Transcriptional regulation of the cardiac-specific *MLC2* gene during *Xenopus* embryonic development

Branko V. Latinkić*, Brian Cooper, Stuart Smith, Surendra Kotecha, Norma Towers, Duncan Sparrow and Timothy J. Mohun†

Division of Developmental Biology, National Institute for Medical Research, The Ridgeway, Mill Hill, London NW7 1AA, UK
*Present address: School of Biological Sciences, Cardiff University, PO Box 911, Cardiff CF10 3US, UK
†Author for correspondence (e-mail: tmohun@nimr.mrc.ac.uk)

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Summary

The mechanisms by which transcription factors, which are not themselves tissue restricted, establish cardiomyocyte-specific patterns of transcription in vivo are unknown. Nor do we understand how positional cues are integrated to provide regionally distinct domains of gene expression within the developing heart. We describe regulation of the *Xenopus XMLC2* gene, which encodes a regulatory myosin light chain of the contractile apparatus in cardiac muscle. This gene is expressed from the onset of cardiac differentiation in the frog embryo and is expressed throughout all the myocardium, both before and after heart chamber formation. Using transgenesis in frog embryos, we have identified an 82 bp enhancer within the proximal promoter region of the gene that is necessary and sufficient for heart-specific expression of an *XMLC2* transgene. This enhancer is composed of two GATA sites and a composite YY1/CArG-like site. We show that the low-affinity SRF site is essential for transgene expression and that cardiac-specific expression also requires the presence of at least one adjacent GATA site. The overlapping YY1 site within the enhancer appears to act primarily as a repressor of ectopic expression, although it may also have a positive role. Finally, we show that the frog *MLC2* promoter drives pan myocardial expression of a transgene in mice, despite the more restricted patterns of expression of murine *MLC2* genes. We speculate that a common regulatory mechanism may be responsible for pan-myocardial expression of *XMLC2* in both the frog and mouse, modulation of which could have given rise to more restricted patterns of expression within the heart of higher vertebrates.

Key words: *Xenopus*, Cardiogenesis, Heart, Myocardium

Introduction

Early heart development is strikingly similar in all vertebrates. Cardiac progenitors are initially specified bilaterally in the mesodermal layer and these subsequently commence differentiation, fuse together and form a linear tube of myocardial muscle. Peristaltic contractions push blood through the inner endocardial vessel that connects to the developing vasculature. This simple arrangement is rapidly transformed by a combination of morphological changes and regional differentiation into the complex architecture of cardiac chambers and valves characteristic of the mature vertebrate heart. Our understanding of these events is rudimentary, but it is apparent that they are driven by a common, underlying genetic program, shared to varying degrees by all metazoans (Harvey, 2002).

Although the identity of several signalling pathways likely to be involved in specifying cardiac fate have been identified (Harvey, 2002; Zaffran and Frasch, 2002), we still have little knowledge of how such signals trigger the onset of cardiac differentiation nor do we understand how distinct programs of differentiation are orchestrated in different regions of the developing heart. Initial studies of cardiac muscle-specific transcription, based largely on cell culture models, have identified candidate regulatory factors that appear to function in a variable but combinatorial manner to drive expression of the terminal differentiation program. However, such factors are apparently distributed quite broadly, both within the developing heart and frequently within the whole embryo, offering few clues as to the basis for regional differentiation in the heart (Bruneau, 2002; Cripps and Olson, 2002).

The scale of this problem has become evident from studies of gene expression in the developing heart of the mouse embryo. These have demonstrated that almost all genes identified as part of the cardiac muscle differentiation program show some regional restriction within the heart, the nature and extent of which frequently changes as cardiogenesis proceeds. To compound this complexity, studies of heart-specific transcription using transgenic models have identified enhancer elements within individual cardiac gene promoters that drive transgene expression in very specific domains within the developing heart. These do not necessarily correspond to any obvious morphological compartment (Habets et al., 2003; Kelly et al., 1999).

Two explanations could account for such findings. One view is that such discrete expression domains may indicate regions that have distinct functional or developmental significance during cardiac morphogenesis. For example, in addition to the different prospective fates along the anteroposterior axis of the initial heart tube, such regional expression patterns probably indicate the importance of patterning in both the dorsoventral

and left-right axes of the initial heart tube (Habets et al., 2003). Indeed, regional expression of several genes early in mouse heart formation, combined with the changing functional characteristics of embryonic myocardium, together form the basis of a compelling model for heart morphogenesis (Christoffels et al., 2000).

From another perspective, the existence of such discrete enhancer elements could testify to the modular nature of the regulatory elements controlling cardiac gene expression. Such an arrangement could reflect the evolutionary diversity of cardiogenesis amongst metazoans. In this view, distinct regulatory modules provide the mechanisms by which functional complexity has been achieved on the basis of a common genetic program (Fishman and Olson, 1997; Habets et al., 2003).

Progress in understanding either the basis for, or the significance of, regional transcription patterns in the developing heart will require detailed and comparative study of individual genes in the myocardial differentiation program. To date, several different families of transcription factors have been implicated in regulating cardiomyocyte differentiation, including members of the Nkx/tinman,family, the MADS factors MEF2 and serum response factor (SRF) and the GATA family of zinc-finger proteins (Bruneau, 2002). In addition, an important role has been identified for myocardin, a regulatory factor whose activity is mediated by protein:protein interactions rather than by direct interaction with specific DNA sequences (Wang et al., 2001).

GATA transcription factors are zinc-finger proteins known to bind DNA and transactivate target genes through the GATA-binding site, (A/T)GATA(A/G) (Ko and Engel, 1993). Based on their expression patterns, the GATA proteins have been divided into two subfamilies: GATA1/2/3, which are primarily expressed in haematopoietic progenitors, but also in the nervous system, and GATA4/5/6 which are broadly expressed in the heart, gut and lungs (Molkentin, 2000). GATA4 has been shown to regulate a number of cardiac-specific genes in vitro, including MHCα, cardiac *TnC* and *ANF* (Grepin et al., 1994; Ip et al., 1994; Molkentin et al., 1994).

SRF is involved in regulation of muscle-specific and growth factor-inducible transcription, binding to the motif CC[A/T]6GG (termed a CArG box or SRE). It has been shown in vitro to interact with GATA4 and Nkx2.5 to regulate transcription of cardiac promoters. These and similar combinatorial interactions between cardiogenic factors, which are present in broader area then the heart, were proposed to provide molecular basis for heart-specific transcription (Bruneau, 2002; Charron and Nemer, 1999; Cripps and Olson, 2002).

The zinc-finger protein YY1 is pleiotropic regulator that can both repress and activate transcription (Thomas and Seto, 1999). In addition, it can cause DNA bending and it has been shown to be involved in chromatin remodelling. In *Xenopus* embryos, YY1 is regulated at the level of nuclear import, being exclusively cytoplasmic during early development, subsequently translocating to the nucleus (Ficzycz et al., 2001). Recent studies have shown that YY1 interacts with GATA4 to synergistically activate transcription of the BNP promoter in cell culture (Bhalla et al., 2001), but also acts to downregulate transcription from the cardiac-specific MHCα promoter (Sucharov et al., 2003).

