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Broad RNA Interference–Mediated Antiviral Immunity and Virus-Specific Inducible Responses in *Drosophila*

Cordula Kemp,*[,1] Stefanie Mueller,*[,1,2] Akira Goto,* Vincent Barbier,* Simona Paro,* François Bonnay,* Catherine Dostert,* Laurent Troxler,* Charles Hetru,* Carine Meignin,* Sébastien Pfeffer,[†] Jules A. Hoffmann,* and Jean-Luc Imler*[,‡]

The fruit fly *Drosophila melanogaster* is a good model to unravel the molecular mechanisms of innate immunity and has led to some important discoveries about the sensing and signaling of microbial infections. The response of *Drosophila* to virus infections remains poorly characterized and appears to involve two facets. On the one hand, RNA interference involves the recognition and processing of dsRNA into small interfering RNAs by the host RNase Dicer-2 (Dcr-2), whereas, on the other hand, an inducible response controlled by the evolutionarily conserved JAK-STAT pathway contributes to the antiviral host defense. To clarify the contribution of the small interfering RNA and JAK-STAT pathways to the control of viral infections, we have compared the resistance of flies wild-type and mutant for Dcr-2 or the JAK kinase Hopscotch to infections by seven RNA or DNA viruses belonging to different families. Our results reveal a unique susceptibility of *hop* mutant flies to infection by *Drosophila* C virus and cricket paralysis virus, two members of the *Dicistroviridae* family, which contrasts with the susceptibility of *Dcr-2* mutant flies to many viruses, including the DNA virus invertebrate iridescent virus 6. Genome-wide microarray analysis confirmed that different sets of genes were induced following infection by *Drosophila* C virus or by two unrelated RNA viruses, Flock House virus and Sindbis virus. Overall, our data reveal that RNA interference is an efficient antiviral mechanism, operating against a large range of viruses, including a DNA virus. By contrast, the antiviral contribution of the JAK-STAT pathway appears to be virus specific. ***The Journal of Immunology*****, 2013, 190: 650–658.**

Viruses represent an important class of pathogens, causing serious concern for human health, as well as important economic losses in crops and animals. Because they replicate inside cells, and rely for the most part on host cell molecular machineries for their replication, viruses pose specific challenges to the immune system. Two major strategies of antiviral resistance have been described. In mammals, viral infection is first detected by pattern recognition receptors of the Toll- and RIG-I–like families that sense the viral nucleic acid and trigger the induction of IFNs and other cytokines (1). These factors activate the production of antiviral molecules, such as protein kinase R or oligo-2′, 5′-adenylate synthetase, that contain the infection and contribute to the activation of the adaptive immune response (2). In plants, viral nucleic acids are recognized by enzymes of the Dicer family, which produce small interfering RNAs (siRNAs) of 21–24 nucleotides. These siRNAs are then loaded onto molecules of the Argonaute (AGO) family and will guide them toward RNAs with complementary sequences; targeted RNAs are then either sliced by AGO, or their translation is inhibited. This RNA interference (RNAi) mechanism provides efficient and sequence-specific protection against viral infections (3).

RNAi also plays an important role in the control of viral infections in insects, as shown by the production of virus-derived siRNAs in infected flies, and the increased susceptibility to viral infection of *Drosophila* mutants for the genes *Dcr-2* and *AGO2* (3–6). In addition, several reports indicate that an inducible response also contributes to the control of viral infections (7–15). We previously showed that infection with *Drosophila* C virus (DCV), a member of the *Dicistroviridae* family, leads to induction of some 130 genes (11). Analysis of the regulation of one of these genes, *vir-1*, revealed the presence of functionally important binding sites for the transcription factor STAT in its promoter. The induction of *vir-1*, as well as several other DCV-induced genes, was found to be dependent on the gene *hopscotch* (*hop*), which encodes the only JAK kinase in *Drosophila*. Furthermore, *hop* mutant flies succumb more rapidly than do wild-type controls, with a higher viral load, to DCV infection (11). The Toll and immune deficiency (Imd) pathways, initially characterized for their role in the control of bacterial and fungal infections, were

*CNRS-UPR9022, Institut de Biologie Moléculaire et Cellulaire, 67084 Strasbourg Cedex, France; [†]CNRS-UPR9002, Institut de Biologie Moléculaire et Cellulaire; 67084 Strasbourg Cedex, France; and [‡]Faculté des Sciences de la Vie, Université de Strasbourg; 67083 Strasbourg Cedex, France

[1]C.K. and S.M. contributed equally to this work.

[2]Current address: Bernhard-Nocht-Institut for Tropical Medicine, Molecular Entomology, Hamburg, Germany

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The sequences presented in this article have been submitted to the Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/) under accession number GSE31542 and to the National Center for Biotechnology Information Small Read Archive (http://www.ncbi.nlm.nih.gov/sra) under accession number GSE41007.

Address correspondence and reprint requests to Prof. Jean-Luc Imler, IBMC-CNRS/Université de Strasbourg, Rue Rene Descartes, Strasbourg, Alsace 67084, France. E-mail address: JL.Imler@unistra.fr

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Abbreviations used in this article: AGO, Argonaute; CrPV, cricket paralysis virus; Dcr-2, Dicer-2; DCV, *Drosophila* C virus; dpi, day postinfection; DXV, *Drosophila* X virus; FHV, Flock House virus; IIV-6, invertebrate iridescent virus type 6; Imd, immune deficiency; MEKK1, MEK kinase 1; RNAi, RNA interference; SINV, Sindbis virus; siRNA, small interfering RNA; *TotM*, *Turandot M*; Upd, unpaired; VSV, vesicular stomatitis virus.

also thought to play a part in the control of viral infections. Whereas the Toll pathway was associated with resistance to the *Drosophila* X virus (DXV) (15), the Imd pathway was implicated in the control of Sindbis virus (SINV) (7) and cricket paralysis virus (CrPV) (9).

Altogether, the data in the present literature point to the involvement of both RNAi and an inducible expression of effector molecules to counter viral infections in insects (5, 16). However, whereas RNAi was shown to contribute to resistance to several RNA viruses (with either single-stranded genomes of both polarities or double-stranded genomes), most studies on the inducible response have so far focused on a single virus. As a result, the global significance of the inducible response for the control of viral infections remains poorly understood. In particular, it is unclear at present if the JAK-STAT pathway is involved in a general antiviral response, providing broad antiviral immunity, or if it acts specifically on a critical step in the replication cycle of a specific virus or virus family. To address this important question, we have compared the resistance of a mutant for the JAK-STAT pathway to infection by seven RNA or DNA viruses. We find that *hop* mutant flies are more susceptible than wild-type controls to infections by the *Dicistroviridae* DCV and CrPV, but exhibit either no or a weak phenotype for other viruses, suggesting that the JAK-STAT pathway–dependent inducible response is virus specific. Genome-wide transcript profiling shows that infection by two other RNA viruses, Flock House virus (FHV; *Nodaviridae*) and SINV (*Alphaviridae*), leads to upregulation of ≥400 genes, which only partially overlap with those induced by DCV. Overall, our data indicate that the siRNA pathway exerts broad antiviral activity and affects both RNA and DNA viruses, with virus-specific inducible responses contributing to the control of viral infections in *Drosophila*.

