Changes in gene expression during stimulation and hatching of the potato cyst nematode *Globodera rostochiensis*

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SUMMARY

The potato cyst nematode (PCN) *Globodera rostochiensis*, like other parasitic nematodes, needs to synchronize its life-cycle with that of its host. This synchrony is achieved by the invasive-stage juvenile remaining dormant within its egg until stimulated to hatch by the presence of root diffusates of its host. Root diffusates may induce changes in gene expression in PCN, some of which may be important in the transition to a parasitic mode of existence. We have used a range of techniques including differential display to examine gene expression during stimulation and hatching of PCN. We find that few changes in gene expression appear to be induced directly by root diffusates. Instead, changes in gene expression seem to occur during or immediately after the hatching process. These results are discussed in the context of the host–parasite relationship.

Key words: differential display, gene expression, *Globodera rostochiensis*, hatching, protein, RNA.

INTRODUCTION

Plant parasitic nematodes are important agricultural pests throughout the world. Estimating the cost of the damage caused by these parasites is difficult, but yield losses alone have been calculated to be worth as much as $77 billion annually (Sasser & Freckman, 1987). Existing control measures are inadequate: natural resistance genes are not available against many species of plant parasites and the use of nematicides is becoming increasingly restricted on environmental grounds. Consequently, alternative control strategies for plant parasitic nematodes are required and knowledge of the mechanisms by which nematodes regulate their growth and development may reveal novel control targets.

Many parasitic nematodes undergo changes in response to stimuli from their hosts, effectively synchronizing host and parasite life-cycles to maximize the chance of successful parasite invasion. This synchronization can be controlled through different stages of the life-cycle in different nematodes. The invasive juvenile stage of *Trichinella spiralis* is sensitive to trypsin and bile at 37 °C (Stewart *et al.* 1987); changes in surface composition are observed in this nematode when exposed to these conditions and the animal follows this response with a series of rapid moults. Many animal parasitic nematodes with insect vectors also undergo rapid changes when transferred to their mammalian hosts (Proudfoot *et al.* 1991). In other nematodes synchrony of parasite and host life-cycles is achieved through dormancy of an unhatched invasive-stage juvenile until conditions are suitable for hatching (Perry, 1989). Examples include *Ascaris suum*, whose eggs remain viable in soil for several years but hatch rapidly in response to conditions found in the alimentary tract of the host. It has been suggested that in this nematode CO_2 affects the permeability of the lipid layers of the egg-shell, allowing the passage of trehalose and various enzymes important in hatching (Clarke & Perry, 1988).

Cysts of the potato cyst nematode, *Globodera rostochiensis*, contain up to 300 eggs, each enclosing an infective 2nd-stage juvenile (J2) which lies dormant in the soil until stimulated to hatch by the presence of host root diffusates (potato root diffusate – PRD). In this species PRD induces a calcium-dependent change in the permeability of the inner lipoprotein membranes of the lipid layer of the egg-shell which results in the release of trehalose from the egg and uptake of water by the incompletely hydrated J2s (Perry, 1989). The active J2 is now able to cut its way out of the egg-shell and begin the search for its host. Exposure to PRD and the resulting hatching process induce other changes in *G. rostochiensis*, including changes in the dorsal gland cells (Perry, Zunke & Wyss, 1989) and in the ultrastructure of the cuticle and the amphids (Jones,

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Perry & Johnston, 1993, 1994), although these changes were not considered to be due to the direct action of PRD. It is also possible, although not previously demonstrated, that changes in gene expression are induced in *G. rostochiensis* during the hatching process. Such changes in gene expression may be important in preparing the nematode for a parasitic mode of existence or may reflect normal developmental processes.

Biochemical and molecular biological studies on plant parasitic nematodes are difficult because of their small size (a J2 is approximately 30 μm × 300 μm). The advent of PCR-based techniques in molecular biology has opened up new areas for study of plant parasitic nematodes. One such technique, differential display (Liang & Pardee, 1992), allows gene expression to be compared in two tissues. A small subset of the mRNAs present in each tissue is first converted to cDNA. The cDNA is then used as a template in a series of PCR reactions using small, randomly chosen primers. Comparison of the reaction products from each tissue on a high resolution gel allows differences to be identified. Bands present in one lane and absent in another can then be cut out and cloned for further analysis.

