

Research article

Enhancer of *split*-related-2 mRNA shows cyclic expression during somitogenesis in *Xenopus laevis*

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Somitogenesis is responsible for production of the segmented body plan typical of vertebrate embryos. The somites are blocks of mesoderm, produced by this process, that give rise to the vertebrae and ribs, the dermis of the skin and all the skeletal muscle of the body. Many genes that regulate somitogenesis have been identified in chick and mouse, whereas considerably fewer are known in *Xenopus laevis*. The expression of *Hairy/Enhancer of split*-related genes is known to cycle during somitogenesis and provides a mechanism for the regular formation of somites. In this project, *in situ* hybridizations were carried out on *bowline*, *Thylacine1*, *Enhancer of split-related-1* (*ESR1*), *ESR2* and *ESR-5* in order to study their expression in relation to somitogenesis. All genes were found to be expressed during somitogenesis, even as early on as late gastrula stages in some cases. In addition, the expression of *ESR2* is shown to be oscillating in the presegmented mesoderm of neurula and early-tailbud embryos. This study has identified *ESR2* as the second known gene (after *esr9*) to show periodic oscillations of gene expression which can be considered as cycling during somitogenesis in *X. laevis*.

Key words: somitogenesis, enhancer of split related, *Xenopus laevis*, cycling, somitomere.

Introduction

The segmented, or metameric, body plan characteristic of all vertebrates is apparent during embryonic development as blocks of mesodermal cells called somites. The generation of these transient somites is a crucial step in development as they not only give rise to the segmented vertebrae of the adult, the dermis of the skin and all of the skeletal muscle of the body, but, moreover, somites also provide positional cues to the migrating neural crest cells. This means that the somites are key players in establishing the overall metameric body plan.

Lying bilaterally to the midline structures (the neural tube and notochord), the somites originate from a region in the posterior of the embryo known as the presegmented mesoderm (PSM). Somites initially form as undetermined balls of epithelial cells initially exhibiting only antero-posterior identity,¹ and later, in response to signals from surrounding tissues including the neural tube, notochord and dorsal ectoderm, the cells will differentiate into the dermatome, sclerotome and myotome which will give rise

to the dermis of the skin, the vertebrae and ribs and all the skeletal muscle of the body, respectively.² Pairs of somites condense from the anterior of the PSM at regular intervals in a strict anterior–posterior manner—a process known as somitogenesis.³

Three major phases of somitogenesis can be described: first, the PSM is created as the paraxial mesoderm cells are produced and organized into two bars of mesenchymal tissue during gastrulation, with one lying either side of the midline. This is followed by the establishment of a segmental pattern and then finally the formation of somitic boundaries.⁴ It is now widely accepted that segmental units called somitomeres exist in the PSM prior to the formation of somites,⁵ and so the PSM can be thought of as two distinct regions: the posterior tailbud domain and the more anterior somitomeric domain, with the boundary between these two regions termed the determination front.⁶

Cooke and Zeeman⁷ first proposed a model for the somitogenesis mechanism, named the clock and wavefront model. The theory behind this model is that there is a molecular oscillator or ‘clock’ with gradients of signalling molecules

interacting to generate dynamic gene expression patterns within the PSM. More recently, Pourquié⁴ explains that in this model the PSM cells are thought to oscillate between a permissive and a non-permissive state for somite formation, as controlled by a cell-autonomous temporal clock. The formation of a somite is thought to be triggered when permissive anterior PSM cells are hit by a 'wavefront' of maturation, which moves posteriorly along the axis of the embryo.

The formation of a somite begins in PSM cells at the determination front, where there is an intersection of the opposing fibroblast growth factor (FGF) posterior–anterior signal⁸ and the retinoic acid (RA) anterior–posterior signal⁹ gradients.¹⁰ FGF8 is thought to produce a moving wavefront, similar to that hypothesized by Cooke and Zeeman;⁷ the overexpression of FGF8 can inhibit somitogenesis by causing an anterior extension of posterior genes, indicating that FGF8 may control the activation of the segmentation programme by maintaining the posterior identity of PSM cells.⁶ Similarly, RA is believed to regulate somite formation as the altered expression of RA causes significant distortions in the size and morphology of somites.⁹ These somite changes are preceded by alterations in a basic helix–loop–helix (bHLH) transcription factor expression during segmentation, which is thought to arise from the altered ability of RA to inhibit the FGF signalling pathway at the determination front. This is achieved by increasing the expression of *MKP3*, a protein known to dephosphorylate, and thus inactivate, *ERK* (a member of the MAPK pathway) which is essential for FGF signalling. Consequently, RA is thought to play a key role in regulating gene expression patterns during segmentation.⁹ The Notch signalling pathway also has a crucial role in somitogenesis, through Notch-dependent intercellular communication. This signalling pathway is mediated through the Hairy/Enhancer of split oscillator (the 'clock'), and couples surrounding cells in order to produce an ordered, synchronized oscillation.¹¹ *Mesp* family genes, including *Mesp2*-like *Thylacine1*, are also bHLH transcription factors that appear to play a crucial role in the segmentation process in chick, mouse, fish and frog, as they are essential for the development of the paraxial mesoderm.¹² Acting upstream of the Notch cascade, they regulate somite boundary positioning at the determination front and establish the somite anterior–posterior polarity.¹³ It is thought that RA signalling is able to regulate somitogenesis by controlling the *Mesp* family genes, evident as *Mesp* promoters are capable of responding to RA signalling.¹² These cells at the determination front are then competent to respond to Notch pathway genes acting as segmentation cues and form somitomeres.

