J. Biochem. 124, 717-725 (1998)

Isolation of the Gene and Characterization of the Enzymatic Properties of a Major Exoglucanase of *Humicola grisea* without a Cellulose-Binding Domain[1]

Shou Takashima,[2] Hiroshi Iikura, Akira Nakamura, Makoto Hidaka, Haruhiko Masaki, and Takeshi Uozumi[3]

Department of Biotechnology, Graduate School of Agricultural and Life Sciences, The University of Tokyo, Yayoi 1-1-1, Bunkyo-ku, Tokyo 113-8657

Received for publication, April 23, 1998

An exoglucanase gene was cloned from a cellulolytic fungus, *Humicola grisea*. DNA sequencing of this gene, designated as *exo1*, revealed that it contained four introns in the coding region. The deduced amino acid sequence of EXO1 was 451 amino acids in length and showed 57.7% identity with that of *H. grisea* cellobiohydrolase 1 (CBH1), but lacked the typical domain structures of a cellulose-binding domain and a hinge region. Transcriptional analysis of the *exo1* and *cbh1* genes showed that the expression of these genes was induced by Avicel, and repressed in the presence of glucose. The *exo1* gene was expressed in *Aspergillus oryzae*, and the recombinant EXO1 protein was purified. EXO1 and CBH1 produced by *A. oryzae* showed relatively higher activity toward Avicel, but showed much lower activity toward carboxymethyl cellulose (CMC) and *p*-nitrophenyl-β-D-cellobioside (PNPC), than *H. grisea* endoglucanase 1 (EGL1). The addition of a cellulose-binding domain and a hinge region to EXO1 caused decreases in its enzymatic activities as well as the deletion of the cellulose-binding domain from CBH1. EXO1 showed relatively weak or no synergistic activity toward Avicel with *H. grisea* endoglucanases, but showed a significant level of apparent synergism with *H. grisea* CBH1 and *Trichoderma reesei* EGLI. CBH1 showed a significant level of apparent endo-exo synergism with *H. grisea* and *T. reesei* endoglucanases. *H. grisea* has at least two different types of major exoglucanase components and shows strong cellulolytic activity through synergism with cellulase components including EXO1 and CBH1.

Key words: cellobiohydrolase, endoglucanase, exoglucanase, *Humicola grisea*, synergism.

Endoglucanases (endo-1,4-β-D-glucanases; EGL, EC 3.2.1.4), cellobiohydrolases (exo-1,4-β-D-glucanases; CBH, EC 3.2.1.91), and β-glucosidases (1,4-β-D-glucosidases, EC 3.2.1.21) are the three major types of cellulolytic enzymes, and cellulose is efficiently hydrolyzed through the synergistic action of these enzymes (*1*). Among the cellulolytic fungi, *Trichoderma reesei* has very strong cellulose-degrading activity, and its cellulase system has been widely studied. Many of the CBHs and EGLs of *T. reesei* have characteristic domain structures, consisting of a catalytic domain, a cellulose-binding domain, and a flexible hinge region between these two domains, which is rich in serine, threonine and proline residues (*2-4*). The cellulose-binding domains of these enzymes are composed of less than 40 amino acid residues, and interact with cellulose through their flat hydrophobic surfaces formed by aromatic amino acid residues (*5*). Deletion of the cellulose-binding domain of *T. reesei* CBHI and CBHII greatly reduced their enzymatic activity toward crystalline cellulose (*6*), indicating that the tight binding to cellulose mediated by the cellulose-binding domain is necessary for efficient hydrolysis of crystalline cellulose by these enzymes. Similar domain structures have been observed in other fungal cellulases, and there are highly conserved amino acid stretches among these enzymes (*7, 8*).

The genus *Humicola* is known to produce many kinds of thermostable cellulases (*9, 10*), and some of the cellulase genes have been cloned (*8, 11*). One of such characterized enzymes, CBH1, of *H. grisea* also has characteristic domain structures, *i.e.* a catalytic domain at the N-terminus, and a cellulose-binding domain and a hinge region at the C-terminus (*8, 12*). *H. grisea* CBH1 showed 60% homology with *T. reesei* CBHI, and 43% homology with *T. reesei* EGLI, suggesting that the genes encoding these enzymes may have evolved from a common ancestral gene.

In this study, we report the isolation of a gene encoding an exoglucanase (*exo1*) from *H. grisea*. Although EXO1

[1] The determined nucleotide sequence has been deposited in the DDBJ, GenBank, and EMBL databases under accession No. AB003105.

[2] Present address: Molecular Glycobiology, Frontier Research Program, The Institute of Physical and Chemical Research (RIKEN), Wako, Saitama 351-0198.

[3] To whom correspondence should be addressed. Phone: +81-3-5684-0387, Fax: +81-3-5684-0387, E-mail: uozumi@mcb.bt.a.u-tokyo.ac.jp

Abbreviations: CBH, cellobiohydrolase; CMC, carboxymethyl cellulose; EGL, endoglucanase; EXO, exoglucanase; PNPC, *p*-nitrophenyl-β-D-cellobioside; PNPG, *p*-nitrophenyl-β-D-glucoside.

showed 57.7% identity with *H. grisea* CBH1, it lacked a cellulose-binding domain and a hinge region. The gene product of *Phanerochaete chrysosporium cbh1-1* also lacks a hinge region and a cellulose-binding domain. But the amount of the *cbh1-1* transcript is very low and the enzymatic properties of CBHI-1 have not been characterized (*13*). On the other hand, transcriptional analysis of the *exo1* gene showed that it is highly transcribed under cellulase-inducing conditions. The enzymatic properties of EXO1 produced by *Aspergillus oryzae*, and the apparent synergistic action between EXO1 and other cellulases as to the hydrolysis of Avicel were investigated. This is the first report of the enzymatic properties of an exoglucanase lacking a hinge region and a cellulose-binding domain.

MATERIALS AND METHODS

Strain, Plasmids, and Media—*H. grisea* var. *thermoidea* IFO9854 was used for DNA isolation. The stock cultures were stored on agar (1.5%) slants of MY medium (2% malt extract, 0.2% yeast extract). For total RNA preparation to construct a cDNA library, spores were inoculated into one liter of the cellulase-inducing medium [5% Avicel, 0.14% $(NH_4)_2SO_4$, 0.2% KH_2PO_4, 0.03% urea, 0.03% $CaCl_2 \cdot 2H_2O$, 0.03% $MgSO_4 \cdot 7H_2O$, 0.1% Bacto peptone, 1% yeast extract, 0.1% Tween 80, 0.0005% $FeSO_4 \cdot 7H_2O$, 0.00016% $MnSO_4 \cdot 4\text{-}5H_2O$, 0.00014% $ZnSO_4 \cdot 7H_2O$, 0.0002% $CoCl_2$, pH 6.8], and grown for 5 days at 37°C with shaking, and then the mycelia were harvested by filtration. For transcriptional analysis, spores were inoculated into 200 ml of MY medium and grown for 24 h. Then the mycelia were collected and washed with water, and then transferred to 10 ml of medium (the cellulase-inducing medium containing 1% Avicel, or 1% glucose, or 1% Avicel+1% glucose) and grown for an appropriate period at 37°C. *A. oryzae* M-2-3 (*argB*$^-$) was used as the host for expression of the cloned cellulase genes and the stock culture was stored on DPY medium (*14*). For fungal transformation, the expression vector, pXB6 (*8*), and its derivatives, and the *argB* containing plasmid, pSal23 (*15*), were used. Czapek-Dox medium was used for fungal transformation (*14*). For the expression of cellulase genes from *H. grisea*, *A. oryzae* transformants were cultivated in CD-P medium for 4 days at 30°C (*14*). *Escherichia coli* JM109 (*16*) was used as the host for the cloning vector, pUC118.

