

Disappearance of Cyclin A Correlates with Permanent Withdrawal of Cardiomyocytes from the Cell Cycle in Human and Rat Hearts

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Abstract

The regulated expression of cyclins controls the cell cycle. Because cardiomyocytes in adult mammals withdraw permanently from the cell cycle and thus cannot regenerate after injury, we examined cyclin expression during development by comparing cyclin A–E mRNA levels in fetal and adult human hearts. Cyclin B mRNA was detectable in adult hearts, although at a level markedly lower than that in fetal hearts. Levels of cyclin C, D1, D2, D3, and E mRNA were essentially identical in the two groups. In contrast, cyclin A mRNA was undetectable in adult hearts whereas cyclin A mRNA and protein were readily detectable in fetal hearts and cardiomyocytes, respectively. We then measured cyclin A mRNA and protein levels in rat hearts at four stages of development (fetal and 2, 14, and 28 d). Cyclin A mRNA and protein levels decreased quickly after birth (to 37% at day 2) and became undetectable within 14 d, an observation consistent with reports that cardiomyocytes stop replicating in rats by the second to third postnatal week. This disappearance of cyclin A gene expression in human and rat hearts at the time cardiomyocytes become terminally differentiated suggests that cyclin A downregulation is important in the permanent withdrawal of cardiomyocytes from the cell cycle. (*J. Clin. Invest.* 1995. 95:2275–2280.) **Key words:** cardiomyocytes • cell cycle • cyclins • gene regulation • heart

Introduction

In mammalian hearts, cardiomyocytes proliferate throughout fetal development and into the early neonatal period (1–8). During the early postnatal period cardiomyocyte division ceases and DNA replication declines quickly (5–8). Because cardiomyocytes have withdrawn permanently from the cell cycle by the time mammals reach adulthood, their cardiac tissue cannot regenerate after injury such as myocardial infarction. A thorough understanding of the mechanisms that regulate this withdrawal could suggest strategies for regenerating damaged cardiac tissue.

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The progress of the cell cycle is regulated by the expression of cyclins and the activation of their associated cyclin-dependent kinases (Cdks)¹ (9–14). Cyclin A is a key molecule in the S and G2/M phases (15–17), cyclin B is important in the G2/M phase (18), and cyclin C, the three D-type cyclins (D1, D2, and D3), and cyclin E have been implicated in the G1 phase (9, 19–22). In both normal and cancer cells (10, 23), the cell cycle is regulated by these cyclins and their attendant Cdks. Recently, several negative regulators of the Cdks have been identified (23–25); these proteins, termed Cdk inhibitory proteins, bind to the Cdk–cyclin complex and inhibit its activity (26–31). The Cdk2–cyclin E complex can be inhibited by p21 and p27, the Cdk4–cyclin D complex by p21 and p16, and the Cdk2–cyclin A complex by p21 only (25).

To determine whether cyclin expression is important in the withdrawal of cardiomyocytes from the cell cycle, we studied cyclin mRNA levels in human fetal and adult hearts. We found no difference between fetal and adult hearts in the mRNA levels of cyclins C–E. In contrast, cyclin A mRNA was undetectable in adult hearts but readily detectable in fetal hearts, as was cyclin A protein in fetal cardiomyocytes. In rats, cardiac cyclin A mRNA and protein levels quickly decreased after birth (to 37% at day 2) and were undetectable after 14 d, in keeping with the observation that cardiomyocytes stop replicating by the second to third postnatal week. Downregulation of cyclin A thus appears to have an important role in the withdrawal of cardiomyocytes from the cell cycle.

Methods

Cloning of human and rat cyclin cDNAs. Human cyclin A, B, C, D1, D2, D3, and E cDNA fragments were amplified from the mRNA of human umbilical vein endothelial cells or HeLa cells by the reverse transcription polymerase chain reaction as described (32, 33). The primers were based on published sequences:

cyclin A, forward 5'-CGTGGACTGGTTAGTTGA-3' and

reverse 5'-ATGGCAAATACTTGAGGT-3' (34);

cyclin B, forward 5'-CAGGCTTCTCTGATGTA-3' and

reverse 5'-TAGCCAGGTGCTGCATAA-3' (18);

cyclin C, forward 5'-TCCATGGCAGGGAACCTT-3' and

reverse 5'-AGATTGGCTGTAGCTAGA-3' (21);

cyclins D1 and D2, forward 5'-GARGTNTGYGARGARCA-3' and

reverse 5'-TCDATYTYTCYTGRCA-3' (20);

cyclin D3, forward 5'-GCTTACTGGATGCTGGAGGTATGTG-3' and

reverse 5'-ACATCTGTAGGAGTGCTGGTCTGG-3' (20);

and cyclin E, forward 5'-TTCTCGGCTCGCTCCAGGAA-3' and

reverse 5'-TGGAGGATAGATTTCTC-3' (22).

