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Inhibition of Retroviral Pathogenesis by RNA Interference

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Summary

Background: RNA interference (RNAi) is a newly discovered cellular defense system that is known to suppress replication of genomic parasites in model organisms. It has been widely conjectured that RNAi may also serve as an antiviral system in vertebrates.
Results: Retroviral infection could be initiated by electroporation of cloned Rous sarcoma virus (RSV) proviral DNA into the developing chick neural tube. Coelectroporation of proviral DNA and short double-stranded RNAs matching sequences of avain retroviruses, which were designed to induce RNAi against RSV, inhibited viral replication. Replication of RSV after electroporation resulted in disruption of embryonic development and early death, but this, too, could be suppressed by RNAi against the RSV genome. RNAi could also inhibit the growth of RSV and HIV in cell culture. Analysis of the step of the retroviral life cycle that is inhibited by RNAi revealed that it primarily prevented accumulation of the viral RNAs synthesized late during infection. RNA genomes introduced in viral particles early during infection were less sensitive.
Conclusions: RNAi can block retroviral infection in vertebrates. The tissue electroporation method described here should allow RNAi to be used widely to study gene function and control of infection in vertebrate animals.

Introduction

Double-stranded RNA can program the selective degradation of sequence-matched RNAs in cells, a process named RNA interference (RNAi) (for recent reviews, see [1–4]). RNAi was first discovered in plants, worms, and other model organisms, but more recently RNAi has been reported in vertebrate cells as well [5–10]. In model organisms, RNAi has been shown to selectively block the replication of genomic parasites such as viruses or transposons [11–13]. Here, we investigate whether RNAi can be directed to inhibit replication of retroviruses in vertebrates.

Genetic and biochemical studies of RNAi have led to a model for its action. Double-stranded RNA, presumably transcribed from DNA of a genomic parasite, is initially cleaved into 21- to 23-base pair small interfering RNAs (siRNAs). These siRNAs are then recruited into a nucleoprotein complex, the RISC complex. The strands of the siRNA are then believed to be unpaired, allowing the message-complementary strand to bind to the targeted mRNA and thereby program its degradation [1–4].

In plants and *C. elegans*, RNAi can also be amplified within cells in a pathway that is dependent on RNA-dependent RNA polymerase (RdRP). According to current models, RdRP extends RNA synthesis along the target message by using the antisense siRNA strand as a primer. The extended duplex RNA is then cleaved into further siRNAs, resulting in amplification of RNAi within a cell. In plants and worms, RNAi also can spread between cells of a tissue or organism [1, 3, 4]. Remarkably, RNAi can even be transmitted through the germline [14].

RNAi has been demonstrated to act as an antiviral system in plants. Mutations in genes encoding the components of the RNAi system cause cells to become more sensitive to infection by some plant viruses, and several plant viruses have been found to encode inhibitors of RNAi [11, 15–17]. The possible activity of RNAi against vertebrate genomic parasites has not previously been reported.

We have developed a convenient system for testing RNAi in vertebrates using electroporation of chick embryos. Nucleic acids are injected into the neural tube 2 days after fertilization, then a current is applied in an orthogonal direction. This introduces nucleic acid into cells near the positive electrode [18]. In initial experiments, we demonstrated that siRNAs against the gene for green fluorescent protein (*gfp*) could inhibit expression of GFP. We then tested whether RNAi could inhibit the replication of a nonpathogenic derivative of Rous sarcoma virus (RSV). Plasmid DNAs encoding the RSV genome were electroporated into embryos along with siRNAs against RSV or control siRNAs, and this allowed for the demonstration of RNAi-mediated control of RSV infection. Electroporation of pathogenic RSV resulted in disruption of the neural tube and death of embryos, but this could be reversed by coelectroporation of RNAi. These data raised the possibility that RNAi may be capable of inhibiting other retroviruses such as HIV. To test this, we characterized RNAi against HIV and RSV in cell culture models. Viral RNA genomes just entering cells were insensitive to RNAi, but, late during infection, the viral genomes were efficiently degraded. Together these data establish that the RNAi system can control the replication of retroviruses and begin to specify the inhibitory mechanism.

