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Differential Expression of the Myocyte Enhancer Factor 2 Family of Transcription Factors in Development: the Cardiac Factor BBF-1 Is an Early Marker for Cardiogenesis

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In the present study, we have used single chicken blastoderms of defined early developmental stages, beginning with the prestreak stage, stage 1 (V. Hamburger and H. L. Hamilton, J. Morphol. 88:49–92, 1951), to analyze the onset of cardiac myogenesis by monitoring the appearance of selected cardiac muscle tissue-specific gene transcripts and the functional expression of the myocyte enhancer factor 2 (MEF-2) proteins. Using gene-specific oligonucleotide primers in reverse transcriptase PCR assay, we have demonstrated that the cardiac myosin light-chain 2 (MLC2) and α-actin gene transcripts appear as early as stage 5, i.e., immediately after the cardiogenic fate assignment at stage 4. Consistent with this observation is the developmental expression pattern of DNA-binding activity of BBF-1, a cardiac muscle-specific member of the MEF-2 protein family, which also begins at stage 5 prior to MEF-2. Differential expression of DNA-binding complexes is also observed with another AT-rich DNA sequence (CArG box) as probe, but the binding pattern with the ubiquitous TATA-binding proteins remains unchanged during the same developmental period. Thus, the cardiogenic commitment and differentiation of the precardiac mesoderm, as exemplified by the appearance of cardiac MEF-2, MLC2, and α-actin gene products, occur earlier than previously thought and appear to be closely linked. The onset of skeletal myogenic program follows that of the cardiogenic program with the appearance of skeletal MLC2 at stage 8. We also observed that mRNA for the MEF-2 family of proteins appears as early as stage 2 and that for CMD-1, the chicken counterpart of MyoD, appears at stage 5. The temporal separation of activation of cardiac and skeletal MLC2 genes, which appears immediately after the respective fate assignments, and those of cardiac MEF-2 and CMD-1, which occur before, are consistent with the established appearance of the myogenic programs and with the acquisition pattern of the two tissue-specific morphological characteristics in the early embryo. The preferential appearance of BBF-1 activity in precardiac mesoderm, relative to that of MEF-2, indicates that these two protein factors are distinct members of the MEF-2 family and provides a compelling argument in support of the potential role of BBF-1 as a regulator of the cardiogenic cell lineage determination, while cardiac MEF-2 might be involved in maintenance of the cardiac differentiative state.

Early embryogenesis involves the establishment of cell lineages and territorial specifications resulting in the onset of differential gene activity which must include, at least initially, the expression of region-specific regulatory factors. The genes encoding these factors in turn mediate the pattern of the downstream tissue-specific gene transcription and thereby constitute the fundamental unit in the regulatory circuit that ultimately leads to morphogenetic changes during development. In the avian embryo, the mesodermal precursors of the cardiac muscle have been mapped and the location, morphogenetic movement, and differentiative transition of these cells have been delineated, beginning with the assignment of cardiogenic fate at stage 4 (≈20 h) (21) in gastrulating embryos (16, 34, 35, 47, 58). As the development proceeds, the precardiac mesodermal cells give rise to a single linear heart tube at about stage 10 (~33 h), at which time the organized myofibrils appear and rhythmic pulsations begin (34, 47). The molecular aspects of these early embryonic changes, especially during the period from the assignment of cardiogenic fate to the onset of morphogenetic differentiation, have not been studied, and it is not clear what developmental and spatial signals responsible for cardiac tissue specification emanate during early embryogenesis. The genes appearing during this critical period, especially those appearing at or immediately before the cardiac fate assignment at stage 4, are the candidates for cardiac lineage-specific regulatory factors. The analysis of the pattern of expression of these genes and their functional characterization would lead to an understanding of the complexities in establishment of the cardiogenic lineage and the mechanism(s) that controls cardiac tissue-specific gene expression.

Muscle differentiation is characterized by activation of transcription of genes specific for, among others, myofibrillar proteins, such as myosin light-chain (MLC) and heavy-chain (MHC) polypeptides, actin, tropomyosin, and troponin, which appear in a developmental stage- and tissue-specific manner (see references 13 and 55 for reviews). In the case of skeletal muscle, MyoD and members of the MyoD family of helix-loop-helix (HLH) proteins (11, 12, 30) play a pivotal role in induction of skeletal myogenic program via interaction with other ubiquitously expressed HLH proteins and subsequent binding to a consensus E-box promoter sequence (see reference 12 for a review). In several muscle genes, surrounding the functional E box are the target sites for other regulatory

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proteins, such as the AT-rich DNA-binding myocyte enhancer factor 2 (MEF-2), which cross-talk with the E-box-binding proteins (8, 15, 18, 52). The muscle creatine kinase gene is one of several examples for the E-box-mediated regulation of transcription (9, 18, 26, 27, 41) in which both MEF-2, a member of the MADS family of transcription factors (60), and the HLH proteins are required for activation of transcription (9). However, in other striated muscle genes, such as the cardiac MLC2 gene, there is no functional E box, and attempts to identify proteins structurally analogous to the MyoD family in cardiac muscle cells have not been successful (43). Thus, the mechanism for activation of cardiac muscle gene expression remains enigmatic, and it would appear that tissue-specific regulatory factors divergent from the myogenic HLH proteins are involved in activation of cardiogenic program. Specific isoforms of MEF-2–MADS proteins, generated through alternatively spliced RNA, have been identified in both heart and skeletal muscles (60) and are implicated in regulation of cardiac muscle-specific myogenic program (4, 12). We have recently identified a new transcription factor (BBF-1) in chicken embryonic cardiac muscle which recognizes the MEF-2 binding site in the cardiac MLC2 promoter but is distinguishable from MEF-2 on the basis of immunoprecipitation with an antibody which recognizes MEF-2 but not BBF-1 (61). Also, unlike MEF-2, which is present in both cardiac and skeletal muscles (18, 42), BBF-1 is restricted to cardiac tissue.