Oscillatory waves of gene expression in the PSM was first observed in chick, where a gene coding for a bHLH transcription factor *c-hairy1*, a chick homologue of the *Drosophila* pair-rule gene *hairy*, exhibited dynamic rhythmic waves of expression, repeating with the formation of each

new pair of somites.¹⁴ Many other cycling genes have now been identified, including *lunatic Fringe*,¹⁵ *Hes1*¹⁶ and *c-Hey2*¹⁷ in chick, *Lunatic fringe*¹⁸ and *Hes7*¹⁹ in mice, *her1* in fish^{20, 21} and *enhancer of split-related-9* (*esr9*) in frogs.²² Hairy/enhancer of split (*HES*) bHLH transcription factors are targets of Notch signalling, while *Lunatic fringe* is a glycosyl transferase required for Notch signalling. These genes all exhibit similar behaviour to *c-hairy1*, indicating the segmentation clock may be conserved among vertebrates. However, there is no obvious rule as to which particular gene(s) in this family will cycle in each species, for example, neither *c-hairy1* or *Lunatic fringe* frog homologs cycle in the *Xenopus* PSM, indicating the conservation of cycling genes may not be as strong as previously thought.²³

Although extensive research has been carried out on somitogenesis in chick and mouse, little is known about oscillating genes in *Xenopus laevis*. The only gene in *X. laevis* that shows the dynamic cycling expression pattern associated with somitogenesis is *esr9*.²² To better understand the process of somitogenesis in frogs, I identified expressed sequence tags representing clones coding for *ESR1*, *ESR2* and *ESR-5* which were used to generate probes for *in situ* hybridization. *ESR-5* is a well characterized gene known to be expressed in the PSM and in the anterior half of the two most posterior somitomeres (S-II and S-III). *ESR1* is a well-documented neural marker²⁴ with expression also seen in the tailbud, highlighting the possibility of its role in somitogenesis. In contrast, there are no documented expression analyses of the *ESR2* gene in *X. laevis*. In addition, the expression pattern of *Thylacine1* and *bowline* mRNA were investigated, as they provide good molecular markers of somitomeres S-II and S-III.

There are many different ways to order and number somitomeres. In this report, somitomeres will be referred to in the same way Moreno and Kintner⁹ do, as S0–S-III with S-III being the most posterior somitomere and S0 the most anterior.

Methods

Obtaining DNA

DNA sequences and clone numbers for required genes were retrieved from the National Center for Biotechnology Information and clones ordered from the National Institute for Basic Biology (NIBB), using both the clone number and DNA sequence. The plasmids were supplied, dried on paper and were extracted by soaking in water, vortexing and heating repeatedly, before storing at -20°C .

Escherichia coli Transformation

Plasmids were introduced to Promega competent *Escherichia coli* cells and shaken in Luria–Bertani (LB) broth before

incubating on antibiotic plates and picking individual colonies to select for transformed *E. coli*. These were then shaken overnight in LB broth with ampicillin to allow growth of the transformed *E. coli* (protocol obtained from <http://www.promega.com/tbs/tb095/tb095.pdf>). A purified plasmid solution was obtained using 'QIAprep Spin Miniprep' according to the manufacturer's protocol (which can be found online: http://www1.qiagen.com/KB/QIAprepMiniprepKit_EN). The concentration of the final plasmid solution was then determined using a NanoDrop spectrophotometer.

Diagnostic DNA Digests

Diagnostic DNA digests using plasmid and gene sequences to identify restriction enzyme (RE) sites were used to check the correct gene was in each plasmid, to determine the orientation of each gene in the plasmid and also the size of the insert. A linearizing RE was then identified for each.

Making Probe Template

DNA template for the probe was generated by mixing 5 µg DNA, 10% 10× buffer and 3% linearizing RE, and incubating at 37°C for 90 min to digest the DNA before checking that all DNA has cut to completion by running on an agarose gel. The digestion was then cleaned up using 'QIAquick Gel Extraction kit' according to manufacturer's protocol, leaving a purified DNA template.

Making Antisense Probe

Antisense RNA probe was generated by mixing 20% transcription buffer, 5% 10× digoxigenin NTP mix (containing 10 mM of each ATP, CTP and GTP, 6.5 mM UTP and 3.5 mM DIG-11-UTP), 10 mM dithiothreitol (DDT), 4% RNasin, 6% appropriate polymerase and 50 µg/ml DNA template, and incubating at 37°C for 2 h to allow adequate time for transcription, before checking the transcript on agarose gel. DNA template was removed from the mixture by adding RNase-free DNase before precipitating the probe with 11% 5 M ammonium acetate and 72% ethanol (EtOH) and leaving overnight at -20°C. The pellet was spun down at 4°C before washing in 70% EtOH, drying and redissolving in dH₂O, to leave a purified antisense RNA probe solution. Probes were hydrolysed, if necessary by adding an RNase-free solution of 40 mM NaHCO₃/60 mM Na₂CO₃ and leaving at 60°C for 20–30 min.

Fertilizations

Mature male *X. laevis* were anaesthetized with 0.05% benzocaine before removing the testes and storing them in normal amphibian media (NAM) (110 mM NaCl, 2 mM KCl, 1 mM Ca(NO₃)₂·4H₂O, 1 mM MgSO₄·7H₂O, 0.1 mM Na₂EDTA). One-fourth to one-eighth of a testicle was macerated in a few drops of NAM to produce a sperm solution. Ovulation was induced in females by injecting

50 units of human chorionic gonadotropin (hCG), 48–72 h before the eggs are needed, then injecting a further 250 units of hCG in the night before the eggs are required. By gently squeezing the female with pressure on her abdomen and lower back, the eggs were expelled, and then the sperm solution was immediately pipetted over the eggs for fertilization. After 5 min, the embryos were flooded with NAM/10 (10% NAM, 5% 0.1 HEPES) and left for at least 40 min to allow for cortical rotation before a 2.5% cysteine solution (pH 7.8) was used to de-jelly the eggs. These were then washed extensively with buffered water to remove all traces of cysteine, followed by one wash in NAM/10 before putting the embryos in trays lined with 1% agarose and covering with NAM/10 for culturing. Fertilized embryos were recognized by their rigidity as the vitelline membrane thickens, and any unfertilized eggs were discarded.