Materials and Methods

Fly strain culture and infection

Oregon-R (*OR*) and *yw* were used as wild-type control flies. The $hop^{M38/msv1}$, $Dcr\text{-}2^{L811fsX}$, and $Dcr\text{-}2^{R416X}$ mutant flies were previously described (17–19). A genomic rescue of the *Dcr-2* gene was established with the Fosmid FlyFos017074 (transgeneome.mpi-cbg.de) inserted at the landing site attP40 (2L), and the transgenic chromosome was recombined with the deficiency Df(2R)BSC45, which uncovers the *Dcr-2* locus. For the rescue experiments, *Dcr-2* mutants were crossed with the deficiency Df(2R) BSC45 or the Df(2R)BSC45–*Dcr-2* rescue line. Flies were fed on standard cornmeal–agar medium at 25˚C. All fly lines were tested for *Wolbachia* infection and cured whenever necessary. Viral stocks were prepared in 10 mM Tris-HCl, pH 7.5, with the exception of vesicular stomatitis virus (VSV), which was used directly from Vero cell culture supernatant [VSV 4×10^9 PFU/ml; DCV 5×10^{10} PFU/ml; CrPV 1×10^9 PFU/ml; FHV 5.5×10^9 PFU/ml; DXV 4.4×10^7 PFU/ml, invertebrate iridescent virus type 6 (IIV-6) 4.4×10^{11} PFU/ml; and SINV 5×10^8 PFU/ml]. Infections were performed with 4- to 6-d-old adult flies by intrathoracic injection (Nanoject II apparatus; Drummond Scientific) with viral particles, indicated in the figure legends. Injection of the same volume (4.6 nL) of 10 mM Tris-HCl, pH 7.5, was used as a control. For bacterial infection, flies were pricked with a thin needle previously dipped in a concentrated overnight culture of *Escherichia coli* and *Micrococcus luteus* in Luria–Bertani medium. Infected flies were then incubated at room temperature, or at 29˚C in the case of $hop^{M38/msv1}$ and the corresponding control flies, and monitored daily for survival, or frozen for RNA isolation and virus titration at the indicated time points.

Cell culture and virus titration

Vero R cells were grown in DMEM (Invitrogen) supplemented with 10% FCS (Biowest), penicillin/streptomycin (Invitrogen), nonessential amino acid mix (Invitrogen), 10 mM pyruvate (Life Technologies), and 200 mM L-glutamine (Invitrogen). Kc167 and S2 cells were grown in Schneider's medium (Biowest) supplemented with 10% FCS, GlutaMAX (Invitrogen), and penicillin/streptomycin (100× mix, 10 mg/ml/10,000 U; Invitrogen). VSV and SINV were titrated from infected flies by plaque assay on Vero R cells. DCV, CrPV, FHV, and IIV-6 were titrated on Kc167 (DCV, CrPV, and FHV) or S2 (IIV-6) cells by the Reed–Muench method to calculate 50% tissue culture–infective dose and converted to PFU with a conversion factor of 0.7.

RNA analysis

Total RNA from infected flies was isolated using TRI Reagent RT bromoanisole solution (MRC), according to the manufacturer's instructions. Total RNA, 1 μg, was reverse transcribed using iScript cDNA Synthesis Kit (Bio-Rad). The reverse transcription was run in the T3000 Thermocycler (Biometra), with the following PCR program: step 1: 65˚C for 5 min, step 2: 4˚C for 5 min, step 3: 25˚C for 10 min, step 4: 42˚C for 60 min, and step 5: 70˚C for 15 min. A total of 100 ng cDNA was used for quantitative real-time PCR, using the iQ Custom SYBR Green Supermix Kit (Bio-Rad). The PCR was performed using the CFX384 Real-Time System (Bio-Rad) with the following program: step 1: 95˚C for 3 min, step 2: 95˚C for 10 s, step 3: 55˚C for 30 s, repeated 39 times from step 2. Primers used for qPCR were as follows: *RpL32* (forward 5′-GACGCTTCAAGGGACAGTATCTG-3′; reverse 5′-AAACGCGGTTCTGCATGAG-3′), *vir-1* (forward 5′-GATCCCAATTTTCCCATCAA-3′; reverse 5′-GATTACAGCTGGGTGCACAA-3′), *drosomycin* (forward 5′-CGTGAGAACCTTTTCCAATATGATG-3′; reverse 5′-TCCCAGGACCACCAGCAT-3′), and *diptericin* (forward 5′-GCTGCGCAATCGCTTCTACT-3′; reverse 5′-TGGTGGAGTGGGCTTCATG-3′). *Turandot M* (*TotM*), *upd*, *upd2*, and *upd3* expression levels were quantified using the Brilliant II QRT-PCR Core Reagent Kit, 1-step (Stratagene). The reaction took place in a total volume of 20 μl, using the Taqman Gene Expression Assay [*TotM* (Dm02362087 s1), *upd* (*os*) (Dm01843792_g1), *upd2* (Dm01844134 g1), *upd3* (custom-designed upd3exon2-ANY), and RpL32 (Dm02151827 g1), all from Applied Biosystems]. We used the 7500 Fast Real-Time PCR System (Applied Biosystems) with following PCR program: step 1: 45˚C for 30 min, step 2: 95˚C for 10 min, step 3: 95˚C for 15 s, step 4: 60˚C for 1 min, repeated 39 times from step 3. In all cases, gene expression was normalized to the ribosomal protein gene *RpL32*.

For IIV-6, the expression of the annotated genes *206R*, *224L*, *244L*, and *261R* was assessed by strand-specific RT-PCR. We used SuperScript III Reverse Transcriptase specifically adapted for gene-specific priming and followed the manufacturer's protocol (Invitrogen). Briefly, primer pairs were designed to amplify regions of the IIV-6 genome exhibiting or not exhibiting a high density of small RNA reads. Total RNA,1 μg, extracted from infected S2 cells was reverse transcribed with 2 pmol of either forward (F) or reverse (R) primer and 200 U of SuperScript III Reverse Transcriptase. The reaction was then incubated for 1 h at 55˚C. Then 1 μl of the resulting cDNA was used to perform 25 cycles of PCR, using Taq DNA polymerase (Invitrogen) and both F and R primers. The primer pairs were as follows: 206R (forward: 5′-AAGGAAAGTGGCGAGTACGA-3′, reverse 5′-AACAAACCCGTTTTCTTCCA-3′); 224L (forward: 5′-CCACCATCACATTGACCTTG-3′, reverse: 5′-ATAAGCGAACCCGAAATCA-3′); 244L (forward: 5′-TGGAAAAGAGTGGTCCCATTT-3′, reverse: 5′-TGTACCTCCCGGAAGATTT-3′); 261R (forward: 5′-CAGCCCCATCCGAATTACTA-3′, reverse: 5′-CTGCAACTGCAGAAATTTGA-3′). The PCR bands were sequenced to verify their viral origin.

Statistical analysis

An unpaired two-tailed Student *t* test was used for statistical analysis of data with GraphPad Prism (GraphPad Software). The p values < 0.05 were considered statistically significant. Survival curves were plotted and analyzed by log-rank analysis (Kaplan–Meier method) using GraphPad Prism (GraphPad Software).

