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Constitutive expression of the *UGA4* gene in *Saccharomyces cerevisiae* depends on two positive-acting proteins, Uga3p and Uga35p

Susana Correa Garcia, Mariana Bermudez Moretti, Alcira Batlle *

Centro de Investigaciones sobre Porfirinas y Porfirias. CIPYP (CONICET, FCEyN, UBA), Ciudad Universitaria, Pabellón II, 2º Piso, 1428 Buenos Aires, Argentina

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Abstract

The first specific precursor of porphyrin biosynthesis is δ-aminolevulinic acid. δ-Aminolevulinic acid enters *Saccharomyces cerevisiae* cells through the γ-aminobutyric acid specific permease Uga4p. It was described that this permease is inducible by γ-aminobutyric acid and its regulation involves several specific and pleiotropic transcriptional factors. However, some studies showed that under certain growth conditions the synthesis of Uga4p was not dependent on the presence of γ-aminobutyric acid. To study the effect of the *trans*-acting factors Uga43p, Uga3p, Uga35p, Ure2p and Gln3p on the expression of *UGA4*, we measured γ-aminobutyric acid and δ-aminolevulinic acid uptake in yeast mutant cells, lacking one of these regulatory factors, grown under different conditions. Experiments analyzing the *UGA4* promoter using a fusion construction *UGA4::lacZ* were also carried out. The results show that the constitutive expression of the *UGA4* gene found in cells under certain growth conditions depends on the presence of Uga3p and Uga35p. In contrast, Gln3p and Ure2p do not seem to have any effect on this constitutive mechanism.

Keywords: δ-Aminolevulinic acid; γ-Aminobutyric acid; Uga4 permease; Transcriptional factor; Yeast; Transport regulation

1. Introduction

The first specific precursor of porphyrin biosynthesis is δ-aminolevulinic acid (ALA). Its transport through plasma membrane has been well characterized in *Saccharomyces cerevisiae* [1]. It has been shown that ALA enters these cells through Uga4 permease [2]. This permease is involved in the transport process of γ-aminobutyric acid (GABA), an amino acid used by *S. cerevisiae* cells as nitrogen source [3]. In this way the entry of ALA, which can not be used as a nitrogen source, is regulated as if it was a nitrogen catabolite uptake. GABA can also be incorporated into the cells through the general amino acid permease (Gap1p) and the proline permease (Put4p) [4].

The synthesis of the Uga4 permease, the GABA specific permease, has been described as inducible in the presence of GABA [4] and it was demonstrated that the induction of Uga4 transporter activity by GABA correlates with strong accumulation of *UGA4* RNA [5]. Induction of this permease requires at least two functional positive-acting proteins, Uga3p and Uga35p (i.e., Dal81p/ DurLp) [6,7]. Uga3p behaves as an inducer-specific factor, while Uga35p is pleiotropic, being required also for allophanate-triggered induction of a number of genes involved in the utilization of ornithine, allantoine and urea [8,9]. The Uga3p and Uga35p act through a GC-rich upstream activating sequence (UAS_{GABA}), essential for the GABA-induced expression; this UAS_{GABA} element is also sufficient to confer to the reporter gene some degree of GABA inducible expression [10].

The promoter region of *UGA4* also contains four adjacent repeats of the heptanucleotide 5′-CGAT(A/T)AG-3′, which constitute an UAS element. This UAS_{GATA} element can potentially confer high levels of expression in the absence of inducer, but this potential activity is inhibited, in uninduced cells grown under conditions of nitrogen derepression by a strong repression mechanism. This repression involves the Uga43p (i.e., Dal80p), a pleiotropic negative regulatory factor [6,11]. There is also evidence that Gln3p, another GATA transcriptional factor, acts in the

* Corresponding author. Tel.: +54 (11) 4812 3357; Fax: +54 (11) 4811 7447; E-mail: batlle@mail.retina.ar

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presence of inductor up-regulating the expression of *UGA4* by competing with Uga43p for binding to the UAS_{GATA} sequence [12,13]. The outcome of this competition influences basal level transcription for inducer-dependent genes. Nitrogen catabolite repression is superimposed on the above regulation; availability of preferred nitrogen sources in some way mediate the ability of Ure2p (GdhCR) to prevent Gln3p from performing its biochemical function in UAS_{GATA}-mediated transcription [14]. A comparison of the deduced Ure2p sequence to sequences in databases reveals a limited homology to glutathione-*S*-transferases, an observation interpreted as suggesting that its biochemical function is one of posttranslational modification [15]. More recently, Ure2p has also been suggested to bind to Gln3p directly [16].

