Differentially expressed midgut transcripts in *Culicoides sonorensis* (Diptera: Ceratopogonidae) following Orbivirus (Reoviridae) oral feeding

C. L. Campbell and W. C. Wilson

USDA, ARS, Arthropod-borne Animal Diseases Research Laboratory, Laramie, USA

Abstract

Understanding the vector insect’s gene expression response to a virus infection may aid design of control measures for arbovirus diseases. *Culicoides sonorensis* is a vector of several agriculturally important pathogens, such as epizootic haemorrhagic disease virus (EHDV) that causes disease in ruminants. Two approaches, differential display and suppression subtractive hybridization, were used to identify 400+ *Culicoides* transcripts that were more abundant in midguts 1 day following an oral meal containing EHDV. Of these, quantitative PCR confirmed seven to be more abundant in virus-fed midguts than controls. One such transcript encodes a putative RNA editase, CsRED1, induced by dsRNA. Transcripts encoding putative receptors involved in cell differentiation included CsLAR, a protein tyrosine phosphatase, and CsFZ2, homologous to the *wingless* receptor in *D. melanogaster*. Transcripts encoding putative translation machinery components included CseIF3, CseIF5A and CsRPS6. Overall, the cDNA fragments identified in this study increased in the midgut at one day postfeeding; by 2 days postfeeding, increases in transcript levels shifted from the midgut to the remainder of the infected midge.

Keywords: genus *Culicoides*, midgut, epizootic haemorrhagic disease virus, differential expression, vector biology.

Introduction

The study of the arthropod-borne virus (arbovirus) infection process within biting insect vectors may help future development of bio-rational control measures for emerging and recurring diseases of man and animals. Biting midges of the *Culicoides* genus are vectors of several agriculturally important pathogens, including the arbovirus epizootic haemorrhagic disease virus (EHDV) (reviewed in Mellor et al., 2000). EHDV is a serious health threat to deer populations (Brodie et al., 1998; Beringer et al., 2000) in the USA. EHDV also causes bluetongue-like disease in cattle; however, laboratory confirmation of this phenomenon has been difficult to obtain (Omori et al., 1969; Abdy et al., 1999). In North America, *Culicoides sonorensis*, previously known as *Culicoides variipennis sonorensis* (Holbrook et al., 2000), is a major insect vector of this arbovirus disease (Foster et al., 1977; Smith & Stallknecht, 1996; Smith et al., 1996).

Arboviruses, such as EHDV, infect susceptible *Culicoides* spp. via a blood meal from an infected host, replicate in midgut epithelial cells, then are transported through the haemocoeel to the salivary glands for subsequent transmission to an uninfected host. *Culicoides* midges vary in their ability to transmit a given viral disease because of various infection or transmission barriers, some of which are heritable (reviewed in Tabachnick, 1991; Fu et al., 1999; Mellor et al., 2000). These barriers are thought to typically occur at the midgut or salivary glands; however, they have not been well defined.

Characterization of transcripts that vary in target tissues during a virus infection should illuminate insect genes that aid or hinder arbovirus morphogenesis and may help define the parameters required for an infection barrier in the refractory insect vector. Recent advances have shown that an insect’s innate immune response differs according to the source of the assault, whether bacterial or parasitic (Dimopoulos et al., 1997; Dimopoulos et al., 2000; Bonnet et al., 2001). The insect’s gene expression response to virus infection may differ depending on whether the infecting agent uses the insect vector as a transmission vehicle,
as with arboviruses, or is pathogenic to the insect itself. Most recent advances in the study of virus infections in insects have focused on entomopathogenic viruses, such as baculovirus and polydnaviruses infections of lepidopterans (Washburn et al., 1995, 2001; Asgari et al., 1996; Volkman, 1997; Shelby et al., 1998; Cui et al., 2000; Hoover et al., 2000). Little work has focused on the insect vector’s response to arbovirus infections. Because arboviruses are thought to be non-pathogenic to the insect host (Sieburth et al., 1991), the effects on gene expression may be distinct from that for entomopathogens.