Genomic DNA Cloning and Sequencing—Unless otherwise stated, standard recombinant DNA techniques were used (*17*). *H. grisea* chromosomal DNA was prepared as described previously (*8*). The amino acid sequences which are conserved among fungal CBHIs (AGAKYGTG and NMLWLDS) were used to design two primers [5′-GCTGGTGCCAAGTACGGTACCGG-3′, which is identical to nucleotide positions +604 to 626 of the *H. grisea cbh1* gene, and 5′-GAGTCGAGCCAGAGCATGTT-3′, complementary to nucleotide positions +1246 to 1265, respectively (*8*)] for use in PCR amplification. PCR was carried out using these primers with *H. grisea* chromosomal DNA as the template, and two DNA fragments of 662 and 822 bp were amplified. The amplified 822 bp fragment was isolated and radiolabeled with [α-^{32}P]dCTP and a random primed DNA labeling kit (Boehringer Mannheim), and used as a probe for the detection of the cellulase gene. Southern hybridization of *H. grisea* chromosomal DNA digested with *Sph*I was performed as described previously (*18*). The hybridizing fragment was cloned into pUC118 using the colony hybridization technique (*17*). DNA sequencing was performed on both strands by the dideoxy sequencing method (*19*), using a single strand-nested deletion kit (TaKaRa) and a *Bca*BEST sequencing kit (TaKaRa).

Isolation of RNA and Synthesis of cDNA—*H. grisea* mycelia were ground in liquid nitrogen, and then total RNA was extracted by the guanidine thiocyanate-CsCl method (*17*). The polyadenylated RNA was purified with an mRNA purification kit (Pharmacia). For Northern blot analysis, total RNA (7 μg) was separated by formaldehyde-agarose gel electrophoresis, transferred to a nylon membrane, and then hybridized with the radiolabeled *cbh1* and *exo1* probes.

Primer extension analysis was carried out with 4 μg of mRNA and radiolabeled primers (5′-GCCACAAGGGCGGCGAGGGT-3′ for the *cbh1* gene, and 5′-CTCTTGATCTGCATCTTG-3′ for the *exo1* gene). After the mRNA and primers had been annealed, extension reactions were performed using a cDNA synthesis system plus kit (Amersham). The products were loaded onto a polyacrylamide gels (6%) containing 8 M urea.

The construction of a cDNA library was performed with 5 μg of the purified mRNA and a cDNA synthesis system plus kit (Amersham).

Construction of Expression Plasmids—Site-directed mutagenesis was performed by the method of Kunkel (*20*) using a Mutan-K kit (TaKaRa). To insert the *exo1* gene into fungal expression vector pXB6, a *Dra*I site was introduced just before the start codon of the *exo1* gene, using the mutagenic primer, 5′-AGCAGTCTTTAAAGATG-3′, and the *Bss*HII site of the *exo1* gene (nucleotide position +257) was disrupted without changes in the coded amino acids using the mutagenic primer, 5′-CCGACTGCGCTCAGAGGTGCG-3′. For the construction of fusion genes from *exo1* and *cbh1*, an *Spe*I site was introduced into the *exo1* gene (nucleotide position +1427) and the *cbh1* gene (nucleotide position +1323) without changes in the coded amino acids using the mutagenic primers, 5′-CCGTGCCCGACCACTAGTGGTGTCCCTGCCG-3′ and 5′-TCAGCAGGGACACCACTAGTGGTCGGGCAGGCACC-3′, respectively. Thus, the *Dra*I-*Bss*HII fragment of the *exo1* gene (nucleotide positions −4 to 1629) containing the *Spe*I site was prepared and blunt-ended, and inserted into the *Bam*HI site of pXB6 using *Bam*HI linker, giving rise to pXB6-EXO1s. For the *cbh1* gene, an *Spe*I site was introduced into the *cbh1* gene on pXB6-CBH1 (*8*) using the above primer, giving rise to pXB6-CBH1s. Then small *Spe*I-*Sph*I fragments of pXB6-EXO1s and pXB6-CBH1s were exchanged with each other, giving rise to pXB6-EXO1CBD and pXB6-CBH1ΔCBD, respectively. For expression of the *H. grisea egl1* and *egl2* genes, pXB6-EGL1 [formerly called pXB6-EGI (*8*)] and pAMYB-EGL2 (*18*) were used, respectively. The expression system for the *H. grisea egl3* and *egl4*, and *T. reesei egl1* and *egl3* genes will be published elsewhere.

Fungal Transformation—Transformation of *A. oryzae* was performed according to the method of Gomi *et al.* (*15*).

Enzyme Assays—Enzyme assays were carried out as described previously (*8, 9*).

Protein Purification—To purify the recombinant cellulases produced by *A. oryzae* transformants, the latter were cultivated for 4 days in CD-P medium and then

culture supernatants were obtained by filtration. The filtrates were buffered by adding a 1/10 volume of 100 mM Tris-HCl buffer, pH 7.5, and then gently mixed for 1 h at room temperature with SuperQ Toyopearl 650M (Tosoh), previously equilibrated with 10 mM Tris-HCl buffer, pH 7.5. After centrifugation, solid ammonium sulfate was added to the supernatants to 40% saturation and then the precipitates were removed by filtration. Samples were loaded onto a column of Phenyl Toyopearl 650M (2.5×5.0 cm, Tosoh), equilibrated with 50 mM Tris-HCl buffer, pH 7.5, 40% saturated with ammonium sulfate. The enzymes were eluted with a linear gradient, 40 to 0% saturation, of ammonium sulfate in the same buffer. The fractions showing cellulase activity were collected and ultrafiltrated with a Centriprep 30 (Millipore), and the purified cellulases were obtained. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed as described by Laemmli (*21*). The protein content was determined with a dye-binding assay kit (Protein assay kit, Bio-Rad) using γ-globulin as the standard.