We have previously reported that the myosin light chain 2 gene provides a sensitive marker for the onset of cardiac muscle differentiation in *Xenopus* embryos (Chambers et al., 1994). In contrast to the *MLC2* genes of amniotes (*MLC2*a and *MLC2*v) that are restricted to the atria or ventricles respectively (Franco et al., 1999), we show that *XMLC2* transcripts are present throughout the entire myocardium, from the onset of cardiac differentiation in the tailbud embryo to the formation of a mature, chambered heart. We also show that the *Xenopus MLC2* promoter faithfully maintains its pan-myocardial expression in transgenic mouse embryos. This suggests that chamber restriction of the mammalian *MLC2* genes may be the result of regulatory controls that have evolved to limit a more ancient pan-myocardial program. Using transgenesis in *Xenopus* embryos, we have found that the combined activities of GATA factors, SRF and YY1 apparently drive pan-myocardial expression of the *Xenopus MLC2* gene.

Materials and methods

Isolation of the *XMLC2* promoter

The *XMLC2* promoter was obtained as a 3057 base pair (bp) *Xba*I-*Hin*dIII fragment from a *Xenopus* genomic library isolate. Its DNA sequence was determined using the dideoxy method. In order to obtain a promoter fragment suitable for use in reporter constructs we removed the 3′ most 48 nucleotides, including the initiation codon (ATG) of the *XMLC2* gene, by exonuclease III digestion (Sambrook, 1989).

XMLC2 promoter-GFP fusion gene constructs

A synthetic GFP reporter gene containing the GFP open reading frame and SV40 polyadenylation (pA) signal was cloned into the *Bgl*II site downstream of the 3kb *XMLC2* promoter. A series of 5′ deletions Δ-1558 bp (*Stu*I) and Δ-681bp (*Eco*RV) were generated by using *Stu*I and *Eco*RV restriction enzymes, respectively. Additional 5′ deletions were generated by PCR using proofreading polymerase and the 3kb reporter plasmid as a template. All primers used consisted of18-20 bp of *XMLC2* sequence with an additional *Eco*RI or *Bam*HI recognition site.

A chimeric *XMLC2*-TK promoter, comprising nucleotides –1558 to –48 bp of the *XMLC2* promoter sequence fused to a thymidine kinase (TK) minimal promoter, was generated by ligating a *Stu*I *XMLC2* fragment to a 161 bp *Sma*I-*Bgl*II TK fragment. Fragments comprising nucleotides –1558 to –249 and –249 to –36 of the *XMLC2* promoter were generated by PCR and used to create chimeric constructs with the minimal promoters of the TK (McKnight and Kingsbury, 1982) or type 5 cytoskeletal actin (Mohun et al., 1987) genes. Other short *XMLC2* fragments (see text and figure legends) were synthesised as oligonucleotides with and cloned into the Asp718 site of the minimal cytoskeletal actin promoter via *Asp*718-compatible ends (Latinkić et al., 2002). One such double stranded oligonucleotide (–122/–85) was also used as a probe for EMSA (see Figs 6 and 7). PCR-mediated mutagenesis was performed by the overlap method (Ho et al., 1989), using proofreading thermostable polymerase. The sequence of all constructs generated by PCR or by insertion of oligonucleotides was confirmed by sequencing, as was the oligonucleotide copy number.

Xenopus embryos, microinjection and transgenesis

Xenopus laevis embryos were obtained and cultured by standard methods and microinjected with linearised DNA or synthetic RNA as described previously (Sive, 2000). Transgenic embryos were generated according to method of Kroll and Amaya (Kroll and Amaya, 1996), with modifications as described (Sparrow et al., 2000b). Successful transgenesis was confirmed by including a γ-*crystallin*/GFP reporter (Offield et al., 2000) together with the test

construct, as described elsewhere (Latinkić et al., 2002). Embryos expressing GFP were analysed either by observation of fluorescence or by whole mount in situ hybridisation to detect GFP mRNA.

Wholemount in situ hybridisation and histology

Whole-mount in situ hybridisation was performed as described (Sive, 2000) with probes specific for GFP (Sparrow et al., 2000a) or *XMLC2* (Chambers et al., 1994). Transverse sections (10 μm) were obtained from stained *Xenopus* embryos after embedding in Paraplast wax. Transgenic mouse embryos were stained for β-galactosidase expression and analysed directly or by cryostat sectioning.

Mouse transgenesis

The 3 kb *Hin*dIII-*Bgl*II fragment containing the *XMLC2* promoter was fused upstream of the β-galactosidase reporter, pPD16.43 (Fire et al., 1990). Transgenic mice were generated by the Biological Services Division of NIMR using standard methods.

EMSA

Embryo extracts were prepared as described (Howell et al., 1999). The following oligonucleotides were synthesised with 5′GTAC overhangs for use as probes or competitors in EMSA reactions: GATA#1 TOP, GTACCTATGCCTGAGATAAGAAGGAGTCG; GATA#1-BOT, GTACCGACTCCTTCTTATCTCAGGCATAG; GATA#2 TOP, GTACCCTTGTGCTCTTATCTCTTCCGTCTG; GATA#2-BOT, GTACCAGACGGAAGAGATAAGAGCACAAGG; GATA#3-TOP, GTACCACTTTCCTGATAAATGAAGTATCCAG; GATA#3-BOT, GTACCTGGATACTTCATTTATCAGGAAAGTG; YY1-TOP, GTACGAGATCTCCCCACCCCTACTCCATGAGAA; YY1-BOT, GTACTTCTCATGGAGTAGGGGTGGGGAGATCTC; SP1-TOP, AATTGACGCTGGGCGGGGTTTG; SP1-BOT, AATTCAAACCCCGCCCAGCGTC.

Double-stranded oligonucleotides (20 ng) were labelled with α-^{32}P-dCTP and Klenow DNA polymerase as described (Sambrook, 1989).

Human SRF (Norman et al., 1988) and XGATA4 (Kelley et al., 1993) were translated in vitro using a coupled transcription-translation system (Promega) according to manufacturers instructions. Binding reactions and competitions were as described previously (Norman et al., 1988). Polyclonal anti-YY1 (Santa Cruz) and anti-XSRF (Chambers et al., 1992) antibodies were used at 1:20 dilution.

Luciferase assays

10 pg of –122/–45/cyt5/luc and RL-TK (Promega) were injected together into the one- or two-cell stage of fertilised *Xenopus* eggs. The DNA was also co-injected with 200 pg of synthetic RNA encoding SRF and/or XGATA4. Animal pole explants were excised at the blastula stage (Sive, 2000) and cultured until control embryos reached stage 13. Firefly and Renilla luciferase assays were performed using the Dual Luciferase Assay kit (Promega), according to the manufacturers recommendations.

