Transcriptional Activation of the p34Nov Gene by cdc2 Promoter Binding Factor/Nuclear Factor-Y in Fetal Rat Ventricular Myocytes

Qingquan Liu,† Huajun Yan,† Nicola J. Dawes, Yujuan Lu, Hong Zhu

Abstract—To determine how myocardial terminal differentiation is regulated by cell cycle control genes, we studied cdc2 expression in rat cardiac muscle and found that cdc2 mRNA and protein levels were reduced in neonatal compared with fetal ventricles and became undetectable in juvenile and adult ventricles. To further determine whether cdc2 downregulation is attributed to a decrease in transcription, transient expression assay was performed using the progressively truncated 6.2-, 1.8-, 1.1-, 0.7-, and 0.1-kb human cdc2 5' flanking regions. All five fragments activated reporter expression in fetal myocytes and were significantly less active in neonatal myocytes. The 0.1-kb fragment showed 65% of the activity of the 6.2-kb fragment. A protein binding site that contains an inverted CCAAT box was identified within the 0.1-kb fragment by DNase I footprint assay and named the cdc2 promoter binding factor (CPBF) site. Point mutations within the CPBF site that abolish CPBF binding significantly decreased both 0.1- and 6.2-kb promoter activities. Competition and antibody supershift assays suggested that CPBF was identical or related to the transcription factor, nuclear factor Y (NF-Y). The 0.1-kb promoter activity was suppressed by a dominant-negative NF-Y mutant in fetal myocytes. Taken together, our results demonstrate that cardiac cdc2 expression is downregulated after birth and turned off when the juvenile stage is attained. A 0.1-kb promoter fragment of cdc2 contains major information for both cdc2 transcriptional activation and suppression in fetal and neonatal myocytes, respectively. NF-Y or its related factor plays a critical role in activating the 0.1-kb cdc2 promoter.

Key Words: myocyte proliferation  ▪ cell cycle control  ▪ transcriptional regulation  ▪ development

In mammalian cycling cells, CDK1 plays an essential role in promoting the G1 to S transition, and cyclin B is required for this CDK1 function.1,2,4 The ubiquitous 34-kD CDK1 is encoded by the cdc2 gene and is highly conserved both structurally and functionally among various cell types and species.5 The transcription of the cdc2 gene is cell cycle–regulated; transcription is activated at the boundary of G1 and S phases and peaks in early M phase. As cells enter the G1 phase, cdc2 transcription is suppressed. Furthermore, cdc2 transcription is turned off in response to growth arrest induced by serum depletion or cell differentiation.3–10 Activation of the growth-arrested cells to reenter the cell cycle requires transcriptional activation of the cdc2 gene. Therefore, regulation of cdc2 transcription plays a critical role in controlling not only progression through the cell cycle but also exit or reentry into the cell cycle.

A number of transcription factors, including E2F, NF-Y, Sp-1, c-myc, c-myb, and cts-2, have been shown to activate cdc2 transcription.7,11–13 NF-Y is a ubiquitous heterotrimERIC transcription factor that activates transcription of a variety of genes by binding to a CCAAT box–containing cis element, which also serves as the core sequence for the binding sites of other transcription factors, such as NF-1 and C/EBP.14 Recently, there has been accumulating evidence showing that NF-Y is also involved in regulating the expression of genes required for cell cycle progression in certain cell types. For instance, the NF-Y binding site is required for the expression of the cdc2, cdc25C, and cyclin A genes in human myeloid leukemia cells and mouse fibroblasts.9,15 The interaction between the cyclin A promoter and NF-Y was confirmed by an antibody specific to one subunit of NF-Y, ie, NF-Yb.15 Furthermore, Chen et al16 have shown that the simian virus 40 large-T antigen activates the cdc2 promoter in CV-1 cells by inducing the DNA binding activity of NF-Y, suggesting that NF-Y also mediates inducible cdc2 expression. Not only does NF-Y activate the expression of these cell cycle control genes, but it also activates genes required for DNA replication during S phase, such as thymidine kinase, ribonucleotide reductase, and topoisomerase IIa.17–19 Interestingly, Isaacs et al19 have shown that the DNA binding activity of NF-Y in mouse fibroblasts is reduced as cells become confluence-arrested, suggesting that NF-Y activity is subjected to cell cycle regulation in these cells. However, the functions of NF-Y in regulating cdc2 expression in cardiac myocytes still remain to be investigated.
In the present study, we demonstrate that cdc2 mRNA expression in rat cardiac muscle is downregulated in early neonatal ventricles, turned off by the juvenile stage, and remains silent in the adult stage. A 0.1-kb cdc2 promoter region contains the major information required for transcriptional activation and suppression in fetal and neonatal cardiac myocytes, respectively. The transcription factor, NF-Y, plays a critical role in activating the 0.1-kb cdc2 promoter in fetal cardiac myocytes by binding to the CPBF site.

**Materials and Methods**

**Northern Blot Analysis**

Northern blot analysis was performed as described previously. Total RNA was isolated from ventricular muscle of fetal (day-15 p.c.), neonatal (day-1 p.p.), juvenile (day-19 p.p.), and adult (2-month-old) rats by the method of Chomczynski and Sacchi, and 50 μg RNA of each sample was resolved on a 1% formaldehyde agarose gel. RNA was transferred from the agarose gel onto a nylon membrane by denaturation in 0.1 M sodium hydroxide at 68°C. The filters were washed four times with 0.1% SDS at 25°C for 20 minutes and three times in 0.3× SSC at 68°C for 20 minutes. The filters were finally exposed to Kodak x-ray films. The RNA blot was probed with 32P-labeled rat cdc2 cDNA and 28S rRNA encoding regions (6.2, 1.8, 1.1, 0.7, and 0.1 kb) were kindly provided by Dr Roberto Mantovani at the University of Milan (Italy). A recombinant plasmid that carries the bacterial β-galactosidase cDNA driven by the CMV promoter was used as an internal control for transfection efficiency. Luciferase and β-galactosidase assays were performed as described previously. For each experiment, three dishes of myocytes were used, and each experiment was repeated four times with different myocyte preparations.

