Transcriptional Regulation of the *Xenopus MyoD* Gene

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1 Summary

MyoD is one of the MRFs (Muscle Regulatory Factors) and it functions to determine the muscle cell fate. The mechanism by which MyoD regulates the muscle development program is very well understood. However, the transcriptional regulation of the *MyoD* gene itself has not studied in *Xenopus*. In this thesis, I have analyzed the transcriptional regulatory mechanism of the *MyoD* gene in *Xenopus* by different approaches, which include transgenic reporter analysis of its *cis*-regulatory elements of *MyoD* transcription and gain-of-function and loss-of-function tests of several potential regulaters.

Here I showed that the expression of the *XmyoD* gene is controlled by a combination of induction, repression and maintenance. One activating motif, one repressing motif and one maintenance motif were found by transgenic reporter analysis. XSRF (*Xenopus* serum response factor) binds with the maintenance enhancer to maintain the expression of *XmyoD* gene. A repetitive DNA sequence was discovered in the -2.8/-2.0kb region in the *XmyoD* genomic sequence. The repetitive DNA sequence in the *XmyoD* gene locus may produce sense and anti-sense transcripts. In addition, several potential regulators have been analyzed. It has been shown that XSEB-4, a direct target of XmyoD protein, is able to induce the expression of *XmyoD* gene. *Xenopus* Ying Yang 1 (XYY1) can repress the expression of the *XmyoD* gene. Lef-1 is necessary and sufficient for the expression of *XmyoD* gene. This provides strong evidence that Lef-1 is the transcription factor of the zygotic Wnt signaling pathway that activates the expression of the *XmyoD* gene.

2 Introduction

2.1 Early development and mesoderm patterning of *Xenopus*

With its rapid embryonic development, large egg size (1–2 mm in diameter) and high numbers of embryos (1,500 per female), *Xenopus* provides a favorable model system for the study of vertebrate development, and it has been used extensively to analyse the events in early embryogenesis (Figure 2.1).

2.1.1 Frog embryology

The frog egg is radially symmetrical and is divided into an animal and a vegetal domain. During embryonic development, the egg is converted into a tadpole containing millions of cells but containing the same volume of material.

Entrance of the sperm initiates a sequence of events: The cytoplasm of the egg rotates about 30 degrees relative to the point of sperm entry. It foretells the future pattern of the animal: its dorsal (D) and ventral (V) surfaces, its anterior (A) and posterior (P), its left and right sides. The haploid sperm and egg nuclei fuse to form the diploid zygote nucleus.

The fertilized egg undergoes a series of mitoses. The first cleavage occurs shortly after the zygotic nucleus forms. A furrow appears that runs longitudinally through the poles of the egg, passing through the point at which the sperm entered. This divides the egg into two halves forming the 2-cell stage. The second cleavage forms the 4-cell stage. The cleavage furrow again runs through the poles but at right angles to the first furrow.

The furrow in the third cleavage runs horizontally but in a plane closer to the animal than to the vegetal pole. It produces the 8-cell stage. The next few cleavages also proceed in synchrony, producing a 16-cell and then a 32-cell embryo. However, as cleavage continues, the cells in the animal pole begin dividing more rapidly than those in the

vegetal pole and thus become smaller and more numerous after midblastula transition (MBT).

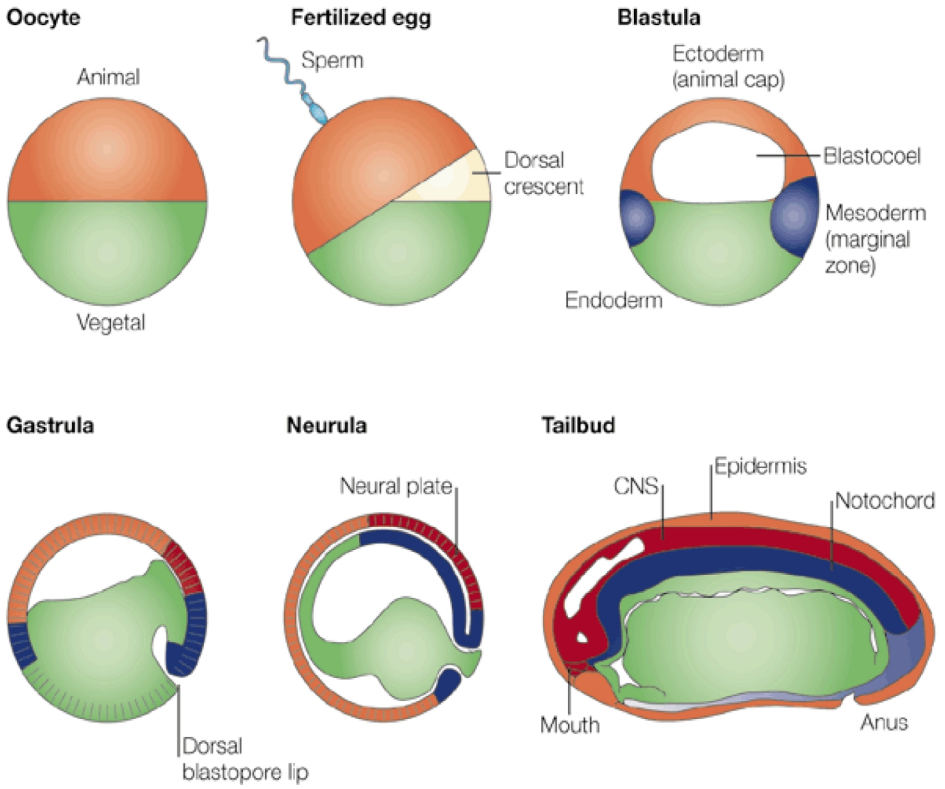

Figure 2.1 The anatomy of *Xenopus* development.
The ovarian oocyte is radially symmetrical and is divided into an animal and a vegetal domain. One hour after fertilization, an unpigmented dorsal crescent is formed in the fertilized egg opposite the sperm entry point. As the embryo rapidly divides into smaller and smaller cells, without intervening growth, a cavity called the blastocoel is formed, which defines the blastula stage. By the late blastula stage (9 h of development), the three germ layers become defined. The ectoderm, or animal cap, forms the roof of the blastocoel. The mesoderm is formed in a ring of cells in the marginal zone, located between the ectoderm and endoderm. At the gastrula stage (10 h), involution of the mesoderm towards the inside of the embryo starts at the dorsal blastopore lip. The morphogenetic movements of gastrulation lead to the formation of the vertebrate body plan, patterning the ectoderm, mesoderm and endoderm. At the neurula stage (14 h), the neural plate, or future central nervous system (CNS), becomes visible in dorsal ectoderm. By the tailbud stage (24–42 h), a larva with a neural tube located between the epidermis and the notochord has formed. The blastopore gives rise to the anus, and the mouth is generated by secondary perforation. (Adapted from De Robertis et al., 2000)

Several hours later, continued cleavage has produced a hollow ball of thousands of cells called the blastula. A fluid-filled cavity, the blastocoel, forms within it. During this entire process there has been no growth of the embryo. There has been no transcription and translation of zygote genes. All of the activities up to now have been run by gene products (mRNA and proteins) deposited by the mother when she formed the egg. After MBT, the zygotic genes start to be transcripted and translated.

The start of gastrulation is marked by the invagination of cells in the region of the embryo that was once occupied by the middle of the dorsal crescent. This produces both an opening (the blastopore) that will be the future anus and a cluster of cells that develops into the Spemann organizer.

As gastrulation continues, three distinct germ layers are formed: ectoderm, mesoderm, endoderm. Each of which will have special roles to play in building the complete animal. Some of these are listed in the table. Muscle is derived from mesoderm.

Ectoderm	Mesoderm	Endoderm
skin	notochord	lining of gut
brain	**muscles**	lining of lungs
spinal cord	blood	lining of bladder
all other neurons	bone	liver
sense receptors	sex organs	pancreas

2.1.2 Mesoderm induction

After the midblastula stage, endoderm releases mesoderm-inducing signals (Wylie et al., 1996; for review see De Robertis, et al., 2000), which has been investigated experimentally by combining explants of vegetal and animal tissue. The established view from embryological studies was that the endoderm releases two signals, one from the ventral endoderm, which induces ventral mesoderm, and a second from the dorsal endoderm (Nieuwkoop center), which induces dorsal mesoderm (Spemann's organizer). A third signal subsequently emanates from the Spemann's organizer to refine the initial dorsal–ventral pattern (Heasman, 1997) (Figure 2.2).

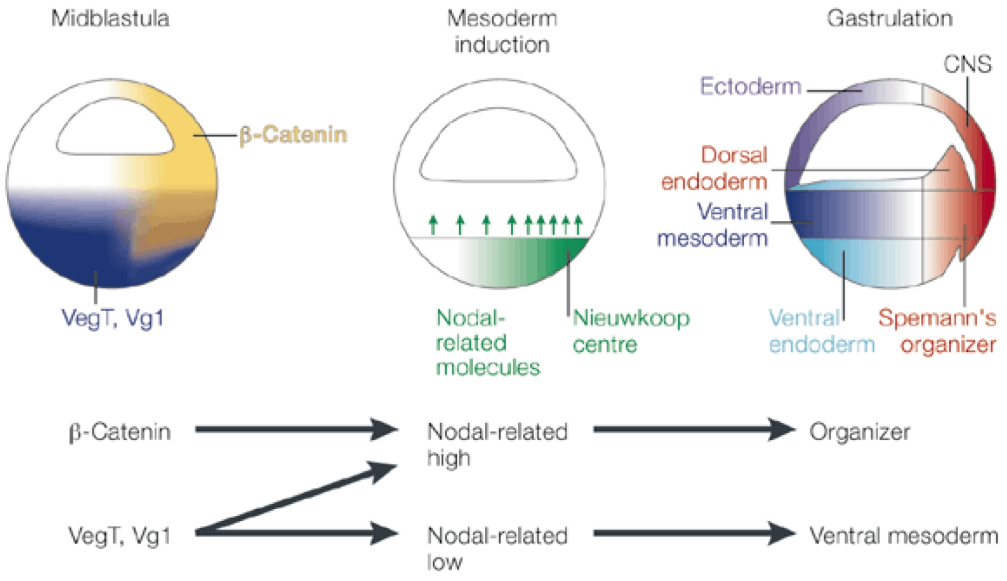

Figure 2.2 Two-step model of mesoderm induction in *Xenopus*.
At the midblastula stage, higher ß-Catenin levels on the dorsal side of the embryo, together with the vegetally located transcription factor VegT and the maternal TGF-ß family growth factor Vg1, generate a gradient of Nodal-related molecules expressed in the endoderm. In turn, this gradient induces the formation of overlying mesoderm: low doses of Nodal-related molecules (Xnrs) lead to the formation of ventral mesoderm, whereas high doses lead to the establishment of Spemann's organizer. Nieuwkoop's centre is the region of dorsal endoderm that induces organizer tissue. At the gastrula stage, the organizer secretes a cocktail of factors that refine the initial patterning. Note that ß-Catenin is widely distributed on the dorsal side, including in derivatives of the three germ layers. (CNS, central nervous system.) (Adapted from De Robertis et al., 2000)

Nodal-related proteins (Xnrs), which belong to the transforming growth factor (TGF)-β family of growth factors, have important functions in mesoderm formation in different species. At the blastula stage, Xnrs are expressed in a dorsal to ventral gradient in endodermal cells (Agius et al., 2000), which is accompanied by the preferential phosphorylation of Smad2 (a downstream effector of TGF-β signaling) on the dorsal side (Faure et al., 2000). Nodal-related proteins function as a morphogen — molecules that directly determine cell fates within a field of cells in concentration-dependent manner — to induce ventral and posterior mesodermal markers at lower doses and progressively more dorsal and anterior markers at higher dosage (Green et al., 1992; Jones et al., 1995; Agius et al., 2000).

The gradient of Xnr expression in the endoderm is thought to be activated by three maternally provided molecules: Vg1, VegT and ß-catenin (Figure 2.2). Vg1, a TGF-β factor, and VegT, a T-box transcription factor, are both localized to the vegetal pole of the *Xenopus* oocyte and are potent inducers of endoderm (Henry & Melton, 1998; Zhang et al., 1998). Depletion of maternal VegT leads to the absence of endoderm. In VegT-depleted embryos, *Xnr* transcription and mesoderm formation are severely inhibited and can be rescued by injection of Xnr mRNA (Kofron et al., 1999). Wild-type embryos microinjected with VegT and Vg1 have only low levels of *Xnr* transcription; however, when ß-catenin is also provided, it cooperates with VegT and Vg1 to achieve the high levels of Xnr expression that cause organizer induction (Agius et al., 2000) (Figure 2.2).

In conclusion, after the midblastula satge, the ß-catenin signal, in combination with other maternal genes, activates a dorsal–ventral gradient of several Nodal-related signals in the endoderm that, in turn, mediate the induction and patterning of the mesodermal layer.

2.2 Muscle development

Skeletal muscle derives from the mesoderm and it has been a paradigm for cell specification and cell differentiation (for review see Perry and Rudnicki, 2000; Pownall et al., 2002). The original cloning of *MyoD* and its characterization as a master regulatory gene for the determination of skeletal muscle, started a new era of research in skeletal myogenesis. This discovery led to the cloning of three other muscle regulatory factors (MRF) namely Myf5, myogenin, and MRF4. In all cases, overexpresison of these factors converts non-muscle cells to the myogenic lineage, demonstrating their role in myogenic lineage determination and differentiation. Furthermore, the ability of each factor to initiate the expression of one or more of the other three suggests they form a cross-regulatory loop.

The MRFs belong to the basic helix-loop-helix (bHLH) superfamily of transcription factors. The HLH domain is responsible for the dimerization of these factors with the

ubiquitously expressed E-proteins, such as E12, E47, HEB, and ITF, and the basic domain is responsible for DNA binding. Heterodimers bind to the consensus E-box (5'-CANNTG-3') DNA sequence motif found in the promoters of many muscle specific genes. The bHLH domains of the MRFs are highly homologous while the amino and carboxyl terminal ends show limited homology. Structurally, the MRFs contain several functionally distinct domains responsible for transcriptional activation, chromatin remodeling, DNA binding, nuclear localization and heterodimerization (for review see Rupp et al., 2002).

2.3 MRF expression in vertebrate embryos

2.3.1 MRF expression in muscle progenitors of the mouse embryo

During mouse development, the MRFs are expressed in a highly regulated spatial and temporal fashion (reviewed in Arnold & Braun, 2000). Gene expression studies using *in situ* hybridization techniques showed that MRF expression occurs in slightly different patterns in epaxial versus hypaxial muscle. *Myf5* is expressed in the dorsomedial portion of the somite at 8 days post coitus (p.c.) and at day 9.5 in the lateral, or hypaxial domain of the somite (Ott et al., 1991). The expression of *Myogenin* is first detected at day 8.5 p.c. and remains detectable throughout fetal development (Sassoon et al., 1989). The expression of *MRF4* is detected transiently between days 10 and 11 and then reexpressed from day 16 onward to become the predominant MRF expressed in adult muscle (Bober et al., 1991; Hinterberger et al., 1991; Hannon et al., 1992). *MyoD* expression is first detected approximately at day 9.75 in the hypaxial somitic domain and continues to be expressed throughout development (Bober et al., 1991; Faerman et al., 1995). In the limb bud, the temporal expression of these factors is slightly different. Although *Myf5* expression is again detected first, it is followed very quickly by *MyoD* and *myogenin* which are detected from day 10.5 onward (Ott et al., 1991; Sassoon et al., 1989). Unlike observations in the somite, *MRF4* is not transiently expressed during limb development, but is first detected at day 16 and becomes the predominant MRF expressed in the adult (Bober et al., 1991, Hinterberger et al., 1991).

2.3.2 MRF expression in muscle progenitors of the frog embryo

Xenopus development is very rapid from fertilized egg to swimming tadpole, which requirs the production of large myotomal swimming muscles. *Xenopus* embryo fate maps have been constructed (Bauer et al., 1994; Dale and Slack, 1987; Keller, 1976), and mesodermal cells have been identified to give rise to the somites that form these myotomal muscles.

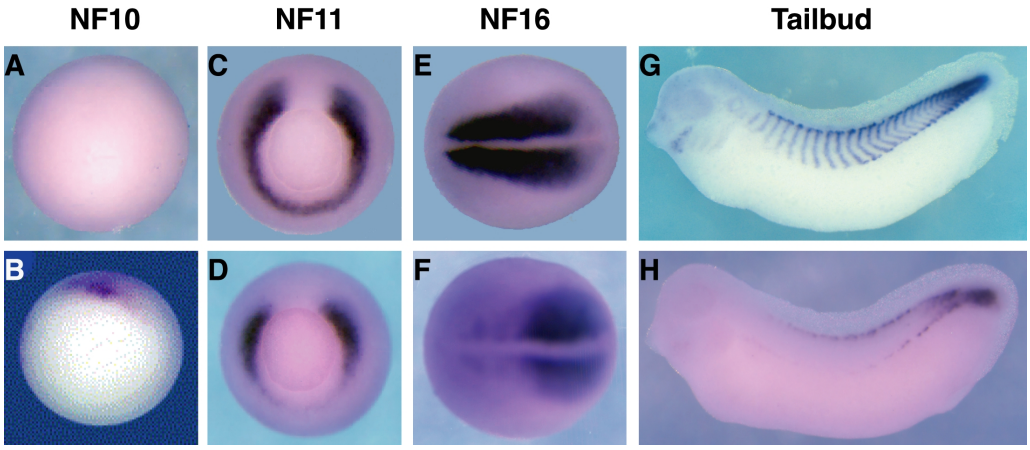

Figure 2.3 *XmyoD* and *Xmyf5* are expressed in myogenic precursors of *Xenopus laevis* embryos. (A) There is no *XmyoD* expression at Nieuwkoop and Farber (NF) stage 10. (B) *Xmyf5* expresses in organizer region at NF 10. (C, D) NF stage 11 gastrula embryos, viewed from the vegetal hemisphere. *XmyoD* (C) is expressed in a broad domain but is restricted from the dorsal organizer region, whereas *Xmyf5* (D) is expressed mainly dorsally at this stage. (E, F) NF stage 16 neurula embryos viewed dorsally. *XmyoD* (E) is expressed in myogenic precursors along the entire anteroposterior axis, whereas *Xmyf5* (F) expression is restricted to a more posterior domain. (G, H) Tailbud stage embryos viewed laterally with anterior to the left. *XmyoD* (G) is expressed in the somite myotome and in myogenic progenitors in the head. *Xmyf5* (H) expression is high in the posterior unsegmented mesoderm and becomes restricted to the dorsal and ventral somite. (A, C, E, G, Xiao and Rupp, unpublished data; B, D, F, H, adapted from Yang et al., 2002)

XmyoD is transiently expressed in all cells at the mid-blastula transition (Rupp & Weintraub, 1991). During early gastrulation, *XmyoD* RNA is upregulated specifically in mesodermal progenitors (Figure 2.3 C). XmyoD protein is detected by mid-gastrulation and after 2h, expression of *cardiac-actin* is activated, which is an early muscle differentiation gene (Hopwood et al., 1989, 1992). *Xmyf5* is also activated during early gastrula stages, and its domain of expression initially extends more dorsally, including

transient expression in Spemann organizer (Figure 2.3B). During later gastrula stages, the expression of *Xmyf5* is restricted to the dorsolateral mesoderm (Figure 2.3D) (Hopwood et al., 1991). It has been shown by cell transplantation studies that cells from myogenic regions of late gastrula embryos, when placed at ectopic ventral sites, can differentiate into muscle cell autonomously (Kato & Gurdon, 1993). Thus mesodermal cells expressing *XmyoD* and *Xmyf5* prior to somite formation are specified and comitted for myogenesis at late gastrula stages. At earlier stages, *XmyoD*- and *Xmyf5*-expressing precursors cannot differentiate ectopically after cell transplantation and therefore require additional signals to maintain their myogenic potential.

2.4 Functional and genetic relationships of the MRFs

The expression of muscle lineage-specificity of MRF genes in vertebrate embryos provide evidence that MRFs are regulators in the specification and differentiation of myogenic progenitor lineages. A framework was established by these findings for genetic and experimental embryological studies of MRF functions in embryos.

2.4.1 Myogenic functions of MRFs

Myf5 and MyoD were identified as dominant regulators of myogenic progenitor specification in tissue culture studies that demonstrated their expression in myoblast progenitors, their myogenic conversion activities in DNA transfection assays, and their autoregulatory functions. However, when expressed ectopically in embryos, MyoD and Myf5 have incomplete myogenic functions. Ectopic expression of *XmyoD* or *Xmyf5* in *Xenopus* animal pole activates chromosomal *XmyoD* and *cardiac-actin*, an early muscle differentiation gene, but muscle differentiation and the expression of the entire array of muscle protein genes are not initiated (Hopwood & Gurdon, 1990; Hopwood et al., 1991), even when these factors are co-expressed with their E12 bHLH partner protein (Rashbass et al., 1992). In transgenic mouse embryos, ectopic expression of *MyoD* in mesoderm and ectoderm lineages (Faerman et al., 1993) or heart (Miner et al., 1992) also led to autoregulation of chromosomal *MyoD* and activation of some contractile proteins

but did not initiate a complete conversion of these lineages into differentiated muscle. Thus at ectopic sites in the embryo, expression of *Myf5* and *MyoD* can initiate some gene regulatory processes of myogenic specification, including autoregulation and expression of early muscle differentiation markers, but not the later regulatory program for muscle differentiation.

2.4.2 Genetic analysis of Myf5 and MyoD functions in progenitor specification

Targeted inactivation of the MRFs has provided a great deal of insight into the nature of lineage determination, lineage maintenance and their genetic relationships. *MyoD* (-/-) and *Myf5* (-/-) mutant mice are viable and fertile (Kaul et al., 2000; Rudnicki et al., 1992); however, *MyoD* (-/-); *Myf5* (-/-) embryos do not form skeletal muscles and die at birth because of respiratory failure (Rudnicki et al., 1993). Thus, compensatory mechanisms allow *MyoD* and *Myf5* to be functionally redundant for skeletal myogenesis, consistent with the closely related structural homology of these bHLH transcription factors and their independent regulation for expression at all sites of skeletal myogenesis in the embryo. This demonstrates that at least one of these factors is required for determining the myogenic lineage during embryonic development.

2.4.3 Myogenin and MRF4 are regulators of muscle differentiation

In contrast to Myf5 and MyoD, Myogenin and MRF4 have differentiation functions. *Myogenin* (-/-) and *MRF4* (-/-) mouse embryos form *Myf5*- and *MyoD*-expressing muscle progenitors, but are deficient in differentiated muscles (Nabeshima et al., 1993; Olson et al., 1996; Venuti et al., 1995). Muscle deficiencies are more severe in *Myogenin* (-/-) embryos than in *MRF4* (-/-) embryos. The specific muscle differentiation requirements for MRF4 are not yet well defined.

Together, these gene targeting experiments have suggested a model (Figure 2.4), in which MyoD and Myf5 act to determine the myoblast lineage, whereas myogenin and MRF4 are important for differentiation and maintenance of the terminally differentiated state in mice (Rudnicki & Jaenisch, 1995).

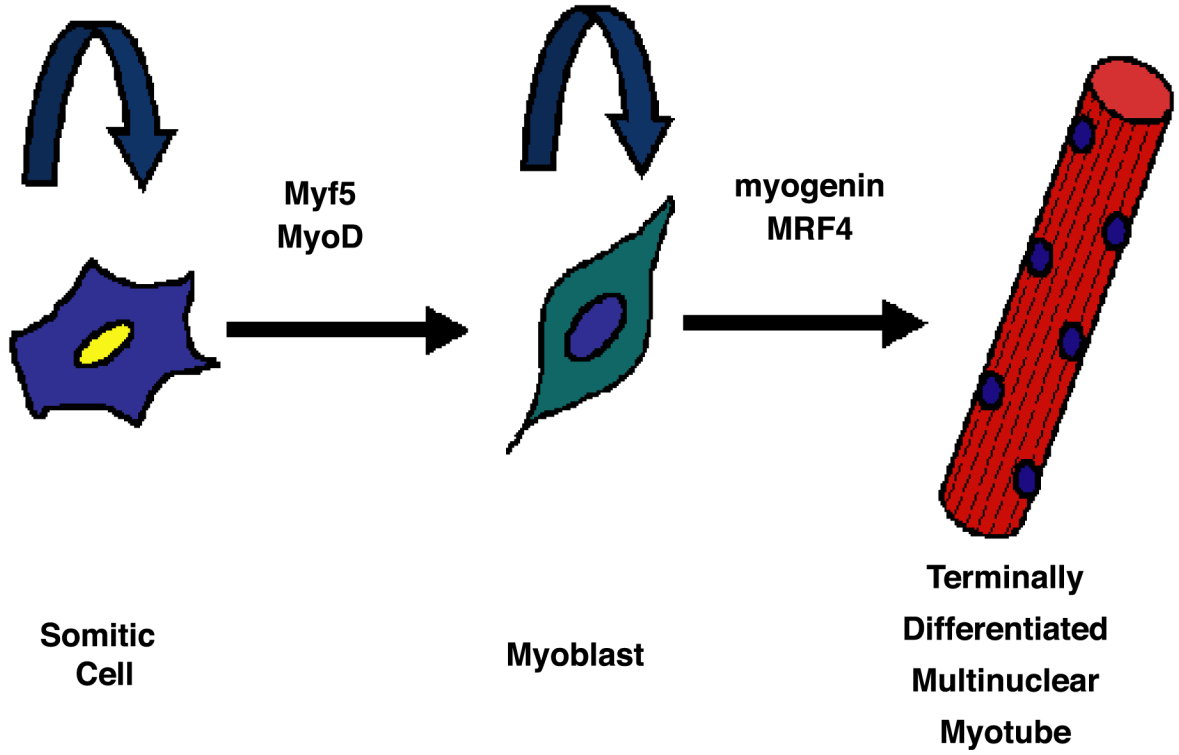

Figure 2.4 Functional and genetic relationships of the MRFs. Gene targeting experiments indicate that Myf5 and MyoD are required for the determination of the myogenic lineage. By contrast, terminal differentiation is dependent upon myogenin and MRF4. (Adapted from Perry and Rudnicki, 2000)

2.5 Myogenic competence

In *Xenopus*, cells from the animal hemisphere are competent to form mesodermal tissues from the morula through to the midgastrula stage (Jones and Woodland, 1987). Loss of mesodermal competence at early gastrula is programmed cell-autonomously, and occurs even in single cells at the appropriate stage (Grainger and Gurdon, 1989). *Xenopus MyoD* is expressed at low levels at the late blastula stage. Subsequently, *MyoD* and *Myf5* expression are induced by secreted growth factors of the TGF-, Wnt- and FGF-families (Steinbach et al., 1998) in prospective muscle, when mesoderm forms at the onset of gastrulation. The timing of *MyoD* induction in *Xenopus* embryonic explant assays is largely independent from the timepoint of inducer application (Steinbach et al., 1998). An independent mechanism ensures that *MyoD* transcription can only be induced until the

midgastrula stage, although signalling pathways, for example that of activin, remain functional (Steinbach et al., 1997). The loss of *MyoD*-inducibility coincides precisely with the disappearance of both muscle-forming competence and general mesodermal competence. Together, these observations describe a window of opportunity to induce *MyoD* transcription of less than two hours, which might reflect the epigenetic state of the *MyoD* locus. Indeed, the ability to induce *MyoD* is under the control of linker histone proteins, which act as transcriptional inhibitors of *MyoD* induction (Steinbach et al., 1997).

2.6 Molecular regulatory mechanisms of *Myf5* and *MyoD*

2.6.1 Modular regulation of *Myf5* and *MyoD* by transcription enhancer cassettes

Transcription enhancers for *Myf5* and *MyoD* in the mouse have been identified by transgenic analysis using *LacZ* reporter genes. Studies of the activities of these enhancers in muscle progenitor lineages in wild-type and mutant embryos have provided insights into regulatory mechanisms for progenitor cell specification and differentiation and have defined a direct, molecular approach to complement functional screens to identify upstream developmental regulators of myogenic specification.

2.6.1.1 Transcription enhancers for *MyoD* expression in muscle progenitors and differentiating muscle in mammals

Two *MyoD* transcription enhancers have been characterized, which are named the core enhancer (CE) and distal regulatory region (DRR). They are located in the 5' upstream regions of the human and mouse *MyoD* genes (Asakura et al., 1995; Goldhamer et al., 1995; Goldhamer et al., 1992; Tapscott et al., 1992). Each of these enhancers has a distinct regulatory function. The core enhancer controls *MyoD* activation in muscle progenitors (Goldhamer et al., 1995), and the DRR controls *MyoD* expression in differentiating muscles (Asakura et al., 1995; J. C. Chen et al., 2001).

Linker-scanner mutagenesis of this 258-bp sequence has identified multiple *cis* elements required for its activity, including an enhancer region specifically required in myotomal lineages that may represent a target for Myf-5-dependent regulation of *MyoD* (Kucharczuk et al., 1999). However, the core enhancer is not sufficient to maintain *MyoD* expression in differentiated skeletal muscle, being down-regulated in fetal and neonatal muscle and essentially inactive in adult muscle. Five kilobases upstream of *MyoD* is a second enhancer, the distal regulatory region (DRR), which is unrelated in sequence to the core enhancer and exhibits largely complementary activity in transgenic mice (Tapscott et al., 1992; Goldhamer et al., 1995; Asakura et al., 1995; J. C. Chen et al., 2001). DRR activity is restricted to differentiated skeletal muscle in vivo (Kablar et al., 1997), which is reflected as a significant delay in DRR-driven transgene expression in several sites of myogenesis relative to the endogenous *MyoD* gene (Asakura et al., 1995). Unlike the core enhancer, the DRR remains active in adult muscle, showing a similar expression profile as the endogenous *MyoD* gene (Chen et al., 2002). Collectively, these data indicate that the core enhancer and DRR have distinct activation and maintenance functions, respectively, that collaborate to establish the dynamic pattern of *MyoD* expression in embryonic and adult skeletal muscle (J. C. Chen et al., 2001).

