Development 129, 2917-2927 (2002)
Printed in Great Britain © The Company of Biologists Limited 2002
DEV1817

A study of mesoderm patterning through the analysis of the regulation of *Xmyf-5* expression

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Accepted 20 March 2002

SUMMARY

Xenopus laevis **has been a particularly useful model organism for identifying factors involved in the induction and patterning of the mesoderm, however, much remains to be learned about how these factors interact. The myogenic transcription factor *Xmyf-5* is the earliest known gene to be expressed specifically in the dorsolateral mesoderm of the gastrula, a domain that is established by the interaction of dorsal and ventral signals. For this reason, we have begun to investigate how the expression of *Xmyf-5* is regulated. We have identified a 7.28 kb *Xenopus tropicalis Xmyf-5* (*Xtmyf-5*) genomic DNA fragment that accurately recapitulates the expression of the endogenous gene. Deletion and mutational analysis has identified HBX2, an essential element, approximately 1.2 kb upstream from the start of transcription, which is necessary for both activation and repression of *Xtmyf-5* expression, implying that positional information is integrated at this site. Electrophoretic mobility shift assays demonstrate that HBX2 specifically interacts with gastrula stage embryonic extracts and that in vitro translated Xvent-1 protein binds to one of its functional motifs. Combined with gain- and loss-of-function experiments, the promoter analysis described here suggests that Xvent-1 functions to repress *Xmyf-5* expression in the ventral domain of the marginal zone. Furthermore, the identification of HBX2 provides a tool with which to identify other molecules involved in the regulation of *Xmyf-5* expression during gastrulation.**

Key words: Xvent-1, *Xmyf-5*, Gastrulation, Repression, Activation, Mesoderm, *Xenopus laevis*, *Xenopus tropicalis*

INTRODUCTION

The mesodermal germ layer contributes cells to the notochord, musculature, kidney and blood and organises the development of the dorsal to ventral and anterior to posterior axes of the body (Harland and Gerhart, 1997). In *Xenopus*, mesoderm is induced around the equator of the embryo at the blastula stages by signals emanating from the vegetal pole. Following this, during the gastrula stages, antagonistic interactions between a second set of signals that arise from the dorsal and ventral domains pattern the mesoderm along its dorsoventral axis. Several signal transduction pathways that function during mesoderm induction and patterning have been identified (for reviews, see Chan and Etkin, 2001; Dale and Jones, 1999; Heasman, 1997). However, we lack a full understanding of how these pathways integrate to pattern the mesoderm. In particular, little is known about how the mesoderm is patterned into its four major compartments, representing the notochord, somites, pronephros and blood.

Xmyf-5 encodes a basic helix-loop-helix (bHLH) transcription factor related to MyoD, myogenin and MRF4, all of which play a role in the development of the somites (Cossu et al., 1996). The pattern of *Xmyf-5* expression during *Xenopus* gastrulation is unique, in that it is the only known gene to be restricted solely to the dorsolateral domain of the marginal zone (Dosch et al., 1997; Hopwood et al., 1991; Jones and Smith, 1998). We believe understanding how the expression of *Xmyf-5* is regulated, will provide important insight into how the mesoderm is patterned, as the dorsolateral domain requires both dorsal and ventral inputs to establish its identity.

To this end we have cloned the *Xmyf-5* gene and studied its regulation in transgenic frog embryos. Here we show that *Xmyf-5* expression is controlled by a regulatory element that binds both activating and repressing homeodomain-containing transcription factors, suggesting that both positive and negative inputs converge onto a single regulatory element.

MATERIALS AND METHODS

Embryo culture, injection and fixation

Embryos were obtained, fertilised and cultured as described (Amaya et al., 1993) and staged according to Nieuwkoop and Faber (Nieuwkoop and Faber, 1967). Injections of 10 nl of appropriately diluted mRNA were made into the marginal zone of a single blastomere of *Xenopus laevis* embryos at the 4 cell stage. Morpholino experiments were performed in *Xenopus tropicalis* embryos by

injecting 2 nl of appropriately diluted morpholino into both blastomeres at the 2 cell stage. At the relevant stage, embryos were fixed at room temperature in MEMFA (Harland, 1991). β-galactosidase activity was assessed as previously described (Bourguignon et al., 1998).

Whole-mount mRNA in situ hybridisation

Whole-mount in situ hybridisation was performed as previously described (Harland, 1991). The following plasmid templates were used to generate in situ hybridisation probes:

RNA	Plasmid	Linearised	Polymerase	Reference
GFP	CSGFP2	*Bam*HI	T7	E. A. (unpublished)
Xlmyf-5	pSP73XlMyf5	*Bam*HI	SP6	(Hopwood et al., 1991)
Xvent-1	pXvent-1	*Sal*I	T7	(Gawantka et al., 1995)
Xtmyf-5	pXTM(3exon)	*Cla*I	T7	see below

A *X. tropicalis*-specific in situ probe was generated by amplifying the third exon of XTM1 using the primers 5′-CCATCGATAAC-CTGACACCTGTCCAAGCC-3′ and 5′-CGGGATCCTTATGCCC-GAGATTTCTCACAA-3′. The PCR product was cut with *Cla*I and *Bam*HI and cloned into pBluescript KS II (Stratagene). Hybridisation was performed at 65°C rather than at 60°C and 0.2× SSC was used in place of 2× SSC for all post-hybridisation washes.

Transgenesis

The restriction enzyme-mediated integration transgenesis protocol was carried out as described (Kroll and Amaya, 1996) with the following modifications. All plasmid transgenes were linearised with *Sal*I. The sperm nuclei were decondensed with 5 μl of high speed egg extract. Either *Xba*I or *Bgl*II was used to induce double strand breaks in the sperm chromatin. The embryos were screened at stage 10.5 for gastrulation defects and only those that were seen to be gastrulating normally were fixed for whole-mount in situ hybridisation.

Isolation of a *X. laevis myf-5* (*Xlmyf-5*) genomic fragment

Approximately 2×10^6 plaques from a *X. laevis* genomic DNA library in λDASH (Leroy and De Robertis, 1992) were screened at high stringency with a probe corresponding to the first 450 bp of the *Xlmyf-5* cDNA (Hopwood et al., 1991). *Eco*RI fragments from one isolate were subcloned into pBluescript KS II. Southern blotting identified a 1.8 kb fragment, containing a portion of the *Xlmyf-5* cDNA. The clone was sequenced and found to correspond to the first 122 bp of the *Xlmyf-5* cDNA and 1739 bp of upstream sequence. This fragment was named XLM1 (*X. laevis myf*-5 fragment 1) (GenBank accession number: AY050250).

Isolation of a *X. tropicalis myf-5* (*Xtmyf-5*) genomic fragment

Approximately 1×10^6 plaques from a *X. tropicalis* genomic DNA library in λFix II (a gift from L. Zimmerman and R. Harland) were screened at high stringency with a probe corresponding to the first 450 bp of the *Xlmyf-5* cDNA (Hopwood et al., 1991). *Xba*I fragments from one of the isolates were subcloned into pBluescript KS II. Southern blotting identified a 7.28 kb clone, containing sequences homologous to the *X. laevis* myf-5 cDNA. The clone was sequenced and found to contain 2671 bp of upstream sequence, the 3 exons and 2 introns of the *Xtmyf-5* gene and 1500 bp of 3′ sequence. This fragment was named XTM1 (*X. tropicalis myf-5* fragment 1) (GenBank accession number: AY050251).

Isolation of *Xtmyf-5* cDNA

In order to isolate the *Xtmyf-5* cDNA, RT-PCR was performed on RNA extracted from *X. laevis* embryos generated by nuclear transplantation with the XTM1 transgene. 2 μg of total RNA from stage 10 embryos was reversed transcribed using AMV reverse transcriptase and random hexamers (Gibco BRL). The *Xtmyf-5* cDNA was then amplified over 29 rounds of PCR using the primers 5′-CC**ATCGAT**GGTACAAGCAGCAGTATTCAGAATG-3′ and 5′-CG**GGATCC**CGCTTTATACGCCGATTTGCTGG-3′. The PCR product was cut with *Cla*I and *Bam*HI and cloned into pBluescript KS.

