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Expression of MEF2 Genes during Human Cardiac Development

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Departments of Bioscience and [2]Pathology, National Cardiovascular Center, Suita 565-8565, and [1]Department of Pathology, Osaka Medical Center of Maternal and Children's Health, Izumi 594-1101

IIDA, K., HIDAKA, K., TAKEUCHI, M., NAKAYAMA, M., YUTANI, C., MUKAI, T. and MORISAKI, T. *Expression of MEF2 Genes during Human Cardiac Development.* Tohoku J. Exp. Med., 1999, **187** (1), 15-23 —— To better understand the reguratory mechanisms in gene expression of human cardiomyocytes, we studied the expression of MEF2 genes encoding transcription factors during the course of cardiac development. Expression of all four MEF2 transcripts (MEF2A, MEF2B, MEF2C, and MEF2D) were detected in all developmental stage of the human heart, while Mef2b transcripts were down-regulated in mouse heart development. Although none of the MEF2 genes, besides mouse Mef2b, exhibited any remarkable quantitative change in their transcripts, qualitative changes in MEF2 transcripts were found during the course of cardiac development. In particular, MEF2D transcripts showed prominent changes by alternative splicing in the perinatal period. MEF2D transcripts containing the 21-base exon (exon b) were predominantly expressed after birth. At the same time, transcripts of the alpha myosin heavy chain (αMHC) gene increased after birth, as the splicing pattern in transcripts of the cardiac troponin T (cTnT) gene changed to decrease the transcripts of cTnT1 after birth. These changes seemed to be correlated with the alternative splicing changes of MEF2 genes, especially MEF2D. The alternative splicing as well as transcriptional regulation in MEF2 genes might be important for regulating the αMHC gene and the maturation of cardiomyocytes. ———— transcription factor; alternative splicing; cardiac development; polymerase chain reaction

Recent studies have revealed that transcription factors play an important role in the differentiation and development of cardiomyocytes (Lyons et al. 1995; Kuo et al. 1997; Lin et al. 1997). Myocyte enhancer factor 2 (MEF2), which is encoded in multiple genes in higher eukaryotes, is one of the transcription factors

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controlling cardiac development and regulates expression of many muscle-specific genes (Yu et al. 1992; Breitbart et al. 1993; McDermott et al. 1993; Martin et al. 1994). Although each MEF2 gene is known to be expressed during the course of cardiac development (Edmondson et al. 1994; Subramanian and Nadal-Ginard 1996) and to produce several isoforms by alternative splicing (Breitbart et al. 1993), functional differences between these isoforms have not been well defined. Furthermore, it has not been well characterized how the regulation of these isoforms is controlled nor how it affects the expression of other genes during cardiac development, though developmental changes of expression of murine Mef2 genes have been studied (Martin et al. 1994; Olson and Srivastava 1996). Here we report our study of the expression of MEF2 genes and other genes, including alpha-cardiac myosin heavy-chain (αMHC) gene, during the course of cardiac development to better understand their function.

Materials and Methods

Isolation of total RNA from human and mouse tissue

Total RNA was isolated from human tissue or cells by TRIzol reagent (Life Technologies, Rockville, MD, USA) according to the manufacturer's protocols. RNAs were isolated from heart and skeletal muscle tissue after informed consent was obtained. Heart tissues were obtained from eleven patients without any cardiac problem, including five from embryos at various stages, and six from neonates, children, and adults. Ten heart samples were obtained from patients with cardiac diseases, including three adults with idiopathic hypertrophic cardiomyopathy (HCM) and seven children from one to three years old with Tetralogy of Fallot (T/F). Skeletal muscle and human embryonal carcinoma cells (NT2) (Andrews 1984) were also used for RNA preparation. Mouse RNAs were also isolated from the hearts at various stages of development as described above.