DNA microarray analysis

For each sample, Tris-injected, DCV-infected (11), and FHV- and SINV-infected, three biologically independent samples comprising 45 male Oregon-R flies were used. RNA extraction, biotinylation, and hybridization to Affymetrix *Drosophila* GeneChip microarrays (Affymetrix) were performed as described (20). The Affymetrix Microarray Suite 5.0 (Affymetrix) or Excel (Microsoft) with a combination of built-in functions and custom formulae was used for data analysis. Raw data were sorted with the "absent-marginal-present flags" generated by the Microarray Suite functions. Although an absent flag might indicate that no mRNA of a particular type was present in a sample, marginal flags and absent flags may indicate problems with the hybridization; therefore, only data points marked as present in at least one replicate were retained. The remaining data mass for each microarray was then normalized to itself, making 1 the median of all the measurements. A gene was considered induced if present in at least one replicate, with a virus/Tris ratio higher than 2 for at least one of the time points. Classification of gene functions was analyzed by David

Bioinformatics Resources 6.7 (21). The data set for FHV and SINV was submitted to the Gene Expression Omnibus database (http://www.ncbi.nlm.nih.gov/geo/) with the accession number GSE31542.

Assembly, sequencing, and analysis of small RNA libraries

The small RNA library of S2 cells and whole flies was constructed as described (22) and sequenced by the Illumina 2G Analyzer. Reads were then aligned to a reference consisting of the IIV-6 genome from the National Center for Biotechnology Information (accession code NC_003038) using the Bowtie program with standard parameters in genome assembly. Reads aligning to the IIV-6 genome with a maximum of one mismatch were retained and analyzed using in-house Perl scripts and Excel. Sequences were submitted to the National Center for Biotechnology Information Small Read Archive (http://www.ncbi.nlm.nih.gov/Traces/sra/sra.cgi?) under the accession number GSE41007.

Results

RNAi provides broad antiviral protection in Drosophila

Several independent studies, including our own, have established that RNAi, and more precisely the siRNA pathway, serves as an efficient host defense against RNA viruses. These include viruses with a single-stranded genome of both (+) and (−) polarity and dsRNA viruses (23–30), and we confirmed that flies mutant for *Dcr-2* died more rapidly than wild-type controls when they were infected with DCV, CrPV, FHV, SINV, VSV (*Rhabdoviridae*), and DXV (*Birnaviridae*) (data not shown). Next, we addressed the question whether the siRNA pathway also participated in the control of a DNA virus infection, and infected wild-type and RNAi mutant flies with IIV-6 (*Iridoviridae*). Infection of *Dcr-2* mutant flies led to a more rapid and intense appearance of blue color, which is characteristic of the accumulation of iridescent viral particles, than in wild-type controls (Fig. 1A). *Dcr-2*$^{-/-}$ flies were significantly more susceptible to IIV-6 infection than were the corresponding wild-type (Fig. 1B). A fraction of *Dcr-2*$^{-/-}$ flies injected with buffer also died in the course of the experiment, confirming the increased sensitivity to stress associated with mutations of the siRNA pathway (31). The decreased survival time correlated with a 20-fold increased viral load in *Dcr-2* mutant flies at 10 d postinfection (dpi) (Fig. 1C). Similar results were obtained when a different null allele of *Dcr-2* was used, and the IIV-6 susceptibility phenotype was rescued by a wild-type genomic *Dcr-2* transgene (Fig. 1D). The *r2d2*$^{-/-}$ and *AGO2*$^{-/-}$ null mutant flies also exhibited increased sensitivity to IIV-6 (Fig. 1E). *AGO2*$^{-/-}$ flies contained more viral DNA than did wild-type controls, confirming that this gene participates in the control of infection (Fig. 1F).

We next sequenced small RNA libraries prepared from IIV-6–infected S2 cells or adult flies. We observed several hundreds of thousands of reads matching the IIV-6 genome in both infected S2 cells and wild-type flies, but not in control noninfected S2 cells (Supplemental Table I). The large majority of these reads had a size of 21 nucleotides, which is characteristic for processing by the RNase Dicer-2 (Dcr-2). This peak was absent from the library prepared from infected *Dcr-2*$^{-/-}$ mutant flies (Fig. 2A). These data indicate that Dcr-2 generates 21-nucleotide IIV-6–derived siRNAs in infected flies, and raise the question of the nature of the substrate used by Dcr-2 in the context of this infection. As previously reported for RNA viruses, the number of reads matching