Isolation of *Xtvent-1* and *Xtvent-2* cDNA

Full-length *X. tropicalis* clones of *Xvent-1* and *Xvent-2* were identified from the *X. tropicalis* EST Database (http://www.sanger.ac.uk/Projects/X_tropicalis/blast_server.shtml). Clones TGas003j13 (*Xtvent-1*) and TGas015g05 (*Xtvent-2*) were picked.

Generation of XTM1 mutant clones

Four different techniques were employed to generate mutations within XTM1. XTM2 was generated by subcloning the *Xtmyf-5* cDNA into XTM1. Other large deletions (XTM3-XTM7) were made by restriction enzyme digests followed by religation. The deletion of the entire HBX2 site (XTM1mHBX2) was achieved using the PCR-based hybrid overlap extension strategy (Ho et al., 1989) using the following primers: **A1** 5′-CAGTTGGTCAGAAACATTGC-3′ and **A2** 5′-**GA-CTATGTCAGATCT**GCAGCCCTCTGCCAAGGTGC-3′ and **B1** 5′-AATGTACAATGCTGGAAAAC-3′ and **B2** 5′-**AGATCTGA-CATAGTC**TACCCTTAACAGTTTCAGTG-3′. The Quikchange™ Site-Directed Mutagenesis Kit from Stratagene (#200518) was used to remove and alter the identity of pairs or groups of four bases in HBX2. The primers shown below and their complements were used to generate the mutations (shown in bold). **XTM1m2-5** 5′-GCACCTTGGCAGAGGGCTGC**GGGG**GGCTTCATTACCACTTT-AATTAC-3′, **XTM1m12-15** 5′-CAGAGGGCTGCTAATGGCTT-C**GGGG**CCACTTTAATTACCCTTAAC-3′, **XTM1m22-23** 5′-GG-CTTCATTACCACTT**GG**ATTACCCTTAACAGTTTCAGTGTTG-3′, **XTM1m22-25** 5′-GCTGCTAATGGCTTCATTACCACTTT**GG-GG**ACCCTTAACAGTTTCAGTG-3′, **XTM1m26-27** 5′-GGC-TTCATTACCACTTTTAT**GG**CCCTTAACAGTTTCAGTGTTG-3′, **XTM1m10-11** 5′-CAGAGGGCTGCTAATGGCT**GG**ATTACCAC-TTTAATTACCC-3′, **XTM1m16-17** 5′-GGGCTGCTAATGGCTTC-ATTA**GG**ACTTTAATTACCCTTAACAG-3′, **XTM1m18-19** 5′-GG-GCTGCTAATGGCTTCATTACC**GG**TTTAATTACCCTTAACAGT-TTC-3′, **XTM1m22-21** 5′-CTGCT-AATGGCTTCATTACCAC-**GG**TAATTACCCTTAACAGTTTCAG-3′ and **XTM1Δ18-19** 5′-GGGCTGCTAATGGCTTCATTACCTTTAATTACCCTTAACAGT-TTC-3′.

Heterologous minimal promoter constructs

mCSKAGFP was generated by cloning a *Xba*I-*Hin*dIII fragment containing the *Xenopus* cytoskeletal actin (CSKA) minimal promoter from the construct pBLCAT3T [a gift of T. Mohun (Mohun et al., 1987)] into the SV40GFP3 vector (Göttgens et al., 2000) from which the SV40 minimal promoter had been removed. To construct **MRRmCSKAGFP, mE1MRRmCSKAGFP** and **mE2MRRmCSKAGFP**, MRRs from XTM1, XTM1m12-15 and XTM1m22-25 respectively were removed by cutting with *Nde*I, end filled and ligated into the vector mCSKAGFP which had been cut with *Asp*718 and end filled.

Electrophoretic mobility shift assay (EMSA) – embryonic extracts

Whole-cell *Xenopus* embryo extracts were prepared from mid-gastrula embryos as described previously (Germain et al., 2000) with the exception that KCl was used at 50 mM rather than 200 mM. The following wild-type (WT) and mutant oligonucleotides probes (mutations are shown in bold) and complementary oligos (not shown) were end-labelled with [γ-^{32}P]ATP using polynucleotide kinase. **WT** 5′-AGAGGGCTGCTAATGGCTTCATTACCACTTTAATTACCCTT-AAC3′, **mE1** 5′-AGAGGGCTGCTAATGGCTTC**GGGG**CCAC-TTTAATTACCCTTAAC3′, **mE2** 5′AGAGGGCTGCTAATGG-CTTCATTACCACTT**GGGG**TACCCTTAAC3′ and **mE1+2** 5′-AGAGGGCTGCTAATGGCTTC**GGGG**CCACTT**GGGG**TACCCTT-AAC3′. Binding reactions were performed at room temperature with

10 μg of protein extract incubated with 1×10^4 cpm of double stranded probe in 20 μl of buffer containing 140 mM KCl, 8 mM $MgCl_2$, 12.5 mM β-glycerophosphate, 1 mM EGTA, 1 mM EDTA and 0.5 μg poly(dI-dC), 2 mM DTT, protease and phosphatase inhibitors for 15 minutes at room temperature. Competition analyses were performed by the addition of unlabelled WT and mE1+2 probes into the initial incubation reaction. The entire reaction was run on 5% polyacrylamide gels in 0.5× TBE at room temperature.

EMSA – in vitro translated proteins

All proteins were transcribed and translated in vitro using the TNT® SP6 Quick coupled reticulocyte lysate system (Promega). The amount of protein produced was assayed by performing a parallel reaction in the presence of [^{35}S]methionine. 1×10^4 cpm of double stranded probe was incubated with 1 μl equivalents of reticulocyte lysate in the presence of 1 μg of poly(dI-dC) in 1× binding buffer (20 mM Tris-HCl pH 7.8, 30 mM KCl, 1 mM $MgCl_2$, 1 mM DTT and 13.2% glycerol) in a total volume of 10 μl. Samples were incubated at room temperature for 10 minutes, followed by a further 20 minutes in the presence of radiolabelled probe. Competition analyses were performed by the addition of unlabelled WT and mE2 probes into the initial incubation reaction. Electrophoresis was performed as outlined above.

Preparation of synthetic mRNA

Capped mRNAs were transcribed using the SP6 Ambion mMessage mMachine® in vitro transcription kit. The plasmid templates used were as follows:

RNA	Plasmid	Linearised	Reference
Xvent-1	CS2Xvent-1	*Not*I	(Gawantka et al., 1995)
Xcad-3	CS2-Xcad3	*Not*I	(Pownall et al., 1996)
Xcad-3VP16	CS2-XcadVP16-A	*Not*I	(Isaacs et al., 1998).

Morpholino oligonucleotides

Antisense morpholino oligonucleotides against Xtvent-1 (CAATAGAGAATCCCTGTTGAACCAT) and Xtvent-2 (TTCC-TGTAGTAGTCCTTGTGTTCAT) were obtained from Gene Tools LLC (USA).

RESULTS

Xmyf-5 expression is restricted to the dorsolateral domain of the marginal zone during gastrulation

As reported previously, the gastrula stage expression of *Xmyf-5* is restricted to two symmetrical wings in the dorsolateral domain of the marginal zone (Fig. 1A) (Hopwood et al., 1991). This is a unique property of *Xmyf-5*, as it is the only known gene whose expression is limited to the dorsolateral domain at this time in development. According to the current model of mesoderm patterning, this domain is established by the interaction of signals emanating from both ventral and dorsal regions of the marginal zone. For this reason, we believe that investigating how the expression of *Xmyf-5* is controlled will provide a useful model to understand how dorsalising and ventralising signals converge to pattern the mesoderm.

