RNA silencing or RNA interference (RNAi) is a highly conserved process in which the introduction or the production of double-stranded RNA (dsRNA) into cells triggers the degradation of mRNAs containing homologous sequences by sequence-specific cleavage of the mRNAs (for review, see e.g. Lecellier & Voinnet, 2004; Voinnet, 2005). The degradation is mediated by 21–24 nt long small interfering RNAs (siRNA), which are produced by the RNase III-like enzyme Dicer and incorporated into the RNA-induced silencing complex (RISC) (Elbashir et al., 2001; Hamilton & Baulcombe, 1999). The RNAi pathway is an ancient evolutionary conserved mechanism, which has been retained in plants, worms, insects and mammals and appears to represent an early form of innate immunity.

Among the different systems in which RNA silencing has been studied, plants, mammalian cells and insects (particularly Drosophila and mosquitoes) represent a significant field, but less is known about ticks and tick cells. Only a few reports described RNAi in ticks, in vitro or in vivo, and these studies describe silencing of specific genes but do not explore the mechanisms (Aljamali et al., 2003; Karim et al., 2004; Narasimhan et al., 2004). Ticks are obligate ectoparasites distributed worldwide and transmit human pathogens, such as the Lyme disease agent, the agent of Ehrlichiosis and arboviruses, some of which are serious human pathogens (i.e. the Crimean-Congo hemorrhagic fever virus). Recently, we showed that the tick cell line ISE6, established from Ixodes scapularis (Munderloh et al., 1994), was able to support the replication of the mosquito-borne Semliki Forest virus (SFV) and recombinant replicon (rSFV) and we found that infection of ISE6 cells with Hazara virus, a tick-borne arbovirus of the family Bunyaviridae (genus Nairovirus) was silenced through RNAi by a rSFV expressing nairovirus sequences (Garcia et al., 2005). The induction of RNAi in tick cells by SFV infection was clearly associated with the synthesis of siRNAs and a Dicer-like activity.

The discovery that plant viruses encode suppressors of gene silencing (Anandalakshmi et al., 1998; Beclin et al., 1998; Brigneti et al., 1998; Kasschau & Carrington, 1998; Vaucheret et al., 1998) provided a strong support that RNAi functions as a natural defence mechanism against viruses (Lindbo et al., 1993; Ratcliff et al., 1999). These inhibitors target various components of the RNAi pathway, thus representing valuable tools for the comprehension of the RNA silencing pathway. Among the best-studied suppressors, the helper component-proteinases (HC-Pro) encoded by potyviruses were shown to inhibit silencing of transgenes in transformed plants, antagonizing silencing in all tissues and targeting a step involved in the maintenance of silencing at or upstream from the production of siRNA (Anandalakshmi et al., 1998; Brigneti et al., 1998; Llave et al., 2000; Mallory et al., 2001). HC-Pro was shown to prevent dsRNA degradation into siRNAs (Bucher et al., 2003; Reavy et al., 2004). As RNA silencing is not limited to the plant...
kingdom, suppressors such as the B2 protein of the Flock house virus (Li et al., 2002; Lingel et al., 2005) have been found in insect and animal viruses and are able to act in cells from heterologous species. Among the inhibitors encoded by mammalian virus, NS1 of Influenza virus, which is acting as an interferon antagonist (García-Sastre et al., 1998), exerts its suppressor activity through its dsRNA-binding domain, in plants, Drosophila and mosquito (Anopheles gambiae) cells (Bucher et al., 2004; Delgadillo et al., 2004; Li et al., 2004). Other non-structural proteins of negative-stranded or ambisense RNA viruses, NSs of Tospovirus Tomato spotted wilt virus (TSWV), NSs of La Crosse virus (genus Orthobunyavirus) and NS3 of Rice Hoja blanca virus (RHBV; genus Tenuivirus) were found to act as suppressors of post-transcriptional silencing of a transgene in mammals and plants (Bucher et al., 2003; Soldan et al., 2005). Interestingly, the NSs of TSWV seem to inhibit only the onset of sense transgene-induced post-transcriptional gene silencing (S-PTGS) but not inverted repeat PTGS (IR-PTGS) (Takeda et al., 2002).

To our knowledge, nothing is known of the ability of viral suppressors to abrogate RNAi in ticks and tick cells. In this work, we investigated whether suppressors of RNA silencing can act efficiently in tick cells and we set up a screening for RNAi inhibitors.

