Cloning and Analyzing of *Xenopus* Mespo Promoter in Retinoic Acid Regulated Mespo Expression

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**Abstract** During vertebrate embryogenesis, presomitic mesoderm cells enter a segmental program to generate somite, a process termed somitogenesis. *Mespo*, a member of the bHLH transcription factor family, plays important roles in this process. However, how *Mespo* expression is regulated remains unclear. To address this question, we isolated a genomic DNA sequence containing 4317 bp of *Mespo* 5′ flanking region in *Xenopus*. Luciferase assays show that this upstream sequence has transcription activity. Transgenic assay shows that this genomic contig is sufficient to recapitulate the dynamic stage- and tissue-specific expression pattern of endogenous *Mespo* from the gastrula to the tailbud stage. We further mapped a 326 bp DNA sequence responding to retinoic acid signaling. These results shed light on how *Mespo* expression is regulated, and suggest that retinoic acid signaling pathways play roles in somitogenesis through regulating *Mespo*.

**Key words** bHLH; paraxial; presomitic; segmental; tailbud; *Xenopus laevis*

Somite is a transient structure in vertebrate embryos derived from paraxial mesoderm [1,2]. The generation of somites, known as somitogenesis, is featured by the sequentially budding off of somites from the rostral end of the presomitic mesoderm (PSM) at regular intervals along the anterior-posterior (AP) axis [3]. Genes responsible for segmentation and cell differentiation within PSM are dynamically expressed. Revealing how signaling pathways coordinatively exert their role in regulating PSM gene expression is of crucial importance in understanding the molecular mechanism of somitogenesis [4,5].

Recent work suggested that Wnt, fibroblast growth factor (FGF) and retinoic acid (RA) signals are all involved in somitogenesis. However, the molecular nature for signals controlling the dynamic expression pattern within PSM has largely remained unknown. *Mespo*, also known as *pMesogenin1* [6], is a bHLH gene specifically expressed in the caudal PSM and has been proposed to be important in the development of the paraxial mesoderm [6,7]. *Mespo* null mutant mouse showed no identifiable somites or segmental patterns in the trunk from the posterior to the forelimbs and severe disruption of gene expression in posterior paraxial mesoderm [8]. In *Xenopus*, overexpression of *Mespo* in PSM caused ectopic expression of *PAPC* that is important in segmental patterning [9]. In animal cap assay, *Xenopus* Mespo can induce presomitic paraxial mesodermal phenotypes [6]. These results indicate that *Mespo* is essential for the development of paraxial mesoderm and establishing segmental patterns. When PSM cells enter the segmental program, *Mespo* expression is down-regulated. However, how *Mespo* expression is regulated is unknown.

Recent findings have highlighted the essential role that RA plays in regulating the development of paraxial mesoderm [10]. Removal of RA in the vitamin-A-deficient (VAD) quail embryos led to embryos with somites that were half the normal size [11]. Homozygous null mutant embryos of Raldh2, an enzyme required for RA synthesis, exhibited abnormally small somites [12,13], while enhancing RA signaling activity by treating *Xenopus* embryos with RA caused caudal shift of the somite boundary [14]. Importantly, it has been shown that RA signaling contri-
butes to segmental patterning by repressing *Mespo* expression in the PSM [14]. Thus, how RA signaling regulates *Mespo* expression becomes critical for understanding the mechanism of segmentation.

To uncover how *Mespo* expression is regulated and how RA signaling represses *Mespo* expression, we isolated *Xenopus Mespo* 5′ upstream regulatory fragment. We found that this genomic contig can recapitulate the endogenous *Mespo* expression pattern from the gastrula to the tailbud stage. *Mespo* promoter responds to RA signaling and a 326 bp DNA sequence is required for this responding.

**Materials and Methods**

**Embryo manipulations and drug treatment**

Preparation and injection of *Xenopus* embryos were carried out as previously described [15]. Embryos were staged according to Nieuwkoop and Faber [16]. All-trans RA (Calbiochem, San Diego, USA) was dissolved in dimethylsulfoxide (DMSO) and used at 1 μM.

**Isolation of the *Xenopus Mespo* promoter**

We cloned the *Xenopus* promoter using the GenomeWalker adaptor system (Clontech, Mountain View, USA). The primer of GSP1 was 5′-TGTGGGCTGGAGGAAGTAGACTCATAG-3′ and the primer of GSP2 was 5′-CTGAGGCCATGGGCTCTGGCTAAGTAGTA-3′. The primers of AP1 and AP2 were based on the GenomeWalker adaptor system. A 4337 bp DNA fragment was obtained. By comparing it with the published *Xenopus Mespo* sequence, we found the 4337 bp DNA fragment has 20 bp overlapping in the 5′-coding region of *Mespo* gene [6]. PCR-amplified fragments without the 20 bp sequence were purified and cloned into XhoI/HindIII digested pGL3 (Promega, Madison, USA), named as p-4317Luc. For the transgenic reporter assays, the luciferase gene was removed with HindIII and XbaI, and replaced by a green fluorescent protein (GFP) cDNA [17]. For luciferase assay, 2 nl solution, containing 25 pg of the various luciferase test constructs and 25 pg of the reference reporter construct pRL-SV40 (Promega), was injected into the animal poles of 4-cell stage embryos.