Cloning the *Xlmyf-5* and *Xtmyf-5* promoters

As a first step towards understanding the regulation of *Xmyf-5* expression, we cloned the *Xmyf-5* gene from *X. laevis*. A probe representing the first 450 bp of the *X. laevis myf-5* (*Xlmyf-5*) cDNA (Hopwood et al., 1991) was used to screen a *X. laevis* genomic DNA library at high stringency. Sequence analysis of an *Eco*RI fragment from one of the phage clones contained the first 122 bp of the *Xlmyf-5* cDNA and 1733 bp of upstream sequence. This fragment was named XLM1 (GenBank accession number: AY050250). As XLM1 contained the first 12 amino acids of the *Xlmyf-5* coding sequence, we generated an in frame fusion of XLM1 with GFP, resulting in the promoter-reporter fusion construct XLM1GFP.

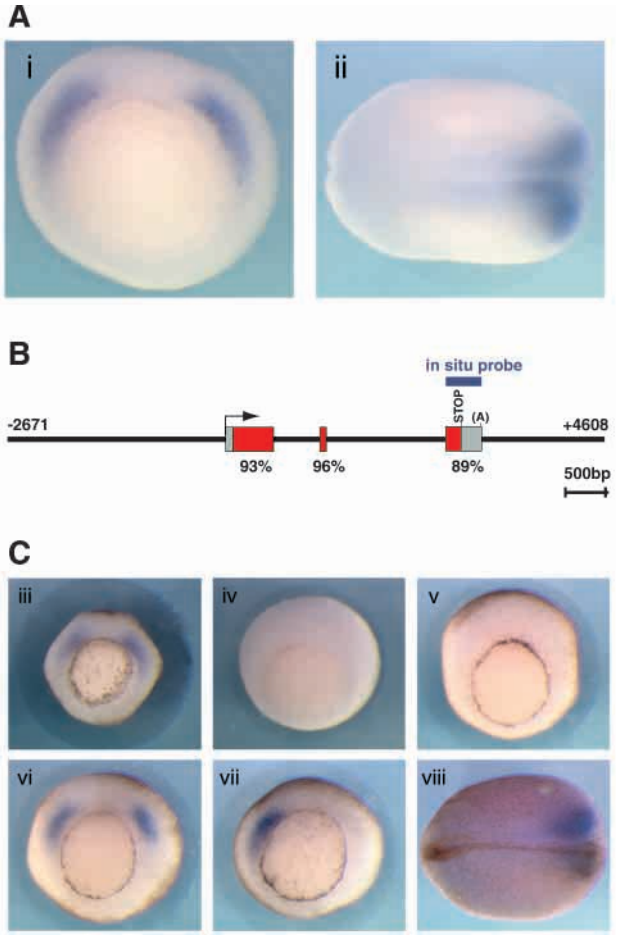

Fig. 1. (A) Expression of *Xmyf-5* at the gastrula and neurula stages. Expression of *Xmyf-5* was assayed in albino embryos by mRNA in situ hybridisation at the early gastrula/stage 10.5 (i) and late neurula/stage 19 (ii). (B) Schematic representation of XTM1, a 7.28 kb genomic fragment containing the *X. tropicalis myf-5* gene. XTM1 is composed of 3 exons of 594 bp, 76 bp and 441 bp (untranslated regions shown in grey and coding sequence in red) separated by introns of 548 bp and 1445 bp. The start of transcription is indicated by an arrow. The percent similarities of the *Xtmyf-5* exons with *Xlmyf-5* are shown below each exon. A *X. tropicalis myf-5*-specific in situ hybridisation probe was raised to the third exon. (C) The expression of XTM1 in transgenic *X. laevis* embryos. (iii) Non-transgenic *X. tropicalis* embryo at stage 10.5 stained with species-specific *Xtmyf-5* probe. (iv, v) Non-transgenic albino (iv) and pigmented (v) stage 10.5 *X. laevis* embryos stained with the species-specific *Xtmyf-5* probe; note lack of staining. (vi, vii and viii) XTM1 transgenic *X. laevis* embryos at stage 10.5 (vi and vii) and stage 19 (ix), stained with the species-specific *Xtmyf-5* probe. Note that the embryo in vii is half-transgenic, demonstrating the specificity of the XTM1 probe. Gastrula embryos are orientated with the dorsal axis pointing up and vegetal pole facing out. The neurula stage embryos are shown with the dorsal axis pointing out and anterior to the left.

We analysed the expression of XLM1GFP in transgenic embryos during gastrulation by whole-mount in situ hybridisation to GFP RNA (fluorescence at the gastrula stages was not detectable). At the early gastrula stage (stage 10.5) XLM1GFP transgenic embryos expressed GFP in two symmetrical wings in the dorsolateral domain of the marginal zone, closely mimicking that of the endogenous *Xmyf-5* gene (data not shown). However, the level of expression was very weak compared to that of the endogenous gene, and the percentage of embryos that expressed GFP with this construct was low. At later stages, expression of GFP mRNA was restricted to the posterior somitic region of the embryo (data not shown), although the expression was broader than the endogenous gene. In contrast to the GFP mRNA expression, GFP fluorescence was detected throughout the somites at the tailbud stages, suggesting that GFP protein is more stable than the mRNA. This implies that assaying for GFP fluorescence to study the regulation of XLM1GFP would not reflect the dynamic nature of its expression and therefore was not ideal to study the regulation of *Xmyf-5* expression.

To overcome the problems associated with using the GFP reporter gene we opted to take a cross-species approach. We therefore cloned the *X. tropicalis myf-5* (*Xtmyf-5*) gene. The same probe that was used to isolate the *Xlmyf-5* promoter was used to screen a *X. tropicalis* genomic DNA library at high stringency. Analysis of a 7.28 kb *Xba*I fragment from one of the phage isolates demonstrated that it contained the three exons and two introns of the *Xtmyf-5* gene along with 2671 bp of 5′ and 1500 bp of 3′ sequence (Fig. 1B). This fragment was named XTM1 (GenBank accession number: AY050251). Although *X. tropicalis* and *X. laevis* are related (Amaya et al., 1998), sequence analysis demonstrated that the third exon of *Xtmyf-5* was sufficiently different from *Xlmyf-5* to allow a species-specific in situ hybridisation probe to be raised (Fig. 1B). The specificity of this probe was confirmed by subjecting both *X. tropicalis* and *X. laevis* embryos to whole-mount in situ hybridisation (Fig. 1C). We could thus study the regulation of the *X. tropicalis myf-5* gene in transgenic *X. laevis* embryos without having to rely on a heterologous reporter gene.

To determine whether the expression profile of XTM1 was the same as the endogenous *Xmyf-5* gene, its regulation was studied in transgenic *X. laevis* embryos. At stage 10.5, the expression of XTM1 was restricted to two symmetrical wings within the dorsolateral domain of the marginal zone (Fig. 1Cvi). At stage 19, the expression of XTM1 became restricted to the posterior somitic and pre-somitic region of the embryo (Fig. 1Cviii). Comparison of Fig. 1A with Fig. 1C reveals that the expression of XTM1 at both the gastrula and neurula stages accurately recapitulates that of the endogenous gene.

The identification of functional domains within XTM1 transgene

As the expression of XTM1 appeared to be initiated and subsequently patterned in the same way as *Xmyf-5*, it provided a foundation for analysing the regulation of *Xmyf-5* expression. To resolve which regions of the XTM1 transgene were responsible for this regulation, a series of deletions were generated (Fig. 2). Constructs in which the introns (XTM2) or sequence 3′ to the gene (XTM3) were removed, were found to express normally (Table 1), suggesting that no elements necessary for the correct expression of XTM1 were contained within these domains.

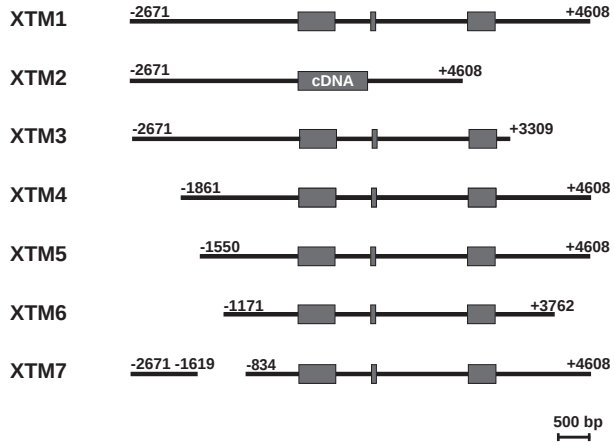

Fig. 2. XTM1 deletion constructs. A schematic representation of the enzymatic deletions made to the XTM1 transgene. The exons are shaded grey and the 5′, intronic and 3′ sequences are shown in black. The extent of the 5′ and 3′ sequences from the start of transcription are shown on each transgene.

