

# Assessment of cardiomyocyte DNA synthesis in normal and injured adult mouse hearts

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**Soonpaa, Mark H., and Loren J. Field.** Assessment of cardiomyocyte DNA synthesis in normal and injured adult mouse hearts. *Am. J. Physiol. 272 (Heart Circ. Physiol. 41): H220–H226, 1997.*—Cardiomyocyte DNA synthesis was examined in normal and injured adult mouse hearts. In preliminary studies DNA synthesis was monitored by [<sup>3</sup>H]thymidine incorporation, followed by autoradiographic analysis of dispersed cell preparations. No synthetic cells were identified when 20,000 ventricular cardiomyocytes from normal adult hearts were examined. A high throughput assay was developed to establish the actual labeling index for the adult mouse heart. The assay utilized [<sup>3</sup>H]thymidine incorporation in transgenic mice which expressed a nuclear-localized β-galactosidase (β-Gal) reporter gene exclusively in cardiac myocytes. Cardiomyocyte DNA synthesis was evidenced by colocalization of β-Gal activity and silver grains in autoradiograms of histological sections. Examination of 180,000 ventricular cardiomyocyte nuclei from normal adult transgenic mice identified a single synthetic nucleus, suggesting a maximum labeling index of 0.0005%. Cardiomyocyte DNA synthesis was next examined in hearts injured by focal cauterization of the left ventricular free wall. Only three synthetic nuclei were identified when 36,000 cardiomyocyte nuclei in the perinecrotic zone of the injured heart were examined. No additional synthetic nuclei were identified when 180,000 nuclei in regions distal to the necrotic zone were examined. These data confirm that cardiomyocyte DNA synthesis in the adult mouse heart is extremely rare and provide baseline data for analyses in genetically modified animals.

terminal differentiation; myocardial regeneration; binucleation; nuclear ploidy

GROWTH OF THE EMBRYONIC HEART is characterized by the proliferation of differentiated, beating cardiomyocytes. After birth, cardiomyocytes withdraw from the cell cycle, and further increases in myocardial mass occur primarily via hypertrophy. It is generally accepted that the ability of adult mammalian cardiomyocytes to proliferate *in vivo* is quite limited (9). This view is supported by the clinical observation that many forms of cardiovascular disease are manifest by irreversible cardiomyocyte loss.

Despite this prevailing view, DNA synthesis and karyokinesis in adult mammalian cardiomyocytes have been observed under certain circumstances. For example, Claycomb and Bradshaw (2) unequivocally demonstrated that a large percentage of cultured adult rat cardiomyocytes synthesize DNA and undergo karyokinesis if maintained *in vitro* for extended periods of time. This observation is consistent with recent reports describing long-term culturing and passaging of human cardiomyocytes (6). It should, however, be noted that in these latter studies, the cultured cells lack the

differentiated phenotype typical of cardiomyocytes *in vivo*.

Although adult cardiomyocyte DNA synthesis has also been observed *in vivo*, the absolute values reported vary markedly. For example, cardiomyocyte labeling indexes ranging from 0 (i.e., 0 labeled cells of 10,000 examined) to 0.45% have been reported in adult rodent hearts (3–5, 10–12). A number of reports have indicated that myocardial injury can induce cardiomyocyte DNA synthesis. Early studies suggested that as many as 6% of the peri-infarct ventricular cardiomyocytes in rats synthesize DNA (8, 17). Cardiomyocyte labeling indexes of 1–2% have recently been reported in rats with either myocardial infarcts or with coronary artery narrowing (3, 10, 11). Cardiomyocyte DNA synthesis was also detected after isoproterenol or work-induced hypertrophy in both mice and rats (14–16). In most of the studies described above, assessment of DNA synthesis relied on thymidine incorporation followed by autoradiographic analysis of tissue sections or alternatively on 5-bromo-2-deoxyuridine incorporation followed by immunofluorescent analyses of tissue sections.

