Helicobacter pylori cag pathogenicity island genes: clinical relevance for peptic ulcer disease development in Brazil

Rejane Mattar, Sergio Barbosa Marques, Maria do Socorro Monteiro, Anibal Ferreira dos Santos, Kiyoshi Iriya and Flair José Carrilho

Department of Gastroenterology, University of São Paulo School of Medicine, São Paulo, SP, Brazil

Correspondence

Rejane Mattar
rmattar@hcnet.usp.br or
rejane-mattar@ig.com.br

The purpose of this study was to verify whether the presence of any of the *Helicobacter pylori* cagPAI genes or segments – *cagA*, *cagA* promoter, *cagE*, *cagM*, *tnpB*, *tnpA*, *cagT* and the left end of the *cag* II (LEC) region – would be a useful marker for the risk of peptic ulcer disease development. *H. pylori* DNA extracted from positive urease tests of 150 peptic ulcer patients and 65 dyspeptic controls was analysed by PCR. Duodenal ulcers were present in 110, gastric ulcers in 23 and both gastric and duodenal ulcers in 17 patients. A significant association ($P < 0.001$) was found between a conserved cagPAI and peptic ulcer disease (34 %). The positivity of the *cagA* gene varied according to the region of the gene that was amplified. The region near to the promoter was present in almost all of the *H. pylori* isolates (97.2 %). The segment from nt 1764 to 2083 and the extreme right end were frequently deleted in the isolates from the controls ($P < 0.01$). The positivity of the promoter region of *cagA* and *cagT*, *cagE*, *cagM* and LEC showed a significant difference between the isolates from peptic ulcer patients and from the controls ($P < 0.01$). Patients usually had moderate gastritis; however, the intensity of the active inflammation was higher in the peptic ulcer group ($P < 0.001$). *cagT*, *cagM*, LEC and the right end terminus of the *cagA*-positive *H. pylori* isolates were associated with a 27-fold, 8-fold, 4-fold and 4-fold risk of peptic ulcer disease, respectively, and may be useful markers to identify individuals at higher risk of peptic ulcer disease development in Brazil.

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INTRODUCTION

Helicobacter pylori colonizes the human stomach causing chronic gastritis. A small number of individuals progress to peptic ulcer or gastric cancer. Multiple bacterial and/or host factors are involved in disease development (Backert *et al.*, 2004; Peek, 2005).

H. pylori clinical isolates are classified into two types according to their degree of pathogenicity. Type I, associated with severe disease pathology, expresses functional VacA (vacuolating cytotoxin A) and contains an insertion of 40 kb of foreign DNA, the *cag* (cytotoxin-associated gene) pathogenicity island (cagPAI). Type II lacks cagPAI, harbours a non-toxic form of VacA and is regarded as less virulent (Censini *et al.*, 1996; Backert *et al.*, 2004).

The cagPAI may be divided into two regions, *cag* I and *cag* II, by a novel insertion sequence (IS605). There are

approximately 16 and 15 open reading frames in *cag* I and *cag* II, respectively (Censini *et al.*, 1996; Akopyants *et al.*, 1998). The cagPAI encodes a type IV secretion system, which delivers CagA into the cytosol of gastric epithelial cells (Covacci & Rappuoli, 2000) through a rigid needle structure covered by Cag7 or CagY, a VirB10-homologous protein, and CagT, a VirB7-homologous protein, at the base (Rohde *et al.*, 2003). CagA is tyrosine-phosphorylated by Src-family kinases (Selbach *et al.*, 2002), triggering the elongated hummingbird phenotype (Higashi *et al.*, 2005) similar to that of hepatocyte growth factor (Segal *et al.*, 1999). Numerous phosphorylation motifs in the *cagA* gene have been identified by specific PCR assays (Owen *et al.*, 2003; Zhang *et al.*, 2005).

Type I isolates also mediate interleukin 8 (IL-8) secretion from gastric epithelial cells by NF- κ B activation (Segal *et al.*, 1997; Glocker *et al.*, 1998; Maeda *et al.*, 2001). *cagE* (Tummuru *et al.*, 1995; Maeda *et al.*, 2001), *cagG* (Hsu *et al.*, 2002), *cagH*, *cagI*, *cagL* and *cagM* are necessary for NF- κ B activation; however, *cagA*, *cagN*, *cagP* and the left end of the *cag* II region (LEC) are not required (Segal *et al.*, 1997; Glocker *et al.*, 1998; Fischer *et al.*, 2001).