Removal of sequence up to –1550 bp (XTM5) had no effect on either the level or pattern of transgene expression (Table 1). However, removal of sequence up to –1171 bp (XTM6) failed to express. To determine whether this failure to express was due to the loss of a single regulatory site or the combined effect of losing several sites that are spread along the length of the upstream region, the effect of specifically deleting the sequence between –1619 bp and –834 bp was assessed (XTM7). XTM7 had no activity in transgenic embryos (Table 1), suggesting that the region –1619 bp to –834 bp contains an activator element or cluster of elements that are necessary for the expression of XTM1. This domain was named the *myf-5* regulatory region (MRR).

The *myf-5* regulatory region

Having shown that the MRR contains elements necessary for activation of XTM1, we next addressed whether these elements

Table 1. Expression of XTM1 deletion constructs

		Incorrect expression			
Construct	Correct expression %	Integrated %	Unintegrated %	No expression %	*n*
XTM1	62	7	4	27	202
XTM2	60	0	12	28	75
XTM3	43	0	12	45	86
XTM4	59	0	19	22	37
XTM5	67	2	0	31	128
XTM6	0	0	26	74	125
XTM7	0	0	17	83	123

Embryos were scored as showing expression in the same pattern as the endogenous *XMyf-5* (correct expression), uniform expression that deviated from the normal pattern of *XMyf-5* (incorrect expression, integrated), highly punctate expression that deviated from the normal pattern of *XMyf-5* (incorrect expression, unintegrated – named this as the pattern was highly reminiscent of the expression observed from injected DNA plasmids), or no expression.

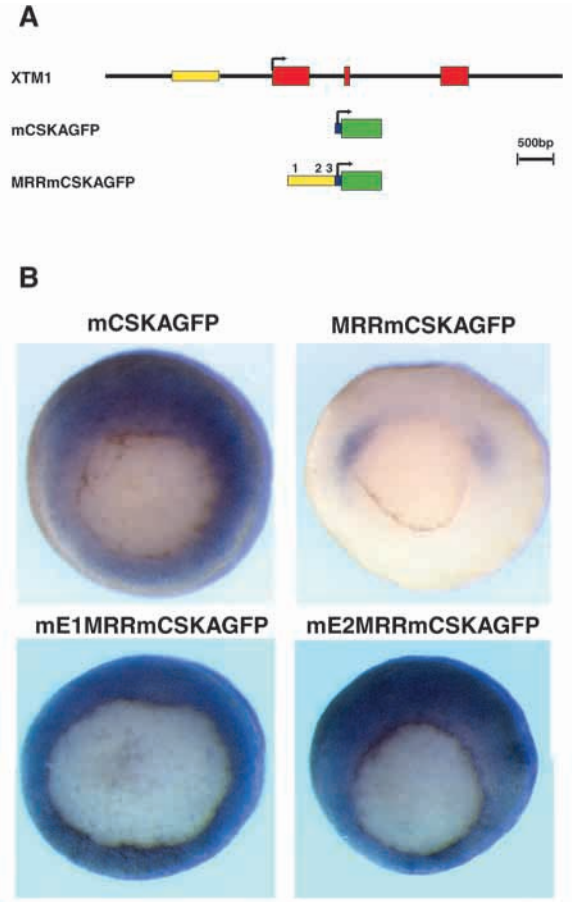

Fig. 3. (A) Schematic representation of the *mCSKA* minimal promoter constructs. The 785 bp MRR (yellow) was cloned in front of the *mCSKA* minimal promoter (101 bp shown in blue) driving the expression of GFP (shown in green) to create the transgene MRRmCSKAGFP. The numbers 1, 2 and 3 illustrate the position of the 3 putative HBX sites. (B) Expression of MRRmCSKAGFP and deletion derivatives at the gastrula stage. Expression was analysed by mRNA in situ hybridisation to GFP. All embryos are orientated with dorsal pointing up and the vegetal pole facing out.

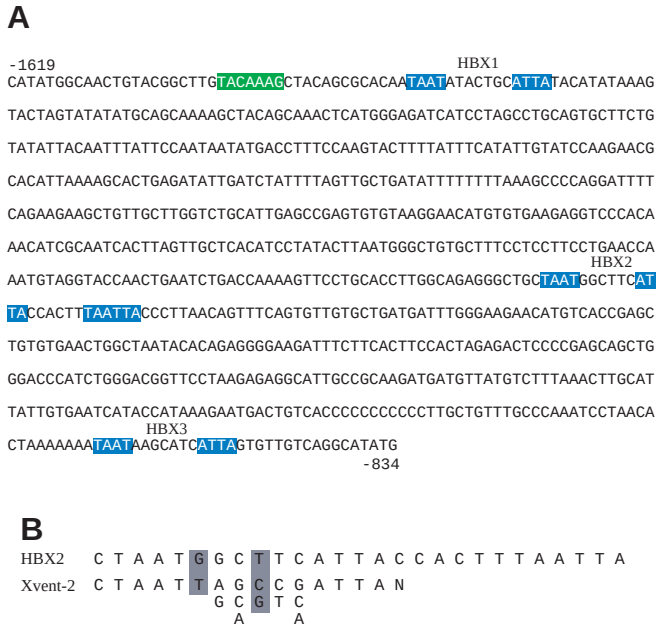

Fig. 4. (A) Putative transcription factor binding sites within the MRR. The MRR is a 785 bp *Nde*I fragment ranging from –1619 bp to –834 bp from the start of transcription. Homology searches identified HBX1, 2 and 3 (blue shading) as being putative binding sites for homeodomain containing transcription factors. A TCF consensus site is shaded green. (B) Comparison of HBX2 with the consensus binding site for Xvent-2 (Trindade et al., 1999). Grey shading highlights differences from consensus sequence.

were also sufficient for the correct expression pattern of *Xmyf-5*. Therefore we determined whether the MRR could confer the *Xmyf-5* expression pattern on a heterologous minimal promoter-reporter construct.

Studying the ability of an element to activate expression requires the use of a minimal promoter that has no endogenous activity. To find such a construct, the minimal promoters from the *simian virus 40* (Göttgens et al., 2000), *Xenopus cytoskeletal actin* (*CSKA*) (Mohun et al., 1987) and zebrafish *floating head* (M. Gomperts and E. A., unpublished) genes were linked to a GFP reporter and their activity monitored in transgenic embryos. All three of the promoters tested expressed at significant levels throughout the early gastrula embryo (data shown for mCSKA promoter only, Fig. 3B), making them unsuitable to test whether the MRR was sufficient to activate expression. However, as the promoters were expressed uniformly and at a high level at the early gastrula stage, they could be used to test if the MRR possessed a repressor capacity.

To examine whether the MRR contained repressor elements, it was cloned in front of the mCSKA promoter (MRRmCSKAGFP) and its activity analysed in transgenic embryos by assaying for GFP expression by in situ hybridisation (Fig. 3B). MRRmCSKAGFP faithfully recreated the expression pattern of *Xtmyf-5* at the gastrula stage, indicating that elements within the MRR are sufficient to repress the mCSKA promoter in areas outside the normal domain of *Xmyf-5* expression. Similar results were obtained with the MRR driving the expression of the SV40 minimal promoter (data not shown). We therefore concluded that the MRR contained elements for both the activation and repression of expression.

Identification of consensus binding sites in the MRR

To identify the factors that mediate the regulatory effects of the MRR, its sequence was examined for the presence of consensus transcription factor binding sites (Fig. 4). Three sites, HBX1, 2 and 3, were identified as having homology to the 5′-TAAT-3′ core motif (ATTA on the other strand) of the homeobox consensus binding site (Gehring et al., 1994). In addition, HBX2 shared significant homology with the consensus binding site of *Xvent-2* (Trindade et al., 1999). Further analysis revealed that HBX2 was highly conserved between *Xtmyf-5* MRR and the similar area within the *Xlmyf-5* gene, where as HBX1 and HBX3 were not.

