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Prenatal Monitoring in a Family at High Risk for Ornithine Transcarbamylase (OTC) Deficiency: A New Mutation of an A-to-C Transversion in Position +4 of Intron 1 of the OTC Gene That Is Likely to Abolish Enzyme Activity

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DNA analysis of a male propositus with ornithine transcarbamylase (OTC) deficiency documented an A-to-C substitution in position +4 of intron 1. No other abnormalities were observed in the OTC gene, or at 563 bp upstream of the 5′ site, which included a promoter region, or at 383 bp downstream of the termination codon, which included a polyadenylation signal sequence. This mutation produces an *Rsa*I site in the sequence, which was used for prenatal monitoring in the fourth and fifth pregnancies. DNA from amniotic cells in the former case were positive for *Rsa*I digestion and the *SRY* gene (sex determinant region Y), indicating hemizygosity for the mutant allele. OTC activity was not measurable, and mRNA of the OTC gene was not detected by Northern blotting in the affected fetal liver. RT-PCR (reverse transcription-PCR) demonstrated only the wild-type allele. Thus, the mutation interferes with RNA processing, and an extremely low amount of normally spliced mRNA for the OTC gene seems to have caused the disease in our patient. The fetus of the fifth pregnancy was a normal male, as confirmed postnatally. © 1996 Wiley-Liss, Inc.

KEY WORDS: ornithine transcarbamylase (OTC), urea cycle, prenatal diagnosis, base substitution

INTRODUCTION

Ornithine transcarbamylase (OTC) (EC2.1.3.3.) deficiency is X-linked, and is the most frequently recorded congenital urea synthesis disorder [Brusilow and Horwich, 1995; Nagata et al., 1991]. Male patients with early onset of the disease usually die in infancy of hyperammonemic coma [Matsuda et al., 1991]. The human cDNA was cloned by Horwich et al. [1984] and the genome structure of the gene was clarified by Hata et al. [1988]. A wide variety of mutant genes include gene deletion, point mutations [Tuchman, 1993; Matsuura et al., 1994], and aberrant splicing with point mutations in introns and exons [Carstens et al., 1991; Matsuura et al., 1995]. Since our ability to treat the disease is limited, prenatal diagnosis plays an important role in genetic counseling in families with OTC deficiency [Old et al., 1985; Matsuda et al., 1989; Spence et al., 1989; Matsuura et al., 1993a]. We report here on a new mutation of an A-to-C transversion in position +4 of intron 1 of the OTC gene, and on prenatal monitoring in a family at high risk for OTC deficiency.

MATERIALS AND METHODS

Family History

In the male propositus, a hyperammonemic attack occurred on the third day of life and he died 5 days later. Enzyme activity and gene analysis were made on the autopsied liver. The maternal grandmother, mother, and sister were carriers of OTC deficiency as determined by a protein-loading test. Seven other males in the family had died neonatally with similar symptoms (Fig. 1A). The fourth and fifth pregnancies were monitored by DNA analysis of amniotic fluid cells obtained in week 14 of gestation, and of chorionic villi obtained in week 10 of gestation, respectively. During the fourth pregnancy the fetus was diagnosed as being hemizygous for the mutation, and a liver specimen was obtained in week 20 of gestation following artificial

