

# Cyclin D1 Overexpression Promotes Cardiomyocyte DNA Synthesis and Multinucleation in Transgenic Mice

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## Abstract

D-type cyclin/cyclin-dependent kinase (CDK) complexes regulate transit through the restriction point of the cell cycle, and thus are required for the initiation of DNA synthesis. Transgenic mice which overexpress cyclin D1 in the heart were produced to determine if D-type cyclin deregulation would alter myocardial development. Cyclin D1 overexpression resulted in a concomitant increase in CDK4 levels in the adult myocardium, as well as modest increases in proliferating cell nuclear antigen and CDK2 levels. Flow cytometric and morphologic analyses of dispersed cell preparations indicated that the adult transgenic cardiomyocytes had abnormal patterns of multinucleation. Histochemical analyses confirmed a marked increase in number of cardiomyocyte nuclei in sections prepared from the transgenic mice as compared with those from control animals. Tritiated thymidine incorporation analyses revealed sustained cardiomyocyte DNA synthesis in adult transgenic hearts. (*J. Clin. Invest.* 1997. 99:2644–2654.) Key words: cardiomyocyte terminal differentiation • cardiac regeneration

## Introduction

Cardiomyocyte death with an ensuing loss of myocardial function is observed in many forms of cardiovascular disease. This pathophysiologic process might be partially abated if the surviving myocardium retained even a limited ability to proliferate. However, it is clear that the proliferative potential of adult mammalian cardiomyocytes (as approximated by studies monitoring DNA synthesis) is quite limited. For example, the cardiomyocyte labeling index in normal adult hearts was observed to be < 0.006% when using tritiated thymidine incorporation assays with transgenic mice expressing a cardiomyocyte-restricted  $\beta$ -galactosidase ( $\beta$ GAL)<sup>1</sup> reporter to mark cardiomyocyte nuclei (1). No changes in cardiomyocyte label-

ing indices were observed after either myocardial infarction (1) or pharmacologically induced hypertrophy (2), consistent with the absence of significant myocardial regeneration in the mouse. Although the absolute frequency of cardiomyocyte DNA synthesis in other species less amenable to genetic manipulation is somewhat more controversial (for review see reference 3), it is generally agreed that the mammalian myocardium lacks a significant capacity for regenerative growth (for review see reference 4).

Given the therapeutic potential of regenerative myocardial growth, considerable effort has been invested to develop strategies to induce cardiomyocyte proliferation. Preliminary efforts towards this end monitored the effects of various growth factors on cultured cardiomyocytes. Although numerous factors have been shown to augment cardiomyocyte DNA synthesis in vitro (5), no factors examined to date have been able to induce sustained proliferation of differentiated cardiomyocytes in fetal or adult cultures. With the advent of gene transfer techniques, it has become possible to test the ability of a specific gene product to augment myocardial proliferation both in vitro and in vivo. For example, retroviral transfection of *v-myc* resulted in the formation of cardiac rhabdomyosarcomas in chick embryos (6), and in sustained proliferation of cultured fetal rat cardiomyocytes (7). In contrast, transgenic mice which expressed high levels of *c-myc* in the heart exhibited only mild developmental hyperplasia which resulted in an approximately twofold increase in cardiomyocyte number, but not in sustained cardiomyocyte proliferation (8, 9). In other studies, primary cultures of neonatal cardiomyocytes prepared from transgenic mice expressing an IGF-1B transgene exhibited enhanced BrdU incorporation as compared with their nontransgenic controls (10). Viral transfection of the E1A oncoprotein induced cardiomyocyte DNA synthesis (and, in the absence of E1B cotransfection, apoptosis) in primary cultures of neonatal rat cardiomyocytes (11). In contrast, overt cardiomyocyte proliferation in vitro and tumorigenesis in vivo have been observed in response to targeted SV40 T antigen expression in primary cardiomyocyte cultures and in transgenic models, respectively (for reviews see references 12 and 13). Although these studies clearly have indicated that forced expression of cellular protooncogenes or transforming oncogenes from DNA tumor viruses can promote cardiomyocyte DNA synthesis, and in some instances proliferation, progress on the identification of genes which might be useful to induce regenerative growth has been slow.

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Received for publication 4 November 1996 and accepted in revised form 10 March 1997.

*J. Clin. Invest.*

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0021-9738/97/06/2644/11 \$2.00

Volume 99, Number 11, June 1997, 2644–2654

1. *Abbreviations used in this paper:* CDK, cyclin-dependent kinase; cTnT, cardiac troponin-T;  $\beta$ -Gal,  $\beta$ -galactosidase; MHC, myosin heavy chain; PCNA, proliferating cell nuclear antigen; RB, retinoblastoma gene product; RT-PCR, reverse transcriptase-PCR; X-GAL, 5-bromo-4-chloro-3-indolyl- $\beta$ -D-galactoside.

Progression through the mammalian cell cycle is regulated at several checkpoints. This highly orchestrated cascade ensures that all requisite activities (i.e., genome reduplication, DNA repair, chromosome segregation, etc.) are completed before initiation of the next step of the cell cycle. Additionally, the presence of multiple checkpoints can provide a mechanism for the identification and subsequent elimination of aberrantly growing (i.e., neoplastic) or genetically compromised cells. Transition through the various checkpoints is regulated in part by the activity of a family of protein kinases (the cyclin-dependent kinases or CDKs) and their activating partners (the cyclins). In most instances, initiation of DNA synthesis requires transit through the so-called restriction point, which is at the G1→S boundary of the cell cycle. Transit through the restriction point is largely regulated by CDK4 and the D-type cyclins (for reviews see references 14 and 15). The importance of D-type cyclins in cell cycle regulation is underscored by the observation that translocations involving the cyclin D1 locus are associated with tumorigenesis in humans.

Previous transgenic experiments have suggested that the cellular phenotypes resulting from cyclin D1 deregulation are largely dependent upon the targeted cell lineage. For example, expression of an MMTV-LTR-cyclin D1 transgene led to constitutive mammary hyperplasia (16). Multifocal lesions and occasional metastasis were observed in older transgenic mice, suggesting a requirement for additional genetic events for overt tumorigenesis to occur. In contrast, no lymphocyte hyperplasia was observed in mice carrying an E $\mu$ -cyclin D1 transgene (17). However, mice carrying both E $\mu$ -cyclin D1 and E $\mu$ -myc transgenes exhibited accelerated lymphoma formation as compared with mice with the E $\mu$ -myc transgene alone (17, 18), once again indicating that cyclin D1 deregulation can cooperate with other genetic events to promote overt tumorigenesis.

This study represents an initial pragmatic effort to test candidate genes which may participate in the regulation of cardiomyocyte DNA synthesis. Transgenic mice which constitutively overexpressed cyclin D1 in the myocardium were produced in order to test the impact of selected D-type cyclin deregulation on cardiomyocyte terminal differentiation. Cyclin D1 overexpression resulted in a concomitant increase in CDK4 levels in the adult myocardium. Flow cytometric and morphometric analyses revealed abnormal patterns of cardiomyocyte multinucleation in the transgenic mice. Thymidine incorporation assays indicated that cyclin D1 overexpression was able to program sustained cardiomyocyte DNA synthesis in adult transgenic hearts. The potential implications of these results on therapeutic myocardial regeneration are discussed.

