Proc. Natl. Acad. Sci. USA
Vol. 91, pp. 6138–6142, June 1994
Agricultural Sciences

Milk composition and lactation of β-casein-deficient mice

(gene targeting/casein micelles)

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Communicated by Neal L. First, December 6, 1993 (received for review October 7, 1993)

ABSTRACT **β-Casein is a major protein component of milk and, in conjunction with the other caseins, it is assembled into micelles. The casein micelles determine many of the physical characteristics of milk, which are important for stability during storage and for milk-processing properties. There is evidence that suggests that β-casein may also possess other, nonnutritional functions. To address the function of β-casein, the mouse β-casein gene was disrupted by gene targeting in embryonic stem cells. Homozygous β-casein mutant mice are viable and fertile; females can lactate and successfully rear young. β-Casein was expressed at a reduced level in heterozygotes and was completely absent from the milk of homozygous mutant mice. Despite the deficiency of β-casein, casein micelles were assembled in heterozygous and homozygous mutants, albeit with reduced diameters. The absence of β-casein expression was reflected in a reduced total protein concentration in milk, although this was partially compensated for by an increased concentration of other proteins. The growth of pups feeding on the milk of homozygous mutants was reduced relative to those feeding on the milk of wild-type mice. Various genetic manipulations of caseins have been proposed for the qualitative improvement of cow's milk composition. The results presented here demonstrate that β-casein has no essential function and that the casein micelle is remarkably tolerant of changes in composition.**

Milk is usually the sole source of nourishment of young mammals, and the milk of domestic animals is an extremely important food source for much of the world's population, both as liquid milk and in a very wide variety of processed forms. Most of the protein in milk is found in the caseins, which are aggregated into large micellar structures that are in colloidal suspension in native milk. Although the detailed structure of casein micelles is not yet established, there are a number of models of micelle structure (for review, see ref. 1). The "calcium-sensitive" caseins (α_{s1}-, α_{s2}-, and β-caseins in cow's milk) are generally thought to be located predominantly within the micelles, and κ-casein is thought to coat the micelle, serving to stabilize the structure. The calcium-sensitive caseins are highly phosphorylated, and calcium phosphate interacts with them via their phosphate groups. In this way the casein micelles carry large amounts of (normally highly insoluble) calcium phosphate into milk and retain it in suspension. A further consequence of the assembly of the caseins into micelles is that the viscosity of milk remains low despite the high protein concentration. Thus, casein micelles are of functional importance for protein and mineral nutrition of the young and in determining the physical properties of milk.

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Selective breeding has been very successful in increasing milk yields of cattle, but this approach has been found to have limited potential for the alteration of milk composition. We and others have previously demonstrated the profound alteration of the protein composition of milk by expression of milk protein genes in transgenic mice (2–4), and the expression of human pharmaceutical proteins in the milk of transgenic mice, rabbits, goats, pigs and sheep has been demonstrated (5–13). In some cases very high yields of active human proteins have been obtained (6, 8, 11–13), and this approach is being commercialized. In addition to pharmaceutical applications, genetic manipulation may be of use for the production of milk with altered nutritional, allergenic, or processing properties. The storage and processing properties of milk are, in large measure, determined by the caseins, and these proteins thus represent a potentially important target for genetic manipulation (14, 15).

There is some evidence that suggests that caseins may possess additional functions. A number of peptides derived by proteolytic cleavage of bovine β-casein and known as β-casomorphins have been shown to possess potent opioid activity and have been suggested to be natural agonists for opioid receptors in the gut (16). In addition to expression in the mammary glands, casein expression has been detected at the RNA level in mouse cytotoxic T-lymphocyte cell lines and in thymus (17). The authors suggested that casein micelles may serve to sequester perforin in cytotoxic T lymphocytes in conditions that prevent its polymerization. The sequestration of perforin may protect the cytotoxic T lymphocytes against perforin-mediated lysis, and casein micelles may function to deliver perforin to the target cells.

To investigate the function of β-casein in milk and to determine whether this protein possesses any other essential function, we generated mice deficient for β-casein by gene targeting in embryonic stem cells. The effects of the mutation on gene expression, milk protein content, and casein micelle structure have been characterized.