Because values reported for adult cardiomyocyte labeling indexes range across several orders of magnitude in both normal and injured rodent hearts (see above and DISCUSSION), the present study sought to accurately establish the cardiomyocyte labeling index in adult inbred mice. Analyses of dispersed cell preparations failed to detect any synthetic cells when more than 20,000 cardiomyocytes from normal adult hearts were examined. Because of the difficulty in screening larger numbers of cardiomyocytes in dispersed cell preparations, a high throughput assay was developed to establish the actual labeling index. This assay employed a transgenic mouse model that expressed a nuclear-localized β-galactosidase (β-Gal) reporter gene exclusively in the myocardium (19). In this system cardiomyocyte labeling indexes were unambiguously ascertained simply by screening for colocalization of β-Gal activity and silver grains in autoradiographs of 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal)-stained heart sections. With the use of this approach, a cardiomyocyte nuclear labeling index was established for normal and injured adult mouse myocardium. These results are discussed within the context of cardiac regeneration after myocardial injury. Moreover, these studies provide useful baseline data for the analysis of genes that impact on cardiomyocyte proliferation and/or terminal differentiation in genetically modified backgrounds (i.e., gain or loss of function transgenic mouse models).

## MATERIALS AND METHODS

**Myocardial damage model.** These studies used 12-week-old C3HeB/FeJ male mice (Jackson Laboratory, Bar Harbor, MA) or, alternatively, 12-week-old MHC-nLAC transgenic male mice (19) maintained in a DBA/2J (Jackson Laboratory) inbred background. Mice were anesthetized (2.5% Avertin, 0.015 ml/g body wt, ip, Fluka Chemicals, Ronkonkoma, NY) and intubated (small animal respirator, 70 cycles/s, tidal pressure 1.2 kPa, Narco Biosystems, Houston, TX). The heart was exposed via an incision at the third intercostal space, and the myocardium was cauterized midway between the apex and base of the heart with a Medi-Pak surgical cautery (General Medical, Richmond, VA). After cauterization, the incision was closed, the pneumothorax was evacuated, and the mice were allowed to recover from anesthesia on a heating pad maintained at 37°C. The mortality rate for the procedure was <5%. All animal manipulations were performed in accordance with institutional guidelines.

**Cardiomyocyte labeling indexes with dispersed cell assay.** Experimental and control mice received three injections of [<sup>3</sup>H]thymidine (200 µCi ip at 28 Ci/mM, Amersham, Arlington Heights, IL) at 6, 6.5, and 7 days postinjury. For analysis of neonatal MHC-nLAC mice, a single injection of [<sup>3</sup>H]thymidine was administered. Mice were killed 4 h after the last isotope injection, and dispersed cell preparations were generated by retrograde perfusion with collagenase, as previously described (18). Animals were heparinized (10 ml/kg ip, Sigma, St. Louis, MO) ~30 min before they were killed by cervical dislocation. Hearts were removed and immediately placed in a beaker of phosphate-buffered saline (PBS, Sigma), trimmed under a dissecting microscope, and then hung by the aorta on 26-gauge cannulas. Hearts were then perfused with PBS (0.5 ml/min at 37°C) containing 0.17% collagenase (type I, Worthington Biochemical, Freehold, NJ). Collagenase was preselected with viability of cardiomyocyte preparations as a criterion. 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. Because the bulk of the ventricle was processed, regional wall analyses were not possible with the dissociated cell preparations. Cell suspensions were immediately placed in several volumes of 10% neutral buffered Formalin. After at least 1 h of fixation, suspensions were filtered through a fine mesh to remove tissue chunks, smeared onto positively charged slides (Superfrost Plus, Fisher, Pittsburgh, PA), and allowed to dry.

For autoradiography, slides of dispersed cardiomyocytes were washed with PBS, stained with 4',6-diamidino-2-phenylindole dihydrochloride (DAPI; 0.28 µM in PBS, 3 min at room temperature, Boehringer Mannheim, Indianapolis, IN), and washed three times in PBS. After the stained slides were dried, they were coated with photographic emulsion (Ilford L.4, 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 (Rochester, NY) for 4 min, washed in water, and fixed in 30% sodium thiosulfate for at least 4 min. Slides were further processed by being washed in water and dehydrated through graded ethanol and xylene, followed by application of a coverslip.

