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

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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 *cre1* gene, which was used to clone the entire gene. It encodes a 402-amino acid protein with a calculated M_r 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-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 RGR1p, was instead identified as a domain occurring in several eucaryotic transcription factors. The presence of the *cre1* 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 eucaryotic 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′-GCGGAG-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 Cre1 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 C_2H_2 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′ 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-β-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 (*cbh1*) shows consensus sequences for binding of a potential CreA-homologue [17]. Deletion of these sequences resulted in glucose derepressed transcription of *cbh1* [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* — Cre1 — 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 strain QM 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 cre1 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 Mig1p of *S.cerevisiae* [5]; 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)TA(C/T)AA(G/A)TG(C/T)CC(C/A/G/T)-3′; and CRE-J2$_{rev}$ 5′-(G/A)TG(G/A/T/C)GC(G/A/T/C)GG(G/C/A)GT(G/A/T/C)GC(G/A)CA(G/A/T/

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C)GG(G/C/A)GT(G/A)TG-3′. These two primers were used to PCR-amplify a *cre1*-corresponding fragment from *T. reesei*. The reaction mixture for PCR contained 50 ng of *T. reesei* chromosomal DNA as a template, 50 pmol of each primer, and 5 units of Vent DNA polymerase (NEB, Beverly, MA) in 10 mM Tris-HCl buffer (pH 8.0), 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM dNTPs. Temperature program was as follows: 10 cycles of 90 s each at 95°C for denaturation, followed by an annealing temperature decreasing linearly from 53°C to 50°C during 60 s and an amplification step at 75°C for 60 s. Subsequently 30 cycles, each consisting of 60 s at 95°C, 60 s at 52°C and 30 s at 75°C, were applied. The amplification program was terminated by one cycle of 95°C for 60 s, 52°C for 60 s and 75°C for 10 min.

The DNA fragments derived from 3 independent PCR amplifications were cloned independently into Bluescript SK+ and sequenced. A random-labeled *Xho*I restriction fragment (360 bp) derived from one of these plasmids (pJOC1/1) was used to screen a chromosomal DNA library of *T. reesei* QM9414 constructed in λGEM-12 (Promega, Madison, WI). Recombinant phage DNA, prepared from a single plaque, was directly sequenced using internal primers according to sequence information from the PCR clone. Oligonucleotides used for upstream and downstream sequencing were: 5′-GCTTCTCGCCCGTGTGGG-3′, and 5′-GCACGACTCTCTGTCCC-3′.

Standard methods were used for plasmid isolation, restriction enzyme digestion, random priming and plaque hybridisation [19]. Sequencing was carried out by the dideoxynucleotide chain termination method [20].

2.3. Preparation of GST: Cre1(−) fusion protein

The 700-bp DNA fragment encoding a protein containing the zinc finger region and the RGR1-like region of *cre1* was released from pJSC1/1 with *ClaI*/*Bam*HI. The protruding ends were filled in with Sequenase (version 2.0, USB, Cleveland, OH) and subsequently into pGEX-4T1 (Pharmacia, Uppsala, Sweden) previously cut with *Eco*RI and the protruding ends filled in with sequenase. The construction was verified by restriction analysis and sequencing. The pGEX-*cre1* plasmid was transformed into *E. coli* LC137 and the expression and purification of the GST:Cre1 fusion protein carried out as described by the manufacturers.

2.4. Preparation of anti-Cre1 antibodies and immunological techniques

A synthetic polypeptide (ELTRHSRIH), chosen from the aa-sequence located at the C-terminal part of the Cre1-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 xyn1* (xylanase I-encoding) gene was used to generate the DNA fragments for EMSA. It was treated with *Nsi*I/*Sau*96I to yield three restriction fragments (I, II and III). A *Bgl*II treatment of fragment I yielded an additional fragment (IV). All fragments were end-labelled with the appropriate [α-^{32}P]dNTP using Sequenase and purified by non-denaturating 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, Cre1so, used for competition experiments, was generated by annealing the two oligonucleotides 5′-ATATTATGCGGA-GACCCCAGAATGTTTCTCC-3′ and 5′-GGAGAAACATTCTGG-GG- 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 completed by Sequenase. Binding assays were performed essentially as described previously [22].

