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Xenopus laevis Ribosomal Protein S1: Evidence for Regulation at the Transcriptional Level

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College of William & Mary - Arts & Sciences

Xenopus laevis Ribosomal Protein S1: Evidence for Regulation at the Transcriptional Level

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Of the Requirements for the Degree of

Master of Arts

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by

Cleve Ray-Dean Sinor

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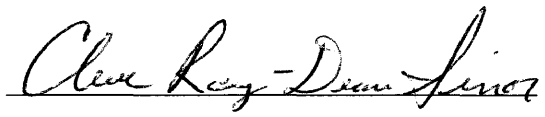
APPROVAL SHEET

This thesis is submitted in partial fulfillment of

the requirements for the degree of

Master of Arts



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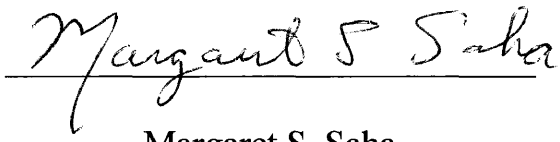
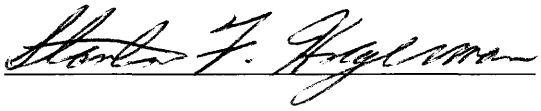


Margaret S. Saha



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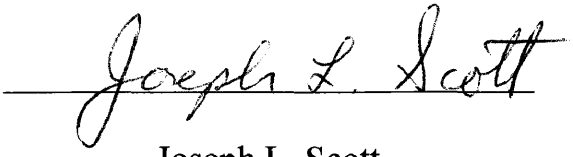

Joseph L. Scott

Lawrence L. Wiseman

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ABSTRACT

The expression and regulation of eukaryotic ribosomal proteins has been investigated intensively over the past thirty years. During this time, findings from various groups have established that eukaryotic ribosomal proteins are controlled at both the post-transcriptional and translational levels. However, regulation of ribosomal proteins at the transcriptional level has only been documented in yeast, and the possibility of ribosomal proteins being regulated at the transcriptional level has not been well addressed in higher eukaryotes. Through the use of whole mount in situ hybridization, we obtained preliminary evidence suggesting that *Xenopus laevis* ribosomal protein S1 (*XLRPS1*) is regulated at the transcriptional level. The differential expression of S1 mRNA, along with the differential translation of S1 transcripts, seems to correlate with tissues undergoing metabolic and structural changes (i.e. differentiation). The fact that S1 mRNA is not expressed in all tissues during embryogenesis could lead to a total reevalution of genes commonly considered to be ubiquitous "housekeeping" genes.

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INTRODUCTION

Embryogenesis in *Xenopus laevis* and other vertebrates is an extremely complex process that requires the active transcription and coordinated expression of thousands of genes. A number of these genes have highly specialized functions and are expressed in very discrete temporal and spatial patterns, for example, the various families of homeobox genes involved in pattern formation. These genes are generally tissue specific, and most if not all are crucial for normal embryonic development. However, many genes are necessary for the proper daily functioning of the cells that make up the organism, and some of these so-called "housekeeping" genes are critical for the accurate expression of the genome. One such group that is absolutely essential for the construction of ribosomes and, therefore, the faithful expression of the genetic code, is the family of ribosomal protein genes.

Ribosomal protein genes have been fairly well characterized in *Xenopus* and other vertebrates. In general, the haploid eukaryotic genome contains one or two copies of each ribosomal protein gene and multiple copies (possibly up to thousands) of each rRNA gene. In *Xenopus* there are at least two copies of each ribosomal protein gene because of tetraploidization which took place around 30 million years ago (Bisbee *et al.*, 1977). Ribosomal protein genes seem to be

scattered throughout the genome and, at least in *Xenopus*, they contain introns (Loreni *et al.*, 1985; Beccari *et al.*, 1986; Mariottini *et al.*, 1993). As a group, the ribosomal protein genes are characterized by a pyrimidine tract at the 5' end that seems to be the major transcription starting point in vertebrates (Loreni *et al.*, 1985; Hariharan *et al.*, 1989), and this 5' pyrimidine motif has been noted in other "housekeeping" genes as well.

The synthesis of new eukaryotic ribosomes involves the regulated expression of over 80 different types of ribosomal protein genes and two major types of rRNA genes. Structurally, the rRNA molecules provide the backbones for the 60S and 40S subunits that comprise a functioning ribosome. In eukaryotes the 60S ribosomal subunit consists of a major 28S rRNA and two minor rRNAs (5.8S and 5.0S), along with nearly 50 distinct ribosomal proteins. The 40S ribosomal subunit is composed of an 18S rRNA and approximately 33 ribosomal proteins. Ribosomal assembly occurs by the sequential binding of groups of ribosomal proteins to rRNA, with each group causing a conformational shift in the rRNA so the next group can bind (Lewin, 1994). The order in which individual ribosomal proteins attach to rRNA has been partially determined in prokaryotic systems, and the specific rRNA binding sites of a few particular ribosomal proteins have been elucidated using nuclease protection assays (reviewed in Zimmermann, 1980). Overall, considerable progress has been made in determining the structure and

assembly of the ribosome, but in spite of this, very little is known about the role ribosomal proteins play in ribosome function.

Although the function of ribosomal proteins in ribosomal activity has been investigated for decades, an answer to this question has remained elusive. Many ideas have been advanced about what confers translational ability to the ribosome, ranging from a view which argues that ribosomal proteins are the key players in translational activity to a view which suggests that rRNA is most responsible for the translation of mRNA transcripts. Presently, the idea that rRNA must play a prominent role in ribosome activity, set forth by Noller (1980), seems to be the most popular. Within this framework, a number of possible ribosomal protein functions have been suggested: 1) ribosomal proteins may stabilize difficult rRNA structures, 2) ribosomal proteins may promote rRNA structural transitions, and 3) ribosomal proteins may provide needed positive charge or other functional characteristics (Draper, 1990). Further research is needed to define the role individual ribosomal proteins (and rRNA) play in translational activity.

From a whole organism point of view, it is necessary to understand the expression and regulation of ribosomal proteins throughout development. Although the developmental expression and regulation of rRNA in *Xenopus* has been well characterized (Reeder and Roan, 1984), only a few of the ribosomal proteins identified thus far have been analyzed to such an extent. However, from

the data collected over the past three decades, a preliminary understanding of ribosomal protein expression and regulation has emerged (reviewed by Amaldi *et al.*, 1989). In general, ribosomal protein mRNA is first synthesized in *Xenopus* around early gastrula stages (St. 8-10) and accumulates to about stage 16 (neurula) (Nieuwkoop and Faber, 1967), at which point mRNA accumulation levels off and remains constant (Pierandrei-Amaldi *et al.*, 1982; Baum and Wormington, 1985). Most of the ribosomal protein mRNAs in these early stages become associated with subpolysomal particles (also known as mRNPs or nonpolysomal particles) and remain inactive in vivo. It is not until early tailbud stages (St.26-30) that these ribosomal protein mRNAs begin to associate with polysomes and become actively translated (Pierandrei-Amaldi *et al.*, 1982). The onset of ribosomal protein mRNA translation at early tailbud stages coincides with a substantial amount of rRNA accumulation (Brown and Littna, 1964), and this fact led early investigators to suggest that the regulation of ribosomal proteins may be controlled by rRNA expression (Hallberg and Brown, 1969). However, recent work with 0-*nu* mutants, first described by Elsdale *et al.* (1958), has provided evidence against this concept. Anucleolate mutants lack the rRNA gene cluster and are unable to synthesize 28S and 18S rRNA; these mutants survive until swimming tadpole stages entirely on the maternally transmitted ribosomal complement, which is approximately 10^{12} ribosomes (Brown and Gurdon, 1964). Studies on *Xenopus* 0-*nu* mutants by

Pierandrei-Amaldi *et al.* (1982) have shown that mature ribosomal proteins are produced even without rRNA synthesis, but these proteins do not accumulate in the embryo because they are unstable in the absence of any rRNA with which to bind. Currently, ribosomal protein and rRNA synthesis is believed to occur independently with some form of translational regulation modulating ribosomal protein synthesis.

Research to date has implicated both a post-transcriptional and a translational mode of regulation in ribosomal protein expression (Pierandrei-Amaldi *et al.*, 1985a). According to Amaldi *et al.* (1989), the cumulative data on ribosomal protein expression suggests that the translational efficiency of ribosomal protein mRNA is influenced by the cellular requirement for new ribosomes. Recent work has identified the 5' untranslated region as an important component in the translational regulation of ribosomal proteins (Mariottini and Amaldi, 1990; Loreni *et al.*, 1993), however, the actual effector of translational regulation is still unknown. The process of post-transcriptional regulation is a bit clearer. It appears that ribosomal proteins modulate ribosomal protein mRNA transcript stability by binding to their own transcripts before intron excision. This binding induces altered mRNA splicing which, at least in the case of ribosomal protein L1, causes the formation of abortive truncated molecules (Bozzoni *et al.*, 1984). Thus, the

effectors of ribosomal protein mRNA post-transcriptional control seem to be the ribosomal proteins themselves.