Results

RNAi Activity in Chick Embryos

We have used electroporation of chick embryos to evaluate the activity of RNAi against avian retroviruses. Short 21- to 23-bp double-stranded siRNAs were designed as described in [19] and were synthesized chemically. In initial studies, we introduced a plasmid encoding *gfp* into the chick neural tube at Hamburger and Hamilton

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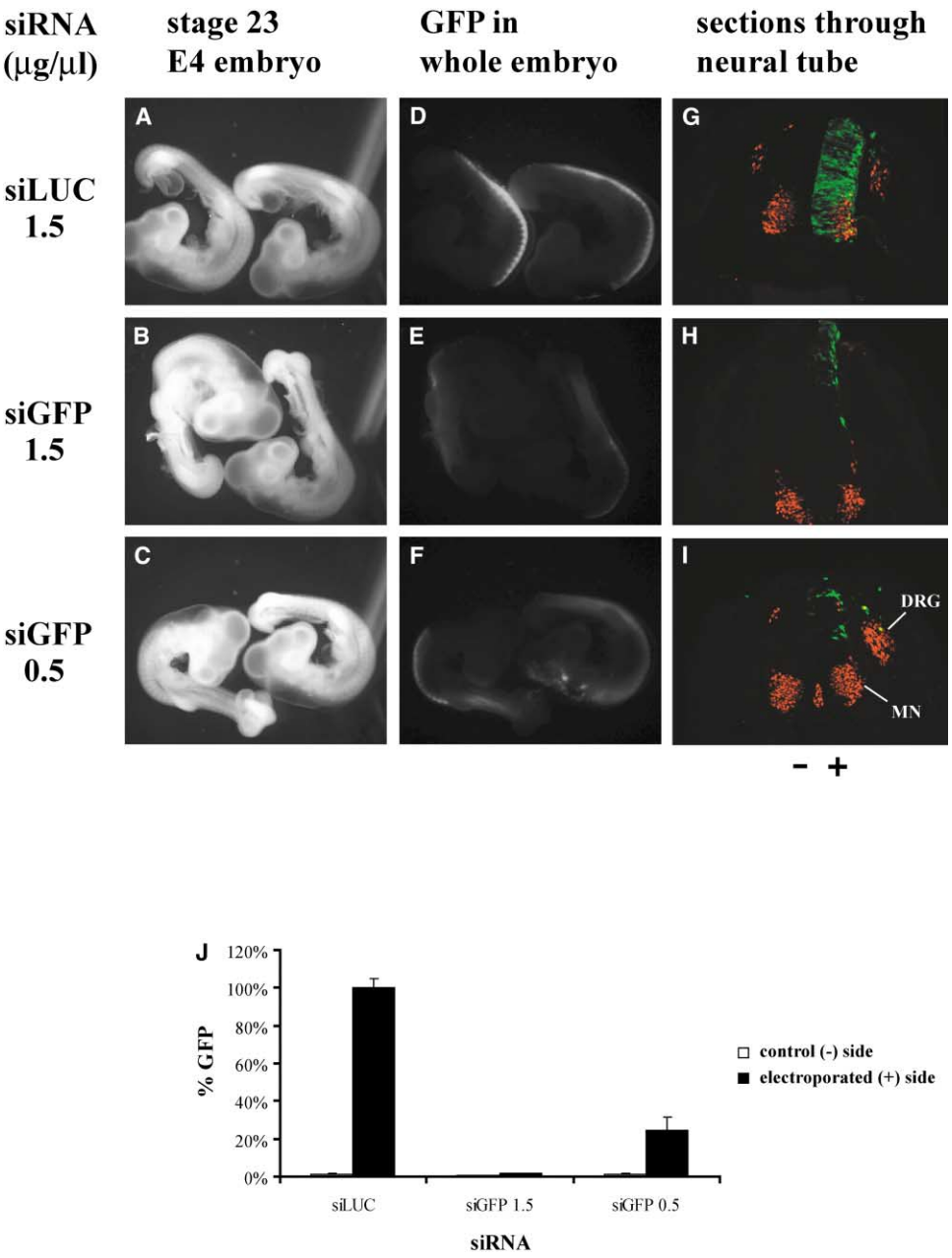


