An interferon regulatory factor-like binding element restricts *Xmyf-5* expression in the posterior somites during *Xenopus* myogenesis

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Abstract The expression of *myf-5*, a key component of myogenic regulatory genes, declines progressively in mature somitic cells during vertebrate myogenesis. Little is known about how this down-regulation takes place. Here we provide evidence that an interferon regulatory factor binding element (IRF element) within the *Xenopus myf-5* promoter is responsible for the elimination of *myf-5* transcription in mature somitic mesoderm of *Xenopus* embryos. We show that this IRF element mediates the down-regulation of *Xmyf-5* transcription in gastrula embryos, and can specifically interact with nuclear proteins of early neurula. Moreover, deletion of this IRF element results in the anterior expansion of reporter gene transcripts within somitic mesoderm in transgenic embryos. Our results, therefore, provide insight into how the negative control of *Xmyf-5* expression takes place. © 2001 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.

Key words: Interferon regulatory factor binding element; Promoter; Gastrula; Myogenesis; *Xenopus myf-5*

1. Introduction

Vertebrate myogenesis is mainly controlled by members of the myogenic regulatory factor (MRF) family, including MyoD, Myf-5, myogenin and MRF4, which are all basic helix-loop-helix transcriptional factors [1–3]. Among these four genes, *myf-5* and *myoD* are expressed earlier than *myogenin* and MRF4, and are therefore believed to play a central role in initiating myogenesis [4]. Mice lacking both *myf-5* and *myoD* die at birth without detectable skeletal muscle or myoblast [5,6], though mice with a targeted knockout of either *myf-5* or *myoD* alone are capable of forming at least some myofiber [7]. In *Xenopus laevis*, ectopic expression of *myf-5* activates both *myoD* and the cardiac *actin* gene in animal cap cells

[8]. When *myf-5* is overexpressed together with *Xenopus myoD* it can also induce enlarged cranial and anterior trunk myotome myocytes and ectopic muscles in the lateral plate and neural tissue [9]. These results suggest that *myf-5*, although it has redundant function with *myoD*, is a muscle initiator.

The expression pattern of *myf-5* is thought to be consistent with its role during the early determination of the myogenic lineage. In *Xenopus*, *myf-5* is first activated in the dorsal region of stage 10 embryos [10,11]. Soon after the onset of gastrulation, the *Xenopus myf-5* (*Xmyf-5*) expression domain shifts to the dorso-lateral marginal zone which is believed to give rise to the anterior somitic mesoderm [9–16]. As gastrulation goes on, *Xmyf-5* expression in the future anterior somitic mesoderm is progressively down-regulated via an unknown mechanism, and *myf-5* transcripts are restricted to the posterior part of the somitic mesoderm [9,11,12]. This character is maintained to tadpole stage with positive signals restricted within the tail [12]. Given that somite formation and maturation in *Xenopus* follow the antero-posterior sequence, the dynamic change of *Xmyf-5* expression pattern is coincident with the process of somitogenesis. A similar observation was made in mouse myogenesis where *myf-5* is activated in newly formed somites prior to the expression of any other muscle regulatory genes and is then down-regulated parallel with the maturation of somites in an anterior-to-posterior order [17].

The dynamic change of the *myf-5* expression pattern thus implies that *myf-5* activity may be only required for the early determination of muscle cells. Precise down-regulation of *myf-5*, followed by the activation of other myogenic regulatory genes such as *myogenin* and MRF4, therefore, might be crucial for the initiation of myogenic differentiation. The question we ask here is, how does this precise elimination of *Xmyf-5* occur.

In this paper, we report our identification of a repressor in the *Xmyf-5* promoter which matches the consensus sequence of the interferon (IFN) regulatory factor (IRF)-like binding element. We show that this putative IRF binding site mediates the down-regulation of *Xmyf-5* transcription in gastrula embryos, and can be bound specifically by nuclear proteins of stage 15 embryos. Moreover, deletion of this putative IRF binding site in *Xmyf-5* promoter results in anterior expansion of reporter gene expression in transgenic embryos. Our results, therefore, provide insight into how the negative control of *Xmyf-5* takes place.