Fixing

Embryos were collected at the required stage after removing their vitelline membranes and were fixed in MEMFA (0.1 M MOPS, 2 mM EDTA, 1 mM MgSO₄ and 3.7% formaldehyde) for 60 min before being washed in 100% EtOH and stored in fresh EtOH at -20°C.

In situ Hybridization

Embryos were rehydrated in a graded series of EtOH washes with PBSAT (PBS, 0.1% Tween) and washed extensively in PBSAT. They were permeabilized with Proteinase K treatment at a concentration of 10 µg/ml 20–35 min. After rinsing in 0.1% triethanolamine (pH 7.8), acetic anhydride was added to make a 0.25% solution, and then again to make a 0.5% solution. After refixing in 3.7% formaldehyde/PBSAT, embryos were washed extensively in PBSAT and then equilibrated in hybridization buffer (50% formamide, 5× SSC, 1 mg/ml total yeast RNA, 100 µg/ml heparin, 1× Denharts, 0.1% Tween, 0.1% Chaps and 10 mM EDTA). After prehybridizing in fresh hybridization buffer for 2 h at 60°C, embryos were incubated overnight in probe solution (probe plus hybridization buffer). Embryos were rinsed twice in hybridization buffer at 60°C to remove any residual probe before a series of rinses were performed, still at 60°C, with 2× SSC (0.1% Tween) and then 0.2% SSC (0.1% Tween) and then at room temperature with maleic acid buffer (MAB: 100 mM MAB, 150 mM NaCl, 0.1% Tween, pH 7.8). Following blocking in MAB, 2% Block (Blocking Reagent; Roche) and 20% heat-treated lamb serum at room temperature for 2 h to prevent non-specific antibody binding, embryos were rocked overnight at 4°C in MAB + Block + HT lamb serum + 1/2000 dilution of sheep anti-digoxigenin antibody coupled to alkaline phosphatase. They were washed several times with MAB to remove any excess antibody and rinsed twice in alkaline phosphatase buffer (100 mM Trizma, 50 mM MgCl₂,

100 mM NaCl, 0.1% Tween, pH9.5) in order to equilibrate embryos before application of the substrate. Embryos were incubated in BM purple for as long as necessary to detect the antibody (between a few hours and a few days), as BM purple substrate turns blue and precipitates in the presence of the antibody. After the colour developed, embryos were washed twice in PBSAT to remove any residual BM purple and fixed overnight in 3.7% formaldehyde/PBSAT. Embryos were later bleached in 5% H₂O₂/PBSAT rolling under bright light until pigment was gone and then washed several times in PBSAT to remove all traces of H₂O₂ before fixing again in 3.7% formaldehyde/PBSAT.

Embryo Photography

Photographs of embryos were taken in PBSA on 1% agarose-lined trays using Spot camera with a Leica MZFLIII stereo-microscope, and Spot Advanced computer software. Pictures were cropped and light levels adjusted using Adobe Photoshop.

Results

Isolation of cDNAs

cDNAs coding for *ESR1*, *ESR2* and *ESR-5* were provided by M.E. Pownall and are being used in other current studies (unpublished data), while *Thylacine1* and *bowline* cDNAs were obtained from NIBB using their unique clone numbers (Table 1). Information about the RE sites of each gene and plasmid details were collected using their sequences (plasmid sequences obtained from the I.M.A.G.E consortium; <http://image.hudsonalpha.org/html/vectors.shtml>) and used to determine both the orientation of the gene and the best linearizing RE to generate a probe template (Table 1). From this information, diagnostic digests were carried out and subsequently, linearized probe templates were generated. Antisense RNA probes were generated from each of these templates for *in situ* hybridization.

Thylacine1 Expression Is Seen Exclusively in S-II and S-III

In situ hybridization using the *Thylacine1* antisense probe reveals the specific expression of this gene in the developing somitomeres. At stage 12, two very faint stripes can be seen above the blastopore, marking the early segmentation in the gastrula embryo (arrowheads, Fig. 1A). By stage 16, *Thylacine1* expression is much stronger and more apparent, marking three somitomeres in this case (Fig. 1B). The marking of more than two somitomeres is not an unexpected result as Sparrow *et al.*²⁵ note that between one and three stripes are usually observed, with two being the most frequent. At stages 26 and 31 (Fig. 1C and D, respectively), two distinct lines can be seen in each, showing expression of *Thylacine1* in S-II and S-III. The more posterior of the two somitomeres seen at stage 31 shows an extension of *Thylacine1* expression ventrally on the tailbud (marked by a black asterisk on Fig. 1D) which has not previously been reported.

