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Expression and Secretion of Barley Cysteine Endopeptidase B and Cellobiohydrolase I in *Trichoderma reesei*

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Localization of expression and secretion of a heterologous barley cysteine endopeptidase (EPB) and the homologous main cellobiohydrolase I (CBHI) in a *Trichoderma reesei* transformant expressing both proteins were studied. The transformant was grown on solid medium with Avicel cellulose and lactose to induce the *cbh1* promoter for the synthesis of the native CBHI and the recombinant barley protein linked to a *cbh1* expression cassette. Differences in localization of expression between the two proteins were clearly indicated by in situ hybridization, indirect immunofluorescence, and immunoelectron microscopy. In young hyphae, native-size recombinant *epb* mRNA was localized to apical compartments. In older cultures, it was also seen in subapical compartments but not in hyphae from the colony center. The recombinant EPB had a higher molecular weight than the native barley protein, probably due to glycosylation and differential processing in the fungal host. As was found with its transcripts, recombinant EPB was localized in apical and subapical compartments of hyphae. The *cbh1* mRNA and CBHI were both localized to all hyphae of a colony, which suggests that the endogenous CBHI was also secreted from these. In immunoelectron microscopy, the endoplasmic reticulum and spherical vesicles assumed to contribute to secretion were labeled by both CBHI and EPB antibodies while only CBHI was localized in elongated vesicles close to the plasma membrane and in hyphal walls. The results indicate that in addition to young apical cells, more mature hyphae in a colony may secrete proteins.

The ability to secrete hydrolytic enzymes into the external medium is a characteristic of filamentous fungi which makes them important as scavengers of plant material in nature and as producers of enzymes in industry. One of the best-known secretors of extracellular proteins is the cellulolytic mesophile fungus *Trichoderma reesei* (reviewed by Nevalainen et al. [25]). Mutant strains can secrete up to 40 g of protein per liter into the growth medium, about 60% of which consists of cellobiohydrolase I (CBHI), produced by one gene (5, 7). After cloning of the gene encoding CBHI, its strong inducible promoter was used for the expression of both homologous and heterologous gene products in the eukaryotic *T. reesei* system (reviewed by Nevalainen and Penttilä [23]).

At the molecular level, very little is known about the secretory pathway, posttranslational modifications, and release of extracellular enzymes from the hyphae into the growth medium in filamentous fungi. Also, the yields of heterologous gene products from *T. reesei* and other fungi have so far been quite low when compared to those of homologous proteins. Since transcription has not been considered to be the main restricting factor in the production of heterologous proteins in filamentous fungi (17), it is probable that they are lost while secreted. Thus, it would contribute to the basic understanding of fungal cell biology and help improve production strains if the sorting, targeting, and excretion of proteins from the hyphae of *T. reesei* were better understood, as is the case for animal cells, yeasts and bacteria (16, 30, 32, 39).

The main secretory route suggested for proteins in filamentous fungi is through the growing hyphal apex (40). Rapid apical growth of hyphae requires an efficient system for exocytosis of cell wall precursors. The proteins destined for secretion are thought to follow the bulk flow and traverse the growing apex (38). Immunoelectron microscopic localization of the homologous endoglucanases in *T. reesei* (35) showed intensively labeled hyphal tips and some label in the hyphal walls. More recent ultrastructural studies with an alkaline xylanase produced by a high-cellulase-secreting mutant of *T. reesei*, Rut-C30, indicated that two major subcellular structures, the endoplasmic reticulum (ER) and secretory vesicles/vacuoles, were involved in secretion (14). No Golgi-like structures were identified in the study.

To increase our understanding of protein processing and excretion of heterologous proteins in fungi, a cDNA of a barley (*Hordeum vulgare*) cysteine endopeptidase B (EPB) has been introduced into *T. reesei* (33). In barley, the secretion of EPB is fast and is apparently a constitutive process in a tissue surrounding a biosynthetically inactive storage compartment of the grain. The secretion occurs through the Golgi complex and inside small vesicles without accumulation (20). The function of the EPB is to hydrolyze storage proteins to amino nitrogen for further use in the seedling. This is a process comparable to the secretion of cellulolytic enzymes of *T. reesei* into its surroundings to hydrolyze polymeric cellulose to oligosaccharides and glucose for growth. Thus, the EPB is a good model for investigating the transcription, translation, and secretion of a foreign protein in *T. reesei*. In the present work, we localize and compare the expression and secretion of EPB and the endogenous CBHI in a recombinant strain, *T. reesei* ALKO3713, which produces both proteins on solid cultivation media.

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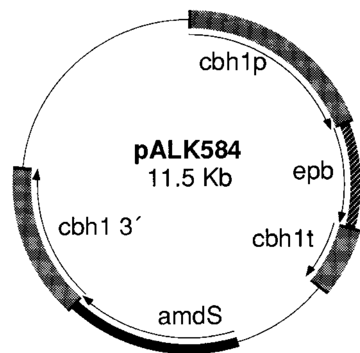

FIG. 1. Physical map of the EPB expression vector pALK584 used to transform the *epb* cDNA in *T. reesei* as described by Saarelainen et al. (33). The acetamidase gene *amdS* is used as a marker for transformation. cbh1p, *cbh1* promoter; cbh1t, *cbh1* terminator.

MATERIALS AND METHODS

Antibodies. EPB, purified from the extract of barley aleurone layers (11), and the polyclonal rabbit antiserum (12) were obtained from D. Ho (Washington University, St. Louis, Mo.). The immunoglobulin G (IgG) fraction of the antiserum (0.34 mg/ml) was separated by protein G-Sepharose (MAbTrap G; Pharmacia LKB Biotechnology, Uppsala, Sweden) and used for immunoblotting and immunolabeling of EPB. Monoclonal antibody against CBHI, CI-261 (1.6 mg/ml) (1), was from Primalco Biotec Ltd., Rajamäki, Finland.