The study of these *trans*-acting factors was carried out in yeast cells from the minimal medium described by Jacobs et al. [17] or from Yeast Nitrogen Base (Difco) medium, which are quite similar. However, when using Vavra's medium [18], regulation of *UGA4* was found to be different. In this medium and using a strain deficient in Uga4p activity, it was shown that GABA can only be incorporated by Uga4p and that the Uga4p synthesis is not dependent on the presence of GABA [19].

The aim of this work was to determine the mechanism leading to a constitutive expression of *UGA4* in Vavra's minimal medium [18]. To this end, we have studied the effect of the transcriptional factors described above in mutant cells lacking one of these regulatory proteins from Vavra's and Jacob's media.

2. Materials and methods

2.1. Strains and growth conditions

The *S. cerevisiae* strains used in this study (Table 1) are isogenic with the wild-type (wt) Σ1278b (*MAT*α) [20] except for the mutations mentioned. These strains were kindly supplied by S. Vissers from the Université Libre de Brussels, Belgium.

Minimal proline medium 1 (MPM1) used was prepared according to the minimal medium described by Vavra and Johnson [18]: in 1 l it contained 1 g $MgSO_4 \cdot 7H_2O$, 4 g KH_2PO_4, 12 g $NaH_2PO_4 \cdot H_2O$, 10 ml vitamin solution (40 mg D-biotin, 1 g calcium pantothenate, 20 g *myo*-inositol, 8.8 g thiamine·HCl, 2.4 g pyridoxine and 500 mg nicotinamide per liter) and 10 ml of trace metal solution (1.73 g $ZnSO_4 \cdot 7H_2O$, 0.722 g $FeSO_4 \cdot 7H_2O$ and 100 mg $CuSO_4 \cdot 5H_2O$ per liter). Final pH is 4.5. Carbon and nitrogen sources were 2% glucose and 10 mM proline, respectively.

Minimal proline medium 2 (MPM2) was prepared according to the minimal medium described by Jacobs et al. [17]: in 1 l it contained 0.7 g $MgSO_4 \cdot 7H_2O$, 1 g KH_2PO_4, 0.4 g $CaCl_2.2H_20$, 0.5 g NaCl, 1 g K_2SO_4, 10.5 g citric acid·H_2O, 10 ml vitamin solution (250 mg D-biotin, 100 mg thiamine·HCl, 1 g *myo*-inositol, 200 mg calcium pantothenate and 100 mg pyridoxine·HCl per liter) and 1 ml of trace metal solution (10 mg H_3BO_3, 1 mg $CuSO_4 \cdot 5H_2O$, 2 mg KI, 4 mg $Na_2MoO_4 \cdot 2H_2O$, 14 mg $ZnSO_4 \cdot 7H_2O$, 10 g citric acid·H_2O, 400 mg $MnSO_4 \cdot H_2O$ and 5 g $FeCl_3 \cdot 6H_2O$ per liter). Final pH is 6.1. Carbon and nitrogen sources were 3% glucose and 10 mM proline, respectively.

2.2. GABA and ALA uptake measurements

GABA and ALA uptake measurements were performed as reported [4]. Briefly, 0.1 mM ^{14}C-GABA (New England Nuclear) or 0.1 mM ^{14}C-ALA (New England Nuclear) was added ($t = 0$) to a culture at an absorbance of 0.200 at 570 nm. At different incubation times 1 ml samples were withdrawn and filtered through S and S 3362 filters (pore size, 0.5–1.5 μm; Schleicher and Schuell GmbH) and the cells were washed three times with 2-ml portions of ice-cold 20 mM potassium phthalate buffer. The dried filters were transferred to vials containing 5 ml of toluene scintillation cocktail and radioactivity was measured in a scintillation counter.

In each experiment for each point duplicates were run and the values presented are the average of three experiments. The deviation of these values from the mean was less than 15%.