Expression Analysis of *bowline* in *X. laevis* Reveals a Novel Expression Pattern in the Somites and Somitomeres

In a stage 12 embryo, *in situ* hybridization using an antisense probe for *bowline* reveals a faint circumblastoporal expression pattern (Fig. 2A). This expression in a gastrula stage embryo has not been described in any literature; in fact, the earliest noted expression pattern is the presence of bilateral stripes in a stage 13 embryo.²⁶ The fact that this expression is circumblastoporal and does not show any specific stripe pattern as observed by Kondow *et al.*²⁶ in stage 13 embryos raises the question of whether this is a true representation of *bowline* mRNA expression in a gastrula embryo, or is background colour that has developed due to the absence of true mRNA expression; further work is needed to decipher this. By stage 16, however, bilateral stripes can be clearly seen marking S-II and S-III (shown by the arrowheads in Fig. 2B). Stage 26 embryos (Fig. 2C and D) and stage 31 embryos (Fig. 2E) both have quite novel

Table 1. Data collected regarding each gene studied

	Gene				
	<i>ESR1</i>	<i>ESR2</i>	<i>ESR-5</i>	<i>Thylacine1</i>	<i>bowline</i>
NIBB/clone number	IMAGE: 5537441	IMAGE: 6955664	XI207h08	XL204i05	XI034g14
Unigene number	XI.8440	XI.12067	XI.14524	XI.54	XI.16077
Plasmid	pCMV. SPORT 6	pCMV. SPORT 6.1	pBluescript sk-	pBluescript sk-	pBluescript sk-
5' clone site	<i>Sall</i>	<i>EcoRV</i>	<i>EcoRI</i>	<i>EcoRI</i>	<i>EcoRI</i>
3' clone site	<i>NotI</i>	<i>NotI</i>	<i>XhoI</i>	<i>XhoI</i>	<i>XhoI</i>
RE for linearizing	<i>EcoRI</i>	<i>XbaI</i>	<i>EcoRI</i>	<i>BglII</i>	<i>SacI</i>
Polymerase	T7	T7	T7	T7	T7
Gene insert size (kb)	1.6	1.1	0.6	1.7	1.8
Transcript size (bp)	600	1100 (hydrolysed to 300)	600	800	1000

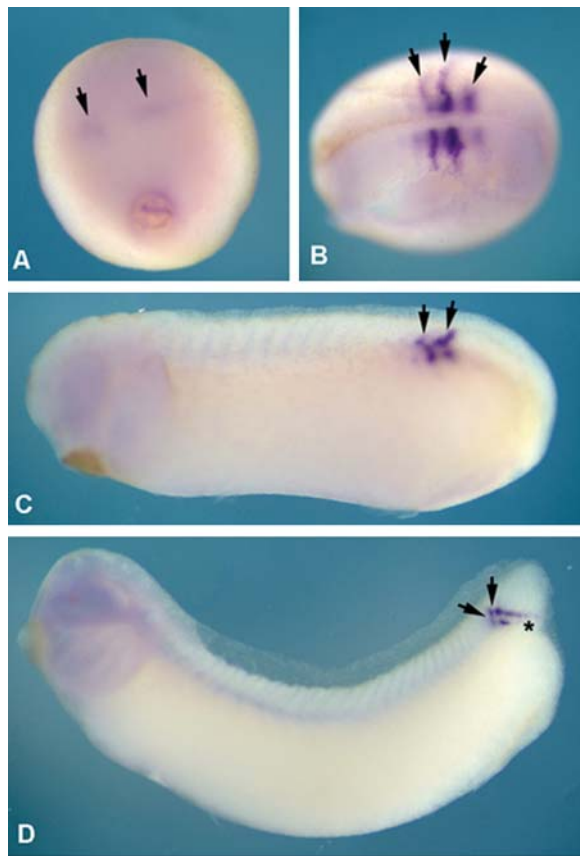


Figure 1. *In situ* hybridization showing the expression of *Thylacine1* in *X. laevis*. (A) Stage 12 vegetal view (dorsal side up), (B) Stage 16 dorsal view, (C) Stage 26 lateral view and (D) Stage 31 lateral view. In (B–D), anterior is to the left. Black arrowheads point to somitomes expressing *Thylacine1* mRNA and the asterisk marks the ventral extension of *Thylacine1* expression in S-III.

expression patterns, significantly different to that described in the literature for similar stages; others describe seeing one to two stripes of expression in the posterior of the embryo, correlating to S-II and S-III, and nothing else.^{26, 27} However, this is not reflected in the *in situ* hybridization studies I carried out for *bowline*. Not only is their considerably strong expression in the stated somitomes, but fairly strong expression can also be seen in the somites. A gap of approximately three to four somitomes/somites can be seen between the most anterior somitome marked, and the most posterior somite marked by *bowline* expression. The reason for this gap in expression is not known as *bowline* expression in the somites has not previously been documented. This observed difference in *bowline* expression is surprising—the cDNA used to create the antisense probe was sequenced and found to be the same as that used by others.²⁶ This study was repeated three times with the same result each time, suggesting that it was not an experimental procedure or similar causing this unexpected result. In addition, while watching the colour development it was

