In situ* Localization of Specific RNAs in Whole Fruiting Colonies of *Schizophyllum commune

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cDNA clones representing eight specific mRNAs abundantly expressed in a dikaryon of *Schizophyllum commune* but not in the progenitor monokaryons, were used to localize these mRNAs in whole colonies by *in situ* hybridization. A genomic clone for 18S rRNA was used to probe for rRNA. The colonies were grown on hybridization membranes, fixed, and treated with RNAase-depleted wall-lytic enzymes from *Trichoderma harzianum* to facilitate permeation of the probes. RNAs in sectors of the colonies were then localized with [³²P]DNA probes. Hybridization at different developmental stages showed that the specific mRNAs were only formed in parts of the colony where fruit-body initials arose. At later stages these mRNAs disappeared from abortive fruit bodies but remained high in fruit bodies continuing development.

INTRODUCTION

Mating of two co-isogenic monokaryotic strains of *Schizophyllum commune* differing only in the alleles of the two incompatibility genes *A* and *B* generates a dikaryon with a genome identical to that of the mates except for the differences in the alleles of the incompatibility genes. The interactions between the different incompatibility alleles causes the hyphal morphology to switch from a monokaryotic type with mononucleated cells to a dikaryotic type with binucleate cells and clamp connections and also prompts the formation of fruit bodies under appropriate environmental conditions (Wessels, 1987). In the dikaryon some specific mRNAs accumulate at high concentrations, temporally and spatially correlated with fruit-body development (Mulder & Wessels, 1986; Wessels *et al.*, 1987; Ruiters *et al.*, 1988).

In the present paper we describe an *in situ* hybridization method developed for the detection of these mRNAs in whole colonies of *S. commune*. Compared to mRNA determinations in RNA extracted from concentric rings of a colony (Ruiters *et al.*, 1988), this method gives a more detailed picture of the spatial distribution of the mRNAs in the colony during development.

METHODS

Organism and culture conditions. A dikaryon of *Schizophyllum commune* constituted by mating strains 4-39 (CBS 341.81, ATCC 44200, *A41B41*) and 4-40 (CBS 341.80, ATCC 44201, *A43B43*) was used throughout. The growth substrate was complete medium (Raper & Hoffman, 1974) solidified with 1.5% (w/v) agar. To obtain colonies suitable for *in situ* hybridization the fungus was inoculated on membranes which were deposited on 25 ml agar medium in 9 cm Petri dishes. Various membranes were tried. Water-permeable cellophane was unsuitable because colonies detached from the membrane after fixation. Cellulose nitrate membranes (BA 85, Schleicher and Schuell) and cellulose acetate membranes (pore sizes 0.45 and 8 µm, Millipore) reduced the linear growth rate of the colonies by 20–40%. Genescreen membranes (NEN) proved satisfactory giving firm attachment of the colony and an only slightly reduced growth rate. The fungus grew through the membrane and to prevent it from growing

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into the agar a cellophane membrane was positioned between the agar and the Genescreen membrane. The cultures were grown in a circulating-air incubator at 24 °C and 95% relative humidity. Dark conditions were obtained in a light-proof box standing in this incubator. Air from the incubator was pumped through the box to maintain the same atmospheric conditions in the box as in the incubator. It was essential to keep the relative humidity at about 95% during growth to maintain medium contact between the membranes and the nutrient agar. The colonies were first grown in darkness at 24 °C for 5 d (colony diameter 20–25 mm) and then transferred to light (1000 lx) to induce the formation of fruit bodies. Within 2 d fruit bodies were seen macroscopically in a ring at the position representing the border of the colony at the moment of the switch to light.

Cell-wall degrading enzymes. Cell-wall degrading enzymes were isolated from the culture fluid of *Trichoderma harzianum* (CBS 354.33) as described by De Vries & Wessels (1972) but with *Penicillium chrysogenum* wall residues as a substrate (Sonnenberg & Wessels, 1987). This enzyme preparation showed considerable RNAase activity: incubating 200 µg total RNA of *S. commune* with 100 µg wall-lytic enzymes in 0.5 ml 10 mM-sodium acetate buffer, pH 6.0, at 37 °C for 1 h converted 43.5% of the RNA into perchloric-acid-soluble products. To remove this RNAase activity, the enzyme powder was dissolved (2 mg ml⁻¹) in 10 mM-sodium acetate, pH 6.0 and mixed with an equal volume of 2% (w/v) bentonite in 10 mM-sodium acetate, pH 6.0. This suspension was left at room temperature for 2 h with occasional shaking. After centrifugation (5 min, 10000 g) the supernatant with no detectable RNAase activity was used immediately or stored at -30 °C.