Reverse transcription-polymerase chain reaction (RT-PCR)

cDNA was synthesized from one microgram of total RNA with a random primer and Superscript II (Life Technologies), according to the manufacturer's directions. A polymerase chain reaction (PCR) was performed on the newly synthesized cDNAs with AmpliTaq DNA polymerase (Perkin-Elmer, Norwalk, CT, USA) and the oligonucleotide primers designed for each cDNA sequence as shown in Table 1. Using various levels of amplification, semi-quantitative changes in the amount of transcripts could be assessed. PCR amplification at the same condition was repeated at least three times to assure the reproducibility, and three different cycle numbers of amplicifation were performed to obtain the relative abundance of transcripts. All primers for MEF2 genes were designed for the 3′ region of each cDNA to distinguish them from other MEF2 transcripts. For the MEF2A, MEF2C, and MEF2D transcripts, the oligonucleotide primers were

TABLE 1. *Oligonucleotide primers used for RT-PCR corresponding to MEF2 genes and sarcomeric genes. Each set of primers was desinged to distinguish respective isoforms produced by alternative splicing. Nucleotide sequences for oligonucleotide primers used in this study*

Gene	Primer	Sequences
MEF2A	5+	5′-GGAATGAACAGTAGGAAACC-3′
	4−	5′-TGAAGCCTTGAAGGGCTGAC-3′
MEF2B	1+	5′-GCCCTCATCATCTTCAACAG-3′
	2−	5′-TCCTCAGGCCCTTCATCCGG-3′
MEF2C	5+	5′-AGCAAGAATACGATGCCATC-3′
	6−	5′-GAAGGGGTGGTGGTACGGTC-3′
MEF2D	1+	5′-GAATGGCTACGTCAGTGCTC-3′
	3−	5′-GCACTGGTCAACTGGTAATC-3′
cTnT	1+	5-GAGCAGAGACCATGTCTGAC-3′
	2−	5′-CTCATTCAGGTCCTTCTCCA-3′
MHC	1+	5′-ACTGAGCAGCTAGGAGAAA-3′
	2−	5′-GGACCGCATCGTCCAGCTGG-3′
GAPDH	1+	5′-CTTCATTGACCTCAACTACA-3′
	2−	5′-AAAGTTGTCATGGATGACCTTG-3′

designed to amplify the region containing the exon that is alternatively spliced. Therefore, the products by RT-PCR showed qualitative transcript changes as well as quantative. We also studied the expression of αMHC gene and the cardiac Troponin T (cTnT) gene by RT-PCR, since they are supposed to be regulated by the MEF2 genes (Molkentin and Markham 1993; Watanabe et al. 1997) and the alternative splicing of the cTnT gene is known to be developmentally regulated (Anderson et al. 1995). The oligonucleotide primers for RT-PCR to detect transcripts for the αMHC gene and the cTnT gene are also shown in Table 1. The oligonucleotide primers were designed to amplify the region containing the alternative spliced exons of the cTnT gene. PCR was performed for 30 cycles, and the RT-PCR products were separated by electrophoresis on a 2.5% agarose gel.

Southern blot

Southern blot hybridization for RT-PCR products was performed to distinguish alternatively spliced MEF2D. Nylon filters transferred from the agarose gels described above were hybridized with the probe 2D5+ (5′-GACTGAGGACCATTTAGATC-3′) to detect only those sequences containing exon b or the probe 2D6+ (5′-GCATCACTTGAACAATGCCC-3′) in order to detect only the human MEF2D sequences without exon b.

Results

MEF2 transcripts in human heart development

Transcripts of all four MEF2 genes were detected by RT-PCR in the human heart at all stages from early embryo to adult (Fig. 1A). The transcripts for each MEF2 gene changed little quantitatively at all developing stages while few or no transcripts for each MEF2 were detected in the embryonal cells. However, the MEF2D transcripts showed qualitative changes during cardiac development, while there was no remarkable qualitative change in the MEF2A, MEF2B, or MEF2C transcripts. Major transcripts for MEF2D before birth were smaller in size than those after birth, suggesting that MEF2D transcripts containing the 21-base mini-exon (exon b) increased and became dominate after birth. Adult skeletal muscle showed only larger transcripts for MEF2D. It was confirmed by Southern analysis that an alternative splicing of exon b in MEF2D was indeed occurred in MEF2 transcripts during human heart development (Fig. 1B). RT-PCR method was used in this study due to limitation of RNA amounts for the embryonic heart since repeated studies of PCR amplification by various conditions could reveal semi-quantitative changes of transcripts as well as qualitative changes by alternative splicing.

Transcripts of sarcomeric genes in human developing heart

During the developmental course, transcripts for αMHC and cTnT also changed. αMHC transcripts gradually increased after birth (Fig. 1C). cTnT transcripts showed isoform switching by alternative splicing (Fig. 1C). Transcripts of both cTnT1 and cTnT3 were expressed in the fetal heart, while cTnT1 transcripts decreased and cTnT3 transcripts increased and dominated in the heart after birth. In the developing human heart, cTnT1 and cTnT3 transcripts were predominantly expressed during fetal periods. After birth, the cTnT1 transcript decreased gradually. αMHC and cTnT were either not expressed or expressed very little, if any, in the skeletal muscle or embryonal cells.

