Regulation of the mRNAs Encoding Proteins of the BMP Signaling Pathway during the Maternal Stages of Xenopus Development

Brian R. Fritz and Michael D. Sheets
Department of Biomolecular Chemistry, University of Wisconsin, Madison, Wisconsin 53706

Activation of the Xenopus bone morphogenetic protein (BMP) pathway is coincident with the onset of zygotic transcription but requires maternal signaling proteins. The mechanisms controlling the translation of mRNAs that encode proteins of the BMP pathway were investigated by using polysome association as an assay for translational activity. Our results indicate that five different mRNAs encoding proteins of the BMP pathway were translationally regulated during Xenopus development. These mRNAs were either not associated or inefficiently associated with polysomes in oocytes, and each was recruited to polysomes at a different developmental stage. The Smad1 and ALK-2 mRNAs were recruited to polysomes during oocyte maturation, whereas the BMP-7 and XSTK9 mRNAs were recruited during the early stages of embryogenesis. The ALK-3 mRNA was not efficiently associated with polysomes during any maternal stage of development and was efficiently recruited to polysomes only after the onset of zygotic transcription. In general, for all stages except oocytes, polysome recruitment was associated with the presence of a 3\(^*\) poly(A) tail. However, there was not an obvious correlation between the absolute length of poly(A) and the efficiency of polysome recruitment, indicating that the relationship between poly(A) tail length and translation during early frog embryogenesis is complex. We further focused on the BMP-7 mRNA and demonstrated that sequence elements within the 3\(^*\) UTR were necessary for recruitment of the BMP-7 mRNA to polysomes and sufficient to direct the addition of poly(A) and activate translation of a reporter during embryogenesis. Interestingly, the BMP-7 mRNA lacks the previously defined eCPE sequences proposed to direct poly(A) addition and translational activation during embryogenesis. The implications of our findings for translational regulation of maternal mRNAs during embryogenesis and for the activation of the BMP pathway are discussed. © 2001 Academic Press

Key Words: translational control; Xenopus embryogenesis; BMP signaling.

INTRODUCTION

The earliest stages of development in both vertebrate and invertebrate organisms proceed in the absence of new mRNA synthesis (Davidson, 1986). Thus, all changes in protein levels during these early stages of embryogenesis are controlled by posttranscriptional mechanisms such as the regulated translation of stored maternal mRNAs (Curtis et al., 1995; Seydoux, 1996; Gray and Wickens, 1998). The translational activation of the appropriate stored mRNAs at the appropriate time after fertilization directs the synthesis of proteins necessary for the normal completion of embryogenesis. In particular, data from invertebrates indicate that many of these translationally regulated mRNAs encode proteins that are regulators of cell-fate decisions. For example, in Drosophila, the regulated translation of the mRNAs encoding the bicoid, hunchback, nanos, and oskar proteins is essential for establishing the normal segmentation and axial patterns in the embryo (Driever and Nusslein-Volhard, 1988; Tautz and Pfaffle, 1989; Gavis and Lehmann, 1994; Kim-Ha et al., 1995). In Caenorhabditis elegans, translational repression of the fem-3 mRNA controls the synthesis of the fem-3 protein, which in turn is required for the switch from spermatogenesis to oogenesis in hermaphrodites (Ahringer et al., 1992; Kraemer et al., 1999; Zhang et al., 1997). These and other examples make it clear that the translational regulation of maternal mRNAs is critical for controlling cell-fate decisions that occur during the early stages of fly and nematode development.

In vertebrates, translational regulatory mechanisms control meiotic maturation, the process by which an oocyte

1 To whom correspondence should be addressed. Fax: 608-262-5253. E-mail: mdsheets@facstaff.wisc.edu.


0012-1606/01 $35.00
Copyright © 2001 by Academic Press
All rights of reproduction in any form reserved.
becomes a fertilizable egg. Both positive and negative regulatory mechanisms control the translation of specific mRNAs during oocyte maturation in frogs and mice (Huarte, 1987; Fox et al., 1989; McGrew, 1989; Hyman and Wormington, 1988; Stutz et al., 1998; Gray and Wickens, 1998; Richter, 1999). Many of these mRNAs encode proteins whose function is to control the progression of meiotic maturation itself (Sheets et al., 1994, 1995; Stebbins-Boaz et al., 1996; Gebauer and Richter, 1997; Tay et al., 2000). Interfering with either the translational mechanisms in general, such as blocking all polyadenylation, or blocking the translation of specific mRNAs, such as the c-mos mRNA, inhibits the synthesis of these key regulatory proteins and inhibits maturation (Kuge and Inoue, 1992; Gebauer et al., 1994; Sheets et al., 1995; Barkoff et al., 1998). Therefore, translational mechanisms control the synthesis of proteins that function to regulate meiotic maturation.

Translational regulatory mechanisms also function to activate the translation of specific mRNAs during the maternal stages of vertebrate embryogenesis, but less is known about these mechanisms in comparison to meiotic maturation (Dworkin et al., 1985; Paris et al., 1988; Paris and Philippe, 1990). Mechanistic information is available for only a few such mRNAs. For example, translation of the maternal CI1 and CI2 mRNAs is activated during the embryonic stages following fertilization. The temporal activation of these mRNAs is regulated by the addition of poly(A) that requires 3'UTR sequence elements distinct from the elements that direct poly(A) addition during maturation, suggesting that different translational regulatory mechanisms operate during oocyte maturation and embryonic development (Simon et al., 1992; Simon and Richter, 1994).

The importance of translational mechanisms for regulating the synthesis of proteins controlling cell-fate decisions during the maternal stages of vertebrate embryogenesis is unclear. To begin addressing these questions, we have focused on the mRNAs encoding proteins of the Bone Morphogenetic Protein (BMP) pathway in the frog, Xenopus laevis. The BMP signal transduction pathway is required for the formation and patterning of specific types of mesodermal and ectodermal tissues (Hogan, 1996; Heasman, 1997; Dale and Jones, 1999). mRNAs encoding many of the proteins of this pathway are present maternally, suggesting that BMP signaling may be regulated by translational mechanisms during early frog development (Nishimatsu et al., 1992a,b; Graff et al., 1994, 1996; Hawley et al., 1995; Suzuki et al., 1997). More recent studies demonstrated that phosphorylated Smad1 protein, which serves as a measure of active BMP signaling, is first detected at the late blastula stage of frog development. Significantly, this Smad1 phosphorylation and activation of the BMP pathway does not require zygotic mRNA synthesis, indicating that all proteins of the BMP pathway have been synthesized from maternal mRNAs (Faure et al., 2000). These results raise the possibility that translational control mechanisms temporally regulate the assembly and potentially the activation of the BMP pathway by controlling the synthesis of BMP pathway proteins from preexisting maternal mRNAs.

In this report, we have measured polysome association to assay the translational regulation of maternal mRNAs encoding a subset of the proteins of the BMP pathway in frog embryos. Significantly, mRNAs encoding components of the BMP pathway, specifically the BMP-7 ligand, the ALK-2, ALK-3, and XSTK9 receptors (putative BMP receptors), and the Smad1 transcription factor, were each recruited to polysomes during frog embryogenesis. Specifically, each of these mRNAs was either not associated or inefficiently associated with polysomes in oocytes and was efficiently recruited to polysomes during oocyte maturation or embryogenesis. In general, each mRNA's recruitment to polysomes was associated with the presence or the addition of a poly(A) tail, consistent with a mechanistic relationship existing between poly(A) elongation and translational activation during embryogenesis. To provide further insights into translational regulatory mechanisms that operate during vertebrate embryogenesis, we focused on the BMP-7 mRNA since it was efficiently recruited to polysomes only during the embryonic stages following fertilization. Our data indicated that the 3'UTR of the BMP-7 mRNA was both necessary and sufficient to recapitulate the regulated translation and polyadenylation of the endogenous BMP-7 mRNA. Together, these data indicate that translational regulation may be important for the assembly and activation of the BMP signaling pathway and provide a foundation for investigating the role that translational mechanisms play in vertebrate embryogenesis.

