Cloning and expression of multiple cellulase cDNAs from the anaerobic rumen fungus *Neocallimastix patriciarum* in *Escherichia coli*

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A cDNA expression library of the rumen fungus *Neocallimastix patriciarum* was made in *Escherichia coli*. Cellulolytic clones were identified by screening on a medium containing carboxymethylcellulose. Restriction mapping and Southern hybridization analysis of selected clones revealed three distinct cellulase cDNAs, designated *celA*, *celB* and *celC*. Studies on the substrate specificity showed that the enzyme encoded by *celA* had high activity towards amorphous and microcrystalline cellulose, while the *celB* and *celC* enzymes had relatively high activity on carboxymethylcellulose, with little activity on microcrystalline cellulose. Analysis of hydrolysis products from defined cellodextrins showed that the *celB* and *celC* enzymes hydrolysed β -1,4-glucosidic linkages randomly, whereas the *celA* enzyme cleaved cellotetraose to cellobiose, and cellopentaose to cellobiose and cellotriose. Cellobiose was also the only product detectable from hydrolysis of microcrystalline cellulose by the *celA* enzyme. Based on substrate specificity and catalytic mode, *celA* appears to encode a cellobiohydrolase, while *celB* and *celC* encode endoglucanases. Northern blot hybridization analysis showed that expression of the three cellulase transcripts in *N. patriciarum* was induced by cellulose.

Introduction

The anaerobic fungus *Neocallimastix patriciarum*, isolated from the sheep rumen, has a high capacity for cellulose degradation and can grow on cellulose as the sole carbohydrate source (Orpin & Munn, 1986; Williams & Orpin, 1987). Cellulose is a major component in the diets of ruminants. The importance of anaerobic rumen fungi in the digestion of cellulose in the rumen ecosystem has been increasingly revealed. It has been suggested that anaerobic fungi may function as initial colonizers of plant fibre in the rumen due to their ability to invade, penetrate and disintegrate plant cell wall fibres not normally accessible to other rumen micro-organisms (Bauchop, 1981; Wood, 1989; Borneman & Akin, 1990; Dehority, 1991). The conversion of cellulose to glucose requires sequential co-operative actions by a family of cellulolytic enzymes which consist of at least three classes: endoglucanases (EC 3.2.1.4), which randomly cleave the internal glucosidic bonds in less

ordered regions of cellulose; exoglucanases (mainly cellobiohydrolases: EC 3.2.1.91), which release cellobiose from the non-reducing ends of cellulose chains; and glucosidases (EC 3.2.1.21), which convert cellobiose to glucose. Many cellulolytic micro-organisms produce multiple forms of cellulases and the production of cellulases shows an inductive response to the presence of cellulose in culture media (see recent reviews by Béguin, 1990; Goyal *et al.*, 1991; Gilkes *et al.*, 1991). Although the cellulase complex of the related species *Neocallimastix frontalis* has been partially characterized (Wood *et al.*, 1988; Li & Calza, 1991), little is known about the cellulase system of *N. patriciarum* except for information on estimations of the enzyme activities using crude enzyme preparations (Williams & Orpin, 1987).

Molecular biological aspects of fungal cellulases have been studied mainly in the aerobic fungi (Shoemaker *et al.*, 1983; Teeri *et al.*, 1983; Chen *et al.*, 1987; Sims *et al.*, 1988; Azevedo *et al.*, 1990). These studies have rapidly elucidated the complexity, structure and regulation of aerobic fungal cellulases. However, molecular characterization of anaerobic fungal cellulases has been hampered by lack of information on the successful purification of individual cellulolytic enzymes from the fungal cellulase complexes. Thus, the preparation of antibodies or

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Abbreviations: CM-cellulose, carboxymethylcellulose; MUC, MUG, methylumbelliferyl cellobioside, methylumbelliferyl glucopyranoside; pNPC, pNPG, *p*-nitrophenyl cellobioside, *p*-nitrophenyl glucopyranoside.

protein microsequencing for the design of oligonucleotide probes has not been possible. Using differential hybridization methods, Raymond *et al.* (1991) have reported isolation of a cDNA clone homologous to a *Trichoderma reesei* cellobiohydrolase I from a cellulose-induced cDNA library of a rumen fungus, *N. frontalis*. However, further work is needed to confirm that the clone encodes a cellulolytic enzyme.