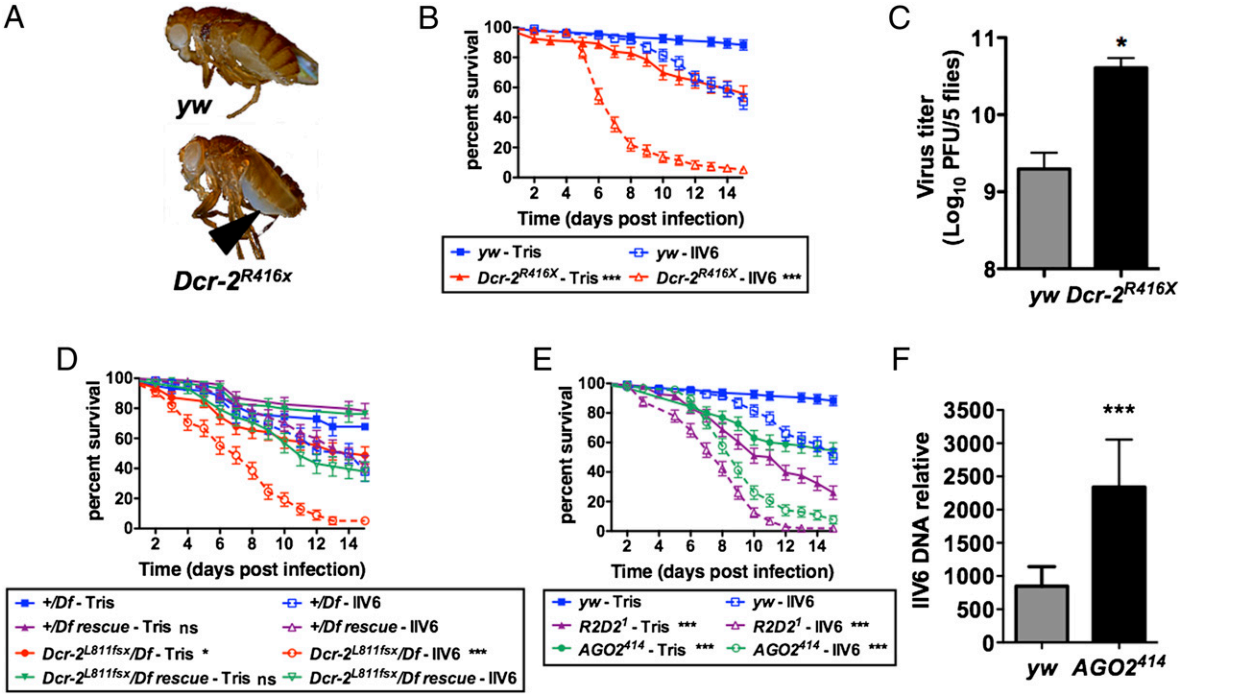

FIGURE 1. Dcr-2 is involved in host defense against the DNA virus IIV-6. (**A**) Upon injection of IIV-6 (5000 PFU) in wild-type (*yw*) and *Dcr-2*^{R416X} mutant flies, typical blue paracrystalline structures appeared earlier in the abdomen (arrowhead) of the mutant flies. Representative individuals 10 dpi are shown. (**B**) Groups of 20 wild-type (*yw*) or *Dcr-2*^{R416X} mutant flies were injected with IIV-6 or Tris, and survival was monitored daily. The difference between the wild-type and *Dcr-2* mutant flies is statistically significant. (**C**) Viral titer in groups of five wild-type (*yw*) or *Dcr-2*^{R416X} mutant flies was monitored 10 dpi. (**D**) Rescue of the hemizygous *Dcr-2*^{L811fsX} for the IIV-6 susceptibility phenotype by a transposon expressing a wild-type *Dcr-2* transgene. *Dcr-2*^{L811fsX} hemizygous flies (*Dcr-2*^{L811fsX}/Df) are significantly more susceptible than *Dcr-2*^{L811fsX} hemizygous flies complemented by a wild-type *Dcr-2* transgene (*Dcr-2*^{L811fsX}/Df rescue). Df is Df(2R)BSC45, a deficiency that fully uncovers the *Dcr-2* locus. All control and genomic rescued flies are in *CantonS* background. (**E**) Survival rate of wild-type (*yw*), *R2D2*^{1}, and *AGO2*^{414} mutant flies upon IIV-6 or Tris injection. (**F**) IIV-6 DNA load was determined by quantitative PCR in four groups of six flies of the indicated genotype at 10 dpi. For all panels, the data represent the mean and SD of at least three independent experiments, and the difference between controls and mutant flies is statistically significant. *$p < 0.05$, ***$p < 0.001$. All experiments are performed at 22°C (A, C, F) or 25°C (B, D, E).

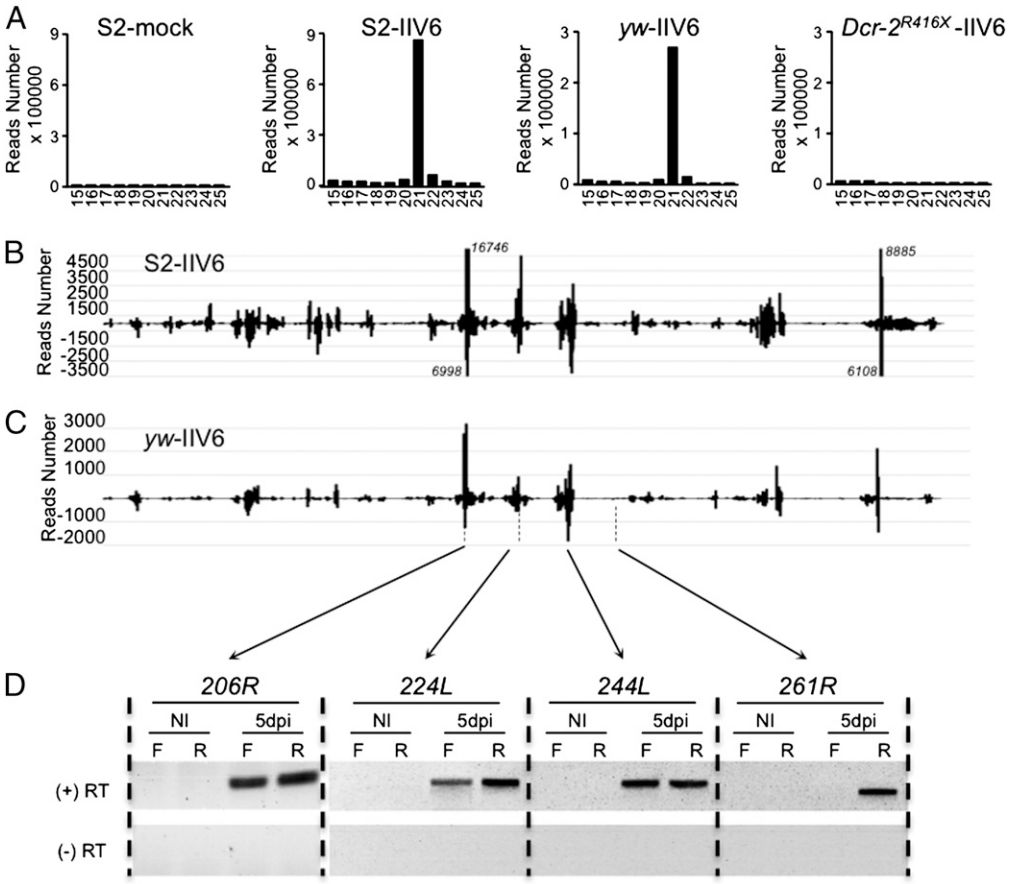

FIGURE 2. Virus-derived siRNAs in S2 cells and *Drosophila* adult flies infected by the DNA virus IIV-6. RNA was extracted 5 dpi from S2 cells infected by IIV-6 (MOI 0.01) and adult wild-type (*yw*) or mutant ($Dcr\text{-}2^{R416X}$) flies injected with IIV-6 (5000 PFU per fly). (**A**) Size distribution of the small RNAs matching the viral genome in S2 cells and adult flies of the indicated genotype. (**B** and **C**) Distribution of the 21-nucleotide siRNAs from the S2 cell (B) and *yw* adult fly (C) libraries along the IIV-6 genome. Each IIV-6–derived small RNA is represented by the position of its first nucleotide. The IIV-6–derived small RNAs matching the upper and lower strand of the DNA genome are respectively shown above (positive reads number) and below (negative reads number) the horizontal axis, which represents the 212482bp genome. In (B), the number of reads for four peaks going off-scale is indicated next to them, in italics. (**D**) Strand-specific RT-PCR with primers corresponding to the annotated viral genes *206R*, *224L*, *244L,* and *261R*. The experiment was performed in the presence (+) or absence (−) of RT. NI, Noninfected; F and R, forward and reverse strand primer used for reverse transcription.

each strand of the viral genome was very similar (Supplemental Table I). However, unlike RNA viruses, the virus-derived siRNAs were not uniformly distributed along the viral genome. Rather, several hotspots were observed, revealing that specific regions of the viral genome generate the siRNAs (Fig. 2B, 2C). These peaks do not correlate with the intensity of transcription of the viral genome, and some highly transcribed regions are located in areas not generating significant levels of siRNAs (32). The strong symmetry of the peaks observed in S2 cells and wild-type flies suggests that these regions are transcribed on both strands and generate dsRNA. Indeed, we could detect bidirectional transcription in the areas of the viral genome covered by the peaks (Fig. 2D). By contrast, transcription of only one strand of the DNA genome was detected for the locus 261R, which is located in a region that does not produce significant amounts of siRNAs. Overall, these results indicate that the siRNA pathway in *Drosophila* can also protect against a DNA virus infection.