We have used a range of techniques, including differential display, to examine the genes expressed and proteins present in *G. rostochiensis* during the hatching process. Dormant (dried) cysts have been compared with those soaked in water and those exposed to the host stimulus (PRD) for various lengths of time. We have also included the hatched nematodes in our comparisons.

MATERIALS AND METHODS

Nematode culture and treatments

Cysts of *G. rostochiensis* Ro1 were from a single generation. They were grown on potato cv. Désirée in pots and stored dry at room temperature (approximately 20 °C) after extraction. In hatching tests, the population used gave over 80% hatch of viable cyst contents when stimulated with potato root diffusate (PRD) (Beane & Perry, 1990). The PRD was obtained by the method of Fenwick (1949) from 10-week-old potato plants (cv. Désirée) grown in sterilized loam pot cultures in a glasshouse. Briefly, a pot containing a plant was watered until the soil was saturated. A further 100 ml of sterile distilled water was then added to the pot and the 100 ml of liquid which consequently flowed through the pot was collected. This PRD was stored in polythene bottles at 4 °C until required, when it was diluted 1 in 4 with sterile distilled water. In hatching tests the above procedure was repeated using pots of soil containing no potato plant and the fluid collected from these pots was used in negative control experiments.

For studies on gene expression changes occurring before, during and after hatching, nematodes at different stages in the hatching process were used. The stages used and methods used to prepare them for experimentation were as follows.

Dry cysts. Cysts were rolled to ensure no contaminating material was present.

Water soaked cysts. Dry cysts were soaked in sterile distilled water for 5 days and rinsed thoroughly in several changes of sterile distilled water to remove any hatched juveniles or contaminating material.

Cysts stimulated for 1 h. Water-soaked cysts, obtained as outlined above, were placed in PRD for 1 h and rinsed in several changes of sterile distilled water to remove hatched J2s.

Cysts stimulated for 24 h. Water-soaked cysts, obtained as outlined above, were placed in PRD for 24 h and then rinsed in several changes of sterile distilled water to remove hatched J2s.

Cysts stimulated for 3 days. Water soaked cysts, obtained as outlined above, were placed in PRD for 3 days and then rinsed in several changes of sterile distilled water to remove hatched J2s.

Hatched J2s. Clean cysts were soaked in sterile distilled water for 5 days as above, rinsed in several changes of sterile distilled water and placed in diluted PRD. Hatched J2s were collected after 24 h or after longer than 3 days in PRD and rinsed again in several changes of water.

Fungal contamination of all nematode material used was minimized by rinsing the cysts thoroughly each day prior to the onset of hatch in several changes of sterile distilled water or PRD as appropriate.

On some occasions nematodes were not used immediately for experiments but were stored at −70 °C until use. Although no differences attributable to storage at −70 °C were observed, as a precaution all comparisons made were between samples which had been stored in the same way before use.

RNA extraction

Cysts or J2s were frozen in liquid nitrogen in a mortar and pestle. They were then crushed thoroughly under 10 vols of solution A (4 M guanidine isothiocyanate, 25 mM sodium citrate, pH 7, 0·5% sarcosyl, 100 mM β-mercaptoethanol). After the powder had thawed, insoluble debris was removed

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by centrifugation at 10000 ***g*** for 10 min. RNA was purified by acid phenol extraction followed by several rounds of precipitation in isopropanol. After the final precipitation, the pellet was washed thoroughly in 70 % ethanol, dried and resuspended in an appropriate volume of DEPC-treated water (Sambrook, Fritsch & Maniatis, 1989). The integrity of the RNA was checked by running a small aliquot on a formaldehyde gel (Sambrook *et al*. 1989) and checking for the presence of the ribosomal RNA bands. The RNA was quantified by comparisons of ribosomal band intensities with those of RNA molecular weight standards or by UV spectrophotometry in a Genequant spectrophotometer (Pharmacia).

cDNA synthesis

Approximately 0·5 μg of total RNA was first treated with DNAse 1 (Life Technologies) for 30 min to remove any contaminating genomic DNA present. After inactivation of the enzyme at 65 °C for 10 min, cDNA was synthesized using Superscript II Reverse Transcriptase (Life Technologies) according to the manufacturer's instructions. The primer used for reverse transcription was the oligonucleotide T_{12}VC, where V is a degenerate position representing any nucleotide except T. After cDNA synthesis the sample was treated with RNAse H for 30 min at 37 °C followed by an incubation at 95 °C for 10 min. The sample was then centrifuged briefly and stored at −20 °C until use.

