INTRODUCTION

The basal mechanisms of innate immunity are surprisingly well conserved between humans and insects. With its well-characterized genetic system, the fruit fly, *Drosophila melanogaster*, has proven to be a useful model organism to study these mechanisms (Hoffmann, 2003; Hultmark, 2003; Leclerc & Reichhart, 2004; Tanji & Ip, 2005). The fruit fly faces many parasites, pathogens and other microorganisms in its natural habitat, rotting fruit. The innate defences against bacteria and fungi in *Drosophila* are now relatively well understood, yet surprisingly little has been done to study how viral infections are controlled. Recent studies have shown that the JAK/STAT pathway plays a role in the response against *Drosophila C virus*, while the Toll signalling pathway may affect the sensitivity to *Drosophila X virus* infection (Dostert et al., 2005; Zambon et al., 2005). The difference in results may be explained by the fact that two different viruses were studied. Better knowledge about *Drosophila* viruses and their biology is needed to develop this field.

Recently, several insect viruses have been sequenced, making it possible to classify them. RNA viruses of the picorna-like superfamily are among the best studied of the insect viruses. Picorna-like viruses are further categorized on the basis of their genome organization. The vertebrate picornaviruses, the members of the family *Picornaviridae*, are positive-strand, single-stranded RNA viruses. They utilize their genomic RNA as an exclusive message for a single polyprotein, from which all viral proteins are produced as the result of processing (Ryan & Flint, 1997). Other viruses of the picorna-like superfamily share a conserved helicase-protease-replicase (H-P-Rep) cassette of replicative proteins with the picornaviruses (Koonin & Dolja, 1993). One heterogeneous group of picorna-like viruses found in insects and other arthropods is usually included in a ‘floating genus’, the genus *Iflavirus*. Their genomes are organized as in the family *Picornaviridae*, with a single open reading frame (ORF) where the structural proteins are encoded 5′ of the H-P-Rep cassette. In contrast, in the family *Dicistroviridae*, the structural proteins are encoded in a second ORF in the 3′ part of their genome. Similar to iflaviruses, dicistroviruses have so far only been found in insects and other arthropods.

Few viruses have been described from *D. melanogaster* and most have not yet been extensively studied and characterized (Ashburner et al., 2005; Brun & Plus, 1980). Three viruses have been classified as picorna-like: *Drosophila* A virus, *Drosophila* P virus and *Drosophila* C virus, but only the latter has been sequenced and has been shown to belong to
the family \textit{Dicistroviridae} (Johnson & Christian, 1998). \textit{Drosophila X virus} is a double-stranded RNA virus of the family \textit{Birnaviridae} (Zambon \textit{et al}., 2005) and \textit{Drosophila} sigma virus, a rhabdovirus with a negative-strand RNA genome, is widely spread in natural populations of \textit{D. melanogaster} and is one of the best-studied viruses in \textit{Drosophila} (Landès-Devauchelle \textit{et al}., 1995). Furthermore, several retrotransposons have been characterized, some of which, like \textit{gypsy}, can also act as independent retroviruses (Kim \textit{et al}., 1994).

In this paper, we describe a picorna-like \textit{Drosophila} virus, Nora virus (‘new’ in Armenian), which represents a new distinct virus family. Unlike other picorna-like viruses, its genome has four ORFs. One encodes a conserved picornavirus-like H-P-Rep cassette of replicative proteins, but the others show no obvious sequence similarity to previously described viruses. Nora virus is present as a persistent infection in several tested laboratory stocks and wild-caught flies.

\section*{METHODS}

\subsection*{Fly stocks}

All adult flies used were 3–5 days old and were reared at room temperature on standard yeast/agar media. The fly stocks used were as follows. Canton S, Oregon R, Nairobi and \textit{e spe\textsuperscript{2} cal TMI} were originally from the Umeå Stock Centre. Zalaszanto was isolated in 2001 by Thomas Werner from wild-caught flies in Hungary. \textit{Relish\textsuperscript{255}, Relish\textsuperscript{16}, Relish\textsuperscript{248}, Relish\textsuperscript{2090} and Relish\textsuperscript{260}} are independent lines generated in a single P element excision experiment (Hedengren \textit{et al}., 1999). Dm1 and Dm2 were recently collected from the vicinity of Umeå by Ines Anderl and Svenja Stöven. The \textit{Drosophila simulans, Drosophila erecta, Drosophila yakuba} and \textit{Drosophila virilis} stocks were all obtained from Jan Larsson, Umeå University.

