Abstract—Cardiomyocytes withdraw from the cell cycle in the early neonatal period, rendering the adult heart incapable to regenerate after injury. In the present study, we report the establishment of a cell-free system to investigate the control of cell cycle reentry in mammalian ventricular cardiomyocyte nuclei and to specifically address the question of whether nuclei from terminally differentiated cardiomyocytes can be stimulated to reenter S phase when incubated with extracts from S-phase cells. Immobilized cardiomyocyte nuclei were incubated with nuclei and cytoplasmic extract of synchronized H9c2 muscle cells or cardiac nonmyocytes. Ongoing DNA synthesis was monitored by biotin-16-dUTP incorporation as well as proliferating cell nuclear antigen expression and localization. Nuclei and cytoplasmic extract from S-phase H9c2 cells but not from H9c2 myotubes induced DNA synthesis in 92% of neonatal cardiomyocyte nuclei. Coincubation in the presence of cycloheximide indicated that de novo translation is required for the reinduction of S phase. Similar results were obtained with adult cardiomyocyte nuclei. When coincubated with both cytoplasmic extract and nuclei or nuclear extracts of S-phase cells, >70% of adult cardiomyocyte nuclei underwent DNA synthesis. In conclusion, these results demonstrate that postmitotic ventricular myocyte nuclei are responsive to stimuli derived from S-phase cells and can thus bypass the cell cycle block. This cell-free system now makes it feasible to analyze the molecular requirements for the release of the cell cycle block and will help to engineer strategies for regenerative growth in cardiac muscle. (Circ Res. 1999;85:294-301.)

Key Words: cell cycle • cardiomyocyte • DNA synthesis
terminally differentiated rat cardiomyocytes can be stimulated to reenter S phase when incubated with extracts from S-phase cells, we established a mammalian myocardial cell-free system, in which DNA synthesis in primary cardiomyocyte nuclei is efficiently induced, to complement currently used molecular genetic approaches. The present study demonstrates that initiation of DNA replication in vitro depends on both cytoplasmic and nuclear factors from S-phase cells and requires de novo protein synthesis. This new myocardial cell-free system is applicable to a variety of biochemical analyses aimed at the molecular dissection of cell cycle control in differentiated cardiomyocytes.

Materials and Methods

Rat Primary Cardiomyocyte Cultures

The care and use of the animals in the present study were in accordance with approved guidelines of the Max-Delbrück-Center (Berlin). Ventricular cardiomyocytes from 2-day-old Wistar rats (Möllergard, Schönwalde, FRG) were isolated and cultivated as described with minor modifications. Briefly, hearts were dissected, minced, and trypsinized. For selective enrichment of cardiomyocytes, cells were then preplated for 1 to 2 hours. The resultant cell suspension (6x10^5 cells) was plated in 100-mm culture dishes. Cells were cultured for 24 hours in the presence of 0.1 mmol/L BrdU (Sigma) to prevent proliferation of nonmyocytes. More than 90% of the cells were myocytes, as evaluated by indirect immunofluorescence staining with an antibody to sarcomeric tropomyosin (CH1, Sigma).

Adult ventricular cardiomyocytes were isolated from 12- to 16-week-old male Wistar rats (250 to 350 g) following basically Powell’s procedure.

**Cell Culture and Synchronization**

H9c2 cells (American Type Culture Collection [Manassas, Va], CRL-1466, a muscle cell line derived from embryonic rat heart tissue and nonmyocytes (derived from preplating of neonatal cardiomyocytes) were grown to 70% confluence in DMEM/F12 (Gibco) supplemented with 10% heat-inactivated FCS (PA), 100 U/mL penicillin, 100 μg/mL streptomycin, and 2 mmol/L L-glutamine (all from Gibco). Cells were synchronized in S phase using a single thymidine (2.5 mmol/L, Sigma) block in culture medium for 18 hours, followed by a release into medium lacking thymidine for 4 hours. Differentiated H9c2 myotubes were obtained by incubation for 10 days without changing the medium.

