Inhibition of iridovirus protein synthesis and virus replication by antisense morpholino oligonucleotides targeted to the major capsid protein, the 18 kDa immediate-early protein, and a viral homolog of RNA polymerase II

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Abstract

Frog virus 3 (FV3) is a large DNA virus that encodes ∼100 proteins. Although the general features of FV3 replication are known, the specific roles that most viral proteins play in the virus life cycle have not yet been elucidated. To address the question of viral gene function, antisense morpholino oligonucleotides (asMOs) were used to transiently knock-down expression of specific viral genes and thus infer their role in virus replication. We designed asMOs directed against the major capsid protein (MCP), an 18 kDa immediate-early protein (18K) that was thought to be a viral regulatory protein, and the viral homologue of the largest subunit of RNA polymerase II (vPol-IIα). All three asMOs successfully inhibited translation of the targeted protein, and two of the three asMOs resulted in marked phenotypic changes. Knock-down of the MCP resulted in a marked reduction in viral titer without a corresponding drop in the synthesis of other late viral proteins. Transmission electron microscopy (TEM) showed that in cells treated with the anti-MCP MO assembly sites were devoid of viral particles and contained numerous aberrant structures. In contrast, inhibition of 18K synthesis did not block virion formation, suggesting that the 18K protein was not essential for replication of FV3 in fathead minnow (FHM) cells. Finally, consistent with the view that late viral gene expression is catalyzed by a virus-encoded or virus-modified Pol-II-like protein, knock-down of vPol-IIα triggered a global decline in late gene expression and virus yields without affecting the synthesis of early viral genes. Collectively, these results demonstrate the utility of using asMOs to elucidate the function of FV3 proteins.

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Introduction

Members of the family Iridoviridae comprise a diverse group of large, double-stranded DNA-containing viruses that include two genera infecting invertebrates (Iridovirus and Chloriridovirus) and three genera targeted to cold-blooded vertebrates (Ranavirus, Lymphocystivirus, and Megalocytivirus) (Chinchar et al., 2005). FV3, the type species of the genus Ranavirus, is the best characterized member of this family, and its study has elucidated the basic features of iridovirus replication (Willis et al., 1985; Chinchar, 2002; Williams et al., 2005). The complete genomic sequences of eleven iridoviruses have been determined including those for FV3 and four additional members of the genus Ranavirus: tiger frog virus (TFV), Singapore grouper iridovirus (SGIV), grouper iridovirus (GIV), and Ambystoma tigrinum virus (ATV) (He et al., 2002; Jancovich et al., 2003; Tan et al., 2004; Song et al., 2004; Tsai et al., 2005). Sequence
analysis indicates that ranaviruses contain ∼100 open reading frames (ORFs) with the potential to encode proteins of >40 amino acids. Approximately one quarter of the ORFs encode proteins of known or presumed function, e.g., the major capsid protein, the viral DNA polymerase, and homologs of ribonucleotide reductase and the two largest subunits of RNA polymerase II. However, more than half of the remaining ORFs encode presumptive proteins with no other matches in the database except to those of other iridovirus genes. Although their function is not known, these ORFs likely encode polypeptides important for ranavirus replication and survival since they are conserved in other family members.

Previous attempts to elucidate FV3 gene function through the use of temperature-sensitive and drug-resistant mutants have met with limited success (Chinchar and Granoff, 1984, 1986). Earlier work identified two complementation groups (CGs) involved with viral DNA synthesis, five CGs with roles in assembly site formation and late gene expression, and 12 CGs that synthesize all classes of viral protein and DNA but fail to assemble infectious virions (Chinchar and Granoff, 1986). Study of these and other mutants elucidated the two-stage model of viral DNA synthesis (Goorha et al., 1981; Goorha and Dixit, 1984). However, attempts at targeting specific genes have been unsuccessful because, unlike poxviruses and herpesviruses, it is not yet possible to introduce mutations into iridovirus genomes by homologous recombination. To circumvent this problem and determine the function of specific viral genes, we describe here a “knock-down” strategy using asMOs.