Fungal strains and cultivation. In the expression plasmid pALK584 (Fig. 1), described by Saarelainen et al. (33), the fragment from the EPB cDNA clone was linked between the *cbh1* promoter and terminator sequences. The *epb* cDNA accommodates the signal, pro-, and mature sequences of the proteinase (12). The *T. reesei* transformant ALKO3713 has only one copy of the gene construct at an ectopic locus and produces CBHI and EPB as described by Saarelainen et al. (33). The transformation host, *T. reesei* Rut-C30 (21), was used as a reference for CBHI production. Fungal strains were cultivated for 1 to 5 days at 28°C on agar plates containing minimal nutrient salts (24) at pH 5.5, supplemented with 1% Avicel-cellulose (Fluka Chemica, Switzerland) and 2% lactose for maximal induction of the *cbh1* promoter. For the Western and Northern blotting, fungal spores were inoculated on Hybond nitrocellulose disks (Amersham, Arlington Heights, Ill.) placed on the agar and grown for 1 to 5 days. Proteins secreted by the colonies were trapped on the nitrocellulose filter (26). For microscopic studies, fungal cultivation was carried out on a polycarbonate film (The Isopore Track-Etched film; pore size, 0.2 μm [Millipore, Bedford, Mass.]) or a cellophane disk (27); both were covered with a thin layer of 2% agarose with minimal salts and 2% lactose to maintain the hyphal orientation.

RNA isolation and Northern blot analysis. Total RNA was isolated from 3- to 4-day-old mycelia of *T. reesei* by using a TRIzol reagent as specified by the manufacturer (Gibco BRL, Gaithersburg, Md.). Total RNA from aleurone layers of barley grain that had germinated for 6 days (20) was isolated by a guanidine-HCl extraction procedure as described by Belanger et al. (2) with modifications described by Koehler and Ho (12). Fungal total RNA (10 μg) and total RNA extracted from barley aleurone layers (5 μg) were used for gel electrophoresis. After electrophoresis on a formaldehyde-agarose gel (18), total RNA was blotted onto a GeneScreen membrane (DuPont NEN, Boston, Mass.), UV cross-linked, and stained with 0.02% methylene blue in 0.5 M sodium acetate (pH 5.2). A 1.26-kb *Hin*dIII-*Eco*RV fragment of the *epb* cDNA clone pHVEP4 (12), a 1.82-kb *Hin*cII fragment of the *cbh1* cDNA clone pTTc01 (28), or a 0.77-kb *Kpn*I-*Eco*RI fragment of a birch ribulose 1,5-bisphosphate carboxylase/oxygenase (Rubisco) cDNA clone (37) was labeled by a random primer method (Ready-To-Go kit; Pharmacia LKB Biotechnology Inc., Piscataway, N.J.). Prehybridization was carried out at 60°C for 1 h in a rapid hybridization solution (QuikHyb; Stratagene, La Jolla, Calif.), after which the probe was added and incubation was continued for another 1 h. The filters were washed at 65°C twice with 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.1% sodium dodecyl sulfate (SDS) and once with 0.1× SSC–0.1% SDS for 30 min each and exposed to a Kodak X-Omat film with an intensifying screen at −70°C for 96 h.

Western blotting. Proteins from mycelia were extracted by a modification of the method of Hurkman and Tanaka (10). Mycelia with membranes were pulverized in liquid nitrogen with a pestle and mortar, transferred into Eppendorf tubes, mixed with extraction buffer, boiled for 5 to 10 min, and centrifuged at 13,000 × *g* for 15 min. The extraction buffer used was 30 mM Tris-HCl (pH 8.5)–20% glycerol–2% SDS–2% β-mercaptoethanol containing 25 μg of pepstatin per ml, E-64 [*trans*-epoxysuccinyl-L-leucylamido(4-guanidino)butane], and 100 mM phenylmethylsulfonyl fluoride as protease inhibitors. The supernatants were collected and stored at −70°C until used. Extracts of barley grains were prepared from seeds germinated for 6 days and stored as above (20). Protein concentrations were measured by Bradford's method (Bio-Rad, Richmond, Calif.) with bovine serum albumin (BSA) as the standard.

Fractionation of proteins by SDS-polyacrylamide gel electrophoresis (15% polyacrylamide) was carried out on a MiniProtean II apparatus (Bio-Rad) as described by Laemmli (15). For Western blot analysis, the proteins were transferred to an Immobilon P membrane (Millipore) and detected with antibodies as described by Marttila et al. (20). The IgG fraction of EPB antiserum was diluted to 3.4 μg/ml and the monoclonal CBHI antibody was diluted to 0.53 μg/ml before use. To control the specificity of the EPB immunoblotting, the EPB antibody was preincubated for 2 h with growth medium containing the recombinant EPB before immunostaining.

In situ hybridization. The quick-freezing, freeze-substitution, and hyphal wall weakening were done as described by Raudaskoski et al. (31). After these treatments, hyphae were rinsed for 5 min with phosphate-buffered saline (PBS) at pH 5.5 and then at pH 7.3. The specimens were placed onto aminoalkylsilane-coated slides (9), which were laid on a thermal block at 40°C. To enhance the penetration of the probe, the hyphae were treated with proteinase K (10 μg/μl; Boehringer, Mannheim, Germany) for 10 min at 37°C and washed briefly with distilled water. Prehybridization and hybridization were done by the method of Marttila et al. (19) with the following modifications. Prehybridization was carried out for 1 h at 40°C in the hybridization buffer (50% formamide, 10% dextran sulfate, 0.3 M NaCl, 1× Tris-EDTA [TE], 1× Denhardt's solution, 10 mM dithiothreitol) containing 20 mg of tRNA per ml from RNase-free *Escherichia coli*. For in situ hybridization, the cDNAs described above were ^{35}S-dATP labeled by the random-priming kit as for Northern blot analysis, giving a specific activity of 1×10^8 to 5×10^8 cpm/μg. The cDNA of Rubisco was used as a control probe. *T. reesei* strains that did not synthesize *cbh1* or *epb* mRNAs were treated with the *cbh1* or *epb* probes as negative controls. The final probe concentrations in the hybridization buffer were 0.5 to 1 ng/μl. Hybridization was carried out for 16 h at 42°C, and posthybridization washes were done at 60°C, twice with 2× SSC for 30 min and once with 0.5× SSC for 1 h. The slides were rapidly dehydrated in ethanol, air dried, and laid on Kodak X-Omat X-ray film for 3 days for preexamination. They were then coated with a Kodak NTB2 nuclear emulsion, diluted 1:1 with distilled water, and exposed for 1 to 3 weeks at 4°C. They were developed in Kodak D19 developer for 3 min and fixed in Kodak Unifix for 5 min.