**Cell Cycle Analysis by Flow Cytometry**

For detection of DNA synthesis, cells were pulse-labeled with 50 μmol/L BrdU for 4 hours and stained with FITC-conjugated ant-BrdU antibody (B4; Becton Dickinson) and propidium iodide (Sigma) (2.5 μg/mL) for DNA content according to the manufacturer’s instructions. Samples (10^5 cells) were analyzed with a flow cytometer (Coulter Epics), and the distribution of cells in the different cell cycle stages was determined using Epics software and Multicycle software (Coulter).

For detection of the percentage of nonmyocyte cells, fixed cultures were permeabilized (15 minutes, 0.5% Triton X-100 in PBS), incubated for 30 minutes in 1% BSA/PBS, and subsequently incubated for 45 minutes with an antibody to sarcomeric tropomyosin (diluted 1:1000 in 1% BSA/PBS, CH1, Sigma). After washing with 0.1% Nonidet P40 and 1% BSA/PBS, cells were incubated with an FITC-conjugated goat anti-mouse IgG antibody (diluted 1:500 in 0.5% Tween and 1% BSA/PBS, Diana), washed again, stained with propidium iodide (Sigma) (2.5 μg/mL) for DNA content, and analyzed as described above. All manipulations were carried out at room temperature.

Preparation of Nuclei and Extracts

For preparation of cytoplasmic extracts, 10^5 cells were washed twice in ice-cold hypotonic buffer (0.01 mmol/L HEPES [pH 7.4] and 1.5 mmol/L MgCl_2) and allowed to swell for 10 minutes on ice. After centrifugation (300g, 4°C, 5 minutes), the pellet was disrupted with 20 to 30 strokes in a Dounce homogenizer using a tight-fitting pestle (Wheaton). Nuclei were pelleted at 5000 rpm for 5 minutes and 4°C in an Eppendorf 5415L centrifuge. The cytoplasmic extract was recentrifuged for 30 minutes at 14 000 rpm and snap-frozen in liquid nitrogen after adding protease inhibitors (0.6 μmol/L aprotinin, 4 μmol/L leupeptin, 3 μmol/L pepstatin, and 2 mmol/L Pefabloc; all from Sigma) and phosphatase inhibitors (25 mmol/L NaF and 1 mmol/L NaVO_3; both from Sigma). Cytoplasmic extract contained 5 to 7 μg/μL protein (determined by BCA protein assay; Pierce).

Pelleted nuclei were resuspended in 5 mL hypotonic buffer with 5 strokes in a Dounce homogenizer and washed twice in PBS. Numbers and integrity of nuclei were determined by staining with Trypan Blue using a hemocytometer. For the preparation of nuclear extracts, pelleted nuclei were resuspended in PBS and sonicated (Bachhofer Sonoplus HD70, MS 72/D), centrifuged, and mixed with inhibitors as described above. Nuclear extracts contained 2 to 4 μg/μL protein.

For chemical isolation of nuclei, 250 μL lysis solution (3% glacial acetic acid and 5% ethylhexadecylmethylammonium bromide in H_2O; Sigma) per 5 mL of hypotonic buffer was added to washed cells, and tubes were shaken every minute for 5 to 10 minutes for cardiomyocytes and 1 to 2 minutes for H9c2 cells.19 The release of nuclei was examined by light microscopy.