asMOs are a class of DNA-like agents composed of purines and pyrimidines linked to 6-member morpholino rings and joined via phosphorodiimideate bonds (Summerton and Weller, 1997; Corey and Abrams, 2001; Summerton, 2003). Because of their unique structure, asMOs resist nuclease degradation and are stable through several rounds of cell division (Dagle and Weeks, 2001). They have been used extensively to knock-down gene expression in developing embryos (Sumanas and Ekker, 2001) and have been shown to inhibit gene expression in cultured cells (Munshi et al., 2002) and viruses (Neuman et al., 2004; Kinney et al., 2005; Deas et al., 2005; Van den Born et al., 2005). Moreover, nearly every asMO targeted to the region immediately surrounding, or upstream, of the translational initiation codon, is able to block gene expression by inhibiting translation (Ekker and Larson, 2001). In addition to blocking viral protein synthesis, asMOs targeted to important control regions have been shown to inhibit viral replication. Here, we describe the use of this approach to determine the role of three FV3 proteins in virus replication: the major capsid protein (MCP), the 18 kDa immediate-early protein (18K), and the largest subunit of the viral homologue of RNA polymerase II (vPol-IIα).

Results

Inhibition of FV3 MCP synthesis

To determine the effect of targeted asMO treatment on FV3 gene expression, we initially focused on the viral MCP. The MCP was chosen because it is the most abundant viral protein and is readily visualized following one-dimensional electrophoresis on SDS-polyacrylamide gels. Lightly confluent monolayers of FHM cells were left untreated, or treated with Endo-Porter (EP) alone, a control MO (CTRL) that does not bind any known message sequence, or an anti-sense MO targeted to the MCP mRNA (anti-MCP MO). The cultures were incubated overnight at 26 °C, and ∼24 h later, they were infected with FV3. One set of replicate cultures was radiolabeled from 7 to 9 h p.i. with [35S]methionine, and viral protein synthesis monitored by SDS-polyacrylamide gel electrophoresis (SDS-PAGE), and viral yields were determined in a replicate set of samples at 24 h p.i. Gel analysis of viral protein synthesis showed a marked drop in MCP expression in the presence of the anti-MCP MO (Fig. 1, lane 4). In contrast, a slight drop in MCP synthesis was seen when FV3-infected cells were treated with EP alone (lane 3) or with a control MO that does not target any known FV3 sequence (lane 5). Phosphorimager analysis of three representative experiments showed that treatment of FV3-infected FHM cells with anti-MCP MO resulted in an ∼80% reduction in MCP synthesis (Fig. 1B). In contrast, exposure to the control MO or to EP alone resulted in only an ∼20% reduction in MCP synthesis. Determination of virus yields from three independent experiments showed a 94% reduction in virus yields compared to untreated FV3-infected cells (Fig. 2). These results clearly indicate that asMOs targeted against the FV3 MCP transcript markedly and specifically reduced synthesis of the cognate protein. Moreover, this study confirmed our expectation that inhibition of MCP synthesis adversely affected FV3 virion formation.

To further examine the effect of inhibiting MCP synthesis, the effects of asMO treatment were examined by transmission electron microscopy. As shown in Fig. 3, FV3 infection induced marked CPE in FHM cells. Consistent with virus replication, nuclei within infected cells showed marked chromatin condensation and margination (compare panel A, mock-infected cells, with panel B, FV3-infected cells). Chromatin condensation and margination were apparent in productively infected cells (panel B) as well as in cells in which virus replication was arrested by treatment with anti-MCP MO (panel C). Chromatin condensation is a hallmark of apoptosis and confirms our earlier study showing that FV3-infected cells undergo apoptosis following productive or non-productive infection of FHM cells (Chinchar et al., 2003). Morphologically distinct viral assembly sites containing non-enveloped virus particles, para-crystalline arrays of virus particles, and virions budding from the plasma membrane were observed in productively infected cells (Fig. 3B). Virion formation was also clearly seen in FV3-infected cells treated with EP alone (Fig. 5A) or with a control MO (data not shown). In contrast, FHM cells pretreated with the anti-MCP MO prior to infection assembled few if any viral particles (Figs. 3C and D). Although viral assembly sites were present in cells treated with anti-MCP MO, they did not contain characteristic icosahedral virions, but rather displayed a series of circular, bent, and elongated structures (designated “atypical elements,” AE) that may represent aberrant or intermediate products of viral assembly (Figs. 3C and D). Collectively, these
results suggest that the MCP is not required for assembly site formation, but that its presence is required for virion morphogenesis.

