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Troponin T Switching in the Developing Rat Heart*

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A monoclonal antibody specific for cardiac troponin T has been used to investigate troponin changes during development in the rat heart. Specificity of the antibody was determined by immunoblot analysis with purified bovine cardiac troponin. In the rat heart, immunoblot analysis shows that anticardiac troponin T reacts with a 42.5-kDa band in fetal ventricles and with a 41-kDa band in adult ventricles. The faster migrating troponin T is present in traces in the fetal heart and increases markedly during the first 2 weeks after birth, concomitantly with the progressive decrease of the slower migrating form that is no longer detectable in the adult. The pattern of reactivity of the monoclonal antibody is not modified by alkaline phosphatase pretreatment, suggesting that the antibody is not specific for a phosphorylated epitope. Conditions known to affect cardiac myosin composition, such as hypothryoidism and hypertrophy secondary to systemic hypertension, do not change the troponin T isoform profile of adult rat ventricles. The expression and accumulation of the adult isoforms of troponin T are not suppressed by propylthiouracil treatment of pregnant and nursing rats.

Distinct isoforms of contractile proteins are expressed in the developing mammalian heart. In the thick filament, both myosin heavy chains (MHCs)[1] and myosin light chains (MLCs) undergo developmental changes in isoform composition. In the rat ventricle, β-MHC, the predominant fetal isoform, is replaced during early postnatal development by α-MHC (1), and atrial/embryonic MLC1 which is present in fetal ventricles disappears after birth (2). In the thin filament, both cardiac and skeletal α-actin isoforms are expressed before birth, whereas only cardiac α-actin is expressed in the adult rat heart (3, 4). Developmental changes of the troponin-tropomyosin systems have been comparatively less investigated. Humphreys and Cummins (5) found no significant change in troponin (Tn) I and only a slight increase in β-tropomyosin in the bovine heart. On the other hand, Solaro *et al.* (6), by SDS-PAGE analysis of myofibrillar proteins, described the presence of a doublet in the region of migration of adult TnI in the dog fetal heart, the slower migrating band decreasing with development. The analysis of developmentally regulated and tissue-specific isoforms of troponin T is complicated by the existence of a large number of variants showing different electrophoretic mobility (7, 8) derived from different genes and from differential splicing of the same gene (9, 10). Specific immunological probes can thus be useful for the study of TnT isoforms (7, 11). Indirect evidence from functional studies suggests that the regulatory proteins of the thin filament are modified during mammalian heart development. Studies on skinned cardiac cells indicate that in the fetal rat ventricle, the myofilaments are more sensitive to Ca^{2+} than in the adult ventricle (12). Furthermore, Ca^{2+} activation of myofilaments is insensitive to acidic pH in the fetal and neonatal dog heart, whereas in the adult heart, acidic pH causes a rightward shift in the relation between pCa and myofibrillar ATPase (6). This effect appears to be due to a reduction in the affinity of troponin C for Ca^{2+}, which was interpreted as resulting from the presence of variants of TnI or TnT.

Using a monoclonal antibody specific for the cardiac TnT, we have obtained direct evidence for developmentally regulated isoforms of this subunit in the rat heart. A preliminary report of these findings has appeared (13).

MATERIALS AND METHODS

Animals—Wistar rats of different ages were used in this study. Systemic hypertension and left ventricular hypertrophy were induced in 150–200-g rats by stenosis of the renal artery (14). Animals killed 2 months after surgery, showing marked accumulation of β-MHC in the ventricular myocardium (14), were used for the study of troponin changes. Thyroidectomized rats were obtained from Charles River Breeding Laboratories, Inc. and were not used until at least 10 weeks after surgery, by which time they showed an almost complete α- to β-MHC transition, as determined by immunoblotting analysis and immunocytochemical staining with antibodies specific for α- and β-MHCs (14, 15). Congenital hypothyroidism was induced by adding 0.02% propylthiouracil (PTU) to drinking water of pregnant rats from 15 days gestation to 30 days postpartum. The effect of this treatment on α- and β-MHC expression was also determined by immunoblotting and immunohistochemistry on the same hearts used for analysis of troponin isoforms.