2.6.1.2 Lineage-specific *Myf5* transcription enhancers

Multiple transcription enhancers control *Myf5* expression, which have been identified by systematic transgenic analysis of a 500-kb sequence region around the mouse *MRF4-Myf5* locus (Carvajal et al., 2001; Hadchouel et al., 2000; Patapoutian et al., 1993; Summerbell et al., 2000; Zweigerdt et al., 1997). These enhancers are distributed in a 90-kb region around the *MRF4-Myf5* locus, and different enhancer cassettes control lineage-specific transcription of *Myf5* in epaxial, hypaxial, limb, and head muscle progenitors. The lineage-specific epaxial, hypaxial, and branchial arch enhancers are located in the *MRF4-Myf5* locus, whereas the limb enhancer and an associated myotome differentiation enhancer are located 58 kb upstream of the locus, embedded in the intronic sequences of a nonmuscle gene (Carvajal et al., 2001; Hadchouel et al., 2000). These enhancers likely regulate the *Myf5* promoter and not the closely linked *MRF4* promoter, based on their

temporal activities, which coincide with the transcriptional activity of *Myf5* and not *MRF4*. MRF4 regulatory elements have been located 5' of the *MRF4* promoter (Black et al., 1995; Naidu et al., 1995; Pin et al., 1997). The close linkage of *MRF4* and *Myf5* and their differential expression in progenitors and differentiated muscle cells predicts the presence of insulator elements (Muller, 2000) to direct specific enhancer activities to the *MRF4* and *Myf5* promoters.

Some analyses of the regulatory elements in the frog *Myf5* locus have been done (Polli & Amaya, 2002; Yang et al., 2002a). Polli and Amaya showed that HBX2, an essential element approximately 1.2 kb upstream from the start of transcription, is necessary for both activation and repression of *Myf-5* expression. Yang et al. (2002a) showed that a distal TCF-3 binding site is necessary to repress the expression of *Myf-5* in non-muscle forming mesoderm (organizer and ventral marginal zone) during gastrulation.

2.7 Developmental signaling that controls MRF expression

2.7.1 Myogenic signals in mice

Several factors are expressed in axial and lateral regions of the developing embryo which are important for somite formation and the determination of cell lineages (Figure 2.5; for review see Perry and Rudnicki, 2000). Axial structures, such as the neural tube and notochord, provide signals necessary for epaxial myogenic determination. By contrast, the hypaxial myogenic lineage is dependent upon signals originating from the lateral plate mesoderm and dorsal ectoderm. Factors secreted from these structures include sonic hedgehog (Shh), Wnts, transforming growth factor-beta (TGF-beta)-like molecules, fibroblast growth factors (FGFs) and bone morphogenic proteins (BMPs). All of these factors regulate myogenic determination and differentiation. However, there are differential effects observed between epaxial and hypaxial musculature.

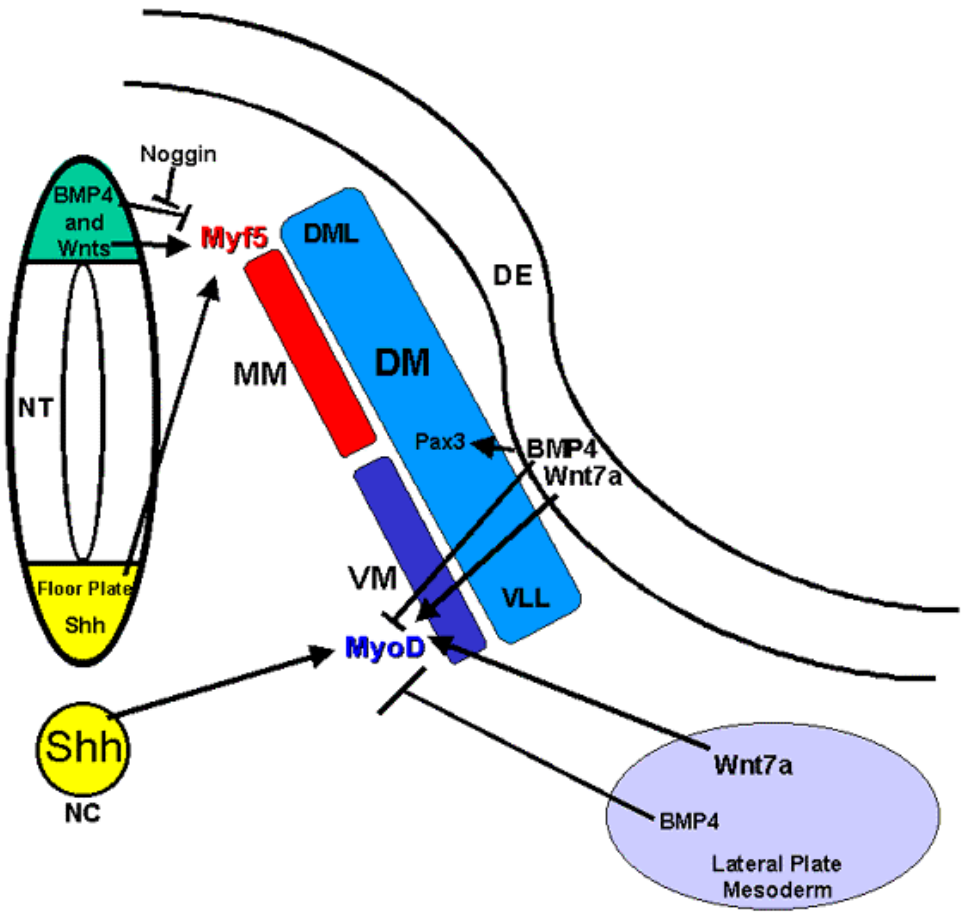

Figure 2.5 Extracellular growth factors which are important for myotomal development. Sonic hedgehog is secreted by both notochord and floor plate which serves to induce *Myf5* expression. Wnts, in particular Wnt1, secreted from the dorsal neural tube similarly induce *Myf5* expression in the epaxial myotome. By contrast, Wnt7a secreted from the dorsal ectoderm induces *MyoD* expression in the ventral myotome. BMP4 secreted from the dorsal ectoderm and lateral plate mesoderm is important for repressing MRF activation and maintaining *Pax3* expression in cells of the dermomyotome and the migrating precursor population in the VLL. Both dorsal neural tube and the DML secrete noggin, inhibiting the repressive effects of BMP4 on myogenesis. DML=dorsomedial lip; VLL=ventrolateral lip; DE=dorsal ectoderm; NT=neural tube; NC=notochord; MM=medial myotome; VM=ventral myotome. (Adapted from Perry and Rudnicki, 2000.)

Sonic hedgehog (Shh) is expressed in the notochord and neural floor plate and has been shown to positively regulate the formation and survival of the dorsal myotome. In association with Shh, several Wnts have been shown to induce myogenesis and are thought to synergistically act with Shh. Interestingly, Wnt-1 induces *Myf5* expression whereas Wnt-7a, which is expressed in the lateral plate mesoderm, induces *MyoD* expression. These results confirm previous studies demonstrating that the neural tube

induces *Myf5* expression while the dorsal ectoderm preferentially activates *MyoD* expression.

The BMPs belong to the TGF-beta family of secreted factors and information obtained thus far shows that these factors negatively regulate myogenesis. Recent experiments strongly suggest that BMP concentration gradients are vital for cells to respond appropriately. Low BMP levels in the limb bud maintain migrating, *Pax3* expressing myogenic precursor cells in a proliferative state and repress myogenesis. By contrast, high BMP concentrations induce cell death. Important aspects of BMP signaling are the patterns of expression of BMPs and their inhibitors follistatin, noggin and chordin. Expression of the BMP antagonist, noggin, in the DML and lateral plate regulates the development of both medial and lateral myogenic lineages (Marcelle et al., 1997). Indeed, ectopic expression of *noggin* in the lateral regions of the embryo represses *Pax3* expression, expands the *MyoD* expression domain and induces myogenesis.

Several FGF and TGF-beta family members have also been implicated in myogenesis. Treatment of cultured myoblasts with these factors suggests they act to stimulate proliferation and repress terminal differentiation. However, in vivo these molecules are important for the formation and terminal differentiation of the dorsal myotome. Neutralizing antibodies to TGF-beta or basic-FGF (bFGF) inhibit myotomal induction by axial structures. Exposure of segmental plate explants to a combination of TGF-beta and bFGF induces myotome formation. TGF-beta acts to specify the cells to the myogenic lineages whereas bFGF acts to promote proliferation and cell survival.

2.7.2 Myogenic signals in *Xenopus*

XmyoD expression in gastrula embryos corresponds precisely to the muscle progenitors that form the somitic myotome. Previous studies showed that the induction of *MyoD* transcription is limited to a small time window that starts after dorsal lip formation (Steinbach et al., 1997; Steinbach et al., 1998; for review see Rupp, et al, 2002). During gastrulation, antagonistic signals emanating from the dorsal and ventral marginal zones

specify the full range of mesodermal derivatives along the dorsal-ventral axis (Harland & Gerhart, 1997). Additional local "community effect" signals are required to maintain *XmyoD* expression and promote cell differentiation (Gurdon, 1988; Gurdon, et al., 1992, Kato and Gurdon, 1993; Standley et al., 2001). The gene regulatory pathways for mesoderm induction have been connected to the FGF, Wnt, BMP and activin signaling pathways that control *XmyoD* and *Xmyf5* activation for the specification and differentiation of myotomal progenitors.

FGF signaling is required for the expression of many mesodermal genes (Amaya et al., 1991, 1993; Lombardo et al., 1998; Schulte-Merker & Smith, 1995; Slack & Isaacs, 1994). Inhibition of FGF signaling in whole embryos results in a dramatic lost of posterior tissues including somites and, thus, muscle (Amaya et al., 1991, 1993). Embryonic FGF (eFGF) has been shown to be sufficient for the expression of *XmyoD* (Fisher et al., 2002), and can act as a community effect signal to maintain high-level *XmyoD* expression and promote muscle differentiation in dispersed cultures of mesoderm cells isolated from the early gastrula embryos (Standley et al., 2001).

XWnt8 is co-expressed with *XmyoD* in the mesoderm at early gastrula stages. Overexpression of *XWnt8* during gastrula stages leads to ectopic *XmyoD* activation across the dorsal midline, where its expression normally is excluded, changing notochord progenitors into myogenic progenitors (Christian & Moon, 1993). Furthermore, dominant-negative forms of XWnt8 inhibit *XmyoD* expression in the early mesoderm (Hoppler et al., 1996), whereas overexpressed ß-catenin increases *XmyoD* expression, establishing ß-catenin as the effector molecule of XWnt8-mediated *XmyoD* activation (Hamilton et al., 2001).

BMP signaling also interacts with XWnt8 in the control of *XmyoD*. BMP4 controls the ventral expression of the Wnt antagonist, *sizzled*, which functions together with the dorsally expressed Wnt inhibitor, *FrzB*, to localize Wnt signaling to the lateral mesoderm, enhancing *XmyoD* expression and muscle progenitor formation (Marom et al., 1999). The timing and spatial localization of *Xmyf5* and *XmyoD* expression in myogenic

progenitors differs, presumably reflecting their control by different signals or combinations of signals. Whereas it has been demonstrated that ß-catenin-mediated Wnt signaling is necessary for the normal expression of *Xmyf5* and *XmyoD* (Hamilton et al., 2001; Shi et al., 2002), BMP signaling has both a positive and a negative role in *Xmyf5* regulation. Overexpression of a dominant-negative BMP4 receptor in embryos abolishes its expression, and low levels of BMP4 signaling can induce ectopic *Xmyf5* expression (Dosch et al., 1997). BMP4 can also repress *Xmyf5*, restricting its expression to the dorsal most mesoderm. Inhibition of BMP activity by dorsally expressed BMP antagonists, i.e. Noggin, Chordin and Follistatin, creates a dorsal-ventral gradient of BMP signaling that localizes *Xmyf5* expression to the dorsal lateral mesoderm of the embryo where muscle progenitors form (Re'em-Kalma et al., 1995).

Activin has also been shown to be sufficient to induce the expression of *XmyoD* in animal cap (AC) assay (Steinbach et al., 1997; Steinbach et al., 1998). To date, no cis-regulatory element of *XmyoD* has been characterized to mediate transcription induction by any of these MIFs. Therefore, the experimental data is still not enough to conclude any of these factors is a direct or indirect input.

2.8 Preliminary work on the transcriptional regulation of the *XmyoDb* gene in *Xenopus*

In order to study the regulation of *XmyoD*, our lab screened a *Xenopus* laevis genomic library using a promoter specific probe. One positive clone was isolated which contained 10.8 kb of sequences flanking the *XmyoDb* gene (Figure 2.6A) from position -6 kb up to position +4829 bp (Steinbach and Rupp, unpublished). Sequence data reveal that the XmyoDb locus contains three exons, which is in agreement with previous data (Leibham et al., 1994). For analysing the *cis*-elements of the *XmyoDb* gene, a GFP reporter gene was cloned into the 5`UTR of *XmyoD* gene at position +107 (Figure 2.6A) and based on this clone other deletion constructs were made.

Initial attempts to study the transcriptional activity of *XmyoD* reporter genes by episomal DNA-microinjection failed to produce specific and consistent results. The injected embyros showed mosaic and ectopic expression of GFP (Otto, 2001 and Figure 2.6B, j).

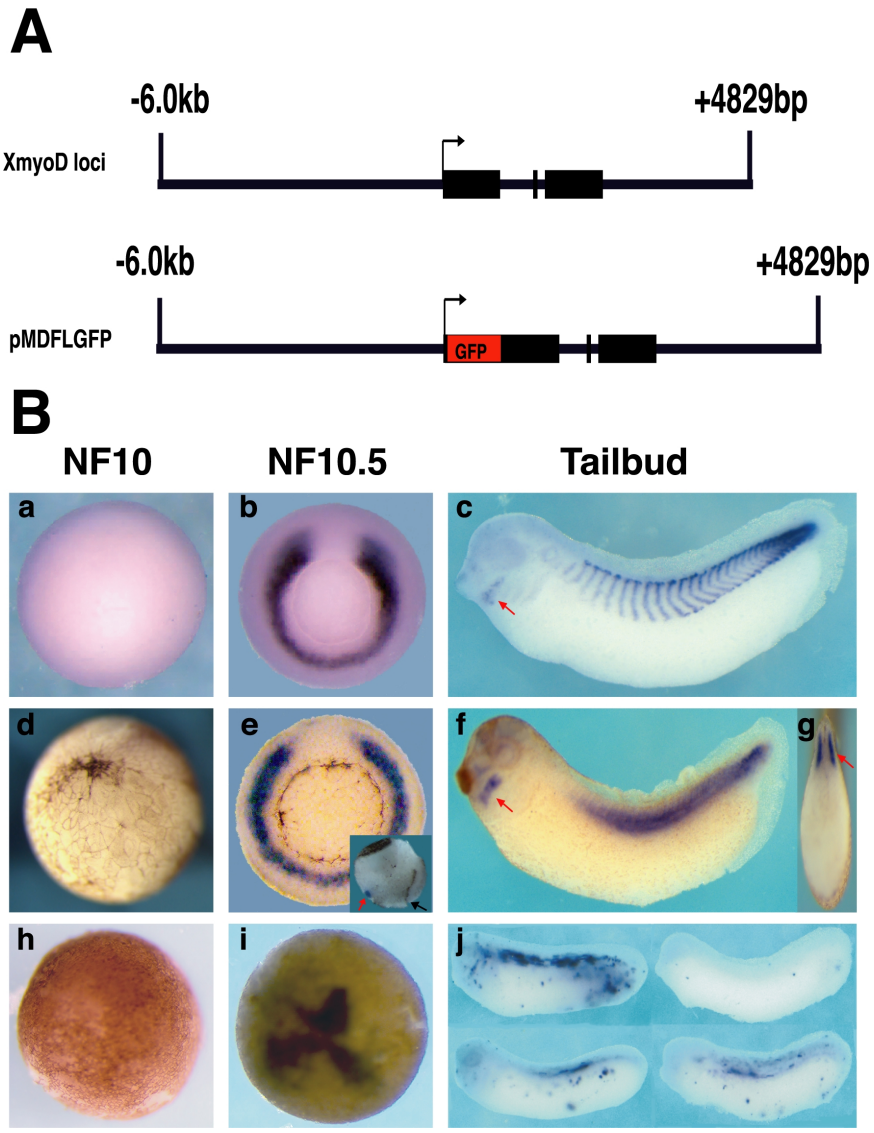

Figure 2.6 Transgenic reporter gene analysis for *XmyoD*. A) Schematic representation of the *XmyoD* locus and the pMD-6.0kb/+4829GFP report plasmid. Black boxes represent the three exons, while red box represent GFP gene. The arrow indicates the transcriptional start site. (B) RNA in situ hybridization analysis. The expression pattern of endogenous *XmyoD* mRNA (a-c) in albino embryo and pMD-6.0/+4.7GFP plasmid (d-g) in transgenic frog embryos at different stages. The section along D-V axis showed that the signal is restricted in the ventral mesodermal cells (insert in e), a black arrow indicates the dorsol lip and a red arrow highlights the *in situ* hybridization signal. (g) Section showed the expression was only in somite muscle tissue (arrow). Ectopic activation of the reporter gene by injected bFGF/Xwnt8 mRNA reflects the timing of induction of the endogenous *XmyoD* gene (compare d,e with h, i). The embryos are orientated with dorsal pointing up and vegetal pole facing out (a,b,d,e), or animal pole facing out (h, i) or head facing left (c, f, j). (Xiao, L., Otto, A. and Rupp, R., unpublished data)

We reported before that chromatin components play a pivotal role in regulating *XmyoDb* transcription (Steinbach et al., 1997). Therefore, we decided to use the transgenesis technique developed by Kroll and Amaya (1996) to introduce GFP reporter constructs into the embryos through restriction enzyme mediated integration (REMI). Because at the gastrula stages, GFP fluorescence intensity was too low to be recorded consistently, and GFP protein is too stable to report the changes of *XmyoD* expression pattern, we documented GFP expression by in situ hybridization in transgenic embryos, and compared the expression pattern with that of endogenous *XmyoD* mRNA in wild-type embryos (Figure 2.6B, a-c). Transgenic embryos containing the full-length reporter construct showed *XmyoD*-like expression during development, i.e., no expression at the onset of gastrulation (NF10), U-shape expression at midgastrula stage (NF10.5) and somite-specific expression at tailbud stages (Figure 2.6B, d-f). Sectioning of these transgenic embryos confirmed that the expression of GFP is restricted to the mesoderm at stage NF10.5 (Insert of Figure 2.6B, e) and to somite at tailbud stages (Figure 2.6B, f and g).

Previous studies showed that the activation of *XmyoD* expression is limited to a small time window that starts after dorsal lip formation (Steinbach et al., 1997; Steinbach et al., 1998; for review see Rupp, et al, 2002). So we asked if the full-length reporter construct does reflect this temporal restriction. To assess this question, transgenic frog embryos, containing the plasmid pMD-6.0kb/+4829GFP, were generated and the cleaving ones were injected with a combination of FGF/Wnt8 mRNA at the 4-cell stage into the animal pole. Under this condition, the reporter gene was activated in the same time window as the endogenous gene (Figure 2.6B, h-i). Therefore, it was concluded that the 10.8 kb contig is sufficient to recapitulate the dynamic stage- and tissue-specific expression pattern of *XmyoD* in transgenic embryos, and therefore it must contain most of *cis*-regulatory elements that controll *XmyoD* transcription (Otto, 2001).

2.9 The aim of this work

As mentioned above, so far, the regulatory mechanisms of the muscle program downstream of XmyoD has been analyzed relatively well, but not the regulation of the *XmyoD* gene itself. So the aim of the study presented here was to analyze the transcriptional regulatory mechanism of *XmyoD* gene in *Xenopus*.

In *Xenopus*, the activation of the myogenic determination factor *XmyoD* in the muscle-forming region of the embryo occurs in response to mesoderm-inducing factors (MIFs). Different members of the FGF, TGF-ß, and Wnt protein families have been implicated in this process (Steinbach et al., 1998), but how MIFs induce *XmyoD* is not known. We expect that the study of the transcriptional regulatory mechanism of *XmyoD* will help us to understand how the muscle linage is specified, and also provide insight into mesodermal patterning.

In order to address the transcriptional regulatory mechanisms of the *XmyoD* gene, I decided to use two approaches:

1. Mapping of the *XmyoDb* *cis*-regulatory elements
   I used the transgenesis technique, developed by Kroll and Amaya (1996), to introduce GFP reporter constructs with serial deletion of genomic fragments from the *XmyoD* gene into the embryos through restriction enzyme mediated integration (REMI). Expression of GFP at various developmental stages were detected by *in situ* hybridization of GFP mRNA in REMI-embryos, and the expression pattern was compared with that of endogenous *XmyoD* mRNA in normal embryos; Fine mapping of *cis*-regulatory elements was accomplished by linker-scanner mutagenesis.

2. Potential protein factors that regulate the expression of *XmyoD* gene
   The main methods I used were gain-of-function and loss-of-function assays to analyze the function of potential protein factors that may regulate the expression of XmyoD gene.

3 Material and methods

3.1 Reagents

Fine chemicals: Fluka, Merck, Sigma, USB.

Bio-chemicals: Agar (Difco); Agarose (Gibco/BRL); Ampicillin, Streptomycin, Bacto trypton, Yeast extract (Difco); Chicken serum, lamb serum (Gibco/BRL); Human choriongonadotrophin (Sigma); Levamisol (Vector Laboratories).

Enzymes and proteins: Alkaline phosphatase (Roche); BSA fraction V, Chymostatin, Leupeptin, Pepstatin (Sigma); DNase I (Stratagene); Klenow enzyme (Roche); MMTV reverse transcriptase (Gibco/BRL); Restriction endonuclease with 10x restriction buffer system (New England Bio Labs, Roche, Fermentas); RNaseA (Sigma); RNAsin (Promega); T3, T7 and SP6 RNA polymerase with 5x incubation buffer (Promega); Taq DNA polymerase with 10x PCR buffer (Perkin Elmer); Pfu polymerase with 10x PCR buffer (Stratagene); Proteinase K (Sigma); RNAse free DNAse I (Promega); Pre-stained protein molecular weight standard low and high range (Gibco/BRL).

Immunochemicals: Sheep anti-mouse IgG coupled with alkaline peroxidase (1:5000, Roche); Monoclonal mouse anti-Myosin heavy chain (MHC) (MF20, 1:5 (Bader et al, 1982)); Sheep anti-Digoxigenin Fab fragment coupled with alkaline phosphatase (Roche); BM Purple solution (Roche).

3.2 Devices

Gel filtration columns QuickSpin G-50 (Roche).

Glass injection needles: Glass 1BBL W/FIL 1.0 mm (World Precision Instrument).

Injection equipment:

Injector Pli-100 (Digitimer Ltd.).

Incubator: Driblock DB1 and DB20 (Teche).

Microneedle Puller P-87 (Sutter Instrument).

Micromanipulator: Mm-33 (Science Products).

Microscopes: Stereomicroscopes Stemi SV6 and Stemi SV11 (Zeiss).

Microsurgery: Gastromaster (Xenotek Engineering).

Nylon membran: Hybond^{TM} N (Amersham).

Software: Adobe Photoshop 6.0; Illustrator 9.0 (Adobe); McVector 6.0 (Oxford Molecular Group); Microsoft Office 98 (Microsoft).

Spectrophotometer: GeneQuant II (Pharmacia Biotech).

Thermocycler: Primus 96 plus (MWG).

Centrifuges: Eppendorf centrifuge 5417C (Eppendorf); Omnifuge 2.0 RS (Haereus); Sorvall RC-5B (Du Pont).

3.3 Nucleic acids

3.3.1 Size standard

DNA standard: GeneRuler™ 1kb DNA ladder (Fermentas). The DNA ladder yields the following 14 discrete fragments (in base pairs): 10000, 8000, 6000, 5000, 4000, 3500, 3000, 2500, 2000, 1500, 1000, 750, 500, 250.

3.3.2 Oligonucleotides

All Oligonucleotides were synthesized by the company MWG Biotec.

3.3.2.1 Oligonucleotides for RT-PCR

Xenopus Histon H4 (Niehrs et. al, 1994):

OS1 (sense): 5' - CGG GAT AAC ATT CAG GGT ATC ACT -3 '

OS2 (anti-sense): 5 ' - ATC CAT GGC GGT AAC TGT CTT CCT -3 '

Random Hexamer:

RR13: 5' - NNNNNC -3 ' (N = G, A, T or C)

Xenopus myoDb (position 662-952, Hopwood et. al, 1989)

RR23 (sense): 5' - AAC TGC TCC GAT GGC ATG ATG GAT TA -3 '
RR24 (anti-sense): 5 ' - AATT GCT GGG AGA AGG GAT GGT GAT TA -3 '

3.3.2.2 Oligonucleotides for cloning

Oligo	Sequence	Position in MD-3200/+4829
XL18	GCGCCCGCGGCTGAGCAGCTACAGGCAAACTG	36-57
XL24	AGGCCTCCCCTTGTGTCCGTGTCTACTC	1713-1734
XL28	GGGGAGGCCTGTGGCCCCCATACTCGGAGG	1742-1761
XL41	GGGGCTCGAGAGATCCACAGCTCTGGGGTCC	3304-3324
AO58	CACGGTGGAAGCTTTCTCCCT	
AO65	TCCCCGCGGCAGTTGGTGTAGTTG	
AO95	CCGCTCGAGCCTGCCTCCTGCTGGT	3'UTR
AO96	CCGCTCGAGGCCCTTGGGATAAGATTT	3'UTR
XL204	GTTTGTTTAACTGACAAATTCCTG	1916-1939 of MDfl
XL205	GGTGTAGTTGCTGGGGGTAC	R&C of 1096-1120 of MDfl
XL206	CAGGAATTTGTCAGTTAAACAAAC	1096-1120 of MDfl
XL207	ACCCCCAGCAACTACACCAAC	R&C 1916-1939 of MDfl

3.3.2.3 Oligonucleotides for mutagenesis

Oligo	Sequence	Mutation
XL37	CCCCTATCCTATCACAAACCTCACAAGTCACAGGAGGGTC	pMDΔ-1.2/-0.9GFP

XL38	GACCCTCCTGTGACTTGTGAGGTTTGTGATAGGATAGGGG	pMDΔ-1.2/-0.9GFP
XL39	GACTATTAACCCACTGTTACCTCACTTCCCACCCAGGAG	pMDΔ-1.8/-1.2GFP
XL40	CTCCTGGGTGGGAAGTGAGGTAACAGTGGGTTAATAGTC	pMDΔ-1.8/-1.2GFP
dRE2a	CCTTCCTTTCCTAGGCCTGGTAGTTTAACCCCTG	pMDΔ-1.8/-1.6GFP
dRE2b	CAGGGGTTAAACTACCAGGCCTAGGAAAGGAAGG	pMDΔ-1.8/-1.6GFP
Linker	AGACTGGGCCCTGTG	
XL43	CCTACAATGAAGCGAATCTGAAAGACTGGGCCCTGTGCACAGGAGGGTCTCACATATTC	LS-1
XL44	GAATATGTGAGACCCTCCTGTGCACAGGGCCCAGTCTTTCAGATTCGCTTCATTGTAGG	LS-1
XL45	GAATCTGAATCTAGTGTCACAAGTAGACTGGGCCCTGTGCATATTCTTGTGTGTTTCACTTG	LS-2
XL46	CAAGTGAAACACACAAGAATATGCACAGGGCCCAGTCTACTTGTGACACTAGATTCAGATTC	LS-2
XL47	CAAGTCACAGGAGGGTCTCAAGACTGGGCCCTGTGTTCACTTGTAGATAAGACTTGAAAG	LS-3
XL48	CTTTCAAGTCTTATCTACAAGTGAACACAGGGCCCAGTCTTGAGACCCTCCTGTGACTTG	LS-3
XL49	GGTCTCACATATTCTTGTGTGTAGACTGGGCCCTGTGGACTTGAAAGGGAATCTCTGAG	LS-4
XL50	CTCAGAGATTCCCTTTCAAGTCCACAGGGCCCAGTCTACACACAAGAATATGTGAGACC	LS-4
XL69	CTTGTGTGTTTCACTTGTAGATAAAGACTGGGCCCTGTGCTCTGAGGAAGTTGAATTAAATTAGG	LS-5
XL70	CCTAATTTAATTCAACTTCCTCAGAGCACAGGGCCCAGTCTTTATCTACAAGTGAAACACACAAG	LS-5
XL71	GTAGATAAGACTTGAAAGGGAATAGACTGGGCCCTGTGATTAAATTAGGAAATTAGCACTTAGC	LS-6
XL72	GCTAAGTGCTAATTTCCTAATTTAATCACAGGGCCCAGTCTATTCCCTTTCAAGTCTTATCTAC	LS-6
XL121	CTCTGGCTTTGATGTGGGCATAATTTGGCATTTGCAGGAAG	mSRE CC-CA
XL122	CTTCCTGCAAATGCCAAATTATGCCCACATCAAAGCCAGAG	mSRE CC-CA
XL123	GTGGGCCTAATTTGGCATTAGCAGGAAGGTGCCGCGTTAATAATG	mE TG-AG
XL124	CATTATTAACGCGGCACCTTCCTGCTAATGCCAAATTAGGCCCAC	mE TG-AG
XL148	CTCTGGCTTTGATGTGGGCCTTATTTGGCATTTGCAGGAAGGTGC	mSRE AA-TA
XL149	GCACCTTCCTGCAAATGCCAAATAAGGCCCACATCAAAGCCAGAG	mSRE AA-TA
XL156	GCTTTGATGTGGGCCTAATTTTTCATTTGCAGGAAGGTGCCGC	mSRE GG-TT

