Linkage analysis of a large inbred family with congenital megaloblastic anemia

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ABSTRACT

Objectives: Megaloblastic anemia during infancy and early childhood often reflects a hereditary disorder of cobalamin's absorption, transport, or intracellular metabolism. There are 3 well defined autosomal recessive syndromes manifesting with megaloblastic anemia due to defects in cobalamin absorption or transport, namely congenital pernicious anemia, Imerslund-Grasbeck syndrome and Transcobalamin II deficiency. The genes responsible for the 3 disorders are gene intrinsic factor (GIF), MGA1 and TCN2, as well as the gene for Transcobalamin I, TCN1 are mapped or cloned, or both.

Methods: We describe the clinical picture of 7 patients from 3 sibships, belong to one large inbred family who presented with megaloblastic anemia during infancy. The mode of inheritance follows an autosomal recessive pattern and the syndrome was completely reversed by parentral vitamin B12 therapy. The ascertainment of the family was carried out in 1998 in the Princess Rhama Children's Hospital, which is affiliated with Jordan University of Science and Technology, Jordan. We performed linkage analysis in this family for genes or regions involved in the above mentioned disorders.

Results: The genes implicated in the etiology of the previously mentioned disorders were excluded from being responsible for the disorder in this family.

Conclusion: The exclusion of the involvement of GIF, MGA1, TCN1 and TCN2 in this family suggests that another gene and its product, involved in cobalamin absorption or transport, remains to be identified. A genome-wide search of the gene implicated in this family may give some insight on that gene, and its function.

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Megaloblastic anemia during infancy and early childhood is quite rare and often reflects a hereditary disorder of cobalamin metabolism. The dietary causes are usually due to folic acid deficiency rather than cobalamin deficiency. All known inborn errors of cobalamin metabolism are autosomal recessive disorders with megaloblastic anemia being a constant and common feature.[1] In addition, each disorder manifests with features specific for the underlying defect.[1] Inborn cobalamin errors usually result from defects in its absorption, transport or its intracellular metabolism.[2] The metabolic pathway of dietary cobalamin starts by its binding to a salivary R-binder protein which is then cleaved off in the upper small intestine by pancreatic enzymes.[1] The released cobalamin is then bound to the gastric intrinsic factor (IF) forming a complex that attaches to a specific surface receptor and cubilin at the mucosa of the distal ileum.[3] Cubilin transports the cobalamin, now released from the IF, across the ileal mucosal cell to the portal circulation.[1] Once it is in the portal compartment, cobalamin is delivered to the cells by transcobalamin I (TCI) and II (TCII), the latter being more efficient and a more rapid delivery agent.[4] The TCN2-cobalamin complex attaches to a receptor that facilitates the entry of cobalamin into the cell by adsorptive endocytosis.[5] Inside the cell, cobalamin is prepared for the execution of its coenzymatic role either by methylation or adenosylation.[1] There are several well-described

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autosomal recessive syndromes that reflect defects in absorption or transport of cobalamin. Congenital pernicious anemia (MIM # 261000) due to absent IF, IF with poor affinity to the intestinal receptor, or IF that is susceptible to an unusual degradation, has been described.[6-10] The gene for IF (GIF) has been cloned using a rat complementary deoxyribonucleic acid (cDNA) probe and localized to chromosome 11 by somatic cell hybrids.[11] This fact, the position of the mouse IF gene (gif) on chromosome 19, and the high degree of homology of synteny between mouse chromosome 19 and human chromosome 11q13, made this band the most likely GIF locus.[12] Congenital megaloblastic anemia with proteinuria (MIM # 261100) has been described independently by Imerslund and Grasbeck et al[13,14] as a syndrome called Imerslund-Grasbeck Syndrome. The gene responsible for this condition (MGA1) was mapped to 10q12.1.[15] Cubilin, the human Cobalamin-IF complex receptor was cloned and mapped to the same chromosomal region.[3] Mutations in the cubilin gene were identified in patients with Imerslund-Grasbeck syndrome providing evidence that mutations in this gene are responsible for the disorder.[16] Transcobalamin II deficiency (MIM # 275350) causes megaloblastic anemia in addition to other symptoms.[17] The gene encoding this protein (TCN2) has been localized by linkage to the P blood group, and by somatic cell hybrids to chromosome 22 then localized by FISH to 22q12-q13.[18-20] The gene encoding for TCI has been localized to the pericentromeric region of human chromosome 11 by in situ hybridization.[21] Both GIF and TCN1 were placed close to each other at 11q13 in further mapping studies of chromosome 11.[22]

We reported here a highly inbred Jordanian family with autosomal recessive megaloblastic anemia, presenting during infancy that was completely reversed by parentral vitamin B12 therapy. Linkage to the 3 loci, which contain the 4 genes (GIF, MGA1, TCN1 and TCN2), was excluded by linkage analysis.