2.3. DNA preparation, manipulation and transformation

Yeast transformation was performed as already described by Gietz and Schiestl [26]. The procedures for growth and transformation of *Escherichia coli* and for preparation of plasmid DNA were essentially based on Sambrook et al. [27].

2.4. Plasmid

The *UGA4::lacZ* fusion gene was constructed by replacing the *Eco*RI–*Hin*dIII fragment of plasmid YEp357 [28] with a PCR-amplified fragment spanning nucleotides −583 to +15 with respect to the ATG initiation codon of *UGA4* (YEp*UGA::lacZ*). The PCR was performed using genomic DNA of strain Σ1278b. The YEp357 (see figure 1 in reference [28]) carries the *URA3* selectable marker, thus complementing the uracil auxotrophy of the yeast strains used. The structures of restriction endonuclease joints and PCR product were verified by DNA sequence analysis. *E. coli* JM109 strain was used to amplify and maintain the plasmids.

2.5. β-Galactosidase assay

A 15-ml culture of exponentially growing cells (A_{570} nm: 0.3–0.7) was collected by centrifugation and resuspended in 1 ml Z buffer [29]. The β-galactosidase activity,

measured according to Miller [29], is expressed as Miller units.

3. Results and discussion

3.1. Uga4 permease activity

3.1.1. GABA uptake

To study the effect of Uga43p, Uga3p, Uga35p, Ure2p and Gln3p on the activity of Uga4p, we measured GABA uptake in yeast cells, lacking one of these regulatory factors, grown on either of MPM1 and MPM2 media. Taking into account that GABA uptake is mediated by three permeases and the aim of the assay was to measure Uga4 activity, incorporation of GABA through Put4 and Gap1 permeases was inhibited. To this end we carried out GABA uptake assays in the presence of proline and citrulline. It is known that in cells growing on proline as the sole nitrogen source, GABA transport is not mediated by Put4 permease [4]. When citrulline, which is incorporated into the cells using the Gap1 permease [21], is in excess, GABA uptake through Gap1 permease is competitively inhibited. Consequently under our experimental conditions GABA would be incorporated mainly through Uga4 permease. In Fig. 1, it is shown that when GABA incorporation is measured in *GAP1* cells from MPM2 in the presence of citrulline, the uptake rate is very low during the first 20 minutes of incubation and thereafter it increases progressively. This might be due to the inducing effect of GABA on the *UGA4* gene expression as previously demonstrated by André et al. [6]. In contrast, when citrulline is not present during uptake measurements,

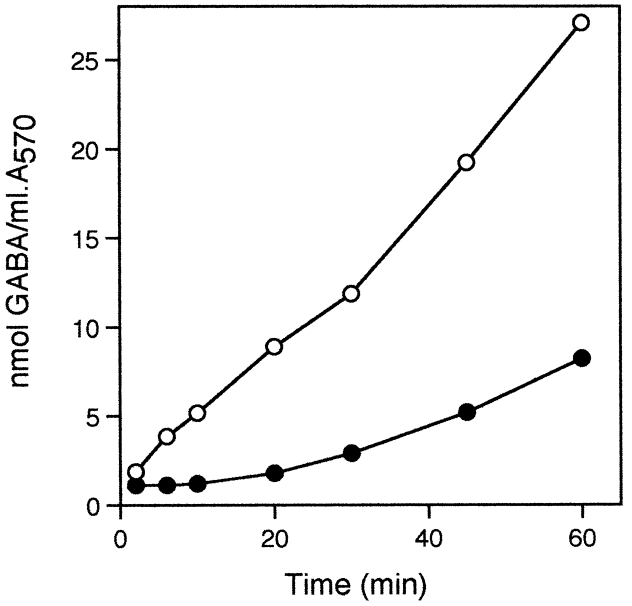

Fig. 1. Time course of GABA uptake in 23344c (*GAP1*) strain grown on MPM2 medium. The assay was carried out in the presence (●) or absence (○) of 2.0 mM citrulline.