Abbreviations: Apcag, *cagA* promoter region; cagPAI, cag pathogenicity island; IL, interleukin; LEC, left end of the *cag* II.

In Brazil, the prevalence of *H. pylori* in the normal population is high (78.8%) (Fujioka *et al.*, 2001). Characterization of a marker to identify patients who are at higher risk of peptic ulcer disease development would be useful for clinical practice, as *H. pylori* genotyping from positive urease tests is easily performed in the laboratory.

METHODS

Patients. *H. pylori* DNA extracted from positive urease tests of 150 peptic ulcer patients and 65 non-ulcer dyspeptic controls, designated by numbers and letters, was used in this study. Controls were randomly selected from 500 consecutive endoscopies. Sixty-one were women (93.8%) and four (6.2%) were men, the sex ratio being 15:1, with a mean age of 38.7 years. In the peptic ulcer group, 92 were women (61.3%) and 58 were men (38.7%), the sex ratio being 1.6:1, with a mean age of 46.8 years. Duodenal ulcers were present in 110, gastric ulcers in 23 and both gastric and duodenal ulcers in 17 patients. These patients had first presentation of the illness and came for diagnosis by upper digestive tract endoscopy. Thus, none of the subjects enrolled had previously taken medications, such as anti-secretory drugs or anti-microbial agents for *H. pylori* eradication, or were receiving non-steroidal anti-inflammatory drugs. This study was approved by the local ethics committee and patients gave written informed consent. Biopsies taken from the antrum and the corpus of the stomach were scored according to the updated Sydney system classification and grading of gastritis (Dixon *et al.*, 1996).

***H. pylori* genotyping.** DNA extraction from gastric biopsies of positive in-house urease tests from the antrum by a salting out procedure and PCR were performed according to previously

reported techniques (Mattar *et al.*, 2005). The PCR primers are listed in Table 1. Amplification was performed under the following conditions for *cagA2* (Covacci *et al.*, 1993): initial denaturation at 94 °C for 5 min, followed by 27 cycles of denaturation at 94 °C for 30 s, annealing at 53 °C for 30 s and extension at 72 °C for 30 s. PCRs of *cagM*, *tnpB* (Kidd *et al.*, 2001), *cagA1* and *cagA3* (Ikenoue *et al.*, 2001) were performed using 40 cycles with an annealing temperature of 52 °C. PCRs of *tnpA* (Kidd *et al.*, 2001), *cagT*, *cagE*, the *cagA* promoter (*Apcag*) (Ikenoue *et al.*, 2001) and LEC (Akopyants *et al.*, 1998) were performed using 40 cycles with an annealing temperature of 55 °C. The final extension at 72 °C was for 10 min. A set of primers (P1 and P2) that amplified a 26 kDa antigen (Ag) gene present in all strains of *H. pylori* (Hammar *et al.*, 1992) was used as a positive PCR control under the following conditions: initial denaturation at 94 °C for 5 min, followed by 40 cycles of denaturation at 93 °C for 1 min, annealing at 57 °C for 2 min and extension at 70 °C for 2 min. Analysis of PCR products was performed by electrophoresis on 2% agarose gel, followed by staining with ethidium bromide.

Statistical analysis. Statistical analysis was performed using χ^2 and Fisher's exact tests, using SPSS. A value of $P < 0.05$ was considered statistically significant. Significant factors were subjected to multivariate stepwise logistic regression analysis.

RESULTS AND DISCUSSION

Amplification of *cagPAI* genes

PCRs yielded high-quality products with the expected sizes and reproducible results (Fig. 1); negative PCRs were repeated for confirmation, along with positive controls.

