A Null Deficiency Allele of $\alpha_1$-Antitrypsin, QO1ladwigshafen, with Altered Tertiary Structure

G. C. Fraizer, M. A. Siewertsen, M. H. Hofker, M. G. Brubacher, and D. W. Cox
Research Institute, The Hospital for Sick Children, Toronto, and the Departments of Paediatrics and Medical Genetics, University of Toronto, Toronto, Ontario, Canada MSG 1X8

Abstract

The most common deficiency allele of the plasma protease inhibitor $\alpha_1$-antitrypsin ($\alpha$1AT) is $PI^*Z$. Some rare deficiency alleles of $\alpha$1AT produce low but detectable amounts of plasma $\alpha$1AT (1–20% of normal), which can be differentiated by isoelectric focusing. Others, designated null (QO) alleles, produce no $\alpha$1AT detectable by routine quantitative methods. We have previously described a method using DNA polymorphisms, haplotypes, and polyacrylamide isoelectric focusing gels, to differentiate various deficiency alleles. Based on haplotypes, we previously identified, in eight patients, five different null alleles, four of which had been previously sequenced. We have now analyzed all 12 null alleles in these eight patients, using allele-specific oligonucleotide probes, and have identified six different null alleles. We have cloned and sequenced one of these, $PI^*QO1ladwigshafen$, which has a base substitution in exon II, replacing isoleucine 92 in the normal sequence with an asparagine. This substitution of a polar for a nonpolar amino acid occurs in one of the $\alpha$-helices and is predicted to disrupt the tertiary structure. A total of 13 different $\alpha$1AT deficiency alleles, 6 of them null alleles, have been sequenced to date. (J. Clin. Invest. 1990; 86:1878–1884.) Key words: protease inhibitor • $\alpha_1$-antitrypsin deficiency • chromosome 14

Introduction

$\alpha_1$-antitrypsin ($\alpha$1AT), a 52-kD plasma glycoprotein, is the primary inhibitor of the serine protease leukocyte elastase (1). $\alpha$1AT is synthesized primarily in the liver, is secreted into the blood, and diffuses into the interstitial fluids (for reviews, see references 2 and 3). $\alpha$1AT is a highly polymorphic protein with more than 60 electrophoretic variants (4, 5). A deficiency of $\alpha$1AT is associated with emphysema and with childhood liver disease (6, 7). The most common deficiency allele of $\alpha$1AT is $PI^*Z$, occurring at a frequency of 0.012 in caucasians (8). The Z allele is associated with a low plasma concentration, less than 20% of normal (8), and with liver inclusions (9) apparently due to the tendency to aggregate in vitro (10).

Alleles associated with the most pronounced deficiency states, in which no serum $\alpha$1AT is detected by routine methods, are designated as null (QO) alleles. The null alleles are rare, with a combined frequency estimated to be $10^{-4}$ among caucasians (11). With the goal of delineating the spectrum of mutations that lead to $\alpha$1AT deficiency, we examined DNA from eight individuals with null alleles. By haplotype analysis, following the algorithm of Cox and Billingsley (11), we identified a unique null allele, designated QO1ladwigshafen in an individual of German ancestry. The QO1ladwigshafen gene was cloned, and sequenced using asymmetric polymerase chain reaction (PCR) amplification. The limitation of the haplotype approach of screening for unique null alleles is that different null mutations may have occurred on the same haplotype background. Therefore, allele-specific oligonucleotide probes were used to further distinguish between different null alleles.

Methods

Protein Studies. Serum $\alpha$1AT was quantified using electroimmunoassay (5, 12). All sera were examined by isoelectric focusing using narrow range ampholine, Pharmalyte pH 4.2–4.9 (LKB, Sweden), followed by immunoprinting (5, 13). By these assays, in combination with family studies or haplotype analysis, eight individuals were identified previously as having a null allele (11).

