Post-transcriptional regulation of *Xwnt-8* expression is required for normal myogenesis during vertebrate embryonic development

by
Qi Tian

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CERTIFICATE OF APPROVAL

This is to certify that the M.S. thesis of

Qi Tian

has been approved

Professor in charge of thesis

[redacted]

Member

[redacted]

Member

Member

Associate Dean for Graduate Studies

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ABSTRACT

The *Xwnt-8* gene is transiently expressed in ventral and lateral mesoderm during gastrulation and plays a critical role in patterning these tissues. Ectopic expression of *Xwnt-8* in dorsal mesoderm after the MBT, by injection of plasmid DNA, ventralizes the fate of these cells. Specifically, their fate of forming the most dorsal structure, the notochord, is changed such that they form a more ventral structure, muscle. Expression of a dominant-negative form of *Xwnt-8* in embryos blocks muscle formation. Thus, both gain-of-function and loss-of-function experiments support the hypothesis that endogenous Xwnt-8 is required to induce the expression of genes involved in specification of ventral and somitic mesoderm. It has also been shown that downregulation of Xwnt-8 following gastrulation may also be important since ectopic expression of Wnts in the somite causes an expansion of the dermomyotome and represses sclerotome formation (Capdevila, 1998).

In the current study, we show that the spatial and temporal pattern of expression of endogenous *Xwnt-8* is regulated, in part, at a post-transcriptional level. We have identified a novel sequence element in the 3' untranslated region of the Xwnt-8 RNA that controls the polyadenylation status of reporter and endogenous Xwnt-8 RNAs, directs rapid RNA degradation beginning precisely at the early gastrula stage, and represses translation of transcripts throughout development. Expression of endogenous *Xwnt-8* is normally

downregulated within lateral (presomitic) mesoderm following gastrulation. We demonstrate that rapid degradation of Xwnt-8 transcripts, mediated by these regulatory elements in the 3' untranslated region, is essential to this process and that downregulation is required to prevent overcommitment of somitic cells to a myogenic fate. These studies demonstrate a role for post-transcriptional regulation of zygotic gene expression in vertebrate embryonic patterning.

CHAPTER 1:

INTRODUCTION

1. *Wnt* family and functions

Studies performed by Nusse and Varmus in 1982 led to the discovery of a locus termed *int-1*(for integration site), that is activated in response to proviral insertion of mouse mammary tumor virus (MMTV) (Nusse and Varmus 1982). Subsequent work identified the gene *int-1* as a proto-oncogene involved in mammary carcinogenesis (Ooyen et al. 1984). In 1987, three groups identified the *wingless* gene, the *Drosophila* homolog of *int-1*(Rijsewijk et al. 1987; Baker 1987; Cabrera et al. 1987). Since then, many genes in this family have been identified. Later, researchers of this field proposed to call *int-1* and related genes as the *Wnt* (the *Wingless*-type MMTV integration site) gene family (Nusse et al. 1991). Up to now, intensive studies have led to the discovery of more than 20 *Wnt* genes from various species including *Xenopus, Drosophila, C. elegans,* zebrafish, mouse and human. Each *Wnt* gene encodes a protein that appears destined for secretion and harbors one or more sites for N-linked glycosylation (Bradley et al. 1990, Gonzalez et al. 1991, Jue et al. 1992, Vincent and Lawrence 1994).

Members of *Wnt* gene family play important roles in regulation of a number of basic developmental processes such as neural development in mouse, axis formation and

myogenesis in *Xenopus*, segmentation in *Drosophila*, and mammary tumorigenesis in mouse (reviewed by Nusse and Varmus 1992, McMahon 1992, Klingensmith et al. 1994).

At present there are many gaps in the knowledge of both the *in vivo* distribution and the normal biological properties of vertebrate Wnt proteins. The relatively poor antibody reagents and low levels of endogenous Wnt protein have hampered attempts to examine normal Wnt expression in vertebrate embryos . Moreover, research on Wnt protein function is slow because of the inability to obtain significant quantities of soluble, biologically active, secreted protein that is free of cell surface or matrix contaminants (Papkoff and Schryver 1990).

Although there exist many difficulties in working with Wnt proteins, several recent advances have shed light on how Wnts function. First, a novel member of the frizzled family in *Drosophila,* Dfz2, was shown to function as a wingless receptor (Wang et al. 1996, Bhanot et al. 1996). Second, two groups independently isolated *Xenopus* Frzb, an antagonist of Wnt signaling. While the amino terminal region of Frzb protein is quite similar to the putative receptor binding domain of the frizzled product, Frzb differs from frizzled proteins in that it lacks all transmembrane domains. This results in a putative secreted Wnt-binding protein, that blocks Wnt protein from binding to its receptor. It is proposed that Frzb is a naturally occurring inhibitor of Wnt signaling (Leyns et al. 1997, Wang et al. 1997). Third, it is found that β-catenin, a component downstream of the Wnt receptor, can translocate to the nucleus and function as a transcriptional activator (Willert and Nusse, 1998). Taken together, these advances greatly improved our understanding of the Wnt signaling pathway.

The current view of Wnt signaling pathway is shown in Fig.0.1. Specifically, after post-translational modification and secretion, Wnts bind to transmembrane receptors (Frizzled), and leads to the hyperphosphorylation of Dishevelled (Dsh). Dsh is a phosphoprotein, which, when Wnt signaling is activated, becomes more highly phosphorylated on serine and threonine residues and relocated to the plasma membrane. That Dsh contains two domains implicated in protein-protein interactions suggests that Dsh may be an adaptor protein required for assembly of a signaling complex, which promotes the inactivation of glycogen synthase kinase 3 (GSK3). GSK3 acts as a constitutive inhibitor of the signaling activity of downstream component β–catenin. GSK3 appears to shorten the halflife of β–catenin by promoting phosphorylation of several sites in the N-terminal portion of β–catenin, leading to its ubiquitination and degradation. As GSK3 suppressed the activity of β–catenin by promoting its degradation, the inactivation of GSK3 in response to WNT signaling leads to the accumulation of β–catenin. The increase in steady-state levels of β–catenin results in its translocation to nucleus and its interactions with HMG-box transcription factors, including LEF, and TCF. Inside the nucleus, β–catenin may alter the ability of LEF/TCF to bend DNA and relieve LEF/TCF-dependent transcriptional repression, and finally activate the transcription of target genes (reviewed by Wodarz and Nusse,1998; Brown and Moon, 1998).

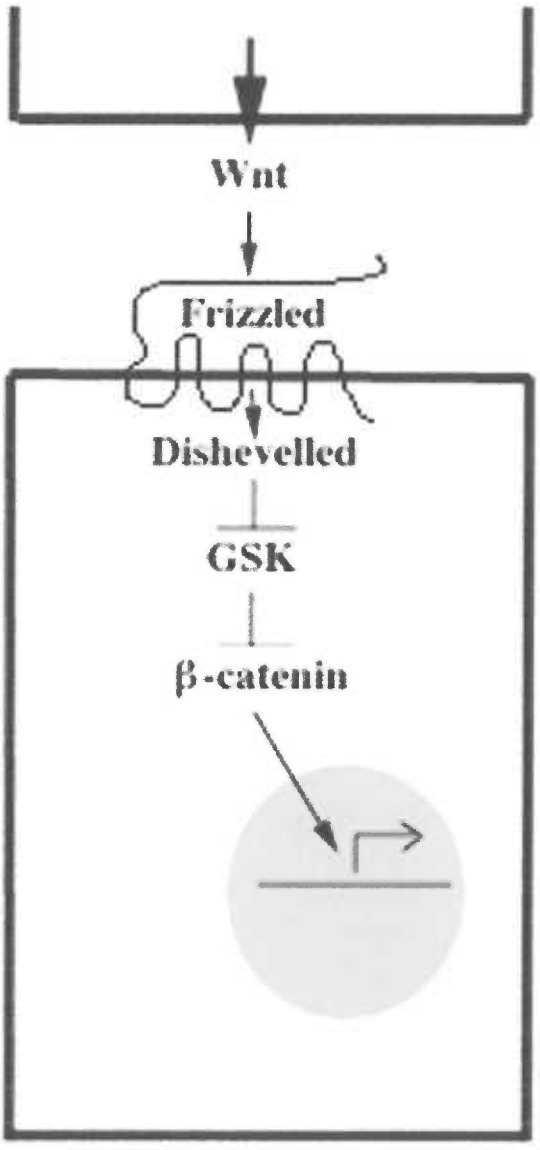


Figure 0.1 Wnt signaling pathway

Although an individual *Wnt* gene has a distinct expression pattern and function, multiple *Wnt* genes show overlapping expression patterns and functional redundancy (Ikeya, 1998), suggesting that multiple members can activate a common intracellular signal pathway. Consistent with this possibility, several independent assays have identified two functionally distinct categories of Wnts. In one set of assays, members of Wnt1 class, including Wnt1, Wnt3a, Wnt8 and Wnt8b, induce complete duplication of the embryonic axis when ectopically expressed in the ventral marginal zone of cleaving frog embryos. Members of the Wnt5a class, including Wnt5a, Wnt4 and Wnt11 do not induce complete axis duplication, but instead produce distinct phenotypic defects when misexpressed in *Xenopus* embryos (Du et al. 1995, Torres et al. 1996, Moon et al. 1997). In another assay, the Wnt1 class, but not the Wnt5a class, has been shown to be capable of transforming C57mg murine mammary epithelial cells (Wong et al. 1994).

2. Role of Xwnt-8 in ventral mesodermal patterning and somite differentiation

Xenopus Xwnt-8 is a member of *Wnt-1* subclass, and can induce the formation of a secondary dorsal axis when misexpressed in early *Xenopus* embryos (Christian et al, 1991). It is thought that Xwnt-8 can mediate the dorsalizing function of the Nieuwkoop center. The Nieuwkoop center is located in vegetal cells on the dorsal side of very early stage embryos, and functions to specify dorsal fate. However, endogenous *Xwnt-8* is not present at the proper time or place to function in early dorsal patterning. Instead, *Xwnt-8* is most abundant during gastrulation at which time expression is confined

primarily to ventral and lateral mesodermal cells. Xwnt-8 plays an important role in ventral and lateral mesodermal patterning after the mid-blastula transition (MBT). The MBT marks a developmental switch that occurs at the twelfth cell cycle. At the MBT, zygotic transcription initiates, cell motility also initiates, and the synthesis of new proteins and activities not present in the unfertilized egg begins. Ectopic expression of *Xwnt-8* in dorsal mesoderm after the MBT, by injection of plasmid DNA, ventralizes the fate of these cells. Specifically, their fate of forming the most dorsal structure, the notochord, is changed such that they form a more ventral structure, muscle (Christian and Moon 1993).

Expression of a dominant-negative form of *Xwnt-8* in embryos blocks expression of *XMyoD* and *Xenopus-posterior (Xpo)* in prospective mesoderm and blocks muscle formation (Hoppler et al. 1996). *XMyoD* encodes a transcription factor with a basic-helix-loop-helix DNA-binding domain, whose expression is restricted to the presumptive somites in the lateral marginal zone of late gastrula stage embryos. XMyoD is responsible for regulating the expression of structural genes that form the differentiated muscle at later stages (Harvey 1990; Hopwood et al. 1989). *Xpo* is a ventral marginal zone marker, which is ubiquitously expressed at low levels during blastula stages and is restricted to the ventrolateral marginal zone by midblastula stage (Sato and Sargent 1991; Amaya et al. 1993). Moreover, ectopic expression of functional *Xwnt-8* in the dorsal marginal zone of the gastrula induces ectopic *XMyoD* and *Xpo*. Thus, both gain-of-function and loss-of-function experiments support the hypothesis that endogenous Xwnt-8 is required to induce the expression of genes involved in specification of ventral and somitic mesoderm (Hoppler et al. 1996). In support of this,

a secreted antagonist of wnt signaling, Frzb, can also block the induction of *MyoD* by *Xwnt-8* and inhibit somite development (Leyns et al.1997; Wang et al. 1997).

