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Identification of a novel cardiac-specific transcript critical for cardiac myocyte differentiation

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SUMMARY

A novel cDNA, pCMF1, which is expressed exclusively and transiently in the myogenic cells of the differentiating chicken heart was isolated and characterized. The full-length cDNA of pCMF1 has one open reading frame encoding 1538 predicted amino acids. While computer analysis predicts the presence of specific structural motifs, the overall sequence of pCMF1 is unique. The pattern of pCMF1 gene expression during heart formation was determined by whole-mount in situ hybridization. pCMF1 is transiently expressed within the myogenic cells of the primitive heart tube from stages 9 to 18 and is not detected in the heart or any other tissue thereafter. A replication-deficient retrovirus was used to mediate pCMF1 antisense expression in cardiogenic mesoderm. These analyses determined that the presence of pCMF1 antisense sequences disrupted myosin heavy chain expression during cardiac mesoderm differentiation. pCMF1 antisense had no effect on myosin heavy chain expression in differentiated cardiac myocytes. These data suggest a potential function for pCMF1 during cardiac myogenesis.

Key words: cardiac myocyte, pCMF1, myogenic cell, chick, heart

INTRODUCTION

In the chicken embryo, the cells that give rise to the heart are some of the first to gastrulate (Garcia-Martinez and Schoenwolf, 1993). After gastrulation, cardiac progenitors have been mapped to mesoderm anterior and lateral to Henson's node at stage 4 [(Hamburger and Hamilton, 1951); Rawles, 1943]. Cells within stage 4 cardiac mesoderm are specified to the cardiogenic cell lineage (Gonzalez-Sanchez and Bader, 1990). The initiation of cardiac muscle-specific gene expression and conversion of progenitors to cardiac myocytes begins at approximately stage 8-9 (Bisaha and Bader, 1991; Han et al., 1992; Yutzey et al., 1994) and progresses from anterior to posterior within the cardiogenic mesoderm in the following stages. All, or most all, cardiomyogenic progenitors are converted into cardiac myocytes by stage 16 and no undifferentiated cardiac myoblasts are thought to exist thereafter (Manasek, 1968; Mikawa et al., 1992). After these stages of development, the increase in number of cardiac myocytes occurs by the division of previously differentiated cardiac myocytes (Manasek, 1968). Thus, the time frame when mesoderm is converted to cardiac myocytes and when cardiac-specific genes are activated in differentiating mesoderm is relatively short in the avian embryo (i.e. stage 8-16). Still, this period represents a unique time in the life history of the cardiomyogenic cell line.

From previous studies on the differentiation of cardiogenic mesoderm (Reviewed in Litvin et al., 1992), one would expect regulatory proteins involved in the activation of cardiac genes to be expressed during this period. In skeletal myogenesis, activation and regulation of skeletal muscle-specific genes are mediated in part by the MyoD family of regulatory proteins (reviewed in Olson, 1993). Although some of the same contractile proteins are expressed in both the skeletal and cardiac muscle lineages, the mRNAs of the MyoD family have not been detected in the heart (Charles de la Brousse and Emerson, 1990; Olson, 1993). Still, using an antiserum (anti-H2) against the second helix of the bHLH domain in MyoD, Litvin et al. (1993) detected nuclear protein(s) which were transiently expressed in the developing heart during the earliest stages of cardiac differentiation (stages 7-16). Thus, this study predicted that proteins immunologically related to the HLH protein family would be expressed during the initial phase of cardiogenesis.

Identification of molecules that regulate or are important in the initial differentiation of cardiogenic cells is essential to our understanding of heart development. In order to isolate proteins reactive with anti-H2, we screened a stage 11 chicken embryonic cDNA expression library with anti-H2 antiserum. 22 cDNA clones were isolated and one of the clones, pCMF1 was analyzed further due to its heart-specific expression pattern. In the present study, we report the full-length cDNA cloning and characterization of pCMF1. The predicted amino acid sequence of pCMF1 represents a new protein that does not share homology to any sequences in the GenBank. A potential nuclear localization sequence is present in the carboxyl terminal sequence of pCMF1. pCMF1 is expressed exclusively in the myocardium and not in the endocardium of

the developing heart. In addition, pCMF1 is not found in the skeletal muscle lineage or in any other cell types. pCMF1 is transiently expressed during heart formation during the period when cardiac-specific genes are activated and cardiac progenitors are converted to cardiac myocytes. Treatment with retrovirus-mediated antisense disruption of pCMF1 function in the stage 4 precardiac mesoderm inhibits myosin expression and implies a role for pCMF1 in cardiac myogenesis.

MATERIALS AND METHODS

cDNA library construction and screening

A directional cDNA expression library was constructed in λEXlox (Novagen) using 10 μg of stage 11 chicken embryo poly(A)+ RNA. Poly(A)+ RNA was isolated using the FASTrack Kit (Invitrogen) from 1000 frozen stage 11 embryos. Approximately 4×10^6 primary plaque forming units were obtained. This library was screened with an antiserum directed against the second helix of MyoD (anti-H2) (Litvin et al., 1993) as previously described (Huynh et al., 1985). Positive clones were isolated and autoexcised as described by the manufacturer (Novagen) into a pEXlox plasmid. The initial screening identified 22 positive clones. pCMF1 (clone 22a-1, Fig. 1A), which was one of the positive clones, was subjected to further characterization based on its expression pattern (see results).

The library was rescreened with ^{32}P-labeled cDNA probes to obtain the full-length pCMF1 cDNA. Three sequential screenings were performed using random primed cDNA probes including the 5′ *Eco*RI-*Eco*RI (250 bp) fragment of p22a-1 (Fig. 1A), the *Hin*dIII-*Eco*RI (250 bp) fragment of p29a-1 (Fig. 1A) and the *Eco*RI-*Hin*dIII (750 bp) fragment of p11 (Fig. 1A). Filters were prehybridized in 50% formamide, 5× SSPE (1× SSPE is 0.18 M NaCl, 1 mM EDTA, 10 mM sodium phosphate (pH 7.5)), 3× Denhardt's (Maniatis et al., 1982), 0.2% SDS and 100 μg/ml denatured Herring testes DNA (Sigma) for 1 hour at 42°C and then hybridized overnight at 42°C with the probes. The filters was rinsed twice at room temperature in 0.2× SSC, 0.2% SDS for 10 minutes and twice at 60°C for 30 minutes. Positive plaques were purified by secondary screening using the same hybridization and washing conditions followed by autoexcision into pEXlox plasmid.

Sequencing and sequence analysis

The insert of each clone (Fig. 1A; p22a-1, p29a-1, p11, p2-11b) was sequenced at least two times in both directions in the pEXlox vector (Novagen) using the dideoxy sequencing method (Chen and Seeburg, 1985) with a modified T7 DNA polymerase (Sequenase 2.0; United States Biochemical Corp.). T7 gene10 primer, Sp6 promoter primer (Novagen) and synthesized oligo primers corresponding to sequences in the pCMF1 cDNA (Operon) were used.

Sequence homology searches were conducted using BLAST in the GenBank databank. Secondary structure analysis was performed by Garnier-Robson (Garnier, 1978) or Chou-Fasman (Fasman, 1989) methods using the DNAstar program for Macintosh. Protein pattern searches were conducted by MacPattern in Prosite database (Fuchs, 1991). The accession number for the pCMF1 nucleotide sequence is GenBank U62026.