Results

XMLC2 is a pan-myocardial marker

The *XMLC2* gene is one of only a few genes that are expressed exclusively in developing cardiac muscle in *Xenopus* embryos (Chambers et al., 1994) and therefore provides a useful marker for studying the onset of cardiogenesis. Expression is restricted to cardiac muscle until adult stages, when it is also detected in the pulmonary vasculature (along with other myocardial markers). In mammals, distinct *MLC2*a and *MLC2*v genes have been identified, differing in the progressive restriction of their expression to atrial and ventricular myocardium respectively (Franco et al., 1999). By sequence comparison, the *XMLC2* appears to be the homologue of *MLC2*a (Chambers et al., 1994) and no amphibian equivalent of *MLC2*v has been identified. However, in contrast to its mammalian counterpart, *XMLC2* expression is not restricted to atrial myocardium during development. Instead, it is uniformly expressed throughout myocardial tissue of the atria, ventricle and outflow tract of the tadpole heart (Fig. 1A,B). Its expression commences in the cardiac mesoderm on the ventral midline of late tailbud embryo, providing a convenient marker with which to visualise the subsequent steps of heart tube formation, looping and chamber formation (Fig. 1C-J).

3 kb *XMLC2* promoter recapitulates embryonic expression of the endogenous gene

To understand regulatory mechanisms directing pan-myocardial expression of *XMLC2*, we isolated a genomic clone that includes 3 kb upstream of the open reading frame. This region includes putative binding sites for GATA, MEF2 and SRF transcriptional regulators, all of which have been implicated in the control of cardiac muscle-specific transcription (Fig. 2). When fused to a GFP reporter, the 3 kb sequence is sufficient to direct strong expression of both GFP mRNA and protein in transgenic tadpoles (5′Δ-2990, Fig. 3A,B). Expression of the transgene, like that of the endogenous *XMLC2* gene, is confined exclusively to the developing myocardium.

Current methods of frog transgenesis cannot easily be used for systematic quantitative comparisons of transgene

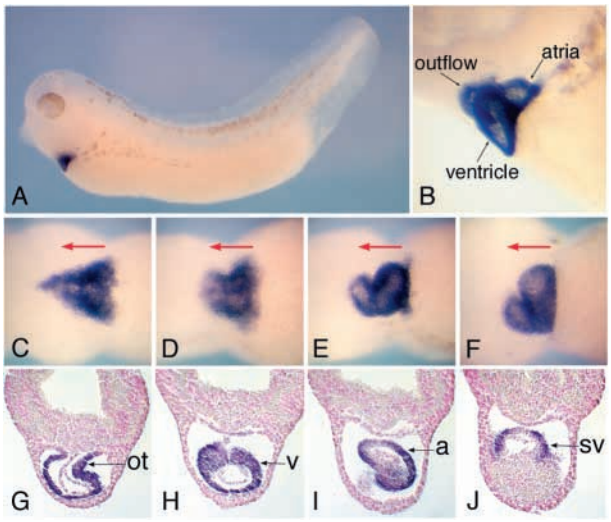

Fig. 1. XMLC2 is expressed throughout the myocardium of developing *Xenopus* tadpole. (A) The myosin light chain 2a gene is expressed throughout the heart of the *Xenopus* tadpole (stage 42). At higher magnification (B), expression is evident in both atrial and ventricular chambers as well as the outflow tract. Ventral views of the heart-forming region reveal XMLC2 expression in bilateral patches of cardiac mesoderm (C) prior to heart tube formation (stage 28). Subsequent formation of a contractile myocardial tube (D; stage 32), looping of the heart tube (E; stage 35) and differentiation of distinct atrial and ventricular chambers (F; stage 42) is clearly visible. Red arrows indicate direction of blood flow (posterior to anterior). (G-J) Transverse sections through the looped heart tube (stage 35) show expression in the myocardial walls of the outflow tract (ot), ventricular (v) and atrial (a) regions and the sinus venosus (sv).

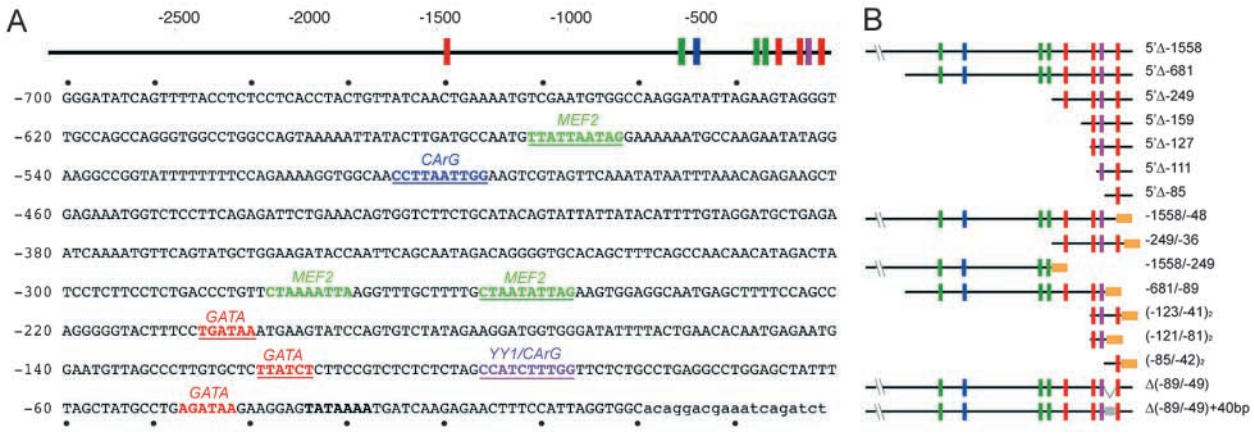

Fig. 2. Sequence of the XMLC2 promoter. (A) The XMLC2 promoter contains GATA-, MEF2- and CArG-binding motifs (shown in red, green and blue, respectively), which are potential binding sites for GATA, MEF2 and SRF transcription factors. An additional proximal motif (purple) combines a YY1 site with a weak SRF-binding site. The proximal 700 nucleotides of promoter sequence are shown, with the TATA box (bold) and 19 nucleotides of exon1 (lower case) indicated. The minimal promoter fragment capable of supporting heart-specific expression in transgenic studies (–123/–41) is highlighted. (B) Promoter regions used in transgenesis experiments. For some constructs, the promoter fragment was fused to a heterologous minimal promoter from the *Xenopus laevis* cytoskeletal actin or Herpes simplex thymidine kinase genes (orange box). All other constructs included to XMLC2a basal promoter and transcription start site.

expression (Mohun et al., 2002) but are better suited for defining sequences that are indispensable for detectable expression and appropriate tissue specificity. To determine the sequences required for myocardial-specific activity of the *XMLC2* promoter, we tested a series of 5′ deletion constructs. Removal of sequences upstream of –249 (including all potential MEF2 sites and an SRF binding motif) had no discernable effect on the specificity of transgene expression and little effect on its level (5′Δ-249; Fig. 3C,D). Further truncation to –159 and –127 removed the most distal of three GATA motifs (GATA#3) and resulted in weaker levels of transgene expression and a lower frequency of expression in transgenic embryos (5′Δ-159 and 5′Δ-127; Table 1). Nevertheless, transgene expression was detectable in the developing tadpole heart. Removal of a further 16 bp of the 5′ sequence (encompassing the GATA#2 motif) resulted in loss of expression in the heart (5′Δ-111; Table 1) indicating that 127 nucleotides of promoter-proximal sequence is indispensable for expression in the heart. This sequence includes two GATA sites (GATA#2 and GATA#1) flanking a combined CArG-like/YY1 site.

