Inducible Activation of c-Myc in Adult Myocardium In Vivo Provokes Cardiac Myocyte Hypertrophy and Reactivation of DNA Synthesis

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Abstract—c-Myc, a protooncogene, mediates both proliferative and cellular growth in many cell types. Although not expressed in the adult heart under normal physiological conditions, Myc expression is rapidly upregulated in response to hypertrophic stimuli. Although Myc is capable of sustaining hyperplastic growth in fetal myocytes, the effects of its re-expression in adult postmitotic myocardium and its role in mediating cardiac hypertrophy are unknown. To determine the effects of de novo Myc activity in adult postmitotic myocardium in vivo, we created a novel transgenic model in which Myc is expressed and inducibly activated specifically in cardiac myocytes. Activation of Myc in adult myocardium was sufficient to reproduce the characteristic changes in myocyte size, protein synthesis, and cardiac-specific gene expression seen in cardiac hypertrophy. Despite the increased cardiac mass, left ventricular function remained normal. Activation of Myc also provoked cell cycle reentry in postmitotic myocytes, which led to increased nuclei per myocyte and DNA content per nuclei. (Circ Res. 2001;89:1122-1129.)

Key Words: cardiac muscle ■ Myc ■ hypertrophy ■ cell cycle ■ cardiac growth

In mice, cardiac myocytes rapidly proliferate during fetal development but typically cease to divide soon after birth.1 Although adult murine cardiac myocytes have lost the ability to proliferate, in many pathological situations they are subjected to increased hemodynamic stress, necessitating compensatory strategies to increase mass. This increase in mass, called hypertrophy, can be initiated by hemodynamic load, hypertrophic agonists, or passive mechanical stretch leading to cytoplasmic signaling cascades and the induction of immediate-early genes such as the transcription factors c-Myc (Myc), Fos, and Jun.2 Myc, which is upregulated in response to virtually all hypertrophic stimuli,3 can induce both proliferation and cellular growth in many cell types. Few studies have investigated the consequences of Myc overexpression in cardiac muscle directly, and none in adult postmitotic myocardium. Transient overexpression of Myc in cultured neonatal cardiac myocytes resulted in increased DNA synthesis and protein content.4 Transgenic (Tg) mice, which overexpressed Myc in fetal myocardium, developed increased ventricular mass secondary to myocyte hyperplasia.5 In these mice, despite the perinatal increases in myocyte number, DNA synthesis reportedly ceased earlier than in wild-type mice.5 The basis for this apparent paradox was not determined. These mice did not display baseline cardiac myocyte hypertrophy, but hypertrophic growth in adult cardiac myocytes was potentiated in response to some, but not all, agonists.7

The concept that Myc could mediate cellular growth distinct from its effects on cell division is relatively new, although several lines of evidence support this hypothesis. In Drosophila, decreased expression of dMyc reduced cell proliferation and cell size.8 In contrast, dMyc overexpression resulted in increased cell size without affecting cell division. More recently, two studies have confirmed that overexpression of Myc in B cells both in vitro9 and in vivo10 is associated with an increase in cell size. Myc regulates multiple candidate gene targets, some of which have been directly linked to cell growth and metabolism.11 Therefore, the upregulation of Myc in cardiac hypertrophy, taken with its known growth-promoting properties, suggests a role for this factor as an intracellular transducer of hypertrophic signals in postmitotic myocardium.

To study the effects of Myc on growth in adult cardiac myocytes we have created a novel Tg model in which Myc is inducibly activated by ligand-dependent nuclear translocation, specifically in cardiac myocytes. Activation of Myc in postmitotic myocardium was sufficient to induce an increase in myocyte size and protein synthesis and to activate a fetal
gene “program.” Activation of Myc also resulted in DNA synthesis leading to increased nuclei number per myocyte and ploidy.

Materials and Methods

Generation and Activation of Tg Mice

The inducible chimeric Myc protein, MycER, has been described.12 α-Myosin heavy chain (α-MHC)–MycER was constructed by cloning the 2.4-kb EcoRI fragment isolated from pBabe-MycER downstream of the murine α-MHC promoter13 (see Figure 1A). The transgene was microinjected into the pronucleus of fertilized eggs from C57BL/6×C3H mice by the UCLA Transgenic Core Laboratory. Tg founders were mated with C3H mice. To activate MycER, 1 mg of 4-hydroxytamoxifen (4-OHT; Sigma) dispersed in peanut oil by sonication was injected intraperitoneally daily. Control littermates were injected with peanut oil alone. Animals were handled in accordance with institutional guidelines.