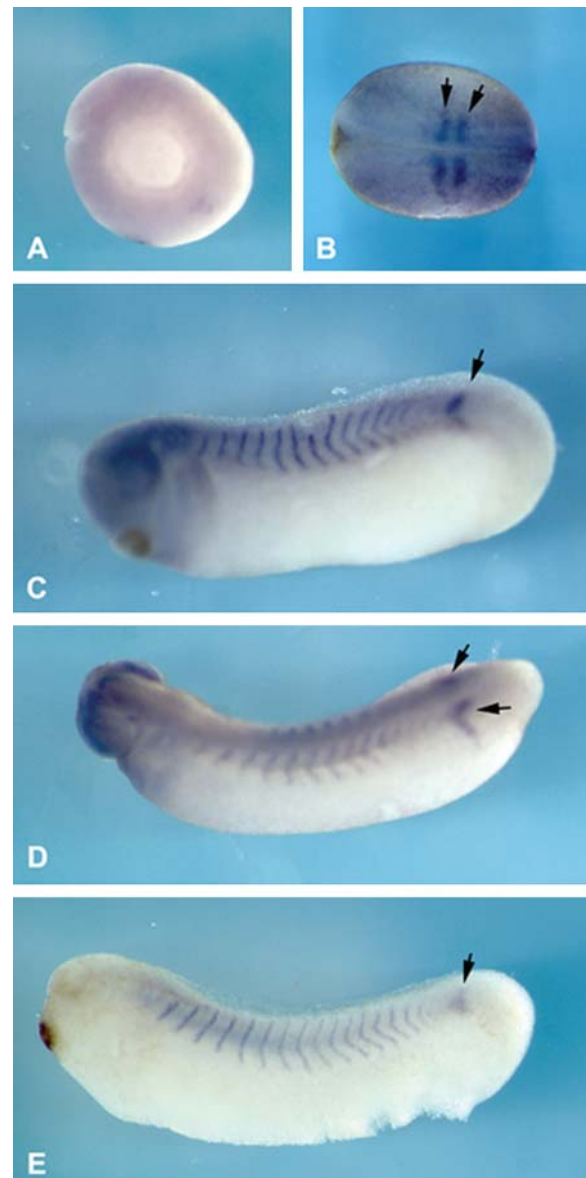


Figure 2. *In situ* hybridization showing the expression of *bowline* in *X. laevis*. (A) Stage 12 vegetal view, (B) Stage 16 dorsal view, (C) Stage 26 lateral view, (D) Stage 26 dorsal view and (E) Stage 30 lateral view. In (B–E), anterior is to the left. Arrowheads mark *bowline* expression in somitomes.

possible to see colour developing in the somites and the somitomes at the same time, thus it was not background occurring due to prolonged exposure to the BM purple substrate.

ESR-5 Transcripts Are Found in the PSM and in One to Three Somitomes in *X. laevis*

Carrying out *in situ* hybridizations with an *ESR-5* antisense probe reveals that this gene is very strongly expressed in the PSM and in the anterior half of S-II and S-III (Fig. 3). During

gastrulation, *ESR-5* expression is seen surrounding the blastopore but is excluded from the dorsal lip (Fig. 3A). In addition to this, two stripes of expression can be seen above the blastopore corresponding to the newly formed somitomeres. Similarly, at stages 16, 26 and 31, strong expression can be seen in the PSM and in one to three somitomeres (Fig. 3B–D, respectively). The dark colouring seen in the head is a common background noise, caused as cavities in the head trap the antisense probe and this should not be considered as *ESR-5* expression. The ventral extension of expression in S-III can be seen here (Black asterisk, Fig. 3D) similar to that seen in *Thylacine1* expression (Fig. 1D). Here, the stripe appears parallel to the PSM highlighting the possibility that this extension of mRNA expression could occur in younger somitomeres which have not yet restricted gene expression laterally, as older somitomeres may have done. This is only one possibility though,

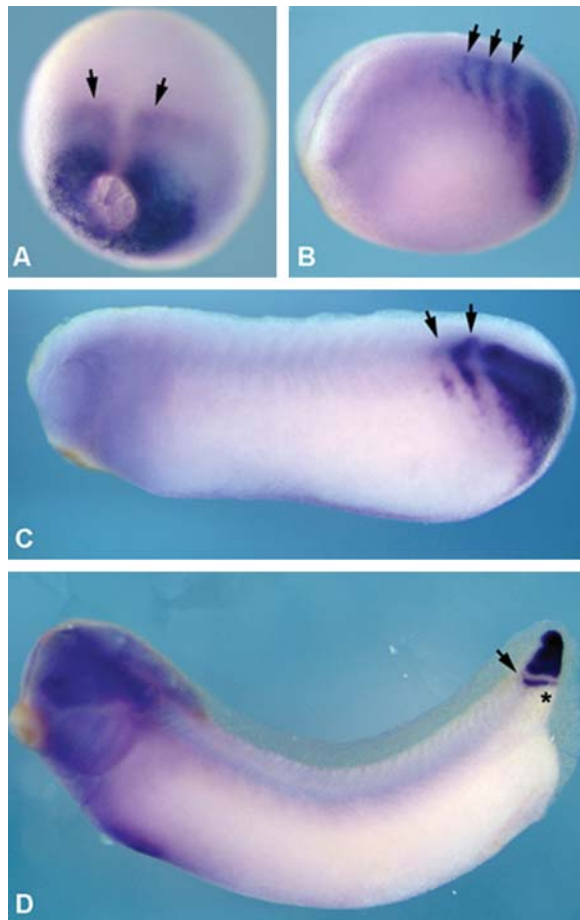


Figure 3. *In situ* hybridization showing the expression of *ESR-5* in *X. laevis*. (A) Stage 12 vegetal view (dorsal side up), (B) Stage 16 lateral view, (C) Stage 26 lateral view and (D) Stage 31 lateral view. In (B–D), anterior is to the left. Black arrowheads point to somitomeres expressing *ESR-5* mRNA and the asterisk marks the ventral extension of *ESR-5* expression in S-III.

and further studies would be needed to decipher this extension of expression.

