MESSENGER RNA TRANSCRIPTS

Dan Cullen

In contrast to DNA, messenger RNA (mRNA) in complex substrata is rarely analyzed, in large part because labile RNA molecules are difficult to purify. Nucleic acid extractions from fungi that colonize soil are particularly difficult and plagued by humic substances that interfere with *Taq* polymerase (Tebbe and Vahjen 1993 and references therein). Magnetic capture techniques have overcome these problems, allowing rapid and efficient purification of microbial DNA and RNA. Magnetic capture involves the use of magnetic beads that are covalently attached to single-stranded DNA, most commonly oligo (dT) chains. The beads are mixed with crude lysates, and hybrid molecules are removed by application of a magnetic field. In the case of beaded oligo (dT) chains, polyadenylated [Poly (A)] RNA, which is a suitable template for reverse-transcription-coupled PCR (RT-PCR), is obtained. When combined with competitive PCR techniques (Gilliland et al. 1990), quantitative assessment of transcript levels is possible. Quantitative transcript analysis is as sensitive and specific as conventional PCR amplification of DNA for identifying fungi. The competitive RT-PCR technique is particularly well suited for differentiating genes within complex gene families such as those encoding the peroxidases, laccases, and cellobiohydrolases of white-rot fungi. In addition, the technique provides a measure of fungal biomass and a glimpse of physiological activities of fungi *in situ.*

The most thoroughly studied gene family includes 10 or more structurally related genes that encode isozymes of lignin peroxidases in *Phanerochaete chrysosporium* (reviewed in Gaskell et al. 1994; Cullen 1997). When the RT-PCR technique was applied to *P. chrysosporium* soil cultures, unusual patterns of peroxidase gene transcription were demonstrated (Lamar et al. 1995). For example, certain lignin peroxidase transcripts, abundant in defined media (Stewart and Cullen 1999), were not expressed in soil during organopollutant degradation. The absence of those peroxidase transcripts argues strongly against a significant role for those genes in the degradation of pentachlorphenol (PCP) and polycyclic aromatic hydrocarbons (Bogan et al. 1996a, 1996b).

IMPORTANT CONSIDERATIONS

Transcripts may not directly reflect protein levels; thus, caution should be exercised in quantitative interpretations of complimentary DNA (cDNA) levels. However, the alternative approach, purification and characterization of fungal proteins, is difficult or impossible in most

instances. Even if a protein is abundant and stable, immunological and/or physical characterization seldom permit unambiguous identification. Individual peroxidase isozymes present in soil cultures of *P. chrysosporium* cannot be quantified, but the total extractable manganese peroxidase activity is correlated roughly with transcript level (Bogan et al. 1996a).

Several precautions must be taken if cDNA is to be quantified reliably. First, excessive thermocycling beyond the reaction's plateau phase should be avoided. This is especially important for quantitative comparisons of cDNAs of substantially different lengths that amplify with different primers (Pannetier et al. 1993). Further, comparisons between separate samples may be affected by varying RNA yields and varying efficiencies of reverse transcription reactions. In such cases, simultaneous amplification of constitutively expressed, endogenous genes may provide an internal standard. Such "housekeeping" genes include glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Harmsen et al. 1992), **b**-actin (Chelly et al. 1988; Horikoshi et al. 1992; Kinoshita et al. 1992), and HPRT (hypoxanthine-guanine phosphoribosyltransferase; Pannetier et al. 1993). Exogenous RNA that differs from the target by the presence of a small intron or restriction site(s) also can be used as an internal standard (Wang et al. 1989; Wang and Mark 1990).