Northern blot analysis

Total RNA used in northern blot analysis was isolated using guanidium thiocyanate/phenol/chloroform extraction and isopropanol precipitations as described by Chomczynski and Sacchi (1987). Equivalent amounts of RNA (10 μg) from tissues of different stages were used for electrophoresis through 1% agarose gels containing 2.2 M formaldehyde and transferred overnight to a nylon filter (Shleicher and Schuell) by passive transfer in 10× SSC [1× SSC is 150 mM NaCl, 15 mM sodium citrate (pH 7.0)]. Hybridization was performed in 50% formamide, 6× SSC, 0.5% SDS, 5× Denhardt's and 100 μg/ml denatured Herring testes DNA (Sigma) at 42°C overnight. The *Hin*dIII-*Hin*dIII fragment of p29a-1 was ^{32}P-labeled by random priming and used as a probe. Hybridized filters were rinsed twice at

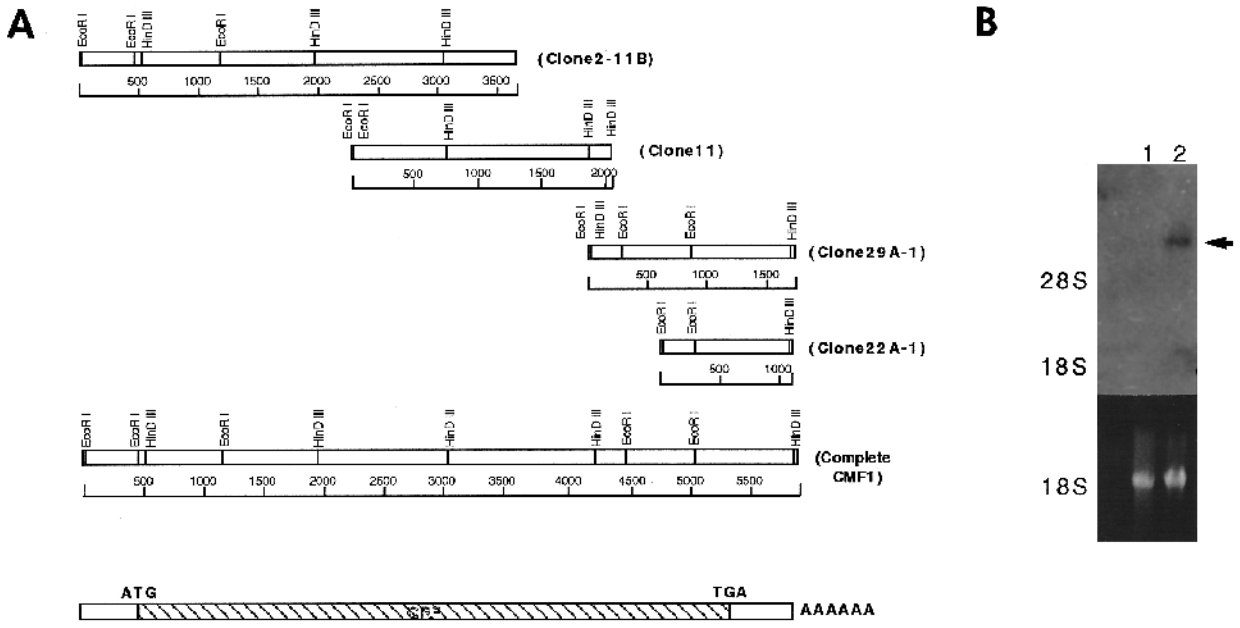

Fig. 1. Characterization of pCMF1. (A) Sequence of the full-length pCMF1 was assembled from four overlapping clones. The predicted amino acid sequence of pCMF1 contains one open reading frame encoding 1538 amino acids. Numbers indicate amino acids. The entire nucleic acid sequence is available through EMBL database library. (B) Northern blot analysis was performed to determine the size of pCMF1 mRNA. Total RNA was isolated from embryonic day 14 pectoralis muscle (lane 1) and stage 11 heart (lane 2). The northern blot was hybridized with the ^{32}P-labeled *Hin*dIII-*Hin*dIII fragments of clone 29a-1 (Fig. 1). The arrow indicates hybridization with a single 6~7 kb pCMF1 mRNA.

room temperature in 0.2× SSC, 0.2% SDS for 10 minutes, then twice at 42°C for 30 minutes and exposed to Kodak XAR film.

Embryos and tissue culture

Chicken embryos were obtained by incubating fertilized White Leghorn chicken eggs (Truslow Farms, Chestertown, MD) in a 37°C incubator under high humidity and staged according to Hamburger and Hamilton (1951). Embryos younger than 3 days were removed from eggs on filter paper rings and rinsed in sterile PBS (150 mM NaCl, 10 mM Na_2HPO_4, 10 mM NaH_2PO_4). Specific cardiogenic regions from stage 4 embryos were dissected on the basis of the fate maps of Rawles (Rawles, 1943). Dissections were conducted with glass needles drawn from capillary tubes (1.0 mm) using a vertical pipette puller (Kopf Instruments). Heart tubes from stage 9 to day 3 embryos were removed from embryos with a tungsten needle. Embryos older than 3 days were transferred directly to 1× PBS in the Petri dish and hearts were dissected with fine forceps. Tissue used for RNA isolation was rinsed with 1× PBS, quickly frozen in liquid N_2 and stored at −70°C. For in vitro cultures, the cardiogenic region was dissected by cutting the mesoderm and endoderm tissue layers leaving ectoderm behind. Cultures of cardiogenic regions (endoderm and mesoderm cell layers) were maintained in 1× M199 (Sigma) with 10% FBS (Hyclone) in Permanox Lab Tek chamber sliders (Nunc) coated with 0.01% collagen I (Sigma) using standard protocols (Gonzalez-Sanchez and Bader, 1990). Embryonic day 6 cardiac myocytes were isolated by dissociating hearts in 0.05% Trypsin (Gibco BRL). Dissociation was facilitated by physical disruption. Primary cardiac myocyte cultures were maintained in 1× M199 with 10% FBS.

Whole-mount in situ hybridization and sections

For whole-mount in situ hybridization, embryos on paper rings were rinsed in sterile 1× PBS and fixed in 4% purified paraformaldehyde ('prill') (Electron Microscopy Sciences, Fort Washington, PA) in PBS at 4°C for 1-2 hours. Embryos were transferred to 70% ethanol and stored at –20°C. The extraembryonic membranes were removed from embryos before hybridization. Digoxigenin UTP-labeled complementary RNA probes were synthesized using the Boehringer Mannheim Genius 4 system. RNA probes synthesized from 1 μg linearized cDNA were resuspended in 0.5 ml hybridization buffer and probes were reused multiple times. Sense and antisense pCMF1 probes were transcribed from p22a-1 cDNA in a linearized pEXlox vectors from T7 or SP6 promoters. Integrity and quantity of RNA probe were determined by agarose gel electrophoresis. Sense and antisense VMHC1 probes were prepared as described previously (Yutzey et al., 1994) and used as described above.

Whole-mount in situ hybridizations of CMF1 and VMHC1 mRNA were carried out as described in Coutinho et al. (1992, 1993) with the modifications described by Yutzey et al (1994). Embryos were incubated in proteinase K (30 μg/ml) for 5-10 minutes at 37°C depending on the stage of development. Antibody was detected using the Genius detection system (Boehringer Mannheim). The color reaction was stopped in 10 mM Tris-HCl (pH 8.0), 1 mM EDTA after 15-30 minutes. When equivalent concentrations of probe were used, VMHC1 signal was always stronger than pCMF1.