The JAK kinase Hopscotch does not confer broad antiviral immunity

To test the contribution of the JAK-STAT pathway in antiviral immunity in *Drosophila*, we injected loss-of-function mutants of the JAK kinase Hopscotch ($hop^{M38/msv1}$) with different ssRNA, dsRNA, and DNA viruses. As previously described, $hop^{M38/msv1}$ mutant flies die more rapidly than do wild-type controls following DCV infection, and contain ∼10-fold more virus (Fig. 3A). By contrast, we did not observe significant differences in survival between wild-type and $hop^{M38/msv1}$ mutant flies upon infection with the alphavirus SINV (Fig. 4A), and the viral titers 2 dpi were not significantly different in wild-type and $hop^{M38/msv1}$ mutant flies (data not shown), indicating that the JAK-STAT pathway does not contribute to resistance to this virus. The $hop^{M38/msv1}$ mutant flies, as well as wild-type flies, also resisted infections by the rhabdovirus VSV and by the nodavirus FHV (Fig. 4B, 4C). A slight reduction in survival was observed in the case of the dsRNA virus DXV (*Birnaviridae*) and the DNA virus IIV-6 (Fig. 4D, 4E). However, the difference between wild-type and $hop^{M38/msv1}$ mutant flies was only statistically significant in the case of DXV infection. Furthermore, we did not observe statistically significant differences in the DXV and IIV-6 viral titers in wild-type and $hop^{M38/msv1}$ mutant flies in the format of our assays (data not shown).

Overall, our data indicate that the JAK-STAT pathway is critical for host defense against DCV, but plays a minor role for DXV and IIV-6 and is essentially dispensable in the case of FHV, SINV, and VSV. We therefore tested CrPV, another member of the *Dicistroviridae* family known to infect *Drosophila*. We observed a decrease in survival and a significant increase in viral titers in CrPV-infected $hop^{M38/msv1}$ mutant flies compared with wild-type flies (Fig. 3B). In conclusion,

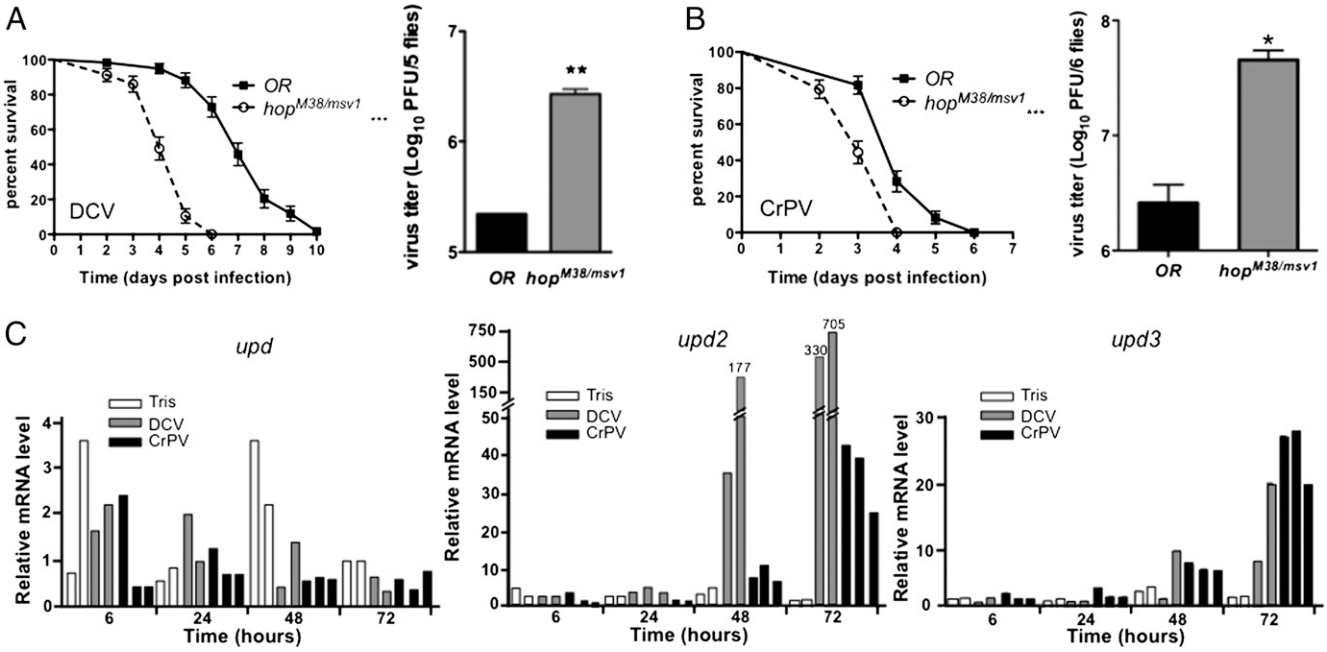

FIGURE 3. The JAK kinase Hopscotch is involved in host defense against DCV and CrPV. (**A** and **B**) Groups of 20 wild-type (OR) or *hopscotch* ($hop^{M38/msv1}$) mutant flies were injected with DCV (500 PFU) (A) or CrPV (5 PFU) (B), and survival was monitored daily. The experiment was repeated three times, and data represent the mean and SD. In the *right panels*, viral titer was determined in groups of five flies 2 dpi for DCV (A) and 1 dpi for CrPV (B). The data represent the mean and SD of three independent experiments, and the difference between wild-type and *hop* mutant flies is statistically significant. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$. (**C**) DCV and CrPV infection triggers induction of the genes *upd2* and *upd3*, which encode cytokines activating the JAK/STAT pathway. Flies were infected with DCV or CrPV, and expression of *upd*, *upd2*, and *upd3* was monitored in groups of 10 flies at the indicated time points by Taqman quantitative PCR. The results of at least two independent experiments are shown.

our data indicate that the JAK-STAT pathway in *Drosophila* confers protection against some viruses—in particular, the *Dicistroviridae*—but does not provide broad antiviral immunity.

Inducible gene expression in FHV- and SINV-infected flies

The above results raised the question of whether an inducible response contributes to host defense against viruses other than DCV and CrPV. We therefore conducted a genome-wide microarray analysis using Affymetrix DNA microarrays to monitor gene expression in flies infected by FHV (2 and 3 dpi) or SINV (4 and 8 dpi), and compared the data with those obtained for DCV infection (1 and 2 dpi). The time points for this analysis were chosen to take into account the different kinetics of replication and colonization of *Drosophila* by the different viruses (11, 24). For each virus, we observed a large overlap between the genes induced at the first and second time points. We then pursued our analysis, focusing on the genes induced either at the first or at the second time point. The microarray data revealed that 487 and 201 genes were induced or

