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Developmental Expression of the Murine Spliceosome-Associated Protein mSAP49

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ABSTRACT **We have isolated the mouse homologue of human spliceosome-associated protein SAP49, mSAP49. mSAP49 contains two RNA recognition motifs (RRM) in the N terminus of the predicted amino acid sequence, and a highly basic C terminus rich in glycine/proline. mSAP49 displayed a plastic of expression in cardiac development. In the adult mouse, mSAP49 is widely distributed, although it was found at relatively lower levels in the heart. *In situ* hybridization analysis of mSAP49 mRNA distribution in staged mouse embryos showed that mSAP49 onset occurs later in the heart than in other embryonic tissues. While mSAP49 expression was found at day 10.0 postconception (pc) in the optic eminence, optic vesicle, hindbrain, and somites, it was not in cardiac structures. mSAP49 was detected in the ventricles at day 11.5, and at day 13.5 it was also detected in the atria. Northern analysis showed that mSAP49 mRNA displayed a peak of expression in the heart at days 14.0–15.0 pc, and its abundance decayed in the adult. This dynamic pattern of cardiac expression suggests that mSAP49 may be contributing to a change in the ratio of spliceosome components during cardiac growth and development, which may have consequences for tissue-specific splicing, RNA stabilization, or translation. *Dev. Dyn. 208:482–490, 1997.*** © 1997 Wiley-Liss, Inc.

Key words: in situ hybridization; expression screening; cardiac differentiation; mouse development; splicing

INTRODUCTION

During vertebrate heart development, the expression of contractile proteins is regulated at both the transcriptional and post-transcriptional levels. For example, during avian and mammalian cardiogenesis, the expression of a single troponin T (cTnT) gene increases and concomitantly the cTnT protein switches from the embryonic to adult isoform (Cooper and Ordhal, 1985; Jin and Lin, 1989). The mechanism for this switch is known to involve a developmentally regulated alternatively spliced transcript (Breitbart and Nadal-Ginard, 1987). Similarly, the expression of a vast array of tropomyosin isoforms during cardiac development, and the switch from non-muscle to muscle isoforms, is also controlled at the level of pre-mRNA splicing (Mullen et al., 1991). In addition, the developmentally regulated switch in contractile protein isoforms appears to correlate with developmental changes in cardiac function (Schiaffino et al., 1993).

At present, relatively little is known regarding developmental changes in spliceosome associated proteins that might mediate these switches during the course of cardiogenesis. The process of pre-mRNA splicing requires the concerted actions of both cis- and trans-acting factors localized in the spliceosome, a ribonucleoprotein (RNP) complex comprising small nuclear RNPs (snRNPs) U1, U2, U4, U5, and U6, and a number of non-snRNP factors (for a review, Moore et al., 1993) of which only a few have been characterized to date. In alternatively spliced transcripts, splice recognition sites that are efficiently used in one cell type may be overlooked by the splicing machinery in other cell types. Therefore, regulated alternative pre-mRNA processing must involve either differences in cis- and trans-acting factors or the existence of additional tissue-specific factors. Splice site choice may not always require the presence of specific factors but can be mediated by differences in the activities or ratios between general splicing factors (Caceres et al., 1994; Mayeda and Krainer, 1992; Valcarcel et al., 1993).

In the course of screening for proteins that bind to the HF-3 negative regulatory element in the ventricular myosin light chain-2 (MLC-2v) promoter (Lee et al., 1994; Ross et al., 1996), we have cloned the murine homologue of the human spliceosome-associated protein SAP49, mSAP49. Spliceosome-associated protein SAP49 is specifically associated with U2 snRNP, interacts with pre-mRNA in a region immediately upstream of the branchpoint sequence in the prespliceosomal complex, and also directly interacts with SAP145, an-

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other U2snRNP-associated protein (Champion-Arnaud, 1994). In this study, we show that mSAP49 transcript accumulation in the heart is temporally regulated during the course of cardiogenesis, thereby suggesting its potential involvement in the developmental regulation of spliceosome activity in the heart.

RESULTS AND DISCUSSION

Isolation of mSAP49 cDNAs and Predicted Protein Sequence

In the search for factors which might interact with the HF-3 element in the ventricular myosin light chain promoter (Lee et al., 1994), we isolated a murine homologue of the human spliceosome-associated factor SAP49 (Champion-Arnaud and Reed, 1994). A stop codon (position 1280nt), a putative polyadenylation signal (nt 1485), and a polyA tract were found, identifying the 3′ end of the mRNA (Fig. 1). The complete mSAP49 cDNA was 1,515 bp long, with a 1,272-bp open reading frame and a predicted 424-amino acid protein product (Fig. 1). The predicted polypeptide contained a C-terminal region that was both glycine (16%) and proline rich (34%) and highly basic, with a predicted isoelectric point of 12.18. The N terminus contained two RNA recognition motifs (RRMs) (Dreyfuss et al., 1993) (underlined in Fig. 1). Predicted polypeptides of mouse and human SAP49 displayed a 100% conservation within the RRM domains and shared high glycine/proline content in the C-terminal region; however, the sequence in this domain was not strictly conserved, with a 47% divergence in a 71-amino acid stretch (bold in Fig. 1). Whether this divergence represents an alternatively spliced form or interspecies variability remains unknown.

