ALTERNATIVE mRNA SPLICE VARIANTS IN *DROSOPHILA* DL2 CELLS FOLLOWING FLOCK HOUSE VIRUS INFECTION

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Abstract

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There is a paucity of information regarding the responses of the insect immune system to viral infection. In other metazoan cells and tissues mRNA splicing variants are frequently associated with viral infection. Here we analyzed transcripts of the DNA repair gene 8-oxoguanine DNA glycosylase generated prior to, and post, infection of *Drosophila melanogaster* macrophage-like DL-2 cells by Flock House Virus (FHV). In mock-infected controls we observed that 2% of the transcripts were incompletely processed, maintaining some but not all introns. Eight hours post-FHV infection of the DL-2 cells, we observed a seven fold increase in the frequency of immature *Ogg1* transcripts. Moreover, there was a change in the introns retained in the transcripts observed compared with mock-infected controls, including completely unspliced transcripts. Surprisingly, the frequency of immature transcripts was reduced to control levels by 12 hours post-inoculation and remained relatively low up to 48 hrs. These results support the conclusion that viral infection may be accompanied by a host-mediated partial inhibition of mRNA splicing factors. This phenomenon has the potential to generate novel splice variants that are neither directly useful to the host nor the infecting virus but have the potential to degrade the transmission of genetic information. To our knowledge this the first report that viral infection may elicit general splicing instability in insects.

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Introduction

Multicellular host organisms have evolved under constant threat of infection from viruses, and consequently they employ numerous anti-infection defenses, such as inflammation, apoptosis, RNA interference, and innate and adaptive immune responses (Tschopp et al. 1998; Dostert et al. 2005; Zambon et al. 2006). At the same time viruses have evolved various strategies to evade host defenses and usurp the cellular machinery for their own reproductive requirements (Li et al. 2002). Understanding the interactions of viruses with their insect hosts is particularly important because insect viruses can cause

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severe problems, both for insects (de Miranda et al. 2008) and for humans, as for example in the case of tick-borne encephalitis virus or the mosquito-born West Nile virus (Brauchli et al. 2008; Orshan et al. 2008). Moreover, elucidation of the interactions between insect viruses and their hosts would help in increasing the efficacy of the many schemes currently underway to use viruses as agents of biological control against insects and for high-throughput production of proteins in insect hosts (Nicholson 2007; Possee et al. 2008).

In order to elucidate host-virus interactions in insects, an appropriate model system is *Drosophila* because it has been the subject of extensive genetic investigations and its gene makeup is largely known (Warren et al. 2006; Lai et al. 2007). *Drosophila* relies on a large repertoire of innate immune defense mechanisms against microbial infections (Lemaitre and Hoffman, 2007). However, with respect to viral infections, RNAi is the only known effector mechanism that has been identified in *Drosophila* (Wang et al. 2006). Microarray analysis of genes expressed in *Drosophila* infected with *Drosophila* C virus (DCV) indicated a transcriptional response to viral infection that differed from bacterial or fungal infection (Dostert et al. 2005). In particular, viral infection was accompanied by an upregulation of the JAK-STAT pathway, which also plays an important role in mammalian viral infections. Several prominent immune reactions and pathways are shared between *Drosophila* and mammals, such as phagocytosis and the Toll-mediated pathways (Lemaitre and Hoffman, 2007). These similarities point to a common evolutionary ancestry in immune reactions to microbial infection and suggest other common reactions. In mammals, viral infection may also be accompanied by changes in host post-transcriptional RNA processing and translation (Adair et al. 2006). Some mammalian DNA and RNA viruses, lacking introns, such as *Herpes simplex* virus, can completely shut down host mRNA splicing machinery without affecting viral gene expression (Muhlemann et al. 2000; Lindberg et al. 2002). On the other hand, vaccinia virus and adenovirus rely on the host splicing machinery to process their genes (Yue et al. 1999; Huang et al. 2002). Conversely, mammalian hosts have been shown to use alternative transcripts to combat viral infection (Dinesh-Kumar et al. 2000; Fridborg et al. 2004). It is not currently known whether these mammalian infection responses also occur in insects.

In this study we have investigated the changes in *Drosophila* post-transcriptional RNA processing following infection by Flock House Virus (FHV). FHV is a positive strand RNA virus belonging to the *Nodaviridae* family and its RNA replication and virion assembly take place on the outer mitochondrial membrane of the infected cells (Ball et al. 1992; Miller et al. 2001). It can infect in several orders of insects, including Coleoptera, Lepidoptera, Diptera, and Hemiptera, as well as a nematode, plants, and yeast (Dasgupta et al. 2007).