Oral infection of Culicoides sonorensis by a closely related orbivirus, bluetongue, results in virus maturation from the basolateral surface of midgut epithelial cells, without concomitant cell lysis, into the extracellular matrix without concomitant cell lysis, into the extracellular matrix and through the basal lamina (Huismans, 1970; Sieburth et al., 1991), a strategy that has also been implicated for other arboviruses (Hoek et al., 1985). Virus particles are transported to other tissues via the haemocoel by an as yet unknown route (Nunamaker et al., 1989). Target tissues include fat body and thoracic muscle, with focused amplification in the salivary glands (Nunamaker et al., 1997). Based on these early studies, we chose to identify midgut transcripts that are differentially expressed early in the course of an EHDV infection. As the highest level of orbivirus load in the Culicoides midgut occurs 3 days after ingestion of a virus meal (Sieburth et al., 1991), characterization of differentially expressed transcripts produced 1–3 days following a virus meal provides a relevant context for analysing the early gene expression response to EHDV. In this study, two approaches were used to identify differentially expressed transcripts, differential display (DD RT-PCR) and a subtractive library. Following candidate isolation and a multistep confirmation process, the differentially expressed transcripts were compared for variations in abundance in midgut tissues at 1 and 2 days postfeeding and in whole midges at 1, 2 and 3 days.

**Results and discussion**

**Insects**

To determine the infection rate, midges were fed a virus meal and held for 13 days prior to virus isolation; in this study, EHDV virus was isolated from 4% of the midges assayed. We chose to assay a strain of Culicoides with the relatively low EHDV infection rate in order to more closely parallel field conditions, because in the field very few (< 0.1%) infected Culicoides are found during EHDV and bluetongue epizootics (Wieser-Schimpf et al., 1996; Smith & Stallknecht, 1996; Smith et al., 1996). In addition, this approach may result in the identification of infection barriers if the induction of certain early transcripts in the refractory midge prevents virus propagation and movement of the virus to tissues beyond the midgut epithelium.

To demonstrate that the initial stages of virus replication occurred, quantitative real-time PCR was used to assess the relative quantity of the EHDV L2 gene in midges following a virus meal. Colony midges were fed a foetal bovine serum meal containing about 5.3 logs TCID50/ml (tissue culture infectious dose 50%) EHDV serotype 1. Real-time PCR values for the L2 gene in pools of 20 midges at 2 and 3 days postfeeding were normalized to values for midges at 1 day postfeeding. Virus replication was demonstrated by a 185-fold increase in L2 levels at 2 days postfeeding. There was no detectable difference in L2 levels between 2 and 3 days postinfection.

**Isolation of differentially expressed transcripts**

Colony midges were fed as described above, and midguts were collected 1 or 2 days later for RNA extraction. Upon screening with 59 primer combinations, differential display analysis produced 29 midgut cDNAs with increased expression levels, relative to the serum-fed control midguts, 1 day following an EHDV meal (Fig. 1A). Preliminary confirmation by differential hybridization analysis (Fig. 1B) reduced this list to 13. Of these, preliminary sequence analysis

![Figure 1](image-url)
designated as CsLAR (insect origin. This 1150 base pair (bp) cDNA fragment was (Fig. 2) and was determined by sequence similarity to be of # A Y095263), passed both the final confirmation process © 2002 The Royal Entomological Society , differential display candidate, CsLAR (G differential expression in midguts from EHDV-fed midges. One real-time PCR was used as a final confirmation of differ-
entiation analysis (Fig. 1B). Twenty-two out of 342 cDNA candidates passed this preliminary screening procedure. Of these, six were confirmed by quantitative real-time PCR (Fig. 2) to be more highly expressed in EHDV-fed midguts when compared to midguts from mides fed an identical meal without virus. Gene names were designated based on
revealed most candidates to be novel sequences or putative microbial transcripts expressed by midgut flora, therefore these were excluded from further examination. Quantitative real-time PCR was used as a final confirmation of differentia-
expression in midguts from EHDV-fed mides. One differential display candidate, CsLAR (GenBank accession # AY095263), passed both the final confirmation process (Fig. 2) and was determined by sequence similarity to be of insect origin. This 1150 base pair (bp) cDNA fragment was designated as CsLAR (Culicoides sonorensis (C. s.) leucocyte antigen receptor-like) due to significant homology to Drosophila and Anopheles LAR nucleotide sequences, 71.7% and 68.5%, respectively.