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2. Materials and methods

2.1. Construction of plasmids

p(−1869/−30)Luc/p(−1869/−30)GFP and pΔIRFLuc/pΔIRFGFP: The 1.8 kb promoter of the *Xmyf-5* 5′ upstream region from −1869 bp to −30 bp was enzyme digested from the 4.858 kb promoter (FL/SK) we previous reported [18] with *Nco*I (blunted by mung bean nuclease) and *Hin*dIII, and was then inserted into the *Sma*I and *Hin*dIII site of pGL3-basic vector to generate p(−1869/−30)Luc.

pΔIRFLuc was generated as follows: Two fragments (−1872/−1410, −1399/−811) were PCR amplified (primers for amplification: upstream, 5′-ttccatgggagatcatctttgcctg-3′, downstream, 5′-cctctagaaatcttcccctctgtgt-3′; upstream, 5′-tcctctagagactccaggagcagct-3′, downstream, 5′-taggatccattgtccggaaacccag-3′). The two fragments were digested with *Xba*I and ligated and inserted into the *Nco*I and *Bgl*II frame of pGL3 Basic to make pΔIRF(−1872/−811). After sequencing, pΔIRF(−1872/−811) was used to replace the sequence from −1872 bp to −811 bp in the wild type *Xmyf-5* promoter to generate pΔIRFSK. And the *Nco*I and *Hin*dIII frame of this mutant promoter was further inserted into the *Sma*I and *Hin*dIII site of pGL3-basic to generate pΔIRFLuc.

Construction of pΔIRFGFP and p(−1869/−30)GFP: For transgenic reporter gene assays, the luciferase gene of p(−1869/−30)Luc was removed with *Xba*I and *Hin*dIII, and replaced by a GFP cDNA[19] to generate p(−1869/−30)GFP. pΔIRFGFP was generated by removing the ΔIRF fragment (−1869/−30) from pΔIRFSK with *Sac*I and *Hin*dIII and cloned into the *Sac*I and *Hin*dIII sites of p(−1869/−30)GFP.

Construction of TK-basicLuc, p(−1869/−819)TK Luc and 5′ or 3′ deletion constructs: TK-basicLuc was generated by inserting the TK promoter sequence of PRL-TK [20] (*Bgl*II and *Hin*dIII frame) into the *Bgl*II and *Hin*dIII sites of pGL3-basic. p(−1869/−819)TKluc was generated by restriction enzyme through releasing the 1.05 kb (−1869/−819) fragment from p(−1869/−30)Luc with *Sac*I and *Bam*HI and then inserting it into *Sac*I and *Bgl*II sites of TK-basicLuc.

Either 5′ or 3′ deletion constructs of p(−1869/−819)TKLuc were further generated according to the restriction sites located within the 1.05 kb fragment: p(−1752/−819)TKLuc by *Eco*RV, p(−1042/−819)TKLuc by *Acc*I, p(−1869/−1158)TKLuc by *Acc*I and p(−1869/−1753)TKLuc by *Eco*RV.

The 5′ or 3′ deletion constructs on the basis of the sequence from −1752 bp to −1158 bp were PCR amplified (primers for 5′ deletion constructs: upstream, 5′-caaagagttcctgcaccttg-3′ for p(−1557/−1158)-TKLuc, 5′-tcctaagagaggcatcgcgg-3′ for p(−1359/−1158)TKLuc; downstream, T3 primer. Primers for 3′ deletion constructs: upstream, T7 primer; downstream, 5′-ccgcgatgcctctcttagga-3′ for p(−1752/−1340)TKLuc; 5′-caaggtgcaggaactctttg-3′ for p(−1752/−1538)-TKLuc). The PCR amplified sequences were further digested with either *Bam*HI or *Kpn*I and inserted into the *Kpn*I (blunted by mung bean nuclease) and *Bgl*II sites of TK-basicLuc to generate p(−1557/−1158)TKLuc and p(−1359/−1158) TKLuc or inserted into the *Kpn*I and *Bgl*II (Klenow) sites of TK-basicLuc to generate p(−1752/−1340)TKLuc and p(−1752/−1538)TKLuc.