The current body of knowledge concerning ribosomal protein synthesis clearly shows that ribosomal proteins are controlled at both post-transcriptional and translational levels. However, the possibility of ribosomal proteins being controlled at the transcriptional level in eukaryotes has not been well addressed. We obtained preliminary evidence suggesting the presence of transcriptional regulation in the synthesis of ribosomal proteins in *Xenopus laevis*. The purpose of this study was to determine whether an isolated ribosomal protein (XLRPS1) is differentially expressed in *Xenopus* at the mRNA level and, if so, characterize its temporal and spatial expression. Such a finding would suggest that ribosomal proteins (or possibly a select group of them) may indeed be regulated at the transcriptional level. Furthermore, it might imply that certain ribosomal proteins are only expressed in select tissues and not ubiquitously as previously thought, thus adding yet another level of control to ribosomal protein gene regulation.

MATERIALS AND METHODS

Embryos

Adult *Xenopus laevis* frogs were purchased from Xenopus I. Embryos were acquired using standard protocols as described by Henry and Grainger (1987) and were raised in 0.1X NAM (Slack, 1984) containing 50 μg/ml gentamycin sulfate.

Construction and screening of cDNA library

A cDNA library was constructed from the anterior region of late neurula embryos (stage 18) as described in Saha and Grainger (1992). In an attempt to isolate possible anterior neural homeobox genes in *Xenopus*, the library was screened at low stringency with a 0.3 kb probe from the mouse homeobox gene *EMX1* (kindly provided by E. Boncinelli), which is an anterior neural marker related to *Drosophila empty spiracles* (Simeone et al., 1992). Initially, the library was plated out on LB agar plates and duplicate plaque lifts were performed with nylon transfer membranes. The agar plates were stored at 4°C, and the membranes were immediately autoclaved for 1 minute at 100°C to lyse the protein coats of the viral particles. The exposed cDNA was cross-linked to the nylon membranes with a Fisher Scientific UV Crosslinker and the membranes were placed in 30% formamide hybridization solution (1 M NaCl, 1% SDS, 50 mM Tris-HCl (pH 7.5), 100 μg/ml salmon sperm DNA, 30% formamide) for 6 hours at 40°C. After the

prehybridization, radiolabeled (^{32}P) *Emx1* probe, constructed using the random hexamer priming reaction of Feinberg and Vogelstein (1983), was added to 30% formamide hybridization solution and placed in sealed plastic bags along with the nylon membranes. Hybridization took place overnight at 40°C in a shaking water bath. The membranes were washed twice for 20 minutes at room temperature in 2X SSC/1% SDS wash solution to remove non-specifically bound probe. The membranes were then mounted on cardboard plates and placed in film cassettes with intensifying screens for overnight exposure at -80°C. The film (Fuji) was developed using standard development procedures.

To isolate positive clones, duplicate films were aligned and promising signals were identified. Plaques corresponding to identified signals were pulled from the agar plates and placed in 1 ml of storage media (SM) (Sambrook *et al.*, 1989) to elute the viral particles. To initiate the secondary screening, 0.5 µl of viral eluate was added to another milliliter of SM to lower the viral concentration. This solution, representing a selected subset of the original cDNA library, was then plated out and the screening procedure as outlined above was repeated. Screening was carried out to the tertiary level where single viral plaques corresponding to the plaques of interest could be isolated.

The excision of pBluescript vector from the λZAPII phage was performed as prescribed by the manufacturer's instructions (Stratagene). The rescued plasmids were grown up on LB/Ampicillin plates and single colonies were isolated

and cultured overnight in LB/Ampicillin media for subsequent isolation of plasmid DNA.

Isolation of plasmid DNA and preparation of subclones

Plasmid DNA was isolated by the alkali lysis method described by Sambrook *et al.* (1989). The resulting plasmid DNA was brought up in 250 μl of TE and stored at 4°C. To confirm the existence of cDNA inserts, the plasmids were cut with the restriction enzyme EcoR I and electrophoresed on 2% agarose gels. Clones containing inserts were identified and one was selected for later characterization.

In order to completely sequence the clone, subclones were made by taking advantage of a BamH I restriction site within the cDNA. BamH I digests and BamH I/EcoR I double digests were performed and the fragments were separated on 2% agarose gels. The three resulting cDNA fragments were ligated into SK+ pBluescript vectors using T4 ligase. Reactions took place overnight at 14°C (Sambrook *et al.*, 1989). The constructed subclones were transformed into a competent cell line (DH5α cells frozen in competent cell buffer) and the cells were checked for plasmid incorporation by an IPTG/X-GAL color selection assay. Single white colonies, indicating plasmid incorporation, were pulled from the plates and cultured in LB/Ampicillin media for future isolation of plasmid DNA.

DNA sequencing

The clone was sequenced using USB Sequenase sequencing kit version 2.0, a dideoxynucleotide chain termination procedure described by Sanger *et al.* (1977). Subclones were made single-stranded and primed at both ends, and the sequencing reactions were carried out for 1 minute at room temperature before nucleotide-specific chain termination. The resulting DNA fragments, radiolabeled with ^{35}S dATP, were electrophoresed on polyacrylamide gels for 2 to 4 hours. Gels were transferred to 3MM blotting paper and placed in a Bio Rad gel dryer for 2 hours at 80°C. The dried gels were placed in film cassettes and exposed overnight to film at room temperature.

Whole mount in situ hybridization

Whole mount in situ hybridization was carried out essentially as described by Harland (1991). Plasmid DNA was linearized with either Cla I or Xba I and transcribed by T3 and T7 RNA polymerase, respectively. Transcription reactions were performed for approximately 2 hours at 37°C in a digoxigenin-11-UTP and ^{3}H dUTP containing nucleotide mix. Tritiated UTP was used to determine nucleotide incorporation by placing 0.5µl of the finished probe on DE-81 filters in duplicate. One filter was washed with phosphate buffer (0.5M Na_2HPO_4) to remove unincorporated radioactivity; the other was left unwashed (Sambrook *et al.*, 1989). Filters were placed in vials and filled with scintillation fluid for

counting in a Beckman scintillation counter. Total counts and incorporated counts were compared to ascertain the quantity of synthesized mRNA.

Following prehybridization, the mRNA probes were placed in in situ hybridization buffer (1 μg probe per 1 ml hybridization buffer - 50% formamide, 5X SSC, 1 mg/ml Torula RNA, 100 μg/ml heparin, 1X Denhart's, 0.1% Tween 20. 0.1% CHAPS, 5mM EDTA) and added to the embryos for overnight hybridization at 60°C. An anti-digoxigenin antibody linked to alkaline phosphatase was then added to the embryos and incubated overnight at 4°C. A series of 1 hour washes removed excess antibody, and NBT (Nitro blue tetrazolium) and BCIP (5-bromo-4-chloro-3-indolyl-phosphate) were added to the embryos in an alkaline phosphatase buffer to start the chromogenic reaction. Embryos were stained at varying intensities and stored in 1X PBS for future analysis.

Histology

Embryos were put through a standard ethanol dehydration series. Solutions of 50%, 70%, 90%, and 100% ethanol were prepared and embryos were placed in each solution for 5 minutes in the described order. Subsequently, embryos were immersed in a 50/50 ethanol/xylene mixture for 10 minutes and placed in 100% xylene for an additional 10 minutes. Paraffin (Paraplast Plus) infiltration was initiated by incubating the embryos in a 50/50 mixture of xylene and paraffin for 30 minutes at 60°C. Complete infiltration was achieved with a final 3 hour, 60°C

incubation in 100% paraffin. Single embryos were placed in paraffin molds, oriented in the anterior-posterior axis, and sectioned at 10 micrometers with an American Optical microtome. Paraffin ribbons were placed on Myer's adhesive treated slides and floated on a layer of water to decompress the sections. Slides were heated on a slide warmer for 6 hours to overnight and subsequently placed in xylene to remove excess paraffin. Permount was used to permanently mount the slides.