To localize hybridization signal to specific cell layers, embryos that

A

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MDEFVLSSSLQVITNKLSELEKICERLQNENTAFASPFKDSKTDGIRDINKMAEEKENII   60
NTNLRTEKAAFQDELLNQSDNSDLRMDCGNKEISFRFKEHSTGSSSDYEDLKLSSEEVKI  120
YFAEIKDKFFSFQNEHMKLYEQHCSMVSKIMELQSCIKILRAENSALSASMSSVHLDSLR  180
ILLSSSQNDTQSKLDETKSTDSLLDVTFSEVSKVDNSCNGGVCKWTDEINQLKNSVEGMS  240
EDAAKVLIEDDHSGHILDSVKELRSSTPSKYNPEGRIKELEMLCQIYEKVIKVLQNQLHI  300
QENMKTEEIQEMKNIMLSERREIDKLQNLSDKKEWQQKLNYLTTEMECKLAEEGKTKNLS  360
FDLKTASPQLQVLNSGSLSLPCTDTENTTQKKNNTLYQLGVPVGNPALRKNKPKLIPVEK  420
TAVGETAVHENTTETTESKLVEAFSNKFHRGEECRNMSGKNTSSSHHDSALSFSNSSIFL  480
SSEDFFENEISTETHQKEIKQKTIEDPKLIYAPEGHKNVDFLMEVKELNSQVDFQNAQLT  540
LENSALIELEETAVEKEENSGIKEKLESASVSKQQLSCGVVSLGKEPEKISAKHNVNKTV  600
LSNTTDTLDDIELTKGNCHDLFLGAEIEQRQTKSEQVNMETHALFVEGNAEILQAKCQQL  660
ERDREINLQTIPSSLQEQLVSVTAERNHISQEQSVILENQKFIGQFYQKIQEKLKELETN  720
KEDSAKLIRRLEREVKIPTNLLETAKSDTDQLSGEKNHLLQNLQSLEKDALSFRLEEEKL  780
QNQVADLNKEKEVLMRESEMMQSKLSALETENSKLSRYLDGLITEKREIAARLNSAQKEV  840
NQMREGMEKLKIKIESDERKTRHIAEKLKENERKSDALLDKVERLERELEMSEENLEDAV  900
VQLATAKAEAETFITEKEEMAEKLKCLQSQLDDLTSQKECLAKDSKEKQERILELESSNL  960
TTAKLLEEKEEEKMQIKDEFENAVLLLKSELKGMSEKLEFSSKEEEFARAKEQVLINQVA 1020
CLEQDKTMIIQFCQFLKNFNIKLDHTRELLAQEVVEYKQKLEEKMQENHAFQQQIKETEE 1080
LSSQLTRMEHEHECWHQEKQKLQNLIVELNLKAQPFSDDGTFPDILNILKVAYNDLEKEL 1140
ESTLCEKTALCKKVNEQTENCIKLEVKLSDAEQAISKLQEEFTMERKKLAEQIQLLQEHS 1200
EQNKIDLHLVVSEKHELTKSLGAVQKELQEKENIMKREISKYQDKQLQTEKEHQNALKEA 1260
NRKNEIQIEVLQEKVNSLESFISSQKLEIEHLKSTKEELNNSLKEANQTLGDLLKLKADN 1320
INTIVQLNKEKEFVQSEVQLWIKSCKQMEQEKEVLQKQLADCEELLKKKDLSVSEKEGTD 1380
ENAITEEIKLELEELQEAVEVKTREANENLEKYCSLIVKYYKQEEANEMLKMQVTLLNGQ 1440
LKQRTSDAVSSSLLNSGNSSTVSSQSDKEMRWDEDTTKPSTKRQRCEDTRKDNGEPRSPL 1500
PETSSKKKRKEDICQNLENQENSDYEPDGLPEVVKRGC. 1538
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B

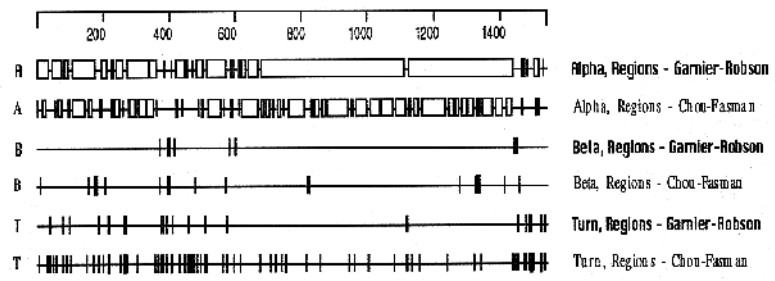


Fig. 2. Predicted amino acid sequence and secondary structure of pCMF1. (A) The predicted amino acid sequence of pCMF1 protein was determined from the open reading frame of full-length pCMF1 cDNA (Fig. 1A) Homology analysis indicates potential leucine-zipper domains (Fig. 2A, underlined) and nuclear targeting sequence (Fig. 2A, bold and underlined).
(B) Secondary structure of pCMF1 was analyzed by either Garnier-Robson (Garnier, 1978) or Chou-Fasman (Fasman, 1989) methods. The sequence was analyzed for its ability to form (a) α-helical region, (b) β-sheet region and (T) turn or loop regions. The predicted secondary structure of pCMF1 is mainly composed of α-helices and turns or loops.

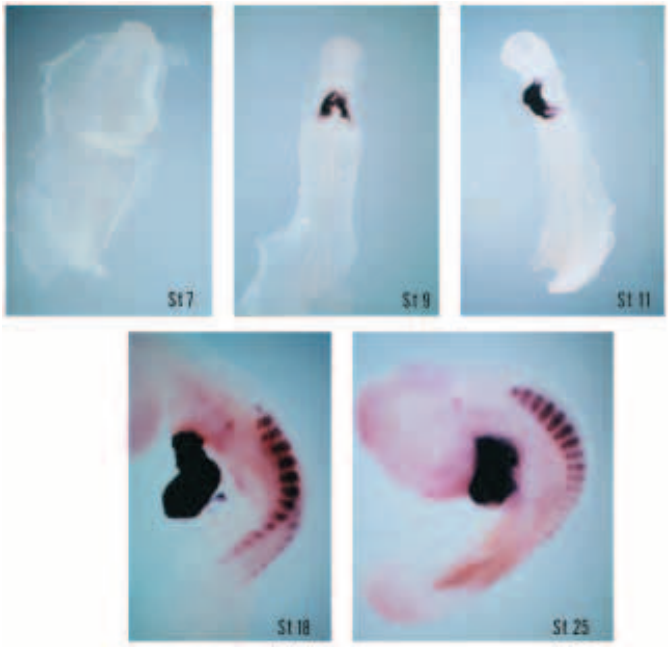


Fig. 3. Differentiation of cardiac progenitors occurs between stages 8 and 16. Whole-mount in situ hybridization of VMHC1 digoxigenin RNA probe with embryos at indicated stages (see Materials and Methods) is presented. VMHC1 expression marks the onset of cardiac differentiation. VMHC1 was first detected in the anterior or cranial parts of the cardiogenic crescent at stage 9 and differentiation progresses posteriorly. At stage 18, VMHC1 expression is detected throughout the differentiated heart and also in the developing somites.

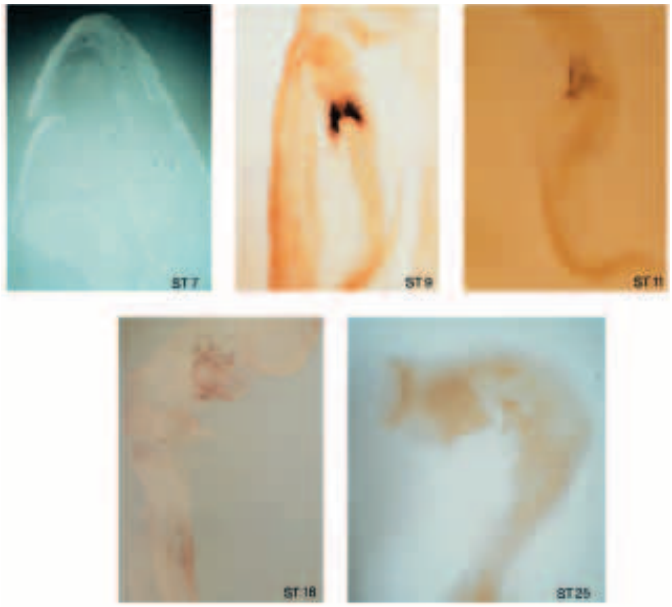


Fig. 4. pCMF1 is expressed in differentiating cardiac myocytes. Whole-mount in situ hybridization of pCMF1 digoxigenin RNA probes with embryos of indicated stages is presented. pCMF1 is first detected at stage 9 in the fusing heart. The expression of pCMF1 progresses posteriorly and, at stage 11, the posterior region of the heart had sharp demarcation of pCMF1-positive and negative cells. At stage 18, pCMF1 staining is seen throughout the heart. After stage 20, pCMF1 staining was no longer detectable. pCMF1 message was never detected in somites.

had been previously analyzed for whole-mount in situ hybridization were dehydrated in alcohol, cleared in xylene and embedded in paraffin. Embryos were cross-sectioned and counter-stained with hematoxylin and eosin.