Studies of the molecular biology of the rumen fungal cellulases would provide useful information not only for our understanding of the complicated cellulase system in these micro-organisms, but also for future genetic manipulation of rumen micro-organisms to improve fibre digestion. In this communication we report, for the first time, the isolation of multiple cellulolytic enzyme cDNAs from a rumen fungus by screening for cellulolytic activities of recombinants from an expression cDNA library constructed in *Escherichia coli*. Expression of these cellulase transcripts in *N. patriciarum* was induced by the presence of cellulose. The enzymic properties of the cloned cellulases were also studied.

Methods

Microbial strains, vectors and culture media. The anaerobic fungus *Neocallimastix patriciarum* (type species) was isolated from a sheep rumen by Orpin & Munn (1986). Two different media were used for the growth of *N. patriciarum*. Medium A was based on the medium described by Phillips & Gordon (1989), except that higher concentrations of yeast extract (0.25%, w/v) and Trypticase (1%, w/v) were used. Medium B, which contains 10% (v/v) rumen fluid, was described previously (Kemp *et al.*, 1984). Glucose or microcrystalline cellulose (Avicel) was used as the sole carbohydrate source, depending upon the purpose of experiment. Host strains for cDNA cloning were *E. coli* PLK-F' and XL1-Blue obtained from Stratagene. *E. coli* strains were grown in L-broth (Sambrook *et al.*, 1989). λ ZAPII vector was obtained from Stratagene and the recombinant phage were grown in *E. coli* strains according to the supplier's instructions.

RNA isolation. Frozen fungal mycelia were ground into fine powder with a mortar and pestle under liquid N₂. Powdered mycelia were homogenized in guanidinium thiocyanate solution [4 M-guanidinium thiocyanate, 0.5% (w/v) sodium lauryl sarcosine, 25 mM-sodium citrate, pH 7.0, 1 mM-EDTA and 0.1 M- β -mercaptoethanol] using a mortar and pestle for 5 min and then further homogenized with a Polytron at full speed for 2 min. Total cellular RNA was prepared from the homogenate either by ultracentrifugation through a CsCl cushion (Sambrook *et al.*, 1989) or by the method of Chomczynski & Sacchi (1987) with the following modifications. The RNA pellet, obtained after acid guanidinium thiocyanate/phenol/chloroform extraction and the first step of 2-propanol precipitation, was suspended in a LiCl/urea solution (6 M-urea, 3 M-LiCl, 1 mM-EDTA, pH 7.6). The suspension was shaken at 4 °C for 1–2 h to remove contaminating protein and DNA. After centrifugation, the RNA pellet was briefly washed once with the LiCl/urea solution, twice with 75% (v/v) ethanol and then dissolved in 10 mM-Tris/HCl/1 mM-EDTA, pH 8.0. The RNA was further purified by extraction with phenol/chloroform and ethanol precipitation. Poly(A)⁺ RNA was selected by oligo(dT)-cellulose chromatography (Sambrook *et al.*, 1989).

General recombinant DNA techniques. DNA isolation, restriction endonuclease digestion, ligation, transformation and preparation of RNA probes were performed according to the procedures described by Sambrook *et al.* (1989).

Construction and screening of the *N. patriciarum* cDNA library. Double-stranded cDNA was synthesized from mRNA isolated from *N. patriciarum* grown on medium B containing 1% (w/v) Avicel for 48 h and ligated with λ ZAPII using a ZAP-cDNA synthesis kit, according to the manufacturer's instructions (Stratagene). A cDNA library of 10⁶ recombinants was obtained. Recombinant phage were screened for cellulolytic activity by plating in 0.7% (w/v) soft agar overlays containing 0.5% (w/v) carboxymethylcellulose (CM-cellulose) and 10 mM-isopropyl β -D-thiogalactopyranoside (IPTG; an inducer for *lacZp*-controlled gene expression). CM-cellulose hydrolysis was detected by the Congo red staining procedure (Teather & Wood, 1982). The cDNA inserts in cellulase-positive phage were recovered in the form of pBluescript SK(–) by *in vivo* excision, according to Stratagene's instructions.