Table 1. Primers used for *H. pylori* genotyping

Region	Primer	Primer sequence (5'→3')	Product size (bp)	Location (nt)	GenBank accession no.
<i>cagM</i>	Cag5	ACAAATACAAAAAGAAAAAGAGGC	586	8022–8046	U60176
	Cag6	ATTTTCAACAAGTTAGAAAAAGCC		8608–8584	U60176
<i>tnpA</i>	Cag10	ATCAGTCCAAAAAGTTTTCTTTCC	344	4280–4305	U60176
	Cag11	TAAGGGGTATATTTCAACCAACCG		4614–4590	U60176
<i>tnpB</i>	Cag8	CGCTCTCCCTAAATTCAAAGAGGGC	569	5080–5104	U60176
	Cag9	AGCTAGGGAAAAATCTGTCTATGCC		5657–5633	U60176
<i>cagE</i>	CagE-F1	GCGATTGTTATTGTGCTGTAG	329	16891–16870	U60176
	CagE-R1	GAAGTGGTTAAAAATCAATGCCCC		16563–16587	U60176
<i>cagT</i>	CagT-F1	CCATGTTTATACGCCTGTGT	301	442–461	U60176
	CagT-R1	CATCACACACCCTTTTGAT		723–742	U60176
<i>cagA3</i>	CagA-F1	AACAGGACAAGTAGCTAGCC	701	2700–2719	AF001357
	CagA-R1	TATTAATGCGTGTGTGGCTG		3400–3381	AF001357
<i>cagA2</i>	CAG-1	AGACAACCTTGAGCGAGAAAG	320	1764–1783	X70039
	CAG-2	TATTGGGATTCTTGGAGGCG		2064–2083	X70039
<i>cagA1</i>	CagA-F2	GATAACAGGCAAGCTTTTGA	349	157–176	AF001357
	CagA-R2	CTGCAAAAGATTGTTTGGCAGA		505–484	AF001357
<i>Apcag</i>	CagA-R2	CTGCAAAAGATTGTTTGGCAGA	730	505–484	AF001357
	AP-F1	GTGGGTAAAAATGTGAATCG		18738–18757	U60176
LEC	LEC-F1	ACATTTTGGCTAAATAAACGCTG	320–550	3920–3942	AC000108
	LEC-R1	TCTCCATGTTGCCATTATGCT		4303–4283	AC000108
Ag*	P1	TGGCGTGTCTATTGACAGCGAGC	298	220–242	M55507
	P2	CGTGCTGGGCATACTTACCATG		500–522	M55507

*Ag, DNA sequence of a species-specific protein antigen of 26 kDa present in all strains of *H. pylori*.

Fig. 1. Agarose gel electrophoresis of the *cagPAI* gene PCR products: *cagA2* (320 bp), *Apcag* (730 bp), *cagE* (329 bp), *cagT* (301 bp), *cagM* (586 bp), *tnpA* (344 bp) from case 45A; *tnpB* (569 bp) from case 39A; LEC (350 bp) from case 45A; and LEC (500 bp) from case 46A. M, 50 bp DNA ladder (Invitrogen) comprising fragments from 50 to 800 bp.

The PCR product size of the LEC varied in length from 320 to 550 bp, due to insertions or deletions just within the left end of the *cag* gene (Akopyants *et al.*, 1998). Most of the studied isolates had a partially deleted *cagPAI*; no *H. pylori* isolate had a totally deleted *cagPAI*.

The profile of *H. pylori cagPAI* genes shows great variability worldwide. Fifty per cent of strains from West India are devoid of the entire island of genes (Kausar *et al.*, 2004), 32 % in France (Audibert *et al.*, 2001), 9 % in Sweden (Nilsson *et al.*, 2003) and the USA (Hsu *et al.*, 2002) and 1 % in Japan (Ikenoue *et al.*, 2001). In the present report, none of the strains showed complete deletion of the *cagPAI* genes. Total deletion of *cagPAI* has been associated with lower production of IL-8 *in vitro* (Audibert *et al.*, 2001; Hsu *et al.*, 2002). However, association of the presence of any gene of the *cagPAI* or the intactness of the *cagPAI* with clinical outcome had been contradictory; some reports have shown an association (Jenks *et al.*, 1998; Ikenoue *et al.*, 2001; Kidd *et al.*, 2001; Nilsson *et al.*, 2003), whilst others have not (Hsu *et al.*, 2002).