## Methods

**Generation of the MHC-CYCD1 fusion gene.** The MHC-CYCD1 transgene was constructed using the transcriptional regulatory sequences of the mouse  $\alpha$ -cardiac myosin heavy chain (MHC) gene and sequences encoding the mouse cyclin D1 (CYCD1) protein (see Fig. 2). The MHC promoter consisted of 4.5 kb of 5' flanking sequence and 1 kb of the gene encompassing exons 1–3 up to but not including the initiation codon (19). The CYCD1 sequences were generated by reverse transcriptase-polymerase chain reaction (RT-PCR) amplification of mouse heart RNA as described (20). The sense primer was located at nucleotide residues 158–187 of the mouse cyclin D1 cDNA (21); the sequence of the primer was 5'-GGCGCCGCCAGACCACAGCCCTCCCCAGACGGCCGCGCC-3'. The antisense primer

was located at nucleotide residues 1046–1075 of the mouse cDNA (21); the sequence of the primer was 5'-GGGTACCCTCAGTGTCCACATCTCGCACGTCGGTGGG-3'. NotI (5') and KpnI (3') restriction sites were incorporated into the amplification primers to facilitate subsequent subcloning. The resulting 930-bp amplification product was subcloned with the TA vector cloning system (Invitrogen, San Diego, CA) according to the manufacturer's protocols. The integrity of the cyclin D1 cDNA was confirmed by sequence analysis; the cyclin D1 nucleic acid sequence and deduced amino acid sequence were identical to those reported previously for the mouse (21). The SV40 early region transcription terminator/polyadenylation site (nucleotide residues 2586–2452, reference 22) was inserted downstream from the CYCD1 cDNA insert.

**Generation of MHC-CYCD1 transgenic mice and myocardial pathology models.** The MHC-CYCD1 plasmid was digested with XhoII and HindIII and the insert purified from an agarose gel using GeneClean glass beads (Bio 101, Vista, CA). Purified insert DNA was microinjected into inbred C3HeB/FeJ (The Jackson Laboratory, Bar Harbor, ME) zygotes using standard methodologies (23). The microinjected embryos were cultured in vitro to the two cell stage, and then reimplanted into pseudopregnant SW/Taconic (Taconic Farms, Germantown, NY) female mice. For all surgeries, mice were anesthetized with 2.5% Avertin (0.015 ml/g body weight, intraperitoneally; Fluka Chemical Corp., Ronkonkoma, NY). All manipulations were performed according to NIH and Institutional Animal Care and Use Guidelines. Pups derived from the microinjected embryos were screened for the presence of the transgene using diagnostic PCR amplification as described (24). Positive animals were then used to establish lineages of transgenic mice. The lineages were maintained in a DBA/2J genetic background (The Jackson Laboratory).

Myocardial hypertrophy was induced by isoproterenol infusion with Alzet minipumps in adult C3HeB/FeJ male mice (7–8 wk old) as described previously (minipump model 2001; Alza Corp., Palo Alto, CA; flow rate of 1  $\mu$ l/h, 0.028 g/ml isoproterenol, reference 25). Hearts were harvested after 7 d of isoproterenol infusion and processed for either RNA or protein isolation (see below). Cardiac hypertrophy was confirmed by image analysis of dispersed cell preparations from randomly selected hearts (41% increase in cardiomyocyte cross sectional area) as described (25). For acute myocardial overload, the upper third of the abdominal aorta of adult C3HeB/FeJ mice (males, 7–8 wk old; The Jackson Laboratory) was exposed through a midline abdominal incision and the vessel was occluded with a pair of hemostats. The occlusion resulted in a stable increase of pressure (40.3 $\pm$ 3.9 mmHg,  $\pm$ SE;  $n$  = 8). Hearts were harvested 60 min after occlusion and processed for either RNA or protein isolation.

**Western blot analyses.** Hearts were homogenized in NP-40 buffer (150 mM NaCl, 5 mM EDTA, 50 mM Tris-HCl, pH 8.0, 1  $\mu$ g/ml aprotinin, 1  $\mu$ g/ml pepstatin, 1  $\mu$ g/ml leupeptin, 50  $\mu$ g/ml TLCK, 50  $\mu$ g/ml PMSF, 100  $\mu$ g/ml TPCK, 1% vol/vol NP-40). The homogenate was cleared by centrifugation at 40,000  $g$  for 10 min, and the protein content of the supernatant was quantitated using a commercial assay (Bio-Rad, Richmond, CA). Samples were separated by size on 12% polyacrylamide gels under denaturing conditions as described (26), and electroblotted to nitrocellulose (Hoefer Scientific, San Francisco, CA) membranes as described (27). The filters were stained with 0.1% naphthol blue-black in 45% methanol and 10% acetic acid to assess the efficiency of transfer. For Western analysis, nonspecific binding was blocked by incubation in block buffer (5% nonfat dry milk, 3% BSA, 0.1% Tween, 1 $\times$  PBS) for 2 h at room temperature. The antibodies used in this study were: anti-cyclin D1 (#sc-450); anti-cyclin D3 (#sc-182); anti-CDK4 (#sc-260); anti-proliferating cell nuclear antigen (anti-PCNA) (#sc-056), anti-CDK2 (#sc-163); anti-cdc2 (#sc-54; all from Santa Cruz Biotechnology, Santa Cruz, CA). In all cases, signal was visualized by the ECL method according to the manufacturer's protocol (Amersham, Arlington Heights, IL).

**Northern blot and RT-PCR analyses.** Tissue samples were homogenized with a polytron in 4.0 M guanidinium thiocyanate, 1%  $\beta$ -mercaptoethanol, and total RNA purified by centrifugation through 5.7 M

CsCl as described (28). RNA samples were quantitated by spectrophotometry at 260 nm. For Northern analysis, total RNA (10 µg) was denatured with glyoxal, separated by size on 1.2% agarose gels, and transferred to Genescreen (DuPont-Merck Pharmaceutical Co., Wilmington, DE) as previously described (25). Probes were radiolabeled by nick translation or with polynucleotide kinase using standard protocols (28). Hybridizations were for 20 h at 65°C for cDNA probes or at 55°C for oligonucleotide probes in 4× SSC, 2× Denhardt's, 0.1% SDS, and 1 mg/ml salmon sperm DNA. Blots were washed at 65°C in 2× SSC, 0.1% SDS, and signal visualized by autoradiography at -70°C with an intensifying screen. 20× SSC is 3 M NaCl, 0.3 M sodium citrate, pH 7.0; 20× Denhardt's is 0.4% (wt/vol) polyvinylpyrrolidone, 0.4% (wt/vol) serum albumin, 0.4% (wt/vol) Ficoll. The probes used were as follows: CYCD1 cDNA (nucleotide residues 158-1075, reference 21); CYCD2 cDNA (nucleotide residues 267-1152, reference 29); CYCD3 cDNA (nucleotide residues 163-1047, reference 30); α-cardiac MHC oligonucleotide (5'-CGAACGTTTATGTTTATTGTGGATTGGCCACAGCGAGGGTCTGCTGGAGAGG-3', reference 31); β-cardiac MHC oligonucleotide (5'-GCTTTATTCTGCTTCCACCTAAAGGGCTGTTGCAAAGGCTCCAGGTCTGAGGGCTTC-3', reference 31); ANF cDNA (nucleotide residues 1-691, reference 32). In all experiments, the integrity of the RNA samples was established by staining the Northern blots with methylene blue as described (28) and/or by Northern analysis with a mouse 18S rRNA probe (5'-TCCATTATTCCTAGTGCGGTATCCAGGAGGATCGGGCCTGCTTT-3', reference 33).

