Expression of the *Aspergillus nidulans* *argB* Gene in *Escherichia coli*

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The *Aspergillus nidulans* *argB* gene coding for ornithine carbamoyltransferase (OTCase) is not expressed in *Escherichia coli*. However, *E. coli* OTCase-deficient strains transformed with plasmids carrying the *argB* gene from *A. nidulans* reverted to prototrophy at a high frequency. In these derivatives the *argB* gene became functional due to DNA rearrangements upstream of the coding sequence. Two types of rearrangement were characterized. One was identified as an insertion of IS2. The second was a deletion that resulted in transcription of the *argB* gene from the Tc^R gene promoter and translation from a newly created ribosome-binding site formed at the junction between the *A. nidulans* and vector DNA sequences.

INTRODUCTION

Attempts to clone eukaryotic genes by complementation of bacterial mutations have been successful only in the case of lower eukaryotes, in which most genes lack introns. Only a limited number of genes have been cloned in this way, indicating that the expression of such genes in bacterial cells is the exception rather than the rule. This is certainly true for genes of the filamentous fungus *Aspergillus nidulans*. We were interested in cloning the *A. nidulans* *argB* gene coding for ornithine carbamoyltransferase (OTCase), the key enzyme in the arginine biosynthetic pathway. Despite many efforts we were unable to clone this gene (as well as several other *A. nidulans* genes) in *Escherichia coli* directly. We finally succeeded in cloning it in yeast and identified it by complementation of the appropriate yeast mutation (Berse *et al.*, 1983). Introduction of the *argB* gene into an OTCase-deficient *E. coli* strain did not result in recovery of prototrophy by the transformed cells. However, strains bearing plasmids containing the *argB* gene reverted to prototrophy at a high frequency. The revertants produced OTCase with the immunological properties of the *A. nidulans* enzyme, and they yielded plasmids capable of rendering OTCase-deficient *E. coli* mutants prototrophic. These plasmids differed in size from the original one as well as among themselves, indicating that structural rearrangements had made expression of the *argB* gene possible.

We believed that a comparison of the structure of the original plasmid with its derivatives, together with an analysis of their transcription and translation products, would yield information on the barriers preventing expression of the *argB* gene in the bacterial host and on the mechanisms by which these barriers could be overcome. In this paper we present results from an analysis of three different plasmids containing the *argB* gene expressed in *E. coli* and describe the character of the changes which made this gene functional.

METHODS

Micro-organisms and growth conditions. The *E. coli* *argF argI* mutant used in this study was C600 OTC⁻Thi⁻HsdR⁻HsdM⁻Δ(*pro-lac*)*argI1* (Crabeel *et al.*, 1979). Plasmids were propagated in *E. coli* strain JA221 (Beggs, 1978). The minicell-producing strain χ1849 [*tonA53* T1⁺ *dapD8 minA1 purE1 supE42* Δ(*gal-uvrB*) λ⁻ *minB2*]

Abbreviation: OTCase, ornithine carbamoyltransferase.

*his-53 nal-25 metC65 T3^r Δ(bioH-*asd*) ilv-227 cycB2 cycA1 hsdR2*] and bacteriophage *λ*cl857 were obtained from the collection of the Department of Virology, Warsaw University. Bacteria were cultivated in L-broth or in M9 medium (Miller, 1972). *Saccharomyces cerevisiae* strain SPU1 carrying the *arg3* mutation, and yeast growth conditions, were described by Berse *et al.* (1983).

Plasmids. The vectors used were pBR327 (Soberon *et al.*, 1980) and pBB29 (Berse *et al.*, 1983). Plasmid pLK5 containing the phage λ P_R promoter (Zabeau & Stanley, 1982) was obtained from EMBL, Heidelberg, FRG. Plasmids pSal43 and pSal43* are derivatives of plasmid pBB116 described by Berse *et al.* (1983). They contain a 2.6 kb fragment of *A. nidulans* DNA with the complete *argB* gene which is functional in yeast. The *A. nidulans* DNA is inserted in opposite orientations into the *SalI* site located within the Tc^R gene of pBB29 (Fig. 1). Plasmids pSal23 and pSal23* do not contain yeast sequences within the vector DNA. They were obtained by removal of two small *EcoRI* fragments from pSal43 and pSal43*, respectively. Derivatives of plasmids pSal43-5, pSal43*-3 and pSal43*-4 (described in Results) were obtained in an analogous way and designated pSal23-5, pSal23*-3 and pSal23*-4, respectively.