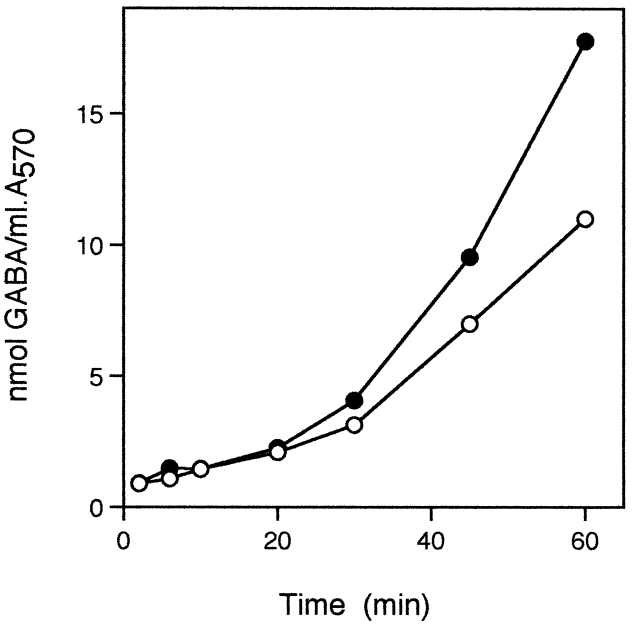

Fig. 2. Time course of GABA uptake in 2512c (*gap1*) mutant strain grown on MPM2 medium. The assay was carried out with (○) or without (●) addition of 2.0 mM citrulline.

GABA incorporation is high from the beginning due to the activity of Gap1 permease.

In the *gap1* mutant, 2512c, the time course of GABA uptake profiles are similar with or without citrulline, presenting low initial uptake rates (Fig. 2). In wt cells, 23344c, grown on MPM2 medium, the activity of Uga4p is inducible by GABA (Fig. 3A) (note that this curve is the same as already shown in Fig. 1) whereas when grown in MPM1 medium this activity is not dependent on the presence of GABA (Fig. 3B). These results agree with those previously presented for the *gap1*Δ mutant, 2512c [19]. As expected, *gln3* cells do not incorporate GABA in MPM2 since Gln3p is essential for GABA induction of the *UGA4* gene [12,13]. However when these cells were grown in MPM1, GABA incorporation is very high from the beginning of the assay (Fig. 3B). These results suggest that Gln3p is not involved in the constitutive expression of the *UGA4* gene.

Although these assays were carried out using a poor

Table 1
Yeast strains used in this study

Strain	Genotype	Reference
Σ1278b	wt	[20]
2512c	*gap1*	[21]
23344c	*ura3*	M. Grenson[a]
30505b	*ura3 gln3*Δ	[6]
26854a	*ura3 ure2*Δ (*gdhCR*)	[22]
30078c	*ura3 uga43*Δ	[23]
26790a	*ura3 uga3*Δ	[24]
CD17	*ura3 uga35*Δ	[25]

[a]Personal communication.

nitrogen source, the lack of Ure2p affects the synthesis of Uga4p, resulting in slightly lower GABA incorporation in MPM2 than in the wt strain, 23344c (Fig. 3A). In MPM1 medium the activity of Uga4p in the *ure2*Δ cells is constitutive (Fig. 3B).

The expression of the *UGA4* gene in *uga43*Δ cells is not dependent on the presence of GABA in MPM2 (Fig. 3A) as already described by Coornaert et al. [23]. The same behavior is observed in MPM1 (Fig. 3B). The expression of the *UGA4* gene in 23344c (wt), 30505b (*gln3*Δ) and 26854 (*ure2*Δ) cells from MPM1 could be due to the absence of Uga43p-mediated repression.

GABA uptake in the *uga3*Δ and *uga35*Δ cells grown in MPM2 is almost undetectable since the activating factors Uga3p and Uga35p are essential to the Uga4p induction by GABA [30,31] (Fig. 3A). When these cells were grown on MPM1, Uga4p activity can be measured although its values are significantly lower than those obtained for the other strains (Fig. 3B). These findings strongly suggest that Uga3p and Uga35p are necessary for constitutive expression of the *UGA4* gene in MPM1.

3.1.2. ALA uptake

Another approach to determine the effect of these transcription factors was to measure ALA uptake. It is known that ALA incorporation is only mediated by Uga4 permease and that it can not induce the synthesis of this protein [2]. So, incorporation of ALA will solely be due to the activity of the Uga4 permease.