In this study, we analyzed the onset of cardiac myogenesis by monitoring the appearance of cardiac muscle-specific MLC2 mRNA in single embryos and that of BBF-1 and MEF-2 binding activities at defined stages of early blastodermal development. We observed that the first discernible cardiac MLC2 gene transcript appears at stage 5 whereas skeletal MLC2 mRNA appears at stage 8. Consistent with this observation is the developmental pattern of the binding activity for BBF-1, distinguishable from cardiac MEF-2, which appears at stage 5 prior to that of MEF-2. Differential appearance of DNA-binding complexes is also observed with another AT-rich DNA sequence (CArG box) (39) as probe, but the binding pattern with the ubiquitous TATA-binding proteins remains unchanged during the same developmental period. We also observed that mRNAs for the MEF-2 family of proteins are present in chicken embryos as early as stage 2. The mRNA for CMD-1, the chicken homolog of MyoD, on the other hand, appears at stage 6. The temporal separation in expression of mRNAs for cardiac and skeletal MLC2, which occurs immediately after the respective fate assignment, and those of CMD-1 and MEF-2 proteins, which occur before, are consistent with the established appearance of the two myogenic programs and the tissue-specific morphological characteristics. The early appearance of BBF-1 binding activity relative to cardiac MEF-2 suggests that BBF-1 is a distinct member of the MEF-2 family with regulatory potential in differentiation of precardiac mesoderm.

MATERIALS AND METHODS

Isolation and microdissection of chicken embryos of defined stages. White Leghorn fresh fertile eggs (SPAFAS, Inc., Norwich, Conn.) were incubated at 37.5°C in 60% relative humidity for appropriate durations to get different developmental stages. The blastoderms were freed from the yolk in Howard's ringer solution and separated from vitelline membranes by gentle jets of saline solution. The embryos were examined under dissecting binoculars at the magnification of ×67 for stage-specific characteristics described by Hamburger and Hamilton (21). Identification of stage 1, the prestreak

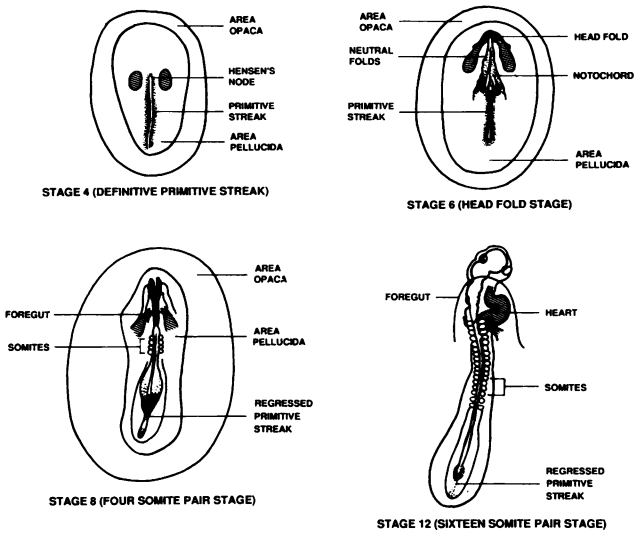

FIG. 1. Drawings illustrating four distinct stages of chicken embryonic development. Embryonic stages are classified according to Hamburger and Hamilton (21). Shaded areas denote HFRs.

embryo, to stage 4, the definitive streak embryo, was based on examination of the appearance and progression of the primitive streak; identification of stage 5 was based on head process formation, that of stage 6 was based on head-fold formation, and stage 7 onwards was identified by the number of somite pairs (Fig. 1). In addition to the number of somite pairs, the developing heart at stage 10 showed pulsation while straight, and the stage 11 embryo had a bent beating heart tube. The stage 12 embryo was characterized by an S-shaped heart. Stage 12 embryos were grouped together for isolation of the heart tubes. Only the beating heart tubes were excised with tungsten needles and used for the preparation of tissue extract (see below).

Isolation of mRNA and first-strand cDNA synthesis. Poly(A)$^+$ RNA was isolated from early-stage chicken embryos by using the Micro-Fast Track mRNA isolation kit (Invitrogen Inc.) according to the manufacturer's protocol. The first-strand cDNA was synthesized from poly(A)$^+$ RNA by oligo(dT) primer and Moloney murine leukemia virus reverse transcriptase (RT) with the first-strand synthesis kit from Stratagene.

PCR assay with mRNA from early embryos. In RT-PCR analysis (51), gene-specific primers were designed from the sequences available in GenBank. PCRs were done in a 50-μl volume containing 2 to 5 μl of the cDNA reaction mixture, 50 pmol of each primer, 200 mM deoxynucleoside triphosphates, and 1.5 U of *Taq* polymerase (Gene Amp; Perkin-Elmer Cetus), at 96°C for 30 s, 55°C for 1 min, and 72°C for 3 min. The authenticity of the amplified products was verified by agarose gel electrophoresis with appropriate markers and by sequence determination when necessary with an automated DNA sequencer (Applied Biosystems).

Preparation of embryonic extracts. Embryonic extracts were prepared by the method of Dignam et al. (10) following modifications by Mohun et al. (40). Typically, 20 to 40 staged embryos were washed briefly in 500 μl of buffer A, containing 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid) (pH 7.9), 10 mM KCl, 1.5 mM $MgCl_2$, 0.5 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 10 μg of aprotinin per ml, and 10 μg of leupeptin per ml, and the

washed embryos were homogenized in 50 to 200 μl of buffer C, containing 20 mM HEPES (pH 7.9), 1.5 mM $MgCl_2$, 420 mM NaCl, 0.2 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, 0.5 mM dithiothreitol, 25% glycerol, 4 μg of aprotinin per ml, and 4 μg of leupeptin per ml. The homogenate was kept on a rocking platform at 4°C for 30 min and then centrifuged in an Eppendorf microcentrifuge at 13,000 rpm for 10 min. The supernatant was again centrifuged at 13,000 rpm for 10 min, aliquoted, and then snap frozen in a dry ice-ethanol bath for storage at −70°C.