Reporter constructs containing severely truncated regions of the *XMLC2* promoter (5′Δ-159, –127, –111, –85) frequently exhibited ectopic expression in the branchial arches, pronephros and ventral side of the embryo (Fig. 3F,G; Table 1) presumably reflecting the loss of regulatory elements that normally restrict *XMLC2* promoter activity to the heart.

Sequences within the proximal 249 nucleotides are indispensable for expression in the heart

To define the 3′ border of the sequence required for expression in heart, we created several constructs containing varying length of the *XMLC2* promoter upstream of a minimal promoter from the *Xenopus* type 5 cytoskeletal actin or the *herpes simplex* thymidine kinase genes. Removal of the most proximal 47 nucleotides (encompassing the GATA#1 motif) had little effect on heart-specific expression (–1558/–48Cyt;

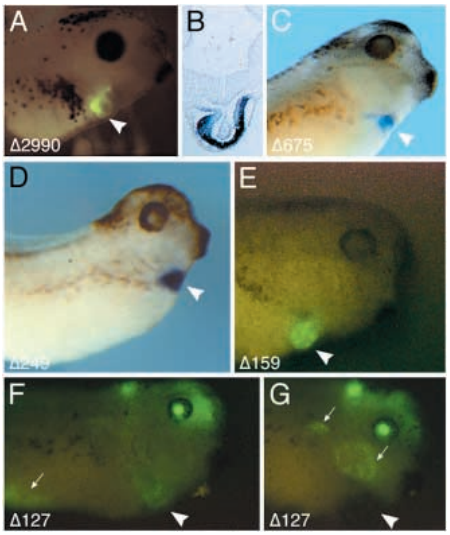

Fig. 3. The XMLC2 promoter recapitulates expression of the endogenous gene in transgenic embryos. Expression of XMLC2-GFP transgenes, assayed by fluorescence (A,E-G) or whole-mount in situ hybridisation for GFP RNA (B-D). Arrowheads indicate the tadpole heart. A GFP transgene containing 2990 bp of promoter sequence directs strong, consistent and uniform expression of GFP protein in the tadpole heart (A). Variation in fluorescence intensity reflects differences in myocardial wall thickness between heart chambers and the stage of contraction. (B) Transverse section through the forming heart tube heart (stage 30), showing that staining for GFP RNA is restricted to the myocardium. Heart-specific expression is retained after 5′ truncation of the promoter to –675 (C) or –249 (D); truncation to –159 retains heart-specific expression, although the level is much reduced (E). Further truncation to –127 yields occasional, weak expression of GFP to the heart and more frequently in ectopic locations (e.g. branchial arches, pronephros, blood island; small arrows). GFP activity in the eyes and hindbrain (F,G) is from the γ-crystallin co-transgene.

Table 1. Results obtained with MLC2 transgene

Transgene construct	Heart/total		Other sites	Fig.
5′ deletions				
5′Δ –2990	15/30			3A
5′Δ –1558	7/12			
5′Δ –681	20/51			3C
5′Δ –249	13/28			3D
5′Δ –159	10/50	(8)†	3 BA, PN	3E
5′Δ –127	5/27*	(3)†	12 BA. PN	3F,G
5′Δ –111	0/8*		6 BA, PN	
5′Δ –85	0/54*		15 BA, S	
Promoter fusions				
–1558/–48 (Cyt/tk)‡	18/98			4A
–249/–36 (Cyt)	3/10			4B
–1558/–249 (Cyt/tk)‡	0/5*			
–681/–89 (Cyt)	0/16*			
$(–123/–41)_2$ (Cyt)	5/5*			4C
$(–121/–81)_2$ (Cyt)	0/12*		3 BA	4D
$(–85/–42)_2$ (Cyt)	0/10*		3 spotty	
Internal deletions				
Int.Δ –89/–49	3/45	(1)†	7 various	
Int.Δ –89/–49 + 40bp	3/7			
Motif mutations				
PM-GATA#3	5/11			
PM-GATA#2	3/8			5A
PM-GATA#1	7/12			
PM-GATA#2,3	3/10*			5B
PM-GATA#1,2	0/12*			5C
CArG-like mut	0/11*			
YY1 mut	2/6*	(0)†	2 BA	6B
CArG-like mut/YY1 mut	3/25*	(0)†	4 BA	6C
CArG mut/YY1 mut	12/15*	(6)†	6 S, BA	

*Experiments performed with γ-crystallin GFP co-transgene. (Only embryos showing cotransgene expression were scored.)

†When GFP expression was detected in other sites as well as the heart, values in parentheses indicate the number of embryos showing heart-only expression.

‡Promoter fragments tested with both *Xenopus laevis* cytoskeletal actin and *herpes simplex* thymidine kinase minimal promoters.

Cyt/tk, cytoskeletal actin or tk minimal promoters; Int.Δ, internal deletion; PM, point mutations; BA, branchial arches; PN, pronephros; S, somites.

Fig. 4A) but a more radical 3′ deletion (–1558/–249) was inactive in our transgenic embryo assay (data not shown). These results demonstrate that sequences upstream of –249 have no independent enhancer activity and indicate that sequences between –249 and –48 in the *XMLC2* promoter can drive heart-specific expression. This minimal region, like that defined by 5′ truncation, encompasses two GATA sites but in this case, however, they lie upstream of the CArG-like/YY1 motif rather than flanking it.

We also found that removal of proximal sequences up to –89 within the promoter abolished cardiac-specific expression from transgenes, leaving only a low level of ectopic expression (–681/–89Cyt; Table 1). The CArG-like/YY1, GATA#2 and GATA#3 motifs all lie upstream of the truncated region and are therefore unaffected by this mutation. Removal of nucleotides –89/–49 from the 681 bp *XMLC2* promoter (IntΔ-89/49; Table 1) severely reduced (but did not completely abolish) heart-specific transgene expression, but this was largely restored by their replacement with heterologous DNA sequence (IntΔ-89/49+40 bp; Table 1). This region may therefore be important for facilitating promoter:enhancer interactions necessary for tissue-specific transcription.

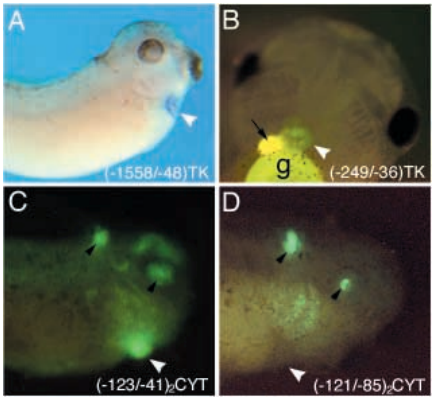

Fig. 4. –123/–41 is sufficient for strong heart-specific expression. Strong, heart-specific expression is obtained with transgenes comprising the XMLC2 promoter region from –1558 to –48 (A) or –249 to –36 (B) fused to the minimal TK promoter. Two copies of an oligonucleotide comprising the sequence from –123 to –41 direct cardiac-specific expression of GFP from the minimal promoter of the cytoskeletal actin gene (C). By contrast, a similar chimeric promoter containing two copies of the promoter region –121 to –85 is insufficient for cardiac expression, giving only ectopic expression in the branchial arches (D). The tadpole heart region is indicated (white arrowhead). GFP activity in the eyes and hindbrain (C,D; black arrowheads) is from the γ-crystallin co-transgene. Intense endogenous fluorescence adjacent to the heart in B comes from the gall bladder (arrowed) and gut (g).