MATERIALS AND METHODS

Gene Targeting. The gene targeting vector (pβMC1neo/TK) includes a 4.7-kb *Sca* I–*Eco*RI fragment of the β-casein gene (18) from a C57BL/6 mouse, with the neomycin-resistance gene from pMC1neo(C) (19) inserted into the *Asp*700I site in exon 2 of the gene in the same orientation. A herpes simplex virus thymidine kinase expression cassette was placed at the 3′ end of the region of homology, for use in positive-negative selection (20). Embryonic day-14 stem cells (21) were electroporated with pβMC1neo/TK at 31.25, 93.75, or 187.5 $\mu g \cdot ml^{-1}$. After 24 hr, the cells were selected with Geneticin (0.3 or 0.5 $mg \cdot ml^{-1}$), and after a further 4 days,

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ganciclovir (2 μM) selection was applied. The enrichment obtained with ganciclovir was 4.4- to 15-fold.

DNA and RNA Analysis. Genomic DNA was prepared from embryonic stem cells and digested by the method of Laird *et al.* (22) and from mouse tails as described (23). RNA was isolated by the method of Chomczynski and Sacchi (24) and redissolved in 100% formamide (25). After electrophoresis, DNA or RNA was blotted onto Hybond-N (Amersham) and hybridized with probes labeled by random priming (26, 27) using the method of Church and Gilbert (28).

Production of Chimeras. Chimeras were produced essentially as described (29) by microinjection of targeted cells into the blastocoel cavity of 3.5-day C57BL/6 × CBA F_2 embryos. Microinjected blastocysts were reimplanted into the uteri of 2.5-day pseudopregnant mice. Chimeras were identified on the basis of the presence of pale patches of fur on an agouti or nonagouti background.

Milk Protein Analysis. Collection of milk and electrophoretic analysis of milk proteins were done essentially as described (2). For quantitative protein analysis, pools were made of equal volumes of milk from 18 wild-type and 18 homozygous mutant mice. Total protein and whey protein concentrations were estimated by the micro-Kjeldahl method as described (30), except that protein concentration was obtained by multiplying the nitrogen value by 6.38 (to take account of the amino acid composition and nonprotein nitrogen content of milk). The factor 6.38 was derived from calibrations with cow milk and may not be entirely accurate for mouse milk. By SDS/PAGE, no casein contamination of the whey fractions was detected. The β-casein concentration was determined by measuring A_{280} of eluates from ion-exchange fast-protein liquid chromatography (31); the extinction coefficient of pure β-casein was found to be 1.4 [1% (wt/vol) solution, 1-cm path length]. Casein micelle sizes were estimated by the dynamic light-scattering method (32, 33).

Analysis of Pup Growth. Homozygous mutant females were mated with wild-type males, and homozygous mutant males were mated with wild-type females. All the pups were thus heterozygous for the β-casein mutation. Entire litters were weighed three times per week (Monday, Wednesday, and Friday) between birth and 11 days of age. Litter weight was found to be linearly dependent upon age over this period; correlation coefficients between weight and age were greater than or equal to 0.96 for all individual litters. The growth rates (slopes) obtained from individual litter weight vs. age regressions were compared across genotypes and litter sizes by using regression analysis. The model included effects for genotype, litter size, and a quadratic effect of litter size with interactions between genotype and litter size terms. Predicted growth rates for each combination of genotype and litter size were then obtained.

RESULTS

A positive-negative selection replacement vector was constructed to target the disruption of the mouse β-casein gene (Fig. 1*A*). The *neo* gene was inserted immediately downstream of the β-casein translation initiation codon, within the secretion signal-coding sequence; translation from this AUG would be predicted to result in expression of a short peptide bearing no resemblance to mature β-casein. This vector was introduced into embryonic day-14 stem cells by electroporation followed by selection with Geneticin and ganciclovir. At the 5′ end of the vector, targeted recombination was predicted to give a 3.8-kb *Sac* I fragment in addition to a 2.7-kb