Gel mobility shift assay. Gel mobility shift assay was done as described earlier (61). Double-stranded DNA fragments obtained by renaturation of chemically synthesized oligonucleotides were radiolabeled at the 5′ end by polynucleotide kinase and [γ-^{32}P]ATP and used for the binding assay. DNA probe (5,000 cpm) and nuclear proteins (5 to 10 μg) were incubated in 10 mM HEPES (pH 7.9)–80 mM NaCl–5 mM $MgCl_2$–0.5 mM EDTA–1 mM dithiothreitol–12.5% glycerol at 4°C for 90 min and were separated on the 6% polyacrylamide gel. For competition, the unlabeled DNA in 100-fold excess was added to the reaction mixture before the addition of nuclear extract. For immunoprecipitation assay, 0.5 μl of preimmune serum or antiserum to RSRFC4 (45) was preincubated with the nuclear extracts for 30 min at 4°C before addition of the DNA probe.

RESULTS

Expression of cardiac and skeletal muscle-specific genes in early embryogenesis. In order to define myogenesis in terms of the onset of tissue-specific expression of cardiac and skeletal genes, we examined single embryos of defined stages by RT-PCR assay (51) to detect the appearance of cardiac and skeletal muscle MLC2 mRNAs with gene-specific DNA primers. As shown in Fig. 2A, the first discernible signals for cardiac (top panel) and skeletal (middle panel) MLC2 mRNAs appear at stages 5 and 8, respectively. No cardiac MLC2 signal was detected before stage 5, even after several additional cycles of amplification. Consistent with the onset of MLC2, cardiac α-actin mRNA was detected at stage 5 and skeletal α-actin mRNA was detected at stage 7 (Fig. 2B and C). On the other hand, the ubiquitous β-actin mRNA was present as early as stage 1 in the same embryo (Fig. 2A, bottom panel). The lack of cross-amplification of skeletal and cardiac cDNAs by the respective DNA primers demonstrates the tissue specificity of the primer sequences used. Multiple embryos of the same stage were examined separately to ensure reproducibility. The amplified products were also sequenced, when necessary, to verify reliability of PCR amplifications (see Materials and Methods). These data thus demonstrate that the cardiac and skeletal muscle differentiation begins immediately after the assignment of the cell lineages at stages 4 and 7, respectively, in developing embryos and well before the onset of morphological differentiation for the respective muscles. The temporal separation of the two transcriptional events (stage 5 and stage 8) is consistent with the established separation in appearance of the two tissue-specific morphological characteristics (stage 10 and stage 13). To our knowledge, this is the first evidence for the presence of muscle-specific gene transcripts in early precardiogenic and presomatogenic cells of the blastoderm. In a previous study (3, 22), ventricular MHC was first detected in stage 7 embryos and the presence of α-actin mRNA was demonstrated in the cardiac tube just prior to myotome formation (53). Subsequently, it was shown that single cells isolated from the precardiogenic area of stage 4 chicken embryos synthesize cardiac MHCs, but only after 2 days of cultivation in vitro (17).