In situ specimens were also treated with *N*-acetylglucosamine-specific rhodamine-conjugated wheat germ agglutinin (WGA; Sigma) to visualize fungal cell walls and septa. The developed slides were washed with PBS (pH 7.3), and rhodamine-WGA (25 μg/ml) in PBS containing 100 μM each $CaCl_2$, $MgCl_2$, and $MnCl_2$ was added to specimens. The slides were incubated for 2 h at 37°C and rinsed with PBS at pH 7.3 and then at pH 8.5. Specimens were then mounted into glycerol-PBS buffer (1:2) containing 1 μg of 4′,6-diamidino-2-phenylindole (DAPI) per ml to visualize the nuclei.

Indirect immunofluorescence microscopy. For protein localization by indirect immunofluorescence microscopy, samples from the *T. reesei* ALKO3713 transformant and host Rut-C30 colonies were quick-frozen and freeze-substituted, and the cell walls were treated as described by Raudaskoski et al. (31). For detection of CBHI and EPB, colonies were transferred into drops of PBS (pH 7.3) containing polyclonal anti-EPB or monoclonal anti-CBHI at dilutions of 17 μg/ml and 3.2 μg/ml in PBS, respectively. The samples were incubated at 37°C for 1 h and then rinsed four times for 5 min with PBS (pH 7.3) containing 0.1% BSA. Samples treated with the polyclonal EPB antibody were further incubated in fluorescein isothiocyanate-labeled goat anti-rabbit IgG (Sigma) diluted to 1:50, and those treated with the antibody against CBHI were incubated in tetramethylrhodamine-5-isothiocyanate (TRITC)-labeled goat anti-mouse IgG (Sigma) diluted to 1:32. Both secondary antibodies were diluted in PBS (pH 7.3) containing 0.1% BSA and incubated for 1 h at 37°C. After incubation, the specimens were rinsed four times for 5 min with PBS (pH 8.5) containing 0.1% BSA. Hyphae were then carefully separated from the membranes under a dissecting microscope and mounted in glycerol-PBS (1:2) (pH 8.5) containing 1 μg of DAPI/ml. The specimens were examined under a microscope equipped with epifluorescence illumination and appropriate filters. Treatment of strains unable to produce EPB or CBHI with the antibodies and replacement of the primary antibody with the dilution buffer were used as controls. Treatments with the rhodamine-WGA lectin were then carried out and the samples were rinsed as described above.

Immunoelectron microscopy. Mycelia from the ALKO3713 transformant and Rut-C30 strain were fixed for 2 h at room temperature in a mixture of 4% (wt/vol) paraformaldehyde and 1% (vol/vol) glutaraldehyde in PBS (pH 7.3) and then rinsed thoroughly with PBS. The fixed samples were incubated at 4°C overnight with rotation in 20% (wt/vol) polyvinylpyrrolidone (M_r, 10,000; Sigma, St. Louis, Mo.) containing 1.84 M sucrose and then frozen in liquid nitrogen as described by Tokuyasu (36). Ultrathin sections were cut at −90°C with the Reichert Ultracut FC4 ultramicrotome equipped with a cryoattachment. Sections were placed on nickel grids coated with Formvar (Monsanto) and carbon.

For immunolabeling, the specimens were first incubated in PBS blocking solution containing 10% (wt/vol) fetal calf serum (Sigma) and 0.12% (wt/vol) glycine for 30 min and then incubated in the IgG fraction for EPB or the monoclonal antibody for CBHI at room temperature. Antibodies against EPB and CBHI were diluted to 25 and 3.2 μg/ml in the blocking solution, respectively. After a 2-h incubation, the grids were washed in PBS-glycine for 25 min, and

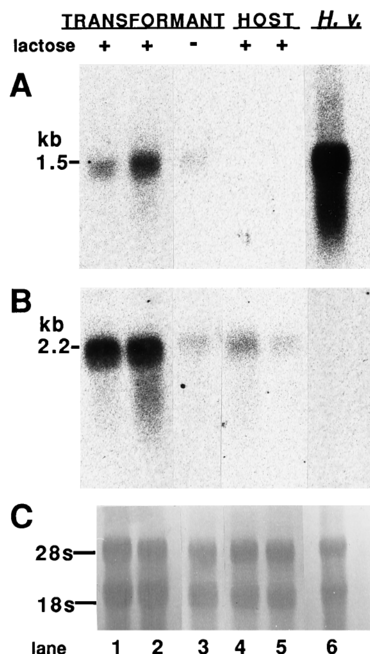

FIG. 2. (A and B) Northern blot analysis of *epb* (A) and *cbh1* (B) expression in the *T. reesei* transformant ALKO3713, the host strain Rut-C30, and the barley (*H. vulgare* [*H.v.*]) aleurone layer. (C) Methylene blue staining of 18S and 28S rRNA from the same samples. Samples (10 μg) of total RNA from *T. reesei* strains (lanes 1 to 5) and samples (5 μg) from the barley aleurone layer (lane 6) were analyzed. The expression of mRNAs is visible in the transformant (lanes 1 to 3) and host (lanes 4 and 5) strains. Two host strain extracts as controls were blotted on the same filter where the transformant samples were located. The label (+) indicates that the growth medium also contained lactose (lanes 1, 2, 4, and 5).

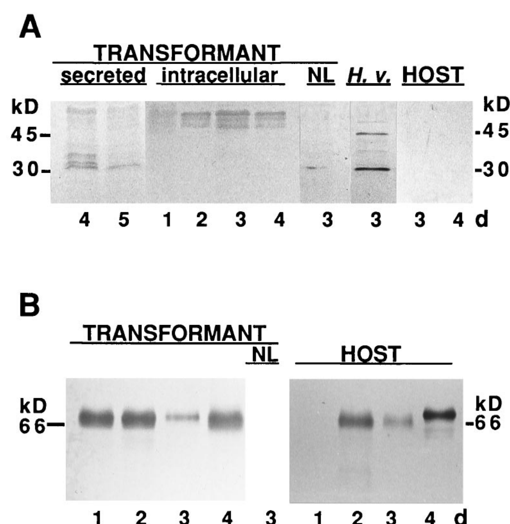

FIG. 3. Western blot analysis of EPB (A) and CBHI (B) production in the *T. reesei* transformant ALKO3713 and host strain Rut-C30 and in germinated barley seeds (*H.v.*) incubated with polyclonal EPB and monoclonal CBHI antibodies. Protein was loaded at 5 μg per lane. The age of the cultures at the time of sampling is indicated in days (d). NL, no lactose. (A) Secreted and intracellular recombinant EPB proteins of transformant ALKO3713. Recombinant protein produced in the absence of lactose (NL) and native EPB from the barley aleurone layer (*H.v.*) are also shown, as is protein extracted from the host strain *T. reesei* Rut-C30. (B) The produced CBHI protein is visible in the transformant and host strains.