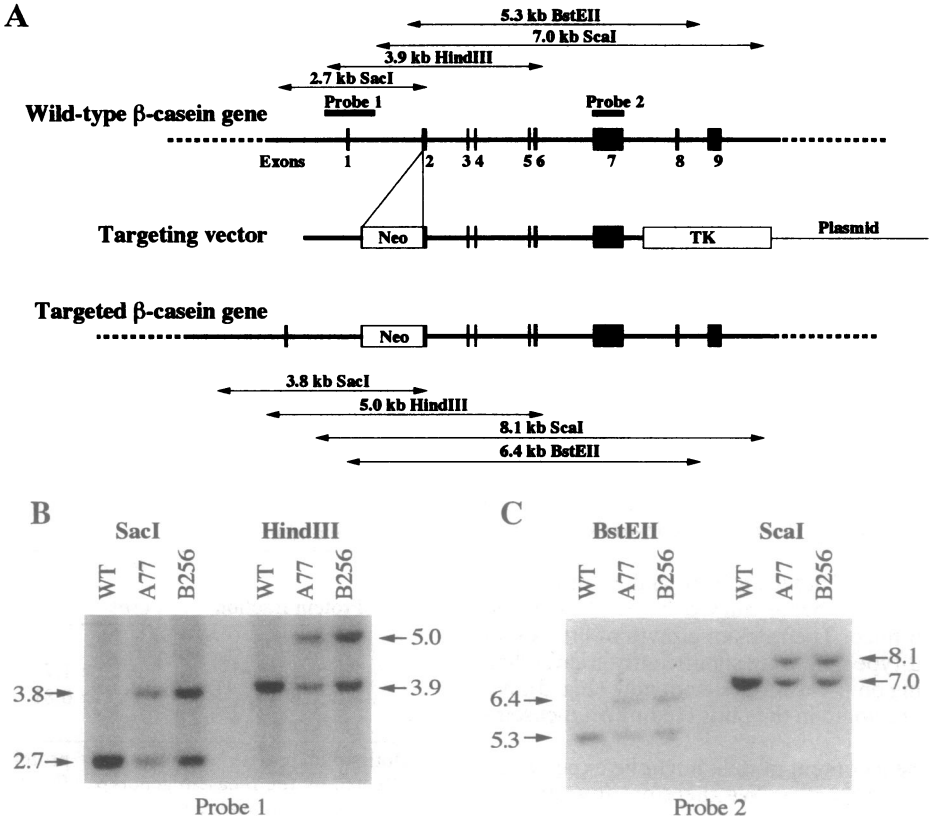

FIG. 1. Targeting of the β-casein gene. (*A*) Structures of the target locus, the targeting vector, and the targeted β-casein gene. Diagnostic restriction fragments and the probes used to detect them are shown. TK, thymidine kinase. (*B*) Southern blot showing the fragments diagnostic of homologous recombination via the 5′ end of the targeting vector. (*C*) Southern blot showing the fragments diagnostic of homologous recombination via the 3′ end of the targeting vector. WT, wild type.

fragment from the wild-type allele, when probed with a 0.9-kb *Hin*dIII–*Sca* I fragment that lies outside the targeting vector and that encompasses exon 1 of the β-casein gene (Fig. 1*A*). DNA from each clone was analyzed by Southern blotting, and the presence of the predicted 3.8-kb *Sac* I fragment indicated that targeted clones were obtained in each of four experiments; 21 targeted clones were obtained from a total of 492 clones analyzed. A number of the clones may have been mixed populations of targeted and wild-type cells or aneuploid because the diagnostic 3.8-kb *Sac* I fragment was of lower intensity than the 2.7-kb fragment. The majority of cells in two clones (A77 and B256) possessed 40 chromosomes, and in these clones the two *Sac* I fragments were of equal intensity. Further analysis of DNA from clones A77 and B256 confirmed the 5′ recombination (Fig. 1*B*) and demonstrated that the 3′ end of the vector had also recombined in the predicted manner (Fig. 1*C*). Chimeras were generated with both of these targeted clones and were mated with MF1 mice. Germ-line transmission of the embryonic stem cell-derived component was obtained from nine chimeric males and one chimeric female, all derived from clone B256. Heterozygous mutant mice were identified by Southern blotting of tail DNA and were interbred. The number of progeny from the intercrosses of $csnb^+/csnb^-$ mice were as follows: $csnb^+/csnb^+$, 57; $csnb^+/csnb^-$, 152; and $csnb^-/csnb^-$, 70; the genotypes of these offspring were determined by Southern blot analysis of DNA from tail biopsies taken at ≈5 weeks of age. Segregation did not deviate significantly from a 1:2:1 ratio (Pearson's goodness of fit test statistic = 3.45 with 2 df, $P > 0.1$).