When the somite is first formed, any of its cells can become any of the somite-derived structures, such as the sclerotome, myotome, and dermatome. However, as the somite matures, the various regions of the somite become committed to forming only certain cell types. The patterning is accomplished by the interaction of several tissues, including the notochord, neural tube, dorsal ectoderm and lateral mesoderm, that form the environment of the somite (Fig.0.2). Research on early chick embryos using

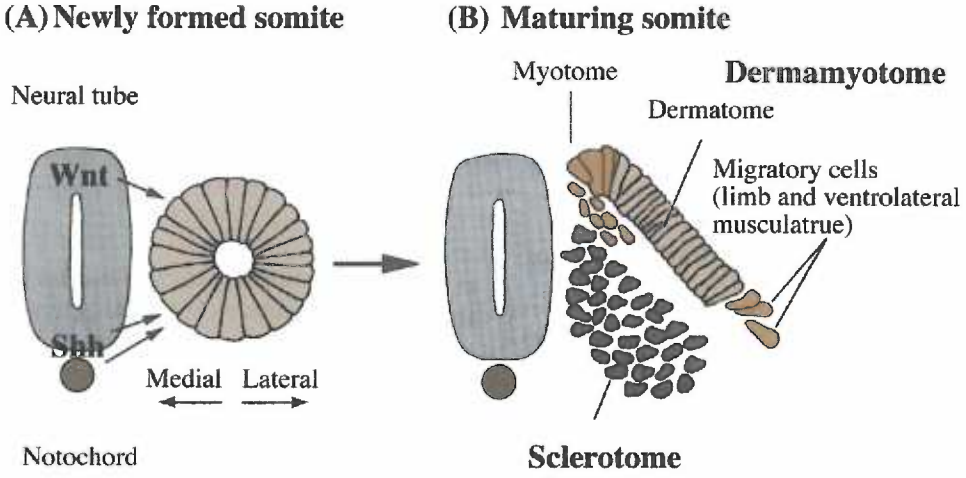


Figure 0.2 Formation of somite structures

the somite explant culture method demonstrated that a combination of signals from the neural tube and the floor plate/notochord synergistically induce the expression of myogenic bHLH genes and myogenic differentiation markers in unspecified somites (Munsterberg and Lassar 1995). Investigations at the molecular level demonstrated that Sonic hedgehog (Shh), which is expressed in the floor plate and notochord, and a subset of Wnt family members (Wnt-1, Wnt-3, and Wnt-4), which are expressed in dorsal regions of the neural tube, mimic the muscle-inducing activity of these tissues (Munsterberg et al. 1995). More evidence emerged from the study of somite development in mouse embryos lacking both Wnt-1 and Wnt-3a. In the absence of these Wnt signals, the myotome is not formed and the expression of a lateral dermomyotome marker gene is expanded more medially (Ikeya and Takada, 1998). These findings led to the proposal that Shh and Wnt signals combinatorially pattern the somite.

It has also been shown that Shh alone induces the expression of a sclerotomal marker Pax-1, and Wnts alone or Wnts together with Shh will induce myotome formation (Munsterberg et al. 1995). When ectopically expressed in the presomitic mesoderm of chick embryos during the time when somites are being patterned, Wnt-1 causes an expansion of the dermomyotome and represses sclerotome formation (Capdevila, 1998). These results indicate that Wnts play an important role in the control of the dorsoventral patterning of the somites, functioning primarily by opposing ventralizing signals such as Shh.

The discoveries described above can be viewed from two sides. One side shows that Xwnt-8 function is required during gastrulation for muscle development. The other side suggests that downregulation of Xwnt-8 following gastrulation may also be

important since ectopic expression of Wnts in the somite causes an expansion of the dermomyotome and represses sclerotome formation (Capdevila, 1998). Thus, downregulation of *Xwnt-8* expression may ensure that not all cells of the somite will form muscle. In summary, although Xwnt-8 is required in presomitic mesodermal cells during gastrulation, it may need to be rapidly cleared from these same cells following this period.

We propose that the temporal and spatial expression of *Xwnt-8* must be finely regulated in order to achieve normal somite development. While Wnt is necessary for myogenesis, up-regulating or prolonging its expression in the entire somite may cause overproduction of myotome at the expense of sclerotome. As described in the following chapter, we have found that expression of *Xwnt-8* is regulated, at least in part, at a post-transcriptional level by elements in the 3' untranslated region (3' UTR) of the *Xwnt-8* mRNA.

3. Regulatory role of 3' UTR of mRNAs

There are numerous examples showing general regulatory roles of the 3' UTR of mRNAs. Firstly, the 3'UTR is involved in regulation of the localization of mRNA (St Johnson 1995). A well-studied case is *nanos (nos)*, whose activity is required for the formation of abdominal segments in the fly embryo. *Cis*-acting sequences required for both mRNA localization and translational repression of unlocalized mRNA are found in the *nos* 3'UTR (Gavis & Lehmann 1992, Gavis and Lehmann 1994). Correct spatial regulation of *nos* mRNA through the 3' UTR sequences is critical for normal pattern

formation. Recently, a protein called Smaug was discovered and shown to bind to the 3'UTR of unlocalized *nos* mRNA and inhibit its translation. At the abdominal region, which is the proper place for nos functioning, Smaug function itself was inhibited (Smibert et al. 1996).

Secondly, the 3'UTR can regulate translation of mRNAs. There are several mechanisms for 3'UTR-mediated regulation of translation. 3'UTR sequences can repress or activate translation by modulating the length of the poly(A) tail, which in turn regulates the rate of translational initiation of mRNA. In concert with cellular factors, 3'UTRs can also directly enhance or repress translational initiation. Factors that bind such sequences may interact with the cap-binding complex or the small ribosomal subunit. Finally, sequences in the 3'UTR can direct mRNAs to specific regions of the cell from which repressor proteins are excluded, thereby activating their translation (for review, see Standart and Jackson 1994, Curtis et al. 1995, Jacobson and Peltz 1996).

Finally, the 3'UTR can regulate the stability of mRNA. In the deadenylation-dependent mRNA decay pathway, these elements may recruit different poly(A) nucleases, resulting in deadenylation. This in turn leads to the decapping of the 5' end of the mRNA and subsequent 5'-to-3' or 3'-to-5' degradation of mRNA (for review, see Decker and Parker 1994, Beelman and Parker 1995, Ross 1996).

The AU-rich element (ARE), defined by the presence of AUUUA motifs, was first identified in the 3'UTR of *c-fos* mRNA and represents the most common RNA destability element among those characterized in vertebrate organisms. AREs direct deadenylation of transcripts followed by rapid degradation. Deletion of the ARE in the 3'UTR of *c-fos* stabilizes the transcripts and converts *c-fos* to a transforming gene

(Meijlink et al. 1985). AU-rich elements are present in a variety of mRNAs including those encoding proto-oncoproteins, nuclear transcription factors, and cytokines (Chen & Shyu 1995).

One family of *Xenopus* maternal mRNAs, *Eg* mRNAs, are deadenylated rapidly and translationally repressed after fertilization. Unlike ARE-containing mRNAs, deadenylated Eg transcripts are not rapidly degraded, but remain stable for approximately 6-7 hours, and are then degraded at the MBT. This delay between deadenylation and degradation of Eg mRNAs could be caused by a limiting amount of one of the components for degradation or by protection of RNA from the degradation machinery (Bouvet et al. 1991). It has also been shown that postfertilization deadenylation of mRNAs is sufficient to cause their degradation at the blastula stage and that only deadenylated RNAs are degraded. Thus, there is a tight relation between deadenylation and degradation (Audic et al. 1997). Recently, the *cis*-sequence for the rapid deadenylation of *Eg5* mRNA has been characterized and named as the embryo deadenylation element (EDEN), the core motif of which is U(A/G) repeats (Paillard et al. 1998).

Many zygotic genes in *Drosophila* are post-transcriptionally regulated by instability elements in their 3'UTRs, such as pair-rule genes (Riedl & Jacobs-Lorena, 1996) and genes for neurogenesis (Lai & Posakony, 1997; Leviten et al. 1997). The instability of these mRNAs is crucial for their correct spatially and temporally expression and for their effects on invertebrate embryonic patterning. In vertebrates, although post-transcriptional regulation of maternal RNAs has been well studied, very little is known about the role of posttranscriptional regulation of zygotic RNA in embryonic patterning.

4. The goals of this project

The expression of *Xwnt-8* is finely regulated, and indirect evidence suggests that the mRNA turns over rapidly (Christian & Moon, 1993). Preliminary studies in the Christian lab suggested that elements in the 3'UTR of the *Xwnt-8* mRNA can regulate transcript stability and translation during embryonic development. The goal of my thesis project was to test the hypothesis that *Xwnt-8* expression is regulated at a post-transcriptional level and to determine the physiological significance of this regulation.

The specific aims are: 1) To test whether *Xwnt-8* mRNA degradation can be regulated by its 3'UTR; 2) To test whether the 3'UTR of *Xwnt-8* mRNA regulates its translation; 3)To elucidate the mechanism by which the 3'UTR affects transcripts; 4) To determine whether the regulation of endogenous *Xwnt-8* expression mediated by its 3'UTR is necessary for normal myogenesis.

These studies may also contribute to understanding of how gene expression is regulated for other members of the *Wnt* gene family, since no similar research has been done for *Wnt* genes. Also, it will be the first demonstration of posttranscriptional regulation of zygotic RNA in patterning vertebrate embryo.

CHAPTER 2: A manuscript submitted to the journal "Development"

Post-transcriptional regulation of *Xwnt-8* expression is required for normal myogenesis during vertebrate embryonic development

Qi Tian, Michael P. Dixon and Jan L. Christian*

*Corresponding author

INTRODUCTION

Wnts are a family of structurally related, secreted proteins that mediate cell-cell signaling. Members of this family play important roles in a number of basic developmental processes including embryonic induction, specification of cell fate and determination of cell polarity (reviewed by Parr and McMahon, 1994; Brown and Moon, 1998; Wodarz and Nusse, 1998).

Within a given vertebrate species, multiple *Wnt* genes have been identified that are expressed in spatially and temporally distinct, yet often overlapping patterns in the embryo. The dynamic pattern of expression of many *Wnt*s suggests that individual gene products are likely to function repeatedly throughout development in patterning distinct organ systems. Indeed, loss-of function mutations in *Wnt* genes in the mouse have identified multiple unique, as well as partially redundant roles for individual Wnt family members. Murine *Wnt-1*, for example, is essential for midbrain patterning (McMahon and Bradley, 1990; Thomas and Capecchi, 1990), and *Wnt-3a* for formation of axial mesoderm (Takada et al., 1994), while both of these genes play a redundant role in promoting proliferation of cell types derived from the dorsal neural tube (Ikeya et al., 1997) and in regulating formation of the dermomyotome (Ikeya and Takada, 1998).

