Different Inducibility of Expression of the Two Xylanase Genes xyn1 and xyn2 in Trichoderma reesei*

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Regulation of formation of the extracellular xylanase system of Trichoderma reesei QM 9414 during growth on xylan, cellulose, and replacement onto a number of soluble inducers was investigated by Northern analysis of xyn1 and xyn2 transcripts and by the use of the Escherichia coli hph (hygromycin B-phosphotransferase-encoding) gene as a reporter. Whereas the xyn1 promoter is active in the presence of xylan and xylose, and virtually silenced in the presence of glucose, the xyn2 promoter enables basal transcription at a low level, but is enhanced in the presence of xylan and xylobiose and also of sorphorose or cellobiose. The respective regulatory nucleotide regions were localized on a 221-base pair fragment and a 55-base pair fragment of the xyn1 and xyn2 5‘-upstream noncoding sequences, respectively. Electrophoretic mobility shift assays, using cell-free extracts, identified induction-specific protein-DNA complexes: one complex of high mobility was observed under basal, noninduced conditions (glucose) with xyn2, which was in part replaced by a slow-migrating complex upon induction by xylan or sorphorose. Both complexes bound to a CCAAT box. With xyn1, the induced complex also binds to a CCAAT box, but this binding is not observed in the presence of the carbon catabolite repressor Cre1, which binds to a nearby located consensus motif.

β-1,4-Xylans are heteropolysaccharides that have a backbone of β-1,4-linked xylopyranosyl residues, which constitute 20–35% of the roughly 830 gigatons of annually formed renewable plant biomass (Timell, 1965). Both pro- and eukaryotic microorganisms can use xylan as a carbon source for growth (Wong et al., 1988). Xylanases of the filamentous fungus Trichoderma reesei received up to date the most attention because of their application in the pulp and paper industry (Viikari et al., 1993, 1994). Their genes (xyn1 and xyn2) have been cloned (Törnroen et al., 1993), and their three-dimensional structure of the encoded proteins has been analyzed (Törnroen et al., 1994; Törnroen and Ruvinen, 1995).

In contrast, the regulation of their formation in Trichoderma has not yet received sufficient attention: xylanases are generally produced together with cellulases during growth of the fungus on macromolecular substrates derived from plant polysaccharides, which inevitably always contain cellulose and xylan. The resulting xylanase to cellulase ratio has been shown to be directly proportional to the xylan to cellulose ratio in the growth substrate (Senior et al., 1989). These data would be consistent with results from induction studies, which showed that xylanase and cellulase biosynthesis in T. reesei is differentially regulated (Hrmova et al., 1986). In contrast, Royer and Nakas (1990) reported that efficient xylanase induction in Trichoderma longibacterium required the simultaneous presence of xylene as well as cellooligosaccharides. In all these studies, the formation of xylanases was determined by enzyme assays only, and since at least endoglucanase I from T. reesei has xylanase activity also (Biely et al., 1991), these results are difficult to interpret.

The regulation of xylanase biosynthesis by T. reesei has not yet been studied on the molecular level. The formation of T. reesei cellulases, for which some data are as yet available, has been shown to be regulated on the level of transcription (El-Gogery et al., 1989; Abrahao-Neto et al., 1995), and it may be reasonable to assume that this is also the case with its xylanases. In this study, we will show that this is the case, and that the expression of xyn1 and xyn2 is regulated by different inducers derived from xylan and cellulose, but both processes involve CCAAT-binding protein-DNA complexes.

EXPERIMENTAL PROCEDURES

Microbial Strains—T. reesei QM 9414 was used throughout this study. Its maintenance and conditions for cultivation have been reported earlier (Kubicek et al., 1988). T. reesei TU-6 (Gruber et al., 1990a) was used for transformation. For experiments with soluble inducers, the replacement technique described by Sternberg and Mandels (1979) was used.

Vector Bluescript II/SK+ (Stratagene, La Jolla, CA) was used for cloning. Escherichia coli LC 137 was obtained from Pharmacia-LKB (Uppsala, Sweden).

Plasmids and Manipulation of DNA—Plasmids pFG1 (Gruber et al., 1990b) and pLMBS3 (Mach et al., 1994, see below) were obtained from our department stock.