Protein and RNA Analysis

Western blots were performed on protein extracts from whole ventricles, according to established protocols.14 Antibodies were obtained from Santa Cruz Biotechnology unless otherwise noted. Immune complex assays for cyclin-dependent kinase (Cdk) activity were performed as described.15 Total RNA was isolated from ventricles using an RNA STAT 60 Kit (Tel-Test, Inc), and Northern blots were performed according to established protocols.16 The universal Myc oligonucleotide probe was 5′-TCAGAGTCGCTGCTGGTGGTGTTGGCGGTGTCTCCTCATG-3′. Other oligonucleotide and cDNA probes used have been reported.16-17 Ribonucleic protection assays were carried out according to the manufacturer’s specifications (Pharmingen).

Histology

Hearts were either freshly frozen or fixed overnight in 4% paraformaldehyde buffered with PBS and routinely processed. 5-Bromo-2′-deoxyuridine (BrdU) labeling was achieved by injecting 50 mg of BrdU per gram of body weight intraperitoneally. To identify DNA synthesis, paraffin-embedded sections were probed with antibodies to BrdU (Zymed). Evidence of apoptosis was assessed by detection of nuclear DNA fragmentation by terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (Trevigen Inc). Histone H3 antibody (Upstate Biotechnology), antibody to sarcomeric MHCs (Chemicon), antibody to cyclin-dependent kinase (Cdk) (Pharmingen), and antibody to myosin heavy chain (α-MHC) (MF20; Developmental Hybridoma Studies Bank), and DAPI were used according to the manufacturer’s instructions. Secondary antibodies were purchased from Molecular Probes.

Animal Studies

Echocardiography on Tg or littermate controls was performed as described.18 At least three independent M-mode measurements per animal were obtained by an examiner blinded to the genotype of the animals. Nuclei were isolated from purified isolated cardiac myocytes as previously described.19 DNA content was quantified on isolated myocyte nuclei using propidium iodide as previously described.14

Results

Creation of Cardiogenic-Restricted, Tamoxifen-Inducible Myc Tg Mice

To determine the consequences of Myc activation in adult, postmitotic myocardium in vivo, we utilized a well-characterized conditional Myc gene (MycER).12 This chimeric protein was created by fusing human Myc to a mutated ligand binding domain of the human estrogen receptor. This mutation renders the ligand binding domain unresponsive to endogenous 17β-estradiol but sensitive to the synthetic estrogen antagonist tamoxifen. In the absence of ligand, the estrogen receptor is normally sequestered in the cytoplasm. On exposure to ligand, the protein translocates to the nucleus and can mediate transcriptional events. Therefore, although MycER is expressed constitutively it remains inactive and cytoplasmically tethered until exposed to tamoxifen. Thus, MycER can be used to trigger Myc-dependent transcription in mammalian cells using tamoxifen, but not by native estrogen.12

As shown in Figure 1B, injection of the Tg construct resulted in two independent Tg lines with similar copy number as demonstrated by Southern blotting (MycER Tg158 and Tg252). Protein lysates from whole ventricles were probed for expression of the transgene (Figure 1C). MycER protein was expressed at low but similar levels in both Tg lines at baseline. To confirm that expression of the MycER
transgene is cardiac-restricted, RNA isolated from multiple tissues from nontransgenic (NTg) and MycER Tg mice were examined (Figure 1D). A transgene-specific probe detected MycER expression solely in Tg158 myocardium. To compare the expression of Myc in our two Tg lines with a physiologically relevant stimulus, we contrasted levels of MycER mRNA in our two Tg lines with the expression of Myc in wild-type mice after 6 hours of pressure overload. As shown, levels of MycER were similar to endogenous levels of Myc seen acutely with pressure overload–induced hypertrophy (Figure 1E).

