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# The RSRF/MEF2 protein SL1 regulates cardiac muscle-specific transcription of a myosin light-chain gene in *Xenopus* embryos

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We have examined the role of two RSRF/MEF2 proteins in the onset of skeletal and cardiac muscle differentiation in early *Xenopus* embryos. In normal development, zygotic expression of SL1 (MEF2D) precedes that of SL2 (MEF2A) by several hours, but neither gene is expressed prior to the accumulation of MyoD and Myf5 transcripts in the somitic mesoderm. Ectopic expression of the myogenic factors in explants of presumptive ectoderm induces expression of both SL1 and SL2, whereas in reciprocal experiments, neither RSRF protein activates the endogenous *myoD* or *Myf5* genes. We conclude that SL1 and SL2 lie downstream of these myogenic factors in the skeletal myogenic pathway. SL1 is distinguished from SL2 in being expressed in the presumptive heart region of the early tailbud embryo, prior to detection of any markers for cardiac muscle differentiation. Furthermore, ectopic SL1 induces the expression of an endogenous cardiac muscle-specific myosin light-chain (*XMLC2*) gene in cultured blastula animal pole explants, whereas SL2 has no comparable effect. These results demonstrate that in addition to a possible role in skeletal myogenesis, SL1 also acts *in vivo* as a regulator of cardiac muscle-specific transcription.

[Key Words: RSRF/MEF-2; myosin light chain; cardiac-specific transcription; *Xenopus*]

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The *RSRF* (or *MEF2*) genes encode a family of sequence-specific DNA-binding proteins that are thought to play an important role in the differentiation of several cell types, including muscle and neuronal tissue. Four different *RSRF* genes have been identified so far, and their transcripts have been detected in a wide variety of vertebrate tissues and cultured cell lines (Pollock and Treisman 1991; Chambers et al. 1992; Yu et al. 1992; Breitbart et al. 1993; Leifer et al. 1993; McDermott et al. 1993). Each gene gives rise to multiple transcripts through alternate splicing, and the RSRF protein family potentially contains many distinct polypeptides. These proteins possess an amino-terminal portion that is virtually invariant throughout the family and encompasses the DNA-binding domain. The remainder of their sequence shows little conservation between family members.

RSRF proteins recognize an A/T-rich DNA sequence motif (Pollock and Treisman 1990; Chambers et al. 1992) and can bind to the target sequence either as homodimers or as heterodimers with other members of the

RSRF family. A similar sequence specificity has been found for a number of previously characterized DNA-binding activities, and it is now clear that these contain RSRF proteins. One such binding activity, termed MEF2, is prevalent in muscle cells (Gossett et al. 1989). MEF2 activity increases during normal differentiation of skeletal myoblasts and is rapidly induced in fibroblasts in response to myogenic conversion by members of the MyoD family (Cserjesi and Olson 1991; Lassar et al. 1991). Sequences matching the RSRF/MEF2-binding site consensus have been found in the regulatory regions of many muscle-specific genes, and for several of these, the functional importance of the MEF2 sites has been established (Braun et al. 1989; Gossett et al. 1989; Horlick and Benfield 1989; Cserjesi and Olson 1991; Wentworth et al. 1991). MEF2 is therefore thought to be a transcriptional regulator that acts in concert with members of the MyoD family to activate skeletal myogenesis. Among its targets are the *myogenin* and *myoD* genes themselves (Edmondson et al. 1992b; Cheng et al. 1993; Yee and Rigby 1993; Leibham et al. 1994), indicating that products of the RSRF gene family play a role in the early steps of muscle differentiation.

Many other cell types also contain DNA-binding ac-

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tivities that cross-react with RSRF-specific antibodies, but little is known about their function. Several studies have described a ubiquitous MEF2-like activity (Hobson et al. 1988; Horlick et al. 1990; Pollock and Treisman 1991; Chambers et al. 1992), consistent with the detection of RSRF transcripts in all cell types so far examined. Putative RSRF-binding sites have been identified in the control regions of several growth factor-induced genes (Pollock and Treisman 1991), raising the possibility that RSRF proteins may play a role in regulating cell proliferation. Among vertebrate tissues, RSRF transcripts are relatively prevalent in cardiac as well as skeletal muscle (Chambers et al. 1992; Navankasattusas et al. 1992; Molkentin and Markham 1993), and MEF2-like motifs are essential for cardiocyte-restricted expression of several genes (Iannello et al. 1991; Nakatsuji et al. 1992; Navankasattusas et al. 1992; Parmacek et al. 1992; Molkentin and Markham 1993). Elevated levels of RSRF transcripts are also found in neural tissue, and expression of one RSRF gene, *MEF2C*, is largely restricted to cortical neurons within the mammalian brain (Leifer et al. 1993; Martin et al. 1993). MEF2-like activities may therefore be important in the differentiation of cardiac muscle and neural cell types.

Little is currently known about the precise function of individual *RSRF* gene products or the role of variants that result from alternative splicing. In vitro, subtle differences have been detected in the binding-site preference of some RSRF proteins (Pollock and Treisman 1991), but no comparable cell type-specific variation in natural RSRF/MEF2-binding sites has been detected so far. In transfected fibroblasts, constitutive expression of *RSRF* genes results in *trans*-activation of reporter genes containing synthetic RSRF/MEF2-binding sites (Yu et al. 1992; Breitbart et al. 1993; McDermott et al. 1993; Leibham et al. 1994), but each member of the family behaves similarly in such an assay. In cultured skeletal myoblasts, members of the *RSRF* gene family are transcribed in a distinct temporal sequence during differentiation (Breitbart et al. 1993), suggesting that at least in these cells, individual RSRF proteins perform distinct functions.

In this study we have examined the relative roles of *SL1* and *SL2*, two *RSRF* genes that are expressed in early embryos of the amphibian *Xenopus laevis* (Chambers et al. 1992). We have found previously that expression of both genes is initially restricted to the newly forming somites of the embryo and is subsequently localized to the myotomal muscle of the tadpole. Zygotic expression of *SL1* (the homolog of mammalian MEF2D) commences in the early gastrula, considerably before any muscle differentiation markers can be detected, whereas *SL2* (*MEF2A*) is activated after the onset of myotomal (skeletal) muscle differentiation.