Expression Analysis of *ESR1* in *X. laevis* Highlights Its Strong Neural Expression and Some Tailbud Expression

In situ hybridization using the antisense probe for *ESR1* revealed a predominantly neural expression pattern. This neural expression is of no great significance to this study as it is not relevant to somitogenesis; however, there is some expression seen in the tailbud region of later stages. At stage 12, expression can be seen around the blastopore in the ventral region of the embryo, and also bilaterally (Fig. 4A); no expression is seen in the dorsal part of the embryo. By stage 16, a clear neural expression can be seen, with stripes of primary neuronal precursors evident on either side of the neural plate and a more anterior neural structure shown (Fig. 4B). In addition to these neural structures, strong expression can be seen in the posterior of the stage 16 embryos, apparently in the tailbud domain. By stages 26 and 31 (Fig. 4C and D, respectively), expression can be seen in many anterior neural structures, including the eye and various parts of the brain. It is also found along the entire length of the neural tube, as verified by transverse sections taken for both stages (Fig. 4E and F, respectively). Black arrowheads on Fig. 4C and D, point to *ESR1* expression in the tailbud domain of stages 26 and 31 embryos, respectively. The gene expression patterns of all similar stage embryos were compared to look for any differences that may indicate the gene was cycling. However, no significant differences were observed at any stage, indicating that *ESR1* mRNA does not cycle during somitogenesis.

ESR2 mRNA Is Seen in Many Neural Structures in Addition to the Tailbud Region in *X. laevis*

At stage 12, expression can be seen in the mesoderm of the ventral half of the embryo, surrounding the blastopore (Fig. 5A). By stage 16, *ESR2* expression can be seen in the posterior paraxial mesoderm of the embryo and also marking some primary neural precursors either side of the neural tube (Fig. 5B). Expression in a stage 26 embryo is predominantly in the tailbud region, but can also be seen in some of the more anterior neural structures including the eye (Fig. 5C). At stage 31, *ESR2* expression is seen in many anterior neural structures, including the eye and brain (Fig. 5D). In addition to this, expression can be seen in the neural tube, notochord and to a lesser extent in the somites. This was verified by studying a transverse section of a stage 31 embryo, which clearly shows *ESR2* expression in these internal structures (Fig. 5E). Tailbud expression is also apparent at this stage, as marked by the arrowhead in Fig. 5D.



Figure 4. *In situ* hybridization showing the expression of *ESR1* in *X. laevis*. (A) Stage 12 vegetal view (dorsal side up), (B) Stage 16 dorsal view, (C) Stage 26 lateral view and (D) Stage 31 lateral view. Transverse sections can be seen for stage 26 (E) and stage 31 (F) embryos showing expression in the neural tube. In (B–D), anterior is to the left.

Variable Expression Patterns in Neurula and Early Tailbud Stage Embryos Highlights the Potential Cycling Nature of *ESR2*

A considerable number of embryos were studied after *in situ* hybridization to look for any sign of dynamic expression which may indicate that *ESR2* mRNA is cycling. There did not appear to be any differences in gene expression patterns for stage 12 or 31 embryos, however, both stage 16 (Fig. 6) and stage 26 embryos (not shown) showed considerably

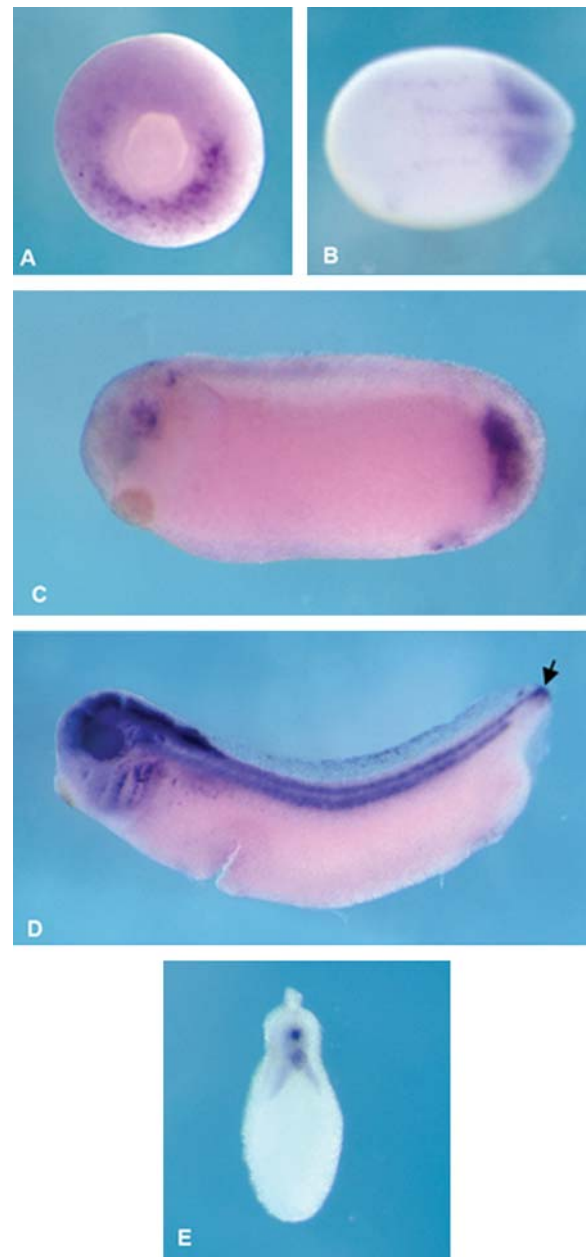


Figure 5. *In situ* hybridization showing the expression of *ESR2* in *X. laevis*. (A) Stage 12 vegetal view (dorsal side up), (B) Stage 16 dorsal view, (C) Stage 26 lateral view and (D) Stage 31 lateral view. In addition, a transverse section for stage 31 can be seen (E) highlighting expression in the neural tube, the notochord and bilaterally in the somites. In (B–D), anterior is to the left. The black arrowhead in D points to tailbud expression.

variable patterns of gene expression, making this an exciting prospect for an oscillating gene.