It is shown in Fig. 4A that high ALA uptake is only detected in the strain *uga43*Δ while there is no ALA incorporation in the other strains, since in MPM2 medium 30078c (*uga43*Δ) is the only strain that can express Uga4p without any addition of GABA to the medium [5,23]. Even though there is no ALA uptake in MPM2 medium in 23344c (wt), 30505b (*gln3*Δ) and 26854 (*ure2*Δ) strains, in MPM1 medium the permease is active in the plasma membrane of these three strains without addition of GABA to the medium, showing a similar behavior to that observed for 30078c (*uga43*Δ) (Fig. 4B). However, ALA incorporation in the CD17 (*uga35*Δ) and 26790a (*uga3*Δ) cells from MPM1 is very low which is in agreement with the results showed in Fig. 3B.

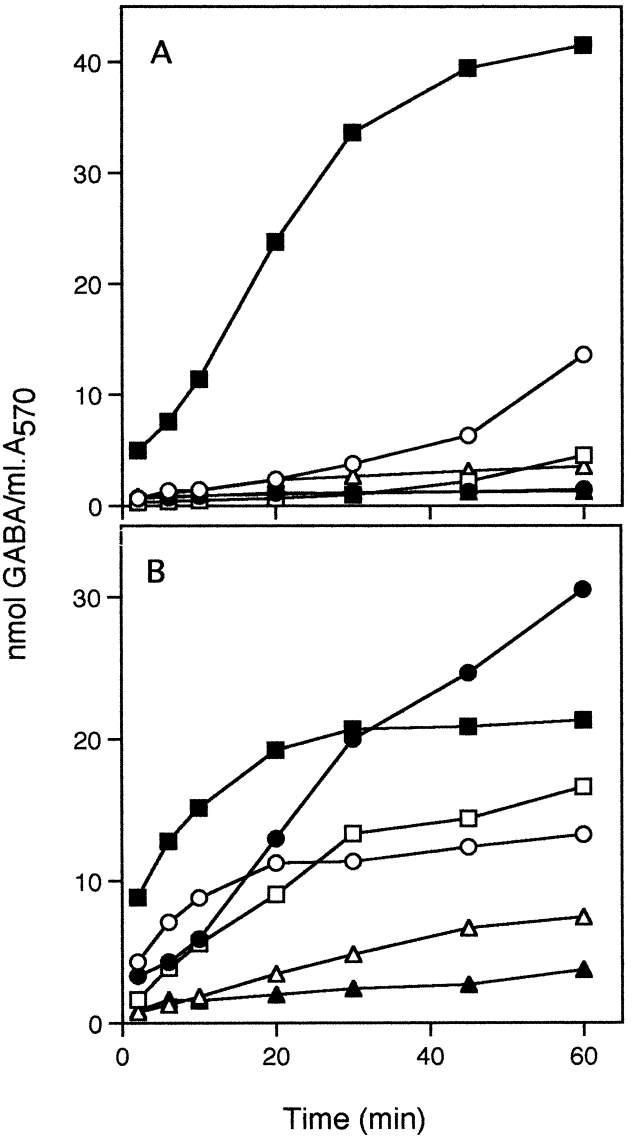

Fig. 3. Time course of GABA uptake in cells grown on MPM2 (A) or MPM1 (B). GABA incorporation was measured in strains 23344c (wt) (○), 30505b (*gln3*Δ) (●), 26854a (*ure2*Δ) (□), 30078c (*uga43*Δ) (■), 26790a (*uga3*Δ) (△) and CD17 (*uga35*Δ) (▲).

Table 2
lacZ expression supported by a *UGA4::lacZ* fusion plasmids

Strain	Relevant genotype	β-Galactosidase activity	
		MPM1	MPM2
23344c	wt	615	75
30505b	*gln3*Δ	630	24
26854a	*ure2*Δ (*gdhCR*)	450	20
30078c	*uga43*Δ	400	885
26790a	*uga3*Δ	57	27
CD17	*uga35*Δ	14	15

Transformants were grown on MPM1 or MPM2. Expression of the *UGA4::lacZ* reporter gene was monitored in the wt and the indicated mutant strains. Units used are those of Miller [29] but are based on 15 ml of cell culture.
In each experiment for each point duplicates were run and the values presented are the average of three experiments. The deviation of these values from the mean was less than 15%.

