Using Double-stranded RNA to Prevent *in Vitro* and *in Vivo* Viral Infections by Recombinant Baculovirus*

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Introduction of double-stranded RNA (dsRNA) into a wide variety of cells and organisms results in post-transcriptional depletion of the homologous endogenous mRNA. This well-preserved phenomenon known as RNA interference (RNAi) is present in evolutionarily diverse organisms such as plants, fungi, insects, metazoans, and mammals. Because the identification of the targeted mRNA by the RNAi machinery depends upon Watson-Crick base-pairing interactions, RNAi can be exquisitely specific. We took advantage of this powerful and flexible technique to demonstrate that selective silencing of genes essential for viral propagation prevents *in vitro* and *in vivo* viral infection. Using the baculovirus *Autographa californica*, a rapidly replicating and highly cytopathic double-stranded DNA virus that infects many different insect species, we show for the first time that introduction of dsRNA from *gp64* and *iei*, two genes essential for baculovirus propagation, results in prevention of viral infection *in vitro* and *in vivo*. This is the first report demonstrating the use of RNAi to inhibit a viral infection in animals. This inhibition was specific, because dsRNA from the polyhedrin promoter (used as control) or unrelated dsRNAs did not affect the time course of viral infection. The most relevant consequences from the present study are: 1) RNAi offers a rapid and efficient way to interfere with viral genes to assess the role of specific proteins in viral function and 2) using RNAi to interfere with viral genes essential for cell infection may provide a powerful therapeutic tool for the treatment of viral infections.

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1 The abbreviations used are: dsRNA, double-stranded RNA; RNAi, RNA interference; AcNPV-GFP, A. californica recombinant baculovirus carrying the GFP and β-galactosidase genes; GFP, green fluorescent protein; pfu, plaque-forming units; m.o.i., multiplicity of infection; Sf21, *S. frugiperda* 21 cell line; X-gal, 5-bromo-4-chloro-3-indolyl-β-D-galactoside; HIV-1, human immunodeficiency virus, type 1; BV, budding viruses; ONPG, α-nitrophenyl-β-D-galactoside; FACS, fluorescence-activated cell sorting, FBs, fetal bovine serum; RT, reverse transcription; PhD, polyhedrin promoter.

Although the effect of dsRNA to prevent viral infections has been tested in cell lines, it remains to be established whether RNAi also works in organisms. To test this hypothesis we used a well-studied insect virus, the *Autographa californica* nucleopolyhedrovirus (AcNPV). This virus is a member of a group (family *Baculoviridae*) of large double-stranded DNA viruses that infect many different insect species (7). The AcNPV GP64 glycoprotein is a major component of the nucleocapsid of budded viruses (BV) and is required for BV entry into host cells by endocytosis (8). Thus, GP64 is a key element for cell-to-cell infection and virus propagation (8). Another gene essential for baculovirus proliferation produces the immediate early protein (IE1). This phosphoprotein regulates the transcription of early viral genes (9). Deletion of the N-terminal domain of IE1 results in the loss of transcriptional activation, and the resulting viruses cannot be replicated (9). Thus, these two proteins are excellent candidates to test if RNAi can prevent viral infection *in vitro* and *in vivo*.

Recombinant AcNPV in conjunction with *Spodoptera frugiperda* (Sf/9 and Sf/21) insect cell lines have been extensively used for the heterologous expression of a wide variety of genes (7). We have produced a recombinant baculovirus containing the genes for the green fluorescent protein (GFP) and β-galactosidase (AcNPV-GFP), which we have used as reporters of cell infection in Sf/21 insect cells and larvae.

Because introduction of dsRNA results in powerful and selective gene silencing in different cells (including insect cells), one would expect that transfecting insect cells with dsRNA from *gp64* prior to virus exposure could prevent virus propagation, because the BV produced by dsRNA-treated cells would lack GP64 protein.

In agreement with this prediction, cells infected with AcNPV-GFP and previously transfected with dsRNA from a portion of *gp64* (dsRNA-*gp64*) do not show GFP fluorescence, and the GP64 protein could not be detected in the membrane of these cells. Because the very late polyhedrin promoter drives GFP production, the lack of GFP expression indicates that dsRNA treatment prevents viral infection in culture. Similar results were obtained with cells transfected with dsRNA from *iei* (dsRNA-*iei*). In this case the inhibition was even stronger as expected when interfering with the synthesis of a transcrip-
n the hemolymph of the larvae from this insect results in Tenebrio mollitor with viral infection. To test if silencing gp64 and 1e1 genes may interfere also with viral genes to assess the role of specific proteins in viral function (reverse genetics in baculovirus) and 2) using RNAi to interfere with viral genes essential for cell infection may provide a powerful therapeutic tool for the treatment of viral infections.