We obtained a rat cyclin A cDNA fragment from the mRNA of rat

1. **Abbreviations used in this paper:** Cdk, cyclin-dependent kinase; PCNA, proliferating cell nuclear antigen.

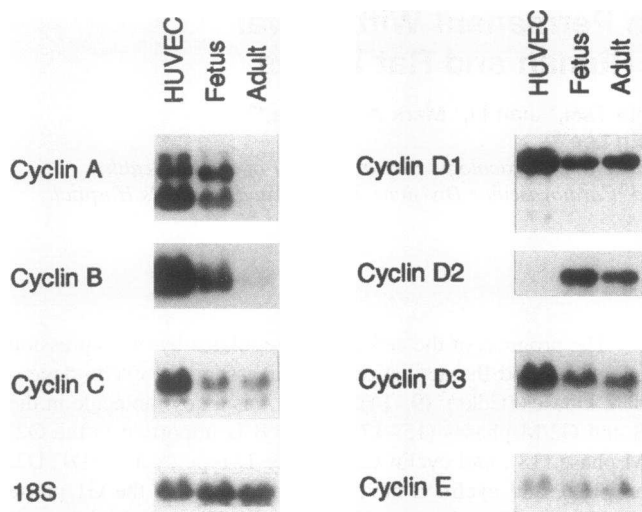


Figure 1. Cyclin mRNA levels in human fetal and adult hearts. Total RNA was extracted from cultured human umbilical vein endothelial cells (HUVEC) or human hearts (Fetus and Adult). Northern blot analyses were performed with 20 μ g of total RNA per lane. After electrophoresis RNA was transferred to nitrocellulose filters, which were hybridized to 32 P-labeled human cyclin probes. Filters were also hybridized to 18S oligonucleotide probes to verify equal loading of RNA in each lane.

aortic smooth muscle cells by the reverse transcription polymerase chain reaction using the same primers for human cyclin A. All PCR fragments were subcloned and their authenticity was confirmed by dideoxy chain termination sequencing (33, 35, 36).

RNA blotting. Total RNA from the ventricles of two 21-wk-old human fetuses was prepared by guanidinium isothiocyanate extraction and centrifuged through cesium chloride (32, 33). (The use of this tissue was approved by the Human Research Committee of the Brigham and Women's Hospital, Boston.) Total RNA from human adult hearts was purchased from Clontech (Palo Alto, CA). Total RNA from rat (Sprague-Dawley) ventricular tissue was obtained at four stages of development: fetal (18–19 d gestation), 2, 14, and 28 d. RNA was fractionated on 1.3% formaldehyde-agarose gels and transferred to nitrocellulose filters, which were hybridized with randomly primed 32 P-labeled cyclin cDNA probes as described (32, 33). The hybridized filters were washed in 30 mM NaCl, 3 mM sodium citrate, and 0.1% sodium dodecyl sulfate at 55°C and autoradiographed on Kodak XAR film at –80°C for 12–48 h or stored on phosphor screens for 6–12 h. To correct for differences in RNA loading, we washed the filters at 80°C in 50% formamide solution to remove the cyclin probes and then rehybridized them with the radiolabeled 18S oligonucleotide (32, 33). Filters were scanned and radioactivity was measured on a PhosphorImager apparatus running the ImageQuant software (Molecular Dynamics, Inc., Sunnyvale, CA).

Western blotting. Cardiac ventricles obtained from Sprague-Dawley rats at various stages of development were homogenized and boiled in

equal volumes of 2 \times sample loading buffer (36, 37). Homogenized samples containing 100 μ g of protein were run on 10% sodium dodecyl sulfate polyacrylamide gels (36, 37). Proteins were electroblotted onto a polyvinylidene difluoride membrane (Immobilon; Millipore Corp., Bedford, MA) in a semi-dry blotting apparatus (Hoefer Scientific Instruments, San Francisco, CA). Filters were incubated with a rabbit polyclonal anti-human cyclin A antibody (38, 39), which was diluted 1:5000 after having been blocked with 3% bovine serum albumin. The filters were then washed and incubated with a 1:5000 dilution of donkey anti-rabbit Ig horseradish peroxidase-conjugated antibody (Amersham Corporation, Arlington Heights, IL). Bound antibody was detected with the Renaissance Chemiluminescence System (DuPont NEN, Boston, MA).