HBX2 is a functional element within the MRR

The homeobox motif, HBX2, was identified in the MRR on the basis of similarity to a *Xvent-2* consensus site (Trindade et al., 1999). To confirm that HBX2 was necessary for the correct

Table 2. Expression of XTM1 mutant constructs

Construct	Correct expression %	Incorrect expression: Integrated %	Incorrect expression: Unintegrated %	No expression %	*n*
XTM1mHBX2	0	0	12	88	60
XTM1m2-5	74	2	0	24	46
XTM1m12-15	0	0	12	88	132
XTM1m22-23	14*	1	15	70	175
XTM1m22-25	2*	0	17	81	184
XTM1m26-27	63	9	0	28	43

Embryos were scored as showing expression in the same pattern as the endogenous *XMyf-5* (correct expression), uniform expression that deviated from the normal pattern of *XMyf-5* (incorrect expression, integrated), highly punctate expression that deviated from the normal pattern of *XMyf-5* (incorrect expression, unintegrated – named this as the patterns were reminiscent of the expression observed from injected DNA plasmids) or no expression. Note the motifs at 12bp-15bp and 22bp-25bp are necessary for expression.

*Expression very weak.

regulation of XTM1, the entire HBX2 site was replaced with random sequence, creating the transgene XTM1mHBX2 (Fig. 5). When tested in transgenic embryos XTM1mHBX2 completely failed to express, suggesting that HBX2 is a functional element in the MRR and that it is involved in at least the activation of expression (Table 2).

Functional motifs within HBX2

HBX2 is a complex site, consisting of 4 TAAT motifs, two of which overlap (Fig. 4). To ascertain which of these motifs mediate the effects of HBX2, each core sequence was mutated in the context of the 7.28 kb XTM1 transgene and their expression analysed. The mutation XTM1m2-5 was expressed in the same pattern as the endogenous *Xmyf-5* gene in transgenic embryos, suggesting that the first TAAT motif is not required for expression (Table 2). XTM1m12-15, a mutation that changed the four bases that make up the second TAAT motif, resulted in the failure of the construct to express in transgenic embryos, indicating that this motif is at least partly responsible for the activation of XTM1 expression.

As the third and fourth motifs within HBX2 overlap, mutations were made to two bases at a time, so that the sequence of one motif was disrupted whilst the other was maintained. Altering the sequence of the third motif significantly reduced the expression of the XTM1m22-23 transgene (Table 2), whereas the XTM1m26-27 mutation, which was specific to the fourth motif, had no effect on either the pattern or level of expression.

The reduction in the level of expression observed upon mutating the third TAAT motif was less pronounced than that caused by the mutation that was specific to the second motif (14% expressing compared to 0% respectively), however, only two bases were altered in this mutation. To assess if the third motif was playing an equivalent role in activating expression, all four of its constituent bases were altered (XTM1m22-25). This resulted in the failure of the transgene to express (Table 2). These experiments demonstrated that the homeobox consensus motifs at nucleotides 12-15 and 22-25 within HBX2 are necessary for the correct regulation of XTM1 expression, therefore they were named Element 1 (E1) and Element 2 (E2), respectively (Fig. 5).

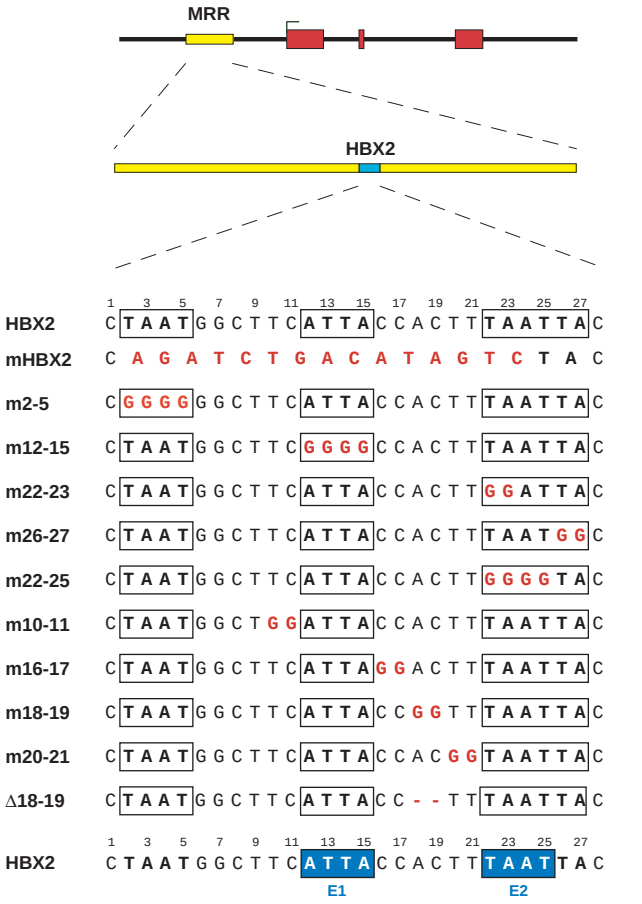

Fig. 5. Mutations within HBX2. The bases within HBX2 were assigned numbers as shown. The MRR is shown in yellow, HBX2 site is in blue, individual TAAT motifs are shown in bold type and are boxed and the mutations are shown in red. The blue shading highlights the two essential elements within HBX2, named element 1 (E1) and element 2 (E2).

E1 and E2 also function as repressor elements

As mentioned above, the similarity of HBX2 to a known repressor's consensus binding site suggested that this region of the MRR might have a repressor function as well as a role in activation. To test this further, E1 and E2 were individually mutated in the context of the MRRmCSKAGFP construct. Mutating them in this context circumvented the loss of expression bought about by removing HBX2's activation function, as the mCSKAGFP transgene has its own basal activity. Mutating either E1 or E2 resulted in the ubiquitous expression of GFP, suggesting that these homeobox motifs are also responsible for repression outside the normal domains of *Xmyf-5* expression (Fig. 3B).

The observation that the elements E1 and E2 within HBX2 are required for both repression and activation suggested that different factors might bind to these sites to mediate the different effects. If this were true, subtle mutations within this location might be expected to selectively interfere with the binding of these factors providing a way in which to separate activator from repressor functions. To test this, mutations in and around E1 and E2 were generated within XTM1 using a PCR-based technique and the effect on expression analysed.

Mutations to nucleotides around E1 and E2 (XTM1m10-11, m16-17, m18-19, m20-21; Fig. 5) or disruption in the spacing between the two elements (XTM1Δ18-19; Fig. 5) failed to affect either the level or the pattern of transgene expression (data not shown).

Transcription factor binding to HBX2

To confirm that HBX2 is able to bind nuclear factors *in vivo*, EMSAs were performed with protein extracts from stage 11 embryos. In these experiments, binding was observed when an oligonucleotide representing a functional HBX2 site was used (Fig. 6A). However, mutation of E1 resulted in the loss of binding. This loss of binding observed upon mutating E1 mimics the result observed in our transgenic assays, where both activation of XTM1 and repression of MRRmCSKAGFP require this element to be intact. Mutations disrupting the TAAT sequence of E2 had no effect on the ability of the embryo extracts to bind to HBX2. Implying that the factors that are responsible for mediating the activation and repression functions of E2 observed in the transgenic studies are unable to interact with HBX2 under the conditions used. The specificity of binding to HBX2 was confirmed by competition analysis in which a 200-fold excess of mutant competitor had no effect on binding, whereas a similar concentration of wild-type competitor completely abolished interaction with the labelled probe.