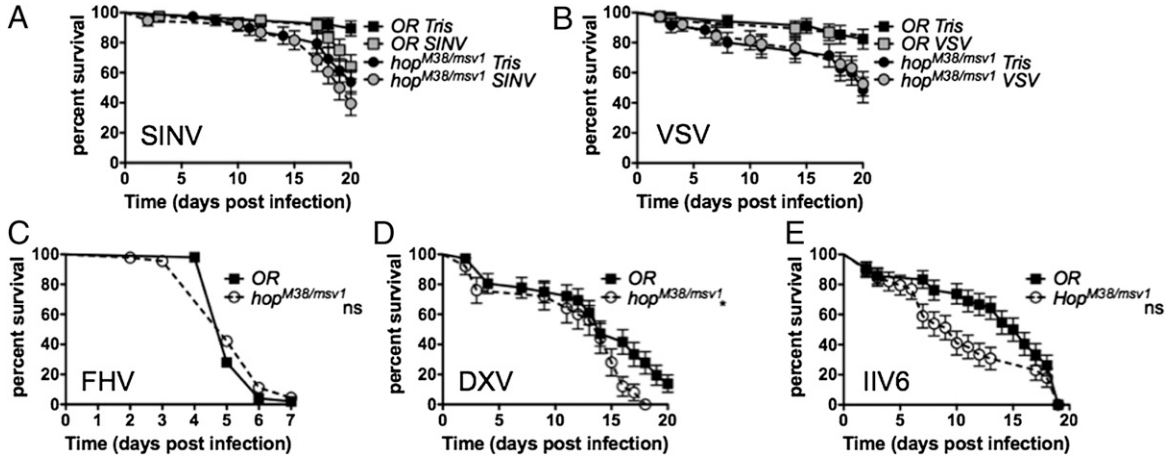

FIGURE 4. Susceptibility of flies mutant for the JAK kinase Hopscotch to infection by SINV, VSV, FHV, DXV, and IIV-6. Groups of 20 wild-type (OR) or *hop* mutant flies were injected with SINV (**A**), VSV (**B**), FHV (**C**), DXV (**D**), or IIV-6 (**E**), and survival was monitored. For VSV and SINV, the Tris buffer control injection is also shown, because *hop* mutant flies exhibited decreased survival at 29˚C after day 16 upon both buffer and virus injection. Kaplan–Meier analysis of the results of at least two independent experiments reveal a statistically significant difference in survival between wild-type and *hop* mutant flies only in the case of DXV. $*p < 0.05$.

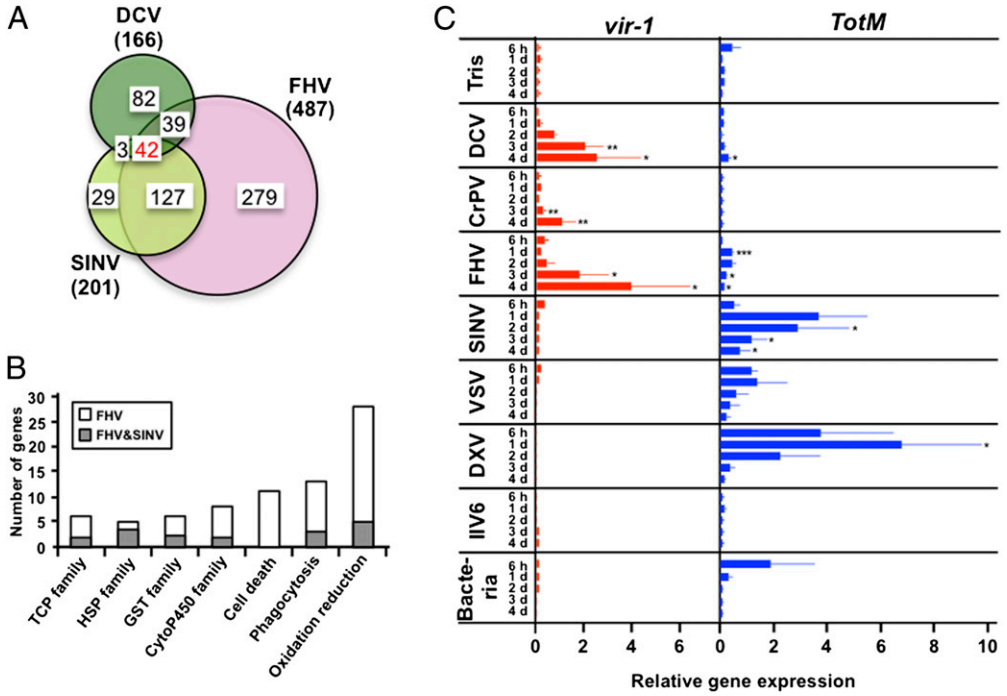

FIGURE 5. Microarray analysis of gene induction following infection by DCV, FHV, or SINV. (**A**) Venn diagram showing the number of upregulated genes (by a factor of at least 2) following infection by the three viruses. The total number of genes regulated by each virus is indicated in parentheses. (**B**) FHV and SINV induce members of the same gene families, but FHV triggers a stronger response. The numbers of genes belonging to seven gene ontology functional categories induced by both FHV and SINV or by FHV only are shown. (**C**) Expression of *vir-1* and *TotM* by quantitative PCR normalized for the expression of the housekeeping gene *RpL32*. Groups of 10 wild-type (OR) flies were injected with Tris buffer or the viruses DCV, CrPV, FHV, SINV, VSV, DXV, or IIV-6 or pricked with a needle dipped in a concentrated pellet of the Gram-positive bacterium *M. luteus* and the Gram-negative bacterium *E. coli*. RNA was extracted at 6 h, 1 d, 2 d, 3 d, and 4 d after challenge. The data represent the mean and SEs of at least two independent experiments. The *p* values were calculated for each time point individually versus the Tris-injected control. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

upregulated by a factor of at least 2 upon infection by FHV and SINV, respectively. When analyzed with the same criteria, 166 genes were induced by DCV (Fig. 5A, Supplemental Table II).

The data of this transcriptomic analysis call for two comments. First, we note that 42 genes were induced by all three viruses (Fig. 5A). We compared this set of genes with microarray studies performed on flies infected by fungi and bacteria (both extra- and intracellular) to identify a potential signature specific for viral infections (Supplemental Table III). We observed that a number of genes, such as *Frost*, are upregulated similarly by all types of infections, suggesting that they are induced by the stress of the infection, rather than by recognition of specific characteristics of the infecting microorganism. Of interest, other genes, such as *Vago*, *Obp99b*, *Mal-B1*, *Nmda1*, *CG8147*, *CG1572*, *l(2)gd1*, *CG14906*, *CG10911*, and *Tsp42EI*, appear to be induced only in response to viral infections, and may represent the core of an inducible antiviral gene expression program. The case of *Obp99b* is particularly striking, as this gene is strongly upregulated by FHV, SINV, and DCV, but inhibited following other types of infection. Clearly, the regulation and function of this molecule deserves further investigation. The genes *CG4680*, *Eip75B*, *Sp7*, and *CG10916* are induced both by the viruses and by the intracellular bacterium *Listeria* (33), suggesting that they may participate in the defense against intracellular intruders (Supplemental Table III).