those treated with the polyclonal EPB antibody were incubated for 60 min with protein A conjugated to 10-nm-diameter gold particles (ICN Immunochemicals, Irvine, Calif.). Protein A-gold was prepared by the method of Slot and Geuze (34) and diluted to 1:125 with blocking solution. For CBHI localization, the grids were treated with anti-mouse IgG conjugated to 10-nm gold particles diluted to 1:50 with blocking solution. After gold labeling, the specimens were washed in PBS for 25 min and in distilled water for 5 min and embedded for 10 min on ice in 1.5% (wt/vol) methylcellulose (25 cP; Sigma) containing 0.4% (wt/vol) uranyl acetate. Control sections were treated with the blocking solution without the primary antibody. The grids were examined with a JEOL 1200EX transmission electron microscope at an acceleration voltage of 60 kV.

RESULTS

In this work, we have studied the simultaneous expression and secretion of a heterologous EPB and homologous cellulase CBHI enzyme in the *T. reesei* transformant strain producing both proteins. Preliminary tests with transformants showed that features of the transformant ALKO3713 made the strain best suitable for our purposes of the expression studies in *T. reesei*. Fungal cultivations were performed on solid media to facilitate the localization of transcription and enzyme secretion.

Expression of CBHI and EPB in *T. reesei*. Northern hybridization of the total RNA isolated from the *T. reesei* transformant strain ALKO3713 with *epb* cDNA showed that the transformant produced *epb*-specific transcripts of the same size as *epb* mRNA found in barley aleurone layers (Fig. 2A). A signal for the natural-size *cbh1* mRNA, about 2.2 kb, was also obtained (Fig. 2B), because integration of the *ebp* construct had not occurred in the endogenous *cbh1* locus (33). The host strain, *T. reesei* Rut-C30, did not produce any *epb* mRNA (Fig. 2A, lanes 4 and 5), and no signal for *cbh1* mRNA was detected in the barley aleuronal RNA (Fig. 2B, lane 6). The presence of lactose in the growth medium significantly increased the synthesis of the *epb* and *cbh1* transcripts in *T. reesei* (Fig. 2A and B; compare lanes 1, 2, and 3), as expected (13, 22).

In the immunoblots of extracts from barley aleurone layers, the mature EPB was seen as a 30-kDa band and its propeptide was seen as a 42.5-kDa band (Fig. 3A). When the EPB antibody was used in Western blotting to detect the EPB produced by the *T. reesei* transformant ALKO3713, a range of secreted polypeptides with molecular masses between 32 and 36 kDa were obtained on days 4 and 5 (Fig. 3A). From days 1 to 4, intracellular *T. reesei* extracts showed considerably larger forms, ranging from about 50 to 70 kDa. The specificity of the antibody used was indicated by the absence of signal when the host strain extract was treated with the antibody (Fig. 3A) or the EPB antibody was preincubated with growth medium containing the recombinant EPB (data not shown). Approximately the same amount of the 67-kDa CBHI protein was produced by both the transformant strain ALKO3713 and its host strain, Rut-C30 (Fig. 3B). Interestingly, the production of CBHI in the transformant started 1 day earlier than in the host strain. The absence of lactose in the growth medium decreased the amounts of EPB and CBHI produced in *T. reesei* (Fig. 3).

In situ localization of *epb* and *cbh1* transcripts in fungal hyphae. In the hyphae of the transformant ALKO3713 grown for 1 to 5 days on Avicel-lactose plates, strong signals were obtained when the transcripts of *epb* and *cbh1* were localized by in situ hybridization (Fig. 4 and 5, respectively). When hybridization was carried out with the cDNA probe of Rubisco for the transformant strain ALKO3713 or with the *epb* probe for the host strain Rut-C30, only low background labeling occurred (Fig. 4A and B).

In 1- to 5-day-old samples, the *epb* transcripts were located only in the apical and subapical cells of hyphae at the edge of the colony. After growth for 16 h, dispersed *epb* transcripts

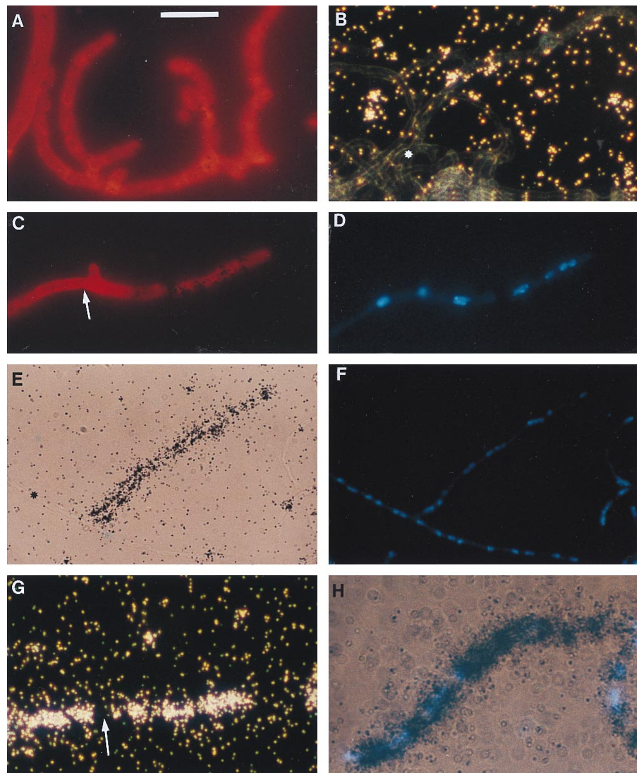

FIG. 4. In situ hybridization analysis for the detection of *epb* mRNAs. Birch Rubisco (A) and *epb* cDNA (B to H) probes to freeze-substituted hyphae of the *T. reesei* transformant (A and C to H) and host strain (B) were used. Hyphal walls (A and C) were stained with rhodamine-labeled WGA lectin, and the nuclei were detected with DAPI (D, F, and H) after the in situ hybridization procedure. (B and G) The field was photographed under dark-field illumination, where silver grains appear bright yellow. (E) Phase-contrast picture without fluorescence. When the specimens were illuminated simultaneously by UV and visible light, the silver grains appeared as black spots against a red background in hyphae stained with rhodamine-labeled WGA (A and C) or between the blue nuclei in DAPI-stained hyphae (H). White arrows mark the position of the septum between the apical and subapical compartments. The white asterisk indicates the hyphae of the host strain. Bars, 10 μm (A to D, G, and H) and 20 μm (E and F).