Northern blotting

Northern hybridization was carried out as described by Sambrook *et al.* (1989) with minor modifications. RNA was electrophoresed through 2% agarose gels containing formaldehyde and stained with ethidium bromide for 15 minutes. Gels were destained for 6 hours to overnight. After destaining, Polaroid pictures were taken and the gels were traced to record the location of rRNA bands. 3MM paper was cut to the proper dimensions and placed in transfer trays, and transfer buffer (0.025M sodium phosphate dibasic (pH 7.0)) was added to the wells to saturate the paper wicks. Gels were placed on top of the wicks, nylon membranes were placed over the gels, and absorbent paper was layered over the membranes. A weight was set on the paper stack to facilitate the transfer of RNA to the nylon membrane. RNA transfer took place for 1 to 2 days. Once the transfer was complete, membranes were immediately subjected to UV light to cross-link the

RNA and immersed in 50% formamide hybridization solution (1 M NaCl, 1% SDS, 50 mM Tris-HCl (pH 7.5), 100 μg/ml salmon sperm DNA, 50% formamide). Prehybridization was carried out for 6 hours at 42°C. A DNA probe was constructed as described by Feinberg and Vogelstein (1983) and added to 50% formamide hybridization solution (1 X 10^6 counts/ml). The membranes were hybridized overnight at 42°C and washed in 2X SSC/1% SDS (2X 20 minutes) to remove excess probe. Hybridized membranes were placed on cardboard plates and put in film exposure cassettes with intensifying screens for overnight exposure at -80°C.

Isolation of Polysomal RNA

Polysomal and nonpolysomal fractions were obtained essentially as described by Baum *et al.* (1988) with minor modifications. Embryos were homogenized in 0.5 ml of polysome buffer (0.3 M KCl, 0.002 M $MgCl_2$, 0.02 M Tris-HCl (pH 7.5), 4 μg/ml polyvinylsulfate, 0.05% (w/v) sodium deoxycholate, 2 mM dithiothreitol, 20 units/ml RNasin, 0.2 mM cycloheximide) and centrifuged for 15 minutes at 4°C in a microcentrifuge. Pellets were discarded, and supernatants were transferred to 15 ml Falcon tubes and brought up in 2 ml of polysome buffer. A cushion of 20% sucrose in polysome buffer was added to Beckman polyallomer ultracentrifuge tubes and the supernatants were laid above the cushions. The tubes were spun at 33,000 rpm for 2 hours in a Beckman

SW41Ti rotor. Supernatants were poured into 15 ml Falcon tubes and 10 ml of ethanol was added to the supernatants to precipitate (4°C) the nonpolysomal RNA. The polyallomer tubes were wiped clean with Kimwipes, and the polysomal pellets were resuspended in 0.5 ml of TENS (0.05 M Tris-HCl (pH 7.5), 0.005 M EDTA, 0.3 M NaCl, 2% SDS) containing 200μg/ml of proteinase K. The tubes were covered and allowed to incubate for 30 minutes at 50°C. The solutions were transferred to microcentrifuge tubes and extracted twice with phenol/chloroform and once with straight chloroform. Polysomal RNA was ethanol precipitated overnight at -80°C.

Precipitated nonpolysomal RNA was pelleted at 10,000 rpm for 15 minutes in a Sorvall HB4 rotor. The nonpolysomal pellets were subjected to the same treatment as described above for the polysomal pellets, and both RNA fractions were spun down in a Sorvall HB4 rotor at 10,000 rpm for 15 minutes. The pellets were rinsed in 95% ethanol and dried in a Savant Speed Vac for 3 minutes. The recovered RNA was resuspended in 80 μl of TE.

RESULTS

Isolation of *XEMX1*

A stage 18 anterior neural cDNA library (Saha and Grainger, 1992) was screened at low stringency with a fragment of the murine homeobox gene *EMX1* (kindly provided by E. Boncinelli). Primary screening of this library, plated out on three dishes at a density of approximately 50,000 plaque forming units (pfu) per 150 mm petri dish, resulted in the isolation of four positive clones. Plaques corresponding to the general area of these four signals were eluted from the agar plugs; this selected subset of the original cDNA library was then plated out for the secondary screening on five 90 mm plates at a density of approximately 10,000 pfu per plate. The secondary screening produced fifteen positive clones which were subsequently used to initiate the tertiary screening. Twenty promising clones were identified from the tertiary screening (thirteen plates at approximately 100 pfu per 90 mm petri dish), and all were digested with EcoR I to determine the presence of cDNA inserts. One was chosen for further characterization on the basis of homology screening signal strength. Restriction enzyme analysis revealed the presence of a 0.8 kb cDNA clone which we called *XEMX1*. In order to ascertain the size of *XEMX1* mRNA transcripts, radioactive probes were constructed and used in Northern blot hybridization experiments. Northern

analysis produced a single transcript of approximately 0.8 kb that is detectable at most stages of *Xenopus* development (Fig. 3).

XEMX1 is *Xenopus laevis* ribosomal protein S1 (*XLRPS1*)

XEMX1 was digested with BamH I and EcoR I to create subclones for DNA sequence analysis. The resulting fragments, approximately 600, 650, and 250 bp in length (see Fig. 1), were sequenced along with the full clone and overlapping regions were aligned to check sequence accuracy.. A Genebank database search revealed (most unexpectedly) that *XEMX1* is 98% identical to *XLRPS1*, a ribosomal protein associated with the 40S small subunit. The partial DNA sequence of the *XEMX1* clone is shown in Fig. 2 along with the published *XLRPS1* sequence.

Developmental expression of *XLRPS1*

To determine if our clone exhibited the same mRNA expression pattern as the published S1 clone (Pierandrei-Amaldi *et al.*, 1982), Northern blot analysis was carried out (Fig. 3). As previously reported, *XLRPS1* mRNA transcripts begin to appear around early gastrula stages (8-10) and are present in all stages thereafter. A small amount of maternal S1 transcripts are detectable in total mRNA isolated from oocytes and, as expected, none are detectable in poly A-

Figure 1. Diagram of subclones generated for DNA sequencing. A schematic of the *XLRPS1* (*XEMX1*) clone is presented with the relative positions of each subclone shown below. The prefix of each subclone denotes whether it was cut with BamH I (B) and/or EcoR I (E). Numbers indicate approximate nucleotide length.

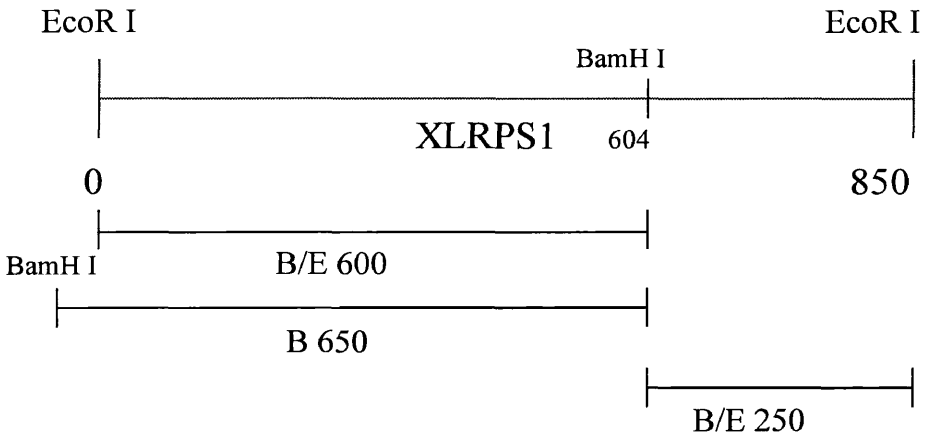
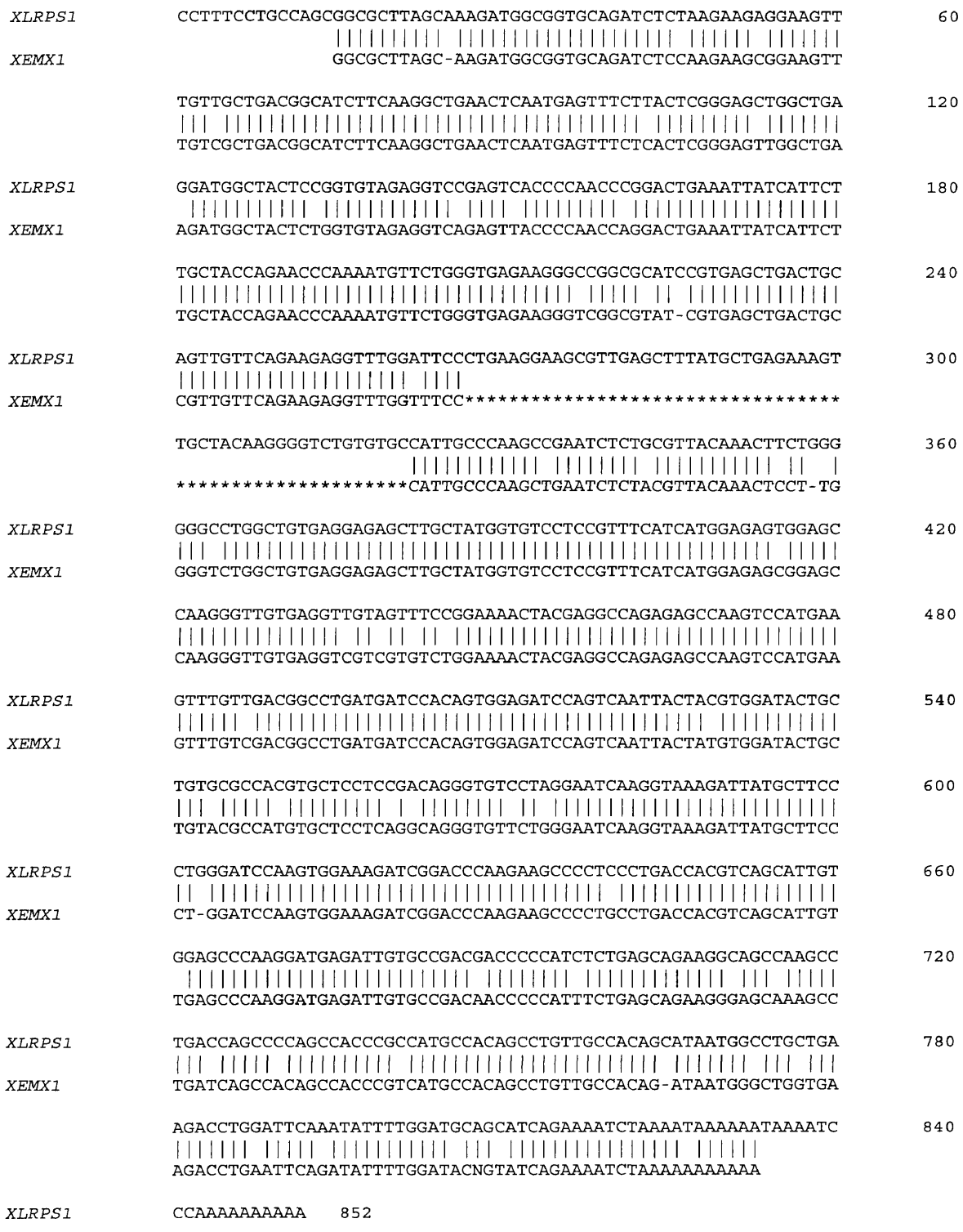