For assessment of cardiac troponin-T (cTnT) isoform expression, first strand cDNA synthesis was performed with 70 ng of total RNA and 100 pmol of random primers in a total volume of 10 µl of 1× PCR (50 mM KCl, 10 mM Tris-HCl, pH 7.5, 1.5 mM MgCl<sub>2</sub>), 1 mM each dNTP, 10 U RNasin (Promega, Madison, WI), and 10 U AMV reverse transcriptase (Boehringer Mannheim Biochemicals, Indianapolis, IN) at 42°C for 60 min. The volume was then adjusted to 50 µl with 1× PCR containing 0.3 µg of the sense and antisense oligonucleotide primers, and *Taq* polymerase (1 U, *Amplitaq*, Perkin-Elmer Corp., Norwalk, CT). The mixture was overlaid with oil and amplified at 94°C (1 min) to 66°C (2 min) to 72°C (3 min) over 35 cycles. The primers used (sense primer 5'-GTGGTGGAGGAGGAACAG-3'; antisense primer 5'-TGGACCATCTCAGCATCTCTGGC-3') spanned a region of the cTnT gene which is alternatively spliced in embryonic (amplification product of 198 bp) and adult (amplification product of 168 bp) hearts (34). After amplification, the products were analyzed by Southern blotting using a radiolabeled probe corresponding to internal cTnT sequences (5'-GGACCAACCTCTTCTGCTTGTTC-3').

**Immune precipitations.** Hearts were homogenized in NP-40 buffer and processed as described above. For immune precipitation, homogenate (1 mg) was preadsorbed with protein A-Sepharose beads (50 µl of a 90 mg/ml suspension, Pharmacia Biotech., Piscataway, NJ) for 30 min at 4°C. The beads were removed by centrifugation, and the preadsorbed homogenate was reacted with 1 µg of antibody in a final adjusted volume of 1 ml (with NP-40 buffer). The mixture was incubated with rocking for 1 h at 4°C. 50 µl of protein A beads was added and the incubation was continued for an additional 30 min. Immune complexes were collected by centrifugation (3,000 g, 3 min, 4°C), washed three times with PBS buffer, and eluted in SDS-PAGE loading buffer for 10 min at 95°C. Samples were resolved on 12% SDS-PAGE gels using standard protocols (26). The samples were electroblotted to nitrocellulose and subjected to Western analyses as described above.

**Dispersed cardiomyocyte preparations and flow cytometry analyses.** Dispersed cardiomyocyte preparations were generated by retrograde perfusion with collagenase as previously described (2). Briefly, animals were heparinized (10 ml/kg, intraperitoneally, Sigma Chemical Co., St. Louis, MO) ~30 min before being killed by cervical dislocation. Hearts were removed, trimmed under a dissecting microscope, hung by the aorta on 26 gauge cannulae, and perfused with PBS (0.5 ml/min at 37°C) containing 0.17% collagenase (type I; Wor-

thington Biochemical, Freehold, NJ). Hearts were perfused until flaccid (45-60 min), and ventricular cells were obtained by removing the lower 75% of the heart, mincing the tissue with scissors, and then triturating with a Pasteur pipette. To monitor nuclear morphology, cell suspensions were fixed in several volumes of 10% neutral buffered formalin for 1 h and smeared onto positively charged slides (Superfrost Plus; Fisher Scientific Co., Pittsburgh PA). After drying, the cells were stained with DAPI (0.28 µM in PBS, 3 min at room temperature; Boehringer Mannheim) and visualized by fluorescent microscopy.

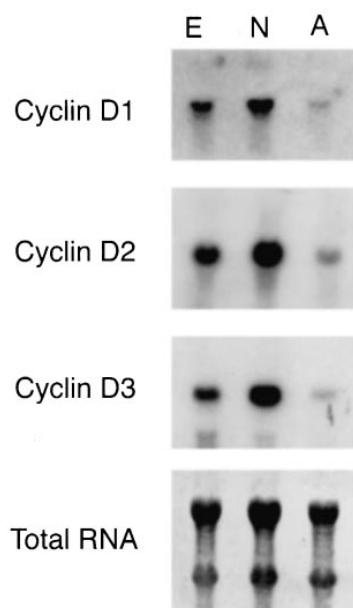
For flow cytometry analysis, aliquots of ~200,000 cells in 1 ml of PBS were incubated with 500 µl of RNase (12 µg/ml in PBS; Boehringer Mannheim) and 500 µl propidium iodide (50 µg/ml in PBS, Sigma Chemical Co.) at room temperature for 30 min. Samples were then analyzed on a FACScan® (Becton Dickinson, San Jose, CA). 60,000 cells were counted in a normal DNA acquisition mode. Data were analyzed on a Macintosh PC using Cell Quest software (Becton Dickinson). In preliminary experiments aimed at validating the flow cytometric analyses, dispersed cell suspensions were prepared from fetal, neonatal, and adult nontransgenic hearts. The samples were then analyzed simultaneously by flow cytometry and by direct visualization under the microscope as described above for cardiomyocyte morphometric analyses. Under the conditions used, cardiac fibroblasts and cardiomyocytes were readily distinguished based on morphologic differences (2). By correlating the changes in flow cytometric profiles with those observed for cardiomyocyte multinucleation in fetal, neonatal, and adult animals, the identity of the cell types contributing to the various peaks in the flow cytometric analyses was readily established. The observation that the content of mono- versus binucleated cells as assessed by the flow cytometric analysis (86.2 vs. 13.8%) in control animals agreed well with those values obtained by visual scoring of similar dispersed cell preparations (85.8 vs. 14.2%, reference 35) further validated the assay.

**Assessment of cardiomyocyte DNA synthesis.** Cardiomyocyte labeling indices were determined using a high throughput thymidine incorporation assay (1). MHC-CYCD1 line 21 mice were crossed with MHC-nLAC mice. The MHC-nLAC mice express a nuclear localized βGAL reporter exclusively in cardiomyocytes (36). Mice from this cross carrying either the MHC-nLAC transgene alone or both the MHC-nLAC and MHC-CYCD1 transgenes were identified and sequestered. To monitor DNA synthesis the control (MHC-nLAC) and experimental mice (MHC-nLAC/MHC-CYCD1 double transgenic mice) received a single injection of [<sup>3</sup>H]thymidine (200 µCi, intraperitoneally, at 28 Ci/mM; Amersham), and were killed 4 h later. The hearts were removed, cryoprotected in 30% sucrose, and embedded and sectioned at 10 µm using standard histologic techniques (37). The sections were postfixed in acetone/methanol (1:1) and overlaid with 1 mg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-GAL), 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, and 2 mM magnesium chloride in PBS. The sections were counterstained with DAPI and washed three times in PBS. After drying, stained slides were coated with photographic emulsion (Ilford L4, Polysciences, Warrington, PA) diluted 1:1 with water, drained, and placed in a light-tight box for 1 wk at 4°C. Slides were then developed in Kodak D-19 (Eastman Kodak, Rochester, NY) for 4 min, washed in water, and fixed in 30% sodium thiosulfate for at least 4 min. Slides were further processed by washing in H<sub>2</sub>O and by dehydration through graded ethanols and xylene, followed by application of a coverslip. Cardiomyocyte DNA synthesis was scored by the colocalization of βGAL activity (blue staining) and silver grains.