To select and elute protein–DNA complexes from EMSA, the gel area corresponding to band 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 xyn1* 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 treatment [24] and the reaction products analysed on a denaturing 6% polyacrylamide-urea sequencing gel.

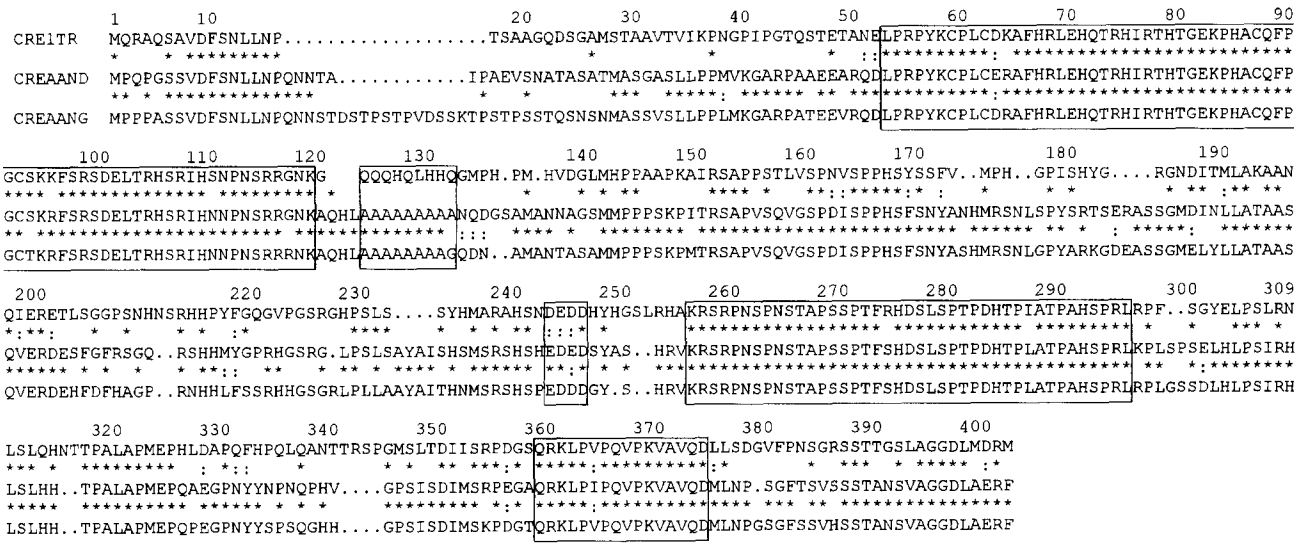

Fig. 1. Putative amino acid-sequence of proteins mediating glucose repression in *Trichoderma reesei, Aspergillus nidulans* and *Aspergillus niger*, Cre1 and CreA, respectively, as deduced from the nt-sequence of the corresponding genes. The nt-sequence of *cre1* had been deposited in the NCBI Database under the accession number U27,356. 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 Cre1, and detection of immunolabelled bands was carried out with an anti-IgG alkaline phosphatase conjugate as described elsewhere [27].

T.reesei	Cre1	KRSRPNSPNSTAPSSPTFRHDSLSPTPDHTPIATPAHSPRL
A.nid.	CreA	KRSRPNSPNSTAPSSPTFSHDSLSPTPDHTPLATPAHSPRL
A.nig.	CreA	KRSRPNSPNSTAPSSPTFSHDSLSPTPDHTPLATPAHSPRL
Mus sp.	NFATc3	..SRPTSP.....SLSPHHSPVPSPGHSPR.
H.sap.	NFAT4	..SRPTSP.....SLSPHHSPVPSPGHSPR.
D.melan.	DMU18130	.....PPPTPSPTASPTAPPYV
Mus sp.	Krox2	.....SPAASSLSSYPSPVATSYPSPA.....
Mus sp.	JS0304	.....ASPAASSLSSYPSPVATSYPSPA.....
D.melan.	ESCA	.....APSSPSDSLGSLSPPPHH.....
H.sap.	Egr1	.....TSYPSPATTSYPSPVPT.....
Mus sp.	Brn-3	.....SPGSSAPAAPSASSPSSS.....
H. sap.	Brn-3b	.....SPGSSAPAAPSASSPSSS.....
X.laev.	XELP100	...RPMSPNVSTPTRP.....
S.cerev.	RGR1p	KNTQLHSPSATVPETTTTQKESLEMVPKDTSAATMTSAPPP

Fig. 2. Alignment of the Cre1- and CreA-sequence between aa_{257}–aa_{297} 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 not scoring in the similarity search (BLAST, NCBI Database). Partial sequence of the yeast RGR1p was aligned manually as the data base showed no scores of sufficient similarity with this protein (see text).