EcoR I
EcoR I
BamH I
XLRPS1
604
0
850
BamH I
B/E 600
B 650
B/E 250

Figure 2. DNA sequence of *XLRPS1*. The published *XLRPS1* sequence is shown along with the partial sequence of the *XEMX1* clone. Vertical hashmarks indicate identical base pairs. The *XEMX1* clone shows 98% homology to *XLRPS1*.


XLRPS1
CCTTTCCTGCCAGCGGCGCTTAGCAAAGATGGCGGTGCAGATCTCTAAGAAGAGGAAGTT 60
XEMX1
GGCGCTTAGC-AAGATGGCGGTGCAGATCTCCAAGAAGCGGAAGTT
TGTTGCTGACGGCATCTTCAAGGCTGAACTCAATGAGTTTCTTACTCGGGAGCTGGCTGA 120
TGTCGCTGACGGCATCTTCAAGGCTGAACTCAATGAGTTTCTCACTCGGGAGTTGGCTGA
XLRPS1
GGATGGCTACTCCGGTGTAGAGGTCCGAGTCACCCCAACCCGGACTGAAATTATCATTCT 180
XEMX1
AGATGGCTACTCTGGTGTAGAGGTCAGAGTTACCCCAACCAGGACTGAAATTATCATTCT
TGCTACCAGAACCCAAAATGTTCTGGGTGAGAAGGGCCGGCGCATCCGTGAGCTGACTGC 240
TGCTACCAGAACCCAAAATGTTCTGGGTGAGAAGGGTCGGCGTAT-CGTGAGCTGACTGC
XLRPS1
AGTTGTTCAGAAGAGGTTTGGATTCCCTGAAGGAAGCGTTGAGCTTTATGCTGAGAAAGT 300
XEMX1
CGTTGTTCAGAAGAGGTTTGGTTTCC**********
TGCTACAAGGGGTCTGTGTGCCATTGCCCAAGCCGAATCTCTGCGTTACAAACTTCTGGG 360
**********CATTGCCCAAGCTGAATCTCTACGTTACAAACTCCT-TG
XLRPS1
GGGCCTGGCTGTGAGGAGAGCTTGCTATGGTGTCCTCCGTTTCATCATGGAGAGTGGAGC 420
XEMX1
GGGTCTGGCTGTGAGGAGAGCTTGCTATGGTGTCCTCCGTTTCATCATGGAGAGCGGAGC
CAAGGGTTGTGAGGTTGTAGTTTCCGGAAAACTACGAGGCCAGAGAGCCAAGTCCATGAA 480
CAAGGGTTGTGAGGTCGTCGTGTCTGGAAAACTACGAGGCCAGAGAGCCAAGTCCATGAA
XLRPS1
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XEMX1
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TGTGCGCCACGTGCTCCTCCGACAGGGTGTCCTAGGAATCAAGGTAAAGATTATGCTTCC 600
TGTACGCCATGTGCTCCTCAGGCAGGGTGTTCTGGGAATCAAGGTAAAGATTATGCTTCC
XLRPS1
CTGGGATCCAAGTGGAAAGATCGGACCCAAGAAGCCCCTCCCTGACCACGTCAGCATTGT 660
XEMX1
CT-GGATCCAAGTGGAAAGATCGGACCCAAGAAGCCCCTGCCTGACCACGTCAGCATTGT
GGAGCCCAAGGATGAGATTGTGCCGACGACCCCCATCTCTGAGCAGAAGGCAGCCAAGCC 720
TGAGCCCAAGGATGAGATTGTGCCGACAACCCCCATTTCTGAGCAGAAGGGAGCAAAGCC
XLRPS1
TGACCAGCCCCAGCCACCCGCCATGCCACAGCCTGTTGCCACAGCATAATGGCCTGCTGA 780
XEMX1
TGATCAGCCACAGCCACCCGTCATGCCACAGCCTGTTGCCACAG-ATAATGGGCTGGTGA
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AGACCTGAATTCAGATATTTTGGATACNGTATCAGAAAATCTAAAAAAAAAAA
XLRPS1
CCAAAAAAAAAA 852
XEMX1

Figure 3. Northern blot analysis of *XLRPS1* expression. Total mRNA was extracted from whole embryos at different stages of development and electrophoresed on formaldehyde gels. The 28S and 18S rRNA bands were used to quantify and equalize the amount of total RNA in the gels (top photo). Autoradiographs revealed the presence of a single 0.8 kb transcript in all stages tested (stage 8 transcripts were visible in gels overloaded with total RNA). As expected, no signal was detected in poly A- mRNA (bottom photo). The drop in S1 signal at stage 37 is probably due to degraded poly A+ mRNA.

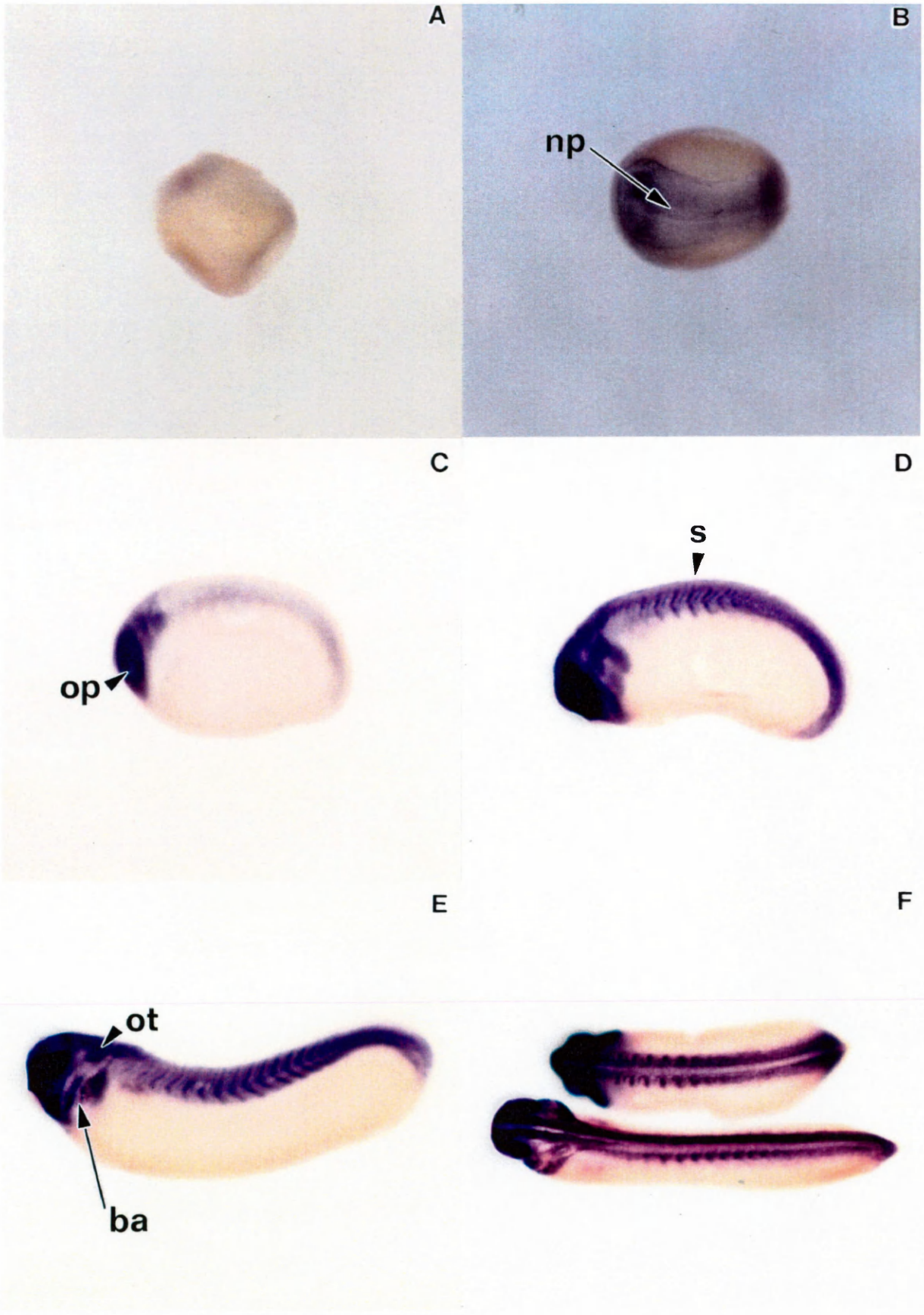
Poly A-
Oocytes
Stage 8
Stage 13
Stage 20
Stage 25
Stage 27
Stage 32
Stage 35
Stage 37
Stage 39
Stage 45
28S-
18S-