A second comment is that the majority of upregulated genes are induced by only one or two of the viruses, revealing virus-specific responses. Of interest, 84% of the genes upregulated by SINV are also induced by FHV, pointing to a strong similarity between the responses to the two viruses. FHV induced a higher number of genes than did Sindbis virus, and only 34% of the genes induced by FHV are also induced by SINV (Fig. 5A). It is intriguing, though, that many of the genes induced solely by FHV, but not by SINV, are members of the same gene families as the genes coinduced by both FHV and SINV. This peculiarity underlines the basic similarities between the transcriptional response to the two viruses. In addition, several genes associated with cell death are induced by FHV, but not SINV, which may reflect the higher virulence of FHV (Fig. 5B, Supplemental Tables II, III). Only 22% and 16% of the genes induced by SINV and FHV, respectively, are also induced by DCV, indicating that DCV, on one hand, and FHV and SINV, on the other hand, trigger different inducible responses (Fig. 5A). We did not detect in our microarrays expression of the genes encoding the unpaired (Upd) cytokines, which activate the JAK-STAT pathway in *Drosophila*. However, quantitative RT-PCR analysis revealed that *upd2* and *upd3*, but not *upd*, are induced or upregulated following DCV and CrPV infection (Fig. 3C).

Virus-specific pattern of gene induction

To further characterize the transcriptional response triggered by different viruses, wild-type flies were injected with DCV, CrPV, FHV, SINV, VSV, DXV, and IIV-6, and gene induction was measured at 6 h postinfection and 1, 2, 3, and 4 dpi. Gene expression was monitored by quantitative RT-PCR, which provides a more accurate quantification of gene expression than does hybridization to short oligonucleotide probes on microarrays (34). We monitored expression of the DCV-induced gene *vir-1* (11) and of *TotM*, which, according to the microarrays, is induced by FHV and SINV infection. We confirmed the induction of *vir-1* by DCV and FHV (11) and detected a milder but significant induction of this gene by CrPV infection. By contrast, no induction of *vir-1* by SINV, VSV, DXV, and IIV-6 was observed (Fig. 5C). For *TotM*,

we confirmed the induction by FHV at different time points. In addition, we observed that *TotM* expression was significantly induced by DCV at late time points of infection (4 dpi). We note that induction of *TotM* by SINV, VSV, and DXV was 10–20 times stronger than the induction by FHV (Fig. 5C). The DNA virus IIV-6 did not induce *TotM* at any measured time point. Interestingly, we observed different profiles for *vir-1* and *TotM* induction after viral challenge. Overall, the viruses that kill wild-type flies rapidly (within 10 d), such as DCV, CrPV, and FHV, were potent inducers of *vir-1*, whereas less pathogenic viruses, such as SINV, VSV, and DXV, did not induce *vir-1*. The opposite trend was observed for *TotM*, which was most potently induced by SINV, VSV, and DXV. The different pattern of induction of *vir-1* and *TotM* suggests that the two genes may be regulated differently, even though both were previously shown to be regulated by the JAK-STAT pathway (11, 17). Indeed, the MAP3K MEK kinase 1 (MEKK1) and the Imd pathways are also known to contribute to the induction of *TotM* induction in some contexts (17, 35).

Some antimicrobial peptide genes were also upregulated according to the microarrays, suggesting an overlap between antiviral immunity and antibacterial–antifungal defenses. We observed an enrichment for genes regulated by the Toll pathway [e.g., the cytokine Spaetzle (Spz) and the antifungal peptides Drosomycine (Drs) and Metchnikowine (Mtk)] in the DCV-specific set of genes (Supplemental Table II). We also noted an enrichment of Imd pathway–regulated genes, such as the antibacterial peptides Attacin-A and -C, Diptericin-B, and the transcription factor Relish, in the genes upregulated by both DCV and FHV. However, when expression of *diptericin* and *drosomycin*—two markers of activation of the Imd and Toll pathways, respectively—was monitored by quantitative RT-PCR, none of the viruses triggered an induction comparable to that of bacterial and fungal infections, although the wounding associated with the injection procedure clearly led to some expression of the genes (Supplemental Fig. 1).

Discussion

We have investigated the involvement of RNAi and the evolutionarily conserved JAK-STAT signaling pathway in the resistance to a panel of seven viruses representing several important families, including the arboviruses SINV and VSV. Our data provide a contrasting picture: on the one hand, a broad antiviral immunity based on RNAi contributing to the defense against both RNA and DNA viruses, and on the other hand, a virus-specific transcriptional response involving the JAK-STAT pathway but playing a critical role only in the case of *Dicistroviridae* infection.

RNAi protects against a DNA virus infection

The present study extends work from several groups, including our own, showing that flies mutant for the siRNA pathway are more sensitive than wild-type flies to a large panel of RNA viruses, and reveals that Dcr-2 is also required for the control of the DNA virus IIV-6. We note, however, that the increase of viral titer in siRNA pathway–mutant flies is not as strong as in the case of some RNA viruses [e.g., VSV (25)]. This finding could reflect either the expression of a viral suppressor of RNAi by IIV-6 or the fact that only a portion of the viral genome is targeted by siRNAs. Indeed, this virus encodes an RNaseIII enzyme, which could cleave siRNA duplexes, as previously reported in plants infected by the sweet potato chlorotic stunt virus (36). The involvement of Dcr-mediated immune responses against DNA virus infections was previously noted in plants, in which secondary structures in the transcribed viral RNAs, or dsRNAs formed from overlapping bi-directional transcripts, can be processed into siRNAs (37, 38). Production of dsRNA from DNA viruses also occurs in animal cells, as demonstrated by the critical role played by the dsRNA receptor TLR3 in the sensing of herpesvirus infection in mammals (39, 40). Our data are consistent with a model whereby dsRNA generated from convergent transcription of the IIV-6 genome is processed by Dcr-2 and triggers RNAi. Thus, we conclude that RNAi provides an efficient and highly specific RNA-based defense against many types of viruses in *Drosophila* and probably other insects. This conclusion parallels the situation described in plants. The vertebrates, which largely rely on the induction of IFNs to counter viral infections, appear to be the exception among multicellular organisms (1). Of interest, however, the DExD/H box helicase domains found in Dcr enzymes and RIG-I–like receptors, which sense the presence of viral RNAs in cells infected by RNA and DNA viruses, are phylogenetically related (10). This finding suggests that an essential domain of a core molecule from the ancestral antiviral response, RNA silencing, was at some point recruited to sense viral RNAs in vertebrates and to subsequently activate a signaling pathway leading to production of IFNs.

Virus-specific induced gene expression in Drosophila

Microarrays are powerful tools to monitor the global transcriptome of infected cells and compare the response to different infections. Despite its limitations for accurate measurements of the magnitude of expression changes, this technology provides useful information on changes in gene expression (34). In this article, using whole-genome Affymetrix microarrays to analyze the transcriptome of flies infected by DCV, FHV, or SINV, we report the existence of virus-specific responses to infection. These results are in keeping with a previous study pointing to autophagy as an antiviral defense mechanism against VSV, but not DCV, infection (14). The three viruses we used belong to different families and present different characteristics that make them valuable for the current study. For example, 1) DCV and FHV replicate rapidly and kill *Drosophila* upon injection, whereas SINV does not at the dose used (11, 24); 2) DCV is a natural pathogen of *Drosophila*, whereas FHV and SINV have not been found in wild *Drosophila* populations (41); 3) FHV and DCV possess, respectively, a strong and moderate viral suppressor of RNAi, whereas SINV presumably does not (28, 42, 43). The three viruses also have different tissue tropism and may be associated with tissue-specific modifications in the physiology of the infected host. For example, FHV was recently shown to be a cardiotropic virus, affected by potassium channels regulating heart function (44), whereas DCV infection causes intestinal obstruction (S. Chtarbanova and J.-L. Imler, manuscript in preparation).