2.2. Transgenesis and whole-mount in situ hybridization

Transgenic *Xenopus* embryos were generated as described by Kroll and Amaya through restriction enzyme-mediated integration (REMI) [21]. Plasmids used for transgenesis were linearized by *Not*I digestion. In situ hybridizations were performed as described by Epstein [22].

2.3. Xenopus embryo manipulation and luciferase assay

Eggs were obtained from *Xenopus* females, fertilized in vitro and cultured as described [23]. Embryonic stages were determined as described previously [24].

A plasmid solution of 5 nl containing 10 pg/nl of promoter constructs and 10 pg/nl of internal control pRL-SV40 (Promega product) was injected into the dorsal-lateral equatorial region of four-cell stage embryos. Injected embryos were harvested at stage 12.5 and luciferase assays were performed according to the instruction of the Dual-Luciferase TM Reporter Assay System (Promega product). Ten embryos were collected for each measurement and embryos from the same fertilization batch were used for each experiment. Each results was repeated at least three times independently.

2.4. Electrophoresis mobility shift assays (EMSA)

The probe for EMSA was end-labeled with the standard method.

The single strand sequence of the probe was: 5′-aggggaagatttcttcacttccactagaga-3′. Nuclear extracts were prepared from *Xenopus* embryos at stage 15 [25]. 15 μg nuclear extract, 2 μg poly(dI-dC) (Pharmacia Biotech) and 4.5 μg bovine serum albumin and various amounts of cold probe (competitor) were mixed and incubated at 25°C for 15 min. Approximately 1 ng probe was added into each reaction and incubated at 25°C for another 25 min. The final concentration of the working solution was 40 mM KCl, 15 mM HEPES (pH 7.9), 1 mM EDTA, 0.5 mM dithiothreitol and 5% glycerol. The reaction products were immediately loaded on a 5% PAGE gel containing 0.5×TBE. Gels were then exposed to X-ray films.

3. Results

3.1. The 2.0 kb Xmyf-5 5′ upstream DNA contains a repressor

Previously we reported our isolation of a 4.858 kb *Xmyf-5* upstream DNA (GenBank, accession number AF212160) and analysis of reporter gene activity directed by serial deletion fragments of the 4.858 kb sequence in late gastrula embryos (stage 12.5) [18]. We mark the 'A' of the ATG start codon of the open reading frame (ORF) as '1'. We showed that the 1.05 kb sequence from −1869 to −819 bp could reduce reporter gene activity, implying that the 1.05 kb fragment might contain a repressor element [18]. To test whether the 1.05 kb sequence (−1869/−819) could play a transcriptional repressive role in a heterologous promoter context, we inserted the 1.05 kb sequence upstream to the TK promoter (see Section 2) for the luciferase assay (Fig. 1A). Two constructs, p(−1869/−819)TKLuc and pTK-basicLuc, were injected into the dor-
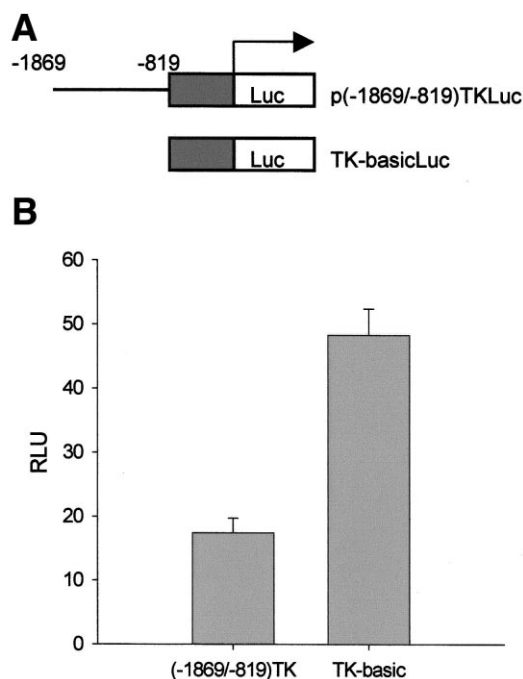