The apparent functional redundancy of some Wnts suggests that multiple members of the Wnt family can activate a common intracellular signal transduction pathway. Consistent with this possibility, several independent assays have identified at least two functionally distinct categories of Wnts, exemplified by Wnt-1 and Wnt-5a

(reviewed by Moon et al., 1997; Wodarz and Nusse, 1998). The Wnt-1 class is defined by the ability to transform C57mg murine mammary epithelial cells, and/or to induce formation of a secondary dorsal axis when misexpressed in *Xenopus* embryos. In contrast, the Wnt-5a class of gene products are unable to transform mammary epithelial cells or to induce a complete axis duplication, but instead produce distinct phenotypic defects when misexpressed in *Xenopus* embryos.

As with other families of multifunctional developmental regulators, the expression and bioactivity of Wnt proteins is tightly regulated. For example, a number of secreted antagonists of Wnt signaling, including cerberus, dickkopf-1 and frizzled related proteins (FRPS), have been identified which appear to function in the extracellular space to prevent Wnts from binding to and activating their cognate receptors (reviewed by Brown and Moon, 1998; Wodarz and Nusse, 1998). In addition, the function of porcupine, a protein resident in the endoplasmic reticulum, is required for production of bioactive wingless (*Drosophila* Wnt-1) protein (Kadowaki et al., 1996). Genetic studies have also revealed a requirement for proteoglycans in wingless signaling (reviewed by Wodarz and Nusse, 1998). These many checkpoints on Wnt activity presumably operate to ensure that individual Wnts are active only when and where they should be, thereby preventing inappropriate tissue patterning or cell type specification.

In the current studies, we explore a further regulatory mechanism that operates to modulate expression of the *Xenopus Wnt (Xwnt)-8* gene. *Xwnt-8* is a member of the *Wnt-1* subclass and, accordingly, can induce the formation of a secondary dorsal axis

when misexpressed in early *Xenopus* embryos (Christian et al., 1991; Smith and Harland, 1991; Sokol et al., 1991). Endogenous Xwnt-8 is not present at the proper time or place to function in early dorsal patterning, but is transiently expressed in ventral and lateral mesoderm during gastrulation. By the early neurula stage, Xwnt-8 transcripts can no longer be detected in lateral mesodermal cells, but persist in ventral cells at least until the tailbud stage (Christian and Moon, 1993). Both gain-of-function and loss-of-function studies have shown that *Xwnt-8* plays an important role in specifying ventral and lateral fate within the newly induced mesoderm. Ectopic expression of *Xwnt-8* in extreme dorsal mesoderm (prospective notochord) during gastrulation causes these cells to adopt a more ventral fate, such that they differentiate as muscle, and causes a secondary loss of anterior structures (Christian and Moon, 1993). Conversely, blocking Xwnt-8 function leads to an enlarged head and to overcommitment to dorsal (notochordal) fate at the expense of lateral mesodermal (muscle) fate (Hoppler et al., 1996).

The dramatic patterning defects caused by deregulation of Xwnt-8 function demonstrate that proper temporal and spatial control of its activity is critical for normal development. Here, we show that expression of *Xwnt-8* is negatively regulated in vivo at the level of RNA stability and translation. Specifically, we have identified elements in the 3' untranslated region (UTR) of the Xwnt-8 transcript that control the polyadenylation status of reporter and endogenous Xwnt-8 RNAs and thereby direct their rapid, stage-specific degradation. These same elements repress translation of

Xwnt-8 transcripts throughout embryonic development. Finally, we demonstrate that 3'UTR-mediated degradation of endogenous Xwnt-8 transcripts within presomitic mesoderm at the end of gastrulation is essential to prevent overcommitment of these cells to a myogenic fate. Collectively, these studies demonstrate a role for post-transcriptional regulation of zygotic gene expression in vertebrate embryonic patterning.

MATERIALS AND METHODS

Embryo culture and manipulation

Xenopus eggs were obtained, and embryos were injected and cultured as described (Moon and Christian, 1989). Embryos were staged according to Nieuwkoop and Faber (Nieuwkoop and Faber, 1967). Animal caps were isolated and cultured as described (Cui et al., 1996).

Plasmid construction and RNA synthesis

The Xwnt-8 cDNA used to generate synthetic RNA in previous studies (pSP64T-Xwnt-8; Christian et al., 1991) includes the first 290 nucleotides of the 3' UTR. pSP64T-X8/UTR- was generated by truncating this cDNA at an AvaI site, thereby removing all but the first 30 nucleotides of the 3' UTR. pSP64T-Vim/UTR+ was generated by subcloning the 3' AvaI-EcoRI fragment of the Xwnt-8 cDNA

downstream of sequence encoding a mutant, myc-tagged form of Vimentin (Vim/UTR-, originally designated V5ΔC-myc, Christian et al., 1990). In order to generate pCS2-UTR derivatives, cDNAs containing portions of the Xwnt-8 UTR were isolated using restriction sites that were naturally present in the 3' UTR of Xwnt-8 cDNAs, or that were introduced by PCR. These fragments were then subcloned downstream of myc coding sequence in the vector pCS2+MT or were subcloned in antisense orientation into the vector pCS2+ (Turner and Weintraub, 1994). Capped synthetic RNA was generated by in vitro transcription of linearized templates using a Megascript kit (Ambion).

Immunostaining, in situ hybridization and histological analysis

Whole mount immunocytochemical analysis, using monoclonal antibody 12/101 to detect muscle and monoclonal antibody 9E10 to detect the myc-epitope, was performed as described (Moon and Christian, 1989). Following immunostaining, some embryos were embedded in paraffin, and 20 μm-thick sections were cut and counterstained with eosin for histological analysis (Christian and Moon, 1993). Whole mount in situ hybridization was performed as described (Harland, 1991) using BM Purple AP Substrate (Boehringer Mannheim).

RT-PCR

RNA was isolated from ten pooled animal caps and reverse transcription-polymerase

chain reaction (RT-PCR) analysis was performed as described (Cui et al., 1996) using the following PCR conditions: 94°C for 5 minutes, followed by a variable number of cycles (determined empirically to be in the linear range for each primer pair using RT product of control embryo RNA) at 94°C for 30 seconds; 55°C for 30 seconds and 72°C for 30 seconds. The sequences of *EF1-α, N-CAM, α-actin, OtxA, Xslug, and Xlhbox6* primers have been reported (Chang and Hemmati-Brivanlou, 1998). PCR products were visualized with a Molecular Dynamics phosphorimager.

Northern blot analysis

RNA was extracted from embryos and analyzed by Northern hybridization as described (Christian et al., 1991).

RNA extraction from polysomal and nonpolysomal fractions

Twenty embryos were homogenized in 500 µl of polysome buffer (300 mM KCl, 2 mM $MgCl_2$, 20 mM Tris-HCl, 4 µg / ml polyvinyl sulfate, 0.05% (V/V) sodium deoxycholate, 2 mM dithiothreitol, 100 units/ml RNasin, and 0.2 mM cycloheximide) and centrifuged at 12,000g for 15 minutes at 4°C. Supernatants were layered over a step gradient of 10% sucrose (0.5ml) and 35% sucrose (0.5ml) in polysome buffer and centrifuged at 100,000g for 2 hours at 4°C. For EDTA control groups, supernatants and sucrose/polysome buffer was adjusted to 20mM EDTA, and cycloheximide was omitted.

RNA was ethanol precipitated from supernatants and then extracted from both fractions

by suspension in 0.5 ml guanidine isothiocyanate solution (4 M guanidine isothiocyanate, 50 mM Tris, pH 7.6, 5 mM EDTA, 0.5 % SDS, and 0.1 M ß-mercaptoethanol), followed by phenol/chloroform extraction and ethanol precipitation. RNA pellets were re-extracted with 1ml of Tri Reagent (Sigma), precipitated with ethanol and resuspended in 40 µl of DEPC-treated H_2O. Six microliters of each RNA was analyzed by Northern hybridization.

Western blot analysis

Proteins were extracted from embryos as described (Moon and Christian, 1989), separated by SDS-polyacrylamide gel electrophoresis, and transferred to PVDF membrane. Membranes were incubated with antibody 9E10 which was detected by chemiluminescence using an ECL kit (Pierce). The same membrane was reprobed with a polyclonal anti-spectrin antibody.

RT-PCR-based polyadenylation assay

RNA was extracted from embryos and reverse transcribed using an XhoI adapter/oligodT primer (Stragene). cDNA was amplified using primers specific for XhoI linker sequence and for sequence present in the 3'UTR of Xwnt-8 (5'-AGGTGGTCTGTTGTAAAT-3') using the following PCR conditions: 94°C for 5 minutes followed by 35 cycles of 94°C

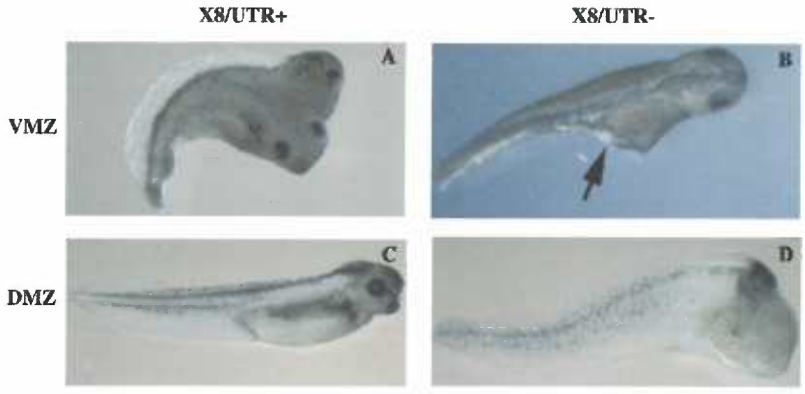
for 1 minute, 55°C for 1 minute and 72°C for 1 minute. Southern blots of PCR products were hybridized with a radiolabled cDNA probe specific for the 3' UTR of Xwnt-8.

RESULTS

Deletion of untranslated sequence from the Xwnt-8 RNA alters its activity in vivo

Injection of 5-20 pg of a synthetic Xwnt-8 RNA that includes the 3' UTR (X8/UTR+) into ventral cells of *Xenopus* embryos induced the formation of a secondary dorsal axis in 98% of injected embryos, and 76% of these induced axes contained a complete set of anterior structures (n=148; Fig. 1A). Ventral injection of 5-20 pg of a Xwnt-8 RNA that lacks most of the 3' UTR (X8/UTR-) also induced formation of a secondary dorsal axis in 93% of injected embryos (n=116; arrow, Fig. 1B), but this axis was anteriorly truncated. Injection of up to 1 ng of X8/UTR+ RNA into dorsal cells of cleaving embryos produced no apparent patterning defects in 80% of embryos (n= 88, Fig. 1C) and led to enlargement of the cement gland in others (data not shown). In contrast, dorsal injection of as little as 5 pg of X8/UTR- RNA led to a loss of anterior structures in 96% of embryos (n=114; Fig. 1D). Histological analysis demonstrated that many of the anteriorly truncated primary and secondary dorsal axes in embryos injected with X8/UTR- lacked notochord (data not shown). The phenotypic defects caused by dorsal injection of X8/UTR- RNA are identical to those observed in embryos injected with a plasmid Xwnt-8 expression construct, which leads to ectopic expression of Xwnt-8

Fig. 1. Differential secondary axis induction and inhibition of anterior development by injected Xwnt-8 RNAs that contain or lack 3' untranslated sequence. Xwnt-8 RNAs that include (X8/UTR+; A, C) or lack (X8/UTR-; B, D) 3' untranslated sequence were injected into the dorsal (DMZ) or ventral marginal zone (VMZ) of four cells embryos which were cultured to the tadpole stage and photographed. Arrow in B indicates partial secondary axis.