A second strategy used a two-stage approach, culminat-
ing in the generation of a midgut subtractive cDNA library. First, a cDNA amplification procedure produced sufficient cDNA from the tiny midge for the subtractive library construction (See Experimental procedures). This strategy has been shown to produce a cDNA pool that is representative of the expressed gene pool of the sample (Zhumabayeva et al., 2001). Secondly, subtractive hybridization and suppression subtractive PCR resulted in enrichment of differ-
etial expression (Melcher et al., 1996). The resulting library was cloned into a plasmid vector and assessed by differential hybridiz-
ation analysis (Fig. 1B). Twenty-two out of 342 cDNA candidates passed this preliminary screening procedure. Of these, six were confirmed by quantitative real-time PCR (Fig. 2) to be more highly expressed in EHDV-fed midguts when compared to midguts from mides fed an identical meal without virus. Gene names were designated based on
significant similarity of the predicted amino acid sequence to known gene products; these are each discussed separa-
tely below. Next, variations in transcript abundance were assessed at various time points following the virus meal.

In the absence of a suitable messenger RNA that could be used as a reference standard, 18S rRNA was chosen. Although other studies have shown that 18S rRNA levels in mosquitoes increase markedly following a blood meal, spike at about 24 h, then decrease to prefeeding levels after about 48 h (Hotchkin & Fallon, 1987; Niu & Fallon, 2000), 18S rRNA remains a valid reference standard, as control and experimental mides were fed and harvested at the same time. To test the validity of the 18S rRNA standard for virus infected Culicoides, we normalized real-time PCR values for 18S rRNA from virus-fed and control mides to those of an unfed female control. Upon assaying three independent groups of mides, 18S rRNA levels increased about three-fold, 1 day (1d) following the proteinaceous serum meal and decreased thereby; however, there was no statistically significant difference between virus infected and control mides (data not shown). This limitation, in addition to the use of the relative expression analytical method (See Experimental procedures), imposes constraints on the conclusions that may be drawn from this study. Because there is no significant difference in 18S rRNA levels between unfed mides and those assayed at 2d and 3d postfeeding, transcript levels may be compared to determine if a virus feeding or proteinaceous meal affects transcript abundance at these time points. However, midguts and whole mides assayed at 1d postfeeding may only be compared with the appropriate control for that day and may not be compared accurately with samples from other time points.

Characterization of transcripts
The 909 bp cDNA fragment CsRED1 (C. s. RNA editase f), GenBank accession # AY095268, was isolated from the subtractive library, found to differentially hybridize to virus-
 fed midgut cDNA pools (Fig. 1B), and confirmed by quantita-
tive real-time PCR (Fig. 2). CsRED1 shares 67% amino acid (a.a.) identity with Drosophila dADAR, adenosine deaminase (Palladino et al., 2000) and 52% identity with human RNA editase, hRED1, for which transcription is typically induced in the presence of double-stranded RNA (Melcher et al., 1996). RNA editases not only respond to the presence of exogenous dsRNA but also act on double-
stranded structures formed in cellular premRNAs and are therefore important in the differential gene splicing (Melcher et al., 1996; O’Connell et al., 1997; Palladino et al., 2000). At one day postfeeding, CsRED1 transcript levels increased dramatically in the midguts of virus-fed mides (Fig. 3A). In whole insects, no significant difference between virus-fed and control mides was seen at 1d postfeeding; however, virus-fed mides showed increased transcript levels over control mides at 2d and 3d postfeeding.

**Figure 2.** Differential expression of transcripts in midgut tissue. cDNAs from midguts harvested 1 day after EHDV meal were assessed by quantitative real-time PCR. Values were normalized to 18S rRNA, then normalized to values for control midgut samples to calculate fold differences in transcript levels. Fold differences in transcript levels were statistically significant, as determined by Student’s T-test, P < 0.05. Values are an average of three independent experiments. Error bars represent standard error.


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Figure 3. A representative example of relative expression analysis. Transcripts vary in whole midges and midguts after oral feeding of EHDV serotype 1. Real-time PCR values were calculated as the mean of six individual PCR reactions, then were normalized to those for 18S rRNA. UF, unfed female; UM, unfed male; 1d, 2d, 3d, whole female midges at 1, 2 or 3 days postfeeding. 1dmg, 2dmg, midguts from females at 1 or 2 days postfeeding. Unfed midges were provided only de-ionized water and were assayed at 2–3 days old. Unfed male data provide a comparison to unfed female data. Error bars represent one standard deviation. This was calculated as a function of the standard deviation for both the sample and 18S rRNA.