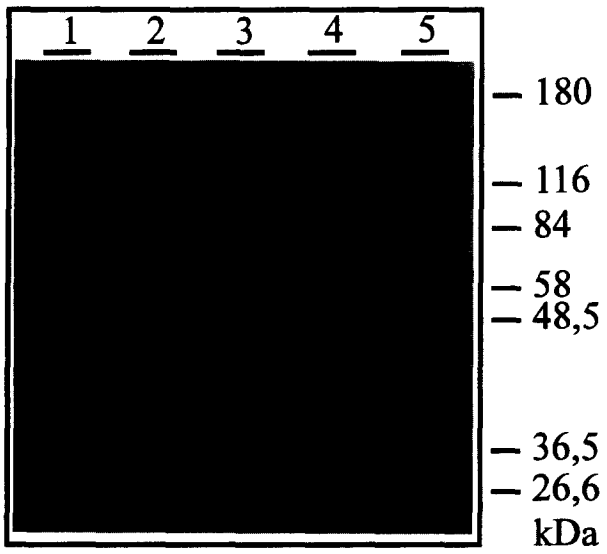

Fig. 3. Presence of the *cre1* gene product in cell-free extracts of *T. reesei* by SDS-PAGE/Western analysis. 5, 20, 50 and 100 μg of cell-free extract were loaded onto tracks 1–4. Polyclonal antibodies against Cre1 were used for immunostaining. Lane 5 shows the relative position of prestained calibration proteins (Pharmacia-LKB, Uppsala, Sweden), that were marked with a pencil after blotting.

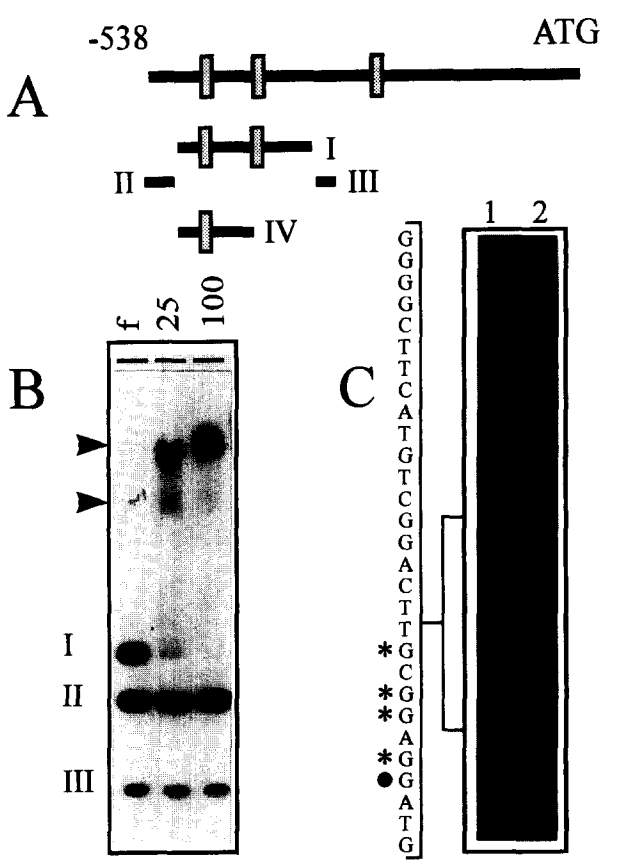

Fig. 4. Binding of GST::Cre1 to a CreA consensus sequence. (A) Schematic drawing of the location of putative CreA-binding sequences (indicated by boxes) within the 538-bp *SalI-XbaI xyn1*-promoter fragment and relative position of the restriction fragments thereof as used for EMSA (B) and footprinting assay (C). (B) EMSA using fragments I, II and III simultaneously by incubating them with buffer only (f), with 25 ng (25), and 100 ng (100) of the GST::Cre1 fusion protein, respectively. Arrowheads indicate the complexes observed. (C) Identification of binding sites within fragment IV by methylation protection footprinting: lanes 1 and 2 indicate results of methylation in the presence and in the absence of the GST::Cre1, respectively. Protected guanidines are indicated by asterisks (*), the hypersensitive guanidin immediately following the binding site is indicated by a dot (●).