RRM containing proteins have one or more copies of the RRM domain in the N terminus and a highly basic auxiliary domain in the C terminus. These proteins were initially reported as RNA-binding proteins, but recently alternate roles, such as DNA binding (DeAngelo et al., 1995) and/or protein–protein interactions (Champion-Arnaud and Reed, 1994), have been postulated for this protein family. We compared the mSAP49 predicted polypeptide sequence with similar proteins in the data bank, in search of a structure–function relationship. Two proteins involved in transcriptional regulation, the stage-specific activator 1 (SSAP-1) from sea urchin and the human liver-specific protein E2BP (DeAngelo et al., 1995; Tay et al., 1992), share two RRM domains and a highly basic C terminus with mSAP49. Other RRM-containing proteins with structural similarities to mSAP49 include the human polyA-binding protein (DNHPA) (Grange et al., 1987), which has four RRM repeats, and the mammalian Elav homolog (King et al., 1994), which has two complete and one incomplete RRM repeat with no accessory C-terminal sequences (Fig. 2A). Amino acid sequences from the entire RRM domain of mSAP49 were aligned to closely related RRM domains (Fig. 2C) using a PILEUP program (GCG, University of Wisconsin), and a dendrogram was constructed representing the relationship among RRM domains, prepared from a multiple alignment (Feng and Doolittle, 1987). From these considerations, it became apparent that the most N-terminal RRM domain in the mSAP49 polypeptide (C1, Fig. 2A) is closest to the most N-terminal sequence in the human polyA-binding protein (D1, Fig. 2A), and the C2 domain is closest to D2 in a manner that seems to be dependent upon their position in the polypeptide sequence. If the criterion followed is the number of repeats, then multifunctional proteins with RNA- and DNA-binding activities are composed of two RRM domains, while RNA-binding proteins may have additional domains.

The fact that we cloned mSAP49 on the basis of its ability to bind to DNA sequences, and previous results with human SAP49, suggests that mSAP49 may be a multifunctional protein. Previous reports have indicated multifunctional roles for several nucleic acid–binding factors. For example, the RNA-binding protein YB1 binds to a cardiac-specific promoter and positively regulates its transcription (Zou and Chien, 1995). In addition, the homeobox-containing protein *bicoid,* initially described as a DNA-binding protein, has been shown to bind to *caudal* (cad) mRNA to control cad translation in a region-specific manner (Rivera-Pomar et al., 1996).

mSAP49 Expression Is Regulated During Mouse Development

To determine the tissue distribution of mSAP49 mRNA, we performed Northern blot analyses with poly A^+ RNA derived from various adult mouse tissues: liver, skeletal muscle, spleen, kidney, brain, and heart. The mSAP49 mRNA (a single 1.5-kb band) was widely distributed, although it was found at relatively low levels in heart, as confirmed after normalization with a GAPDH probe (Fig. 3A). To further establish that this differential pattern of expression was not simply a function of the quality of the cardiac RNA, a cDNA encoding MLC-2v, a marker for ventricular muscle, was hybridized with the same Northern blot. A specific MLC-2v signal was present in the heart and at lower levels in skeletal muscle (Fig. 3A), consistent with the expression pattern of MLC-2v in adult tissues previously described (Lee et al., 1992).

Embryonic stem cells (ES cells) can aggregate and form spontaneously beating embryoid bodies (EBs) which sequentially express cardiac-specific markers and serve as a model system to study cardiogenesis (Miller-Hance et al., 1993). To determine the onset of appearance of the mSAP49 message, a semi-quantitative reverse transcriptase/polymerase chain reaction (RT/PCR) was performed at sequential days during EB development. An mSAP49-specific band was in both

Fig. 1. (overleaf) mSAP cDNA nucleotide sequence and predicted protein sequence. The two RNP domains are underlined. An in-frame stop codon is marked by *. The putative polyadenylation signal at the 3′ end of the transcript is also underlined.