Haplotype analysis. These individuals were classified using the algorithm for limited haplotype analysis as described (11). The restriction enzymes used to determine the polymorphic sites forming the haplotypes are listed in Table I in an order that represents the location of the polymorphic sites of the $\alpha$1AT gene and the related PI-like (PIL) gene. Probes used, designated 6.5 (PAT 6.5) and 4.6 (PAT 4.6) (Fig. 1), were provided by S. L. C. Woo. The last five restriction sites listed, not contained within the 6.5 probe, represent polymorphisms in the PIL gene (unpublished data), located 8 to 12 kb downstream of the $\alpha$1AT gene (16, 17). The previous identification of the unique $PI^*Z$ haplotype in 55 of 58 individuals examined (14) allowed the assignment of the complete QO1ladwigshafen (QO1ud) haplotype in the proband, a QO1udZ compound heterozygote. The QO1ladwigshafen allele was initially identified as a unique null allele based on its haplotype.

Gene cloning. High molecular weight DNA suitable for cosmid cloning was extracted from leukocytes from the proband (18). DNA was partially digested with Mbo I, fractionated by sucrose density gradient centrifugation (19) and fractions containing 23 to 50 kb DNA fragments were ligated to the double cos site vector, C2RB (20). Clones were screened (21) with an exon I-specific probe (Fig. 1). Positive clones were digested with Eco RI to determine the integrity of the full-length genes. QO1ladwigshafen clones were distinguished from Z clones by two restriction site differences using either Msp I with probe 4.6 or a double digest using Bst EII and Pst I with probe 6.5. Locations of the two polymorphic sites are indicated (Fig. 1).

Sequencing. Cosmid DNA containing the full-length gene was prepared from one QO1ladwigshafen clone (22). Single-stranded DNA was prepared for each of the coding exons (II through V) by asymmetric PCR amplification as used on genomic DNA (23), except that 15–150 pg of cosmID DNA was amplified. 11 synthetic primers were used and 7 regions were amplified (Fig. 1). Amplification primers were as fol-
Table I. DNA Haplotypes of a QOludwigshafen Z Heterozygote as Defined by 12 Polymorphic Restriction Sites

<table>
<thead>
<tr>
<th>Allele</th>
<th>4.6</th>
<th>6.5</th>
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<tbody>
<tr>
<td>S</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Msp</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>A</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>A 3/2</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>M/B</td>
<td>M</td>
<td>T</td>
</tr>
<tr>
<td>A 5/7</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>T</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Bgl</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>A 1/4</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>M</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>RI</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>T</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

* Polymorphic restriction enzyme sites are as follows: S, Sst I; Msp, Msp I; A, Ava II; M, Mae III; B, Bst E II; T, Taq I; Bgl, Bgl II; RI, Eco RI; +, presence of site; -, absence of site. 

1 Alleles are as described (11, 14, 15). 

2 The QOludwigshafen (QO lud) haplotype was initially deduced from the assignment of the proband's Z haplotype, based upon the previous identification of a unique Z haplotype in 55 of 58 individuals examined (53).

... (remainder of text)
associated haplotype is identical to the most common M2 haplotype for all enzymes tested. The same M2 haplotype is also associated with the Mmalton deficiency allele (25).

Cloning and sequencing. Six cosmid clones were purified; full-length genes were obtained in five as determined by digestion with Eco RI and hybridization with the probes 4.6 and 6.5. The absence of the polymorphic Msp I site in the intron 3' of exon 1 and the presence of the polymorphic Bst EII site in exon III (Fig. 1 A) differentiated the QO ludwigshafen clone from the Z clone.

Sequence was obtained for all five exons, all intron/exon junctions and the hepatocyte transcriptional control region (29) and compared with the genomic sequence of S (30) and cDNA of M2 (31). A single T to A base transition in exon II was the only mutation found (Fig. 2), except for the mutations characteristic of S and M2. The substitution results in an altered amino acid 92. The other differences observed were the absence of the S mutation (valine was found at amino acid 264) and the presence of the asparagine at 376 and histidine at 101 as in M2 (31).