**MATERIALS AND METHODS**

**Collection of Oocytes, Eggs, and Embryos**

Oocytes were harvested from anesthetized females (0.02% benzocaine) and manually defolliculated in 1× oocyte ringer (100 mM NaCl, 1.8 mM KCl, 1 mM MgCl₂, 2 mM CaCl₂, 5 mM NaHCO₃, pH 7.0). Oocytes were collected, frozen immediately on dry ice, and stored at −80°C. Eggs were harvested from females injected with 650 units of chorionic gonadotropin (Sigma). For fertilization, eggs were collected in a glass dish and mixed with macerated testes. After 10 min, the dish was filled with 0.25× MMR, and embryos were allowed to develop at room temperature. Eggs and embryos were dejellied with 2% cysteine-HCl, pH 8.0, frozen immediately on dry ice, and stored at −80°C.

**Total RNA Isolation from Xenopus Oocytes, Eggs, and Embryos**

RNA was isolated from Xenopus oocytes, eggs, or embryos by Trizol extraction (Gibco/BRL) following the manufacturer's instructions, with the addition of a phenol/chloroform extraction prior to the precipitation of RNA with isopropanol. RNA was resuspended in DEPC-treated water and stored at −80°C.
Polysome Fractionation

Polysomes were isolated from Xenopus oocytes, eggs, and embryos by using standard procedures with the following modifications (Wormington, 1991). Polysome buffer (PB, 300 mM KCl, 2 mM MgCl₂, 20 mM Tris-HCl, pH 7.5, 4 μg/mL polyvinylsulfate) was prepared on ice and allowed to equilibrate at 4°C from 4 to 24 h prior to use. Immediately prior to use, polysome buffer was supplemented with sodium deoxycholate to 0.5% (from freshly prepared 10% w/v stock), dithiothreitol (DTT) to 4 mM and rRNAasin (Promega) to 25 U/mL. Twenty oocytes, eggs, or embryos were homogenized on ice in a cold room (4°C) in 500 μL of polysome buffer (PB). An additional 500 μL of PB was added to the homogenate, mixed by pipetting several times, and transferred to a second eppendorf tube. Homogenates were spun in a microfuge at 12,000g, 4°C for 15 min. The supernatant from the clearing spin was removed, taking care not to disturb the postmitochondrial pellet, and transferred to a 15-mL falcon tube containing either 2 mL of PB or 2 mL of PB plus 15 mM EDTA. Diluted supernatants were layered over 2.5 mL of PB, or PB + EDTA, containing 25% (w/v) sucrose and subjected to ultracentrifugation at 144,000g, 4°C for 2 h, in a Beckman SW55Ti rotor. Following ultracentrifugation, the supernatant fractions were recovered, taking care not to disturb the pellet, and transferred to sterile 10-mL centrifuge tubes. RNA was isolated from the non-polyosomal supernatant fractions with two extractions using an equal volume of phenol/chloroform. The RNA was precipitated overnight with 2.5 volumes of ethanol, 1/10th volume of 5 M NaCl, and 70 μg of glycogen at −20°C. The RNA was further purified by addition of an equal volume of 8 M LiCl and precipitation at −20°C for at least 4 h. The precipitate was spun in a microfuge at 10,000g, 4°C for 30 min, washed with 150 μL of cold 70% ethanol, resuspended in DEPC-treated water, and stored at −80°C. RNA was isolated from the polysomal pellet fractions by Trizol extraction as outlined above.

Three important controls were performed to ensure the consistency and validity of the polysome fractionation and analysis. First, to ensure that the mRNA pelletting was due to its association with polysomes, each fractionation experiment was repeated with the addition of EDTA to the polysome buffers. EDTA chelates Mg²⁺ions, causing polysomes to dissociate and release bound mRNAs into the supernatant fraction. Therefore, mRNAs were only considered to be associated with polysomes if they were found in the supernatant fraction in the presence of EDTA. Second, the amount of each mRNA in the combined polysome and nonpolysome fractions was compared to the amount of each mRNA present in unfractionated material for each stage of development. This control ensured that substantial amounts of material were not lost during fractionation. Only experiments where the amount of each message in the combined polysomal pellet and nonpolysomal supernatant was at least 75% of the unfractionated starting material were used for analysis, with the exception of oocyte samples. The discrepancy in amounts of mRNA between total and fractionated samples in oocytes results from the strong association of mRNAs with an unidentified component of the oocyte, possibly the cytoskeleton. This association causes mRNAs to fractionate into the postmitochondrial pellet (PMP) during extract preparation (Fritz and Sheets, unpublished data). All mRNAs that we have analyzed, including actively translated messages such as the actin and ribosomal protein mRNAs, are associated with the PMP of oocytes but not the PMP from eggs and embryos. Furthermore, the association of mRNAs with the PMP from oocytes is unaffected by changing the composition of the polysome isolation solution, including variations in Mg²⁺ concentration, ionic, and nonionic detergents at low and high concentrations, and the presence of EDTA during homogenization (data not shown). Third, each polysome fractionation experiment was analyzed with respect to two mRNAs, the D7 and cytoskeletal actin mRNAs, whose translational behavior has been well characterized (Dworkin et al., 1985; Ballantine et al., 1979; Sturgess et al., 1980). All samples from polysome isolation experiments were analyzed for the correct fractionation of the D7 and cytoskeletal actin mRNAs and the only samples considered for further analysis were those in which the D7 and actin mRNAs fractionated correctly.

Fractionation of RNA According to poly(A) Tail Length

To estimate the length of poly(A) tails present on specific mRNAs, thermal elution from oligo dT was used to separate RNAs into three fractions based on the length of their poly(A) tails. Total RNA from Xenopus oocytes, eggs, and embryos was fractionated with respect to poly(A) tail length by using biotinylated oligo(dT)₂₅, streptavidin-coated magnetic particles, and a modification of the manufacturer’s protocol (PolyATract IV, Promega). All aliquots of total RNA (160 μg) were heated at 65°C for 10 min and then supplemented with 13 μL of 20X SSC and 3 μL of oligo(dT)₂₅ (50 pmol/μL). The RNA was cooled quickly on ice (90 s), and added to the washed streptavidin-coated magnetic particles (washed and transferred to siliconized eppendorf tubes). The RNA bound to the particles was separated from unbound RNA by using a magnet provided by the manufacturer. The unbond, or poly(A⁻) supernatant fraction was recovered and supplemented with glycogen (60 μg) and yeast RNA (20 μg), and frozen immediately on dry ice. Poly(A⁺) RNA was eluted from the magnetic particles by using two successive 10-min washes of increasing stringency. The first wash consisted of 0.1X SSC at 30°C and the second wash was 0.1X SSC at 35°C. Each wash solution was supplemented with glycogen and yeast RNA immediately prior to resuspension of the magnetic particles and incubation at the appropriate elution temperature. After each incubation, the supernatant fractions were recovered and frozen immediately on dry ice. The RNA in all fractions was extracted twice with an equal volume of phenol/chloroform, precipitated with ethanol and NaCl overnight at −20°C and resuspended in DEPC-treated water. To confirm that this strategy separated RNAs based on poly(A) tail length, a sample containing a 93-nucleotide32P-labeled cyclin RNA was injected into oocytes where it receives a short poly(A) tail (see Fig. 5D, lane 6). Some of the injected oocytes were matured, causing the poly(A) tail of the labeled cyclin RNA to elongate to over 200 nucleotides during maturation (Fig. 5D, lane 7). RNAs isolated from injected oocytes and matured oocytes were mixed with uninject cyclin B1 RNA to generate a sample where the cyclin B1 RNA possessed a complete range of poly(A) lengths that varied in length from 0 to over 200 nucleotides (see Fig. 2A, lane 2). The polyadenylated RNA was supplemented with total RNA from oocytes, fractionated as described above, and analyzed by denaturing polyacrylamide gel electrophoresis and autoradiography.