**Cardiomyocyte labeling indexes with X-Gal histochemical assay.** Cardiomyocyte labeling indexes were also determined with histological sections from MHC-nLAC transgenic mice. These animals carry a fusion gene composed of the α-cardiac myosin heavy chain promoter and a nuclear-localized β-Gal reporter and exhibit nuclear β-Gal activity exclusively in cardiomyocytes. Generation and preliminary characteriza-

tion of the MHC-nLAC mice have been described (19). Injured and control MHC-nLAC mice received five injections of [<sup>3</sup>H]thymidine (200 µCi ip at 28 Ci/mM, Amersham) at 5, 5.5, 6, 6.5, and 7 days postinjury. Mice were killed 4 h after the last isotope injection, and hearts were removed, cryoprotected in 30% sucrose, embedded, and sectioned at 10 µm with standard histological techniques (1). To localize regions of myocardial damage, sections were stained with hematoxylin and eosin (H&E) according to the manufacturer's specifications (Sigma). To localize cardiomyocyte nuclei, sections were postfixed in acetone/methanol (1:1) and overlaid with 1 mg/ml X-Gal, 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, and 2 mM magnesium chloride in PBS. The sections were counterstained with DAPI, and after the sections were dried, autoradiographic emulsion was applied and processed as described above.

**Cell counting.** Labeling indexes were determined with a Leitz microscope with a ×40 objective. For dispersed cell preparations, counting of cardiomyocytes that appeared to be intact and separate was initiated in one corner of the slide and continued in lengthwise rows. For analyses with MHC-nLAC transgenic mice, sections were examined for colocalization of silver grains and β-Gal activity. The perinecrotic zone was defined as a 500-µm-wide region of the surviving myocardium immediately bordering the myocardial scar. To determine whether myocardial injury induced reactive cardiomyocyte growth, dispersed cell preparations from control and injured animals were stained with Masson's trichrome, and images of the cardiomyocytes were captured on a Leitz Labrolux microscope outfitted with a Sony CCD/RGB camera. Video output was fed to a Macintosh Quadra 900 computer and analyzed with National Institutes of Health Image 1.47 software.

## RESULTS

The accurate assessment of cardiomyocyte DNA synthesis is dependent on the unambiguous identification of myocyte nuclei. Our initial efforts to establish the labeling index in normal adult mouse hearts, therefore, relied on autoradiographic analysis of dispersed cell preparations, because cardiomyocyte nuclei can readily be identified on the basis of cellular morphology (Fig. 1A). C3HeB/FeJ adult male mice received three injections of [<sup>3</sup>H]thymidine at 12-h intervals. The mice were killed 2 h after the final thymidine injection, and the hearts were harvested. Dispersed cell preparations generated by retrograde collagenase perfusion were affixed to a microscope slide, stained with DAPI to facilitate nuclear visualization, and subjected to autoradiography. Examination of 20,000 cardiomyocytes failed to identify any synthetic cells, in agreement with our previous results (Table 1A, see also Ref. 18). In control experiments, cardiomyocyte DNA synthesis was readily detected in dispersed cell preparations from neonatal mice, thus validating the assay (see below).

Although these studies suggested an upper limit of 0.005% for the cardiomyocyte labeling index in adult mice, this value was somewhat provisional because no labeled cells were identified. Because of the difficulties encountered when larger numbers of cardiomyocytes with the dispersed cell assay were examined, we sought to develop a high throughput assay that would yield a more accurate determination of the labeling index. This assay relied on a transgenic mouse model (designated