The RT-PCR analysis is applicable to an array of substrata and fungal species. The only prerequisite is nucleotide sequence data of a transcribed region or at least partial coding sequences, flanking intron(s). Although most genes characterized from filamentous fungi contain introns, some do not. In those cases, competitive templates can be constructed with a restriction site not present in the cDNA target. Following PCR amplifications, the products are digested prior to gel fractionation and estimation of the equivalence point. With minor modifications of the soil extraction protocol, we have detected *P. chrysosporium* transcripts in colonized wood chips and in Lambert spawn inoculum, a mixture of sawdust and grain (Lamar et al. 1995; Janse et al. 1998; Vallim et al. 1998; Wymelenberg et al. 2002). All known peroxidase and cellulase transcripts of *P. chrysosporium* in aspen wood chips (Janse et al. 1998; Vallim et al. 1998) have been quantified and shown to differ dramatically from those of *P. chrysosporium* in soil (Bogan et al. 1996a, 1996b) or in defined media (Stewart and Cullen 1999). Laccase transcripts of *Trametes hirsutas* (Kojima et al. 1990) also have been amplified from colonized soil (D. Cullen, unpublished data). In contrast, no peroxidase transcripts were detected in soil samples collected from a hazardous waste site in Brookhaven, Mississippi (Lamar et al. 1994). Peroxidase transcript patterns of *P. chrysosporium,* however, were similar in presterilized and nonsterilized cultures (Bogan et al. 1996b).

Numerous modifications can be envisioned. Magnetic capture need not be confined to polyadenylated regions. Magnetic beads can be attached to virtually any oligonucleotide sequence, including those complementary to abundant ribosomal RNAs or to specific DNA sequences (Jacobsen 1995). Enormous amounts of sequence data are accessible for the small-subunit ribosomal RNA of fungi, so that species-specific primers and probes could be readily designed and tested.

Recent advances in fungal genomics and in high-throughput transcript analysis offer new opportunities. A high-quality draft genome of *P. chrysosporium* has been completed and is freely available from the U.S. Department of Energy's Joint Genome Institute (see Appendix 111). Thus, it is now possible to construct microarrays representing all predicted genes (>10,000) and to examine their relative levels under a wide range of conditions. Impediments to this approach include the high costs associated with either DNA- or oligonucleotide-printed arrays. Also, the submicrogram amounts of Poly (A) RNA typically captured from sparsely colonized soil, litter, or wood are inadequate for direct labeling with fluorochromes. However, several mRNA amplification strategies have been devised to overcome limiting quantities of RNA (Iscove et al. 2002; Schoor et al. 2003; Wang et al. 2003). Another increasingly popular method for quantitative transcript analysis is reverse-transcription, real-time PCR (Heid et al. 1996). By continuously monitoring PCR reactions, the key log-linear cycles can be identified precisely in real-time adapted thermocyclers. Competitive and real-time PCR are similar in sensitivity and specificity (Freeman et al. 1999), although equipment and operational costs are considerably higher for real-time PCR approaches. The methodology described below focuses on reverse-transcription, competitive PCR, although the protocols for Poly (A) RNA purification yield mRNA suitable for microarrays or real-time PCR.

