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The C-terminus of the 14-kDa subunit of ubiquinol-cytochrome-c
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is involved in the assembly of a functional enzyme

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Disruption of QCR7, the gene encoding the 14-kDa subunit of ubiquinol-cytochrome-c oxidoreductase of the yeast *Saccharomyces cerevisiae*, results in an inactive enzyme which lacks holo-cytochrome *b* and has severely reduced levels of apo-cytochrome *b*, the Rieske Fe-S protein and the 11-kDa subunit [Schoppink, P. J., Berden, J. A. & Grivell, L. A. (1989) *Eur. J. Biochem.* 181, 475–483].

An episomal system was developed to study the effect on complex III of transformation of *in vitro* mutagenised QCR7 genes to a QCR7⁰ mutant. Transformation of a gene (TNT1) in which the 12 C-terminal residues are replaced by 3 amino acids encoded by an oligonucleotide containing a stop codon in all three reading frames (STOP-oligonucleotide), only leads to partial complementation of the respiratory capacity of the yeast strain. The amounts of apo-cytochrome *b*, the Rieske Fe-S protein and the 11-kDa subunit are reduced and enzymic activity, together with the amount of holo-cytochrome *b*, is lowered to about 40% of that of the wild type, indicating a normal turnover number of the mutant enzyme.

Transformation of the QCR7⁰ mutant with another gene (TNT2) encoding the first 96 residues of the 14-kDa subunit fused to 9 amino acids encoded by the STOP-oligonucleotide, leads to a phenotype almost indistinguishable from that of the QCR7⁰ mutant.

The role of the charged C-terminus of the 14-kDa (and the 11-kDa) subunit in the assembly of a functional complex III is discussed.

Ubiquinol-cytochrome-c oxidoreductase (complex III or the bc₁ complex) is an oligomeric respiratory-chain enzyme, which invariably consists of at least three subunits: cytochrome *b*, cytochrome *c*, and the Rieske Fe-S subunit. These subunits, which carry the prosthetic groups, are functionally well characterised and have been shown to be essential for the activity of the enzyme. Fully active three-subunit bc₁ complexes can be isolated from bacteria such as *Paracoccus denitrificans* and *Rhodospirillum rubrum* [1, 2]. All known eukaryotic bc₁ complexes contain additional subunits, implying a more complicated situation in these cases, but in spite of a great deal of experimental effort the precise physiological role of these extra subunits remains enigmatic ([3, 4] and references therein). The bc₁ complex of the yeast *Saccharomyces cerevisiae* contains six or seven additional subunits which are named, in order of descending molecular mass the 44-, 40- (also called core I and core I₁ subunits), 17-, 14-, 11-, 8.5- and 7.3-kDa subunits [S-111.

The 14-kDa subunit VII of yeast complex III shows at least 45% identity to the 14-kDa subunits of human, rat and bovine complex III [12]. Initially these 14-kDa subunits were thought to be ubiquinone(Q)-binding proteins since both in yeast and bovine heart complex III a subunit running as a protein of approximately this molecular mass was able to bind a radiolabelled azido-Q analogue [13, 14], but due to the different electrophoresis conditions applied and to their anomalous running behaviour, the identification of these proteins was not straightforward.

Berden et al. [15] concluded that for the bovine heart bc₁ complex it was subunit 7, currently known as the 9.5-kDa subunit, that was labeled by azidoquinone. Recently it was unambiguously confirmed that in the beef heart complex it is not the 14-kDa subunit but the 9.5-kDa subunit which binds azido-Q, while also the binding domain was established [16]. Since this 9.5-kDa subunit shows low but significant similarity to the 11-kDa [17], but not to the 14-kDa subunit of yeast complex III, the role of this latter subunit in Q binding remains unclear although some authors still claim it to be the Q-binding protein of complex III [18, 19].

The 14-kDa subunit was shown to be essential for the assembly of yeast complex III. A mutant in which the QCR7, the gene coding for the 14-kDa subunit, was disrupted, was unable to grow on non-fermentable carbon sources, due to an inactive bc₁ complex [20]. It was shown that the mutant

Abbreviations. Q₁H₂, 2,3-dimethoxy-5-methyl-6-geranyl-1,4-benzoquinol; Q, ubiquinone.