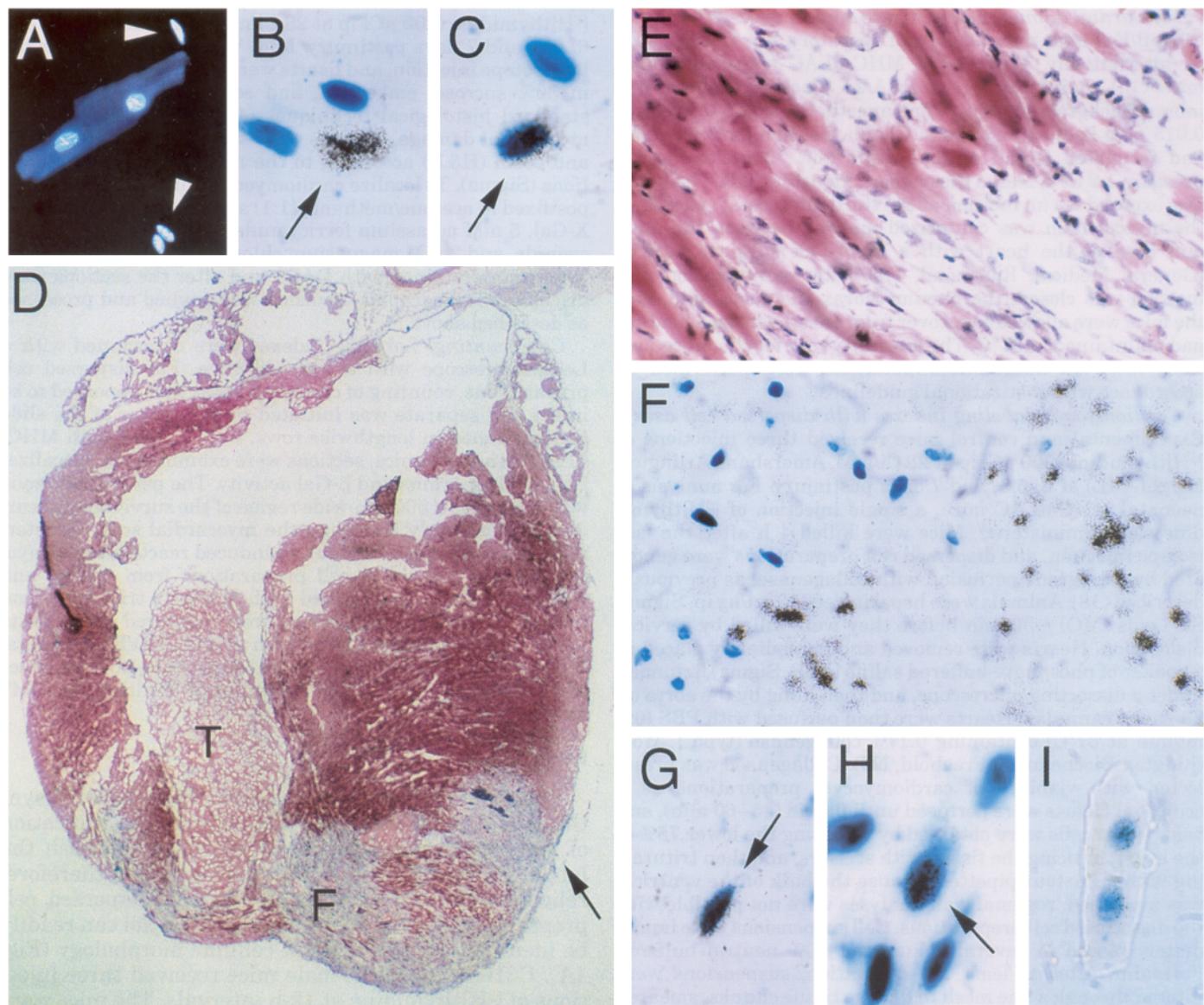


Fig. 1. Assessment of cardiomyocyte DNA synthesis in normal and injured adult mouse hearts. *A*: typical nonsynthetic adult cardiomyocyte prepared by retrograde collagenase perfusion and visualized via 4',6-diamidino-2-phenylindole dihydrochloride (DAPI) epifluorescence. A binucleated cardiomyocyte is shown, and several fibroblast nuclei are evident (arrowheads). *B*: autoradiograph of a synthetic fibroblast nucleus in an adult MHC-nLAC transgenic heart. Section was stained with 5-bromo-4-chloro-3-indolyl- $\beta$ -D-galactoside (X-Gal) before autoradiography, and blue signal demarcates cardiomyocyte nuclei. Note presence of silver grains over a fibroblast nucleus (arrow) but not cardiomyocyte nuclei (presence of fibroblast nucleus was confirmed by DAPI epifluorescence). *C*: synthetic adult ventricular cardiomyocyte from a normal adult heart. Note colocalization of silver grains and  $\beta$ -galactosidase ( $\beta$ -Gal) activity (arrow). *D*: survey micrograph of an injured MHC-nLAC transgenic mouse heart stained with hematoxylin and eosin. Note localized necrotic damage at site of cauterization (arrow). A large thrombus (T) is seen in right ventricular chamber, and areas with extensive fibrosis (F) are seen distal to site of cauterization. *E*: high-power micrograph of perinecrotic zone of same heart depicted in *D*. Note difficulty in unambiguously identifying cardiomyocyte vs. fibroblast nuclei. *F*: high-power view of a similar field on an adjacent section to that depicted in *E*, stained with X-GAL. Note absence of  $\beta$ -Gal activity (and consequently cardiomyocyte nuclei) in injured myocardium. This section was also processed for autoradiography; extensive fibroblast DNA synthesis is apparent in injured myocardium. *G*: high-power view of a synthetic ventricular cardiomyocyte nucleus (arrow) in perinecrotic zone of an MCH-nLAC mouse. Note superimposition of  $\beta$ -Gal activity and silver grains. *H* and *I*: comparison of cardiomyocyte labeling index in MHC-nLAC neonatal mice, as determined by X-Gal histochemistry assay (*H*) vs. dispersed cell preparations (*I*). Arrow in *H* indicates a synthetic neonatal cardiomyocyte nucleus in an X-Gal-stained section. *I*: synthetic neonatal cardiomyocyte using dispersed cell assay. Lower density of silver grains in dispersed cells vs. histological sections was reproducible and presumably reflects partial quenching of thymidine signal due to presence of an intact cell.

