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In-situ Hybridization to Messenger RNA in *Heterodera glycines*[1]

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Abstract: A method is presented for in-situ hybridization to mRNA in second-stage juveniles (J2) of the soybean cyst nematode *Heterodera glycines*. The protocol was developed using a digoxigenin-labeled RNA probe transcribed from cDNA of a cellulase gene that was known to be expressed in the subventral esophageal glands of *H. glycines*. Formaldehyde-fixed J2 were cut into sections with a vibrating razor blade to make the inside of the nematodes accessible for probing. These nematode fragments then were hybridized in suspension with riboprobe, and labeled with an alkaline phosphatase-conjugated antibody to digoxigenin. Staining with nitroblue tetrazolium and bromo-chloro-indolyl phosphate revealed a highly specific hybridization signal to mRNA within the cytoplasm of the subventral gland cells, using this specific antisense probe. This in-situ hybridization protocol will be useful for the characterization and identification of esophageal gland secretion genes in plant-parasitic nematodes, among other applications.

Key words: cellulase gene, digoxigenin RNA probe, esophageal gland, *Heterodera glycines*, in-situ hybridization, nematode.

Secretions from the esophageal glands are considered to play an important role in the interaction between plant-parasitic nematodes and their hosts (Hussey, 1989). Recently, four mRNAs were identified from secretory cellulase genes that are expressed in the subventral esophageal glands of second-stage juveniles (J2) of *Heterodera glycines* and *Globodera rostochiensis* (Smant et al., 1998). We have used an antisense RNA probe to one of these cellulase mRNAs (*Hg-eng-2*) to develop a procedure for in-situ hybridization to mRNA in J2 of *H. glycines* (Smant et al., 1998). Here we present a complete description of this in-situ hybridization procedure.

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MATERIALS AND METHODS

Nematodes: Heterodera glycines inbred line OP50 (Dong and Opperman, 1997) was grown on soybean cv. Corsoy in a greenhouse. Juveniles were hatched from eggs in 3 mM $ZnSO_4$ at 26 °C, and were purified with sucrose centrifugation (De Boer et al., 1996).

Riboprobe synthesis: The sequence representing nucleotides 273 to 447 of the *Hg-eng-2* cDNA (E. L. Davis, Genbank accession AF006053) was subcloned into the Eco RI cloning site of a modified pBlueScript II transcription vector, pBST (D. Hermsmeier and T. J. Baum, unpubl.). An antisense riboprobe was synthesized in vitro from this subcloned cDNA fragment according to standard procedures (Boehringer Mannheim, Indianapolis, IN). A sense probe control also was synthesized in a separate reaction. Both probes were labeled by adding digoxigenin-11-UTP (Boehringer Mannheim) to the transcription reaction.

Permeabilization: About 70,000 J2 of *H. glycines* were fixed with 2% paraformaldehyde in M9 buffer (Brenner, 1974) for 18 hours at 5 °C, followed by 4 hours in fixative at 22 °C. A cutting device was fabricated by taping a single-edge razor blade (thickness 0.25 mm) to the side of a vibrating aquarium air pump, with the edge of the blade protruding 4 mm above the top surface of the pump. The fixed J2 were resuspended in

450 µl of 0.1× fixative (0.2% paraformaldehyde in 0.1× M9 buffer), and an aliquot of 150 µl was pipetted onto a microscope slide. The vibrating razor blade was placed vertically on the slide and moved slowly back and forth across the nematode suspension until most J2 were cut into 2 to 5 fragments. The J2 sections were rinsed off the slide with 0.1× fixative, and the cutting procedure was repeated until all J2 were processed. The J2 sections were washed twice in M9 buffer and incubated in 0.5 mg/ml proteinase K (Life Technologies, Grand Island, NY) in M9 buffer at 22 °C for 22 minutes. Following a wash in M9 buffer, the J2 were pelleted, frozen on dry ice for 20 minutes, and incubated on dry ice in methanol for 1 minute followed by acetone for 1 minute (Atkinson et al., 1988). Following pelleting at 15,000*g*, the J2 sections were rehydrated in 20% acetone in water at 22 °C for 20 minutes.