**Site-Directed Mutagenesis**

To introduce each set of point mutations into the CPBF site in the 0.1-kb cdc2 promoter, PCR was performed using four primers, two of which were complementary to each other and contained the desired mutations (Table). The 0.1-kb cdc2 promoter fragment was first isolated from the original luciferase containing plasmid and then subcloned into pBluescript as an SmaI–SacI fragment. The upstream part of the 0.1-kb cdc2 promoter was amplified by PCR using the pBluescript reverse primer and the antisense strand of mutant CPBF oligonucleotide, and the downstream part was amplified by PCR using the sense strand of the mutant CPBF oligonucleotide and the pBluescript T7 primer. PCR amplification was performed with 30 cycles consisting of 95°C for 30 seconds, 55°C for 1 minute, and 72°C for 30 seconds, followed by one cycle at 72°C for 10 minutes. Two PCR products were then mixed, and PCR amplification was repeated using the pBluescript reverse primer and T7 primer with the same cycles. The PCR product was purified, restricted with SmaI and SacI, and subcloned into the original luciferase plasmid. The specific point mutations were confirmed by double-stranded plasmid sequencing of the entire promoter region. The point mutations were also introduced into the 6.2-kb cdc2 promoter by replacing the SmaI–SacI fragment with the mutant the SmaI–SacII fragment. The mutant plasmids were transfected into RVM, and the luciferase activity was measured.

**Nuclear Extract Preparation**

Nuclear extracts were prepared from cultured cells as previously described with some minor modifications. Briefly, cells were resuspended in ice-cold buffer A (10 mM HEPES [pH 7.9], 10 mM KCl, 0.1 mM EDTA, 1 mM DTT, and 0.5 mM phenylmethylsulfonyl fluoride) after harvesting. Cells were lysed by a discontinuous Percoll (Pharmacia Biotechnology Inc) gradient to obtain myocardial cell cultures with >95% myocytes, as assessed by immunofluorescence with an antibody directed against ventricular myosin light chain-2. Fetal and neonatal cardiac myocytes were plated on 35-mm dishes coated with laminin (Sigma). Ventricular myocytes were cultured in DMEM with 10% FBS and 20% horse serum at a density of 1.0×10^5 cells per 35-mm dish chamber for 16 hours. The cardiac myocytes were transfected with various recombinant plasmids according to calcium phosphate precipitation method described by Chen and Okayama. The recombinant plasmids that carry firefly luciferase cDNA and the progressively deleted human cdc2 promoter regions (6.2, 1.8, 1.1, 0.7, and 0.1 kb) were kindly provided by Dr Teresa L. Born at the University of California at San Diego. A plasmid that expresses a dominant-negative NF-Ya mutant (NF-YA29) was kindly provided by Dr Roberto Mantovani at University of Milan (Italy). A recombinant plasmid that carries the bacterial β-galactosidase cDNA driven by the CMV promoter was used as an internal control for transfection efficiency. Luciferase and β-galactosidase assays were performed as described previously. For each experiment, three dishes of myocytes were used, and each experiment was repeated four times with different myocyte preparations.

**Isolation and Culturing of Fetal and Neonatal Rat Ventricular Myocytes and DNA Transfection**

The isolation and culture of fetal and neonatal rat ventricular myocytes are described previously. The only rat strain used for all the studies. Myocytes were dispersed from the ventricles of rat fetuses of 15-day gestation or 1-day-old neonates by digestion with collagenase II (Worthington) and pancreatin (GIBCO BRL) at 37°C. Myocytes were further purified by a discontinuous Percoll (Pharmacia Biotechnology Inc) gradient to obtain myocardial cell cultures with >95% myocytes, as assessed by immunofluorescence with an antibody directed against ventricular myosin light chain-2. Fetal and neonatal cardiac myocytes were plated on 35-mm dishes coated with laminin (Sigma). Ventricular myocytes were cultured in DMEM with 10% FBS and 20% horse serum at a density of 1.0×10^5 cells per 35-mm dish chamber for 16 hours. The cardiac myocytes were transfected with various recombinant plasmids according to calcium phosphate precipitation method described by Chen and Okayama. The recombinant plasmids that carry firefly luciferase cDNA and the progressively deleted human cdc2 promoter regions (6.2, 1.8, 1.1, 0.7, and 0.1 kb) were kindly provided by Dr Teresa L. Born at the University of California at San Diego. A plasmid that expresses a dominant-negative NF-Ya mutant (NF-YA29) was kindly provided by Dr Roberto Mantovani at University of Milan (Italy). A recombinant plasmid that carries the bacterial β-galactosidase cDNA driven by the CMV promoter was used as an internal control for transfection efficiency. Luciferase and β-galactosidase assays were performed as described previously. For each experiment, three dishes of myocytes were used, and each experiment was repeated four times with different myocyte preparations.

**Oligonucleotide Sequence**

<table>
<thead>
<tr>
<th>Oligonucleotide</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>CPBF</td>
<td>GTAGCTGGGCTCTGATTTGCTGCTTTTGA</td>
</tr>
<tr>
<td>NF-Ya</td>
<td>GGAATTTTCTGATTGTTAAAGTCTCGAT</td>
</tr>
<tr>
<td>C/EBP</td>
<td>TGCAGATGGCCAATATCGCAAGATGC</td>
</tr>
<tr>
<td>DSC</td>
<td>TACGGGCTACCCGATGTTGGAATC4GGG</td>
</tr>
<tr>
<td>NF-10</td>
<td>TTTTTTCCCTAAGGCCCAATATGCTAGC</td>
</tr>
<tr>
<td>CMM1</td>
<td>GTAGCTGGGCTCTGACCGGCTGCTTTTGA</td>
</tr>
<tr>
<td>CMM2</td>
<td>GTAGCTGGGCTCTGATTTGCTGCTTTTGA</td>
</tr>
<tr>
<td>CMM3</td>
<td>GTAGCTGGGCTCTGATTTGCTGCTTTTGA</td>
</tr>
<tr>
<td>CMM4</td>
<td>GTAGCTGGGCTCTGATTTGCTGCTTTTGA</td>
</tr>
</tbody>
</table>