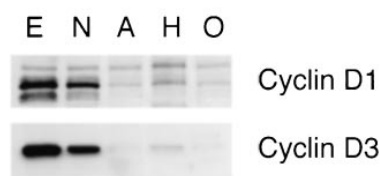
## Results

Given their central role in the initiation of DNA synthesis, the relative levels of the D-type cyclins were monitored during myocardial development. In preliminary Northern blot analyses cyclin D1, D2, and D3 mRNAs were readily detected in fe-

A. D-Type Cyclin:  
Northern Analyses



B. D-Type Cyclin:  
Western Analyses



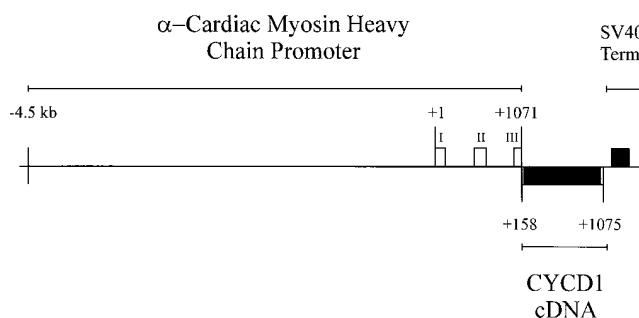
**Figure 1.** Cardiac D-type cyclin expression. (A) Northern blot analyses of cyclin D1, D2, and D3 in RNA prepared from embryonic day 15 (E), neonatal day 1.5 (N), or adult (A) hearts. The quantity and quality of the RNAs were established by staining the blots with methylene blue (Total RNA). (B) Western blot analysis for cyclin D1 and cyclin D3 expression in protein prepared from embryonic day 15 (E), neonatal day 1.5 (N), or adult (A) hearts. Protein prepared from adult hearts with isoproterenol-induced hypertrophy (H) or acute aortic overload (O) was also examined.

tal hearts (Fig. 1 A). In each case, the levels of D-type cyclin mRNA were increased in neonatal hearts, and all were markedly decreased in adult hearts (Fig. 1 A). Western blot analyses were performed to monitor D-type cyclin protein level at the same stages of development. Interestingly, the levels of both cyclin D1 and cyclin D3 protein were high in the fetal heart, somewhat lower in neonatal hearts, and dramatically reduced in adult hearts (Fig. 1 B). The cardiomyocyte labeling indices (i.e., the percentage of cells undergoing DNA synthesis) at these approximate times of development were  $26.3 \pm 0.75\%$  (embryonic day 15),  $2.8 \pm 0.85\%$  (neonatal day 1), and  $< 0.0005\%$  (adult, reference 35). No significant increases in cyclin D1 or D3 protein were observed in hearts with isoproterenol-induced hypertrophy or after 2 h of acute myocardial overload (Fig. 1 B). These results underscore the importance of assessing protein levels, as the amount of cyclin D1 protein in fetal hearts was discordant with the mRNA levels. Unfortunately, the commercially available anti-cyclin D2 antibodies failed to react with homogenate prepared from fetal mouse hearts, thereby precluding assessment of cyclin D2 protein levels during cardiac development.

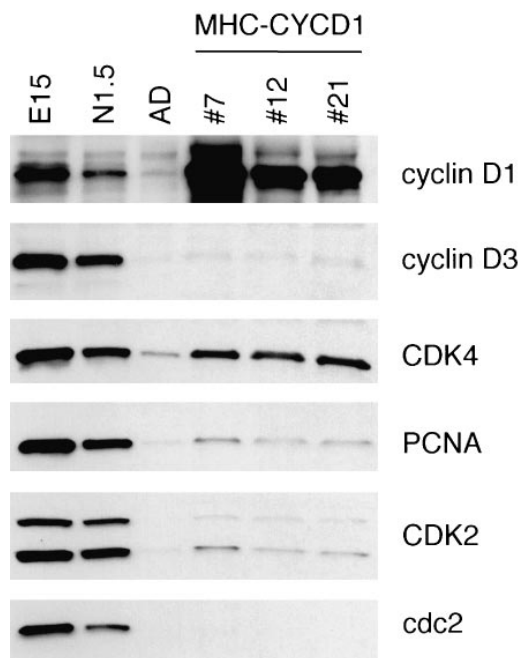
The temporal pattern of expression observed for cyclin D1 and cyclin D3 protein levels was consistent with a potential role in cardiomyocyte DNA synthesis. In this study, transgenic mice which constitutively express cyclin D1 in the heart were generated in order to directly examine the potential impact on the cardiomyocyte cell cycle. The transgene (designated MHC-CYCD1, Fig. 2) consisted of the mouse  $\alpha$ -cardiac myosin heavy chain promoter and a cDNA encoding the mouse cyclin D1 gene product. 24 transgenic mice were identified when 72 pups derived from microinjected embryos were screened. No obvious morbidity was apparent in the MHC-CYCD1 founder mice. Eight mice randomly selected and placed in breeding cages ultimately gave rise to six transgenic lineages. Three of

these lineages were selected for detailed analysis. Transgene expression was initially established by Western blot analyses (Fig. 3). High levels of cyclin D1 protein were present in the hearts of adult transgenic mice from MHC-CYCD1 lines 7, 12, and 21. Western blot analyses failed to detect elevated cyclin D1 levels in all other tissues examined, consistent with the previously described myocardial specificity of the  $\alpha$ -cardiac myosin heavy chain promoter (Koh, G.Y., unpublished data; 19, 36).

Expression of several other cell cycle regulatory proteins was monitored in the hearts of adult MHC-CYCD1 transgenic mice. Cyclin D3 protein levels were not altered by the presence of the MHC-CYCD1 transgene (Fig. 3). In contrast, expression of CDK4 was elevated in the MHC-CYCD1 transgenic hearts as compared with nontransgenic controls. This



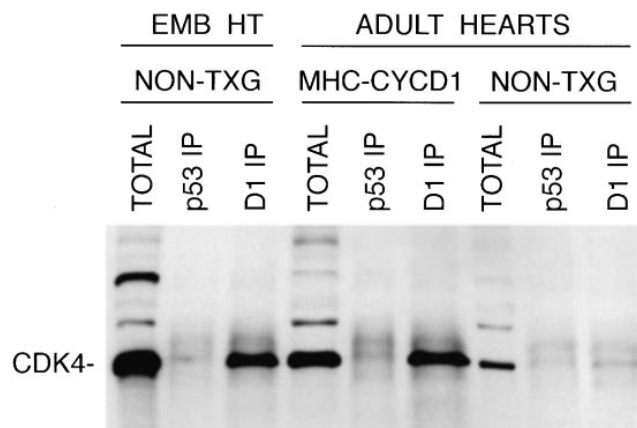
**Figure 2.** Structure of the MHC-CYCD1 transgene. The MHC promoter consisted of 4.5 kb of 5' flanking sequence and 1 kb of the gene encompassing exons 1–3 up to but not including the initiation codon. The cyclin D1 cDNA encompasses nucleotide residues 158–1075. The SV40 early region transcription terminator (nucleotide residues 2586–2452) was inserted downstream from the cyclin D1 cDNA.



**Figure 3.** Cell cycle regulatory gene expression in MHC-CYCD1 transgenic mice. Protein or RNA was prepared from the hearts of adult mice from MHC-CYCD1 lines 7, 12, and 21. Control samples from nontransgenic embryonic, neonatal, and adult mice are included for comparison. Western blot analyses for cyclin D1, cyclin D3, CDK4, PCNA, CDK2, and *cdc2* expression are shown. Note the increased expression of cyclin D1 and CDK4 in the samples from the transgenic mice.

finding was of particular interest as cyclin D1 is an activating partner of CDK4, which in turn is the principal cyclin-dependent kinase at the G1→S cell cycle checkpoint. In nontransgenic animals, CDK4 was expressed at high levels in embryonic hearts, at lower levels in neonatal hearts, and at very low levels in adult hearts (Fig. 3). PCNA is a component of the DNA replication fork, and is required for both DNA synthesis and repair. PCNA is also known to bind to the CDK4/cyclin D1 complex (38). A modest increase in PCNA levels was observed in the hearts of adult MHC-CYCD1 transgenic mice (Fig. 3). In nontransgenic animals, PCNA was expressed at high levels in embryonic and neonatal hearts, but only at very low levels in adult hearts. Expression of CDK2 and *cdc2* was also monitored. CDK2 functions late in the G1→S cell cycle checkpoint, while *cdc2* functions primarily at the G2→M cell cycle checkpoint. CDK2 protein levels were observed to be slightly upregulated in the hearts of the transgenic mice, while *cdc2* protein was not detectable.