RESULTS AND DISCUSSION

Cloning of the exo1 Gene—Comparison of the amino acid sequences of several fungal CBHI-type enzymes showed the existence of small but highly conserved amino acid stretches within the catalytic domain and the cellulose-binding domain (*2, 7, 8*). Our strategy for cloning cellulase genes of *H. grisea* was based on the assumption that there may be enzymes having such conserved regions other than CBHI-type enzymes. So we used two nucleotide sequences corresponding to the highly conserved stretches within the catalytic domain of CBHI-type enzymes (shown in Fig. 2) as primers for PCR. Two amplified products, of 662 bp and 822 bp, were obtained on PCR, and partial sequence analysis of these DNA fragments showed that the 662 bp fragment corresponded to a portion of the *H. grisea cbh1* gene, but on the other hand the 822 bp fragment seemed to encode a portion of another cellulase which shows some similarity with *H. grisea* CBH1. Therefore, the 822 bp

```
GGATCCGGTAAGCGCATACGGGATTTGATATACAGCATCAATTGCCCGACCCATCCGCGTGTAACCTCCAGCCCGCCAAG   -830
ACTCCCGCCACCGGGCCAACTGCCGGGGCCGAGCCCGGCCAAGACGTCGGAGCCGGCCAATGGGGAAAGCTGGGTCGTGG   -750
CCGGCAAATATGGGGTAGGCCGGGAACACACGTTGATGCTCGGTGAGGGCCGACGAATTGATTAATCCATATTACTTCA   -670
CAACAGTAAATTGCAACACCCTCTCATCAGGGCATTCTCGTAGATGAACTCGCGGGACCTACTGCAGGCTCTGCTGCAAC   -590
TGCGGGGGCCCCAGTCTCCGACTCTCCGTTACTGGCCGCCCAGCTCCCGACCGGAAAAAAAAAGTCAAGTCGGCGGAACGCA   -510
CGACCGGCGACTGGCAGCGGCGCGCGGACAGCCGCTGCCGGGGGGGGATAGCTAGGCTCGATGCGCCCGTTCAGGTAGCAA   -430
CGTGGACGAGATCTCCGGGGGATGCAGCGGGCGCATACCCCAGATTTCCAGGGGCGTTTAATCTATCTCGGTCTTGGGGC   -350
CCGTCTGGGCGGAGTGCGGCCAAGAAGACTCGCGTCCTTGTCTCCAATAACGACGAAGCGACTGTTGGCTGATCAATTAG   -270
CTGGCGATGGGTCTGTGGTATGGAACGTCGGCTGAGTCTTCCATCTCCCACCGTAGACGTGTTCCGCGGATCAAGGTCTC   -190
CCGCTCCGTAACCGCCCAGGTGGCTCGGTTCTTGATGATGGGAAAGGGGCCGACGGCAGTATAAAGAGCCATGGAAGCAT   -110
CCCTCGAGGCCGGAAGGAAATCTTGCTCGAGCCACCGCAGTTCGACTTGTCTTATCGATCTGAGCAGCAGTTGACCGGTCTT    -30
CTCTGTCATCTCAGCAGCAGTCTTTCAAGATGCAGATCAAGAGCTACATCCAGTACCTGGCCGCGGCTCTGCCGCTCCTG      51
                          M  Q  I  K  S  Y  I  Q  Y  L  A  A  A  L  P  L  L          17
AGCAGCGTCGCTGCCCAGCAGGCCGGCACCATCACCGCCGAGAACCACCCCAGGATGACCTGGAAGAGGTGCTCGGGCCC    131
S  S  V  A  A  Q  Q  A  G  T  I  T  A  E  N  H  P  R  M  T  W  K  R  C  S  G  P     44
CGGCAACTGCCAGACCGTGCAGGGCGAGGTCGTCATCGACGCCAACTGGCGCTGGCTGCACAACAACGGCCAGAACTGCT    211
 G  N  C  Q  T  V  Q  G  E  V  V  I  D  A  N  W  R  W  L  H  N  N  G  Q  N  C  Y    71
ATGAGGGCAACAAGTGGACCAGCCAGTGCAGCTCGGCCACCGACTGCGCGCAGAGGTGCGCCCTCGACGGTGCCAACTAC    291
  E  G  N  K  W  T  S  Q  C  S  S  A  T  D  C  A  Q  R  C  A  L  D  G  A  N  Y     97
CAGTCGACCTACGGCGCCTCGACCAGCGGCGACTCCCTGACGCTCAAGTTCGTCACCAAGCACGAGTACGGCACCAACAT    371
Q  S  T  Y  G  A  S  T  S  G  D  S  L  T  L  K  F  V  T  K  H  E  Y  G  T  N  I    124
CGGCTCGCGCTTCTACCTCATGGCCAACCAGAACAAGTACCAGATGTTCACCCTGATGAACAACGAGTTCGCCTTCGATG    451
 G  S  R  F  Y  L  M  A  N  Q  N  K  Y  Q  M  F  T  L  M  N  N  E  F  A  F  D  V   151
TCGACCTCTCCAAGGTTGAGTGCGGTATCAACAGCGCTCTGTACTTCGTCGCCATGGAGGAGGATGGTGGCATGGCCAGC    531
  D  L  S  K  V  E  C  G  I  N  S  A  L  Y  F  V  A  M  E  E  D  G  G  M  A  S     177
TACCCGAGCAACCGTGCTGGTGCCAAGTACGGCACGGGCgtacgttctctccgtcccgccccctaccaaaagtatgactcg    611
Y  P  S  N  R  A  G  A  K  Y  G  T  G                                              190
tgctgacgtttgacagTACTGCGATGCCCAATGCGCCCGTGACCTCAAGTTCATTGGCGGCAAGGCCAACATTGAGGGCT    691
                Y  C  D  A  Q  C  A  R  D  L  K  F  I  G  G  K  A  N  I  E  G  W   212
GGCGCCCGTCCACCAACGACCCCAACGCCGGTGTCGGTCCCATGGGTGCCTGCTGCGCTGAGATCGACGTTTGGGAGTCC    771
  R  P  S  T  N  D  P  N  A  G  V  G  P  M  G  A  C  C  A  E  I  D  V  W  E  S     238
AACGCCTATGCTTATGCCTTCACCCCCCACGCCTGCGGCAGCAAGAACCGCTACCACATCTGCGAGACCAACAACTGCGG    851
N  A  Y  A  Y  A  F  T  P  H  A  C  G  S  K  N  R  Y  H  I  C  E  T  N  N  C  G   265
TGGTACCTACTCGGATGACCGCTTCGCCGGCTACTGCGACGCCAACGGCTGCGACTACAACCCCTACCGCATGGGCAACA    931
 G  T  Y  S  D  D  R  F  A  G  Y  C  D  A  N  G  C  D  Y  N  P  Y  R  M  G  N  K   292
AGGACTTCTATGGCAAGGGCAAGACCGTCGACACCAACCGCAAGTTCACgtaagttccctggccgcctcttcgacgacgc   1011
  D  F  Y  G  K  G  K  T  V  D  T  N  R  K  F  T                                  308
agaatgtccggatgctgacccagaacagCGTTGTCTCCCGCTTCGAGCGTAACAGGCTCTCTCAGTTCTTCGTCCAGGAC   1091
                          V  V  S  R  F  E  R  N  R  L  S  Q  F  F  V  Q  D          325
GGCCGCAAGATCGAGGTGCCCCCTCCGACCTGGCCCGGCCTCCCGAACAGCGCCGACATCACCCCTGAGCTCTGCGATGC   1171
G  R  K  I  E  V  P  P  P  T  W  P  G  L  P  N  S  A  D  I  T  P  E  L  C  D  A   352
TCAGTTCCGCGTCTTCGATGACCGCAACCGCTTCGCCGAGACCGGTGGCTTCGATGCTCTGAACGAGGCCCTCACCATTC   1251
 Q  F  R  V  F  D  D  R  N  R  F  A  E  T  G  G  F  D  A  L  N  E  A  L  T  I  P   379
CCATGGTCCTTGTCATGTCCATCTGGGATGACgtatgtggcaccaacctccaaccgggcatgagacctgtactgacgtgt   1331
  M  V  L  V  M  S  I  W  D  D                                                    389
cttgacagCACCACTCCAACATGCTCTGGCTCGACTCCAGCTACCCGCCCGAGAAGGCCGGCCTCCCCGGTGGCGACCGT   1411
        H  H  S  N  M  L  W  L  D  S  S  Y  P  P  E  K  A  G  L  P  G  G  D  R      413
GGCCCGTGCCCGACCACCTCTGGTGTCCCTGCCGAGGTCGAGGCTCAGTACCCCGATGCgtacgttactaccgccgctgc   1491
G  P  C  P  T  T  S  G  V  P  A  E  V  E  A  Q  Y  P  D  A                       433
atctgcaaaaaatacCggtgctaaccattgtgcagTCAGGTCGTCTGGTCCAACATCCGCTTCGGCCCCATCGGCTCGAC   1571
                                Q  V  V  W  S  N  I  R  F  G  P  I  G  S  T          448
CGTCAACGTCTAAGCTATCACGGCTCAAAATCAGCGCCGCTCTGCTCGTCCTGTTCGGCGCGCCAGTAGGGGGATATGGG   1651
 V  N  V  *                                                                       451
GCATTTCTTTGTTCAAGCATTTTTCTCTTCGTCCTGCTACATATTGAGATTGTGTATCGTATGCACGCGT              1721
```