Monoclonal Antibodies—BALB/c mice were immunized with a myofibrillar preparation from hypothyroid rabbit ventricle (15) using the protocol described by Cianfriglia *et al.* (16). Spleen cells were fused with NS-O myeloma cells essentially as described by Galfré and Milstein (17). Hybridoma supernatants were first screened by indirect immunofluorescence using sections of composite blocks of rat cardiac and skeletal muscle and subsequently by immunoblotting with whole tissue extracts. Selected hybridomas were cloned by limiting dilution and grown as ascites in pristane-primed mice. Immunoglobulin classes were determined by enzyme immunoassay using specific antisera (Miles Laboratories Inc.). The mAb used in this study (RV-C2) was found to be IgG_{2b}. It was purified from ascites fluid by caprylic acid precipitation (18); purity of Ig was monitored by SDS-PAGE.

SDS-PAGE and Immunoblotting—Troponin was isolated from bovine atria or ventricles using the procedure of Tsukui and Ebashi (19) as modified by Lin and Cassim (20). Whole tissue extracts were prepared by homogenizing samples of cardiac muscle in Laemmli buffer (21).

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[1] The abbreviations used are: MHCs, myosin heavy chains; MLCs, myosin light chains; mAb, monoclonal antibody; PTU, prophylthiouracil; Tn, troponin; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

Proteins were separated in SDS-polyacrylamide (12.5%) gels (21) and stained with Coomassie Blue. For estimation of molecular masses, the low molecular mass protein standard kit (Bio-Rad) containing phosphorylase (92.5 kDa), bovine serum albumin (66.2 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa), soybean trypsin inhibitor (21.5 kDa), and lysozyme (14.4 kDa) was used. Duplicate gels were electrophoretically transferred to nitrocellulose sheets as described by Towbin *et al.* (22). The blots were first incubated with monoclonal antibody at a concentration of about 30 ng/ml and then with peroxidase-conjugated rabbit anti-mouse Ig antibody (Dakopatts) and developed with diaminobenzidine in the presence of imidazole (23).

Purified bovine troponin was dephosphorylated by alkaline phosphatase treatment as described by Perry and Cole (24). Rat cardiac tissue extracts were dephosphorylated after transfer to nitrocellulose paper as described by Sternberger and Sternberger (25). Blots were incubated at 32 °C for 2.5 h in 0.1 M Tris-HCl, pH 8.0, containing 30 μg/ml calf intestinal alkaline phosphatase (Boehringer Mannheim, 2700 units/mg).

Immunohistochemistry—Immunoperoxidase staining of cryosections of unfixed cardiac muscle was performed as described previously (26).

RESULTS

Specificity of mAb for Cardiac TnT—The specificity of the antibody is illustrated in Figs. 1 and 2. Purified bovine cardiac troponin consists of two closely spaced TnT bands (8, 27, 28), whereas TnI and TnC migrate as a single band. RV-C2 reacts specifically with both TnT isoforms. In contrast, a single TnT band with an apparent M_r of about 41,000 is recognized by this antibody in rat ventricular extracts. Alkaline phosphatase treatment of purified bovine troponin does not change this pattern of reactivity, suggesting that the antibody is not specific for a phosphorylated epitope (Fig. 2). Antibody binding to rat TnT is likewise unchanged by alkaline phosphatase treatment after transfer to nitrocellulose paper (data not shown).