Next, we studied the transcripts for MEF2 genes, αMHC and cTnT genes in hypertrophic hearts due to HCM or congenital anomaly (T/F), but we found no obvious change in those transcripts (data not shown).

Mouse MEF2 expression in heart development

To check if MEF2 transcripts change similarly in other animals, mouse hearts at various developmental stages were also studied. As we reported previously (Morisaki et al. 1997), mouse Mef2b transcripts were detected in the early stages of the embryonic heart and then down-regulated during the course of development (Fig. 2A). We also observed qualitative changes in transcripts Mef2a as well as Mef2d in the developing heart (Fig. 2A). Prominent changes of mouse Mef2d transcripts were found in the developing mouse heart as seen in the human heart.

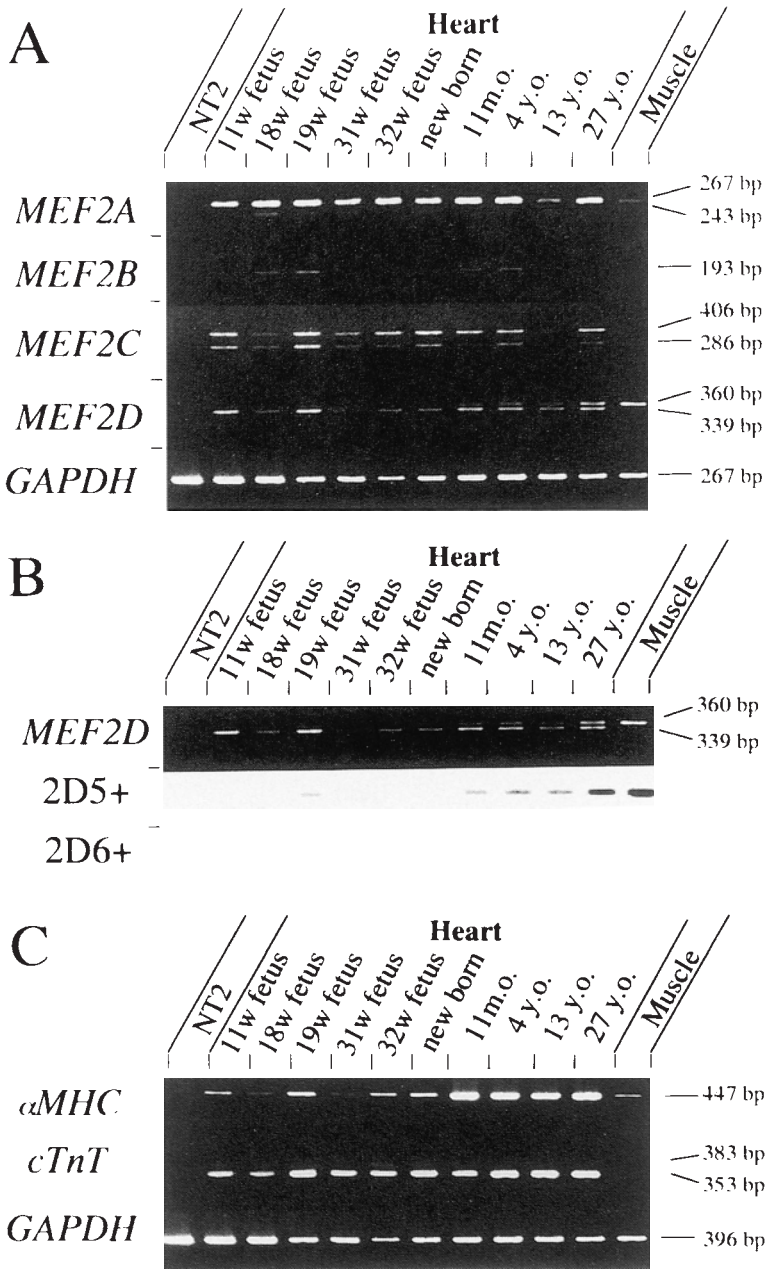

Fig. 1. Transcripts for MEF2 genes and sarcomeric genes in human developing heart. A) Transcripts for all MEF2 genes (MEF2A, MEF2B, MEF2C and MEF2D) were detected by RT-PCR. Different sizes of PCR products represented alternative spliced products foreach MEF2 gene. MEF2D transcripts containing 21-base exon (exon b) (360 bp) predominantly expressed after birth, while MEF2D transcripts without exon b (339 bp) predominantly expressed in embryonal period. B) MEF2D transcripts in human developing heart. MEF2D transcripts were studied by Southern blot. Relative ratio of MEF2D transcripts containing exon b to MEF2D transcripts without exon b exactly changed during perinatal period. C) Transcripts of sarcomeric genes in human developing heart. Transcript of αMHC gradually increased after birth. Transcripts of cTnT1 (383 bp) and cTnT3 (353 bp) predominantly expressed during embryonal periods. After birth, transcript of cTnT1 (383 bp) decreased gradually. These change of trascripts of cTnT isoform were due to alternative splicing of cTnT gene.