Having shown that asMOs are capable of blocking the synthesis of specific viral proteins without triggering off-targeting effects such as those sometimes observed following treatment with short, interfering RNAs (siRNA), we turned our attention to two viral gene products whose functions have yet to be elucidated, an abundant 18 kDa immediate-early protein (18K), and the largest subunit of the viral homologue of host RNA polymerase II (vPol-IIα).

Inhibition of 18 K synthesis does not affect late gene expression or viral assembly

The FV3 18 kDa immediate-early protein is a relatively abundant protein and, by analogy to immediate-early proteins in other virus families, was thought to play an important regulatory role in FV3 biogenesis (Willis et al., 1984). However, in the absence of known mutants, its precise function in FV3 replication is not known. To ascertain the role of the 18K protein in FV3 morphogenesis, we exposed FHM cells to an anti-18K MO, and 24 h later infected them with FV3. As before, replicate cultures were monitored by SDS-PAGE of viral proteins radiolabeled from 7 to 9 h p.i., transmission electron microscopy, and by determining viral yields at 24 h p.i. As shown in Fig. 4A, treatment of cells with anti-18K MO blocked the synthesis of the 18 kDa protein (lane 5). Synthesis of the 18K protein was not affected by treatment with EP alone (lane 4), or a control asMO (lane 6). EP did not affect translation in mock-infected cells (compare lanes 1 and 2). Contrary to our expectations, inhibition of 18K synthesis had no effect on the synthesis of non-targeted viral proteins (Fig. 4A) nor did it reduce virus yields beyond that seen following treatment with EP alone or EP plus the control MO (Fig. 2) suggesting that, at least in FHM cells, the 18K protein did not play an essential role in virus replication. Quantification of 18K yields in three independent experiments showed that treatment with the anti-18K MO resulted in an ~80% reduction in its synthesis (Fig. 4B). In contrast, treatment with EP alone, a control MO, or the anti-MCP MO reduced translation of 18K by only 20–30%. Transmission electron micrographs (TEM) of treated cells subsequently infected with FV3 showed that anti-18K MO affected neither assembly site formation nor virion biogenesis (Figs. 5C and D). Inspection of electron micrographs showed that FV3-infected cells treated with the anti-18K MO were essentially identical to untreated, or EP-treated, FV3-infected cells (compare Figs. 3B and 5A with Fig. 5C). Taken together, the above results indicate that synthesis of the 18K protein is not required for virus replication in FHM cells and do not support the suggestion that 18K is an essential regulatory factor.

Fig. 1. Effect of anti-MCP MO on protein synthesis in FV3-infected cells. (A) Gel analysis. Lightly confluent monolayers of FHM cells were exposed to Endo-Porter (EP) alone or EP plus the indicated asMOs and incubated overnight. Twenty four hours later, replicate cultures were infected with FV3 at an MOI of 20 PFU/cell and viral proteins were radiolabeled from 7–9 h.p.i. with [35S] methionine. Radiolabeled viral proteins were separated by electrophoresis on 15% SDS-polyacrylamide gels and visualized by autoradiography. The molecular weights of marker proteins (in kDa) are shown to the left of the gel, and the position of the MCP is indicated by an arrow. Untreated, mock-infected FHM cells are shown in lane 1. The panel above the figure indicates whether the cultures were infected or not, the presence of the transfection reagent, and the identity of the MO. (B) Phosphorimager analysis. The level of MCP synthesis was determined by Phosphorimager analysis following treatment of FHM cells with EP alone, a control MO, or the anti-MCP MO. The level of MCP synthesis in untreated, FV3-infected FHM cells is indicated in the column marked “none.” The results of three independent experiments are shown.
Inhibition of late viral protein synthesis follows targeting of vPol-IIα

