Cre1, the carbon catabolite repressor protein from *Trichoderma reesei*

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Received 22 September 1995; revised version received 23 October 1995

**Abstract** In order to investigate the mechanism of carbon catabolite repression in the industrially important fungus *Trichoderma reesei*, degenerated PCR-primers were designed to amplify a 0.7-bp fragment of the crel gene, which was used to clone the entire gene. It encodes a 402-amino acid protein with a calculated Mr of 43.6 kDa. Its aa-sequence shows 55.6% and 54.7% overall similarity to the corresponding genes of *Aspergillus nidulans* and *A. niger*, respectively. Similarity was restricted to the aa-region containing the C2H2 zinc finger and several aa-rich regions rich in proline and basic amino acids, which may be involved in the interaction with other proteins. Another aa-region rich in the SPXX-motif that has been considered analogous to a region of yeast RGRlp, was instead identified as a domain occurring in several eukaryotic transcription factors. The presence of the crel translation product was demonstrated with polyclonal antibodies against Cre1, which identified a protein of 43 ± 2 kDa in cell-free extracts from *T. reesei*. A Cre1 protein fragment from the two zinc fingers to the region similar to the aa-sequence of eukaryotic transcription factors, was expressed in *Escherichia coli* as a fusion protein with glutathione S-transferase. EMSA and in vitro footprinting revealed binding of the fusion protein to the sequence 5'-SYGGRG-3', which matches well with the *A. nidulans* consensus sequence for CreA binding (5'-SYGGRG-3'). Cell-free extracts of *T. reesei* formed different complexes with DNA-fragments carrying this binding sites, and the presence of Crel and additional proteins in these complexes was demonstrated. We conclude that *T. reesei* Cre1 is the functional homologue of *Aspergillus* CreA and that it binds to its target sequence probably as a protein complex.

**Key words:** *Trichoderma reesei*; Transcription factor; DNA-binding protein; Cloning; Carbon catabolite repression; creA

1. **Introduction**

Carbon catabolite repression in microorganisms is a means to control the synthesis of a range of enzymes required for the utilization of less favoured carbon sources when more readily utilized carbon sources are present in the medium. Several genes participating in this process have been identified in *Saccharomyces cerevisiae* [1,2]. In the multicellular fungi, the creA gene cloned from *Aspergillus nidulans* [3] and *A. niger* [4] is the only hitherto regulatory gene known to mediate carbon catabolite repression. It encodes a DNA-binding protein containing a two-zinc-finger domain of the C2H2 class, which mediates 84% similarity to MIG1 from *S. cerevisiae*, which is also involved in glucose repression of *SUC2*, *GAL1*, *GAL4* and *GAL10* transcription [5,6]. The sequence 5'- SYGGRG-3' has been proposed as a consensus for CreA-binding [7]. Unlike MIG1, however, CreA contains an additional domain downstream of the zinc-finger, which has been reported to bear high similarity to *S. cerevisiae* RGR1 [8,9], and whose function is unknown. Since its cloning and sequencing, molecular evidence has been presented for an involvement of CreA in the catabolite repression of transcription of genes involved in proline utilization [7], ethanol metabolism [10,11] and polysaccharide hydrolysis [12] in *A. nidulans*.

Nothing is known as yet on the mechanism of carbon catabolite repression in other fungi. The filamentous fungus *Trichoderma reesei* is an industrially important producer of several extracellular enzymes, including a highly active cellulase [13] and hemicellulase enzyme system [14]. The formation of some of these enzymes (e.g. cellobiohydrolase I; endo-B-1,4-xylanase I) is repressed by glucose [15,16]. It has been reported that the 5'-upstream nt-sequence of the *T. reesei* gene encoding cellobiohydrolase I (cbhl) shows consensus sequences for binding of a potential CreA-homologue [17]. Deletion of these sequences resulted in glucose derepressed transcription of cbhl [17]. It is therefore possible that carbon catabolite repression in *T. reesei* occurs by a mechanism similar to that existant in *Aspergillus*. However, the presence of a DNA-binding protein in *T. reesei* similar to CreA has not yet been published. As a first step towards understanding the mechanisms and cloning of the genes involved in carbon catabolite repression in *T. reesei*, we demonstrate here the presence of a creA homologue in *T. reesei* — Crel — and provide evidence that the native gene product is a DNA-binding protein, thereby showing that the mechanisms of carbon catabolite repression have been basically conserved in the ascomycetous classes of Pyrenomycetes and Plectomycetes.