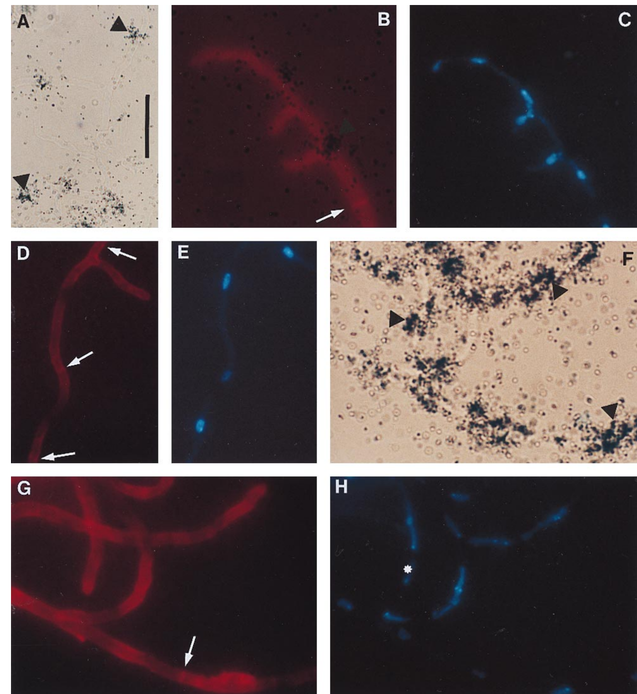

FIG. 5. In situ hybridization analysis for the detection of *cbh1* in freeze-substituted hyphae of the *T. reesei* transformant. Hyphal walls (B, D, G) and nuclei (C, E, H) were stained as in Fig. 4. Arrows indicate septa. The asterisk marks the outermost nucleus in an apical cell. The triangles point to the aggregate of *cbh1* transcripts. Bars, 20 μm (A) and 10 μm (B to H).

appeared as associated with the nuclei in the apical cells but not in the extreme tip region of the hyphae (Fig. 4C and D). In 3- and 4-day-old samples (Fig. 4H), when a strong signal for *epb* transcripts was obtained by Northern hybridization (Fig. 2A), *epb* mRNA was also localized in the subapical compartments of the hyphae. At this stage, the *epb* transcripts were visualized mainly in the branches of the subapical compartments (Fig. 4E and G) which contained several nuclei (Fig. 4F) that possibly participated in the transcription. After 3 days, the intensity of the hybridization increased (Fig. 4H) and transcripts were no longer detected solely in the branches but also in the main hypha from which the branches originated. Due to extremely high levels of the signal in both apical and subapical compartments, the nuclei were difficult to distinguish among the transcripts. The described labeling for *epb* transcripts was represented by over 90% of 200 randomly selected apical and subapical cells in each of 11 inspected colonies of the transformant ALKO3713. The signal was totally absent in the cells of the central area of each analyzed colony.

The signal for the *cbh1* mRNA was quite weak and somewhat clustered in the mycelia of the transformant ALKO3713 in the samples grown for 1 and 2 days (Fig. 5A and B). In the apical cells, *cbh1* mRNAs appeared to form larger aggregates than did *epb* transcripts. Similarly to the *epb* signal, these were absent from the extreme apex of hyphae (Fig. 4C and 5B). In 3- to 5-day-old samples, the *cbh1* signal was strong in the entire mycelium including the old hyphal cells (Fig. 5D, F, and G). Similar signals for *cbh1* transcripts were also detected in the host strain Rut-C30 (data not shown). In all 13 colonies of the transformant ALKO3713 and the host strain Rut-C30 inspected, over 95% of young and old hyphal cells throughout each colony had the characteristic signal for *cbh1* mRNA.

Localization of EPB and CBHI proteins in hyphae. Labeling of the EPB protein in the *T. reesei* transformant ALKO3713 showed that the recombinant cysteine proteinase was located in the hyphae at the colony edges. In the samples from 1-day-old colonies, the signal for EPB was seen only at the apex of the hyphae (Fig. 6B). In 3-day-old samples, the label was seen as bright spots in the apical cells and their branches (Fig. 6E), as well as in the adjacent subapical cells (data not shown). The bright spots, visualized with fluorescein, were interpreted to represent vesicle-like structures containing EPB protein. The hyphal structure was well preserved after the immunolabeling procedure. The hyphae accommodated several nuclei, and a certain distance was always observed between the tip and the nucleus closest to it (Fig. 6D and G). Similar hyphal structures were also visualized in the host strain Rut-C30 but without a signal for EPB after the immunolabeling process (Fig. 6F to H). Over 95% of 200 randomly chosen apical and subapical cells gave the positive label for EPB in each of 12 colonies of the transformant ALKO3713. The signal was completely absent in the cells of the central area of each analyzed colony.

In the transformant strain ALKO3713, which produced both CBHI and EPB, a signal for CBHI was visible throughout the mycelium of 1-day-old samples. The signal was clear also in the apical cells (Fig. 6I). After 2 days, stronger labeling was seen