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  TTTCGCCATGGCTGCCGGACCGATCTCCGAACGGAATCAGGATGCCACGGTGTACGTG   58
        M  A  A  G  P  I  S  E  R  N  Q  D  A  T  V  Y  V    17

GGAGGTCTAGACGAGAAAGTGAGCGAGCCACTGCTATGGGAGCTCTTTCTCCAGGCAGGG  118
 G  G  L  D  E  K  V  S  E  P  L  L  W  E  L  F  L  Q  A  G    37

CCAGTGGTCAACACCCACATGCCCAAGGACAGAGTCACTGGCCAGCACCAGGGCTATGGC  178
 P  V  V  N  T  H  M  P  K  D  R  V  T  G  Q  H  Q  G  Y  G    57

TTTGTTGAATTCCTGAGCGAGGAAGATGCCGACTATGCCATTAAGATTATGAACATGATC  238
 F  V  E  F  L  S  E  E  D  A  D  Y  A  I  K  I  M  N  M  I    77

AAACTCTATGGAAAGCCAATACGGGTGAACAAGGCCTCAGCTCACAACAAAAACCTGGAT  298
 K  L  Y  G  K  P  I  R  V  N  K  A  S  A  H  N  K  N  L  D    97

GTGGGAGCCAACATTTTCATTGGAAATCTGGACCCAGAAATTGATGAAAAGCTGCTTTAC  358
 V  G  A  N  I  F  I  G  N  L  D  P  E  I  D  E  K  L  L  Y   117

GATACTTTCAGCGCCTTTGGAGTCATCCTACAGACCCCCAAGATCATGCGGGACCCTGAC  418
 D  T  F  S  A  F  G  V  I  L  Q  T  P  K  I  M  R  D  P  D   137

ACAGGCAACTCCAAGGGTTACGCCTTCATTAATTTTGCCTCCTTCGATGCTTCGGATGCA  479
 T  G  N  S  K  G  Y  A  F  I  N  F  A  S  F  D  A  S  D  A   157

GCAATTGAGGCCATGAACGGGCAGTACCTGTGTAACCGTCCTATCACTGTGTCTTATGCC  538
 A  I  E  A  M  N  G  Q  Y  L  C  N  R  P  I  T  V  S  Y  A   177

TTCAAGAAGGACTCTAAGGGTGAACGCCATGGCTCAGCAGCTGAAAGACTCCTGGCAGCC  598
 F  K  K  D  S  K  G  E  R  H  G  S  A  A  E  R  L  L  A  A   197

CAGAACCCGGCTGTCCCAGCTGACCGCCCTCACCAGCTGTTTGCCGATGCACCCCCTCCG  658
 Q  N  P  A  V  P  A  D  R  P  H  Q  L  F  A  D  A  P  P  P   217

CCCTCTGCCCCCAATCCCGTGGTTTCATCCCTGGGTTCTGGGCTTCCTCCACCAGGCATG  718
 P  S  A  P  N  P  V  V  S  S  L  G  S  G  L  P  P  P  G  M   237

CCGCCTCCTGGCTCTTTTCCACCTCCAGTGCCACCTCCTGGGGCCCTCCCTCCTGGGATT  778
 P  P  P  G  S  F  P  P  P  V  P  P  P  G  A  L  P  P  G  I   257

CCCCCAGCCATGCCCCCACCACCTATGCCACCTGGGGCTGGAGGACATGGCCCCCCATGC  838
 P  P  A  M  P  P  P  P  M  P  P  G  A  G  G  H  G  P  P  C   277

AGCAGGAACTCCAGGGGCTGGACATCCTGGTCACGGACATTCACATCCTCATCCATTCCA  898
 S  R  N  S  R  G  W  T  S  W  S  R  T  F  T  S  S  S  I  P   297

CCAGGTGGGATGCCCCATCCAGGGATGTCCCAGATGCAGCTGGCCCACCATGGCCCCCAT  958
 P  G  G  M  P  H  P  G  M  S  Q  M  Q  L  A  H  H  G  P  H   317

GGCCTAGGACACCCCCATGCTGGGCTCCGGGCTCTGGGGGGCAGCCACCACCTCGGCCAC 1018
 G  L  G  H  P  H  A  G  L  R  A  L  G  G  S  H  H  L  G  H   337

CTCCTGGAATGCCTCCATCCTGGACCTCCTCCAATGGGCATGCCCCCCCGAGGGCCTCCT 1078
 L  L  E  C  L  H  P  G  P  P  P  M  G  M  P  P  R  G  P  P   357

TTTGGATCTCCCATGGGTCACCCAGGTCCCATGCCTCCACACGGCATGCGTGGGCCTCCT 1138
 F  G  S  P  M  G  H  P  G  P  M  P  P  H  G  M  R  G  P  P   377

CCATTGATGCCCCCTCATGGATACACGGGTCCTCCAAGACCCCCTCCCTATGGCTACCAG 1198
 P  L  M  P  P  H  G  Y  T  G  P  P  R  P  P  P  Y  G  Y  Q   397

CGGGGGCCCCTCCCGCCACCCAGACCCACTCCACGGCCCCCAGTTCCACCTCGTGGTCCT 1258
 R  G  P  L  P  P  P  R  P  T  P  R  P  P  V  P  P  R  G  P   417