RNA Blot Hybridization Analysis

RNA for analysis was treated with deionized glyoxal and subjected to electrophoresis through 1% agarose (Seakem LE), 10 mM...
sodium phosphate (pH 7.0) at 50 volts for 3–4 h with recirculation (Sheets et al., 1994). RNA was transferred to Pall Biodyne A nylon membranes via capillary action and 20× SSC for 16–24 h. RNA was fixed to membranes by UV crosslinking and baking at 80°C for 2 h. Glyoxal adducts were removed by boiling the filters in 20 mM Tris, pH 8.0, for 3 min. Single-stranded antisense DNA probes were generated with asymmetric PCR using either phage promoter primers or template-specific primers (100 pmol), 1 μg of template DNA, 0.2 mM dTTP, dGTP, and dATP, 0.02 mM dCTP and 50–150 μCi of [α-32P]dCTP per 50-μL reaction. Probes were purified by S-400 columns as recommended by the manufacturer (Pharmacia). Filters were prehybridized at least 1 h at 65°C in hybridization solution (1% BSA, 7% SDS, 0.25 mM NaPhosphate, pH 7.2, and 1 mM EDTA). Following replacement of the hybridization solution, filters were hybridized overnight at 65°C with 0.7× 106 cpm/mL of probe. Hybridized filters were washed three times for 30 min in 150–250 mL wash solution (5% SDS, 40 mM NaPhosphate, pH 7.2, and 1 mM EDTA). Following replacement of the hybridization solution, filters were hybridized overnight at 65°C with 0.7× 106 to 3.5× 108 cpm/mL of probe. Hybridized filters were washed three times for 30 min in 150–250 mL wash solution (5% SDS, 40 mM NaPhosphate, pH 7.2, and 1 mM EDTA) at 65°C. Longer wash times (up to 16 h) were necessary when filters were simultaneously hybridized with multiple probes. Hybridization signals were quantified using a PhosphorImager (Molecular Dynamics).

**Oocyte Injection, Maturation, and Activation**

Oocytes were injected with 25 ng of HPLC-purified DNA oligonucleotide (Operon). The sequence of the antisense oligonucleotide used was 5′-GACGTGTAAAAACAGAATAACTGC-3′, and this sequence is complimentary to the 5′ end of the BMP-7 3′UTR, immediately downstream of the stop codon (Nishimatsu et al., 1992b). The sense oligonucleotide used was 5′-GCAGTTAT-TCTGTTTTTACAGTC-3′, and this oligonucleotide was injected as a negative control. In each experiment, oocytes were injected twice with oligonucleotide on opposite sides of the animal pole and incubated at room temperature for 2 h. Some of the injected oocytes were matured into eggs by treatment with progesterone (10 μg/mL), and the eggs were activated by treatment with the Ca2+ ionophore A23187 (10 μM). Eggs were incubated in ionophore until cortical contraction was observed (usually within 7–10 min), and were washed three times with 1× MMR and maintained in 0.25× MMR for 5 h.

**Injection of BMP7 3′UTR and In Vivo Polyadenylation Assay**

The 3′UTR of BMP7 mRNA was obtained by 3′RACE (Gibco/BRL) using total RNA from stage 7 frog embryos and a gene-specific primer with the sequence 5′-CAAGCGTGGTGTTGGCATTGCAG-3′. PCR products were cloned into the EcoRV site of pSTBlue-1 (Novagen) and sequenced. A DNA fragment containing the BMP-7 3′UTR was subsequently cloned into the KpnI and HindIII sites of the pSTBlue-1 plasmid to generate pSTBM7-3′UTR. This plasmid was linearized with HindIII and transcribed in vitro by using T7 RNA polymerase (Epitext) in the presence of 6.3 mM ATP and CTP, 1.5 mM UTP and GTP, 3 mM monomethylated cap analog (NEB), and 200 μCi of [γ-32P]UTP. This generated a 360-nucleotide 32P-labeled RNA consisting of the BMP-7 3′UTR. As a control, cyclin B1 3′UTR RNA was transcribed from plasmid pMDS999 linearized with XbaI using T7 RNA polymerase to generate an RNA of 94 nucleotides (Sheets et al., 1989). 32P-labeled RNAs were gel-purified through 6% polyacrylamide, 7 M urea, 1× TBE at 250 V, 4°C for 4 h, excised from the gel and eluted overnight into 300 μL of elution buffer (0.5 M ammonium acetate, 1 mM EDTA, 0.2% SDS) at room temperature. Eluted RNAs were purified by phenol/chloroform extraction followed by ethanol precipitation a total of three times. The purified RNAs were resuspended in DEPC-treated water at a concentration of ~0.2 μM. RNAs were injected into Xenopus oocytes in a volume of 10 nl (~2 fmol/oocyte). RNAs were isolated from oocytes, matured oocytes, and activated eggs (5–10 ea.) by homogenization in 20 μL/oocyte of buffer containing: 200 mM Tris, pH 8.0, 25 mM EDTA, 300 mM NaCl, 2% SDS. The homogenate was extracted twice with phenol/chloroform, ethanol precipitated, and resuspended in 20 μL of DEPC-treated water. Labeled RNAs were analyzed by denaturing PAGE (electrophoresed through 6% polyacrylamide, 7 M urea, 1× TBE) and autoradiography.

**Injection of Luciferase Reporter mRNAs and Luciferase Assay**

The BMP7 3′UTR was PCR amplified from pSTBM7-3′UTR with the primers CCATGGATCCAATAGCAGTTATTCT-GTTTTT and AAAAAAGTCAGATCCTGTTACTAAAATTGT GTTTAAT. The resulting DNA fragment was cloned into the BglII and KpnI sites of the pTFLucBglII plasmid to generate pLucBM7 3′UTR (Gallie, 1991). To generate mRNAs encoding luciferase and luciferase/BMP7 3′UTR, plasmid templates were then linearized by digestion with BglII, and transcribed in vitro by using T7 RNA polymerase as described above (nucleotide concentrations of 7.5 mM (ATP, CTP, and UTP) and 1.5 mM GTP, and omitting 32P-labeled UTP). As a control, luciferase/cyclin B1 3′UTR mRNA (Sheets et al., 1994) was prepared from the pTFLuc/cyclin B1 plasmid linearized with BamHI. mRNAs were purified with Microcon YM-10 concentrators (Millipore) and resuspended in DEPC-treated water. Each RNA was diluted to a concentration of 50 nM, and injected into oocytes in a 10-nL volume (0.5 fmol/oocyte). Oocytes were matured into eggs, and the eggs were activated as described above. Cell extracts were prepared from samples of 5–10 oocytes, eggs, or activated eggs by homogenization on ice in 50 μL/cell of 1× Cell Culture Lysis Reagent (Promega) and centrifuged at 12,000 g, 4°C for 10 min. The clear supernatant (20 μL) was aliquoted into assay tubes (5 replicates/sample), 100 μL of luciferase assay reagent (Promega) was added, and each tube was then immediately analyzed in a Berthold Lumat LB9501 luminometer (12 s assay time).

**Embryo Dissection**

**Anterior/posterior dissections.** Embryos with regular cleavage planes and asymmetric pigmentation differences were selected at the four-cell stage. The lightly pigmented side representing the prospective anterior side of the embryo was marked with nile blue sulfate. When the embryos reached the blastula stage, stage 7, the vitelline membranes were removed and the embryos were cut vertically into anterior and posterior halves, formerly called the dorsal and ventral halves, respectively (Lane and Sheets, 2000; Lane and Smith, 1999). Some of the embryos were left intact to develop to the gastrula stage. These control embryos were examined to ensure that the nile blue marks coincided with the early blastopore lip that delineates the anterior midline of the embryo.