Table 1. Cardiomyocyte labeling indexes in control and injured adult murine myocardium

n	Chamber	[ <sup>3</sup> H]Thymidine Positive Nuclei	Total Nuclei Screened	Labeling Index, %	
<i>A. Assessment of DNA synthesis in control and injured adult murine myocardium via dispersed cell counts</i>					
Control heart	2	Ventricle	0	20,000	≤0.005
Injured hearts	3	Ventricle	0	20,000	≤0.005
<i>B. Assessment of DNA synthesis in control and injured adult murine myocardium via X-Gal histochemistry at 7 days postinjury</i>					
Control hearts	4	Ventricle	1	180,000	0.0006
		Atrium	0	18,000	≤0.0055
Injured hearts	4	Ventricle (bordering damaged region)	3	36,000	0.0083
		Ventricle (distal to damaged region)	0	180,000	≤0.0006
		Atrium	1	18,000	0.0055
<i>C. Assessment of DNA synthesis in injured adult ventricular myocardium via X-Gal histochemistry at 1 and 3 days postinjury</i>					
1 Day postinjury	4	Ventricle (bordering damaged region)	0	36,000	≤0.0028
		Ventricle (distal to damaged region)	0	180,000	≤0.0006
3 Days postinjury	4	Ventricle (bordering damaged region)	0	36,000	≤0.0028
		Ventricle (distal to damaged region)	0	180,000	≤0.0006
Preparation		n		Labeling Index	
<i>D. Comparison of dispersed cell assay vs. X-Gal histochemistry assay for assessment of cardiomyocyte labeling indexes in neonatal day 7 MHC-nLAC transgenic mice</i>					
Dispersed cells (n = 1,000 nuclei)		2		1.8 ± 0.285*	
X-Gal histochemistry (n = 1,000 nuclei)		2		1.6 ± 0.506*	

\*Labeling index values under D are means ± SE in percentages; n, no. of mice. X-Gal, 5-bromo-4-chloro-3-indolyl-β-D-galactoside.

MHC-nLAC) in which the α-cardiac myosin heavy chain promoter drives the expression of a nuclear-localized β-Gal reporter gene. Cardiomyocyte-specific nuclear β-Gal activity was readily detected in these animals by histochemical staining with X-Gal. Previous studies using dispersed cell preparations indicated that virtually 100% of the cardiomyocytes exhibited β-Gal activity (19). Cardiomyocyte DNA synthesis in the transgenic animals was unambiguously assessed simply by scoring for colocalization of β-Gal activity and silver grains in autoradiograms of X-Gal-stained histological sections.

Normal adult MHC-nLAC transgenic mice were given five injections of [<sup>3</sup>H]thymidine at 12-h intervals. Examination of autoradiograms of X-Gal-stained sections revealed that the vast majority of thymidine incorporation was localized to fibroblast nuclei (Fig. 1B, arrow). The fibroblast nuclei were independently visualized by DAPI epifluorescence (not shown). Fibroblast labeling indexes of ~2% were observed, in good agreement with our previous results obtained with dispersed cell preparations. Examination of 18,000 atrial cardiomyocyte nuclei in sections prepared from normal transgenic adult hearts failed to detect any DNA synthesis. However, examination of 180,000 ventricular cardiomyocyte nuclei identified a single synthetic nucleus (Fig. 1C, arrow). Silver grains in the autoradiograph clearly colocalized with nuclear β-Gal activity. To rule out the possibility that the observed autoradiographic signal was due to the superimposition of a synthetic fibroblast nucleus on a nonsynthetic cardiomyocyte nucleus, the section was examined via confocal microscopy; only the cardiomyocyte nucleus was observed. Thus cardiomyocyte DNA synthesis could be detected in the normal adult heart, albeit at an exceedingly low frequency.