We have analyzed splice variant transcripts of the *Drosophila melanogaster* 8-oxoguanine DNA glycosylase (*Ogg1*) gene following infection of macrophage-like *Drosophila* line 2 cells (DL2) with FHV. *Ogg1* is a DNA repair gene that is neither directly involved in host antiviral defense nor evidently useful to an RNA virus. It was selected to enable us to determine whether splicing instability is targeted to loci associated with viral infection and defense or whether its effects are broader. In this communication we report changes in the frequency and types of partially spliced *Ogg1* transcripts during FHV infection. We suggest that some of the splice variants observed during infection may be the result of infection-associated reduction in splicing efficiency.

Methods and Materials

Cell Line and Viral Infection

DL2 cells were obtained from ATCC, and cultured in Schneider's *Drosophila* media (SDM) supplemented with 9% heat inactivated fetal bovine serum and 100 µg/mL kanamycin (henceforth referred to as media) (Schneider 1972). DL2 cells were grown at 22ºC without CO_2 and subcultured every 3 days. The cells were infected with Flock House Virus (FHV) (gift from Dr. David Miller) as follows: Subconfluent DL2 were gently dislodged and pelleted by centrifugation (300g). Cells were then resuspended in media and counted with a haemocytometer. Cell viability was determined with Trypan blue exclusion to ensure viability greater than 90%. Cell concentration was adjusted to 10^7 cells/ml with SDM and FHV added at a multiplicity of infection (MOI) of 10. Following inoculation cells were incubated at room temperature with occasional gentle mixing for 1 h. Cell density was then adjusted to 10^6 cells per ml with SDM, and incubated at 22ºC without CO_2 exchange. A mock infection was performed on a replicate culture. The mock infected cells were treated as described above except the inoculum did not contain FHV. The cell morphology in all cultures was periodically monitored by light microscopy up to 72 h post infection. Under our infection protocol conditions, 95% of DL2 cells are infected by 8 h post inoculation and maximum viral RNA replication takes place between 4 and 16 h post inoculation (Dr. David Miller, pers. comm.).

RNA Extraction and RT-PCR

Total RNA was extracted according to the manufacturer's instructions using QIAGEN Easy RNA extraction kit from $2x10^6$ cells at 8, 12, 20 and 44 h post infection from the infected cells and at 8 h from the mock-infected control. The RNA was then treated with DNAse for 30 min at 37 ºC followed by 1 h incubation at 80 ºC to degrade genomic DNA. *Ogg1* was amplified from RNA using QIAGEN one step RT-PCR according to the manufacturer's instructions. The primers used were forward 5'- CGGGATCCAT GAAGGCTGTTTTAC and a reverse 5'-TAGATAAGAT CACTTTTTAGG.

Splice Variant Characterization and Frequency Calculation

PCR products were cloned into pGEM-T easy vector (Promega) and individual *Ogg1* transcripts were characterized by PCR, *Sal*I restriction endonuclease analysis, and DNA sequencing as previously described (Skandalis et al. 2004). Splice variant frequency was calculated as the fraction of the transcripts characterized that exhibited an alternative splicing pattern and it was reported as a percentage.

Results

Cell Morphology and Cytopathic Effect

FHV inoculated and mock infected DL2 cells were periodically monitored by light microscopy for morphological changes. DL2 cells adhered loosely to tissue culture plates during growth as previously observed (Schneider 1972). Adherence to culture plates decreased with time following FHV inoculation and gross morphological changes