Figure 1. RNA Interference in Chick Embryos

(A–F) A plasmid encoding *gfp* (3 μg/μl, p156RRLsinPPTCMVGFPPRE, described in [35]) was pipetted into the neural tube of a 2-day-old chick embryo together with the indicated siRNA. Nucleic acids were introduced into tissue proximal to the positive electrode by electroporation essentially as described [21]. Embryos (day 4 postfertilization) are shown in (A)–(C), and the fluorescence signal is shown in (D)–(F) ("GFP in whole embryo").

(G–I) Present transverse sections through the spinal cord. The GFP signal is green. Tissue was stained with an antibody against Isl1/2 to reveal motor neurons (red); note that the DRG is a segmental structure and so does not appear in every section. The plus and minus signs indicate the positive and negative electrodes.

(J) GFP signal intensity was quantitated with ImageQuant. The electroporated (+) and nonelectroporated (−) halves of the neural tube are compared.

(H.H.) stage 11 (approximately E2, [20]) together with siRNA against *gfp* (siGFP). As a control, siRNAs against irrelevant sequences were also tested to document the specificity (an assay with siLUC, against firefly luciferase RNA, is shown). Tissue electroporation efficiently introduced the nucleic acids into the half of the neural tube proximal to the positive electrode and occasionally into neural crest cells that migrate from the neural tube and differentiate into structures such as sensory neurons of the dorsal root ganglion (DRG) [21].

Two days after electroporation, embryos were analyzed by whole-mount fluorescence microscopy (Fig-