***cagPAI* gene positivity and risk of peptic ulcer disease**

Fifty-three (24.7%) of the *H. pylori* isolates studied were positive for all of the *cagPAI* genes: two isolates (3 %) from the controls and 51 (34 %) from the peptic ulcer group. The number of peptic ulcer strains (34 %) positive for the entire set of *cagPAI* genes was low in comparison with that in other countries, such as the USA (88 %; Hsu *et al.*, 2002), Japan (100 %; Ikenoue *et al.*, 2001) and South Africa (78 %; Kidd

et al., 2001). The higher frequency of deletions in the *cagPAI* of Brazilian peptic ulcer *H. pylori* isolates could be a geographical trait. However, the presence of these genes overall had a significant association with peptic ulcers ($P < 0.001$), although this was not an independent risk factor for peptic ulcer disease (see Table 4) and there was no significant association with intensity of gastritis (see Table 6). Conversely, colonization with intact *cagPAI*-type strains has been associated with a fivefold increased risk for diagnosis of duodenal ulcers or gastric cancer (Nilsson *et al.*, 2003).

The *cagA* gene has been considered to be a marker of the presence of the *cagPAI* (Censini *et al.*, 1996) and has been associated with peptic ulcer disease (Rudi *et al.*, 1998). Thus, three different segments of the *cagA* gene were analysed, nt 157–505 (*cagA1*), nt 1764–2083 (*cagA2*) and nt 2700–3400 (*cagA3*). The positivity of the *cagA* gene depended on the region of the gene under evaluation, as shown in Table 2. The segment close to the promoter was present in almost all of the *H. pylori* isolates, from peptic ulcer (96.7 %) and controls (98.5 %). However, the segment in the middle of the *cagA* gene (*cagA2*) and the right end terminus (*cagA3*) were not detected in the majority of the isolates from the controls in relation to the peptic ulcer isolates ($P < 0.01$). Kausar *et al.* (2004) reported that the extreme right end of the *cagA* gene was frequently found to be rearranged in all African isolates compared with those from other countries, although the proximal portion of this gene was present in 100 % of the African isolates. Interestingly, Brazilians are a mixture of races with an important contribution from

Table 2. Positivity of *cagPAI* genes in *H. pylori* isolates from patients with peptic ulcers and from control patients

Gene	No. of peptic ulcer patients (%)	No. of controls (%)	Total no. (%)	P value
LEC	87 (58)	21 (32.3)	108 (50.2)	<0.01
<i>cagT</i>	147 (98)	38 (58.5)	185 (86)	<0.01
<i>tnpA</i>	105 (70)	32 (49.2)	137 (63.7)	<0.01
<i>tnpB</i>	24 (16)	5 (7.7)	29 (13.5)	
<i>cagM</i>	117 (78)	16 (24.6)	133 (61.9)	<0.01
<i>cagE</i>	144 (96)	48 (73.8)	192 (89.3)	<0.01
<i>Apcag</i>	147 (98)	41 (63.1)	188 (87.4)	<0.01
<i>cagA1</i>	145 (96.7)	64 (98.5)	209 (97.2)	
<i>cagA2</i>	113 (75.3)	17 (26.2)	130 (60.5)	<0.01
<i>cagA3</i>	78 (52)	18 (27.7)	96 (44.7)	<0.01
Total	150	65	215	

Table 3. Relationship between independent factors and risk of peptic ulcer disease for patients with *H. pylori* infection

Factor	P value	Odds ratio	95 % CI
<i>cagT</i>	0.0001	27.329	4.535–164.687
Male gender	0.0001	9.731	2.711–34.927
<i>cagM</i>	0.0001	8.081	2.985–21.878
LEC	0.003	4.366	1.676–11.375
<i>cagA3</i>	0.005	4.204	1.546–11.434

Africans. Nevertheless, this peculiarity was not a geographical trait, but rather an indication of a fourfold risk of peptic ulcer disease development (Table 3).

The right end of the *cagA* gene (GenBank accession no. AF001357) encompasses the C-terminal CagA variable-region EPIYA tyrosine phosphorylation motifs (Covacci *et al.*, 1993; Stein *et al.*, 2002; Zhang *et al.*, 2005), which, by tyrosine phosphorylation (Selbach *et al.*, 2002), promote a growth factor-like response with intense cytoskeletal rearrangements, cell elongation effects and increased cellular motility (Stein *et al.*, 2002; Higashi *et al.*, 2005). The frequent deletion of *cagA3* in *H. pylori* isolates from the controls would render these strains less pathogenic. Nevertheless, as this region shows variability, sequence divergence at the primer annealing site could cause a negative PCR result. Thus we shall further analyse the C-terminal *cagA* variable region by nucleotide sequencing of Brazilian *H. pylori* isolates and analyse the number of EPIYA tyrosine phosphorylation motifs.