Methods.

Patient evaluation. We identified one large inbred family in which multiple sibs in several sibships had megaloblastic anemia. The family was identified through the clinical genetics and pediatrics hematology services provided by the Princess Rahma Teaching Hospital, Irbid, Jordan. Detailed family history data and 8 generation pedigree were obtained **(Figure 1)**. Family members who agreed to participate in the study had blood drawn after an informed consent was obtained from each individual or their legal guardian. The affected family members are currently being followed up in the pediatrics or hematology clinical service of the hospital where they receive their continued care.

Genotyping and linkage analysis. Deoxyribonucleic acid was extracted from leukocytes in venous blood by standard procedures. Three sets of microsatellite DNA marker known to be on 3 chromosomal regions containing the MGA1 locus (chromosome 10), the IF and TCN1 loci (chromosome 11) and TNC2 locus (chromosome 22) were selected[23] **(Table 1)**. Amplification of these markers was performed with 40 ng (2 ul) of template DNA, in an 8.4 ul polymerase chain reaction (PCR) mixture containing 1.25 ul of PCR buffer (100 mM Tris-HCl, pH 8.8; 500 mM KCl; 15 mM MgCl2; 0.01% w/v gelatin), 200 uM each dATP, dCTP, dGTP and dTTP, 2.5 pmol of each forward and reverse primers and 0.25 U of Taq polymerase (Boehringer). The reaction mixture was subjected to 35 cycles of 94°C for 30 seconds, 55°C for 30 seconds and 72°C for 30 seconds. Products were analyzed on 6% denaturing polyacrylamide gels (8 M urea). The polyacrylamide gels were silver-stained using the protocol of Bassam et al.[24]

Statistical analysis. Logarithm of odds (LOD) score analysis was performed using MLINK of the Linkage 5.1 computer program package.[25] The analysis was carried out assuming autosomal recessive inheritance with full penetrance and no phenocopies. A disease allele frequency of 0.001 was used in the analysis and the allele frequencies for the markers were assumed to be equal, since the true allele frequency for this population is unknown. A LOD score of -2 or below was used as evidence of exclusion of linkage. The Marshfield genetic map was used to obtain the genetic location of the markers used in the analysis.[23] The approximate genetic location of the markers is shown in **Table 1**.

Results.

Clinical picture. Individual VIII.3. This individual, proband, presented at the age of one year, to his local physician, with poor feeding, pallor and diarrhea and was found anemic. He was treated

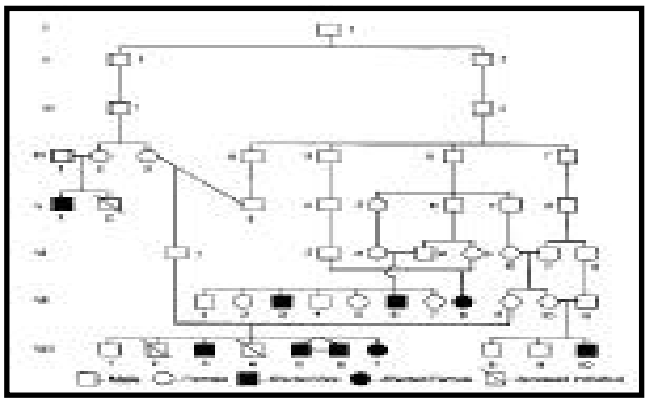

Figure 1 - Pedigree of the family showing several consanguinity loops and showing the proband and other affected individuals. Individuals V.1 and VII.8 were not examined but reported by the family to be affected by the same disorder. Individuals VIII.2 and VIII.4 probably had the same disorder but died before being clearly diagnosed.