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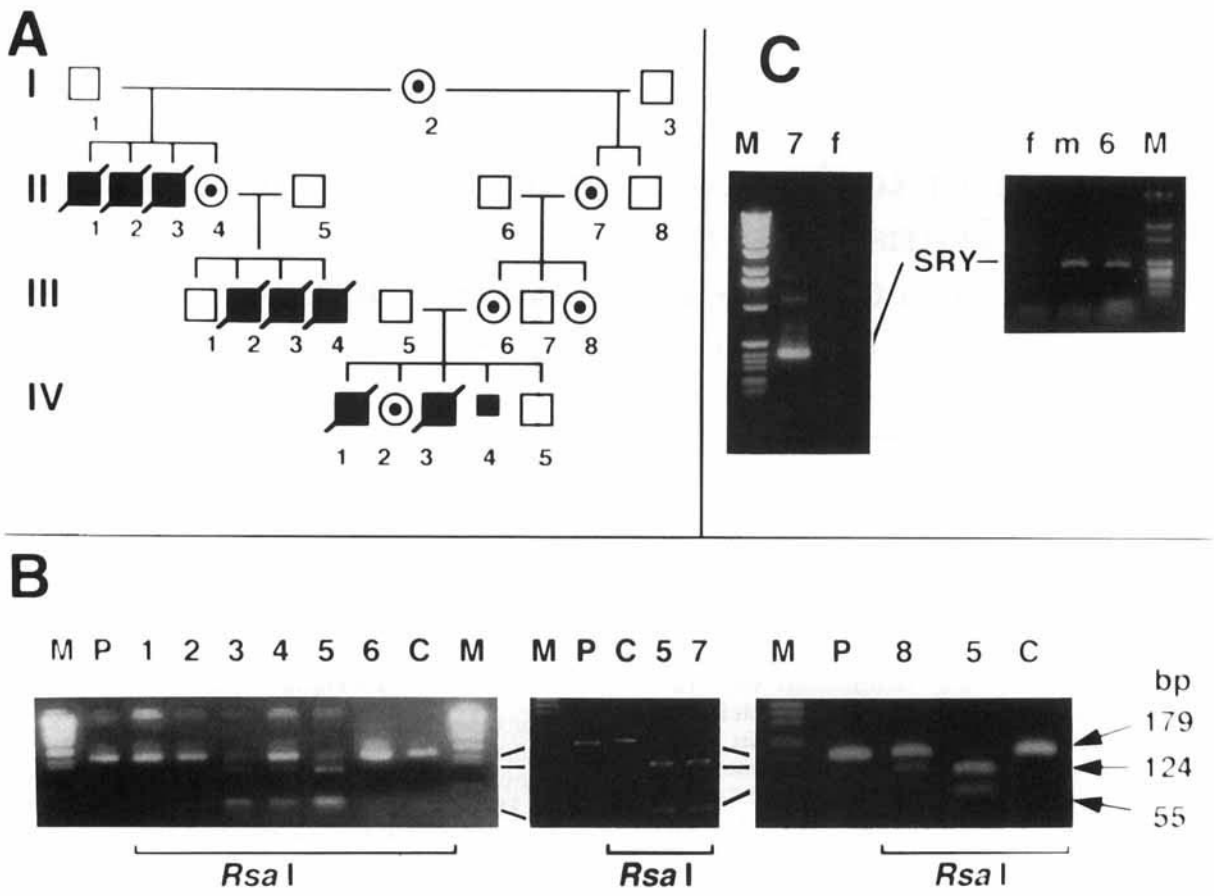

Fig. 1. Pedigree analysis and prenatal monitoring. **A:** Pedigree of a Japanese family with OTC deficiency. **B:** Gene tracking in the family. OTC gene fragments spanning exon 1 were amplified using PCR and digested with *Rsa*I. The 179-bp fragment was cleaved into 124-bp and 55-bp fragments in the mutant allele. The DNA fragments were electrophoresed in 4% NuSieve gel. **Lane M,** marker; **lane P,** undigested fragment; **lane C,** control; **lane 1,** III-5; **lane 2,** III-6; **lane 3,** IV-1; **lane 4,** IV-2; **lane 5,** IV-3; **lane 6,** IV-5; **lane 7,** IV-4; **lane 8,** III-8. **C:** SRY gene detection in the fetus. m, male; f, female.

abortion. As a control, a liver specimen of a similar gestational age was obtained from an aborted fetus with a chromosomal abnormality.

Analysis of OTC Gene by Polymerase Chain Reaction (PCR)