METHODS

An array of transcripts has been quantified from *P. chrysosporium* growing in presterilized soil, using magnetic capture of Poly (A) RNA followed by competitive RT-PCR (Lamar et al. 1995; Bogan et al. 1996a, 1996b). The RT-PCR technique used to obtain *P. chrysosporium* GAPDH transcripts from 4-day-old soil culture is illustrated in Figure 6.9. Approximately 10 g of *P. chrysosporium*-colonized soil are wrapped in Miracloth (Calbiochem-Novabiochem Corporation; see Appendix IV), snap frozen in liquid nitrogen, and ground in a mortar and pestle precooled with dry ice. During grinding, 10 ml of extraction buffer (4 M guanidinium thiocyanate, 0.1 M Trizma base, 1% dithiothreitol, and 0.5% Sarkosyl, pH
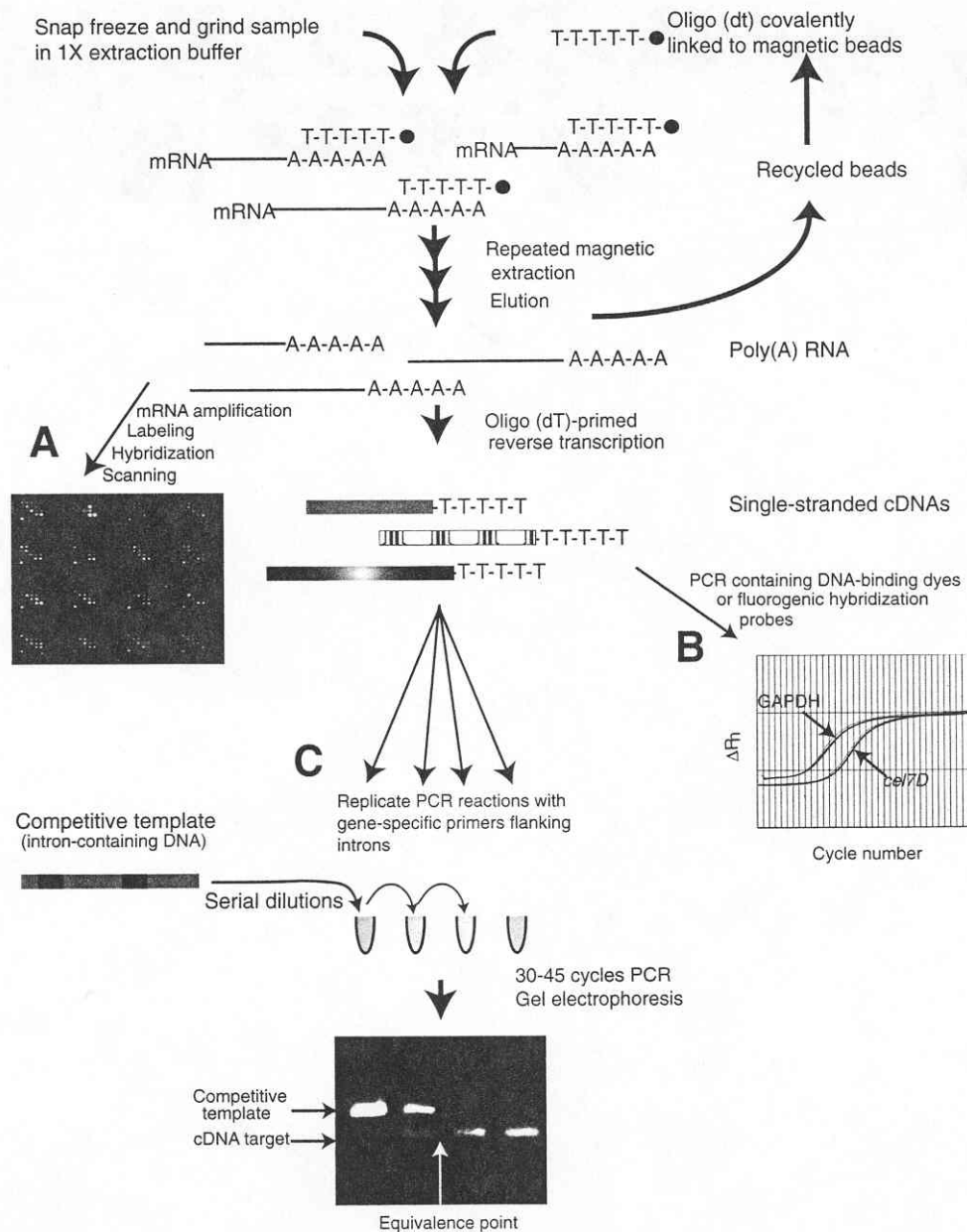
FIGURE 6.9 Protocol used for magnetic capture of Poly (A) RNA from colonized soil or wood, and alternative approaches to quantifying transcripts. Similar capture methods have been used for Phanerochaete chrysosporium (Lamar et al. 1995) and yeasts (Tebbe et al. 1995) in soil. (A) Microarray of subset of *P. chrysosporium* peroxidase and cellulase genes. Typically, hybridization involves combining two samples labeled with Cy3 and Cy5 fluorescent dyes (for review see Knudsen 2002). (B) Amplification plots of real time PCR of GAPDH and cellulase gene, *cel*7D. The GAPDH transcripts are more abundant. (C) Competitive PCR of GAPDH. Aliquots of the total complementary DNA (cDNA) pool were subjected to competitive PCR (Gilliland et al. 1990; Stewart et al. 1992). Competitive template, in the form of an intron-containing genomic clone, was added in 10-fold serial dilutions from 10 to 0.1 fg. The far right lane shows the cDNA target only (no competitive template reaction). The approximate equivalence point is 0.05 fg.