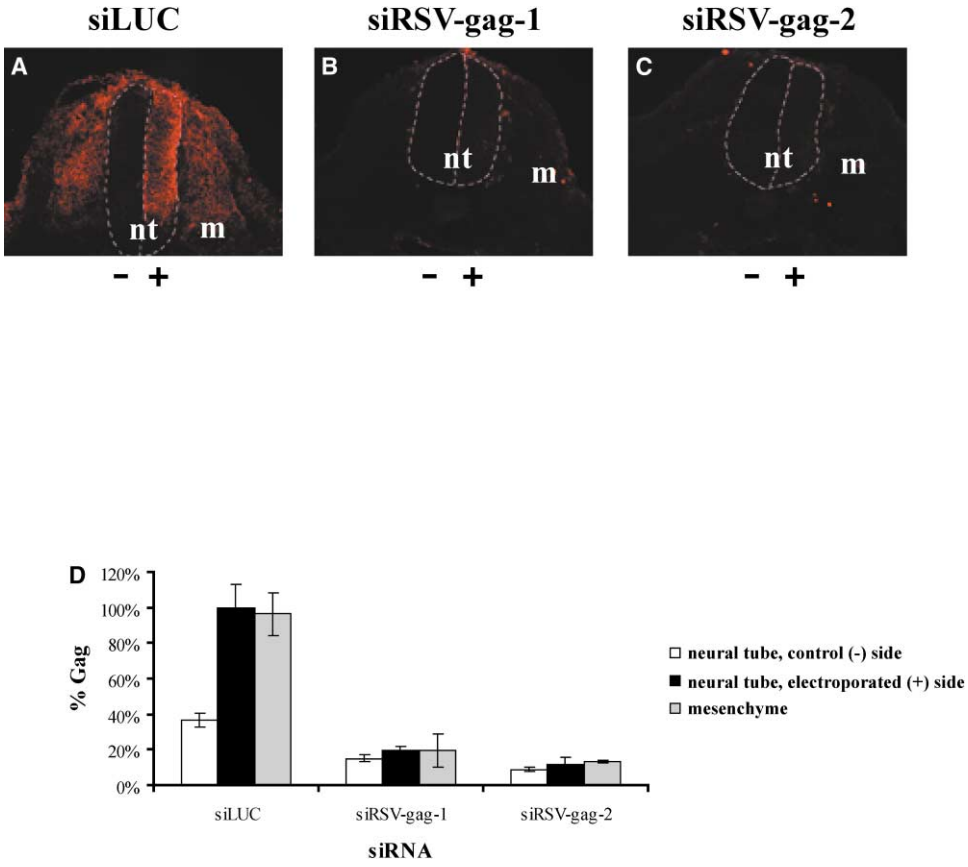


Figure 2. Inhibition of RSV Replication by RNA Interference

(A–C) Embryos were electroporated with a cloned RSV genome (RCASBP[B], [22]), and the siRNA is indicated at the top of the figure at day 2 postfertilization. At day 4, embryos were harvested, sectioned transversely through the spinal cord, and stained with an RSV Gag antibody. Gag-positive staining is shown in red. Spreading infection is seen with the control (A) siLUC RNA, but not with (B) siRSV-gag-1 or (C) siRSV-gag-2.

(D) Images were quantitated with ImageQuant; the electroporated (+) and nonelectroporated (−) halves of the neural tube (nt) are compared with the adjoining mesenchyme (m).

ures 1A–1F). Extensive fluorescence was seen in the neural tube of the control siLUC-treated embryos (Figures 1A and 1D) and in embryos electroporated with the *gfp* plasmid only (data not shown). Treatment with 1.5 μg/μl siGFP, in contrast, greatly abrogated the fluorescent signal (Figures 1B and 1E). Treatment with lower doses of siGFP (0.5 μg/μl) resulted in weaker inhibition (Figures 1C and 1F). Treatment with either RNA strand alone did not inhibit the appearance of the fluorescent signal, indicating that the RNAs are not working by an antisense mechanism (data not shown). Embryos were then sectioned through the spinal cord, and GFP expression was assayed by fluorescence microscopy (Figures 1G–1I, green signal). The intensity of GFP fluorescence is quantitated in Figure 1J. An intense GFP signal is seen in the electroporated half of the embryo, and the signal is reduced in a concentration-dependent fashion by coelectroporation of siGFP. To visualize the tissue organization and assess the specificity of RNAi, embryos were also stained with an antibody recognizing the endogenous nuclear proteins Isl1 and Isl2 expressed by motor neurons and DRG (Isl1/2, Figures 1G–1I, red signal). No increase in embryo mortality was observed due to the addition of RNAi up to a concentration of 3 μg/μl, and motor neuron differentiation proceeded normally, as monitored by antibody staining for Isl1/2. These data indicate that the chick neural tube contains the machinery for RNAi and that RNAi can be elicited without toxicity by tissue electroporation of siRNAs.

Control of RSV Infection by RNAi

We next investigated whether RNAi could inhibit retroviral replication. As a model virus, we used RCASBP(B), which is a derivative of RSV modified for use as a retroviral vector by removal of the *src* oncogene [22]. RCASBP(B) was chosen because it is known to be competent for replication in the chick neural tube. Embryos were electroporated with a plasmid encoding the RCASBP(B) genome and either of two siRNAs against RSV Gag (siRSV-gag-1 and siRSV-gag-2) or siLUC-1 as a nonspecific siRNA control. Electroporation was carried out in embryos 2 days after fertilization and was analyzed at day 4 by sectioning and staining with an antibody against RSV Gag.