Sequences of the complementary strands are not shown. *Purchased from Santa Cruz Biotechnology.
10% glycerol, 0.15 mmol/L spermine, 0.5 mmol/L spermidine, and the same panel of protease inhibitors as in the homogenization buffer). The nuclei were pelleted by centrifugation and resuspended in 10 to 50 vol buffer A (10 mmol/L HEPES [pH 7.9], 1.5 mmol/L MgCl₂, 10 mmol/L KCl, 0.5 mmol/L DTT, and the same panel of protease inhibitors as in the homogenization buffer). The nuclei were pelleted in a microfuge and resuspended in 1 vol buffer B (20 mmol/L HEPES [pH 7.9], 25% glycerol, 0.55 mol/L NaCl, 1.5 mmol/L MgCl₂, 0.2 mmol/L EDTA, 0.5 mmol/L DTT, and the same panel of protease inhibitors as in the homogenization buffer). The nuclear proteins were extracted in buffer B by three cycles of freeze-thaw. The extracts were centrifuged in a microfuge at top speed for 10 minutes, and the supernatant was dialyzed in 1 L of dialysis buffer (40 mmol/L HEPES [pH 7.9], 15 mmol/L NaCl, 0.5 mmol/L EDTA, 0.5 mmol/L PMSF, 0.5 mmol/L DTT, and 20% glycerol) at 4°C overnight. The dialyzed nuclear extracts were stored in small aliquots at −80°C.

**Gel Mobility Shift Assay**

Gel mobility shift assay was performed as previously described with some minor modifications. Ten micrograms of nuclear extract was preincubated with 4 μL of 5× binding buffer (200 mmol/L KCl, 75 mmol/L HEPES [pH 7.9], 5 mmol/L EDTA, 2.5 mmol/L DTT, 25 mmol/L MgCl₂, and 25% glycerol) and 4 μg of poly(dI-dC) in a final volume of 18 μL for 30 minutes on ice. The DNA fragment was end-labeled by either T4 DNA kinase or Klenow fragment with either [γ-³²P]ATP or [α-³²P]dATP, 20,000 cpm of the labeled probe (2 μL) was added to the assay mixture, and the mixture was incubated for 30 minutes on ice. The reaction was resolved on a 5% polyacrylamide gel. For competition assay, molar excess of unlabeled competitor DNA fragments at various fold levels was added to the preincubation reaction. For antibody “supershift” assay, 1 μL of antibodies against either NF-Ya or NF-Yb was used in each DNA-protein binding reaction.

**DNase I Footprint**

A DNase I footprint assay was performed according to the previously described method with minor modifications. Ten micrograms of nuclear extract was preincubated with 4 μL of 5× binding buffer (200 mmol/L KCl, 75 mmol/L HEPES [pH 7.9], 5 mmol/L EDTA, 2.5 mmol/L DTT, 25 mmol/L MgCl₂, and 25% glycerol) and 4 μg of poly(dI-dC), 25 μg of nuclear extracts in a final buffer concentration of 25 mmol/L Tris-HCl [pH 7.9], 6.25 mmol/L MgCl₂, 0.5 mmol/L EDTA, 50 mmol/L KCl, 0.5 mmol/L DTT, 10% glycerol, and 2% polyvinyl alcohol. After dilution with an equal volume of a mixture containing 5 mmol/L CaCl₂, 10 mmol/L MgCl₂, DNA was then digested for 1 minute at room temperature with freshly diluted DNase I (Worthington). After removing proteins by phenol-chloroform extraction, DNA was resolved on a 6% polyacrylamide–8 mol/L urea gel. A ladder of T-sequencing reaction using the same template was used as the molecular weight standard.

**Methylation Interference Assay**

Methylation interference assay was performed according the previously described method with minor modifications. The sense or antisense strand of oligonucleotide was end-labeled by T4 DNA kinase with [γ-³²P]ATP and annealed to the complementary strand. The labeled probe was treated with dimethyl sulfate and used for preparative gel mobility shift DNA-binding assay. The free and protein-bound probes were excised from the gel and eluted. The eluted DNAs were extracted with phenol-chloroform and precipitated with ethanol. They were then cleaved in 100 μL of 1 mol/L piperidine at 90°C for 30 minutes, and the piperidine was removed by repeated addition of water and lyophilization three times. The free and bound probes were then resolved on a 12% polyacrylamide–8 mol/L urea gel.

**Statistical Analysis**

Values of experimental data were expressed as mean±SEM. Statistical analysis was performed using the Mann-Whitney U test. Mann-Whitney probability values (P) were for the significance of differences in the luciferase activities expressed from various cdc2 promoters in fetal myocytes, from the 0.1-kb cdc2 promoter between fetal and neonatal myocytes, or from the wild-type and mutant 0.1-kb cdc2 promoters in fetal myocytes. Results were considered significant at P<.05.

**Results**

cdc2 mRNA Expression Is Downregulated in Ventricular Muscle During Postnatal Development

To assess cdc2 mRNA expression in ventricular muscle during development, the steady-state levels of cdc2 mRNA in fetal (day-15 p.c.), neonatal (day-1 p.p.), juvenile (day-19 p.p.), and adult (2-month-old) ventricular muscle were determined by Northern blot analysis. As shown in Fig 1A, a single cdc2 mRNA species of ≈2.0 kb was detected in fetal ventricular muscle. In other cell types, such as HeLa cells, however, cdc2 transcription is initiated from two major sites, which result in a 2.0-kb and a 1.4-kb mRNA. The cdc2 mRNA level, measured by densitometer scanning, was decreased by ≈3-fold in neonatal ventricular muscle, where myocytes are in the process of withdrawing from the cell cycle. In juvenile and adult rat ventricles, where myocytes have become permanently postmitotic, cdc2 mRNA becomes undetectable. These results demonstrate that cdc2 mRNA expression in ventricular muscle...
cdc2 Expression in Cardiac Muscle

is downregulated as early as day 1 after birth and that at the juvenile stage, cdc2 mRNA expression is turned off. Consistently, cdc2 protein showed a temporal pattern almost identical to that of cdc2 mRNA (Fig 1B). The temporal correlation between downregulation of cdc2 mRNA and protein and permanent cessation of myocyte proliferation suggests that downregulation of cell cycle control genes could play a critical role in myocyte withdrawal from the cell cycle.