Poly A-
Oocytes
Stage 8
Stage 13
Stage 20
Stage 25
Stage 27
Stage 32
Stage 35
Stage 37
Stage 39
Stage 45
0.8kb-

RNA. A drop in S1 signal strength at stage 37 is probably due to degraded polyA+ mRNA and not indicative of what takes place in vivo.

To further characterize the temporal expression of *XLRPS1* as well as analyze its spatial expression, whole mount in situ hybridization was performed on embryos fixed at various stages of development (Fig. 4). Using this technique, *XLRPS1* mRNA transcripts first appear at early neurula stages (stage 14) as a diffuse stain corresponding to the dorsal side of the embryo. Staining remains diffuse and localized in the dorsal region until later neurula stages (stages 18-20) when signal becomes more intense in the cephalic area. By tailbud stage (stage 25), *XLRPS1* transcripts are evident along the entire dorsal side with intense staining in the anterior region. In particular, the branchial arches are well delineated as are the optic and otic vesicles (see Fig. 5). Signal appears to be absent in the neural tube, however, with only the somitic mesoderm staining along the embryos' entire dorsal length. At late tailbud stages the pronephros and pronephric ducts begin to stain along with the ventral ectoderm, and a characteristic five spot repeating pattern appears on the lateral sides of the embryo (see Fig. 5). This blotchy lateral pattern may be involved with the forming pronephros, but it is more probable that this staining pattern correlates with mesomeres or an as yet unknown anatomical structure. At all stages endodermal tissue is practically negative for S1 mRNA expression.

Figure 4. *XLRPS1* whole mount in situ hybridization performed on embryos fixed at different developmental stages. (A) Stage 12 (gastrula). No evident staining. (B) Stage 15 (early neurula), dorsal view. The arrow points to staining in the neural plate (**np**). (C) Stage 20, lateral view. The head region is intensely stained, particularly in the optic placode (**op**). (D) Stage 25 (pre-tailbud), lateral view. The somites (**s**) begin to stain heavily. (E) Stage 28 (early tailbud), lateral view. The branchical arches (**ba**) are well delineated as are the otic vesicles (**ot**). (F) Stages 25 and 28, dorsal views. *XLRPS1* staining appears absent in the neural tube.


A
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np
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D
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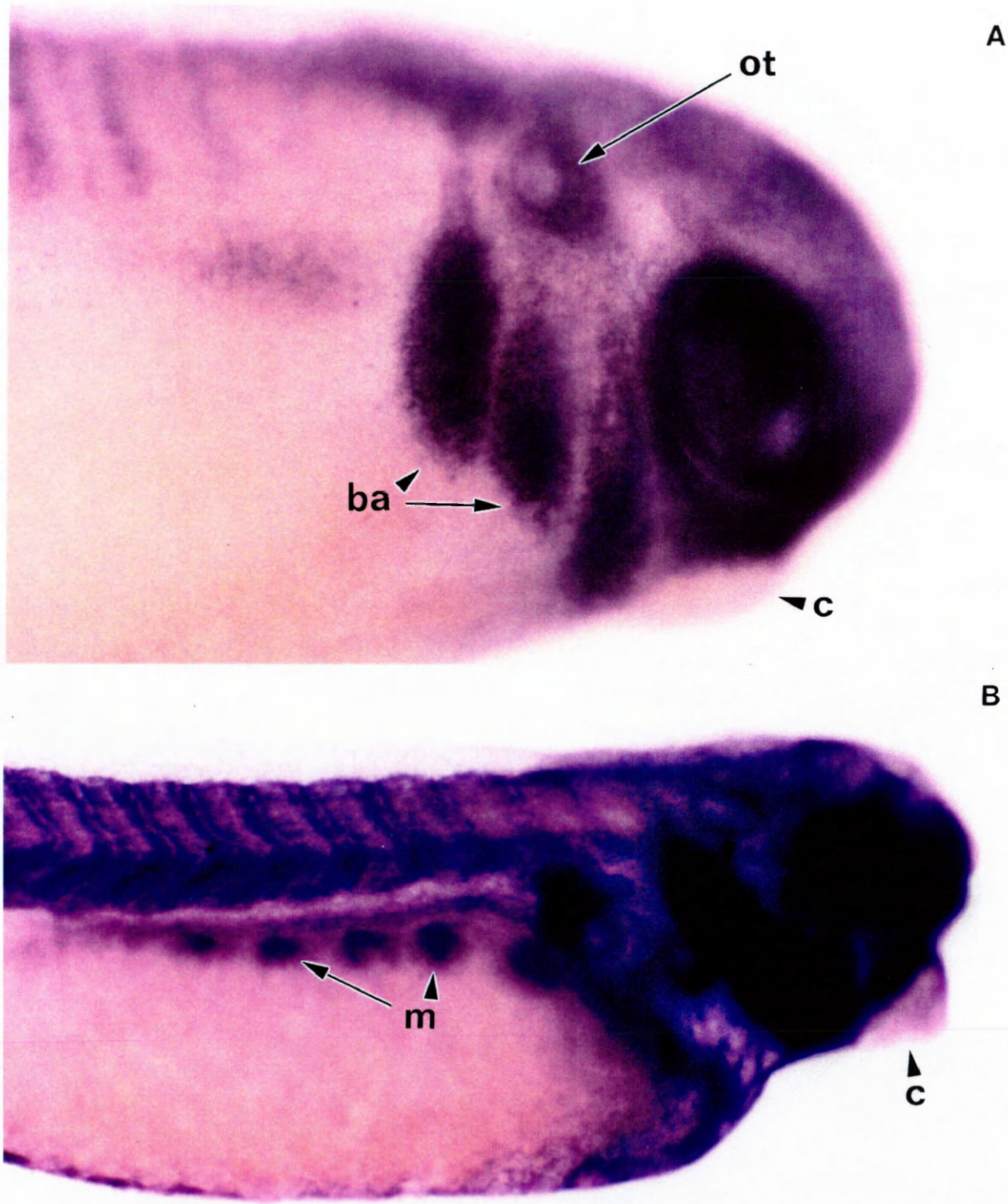
ba

Figure 5. *XLRPS1* whole mount in situ expression in stage 27 and stage 37 embryos. (A) The branchial arches (**ba**) are well defined in this stage 27 embryo. Staining in the otic region (**ot**) is visible while no signal is present in the cement gland (**c**). (B) The five spot blotchy pattern corresponding to mesomeres (**m**) is evident in this stage 37 embryo. Again, the cement gland is negative for *XLRPS1* expression.