Two putative transmembrane receptors were isolated. The CsLAR cDNA clone, isolated by differential display, is homologous to a family of receptor protein tyrosine phosphatases responsible for cell proliferation (Streuli et al., 1989). The predicted amino acid (a.a.) sequence shows 84% identity and 91% similarity to DLAR, a protein tyrosine phosphatase responsible for actin bundle formation during nerve axon and follicular epithelium differentiation in Drosophila melanogaster (Krueger et al., 1996; Frydman & Spradling, 2001). The cell surface receptor, DLAR, has three immunoglobulin-like C2-type domains and 16 fibronectin type III domains, iv which the extracellular matrix and two phosphatase domains in the cytoplasm (Streuli et al., 1989). Mammalian and Drosophila LAR homologues have multiple signal transduction functions ranging from actin bundling and cell differentiation to cell proliferation and apoptosis (Ruoslahti & Reed, 1994; Katsura et al., 1995; Pulido et al., 1995a,b; Serra-Pages et al., 1995; Krueger et al., 1996; Weng et al., 1998; Frydman & Spradling, 2001). The CsLAR cDNA fragment’s predicted amino acid sequence spans 383 residues and encodes the putative cytoplasmic domain. CsLAR transcript levels are elevated, relative to the control, in the virus-fed midguts at 1d. Control midguts show an apparent increase compared to infected midguts at 2d postfeeding. Although the reasons for this are unclear, perhaps the proteinaceous meal induces transcript levels at 2d postfeeding, and that induction occurs earlier in infected midguts. In contrast, whole midges showed no difference in transcript levels at 1d, but have an apparent increase in virus-fed samples over controls at 2d and 3d (Fig. 3B).

A second provisional midgut receptor, named CsFZ2 (C. s. frizzled 2), is more highly expressed in midgut tissue following an EHDV meal (Fig. 2). The CsFZ2 2757 bp cDNA fragment, GenBank accession #AY095265, named for its homology to the human frizzled-2 domain, contains a predicted translation product that is 51% identical to the carboxyl terminus of frizzled2 (DFZ2) protein of D. melanogaster. DFZ2, localized to the posterior midgut primordium, is a cellular receptor of the wingless transactivator, and thereby participates in signal transduction of cell differentiation events (Nakato et al., 1995; Bhanot et al., 1996; Tsuda et al., 1999; Axelrod, 2001; Lecourtois et al., 2001; Zecca & Struhl, 2002). CsFZ2 exhibits elevated transcript levels in the virus-fed midgut at 1d and 2d postfeeding, with greater abundance in the infected whole midge, compared to the serum-fed control at 3 days postfeeding (Fig. 3C).

Another differentially expressed transcript, potentially involved in signal transduction, is CsSAC2, GenBank accession #AY095266, named for its homology to the human Sac domain-containing gene, hSAC2 (Minagawa et al., 2001). A Sac domain is a protein motif indicative of inositol polyphosphate phosphatase activity. The human SAC2 gene product, a type II inositol phosphatase, is thought to act on lipid substrates that may regulate functions as diverse as membrane trafficking and cell proliferation (Minagawa et al., 2001). The 318 bp cDNA fragment CsSAC2 has a predicted translation product that shares 30% a.a. identity with a yeast protein, Sac1p. Yeast Sac1p regulates both the actin cytoskeleton and the secretory system (Whitters et al., 1993; Minagawa et al., 2001). CsSAC2 displayed a nearly four-fold increase in transcript abundance in the virus-fed midgut, relative to the control, at 1d postfeeding (Fig. 2); however, by 2d, control levels are higher than those of infected midguts. In this study, this is unclear, one might speculate that a proteinaceous meal induces transcript levels at 2d postfeeding, and that induction occurs earlier in infected midguts. At 1d postfeeding, transcript levels in the virus-fed midge are less than the control (Fig. 3D). This is overcome in subsequent days, 2d and 3d, when CsSAC2 transcripts are more abundant in virus-fed whole midges.

Several differentially expressed transcripts are homologous to molecules of the translational machinery. One candidate was designated as CsRPS6 (C. s. Ribosomal protein subunit 6), GenBank accession #AY095266, because the predicted translation product of the 807 bp cDNA fragment shares 61% a.a. identity with the carboxyl terminus of ribosomal protein subunit 6 from Aedes albopictus. In Niu et al.’s study of A. albopictus fat body transcript levels of this major ribosomal protein were similar to those of the uninfected female for the first day following a blood meal with reduction 2d after feeding (Niu & Fallon, 2000). In this study, CsRPS6 levels in the virus-fed midguts increased two-fold over controls 1d postfeeding (Fig. 2), with no significant difference between control and virus-fed midguts at 2d postfeeding (Fig. 3E). CsRPS6 transcript levels in virus-fed midges were reduced relative to the control, whereas by 2d and 3d postfeeding, the EHDV-fed whole midge showed a significant increase in expression (Fig. 3E).