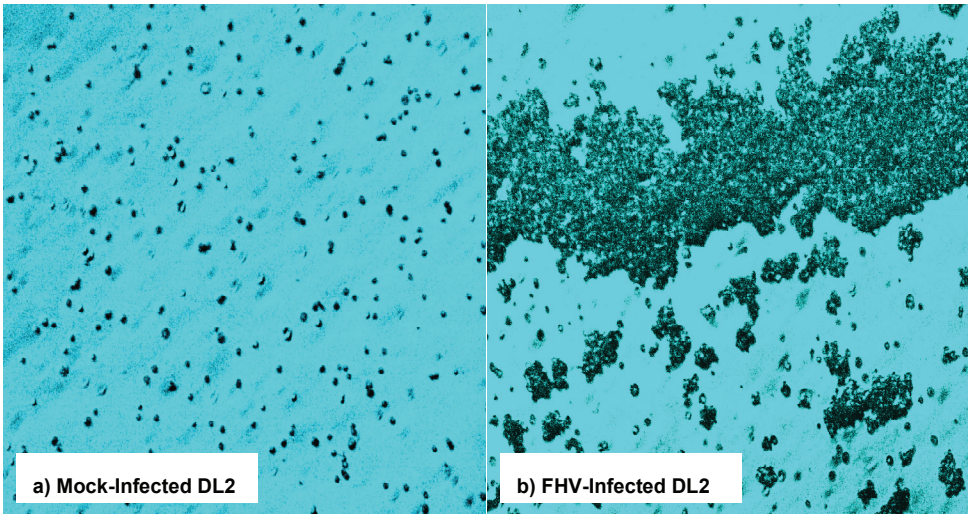

FIGURE 1. DL2 cell cultures 72h post infection (400x magnification). a) Mock-infected DL2 b) FHV-infected DL2.

were evident in the infected cells 72 h post inoculation (Fig. 1b), at which point the infected cells aggregated into a mass and became individually indistinct. By contrast the mock-infected control DL2 cell culture showed no differences in morphology or growth pattern compared to cells grown under normal conditions (Fig. 1a). The morphological changes of the infected cells were consistent with cytopathic cell death and no morphological changes associated with apoptosis were observed.

Cloning of *Ogg1*

The *Drosophila Ogg1* gene consists of 4 exons and 3 introns totaling 1476 nucleotides. The spliced *Ogg1* transcript is 1298 nucleotides in length. In our analysis of alternative *Ogg1* transcripts, the small size of the introns represented a potential problem since it was possible to amplify genomic *Ogg1* sequences directly from contaminating genomic DNA rather than from reverse transcribed mRNA. To avoid this problem, all RNA preparations were treated with DNAse to degrade any contaminating genomic DNA and then each RNA preparation was tested by PCR to confirm that it could not support *Ogg1* amplification without reverse transcribing mRNA (Fig. 2b).

Splice Variant Analysis

The effect of FHV infection on DL2 splicing was assessed at 8, 12, 20, and 44 hrs post FHV inoculation. Based on previous observations, approximately 95% of the cells are infected 8 h following inoculation with maximal viral RNA synthesis occurring approximately 20 h post inoculation. Amplified *Ogg1* transcript sequences were successfully obtained from each time point (Fig. 2a) and cloned into *E.coli* DH5α cells.

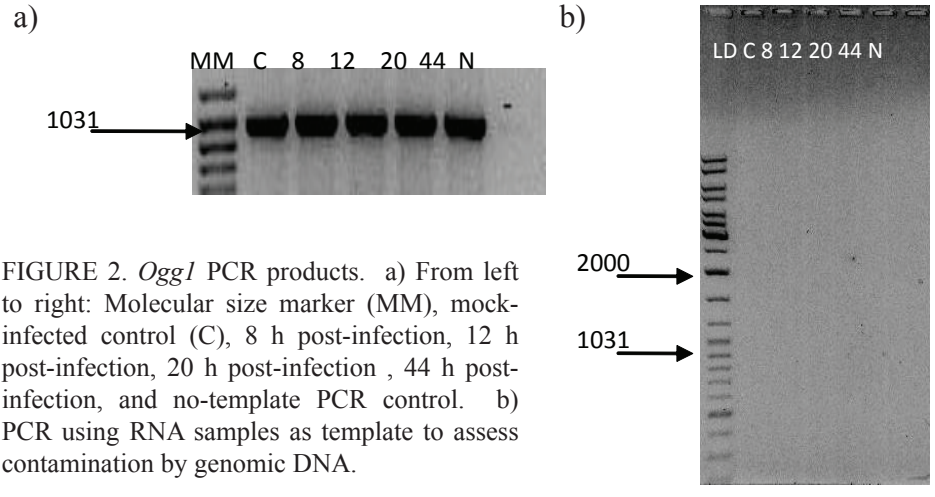

FIGURE 2. *Ogg1* PCR products. a) From left to right: Molecular size marker (MM), mock-infected control (C), 8 h post-infection, 12 h post-infection, 20 h post-infection , 44 h post-infection, and no-template PCR control. b) PCR using RNA samples as template to assess contamination by genomic DNA.

Individual cloned transcripts from each stage of infection were analyzed by restriction digest analysis and sequenced to determine the type and numbers of splice variants generated by *Ogg1*.

