrids indicates that it primarily localizes to the zebrafish chromosome corresponding to linkage group VIII. This repetitive DNA sequence will be useful for cloning zebrafish chromosome fragments out of the somatic cell hybrids. This will help in the understanding of the organization of zebrafish chromosomes and may increase our knowledge of the conservation of chromosome structure in vertebrates.

INTRODUCTION

The zebrafish *Danio rerio* has become increasingly popular as a model organism for vertebrate development and genetics. The establishment of genetic methods, and large scale mutagenesis, has identified mutations that affect the development of this organism (Streisinger et al., 1981; Kimmel, 1989; Rossant and Hopkins, 1992; Mullins and Nüsslein-Volhard, 1993; Solnica-Krezel et al., 1994). The production of a genetic linkage map of the zebrafish genome consisting of random amplified polymorphic DNAs (RAPDs), simple sequence repeats (SSR), and cloned genes has aided in the analysis of zebrafish mutations (Postlethwait et al., 1994). Recently, a panel of zebrafish/mouse somatic cell hybrids have been produced, which contain one or several zebrafish chromosomal fragments within a mouse genetic background (Ekker et al., 1996). Like the panels of human/mouse and mouse/hamster somatic cell hybrids which greatly assisted the mapping of both the human and mouse genomes (Ringertz and Savage, 1976; Ruddle, 1981), the zebrafish/mouse somatic cell hybrid panel promises the same.

The human Alu sequences provided an excellent tool to extract human genomic clones from the mouse DNA background in human/mouse hybrid libraries. Moreover, fluorescent *in situ* hybridizations (FISH) by Ekker et al. (1995) has suggested however, the presence of an interspersed repetitive sequence similar to the human Alu and mouse B1 interspersed repetitive sequences (Jagadeeswaran et al., 1981; Kominami et al., 1983; Deininger and Daniels, 1986). We would therefore be interested in the cloning, and eventual use, of these repetitive sequences for genomic analysis.

Repetitive DNA sequences can account for 20% to 70% of the total genomes for most species (Corneo et al., 1967; Birnstiel et al., 1968; Davidson and Britten, 1973; Fowler and Skinner, 1985; Varley et al., 1990). These repetitive DNA sequences have been divided into three major groups: satellite DNAs, short and long interspersed repetitive sequences (SINE and LINE), and multigene families (gene duplications) (Skinner, 1977; Brutlag, 1980; Jelinek and Schmid, 1982; Singer, 1982; Deininger and Daniels, 1986; Nanda et al., 1992). Repetitive DNAs, primarily satellite type sequences, have been identified in a number of fish species including zebrafish (Datta et al., 1988; Moyer et al., 1990; Wright, 1989; Denovan and Wright, 1990; Franck et al., 1991; He et al., 1992; Ekker et al., 1992). These satellite sequences occur primarily as tandem arrays within the genome and have a relatively short monomeric base sequence (John and Miklos, 1979). While little is known about the functional role of these repetitive DNA sequences, it has been suggested that they play an important role in chromosomal organization (John and Miklos, 1979; Jelinek and Schmid, 1982). Many of the cloned satellite type sequences are located exclusively in centromeric heterochromatin of all chromosomes.

Several sequences have also been identified to be associated with the sex chromosomes in a wide range of animals (Singh et al., 1981; Epplen et al., 1982; Singh et al., 1984; Jones and Singh, 1985; Hochstenbach et al., 1993).

A number of cloned repetitive elements in zebrafish have also been cloned which show structural and sequence characteristics of known transposable elements (Izsvák et al., 1995; Izsvák et al., 1996; Shimoda et al., 1996a; Shimoda et al., 1996b). To date, approximately 15-20% of the zebrafish genome is comprised of these known types of repetitive DNA sequences. In order to further characterize the organization of zebrafish chromosomes, to assist in the analysis of zebrafish/mouse somatic cell hybrids, we have cloned two new satellite type repetitive DNA sequences in the zebrafish. Using (FISH) we were able to show the chromosomal organization of three previously known repetitive sequences which together represent greater than 15% of the zebrafish genome. One of these zebrafish satellite-like sequences primarily localizes, and is interspersed on a single chromosome from zebrafish ZF4 cells. By using a panel of zebrafish X mouse somatic cell hybrids which were generated using zebrafish ZF4 cells, we were able to determine that linkage group VIII is highly enriched in this sequence.