Fig. 1. The 1.05 kb DNA sequence (−1869/−819) of the *Xmyf-5* promoter contains a repressive regulatory element. A: Schematic diagram of p(−1869/−819)TKLuc and TK-BasicLuc. Solid rectangle, TK promoter; open rectangle, the open reading frame of the luciferase gene. B: The *cis*-element within the sequence from −1869 bp to −819 bp plays a repressive role in a heterologous context. Constructs were injected into the dorsal-lateral marginal zone of four-cell stage embryos and harvested at stage 12.5 for luciferase activity. The experiment was independently repeated three times, and a representative result is shown. Each bar is the mean of three replicates+S.D.

sal-lateral marginal zone of four-cell stage embryos and the reporter gene activities were measured in stage 12.5 when endogenous *Xmyf-5* peaks. As shown in Fig. 1B, luciferase activities from p(−1869/−819)TKLuc-injected embryos only reached about 30% of control embryos injected with pTK-basicLuc. These results suggested that a repressor is located within the 1.05 kb sequence (−1869/−819) which could behave similarly in the heterologous promoter context as in the *Xmyf-5* promoter: it led to an inhibition of transcriptional activity.

3.2. An IRF-like binding element within the Xmyf-5 promoter region plays a repressive role in the transcriptional regulation of Xmyf-5