Deletion of 3' region of the cDNA inserts in *celA*, *celB* and *celC*. This was accomplished by digestion of recombinant plasmids with *Xho*I and the enzyme at the upstream restriction site as indicated in Fig. 1. After filling in with the Klenow fragment, the truncated cDNA was circularized using T₄ DNA ligase.

Southern blot hybridization. λ DNA from the cellulase-positive clones was purified by a rapid mini-preparation method as follows. One millilitre of phage lysate from liquid culture was incubated with RNAase A (10 μ g ml^{–1}) at 37 °C for 1 h and with proteinase K (1 mg ml^{–1}) at 37 °C for 3 h and then extracted with phenol/chloroform. The DNA was precipitated by ethanol, digested with *Eco*RI and *Xho*I (the cDNA cloning sites), fractionated by electrophoresis on 1% (w/v) agarose gel and blotted onto Hybond N membrane (Amersham). Procedures for hybridization and signal detection were as described previously (Xue & Morris, 1992), using digoxigenin-labelled RNA probes prepared from the 3'-region-deleted cDNAs. Hybridization was carried out at 50 °C in a hybridization mixture of 50% (v/v) formamide, 0.8 M-NaCl, 50 mM-sodium phosphate (pH 7.2), 4 mM-EDTA, 0.2% (w/v) SDS, 5 $\times$ Denhardt's solution, 0.2 mg yeast RNA ml^{–1}, 0.2 mg herring sperm DNA ml^{–1} and 20 ng digoxigenin-labelled RNA ml^{–1} (1 $\times$ Denhardt's solution is 0.02% bovine serum albumin, 0.02% Ficoll, 0.02% polyvinylpyrrolidone). High-stringency washing was performed in 0.1 $\times$ SSC/0.1% (w/v) SDS at 68 °C (1 $\times$ SSC is 0.15 M-NaCl, 15 mM-sodium citrate).

Northern blot hybridization. Poly(A)⁺ RNA was fractionated by electrophoresis on 1.2% (w/v) agarose/2.2 M-formaldehyde gel and transferred to Hybond N membrane. Poly(A)⁺ RNA on the membrane was hybridized to digoxigenin-labelled RNA probes as described previously (Xue & Morris, 1992). Hybridization was carried out at 57 °C in the above hybridization mixture and final post-hybridization washing was performed in 0.1 $\times$ SSC/0.1% (w/v) SDS at 68 °C.

Enzyme assays, cellulose-binding studies and product identification. *E. coli* cells harbouring the recombinant plasmids were grown in LB medium to the end of the exponential phase in the presence of 2 mM-IPTG. Crude cell lysates prepared according to Schwarz *et al.* (1987) were used for enzyme assays as described by Hazlewood *et al.* (1990). For quantitative assays, the enzyme preparations were incubated at 39 °C for 30–60 min in 50 mM-sodium citrate (pH 5.7) with the following substrates: 0.5% (w/v) CM-cellulose (low viscosity, Sigma), 1% (w/v) amorphous cellulose (H₃PO₄-swollen Sigmacell type 50), 1% (w/v) Avicel (Merck), 0.05% (w/v) *p*-nitrophenyl cellobioside (pNPC, Sigma), *p*-nitrophenyl glucopyranoside (pNPG, Sigma) and 0.5% (w/v) oat spelt xylan (Sigma). Enzyme activities were measured under the conditions of reactions within the range of linear response to the

incubation time and the amount of the enzyme added. The cell lysate prepared from *E. coli* harbouring non-recombinant pBluescript was used as control. Protein concentrations were determined by dye-binding assay using Bio-Rad protein assay kit II according to the supplier's instructions.

For assays of cellulose-binding capacity of the cloned cellulase, cell lysates were incubated with 200 μ l of pre-washed 5% (w/v) Avicel in 50 mM-sodium citrate (pH 5.7) at 0 °C with continuous shaking for 1 h. The unbound protein was removed after centrifugation and the Avicel pellet was washed three times with 50 mM-sodium citrate (pH 5.7). The bound cellulase was assayed for enzyme activity as above.