Mutational analysis combined with transgenesis has demonstrated the importance of E1 and E2 in establishing the correct pattern of *Xmyf-5* expression during gastrulation. For this reason, we were interested in identifying the transcription factors that bind to these motifs and thereby identify candidate proteins that may directly regulate *Xmyf-5* expression. To achieve this we tested a range of in vitro translated proteins for their ability to bind to HBX2 in electrophoretic mobility shift assays (EMSA). The proteins, Xcad-1, Xcad-2, Xcad-3, Xvent-1, Xvent-2, Xnot and Xmsx-1 were chosen on the basis of them possessing a homeodomain and being expressed at the right time and place to suggest a role in the regulation of *Xmyf-5* expression. Of the proteins tested, the members of the *Xenopus* caudal family, Xcad-1 (not shown), Xcad-2, Xcad-3 and Xvent-1, were shown to interact with a radiolabelled probe representing HBX2 (Fig. 6B), thus implying, that these proteins may directly regulate *Xmyf-5* expression and they were therefore studied further.

Caudal regulation of *Xmyf-5* expression

The members of the *Xenopus* caudal protein family have previously been shown to act as transcriptional activators and were therefore candidates to mediate the activation of *Xmyf-5* expression which was lost upon mutating HBX2 in the context of XTM1. To study this possibility further, mRNA encoding both wild-type Xcad-3 and a VP16 dominate active version of the protein were injected into a single dorsal blastomere at the 4 cell stage and the effect on *Xmyf-5* expression analysed by mRNA in situ hybridisation (data not shown). It would be expected that if Xcad-3 does directly activate *Xmyf-5* expression, that a dominantly active version would do the same. However, with both wild-type and the VP16 versions of Xcad-3, *Xmyf-5* expression was repressed, implying that the regulation observed was indirect. For this reason the regulation of *Xmyf-5* expression by Xcad-3 was not studied any further.

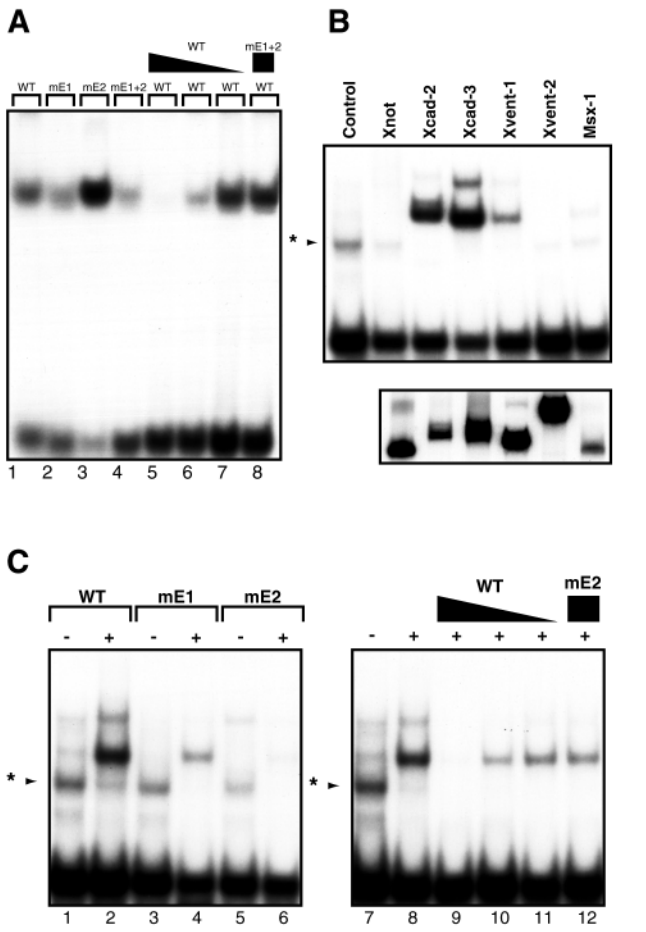

Fig. 6. (A) EMSA of embryonic extract binding to HBX2. Binding was performed with wild type (WT) and mutant HBX2 (mE1, mE2 and mE1+2) probes. Competition was performed with 200×, 50× and 12.5× excess of unlabelled WT probe (lanes 5-7 respectively), and 200× excess of unlabelled mE1+2 competitor (lane 8). (B) EMSA of in vitro translated protein binding to HBX2. Of the proteins tested, Xcad-2, Xcad-3 and Xvent-1 were found to bind to HBX2. The lane marked Control represents the interaction between the HBX2 probe and uncharged reticulocyte lysate. *Denotes nonspecific DNA binding. Shown below is an autoradiograph of in vitro transcribed proteins, following reticulocyte transcription/translation reaction in the presence of [^{35}S]methionine. (C) Interaction of Xvent-1 with E1 and E2. Left hand panel shows the binding of Xvent-1 to wild-type (WT) and mutant HBX2 probes (mE1 and mE2). Competition analyses are shown in the right hand panel. Competition was performed with 200×, 50× and 12.5× excess of unlabelled WT probe (lanes 9-11 respectively), and 200× excess of unlabelled mE2 competitor (lane 12). *Denotes nonspecific DNA binding.

The repressor Xvent-1 binds to E2

Another factor identified as a candidate to directly regulate *Xmyf-5* expression by interacting with HBX2 was Xvent-1(Gawantka et al., 1995) also called PV.1 (Tidman Ault et al., 1996). Indeed, HBX2 was identified by its its high degree of homology to a consensus site for Xvent-2 (Trindade et al., 1999) also called Xom (Ladher et al., 1996), Vox (Schmidt et al., 1996) and Xbr-1 (Papalopulu and Kintner, 1996), which itself shares significant homology to Xvent-1(Onichtchouk et al., 1996). It was therefore suprising that in vitro translated

Xvent-2 protein did not bind to HBX2. However, as Xvent-1 was shown to interact with HBX2, we elected to further investigate its binding requirements.

EMSAs were repeated with in vitro translated Xvent-1 using radiolabelled probes in which E1 and E2, the essential motifs within HBX2, were mutated (Fig. 6C). In these experiments Xvent-1 was seen to interact with the wild-type probe and to a lesser extent the E1 mutant probe, however, no binding to the probe in which E2 was mutated was observed. This suggests that Xvent-1 normally binds to E2 to mediate part of its function and implies that Xvent-1 may directly regulate *Xmyf-5* expression in vivo. The specificity of the interactions was further assessed by competition analysis, which showed that a 200-fold excess of mutant competitor had no effect on Xvent-1 binding to the wild-type probe.

Over-expression of Xvent-1 inhibits *Xmyf-5* expression in the dorsolateral domain

Previous reports have shown that Xvent-1 functions as a transcriptional repressor (Onichtchouk et al., 1998). This data combined with that outlined above, which shows that Xvent-1 binds to an element within the *Xmyf-5* promoter that is required for repression outside the normal pattern of *Xmyf-5* expression, suggests that Xvent-1 may function to directly repress *Xmyf-5* expression. To further investigate this possibility, the gastrula stage expression of *Xmyf-5* and *Xvent-1* were compared by mRNA in situ hybridisation. The expression of *Xvent-1* and *Xmyf-5* within the marginal zone were found to be mutually exclusive, with *Xvent-1* being restricted to the ventral domain whereas the expression of *Xmyf-5* was limited to the dorsolateral domain (Fig. 7A). These patterns of expression support a model in which the coexpression of *Xmyf-5* and *Xvent-1* are incompatible and combined with the observation that Xvent-1 binds to a repressor element within the *Xmyf-5* promoter purport a model whereby Xvent-1 directly represses *Xmyf-5* expression.

If one of the functions of Xvent-1 is to repress *Xmyf-5* in the ventral domain, then it would be expected that mis-expressing Xvent-1 in the dorsolateral domain should down-regulate *Xmyf-5* transcription. To ascertain if this was the case, Xvent-1 mRNA was injected into a single dorsal blastomere at the 4 cell stage and the effect on *Xmyf-5* expression analysed at gastrulation. In 94% of the embryos injected the expression of *Xmyf-5* was down-regulated (n=31) (Fig. 7B). This evidence combined with that from binding studies supports a role for Xvent-1 in directly repressing *Xmyf-5* expression.