To address which sequence within this 1.05 kb fragment might mediate the repressor activity, serial deletion constructs were generated on the basis of p(−1869/−819)TKLuc according to the naturally occurring restriction sites located within the 1.05 kb sequence (Fig. 2A), and their activities were tested in the dorsal-lateral marginal zone as described above. As shown in Fig. 2B, reporter constructs containing the 595 bp sequence between −1752 and −1158 bp (p(−1752/−819)TKLuc and p(−1869/−1158)TKLuc) showed significantly lower activities than those without this 595 bp sequence (p(−1042/−819)TKLuc and p(−1869/−1753)TKLuc). Therefore, the repressor of interest is likely located within this 595 bp fragment (−1752/−1158 bp). We thus generated further deletion constructs by PCR on the basis of this 595 bp sequence and again assayed the reporter gene activities in the late gastrula stage (Fig. 2A). Significantly, the activities of two reporter constructs, p(−1752/−1340)Luc and p(−1557/−1158)Luc, were remarkably lower than those of the other two reporter constructs, p(−1752/−1538)Luc and p(−1359/−1158)Luc, suggesting that the 198 bp fragment from −1557 bp to −1360 bp is indispensable for the repressive activity (Fig. 2C). Thus, the 198 bp sequence (−1557/
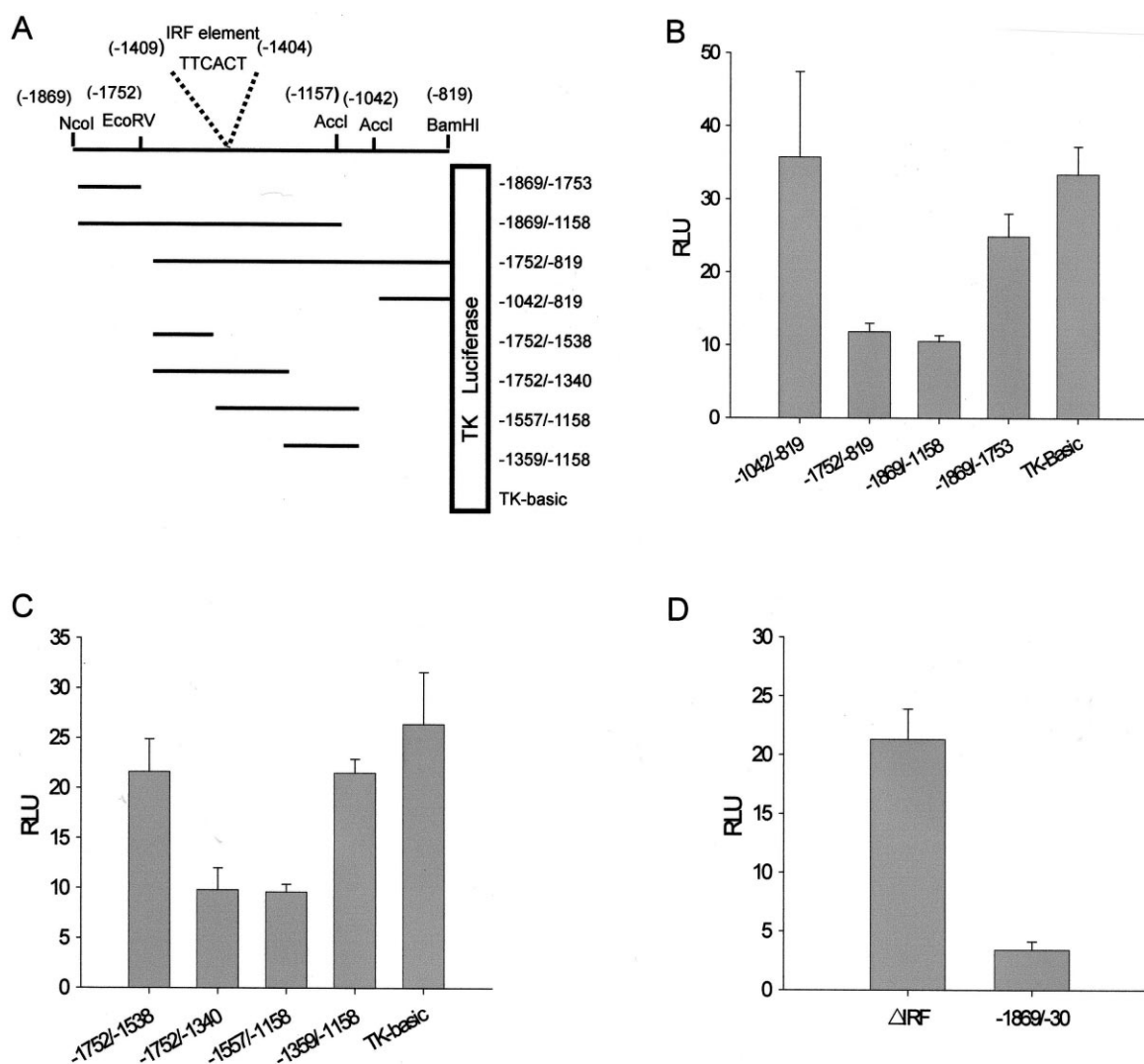

Fig. 2. An IRF element within the 1.05 kb *Xmyf-5* upstream sequence plays a repressive role in the regulation of *Xmyf-5* in late gastrula embryos. A: Diagram of *Xmyf-5* promoter deletion constructs used in this study. Each horizontal line represents the relative length of the upstream sequence of *Xmyf-5*. Each deletion fragment was fused upstream to a luciferase reporter gene driven by TK promoter. B: 595 bp sequence between −1752 bp and −1158 bp represses reporter gene activities directed by TK promoter. C: 198 bp sequence between −1557 bp to −1360 bp is sufficient to decrease reporter gene activities directed by the TK promoter. D: An IRF element (−1409/−1404) within the *Xmyf-5* upstream sequence significantly decreases transcriptional activity of *Xmyf-5*. Luciferase assay was performed as described in Fig. 1.