Overall, our analysis of *Ogg1* splice variants in *Drosophila* revealed five different transcripts (labeled SV1-5) in addition to the wild type (WT), all of which maintained some or all introns (Fig. 3). However, different combinations of splice variants were observed under different conditions. Characterization of 95 transcripts recovered from mock-infected DL2 revealed, in addition to WT, two types of splice variants: SV4, which retained introns 3 (frequency 1.1%) and SV2, which retained introns 2 and 3 (frequency 1.1%) (Fig. 4). Eight hours following FHV inoculation the frequency of splice variants increased to 15% (60 transcripts characterized). Of the three splice variants characterized, two were not detected in the control cells: SV1, which was completely un-spliced, retaining all introns (frequency 6.7%) and SV3 that retained introns one and two (frequency 3.3%). The third splice variant observed, SV4, was also present in the mock-infected control and at 8 hrs increased to 5%. Twelve hours post-infection the overall frequency of splice variants was less than at eight hours and similar to the frequency observed in uninfected cells (2.2 % of 95 transcripts characterized). The transcripts detected were SV4 and a novel variant, SV5, which retained intron 2. Twenty hours post-infection only one variant was detectable, SV5, with a frequency of 2.7% (N=74 transcripts). Finally, 44 hours post-infection the frequency of splice variants was elevated to 5.3% (N=95 transcripts) which was approximately double the frequency observed in the mock-infected cells but still 3-fold lower than the frequency observed 8 hours post-infection. The splice variants detected were SV1 (1.1%), SV2 (1.1%), SV3 (1.1%), and SV4 (2.1%) (Fig. 4).

Then frequencies of splice variants at 12 and 20 hrs were similar to the ones observed in mock-infected controls, but the types of variants differ (Fig. 4). Splicing of intron 2 exhibited a unique dependence on splicing of intron 1. In the hundreds of

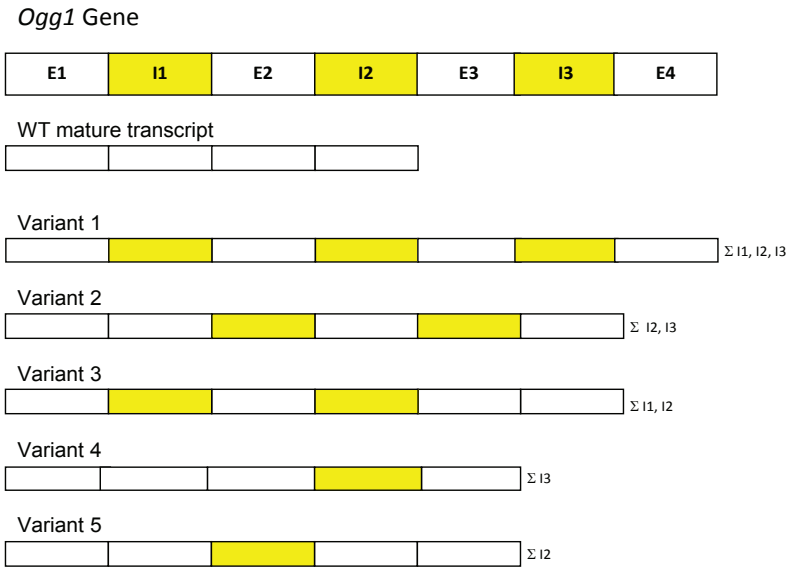

FIGURE 3. The structure of the *Drosophila Ogg1* gene and the types of splice variants detected in the infected and control DL2 cells. Not all variants were present in all conditions.

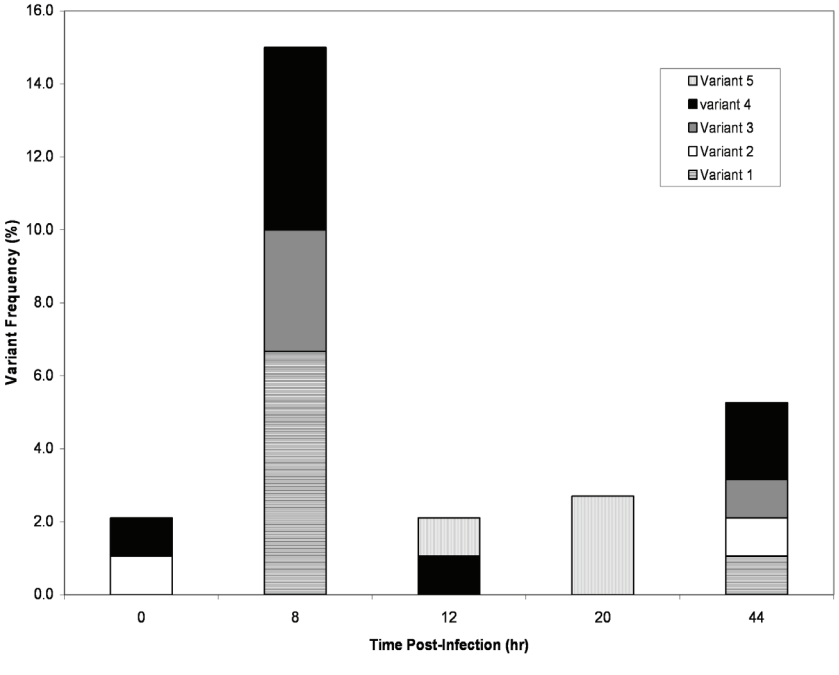

FIGURE 4. *Ogg1* splice variant frequencies pre and post infection in DL2 cultures. The mock infected control frequencies are indicated at time 0.