Enzymes. Ubiquinol-cytochrome c oxidoreductase (EC 1.10.2.2); cytochrome c oxidase (EC 1.9.3.1).
lacked holo-cytochrome b and had severely reduced levels of the Rieske Fe-S protein and the 11-kDa subunit. From the phenotype of this QCR7" mutant and the similar phenotypes of the QCR8" mutant [21] and mutants lacking functional cytochrome b [8], it was concluded that the 14-kDa subunit, together with the 11-kDa subunit and cytochrome b, forms a pre-assembly complex which is sensitive to endogenous proteases until association with the protease-resistant subcomplex formed by the two core subunits has taken place. Since this association is prevented in the QCR7", the QCR8" and the cytochrome b mutants, the remainder of the cytochrome b subcomplex is degraded when either of these subunits is missing [22, 23].

The pleiotropic effects, caused by the disruption of the QCR7" gene, preclude analysis of the possible mechanistic involvement of this subunit in the activity of the enzyme. Indications of an involvement of the 14-kDa subunits in the activity of the bc1 complexes of yeast [24] and beef-heart [25] came from studies using specific antibodies directed against these subunits. It was shown that these antibodies were able to inhibit complex III activity. In the bovine heart enzyme the complex had to be delipidated prior to incubation with the antibodies, indicating that the catalytic-site-related epitopes are not exposed when phospholipids are present, which seems to be in contrast with the situation in yeast. From their results the authors concluded that the 14-kDa subunit faces the matrix side of the enzyme, which is in agreement with the results obtained by our group [26], and that it is not involved in the Qo site [25], but in the Qo site [24].

Previously Schoppink et al. [20] showed that the pleiotropic properties of the QCR7" mutation could be relieved by a wild-type gene replacement but no systematic efforts were made to establish an episomal system. To study the effect on wild-type gene replacement but no systematic efforts were  

Plasmids

Single-copy and multi-copy shuttle plasmids YCplac33 and YEplac195 [27] were used as carriers of the wild-type or the mutated QCR7" genes.

The wild-type QCR7" gene was liberated as a 1.3-kb BamHI–XbaI fragment, which does not contain the complete QCR7" promoter [20], from plasmid pEMBL9-14k (Fig. 1a) and cloned into the polylinker of the plasmids YCplac33 and YEplac 195 [24], digested with the same enzymes, giving plasmids YCp-QCR7 and YEp-QCR7 (Fig. 1b).

Plasmid M13mp8 was used to obtain Bal31 deletion clones and to use its HindIII site, which is at the 3' end of both its HindIII (used to obtain mutant TNT2) and PstI site (used to obtain mutant TNT1).

Plasmid pKTH601 [28] contains a STOP-oligonucleotide between its HindIII and BamHI site. This STOP-oligonucleotide encodes a TGA stop codon in all three reading frames. Truncated QCR7" genes were liberated from M13mp8 as HindIII fragments and ligated into the HindIII site of pKTH601, thereby generating a stop-codon behind the truncated genes when properly oriented.

Isolation of mitochondria and immunoblotting

Mitochondria were isolated as described earlier [29]. Mitochondrial proteins were separated on SDS/polyacrylamide slab gels according to [30] and blotted according to [31]. After incubation of the blots with antibodies directed against specific subunits of complex III [26], the antigen/antibody complexes were made visible with the horseradish peroxidase colour-development assay [32]. The antisera used were raised in rabbits or mice.

Spectral analysis and complex III assay

Spectral measurements were carried out at room temperature in an Aminco dual-wavelength spectrophotometer model DW2. Concentrations of cytochromes were determined using the following absorption coefficients and wavelengths for the reduced minus oxidised proteins: 24.0 mM⁻¹ cm⁻¹ at 605–630 nm for cytochrome aa₃ [33], 20.1 mM⁻¹ cm⁻¹ at 550–540 nm for cytochromes c₁+c₃ [34], 28.5 mM⁻¹ cm⁻¹ at 562–575 nm for cytochrome b [35]. The ubiquinol-cytochrome-c oxidoreductase assay was performed spectrophotometrically at 25°C by measuring the reduction of 18 µM horse-heart ferricytochrome c at 550–540 nm by 25 µM 2,3-dimethoxy-5-methyl-6-geranyl-1,4-benzoquinol (QH₂). The buffer used contained 20 mM potassium phosphate, pH 7.4, 2 mM EDTA and 0.5 mM potassium cyanide.