Genomic DNA was isolated from liver specimens, amniotic fluid cells, chorionic villi, or whole blood samples, as described previously [Hata et al., 1988]. Exons 1–10 and adjacent introns were amplified using PCR, for which 10 paired oligonucleotides were synthesized [Matsuura et al., 1993b, 1994]. In addition, 563 bp upstream of the 5′ site, which included a promoter region, and 383 bp downstream of the termination codon, which included two potential polyadenylation signal sequences [Hata et al., 1988], were also amplified separately. For these procedures, two pairs (p-1, 5′-GAGCCCCAGGACTGAGATATTTTTA-3′; p-2-E, 5′-TATGCCAAGCTCCACCGCTGTGTAT-3′; p-1-B, 5′-TGAGGAGGCCAGGCAATAAAAGAGT-3′; p-2-B, 5′-GTTTAACAGGATCCTCAGA-TTAAAC-3′) and one pair (3′-1, TACTCACCTCAGCTCCAGAAG-3′; 3′-2, TTACCAGTAGCTGACTGCCTG-3′) of oligonucleotides covering each region were synthesized, respectively [Hata et al., 1988]. Conditions used for amplification were as follows: 1-min denaturation at 94°C; 1-min annealing at 37°C or 55°C; and 1-min polymerization at 72°C for 35 cycles, using a reaction buffer of 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% (w/v) gelatin, 0.16 μg dNTPs, 10 pmol of each primer, 0.1 μg DNA, and 0.2 U Taq DNA polymerase (Boehringer Mannheim, Tokyo, Japan) in a reaction volume of 20 μl. Amplified DNA was subcloned, and nucleotide sequences were determined using a T7 sequence kit (United States Biochemical Corporation, Cleveland, OH).

Northern Blot and cDNA Analysis

Total RNA was isolated from fetal liver, using the guanidium thiocyanate/cesium chloride centrifugation method. Ten micrograms of total RNA were denatured with 3% formaldehyde, separated by electrophoresis using a 1.0% agarose gel, and transferred to a nitrocellulose membrane. The filter was hybridized with a ^{32}P-labeled full-length human OTC cDNA probe and a hu-

man arginase cDNA probe [Haraguchi et al., 1991]. Prehybridization, hybridization, and washing were done as described elsewhere [Hoshide et al., 1993]. Autoradiography was performed using an intensifying screen for 24 hr at −70°C.

First-strand cDNA was generated from 10 μ total RNA, using Moloney murine leukemia virus reverse transcriptase (Bethesda Research Laboratories, Gaithersburg, MD) with specific antisense oligonucleotide primers. PCR amplification of cDNA was performed using different methods (Fig. 2), as described elsewhere [Matsuura et al., 1995]. Amplified cDNA was subcloned and sequenced, as described above.

Detection of SRY (Sex Determinant Region Y) Gene

Oligonucleotide primers for SRY were synthesized for PCR amplification, and PCR was performed as described elsewhere [Tanoue et al., 1992].

Enzyme Measurement

OTC and carbamylphosphate synthetase I in liver specimens were measured by the method of Brown and Cohen [1959].

RESULTS

Gene analysis of the propositus revealed an A-to-C substitution in position +4 of intron 1 of the OTC gene (Fig. 3). Sequences covering 563 bp upstream of the 5′ site, which included a promoter region, other exons, and exon/intron junctions, and 383 bp downstream of the termination codon, which included two potential polyadenylation signal sequences (AATAAA), were intact (data not shown). The mutation led to a restriction endonuclease *Rsa*I site in the sequence. Therefore, DNA amplification was done so as to cover the mutation sequence in intron 1, using 1-1-B and 1-2 oligonucleotide primers (Fig. 1B), followed by *Rsa*I digestion, for gene tracking. Amplified DNA of the mother, maternal grandmother, and sister revealed three fragments, undigested (179 bp) and digested (55 bp and 124 bp), indicating that they were heterozygous for the mutant allele. In a dead brother and the fetus of the fourth pregnancy, as in the propositus, the amplified DNA was cleaved into 55 bp and 124 bp fragments by *Rsa*I digestion. The sample from the fetus in the fifth pregnancy was undigested. The SRY gene was positive in both fetuses (Fig. 1C). Thus, the fourth fetus was hemizygous for the mutant gene, and this diagnosis was confirmed by studying liver specimens after abortion of the fetus. The fifth fetus was a normal male, as confirmed postnatally.

Hepatic OTC activity was not detected in the affected fetus, and was 72.4 μmol/mg protein/hr in the age-matched control, while hepatic carbamyl phosphate synthetase I (CPSI) activity was similar in the affected and control fetuses (11.7 vs. 14.4 μmol/mg protein/hr, respectively). In Northern blot analysis of the OTC mRNA, no signal was detected in the affected fetus and a single band was visible in the control fetus, although the amount of arginase mRNA was similar in both samples (Fig. 4). RT-PCR products of OTC mRNA covering exons 1–2 and exons 1–3 were seen in the affected fetus, each with a single band (Fig. 5). All the subcloned samples showed only normal splicing products. To detect possible aberrant splicing products containing a part of the intron sequence, RT-PCR was repeated using primers covering the 5′ site of exon 1 to position +32 of intron 1, and from position −32 of intron 1 to exon 3; no significant product was obtained with either procedure (data not shown).