In addition to RPS6, two putative translation initiation factors were identified. CseIF3 (C. s. elongation initiation factor subunit 3), a 486 bp cDNA fragment with GenBank accession #AY095269, contains a predicted translation product with 66% a.a. identity with carboxyl terminus of Drosophila elf1F subunit 3. For numerous viruses, elf3 assists in ribosomal re-entry at internal translation start sites (reviewed in Pestova et al., 2001). Orbiviruses have an alternate translation initiation site in the S10 genome segment encoding the NS3 and NS3A proteins (French et al., 1989), and therefore may be the target of the CseIF3 protein. CseIF3 transcript levels in EHDV-fed midges were 2.4-fold higher than controls after 1d (Fig. 2) and were elevated in whole midges at 2d and 3d postfeeding (Fig. 3F).

The second provisional translation machinery component identified in this study, CseIF5A (C. s. elongation initiation...
CseIF5A transcripts are nearly three-fold more abundant in In contrast, our study showed that steady state levels of release from cells occurs through budding or extrusion et al., 1990), it is unclear whether actin is required for viral infection lies in the proposed roles in actin organization and the increase in actin transcript abundance in virus-fed midguts suggests that actin organization is affected by EHDV infection of the midgut.

Further studies of the midge gene expression response in other target tissues beyond the midgut, as well as characterization of the genes isolated here, will be required to understand more fully how the resistant or susceptible biting midge transcription profile changes in response to an arbovirus infection and to determine a causal relationship to vector competence.

Conclusion

One day following an EHDV meal, certain transcripts in the midgut transiently increased over basal levels during the early stages of viral replication. The lack of a significant increase over controls in infected 2d midguts suggests that increased production of these transcripts is no longer necessary following viral replication. This transient increase in transcript levels shifts from the midgut to the whole midge by 2d and 3d postfeeding. Characterization of other virus products that may be commandeered for viral replication and morphogenesis. CsRED1 may fall into this class. CseIF3 is also a candidate for this role, possibly participating in the production of alternate viral translation products. Upon further characterization, other genes may fall into this class as well.

A second class of transcripts, CsF22 and CsLAR, may become more abundant for initiation of turnover of the infected midgut epithelium; however, this hypothesis contradicts reports suggesting a lack of cytopathology in orbivirus infected Culicoides midguts (Sieburth et al., 1991), as well as other evidence indicating that orbivirus particle release from cells occurs through budding or extrusion through the cell membrane without an apparent increase in cell death (Hyatt et al., 1989).

An alternative possibility for CsLAR function during EHDV infection lies in the proposed roles in actin organization for homologues of both CsLAR and CsSAC2 (Whitters et al., 1993; Fryman & Spradling, 2001). Although microscopic evidence indicated that EHDV particles associate with the cytoskeleton in mammalian cell culture (Nunamaker et al., 1990), it is unclear whether actin is required for viral particle maturation in insects. In our study, relative levels of actin transcripts were higher in EHDV-fed midguts than in serum-fed controls (data not shown). The combined increase in abundance of these two gene products with proposed involvement in actin organization and the increase in actin transcript abundance in virus-fed midguts suggests that actin organization is affected by EHDV infection of the midgut.

Experimental procedures

Insects

Culicoides sonorensis were reared at the colony maintained at the Arthropod-borne Animal Diseases Research Laboratory, Laramie, Wyoming, USA. The specimens used were of the Van Ryn strain, originally isolated from field specimens collected in 1995 in California (Brad Mullens, University of California, Riverside, CA, USA). 2–3-day-old insects, provided de-ionized water ad libitum since eclosion, were allowed to feed on a meal of fetal bovine serum, EHDV serotype 1, 100 μg/ml phenol red sodium salt (Sigma), and phagostimulants ATP sodium salt, 50 μM, pH 7.0, and sodium bicarbonate, 0.37 mg/ml (Nunamaker et al., 2000). The meal virus titre was about 5.3 log TCID₅₀/ml (Karber method). TCID₅₀ is the infectious dose which results in 50% cell death in a 96-well cell culture assay format (Payment & Trudel, 1993). In order to closely approximate the virus meal, the meal for control midges contained a 1 : 1 mixture of cell culture medium and buffered lactose peptone added in place of the virus stock; these reagents are commonly used to dilute virus stocks during preparation. Midges were fed and maintained according to the methods of Hunt (Hunt et al., 1989; Hunt & McKinnon, 1990) at 27 °C. When held for 2 weeks at 20 °C prior to processing, this insect strain had an infection rate of 4.3%, as determined by cell culture assay for EHDV serotype 1 (Nunamaker et al., 2000).