EXPERIMENTAL PROCEDURES

Reagents—All salts were analytical grade purchased from Sigma (St. Louis, MO). 5-Bromo-4-choro-s-indolyl-β-D-galactoside (X-gal), obtained from Sigma (St. Louis, MO); o-Nitrophenyl-β-D-galactoside (ONPG) was purchased from Research Organics Inc. (Cleveland, OH). The enhanced green fluorescent protein (GFP) vector (pEGFP-N1) was purchased from Clontech (Palo Alto, CA).

Cell Culture—Insect S2/1 cells were obtained from Invitrogen (San Diego, CA) and cultured in Grace's medium (Sigma, St. Louis, MO) for polyhedrin the entire promoter sequence (GenBankTM X06637). The sequence, including the fragment of the first 619 nucleotides from the promoter using the Megascript kit (Ambion, Austin, TX), following the manufacturer's instructions (Invitrogen). After two plaque assays, the pBB4 from GFP (Clontech, Palo Alto, CA) was cloned in the pBB4 vector from Sigma (St. Louis, MO).

Control uninfected cells were used to adjust the number of GFP-positive cells/well, allowed to attach for 30 min, and infected 1 h at 27°C with serial dilutions (in 1 ml of Grace media, 10% FBS) of the supernatant obtained from transfected in infected cells, as indicated in the figure legend (see Fig. 2). After the incubation period the media was aspirated and 2 ml of Grace media, 10% FBS, 1.5% low melting point agarose (Invitrogen) was added. Finally, 1 ml of Grace media with 10% FBS was added over the agarose. Cells were monitored for GFP-positive plaque formation after 72 h. The GFP-positive plaques were quantified, and the number of GFP-positive plaques by dilution reciprocal. All dilutions were repeated at least twice.

RNA Purification and RT-PCR Analysis—Total RNA was isolated from dsRNA-transfected or untreated cells using TRIzol reagent (Invitrogen, Carlsbad, CA) following the manufacturer's recommended protocol. All RNA samples were quantified by spectrophotometry and run on agarose gel to check the quality and integrity of total RNA. To avoid DNA contamination, 5 μg of total RNA was treated with 15 units of DNase I RNAse-free (Ambion, Austin, TX) for 20 min at 37°C. After a phenol-chloroform extraction, reverse transcriptions reactions were done with the SuperScript™ One Step RT-PCR system from Invitrogen using一组 specific primers (0.2 μM), β-Acin: 5'-GATATGGCGAGAAGTCGGCACCAC-3' and 5'-TGCGGCAAGGGCGTAGCC-3'; gp64: 5'-G' GAAAAGCAGTGCCTGTCGTA-3' and 5'-TATATGCGAGACGACTGGCAACGGGCAAATGT-3'; 1e1: 5'-GGGCCCCGAATTTAATGCGGT-3' and 5'-GGGACTTATGCGGCCAGTT-3'; 3'-GATUCCGGCTTGGCGACGAG-3'. For each RT-PCR reaction, 200 ng of RNA were used in a 50-μl reaction. A retrotranscription step (50°C for 30 min and 94°C for 2 min) was followed by 21 cycles of denaturation at 94°C for 15 s, annealing at 55°C for 30 s, and extension at 72°C for 50 s. To ensure that there was no DNA contamination in the RT-PCR experiments, controls without reverse transcriptase were performed in all cases. In all cases, PCR products were resolved by 1.5% agarose gel electrophoresis and analyzed after ethidium bromide staining in a Typhoon 8600 variable mode imager (Amersham Biosciences, Piscataway, NJ).

Electron Microscopy—Sections (1 μm) were fixed with 3% glutaraldehyde in saline buffer for 2 h at room temperature. The sections were then rinsed with saline and embedded in epon. The blocks were cut into 1 μm sections and stained with 1% uranyl acetate and 0.5% lead citrate. The sections were observed using a transmission electron microscope (Jeol, JSM-5410LV) at low vacuum.