−1360) of *Xmyf-5* upstream contains a *cis*-regulatory element which mediates the transcriptional repression of *Xmyf-5* in late gastrula.

Consensus sequences of several known *cis*-regulatory elements were found in this 198 bp fragment (−1557 bp/−1360 bp). Among them, 5′-TTCACT-3′ (−1409/−1404) matches the consensus of the IRF element perfectly [26,27]. The IRF element has been classically found in the promoters of *IFN-α* and *IFN-β*, and can act as either enhancer or repressor through interacting with transcriptional factors of the IRF family [26,28–31]. And IRF elements have been identified from promoters of several genes other than *IFN-α* and *IFN-β* [32]. Recently, an IRF element within the vascular cell adhesion molecule-1 (*VCAM-1*) promoter has been identified to mediate the transcriptional activation of *VCAM-1*, which is important for mouse myogenesis. This observation raises the possibility that the putative IRF element might be responsible for the down-regulation of *Xmyf-5* in gastrula embryos. To address this hypothesis, we generated the construct pΔIRFLuc in which the putative IRF element was removed (see Section 2). Interestingly, when the reporter gene activity was tested in late gastrula embryos, embryos injected with pΔIRFLuc indeed showed a higher reporter gene activity (about four-fold) than that of control embryos injected with p(−1869/−30)Luc (Fig. 2D). These results thus suggested that this putative IRF binding site in *Xmyf-5* promoter may act as a transcriptional repressor to down-regulate *Xmyf-5* activity in the late gastrula stage.

Fig. 4. The IRF element within the *Xmyf-5* promoter region is responsible for the elimination of *Xmyf-5* activity in the anterior part of the somitic mesoderm. A, B: GFP signal exhibits a posterior localization expression pattern in p(−1869/−30)GFP REMI embryos. GFP was detected in the posterior part of the presomitic mesoderm of late gastrula (A); dorsal view with anterior facing up. At tadpole stage, GFP signal appears in the tail region (B). C, D: Removing the IRF element from the *Xmyf-5* promoter results in GFP signal extending anteriorly within the somitic mesoderm in pΔIRFGFP REMI embryos. GFP signal spreads to the anterior part of the somitic mesoderm at late gastrula and signal occupies the whole somitic mesoderm adjacent to the notochord with some signal in the neural ectoderm (C). Dorsal view with anterior facing up. GFP is expressed both in the trunk somites and in those in the tail region at tadpole stage with some signal in the eyes (D).

3.3. The IRF element can specifically interact with nuclear proteins of early neurula

Transcriptional regulation often needs physical interaction between the transcriptional regulator and its specific binding site. To address whether the IRF element we isolated in the *Xmyf-5* upstream region is involved in protein–DNA interaction during myogenesis, we performed EMSA. A probe with sequence encompassing the putative IRF element was used (see Section 2). As shown in Fig. 3, this probe was bound by nuclear proteins extracted from nuclei of early neurula (stage 15), as judged by three shift complexes. These interactions are specific, since increasing amount of competitor, the 'cold' probe, was able to interfere with the binding between the 'hot' probe and the nuclear proteins. The probe–protein complex was significantly reduced or completely abolished when 100- or 1000-fold of the competitor was incubated with the nuclear protein before the hot probe was added, respectively (Fig. 3). The EMSA data, therefore, indicated that this putative IRF binding element mediates the down-regulation of *Xmyf-5* probably through the specific interaction with nuclear proteins of early neurula.