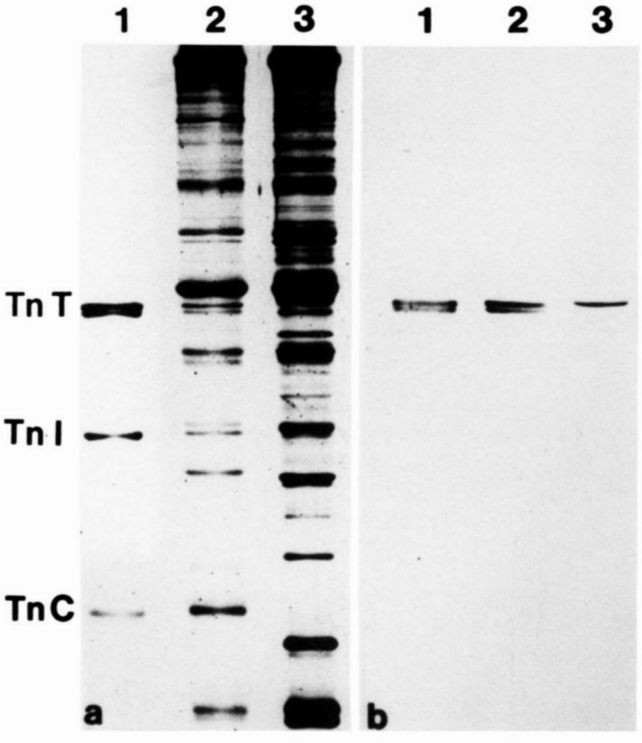

FIG. 1. **Specificity of anti-TnT antibody assessed by immunoblotting.** Purified cardiac troponin (*lane 1*) and total tissue homogenates of adult bovine ventricles (*lane 2*) and adult rat ventricles (*lane 3*) were separated by SDS-PAGE on 12.5% gels and either stained with Coomassie Blue (*a*) or transferred to nitrocellulose and reacted with mAb RV-C2 (*b*). Bound antibody was visualized by peroxidase-conjugated anti-mouse IgG antibody as described under "Materials and Methods." The positions of troponin subunits, TnT, TnI, and TnC, are indicated to the *left*.

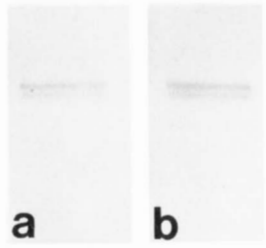

FIG. 2. **Effect of alkaline phosphatase pretreatment on reactivity of anti-TnT antibody.** Immunoblot is shown of untreated (*lane a*) and alkaline phosphatase treated (*lane b*) purified bovine cardiac troponin. Both samples were run on SDS gels, transferred to nitrocellulose, and incubated with anti-TnT antibody. The reactivity pattern is unchanged by phosphatase treatment. Only the portions of the blots corresponding to the TnT subunits are shown.

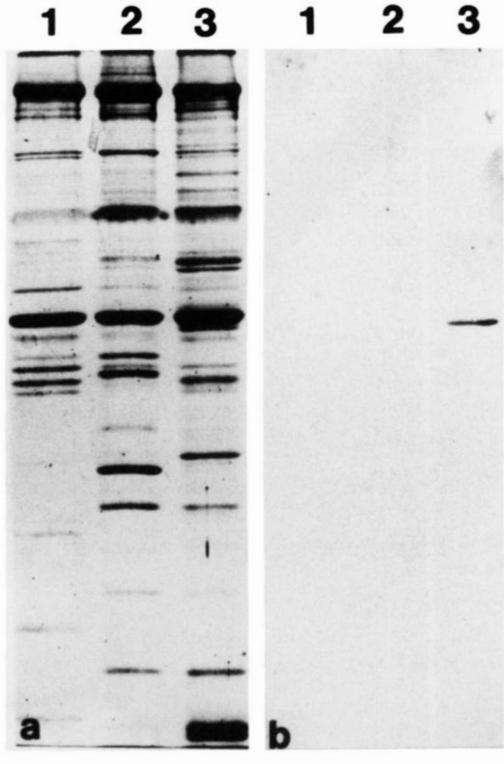

FIG. 3. **Reactivity of anti-TnT antibody with skeletal and cardiac muscles.** Immunoblots are shown of tissue extracts from adult rat tibialis anterior muscle (*lane 1*), soleus muscle (*lane 2*) and ventricle (*lane 3*) with antitroponin antibody. *a*, Coomassie Blue-stained gel; *b*, duplicate gel after transfer to nitrocellulose and reaction with mAb RV-C2.

On immunoblots, anti-TnT antibody does not react with troponins from the fast tibialis anterior and slow soleus muscles (Fig. 3). Accordingly, the antibody stains cardiac but not skeletal muscle in cryosections of adult rat tissues processed for immunoperoxidase (data not shown). As shown in Fig. 4, the antibody shows an identical reactivity pattern with atrial and ventricular muscle extracts.