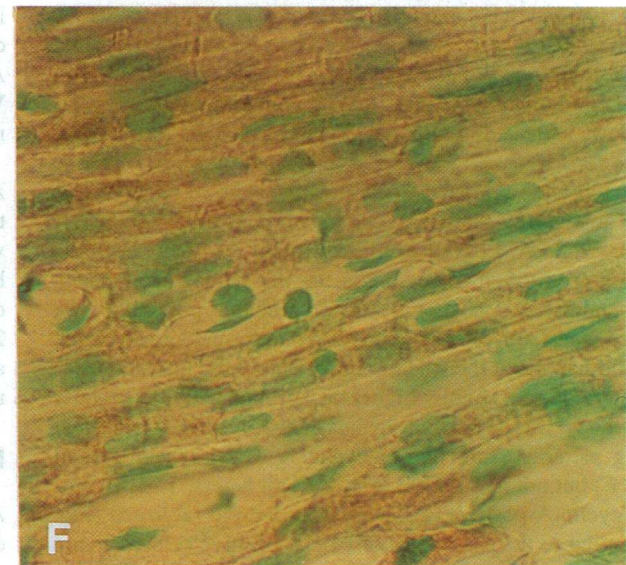
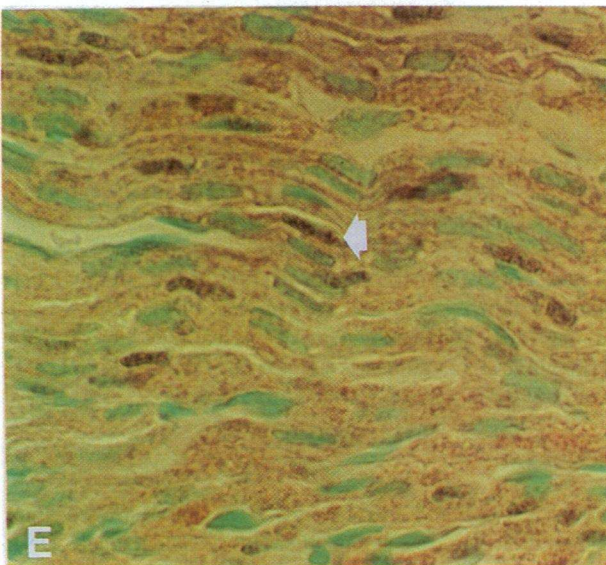
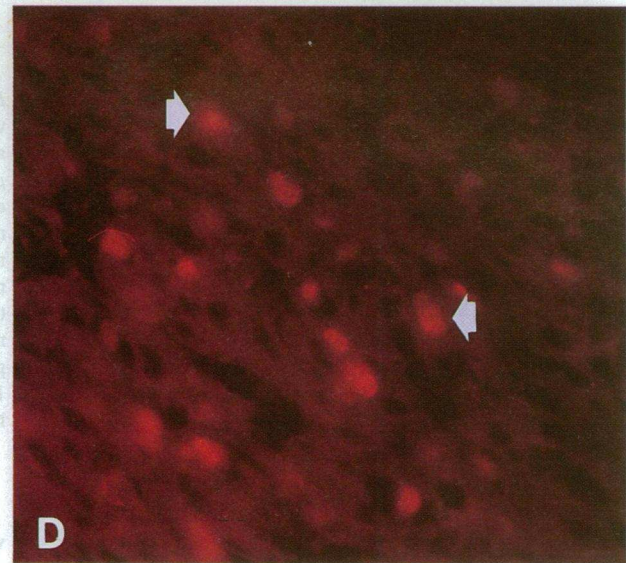
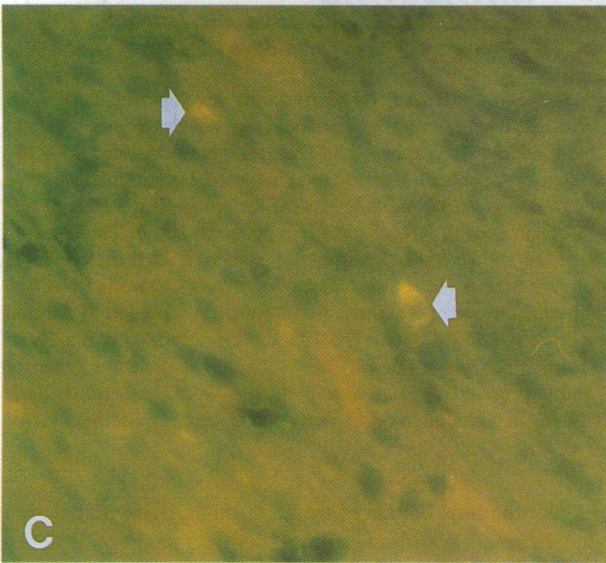
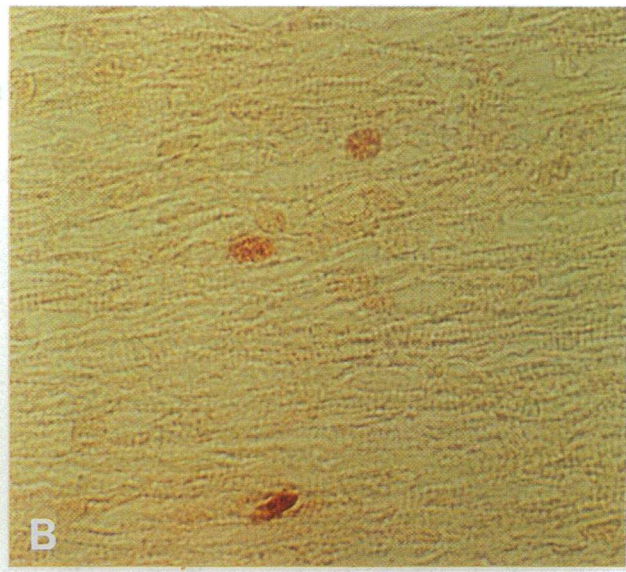
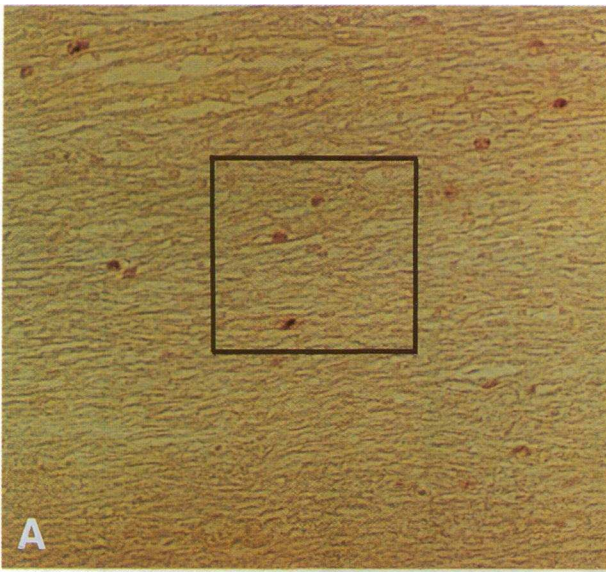
Immunocytochemistry. Human fetal heart (20-wk-old) specimens in methyl Carnoy's fixative were processed for paraffin embedding in an automated system (Hypercenter XP, Shandon Scientific Ltd). Male rats (2 or 14 d old) were perfused with 4% paraformaldehyde, and the hearts were removed and embedded in paraffin or O. C. T. (optimum cutting temperature) compound (Miles, Elkhart, IN). Both human and rat heart specimens were cut at a thickness of 5 μ m. Immunocytochemical analysis of cyclin A and proliferating cell nuclear antigen (PCNA) was performed as described (40, 41). Briefly, the tissue was incubated with a rabbit polyclonal anti-human cyclin A antibody (20 μ g/ml, UBI, Lake Placid, NY) or mouse monoclonal anti-PCNA antibody (3 μ g/ml, Oncogene Science, NY) for 1 h at room temperature and then overnight at 4°C. Staining was visualized by an immunoperoxidase technique, with diaminobenzidine used as chromogen to yield a brown reaction product (40). The tissue was counter-stained with methyl green to visualize the nuclei.