with iron without response and his symptoms waxed and waned until he was 3.5-years-old when he was eventually hospitalized and worked up. At that time his growth was delayed, his pallor was severe and the rest of his symptoms, such as poor feeding and diarrhea, still present. His hemoglobin was 7.2 gm/dl, his platelet count was low ($14x10^9$/l) and his serum vitamin B_{12} was low (42 pg/ml). The bone marrow examination showed megaloblastic myelogenesis and erythropoeisis. The serum iron, the total iron binding capacity and the glucose-6-phosphate dehydrogenase (G-6PD) enzyme activity level were normal. The osmotic fragility test was read as normal. The urine analysis at that time was normal with no evidence of proteinuria. The family history showed an unexplained death of one sibling (VIII.2) with anemia at the age of 3-years. He was then treated with parentral vitamin B_{12} and oral folic acid with remarkable response. Now, at the age of 14 years, he is completely normal with the exception of being slightly short. All the blood indices are normal. While he was on treatment, several urinalyses showed proteinuria, although this was not a consistent finding, and the urine pH was around 5. The serum amino-acid profile, carried out on treatment, was normal. Also, the cyanide nitroprusside test carried out on urine was reported as negative.

Individuals VIII.5 and VIII.6. These male twins, definitely identical, showed the same symptoms around similar age as their older brother. They had low vitamin B_{12} levels and were started on parentral vitamin B_{12} therapy. They attained normal growth parameters. The diagnosis was easily achieved due to the family history.

Individual VIII.7. This young female was diagnosed early at the age of one year, when she was checked-up to see whether she had the same condition as her siblings. She was started on parentral vitamin B_{12} therapy with excellent response.

Individual VIII.10. This individual was diagnosed early at the age of one year, after presenting with megaloblastic anemia. He was diagnosed immediately and started on parentral vitamin B_{12} therapy with excellent response.

Individuals VII.3 and VII.6. These 2 siblings, from the same sibship, were diagnosed with the same disorder as their relatives without any extensive laboratory testing. They were started on parentral vitamin B_{12} therapy with good response.

Linkage analysis. Since this family is highly inbred, it is expected that the region where the disease gene lies, the markers will be homozygous. The absence of homozygosity is an indicator for absence of linkage to the disease gene regions

Table 1 - Markers used and their approximate location.

CH	Marker	Name	Location cM
10	D10S2325	GAAT5F06	32.8
10	D10S1440	GTAT11CO3	40.4
10	D10S1423	GATA70E11	46.8
10	D10S611	GATA3G07	55
10	D10S1426	GATA73E11	59.5
10	D10S1225	ATA24F10	80.7
11	D11S1999	GATA23F06	17.1
11	D11S2370	GATA82A06	37.6
11	D11S1392	GATA6B09	43.1
11		GATA152F11	48.3
11	D11S1393	GATA6C04	54.7
11	D11S1385	GATA2A01	57.8
11		ATA9B04	58.4
11	D11S2006	GATA46A12	59.2
11	D11S1975	GAAT1B01	71.6
11	D11S2371	GATA90D07	76.1
11		GATA2D11	83.8
11	D11S1989	ATA10D05	84.3
11		ATA21A09	84.3
11	D11S2002	GATA30G01	85.4
11	D11S2015	GATA63C08	87.8
11	D11S1986	GGAA7G08	105.7
22	D22S685	GATA6F05	32.3
22	D22S683	GATA11B12	36.2
22	D22S445	GGAT3C10	45.8

CH - chromosome, cM - centimorgan

Table 2 - The maximum LOD score and the region excluded by each marker.