For analysis of hydrolysis products of cellulosic substrates, crude *E. coli* cell lysates containing the cloned cellulases were spin-dialysed to remove small molecules using Centricon tubes (Amicon). The dialysed enzyme preparations were incubated at 39 °C in 50 mM-sodium citrate (pH 5.7) with 1% (w/v) Avicel for 20 h or 2 mg ml⁻¹ cellodextrins containing 3–6 glucose units. In order to examine the intermediate and end hydrolysis products of cellodextrins, samples were taken at five incubation times (30 min, 1 h, 2 h, 4 h and 20 h), using appropriate amounts of enzymes to ensure partial as well as complete digestion. Hydrolysis products of cellulosic substrates were identified by thin-layer chromatography (TLC) using silica gel plates and a solvent system of ethyl acetate/water/methanol (8:3:4, by vol.). The positions of sugars on the plate were visualized by spraying with the diphenylamine reagent as described by Lake & Goodwin (1976) and authentic cellodextrins (Merck) were used for identification.

Results and Discussion

Isolation of cellulase cDNAs from *N. patriciarum*

A cDNA expression library, prepared from *N. patriciarum* grown on crystalline cellulose (Avicel) as the sole carbohydrate source, was made in *E. coli* using the λ ZAPII vector. A total of 4×10^5 plaques were screened for expression of cellulolytic activity on CM-cellulose plates. Two hundred CM-cellulose-positive plaques were isolated. Initial characterization of the selected bacteriophage clones by restriction mapping and cross-hybridization revealed at least three classes of cellulase cDNAs. Restriction maps of three cellulase cDNA sequences (the longest cDNA insert for each type), designated *celA* (2.0 kbp), *celB* (1.7 kbp) and *celC* (1.6 kbp) respectively, are shown in Fig. 1. Southern hybridization analysis showed that these three cDNA inserts did not cross-hybridize to each other (Fig. 2), using nucleic acid probes prepared from *celA* and *celC* clones with the 3' regions of the cDNA inserts removed by digestion with *XhoI* and the enzyme at the upstream restriction site (see Fig. 1). Therefore, these cellulase cDNAs are from three distinct cellulase genes in *N. patriciarum* and there is no significant sequence homology among them.

To estimate the transcript sizes of the cDNAs, Northern hybridization was performed using nucleic acid probes consisting of 3'-region-deleted cDNA inserts. As shown in Fig. 3, *celA* hybridized to a single 2.1 kb *N. patriciarum* RNA species and *celC* to a major

Fig. 1. Restriction maps of *celA*, *celB* and *celC* and the 3'-region-deleted subclones used for generating RNA probes. The cDNA inserts are indicated by a rectangle. The direction of transcription is shown by a straight arrow. The 3'-region-deleted cDNA inserts (*celA'*, *celB'* and *celC'*) are indicated by a solid bar.

Fig. 2. Cross-hybridization of three cellulase cDNA inserts by Southern blot analysis. Plasmids containing *celA* (A), *celB* (B) and *celC* (C) were cut with *EcoRI* and *XhoI* (the cDNA cloning sites) and fractionated on 1% (w/v) agarose gel. Digoxigenin-labelled RNA probes generated from 3'-region-deleted cDNA clones were used for hybridization: *celA'* probe, left blot; *celC'* probe, right blot. Large arrows indicate the cDNA inserts being hybridized. The bands indicated by small arrows are the cloning vector being hybridized, as the RNA probes contain part of the sequence from the vector. Numbers on the margins indicate the sizes, in kbp, of molecular markers (*BstEII* fragments of λ DNA).

Fig. 3. Northern blot hybridization of *N. patriciarum* RNA. RNA was prepared from *N. patriciarum* grown on medium B containing 0.5% (w/v) Avicel and fractionated on 1.2% (w/v) agarose/2.2 M-formaldehyde gel. Digoxigenin-labelled RNA probes generated from 3'-region-deleted cDNAs (*celA'*, *celB'* and *celC'*) were used for hybridization. Numbers on the left margin indicate the sizes, in kb, of RNA molecular markers (Boehringer).

transcript of 2.2 kb, while *celB* hybridized to two RNA species (with an equal intensity) of 2.0 and 2.2 kb, respectively, despite the high-stringency conditions employed (see Methods). Northern hybridization analysis suggests that *celA* cDNA is almost full-length. Although *celB* and *celC* contain only three-quarters of the full-length cDNA sequence, they still generated functional enzymes, presumably as fusion proteins which contain N-terminal sequence of β -galactosidase (41 amino residues) encoded by the cloning vector. Thus, the missing 5'-regions of *celB* and *celC* are not essential for these cellulases to correctly fold into catalytically active enzymes. However, it is possible that the specific activities of the cloned cellulases may differ from those of the native enzymes. To our knowledge, this is the first report of the isolation of fungal cellulase cDNAs based upon the expression of recombinant bacteriophage clones in *E. coli*. The isolation of multiple cellulase cDNAs from the rumen fungus has proven the efficiency of this approach.