A second series of experiments was initiated to determine whether myocardial injury induces cardiomyocyte DNA synthesis. The left ventricular free wall was injured by cauterization. Gross examination of the hearts 7 days postinjury revealed the presence of a necrotic zone at the site of cauterization (Fig. 1D, arrow). In addition, pronounced blanching of the myocardium was evident in the region distal to and apically located from the cauterization site. The appearance and location of the blanching were consistent with ischemic myocardial damage resulting from disruption of the underlying vasculature at the site of cauterization. The extent of myocardial damage was readily detected in histological sections; as much as 50% of the left ventricular free wall was affected. Visualization of regions with ischemic damage under high magnification revealed the presence of extensive fibrosis with limited monocyte infiltration. A transitional area between injured and apparently healthy myocardium (designated the perinecrotic zone) was also apparent (Fig. 1E). The absence of X-Gal staining in the regions with ischemic damage confirmed the presence of fibrosis (Fig. 1F). To determine whether the cauterization injury induced hypertrophic growth of the surviving myocardium, dispersed cell preparations were generated from four control and four injured hearts. The cells were then stained with Masson's trichrome, and the cardiomyocyte cross-sectional area was measured by image analysis. Because the lower 75% of the entire ventricle was processed, cells deriving from the intra-ventricular septum and the left and right free walls were represented in the analysis. A 20% increase in mean ventricular cardiomyocyte surface area was observed in cells prepared from cauterized animals, con-

firmed that this injury was able to induce reactive cardiomyocyte growth (Table 2).

To monitor DNA synthesis, MHC-nLAC transgenic animals received five injections of [<sup>3</sup>H]thymidine at 5, 5.5, 6, 6.5, and 7 days postinjury. The hearts were then harvested, sectioned, stained with X-Gal, and processed for autoradiography. Figure 1G shows a synthetic ventricular cardiomyocyte nucleus (*arrow*) in the perinecrotic zone located apically from the cauterization site. A total of three synthetic nuclei were identified when 36,000 X-Gal-positive perinecrotic cardiomyocyte nuclei were examined (Table 1B). In each instance, the silver grains were observed to be colocalized with β-Gal activity, and only the cardiomyocyte nucleus was detected on visualization by confocal microscopy. Ventricular cardiomyocyte DNA synthesis was also evaluated in regions distal to the injured myocardium; examination of 180,000 X-Gal-positive nuclei failed to identify any additional synthetic cells. DNA synthesis was detected in a single atrial cardiomyocyte when 18,000 nuclei from an injured MHC-nLAC mouse were examined (Table 1B). To rule out the possibility that cardiomyocyte DNA synthesis may have occurred at earlier time points, a second series of MHC-nLAC mice was examined. These animals received a single injection of [<sup>3</sup>H]thymidine at 1 or 3 days postcauterization. The hearts were then harvested, sectioned, stained with X-Gal, and processed for autoradiography. No synthetic cardiomyocyte nuclei were detected in the perinecrotic zone ( $n = 36,000$  nuclei) nor in the distal region ( $n = 180,000$  nuclei) at either 1 or 3 days postinjury (Table 1C).

Collectively, these data suggest that myocardial damage can induce cardiomyocyte DNA synthesis in the adult murine heart, albeit at an exceedingly low frequency. The results and calculated labeling indexes for atrial and ventricular cardiomyocytes in both control and injured transgenic hearts are summarized in Table 1. It is of interest to note that the density of silver grains over synthetic cardiomyocyte nuclei was similar to that over synthetic fibroblast nuclei (compare Fig. 1, B with C). Presently, it is not clear if cardiomyocyte DNA synthesis ultimately results in polyploidization, karyokinesis, or cytokinesis.