By use of microinjection of synthetic RNAs into developing embryos, we have tested the relative abilities of ectopic RSRF and MyoD proteins to activate skeletal and cardiac differentiation markers in embryo explants. Our results demonstrate that both RSRF proteins lie below XMyoD and XMyf5 in the skeletal muscle regulatory

hierarchy. Neither RSRF protein induces the expression of skeletal muscle markers in cultured explants, but *SL1* can be distinguished from *SL2* by its ability to induce the expression of a myosin light-chain (*MLC2*) gene. In normal development, transcripts of the *MLC2* gene are localized exclusively to the developing heart of the tadpole and are present in both atrial and ventricular muscle. We show that *SL1* transcripts accumulate in the presumptive heart region of the tailbud embryo, prior to the onset of cardiac muscle differentiation. These results demonstrate that *SL1* is a transcriptional regulator of cardiac muscle differentiation and provide the first direct evidence that individual members of the RSRF/MEF2 family have distinct regulatory functions in vivo.

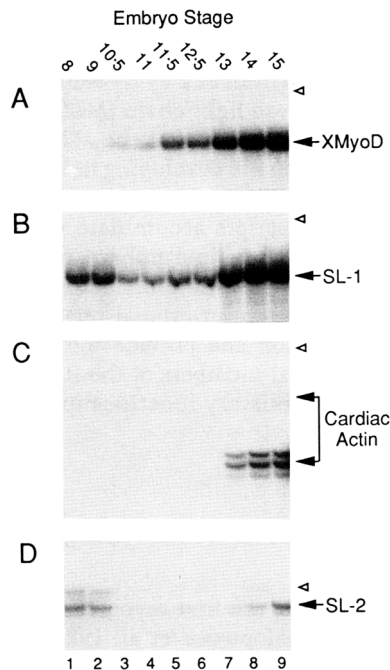
## Results

### *XMyoD* is expressed prior to the *RSRF* genes during gastrulation

In *Xenopus* embryos, the first myogenic factor to be expressed is XMyoD (Hopwood et al. 1989, 1991; Harvey 1990, 1991). Transcripts accumulate in the newly formed mesoderm of the early gastrula embryo, and XMyoD protein can be detected in the nuclei of the dorsal, presomitic mesoderm (Hopwood et al. 1992). In situ hybridization studies have shown that zygotic expression of the *Xenopus RSRF/MEF2* gene *SL1* also commences during gastrulation, and in later stages its transcripts are also localized in the somitic mesoderm (Chambers et al. 1992). The *XmyoDa* gene contains a MEF2-binding site coincident with the TATA motif and constitutive expression of *RSRF* genes activates a minimal *XmyoDa* promoter in COS-1 cells (Leibham et al. 1994). These results raise the possibility that RSRF proteins may be responsible for *XmyoD* expression in the presomitic mesoderm of early gastrulae.

To investigate this possibility, we first determined the relative order of *XmyoD* and *RSRF* gene expression in synchronous embryos from blastula to neurula stages, by use of an RNase protection assay (Fig. 1). Cardiac actin transcription was monitored in the same samples, because in vertebrate embryos this gene is expressed in both cardiac and skeletal muscle. In amphibians, it provides a sensitive marker for the onset of terminal differentiation of myotomal (skeletal) muscle that forms the tadpole body musculature (Mohun et al. 1984).

*XmyoD* transcripts were first detected in early gastrulae (stage 10.5), several hours before those of cardiac actin (Fig. 1, cf. A and C). In contrast, transcripts of the *SL1* gene are present at all stages of early development (Fig. 1B). In the blastula embryo (stage 8), these derive from a maternal store, present in both oocytes and the unfertilized egg (Chambers et al. 1992). During subsequent development, the level of maternal transcripts declines, and an increase is first detected in mid-gastrulation (stage 11.5), indicating that zygotic transcription of *SL1* commences before that of differentiation markers such as the cardiac actin gene but after the accumulation of *XmyoD* transcripts in the presomitic mesoderm. It also



**Figure 1.** Sequential expression of *XmyoD* and the *RSRF* genes during early development. RNAs from successive stages of development from blastula (stage 8) to mid-neurula (stage 15) were analyzed by RNase protection assay for the presence of *XmyoD* (A), *SL1* (B), cardiac actin (C), and *SL2* (D) transcripts. Total RNA (10  $\mu$ g) was used for each assay. For each probe, the positions of protected fragments (solid arrows) and undigested probe (open triangles) are indicated.

follows the first detection of XMyoD protein in mesodermal cell nuclei (Hopwood et al. 1992).

*SL2* transcripts are also inherited by the fertilized egg, but their level falls sharply between blastula and gastrula stages. However, unlike *SL1*, accumulation of *SL2* mRNA does not begin until neurulation (stage 14), several hours after terminal differentiation of somitic muscle has commenced (Fig. 1, cf. C and D). This suggests that the two *RSRF* genes have distinct functions in the differentiation of axial (skeletal) muscle.

#### Activation of the *RSRF* genes by the myogenic factors

Ectopic expression of genes in *Xenopus* embryos provides a powerful assay to investigate gene function, and we have used this approach to test directly the regulatory interactions between the *RSRF* and *myoD* gene families. Fertilized eggs were injected with synthetic RNAs and animal pole explants subsequently isolated from blastula (stage 8) embryos. During normal development, animal pole tissue of the blastula contributes to ectodermal and neural tissue (Keller 1975), and in explant culture it forms balls of epidermal tissue. Explants from uninjected and injected embryos were cultured until neurula stage and the expression of a variety of muscle-specific genes monitored by RNase protection assay.

Synthetic RNAs encoding the myogenic factors

MyoD, Myf5, and myogenin were injected with an equal amount of *Xenopus E12* RNA to potentiate their phenotypic effects (Rashbass et al. 1992). The *RSRF* RNAs were injected individually, either alone or together with *XmyoD* and *XE12*. They were also tested as an equimolar mixture.