Hybridization probes. The probes were cDNA sequences cloned in pGEM-1 (Promega Biotec) representing mRNAs specifically present in fruiting dikaryons (Sc-1, -2, -4, -5, -6, -7, -9, and -14) and one mRNA (Sc-3) present in both monokaryotic and dikaryotic mycelium (Mulder & Wessels, 1986). A 1.8 kbp *EcoRI/XbaI* genomic clone containing a part of the 18S ribosomal RNA gene of *S. commune* was isolated (Ruiters & Wessels, 1989) and used as a probe for rRNA. The vector pGEM-1 was used as a control.

Labelling of the plasmid. Plasmid DNA was labelled by nick-translation with [α -³²P]dCTP as described by Meinkoth & Wahl (1984) with a slight modification according to Mulder & Wessels (1986). Specific activity was about 5×10^7 d.p.m. µg⁻¹.

In situ hybridization of colonies. Cultures at various developmental stages were fixed by pouring 15 ml 4% paraformaldehyde in PBS (phosphate-buffered saline: 137 mM-NaCl, 1.5 mM-KH₂PO₄, 7 mM-Na₂HPO₄, 7 mM-KCl, pH 7.4) on the membrane-grown colony in a Petri dish. To improve penetration of the fixative a vacuum was applied for 15 min and fixation was continued for another 15 min at ambient pressure. The membrane with adhering colony was lifted off the cellophane-covered agar plate, washed three times in PBS (5 min) after which the membrane was blocked off with 3% BSA in PBS for 4 h at 65 °C. (This was necessary because omission of this step caused binding of wall-degrading enzymes to the membrane in the next step, preventing adequate permeabilization of the walls.) The filter was then washed in 10 mM-sodium acetate, pH 6.0, for 5 min. Treatment with cell-wall degrading enzymes (1 mg ml⁻¹ in 10 mM-sodium acetate, pH 6.0) proceeded overnight (16–18 h) at 37 °C. The membrane was washed twice (5 min) in PBS and again blocked off with 3% BSA in PBS at 65 °C for 1 h. (This second blocking treatment was necessary to prevent all background absorption of probes.) The membrane with colony was then cut into 30° sectors which were marked. Four filter sectors to be hybridized with one specific probe were sealed in one polyethylene bag containing 5 ml pre-hybridization buffer, pH 8.0 (50%, v/v, formamide; 0.3 M-NaCl; 10 mM-Tris; 1 mM-EDTA; 1% SDS and 100 µg herring sperm DNA ml⁻¹). Pre-hybridization was done at 42 °C for 2 h. Hybridization was then done at 42 °C for at least 16 h in the same solution after adding 10 ng of ³²P-labelled probe per ml of hybridization buffer. Before addition, the probe was boiled for 3 min and cooled on ice. After hybridization the filters were washed in 2 × SSC (1 × SSC is 0.15 M-NaCl, 15 mM-sodium citrate, pH 7.0) plus 0.1% SDS, twice for 5 min at room temperature and twice for 30 min at 65 °C. The hybridized filters were then positioned so as to image the original colony and exposed to Kodak XAR-5 film for an appropriate time at -70 °C using one intensifying screen.

For re-hybridization of the filters with the 18S rDNA clone, hybridized filters were stored at -70 °C for 4 weeks (twice the half-life of the ³²P label). Re-hybridization was then started at the pre-hybridization step in the protocol given above.

Scanning profiles of whole colony hybridizations. After hybridization of the membrane colonies with the specific cDNA clones and recording the results on X-ray film, the colonies were stored for 4 weeks at -70 °C to lower their radioactivity, and then rehybridized with the 18S rDNA clone. The X-ray film recordings of the two hybridizations were scanned with a Joyce Loeb microdensitometer along three different radii for each mRNA hybridization and along corresponding radii for the rRNA re-hybridization. The signals were averaged and corrected for differences in exposure time and specific activities of the probes. The ratio of the corrected signals, i.e. the relative abundances of the mRNAs, was then plotted along the radius of the colony.

RESULTS

In situ hybridization of RNA in colonies

Fig. 1(a) shows a 90° sector of a 5-d-old dark-grown colony of *S. commune* hybridized to the 18S rDNA clone according to the protocol outlined in Methods but without the treatment with

Fig. 1. Autoradiographs of sectors from a 5-d-old dark-grown culture of *S. commune* on a Genescreen membrane hybridized to an 18S rDNA clone. (a) Not treated with wall-lytic enzymes; (b) pretreated with crude cell-wall lytic enzymes; (c and d) pretreated with RNAase free cell-wall lytic enzymes but (d) additionally treated with RNAase. Exposure time, 4 d.

wall-lytic enzymes. No hybridization signal is visible. Inclusion of a treatment with a non-purified wall-lytic enzyme preparation resulted in a weak hybridization signal near the inoculum (Fig. 1b). A strong hybridization signal, particularly at the growing edge of the colony, was present in a sector incubated with a bentonite-treated enzyme preparation (Fig. 1c). When a sector incubated with this RNAase-free wall-lytic enzyme preparation was subsequently treated with 2 mg ml^{-1} RNAase A and 600 U ml^{-1} RNAase T₁ at 37°C for 2 h, a very weak hybridization signal was obtained (Fig. 1d). In none of these treatments was a hybridization signal seen when the vector pGEM-1 without insert was used as a probe (not shown). These results show that permeabilization of the walls prior to hybridization was necessary and that the signal was derived from an RNA specific to the probe.