Fig. 1. **Nucleotide sequence of the *H. grisea exo1* gene and the deduced amino acid sequence.** The nucleotides are numbered from the translational initiation site. The intron sequences are shown in lowercase letters. The TATA sequence is shown by the waved underline. CAAT sequences are underlined. Identical or similar sequences to the consensus sequences for binding to a catabolite repressor, *Aspergillus nidulans* CREA, are shown by the bold-underlines. The major transcription start sites are boxed.

fragment was used as a probe to isolate a genomic clone of the cellulase gene of *H. grisea*. Southern hybridization of *H. grisea* chromosomal DNA digested with *Sph*I was performed, and a 8.8 kb fragment was found to uniquely hybridize with the probe and so this fragment was cloned into pUC118 using the colony hybridization technique. Restriction analysis of this clone suggested that a part of it, a 2.6 kb *Bam*HI-*Mlu*I fragment, contained a cellulase gene, and this fragment was sequenced on both strands by the dideoxy sequencing method (Fig. 1). The sequence contained a coding region of 1584 nucleotides interrupted by four introns of 57, 61, 56, and 56 nucleotides, which were confirmed by cDNA sequencing. As the *H. grisea cbh1* gene and other fungal *cbh1* genes contain only one or two introns in their coding regions, the genomic structure of this gene is unique in containing four introns. The translation product of the coding region should be 451 amino acids in length with a molecular mass of 49,599 Da. The deduced amino acid sequence of this gene shows 57.7% identity with that of *H. grisea* CBH1, and 50.7% identity with that of *T. reesei* CBHI, but lacks a typical hinge and a cellulose-binding domain (Fig. 2). The deduced amino acid sequence of this gene also shows 45.6% identity with that of *P. chrysosporium* CBHI-1 (*13*), which also lacks a hinge and a cellulose-binding domain. The codon usage of this gene is similar to that of *H. grisea* cellulase genes, as cytosine and guanine are preferred as the third letter of codons, and there is a strong bias against adenine as the third letter of

```
H.grisea EXO1            1:MQIKSYIQYLAAALPLLSSVAAQQAGTITAENHPRMTWKRCSGPGNCQTVQGEVVIDANM 60
H.grisea CBH1            1:*----RTAKF*TLAA*VA*A*****CSL*T*R**SLS**K*TAG*Q*****ASITL*S*W 56
T.reesei CBH1            1:*-----YRK**VISAF*ATAR**S*C*LQS*T**PL**QK**SG*T*TQQT*S******W 55
P.chrysosporium CBHI-1   1:*VDIQIATF*--L*GVV-G*****V**YIP****LLATQS*TAS*G*T*SSSKI*L***R 57

H.grisea EXO1           61:RWLHNN-G-QNCYEGNKWTSQ-CSSATDCAQRCALDGANYQSTYGASTSGDSLTLKFVTK 117
H.grisea CBH1            57:**T*QVS*ST***T****DTSI*TD*KS***N*CV***D*T****IT*N****S****** 116
T.reesei CBH1            56:**T*ATNSST***D**T*S*TL*PDNET**KN*C****A*A****VT***N**SIG***Q 115
P.chrysosporium CBHI-1   58:**I*STL*TTS*LTA*G*DPTL*PDGIT**NY*****VS*S****IT***SA*R*Q***- 116

H.grisea EXO1          118:HEYGTNIGSRFYLMANQNKYQMFTLMNNEFAFDVDLSKVECGINSALYFVAMEEDGGMAS 177
H.grisea CBH1            117:GQ*S**V***T***DGED***T*E*LG***T****V*NIG**L*G*****S*DA***LSR 176
T.reesei CBH1            116:SA-QK*V*A*L****SDTT**E***LG***S****V*QLP**L*G*****S*DA***VSK 174
P.chrysosporium CBHI-1 117:---*******VF***DDTH*RT*Q*L*Q*L*****V**LP**L*G*******DA***KSK 173

H.grisea EXO1          178:YPSNRAGAKYGTGYCDAQCARDLKFIGGKANIEGWRPSTNDPNAGVGPMGACCAEIDVWE 237
H.grisea CBH1            177:**G*K**************P**I***N*E******TG********A*RY*T**S*M*I** 236
T.reesei CBH1            175:**T*T***********S**P******N*Q**V***E**S*NA*T*I*GH*S**S*M*I** 234
P.chrysosporium CBHI-1 174:**G************S**P**VQ**N*Q**VQ**N-A*S-ATT*T*SY*S**T*L*I** 231

H.grisea EXO1          238:SNAYAYAFTPHACGSKNRYHICETNNCGGTYSDDRFAGYCDANGCDYNPYRMGNKDFYGK 297
H.grisea CBH1            237:A*NM*T*****P*-TIIGQSR**GDS******NE*Y**V**PD***F*S**Q***T**** 295
T.reesei CBH1            235:A*SISE*L***P*-TTVGQE***GDG*******N*YG*T**PD***W****L**TS***P 293
P.chrysosporium CBHI-1 232:A*SN*A*L***T*TNNAQTR-*SGS**--*-*-N--T*F***D***F*SF*L**TT*L*A 284

H.grisea EXO1          298:G--KTVDTNRKFTVVSRF---ER-N--RLSQ---FFVQDGRKIEVPPPTWPGLPNSADIT 346
H.grisea CBH1            296:*--M****TK*I***TQ*L---KDANGD*GEIKR*Y****-**IPNSESTIPGVEGNS** 349
T.reesei CBH1            294:*SSF*L**TK*L***TQ*-----ETSG--A-INRYY**N*-VTFQQ*NAEL*SYSGNELN 344
P.chrysosporium CBHI-1 285:*--MS***TKT****TQ*ITSDNTSTGN*TEIRR*Y**N*NV*PNSVVNVT*IGAVNS** 342

H.grisea EXO1          347:PELCDAQFRVFDDRNRFAETGGFDALNEALTIPMVLVMSIWDDHHSNMLWLDSSYPP-EK 405
H.grisea CBH1            350:QDW**R*KVA*G*IDD*NRK**MKQMGK**AG**********A********TF*V-DA 408
T.reesei CBH1            345:DDY*T*EEAE*GG-SS*SDK**LTQFKK*TSGG******L***YYA*******T**TN*T 403
P.chrysosporium CBHI-1 343:DPF*SQ*KKA*IET*Y**QH**LAQ*GQ**RTG***AF**S**PANH******NF**SAN 402

H.grisea EXO1          406:AGLPGGDRGPCPTTSGVPAEVEAQYPDAQVVWSNIRFGPIGSTVNV---------------- 451
H.grisea CBH1            409:**K**AE**A*************EA*NSN**F************AGLPGAGNGGNNGGNP 468
T.reesei CBH1            404:SST**AV**S*S*S*****Q**S*S*N*K*TF***K*******--GNP-SG-GNPGGN- 458
P.chrysosporium CBHI-1 403:PAV**VA**M*SI***N**D*GILN*SPY*SFL**K**S**T*FRPA---------------- 449

H.grisea EXO1          451:------------------------------------------------------------
H.grisea CBH1            469:PPPTTTTSSAPATTTTASAGPKAGFWQQCGGIGFTGPTQCEEPYTCTKLNDWYSQCL 525
T.reesei CBH1            459:-PGTTTTRR-PATTTGSSPGPTQSHYGQCGGIGYSGPTVCASGTTCQVLNPYYSQCL 513
P.chrysosporium CBHI-1 449:------------------------------------------------------------
```