XL157	GCGGCACCTTCCTGCAAATGAAAAATTAGGCCCACATCAAAGC	mSRE GG-TT
XL158	GGGAATCTCTGAGGAAGTTGAAGACTGGGCCCTGTGTAGCACTTAGCTCTGGGACTCC	LS-7
XL159	GGAGTCCCAGAGCTAAGTGCTACACAGGGCCCAGTCTTCAACTTCCTCAGAGATTCCC	LS-7
XL160	GGAAGTTGAATTAAATTAGGAAATAGACTGGGCCCTGTGGGACTCCCGGTACCAAGCG	LS-8
XL161	CGCTTGGTACCGGGAGTCCCACAGGGCCCAGTCTATTTCCTAATTTAATTCAACTTCC	LS-8
XL162	GGAAATTAGCACTTAGCTCTGAGACTGGGCCCTGTGAGCGAAATCTTTGGGATCCCAGAAG	LS-9
XL163	CTTCTGGGATCCCAAAGATTTCGCTCACAGGGCCCAGTCTCAGAGCTAAGTGCTAATTTCC	LS-9
XL164	CTCTGGGACTCCCGGTACCAAGACTGGGCCCTGTGATCCCAGAAGGTGCGAATG	LS-10
XL165	CATTCGCACCTTCTGGGATCACAGGGCCCAGTCTTGGTACCGGGAGTCCCAGAG	LS-10
XL185	GCGTTAATAATGAGTTTTTAGCTTCTGGGATCCAGCAAGTTCTATGGTCACAGGAG	mFAST site in –1.8/-1.6kb
XL186	CTCCTGTGACCATAGAACTTGCTGGATCCCAGAAGCTAAAAACTCATTATTAACGC	mFAST site in –1.8/-1.6kb
XL187	CAGGAGATGACAAGAGCCTCTGGGGATCCTGTGGGCCTAATTTGGCATTTGC	mLEF in –1.8/-1.6kb
XL188	GCAAATGCCAAATTAGGCCCACAGGATCCCCAGAGGCTCTTGTCATCTCCTG	mLEF in –1.8/-1.6kb
XL210	CTGGCAAATGGAAATTAAACGAAAGGATCCCTCTGGGGCCCCCCAGCC	mLEF in promoter
XL211	GGCTGGGGGGCCCCAGAGGGATCCTTTCGTTTAATTTCCATTTGCCAG	mLEF in promoter

3.3.3 Plasmids

3.3.3.1 Plasmids for *in vitro* transcription

Plasmid	**Linearized by**	**Polymerase**	**Reference**
pSP64TXeFGF	Acc I	SP6	Isaacs et al., 1994
pCS2+Xcad3	Not I	SP6	Isaacs et al., 1998
pCS2+Xcad3-VP16	Not I	SP6	Isaacs et al., 1998
pCS2+Xcad3-EnR	Nsi I	SP6	Isaacs et al., 1998
pCS2+mtXSRF	Not I	SP6	Oliver Nentwich, Nordheim Lab
pCS2+XSRF1-350EnRmt	Ehe I	SP6	Oliver Nentwich, Nordheim Lab
pCS2+MT6 Xseb4-WT	Not I	SP6	Rupp Lab

pCS2+MT6 Xseb4-ΔN	Not I	SP6	AA50, Rupp Lab
pCS2+MT6 Xseb-ΔC	Not I	SP6	AA48, Rupp Lab
pCS2+MT6 long mSeb4	Not I	SP6	AA55, Rupp Lab
pCS2+MT6 short mSeb4	Not I	SP6	AA56, Rupp Lab
pCS2+EnR-YY	Not I	SP6	Satijn et al., 2001

3.3.3.2 Plasmids for dig-labeled RNA in situ hybridization probes

Plasmid	**Linearized by**	**Polymerase**	**Primers**	**References**
pRR3 (XmyoDb)	Hind III	T7		Antisense probe
pCS2+GFP2	BamH I	T7		Antisense probe
TOPOII XLC04	Not I	SP6	XL18, XL24	Sense probe
TOPOII XLC04	Hind III	T7	XL18, XL24	Antisense probe
TOPOII XLC09	Apa I	SP6	XL28, XL41	Sense probe
TOPOII XLC09	Sac I	T7	XL28, XL41	Antisense probe
TOPOII XLC14	Not I	SP6		No Document
TOPOII XLC14	Hind III	T7		ND
TOPOII XLC15	Not I	SP6		ND
TOPOII XLC15	Hind III	T7		ND
TOPOII XLC16	Not I	SP6	XL204, XL18	ND
TOPOII XLC16	Hind III	T7	XL204, XL18	ND
TOPOII XLC17	Not I	SP6	XL205, XL24	ND
TOPOII XLC17	Hind III	T7	XL205, XL24	ND
TOPOII XLC18	Not I	SP6	XL206, XL207	ND
TOPOII XLC18	Hind III	T7	XL206, XL207	ND
Xnot10	Hind III	T7		von Dassow et al., 1993

XpoHK	Hind III	T7		Sato and Sargent, 1991

3.3.3.3 Reporter gene constructs

Plasmid	Primers	Template	Contructed by
pMD-2100/+4829 GFP	AO58, AO65	pMD-3200/+4829GFP	Digested by SacII and Hind III
pMD-3200GFP3'UTR	AO95, AO96	pMD-3200/+4829GFP	Digest by Xho I
pMDD-1770/-1200GFP	XL39, XL40	pMD-3200/+4829GFP	Site-Directed Mutagenesis
pMDD-1200/-840GFP	XL37, XL38	pMD-3200/+4829GFP	Site-Directed Mutagenesis
pMDD-1770/-1586GFP	dRE2A, dRE2b	pMD-3200/+4829GFP	Site-Directed Mutagenesis
pMDLS1	XL43, XL44	pMD-3200/+4829GFP	Site-Directed Mutagenesis
pMDLS2	XL45, XL46	pMD-3200/+4829GFP	Site-Directed Mutagenesis
pMDLS3	XL47, XL48	pMD-3200/+4829GFP	Site-Directed Mutagenesis
pMDLS4	XL49, XL50	pMD-3200/+4829GFP	Site-Directed Mutagenesis
pMDLS5	XL69, XL70	pMD-3200/+4829GFP	Site-Directed Mutagenesis
pMDLS6	XL71, XL72	pMD-3200/+4829GFP	Site-Directed Mutagenesis
pMDLS7	XL158, XL159	pMD-3200/+4829GFP	Site-Directed Mutagenesis
pMDLS8	XL160, XL161	pMD-3200/+4829GFP	Site-Directed Mutagenesis
pMDLS9	XL162, XL163	pMD-3200/+4829GFP	Site-Directed Mutagenesis
pMDLS10	XL164, XL165	pMD-3200/+4829GFP	Site-Directed Mutagenesis
mSRE AA-TA	XL148, XL149	pMD-3200/+4829GFP	Site-Directed Mutagenesis
mSRE CC-CA	XL121, XL122	pMD-3200/+4829GFP	Site-Directed Mutagenesis
mSRE GG-TT	XL156, XL157	pMD-3200/+4829GFP	Site-Directed Mutagenesis
mE TG-AG	XL123, XL124	pMD-3200/+4829GFP	Site-Directed Mutagenesis

3.3.3.4 Plasmids for transgenesis

Plasmid	Digestion by	Reference
pCS2+XSRF1-350EnRmt	Ehe I	Oliver Nentwich
pCSGFP2	Not I	Zernicka-Goetz et al., 1996
PCS2+Lef-1-VP16	Not I	Yang et al., 2002b
PCS2+Lef-1-EnR	Asp 718	Yang et al., 2002b

3.4 Bacteria manipulation

Preparation of competent cells and transformation have been performed according to standard methods (Sambrook et al., 1989).

3.5 Embryological Methods

3.5.1 Solutions

Cystein: 2% L-Cystein in 0.1x MBS (pH 7.8 at 23°C, adjusted with 5 M NaOH).
Human Choriongonadotropin (HCG): 1000 I.U./ml HCG in ddH_2O.
MEMFA: 0.1 M MOPS, 2 mm EGTA, 1 mm $MgSO_4$; 3.7% formaldehyde (pH 7.4 at 23°C).
1xModified Barth's Saline (MBS): 5 mm HEPES, 88 mm of NaCl, 1 mm of KCl, 0.7 mm $CaCl_2$, 1 mm $MgSO_4$, 2.5 mm $NaHCO_3$ (pH 7.6 at 23°C). Add the $CaCl_2$ before use.
0.1xMBS/Gentamycin: 0.1xMBS +10 ug/ml Gentamycin, in cell culture water.
0.5xMBS/BSA: 0.5 x MBS, 1mg/ml BSA, 10 ug/ml Gentamycin, in cell culture water.
0.5xMBS/CS: 0.5 x MBS with 20 % chicken serum, stored at -20°C.
1xMarc's Modified Ringer's Solution (MMR): 0.1 M NaCl, 2 mm of KCl, 1 mm $MgCl_2$, 2 mm $CaCl_2$, 5 mm HEPES (pH 7.5 at 23°C)
0.1xMMR: 0.1xMMR + 10 ug/ml Gentamycin, desolved in medium water.

0.1xMMR/Ficoll: 0.1xMMR, 6 % (w/v) Ficoll, 10 ug/ml Gentamycin, desolved in cell culture water.
0.4xMMR/Ficoll: 0.4xMMR, 6 % (w/v) Ficoll, 10 ug/ml Gentamycin, desolved in cell culture water.

3.5.2 Experimental animals

Adult wild-type (Nasco) and albino (Xenopus I) *Xenopus laevis* frog were used. The frogs were held with a water temperature of 16-19°C and a population density of 5 L water per frog. Feeding takes place three times weekly.

3.5.3 Superovulation of the female *Xenopus laevis*

Xenopus laevis females were stimulated to lay eggs by injection of 500 units of human chorionic gonadotropin (Sigma) into the dorsal lymph sac and incubate at 15-23 °C over night. Egg laying started about 12 hours later.

3.5.4 Preparation of testis

A male was anaesthetized in 0.1 g 3-Aminobenzoesaeureethylester per 100 ml ddH_2O for 20 min, cooled down in ice and killed by decapitation. The two testes were taken from the abdominal cavity by opening and pulling the yellow fat body, with which they are connected by connective tissues. Before use the testis were stored in MBS/CS plus antibiotics for maximal 7 days.

3.5.5 *In vitro* fertilization of eggs and culture of the embryos

In vitro fertilization was performed by mincing a piece of testis and mixing it with freshly-laid eggs. Afterwards they are cultured in 0.1 x MBS at 15-23°C in 110 mm Petri plates.

3.5.6 Dejelly with cystein solution

In order to prepare eggs for the injection with DNA or mRNA (A) or for the generation of transgenic embryos (B), after approx. 1 hpf (A) or after the ovulation (B) the jelly coat should be removed in a 2% cystein buffer for 5 min under agitation. Embryos were then washed three times with 0.1x MBS and were cultured in 0.1 x MBS (0.4 x MMR for (B)) at 15-23°C.

3.5.7 Injection of embryos

The glass injection needles were pulled with Microneedle Puller (Sutter Instrument, model P-87). They were filled with 1-2 µl nucleotide acid containing solution shortly before the injection. The needles were placed into the holder of the injection equipment (Medical System, model Pi-100). Adjusting the injection volume was done via gradual breaking of the needle tip and choice of the injection pressure and/or the injection duration. Totally 2.5 to 10 nl nucleotide solution was injected per embryo. Embryos were generally injected at 2-8-cell stage. After injection, embryos were incubated in 0.1X MBS at 15-23 °C until the desired developmental stages. The medium was changed every day to increase survival.

3.5.8 Preparation of explants

For the preparation of explants, injected embryos were transferred in 3 cm cell culture dishes covered with 1% agarose, medium was 0.5 x MBS with BSA. Afterwards the animal caps were taken with Gastromaster (Xenotek Engineering) and transferred individually into a 96-well plate covered with 50 µl 1% Agarose and filled with 0.5 x MBS.

3.6 Histological methods

3.6.1 Solution

AP buffer: 100 mM Tris HCl (pH 9.5), 50mM $MgCl_2$, 100 mM NaCl, 0.1% Tween 20, 5 mM Levamisole.

AP staining solution: 4.5 µl NBT, 3.5 µl BCIP in 1 ml AP buffer.

MEMFA: 0.1 M MOPS, 2 mm EGTA, 1 mm $MgSO_4$, 3.7% formaldehyde (pH 7.4 at 23°C).

PBS: 137 mm of NaCl, 2.7 mm of KCl, 8 mm Na_2HPO_4, 1.7 mm KH_2PO_4 (pH 7.2 at 23°C).

PBT: PBS plus 2 mg/ml BSA plus 0.1% (v/v) triton-X-100.

X-Gal: 1 mg/ml X-Gal in DMF.

X-Gal staining solution: 50 mm $K_3Fe(CN)_6$, 50 mm $K_4Fe(CN)_6$, 1 mg/ml X-Gal (dissolved in DMF) in X-Gal Washing buffer.

X-Gal washing buffer: 0.1 M phosphate buffers, 2 mm $MgCl_2$, 0.1% sodium desoxycholat, 0.02% (w/v) NP40 (pH 7.3 at 23°C).

3.6.2 Fixation of embryos

Embryos were fixed in MEMFA for 1 h under rotation on a vertical wheel. The Embryos was washed 2 times with PBS. Embryos for the immunohistochemistry were finally washed with methanol. Embryos for in situ hybridizing were incubated for several hours in 100% ethanol.

3.6.3 Immunocytochemistry

1. Fix embryos in MEMFA for one hour at room temperature with rotation. Rinse with PBS, replace with methanol.
2. Rehydrate by 80%, 50%, 0% methanol in PBS. Do 1 x 5 min wash with PBS.
3. Wash in PBT for 15 min.
4. Block protein binding sites by incubating embryos in PBT plus 10% heat inactivated goat serum at room temperature for 15-30 min.
5. Remove blocking solution and add primary antibody (1 µl MF20 from mouse in 1 ml PBT to 1:1000 dilution), incubated overnight at 4 °C if sensitivity is not a problem.

6. Wash with at least five changes, 5 ml per wash, an hour (30-60') each, of PBT.
7. Block as above 15 to 30'.
8. Secondary antibody coupled with AP is added in blocking solution. Incubate at 4°C overnight.
9. Wash 5 times, 60' each, 5 ml per wash PBT.
10. Wash twice in AP buffer 5-20'.
11. Staining in 1 ml staining solution in dark.
12. After staining, wash in PBS, fix O.N in methanol and store in PBS at 4 °C.

3.6.4 LacZ staining

1. Fix embryos in MEMFA for 1 h.
2. Rinsing with 1× PBS twice for 10 min each,
3. Wash the embryos with X-gal washing solution with shaking for 3 times and 30 min each.
4. stain for 20-120 min at room temperature until the blue color appears.
5. Rinse the embryos with 100% ethanol twice for 5min each, afterwards store them at –20ºC.

3.7 SDS-PAGE and Western blotting

SDS-PAGE (SDS-polyacrylamide gel electrophoresis) and Western blotting were carried out according to standard protocols (Sambrook et al., 1989), and signals were detected by enhanced chemiluminisence (Amersham).

3.8 Molecular biological methods

3.8.1 Solutions

AB buffer: 80% (v/v) TBSX, 15% (v/v) heat-inactivated lamb serum, 5% (v/v) *Xenopus* egg extract.

DEPC-H_2O: ddH_2O with 0.1% (v/v) Diethylpyrocarbonat (DEPC) agitated at 23°C over night and autoclaved afterwards.

10 mm DIG NTP mixture: 10 mm CTP, GTP, ATP, 6.5 mm UTP and 3.5 mm Dig-11-UTP.

Hybridizing solution: 5 x SSC, 50% (v/v) formamide, 1 % (w/v) Boehringer block, 0.1 % (w/v) Torula RNA, 0.01 % Heparin, 0.1 % Tween-20, 0.1 % CHAPS, 5 mm EDTA.

Lamb Serum: Heat-inactivated lamb serum (30 min with 56°C), stored at -20°C.

MEMFA: 0.1 M MOPS, 2 mm EGTA, 1 mm $MgSO_4$, 3.7% formaldehyde (pH 7.4 at 23°C).

PBS: 137 mm of NaCl, 2.7 mm of KCl, 8 mm Na_2HPO_4, 1.7 mm KH_2PO_4 (pH 7.2 at 23°C).

PBSw: 1 x PBS, 0.1 % Tween-20 (pH 7.5 at 23°C).

PCI: 50% (v/v) phenol, 48%(v/v) chloroform, 2% (v/v) isoamyl alcohol.

PCR buffer (Taq): 10 mm of trichloroethylene HCl, 50 mm of KCl, 1.5 mm $MgCl_2$ (pH 8.4 at 23°C).

PCR buffer (Pfu): 10 mm of trichloroethylene HCl, 10 mm of KCl, 2 mm $MgCl_2$, 10 mm $(NH4)_2SO_4$, 0.1% (v/v) triton X-100, 100 ug/ml nuclease free BSA in DEPC H_2O (pH 8.4 at 23°C).

5x RNA polymerase transcriptions buffer: 200 mm of trichloroethylene HCl, 30 mm $MgCl_2$, 10 mm of spermidin, 50 mm of NaCl (pH 7.5 at 23°C).

20 x SSC: 3 M NaCl, 0.3 M sodium citrate (pH 7.0 at 23°C).

TBS: 50 mm of trichloroethylene HCl, 150 mm of NaCl (pH 7.5 at 23°C).

TBSX: 1 x TBS, 0.1% triton X-100 (pH 7.5 at 23°C).

TE: 1 mm EDTA, 10 mm of tris HCl (pH 8.0 at 23°C).

TBE: 100 mm of trichloroethylene HCl, 83 mm borate, 0.1 mm EDTA (pH 8.6 at 23°C).

Transcription buffer: 20 mm of Tris HCl, 10 mm of Spermidin, 3 mm $MgCl_2$, 50 mm of NaCl (pH 7.5 at 23°C) in DEPC H_2O.

Xenopus egg extract for in situ hybridization: dejelly unfertilized eggs with 2% cystein, wash 3 times, add 1 volume of PBS then lysis by 10 strokes of a Dounce homogenisators,

and centrifuged (7500 x g, Sorvall Rc-5b, rotors Ss-34, 10000 rpm, 4°C, 10 min). The supernatant was transferred into a fresh centrifuge tube and recentrifuged twice under the same conditions. The supernatant was aliquoted and stored at -20°C.

3.8.2 Isolation of nucleic acids

3.8.2.1 Mini-preparation with Qiagen kit

Plasmid DNAs mini-preparations were carried out using Qiagen mini-preparation kits .

3.8.2.2 Isolation of RNA

The embryos or explants were collected at the proper developmental stage in 1.5 ml eppendorf tubes, 3 animal caps or 3 whole embryos in one tube. Pipette off the buffer as much as possible and add Trizol (GibcoBRL), 30µl per explant and 100µl per embryo, vortex for 5-10 min at room temperature. If not used immediately, Trizol samples can be stored at -70°C. Add 2µl per 10µl of chloroform, vortex, spin 5 min at 4°C with maximal speed. Take the upper phase, precipitate with 0.5 volume isopropanol/tRNA, mix, keep at -20°C for at least 30 minutes. Spin at 4°C for 20 minutes. Wash the pellet with 70% ethanol, keep at room temperature for 5-10 minutes. Spin, pour off ethanol, dry briefly, dissolve in DEPC-treated H_2O, 2 µl per explantat and 25 µl per embryo.

3.8.3 Analysis and manipulation of nucleic acids

3.8.3.1 Gel electrophoresis of nucleic acids

DNA or *in vitro* synthesized RNA was isolated in horizontal agarose gel. Depending upon fragment size, 0.7 or one percent agarose gel were used. 0.5x TBE buffer was used. After electrophoresis the gels were photographed. 1 kb DNA ladder was used as size standard.

3.8.3.2 Isolation of DNA fragments from agarose gel

In order to isolate DNA fragments after electrophoresis from agarose gel, the appropriate band were cut out under long-wave UV light. The DNA was extracted from the gel with Qiagen gel-extraction kit.

3.8.3.3 Cloning methods

The cloning of DNA has been performed according to standard methods (Sambrook et al., 1989).

3.8.4 Polymerase chain reaction (PCR)

3.8.4.1 PCR amplification of DNA fragments for cloning

The reaction was accomplished in a total volume of 50 µl. The reaction mixture contained 100 ng of the DNA template, 25 pmol eash primer, 0.5 mm dNTPs, 1 U turbo-Pfu polymerase or Taq polymerase and 1x buffer. The programe is: 95ºC 1 min, 55ºC 1 min, 72ºC 1 min/kb, 35 cycles. The PCR products were digested with suitable endonuclease, seperated on agarose gel and isolated the desired DNA fragment.

3.8.4.2 RT-PCR

In RT-PCR assay, RNA was initially reverse-transcribed to yield cDNA, the cDNA samples were normalized by PCR amplification of housekeeping genes, such as H4 (histone 4), and then the desired target cDNA species were amplified using specific primers. PCRs were carried out in the exponential phase of amplification and PCR samples were loaded side by side in the agarose gel to compare their intensity.

3.8.5 *In vitro* transcription

3.8.5.1 *In vitro* reverse transcription

A cDNA pool was generated from total cellular RNA by using of random oligo nucleotide and reverse transcriptase (RTase) (Steinbach and Rupp, 1999). The reaction was accomplished in a total volume of 10 µl. The reaction beginning contains 1/10 volume of total RNA, 1 x RT buffer for RTase, 10 mm DTT, 0.5 mm dNTPs, 10 U RNAsin and 100 pmol random hexamer primers as well as 200 U RTase. The samples was incubated at 55°C for 30 min and cooled down to 4ºC. The synthesized cDNA were stored at -20ºC.

3.8.5.2 *In vitro* transcription for microinjection

Capped mRNAs used for microinjection were *in vitro* transcripted with RNA polymerase. Reactions were set up as following: in a total volume of 50 µl, 2.5 ug linearized plasmid DNA, 1 x transcription buffer, 0.5 mm dNTPs, 2.5 mm RNA cap structure analogue, 10 mm DTT, 20 U RNAsin and 40 U Sp6 or 60 U T3 or T7 RNA Polymerase, incubate at least for 2.5 hours at 37°C. Subsequently, digeste the templates with 10 U RNAse free DNAse I for 30 min at 37°C. The volume was filled up with DEPC ddH_2O to 90 µl, extracted and centrifuged with 1 volume of PCI (13500 x g, Eppendorf centrifuge 5415C, 14000 rpm, 23°C, 5 min). About 80 µl the supernatant was applied on the QuickSpin column and eluted by centrifugation (1100 x g, 23°C, 4 min). The elute was again centrifuged (13500 x g, 23°C, 5 min), and transfer supernatant into a new 1.5 ml reaction tube and precipitate RNA with 0.1 volume of 3 M NaAc and 2.5 volume ice-cold 100% ethanol for 20 min at -20°C. After a centrifugation (13500 x g, 4°C, 20 min), the pellet was washed with 0.5 ml 70% ethanol, air-dried and dissolved in 10 µl DEPC ddH_2O. The concentration was determined by GeneQuant II (Pharmacia Biotech).

3.8.5.3 *In vitro* transcription of dig labeled RNA probes

Plasmide was linearized and generated antisense RNA by employment RNA polymerases. The reactions were set up as following: in a total volume of 20 µl, 1 µg

linearized plasmid DNA, 1x transcription buffer, 0.1 mm Dig NTPs, 20 U RNAsin and 20 U SP6 or T3 or T7 RNA Polymerase, incubated at 37°C for 2 h.

3.8.6 Site-directed mutagenesis

Site-directed mutagenesis is done with the QuikChange site-directed mutagenesis kit (Stratagen, Cat. 200518) according the instruction manual. In brief (as shown in Figure 3.1), the QuickChange site-directed mutagenesis method is performed using Pfu DNA polymerase and a temperature cycler. The basic procedure utilizes a supercoiled double-

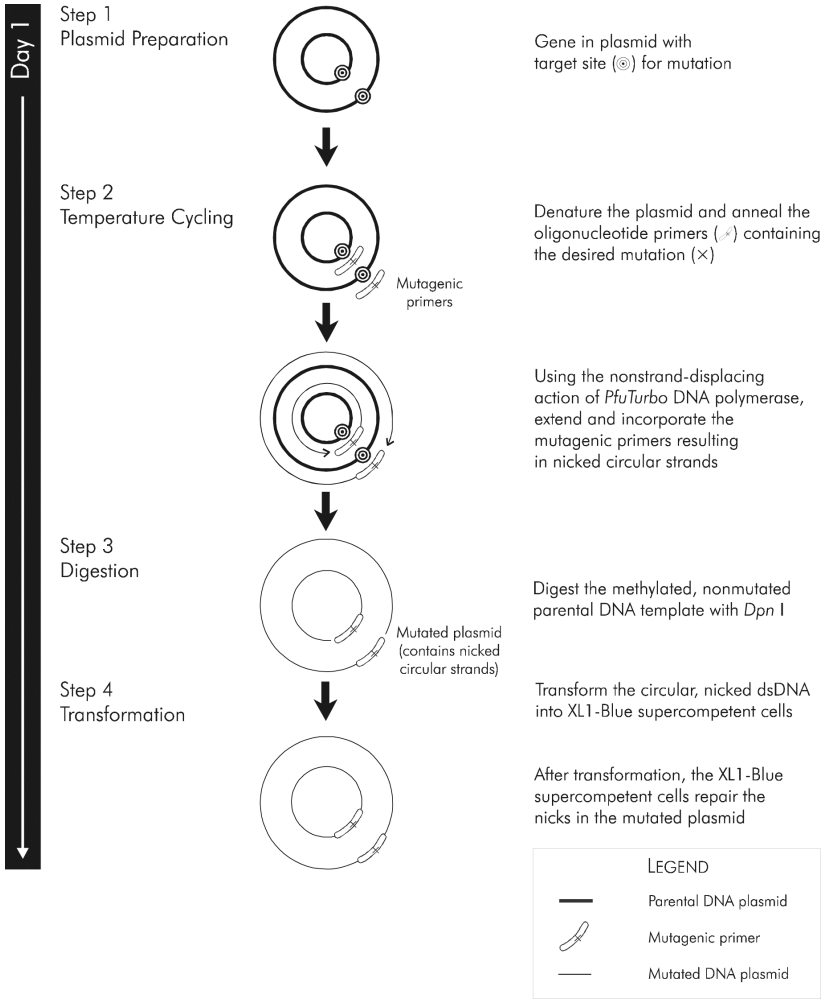

Figure 3.1 Overview of the site-directed mutagenesis method. (Adapted from the instruction manual of QuikChange site-directed mutagenesis kit)

stranded DNA (dsDNA) template (50 ng) and two synthetic oligonucleotide primers (100 ng each) containing the desired mutation. The oligonucleotide primers, each complementary to opposite strands of the vector, are extended during temperature cycling by Pfu DNA polymerase. Incorporation of the oligonucleotide primers generates a mutated plasmid containing staggered nicks. Following temperature cycling (96°C 1min, 55°C 1 min, 72°C 2min/kb, 20 cylces), the product is treated with DpnI. The DpnI endonuclease (target sequence: 5´-Gm^{6}ATC-3´) is specific for methylated and hemimethylated DNA and is used to digest the parental DNA template and to select for mutation-containing synthesized DNA. DNA isolated from almost all E. coli strains is dam methylated and therefore susceptible to DpnI digestion. The nicked vector DNA containing the desired mutations is then transformed into XL1-Blue supercompetent cells. After the transformation, the competent cells repair the nicks in the mutated plasmid. The small amount of starting DNA template required to perform this method, the high fidelity of the PfuTurbo DNA polymerase, and the low number of thermal cycles all contribute to the high mutation efficiency and decreased potential for generating random mutations during the reaction. The mutated plasmid were conformed by restriction enzyme digestion or sequencing.

3.8.7 RNA in situ hybridization

The Expression of mRNAs in embryos was proved by in situ hybridization.

Day 1:

1. Rehydrate the fixed embryos in 80%, then 50% EtOH in PBSw.
2. Wash 3x in PBSw.
3. Treat with 10 ug/ml Proteinase K in PBSw (1 ml per vial) for 30 min at r.t.
4. Rinse with PBSw.
5. Wash 2x in PBSw, 5 min each
6. Fix with 4% PFA in PBSw (0.5ml per vial). Stand for 5 min, then rock for 15'.
7. Short rinse with PBSw.

8. Wash 5x in PBSw.
9. Incubation in 50% PBSw/50% hybridization solution, followed by 100% hybridization solution for about 3 min each step without agitation at RT.
10. Add 0.5 ml of fresh hybridization solution.
11. Prehybridization: incubate at 65°C for 1 hour.
12. Prehybridization: incubate at 60°C for 2-6 hours.
13. Heat 30 ng of probe to 95°C for 2-5 min. Add to 100 ml of prewarmed hybridization solution.
14. Add the probe to the embryos in prehybridization solution.
15. Incubate at 60°C overnight (hybridization).

Day 2:

16. Prewarm 2xSSC/0.1% CHAPS to 37°C, and 0.2xSSC/0.1% CHAPS to 60°C.
17. Short sequential washes of embryos at 60°C with:
    a) hybridization solution
    b) 50% hybridization solution/50% 2xSSC/0.1% CHAPS
    c) 2xSSC/0.1% CHAPS
18. Wash 2x in 2xSSC/0.1% CHAPS for 30 min at 37°C.
19. Short rinse with 0.2xSSC/0.1% CHAPS
20. Wash 2x in 0.2xSSC/0.1% CHAPS for 30 min at 60°C.
21. Rinse in 50% TBS/50% 0.2xSSC/0.1% CHAPS.
22. Wash once in TBS.
23. Rinse in TBS/0.1% Triton X-100 (=TBSX).
24. Incubate in antibody buffer (0.5 ml per vial) for 2 h at 4°C.
25. In parallel, preabsorb AP-conjugated anti-DIG antibodies (1/5000 diluted) against *Xenopus* proteins present in antibody solution.
26. Add 0.5 ml of preabsorbed antibody solution to embryos (i.e. final dilution of antibodies is 10^{-4}).
27. Incubate O. N. at 4°C.

Day 3:

28. Rinse with TBSX
29. Wash 6x in TBSX for 1 hour each.
30. Rinse with AP-buffer.
31. Equilibrate in AP-buffer for 15 min.
32. Replace AP-buffer with 0.5 ml BM-Purple staining solution.
33. Incubate overnight at R.T. in dark with slight rocking (color reaction).

Day 4:

34. Stop reaction by washing twice in PBS for 10 min at R. T. with rocking.
35. Refix embryos in MEMFA for 2 hours, short rinse with H_2O or PBS, store in H_2O or PBS at 4°C.

