A Potent and Specific Morpholino Antisense Inhibitor of Hepatitis C Translation in Mice

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Hepatitis C virus (HCV) is an RNA virus infecting one in every 40 people worldwide. Current treatments are ineffective and HCV is the leading cause of liver failure leading to transplantation in the United States and Europe. Translational control of HCV is a prime therapeutic target. We assessed the inhibitory potential of morpholino phosphoramidate antisense oligonucleotides (morpholinos) on HCV translation by codelivering them with reporter plasmids expressing firefly luciferase under the translational control of the HCV internal ribosome entry site (IRES) into the livers of mice. Real-time imaging of HCV IRES luciferase reporter messenger RNA (mRNA) translation in living mice showed that a 20-mer complementary to nucleotides 345-365 of the IRES inhibited translation by greater than 95% for at least 6 days and showed mismatch specificity. No significant nonspecific inhibition of a cap-dependent luciferase or encephalomyocarditis virus (EMCV) IRES luciferase reporter translation was observed. Inhibition by the 20-mer morpholino was dose dependent, with 1 nmol/mouse giving the highest inhibition. In conclusion, morpholino antisense oligonucleotides are potent inhibitors of HCV IRES translation in a preclinical mouse model; morpholinos have potential as molecular therapeutics for treating HCV and other viral infections. The *in vivo* model described is a broadly applicable, straightforward, and rapid readout for inhibitor efficacy. As such, it will greatly facilitate the development of novel therapeutic strategies for viral hepatitis. Notably, the level of antisense inhibition observed in this *in vivo* model is similar to the maximal inhibition we have obtained previously with RNA interference in mice. (HEPATOLOGY **2003;38:503-508.**)

Cap-independent translation of the hepatitis C virus (HCV) polyprotein initiates at a highly structured internal ribosome entry site (IRES) at the 5′ end of the genomic RNA. The conserved HCV IRES is a likely target for effective therapeutic intervention. Extensive effort has been devoted to the development of antisense oligonucleotide,[1-7] ribozyme,[8-10] and DNA ribonuclease[11] inhibitors targeting HCV IRES sequences that are accessible for nucleic acid hybridization.

Although *in vitro* assays have been invaluable in the early evaluation of inhibitors, work by our group[12] and that of others[13,14] suggests that cell-free systems may not faithfully recapitulate translation from the HCV IRES. A recent report that HCV IRES translation varies with cell cycling[15] also raises concern that rapidly dividing cells in tissue culture may not accurately model the normally quiescent human liver. Therefore, model systems that allow evaluation of pharmacokinetic parameters such as inhibitor bioavailability, toxicity, and systemic clearance in the complex milieu of a mammalian organism would be useful. Preclinical small animal models for testing nucleic acid inhibitors of HCV will help move candidate inhibitors into the clinic. Recently, a mouse xenotransplantation model was reported that supports HCV viral replication in transplanted human liver cells.[16] Although very exciting, this system requires access to fresh human liver tissue. A vaccinia virus expressing a HCV IRES luciferase fusion also has been used as a model system for evaluating antisense inhibitors *in vivo*.[4] In this model, a phosphorothioate and a 5-methylcytidine antisense oligonucleotide moderately reduced expression from the HCV IRES (~50% and 80%). However, nonspecific effects

Abbreviations: HCV, hepatitis C virus; IRES, internal ribosome entry site; morpholinos, morpholino phosphoramidate antisense oligonucleotides; EMCV, encephalomyocarditis virus; mRNA, messenger RNA; MM, mismatched bases; RNAi, RNA interference.

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Supported by National Institutes of Health grants AI-99-007 (M.A.K.) and R24CA92862 (C.H.C.), and the Hutchison Program for Translational Research, Stanford University, CA.

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0270-9139/03/3802-0028$30.00/0
doi:10.1053/jhep.2003.50330

were observed with a control phosphorothioate oligonucleotide and nontarget sequences were inhibited by the 5-methylcytidine antisense oligonucleotide. Because of nonspecific effects such as these, there is considerable interest in testing new backbone chemistries, such as morpholinos, which could be more specific.