2. **Experimental**

2.1. **Strain, cloning vector and plasmid**

*Trichoderma reesei* strainQM 9414 (ATCC 26921) was used throughout this study and maintained on malt agar. Bluescript II/SK+ (Stratagene, La Jolla, CA) and *E. coli* LC 137 (Pharmacia-LKB, Uppsala, Sweden) were used as cloning and plasmid vectors, respectively.

2.2. **Cloning of the T. reesei crel gene**

Fungal genomic DNA was isolated as described elsewhere [18]. Degenerated oligonucleotide primers were designed according to the highly conserved Zn-finger domains of: (a) the CreA-proteins of *A. nidulans* [3] and *A. niger* [4] and Miglp of *S. cerevisiae* [3]; and (b) a C-terminal region of *A. nidulans* and *A. niger* CreA showing similarity to the *S. cerevisiae* RGR1p [9] protein. The nt-sequence of the primers was: CRE-J1 5' -CC(C/A/G/T)CG(C/A/G/T)CC(C/A/G/T)CC(A/T)AA(G/A)TG(C/T)CC(C/A/G/T)-3'; and CRE-J2r 5' -(G/A)TG(G/G/ A/T/C)GC(G/A/T/C)GG(G/C/A)GT(G/A/T/C)GC(G/C/A)CA(G/A/T/...
2.3. Preparation of GST: CreI(-) fusion protein

The 700-bp DNA fragment encoding a protein containing the zinc finger region and the RGR1-like region of CreI was released from pJSCI/1 with Clal/BamHI. The protruding ends were filled in with Sequenase. The construction was verified by restriction analysis and sequencing. The nt-sequence of the coding strand to a corresponding oligonucleotide of the complementary strand and filling

2.4. Preparation of anti-CreI antibodies and immunological techniques

A synthetic polypeptide (ELTRHSRHI), chosen from the sequence located at the C-terminal part of the CreI-Zn-finger domain, was synthesized. After coupling onto Tentagel MAP (Rapp Polymere, Vienna, Austria), 10 mg of the aduct were used to raise polyclonal antibodies in rabbits. Antisera were purified according to standard methods [21].

2.5. Preparation of cell-free extracts and electrophoretic mobility shift assay (EMSA)

The growth of T. reesei on glucose-minimal medium and the preparation of cell-free extracts for use in EMSA has been described previously [22]. A 538-bp fragment of the promoter of the T. reesei xynl (xylose 1-encoding) gene was used to generate the DNA fragments for EMSA. It was treated with NdeI/SalI to yield three restriction fragments (I, II and III). A BgIII treatment of fragment I yielded an additional restriction (IV). All fragments were end-labelled with the appropriate [α-32P]dNTP using Sequenase and purified by non-denaturing PAGE. Double stranded oligonucleotides were prepared by annealing the oligonucleotide of the coding strand to a corresponding oligonucleotide (at least 4 nucleotides shorter) of the complementary strand and filling in the protruding ends using Sequenase. The synthetic, double-stranded oligonucleotide, CreI so, used for competition experiments, was generated by annealing the two oligonucleotides 5'-ATATTATGCGGAGGCCCGAATGTTTCTCC-3' and 5'-GGAGAAACATTCTGG-CCAGACCAGAATGTTTCTCC-3' in 50 mM Tris-HCl pH 7.5, 100 mM NaCl, incubating for 2 min at 90°C, and cooling to 4°C. The second strand was then competed by Sequenase. Binding assays were performed essentially as described previously [22]. To select and clute protein-DNA complexes from EMSA, the gel was air-dried, binding to DNA visualized on the X-ray film was cut out, suspended in 0.5 ml of SDS-PAGE sample buffer [23] and slowly shaken (50°C, 18 h). Solubilized proteins were precipitated with 2 vols. of 96% (v/v) ethanol (1 h, −70°C). The pellets were recovered by centrifugation (20 min, 10,000 × g, 4°C), solubilized in 20 μl of SDS-PAGE sample buffer, and stored at 4°C until use.

2.6. Methylation protection footprinting assays

End-labelled fragment IV of T. reesei xynl promoter, was used for footprinting experiments. Binding reactions were performed as described above, and DNA was partially methylated by incubation in 0.25% (w/v) dimethyl-sulfate in 50 mM sodium cacodylate pH 8.0 and 1 mM EDTA at 25°C for 5 min. Free and bound DNA fragments were separated by PAGE, located on the gels by autoradiography and electroeluted onto DEAE membranes (Schleicher and Schuell, Dassel, Germany). After purification by phenol/chloroform treatment, they were cleaved by piperidine and the reaction products analysed on a denaturing 6% polyacrylamide-urea sequencing gel.