As an additional control for the experiments in this study, results obtained from the dispersed cell assay were compared directly with those obtained from the transgenic mouse assay. Neonatal MHC-nLAC littermates (7 days old) received a single injection of [<sup>3</sup>H]thy-

midine and were processed either for dispersed cell analyses or X-Gal histochemistry (Fig. 1, H and I, respectively). Comparison of the resulting autoradiographs revealed a cardiomyocyte labeling index of ~2.0% with either assay (Table 1D). These results further validate the two assays employed in this study and confirm that synthetic cardiomyocyte nuclei can readily be scored in dispersed cell preparations. Finally, it is a formal possibility that cardiomyocyte DNA synthesis in the injured transgenic hearts might be masked by downregulation of the α-cardiac MHC promoter (and consequently the β-Gal reporter). This is an unlikely scenario, because the β-Gal signal observed in the synthetic cardiomyocytes was comparable with that seen in nonsynthetic cells (compare, for example, Fig. 1, B vs. G). This potential caveat was further addressed when ventricular cardiomyocyte DNA synthesis in injured adult hearts was monitored by the dispersed cell assay. As would be predicted by the transgenic mouse results, no synthetic cells were identified when 20,000 cardiomyocytes (corresponding to ~40,000 nuclei) were examined (Table 1A).

## DISCUSSION

Accurate assessment of cardiomyocyte DNA synthesis is dependent on the reliable identification of myocyte nuclei, particularly in instances in which the frequency of synthetic cells is low. The goal of the present study was to establish the level of DNA synthesis in the adult mouse heart with methods that permitted the unambiguous identification of cardiomyocyte nuclei. Dispersed cell preparations that relied on the enzymatic separation of cardiomyocytes and fibroblasts failed to identify any synthetic myocytes when >20,000 cells from normal hearts were examined. Because this result suggested that any cardiomyocyte DNA synthesis in the adult mouse heart occurs at extremely low frequencies, a high throughput labeling index assay using transgenic mice was developed. This assay identified only one and three synthetic ventricular cardiomyocyte nuclei when 180,000 cardiomyocyte nuclei were examined from normal and injured transgenic mice, respectively. In the injured hearts, the labeled cardiomyocytes were localized to perinecrotic regions. A lone labeled atrial cardiomyocyte was also identified in the injured heart preparations. No cardiomyocyte mitotic figures were detected during any of the analyses, consistent with the extremely low labeling indexes. In control experiments comparable labeling indexes were obtained for neonatal transgenic cardiomyocyte preparations with either the dispersed cell assay or β-Gal histochemical assay.

Although previous studies indicated that cardiomyocytes can synthesize DNA in the adult rodent heart, the absolute values reported varied markedly. For example, studies from our group failed to identify any synthetic cells when 15,000 cardiomyocytes from dispersed cell preparations were examined, suggesting a labeling index of ≤0.006% (18). In contrast, other groups reported labeling indexes ranging from 0.01 to

Table 2. Cautery injury induces hypertrophic cardiomyocyte growth in surviving myocardium

	Control Hearts	Injured Hearts
Mean	$367.46 \pm 13.0$	$468.19 \pm 16.4$
	$399.87 \pm 12.9$	$574.45 \pm 18.0$
	$464.55 \pm 13.9$	$515.15 \pm 15.2$
	$395.09 \pm 13.2$	$396.19 \pm 13.0$
	$406.74 \pm 13.7$	$488.50 \pm 17.0^*$

Values are mean cardiomyocyte cross-sectional area ± SE (arbitrary units). For each mouse, 100 randomly selected cardiomyocytes were scored. \*Significantly different from the control,  $P < 0.001$ .

0.035% for adult mouse cardiomyocytes (5, 13). These latter studies relied on autoradiographic analysis of heart sections from mice receiving injections of [<sup>3</sup>H]thymidine. Cardiomyocyte labeling indexes as high as 0.2% were reported for normal adult rats with a 5-bromo-2-deoxyuridine incorporation assay (3, 10, 11) and 0.45% with thymidine incorporation (7). The cardiomyocyte labeling index obtained for normal adult mice in the present study (1 nucleus per 180,000 nuclei examined or 0.0006%) was >20-fold lower than previous estimates for the mouse heart and >900-fold lower than those suggested for adult rats. It should further be noted that because only one thymidine-positive nucleus was detected, the labeling index for normal murine myocardium obtained here may be an overestimate. The failure to find any synthetic ventricular cardiomyocyte nuclei when 180,000 nuclei outside of the perinecrotic region were examined in injured transgenic hearts would tend to support this view (see below).