Hybridization of the QO ludwigshafen ASO to polymerase chain reaction amplified DNA. The QO ludwigshafen mutation was confirmed by oligonucleotide hybridization after PCR amplification of both the cloned allele and genomic DNA from the proband (Fig. 3). Since the QO ludwigshafen haplotype is identical to both the haplotype associated with the normal variant M2 and the Mmalton deficiency allele (25), it was important to determine if the mutation was unique to the QO ludwigshafen allele. Genomic DNA from six independent M2 haplotypes, other deficiency alleles having the same M2 haplotype, the proband, and his daughter were amplified along with the QO ludwigshafen cosmid control and a subclone containing a normal exon II. The QO ludwigshafen ASO hybridized to amplified genomic DNA from the proband and his daughter, confirming the inheritance of this null allele. Conversely, the QO ludwigshafen ASO did not hybridize to the normal control, normal M2, Mmalton deficiency, or QO mat-tawa alleles.

![Figure 3. QO ludwigshafen allele ASO hybridization to amplified genomic DNA. (A) Arrows indicate the location of the 5' and 3' PCR amplification primers which flank the substitution. Sequences of the normal and QO ludwigshafen ASO probes are listed (B) The QO ludwigshafen ASO or its normal counterpart were hybridized to DNA amplified by PCR. Genotypes tested are indicated above each lane: M1, M2, M3, Z, QO bellingham (QObel), QO cedarland (QOced), QO bolton (QObol), QO ludwigshafen (QO lud), and Mmalton (Mmal). Plasmid clones containing exon II from either the QO ludwigshafen or the normal allele were amplified for homozygous controls.](image)

Identification of QO ludwigshafen with the restriction enzyme Xho II. The T → A transition of QO ludwigshafen results in the loss of an Xho II site. The PCR amplification product of exon II was 254 bp in length. DNA without the QO ludwigshafen allele was predicted to produce products of 216 and 38 bp, whereas DNA with the QO ludwigshafen allele would remain intact at 254 bp. The controls used were an Mmalton cosmid clone determined to be normal in this region by sequencing (25) and the QO ludwigshafen cosmid clone known to contain the mutation by sequence analysis. Amplified DNA from the proband and his daughter both showed, on agarose electrophoresis, fragments of 254 and 216 bp. This is consistent with their heterozygous state at the a1AT locus, QO ludZ, and QO lud M1, respectively (Fig. 4).

Examination of other null alleles. In our eight patients with QO alleles we had identified five different haplotypes associated with a QO allele. Of the 12 QO alleles, seven had the M1 val213 haplotype associated with the QO bellingham mutation (11), so we determined if all seven carried the QO bellingham allele (data summarized in Table II). This was done by hybridization with the QO bellingham ASO after PCR amplification of genomic DNA (Fig. 5). Six of the seven alleles were QO bellingham DNA samples from five normal individuals having haplotypes identical to that of QO bellingham, and were amplified along with control clones containing exon III from either the QO bellingham or the QO ludwigshafen allele, the latter being normal in this region. The QO bellingham ASO did not hybridize to any of the normal alleles, including those with an M1 ala213 haplotype or with M1 val213 (the ancestral allele for M2), or to any of the deficiency alleles with haplotypes different from M1 val213. Based on haplotype analysis, we had previously suggested that the second QO allele found in

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**Figure 2.** Sequence of the region around the QO ludwigshafen mutation in exon II, obtained by the dye deoxyribonucleotide chain termination method. Codon numbers and corresponding amino acids in the mature protein are indicated. The sequence of the QO ludwigshafen allele is identical to that for M2 except for the T → A alteration (depicted in bold), resulting in the substitution of asn for ile 92.
the proband in association with $QOhongkong$ (32) might be a $QObellingham$ allele (11), but this is not the case, as demonstrated by ASO hybridization. We have tentatively designated the different allele as $QOhongkong2$. Another potential $QObellingham$ allele, found in combination with the deficiency allele $Mmalton$, was demonstrated to be a different null allele, which we designated $QOcedarisland$ according to nomenclature guidelines (28). ASO hybridization confirmed the presence of the $QObellingham$ allele in four individuals, two of whom were homozygous for the $QObellingham$ allele (data not shown) and two heterozygous for other deficiency alleles.