Spectral and kinetics data were analysed using Popspec software on an Atari 1040ST computer coupled to the spectrophotometer via the Poptronics 4 channel A/D converter.

Myxothiazol and antimycin were dissolved in absolute ethanol and the concentrations were determined spectrophotometrically as described [36, 37].
positive phenotype. We decided to use this construct for our further experiments.

**RESULTS**

**Episomal complementation of yeast strain D11-QCR7**

Results obtained earlier in our laboratory with a QCR7 disruption mutant in the wild-type strain HR2 showed that functional complementation was only possible after reinteg-

ration of the QCR7 gene into its original locus [20]. To be able to select for the URA3 gene, present on the plasmids YCplac33 and YEplac195 [27], we decided to make a new QCR7 null mutant in the wild-type strain D11, using the knock-out construct described in [20].

Transformation of the multi-copy and single-copy plasmids YEplac-QCR7 and YCp-QCR7 (see Materials and Methods and Fig. 1b) to DL1-QCR7 resulted in successful episomal complementation, since the transformants displayed both uracil prototrophy and a glycerol-positive or ethanol-

positive phenotype. Western blot analysis on mitochondrial protein isolated from 0.3% glucose, 2% galactose-grown cells of D11, D11-

QCR7, and from null mutants transformed with either YCp-

QCR7 or YEplac-QCR7 showed that the D11-QCR7 mutant has the same phenotype as described for the HR2-QCR7 mutant [20] and that, due to the absence of a complete QCR7 promoter [20], complementation of the QCR7 strain with the single copy plasmid YCp-QCR7 does not completely restore the levels of cytochrome b, the Rieske FeS-protein and the 11-kDa subunit to the wild-type levels, while complementation with the multi-copy plasmid YEplac-QCR7 does (data not shown, but see Fig. 5).

These results were confirmed by measuring the specific activity of complex III in the mitochondria and by measuring the amounts of the cytochromes via optical spectra. Compl-

ementation of the QCR7 mutant with the single-copy plasmid restores the specific activity and the holo-cytochrome b content to only approximately 70% of that of the wild-type and the multi-copy transformant (data not shown).

Since we now were able to complement a QCR7 mutant to the wild-type level with the QCR7 gene present on the multi-copy vector YEplac195, we decided to use this construct for our further experiments.

**Construction of C-terminal deletion mutants**

To construct QCR7 genes with truncated 3' ends we made use of the plasmid pKTH601 [28], which contains a STOP-oligonucleotide ligated between its HindIII and BamHI site. YEplac-QCR7 (Fig. 1b) was linearised with XbaI, the position of its site being approximately 650 nucleotides downstream of the QCR7 stop codon, and treated with exonuclease Bal31 to shorten the 3' end of the QCR7 gene. After generating blunt ends, the truncated QCR7 genes were liberated from YEplac-QCR7 with BamHI and subcloned into BamHI/HincII digested M13mp8, to obtain a HindIII site at the 3' end of the QCR7 gene (Figlc). Several plasmids obtained in this
Fig. 3. Analysis of the growth characteristics of the wild type, the wild-type transformant, the TNT1 and the TNT2 transformants and the QCR7" strain. Cells of the different yeast strains were streaked on plates containing glucose-rich medium (YPD), minimal-glucose medium supplied with histidine and uracil (+His+Ura), minimal-glucose medium supplied with only histidine (+His) or glycerol-rich medium (EthGly). See Materials and Methods for details of the composition of the media.

way were sequenced to determine the length of the truncated QCR7 genes.

The longest QCR7 gene present in this library appeared to miss 94 nucleotides of the coding sequence. This gene, which was named TNT2, was liberated from M13-mp8 as a HindIII fragment and ligated into pKTH601 digested with the same enzyme, after which correctly oriented inserts were selected (Fig. 1d).

To construct a QCR7 gene with a shorter deletion at the 3' end, YEp-QCR7 was digested with PstI and this PstI fragment (missing the last 43 nucleotides of the QCR7 gene) was ligated into M13mp8 digested with the same enzyme. Plasmids with inserts in the correct orientation were digested with HindIII and the fragment containing this truncated QCR7 gene (which was named TNT1) was ligated into pKTH601 linearised with HindIII. Again plasmids with the inserts in the correct orientation were selected.