All three myogenic factors were equally effective in activating the cardiac actin gene (Fig. 2A, lanes 6–8), which is a direct target for *trans*-activation via an E-box site within its promoter (Taylor et al. 1991). Their injection also resulted in the activation of endogenous *SL1* and *SL2* expression (Fig. 2B,C, lanes 6–10). Coinjection of *SL1* or *SL2* with *XmyoD/XE12* had no detectable effect on the levels of cardiac actin transcripts (Fig. 2A, lanes 9, 10).

In reciprocal experiments, ectopic expression of *SL1* or *SL2* in animal pole explants had no comparable effect. Neither caused the activation of endogenous muscle-specific markers, such as the cardiac actin (Fig. 2A, lanes 2–5) *Xmyf5* or *XmyoD* genes (data not shown), nor did they activate the expression of the endogenous *RSRF/MEF2* genes (Fig. 2B,C). Both injected RNAs could be detected at the end of the culture period (Fig. 2B, lanes 2, 5; Fig. 2C, lanes 4, 5), and their stability was comparable with that of the synthetic myogenic factor RNA (data not shown).

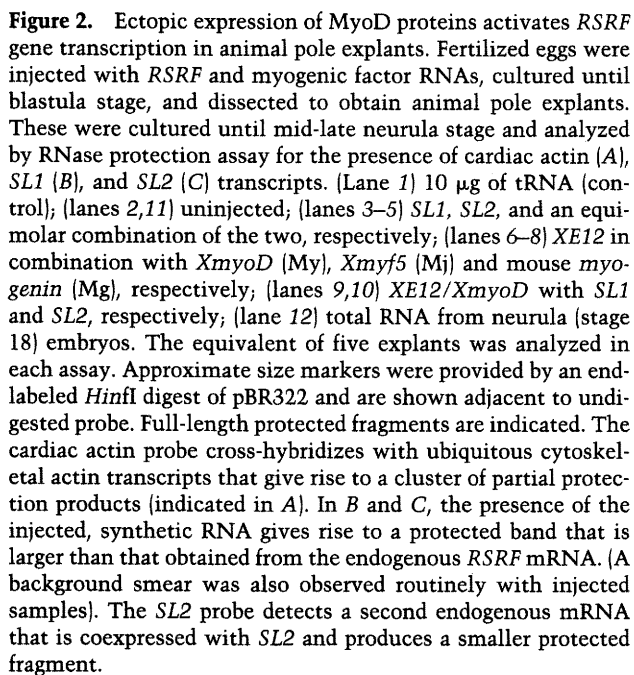
Taken together with the order of expression during normal development, these results demonstrate that the two *Xenopus RSRF/MEF2* genes lie downstream of the myogenic factors in the regulatory hierarchy controlling myotomal muscle differentiation. We found no evidence from our injection experiments for a role of these factors in activating expression of the myogenic factors, nor did they activate skeletal myogenesis in presumptive ectodermal tissue, whether injected alone or in combination with *XmyoD* and *XE12*.

#### *SL1* is expressed in the prospective heart region of the *Xenopus* embryo

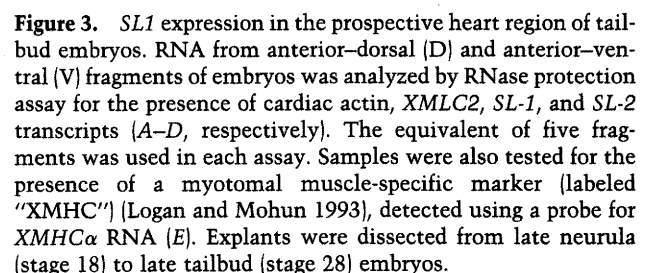
Among adult frog tissues, expression of *SL1* and *SL2* is relatively high in skeletal muscle, brain, and heart (Chambers et al. 1992). In the embryo, neither gene is expressed in the developing nervous system until the swimming tadpole stage and cannot therefore be involved in early stages of neural differentiation (A. Chambers and T. Mohun, unpubl.). To test whether they may play a role in cardiac myogenesis, we took advantage of the relatively late onset of cardiogenesis in amphibians compared with mammals or birds. In *Xenopus*, the presumptive heart region lies on the ventral side of neurula and tailbud embryos, well separated from the dorsal, axial muscle of the myotomes (Sater and Jacobson 1990). The cardiac mesoderm is specified to form muscle tissue (Sater and Jacobson 1989) even though terminal differentiation markers are not detected until the late tailbud (stage 26) (Logan and Mohun 1993).

We dissected anteroventral fragments containing the cardiac mesoderm from neurula stage onward and exam-





Both *SL1* and *SL2* were expressed in dorsal explants, consistent with the somite and myotome-specific localization of transcripts detected in whole-mount in situ



hybridization studies. *SL1* transcripts show a marked gradient of expression, concentrated in more recently formed, posterior somites (Chambers et al. 1992). This accounts for the progressive decline in their abundance in anterior, dorsal explants during development (Fig. 3C). From the onset of cardiac muscle differentiation (stage 25), *SL1* expression was evident in ventral explants and in longer exposures, transcripts could be detected as early as stage 21. In contrast, *SL2* mRNA was entirely restricted to dorsal explants and accumulated steadily between neurula and tailbud stages (Fig. 3D).

The two *Xenopus* *RSRF* genes therefore showed distinct temporal and spatial patterns of expression in the muscle of early embryos. Although both were expressed during skeletal myogenesis, the *SL1* gene was also activated in the presumptive heart region, prior to the onset of cardiac muscle differentiation.

#### *XMLC2 is a marker for cardiac muscle differentiation*

Because *SL1* was expressed in the region comprising the heart anlagen, we wished to test whether ectopic expression of the *Xenopus* *RSRF* genes had any effect on genes expressed in cardiac myogenesis. A potential target for regulation by *RSRF*/MEF2 factors is the *MLC2* gene. In mammals, this gene contains an A/T-rich motif within its promoter that is essential for transcription in transfected primary cardiocytes (Navankasattusas et al. 1992). To obtain a specific probe for the *Xenopus* *MLC2* gene,

we constructed an adult heart cDNA library and screened it with a probe from the human *MLC2a* cDNA (Hailstones et al. 1992). A composite, full-length cDNA sequence derived from this screen is shown in Figure 4A. This sequence contains a single open reading frame that encodes a regulatory myosin light chain polypeptide. The amino acid sequence shows 60–70% identity with other vertebrate *MLC2* proteins and is most closely related to the human atrial-specific *MLC2* isoform (Fig. 4B).

