Triiodothyronine-mediated myosin heavy chain gene transcription in the heart

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Triiodothyronine-mediated myosin heavy chain gene transcription in the heart. Am J Physiol Heart Circ Physiol 284: H2255–H2262, 2003. First published February 27, 2003; 10.1152/ajpheart.00860.2002.—We developed an RT-PCR assay to study both the time course and the mechanism for the triiodothyronine (T3)-induced transcription of the α- and β-myosin heavy chain (MHC) genes in vivo on the basis of the quantity of specific heterogeneous nuclear RNA (hnRNA). The temporal relationship of changes in transcriptional activity to the amount of α-MHC mRNA and the coordinated regulation of transcription of more than one gene in response to T3 are demonstrated here for the first time. Quantitation of α-MHC hnRNA demonstrated that T3 induced α-MHC transcription in hypothyroid rats within 30 min of a single injection of T3 (0.5 μg/100 g body wt). Maximal transcription rates (155% ± 15.8 of euthyroid values) occurred 6 h after injection and subsequently declined in parallel with serum T3 levels. The transcription of β-MHC was reduced to 86% of peak hypothyroid levels 6 h after a single T3 injection and reached a nadir of 59% of hypothyroid levels at 36 h. Analysis of the time course of T3-mediated induction of α-MHC hnRNA and repression of β-MHC hnRNA indicates that separate molecular mechanisms are involved in the coordinated regulation of these genes.

Cardiac contractility; heterogeneous nuclear ribonucleic acid; thyroid hormone; thyroid disease

THYROID HORMONE EXERTS profound effects on the heart and cardiovascular system (19). Many of the cardiac effects of triiodothyronine (T3), the biologically active form of the hormone, are mediated at the level of gene transcription. After transport to the myocyte nucleus, T3 binds to thyroid hormone nuclear receptors (TRs), which in turn bind to T3 response elements located within the 5′ flanking regions of T3-responsive genes (5, 9, 12). TRs act in a bimodal fashion by activating transcription in the presence of T3 by recruiting coactivator complexes and repressing transcription in the absence of ligand by recruiting corepressor complexes (15, 24, 39). Two distinct genes encode the family of TRs, and in the mammalian heart two splice variants of the TRα gene, TRα-1 and TRα-2, and primarily one splice variant of the TRβ gene, TRβ1, are expressed (14, 23, 35). There are multiple T3-regulated cardiac-specific genes including α-myosin heavy chain (MHC), β-MHC, phospholamban (PLB), and sarcoplasmic reticulum Ca2+-ATPase (SERCA2) (19, 28, 36).

Hypothyroidism results in decreased expression of positively regulated T3-responsive cardiac genes such as α-MHC and SERCA2, whereas the expression of negatively regulated T3-responsive genes such as β-MHC and PLB is increased (9, 16, 28). The shift in cardiac phenotype of rodents with hypothyroidism determined in large part the decrease in both systolic and diastolic contractile function (1, 9, 14). Treatment with thyroid hormone restores the normal expression of these genes, increases cardiac mass, and improves contractile function (3, 7, 18, 22, 38). The kinetics of gene activation and repression in the heart have not been fully investigated (9, 19, 28). Various lines of evidence have suggested that the cardiac response to T3 treatment results from changes in the rate of transcription of cardiac-specific genes, including nuclear run-on assays for α- and β-MHC expression performed on nuclei isolated from euthyroid rat hearts (4, 9, 17, 29). However, experiments were not designed to specifically measure the effects of T3 treatment (4). Direct gene transfer of an α-MHC promoter/luciferase reporter plasmid into hearts of hypothyroid rats demonstrated thyroid hormone regulation of α-MHC expression as measured by reporter gene activity (17, 30). Those measurements of α-MHC promoter activity required injection of a recombinant plasmid into the ventricular tissue and quantitation of luciferase activity several processing steps downstream from transcription (30), thereby precluding studies of the initial transcriptional response to thyroid hormone. Although nuclear run-on studies and direct gene transfer with α-MHC promoter/luciferase reporter plasmids have demonstrated a transcriptional mechanism for thyroid hormone regulation of α-MHC expression, these studies are not technologically capable of demonstrating the kinetics of α-MHC induction by T3 or the coordinated expression of two thyroid hormone-responsive genes as we report here.