Although the region of the *cagA* gene close to the promoter was frequently found in isolates from both peptic ulcers and controls, *Apcag* showed a significant difference between isolates from peptic ulcers (98 %) and from controls (63.1 %), as shown in Table 2. Similar results were observed in Japanese *H. pylori* isolates (Ikenoue *et al.*, 2001) that lacked the CagA protein in spite of being *cagA*-positive (Maeda *et al.*, 1999). This contrasts with a report by Kauser *et al.* (2004), which showed that the *cagA* promoter was rearranged more frequently in isolates linked to severe pathology than in isolates responsible for a more benign outcome worldwide.

Table 4. Distribution of patients according to gender

Gender*	No. of peptic ulcer patients (%)	No. of controls (%)	Total no. (%)
Female	92 (61.3)	61 (93.8)	153 (71.2)
Male	58 (38.7)	4 (6.2)	62 (28.8)
Total	150	65	215

* $P < 0.001$.

Most of the peptic ulcer disease isolates retained *cagT* (98 %), *cagE* (96 %) and *cagM* (78 %) ($P < 0.01$; Table 2). In Japan (Ikenoue *et al.*, 2001), strains with partial or totally deleted *cagPAI*, which lacked both *cagE* and *cagT*, were found more frequently in more patients with chronic gastritis only than in patients with peptic ulcer disease or gastric cancer. In England (Kauser *et al.*, 2005), the majority of ulcer disease strains retained the *cagT* and *cagE* genes. In fact, *cagT*, which was positive in 58.5 % of the isolates from the controls, was associated with a 27-fold risk and *cagM* with an 8-fold risk of developing peptic ulcer disease (Table 3). CagT is found at the base of the outgrowing pilus of the type IV secretion system supposed to be responsible for binding to a cellular receptor to induce IL-8 secretion and for ejecting CagA (Rohde *et al.*, 2003). CagE and CagM are absolutely necessary for transcription factor NF- κ B activation, which mediates IL-8 secretion (Glocker *et al.*, 1998).

The LEC was positive in only 58 % of the peptic ulcer *H. pylori* isolates and in 32.3 % of the controls ($P < 0.01$; Table 2). Interestingly, the LEC presented a fourfold risk similar to the right end terminus of *cagA* (Table 3). In South African *H. pylori* isolates, the entire *cag* II region, including the LEC, was undetectable in 23 % of gastritis-only isolates but in only 8 % of peptic ulceration isolates (Kidd *et al.*, 2001). The LEC (Hp0520/ORF6; Covacci & Rappuoli, 2000) was not necessary for either translocation of CagA or IL-8 induction (Fischer *et al.*, 2001); however, it might be involved in the pathogenesis of peptic ulceration. Conversely, the LEC is rearranged more frequently in isolates linked to severe pathology worldwide (Kauser *et al.*, 2004).

Table 5. Histology of the antrum collected from patients with peptic ulcers and from controls

Histology*	No. of peptic ulcer patients (%)	No. of controls (%)	Total no. (%)
Normal	—	5 (7.7)	5 (2.3)
Mild	15 (10.1)	25 (38.5)	40 (18.8)
Moderate	119 (80.4)	34 (52.3)	153 (71.8)
Marked	13 (8.8)	1 (1.5)	14 (6.6)
Atrophic	1 (0.7)	—	1 (0.5)
Total	148*	65	213

*In two patients of the peptic ulcer group, biopsies were not taken for histology before *H. pylori* eradication. $P < 0.001$.