Differential displays

Differential displays were set up essentially according to the method of Liang & Pardee (1992). PCR reactions were set up containing 1 × *Taq* buffer, 1·5 mM $MgCl_2$, 20 μM dNTPs, 1 μM T_{12}VC primer, 1 μM random oligonucleotide, 1 μl cDNA and 1 unit *Taq* DNA polymerase (Promega). The random oligonucleotide used varied depending on the experiment being carried out but was always a 10 mer from one of the Operon kits (Operon). A small amount (approx. 2·5 μCi) of alpha^{32}P dATP (NEN) was also included in the reaction to allow visualization of the reaction product. Then 40 cycles of synthesis were carried out with an annealing temperature of 42 °C on a Hybaid Omnigene thermal cycler. Negative controls (PCR reactions containing no template, PCR reactions set up with cDNA synthesized in the absence of Reverse Transcriptase) were used throughout.

PCR products were separated on 7 % polyacrylamide gels, containing urea and TBE, made and run according to standard protocols (Sambrook *et al*. 1989). Gels were fixed in 10 % acetic acid/10 % methanol, rinsed in water and dried onto 3MM paper (Whatmann) and PCR products were then visualized by exposing the dried gels to X-ray film overnight.

Cloning and analysis of differentially amplified gene fragments

Some apparently differentially expressed gene fragments were cloned for further analysis. Bands of interest were cut out of dried gels and boiled in 20 μl of sterile distilled water. An aliquot (5 μl) of the fluid was then used in a PCR reaction under the same conditions and with the same primer combination used in the display reaction which generated the product of interest. After checking an aliquot of the PCR products on a 2 % agarose gel, the remainder was cloned into the pCR-Script SK+ vector using the pCR-Script cloning kit (Stratagene) according to the manufacturer's instruction manual. Subsequent screening and sequencing was carried out using standard protocols (Sambrook *et al*. 1989).

Protein analysis

Cysts or nematodes, prepared as described above, were ground in TE buffer (10 mM Tris, 0·1 mM EDTA) in a 0·5 ml microcentrifuge tube in the presence of 0·25 mM Pefabloc (Boehringer Mannheim). The resulting fluid was then centrifuged briefly to remove insoluble debris and stored at −70 °C until use. Protein concentration was determined using the Coomassie Plus protein assay reagent (Pierce), a protocol based on the Bradford assay, with absorbance readings taken at 595 nm and compared to a standard curve generated using known concentrations of BSA. Equal quantities of proteins from the stages being examined were separated using a Phastgel system (Pharmacia) according to the manufacturer's instructions. Proteins were separated on 12·5 % SDS polyacrylamide gels or on pH 3–9 polyacrylamide isoelectrofocussing gels. Gels were silver stained in the Phastgel unit as recommended in the manufacturer's instruction manual.

Primers

The sequences of primers used in this study were: OPG 2: GGCACTGAGG, OPG 3: GAGCCCTCCA, OPG 6: GTGCCTAACC, OPG 8: TCACGTCCAC, OPG 11: TGCCCGTCGT, OPG 12: CAGCTCACGA, OPG 16: AGCGTCCTCC.

RESULTS

Differential displays

RNA was obtained in approximately equal quantities from each of the groups of nematodes examined (data

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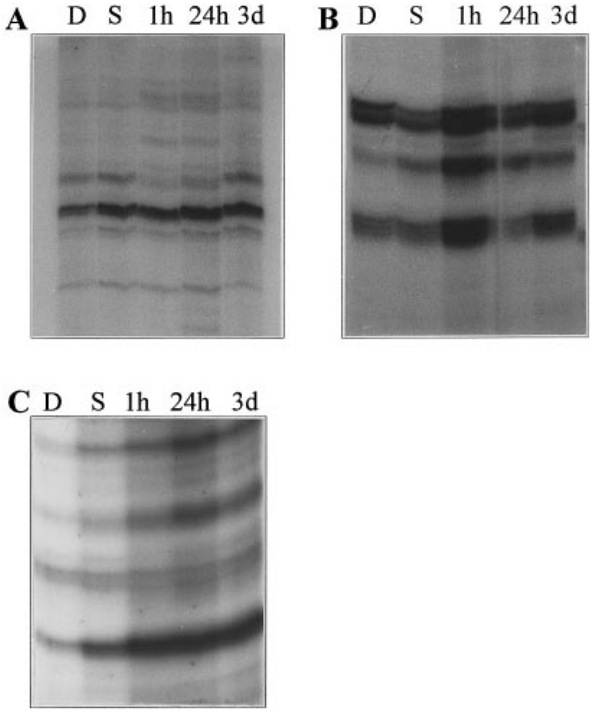