CTTCGGGGCCCACTTCCTCAGTAAATTGCCATCCTTTGTCTCCTCCTTTTATCCTCCCTT 1318
 L  R  G  P  L  P  Q  *                                       424

TATCTTCATTTCTTGGACCAATCAGAGATGCTGTAGCTTGTCAAGGCTAATGTACTGACC 1368

CTTCTCAGTAACTCTGTCCCCATTCCAATGTCATGTTTTCCACTGGGGATTTTCTTCATT 1438

TTTATTTTAAAATGTTGGTCCTAATTTCTTTACACATGTATAGAAAAATAAAACTATGCT 1498

TCTTGTTTTAAAAAAAA                                            1515
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Fig. 1.

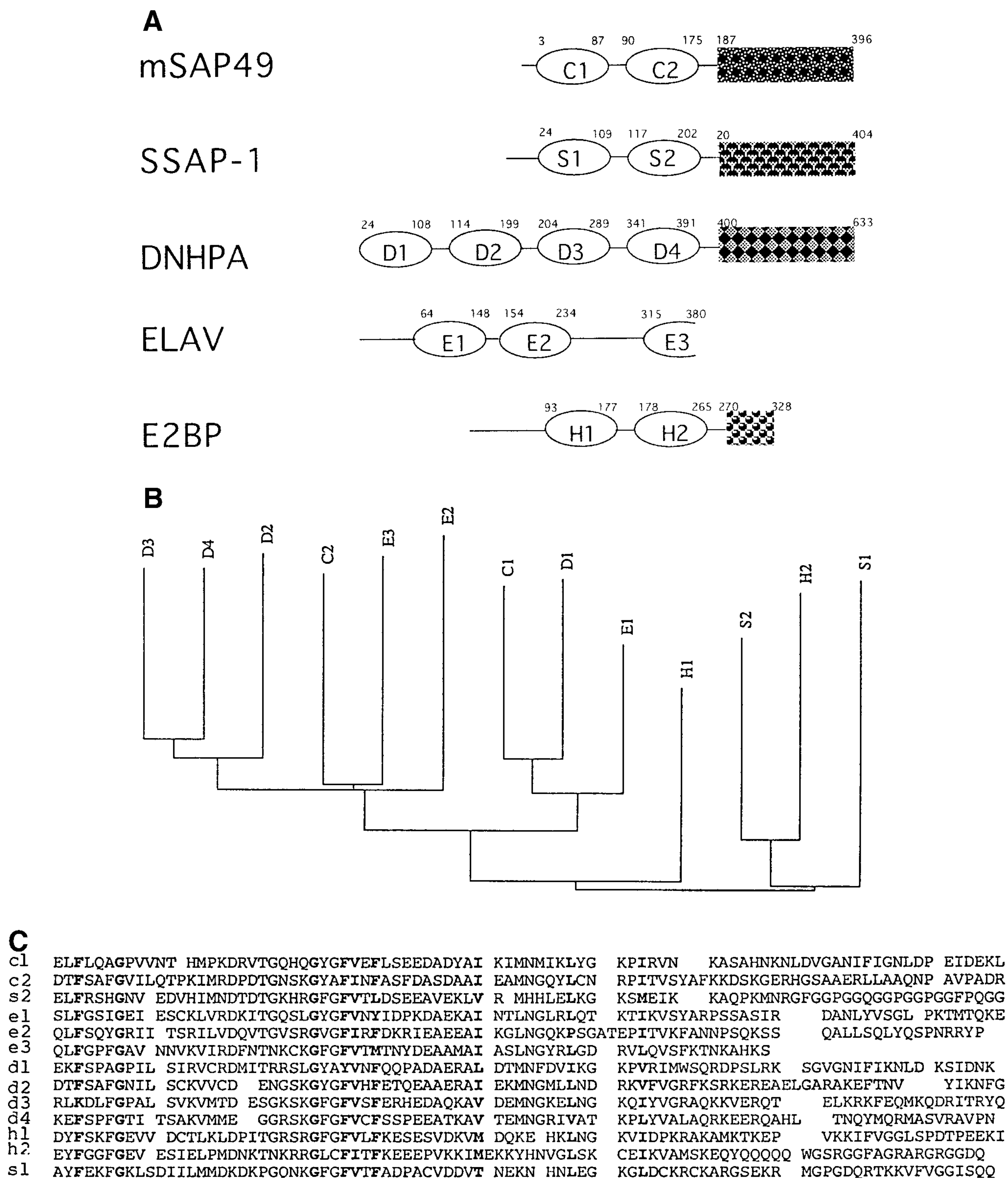

Fig. 2. **A:** Schematic representation of the mSAP49 predicted protein and comparison with other related gene products. SSAP-1: Stage-specific activator 1 (DeAngelo et al., 1995). DNHPA: (Grange et al., 1987) ELAV: Human *elav* (King et al., 1994). Liver-specific E2BP (Tay et al., 1992). The RRM domains are represented in circles. The auxiliary C-terminal domains are boxed. Numbers indicate amino acid position. **B:** Dendogram that represents the relationship among RRM domains prepared from a multiple alignment (Feng and Doolittle, 1987) and summarizes the similarity among the RRM-containing proteins by sequence. **C:** Sequence comparison of RRM domains, inter-mSAP49 and intra–other related proteins: The two RRM domains in mSAP49 (C1 and C2), in SSAP-1 (S1 and S2), in E2BP (H1 and H2), the four domains in DNHPA (D1–D4), and the three domains in Human Elav (E1–E3) are aligned.

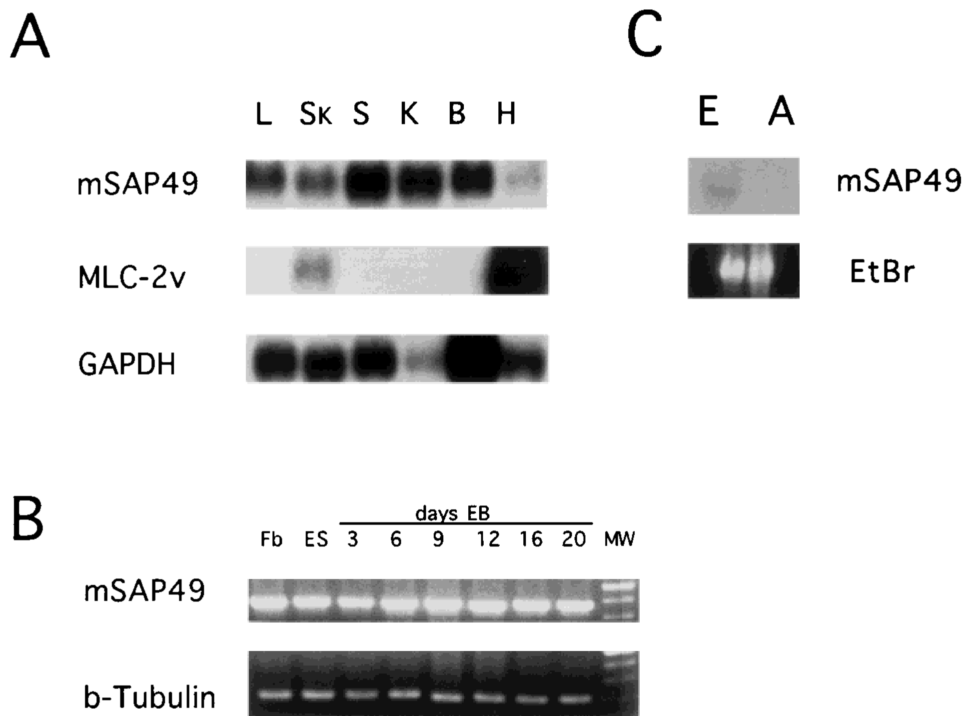