**Activation of Myc Results in Increased Cardiac Mass**

Both lines of MycER Tg mice appeared healthy and bred normally. We utilized 8-week-old MycER Tg or NTg mice throughout the study, comparing tamoxifen-treated mice of both genotypes with vehicle-treated littermate controls. Postmortem examination of multiple organs including heart, lung, liver, and kidney revealed pathologic changes only in the hearts of 4-OHT–stimulated MycER mice. At baseline, hearts from MycER Tg158 mice were indistinguishable from those of wild-type mice by either size or histology (Figures 2A and 2B). 4-OHT treatment itself had no effect on body weight or cardiac mass in NTg mice (Figure 2B). However, activation of MycER Tg158 mice for 1 week resulted in a 41.1% increase in cardiac mass normalized to body weight compared with treated NTg mice or MycER mice at baseline (6.03 ± 0.15 versus 4.26 ± 0.15 or 4.25 ± 0.13 mg/g, \( P < 0.0001 \); Figure 2B). Consistent with this finding, perfusion-fixed ventricles demonstrated increased wall thickness grossly (Figure 2A, c versus a and b) and a marked increase in fiber width at higher magnifications (Figure 2A, f versus d and e). This was not accompanied by fibrosis. No gender difference was detected with respect to growth response after 4-OHT treatment. To confirm that the ability of Myc to induce an increase in cardiac mass was not related to spurious insertion effects of the transgene, the two independent lines were systematically compared. As shown in Figure 2C, activation of Myc for 7 days was able to induce an increase in cardiac mass, in both MycER Tg lines 158 and 252, of 40.4% and 44.7%, respectively, when compared with
treated NTg mice. Although all results were confirmed in both Tg lines, results for the remainder of this article are reported for MycER Tg158 because the effects of Myc activation were similar in the two lines.

To determine whether the observed increase in cardiac mass was accompanied by the characteristic changes in cardiac-specific gene expression seen with hypertrophy, total RNA from ventricular muscle was examined (Figure 2D). Genes normally expressed only in fetal myocardium, atrial natriuretic factor, skeletal α-actin, and β-MHC were highly upregulated in MycER Tg ventricles treated with 4-OHT. Levels of cardiac α-actin were minimally decreased whereas levels of myosin light chain 2V and the constitutive control GAPDH were similar in both genotypes with and without 4-OHT. Therefore, activation of Myc alone is sufficient to induce changes in both cardiac mass and gene expression that are characteristic of cardiac hypertrophy.

**Left Ventricular Function Is Normal in MycER Mice Treated With 4-OHT**

Many Tg models of cardiac hypertrophy are associated with abnormalities in cardiac function. To determine whether the phenotypic changes associated with MycER activation alter myocardial function, transthoracic echocardiograms were performed on MycER Tg or NTg. After 1 week of treatment with 4-OHT, MycER mice demonstrated a significant increase in posterior wall thickness (0.86 ± 0.06 mm, 0.7 ± 0.05 mm, P < 0.05) and intraventricular septal thickness (0.82 ± 0.05 versus 0.7 ± 0.06 mm, P < 0.05) when compared with treated NTg mice; however, no significant differences in functional parameters as measured by aortic ejection time, left ventricular fraction shortening, or circumferential shortening velocity were seen (data not shown). To determine whether left ventricular dysfunction would develop after longer treatment intervals, myocardial function was determined after 3 weeks of tamoxifen stimulation (Table). Again, although treated MycER mice demonstrated increased myocardial mass (6.05 ± 0.18 versus 4.54 ± 0.25 mg/g, P < 0.005) and posterior wall thickness (0.90 ± 0.07 versus 0.69 ± 0.05 mm; P < 0.05) when compared with treated NTg mice, all measurements of myocardial function were normal. 4-OHT had no significant effect on any of the parameters measured in NTg mice.

**Myc Induces Hypertrophy and G1 Exit in Cardiac Myocytes**

To determine whether the increase in cardiac mass observed in treated MycER mice was secondary to increased myocyte size, we isolated adult cardiac ventricular myocytes after 7 days of treatment (Figure 3A). Myocyte size in NTg and untreated MycER mice was indistinguishable. Activation of Myc resulted in a 33% increase in relative cross-sectional area of isolated MycER myocytes compared with NTg littermates (1.33 ± 0.04 versus 1.0 ± 0.04, P < 0.005, Figure 3B). Therefore, activation of Myc alone is sufficient to induce cardiac myocyte hypertrophy in adult myocardium in vivo.