Insect Infection—T. mollitor (mealworm beetle) larvae were obtained at a local pet store and maintained in the laboratory in a plastic container. Larvae were injected in the hemolymph with 0.1 μg of the

Confocal Microscopy—Infected S2/1 cells were fixed 48 h postinfection with a buffer containing 3% paraformaldehyde Sigma (St. Louis, MO) as previously described (11) and incubated with the specific GP64 antibody (AvG5) eBioscience (San Diego, CA). A second anti-mouse antibody conjugated with rhodamine (Zymed, San Francisco, CA) was used to detect gp64-specific fluorescence. Cells were analyzed using a Bio-Rad confocal system (Bio-Rad, Hercules, CA) attached to a Nikon Diaphot inverted microscope equipped with a 60× oil-immersed objective. GFP fluorescence was obtained after exciting the cells with 395 nm and reading fluorescence at 540 nm (green channel). For gp64 detection, the excitation wavelength was 570 and emission was collected at 590 nm (red channel). Double labeling was observed in yellow, the combination of green (GFP) and red (GP64) channels.

Silencing a Viral Infection with Double-stranded RNA

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Cell Culture—Insect S2/1 cells were obtained from Invitrogen (San Diego, CA) and cultured in Grace's medium (Sigma, St. Louis, MO) supplemented with tetrachloroethylene, yeastolate, 2-μm g-t-glutamic acid, 10% heat-inactivated fetal bovine serum, 100 μg/ml streptomycin, and 250 ng/ml amphotericin B (Invitrogen, Grand Island, NY).

Production of Recombinant AcNPV-GFP Baculovirus—The cDNA from GFP (Clontech, Palo Alto, CA) was cloned in the pBB4 vector (Invitrogen, Carlsbad, CA) next to the polyhedrin promoter. The pBB4-GFP vector was recombinated with linear AcNPV DNA following the manufacturer's instructions (Invitrogen). After two plaque assays, the recombinant baculovirus containing the GFP (AcNPV-GFP) was amplified twice and the titer was determined by plaque assay. This recombinant baculovirus was used in the studies described in this work.

dsRNA Synthesis—Double-stranded RNA was synthesized in vitro using the Megascript kit (Ambion, Austin, TX), following the manufacturer's instructions. Briefly, nucleotides 109330–109949 were amplified by PCR from the entire virus genome. This sequence corresponds to the first 619 nucleotides from gp64 as verified by sequence analysis. The sequence was cloned in the pFD129.36 vector multiple cloning site, which is flanked by two T7 opposing promoters (10). The T7 promoter sequence, including the fragment of gp64 was amplified by PCR using universal T7 primers, and this sequence was used for the in vitro transcription to obtain the dsRNA with the T7 polymerase. For the synthesis of dsRNA from GFP, the entire gene sequence was used and for polyhedrin the entire promoter sequence (GenBank™ X06837). The Ie1 gene is formed from a promoter sequence and the sequence resulting in the transcription of Ie1 activator protein. The Ie1 RNA was synthesized from nucleotides 19-470 (ATG is in position 1) corresponding to the N-terminal domain from the Ie1 protein. For single-stranded RNA production, the vector was linearized with HindIII, and in vitro transcription was performed (positive strand). The antisense strand was synthesized after linearizing the vector with EcoRI. After two plaque assays, the pBB4-GFP vector was recombinated with linear AcNPV DNA following the manufacturer's instructions (Invitrogen). After two plaque assays, the recombinant baculovirus containing the GFP (AcNPV-GFP) was amplified twice and the titer was determined by plaque assay. This recombinant baculovirus was used in the studies described in this work.

Fluorescence-activated Cell Sorting—The percentage of infected cells (GFP-expressing cells) and fluorescence intensity was assessed by FACS (FACSCalibur, BD Biosciences) analysis 48 h postinfection using the multiplicity of infection (m.o.i.) reported in the figure legends. Control uninfected cells were used to adjust the number of GFP-positive cells and mean fluorescence (20,000 events/sample). Acquisition and analysis of the FACs data were performed using CellQuest software (BD Biosciences, Palo Alto, CA).
different dsRNAs nude or with distilled water (control). 24 h later larvae were injected again in the hemolymph with 2 μl of AcNPV-GFP viral stock with a titer of 1 × 10^7. Dead insects were assayed for GFP fluorescence and β-galactosidase activity. To assess the GFP fluorescence, injected insects were explored with the confocal microscope using a low magnification objective (10×; Nikon). For β-galactosidase activity, the insects were injected in the hemolymph with 2 μl of 0.05% X-gal in MeSO.