Morpholinos are a promising, third-generation, antisense molecule that possess favorable hybridization, nuclease stability, and toxicity profiles.[17] Morpholinos do not prevent gene expression by activation of RNase H, rather, they function by translational arrest,[17] making them well suited for targeting the HCV IRES. Recently, a morpholino oligonucleotide complementary to start codon region of the HCV IRES was shown to reduce translation in rabbit reticulocyte lysates by greater than 80%.[7] To date, however, there have been few reports of morpholino efficacy in mammals.[18] A small animal model that uses a noninvasive assay for rapid and repeated measurements of HCV IRES inhibition would greatly accelerate the *in vivo* evaluation of morpholino oligonucleotides as therapeutic. For this purpose we used *in vivo* bioluminescent imaging to assess translation from *in vivo* transfected plasmids. The applicability of this methodology extends beyond the development of HCV inhibitors because antisense inhibitors against any target sequence could be evaluated rapidly using this accessible imaging modality and existing expression plasmids.

Materials and Methods

Oligonucleotides and Plasmids. Morpholino phosporamidate oligonucleotides were synthesized by GeneTools (Philomath, OR). pHCV Dual Luc and pEMCV Dual Luc were kindly provided by Alissa Lancaster and Mark Carter, respectively. pGL3-control was purchased from Promega Corp. (Madison, WI). All plasmids were purified with Endo-Free Megaprep kits (Qiagen, Hilden, Germany). Luciferin was purchased from Biosynth International (Naperville, IL).

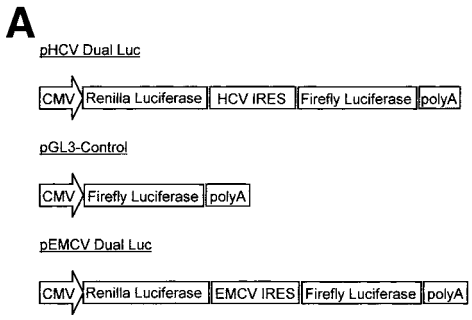
Hydrodynamic Transfection and Imaging. Morpholino oligonucleotides and 5 μg of indicated plasmid were added to a final volume of 1.8 mL of phosphate-buffered saline and steadily injected in the tail vein of mice over 4 to 5 seconds (N = 4-6 animals per group). Reproducibility of hydrodynamic transfection is highly dependent on the quality of injection and requires some practice. It appeared that the rate of injection needed to be consistent from injection to injection.[19] Animals that did not receive the entire volume in a single bolus were not imaged. At indicated times, 80 μL of 30 mg/mL luciferin was injected intraperitoneally. Ten minutes after injection, live anesthetized mice were imaged for 2 or 5 minutes. An imaging system based on an ultra-sensitive, cooled, charge-coupled device camera (Xenogen Corp., Alameda, CA) was used as previously described.[20] Image data were acquired and analyzed using LivingImage software (Xenogen Corp). Raw values are reported as relative detected light per minute and standard errors of the mean for each group (N = 4-6 animals) are shown. The 18 to 22 g female BALB/c mice were obtained from Jackson Laboratory (Bar Harbor, ME). Animals were treated according to National Institutes of Health Guidelines for Animal Care and the Guidelines of Stanford University.

Results and Discussion

To develop a model to test morpholino antisense oligonucleotide inhibitors of HCV IRES translation, we used hydrodynamic transfection.[12,19,21] Hydrodynamic transfection leads to efficient transfer of nucleic acids, primarily to the liver of mice. Previously, we used this method to test RNA interference inhibitors of HCV in mice.[22] The plasmid pHCV Dual Luc encodes a bicistronic messenger RNA (mRNA) in which the HCV IRES drives cap-independent translation of firefly luciferase from the second cistron.[23] Morpholino oligonucleotides were codelivered with this target plasmid (Fig. 1). Translation from the IRES was monitored using whole-body imaging of *in vivo* firefly luciferase activity,[24] a method we have used previously to monitor HCV IRES translational initiation.[12] Before imaging, animals were anesthetized and received an intraperitoneal injection of luciferin (150 mg/kg), the substrate for luciferase. The number of mice per group was 4 to 6, and standard errors for each group are shown.