Fetal Ventricles Contain a Distinct TnT Isoform—The pattern of reactivity of the antibody was found to vary in the rat heart during development. As shown in Fig. 5, a TnT isoform with a higher M_r value (~42,500) is identified by RV-C2 in ventricles from 18-day rat fetus. This different pattern of reactivity is not modified by alkaline phosphatase treatment

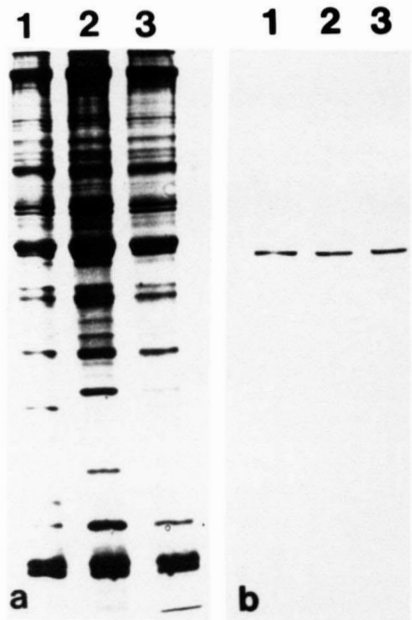

FIG. 4. **Reactivity of anti-TnT antibody with atrial and ventricular myocardia.** Immunoblots are shown of tissue extracts from rat atria (*lane 1*), ventricles (*lane 2*), and a mixture of atria and ventricles (*lane 3*). *a*, Coomassie Blue-stained gel; *b*, duplicate gel after transfer to nitrocellulose and reaction with mAb RV-C2.

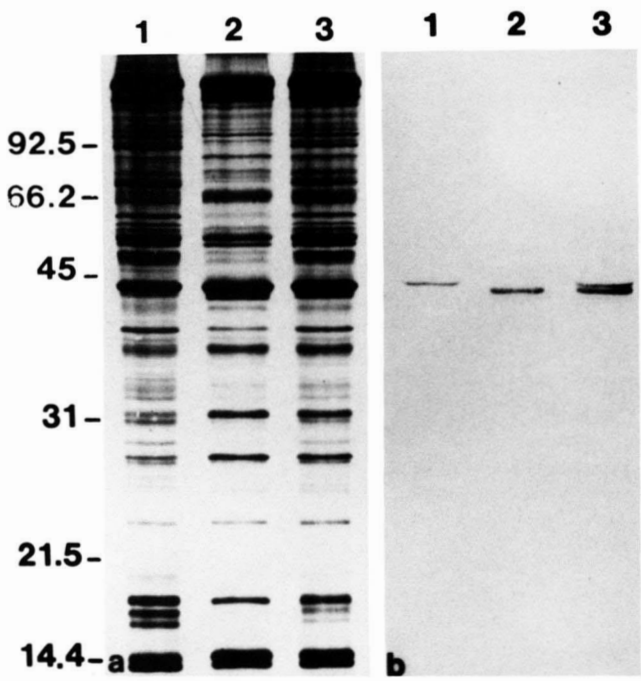

FIG. 5. **Reactivity of anti-TnT antibody with fetal and adult ventricles.** Immunoblots are shown of ventricular extracts from 18-day fetus (*lane 1*), 90-day rat (*lane 2*), and a mixture of fetal and adult ventricles (*lane 3*). *a*, Coomassie Blue-stained gel; *b*, duplicate gel after transfer to nitrocellulose and reaction with mAb RV-C2. The positions of molecular weight ($\times 10^{-3}$) markers are indicated to the left.

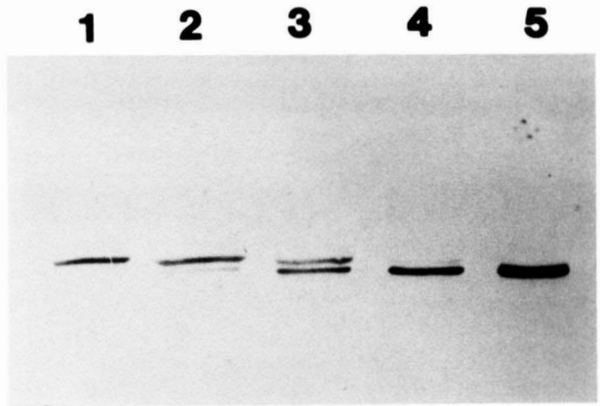

FIG. 6. **Changes in pattern of reactivity of anti-TnT antibody during development.** Immunoblotting analysis is shown of ventricular tissue extracts from 18-day fetus (*lane 1*) and 1-day (*lane 2*), 7-day (*lane 3*), 15-day (*lane 4*), and 30-day (*lane 5*) postnatal rats. Only the areas of the blots corresponding to the position of TnT are shown.