By use of an RNase protection assay, *XMLC2* expression was first detected in tailbud embryos, and the level of transcripts increased during subsequent development. In whole embryos, *XMLC2* RNA could be detected from stage 26 (Fig. 5, lanes 2–10), but in anteroventral explants containing the prospective heart region, activation of the gene was detected a few hours earlier (Fig. 3B). No *XMLC2* transcripts were detected in dorsal axial tissue (enriched for skeletal muscle tissue) nor in adult skeletal muscle tissue (Fig. 5, lanes 11, 17). In contrast, transcripts were highly abundant in the tadpole and adult heart (Fig. 3B; Fig. 5A, lanes 11, 12, 15, 16). Treatment of blastula animal pole explants with the mesoderm-inducing factor activin A resulted in activation of the *XMLC2* gene in a dose-dependent manner (Fig. 5A, lanes 13, 14), consistent with the induction of cardiac muscle (Logan and Mohun 1993). By use of whole-mount RNA in situ hybridization (Fig. 6) we confirmed that the *XMLC2* gene is expressed exclusively within the developing tadpole

A

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      . . . . . M A S R K A A G K A A A K R A Q R G S S N V F S M F E Q S O I Q
GGACGAAGAAGACACTAGAGAACATGGCCAGCAGAAAAGCTGCAGGAAGGGCGCTGCCAAGCTGCAGCGAGGCTCCTCTAATGTCTCTCCATGTTTGGAGCAGTCACAGATTCAA 120
E F K E A F S C I D Q N R D G I I S K P D L K E T Y M Q L G K M N V N E D E L D
GAATTTAAGGAGGCAATTCAGCTGTATCGACCAAAACCGTATGGGATCATAAGCAAAACCTGACCTCAAGAGAACATATATGCAATTGGGTAAAGATGAACGTGAATGAGGATGAGCTGGAT 240
E M L K E G K G P I N F T V F L S L F G E K L N G T D P E D S I L S A F K I L D
GAGATGCTCAAGAGGGCAAGGACCCATTAACTTCACAGCTCTTCCTGCTGTGTGGCGAGAAGCTTAATGGAAGCGACCCAGAGACTCAATCTCAGTCGATCAAAATCTCTTGAC 360
P N A T G N I N K D E L K L L L M T Q A D K F S D E E V D Q M F A V T P I D V A
CCCAACGCACTGGCAATATCAACAAAGATGAATTAAGCTGCTCTCATGACACAGCAGCAAAATTCAGTGACGAGGAGGTAGACAGATGTTTGTGTGACCCCAATTGACGTGGCA 480
G N I D Y K S L C Y I I T H G D E K E D S
GGGAATATTGACTCAAGTCACTGTGTTATATCATCAGCCATGGAGATGAGAAAGAAGACTCATAAATCCAGCTAGAAATAAATGACATGTTCTGGAAGTCCCCATTCCATCTCTTG 600
ACTTTATTTCTGATTCTGTATATAGGGCTCCGTGGGATGTCGGAGAAAATGTTCCAGAAAATTTCTTGACTCTATAAATGAGACTTAAATGAACAGTTCGGTGTAAAGATGGAACTG 720
GGTGGTGGATAGGCTGTGCAAGCTGAATTTTCTGCTGTGGTGTAGTGTAGCAAGGATGTGGTGTAGGAGCTGGGTGGCTGATGTGTGGCATAGTAGCCAGAGCACTACTTCCT 840
GCTTTTCAGCTCTAAGTCCGAGTTAGTCTGACCTTGAAGGGGCCACATGGGACATAAATTCAGTGAGTTTGAATTTGATCCTCAGAAATCCAGCTCAGATACAAATGCAGCTGT 960
CATGCCCATTTGGCCCCCCCCAAGTCACTGATTGTTAGTGAAGTGAACCAATCAGTGGAAACCAAGAGATCTGAAAGCAGTAGTGTAGTGTCTGGCTATTGTGTGCAGATCCAG 1080
TCGCTCCAGCCTTTGTGCTATGCAATTTTGGCTAACTGACTATATGGAAACATTTTATTTTGCACAGCTATCTATTTGCCAGTTTTTATTTTAACTAGTAATTCCTTTAAAG 1200
ACCTAAAAATGTCACCTGCCGACCTATAACATGGTAATACCACAATTTTCAAGATATAATGAGTGACCTGATTCATATATTAATTAAGTATTATTAACTAGTAAAAAATTAATTAAG 1320

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**Figure 4.** (A) Nucleotide sequence of a *Xenopus* *MLC2* cDNA. The nucleotide sequence is derived from two, overlapping cDNA isolates.  $\lambda$ XMLC2-5 contained the entire sequence shown except exon 5 (nucleotides 393–441). This was present in a partial cDNA,  $\lambda$ XMLC2-12, which lacks the first 260 nucleotides of the sequence. The predicted XMLC2 polypeptide sequence is shown, as is a putative poly(A) addition sequence (bold). (B) Comparison of XMLC2 and other vertebrate MLC2 proteins. The XMLC2 polypeptide sequence is aligned with the human atrial (Hailstones et al. 1992), human ventricular (EMBL accession S22101), chick (Winter et al. 1985), and rat (Henderson et al. 1989) *MLC2* sequences. These are arranged in order of similarity to the frog sequence as determined by use of the UWGCG Pileup program. Only residues that differ from the frog sequence have been shown, and gaps (dashes) have been introduced to permit optimal alignment.