Localization of RNAs during fruit-body development

^{32}P -Labelled DNA probes for the various mRNAs known to be elevated in the dikaryon at the time and place of fruiting (Mulder & Wessels, 1986; Ruiters *et al.*, 1988) were used to estimate local concentrations of these RNAs in sectors of whole colonies induced for fruit-body development.

Fig. 2 shows the results of *in situ* hybridization at five developmental stages. At the moment of transfer of a 5-d-old dark-grown colony to the light to induce fruit bodies none of the probed mRNAs was visible (Fig. 2b, 0 h), although prolonged exposure showed the presence of low concentrations of mRNAs Sc-3 and Sc-4 throughout the colony (not shown). After 12 h of illumination Sc-4 and Sc-3 were located at a position representing the colony edge at the moment of transfer to the light (Fig. 2b, 12 h), where fruit-body development started (microscopic aggregates, not shown). Macroscopically, a fruit-body ring was clearly distinguishable after 24 h (Fig. 2a, 24 h) and all fruiting-associated mRNAs were elevated in the ring except Sc-14 (Fig.

2b, 24 h). mRNAs Sc-3 and Sc-4 also became elevated in a newly established ring of initials sometimes arising outside the original ring of primordia (Fig. 2b, 72 h). Primordia also arose at the site of the inoculum (Fig. 2a, 72 h). After 72 h of illumination all dikaryon-specific mRNAs were detected at places where primordia were present (Fig. 2b, 72 h). After 96 h of illumination some fruit bodies continued development while others became abortive. The specific mRNAs disappeared from these abortive fruit bodies (also identified by their loss of rRNA; see below) but remained high in fruit bodies continuing development.

Up to 24 h after transfer to the light 18S rRNA was present throughout the colony with the highest concentration in the outer region, presumably the metabolically most active part of the colony (Fig. 1; Fig. 2c, 0–24 h). At 72 h after transfer, 18S rRNA became concentrated in the fruit-body primordia with a loss of hybridization signal in the vegetative mycelium (Fig. 2c, 72 h). After 96 h of illumination the 18S rRNA signal was present in developing fruit bodies but absent from abortive primordia. In a representative case of 203 visible primordia only 60% showed an 18S rRNA hybridization signal at this time of development.

Scanning profiles of whole-colony hybridizations

Colonies were grown for 7 d in darkness or for 5 d in darkness followed by 2 d in light to induce fruit bodies. After hybridizations with the specific cDNA clones and re-hybridization with the rDNA clone the ratio of the signals obtained in the two hybridization experiments were plotted along the radii of the colonies (see Methods). The pattern shown in Fig. 3 was found in three independent experiments. It appears that not only the absolute amounts (as recorded in Fig. 2) but also the relative abundances of the probed mRNA (mRNA/rRNA) were generally highest in the primordial ring induced by light and remained low in darkness. (Note that the absolute concentration of the mRNAs is not reflected in the curves; see Discussion.) mRNA Sc-9 also seemed to be elevated at the margin of the colonies, especially in dark-grown colonies. It should be noted, however, that the ratio of specific mRNA/18S rRNA at the margin becomes very imprecise because both signals fall rapidly to very low values at the low density of hyphae at the margin. Therefore, in Fig. 3, values for the extreme margin of the colonies are not given.

DISCUSSION

In situ hybridizations are mostly done on sectioned tissues exposing the cytoplasm to the nucleic acid probes, e.g. in animals (Akam, 1983; Cox *et al.*, 1984; Hafen *et al.*, 1987) and plants (Cornish *et al.*, 1987; Martineau & Taylor, 1986; Schmelzer *et al.*, 1988; Smith *et al.*, 1987). Whole colonies of *Saccharomyces cerevisiae* have been treated with Zymolase to digest the walls and then hybridized to probes to quantitate mRNA (Yu & Gorovsky, 1986). Similar procedures have been described to detect specific DNA sequences in yeast (Blanc *et al.*, 1987; Hinnen *et al.*, 1978) and filamentous fungi (Crowley & Oliver, 1987; Stohl & Lambowitz, 1983). However, our aim was to devise a method for localization of RNAs in particular parts of a colony. We adapted a procedure previously developed for *in situ* hybridizations in sectioned fungal tissues which also contain intact walls posing a barrier to permeation of the probes (Ruiters & Wessels, 1989). To permeabilize the walls we used an enzyme preparation derived from *Trichoderma harzianum* known to be effective in releasing protoplasts from *S. commune* and a variety of other basidiomycetes and ascomycetes (De Vries & Wessels, 1973), a capacity only slightly reduced by treatment of the enzyme preparation with bentonite to remove RNAase (not shown). The hybridization signals were clearly due to DNA/RNA hybridization, and not to DNA/DNA