RESULTS

Selective Inhibition of Baculovirus Infection of Sf21 Cells by dsRNA from gp64 and ie1—We explored the effect of transfecting Sf21 cells with different dsRNAs prior to AcNPV-GFP infection. For these experiments we used four different multiplicities of infection (m.o.i.), 0.05, 0.5, 1, and 5. Fig. 1 illustrates a representative example of the effect of different dsRNAs on the fluorescence of GFP at am m.o.i. of 0.05 obtained 72 h postinfection. As illustrated in this example, the fluorescence of GFP was greatly reduced by transfecting the cells (48 h prior to AcNPV-GFP infection) with dsRNA from gfp or gp64 but not with dsRNA from the polyhedrin promoter used as control. At this time of postinfection, 90.2 ± 2.1% (n = 5) of AcNPV-GFP-treated cells show high GFP fluorescence when compared with control (uninfected) cells. Transfecting the cells with dsRNA-gp48 48 h prior to AcNPV-GFP infection resulted in significant reduction of GFP fluorescent cells to 9.1 ± 0.7% (n = 5). Exposing the cells to dsRNA from gp64 (dsRNA-gp64) prior to AcNPV-GFP infection resulted also in an important reduction in the number of GFP fluorescent cells to 8.3 ± 0.5% (n = 5). Transfecting the cells with dsRNA from the polyhedrin promoter (dsRNA-PhP) did not affect significantly the GFP fluorescence, showing values of 89.1 ± 1.2% (n = 5).

These results show that introduction of dsRNA from the gp64 gene prevents GFP fluorescence induced by recombinant baculovirus. Because the very late polyhedrin promoter (PhP) drives GFP production, the lack of GFP expression indicates that dsRNA-gp64 treatment prevents viral infection in culture. Cells transfected with dsRNA-gp64 did not show the β-galactosidase activity of the second reporter gene introduced in the recombinant AcNPV-GFP (Fig. 2B). The early-to-late (P_ETL) promoter drives β-galactosidase production. Thus, using these two reporter genes one can monitor early and very late viral gene expression. Transfecting insect cells with dsRNA-gfp prevents GFP fluorescence by favoring GFP mRNA degradation, but as expected, this does not appear to affect viral infection. Cells that interfered with dsRNA-gfp showed β-galactosidase activity, indicating the success of typical viral infection (Fig. 2B). Furthermore, recombinant AcNPV-GFP is a lytic baculovirus, therefore, cells infected with AcNPV-GFP will lyse eventually. Even at a low m.o.i. of 0.05, all insect cells infected with AcNPV-GFP and treated with dsRNA-gfp were lysed 7 days after infection.

Fig. 2 shows all the RNAi experiments performed at m.o.i. values of 0.05, 0.5, 1, and 5. In this figure the percentage of GFP-fluorescent cells is presented in panel A, and the β-galactosidase activity from the same cells is shown in panel B. As illustrated in this figure, cells treated with dsRNA from gp64 showed low levels of GFP fluorescence and β-galactosidase activity at 0.05 and 0.5 m.o.i. At m.o.i. 1 and 5 dsRNA-gp64 did not prevent GFP fluorescence or β-galactosidase activity from AcNPV-GFP-infected cells. This result is somewhat expected, because all cells would be infected by the initial virus application and do not depend on the production of viral progeny. When using m.o.i. of 0.05 (one viral particle for every 20 cells), the infection of the rest of the cells not infected by the initial viral application would depend upon production of new viruses by the cells initially infected. Because cells are transfected with dsRNA-gp64, one would expect that the GP64 protein could not be produced and the new viruses would lack this protein. At m.o.i. 0.05, dsRNA-gp64 strongly inhibits GFP fluorescence (Fig. 2A) and β-galactosidase activity (Fig. 2B). These results are consistent with previous studies found in the literature indicating the gp64 gene deletion result in noninfective baculoviruses (8). In contrast to these results, transfecting the cells with dsRNA-ie1 inhibited GFP fluorescence and β-galactosidase activity even at m.o.i. values of 1 and 5 (Fig. 2). The IE1 transcriptional factor is essential for initiating the transcription of several early baculovirus genes. In fact, deletion of the ie1 gene results in viruses that cannot be replicated (9).
fore, the results presented here are consistent with the idea that interfering with the production of the initial transcriptional activator for viral genes (ie1) would prevent the production of viral proteins and interfere with viral replication.