Transformation. *E. coli* was transformed as described by Dagert & Ehrlich (1979). Yeast transformation was performed according to the method of Hinnen *et al.* (1978).

DNA isolation. Plasmid DNA was isolated by the technique of Clewell & Helinski (1969) and purified by caesium chloride/ethidium bromide centrifugation. Total *E. coli* DNA was extracted by the method described by Cosloy & Oishi (1973). DNA fragments were recovered from agarose or polyacrylamide gels according to Maxam & Gilbert (1977).

Blot hybridization. DNA-DNA hybridization was done by the method of Southern (1975). DNA-RNA hybridization was performed according to Thomas (1980). DNA used as a probe was labelled with [α -³²P]dATP or [α -³²P]dCTP by nick-translation as described by Mackey *et al.* (1977).

In vitro transcription. The reaction was carried out following Lee & Yanofsky (1977) with slight modifications. The reaction mixture (50 μ l) contained 5 μ g plasmid DNA, 2.5 units of RNA polymerase and each nucleotide triphosphate at a concentration of 0.2 mM. Transcripts purified by DNAase I treatment and phenol extraction were separated in an agarose gel according to Lehrach *et al.* (1977).

Analysis of protein synthesis in minicells. *E. coli* minicells were purified following the procedure of Reeve (1979). Proteins synthesized in minicells were labelled using a ¹⁴C-labelled amino acids mixture as described by Roozen *et al.* (1971). Proteins were separated in 12.5% (w/v) polyacrylamide-SDS gels according to Laemmli (1970). Fluorography was done by the method of Bonner & Laskey (1974).

OTCase assay. OTCase activity was assayed according to Cybis *et al.* (1972).

DNA sequencing. The fragment of pSal43-5 was sequenced by the method of Maxam & Gilbert (1980). In the case of pSal43*-3, M13 cloning and sequencing by the Sanger method were done according to the Amersham manual.

RESULTS

Selection of plasmids with the A. nidulans argB gene expressed in E. coli

The *E. coli* double mutant *argF argI*, which lacks OTCase activity, was transformed with plasmids pSal43 and pSal43*, which carry the *A. nidulans argB* gene in opposite orientations with respect to the vector (see Fig. 1). The transformants required arginine for growth but reverted to prototrophy at a frequency of 10⁻³ in the case of pSal43 and of 10⁻⁵ in the case of pSal43*. Plasmid DNA isolated from seven Arg⁺ colonies was analysed by agarose gel electrophoresis. Six out of seven plasmids differed significantly in size from the parental plasmid and three of them were investigated further. The first plasmid (named pSal43-5) originated from pSal43 and was smaller than it. Two others (pSal43*-3 and pSal43*-4) were derived from pSal43* and were bigger than it. All three derivative plasmids were able to suppress the *argF* and *argI* mutations in *E. coli*, and they retained the ability to complement the *arg3* mutation in yeast. Yeast DNA sequences were removed from the vector part of pSal43, pSal43* and their derivatives, to yield a series of corresponding plasmids (see Methods). As expected, plasmids pSal23-5, pSal23*-3 and pSal23*-4, like their counterparts pSal43-5, pSal43*-3 and pSal43*-4, suppressed the *argI* and *argF* mutations in *E. coli*. The activity of the OTCase in *E. coli* strains bearing these plasmids was substantially higher than that in strains carrying plasmids pSal23 and pSal23*, which carry the *argB* gene in its original cloned form (Table 1). It is worth noting that the OTCase activity present in the *E. coli* strains bearing pSal23 and pSal23* is not sufficient to allow the growth of these strains on the arginine-free medium. The enzyme was very stable in *E. coli*, as the activity showed no decrease after 48 h incubation of bacteria in the presence of chloramphenicol.

Fig. 1. Restriction maps of pSal43 and pSal43*. pBR327 sequences are represented by the double open lines, *A. nidulans* DNA by the solid bars and yeast DNA by the hatched areas. The position of the *argB* gene within the *A. nidulans* DNA is indicated. Abbreviations: B, *Bam*HI; BII, *Bgl*II; E, *Eco*RI; H, *Hind*III; P, *Pst*I; S, *Sal*I.