The pRAMB series of reporter plasmids was developed from plasmid pUC19 and generally contained the E. coli hph (hygromycin B-phosphotransferase-encoding) gene fused to the T. reesei cbb2 3′-noncoding regions as reporter and the T. reesei pyrD gene (Gruber et al., 1990b) as a marker for transformation. To construct pRAMB, oligonucleotides CKT 085 (5′-ATGCCAGGCGCGCCATTCTGACGCTGTTCC-3′), which represent sequences 5′-354 to 5′-313 and 1 to 23 in xyn1, respectively, were used to amplify a 534-bp fragment from the xyn1 5′-noncoding sequences, thereby also generating additional XhoI/SalI and XbaI terminal sites. This fragment was used to replace a SauII/XbaI

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1 The abbreviations used are: bp, base pair(s); kb, kilobase(s); EMSA, electrophoretic mobility shift assay.
fragment of the phiI promoter in pRLM<sub>exp</sub>30 (Mach et al., 1994), thereby fusing it to the E. coli hph gene, followed by the T. reesei chb2 terminator. Finally, a 2.7-kb SalI fragment containing the T. reesei pyr4 gene (Gruber et al., 1990b) was inserted into a single XhoI site, to yield pRAMB1.

To yield pRAMB2, a 1075-bp fragment of the 5′-noncoding sequences of xyn2 was amplified using oligonucleotides CTK 085 (5′-GATCGCACCTCGCATCCGG-3′; corresponding to sequences from –1075 to –1053) and CTK 086 (5′-GATCTGCGTATGATGTCTTTC- GCCCTACG-3′; corresponding to sequences from –1 to –23), which included an additional 3′-XhoI site to facilitate cloning. The amplicon was cleaved with SalI/XhoI to yield a 224-bp SalI/SalI and a 484-bp SalI/XhoI fragment, which was used to replace the SalI/XhoI xyn1 fragment in pRAMB1 to yield pRAMB2.

Deletion Analysis—pRAMB1321 and pRAMB1100, pRAMB1 analogues carrying 5′ deletion variants of the xyn1 5′-upstream sequences, were constructed as follows: pRAMB1 was digested with HpaII/SalI, and the resulting 1.3-kb fragment (containing the hph structural gene and part of the xyn1 5′-upstream sequences) was fused to a 6.3-kb SalI/SalI fragment of pRAMB1 after filling in the SalI protruding ends with Sequenase version 2.0 (U. S. Biochemical Corp., Cleveland, OH). This plasmid contains the xyn1 5′-upstream sequences from –1 to –321 and was named pRAMB1321. To construct pRAMB1100, pRAMB1 was cleaved with EcoRI, the resulting 350-bp fragment was treated with Sequenase version 2.0 (U. S. Biochemical Corp., Cleveland, OH), and subsequently cleaved with XbaI. The resulting 100-bp fragment was then used to replace the xyn1 fragment in pRAMB1, by removal of the original xyn1 fragment by a SalI digestion, followed by the generation of blunt ends with Sequenase and subsequent cleavage with XbaI. pRAMB2316, pRAMB2235, and pRAMB2318, pRAMB2 analogues carrying 5′ deletion variants of the xyn2 5′-upstream sequences were constructed as follows: pRAMB2316 was obtained via ligation of a 1.3-kb XhoI/SalI fragment of pRAMB2 to a 6.3-kb SalI/SalI fragment of pRAMB2. To construct pRAMB2235, pRAMB2 was digested with SalI/XbaI, the resulting 7.6-kb fragment was isolated, the ends were made blunt with Sequenase version 2.0 (U. S. Biochemical Corp.), and the fragment ligated to give pRAMB2235. For construction of pRAMB2180, a 1.2-kb HindIII/SalI fragment of pRAMB2 was isolated, and the HindIII site was blunt-ended with Sequenase version 2.0 (U. S. Biochemical Corp.). This fragment was then ligated to a 6.3-kb SalI/SalI fragment of pRAMB2, whose SalI site had previously been blunt-ended with Sequenase version 2.0. All vector constructs were verified via sequencing by the dye-oxyxynuclease chain termination method (Sanger et al., 1977).