transcripts analyzed a variant maintaining intron 1 while intron 2 was spliced out was never observed. In other words splicing of intron 2 was predicated upon prior splicing of intron 1. By contrast variants were identified with intron 3 spliced out while introns 1 and 2 were retained indicating that prior splicing of intron 1 is not a prerequisite for intron 3 removal.

Discussion

Some mammalian DNA and RNA viruses, lacking introns, such as *Herpes simplex* virus can completely shut down host mRNA splicing machinery without affecting viral gene expression (Muhlemann et al. 2000; Lindberg et al. 2002). On the other hand, Vaccinia virus and Adenovirus rely on the host splicing machinery to process their genes (Yue et al. 1999; Huang et al. 2002). Conversely, mammalian hosts have shown to use alternative transcripts to combat viral infection (Dinesh-Kumar et al. 2000; Fridborg et al. 2004).

Here we analyzed splice variant transcripts of the *Drosophila* gene *Ogg1* following FHV infection of macrophage-like *Drosophila* DL2 cells. FHV does not itself undergo splicing, thus eliminating the possibility that the splicing machinery was manipulated for viral infection. *Drosophila* DL-2 cell lines were utilized since they exhibit macrophage-like genotypes, with receptors capable of detecting viral RNA and initiating native immune response (Shields et al. 1975; Rehli et al. 2003).

All *Ogg1* splice variants identified in this study maintained some or all introns. Since transcripts with un-excised introns were observed in the FHV infected as well as in the mock-infected control cells it is unlikely that the transcripts represented infection-related disregulation of splicing. These observations indicated that the transcripts detected in *Drosophila* cells were not genuine alternative transcripts but rather immature transcripts awaiting further splicing. Even though splicing is normally co-transcriptional, splicing can occur post-transcriptionally (Aguilera 2005). In the control, non-infected cells, the intron retention pattern of the splice variants (retention of intron 3 or introns 2 and 3) was consistent with sequential removal of introns. However, in the FHV infected cells intron removal was not sequential, with some transcripts retaining intron 2 or, introns 1 and 2. Unspliced transcripts were also detected indicating that following infection splicing is not co-transcriptional.

Overall, splicing was adversely affected by FHV infection in *Drosophila* DL2 cells. Following FHV inoculation, we observed an accumulation of immature *Ogg1* transcripts suggesting a retardation of host gene splicing. This observation and the shift in the types of transcripts observed during infection implicate changes in the regulation or localization of some splicing factors as the likely mechanism responsible. These changes were likely host-directed since the simple FHV lacks the molecular machinery to implement changes in splicing.

The results support the conclusion that viral infection may be accompanied by host-produced splicing instability, which has the potential to generate novel splice variants not associated with the generation of useful splice variants for either the host or the infecting virus. Nonetheless, this instability has the potential of degrading the

transmission of genetic information of both the host and the virus. In mammalian systems, it appears that splicing of host genes disrupts viral infection. *Herpes simplex* virus, vaccinia virus and adenovirus decrease splicing of cellular pre-mRNAs by inducing hypo-phosphorylation of the human ASF/SF2 protein, a member of the evolutionary conserved SR family of splicing factors (Kanopka et al. 1996; Huang et al. 2002; Sciabica et al. 2003). Differential expression of several host splicing factors was also observed following cytomegalovirus infection (Adair et al. 2004). Rous sarcoma virus (RSV) contains a negative regulator of viral splicing, and is has been hypothesized that the suppression of splicing benefits the virus by increasing the pool of unspliced viral RNA that will be packaged as progeny genome (Maciolek and McNally 2007).

To our knowledge this is the first report that infection may be accompanied by a general splicing instability in *Drosophila*. Given the similarities in immune responses between mammals and *Drosophila*, reviewed in (Muller et al. 2008) it may have far reaching implications about metazoan defenses against infection. It would be a great interest for infection treatment to determine in future experiments whether this phenomenon constitutes an adaptive defensive strategy by the host or simply a by-product of other anti-viral responses.

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