Although the precise mechanisms of FV3 RNA synthesis are not known, the current model suggests that synthesis of immediate-early (IE) viral mRNA is catalyzed in the nucleus by host RNA polymerase II in a reaction-dependent upon one or more virion proteins (Chinchar, 2002; Williams et al., 2005). Later, either within the cytoplasm of infected cells or within viral assembly sites, a virus-modified, or a novel virus-encoded polymerase, transcribes late (L) viral messages. Sequencing studies indicate that iridoviruses encode proteins with marked homology to the two largest subunits of host RNA polymerase II and support the view that late viral transcription is catalyzed by a novel virus-encoded, or virus-modified, RNA polymerase (Jancovich et al., 2003; Tan et al., 2004; Williams et al., 2005). While plausible, formal proof of this hypothesis has heretofore been lacking. To determine if the viral homologue of the largest subunit of Pol II is required for late viral gene expression, we targeted vPol-IIα production using an anti-vPol-IIα asMO. We reasoned that, if vPol-IIα expression were
inhibited, we should detect a marked inhibition of late viral protein synthesis without a corresponding decrease in early viral gene expression.

To determine the effect of vPol-IIα knock-down on late viral gene expression, FHM cells were treated or not with asMOs, and 24 h later infected with FV3. Cultures were radiolabeled from 7 to 9 h p.i., and viral proteins analyzed by SDS-PAGE. In these experiments, four different MOs were used: one that was antisense to the MCP transcript (positive control), another positive control that targeted the 18K protein, a control MO that does not target any known message (negative control), and one that targeted the vPol-IIα message. As shown in Fig. 6A, late gene expression was clearly evident in untreated FV3-infected cells as indicated by the presence of the MCP as well as several other characteristic late proteins (lane 2). As noted previously, treatment with EP alone (lane 3) or with the control MO (lane 7) had a slight inhibitory effect on both early and late viral gene expression. As expected, treatment with asMOs targeted to the MCP and 18K proteins blocked their synthesis, but had little to no effect on the production of other viral proteins (lanes 4 and 6, respectively). In contrast, treatment with an asMO targeted to vPol-IIα (lane 5) had a marked effect on late gene expression (indicated by the asterisks) while showing little if any inhibition of early viral genes. Early gene expression (represented by the bands marked E1, E2, and 18K) was as high, if not higher, than that seen in FV3-infected cells treated with the anti-MCP MO (compare lanes 4 and 5), whereas late gene expression was markedly inhibited following exposure to anti-vPol-IIα MO. Phosphorimager analysis of three independent experiments confirmed these expectations. Synthesis of vPol-IIα was reduced by ∼80% after treatment with anti-vPol-IIα MO (Fig. 6B). In addition, MCP expression was reduced by ∼70% following treatment with anti-vPol-IIα (Fig. 6C), whereas 18K expression was slightly elevated in cells treated with anti-vPol-IIα (Fig. 6D). Consistent with the drop in late gene expression, treatment of FV3-infected cells with an anti-vPol-IIα MO reduced viral yields by ∼98% (Fig. 2). In support of these studies, TEM analysis confirmed that treatment of FV3-infected cells with the anti-vPol-IIα MO inhibited virion formation, but not assembly site formation (Fig. 5B). Treated cells showed large, clearly defined assembly sites that often contained an electron dense area that likely corresponds to viral DNA. Despite the marked reduction in MCP synthesis, assembly sites within anti-vPol-IIα treated cells did not show the AEs seen following treatment with an anti-MCP MO, suggesting that their formation depends on the presence of additional late proteins besides the MCP. Collectively, these biochemical and ultrastructural results are consistent with the hypothesis that the largest subunit of the viral homologue of Pol II plays a key role in late viral gene expression.