Table 1. Activity on various substrates of cloned cellulases expressed in *E. coli*

Crude cell lysate preparations were used for the measurement of enzyme activities as described in Methods. The values given are representative of at least three assays and are expressed as nmol product (reducing sugar or *p*-nitrophenol released) min^{-1} (mg protein) $^{-1}$. The values in parentheses are percentages of the activity of each clone on CM-cellulose.

Substrate	<i>celA</i>	<i>celB</i>	<i>celC</i>
CM-cellulose	15.1 (100)	53.6 (100)	20.6 (100)
Avicel	26.8 (177)	1.4 (2.6)	0.9 (4.4)
Amorphous (H ₂ PO ₄ -swollen) cellulose	431 (2854)	6.17 (12)	19.6 (95)
Xylan	ND	ND	ND
pNPG	ND	ND	ND
pNPC	ND	1.31 (2.4)	ND

ND, Not detectable [<0.1 nmol product min^{-1} (mg protein) $^{-1}$].

Substrate specificity

Substrate specificity was initially characterized by assay using agar plates containing CM-cellulose, methylumbelliferyl cellobioside (MUC), methylumbelliferyl glucopyranoside (MUG) and xylan. In addition to the CM-cellulose-hydrolysis activity of *celA*, *celB* and *celC* clones, the *celB* enzyme also had weak MUC-hydrolysing activity. All clones showed no activity towards MUG (a substrate for β -glucosidase activity), but showed xylanolytic activity (although the hydrolysis zones were relatively weak) after Congo red staining. Quantitative analysis of the cloned cellulolytic enzymes on various substrates is shown in Table 1. The *celA* enzyme possessed the highest activity towards amorphous (H₃PO₄-acid swollen) and microcrystalline cellulose. In contrast, the *celB* and *celC* enzymes possessed high activity for hydrolysis of CM-cellulose and little activity towards microcrystalline cellulose. None of the three cellulases hydrolysed pNPG, confirming that they possess no β -glucosidase activity. Among the three cellulases, only *celB* showed some activity towards pNPC. These results are consistent with plate assays using MUG and MUC. However, no detectable reducing sugar was produced by these enzymes incubated with xylan. This is in contrast to the xylan plate assay, which clearly showed that the enzymes were all able to degrade xylan to xylose polymers of a sufficiently short length so as to be no longer stained with Congo red (data not shown). Presumably, the enzymes attack xylan by endo-type action, but do not hydrolyse, or hydrolyse at a very slow rate, short-chain xylan polysaccharides. Many cellulases have been reported to possess some xylanase activity or vice versa (Gilkes *et al.*, 1984; Goyal *et al.*,

Fig. 4. Analysis of hydrolysis products of the *celA*, *celB* and *celC* enzymes on cellulosic compounds. (a) Crude cell lysates were prepared from *E. coli* harbouring recombinant plasmids (*celA*, *celB* and *celC*) and the small molecules were removed by spin-dialysis using Centricon-10 tubes (Amicon). The enzyme preparations were incubated with cellodextrins (2 mg ml⁻¹): cellotriose (G₃), cellotetraose (G₄) and cellopentaose (G₅) or with 1% (w/v) Avicel (C) as described in Methods. Products were identified by TLC. Complete hydrolysis of cellodextrins by the *celA* enzyme and partial hydrolysis by the *celB* and *celC* enzymes are shown. Authentic cellodextrins (S) are shown in the rightmost lane. (b) Illustration of the catalytic mode of the three cloned enzymes on cellotetraose.

1991; Flint *et al.*, 1991). Whether cross-specificity between cellulases and xylanases is due to the presence of different catalytic sites has yet to be determined. It is possible that such enzymes have the same catalytic site for cleavage of both substrates, owing to similarity of the bonds (β -1,4-glycosidic linkages) in cellulose and xylan.