3.4. The IRF element is sufficient to eliminate Xmyf-5 activity in the anterior somitic mesoderm

To address the functional significance of this IRF element during the fine spatial patterning of *Xmyf-5* expression, we used REMI to introduce GFP reporter constructs (p(−1869/−30)GFP and pΔIRFGFP) into the embryos [21]. Expression of GFP was detected by in situ hybridization in stage 12.5 when endogenous *Xmyf-5* begins its posterior localization within the presomitic mesoderm, and stage 30.
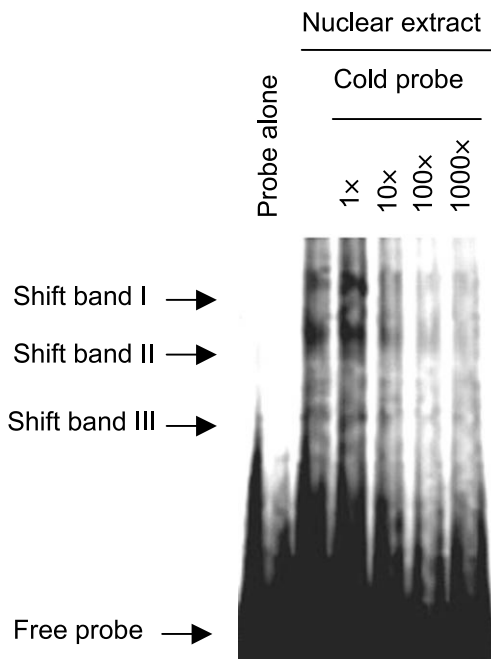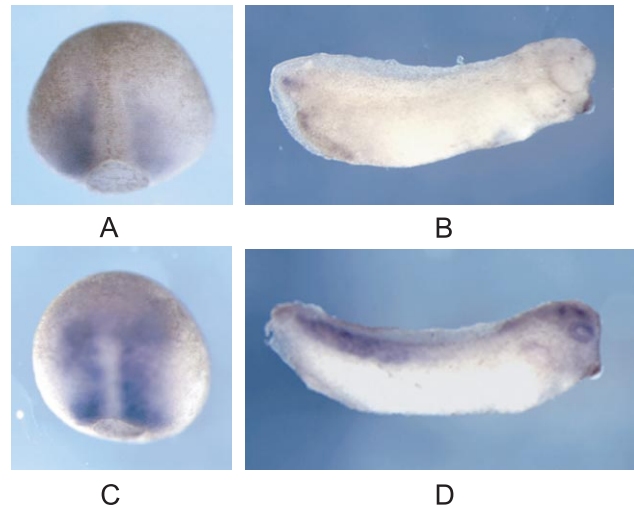

Fig. 3. The IRF element within the *Xmyf-5* promoter region specifically interacts with the nuclear proteins from early neurula (stage 15). A copy of the sequence from −1422 bp to −1393 bp of *Xmyf-5* upstream DNA that encompassed the IRF element was radiolabeled and then incubated with nuclear extract from stage 15 embryos. Three probe–protein complexes were resolved by gel electrophoresis. Shift bands are denoted by arrows. When in the presence of the 'cold' probe as competitor, 1000-fold molar excess of 'cold' probe completely blocks the formation of complex between nuclear proteins and the 'hot' probe.

In p(−1869/−30)GFP REMI embryos, GFP transcripts were restricted to the posterior somitic mesoderm at stage 12.5 (Fig. 4A). In keeping with the posterior restriction of reporter gene expression, GFP signal was visible in the tail region of the tadpole stage embryos (Fig. 4B). The posterior localization of reporter gene transcripts within the presomitic mesoderm in p(−1869/−30)GFP REMI embryos at these stages is quite similar to endogenous *Xmyf-5* mRNA in normal embryos [12].

In contrast, the GFP expression pattern in pΔIRFGFP REMI embryos is impressively distinct from the pattern in p(−1869/−30)GFP REMI embryos. As shown in Fig. 4C, GFP signal in pΔIRFGFP REMI embryos spread to the anterior part of the presomitic mesoderm in late gastrula and the signal occupied the whole somitic mesoderm laterally localized to the notochord. The GFP signal extended even very anteriorly, to the tissue that might be the rostral neural ectoderm (Fig. 4C). Consistent with the observation that GFP signal could extend anteriorly in late gastrula, GFP was detected in both the trunk somites and those in the tail region at tadpole embryos with some signal in the eyes, a rostral neural ectoderm derivative (Fig. 4D).