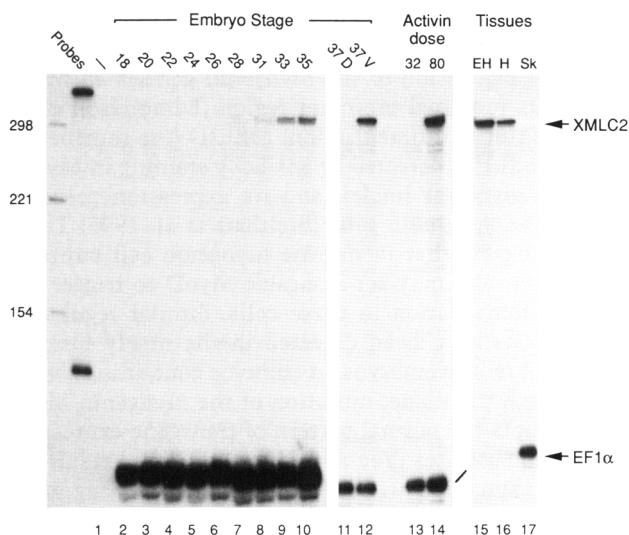
B

XMLC2	MASRKAAGK---AAAKRAQGRSSNVFSEFQSQIQEFKEAFSCIDQNRDGIISKPLKETYMQLGKMNVDDELDEMLKEKGKPIINFVFL
human MLC2a	.....GTRGV..T.Q.....A.....C.A..RE..S...VS.PEE...A...Q.....E
human MLC2v	.....TKKKE--GG.L...A.....N...T.....TIM...F.D.E..KD..AS...T..KD...A...AS...M...
chick MLC2	.....PKKAKRKRIEGAN.....A.....TIM...F.D.A..RD.FAA..RL..KNE.I.E.I..AP.....
rat MLC2	.....SPKKAKRKLEGG..-.....T.....TIM...A.F.D.N..RD.FAA..RV..KNE.I.E.I..AP.....

XMLC2	SLFGEKINGTDPEDSILSAFKILDPNATGINKDELKLLMTQADKFSDEEVDQMFAVTPIDVAGNIDYKSLCYIITHGDEKEDS
human MLC2a	T.....S.....EA..S..RMF..SGK.VV...F.Q..L.....PA..E.....L..M.L.....C.....E
human MLC2v	N.....S...A..ET..N...M...DGK.K...EYL.R...S...MTA.....QFAS.....L..A.S.V...E...E
chick MLC2	TM.....K.A...ET..N...VF...EGK...LKSAYI..EM...EGR..Q..I.....AF..P..S...L..N.VHV...E..D
rat MLC2	TM.R...K.AG...ETL.N...VFA.R.RRITGA.CVQEM.T...ER..K.T...AF..PN.T...L..N.VH...E...D





**Figure 5.** *XMLC2* expression during development. Total RNA from embryos, cultured explants, and tissues was tested for *XMLC2* mRNA by use of an RNase protection assay. (Lane 1) tRNA control; (lanes 2–10) two embryos from neurula to tadpole stages of development (stages 18–35, as indicated); (lanes 11,12) five anterior–dorsal (D) and anterior–ventral (V) regions of swimming tadpoles (stage 37); (lanes 13,14) the equivalent of five blastula animal pole explants treated with 32 or 80 U/ml of activin A and cultured until stage 42; (lanes 15–17) two tadpole (stage 42) heart tubes (EH), 0.5  $\mu$ g of adult heart (H), and 10  $\mu$ g of adult skeletal muscle (Sk). As an internal control, a probe for *EF1 $\alpha$*  mRNA was included in each assay. An end-labeled *HinfI* digest of pBR322 was used for approximate size markers.

heart and was entirely absent from any skeletal muscle tissue at all embryonic stages examined.

#### *XMLC2* expression is activated by *SL1* in embryo explants

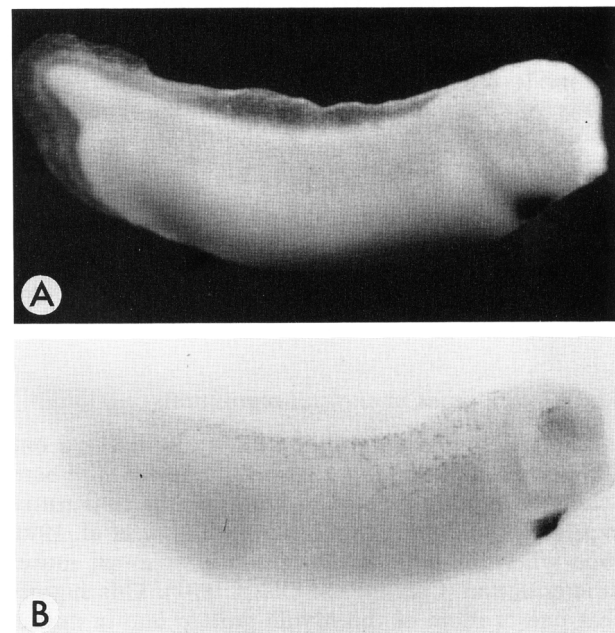
These results demonstrate that *XMLC2* mRNA provides a tissue-specific marker for cardiac muscle in *Xenopus* embryos. We therefore tested whether *XMLC2* expression could be activated by ectopic expression of the *RSRF/MEF2* genes in blastula animal pole explants. High levels of *XMLC2* RNA were found in explants from *SL1*-injected embryos but were undetectable in those from uninjected siblings (Fig. 7, cf. lanes 2 and 3). Interestingly, because the cultured explants were harvested when control embryos reached mid- to late-neurula stage, *SL1*-induced *XMLC2* transcription occurred many hours before the onset of cardiac muscle differentiation in uninjected embryos (Fig. 7, cf. lanes 3 and 5 with lanes 6 and 7). Neither the cardiac actin nor the cardiac muscle-specific *XMHC $\alpha$*  gene was activated in *SL1*-injected explant tissue (Fig. 2, data not shown), indicating that *XMLC2* expression was unlikely to be the result of cardiomyogenic conversion in this tissue. Rather, the *XMLC2* gene is probably a direct target for *trans*-activation by the RSRF protein.