Production of pCMF1antisense-CXL retrovirus

Replication incompetent CXL, pCMF1 antisense-CXL and pCMF1sense-CXL retroviruses were employed in this study (Fig. 6). CXL is a *lacZ*-containing retrovirus that has been described previously (Mikawa et al., 1991, 1992). It is derived from the spleen necrosis virus (SNV) (Dougherty and Temin, 1988) by replacement of viral structural genes, *gag*, *pol* and *env* with the bacterial β-galactosidase gene, *lacZ*. pCMF1 antisense-CXL retrovirus contains antisense sequence of pCMF1 derived from clone 29a-1 (Fig. 1). This DNA was chosen for construction of the antisense retrovirus because computer analysis determined that clone 29a-1 sequence (carboxyl coding and 3′ UTR of pCMF1) was the most unique or non-homologous portion of pCMF1. The 1.7 kb cDNA fragment from clone 29a-1 of CMF1 (Fig. 1) was excised by *Hin*dIII digestion and engineered for cloning by adding *Sal*I and *Sma*I cloning sites to the 3′end and 5′end respectively. The engineered fragment was inserted into *Sal*I and *Sma*I sites 5′ to the *lacZ* gene of CXL in the antisense orientation (Fig. 6A). To control for the non-specific inhibition of differentiation by addition of eukaryotic DNA sequences delivered by retrovirus, clone 29a-1 was cloned to sense orientation into CXL.

Control and experimental retroviruses were produced in D17.2G cells as described previously (Mikawa et al., 1991). D17.2G cell line is the SNV packaging cell line that expresses the viral structural genes (Dougherty and Temin, 1988). D17.2G cells were cotransfected with pCXL, pCMF1 antisense-CXL, and pCMF1 sense-CXL and pGEM4SV40neo. Virus-producing cell lines were isolated from the G418-resistant population. Viral stocks were harvested from the supernatant of confluent cultures and concentrated by centrifugation at 15,000 revs/minute for 90 minutes. The titer of concentrated virus was assayed by infecting D17 cells in the presence of 10 μg/ml polybrene and subsequent staining with X-gal. A titer of 5×10^6 to 10^8virions/ml was usually obtained with CMF1antisense-CXL.

To analyze the effects of pCMF1 antisense in cardiac differentiation, explants of stage 4 cardiac mesoderm or day 6 primary cardiac myocytes were infected with the viruses. These cultures were incubated with concentrated viruses in the presence of 10 μg/ml polybrene for 2 hours and then transferred into fresh medium for 48 hours. Cultures were then fixed and analyzed using immunohistochemical techniques with antibodies directed against myosin heavy chain (MF20) and β-gal (anti-β-gal antibody).

Immunochemistry

Antibodies used for immunochemical analysis were MF20, a monoclonal antibody that reacts with sarcomeric myosin heavy chain (Bader et al., 1982) and anti-β-gal antibody (rabbit polyclonal antibody; 5′→3′). Double immunofluorescent studies were conducted as follows. Cultures of explants or cells were fixed in 2% paraformaldehyde in 1× PBS for 15 minutes, washed twice in 1× PBS for 10 minutes and made permeable with 0.1% Triton X-100 in 1× PBS. The cultures were blocked with 1% BSA for 30 minutes before incubating with MF20 for 2 hours at room temperature. The samples were rinsed then several times with 1× PBS and incubated with Texas Red-conjugated goat anti-mouse IgG (Jackson ImmunoResearch Laboratories, Inc.) for 30 minutes. The same washing and blocking procedures were performed before the samples were incubated with the rabbit antibody to β-gal protein (5′→3′) for 45 minutes at 37°C. The anti-β-gal antibody was detected by incubating with biotinylated anti-rabbit IgG (Vector Lab) for 45 minutes at 37°C and visualized by incubating with fluorescein isothiocyanate (FITC)-conjugated avidin

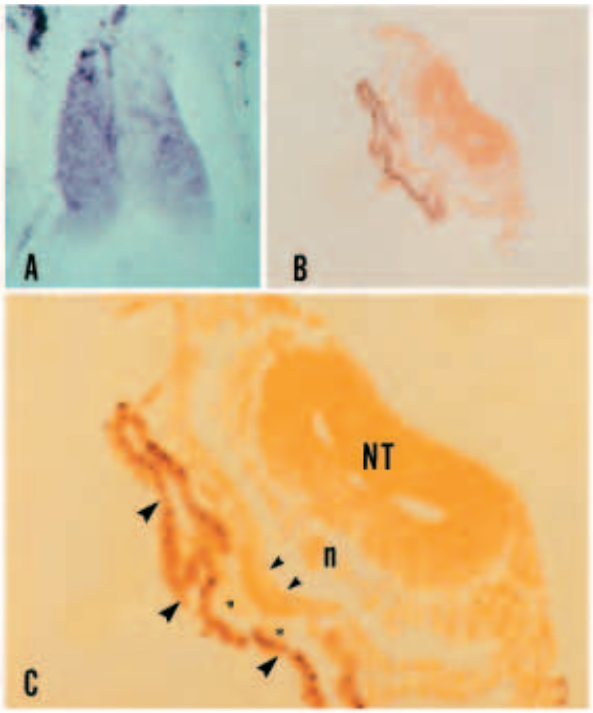


Fig. 5. pCMF1 expression is localized to the myocardium of the heart. (A) pCMF1 expression in a stage 9 whole-mount embryo is given at high power. (B) A transverse section across the cardiac region of the stage 9 embryo in A is shown. This low-power magnification shows that pCMF1 staining is specific for the heart. The edge artifact seen in some whole-mount preparations is lost during tissue processing. (C) The larger arrowheads point to stained myocardium. The small arrowheads point to pharyngeal endoderm and endocardium is indicated by an asterisk. Note that pCMF1 mRNA is detected only in the myocardium. pCMF1 mRNA is not present in endocardium or pharyngeal endoderm. NT, neurotube; n, notochord.

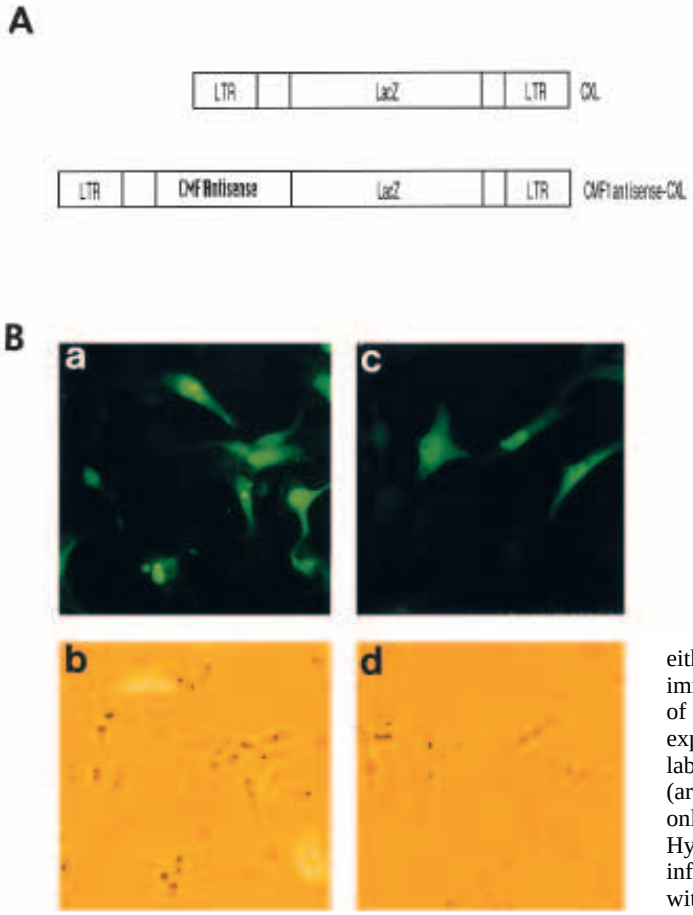
D (Vector Lab) in 100 mM $NaHCO_3$, 150 mM NaCl at room temperature for 30 minutes. Stained samples were mounted in 50% glycerol/1× PBS, visualized and photographed with an epi-fluorescence microscope (Nikon).

The number of anti-β-gal antibody-positive cells (FITC-labeled), representing the number of cells infected with virus, were counted in each explant experiment in the areas where the cells were arranged as a single layer. MF20 reactivity was determined for the infected cells infected in control and experimental groups. The percentage of MF20-positive cells in the total number of virus-infected cells was calculated in each experiment for control and experimental retrovirus samples. The difference in the percentage of MF20-positive cells in virus-infected cells between control CXL and pCMF1antisense-CXL and pCMF1 sense-CXL were determined and analyzed statistically.