Discussion

The aforementioned results indicate that asMOs are appropriate tools for transiently and specifically inhibiting (i.e., knocking down) expression of ranavirus genes. As with siRNAs, they offer the potential of selectively inhibiting the synthesis of individual viral gene products and assessing the effect of inhibition on viral biogenesis. However, whereas sequences targeted by siRNAs may be found anywhere within a given message, asMOs targeted to the region preceding, or immediately surrounding, the initiation codon readily inhibit
Furthermore, while siRNAs inhibit translation by triggering the degradation of the targeted message (Hannon, 2002; Meister and Tuschl, 2004), asMOs bind mRNA and inhibit translation by impeding progression of the 40S ribosomal complex (Ekker and Larson, 2001). Although properly designed asMOs block translation of targeted messages, one disadvantage is that assessment of their effectiveness requires detecting a decrease in the synthesis of the targeted protein either by SDS-PAGE, immune precipitation, or Western blotting. Thus, if the targeted protein cannot be identified by one of the three aforementioned techniques, and if a marked alteration in viral macromolecular synthesis or yield cannot be observed, it would not be possible to determine if the asMO failed to inhibit synthesis of the targeted protein or if the protein in question is non-essential for virus replication. As shown above, we were able to specifically inhibit the translation of three viral messages and infer their function by biochemical and ultrastructural changes in treated, infected cells.

Our results following targeting of the MCP of FV3 are similar, but not identical, to those of Xie et al. (2005) who showed, using short hairpin RNAs (shRNAs) and siRNAs targeted to the MCP of tiger frog virus (TFV), that degradation of mRNAs encoding the MCP of TFV was accompanied by corresponding declines in cytopathic effect and viral yields. While our results using an anti-MCP MO confirm the drop in virus titer seen by Xie et al., we observed that treatment with anti-MCP MOs resulted in the generation of atypical elements (AEs) within viral assembly sites. It is not clear if these structures represent assembly intermediates that accumulate in the absence of wild-type levels of MCP synthesis or if they are aberrant structures that are dead end products of virion assembly. Low levels of similar AEs are sometimes seen in cells productively infected with ranaviruses (Huang et al., 2006; AD Hyatt, personal communication). It is not clear why AEs were seen following anti-MCP MO treatment but were not detected following exposure to an siRNA or shRNA targeted against the MCP. It is unlikely that the difference is due to the fact that Xie et al. (2005) studied TFV and we used FV3 since these are very similar viruses that may represent strains of the same viral species (Chinchar et al., 2005). Despite these differences, these proof-of-concept experiments demonstrate the utility of using antisense technologies to elucidate the function of individual viral proteins. However, one limitation common to both antisense methodologies is that they are only effective against proteins...
that are essential for virus replication. If a viral gene product (e.g., an immune evasion protein) is required only for growth in vivo, or if a viral protein is only needed for replication in some cell lines (e.g., a host range, or efficiency function), then asMOs and siRNAs may not reduce viral yields in vitro. Despite this drawback, asMOs and siRNAs will likely provide complimentary methodologies to inhibit the synthesis of specific viral genes in vitro and thus infer their function.

The results reported herein demonstrate that asMOs targeted to specific viral mRNAs inhibit translation of those messages and result in various downstream effects ranging from no apparent effect, in the case of the anti-18K asMO, to the inhibition of late viral gene expression and virion assembly, in the case of the anti-vPol-IIα and anti-MCP asMOs, respectively. Specifically, results using an asMO targeted to the largest subunit of the viral homolog of Pol II provide the first supportive evidence that a virus-encoded or virus-modified polymerase is responsible for late ranavirus gene expression. However, it is not known if the polymerase responsible for transcribing late viral messages is a completely virus-encoded enzyme or a chimera of...