Proximal GATA sites are required for activity of *XMLC2* promoter

Together, the results from deletion mutants indicate that the proximal region of the promoter, which includes the three GATA motifs, is important for myocardial expression of the *XMLC2* promoter. To investigate the role of GATA motifs further, we mutated them singly and in combination and tested the effect on the normally robust expression driven by the 5′-681 promoter (PM-GATA series, Table 1). Mutation of any single GATA site (Fig. 5A and data not shown; Table 1) had little effect on transgene expression, demonstrating that none of the GATA sites is indispensable. Simultaneous inactivation of GATA#2 and GATA#3 reduced, but did not abolish, cardiac-specific expression of the reporter (Fig. 5B) but mutation of GATA#1 together with GATA#2 abolished activity of the promoter (Fig. 5C).

The simplest interpretation of these results is that promoter activity requires the presence of at least one of the two proximal sites (GATA#1 and GATA#2) along with at least one other. Limited functional redundancy accounts for similar activity from transgenes containing GATA#1 with GATA#3 and GATA#2 with GATA#3 (Table 1). Consistent with this, each of the GATA motifs is capable of binding GATA4 protein in vitro as judged by EMSA assay, GATA#1 and GATA#2 having much higher affinity than the more distal GATA#3 (Fig. 5D,E).

Inspection of the *XMLC2* promoter sequence reveals that there are five additional GATA-like sites within the proximal 681 nucleotides of promoter, each comprising only the four nucleotide core of the binding site consensus (A/T GATA (A/G). Two of these lie between the GATA#2 and GATA#3 and

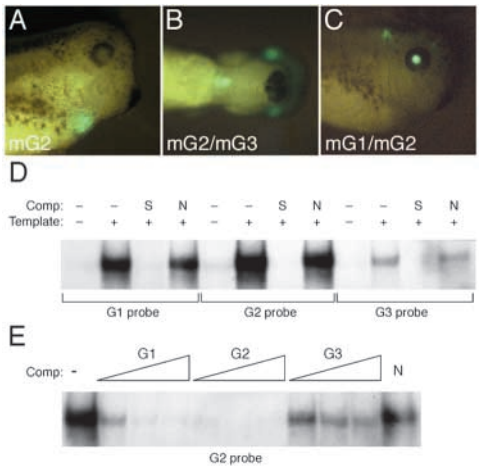

Fig. 5. Proximal, high-affinity GATA sites are critical for expression of the XMLC2 promoter. Heart-specific expression is unaffected by targeted mutation of individual GATA sites, such as GATA#2 (A). When both GATA#2 and GATA#3 sites are inactivated, expression in the heart is maintained, but it is weak (B; ventral view). Mutation of both GATA#1 and GATA#2 abolishes activity of the promoter (C). Note that transgenesis in this case is confirmed by expression of the co-transgene gamma-crystallin in the eye and hindbrain.
(D) GATA#1 and GATA#2 have greater affinity for GATA4 than the more distal GATA#3. S, specific competitor (unlabelled probe); N, non-specific competitor (both used at 100× molar excess).
(E) Competition assay, using 20, 50 and 100× molar excess of competitors confirms this result.

it is conceivable that their presence in the GATA#2/GATA#3 double mutant supports the limited expression of this transgene.

XMLC2 promoter –123/–41 region is sufficient for heart expression

Having established that regions encompassing a combination of GATA and CArG-like/YY1binding sites are necessary for heart-specific expression of transgenes, we next tested whether these sequences were sufficient to confer this expression on a transgene containing a heterologous basal promoter. Two copies of the sequence from –123 to –41 were cloned in front of a transgene comprising the minimal promoter from the *Xenopus* cytoskeletal actin gene driving GFP. This resulted in strong, heart-specific expression in transgenic embryos, demonstrating that the sequence –123/–41 is both necessary and sufficient for heart expression (Fig. 4C). Similar tests using a shorter sequence (–121/–81) resulted in expression of the transgene only in the branchial arches (Fig. 4D). This result suggests that the minimal heart enhancer sequence requires both GATA binding sites. Consistent with this interpretation, a transgene containing the promoter region –85/–42 was inactive in the tadpole heart (Table 1).

CArG-like and YY1 sites are important for strong heart-restricted *XMLC2* promoter activity

We next examined the contribution of the CArG-like/YY1 site lying within the minimal heart enhancer. As YY1 has relaxed sequence requirements for binding (Yant et al., 1995), we first used a gel shift assay to establish whether the motif in the proximal *XMLC2* promoter could indeed bind *Xenopus* YY1. Using a tadpole nuclear or whole cell extract with the *XMLC2* probe, we obtained a specific complex that was competed by the presence of unlabelled YY1-binding site sequence and blocked by anti-YY1 antibody (Fig. 6A). By contrast, only very low levels of complex were formed between the overlapping CArG-like sequence and recombinant serum response factor (Fig. 7A), a result that is unsurprising given the single base mismatch between the *XMLC2* motif and the consensus binding site ($CC(A/T)_6GG$) identified for this protein (Pollock and Treisman, 1990).

To assess the possible role of SRF or YY1 binding in regulating expression of the *XMLC2* gene, we tested the effect of point mutations within the CArG-like/YY1 sequence on transgene expression. Mutations that inactivated SRF binding without affecting the overlapping YY1 site blocked all detectable expression of the transgene, indicating the importance of the CArG-like motif despite its low affinity for SRF (Table 1). Mutations blocking YY1 binding gave more