A 0.1-kb cdc2 5′ Flanking Region Confers Reporter Gene Expression in Fetal Ventricular Myocytes

To determine whether the decrease in ventricular cdc2 mRNA level in day-1 neonatal myocytes is due to reduced transcription, a transient expression assay was performed. The recombinant plasmids that carry a firefly luciferase reporter gene driven by progressively deleted 5′ flanking regions of the human cdc2 gene were transfected into cultured fetal ventricular myocytes. The schematic structures of these overlapping cdc2 5′ flanking regions are illustrated in Fig 2A. The relative promoter activities were determined by measuring luciferase activities in the lysates of transfected cells. All five cdc2 chimeric plasmids had detectable luciferase reporter gene expression in cultured fetal myocytes (Fig 2B). A plasmid that carries the promoterless luciferase cDNA was used as a negative control, and no luciferase activity was detected after transfection (data not shown). In spite of the large differences in lengths, differences in promoter activity were <2-fold among these five fragments. The shortest 0.1-kb fragment contains 65±17% (mean±SEM) promoter activity of the longest 6.2-kb fragment, suggesting that the major information required for basal level cdc2 expression is located within the 0.1-kb fragment. Sequence analysis revealed two inverted CCAAT boxes (IC box) that are located at −46/−42 and −78/−74 within the 0.1-kb cdc2 promoter. Although it has been shown that the IC boxes in the human cdc2 gene are important for cdc2 transcriptional activation in human myeloid leukemia cells, it is not clear whether the IC boxes are required for cdc2 transcriptional activation in fetal ventricular myocytes.

Since the steady-state level of cdc2 mRNA is lower in neonatal muscle compared with fetal muscle, we next determined whether the 0.1-kb cdc2 promoter also contains sufficient sequence to elicit developmental transcriptional downregulation. To that end, we compared the relative 0.1-kb cdc2 promoter activities as well as the 6.2-, 1.8-, 1.1-, and 0.7-kb cdc2 promoter activities in neonatal and fetal myocytes by transient expression assay. Fig 2C shows that the activities of all five cdc2 promoters in neonatal myocytes were 3- to 4-fold lower than in fetal myocytes; these data are consistent with the data of Northern blot analysis. Therefore, the 0.1-kb cdc2 promoter may contain essential cis regulatory elements that are involved in both transcriptional activation in fetal myocytes and downregulation in neonatal myocytes.

A Single Protein Binding Site Containing the Upstream IC Box Is Identified Within the 0.1-kb cdc2 Promoter

To detect nuclear proteins that interact with the 0.1-kb cdc2 promoter, a gel mobility shift assay was performed using nuclear extracts prepared from fetal ventricular myocytes. The 0.1-kb cdc2 promoter fragment was end-labeled with 32P and used as a probe for DNA-protein interaction. As shown in Fig 3, a single prominent DNA-protein complex was detected with fetal myocyte nuclear extracts. To assess the specificity of this DNA-protein interaction, binding competition was performed with 50-, 100-, and 200-fold molar excess of either unlabeled 0.1-kb cdc2 promoter fragment or a nonspecific DNA of the same size. The DNA-protein complex was completely competed away by the unlabeled 0.1-kb cdc2 promoter fragment at 50-, 100-, and 200-fold molar excess; however, the nonspecific DNA fragment had little effect on the DNA-protein complex (Fig 3). These results indicate that the observed DNA-protein interaction is sequence specific. A faint band was also observed below the prominent band, which may represent a degradation product of the top band or a second protein binding to the 0.1-kb fragment with a lower affinity. This faint band also seems to represent specific DNA-protein interaction because it was only competed away by the unlabeled 0.1-kb cdc2 promoter fragment. The prominent DNA-binding factor was named CPBF.
Figure 3. Detecting nuclear proteins that interact with the 0.1-kb cdc2 promoter by gel mobility shift assay. The 0.1-kb fragment was end-labeled by Klenow with $^{32}$P and incubated with nuclear extracts prepared from isolated fetal ventricular myocytes. For binding competition assay, 50-, 100-, or 200-fold molar excess of unlabeled 0.1-kb fragment (self) or a nonspecific DNA (NSD) fragment of the same size derived from pBlue-script SK was added to the DNA-protein binding reactions. F indicates free probe; 0, the probe incubated with nuclear extracts. The sequence of the 0.1-kb cdc2 promoter is shown in the right panel. Two IC boxes are outlined, and the protected region from DNase I digestion (Fig 4, lane T). The same radiolabeled template was digested by DNase I in the absence of nuclear extracts and used as a control (Fig 4, lane F). A single region from –92 to –67 was protected by the nuclear extracts from DNase I digestion (Fig 4, lanes B). This protected region encompasses the upstream IC box (–78/-74), whereas the downstream IC box (–46/-42) was not protected (Fig 4, right panel). Therefore, if the upstream IC box is directly involved in this DNA-protein interaction, its flanking sequences may also be important for the DNA-protein interaction. Since the region from –92 to –67 seems to be the only protected region, we hypothesized that it serves as the CPBF binding site. To test this hypothesis, the following two binding competition assays were performed. First, a gel mobility shift assay was performed using a radiolabeled oligonucleotide derived from the putative CPBF binding site (–92 to –67) as the probe (see the Table for the sequence). Prominent and lower faint DNA-protein bands were observed with fetal myocyte nuclear extracts (Fig 5, left panel). Both bands represent specific DNA-protein interactions, since both were only competed away with 100-fold molar excess of unlabeled CPBF oligonucleotide. A random oligonucleotide at the same molar excess did not have any effects on these DNA-protein complexes. Second, a gel mobility shift assay was performed with the end-labeled 0.1-kb cdc2 promoter fragment as a probe and the unlabeled CPBF oligonucleotide as a competitor. The DNA-protein complex formed with fetal myocyte nuclear extracts was effectively competed away with 10-fold molar excess and completely competed away with 40- and 200-fold molar excess of the CPBF oligonucleotide (Fig 5, right panel). The above results unambiguously prove that the nuclear extract–protected region from –92 to –67 contains the CPBF binding site.