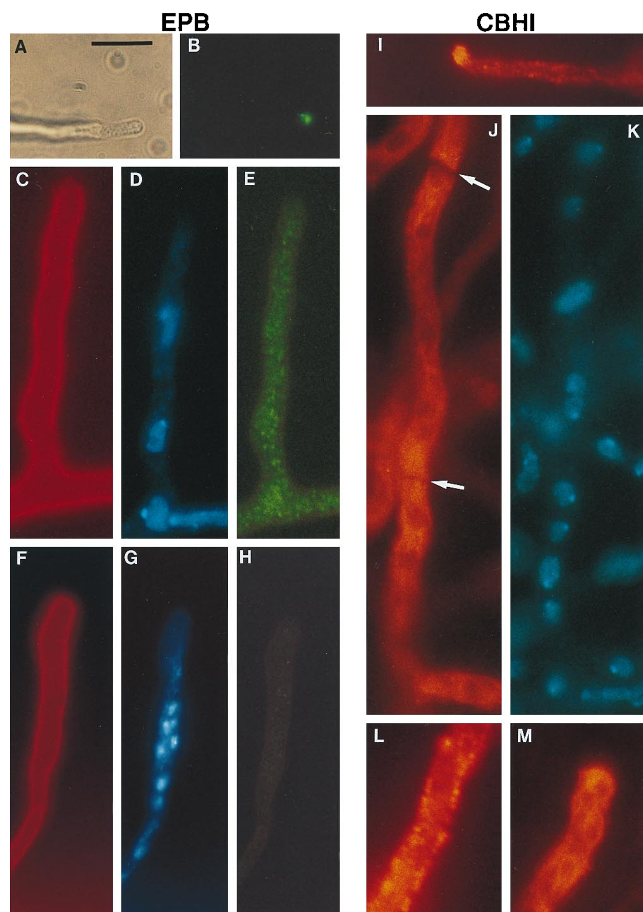

FIG. 6. Indirect immunofluorescence microscopy of EPB (A to H) and CBHI (J to M) proteins in the freeze-substituted hyphae of the *T. reesei* transformant (A to E and I to K) and the host (F to H, L, and M). Hyphal wall (C and F) and nuclear (D, G and K) staining was done as in Fig. 4 and 5. White arrows denote septa (J). Bars, 10 μm (A, B, and F to I) and 5 μm (C to E and J to M).

throughout the mycelium both at hyphal tips and in areas behind them. The signal was also seen in association with hyphal walls but not with the septa (Fig. 6I and J). Nuclei occurred as dark areas surrounded by the labeled CBHI, as shown in an example from the older parts of the mycelium (Fig. 6J and K). In the host strain Rut-C30, a strong signal for CBHI was frequently obtained close to the hyphal wall (Fig. 6L) and labeling for CBHI was detected in the entire mycelium including apical cells (Fig. 6M). Each of 10 inspected colonies of the transformant ALKO3713 and the host strain Rut-C30 had the characteristic label for CBHI in over 99% of all the young and old hyphal cells throughout the colony.

Subcellular localization of EPB and CBHI. A more detailed localization of EPB and CBHI was carried out by immunoelectron microscopy investigation of cryosections from the transformant strain *T. reesei* ALKO3713 (Fig. 7 and 8). A common ultrastructural feature of the host strain Rut-C30 and the recombinant strain ALKO3713 was the large amount of ER. This has also been reported in a previous ultrastructural study of the host strain Rut-C30 (7).

In the immunoelectron microscopic investigation, the recombinant EPB was localized in the ER and small vesicles, which had perhaps been budded from the ER. All hyphae in which the label was found had thin hyphal walls, which suggested that they were from the young parts of the mycelial colony (Fig. 7A to D). Vesicles with the label were also detected in the proximity of the plasma membrane. Characteristic of EPB immunolabeling was the occurrence of the label in conspicuous aggregates next to the ER. No membrane surrounding the aggregation or individual immunogold particles could be distinguished even at high magnification (Fig. 7D). EPB was also localized in the area close to flat, tubular endomembraneous cisternae that seemed to be associated with the plasma membrane and to open to the cell wall (Fig. 7E). The function of these structures is unknown, but they resemble the membrane compartments shown to be responsible for Golgi functions in wild-type yeast cells (29). In control sections treated with buffer instead of the EPB antibodies, only sporadic labeling was observed (Fig. 7F).

In the ALKO3713 strain, CBHI was associated with the ER throughout the cross sections of young (Fig. 8A, E, and F) and old (Fig. 8B to D) hyphae, where the age was deduced from the thickness of the cell wall. CBHI label was also detected in the hyphal wall but not in the septa (Fig. 8B to D), which was consistent with the indirect immunofluorescence microscopic results. In the hyphae, the label for CBHI was often present inside elongated vesicles adjacent to the septum and cell wall (Fig. 8E and F). In addition, small spherical vesicles which resembled those detected by the EPB antibody were labeled. Electron-dense putative protein bodies surrounded by the membrane did not give any positive signal for CBHI (Fig. 8D).

DISCUSSION

In this study, we have focused on visualization of the expression and structures involved in the secretion of the main endogenous CBHI enzyme and a heterologous barley (*H. vulgare*) EPB in *T. reesei*. This was possible in the selected transformant ALKO3713, since the integration of the expression cassette had not disrupted the *cbh1* locus. Thus, the strain produced both CBHI and EPB, of which about 50 mg/liter was found in the culture medium, when grown in shake flask cultures (33). In strain ALKO3713, the level of recombinant *epb* transcripts was only slightly lower than that of endogenous *cbh1* transcripts. This supports the idea that transcription may not be the main restricting event in the production of heterologous proteins in filamentous fungi (17).

The cDNA sequence of the EPB used for expression encodes a preproenzyme of 42.5 kDa. During processing, the 2.5-kDa signal sequence and a propeptide of 12.3 kDa are cleaved off to give the mature form of 25.7 kDa. On SDS-polyacrylamide gel electrophoresis, the mature enzyme migrates with a size of about 30 kDa (12). In Western blots presenting EPB produced by *T. reesei* ALKO3713, the EPB antibody recognized intracellular high-molecular-weight polypeptides and multiple forms of secreted recombinant EPB. The 42.5-kDa preproform of the 30-kDa mature enzyme was not seen in the transformant *T. reesei*, and the secreted form was slightly larger than the mature barley enzyme. However, the secreted EPB from *T. reesei* is enzymatically active (33), which indicates that the fungus is capable of processing at least part of the enzyme into an active form. An explanation for the large EPB polypeptides could be the glycosylation of the barley enzyme in the fungal host. Our preliminary in vitro deglycosylation studies suggest that the recombinant protein, unlike in *H. vulgare*, is indeed N glycosylated (25a).