MATERIALS AND METHODS

Construction of a zebrafish genomic library and screening for highly repetitive sequences.

Genomic DNA from zebrafish *Danio rerio* was prepared as described (Westerfield, 1989) and digested to completion with Tsp509I, or HindIII (NEB). The resulting DNA fragments were cloned in the EcoRI site of pBluescript KS+ vector (Stratagene) and the HindIII site of pUC19. The ligation mixture was used to transform DH5a Ultra-competent *E. coli* (Gibco/BRL) which were plated at a resulting density of 5000 colonies per 150 mm plate. The bacterial colonies were transferred to Hybond N+ nylon membrane (Amersham).

Zebrafish genomic DNA digested with Eco RI, Pst I, and Pvu II was labeled with [32P]-dCTP using the random hexamer method. This probe was used to screen the colony lifts. Colonies with the most intense hybridization signal, which should represent repetitive sequences, were picked for further analysis.

Southern and dot blot analysis.

For southern analysis, zebrafish genomic DNA was digested to completion with the appropriate restriction endonuclease according to the manufacturer instruction (NEB). The digested DNA was separated by electrophoresis on a 1.2% agarose gel and transferred onto Hybond N+ membrane (Amersham) according to Southern (1975). Dot blot analysis was performed by applying 100 ng undigested DNA directly to PALL nylon membrane (Gibco/BRL). Blots were hybridized to 32P-random primer labeled probes and washed in 0.1X SSC and 0.1% SDS at room temperature. Blots were exposed to x-ray film and imaged and densitometrically analyzed using a Bio-Rad GS-525 Molecular Imaging System and Molecular Analyst software.

Fluorescence in situ hybridization.

The repetitive sequence probes were biotinylated using the BRL Bioprime DNA labeling system. Unincorporated biotinylated nucleotides were removed by ethanol precipitation. Metaphase chromosome spreads of ZF4 and LFF (zebrafish fibroblast-like) cells and zebrafish-mouse hybrid cells were prepared according to standard techniques (0.05 ug/ml. colcemid for 2 hours, followed by a hypotonic solution of 1% sodium citrate for 20 minutes at 37°C. Slide preparations were warmed overnight at 55°C, then stored at -20°C until use. Prior to hybridization, slides were dehydrated by passing through a 70%, 90%, and 100% ethanol series for 2 minutes each, denatured in 70% de-ionized formamide/2X SSC for 2 minutes at 70°C, and dehydrated again in ethanol. The hybridization mixture (50% de-ionized formamide, 10% dextran sulfate, 2X SSC, 100 ng of biotinylated probe, and 1 ug of salmon sperm DNA) was denatured at 75°C for 10

minutes before applying 10 ul to each slide. The slides were covered with a coverslip, sealed with rubber cement, and incubated overnight at 37°C in a humidified chamber. The slides were washed at 37°C in 50% de-ionized formamide/2X SSC for 10 minutes followed by a 10 minute wash in 2X SSC. Avidin-FITC detection was performed using the Oncor detection kit of biotin labeled probes. The slides were counter-stained with propidium iodide in antifade and observed using a fluorescein filter combination on a Zeiss Axioskop microscope.

RESULTS AND DISCUSSION

Zebrafish repetitive DNA sequences were cloned by screening a plasmid genomic library of short genomic DNA fragments with a probe made from total genomic DNA digested to completion with Kpn I, Pst I, and HinfI. Colonies corresponding to the strongest signals were isolated and analyzed further.

In the sequencing of many clones we obtained two previously unknown zebrafish repetitive elements designated ZTR8 and ZTR22, and three sequences which were previously cloned - SR1, DANA and ZFRAL (Ekker et al., 1992; Izsvák et al., 1996; He et al., 1992, respectively). Similar to SR1 and ZFRAL, Southern analysis of restriction enzyme digested zebrafish DNA with ZTR8 and ZTR22 probes produced a ladder pattern (Figure 1 - Southern blots of ZTR8 and ZTR22). This pattern is consistent with repetitive sequences which are organized in the genome as tandem arrays. However, the pattern obtained from probing completely digested DNAs with the ZTR8 probe only revealed a single monomer band. The preservation of the HindIII sites in most of the repeats throughout the genome suggests that this repetitive sequence evolved relatively recently since mutations over time would destroy some of these sites (as evident by the ladder produced by ZTR22).