DNA Synthesis Assay and Immunofluorescence

Unless specified otherwise, all solutions were prepared in PBS with 0.68 mmol/mL CaCl_2 and 0.49 mmol/mL MgCl_2, all antibodies were diluted in blocking buffer as indicated, and all manipulations were carried out at room temperature. Chemically isolated cardiomyocyte nuclei (1 to 1.5x10^5 neonatal, 0.5 to 1x10^5 adult) were immobilized onto poly-L-lysine (Sigma)–coated coverslips by centrifugation for 8 minutes at 600 rpm and 4°C in a Beckman CS-6R centrifuge. Nuclei were then washed with ice-cold PBS and subsequently covered with 30 μL cytoplasmic extract (substituted with PBS for control reactions), 20 μL mechanically isolated H9c2 nuclei (2 to 2.5x10^5), or 30 μL nuclear extract and 20 μL of a buffered nucleotide mix (40 mmol/L K-Hepes [pH 7.8], 7 mmol/L MgCl_2, 3 mmol/L ATP, 0.1 mmol/L each of GTP, CTP, UTP; 0.1 mmol/L each of dATP, dGTP, dCTP; 7.5 μmol/mL biotin-16-dUTP; 0.5 mmol/L DTT; 40 mmol/L creatine phosphate, and 0.5 μg/μL phosphorecatine kinase; all from Boehringer Mannheim) and incubated for 2 hours (neonatal) or 3 to 4 hours (adult) at 37°C in a humidified atmosphere of 95% O_2/5% CO_2.

For detection of DNA replication foci, chemically isolated immobilized nuclei were washed twice with ice-cold PBS, permeabilized twice at 4°C for 30 seconds with 0.5% Triton X-100 in CSK buffer (100 mmol/L NaCl, 300 mmol/L sucrose, 3 mmol/L MgCl_2, and 10 mmol/L PIPES [pH 6.8]), and then fixed for 5 minutes in −20°C methanol. Nuclei were then incubated for 20 minutes in 5% goat serum/0.2% (vol/vol) Tween 20 and 20% mouse monoclonal IgG2a antibody to proliferating cell nuclear antigen (PCNA) for 1 hour (PC10, diluted 1:40; Diana). Nuclei were washed 4 times with 0.1% (vol/vol) Nonidet P-40, incubated with an FITC-conjugated goat anti-mouse IgG antibody (diluted 1:50; Diana) for 1 hour, washed 3 times, and mounted (13.3% [wt/vol] Mowial 4-88, 34% [vol/vol] glycerol, and 2.5% [wt/vol] DABCO in Tris-HCl [pH 8.5]).

To assess DNA synthesis, biotin-16-dUTP incorporation was used. Chemically isolated, immobilized nuclei were fixed with 3.7% (vol/vol) formaldehyde for 10 minutes. DNA was denatured with 2 N HCl/0.5% (vol/vol) Triton X-100 for 20 minutes and neutralized with 0.1 mol/L Na_2B_4O_7 [pH 8.5]. Afterward, nuclei were blocked in 0.5% (vol/vol) Tween 20/1% (vol/vol) BSA for 30 minutes, incubated with Texas Red streptavidin (diluted 1:100 in 0.5% Tween 20; Amersham) for 45 minutes, and washed 3 times with PBS containing 0.1% (vol/vol) Nonidet P-40. For visualization of DNA, Hoechst...
Synthesis in G1 phase but not G2 phase nuclei. To test cell-free system have shown that S-phase nuclei induce DNA synthesis in neonatal cardiomyocyte nuclei and differentiated H9c2 myotubes. Immunofluorescence analysis showed that 92% of isolated cardiomyocyte nuclei were stained with an antibody to sarcomeric tropomyosin to discriminate between myocytes and nonmyocytes. After the extraction of the Triton X-100 soluble fraction of PCNA, <1% of immobilized cardiomyocyte nuclei were stained positively before (data not shown) and after coincubation with their own cytoplasm (Figure 2A).

Both methods demonstrate that <4% of cardiomyocytes synthesize DNA under our conditions. The difference of S-phase cells as detected by propidium iodide and PCNA staining is relatively minor and could be due to the following reasons: (1) cardiomyocyte nuclei are more resistant to the isolation procedure than noncardiomyocytes, resulting in an additional enrichment of cardiomyocyte nuclei; (2) the threshold for detection by immunofluorescence is confounded by partial inactivation of the epitope by the isolation procedure and fixation; and (3) small populations are often overestimated by flow cytometry analysis.