Fig. 2. *In situ* hybridization of 5-d-old dikaryotic colonies of *Schizophyllum commune* after transfer to the light. Column (a), morphological appearance of the colonies at various times after transfer. The extreme margin of the colonies is indicated by stippling in columns (a) and (b). Column (b), autoradiographs of the same colonies after hybridization of 30° sectors with the specific clones for mRNAs Sc-1, -2, -3, -4, -5, -6, -7, -9, -14, and 18S rRNA (R), the vector without insert (–), and a blank without DNA during hybridization (0) (exposure time 4 d). Column (c), autoradiographs of the same sectors shown in (b) but after rehybridization with an 18S rDNA clone (exposure time 1 d).

Fig. 3. Relative abundance of mRNAs (mRNA concn/rRNA concn) along radii of colonies as estimated by scanning autoradiographs after hybridizations to cDNA clones followed by rehybridization to an 18S rDNA clone (as described in Methods). The numbers of the mRNAs traced are shown to the right. The upper profiles (R) show the distribution of 18S-rRNA in the colonies. The colonies were grown for 7 d in the dark (left-hand column) or for 5 d in the dark and then in the light for 2 d (right-hand column).

hybridization, because no signal occurred after RNAase treatment and the signals were confined to specific parts of the colonies.

The estimated amount of RNA in the hybridized sectors of the colonies was 50–100 µg. From previous measurements on the abundance of the probed mRNAs (Mulder & Wessels, 1986; Ruiters *et al.*, 1988) we estimate that in the hybridized 30° colony sectors the more abundant probed mRNAs amounted to 50–100 ng. Because each sector was hybridized to 12.5 ng of probe containing about 2.5 ng of specific insert DNA, this probably resulted in non-excess hybridization conditions for most of the mRNAs and certainly for the 18S rRNA. Consequently, the signals obtained cannot be taken as a measure of the absolute amounts of the various probed RNAs but merely reflect differences in the local abundance of specific RNAs in the colony. Under these conditions the access of the probes to cytoplasmic RNA becomes very critical. Because it is difficult to assess possible differences in wall permeabilization by the wall-lytic enzymes in the different parts of the colony this introduces uncertainty about the significance of the height of the signal. However, we feel that this was not a serious problem in the present hybridizations because the 18S rRNA probe hybridized to all parts of the colony.

The detection and localization of dikaryon-specific mRNA by *in situ* hybridizations gave results in general agreement with those obtained in previous experiments using hybridizations to RNA extracted from concentric rings of colonies (Ruiters *et al.*, 1988; Yli-Mattila *et al.*, 1989). Light simultaneously induced the dikaryon-specific mRNAs and the fruit bodies in a ring (approximately 5 mm wide) just inside the margin of the colony as present at the moment of transfer to light. However, the *in situ* colony hybridizations presented here give much more detailed information about this correlation. (i) The mRNAs were not uniformly produced in the

colony ring but only at locations where fruit-body initials actually formed. (ii) The mRNAs became concentrated in primordia which continued development but disappeared from abortive primordia. This coincided with the general drop in the concentrations of these mRNAs in the ring as measured in extracted RNA (Ruiters *et al.*, 1988). (iii) The developing fruit-body primordia also accumulated rRNA whereas the amount of rRNA in the abortive primordia and in the vegetative mycelium drastically diminished at the same time. Only the vegetative mycelium at the growing edge of the colony maintained a relatively high concentration of rRNA. Apparently, the developing fruit bodies drew on abortive fruit bodies and vegetative mycelium for rRNA. We suspect that this is accomplished through controlled hydrolysis of rRNA and transport of nucleotides to the developing fruit bodies. It was concluded earlier that after depletion of nutrients growth of fruit bodies is accompanied not only by controlled degradation of RNA but also of protein, glycogen and cell-wall components in the vegetative mycelium and abortive primordia to supply the building blocks for the developing fruit bodies (Wessels, 1965; Wessels & Sietsma, 1979). It now appears that all these processes may also take place in colonies still growing at the margin where external nutrients are available.

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