Figure 6 shows a series of stage 16 embryos aligned to demonstrate a progressive mRNA expression pattern, similar to that seen for *esr9*.²² Figure 6A–C shows the first stage of the progression, with *ESR2* expression in the PSM of these embryos, but not in any somitomeres. Figure 6A and B

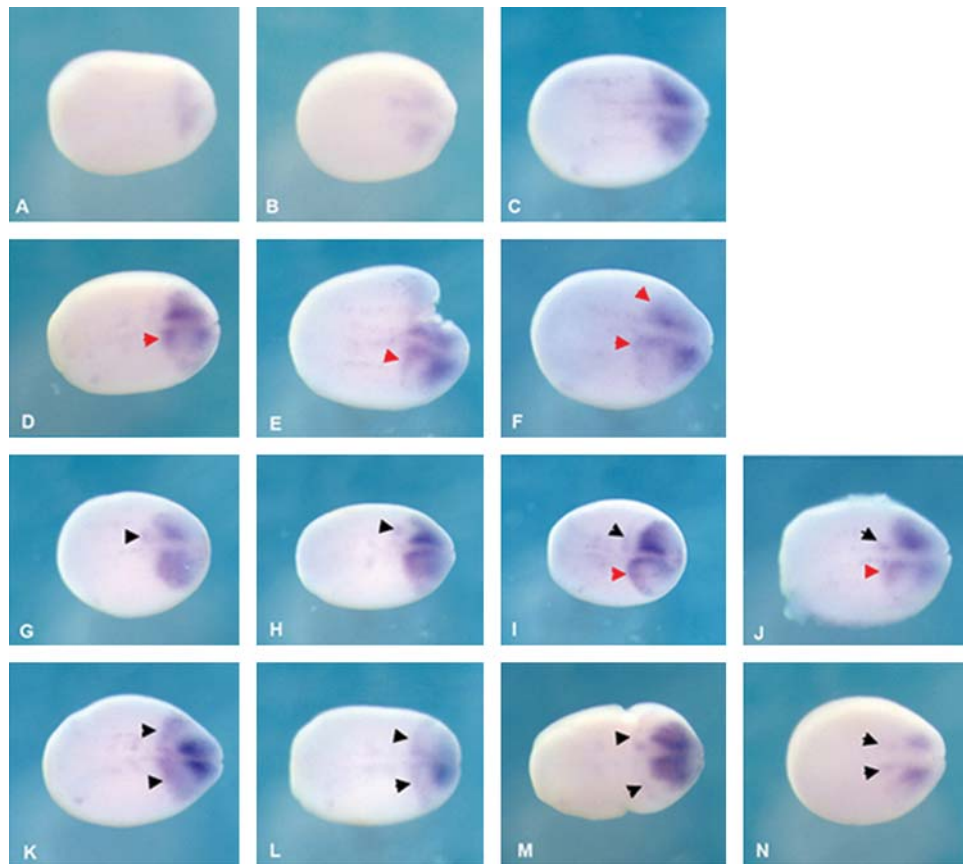


Figure 6. *In situ* hybridization showing the dorsal view of stage 16 embryos with different *ESR2* gene expression patterns. (A–C) *ESR2* expression in the PSM, but no fully or partially separated somitomeres can be seen. (D–F) Expression in the PSM, and red arrowheads here point to partially separated somitomeres. (G–H) Expression in the PSM and in one fully separated somitomere (black arrowheads). (I–J) Expression in one fully separated somitomere (black arrowheads), and in the PSM with one somitomere partially separated (red arrowheads). (K–N) Expression in the PSM and in two fully separated somitomeres each (black arrowheads). In all pictures, anterior is to the left.

shows considerably weaker expression in the PSM than Fig. 6C, but similar neural expression, so this may reflect an earlier stage of this expression cycle. Figure 6D–F each shows expression in the PSM and in one or two somitomeres of each embryo, as marked by red arrowheads. The expression in these somitomeres appears to be continuous with the PSM at the midline, while others (black arrows) do not. This may represent different stages of somitomere formation. In the next stage of this series (Fig. 6G and H), somitomeres exhibiting mRNA expression separately to the expression in the PSM can be seen on only one side of the embryo (as shown by black arrowheads), while Fig. 6I and J shows a combination of continuous (red arrowheads) and separate (black arrowheads) expression in somitomeres with relation to the PSM. Figure 6K–N shows the next stage in the process, with one somitomere either side of the midline displaying *ESR2* mRNA expression separate to that in the posterior PSM (shown by black arrowheads).

Discussion

Expression of *Thylacine1* and *ESR-5* Can Be Seen in Somitomeres during Gastrulation

One pair of somitomeres can be seen during gastrulation to express both *Thylacine1* and *ESR-5*, as shown by black arrowheads in Figs 1A and 3A, respectively. There are no published reports of gene expression in segmented mesoderm being observed prior to neurula stages and so *Thylacine1* and *ESR-5* expression in the late gastrula may be marking the initial segmentation of mesoderm in the embryo. Interestingly, *bowline* expression is not restricted to somitomeres at this same stage (Fig. 2A), even though *bowline* mRNA is known to be expressed in the same somitomeres as *ESR-5* and *Thylacine1*.²⁶ The reason for not seeing any somitomere stripes at this stage may be as simple as there being widespread *bowline* expression circumblastoporally. Although this may be the case as there appears to be weak

circumblastoporal expression (Fig. 2A), the colour seen is faint and without defined margins, so was initially considered to be background colour. One consideration to make is that the *bowline* antisense probe used for this *in situ* hybridization appeared less defined in comparison to other probes when run on an agarose gel. This may cause the unexpected expression pattern by either creating the suspected circumblastoporal background noise or failing to detect any small amounts of *bowline* mRNA in the segmented mesoderm. Further investigation is needed to clarify the expression of *bowline* mRNA at gastrula stage, and if there truly is no somitomere expression found, to determine why this is.