Fig. 1. Differential display gels showing products generated from nematodes subjected to various treatments before hatching. No differences are observed after soaking in water or potato root diffusate (see Materials and Methods section). Products generated using primers dTVC and OPG 12 (A), OPG 16 (B) and OPG 8 (C). D, Dry cysts; S, cysts soaked in water for 5 days; 1 h, water-soaked cysts placed in PRD for 1 h; 24 h, water-soaked cysts placed in PRD for 24 h; 3d, water-soaked cysts placed in PRD for 3 days.

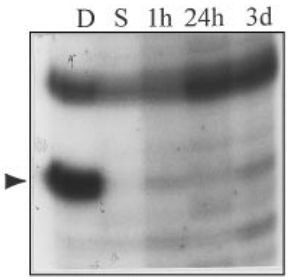

Fig. 2. Differential display gel showing a band representing a gene apparently expressed specifically in dry cysts (arrowhead). Products generated using primers dTVC and OPG3. Lanes as Fig. 1.

not shown). Initial differential display experiments focused on comparisons of unhatched nematodes stimulated with PRD for different lengths of time. Comparisons of dry cysts, water-soaked cysts and cysts soaked in PRD for 1 h, 24 h or 3 days showed that few changes in gene expression occurred prior to hatching, even after prolonged exposure to PRD. Fig. 1 shows examples of regions of typical differential display gels obtained using these tissues. Even though many display reactions were carried out with a large number of primers (over 25 combinations), no changes in gene expression which

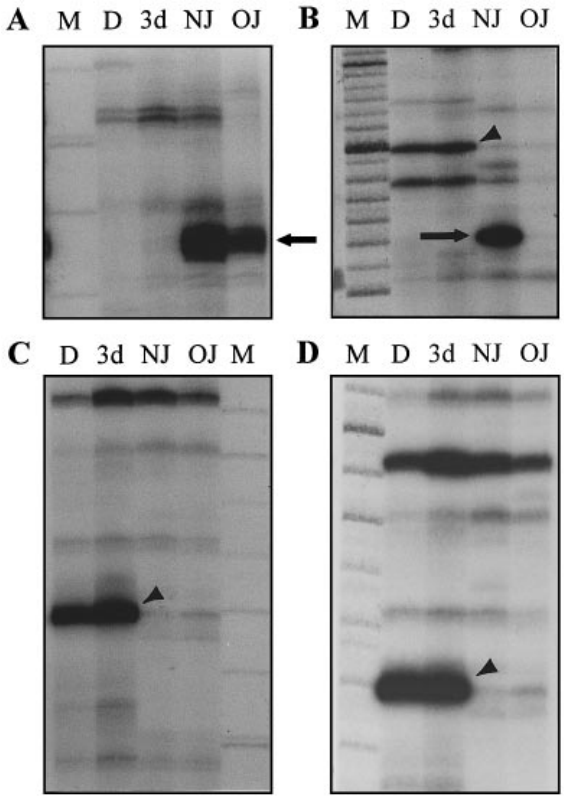

Fig. 3. Differential display gels comparing hatched and unhatched nematodes. Bands representing genes expressed specifically in hatched nematodes (arrow) are observed (A). A band specific to nematodes hatched in the previous 24 h (arrow) was also observed (B). Bands representing genes apparently down-regulated during the hatching process (arrowheads) are also observed (B–D). Products were generated using primers dTVC and OPG 2 (A), OPG 11 (B), OPG 6 (C) and OPG 8 (D). M, Marker; D, Dry cysts; 3d, water-soaked cysts placed in PRD for 3 days; NJ, J2s hatched within previous 24 h; OJ, J2s hatched up to 3 days previously.

were attributable to exposure to PRD were found. However, when examining gels a few reproducible differences in banding patterns were observed (Fig. 2) indicating the presence of genes apparently specifically expressed in dry cysts. Cloning and sequencing of this, and other similar small fragments of DNA revealed no continuous open reading frame and no significant homology to any sequences in the database (see below).