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Although it has been demonstrated that α-MHC mRNA content increases in the hypothyroid cardiac myocyte in response to thyroid hormone (3, 28), the temporal relationship of changes in transcriptional activity to the amount of α-MHC mRNA or of the coordinated regulation of transcription of more than one gene in response to T₃ have not been established. We have developed a novel RT-PCR-based assay that requires small amounts of RNA and can be used to test for the transcriptional regulation of multiple genes at multiple time points. We used this assay to simultaneously determine the transcription rate of the α- and β-MHC genes in response to T₃ in hypothyroid animals. This study was designed to investigate the coordinated transcriptional response of the cardiac myocyte to T₃ administration in vivo to further identify the role of gene transcription and the mechanisms involved in this process over an entire physiological range of serum T₃ concentrations.

MATERIALS AND METHODS

Animal protocols. Adult male Sprague-Dawley rats (4–6 wk of age) were obtained from Taconic Farms (Germantown, NY). Animals were rendered hypothyroid by surgical thyroidectomy (Tx). Seven days after surgery, hypothyroidism was confirmed by analysis of serum total T₃ levels by RIA (Diasorin, Stillwater, MN). Rats were given a single intramuscular injection of 1 μg of T₃ (ICN Biomedicals, Aurora, OH) in 0.2 ml of PBS and were killed 0.5, 2, 6, 12, 24, 36, 48, and 72 h after injection. Hearts were quickly excised and weighed, and left ventricles (LVs) including septum were rapidly frozen in liquid nitrogen and stored at −80°C until being extracted for RNA. Three animals were used for each time point. RNA purified from four euthyroid animals was pooled and used as the reference sample in the RT-PCR analysis. The investigation conformed to the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (NIH Publication No. 85-23, Revised 1985).

Total RNA isolation. Total RNA was extracted from frozen LV samples as we described previously (3). The integrity of RNA was confirmed by electrophoretic resolution of the RNA and visualization of the ribosomal RNA subunits by ethidium bromide intercalation (3).

RT-PCR-based transcription assay of heterogeneous nuclear RNA. The current report demonstrates the measurement of transcription by quantitation of the primary transcript. The first product of transcription is the primary transcript, or heterogeneous nuclear RNA (hnRNA), which includes introns as well as exons. Total RNA from the hearts of experimental animals was used in a reverse transcription reaction with a primer that annealed to a sequence within the intron, therefore amplifying only the hnRNA (10). Treatment with DNase I ensured that no amplification of the cardiac genomic DNA occurred. The RT-PCR protocol is outlined in Fig. 1. Fifty micrograms of total RNA was treated with DNase I and the RNeasy mini protocol for RNA Cleanup (Qiagen, Valencia, CA). RNA concentration was determined spectrophotometrically. RT-PCR was performed with 2 μg of total LV RNA and reverse primers that annealed to the first intron of the α-MHC gene (α-MHC-949R: 5′-GACACAGAAGAAGGAAAGGAT-3′; GenBank accession no. AH002207; Ref. 25) and to the first intron of the β-MHC gene (β-MHC-614F: 5′-CTGCTGTTTCCATCCCTAGAAGTT-3′; GenBank accession no. X16291). These primers amplified only α- or β-MHC hnRNA. Separate RT reactions were done with the α-MHC reverse primer and the intronic reverse primer for β-actin hnRNA (β-actin-492R: 5′-GGATAACGTGCACACACTCTC-3′; GenBank accession no. V01217; Ref. 28). Reactions were then set up that contained both reverse primers with β-actin hnRNA as an internal control for each sample. The individual reactions ensured that reverse transcription reactions with both reverse primers accurately amplified both α-MHC and β-actin hnRNA fragments. RNA and 25 pmol of primer reverse (36-μl volume) were annealed in a 65°C water bath for 5 min and then allowed to cool at room temperature for 20 min. Reactions were centrifuged briefly, and 5× RT reaction buffer, 1 μl of Moloney murine leukemia virus RT (Promega, Madison, WI), 1 μl of RNasin (Promega), and dNTPs (Amersham Pharmacia, Piscataway, NJ; final concentration of 1 mM each) were added for a total reaction volume of 50 μl. Reactions were incubated for 60 min at 37°C and then stopped at 94°C for 6 min.