3.9 Generation of transgenic embryos by restriction enzyme meadiated integration (REMI)

3.9.1 Introduction

Kroll and Amaya recently developed a very efficient method for generating transgenic frog embryos (Kroll and Amaya, 1996). Briefly the protocol involves the following steps: 1. Sperm nuclei are incubated with linearised plasmid DNA. 2. After a short incubation, a high-speed interphase egg extract and a small amount of the restriction enzyme are added to the sperm nuclei and plasmid mixture. The extract partially decondenses the sperm chromatin but does not promote replication and the restriction enzyme stimulate recombination and integration by creating double stranded breaks in the sperm chromatin. 3. After the plasmid-treated nuclei are incubated for a brief period in the interphase extract, the mixture is diluted and the nuclei are transplanted into unfertilized eggs, resulting in the production of transgenic embryos.

3.9.2. High speed extract preparation

The high speed extract preparation is largely based on Murray (1991). Briefly, a crude cytostatic factor (CSF) arrested egg extract (cytoplasm arrested in meiotic metaphase) is prepared. Calcium is added to drive the extract into interphase and a high speed spin is performed to obtain a purer cytoplasmic fraction. Cytochalasin is omitted because it interferes with normal development. Although a crude extract can be used for making transgenic embryos, high speed extracts have several advantages over crude extracts. High speed extracts promote decondensation of sperm nuclei but do not promote DNA replication; high speed extracts cannot progress through the cell cycle; high speed extract can be stored frozen (at -80°C) for many months before use; and sperm nuclei are more easily mixed in high speed extracts which do not contain actin polymers.

1) The evening before the extract preparation, inject each frog with 500U HCG. The frogs are then placed at 18-19°C overnight (12-14 hours). The next morning the egg quality from each container is screened before mixing all the eggs and starting the extract preparation. All the eggs released from a frog which lays mottled, lysing or dying eggs are left out of the extract preparation.

2) All solutions should be prepared before beginning the extract preparation since the procedure should be carried through all steps promptly once it is initiated; optimally, the high speed spin should begin within 45-60 minutes of dejellying the eggs.

3) Overnight the frogs should have laid eggs into the 2 liters of 1X MMR. The high salt in 1X MMR should keep the eggs unactivated and healthy. Manually expel any remaining eggs from each frog into the 2 liter of 1X MMR. Remove the frogs and transfer the eggs from each container into separate 500ml beakers containing around 50ml of 1X MMR. If the eggs look unhealthy, eliminate the eggs from the prep.

4) Remove as much MMR as possible from the eggs. Dejelly eggs in 2% cysteine in XB salts (no HEPES/sucrose) at pH 7.8. Add a small amount at a time, swirl eggs, and partially replace with fresh cysteine several times during dejellying. Dejellying should be

performed separately for different batches of eggs and batches which show breakage or egg activation during dejellying should be discarded.

5) Once the eggs from all the batches have been dejellied, the remaining good eggs are pooled and washed thoroughly in XB (with HEPES/sucrose). We use about 125 ml for each wash and do 4 washes.

6) Wash eggs in CSF-XB with protease inhibitors. We do two 35 ml washes.

7) Using a wide-bore pasteur pipette, transfer eggs into Beckman ultraclear tubes. For these volumes, we typically use 14X95 mm tubes (Catalog #: 344060; Beckman, Fullerton, CA 344057). If multiple tubes will be used, try to transfer an equal volume of eggs per tube. Remove as much CSF-XB as possible and replace with about 1 ml of Versilube F-50.

8) Spin in a clinical centrifuge at room temperature for about 60 seconds at 1000 rpm (150g) and then 30 seconds at 2000 rpm (600 g). Eggs should be packed after this spin but unbroken. Versilube F50 should replace the CSF-XB between the eggs and an inverted meniscus between the Versilube and displaced CSF-XB should be clearly visible. Remove the excess CSF-XB and Versilube F50 and then balance the tubes.

9) Spin the tubes in rubber adapters for 10 minutes at 10,000 rpm at 2°C in Sorvall HB-4 or similar swinging bucket rotor to crush the eggs. The eggs should be separated into three layers: lipid (top), cytoplasm (center), and yolk (bottom). Collect the cytoplasmic layer from each tube with an 18 gauge needle by inserting the needle at the base of the cytoplasmic layer and withdrawing slowly. Transfer cytoplasm to a fresh Beckman tube on ice. If large volumes of darkly pigmented eggs are used, the cytoplasmic layer may be greyish rather than golden at this step. After a second spin to clarify this extract, it should be golden.

10) Estimate the volume of extracts and add the appropriate amount of protease inhibitors to the isolated cytoplasm (do not add cytochalasin); recentrifuge the cytoplasm in Beckman tubes for an additional 10 minutes at 10,000 rpm to clarify, again using a swinging bucket rotor. Collect the clarified cytoplasm as before.Expect to get about 0.75-1 ml cytoplasm per batch of eggs collected from one frog.

11) Add 1/20th volume of the ATP-regenerating system (Energy Mix). Transfer the clarified cytoplasm into TL100 tubes thick wall polycarbonate tubes (Beckman 349622). Tubes hold about 4 ml each and should be at least half full.

12) Add $CaCl_2$ to each tube to a final concentration of 0.4mM; this inactivates CSF and pushes the extract into interphase. Incubate at room temperature for 15 minutes then balance for the high speed spin.

13) Spin tubes in a Beckman tabletop TL-100 ultracentrifuge in a TL100 rotor (fixed angle) at 70,000 rpm for 1.5 hours at 4°C.

14) The cytoplasm will fractionate into four layers, top to bottom: lipid, cytosol, membranes/mitochondria, and glycogen/ribosomes. Remove the cytosolic layer from each tube (about 1 ml if 2-3 ml was loaded into the tube) by inserting a syringe into the top of the tube through the lipid layer. Transfer this fraction to fresh TL-100 tubes and spin again at 70,000 for 20 min. at 4°C.

15) Aliquot the high speed cytosol supernatant into 15 µl aliquots in 0.5ml Eppendorf tubes. Quick freeze aliquots in liquid nitrogen and store at -80°C until use. We typically obtain 2-3 ml of high speed cytosol from preparations of this scale. Sperm nuclei should be incubated in an aliquot of extract and stained with Hoechst to determine whether extract is active in decondensation. Active interphase extract should visibly swell sperm nuclei (thicken and lengthen) within 10-15 minutes of addition to extract at room temperature.

3.9.3 Sperm nuclei preparation (largely based on Murray, 1991)

1) Anaesthetise a male by immersion in 0.1% aminobenzoic acid ethyl ester (Tricaine; MS222) (Sigma A-5040) with 0.1% sodium bicarbonate for at least 20 minutes and then kill it. Cut through the ventral body wall and musculature and lift the yellow fat bodies to isolate the two testes which are attached to the base of the fat bodies one on each side of the midline. Remove the testes with dissecting scissors and place them in a 35mm tissue culture dish containing cold 1X MMR. We routinely isolate sperm nuclei from one testis and use the other for *in vitro* fertilisations. Inspect the two testes and isolate the one with less blood contamination for the nuclear prep. If a large prep is required, testes from two to four males can be used. The final resuspension volume should be increased accordingly.

2) Rinse the testis in three changes of cold 1X MMR.

3) Using fine forceps, remove any remaining fat body and excess blood. Do not try to remove the blood vessels. Rather, puncture holes in the largest vessels and gently push the blood out. Take care not to puncture the testis as this releases sperm.

4) Place the cleaned testis in another 35mm tissue culture dish with 5 ml of cold 1X NPB + protease inhibitors for 2 to 5 minutes.

5) Transfer the testis to a dry 35mm tissue culture dish, and macerate the tissue well (until clumps are no longer visible to the naked eye) with a pair of clean forceps.

6) Resuspend the macerated testes in 2 ml of cold 1X NPB + protease inhibitors by pipetting the mixture up and down through a sterile, disposable 5 ml pipette.

7) Squirt the sperm suspension through two-four thicknesses of cheesecloth placed into a funnel and collect the solution into a 14 ml sterile culture tube (Falcon 2059; 17 x 100 mm). Rinse the dish with an additional 3 ml of cold 1X NPB + protease inhibitors and

add to the cheesecloth. After adding 5 ml more (10 mls total) of cold 1X NPB + protease inhibitors use a gloved hand to fold the cheesecloth and squeeze any remaining liquid through the funnel into the 14 ml tube. We usually end up with 9 ml of sperm suspension in the tube.

8) Centrifuge the sperm suspension at 3,000 rpm for 10 min. at 4°C (we use a Sorvall HB-4 or similar swinging bucket rotor fitted with the appropriate adapters). The sperm pellet should be white, fairly compact. Usually we have some blood contamination which can be seen in the center of the pellet. During the spin, allow 1 ml of 1X NPB + protease inhibitors to equilibrate to room temperature.

9) Decant the supernatant and resuspend the sperm pellet in 9 ml of cold 1X NPB + protease inhibitors and repellet by centrifugation at 3,000 rpm, 10 min., 4°C. During this spin dissolve 1 mg of L-a-lysophosphatidylcholine (Lysolecithin) (Sigma L-4129) in 100 µl (10mg/ml) of H_2O at room temperature. Lysolecithin will not remain in solution below room temperature.

10) Decant the supernatant and resuspend the sperm pellet in the 1 ml of 1X NPB + protease inhibitors that has equilibrate at room temperature and add 50µl of 10mg/ml lysolecithin. Mix gently and incubate for 5 minutes at room temperature.

11) Add 10 ml cold 1X NPB +3% BSA + protease inhibitors to the suspension and centrifuge at 3,000 rpm, 10 min., 4°C. At the end of this spin the pellet should now be wider and more loose than before. In addition the pellet should no longer have redness. The looseness and the loss of haemoglobin mean that the pellet now contains nuclei rather than intact cells.

12) Decant the supernatant and resuspend the pellet in 5 ml cold 1X NPB + 0.3% BSA (no protease inhibitors), mix gently by pipetting up and down, and centrifuge at 3,000 rpm, 10 min., 4°C.

13) Decant the supernatant and resuspend the pellet in 250µl of 1X NPB + 30% (w/v) glycerol + 0.3% BSA (Sperm Storage Buffer) and transfer suspension into a 1.5 ml Eppendorf tube. Store at 4°C and use for transgenesis for up to 48 hours.

14) Cut the tip of a yellow tip with a razor blade and mix the sperm nuclei suspension by pipetting up and down. Remove 2 µl and dilute into 200 µl of sperm dilution buffer (i.e. 1:100 dilution). Add 2 µl of a 1:100 diluted Hoechst stock and transfer the diluted sperm nuclei to a hemacytometer for counting. Visualise the sperm nuclei under a fluorescence microscope using a DAPI/Hoechst filter set. For a 1:100 dilution of our sperm nuclei stock, we typically obtain counts of 125-200 (X10 4 nuclei/ml). At this concentration, the undiluted stock contains 125-200 nuclei/nl. If your sperm stock is substantially less concentrated (i.e.. a count of <100 for a 1:100 dilution), repellet the sperm nuclei at low speed (or allow the nuclei to settle over a few hours) and resuspend in a smaller volume of sperm storage buffer. We store the fresh nuclei overnight at 4°C and after extensive mixing by pipetting up and down with a cut yellow tip, we freeze 40 µl aliquots in liquid nitrogen and store the frozen nuclei at -80°C. One aliquot is thawed for each day of transgenesis.

3.9.4 Preparation of DNA, needles and equipment

3.9.4.1. Preparation of linearised DNA

Digest DNA using standard conditions.

3.9.4.2 Preparation of injection needles for nuclear transplantation

These needles are unlike standard needles used for DNA and RNA injection in that the diameter of the point is an order of magnitude larger to allow nuclei to pass through without shearing. 30 µl Drummond micropipets (Fisher, cat. #: 21-170J) are used to make the needles. It is important to pull needles with gentle slopes at the tip. This makes it easier to clip the needles at the desired diameter and also they cause less damage to the

eggs during transplantation. We currently use a Flaming/Brown Micropipette Puller Model P-87 (Sutter Instruments). Clip the needle with forceps to produce a bevelled tip of 80-100 µm diameter using the ocular micrometer of a dissecting microscope or a stage micrometer for measurement. It is essential that the tip be this wide or nuclei passing through will be damaged. When clipping tips, it often helps to use forceps with slightly unmatched tips and to pull outward at a 20 or 30 degree angle from the needle as the forceps contacts the needle. Treat the inside of needles with Sigmacote (Sigma SL-2) to prevent shearing of sperm nuclei flowing through the needle (needles can be coated 10 minutes to several months before use). Attach approximately 1 cm Tygon tubing (R-3603 1/32"; Fisher, cat. #: 14-169-1A) to the end of a plastic pipetteman (200µl) tip and use the pipetteman to draw up Sigmacote; then attach the other end of the tubing to the injection needle (see below). Depress the pipetteman plunger to force Sigmacote through the needle until a few drops emerge from the tip then release the pipetteman plunger to withdraw most of the solution. Rinse needle with at least 200ml of water. Make sure to remove all the liquid from the needle as any remaining liquid will block flow of nuclei into the needle.

3.9.4.3 Agarose-coated injection dishes

Pour 2.5% agarose in 0.1X MMR into 60mm petri dishes. Before the agarose solidifies, place small weigh boats on the agarose so that as the agarose solidifies, a square depression in the agarose remains. The depression will accommodate ~500 eggs. Wrap the dishes in parafilm and store at 4°C until use.

3.9.4.4 Transplantation apparatus

Most commercial injection apparatuses used for DNA and RNA injections which are based on air pressure are unsuitable for nuclear transplantations, due to the large difference in needle tip size. Flow through the 5 µm needle tips used for fluid injections is controllable at fairly high pressures. However, with these standard systems it is usually not possible to obtain an extremely low positive pressure and gentle, controlled flow required to deliver an intact nucleus in a small volume (10-15 nl) through a 80-100 µm

needle tip. Two people can transplant nuclei at the same time. The oil that we use to fill the system is Mineral Oil (Embryo Tested) from Sigma (Cat. No. M-8410). The big advantage of using an infusion pump is that we can adjust it to any desirable flow rate. We have ours set at 10nl/sec.

3.9.4.5 Transgenesis by sperm nuclear transplantation into unfertilised eggs

1) The night before eggs are needed for transplantations, inject 2 adult female frogs in the dorsal lymph sac with 500U Human Chorionic Gonadotropin (HCG) and incubate at 19°C for 10-12 hours. 2) Remove a 1ml aliquot of Sperm Dilution Buffer (SDB) from the freezer and allow it to warm to room temperature.

3) Make up 500ml of 2% cysteine in 1X MMR pH 7.8 (with 5N NaOH).

4) Fill agarose coated injection dishes with 0.4X MMR + 6% Ficoll

5) Set up a reaction:
4 µl sperm stock (~4 X 10 5 nuclei)
2.5 µl linearized plasmid (100 ng/µl)
Incubate 5min at room temperature.
Meanwhile:
Obtain a 15ml aliquot of high speed extract from -80 freezer and allow to thaw to room temperature (only takes a few minutes). Keep aliquot on ice for the day.

Make the following mixture:
18 µl SDB
2 µl extracts
2 µl 100 mM MgCl2 (add to 5 mM final at all steps to aid enzyme action)
0.5 µl of a ~1:10 dilution of Sal I or Not I to the extracts (depending on the construct).
Mix well.

After 5 minute incubation of DNA with sperm nuclei add the extract:enzyme:$MgCl_2$ mixture to the sperm nuclei.
Mix the reaction by gentle pipetting (using a clipped yellow tip).
Incubate 15 min. at room temperature.

6) While sperm are swelling in reaction mixture, collect eggs from the frogs and dejelly them in 2 % cysteine hydrochloride in 1X MMR (pH 7.8 with NaOH). Wash the eggs thoroughly in 1X MMR. Transfer dejellied eggs into agarose coated dishes containing 0.4X MMR with 6% ficoll and gentamycin. We try to fill the depression square with eggs. After about 5 minutes in 0.4X MMR + 6% Ficoll the eggs will pierce easily.

7) After the 10-15 minute incubation with extracts, mix the sperm nuclei gently by pipetting up and down with a cut of yellow tip. Then transfer 5 ml of mixture into 150 ml of SDB that is already at room temperature. At this point do not mix the nuclei. Mixing at this point is likely to shear the nuclei. Allow the sperm nuclei:extract mixture to slowing equilibrate with the SDB over the span of a few minutes.

8) Using a piece of Tygon tubing attached to a yellow tip (as previously described for Sigmacoting needles) draw up the dilute sperm suspension, mix gently by pipetting up and down and then draw up the dilute sperm nuclei and detach the yellow tip from the pippetman (try not to create or leave bubbles in the tygon tubing as these may damage nuclei). Be careful to keep the yellow tip horizontal or the nuclei will dribble out. Now back load a needle by attaching it to the tygon tubing and raising the angle slightly so that the nuclei flow gently into the needle. As long as no liquid is present at the tip of the needle the nuclei should flow easily by simple gravity. Once the needle has backfilled completely with nuclei, detach the needle and attach it to the tygon tubing filled with mineral oil that is connected to the Harvard Apparatus infusion pump. If two people of injecting load another needle as before. Place the yellow tip with the remaining nuclei aside horizontally in case you need to load another needle.

9) Transplant sperm nuclei into unfertilised eggs.

Start the flow in the infusion pump and begin injecting eggs, keeping the needle inside each egg for approximately one second. Move the needle fairly rapidly from egg to egg, piercing the plasma membrane of each egg with a single, sharp motion then drawing the needle out more slowly. The angle of the needle should be perpendicular to the membrane surface (rather than glancing) to avoid tearing the plasma membrane. If the needle becomes clogged with cytoplasm, bring the tip to the air-liquid interface of the dish. Sometimes the surface tension of the interface removes the cytoplasm plug in the end of the needle. If a needle tip is too narrow, or if it becomes partially clogged with debris during transplantations, the injected nuclei will be damaged during transplantation, resulting in aneuploid or haploid embryos. You can determine whether your sperm dilution and the flow rate used for injections were appropriate by watching the first cleavage of the transplanted eggs. If few of the eggs received a nucleus, the frequency of cleavage will be low; one fifth to one third of our transplantations typically result in normally cleaving embryos. Eggs that were injected with more than one nucleus will divide at the time of first cleavage abnormally into three or four (or more) cells. Many of these embryos will develop to blastula stages, but most fail during gastrulation; in some, a region of the embryo will fail to cellularize and die. Eggs injected with multiple nuclei which do gastrulate usually do so abnormally; typically, blastopore closure is incomplete resulting in embryos that form two wings of somites and neural tissue on each side of the exposed yolky tissue lying in the center of the trunk. This type of gastrulation failure is common to stressed or unhealthy embryos (particularly embryos derived from ‘soft’ eggs).

10) When the embryos have reached the 4-cell stage, gently separate them from uncleaved eggs and transfer them to a separate dish of 0.1X MMR + 6%Ficoll +50µg/ml gentamycin. Do not be fooled by speudocleaveages. Only keep embryos that appear like normal, healthy 4-cell embryos. We commonly culture transplanted embryos in 24-well tissue culture dishes with about 5 embryos per well, since culturing embryos at high density can compromise their health. It is also important to remove dying embryos promptly since they also can compromise the health of their siblings. When embryos are around stage 12, media is replaced with 0.1X MMR + 50µg/ml gentamycin without

Ficoll. Because of the large needle tip used for transplantations, embryos often develop large blebs at the site of injection. These blebs occur when cells are forced out of the hole left in the vitelline membrane at the injection site but they generally do not affect development. The blebs usually fall at the neurula or tailbud stages, but they can be removed manually once the embryos have reached the late blastula stage.

4 Results

4.1 Analysis of *XmyoD cis*-regulatory elements

4.1.1 The expression of *XmyoD* at NF10.5 is regulated by multiple elements

As the expression of pMD-6.0kb/+4829GFP recapitulates the dynamic stage and tissue-specific expression pattern of *XmyoD*, it provided a basis for analyzing the *cis*-regulatory elements of *XmyoD* expression. To resolve which regions of the pMD-6.0kb/+4829GFP were responsible for the regulation, a series of truncation constructs were generated (Figure 4.1A).

As shown in Figure 4.2 and Table 4.1, the pMD-704/+4829GFP construct containing promoter proximal regions and 3' downstream region of the *XmyoD* gene, did not show any expression in mesoderm at stage NF10.5, but showed a high level of non-specific GFP mRNA expression mainly in ectoderm (Figure 4.2A). At tailbud stages, most transgenic embryos lost the expression (Figure 4.2B), none of the remaining embryos showed somite specific expression. Therefore, it is concluded that the promoter region of *XmyoD* is not active in mesoderm, however it is very active in ectoderm. The correct expression of *XmyoD* in *Xenopus* needs at least a mesoderm-activation element and an ectoderm-repression element.

The expression of pMD-840/+4829GFP was mainly restricted to the dorso-lateral domain of the marginal zone at NF stage 10.5 (Figure 4.2C and Table 4.1). The RNA *in situ* hybridization signals were quantitatively very weak. Only in a minor fraction of the embryos did the reporter expression spread to the ventral side, as is characteristic for the endogenous *XmyoD* mRNA. Importantly, with this construct the non-specific expression of pMD-704/+4829GFP in the ectoderm was extinguished and mesoderm specific expression was initialized. It is expected that there must be at least two motifs in the region of -840/-704bp: one repressing element to extinguish the non-specific expression in ectoderm and one activating element to initialize the mesoderm specific expression.

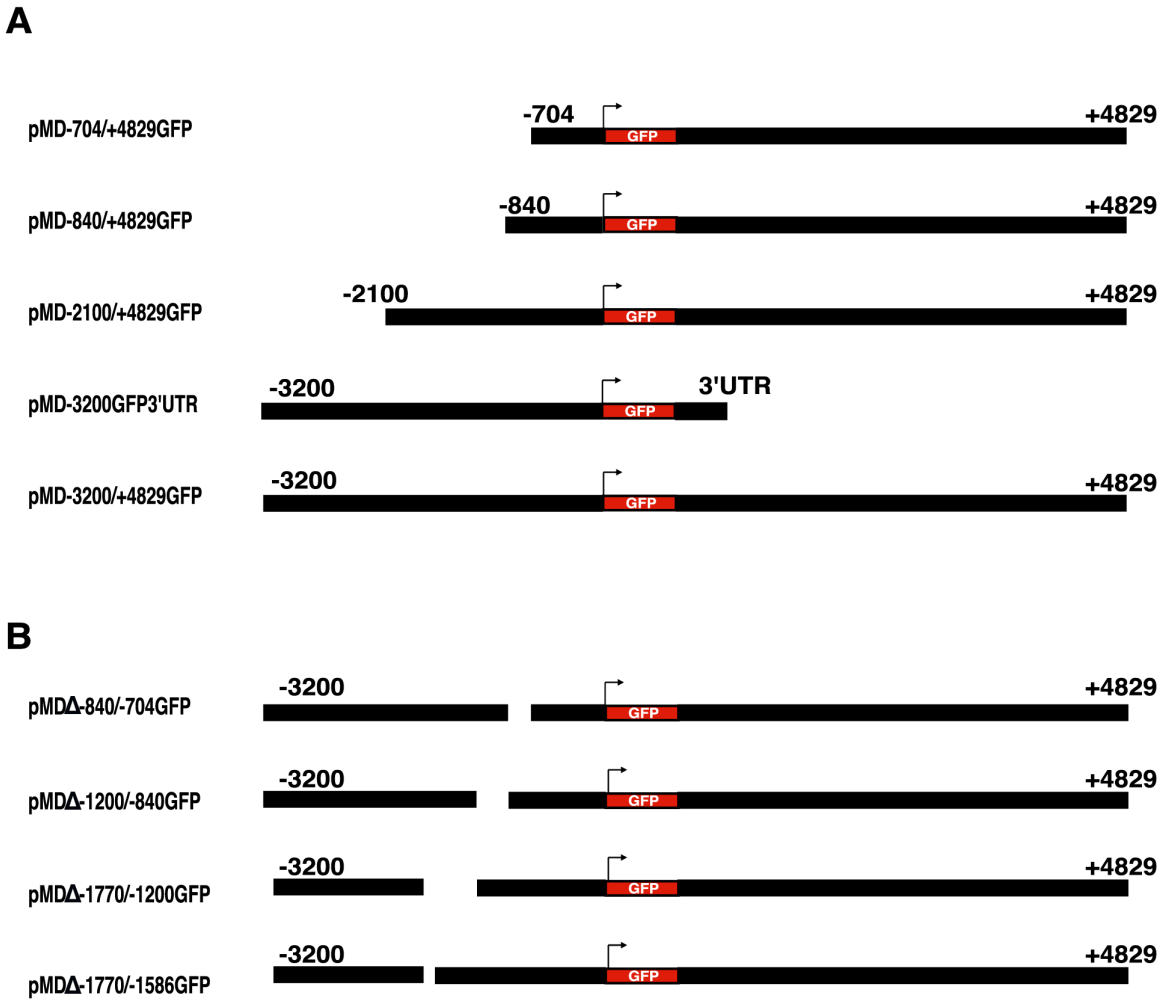

Figure 4.1 Schematic representation of the deletion constructs. The black bars represent *XmyoD* genomic DNA; Red bars represent GFP gene. The arrow indicates the transcription start site. The extent of the 5' and 3' sequences from the start of transcription are shown.

When additional sequences between -2100 and -840 bp were included in the reporter construct, GFP-positive signals extended ventrally around the blastopore on the ventral side (Figure 4.2E and Table 4. 1). This expression pattern was very similar to the endogenous *XmyoD* expression, although the average reporter gene expression level was somewhat weaker when compared to the full-length construct (pMD-6.0kb/+4829GFP).

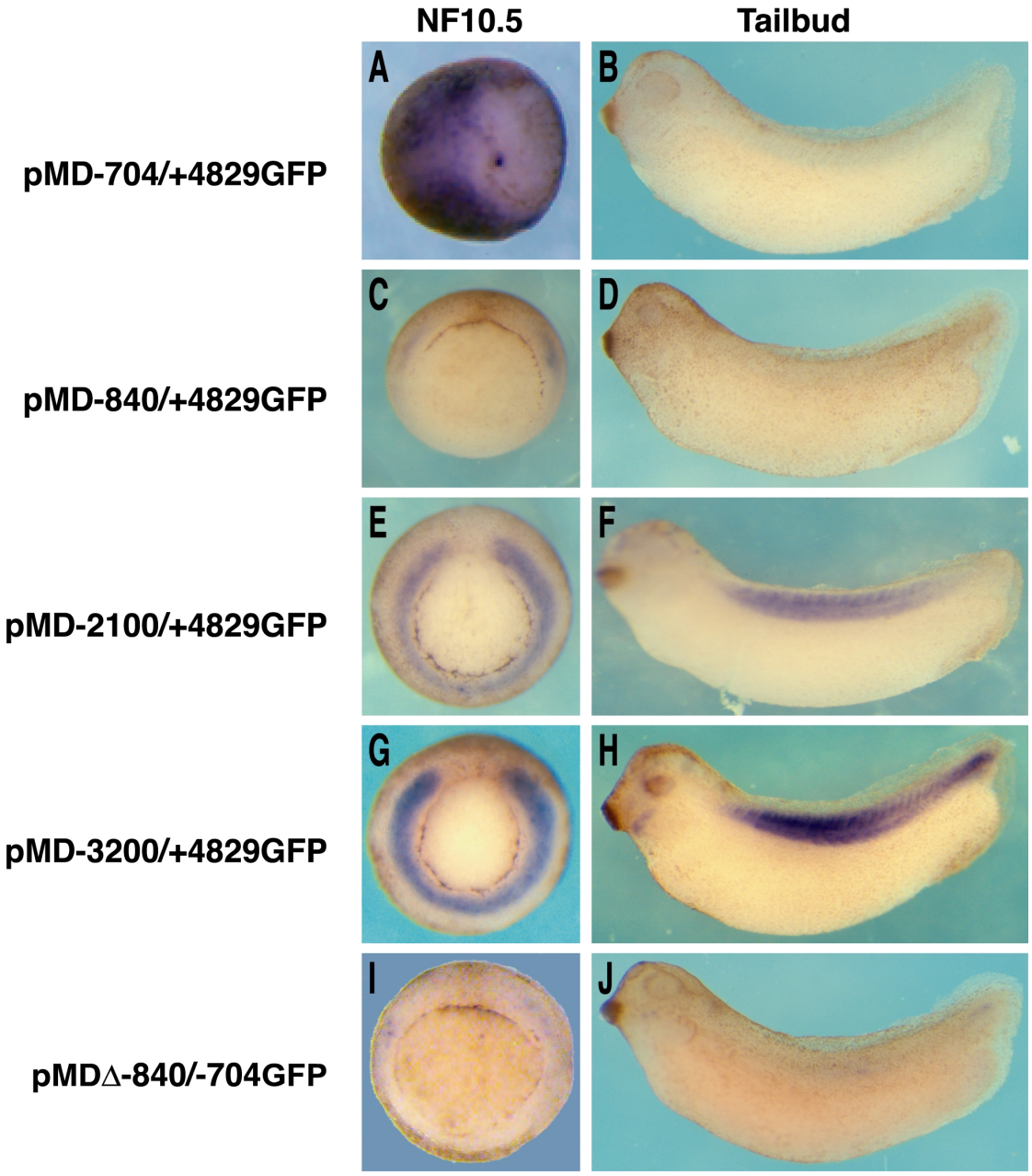

Figure 4.2 The expression of the deletion constructs at different stages. The names of the construct are indicated on the left side of the pictures, and the stage is indicated above the pictures. (A) The pMD-740/+4829GFP did not show any expression in mesoderm at stage NF10.5, but showed a high level of non-specific GFP mRNA expression mainly in ectoderm. At tail bud stage, most embryos lost the expression (B). The expression of pMD-840/+4829GFP was mainly restricted to the dorsal-lateral domain of marginal zone at NF 10.5 (C), and the expression is gone at tail bud stage (D). When additional sequence between -2100 and -840 bp were included in the promoter, GFP-positive signals extended ventrally and frequently surrounded the blastopore on the ventral side (E). PMD-3200/+4829GFP was indistinguishable from pMD-6.0kb/+4829GFP (G and H). pMDΔ-840/-704GFP showed heavy reduction of the mesoderm specific expression, and incorrect expression increase significantly (I), the expression disappeared at tail bud stage (J). The embryos are viewed from vegetal pole with dorsal up (C, E, G, I), or laterally (A, B, D, F, H, J).