We chose to use a recombinant SFV to express heterologous proteins and to induce RNAi in tick cells (García We chose to use a recombinant SFV to express heterologous RNAi inhibitors.

To ascertain that the downregulation in luciferase expression is due to RNAi, likewise, ISE6 cells infected with SFV-Luc (or the control SFV-1) were collected at 2 days p.i. and total cellular RNA was extracted with TRIzol (Invitrogen). The presence of luciferase-specific siRNAs was assayed by RNase protection using a 32P-labelled RNA probe in vitro transcribed with SP6 RNA polymerase from a pGEM plasmid containing the cDNA sequence encompassing the 5’ terminal 106 nt of the firefly luciferase mRNA. RNase this activity was highly expressed at 1 day p.i. but the level of expression decreased progressively from 2 to 4 days p.i. (Fig. 1a). Northern blots were carried out to analyse the viral genomic and 26S subgenomic RNAs. A significant decrease in SFV subgenomic mRNA was detected (data not shown), explaining reduction in protein expression.

Fig. 1. (a) Kinetics of luciferase expression in ISE6 cells. ISE6 cells were infected at a m.o.i. of 5 with SFV-Luc or SFV-1 as a negative control. Cells were collected at 1, 2, 3 and 4 days p.i. and luciferase activity was determined. (b) Detection of siRNAs by RNase protection assay. RNAs from ISE6 cells infected with SFV-Luc (lane 1) or SFV-1 (lane 2) and harvested 2 days p.i. were hybridized with a luciferase-specific probe, treated with RNase and analysed in a 15% denaturing polyacrylamide gel. The RNase treated unprotected probe was analysed (lane 3). The positions of 32P-labelled RNA markers (20 and 30 nt) are indicated.
indicated that the viral proteins identified as suppressors in expressing the suppressor (Fig. 2b). Altogether, the data SFV-Luc subgenomic mRNA was clearly increased in cells activity from the SFV replicon. Additionally, the level of reporter activity at this time, indicating a lack of suppressor co-infection with SFV-1 led to an almost identical level of It should be noted that infection with SFV-Luc alone or <0

To investigate the ability to suppress RNAi in this system, we selected three different viral suppressors known as inhibitors in plants and/or mosquito cells: NS1 of influenza virus (NS1inf), NSs of TSWV (NSsTSWV) and HC-Pro of Zucchini yellow mosaic virus (ZYMV; HC-ProZYMV). The NS1 ORF of A/PR/8/34 strain and the TSWV NSs sequence were amplified from plasmids (Bucher et al., 2004), the HC-Pro ORF was derived from a plasmid containing ZYMV cDNA sequences, strain NAT [kindly provided by C. Desbiez, Institut National de la Recherche Agronomique (INRA), Montfavet, France and C. Antoniewski, Institut Pasteur, Paris, France]. The three rSFVs, SFV-NS1inf SFV-NSsTSWV and SFV-HC-ProZYMV, were generated by inserting the specific cDNA in the SmaI site of the pSFV-1, after RT-PCR using oligonucleotides the sequences of which are available on request. Expression of these proteins in ISE6 cells infected with the corresponding rSFV was confirmed by indirect immunofluorescence assay using specific antisera (data not shown).

To evaluate the effect of the viral proteins on the suppression of RNAi in tick cells, we co-expressed the viral protein together with luciferase by co-infecting cells with SFV-Luc and SFV-NS1inf SFV-NSsTSWV, SFV-HC-ProZYMV or SFV-1 as a control. Infections were carried out at an m.o.i. of 5 for each virus. The luciferase activity was determined at 3 days p.i., an incubation time when RNA silencing was effective in cells infected with SFV-Luc alone (see Fig. 1). Co-expression of NS1inf NSsTSWV or HC-ProZYMV and firefly luciferase strongly increased the luciferase activity compared with the control in which SFV-1 was co-infected with SFV-Luc (Fig. 2a). Co-expression of SFV-NS1inf SFV-NSsTSWV or SFV-HC-ProZYMV stimulated the level of luciferase activity by a factor of 4-5-, 2-5- and 1-5-fold (P<0.001, P<0.01, P<0.01, respectively, for the Student’s t test), respectively. It should be noted that infection with SFV-Luc alone or co-infection with SFV-1 led to an almost identical level of reporter activity at this time, indicating a lack of suppressor activity from the SFV replicon. Additionally, the level of SFV-Luc subgenomic mRNA was clearly increased in cells expressing the suppressor (Fig. 2b). Altogether, the data indicated that the viral proteins identified as suppressors in plants and insect cells are also able to abrogate RNA silencing in tick cells.