Table 1. OTCase activity in *E. coli* strains carrying plasmids with the *A. nidulans argB* gene

<i>E. coli</i> strain	Plasmid	OTCase activity*	
		M9 medium	M9 medium with arginine (10 µg ml ⁻¹)
C600 OTC ⁻ (<i>argF argI</i> mutant)	pSal23	—	0.14 ± 0.015
	pSal23*	—	0.09 ± 0.01
	pSal23-5	2.82 ± 0.41	1.93 ± 0.28
	pSal23*-3	3.23 ± 0.135	2.37 ± 0.41
	pSal23*-4	1.23 ± 0.15	1.51 ± 0.13
	pSal23-50	—	0.00
JA221 (wild-type)	pBR327	—	0.00
	—	13.80 ± 0.60	0.22 ± 0.04

* Expressed as mm-citrulline h⁻¹ (mg protein)⁻¹. The values are means of at least three independent assays, ± SE.

DNA insertion leading to the expression of the *A. nidulans argB* gene in *E. coli*

The restriction patterns of pSal43* and its derivative pSal43*-3 were compared. The 1.8 kb *Sal*I fragment of pSal43* (see Fig. 1) was replaced by a 3.1 kb fragment in pSal43*-3, indicating that a DNA sequence of about 1.3 kb had been inserted into the *A. nidulans* DNA. In order to elucidate the origin of this insertion, the DNA of pSal43*-3 was hybridized to restriction fragments of total *E. coli* DNA. pSal43*-3 revealed strong homology to five *Bgl*II fragments (Fig. 2, lane B). This result suggested that the extra DNA present in pSal43*-3 was derived from the *E. coli* genome and could be an IS element. IS2 was the favoured candidate because its size is approximately that of the insert (1327 nucleotides; Ghosal *et al.*, 1979), it occurs in five copies in the genomes of most *E. coli* strains (Calos & Miller, 1980), and it is known to promote expression of adjacent genes (Kleckner, 1981). To test this supposition we used the M13 system to recloned the 0.6 kb *Hind*III fragment of pSal43*-3 containing the right-end portion of the inserted *E. coli* DNA (see Fig. 3). The sequence of the 175 nucleotides to the right of the *Hind*III site and within the insert was determined by the Sanger method and was found to be completely homologous with that of IS2 as reported by Ghosal *et al.* (1979). The IS2 element in pSal43*-3 is inserted in orientation II (Ghosal *et al.*, 1979), about 500 nucleotides from the 5' end of the *argB* gene.

Another pSal43* derivative, pSal43*-4, was also hybridized to total *E. coli* DNA; it gave the same pattern of hybridization as pSal43*-3 (Fig. 2). Moreover, pSal43*-4 showed the same *Alu*I and *Msp*I restriction sites as pSal43*-3 (Fig. 3). This result indicated that IS2 is also present in this plasmid. However, the results of restriction mapping (not shown) revealed that, besides the IS2 insertion, other rearrangements had occurred in this plasmid DNA. pSal43*-4 was a doublet

Fig. 2. Autoradiogram of Southern blots of restriction fragments of *E. coli* genomic DNA probed with DNA of pSal43* and its derivatives. The *E. coli* DNA was digested with *Bgl*II, transferred onto nitrocellulose and hybridized to 32 P-labelled DNA of pSal43* (lane A), pSal43*-3 (lane B) and pSal43*-4 (lane C). Bacteriophage λ DNA cleaved with *Hind*III was used as a DNA size marker.

Fig. 3. Restriction maps of the *A. nidulans* DNA and flanking sequences within plasmids pSal43*, pSal43*-3, pSal43*-4 and pSal43-5. The portion of DNA present in pSal43 and deleted from pSal43-5 is shown above the map of the latter plasmid. The double open lines represent pBR327 sequences, the solid bars *A. nidulans* DNA, and the hatched areas IS2 sequences. The zig-zag lines indicate the DNA fragments used as probes in hybridization with *in vitro* transcription products. Abbreviations: A, *Alu*I; AI, *Ava*I; B, *Bam*HI; BI, *Bgl*I; BII, *Bgl*II; D, *Dde*I; E, *Eco*RI; H, *Hind*III; M, *Msp*I; P, *Pst*I; R, *Rsa*I; S, *Sal*I. Restriction sites for *Alu*I, *Dde*I, *Msp*I and *Rsa*I are shown only in the regions of the maps within parentheses. D* indicates the *Dde*I site created as a result of the deletion in pSal43-5.