Transformation—E. coli transformations were carried out according to standard techniques (Sambrook et al., 1989). Transformation of T. reesei TU-6 was carried out as described by Gruber et al. (1990b).

Enzyme Assays—Xylanase activity was assayed as described previously (Ferroni et al., 1992), using Lentinus xylan (Lentinus AG, Austria) as substrate. Activities are given as units, 1 unit being defined as the release of 1 pmol of reducing sugar per min under these conditions. Protein concentrations in the culture filtrate were determined by the dye-binding procedure (Bradford, 1976).

Hygromycin B Resistance Assay—T. reesei transformants were analyzed for hygromycin B resistance by means of a plate assay, which measures the increase in colony diameter at different hygromycin B concentrations. To obtain linear growth rates, transformants were pregrown on the same carbon source and agar plugs (4-mm diameter) overgrown with mycelium from the growing front were excised, placed in the middle of fresh plates, and incubated at 28°C in the dark. The rates of increase in colony diameter were calculated from at least three measurements during this incubation.

**TABLE I**

| Putative inducer | Xylanase activity 9 h | Xylanase activity 24 h |
|------------------|-----------------------|------------------------|
| None             | 0.03 (±0.03)          | 0.08 (±0.04)           |
| Xylan            | 0.16 (±0.05)          | 0.68 (±0.10)           |
| Xylobiose        | 0.07 (±0.04)          | 0.28 (±0.08)           |
| Sophorose        | 0.44 (±0.08)          | 1.30 (±0.26)           |
| Xylobiose plus cellobiose | 0.49 (±0.06) | 0.97 (±0.24) |
| Xylose           | 0.28 (±0.05)          | 0.38 (±0.08)           |
| Xylose plus xylulose | 0.18 (±0.04) | 0.38 (±0.05) |
| Xytil            | 0.04 (±0.04)          | 0.09 (±0.05)           |
| Arabinose        | 0.03 (±0.03)          | 0.08 (±0.04)           |
| Arabitol         | 0.03 (±0.03)          | 0.08 (±0.05)           |

**FIG. 1.** Northern slot-blot analysis reporting the expression of xyn1 and xyn2::hph (A) and xyn2::hph fusions (B), respectively. act1 indicates the act controls. Slopes corresponding to the different inducing or noninducing sugars (G, glucose; Xo, xylulose; So, sophorose; Xb, β1,4-xylobiose) were loaded with total RNA in the following concentrations: 5, 2, 0.5, and 0.005 μg.

**RESULTS**

Induction of Xylanase Activity—Preliminary studies in our laboratory showed that cultivation of T. reesei on xylan resulted in highest xylanase activities, whereas those on cellulose were considerably lower. Even lower activities were observed on lactose, and no xylanase activity at all could be detected upon cultivation on glucose (data not shown). Since xylan and cellulose are polysaccharides which cannot be taken up by the fungus, the induction of xylanase activity is likely mediated by
The Expression of the Two Major Xylanase-encoding Genes xyn1 and xyn2 Is Mediated by Different Inducers—Northern analysis was carried out to study expression of the two xylanase genes (Fig. 1). A different pattern of induction was apparent: whereas xyn1 mRNA was detected only in mycelia transferred to xylose, xyn2 mRNA was formed under all conditions tested, and xyn2 expression was also detected on glucose (Fig. 1). This indicates that the two xylanase genes are differently regulated and hence their translation products contribute to different extents to the activities induced by the various carbon sources.

Identification of Nucleotide Regions in the 5' Upstream Sequences of xyn1 and xyn2 Responsible for Transcriptional Regulation—To identify nucleotide motifs responsible for the induction of xyn1 and xyn2 gene expression, we first fused the 538 and 944 bp of the respective promoters to the E. coli hph (hygromycin B phosphotransferase-encoding) gene as a reporter and studied whether the formation of hph-mRNA from this fusion parallels that of xyn1 and xyn2. The results, documented in Fig. 1, A and B, prove that this is the case and that the respective promoter fragments therefore carry the information required for the induction of transcription of these two genes.