The tandem repeat ZTR8 is A+T rich (72.5%) and has 191 bp HindIII monomer unit (Fig 2a). Two short (7 bp) direct repeats, CAATTTT, were found at position 36-42, and 127-134. The repeat ZTR22 has a HindIII monomer length of 183 bp, and contains a relatively high G+C content (50%) (Fig 2b). Following a series of dot blot hybridizations of serial dilutions of probe and zebrafish genomic DNA we determined that these two repetitive sequences each represent approximately 0.05-0.1% of the total zebrafish genome (data not shown). A sequence similarity search indicates that the ZTR22 repetitive sequence shows a high nucleotide identity of 86-91% to a repetitive sequence found in several different carp species (GenBank #X63146, X63148, X63145). These fish belong to the same family Cyprinidae as zebrafish. This sequence was also shown to be very similar (80-90% identity) to a large number of vertebrate 5S rRNA sequences suggesting this repeat may have evolved within the family Cyprinidae from 5S rRNA sequences. Such evolution has been reported for a tRNA-derived short interspersed DNA element associated with mutational activities in the zebrafish (Izsvák et al., 1996).

In order to determine the overall organization, localization and interspersion of these sequences, we performed fluorescent *in situ* hybridization (FISH) using ZTR8, ZTR22, SR1, DANA and ZFRAL probes to metaphase spreads of the zebrafish fibroblast-like cell line ZF4 (Driever et al., 1993). We were unable to obtain an observable hybridization signal using both the ZTR8 and ZTR22 probes. This result is

probably indicative of the interspersed organization of these repeats since we would expect to observe a hybridization signal if these repeats were highly localized to a single portion of the chromosomes, for example to the centromeres. FISH with the SR1 revealed that this sequence is exclusively located at the centromere of all chromosomes (Figure 3a). We have used this sequence previously, along with a total zebrafish genomic DNA probe, to confirm and identify the presence of zebrafish chromosomal segments in zebrafish X mouse somatic cell hybrid clones (Ekker et al., 1996). The ZFRAL sequence also hybridized to all chromosomes at or near the region of the centromeres. However, most interesting is the observation that this probe also completely paints a single chromosome in its entirety (Figure 3b).

To identify the chromosome to which the ZFRAL sequence hybridizes to in its entirety, we performed dot blot analysis to DNAs extracted from a panel of zebrafish/mouse somatic cell hybrids. An example of this analysis is shown in Figure 4. The panel consisted of hybrids which contain zebrafish chromosomal elements corresponding to all of the 25 zebrafish linkage groups, previously determined by Southern blot and PCR analysis (Ekker et al., 1996). The linkage groups analyzed correspond to the linkage groups of Postlethwait et al. (1994). Strong hybridization of the 32P-labeled repeat is apparent only in hybrids that have been shown to contain the chromosome corresponding to linkage group VIII. The signal intensity for the hybrid DNAs containing linkage group VIII were 5-200X stronger than the signal intensity of hybrid DNAs that did not contain markers for zebrafish linkage group VIII(not shown)(Ekker et al., 1996). The high signal intensity of the dot blots suggests that the FISH painted chromosome belongs to linkage group VIII. In order to further clarify the identification of this FISH painted chromosome, we performed FISH using this repetitive element on metaphase spreads of different hybrid cell lines containing the zebrafish chromosome corresponding to linkage group VIII (Figure 5a). The repetitive element probe completely painted the zebrafish chromosome segments in a manner similar to the total zebrafish probe (Fig 5b). Since FISH on metaphase spreads of ZF4 cells indicated hybridization only near the centromere on most chromosomes, this result is also suggestive that this repetitive element is interspersed throughout chromosome VIII.