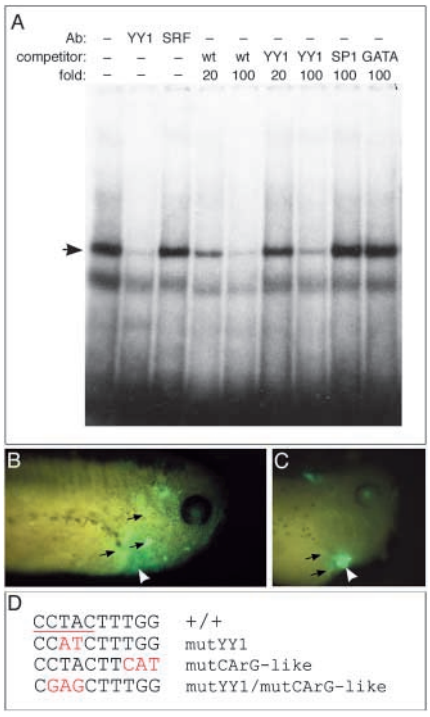

Fig. 6. The overlapping CArG-like and YY1 sites are required for strong and restricted expression of the XMLC2 promoter in the heart. (A) A binding activity present in tadpole extracts binds the combined CArG-like/YY1 site in proximal XMLC2 promoter (–122/–85). This activity (arrow) was identified as YY1 as it is blocked by a specific antiserum to YY1, but not by anti-SRF antibodies. The same complex is specifically competed by an unlabelled YY1 site, but not by SP1 or GATA sites. (Ab, antibody; Fold, fold molar excess of competitor; wt, unlabelled probe). (B,C) Mutation of theYY1 site alone (B) or in combination with the CArG-like/YY1 site (C) results in variable (though generally weaker) and less uniform expression of GFP in the heart (arrowhead), as well as in ectopic expression (arrows). Comparison of the two panels indicates the variability in expression obtained with these mutations. (D) The sequence of CArG-like/YY1 site and mutated versions tested by transgenesis (see Table 1). The YY1 motif in the wild-type sequence is underlined; mutated residues are shown in red.

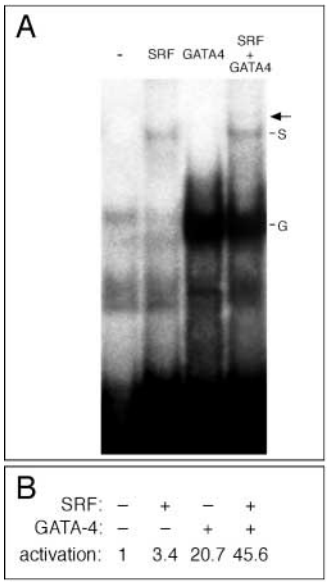

Fig. 7. SRF and GATA4 can simultaneously bind XMLC2 promoter in vitro, and can synergistically activate an XMLC2-luciferase reporter in animal pole explants. (A) The CArG-like site in –122/–85 promoter fragment binds SRF only weakly (complex labelled S); GATA4 protein binds efficiently to the GATA#2 site present in the same probe (labelled G); simultaneous binding of SRF and GATA4 can be detected (arrow). (B) Overexpression of SRF activates an XMLC2(–123/–41) cyt.actin-luciferase reporter only weakly in animal pole explants, while ectopic GATA4 gives strong activation. Co-expression of both factors leads to synergistic activation. Animal poles were excised at stage 9 and collected after three hours of incubation at 23°C. Firefly luciferase activity was normalised using coinjected tk-Renilla luciferase as a control and was set at 1 for XMLC2(–123/–41) cyt.actin-luciferase in the absence of SRF or GATA4. Values shown represent a single experiment; a second experiment gave similar results.

complex results, transforming the consistently strong, heart-specific expression characteristic of the 5′-681 promoter into much more variable expression that was accompanied by ectopic transgene activity in the branchial arches and pronephros (Fig. 6B, Table 1 and data not shown). Finally, simultaneous mutation of both YY1 and CArG-like motifs also resulted in similar ectopic expression (Fig. 6C).

From these results we conclude that both the YY1 and CArG-like motifs are necessary for heart-specific expression from the *XMLC2* promoter. The CArG-like sequence is important for any activity from the promoter while the YY1 site is necessary for suppression of ectopic expression. In the absence of a functional YY1-binding site, transgene expression encompasses not only the heart, but also other regions (such as branchial arches and pronephros) perhaps as a result of the more widespread expression of GATA factors. Our studies also suggest that in the absence of a functional YY1 site, levels of transgene expression within the heart are significantly reduced, indicating that YY1 binding may also have a second role as a positive regulator of heart-specific transcription. This would be consistent with other studies that have identified multiple roles for the YY1 protein (see Discussion).

GATA4 and SRF activate the *XMLC2* promoter synergistically

Using a probe comprising the CArG-like motif and the adjacent GATA#2 site (–122/–85), we found that SRF and GATA4 can simultaneously bind in vitro to this region of the *XMLC2* promoter (Fig. 7A). Individually, the importance of their binding is clear from our studies of transgene expression. We next examined whether their combined effect as transcriptional activators was additive, or whether simultaneous binding resulted in cooperative stimulation of transcription. Using an animal cap explant assay, we tested the capacity of SRF and GATA4 to transactivate a luciferase reporter driven by the minimal heart enhancer region (–123/–41) fused to a heterologous basal promoter. Overexpression of SRF gave only modest activation of the reporter, while ectopic GATA4 expression was much more effective (Fig. 7B). Simultaneous over-expression of both factors clearly resulted in synergistic activation, suggesting that functional interaction between a GATA factor (perhaps GATA4) and SRF could play an important role in *XMLC2* regulation. If such interactions occur in vivo, their spatial requirements must be flexible because our mapping studies demonstrate functional redundancy between the GATA#2 and GATA#1 sites in the promoter (Fig. 5).

XMLC2 promoter directs pan-myocardial expression in transgenic mice

Because the apparent counterpart of *XMLC2* in mammals is expressed only in atrial myocardium, the regulatory elements that direct pan-myocardial expression of *XMLC2* in the developing tadpole could represent a specific adaptation of the cardiogenic program in amphibians. Alternatively, they might constitute a regulatory mechanism conserved during vertebrate evolution and perhaps modified in mammals to provide more restricted domains of expression within the heart. To investigate this further, we tested the expression of an *XMLC2*-lacZ transgene in transgenic mice.

Using the entire 3 kb of *XMLC2* promoter sequence, three founder lines were obtained and in each case, staining for *lacZ* was first detected within the cardiac crescent (E7.5-8.0). In subsequent development, intense staining was detected throughout the linear and looped heart tube (Fig. 8A-G,J) and later in all four chambers of the embryonic heart (Fig. 8H,K). Such pan-myocardial expression was maintained in the chamber walls of the neonatal and adult mouse heart (Fig. 8I,L) but absent from the coronary arteries, valves and aorta (Fig. 8L,N,O). These results demonstrate that the regulatory mechanisms driving precise, pan-myocardial expression of *XMLC2* in the tadpole are retained in the mouse.

Discussion

In this report we have established that the heart-specific expression of *XMLC2* is achieved by a relatively short and compact promoter element spanning nucleotides –123 to –41. Within this heart element (HE), we have identified two GATA sites and a composite YY1/CArG-like site that are essential for promoter activity in transgenic assays.