Differential display reactions comparing unhatched with hatched nematodes revealed many more differences in gene expression (Fig. 3). Bands were amplified from J2s, both 24 h and several days after hatching, which were not amplified from dry cysts or cysts soaked for 3 days in PRD (Fig. 3A). A number of bands which were specific to J2s hatched within the previous 24 h were also amplified (Fig. 3B). Examples of genes apparently downregulated during the hatching process were also found (Fig. 3C and D), as demonstrated by fragments amplified from dried cysts and those soaked for 3 days in root

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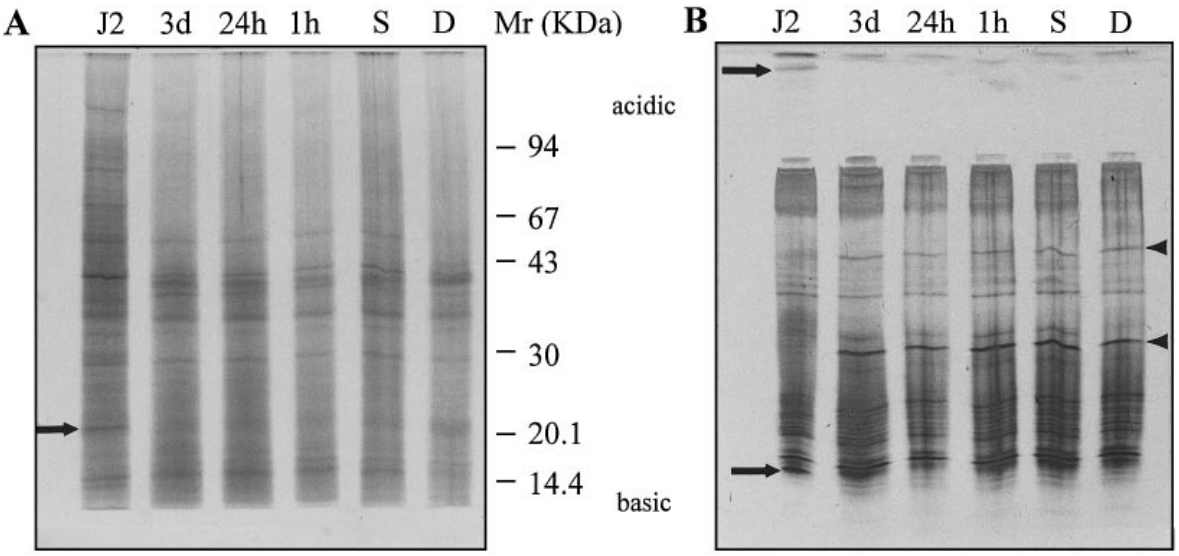

Fig. 4. SDS–PAGE (A) and IEF (B) gels of proteins from nematodes at various stages in the hatching process. The patterns observed in differential display experiments are confirmed with many differences observed between hatched and unhatched nematodes but none between unhatched nematodes subjected to various treatments. Bands are present in unhatched nematodes which are absent from hatched nematodes (arrowheads) and other bands are present in hatched nematodes which are not observed in unhatched nematodes (arrows). D, Dry cysts; S, Cysts soaked in water for 5 days; 1 h, water-soaked cysts placed in PRD for 1 h; 24 h, water-soaked cysts placed in PRD for 24 h; 3d, water-soaked cysts placed in PRD for 3 days; J2, hatched second-stage juveniles.

diffusate which were not detected in reactions using hatched J2s. The ease with which it was possible to amplify differentially expressed gene fragments when comparing hatched and unhatched nematodes (many of the primer combinations used gave differential banding patterns) contrasts with experiments comparing unhatched juveniles at different stages in the stimulation process where dozens of primer combinations were tested and only 1 or 2 gave reliable differences.

Attempts to further characterize genes represented by these fragments proved unsuccessful. After cloning and sequencing of many fragments of DNA eluted from bands apparently representing differentially expressed genes, no significant matches to known genes were found in database searches. Many of the fragments isolated were extremely AT rich and contained no open reading frames which gave matches from the database. This problem was accentuated as many of the fragments isolated were extremely short (> 200 bp) making meaningful comparisons difficult. It was also clear from the cloning and sequencing experiments that each band on a differential display gel often contained more than 1 species of DNA of the same size, further complicating analysis.