\subsection*{Virus purification}

\textit{Relish\textsuperscript{2090}} flies were homogenized in 10 ml NT buffer (100 mM NaCl, 10 mM Tris/HCl, pH 7·4) and clarified by centrifugation at 4500 g for 20 min. Supernatant was extracted with an equal volume of 1,1,2-trichlorotrifluoroethane before the aqueous phase was layered over a discontinuous CsCl gradient (1-5% CsCl according to the manufacturer’s instructions. Using the initial primer combinations and cloned by TA cloning using the pCR2.1-TOPO vector (Invitrogen). The vector was then transformed into TOPO-10 competent cells (Invitrogen) and sequenced using the Big Dye sequencing kit (Perkin Elmer Life Sciences) with either vector- or virus-specific primers. For 3’-RACE, we used an oligo(dT) primer, as described in the user manual. cDNA was amplified and sequenced as described above.

\subsection*{RNA preparation, Northern blot analysis and hybridization}

\textit{Relish\textsuperscript{255}} total RNA was prepared using the Aurum total RNA kit (Bio-Rad). For Northern blots, 15 μg total RNA per lane was run on a 1% agarose gel containing formaldehyde along with 0·24–9·5 kb RNA size standards (Invitrogen). Hybridization was performed under high-stringency conditions (50% formamide, 42°C). Virus probe was made from a 1·65 kb PCR fragment, after labelling with the Rediprime II kit (Amersham Pharmacia), according to the manufacturer’s instructions. After hybridization, the filters were washed and the radioactivity monitored using a PhosphorImager (Storm; Molecular Dynamics).

\subsection*{DNA preparation and Southern blot analysis}

DNA was isolated from Canton S and \textit{Relish\textsuperscript{255}} flies essentially as described by Hamilton \textit{et al}., (1991). Approximately 5–10 μg aliquots (less for Canton S) of genomic DNA were digested with EcoRI or BamHI. The digested DNA was separated on a 0·7% agarose gel and blotted onto a Hybond membrane (Amersham Biosciences). Probe hybridization and detection was carried out as for Northern blot analysis. Different concentrations of a control plasmid containing the probe sequence were run on the same blot as a standard.

\subsection*{Preparation and hybridization with ssRNA probes}

The pCR2.1-TOPO \textit{in vitro} transcription system (Invitrogen) was used to synthesize radioactively labelled probes from the T3 and T7 promoters. Linearized plasmid DNA was made by cutting the end of the QRT-PCR plasmid (see below) and used as a template in the transcription reaction. Briefly, a 20 μl reaction mixture containing 1 μg linearized DNA template, 1× transcription buffer (Roche), RNAse inhibitor (20 U), 1 mM each ATP, GTP and CTP, T3 or T7 RNA polymerase (40 U) and 1·5 mM \textsuperscript{32}P]-UTP (10 nCi ml\textsuperscript{-1}; 3000 Ci mmol\textsuperscript{-1}; Amersham) was incubated at 37°C for 2 h. After incubation, the template DNA was removed by DNase I digestion (20 U) at 37°C for 30 min. Unincorporated nucleotides were removed on a Sepharose G-50 column (Roche) according to the manufacturer’s instructions. Hybridization of Northern blots with ssRNA probes was done by pre-hybridization for 2 h at 55°C. Hybridization was performed in the same pre-hybridization solution with the addition of the \textsuperscript{32}P]-labelled ssRNA, essentially as described by Jiang \textit{et al}., (1987). After hybridization, the filters were washed and the radioactivity monitored using a PhosphorImager as above.

\subsection*{Treatment with RNase I}

Total RNA from \textit{Relish\textsuperscript{255}} was treated with RNase I (BioLabs) for 30 min at 37°C, followed by heat inactivation for 20 min at 72°C. RNA was precipitated using 4 M LiCl and 100% ethanol for 30 min at −80°C, washed with 70% ethanol and resuspended in DEPC-treated water. The presence of virus was tested by RT-PCR amplification (reverse transcription at 55°C for 10 min, followed by 95°C for 5 min and amplification for 40 cycles at 95°C for 10 s, followed by 30 s of annealing/extension at 58°C) using the same primers as described below.