Fig. 3. **A:** Northern blot using 2.5 μg polyA$^+$ mRNA per lane from adult mouse tissues. Right to left: Liver, skeletal muscle, spleen, kidney, brain, and heart. The probes with which the same filter was hybridized are indicated in the left side of the panel. **B:** Reverse transcriptase/ polymerase chain reaction from different days of embryoid bodies (EB) development. Days are indicated in the upper panel. ES, Stem cell; Fb, fibroblasts. **C:** Northern blot using 30 μg of total RNA from a pool of embryonic hearts (E) or adult mouse heart (A) hybridized with the full-length mSAP49 probe. Ethidium bromide (EtBr) staining is shown to control for loading and RNA integrity. Note that panel A contains isolated polyA$^+$ while panel C shows hybridization signal using total RNA.

fibroblasts and ES cells, and no significant changes in expression were detected following the differentiation of ES cells into beating EBs (Fig. 3B). These results suggest that mSAP49 onset is very early and that its expression might not be regulated. Alternatively, they may represent a failure of the EB system to detect developmental modulation of non–cardiac-specific genes. In Northern blot experiments using total RNA from isolated embryonic hearts at days 14–15 pc and adult heart, mSAP 49 was expressed at higher levels in cardiac embryonic tissue relative to adult heart (Fig. 3C), thus suggesting that mSAP mRNA content varies with cardiac development.