DISCUSSION

A novel mutation of an A-to-C transversion at position +4 of intron 1 of the OTC gene was found in the propositus. The maternal grandmother, mother, and sister were suspected to be carriers of OTC deficiency, as deduced from protein-loading tests. In addition, 7 other male members of the family were likely to have been affected, based on the course of their disease.

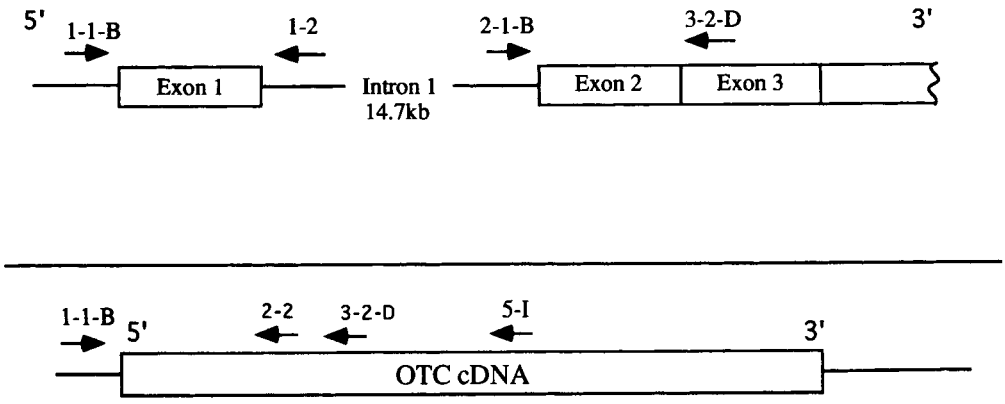

Fig. 2. RT-PCR for human liver OTC cDNA. Arrows indicate orientation and region of primers used for first-strand cDNA and PCR amplification. Sequences of primers are as follows: 5-I, 5′-ATGGTACAAATCTGACA-3′; 3-2-D, 5′-ACTTCCCTTGCAATAAAGGCAAATA-3′; 2-2, 5′-CAGATCTGCTGATAGCCAT-3′; 2-1-B, 5′-CACCATAGTACATGGTGTTTTCTGA-3′; 1-1-B, 5′-GAGTTTTCAAGGGCATAGAATCGTC-3′; 1-2, 5′-AGTTTTATGCATCACCATGATTCCT-3′.