In agreement with all these results, the baculoviruses isolated from the supernatant of dsRNA-gp64- and dsRNA-ie1-treated cells were less infective when compared with untreated cells or cells transfected with dsRNA-gfp prior to baculovirus infection (Fig. 2C). As expected from cells exposed to dsRNA-gp64, only baculoviruses collected from cells infected with low m.o.i. values (0.05 and 0.5) showed reduced plaque-forming units (pfu). At m.o.i. 1 and 5 the baculoviruses produced by dsRNA-gp64-treated cells showed pfu indistinguishable from cells not exposed to dsRNA or cells treated with dsRNA-gfp (Fig. 2C). Notice that the effect of dsRNA is specific for the gene explored, because cells exposed to dsRNA-gfp showed a significant reduction of GFP fluorescence but not β-galactosidase activity. As expected from these experiments, baculoviruses isolated from dsRNA-gfp-treated cells showed pfu similar to cells not exposed to any dsRNA. The RNAi effects observed are specific for the double-stranded RNA, because transfecting the cells with single-stranded RNA prior to baculovirus infection did not alter the time course of viral infection and did not prevent GFP fluorescence or β-galactosidase activity (data not shown). A small reduction in GFP fluorescence was obtained with the negative single RNA strand of gp64 of 20 ± 5% compared with the large reduction of 92 ± 7% obtained with dsRNA-gp64 (n = 6). This result demonstrates that the inhibition induced is specific for the double RNA strand, as expected from an RNAi phenomenon.

dsRNA-gp64-treated Cells Do Not Express GP64 Glycoprotein—Confocal microscopy studies using a specific GP64 antibody showed protein localization at the cell plasma membrane. The pattern of localization of GP64 contrasted with the generalized fluorescence of GFP, as expected for a soluble protein. Fig. 3 shows representative images obtained with a rhodamine-labeled antibody for GP64 (red channel) and GFP fluorescence (green channel). As illustrated in the figure, the transfection with dsRNA-gf prevented GFP fluorescence but did not affect GP64 expression of insect cells infected with AcNPV-GFP at m.o.i. 0.05. Transfecting the cells with dsRNA-gp64 eliminated the fluorescence of both GFP and GP64, leaving only a few cells showing both signals. These cells may reflect cells infected by the initial application of recombinant AcNPV-GFP. Interestingly, when AcNPV-GFP was used at an m.o.i. of 1, dsRNA-gp64 could not prevent GFP fluorescence, however, the transfection prevented GP64 protein production, as illustrated by the lack of red fluorescence in Fig. 3. This result is consistent with the data presented in Fig. 2, suggesting that the initial viral application can infect most of the cells (therefore the GFP production is intact), yet the cells do not produce GP64 protein. The transfection of cells with dsRNA-ie1 prevented GFP and GP64 production even at an m.o.i. of 1 (Fig. 3). Only a few cells per field showed both green and red fluorescence, because not all the cells were efficiently transfected (Fig. 3, bottom panel to the left). This result is also consistent with the findings using FACS. Transfecting the cells with dsRNA-PhP did not alter the GP64 and GFP fluorescence, showing values similar to those obtained from cells exposed to recombinant AcNPV-GFP alone (Fig. 3).
Southern blotting analysis illustrated in Fig. 4A confirmed the selective RNAi effect observed with dsRNAs. The upper panel shows the amount of GP64 and GFP proteins obtained at three different m.o.i. values (1, 5, and 10) in the absence of dsRNA. For GP64, two bands were observed reproducibly in all assays. These bands may represent different glycosylation forms of the protein previously described (14).

The second panel in Fig. 4A shows the GP64 and GFP protein contents from cells transfected with dsRNA-gfp 48 h prior to infection. Notice the reduction of GFP protein content at m.o.i. 1 and 5, whereas the GP64 protein content was unaltered. The third panel in Fig. 4A shows the effect of transfecting the cells with dsRNA-gp64 prior to viral infection. In this case, GP64 protein content was significantly reduced at an m.o.i. of 1. At m.o.i. 5 and 10 substantial GP64 protein was detected. These results are consistent with the data previously shown using FACS and confocal microscopy.