\subsection*{Quantitative real-time RT-PCR (QRT-PCR)}

QRT-PCR was performed in duplicate, using the SYBR Green detection system (Bio-Rad) in the \textit{iCycler} \textit{iQ} Thermal Cycler (Bio-Rad). The primers used produced a product of 141 bp and were: 5’-AACCTGTAGCAAT-CCTCTCAAG-3’ (forward) and 5’-TCTTGTCCGGTGATCTGG-TATGC-3’ (reverse). The results were quantified by comparison with a dilution series of \textit{in vitro}-transcribed RNA from a viral subclone, the QRT-PCR plasmid. This plasmid contains a 370 bp RT-PCR
Multiple sequence alignment was done with CLUSTAL_W (Jean-FISH server at Umeå University (http://babel.ucmp.umu.se/fish/). and was relatively A-rich (34 % A, 26 % U, 22 % G, 18 % C).

The sequenced RNA genome of Nora virus consisted of 11879 nt, but we could not exclude the possibility that the virus could be detected on a Southern blot, at a detection level of about 0.01 viral genomes per Drosophila genome (Fig. 1d).

The sequence contained four ORFs (Fig. 2). The second ORF from the 5’ end, ORF2, was the largest, encoding 2105 aa. It encoded a picornavirus-like H-P-Rep cassette, which included an RNA-dependent RNA polymerase, an RNA helicase and a protease. The polymerase domain was the most conserved and we could identify all eight consensus motifs typical of RNA-dependent RNA polymerases of ‘supergroup 1’ (Koonin & Dolja, 1993), which includes the picorna-like viruses. A pairwise comparison shows 27 %
sequence identity to the honeybee sacbrood virus (GenBank accession no. AF092924) over a stretch of 407 residues. The RNA helicase domain was more similar to that of vertebrate proteins (GenBank accession no. AF092924) over a stretch of 407 residues. The lower panel shows conserved domains in the four ORFs and it is therefore possible that ORF2 and ORF3 are translated after ribosomal frameshifting (Dreher & Miller, 2006). Between ORF3 and ORF4 there are 85 nt of non-coding RNA, which may act as an internal ribosome entry site for the translation of ORF4. However, neither this region nor the upstream regions of the other ORFs are obviously related to known internal ribosome entry sites and they have little potential to form stem–loop structures.

We used the sequences of the most conserved domains, the polymerase and the helicase, to investigate the possible phylogenetic relationship between Nora virus and other picorna-like viruses. Fig. 3 showed that Nora virus is not closely related to any of the major families of picorna-like viruses. Strikingly, the structural proteins of the latter did not seem to be conserved in Nora virus. Furthermore, its almost 12 kb positive-strand RNA genome is considerably larger than that of most picorna-like viruses, which typically range from 7 to 10 kb. This suggests that the virus belongs to a new, previously undescribed, family of viruses.

How the four ORFs are translated is an interesting question. There are short overlaps of 7 and 26 nt between the first three ORFs and it is therefore possible that ORF2 and ORF3 are translated after ribosomal frameshifting (Dreher & Miller, 2006). Between ORF3 and ORF4 there are 85 nt of non-coding RNA, which may act as an internal ribosome entry site for the translation of ORF4. However, neither this region nor the upstream regions of the other ORFs are obviously related to known internal ribosome entry site sequences and they have little potential to form stem–loop structures.

Surprisingly, persistent Nora virus infections do not give rise to any obvious pathology and even the high-titre stocks

**Ubiquitous presence of Nora virus in fly stocks**

Using Northern blot analysis, we were able to detect Nora virus in a few of our D. melanogaster laboratory stocks (data not shown). However, RT-PCR experiments suggested that low levels of the virus were in fact present in most or all of our laboratory stocks and in Schneider’s SL2 cells. Using QRT-PCR, we found that viral titres varied enormously, from less than $10^4$ to more than $10^{10}$ viral genomes per fly, as exemplified in Fig. 4(a). Most wild-type and mutant stocks had low titres, but high titres were found in a few mutants, including the P element excision mutant Relish$^{E20}$ (Hedengren et al., 1999). This was probably not caused by the mutation in the Relish gene, as similar titres were found in other P element excision stocks that were generated in the same study, including the precise excision line Relish$^{E23}$, which retains a wild-type Relish gene. The virus was not endemic to our laboratory, as we could also detect it in stocks from other laboratories, in some cases at high levels (results not shown), as well as in recently wild-caught animals (Dm1 and Dm2 in Fig. 4b). The wild-type Canton S stock usually had low levels of the virus, but in a few experiments flies with a high titre were found in this stock (Fig. 4c). We also detected the virus in D. simulans, a close relative of D. melanogaster, but not in the more distantly related species D. yakuba, D. erecta and D. virilis (Fig. 4b).