**Animal/vegetal dissections.** After removal of the vitelline membranes, stage-7 embryos were cut horizontally along the pigment boundary to generate animal and vegetal halves.
RESULTS

The Maternal mRNAs Encoding Proteins of the BMP-Signaling Pathway Are Efficiently Recruited to Polysomes during Oocyte Maturation and Embryogenesis

To test whether the translation of mRNAs encoding proteins of the BMP signaling pathway is regulated during the maternal stages of Xenopus development, we quantitatively analyzed the association of these mRNAs with polysomes. Extracts prepared from Xenopus oocytes, unfertilized eggs, and embryos were separated into polysomal (P) and nonpolysomal (N) fractions by using standard methodologies (Wormington, 1991). Each fraction was analyzed with quantitative RNA blot hybridization to detect the presence of mRNAs encoding the BMP-7 ligand, two closely related putative BMP type I receptors (ALK-2 and ALK-3), a BMP type II receptor (XSTK9), and a BMP-specific transcription factor (Smad1) (Nishimatsu et al., 1992a,b; Graff et al., 1994, 1996; Hawley et al., 1995; Thomsen, 1996; Suzuki et al., 1997). A representative sample of these results is shown in Fig. 1A, and a quantitative summary of at least three independent experiments is shown in Fig. 1B.

All five of the maternal mRNAs encoding proteins of the BMP pathway were inefficiently associated with polysomes and present in the nonpolysomal (N) fraction from oocytes (Fig. 1A, compare lanes 2 and 3; Fig. 1B). No BMP-7 mRNA was detected in the polysomal fraction from oocytes, whereas only small amounts of the Smad1, ALK-2, and ALK-3 mRNAs were associated with polysomes in oocytes (Fig. 1A, compare lanes 2 and 3; and Fig. 1B). The small amount of XSTK9 mRNA in the polysomal fraction from both oocytes and eggs (Fig. 1, lanes 2 and 5) was not due to a bona fide association with ribosomes, since this interaction was not disrupted by the presence of EDTA (data not shown).

As controls for these experiments, the polysome association of the D7 and cytoskeletal actin mRNAs, two mRNAs whose translation has been characterized, were analyzed. Previous studies demonstrated that the D7 mRNA is not loaded onto polysomes in oocytes, but translated in eggs, and our results are consistent with these findings (Fig. 1A, lanes 2 and 3, Fig. 1B) (Dworkin et al., 1992a, 1992b; Graff et al., 1994, 1996; Hawley et al., 1995; Thomsen, 1996; Suzuki et al., 1997). A representative sample of these results is shown in Fig. 1A, and a quantitative summary of at least three independent experiments is shown in Fig. 1B.

The poly(A) Tails of the mRNAs Encoding Proteins of the BMP Signaling Pathway Change in Length during Oocyte Maturation and Embryogenesis

We find that the ALK-2 and Smad1 mRNAs were efficiently recruited to polysomes during oocyte maturation and embryogenesis, whereas the BMP-7 and XSTK9 mRNAs were efficiently recruited to polysomes only during embryogenesis. One mechanism that governs the translational activation of maternal mRNAs in Xenopus and other species is the regulated elongation of their poly(A) tails (Fox et al., 1989; McGrew et al., 1989; Gray and Wickens, 1998; Richter, 1999). To determine whether this mechanism was responsible for the translational activation of the mRNAs encoding proteins of the BMP pathway, we analyzed the length of these mRNAs' poly(A) tails at different stages of maternal development.

The poly(A) tails of specific mRNAs can be measured by using RNAseH cleavage combined with high resolution RNA blot hybridization or PCR-based strategies (Mercer and Wake, 1985; Sheets et al., 1994; Sales and Strickland, 1995). However, these approaches require that the 3'UTR sequence of the mRNA to be analyzed must be known. For the Smad1, ALK-3, and XSTK9 mRNAs, the sequences of the 3'UTRs have not been determined. Therefore, we used a strategy to analyze mRNA poly(A) tails that was independent of 3'UTR sequence information (Simon et al., 1996).

To estimate the length of poly(A) tails present on specific mRNAs, thermal elution from oligo(dT) was used to separate mRNA samples into three fractions; a poly(A)- fraction, a poly(A)+ fraction eluted at 30°C that contains poly(A) mRNAs encoding proteins of the BMP pathway were inefficiently associated with polysomes and present in the nonpolysomal (N) fraction from oocytes (Fig. 1A, compare lanes 2 and 3; Fig. 1B). The small amount of XSTK9 mRNA in the polysomal fraction from both oocytes and eggs (Fig. 1, lanes 2 and 5) was not due to a bona fide association with ribosomes, since this interaction was not disrupted by the presence of EDTA (data not shown).

As controls for these experiments, the polysome association of the D7 and cytoskeletal actin mRNAs, two mRNAs whose translation has been characterized, were analyzed. Previous studies demonstrated that the D7 mRNA is not loaded onto polysomes in oocytes, but translated in eggs, and our results are consistent with these findings (Fig. 1A, lanes 2 and 3, Fig. 1B) (Dworkin et al., 1992a, 1992b; Graff et al., 1994, 1996; Hawley et al., 1995; Thomsen, 1996; Suzuki et al., 1997). A representative sample of these results is shown in Fig. 1A, and a quantitative summary of at least three independent experiments is shown in Fig. 1B.

The poly(A) Tails of the mRNAs Encoding Proteins of the BMP Signaling Pathway Change in Length during Oocyte Maturation and Embryogenesis

We find that the ALK-2 and Smad1 mRNAs were efficiently recruited to polysomes during oocyte maturation and embryogenesis, whereas the BMP-7 and XSTK9 mRNAs were efficiently recruited to polysomes only during embryogenesis. One mechanism that governs the translational activation of maternal mRNAs in Xenopus and other species is the regulated elongation of their poly(A) tails (Fox et al., 1989; McGrew et al., 1989; Gray and Wickens, 1998; Richter, 1999). To determine whether this mechanism was responsible for the translational activation of the mRNAs encoding proteins of the BMP pathway, we analyzed the length of these mRNAs' poly(A) tails at different stages of maternal development.

The poly(A) tails of specific mRNAs can be measured by using RNAseH cleavage combined with high resolution RNA blot hybridization or PCR-based strategies (Mercer and Wake, 1985; Sheets et al., 1994; Sales and Strickland, 1995). However, these approaches require that the 3'UTR sequence of the mRNA to be analyzed must be known. For the Smad1, ALK-3, and XSTK9 mRNAs, the sequences of the 3'UTRs have not been determined. Therefore, we used a strategy to analyze mRNA poly(A) tails that was independent of 3'UTR sequence information (Simon et al., 1996).

To estimate the length of poly(A) tails present on specific mRNAs, thermal elution from oligo(dT) was used to separate mRNA samples into three fractions; a poly(A)- fraction, a poly(A)+ fraction eluted at 30°C that contains poly(A) mRNAs encoding proteins of the BMP pathway were inefficiently associated with polysomes and present in the nonpolysomal (N) fraction from oocytes (Fig. 1A, compare lanes 2 and 3; Fig. 1B). The small amount of XSTK9 mRNA in the polysomal fraction from both oocytes and eggs (Fig. 1, lanes 2 and 5) was not due to a bona fide association with ribosomes, since this interaction was not disrupted by the presence of EDTA (data not shown).

As controls for these experiments, the polysome association of the D7 and cytoskeletal actin mRNAs, two mRNAs whose translation has been characterized, were analyzed. Previous studies demonstrated that the D7 mRNA is not loaded onto polysomes in oocytes, but translated in eggs, and our results are consistent with these findings (Fig. 1A, lanes 2 and 3, Fig. 1B) (Dworkin et al., 1985). The cytoskeletal actin mRNA is translationally active in oocytes but not in eggs, and this mRNA is present in the polysomal fraction in oocytes but not eggs (Fig. 1A, lanes 2 and 3, Fig. 1C) (Ballantine et al., 1979; Sturgess et al., 1980). Therefore, the analysis of these mRNAs, whose translational behavior has been previously characterized, indicates that this polysome assay accurately reflects the translation activity of the mRNAs analyzed.

During maturation of oocytes to eggs, a significant fraction of the ALK-2 and Smad1 mRNA populations became loaded onto polysomes (Fig. 1A, compare lanes 2 and 5; Fig. 1B: 22% of the ALK-2 mRNA and 53% of the Smad1 mRNA was associated with polysomes in unfertilized eggs). A small amount of BMP-7 mRNA was also associated with polysomes in eggs (6%). This mRNA is completely released from the polysomal pellet by the presence of EDTA, indicating that the fractionation of this mRNA is due to an authentic association with polysomes (data not shown). In contrast, the ALK-3 and XSTK9 mRNAs remained predominantly in the nonpolysomal fraction in eggs (Fig. 1A, compare lanes 2 and 5).