Fig. 1. Putative amino acid-sequence of proteins mediating glucose repression in Trichoderma reesei. Aspergillus nidulans and Aspergillus niger, CreI and CreA, respectively, as deduced from the nt-sequence of the corresponding genes. The nt-sequence of creI had been deposited in the NCBI Database under the accession number U27336. Boxes represent regions of high similarity between all three organisms, as explained in the text. Asterisks indicate identical, and a double-point conserved amino acids.
2.7 Electrophoresis and Western analysis

Samples were boiled in sample buffer [23] for 5 min, and subjected to SDS-PAGE in 10% polyacrylamide gels [23]. The separated proteins were either visualized by silver staining [25] or blotted to nitrocellulose [26]. Membranes were treated with the antibodies against Crel, and detection of immunolabelled bands was carried out with an anti-IgG alkaline phosphatase conjugate as described elsewhere [27].

Fig. 2. Alignment of the Crel- and CreA-sequence between aa257-aa297 as shown in Fig. 1, with that from various eukaryotic transcription factors. Shades boxes represent identical amino acids which occur at the same position within this stretch. Dots represent other amino acids scoring in the similarity search (BLAST, NCBI Database). Partial sequence of the yeast RGRlp was aligned manually as the database showed no scores of sufficient similarity with this protein (see text).

Fig. 3. Presence of the crel gene product in cell-free extracts of T. reesei by SDS-PAGE/Western analysis. 5, 20, 50 and 100 μg of cell-free extract were loaded onto tracks 1-4. Polyclonal antibodies against Crel were used for immunostaining. Lane 5 shows the relative position of prestained calibration proteins (Pharmacia-LKB, Uppsala, Sweden), that were marked with a pencil after blotting.

3. Results and discussion

The Aspergillus CreA-gene products are characterized by two conserved protein domains, which reveal a high similarity to DNA-binding proteins from other organisms, e.g. the Zn-finger region and the region with similarity to a region in yeast RGR1. Our strategy to clone the T. reesei crel gene was based on the assumption that these homologous regions may also occur in Crel, and we used degenerated consensus nucleotide sequences corresponding to the most conserved aa-stretches as primers for PCR-amplification. Three major amplification products of approximatively 350-bp, 700-bp and 900-bp size were obtained. Since the 700-bp fragment corresponded well to the expected size of a putative crel fragment, three 700-bp PCR amplicons were cloned and two of them were sequenced. Both PCR products revealed identical sequences and showed high similarity to the Aspergillus CreA. One of these fragments was used as a probe to isolate a 12-kb chromosomal clone of T. reesei crel, which was sequenced. A 1206-bp intronless ORF was found.
The deduced *T. reesei* Cre1 protein (Fig. 1) has a calculated $M_r$ of 43.6 kDa and its aa-sequence shows high similarity to that of the CreA proteins from *A. nidulans* and *A. niger* [3,4] (55.6% and 54.7%, respectively). A sequence comparison between the deduced Cre1 and CreA proteins of these three fungi revealed several regions of significant identity that may be essential for the function of these proteins. These regions included the two C2H2-type Zn-fingers (aa55-aa211), and the region with the sequence containing only acid amino acids (DEDD), preceding the motif, that typically occurs at high frequency in gene regulatory proteins [29]. A unique characteristic of Crel is a stretch of eight glutamine-histidine residues at the same position where an alanine-rich stretch occurs in *A. nidulans* and *A. niger*. Glutamine-rich aa sequences have also been implicated in protein–protein interactions [31].

In order to demonstrate the occurrence of the crel gene product in *T. reesei*, we have raised antibodies and used them to detect Cre1 by Western analysis (Fig. 3). When amounts of 0.5–10 µg protein were loaded per track, the antibodies recognized one major band of about 43 (±2) kDa and a few additional, but fainter bands. The ratio between the 43 kDa band and the others was not altered when different amounts of protein were applied to the gels. Therefore the additional protein bands are specifically recognized by the antibody and should contain epitopes similar to the one used for immunization. These results indicate that crel is transcribed and translated in *T. reesei* and its gene product occurs in cell-free extracts from mycelia grown in glucose-containing media.