For double-immunofluorescent staining, human fetal heart tissue sections were incubated with a mixture of primary antibodies—anti-human cyclin A (40 μ g/ml) and anti-PCNA (6 μ g/ml) or anti-human cyclin A (40 μ g/ml) and anti-desmin (monoclonal, 1:20 dilution, Amersham, Buckinghamshire, England)—for 1 h at room temperature and overnight at 4°C. After having been washed three times with 1 \times PBS, the tissue sections were incubated with a mixture of two secondary antibodies: fluorescein isothiocyanate-conjugated goat anti-rabbit IgG (1:20; Boehringer Mannheim, Indianapolis, IN) and rhodamine-conjugated goat anti-mouse IgG (1:20, Boehringer Mannheim). Cyclin A and PCNA or cyclin A and desmin were visualized in a camera-equipped, Nikon immunofluorescence microscope. Two photographs of the double-labeled sections were taken sequentially: first at the wavelength specific for fluorescein isothiocyanate fluorescence and then (without changing the slide) at the wavelength specific for rhodamine fluorescence. Cyclin A immunoreactivity stained yellow and PCNA or desmin immunoreactivity stained red.

Results

As a positive control, total RNA prepared from cycling human umbilical vein endothelial cells was hybridized with cyclin probes. We detected transcripts of cyclins A, B (B1), C, D1, D3, and E in total RNA prepared from human umbilical vein endothelial cells (Fig. 1), and the size of each transcript was as

Figure 2. Expression of cyclin A protein in human and rat cardiomyocytes. Cyclin A immunoreactivity was detected in heart tissue by single-immunoperoxidase methods (A and B, human tissue; E and F, rat tissue) and dual-immunofluorescent methods (C and D, human tissue). Arrows denote nuclei stained positively with cyclin A or PCNA antibodies. (A) Low magnification micrograph (\times 230) showing the distribution of cyclin A (brown spots) in human fetal heart tissue. (B) High magnification micrograph (\times 700), corresponding to the area defined by the square in A, showing striated morphology and large nuclei of cardiomyocytes in human fetal heart tissue. Three cardiomyocyte nuclei positive for cyclin A are visible. (C) Two cells from a human fetal heart stained with antibody to cyclin A (yellow, arrows). (D) Same two cyclin A-positive cells in C stained with antibody to PCNA (red, arrows). (E) High magnification micrograph (\times 700) of a 2-d-old rat heart section showing many nuclei stained with antibody to cyclin A. The arrow shows a representative nucleus positive for cyclin A. (F) High magnification micrograph (\times 700) of a 14-d-old rat heart section showing no cyclin A-positive nuclei.



reported (18, 20–22, 34). In contrast with lymphocytes and macrophages (20), cyclin D2 was not expressed in human umbilical vein endothelial cells.

Cyclin A, B (B1), C, D1, D2, D3, and E messages could be detected readily in RNA from human fetal hearts (Fig. 1). Although there were no significant differences between adult and fetal hearts in cyclin C, D1, D2, D3, and E mRNA levels, cyclin B mRNA levels decreased. Most interesting was the difference between fetal and adult hearts in the expression of cyclin A mRNA. Cyclin A mRNA was readily detectable in fetal hearts after the filters had been exposed to XAR film for 12 h (Fig. 1); however, even after 3 d of exposure cyclin A mRNA could not be detected in heart tissue from adults (data not shown). (The two sizes of cyclin A mRNA visible in Fig. 1 are due to the use of alternative polyadenylation signals (42).

Although we detected cyclin A mRNA in the human fetal heart, we were not sure which cell type expressed it. To determine whether cyclin A protein was present in fetal cardiomyocytes we incubated paraformaldehyde-fixed cryostat sections with an anti-cyclin A antibody. At a low magnification cyclin A immunoreactivity was detectable in the fetal heart (Fig. 2 A), although only 4% of the cells expressed the protein. At high magnification cardiomyocytes were easily identified by their striated pattern and large nuclei (Fig. 2 B). Fig. 2 B shows three representative cardiomyocytes immunoreactive for cyclin A. Also, cyclin A immunostaining was localized to the nuclear region of cardiomyocytes, consistent with previous reports that cyclin A and Cdk2 localize to nuclei of other cell types, particularly at sites of DNA replication (43, 44). Because desmin localizes to the Z bands of fetal and adult cardiomyocytes (45) it makes a good marker for them. We also performed dual-immunofluorescent studies with anti-desmin and anti-cyclin A antibodies and confirmed that the cells that stained positive for cyclin A were cardiomyocytes (data not shown). The specificity of the antisera was confirmed by incubation with irrelevant antibodies, such as anti-LHRH antibody, or normal goat serum. No immunostaining could be detected when tissue was incubated with these irrelevant antibodies (data not shown).