When more upstream sequences were included in the promoter, the GFP signals increased in strength, but the pattern did not change compared with pMD-840/+4829GFP. pMD-3.2kb/+4829GFP was indistinguishable from pMD-6.0kb/+4829GFP. When the 3' region was removed in pMD-3.2/3'UTRGFP construct, the expression pattern of GFP was identical to that of pMD-6.0kb/+4829GFP at the gastrula stage (data not shown), although staining was less intense. But at tailbud stages, the expression was not maintained well. So 3' region was included in all other constructs.

Addition of sequence upstream of position –704 resulted on average in a much stronger reporter expression in the mesoderm. Therefore, it is concluded that multiple activating elements exist in the *XmyoD* regulatory region, and at least one repressing element to repress expression in ectoderm. The enhancers and silencers cooperate to define the expression domain during the development of *Xenopus*.

Table 4. 1 Expression of deletion constructs at stage NF10.5

Construct	Correct Expression	Incorrect Expression	No Expression	n	Average level of *XmyoD*-like expression∗
pMD-3200/+4829GFP	77%	8%	15%	117	+++
pMD-704/+4829GFP	0%	86%	14%	59	-
pMD-840/+4829GFP	46%	6%	48%	46	+
pMD-2100/+4829GFP	67%	6%	27%	66	++
pMD-3200/3'UTRGFP	38%	1%	61%	95	++
pMDΔ-840/-704GFP	27%	50%	23%	131	+
pMDΔ-1200/-840GFP	61%	2%	31%	152	++
pMDΔ-1770/-1200GFP	51%	1%	48%	140	++
pMDΔ-1770/-1586 GFP	38%	6%	56%	143	++

Embryos were scored as showing expression in the same pattern as the endogenous *XmyoD* (correct expression), expression that does not locate in mesoderm but in ectopic place (incorrect expression) or no expression.

∗ represents average level of *XmyoD*-like expression for all transgenic embryos from a given construct. The level of expression was rated on a scale of – to +++, with – representing no expression, while +++ representing the expression level of full length construct (pMD-6.0kb/+4829GFP).

4.1.2 The -840/-704 region contains both activating and repressing elements

Because multiple *cis*-regulatory elements exist and cooperate to control the strength of expression, it is difficult to conclude what regions are more important than others from the above results. In order to further narrow down the main activating motif and repressing motif, reporter constructs were designed that contained several internal deletions (Figure 4.1B).

The transgenic embryos of pMDΔ-1200/-840GFP, pMDΔ-1770/-1200GFP and pMDΔ-1770/-1586GFP did not reveal any changes of the expression pattern at stage NF10.5, only the staining intensity was reduced slightly (Table 4.1). The reporter construct pMDΔ-840/-704GFP, however, showed a significant reduction of the mesoderm specific expression, and at the same time an increase in incorrect expression (Figure 4.2I and Table 4. 1). When the -840/-704bp region was added to the promoter in pMD-840/+4829GFP, it is showed that this region is sufficient to repress the non-specific expression in ectoderm and initiated the specific *XmyoD*-like expression (Figure 4.2C and table 4.1). When the -840/-704bp region was deleted in the context of the –3200/+4829GFP construct, although all the other regions are present, most of the mesoderm expression disappeared and incorrect expression increased significantly. Therefore, -840/-704 region is essential for the correct spatial induction of *XmyoD*. Due to this activity, I named this region the MIE-element (*MyoD* Induction Enhancer).

4.1.3 Fine-scale mapping of MIE

Based on the above results, we expected that there were at least one activating element and one repressing element within the MIE. I further employed a linker-scanning mutation strategy to perform an unbiased search for important DNA motifs in the MIE. Mutations were created such that 15bp blocks of genomic sequence were replaced by a 15bp linker sequence (Kucharczuk et al., 1999; Figure 4.3A). In Figure 4.3B, the positions of the 10 linker-scanning mutations are demarcated by vertical lines above the sequence.

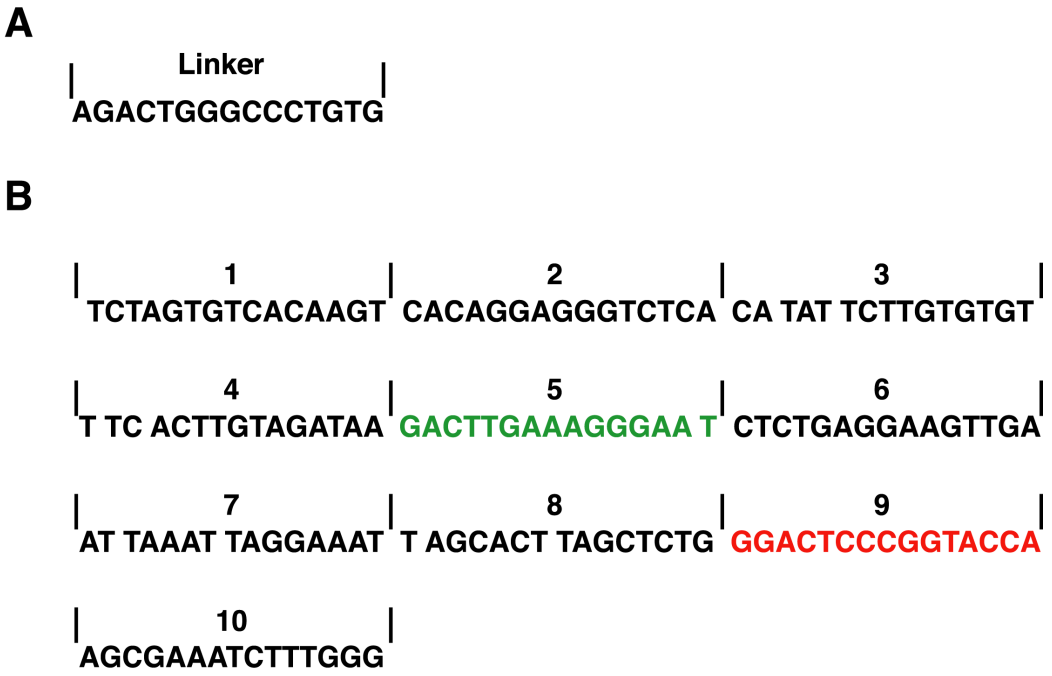

Figure 4.3 (A) The linker sequence with a Apa I site in the middle. (B) Linker-scanning mutations were created such that 15bp blocks of genomic sequence were replaced by a 15bp linker sequence. Vertical lines above the sequence demarcate positions of the 10 linker-scanning mutations. The green sequence indicates the motif for activation, and the red sequence indicates the motif for repression.

The results of linker-scanning assay are shown in Figure 4.4 and Table 4.2. The reporter gene expression of LS-2, LS-3, LS-6, LS-10 mutation constructs were indistinguishable from that of the pMD-6.0kb/+4829GFP construct (Table 4. 2).

The LS-5 mutation construct resulted in significant reduction of transgenic embryos showing reporter expression at stage NF10.5 and at tailbud stages (Figure 4.4A,B and Table 4. 2). The remaining GFP-positive embryos showed only a very weak RNA-signal, restricted to the dorsolateral marginal zone. This is similar to the result of the pMDΔ-840/-704GFP construct (Figure 4.2C). From this, I conclude that the DNA sequences mutated in the LS-5 construct are required for most of the transcriptional activation input attributed to the MIE.

The LS-9 mutation construct resulted in precocious expression of reporter gene before stage NF10 (85%, n=33) (Figure 4.4 C) and a stronger and expanded expression of reporter gene at stage NF10.5 (Figure 4.4 D). The expression expanded to the posterior wall (PW) of the neuroenteric canal (Beck, and Slack, 1998) at tailbud stages (Figure 4.4 E-F and Table 4.2). It has been shown that MyoD induction depends on the developmental age of the induced cells, rather than on the type or time point of inducer application (Steinbach et al, 1998 and Figure 2.6B, h, i). The precocious expression of reporter gene before NF10 indicated that the LS-9 motif may contain an important cis-regulatory element that controls the timing of *XmyoD* expression.

The reporter gene expression of LS-4, LS-7 and LS-8 constructs also showed a slight reduction in intensity. The LS-1 construct showed a slight increase in expression intensity. Considering there are multiple enhancers located outside of the MIE, the LS mutants were constructed in the context of more than 8kb genomic sequence (-3200/+4829), only 15bp exchange results in significant change of the expression. This indicates that both LS-5 and LS-9 motifs are indispensable for the correct spatiotemporal induction of *XmyoD* transcription.

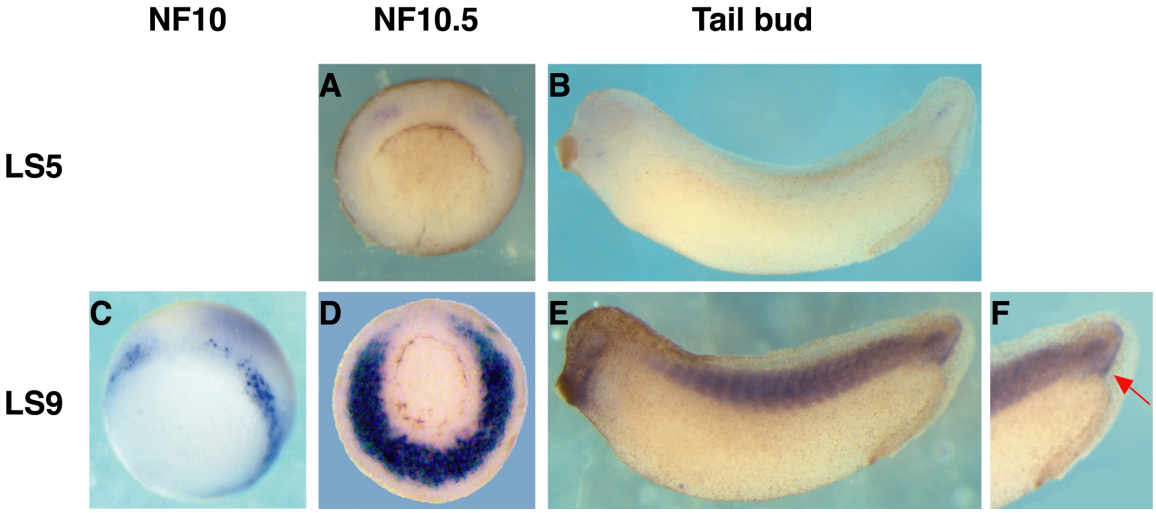

Figure 4.4. LS-5 mutation construct results in a loss of most of the expression of reporter gene (A, B); while LS-9 mutation construct resulted in precocious expression of reporter gene before NF10 (C) and a stronger and expanded expression of reporter gene at NF 10.5 (D); The expression expanded to the posterior wall of the neuroenteric canal (arrow in F) at tail bud stage (E, F). The embryos are orientated with dorsal pointing up and vegetal pole facing out (A, C, D), or head facing left (B, E, F).

Table 4. 2 Expression of linker-scanning mutation constructs at stage NF10.5

Construct	Correct Expression (%)	Incorrect Expression (%)	No Expression (%)	n	Average level of *XmyoD*-like expression∗
PMDLS-1	81	16	4	57	++++
PMDLS-2	72	15	12	65	+++
PMDLS-3	66	23	11	44	+++
PMDLS-4	62	19	19	63	++
PMDLS-5	38	38	24	45	+
PMDLS-6	69	20	10	49	+++
PMDLS-7	58	28	14	36	++
PMDLS-8	52	19	28	42	++
PMDLS-9	86	5	9	65	+++++
PMDLS-10	67	11	21	61	+++

Embryos were scored as showing expression in the same pattern as the endogenous *XmyoD* (correct expression), expression that does not locate in mesoderm but in ectopic place (incorrect expression) or no expression.
∗Represents average level of *XmyoD*-like expression for all transgenic embryos from a given construct. The level of expression was rated on a scale of – to +++++, with – representing no expression, while +++representing the expression level of full length construct (pMD-6.0kb/+4829GFP).

4.1.4. MIE is essential for eFGF to active *XmyoD* at NF 10.5

A recent paper reported that eFGF is an inducer of *XmyoD* in an animal cap (AC) assay (Fisher et al., 2002), so we asked which region in the *XmyoD* promoter is responsible for the induction by eFGF.

A total of 8pg eFGF mRNA was injected into the animal pole (AP) region of wild type or transgenic embryos at the 4-cell stage, and the expression of endogenous *XmyoD* or *GFP* reporter gene was assayed by RNA *in situ* hybridization at stage NF 10.5. At this stage, 86% (n=14) of wild type embryos showed strong expression of *XmyoD* in the AP (Figure 4.5A and Table 4.3). Similarly, 83% (n=23) of transgenic embryos of pMD-3200/+4829GFP construct also showed strong expression of the GFP gene in the AP (Figure 4.5B and Table 4.3). In contrast, only 8% (n=59) of transgenic embryos of pMDΔ-840/-704GFP construct showed strong expression, the majority (92%, n=59) showed very low or no expression of GFP gene in the AP (Figure 4.5C). Another internal deletion construct, pMD-1770/-1586GFP, showed a moderate activation of the GFP gene

by eFGF in the AP (Figure 4.5D), 60% (n=52) of the transgenic embryos showed a strong expression of GFP in the AP. I also assayed if the LS-5 motif played a role in the response to the induction of eFGF. In this experiment, 27% (n=30) embryos transgenic with the LS-5 construct showed strong expression of the GFP gene in the AP under the induction of eFGF (Figure 4.5E).

Three conclusions can be drawn from these experiment. First, that there are multiple response elements for eFGF signaling in *XmyoD* regulatory region because both deletion constructs pMDΔ-840/-704GFP or pMDΔ-1770/-1586GFP showed a reduction of the activation of GFP by eFGF. Second, the -840/-704 region (MIE) is essential for the induction of *XmyoD* by eFGF signal. Third, the LS-5 motif accounts for most of the reduction caused by deletion of -840/-704bp. This indicates LS-5 motif is very important for the induction by eFGF.

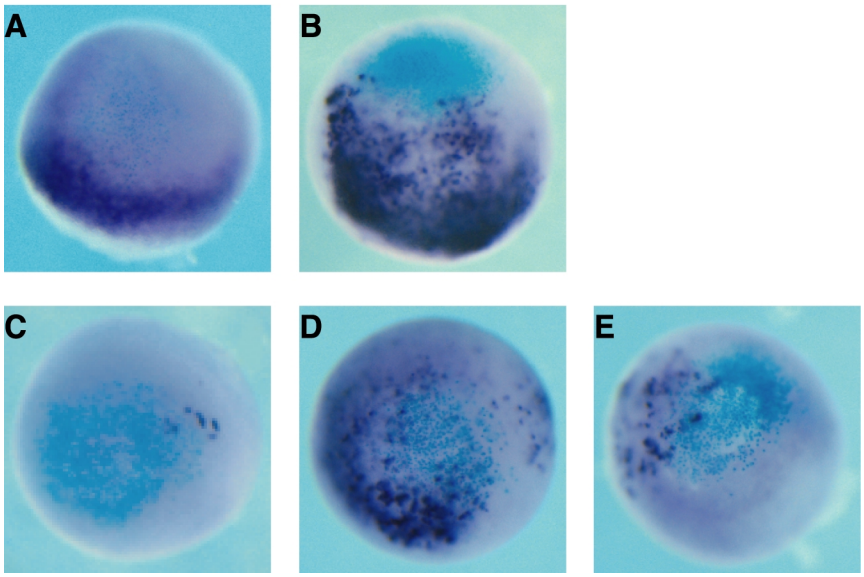

Figure 4.5 MIE is essential for eFGF to induce the reporter gene at NF 10.5.
A total of 8pg eFGF mRNA was injected in the animal pole of wild type embryos or transgenic embryos at the 4-cell stage. The expression of either *XmyoD* or *GFP* was assayed by *in situ* hybridization at NF 10.5. Wild type embryos showed strong ectopic expression of *XmyoD* in AP (A), transgenic embryos of pMD-3200/+4829GFP construct (B) and pMDΔ-1770/-1586GFP construct (D) also showed ectopic expression of *GFP*, but transgenic embryos of pMDΔ-840/-704GFP (C) or LS-5 constructs (E) showed very low or no ectopic expression of *GFP*. LacZ staining (blue) indicates the injection position of eFGF mRNA. The embryos are orientated with animal pole facing out.

Table 4.3 eFGF regulates the expression of *XmyoD*

Transgene or Endogenous *XmyoD*	Strong Induction	No Induction or almost no Induction	n
Endogenous *XmyoD*	86%	14%	14
pMD-3.2/+4829GFP	83%	17%	23
pMDΔ-840/-704GFP	8%	92%	59
pMDΔ-1770/-1586GFP	60%	40%	52
LS-5	27%	73%	30

4.1.5 Xcad-3 as potential regulator of *XmyoD* transcription

The DNA sequences mutated in LS-5 and LS-9 were used as queries for the TRANSFAC database (www.molsun1.cbrc.aist.go.jp) to identify potential binding sites for sequence-specific DNA-binding proteins. No potential motif was indicated in the LS-9 region, however, a consensus cdxA binding site was found in the LS-5 sequence. The sequence of the oligonucleotide used for linker scanner mutagenesis does not contain any similarity to the cdxA consensus binding site.

CdxA is a member of the caudal protein family (Margalit et al., 1993 and Pillemer et al., 1998). The members of the *Xenopus* caudal protein family have previously been shown to be downstream of eFGF signaling and act as transcriptional activators for posterior genes (Pownall et al. 1996). Therefore, they could be candidates to mediate the activation of *XmyoD* expression, which was lost upon mutation of the CdxA binding site motif by the LS-5 mutation. To study this possibility further, mRNAs encoding wild-type Xcad3 (a member of the caudal protein family in *Xenopus*), a VP16 dominate active version of Xcad3 and an EnR dominate negative version of Xcad3 were injected unilaterally into the lateral marginal zone at the 2-cell stage and the effect on endogenous *XmyoD* expression

was analyzed by mRNA *in situ* hybridization. It would be expected that if Xcad3 does directly activate *XmyoD* expression, the dominant active Xcad3 variant would not negatively interfere, and perhaps hyperactivate *XmyoD* expression, while the dominant negative variant would inhibit *XmyoD* transcription. However, with all these versions of Xcad3, *XmyoD* expression was repressed (Figure 4. 6 and Table 4. 4).

There are some reports that if a protein is expressed at different stages, that is, before MBT or after MBT, that protein may possess different functions (Hoppler et al., 1996). To test whether or not Xcad3 played a different role after MBT, Xcad3 and Xcad3-EnR plasmids were injected to achieve expression starting after MBT. Even under these circumstances, Xcad3 and Xcad3-EnR repressed *XmyoD* expression (Figure 4. 7 and Table 4. 4). These results imply that the observed interference was indirect. For this reason the possible regulation of *XmoyD* expression by Xcad-3 was not studied further.

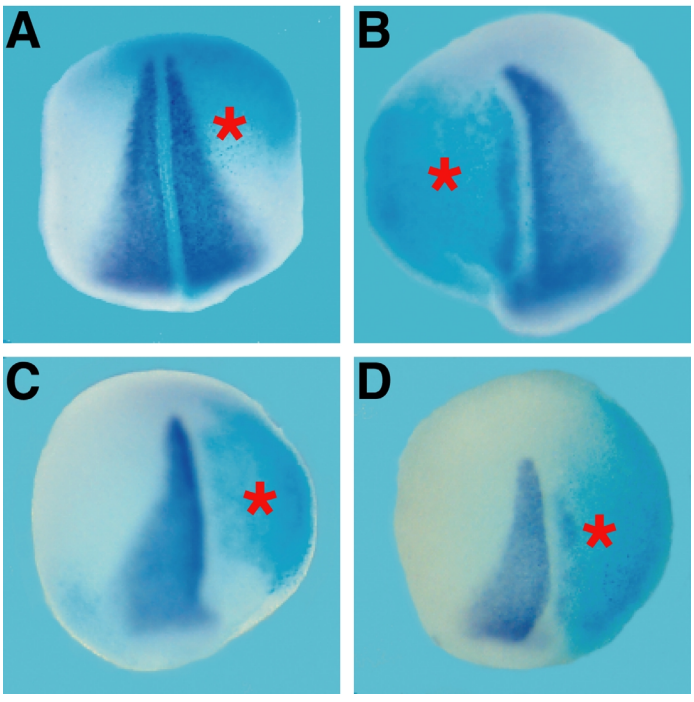

Figure 4.6 Xcad3 affects endogenous *XmyoD* expression when it is expressed before MBT by mRNA injection. 100 pg Xcad3, Xcad3-EnR, Xcad3-VP16 mRNA were injected in LMZ unilaterally at 2-cell stage. *XmyoD* gene expression was analyzed at stages NF 12.5 by whole-mount RNA *in situ* hybridization. All three variants of Xcad3 repressed *XmyoD* expression (B-D). As a control, injection of *LacZ* mRNA alone did not change the expression of *XmyoD* (A). All the embryos are viewed from the dorsal side with anterior facing up. *LacZ* staining (blue) indicates the injected side (also marked by red star).

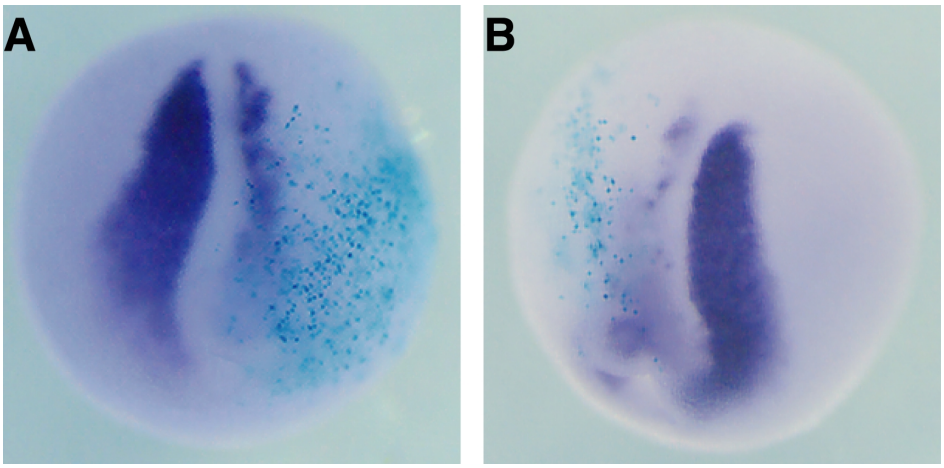

Figure 4.7 Xcad3 affects endogenous *XmyoD* transcription when it is expressed after MBT by plasmid injection. 50 pg pCS2Xcad3 (A), pCS2Xcad3-EnR (B) plasmids DNA were injected in LMZ unilaterally at 2-cell stage. *XmyoD* gene expression was analyzed at stages NF 12.5 by whole-mount RNA *in situ* hybridization. Both Xcad3 variants repressed *XmyoD* expression (A, B). All the embryos are viewed from the dorsal side with anterior facing up. Staining for coinjected lacZ mRNA (blue) indicates injected region.

Table 4. 4 Xcad3 effects on *XmyoD* expression

Injection	Normal or almost normal	Weaker and/or more restricted	Stronger and/or expanded	n
Xcad3-WT mRNA 100pg	2%	98%		58
Xcad3-VP16 mRNA 100pg	4%	96%		54
Xcad3-EnR mRNA 100pg	2%	98%		43
LacZ mRNA 500pg	97%	3%		61
PCS2+ Xcad3-WT plasmid 100pg	9%	91%		32
PCS2+ Xcad3-EnR plasmid 100pg	17%	74	9%	23

4.1.6 A serum response element (SRE) is essential to maintain *XmyoD* transcription

An SRE box and an E-box were found in -1770/-1586bp region of *XmyoD* regulatory region. I hypothesize that SRF may bind with this SRE box and regulate the expression of

XmyoD. Serum response factor (SRF) is a transcription factor, which binds to a serum response element (SRE) associated with a variety of genes including immediate early genes such as *c-fos*, *fosB*, junB, *egr-1* and *-2*, neuronal genes such as *nurr1* and *nur77* and muscle genes such as *actins* and *myosins* (reviewed in Chai and Tarnawski, 2002). SRF controls cell growth and differentiation, as well as muscle development and function. Its biological function is best elucidated for myocardium. Specific cardiac SRF transgenesis demonstrated that overexpression of SRF caused hypertrophic cardiomyopathy in mouse and the mouse died of heart failure within 6 months after birth (Zhang et al., 2001). Other transgenic data suggested that SRF was needed for embryogenesis and early development (Arsenian et al., 1998). Since SRF is an important regulator of numerous genes involved in cell growth and differentiation, including muscle components, SRF may also play a crucial role in the regulation of *XmyoD*.

In order to test this hypothesis, I made the mutation in the SRE box and performed a transgenic reporter assay and a loss-of-function assay of SRF by expression of XSRF-EnR, a dominant negative version of XSRF.

The consensus sequence of an SRE box is $CC(A/T)_6GG$ (Chai and Tarnawski, 2002). To test the function of this motif, I made two kinds of mutations in the context of pMD-3200/+4829GFP: a neutral mutation (AA-TA, Figure 4.8), which does not interfere with SRF-binding, and several mutations that abolish specific binding by SRF. These loss-of-function mutations were generated such that the flanking CC or GG motif of the consensus binding site were altered (Figure 4.8). At stage NF10.5, 81% (n=64) of transgenic embryos carrying the CC-CA neutral mutation of the SRF binding site showed the normal expression of GFP gene and 78% (n=83) of embryos still showed expression of GFP at tailbud stages. This indicated that almost all of the embryos maintained expression of GFP gene throughout development. At stage NF10.5, 77% (n=61) of embryos containing the CC-CA mutation expressed GFP mRNA normally, but only 45% (n=42) of the embryos showed expression at tailbud stages (Table 4.5). This showed that the expression of GFP could not be maintained in 32% of the embryos of CC-CA mutation. At stage NF10.5, 75% (n=60) of the GG-TT mutation containing transgenic

embryos showed normal expression, but only 47% (n=97) of the embryos expressed the reporter gene at tailbud stages. This showed that expression of the GFP gene could not be maintained in 28% of the embryos containing the GG-TT mutation transgene.

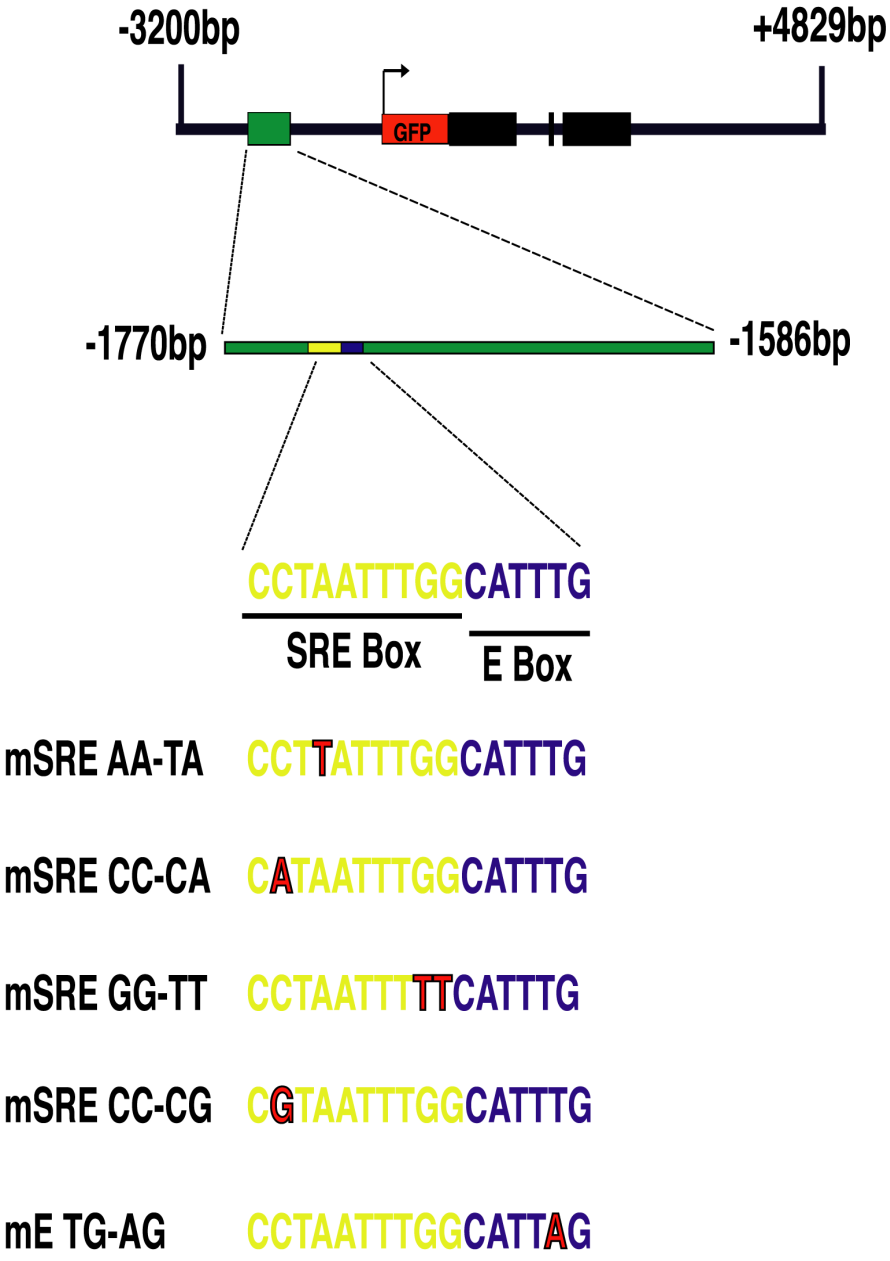

Figure 4.8 Schematic representation of the SRE-mutation constructs. The black bars represent the *XmyoD* genomic DNA, the thicker black bar represent the exons, and the red bar represents the *GFP* gene. The arrow indicates the transcription start site, the green box represent the –1770/-1586 fragment. The extent of the 5' and 3' sequences from the start of transcription are shown. The SRE box is shown in yellow, E box is shown in blue. The mutations are shown in red. The names of the mutation constructs are listed on the left side of the sequences.

