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The glucose repressor CRE1 from *Sclerotinia sclerotiorum* is functionally related to CREA from *Aspergillus nidulans* but not to the Mig proteins from *Saccharomyces cerevisiae*

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Abstract We isolated the putative glucose repressor gene *cre1* from the phytopathogenic fungus *Sclerotinia sclerotiorum*. *cre1* encodes a 429 amino acid protein 59% similar to the carbon catabolite repressor CREA from *Aspergillus nidulans*. In addition to the overall amino acid sequence relatedness between CRE1 and CREA proteins, *cre1* can functionally complement the *A. nidulans creAd30* mutation as assessed by repression of the alcohol dehydrogenase I gene expression. The CRE1 region carrying the two zinc fingers is also very similar to the DNA binding domains of the *Saccharomyces cerevisiae* glucose repressors Mig1p and Mig2p. Despite the presence in the CRE1 protein of several motifs involved in the regulation of Mig1p activity, *cre1* cannot complement *mig* deficiencies in *S. cerevisiae*. These data suggest that glucose repression pathways may have evolved differently in yeasts and filamentous fungi.

Key words: CRE1; CREA; Mig1p; Zinc finger protein; Glucose repression; *Sclerotinia sclerotiorum*

1. Introduction

Filamentous fungi are able to grow under a variety of environmental conditions and have evolved regulatory mechanisms to ensure appropriate adaptive responses. Carbon catabolite repression is a wide domain regulatory system that controls the synthesis of a wide range of enzymes and represses the utilization of carbon sources when a more convenient source such as glucose is present in the growth medium. The mechanisms involved in glucose repression have been extensively investigated in *Saccharomyces cerevisiae*. Among the several components of the regulatory pathway that have been identified, Mig1p is the key regulator in glucose repression [1,2]. Mig1p is a Cys_2His_2 zinc finger-containing protein that binds to the promoters of glucose-repressed genes and recruits the general repressors Ssn6p and Tup1p [3,4]. Mig1p function is regulated in response to glucose. The roles of an opposing kinase and a phosphatase in glucose repression suggest that Mig1p function may be regulated by phosphorylation [4,5]. When glucose is absent, Mig1p activity is inhibited by the protein kinase Snf1p [6,7] which is activated under these conditions. Another glucose-activated repressor, Mig2p, that contains two zinc fingers highly similar to Mig1p has recently been characterized. However, Mig2p seems to be of little importance in glucose repression, regulating the invertase encoding gene *SUC2* but having little effect on other glucose-regulated genes [8]. Mig1p and Mig2p bind to similar sites but several differences exist between these glucose repressors as Mig2p is not inactivated by Snf1p and its expression is not regulated by glucose [9].

In filamentous fungi, genetic analyses have indicated that the major regulatory gene controlling glucose repression encodes a DNA binding protein which exhibits a strong similarity to Mig1p [10]. The *creA* gene of *Aspergillus nidulans* encodes a protein containing two Cys_2His_2 zinc fingers strikingly similar to those of Mig1p [10,11]. The recent identification of similar wide domain genes *cre1* from *Trichoderma harzianum* and *T. reesei* [12–14] and *crr1* from *Metarhizium anisopliae* [15] suggests that this regulatory mechanism is widely conserved amongst fungi. Mig1p, Mig2p and CRE each contain two zinc finger motifs closely related to a family of GC box binding proteins that includes the human Wilms' tumor and Sp1 proteins [16]. Binding of the yeast and fungal repressors requires a GC box with the consensus sequences 5′-SYGGGG-3′ and 5′-SYGGRG-3′ (S = C or G, Y = C or T and R = A or G) respectively [17,18].

Sclerotinia sclerotiorum is a ubiquitous necrotrophic fungus which is able to infect a wide range of plants, and to secrete a number of pectinolytic enzymes which macerate plant tissues [19]. Polygalacturonase activity is encoded by a multigene family [20,21] which includes numerous isoforms whose synthesis is induced by pectic polymers and repressed by glucose [22,23]. Analysis of the promoter sequences of several *pg* genes has revealed the presence of several copies of the CREA binding site (5′-SYGGRG-3′). In vitro, DNA binding assays have demonstrated that the fusion protein GST::CREA (corresponding to the glutathione *S*-transferase fused to the CREA zinc finger domain) binds to the different regions of the *pg1* promoter containing the 5′-SYGGRG-3′ sequence [24]. These data suggested that *pg* regulation may be modulated by a homolog to the *creA* gene.