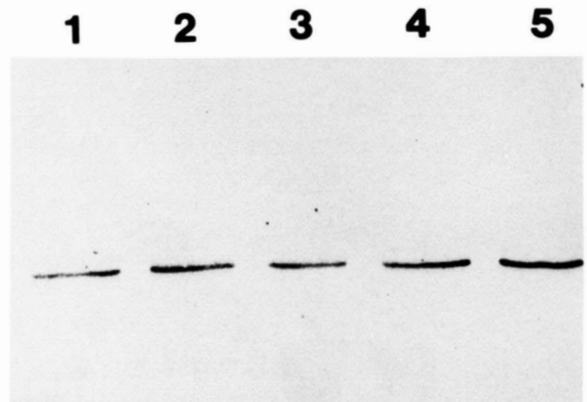

FIG. 7. **Reactivity of anti-TnT antibody with hypertrophied and hypothyroid ventricles.** Immunoblot is shown of ventricular extracts from normal 30-day rat (*lane 1*), rat with pressure overload-induced cardiac hypertrophy (*lane 2*), hypothyroid rat (*lane 3*), a mixture of normal and hypertrophied ventricles (*lane 4*), and a mixture of normal and hypothyroid ventricles (*lane 5*).

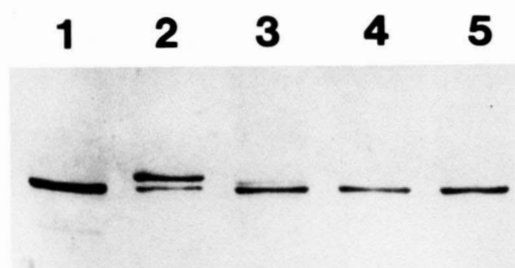

FIG. 8. **Reactivity of anti-TnT antibody after PTU treatment of pregnant rats.** Immunoblot is shown of ventricular extracts for normal 30-day rat (*lane 1*) and PTU-treated 4-day (*lane 2*), 15-day (*lane 3*), 30-day (*lane 4*), and 60-day (*lane 5*) rats. Note that the low mobility TnT band is still predominant in the PTU-treated 4-day sample, but is barely seen by 15 days and disappears at subsequent stages.

of the blots (data not shown). The time course of troponin changes in developing ventricles is illustrated in Fig. 6. In fetal ventricles, a minor band of higher mobility co-migrating with adult-type TnT is barely seen in blots from overloaded gels. This band is clearly seen by day 1 and becomes the major component by day 7. The fetal, lower mobility TnT isoform shows a rapid decrease during the first 2 weeks postpartum: it is still seen as a faint band by day 15, but is not detectable by day 30.

Expression of TnT Isoforms Is Unchanged in Hypertrophied and Hypothyroid Heart—Hypothyroidism and hypertrophy secondary to hemodynamic overload have been shown to influence the expression of MHC isoforms in rat ventricles: α-MHC, which predominates in the adult, is deinduced in

both conditions, with a concomitant induction of β-MHC, the predominant fetal isoform (1, 14, 29). We have therefore compared the TnT profile from normal, hypertensive, and hypothyroid animals to determine whether troponins undergo a similar reversion to the fetal isoform. Chronic hypertension induced by renal artery stenosis and hypothyroidism were found to cause replacement of α-MHC by β-MHC in the ventricular myocardium, as determined by immunoblotting and immunohistochemical analysis with specific antibodies (data not shown). However, samples from the same hearts show no significant difference in the pattern of reactivity with the anti-TnT antibody when compared to controls (Fig. 7). No trace of fetal-type TnT can be detected in these samples even in blots from overloaded gels.