A
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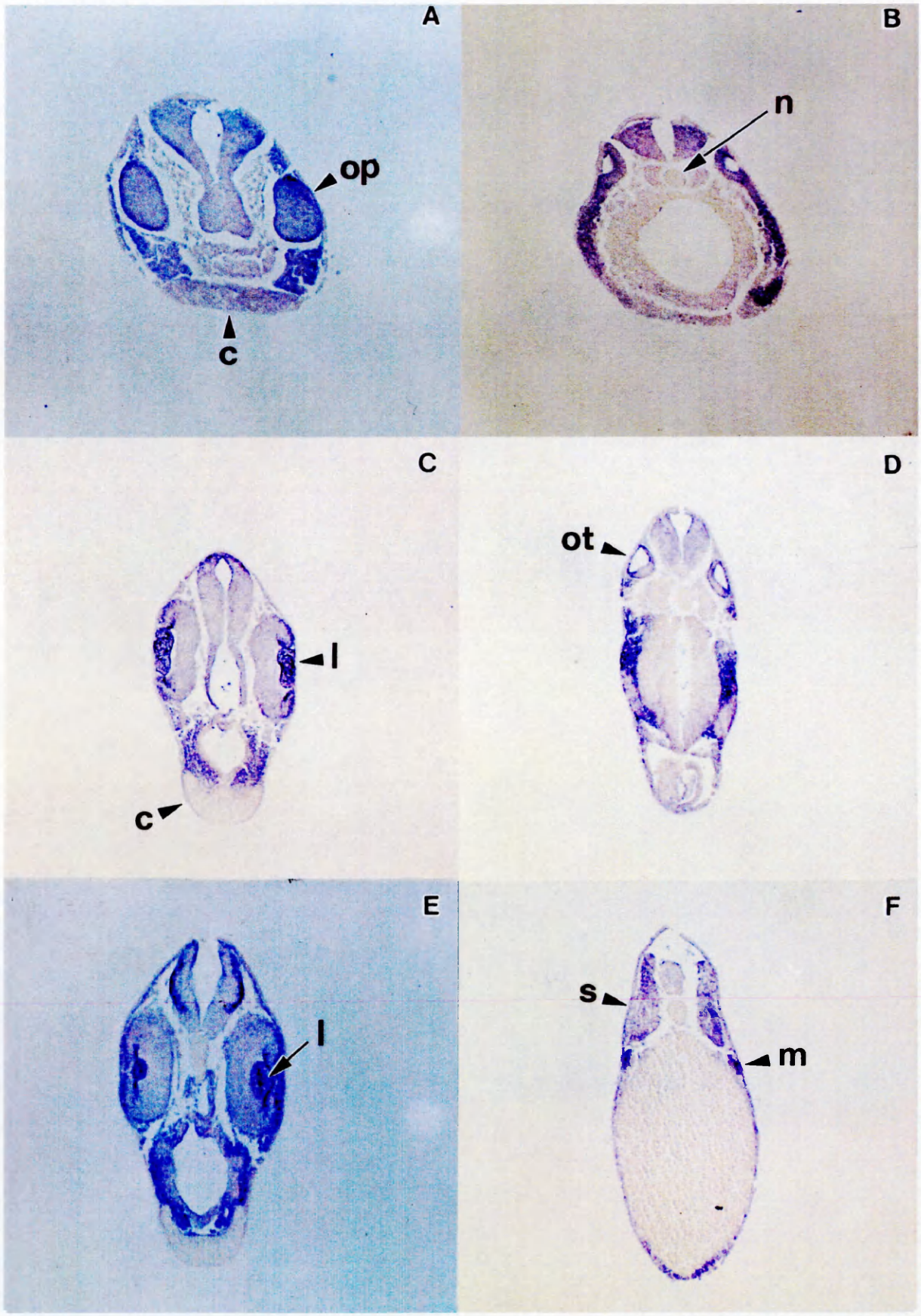
In order to gain a better understanding of *XLRPS1* expression within the embryo, transverse sections of stained embryos were cut and mounted on slides for analysis (Fig. 6). Staining becomes evident at stage 18 where signal is confined to a dorsal margin that includes sensorial ectoderm and lateral mesoderm. The dorsal neural tube is also lightly marked, which was not apparent in our whole mount specimens, and the outer ectoderm is unstained. By tailbud stages, the head region becomes markedly stained with the most pronounced signal located in the neural retina. The cement gland, positioned on the ventral side of the head, is entirely negative. More posterior, the dorsal neural tube and otic vesicles are noticeably stained as are the lateral mesodermal bands. The notochord and endodermal structures are unstained. In the caudal region, *XLRPS1* transcripts are clearly visible in the somites and detectable in the neural tube. However, notochord and endoderm are completely negative. At swimming tadpole stages (stages 33-37), staining becomes pronounced in the lenses with a diffuse signal located throughout the remainder of the head region. The cement gland remains negative for S1 mRNA expression. Staining in the otic region is similar to stage 25 embryos except that the lateral mesoderm no longer appears as bands but assumes a more complex pattern. Posterior sections reveal staining in the somites and ventral ectoderm and, in addition, staining in the lateral mesoderm that corresponds to the blotchy pattern observed in whole mount embryos (possibly mesomeres). Again, the endoderm is totally negative for *XLRPS1* mRNA expression.

Figure 6. Transverse sections of embryos analyzed for *XLRPS1* expression. In situ hybridization was performed on embryos fixed at different developmental stages then sectioned along the anterior-posterior axis. (A) Stage 24 (pre-tailbud) embryo sectioned through the eye region. The optic placode (**op**) is stained intensely in the region of the neural retina. No staining is evident in the cement gland (**c**). (B) Stage 24 embryo sectioned through the otic region. The hindbrain, otic vesicles, and lateral mesoderm are stained. No staining appears in the notochord (**n**). (C) Stage 33 (late tailbud) embryo sectioned through the eye region. The cement gland (c) remains negative for S1 expression. The lens (**l**) is well marked. (D) Stage 33 embryo sectioned through the otic region. The otic vesicles (**ot**) are still stained and the lateral mesoderm displays dynamic expression. (E) Stage 38 (swimming tadpole) embryo sectioned through the eye region. The lens (**l**) remains stained while the cement gland remains unstained. (F) Stage 33 embryo sectioned through the posterior region. The somites (**s**) are well marked and the mesomeres are stained intensely.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XLRPS1 expression and regulation in isolated tissues

The expression and regulation of ribosomal proteins in the whole embryo has been investigated intensively over the past decade, however, no studies have analyzed ribosomal protein expression and regulation in select tissues. To address this issue and further investigate the expression and regulation of S1, 50 embryos at stages 24 and 30 were dissected into head, somite, and ventral tissues as shown in Figs. 7 and 8. These regions were selected because of the apparent differential expression of S1- high in the head, moderate in the somites, and virtually none in the ventral endoderm - as determined by in situ hybridization. Northern blot analysis from these tissues showed that S1 mRNA is highest in the head region at stage 30 (Fig. 9). In the somite region, signal is apparent but approximately threefold lower than in the head region; no signal is detectable at stage 30 in the ventral tissue. At stage 24, S1 transcripts were undetectable in all regions. This is probably due to the smaller amounts of total mRNA extracted from the isolated tissues as compared to the total mRNA extracted from the whole embryos.

To determine whether S1 is actively translated in particular tissues at certain stages of development, polysomal and nonpolysomal mRNA fractions were isolated (see Materials and Methods). Polysomal mRNA fractions contain all the mRNAs that are being actively translated while, conversely, nonpolysomal fractions contain all inactive mRNAs not associated with fully functional ribosomes. Isolation of polysomal and nonpolysomal mRNA and subsequent

Figure 7. Diagram of dissections performed on stage 24 (pre-tailbud) embryos. Each embryo was sectioned in the following manner and the resulting head, somite, and ventral tissues were used for subsequent Northern analysis.

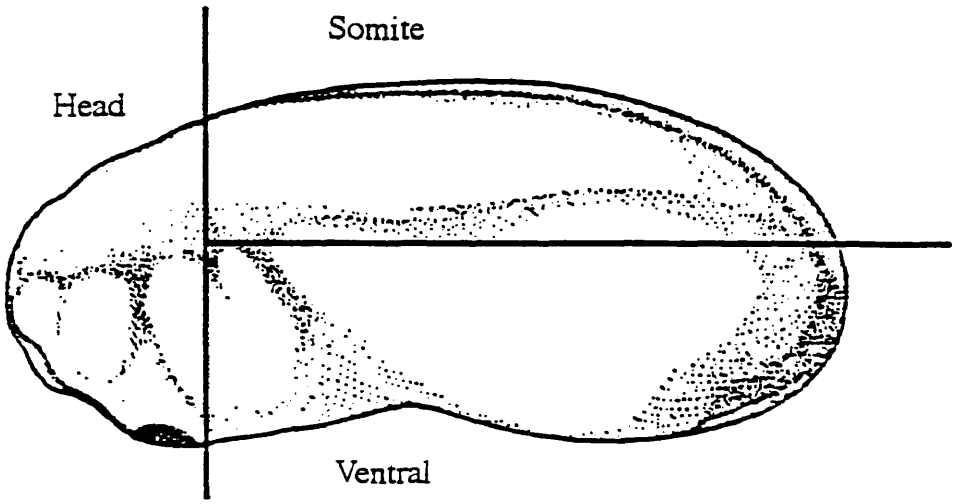

Somite
Head
Ventral

Figure 8. Diagram of dissections performed on stage 30 (tailbud) embryos. Each embryo was sectioned in the following manner and the resulting head, somite, and ventral tissues were used for subsequent Northern analysis.