*SL2* RNA consistently failed to elicit comparable

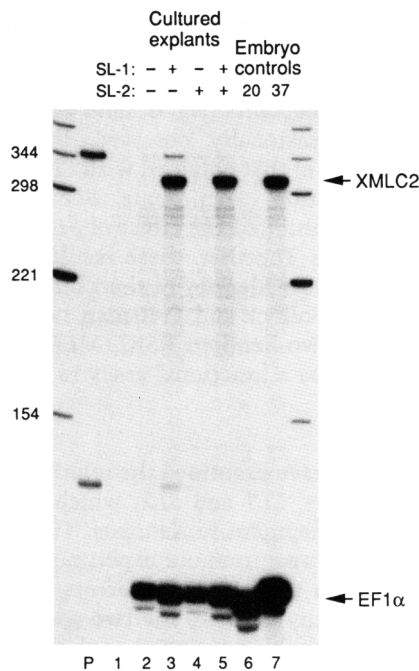
*XMLC2* expression despite the abundance of *SL2* protein in explants from injected embryos (data not shown). In most experiments, *XMLC2* transcripts were entirely undetectable in the explants (Fig. 6, lane 4). Low level expression was occasionally observed, but this corresponded to <10% of that obtained with *SL1*-injected embryos. In coinjection experiments, *SL2* RNA neither potentiated nor inhibited the effect of *SL1* RNA (Fig. 7, cf. lanes 3 and 5). Together, these results demonstrate that despite the near identity of their binding site preferences *in vitro* (Pollock and Treisman 1991; Chambers et al. 1992), the two *Xenopus* RSRF/MEF2 proteins can be distinguished by a functional assay *in vivo*.

#### Discussion

In this study we have examined the relative roles of two *RSRF/MEF2* genes, *SL1* and *SL2*, which are expressed during early development in *Xenopus*. Three main conclusions can be drawn from our experiments. First, both genes lie beneath the myogenic factors in the muscle regulatory hierarchy. Second, the two genes can be distinguished not only by the timing of their activation in the somitic mesoderm, but also by their spatial patterns of expression after neurulation. The *SL1* gene is activated in the presumptive heart region of the neurula embryo, and its product acts as a transcriptional regulator of



**Figure 6.** *XMLC2* is a marker for terminal differentiation of cardiac muscle in *Xenopus* embryos. The distribution of *XMLC2* RNA was examined by use of albino embryos and digoxigenin-labeled probes. Staining was detected first in stage 28/29 tadpoles in the presumptive heart region and was subsequently confined to the developing heart tube. A stage 32 embryo is shown before (a) and after (b) clearing. In later stages, staining was detected in both atrial and ventricular chambers of the beating tadpole heart. No staining was detected with a sense control probe.



**Figure 7.** Ectopic expression of *SL1* activates *XMLC2* gene transcription in animal pole explants. Cultured animal pole explants from injected embryos were analyzed by RNase protection at the neurula stage for expression of the heart-specific *XMLC2* gene. Levels of *EF1α* mRNA were monitored in the same assay. (P) Undigested probe; (lane 1) 10 μg of tRNA; (lane 2) control explants (uninjected); (lanes 3–5) explants from embryos injected with *SL1* and *SL2* RNA, individually or in combination (as indicated); (lanes 6, 7) 5 μg of late neurula (stage 20) and tadpole (stage 37) RNA, respectively. (Size markers as in earlier figures).

the cardiomyogenic program. Last, the cardiac muscle-specific *XMLC2* gene is a target for *trans*-activation by *SL1* but not *SL2* protein, demonstrating that individual *RSRF* proteins possess distinct functions *in vivo*.

#### *RSRF/MEF2 genes in skeletal myogenesis*

In skeletal myoblasts, *MEF2* activity is detected only after expression of *MyoD* family members. The same activity is rapidly induced in fibroblasts by constitutive expression of the myogenic factors (Lassar et al. 1991), even when the cells are incapable of terminal muscle differentiation (Cserjesi and Olson 1991). This suggests that one or more of the *MyoD* protein family directly activate the *RSRF/MEF2* genes. Taken together with the identification of potential *MEF2*-binding sites in the regulatory regions of many muscle-specific genes, these results have led to a model in which the *RSRF/MEF2* genes lie downstream of the myogenic factors and coordinate activation of *MEF2*-regulated, skeletal muscle genes (Olson 1992).

There is also evidence to suggest that *MEF2*-like proteins regulate expression of the myogenic factors themselves and thus mediate a positive feedback loop that

may be important in establishing the myogenic phenotype. In myotube cultures, an important regulatory element for expression of the *myogenin* gene is an *MEF2* site in its proximal promoter region (Edmondson et al. 1992a). Prior to differentiation, *MEF2D* (the murine homolog of *SL1*) is detected by antibody staining in *MyoD*-positive myoblast nuclei, and its expression precedes that of the *myogenin* gene (Breitbart et al. 1993). These results suggest that in murine myogenic cell cultures, *MEF2D* protein may act alongside *MyoD* to trigger terminal differentiation in these cells. Similar regulatory interactions have been detected in the newly forming muscle of mouse embryos. In embryos containing a *myogenin-lacZ* transgene, mutation of the *myogenin* *MEF2* site disrupts the normal pattern of transgene expression within the somites (Yee and Rigby 1993) and results in delayed expression within the limb buds (Cheng et al. 1993).

In cultured cell lines, the onset of myogenic differentiation is signaled by *myogenin* expression, and an essential role for this protein is confirmed by studies of transgenic mice lacking the *myogenin* gene (Hasty et al. 1993). If *RSRF/MEF2* proteins activate *myogenin* expression, we might expect that overexpression of *RSRF/MEF2* genes in cell lines may trigger myogenic differentiation. However, in *Xenopus* embryos, myogenesis occurs without any detectable *myogenin* expression, and for *RSRF* proteins to play a similar role, they must activate expression of *XmyoD* and/or *Xmyf5*. A *MEF2* site has been identified in the *Xenopus myoD* gene promoter (Leibham et al. 1994), but the role of this motif in normal myotome differentiation is unknown.