Figure 2A shows an image of light emitted from a representative mouse 3 hours after transfection with 5 μg of pHCV Dual Luc reporter alone. Bioluminescent images are comprised of a pseudocolor image representing intensity of emitted light (red most and blue least intense) superimposed on a gray-scale reference image (for orientation). Figure 2B shows greatly reduced light emitted from a representative mouse that received 5 μg of pHCV Dual Luc as well as 1 nmol of a 25-mer morpholino oligonucleotide (25-mer) complementary to nucleotides 345-370 of the HCV IRES. The observed reduction in light emitted was not due to nonspecific morpholino toxicity because a 25-mer reverse complement oligonucleotide (25-mer RevC) (Fig. 2C) did not reduce the amount of light emitted.

One advantage of whole-body *in vivo* imaging is the ability to measure biologic effects accurately and rapidly, repeatedly, in the same animal over time. This reduces experimental variability and the number of mice required for a given study. Fig. 3A shows the luciferase activity



B

25-mer	5'-TCTTTGAGGTTTAGGATTCGTGCTC-3'
25-mer RevC	5'-GAGCACGAATCCTAAACCTCAAAGA
25-mer-4MM	5'-TCTTAGAGGATTAGGTTTCGAGCTC-3'
20-mer	5'-GAGGTTTAGGATTCGTGCTC-3
20-mer-4MM	5'-GAGATTTACGATTCATGATC-3

Fig. 1. (A) Schematic of pHCV Dual Luc, pEMCV Dual Luc, and pGL3-control reporter transcripts. Firefly luciferase activity was monitored. For technical reasons, Renilla luciferase activity is difficult to monitor. (B) The sequence of morpholino antisense inhibitors is shown. 25-mer and 20-mer oligonucleotides are complementary to nucleotides 345-370 and 345-365, respectively. Mismatched nucleotides are underlined.

(relative to the reporter alone) at 3, 48, 96, and 144 hours posttransfection for cohorts of mice (n = 4) that received the 1 nmol of the 25-mer or the 25-mer RevC. The 25-mer but not the 25-mer RevC reduced the amount of light emitted by greater than 95% for 6 days. These data showed that the 25-mer morpholino targeting the HCV IRES was a potent and long-lasting inhibitor of HCV IRES translation.

A crucial consideration in the design of antisense inhibitors is specificity. One parameter that affects specificity is the relative accessibility to hybridization of the target sequence versus the accessibility of targets having undesired homology to the intended target. The target site for the 25-mer was chosen because of its proximity to the AUG start codon and because *in vitro* mapping and functional studies had shown previously that this site was accessible to hybridization.[2-5,9,25] The affinity of the oligonucleotide for the intended and mismatched target sequences also influenced specificity. Ideally, an antisense oligonucleotide should dissociate slowly when bound to its desired target yet have a significant dissociation rate when bound to inappropriate mismatched targets that share homology with the intended target. The dissociation rate can be modulated by changing oligonucleotide length to find a window that provides both potency and specificity. It should be noted that for any given accessible target site, extending the oligonucleotide length beyond a certain point will lead to a loss of mismatch specificity.

To test if the 25-mer displayed mismatch specificity, an oligonucleotide containing 4 mismatched bases (25-4MM) was tested for its ability to inhibit translation from the HCV IRES in mice. At a dose of 1 nmol, 25-mer-4MM reduced the amount of light emitted to the same degree as the perfect complement 25-mer (Fig. 3A). This suggested the affinity of the 25-mer was too high to provide mismatch specificity. This raised the possibility that the 25-mer could inadvertently inhibit nontarget genes.

In an effort to obtain potency while maintaining specificity, a shortened 20-mer morpholino oligonucleotide (20-mer) complementary to nucleotides 345-365 of the HCV IRES and a 20-mer with 4 mismatched bases (20-mer-4MM) were tested. The 20-mer reduced the amount of emitted light by greater than 95% at 3, 48, 96, and 144 hours (Fig. 3B), an amount similar to that observed with the 25-mer. In contrast, the 20-mer-4MM did not significantly reduce emitted light (Fig. 3B). These results showed that the 20-mer was a potent and specific inhibitor of HCV IRES–mediated translation in mice.