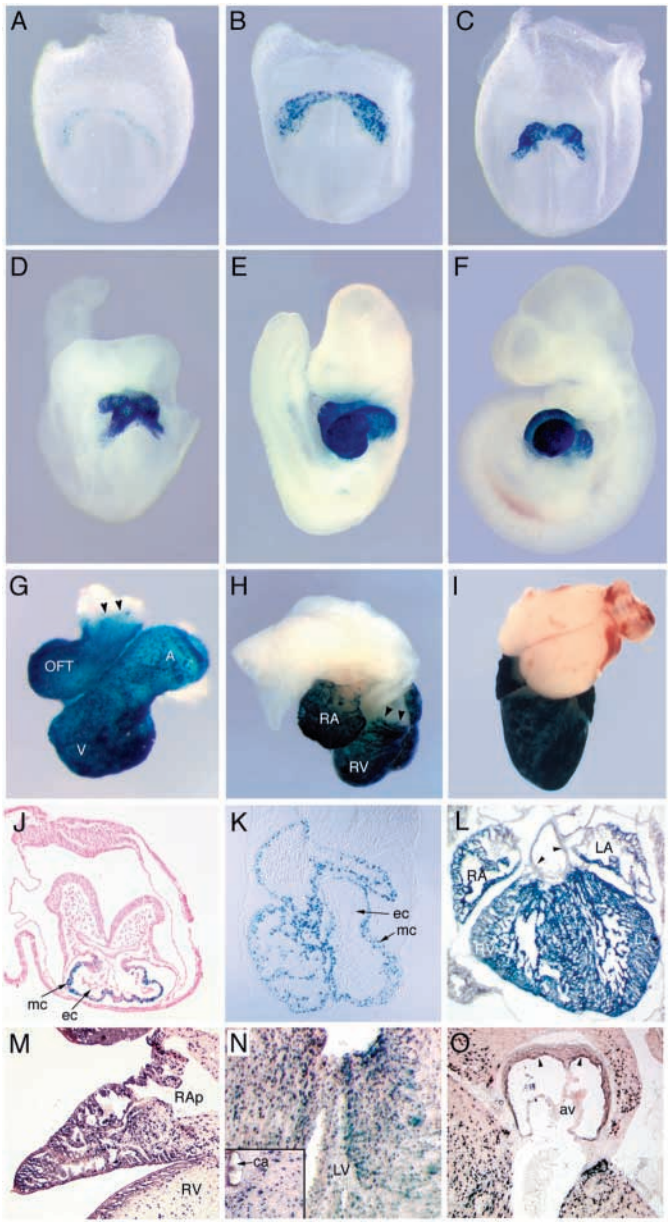

Fig. 8. Pan-myocardial expression of XMLC2-*lacZ* in transgenic mice. (A,B) E7.5-8.0 (early and late respectively) showing nuclear localised staining in the cardiac crescent; (C,D) E8.0-8.5 (early and late respectively) showing staining throughout the linear heart tube; (E) E9.5; (F) E10.5; (G) isolated heart tube from E9.5. The atrial (A), ventricular (V) and outflow tract (OFT) regions are indicated; (H) oblique frontal view of isolated heart from E10.5. The right atrium (RA) and right ventricle (RV) are marked. Note the abrupt boundary in staining in the outflow tract (arrowheads); (I) neonatal heart with attached thymus. (J) Transverse wax section (6 μm) through the newly formed linear heart tube (~E8.0); (K) Cryostat section (10 μm) through the E10.5 heart. In each case, expression is confined to nuclei of the myocardium (mc) and is entirely absent from the endocardium (ec). (L) Cryostat section through the E16.5 heart (8 μm). High levels of expression are detected throughout the myocardium of all four heart chambers but absent from the walls of the great vessels, such as the aorta (arrowheads). (M) Nuclear staining is detected in the myocardium of the right atrial appendage (RAp) and right ventricle (RV). (N) A noticeably higher density of stained nuclei is evident in the myocardium of the left ventricle (LV) but absent from the coronary arteries (inset; ca). (O) Staining is also absent from tissue of the aortic valve (av) and the wall of the aorta (arrowheads).

Combinatorial regulation of the XMLC2 promoter in embryos

Although we have identified the binding sites within the HE that are required for its activity, we do not yet have unequivocal identification of the factors that interact with this region of the promoter in vivo. The GATA sites within HE are likely to be targets for GATA factors 4/5/6, and we have shown that in vitro these factors can bind the *XMLC2* promoter (Fig. 5 and data not shown). Similarly, we detected YY1 in embryonic extracts that is capable of binding to the putative YY1 site within the HE (Fig. 6). Finally, the overlapping CArG-like site bound SRF with the low affinity that might be predicted from its variant sequence (Fig. 7) (Pollock and Treisman, 1990). Assuming that the factors binding the HE in vivo are those suggested from our in vitro experiments, the most important implication of our results is that cardiac restricted activity of the HE results from combinatorial interactions of factors which are themselves not tissue specific.

GATA4/5/6 factors are expressed in many endodermal cell types as well as in the heart. They are transcriptional activators whose activity is regulated at multiple levels, including post-translational modifications and interactions with other proteins (Molkentin, 2000). As GATA factors are not restricted to a particular tissue, the regulation of their activity, in particular through numerous binding partners, has been proposed to provide specificity to their action. In the context of cardiac-specific transcription, GATA4 has been shown to interact with Nkx2.5, MEF2 and SRF. This latter interaction may play a role in regulation of *XMLC2* as well. We observed synergism between SRF and GATA4, acting through GATA#2 site (Fig. 7). The only area of overlap of expression of GATA4 and SRF is in the heart, and the observed interaction between these two factors may provide a basis for cardiac-specific expression of *XMLC2*.

The role of YY1 in regulation of XMLC2

The HE contains a binding site for YY1 factor, which overlaps with the CArG-like site. Inactivation of the YY1 site leads to broadening and weakening of *XMLC2* promoter activity. YY1 is known to act as both a repressor and an activator in different contexts and our results can be interpreted in light of these activities. Repressor activity of YY1 might be involved in preventing expression in tissues other than cardiac muscle. More surprising was our finding that mutation of the YY1 site frequently led to weaker expression in the heart, indicating that YY1 might also be positively regulating expression of *XMLC2*. We note that YY1 has previously been shown to modulate the activity of Fos SRE by promoting loading of SRF (Natesan and Gilman, 1995) and it is conceivable that such a mechanism may also be operating at the YY1/CArG-like site within *XMLC2* promoter. The affinity of CArG-like site for SRF is inherently low (Fig. 7), and it will be interesting to establish whether the affinity is altered in the presence of YY1. Ternary complex formation between GATA4 and SRF may additionally stabilise

the association of SRF with *XMLC2* promoter (Figs 6 and 7) (Belaguli et al., 2000).

It is interesting to speculate that a dependence on YY1 for efficient binding of SRF at the low affinity CArG-like site, could result in the HE providing a more versatile regulatory element than could be obtained simply with a high affinity CArG site. In addition to promoting positive regulation by enhancing SRF loading onto the *XMLC2* promoter in myocardial cells, the presence of YY1 repressor at the promoter may also effectively suppress ectopic activity in the somites, which contain high levels of SRF (Latinkić et al., 2002). The potential for ectopic expression in the somites is clearly revealed by mutations that simultaneously abolish YY1 binding and transform the CArG-like motif into a high affinity SRF-binding site (Table 1; Fig. 6). Thus, both the positive and negative activities of YY1 might be mediated via their effects on SRF.