In addition to myocyte hypertrophy, histological assessment of stimulated MycER myocardium also demonstrated enlarged hyperchromatic myocyte nuclei and a suggestion of increased nuclei per myocyte (Figure 2A-f). To confirm this, we counted the number of nuclei per cell on dissociated cardiac myocytes and performed flow cytometry on nuclei isolated from these purified myocytes. 4-OHT treatment increased the percentage of myocytes with three or more nuclei per myocyte in MycER hearts compared with NTg or untreated MycER littermates (46.6 ± 4.9 versus 32 ± 0.3 or 31.7 ± 3.6, P < 0.05; Figure 3C) with a corresponding decrease in the percentage of myocytes with two nuclei. Induction of Myc activity also resulted in increased ploidy. The percentage of cardiac nuclei having 2N DNA content from 4-OHT–treated MycER mice was decreased compared with NTg or untreated MycER littermates (67.4 ± 2.5 versus 82.2 ± 1.8 or 80.7 ± 0.3%, P < 0.001; Figure 3C) with a corresponding increase in 8N nuclei (7.1 ± 0.7 versus 1.3 ± 0.5 or 1.7 ± 0.2%, P < 0.001; Figure 3D). Thus, MycER was sufficient to trigger a change in both parameters of DNA per cell: nuclei per myocyte and DNA per nucleus.

**Myc Activation In Vivo Reactivates DNA Synthesis**

To account for the nuclear changes in MycER mice, DNA synthesis was examined using BrdU labeling of synthetic nuclei. As shown in Figure 4A, nuclei from treated MycER mice incorporated BrdU. To quantify the DNA synthesis, we determined the percentage of cardiac nuclei labeling with BrdU. Myc activation induced a 50-fold increase in S-phase nuclei (BrdU−) in ventricular myocytes from stimulated MycER compared with NTg mice (0.94 ± 0.03 versus 0.2 × 10−3 ± 0.37 × 10−3%, P < 0.001; Figure 4A). There was no significant difference between NTg and untreated MycER mice. Phosphorylation on serine-10 of histone H3, a marker of cyclin B/cdc2 kinase activity, was also observed only in cardiac myocyte nuclei from activated MycER mice (Figure 4B-c). Provoking G1 exit in
adult cardiac myocytes has often been accompanied by apoptosis, and Myc itself is a well-known trigger of programmed cell death. To determine whether activation of Myc in this model was associated with apoptosis, terminal deoxynucleotidyltransferase-mediated dUTP nick-end labeling (TUNEL) staining and DNA laddering were performed (data not shown). Although DNase-treated control sections were positive (Figure 4B-d), sections from 4-OHT-treated NTg or MycER Tg mice did not display TUNEL-positive nuclei (Figure 4B, e and f). Likewise, 4-OHT-treated MycER ventricles showed no evidence of DNA laddering (data not shown). Thus, ligand-dependent activation of Myc function provokes G1 exit in postmitotic ventricular myocytes without inducing apoptosis.

Figure 3. Activation of MycER induces cardiac myocyte hypertrophy and nuclear changes. A, Adult cardiac myocytes were isolated from ventricles of MycER Tg or NTg mice and stained with DAPI. Bar=50 μm. B, Myocyte cross-sectional area was determined for each genotype and presented relative to NTg mice; n=4 for each group. C, Number of nuclei per myocyte quantified for each group (n=3). D, Flow cytometry was performed on propidium-stained nuclei isolated from purified adult cardiac myocytes; n=4 for each group. **P<0.001; *P<0.05 vs NTg or untreated MycER littermates.

Figure 4. Myc activation induces DNA synthesis. A, Low- and high-powered magnifications of myocardial sections from 4-OHT-treated MycER mice probed for BrdU. Percentage of BrdU-positive nuclei was quantified (n=5). Bar=50 μm. B, Immunofluorescent detection of M phase-specific marker, phosphorylated histone H3 (green; panels a through c) in cardiac myocytes identified with MF20 (red) from NTg (a) or 4-OHT-treated MycER mice at low (a) and high magnifications (c; bar=100 μm) and high magnifications (c; bar=50 μm). Nuclei were stained with DAPI (blue). Positive-staining nuclei were seen only in 4-OHT-treated MycER Tg mice (b and c; white arrowhead). Presence of Myc-induced apoptosis was assessed by TUNEL staining. Control DNase-treated sections (d) and NTg (e) or MycER Tg (f) mice are shown. No apoptosis was detected. *P<0.005 vs NTg or untreated MycER littermates.
Activation of Myc Upregulates Cdk2 Activity in Adult Myocardium