X8/UTR+
X8/UTR-
VMZ
DMZ
A
B
C
D

protein after the onset of zygotic gene expression at the midblastula transition (MBT, Christian and Moon, 1993). These defects are not produced by injection of X8/UTR+ RNA, raising the possibility that regulatory elements in the 3'UTR destabilize or inhibit translation of these transcripts after the midblastula stage.

To further test the hypothesis that the 3' UTR of the Xwnt-8 RNA can negatively regulate Xwnt-8 activity in vivo, we directly compared the ability of Xwnt-8 protein translated from microinjected X8/UTR+ and X8/UTR- RNAs to posteriorize neural tissue and to induce neural crest. Previous studies have shown that *Xenopus* ectodermal explants form neural tissue that is anterior in character when made to ectopically express the BMP antagonist, noggin. This tissue can be repatterned as posterior, or converted to neural crest by addition of members of the Wnt-1 class of proteins (McGrew et al., 1995; Saint-Jeannet et al., 1997; Chang and Hemmati-Brivanlou, 1998; LaBonne and Bronner-Fraser, 1998). Since these Wnt-dependent patterning events take place during gastrulation, they provide sensitive assays for Wnt function after the midblastula stage.

RNA encoding noggin (150 pg) was injected either alone, or together with X8/UTR+ or X8/UTR- RNA (10 pg), into 2-cell embryos as illustrated above Fig. 2. At the blastula stage, ectodermal explants (animal caps) were isolated, cultured until sibling embryos reached the tailbud stage (stage 25) and analyzed for expression of specific marker genes by semi-quantitative RT-PCR. Injection of noggin (Nog) RNA induced the expression of the general and anterior neural-specific genes, *NCAM* and *OtxA*, respectively, but did not induce expression of markers of posterior neural tissue

(*Xlhbox6*), neural crest (*Xslug*) or dorsal mesoderm (*muscle actin*) (Fig. 2A). This pattern of gene expression was not altered when X8/UTR+ RNA was co-injected along with noggin except that expression of *Xlhbox6* was weakly induced. In contrast, co-injection of X8/UTR- RNA along with noggin led to a marked suppression of expression of the anterior neural-specific gene, *OtxA*, and to a concomitant induction of expression of posterior neural- (*Xlhbox6*) and neural crest-specific (*Xslug*) genes.

We also compared the ability of X8/UTR- and X8/UTR+ to specify neural crest in whole embryos, using *Xtwist* as a marker. X8/UTR+ or X8/UTR- RNA (10 pg) was injected near the animal pole of one blastomere of two cell embryos and expression of *Xtwist* was analyzed by in situ hybridization at the midneurula stage (stage 17). X8/UTR+ injected embryos (Fig. 2B, left panel) showed symmetric expression of *Xtwist* along the lateral edges of the anterior neural plate in the prospective neural crest region, as did uninjected embryos (data not shown). In contrast, injection of X8/UTR- RNA, led to a unilateral expansion of the *Xtwist* expression domain (Fig. 2B, right panel, arrow). Collectively, these results demonstrate that protein translated from injected Xwnt-8 transcripts containing the 3' UTR can act prior to the midblastula stage to induce dorsal development, but is much less active or is inactive in patterning neural tissue, which occurs after the onset of gastrulation. Xwnt-8 alone, among various members of the Wnt-1 class of gene products, has been reported to be incapable of posteriorizing neural tissue or inducing neural crest (Saint-Jeannet et al., 1997; Chang and Hemmati-Brivanlou, 1998). This may be due to insufficient levels of Xwnt-8

Fig. 2. Xwnt-8 RNAs that lack the 3' UTR can posteriorize neural tissue and induce neural crest whereas RNAs that include the 3' UTR cannot. (A) Animal caps were isolated from uninjected embryos or from embryos injected with noggin (Nog) RNA alone, or in combination with X8/UTR+ or X8/UTR- RNAs. Ectodermal explants were cultured to stage 25 and expression of pan-neural *(NCAM)*, anterior neural *(OtxA)*, posterior neural *(Xlhbox6)*, neural crest *(Xslug)*, dorsal mesodermal *(muscle actin)* and ubiquitously expressed *(EF1-α)* genes were analyzed by RT-PCR. (B) X8/UTR+ (left panel) or X8/UTR- (right panel) RNA was injected into the animal pole of one blastomere of two-cell embryos and expression of the neural crest marker Xtwist was analyzed by in situ hybridization at stage 18. Arrow indicates expanded domain of Xtwist staining.

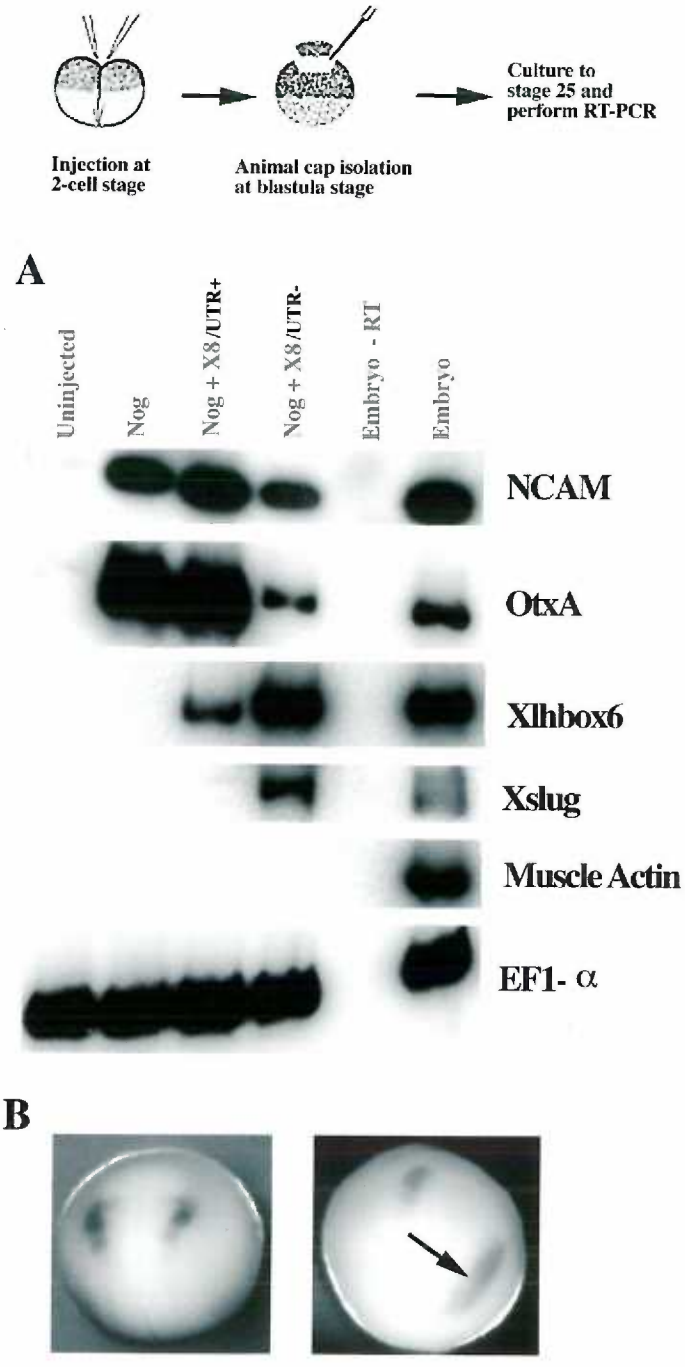

Culture to
stage 25 and
perform RT-PCR
Injection at
2-cell stage
Animal cap isolation
at blastula stage
A
Uninjected
Nog
Nog + X8/UTR+
Nog + X8/UTR-
Embryo - RT
Embryo
NCAM
OtxA
Xlhbox6
Xslug
Muscle Actin
EF1- α
B

Fig. 2

protein being synthesized from injected RNAs that include the 3' UTR, rather than reflecting true functional differences. Our results are consistent with the possibility that the Xwnt-8 3' UTR can negatively regulate transcript stability or translation in vivo in a temporally restricted fashion, beginning after the midblastula stage of development.

The Xwnt-8 3' UTR negatively regulates transcript stability in developing embryos beginning at the early gastrula stage

To directly test whether the 3' UTR of Xwnt-8 can regulate transcript degradation in a temporally restricted fashion, we compared the in vivo stability of reporter transcripts that either included (Vim/UTR+) or lacked (Vim/UTR-) the Xwnt-8 3' UTR (These constructs were made by Michael Dixon in Christian Lab). Each of these transcripts encodes a non-functional myc-epitope tagged reporter protein (illustrated above Fig. 3). Vim/UTR- or Vim/UTR+ RNA (500 pg) was injected into one-cell *Xenopus* embryos and the persistence of each injected transcript was assayed by Northern blot analysis of RNA collected from developmentally staged embryos (Northern analysis was performed by Jan Christian). Roughly equivalent amounts of each RNA were detected shortly after injection (one-cell stage) and Vim/UTR- transcripts persisted at nearly steady state levels at least until stage 17 (Fig. 3). In contrast, although Vim/UTR+ transcripts were stable through the early gastrula stage (stage 10) they were almost completely degraded by the early neurula stage (stage 14). Further analysis demonstrated that Vim/UTR+ transcripts were rapidly degraded shortly after the onset of gastrulation, becoming nearly

Fig. 3. The 3' UTR of Xwnt-8 induces the degradation of a heterologous transcript beginning after the onset of gastrulation. Vim/UTR+ or Vim/UTR- RNA was injected into one-cell embryos. RNA was extracted from 10 embryos in each experimental group and from uninjected (control) embryos at the indicated developmental stages. A Northern blot containing 15 μg of each RNA was hybridized with a radiolabeled Vim antisense probe. The Vim probe detects endogenous vimentin RNA and thus signal is observed in control lanes by the late gastrula stage.

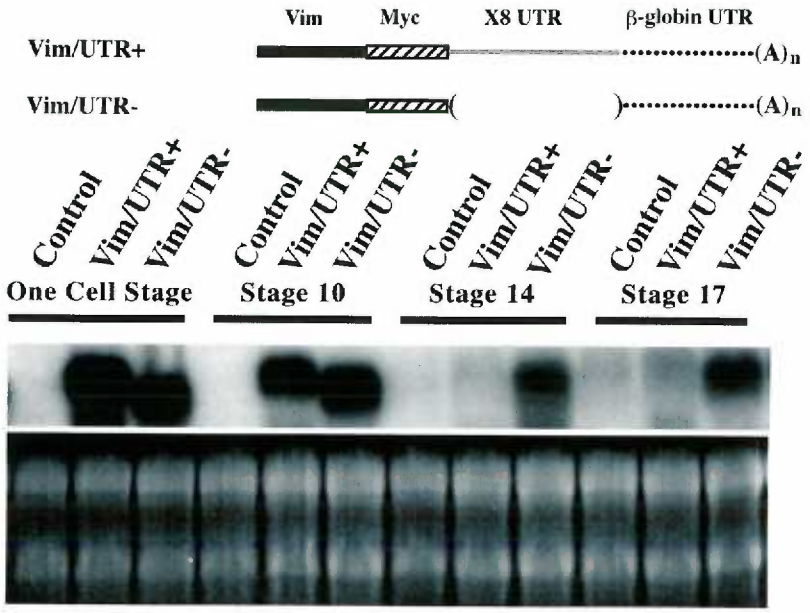

Vim
Myc
X8 UTR
β-globin UTR
Vim/UTR+
(A)n
Vim/UTR-
(A)n
Control
Vim/UTR+
Vim/UTR-
Control
Vim/UTR+
Vim/UTR-
Control
Vim/UTR+
Vim/UTR-
Control
Vim/UTR+
Vim/UTR-
One Cell Stage
Stage 10
Stage 14
Stage 17

Fig. 3

undetectable by stage 10.5 (data not shown and Fig. 4). These data demonstrate that elements in the Xwnt-8 3' UTR can destabilize a heterologous transcript in *Xenopus* embryos, beginning at the early gastrula stage.