8.0) are added in 2-ml aliquots. The slurry is centrifuged at $500 \times$ g for 8 minutes. The supernatant is centrifuged a second time at $500 \times$ g for 8 minutes, and approximately 6 ml of final supernatant is mixed with two volumes of binding buffer (100 mM Trizma base, 400 mM LiCl, and 20 mM EDTA, pH 8.0). Tubes are centrifuged at 8000 $\times$ g for 2 minutes, and each supernatant is added to 1.5 mg of oligo(dT)25 Dynabeads (Dynal Biotech, Appendix IV) that previously have been washed in binding buffer. The mixture hybridizes for 30 minutes on ice.

Bound Poly (A) RNA is then isolated by magnetic concentration with a Dynal Magnetic Particle Concentrator MPC-1, resuspended in 500 ml of washing buffer (10 mM Trizma base, 0.15 M LiCl, and 1 mM EDTA, pH 8.0), and washed twice after retrieval with the smaller MPCE-1 model magnetic particle concentrator. Poly (A) RNA is eluted at 65°C for 2 minutes in 2 mM EDTA, pH 8.0. Dissociated Dynabeads can be recovered by magnetic concentration and regenerated according to the manufacturer's recommendations. RNA is precipitated with 2 volumes of ethanol and stored at −20°C. Typically, yields of Poly (A) RNA from soil or wood samples are sufficient for quantifying dozens of transcripts by either real-time or competitive PCR. (Fluorochrome labeling of mRNA for microarrays would require amplification.)

Oligo(dT) 15 may serve as primer in reverse transcription reactions (Lamar et al. 1995; Bogan et al. 1996a, 1996b; Janse et al. 1998; Vallim et al. 1998; Fig. 6.9). The resulting single-stranded cDNA pool is a suitable source for PCR amplification of most expressed genes. Alternatively, a gene-specific "downstream" primer can be used to optimize sensitivity and reduce nonspecific PCR amplifications.

In the example shown in Fig. 6.9, competitive PCR was used to assess relative levels of GAPDH cDNA (Gilliland et al. 1990; Stewart et al. 1992). In brief, replicate PCR reactions, each involving 21 pmol of GAPDH-specific primers, are prepared, and known concentrations of a competitive template are added as a serial dilution to each reaction tube. In the GAPDH example illustrated in Fig. 6.9, the competitive template is an intron-containing genomic clone in plasmid pCRII (Invitrogen Corporation; Appendix IV). However, any vector, cosmid, or liter subclone of GAPDH would be adequate provided that the entire amplification target is included and the region contains at least one intron. A variety of thermocyling regimens can be used. For the GAPDH amplification shown, reactions are subjected to an initial cycle of denaturation (6 minutes, 94°C), annealing (2 minutes, 54°C), and prolonged extension (40 minutes, 72°C), followed by 35 cycles of denaturation (1 minute, 94°C), annealing (2 minutes, 54°C), and extension (5 minutes, 72°C). A final 15-minute extension at 72°C is also included.

Following amplification, the target cDNA and competitive template are size fractionated on agarose gels. The initial concentration of GAPDH cDNA is determined by estimating the dilution point at which the target cDNA and competitive template are equivalent. Typically, 10-fold or 5-fold serial dilutions are used initially to approximate cDNA concentrations. When necessary, increased resolution may be achieved using many smaller dilutions (e.g., 1-fold or 2-fold). Increased resolution of the equivalence point is also attained by scanning the ethidium bromide-stained gels and quanti-fying band intensity using imaging software such as NIH *Image* (version 1.58 or later; see Appendix IV). To confirm the identity of PCR products, agarose gels can be blotted to nylon membranes and probed with ^{32}P-labeled oligonucleotides specific for cDNA targets (Stewart et al. 1992).

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Front cover: The slime mold *Physarum roseum* on a decaying leaf. Slime molds are fungal-like organisms traditionally studied by mycologists. Photo by Ray Simons.

Back cover: A species of *Mycena* found in Yunnan, China. Species of the mushroom genus Mycena are commonly encountered throughout the world. Photo by Gregory M. Mueller.