Next we sought to determine the minimum dose of 20-mer required for significant inhibition of HCV IRES translation. Figure 3C shows the percent inhibition observed at 48 hours for groups of mice that received 0, 1, 10, 100, or 1,000 pmol of the HCV-specific 20-mer. The degree of inhibition increased with increasing doses of 20-mer administered, with maximal inhibition at the highest dose tested, 1,000 pmol. The mismatch specificity experiments were conducted at a dose of 1,000 pmol. Thus, we identified the minimum dose at which the 20-

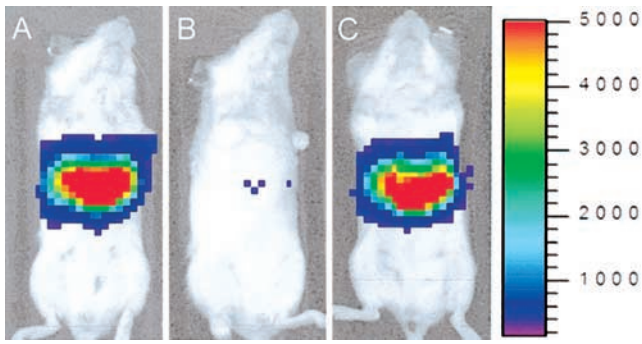


Fig. 2. Representative image of light emitted from a mouse 3 hours after transfection with (A) 5 μg of pHCV Dual Luc reporter alone, as well as (B) 1 nmol of a 25-mer morpholino oligonucleotide (25-mer) or (C) 1 nmol of a 25-mer reverse complement oligonucleotide. Significant reduction of light emitted is seen in the presence of the 25-mer.