To further characterize DNA-protein interaction at the CPBF site, we used a methylation interference assay to define those G or A nucleotides within the CPBF site that made close contact with CPBF. The CPBF oligonucleotide that was

Figure 4. Localization of the CPBF binding site within the 0.1-kb cdc2 fragment by DNase I footprint assay. The 0.1-kb fragment was end-labeled at the sense strand by T4 DNA kinase with $^{32}$P and incubated with nuclear extracts prepared from isolated fetal ventricular myocytes. After partial digestion with DNase I, the reaction was resolved on a 6% sequencing gel. The protected region from –67 to –92 was indicated by a vertical bar on the right side of the autoradiograph. T indicates the T dideoxynucleotide–sequencing ladder using a primer derived from the very 5’ end of the sense strand; F, probe digested with DNase I without nuclear extracts; and B, probe digested with DNase I after being incubated with nuclear extracts. The sequence of the 0.1-kb cdc2 promoter is shown in the right panel. Two IC boxes are outlined, and the protected region is underlined.

Figure 5. Binding competition assay. In the left panel, the double-stranded oligonucleotide derived from the region of –67 to –92 (CPBF) was end-labeled by T4 DNA kinase with $^{32}$P and used as a probe for gel mobility shift assay. For binding competition assay, 100-fold molar excess of unlabeled CPBF or a random oligonucleotide (RO) was added to the DNA-protein binding reactions. In the right panel, the 0.1-kb cdc2 promoter fragment was end-labeled by Klenow with $^{32}$P and used as a probe for gel mobility shift assay. For binding competition assay, 10-, 40-, and 200-fold molar excess of CPBF oligonucleotide was added to DNA-protein binding reactions. F indicates free probe; 0, the probe incubated with nuclear extracts in the absence of competitors.

To locate the CPBF binding site within the 0.1-kb cdc2 promoter, a DNase I footprint assay was performed. The sense strand of the 0.1-kb cdc2 promoter was end-labeled with $^{32}$P and used as a template for DNA-protein interaction. An oligonucleotide derived from the very 5’ end of the end-labeled strand was used as a primer for dideoxynucleotide sequencing reaction, and the T-sequencing reaction ladder was used as a molecular weight standard (Fig 4, lane T). The same
end-labeled at either the sense or antisense strand was subjected to G-sequencing reaction, and the corresponding resultant sequencing ladder was used as the molecular weight standard (Fig 6, lanes G). As shown in the right panel of Fig 6, three nucleotides at the CPBF contact points were identified on the sense strand: one relatively strong contact point at $-78$ (A) and two weak contact points at $-75$ (G) and $-74$ (G). Similarly, three nucleotides at CPBF contact points on the antisense strand were identified: two relatively strong contact points at $-77$ (A) and $-76$ (A) and one weak contact point at $-80$ (A) (Fig 6, left panel). The CPBF contact points on both strands are located both inside and outside the IC box (Fig 6, bottom panel), further indicating that the flanking sequences of the upstream IC box are important for CPBF binding.

To directly prove that these contact points are actually required for CPBF binding, binding competition was performed using a radiolabeled 0.1-kb cdc2 promoter fragment as a probe and both wild-type and mutant CPBF oligonucleotides as competitors. As illustrated in the Table, two mutant CPBF oligonucleotides were synthesized; these contained point mutations either at the strong contact point (CMM1) or weak contact point (CMM2). Since methylation interference assay only detects the contact points at G or A nucleotides, we also introduced two sets of point mutations into the CPBF site at $-70$ and $-68$ (CMM3) or at $-69$ and $-67$ (CMM4), where the corresponding T or C nucleotides could possibly serve as the contact points. As shown in Fig 7, CMM1 failed to compete away the CPBF DNA–protein complex at 10-, 40-, and 200-fold molar excess. CMM2 partially competed away the CPBF DNA–protein complex at 10- and 40-fold molar excess and completely competed away the complex at 200-fold molar excess. The difference in competition efficiency between CMM1 and CMM2 is consistent with the notion that strong contact points play a more important role in mediating DNA–protein interaction. Interestingly, CMM3 failed to compete away the complex at 10-, 40-, and 200-fold molar excess, whereas CMM4 competed nearly as efficiently as the wild-type CPBF oligonucleotide. Therefore, the C nucleotide at $-70$ and T nucleotide at $-68$ on the sense strand could function as the critical contact points for CPBF binding, whereas the two T nucleotides at $-69$ and $-67$ on the sense strand may not be so essential for CPBF binding. Since the C nucleotide at $-70$ and T nucleotide at $-68$ on the sense strand are both outside the IC box, the above results offer further evidence that flanking sequences of the upstream IC box are important for CPBF binding.

