Clinical Variability of Fanconi Anemia (Type C) Results From Expression of an Amino Terminal Truncated Fanconi Anemia Complementation Group C Polypeptide With Partial Activity

By Takayuki Yamashita, Nan Wu, Gary Kupfer, Cristin Corless, Hans Joenje, Markus Grompe, and Alan D. D’Andrea

Fanconi anemia (FA) is an autosomal recessive disease characterized by congenital anomalies, aplastic anemia, and cancer susceptibility. Mutations within the FA complementation group C (FAC) gene account for approximately 14% of diagnosed FA cases. Two mutations, one in exon 1 (delG322) and one in exon 4 (IVS4 + 4 A to T), account for 90% of known FAC mutations. The delG322 mutation results in a mild FA phenotype, while the IVS4 + 4 A to T mutation results in a severe FA phenotype. To determine the molecular basis for this clinical variability, we analyzed patient-derived cell lines for the expression of characteristic mutant FAC polypeptides. All cell lines with the delG322 mutation expressed a 50-kD FAC polypeptide, FRP-50 (FAC-related protein), shown to be an amino terminal truncated isoform of FAC reinitiated at methionine 55. All cell lines with the IVS4 + 4 A to T mutation lacked FRP-50. Overexpression of a cDNA encoding FRP-50 in an FA(C) cell line resulted in partial correction of mitomycin C sensitivity. In conclusion, expression of an amino terminal truncated FAC protein accounts, at least in part, for the clinical heterogeneity among FA(C) patients.

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MATERIALS AND METHODS

Cell lines and culture conditions. Epstein-Barr virus (EBV)-transformed normal and mutant FA lymphoblast lines have been previously described.13 Cells were maintained as suspension cultures in RPMI media supplemented with 15% heat-inactivated fetal calf serum (FCS). They were grown at 37°C in a humidified, 5% CO2-containing atmosphere.

Development of specific anti-FAC antiserum. A polyclonal antiserum against the carboxy terminus of FAC (amino acid 281-558) has previously been described.14 For affinity-purification, the 3’ end of the FAC cDNA (amino acids 502-558) was subcloned into the pGEX2T vector (Pharmacia), maintaining the glutathione-S-transferase (GST) protein and FAC in frame. This GST fusion protein (GST-FAC-C1) was synthesized and coupled to CNBr-activated Sepharose 4B via its primary amino group. The anti-FAC antiserum was first depleted of antibodies against GST by passage over a GST-Sepharose 4B column. The flow-through was then applied to the GST-FAC-C1 Sepharose 4B column. After extensive washing with phosphate-buffered saline (PBS) (pH 7.4), the antibody against FAC-C1 (C1 antibody) was eluted from the GST-FAC-C1-Sepharose 4B column with 0.1 mol/L glycine and 1 mol/L acetic acid (pH 2.4), dialyzed against a large volume of PBS (pH 7.4), and concentrated.

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Fig 1. Mutations of the FAC polypeptide found in patients with FA (Group C). The wild type FAC polypeptide (558 amino acids) is shown schematically at the top. The indicated FAC alleles (shown at the right) are predicted to encode the FAC mutant polypeptides shown. The IVS4+4A to T allele encodes a FAC mutant with an in frame deletion of the 37 amino acids encoded by exon 4. The delG(322) mutation encodes a truncated FAC polypeptide with a frame shift and a premature STOP codon at amino acid 44. A hatched bar shows the frameshifted region of the protein.