Polyosome analysis was repeated by using extracts from Xenopus embryos to determine whether the mRNAs encoding proteins of the BMP pathway were actively translated during the postfertilization stages of Xenopus development, (Fig. 1A, lanes 7–14, Fig. 1B). The fractions of the BMP-7 and XSTK9 mRNAs associated with polysomes increased after fertilization (Fig. 1A, stage 2 embryos, compare lanes 5 and 8, Fig. 1B) and increased significantly by the early blastula stages (Fig. 1A, stage 7 embryos, compare lanes 8 and 11, Fig. 1B). Furthermore, fractions of the Smad1 and ALK-2 mRNAs associated with polysomes increased after fertilization and during embryogenesis. In contrast, the association of ALK-3 mRNA with polysomes was not efficient at any stage examined (Fig. 1A, lanes 2, 5, 8, and 11, Fig. 1B). Thus, the BMP-7 and XSTK9 mRNAs are efficiently recruited to polysomes during Xenopus embryogenesis, while the ALK-3 mRNA is inefficiently associated with polysomes in oocytes, eggs, and maternal embryonic stages.

Copyright © 2001 by Academic Press. All rights of reproduction in any form reserved.
mRNAs with short to intermediate poly(A) tails, and a poly(A) + fraction eluted at 35°C that contains mRNAs with long poly(A) tails. To demonstrate the effectiveness of this strategy, we fractionated a 32P-labeled cyclin B1 3'UTR that possessed a distribution of poly(A) lengths, from 0 up to greater than 200 nucleotides in length (Fig. 2A, lane 2 compare with uninjected and unpolyadenylated RNA in lane 1). This RNA was applied to oligo(dT), and the RNA that bound was eluted with a 30°C wash followed by a 35°C wash. Analysis of each fraction by denaturing polyacrylamide gel electrophoresis indicated that the unbound fraction contains unpolyadenylated RNA and RNAs with very short poly(A) tails less than 30 nucleotides in length (Fig. 2A, lane 3). The 30°C fraction predominantly contained

**FIG. 1.** The maternal mRNAs encoding proteins of the BMP-signaling pathway were efficiently recruited to polysomes during oocyte maturation and embryogenesis. (A) Polysomal (P) and nonpolysomal (N) fractions were prepared from *Xenopus laevis* oocytes (lanes 1–3), eggs (lanes 4–6), two-cell stage embryos (lanes 7–9), and stage-7 blastula embryos (lanes 10–14). RNA was isolated from unfractonated samples (T), the polysomal (P) and nonpolysomal supernatant (N) fractions and analyzed by RNA blot hybridization. Probes were used to detect mRNAs encoding the *Xenopus* ALK-2 type I receptor, the ALK-3 type I receptor, the BMP7 ligand, the Smad1 transcription factor, the XSTK9 type II receptor, the D7 and cytoskeletal actin proteins. A representative RNA blot analyzing each mRNA at each stage of development is shown. An example of the EDTA control experiment is shown with st. 7 RNA fractionation (lanes 13 and 14). mRNAs which are genuinely associated with polysomes will fractionate with the polysomal pellet in the absence of EDTA and with the nonpolysomal supernatant in the presence of EDTA. (B) The fraction of each mRNA population that was polysome associated at different stages of *Xenopus* development. Polysomes were isolated from 12 sets of oocytes, 8 sets of eggs, and 5 sets of embryos, and each RNA was analyzed in each experiment. The RNA blots from each experiment were scanned with a PhosphorImager (Molecular Dynamics), and the average polysome association was calculated (% in pellet – [% in pellet + EDTA]) for each mRNA and plotted for each stage of development.
FIG. 2. The poly(A) tails of mRNAs encoding proteins of the BMP signaling pathway changed in length during oocyte maturation and embryogenesis. (A) To estimate the length of poly(A) tails present on specific mRNAs, thermal elution from oligo(dT) was used to separate mRNA samples into three fractions; a poly(A)^- fraction, a poly(A)^+ fraction eluted at 30°C that contains mRNAs with short to intermediate poly(A) tails, and a poly(A)^+ fraction eluted at 35°C that contains mRNAs with long poly(A) tails. To demonstrate this strategy, we fractionated a radiolabeled cyclin B1 3’UTR RNA that possessed a distribution of poly(A) tail lengths, from 0 up to greater than 200 nucleotides in length (lane 2, polyadenylated cyclin B1 3’UTR RNA, compare with the uninjected and unpolyadenylated cyclin B1 3’UTR RNA in lane 1). The RNA in each fraction was analyzed by denaturing polyacrylamide gel electrophoresis. Lane 3, the unbound poly(A)^- fraction; lane 4, the 30°C poly(A)^+ fraction; and lane 5, the 35°C poly(A)^+ fraction. The position of RNA molecular weight markers is indicated at the right. (B) The poly(A) tails of the mRNAs encoding proteins of the BMP signaling pathway change in length during oocyte maturation and embryogenesis. Total mRNA (T) from oocytes, eggs, and embryos were fractionated according to poly(A) tail length using thermal elution from oligo(dT) into unbound (–), 30°C, and 35°C fractions. Each fraction was analyzed for the presence of the BMP-7, ALK-2, ALK-3, Smad1, XSTK9, cyclin B1, and cytoskeletal actin mRNAs by using RNA blot hybridization. A representative sample of the RNA blot hybridization results for ALK-2, ALK-3, cyclin B1, and cytoskeletal actin mRNAs is shown. (C) Quantitative summary of poly(A) length changes. mRNAs were analyzed at each stage of development at least three times. RNA blots were scanned with a PhosphorImager (Molecular Dynamics). The average amount of each mRNA in the three fractions was plotted for each stage of development.
translational regulation of the BMP pathway

During oocyte maturation and these changes have been
in the cyclin B1 and cytoskeletal mRNAs. The poly(A) tails of the
in oocytes and eggs were separated into these three fractions
and the fractions were analyzed for the presence of the
cyclin B1 and cytoskeletal mRNAs. The poly(A) tails of the
cyclin B1 and cytoskeletal actin mRNAs change in length
during oocyte maturation and these changes have been
well-characterized (Sheets et al., 1994). The cyclin B1
mRNA in oocytes possesses a distribution of poly(A) tails
approximately 30 nucleotides long that increases to 250
nucleotides during oocyte maturation. Using thermal elu-
tion from oligo(dT), we found that the cyclin B1 mRNA
from oocytes was distributed between the unbound and the
30°C poly(A) fraction, while the cyclin B1 mRNA from
eggs was present predominantly in the 35°C poly(A) fraction (Fig. 2B, lanes 3 and 8, Fig. 2C). The cytoskeletal
actin mRNA from oocytes was present in the
30°C poly(A) fraction, and this mRNA was present in the
poly(A)– fraction from eggs (Fig. 2B, lanes 3 and 6).
Therefore, these two experiments indicate that mRNAs
that have short to intermediate poly(A) tails, approximately
30–100 nucleotides in length, elute into the 30°C fraction,
while mRNAs that have long poly(A) tails of greater than
100 nucleotides in length elute into the 35°C fraction.