Notably, these stage-specific expression patterns were observed in the vast majority of pΔIRFGFP REMI embryos (10/16) and p(−1869/−30)GFP REMI embryos (15/19), with almost no apparent ectopic expression.

By comparing the GFP expression patterns in p(−1869/−30)GFP and pΔIRFGFP REMI embryos, we concluded that the IRF-like binding element within the 1.8 kb (−1869/−30) *Xmyf-5* promoter region is sufficient to eliminate *Xmyf-5* activity in the anterior somitic mesoderm. The transgenic result also indicated that the IRF element might be required for the exclusion of *Xmyf-5* transcripts from the neural ectoderm.

4. Discussion

As one of the key components of myogenic regulatory genes, *Xmyf-5* is expressed persistently in the muscle precursor cells during myogenesis, like other members of the myogenic regulatory gene family [9–12]. However, the transcripts of *Xmyf-5* are more posteriorly localized than those of other members of myogenic regulatory genes. The posterior restriction of *Xmyf-5* expression first appears at late gastrula, and peaks at neurula, when *Xmyf-5* transcripts can only be detected in the presumptive tail region [9]. The loss of *Xmyf-5* transcripts is correlated with differentiation of determined muscle cells. There are two possible mechanisms that control this posterior restriction of *Xmyf-5* expression: the anterior muscle lineage may gradually lose the activator of *Xmyf-5*, or a transcriptional repressor of *Xmyf-5* may be progressively turned on during myogenesis.

The results of our study provide the first direct evidence to support the second hypothesis. We show that an IRF element within 1.8 kb (−1869/−30) of the *Xmyf-5* promoter region is responsible for the elimination of *Xmyf-5* transcripts in the anterior somitic mesoderm. Our conclusion comes from the following four results. First, transgenic results show that the 1.8 kb (−1869/−30) *Xmyf-5* promoter can direct reporter gene expression in the posterior somitic mesoderm. And loss of the IRF element within the 1.8 kb *Xmyf-5* promoter region leads to reporter gene transcripts extending to the anterior somitic mesoderm. Moreover, the deletion of the IRF element from the 1.8 kb *Xmyf-5* promoter results in a significant increase of *Xmyf-5* reporter construct activity in late gastrula embryos. Finally, the nuclear proteins of early neurula specifically bind to this IRF element. Interestingly, when the probe encompassing the IRF element was incubated with nuclear proteins of late gastrula, only very weak binding was observed (data not shown), indicating that the formation of IRF element–protein complex is developmentally regulated. In other words, transcriptional regulators responsible for the elimination of *Xmyf-5* activity through the IRF element may be activated from gastrula and their activities keep on increasing during neurula when endogenous *Xmyf-5* transcripts are strongly decreased in the anterior somites. Taken together, these data evidently demonstrate that the IRF element is sufficient for repressing *Xmyf-5* in the anterior somitic mesoderm during myogenesis.

The posterior restriction expression pattern of the reporter gene under the control of the 1.8 kb (−1869/−30) *Xmyf-5* promoter is quite similar to the endogenous *Xmyf-5* gene during myogenesis [8,11,12]. Our identification and functional analysis of *cis*-regulatory elements required for the dorsolateral activation of *Xmyf-5* at early gastrula will be published elsewhere.

Given that IRF-2 protein is important for mouse muscle cell development through regulating expression of *VCAM-1*, though it acts as a transcriptional activator there, we cannot exclude the possibility that the nuclear protein binding to the IRF element within the *Xmyf-5* upstream region is one of the IRF family members, like IRF-2 [32]. Further studies are required to determine the identities of proteins interacting with the IRF element within the *Xmyf-5* promoter and the relationship between these proteins and the genes involved in dorsal-ventral patterning, such as *bmps* and *Xwnt-8* [33,34].

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