**Discussion**

The null alleles of $a1$AT described to date, which have less than 1% of the normal concentration of $a1$AT, have base substitutions, deletions, or insertions that result in the generation of a premature translational stop codon. However, other possible mechanisms for generating null alleles are promoter/enhancer defects, splice site mutations, or critical amino acid substitutions. The $QOludwigshafen$ mutation is a T to A transversion in exon II, resulting in replacement of isoleucine 92 by asparagine. A comparison of amino acids found at the equivalent of position 92 in human and baboon $a1$AT, human $a1$-antichymotrypsin, human antithrombin III, chick ovalbumin, and rat angiotensin reveals that uncharged nonpolar resi-

**Table II. Null Alleles Analyzed by Hybridization with Probes**

<table>
<thead>
<tr>
<th>Allele</th>
<th>Number observed*</th>
<th>Haplotype derivation</th>
<th>ASO verification</th>
</tr>
</thead>
<tbody>
<tr>
<td>$QOludwigshafen$</td>
<td>1</td>
<td>M2</td>
<td>+ASO $QOlud$</td>
</tr>
<tr>
<td>$QObolton$</td>
<td>1</td>
<td>$M1val213 = Taq$</td>
<td>+ASO $QObolton$</td>
</tr>
<tr>
<td>$QObellingham$</td>
<td>6</td>
<td>$M1val213$</td>
<td>+ASO $QObel$</td>
</tr>
<tr>
<td>$QOmatthewa$</td>
<td>1</td>
<td>M3</td>
<td>−ASO $QObel$</td>
</tr>
<tr>
<td>$QOhongkong1$</td>
<td>1</td>
<td>$M2^2$</td>
<td>−ASO $QObel$</td>
</tr>
<tr>
<td>$QOhongkong2$</td>
<td>1</td>
<td>$M1val213^3$</td>
<td>−ASO $QObel$</td>
</tr>
<tr>
<td>$QOcedarisland$</td>
<td>1</td>
<td>M2</td>
<td>−ASO $QOlud$</td>
</tr>
</tbody>
</table>

* Independent allele observations in unrelated individuals.

† Rare $M_2$; haplotype for Europeans, frequency in Asian population unknown.

‡ Derivation based on European haplotypes may not apply to Asian population.

**Figure 4.** Identification of $QOludwigshafen$ by digestion with Xho II. Genomic DNA was amplified by PCR, producing a 254-bp fragment. Amplification products were run in the agarose gels as controls (−X) or after digestion with Xho II (+X). The T to A mutation in $QOludwigshafen$ destroys an Xho II site in exon II. Genotypes tested are indicated, abbreviations are as in Figs. 3 and 4. Plasmid clones containing exon II from $QOlud$ or a normal allele were digested and used as homozygous controls.

**Figure 5.** $QObellingham$ ASO hybridization to amplified genomic DNA. (A) Arrows indicate the location of the ASO probes and the 5′ and 3′ PCR amplification primers which are separated by 394 bases and flank the substitution. Sequences of the normal and $QObellingham$ ASO probes are listed. (B) The $QObellingham$ ASO or its normal counterpart was hybridized to DNA samples amplified by PCR. Haplotypes tested are indicated above each lane: M1, M2, Z, $QOmatthewa$ ($QOmat$), $QOhongkong$ ($QOhon$), and $QObellingham$ ($QObel$). Plasmid clones containing exon III from either the $QObellingham$ or the normal allele were amplified for homozygous controls.
determines these alleles based on ASOs in wigshafen. Examination of the three-dimensional model, based on studies of Loeberman et al. (34), using both a computer image and a physical model of crystallized α-lAT, reveals that amino acid 92 lies in a hydrophobic pocket, along with methionine 63 and phenylalanine 82. The hydrophilic asparagine would result in a sharp twisting of the α-helix D, with resulting distortion of the tertiary structure. Although the disruption could result in impaired folding as occurs in Z α-lAT, the complete absence of protein suggests the QOlugwigshafen protein is destabilized. Normal mRNA is expected in this type of mutation.

This type of mutation, which identifies critical regions for protein conformation, has been described for other null phenotypes such as found for Factor IX (35), phenylalanine hydroxylase (36), and hypoxanthine phosphoribosyltransferase (37).

