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Molecular investigations of orbivirus/vector interactions

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Summary

Defining predictors for insect-transmitted virus (arbovirus) disease cycles requires an understanding of the molecular interactions between the virus and vector insect. Studies of orbiviruses from numerous geographic regions have indicated that virus genes are affected by insect population differences. Therefore, the authors have initiated genetic studies of *Culicoides sonorensis*, isolating cDNAs for characterisation of differential insect gene expression, as well as a gene discovery project. Previous work identified insect transcripts elevated in orbivirus-infected female midguts at one day post infection (pI). Here, we report cDNAs that were more abundant in midguts two days following an epizootic haemorrhagic disease virus feeding, as well in head/salivary glands at three days pI. Of the cDNAs identified in midguts at two days pI, three encode translational machinery components, and three encode components that affect cellular structural features. Of the differentially expressed salivary gland cDNAs, only one was homologous to a previously identified gene, a putative odorant binding protein.

Keywords

Bluetongue – *Culicoides sonorensis* – Cytoskeleton – Differential gene expression – Ribosomal protein subunit – Sensory appendage protein – Subtractive library – Translation – Vector.

Introduction

Bluetongue (BT) virus (BTV) infection of livestock in the United States of America (USA) causes limited clinical losses. However, there is continued concern because of the potential danger of importing exotic virus strains of unknown virulence to livestock in the USA. Mounting virus phylogenetic analyses have resulted in the delineation of orbiviruses into geographic types (2, 6, 7, 19, 22) potentially caused by evolutionary pressure from regional vector populations. BTV gene segments appear to evolve independently in a host-specific fashion suggesting that both invertebrate and vertebrate hosts influence genetic selection (6). To better define possible interactions between orbiviruses, such as BTV and epizootic haemorrhagic disease virus (EHDV), and the insect vector host, the Arthropod-Borne Animal Diseases Research Laboratory has focused on the primary vector species encountered in the USA, *Culicoides sonorensis*. These studies will provide the basis for comparative genomic studies as well as investigations of specific interactions between insect vector proteins and exotic viruses. Eventually information gathered from these studies should provide insight into risk assessment for importation of exotic vector species or virus strains and provide targets for genetic manipulations to increase virus resistance and the development of new control strategies for interrupting insect-transmitted virus (arbovirus) disease cycles.

The authors have chosen several approaches to characterise both genetic and environmental factors that may influence the ability of *Culicoides* spp. to amplify and transmit an arbovirus, as follows:

1) identification of differentially expressed transcripts in orbivirus-infected midge target tissues most relevant to virus infection
2) a gene discovery project of midguts and salivary glands to identify Culicoid genes for further study
3) characterisation of possible environmental factors that, combined with the Culicoid genetic characteristics, determine vector competence.

This paper, however, focuses on the identification of differentially expressed insect cDNAs in target tissues and outlines our developing efforts towards a *C. sonorensis* tissue-specific gene discovery project.

The purpose of the differential expression studies was to identify cDNAs that might comprise possible barriers to orbivirus infection in *Culicoides* spp. and to

develop markers to assess differences between resistant and susceptible vector populations. The primary focus was on those genes expressed early in the infection process because the encoded proteins are likely to be important in affecting virus infection and may include insect gene products that assist in virus replication or during attachment. A previous report from this laboratory described seven of eight midge cDNAs that had elevated transcript levels in EHDV-infected midge midguts compared to serum-fed controls one day following a virus meal (9).

The majority of the cDNAs identified in the previous study comprise three major groups, those that were homologous to genes coding for translation machinery components (RPS6, eIF3, eIF5A), those potentially involved in cellular differentiation (LAR, FZ2) or those putatively associated with actin (LAR, SAC, actin) (9). Based on the general understanding of orbivirus replication in host cells (18), we proposed that host translation factors, such as those translation elongation factors and the ribosomal binding subunit listed above, are recruited for viral replication. We also postulated that the putative actin-binding cDNAs and actin might be involved in virus movement such as has been shown for other viruses such as West Nile virus (12).