Subsequently, we studied mSAP49 mRNA expression in the developing mouse by whole mount in situ hybridization utilizing staged embryos at early days of development. We did not detect mSAP49 mRNA in the mouse embryo at days 7.5–8.0 pc (Fig. 4B), while the atrial MLC-2A message, used as a control for RNA integrity, was seen in the fusing cardiac tubes (Fig. 4A). At day 10.0 pc, mSAP49 mRNA was first detected in the optic eminence and optic vesicle (Fig. 4F). Ridges on the inside of the 4th ventricle (Fig. 4G) and a weaker signal in the hindbrain and somites were also found (Fig. 4C,D). At day 11.0 pc, limb and somites appeared strongly stained (Fig. 4E,F), but no mSAP49 message was detected in the heart or in the fore- and midbrain structures (Fig. 4E). Longer incubation of parallel embryo samples confirmed mSAP49 expression in the somites and the negative mSAP49 staining in the heart and brain (Fig. 4C,D). mSAP49 was first detected in the heart at day 11.5 pc, when it was restricted to the ventricular myocardium. At day 14 pc hybridization signal was also detected in the atria (Fig. 4H).

The above results suggest a dynamic pattern of mSAP49 expression during cardiogenesis. To complement the in situ analysis and quantitatively determine the kinetics of mSAP49 mRNA abundance in heart development, we performed Northern-blot analysis using 30 μg of RNA from embryonic heart day 11.0 to the adult cardiac tissue (Fig. 5). After normalization of the signal by the use of a GAPDH control probe, we determined that mSAP49 displayed a peak of expression at embryonic day 14–15.0 pc, and its expression decayed dramatically at day 17.0 pc to reach levels comparable to the adult in the neonate stage.

Human SAP49 was initially cloned as an spliceosome-associated factor although its role in splicing is not completely understood. An essential or general factor of the spliceosome would be expected to be expressed ubiquitously during mammalian development and in a housekeeping manner in the adult. The pattern of mSAP49 mRNA expression during cardiac chamber development suggests that mSAP49 expression is not