The QOlugwigshafen haplotype is indistinguishable from that of one of the M2 haplotypes (11, 15), the background of the allele on which the mutation must have occurred. As predicted from haplotype data, the changes at amino acids 101 and 376 associated with M2 (31) are found in the QOlugwigshafen sequence. To verify that the substitution in exon II of the QOlugwigshafen allele is not characteristic of M2, which constitutes 10% of the normal M alleles, we hybridized the QOlugwigshafen mutant oligonucleotide to PCR amplified DNA of the identical M2 haplotype. The QOlugwigshafen allele was clearly distinguished from the M2 alleles and from the normal M1 or M3 alleles. Although direct sequencing of amplified exons can be carried out from genomic DNA, the prior cloning allows unambiguous assignment of the common mutations that occur in several exons. Since rare deficiency states will frequently occur in genetic compounds, correct matching of exons will allow ambiguity from the direct genomic sequencing.

We have previously described a method for identifying null alleles based upon limited haplotype analysis (11), which can identify heterozygosity even in the absence of family studies. Haplotype analysis revealed heterozygosity in six of the eight individuals in our series who had null α-lAT alleles. Two of these were heterozygous with P1*Z, two with P1*Mmalton, and two individuals were compound heterozygotes for different null alleles, as demonstrated by ASO hybridization. After determining the haplotype from which each null allele was derived, ASOs for those null alleles were hybridized to other null alleles with a similar haplotype background. Using this approach we have identified the likely presence of two new null alleles: QOHongkong2 and QOcedariland. At least three deficiency mutations must have occurred on the same M2 haplotype background: Mmalton, QOlugwigshafen, and QOcedariland.

ASO hybridization verified the presence of QObellingham alleles in the homozygous state in one individual of Norwegian and one of Dutch descent. The former individual had been provisionally identified as homozygous for the QOoslo allele (2), which is therefore identical to QObellingham. QObellingham is apparently the most common of the null alleles found in individuals of European descent. Haplotype analysis is hampered when the individual being studied is of a different ethnic origin than that for which haplotype data are available, as for QOhongkong (i.e., hongkong1). By sequence analysis (32), this allele must have been derived from an M2 haplotype, but the haplotype does not resemble the common M2 haplotype of European populations (15). Our haplotype analysis for QOhongkong1 was incomplete because phase could not be assigned to the one RFLP showing heterozygosity in this individual (4.6 probe and Ava II digestion). ASO hybridization allowed us to distinguish the new null allele (QOhongkong2), from the QObellingham allele in the compound heterozygote, despite haplotypes that differ at no more than one polymorphic restriction site. We have not used ASO hybridization for the QOgranite falls allele (38) because we have found this mutation results in the loss of a Mae II site in exon II (11) and none of the alleles we have examined have lost that site. Furthermore, the haplotype on which the QOgranite falls mutation occurred is the M1ala213 haplotype, which is not present with any of the null alleles in our series.

Since the combined frequency for all the null alleles is estimated, based on unbiased ascertainment, to be 1.4 × 10⁻⁴ (8), the individual null alleles are even more rare. We have observed the QOlugwigshafen allele in only one pedigree to date. This has been the case for all other null alleles described except for the QObellingham allele (26) which has been observed in several different pedigrees of European origin. Of four alleles ascertained without bias in Canadian caucasians, two were QObellingham, which is therefore estimated to have an allele frequency of 7 × 10⁻⁵.

Six QQ alleles have now been sequenced (Table III). QOcardiff (41) is not included, as the reported change of asparagine 256 to valine is the same as the mutation described for the P1*P allele (42), coding for a somewhat unstable protein associated with ~30% of the normal serum concentration of