of pSal43* with IS2 inserted within one of the *A. nidulans* DNA fragments, and it also had a deletion of 2.5 kb adjacent to IS2. This plasmid contained two copies of the *argB* gene, one indistinguishable from that present in pSal43* and the other with IS2 located about 20 nucleotides from the 5' end of this gene (Fig. 3). To check which copy was responsible for the expression of the *argB* gene in *E. coli*, the 1.65 kb *Hind*III fragment containing part of the *argB* gene and the large portion of IS2 was ligated with the 9.6 kb *Hind*III fragment of pSal43 containing the rest of the *argB* gene and the vector sequences (see Figs 1 and 3). The new plasmid

$\downarrow$ *Msp*I (533)
 GGG GAC TGT TGG GCG CCA TCT CCT TGC ATG CAC CAT TCC TTG CGG
 45
 $\downarrow$ *Dde*I
 CGG CGG TGC TCA GTG GAT GCC CTC GTT CTG TCC ACA ATT TCT TTC
 90
 CGC CCC AAG TCT TTT AAG TTC TTT AAC ATC TAT ATT CTT GCA CTT
 135
 $\downarrow$ *Rsa*I
 CCA ATG GCA TCC CTT CGC TCC GTA CTC

Fig. 4. Nucleotide sequence of the fragment of pSal43-5 comprising the new junction between the *A. nidulans* DNA and pBR327 DNA. The sequence of pBR327 (Sutcliffe, 1979) is shown in italics. The sequence begins from the *Msp*I site at the position 533 in pBR327. The AUG postulated as the initiation site for OTCase synthesis in *A. nidulans* (B. Berse, unpublished) is boxed. The putative prokaryotic ribosome-binding site is underlined.

Fig. 5. Construction of plasmids pSal23-50 (a) and pSal23-51 (b). ■ indicates the promoter of the *Tc^R* gene; ● indicates the *P_R* promoter of phage λ . In plasmid pLK5 the double open line represents the pBR322 sequence. Other designations and abbreviations are as for Fig. 1.

was able to complement the *argF* and *argI* mutations in *E. coli*. This result proved that the expression of the *argB* gene in *E. coli* bearing plasmid pSal43*-4 was connected with the presence of IS2 in front of the coding sequence.

Deletion leading to the expression of the *argB* gene in *E. coli*

Plasmid pSal43-5 was about 1.1 kb smaller than the original plasmid pSal43. Comparison of the restriction patterns of these plasmids revealed that the deletion comprised sequences of the original *Tc^R* gene and neighbouring *A. nidulans* DNA sequences of about 1 kb (Fig. 3). Analysis of the nucleotide sequence of the DNA fragment which included the new junction between the *A. nidulans* DNA and the vector DNA revealed that 58 nucleotides of the deleted fragment came from pBR327 and the rest (about 1100 nucleotides) from the *A. nidulans* DNA (Fig. 4).

As a result of the deletion, the *argB* gene was closer to the promoter of the *Tc^R* gene. To test whether the transcription of the *argB* gene in pSal43-5 is under the control of the *Tc^R* gene promoter, a DNA fragment containing the 5' end of the *Tc^R* gene was removed from this plasmid (Fig. 5a). The resulting plasmid (pSal23-50) was unable to suppress the *argF* and *argI* mutations in *E. coli* and no OTCase activity was detected in cells carrying this plasmid (Table 1).

Fig. 6. *In vitro* transcription products of plasmids pSal23 (lanes A, B and C), pSal23-5 (lane D) and pSal23-50 (lane E) probed with the *argB* gene and with DNA fragments adjacent to the Tc^R gene. Transcripts were separated on a formaldehyde-agarose gel, transferred to nitrocellulose and hybridized to the following ³²P-labelled probes: the 0.4 kb *AluI*–*Bgl*II fragment of the *argB* coding sequence (lanes C, D, E), the 0.65 kb *Eco*RI–*Pst*I fragment adjacent to the 5' end of the Tc^R gene (lane A), and the 0.9 kb *Ava*I–*Bgl*II fragment adjacent to the 3' end of the Tc^R gene (lane B). The positions of these DNA fragments are shown on Fig. 3. For calibration, *A. nidulans* RNA was run on the same gel (lane F); the band corresponding to the OTCase mRNA (1500 nucleotides) is visible on the autoradiogram (the hybridization probe was the same as in lanes C, D and E). The positions of the two rRNA classes (3300 and 1800 nucleotides) seen on the gel are marked.