Fig. 6. Effect of anti-vPol-IIα MO on viral gene expression. (A) SDS-polyacrylamide gel electrophoresis. FHM cells were treated overnight with the indicated combinations of EP and MOs and mock-infected (lane 1) or infected with FV3 (lanes 2–7). Protein synthesis was monitored from 7 to 9 h p.i. by labeling with [35S]methionine and separating the proteins by electrophoresis on 15% SDS-polyacrylamide gels. The positions of the MCP (a late protein) and 18K (an early) protein are indicated, as are the positions of two other early proteins (designated E1 and E2), and four late proteins identified by asterisks. Molecular weight markers are shown to the left of the figure. (B–D) Phosphorimager analysis. The effects of treatment with the indicated antisense morpholino oligonucleotides on MCP synthesis (B), 18K synthesis (C), and vPol-IIα synthesis (D) are shown. The results of three independent experiments are shown.
host and viral subunits. Moreover, it remains to be determined (1) if late in infection the transcription of early viral genes is also catalyzed by the novel polymerase, (2) in which cellular compartment (nucleus, cytoplasm, or viral assembly sites) the novel polymerase is found, and (3) the composition of the viral polymerase. To answer these questions, we are currently attempting to develop an antibody against vPol-IIα so that we might determine its cellular location and molecular composition. The observation that viral assembly sites form in cells pretreated with anti-vPol-IIα (Fig. 5B) indicates that assembly site formation does not depend on late gene expression and likely requires one or more early gene products in addition to viral DNA. The large size of the assembly sites in treated cells is surprising given that virion assembly is not taking place. The electron dense central core seen in Fig. 5B may represent viral DNA, but it remains to be determined if viral DNA synthesis continues in treated cells in the absence of virion assembly. Clearly, enlargement of assembly sites does not depend upon ongoing virion assembly and may depend on other factors such as continued viral DNA synthesis or the presence of one or more early viral proteins. Furthermore, the absence of the AEs within cells treated with anti-vPol-IIα suggests that their formation is not due solely to the absence of the MCP since MCP synthesis is inhibited to comparable levels by both anti-MCP MO and anti-vPol-IIα inhibited to comparable levels by both anti-MCP MO and anti-vPol-IIα. Thus, it appears that at least one other late protein (whose synthesis is inhibited by anti-vPol-IIα) is required for formation of AEs.

While results with asMOs targeted to the MCP and vPol-IIα confirmed previous models of ranavirus replication, the results obtained with an asMO targeted to the 18K immediate-early protein were unexpected. Earlier work showed that 18K was a relatively abundant protein that was expressed early in ranavirus infection, and under conditions where ranavirus DNA and late protein synthesis was inhibited (Willis et al., 1984, 1985). While the function of 18K was unknown, its abundance and temporal class suggested that it was likely an important regulatory protein. For example, the four alpha (i.e., immediate-early) proteins of herpes simplex virus type 1 are regulatory proteins that are required for the synthesis of beta (delayed early) and gamma (late) genes (Roizman and Sears, 1996). If 18K were to function in an analogous manner, one would have expected that inhibition of its synthesis should have resulted in numerous downstream effects, including the inhibition of late gene expression. Instead, we observed that, with the exception of the 18K protein itself, viral protein synthesis was not adversely affected by pretreatment with the anti-18K MO. Thus, at least in FHM cells, this protein appears to be non-essential for replication. BLAST analysis of the FV3 18K protein indicates that it is conserved among members of the genus Ranavirus, but is not found among other genera (Lymphocystivirus, Megalocytivirus, or Iridovirus) within the family. Among ranaviruses, 18K sequence identity/similarity ranges from 46 to 100%. PsiBLAST analysis (Altschul et al., 1997) detected limited sequence identity/similarity to an intercapsomeric triplex protein of carp pox herpesvirus and the adenovirus hexon protein, suggesting that 18K may be a virion protein. Attempts to determine if the domain structure of 18K was present in other proteins were unsuccessful, that is, there were no “hits” using the Conserved Domain Architecture Retrieval Tool (CDART) or Conserved Domain Searches within NCBI. Since there are several virion proteins within the size range of 18K, confirmation that 18K is a bona fide virion protein must await the development of specific antisera. Collectively, these data indicate that 18K is a protein unique to the genus Ranavirus and further suggests that it might be a virion-associated protein that is only required in some cell lines, or a viral protein that is only required for growth in vivo.