<table>
<thead>
<tr>
<th>Allele</th>
<th>Ethnic origin</th>
<th>Altered exon</th>
<th>Base change</th>
<th>Amino acid change</th>
<th>Premature stop</th>
<th>mRNA present</th>
<th>Reference</th>
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</thead>
<tbody>
<tr>
<td>QObellingham</td>
<td>Dutch, other</td>
<td>III</td>
<td>A → T</td>
<td>217 Lys → stop</td>
<td>217</td>
<td>no</td>
<td>26</td>
</tr>
<tr>
<td>QObolton</td>
<td>British</td>
<td>V</td>
<td>delC</td>
<td>362 3glu → arg</td>
<td>373</td>
<td>—</td>
<td>39</td>
</tr>
<tr>
<td>QOgranitefalls</td>
<td>American, white</td>
<td>II</td>
<td>delC</td>
<td>160 tyr → stop</td>
<td>160</td>
<td>no</td>
<td>38</td>
</tr>
<tr>
<td>QOhongkong1*</td>
<td>Oriental</td>
<td>IV</td>
<td>delTC</td>
<td>319 ser → arg</td>
<td>334</td>
<td>yes</td>
<td>32</td>
</tr>
<tr>
<td>QOmattawa</td>
<td>French</td>
<td>V</td>
<td>insert T</td>
<td>353 leu → phe</td>
<td>376</td>
<td>yes</td>
<td>40</td>
</tr>
<tr>
<td>QOlugwigshafen</td>
<td>German</td>
<td>II</td>
<td>T → A</td>
<td>92 ile → asn</td>
<td>none</td>
<td>—</td>
<td>this study</td>
</tr>
</tbody>
</table>

* Previously called QOhongkong, number has been added because a second null allele (QOhongkong2) has been identified in the same individual, according to haplotype analysis.
a1AT. The presence of other differences from P has not been excluded. The five previously sequenced null alleles all have premature termination codons. Only two of these null alleles retain the active site of a1AT at amino acid 358. The QObelievingham alleles (26) and QOgrantiefalls (43) are apparently associated with unstable transcripts, as no mRNA could be isolated from alveolar macrophages obtained from lung lavage, a normal source of a1AT mRNA. In contrast, the QOmtawata allele is associated with stable mRNA but no protein is secreted in in vitro expression assays (40). The QOhongkong allele codes for a protein that is not transported from the rough endoplasmic reticulum to the Golgi apparatus but is apparently too unstable to form inclusions (32).

The null alleles lie at the extreme of a spectrum of alleles that result in various degrees of deficiency. Seven distinct deficiency alleles, which produce detectable plasma protein product, (3–18% of normal) have also been sequenced and do not have premature termination codons as are found in the null alleles. Three of the four deficiency alleles associated with inclusion bodies have been sequenced. The PI Z mutation (glutamine to lysine at amino acid 342) (44, 45) and the Mmalton (25) and Mnichinin (46) mutations (deletion of phenylalanine at amino acid 51/52) result in proteins that readily aggregate in vitro and form stable inclusion bodies in the liver. The phenylalanine deletion of the latter two PI types occurred on different backgrounds, M2 and Mival213, respectively. Similarly, the PI Z mutation has occurred on an M2 background in Ztunbridgewells (47), which appears to be identical to PI*Zaugsberg (48). Two of the deficiency alleles, Mheerlen (49) and Mprocida (50), have amino acid substitutions involving a proline and therefore likely form unstable proteins. The Mheerlen substitution would change the bend of the β-sheet B4; the Mprocida substitution would disrupt α-helix A. Neither are associated with liver inclusion bodies (50, 51). Mminalsprings, which shows a modest reduction in concentration, is also functionally deficient (14% of normal) (52). PI*Mduarte and PI*Ztunbridgewells could be the same from protein studies but are not sequenced (11).

Clinical implications for lung destruction may be similar for all of the deficiency variants, except that there is evidence that even the low amount of a1AT produced in PI ZZ homozygotes is protective in comparison with PI QO homozygotes (53). Liver disease has only been described in association with PI*Z (7) and PI*Mmalton (54). Further studies of patients with rare deficiency alleles may eventually help clarify whether the plasma deficiency or liver accumulation of a1AT is responsible for liver disease.

Haplotype and sequencing studies have now demonstrated a minimum of 15 different deficiency alleles, with different types of molecular defects. Characterization of the spectrum of mutations leading to a1AT deficiency allows delineation of amino acid residues critical for stability, for normal secretion, and for normal function.

Acknowledgments

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