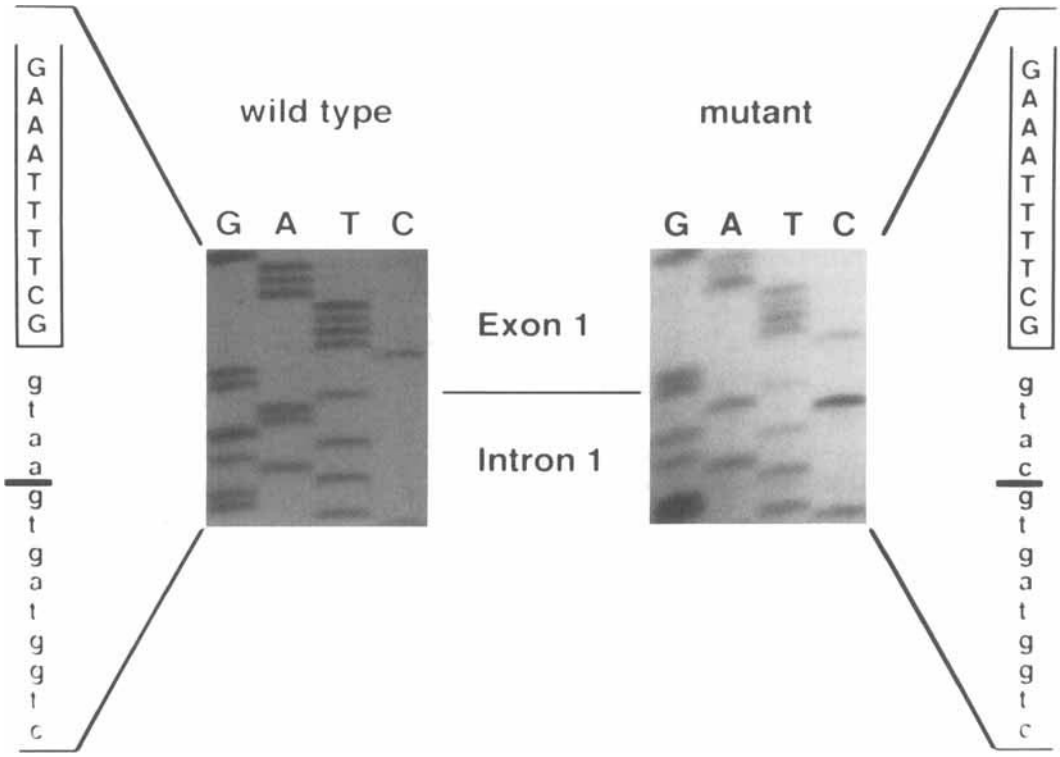

Fig. 3. Partial nucleotide sequences of wild-type and mutant genomic DNA. Each autoradiogram shows the genomic sequence encompassing the mutation of the exon 1/intron 1 junction in the OTC gene. Mutation is underlined.

Thus, this family is at high risk for OTC deficiency. The mutation resulted in an *Rsa*I digestion site in the sequence. Therefore, PCR-*Rsa*I digestion was used for gene tracking, as well as for prenatal monitoring. As shown in Figure 1, a dead brother and the fetus from the fourth pregnancy were hemizygous for the mutant allele, and the maternal grandmother, mother, and sister were heterozygous for the mutant allele. Since the liver sample of the propositus had been kept frozen for a considerable period, only a freshly obtained liver from an aborted fetus was studied. Urea cycle enzymes in fetuses are known to be less active than those in normal children, due to delayed development [Oyanagi et al., 1980]. Accordingly, a liver specimen from an age-matched fetus was used as control. Hepatic OTC activity was absent, and the mRNA in the affected fetus was undetectable by Northern blot analysis, whereas both were clearly detected in the control. An RT-PCR procedure, which is highly sensitive for mRNA detection, revealed a single visible band in the affected fetal liver specimen. The sequence of the subcloned samples showed only normally spliced mRNA.

Substitutions of G-to-T, G-to-C, and G-to-A at position +5 of intron 1, and a substitution of T-to-C at position +6 of intron 1 of human β-globin genes, have all been found in case of β-thalassemia [Treisman et al., 1983; Lapoumeroulie et al., 1986; Atweh et al., 1987]. In a patient with a G-to-T substitution at position +5 of intron 1, Atweh et al. [1987] observed a decreased use of the usual intron 1 donor splice site, as in our patient, and activation of at least three surrounding cryptic splice sites in the β-globin gene. They demonstrated that after transfer of the mutant gene (7.5 kb) to Hela cells and expression in these cells, the RNA products showed partial inactivation of the normal donor splice site of intron 1, and activation of two major cryptic splice sites. In other G-to-C and G-to-A substitutions at position +5, abnormal mRNA processing was shown to be responsible for a reduced level of β-globin mRNA [Treisman et al., 1983; Lapoumeroulie et al., 1986]. In a case of glutaryl CoA dehydrogenase deficiency, Greenberg et al. [1995] found that a G-to-T transversion at position +5 of intron 1 resulted in normally-spliced and 26-bp-deleted mRNAs. In our subject, aberrant splicing with cryptic sites was not evident. Due to the extreme length of intron 1 (14.9 kb in the OTC gene vs. 130 bp in the β-globin gene), an in vitro study using cultured cells, as performed by Atweh et al. [1987], was not feasible.

To search for another explanation for the complete absence of splicing in intron 1, we repeated RT-PCR covering exon 1 to position +33 of intron 1, and from position −33 of intron 1 to exon 3. However, no significant product was seen. Still, when the length of intron 1 of the OTC gene is taken into account, the possibility of aberrant splicing cannot be excluded.