The ZF4 cell line however, is problematic for this type of genomic organizational analysis since it is apparent that it is a pseudoploid cell line and may contain large chromosomal rearrangements. We have generated in our lab, a zebrafish embryonic cell line (LFF) which contains a normal chromosome complement of 50 (2N). FISH with the SR1 probe on this cell line again indicates localization at the centromeres of all chromosomes (Figure 6a). Localization at or near the centromere was also observed

using the ZFRAL probe, however in these cells we did not observe a completely painted chromosome as we did in the ZF4 cells (Figure 6b). The difference in the results between ZF4 and LFF cell lines is difficult to interpret. Analysis of the strongly FISH and dot blots ZFRAL positive somatic cell hybrids (discussed above) suggests enrichment of ZFRAL along the entire length of linkage group VIII. By PCR analysis of these hybrids we know that the normal arrangement of known genes and markers for linkage group VIII does not appear to be altered (Egger et al., 1996; unpublished data). We can also not evoke a mechanism (transposition for example) that could account for this enrichment on a single chromosome of the ZF4 cell line. It is intriguing that in some organisms, chromosome-specific repetitive sequences have been observed to be associated with the sex-chromosomes. It is formally possible that these two different cell lines were generated from zebrafish embryos of the opposite genetic sex. We have tested this hypothesis by Southern analysis of restriction enzyme digested DNAs from female and male adult zebrafish (data not shown) and were unable to observe any difference in hybridization between the two sexes. While this result does appear to be negative, because of possible signal quenching from hybridization to the centromeres of all chromosomes or the apparent epigenetic effects on sex determination in zebrafish, we can not reject the possibility that this sequence is associated to a primitive sex chromosome similar to that observed in other organisms.

The repetitive sequence DANA represents approximately 10% of the zebrafish genome (Izsvák et al., 1996). FISH with this sequence shows labeling along the entire extent of all chromosomes (Figure 7).

Interestingly, there is an apparent lack of signal in the region of the centromere where we previously showed hybridization by SR1 and ZFRAL repetitive sequences. The high copy number and degree of interspersed of the DANA repeat throughout the genome, similar to the human Alu repeats, would make this sequence useful for retrieving zebrafish DNA clones from libraries produced from mouse X zebrafish somatic cell hybrid DNAs.

Figure 1 - Southern blots of HindIII digested zebrafish DNA hybridized with ZTR8 (P-partial digestion; C-complete digestion) and ZTR22 repetitive sequence probes display a ladder pattern characteristic of satellite-type sequences occurring as tandem repeats.

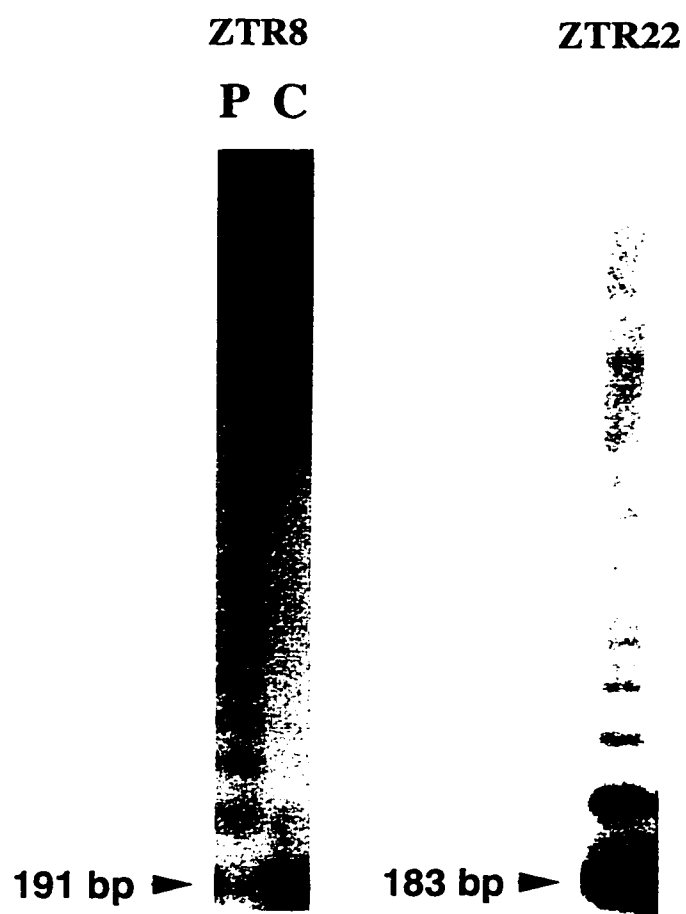


Figure 1

Figure 2- DNA sequence of HindIII monomers of the tandem repeats ZTR8 (A) and ZTR22 (B). The sequence of the ZTR8 repeat contains two short internal repeats of the sequence, CAATTTT (UNDERLINED).