We have isolated and characterized such a gene, *cre1*, which shows significant homology to the glucose repressors from other filamentous fungi and yeasts. Complementation of loss of function mutants from *A. nidulans* and *S. cerevisiae* revealed that CRE1 is a protein functionally related to CREA but not to Mig1p and Mig2p, suggesting that glucose repression has evolved differently in filamentous fungi and yeasts.

2. Materials and methods

2.1. Strains and growth conditions

S. sclerotiorum S5 strain was maintained on PDA (potato dextrose agar, Merieux).

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The *Escherichia coli* Sure R strain (Stratagene) was used for cloning and plasmid propagation. *E. coli* P2392 was used as host for bacteriophage lambda EMBL3 and was grown in NZY medium. DNA from lambda clones was purified by standard techniques and cloned into pUC18 [25].

A. nidulans creAd30 biA1 argB2 provided by B. Felenbok was cultivated in a complete medium [26]. *S. cerevisiae* strains H172 (*leu2::(GAL1:TPK2, HIS3)*, H174 (*mig1-Δ1::LEU2*) and H734 (*mig1-Δ1::LEU2, mig2::HIS3*) were provided by H. Ronne. They are all congenic to W303-1A and therefore contain the *MAT***a** *SUC2 ade2-1 can 1-100 his3-11, 15 leu2-3, 112 trp1-1 ura3-1* markers. Yeasts were grown at 30°C on standard synthetic minimal medium (yeast nitrogen base without amino acids (Difco) 0.67%, carbon source 2%) lacking the appropriate amino acids.

2.2. Molecular cloning of the cre1 gene

Genomic DNA was isolated as previously described [24]. For amplification of a *cre1* fragment from the genomic DNA of *S. sclerotiorum* two mixed degenerate oligonucleotide primers were used. Their sequences were: 5′-GTCGGATCCCAYGCNTGYCARTTYCCNG-G-3′ (HACQFPG) and 5′-CTGAAGCTTGGNGTRTGRTCNGGN-GT-3′ (TPDHTP) where N indicates an equimolar mixture of all four nucleotides, R of A and G, and Y of C and T. The reaction mixture for PCR contained 30 ng of genomic DNA as a template, 100 pmol of each primer and 1.25 U of *Taq* DNA polymerase in PCR buffer (Tris-HCl 10 mM pH 9, KCl 50 mM, $MgCl_2$ 1.5 mM, Triton X-100 0.1%, bovine serum albumin (BSA) 0.2 mg/ml; Appligene). Amplification was initiated by 10 cycles of 90 s each at 95°C for denaturation, followed by an annealing temperature decreasing linearly from 53 to 50°C during 60 s and an amplification step at 75°C during 60 s. Subsequently 30 cycles were applied, each consisting of 60 s at 95°C, 60 s at 52°C and 30 s at 75°C. The amplification program was terminated by a 10 min extension step at 75°C.

The major DNA fragment of 650 bp amplified along with several weakly amplified fragments was digested and isolated by absorption to glass silica beds (Geneclean II, Bio 101), then cloned into *Bam*HI-*Hin*dIII digested Bluescript SK^+ and sequenced. The identity of the 650 bp DNA fragment was established by sequencing and comparison of the translated sequence to those of known glucose repressors. The cloned PCR fragment was labeled with [α^{32}P]dCTP and used as a probe to screen a genomic library of *S. sclerotiorum* constructed in the EMBL3 lambda phage [27]. A 3 kb *Xba*I-*Eco*RI genomic DNA fragment carrying the *cre1* entire coding region, including 1.1 kb of DNA to the 5′ end of the predicted open reading frame and 0.8 kb to its 3′ end, was cloned into pUC18 to give the pGV2 plasmid.

2.3. General DNA techniques

Standard methods were used for plasmid isolation, restriction enzyme digestion, random priming and plaque hybridization [25]. DNA sequencing of plasmid clones was accomplished by the dideoxy chain termination method [28], using either universal primers or oligonucleotide primers to yield complete sequences from both strands and according to the manufacturer's recommendations (Pharmacia Biotech). Sequence analysis was done with the CLUSTALW PILEUP program of the Wisconsin program package.