Finally, the lower panel of Fig. 4A illustrates the effect of transfecting cells with dsRNA-ie1. Under these conditions, both GFP and GP64 proteins were dramatically reduced at all m.o.i. values explored. Consistent with these findings, cells transfected with dsRNA-ie1 and later infected with AcNPV-GFP could be maintained in culture for several months, even at m.o.i. 10. Insect cells divided normally like uninfected cells. On the contrary, cells transfected with dsRNA-gp64 or dsRNA-gfp and infected with an m.o.i. of 5 or higher could not be maintained in culture more than a week. After 7 days in culture only cellular debris was observed in the culture, indicative of cell lysis as a result of the baculovirus infection.

To determine if the reduction in protein content in cells transfected with dsRNAs was the result of depletion of the specific mRNA, we performed RT-PCR experiments using primers specific for gp64, ie1, gfp, and β-actin from sf21 cells (see “Experimental Procedures”). Fig. 4B illustrates a representative experiment obtained at an m.o.i. of 1. Uninfected cells (NI) showed the presence of β-actin mRNA, and this was confirmed by sequence analysis of the fragment amplified by the RT-PCR experiment. As expected, in uninfected cells gfp, gp64, and ie1 were not detected. Interestingly, infection with recombinant baculovirus resulted in reduction of mRNA for β-actin. This result can be explained by the fact that, at the late phase of baculovirus infection, the majority of mRNAs are produced by the baculovirus α-aminotransferase RNA polymerase (15). The β-actin mRNA is observed again in cells that were interfered with dsRNA from gp64 and ie1. In these experiments we observed again the specificity of the RNAi effect; notice that interfering with gp64 (dsRNA-gp64 panel) significantly reduced the mRNA from gp64 but did not affect the ie1 mRNA. As expected, interfering with ie1 (dsRNA-ie1) significantly reduced the amount of gfp, gp64, and ie1 mRNAs, and β-actin levels were restored to normal due to the inhibition of viral infection. These results demonstrate that the reduction in protein content originates from the depletion of the respective mRNA.

Depleting the ie1 mRNA turned out to be a very efficient way to interfere with baculovirus infection. In fact, cells transfected with dsRNA-ie1 and later infected with AcNPV-GFP showed morphological characteristics of uninfected cells, as illustrated in Fig. 5. Panel A shows the typical morphology of Sf21 insect cells infected with recombinant AcNPV-GFP. Notice the large amount of viral particles inside the cell nucleus. In contrast, no viral particles could be detected in dsRNA-ie1-treated cells as illustrated in Fig. 5B. Purification of virions from supernatants of infected cells (see “Experimental Procedures”) allowed the observation of multiple viral particles (Fig. 5C). In contrast,
Silencing a Viral Infection with Double-stranded RNA

Viral particles were extremely difficult to find in supernatants obtained from dsRNA-ie1-treated cells (Fig. 5D). These viral particles did not show any reproducible characteristic that could separate them from particles isolated from dsRNA-ie1-untreated cells. These viral particles may come from the initial viral infection or could be produced by cells that were not efficiently transfected with dsRNA-ie1. These results are consistent with the low pfu obtained with the supernatant of dsRNA-ie1-treated cells (Fig. 2C). The very low titer found in the supernatant of dsRNA-ie1- and dsRNA-gp64-treated cells confirms the poor viral content observed with the electron microscope (Fig. 5D). Interestingly, it has been previously shown that GP64 glycoprotein is required for virus budding from the cell membrane (16). In fact, this glycoprotein is acquired by virions during budding through the plasma membrane of the infected insect cell, representing the final step in virus assembly (14).