Destabilization of transcripts requires de novo protein synthesis

To determine whether the onset of degradation of Vim/UTR+ transcripts requires new protein synthesis at the gastrula stage, the persistence of injected Vim/UTR- and Vim/UTR+ transcripts was compared in embryos cultured in the absence or presence of cycloheximide (5 μg/ml) beginning at the late blastula (stage 9) or early gastrula (stage 10) stages. As shown in Fig. 4 (Northern analysis was performed by Jan Christian), in the absence of cycloheximide (CHX -), Vim/UTR- transcripts persisted at steady state levels throughout gastrulation whereas Vim/UTR+ transcripts remained stable through the early gastrula stage (stage 10) and were rapidly degraded by stage 10.5 (compare VIM signal in lanes 5 and 9). Incubation in cycloheximide beginning at stage 9 blocked degradation of Vim/UTR+ transcripts (compare lanes 9 and 10) while incubation beginning at stage 10 did not (compare lanes 9 and 11).

To determine whether steady state levels of endogenous Xwnt-8 transcripts are altered by inhibition of protein synthesis, the same filter was rehybridized with an antisense Xwnt-8 riboprobe (Fig. 4, Xwnt-8). Incubation in cycloheximide had no effect on the accumulation of endogenous Xwnt-8 transcripts at stage 10, led to a slight increase in accumulation by stage 10.5 (compare lanes 9 and 10, 12 and 13) and to a

Fig. 4. Xwnt-8 3' UTR-mediated destabilization of transcripts requires de novo protein synthesis. Vim/UTR+ or Vim/UTR- transcripts were injected into one-cell embryos which were then cultured in the presence or absence of cycloheximide (CHX) as indicated [CHX: incubated in the absence (-) of CHX, or in the presence of CHX beginning at stage 9 (9) or 10 (10)]. RNA was extracted from 10 embryos in each experimental group (RNA: con; uninjected controls, UTR+; Vim/UTR+ injected, UTR-; Vim/UTR- injected) at the indicated developmental stages and the persistance of injected RNA was assayed by Northern blot hybridization using a Vim antisense riboprobe (VIM). The same filter was rehybridized with an antisense Xwnt-8 riboprobe (Xwnt-8).

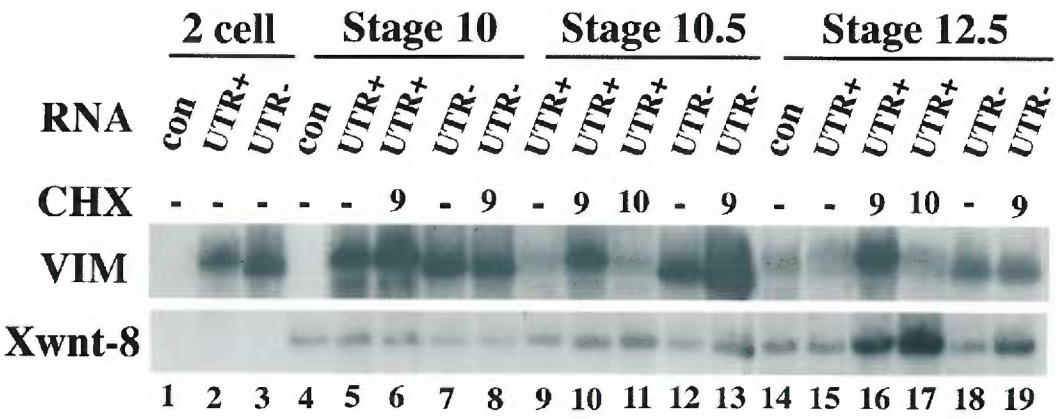

2 cell
Stage 10
Stage 10.5
Stage 12.5
RNA
con
UTR+
UTR+
UTR-
UTR-
UTR+
UTR+
UTR+
UTR-
UTR-
con
UTR+
UTR+
UTR+
UTR-
UTR-
CHX
- - - - - 9 - 9 - 9 10 - 9 - - 9 10 - 9
VIM
Xwnt-8
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19

Fig. 4

much larger increase by stage 12.5 (compare lanes 15 and 16, 15 and 17). These results are consistent with the possibility that 3' UTR-mediated degradation of Xwnt-8 transcripts requires a protein(s) that is (are) synthesized shortly before the onset of gastrulation, or that is (are) extremely labile.

The 3' UTR of Xwnt-8 negatively regulates translation of reporter RNAs

To determine whether the 3'UTR of Xwnt-8 can regulate translation of reporter RNAs, Vim/UTR+ and Vim/UTR- transcripts (These constructs were made by Michael Dixon in Christian Lab) were injected near the dorsal midline of cleaving embryos and synthesis of epitope-tagged reporter protein was analyzed by whole mount immunostaining of staged embryos (Whole mount immunostaining was performed by Michael Dixon in Christian Lab), and by Western blot analysis of embryonic extracts, using a monoclonal antibody specific for the myc-epitope. As shown in Fig. 5A, Vim/UTR- transcripts were translated to produce abundant immunoreactive protein (arrows) that was readily detected by the blastula stage (stage 7). In contrast, weak or no immunoreactivity was detected in blastula stage embryos injected with Vim/UTR+ RNA, despite the presence of abundant Vim/UTR+ transcripts at least until the gastrula stage (Fig. 3 and 4). Inhibition of translation of Vim/UTR+ was also apparent when transcripts were targeted to ventral cells or to other regions of the embryo (data not shown). Western blot analysis confirmed that Vim/UTR- transcripts were translated in vivo to yield a 55 Kd protein that was readily detected by the blastula stage (stage 7) and persisted at least until the neurula

Fig. 5. The 3'UTR of Xwnt-8 inhibits translation of a reporter transcript in developing embryos. (A) Vim/UTR+ or Vim/UTR- RNAs were injected near the dorsal side of cleaving embryos and synthesis of Vim protein was analyzed by immunostaining whole embryos at the indicated stages with antiserum specific for the myc-epitope tag present in the Vim reporter protein. Arrows indicate specific staining. (B) Vim/UTR+ or Vim/UTR- RNAs were injected into one-cell embryos and total proteins were extracted from injected and uninjected (control) embryos at the indicated stages. A Western blot containing three embryo equivalents of each extract was probed with anti-myc antiserum and was reprobed with an antibody specific for spectrin to demonstrate the presence of protein in each lane.

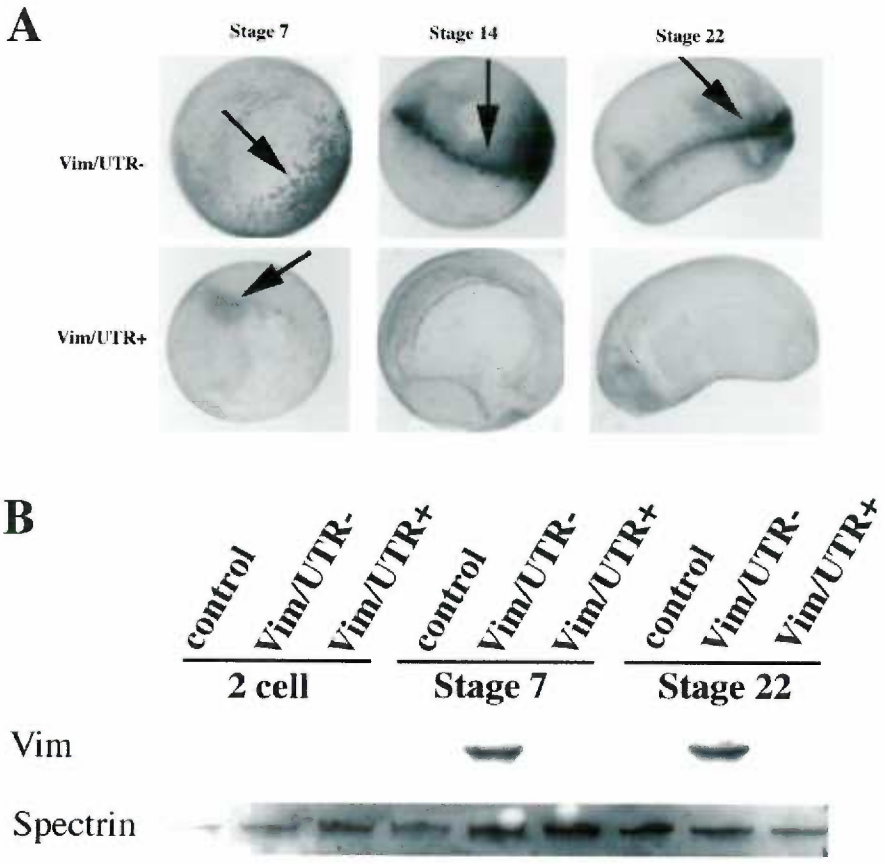

A
Stage 7
Stage 14
Stage 22
Vim/UTR-
Vim/UTR+
B
control
Vim/UTR-
Vim/UTR+
control
Vim/UTR-
Vim/UTR+
control
Vim/UTR-
Vim/UTR+
2 cell
Stage 7
Stage 22
Vim
Spectrin

Fig. 5

stage (stage 22, Fig. 5B). In contrast, protein generated by translation of Vim/UTR+ transcripts was only detected when blots were overexposed, being many fold less abundant than protein generated by translation of Vim/UTR- (not visible on exposure shown in Fig. 5B). These data show that the 3' UTR of Xwnt-8 can inhibit the translation of a heterologous reporter RNA in developing *Xenopus* embryos and that this inhibition is not temporally or spatially regulated.

Identification of sequence elements that negatively regulate RNA stability and translation

To begin to identify sequence elements in the 3' UTR of Xwnt-8 that negatively regulate RNA stability or translation, we generated deletion mutant forms of the UTR and analyzed their ability to repress translation and to destabilize reporter transcripts in vivo (These experiments were performed by Jan Christian). The 3' UTR of Xwnt-8 is 436 nucleotides in length and contains a canonical polyadenylation signal, AAUAAA (Fig. 6A, bold and italicized), located 10 nucleotides upstream of the polyadenylate [poly(A)] tract. Three deletion mutant forms of the UTR, one consisting of nucleotides 27 to 436 (UTR, sequence between asterisks in Fig. 6A), a second consisting of nucleotides 92 to 287 (UTR1, underlined in Fig. 6A), and a third consisting of nucleotides 132 to 287 (UTR2, sequence between arrows in Fig. 6A) were subcloned downstream of sequence encoding six copies of a myc epitope tag (MT). Synthetic MT RNAs with no UTR, or containing deletion mutant forms of the Xwnt-8 3' UTR (illustrated schematically in Fig.