Both the TNT1 and the TNT2 insert were liberated from pKTH601 by digestion with EcoRI and BamHI and ligated into the multi-copy shuttle vector YEplac195 digested with the same enzymes.

Fig. 2 shows the amino acid sequence encoded by the wild-type QCR7 gene (Fig. 2A) and by the two truncated genes TNT1 (Fig. 2B) and TNT2 (Fig. 2C), as determined after a final sequence analysis.

Properties of the transformants

Fig. 3 shows the growth characteristics of the D11 wild type, the YEpl-QCR7 transformant, the TNT1 and TNT2 transformants, and the QCR7" strain on different solid media, after one week of growth. It can be seen that all the yeast strains grow on glucose-rich medium (Fig. 3) and that all but the wild-type grow on minimal-glucose medium without leucine (Fig. 3), indicating the presence of the LEU2 gene at the QCR7 locus. The same medium, but now without both leucine and uracil (Fig. 3), shows that complementation has taken place in the transformants without re-integration of the QCR7 gene. When the strains are grown on ethanol/glycerol plates (Fig. 3), the D11 wild-type and the YEpl-14k transformant grow at approximately equal rates, as indicated by the size of the colonies. The TNT1 transformant grows slower while the TNT2 transformant, and of course the QCR7" strain, do not grow at all.

Fig. 4 shows the result of a Western blot analysis of mitochondria from 0.3% glucose, 2% galactose grown cells of D11, the YEpl-QCR7 transformant, the QCR7" mutant and the TNT1 and TNT2 transformants. Equal amounts of wild-type and mutant mitochondrial protein were separated on a SDS/polyacrylamide gel and blotted onto nitrocellulose paper to determine the steady-state levels of the complex III subunits. It is shown in Fig. 4, lanes 2 and 3, that in the blots from the TNT1 and TNT2 transformants there is no band visible which can be attributed to the truncated 14-kDa proteins. It should be noted that such bands are also invisible when Western blots of total cell lysates of the transformants are incubated with these antibodies. It is also seen that the amounts of apo-cytochrome b, the Rieske Fe-S protein and
Fig. 5. Spectral analysis of the cytochrome content of mitochondria isolated from wild-type, wild-type transformant, the TNT1 and TNT2 transformant, and the QCR7" strain cells. Mitochondrial protein was isolated from cells grown on glycerol medium. The difference spectra were recorded at room temperature, protein concentrations were 4.5 mg/ml for the wild type, the wild-type transformant and the TNT1 transformant, 5 mg/ml for the TNT2 transformant and 6 mg/ml for the QCR7" mutant. (A) Difference spectrum of succinate-reduced mitochondria in the presence of antimycin, minus ferricyanide-oxidized mitochondria. (B) Ascorbate+KCN were added to the sample cuvettes of traces 1; the reference cuvettes remained the same. Traces 1, mitochondrial protein isolated from the QCR7" strain; traces 2, mitochondrial protein isolated from the TNT2 transformant strain; traces 3, mitochondrial protein isolated from the TNT1 transformant strain; traces 4, mitochondrial protein isolated from the YEp-QCR7 transformant strain; traces 5, mitochondrial protein isolated from the wild-type strain.

The 11-kDa protein are diminished in mutant TNT1 (Fig. 4) compared to the amounts of these proteins in the wild type and the YEp-QCR7 transformant (Fig. 4). Comparison of the TNT2 transformant with the QCR7" mutant (Fig. 4) shows that the amounts of all the detected bc1 subunits are approximately equal. Furthermore a band just below cytochrome b is visible in the TNT2 transformant which probably is a breakdown product of the former. Since this band is not visible in the QCR7" mutant this could mean that the proteolytic breakdown of the pre-assembly complex formed between cytochrome b and the 14-kDa and the 11-kDa subunits is slower in the presence of the truncated 14-kDa protein than when no 14-kDa subunit is present.