Five microliters of the RT reaction product was used for subsequent amplification of a 335-bp fragment of α-MHC by PCR with the reverse primer (α-MHC-949R) and a forward primer that annealed to the first exon (α-MHC-614F: 5′-ATTTCCTCCATCCCTAGAAGTT-3′; Ref. 25). Amplification of a 312-bp fragment of β-MHC was done with the reverse primer (β-MHC-1456R) and a forward primer that annealed to sequences within exon 1 (β-MHC-1144F: 5′-TGAGCATTCTGAGTGTCC-3′; GenBank accession no. X16291). These primers amplified only α- or β-MHC hnRNA. Separate RT reactions were done with the α-MHC reverse primer and the intronic reverse primer for β-actin hnRNA (β-actin-492R: 5′-GGATAACGTGCACACACTCTC-3′; GenBank accession no. V01217; Ref. 28). Reactions were then set up that contained both reverse primers with β-actin hnRNA as an internal control for each sample. The individual reactions ensured that reverse transcription reactions with both reverse primers accurately amplified both α-MHC and β-actin hnRNA fragments. RNA and 25 pmol of primer reverse (36-μl volume) were annealed in a 65°C water bath for 5 min and then allowed to cool at room temperature for 20 min. Reactions were centrifuged briefly, and 5× RT reaction buffer, 1 μl of Moloney murine leukemia virus RT (Promega, Madison, WI), 1 μl of RNasin (Promega), and dNTPs (Amersham Pharmacia, Piscataway, NJ; final concentration of 1 mM each) were added for a total reaction volume of 50 μl. Reactions were incubated for 60 min at 37°C and then stopped at 94°C for 6 min.

Figure 1. Protocol for RT-PCR of rat left ventricular (LV) total RNA for amplification of α- and β-myosin heavy chain (MHC) heterogeneous nuclear (hn)RNA. Forward (F) primer for α-MHC is 614F; reverse (R) primer is 949R; F primer for β-MHC is 1144F, R primer is 1456R. Both R primers anneal to the 1st intron of the respective genes.
0.5 μl (2.5 U) AmpliTaq Gold enzyme (Perkin Elmer, Foster City, CA), and dNTPs (0.1 mM final concentration each) in a total volume of 50 μl. After an initial activation step at 94°C for 10 min, amplification was done with 30 cycles: melting step at 94°C for 45 s, annealing at 55°C for 1 min, and extension at 72°C for 1 min. Ten microliters of the PCR reaction product was run on a 2% agarose gel with ethidium bromide and quantitated by densitometry with BioRad Quantity 4.2.2 software. All RT reactions were done in duplicate. PCR products were sequenced with an ABI prism 3100 Genetic Analyzer (Applied Biosystems/Hitachi).

Validation of RT-PCR assay. We determined the optimum PCR cycle number for the hnRNA content in the LV samples. PCR products after 15, 20, 25, 30, 35, and 40 reaction cycles were resolved by electrophoresis and quantified. Plotting PCR product content vs. cycle number showed that a cycle number of 30 fell within the linear range of amplification and was therefore chosen for subsequent analyses (Fig. 2). One, two, and three micrograms of total LV RNA from euthyroid and hypothyroid rats were assayed for hnRNA. hnRNA levels in hypothyroid animals were low to undetectable in 1, 2, or 3 μg RNA. In contrast, the concentration of α-MHC hnRNA from euthyroid animals increased linearly with an increase of total RNA used in the RT reaction as shown in Fig. 3. To maintain linearity of the assay, 2 μg of total LV RNA was subsequently used in each RT reaction. Because the primary transcript (hnRNA) is identical in sequence to the DNA, we routinely use DNase treatment of RNA samples to ensure that no DNA is amplified by PCR. To further demonstrate that the DNase treatment was complete, additional control RT-PCR reactions were performed on total RNA that was not DNase treated as well as RNA samples that were RNase treated to demonstrate that only the specific hnRNA was the template for the amplification reaction.

To verify the specificity of this assay, total RNA purified from two non-cardiac muscle sources [extensor digitorum longus (EDL) and soleus] that do not express α-MHC mRNA or protein was assayed. RT-PCR reactions using primers specific for α-MHC hnRNA showed no product in both EDL and soleus muscle RNA (Fig. 4).