Protein profiles

Protein gels confirmed the patterns observed with differential displays (Fig. 4), with many differences observed between hatched J2s and unhatched nematodes but few if any differences observed between the unhatched nematodes at different stages in the stimulation process. Protein bands were observed in unhatched nematodes that were absent from hatched nematodes and other bands were observed which were present in hatched nematodes but absent in unhatched nematodes. This reflects the patterns observed in the differential display experiments, although it should be noted that some bands present in the unhatched nematodes but absent in the hatched J2s probably represent cyst or egg proteins and are therefore unlikely to be of biological significance.

DISCUSSION

The observation that PRD does not affect gene expression seems at first surprising given the changes apparently induced by this stimulus. A range of explanations can be offered for these results.

It is possible that PRD does induce changes in gene expression but that technical limitations have precluded the detection of such changes. More primer combinations used in further differential display reactions might uncover differences attributable to stimulation with PRD. However, many primer combinations were used in the present work in an attempt to amplify fragments from as many expressed genes as possible. Moreover, results obtained with protein gels reflect the patterns we observed with differential displays. It is also possible that the differential display technique is simply not sensitive enough to detect important quantitative changes in gene expression or to detect extremely

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rare messages. Indeed, evidence is emerging that differential display preferentially detects changes in abundant or moderately expressed genes (Bertioli *et al*. 1995). Despite our attempts to overcome this problem, it is still possible that subtle, but important, changes in gene expression have been missed.

Another possibility is that our results reflect what is actually occurring in the parasite; that changes in gene expression occur mainly during or after hatching. Indeed some evidence exists to support this theory. First, some structural changes in *G. rostochiensis* occur during the hatching process rather than as a result of stimulation while in the egg (Jones *et al*. 1993). Also, it has been shown recently that changes important in pathogenesis occur in naturally hatched *G. rostochiensis* in response to phytohormones (Duncan *et al*, unpublished observations). This is important as it demonstrates that the nematode is responsive to other host stimuli after initial exposure to PRD and subsequent hatching, and that PRD itself may not induce changes in the nematode which prepare it for the parasitic part of its life-cycle.

What are the changes in gene expression observed during or after hatching likely to reflect? In studies of the life-cycle of *Caenorhabditis elegans* it has been shown that as long as food is not limiting the nematode life-cycle proceeds without pause: there is a continuous cycle of growth and moulting. Recent experiments examining expression patterns of various collagen genes demonstrated that as soon as one moult is completed synthesis of cuticle components for the next moult begin (Johnstone & Barry, unpublished observations). This suggests that *C. elegans* develops and moves through the different phases of its life-cycle as quickly as possible. If a similar situation exists in *G. rostochiensis* the changes in gene expression observed here may simply reflect normal developmental processes. Development in the invasive J2 may be able to proceed as it contains lipid reserves which it uses until feeding begins. In this scenario, the nematode life-cycle can be viewed as being 'on hold' (i.e. quiescent) while the nematode is in the egg, exposure to PRD gives the nematode a signal to continue its development.

Clearly the next step is for us to characterize the genes whose expression patterns we have observed to change during hatching. We have cloned and sequenced a number of fragments of DNA isolated from differential display gels and carried out Northern blots using these cloned fragments as probes. However, several intrinsic limitations of the differential display technique make it extremely difficult to obtain information about the gene from which the displayed fragment was generated. Firstly, the fragments produced are extremely small making meaningful comparisons from the database extremely difficult. Secondly, since one of the primers in differential display reactions has to bind to the extreme 3′ end of messages (the poly-A tail) and since the products generated are small, many of the fragments displayed are sequences of 3′ untranslated regions of mRNAs from which it is impossible to tell anything about the nature of the coding region of the gene. Furthermore, the 3′ untranslated regions of mRNAs can vary between different messages generated from the same gene making Northern blotting difficult and sequence comparisons meaningless. Consequently, although differential display may be productive with other systems we are currently focusing our efforts on other techniques in order to isolate genes important in pathogenesis (Jones *et al*. and Duncan *et al*. unpublished observations).

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