2.4. Fungal and yeast transformation

A. nidulans creAd30 biA1 argB2 was co-transformed with the *A. nidulans argB* harboring plasmid pFB39 and the pGV2 vector. Transformation was carried out essentially as described by Tilburn and al. [29] except for the use of Glucanex (SEPCA) as lytic enzymes and BSA respectively at 30 mg and 5 mg/g dried mycelium, for protoplast production. Transformants were first selected on an arginine-lacking minimal glucose medium supplemented with biotin, using NH_4Cl as nitrogen source, and then tested for their ability to grow on the same medium supplemented with 2.5 mM allyl alcohol.

Plasmids were constructed to transform the yeast mutants by electroporation [30]. A *cre1* fragment carrying the entire coding region and 0.8 kb of the terminator was amplified by PCR from pGV2 plasmid using the oligonucleotide primers 5′-CACTCTAGACACT-TATTCGCTGCGG-3′ and 5′-CCCATCTATTTGTTAC-3′ corresponding to the genomic sequences located respectively 11 bp upstream of the start codon and 873 bp downstream of the stop codon. The reaction mixture was as described above except for the use of 5 ng of pGV2 as a template. Amplification was initiated by a 90 s denaturation step at 95°C, followed by 30 cycles each consisting of 60 s at 95°C, 60 s at 47°C and 180 s at 75°C. The amplification program was terminated by a 10 min extension step at 75°C. The PCR product was then digested with *Xba*I and *Eco*RI restriction enzymes and placed under the control of the *MET3* promoter in the episomal multicopy pJO177 vector carrying the ampicillin resistance gene and the *URA3* marker. Transformants were selected on uracil-lacking glucose media. *cre1* gene expression was induced on methionine-lacking media. H172 and H174 transformants obtained with the pJO177 vector containing the *cre1* gene were respectively tested for their ability to grow on galactose and raffinose with or without 2-deoxyglucose minimal media. H734 cells were transformed with both the *cre1* carrying pJO177 vector and the *SUC2-lacZ* reporter plasmid pJO27 harboring the ampicillin resistance gene and the *TRP1* marker [6].

β-Galactosidase activity of the transformants selected on uracil- and tryptophan-lacking minimal glucose medium was tested in permeabilized cells grown to mid-log phase in 4% glucose medium (repressed cultures) or shifted for 3 h to 5% glycerol and 0.05% glucose medium (derepressed cultures). β-Galactosidase activities presented in the text are averages of at least three assays performed with three independent transformants.

3. Results and discussion

3.1. Isolation and characterization of cre1 from S. sclerotiorum

Degenerate oligodeoxynucleotide primers were designed on the basis of alignment of *Aspergillus* CREA sequences [10,31]. Primers corresponding to amino acids HACQFPG and TPDHTP in the deduced peptide sequence of *creA* allowed amplification of a major DNA fragment of 650 bp whose sequence was homologous to the translated sequence of fungal glucose repressors (not shown).

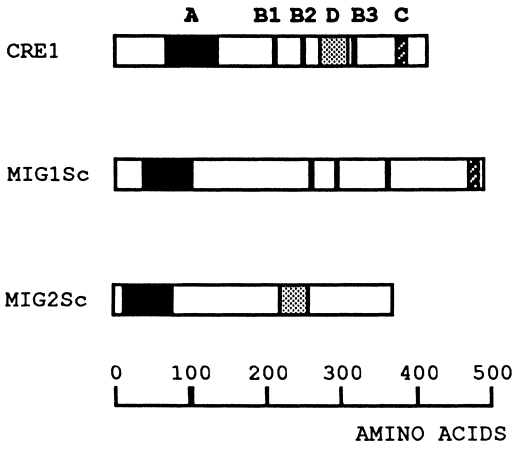
The 650 bp PCR fragment was then used as a probe to screen a genomic library constructed in the lambda phage EMBL3. Seven positives clones were obtained from 4×10^4 recombinant phages screened. The clones were restriction mapped and shown to contain a common 1.1 kb *Sal*I-*Eco*RI fragment that hybridized to the PCR fragment. Overlapping fragments were subsequently isolated and the sequence of the gene was determined (EMBL Nucleotide Sequence Database accession number AJ000976). The *cre1* gene has an intronless open reading frame of 1287 bp encoding a protein of 429 amino acids. The deduced *S. sclerotiorum* CRE1 protein has a calculated M_r of 47 kDa.