A-ZTR8

5' AAGCTTTCTTTAAAAATAGTTTTTCCTCTTAAACACAATTTTTT
TATACCAAATTTTCACTTGATTACATAAGGTATATGTTTTGATAA
ATAGTACTTACTTTCAACATACCTTCITTATGCTCATTCAATTTTAT
TTGGTGGTGTAAGTAGGAAAAGTGGTTGGTCAATGGTTTTAG
CTTAGAAGCTT 3'

B-ZTR22

5' AAGCTTTTCGAAGTGCTTCATTTGGCAGCTGATTTAAATAGCCA
ACGCTTGACGTCCTCTTTGCTTACGGCCATACCACCCTGGAAATG
CCCGATCTCATCTGAACTCGGAAGTAAAGCAGGGTCGGGCCTGGT
TAGTACTTGGATGGGAGACCGCCTGGGAATACCAGGTGCTGTAAG
CTT 3'

Figure 3 - Fluorescent *in situ* hybridization of repetitive sequences SR1 (a) and ZFRAL (b) to chromosome spreads from zebrafish ZF4 cells.

Figure 4- Dot blot hybridization of the repetitive sequence ZFRAL to DNAs from a number of zebrafish X mouse somatic cell hybrids. A very strong hybridization signal is observed with ZFB27 which has been shown to contain a large zebrafish chromosome segment of linkage group VIII.

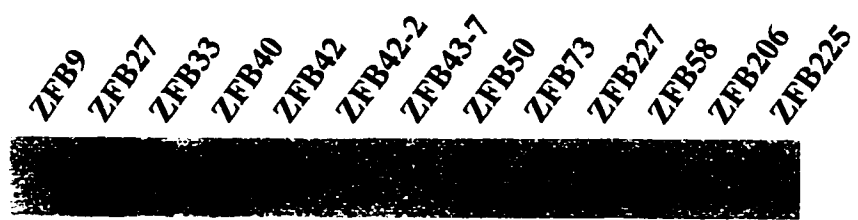


Figure 4

Figure 5 - Fluorescent *in situ* hybridization of repetitive sequence ZFRAL (a) and a total zebrafish DNA probe (b) to chromosome spreads from a zebrafish X mouse somatic cell hybrid containing zebrafish linkage group VIII.

a

b

Figure 6 - Fluorescent *in situ* hybridization of repetitive sequence SR1 (a) and ZFRAL (b) to chromosome spreads from a zebrafish embryonic cell line LFF.