Further evidence that the *A. nidulans argB* gene in plasmid pSal43-5 is transcribed from a prokaryotic promoter was obtained by replacing the Tc^R gene promoter with the P_R promoter of λ phage (Fig. 5b). The resulting plasmid (pSal23-51) was introduced into *E. coli* C600 OTC⁻ lysogenized with λ cI857 (a mutant with a temperature-sensitive repressor). The transformants did not grow on a medium without arginine at 30 °C, while they grew well at 37 °C. OTCase activity was 30 times higher in the cells cultivated at 37 °C than in those grown at 30 °C. This result showed that in plasmid pSal23-51 the *A. nidulans argB* gene is controlled by the λ promoter.

To determine whether the transcription of the *argB* gene from the bacterial promoter occurred as a result of the deletion or was already present in the original plasmid, *in vitro* transcripts of plasmids pSal23, pSal23-5 and pSal23-50 were analysed. The transcripts were separated by agarose gel electrophoresis under denaturing conditions, transferred onto nitrocellulose and hybridized with a ³²P-labelled 0.4 kb fragment of the *argB* gene (the *AluI*–*Bgl*II fragment, see Fig. 3). Only one predominant transcript, about 3000 nucleotides in size, was synthesized from pSal23-5 (Fig. 6). This is the size expected for the transcript that begins and finishes at the appropriate signals of the Tc^R gene. This transcript was not observed when pSal23-50 (which does not contain the Tc^R gene promoter) was used as the DNA template. In the case of pSal23, several transcripts were obtained. One of them, about 4000 nucleotides in size, corresponds to a possible mRNA extending from the promoter to the terminator of the Tc^R gene. This transcript showed no homology to pBR327 DNA fragments adjacent to the 5' and 3' ends of the Tc^R gene (Fig. 6, lanes A and B). It seems, therefore, that the Tc^R gene promoter of pSal23 is used for transcription of the *argB* gene, but the plasmid is still unable to complement the OTCase deficiency in *E. coli*, unlike its functional derivative pSal23-5. Thus the high expression of the *argB* gene of plasmid pSal23-5 (and pSal43-5) is not only due to efficient transcription.

A deletion could increase the level of translation of the *argB* gene by making available a ribosome-binding site present in the vector or by creating a new one. To investigate this, we

Fig. 7. Proteins synthesized in *E. coli* minicells carrying plasmids pSal23-50 (lane B), pSal23-51 (lane C) and pSal23-5 (lane D). ^{14}C -labelled proteins were isolated from minicells, separated in an SDS-polyacrylamide gel and visualized by fluorography. Proteins isolated from minicells containing no plasmid were run in the gel as a control (lane A). The position in the gel of standards and of the OTCase isolated from *A. nidulans* mycelium is indicated. The arrow indicates the band corresponding to the *argB* gene product in *E. coli*.

compared the OTCase synthesized in *E. coli* minicells with that present in *A. nidulans* mycelium. Strain λ 1849 was transformed separately with plasmids pSal23-4, pSal23-50 and pSal23-51 and minicells were isolated. Proteins synthesized in the minicells were labelled with ^{14}C , separated on a polyacrylamide gel and visualized by fluorography. OTCase isolated from *A. nidulans* mycelium (the procedure of isolation will be published elsewhere) was run on the same gel (Fig. 7). In extracts from minicells carrying pSal23-5 or pSal23-51 one additional 53 kDa protein band was present, which was not observed in the case of pSal23-50. We concluded that this band corresponds to the OTCase, because the only difference between pSal23-50 and the two other plasmids is the absence of a bacterial promoter for transcription of the *argB* gene in the former plasmid. The OTCase detected in the minicells was about 4 kDa larger than that isolated from *A. nidulans* mycelium, suggesting that in *E. coli* the translation of the *argB* gene starts upstream of the initiation site used in *A. nidulans*. Analysis of the nucleotide sequence of this region in pSal43-5 revealed the presence of a sequence that resembles the prokaryotic ribosome-binding site (Fig. 4). This sequence comprises a GTG codon preceded (with a gap of six nucleotides) by the sequence GGCGG, which closely resembles the Shine-Dalgarno sequence GGAGG (Kozak, 1983). The proposed ribosome-binding site is located at a junction between *A. nidulans* and pBR327 DNA: the GTG initiation codon comes from the *A. nidulans* DNA and the Shine-Dalgarno sequence from the vector. The use of this site, created by the deletion, to initiate the translation of the *argB* gene in *E. coli* could explain the efficient expression of this gene in cells carrying plasmid pSal43-5.