Our previous study has been extended in this paper to describe elevated transcripts in midguts and heads/salivary gland tissues later during the infection cycle to better characterise the progression of orbivirus infection in the female midge midgut. Midguts were analysed for elevated transcript levels at two days post infection (pI). As orbiviruses are expected to escape from the midgut milieu by three days pI (21), we chose to identify elevated transcripts in the distal head/salivary gland tissues during this time. Reverse Northern blot analysis provided preliminary confirmation of cDNAs from subtracted libraries designed to enrich for elevated transcripts in tissues of EHDV-infected midges. These analyses resulted in the preliminary identification of 14 transcripts from midguts at two days pI and 4 transcripts from heads/salivary glands at three days pI that are more abundant during EHDV infection.

Methods

Detection of differential transcript levels

Midges were fed an EHDV-containing (6.0 $\log_{10}$TCID$_{50}$/ml) meal in foetal bovine serum plus phagostimulants, whereas control midges were fed a similar meal without virus (9). Midges were held for two or three days at 27°C prior to tissue dissection and processing. At two days following the virus meal, midguts were dissected from both groups of midges and total RNA was extracted as previously described (9). At three days following the virus meal, heads with salivary glands from female midges were removed and RNA was extracted. Total RNA was used to prepare subtracted libraries (BD Biosciences-Clontech) and cDNA inserts were cloned into PCR TOPO 4.0 sequencing plasmids. Reverse Northern blots (13) using Alk-Phos Direct labelling (Amersham) revealed cDNAs in virus-infected midguts or heads/salivary glands, more abundant than those of serum-fed controls. cDNAs were sequenced and subjected to tBLASTx analysis for preliminary identification based on sequence similarity (1).

Expressed sequence tag gene discovery project

RNA was prepared from either serum-fed colonised *C. sonorensis* female midguts or from salivary glands removed from 2- to 4-day-old naive females. All tissues were dissected and stored in RNAlater (Ambion). The midgut cDNA library was prepared from poly A+ RNA and cloned into pSPORT1 (Life Technologies). The salivary gland library was generated by PCR amplification of total RNA and cloned into pDNR-lib plasmids (Clontech).

Results and discussion

A total of 279 cDNAs were analysed by reverse Northern blot for the two-day-midgut subtracted library. Only those cDNAs that showed increased intensity of hybridisation over serum-fed controls, following two independent reverse Northern blots, were analysed further. Of these, 14 cDNAs showed a stronger intensity of hybridisation when probed with alkaline phosphatase-labelled EHDV-infected total midgut cDNAs versus serum-fed total midgut cDNAs (data not shown). These cDNAs are listed in Table I. The number of elevated transcripts comprised only 5% of the number of cDNAs screened suggesting that orbivirus infection affects a relatively small number of insect transcripts (24, 25). These data indicated that increased transcript levels during EHDV infection of *Culicoides* comprise a small group of cDNAs coding for three major groups of putative proteins: translation factors, proteins affecting cellular structural components and putative effectors of cellular differentiation.

Translation factors constitute one group of cDNAs more abundant in EHDV-infected midguts at two days pI. Similar to the findings previously reported (9), elevated transcripts at two days pI include two ribosomal protein subunits (RPL32, RPL1) (Table I). Many viruses, including orbiviruses, repress overall host cell metabolism, including transcription and

Table I
Midge midgut transcripts elevated at two days post infection

cDNA	Accession number	bp	Homology	GI number	E-value	Protein domain
402	AY686211	342	Gelsolin (GEL)	24643859	1E -042	–
450	AY686215	347	Unknown	34850146	1E -007	Zinc finger, zf-C2H2 0.0013
518	AY686208	681	*Drosophila melanogaster* cue-related	24655246	1E -021	EGF-laminin, ferredoxin, LDL_receptor b, 3E-07
529	Not submitted to GenBank	614	Novel	–	–	–
535	AY686210	465	BTB domain protein	24641897	6E -019	–
536	Not submitted to GenBank	237	TRY1-like	2317914	1E -005	–
537	Not submitted to GenBank	386	Ribosomal protein L 32 (RPL32)	31241002	3E -044	Ribosomal L32, 3.8E-21
566	Not submitted to GenBank	661	Novel	–	–	–
616	AY686207	210	Clathrin heavy chain	31205808	1E -038	–
617	AY686209	507	eIF 3 subunit 8	31228735	2E -053	Ferredoxin, eIF3c N, 6.5E-09
628	AY686212	349	Ribosomal protein L 1 (RPL1)	31207396	4E -037	Ribosomal L4, 1.7E-05
630	AY686216	648	Tropomysin-like	24647088	3E -006	–
638	Not submitted to GenBank	327	Novel	–	–	–
639	AY603561	295	COX III	27657059	1E -040	–

cDNA complementary DNA
bp base pair ('bp' indicates cDNA fragment length)
GI GenInfo identifier
– no match was identified.