**Mutations That Abolish CPBF Binding Significantly Decrease the 0.1-kb cdc2 Promoter Activity**

To determine whether CPBF actually activates the 0.1-kb cdc2 promoter by binding to the CPBF site, mutations were introduced into the CPBF site within the 0.1-kb cdc2 promoter by site-directed mutagenesis. The locations of these mutations are identical to those in the mutant CPBF oligonucleotides, ie, CMM1, CMM2, CMM3, and CMM4, used for the binding competition assay as described above. Therefore, we used the same nomenclature for the mutant 0.1-kb cdc2 promoters. The effects of these mutations on the 0.1-kb cdc2 promoter activity were determined by transient expression assay in fetal myocytes. Recombinant plasmids that carry the luciferase reporter gene driven by CMM1, CMM2, CMM3, or CMM4 0.1-kb cdc2 promoter were transfected into fetal myocytes, and the relative promoter activities were compared with those of the wild-type 0.1-kb cdc2 promoter. The
mutations significantly decreased the 6.2-kb promoter activity in fetal ventricular myocytes (FM). A, The point mutations described in Fig 7 were introduced into the 0.1-kb cdc2 promoter fragment by site-directed mutagenesis (see "Materials and Methods"). The recombinant plasmids that carry luciferase cDNA driven by either the wild-type (W.T.) or the mutant 0.1-kb cdc2 promoter were transfected into FM, and the promoter activity of the W.T. promoter was arbitrarily set as 100%. The difference in promoter activity between the W.T. and CMM1, CMM2, and CMM3 mutant promoters is significant ($P<.05$, $n=4$), whereas the activity of CMM4 mutant promoter is not significantly different from the W.T. promoter. B, The activities of CMM1 and CMM3 mutant promoters in FM were compared with those of CMM1 and CMM3 mutant promoters in neonatal myocytes (NM). The activities in FM were arbitrarily set as 100% (the striated bars). No significant difference in promoter activity was observed between FM and NM. C, The CMM1 and CMM3 mutations were introduced into the 6.2-kb cdc2 promoter, and their effects on promoter activity were determined as described in panel A. Both CMM1 and CMM3 mutations significantly decreased the 6.2-kb promoter activity in FM ($P<.05$, $n=4$).

The effects of point mutations in the CPBF site on cdc2 promoter activities in fetal ventricular myocytes (FM). A. The point mutations described in Fig 7 were introduced into the 0.1-kb cdc2 promoter fragment by site-directed mutagenesis (see "Materials and Methods"). The recombinant plasmids that carry luciferase cDNA driven by either the wild-type (W.T.) or the mutant 0.1-kb cdc2 promoter were transfected into FM, and the promoter activity of the W.T. promoter was arbitrarily set as 100%. The difference in promoter activity between the W.T. and CMM1, CMM2, and CMM3 mutant promoters is significant ($P<.05$, $n=4$), whereas the activity of CMM4 mutant promoter is not significantly different from the W.T. promoter. B. The activities of CMM1 and CMM3 mutant promoters in FM were compared with those of CMM1 and CMM3 mutant promoters in neonatal myocytes (NM). The activities in FM were arbitrarily set as 100% (the striated bars). No significant difference in promoter activity was observed between FM and NM. C. The CMM1 and CMM3 mutations were introduced into the 6.2-kb cdc2 promoter, and their effects on promoter activity were determined as described in panel A. Both CMM1 and CMM3 mutations significantly decreased the 6.2-kb promoter activity in FM ($P<.05$, $n=4$).

Figure 8. Effects of point mutations in the CPBF site on cdc2 promoter activities in fetal ventricular myocytes (FM). A, The point mutations described in Fig 7 were introduced into the 0.1-kb cdc2 promoter fragment by site-directed mutagenesis (see "Materials and Methods"). The recombinant plasmids that carry luciferase cDNA driven by either the wild-type (W.T.) or the mutant 0.1-kb cdc2 promoter were transfected into FM, and the promoter activity of the W.T. promoter was arbitrarily set as 100%. The difference in promoter activity between the W.T. and CMM1, CMM2, and CMM3 mutant promoters is significant ($P<.05$, $n=4$), whereas the activity of CMM4 mutant promoter is not significantly different from the W.T. promoter. B, The activities of CMM1 and CMM3 mutant promoters in FM were compared with those of CMM1 and CMM3 mutant promoters in neonatal myocytes (NM). The activities in FM were arbitrarily set as 100% (the striated bars). No significant difference in promoter activity was observed between FM and NM. C, The CMM1 and CMM3 mutations were introduced into the 6.2-kb cdc2 promoter, and their effects on promoter activity were determined as described in panel A. Both CMM1 and CMM3 mutations significantly decreased the 6.2-kb promoter activity in FM ($P<.05$, $n=4$).

Biochemical Evidence Suggests That CPBF and NF-Y Are Identical or Closely Related

As described above, the CPBF site contains an IC box, which serves as the core sequence of binding sites for transcription factors such as NF-Y, C/EBP, and NF-1. Therefore, it is possible that CPBF is related or identical to one of these factors. To assess the relationships of CPBF to NF-Y, C/EBP, and NF-1, a binding competition assay was performed using radiolabeled CPBF oligonucleotide as probe and the oligonucleotides containing the consensus binding sites of NF-Y, C/EBP, and NF-1 as competitors (see the Table for sequenc.

In this assay, the wild-type CPBF oligonucleotide and mutant CPBF oligonucleotide, CMM1, were used as positive and negative control competitors, respectively. As shown in Fig 9, the NF-Y consensus binding site competed away the CPBF DNA-protein complex as efficiently as the wild-type CPBF oligonucleotide at 40-fold molar excess. The oligonucleotides of NF-1 and C/EBP consensus binding sites had little effect on the CPBF DNA-protein complex at the same molar excess. These results suggest a greater similarity between CPBF and NF-Y than between CPBF and C/EBP or NF-1. Interestingly, an oligonucleotide derived from the region of −59 to −31 in the 0.1-kb cdc2 promoter, which contains the downstream IC box (−46/−42, DSC), also effectively competed away the CPBF DNA-protein complex. Furthermore, we performed a gel mobility shift assay using the radiolabeled consensus NF-Y binding site as the probe, which is the same length as CPBF oligonucleotide (Fig 10, bottom panel). For direct comparison, results of competition assay using radiolabeled CPBF oligonucleotide as a probe and unlabeled CPBF or NF-Y oligonucleotide as a competitor were included. A single DNA–protein complex was detected using NF-Y probe with fetal myocyte nuclear extracts, which has the same mobility as the CPBF DNA–protein complex (Fig 10). In addition, the NF-Y DNA–protein complex was completely competed away by unlabeled CPBF or NF-Y oligonucleotide at 40-fold molar excess. On the basis of these results, we hypothesized that CPBF was identical or closely related to NF-Y.