CH	Marker	Name	Theta	LOD*	Region*
10	D10S2325	GAAT5F06	0.28	0.1559	5.27
10	D10S1440	GTAT11CO3	0.5	0	7.54
10	D10S1423	GATA70E11	0.5	0	11.16
10	D10S611	GATA3G07	0.19	0.4972	1.01
10	D10S1426	GATA73E11	0.5	0	5.27
10	D10S1225	ATA24F10	0.5	0	11.16
11	D11S1999	GATA23F06	0.5	0	15.06
11	D11S2370	GATA82A06	0.5	0	13.72
11	D11S1392	GATA6B09	0.45	0.0093	6.39
11		GATA152F11	0.47	0.0058	8.72
11	D11S1393	GATA6C04	0.06	1.25	marker
11	D11S1385	GATA2A01	0.5	0	2.04
11		ATA9B04	0.5	0	2.04
11	D11S2006	GATA46A12	0.49	0.003	7.54
11	D11S1975	GAAT1B01	0.5	0	7.54
11	D11S2371	GATA90D07	0.35	0.0904	3.09
11		GATA2D11	0.4	0.0305	5.27
11	D11S1989	ATA10D05	0.5	0	9.92
11		ATA21A09	0.49	0.0001	5.27
11	D11S2002	GATA30G01	0.12	0.6967	marker
11	D11S2015	GATA63C08	0.34	0.0772	6.39
11	D11S1986	GGAA7G08	0.2	0.0418	marker
22	D22S685	GATA6F05	0.5	0	9.92
22	D22S683	GATA11B12	0.49	0.0001	5.27
22	D22S445	GGAT3C10	0.22	0.2229	1.01

* - LOD score and the region excluded by each marker are measured in centimorgan [cM]. LOD - logarithm of odds, CH - chromosome