Table 1. Genotype of FA(Ch) Lymphoblast Lines

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Allele 1</th>
<th>Allele 2</th>
<th>Phenotype*</th>
<th>FRP-50</th>
<th>Full-Length FACC</th>
<th>Truncated FACC</th>
</tr>
</thead>
<tbody>
<tr>
<td>NSK</td>
<td>wt</td>
<td>wt</td>
<td>Normal</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>VU168</td>
<td>wt</td>
<td>wt</td>
<td>Normal</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>PD7</td>
<td>del(G322)</td>
<td>wt</td>
<td>Normal</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>VU003</td>
<td>del(G322)</td>
<td>wt</td>
<td>Normal</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>PD4</td>
<td>del(G322)</td>
<td>C to T (800)</td>
<td>Mild FA (1)</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>VU001</td>
<td>del(G322)</td>
<td>del(G322)</td>
<td>Mild FA (1)</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>VU002</td>
<td>del(G322)</td>
<td>del(G322)</td>
<td>Mild FA (2)</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>VU158</td>
<td>del(G322)</td>
<td>del(G322)</td>
<td>Mild FA (1)</td>
<td>+</td>
<td>-</td>
<td>-</td>
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<tr>
<td>VU166</td>
<td>del(G322)</td>
<td>del(G322)</td>
<td>Mild FA (1)</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>VU239</td>
<td>del(G322)</td>
<td>IVS4 + 4A to T</td>
<td>Mild FA (3)</td>
<td>+</td>
<td>-</td>
<td>-</td>
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<td>del(G322)</td>
<td>Mild FA (2)</td>
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<td>ND</td>
<td>ND</td>
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<tr>
<td>PD153</td>
<td>IVS4 + 4A to T</td>
<td>IVS4 + 4A to T</td>
<td>Severe FA (5)</td>
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<td>-</td>
<td>-</td>
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<tr>
<td>4510C</td>
<td>IVS4 + 4A to T</td>
<td>IVS4 + 4A to T</td>
<td>Severe FA</td>
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<tr>
<td>PD77</td>
<td>IVS4 + 4A to T</td>
<td>IVS4 + 4A to T</td>
<td>Severe FA</td>
<td>-</td>
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<td>-</td>
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<tr>
<td>GM449</td>
<td>IVS4 + 4A to T</td>
<td>IVS4 + 4A to T</td>
<td>Severe FA</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<tr>
<td>PD149</td>
<td>IVS4 + 4A to T</td>
<td>IVS4 + 4A to T</td>
<td>Severe FA (4)</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>BD6215</td>
<td>C to T (800)</td>
<td>C to T (800)</td>
<td>Severe FA (4)</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<tr>
<td>PD182</td>
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<td>IVS4 + 4A to T</td>
<td>Severe FA (4)</td>
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<td>IVS4 + 4A to T</td>
<td>Severe FA (4)</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>PD162</td>
<td>IVS4 + 4A to T</td>
<td>IVS4 + 4A to T</td>
<td>Severe FA (5)</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

*IVS4 + 4A to T indicates a single base change in the fourth intronic base, changing the sequence from A to T.

Abbreviation: ND, immunoprecipitation results not available.

* Simplified severity score (Auerbach et al1). A score of less than 3 is considered a mild phenotype.

† Multiple skeletal abnormalities (exact clinical data not available).
have been identified within the FAC gene.\textsuperscript{12,15,19} The most frequent mutations are the IVS4 + 4 A to T mutation, which results in the loss of exon 4\textsuperscript{4,15} and the delG322 mutation, which results in a frameshift and a premature STOP codon in exon 1.\textsuperscript{8,12} Together, these two mutations account for approximately 90\% of known FA(C) mutations (Fig 1).

Initially, we compared the clinical severity of several FA patients by using the clinical score of Auerbach et al\textsuperscript{11} (Table 1). This score was originally described for distinguishing FA patients from non-FA patients with aplastic anemia and, more recently, was shown to be proportional to the severity of the FA phenotype.\textsuperscript{12} The score is calculated by adding one point each for growth retardation, birthmarks, kidney and urinary tract abnormalities, microphthalmia, low platelets, or thumb and radial abnormalities and by subtracting one point each for learning disabilities and other skeletal abnormalities. The score thus ranges from -2 to +6; a score below 3 is defined as a mild phenotype. There is no evidence that the score is a useful predictor of bone marrow failure or cancer susceptibility.

Based on this clinical score, we compared the severity of patients with either the IVS4 + 4 A to T mutation or the delG322 mutation (Table 1). A mild FA clinical score (<3) correlated with the homozygous delG322 genotype, while a severe FA clinical score (>3) correlated with the homozygous IVS4 + 4 A to T genotype (P = .0008).

We next analyzed FAC polypeptides expressed in EBV-transformed lymphoblast lines derived from normal adult
controls and FA patients (Fig 2). A polyclonal antiserum that recognizes the carboxy terminus of FAC (C1 antibody) immunoprecipitated the 60-kD FAC polypeptide from a normal control cell line (Fig 2A, lane 1), as previously described.\textsuperscript{16,20,21} In addition, the antiserum specifically immunoprecipitated a 50-kD (FAC-related protein, or FRP-50) and a 150-kD (FRP-150) polypeptide. Using immunoprecipitation, we next screened multiple EBV immortalized lymphoblast lines, derived from patients with either exon 1 or exon 4 mutations in the FAC gene (Fig 2B and 2C). Cell lines from patients with delG322 did not express full length FAC, but did express FRP-50 (Fig 2B, lane 7 and Fig 2C, lanes 4, 6, 8, and 10). Cell lines from patients with IVS4 + 4 A to T did not express full length FAC, but did express a 55-kD truncated protein (Fig 2B, lanes 3, 5 and Fig 2C, lane 12), consistent with the in frame deletion of 37 amino acids from the FAC polypeptide.\textsuperscript{15}