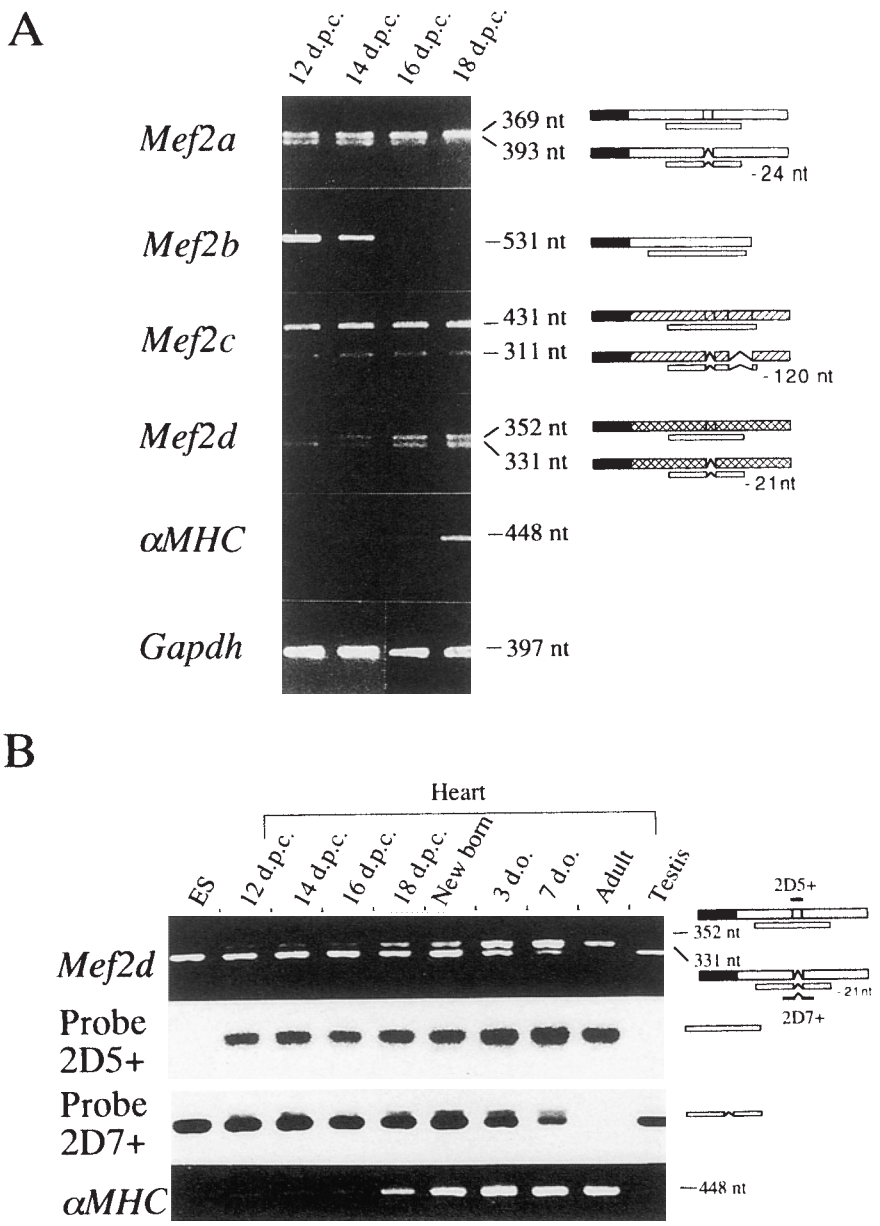

Fig. 2. Transcripts for Mef2 genes and α MHC gene in mouse developing heart. A) Transcripts for all Mef2 genes (Mef2a, Mef2b, Mef2c and Mef2d) and α MHC gene were detected by RT-PCR. Different sizes of PCR products represented alternative spliced products for each Mef2 gene. Larger Mef2a and Mef2d transcripts predominantly expressed in late embryonal heart, while smaller Mef2a and Mef2d transcripts predominantly expressed in earlier embryonal period. Transcript of αMHC increased during the course of development. B) Mef2d transcripts in mouse developing heart. Mef2d transcripts were identified by Southern blot analysis. Oligonucleotide 2D7+ corresponding to mouse Mef2d transcripts without exon b was used to detect Mef2d transcripts without exon b. Relative ratio of Mef2d transcripts containing exon b to Mef2d transcripts without exon b changed during perinatal period as transcript of αMHC increased.

Using Southern blot hybridization we confirmed that this change was due to alternataive splicing and could be correlated with the up-regulation of αMHC expression as well (Fig. 2B).

Discussion

Human MEF2 genes are known to be expressed in differentiated skeletal and cardiac muscles and to have multiple isoforms by alternative splicing within the transcription activation domain (Yu et al. 1992; Breitbart et al. 1993; McDermott et al. 1993). In contrast, the mouse Mef2 gene family, of which four genes have also been identified, have a unique member, mouse Mef2b, with properties different from the human MEF2B (Morisaki et al. 1997). Therefore, it is intriguing to clarify the expression profile of the human MEF2 gene family during cardiac development. In addition, we tried to identify the role of MEF2 genes in the perinatal heart, since relatively little is known about their mechanism for the terminal maturation of cardiac myocytes in these periods.

The human MEF2B gene is expressed in the heart throughout the developing stages including adult, while the mouse Mef2b gene is expressed in the early embryonic heart but then down-regulated according to the maturation of heart. Although the functional differences between human MEF2B and mouse Mef2b have not been well defined, the mouse Mef2b gene might have a distinct role in cardiac development.