Fig. 2. **Sequence comparison of *H. grisea* EXO1 with other CBHI-type exoglucanases.** Asterisks denote identical residues with *H. grisea* EXO1. The hinge regions are underlined and the cellulose-binding domains are shown by the waved underline. The PCR primers were derived from the overlined amino acid sequences.

codons. The sequence homology and enzymatic properties of the gene product (see below) suggested that this gene encodes a novel exoglucanase of *H. grisea*, which we tentatively designated as the *exo1* gene.

Transcriptional Analysis of the exo1 and cbh1 Genes—Among cellulases produced by cellulolytic fungi, it has been considered that exoglucanases, in particular cellobiohydrolases, are the main cellulase components because they can hydrolyze the crystalline structure of cellulose. It has also been considered that the cellulose-binding domain of these enzymes is functionally important in the binding to cellulose, resulting in efficient hydrolysis. The main cellulase components of *T. reesei* are CBHI, CBHII and EGLI, their amounts reaching about 60, 20, and 10% of the total secreted protein, respectively (*22*). Each of these highly produced cellulases has a cellulose-binding domain. In the *T. reesei* cellulase system, CBHI and CBHII play important roles in the efficient degradation of crystalline cellulose.

Studies on the cellulase system of the white rot fungus, *P. chrysosporium*, have shown that this fungus has at least six genes exhibiting significant homology to the *T. reesei cbh1* gene (*23*). Among these genes, *cbh1-4* is the most highly expressed gene and its gene product also has a cellulose-binding domain (*24*). The gene product of *cbh1-1* lacks a hinge and a cellulose-binding domain, and the amount of the *cbh1-1* transcript is very low as compared to that of the *cbh1-4* transcript [less than 5×10^{-4} (*13, 24*)], suggesting that the gene product of *cbh1-1* contributes little to the hydrolysis of cellulose.

To determine the transcriptional levels of the *exo1* and *cbh1* genes of *H. grisea*, total RNA was prepared from mycelia grown on 1% glucose, 1% Avicel (microcrystalline cellulose), or 1% glucose+1% Avicel as a carbon source after 15 h cultivation. Northern blot analyses showed that both cellulase genes were highly transcribed when the mycelia were grown on 1% Avicel as a carbon source, but repressed when they were grown on the glucose-containing media (Fig. 3, A and B). This indicates that the expression of both cellulase genes is regulated through carbon catabolite repression. Within the determined 5′ upstream sequence of the *exo1* gene, there are eight 6-bp sites which are identical or similar to the consensus sequence (5′-SYGGRG-3′) for the binding of a catabolite repressor in *Aspergillus nidulans* CREA (*25*). We have cloned the *creA* homologue gene from *H. grisea*, and have shown by means of *in vitro* binding assays that the CREA homologue protein binds specifically to at least one of these 6-bp sites (unpublished results), suggesting that the 6-bp elements play an important role in the carbon catabolite repression of the *exo1* gene.

The level of transcription of the *exo1* gene was higher than that of the *cbh1* gene at an early stage of cultivation. The transcription of both genes was at almost equally high levels at a late stage of cultivation (5 days, data not shown). Under cellulase-inducing conditions, the corresponding protein bands of EXO1 and CBH1 could be detected on SDS-PAGE of the culture supernatants (Fig. 3C). *H. grisea* produces many cellulase components (*8, 9, 18*), among which, CBH1 and EXO1 seem to be two of the major exoglucanase components of *H. grisea* so far.

We also determined the transcription start sites of the *exo1* and *cbh1* genes by primer extension analyses (Fig. 4). The main transcription start sites of the *cbh1* gene were located at 78 (A), 75 (A), and 69 (A) nucleotides upstream from the translational initiation site. The most major transcription start site was located 75 nucleotides upstream from the translational initiation site. On the other hand, the main transcription start sites of the *exo1* gene were located at 81 (A), 77 (A), 70 (G), 69 (T), and 58 (A)

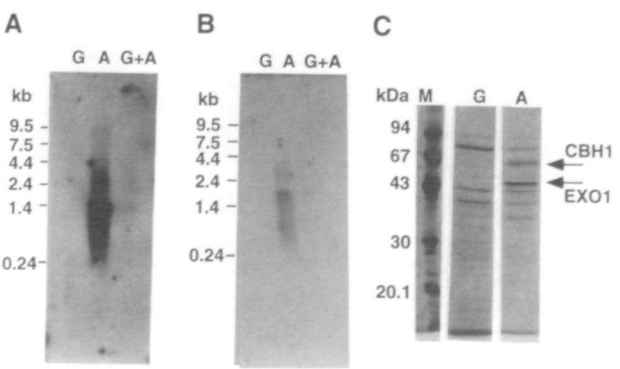

Fig. 3. **Transcriptional analysis of the *exo1* and *cbh1* genes.** Northern blot analysis was performed with the *exo1* transcript (A) and the *cbh1* transcript (B). The mycelia were grown for 15 h in a medium containing 1% glucose (G), or 1% Avicel (A), or 1% glucose+1% Avicel (G+A), and then total RNA was extracted from the mycelia and used for Northern hybridization. C: SDS-PAGE analysis of the culture supernatant. The mycelia were grown for 22 h in a medium containing 1% glucose (G), or 1% Avicel (A), and 30 μl of the culture supernatant was analyzed by SDS-PAGE. M, molecular weight markers.

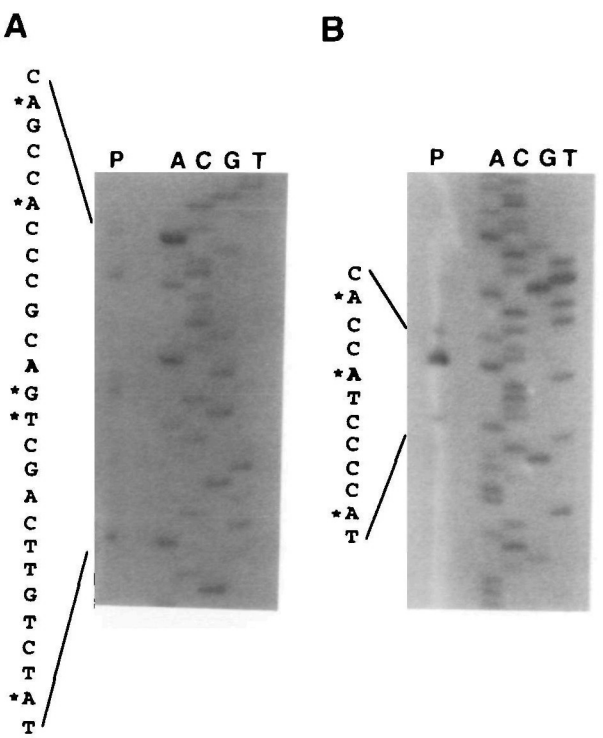

Fig. 4. **Primer extension analysis of the transcription start sites of the *exo1* (A) and *cbh1* genes (B).** The primer-extended product (P) was run on a sequence gel along with a sequencing reaction product using the same primer. Asterisks denote the major transcription start sites.

nucleotides upstream from the translational initiation site. Unlike the *cbh1* gene, the level of transcription from each site was almost the same except that the level of transcription from the site 70 nucleotides upstream from the translational initiation site was rather low. It may be possible that the differences observed in the distribution of the transcription start sites of these two genes are related to the regulation of gene expression.