Table 1 summarizes the immunoprecipitation results and corresponding clinical phenotype for multiple cell lines from normal controls and patients. Cell lines derived from four normal controls and six patients with the delG322 mutation expressed FRP-50. In contrast, all five cell lines from patients with homozgyous exon 4 mutations lacked the FRP-50 polypeptide. Among cell line tested, the presence of FRP-50 correlated with the homozgyous delG322 genotype, while the absence of FRP-50 correlated with the homozgyous IVS4 + 4 A to T genotype (\textit{P} = .008).

Most cell lines from mild FA patients (5 of 7) were homozgyous for delG322. Two additional patients were compound heterozygotes. For PD4, one allele was delG322, and the second allele was (C to T [808]). For VU239, one allele was delG322, and the second allele was IVS4 + 4 A to T. Patients who are homozgyous for (C to T [808])\textsuperscript{19,20} or IVS4 + 4 A to T\textsuperscript{15} have severe disease. Interestingly, the two compound heterozygotes had mild FA and endogenous FRP-50 expression. Taken together, these results suggest that the delG322 allele is dominant over the IVS4 + 4 A to T allele or the (C to T [808]) allele and confers a mild phenotype.

\textbf{The FRP-50 polypeptide is encoded by the FAC gene.} By several criteria, FRP-50 seems to be derived directly from the FAC gene, either as a splice variant or as an internal translation initiation product.\textsuperscript{23,24} First, FRP-50 is directly immunoprecipitated with the anti-FAC antiserum, even in the absence of full-length FAC polypeptide (Fig 2B, lane 7, and Fig 2C, lanes 4, 6, 8, and 10). Therefore, FRP-50 is not an associated protein of FAC or a degradation product of FAC but rather, shares an epitope with FAC. Second, FRP-50 was not present in cells where both FAC alleles contained mutations in exon 4 (Fig 2B, lanes 3 and 5 and Fig 2C, lane 12).

To confirm that FRP-50 is derived from the FAC gene, we prepared two discrete antisera against FAC, directed against different linear epitopes (Fig 3). Both C1 and C3 antisera immunoprecipitated FAC and FRP-50 (Fig 3, lanes 2 and 4, respectively), suggesting that these two proteins are highly related isoforms. In contrast, C1, but not C3, recognized an epitope of FRP-150 (lane 2 v lane 4), suggesting that FRP-150 and FAC are only related in the C1 epitope. Immunoprecipitation of FAC and FRP-50 by C1 antibody was competed by an excess of GST-FAC-C1 (lane 3), but not by GST (data not shown). Immunoprecipitation of FAC and FRP-50 by C3 antibody was competed by GST-FAC, containing amino acids 281-558 of FAC (lane 5), but not by GST-FAC-C1 (lane 6), confirming that C1 and C3 antisera recognize non-overlapping epitopes. FRP-50 from PD4 cells (Table 1) also is recognized by the C1 and C3 antibody (data not shown). Taken together, these results demonstrate that FAC and FRP-50 share two discrete epitopes and that FRP-50 is probably encoded by the FAC gene.

\textbf{FRP-50 is an isoform of the FAC polypeptide, initiated internally at methionine 55.} Because FAC and FRP-50 have related epitopes at the carboxy terminus, we reasoned that FRP-50 is an isoform of FAC that lacks the normal amino terminus encoded by exon 1. To test this hypothesis, we generated mutant FAC cDNAs by in vitro mutagenesis (Fig 4). These cDNAs encode FAC polypeptides with amino terminal truncations. The T1, T2, and T3 cDNAs encode FAC polypeptides that initiate at methionine 48 (M48), me-
thionine 55 (M55), and methionine 350 (M350), respectively (Fig 4A). An MMC-sensitive, type C FA cell line, HSC536N, was transfected with the cDNAs encoding either full-length or truncated FAC polypeptides. Transfected cells were selected in hygromycin and assayed for the presence of the mutant polypeptides (Fig 4B and C). Untransfected HSC536N cells expressed a mutant FAC polypeptide (60 kD) and mutant FRP-50, (Fig 4B, lane 2), as previously described.10 Both FAC and FRP-50 in these cells have an L554P missense mutation and have no functional activity.15 Cells transfected with full-length FAC cDNA had increased expression of both FAC and FRP-50 (Fig 4B, lane 4), indicating that both FAC and FRP-50 are derived from the full-length cDNA. In contrast, the expression of FRP-150 remains unchanged following transfection (compare lanes 2 and 4). Cells transfected with pREP-FAC(T1), pREP-FAC(T2), and pREP-FAC (T3), expressed proteins of 52 kD, 50 kD, and 25 kD, respectively (Fig 4B and C). The T2 FAC polypeptide had the same electrophoretic mobility as FRP-50, suggesting that these polypeptides are identical. Consistent with these results, the in vitro transcription and translation of the full-length FAC cDNA resulted in synthesis of both FAC and FRP-50 (data not shown).