Myc has been implicated in regulating multiple genes involved in cell cycle regulation.11 In particular, cyclin D1 and D2, along with their kinase partner Cdk4, have been shown to be critical for Myc-mediated cell cycle progression.25,26 Activation of the cell cycle through induction of cyclin D occurs in large part by the titration of Cip/Kip proteins away from Cdk2 resulting in increased Cdk2 activity.25,26 To determine whether Myc could upregulate these factors in postmitotic cardiac myocytes, we performed ribonucleic protection assays on ventricular RNA. As shown, Myc specifically activates transcription of cyclin D2 and Cdk4 (Figure 5A). This upregulation was confirmed by examining protein lysates in which, in addition to cyclin D2 and Cdk4, proliferating cell nuclear antigen expression was also detected (Figure 5B). Interestingly, although transcripts of Cdk2 were unchanged, Cdk2 activity was strongly induced (Figure 5C). Therefore, titration of Cdk inhibitors away from Cdk2 by cyclin D2–Cdk4 complexes is a likely mechanism for the effects of Myc in cardiac muscle given that Cdk4 activity increased minimally (Figure 5C).25 However, because Cdk2 activity is necessary but not sufficient for G1 exit in cardiac myocytes, Myc must activate other effectors in addition to Cdk2.15

Myc Upregulates Genes Involved in Ribosome Biogenesis and Protein Translation

Recently, it has been suggested that Myc may have a direct effect on protein synthesis by upregulating large- and small-subunit ribosomal and nucleolar genes.17 To determine whether this mechanism might explain, at least in part, the increase in myocyte size observed in MycER mice, Northern analysis of total RNA prepared from ventricles of NTg or MycER Tg mice. Northern analysis of total RNA prepared from ventricles of NTg or MycER mice stimulated with 4-OHT for the indicated times was performed (Figure 6). Several ribosomal genes and genes that encode proteins engaged in ribosome biogenesis and protein translation are upregulated including nucleophosmin (B23), nucleolin (C23), L3, L23, and L35 by comparison with 18S rRNA and GAPDH expression as controls for specificity and for RNA loading. At least one of these genes, nucleolin, is known to be a direct transcriptional target of Myc.27

Discussion

Although much research effort has focused on elucidating the signaling pathways that activate the fetal gene “program” in hypertrophic cardiac growth, the transcriptional regulation of the actual growth process itself is poorly understood. Myc is a likely candidate to directly mediate hypertrophic cardiac growth given that it is critical for cellular growth in other cell types and is induced by virtually all interventions that lead to cardiac hypertrophy either in cultured cells or the intact myocardium. The present study establishes that activation of Myc alone is sufficient to induce hypertrophic growth in the ventricles of adult mice in vivo. Myc also induced characteristic changes in cardiac-specific gene expression whereby a fetal program was reactivated. Interestingly, the increase in cardiac mass was not associated with contractile dysfunction as has been seen in many Tg animals with cardiac hypertrophy.22,28 This may be related to the relatively short time course of these experiments in relationship to many other heart failure models. Longer-term experiments are in progress to determine the effects of prolonged Myc activation on myocardial growth and function.

The molecular pathways that are required for Myc-induced hypertrophic growth remain uncertain; however, the ability of Myc to directly regulate protein synthesis by upregulating ribosomal and nucleolar genes may account for this effect, at least partially. The conclusion that Myc can regulate mammalian cell size in vivo by means of such a pathway has been suggested by studies of Myc gene transfer to the liver.17 Although regulation of protein translation in the development of cardiac hypertrophy has been well established,29 the link to signaling pathways was unknown. Our results are the first conclusive demonstration that Myc can upregulate these

![Figure 5](https://www.ahajournals.org/doi/10.1161/CIRCRESAHA.105.151783)

**Figure 5.** Myc upregulates G1 cyclins and Cdns in adult myocardium. A, Representative ribonucleic protection assay performed on total RNA prepared from ventricles of mice with indicated genotypes stimulated with 4-OHT or vehicle. B, Western blots of protein lysates from NTg or MycER Tg mice. C, Representative Cdk assays performed on protein lysates from indicated mice with and without 4-OHT treatment.