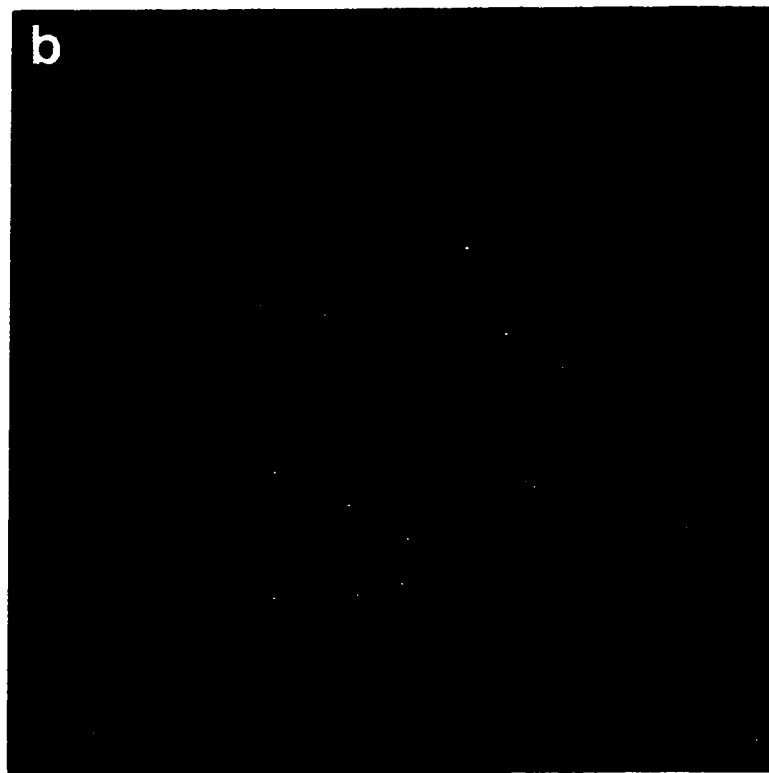
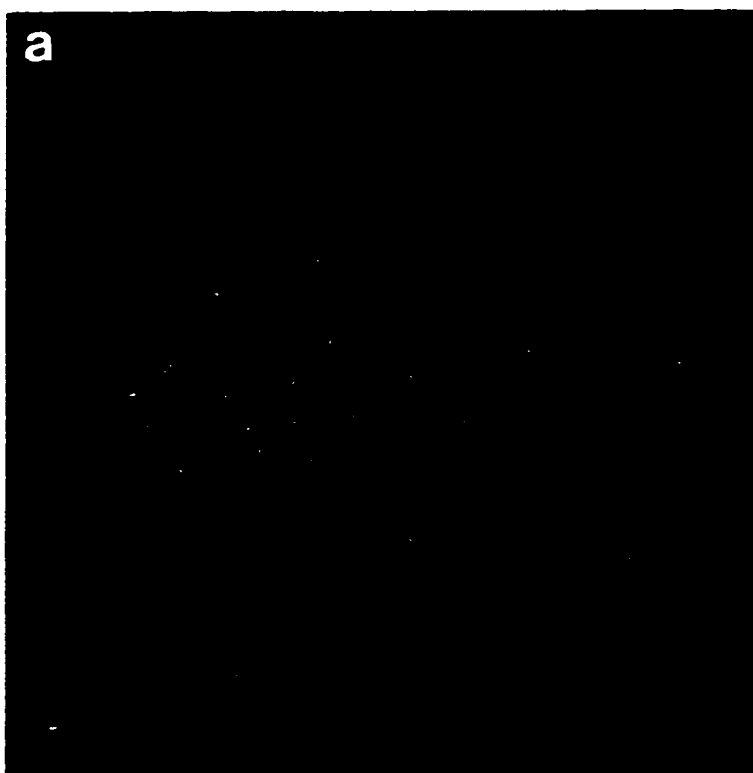
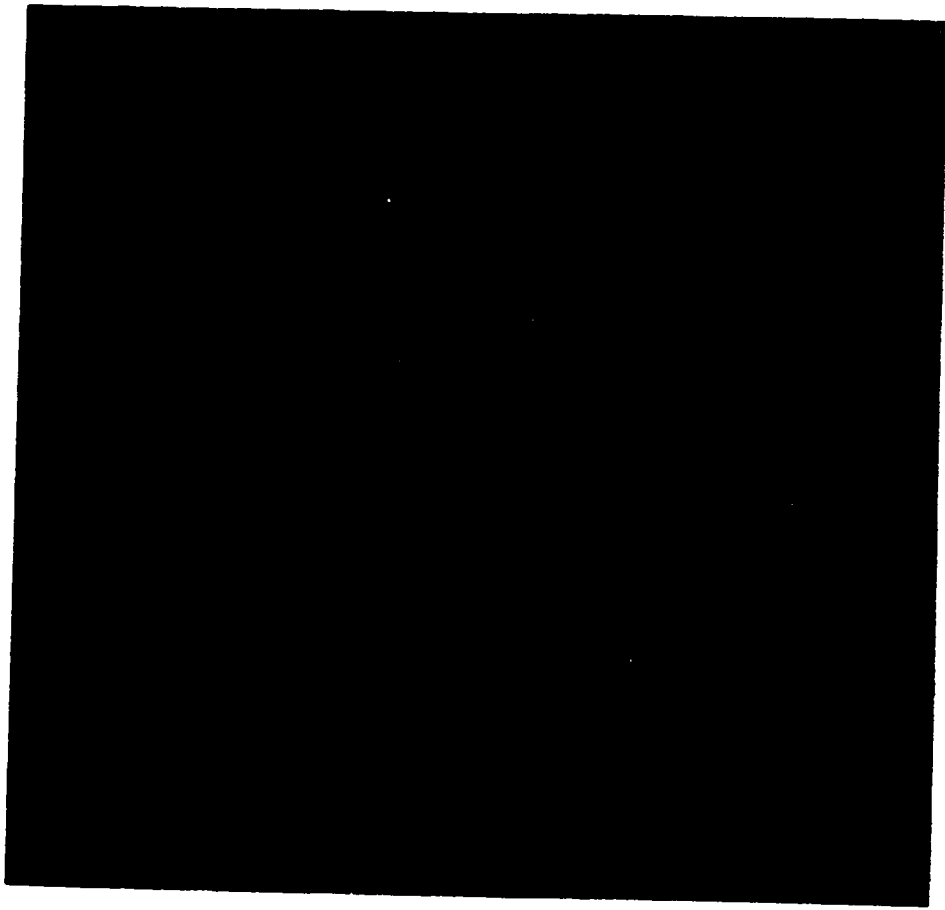


Figure 7- Fluorescent *in situ* hybridization of repetitive sequence DNA to chromosome spreads from a zebrafish embryonic cell line LFF.