To determine if cyclin D1 and CDK4 form a stable complex in the myocardium, total protein, anti-p53 immune complex, and anti-cyclin D1 immune complex were prepared from the hearts of embryonic nontransgenic mice, from the hearts of MHC-CYCD1 adult transgenic mice, and from the hearts of adult nontransgenic mice. The samples were displayed on a polyacrylamide gel, transferred to nitrocellulose, and probed with an anti-CDK4 antibody. As expected, a strong CDK4 signal was observed in the total protein and in the anti-cyclin D1 immune complex samples prepared from embryonic nontransgenic hearts (Fig. 4). In contrast, no CDK4 signal was observed



**Figure 4.** Cyclin D1/CDK4 complex is present in the hearts of adult MHC-CYCD1 transgenic mice. Total protein, anti-p53 immune complex, and anti-cyclin D1 immune complex from embryonic nontransgenic, adult transgenic (line MHC-CYCD1-21), and adult nontransgenic hearts were displayed on polyacrylamide gels, transferred to nitrocellulose, and probed with an anti-CDK4 antibody. Cyclin D1/CDK4 complex was present in nontransgenic embryonic hearts and in MHC-CYCD1 adult transgenic hearts, but not in nontransgenic adult hearts.

in the anti-p53 immune complex sample, which served as a negative control. Thus, cyclin D1/CDK4 complex is present in embryonic hearts. Similarly, a strong CDK4 signal was observed in the total protein sample and in the anti-cyclin D1 immune complex sample prepared from the transgenic animals, indicating the presence of cyclin D1/CDK4 complex in the adult MHC-CYCD1 hearts (Fig. 4). In contrast, only very low levels of cyclin D/CDK4 complex were detected in the nontransgenic adult hearts.

The data presented above indicated that several components of the cell cycle regulatory cascade necessary for transit through the restriction point of the cell cycle (namely cyclin D1, CDK4, PCNA) were expressed in the hearts of adult MHC-CYCD1 transgenic mice. Flow cytometry was used to determine if cyclin D1 deregulation impacted on the DNA content of adult transgenic cardiomyocytes. The assay used dispersed cell preparations, and took advantage of the fact that terminally differentiated cardiomyocytes are multinucleated, whereas other cells present in the adult heart are mononucleated. Flow cytometric analysis of dispersed cell preparations from normal adult mouse hearts revealed the presence of two prominent populations of cells (Fig. 5A). The first population of cells exhibited a DNA content equivalent to 2C, and the second population of cells exhibited a DNA content equivalent to 4C. Comparative analyses of data obtained by flow cytometry to that obtained by direct visualization of dispersed cell preparations established that the 2C peak was comprised predominately of mononucleated fibroblasts whereas the 4C peak was comprised predominately of binucleated cardiomyocytes (see Methods). Smooth muscle cells and mononucleated cardiomyocytes were presumably also present in the 2C peak (mononucleated cells comprise 5% of the total cardiomyocyte population, reference 2). A small population of cells with a DNA content equivalent to 6C (trinucleated cardiomyocytes) was also detected by this assay in nontransgenic adult hearts.

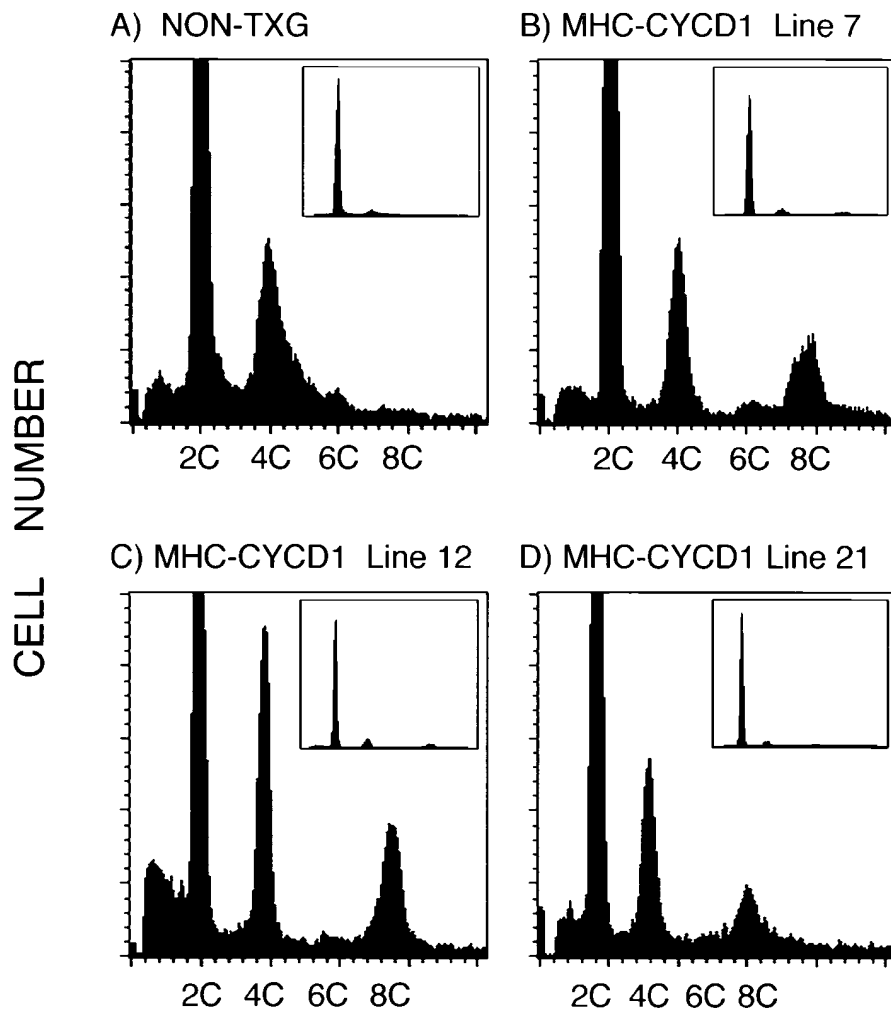


Figure 5. Flow cytometry analysis of dispersed cell preparation from the hearts of adult nontransgenic or MHC-CYCD1 transgenic mice. Dispersed cell preparations were generated by retrograde collagenase perfusion of nontransgenic and MHC-CYCD1 line 7, 12, and 21 adult hearts. The cells were stained with propidium iodide and analyzed on a Becton Dickinson FACScan<sup>®</sup>. 60,000 cells were counted for each sample. The relative positions of 2C, 4C, 6C, and 8C chromosome equivalents are indicated. Insets show non-scaled plots of the flow cytometry profiles.

Dispersed cell preparations from MHC-CYCD1 lines 7, 12, and 21 were analyzed with the flow cytometry assay. Once again, populations of cells with DNA content equivalents of 2C and 4C (mononucleated cells and binucleated cardiomyocytes, respectively) were present. In addition, a very prominent population of cells with a DNA content equivalent of 8C was present in each transgenic heart (Fig. 5, B–D). This population of cells comprised as much as 60% of the cardiomyocytes present in the hearts of adult MHC-CYCD1 transgenic mice. Dispersed cell preparations from MHC-CYCD1 line 21 transgenic hearts were stained with DAPI and visualized directly in order to determine if the elevated DNA content represented an increase in cardiomyocyte nuclear ploidy, or alternatively if there was an increase in the number of nuclei present in the cardiomyocytes. Microscopic analysis of the dispersed cell preparations revealed the presence of essentially normal appearing binucleated cardiomyocytes (Fig. 6 A), as well as cardiomyocytes with abnormal nuclear content. For example, cardiomyocytes with apparently partially replicated nuclei (Fig. 6, B and D, arrows) were readily observed. Other cells with abnormally long nuclei suggestive of nuclear replication in the absence of karyokinesis (Fig. 6, D–F, arrowheads) were also seen. The rather broad 8C peaks observed by flow cytometry analyses (Fig. 5, B–D) were probably attributable to

the cardiomyocytes with abnormal nuclear content. Numerous relatively normal appearing tetra- and pentanucleated cardiomyocytes were also observed in the dispersed cell preparations from the transgenic hearts (Fig. 6, C and G).