In situ hybridization experiments indicated that in the transformant ALKO3713, the *epb* mRNA was located mainly in the young hyphae while the *cbh1* mRNA also appeared in the old compartments of the colony. Occurrence of the transcripts in apical compartments which are involved in the growth of the

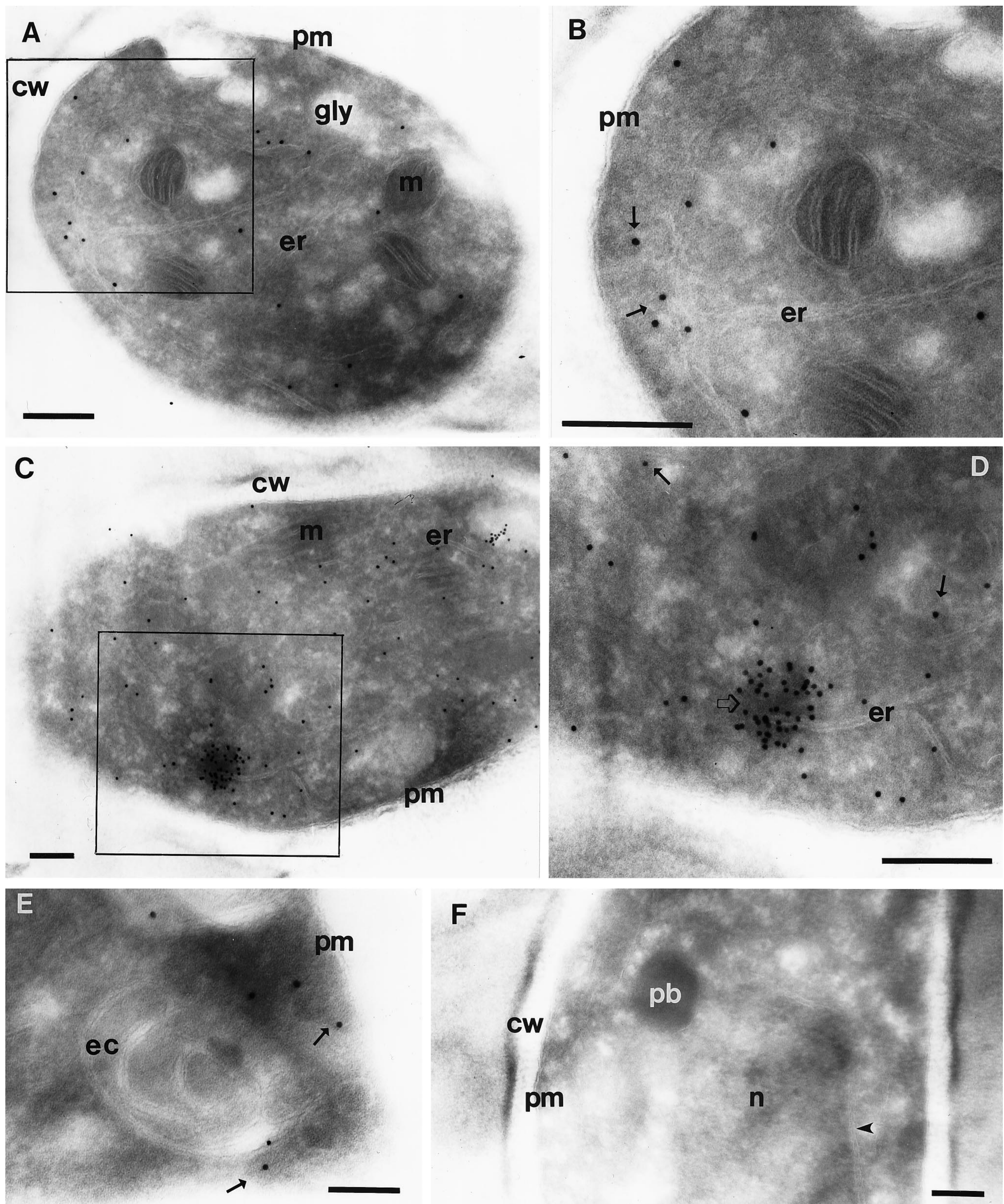

FIG. 7. Intracellular localization of EPB in *T. reesei* transformant grown on Avicel-lactose medium for 2 days. Ultrathin cryosections were treated with the EPB antibody followed by protein A-conjugated 10-nm-diameter gold particles. Arrows point to membrane-coated vesicles (B and E). The open arrow indicates an EPB aggregate adjacent to the ER (C). The arrowhead marks the nuclear envelope (F). cw, cell wall; er, endoplasmic reticulum; gly, glycogen granules; m, mitochondrion; pm, plasma membrane; ec, endomembraneous cisterna; n, nucleus; pb, protein body. Bars, 0.2 μm (A to D and F) and 0.1 μm (E).