CreA from *A. nidulans* has been shown to bind to the consensus sequence 5'-SYGGRG-3' [7]. In order to investigate whether Cre1 binds to a similar consensus nt-sequence, we have overexpressed the 0.7-kb PCR-fragment of crel as a GST-fusion protein in *E. coli*, and investigated its binding by EMSA and methylation protection footprinting. A SalI/BglII-fragment of the *xynl* (xylanase I-encoding) promoter, corresponding to nt(-538)–(-390; fragment I) and containing several putative *A. nidulans* CreA consensus binding sites (Mach et al., unpublished), was used as target DNA. Other promoter fragments not containing this sequence (fragments II and III) were used as control. Fig. 4A shows the results from EMSA with two different concentrations of the GST::Cre1 fusion protein. Only fragment I, which contains putative Cre1 binding sites, resulted in gel retardation; no shift was observed with fragments lacking such sequences (fragments II and III). Methylation footprinting (Fig. 4B) shows that the GST::Cre1 fusion proteins protects guanines within the sequence 5'-GGCGGAG-3' that matches the consensus for GST::CreA [7]. Therefore Cre1 might establish its contact with DNA in the same way as CreA. It shall be noted that the GST::Cre1 fusion protein is considerably larger than the GST::CreA-fusion protein used by [7], which indicates that the presence or absence of additional protein domains does not alter the DNA-binding of the purified protein in vitro. This was confirmed by using a shorter GST::Cre1-fusion protein for footprinting (data not shown).

To determine if the native crel gene product also binds to the target sequences identified in vitro, cell-free extracts of *T. reesei* grown on glucose were used for EMSA. Fig. 5 shows that the extracts give rise to four major complexes of different mobility. To determine which one of these complexes was produced by Cre1 binding, a 50-fold molar excess of an unlabelled double-stranded synthetic oligonucleotide (Crelso) and 250 ng of calf thymus DNA were used to specifically and nonspecifically compete with the labelled DNA, respectively. It is important to note that the nt-sequences flanking the Crel-target site in the *xynl* promoter are absent in Crelso and, hence, competition will specifically occur for the binding to the Crel target sequence. As shown in Fig. 5A (lane 3), complexes a and d disappear upon specific but remain present upon unspecific

![Fig. 5. DNA-binding of Crel present in cell-free extracts of *T. reesei*.](image)

(A) EMSA was carried out with fragments I, II and III (cf. Fig. 4) of the *xynl* promoter. 'f' indicates buffer only. 100 µg protein of the cell-free extract were used in the tracks indicated as D100, sc and ise, and 10 µg in track D10. Track sc indicates a specific competition experiment, in which 250 µg of non-labeled double-stranded oligonucleotide crelso was also included. ise indicates a non-specific competition experiment, in which 250 µg of calf thymus DNA (Boehringer-Mannheim, Mannheim, Germany) was included. Complexes which were specifically competed by Crelso are indicated by arrows. (B) SDS-PAGE/Western analysis of proteins participating in two complexes (one specific, one non-specific) from (A) as indicated by connecting lines. Lane 1 shows the relative position of prestained calibration proteins (Pharmacia-LKB, Uppsala, Sweden) that were marked with a pencil after blotting.
competition, suggesting that binding to a Cre1 target sequence is involved in the formation of these two complexes. Proteins present in the competitive complex d and in the non-competitive complex c were eluted from the gel and analyzed by SDS-PAGE and Western analysis (Fig. 5B). Although several proteins were detected by silver-staining in the specific as well as in the inspecific EMSA-complex (data not shown), only proteins eluted from the specific, competitive complex showed the presence of Cre1 on Western blots. It is interesting to note that the 43-kDa Cre1 protein band was accompanied by a 86 kDa form, which could be a dimer of Cre1. We conclude from all these data that the Cre1 gene product occurs in this complex and it is able to bind to its target sequence in its native form. The detection of several other proteins in this DNA-protein complex and its very slow mobility suggests that binding of Cre1 involves a multicomponent protein complex. This is in accordance with the fact that the putative Cre1 from T. reesei contains ss-sequences typically involved in protein-protein interactions. This putative protein-protein interaction involving Cre1 may be in analogous to the proposed interaction of SSN6p and TUP1p to MIG1p in yeast [32].

In summary, we have demonstrated that T. reesei contains a homologue of the Aspergilli CreA catabolite repressor protein, designated Cre1, and its DNA binding properties are basically similar to those of CreA. It is conceivable that similar genes occur also in other multicellular ascomycetes and the strategy used in this paper may also be applicable for cloning of this gene in other organisms.

Acknowledgements: Work in the laboratory in Innsbruck was supported by an Otto Loewi stipendium to J.S. (K077-MOB). Thanks are due to Dr. H. Haas for his help in the preparation of the antibodies, to Dr. Matteo Lorito for critically reading the manuscript, and to Dr. B. Felenbok for providing the plasmid containing the GST::CreA fusion. This work was supported by a grant from the Austrian Science Foundation (P 10793-GEN) to C.P.K.

References