The flow cytometry and isolated cell morphometry analyses indicated that cyclin D1 deregulation can augment cardiomyocyte DNA content and/or nuclear number. A thymidine incorporation assay (1) was used to determine if cardiomyocyte DNA synthesis persisted in the adult transgenic animals. This assay used a second transgenic mouse model (designated MHC-nLAC) which expresses a nuclear-localized  $\beta$ GAL reporter gene exclusively in the myocardium (36). Accurate cardiomyocyte tritiated thymidine labeling indices can be readily obtained with these animals simply by screening for colocalization of  $\beta$ GAL activity and silver grains in autoradiographs of X-GAL-stained heart sections (1). To monitor the effect of cyclin D1 overexpression on cardiomyocyte DNA synthesis, MHC-CYCD1 line 21 mice were crossed with MHC-nLAC mice, and animals carrying either the MHC-nLAC transgene alone or both the MHC-nLAC and the MHC-CYCD1 transgenes were identified and sequestered. When the mice reached three mo of age, they received a single injection of tritiated thymidine and were killed 4 h later. The hearts were removed, sectioned, stained with X-GAL, and processed for autoradiog-



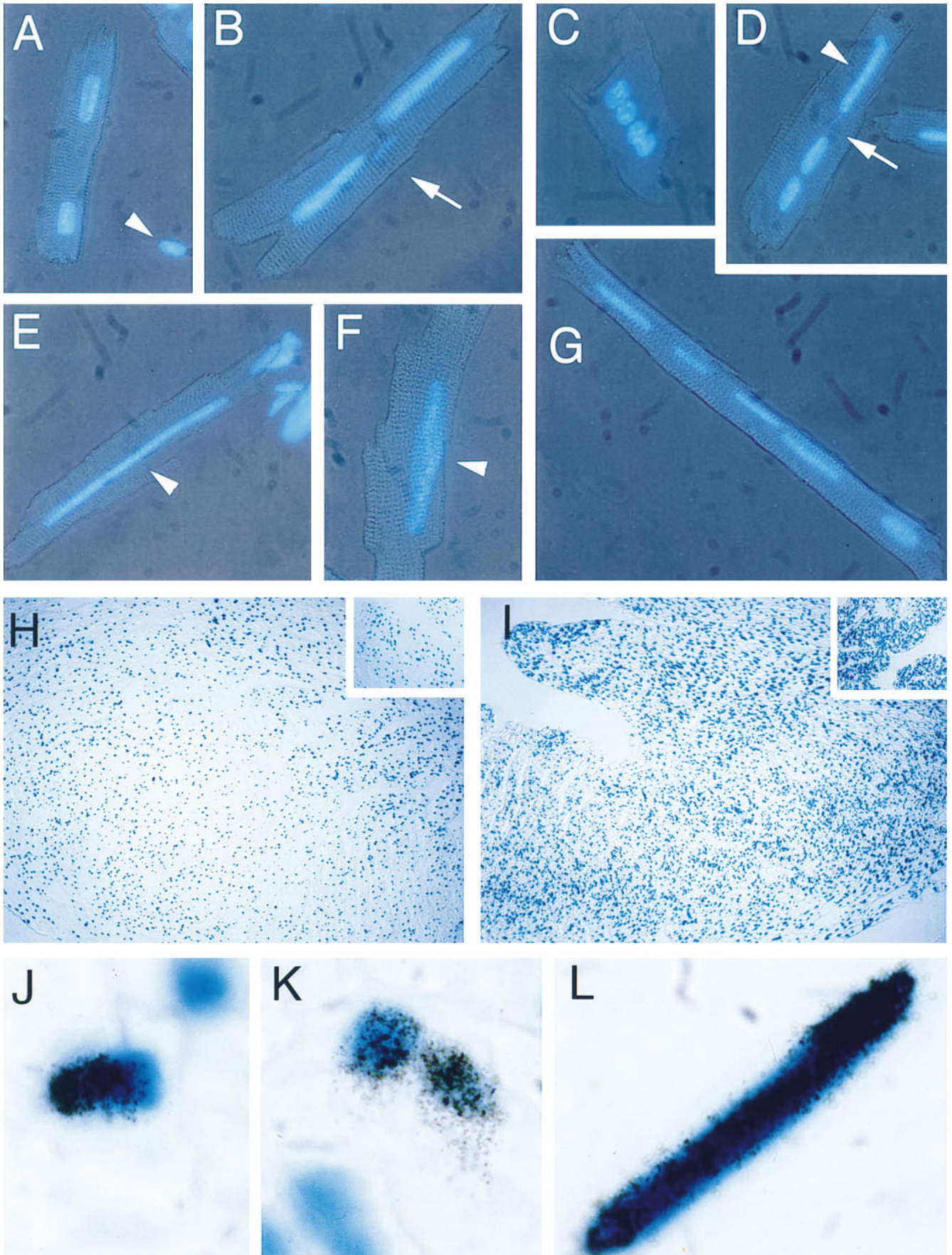


Table I. Cardiac Attributes of MHC-CYCD1 Transgenic Mice

	Nontransgenic	MHC-CYCD1	% Change relative to nontransgenic	<i>P</i> *
Heart weight (mg)	157±7.1	202±17.7	128	< 0.05
Body weight (g)	24.4±0.81	22.6±1.49	93	NS
Heart/Body (mg/g)	6.3±0.19	8.9±0.28	141	< 0.02
<i>n</i>	4 mice	4 mice		
Ventricular cardiomyocyte DNA synthesis (No. tritiated thymidine positive nuclei total nuclei screened):				
3-month-old mice	1/270000	112/270000 <sup>‡</sup>	16768	< 0.001
10-month-old mice	ND	66/120000 <sup>‡</sup>	ND	ND

\*Determined by Student's *t* test. <sup>‡</sup>Not statistically different via Fisher's exact test.

raphy. Comparison of low-power views of the control (MHC-nLAC) and experimental (MHC-nLAC/MHC-CYCD1) mice clearly revealed an increased number of cardiomyocyte nuclei in the ventricles of mice overexpressing cyclin D1 (Fig. 6, *H* and *I*), consistent with the results obtained by the flow cytometry and dispersed cell assays. This increase in nuclear number was also apparent in the atrium of the transgenic mice (see Fig. 6, *insets*).