Southern blot analysis of *S. sclerotiorum* genomic DNA showed that the PCR fragment hybridized to a single fragment when DNA was digested with *Eco*RI, for which no internal site was present in the cloned fragment. Two hybridizing restriction fragments were detected when Southern analyses were performed following genomic DNA digestion with *Bam*HI or *Bgl*II which cut once in the PCR fragment (not shown). This indicates that only one CREA-homologous glucose repressor encoding gene can be detected in the genome of *S. sclerotiorum*.

3.2. Structural comparison between CRE1 and other CRE and Mig proteins

The degree of amino acid sequence identity between CRE1 and the glucose repressors from other filamentous fungi varied from 55 to 61% (59% to CREA from *A. nidulans*). Several regions of significant identity are found outside the conserved zinc finger regions (Fig. 1).

Comparison between CRE1 and Mig1p revealed very little similarity outside the highly conserved DNA binding domains which are almost identical in fungi and yeasts. However, several domains of the Mig proteins were recognized on the CRE

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A. CRE1    67 LPRPYKCPLCEKAFHRLEHQTRHIRTHTGEKPHACLFPGCTKRFSRSDELTRHSRIHNN--PNSRRSNKTQ 135
   CREA    60 LPRPYKCPLCERAFHRLEHQTRHIRTHTGEKPHACQFPGCSKRFSRSDELTRHSRIHNN--PNSRRGNKAQ 128
   MIG1Sc  34 APRPHACPICHRAFHRLEHQTRHMRIHTGEKPHACDFPGCVKRFSRSDELTRHRRIHTNSHPRGKRGRKKK 104
   MIG2Sc  13 ENRPFRCDTCHRGFHRLEHKKRHLRTHTGEKPHHCAFPGCGKSFSRSDELKRHMRTHTG--QSQRRLKKAS  81
                 **. *  *...******. **.* ******* * **** * ******* ** * *..    ..*  * .

B.1) CRE1    221 QVERDSSTTANH 232
     CREA    217 QVERDESFGFRS 228
     MIG1Sc  272 ILPRPRSLTDFQ 283

  2) CRE1    260 AMTRAYSHEEDD 271
     CREA    256 SMSRSHSHEDED 267
     MIG1Sc  305 QLKRPSSVLSLN 316

  3) CRE1    329 PGIRNLSLHHTP 340
     CREA    326 PSIRHLSLHHTP 337
     MIG1Sc  375 MLSRSTSGTNLH 386

C. CRE1    389 VPQAP-VAV 396
   CREA    379 IPQVPKVAV 387
   MIG1Km  529 LPSLRSLDI 537
   MIG1Sc  490 LPPIRSLPL 498

D. CRE1    279 KRSRPNSPMSTAPSSPTFSHD---SLSPTPDHTPLATPA---HSP 317
   CREA    275 KRSRPNSPNSTAPSSPTFSHD---SLSPTPDHTPLATPA---HSP 313
   MIG2Sc  227 HQT--ASP-SVAKQQKTFAHSLASALSTLQKRTPVSAPSTTIESP 268
                  ...   ** *.* .. **.*.   .**    .**...*. . .**
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Fig. 1. Overall structural homology between the fungal and yeast glucose repressors. Comparison of the predicted amino acid sequence of *cre1* from *S. sclerotiorum* (CRE1) with the corresponding sequences of *creA* from *A. nidulans* (CREA), *mig1* from *S. cerevisiae* (MIG1Sc) or *K. marxianus* (MIG1Km) and *mig2* from *S. cerevisiae* (MIG2Sc) was performed with the LALIGN program from the FASTA package. Amino acids that are conserved in all proteins are marked by asterisks. Dots indicate conservative replacements. Dashes indicate gaps introduced to achieve optimal alignment. Amino acids are numbered relative to the translation start point. A: Sequence alignment of the zinc finger domain. Basic amino acids at the N-terminal end of the DNA binding domain are shown in bold. B: Comparison of the CRE1 and CREA Snf1p putative phosphorylation sites to the Snf1p motifs recognized in Mig1p from *S. cerevisiae*. The key residues are shown in bold. C: Comparison of the CRE1 and CREA N-terminal regions to the effector domain of Mig1p from *K. marxianus* and *S. cerevisiae*. Hydrophobic amino acids at positions homologous to those of the hydrophobic residues required for the yeast repressor activity are shown in bold. D: Sequence alignment of the Mig2p homologous domains.