Fig. 6. Deletion analysis of the Xwnt-8 3'UTR identifies a 194 nucleotide regulatory region. (A) Nucleotide sequence of the Xwnt-8 3' UTR with deletion mutant forms indicated. UTR consists of sequence between asterisks, UTR1 sequence is underlined and UTR2 consists of sequence between the two arrows. (B) Reporter RNAs used to test the function of the three deletion mutants indicated in (A) are shown schematically. Black box represents myc coding sequence, black line, Xwnt-8 3' UTR and open box, SV40 polyadenlyation signal. Reporter RNAs were injected into one-cell *Xenopus* embryos and post-gastrula stage stability, and midblastula stage translation, were assayed by Northern analysis and whole mount immunostaining as described in the legends to Fig. 3 and 5.

A

CGGCAUUCCAGAAAAUG UCUCCGACCUCAACUCGGGC CAGUGGAUUUGUUUGCCUUC 57

AGGAUUUCUUCCUUAAUUUA UGGACUUAAAGUAACGUCAU UAAAAGUAUAAGCUAUAAAG 117

GUGGUCUGUUGUAAAUAUUA AUAUAUAUGCACUUAUGAAU AUUCAUUAUAACCCCGUGUA 177

GAGAUUUCUACACCGCAGAG UAUUCCAUGAAACAGAAACA UGCUUCCGUUCCGAAGGAUA 237

UGUUGAUUUGAUGAACAAUA UAUUAAUUAAUUAAAUGGGU CUUUUUCAUGAAUUCAUAAA 297

CGAUUGGUCUGUCCUGAGAU AUCGGGAGAUAUAUAUAUAU AAAUAUGGAAUUUGCAGACA 357

UUCUUUGCAUUGCAUCCAUU CAGUUUUUUCUGUGCUCAUG UUUUAUGAACAUCAUCAGUA 417

UUU***AAUAAA***AAUAUUUUAC(A)$_n$

B

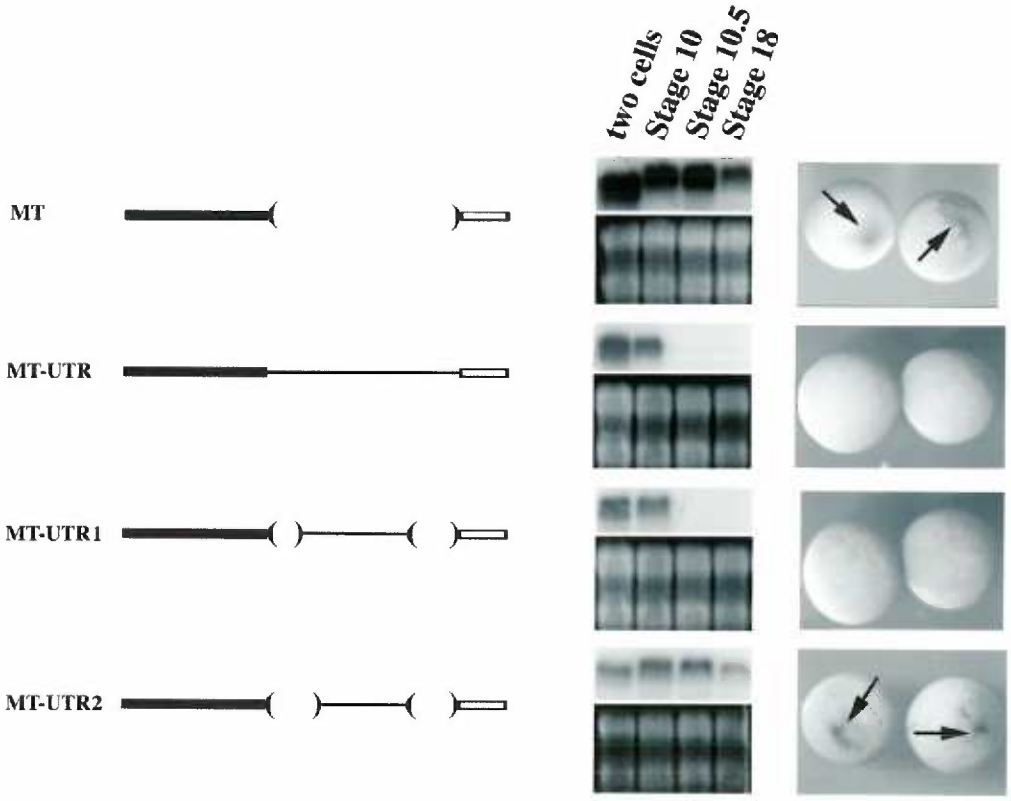


Fig. 6

6B) were injected into *Xenopus* embryos and the translation and stability of each transcript was examined by immunostaining and Northern blot analysis, respectively, as described in the previous section. MT and MT-UTR2 transcripts persisted at least until the mid-neurula stage (stage 18) and generated abundant immunoreactive protein at the midblastula stage (Fig. 6B). In contrast, MT-UTR and MT-UTR1 transcripts were rapidly degraded following the onset of gastrulation and immunoreactive protein was rarely detected in embryos injected with these transcripts (Fig. 6B). These studies identify a 194 nucleotide stretch of the Xwnt-8 3' UTR (UTR1) that is sufficient to destabilize and repress translation of reporter transcripts in *Xenopus* embryos, and demonstrate that the first 40 nucleotides of this sequence are essential for this negative regulation.

The majority of endogenous Xwnt-8 RNAs are not associated with polysomes

The above results demonstrate that elements in the 3' UTR of Xwnt-8 can repress translation of reporter transcripts. We used a polysome recruitment assay to determine whether these same elements inhibit translation of endogenous Xwnt-8 transcripts. As shown in Fig. 7A, the majority of endogenous Xwnt-8 transcripts were found in the non-polysome (supernatant) fraction, suggesting that Xwnt-8 RNA is inefficiently translated in vivo. As a positive control, we also analyzed polysomal recruitment of MT-UTR2 RNA following injection into *Xenopus* embryos. MT-UTR2 transcripts were

Fig. 7. Endogenous Xwnt-8 RNA is inefficiently translated. (A) RNA from stage 11 embryos was separated into polysomal (pellet) and non-polysomal (supernatant) fractions. As a control, some samples were adjusted to 20mM EDTA which dissociates RNA from ribosomes. The presence of Xwnt-8 transcripts in each fraction was analyzed by Northern blot hybridization. (B) Northern blot analysis of RNA extracted from MT/UTR2-injected embryos at stage 9 and separated into polysomal and non-polysomal fractions.

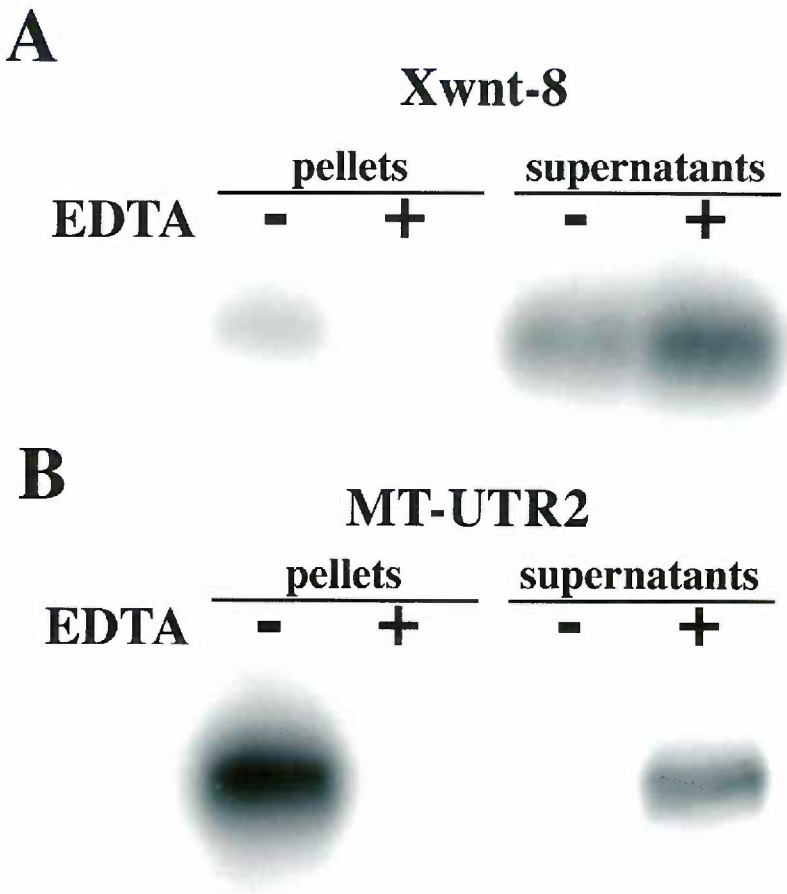

A
Xwnt-8
pellets
supernatants
EDTA
-
+
-
+
B
MT-UTR2
pellets
supernatants
EDTA
-
+
-
+

Fig. 7

found solely in polysomal (pellet) fractions (Fig. 7B), consistent with our previous finding that the portion of the Xwnt-8 3' UTR contained in MT-UTR2 is not sufficient to repress translation. In all experiments, addition of EDTA led to the release of transcripts from polyribosomal pools. These results are consistent with the hypothesis that sequence elements in the 3' UTR of endogenous Xwnt-8 transcripts inhibit translation of these mRNAs in vivo.

Transcripts containing the 3' UTR of Xwnt-8 have a short poly(A) tail

One mechanism by which UTRs of RNAs control transcript stability and/or translation is by regulating the length of the 3' poly(A) tail (Jacobson and Peltz, 1996). We used a PCR-based poly(A) assay (Robbie et al., 1995, illustrated schematically in Fig. 8A) to determine whether elements in the Xwnt-8 3' UTR can regulate polyadenylation of reporter transcripts, and to assay the polyadenylation status of endogenous Xwnt-8 transcripts. Given that UTR1, but not UTR2, confers early degradation and blocks translation of a reporter transcript (Fig. 6B), we compared the polyadenylation status of MT-UTR1 and MT-UTR2 transcripts following injection into *Xenopus* embryos. As shown in Fig. 8B, amplification of MT-UTR1 cDNAs produced a single compact band of PCR products, consistent with the presence of a short poly(A) tail. In contrast, amplification of MT-UTR2 yielded a heterogeneous smear of products that range in size up to several hundred nucleotides longer than the MT-UTR1 products, consistent with the presence of a long poly(A) tail. RT-PCR analysis of endogenous Xwnt-8 transcripts

Fig. 8. RNAs bearing the Xwnt-8 3'UTR have a short poly(A) tail. (A) Design of RT PCR assay for polyA tail length. RNA was reverse transcribed using an oligo-dT/adapter primer and the resultant cDNA amplified using primers that anneal to the 3' adapter sequence and sequence in the Xwnt-8 3' UTR. (B) MT-UTR1 or MT-UTR2 transcripts were injected into Xenopus embryos and the poly(A) tail length of each transcript was assayed by RT PCR amplification of RNAs extracted from injected embryos at stage 9 in the presence (+) or absence (-) or reverse transcriptase (RT). Specific products were detected by hybridizing Southern blots with a radiolabeled Xwnt-8 UTR probe. (C) RT-PCR analysis of polyA tail length of endogenous Xwnt-8 transcripts at the indicated developmental stages.