Although the differences in cardiomyocyte-labeling index presented above at first glance might appear to be insignificant, it is important to resolve this issue because a synthetic rate of 0.1–0.2% could account for a significant degree of cardiomyocyte reduplication over a life span. In contrast, a labeling index of 0.0005% would have a negligible impact on cardiomyocyte content. It is difficult to reconcile the differences in the extent of adult mouse cardiomyocyte DNA synthesis reported previously to the data presented here. It is unlikely that labeling indexes would vary greatly for different strains of inbred mice. Indeed, no overt differences in cardiomyocyte-labeling index were detected when three different inbred lines were examined via the dispersed cell assay (18). However, strain and/or species differences might explain some of the discrepancies reported for adult mouse versus rat cardiomyocyte DNA synthesis. One cannot rule out the possibility that the regulation of cardiomyocyte cell cycle withdrawal is more lax in rats than in mice and that these differences might account for the persistent cardiomyocyte DNA synthesis reported for adult rats. *A priori*, one might expect that such a situation would predispose rats to myocardial tumors. We are, however, unaware of any data suggesting an increased prevalence of myocardial tumors in rats compared with mice. An equally important consideration is the fact that the present study relied on methods that permitted unambiguous identification of cardiac myocyte nuclei (namely, dispersed cell preparations or the MHC-nLAC transgenic mice), whereas many of the previous studies relied on the analysis of intact, nontransgenic tissue sections. It is, nonetheless, a formal possibility that the cardiomyocyte labeling indexes reported here may be underestimates, because both assays relied on recognition of a differentiated phenotype (*i.e.*, retention of cellular morphology for the dispersed cell assay and  $\alpha$ -cardiac myosin heavy chain promoter activity for the X-Gal histochemistry assay). This issue notwithstanding, trivial arguments (*e.g.*, membrane instability or incomplete penetrance of transgene expression in synthetic cells) do not readily explain the absence of significant

levels of adult cardiomyocyte DNA synthesis observed in the present study because neonatal control animals had similar cardiomyocyte labeling indexes with dispersed cell preparations and X-Gal histochemistry. Furthermore, the robust X-Gal signal present in the limited number of synthetic adult cells that were identified suggests that activity of the  $\alpha$ -cardiac myosin heavy chain promoter is not incompatible with cardiomyocyte DNA synthesis in the transgenic mouse assay. This view is further supported by the  $\beta$ -Gal activity observed in synthetic neonatal MHC-nLAC mice.

Previous studies also suggested that myocardial injury can induce cardiomyocyte DNA synthesis in the perinecrotic regions of adult mouse hearts, and labeling indexes as high as 0.5% were reported (12). Even higher cardiomyocyte labeling indexes (1–2%) were reported in rats with myocardial infarcts or coronary artery narrowing (3, 10, 11). Marked atrial cardiomyocyte DNA synthesis was also reported in response to ventricular injury in both adult mice (12) and rats (14). Once again, the ventricular cardiomyocyte nuclear labeling index obtained in the present study (3 nuclei out of a total of 216,000 screened or 0.0014%) was markedly lower than that reported previously for both mice (~300-fold lower) and rats (~600- to 1,200-fold lower). Furthermore, the response of atrial cardiomyocytes to ventricular injury was virtually nonexistent in the present study. It is important to note that previous studies suggested that the extent of hemodynamic perturbation might impact on the degree of cardiomyocyte labeling in injured hearts (3, 10, 11). Despite the lack of data pertaining to cardiac function in the injured mouse hearts, the presence of a significant degree of hypertrophic cardiomyocyte growth, as assessed by image analysis of dispersed cell preparations, provides compelling evidence of a physiological response to the cauterization injury. Indeed, in some of the injured animals the mean cardiomyocyte cross-sectional area was 40% greater than the values observed in control animals (Table 2). These values compare favorably with the ventricular hypertrophy in rats with coronary artery narrowing (21 and 49% increase in mean cardiomyocyte volume in the right and left ventricles, respectively, as estimated by morphometric analyses; see Ref. 3). Once again, issues pertaining to species differences should also be considered as potential explanations for the observed differences.

In summary, the data presented here support the notion that cardiomyocyte DNA synthesis can occur in the adult mammalian heart, albeit at exceptionally low levels. Although no evidence for karyokinesis and/or cytokinesis was detected, the small number of labeled cells observed in the present study was insufficient to rule out this possibility. Nonetheless, in light of the exceedingly low labeling indexes observed for both normal and injured hearts, it is obvious that any resulting increase in myocardial mass would have a negligible effect on myocardial performance, even in the event that each synthetic cell was able to complete cytokinesis. These studies also provide important base-

line data for the assessment of the effects of gene transfer on cardiomyocyte DNA synthesis in both normal and injured mouse myocardium.

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