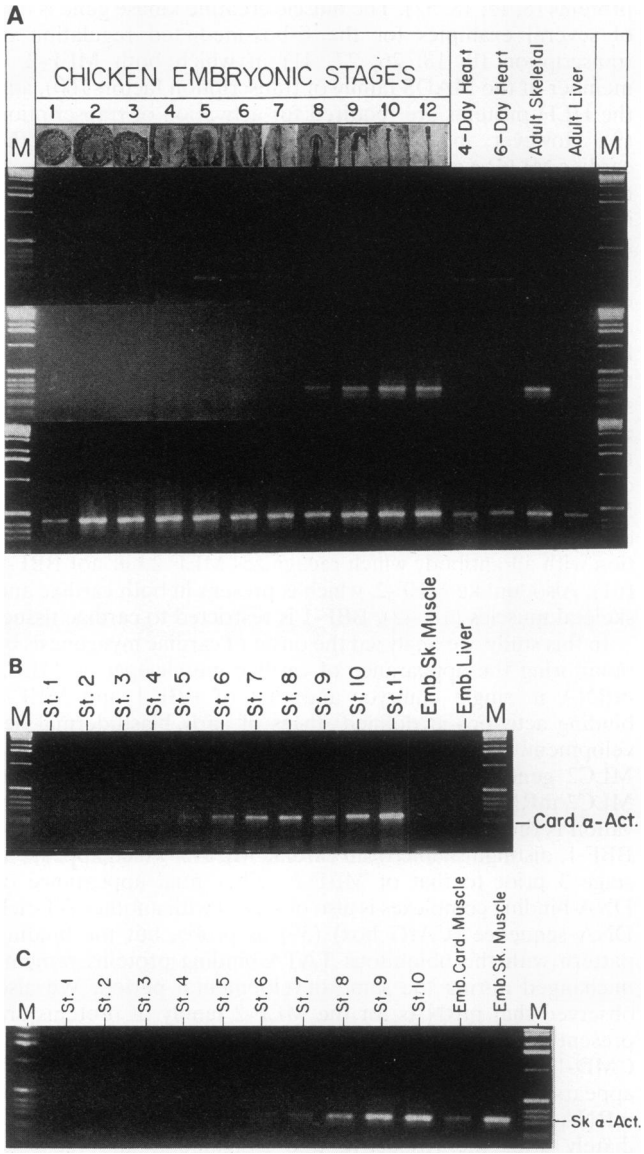

FIG. 2. RT-PCR analysis of muscle gene expression in early chicken embryos. (A) Poly(A)$^+$ RNAs isolated from a single chicken blastoderm at various stages of development (shown on top) were subjected to RT-PCR analysis with gene-specific DNA primers. The top panel shows the developmental expression of cardiac MLC2 mRNA (forward primer, GAGGAGATCGATCAGATG; reverse primer, ACTTTATTGCGTGTGGCTGC), the middle panel shows skeletal MLC2 mRNA expression (forward primer, CTTCACCCCCG AGGAGAT; reverse primer, TTAATTGGGATAGCCGGG), and the bottom panel shows β-actin mRNA (forward primer, GTTTG AGACCTTCAACACC; reverse primer, CCAAGAAAGATGGCTG GAAG). The PCR products were analyzed on a 1.5% agarose gel stained with ethidium bromide. The marker lanes are 1-kb DNA ladder (Gibco-BRL). (B) RT-PCR analysis of expression of cardiac α-actin mRNA from developmental stages as indicated (forward primer, GCGGGCTTTGCTGGGGACGA; reverse primer, TCAGG GGCCACACGGAGTTC). (C) RT-PCR analysis of expression of skeletal α-actin mRNA from developmental stages (forward primer, TATGGAGTCCGCTGGGATCC; reverse primer, ATCTTCGTAA ATGCCACCTG).

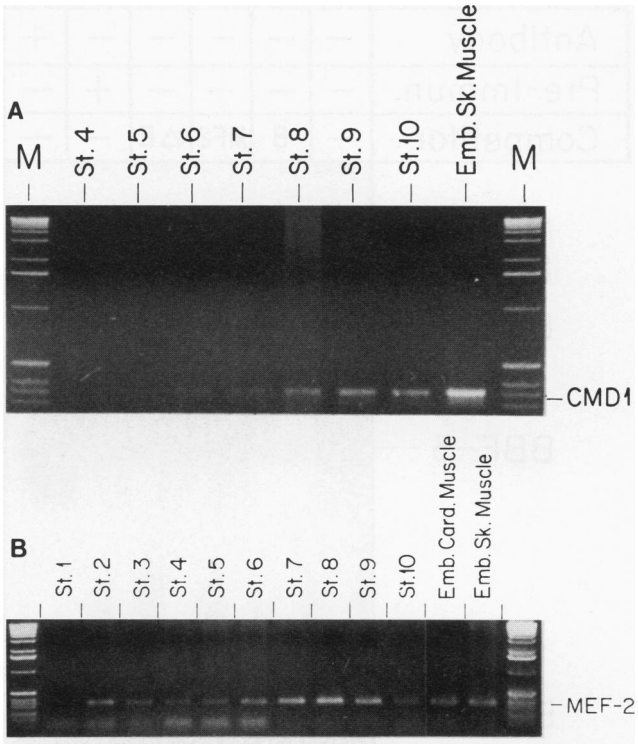

FIG. 3. Expression of myogenic regulatory factors in developing chicken embryos assayed by RT-PCR analysis. Poly(A)$^+$ RNAs isolated from a single chicken blastoderm at various stages were analyzed by RT-PCR assay with primers specific for CMD-1 mRNA (A), the chicken homolog of MyoD (forward primer, CAGATTGCTTC CCCCACCAA; reverse primer, TCCCTATTCTCCAAAGCCCA), and for a conserved sequence for cDNA for MEF-2-like proteins (B) (forward primer, GATTTCCACTCTCCTGT; reverse primer, TTAC CATGGGACATCT; see text and legend to Fig. 4 for details). The PCR products were analyzed on a 1.5% agarose gel stained with ethidium bromide as for Fig. 2. M in panel A and unlabeled lanes in panel B denote 1-kb DNA ladder used as size marker.

Developmental expression of mRNAs for chicken CMD-1 and the MEF-2 family of proteins. The expression of myogenic HLH determination genes has become a criterion for defining the skeletal myogenic lineage, but the cardiogenic counterparts of the MyoD family have not been identified to date. All members of the MyoD family have been detected first in the somites and subsequently in limb buds during muscle fiber formation (53). In some cases, one or another homolog of the MyoD family has been found just prior to compartmentalization of somites (7, 44). Using a DNA primer specific for CMD-1 (32), the chicken skeletal muscle homolog of MyoD, we analyzed (Fig. 3A) single blastoderms at defined stages as above and observed that the first signal for CMD-1 can be detected as early as stage 5, i.e., prior to the appearance of the first somite and the onset of transcription of the skeletal MLC2 gene at stage 8 (Fig. 2A). This is consistent with the expected role of HLH proteins in programming skeletal myogenesis. The MyoD homolog in *Xenopus laevis*, XMyoD, was also reported to be present in mesoderm prior to somite formation (25). Since we did not detect CMD-1 during precardiac developmental stage 4 or earlier, we conclude that CMD-1 is not involved in specification of cardiogenic lineage.

Unlike the MyoD family of proteins, there is no marker available for cardiogenic programming of embryonic cells.

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AGCCCCCGCA GGAGACGNAG NCCCCACAGA CCCCGCAGNA GACGNCGCAG GGCCAGGAGA
AGCCGCCGCC ACCACCGCAG CCCCAGCCAC AACCCCCGCA GCCCCAGCCC CGACAGGAAA

TGGGNCGCTC TCCCGTCGAC AGGCTCAGAA GGTACAGCAG GTCCTACGAC GGGAGGGAAC
TGGGGCGCTC CCCTGTGGAC AGTCTGAGCA GCTCTAGTAG CTCCTATGAT GGCAGTGATC

NGGAGGACCC TCGGAGNGAT TTCCACTCTC CTGTNCTGGT GGGNAGGGCG NCCAATTCAN
GGGAGGATCC ACGGGGCGAC TTCCATTCTC CAATTGTGCT TGGCCGACCC CCAAACACTG

AAGATAGGGAGAGTCCGTCAGTAAAACGAATGAGGATGGACACGTGGGTGACATAAGCTT
AGGACAGAGAAAGCCCTTCTGTAAAGCGAATGAGGATGGACGCGTGGGTGACCTAAGGCT

GCGGTGTTGC CTCTTCAGTT TTGTGTTCTC AAAATGAACT GTCCTGACAT ATCTAAATTT
TCCAAGCTGA TGTTTGTACT TTTGTGTTTC TGCAGTGACC TGCCCTACAT ATCTAAATCG