Injecting Insects with dsRNA from gp64 or ie1 Prevents Baculovirus Infection in Vivo—To test if dsRNA from gp64 or ie1 could protect against AcNPV-GFP infection in vivo, we used the larvae from the insect T. mollitor as a bioassay. Injection in the hemolymph with 2 μl of AcNPV-GFP resulted in death of over 97% of the larvae within the following 7 days (68 dead of 70 injected). Dead insects showed high GFP fluorescence assessed by confocal microscopy (Fig. 6, inset box in the upper left corner of each panel) and high β-galactosidase activity, as illustrated in Fig. 6 (AcNPV-GFP, upper panel to the left). Uninfected larvae or larvae injected with distilled water did not show GFP fluorescence or β-galactosidase activity (H2O, upper panel to the right), and over 95% of the insects survived the injection (4 dead of 60 injected). Larvae injected with 0.1 μg of nude dsRNA-ie1 24 h prior to the injection with AcNPV-GFP survived the baculovirus infection (6 dead of 80 injected). These insects did not show GFP fluorescence or β-galactosidase activity. Similar results were obtained with larvae pretreated with 0.1 μg of nude dsRNA-gp64, where 10 insects died of the 61 injected with AcNPV-GFP (data not shown). In contrast, injection with 0.1 μg of nude dsRNA-PhP did not protect against the subsequent injection with AcNPV-GFP. As expected, over 97% of the larvae died within the subsequent 7 days (34 dead of 40 injected, data not shown). Similarly, insects pretreated with dsRNA-gf and later challenged with AcNPV-GFP died within the next 7 days (58 dead of 62 injected). Interestingly, these insects did not show GFP fluorescence.
(inset in the lower panel to the left) but did show high levels of \( \beta \)-galactosidase activity (lower panel to the left).

DISCUSSION

RNAi is a recently discovered phenomenon, which is rapidly becoming a powerful tool for selectively depleting mRNA, thus resulting in reduction of the specific protein. This phenomenon has been found in worms, flies, several insects, plants, and mammals (1). The RNAi molecular mechanism involves several protein complexes, including the RNA-induced silencing complex responsible for the degradation of the homologue mRNA and RNA-dependent RNA polymerases that synthesize new dsRNAs and amplify the RNAi signal (1). Proteins essential for the RNAi phenomenon such as Dicer and Argonaute are highly conserved in fungi, plants, and animals. Although the exact physiological role of RNAi in animals is not known to the present day, recent evidence suggests that RNAi may be involved in organism development, germ line fate, and host defenses against transposable elements and viruses (17).

Although it is not clear that RNAi may function as a defense mechanism in mammals, recent experimental evidence shows that modulation of HIV-1 replication in human cell lines by dsRNA is possible (5). In a different study, dsRNA conferred viral immunity in human cells against poliovirus (6). Whether this is a physiological function for RNAi or not, this recent discoveries open the possibility of using dsRNA to treat or prevent viral infections.

One important point that needs to be explored is whether the introduction of dsRNAs in mammals provides effective defense against systemic infections. The adequate distribution of the dsRNAs in the organism and the possible secondary effects of introducing dsRNAs in humans are two of several issues that would have to be solved before this technique is employed efficiently to prevent or treat viral infections. Interestingly, it has been recently shown that intravenous introduction of small interfering dsRNA in adult mice silences luciferase activity and endogenous Fas expression in vivo (18), which demonstrates that systemic application of dsRNA is feasible and effective for gene silencing in organisms.

In the present study we have used the rapidly replicating and highly cytolitic DNA virus A. californica in combination with the insect cell line S/21 from S. frugiperda to assess the protective role of dsRNA from genes essential for baculovirus
propagation. We show here for the first time that dsRNAs from segments of two genes essential for baculovirus propagation prevent virus infection in vitro and in vivo. We have demonstrated that this effect is specific for the dsRNA sequence used, because we can eliminate GFP fluorescence (one of the reporter genes present in the recombinant baculovirus) without affecting the time course of viral infection or the activity of β-galactosidase (the second reporter gene). Transfecting the insect cells with dsRNA from gp64 prevents viral infection when using low m.o.i. values. On the other hand, dsRNA from the ie1 gene prevented viral infection even when high m.o.i. values were utilized. Using the larva from the insect T. mollitor, we have shown that injecting dsRNAs from gp64 or ie1 confers resistance to recombinant baculoviruses. This protective effect was not obtained with dsRNA from gfp or the polyhedrin promoter, which were two dsRNA sequences used as controls. Even though injecting dsRNA from gfp prevented GFP fluorescence in insects, it did not stop viral infection by recombinant baculovirus. As far as we know, this is the first report showing the protective effects of RNAi in vivo against a viral infection in animals. The protective effects of dsRNA against viral infections have been previously observed in plants (3).