sequences (Fig. 1). The region which extends beyond the Cys_2His_2 motifs includes a stretch of basic amino acids, similar to the organization observed in Mig1p (Fig. 1A). Four motifs **RXXS**, a common core site shared by several serine protein kinases, which has been proposed as the likely phosphorylation site involved in regulation of Mig1p activity [6,7,32], are present in CRE1. Three of these motifs, at positions 224, 263 and 332, are preceded by a hydrophobic residue as the phosphorylation sites of AMPK [33] (Fig. 1B). They are similar to the consensus sequence ΦXRXXSXXXΦ (where Φ represents a hydrophobic residue) for phosphorylation of Mig1p by the protein kinase Snf1p as defined by Dale et al. [34]. These phosphorylation sites are also found in the other CRE proteins, and they have a relative position similar to that of the sites found in Mig1p at positions 275, 308 and 378 (Fig. 1B). They may be of importance as it has been found that Mig1p with serines at position 278, 311 and 381 replaced by alanines is not further inactivated by the protein kinase Snf1p in the absence of glucose [7,35]. The carboxy-terminal region $\mathbf{V}_{389}$**PQAPVAV**$_{396}$ is rich in hydrophobic amino acids, like the $\mathbf{L}_{529}$**PSLRSLDI**$_{537}$ motif found in Mig1p from *Kluyveromyces marxianus*, conserved in *K. lactis* and in *S. cerevisiae*

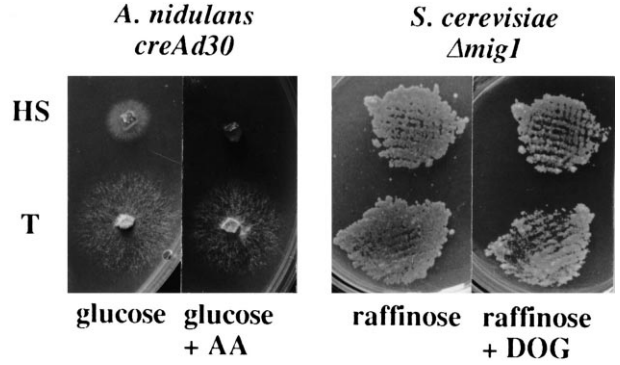

Fig. 2. Complementation experiments of a *creA* mutant of *A. nidulans* and a *mig1* mutant of *S. cerevisiae* with the *S. sclerotiorum cre1* gene. Left: Growth of the *A. nidulans creAd30* host strain (HS) and a selected transformant (T) on 2% glucose medium in the absence or presence of 2.5 mM allyl alcohol (AA). Right: Growth of the *S. cerevisiae Δmig1* host strain H174 (HS) and a selected transformant (T), first grown on 2% glucose medium, then replicated to 3% raffinose medium in the absence or presence of 20 μg/ml 2-deoxyglucose (DOG).

Mig1p, and required together with the zinc finger domain for mediating repression [6,36] (Fig. 1C).

Comparison of the CRE1 deduced polypeptide sequence to Mig2p revealed that outside the DNA binding domains, a CRE1 region (aa 279–317) highly conserved in fungal glucose repressors exhibits 70% homology to a region of Mig2p (Fig. 1D).

The low level of similarity observed between Mig1p, Mig2p and CRE homologs outside the zinc fingers may represent their divergent phylogenetic relationship. While these regulatory proteins have similar functions, the presence of a limited number of common motifs in Mig proteins and CRE homologs may indicate that these motifs are important for the repressor activities.