Fig. 5A and B and Table 1 show the results of the spectral and the kinetics analyses of the different yeast strains. The difference spectra of Fig. 5A show that in the presence of antimycin and succinate no holo-cytochrome b can be detected in the TNT2 transformant (and of course in the QCR7" mutant), while the amount of holo-cytochrome b in transformant TNT1 is clearly less than that of the wild type and the YEp-QCR7 transformant. Calculation of the turnover number of complex III according to the amount of cytochrome b and the specific complex III activity measured in the mitochondria (Table 1), shows that this is approximately the same in the wild type, the YEp-QCR7 transformant and the TNT1 transformant, showing that in the TNT1 transformant the assembly but not the kinetics of the enzyme is impaired. Fig. 5B shows that the levels of cytochromes \( c + c_1 \) are slightly less in the transformants compared to the wild-type (see also Table 1), while the amounts of cytochrome \( aa_3 \) are equal. The QCR7" mutant shows less of the cytochromes \( c + c_1 \) and especially the reduction of the amount of cytochrome \( aa_3 \) is evident. This was also reported for the QCR7" mutant of HR2 [20] where the low amount of cytochrome \( aa_3 \) was thought to be due to the growth of the null-strain on maltose. The fact that such a phenotype is not found in a QCR8" mutant [21] and, more important, in the TNT2 transformant, which is otherwise indistinguishable from the QCR7" mutant, may point to a more causal relation between the N-terminal part of the 14-kDa subunit and the level of cytochrome \( aa_3 \).

We have also studied the effect of the specific \( Q_{0o} \)- and \( Q_{0a} \)-site inhibitors myxothiazol and antimycin on mitochondria of the TNT1 transformant as compared to those of the wild-type. Aliquots of mitochondria, calculated to have equal amounts of complex I11, were incubated for 5 min with varying amounts of either of the inhibitors, prior to measuring the complex III activity. No differences were found in the affinities for myxothiazol or antimycin between the wild type and the mutant complex III (data not shown).

DISCUSSION

QCR7, the gene coding for the 127-amino-acid 14-kDa subunit of the bc1 complex of \textit{S. cerevisiae}, is a single-copy nuclear gene located on chromosome IV [20]. Disruption of this gene leads to a yeast strain unable to grow on non-fermentable carbon sources, due to an improperly assembled and inactive complex III.
Both have an inactive respiratory chain, this possibility can include introduction of the mutated genes into the null mutant. The presence of a large N-terminal part of the 14-kDa subunit encoding its first 66 amino acids, fused to residues encoded in electron transport, but is relevant for assembly. The C-terminus of this subunit is therefore not directly involved in functional complex I11 in mitochondria of this yeast strain. The paired kinetics of the enzyme but to a lower amount of functional complex I11. We conclude that the lower specific complex I11 activity in mitochondria of aer 1401 (but see aer 181) that complementation of the QCR7" mutant was previously only achieved after re-integration of the QCR7 gene into its original locus [20].

In this report we describe the episomal complementation of the D11-QCR7" mutant. It is shown that full complementation was achieved by using a multi-copy, instead of a single-copy, shuttle plasmid.

Episomal complementation of the QCR7" strain enabled us to start further investigation of the function of the 14-kDa protein by in vitro mutagenesis of its gene followed by re-introduction of the mutated genes into the null mutant.

Complementation of the QCR7" strain with a gene (TNT1) coding for a protein in which the 12 C-terminal residues (EKDELDNIEVSK) are replaced by the sequence LAD, leads to a partial restoration of the wild-type phenotype. The specific complex III activity in mitochondria of this strain is only about 40% of that of the wild type but since the assembly of the enzyme is also affected in this mutant (homo-cytochrome b is 36% of the amount present in the wild-type mitochondria, while the Rieske Fe-S protein and the 11-kDa subunit are less diminished), we conclude that the lower specific complex III activity is not due to impaired kinetics of the enzyme but to a lower amount of functional complex III in mitochondria of this yeast strain. The C-terminus of this subunit is therefore not directly involved in electron transport, but is relevant for assembly.