ATNAATAAGGACATGAAATAAGTATATTTATATGTATATACATACGTGGATATATATATC
GTAAATAAGGACATGAGTTAAATATATTTATATGTACATACATATATATCCCTTTACA

TTCACATGCA TATATATGTG CTAGTGTGTG TATGTGTGGG TGTATCATCA GGCACTTATT
TATATATGTA TGTGGGTGTG AGTGTGTGTG TAGCATACAC AGAGTGTTAC ATACACAGAA

ACAAACTTCT TGTATAGCTG NAGATGTCCC ATGGTAA    BBF-1/MEF-2 Chicken
TCAGGCACTT ACCTGCAAAC TCCTTGTAGG TCTGCAG    MEF-2 human
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FIG. 4. Partial nucleotide sequence of the candidate cardiac chicken MEF-2 cDNA (upper line), isolated in our laboratory, is compared with that of the human homolog (lower line, bp 1703 to 2159). The cDNA was isolated from the embryonic chicken cardiac cDNA library with RSRFC4 cDNA (45) as probe (unpublished data). The conserved nucleotides are shown in boldface. The oligonucleotides used for the PCR are underlined.

Since MEF-2 proteins exist in both cardiac and skeletal muscles, and at least one member of this protein family, BBF-1, is cardiac muscle specific (61), we examined the expression of mRNA for MEF-2 proteins. We have recently isolated a cDNA clone by screening the 72-h chicken embryonic cardiac cDNA expression library (unpublished work) with human RSRFC4 cDNA (45) as probe. Partial sequence of the cDNA clone revealed that it has a significant homology with human MEF-2 cDNA clone (60) (Fig. 4). Using DNA primers encompassing this domain, we observed (Fig. 3B) that mRNA transcripts for the MEF-2 family of proteins are present in very early embryos, before the appearance of CMD-1 mRNA, beginning at the pregastrulation stage, stage 2. Although at present it is not possible to distinguish between mRNAs for different members of cardiac MEF-2, the evidence (see below) that functional expression of BBF-1 precedes that of MEF-2 presents a compelling argument that expression of mRNA for BBF-1 must precede that of MEF-2 mRNA.

Differential expression of MEF-2 and BBF-1 DNA-binding activities in early development. To examine the possibility whether the functional expression of BBF-1 and MEF-2 DNA-binding activities is developmentally regulated in harmony with the appearance of MLC2 mRNA, we isolated protein extracts from embryos of defined stages and tested for DNA-protein complex formation in gel mobility shift assay with element B sequence (MEF-2 site) (Fig. 5) in cardiac MLC2 promoter as probe. As shown in Fig. 6, a defined BBF-1–DNA complex appears first at stage 5, with a barely detectable signal at stage 4. The MEF-2-like activity, on the other hand, begins to appear only at stage 8 and becomes defined at stage 12. The origin of an additional faster-moving band at stage 8 is not presently known. MEF-2 complex, on occasion, is known to produce a doublet in gel shift assay. To ascertain whether MEF-2 and BBF-1 from early embryos are the same as those obtained from the differentiated heart muscle, we examined the immunological and DNA recognition properties of these proteins. Figure 7 shows that preincubation of extracts isolated from stage 12 embryos with antibody RSRFC4 (45) eliminated MEF-2 without affecting BBFs, as observed earlier (61). Competition with MEF-2 DNA eliminated both MEF-2 and

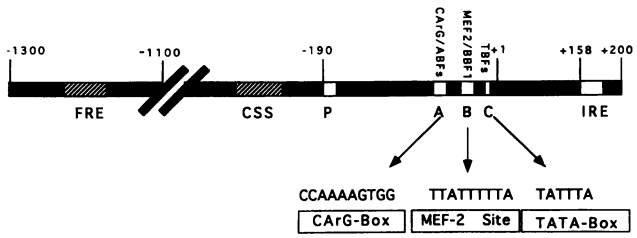

FIG. 5. Functional sequence domains in cardiac MLC2 promoter. The functional sequence domains in MLC2 promoter were delineated (19, 46, 54, 61). The sequences of element A, B, and C are indicated. Element P is a positive regulator (61); element CSS is cardiac muscle-specific sequence, which represses cardiac MLC2 transcription in skeletal muscle (54); and FRE is Fos-responsive element (19). TBFs, TATA-binding proteins.

BBF-1 complexes without affecting BBF-2 and BBF-3. Competition with mutant DNA, in which element B (MEF-2 site) core sequence was deleted, did not affect MEF-2 and BBF-1 complex formation, although BBF-2 was eliminated. These findings are consistent with previous studies (61) which utilized nuclear extracts from 12-day-old differentiated heart tissue. Likewise, the binding to B-DNA (MEF-2) site was dependent upon the element B (MEF-2) site core sequence, as single site mutations in the core sequence were ineffective in inhibiting both MEF-2 and BBF-1 (data not shown). These results thus demonstrate that MEF-2 and BBF-1 from early heart tube of