Morpholino knock-down of Xvent function

The mutually exclusive patterns of *Xmyf-5* and *Xvent-1* expression, the repression of *Xmyf-5* expression observed upon over-expression of *Xvent-1* and the binding of Xvent-1 to a repressor element in the *Xmyf-5* promoter suggest that Xvent-1 functions to directly repress *Xmyf-5* expression. If this were true, disrupting Xvent-1 function would be expected to result in an expansion of *Xmyf-5* expression into the ventral domain, the normal region of *Xvent-1* expression. To disrupt gene function in *Xenopus*, we made use of antisense morpholino oligonucleotides (Heasman et al., 2000; Nutt et al., 2001). These are short, stable single stranded nucleotides that prevent mRNA translation by hybridising to the region

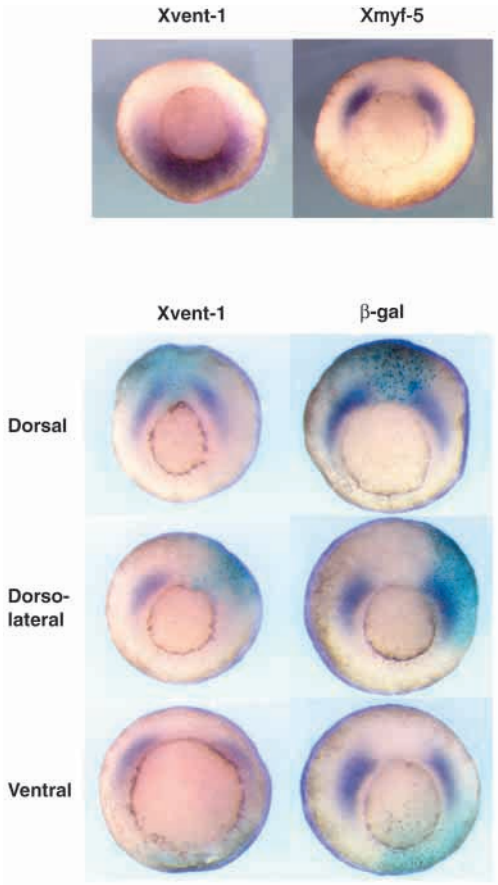

Fig. 7. (A) Endogenous expression of *Xvent-1* and *Xmyf-5*. (B) The effect of Xvent-1 mis-expression on *Xmyf-5*. Single blastomeres of 4 cell stage embryos were injected with 250 pg Xvent-1 capped mRNA along with the lineage tracer β-gal (shown in blue). *Xmyf-5* expression was then assayed at stage 10-11 by mRNA in situ hybridisation (purple). Embryos are orientated with dorsal pointing up and vegetal pole facing out.

around the initiation ATG and thus blocking ribosomal access. *X. laevis* is pseudotetraploid and has paralogues of many of its genes, it is therefore important to target morpholinos against both paralogues to ensure complete knockdown of function. To avoid this complication, we carried out all of the morpholino experiments described here in the diploid *X. tropicalis*.

To test whether preventing Xtvent-1 translation would cause *Xtmyf-5* expression to expand into the ventral domain, we injected both cells of a two cell stage embryo with a Xtvent-1 morpholino and assayed the expression of *Xtmyf-5* by mRNA in situ hybridisation. In these experiments, 26% of the embryos showed expansion of *Xtmyf-5* expression into the ventral domain (Fig. 8A). 26% is a relatively low number of embryos to be affected and could be explained in at least two ways. Firstly, the dose of the Xtvent-1 morpholino may be too low to have a complete effect or secondly, another factor may be acting in concert with Xtvent-1 to repress *Xtmyf-5* expression in the ventral domain.

To test the first possibility, double the dose of the Xtvent-1 morpholino was injected and the effect on *Xtmyf-5* expression assayed. Again, only a relatively low percentage of embryos

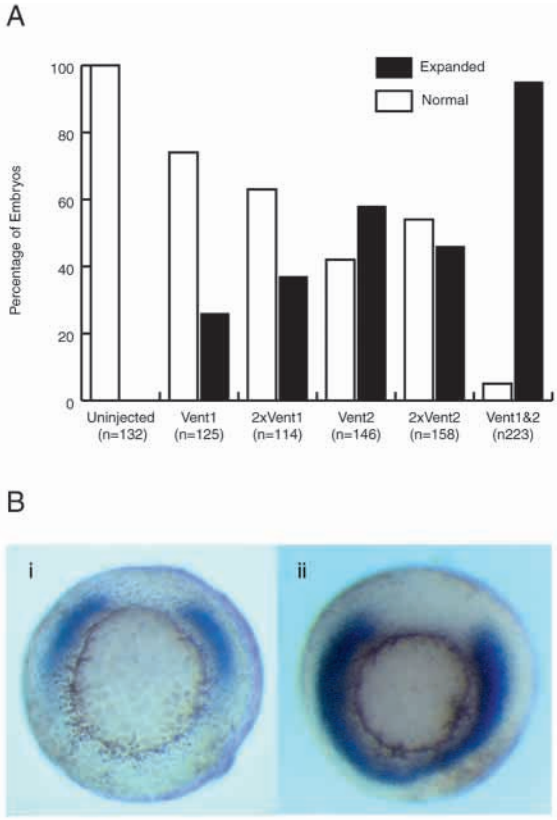

Fig. 8. The effect of Xtvent knockdown on *Xtmyf-5* in *X. tropicalis* embryos. (A) Percentage of embryos showing normal (white) or expanded (black) *Xtmyf-5* expression following injection with antisense Xtvent-1 and Xtvent-2 morpholinos. Single doses were 2 ng/embryo, and double doses were 4 ng/embryo, *n*=number of embryos scored. (B) *Xtmyf-5* expression in wild-type embryo (i) and embryo injected with both Xtvent-1 and Xtvent-2 antisense morpholino (ii).

(37%) showed expansion of *Xtmyf-5* expression into the ventral region of the marginal zone, suggesting that another factor maybe acting along with Xtvent-1 to repress *Xtmyf-5* expression.

A good candidate for such a factor is Xvent-2, as it has previously been shown to act synergistically with Xvent-1 (Onichtchouk et al., 1998). To investigate the relationship between Xtvent-2 and *Xtmyf-5*, the function of Xtvent-2 was again disrupted using morpholino technology. As with Xtvent-1, the effect of disrupting Xtvent-2 function resulted in the expansion of *Xmyf-5* expression into the ventral domain in only a portion of the embryos (58%). However, when the function of both Xtvent-1 and Xtvent-2 were disrupted simultaneously, 95% of the embryos showed expansion of *Xtmyf-5* expression into the ventral domain (Fig. 8B). To confirm this effect was due to a synergistic interaction between Xtvent-1 and Xtvent-2 rather than a dose effect, a double dose of anti-Xtvent-2 was injected. As with Xtvent-1, disrupting the translation of Xtvent-2 with double the dose of Xtvent-2 morpholino had a similar effect to that observed with a single dose. These results suggest that Xtvent-2 activity is required for Xtvent-1 to directly repress *Xtmyf-5* in the ventral domain.

DISCUSSION

Studying *Xmyf-5* regulation using a cross species approach

The pattern of *Xmyf-5* expression during *Xenopus* gastrulation is unique in that it is the only known gene to be restricted to the dorsolateral domain of the marginal zone. We believe understanding how the expression of *Xmyf-5* is regulated will provide important insights into how the mesoderm is patterned, as the dorsolateral domain requires both dorsal and ventral inputs to establish its identity. To investigate the control of *Xmyf-5* expression, we have established a cross-species approach by analysing the regulation of the *X. tropicalis myf-5* gene (*Xtmyf-5*) in transgenic *X. laevis* embryos. This approach has enabled us to rapidly screen large numbers of transgenes and identify a region of the *Xtmyf-5* promoter, named a myf-5 regulatory region (MRR), that is required for both activation of expression and repression outside the normal domains of *Xmyf-5* transcription.

myf-5 regulatory region (MRR)

The promoter studies described here have identified the MRR, a region of the *Xtmyf-5* gene located between –834 bp and –1619 bp upstream from the start of transcription. Deletion of this region in the context of a 7.28 kb *Xtmyf-5* genomic fragment eliminates expression in transgenic embryos, implying that the MRR contains elements required for the activation of expression. In addition, when the MRR is placed upstream of a heterologous promoter that has ubiquitous basal activity, expression is repressed outside the normal pattern of *Xmyf-5* expression. This suggests that the MRR also contains elements that are involved in the repression of *Xmyf-5* outside its normal expression domains. Scanning the MMR for potential transcription factor binding sites led to identification of a homeobox consensus motif, which we called HBX2. This site has significant similarity to a consensus binding site for Xvent-2 (Trindade et al., 1999), which is expressed in the gastrula embryo in the dorsolateral and ventral mesoderm (Onichtchouk et al., 1996).