Introduction of a second truncated protein (encoded by the TNT2 gene) consisting of the first 96 residues of the 14-kDa subunit fused to the sequence DLQQPSLLID, leads to a phenotype almost indistinguishable from that of the QCR7" mutant. The one obvious difference we found was, however, most remarkable: in contrast to the QCR7" mutant, the TNT2 transformant has a normal amount of cytochrome aa3. A low amount of cytochrome aa3 was also reported for the original QCR7" mutant of the HR2 yeast strain [20] and was thought to be the aspecific result of growth on the fermentable carbon source maltose in the presence of an inactive respiratory chain. Since both the QCR7" mutant and the TNT2 transformant were grown on the same carbon source and since both have an inactive respiratory chain, this possibility can be eliminated. Clearly further experiments are needed to establish this interesting and possibly direct relation between the presence of a large N-terminal part of the 14-kDa subunit and the amount of cytochrome aa3.

It was shown in [40] (but see [18]) that complementation of a QCR8" strain with a truncated copy of the QCR8 gene encoding its first 66 amino acids, fused to residues encoded by the STOP-oligonucleotide, also severely impaired the assembly of the enzyme. Comparison of the sequences of the 14-kDa and the 11-kDa subunit shows no sequence or structural similarities apart from their C-termini. The C-termini of both the 14-kDa and the 11-kDa subunit are hydrophilic, contain many charged residues (10 out of the last 20 for the 14-kDa and 8 out of the last 20 for the 11-kDa subunit) and are predicted to form an a-helical structure.

Since both the 14-kDa and 11-kDa subunits are synthesised without a cleavable signal sequence and since the sequences involved in the addressing of these subunits to the mitochondria are unknown, it cannot be a priori excluded that their C-termini contain such sequences. This possibility could not be investigated since the antibodies used do not recognise the truncated 14-kDa (this report) and 11-kDa subunits [40]. Nevertheless, since most mitochondrial proteins contain their signal sequences within the N-terminal part of the protein and since the C-termini of the 14-kDa and the 11-kDa subunits do not contain any features typical of N-terminal targeting sequences [41] we favour the opinion that the assembly but not the import is impaired in these C-terminal deletion mutants.

According to the model of Berden et al. [22] (see also Crivellone et al. [23]), assembly of complex III involves three subcomplexes and the Rieske Fe-S subunit. One of these subcomplexes is formed between cytochrome b, the 14-kDa and the 11-kDa subunit. Both the 14-kDa and the 11-kDa subunit are strongly associated with cytochrome b and the mitochondrial inner membrane [8] without possessing hydrophobic stretches long enough to be predicted as membrane spanning. It is therefore very likely that the association with the membrane is accomplished via interaction of the hydrophobic patches of the 14-kDa and the 11-kDa subunits with the hydrophobic residues of cytochrome b. A similar role of the hydrophilic charged C-termini of the 14-kDa and 11-kDa subunits could very well be the docking of the more hydrophilic subcomplexes (one consisting of the 44-kDa and 40-kDa subunits and one consisting of cytochrome c1, the 17-kDa and 7.3-kDa subunits) to this hydrophilic and protease-sensitive cytochrome b subcomplex, thereby forming, together with the Rieske Fe-S protein and the 8.5-kDa subunit, whose role in the assembly of complex III is unknown, the mature complex III.

In the presence of the truncated subunit as encoded in the TNT1 transformant, the cytochrome b subcomplex is formed and stays protease sensitive until the core subcomplex has associated with it. Due to the partial removal of the hydrophilic C-terminus in this mutant, the association with the protease-resistant core subunit subcomplex may proceed less efficiently than in the wild type, leading to proteolysis of the unassembled cytochrome b subcomplex and therefore a lower amount of functional complex III.
In the case of mutant TNT2 the cytochrome b subcomplex may still be formed, which can be hypothesised from the appearance of the cytochrome b breakdown product in Fig. 4, lane 2, suggesting a slower proteolysis of the cytochrome b subcomplex in this mutant than in the 14-kDa0 mutant. The formation of this cytochrome b subcomplex could then somehow influence the levels of cytochrome aa3, leading to an increased amount of these cytochromes compared to the null mutant.

Clearly further research is needed to test the validity of this (hypothetical) model. The way this might be achieved is through the generation of mutations in hydrophilic and charged domains of the core subunits to establish whether such mutations could prevent the association between the core subcomplex and the cytochrome b subcomplex. If this is indeed the case, interaction between these domains and the hydrophilic C-terminus of the 14-kDa subunit should be investigable by analysis of second-site suppressor mutations.

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