There are reports that SRE may cooperate with an E-box (Catala et al., 1995), so I also mutated the adjacent E-box (Figure 4.8). The result showed that this E box is neither necessary for activation nor for maintenance of expression (Table 4. 5). Therefore, it is concluded that this SRE contributes to the maintenance of *XmyoD* expression. When it is mutated, about 30% of embryos lost the maintenance of the expression of the reporter gene, while the adjacent E-box is dispensable. Both *XmyoD* mRNA and protein have short half-lives (about 30-60min. Thayer et al., 1989). Considering that the GFP mRNA is quite stable, and therefore disappears only slowly from the embryos, which initiated correctly reporter gene transcription at the gastrula stage, the real impact of the SRF-site on maintenance of *XmyoD* transcription may be underestimated based on these statistics. Interestingly, the SRF-box mutant CC-CG, showed a much stronger reduction of the reporter gene expression at tailbud stages (Table 4.5). This may be due to the production of a CG-dinucleotide, which could be a site of DNA methylation. It also implies that methylation may play a role in the regulation of *XmyoD*.

Table 4. 5 SRE site is necessary to maintain the expression of the GFP

Transgene Mutation	Expression at NF10.5		Expression at NF 28	
	Percentage	n	Percentage	N
mSRE AA-TA	81%	64	78%	83
mSRE CC-CA	77%	61	45%	42
mSRE GG-TT	75%	60	47%	97
mSRF CC-CG	89%	36	22%	27
mE	70%	43	80%	25

4.1.7 Effects of SRF-interference analysis on *XmyoD* expression

To complement the SRE-mutation analysis described above, I performed loss-of-function assays by injecting RNA coding for a dominant-negative XSRF protein variant, i.e., XSRF-EnR. At the 2-cell stage, 100pg XSRF-EnR mRNA or wild type XSRF mRNA was injected into the lateral marginal zone (LMZ). The embryos were fixed at stage NF12 and *XmyoD* expression was analysed by *in situ* hybridization. If SRF protein would bind to XmyoD's maintenance enhancer, i.e., the SRE, injection of SRF-EnR mRNA should reduce XmyoD expression, preferentially at late developmental stages, or perhaps interfere with its transcriptional induction at the gastrula stage. In contrast,

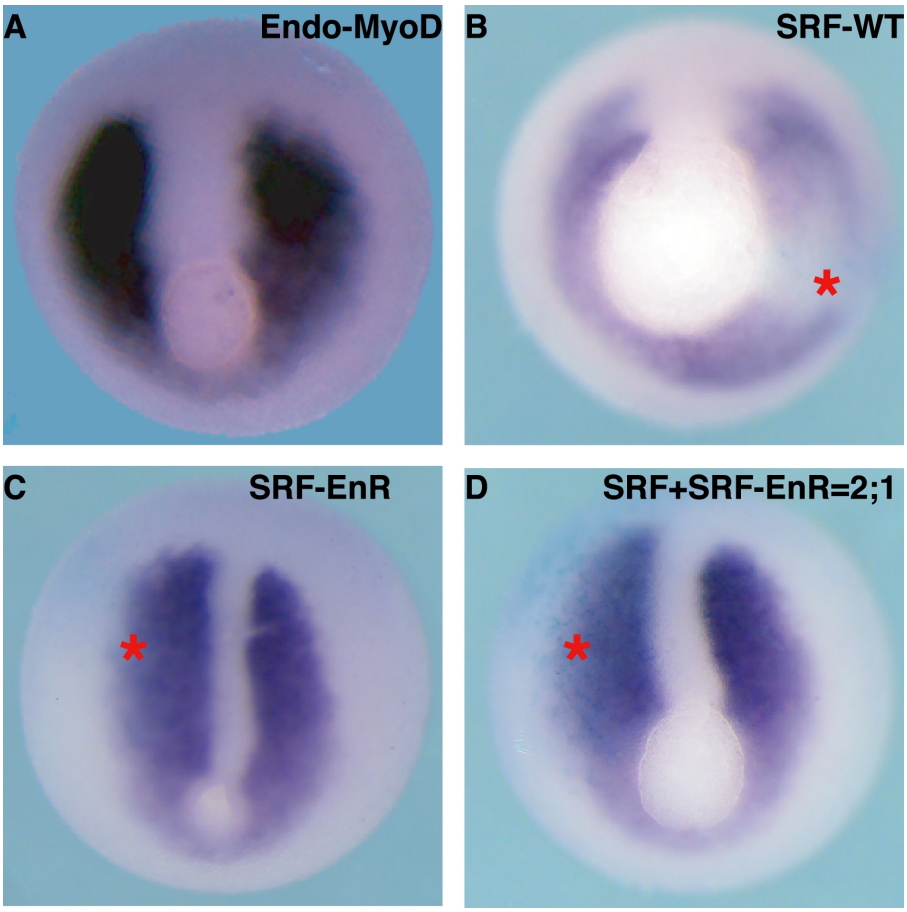

Figure 4.9 XSRF and/or XSRF-EnR mRNA were co-injected with lacZ mRNA in LMZ unilaterally at 2-cell stage. *XmyoD* gene expression was analyzed at stage NF 12 by whole-mount RNA *in situ* hybridization. A) The expression pattern of endogenous *XmyoD* at NF12. B) The overexpression of WT-XSRF had two effects on *XmyoD* expression: the expression was repressed in the blue field where the mRNA was injected, but the expression of *XmyoD* was stronger and expanded adjacent the blue field. C) XSRF-EnR enlarged the expression domain of *XmyoD*. D) The enlarged expression of *XmyoD* could not be repressed by injection of 2 times of XSRF mRNA. The blue staining marks the injection site (red star). The embryos are viewed from vegetal pole with anterior facing up.

Table 4. 6 XSRF effects on *XmyoD* expression

mRNA Injection	Normal or almost Normal	Weaker and/or more Restricted	Stronger and/or Expanded	n
XSRF-WT 200pg	14%	86%		28
XSRF-EnR 200pg	11%	2%	87%	53
XSRF-WT 100pg XSRF-EnR 200pg			100%	16

overexpression of wild type SRF protein alone should have no effect on XmyoD mRNA levels. Coinjection of SRF-EnR with wt SRF should rescue XmyoD expression. Surprisingly, I found that SRF-EnR enlarged the XmyoD expression domain to the neurula stage (Figure 4.9C). Wild type SRF protein, however, inhibited XmyoD expression in the injected region and at the time caused expansion of the XmyoD domain directly adjacent to the injection site (Figure 4.9B). Furthermore, coinjection of mRNA for wild type SRF and SRF-EnR at a 2:1 molar ratio failed to revert the SRF-EnR phenotype of a broadened XmyoD domain (Figure 4.9D). SRF protein is maternally expressed in the *Xenopus* egg.

In line with the mouse SRF (-/-) phenotype, which indicates a very early function for SRF in mesoderm formation, we suspected that the above effects on XmyoD expression, which were achieved by mRNA injection, could be the result of interference with maternal SRF functions, rather than later functions during mesodermal patterning. In order to circumvent these problems, I expressed SRF-EnR by transgenesis after the MBT.

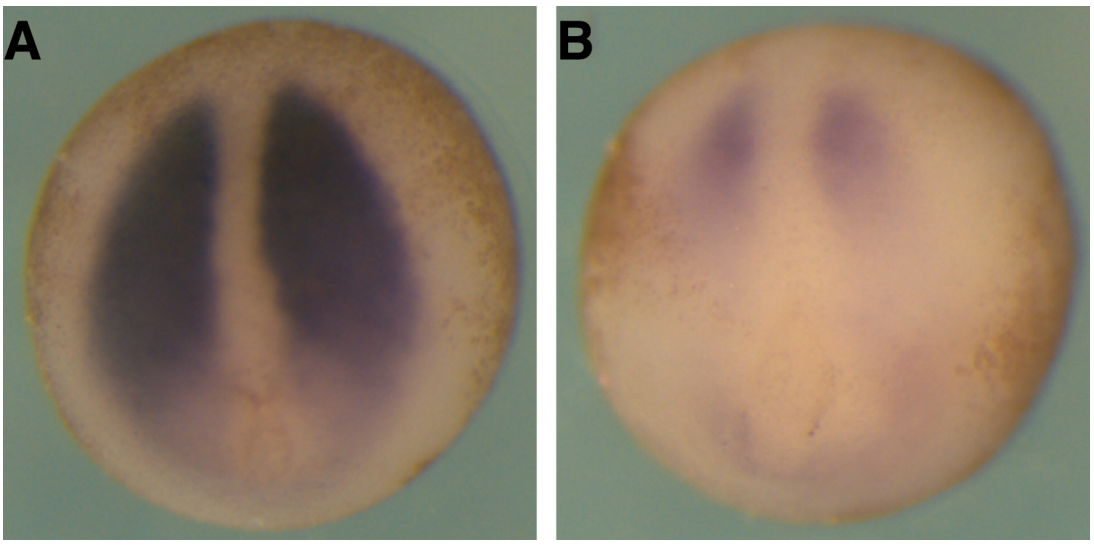

Figure 4.10 Transgenic embryos were produced and *XmyoD* expression was analyzed at stages NF 12.5 by whole-mount RNA *in situ* hybridization. A: The embryo without a transgene of XSRF-EnR showed normal *XmyoD* expression. B: The expression of *XmyoD* was repressed in XSRF-EnR transgenic embryo. All the embryos are viewed from the dorsal side with anterior facing up.

Table 4.7. XSRF-EnR repress the expression of *XmyoD*

Marker	Transgenesis	Fluorescence	Normal/near normal	Weaker	n
XmyoD	PCS2+GFP + PCS2+XSRF-EnR	-	100%		15
		+	36%	64%	56

I generated double-transgenic embryos with pCS2+GFP and pCS2+XSRF-EnR plasmids. In this experiment, GFP-expression identifies REMI-embryos, which integrated and express the transgenes. Independent studies in our and other labs has demonstrated co-expression of marker genes in double-transgenic embryos to occur at a frequency ≥ 96% (Otto and Rupp, unpublished result; Hamilton et al., 2001). The embryos were fixed at stage NF12.5, sorted into two groups: GFP positive (GFP+) and GFP negative (GFP-) and subjected to RNA *in situ* hybridization for endogenous *XmyoD* mRNA. In the non-transgenic embryos (GFP-), the expression of *XmyoD* was normal (Figure 4.10A and Table 4. 7). In contrast, the GFP+ embryos, which express SRF-EnR, showed a much reduced *XmyoD* expression (Figure 4.10B and Table 4.7). This indicated that XSRF-EnR largely down-regulated the expression of *XmyoD*. The expression pattern of *XmyoD* in these embryos (Figure 4.10B) was very interesting. The most anterior and posterior domains showed more *XmyoD* mRNA than the region in between. While the reason for this graded response of XmyoD transcription to constitutively expressed SRF-EnR is unclear, it clearly shows that SRF-EnR does not interfere with the induction of XmyoD transcription, but inhibits the maintenance of *XmyoD* after the induction. This observation supports our hypothesis that XSRF binds with the SRE box and maintains the expression of *XmyoD*, so the dominant-negative XSRF, XSRF-EnR, also binds with the same box and represses the maintenance of *XmyoD* expression.

4.1.8 Analysis of TCF binding site and FAST binding site

Wnts and Nodals are important mesoderm induction factors. In order to test the role of these factors in XmyoD transcription, mutation constructs were generated that contained mutations in either the TCF or FAST binding sites in the -1.8/-1.6kb region. TCF is a transcription factor of Wnt signaling (Molenaar et al., 1996). FAST is a transcription factor of Nodal signaling (Hill, 2001). A second TCF site (300 bp upstream to the transcription start site of *XmyoD*) was also mutated. No significant change in expression level of the reporter gene was observed in any of these mutation constructs (Table 4. 8).

Table 4. 8 Expression of mutation constructs at stage NF10.5

Construct	Correct expression	Incorrect expression	No expression	n
mTCF in -1.8/-1.6	61%	7%	32%	122
mFAST in -1.8/-1.6	69%	7%	24%	114
mTCF (–300bp)	65%	8%	28%	65

4.1.9 Non-coding RNA transcripted in 5' region of *XmyoD* genomic sequence

Randomly integrated transgenes receive input from the genomic environment of their integration site, leading to partial ectopic expression. The observed very high frequency of correct transcriptional regulation without significant ectopic expression of the pMD-3200/+4829GFP transgene (77%, n=117) is, therefore, quite unusual. In particular, each of the transgenic embryos represents independent integration events (Kroll and Amaya, 1996). This suggests the presence of some epigenetic regulatory elements, which uncouple the transgene from influence of the surrounding DNA, such as locus control region (Engel and Tanimoto, 2000), insulator elements (Bell and Felsenfeld, 1999) or cellular memory modules (Cavalli and Paro, 1998). In many cases, non-coding transcripts have been found to originate from the neighborhood of these elements, and these non-coding RNAs may be involved in the function of these elements (Rank et al., 2002). Given the exceptional reliability of correct spatiotemporal regulation of the full length *XmyoD* transgene, we decided to search for genomic transcripts in the vicinity of the

XmyoD transcriptional unit by RNA *in situ* hybridization. For this purpose, I generated a series of plasmid templates from the upstream region of the *XmyoD* gene, which were used to generate both sense and antisense RNA specific *in situ* hybridization probes. An overview of these constructs is shown in Figure 4.11.

In the first round of the experiment, I used the sense and anti-sense probes of XLC14, XLC15, XLC04, and XLC09. Surprisingly, both sense and anti-sense of XLC04 detected some transcripts (Figure 4. 12, Figure 4. 13). In contrast, none of the other probes (XLC14, XLC15 and XLC09) detected any transcript. I further narrowed down the transcript by splitting the XLC04 fragment into three shorter fragments: XLC16, XLC17 and XLC18. In that series of RNA in situ hybridizations, only XLC18 detected the same expression patterns of transcripts as XLC04 did. This result suggests that the XLC18 fragment in the upstream region of *XmyoD* produce both sense and anti-sense transcripts, but the flanking regions do not produce transcript.

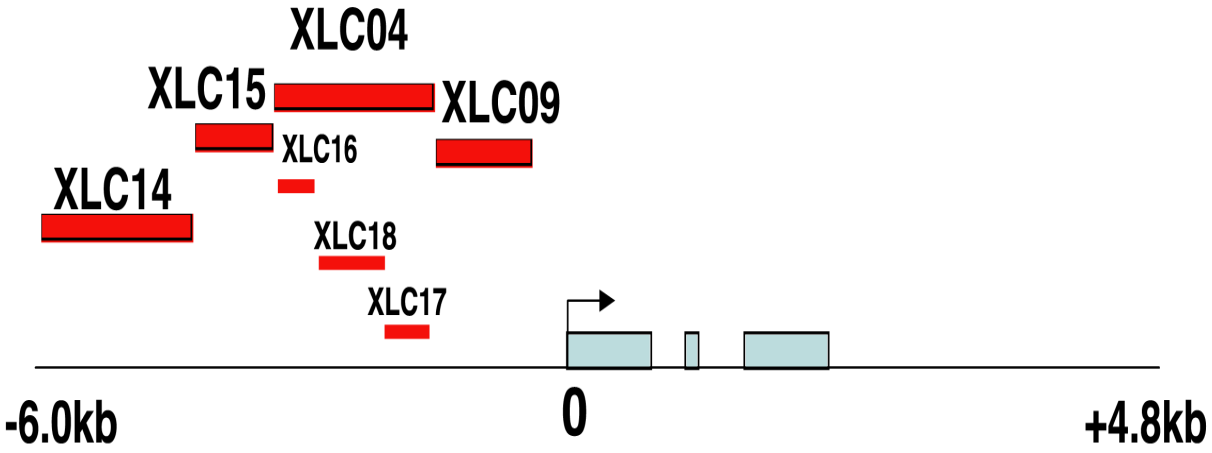

Figure 4.11 Schematic representation of dig-labeled probes. The blue bars represent the *XmyoD* exon, the red bars represent the DNA fragment to synthesize dig-labeled probes for *in situ* hybridization and the arrow indicates the transcription start site. For each fragment I synthesized sense and anti-sense probes. The regions of the fragments are: XLC04: -3.2/-1.5kb; XLC09: -1.5/-0.4kb; XLC14: -5.7/-3.9kb; XLC15: -3.9/-3.2kb; XLC17: -2.0/-1.5kb; XLC18: -2.8/-2.0kb; XLC16: -3.2/-2.8kb.

The expression pattern of the non-coding RNA detected by anti-sense probe is shown in Figure 4.12. The anti-sense probe detected a maternally expressed RNA in the animal pole region. After the zygotic expression starts, the signal localizes to the nucleus. The RNA can be seen in the nucleus of every cell in the embryo at stage NF10. This RNA

represents the recent onset of zygotic transcription of these units. At stage NF11 the RNA is dispersed or degraded. At stage NF14 there is no clear expression pattern. At stage NF18 the anterior part of the embryo shows stronger staining than any other part in the body. After stage NF 28 strong staining can be seen in the epidermis and in the cells between the myotomes.

Figure 4.13 shows the expression pattern of the non-coding RNA detected by the sense probe. Before MBT, the sense probe detected a maternal expressed RNA in the vegetal cortex. After MBT, the zygotic expression starts and the staining signal localizes in the nucleus, similar the transcript detected by anti-sense probe. At stage NF10, the RNA can be seen in the nucleus of all the cells. At stage NF11, the RNA is dispersed or degraded. At NF14, no clear expression pattern can be seen. At stage NF18, the anterior part of the embryo shows stronger staining than the other parts of the embryo. At stage NF26, a very strong staining can be seen in epithelia. After stage NF 28, strong staining can be seen in the cells between the myotomes and the staining also can be seen in epidermis.

Both sense and anti-sense transcripts have the same expression pattern at stage NF10 and disappear at stage NF11. This may reflect hybridization of the two transcripts at NF10 and formation of double strand RNA (dsRNA). The dsRNA may be broken into small pieces by RNAi (RNA interference) machinery (Jenuwein, 2002). The RNA probes used in these *in situ* hybridization experiments only recognize single stranded RNA (ssRNA), but not the dsRNA. So what I detected are the expression patterns of transcripts existing as ssRNA.

4.1.10 The transcripts are produced by a repetitive sequence

A DNA repetitive sequence in XLC04 and XLC18 was identified through a BLAST search. It showed high similarity with *Xenopus* genomic sequence named *Xenopus leavis* short interspersed repeat transcripts (Xlsirt) reported by Spohr et al. (1984). They are 93% identical (Figure 4.14) and contain eight copies of a tandemly repeated unit of 79-81 nucleotides.

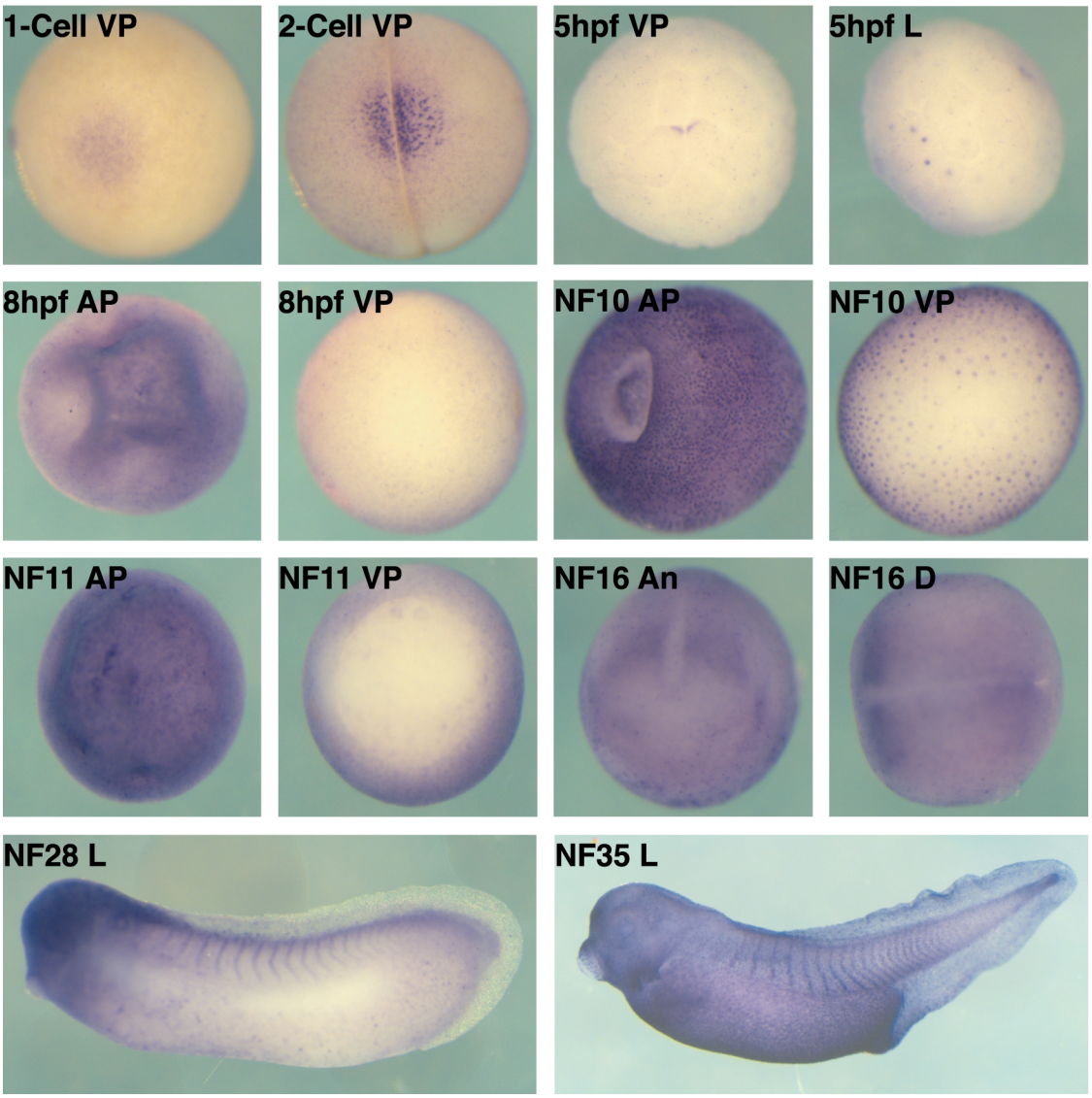

Figure 4. 12. The expression pattern of the repeats detected by XLC04 anti-sense probe. The stages of the embryos are indicated in the top left of the picture. VP=vegetal pole view; L=lateral view; AP=animal pole view; An=Anterior view; D=dorsal view.

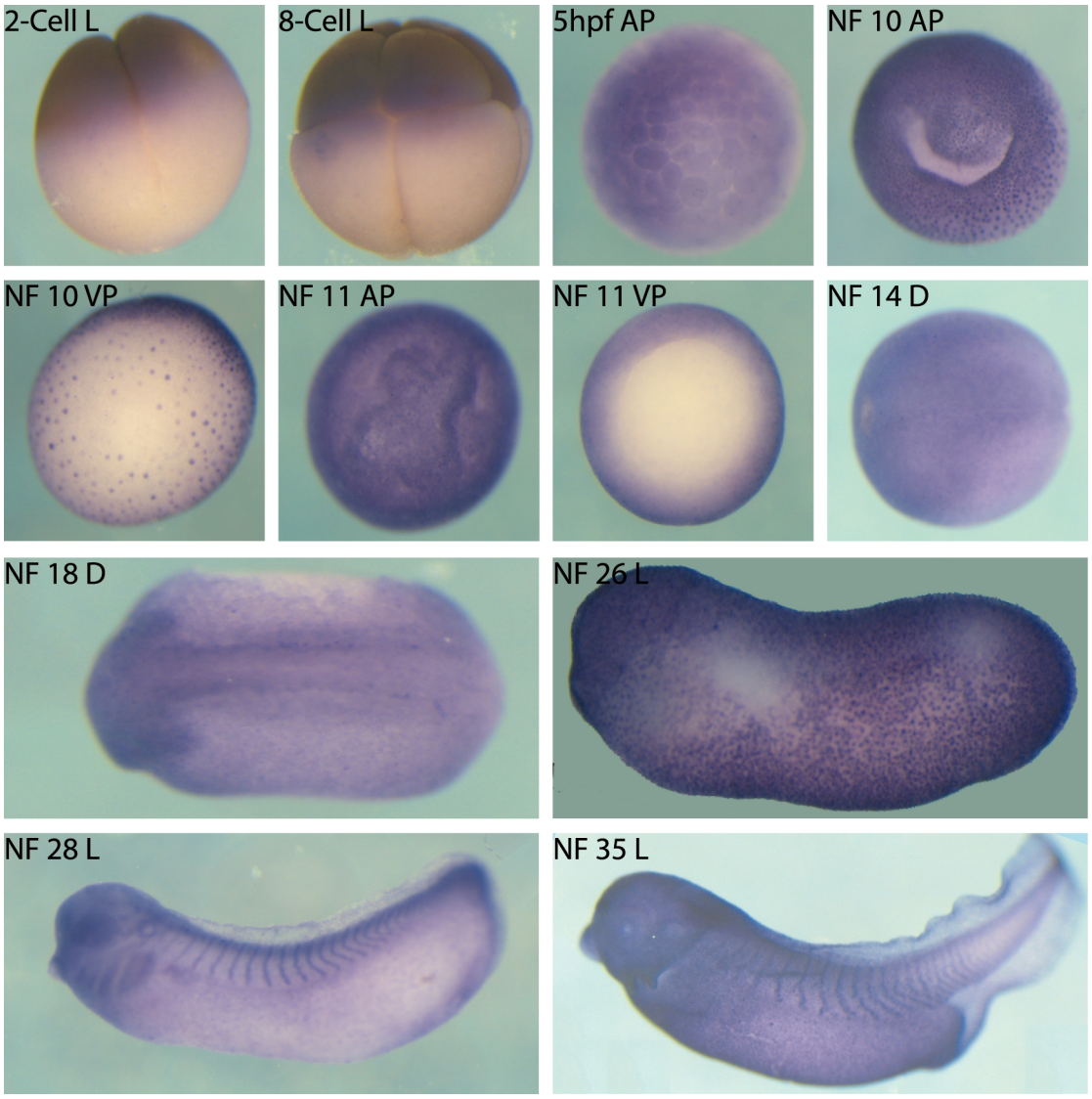

Figure 4.13 The expression pattern of the repeats detected by XLC04 sense probe. The stages of the embryos are indicated in the top left of the picture. VP=vegetal pole view; L=lateral view; AP=animal pole view; An=Anterior view; D=dorsal view.

The DNA repetitive sequence in the *XmyoD* locus was given the name MyoD's Xlsirt (M-sirt). M-sirt might produce sense and anti-sense transcripts (Figure 4.12, 4.13). The flanking regions of Xlsirt and M-sirt are unique and do not produce transcript.

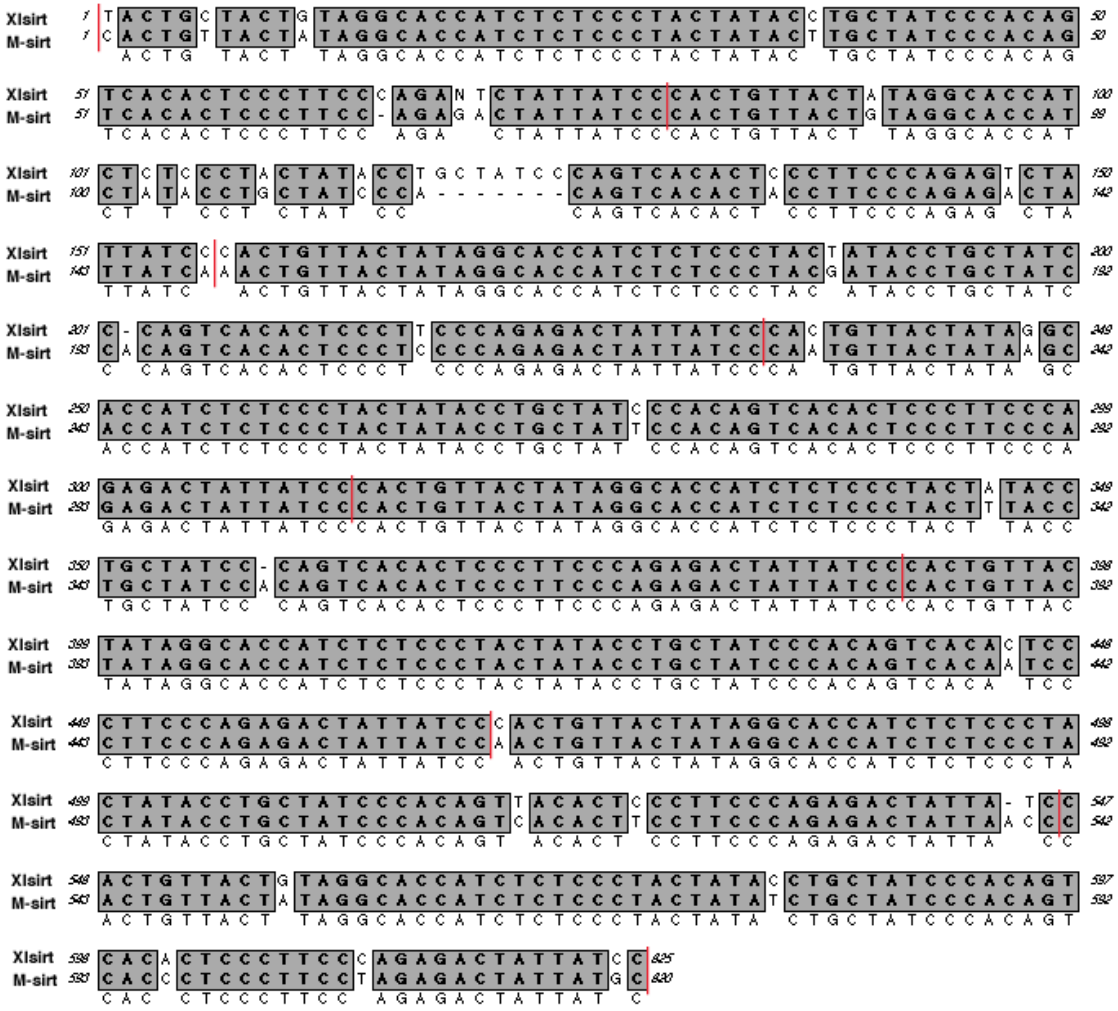

Figure 4.14. Sequence alignment of Xlsirt and M-sirt (ClustalW software). Dark shading: identical nucleotides. The red vertical lines indicate the boundary of the repeated units.