Zygotic expression of the *RSRF* genes within the somitic mesoderm only appears to commence after expression of *XmyoD* and *Xmyf5*, indicating that the rapid accumulation of *RSRF/MEF2* factors in the somitic mesoderm is unlikely to be responsible for initial activation of the myogenic regulators. As in cultured skeletal myoblasts, *SL1(MEF2D)* is expressed prior to muscle-specific markers, whereas *SL2(MEF2A)* is expressed only after terminal differentiation has begun. We cannot exclude a role for maternally inherited *SL1* or *SL2* protein in the onset of *XmyoD* and *Xmyf5* expression, but if this is the case, there must also be mechanisms to restrict their activity to the presomitic mesoderm because these factors are distributed throughout the early embryo. In a direct test of their roles, ectopic expression of *SL1* and *SL2* failed to activate the endogenous *XmyoD* and *Xmyf5* genes in animal pole tissue, whereas both *RSRF* genes were activated by ectopic expression of *Xenopus* and murine *myoD* family members. We do not think this difference results from insufficient accumulation of *RSRF* protein in the animal pole cells of *SL1*- or *SL2*-injected embryos because both proteins are readily detected in the explants and their level is manyfold greater than the level of maternal *RSRF* protein in normal embryos (D. Sparrow and T. Mohun, unpubl.). These results argue strongly that factors other than *SL1* or *SL2* activate the *XmyoD* and *Xmyf5* genes *in vivo*. Our experiments do not exclude other roles for the *RSRF/MEF2* proteins in



regulating expression of the *myoD* family. It is entirely possible that the accumulating SL1 and SL2 proteins act to maintain or indeed amplify expression of *XmyoD* and *Xmyf5* within the somitic mesoderm.

In *Xenopus* embryos, ectopic expression of myogenic factors is insufficient to induce muscle formation in the prospective ectodermal tissue derived from animal pole explants (Hopwood and Gurdon 1990). It does, however, result in the activation of E-box-regulated genes (such as cardiac actin), and this effect is potentiated by coexpression of XE12 (Rashbass et al. 1992). We interpret this to mean that endogenous genes that are activated by exogenous myogenic factors constitute direct *trans*-activation targets for these proteins. On this basis, it is likely that muscle-specific expression of the *RSRF* genes is attributable to the presence of E-box sites within their regulatory regions. In *Xenopus*, these would be binding sites for XMyoD and/or XMyf5 because XMRF4 is only expressed later in development (Jennings 1992). Interestingly, ectopically expressed mouse myogenin is as effective in *trans*-activating the *RSRF* and cardiac actin genes as the other myogenic factors, even though the *Xenopus myogenin* gene is not apparently expressed at any stage. We do not know whether this indicates a limitation in the usefulness of the ectopic expression assay or simply testifies to functional redundancy among members of the MyoD family.

#### *RSRF/MEF2 in cardiac myogenesis*

The possibility that RSRFs may play a role in cardiac myogenesis was first suggested by the studies of Chien and co-workers who identified an RSRF/MEF2-like binding site essential for expression of the rat *MLC2* gene in primary cardiocytes (Zhu et al. 1991; Navankasattusas et al. 1992). Similar sequences are required for cardiac muscle-specific expression of the chick cardiac troponin T (Iannello et al. 1991), mouse troponin C (Parmacek et al. 1992), rat *MHC $\alpha$*  (Molkentin and Markham 1993), and human phosphoglycerate mutase (Nakatsuji et al. 1992) genes. RSRF/MEF2-binding activity is prevalent cardiac muscle cell extracts (Chambers et al. 1992; Navankasattusas et al. 1992; Molkentin and Markham 1993), and immunofluorescence studies have identified RSRF/MEF2 proteins in nuclei of primary cardiocytes (Breitbart et al. 1993).

We have found that during normal development, *SL1* mRNA accumulates in an anteroventral region of the *Xenopus* neurula embryo. This encompasses the cardiac mesoderm, which only differentiates several hours later. In functional assays, ectopic expression of *SL1* in explants of presumptive ectoderm causes activation of the cardiac muscle-specific *MLC2* gene. Together, these results demonstrate unequivocally that SL1 is an early regulator of cardiac muscle differentiation.

The *XMLC2* gene that we have identified is most closely related in sequence to the human *MLC2a* gene, about which little is known (Hailstones et al. 1992). *MLC2a* expression is restricted exclusively to fetal atrial muscle, whereas the *XMLC2* transcripts are abundant in

cardiac tissue throughout development and are present in both atrial and ventricular muscle (T. Mohun, unpubl.). *XMLC2* activation is not the result of cardiomyogenic conversion of ectodermal tissue because neither of the other two markers for cardiac muscle (cardiac actin and *XMHC $\alpha$*  genes) is expressed in the explants. This specificity suggests that the *XMLC2* gene is a direct target for *trans*-activation by the SL1 protein. Preliminary studies of the *XMLC2* gene promoter have identified two adjacent motifs that match the RSRF/MEF2 consensus-binding site, but their functional role has yet to be tested. The absence of any *XMHC $\alpha$*  expression in *SL1*-injected explants is surprising because cardiocyte-specific expression of the rat homolog is dependent on a MEF2-binding site in its promoter (Molkentin and Markham 1993). Other proteins in addition to RSRFs have been identified that bind to MEF2-like sequences (Cserjesi et al. 1992; Zhou et al. 1993; Zhu et al. 1993), and it is possible that one of these regulates the *MHC $\alpha$*  gene. Alternatively, cardiac-specific expression of the *Xenopus MHC $\alpha$*  gene may result from other regulatory mechanisms.

In earlier studies, we found that maternally derived RSRF/MEF2 activity declines during early development and is undetectable in animal pole explants after overnight culture. (Chambers et al. 1992). Ectopic expression of *SL1* in such explants activates the *XMLC2* gene but does not similarly activate the *SL2* gene. The *XMLC2* gene must therefore be *trans*-activated either by an SL1 homodimer or by SL1 in association with another partner that is normally present in presumptive ectodermal cells. In either case, it is intriguing that the *XMLC2* gene is expressed only in cardiac muscle, because in the early embryo, *SL1* is expressed at high levels in somitic muscle in early embryos. One possibility is that somitic mesoderm contains a negative regulator (perhaps induced by XmyoD or XMyf5) that blocks *XMLC2* gene activation during skeletal muscle differentiation. In adult tissues, analogous mechanisms must exist to restrict *XMLC2* expression because *SL1* is widely expressed and is relatively abundant in both spleen and brain.