RESULTS

Cloning and sequence analysis of pCMF1

To identify proteins in the differentiating heart (stage 7-16) that



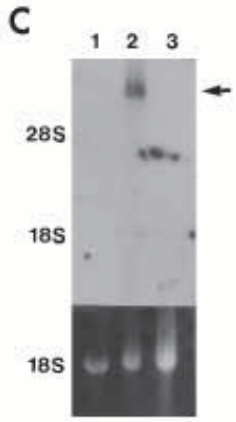


Fig. 6. Construction of pCMF1antisense-CXL recombinant retrovirus. (A) A schematic diagram of the parental CXL retrovirus genome (top) is given CXL retrovirus has *lacZ* gene under control of the SNV LTR (see Materials and Methods). pCMF1antisense-CXL retrovirus (bottom) contains approximately 1.5 kb of pCMF1 (clone 29a-1, Fig. 1) in antisense orientation 5′ to *lacZ* coding sequences. (B) Analysis of β-gal protein expression in retrovirus-infected D17 cells. β-gal protein expression was evident in D17 cells infected with either CXL (a) or pCMF1antisense-CXL (c) as determined by immunostaining with anti-β-gal antibody. Corresponding phase pictures of a and c are given in b and d. (C) Analysis of retrovirus mRNA expression in retrovirus-infected D17 cells. When probed with a ^{32}P-labeled *Hin*dIII/*Hin*dIII fragment of pCMF1 (Fig. 1), a ~6.8 kb band (arrow) corresponding to the predicted size of pCMF1-*lacZ* mRNA was only detected in D17 cells infected by pCMF1antisense-CXL (lane 2). Hybridization was not detected in D17 cells alone (lane 1) or in D17 cells infected with the parental CXL (lane 3). Equal loading was demonstrated with ethidium bromide staining of 18S ribosomal RNA.

were antigenically reactive with the H2 antiserum, a stage 11 chicken cDNA expression library was screened with anti-H2. From 22 immunoreactive clones, clone p22a-1 (Fig. 1A) was chosen for further analysis because of its spatial and temporal expression pattern (described below). The 1.1 kb cDNA insert of clone22a-1 was autoexcised and sequenced. The sequence revealed one major uninterrupted open reading frame extending to the 5′ end of the clone with a poly(A) adenylation addition site and poly(A) tail at the 3′ end. While homology with the entire 20 amino acid second helix immunogen was not observed, a potential epitope for the antiserum with one conservative substitution was recognized at position 1372 aa→1379 aa in a region predicted to be an α-helical structure. No other portion of the molecule was immunoreactive with anti-H2. No 5′ ATG start codon was found in this open reading frame and northern blot analysis (Fig. 1B) indicated that p22a-1 hybridizes to a single message approximately ~6 kb in size with RNA from stage 11 chicken hearts but not in embryonic skeletal muscle. Thus, subsequent rounds of cloning using the 5′ sequences of newly derived cDNAs as probes were conducted to obtain the full-length pCMF1 cDNA. A total of four overlapping cDNA clones were isolated that represent the full-length cDNA of pCMF1 (Fig. 1A). The entire non-overlapping sequence of these four clones together is 5850 bp and contains a single open reading frame with an in frame ATG start codon at position 603 bp and TGA stop codon at position 5217 bp. This open reading frame encodes a predicted protein of 1538 amino acids with an estimated relative molecular mass of 177×10^3.

The predicted amino acid sequence of pCMF1 (Fig. 2A) was compared with sequences in GenBank using BLAST. No significant homology to any reported protein was identified over the entire length of pCMF1 amino acid sequence. The secondary structure of pCMF1 (Fig. 2B), predicted by either Garnier-Robson (Garnier, 1978) or Chou-Fasman (Fasman, 1989) methods, suggested that α-helices and loose turns or loops are the main components of its structure. These searches identified limited potential for β-sheet structure predicted from the entire amino acid sequence of pCMF1. The amino acid sequence of pCMF1 was searched for conserved stretches of amino acid motifs or patterns in the PROSITE database using the MacPattern software (Fuchs, 1991). This search found a sequence of basic amino acids (KKKRK, 1506-1510 bp) with a second site of basic amino acids (RK, 1490-1491 bp) located 14 positions upstream which is homologous to the well-characterized bipartite nuclear targeting sequence of SV40 large T antigen (Dingwall and Laskey, 1991). Cloned sequences containing this site direct nuclear localization of reporter proteins in transfected HeLa cells (Pabon and Bader, unpublished data). The identification of the potential nuclear targeting sequence was consistent with our previous observation that the proteins antigenically related to the anti-H2 serum in the developing heart have a nuclear localization (Litvin et al., 1993). In addition, leucine-zipper motifs (Landschulz et al., 1988) were found in three locations in pCMF1 sequence (696-717 bp; 808-829 bp; 1015-1050 bp) (Fig. 2A). Thus, while pCMF1 contains many predicted helices and loops, strong homology to the skeletal myogenic bHLH family was not observed. Computer analysis of the database suggested that pCMF1 codes for a novel protein.

Expression of pCMF1 is restricted to the cardiomyogenic lineage during the early stages of heart formation

The expression of cardiac muscle-specific genes such as VMHC1 (Ventricular Myosin Heavy Chain 1) marks the onset of cardiac myogenesis in avian embryos (Bisaha and Bader, 1991; Yutzey et al., 1994). Whole-mount in situ hybridization with a digoxigenin-labeled probe complementary to VMHC1 is presented in Fig. 3 to demonstrate the pattern of myogenic differentiation in the developing chicken embryo. The expression of VMHC1 was detected at high levels at stage 9 (Fig. 3, stage 9) as cardiac myogenic differentiation proceeds from anterior to posterior within cardiogenic mesoderm. Differentiation proceeds posteriorly as the cardiac primordia fuse at midline and the heart begins to loop. VMHC1 expression expands posteriorly and is maintained throughout the entire developing heart (Fig. 3, stages 11 and later) and persists in the heart throughout later stages of development. VMHC1 was also found in the developing somites (Bisaha and Bader, 1991; Fig. 3, stages 18 and 25) and marks the onset of differentiation in skeletal myogenic cells.

Next, we determined the expression pattern of pCMF1 mRNA in developing chicken embryos by whole-mount in situ hybridization with digoxigenin labeled probe complementary to the 1.1 kb cDNA fragment of p22a-1 (Fig. 4). pCMF1 mRNA was first detected at stage 9 with higher levels of expression in the anterior regions of the bilateral cardiogenic mesoderm (Fig. 4, stage 9). pCMF1 expression was observed to expand posteriorly along the anteroposterior axis of the heart with the increasing age of the embryo (Fig. 4, stage 11). Later, expression of pCMF1 was observed within the entire, fused heart tube including the posterior, atriogenic regions of the heart until approximately stage 18 (Fig. 4). After stage 20, pCMF1 expression was not detected by in situ hybridization in the heart or in any other part of the embryo. At all stages tested, expression of pCMF1 was confined to the developing heart. pCMF1 message was not detected in any other types of tissue including skeletal myogenic cells of the somites and limb buds (Fig. 4). Thus, pCMF1 was expressed only in the cardiogenic cells during early heart development when the heart was first differentiating.

In order to define clearly the cell layer that expresses the pCMF1 message, embryos previously stained by whole-mount in situ hybridization were examined in cross section (Fig. 5). At these early stages, the myogenic component of the heart is seen as a single cell layer epithelium (Han et al., 1992; Linask, 1992). As seen in Fig. 5, pCMF1 expression was confined to the myocardial epithelium. The absence of staining in the endocardium (Fig. 5, asterisk) and anterior pharyngeal endoderm (Fig. 5, small arrowhead) was in contrast to the expression patterns reported for GATA and tinman (also known as CSX or NK2.5) transcripts in the early heart. These two markers are expressed in the myogenic component but also in either the endocardium or anterior pharyngeal endoderm (Arceci et al., 1993; Komuro and Izumo, 1993; Lints et al., 1993).