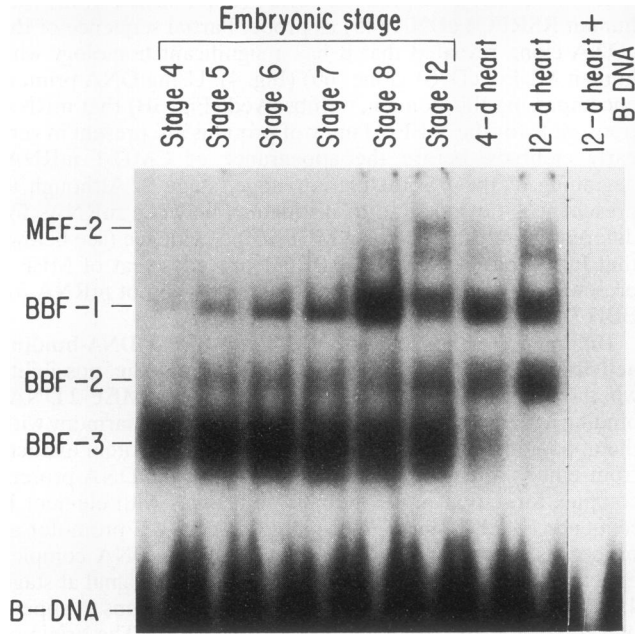

FIG. 6. Developmental expression of MEF-2 site (element B)-binding proteins by gel mobility shift assay. Embryonic extracts prepared from various stages of chicken embryos were assayed for binding to a synthetic 30-bp oligonucleotide containing element B (MEF-2 site) of the chicken cardiac MLC2 promoter (−50 to −42) (Fig. 5). Four major complexes, identified as MEF-2, BBF-1, BBF-2, and BBF-3, bind to the AT-rich core sequence of element B (MEF-2 site) probe. The stages of embryonic development and embryonic ages at which extracts were isolated are indicated on top. Stages were assigned on the basis of correspondence of time of incubation with morphological parameters observed in our laboratory.

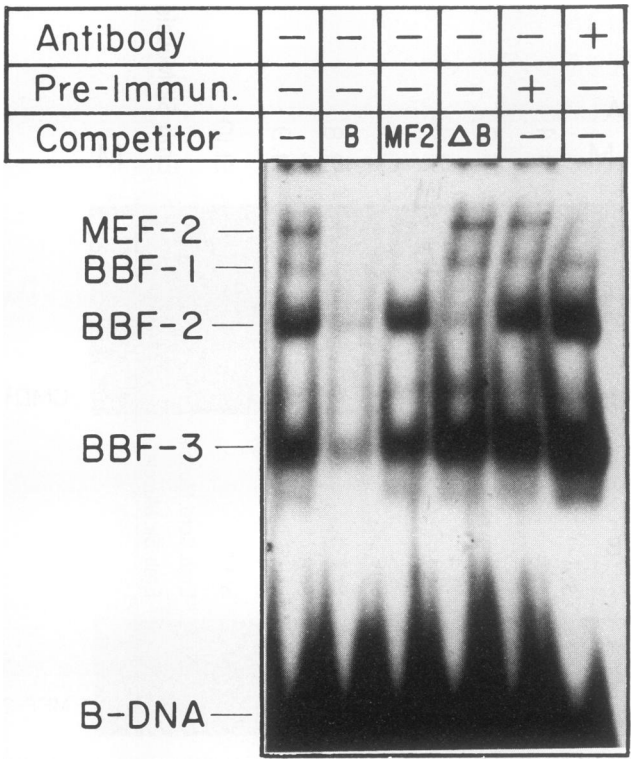

FIG. 7. BBF-1 and MEF-2 are two distinct DNA-binding proteins. Gel mobility shift assay was done with stage 12 chicken embryonic extracts with MEF-2 site (element B) oligonucleotide as probe. Competition was done with an oligonucleotide containing MEF-2 sequence (MF2) from the muscle creatine kinase enhancer, or the oligonucleotide containing the mutated element B sequence (ΔB) and element B-DNA. Extracts were also preincubated either with an antibody against RCSRFC4 (45) or with the preimmune serum prior to gel shift assay with the element B DNA as probe as described in Materials and Methods.

stage 12 embryos are the same as those obtained from differentiated heart tissues of 12-day-old embryos. With the MEF-2 site DNA of the muscle creatine kinase promoter as probe and extracts from stages 6 and 9 of chicken embryos, a previous study (33) also noted the presence of a complex, with mobility similar to that of BBF-1, in stage 6 and stage 9 extracts, but the MEF-2-like complex was found only in stage 9 embryos. BBF-2 and BBF-3 are also differentially regulated during development (Fig. 6). The developmental expression of BBF-2 was similar to that of BBF-1, but BBF-3 was down-regulated after reaching a maximum at about stage 6. The tissue specificity of these proteins, however, is not yet established.

For comparison, we also examined the gel mobility shift patterns in single embryos with another AT-rich DNA (element A), the CArG box (39), and the TATA box (element C) (Fig. 5) in cardiac MLC2 promoter as probes. The appearance of the CArG-binding proteins is also differentially regulated during development with some similarity to that of element B-binding pattern (Fig. 8A) while the pattern of the ubiquitous TATA-binding proteins remained unchanged throughout the same developmental period (Fig. 8B), indicating that the developmental changes observed for MEF-2 and CArG-binding proteins (MEF-2 and BBF-1) in gel shift assays were not due to nonspecific losses in proteins during experimental procedures.