'Homology' indicates the proposed molecular function of the most closely related gene (GenBank accession number) of known function available in GenBank using a tBLASTx search

'E-value' indicates the confidence level of the homology assignment

Protein domains were determined by Prosite or Pfam search; Pfam searches are accompanied by an E-value

translation of host mRNAs (18). However, drawing a parallel in another virus system, host cells infected with poliovirus exhibit 'un-repressed' translation of ribosomal protein subunits (10) such as RPL32 identified in this study (Table I), in addition to post translational modification of RPS6 (9), identified in our previous report. These parallels in another virus system corroborate the supposition that these proteins are recruited for virus replication; however, further studies are required to confirm this hypothesis.

A second category of cDNAs enriched in EHDV-infected midguts at two days pI were cDNAs encoding proteins that affect cellular structural features. As stated previously, several putative actin-binding cDNAs were isolated from midguts one day pI (9). Many types of viruses recruit actin for various steps of viral propagation (reviewed in 14). At two days pI, a cDNA encoding the *Culicoides* homolog of gelsolin was isolated. Gelsolin is an actin filament severing protein (3). Perhaps gelsolin participates in viral particle release from the cytoskeletal matrix during the replicative process. Other cDNAs encoding structural proteins include a tropomyosin-like cDNA enriched in EHDV-infected midge midguts (Table I). The function of tropomyosin as a contractile fibre does not provide any clues into its possible function in assisting or preventing viral propagation.

A *Culicoides* homolog to clathrin heavy chain was also enriched in EHDV-infected insect midguts (Table I). Although clathrin is known for virus endocytosis (15), research of a closely related orbivirus, BT, suggests, indirectly, that perhaps this molecule could be involved in non-lytic virus release from insect cells. The BT NS3 protein directly interacts with calpactin of the annexin II complex (4). Annexin II complexes associate with clathrin heavy chain *in vivo* (23), and clathrin heavy chain participates in exocytosis (16, 17). The enrichment of the clathrin heavy chain homolog in EHDV-infected midguts suggests that EHDV may stimulate and use a non-lytic viral egress in insect cells.

The last group of cDNAs enriched in midguts by EHDV infection is associated with cellular

differentiation. This includes a *Culicoides* homolog of the *Drosophila cue* gene. Although not well studied, the *Drosophila cue* homolog has predicted epidermal growth factor (EGF)/laminin protein domains that participate in sperm differentiation and maturation (11). The nature of its participation during virus infection is unclear. Nevertheless, the *Culicoides cue* gene (cDNA 518) is the third differentially expressed cDNA potentially encoding an effector of cellular differentiation. This follows the earlier expression of cDNAs for CsFZ2, coding for the putative *Culicoides* homolog of the WNT receptor, and CsLAR, encoding a putative temporally expressed cell adhesion molecule (9).

Of the head/salivary gland library, 157 cDNAs were analysed by reverse Northern analysis, and 4 transcripts from EHDV-infected insects had consistently stronger hybridisation intensities than serum-fed controls following several rounds of reverse Northern analysis (Table II).

Only one of the four differentially expressed cDNAs, number 818, was similar to any known genes. cDNA 818 is most closely related to a putative odorant binding protein that may possess pheromone binding activity (5). This is the first report of increased levels of a cDNA encoding odorant-binding protein during an orbivirus infection. This finding lends credence to the idea that behavioural studies of virus-infected insect vectors may provide insight into the evolutionary relationships between viruses and insect vectors. Perhaps EHDV infection increases the host-finding behaviour similar to the previous report of *Plasmodium* infection increasing the biting rate of mosquitoes (20).

Taken together, these data suggest that increased transcript levels during EHDV infection of *Culicoides* comprise a small group of cDNAs coding for three major groups of putative proteins: translation factors, putative modifiers of cellular differentiation and those affecting cellular structural components. When considered in the context of current knowledge of the corresponding homologs in other organisms, collectively, these cDNAs are forming a picture of the nature of the gene expression response of the insect to an orbivirus infection. The apparent absence of immune response genes in these screens might suggest a synergistic relationship between Culicoids and orbiviruses. Alternatively, immune response genes may not have been detected for a variety of reasons; for example, they may act transiently or may be regulated post-transcriptionally or post-translationally.