4.2 Analysis of potential regulators of *XmyoD* expression

4.2.1 XSEB-4

RNA binding proteins play key roles in the regulation of gene expression (reviewed in Burd and Dreyfuss, 1994). XSEB-4 codes for a putative RNA binding protein containing a single RNA recognition motif (RRM) (Fetka et. al, 2000) and has been identified as a

direct target gene of XmyoD protein (Jasper, 1998). XSEB-4 shares 60-65% identity with the mammalian SEB-4 proteins. XSEB-4 is strongly expressed maternally. Zygotic transcription is initiated in the early gastrula embryo in paraxial mesoderm that is fated to give rise to somites. During the course of gastrulation and neurulation, XSEB-4 expression in somitic paraxial mesoderm is centered within the *XmyoD* expression domain. As development proceeds, XSEB-4 expression is in addition initiated in the cardiac primordium and the lens vesicle. In the heart, expression is confined to the myocardium (Fetka et. al, 2000). Thus, the RRM-containing putative RNA binding protein XSEB-4 is a good candidate to regulate *XmyoD* during embryonic development in *Xenopus*.

4.2.1.1 The subcellular localization of XSEB-4

To determine the subcellular localization of XSEB-4, I injected mRNA of different version of myc-tagged XSEB-4 at the 4-cell stage in the animal pole. At stage NF9, the embryos were fixed in 1X MEMFA to perform immunocytochemistry (ICC) or collected for western blotting to determine the protein level. ICC and western blotting were performed with anti-myc antibody.

XmyoD (RR 107 is the plasmid containing *XmyoD* gene) is used as a control, it mainly localized in the nucleus in lateral marginal zone (Rupp et al., 1994), and slight cytoplasm expression also could be observed. This is also true for mLong (long version of mouse SEB-4), mShort (short version of mouse SEB-4), XSEB-4WT (wild type of *Xenopus* SEB-4), and XSEB-4ΔC (C terminal truncated version of *Xenopus* SEB-4). XSEB-4ΔN mainly localized to cytoplasm (Figure 4.15). This suggests the presence of a NLS (Nuclear Localization Signal) in the N-terminal region. I also observed that XSEB-4ΔN is around 10 times more unstable than XSEB-4WT or XSEB-4ΔC (Figure 4. 16), perhaps the result of its altered cellular localization.

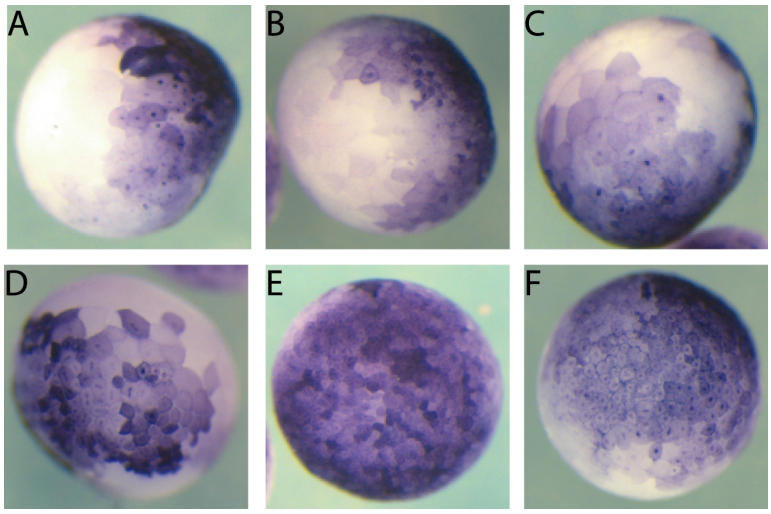

Figure 4.15 The subcellular localization of XSEB-4.
To determine the subcellular localization of XSEB-4, 150pg mRNA of different version of myc-tagged SEB-4 were injected in animal pole at 4-cell stage. At NF9, the embryos were fixed in 1X MEMFA, ICC was performed with anti-myc antibody. A: XmyoD mainly localized in the nucleus in lateral marginal zone, while slight cytoplasm expression also could be observed. This is also true for mLong (long version of mouse SEB-4) (B), mShort (short version of mouse SEB-4) (C), XSEB-4WT (wild type of *Xenopus* SEB-4) (D), and XSEB-4ΔC (C terminal truncated version of *Xenopus* SEB-4) (F). XSEB-4ΔN mainly localized in cytoplasm (E). Abbreviations: mLong: long version of mouse SEB-4; mShort: short version of mouse SEB-4; XSEB-4WT: wild type of *Xenopus* SEB-4; XSEB-4ΔC: C terminal truncated version of *Xenopus* SEB-4; XSEB-4ΔN: N terminal truncated version of *Xenopus* SEB-4.

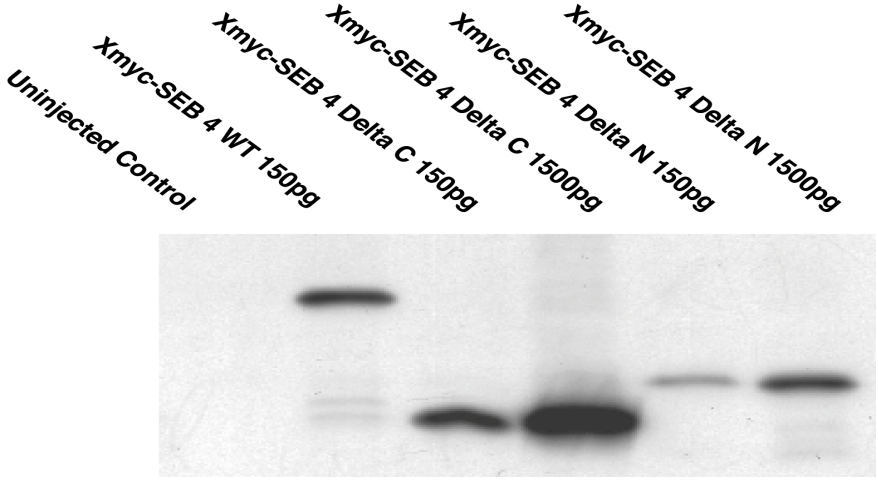

Figure 4.16 150 and/or 1500 pg mRNA of different version of myc-tagged XSEB-4 were injected in animal pole at 4-cell stage. The embryos were collected for western blotting to determine the protein level at stage NF9.

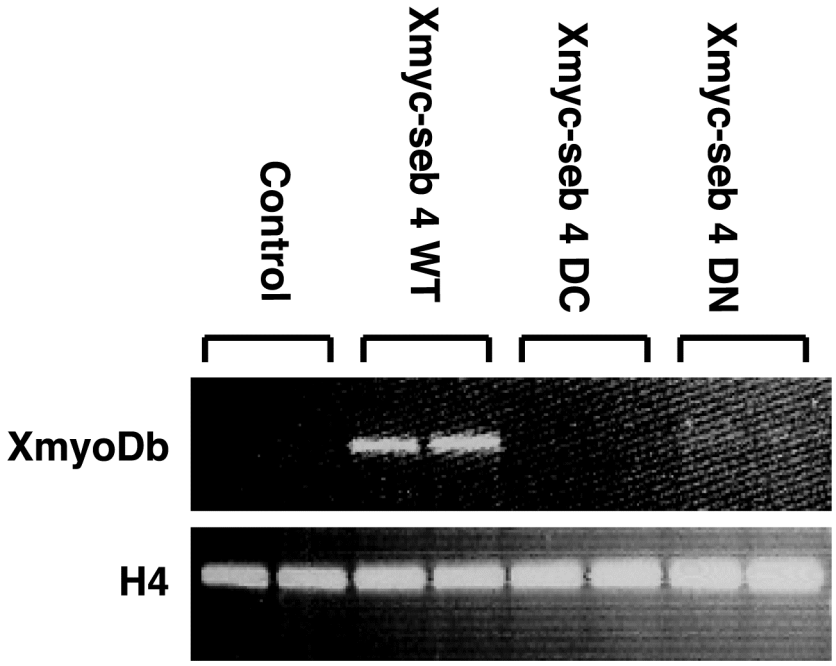

Figure 4.17 150 pg different version of XSEB-4 mRNA were injected in the animal pole at 4-cell stage, and the ACs were explanted at NF 9 and lysed by Trizol (GibcoBRL) at NF 18. The expression of *XmyoD* was assayed by RT-PCR. The ACs were separated into 2 groups for every injection. Only the injection of wild type of XSEB-4 mRNA resulted in the induction of *XmyoD*. DC=Delta C; DN=Delta N.

4.2.1.2 XSEB-4 induces *XmyoD* expression in animal cap assay

I further checked the ability of XSEB-4 to induce *XmyoD* expression in the animal caps (ACs). Different version of XSEB-4 mRNA were injected in the animal pole at the 4-cell stage, and the ACs were explanted at stage 9. ACs were lysed by Trizol (GibcoBRL) at stage NF 18. The expression of *XmyoD* was assayed by RT-PCR. There was no *XmyoD* expression in control Acs. Wild type XSEB-4 did induce the expression of *XmyoD*, but XSEB-4ΔN and XSEB-4ΔC could not induce *XmyoD* (Figure 4. 17). This indicated that XSEB-4 is sufficient to induce the expression in ACs, and both the N- and C- terminal domains of XSEB-4 are necessary for the activity of XSEB-4 to induce *XmyoD*.

4.2.2 YY1

The transcription factor Yin Yang 1 [YY1 (also known as, NF-E1, UCRBP, and CF1)] is a 65 kDa member of the GLI-krueppel family of zinc finger transcription factors and is a homolog of the Drosophila polycomb group (PcG) protein pleiohomeotic (Pho). Satijn et al. (2001) reported that YY1 interacts specifically with the human PcG protein EED, while PcG proteins form multimeric protein complexes that are involved in the heritable repression of genes. When the *Xenopus* homolog of YY1 or YY1-EnR are expressed ectopically in *Xenopus* embryos, ectopic neural axes formed, but no mesoderm was induced (Satijn et al., 2001). We found a potential YY1 binding site near the transcription start site of XmyoD. It is possible that YY1 functions with polycomb group proteins to maintain the silence of *XmyoD* in the region where *XmyoD* should not be expressed.

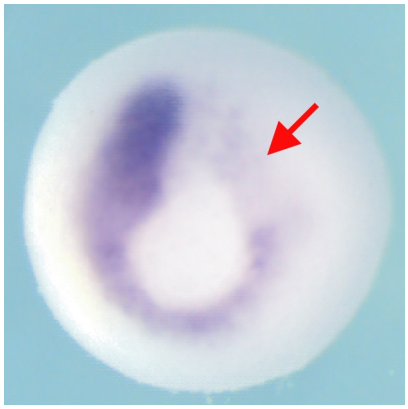

Figure 4. 18 XYY1-EnR represses the expression of *XmyoD*. 100 pg XYY1-EnR mRNA was injected in LMZ unilaterally at 2-cell stage. *XmyoD* gene expression was analyzed at stages NF 12 by whole-mount RNA *in situ* hybridization. The expression of *XmyoD* was repressed in the injected side (Red arrow). The embryo is viewed from the vegetal pole with dorsal side facing up.

In order to determine the function of YY-1 on the expression of *XmyoD*, 100 pg of XYY1-EnR mRNA was injected in LMZ unilaterally at the 2-cell stage. *XmyoD* gene expression was analyzed at stage NF 12 by whole-mount RNA *in situ* hybridization. The expression of *XmyoD* was repressed in the injected side (93%, n=60) (Figure 4.18). Therefore, it can be concluded that YY-1 functions to repress the expression of *XmyoD*.

4.2.3 Lef-1

The canonical Wnt pathway has been studied extensively during the past decade. It has been shown that maternal Wnt signaling activity induces the Spemann organizer (for review see De Robertis et al, 2000). Zygotic Wnt/ß-catenin signaling is essential for the ventro-posterior development and the activation of *XmyoD* (Hoppler et al., 1996). TCF-3 is dispensable for the activation of *XmyoD* (Hamilton et al., 2001). Hamilton et al. (2001) showed that the maternal and zygotic Wnt signaling could employ different transcription factors. It is presumed that the zygotic Wnt signaling functions via Lef-1instead of TCF-3.

Since it has been shown that the protein produced from a transgene is synthesized as early as stage 10 (Kroll and Amaya, 1996), I generated transgenic embryos that expressed either dominant active or dominant negative forms of Lef-1 to interfere with the activity of Lef/Tcf proteins zygotically. I generated pCS2+Lef-1-VP16 and pCS2+GFP double-transgenic embryos. In this case, GFP expression served as an indicator of successful transgenesis (Hamilton et al., 2001). Transgenic embryos with fluorescence were sorted out. The majority of these transgenic embryos failed to undergo epiboly. As a result of this morphogenetic failure, the ectoderm of these embryos shrank and formed a small cap upon a mass of vegetal cells (Table 4. 9, Figure 4.19E). Surviving embryos developed with severe anterior defects and a shortened dorsal axis (Table 4. 9; Figure 4. 19C). When such embryos were assayed at mid-gastrula for marker gene expression, most of them showed ectopic expression of ventral and lateral mesodermal markers (*XmyoD*, *Xpo*; Figure 4. 20E and 20F, compare with control embryos in Figure 4. 20B and 20C), which extended dorsally and occupied the organizer region. However, the expression of the notochord-specific dorsal mesodermal marker, *Xnot* was unchanged (Figure 4. 20D, compare with 20A).

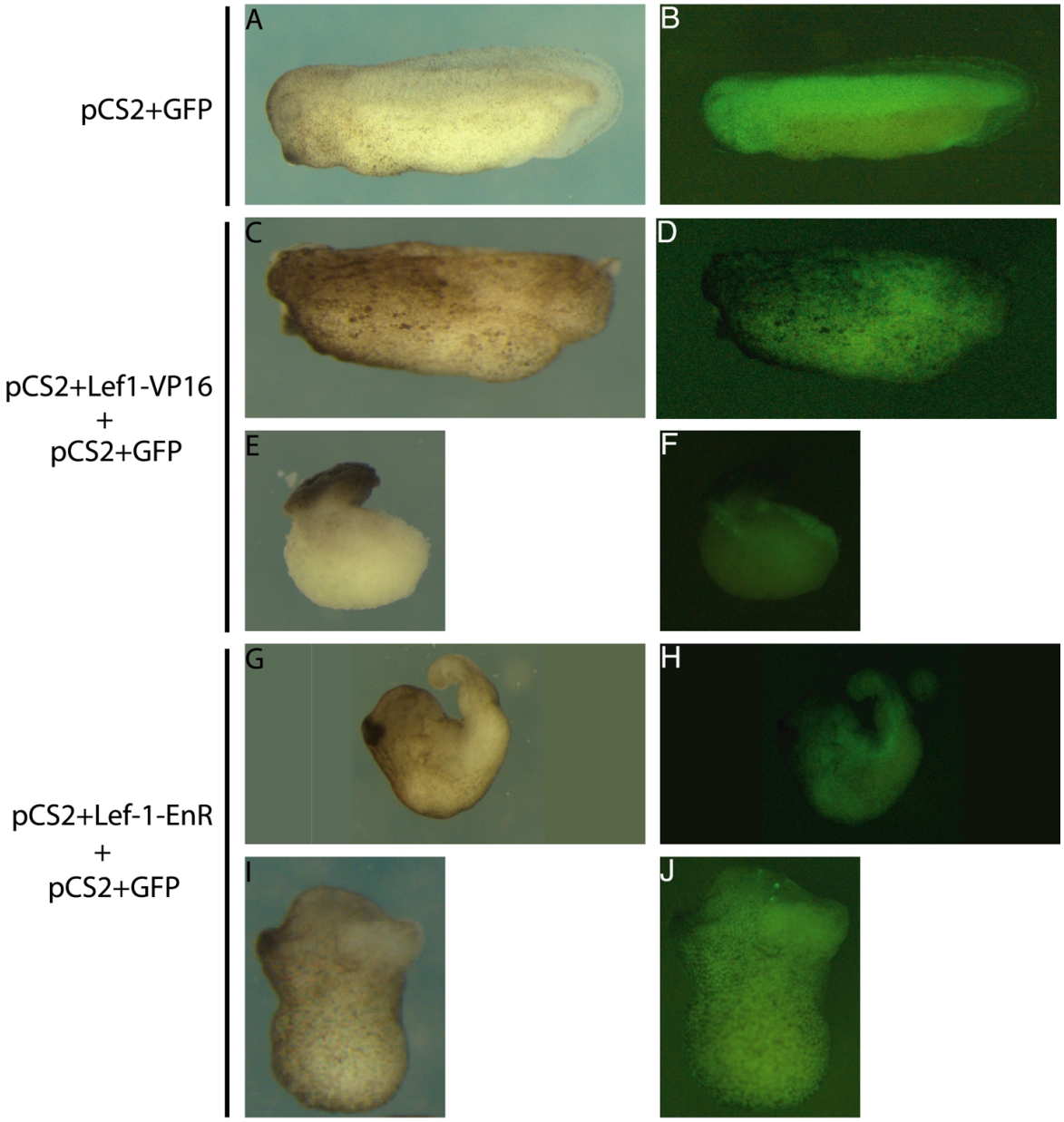

Figure 4.19 Loss- or gain-of Lef-1 activity results in severe developmental defects
C-F: PCS2+GFP and p-Lef-1-VP16 co-transgenic embryos were analyzed by morphology at stage 29. Transgenic embryos either fail to undergo epiboly (E) or have a ventro-posteriorized phenotype (C). D, F: The same embryos as in (C) and (E) are observed under UV light. G-J: pCS2+GFP and p-Lef-1-EnR co-transgenic embryos were analyzed by morphology at stage 29. The trunks and tails are totally absent from transgenic embryos, while the heads develop normally (G, I). H, J, the same embryo as in (G) is observed under UV light. As a control, transgenic embryos with GFP over-expression alone develop into well-organized embryos (A, B). All the embryos are lateral view, with anterior to the left.

Table 4. 9 Lef-1 dependent zygotic Wnt signaling ventro-posteriorizes the embryo

Transgenesis	GFP	Normal and almost Normal	Trunk Defect	No Head	No Tail	Failure in Gastrulation	Other Phenotype	n
pCS2+GFP	-	100%						9
	+	81%	12%					63
Lef-Vp16 and pCS2+GFP	-	100%						8
	+	18%	13%	13%		55%		82
Lef-EnR and pCS2+GFP	-	100%						21
	+	23%	50%	1%	25%		1%	108

In contrast to gain-of Lef-1 activity, Lef-1-EnR transgenic embryos developed severe posterior defects but normal head structure (Table 4.9, Figure 4.19G). In the extreme cases, the tails and trunks of transgenic embryos were completely absent (Figure 4.19I). Loss of Lef-1 activity leads to reduced expression of *XmyoD* and *Xpo* (Table 4. 10 and Figure 4.20H and 20I, compare with 20B and 20C) while *Xnot* expression is unaffected (Table 4. 10 and Figure 4.20G, compare with 20A). These results are strictly dependent on loss- or gain-of Lef-1 activity because expression of GFP alone did not cause this phenotype (Table 4.10 and Figure 4.20A-C). In a recent study, Roel et al. (2002) generated similar anteriorized embryos by using a mopholino to deplete XLef-1. From these results, it is concluded that Lef-1 proteins are not only sufficient but also necessary for the expression of *XmyoD* and ventro-posterior development in *Xenopus* embryos.

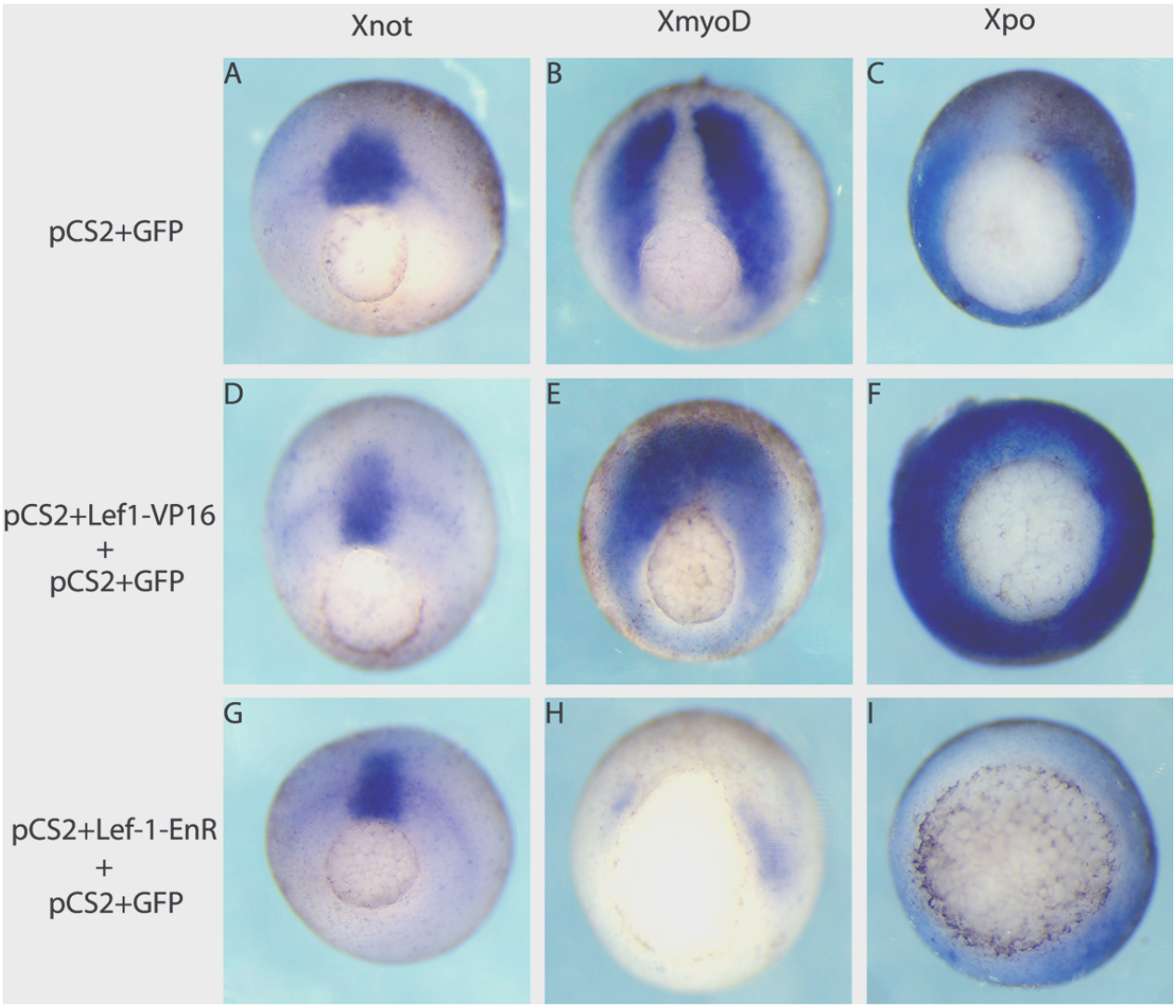

Figure 4. 20 Loss- or gain-of Lef-1 activity results in change of the expression of ventral and lateral markers.
D-F: PCS2+GFP and p-Lef-1-VP16 co-transgenic embryos were analyzed by molecular marker expression at stage 11. Analysis of molecular markers show the ectopic expression of ventral and lateral markers *XmyoD* (E) and *Xpo* (F) in the dorsal midline (compared with B, C) and unchanged the expression of dorsal marker *Xnot* (D, compared with A). G-I: PCS2+GFP and p-Lef-1-EnR co-transgenic embryos were analyzed by molecular marker expression at stage 11, the expression of *Xnot* is comparable with the control expression (G, A). The reduction of ventro-lateral gene expression of *XmyoD* and *Xpo* is consistent with the reduced ventro-posterior development (H, I compared with B, C). As a control, transgenic embryos with GFP over-expression alone show normal expression patterns of indicated molecular makers (A-C). All are vegetal view, with dorsal up.

Table 4. 10. Xlef-1 Interferes with ventral and lateral mesoderm patterning

Marker	Transgenesis	Weaker and/or more restricted	Normal or near normal	Stronger and/or expanded	n
XmyoD	PCS2+GFP	10%	90%		30
	Lef-1-VP16		21%	79%	19
	Lef-1-EnR	76%	24%		33
Xpo	PCS2+GFP	7%	83%	10%	29
	Lef-1-VP16		18%	82%	22
	Lef-1-EnR		46%	54%	35
Xnot	PCS2+GFP	22%	78%		23
	Lef-1-VP16	20%	80%		20
	Lef-1-EnR	18%	82%		33

5 Discussion

In *Xenopus*, the activation of the myogenic determination factor XmyoD in the muscle-forming region of the embryo occurs in response to mesoderm-inducing factors (MIFs). Different members of the FGF, TGF-beta, and Wnt protein families have been implicated in this process (Steinbach et al., 1998), but how MIFs induce the expression of *XmyoD* is not known. The expression of *XmyoD* is thought to have three stages: ubiquitous low-level transcription at the MBT; full activation at mid-gastrula stage in myogenic precursors; maintenance after full activation in myogenic precursors. The expression of *XmyoD* at these three stages may involve different mechanisms, which are still unknown. The *cis*-regulatory elements of the *XmyoD* gene are the intermediary between the mesoderm-inducing factors and transcription of the *XmyoD* gene. In order to understand the regulatory mechanisms of *XmyoD*, the *cis*-regulatory elements of the *XmyoD* gene were characterized by a transgenic reporter assay. Several *cis*-elements were characterized, including one induction enhancer motif, one silencer motif and one maintenance enhancer motif. Several potential protein factors, which may regulate the expression of *XmyoD*, were also analyzed in this study.

5.1 Methodological considerations

Transgenic reporter analysis by REMI (Restriction Enzyme Mediate Intergration) in *Xenopus* provides a good method to search for *cis*-regulatory elements. Unlike embryos injected with plasmids, transgenic embryos show the correct spatial and temporal regulation of the integrated promoter constructs (Lerchner et al., 2000, Polli and Amaya, 2002, Yang et al., 2002 and Figure 2.6B). One of the great advantages of this system over transgenesis in mice or zebrafish is that the transgene is integrated into the male genome prior to fertilization, therefore the resulting embryos are not chimeric and breeding of animals is not required. This technique permits large scale transgenesis in *Xenopus*. When this method is used to study the regulation of promoters of genes, the regulatory region of "the gene of interest" is ligated to a reporter gene like GFP, which is nontoxic, and does not interfere with the development of the embryo. The expression of

the reporter gene is co-regulated with the endogenous gene during the normal development of the embryos. The expression of GFP can be analyzed by either GFP microscopy or *in situ* hybridization. When the results of transgenic embryos harboring different presumptive *cis*-regulatory elements are compared, information can be obtained about the location and function of these elements.

Expression of the *XmyoD* gene in mesoderm at mid-gastrula stages may be achieved by induction of MIFs and XmyoD autocatalysis afterwards (Steinbach et al., 1998). The co-regulation of reporter gene and endogenous *XmyoD* also raises a disadvantage: even if the *cis*-element responsible for induction is missing from the reporter constructs, the correct expression of the reporter gene can be achieved as long as the *cis*-element responsible for XmyoD's autocatalytic loop is present. Direct autocatalysis would involve either E-boxes (the binding motif of bHLH protein like MyoD, Perry and Rudnicki, 2000) or MEF-2 binding sites, since MyoD can transactivate transcription through protein-protein interactions with MEF-2 family members (Molkentin et al., 1995). However, the MIE element contains neither of these binding sites. Thus, the LS-5 motif represent an essential regulatory element for the induction of *XmyoD* transcription, independent from MyoD's autocatalytic circuitry.

To begin the search for *cis*-regulatory elements, a series of truncation and deletion constructs were assayed by transgenic reporter analysis. Second, a linker-scanning strategy was used to define discrete motifs of the enhancer or silencer. It should be noted that all the linker-scanning mutations were tested within the context of –3200/+4829bp fragment. This genomic region is able to recapitulate all major aspects of endogenous *XmyoD* regulation. It should also be noted that *cis*-regulatory elements may have redundant functions (see e.g. Chen et al., 2002). Therefore, some elements may be missed if their functions are compensated by other *cis*-elements. Consequently, all elements identified by REMI in this study have dominant activaties.

The REMI transgenesis technique also provides a tool for researchers to target gene expression to specific cell types in *Xenopus*. Compared with REMI, mRNA and episomal

DNA plasmid injections lack temporal and spatial specificity. For example, DNA plasmids which use tissue-specific promoters to drive expression are poorly regulated when injected into *Xenopus* embryos and lead to mosaic expression. Thus, if one is interested in targeting or labeling a particular embryonic region, tissue or cell type, neither of these techniques provides the desired level of specificity. With the REMI technique, we can achieve this. For example, in this study, it has been shown that the –3200/+4829bp region of the *XmyoD* gene is sufficient to drive expression of the reporter gene in a manner very similar to endogenous *XmyoD*. The *XmyoD* regulatory region, which has been characterized by REMI in this work, provides a powerful tool for targeted expression of regulater genes or interference variants to test the myogenic regulatory network *in situ* at the time of muscle determination.

5.2 *Cis*-regulatory elements that regulate the expression of *XmyoD*

5.2.1 Expression of *XmyoD* is regulated through activation and repression

It has been demonstrated that the proximal promoter sequences of *XmyoD* (up to –0.7kb) leads to activation of reporter gene expression in ectoderm, but not in mesoderm. This indicated that the correct expression of *XmyoD* requires repression in ectoderm and activation in mesoderm. It has been further shown in this work that multiple enhancer elements in the *XmyoD* regulatory region contribute to full activation of *XmyoD*. The region spanning position -840 to -704 of the *XmyoD* locus appears to have significant importance on the regulation of *XmyoD*. We call this region MIE (MyoD induction enhancer). Specifically, the MIE region contains a repressing (LS-9) and a activating (LS-5) elements (Figure 4.5). Taken together, the correct expression of *XmyoD* is regulated by a combination of activation and repression.