Female mice of the three genotypes were mated for analysis of mammary gene expression and of milk composition. Northern blot analysis was performed on RNA isolated from mammary glands at midlactation (11 days postparturition). Heterozygous mice consistently expressed β-casein mRNA at a lower level than wild-type mice, and in homozygous mutant mouse mammary glands there was no detectable β-casein mRNA (Fig. 2*A*). Hybridization of Northern blots with a neo probe, to detect transcripts from the targeted allele, gave some diffuse signal but no discrete bands (data not shown); control experiments eliminate the possibility that this signal was due to degradation of the RNA or to contamination of the RNA with genomic DNA (data not shown).

Milk was collected from 11-day lactating mice and analyzed by SDS/PAGE: β-casein was found to be absent from the milk of homozygous mutant mice and to be expressed at a lower level by heterozygotes than by wild-type animals (Fig. 2*B*). No other differences were evident from this analysis. To investigate the effects of the mutation on the total protein content of milk, micro-Kjeldahl analysis was performed on milk from wild-type and homozygous mutant mice. Milk from β-casein-deficient mice contained significantly less protein than milk from wild-type mice (Table 1). Similar measurements on whey revealed an increase in the whey protein content of milk from β-casein-deficient mice (Table 1).

We followed the growth between birth and 11 days after birth, of litters that were being nursed by wild-type and homozygous mutant mice. The rates of growth of litters that were feeding on wild-type milk were significantly greater than those of litters feeding on β-casein-deficient milk (Fig. 3). No other differences were noted in the pups feeding on β-casein-deficient milk.

Perturbing the casein content of milk might be expected to influence its physical properties. We therefore investigated the effects of the mutation on one such property: the sizes of casein micelles. The casein micelles in the milk of homozygous mutant mice were significantly smaller than those in wild-type mouse milk, and micelles from heterozygous mice were intermediate in size (Table 2).

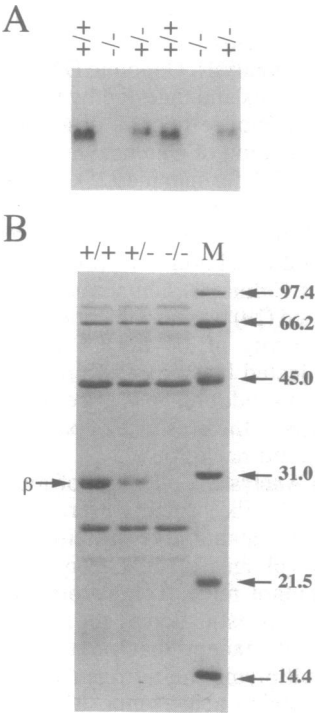

FIG. 2. RNA and protein expression. (*A*) Northern blot analysis of mammary gland RNA from wild-type (+/+), heterozygous (+/−), and homozygous (−/−) β-casein-deficient mice, hybridized with a β-casein cDNA probe (exons 7–9). (*B*) SDS/PAGE analysis of milk proteins. Milk from wild-type (+/+), heterozygous (+/−), and homozygous (−/−) β-casein-deficient mice was diluted, and fat was removed. The equivalent of 75 nl of milk was loaded in each lane and was electrophoresed together with molecular weight markers (M); the gel was stained with Coomassie blue.

DISCUSSION

We have shown that β-casein is not required for viability or fertility of either homozygous mutant mice or of mice feeding on β-casein-deficient milk. Because casein micelles are assembled in the mammary glands of β-casein mutant mice, it is likely that any assembly of micelles elsewhere would not be affected. For this reason, the analysis undertaken did not address the proposed (17) function of casein micelles in cytotoxic T lymphocytes. No increase in disease susceptibility of the mutant mice was evident. The apparent reduction in growth of pups sucking milk of $csnb^-/csnb^-$ mice may be a consequence of the reduced protein content of the milk, although we cannot exclude other possibilities—such as alterations in calcium delivery to the pups or on the digest-

Table 1. Protein content of milk and of milk fractions of wild-type and mutant mice