To localize the promoters of the two xylanase genes in more detail, we performed 5'-deletions of the xyn1 and xyn2 5' upstream fragments and investigated the effect of these deletions on the formation of hygromycin B resistance. Since there is not yet a targeted integration system available in T. reesei, a population of 8–15 transformants was investigated for every deletion, in order to account for position and multicopy effects. In xyn1 (Fig. 2A), a removal of a region between –538 and –321 resulted in a complete loss of expression on every carbon source tested, except for very low growth on glucose in the presence of 100 µg/ml hygromycin B, indicating a very low constitutive level of expression. This level of expression was still detectable when the 5' sequences were further shortened to –100.

A similar analysis with xyn2 showed that a fusion of hph with a 235-bp promoter fragment resulted in a full pattern of regulation of hph expression, whereas virtually all regulation was lost in fusions with a 180-bp fragment (Fig. 2B). This suggests that the regulatory elements are located between –235 and –180. No constitutive level of expression of the 180-bp truncated promoter could be detected even at very low hygromycin B concentrations.

The sequence of the two nucleotide fragments shown to be relevant to xyn1 and xyn2 gene expression is given in Fig. 3, A and B; both xyn1 and xyn2 contained consensus sequences for binding of the Cre1 catabolite repressor protein (Cubero and Scazzocchio, 1994; Strauss et al., 1995, Mach et al., 1996). Also CCAAT boxes, which have been shown to be functionally involved in the induction of cbh2 gene expression,2 were present.
in both promoters. No other nucleotide sequences with sufficient similarity to known DNA-binding targets were detected.
The low constitutive level of \textit{xyn2} may be due to a TATA box located at 290.

Binding of Proteins to the \textit{xyn1} and \textit{xyn2} Promoters—In order to provide evidence that the regions of \textit{xyn1} and \textit{xyn2} described above are responsible for the binding of respective transcriptional activators, cell-free extracts were prepared from \textit{T. reesei} mycelia, grown under various inducing and non-inducing conditions, and used in EMSA with the DNA-fragments determined as relevant \textit{in vivo}. Different results were obtained for \textit{xyn1} and \textit{xyn2}, respectively. The \textit{xyn1} promoter formed two protein-DNA complexes of relatively low mobility (i.e. high \(M_r\)) with cell-free extracts from xylan (or xylose, data not shown)-induced cultures. Addition of an excess of unlabeled oligonucleotide CKTAAT, which contains a CCAAT motif, resulted in the removal of these two complexes and in the formation of a single complex of almost as fast mobility as the free 200-bp fragment (Fig. 4, \textit{A} and \textit{B}). Since the addition of a similar oligonucleotide (CKTTTT), in which only the CCAAT motif was changed to CCTTT, had no effect, we conclude that the two inducing complexes are bound to the CCAAT box at \(2430\). An even slower migrating DNA-protein complex was obtained with cell-free extracts from mycelia grown on glucose, whose appearance was not eliminated by the addition of an excess of oligonucleotide CKT\textsc{ttt}, which contains a CCAAT motif, but not CKT\textsc{aaa} and is therefore due to binding to the CCAAT box at \(216\). Upon incubation of the 55-bp DNA fragment with cell-free extracts from induced cultures (xylan, Fig. 4, \textit{A} and \textit{B})), one additional complex of slower mobility was observed. This complex was not seen in the presence of an excess of a cold CCAAT fragment and, therefore, also binds to the CCAAT box. However, whereas no competition was observed by the addition of nonspecific DNA, the addition of an excess of CKTTTT bearing the CCTTT motif equally prevented complex formation. Since the CCAAT/CCTTT boxes are the only regions of similarity between the 55-bp \textit{xyn2} promoter fragment and oligonucleotides CKTAAT and CKTTTT, we conclude that the induced protein complex binds DNA at the CCAAT motif in a different way than the constitutive protein complex.