Cardiac nuclei were isolated by a modification of the protocol published by Liew et al. (25). Hearts were excised, minced, and homogenized in 10 volumes of buffer containing 0.25 M sucrose, 10 mM Tris-HCl, pH 7.4, 10 mM MgCl2, 0.1 mM PMSF, 1 mM DTT, protease inhibitors (10 μg/ml each antipain, leupeptin, aprotinin, and benzamidine), and 2 mM vanadyl ribonucleoside complex with a Potter-Elvehjien homogenizer. Homogenate was then filtered through sterile
gaue and centrifuged at 600 g to obtain a crude nuclear pellet. The pellet was resuspended in 10 ml of homogenization buffer containing 2.2 M sucrose, rehomogenized, and centrifuged at 150,000 g at 4°C for 90 min. The purified nuclei were collected at the bottom of the tube, resuspended in 0.25 M sucrose homogenization buffer, and counted with a hemocytometer. The number of ventricular nuclei recovered was 10–17 × 10⁶ per heart, and the purity of the nuclei was confirmed by phase-contrast microscopy. For nuclear RNA isolation, pelleted nuclei were resuspended in guanidinium thiocyanate and RNA extracted as described (3). Purified RNA was used for RT-PCR as described in RT-PCR-based transcription assay of heterogeneous nuclear RNA.

Real-time quantitative PCR. α-MHC hnRNA was also quantitated by real-time PCR with the ABI PRISM 7700 (Perkin Elmer Life Sciences), and results were analyzed with the accompanying software. First-strand synthesis was performed with RT-PCR as described in RT-PCR-based transcription assay of heterogeneous nuclear RNA with 0.5 μg of total RNA and 37.5 pmol of reverse primer. The primer-probe set for α-MHC hnRNA was as follows: α-MHC R (5′-AAGTCGCGCTCTCCCTCC-3′) annealed within the second intron, α-MHC F (5′-GCAAGTGCTACTGGGAAAATC-3′) annealed within the second exon (also the first translated region), and the α-MHC probe (5′-AAAACGGAAGGATGTGCAATGGTGG-3′) labeled with TAMRA extended across the intron/exon boundary. The primer-probe set for GAPDH mRNA was as follows: GAPDH R (5′-GGCTCTCTTCTGTCTCAGTATC-3′), GAPDH F (5′-GGCTCTACATGGCCCTCCA-3′), and GAPDH probe (TET/TAMRA) (5′-AGTAAAGAAACCCTTGGCCACCGG-3′). The real-time PCR cycle used was 50°C (2 min), 95°C (10 min), followed by 45 cycles of 95°C (30 s) and 60°C (1 min). Amplification of fully processed α-MHC mRNA by RT-PCR. A reverse primer that annealed to the 3′ untranslated sequence of α-MHC, a region that differs from β-MHC (α-MHC-5892R: 5′-GTGAGGATCCACACCCG-3′) was used as described in RT-PCR-based transcription assay of heterogeneous nuclear RNA with 2 or 100 ng of total RNA from each animal. PCR with the same reverse primer and forward primer (α-MHC-5593F: 5′-CTACAGACAGAAGACACGAG-3′) amplified a region 299 bp in length (27). The PCR reactions were as described above but were run for 25 cycles, which we determined to be within the linear range.

Statistical analysis. All data are expressed as means ± SE. Statistical differences between values were evaluated by Student’s t-test with significant probability at P < 0.05.

RESULTS

Cardiac mass and thyroid status. Compared with the euthyroid control animals with a heart weight-to-body weight ratio (HW/BW, mg/g) of 3.42 ± 0.16, the hypothyroid animals had a 26% reduction in HW/BW to 2.52 ± 0.05, similar to that previously described (3). This difference in ratio was the result of a significant decrease in total cardiac and LV mass in the hypothyroid rats. Total heart weight for euthyroid animals was 705 ± 32 mg vs. 507 ± 10 mg for hypothyroid animals (P < 0.01). LV mass was 520 ± 25 and 400 ± 5 mg for euthyroid and hypothyroid animals, respectively (P < 0.01). After injection of 1 μg of T3, there was no significant change in absolute heart weight or HW/BW at any of the time points studied (3).