Protein fraction	Protein content, mg·ml^{-1} ± SEM	
	$csnb^+/csnb^+$	$csnb^-/csnb^-$
β-casein	21	0
Total protein*†	97.1 ± 1.71	87.3 ± 0.89
Whey protein*‡	18.5 ± 0.81	22.0 ± 0.54
Total caseins§	78.6	65.3

*Both total protein and the whey protein content of milk were affected by the β-casein genotype ($P < 0.001$; Student's t test).
†SEMs were calculated from the variation among six mice of each genotype.
‡SEMs were calculated by assuming the same coefficient of variation for whey protein as for total protein.
§This value was calculated from the values for total protein and whey protein by subtraction.

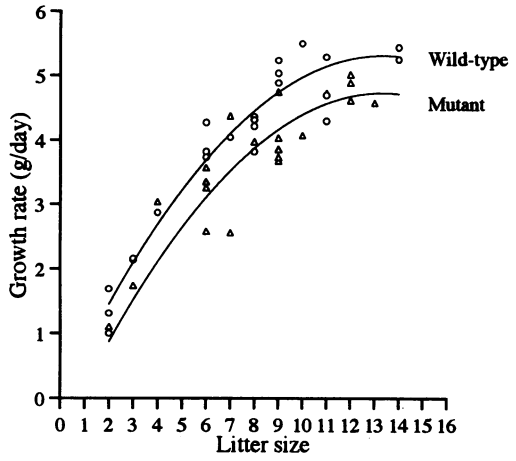

FIG. 3. The effect of maternal genotype on litter growth rate. The graph shows growth rates from birth to 11 days of age for litters feeding on wild-type (○) and homozygous β-casein mutant (△) mouse milk. All pups were heterozygous for the β-casein mutation. The curves have common linear and quadratic coefficients, which are 0.80 (±0.06) and −0.030 (±0.004), respectively. The intercept for wild type is −0.032 and for homozygous mutants is −0.61 (The SE of the difference is 0.12, the intercepts are significantly different; $P < 0.01$, Student's t test with 40 df). The litter sizes were 7.4 ± 0.8 ($n = 23$) and 8.3 ± 0.6 ($n = 21$) for wild-type and homozygous groups, respectively.

ibility of milk, or nonnutritional effects, such as loss of casomorphin activity.

During lactation, milk protein mRNAs are extremely abundant in the mammary gland. In the rat at mid-lactation, β-casein mRNA constitutes ≈20% of poly(A)$^+$ mRNA (34); both transcription of milk protein genes and stability of milk protein mRNAs are regulated (35). The absence of abundant transcripts from the mutated β-casein gene could be due to an effect on transcription, stability, or both. From the data, we cannot distinguish between these possibilities, although the detection of neo transcripts demonstrates that the mutant gene is transcribed. Antisense transcription of the endogenous mouse β-casein gene has been detected in cultured mammary cells (18), raising the possibility that the neo RNA detected may not derive from transcription originating at the β-casein promoter. The dosage effect seen in the heterozygous mutant mice shows that the absolute level of β-casein mRNA is not regulated, suggesting that the regulation of milk protein gene expression is more general than specific.

We previously found that expression of sheep β-lactoglobulin in transgenic mice did not result in any increase in total milk protein concentration, despite the fact that β-lactoglobulin was estimated to constitute 29% of milk protein (36). Thus, in the mouse, there is a physiological limitation on the rate of milk protein synthesis and/or secretion that, under the conditions of the experiment, could not be overcome by augmenting milk protein gene expression. In homozygous β-casein-deficient mice, the absence of β-casein mRNA is accompanied by an overall decrease in the total protein content of the milk, suggesting that mRNA availability is now limiting the secretion of milk protein. Together, these data suggest that the mRNA levels are controlled such that there is normally little or no mRNA in excess of the glands' capacity to use it. Although β-casein constitutes >20% of the protein in the milk of wild-type mice, the overall reduction in total milk protein in homozygous mutant mice was only 10%. The absence of β-casein expression is thus compensated for by increased secretion of other milk proteins. Consistent with this is the finding that the whey protein concentration was higher in the milk of mutant mice than in wild-type mice. There are no major changes in the relative concentrations of proteins other than β-casein (Fig. 2*B*), showing that the compensation does not occur by a disproportionate increase in the concentration of one component but is shared among various proteins. This result suggests that the compensation that occurs may not result from a specific regulatory mechanism but may simply reflect the metabolic consequences of mRNA depletion. This result is consistent with the effect of β-casein gene dosage.