\textbf{DISCUSSION}

Expression of \textit{xyn1} and \textit{xyn2} in \textit{T. reesei} is regulated in a different way: whereas \textit{xyn1} expression is triggered by the presence of xylan and its final degradation product xylose only, that of \textit{xyn2} is also initiated upon growth on cellulose and supply of the cellulase-inducer sophorose. Differences in the
regulation of xyn1 and xyn2 transcription were also noted in the inducibility of xyn1 and xyn2 by xylan degradation products: whereas expression of xyn1 was observed upon incubation of mycelia with xylose but not with xylulose, xyn2 expression was triggered by xylose only. Both compounds accumulate one of its catabolites such as xylitol, xylulose etc.). The role of different intracellular steady-state concentrations of xylose (or reflect a triggering of xylan-dependent signal transduction by xyn2 transcription by xylose and xylobiose, respectively, may the inducibility of xyn1 and xyn2 by xylan degradation products, and the protein components involved must therefore, at least in part, be different. While a number of diverse CCAAT-binding proteins has been described in mammalian cells (Chodosh et al., 1988; Benoist and Mathis, 1990), the HAP2/HAP3/HAP5 proteins of Saccharomyces cerevisiae (Forsburg and Guarente, 1989; McNabb et al., 1995) and the homologues in Schizosaccharomyces pombe (Olesen et al., 1991) and Kluyveromyces lactis (Mulder et al., 1994) are the only components known from fungi. The A. nidulans hapC (HAP3 homologue) has recently been cloned (Papagiannopoulos et al., 1995), and we thus assume its presence also in T. reesei by analogy. In A. nidulans amdr, the CCAAT box is responsible for the basal transcriptional level (van Heeswijck and Hynes, 1991), and the present data suggest that this also seems to be the case in T. reesei xyn2.

The simplest model to explain the regulation of xyn2 would be to postulate the binding of a Hap2/Hap3 (and eventually Hap5) complex to its promoter under basal conditions, which associates with additional components upon induction by xylan or cellulose. This model basically also can be applicable to the regulation of xyn1, yet is complicated by the apparent involvement of additional factors. The lack of binding of protein extracts from glucose-grown cultures to the CCAAT motif and the concomitant observation of a Cre1-DNA complex would be a typical example of carbon catabolite repression. This coincides with our findings (Mach et al., 1996) that functional impairment of the Cre1 target sequence in xyn1 in vivo allows the gene to be constitutively expressed at a level comparable to that of basal xyn2 transcription. As the CCAAT box and the Cre1-binding consensus are separated by only 31 bp and the Cre1-binding site lies downstream of the CCAAT box, repression may act either by competition for binding sites or by inhibition of contact with the RNA-polymerase II initiation complex. Upon induction, binding of the CCAAT-protein complex instead of the Cre1-DNA complex is observed. We do not know whether this requires a functional inactivation of the Cre1 complex or a gain of binding strength of the CCAAT-binding complex. From these data, we assume as a model for further work that the expression of xyn1, in contrast to that of xyn2, is regulated by competition of DNA-binding complexes for binding at the Cre1 consensus sites and the CCAAT box.

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Using 5′-deletion analysis and EMSA, the nucleotide areas responsible for regulation of xyn1 and xyn2 transcription were localized. Evidence was obtained that the induction of xyn1 and xyn2 transcription involves the binding of nuclear proteins to a

CCAAT box. CCAAT boxes have been observed in the regulatory regions of several fungal genes, and their function has been proven in Aspergillus nidulans amdr (van Heeswijck and Hynes, 1991) and γA (Aramayo and Timberlake, 1993) and in T. reesei cellubiohydrolase II-encoding gene cbh2. In the latter case, site-directed mutagenesis of the CCAAT box revealed that this motif mediates induction by cellulose and sophorose, which fits perfectly to the present results. However, transcription of cbh2 is not triggered by xylan or its degradation products, and the protein components involved must therefore, at least in part, be different. While a number of diverse CCAAT-binding proteins has been described in mammalian cells (Chodosh et al., 1988; Benoist and Mathis, 1990), the HAP2/HAP3/HAP5 proteins of Saccharomyces cerevisiae (Forsburg and Guarente, 1989; McNabb et al., 1995) and the homologues in Schizosaccharomyces pombe (Olesen et al., 1991) and Kluyveromyces lactis (Mulder et al., 1994) are the only components known from fungi. The A. nidulans hapC (HAP3 homologue) has recently been cloned (Papagiannopoulos et al., 1995), and we thus assume its presence also in T. reesei by analogy. In A. nidulans amdr, the CCAAT box is responsible for the basal transcriptional level (van Heeswijck and Hynes, 1991), and the present data suggest that this also seems to be the case in T. reesei xyn2.

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