3.2. LacZ expression driven by the UGA4 promoter

Experiments analyzing the *UGA4* promoter using a *UGA4::lacZ* fusion gene were carried out. For this purpose the strains were transformed with a YEp*UGA::lacZ* plasmid and the activity of the reporter gene, *lacZ*, was measured in both MPM1 and MPM2 media. In Table 2 it is shown that β-galactosidase activity in wt, *gln3*Δ and *ure2*Δ mutant cells from MPM1 is at least 10-fold higher

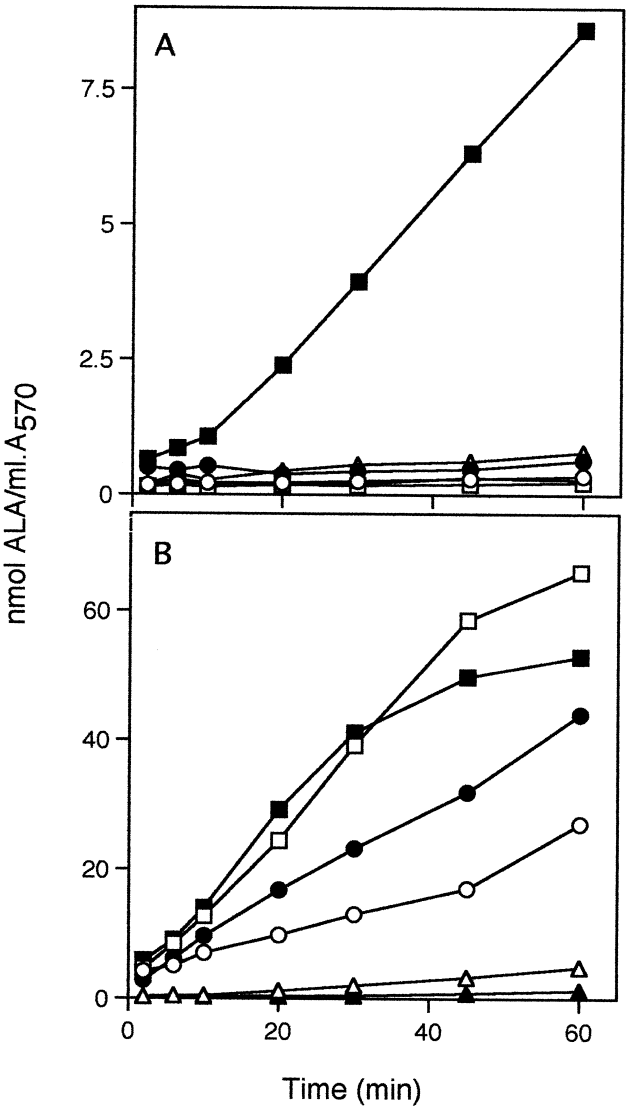

Fig. 4. Time course of ALA uptake in cells grown on MPM2 (A) or MPM1 (B). ALA incorporation was measured in strains 23344c (wt) (○), 30505b (*gln3*Δ) (●), 26854a (*ure2*Δ) (□), 30078c (*uga43*Δ) (■), 26790a (*uga3*Δ) (△) and CD17 (*uga35*Δ) (▲).

than the basal levels detected in MPM2 suggesting that in MPM1 there is a significant expression of Uga4p which is independent on the presence of GABA. However, in both *uga3*Δ and *uga35*Δ mutant cells, the activity of β-galactosidase is quite similar in both media.

In summary, the results here presented clearly show that the constitutive expression of the *UGA4* gene found in cells grown in MPM1 medium depends on the presence of the two specific and pleiotropic positive regulatory factors, Uga3p and Uga35p, respectively. In contrast, Gln3p and Ure2p do not seem to have any effect on this constitutive mechanism. The absence of Uga43p leads to a constitutive expression of the *UGA4* gene in both media.

Further studies are needed to establish which differences between both media are involved in the regulation mechanism of the *UGA4* gene expression. One simple way to explain these findings would be to assume the presence of an intracellular pool of inducer in cells grown in MPM1, which could be due for example, to an increased activity of glutamate decarboxylase in cells grown in MPM1. Another possibility could be the presence of another unknown effector which acts on a regulatory transcription element.

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