Total mRNA from oocytes, eggs, and embryos was frac-
tionated according to poly(A) tail length using thermal elu-
tion from oligo(dT), and each sample was analyzed for the
presence of the BMP-7, ALK-2, ALK-3, Smad1, and
XSTK9 mRNAs using RNA blot hybridization. This analy-
sis indicated that the poly(A) tails of these mRNAs undergo
differences in length during oocyte maturation and the embry-
onic stages of Xenopus development. A representative
sample of the RNA blot hybridization results is shown in
Fig. 2B, and a quantitative summary is shown in Fig. 2C.
In oocytes, the ALK-2, BMP-7, XSTK9, and Smad1 mRNAs
predominantly fractionated to the 30°C fraction and there-
fore had short to intermediate length poly(A) tails between
30 and 100 nucleotides (Fig. 2B, lane 3, and Fig. 2C). In eggs,
the majority of the Smad1 mRNA (65%) fractionated to the
35°C fraction and therefore possessed a long poly(A) tail of
greater than 100 nucleotides, indicating that it had under-
gone poly(A) elongation during maturation. In contrast, the
majority of the XSTK9 and BMP-7 mRNAs from eggs were
found in the poly(A)– fraction, indicating that these
mRNAs had been deadenylated during maturation. A sig-
ificant proportion of the ALK-2 mRNA from eggs was also
present in the poly(A)– fraction, indicating that it had been
deadenylated during maturation. However, 20% of the
ALK-2 mRNA retained a short poly(A) tail and remained in
the 30°C fraction (Figs. 2B and 2C). The amount of ALK-2
mRNA present in the 30°C fraction (20%) was very similar
to the proportion of this message associated with poly-
somes at this stage (Figs. 1A and 1B). In stage-7 embryos, at
least 50% of the ALK-2, XSTK9, and BMP-7 mRNAs were
present in the combined 30° and 35° fractions, indicating
that these mRNAs possessed short to long poly(A) tails and
had undergone poly(A) elongation following fertilization. In
contrast, the vast majority of the ALK-3 mRNA was present
in the unbound poly(A)– fraction at all stages analyzed and
therefore this mRNA either lacked or possessed a very short
poly(A) tail. Thus, with the exception of the ALK-3 mRNA,
the poly(A) tails of all the mRNAs analyzed changed during maturation and the maternal stages of embryogenesis.

Polyosomal Association of the ALK-3 mRNA
Increased after the Onset of Zygotic Transcription

The ALK-3 receptor protein is postulated to be necessary
for BMP signal transduction that regulates cell differen-
tiation during Xenopus embryogenesis (Graff et al., 1994;
Suzuki et al., 1995). However, we found that the ALK-3
mRNA was poorly associated with polysomes throughout
the maternal stages of embryogenesis, suggesting that little
if any of the ALK-3 mRNA is translated during these stages
(Figs. 1A and 1B). Therefore, to determine whether the
ALK-3 mRNA was ever efficiently translated during em-
byogenesis, the association of the ALK-3 mRNA with polysomes was analyzed from samples of embryos at later
stages of development: the blastula stages 7, 9, and the early
gastrula stage 10°, respectively. Zygotic transcription com-
ences between stage 7 and 9, and stage 10° marks the
beginning of gastrulation (Newport and Kirschner, 1982).
The ALK-3 mRNA was inefficiently associated with poly-
somes in stage-7 and -9 embryos (less than 10%, Fig. 3A,
lanes 2 and 5, Fig. 3B), while the ALK-2 and BMP-7 mRNAs
in these same embryos were efficiently loaded onto poly-
somes. However, by the gastrula stage, approximately 50% of
the ALK-3 mRNA population was recruited to polysomes
(Fig. 3A, lane 8, Fig. 3B). Therefore, the ALK-3 mRNA is
recruited to polysomes only after the onset of zygotic
transcription and coincident with or before the onset of
gastrulation.

Polysome Association of the ALK-2 and BMP-7
mRNAs Was Not Spatially Restricted

The current model for the regulation of cell differentia-
tion by the BMP pathway during Xenopus development postulates that BMP signaling is restricted to cells fated to
form the posterior tissues of the Xenopus embryo (Hogan,
1996; Heasman, 1997; Dale and Jones, 1999). One possible
mechanism of restricting BMP signaling in the embryo
would be to restrict the translation of the mRNAs encoding
proteins of the BMP pathway to specific embryonic cells.
This type of spatially restricted mRNA translation has been
observed for the Xenopus FGF receptor (Cornell et al.,
1995). Therefore, to determine whether the translation of
Polysome Recruitment during Embryogenesis

mRNAs encoding proteins of the BMP pathway was restricted to specific embryonic cells, the polysome association of the ALK-2 and BMP-7 mRNAs in specific embryonic tissue was analyzed. Stage-7 Xenopus embryos were dissected into either anterior–posterior halves, formerly referred to as the dorsal–ventral halves, respectively (Lane and Sheets, 2000; Lane and Smith, 1999) or dissected into animal–vegetal halves. Polysomes were isolated from each sample and the associated mRNAs were assayed by quantitative RNA blot hybridization. Representative results from these experiments are shown in Fig. 4A, and a quantitative summary of two experiments is presented in Fig. 4B. The ALK-2 and BMP-7 mRNAs were associated with polysomes to similar extents in anterior–posterior regions of the stage-7 Xenopus embryo (Fig. 4A, compare lane 1 with lanes 3 and 5). Comparison of the animal–vegetal regions reveals a slight enhancement of polysome recruitment for mRNAs in embryonic cells of the animal half (Fig. 4A, lanes 7–10, Fig. 4B). However, this increased polysome association was not restricted to the ALK-2 and BMP-7 mRNAs, as a similar increase in polysome loading was observed for the D7 mRNA in these cells. Thus, the polysome association of the BMP-7 and ALK-2 mRNAs was not spatially restricted to particular cells of the embryo.

The 3'UTR of the BMP7 mRNA Was Necessary for Polysome Recruitment during Embryogenesis

To test whether the translational activation of the endogenous BMP-7 mRNA during embryogenesis required sequence elements in its 3'UTR, we specifically amputated the 3'UTR from the coding region of the BMP-7 mRNA. For this experiment, we took advantage of the endogenous RNaseH activity present in Xenopus oocytes and microinjected an antisense DNA oligonucleotide that was complimentary to sequences immediately 3’ of the translational stop codon within the BMP-7 mRNA’s 3’UTR. Analysis of total RNA from oocytes injected with the BMP-7 antisense DNA oligonucleotide using RNA blot hybridization indicated that the BMP-7 mRNA from injected oocytes had an increased mobility compared to this mRNA from uninjected oocytes (Fig. 5A, compare lane 1, uninjected, to lane 2, injected with the antisense DNA oligonucleotide). The increased mobility of the BMP-7 mRNA from oocytes injected with the antisense DNA oligonucleotide is consistent with the reduction in length that would occur with the removal of the 340 nucleotide 3’UTR and a short poly(A) tail from the full-length 2-kb BMP-7 mRNA (Fig. 5A, compare lanes 1 and 2) (Nishimatsu et al., 1992b; Hawley et al., 1995). The effect of the injected antisense DNA oligonucleotide was specific for the BMP-7 mRNA; the length of the ALK-2 mRNA from the same injected cells was unaffected by the antisense BMP-7 DNA oligonucleotide (Fig. 5A, lanes 1 and 2). Injection of a control DNA oligonucleotide in the sense orientation did not affect the length of the BMP-7 mRNA (data not shown). Therefore, injection of the antisense BMP-7 DNA oligonucleotide into oocytes specifically amputates the 3’UTR from the endogenous BMP-7 mRNA, leaving the coding region perfectly stable similar to what was observed following the removal of the c-mos mRNA’s 3’UTR (Sheets et al., 1995).

To determine whether translational activation of the endogenous BMP-7 mRNA depended upon its 3’UTR, we analyzed the polysome recruitment of the BMP-7 mRNA after removal of its 3’UTR. Oocytes injected with the antisense DNA oligonucleotide, oocytes injected with a control DNA oligonucleotide in the sense orientation, and uninjected oocytes were matured into eggs with progesterone. These eggs were activated with the calcium ionophore A23187 to initiate the events of embryogenesis normally triggered by fertilization, polysomal (P), and nonpolysomal (N) fractions were isolated from each sample, and the associated mRNAs were analyzed by quantitative RNA blot hybridization. 