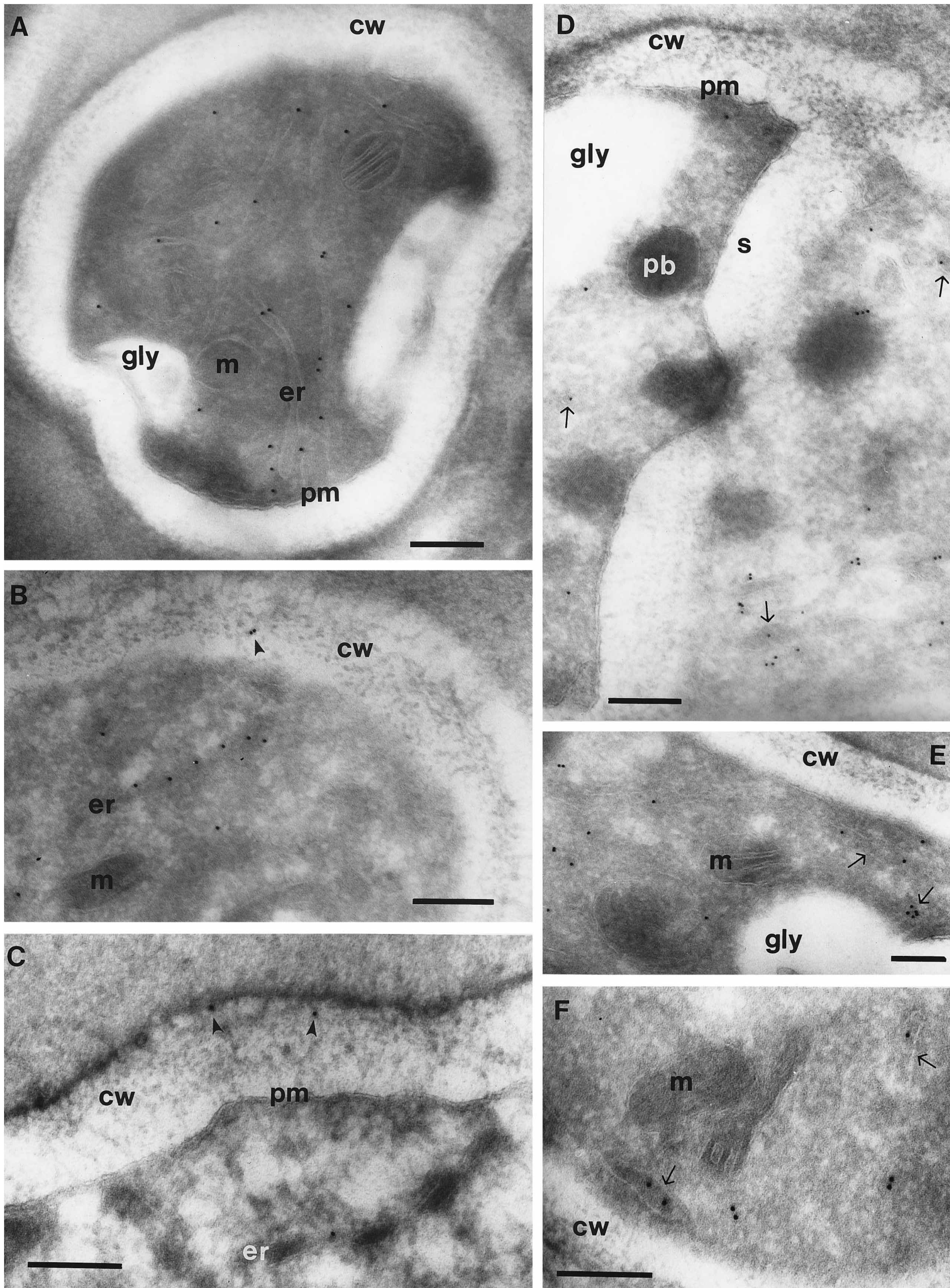

FIG. 8. Intracellular localization of CBHI in the *T. reesei* transformant grown on Avicel-lactose medium for 2 days. Ultrathin cryosections were treated with monoclonal CBHI antibody followed by anti-mouse IgG-conjugated 10-nm-diameter gold particles. Arrowheads indicate the label for CBHI (B and C). Arrows point to various labeled vesicles (D to F). cw, cell wall; er, endoplasmic reticulum; gly, glycogen granules; m, mitochondrion; pb, protein body; pm, plasma membrane; s, septum. Bar, 0.2 μm.

hyphae suggests that nuclei which are closely coupled to the progression of the cell cycle (4) are also involved in the production of transcripts of secreted proteins. The *epb* mRNA observed in subapical compartments and that of *cbh1* in the hyphae from the center of the colony could represent stable transcripts originating at the time these compartments belonged to the actively growing parts of the colony. If this is the case, the absence of *epb* mRNA from the old hyphae could indicate that it is less stable than the endogenous *cbh1* mRNA.

In the *T. reesei* transformant studied, EPB and CBHI were generally localized in the same hyphal compartments as their transcripts, except that both proteins were also found at the very apex of the hyphae, where transcripts were never detected. This means that transport of the proteins in the hyphae must take place from the site of synthesis, for example, to the tip. In yeast and animal cells, transport through the secretory pathway occurs in transport vesicles (30). In the immunogold-labeled cryosections of hyphae from ALKO3713, EPB and CBHI were localized in the ER and close to it in vesicles, which could represent transport vesicles leaving the ER. Only a few structures comparable to Golgi compartments in yeast (29) have been identified. The lack of data about the morphology of the Golgi compartments in *T. reesei* made it difficult to deduce the part of the secretion pathway targeting to the plasma membrane, although vesicles containing EPB and CBH were distinguished close to it.

Electron-dense elongated vesicles were found to be only CBHI labeled in both strains studied. These elongated vesicles could originate from Golgi bodies (8). The tubular elongated vesicles could form a part of the secretion mechanism for CBHI and represent an ultrastructural counterpart to structures with strong fluorescence seen close to the plasma membrane in the indirect immunofluorescence microscopic studies. In the labeled cryosections, CBHI was also observed inside the hyphal wall but not in the septa. These results, together with the occurrence of the *cbh1* transcripts and the CBHI protein in all hyphae independent of age, suggest that each hyphal compartment throughout the *T. reesei* mycelium has the ability to secrete the CBHI protein. This may partially explain the gram-level yields of CBHI protein excreted in the cultivation medium and suggests a complementary mechanism for fungal protein secretion in addition to the bulk flow associated with polarized apical growth of hyphae (38). An alternative protein secretion pathway in *T. reesei* hyphae could, for example, function with a translocator resembling the P glycoprotein on the plasma membrane as suggested for *Aspergillus* (3).

Unlike the endogenous CBHI, immunolocalization results for the heterologous EPB suggest that the main secretion of EPB takes place at the hyphal apex. The EPB label was detected only in the youngest part of the colony, and no label was seen next to the plasma membrane along the lateral walls of hyphae by indirect immunofluorescence microscopy or in the hyphal walls in labeled cryosections. In the immunoelectron microscopic investigation, aggregates of EPB-labeled granules were found adjacent to the ER. Whether these EPB aggregates are ultrastructural equivalents of the large proteins in the intracellular fraction revealed by Western blotting, perhaps representing poorly secretable/unsecretable EPB, remains to be clarified.

In summary, this is the first report on a microscopic study of simultaneous expression and secretion of a native protein and a foreign protein in a filamentous fungus. We were able to show that the translational sites of EPB and CBHI correspond to their transcriptional locations, which are different for the recombinant EPB and the native CBHI. The *cbh1* gene is expressed and protein is secreted throughout the mycelium, whereas expression of the recombinant EPB occurs only in the apical and subapical cells. Thus, CBHI could contain a signal(s) promoting effective secretion from the fungal cell. We plan to explore this further with the aid of different fusion proteins. Comparison of the levels of transcripts for the immunoreactive EPB and the endogenous CBHI showed that the efficiency of translation of the recombinant mRNA was reasonably high and thus will not explain the low yields of the secreted recombinant EPB. The most likely reasons for this include degradation, incomplete processing, and/or activation of the barley protein produced in the fungus. In future studies, we will focus on the processing of EPB in *Trichoderma* hosts.

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