An interesting result from our studies is the high efficacy to silence genes obtained with long dsRNAs. It has been previously recognized that in nematodes and Drosophila, long dsRNA sequences are as effective as short dsRNA sequences contrary to mammals, where dsRNAs only 21 nucleotides long are functional. The highly efficient RNAi observed in the present study was obtained with a single transfection. This was possible after optimizing the type of lipids used for transfection and the amount of dsRNA used in the mixture (see “Experimental Procedures”). Previous studies using null mutants of gp64 gene have shown that the mutant baculovirus cannot be replicated, providing evidence of the important role-played by GP64 protein in cell-to-cell infection (8). Our results are in agreement with these original reports, because interfering with GP64 protein synthesis results in baculoviruses that cannot infect insect cells. Only when high m.o.i. values are used, where virus infection does not depend on generation of new progeny, did we observe normal GFP production and β-galactosidase activity from infected cells. However, even when these cells produced GFP and β-galactosidase, no GP64 protein was detected by confocal microscopy or Western blotting analysis. These results suggest that the new viral particles produced by the cells interfered with dsRNA-gp64 would lack GP64 protein. In agreement with this prediction, viruses collected from the supernatant of dsRNA-gp64-treated cells and infected with low m.o.i. values showed significantly reduced pfu as compared with control. The possibility of producing viral particles missing specific proteins provides a powerful tool for reverse genetics with viruses. Using this technique one could rapidly explore the role of single or multiple genes in virus morphogenesis, assembly, and transport. A rapid method for screening the role of specific proteins in virulence may be possible also. Several groups have shown genome-wide RNAi to deplete proteins from entire chromosomes in the nematode Caenorhabditis elegans (19). Such genome-wide RNAi could easily be implemented for entire virus genomes, especially with large complex genomes such as the A. californica 134-kb genome.

Finally, in the genomic era where scientists are gathering large amounts of information about the sequence of many genomes (20), we are learning that the next big challenge will be finding rapid and efficient tools to determine the role played by thousands of genes with unknown function. RNAi might be one of such tool (21).

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REFERENCES

1. Sharp, P. A. (1999) Genes Dev. 13, 139–141
2. Hammond, S. M., Caudy, A. A., and Hannon, G. J. (2001) Nat. Rev. Genet. 2, 110–119
3. Waterhouse, P. M., Wang, M. B., and Lough, T. (2001) Nature 411, 834–842
4. Finnegan, E. J., Wang, M., and Waterhouse, P. (2001) Curr. Biol. 11, R99–R102
5. Jacque, J. M., Triques, K., and Stevenson, M. (2002) Nature 418, 435–438
6. Gitlin, L., Karelsky, S., and Andino, R. (2002) Nature 418, 430–434
7. Jackson, J. A. (1991) Bioprocess. Technol. 13, 402–413
8. Monsma, S. A., Oomens, A. G., and Blissard, G. W. (1996) J. Virol. 70, 4607–4616
9. Kovacs, G. R., Guarino, L. A., and Summers, M. D. (1991) J. Virol. 65, 5281–5288
10. Timmons, L., and Fire, A. (1998) Nature 395, 854
11. Reyes-Cruz, G., Vazquez-Prado, J., Muller-Esterl, W., and Vaca, L. (2000) J. Cell. Biochem. 76, 658–673
12. Molinari, J. L., Tato, P., Rodriguez, D., Solano, S., Rubio, M., and Sepulveda, J. (1998) Parasitol. Res. 84, 173–180
13. Perfiri, E., Evans, T., Bollag, G., Clark, R., and Hancock, J. F. (1995) Methods Enzymol. 255, 13–21
14. Oomens, A. G., Monsma, S. A., and Blissard, G. W. (1995) Virology 209, 592–603
15. Huh, N. E., and Weaver, R. F. (1999) J. Gen. Virol. 71, 195–201
16. Oomens, A. G., and Blissard, G. W. (1999) Virology 254, 297–314
17. Dernburg, A. F., and Karpen, G. H. (2002) Cell 111, 159–162
18. McCaffrey, A. F., Meuse, L., Pham, T. T., Conklin, D. S., Hannon, G. J., and Kay, M. A. (2002) Nature 418, 38–39
19. Maeda, F., Kohara, Y., Yamamoto, M., and Sugimoto, A. (2001) Curr. Biol. 11, 171–176
20. Ueda, R. (2001) J. Neurogenet. 15, 193–204
21. Kawasaki, P. E., and Coulson, A. (2000) Parasitol. Today 16, 347–349