FIG. 3. The polysome association of the ALK-3 mRNA increased after the onset of zygotic transcription. (A) Polysome analysis of mRNAs from blastula (maternal) and early zygotic stages of frog development. Polysomes were isolated from st. 7 blastula embryos (lanes 1–3), st. 9 late blastula embryos (lanes 4–6), and st. 10+ early gastrula embryos (lanes 7–11). RNA from unfractonated embryos (T), polysomal (P), and nonpolysomal (N) fractions were analyzed by RNA blot hybridization. An EDTA control experiment is shown for the st. 10+ RNA fractionation (lanes 10 and 11). (B) The fraction of each mRNA population that is polysome associated at different stages of frog development. This experiment was performed two to four times, depending on the mRNA analyzed. The RNA blots from each experiment were scanned with a PhosphorImager (Molecular Dynamics), and the average polysome association was calculated for each mRNA and each stage of development.
hybridization. Amputation of the 3'UTR from the endogenous BMP-7 mRNA completely inhibited the association of this mRNA with polysomes in activated eggs (Fig. 5B, compare lanes 1 and 3). Importantly, the BMP-7 mRNA was efficiently loaded onto polysomes in activated eggs that were uninjected or injected with a control (sense orientation) DNA oligonucleotide (Fig. 5B, lanes 1 and 5). Furthermore, the effect of the injected antisense DNA oligonucleotide was specific for the BMP-7 mRNA, since polysome loading of the ALK-2 mRNA (Fig. 5B, lanes 1–6) or other mRNAs such as Smad1 and D7 (data not shown) occurred normally in the injected cells. Thus, the 3'UTR of the endogenous BMP-7 mRNA contains important regulatory sequences that are necessary for the polysome association of this mRNA during the post-fertilization stages of embryogenesis.

The previous experiment demonstrates that the BMP-7 mRNA's 3'UTR was necessary for polysome association and translational activation of the BMP-7 mRNA during embryogenesis, but this experiment does not test sufficiency. To demonstrate that the BMP-7 3'UTR was also sufficient for translational activation, a reporter mRNA in which the BMP-7 mRNA's 3'UTR was fused 3' to the coding region of the luciferase gene was generated (Fig. 5C). Two other mRNAs were used as controls, a second mRNA in which the luciferase gene was fused to the 3'UTR from the cyclin B1 mRNA and a third mRNA consisting of the luciferase coding region alone (Gallie, 1991; Sheets et al., 1994) (Fig. 5C). Equal amounts of each mRNA were injected into oocytes, some of the oocytes were matured into eggs, and some of the eggs were activated with ionophore. Extracts were prepared from the injected cells and luciferase activity was quantitatively measured. The ratio of luciferase activity in matured oocytes (eggs) versus oocytes and the ratio of luciferase activity in activated eggs versus oocytes was calculated for each injected mRNA and graphed (Fig. 5C). Significantly, the 3'UTR of the BMP-7 mRNA stimulated translation of the luciferase reporter only in activated eggs, but not in matured oocytes. In contrast and as previously demonstrated, the 3'UTR of the cyclin B1 mRNA stimulated reporter translation during maturation and after egg activation (Sheets et al., 1994).

FIG. 4. Polysome association of the BMP-7 and ALK-2 mRNAs was not spatially regulated. (A) Polysome analysis of the BMP7, ALK-2, and D7 mRNAs in dissected halves of blastula stage embryos. St. 7 Xenopus embryos were dissected into animal and vegetal halves or into anterior and posterior halves (Lane and Sheets, 2000; Lane and Smith, 1999), formerly referred to as the dorsal and ventral halves, and polysome analysis was performed on whole embryos and the isolated halves. RNA was extracted from the polysomal (P) and nonpolysomal (N) fractions from each sample and analyzed by RNA blot hybridization. (B) The fraction of each mRNA population that is polysome associated in the different halves of st. 7 embryos. This experiment was performed twice, the RNA blots were scanned with a PhosphorImager (Molecular Dynamics), and the average amount of each mRNA in the polysomal fraction was plotted for each sample.
These results demonstrate that the 3'UTR of the BMP-7 mRNA is sufficient to activate translation of the reporter and recapitulate the translational regulation observed for the endogenous BMP-7 mRNA as measured by polysome recruitment.

The 3' UTR of the BMP-7 3' UTR Contains Sequence Elements that Can Direct the Addition of a poly(A) Tail during the Postfertilization Stages of Development

The poly(A) tail of the BMP-7 mRNA is removed or shortened to less than 30 nucleotides during oocyte maturation, and a short to intermediate length poly(A) tail is added to this mRNA during the embryonic stages following fertilization (Figs. 2B and 2C). mRNAs that receive poly(A) during vertebrate embryogenesis contain sequence elements called eCPEs in their 3'UTRs that, in conjunction with the AAUAAA sequence, direct poly(A) addition (Simon et al., 1992; Simon and Richter, 1994). The 3'UTR of the BMP-7 mRNA does not contain any readily identifiable eCPE elements (Hawley et al., 1995). Therefore, to test if sequences in the BMP-7 mRNA's 3'UTR were sufficient to direct poly(A) addition during embryogenesis, a 32P-labeled RNA consisting of the BMP-7 3'UTR was injected into oocytes. Some of the oocytes were matured into eggs and some of the eggs were activated to initiate the events of fertilization. RNA was isolated from injected oocytes, matured oocytes, and activated eggs and analyzed by denaturing polyacrylamide gel electrophoresis (Fig. 5D, lanes 1–4).

FIG. 5. The 3'UTR the BMP-7 mRNA is necessary for polysome association of the endogenous BMP-7 mRNA and sufficient to activate translation of a reporter mRNA and direct polyadenylation. (A) Removal of the 3'UTR from the endogenous BMP-7 mRNA does not affect the stability of the coding region. A DNA oligonucleotide complimentary to the 5' end of the BMP-7 mRNA's 3'UTR was injected into oocytes. Total RNA isolated from uninjected controls (lane 1), and the antisense DNA oligonucleotide injected cells (lane 2) was analyzed by RNA blot hybridization by using probes to the BMP-7 and ALK-2 mRNAs. (B) The 3'UTR from the endogenous BMP-7 mRNA is necessary for polysome association of the BMP-7 mRNA during the embryonic stages following fertilization. Oocytes injected with the antisense DNA oligonucleotide (lanes 3 and 4), oocytes injected with the control DNA oligonucleotide in sense orientation (lanes 5 and 6), and uninjected oocytes (lanes 1 and 2) were matured into eggs with progesterone. These eggs were activated with the calcium ionophore A23187 to initiate the events of embryogenesis normally triggered by fertilization. Polysomal (P) and nonpolysomal (N) fractions were isolated from each sample and the associated mRNAs were assayed by quantitative RNA blot hybridization. This experiment was performed twice and a representative result is shown. (C) The 3'UTR of the BMP-7 mRNA is sufficient to temporally activate the translation of a reporter. Luciferase/BMP-7 3'UTR, luciferase/cyclin B1 3'UTR, and luciferase mRNAs were injected into frog oocytes. The oocytes were matured into eggs with progesterone and some of the eggs were activated with ionophore. Extracts were prepared from the injected oocytes, eggs, and activated eggs and luciferase activity was determined. The ratio of luciferase activity in eggs versus oocytes and activated eggs versus oocytes was calculated and plotted. The data represent the average of two separate experiments. (D) The 3'UTR of the BMP-7 mRNA is sufficient to temporally regulate poly(A) addition. Radiolabeled RNAs consisting of the BMP-7 3'UTR or the cyclin B1 3'UTR were injected into frog oocytes. Some of the oocytes were matured into eggs with progesterone and some of the eggs were activated with calcium ionophore. RNA was extracted from each cell type and analyzed by denaturing polyacrylamide gel electrophoresis. Lane 1, BMP-7 3'UTR RNA not injected; lane 2, BMP-7 3'UTR RNA after injection into oocytes; lane 3, BMP-7 3'UTR RNA from eggs; lane 4, BMP-7 3'UTR RNA from activated eggs; lane 5, cyclin B1 3'UTR RNA not injected; lane 6, cyclin B1 3'UTR RNA after injection into oocytes; lane 7, cyclin B1 3'UTR RNA from eggs; lane 8, cyclin B1 3'UTR from activated eggs. The position of RNA molecular weight markers is indicated at the right.
The majority of the BMP-7 3'UTR from activated eggs increases in size by 40–100 nucleotides (Fig. 5D, lane 4). This increase in size is due to the addition of poly(A) as shown by oligo(dT) chromatography (data not shown). In contrast, a small proportion of the BMP-7 3'UTR increased in size by ~20 nucleotides in matured oocytes (Fig. 5D, lane 3), while the RNA in oocytes did not significantly increase in size (Fig. 5D, lane 2). As a control, a 32P-labeled RNA consisting of the 3'UTR of the cyclin B1 mRNA was injected into oocytes and the polyadenylation of this RNA in oocytes, matured oocytes, and activated eggs was analyzed in parallel. As previously demonstrated, this RNA receives a very short poly(A) tail in oocytes that is elongated to 250 nucleotides during oocyte maturation (Fig. 5D, lanes 5–8) (Sheets et al., 1994). These results demonstrate that the BMP-7 3'UTR contains sequence elements that can direct the addition of a poly(A) tail during the postfertilization stages of development.