To further investigate the relationships between CPBF and NF-Y, an antibody supershift assay was performed. NF-Y is a heterotrimeric transcription factor that is composed of NF-Ya, NF-Yb, and NF-Yc subunits. We reasoned that if CPBF is identical to NF-Y, antibodies against one of the three NF-Y subunits would either block the formation of the CPBF DNA–protein complex or decrease its mobility by physically associating with the corresponding NF-Y subunit. Therefore,
we tested the effects of antibodies against either NF-Ya or NF-Yb on the CPBF DNA-protein complex by gel mobility shift assay.31 As shown in Fig 11A, when antibodies against NF-Ya were incubated with CPBF oligonucleotide and fetal myocyte nuclear extracts, the CPBF DNA-protein complex was “supershifted” to a higher position in the polyacrylamide gel, indicating a decrease in mobility of the DNA-protein complex (FVNE, lane +). The preimmune serum did not have any effects (FVNE, lane −). When the same experiment was performed with NF-Yb antibodies (Fig 11B), the CPBF DNA-protein complex was also supershifted (FVNE, lane +). In fact, the CPBF DNA-protein complex associated with NF-Yb antibodies hardly entered the gel (Fig 11B). The preimmune serum did not have any effects (FVNE, lane −). The above results indicate that CPBF contains NF-Ya and NF-Yb subunits. Altogether, our results further prove that CPBF in fetal myocytes is identical or closely related to NF-Y.

To functionally demonstrate that NF-Y or its related transcription factor activates the 0.1-kb cdc2 promoter in fetal myocytes, cotransfection was performed using the 0.1-kb cdc2 promoter/luciferase plasmid and a dominant-negative NF-Ya mutant (NF-YA29) plasmid. It has been shown that overexpression of NF-YA29 in other cell types can specifically suppress NF-Y-activated transcription.32 Luciferase expression from the 0.1-kb cdc2 promoter in fetal ventricular myocytes was suppressed by expression of NF-YA29 in a dosedependent manner (Fig 11C), whereas the control plasmid lacking NF-YA29 cDNA did not have any effects (data not shown). This result further indicates that NF-Y or its related transcription factor activates the 0.1-kb cdc2 promoter in fetal myocytes.

Possible NF-Y Involvement in Downregulation of cdc2 Transcription During Postnatal Development

Since NF-Y or its related transcription factor plays a critical role in activating the 0.1-kb cdc2 promoter in fetal ventricular myocytes, it is possible that the decrease in 0.1-kb cdc2 promoter activity in neonatal myocytes is induced by a decrease in NF-Y activity. To test this possibility, we compared NF-Y DNA binding activities in fetal, neonatal, and juvenile ventricular muscle by gel mobility shift assay using CPBF oligonucleotide as a probe. To our surprise, NF-Y in the nuclear extracts prepared from fetal (FVNE) and neonatal (NVNE) ventricular muscle was highly comparable, as judged by intensity, mobility, and immune reactivity to NF-Y antibodies (Fig 11A and 11B). This result suggests that NF-Y may not be involved in the initial downregulation of cdc2 transcription at the early neonatal stage. The quantity of NF-Y from the nuclear extracts of juvenile ventricular muscle (JVNE) was also comparable to that from fetal ventricular muscle, except its mobility is faster than its counterpart from fetal and neonatal ventricular muscle. This difference in mobility was reproducible with five different preparations of ventricular nuclear extracts. Higher mobility of juvenile ventricular NF-Y suggests that it may lack a subunit, which could result in the inactivation of NF-Y. Since the NF-Y DNA-protein complex from juvenile ventricular muscle can also be supershifted by antibodies against either NF-Ya or NF-Yb (Fig 11A and 11B), it is possible that NF-Yc becomes absent in juvenile ventricular muscle. Western blot analysis with anti-

Figure 11 Antibody supershift assay. FVNE, NVNE, JVNE, and the end-labeled CPBF oligonucleotide were used for gel mobility shift assay. A and B, To determine immune reactivity of CPBF to antibodies against either NF-Ya (A) or NF-Yb (B) was added to DNA-protein binding reactions (+), which were run next to the corresponding controls with preimmune serum (−) on a 5% polyacrylamide gel. F indicates free probe. C, Dominant-negative NF-Ya mutant protein is expressed from NF-YA29 plasmid. Five micrograms of 0.1-kb cdc2 promoter/luciferase plasmid was cotransfected with 0, 1, 2.5, 5, and 7.5 µg of NF-YA29 plasmid into fetal myocytes in each dish. A dominant-negative NF-Ya mutant protein is expressed from NF-YA29 plasmid. D, Protein levels of NF-Ya and NF-Yb in fetal (F), neonatal (N), juvenile (J), and adult (A) rat ventricular tissue. Western blot analysis was performed as described in Fig 1B. NF-Ya and NF-Yb proteins were detected with antibodies against NF-Ya and NF-Yb.
bodies against NF-Ya or NF-Yb showed that indeed the protein levels of both NF-Ya and NF-Yb are identical in fetal, neonatal, juvenile, and adult rat ventricular muscle (Fig 11D). However, this hypothesis cannot be directly tested because of the lack of antibodies against NF-Yc. Interestingly, the juvenile ventricular NF-Y complex comigrates with the faint lower NF-Y DNA-protein complex observed with FVNE and NVNE (Fig 11A and 11B). This faint lower band displayed identical sequence specificity and immune reactivity to antibodies against either NF-Ya or NF-Yb (Figs 3, 5, 11A, and 11B). Thus, the lower faint band could possibly represent the fraction of NF-Y in fetal and neonatal ventricular muscle that only contains NF-Ya and NF-Yb.

Discussion

Does Downregulation of cdc2 Transcription Contribute to Inducing Cardiac Myocytes to Withdraw From the Cell Cycle and/or Preventing Terminally Differentiated Cardiac Myocytes From Reentering the Cell Cycle?