Comparison of the transcriptomes of the flies infected by the three viruses revealed more similarities between FHV and SINV than between each of these and DCV. This may reflect the co-evolution of DCV with its host, and the fact that this virus may have learned to ward off the antiviral arsenal of its host. Indeed, DCV induces fewer genes than does FHV, even though the two viruses replicate with similar kinetics and lead to the rapid death of the flies. The genes induced by FHV and SINV encode chaperonins (Tcp or Hsp), glutathione transferases, cytochrome P450s, stress markers (Tot family), thioester-containing proteins, and cytoskeletal regulators, suggesting an involvement of oxidative stress and phagocytosis in the response to these viruses. The two viruses also upregulate the gene *egghead* (*egh*), which encodes a molecule involved in the uptake of dsRNA and antiviral immunity (27). Despite the large overlap between the genes upregulated by FHV and SINV, the former induce a more intense transcriptional response than the latter. This observation may reflect the more aggressive replication of FHV in *Drosophila*. Indeed, the genes specifically induced by FHV include not only additional members of the families mentioned above (Hsp, Tcp, Gst, cytP450, thioester-

containing proteins), supporting the idea of a more intense response, but also genes associated with cell death. In addition, FHV upregulates several molecules previously connected to innate immunity in *Drosophila*, such as Hel89B (45), POSH (46), or MEKK1 (35), or molecules that may downmodulate the strong response to virus infection (e.g., the genes *CG9311* and *Pez*, encoding tyrosine phosphatases). Finally, we note that FHV induced eight genes encoding factors with RNA binding domains, including four DExD/H box helicases, which may participate in the sensing and neutralization of viral nucleic acids. This specificity may reflect a response of the host to counter the effect of the strong suppressor of RNAi B2, a dsRNA-binding protein (47).

An intriguing aspect of the transcriptome of virus-infected flies is the upregulation of genes regulated by the Toll and Imd pathways. We observed an enrichment of Toll pathway target genes induced in flies infected by DCV, but not FHV or SINV, suggesting that DCV infection triggers this pathway. Among the genes induced by DCV, but not by the two other viruses, we also note the presence of *Ect4*, which encodes a TIR domain cytoplasmic molecule. The mammalian ortholog of this gene, SARM, was proposed to participate as a negative regulator of TLR signaling in some antiviral defenses (48). Two other genes regulated by DCV and possibly establishing a connection between RNA silencing and the inducible response are worth mentioning: *headcase* was identified in a screen as a regulator of the siRNA pathway (49), whereas *CG9925* encodes a protein with a Tudor domain, a characteristic of several components of the Piwi-interacting RNA pathway (50).

Unlike the Toll-regulated genes, several genes regulated by Imd were induced in flies infected by DCV or FHV, although not by SINV. The Toll and Imd pathways play a well-characterized role in the regulation of bacterial and fungal infections, through the regulation of genes encoding antimicrobial peptides. These genes are also upregulated by viral infection, although not significantly, compared with buffer injection. This low level of induction most likely explains our inability to detect antimicrobial peptides in the hemolymph of DCV-infected flies (51). Although not formally establishing that the Toll and Imd pathways participate in the antiviral response, these results certainly do not rule out such a role (7, 9, 15). Alternatively, induction of the antimicrobial genes may involve the transcription factor FOXO, a known regulator of stress resistance, and may occur independently of the Toll and Imd pathways (52). Whatever the mechanism of induction, the biological significance of this weak induction of molecules normally active in the micromolar range is unclear. One possibility is that the *Drosophila* antimicrobial peptides carry additional functions that do not require high-level expression. For example, some mammalian β-defensins play a dual role in innate immunity and, in addition to their antibacterial properties, interact with chemokine receptors with affinities in the nanomolar range, thus mediating chemoattraction of phagocytic cells (53).

Dicistroviridae-*specific contribution of the JAK-STAT pathway to antiviral immunity*

An unexpected finding reported in this article is that *hop* mutant flies have a clear phenotype for DCV and CrPV, but not for the other viruses tested. This observation indicates that the JAK-STAT pathway, in addition to RNAi, participates in host defense against members of the *Dicistroviridae* family. DCV infection leads to induction of the genes encoding the cytokines Upd2 and Upd3, which may subsequently activate the JAK-STAT pathway in noninfected cells, triggering an antiviral program of gene expression. Altogether, our results highlight that the contribution of the inducible response to the control of DCV is similar to that of RNAi, as flies mutant for either RNAi or the inducible JAK-STAT pathway succumb to infection 2–3 d before the controls, with an ∼10-fold increase in viral titer.

Interestingly, even though *hop* mutant flies appear to be specifically sensitive to *Dicistroviridae*, other viruses activate the JAK-STAT pathway. Indeed, we observed a slight increase in the lethality of *hop* mutant flies postinfection with DXV and IIV-6. In *Aedes* mosquitoes, the JAK/STAT pathway was also shown to activate a defense against Dengue, a member of the *Flaviviridae* family (54). We also note that the JAK-STAT pathway–regulated gene *vir-1* (11) is induced by DCV and CrPV, but also FHV, even though *hop* mutant flies resist FHV infection much as do wild-type flies. One hypothesis to explain this apparent paradox is that some genes may be induced in a JAK-STAT–independent manner in the context of viral infections. For example, the gene *TotM*, which is induced by several viruses normally resisted by *hop* mutant flies, can be induced by the MEKK1 pathway, in addition to the JAK-STAT pathway (35). Indeed, we observed that *TotM* remains fully induced by FHV and SINV in *hop* mutant flies (C. Dostert and J.-L. Imler, unpublished observations). However, this hypothesis cannot account for the induction of *vir-1* by FHV, because it is strongly reduced in *hop* mutant flies (C. Dostert and J.-L. Imler, unpublished observations). This finding suggests that some aspects of the JAK-STAT–induced response may be redundant of other defenses for FHV, but not for DCV. The fact that FHV triggers a stronger transcriptional response than does DCV (Fig. 5) is consistent with this hypothesis.

A key question pertains to the nature of the receptor detecting *Dicistroviridae* infection and triggering the JAK-STAT–dependent inducible response. Our data point to the induction of a specific subset of genes, including the JAK-STAT–regulated gene *vir-1* (11), by fast-killing viruses such as DCV and CrPV, but also FHV, which replicate rapidly to high titers upon injection in flies. Of note, *vir-1* induction is not affected in flies expressing the dsRNA-binding protein B2, or in *Dcr-2* mutant flies, indicating that this gene is not induced following sensing of dsRNA (10). This finding suggests that sensing tissue damage and/or cell death could contribute to this inducible response, a hypothesis corroborated by the association of the JAK-STAT pathway with the cellular response to a variety of stresses (17, 55–57).

In conclusion, our data confirm that, beyond RNAi, an inducible response contributes to the control of some viral infections in *Drosophila*. However, this response is complex, and great care should be exercised before generalizing the results obtained with one single virus species. This unexpected complexity probably reflects the intricate association of viruses with their host cells in different tissues, their different strategies of replication or protein expression, or their acquisition of suppressors of host defense.

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Disclosures

The authors have no financial conflicts of interest.

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