HBX2

Specific mutations either removing the entire HBX2 site or altering two of the TAAT core motifs that constitute it (named E1 and E2), resulted in the loss of expression in the context of the entire 7.28 kb transgene and loss of repression in the context of the MRR controlling the expression of a heterologous promoter. This suggested that HBX2 confers some of the activation and repression functions ascribed to the MRR. The dual role of HBX2 implies that it is a site where positional information converges on the *Xmyf-5* promoter to establish the correct pattern of *Xmyf-5* expression. For this reason, we were particularly interested in identifying what transcription factors mediate these effects. The ability of HBX2 to interact with endogenous nuclear factors was confirmed by EMSA. Indeed, mutations effecting E1 abolished binding mimicking the loss of regulation observed in the transgenic experiments.

Binding studies with a number of homeodomain-containing proteins present during the gastrula stages identified Xvent-1 and the *Xenopus* caudal proteins as factors that bind to the E2 motif within the HBX2 element, thus implicating them as direct regulators of *Xmyf-5* expression.

We were unable to utilise super-shift experiments to confirm whether endogenous Xvent-1 or the caudal proteins from embryonic extracts could bind to HBX2, as there are currently no antibodies available to these proteins. Attempts to use myc-tagged versions of the proteins were unsuccessful, as we found that tagging the proteins either in the N or C terminus severely impaired their ability to interact with the HBX2 in vitro.

Vent regulation of *Xmyf-5* expression

EMSAs identified Xvent-1 as a transcription factor that binds to E2, and therefore as a candidate to directly regulate *Xmyf-5* expression. Xvent-1 is a transcriptional repressor (Onichtchouk et al., 1998), and was shown to be expressed in a pattern adjacent to Xmyf-5 but which did not to overlap with *Xmyf-5*. This evidence implied that Xvent-1 binds to E2 to mediate its repressive functions and thus prevent the expression of *Xmyf-5* in the ventral domain, a phenotype that is observed when E2 is mutated. This is supported by the observation that mis-expressing Xvent-1 in the dorsolateral domain results in the repression of *Xmyf-5* expression and that knock down of Xvent-1 protein causes the expansion of *Xmyf-5* into the ventral domain.

The expansion of *Xmyf-5* expression when Xvent-1 was knocked down was observed in only a proportion of embryos tested and subsequent experiments suggested that the simultaneous disruption of the activity of the related factor Xvent-2 was required in order to generate a phenotype in the majority of embryos. We wanted to ascertain whether Xvent-2 also regulates *Xmyf-5* expression directly by binding to HBX2. Xvent-2 had previously been tested for its ability to interact with HBX2 in EMSAs, however, no binding was observed. This was somewhat suprising as HBX2 was first identified by its high degree of identity with a Xvent-2 consensus binding site (Trindade et al., 1999). It is possible that Xvent-2 directly regulates *Xmyf-5* expression by binding to HBX2 and we failed to observe this interaction because Xvent-2 requires a form of modification that is not provided when it is translated in rabbit reticulocyte lysate. This modification may be in the form of phosphorylation, as Xvent-2 has been shown to be a target of cdc2/cyclin B1 (Stukenberg et al., 1997). The regulation of *Xmyf-5* expression by Xvent-2 may also be through regulatory element other than HBX2.

It is also possible that Xvent-2 controls *Xmyf-5* expression indirectly, via a factor that acts in concert with Xvent-1 to repress transcription in the ventral domain. This would explain why it is necessary to simultaneously repress Xvent-1 and Xvent-2 activity in order to obtain a complete expansion of *Xmyf-5* expression into the ventral domain.

Model of *Xmyf-5* regulation

The identification of HBX2, an element that is required for both the activation and repression of *Xmyf-5* expression, suggests that this element receives both positive and negative inputs and is therefore a site where positional information is integrated to generate the correct pattern of *Xmyf-5* expression. Mutating the motifs that constitute HBX2 in the context of the MRR driving a heterlogous promoter, disrupted the MRR's capacity to repress expression outside the normal domain of *Xmyf-5* transcription resulting in ubquitous expression. This implied that HBX2 normally binds factors that prevent expression in all regions of the embryo apart from the dorsolateral domain.

Whether the *Xmyf-5* gene has the potential to be activated in all areas of the embryo but is refined by dominant repression, resulting in expression only in the dorsolateral domain, is not clear. However, the identification of Xvent-1 as a repressing factor that binds to HBX2 suggests that *Xmyf-5* expression is normally actively and directly repressed at least in the ventral domain. Xvent-1 was shown to require an intact E2 motif within HBX2 in order to bind, however, HBX2 is composed of two core TAAT motifs both of which are necessary for repression and activation of expression and in order for HBX2 to interact with nuclear extracts. It is likely therefore that several factors bind to HBX2 via both E1 and E2 in order to generate the stereotypical pattern of dorsolateral domain expression of *Xmyf-5*.

One potential way in which *Xmyf-5* expression may be activated is via the members of the caudal protein family. These homeodomain-containing proteins are transcriptional activators that are expressed throughout the marginal zone and have also been shown to bind to HBX2 identifying them as candidates to mediate its activator function. However, over-expression of wild-type and dominant active forms of Xcad-3 were seen to repress *Xmyf-5* transcription, suggesting that the caudal proteins do not directly activate *Xmyf-5* expression.

In conclusion, the work in this paper has identified a promoter element (HBX2) that is required for both activation and repression of *Xmyf-5* expression and has two functional motifs (E1 and E2). E2 interacts with the transcriptional repressor Xvent-1 and is partly responsible for repressing expression in the ventral domain, implying part of the mechanism that generates the normal pattern of *Xmyf-5* expression requires Xvent-1 to directly repress *Xmyf-5* in the ventral domain. Furthermore, we have identified a protein or protein complex from gastrula stage embryo extracts, which binds to E1.

Identifying this protein(s) will provide important evidence both as to how the expression of *Xmyf-5* is controlled and how positional information is integrated to establish the dorsolateral domain of the marginal zone. However, it is unlikely that HBX2 provides all of the positional information required to establish the correct pattern of *Xmyf-5* expression during gastrulation. Indeed, a recent study has identified a distal TCF-3 binding motif that is essential in regulating *Xmyf-5* expression at the gastrula stage (Yang et al., 2002). We also identified a TCF consensus in the MRR (Fig. 4A), however, deletion of this putative site had no effect on either the level or pattern of transgene expression, implying that this region is not necessary for *Xmyf-5* expression during gastrulation.

In contrast to work in the mouse (Carvajal et al., 2001; Hadchouel et al., 2000; Summerbell et al., 2000; Zweigerdt et al., 1997), we have found that the regulation of *Xmyf-5* expression in early frog embryos is relatively simple, requiring a few elements within 2 kb of the start of transcription. However, it is important to note that our work has focused exclusively on the regulation of *Xmyf-5* expression during the gastrula stages, when it is first expressed. During later stages of development, *Xmyf-5* expression becomes more complex, and this regulation is likely to involve the activity of, as yet, unidentified control elements.

We would like to thank J. B. Gurdon, E. de Robertis, H. Isaacs, D. Kimelman, S. Hoppler, A. Fainsod, T. Mohun, B. Göttgens, C. Niehrs, R. Harland and L. Zimmerman for providing essential reagents and X. Ding and R. Rupp for communication of results prior to publication. We acknowledge the Sanger Centre (Hinxton, UK) for providing the *X. tropicalis* EST sequences used in this work. We would also like to thank the members of the Amaya lab for many helpful discussions in the preparation of this manuscript. A Wellcome Trust Prize PhD Studentship (M. P.) and National Institutes of Health Grant RR13221 and Wellcome Trust Senior Research Fellowship (E. A.) supported this work.

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