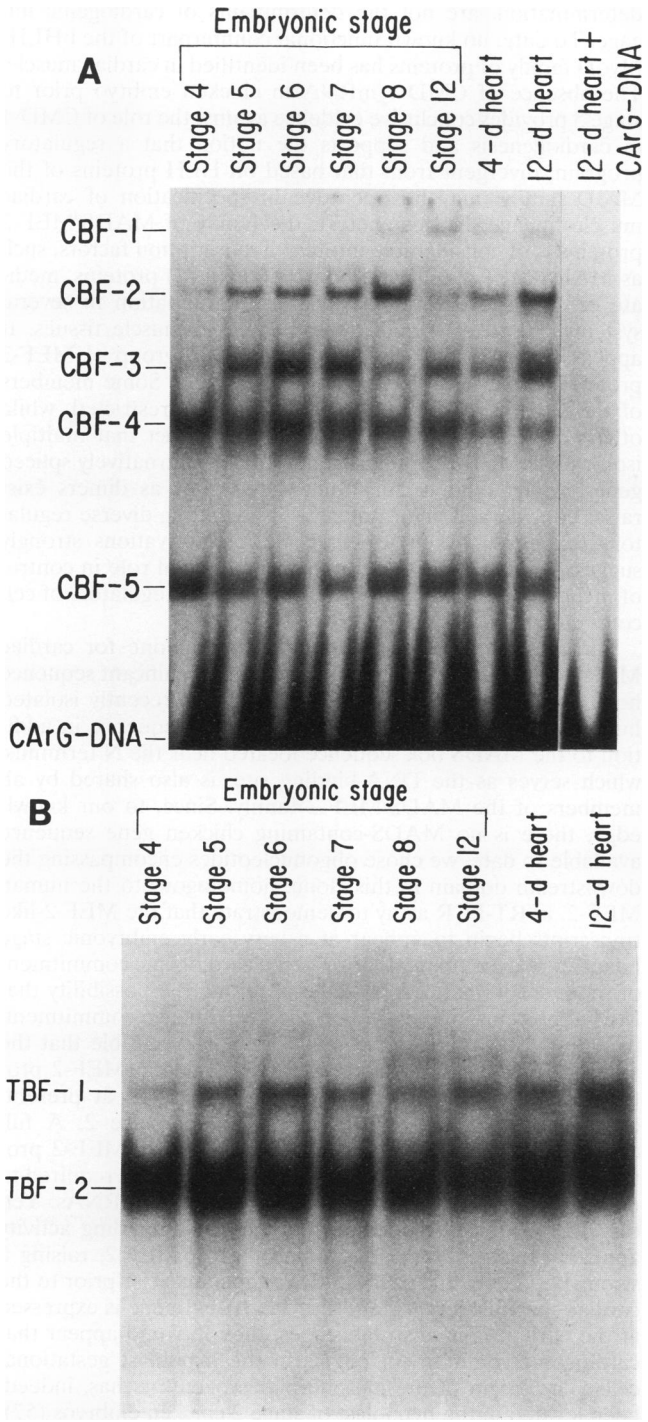

FIG. 8. (A) Development expression of CArG-binding proteins (CBFs). Gel mobility shift assays were done with CArG (element A) DNA as probe as for Fig. 6. (B) Development expression of TATA-binding proteins (TBFs). Gel mobility shift assay was done with the TATA sequence (element C) (Fig. 5) as for Fig. 6.

MEF-2 and BBF-1 in early embryos are localized in early embryonic heart tube only. Since MEF-2 proteins are known to be present in both skeletal and cardiac muscle tissues (9, 42), we asked the question whether the MEF-2 activity observed in early embryos above is of cardiac origin. For this purpose, we

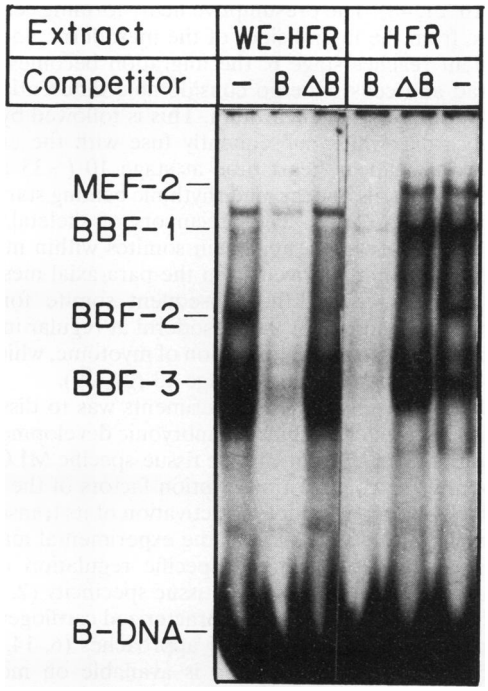

FIG. 9. BBF-1 and MEF-2 are expressed only in the embryonic heart tube region of the stage 12 embryo. Protein extracts were prepared from the HFR of the stage 12 embryo and from the embryo free of HFR (see text). The extracts were assayed by gel shift assay with element B (MEF-2 site) DNA as probe. In order to ascertain the specificity of complexes, competitions were done with a 100-fold excess of nonradioactive element B DNA or an oligonucleotide containing a mutated element B sequence (61). WE, whole embryo.

isolated the heart tubes from several embryos at stage 12 of development (Fig. 1), at which time a well-defined heart tubular structure can be identified and excised microscopically free of the rest of embryonic tissues. Protein extracts pooled separately from several stage 12 heart tube (heart-forming region [HFR]) and the remaining embryos (whole embryo-HFR) were compared for DNA binding. As shown in Fig. 9, the gel shift pattern obtained from the heart tube region (HFR) alone is identical to that observed in extract from whole embryos (Fig. 6 and 7). The extract isolated from the remaining (heart-free) embryos (whole embryo-HFR), on the other hand, lacked both MEF-2 and BBF-1, but contained BBF-2 and BBF-3. As before, competition with element B eliminated all complexes, but B-DNA with core sequence deleted (ΔB) was ineffective, except for BBF-2. These data demonstrate unequivocally that both MEF-2 and BBF-1 of stage 12 embryos are of cardiac origin, as they are produced exclusively in the early heart tube. Yet, these two MEF-2 family members are distinct proteins distinguishable by their differential expression in early development. The lack of MEF-2 activity in HFR-free embryos would indicate that the level of noncardiogenic MEF-2 is either below the detection level for DNA-binding assay or absent at this stage. Indeed, Breitbart et al. (5) have observed that despite the ubiquitous distribution of the MEF-2 family of mRNAs, these proteins are highly restricted to cell types that harbor their activity.

DISCUSSION

In avian embryos, cells of the lateral plate mesoderm are committed to cardiogenic lineage at stage 4 of development

(Fig. 1) (16, 21, 48). The presumptive heart-forming cells begin to migrate from the lateral sides of the mesoderm, and as the development reaches stage 6, the migration becomes medial and rostrad and cells undergo considerable differentiation to form epicardium and endocardium. This is followed by fusion of epimyocardia, which subsequently fuse with the endocardium to form a linear heart tube at stage 10 (~33 h) (35). Organized myofibrils appear, and rhythmic beating starts while the fusion is in progress. The precursors of skeletal muscle fibers, on the other hand, appear in somites within myotome (24). The first somite, derived from the para-axial mesoderm, appears at stage 7, and the subsequent somite formation results from the budding of the mesoderm at regular intervals. Further changes lead to the formation of myotome, which gives rise to skeletal muscle at about stage 13 (24, 29).