Expression of the Cellulase Genes—In our previous study, we expressed the *H. grisea cbh1* and the *egl1* genes in *Aspergillus oryzae*, and revealed their enzymatic properties (*8*). In order to characterize and compare the enzymatic properties of *H. grisea* EXO1, we also used the expression system for *A. oryzae* (*14*) and constructed an expression vector, pXB6-EXO1s. The deduced amino acid sequence of the *exo1* gene predicted that EXO1 lacks the typical domain structures of a hinge region and a cellulose-binding domain. On the other hand, *H. grisea* CBH1 has such domain structures. So we also constructed the expression vectors of fusion genes from *exo1* and *H. grisea cbh1* (pXB6-EXO1CBD and pXB6-CBH1⊿CBD), and analyzed the effects of addition and deletion of a hinge region and a cellulose-binding domain. EXO1CBD is an enzyme which consists of the catalytic domain of EXO1, and the hinge region and cellulose-binding domain from CBH1. CBH1⊿CBD is an enzyme which consists of the catalytic domain of CBH1, and the C-terminal region of EXO1, and therefore lacks a hinge region and a cellulose-binding domain (Fig. 5A). These expression vectors (20 μg, each), including pXB6-CBH1s for the expression of the *cbh1* gene and pXB6-EGL1 for the expression of the *egl1* gene, were introduced into *A. oryzae* M-2-3, an arginine-auxotroph, by co-transformation with 20 μg of an *argB*-containing plasmid, pSal23 (*15*). The expression of these cellulase genes is regulated by the Taka-amylase promoter, and is induced on the addition of maltose as a carbon source. The arg^+ transformants were isolated and grown in 10 ml of CD-P medium (containing maltose as a carbon source) for 4 days at 30°C, and then assayed for enzyme activity toward *p*-nitrophenyl-β-D-cellobioside (PNPC). The clones showing the highest activity of each enzyme were selected from the transformants and used for enzyme production. From the culture supernatants of these clones after 4 days cultivation in CD-P medium, each enzyme was purified by processes involving SuperQ Toyopearl and Phenyl Toyopearl.

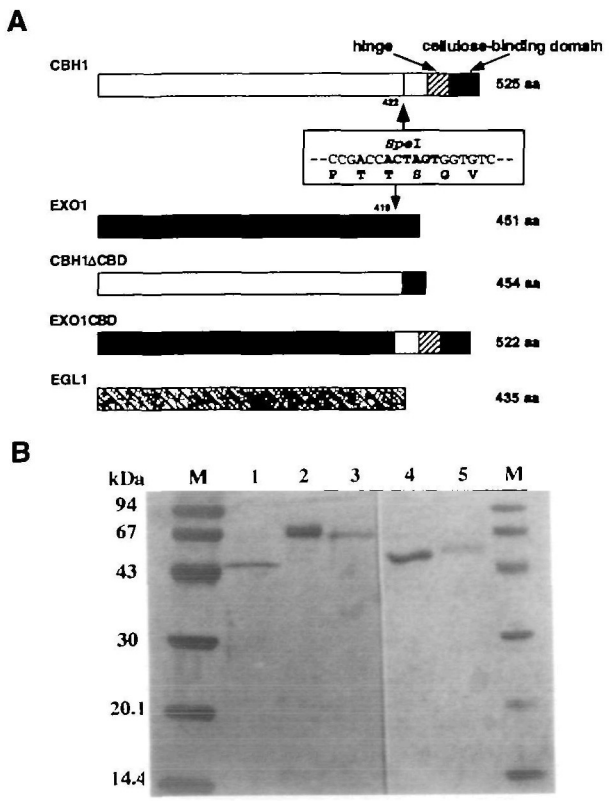

Fig. 5. **Schematic representation of the construction of recombinant cellulases (A), and SDS-PAGE of the purified recombinant cellulases produced by *A. oryzae* (B).** Lanes M, molecular mass standards; lane 1, EXO1; lane 2, CBH1; lane 3, EXO1CBD; lane 4, CBH1⊿CBD; lane 5, EGL1.

Enzymatic Properties of the Recombinant Cellulases Produced by A. oryzae—The purified enzymes are shown in Fig. 5B. The molecular masses of EXO1 and CBH1⊿CBD estimated by SDS-PAGE were each about 50 kDa, corresponding to the molecular masses determined from the deduced amino acid sequences. On the other hand, the molecular masses of CBH1 and EXO1CBD estimated by SDS-PAGE were each about 67 kDa, which is about 11 kDa higher than those determined from the deduced amino acid sequences. Periodic acid-Schiff staining of these enzymes showed that the differences between the molecular masses on SDS-PAGE and those determined from the deduced amino acid sequences were due to the glycosylation of these enzymes (data not shown).

Using PNPC as a substrate, the optimal temperature, thermal stability, optimal pH, and pH stability were measured. The optimal temperatures for the reactions of EXO1, CBH1⊿CBD, CBH1, and EXO1CBD were 65, 65, 60, and 60°C, respectively. EXO1, CBH1⊿CBD, CBH1, and EXO1CBD retained more than 80% relative activity on heating up to 65, 60, 55, and 55°C for 10 min, respectively. There was a tendency that the optimal temperatures and thermal stabilities of the enzymes without a hinge region or a cellulose-binding domain were higher than those of the corresponding enzymes with a hinge region and a cellulose-binding domain.

The optimal pHs of these enzymes were pH 5.0, and these enzymes were stable at least within the range of pH 3.0–10.0 at 4°C for 20 h.

TABLE I. **Substrate specificities of the recombinant cellulases.**

	CBH1	CBH1⊿CBD	EXO1	EXO1CBD	EGL1
	(U/mg)				
Avicel	0.145	0.0275	0.0935	0.0521	0.00935
CMC	0.262	0.156	0.468	0.0169	15.4
Cellobiose	0.113	0.0169	0.258	0.00923	0.0588
Cellotriose	0.163	0.0497	0.280	0.0144	0.136
Cellotetraose	0.192	0.0654	0.368	0.0708	0.311
Cellopentaose	0.213	0.0967	0.449	0.110	0.349
Cellohexaose	0.220	0.0963	0.490	0.109	0.358
PNPG	0.00586	0.00521	0.0459	0.0126	0.0101
PNPC	0.0543	0.0425	0.0445	0.0335	0.964

CMC, carboxymethyl cellulose; PNPG, *p*-nitrophenyl-β-D-glucoside; PNPC, *p*-nitrophenyl-β-D-cellobioside.