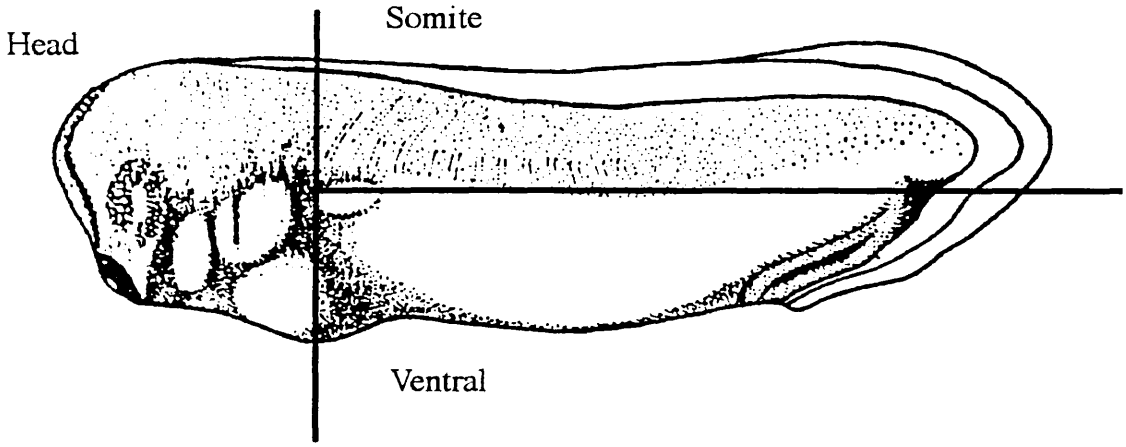
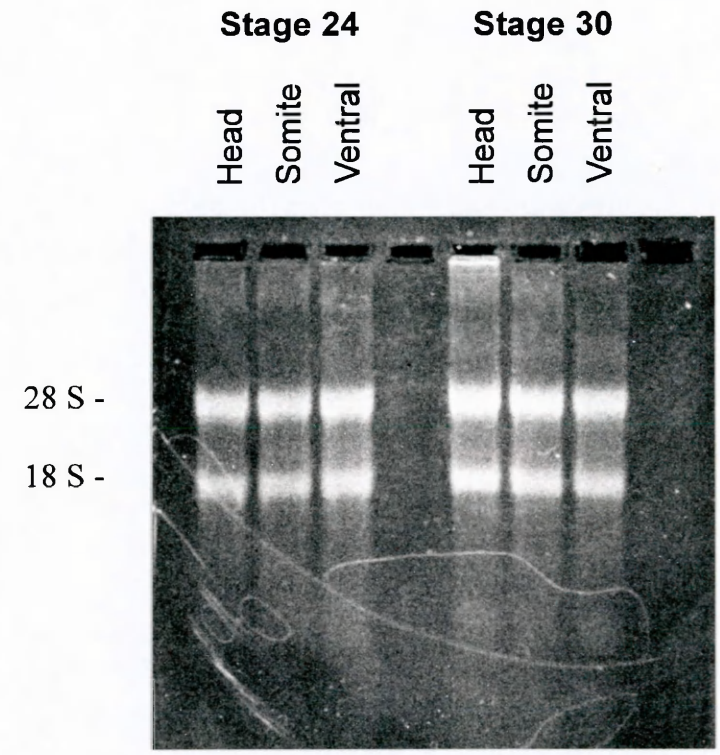
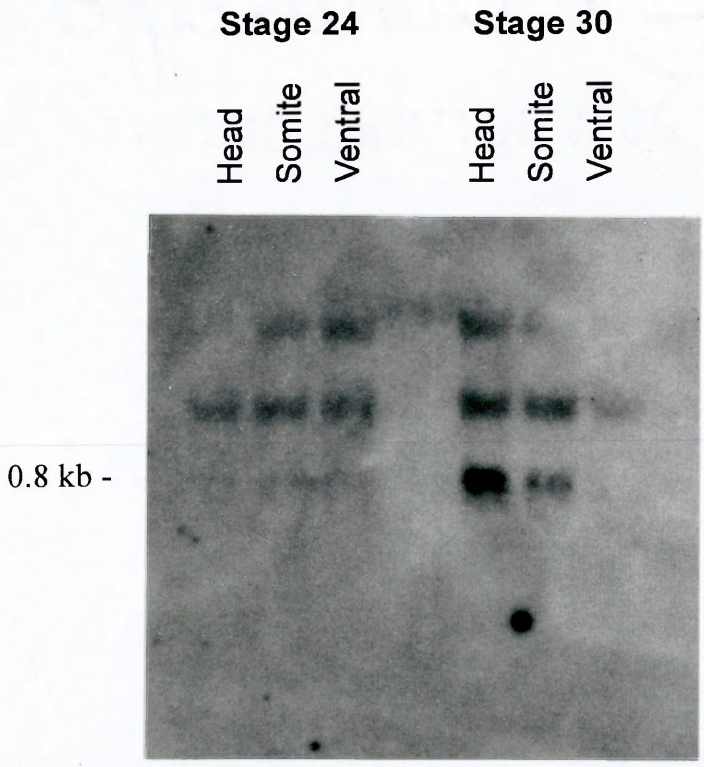

Somite
Head
Ventral

Figure 9. Northern blot analysis of isolated tissue mRNA. Total mRNA corresponding to head, somite, and ventral tissue was extracted from stage 24 and stage 30 embryos and subsequently electrophoresed on formaldehyde gels. The 28S and 18S rRNA bands were used to quantify and equalize the amount of total RNA in the gels (top photo). Autoradiographs revealed *XLRPS1* probe hybridizing with a single 0.8 kb transcript in the head and somite RNA fractions at stage 30 (bottom photo). S1 expression at stage 30 appears to be threefold higher in the head region than in the somite region, with no expression evident in the ventral tissue. No hybridization is apparent at stage 24.


Stage 24
Stage 30
Head
Somite
Ventral
Head
Somite
Ventral
28 S -
18 S -


Stage 24
Stage 30
Head
Somite
Ventral
Head
Somite
Ventral
0.8 kb -

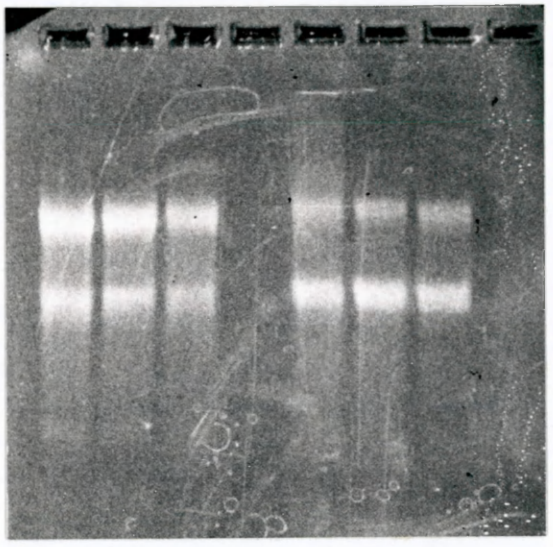
Northern analysis revealed that most S1 mRNA transcripts in the head are being translated by stage 30 (Fig. 10). In the somite region, it appears that less than half of the total S1 mRNA complement is on polysomes and being actively translated, while no mRNA transcripts are detectable in the ventral polysomal fraction. In addition, S1 transcripts are present at varying levels in all three nonpolysomal fractions at stage 30, with the highest level of inactive S1 mRNA located in the somitic tissue. Again, S1 transcripts were not detected in either fraction from all regions at stage 24 (data not shown). It is interesting to note that while there is a detectable S1 signal in the ventral nonpolysomal fraction at stage 30, this signal is very low and could be accounted for by the ventral ectoderm, which does express S1 transcripts during the late tailbud stages of *Xenopus* embryogenesis.

Figure 10. Northern blot analysis of polysomal/nonpolysomal mRNA fractions. Polysomal and nonpolysomal fractions were isolated from head, somite, and ventral tissues of stage 30 embryos and subsequently electrophoresed on formaldehyde gels. The 28S and 18S rRNA bands were used to quantify and equalize the amount of total RNA in the gels (top photo). Autoradiographs revealed *XLRPS1* probe hybridizing with a single 0.8 kb transcript in all fractions except ventral polysomal (bottom photo). It appears that 90% of S1 mRNA in the head region is associated with polysomes while less than half of the total S1 complement is on polysomes in the somite region. The small amount of S1 mRNA detected in the ventral nonpolysomal fraction may be due to ventral ectoderm, which does show *XLRPS1* expression at late tailbud stages.