A Novel Expression Pattern Is Seen for *bowline* Which May Be Linked to a Negative Feedback Loop of Gene Expression

In previous experiments, *bowline* expression has only been seen in the two most posterior somitomeres, S-II and S-III.^{26, 27} Expression in these somitomeres is apparent in my studies, but in addition to this, expression is also seen in the somites (Fig. 2). Interestingly, there is a gap the size of approximately three somitomeres/somites between the most anterior somitomere and the most posterior somite expressing *bowline* mRNA. As *bowline* expression has not been documented in the somites before, the reason for this gap is also unknown. Kondow *et al.*²⁸ have found that although the *bowline* mRNA is located in S-II and S-III, the functional protein is found in S-I and S0. This late translation of *bowline* mRNA may account for the gap seen between the somites and the somitomeres. They go on to explain that *bowline* expression is activated by Tbx6²⁷ and once the Bowline protein reaches a threshold concentration Tbx6 is converted from a transcriptional activator to a transcriptional repressor by associating with X-Grg4 (a corepressor member of the Groucho family) via Bowline.²⁸ This new transcriptional repressor turns off *bowline* mRNA expression and may be the mechanism responsible for repressing *bowline* expression in the older and more anterior somitomeres, S-I and S0.

Previously Unstudied *ESR2* Shows Expression in Both Mesoderm and Ectoderm

Expression analysis of *ESR2* has revealed expression in both mesodermal and ectodermal tissues. Neuronal expression is very obvious in stages 16, 26 and 31 (Fig. 5B–D). Strong expression in the neural tube, the brain and in the eye at stage 31 highlights a strong presence in ectodermal derivatives, while expression in the notochord, somites and PSM demonstrate a strong mesodermal presence as well (Fig. 5D and E). At stage 26, expression appears to be more restricted to the mesoderm, with strong expression in the PSM and in a few somites while neural expression falls mainly in the eye (Fig. 5C). Primary neuronal precursors can be seen to express *ESR2* at stage 16, as can the PSM (Fig. 5B), whereas circumblastoporal expression at stage 12 does not

discriminate between the mesodermal and ectodermal tissues (Fig. 5A). This expression in both the mesoderm and ectoderm makes the expression pattern of *ESR2* somewhat similar to that of both *ESR1*²⁴ (Fig. 4) and *esr9*.²²

The Dynamic Expression Pattern of *ESR2* at Stages 16 and 26 Correlates to that of a Known Cycling Gene, *esr9*

The dynamic expression pattern of *ESR2* seen in stage 16 embryos (Fig. 6) and stage 26 embryos (not shown) appears to show cyclic oscillations, similar to the way *esr9* mRNA cycles²²—an exciting discovery as this makes *ESR2* the second potential oscillating gene in *X. laevis*. The cycle begins with expression restricted to the PSM, before the mesoderm forms somitomeres (Fig. 6A–C). These somitomeres initially show striped *ESR2* expression continuous at the midline with expression in the posterior PSM (Fig. 6D–F). This expression then appears to separate at the midline from that in the PSM, and as the somitomeres mature they will eventually stop expressing *ESR2* altogether (Fig. 6G–M). This cycle will then start again with expression restricted to the PSM (Fig. 6N).

Many embryos were observed to be asymmetrical with regards to their expression of *ESR2*, including both stage 16 (Fig. 6) and stage 26 embryos (not shown), but this asymmetry never seemed to exceed more than one somitomere in either stage. This highlights the autonomy of PSM cells either side of the midline, and indicates that oscillations between either side of the midline may be regulated independently. Asymmetry in gene expression has also been observed in *esr9*²² and in *hairy2a*²⁹ in *X. laevis* while mouse and chick expression is symmetrical.

Conclusion and Further Studies

This study has identified *ESR2* as the second potential gene in *X. laevis* to show periodic oscillations of gene expression during somitogenesis. However, other experiments are required to show definitively whether or not *ESR2* is cycling in the *Xenopus* PSM. Explanting the PSM of the embryo either side of the midline and fixing at varying time points would reveal whether or not *ESR2* mRNA oscillates autonomously, and thus whether or not *ESR2* is a true cyclic gene. Experiments similar or the same as this are routinely carried out in other studies to identify cyclic genes.^{14, 16, 17, 19} In addition, periodic oscillations of both *Hes1* in chick and *ESR9* in *X. laevis* have been shown by application of cyclohexamine to be independent of protein synthesis,^{14, 22} and so replication of these experiments to look at the effect of blocking protein synthesis on *ESR2* mRNA expression may also help elucidate whether or not this gene is truly cycling. An in depth study into the structure of the *ESR2* gene and its regulatory elements would be beneficial to this research also by revealing activators and/or repressors of *ESR2* expression, hopefully confirming its

role in the Notch pathway and possibly linking its expression to that of genes known to be involved in the segmentation of somitomeres, such as *bowline* and *Thylacine1*. In addition, the use of an antibody to detect protein localization for ESR2 would be interesting in order to see if there is dynamic localization of the protein as well as the dynamic mRNA expression. Unfortunately, these further studies were not carried out due to time constraints but would be a good starting point to further understand the process of somitogenesis in *X. laevis*.

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