DISCUSSION

In this report, polysome analysis was used to systemati-
cally evaluate the translational activity of maternal mRNAs encoding proteins of the BMP signaling pathway in Xenopus laevis. Significantly, the polysome association of every mRNA examined was regulated during embryogenesis, consistent with the view that the BMP pathway plays a critical role in regulating early vertebrate development. In addition, the presence of a poly(A) tail correlated well with changes in polysome association that occurred after oocyte maturation and fertilization, indicating that regulated polyadenylation may play a role in controlling the translation of this mRNA family. Analysis of the 3'UTR of the BMP-7 mRNA indicated that this element was both necessary and sufficient to recapitulate the poly(A) tail changes and translational regulation of the endogenous BMP-7 mRNA. Taken together, these data provide evidence for the role of translational control in the assembly of a functional BMP signaling pathway during vertebrate embryogenesis.

Poly(A) Tails and Translational Regulation of BMP Signaling Pathway mRNAs

In general, the presence or lengthening of a poly(A) tail correlated with changes in polysome recruitment of specific mRNAs in the BMP signaling pathway. For example, the poly(A) tail of Smad1 mRNA was lengthened during maturation, while this message was efficiently recruited to polysomes. The poly(A) tail of BMP-7 mRNA was not significantly lengthened until after fertilization at a time that corresponds well with its efficient recruitment to polysomes. In contrast, during the maternal stages of development, the ALK-3 mRNA was not polyadenylated, nor was it efficiently recruited to polysomes. However, other data indicate that there is not a simple or quantitative relationship between poly(A) tail length and the translation of the BMP pathway mRNAs. For example, the ALK-2 mRNA is recruited to polysomes during oocyte maturation and yet there is no obvious net increase in its poly(A) tail length. It is notable, however, that the amount of ALK-2 mRNA that possessed a short to intermediate poly(A) tail in eggs was very similar to the proportion of this message that was associated with polysomes at this stage. In addition, polysome association in oocytes does not appear to correlate strongly with poly(A) tail length at all. In these cells, additional mechanisms may exist to repress or activate the translation of specific mRNAs. Thus, although poly(A) tail changes may be important in the translational regulation of BMP pathway mRNAs, the mechanisms underlying this control are complex and require further investigation.

Regulated poly(A) tail changes that control translational activation require sequences within the 3'UTRs of mRNAs from a variety of organisms including Xenopus. For example, regulated poly(A) tail shortening during vertebrate oocyte maturation requires the AAUAAA sequence and specific elements called CPEs (Cytoplasmic Polyadenylation Elements) in the mRNAs' 3'UTRs (Gray and Wikens, 1998; Richter, 1999). Both the ALK-2 and Smad1 mRNAs were recruited to polysomes during oocyte maturation concomitant with the retention (ALK-2) or elongation (Smad1) of their poly(A) tails. Although the complete sequence of the Smad1 3'UTR is not known, the sequence of the ALK-2 3'UTR reveals the presence of several putative CPEs (Suzuki et al., 1997). Thus, it is probable that poly(A) regulation and translational activation of these mRNAs is related to CPE sequences within their 3'UTRs.

In contrast to the polyadenylation that occurs during maturation, less is known about the regulated polyadenylation that occurs after fertilization. Most information has come from studies examining the regulated translation and polyadenylation of two mRNAs after fertilization, the C11 and C12 mRNAs that encode proteins of unknown function (Dworkin et al., 1985; Paris et al., 1988; Paris and Philippe, 1990). These mRNAs require embryonic CPEs (eCPEs) in their 3'UTRs that are distinct from maturation-type CPEs (Simon et al., 1992; Simon and Richter, 1994). In addition, recent studies have identified an additional 3'UTR sequence that controls poly(A) addition during embryogenesis, the C-rich element (Paillard et al., 2000). We demonstrated that the 3'UTR of the BMP-7 mRNA was sufficient to direct polyadenylation and translational activation after fertilization and was required for the polysome recruitment of the endogenous BMP-7 mRNA after fertilization. These results suggest that the BMP-7 mRNA may be regulated by mechanisms similar to those that regulate the C11 and C12 mRNAs. Interestingly, however, the 3'UTR of the BMP-7 mRNA does not contain any obvious eCPE or C-rich elements (Hawley et al., 1995), suggesting that novel sequence elements or diverged eCPE variants may control both polyadenylation and polysome recruitment of this mRNA after fertilization.
Translational Control and the Regulation of BMP Pathway Function

Our data provide evidence that translation of BMP pathway mRNAs may be temporally regulated during vertebrate embryogenesis. Temporally and quantitatively regulated polysome association may be one mechanism to ensure the appropriate assembly and activation of the BMP pathway. For example, the Smad1 and ALK-2 mRNAs are recruited to polysomes earlier and to a greater extent than the BMP-7 ligand mRNA. Although the significance of why the BMP pathway is not activated before the onset of zygotic transcription is unknown (Faure et al., 2000), it is possible that precocious activation of the receptor could be detrimental to embryogenesis. Thus, the translational activation of the ALK-2 and Smad1 mRNAs followed by the later translational activation of the BMP-7 ligand mRNA may contribute to controlling the timing of pathway activation and ensure that the pathway is assembled appropriately before the ALK-2 receptor can be stimulated efficiently by ligand.

One interesting exception to the pattern of polysome recruitment observed for most BMP pathway mRNAs was the postzygotic recruitment to polysomes of the ALK-3 mRNA. Unlike the other mRNAs of the BMP pathway, the ALK-3 mRNA was inefficiently associated with polysomes prior to zygotic transcription. BMP pathway signaling requires both a type I and type II BMP receptor (Hogan, 1996; Dale and Jones, 1999). XSTK9 is a type II receptor and both ALK-2 and ALK-3 are type I receptors (Nishimatsu et al., 1992a; Graff et al., 1994; Suzuki et al., 1997). Since the ALK-2 and XSTK9 mRNAs are efficiently associated with polysomes during maternal embryogenesis, it is possible that it is these two receptors that are responsible for the earliest signaling that occurs through the BMP pathway. In contrast, the ALK-3 receptor, which is efficiently recruited to polysomes only after zygotic transcription, may become important to BMP pathway signaling only after zygotic transcription begins and synthesis of mRNAs encoding additional BMP ligands increases. Resolution of these issues, and the role that translational control contributes to this possible regulation, will require further experimentation. However, the ability to specifically ablate and inhibit polysome recruitment of maternal mRNAs should allow the contribution of translational control to this important issue to be addressed.

ACKNOWLEDGMENTS

We thank Ken Cho, Jon Graff, Doug Melton, Ali Hemmati-Brivanlou, and Naoto Ueno for providing cDNA clones. Lisa Abler, Connie Lane, Tracy Smith, and Jason Weyers provided helpful input. We thank Catherine Fox for her support, insights, and editorial prowess, and the Fox lab for their view from the yeast world. This work was supported by grants from the Beckman Foundation and the Pew Scholars in Biomedical Sciences Program. B.F. was supported by a Biotechnology Training Grant to the University of Wisconsin-Madison (NIH ST32 GM08349).

REFERENCES


Received for publication December 15, 2000
Accepted May 15, 2001
Published online June 27, 2001