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Appendix 3: Curriculum Vitae - C. Cristofre Martin

Curriculum Vitae

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Research Assistant, Dr. Marianne Steggle (Supervisor) Department of Fine Arts, Acadia University, Wolfville, Nova Scotia. Duties: field study cataloging and surveying of British sculptures in India (Madras - New Delhi). May 1988 - August 1988.

Research Assistant, Dr. Richard Gordon (Supervisor) Department of Botany, Radiology, Physics & Electrical Engineering. Duties: electron microscopy, light microscopy, image processing, establishment, maintenance and breeding of axolotl colony (100+ animals). September 1988 - May 1992.

Board of Directors (Research Co-ordinator, Treasurer), Spina Bifida Association of Manitoba, Winnipeg, Manitoba. Editor: Spina Bifida Association of Manitoba Newsletter. December 1990 - November 1993.

Researcher, Dr. John Stewart (Supervisor) Department of Botany (Head), University of Manitoba. Duties: gas chromatography for CO₂ production from soil and peat moss samples, herbicide bioassay on Black spruce, production and analysis of a questionnaire on the Manitoba wild rice industry (in conjunction with the Department of Natural Resources - Lands Branch). September 1991 - May 1992.

Teaching Assistant, Dr. Norm Kenkel and Dr. Isobel Waters (Supervisors), Department of Botany, University of Manitoba. Principles of Ecology. January 1992 - April 1992.

Teaching Assistant, Dr. Sam Badour & Dr. Larry Van Caeselle (Supervisor), Department of Botany, University of Manitoba. Introduction of Genetics I & II. 1992 - 1994.

Lecturship, Dr. Fransisco Madrid (Supervisor), Continuing Education (Adult education), Department of Education. Introduction of Genetics I. 1994.

Lectures:

R. Gordon, C.C. Martin & N.K. Bjorklund (1991). A wave of cell differentiation that forms the brain, with genetic and evolutionary consequences. *Physiology Lecture, Faculty of Medicine, University of Manitoba*, May 7,1991.

Department of Botany, Genetics II 1.346., Supervisor Dr. R. McGowan, 1993

Awards:

Northern Studies Training Program 1992 - \$4,100 (Declined)

Department of Zoology Special Scholarship, Univ. of Manitoba 1992 - \$4,000

Department of Zoology Special Scholarship, Univ. of Manitoba 1993 - \$4,000

NSERC Post-Graduate Scholarship 1994-96 - \$34,800

University of Ottawa Excellence Scholarship 1994-97 - \$18,150

University of Ottawa Research Grant 1995-96 - \$3,000

University of Ottawa Travel Grant 1996 - \$400

Ontario Graduate Scholarship 1996-97 - \$10,000

Departmental Seminar Award, Dept. Anat. & Neuro., Univ. of Ottawa 1996 - \$50

HSURC Post-doctoral Fellowship, Univ. of Sask. 1997-1998 - \$26,500 (Declined)

NSERC Post-doctoral Fellowship, Temple Univ. 1997-99 - \$60,000

Degrees Obtained/In Progress:

<u>Degree</u>	<u>University</u>	<u>Year</u>
B.Sc.	Department of Zoology University of Manitoba	1992 (Dean's Honor List)
M.Sc.	Department of Zoology University of Manitoba	1994
Ph.D.	Dept. of Anatomy & Neurobiology University of Ottawa	1997

Extracurricular Training:

PADI open water SCUBA diving (certified) (1989) #C73,533

Electron microscopy Technique (1989-1990): Trained by Garry Burgess and Dr. Larry Van Caessle in theory, fixation, embedding, staining, sectioning, and imaging. Independent use of: Phillips 420, Hitachi, AEI, and Zeiss electronmicroscopes.

Canadian Council on Animal Care. Using of Laboratory Animals for Scientific Research. University of Manitoba. 1993

Confocal Microscopy (1995). University of Ottawa, Confocal Microscopy Facility.