Comparison of virion morphogenesis, viral protein synthesis, and viral yields showed that EP, and EP plus a control MO, did not prevent the formation of viral assembly sites (Fig. 5A and data not shown), but did trigger a modest (~20–30%) drop in protein synthesis and a more marked reduction (~65%) in virus yields (Fig. 2). We do not know the basis for this differential affect on protein synthesis and viral yields, but speculate that it may involve interaction of the transfection reagent with the cell membrane. It should be noted that the inhibitory effect triggered by the three specific asMOs was markedly greater than the non-specific effect induced by EP or EP plus the control MO.

The methodology outlined above, in conjunction with siRNA approaches currently under development for FV3, should allow us to assess the roles of iridovirus genes of unknown function in vitro. Because gene “knock-out” experiments are labor-intensive and the technology (i.e., homologous recombination) required to generate viral knock-outs has not yet been successfully applied to iridoviruses, alternate means of assessing gene function are needed. asMOs could fill this gap and provide a straightforward way to screen genes of interest and determine their roles in virus replication. Since iridoviruses straddle the middle ground between the cytoplasmic poxviruses and nuclear viruses such as herpesviruses (Iyer et al., 2001; Allen et al., 2005), elucidating the function of gene products that mediate viral biogenesis should not only advance understanding of ranavirus biology, but also further our understanding of viral evolution (Stasiak et al., 2003).

Methods

Cells and virus

FV3 was propagated in fathead minnow cells (FHM) grown in Dulbecco’s modified Eagle’s medium (DMEM) containing 4% fetal calf serum (D4) at 26 °C in a humidified incubator in 95% air and 5% CO₂. Viral titers were determined by TCID₅₀ assay on FHM monolayers.

Antisense morpholinos

asMOs and delivery agents were purchased from GeneTools (Philomath, OR) and were used according to the manufacturer’s protocol with slight modification. Two different types of morpholino oligonucleotides and delivery agents were utilized. In our initial experiments, special delivery (SD) asMOs were used along with the delivery agent ethoxylated polyethyleneimine (EPEI, Morcos, 2001). SD asMOs consist of a pre-paired duplex of an asMO and a partially complementary DNA.
Table 1  
Morpholino oligonucleotides

<table>
<thead>
<tr>
<th>Oligonucleotide</th>
<th>Sequence (5'→3')</th>
<th>Position*</th>
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<tr>
<td>Anti-18Kb</td>
<td>GCA CAG GTA GGC TTG GAT CAT GGC C</td>
<td>+2→+27</td>
</tr>
<tr>
<td>Anti-Pol-IIb</td>
<td>AGA TTT AGA TGC AAA CAT TTC CAT G</td>
<td>-1→+24</td>
</tr>
<tr>
<td>Anti-MCPb</td>
<td>TGA ACC AGT TAC AGA AGA CAT TTC C</td>
<td>-4→+21</td>
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<tr>
<td>CTRL MOc</td>
<td>CCT CTT ACC TCA GTT ACA ATT TAT A</td>
<td>Not applicable</td>
</tr>
</tbody>
</table>

* The position of the morpholino is given relative to the translational start codon with the A of the AUG start codon designated as +1. The sequence complementary to the start codon is underlined.

b These morpholino oligonucleotides are antisense to viral mRNA and will inhibit translation by binding the message.

c This is a control MO that does not target any known sequence and thus serves as a negative control.