Coincubation with nuclei and cytoplasmic extract from S-phase H9c2 cells but not from differentiated H9c2 myotubes induces DNA synthesis in neonatal cardiomyocyte nuclei. Studies using mammalian cell fusion experiments or a human cell-free system have shown that S-phase nuclei induce DNA synthesis in G1 phase but not G2 phase nuclei. To test whether S phase could also be induced in rat cardiomyocyte nuclei, we chose H9c2 cells, a muscle cell line from the same species, to prepare S-phase cytoplasmic extracts and nuclei. Synchronization of H9c2 cells was confirmed by flow cytometry using sarcomeric tropomyosin antibody and propidium iodide to detect total DNA and analyze by flow cytometry. For distribution of cells, gates were set as indicated and analyzed by Epics software. A, Percentage of cells in the respective cell cycle stage. B, Percentage of tropomyosin-negative nonmyocytes and tropomyosin-positive cardiomyocytes. C, Percentage of neonatal cardiomyocytes in S phase. A representative result of 3 independent experiments is shown.

Results
Characterization of S-Phase Cells in Primary Rat Neonatal Ventricular Cardiomyocyte Cultures
Primary rat neonatal ventricular cardiomyocyte cultures were analyzed by flow cytometry using sarcomeric tropomyosin antibody and propidium iodide to determine the percentage of nonmyocytes and cardiomyocytes in S phase. In the unpurified cell population (without preplating), 13.6% of cells were in S phase, whereas only 6.3% were in S phase in the purified preparations (with preplating, Figure 1A). By preplating, the percentage of nonmyocytes could be reduced from 48.7 to 9.5% (Figure 1B). Around 4% of cardiomyocytes were in S phase (Figure 1C).

As a marker for S phase, we used indirect immunostaining for PCNA and fluorescence microscopy analysis. In proliferating cells, PCNA cycles between a chromatin-bound, detergent-insoluble state in S phase and a diffuse-soluble state when DNA is not being synthesized. After the extraction of the Triton X-100 soluble fraction of PCNA, <1% of immobilized cardiomyocyte nuclei were stained positively before (data not shown) and after coincubation with their own cytoplasm (Figure 2A).

Both methods demonstrate that <4% of cardiomyocytes synthesize DNA under our conditions. The difference of S-phase cells as detected by propidium iodide and PCNA staining is relatively minor and could be due to the following reasons: (1) cardiomyocyte nuclei are more resistant to the isolation procedure than noncardiomyocytes, resulting in an additional enrichment of cardiomyocyte nuclei; (2) the threshold for detection by immunofluorescence is confounded by partial inactivation of the epitope by the isolation procedure and fixation; and (3) small populations are often overestimated by flow cytometry analysis.
In Figure 4B, the subnuclear PCNA pattern of replication foci in cardiomyocyte nuclei highly resembles that found in S-phase H9c2 nuclei (Figure 4C). These results indicate that cardiomyocyte nuclei undergo a normal S phase with PCNA replication patterns indistinguishable from intact cycling cells.

For the Induction of DNA Synthesis in Cardiomyocyte Nuclei, De Novo Protein Synthesis in Conjunction With Both Nuclear and Cytoplasmic Factors Is Necessary

To evaluate whether the translation machinery necessary of S-phase H9c2 cytoplasm is required for DNA replication, we included cycloheximide in the coincubation experiments. Cardiomyocyte nuclei and S-phase H9c2 nuclei were immobilized together and incubated with S-phase H9c2 cytoplasmic extract in the presence or absence of cycloheximide (10 μg/mL). Under these conditions, DNA synthesis proceeded normally in H9c2 nuclei, which had been synchronized in S phase (Figure 5A and 5B, lanes g and h). In contrast, the efficiency of initiation of DNA synthesis was reduced to 20% in cardiomyocyte nuclei (Figure 5A and 5B, lanes e and f).