To confirm that the cardiomyocytes that stained positive for cyclin A were cycling cells, we performed dual-immunofluorescent staining with anti-human cyclin A and anti-PCNA antibodies. PCNA has been used before as a marker for cycling cells, including cardiomyocytes (46–50). Fig. 2 C shows two cells from a human fetal heart stained with antibodies to cyclin A (yellow, indicated by arrows). These same cells also stained positive for PCNA (Fig. 2 D, red, indicated by arrows). These results suggest that the cyclin A-positive cells were cycling. It is also noteworthy that more cells stained positive for PCNA than for cyclin A. Loss of cyclin A protein may have been the result of postmortem degradation, as the human fetal hearts were obtained ~ 2 h after death. To test this possibility, we perfused anesthetized rats with 4% paraformaldehyde while alive and harvested their hearts immediately for immunohistochemistry. Despite slightly higher background activity (because the antibody was generated against human cyclin A), nuclear staining patterns distinctive of cyclin A were seen in 2-d-old (Fig. 2 E) but not in 14-d-old (Fig. 2 F) rat hearts. The percentage of cyclin A-positive cells was 20 in 2-d-old rat hearts (Fig. 2 E), similar to the percentage of PCNA-positive cells (data not shown). Thus, the difference in the percentage of cyclin A-positive cells in human and rats was more likely due to postmortem degradation of cyclin A protein.

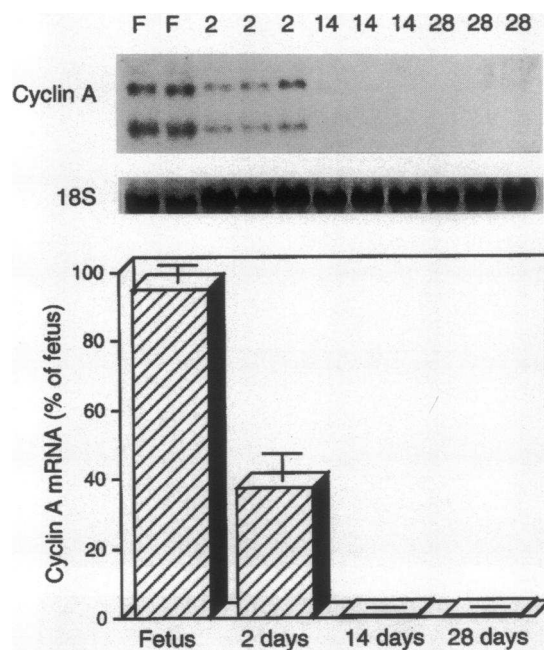


Figure 3. Cyclin A mRNA in rat hearts during development. Total RNA was extracted from rat ventricles at four stages of development (fetal and 2, 14, and 28 d). Northern blot analyses were performed with 15 μ g of total RNA per lane. Because fetal rat ventricles are small we were obliged to pool tissue from three rats in two groups (for a total of 6 rats) to obtain enough RNA for the two lanes marked F. The lanes marked 2, 14, and 28 contained total RNA isolated from three individual rats for each time point, run separately. After electrophoresis RNA was transferred to nitrocellulose filters, which were hybridized to 32 P-labeled rat cyclin A and 18S probes. (The 18S autoradiogram is from a PhosphorImager.) The signal intensity for cyclin A was divided by that for 18S, and the corrected values were plotted as a percentage of the fetal heart value (first lane marked F). Values represent means \pm SE.

To plot the time course of cyclin A mRNA downregulation we studied ventricular tissue from rats at four stages of development (Fig. 3). A high level of cyclin A mRNA could be detected in hearts obtained from rat fetuses (18–19 d gestation or ~ 2 d before birth). However, in hearts from 2-d-old rats the cyclin A mRNA level decreased to 37% that in hearts from rat fetuses. We could not detect any cyclin A mRNA in 14- and 28-d-old rat hearts.