The purified cellulases were examined as to their substrate specificities (Table I). EXO1 and CBH1 showed relatively high activity toward microcrystalline cellulose, Avicel, but showed very low activity toward carboxymethyl cellulose (CMC) and PNPC, compared to endoglucanase 1 (EGL1). The deletion of the hinge region and cellulose-binding domain from CBH1 greatly reduced its activity toward Avicel. In addition, CBH1⊿CBD showed relatively lower activities toward CMC and cellooligosaccharides than CBH1, suggesting that the cellulose-binding domain is necessary for the efficient hydrolysis of soluble cellulosic substrates as well. Although we expected EXO1CBD to

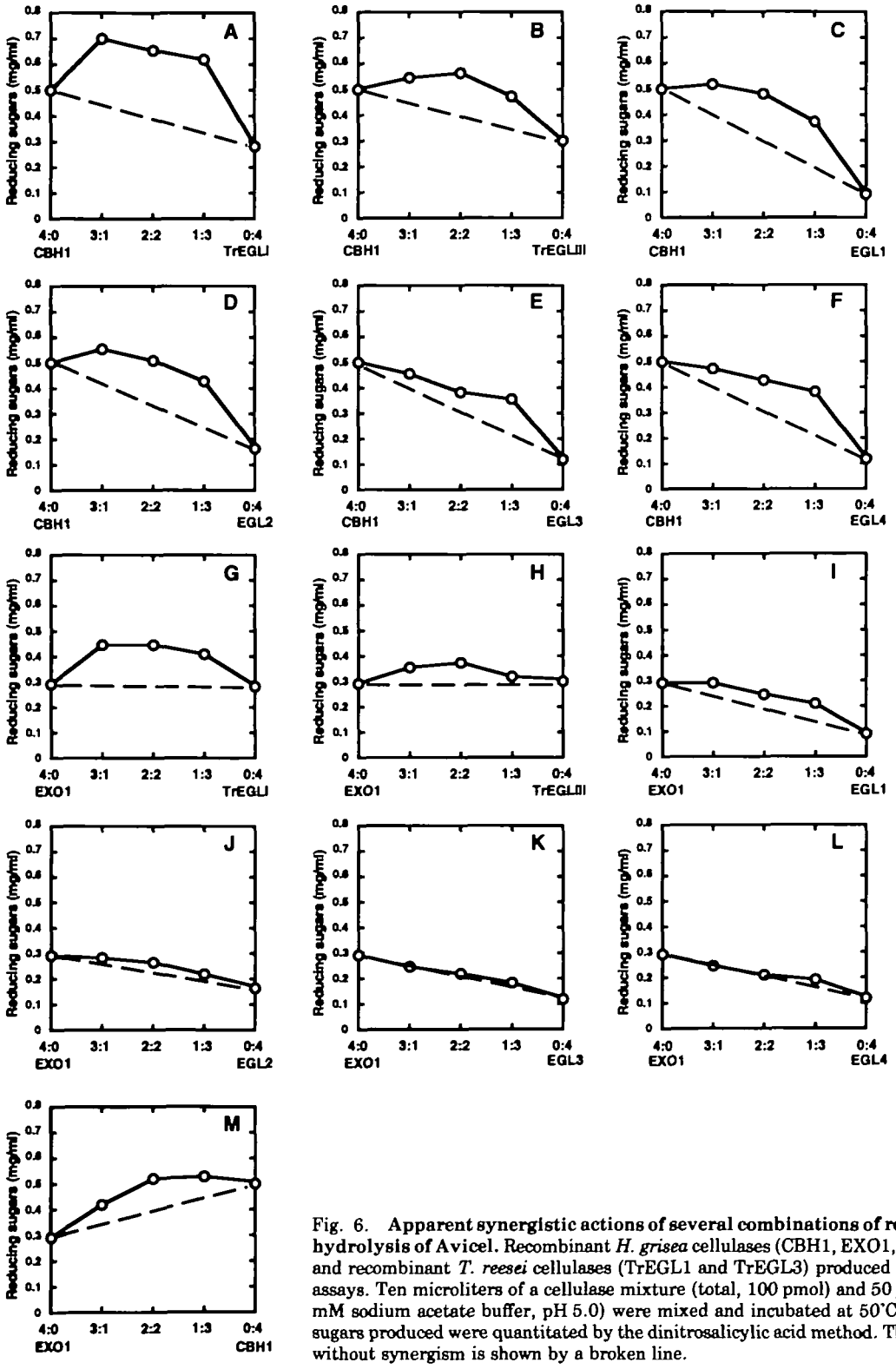

Fig. 6. **Apparent synergistic actions of several combinations of recombinant cellulases as to the hydrolysis of Avicel.** Recombinant *H. grisea* cellulases (CBH1, EXO1, EGL1, EGL2, EGL3, and EGL4) and recombinant *T. reesei* cellulases (TrEGL1 and TrEGL3) produced by *A. oryzae* were used in these assays. Ten microliters of a cellulase mixture (total, 100 pmol) and 50 μl of a 1% Avicel suspension (50 mM sodium acetate buffer, pH 5.0) were mixed and incubated at 50°C for 20 h, and then the reducing sugars produced were quantitated by the dinitrosalicylic acid method. The expected sum of two activities without synergism is shown by a broken line.

show some positive effect of the addition of a hinge and a cellulose-binding domain as to the hydrolysis of cellulosic substrates, no such positive effect was observed. There might be some negative effect due to the chimeric structure of EXO1 and the added cellulose-binding domain. In the C-terminal region of CBH1⊿CBD and EXO1CBD, eight amino acid residues are substituted, as compared with those in the original enzymes, as well as the deletion or addition of a hinge region and a cellulose-binding domain, respectively. So it may be possible that some of these eight amino acid residues play important roles in the hydrolysis of cellulosic substrates or the maintenance of the three-dimensional structure of each enzyme. Further studies with other constructs seem to be needed for elucidation of the effects of the addition of a hinge region and a cellulose-binding domain to EXO1.

Among the cellooligosaccharides tested so far, these enzymes preferred, as substrates, longer species up to cellohexaose. Thin-layer chromatographic analyses showed that the main product produced by EXO1 from cellooligosaccharides and Avicel was glucose, and the degradation profiles on Avicel and CMC of EXO1 were those an exo-mode enzyme (data not shown), suggesting that this enzyme hydrolyzes cellulosic substrates exowise glucose unit by glucose unit. EXO1 also showed significant activity toward *p*-nitrophenyl-β-D-glucoside (PNPG). Therefore, we tentatively designated the cloned gene as the *exo1* gene, although there remains a possibility that the cloned gene encodes a kind of cellodextrinase, judging from the substrate specificity of EXO1.

Synergistic Hydrolysis of Avicel by Recombinant Cellulases—The production by EXO1 is almost the same as or higher than that by CBH1 under cellulase-inducing conditions, indicating that EXO1 is a major cellulase component involved in the hydrolysis of crystalline cellulose. But the activity of EXO1 toward crystalline cellulose is lower than that of CBH1, suggesting that EXO1 is an enzyme which only shows strong activity in combination with other cellulases through a synergistic action, rather than acting singly on crystalline cellulose. So the apparent synergistic action of several combinations of recombinant cellulases toward Avicel was investigated (Fig. 6). In these assays, the conversion of oligosaccharides released from Avicel to glucose, which does not account for the enhanced degradation of the crystalline structure of Avicel, was also examined. We could observe some tendencies as to efficient hydrolysis of cellulosic substrates due to the effect of a combination of two enzymes. CBH1 showed a significant level of apparent synergistic activity with all the recombinant cellulases we examined in this study, and its apparent synergistic action was dependent on the enzyme composition (Fig. 6, A-F). On the other hand, EXO1 showed weak or no synergistic activity with the recombinant endoglucanases we examined in this study (Fig. 6, H-L), except for showing a significant level of apparent synergistic activity with *T. reesei* EGLI (Fig. 6G). Probably, there are some endoglucanases in the *H. grisea* cellulase system that show strong synergism with EXO1, such as a *T. reesei* EGLI-type enzyme. A significant level of apparent synergism between EXO1 and CBH1 was also observed (Fig. 6M), suggesting that the exo-exo synergistic action of these enzymes is necessary for the efficient hydrolysis of crystalline cellulose.