![Figure 6](https://www.ahajournals.org/doi/10.1161/CIRCRESAHA.105.151783)

**Figure 6.** Myc upregulates genes involved in ribosome biogenesis and protein translation. Total ventricular RNA from NTg and MycER ventricles treated for indicated number of days was probed with specified probes B23 (nucleophosmin), C23 (nucleolin), L3, L23, L35, and GAPDH.
proteins and regulate cell size even in postmitotic cells. The relative importance of these factors versus the multiple other Myc-regulated genes remains to be determined.11

A less expected finding of this model was the observation that Myc activation in adult murine myocardium could trigger DNA synthesis with resulting endoreduplication. This increase in nuclei number and ploidy was sufficient to account for all of the DNA synthesis we observed. Despite examination of multiple sections from treated MycEER ventricles, no evidence of myocyte cytokinesis was seen. Likewise, overexpression of Myc in vitro did not increase myocyte number (data not shown), implying that although Myc is sufficient to induce cell cycle reentry it is not associated with myocyte cytokinesis. The basis for this effect is unclear, particularly given that induction of endogenous Myc in response to hypertrophic stimuli is not normally associated with DNA synthesis in mice.30 In contrast to mice, DNA synthesis has been reported in adult human myocardium after myocardial infarction.31 In this study the rate of myocyte Ki-67 staining (a marker of cycling cells) ranged from 1% to 4%, which is similar in magnitude to that seen in our Myc-induced mice. Although the authors felt that these findings signified cardiac myocyte division, an equally plausible explanation is that DNA synthesis resulted in endoreduplication or nuclear division similar to that seen in our mouse model. Although not a common feature of hypertrophy or cardiomyopathy in mice, increased ploidy level and number of nuclei per myocyte have been repeatedly identified in human hearts after myocardial injury.32,33 The basis for this apparent species-related difference is unknown, but it is interesting to note that Myc has been reported to be persistently upregulated in some human cardiomyopathies, raising the possibility that Myc might induce these changes in the human heart as well.34

The finding that Myc could induce both hypertrophic growth and DNA synthesis in adult myocardium differs from a previous Tg model that overexpressed Myc in the heart, although they are consistent with the effects of Myc in a variety of other models.8–10 The previous Myc Tg mice demonstrated cardiac myocyte hyperplasia; however, this all occurred in utero, and cell cycle exit actually occurred sooner in Tg mice.6 Furthermore, no baseline phenotypic abnormalities were identified in the adult ventricle that can be ascribed to Myc expression. A phenotype in adult myocardium was only uncovered after stimulation with select hypertrophic agonists, which demonstrated enhanced hypertrophic growth. A simple explanation for the differences between the two models is that the differential temporal pattern of expression led to developmentally dependent effects. However, it should be noted that the Tg models have many other differences. In the mice described by Jackson et al,5 the expression of Myc was constitutively driven by the Rous sarcoma virus promoter. Although expression of mRNA was demonstrated in the adult ventricle, the cell type was not characterized nor was protein expression documented. This is not a trivial point because the half-life of Myc protein is very short in quiescent cells.35 Likewise, it is impossible to know what compensatory changes occurred in the cardiac myocytes in response to the constitutive Myc expression during cardiac development. To this end, Myc overexpression has been shown to upregulate factors capable of limiting cell cycle progression in primary cells.36 This mechanism may account for the observation that Myc ultimately accelerated cell cycle exit in prior Tg models when expressed in the myocardium for prolonged periods of time.6

In summary, we have created a novel inducible Tg model of cardiac hypertrophy and cell cycle reentry in postmitotic cardiac myocytes. This model should prove useful to identify Myc-induced genes required for its growth effects and the molecular mechanisms that mediate endoreduplication and limit cytokinesis in adult cardiac myocytes. Although additional studies will be essential to monitor cardiac structure and function at much later intervals after Myc activation, the preservation of pump function here differs from other genetic models of induced cardiac growth and raises the long-term prospect of beneficial growth, as a tool, for cardiac repair.

Acknowledgments

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