During the neonatal period, thyroid hormone has been shown to influence the expression of several proteins. In particular, β- to α-MHC switching, which occurs in the perinatal and early postnatal periods coincides with the increase in serum level of thyroid hormone and is completely inhibited by PTU treatment of pregnant rats (30). We have confirmed this result using immunoblotting and immunohistochemical studies with specific antimyosin antibodies (data not shown). On the other hand, as shown in Fig. 8, PTU treatment does not appear to influence the replacement of fetal TnT by adult-type TnT.

DISCUSSION

The results show that at least two distinct isoforms of cardiac TnT are present in the fetal and adult rat heart: the two isoforms share a common epitope recognized by mAb RV-C2, but differ in the electrophoretic mobility. The results do not exclude the possibility that still other TnT variants with apparent molecular weight similar to that of the two isoforms described here may be expressed in cardiac muscle. Two TnT isoforms with the same apparent molecular weight have been identified in the adult rabbit heart (31).

Cooper and Ordahl (10) have shown that in the chick heart, a single troponin T gene generates embryonic and adult isoforms via developmentally regulated alternative splicing. The TnT mRNA expressed in the embryonic heart differs by the inclusion of a single exon coding for a short highly acidic peptide near the amino terminus (10, 32). It is likely that alternative splicing of the cardiac TnT gene occurs in the mammalian heart as well (27, 31). The analogy is further supported by the finding that embryonic cardiac TnT is also expressed in developing skeletal muscle both in the chick (10) and the rat.[2] It has been hypothesized that the embryonic TnT isoform may play a role during sarcomere assembly and may be associated with noncontractile myofibrils (10). However, this interpretation is not consistent with the finding that this isoform is predominant in the late fetal and neonatal rat heart, at a stage when contractile function is well developed, and persists even in the adult stage in the bovine heart.

The time course of cardiac TnT switching during development is similar to that of the β- to α-MHC transition (1) and of the decrease of skeletal α-actin (3) and β-tropomyosin (34) mRNAs in the developing rat ventricle. Thus, multiple changes of both thick and thin filament proteins take place in the rat heart in an apparently coordinated manner during the first weeks after birth. It is of interest to contrast these changes with those occurring in larger mammals. In the bovine and human heart, there is no significant β- to α-MHC transition during development (15, 35, 36), and both skeletal α-actin (4) and β-tropomyosin (37, 38) persist in the adult; accordingly, both TnT isoforms are present in the adult. Only atrial/embryonic MLC1 disappears with development in the bovine ventricles (39).

The observed temporal correlation in the developmental changes of contractile protein isoforms should not be taken as evidence for a common pattern of regulation of the corresponding gene families. Previous studies point to uncoordinated regulation of contractile protein genes in response to hormonal and mechanical stimuli. Thyroid hormones play a major role in the postnatal induction and maintenance of α-MHC in the rat ventricular myocardium (30), whereas neither skeletal α-actin nor β-tropomyosin genes are induced in hyper- and hypothyroid animals (34). We show here that TnT composition is also unchanged in hypothyroid rats and that TnT switching in neonatal rat ventricles is not prevented by inhibiting thyroid hormone synthesis with PTU, suggesting that thyroid hormones are not involved in the regulation of TnT isoform expression. Our results also indicate that the TnT isoform profile is not modified by chronic pressure overload, which is known to induce a marked and persistent transition from the normal adult α-MHC to the fetal β-MHC isoform (29). However, it remains to be established whether there is a transitory expression of fetal TnT during the early stages after imposition of a pressure overload. It has recently been reported that the mRNAs encoding skeletal α-actin and β-tropomyosin are re-expressed in the rat heart within 2 days after aortic coarctation, but return to negligible control levels at later stages (34, 40).

The functional significance of the developmental changes of cardiac TnT remains to be established. The two isoforms of bovine cardiac TnT have been shown to confer different Ca^{2+} sensitivities in a reconstituted system of contractile proteins (28). It is possible that the troponin changes described here may account for the differences in Ca^{2+} and pH sensitivity between fetal and adult myofilaments (6, 12). Developmental changes in cardiac contractility and in the response of the mammalian heart to various inotropic stimuli, such as paired pulse stimulation, post-extrasystolic potentiation, and β-adrenergic stimulation, have also been described (33, 41, 42). These differences in function between fetal and adult hearts may be related to fetal-to-adult isoform switching of the TnT and TnI (6)[3] subunits of the troponin complex.

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