H. grisea has at least two different types of major exoglucanase components, EXO1 and CBH1. It is interesting that EXO1, which lacks a cellulose-binding domain and has restricted synergistic ability, seems to greatly contribute to the hydrolysis of cellulosic substrates, including crystalline cellulose, judging from the high production under cellulase-inducing conditions. Probably, exoglucanases with different exo-mechanisms as to crystalline cellulose are necessary for the efficient hydrolysis of crystalline cellulose, like the synergism of the combination of *T. reesei* CBHI and CBHII. Although the level of the synergistic effect of each enzyme varied, *H. grisea* shows strong cellulolytic activity through the synergism between its cellulase components, including EXO1 and CBH1. The molecular genetics of the *H. grisea* cellulase system will provide a novel model of a fungal cellulase system.

We wish to thank Dr. K. Kitamoto for generously providing the expression system for *A. oryzae*. This work was performed using the facilities of the Biotechnology Research Center, The University of Tokyo.

REFERENCES

1. Henrissat, B., Driguez, H., Viet, C., and Schülein, M. (1985) Synergism of cellulases from *Trichoderma reesei* in the degradation of cellulose. *Bio/technology* **3**, 722-726
2. Shoemaker, S., Schweickart, V., Ladner, M., Gelfand, D., Kwok, S., Myambo, K., and Innis, M. (1983) Molecular cloning of exo-cellobiohydrolase I derived from *Trichoderma reesei* strain L27. *Bio/technology* **1**, 691-696
3. Chen, C.M., Gritzali, M., and Stafford, D.W. (1987) Nucleotide sequence and deduced primary structure of cellobiohydrolase II from *Trichoderma reesei*. *Bio/technology* **5**, 274-278
4. Penttilä, M., Lehtovaara, P., Nevalainen, H., Bhikhabhai, R., and Knowles, J. (1986) Homology between cellulase genes of *Trichoderma reesei*: complete nucleotide sequence of the endoglucanase I gene. *Gene* **45**, 253-263
5. Linder, M., Mattinen, M.-L., Kontteli, M., Lindeberg, G., Ståhlberg, J., Drakenberg, T., Reinikainen, T., Pettersson, G., and Annila, A. (1995) Identification of functionally important amino acids in the cellulose-binding domain of *Trichoderma reesei* cellobiohydrolase I. *Protein Sci.* **4**, 1056-1064
6. Tomme, P., Van Tilbeurgh, H., Pettersson, G., Van Damme, J., Vandekerckhove, J., Knowles, J., Teeri, T., and Claeyssens, M. (1988) Studies of the cellulolytic system of *Trichoderma reesei* QM9414. Analysis of domain function in two cellobiohydrolases by limited proteolysis. *Eur. J. Biochem.* **170**, 575-581
7. Sims, P., James, C., and Broda, P. (1988) The identification, molecular cloning and characterization of a gene from *Phanerochaete chrysosporium* that shows strong homology to the exo-cellobiohydrolase I gene from *Trichoderma reesei*. *Gene* **74**, 411-422
8. Takashima, S., Nakamura, A., Hidaka, M., Masaki, H., and Uozumi, T. (1996) Cloning, sequencing, and expression of the cellulase genes of *Humicola grisea* var. *thermoidea*. *J. Biotechnol.* **50**, 137-147
9. Takashima, S., Nakamura, A., Masaki, H., and Uozumi, T. (1996) Purification and characterization of cellulases from *Humicola grisea*. *Biosci. Biotechnol. Biochem.* **60**, 77-82
10. Hayashida, S. and Yoshioka, H. (1980) Production and purification of thermostable cellulases from *Humicola insolens* YH-8. *Agric. Biol. Chem.* **44**, 1721-1728
11. Dalbøge, H. and Heldt-Hansen, H.T. (1994) A novel method for efficient expression cloning of fungal enzyme genes. *Mol. Gen. Genet.* **243**, 253-260
12. Azevedo, M.O., Felipe, M.S.S., Astolfi-Filho, S., and Radford, A. (1990) Cloning, sequencing and homologies of *cbh-1* (exoglucanase) gene of *Humicola grisea* var. *thermoidea*. *J. Gen. Microbiol.* **136**, 2569-2576

13. Covert, S.F., Wymelenberg, A.V., and Cullen, D. (1992) Structure, organization, and transcription of a cellobiohydrolase gene cluster from *Phanerochaete chrysosporium*. *Appl. Environ. Microbiol.* **58**, 2168-2175
14. Tsuchiya, K., Nagashima, T., Yamamoto, Y., Gomi, K., Kitamoto, K., Kumagai, C., and Tamura, G. (1994) High level secretion of calf chymosin using a glucoamylase-prochymosin fusion gene in *Aspergillus oryzae*. *Biosci. Biotechnol. Biochem.* **58**, 895-899
15. Gomi, K., Iimura, Y., and Hara, S. (1987) Integrative transformation of *Aspergillus oryzae* with a plasmid containing the *Aspergillus nidulans argB* gene. *Agric. Biol. Chem.* **51**, 2549-2555
16. Yanisch-Perron, C., Vieira, J., and Messing, J. (1985) Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. *Gene* **33**, 103-119
17. Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
18. Takashima, S., Nakamura, A., Masaki, H., and Uozumi, T. (1997) Cloning, sequencing, and expression of a thermostable cellulase gene of *Humicola grisea*. *Biosci. Biotechnol. Biochem.* **61**, 245-250
19. Sanger, F., Nicklen, S., and Coulson, A.R. (1977) DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. USA* **74**, 5463-5467
20. Kunkel, T.A. (1985) Rapid and efficient site-specific mutagenesis without phenotypic selection. *Proc. Natl. Acad. Sci. USA* **86**, 6138-6141
21. Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680-685
22. Uusitalo, J.M., Nevalainen, K.M.H., Harkki, A.M., Knowles, J.K.C., and Penttilä, M.E. (1991) Enzyme production by recombinant *Trichoderma reesei* strains. *J. Biotechnol.* **17**, 35-50
23. Covert, S.F., Bolduc, J., and Cullen, D. (1992) Genomic organization of a cellulase gene family in *Phanerochaete chrysosporium*. *Curr. Genet.* **22**, 407-413
24. Wymelenberg, A.V., Covert, S., and Cullen, D. (1993) Identification of the major cellobiohydrolase of the white rot fungus *Phanerochaete chrysosporium*. *Appl. Environ. Microbiol.* **59**, 3492-3494
25. Cubero, B. and Scazzocchio, C. (1994) Two different, adjacent and divergent zinc finger binding sites are necessary for CREA-mediated carbon catabolite repression in the proline gene cluster of *Aspergillus nidulans*. *EMBO J.* **13**, 407-415