The purpose of the present experiments was to dissect the molecular events in early chicken embryonic development with respect to the expression of cardiac tissue-specific MLC2 gene transcript and the cognate transcription factors of the MEF-2 family which play a pivotal role in activation of its transcription (61). Ventricular MLC2 gene was the experimental marker of choice for elucidation of tissue-specific regulation of gene activity, as it exhibits strict cardiac tissue specificity (2, 50, 54). Although previous studies have characterized cardiogenesis by immunochemical and/or molecular approaches (6, 14, 17, 23, 37, 49, 58, 59), little information is available on molecular events in early stages of development during which the committed precardiac mesoderm enters cardiogenic differentiation. With a monoclonal antibody specific for sarcomeric MHCs, it was recently reported that MHC is the earliest muscle marker protein detectable as early as stage 7 in chicken development, i.e., approximately 8 h prior to myofibrillogenesis (22). Our studies, employing the sensitive RT-PCR assay and single embryos of defined developmental stages, demonstrate that both cardiac MLC2 and α-actin mRNAs can be discerned as early as stage 5, i.e., immediately after the cardiogenic fate assignment at stage 4 and at least 15 h prior to stage 9 and after, at which time myofibril formation begins. The short developmental interval (~1 h) between the cell commitment stage and activation of these genes leads one to speculate that these two events might be mediated by closely linked regulatory pathways and that the signal(s) which initiates cardiac tissue-specific gene expression becomes operative much earlier than was previously expected (59). This would also be consistent with the interpretation that specification of the precardiac mesoderm and initiation of cardiogenic differentiation are temporally linked and may involve a common regulatory factor(s). Other characteristics of cardiac development, such as myofibril assembly and production of extracellular matrix, etc., must follow the expression of tissue-specific genes.

The onset of skeletal myogenic program follows that of cardiogenic program as exemplified by the appearance of skeletal MLC2 mRNA at stage 8 in comparison with cardiac MLC2 mRNA at stage 5 (Fig. 2). That the temporal relationship between the appearance of these two mRNAs is physiologically relevant is demonstrated by the correspondence in appearance of skeletal muscle differentiation (stage 13) (24) with myofibrillogenesis (stage 10), at which time cardiac muscle contraction begins. This correlation and the fact that there are common genes expressed in skeletal and cardiac muscles would predict the existence of similar, if not identical, mechanisms for control of gene transcription in these two closely related tissue types. Yet, the cues responsible for activation of the respective myogenic lineages are different, since the bHLH proteins of MyoD, the hallmark for skeletal muscle lineage determination, are not the determinants of cardiogenic lineage. To date, no known functional counterpart of the bHLH-MyoD family of proteins has been identified in cardiac muscle. The absence of CMD-1 mRNA in chicken embryo prior to stage 5 provides conclusive evidence against the role of CMD-1 in cardiogenesis and supports the notion that a regulatory program, divergent from that based on HLH proteins of the MyoD family, may be operative in specification of cardiac muscle lineage. In this context, the family of MADS/MEF-2 proteins is of considerable interest. Transcription factors, such as MADS/MEF-2 (60) and homeodomain (8) proteins, mediate cell lineage determination and differentiation in several systems (20, 28, 56) and are expressed in muscle tissues. It appears that MEF-2 activity is caused by a group of MEF-2 proteins, rather than by a single polypeptide. Some members of the MEF-2 family of proteins are tissue restricted, while others are ubiquitous (4, 31, 36, 38). The fact that multiple isoforms of MEF-2 proteins, generated by alternatively spliced genes, which bind to functional DNA sites as dimers exist raises considerably their potential in executing diverse regulatory functions. Taken together, these observations strongly suggest that the MEF-2 proteins play a pivotal role in control of myogenesis and point to their potential as regulators of cell commitment (1, 4, 12, 36, 38).

Partial sequence of a candidate cDNA clone for cardiac MEF-2 isolated in our laboratory shares a significant sequence homology in the C terminus (Fig. 4) with a recently isolated human MEF-2 cDNA clone (60). Another sequence in addition to the MADS box sequence located near the N terminus, which serves as the DNA-binding site, is also shared by all members of the MADS/MEF-2 family. Since, to our knowledge, there is no MADS-containing chicken gene sequence available to date, we chose oligonucleotides encompassing the downstream domain in this clone, homologous to the human MEF-2, in RT-PCR assay to demonstrate that the MEF-2-like transcripts begin to appear at a very early embryonic stage (stage 2) of development, prior to the cardiogenic commitment of the mesodermal cells at stage 4, raising the possibility that MEF-2 proteins are involved in early cell lineage commitment, including the cardiogenic commitment. It is possible that the amplified products contain sequences of several MEF-2 proteins, including noncardiac MEF-2. We cannot, at present, establish that BBF-1 mRNA is present at stage 2. A full characterization of several candidate clones for MEF-2 proteins presently in progress in our laboratory will be required to distinguish between BBF-1 and cardiac MEF-2 mRNAs. Yet, our data establish unequivocally that BBF-1 binding activity appears at stage 4 or 5, preceding that of MEF-2, raising a strong possibility that BBF-1 mRNA appears at or prior to the cardiac specification at stage 4. If the BBF-1 gene is expressed at the early primitive streak stages, then it would appear that cardiogenic commitment begins in the hypoblast gestational cells. The origin of the mesodermal progenitors has, indeed, been located in the hypoblast of stage 2 chicken embryos (57). We, therefore, view BBF-1 as a crucial member of the MEF-2 family of proteins which influences the downstream events via activation of cardiac muscle gene transcription and serves as a determinant of cardiogenic cell lineage.

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S.G. and P.Q. contributed equally to this paper.

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