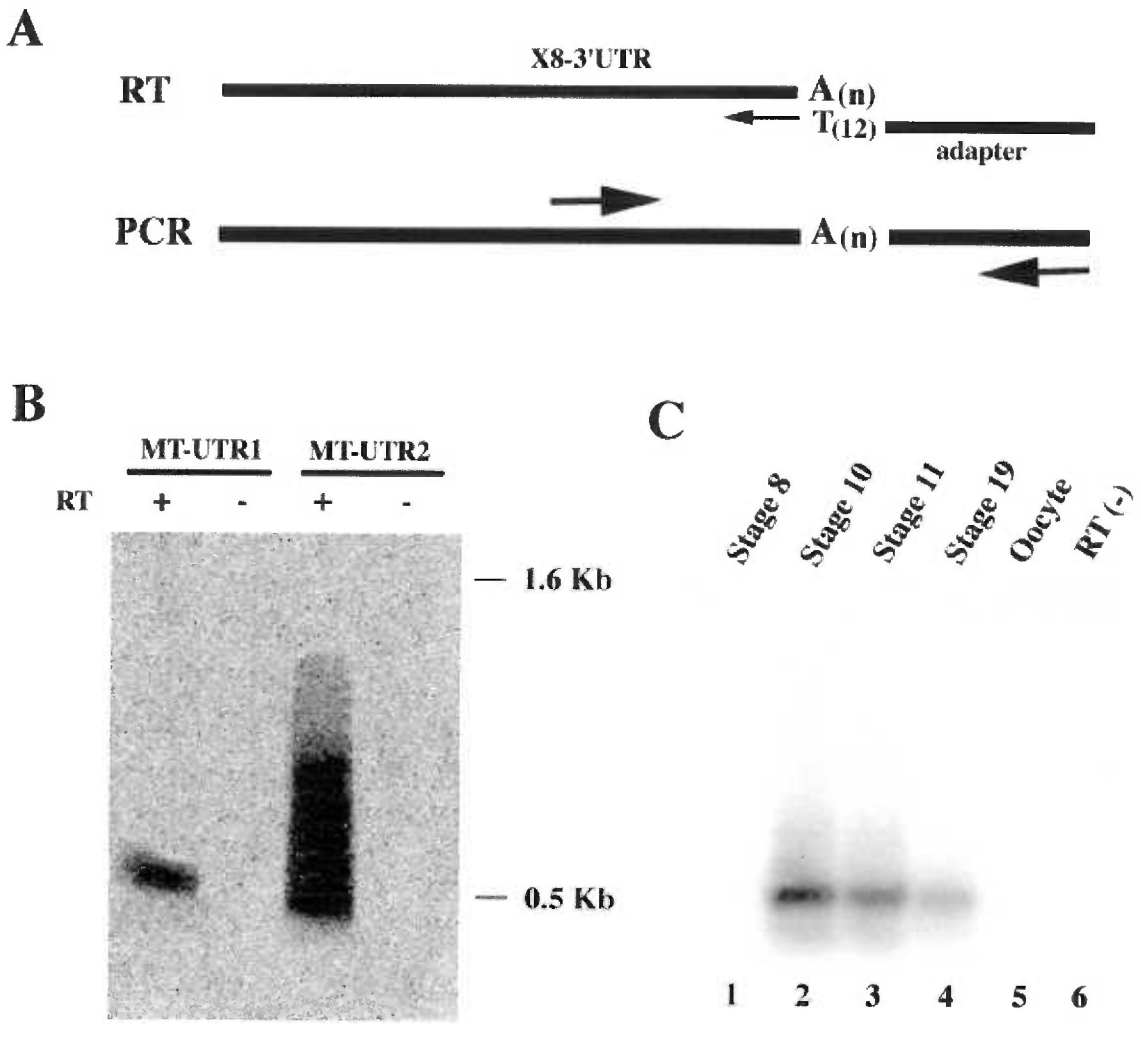

A
X8-3'UTR
RT
A(n)
T(12)
adapter
PCR
A(n)
B
MT-UTR1
MT-UTR2
RT + - + -
1.6 Kb
0.5 Kb
C
Stage 8
Stage 10
Stage 11
Stage 19
Oocyte
RT (-)
1 2 3 4 5 6

Fig. 8

yielded a single compact band at all developmental stages that *Xwnt-8* is expressed (Fig. 8C), suggesting that these transcripts possess a short poly(A) tail. Taken together, our results are consistent with the hypothesis that elements in the Xwnt-8 3' UTR repress translation and destabilize RNAs by repressing polyadenylation, or by promoting deadenlyation.

3'UTR-mediated degradation of Xwnt-8 RNA is essential for normal patterning of somites

Endogenous Xwnt-8 transcripts are present in ventral and lateral mesodermal cells during gastrulation but are rapidly downregulated in lateral cells, which will give rise to the somites, shortly thereafter (Christian and Moon, 1993, illustrated schematically above Fig. 9, Xwnt-8 expressing cells shaded pink, regions fated to form somites hatched). To begin to test whether this downregulation involves UTR-mediated destabilization of endogenous transcripts, we asked whether ectopic expression of an RNA that is complementary to the regulatory region of the Xwnt-8 3' UTR (UTR1) leads to prolonged expression of endogenous Xwnt-8 transcripts in presomitic mesodermal cells. Antisense UTR1 RNA or, as a negative control, UTR2 RNA (1 ng) was co-injected together with RNA encoding ß-galactosidase (which serves as a lineage tracer) near the dorsolateral marginal zone on one side of four cells embryos, as indicated in the schematic above Fig. 9. This targets the transcripts primarily to presomitic and somitic mesoderm (hatched in schematic) on one side of the embryo. Injected embryos were

Fig. 9. UTR-mediated degradation of endogenous Xwnt-8 transcripts is required for normal myogenesis. (A) Synthetic RNAs complementary to UTR2 (A') or UTR1 (B', C') were co-injected with ß-galactosidase RNA into the dorsolateral marginal zone on one side of four-cell embryos as illustrated. This injection targets transcripts to regions of the embryo fated to form somites (hatched) on one side of the body. Embryos were stained for ß-galactosidase activity and then hybridized with digoxigenin-labeled Xwnt-8 probe (purple stain). Staining of Xwnt-8 positive cells in the nervous system (arrowheads) and dorsolateral regions (arrows) is indicated. (C) Embryos were injected with antisense UTR2 (left panel) or UTR1 (right panel) RNAs as illustrated in panel A, cultured to the tailbud stage, stained for ß-galactoside(blue) and then immunostained with a muscle specific antibody (brown stain). Paraffin sections of stained embryos are shown. Blocks of muscle on the injected (arrow) and uninjected (arrowhead) side of each embryo are indicated.

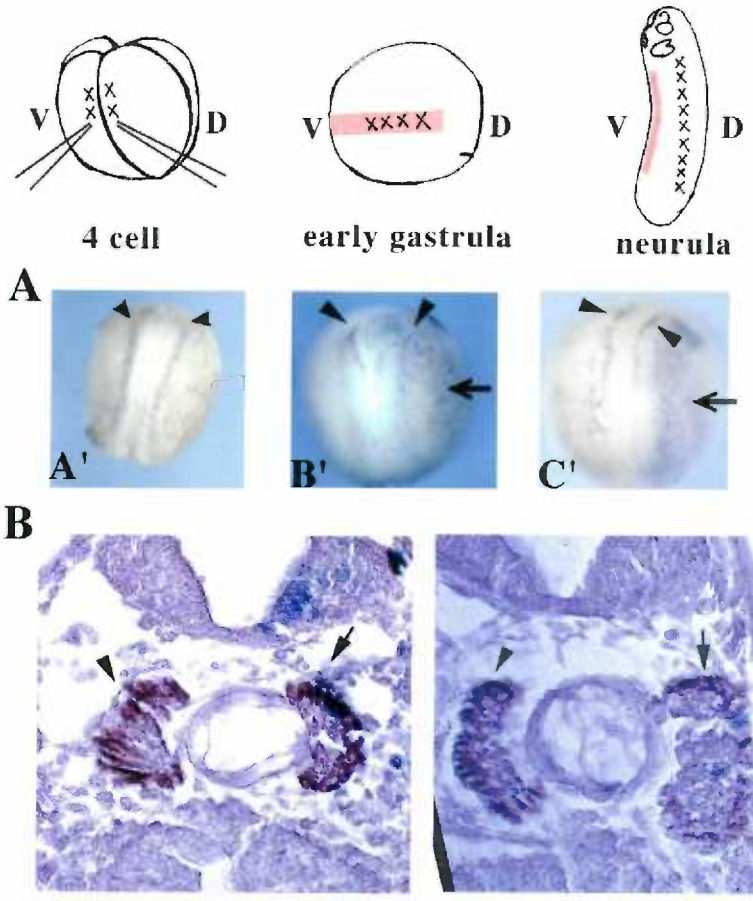


Fig. 9

stained for ß-galactosidase activity at the mid-neurula stage (stage 18), following which endogenous Xwnt-8 transcripts were detected by whole mount in situ hybridization. Embryos injected with antisense UTR2 RNA showed expression of Xwnt-8 in a subset of neural cells (Fig. 9A, A', arrowheads) and in ventral mesodermal cells (not visible in dorsal view), as did uninjected embryos (data not shown). Embryos injected with antisense UTR1 showed Xwnt-8 staining in ventral and neural (Fig. 9A, B' and C', arrowheads) cells but Xwnt-8 transcripts were also detected in dorsolateral (somitic) mesodermal cells on the ß-galactosidase-expressing side of many embryos (Fig. 9A, B' and C', arrows). This result is consistent with the hypothesis that UTR-mediated destabilization of Xwnt-8 transcripts is essential for downregulation of *Xwnt-8* expression in somitic mesoderm following gastrulation.

To determine whether post-transcriptional downregulation of Xwnt-8 expression is necessary for normal patterning of the somites, we analyzed differentiation of one somitic derivative, namely muscle, in embryos in which expression of Xwnt-8 was upregulated by unilateral targeted injection of antisense UTR1 RNA, or by injection of a Xwnt-8 expression plasmid. Embryos were injected as described above, cultured to stage 32, stained for ß-galactosidase activity, immunostained with a muscle-specific antibody, embedded in paraffin and sectioned for histological analysis. Embryos injected with antisense UTR2 RNA formed bilaterally symmetric blocks of muscle (Fig. 9B, left panel) on the injected (arrow) and uninjected (arrowhead) side of the embryo at all anterior-posterior levels that were examined. In contrast, in a subset of embryos

made to express antisense UTR1 RNA, the myotome was much larger on the injected side of the embryo (Fig. 9, right panel, arrow) relative to that on the uninjected side (arrowhead). An identical unilateral enlargement of the myotome was observed following injection of a Xwnt-8 expression plasmid that lacks the 3' UTR, but was not observed following injection of an expression plasmid that includes UTR sequence (data not shown). These results suggest that rapid, 3'UTR-mediated degradation of endogenous Xwnt-8 transcripts in the somitic mesoderm following gastrulation is essential to prevent overcommitment of these cells to a myogenic fate.