**Transgenesis**

Transgenic *Xenopus* embryos were generated as described previously [18]. Plasmids used for transgenesis assay were linearized by NotI digestion. Three independent rounds of transgenesis were performed for each plasmid.

**In situ hybridization**

Whole-mount *in situ* hybridization was performed as previously described [19] using Digoxigenin (DIG)-labeled probes. For bleaching of pigmented embryos, hybridized embryos were treated with bleaching solution (0.5×SSC with 1% hydrogen peroxide and 5% formamide) under a fluorescent light.

**Results**

**Isolation of the *Xenopus* promoter**

As a first step to understand the regulation of *Mespo* expression, we isolated the *Xenopus Mespo* upstream regulatory sequence. Since there was no available *Xenopus* genomic sequence information, we performed adaptor PCR according to the published protocol [20], and got a 4317 bp fragment upstream the transcription starting code ATG of the *Mespo* gene [Fig. 1(A)]. To test whether this fragment had a transcription activity, we carried out luciferase assay. The reporter construct of p-4317Luc was injected into animal pole of each blastomere of 4-cell stage embryos, and animal caps were dissected at stage 8.5 and harvested for reporter gene assay at stage 12.5. As shown in Fig. 1(B), the reporter gene activity is increased in animal caps injected with reporter construct p-4317Luc, indicating that this genomic contig has a transcription activity. This experiment was repeated three times.

![Fig. 1](image_url)

**Molecular cloning of *Xenopus Mespo* upstream DNA**

(A) The isolated 4317 bp genomic DNA contains upstream sequence of *Xenopus Mespo*. The transcription start site is marked as +1. (B) Luciferase activities of the indicated constructs injected into animal pole of each blastomere of 4-cell stage embryos. Animal caps were dissected at stage 8.5 embryos, and luciferase activities were measured at stage 12.5. ORF, open reading frame; RLU, relative light unit.
The 4317 bp genomic contig encompassed major cis-regulatory elements controlling Mespo transcription

Transgenesis is widely used for in vivo analysis of promoter elements [18]. To investigate the regulatory activity of the 4317 bp genomic contig, we performed transgenesis to introduce the 4317 bp GFP reporter construct (p-4317GFP) into Xenopus embryos. GFP mRNA expression in transgenic embryos was detected by in situ hybridization and compared with that of endogenous Mespo mRNA in normal embryos (Fig. 2).

Endogenous Mespo expression was first detected at the gastrula stage, and predominantly localized in the ventral-lateral mesoderm, but was absent in dorsal mesoderm [Fig. 2(A,B)] [7]. At the neurula stage, Mespo expression is in the PSM [Fig. 2(C)] and localized to the most caudal PSM [Fig. 2(D)] [7] compared with Thylacine 1 (Thy) that is expressed in somitomeres that lies in the rostral PSM [Fig. 2(D)] [21].

The expression pattern of the p-4317GFP reporter gene was very similar to that of the endogenous Mespo expression [Fig. 2(E–H)]. At the gastrula stage, the expression of GFP mRNA is restricted to the ventral-lateral mesoderm [Fig. 2(E,F)]; at the neurula stage, GFP mRNA is localized at the PSM [Fig. 2(G)] and restricted to the caudal PSM compared with Thy expression [Fig. 2(H)]. This was observed for the majority of p-4317GFP transgenic embryos (>60%, n>25/stage). All the experiments were repeated three times.

The spatial- and temporal-restricted expression of Mespo in PSM is under the control of multiple inputs. As the p-4317GFP reporter gene accurately recapitulates that of the endogenous Mespo expression pattern at gastrula and neurula stages, we thus concluded that the major cis-regulatory elements responsible for regulating Xenopus Mespo expression are included within this 4317 bp genomic contig.

The Mespo upstream region responds to RA signaling

Mespo is required for specification and segmentation of the paraxial mesoderm [8]. Evidence supports the issue that RA signaling is involved in regulating Mespo expression [14]. However, the exact molecular nature that RA signaling regulates Mespo expression remains unclear. In embryos that underwent somitogenesis, RA activity was dynamically changed, especially in the rostral end of PSM where Mespo expression was sharply down-regulated. We then treated embryos with RA and noticed that the expression of Mespo was rapidly reduced when the activity of RA signaling was increased [Fig. 3(B)], compared with control embryos [Fig. 3(A)] [14]. We then asked whether the p-4317GFP reporter construct responded to RA signaling. By treating p-4317GFP transgenic embryos with RA, we found that GFP mRNA expression was decreased [Fig. 3(D)] compared with the DMSO-treated transgenic embryos.