Heterologous expression of FRP-50 partially complements the MMC sensitivity of an FA(C) cell line. Because FRP-50 expression correlates with the mild FA phenotype, we reasoned that FRP-50 might have functional activity in vivo. To assess the functional activity of FRP-50 and other mutant FAC polypeptides, we analyzed their ability to complement the MMC-sensitivity of a severe type C FA cell line, as previously described.16 HSC536N cells, transfected with wild-type FAC, demonstrated enhanced cellular resistance to MMC, similar to normal lymphoblasts (Fig 5). HSC536N cells, transfected with the T3 mutant, an
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FAC(L554P) mutant, or mock-transfected remained sensitive to MMC. Interestingly, overexpression of both the T1 and T2(FRP-50) mutant polypeptides resulted in partial correction of the MMC sensitivity of the FA(C) cell line.

To verify the partial functional activity of T2(FRP-50), we designed a second in vitro complementation assay (Fig 6). This assay exploits the fact that FA cells, unlike normal cells, accumulate in the G2 phase of the cell cycle following low-dose exposure to MMC. Control cells (HSC536N-mock) and cells transfected with wild-type FAC (HSC536N-wt FAC) showed a similar cell cycle distribution in the absence of MMC (Fig 6A and C). After treatment with a low concentration of MMC (0.1 μmol/L) for 48 hours, HSC536N-mock cells accumulated in the G2 phase of the cell cycle (Fig 6B). In contrast, HSC536N-wt FAC cells did not accumulate in G2 to the same extent (Fig 6D). The reduction of MMC-induced G2 accumulation by wild-type FAC expression was defined as a 100% correction. Transfection with the mock pREP4 vector resulted in a 0% correction.

Transfected HSC536N cells expressing either wild-type or amino terminus truncated FAC polypeptides were next analyzed by this functional assay (Table 2 and Fig 7). In the absence of MMC, all transfected cell lines had similar cell cycle distributions (Table 2). Following treatment with MMC for 24 hours, HSC536N-T1 and HSC536N-T2 cells corrected MMC-induced G2 accumulation by 37% and 49%, respectively (Fig 7). Cells transfected with vector alone, the T3 mutant, or a full-length FAC(L554P) mutant accumulated in G2 phase similar to parental untransfected cells. Taken together, these data are concordant with the cytotoxicity data (Fig 5) and demonstrate that the amino terminus of FAC can be truncated with only partial loss of FAC function.

DISCUSSION

In the current work, we have analyzed FAC polypeptides expressed in cell lines from FA patients with variable clinical severity. Cell lines derived from patients with delG322 and mild disease expressed a 50-kD protein (FRP-50). Cell lines derived from patients with IVS4 +4 A to T and severe disease did not express FRP-50. We have demonstrated that FRP-50 is an amino terminal truncated FAC isoform, encoded by the FAC gene. Moreover, we have demonstrated that ectopic overexpression of FRP-50 partially corrects the MMC sensitivity of severe FA(C) cells. Taken together, these data suggest that the expression of FRP-50 accounts, at least in part, for the mild phenotype of FA patients with exon 1 mutations in the FAC gene.

The FAC gene directs the synthesis of the FRP-50 polypeptide by one of two possible mechanisms. First, an alternative splicing mechanism could generate an FAC mRNA that lacks exon 1 sequences. Second, a ribosomal skip mechanism could generate a truncated protein (FRP-50) that initiates at an internal methionine, downstream of methionine 1 (M1) (Fig 4). In either mechanism, the region of exon 1 that contains an in-frame STOP codon would be omitted, and a continuous reading frame would be generated that encodes FRP-50.