Comparison between the corresponding stages of whole-mount in situ hybridizations using CMF1 and VMHC1 probes (Figs 3, 4) shows that the pCMF1 expression pattern is similar to that of VMHC1 in the cardiogenic region. The expression

of pCMF1 is different from VMHC1 in that pCMF1 expression is transient in the heart and is not detected in the skeletal myogenic lineage. The cardiac-specific expression pattern of pCMF1 and its appearance in the initial phase of cardiac differentiation suggested that pCMF1 may have a role in early cardiac myogenesis.

pCMF1 antisense inhibits myosin expression during cardiac muscle differentiation

The heart specific expression pattern and sequence analysis of pCMF1 suggested a possible role for pCMF1 in cardiac myogenesis. In an effort to determine the function of pCMF1 in cardiac muscle differentiation, pCMF1 function was disrupted using a replication incompetent retrovirus. Viral constructs are shown in Fig. 6A. pCMF1antisense-CXL contains one copy of clone 29a-1 (Fig. 1A) sequence in antisense orientation in the parental virus. The LTR of pCMF1antisense-CXL initiates transcription of the viral mRNA, which includes antisense 29a-1 sequences and the *lacZ* gene. The subsequent translation of this message should produce β-gal protein. In this way, it would be possible to identify infected cells with the anti-β-gal antibody and determine whether these cells differentiated using MF20, an antibody against a known marker of myogenic differentiation.

Before use with embryonic chick cells, the ability of virus-producing cells to transcribe the hybrid mRNA and make β-gal protein was tested in D17 cells. D17 cells were infected by either CXL or pCMF1antisense-CXL. Infected cultures were either lysed to isolate mRNA by northern blot analysis or fixed for staining with anti-β-gal antibody. As seen in Fig. 6C, a ~6.8 kb mRNA corresponding to the predicted size of pCMF1antisense-*lacZ* hybrid mRNA was detected in pCMF1antisense-CXL when pCMF1 was used as the probe. As predicted, the message was not detected in CXL-infected D17 cells or D17 cells alone. The ability of infected cells to make β-gal protein was tested using immunochemical methods. Both pCMF1antisense-CXL- and CXL-infected cells made β-gal protein as detected by immunostaining with anti-β-gal antibody (Fig. 6C). Thus, the present data show that pCMF1antisense-CXL virus transcribes a stable viral RNA that contains pCMF1antisense sequences and is capable of producing β-gal protein. The stability of β-gal protein produced in pCMF1 sense-CXL is demonstrated below (Fig. 8).

Cardiac muscle differentiation as defined by cardiac muscle-specific gene expression is initiated at stage 8-9 during chicken embryogenesis (Han et al., 1992; Yutzey et al., 1994). Cardiomyogenic cells can be isolated from anterior mesoderm prior to differentiation at stage 4. These cells, which are highly proliferative, differentiate into cardiac myocytes approximately 48 hours later (Gonzalez-Sanchez and Bader, 1990). Thus, undifferentiated cardiogenic mesoderm could be infected and high levels of control or pCMF1antisense-CXL message can accumulate prior to initiation of cardiac-specific gene expression. In order to determine whether pCMF1 antisense affects the differentiation of cardiac progenitors, cardiogenic mesoderm from stage 4 embryos (Fig. 7A) was incubated with pCMF1antisense-CXL retrovirus. Explants were allowed to grow for 48 hours and were then processed for double immunofluoresence with MF20 and anti-β-gal antibody. As controls, the parental CXL and pCMF1 sense-CXL retroviruses were used to monitor the non-specific effects of infection, and transcription and translation of exogenous sequences.

In both control and pCMF1antisense-CXL-infected stage 4 cardiac mesodermal explants, uninfected mesodermal cells differentiate into MF20-positive myocytes after 48 hours (Fig. 7B upper, arrowhead). [In addition, it should be noted that endoderm and non-cardiogenic mesoderm were present in these cultures. Consequently, non-myogenic cells were also infected in both control and experimental groups. For this reason, CXL-infected cells in control experiments never approached 100% in their MF20 staining (see Fig. 7C).] In control CXL-infected cultures, the percentage of β-gal-positive cells that were also MF20 positive ranged from approximately 20-60% (Fig. 7C). This indicated that viral infection and expression of β-gal protein did not interfere with cardiac muscle-specific gene expression.

In contrast to CXL-infected cultures, pCMF1antisense-CXL-infected cultures have few β-gal-positive cells that were positive for MF20 (Fig. 7B). These β-gal-positive/MF20 negative (i.e. infected/undifferentiated) cells were often surrounded by β-gal negative/MF20-positive (i.e. uninfected, differentiated) cells (Fig. 7B). The percentage of MF20-positive cells in all virally infected cells was determined for both pCMF1antisense-CXL or CXL groups. The results of six experiments are summarized in Fig. 7C. As seen in Fig. 7C, the percentage of cells positive for both MF20 and β-gal in pCMF1antisense-CXL-infected cultures was significantly decreased when compared to CXL-infected control cultures. The difference between CXL and pCMF1antisense-CXL-infected samples for six matched experiments is statistically significant with $P<0.01$. To control further for the nonspecific effects of introduced eukaryotic sequences on myogenic differentiation, pCMF1 sense-CXL contructs were used to infect cardiogenic mesoderm. As seen in Fig. 8, cells infected with this retrovirus expressed high levels of sarcomeric MHC. Matched experiments showed that 29.4% (83/282) of CXL-infected cells were also MF20-positive while 23.8% of pCMF1 sense-infected cells were MF20-postive with no statistically significant difference between these groups. These data suggested cardiac myocyte differentiation did not occur at high frequency when cells were infected with pCMF1antisense-CXL but did when control viruses were used.

pCMF1 message is expressed exclusively during the initial phases of heart development and, thus, one might predict that the presence of pCMF1 antisense would have little effect on the expression and/or accumulation of contractile gene products in differentiated cells. To test this hypothesis, pCMF1antisense-CXL was used to infect primary cultures of embryonic day 6 (HH stage 29) cardiac myocytes. At this stage of development, all cardiomyogenic progenitors have differentiated. Differentiated cardiac myocytes remain mitotic. Freshly prepared primary cardiac myocyte cultures were incubated immediately with concentrated pCMF1antisense-CXL or CXL retroviruses. After removal of virus, the cells were cultured for an additional 2 days and processed for MF20 and anti-β-gal immunofluoresence. Under these experimental conditions, cells were stained positively for both MF20 and anti-β-gal antibodies in both CXL and pCMF1antisense-CXL-infected samples (Fig. 9A). The percentage of infected cells that were MF20 positive was determined for both groups. The results of two such experiments are summarized in Fig. 9B.