Binding of *celA* enzyme to microcrystalline cellulose

The high activity of the *celA* enzyme towards crystalline cellulose is of interest in view of the low activity of many bacterial cellulases on this substrate (Robson & Chambliss, 1989). Structural studies have revealed that many cellulases generally consist of at least two distinct functional domains: a catalytic domain and a cellulose-binding domain (Béguin, 1990; Gilkes *et al.*, 1991; Goyal *et al.*, 1991). To test whether the *celA* enzyme possesses cellulose-binding capacity, a comparative assay of its

activity with or without prior adsorption to crystalline cellulose was conducted. The amount of reducing sugar released from Avicel after adsorption of the enzyme to Avicel followed by extensive washing of the enzyme-substrate complex was 13.6 μ g glucose equivalent min⁻¹ per ml of the enzyme extract, compared to 17.6 μ g min⁻¹ per ml for the enzyme directly added. The high recovery (77%) of the enzyme after adsorption and washing suggests that the cellulase encoded by *celA* contains a strong cellulose-binding domain.

Catalytic mode

Distinction between endoglucanase and cellobiohydrolase (exoglucanase) is theoretically simple according to the definition of cellulases (Webb, 1984), but in practice often comes into confusion. This is due not only to the unavailability of specific substrates for cellobiohydro-

lase, but also to the fact that some cellulases possess a mixed type of catalytic action (Kanda & Nisizawa, 1988; Wood *et al.*, 1988). However, CM-cellulose has commonly been used as a substrate for identifying endoglucanases and Avicel for cellobiohydrolases. More recently, some investigators have tentatively used hydrolysis of MUC and pNPC as an indication of cellobiohydrolase activity (see review by Robson & Chambliss, 1989; Goyal *et al.*, 1991).

Based on the substrate specificities shown in Table 1, these cloned cellulases can not be clearly classified. In order to clarify the catalytic mode of the cloned cellulases, hydrolysis products of cellodextrins and cellulose were analysed by TLC. As shown in Fig. 4(a), the *celA* enzyme released only cellobiose (G_2) from cellotetraose (G_4), G_2 and cellotriose (G_3) from cellopentaose (G_5) and cellohexaose (G_6) (data not shown for G_6) and did not hydrolyse cellotriose even after prolonged incubation. In contrast to *celA*, the complete hydrolysis products by the *celB* and *celC* enzymes on G_3 , G_4 and G_5 were G_2 and glucose (G_1). The intermediate products were also detected: G_3 from G_4 hydrolysis, and G_3 and G_4 from G_5 hydrolysis in the early stages of the reaction (see Fig. 4a). The hydrolysis patterns of the three cellulases on cellotetraose are illustrated in Fig. 4(b). The catalytic mode of the *celB* and *celC* enzymes is different from that of *celA* and they both randomly cleaved β -1,4-glucosidic linkages of cellodextrins. As the *celA* enzyme had high activity towards microcrystalline cellulose, its hydrolysis products were also analysed and found to be almost exclusively cellobiose. No cellotriose or higher oligocellulosic compounds were detectable. This indicates that the *celA* enzyme appears to cleave cellulose by cellobiose units (presumably from the non-reducing ends of cellulose chains), as endo-type cellulose degradation would accumulate an appreciable amount of cellotriose, which is not degraded by *celA* enzyme. The catalytic mode of *celA* enzyme on defined cellodextrins also exhibited the cellobiohydrolase-like property, for it did not cleave the bonds in a random manner as endoglucanases do. The *celA* enzyme cleaved G_4 and G_5 by removing a cellobiose unit, although it also split G_6 into two G_3 molecules. This contradictory behaviour has also been observed with cellobiohydrolases isolated from *Penicillium pinophilum* (Claeysens *et al.*, 1989) and *Clostridium thermocellum* (Morag *et al.*, 1991). It remains to be determined whether the unique symmetrical position of the third bond in G_6 is a special temptation for some cellobiohydrolases. Analysis of the pattern of celloheptaose hydrolysis would clarify the issue (unfortunately, this compound is not commercially available). Collectively, these results suggest that *celB* and *celC* encode endoglucanases, while *celA* encodes a cellobiohydrolase despite possessing weak activity on CM-cellulose