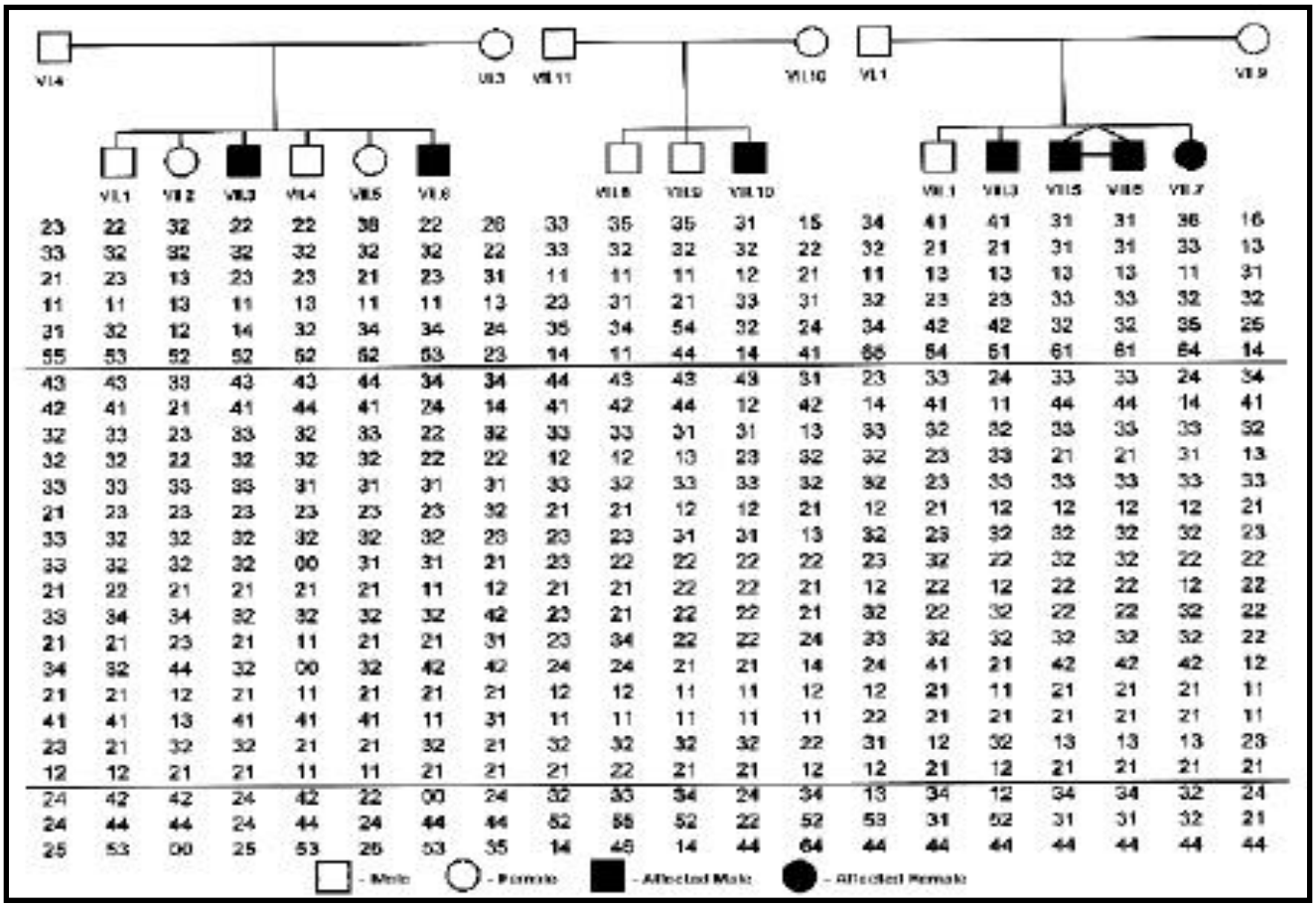

Figure 2 - Partial pedigree showing the 3 sibships of the affected individuals and their parents. The genotypes for all selected markers are shown for each individuals. The markers are arranged similar to Tables 1 & 2, starting with chromosome 10 markers, then chromosome 11 and then chromosome 22 and from the telomere of the short arm towards the telomere of the long arm. The transverse lines separate the haplotypes for each chromosome.

(Figure 2). In addition, linkage analysis produced LOD scores, which were less than -2 across these chromosomal regions, therefore excluding linkage **(Table 2)**. **Table 2** shows the recombination fraction for each marker at which the maximum LOD score was obtained and the region excluded by each marker. **Figure 3** provides a diagrammatic representation of the regions excluded by each marker.

Discussion. The inheritance of the megaloblastic anemia in this family is clearly autosomal recessive. This is supported by the presence of multiple affected individuals in 2 of the sibships, the normal parents, and the relation between the 3 sibships with affected individuals. The extensive consanguineous marriage in this family provides further evidence for the autosomal recessive mode of inheritance. The assumption that the megaloblastic anemia present in affected individuals in this family is related to a cobalamin metabolic defect is based on several facts; the most important is the curative effect of parentral vitamin B_{12} therapy. In addition, the clinical picture with the particular age at presentation and the low serum vitamin B_{12} levels in affected members point to cobalamin metabolic problem. The inconsistent presence of proteinuria in some patients, even on treatment, is suggestive of Imerslund-Grasbeck syndrome, but is not conclusive for the final diagnosis. It was difficult to exclude congenital pernicious anemia based on clinical grounds only. The Schilling test is not performed in Jordan due to hazards of radioactivity. The less severe clinical picture, the absence of other clinical findings, the low vitamin B_{12} levels, and the age at presentation, suggested the exclusion of TCII deficiency. However, the 3 above-mentioned conditions were considered when the search for linkage was performed. The 3 chromosomal regions chosen in this study contain 4 genes were GIF, MGA1, TCN1 and TCN2, which have a confirmed role in the absorption and transport of vitamin B_{12}. Alterations in 3 of these genes, GIF, MGA1 and TCN2, are known to produce syndromes with megaloblastic