Dissections and RNA extractions

Midguts were dissected in RNAlater (Ambion) one day (22–30 h) or two days (48–54 h) following the virus meal. Midgut or carcass pools of 20–50 were processed for RNA extraction. Whole midges were stored in liquid nitrogen prior to RNA extraction and processed in pools of 10–20. Total RNA was isolated using Bio101 RNAid Plus kit. Total RNA was treated with Amplification grade DNase I (Invitrogen) according to manufacturer’s recommendations to remove genomic material prior to RT-PCR. Successful DNase I treatments were confirmed by lack of PCR amplification of an amplicon for an RT reaction without reverse transcriptase.

Differential display RT-PCR

First strand synthesis (RT rxn) of the complementary DNA (cDNA) strand was accomplished using a two anchor oligo dT primer
of the band of interest using the original primers to confirm size, sequences were initially verified by gel purification, re-amplification DNA bands were extracted from the gel for cloning. Candidate fed midgut cDNA showed differentially expressed gene fragments. Comparisons of RT-PCR reactions of virus-fed midgut cDNA vs. serum-fed midgut cDNA showed differentially expressed gene fragments. DNA bands were extracted from the gel for cloning. Candidate sequences were initially verified by gel purification, re-amplification of the band of interest using the original primers to confirm size, cloning into a sequencing vector, and partial sequencing.

Subtractive library
DNaseI-treated total RNA was used for preparation of amplified cDNA (SMART cDNA synthesis kit, Clontech) according to manufacturer’s recommendations. One deviation from the recommended procedure was to use Chroma-spin 300 columns for size exclusion rather than Chroma-spin 1000 columns, to promote isolation of relatively small cDNAs. The SMART cDNA synthesis kit allowed us to generate material of sufficient quantity for subtractive library procedures (PCR-Select cDNA Subtraction kit, Clontech), according to manufacturer’s recommendations. Subtraction candidates were cloned into the pCR-TOPO 4.0 sequencing plasmid (Invitrogen), transformed into E.coli and colony purified.

Differential display and subtractive library clone inserts were PCR amplified directly from recombinant E.coli colonies (Corton & Gustafsson, 1997). The resulting double-stranded cDNA fragments were spotted on to Nytran Supercharged membranes (Schleicher and Schuell) using a vacuum manifold apparatus (Schleicher and Schuell) using a vacuum manifold apparatus (Schleicher & Schuell) using a vacuum manifold apparatus (Schleicher & Schuell) using a vacuum manifold apparatus (Schleicher & Schuell) using a vacuum manifold apparatus (Schleicher & Schuell) using a vacuum manifold apparatus (Schleicher & Schuell) using a vacuum manifold apparatus (Schleicher & Schuell). Duplicate blots were prepared as per Corton et al. and probed with either control midgut cDNA pools or EHDV-fed midgut cDNA pools that had been conjugated to alkaline phosphatase. Hybridization procedures followed manufacturer’s recommendations for chemiluminescence using Alk-phos Direct probe labelling kit (New England Nuclear, Life Technologies), and Schuell) using a vacuum manifold apparatus (Schleicher and Schuell) using a vacuum manifold apparatus (Schleicher & Schuell) using a vacuum manifold apparatus (Schleicher & Schuell) using a vacuum manifold apparatus (Schleicher & Schuell) using a vacuum manifold apparatus (Schleicher & Schuell). The resulting double-stranded cDNA fragments were probed with either control midgut cDNA pools or EHDV-fed midgut cDNA pools.

Sequence analysis
Clones in pCR-TOPO 4.0 plasmids (Invitrogen) were sequenced using T3, T7, M13 forward, or M13 reverse sequencing primers on an ABI 3100 automated sequencer (Applied Biosystems) with dRhodamine Ready Reaction sequence reagent (Applied Biosystems). Sequences were assembled using Vector NTI software (Informax), conceptually translated, and analysed for similarity to known sequences using Blast, Blastp or Blastx programs (Altschul et al., 1990). Sequences were aligned using the FAST algorithm to determine similarities.

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References


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