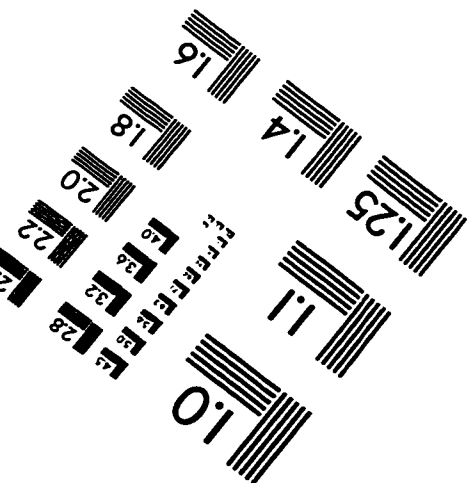
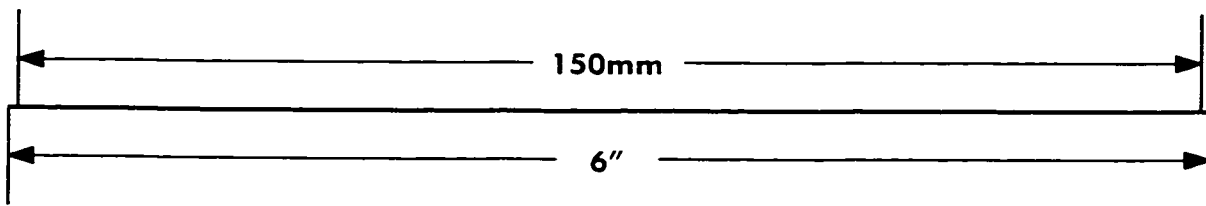
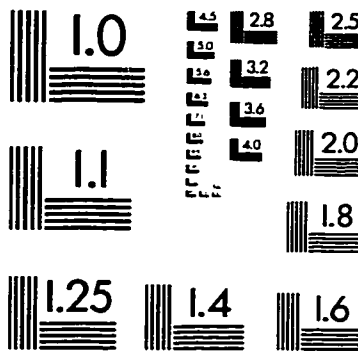
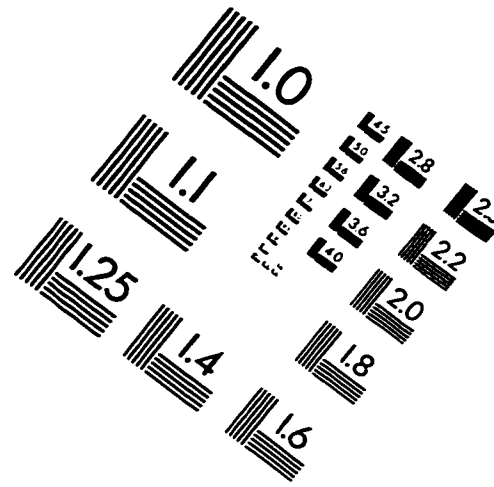
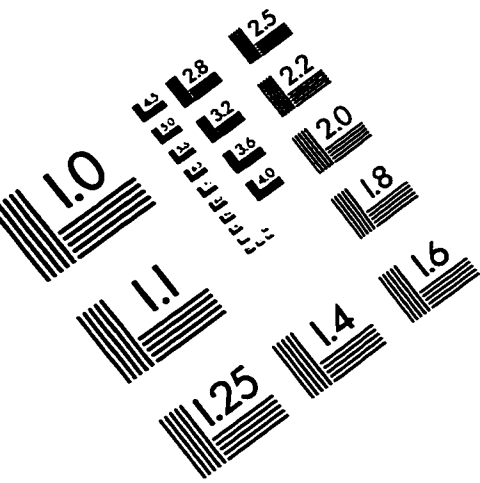
Center for University Teaching, University of Ottawa:
Workshop: The Lecture - A Tool to be Mastered. October 1995

Extracurricular Activities:

Judge, Manitoba Schools Science Symposium. University of Winnipeg, Winnipeg, Manitoba. May 5-6, 1990 and April 26-27, 1991.

Spokesperson, "Let's Talk Science" Program, University of Ottawa, Ottawa, Ontario. Science outreach program to high school students. 1994-1996.

IMAGE EVALUATION TEST TARGET (QA-3)



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