Serum T3 levels were determined at 8 days after thyroidectomy (Tx), at time 0 and at the times indicated after T3 treatment. All Tx animals were determined to be chemically hypothyroid, with T3 levels <20 ng/dl (29). The range of normal serum T3 levels in euthyroid control animals was between 80 and 120 ng/dl (29). After treatment with 1 μg T3, serum T3 levels appeared to peak within 30 min at 493 ± 20 ng/dl and fell to <50 ng/dl by 24 h (Fig. 5). The half-life of T3 in vivo was determined to be 7 h after T3 treatment (Fig. 5, inset), similar to that previously published by Goslings et al. (13).

Measurement of α-MHC hnRNA. The content of hnRNA in 2 μg of purified total LV RNA was measured for each animal at the indicated time points after T3 injection. The amount of the 335-bp PCR fragment of MHC hnRNA was determined by densitometry and expressed in arbitrary densitometric units.

The results of the time course of α-MHC hnRNA expression after T3 treatment are shown in a typical gel illustrating results from the indicated time points in Fig. 6A and quantitated in Fig. 6B. The transcription of α-MHC is initiated as early as 30 min after a single injection of T3, with a significant rise in the level of α-MHC hnRNA (P < 0.05 vs. time 0) to a level that was 17 ± 3% of the euthyroid standard. The maximum content of hnRNA was reached at 6 h after injection, to a level of 135 ± 16% of euthyroid (Fig. 6). The content of α-MHC hnRNA had declined to 42% of peak levels by 36 h after treatment (simultaneous serum T3 = 25 ng/dl) and continued to decline almost linearly at 48 and 72 h.

We used a second set of primers designed to amplify hnRNA of β-actin, a non-T3-responsive gene, as an internal control. Primer sets for α-MHC and β-actin
hnRNA were assayed together for euthyroid, 6 h after T₃, and hypothyroid RNA samples. These time points represent the lowest and highest levels of H9251-MHC detected. Although H9252-actin hnRNA levels did not change, expression of H9251-MHC hnRNA in each of these samples with both primer sets was the same as that observed with a single primer set. A representative gel is shown in Fig. 7.

Total RNA from selected critical time points, 2, 6, and 12 h after T₃ treatment, as well as hypothyroid controls was used in real-time PCR for quantitative analysis of H9251-MHC hnRNA. H9251-MHC hnRNA content is expressed as the fold difference between a given sample and hypothyroid levels and was calculated after normalization to GAPDH. GAPDH cycle number for all samples submitted for analysis was 24.97 ± 0.22, confirming that GAPDH is a non-T₃-responsive gene. As shown in Fig. 8, quantitative analysis of H9251-MHC hnRNA by real-time PCR concurs with the results of the transcription assay described in Figs. 6 and 7.

hnRNA is found exclusively in the nucleus of the cell, whereas only properly spliced and processed mRNA leaves the nucleus and enters the cytoplasm (21). We used RNA prepared from freshly isolated cardiac nuclei as well as total RNA prepared from cardiac tissue to confirm that the assay and these primers are specific for hnRNA. This transcription assay for H9251-MHC hnRNA demonstrated relative concentrations of 27.5 units/2 μg nuclear RNA and 2.25 units/2 μg total RNA. This corresponds to an ~12-fold increase in the amount of euthyroid H9251-MHC hnRNA from nuclear RNA compared with total cellular RNA, similar to expected results, because ~14% of the total cellular RNA is found in the nucleus at any given time including all of the hnRNA and a small percentage of the processed mRNA. As a result, the concentration of any hnRNA species will be at least sevenfold higher in purified nuclear RNA than in total cellular RNA.

Measurement of β-MHC hnRNA. The content of β-MHC hnRNA in total LV RNA was measured at the indicated time points and compared with hypothyroid samples, which were used as a standard maximum.
The results of the time course of β-MHC hnRNA expression after T3 treatment are shown in Fig. 9. The first measurable decrease in β-MHC expression of 14 ± 2% was detected at 6 h after injection (P < 0.05). The levels continued to decline in response to a single injection of T3 and reached a nadir of 59 ± 2% of pretreatment levels at 36 h. By 72 h after T3 treatment, when serum T3 levels had fallen to <20 ng/dl (Fig. 5), β-MHC hnRNA expression had returned to levels not different from those at time 0 (95 ± 7% of hypothyroid).