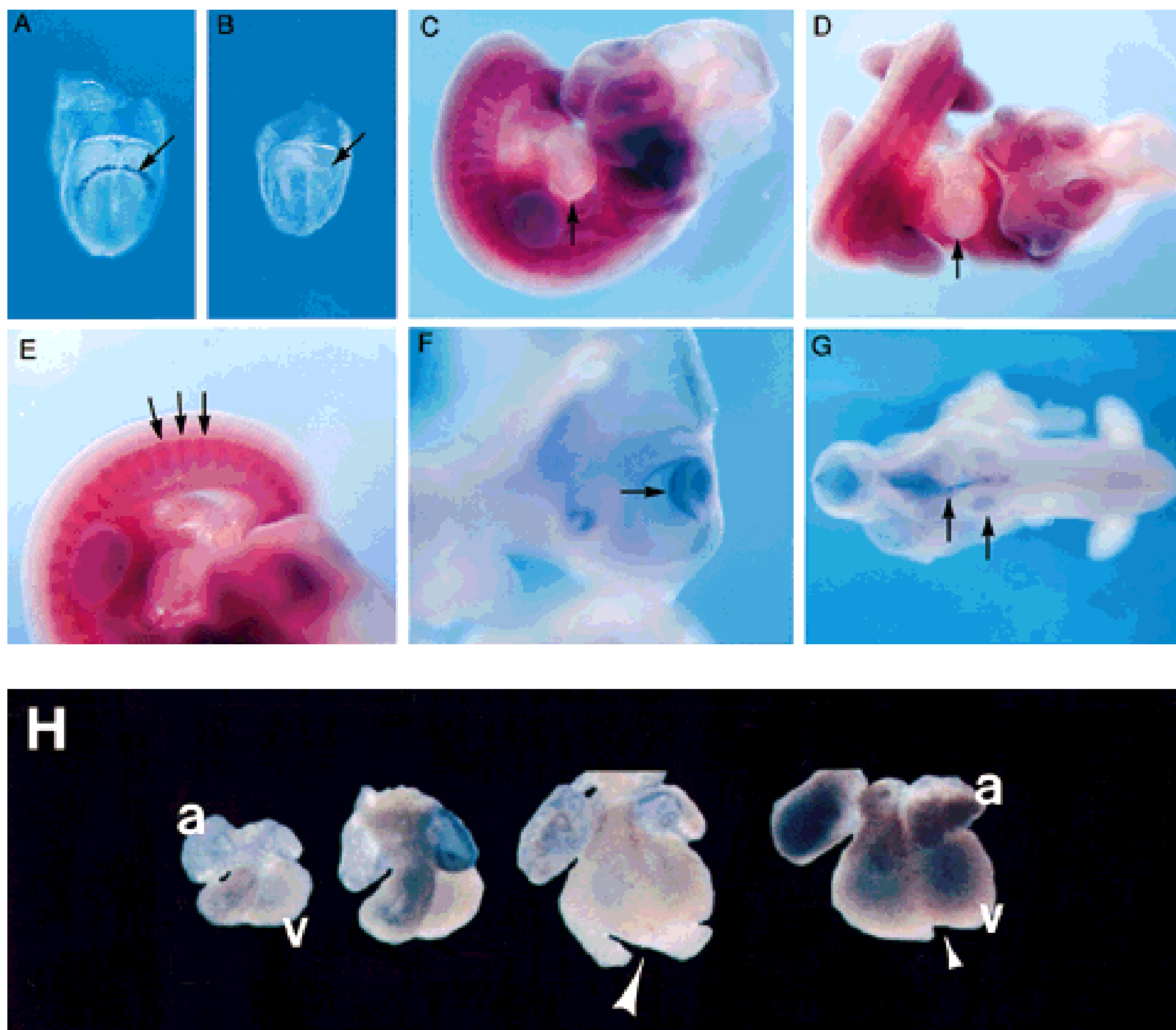

Fig. 4. Whole-mount in situ hybridization in day 8.0 pc (**A,B**) and day 10.0 pc (**C–G**) mouse embryos, hybridized with (A) an MLC-2A probe, to control for RNA integrity or (B–G) mSAP49. Arrows indicate hybridization signal in somites (E), optic eminence (F), and ridges in the forth ventricle (G). No detectable hybridization signal was observed in the heart (arrows in C and D). **H:** Whole mount in situ hybridization on isolated embryonic hearts. Left to right: e11.5pc, e12.5pc, e13.5pc, and e14pc. a, atrium; v, ventricle; arrowheads, small incision in the tissue.

required in the processing of early cardiac messages. Nevertheless, the transient increase in the mRNA abundance of this pre-spliceosome component coincides with the time frame in which a battery of cardiac proteins change from the embryonic to the adult isoforms (for reviews, Schiaffino et al., 1993; Lyons, 1994), and it should be considered in this context. Contractile protein transcripts are in the cardiac tube at the onset of pumping function. At day 8.5 pc of the mouse embryo, mRNAs for the sarcomeric proteins myosin heavy chain α and β, myosin light chain 1A and 1V, cardiac α-actin, tropomodulin, tropomyosin, α-actinin, titin, and desmin, and the metabolic B-creatine kinase are already detected in the myocardium (Lyons et al., 1990; Jockusch et al., 1984; Shaart et al., 1989). Nevertheless, the transition between this early pump to the mature cardiac system involves the expression of different myofibrillar, membrane, and cytosolic proteins isoforms than those observed in the embryo. This developmental switch is mediated both by differential splicing of single primary transcripts and transcriptional induction of the adult isoforms.

At day 12.5 pc in the mouse the muscle-specific creatine kinase (MCK) transcript is expressed in the myocardium (Lyons et al., 1991). Cardiac troponin I (cTnI) transcripts are detectable in the heart at embryonic day 11 pc in the rat embryo but the protein product is not detected until day 18 pc, suggesting a post-transcriptional mechanism involved in the regulation of (cTnI) expression (Gorza et al., 1993). Troponin T

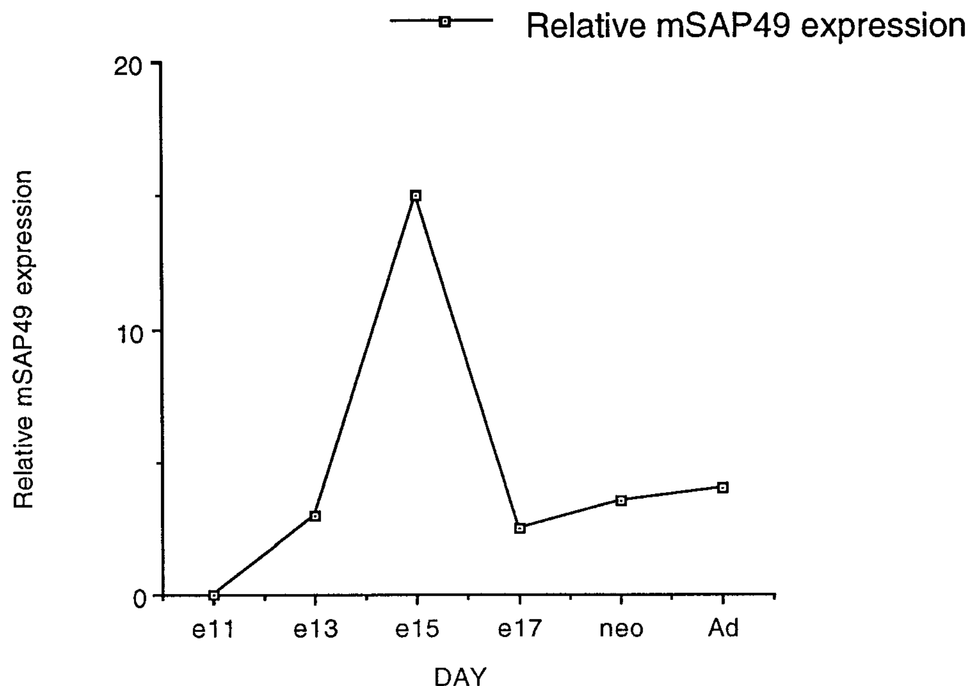

Fig. 5. Kinetics of mSAP49 expression in the heart during development. Densitometry analysis of mSAP49 mRNA abundance in isolated hearts is plotted. Measures were taken at embryonic days 11.0 pc, 13.0 pc, 15.0 pc and 17.0 pc, neonatal and adult mouse hearts. Data were normalized using a GAPDH control probe.