For the *Xbra* and *Xmyf-5* gene, it was also found that the regulations are dependent on a complex interplay of repression and activation (Lerchner et al., 2000, Polli and Amaya, 2002, Yang et al., 2002). These suggested that the restriction of *XmyoD, Xbra, Xmyf-5*

expression to the mesoderm of the early *Xenopus* embryo is achieved by general activation followed by repression in ectoderm.

LS-9 motif has been shown to be a silencer. However, the LS-9 motif does not account for all the repression. Because if LS-9 is the only silencer, we should expect that the expression of LS-9 mutation construct should be very high in ectoderm but not in mesoderm, like that of the short promoter. In fact, the LS-9 mutation construct only showed precocious expression in mesoderm at NF10, expanded expression of reporter gene at NF10.5 and ectopic expression in the posterior wall (PW) of the neuroenteric canal at tail bud stage (Figure 4.4 C-F). Therefore, additional silencers should be further characterized, they may co-operate with LS-9 motif to silence the ectopic expression in ectoderm.

5.2.2 LS-5 motif

Analysis of the LS-5 mutation construct showed a loss of most of the expression of the reporter gene at NF10.5 and at tail bud stages (Figure 4.4 A, B and Table 4.2). Therefore, the LS-5 motif mediates an essential activating function of XmyoD induction.

To ascertain, whether any potential protein binding site is found within the LS-5 region, we searched for putative binding sites and discovered that a consensus CdxA binding site lies within the LS-5 motif. CdxA is a member of the caudal protein family. In *Xenopus*, members of the caudal protein family have previously been shown to be downstream of eFGF signaling and act as transcriptional activators for the posterior genes (Isaacs et al., 1998). However, the gain-of-function and loss-of-function analysis of the caudal related gene Xcad-3 did not give us expected result. This means that the LS-5 motif should be bound by other transcription activator.

A recent paper characterized eFGF as an inducer of *XmyoD* expression (Fisher, et al., 2002). This prompted me to ask which region in the *XmyoD* genomic sequence is responsible for the induction by eFGF.

It is shown in this study that eFGF is able to induce *XmyoD* expression by mRNA injection in the animal pole. It is also found that both the -840/-704 bp region and the LS-5 motif are essential for the induction. The -1770/-1586bp region also contributes to the induction (Figure 4.5 and Table 4.3). So it is concluded that both the -840/-704 region and the LS-5 motif are essential for the induction of *XmyoD* by eFGF signaling; LS-5 motif accounted for most of the reduction caused by deletion of -840/-704 region. Taken together, this indicated the LS-5 motif is the most important enhancer for the induction of *XmyoD* by eFGF.

Although it is shown that LS-5 motif is essential for the induction of *XmyoD* by eFGF, it is difficult to distinguish whether the LS-5 motif is bound by a factor which is induced by eFGF signaling or if the LS-5 motif is bound by an essential factor, which functions in parallel or downstream, but independent from eFGF signaling. In order to test these two possibilities, we should construct a plasmid in which a TK minimal promoter is driven by the LS-5 motif. We can inject eFGF mRNA in the transgenic embryos that contain this construct to test whether or not the LS-5 motif responds to eFGF signaling.

While it is clear that LS-5 is necessary to drive the expression of the reporter gene, it remains unclear whether or not the LS-5 motif is sufficient to achieve a correct expression pattern of the reporter construct. In order to test this possibility, it may be necessary to construct the LS-5 motif with a minimal promoter to drive a reporter gene, and analyze the expression of the reporter gene by REMI. If this construct shows an *XmyoD*-like expression pattern, then we can conclude that the LS-5 motif is also sufficient for the expression of *XmyoD* gene.

5.2.3 LS-9 motif

5.2.3.1 The LS-9 motif functions as a silencer

The LS-9 mutation construct showed precocious expression of the reporter gene before stage NF10 and stronger, expanded expression of the reporter gene at stage NF10.5. At

tailbud stages, expression was also observed in the posterior wall (PW) of the neuroenteric canal (Figure 4.4 C-F), which does not produce muscle. It is therefore concluded that the LS-9 region contains an element that mediates transcription repression.

Two possible mechanisms exist by which the LS-9 motif silences the ectopic expression of *XmyoD*. First, the LS-9 motif may be bound by a protein factor, which functions as a transcriptional repressor to repress the ectopic expression of *XmyoD*. Alternatively, the LS-9 motif may not be bound by transcription factor. Instead, the CpG dinucleotide, located in the LS-9 motif may be methylated, which may serve as an epigenetic mark for the repression. Methylation occurring at CpG-dinucleotides in LS-9 may result in the formation of transcriptionally silent heterochromatin. The enhancer motif, LS-5, that is located 45 bp upstream of LS-9 may be affected by the neighboring heterchromatin and thus rendered inaccessible by transcriptional activators. In support of this argument, the CpG-dinucleotide in the LS-9 motif is the most upstream of several CpG motif in the third CpG island in *XmyoD* gene locus, the LS-10 motif contains the second CG dinucleotide of this CpG island. However, the mutation of the LS-10 motif did not result in any change of the reporter gene expression compared with that of the wild type construct (Table 4.2). These observations may reflect that the first CpG dinucleotide in a CpG island is more important for the initiation, relay, or propagation of the methylation.

5.2.3.2 Temporal regulation of the *XmyoD* gene

XmyoD can be induced in animal caps (ACs) by several mesoderm-inducing factors (MIFs) like Activin or Wnt8/bFGF (Steinbach et al, 1998). It has been shown that MyoD induction depends on the developmental age of the induced cells, rather than on the type or time point of inducer application (Steinbach et al, 1998 and Figure 2.6B, h, i). This implies that the expression of *XmyoD* is tightly controlled by a timing device.

In wild type embryos, *XmyoD* expression starts at stage NF10.5. The pMD–3200/+4829GFP reporter constructs also recapitulate the timing of *XmyoD*

expression. However, mutation of the LS-9 motif in the reporter construct resulted in precocious expression of the reporter gene before NF10 (Figure 4.4 C). This indicated that the LS-9 motif may contain the timing device, or a component of it, which controls the expression of *XmyoD*. The mechanism by which LS-9 serves as a timing device is currently under investigation.

5.2.3.3 Xvents may regulate *XmyoD* transcription via LS-9

Xvent-1 and Xvent-2 are homeobox genes. They are expressed in the ventral and lateral marginal zones of *Xenopus* embryos and are excluded from most of the dorsal regions at mid-gastrula stages. At tail bud stages, *Xvents* are expressed in the posterior wall (PW) of the neuroenteric canal. They have been shown to be an immediate response gene of BMP-4 signaling and function in BMP signaling as transcriptional repressors (Onichtchouk et al, 1998). Xvent-2 can also act as an activator, e.g., BMP-autoactivation (Schuler-Metz et al., 2000). The expression of *Xvents* overlaps with the ventral domains of *XmyoD* expression pattern at mid-gastrula stages, at the same time the ventral domains of the *XmyoD* expression pattern are weaker in expression intensity than the dorsal domains. The expression of *XmyoD* is excluded from the posterior wall of the neuroenteric canal where Xvents are expressed. These data imply that Xvents may be negative regulators of *XmyoD* expression.

When the LS-9 motif is mutated, the reporter gene showed expanded expression at stage NF10.5, the expanded region included the ventral marginal zone, a region where Xvents are expressed. The ectopic expression of the LS-9 mutant construct also showed an overlap with the expression of Xvent-1 and Xvent-2 in the PW of the neuroenteric canal at tailbud stages (Gawantka et al., 1998). Therefore, *XmyoD* may be regulated by BMP signaling through Xvent-1 and Xvent-2 via the LS-9 motif. However, there is no homeobox found in LS-9 motif. This implies that the Xvents may regulate *XmyoD* indirectly or that Xvents bind a non-canonical DNA motif.

5.2.4 Maintenance enhancer

It has been found that maintenance of *XmyoD* expression plays an important role in *Xenopus* myogenesis after induction of *XmyoD* expression (Kato and Gurdon, 1993). Standley et al. (2001) reported that a secreted factor, eFGF, is sufficient for the maintenance of *XmyoD* expression, but the mechanism has not yet been elucidated.

It has been shown in this study that *Xenopus* serum response factor (XSRF) is likely to be the transcription factor that binds with an SRE box in the 5' genomic sequence of *XmyoD* and functions to maintain the expression of *XmyoD*. When the SRE box was mutated in a way to inhibit SRF binding, about 80% of embryos containing the mutation expressed the reporter gene normally at stage NF10.5, however, only about 50% of embryos showed some, usually much weaker, expression at tailbud stages. This indicated that around 30% of embryos lost maintenance of the reporter gene expression. It has also shown that a dominant negative version of XSRF, XSRF-EnR, largely down-regulated the expression of *XmyoD* when it is expressed after MBT. Therefore, these data indicate that XSRF binds with the SRE box and maintains the expression of *XmyoD* during development.

Experiments using an electrophoretic mobility shift assays (EMSA) confirmed that XSRF can bind the SRE box *in vitro* (Oliver Nentwich, unpublished data), and a CHIP (Chromatin Imunoprecipitation) assay confirmed that XSRF binds with *XmyoD* 5' genomic sequence *in vivo* (Oliver Nentwich and Katrin Mansperger, unpublished data). It has also been shown that XmyoD up-regulates the expression of XSRF (Armstrong and Rupp, unpublished data). Taken together, these data suggest that XmyoD first up-regulates the expression of XSRF and subsequently XSRF maintain the expression of XmyoD.

Since both a secreted factor, eFGF, and a transcription factor, XSRF, function to maintain the expression of *XmyoD*, it would be interesting to investigate if XSRF functions downstream of eFGF pathway in the maintenance of *XmyoD* expression.

5.3 The expression pattern of M-sirt is correlated with the inactive state of *XmyoD* gene

Transcripts of DNA repetitive sequence, which are localized in the -2.8/-2.0kb region of the *XmyoD* genomic sequence, were detected by *in situ* hybridization. We named this DNA repetitive sequence as M-sirt (MyoD's Xlsirt). The M-sirt region contains eight copies of a tandemly repeated unit of 79-81 nucleotides and shows 93% sequence identity with Xlsirt (Spohr et al., 1984) (Figure 4.14). M-sirt produces both sense and antisense transcripts and the expression pattern of M-sirt is correlated with the inactive state of the *XmyoD* gene (Figure 2.6B, a-c): the transcripts exist in the nucleus in all cells before the induction of *XmyoD* at stage NF 10, but at stage NF10.5, when the expression of *XmyoD* is upregulated in the mesoderm, the non-coding transcripts of M-sirt are dispersed or degraded (Figure 4.12, 4.13). Later on, the transcripts of M-sirt never overlap with the *XmyoD* expression domains. After the tailbud stage, M-sirt is expressed in the epidermis and between the somite myotome, while *XmyoD* is expressed in somite myotome. Therefore, there is a clear correlation between the transcripts of M-sirt and the inactive state of *XmyoD* both spatially and temporally.

Since M-sirt shows 93% sequence identity with Xlsirt, it raises the question of how specific RNA *in situ* hybridization is and whether the RNA-signals indeed originate from the *XmyoD* locus. Polli and Amaya (2002) reported that *in situ* hybridization can clearly distinguish the *Xenopus leavis* myf5 mRNA from *Xenopus tropicalis* myf5 mRNA although they possess 90% sequence identity in the probe region.

To further verify the specificity of the M-sirt *in situ* hybridization probe, a second *in situ* hybridization could be performed using a second, closely related Xlsirt sequence. If non-identical *in situ* hybridization staining patterns would be obtained, then the specificity of the probe would be further supported. RT-PCR experiment with M-sirt specific primers also should be done to prove that M-sirt is actively transcribed.

5.4 Potential protein factors that regulate the expression of *XmyoD*

The expression of a gene is regulated at several levels. Beside *cis*-regulatory elements, protein factors also play important roles. In this study, several potential regulatory factors of *XmyoD* were analyzed.

5.4.1 Putative RNA binding protein XSEB-4 is able to induce *XmyoD* expression in an animal cap assay

RNA binding proteins play diverse roles in the regulation of RNA metabolism in vertebrates and invertebrates (Burd and Dreyfuss, 1994). XSEB-4, a direct transcriptional target of XmyoD protein (Jasper, 1998), has a putative RNA recognition motif (RRM). It is shown in this study that XSEB-4 is mainly localized in nuclei and able to induce the expression of *XmyoD* in an animal cap assay. However, which RNA is bound by XSEB4 is unknown. The mechanism by which XSEB4 regulate *XmyoD* expression is under analysis.

5.4.2 XYY1

Yin Yang 1 (YY1) is a multifunctional transcription factor that acts as an activator, repressor, or initiator of transcription of numerous genes (Ficzycz et al., 2001). One potential YY1 binding site is found in XmyoD's promoter region. Satijn et al. (2001) showed that XYY1 functions as a repressor to induce ectopic neural tissue. It is shown in this study that XYY1-EnR is able to inhibit the expression of *XmyoD*. However, Ficzycz et al. (2001) reported that YY1 does not appear in the nuclei of cleavage stage embryos, nor in gastrula stage embryos several hours past the MBT, nor in neurula stage embryos. This observation ruled out the possibility that XYY1 functions as transcription factor to regulate the expression of *XmyoD*. It also implies that XYY1 regulates *XmyoD* expression indirectly. Further analysis should be done to characterize the mechanism that how XYY1 regulates the expression of *XmyoD*.

5.4.3 Lef-1 is necessary and sufficient for the expression of *XmyoD*

Zygotic Wnt signaling has been shown to be involved in dorsoventral mesodermal patterning in *Xenopus* embryos, but how it regulates *XmyoD* expression in the lateral mesodermal domains is not clear. Overexpression of XWnt8 during gastrula stages leads to ectopic *XmyoD* activation across the dorsal midline, where its expression is normally excluded (Christian & Moon 1993). Furthermore, dominant-negative forms of XWnt8 inhibit *XmyoD* expression in the early mesoderm (Hoppler et al. 1996), whereas overexpressed ß-catenin increases *XmyoD* expression, thus establishing ß-catenin as the transcription factor effector for XWnt8-mediated *XmyoD* activation. Interestingly, TCF-3 is not necessary for the activation of *XmyoD* (Hamilton et al. 2001). So ß-catenin must co-operate with other transcription factors to activate *XmyoD*. This study showed that Lef-1 is both necessary and sufficient to drive the expression of *XmyoD* (Figure 4.20), which provides strong evidence that Lef-1 is a transcription factor of zygotic Wnt signaling that activates the expression of *XmyoD*.

5.5 Conservation of MyoD regulation

How conserved is the *MyoD* regulation among the different species? In human and mouse, a 258 bp element located 20 kb upstream of the *MyoD* gene had been isolated. This so-called core enhancer can drive the expression of a LacZ reporter in the myotome (Goldhamer et al., 1992; Goldhamer et al., 1995). By linker scanning mutations, one element has been isolated that is dependent on *Myf-5* activation (Kucharczuk et al., 1999). No additional activator of the core enhancer has been found so far, although the element can weakly activate the LacZ reporter in a *Myf-5/MyoD* double knock-out background (Kablar et al., 1999). The function of the core enhancer is independent of the promoter because it functions with a heterologous TK-promoter (Goldhamer et al., 1992; Goldhamer et al., 1995). An additional regulatory region named DRR was found 5 kb upstream of the mouse *MyoD* gene (Asakura et al., 1995). The DRR also activates the LacZ reporter in the myotomes but is not active in the *Myf-5/MyoD* double knock out

background, indicating that the element is necessary for the maintenance of *MyoD* expression (Asakura et al., 1995; Kablar et al., 1999).

In summary the data from the other species show that *MyoD* expression is regulated by activation rather than inhibition and that only a few (possibly two) elements are necessary for the correct regulation of *MyoD*. This is in contrast to the results of this study because many different regulatory regions have been isolated which have both repression and activation functions. Moreover, there is no homology detected between the mouse core enhancer and the sequences of the frog *myoD* locus. In conclusion, the regulation of *MyoD* expression differs among different species. One likely reason for these apparent differences is that it is a result of adaptation to the developmental environment. The placental development of mammals allows for a comparatively slow growth, while the extracorporal development of the frog requires much faster growth. This rapid accumulation of muscle could be induced by the early activity of *XmyoD*, which helps to build up fast growing muscle.

5.6 A model of the epigenetic regulation of *XmyoD*

The transcripts of M-sirt were detected by *in situ* hybridization, and the expression pattern of M-sirt is correlated with the inactive state of *XmyoD* gene (Figure 4.12, 4.13 and Figure 2.6B, a-c) both spatially and temporally. This raises the question: what are the functions of M-sirt and its non-coding transcripts?

Several recent papers have provided insight into this issue by demonstrating the presence of short RNA transcripts that are complementary to repeats in centromeres and elsewhere in the genome (reviewed by Jenuwein, 2002). Jenuwein (2002) has proposed a model based on these observations, which stated that DNA repeats may be nuclear foci for the generation and accumulation of short heterochromatic RNAs (shRNAs). These RNAs are important for epigenetic processes associated with initiation and formation of heterochromatin (Figure 5.1).

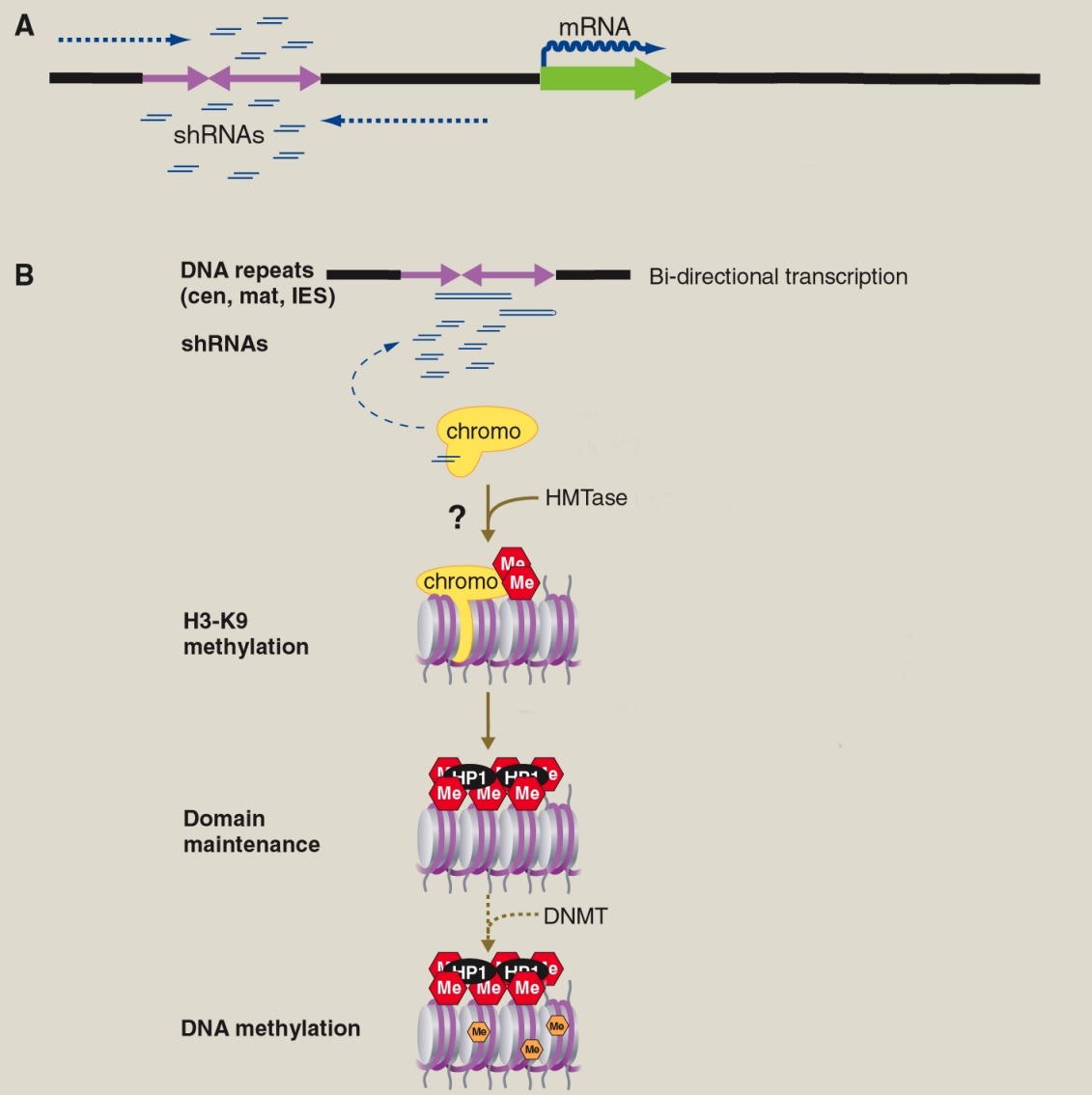

Figure 5.1 Model for heterochromatin formation by shRNA. (A) Shown is a hypothetical genomic region (black) containing DNA repeats (purple) and a gene or transcription unit (green). Promoter-driven mRNA is indicated by the wavy line (dark blue), and spurious intergenic transcription is represented by the blue dashed lines. (B) Simplified model highlighting the hierarchy of processes that induce epigenetic transitions triggered by shRNAs originating from DNA repeats. (DNMT, DNA methyltransferase.) (Adapted from Jenuwein, 2002)

According to this model, M-sirt and its non-coding transcripts may function as a signal to start the methylation of both histones and DNA to form heterochromatin. Interestingly, four CpG islands are found near the important regulatory elements of *XmyoD* (Figure 5.2). These four CpG islands are potential targets of DNA methylation. Among these four CpG islands, three are between the repeats and the transcription start site of *XmyoD* and one is downstream to the transcription start site of *XmyoD*. The SRE box (maintenance enhancer motif) is shortly upstream of the most distal CpG island. The LS-9 motif (Silencer) contains the first CpG dinucleotide of the third CpG island. The LS-5 motif, which is the main enhancer, is immediately upstream of the third CpG island. The LS-9 motif may contain binding sites for a transcription repressor or be important for its CpG motif. The observation implies that the CpG dinucleotide may be important for the correct expression of *XmyoD*.

When we connect the observations regarding transcripts of M-sirt, CpG islands and the inactive state of *XmyoD*, a picture begins to emerge (as shown in Figure 5.2): the transcripts of the repeats function as a signal to start the methylation of both histones and DNA, and control the accessibility of the enhancers of *XmyoD*, then regulate the timing and maintenance of *XmyoD* expression.

The M-sirt transcripts may hybridize to each other and form double stranded RNA and function as a signal to start the methylation of CpG islands and heterochromatin formation. The methylation of *XmyoD* locus may finish by stage NF10.5, leaving the enhancers (LS-5) unaccessible. This corresponds to the point in time when cells lose the competence to form muscle (Steinbach, et al., 1997).

After XmyoD is up-regulated at stage NF10.5 in mesoderm, XmyoD up-regulates XSRF (Armstrong and Rupp, unpublished data). XSRF somehow can prevent the process of methylation to maintain the accessibility of the LS-5 motif and further maintain the expression of *XmyoD* at later stages.

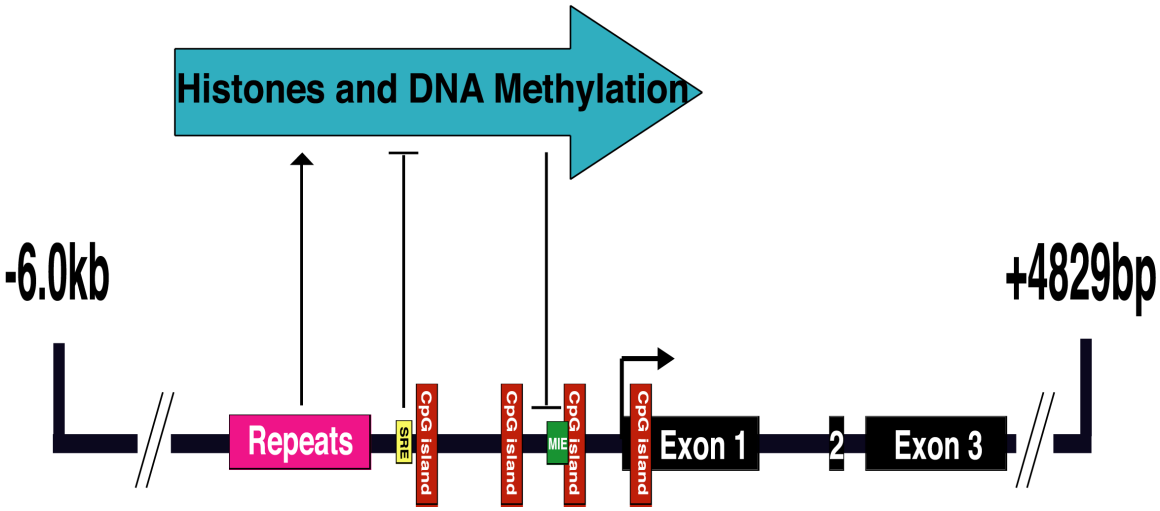

Figure 5.2 The model of the epigenetic regulation of *XmyoD*. The M-sirt (pink box) produces transcripts and function as a signal to start the methylation of the four CpG islands (red boxes). The DNA methylation and heterochromatin formation inhibit the accessibility to MIE (green box) and further inhibit the transcription of *XmyoD*. SRF may bind with the SRE (yellow box) to block the process of methylation.

5.7 Conclusion and outlook

In this study, the transcriptional regulation of *XmyoD* in *Xenopus* was analyzed. Three *cis*-regulatory motifs were defined: an induction enhancer (the LS-5 motif), a silencer (the LS-9 motif) and a maintenance enhancer (the SRE box). M-sirt, a repetitive DNA sequences, was discovered in -2.8/-2.0kb region in the *XmyoD* genomic sequence. M-sirt is likely to produce sense and anti-sense transcripts. These may provide an important epigenetic mark to regulate the expression of *XmyoD*. Several protein factors have also been shown to regulate the expression of *XmyoD* including XSRF, Xcad-3, XSEB-4, XYY-1 and Lef-1.

This study provided important information for the understanding of *XmyoD* gene expression, muscle development and mesoderm patterning. It will be interesting to determine the protein factors that bind with the LS-5 and LS-9 motifs. The methods we can use include yeast one-hybrid screening (Sieweke, 2000) or screening phage expression libraries with these two DNA motifs (Zhang et al., 2002). In order to confirm if the CpG dinucleotide in the LS-9 motif is important, we may mutate the CG into CC or

GG, and check the expression of reporter gene by REMI. We also can produce more CpG dinucleotides in the LS-9 motif or other regions, like the LS-6 motif, and assay for the effects on expression.

It would also be interesting to test the model of the epigenetic regulation of *XmyoD* (shown in Figure 5.2). In order to test this model, we could analyze whether there is correlation between demethylation of the *XmyoD* locus and *XmyoD* gene expression. Experiments could also be done to test if the overexpression or knockdown of M-sirt transcripts affects the expression of *XmyoD*. The best method to address the function of the repeats in *XmyoD* locus is to make a targeted deletion of the repeats from the *XmyoD* locus by homologous recombination (Chen et al., 2002). Although this method has not been established in *Xenopus*, it has been worked out in numerous other species. When we have a frog line that harbors a targeted deletion of the M-sirt in *XmyoD* gene locus, we can easily analyze the phenotype of the frogs and the expression of *XmyoD* gene to get information of the function of M-sirt. If this model is proven true, we will gain very valuable information to understand the regulation of the *XmyoD* gene expression and mesoderm patterning.

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Abbreviations

A	adenine
Ab	anti-body
AC	animal caps
AP	alkaline phosphatase
APS	Ammoniumperoxidisulfat
BCIP	5-Bromo-4-chloro-3-indolyl-phosphat
BMP	bone morphogenic protein
bp	base pair
BSA	bovine serum albumin
C	cytosine
CHAPS	3[(3-cholamidopropyl)-dimathylammonio]-1-propansulfonat
CNS	central nervous system
CS	chicken serum
D	dorsal
ddH_2O	bidestilled water
DE	dorsal ectoderm
Dig	digoxygenin
DML	dorsomedial lip
DTT	dithiothreitol
EDTA	ethylendiamintetraacetat
eFGF	embryonic fibroblast growth factor
EnR	engrailed repressor domain
FGF	fibroblast growth factor
G	guanine
Gal	galactosidase
h	hours
HCG	humanes choriongonadotropin
HEPES	N' (2-Hydroxyethyl)-piperazin-N`-2-ethansulfonsaeure
HMG	high mobility group
hpf	hours past fertilisation
HSV	herpes simple virus
ICC	Immunocytochemistry
kb	kilo base pair
LMZ	lateral marginal zone
LS	linker-scanner
MBS	modified Barth`s salt solution
MIE	MyoD induction enhancer
MIF	mesoderm induction factors
min	minutes
MHC	myosin heavy chain
MM	medial myotome
MMR	Marc's modified ringer's
MOPS	morpholinopropansulfonsaeure
mpcs	myogenic progenitor cells

M-sirt	MyoD's Xlsirt repeats
N	arbitrary nucleotide
NC	notochord
NF	Nieuwkoop and Farber stage
NLS	Nuclear Localization Signal
NT	neural tube
O.N.	over night
PAGE	polyacrylamid gel electrophoresis
PBS	phosphates buffered salt solution
p. c.	post coitum
PcG	polycomb group
PCI	phenol/chloroform/iso amyl alcohol
PCR	polymerase chain reaction
PMSF	paramethylsulfonylfluirid
PW	posterior wall
R & C	reverse and complement
REMI	restriction enzymes mediated integration
RNase	ribonuclease
RRM	RNA recognition motif
RT	reverse transcription or room temperature
s	second
SDB	sperm dilution buffer
SDS	sodiumdodecylsulfate
shh	sonic hedgehog
shRNAs	short heterochromatic RNAs
SSB	sperm storage buffer
T	thymine
TEMED	N,N,N,N-Tetramethylethylendienamin
TGF	transforming growth factor
TK	Thymidin Kinase
UTR	untranslated region
V	ventral
VLL	ventrolateral lip
VM	ventral myotome
WT	wild-type

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