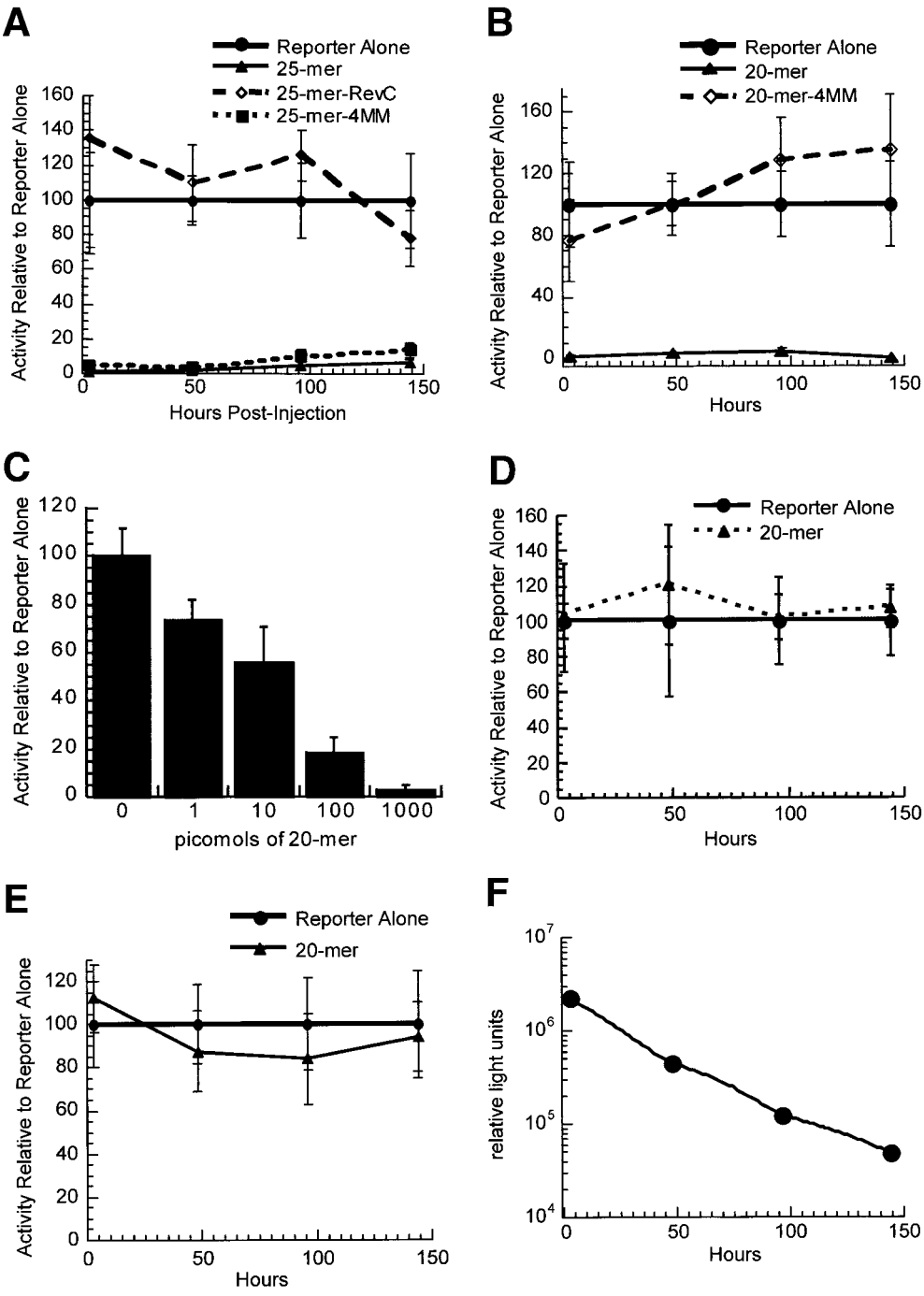


Fig 3. Quantitation of light emitted as a result of firefly luciferase reporter transcript translation (relative to the reporter alone group). (A, B, D, E, and F) Oligonucleotide-treated cohorts received 1 nmol of morpholino oligonucleotide. Standard errors are indicated. (A) Light emitted by mice that received the 25-mer was reduced by greater than 95% for 6 days. Light emitted from mice that received 25-mer reverse complement (25-mer-RevC) was not reduced significantly, showing that morpholino oligonucleotides are not nonspecifically toxic. However, treatment with 25-mer-4MM reduced light emission to the same degree as the 25-mer, indicating that the 25-mer is too long to exhibit mismatch specificity. ●, Reporter alone; ▲, 25-mer; ◇, 25-mer-RevC; ■, 25-mer-4MM. (B) Light emitted by mice that received the 20-mer was reduced by greater than 95% for 6 days. This inhibition is equivalent to that observed with the 25-mer. Inhibition by the 20-mer shows mismatch specificity because a 20-mer containing 20-mer-4MM does not significantly reduce emitted light. ●, Reporter alone; ▲, 20-mer; ◇, 20-mer-4MM. (C) Inhibition of the HCV IRES translation by the 20-mer is dose dependent over a range of 0, 1, 10, 100, or 1,000 pmol per mouse. The 20-mer does not inhibit translation from (D) a cap-dependent reporter that does not contain the HCV IRES (pGL3-contol), or (E) from a reporter in which the EMCV IRES drives translation of the luciferase gene (D and E: ●, reporter alone; ▲, 20-mer). (F) Signal from the reporter pHCV Dual Luc reporter alone decreases over time due to loss of plasmid or silencing of the reporter.

mer morpholino potently inhibited HCV IRES translation while maintaining specificity.

To further test the specificity of the 20-mer, 2 additional reporter constructs were used. The first was pGL3-control (Promega), which encodes a monocistronic luciferase mRNA that lacks an IRES and is translated in a cap-dependent manner. The second control reporter was identical to pHCV Dual Luc except that the HCV IRES was replaced with the EMCV IRES.[26] As shown in Figs. 3D and 3E, respectively, the 20-mer did not significantly inhibit translation from either reporter. These data provided additional evidence for the specificity of the 20-mer, and indicated that it does not nonspecifically target cap-dependent translation, the luciferase gene, or cap-independent translation from other IRES sequences. This is in contrast to HCV IRES targeting phosphorothioate and 5-methylcytidine antisense oligonucleotides, which showed significant nonspecific effects in previous studies *in vivo*.[4]

Expression from the reporter plasmids used in this study diminished with time. This may be due to loss of

DNA or silencing of the promoter. This decrease was seen easily when the data for the reporter alone control was plotted as relative light units emitted over time (Fig. 3F). Beyond 6 days, we judged the signal-to-noise ratio to be inadequate to accurately determine the percent inhibition (less than a 100-fold dynamic range). Morpholino oligonucleotides are highly nuclease resistant[17] and no significant decrease in inhibition was seen over the 6-day time course. *In vitro* studies indicated that morpholinos are completely resistant to nucleases,[27] suggesting that inhibition by the 20-mer might persist for longer than 6 days. We are currently working to develop persistent HCV IRES luciferase reporter plasmids to determine the duration of morpholino antisense inhibition of HCV IRES translation.