Factors from S-phase H9c2 cells needed for the induction of DNA synthesis in cardiomyocytes could be located in either the nucleus or the cytoplasm. To address this question, cardiomyocyte nuclei were incubated with either S-phase H9c2 nuclei and PBS or S-phase H9c2 cytoplasmic extract alone. In neither case could initiation of DNA synthesis be observed in cardiomyocyte nuclei (Figure 5A and 5B, lanes e and f). These data indicate that the induction of DNA synthesis in cardiomyocyte nuclei requires components from both nuclei and cytoplasmic extracts. However, the possibility that factors are leached out of the nucleus during the preparation of cytoplasmic extracts cannot be ruled out.
Next, neonatal cardiomyocyte nuclei were coincubated with S-phase H9c2 nuclei and cytoplasmic extracts from different cell cycle stages. Coincubation with S-phase H9c2 cytoplasmic extract resulted in the efficient induction of DNA synthesis in 92% of cardiomyocyte nuclei (Figure 5B, lane c). Coincubation with differentiated H9c2 myotube cytoplasmic extract failed to do so (Figure 5B, lane d). These data indicate that S-phase–specific factors in addition to the translation machinery are required for the reinitiation of DNA synthesis in cardiomyocyte nuclei.

**Induction of DNA Synthesis in Adult Cardiomyocyte Nuclei**

Neonatal cardiomyocytes are not fully differentiated. Furthermore, in vivo, they are still able to undergo DNA synthesis and mitosis, giving rise to binucleated myocytes. Therefore, we wanted to test whether fully differentiated adult cardiomyocytes could be induced to reenter S phase or had irreversibly lost the ability to synthesize DNA. Coincubation of adult rat cardiomyocyte nuclei with their own cytoplasm (Figure 6A and 6D, lane a) or with nuclei and cytoplasmic extract of differentiated H9c2 myotubes (Figure 6B and 6D, lane b) did not induce DNA synthesis. However, in 72% of adult cardiomyocyte nuclei, DNA synthesis was induced upon coincubation with nuclei and cytoplasmic extract from S-phase H9c2 cells (Figure 6C and 6D, lane c).

**Coincubation With Nuclei and Cytoplasmic Extract From S-Phase Primary Rat Cardiac Nonmyocytes Induces DNA Synthesis in Adult Cardiomyocyte Nuclei**

H9c2 myocytes are immortalized cells that may express factors not present in normal primary cells. To test whether factors present in primary cells suffice to induce S phase, we analyzed the effect of extracts from primary proliferating cells on isolated cardiomyocyte nuclei in our coincubation system. Cardiac fibroblasts that represented the vast majority of nonmyocytes attaching to the bottom of culture dishes during the preplating procedure were isolated and synchronized in S phase (Figure 7). Seventy-six percent of adult cardiomyocyte nuclei underwent DNA synthesis when coincubated with nuclear and cytoplasmic extract from S-phase nonmyocytes, but neither the nuclear nor the cytoplasmic...
extract alone exhibited a significant induction of DNA synthesis (Figure 8A and 8B). The induction of biotin-16-dUTP incorporation correlated well with the appearance of the characteristic PCNA replication–associated pattern (Figure 8C), as it was observed before in neonatal rat cardiomyocytes using H9c2 S-phase nuclei and cytoplasmic extracts (Figure 4A and 4B).

**Discussion**

In the present study, we describe the establishment and characterization of a mammalian myocardial cell-free system to investigate the cell cycle control of DNA replication in nuclei from differentiated cells. We show that nuclei and cytoplasmic extract from S-phase H9c2 cells but not from differentiated H9c2 myotubes efficiently initiate DNA synthesis in terminally differentiated cardiomyocyte nuclei. Furthermore, we demonstrate that the induction of DNA synthesis in cardiomyocyte nuclei depends on both nuclear and cytoplasmic factors in conjunction with de novo protein synthesis.