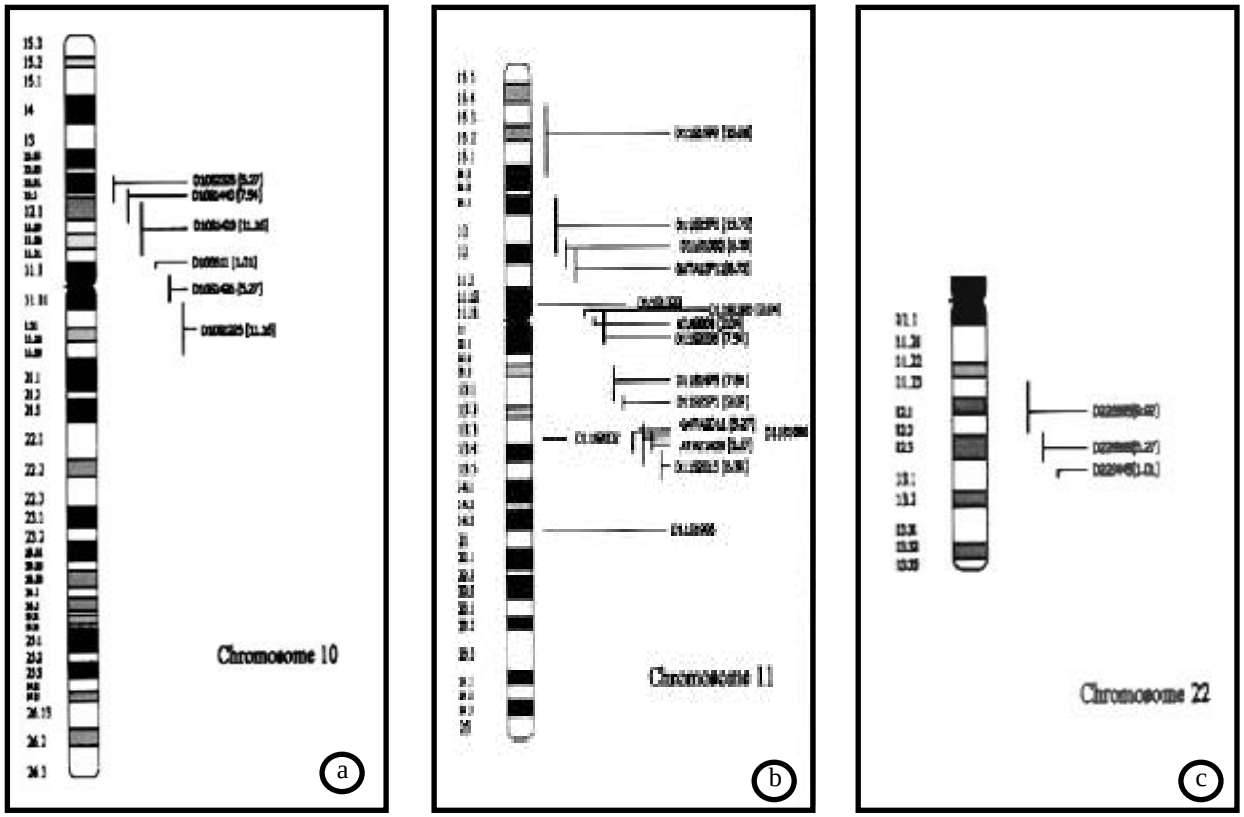

Figure 3 - Diagrammatic representation of the regions excluded by linkage on **a)** chromosome 10 **b)** chromosome 11 and **c)** chromosome 22. Numbers in parenthesis are the distance excludes by each marker in centimorgan. The overlapping coverage ensures comprehensive saturation of the region of linkage for the genes or loci involved in the disorders.

anemia as one of the clinical features.[7-10,16,17] Linkage to the 3 regions in this family is excluded by absence of homozygosity in the affected individuals and confirmed by the significant exclusion LOD scores. The exclusion of linkage in this family to the 3 regions that harbor 4 genes involved in cobalamin metabolism have 2 alternative explanations. First, the disorder in this family is evidence for genetic heterogeneity of the syndromes associated with cobalamin metabolic errors that would reflect a missing step in the absorption or transport pathways, or both, of dietary cobalamin. Second, the information regarding the location of one of these 4 genes is erroneous. The MGA1 and the TCN2 genes have loci that are clearly correct as it is supported by identification of mutations in specific genes within the region of linkage. The localization of the GIF to chromosome 11 has been confirmed, but its localization to the band 13 on the long arm is only suggested by the high homology of synteny between the mouse chromosome 19 where the gif is located, and this specific human band.[12] There is data supporting its locus at 11q13 but the methodology is not very clear.[22] We chose 16 consecutive microsatellite markers, somewhat evenly spaced and covering almost two-thirds of chromosome 11, especially the pericentromeric region and 11q13. Two of the markers (D11S2006 and D11S1975) are reported to be flanking an approximately 10 centimorgan region, which contains both GIF and TCN1.

The exclusion of linkage to the 3 loci prompts a genome-wide search for the locus of the gene responsible for the disorder in this family. This would serve the identification of a gene whose product plays a role in the absorption or transport of cobalamin. The identification of the locus should provide a reasonable tool for genetic testing in this family to be used in carrier identification for premarital counseling and possibly prenatal diagnosis. It may also provide some insight into completing the metabolic pathway of cobalamin.

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