Other MEF2 genes show qualitative transcript changes during the course of cardiac development, especially in MEF2D in the perinatal period. Both in humans and mice, MEF2D transcripts with the 21-base exon (exon b) are up-regulated according to cardiac maturation and finally become dominate in the adult heart, while MEF2D transcripts without exon b are dominate in the early embryonic heart. In human, variable but less abundant expression of MEF2D transcripts with exon b was observed in the mid gestational fetuses. In mice, Mef2a also shows an increasing expression of the transcripts containing a small exon, while the embryonic heart expresses dominantly Mef2 transcripts missing this small exon. However, the total amount of transcripts in each MEF2 member, except mouse Mef2b, do not exhibit remarkable changes during the course of cardiac development.

Our results indicate that alternative splicing of MEF2D is regulated during the perinatal period of human and mice development. In the same period, the amount of αMHC transcripts have been shown to be up-regulated. αMHC gene expression has been reported to be regulated by an MEF2 element and a thyroxin responsive element (TRE), both of which are reported to be necessary for full promoter activity. Although it is known that the thyroxin level is up-regulated in the perinatal period, the MEF2 element should also work to give a higher expression of the αMHC gene. Since the total amount of MEF2 gene products do not seem to change very much in this period, qualitative changes seen in MEF2D might play an important role for the correct regulation of the αMHC gene. However, the functional difference of the MEF2 protein with or without an exon b region was not clearly shown in vivo, although the previous report indicated

that both mouse and human MEF2D genes are active for transactivation in the transfection analysis.

Since cardiomyocytes are known to manifest dramatic changes, stopping proliferation, regulation of alternative splicing in MEF2 genes could be one of the important mechanisms for cardiomyocyte maturation in the perinatal period. Regulation of alternative splicing in the perinatal period should be a rather common pathway since both MEF2D genes and the cTnT gene show qualitative changes of transcripts in this period by alternative splicing. In other situations, including pathological conditions such as cardiac hypertrophy, transcriptional changes of the αMHC gene have been reported. Therefore, some of the hypertrophic changes in the heart seem to be regulated in a similar manner as seen in the embryonic stages but not by the same way.

Based on these observations, we propose that the alternative splicing as well as transcriptional regulation in MEF2 genes might be important for cardiomyocyte development, although further studies would be required to confirm our speculation.

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