We performed Western blot analysis to confirm that cyclin A protein was downregulated after birth (Fig. 4). The antiserum to cyclin A recognized a 58-kD band in cycling HeLa cells that were used as a positive control (38, 39). Although we detected high levels of cyclin A protein in fetal rat hearts, protein levels declined within 2 d of birth. Protein was undetectable 14 and 28 d after birth. These coincident decreases in cyclin A mRNA and protein levels in rat hearts suggest that cyclin A is downregulated between 2 and 14 d after birth.

Discussion

Although cardiac and skeletal muscle are both striated, their differentiation patterns are distinct. In skeletal muscle DNA synthesis and activation of muscle-specific genes are mutually exclusive (5, 51, 52); in cardiac muscle proliferation and differentiation occur simultaneously (8). Thus, the molecular mecha-

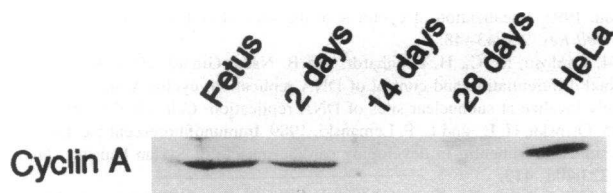


Figure 4. Cyclin A protein in rat hearts during development. Total protein extracts were obtained from fetal and neonatal rat ventricles. Protein from HeLa cells served as controls. Extracts were separated by sodium dodecylsulfate polyacrylamide gel electrophoresis according to standard protocols (36) and transferred to a polyvinylidene difluoride membrane. Cyclin A protein was detected with rabbit anti-human cyclin A antibody (1:5000 dilution).

nisms that control proliferation and terminal differentiation in these two striated cell types may be quite distinct (5–7, 53). Although several molecules have been implicated in the terminal differentiation of skeletal muscle cells (5, 51, 52, 54), very little is known about the molecular mechanisms regulating the terminal differentiation of cardiac muscle cells and their withdrawal from the cell cycle (5, 49, 55).

Despite the importance of cyclins in regulating the progress of the mammalian cell cycle, there has been no report on cyclin gene expression in cardiomyocytes during terminal differentiation. In this report we have shown that the mRNA levels of the G1 cyclins (C, D1, D2, D3, and E) in fetal and adult hearts are essentially identical (Fig. 1). In contrast, cyclin B mRNA expression decreases markedly and cyclin A mRNA expression is suppressed in adult compared with fetal hearts (Figs. 1 and 3). It has been shown that by day 15 of postnatal rodent development essentially all cardiomyocytes have exited the cell cycle (8, 56). Our observations about the time course of cyclin A mRNA downregulation (Fig. 3) correlate well with the withdrawal of cardiomyocytes from the cycle. Moreover, cyclin A is required for the S and G2/M phases of the cell cycle (16, 17, 57). Inhibition of cyclin A synthesis or activity—by microinjection of plasmids encoding antisense cyclin A cDNA or by anti-cyclin A antibodies— inhibits DNA synthesis and mitosis in fibroblasts (16, 17). Therefore the complete disappearance of cyclin A in adult hearts may have an important role in the withdrawal of adult cardiomyocytes from the cell cycle.

It is interesting to note that levels of the G1 cyclins (D and E) in adult hearts remained elevated whereas those for cyclin A declined dramatically. Although we cannot exclude the possibility that noncardiomyocytes contributed to these high G1 cyclin levels, this situation is strikingly similar to that seen in senescent human fibroblasts (58), in which cyclin A was undetectable and cyclins D and E were expressed at high levels. In this regard terminal differentiation and senescence may share regulatory pathways controlling cell cycle progression.

It has been reported that endogenous cAMP concentrations increase progressively in rat hearts during late fetal and early neonatal development (59). Furthermore, exogenous cAMP can cause premature withdrawal of cardiomyocytes from the cell cycle (59). The human cyclin A promoter has recently been cloned and a cAMP-responsive element has been identified 73 bp 5' to the major transcription start site (42, 60). It will be interesting to test whether cAMP downregulates the expression of the cyclin A gene in cardiomyocytes.

To our knowledge this is the first report that cyclin A disap-

pears in adult cardiomyocytes. Elucidation of the molecular mechanisms regulating downregulation of cyclin A gene expression in adult cardiomyocytes will provide further insight into their permanent withdrawal from the cell cycle. Only when the mechanisms regulating this withdrawal are fully understood can we design strategies for regenerating damaged cardiac tissue.

Acknowledgments

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