Ventricular cardiomyocyte DNA synthesis was readily detected in the hearts of adult MHC-CYCD1 transgenic mice, as evidenced by the presence of silver grains over βGAL positive nuclei (Fig. 6 *J*). Ventricular cardiomyocyte labeling indices approaching 0.05% were observed for the transgenic mice, while the labeling index in control animals was < 0.0003% (Table I). It is of interest to note that silver grains were occasionally observed over two closely opposed cardiomyocyte nuclei in the MHC-CYCD1 transgenic hearts (Fig. 6 *K*). Moreover, the relative density of the silver grains over such closely opposed nuclei was diminished as compared with other synthetic nuclei (compare Fig. 6, *J* and *K*). This result is consistent with karyokinesis of a recently synthetic nucleus, however pulse-chase experiments are required to directly prove the point. DNA synthesis was also observed in abnormally long cardiomyocyte nuclei similar to those identified in the dis-

persed cell assay (compare Fig. 6, *E* and *L*). To determine if cardiomyocyte DNA synthesis persisted in older animals, similar analyses were performed on 10-mo-old MHC-nLAC/MHC-CYCD1 transgenic mice. Labeling indices of 0.055% were detected in the old mice (Table I), indicating that the capacity for cardiomyocyte DNA synthesis was retained in aged animals. It is also of interest to note that there was an ~ 40% increase in the heart weight to body weight ratio in the MHC-CYCD1 transgenic mice as compared with age-matched control animals (Table I). In preliminary analyses, hemocytometer cell counts of dispersed cell preparations revealed an approximately twofold increase in the total number of cardiomyocytes in 14-d-old transgenic mice as compared with their nontransgenic littermates ( $3.7 \times 10^6 \pm 1.78 \times 10^5$  vs.  $1.7 \times 10^6 \pm 1.22 \times 10^5$ , *n* = 8). The increase in cardiomyocyte number undoubtedly contributes to the increased heart weight to body weight ratio observed in the adult transgenic mice.

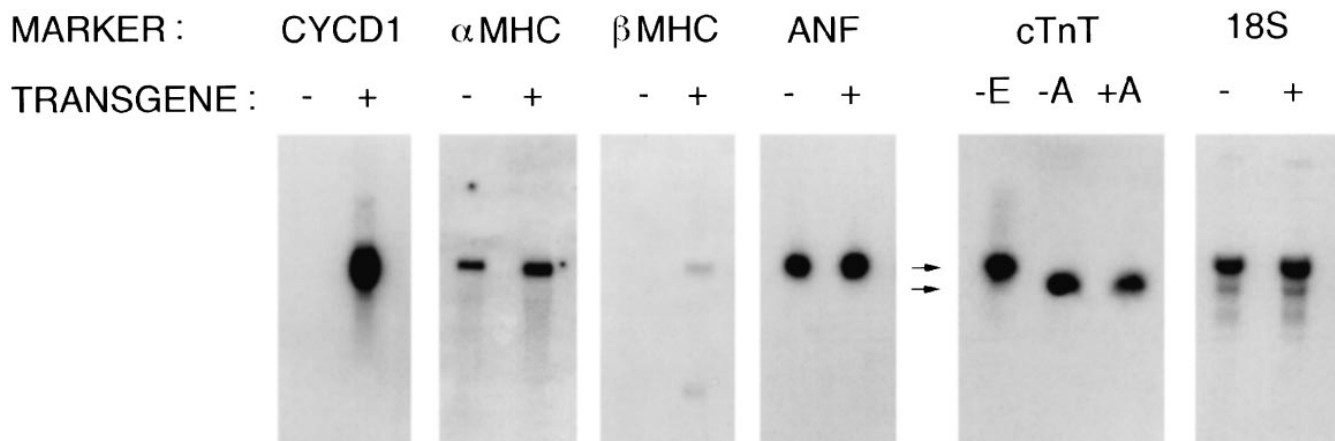
Previous studies have shown that forced expression of cyclin D1 can block skeletal myoblast differentiation in vitro, in part by blocking the ability of myoD to transactivate skeletal muscle promoters (39, 40). Therefore, we examined several myocardial markers in adult MHC-CYCD1 mice to determine if cyclin D1 deregulation had a similar effect on myocardial differentiation. Northern blot analyses of RNA prepared from MHC-CYCD1 transgenic hearts revealed essentially normal levels of α-cardiac MHC and atrial natriuretic factor mRNA, and a slight induction of β-cardiac MHC mRNA (Fig. 7). RT-PCR analyses indicated that the MHC-CYCD1 transgenic animals expressed the adult cardiac troponin T isoform. Thus, constitutive cyclin D1 expression does not negatively impact on the expression of muscle-specific markers in the adult heart.

## Discussion

D-type cyclin/CDK protein kinase activity is required for transit through the restriction point. Once past this G1→S checkpoint, the cell commits to a new round of DNA synthesis (for reviews see references 14 and 15). The direct correlation observed between myocardial cyclin D1 and D3 protein levels and the relative levels of DNA synthesis during cardiac development suggested that these proteins may participate in cardiomyocyte cell cycle regulation. This notion was partially

Figure 6. Assessment of dispersed cell morphology and DNA synthesis in cardiomyocytes from adult MHC-CYCD1 transgenic mice. (A–G) Dispersed cell preparations from adult MHC-CYCD1 transgenic mice stained with DAPI and photographed under fluorescent illumination. Magnification for each panel is the same; the bar in A is 1 μm. (A) Binucleated transgenic cardiomyocyte with normal nuclear morphology. Arrow indicates the presence of a cardiac fibroblast. (B) Transgenic cardiomyocyte with two normal and one abnormal (arrow) nucleus. (C) Tetranucleated transgenic cardiomyocyte. (D) Trinucleated transgenic cardiomyocyte. Note the abnormally large (arrowhead) and apparently partially replicated (arrow) nuclei. (E) Transgenic cardiomyocyte with abnormally elongated nucleus (arrow). (F) Transgenic cardiomyocyte with heterogeneously staining chromatin (arrow). (G) Transgenic cardiomyocyte with five nuclei of variable size. (H–L) Assessment of DNA synthesis in adult MHC-CYCD1 transgenic mice. MHC-CYCD1 mice were crossed with MHC-nLAC mice, and mice carrying the MHC-nLAC transgene alone or both the MHC-nLAC and the MHC-CYCD1 transgenes were identified and sequestered for assessment of DNA synthesis. (H) Survey photomicrograph of the interventricular septum of an adult MHC-nLAC transgenic mouse stained with X-GAL. Note relative density of the cardiomyocyte nuclei (blue staining). Inset shows a section of the atrial appendage from the same heart, stained with X-GAL. (I) Survey micrograph of the interventricular septum of an adult mouse carrying both the MHC-nLAC and the MHC-CYCD1 transgenes, stained with X-GAL. Note the marked increase in nuclear density as compared with the control littermate shown in H. Inset shows a section of the atrial appendage from the same heart; once again the increased cardiomyocyte nuclear density is apparent in the double transgenic animals. (J–L) Autoradiographs of sections prepared from transgenic mice carrying both the MHC-nLAC and the MHC-CYCD1 transgenes. The mice received a single injection of tritiated thymidine and were killed 4 h later. The hearts were sectioned, stained with XGAL, and processed for autoradiography. Bar indicates 1 μm. (J) Cardiomyocyte DNA synthesis is readily observed in the hearts of the adult transgenic mice; note the presence of silver grains over the blue nuclei. (K) Two putative daughter cardiomyocyte nuclei. Note that the silver grain intensity is approximately half of that observed for synthetic nucleus depicted in J. (L) Synthetic transgenic cardiomyocyte with markedly abnormal nuclear morphology.





**Figure 7.** Expression of cardiomyocyte contractile proteins in MHC-CYCD1 transgenic mice. Total RNA prepared from adult nontransgenic (–) and MHC-CYCD1 transgenic (+) hearts was hybridized with probes for cyclin D1,  $\alpha$ -cardiac MHC,  $\beta$ -cardiac MHC, or 18s rRNA. For analysis of cTnT expression, RNA prepared from nontransgenic embryonic (–E) and adult (–A) heart, as well as adult transgenic hearts (+A) was subjected to RT-PCR analysis. The primers were selected so as to permit differentiation between the amplification products generated from the embryonic (198 bp) and adult (168 bp) cTnT isoforms. The amplification products were visualized by Southern blot analyses using an oligonucleotide probe corresponding to a common internal sequence in the embryonic and adult isoforms.

supported by the sustained cardiomyocyte DNA synthesis observed in adult transgenic mice with deregulated cyclin D1 expression. Morphologic and histochemical analyses revealed an increase in cardiomyocyte multinucleation in the transgenic animals. Moreover, tritiated thymidine incorporation analyses demonstrated the capacity for sustained cardiomyocyte DNA synthesis in the hearts of adult transgenic animals. Despite this obvious effect on cardiomyocyte terminal differentiation, the transgenic animals continued to express contractile protein isoforms typical for normal adult mouse hearts.