Hybridization: The hybridization buffer contained 50% deionized formamide, 4× SSC (diluted from 20× SSC, which is 3 M NaCl, 0.3 M sodium citrate; pH 7.0), 2% sodium dodecyl sulfate (SDS), 1% blocking reagent (Boehringer Mannheim), 0.1× maleic acid buffer (see below), 0.2 mg/ml Ficoll 400, 0.2 mg/ml polyvinylpyrrolidone, 0.2 mg/ml bovine serum albumin, 1 mM ethylene-diaminetetraacetic acid (EDTA), 0.2 mg/ml fish sperm DNA (Acros Organics, Pittsburgh, PA), and 0.15 mg/ml yeast tRNA. The rehydrated, permeabilized nematode sections were pre-hybridized in 250 µl hybridization buffer at 55 °C for 1 hour. A 5- to 10-µl pellet of pre-hybridized nematode sections was resuspended in 250 µl of fresh hybridization buffer containing unpurified, unhydrolyzed riboprobe in a 1:2,000 dilution, which corresponds to an estimated probe concentration of 300 ng/ml. Hybridization was performed overnight at 55 °C. The J2 sections then were washed three times for 15 minutes with 4× SSC at 55 °C and once with NTE buffer (0.5 M NaCl, 10 mM Tris-HCl, 1 mM EDTA; pH 8.0) for 10 minutes at 37 °C. Unhybridized riboprobe was digested with RNAse A (1 hour at 37 °C; 60 µg/ml in NTE buffer), and the J2 were washed three times for 20 minutes in 0.1× SSC, 0.1% SDS at 55 °C.

Detection: Unless specified otherwise, incubations were at 22 °C. Following a wash in maleic acid buffer (0.1 M maleic acid, 0.15 M NaCl; pH 7.5), the J2 sections were incubated 30 minutes in blocking solution (1% blocking reagent in maleic acid buffer). The J2 then were labeled for 2 hours with alkaline phosphatase-conjugated sheep anti-digoxigenin Fab antibody fragments (Boehringer Mannheim) diluted 1:1,000 (750 mU/ml) in 0.5 ml blocking solution. Following three 15-minute washes in maleic acid buffer with 0.05% Tween-20 and a brief wash in detection buffer (0.1 M Tris-HCl, 0.1 M NaCl, 50 mM $MgCl_2$; pH 9.5), the J2 were stained overnight at 5 °C without agitation in 250 µl detection buffer with 337 µg/ml nitroblue tetrazolium and 175 µg/ml 5-bromo-4-chloro-3-indolyl phosphate. Staining was stopped by two washings in water. The J2 were examined with differential interference contrast microscopy.

Incubations: Unless otherwise specified, J2 were incubated in 1.5-ml microcentrifuge tubes mounted on a rotator, using 1 ml solution per step. To change solutions, J2 were pelleted at 7,000*g* for 40 seconds.

RNAse treatment: mRNA was digested as a separate negative control treatment immediately before the proteinase K treatment. J2 were incubated in RNAse A as described above, and washed three times with NTE buffer.

Results and Discussion

Hybridization of J2 with the antisense probe to *Hg-eng-2* mRNA revealed a highly specific binding within the cytoplasm of the two subventral esophageal gland cells, without any background staining (Fig. 1A). Usually, the alkaline phosphatase stain was intensely black throughout both gland cells, and weak or absent in the gland extensions. Signal was not observed in a parallel hybridization with the sense probe (Fig. 1B). Treatment of J2 with RNAse A before hybridization with the antisense probe completely