**DISCUSSION**

Here, we have reported the identification of a new virus that is persistently infecting many or possibly all stocks of D. melanogaster. Although Nora virus is clearly picorna-like, it differs radically from previously described viruses. It has four ORFs, only one of which showed significant similarity to known picorna-like viruses. Strikingly, the structural proteins of the latter did not seem to be conserved in Nora virus. Furthermore, its almost 12 kb positive-strand RNA genome is considerably larger than that of most picorna-like viruses, which typically range from 7 to 10 kb. This suggests that the virus belongs to a new, previously undescribed, family of viruses.
appeared healthy. We have no explanation for the large differences in viral titres among different stocks, but they did not appear to be related to the genotype of the fly. As the virus was first detected in material from bacterially infected flies, we tested whether it could be induced by bacterial infection or other types of stress, but the results were negative (data not shown). It is possible that we are dealing with more than one viral strain. The viral sequence shown here was derived from the Relish mutant stock, which has a high viral titre. The cDNA clones we isolated from Canton S flies differed only minimally from this sequence and may also correspond to high-titre virus. In general, our Canton S wild-type stock harbours low numbers of Nora virus, but occasionally we have also found increased levels in this stock. This is probably the reason why the virus was first detected in our differential display screen and why it was well represented in the two independent cDNA libraries. We also sequenced a 113 bp fragment of a low-titre virus and did not find any differences. It would be interesting to sequence the entire genome of a low-titre virus to investigate whether there is any difference, but this is a more demanding task.

An important question is in what form Nora virus remains dormant in the flies and how it is transmitted. It is obviously not integrated into the genome, as no DNA form was detected. There are now several examples of viruses that are able to cause silent, persistent infections, but the mechanisms involved are still poorly understood (Oldstone, 2006). In fact, it has recently become apparent that this phenomenon is both common and important. For instance, human picornaviruses, such as poliovirus and coxsackieviruses,

Fig. 3. Phylogenetic relationship between Nora virus and other picorna-like viruses. Maximum-parsimony trees were generated for the sequences of the predicted polymerase and helicase domains, using bootstrap analysis with 500 replications. The percentage bootstrap support is indicated for branches that are supported in at least 50% of the replications. The analysis included all arthropod viruses with significant sequence similarity to Nora virus in a BLAST search, as well as a selection of best-fitting plant and vertebrate viruses.
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REFERENCES


Fig. 4. Quantitative analysis of Nora virus RNA in different *Drosophila* stocks using QRT-PCR. (a) *D. melanogaster* from laboratory stocks. Canton S, Oregon R, Nairobi and Zalasanzto are wild-type stocks. e spz2 ca/TM1 is a recessive mutant in the Toll signalling pathway; here a mixture of heterozygous and homozygous flies was assayed. *Rel* E220 and *Rel* E238 are *Relish* deletion mutants, *Rel* E221 and *Rel* E226 are shorter deletions that do not affect the *Relish* gene and *Rel* E23 is a wild-type precise excision stock. (b) Different *Drosophila* species and wild-caught *D. melanogaster*, Dm1 and Dm2. ND, Not detectable. (c) An example of a case when Canton S flies from two independent vials showed increased viral levels.

are able to cause persistent infections and this can lead to late-onset pathological complications such as myocarditis and post-polio syndrome (Julien et al., 1999; Klingel et al., 1992; Pelletier et al., 1998). The presence of a viral reservoir in the population may also have serious epidemiological consequences. The adaptations of these viruses for persistence have mainly been studied in tissue culture systems (Calvez et al., 1993; Pelletier et al., 1998). Using Nora virus, it will now be possible to take advantage of *Drosophila* genetics to study the interactions in vivo between a persistent virus and its host.