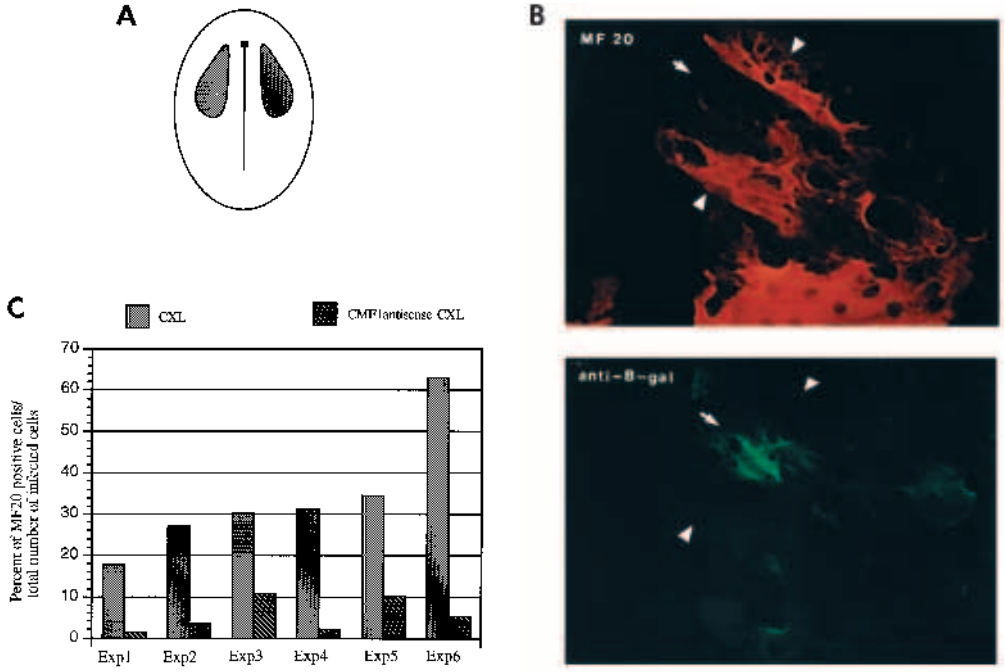


Fig. 7. MHC expression is inhibited in pCMF1antisense-CXL-infected cardiogenic mesoderm. (A) Stage 4 cardiac mesoderm (shown as the shaded area) was infected with control CXL or pCMF1antisense-CXL retroviruses, cultured for 2 days in vitro and processed for double immunofluoresence with MF20 antibody (Texas red) and anti-β-gal-antibody (FITC). (B) A typical culture infected with pCMF1antisense-CXL is shown. The top picture shows MF20 staining of differentiated cardiogenic mesoderm. The bottom picture indicates the pattern of retroviral infection revealed by anti-β-gal staining. Note that pCMF1antisense-CXL-infected cells (bottom, arrow, anti-β-gal-positive) do not express MHC (top, arrow, MF20 negative), while the non-virally-infected cells (bottom, arrowheads, β-gal-negative) differentiate and express MHC (top, arrowheads, MF20-positive). (C) The inhibition of MHC expression in differentiating stage 4 cardiac mesoderm by pCMF1antisense-CXL is statistically significant. Stage 4 cardiac mesoderm infected with either control CXL or pCMF1antisense-CXL retroviruses were cultured for 2 days and double immunostained with MF20 antibody and anti-β-gal-antibody as in Fig. 7B. The total number of infected cells (anti-β-gal-positive) was determined for each experiment. The percentage of infected cells that were differentiated cardiac myocytes (MF20-positive) was determined for control and experimental samples in each paired experiment. Many non-myogenic cells are infected in both control and experimental conditions. Six independent experiments are shown. The data show that the MHC-positive phenotype does not emerge in pCMF1antisense-CXL-infected samples but does in CXL-infected cells. The differences in percentages are statistically significant with P values <0.01 for all six experiments.

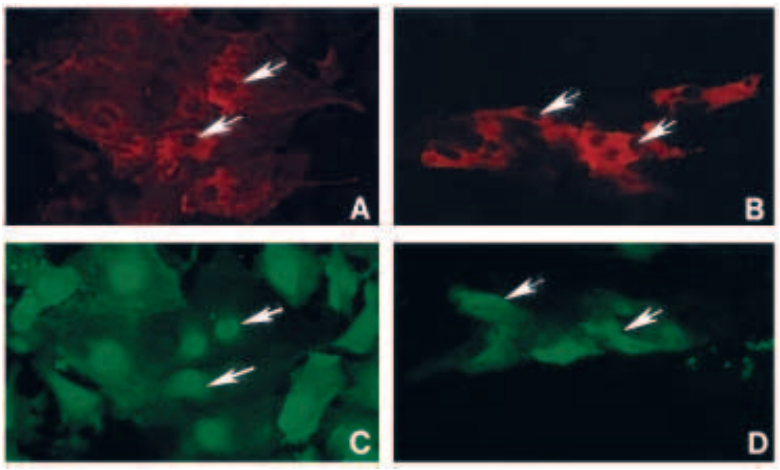
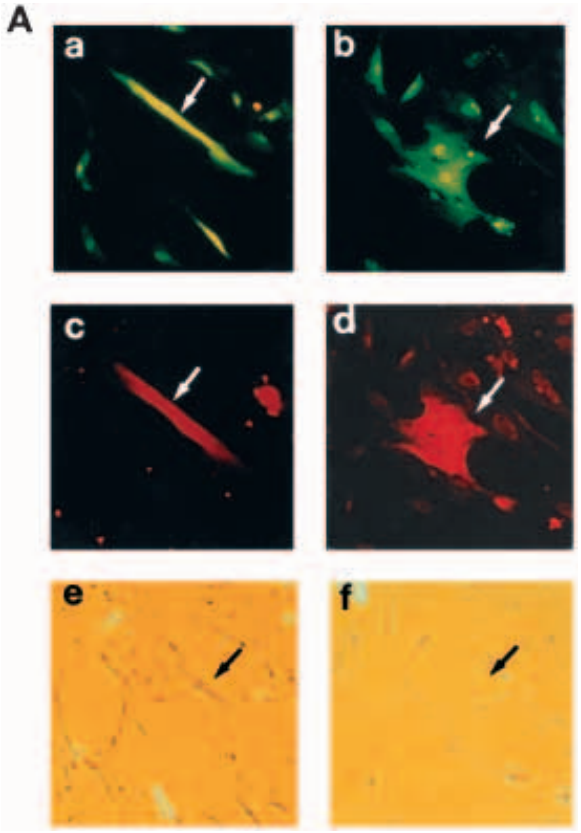


Fig. 8. MHC expression is not inhibited in pCMF1 sense-CXL-infected cardiogenic mesoderm. This experiment was conducted as described for pCMF1 antisense-CXL except the inserted DNA was in sense orientation. CXL was used in matched studies. CXL-infected cells express sarcomeric MHC (A) and β-gal protein (C). pCMF1 sense-CXL-infected cells are also MF20-positive (B) and β-gal-positive (D). Arrows indicate cells doubly labeled.

Statistical comparison of double immunostaining results determined that there was no significant difference observed between pCMF1antisense-CXL- and CXL-infected cultures (Fig. 9B). It should be noted that the percentage of retrovirally infected myocytes is relatively low with both viruses when compared to the situation in cardiogenic mesoderm. The reason for this occurrence is that avian cardiac myocytes are relatively non-proliferative at this stage of development while contaminating non-myogenic cells are mitotically active (Clark and Fischman, 1983). In addition, pCMF1antisense-CXL infection did not inhibit skeletal myogenic differentiation (Wei and Bader, data not shown). These data suggested that pCMF1 antisense does not inhibit MHC expression in differentiated cardiac myocytes and that the inhibitory effect of pCMF1 antisense on stage 4 cardiac mesoderm were not due to a non-specific toxic effect of pCMF1 antisense on muscle-specific protein expression.

DISCUSSION

The full-length cDNA cloning of pCMF1 and the determina-



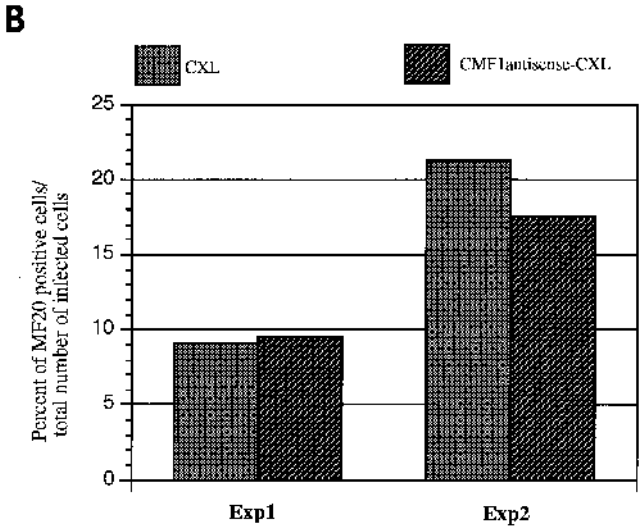


Fig. 9. MHC expression is not inhibited in pCMF1antisense-CXL-infected, differentiated cardiac myocytes. Embryonic day 6 cardiac myocytes were infected with CXL or CMF1antisense-CXL, cultured in vitro and double immunostained with MF20 antibody (Texas red) and anti-β-Gal-antibody (FITC). (A) pCMF1antisense-CXL and parental CXL-infected cardiac myocytes express MHC. (a,c,e) Anti-β-gal antibody, MF20 and phase pictures of a pCMF1antisense-CXL-infected culture; (b,d,f) corresponding pictures of a CXL-infected culture. The arrows indicate cells that were both MF20- and anti-β-gal-positive in each culture. (B) The percentage of MF20-positive cells in the total number of infected cells was calculated and compared between CXL and pCMF1antisense-CXL-infected samples. Two paired experiments are summarized. The differences in the percentage are statistically equivalent (*P* values: 0.9, 0.4 and 95% confidence intervals: (−5%, +6%); (−5%, 12%) respectively).