DISCUSSION

In this paper we have described the characterization of two types of DNA rearrangements that resulted in the activation of the *argB* gene from *A. nidulans* in *E. coli*. In one case there was an insertion and in the other case a deletion of DNA sequences.

The insertion present in two independently isolated plasmids was found to be IS2. In both cases IS2 was inserted near the 5' end of the *argB* gene, but in two different sites. This result is analogous to those obtained for other fungal genes cloned in *E. coli* (Walz *et al.*, 1978; Brennan & Struhl, 1980). In these examples, as well as in our case, IS2 was in orientation II relative to the activated gene. IS2 located upstream of a gene and in orientation II is known to be responsible for switching on or increasing the level of transcription of many bacterial genes (Kleckner, 1981; Glansdorff *et al.*, 1981). IS2 has been postulated to be a donor of the -35 element of the prokaryotic promoter (Jaurin & Nomark, 1983). Although we did not establish rigorously that IS2 is the source of the efficient promoter of the *A. nidulans argB* gene in *E. coli*, this seems to be a plausible interpretation. In plasmids containing IS2 the *argB* gene is situated such that it cannot be transcribed from any of the promoters identified in the vector (Stüber & Bujard, 1981; Queen & Rosenberg, 1981).

The second DNA rearrangement studied was identified as a deletion of DNA sequences adjacent to the 5' end of the *argB* gene. Transcription of the *argB* gene was dependent on the Tc^R gene promoter of the vector. *In vitro* experiments suggested, however, that this promoter can be used for transcription of the *argB* gene not only in the plasmid with the deletion but also in the parental plasmid which is unable to suppress the OTCase deficiency in *E. coli*. Therefore the deletion seems to affect post-transcriptional steps of *argB* gene expression. One possibility is that it results in an increase of the stability of the mRNA for OTCase. A second possibility is that the deletion affects the efficiency of translation of the *argB* gene. Sequence analysis indicated that the deletion created a prokaryotic ribosome-binding site at the junction between the *A. nidulans* and the vector DNA. This site is located 81 nucleotides upstream of the codon postulated to initiate translation of the *argB* gene in *A. nidulans*. If this putative ribosome-binding site is used in *E. coli*, the OTCase present in bacteria would be about 3 kDa larger than that synthesized in *A. nidulans*. The difference in size between the OTCase isolated from *E. coli* cells and *A. nidulans* mycelium could be even greater because the *A. nidulans* enzyme is known to be located in mitochondria (unpublished results) and is probably truncated during transport through the mitochondrial membrane, as are many other mitochondrial proteins (Schatz & Butow, 1983), including a human OTCase (Horwich *et al.*, 1984). The results presented in this paper show that the OTCase synthesized in *E. coli* minicells is about 4 kDa larger than that isolated from *A. nidulans* mycelium. This difference is consistent with the expected one and suggests that the putative ribosome-binding site created by the deletion is used for the initiation of translation of the *argB* gene in *E. coli*.

The results presented in this paper show that there are several barriers preventing the efficient expression of the *argB* gene in *E. coli*. One obstacle is the lack of proper signals for transcription of the *argB* gene. This could be overcome by using prokaryotic promoters on the vector or introduced by IS2. Other barriers are not so strong, because in plasmids with an IS2 insertion the increased efficiency of the transcription is enough to make the *argB* gene functional. Nevertheless, transcription from the Tc^R gene promoter is not sufficient for the effective expression of this gene and an increase in translation efficiency is also necessary.

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