#### *Specificity of RSRF function*

In transfection studies, all members of the *RSRF/MEF2* family *trans*-activated reporter genes containing a pair of synthetic binding sites adjacent to a basal promoter. Furthermore, similar activity was obtained with all of the splice variants examined. This approach is therefore of little use in assessing the individual roles of each RSRF/MEF2 protein. In contrast, ectopic expression in embryo explants provides an assay that can reveal functional differences between the SL1 and SL2 proteins. It will therefore be useful in identifying the basis for specificity of RSRF/MEF2 protein function.

SL1 and SL2 proteins differ in only 8 of 91 residues across their DNA-binding domains, and their consensus-binding sites *in vitro* are virtually identical. For this reason it is puzzling that coexpression of *SL2* had no effect on the activity of *SL1*. If both proteins bind similar sites

in vivo, we might expect that SL2 would act as competitive inhibitor of SL1, blocking activation of the *XMLC2* gene. Alternatively, interaction of SL1 with other factors on the *XMLC2* promoter might stabilize binding or produce a complex with higher affinity for the binding site. Characterization the *XMLC2* promoter should clarify this issue.

## Materials and methods

### Embryo culture, dissection, and injection

Synchronous embryos were obtained by artificial fertilization and cultured in  $0.1 \times$  NAM at 18–21°C. These were staged according to Nieuwkoop and Faber (1956). Blastula animal pole explants were dissected from stage 8 embryos and cultured in  $3/4 \times$  NAM, in the presence or absence of activin A. Dorsal and ventral explants were dissected from neurula and tailbud embryos as described previously (Logan and Mohun 1993). For RNA injections, ~5 ng of synthetic RNA was injected into fertilized eggs at the one- or two-cell stage, with an injection volume of 8–12 nl per blastomere. Injections were carried out in  $3/4 \times$  NAM containing 3% Ficoll, and embryos were transferred to  $3/4 \times$  NAM after ~1 hr.

### RNA synthesis in vitro

Fragments containing the entire *SL1*- and *SL2*-coding sequences (nucleotides 235–1968 and nucleotides 205–1920, respectively; Chambers et al. 1992) were prepared by PCR and cloned via *NcoI* ends into the transcription vector pSP64T(*Nco*) to produce pSL1.64T and pSL2.64T. The resulting plasmids contain the RSRF-coding sequence flanked by the human  $\beta$ -globin leader and a portion of the *Xenopus*  $\beta$ -globin 3'-untranslated region. Templates for RNA synthesis were prepared by linearization with *EcoRI*. The mouse myogenin-coding sequence was also amplified by PCR and cloned via linkers as a *BglIII*–*BamHI* fragment into the transcription vector pSP64T (Melton et al. 1985) to give pMg.64T. The DNA sequence of each construct was verified by use of specific primers. Capped RNA for injection into eggs was synthesized in vitro (Krieg and Melton 1984) with SP6 RNA polymerase. *XmyoD*, *Xmyf5*, and *XE12* RNA were prepared similarly with *XhoI*-linearized templates from pSP64T-*XmyoD* (Hopwood and Gurdon 1990), pSP64T-*Xmyf5* (Hopwood et al. 1991), and pSP64-*XE12* $\beta$ M (Rashbass et al. 1992). Several different preparations of each synthetic RNA were used for embryo injection experiments.

### RNA preparation and assay

RNA was prepared from embryos and adult tissue as described previously (Mohun et al. 1984). The following templates were used to prepare RNA probes for use in RNase protection assays. For *XmyoD*: pSP73–5bG/*XmyoD* comprises a 295-nucleotide *HindIII*–*SalI* fragment of pSP64T-*XmyoD* subcloned into pSP73. An RNA probe was prepared with SP6 RNA polymerase and *XhoI*-linearized template DNA. For *SL1* and *SL2*: pSP73.SL-1 and pKS.SL-2 $\Delta$ 71 (Chambers et al. 1992) were used in Figures 1 and 2. To distinguish endogenous from synthetic RNAs (Fig. 3), templates were prepared from plasmids pSP73.SL1(64T) and pSP65.SL2(64T) by linearization with *HindIII*. These were derived from pSL1.64T and pSL2.64T and contain the entire human  $\beta$ -globin 5'-untranslated region fused to 405 and 304 nucleotides of the *SL1*- and *SL2*-coding sequences, respectively. Templates for cardiac actin [*pSP $\alpha$ 1*] (Mohun et al.

1988), *XMHC $\alpha$*  [pXMHC $\alpha$ 1/3] (Logan and Mohun 1993), and *EF1 $\alpha$*  (Sargent and Bennett 1990) probes have been described previously. *XMLC2* transcripts were detected with a probe prepared from pXMLC2 $\Delta$  spanning nucleotides 1–309 of the *XMLC2* cDNA sequence. This was synthesized using *EcoRI*-linearized template DNA and T7 RNA polymerase.

### Whole-mount RNA in situ hybridization

Albino embryos (stages 22–46) were used for whole-mount in situ hybridization as described by Harland (1991) using digoxigenin-labeled probes. The antisense probe was identical to that used for RNase protection assays (see above). A sense control probe was synthesized from *HindIII*-linearized pXMLC2 $\Delta$  template by use of T3 RNA polymerase.

### Isolation of *XMLC2* cDNA

A directional cDNA library was created by use of the UniZapII kit (Stratagene) and screened with an oligo-labeled probe containing the entire coding region of the human *MLC2a* cDNA (Hailstones et al. 1992). Several cDNA positively hybridizing clones were analyzed and found to contain overlapping regions of a *Xenopus* *MLC2* cDNA. pXMLC2-5 consisted of an apparently full-length cDNA that subsequently proved to be a splice variant in which exon 5 is absent, resulting in a frameshift mutation. pXMLC2-12 consisted of a partial cDNA derived from the same gene and including exon 5 (see Fig. 4A). The entire *XMLC2* cDNA sequence was constructed as a composite from the two cDNAs, which were sequenced by the shotgun procedure. The composite sequence was analyzed using the STADEN (Staden 1982, 1984) and UWGCG (Devereux et al. 1984) programs.

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