The results presented in the present study have shown that cardiac cdc2 expression is downregulated after birth and turned off at the juvenile stage. A 0.1-kb cdc2 promoter can mediate transcriptional activation and suppression of a reporter gene in fetal and neonatal myocytes, respectively. The transcription factor NF-Y or its closely related factor plays a critical role in activating the 0.1-kb cdc2 promoter. In laboratory rats, withdrawal of cardiac myocytes from the cell cycle is completed by the end of the third week after birth. The results of Northern blot analysis indicate that the cdc2 mRNA expression is downregulated shortly after birth and is turned off by the end of the third week after birth. The temporal correlation between cdc2 mRNA expression and withdrawal of cardiac myocytes from the cell cycle suggests possible involvement of cdc2 downregulation in myocyte withdrawal from the cell cycle. Although the function of CDK1 encoded by the cdc2 gene is primarily to promote the G2 to M transition, cdc2 transcription is activated at the early S phase and suppressed at the G1 phase. Since ≈95% of rat terminally differentiated cardiac myocytes are arrested in G0 and G1 phases, a G1 blockade that blocks the entry into S phase may be established in these myocytes. Therefore, the factors that downregulate cdc2 mRNA expression in neonatal cardiac myocytes could participate in the establishment of G1 blockade. It has been shown recently that this G1 blockade can be overcome or bypassed by two adenovirus nuclear oncoproteins, E1A and E1B. E1A protein can disrupt the functions of a family of growth suppressor proteins collectively known as the pocket proteins, which block the G1 to S transition. One well-established mechanism by which these pocket proteins suppress G1 to S transition is to bind and sequester the transcription factor E2F. Therefore, pocket proteins could possibly become constitutively active in cardiac myocytes during terminal differentiation and block the entry into S phase. As a result, cardiac myocytes become permanently growth-arrested at the G1 phase and some subsequently enter the G0 phase. As a further proof, Kirshenbaum et al have reported that the overexpression of the human E2F-1 gene can completely mimic the effect of E1A/E1B overexpression in reactivating DNA synthesis in neonatal rat ventricular myocytes. However, it is not clear whether overexpression of E1A/E1B or E2F-1 can reactivate cdc2 expression in terminally differentiated cardiac myocytes. As described above, cdc2 transcription is suppressed by E2F-independent mechanisms on entry into the G1 phase or by growth arrest in a number of cell types. Consistently, our data in the present study indicate that the activity of the 0.1-kb cdc2 promoter that lacks the E2F site is downregulated in cardiac myocytes at an early neonatal stage in parallel to the downregulation of endogenous cdc2 mRNA. Therefore, it is possible that factors in addition to E2F may be involved in suppression of cardiac cdc2 transcription during terminal differentiation and could be critical components of the G1 blockade.

Does NF-Y Serve as a General Transcription Factor for Cell Cycle Control Genes in Cardiac Myocytes?

In cycling cells, genes involved in progression through the same phases of the cell cycle are generally regulated in a coordinated fashion through common transcription factors, such as E2F. Recent studies in various cell types have indicated that NF-Y may also serve as such a common transcription factor for a number of cell cycle control genes. For instance, the activities of cdc25C, cyclin A, and cyclin B are required for CDK1 to control G2 to M transition; thus, the expression patterns of these three genes in cycling cells are very similar. They are activated at either early S phase (cdc2 and cyclin A) or late S phase (cyclin B and cdc25C) and become suppressed in early G1 phase. The cdc2, cyclin A, and cdc25C genes all contain NF-Y sites in their promoters, which are required for their transcriptional activation at S phase. At G1 phase, NF-Y sites seem to be suppressed by two downstream negative regulatory elements, CDE and CHR, collectively known as the R box in the cdc2 promoter. Zwicker et al have shown that mutations at either the CDE or CHR site lead to almost complete loss of cell cycle regulation of these three genes. The molecular mechanisms by which the CDE and CHR sites suppress NF-Y sites at the G1 phase still remain to be elucidated. Thus, in proliferating cells, NF-Y activates the expression of the cdc2, cyclin A, and cdc25C genes in the S phase, and this activation is suppressed by two downstream negative regulatory elements in the promoters. Yoshizumi et al have shown that the expression of cyclin A during myocardial development is quite similar to that of the cdc2 gene: it is downregulated after birth and turned off at the juvenile stage. It seems likely that the cdc2 and cyclin A genes, and possibly other cell cycle control genes, are regulated by common transcription factors, such as NF-Y, in fetal cardiac myocytes. Permanent downregulation of the genes encoding NF-Y subunits, eg, NF-Yc, could play a particularly important role in reducing the expression of these cell cycle control genes during postnatal myocardial development. Thus, a study of the regulation of NF-Y expression, in particular, the NF-Yc subunit, in cardiac muscle during terminal differentiation would be an important contribution toward fully understanding cell cycle control in cardiac myocytes.
Acknowledgments

DrS Yan, Liu, Dawes, Lu, and Zhu are supported by the Theodore C. Laubisch Research Fund (University of California, Los Angeles), a Grant-in-Aid from the American Heart Association, National Center (No. A94–2457A–00), and the First Award Fund from the National Heart, Lung, and Blood Institute (R29 HL–56183, 1A1). The authors would like to thank Dr Teresa L. Born for providing the recombinant plasmids carrying the five progressively deleted human cdc2 5′-flanking regions; Dr Roberto Mantovani for providing the antibodies against NF-Ya and NF-Yb, ie, the NF-YA29 plasmid; and Drs Susanne B. Nicholas, Fuhua Chen, Kenneth D. Philipson, and Robert S. Ross for critically reading this manuscript.

References

Transcriptional Activation of the p34^cdc2\(^2\) Gene by cdc2 Promoter Binding Factor/Nuclear Factor-Y in Fetal Rat Ventricular Myocytes

Qingquan Liu, Huajun Yan, Nicola J. Dawes, Yujuan Lu and Hong Zhu

doi: 10.1161/01.RES.82.2.251

_Circulation Research_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 1998 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/content/82/2/251

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in _Circulation Research_ can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to _Circulation Research_ is online at:
http://circres.ahajournals.org//subscriptions/