FV3 protein synthesis and virus yields

FHM cells were grown as lightly confluent monolayers (∼1.6×10⁶ cells/plate) in 35 mm tissue culture dishes. On the day of assay, the media was removed from each well by aspiration and replaced with 1 ml of D4. Subsequently, 8 μl of Endo-Porter and 20 μl of the indicated asMO (final concentration 10 μM) were added, and the cultures incubated at 26 °C overnight. The following day (∼24 h after treatment with asMOs), the cultures were infected with FV3 at a MOI of ∼20 PFU/cell. Replicate cultures were radiolabeled from 7 to 9 h post-infection (p.i.) in methionine-free Eagle’s minimum essential medium with Earle’s salts containing 20 μCi/ml [35S] methionine (Redivue Pro-mix, GE Healthcare). At 9 h p.i., the radiolabeling medium was removed, and cell lysates were prepared by disrupting the cells in 300 μl Direct Sample Buffer (125 mM Tris–HCl, pH 6.8, 10% glycerol, 2% SDS, 0.02% 2-mercaptoethanol, 0.01% bromophenol blue). Radiolabeled proteins were separated by electrophoresis on 12 or 15% SDS-polyacrylamide gels (Laemmli, 1970) and visualized by autoradiography using Kodak XAR film. The level of target protein inhibition was determined using a Molecular Dynamics Fast Scan Series 400 Phosphorimager and Image Quant software version 5.2 (GE Healthcare). To control for differences in loading, the final values were normalized based on the levels of two non-targeted viral proteins. Normalization could not be performed on cells treated with the anti-vPol-IIb MO because, as stated below, late protein synthesis was globally inhibited, and early protein synthesis was slightly enhanced. To determine virus yields, replicate cultures were infected at an MOI of ∼5 PFU/cell. After allowing 1 h for adsorption, non-attacked virus was removed by washing and the cultures incubated at 26 °C in D4. The infection was stopped at 24 h p.i., and the samples were frozen and thawed three times to release cell-associated virions, clarified by centrifugation, and the virus titers determined by TCID₅₀ assay. TCID₅₀ assays were performed in 96-well plates using confluent FHM monolayers. Virus was serially diluted 10-fold and after ∼7–10 days TCID₅₀ titers were determined using the Karber method (Blake and O’Connell, 1993).

Transmission electron microscopy

Replicate cultures (2, 60 mm dishes/sample) were treated with asMOs as previously described and infected at an MOI of ∼20 PFU/cell. At 9 h p.i., the media was removed, and cells were rinsed gently with 0.1 M sodium phosphate, pH 7.4. The cells were scraped into fresh buffer, pelleted, fixed in 2% glutaraldehyde in 0.1 M sodium phosphate buffer, and stored at 4 °C until processed for electron microscopy. At that time, the samples were post-fixed in 1% osmium tetroxide and stained with 2% uranyl acetate in de-ionized water. The samples were dehydrated in a stepwise fashion in ethanol, the ethanol was removed by treatment with 100% propylene oxide, and the samples were post-fixed in 1% osmium tetroxide and stained with 2% uranyl acetate in de-ionized water. The samples were floated onto 200 mesh copper hex grids (Electron Microscopy Sciences, Fort Washington, PA), counter-stained with lead citrate, and examined using a Leo 906 (Zeiss) transmission electron microscope.

Statistical analysis

Viral yields achieved in three separate experiments were analyzed using a randomized block design ANOVA to assess differences in titers among treatment categories. To determine statistical significance at the α=0.05 level, pairwise treatment comparisons were made using REGWQ, a statistical method shown to outperform other multiple range tests (Ramsey, 1978). For illustrative purposes, simultaneous 95% confidence intervals were displayed using Fisher’s least-significant-difference test (LSD) since multiple range tests, such as REGWQ, do not estimate confidence intervals. It should be noted the results from REGWQ and LSD analyses are in agreement.

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References