We have used hydrodynamic transfection to develop a preclinical small animal model for testing inhibitors of HCV IRES–mediated translation in living mice. Real-time imaging in living mice allowed us to make serial measurements in the same groups of mice. Using this model, we showed that morpholino antisense oligonucleotides can potently inhibit translation from the HCV IRES in mice. Oligonucleotide length was optimized to maintain specificity as well as potency in the complex intracellular milieu of the mammalian liver. Inhibition by the optimized 20-mer was dose dependent, showed mismatch specificity, and did not nonspecifically target cap-dependent translation of the luciferase gene or translation from the EMCV IRES. This was in contrast to a previous study *in vivo* in which nonspecific effects were observed with phosphorothioate and 5-methylcytidine antisense oligonucleotides.[4]

Antisense gene inhibition has been used extensively *in vitro*, in culture, and, to a more limited extent, *in vivo*. Recently, the first antisense therapeutic was approved for clinical use in humans. However, many hurdles remain before the use of antisense therapeutics can become widespread. These include: (1) efficient delivery of antisense to target organs; (2) development of nontoxic, nuclease stable chemistries with favorable hybridization characteristics; (3) identification of accessible and efficacious mRNA target sites; and (4) development of small animal model systems for easily testing antisense oligonucleotides. Hydrodynamic transfection with morpholino oligonucleotides and use of bioluminescent imaging for assessing outcome has allowed us to satisfy all 4 of these conditions in our mouse model.

As with many other pharmaceutical entities, delivery remains the last obstacle. It is clear from our study that if morpholino oligonucleotides can be delivered efficiently to the appropriate intracellular compartment, they can be extremely efficient inhibitors. Some success has been reported in the effort to adapt hydrodynamic transfection for application in primates.[28] However, it seems likely that other delivery methods will need to be used for most applications. It may be possible to deliver antisense oligonucleotides by coupling them to cell-permeable peptides. If efficient delivery can be accomplished, antisense may find wide application in the treatment of diverse diseases.

Recently, there has been significant interest in the use of RNA interference (RNAi) inhibitors of gene expression. A frequently asked question is whether RNAi has significant advantages over antisense. In previous studies conducted in our laboratory, we used hydrodynamic transfection to codeliver luciferase reporter plasmids along with small interfering RNAs or plasmids expressing short hairpin RNAs that trigger RNAi targeting the luciferase transcript. Transfection of mice with approximately 6 nmols of small interfering RNAs or with a plasmid expressing short hairpin RNAs decreased luciferase gene expression by an average of 81% and 93%, respectively, at day 3.[22] For comparison, transfection of mice with 1 nmol of 20-mer morpholino in the present study resulted in greater than 95% reduction in gene expression. Direct comparisons between antisense inhibition and RNAi *in vivo* are difficult because the serum stabilities of small interfering RNAs, short hairpin RNA expression plasmids, and morpholino oligonucleotides may be quite different; therefore, it is unclear what the final intracellular concentration of the antisense and RNAi triggers was in these experiments. However, it is clear from our data that if sufficient amounts of antisense oligonucleotides can be delivered to the appropriate subcellular compartment, antisense inhibition of gene expression can be as potent as RNAi. RNAi is thought to catalytically trigger degradation of target mRNAs before translation. However, this degradation is not complete because residual translation is observed. Morpholinos act at a different step in gene expression, by blocking translational initiation. It is therefore possible that morpholino antisense and RNAi could act synergistically to result in even greater decreases in gene expression.

Acknowledgment: The authors thank Ramona Gonzales for critical review of the manuscript. The authors also thank Peter Sarnow, Alissa Lancaster, and Mark Carter for generously providing plasmids.

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