(TnT) undergoes a perinatal switch from the larger acidic embryonic form to the smaller, basic adult form (Jin and Jin, 1988), which involves differential splicing. Dystrophin gene transcripts reach peak levels around mouse embryonic day 15 pc (Houzelstein et al., 1992). These changes have important functional and pathological implications. For example, a fetal TnT isoform is increased in human heart failure, suggesting a decrease in ATPase activity in the failing heart (Anderson et al., 1992).

We provide the first evidence to date that a spliceosome component (mSAP49) can be developmentally regulated in both a spatial and temporal fashion. The onset of mSAP49 expression in the heart is initially restricted to the ventricle and only later is seen in the atria, again suggesting spatial specificity. The fact that this regulation coincides temporally and spatially with the onset of differential expression of a wide variety of alternatively processed transcripts during cardiac development may be more coincidental. A direct test of this notion would require transgenic approaches to express mSAP49 early in the heart tube under the control of a promoter that will drive expression of mSAP49 early in the heart tube, e.g., MLC2v. The determination of mSAP49 binding sites in vitro using a SELEX approach (Siomi et al., 1993) along with a systematic determination of the developmental expression of the isolated spliceosomal components will shed important information on the molecular mechanisms that regulate tissue maturation.

EXPERIMENTAL PROCEDURES

Screening of cDNA Libraries

To isolate factors that bind to the HF3 site in the MLC-2v promoter, 2×10^6 plaque-forming units (pfu) λgt11 from a rat neonatal ventricle cDNA expression library (Zhu et al., 1993) were screened with a 5′ end-labeled HF-3 oligo 5′ AGCTTCCACTGTCTCTTTAAC-CTTGAAGCATTTTTA3′, from positions −211 to −176 of the rat MLC-2v promoter (Lee et al., 1994). To obtain a double-stranded probe, the sense strand was end-labeled and hybridized with a tenfold excess of cold antisense strand by heating to 94°C for 5 min and gradually cooling to room temperature. Subsequently, the HindIII cohesive ends of the probe were ligated using T4 DNA ligase (Gibco). The probe was purified over a G25 Sepharose column and its size evaluated by agarose gel electrophoresis. Southwestern screening of the rat neonatal heart λgt11 cDNA library was performed as described elsewhere (Zou and Chien, 1995). For sequence analysis, positive clones were plaque purified and subcloned into the NotI site of pBluescript II SK(+) (Stratagene). To isolate full-length cDNAs, conventional DNA/DNA hybridization was performed using the primary cDNA clone to probe a mouse 14-day embryoid body cDNA library in Lambda gt11 (kindly provided by Dr. J. Robbins).

Northern Blotting Analyses

Two and a half micrograms of $polyA^+$ RNA from different adult mouse tissues—liver, spleen, skeletal

muscle, kidney, brain, and heart (Clontech)—or 30 μg of total RNA from a pool embryos, were separated on a formamide agarose gel, transferred to a nylon filter (N-Hybond, Amersham), fixed by UV cross-linking, and hybridized with 3×10^6 cpm/ml of the 32-P random-labeled 1.5-kb full-length NotI fragment, following the manufacturer's directions (Amersham). Washes were performed under moderate stringency (0.2× standard saline citrate, 65°C). Densitometry analysis of the blots was performed using the IS-1000Digital Imaging System (Alpha Innotech Corporation, San Leandro CA).

RT/PCR

In vitro differentiation of ES cells, polymerase chain reaction, and analysis of the amplified products were performed as previously described (Miller-Hance et al., 1993).

In Situ Hybridization

The full-length mSAP49 was cloned into pBluescript SKII+ (Stratagene), cut with HaeII. An FITC-labeled cRNA probe was generated by in vitro transcription with T7 RNA polymerase and the 10× DIG RNA labeling mixture obtained from Boehringer Mannheim (Indianapolis, Illinois), to yield an antisense riboprobe of 373 nt. Whole-mount in situ hybridization was performed using a modification of the procedure described by Christiansen et al. (1995). Specimens were photographed submerged in PBS on a 1% agarose petri dish with a stereomicroscope (Zeiss SV6), and slides were digitized. Figures were composed in Adobe Photoshop 3.0, QuarkXPress 3.31 and printed by Fujix Pictography.

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