tion of its sequence suggest that a novel gene product has been cloned. The predicted amino acid sequence of pCMF1 does not share significant homology with any sequence in the GenBank. The search of common protein motifs within pCMF1 suggests that a major structural element of pCMF1 is a series of helices with intervening loops. Whole-mount in situ hybridization with pCMF1 demonstrates that pCMF1 expression is restricted to the myogenic cell layer of the heart and is not detected in other tissues. Additionally, pCMF1 is transiently expressed during the period when cardiac progenitors differentiate into cardiac myocytes. Finally, when cells of stage 4 cardiac mesoderm are infected with a retrovirus that mediates pCMF1 antisense expression, cardiac-specific contractile gene expression is suppressed. These data suggest a potential role for pCMF1 in cardiac myogenesis.

pCMF1 is a novel gene product

Homology analysis of pCMF1 amino acid sequence shows that pCMF1 does not have significant homology with published sequences. Thus, pCMF1 is a novel gene product. While the cDNA clone of pCMF1 was isolated through its reactivity with the anti-H2 serum directed against the second helix of the bHLH domain in MyoD, the predicted amino acid sequence of CMF1 does not show primary sequence homology with the MyoD protein family of transcription factors. The recent studies of Srivastava et al (1995) have shown the presence of bHLH molecules, HAND proteins, in the heart and it is possible that anti-H2 recognizes these proteins also. Sequence analysis identifies a potential epitope located in a predicted helix of the immunoreactive p22a-1 clone. Alternately, immunoreactivity of H2 with pCMF1 protein may be determined by an epitope(s) on local surface structures of protein rather than primary sequence as described (Harlow and Lane, 1988). In any case, strong primary sequence homology with skeletal myogenic bHLH proteins is not observed in pCMF1.

In a search for common protein motifs within pCMF1, several interesting characteristics were identified. First, computer analysis of pCMF1 amino acid sequence predicted a secondary structure primarily composed of α-helices and intervening loops. The presence of α-helical motifs predicted in the secondary structure of pCMF1 may suggest a potential for interaction of pCMF1 with other proteins containing α-helices. The α-helices in the bHLH domain of MyoD and other members of HLH proteins have been shown to function as dimerization domains mediating the interaction with bHLH proteins as well as other transcription factors in order to affect gene expression (Murre et al., 1989; Lassar et al., 1989; Bengal et al., 1992). Indeed, our preliminary studies suggest that the carboxyl region of pCMF1 contained within clone p22a-1 (Fig. 1A) can interact with E2A proteins and will bind the muscle creatine kinase enhancer (Litvin and Bader, unpublished data). In addition, the leucine zipper motifs identified in the amino acid sequence of pCMF1 could mediate protein interactions. As leucine zipper motifs are known to form homo- and heterodimers among transactivators such as *fos* and *jun* (Landschulz et al., 1988; O'Shea et al., 1989; Busch and Sassone-

Corsi, 1990), the potential for pCMF1 to interact with itself or other proteins exists through these domains. Lastly, a potential bipartite nuclear targeting sequence was observed in the carboxyl region of pCMF1. This sequence shares significant homology with other known nuclear targeting domains (Dingwall and Laskey, 1991) and directs nuclear localization of reporter proteins (Pabon and Bader, unpublished data) which is consistent with our previous observation of nuclear localization of anti-H2-reactive proteins in the developing heart (Litvin et al., 1993). While these predicted properties imply that pCMF1 might have either a direct or indirect role in the regulatory events of gene expression, direct transactivation analysis and protein localization studies will be necessary to clarify these issues.

The heart-specific expression pattern of CMF1 and its potential role in cardiogenesis

In the present study, differentiation of cardiac myocytes in the avian embryo was monitored by in situ hybridization using VMHC1 as a probe (Fig. 3). Cardiomyogenic differentiation is detected at stage 9 in the anterior portion of the paired cardiogenic primordia. Differentiation proceeds posteriorly with the conversion of mesoderm to differentiated myocytes along the anteroposterior axis. All, or nearly all, cardiac progenitors have differentiated into cardiac myocytes by stage 16 (Manasek, 1968; Yutzey et al., 1994). After this time, new myocytes are added to the heart by the proliferation of previously differentiated cells (Manasek, 1968).

The expression of pCMF1 is restricted to the time frame when mesoderm is converted to cardiac myocytes. In addition, the expression of pCMF1 proceeds in an anterior-to-posterior manner during these stages (Fig. 4) and is specific to the cardiomyogenic epithelium (Fig. 5). Other markers of early cardiac progenitors such as *tinman*/NK2.5/CSX, MEF2c and GATA-4 appear to have a broader distribution of expression in vertebrate embryos (Lints et al., 1993; Kelley et al., 1993; Komuro and Isumo, 1993; Edmondson et al., 1994). The unique temporal and spatial expression pattern of pCMF1 suggests that pCMF1 may be involved either directly or indirectly in the initiation of cardiac muscle differentiation.

As an initial attempt to determine the function of pCMF1, we sought to disrupt pCMF1 function during the period of its expression. We chose this general approach because analysis of pCMF1 sequence and its predicted secondary structure suggested it was a novel protein and, thus, its possible function in development must be analyzed in its broad terms. Additionally, the unique expression pattern suggested that pCMF1 may function during the initial period of myocyte differentiation. The antisense approach was used because of the limitations of genetic manipulation in avian embryogenesis. Use of dominant-negative strategies was impossible at present as we can not predict which regions of the entire sequence will confer essential functions. In addition, this situation confounds the use of sense controls in the present retroviral studies. A replication-incompetent retrovirus was chosen because of the small time frame available between the generation of cardiogenic cells and their differentiation (Gonzalez-Sanchez and Bader, 1990; Han et al., 1992). The antisense retroviral approach used here has been shown to inhibit translation of mRNA in developing myogenic cells, presumably by hybridizing with the target mRNA, and to disrupt the program of myogenesis (Mikawa et al., 1991). MF20 was chosen as the test marker antibody because it recognizes all sarcomeric MHCs (Bader et al., 1982). Remembering that the embryonic chicken heart expresses multiple MHCs (Evans et al., 1988), MF20 staining may then serve as a general marker of cardiomyogenic differentiation. At present, we have not yet generated a pCMF1-specific antibody. This necessitated a statistical approach to demonstrating that the presence of pCMF1antisense sequence over multiple experiments disrupted the normal differentiative process. Statistical analysis demonstrated that the presence of pCMF1antisense-CXL retrovirus alone correlated with disruption of the cardiomyogenic process.

The present data suggest that introduction of pCMF1 antisense into stage 4 cardiogenic mesoderm significantly decreased the expression of MHC in infected cells as evidenced by the lack of MF20 staining (Fig. 7). Under the present experimental conditions, this effect is specific to the introduction of pCMF1 antisense sequences as the presence of the viral genome, sense sequences and β-gal protein do not inhibit expression of MHC (Figs 7, 8). This effect was found to be statistically significant over a group of six matched experiments. The possibility that this effect is due to toxicity of pCMF1 antisense on the production of MHC protein may also be excluded since the expression of pCMF1 antisense has no effect on the expression of MHC protein in embryonic day 6 cardiac myocytes (Fig. 9) and in skeletal myoblasts (Wei and Bader, unpublished data). These data also suggest that the inhibitory effect of pCMF1 antisense is not due to a non-specific of pCMF1 antisense on myogenic cells and that the inhibitory effect of pCMF1 is transient. Taken together, the present study suggests a role for pCMF1 in the differentiation of cardiac myocytes. While our data show that pCMF1 antisense disrupts cardiomyogenic differentiation, at present we can not determine whether pCMF1 interacts directly with muscle-specific genes or whether its influence on the expression and/or accumulation of muscle-specific gene products is indirect in nature. In addition, it is possible that pCMF1 antisense may interact with other sequnces highly related to pCMF1. The generation of a pCMF1-specific antibody and analysis of the potential transactiving ability of pCMF1 may further determine the precise function of pCMF1 in cardiomyogenesis.

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