Determination of fully processed α-MHC mRNA accumulation. To determine that the hnRNA analysis was predictive of changes in mature mRNA, we measured the cellular accumulation of fully processed α-MHC mRNA in LV tissue in the RT-PCR assay as described. Negligible amounts of fully processed, mature α-MHC mRNA were detected in Tx animals. After injection of T3, mature α-MHC mRNA was first detected at 30 min, with peak levels accumulating by 24 h and thereafter declining (Fig. 10).

DISCUSSION

Although prior studies suggested that T3 acts at the cardiac myocyte nucleus to directly regulate specific gene transcription, definitive evidence for this premise has not been established because this requires a direct measure of transcriptional activity (17, 30, 33). The use of reporter genes to study transcription has inherent shortcomings due to the latent period of reporter protein accumulation, thus making it impossible to study the kinetics of α-MHC induction by T3 with this methodology. In addition, the coordinated expression of two thyroid hormone-responsive genes has not been demonstrated previously. Therefore, we developed an RT-PCR-based assay of hnRNA content to quantitate the rate of appearance of myocyte-specific primary gene transcripts. This assay for both α- and β-MHC hnRNA was determined to be sensitive, reliable, reproducible, and specific for myocyte gene expression.

The initiation of α-MHC transcription in the hearts of hypothyroid animals was evident by 30 min after a single T3 dose and suggests that T3 was taken up by cardiac myocytes in parallel with the rise in serum T3 (11). This observation is the earliest in vivo cardiac-specific nuclear event reported to date (33). Repression of β-MHC transcription was first detectable at 6 h after injection, still 86 ± 2% of hypothyroid levels, at a time when α-MHC transcription was fully activated. An increase in the accumulation of fully processed mature α-MHC mRNA was first detectable at 30 min after T3 injection with a sensitive RT-PCR assay of 100 ng of total RNA. The accumulation of mRNA peaked at 24 h and then began to decline as the serum T3 levels and transcription began to fall. Analysis of the rate of decline of α-MHC hnRNA and mRNA indicated apparent half-lives of ~20 and 14 h, respectively. Although there are inherent limitations in establishing a half-life for hnRNA or mRNA in vivo as problems exist in calculating an “absolute” half-life because of complications such as RNA stability and incomplete cessation of transcription, cell culture experiments can only approximate what occurs in vivo.
The temporal relationship between the T₃-mediated induction of α-MHC and repression of β-MHC transcription appears to be separate and distinct. The sequence of events involved in the induction of the positively regulated α-MHC is a two-step process involving recruitment of coactivators including proteins in the thyroid hormone-associated protein (TRAP) and nuclear receptor coactivator (NCoA) families subsequent to recruitment of histone acetyl transferases that remodel chromatin (24, 39). The present data show that this process of induction is initiated within minutes. In contrast, the molecular events involved in the repression of β-MHC are less well understood (15, 29, 37). The first measurable decline in the transcription of β-MHC was not evident until 6 h after administration of T₃, when α-MHC hnRNA levels were already maximal. The difference in response time for each gene suggests that different molecular mechanisms are at work (39). The repression of β-MHC transcription likely requires the recruitment of multiple coregulators/corepressors, such as nuclear receptor corepressor (NCoR) and histone deacetylases (HDACs), that may act at some site other than directly on the 5′ region of the β-MHC promoter (31, 37). Additional experiments are necessary to elucidate the molecular mechanisms involved in β-MHC regulation by T₃.

This is the first demonstration of the temporal association between serum T₃ level, α-MHC gene transcription, and mature mRNA content in the cardiac myocyte (33, 34). These in vivo animal data provide support for recent human studies in which the cardiac effects of T₃ are seen rapidly after hormone administration (6, 20).

In summary, these studies emphasize that both positively and negatively T₃-mediated cardiac-specific gene transcription are sensitive to changes in serum levels of T₃ over the entire range of thyroid function; however, the T₃-mediated repression of β-MHC transcription is a slower, more complex process than the induction of α-MHC. These studies further support the concept that the cardiac phenotype is regulated rapidly by T₃ at the level of gene transcription (2, 8, 19).

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