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 cre1* gene was based on the assumption that these homologous regions may also occur in Cre1, 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 *cre1* 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 cre1*, which was sequenced. A 1206-bp intronless ORF was found.

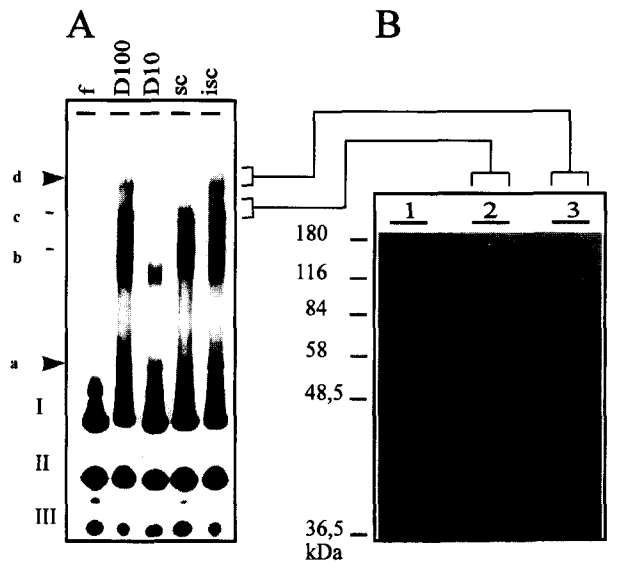

Fig. 5. DNA-binding of Cre1 present in cell-free extracts of *T. reesei*. (A) EMSA was carried out with fragments I, II and III (cf. Fig. 4) of the *xyn1* promoter. 'f' indicates buffer only. 100 μg protein of the cell-free extract were used in the tracks indicated as D100, sc and isc, and 10 μg in track D10. Track sc indicates a specific competition experiment, in which 250 ng of non-labeled double-stranded oligonucleotide cre1so was also included. isc 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 Cre1so 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.

The deduced *T. reesei* Cre1 protein (Fig. 1) has a calculated M_r. of 43.6 kDa and ist 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 C_2H_2-type Zn-fingers (aa_{55}–aa_{121}), and the region with the claimed [4] similarity to yeast RGR1p (aa_{258}–aa_{296}). However, RGR1p showed only low similarity to this region, when searched via BLAST server or the NCIB Databank, and we found a number of other eucaryotic transcription factors, who exhibited a considerably higher similarity than RGR1p (Fig. 2). Therefore, we conclude that this domain is characteristic of a particular type of DNA-binding proteins and not specific for yeast RGR1p. This is supported by the fact that the SPXX-motif, that typically occurs at high frequency in gene regulatory proteins [28], is also abundant in this domain. It is, therefore, doubtful whether CreA and Cre1 fulfill the functions of RGR1p, as has been speculated previously [4]. A short sequence containing only acid amino acids (DEDD), preceding this domain, was also found in Cre1. Other regions of significant similarity between Cre1 and CreA are characterized by a high percentage of proline residues (e.g. the region around aa_{165}, aa_{320} and aa_{370}). The preponderance of basic amino acids within these proline-rich sequences is reminiscent of the polyproline (PP II) helices binding to the SH3 domains of higher eucaryotic proteins [29] that interact with other signal transducing proteins [30]. A unique characteristic of Cre1 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 occurence of the *cre1* 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 *cre1* 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 *cre1* as a GST-fusion protein in *E. coli*, and investigated its binding by EMSA and methylation protection footprinting. A *SalI*/*BglII*-fragment of the *xyn1* (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′-GCGGAG-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 *cre1* 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 (Cre1so) 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 Cre1-target site in the *xyn1* promoter are absent in Cre1so and, hence, competition will specifically occur for the binding to the Cre1 target sequence. As shown in Fig. 5A (lane 3), complexes a and d disappear upon specific but remain present upon unspecific

competition, suggesting that binding to a Cre1 target sequence is involved in the formation of these two complexes. Proteins present in the competible complex d and in the non-competible complex c were eluted from the gel and analyzed by SDS-PAGE and Western analysis (Fig. 5B). Although several protein bands 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, competible 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 aa-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.

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