Numerous cell culture studies have indicated that cyclin D1 overexpression accelerates G1 phase (41–43). In this regard it is of interest to note that cardiomyocyte DNA synthesis does occur in normal adult mouse hearts, albeit at an exceedingly low frequency (labeling indices of < 0.0005%, reference 1 and Table I). However, it is unlikely that the increased cardiomyocyte DNA synthesis observed in the MHC-CYCD1 transgenic mice results only from shortening of the G1 phase of adult cardiomyocytes, as the transgenic labeling indices were > 150-fold greater than those observed in control hearts (Table I). It is also unlikely that the observed DNA synthesis results from titration of cyclin/CDK inhibitors, given that no overt myocardial phenotype was detected in mice lacking p21 (44), nor in mice lacking both p16 and p19 (45). However, we cannot rule out the possibility that a combinatorial effect resulting from cyclin D1 binding to multiple cyclin/CDK inhibitors might contribute to the phenotype observed in the MHC-CYCD1 mice.

The observation that the cyclin D/CDK4 complexes can phosphorylate the retinoblastoma gene product (RB) and thereby disrupt RB-E2F binding (46, 47) led to the suggestion that the D-type cyclins are likely to be key regulatory factors for transit through the restriction point. However, previous studies from this laboratory have shown that RB is present only at exceedingly low levels in the mouse heart (25). Moreover, no correlations between RB phosphorylation status and cardiomyocyte DNA synthesis were detected in Western blots of total heart protein prepared from various stages of myocardial development (35). Preliminary analyses indicated that the

low levels of RB present in hearts of both neonatal and adult MHC-CYCD1 transgenic mice is in the hypophosphorylated form (Soonpaa, M.H., manuscript in preparation). This finding suggests that RB is not present in the cardiomyocytes, or alternatively that it is somehow sequestered in a way which prevents phosphorylation by cyclin D1/CDK4. It has previously been suggested that p107 may play a more relevant role during myocardial development. This suggestion was based largely on the concordance observed between cardiomyocyte DNA synthesis and p107 transcript levels (25, 35), and on the presence of SV40 T antigen/p107 complex (but not T antigen/RB complex) in cultures of transformed cardiomyocytes (25, 48). In this regard it is important to note that cyclin D1/CDK4 complex can phosphorylate p107 in vivo with a concomitant release of active E2F (49). The in vitro specificity of the cyclin D1-associated kinase activity present in the transgenic hearts is currently under investigation.

The cardiomyocyte labeling index for adult MHC-CYCD1 mice was calculated to be  $\sim 0.05\%$  (Table I). Assuming that this DNA synthesis culminated with karyokinesis, and that karyokinesis was completed in 24 h, over the course of 2 yr (the average life span of a mouse) only 36.5% ( $365 \times 2 \times 0.05$ ) of the cardiomyocytes would have undergone karyokinesis. However, the flow cytometry analyses indicated that as many as 60% of the cardiomyocytes in 3-mo-old transgenic mice had a DNA content in excess of 4C (the typical DNA content for a binucleated adult mouse cardiomyocyte). This finding suggests that a large proportion of the cardiomyocyte nuclear anomalies observed in the MHC-CYCD1 mice arises before adult life, rather than as a cumulative effect resulting from the sustained, albeit low, levels of DNA synthesis observed in the adult mice. In support of this, abnormal multinucleation profiles similar to those seen in adult MHC-CYCD1 transgenic mice were also observed in preliminary flow cytometry analyses of 14-d-old transgenic animals (Soonpaa, M.H., and M.T. Franklin, unpublished observation). It should be reiterated that the ultimate fate of the adult cardiomyocytes undergoing DNA synthesis in the adult MHC-CYCD1 transgenic mice is

presently unknown. Comprehensive pulse–chase experiments will be required to address this issue.

The differential response of skeletal and cardiac muscle to cyclin D1 overexpression deserves comment. Cyclin D1 is expressed at high levels in replicating skeletal myoblasts, but is rapidly downregulated upon myogenic differentiation (39). Forced expression of cyclin D1 in skeletal myoblasts blocked myogenic differentiation (50), blocked transactivation of skeletal muscle specific promoters, and resulted in hyperphosphorylation of myoD (40). In contrast, forced expression of cyclin D1 in the heart did not block the expression of muscle-specific genes (Fig. 6). Importantly, the adult transgenic hearts expressed  $\alpha$ -cardiac MHC and adult cTnT. Expression of these genes, along with the absence of markedly abnormal atrial natriuretic factor and  $\beta$ -cardiac MHC expression, indicates that the normal program of contractile protein isoform switching which accompanies cardiomyocyte terminal differentiation is retained in the transgenic mice, despite the sustained DNA synthesis and increased myocardial mass. Although these data suggest a fundamental difference in the response of cardiac and skeletal myocytes to cyclin D1 overexpression, several potential caveats should be considered. For example, in the skeletal model cyclin D1 expression was targeted to myoblasts, whereas expression in the transgenic hearts occurred after cardiogenic induction. As such, the timing of cyclin D1 overexpression relative to myogenic induction differed in the two models. Although cyclin D1 overexpression was targeted uniformly across the myocardium, it is important to reiterate that only a small percentage of the cardiomyocytes was observed actively synthesizing DNA. This suggests that expression of other factors required for the initiation of DNA may be limited to a subset of the cardiomyocytes present in the adult heart. Similarly, if the effect of cyclin D1 overexpression on contractile protein promoter activity was limited to those cells exhibiting DNA synthesis, it is unlikely that shifts in contractile protein expression would have been detected by Northern analyses, given the small number of cells involved. These issues notwithstanding, it is clear that cyclin D1 overexpression per se does not significantly impact on contractile protein expression in adult hearts.

The ability to induce regenerative cardiomyocyte growth could potentially impact on the pathophysiological sequelae of a variety of cardiovascular diseases. It is clear from the results presented here that cyclin D1 deregulation can program cardiomyocyte DNA synthesis in adult hearts. Intriguingly, the expression of other cell cycle regulatory proteins known to act in concert with cyclin D1 (i.e., CDK4 and PCNA) was induced in the transgenic hearts. However, it should be reiterated that the cardiomyocyte labeling indices in the adult transgenic animals were quite low, despite the presence of high levels of cyclin D1/CDK4 complex. Although the bulk (> 60%) of the adult cardiomyocytes exhibited an 8C DNA content and a multinucleated phenotype, it presently is not clear if they retained the ability to undergo karyokinesis. In this regard it will be informative to test additional cell cycle regulatory proteins known to act at other stages of the cell cycle (i.e., G<sub>2</sub>→M and/or cytokinesis) to determine if combinatorial approaches can be used to induce overt cardiomyocyte cell division in the adult heart.

## Acknowledgments

We thank Qian Chen and Dorothy Field for excellent technical assistance.

This work was supported by National Heart, Lung, and Blood Institute grant HL-06308, and by a grant from Bristol-Myers Squibb. This work was done during the tenure of an Established Investigatorship from the American Heart Association (L.J. Field). M.H. Soonpaa and G.Y. Koh were supported by Grants in Aid from the American Heart Association, Indiana Affiliate. L. Pajak was the recipient of a Postdoctoral Fellowship from the American Heart Association, Indiana Affiliate.

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