3.3. *CRE1 is functionally related to CREA in A. nidulans*

A 3 kb fragment containing the entire coding region and the promoter sequence of *cre1* was cloned into pUC18 to give the plasmid pGV2. This vector was used in combination with the plasmid pFB39 carrying the selection marker *argB* to co-transform the *A. nidulans creAd30 argB2 biA1* mutant. About 50% of the transformants selected on an arginine-lacking medium were able to grow in the presence of 2.5 mM allyl alcohol and glucose. This indicates that CRE1 mediates glucose repression of the alcohol dehydrogenase I encoding gene preventing conversion of allyl alcohol into acrolein which inhibits growth of the glucose non-repressed host strain (Fig. 2). Complementation of CREA loss of function by CRE1 was supported by genetic and molecular analyses. The presence of *cre1* in the allyl alcohol-resistant transformants was revealed by Southern blotting and confirmed by PCR using *cre1*-specific primers (not shown). The resistance phenotype was not due to the inactivation of the ethanol regulon by insertion of the transforming plasmid as the transformants were able to grow on an ethanol minimal medium. Thus in addition to the overall amino acid sequence relatedness between CRE1 and CREA proteins, CRE1 can functionally complement the *A. nidulans creAd30* mutation.

3.4. *cre1 does not complement mig deficiencies in S. cerevisiae*

In order to establish whether CRE1 could be functionally related to Mig1p and Mig2p, we determined whether *cre1* was able to complement glucose repression deficiency in yeast. The *cre1* coding region was cloned into the episomal plasmid pJ0177 under the control of the *MET3* promoter and used to transform two *Δmig1 S. cerevisiae* mutants. The strain H172 carries the *GAL1:TPK2* construction whose expression is induced by galactose and repressed by Mig1p in the presence of glucose. Overproduction of TPK2, a catalytic subunit of cAMP-dependent protein kinase, is lethal in yeast. H172 transformants did not survive on galactose medium, demonstrating that CRE1 cannot counteract *GAL1* promoter activation and cannot restore the Mig1p repressor function in the absence of glucose. This result was confirmed when *cre1* was introduced in the *Δmig1* strain H174, able to grow on a raffinose medium even in the presence of 2-deoxyglucose (DOG), a gratuitous inducer of glucose repression in yeast. *cre1* was unable to repress the expression of *SUC2* as the transformants were able to grow on the raffinose and raffinose+DOG media. The *cre1* gene expression in the transformants was confirmed with the detection of CRE1 amongst intracellular yeast proteins analyzed by SDS-PAGE and Western blot using antibodies raised against the zinc finger domain (not shown). The *cre1*-carrying plasmid pJ0177 was also used with pJ027 harboring the *SUC2-lacZ* reporter construct, to co-transform the *Δmig1 Δmig2* deleted strain (H734). β-Galactosidase activities of the host strain (*Δmig1 Δmig2*) and of the transformants (*Δmig1 Δmig2 cre1*) grown under repressing conditions (4% glucose) were similar (13 ± 1 and 12 ± 1 units respectively). When the yeast cells were grown under derepressing conditions (5% glycerol and 0.05% glucose), the β-galactosidase activity of the transformants was slightly reduced (33 ± 1 units) compared to the activity of the host strain (42 ± 6 units), confirming that *cre1* cannot fully complement *mig* deficiencies to induce glucose repression.

DNA binding activity of Mig1p is regulated by Snf1p and repression depends on the interaction of Mig1p with Tup1p and Ssn6p. Fungal genes homologous to the yeast genes involved in the Mig1p repression pathway have been identified. In *Neurospora crassa*, *rco-1* is similar to Tup1p but does not seem to be involved in glucose repression [37]. The inability of *cre1* to complement *mig* mutants indicates that glucose repression, mediated by similar zinc finger proteins, may involve different pathways and that homologous genes may have different functions in yeast and filamentous fungi. One cannot exclude that several glucose repressors may exist in filamentous fungi as in yeast and that the *mig* homologous gene has not yet been characterized. Identification of other fungal homologs of genes involved in the yeast glucose repression pathway is now in progress. We have isolated a Snf1p homologue in *S. sclerotiorum* (Vacher et al., unpublished). Functional analysis of these genes will provide complementary information concerning the pathway of carbon catabolite repression in fungi.

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