Besides acting as a transcription factor, YY1 has chromatin remodelling activity and is known to cause DNA bending in vitro (Natesan and Gilman, 1993). We have no direct evidence for involvement of chromatin remodelling in regulation of *XMLC2* promoter at the present. However, we note that several of our observations point to the potential involvement of chromatin architecture in regulation of *XMLC2*. The apparent structural role of the element between the GATA#1 and CArG-like/YY1 sites (–89/–49 region) strongly suggest that activity of the *XMLC2* promoter depends on its spatial organisation. The function of YY1 might be affected by deleting the region –89/–49. We have observed similar effects of mutating the YY1 site and of reducing the spacing between the basal promoter and CArG/YY1 site (Table 1, Δ –89/–49). Both mutations lead to weakening and broadening of the *XMLC2* promoter activity in the head region of transgenic embryos. This interpretation is supported by previous studies that have shown spacing-and orientation-dependent activity of YY1 (Natesan and Gilman, 1993). Finally, we have observed that the *XMLC2* promoter can compensate for a loss of any single GATA site, presumably by relying on the remaining GATA sites (Fig. 5). Such compensation requires new interactions between active elements and the basal promoter and presumably depends on changes in chromatin conformation.

Our finding that YY1 apparently participates in regulating heart-specific expression of *XMLC2* transgenes provides at least some explanation for the absence of *XMLC2* transcription in axial muscle of the embryo. Cell culture studies with the chick cardiac *MLC2* gene have also provided evidence for other inhibitory factors that may block transcription in skeletal muscle cells (Dhar et al., 1997) and it remains to be seen whether such factors also regulate *XMLC2* expression in the developing embryo.

In the present study, we could not reduce the sequence requirements for heart expression beyond the HE, as transgenes containing only its subregions, –122/–85 and –80/–45 are not expressed in the heart. This strongly suggests that the sites present in the two halves of HE interact to create a new composite function. According to our results, the –80/–45 region provides two elements: a single functional enhancer element, GATA#1, and –89/–49, whose role is structural. One possibility is that the GATA#1 site, which has a similar affinity for GATA4 factor as GATA#2 site (Fig. 5), is nevertheless unique and cannot be functionally substituted by the GATA#2 site. We believe it more likely that the intervening sequence –89/–49 is required to maintain optimal spatial organisation of the promoter-enhancer interaction. It will be of interest to determine the molecular basis for these observations in the future.

The role of the CArG-like site

One striking result of our study is the absolute requirement of the proximal *XMLC2* promoter for the low-affinity CArG-like box within the HE. Mutation of this element created an inactive promoter even in the presence of 671 bp of proximal promoter sequence (Table 1). Although expression from the *XMLC2* promoter is likely to depend on cooperative interactions between multiple factors, it is nevertheless surprising that elimination of one binding site has such a dramatic effect. The powerful transcriptional activator myocardin acts via direct interactions with SRF (Wang et al., 2001) and one possibility is that elimination of the CArG-like site prevents myocardin from activating transcription of the reporter gene. However, it has also been suggested that myocardin may require multiple SRF binding sites for activity (Wang et al., 2001) and if so, its involvement in *XMLC2* expression may be questioned. An alternative explanation might be that in CArG-like mutants, the repressor activity of YY1 predominates. Consistent with this, simultaneous mutation of both CArG-like and overlapping YY1 motifs uncovers residual promoter activity that includes expression in the heart (Fig. 6).

MEF2 factors and XMLC2 transcription

Our studies of *XMLC2* gene transcription were initially prompted by the observation that ectopic expression of the MEF2 factor, MEF2D, in *Xenopus* animal cap explants resulted in precocious activation of the endogenous *XMLC2* gene (Chambers et al., 1994). As MEF2D is expressed in the presumptive heart region, these results suggested that MEF2D might play an important role in regulating transcription of *XMLC2*. Members of the MEF2 family are also highly expressed in axial, somitic muscle of the embryo and these findings therefore left the absence of *XMLC2* expression in the myotomes unexplained. Several potential MEF2 binding sites are present in the 3 kb of *XMLC2* promoter (Fig. 2) and each of these can bind MEF2 factors in vitro (B.C. and T.M., unpublished). However, in our current study, we have found that the MEF2 binding sites are dispensable for promoter activity (Fig. 3). This could indicate that the earlier results were an artefact resulting from inducing high levels of a transcriptional activator capable of binding the *XMLC2* promoter. Alternatively, the current results could simply reflect the nature of our transgenic assay, which only provides unequivocal evidence for binding sites that are indispensable for transgene activity. Furthermore, MEF2 factors may still play a role in regulation of *XMLC2* promoter constructs in which MEF2-binding sites have been eliminated, as MEF2 proteins are capable of forming a complex with DNA-bound GATA4, without binding the DNA itself (Morin et al., 2000).

Pan-myocardial expression of XMLC2

Despite repeated efforts, only a single *MLC2* gene has been isolated in *Xenopus* and cDNA screening suggests that a single gene is also present in the urodele amphibian, *Ambystoma mexicanum* (T.M., unpublished results). In both cases, the gene

is expressed throughout the entire myocardium, even after differentiation of distinct atrial and ventricular chambers. By contrast, the mammalian *MLC2*a and *MLC2*v genes are progressively restricted in their domains of expression during cardiogenesis, yielding reciprocal patterns of expression in the atria and ventricles, respectively. The precise evolutionary relationship between the amphibian and mammalian genes is unclear although within their coding regions, the amphibian genes most closely resemble the *MLC2*a sequence. There is only limited similarity between the mouse *MLC2*a and *Xenopus MLC2* genes in the proximal promoter region, most notably common GATA and CArG sites in the proximal region (data not shown).

Our transgenic studies demonstrate that whatever the precise mechanisms driving pan-myocardial expression of *XMLC2* in the tadpole, the same regulatory controls are present in the mouse embryo even though the endogenous *MLC2*a and *MLC2*v genes are not themselves expressed in this manner. On the basis of this, it is tempting to speculate that the pan-myocardial program retained in modern amphibians represents an ancient regulatory program. The regional patterns of expression shown by the mammalian *MLC2* genes could indicate that the pan-myocardial program has been lost; however, the expression of the *XMLC2* transgene in mouse embryos suggests otherwise. The alternative is that the pan-myocardial program has been transformed in mammals by additional regulatory controls that restrict expression of individual *MLC2* genes to particular regions of the myocardium. If this hypothesis is correct, it should be possible to identify elements within, for example, the murine *MLC2*a promoter, which might impose atrial-specific expression on the *XMLC2* promoter. A similar type of analysis has, for example, recently demonstrated that the *ANF* promoter can dominantly impose transcriptional repression of transcription on the cardiac Troponin I promoter in the atrio-ventricular canal of the embryonic mouse heart (Habets et al., 2002).

Conclusions

Our studies show that myocardial-specific transcription from the *Xenopus MLC2* promoter depends upon a remarkably small region of the proximal promoter and have indicated at least some of the likely DNA-binding factors involved. It should now be possible to examine the precise interactions of these regulators and the participation of any co-factors in establishing cardiac muscle-specific transcription during development of the tadpole heart. Our finding that pan-myocardial expression of *XMLC2* transgenes is conserved between frogs and mice indicates that such regulatory mechanisms are likely to be conserved among vertebrates.

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