![Fig. 2 p-4317GFP reporter gene expression in transgenic embryos](http://www.abbs.info; www.blackwellpublishing.com/abbs)
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Fig. 3 Retinoic acid (RA) inhibition of Mespo reporter gene
Control embryos treated with dimethylsulfoxide (DMSO) (A) or RA (B) at stage 15 for 2 h, then the expression of endogenous Mespo was detected by whole mount in situ hybridization with Mespo antisense RNA probe. p-4317GFP transgene embryos were treated with DMSO (C) or RA (D) at stage 15 for 2 h, then the expression of green fluorescent protein (GFP) gene was detected by whole mount in situ hybridization with GFP antisense RNA probe. Numbers in the right corner represent the ratio of embryos with positive signals. In all cases, head is toward left.

Fig. 4 A 326 bp Xenopus upstream DNA sequence is required for responding to RA signaling
Since RA regulates downstream gene expression by directly binding to their conserved cis-elements with RA receptors, we searched for a potential RA receptor binding site in the Mespo promoter by MathInspector of Genomatix (http://www.Genomatix.com), but none were found. In order to define which region is needed for responding to RA signaling, we deleted the Mespo promoter to check reporter gene activity. The various deletions of the Mespo promoter are shown in Fig. 4(A). In animal cap assays, we found that the luciferase activities of the p-511Luc, p-837Luc, p-1107Luc and p-1500Luc were nearly the same with only a slight increase [Fig. 4(B)]. It indicates that no obvious enhancer exists in the 4317 bp upstream sequence of the Mespo promoter. Since animal cap cells are designated to form epidermis and neural tissues but not mesoderm tissues, we injected the various reporter constructs into the ventral marginal zone (VMZ), which will later form PSM so that the luciferase activity driven by various promoter constructs will much more
faithfully reflect the endogenous situations. We performed injections at 4-cell stage and cultured embryos to stage 15, then treated embryos with either DMSO or RA for 2 h. We detected the luciferase activity and found that the luciferase activity in the cases of either p-4317Luc, p-1500Luc, p-1107Luc or p-837Luc treated with RA was decreased to about half of that in DMSO-treated embryos [Fig. 4(C–F)], while the luciferase activity of the p-511Luc construct in RA-treated embryos was the same as that in DMSO treated embryos [Fig. 4(G)]. All the experiments were repeated three times. These results indicate that the p-511Luc construct did not respond to RA signaling and the 326 bp DNA sequence is required for responding to RA signaling.

Discussion

In the present study, we showed that a 4317 bp regulatory fragment of Xenopus Mespo contains major cis-regulatory elements that control the specific expression pattern of endogenous Mespo from the gastrula to the tailbud stage. We also showed that a 326 bp DNA sequence is required for responding to RA signaling.

The bHLH family transcript Mespo operates as a regulator of paraxial mesoderm specification and segmentation. Mespo is expressed in the unsegmented PSM and its expression is reduced when these cells enter the segmentation program [14]. Thus, the regulation of Mespo expression in PSM is important in segmental patterning. As multiple factors might be involved in the regulation of the spatial- and temporal-specific expression of Mespo in PSM, it is critical to identify a regulatory sequence that can faithfully recapitulate Mespo expression spatially and temporally. That means that the regulatory elements responsible for all of the input signals must be included. Therefore, the Mespo promoter we have cloned that correctly recapitulates the endogenous Mespo expression from the gastrula to tailbud stage allows us to study how signaling pathways regulate Mespo expression.

Evidence has suggested that RA signaling was involved in the regulation of Mespo expression [14]. Enhancing RA signaling activity down-regulated the expression of Mespo. However, the molecular nature that RA signaling regulates Mespo expression remains unclear. RA signaling can regulate gene expression by directly binding to the cis-elements with its receptor or by interacting with other signaling pathways. Although we did not find a potential RA receptor binding site in the 4317 bp genomic contig by a search of alignment using a computer, we did find that the 326 bp DNA sequence was required by luciferase assay. The luciferase assay was performed by injection of constructs into VMZ, which is the exact place that will later form PSM. In this context, the 326 bp sequence was required for RA-induced reduction of Mespo expression. It pointed to a possibility that endogenously RA signaling might interact with other signaling pathways to regulate Mespo expression. Further work is needed to uncover how RA is involved in repressing Mespo expression in a spatial and temporal specific manner.

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References

14. Moreno TA, Kintner C. Regulation of segmental patterning by retinoic acid

http://www.abbs.info; www.blackwellpublishing.com/abbs
16 Nieuwkoop PD, Faber J. Normal Table of Xenopus Laevis. North Holland: Amersterdam 1967
21 Sparrow DB, Jen WC, Kotecha S, Towers N, Kintner C, Mohan TJ. Thylacine 1 is expressed segmentally within the paraxial mesoderm of the Xenopus embryo and interacts with the Notch pathway. Development 1998, 125: 2041–2051