To prepare for future studies of the molecular interactions of the arbovirus and vector insect, we have undertaken a gene discovery expressed sequence tag (EST) project designed to increase our repertoire of *Culicoides* cDNAs. The focus is on the primary tissue barriers to vector infection, replication and transmission. The growing dataset of EST sequences from *C. sonorensis* midgut and salivary glands are being analysed, categorised and annotated. In preliminary annotation, over 874 unique gene alleles have already been identified (data not shown).

Table II
Midge head/salivary gland transcripts elevated at three days post infection

cDNA	Accession number	bp	Homology	GI number	E-value	Protein domain
752	AY686217	825	Novel	–	–	Anaphylatoxin, VWFC
818	AY686213	715	SAP1, sensory appendage protein (antennae)	19071285	7E -046	OS-D, 1E -64
825	Not submitted to GenBank	476	Novel	–	–	
829	Not submitted to GenBank	437	Novel	–	–	–

cDNA complementary DNA
bp base pair ('bp' indicates cDNA fragment length)
GI GenInfo identifier
OS-D insect pheromone-binding family
VWFC a type of C-terminal cystine knot
– no match was identified

'Homology' indicates the proposed molecular function of the most closely related gene (GI number) of known function available in GenBank using a tBLASTx search

'E-value' indicates the confidence level of the homology assignment

Protein domains were determined by Prosite or Pfam search; Pfam searches are accompanied by an E-value

The 25% proportion of digestive enzymes amongst the individual EST sequences is indicative of the presence of redundant transcripts, as would be expected from midges that had recently received a serum meal (Fig. 1).

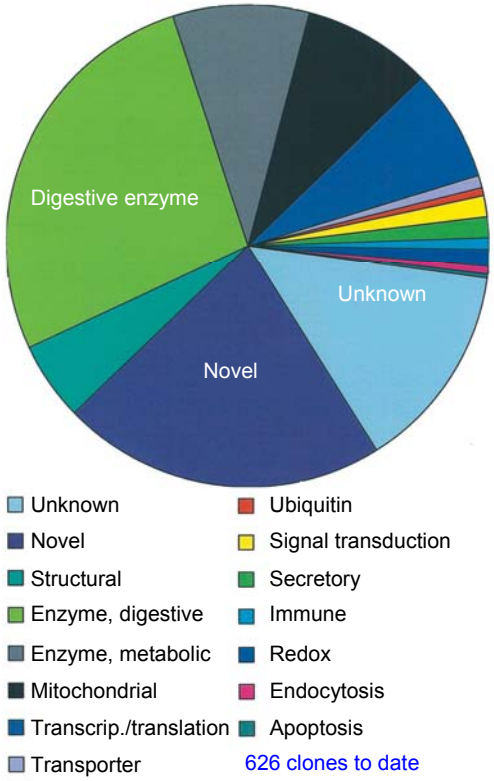

Figure 1
Expressed sequence tag (EST) pilot gene discovery project showing the relative frequency of various gene categories identified from initial EST sequences
cDNAs were prepared from serum-fed colony *Culicoides* midgut mRNAs
Homology to known genes was determined by tBLAST× search of NCBI public database
'Unknown' genes represent those that are homologous to genes in the public databases with no assigned molecular function
'Novel' cDNAs represent those that do not have significant homology to any genes in GenBank

Most genes identified in the differential expression studies were not isolated in the EST gene discovery project. Among the 21 confirmed or provisional differentially expressed cDNAs identified from midguts at one and two days pI, only RPS6 and translation factor eIF4A were also isolated from the serum-fed midgut cDNA library. Therefore, the differentially expressed cDNAs may represent rare transcripts that would not otherwise be isolated except through a subtractive method as described here. The development of a *C. sonorensis* tissue-specific EST database and the combined application of modern and classical arbovirology will move BTV and EHDV vector ecology/competence studies into a new era. These studies are presumably defining genetic markers associated with vectorial capacity. Additional markers are also being generated that allow assessment of the environmental factors involved in vector competence (8). When combined, this growing set of reagents should provide opportunities for assessing the risk of virus infection within a population, persistence in a geographical location via overwintering insects, and potential for geographical movement. In addition, novel targets for interrupting the orbiviral transmission cycle could be identified for potential development of therapeutic agents.

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