The molecular mechanisms responsible for the arrest of cardiomyocyte proliferation during the postnatal period are largely unknown. Earlier studies have shown that the activity of cardiomyocyte DNA polymerase α decreases during the first postnatal weeks.23–25 Recently, cyclin-dependent kinase (cdk) activities in cardiomyocytes were also reported to decline during the early neonatal period.26–28 In particular, the phosphotransferase activity of cdk4 governing the G1 phase of cell cycle is detectable until only day 1, whereas the cdk2 activity, regulating the G1–S-phase transition, decreases markedly after day 2. Although FCS, for example, upregulates G1 and G1–S-phase cyclins, activates cdk4, cdk2, and cdc2, and induces phosphorylation of the retinoblastoma
Increased DNA synthesis in adult rat ventricular cardiomyocytes by E1A oncoprotein.

Figure 8. Induction of DNA synthesis in adult rat ventricular cardiomyocyte nuclei immobilized on coverslips. DNA replication was evaluated as described in Figure 2A. Immunofluorescence microscopy analysis of cardiomyocyte nuclei after coincubation with nuclear and cytoplasmic extract of S-phase nonmyocytes. B, Quantitative analysis of the biotin-16-dUTP incorporation after coincubation: with both nuclear and cytoplasmic extract of nonmyocytes synchronized in S phase (a), with only nuclear extract (b), and with only cytoplasmic extract (c). Analysis was performed as in Figure 2D. Results from 3 independent experiments are shown. Data are mean ± SEM. C, Characteristic PCNA replication-associated patterns in adult cardiomyocyte nuclei analyzed by immunofluorescence microscopy after in situ extraction. Nuclei were coincubated for 3 hours with nuclear and cytoplasmic extract from nonmyocytes synchronized in S phase. Scale bar = 5 μm.

Our results indicate that, in principle, the cell cycle block in cardiomyocytes is reversible. They also suggest that no dominant inhibitory factors are present, although we cannot exclude the possibility that they are titrated out. The isolation procedure could also affect the level and/or functional status of inhibitors in the nuclei, which, by itself, however, is not sufficient to enter S phase. The induction of DNA synthesis in this system could also be due to the factors that are missing for the execution of DNA replication are directly supplied by the addition of S-phase nuclei and cytoplasmic extract.

The basic components of the DNA replication machinery have been mostly identified by use of an in vitro viral DNA replication system. One of the central proteins of the DNA replication machinery is PCNA. Our results show that PCNA is present at very low to undetectable levels in cardiomyocyte nuclei, which is in agreement with previous studies in which PCNA protein was not found in adult cardiomyocytes. Most replisome components are organized into megadalton complexes and tightly tethered to the nuclear matrix during S phase. Therefore, it is unlikely that replication factors such as PCNA, which are localized exclusively in the nucleus, would translocate from the S-phase H9c2 nuclei to cardiomyocyte nuclei. The importance of de novo translation for the induction of DNA synthesis is supported by our experiments using cycloheximide. PCNA accumulation was blocked and initiation of DNA synthesis was inhibited in cardiomyocyte nuclei, but elongation phase of DNA replication was not influenced in nuclei from H9c2 cells, which were already in S phase and had already accumulated the necessary proteins. However, we cannot exclude the possibility that PCNA or other cell cycle regulators were transcribed in the S-phase nuclei, translated by the cytoplasmic extract, and then translocated to the cardiomyocyte nuclei.

The mammalian myocardial cell-free system described in the present study now makes it feasible to study the transition of nuclei from the differentiated state into S phase and to analyze the requirements with a variety of biochemical techniques, such as fractionation of cellular extracts, addition of specific inhibitors, immunodepletion of candidate proteins, and extract mixing experiments.

Acknowledgments
This work was supported in part by the Deutsche Forschungsgemeinschaft Grant Le721/2-1 (M.C.C., H.L.) and by the Max Delbrück Center for Molecular Medicine (R.v.H., M.C.C.). The authors wish to thank Marlies Grieben for her excellent technical assistance.

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A Mammalian Myocardial Cell-Free System to Study Cell Cycle Reentry in Terminally Differentiated Cardiomyocytes
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Circ Res. 1999;85:294-301
doi: 10.1161/01.RES.85.3.294

Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7330. Online ISSN: 1524-4571

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