Stage 30

Polysomal **Nonpolysomal**

Head Somite Ventral Head Somite Ventral



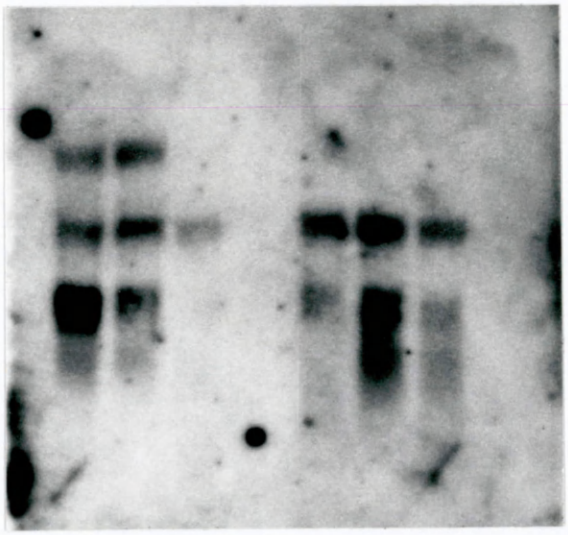
28 S -

18 S -

Stage 30

Polysomal **Nonpolysomal**

Head Somite Ventral Head Somite Ventral



0.8 kb -

DISCUSSION

The prokaryotic *E. coli* system served as a paradigm for early work on *Xenopus* ribosomal protein synthesis. In this system, ribosomal protein genes are arranged in unique multigene transcriptional units (polycistronic operons) that are regulated at both the transcriptional and translational levels (Dean and Nomura, 1980; Lindahl *et al.*, 1983). It appears as though one particular protein within each operon acts as an autogenous regulator of all the ribosomal protein genes located within the same operon. For example, it has been established by the Nomura group that the ribosomal proteins S4, S8, and L1 have specific inhibitory effects at the translational level on ribosomal proteins located within their own operons - the α, *spc*, and L11 operons, respectively (Yates *et al.*, 1980). In addition, the Lindahl and Zengel group discovered that the ribosomal protein L4 acts as the transcriptional feedback repressor for the eleven genes of the S10 transcriptional complex (Lindahl *et al.*, 1983). Findings from these two groups and others have established autogenous control as a fundamental process by which prokaryotic ribosomal proteins are regulated.

The autogenous regulation of ribosomal protein synthesis in prokaryotes led some groups to investigate whether the same type of regulation occurs in eukaryotes. From studies using *Xenopus laevis* as a model system, it appears that

ribosomal proteins do in fact autogenously regulate themselves in eukaryotes by binding to their own mRNA transcripts at the post-transcriptional level (reviewed by Amaldi *et al.*, 1989), and this binding seems to produce immature mRNA fragments by disturbing the proper splicing of transcripts. In contrast, it has been shown that ribosomal protein regulation in *Xenopus* is not autogenous at the translational level (Pierandrei-Amaldi *et al.*, 1985b). Work conducted by the Amaldi group has revealed that translational regulation is probably modulated by some component of the 5' untranslated region of the ribosomal protein mRNAs (Mariottini and Amaldi, 1990). Although the prokaryotic and eukaryotic systems share an autogenous mechanism of control at some level of ribosomal protein regulation, one obvious difference between these two systems is the lack of any evidence of transcriptional control among higher eukaryotes.

Transcriptional control of ribosomal proteins in eukaryotes has been documented in yeast (Robash *et al.*, 1981). However, no data has been obtained that would indicate a transcriptional level of control of ribosomal proteins in higher eukaryotes. The purpose of this study was to determine whether ribosomal proteins are differentially expressed within a multicellular organism during embryogenesis, which would suggest that transcriptional regulation is taking place. To accomplish this, we performed in situ hybridization experiments to ascertain whether the ribosomal protein S1 in *Xenopus* is differentially expressed at the

mRNA level. The results of our in situ hybridization experiments show that XLRPS1 is expressed in select tissues throughout embryogenesis. S1 expression is dynamic, but is highest in mesodermal tissue of the head and somite regions. A moderate amount of S1 expression is noticeable in ectodermal tissue while virtually no expression is observed in endodermal tissue. It is interesting to note that S1 expression is absent from anatomical structures that have a transitory existence in the developing embryo, such as the cement gland and notochord. These tissues may possess all the maternally derived ribosomes needed to carry out their functions and, therefore, may have no need for S1 mRNA. It is also possible that these tissues do not need S1 protein to create fully functional ribosomes. The overall pattern of S1 expression in *Xenopus* is also quite interesting in that it seems to correlate with tissues undergoing metabolic and structural changes (i.e. differentiation). This correlation can be rationalized because such tissues would most likely deplete their maternal store of ribosomes quickly and require the active synthesis of new ribosomes at the earliest possible time. Even though ribosome biosynthesis does not begin until early tailbud stages (stages 26-30), a large pool of ribosomal protein mRNA would ensure the rapid production of new ribosomes in metabolically active tissues.

Our preliminary findings from in situ analysis showed that S1 transcripts are differentially expressed within the developing *Xenopus* embryo. To confirm

this result, Northern blot hybridization was carried out on mRNA isolated from select tissues (see Results). Northern analysis revealed that S1 mRNA is present in the head and somite regions but is essentially absent in the ventral region, thus confirming the data obtained from the in situ experiments. The absence of XLRPS1 transcripts in the ventral endoderm raises the possibility that ribosomal proteins (or a select group of them) are not ubiquitously expressed, but rather are expressed in only select tissues. Confirmation of this possibility would add another level of control in the regulation of eukaryotic ribosomal proteins and raise doubts about the ubiquitous expression of other so-called "housekeeping" genes. However, this possibility is beyond the scope of this paper and should be addressed in future experiments.

The finding that S1 mRNA is differentially expressed during embryogenesis led us to ask whether transcripts isolated from select tissues are translated in a manner consistent with previous data on S1 (Pierandrei-Amaldi *et al.*, 1982). Using total mRNA extracted from whole embryos, the Amaldi group found that S1 transcripts are located only in nonpolysomal fractions at stage 15. By stage 26, only a small fraction of the total S1 complement is found on polysomes and are being actively translated, and it is not until stage 31 that more than 50% of the total S1 mRNA pool is loaded onto polysomes (Pierandrei-Amaldi *et al.*, 1982). Our findings at stage 30, taken as a whole, are in agreement with the

latter results. However, the distribution of S1 mRNA on polysomes between tissues is markedly different. In the head region as much as 90% of S1 transcripts appear to be loaded onto polysomes, with less than half of the S1 mRNA loaded onto polysomes in the somite region. No XLRPS1 transcripts appear on polysomes in the ventral region at stage 30. This data, taken in conjunction with earlier work concerned with the translational regulation of S1 and other ribosomal proteins, seems to suggest that a threshold level of ribosomal protein transcripts is needed to allow loading of these transcripts onto polysomes. This threshold level may be determined by some component of the 5' untranslated region on ribosomal protein mRNAs, which has been implicated in the translational regulation of ribosomal proteins (Mariottini and Amaldi, 1990; Loreni *et al.*, 1993). The possibility also exists that some other factor may be responsible for the apparent threshold level loading of mRNA transcripts onto polysomes.

Future Directions

The data presented in this paper represents a preliminary investigation into the possible regulation of ribosomal protein genes by a transcriptional mechanism. Our findings suggest that ribosomal proteins are regulated in some fashion at the transcriptional level, however, more research is needed to determine if transcriptional regulation is a fundamental regulatory mechanism in the expression

of eukaryotic ribosomal proteins. To begin, additional ribosomal proteins from both subunits should be used in in situ experiments to determine whether the expression pattern observed for S1 is characteristic of ribosomal proteins in general. Such evidence would bolster the idea that ribosomal proteins are transcriptionally regulated. However, if other ribosomal proteins display unique differential expression patterns, such data would suggest that ribosomes from particular tissues may not be composed of an identical complement of ribosomal proteins, further supporting the idea that ribosomal proteins are transcriptionally regulated.

It would also be interesting to see if other "housekeeping" genes are differentially expressed within *Xenopus*. It has been established that elongation factor 1α, a translational factor sometimes reported to be translationally regulated, shares similarities in it's 5' untranslated region with the ribosomal protein mRNAs. Since this protein appears to be regulated in much the same way as ribosomal proteins (Loreni *et al.*, 1993), an analysis of its temporal and spatial expression would be useful in determining if transcriptional regulation is a common mechanism in the control of ribosomal protein synthesis and, furthermore, a common mechanism in the control of so-called "ubiquitous" genes in general. This could lead to a total re-evaluation of all the genes that are commonly considered to be ubiquitous "housekeeping" genes.

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