As NSs of La Crosse virus (family Bunyaviridae, genus Orthobunyavirus) was recently described also to be an RNAi suppressor in human cells (Soldan et al., 2005), the NSs protein of Rift Valley fever virus (RVFV) was assayed for potential RNAi suppressive activity using the aforementioned tick-cell system. This experiment was carried out on the basis that the NSs of bunyaviruses share structural and functional similarities as they are encoded by the S segment.

**Fig. 2.** (a) Effect of RNAi suppressors during siRNA processing. ISE6 cells were infected, as indicated, with SFV-Luc, SFV-Luc and SFV-1, SFV-NS1inf, SFV-NSsTSWV or SFV-HC-Pro and collected at 3 days p.i. Luciferase activity was expressed as percentage of the value obtained in cells infected with SFV-Luc and SFV-1. Data represent the mean values and standard deviations from triplicates. *, P<0.01; **, P<0.001 for the Student’s t test. (b) Northern blot analysis of RNA synthesized in ISE6 cells, mock infected (M), infected with SFV-1 (lane 1) or co-infected with SFV-Luc and SFV-1 (lane 2) or SFV-Luc and SFV-NS1 (lane 3). RNAs were extracted with TRIzol at 3 days p.i. and analysed by Northern blot using a 32P-labelled luciferase-specific probe.
and are involved in virulence (Blakqori & Weber, 2005; Bridgen et al., 2001). Notably, RVFV NS is a strong inhibitor of cellular transcription and an antagonist of interferon expression (Bouloy et al., 2001; Le May et al., 2004). Additionally, NSs of TSWV and RVFV, like the established RNA silencing suppressor protein NS3 of the tenuivirus RHBV (Bucher et al., 2003) are encoded by the same genomic organization using an ambisense strategy. However, no RNAi suppressive activity was observed in tick cells expressing RVFV NSs, confirming data obtained in plants and human 293T cells (E. Bucher, J. van der Velden, P. de Haan and M. Prins, unpublished). This suggests that the NSs proteins of bunyaviruses have evolved differently.

Viral suppressors have been described to abrogate RNA silencing at different steps of the RNAi pathway, some of them blocking the generation of the Dicer cleavage products, and others inhibiting a step further down. To determine at which step the viral suppressor acts, ISE6 cells were first infected with SFV-1 to induce the production of siRNAs and subsequently superinfected with SFV-Luc and rSFV expressing the suppressor. To validate the experimental conditions, we first verified that RNA silencing was established. When SFV-1 infected cells were superinfected with SFV-Luc at day 1, and reporter activity measured at day 4 p.i. (i.e. 3 days after superinfection), a strong reduction of reporter activity was observed in these cells (Fig. 3) compared with non-SFV-1-infected-SFV-Luc-supersinfectected cells. A reduction in the amount of subgenomic mRNA was similarly observed by Northern blot using a luciferase-specific probe (not shown). Together, these data clearly indicated that SFV-1 was the effector of RNA silencing. When cells infected with SFV-1 were superinfected with SFV-Luc and SFV-NS1, -NSs or -HC-Pro, the level of reporter activity clearly indicated that the presence of NS1 and, to some extent, of NSs partially, but significantly (Student’s t test; \( P<0.01 \)), restored the level of the reporter gene expression by a factor 3 and 1-5, respectively (Fig. 3). Interestingly, under these conditions and in contrast with the experiment described in Fig. 2, HC-Pro had no inhibitory effect on RNA silencing. The results obtained with HC-Pro are in accordance with published data showing that this protein acts at a maintenance step at or upstream of the production of siRNA (Llave et al., 2000; Mallory et al., 2001). For the NS1 protein, the partial inhibition of RNAi is associated with the protection of the luciferase mRNA against degradation (not shown), suggesting that NS1 inhibits RNAi in tick cells as in other cells, by sequestering the siRNAs through its RNA-binding activity (Bucher et al., 2004; Li et al., 2004).

In conclusion, the system described here could be useful to study other RNAi suppressors in tick cells.

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References


Suppressors of alphavirus replicon induced RNAi