Table 6. Relationship between the positivity of *cagPAI* genes or segments and histological findings (intensity of gastritis) of the antrum from peptic ulcer patients and controls

<i>cagPAI</i> gene/segment	No. of patients (%) positive for <i>cagPAI</i> gene/segment with histology:						P value
	Normal (n=5)	Mild (n=40)	Moderate (n=153)	Marked (n=14)	Atrophic (n=1)	Total (n=213)	
LEC	—	19 (47.5)	81 (52.9)	6 (42.9)	1 (100)	107 (50.2)	
<i>cagT</i>	2 (40)	26 (65)	140 (91.5)	14 (100)	1 (100)	183 (85.9)	<0.05
<i>tnpA</i>	3 (60)	24 (60)	96 (62.7)	11 (78.6)	1 (100)	135 (63.4)	
<i>tnpB</i>	—	5 (12.5)	22 (14.4)	2 (14.3)	—	29 (13.6)	
<i>cagM</i>	—	14 (35)	104 (68)	12 (85.7)	1 (100)	131 (61.5)	<0.05
<i>cagE</i>	3 (60)	31 (77.5)	142 (92.8)	13 (92.9)	1 (100)	190 (89.2)	<0.05
<i>Apcag</i>	1 (20)	28 (70)	142 (92.8)	14 (100)	1 (100)	186 (87.3)	<0.05
<i>cagA1</i>	5 (100)	38 (95)	149 (97.4)	14 (100)	1 (100)	207 (97.2)	
<i>cagA2</i>	1 (20)	13 (32.5)	101 (66)	12 (85.7)	1 (100)	128 (60.1)	<0.05
<i>cagA3</i>	1 (20)	12 (30)	75 (49)	5 (35.7)	1 (100)	94 (44.1)	
Intact <i>cagPAI</i>	5 (100)	8 (20)	40 (26.1)	3 (21.4)	1 (100)	52 (24.4)	

The IS605 insertion sequence (*tnpA* + *tnpB*) was intact in 19 of the 215 (8.8%) *H. pylori* isolates. The *tnpA* gene was found more frequently in *H. pylori* isolates from peptic ulcer patients (70%) than in the isolates from the controls (49.2%) ($P < 0.01$). The *tnpB* gene was usually negative in both groups ($P = 0.129$). On the basis of amino acid sequence similarities, *tnpA* and *tnpB* could encode transposases and could mediate deletions within the *cag* I region (Censini *et al.*, 1996). In contrast, peptic ulcer isolates, which were usually *tnpA*-positive, had *cagPAI* genes that were more intact than the controls. Other reports (Akopyants *et al.*, 1998; Jenks *et al.*, 1998) have detected partial deletions of *tnpA* and *tnpB* in the majority of *H. pylori* strains; therefore, the *cagPAI* is generally present as a single block of genes.

The distribution of patients according to gender (Table 4) showed a preponderance of women in both groups. However, women with *H. pylori* infection more often (93.8%) had normal endoscopy compared with men (6.2%) ($P < 0.001$). Analysis of the peptic ulcer group alone suggested that, unexpectedly, there was a female predominance; however, the control group analysis demonstrated that although the ratio of females to males in the peptic ulcer group was 1.6:1, women more commonly had non-ulcer dyspepsia when colonized by *H. pylori*. The risk of male gender for development of peptic ulcers was 9.7-fold; actually, the incidence of peptic ulcer formation was usually higher in men (Dong *et al.*, 2004). Gender differences have been observed in rats with chemically induced gastritis or gastric ulcers; females had a significantly faster ulcer-healing rate due to more proliferating cells and higher PGE₂ levels (Liu *et al.*, 2001).

cagPAI genes and histological findings

DNA for *H. pylori* analysis was extracted from the antrum where the intensity of gastritis was higher; consequently, the histological findings presented in Table 5 were from the antrum and showed that peptic ulcer patients had a

more-intense gastritis than the controls ($P < 0.001$). The intensity of gastritis of both groups together was associated with the presence of *cagT*, *Apcag*, *cagE*, *cagM* and *cagA2* ($P < 0.05$) (Table 6). The right end termini of *cagA* (*cagA3*) and the LEC, although associated with a fourfold risk of peptic ulcer development, had no significant association with intensity of gastritis. Interestingly, *cagT* (Rohde *et al.*, 2003), *cagE*, *cagM* (Glocker *et al.*, 1998) and *cagA* (Stein *et al.*, 2002; Higashi *et al.*, 2005) are the genes involved in *H. pylori* induction of gastric epithelial morphological alterations and inflammation.

We conclude that *cagPAI* (*cagT*, *cagM* and *cagA*) genotyping may be useful in clinical practice to indicate dyspeptic non-ulcer patients for *H. pylori* eradication in a country with a high prevalence of *H. pylori* infection in the normal population.

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