While we cannot rigorously distinguish between these possibilities, our data strongly support the mechanism of internal translation initiation. First, FRP-50 has the same molecular weight as the T2(M55) polypeptide that is expressed in transfected cells (Fig 4C) or synthesized by in vitro translation (data not shown). Second, increased levels of both FAC and FRP-50 are found in cells transfected with a full length FAC cDNA that contains no intron sequences (Fig 4B, lane 4). Third, the DNA sequence surrounding codon 55 (AAGAGATGG) resembles a Kozak consensus sequence (CCACCAATGG), particularly at bases -3 and +1, and is, therefore, a plausible site for translation reinitiation. Finally, the delG322 mutation in codon 22 (Fig 1) is found upstream of M55, and translation of FRP-50 is not affected by this mutation. In contrast, the IVS4 +4 A to T mutation is downstream of M55 and translation initiation at M55 would not yield a functional FAC isoform. Several mammalian genes have been described that generate protein isoforms by alternative initiation of translation. For instance, internal translation initiation can account for a comparatively mild variant of Duchenne Muscular Dystrophy. In these cases, translation of carboxy terminal polypeptides from downstream start codons account for the milder phenotype.

Primary cells and immortalized cell lines, derived from patients with either delG322 or IVS4 +4 A to T mutations, exhibit similar sensitivity to MMC in vitro (A. D’Andrea, unpublished results, June 1995). The endogenous low-level expression of FRP-50 in cells with the delG322 mutation, therefore, does not improve their cellular sensitivity to MMC. The partial correction of MMC sensitivity by the T2 mutant observed in the cytotoxicity assay (Fig 5) and in the G2 accumulation assay (Fig 7), may, therefore, result from overexpression of FRP-50 in transfected cell lines. Still, overexpression of other mutant FAC polypeptides such as FAC (T3) (Fig 4) and FAC(LS54P) fails to even partially correct the MMC sensitivity of the HSC536N cells. Taken together, these
results suggest that the endogenous FRP-50 levels in cells with delG322 has no effect on cellular MMC sensitivity in vitro, but a measurable protective effect against severe phenotypic features of FA.

Table 2. Effect of Mitomycin C on G2+M Percentage of Flow Histograms From Transfected HSC536N Cells

<table>
<thead>
<tr>
<th>cDNA Transfected</th>
<th>MMC 0.1 μmol/L</th>
<th>+MMC 0.1 μmol/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mock</td>
<td>23.3</td>
<td>40.2</td>
</tr>
<tr>
<td>FACC</td>
<td>19.3</td>
<td>27.1</td>
</tr>
<tr>
<td>T1</td>
<td>22.7</td>
<td>36.3</td>
</tr>
<tr>
<td>T2</td>
<td>19.7</td>
<td>33.8</td>
</tr>
<tr>
<td>T3</td>
<td>22.5</td>
<td>39.2</td>
</tr>
<tr>
<td>FACC (L554P)</td>
<td>18.4</td>
<td>39.8</td>
</tr>
</tbody>
</table>

HSC536N cells were transfected with the indicated cDNA. The percentage of cells in G2+M was determined by the computer program CELLFIT. Transfection with the indicated cDNAs was performed three times, with similar results obtained each time.

Other mutations in the FAC gene may account for mild or even subclinical cases of FA. As discussed above, patients with the delG322 mutation frequently have no skeletal abnormalities and develop hematologic disease later. It is possible that even more subtle mutations in the FAC gene could account for the later onset of aplastic anemia or leukemia. The actual incidence of FA, therefore, may be higher than previously calculated. Also, because the DEB test is a highly sensitive and specific test, the test should be employed for more patients with sporadic onset of aplastic anemia or acute myeloid leukemia, even in the absence of congenital abnormalities.

Finally, the cellular function of the FAC polypeptide remains unknown. FA genes have been proposed to play a role in DNA repair, cell cycle regulation, cellular response to oxidative stress, and prevention of apoptosis. As FRP-50 has partial functional activity and lacks the amino terminal 54 amino acids, a critical functional domain must exist in the more carboxy portion of the protein.
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Fig 7. Heterologous overexpression of FRP-60 partially complements the MMC sensitivity of a FA cell line. Data from Table 1, representing the percentage of cells in G2+M following MMC treatment, was normalized to demonstrate the percent correction of G2 phase accumulation. By definition, transfection with the cDNA encoding FAC (wt) corrected the MMC sensitivity of HSC536N cells by 100%. Vector alone (mock) did not correct the MMC sensitivity of HSC536N cells (0%). Mutant FAC polypeptides T1, T2, T3, and FAC (L954P) corrected the MMC sensitivity to 37%, 45%, 8%, and 3% of FAC (wt), respectively. Percent correction was calculated as [%G2(mock) - %G2(test)] + [%G2(mock) - %G2(FAC)].

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