Fig. 5. Expression of three cellulase transcripts in *N. patriciarum* grown on cellulose or glucose medium with or without rumen fluid. RNA was prepared from *N. patriciarum* grown either on medium A (rumen fluid-free) with 0.5% (w/v) crystalline cellulose, Avicel (C) or 1% (w/v) glucose (G) at 39 °C for 4 d; or on medium B (containing 10% w/v, rumen fluid) with 0.5% (w/v) Avicel (C) or 1% (w/v) glucose (G) at 39 °C for 48 h (the reason for the short culture time is that the fungus grew much faster in medium B than medium A). One microgram of each poly(A)⁺ RNA preparation was fractionated on 1.2% (w/v) agarose/2.2 M-formaldehyde gel. Digoxigenin-labelled RNA probes generated from 3'-region-deleted cDNA clones (*celA'*, *celB'* and *celC'*) were used for hybridization.

(in comparison with activity on amorphous cellulose) and no activity on MUC. The enzymic properties of the cellobiohydrolase encoded by *celA* are similar to those of cellobiohydrolase II isolated from *Trichoderma reesei* and the major cellobiohydrolase from *Clostridium thermocellum*, which are unable to hydrolyse MUC (Penttilä *et al.*, 1988; Morag *et al.*, 1991) and may also possess some activity on CM-cellulose (Kyriacou *et al.*, 1987; Morag *et al.*, 1991). In view of the CM-cellulose-hydrolysing activity observed, the cellobiohydrolase encoded by *celA* must also be able to attack the internal glucosidic linkages of cellulose chains, though at a much slower rate.

Regulation of cellulase gene expression in *N. patriciarum*

Isolation of multiple cellulase cDNAs provides an opportunity to study the regulation of cellulase gene expression in the rumen fungus, using nucleic acid probes generated from these clones. Northern hybridization analysis showed that expression of all three cellulase transcripts was very low in the fungus grown in glucose medium without rumen fluid (Fig. 5). However, unlike the aerobic fungus *Trichoderma reesei*, where the cellulase mRNA studied was undetectable in the cells grown

on glucose medium (El-Gogary *et al.*, 1989; Messner & Kubicek, 1991), this low level of expression in *N. patriciarum* was detectable even after three passages through the glucose medium. As shown in Fig. 5, the levels of all three cellulase transcripts were strongly induced by the presence of cellulose in the rumen-fluid-free medium. Indeed, both transcripts detected by the *celB* probe under high-stringency conditions are regulated by cellulose, suggesting that they are probably transcribed from the same gene, but are either processed differently (alternative splicing) or initiated from different promoters (dual transcriptional initiation sites). However, they could also result from two homologous genes.

Most interestingly, we observed that the mRNA levels of all three cellulases were high in the fungus grown in the medium containing 10% (w/v) rumen fluid with the presence of either glucose or cellulose (Fig. 5). Presumably, the rumen fluid contains components of incompletely digested cellulosic substances which are able to induce cellulase mRNA expression. It is intriguing that an insoluble substrate (cellulose) can regulate enzyme synthesis without entering the cell. It has been speculated that the constitutive level of cellulase synthesis, though low, generates small soluble molecules which may be able to trigger the higher levels of cellulase gene expression (Robson & Chambliss, 1989; Béguin, 1990). The ability of small molecules such as sophorose (a disaccharide) to induce cellulase synthesis has been demonstrated (El-Gogary *et al.*, 1989). It remains to be determined which compounds in the rumen fluid induce the cellulase mRNA transcription. It has been suggested that glucose at the concentration of 1% (w/v) represses cellobiohydrolase I mRNA transcription in an aerobic fungus, *Trichoderma reesei* (El-Gogary *et al.*, 1989). Addition of glucose to the medium containing rumen fluid did not appear to reduce the levels of any of the three cellulase transcripts in *N. patriciarum*. However, it is possible that the effective concentration of glucose for repression of cellulase transcripts may differ between *T. reesei* and *N. patriciarum* and may not be maintained during cultivation (this may especially present a problem if the turnover rates of these cellulase transcripts are very high). Further investigation is needed to clarify these issues.

In summary, we have isolated three distinct cDNAs encoding one cellobiohydrolase and two endoglucanases from the rumen fungus *N. patriciarum*, which were functionally expressed in *E. coli*. Expression of these cellulase transcripts in the fungus is induced by cellulose.

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