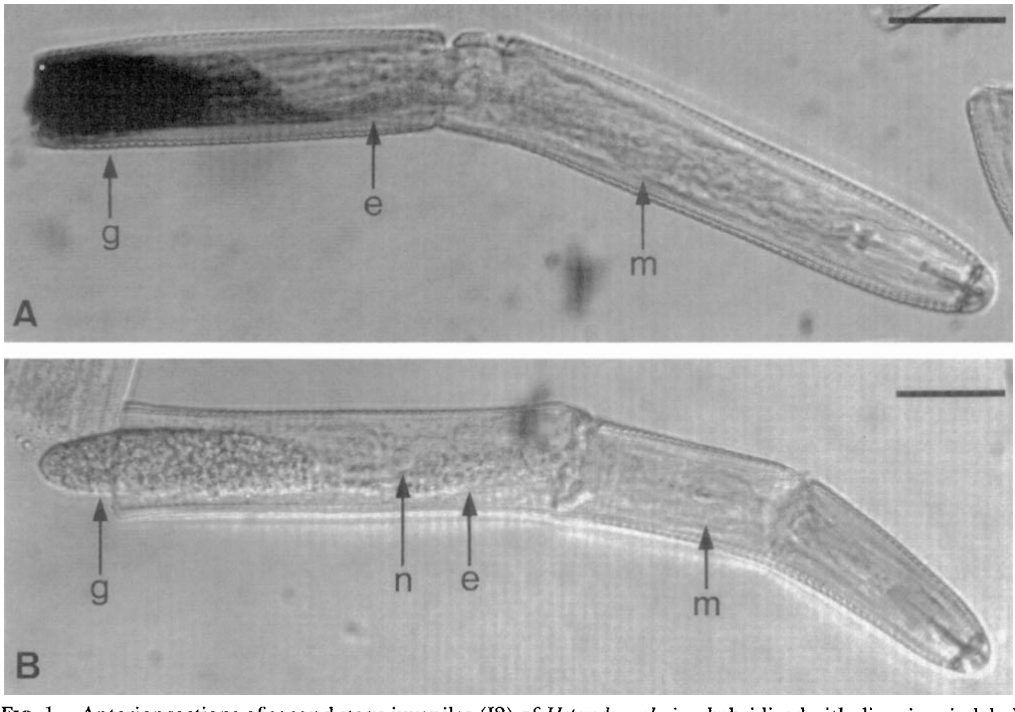

FIG. 1. Anterior sections of second-stage juveniles (J2) of *Heterodera glycines* hybridized with digoxigenin-labeled riboprobes derived from the *Hg-eng-2* cellulase cDNA. A) Alkaline phosphatase staining shows specific binding of the antisense probe to the cytoplasm of the subventral esophageal glands (g). The subventral gland extensions (e) remain unstained. B) Alkaline phosphatase staining is absent in J2 that have been incubated with the sense probe. m, metacorpus; n, nucleolus of dorsal esophageal gland. Bars: 20 μm.

prevented staining of the subventral glands, demonstrating that this probe hybridized specifically to mRNA.

Immunolabeling has shown that, in J2 of *H. glycines*, the *Hg-eng-2* cellulase is produced only in the subventral esophageal glands (Smant et al., 1998). This gland-specific expression of the *Hg-eng-2* gene is confirmed by our in-situ hybridization results. As such, the in-situ hybridization procedure presented here reliably reflects the spatial pattern of protein expression determined independently by immunocytochemistry.

The in-situ protocol used here for *H. glycines* differs from published methods for in-situ hybridization to whole life stages of *Caenorhabditis elegans*. The nematode cuticle forms a barrier for the entry of macromolecular probes, and in *C. elegans* this impermeability can be overcome by a combination of physical and chemical treatments that render the cuticle permeable (Albertson et al., 1995; Ray and McKerrow, 1992). However, attempts to apply such permeabilization treatments to intact formaldehyde-fixed J2 of *H. glycines* were not effective. We therefore decided to follow a tissue permeabilization procedure that was developed for immunolabeling of plant-parasitic nematodes (Atkinson et al., 1988; De Boer et al., 1996). This procedure involved cutting of fixed nematodes into sections. A modification for the in-situ protocol was that the fixed J2 were no longer dried and frozen prior to cutting. Instead, the J2 were cut in dilute formaldehyde fixative and used immediately for hybridizations. This procedure reduces degradation of mRNA and allows better tissue penetration of both probe and antibody.

The degree of formaldehyde fixation proved to be critical for the in-situ hybridization. With overnight fixation at 5 °C, the J2 tissues often were digested too severely by the subsequent proteinase K treatment. Overnight fixation at 22 °C visibly produced better tissue fixation but led to decreased

staining signals because of a reduced tissue permeability. An optimal fixation schedule for J2 was obtained with overnight incubation at 5 °C followed by 4 hours at 22 °C.

The mRNA in-situ hybridization protocol described here for J2 of *H. glycines* should be adaptable also to other life stages and species of plant-parasitic nematodes. In fact, preliminary results have already demonstrated successful application to other *H. glycines* life stages (unpublished). Adaptations may require a change in the parameters for formaldehyde fixation or proteinase K treatment. Furthermore, it may be necessary to change hybridization temperature when using the protocol with different probes.

It can be expected that the present in-situ protocol will be useful for the identification of additional esophageal gland secretion genes of *H. glycines* and for examining gene expression patterns in plant-parasitic nematodes.

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