The region upstream of the 5′ site of the OTC gene, including a promoter region, and potential polyadeny-

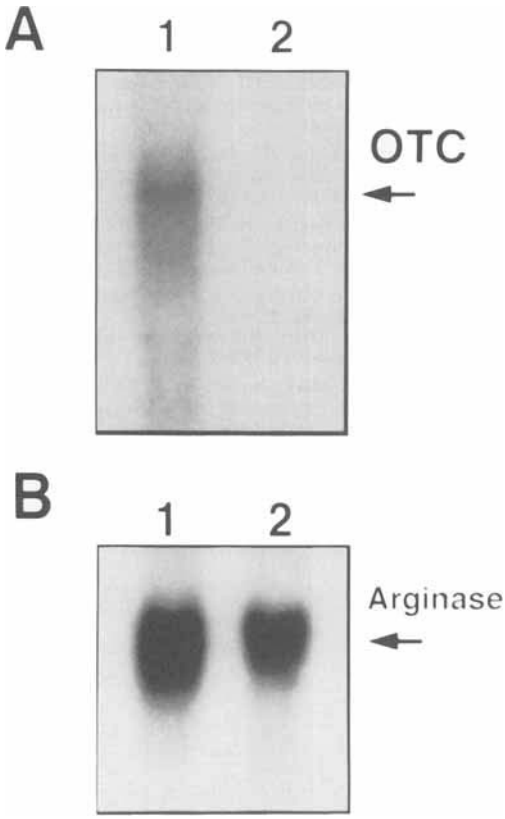

Fig. 4. Northern blot analysis of RNA obtained from a human fetal liver. **Lane 1,** 10 μg of total RNA from control fetal liver. **Lane 2,** 10 μg of total RNA from patient's fetal liver. The blot was probed with labeled human OTC cDNA **(A)** and arginase cDNA **(B).** Arrow indicates size of the human OTC mRNA and arginase mRNA.

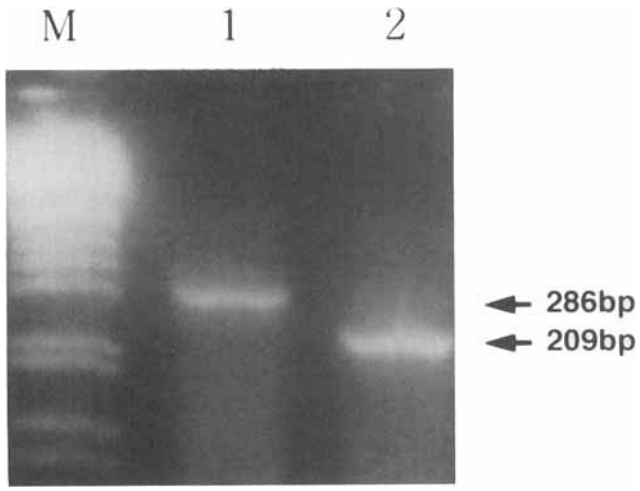

Fig. 5. RT-PCR analysis of the Japanese patient's OTC mRNA. Total RNA (10 μg) extracted from fetal liver was converted to cDNA by reverse transcription and subjected to 35 cycles of PCR. DNA fragments were electrophoresed in 4% NuSieve gel. **Lane 1,** PCR product of OTC mRNA covering the 5′ noncoding region to exon 3 (primers 1-1-B and 3-2-D). **Lane 2,** PCR product of OTC mRNA covering the 5′ noncoding region to exon 2 (primers 1-1-B and 2-2).

lation signal sequences in the affected fetus were analyzed, since mutations of these regions in the β-globin gene (A-to-G substitution in the ATA box) and the α-globin gene (A-to-G substitution in the AATAAA sequence) result in β- and α-thalassemia accompanied by a low amount of normally-spliced mRNA, respectively [Orkin et al., 1983; Higgs et al., 1983]. Again, we observed no abnormalities in these regions. Although the precise mechanism of mRNA processing was not clear in our patient, it is likely that the A-to-C substitution in position +4 of intron 1 of the OTC gene interferes with splicing events and leads to an extremely low amount of normal mRNA, and this may explain the undetectable OTC activity.

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