Table 2. Reduction of casein micelle sizes in β-casein mutant mice

Micelle diameters, nm ± SEM		
csnb$^+$/*csnb*$^+$ (*n*)	*csnb*$^+$/*csnb*$^-$ (*n*)	*csnb*$^-$/*csnb*$^-$ (*n*)
280.5 ± 3.7 (12)	268.6 ± 3.6 (11)	255.8 ± 2.3 (14)

Diameters of casein micelles in the milk of mice of the three genotypes differ significantly from each other ($P < 0.01$, one-way ANOVA and Student's t test).

For the dairy industry, milk with increased protein content would be highly desirable. If the physiology of milk protein secretion in livestock is similar to that of mice, it may be difficult to obtain milk with an increased protein content by genetic manipulation. There is some evidence that in cattle the capacity for protein secretion is limited: the A allele of β-lactoglobulin is associated with a significantly increased level of β-lactoglobulin but at the expense of α-lactalbumin and casein (37). Alteration of the biochemical and biophysical properties of milk will be more easily accomplished, although ideally such improvements should not adversely affect yield and gross composition. If in other species, compensation of protein secretion occurs similar to that observed in the mutant mice, it is possible that reduced expression of some milk protein genes will not result in reduction of total milk protein concentration.

The finding that β-casein-deficient milk contains casein micelles demonstrates that although β-casein is normally a major component of the micelle, this protein is entirely dispensible for micelle assembly and secretion. This is consistent with the extremely high degree of sequence divergence among β-caseins of different species. However, β- and κ-caseins are the only caseins that have been found in the milk of all species analyzed (38), suggesting some specific role for β-casein. Although micelle stability was not investigated directly, any major destabilizing effects would have been obvious because the caseins would precipitate. There was no evidence of such instability of micelles in β-casein-deficient milk, demonstrating that β-casein plays no major role in stabilizing micelles at temperatures of 4°–37°C.

The mean diameter of β-casein-deficient casein micelles was found to be smaller than that of micelles from wild-type mouse milk; the micelles of heterozygotes are intermediate in size. The magnitude of the reduction in micelle diameter is consistent with that expected if the volumes contributed to the micelles by the caseins are in proportion to their respective concentrations. This result suggests that the number of micelles is not appreciably affected by the absence of β-casein; again, this argues against any specific role of β-casein in micelle assembly. In cows' milk, small micelles have a higher ratio of κ-casein to β-casein than large micelles (30, 39), consistent with κ-casein being located on the surface. The effects of the β-casein mutation on casein micelle size are thus probably due to a change in the ratio of κ-casein to calcium-sensitive caseins. In bovine milk, small micelles have been found to possess greater heat stability than large micelles (40). Whether the casein micelles from β-casein-deficient mice have increased heat stability is not yet known.

The insensitivity of casein micelle assembly to major changes in composition augurs well for the production of milk with altered properties by genetic manipulation of livestock, although it remains possible that expression of modified

caseins [for example with increased phosphorylation (15)] may interfere with micelle assembly or may destabilize casein micelles. Given the time scale, difficulty, and expense of genetic manipulation of livestock, before undertaking such manipulations it may be prudent to model them in mice. To this end, it would be desirable to generate mice in which the mouse casein genes are replaced by their bovine counterparts. Finally, targeting of transgenes designed for expression of pharmaceutical proteins in milk into the casein locus may enhance the levels of expression by providing a positive position effect (41).

We thank Prof. Jeffrey Rosen for his kind gift of β-casein clones and for communicating unpublished information. We thank Ray Ansell, Lorraine Dobbie, Frances Thompson, Audrey Peter, John Verth and his staff, and Roberta Wallace for excellent technical assistance; Kenneth Dobie for help and advice; and John Clark for support and discussions. This work was supported, in part, by Grant TAP2B from the Agricultural and Food Research Council. S.K. was in receipt of an Indian National Scholarship and an Overseas Research Student Award from the Committee of Vice Chancellors and Principals.

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