DISCUSSION

Negative post-trancriptional regulation of *Xwnt-8* expression mediated by novel sequence elements in the 3' UTR of Xwnt-8 RNAs

In this study, we have investigated the role of post-trancriptional regulatory mechanisms in controlling embryonic expression of the zygotically transcribed gene, *Xwnt-8*. Our results demonstrate that elements in the 3' UTR of Xwnt-8 RNAs negatively regulate adenylation, repress translation, and lead to stage-specific degradation of transcripts.

Regulation of RNA stability is a feature of many transiently expressed genes, including maternally expressed embryonic genes (reviewed by Wickens et al., 1997). The mechanism by which such transcripts are targeted for degradation, and the sequence elements that control their stability, have been studied extensively. Two of the best characterized determinants of RNA instability in vertebrate organisms are the AU-rich element (ARE), found in RNAs encoding many short lived cytokines and oncogenes,

and the embryo deadenylation element (EDEN) that directs degradation of a class of maternally encoded RNAs in *Xenopus* embryos. AREs and EDENs are located in the 3' UTR of transcripts and both direct deadenylation as the first step in RNA decay. AREs are characterized by the presence of one or more AUUUA motif (reviewed by Jacobson and Peltz, 1996) while the EDEN is composed of a minimum of four UA/G repeats (Audic et al., 1998; Paillard et al., 1998).

Given that Xwnt-8 transcripts are deadenylated and destabilized by elements in the 3' UTR, it is possible that this represents an EDEN- or ARE-mediated event. The Xwnt-8 RNA contains a potential EDEN motif [$(UA)_6$; residues 327-338 in Fig. 6A)] as well as a potential ARE (AUUUA, residues 417-421 in Fig. 6A) within the 3' UTR. Neither of these motifs is required for deadenylation or degradation, however, since MT-UTR1 RNAs, which lack these sequences, are not adenylated and are rapidly degraded during gastrulation. A second potential EDEN [$(UA/G)_4$, residues 139-146 in Fig. 6A] is located within the portion of the Xwnt-8 3'UTR included in MT-UTR1 transcripts, consistent with the possibility that it contributes to their instability. This EDEN is not sufficient to mediate instability, however, since it is also contained within MT-UTR2 transcripts, which have a long poly(A) tail and remain stable throughout development. Our results suggest that Xwnt-8 transcripts contain a novel instability determinant(s).

Xwnt-8 3' UTR-mediated deadenylation (or inhibition of adenylation) is temporally uncoupled from transcript degradation as evidenced by the fact that deadenylation of reporter transcripts is apparent many hours prior to the time that these RNAs are degraded. A similar uncoupling of deadenylation and degradation of maternally encoded RNAs in *Xenopus* embryos has been reported. Specifically, reporter transcripts bearing either an ARE (Voeltz and Steitz, 1998) or an EDEN (Paris et al.,

1988; Duval et al., 1990) are deadenylated immediately after fertilization but remain stable until the MBT. Synthetic RNAs that are injected into embryos as poly(A)$^-$ transcripts are also protected from degradation until the MBT, thereby demonstrating that post-MBT degradation is dependent solely on deadenylation, and does not require specific sequence information (Audic et al., 1998). It is possible that a component of the degradation machinery is first expressed at the MBT.

Although Xwnt-8 3' UTR-mediated transcript degradation is mechanistically similar to that of previously characterized maternal transcripts, the timing of transcript degradation is quite different. In *Xenopus,* degradation of deadenylated maternal transcripts begins shortly after the MBT (Paris et al., 1988; Duval et al., 1990), which occurs at six to seven hours post-fertilization. In contrast, reporter transcripts bearing the Xwnt-8 3' UTR remain stable through the onset of gastrulation and are then rapidly degraded at approximately 11 hours of development (stage 10.5). The observation that inhibition of protein synthesis does not affect accumulation of Xwnt-8 transcripts until stage 10.5 suggests that endogenous Xwnt-8 transcripts are similarly stable until gastrulation begins. This delay in the onset of degradation of Xwnt-8 RNAs, relative to that of maternal transcripts, may be critical for normal patterning. Specifically, transcription of the *Xwnt-8* gene initiates shortly after the MBT (Christian et al., 1991) and normal patterning of ventral and lateral mesoderm requires that sufficient Xwnt-8 protein accumulate to activate the Wnt signal transduction pathway within these cells during gastrulation (Hoppler et al., 1996). Since Xwnt-8 transcripts are inefficiently translated, most likely due to their short polyA tail, a mechanism that operates to delay degradation of these RNAs may be critical to enable transcripts to accumulate to high enough levels to generate sufficient protein for normal patterning. It is possible that sequence elements in the Xwnt-8 3' UTR protect the deadenylated transcripts from the

default pathway of rapid degradation at the MBT, although further experiments will be required to test this hypothesis.

Destabilization of Xwnt-8 transcripts requires ongoing protein synthesis

Inhibitors of protein synthesis have been shown to stabilize a wide variety of labile RNAs in mammalian cells (reviewed by Sachs, 1993; Ross, 1997) and in *Xenopus* (Duval et al., 1990), and several explanations for this phenomena have been suggested. Firstly, it has been hypothesized that nucleases or other factors involved in degradation are ribosome-associated and that ongoing translation of an RNA is required for its breakdown. This explanation is unlikely to account for stabilization of Xwnt-8 transcripts in the presence of cycloheximide since the majority of these RNAs are not associated with polyribosomes. Secondly, it is possible that degradation of Xwnt-8 transcripts is mediated by a newly synthesized protein encoded by an RNA that is first translated at the onset of gastrulation. This also does not seem probable since endogenous Xwnt-8 transcripts are stabilized even when cycloheximide is applied beginning at the mid-gastrula stage (stage 11.5, Christian, unpublished data). Finally, the rapid turnover of Xwnt-8 transcripts may require a labile RNA-degrading factor that requires ongoing synthesis to remain active.

Destabilization of Xwnt-8 transcripts is required for normal patterning of somites.

Our results demonstrating that inhibition of Xwnt-8 transcript destabilization leads to an overcommitment of somitic cells to a myogenic fate are consistent with previous studies showing a role for Wnt signaling in formation of the dermomyotome (reviewed by Yamaguchi, 1997). Myogenic fate is specified by a cascade of inductive and patterning signals. In *Xenopus*, the first set of signals operates during gastrulation when BMP

antagonists, such as noggin and chordin, are secreted by organizer cells and dorsalize adjacent lateral (presomitic) mesoderm. In the absence of these signals, lateral mesoderm adopts a more ventral fate and is unable to form muscle (reviewed by Harland and Gerhart, 1997). *Xwnt-8* is expressed throughout the ventral and lateral mesoderm during this time and functions to antagonize dorsalizing signals provided by the organizer (Christian and Moon, 1993). In the absence of Xwnt-8 function, lateral mesodermal cells fail to express early markers of myogenic fate, such as MyoD, and muscle differentiation is repressed (Hoppler et al., 1996). Thus, *Xwnt-8* is required for the initial specification of presomitic fate during gastrulation. Following gastrulation, *Xwnt-8* is no longer expressed in the paraxial mesoderm, although expression in the ventral mesoderm persists.

In addition to specifying presomitic fate, *Wnts* participate in the subsequent dorsoventral patterning of the segmental blocks of paraxial mesoderm, the somites, from which skeletal muscle is derived. During neurulation, individual somites begin to segregate away from the presomitic mesoderm and are patterned along their dorsoventral axis by signals originating from adjacent tissues, such as the neural tube and notochord (reviewed by Yamaguchi, 1997). The ventral portion of each somite will give rise to sclerotome, the source of cartilage, while the dorsal portion of each somite will form the dermomyotome and give rise to muscle precursors and other cell types. Sonic hedgehog, secreted from the notochord and floor plate, is required for formation of the sclerotome (Chiang et al., 1996) while Wnt-1 and Wnt-3a, secreted from the dorsal neural tube, play an essential role in the formation of the dermomyotome and in the differentiation of myotomal cells (Ikeya and Takada, 1998).

Given that dorsally restricted Wnt signals are required for formation of the dermomyotome during neurulation, it is understandable that prolonged expression of *Xwnt-8* throughout the somitic mesoderm leads to an overcommitment to myogenic fate.

Indeed, previous studies have shown that ectopic expression of Wnt-1 within the presomitic mesoderm during the time that somites are being patterned causes an expansion of the dermomyotome and represses sclerotome formation (Capdevila et al., 1998). Thus, although Xwnt-8 is required in presomitic mesodermal cells during gastrulation, it must be rapidly cleared from these same cells following this period. Our results demonstrate that transcriptional downregulation of expression of *Xwnt-8* within the early mesoderm is not sufficient to allow proper patterning of the somites, and reveal an essential role for regulated RNA stability and/or translation in this process.

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SUMMARY AND FUTURE DIRECTION

The *Xwnt-8* gene is transiently expressed in ventral and lateral mesoderm during gastrulation and plays a critical role in patterning these tissues. In the preceding studies, we have shown that the spatial and temporal pattern of expression of endogenous *Xwnt-8* is regulated, in part, at a post-transcriptional level. We have identified a novel sequence element in the 3' untranslated region of the Xwnt-8 RNA that controls the polyadenylation status of reporter and endogenous Xwnt-8 RNAs, directs rapid RNA degradation beginning precisely at the early gastrula stage, and represses translation of transcripts throughout development. Expression of endogenous *Xwnt-8* is normally downregulated within lateral (presomitic) mesoderm following gastrulation. We have demonstrated that rapid degradation of Xwnt-8 transcripts, mediated by these regulatory elements in the 3' untranslated region, is essential to this process and that downregulation is required to prevent overcommitment of somitic cells to a myogenic fate. These studies have demonstrated a role for post-transcriptional regulation of zygotic gene expression in vertebrate embryonic patterning.

Specifically, we identified a 194 nucleotide stretch of the Xwnt-8 3' UTR (UTR1) that is sufficient to destabilize and repress translation of reporter transcripts in *Xenopus* embryos; additionally, we demonstrated that the first 40 nucleotides of this sequence are essential for this negative regulation. Further deletion experiments and experiments with introduction of various point mutations can now be done to demonstrate the minimum regulatory sequence.

Since we have shown that destabilization of Xwnt-8 transcripts requires ongoing protein synthesis, there is at least one protein being made that facilitates mRNA turn over. Perhaps this protein interacts directly with the Xwnt-8 transcript. To explore this

possibility, the following experiment can be done: UV-crosslinking of protein to 3' UTR sequence (especially UTR1 sequence), followed by purification and characterization of the crosslinked protein. A logical direction for future work is to resolve the relative contributions of primary sequence and structure in the recognition of 3' UTR element by proteins, perhaps by determining co-crystal structures. Also, protein-binding experiments could be utilized to test how antisense UTR1 works. The idea that the antisense oligonucleotides anneals to UTR1 and interferes with the protein-binding ability of UTR1 might be tested *in vitro*.

One possible mechanism by which Xwnt-8 RNA is degraded rapidly is 3' UTR-mediated cytoplasmic deadenylation, i.e., the mRNA is adenylated in the nucleus but is deadenylated and degraded shortly after it is exported from nucleus to cytoplasm. To begin to investigate this possibility, one might separate nuclear RNA from cytoplasmic RNA, and observe the polyadenylation status of Xwnt-8 transcripts in each fraction.

Analysis of secondary structure of the Xwnt-8 3' UTR (UTR1) and of the mutual effects of this novel regulatory element with other destabilization elements will tell us more about the mechanisms through which the Xwnt-8 3' UTR functions.

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