After treatment with the RCASBP(B) plasmid only (not shown), or with RCASBP(B) and the siLUC-1 control

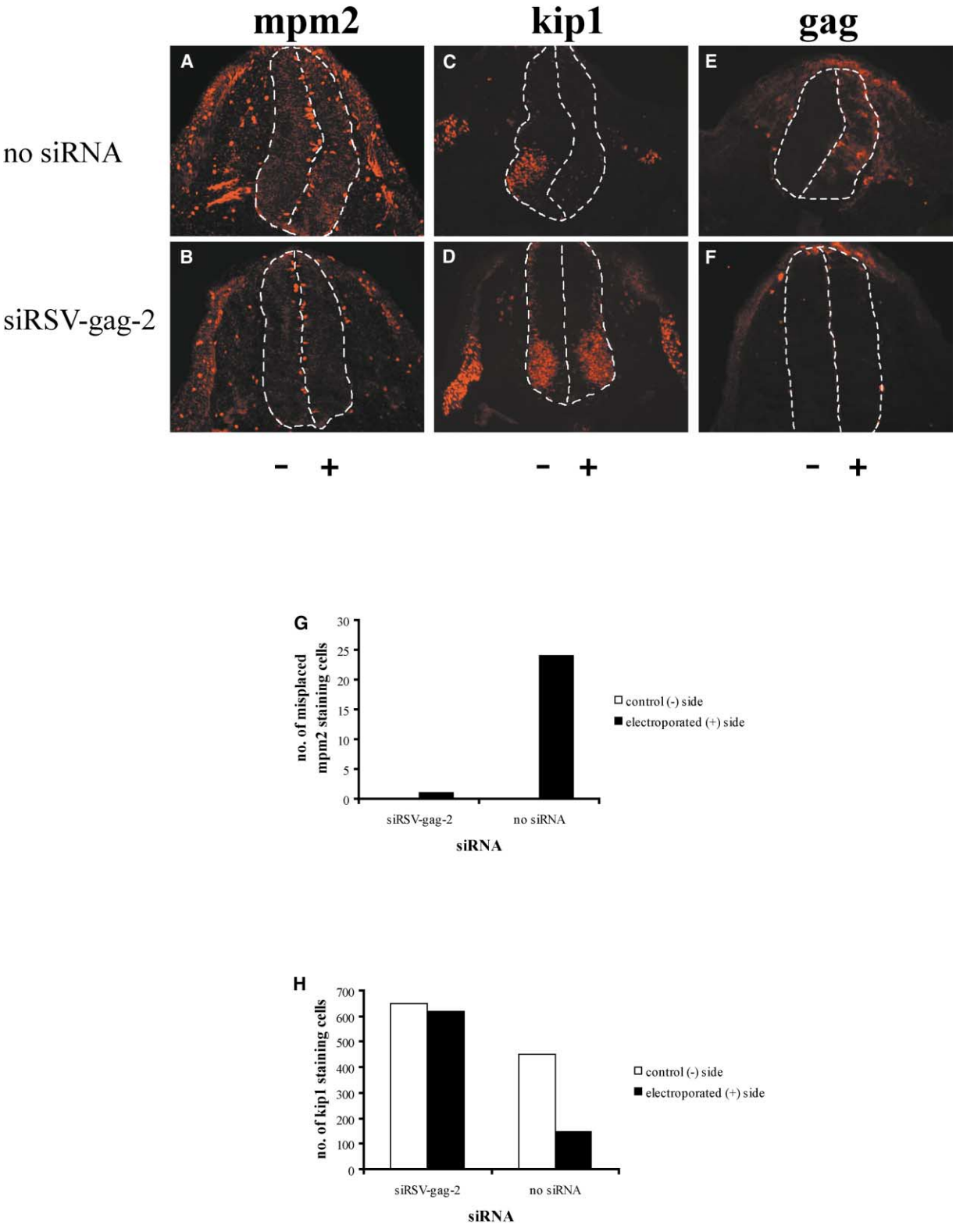


Figure 3. Inhibition of RSV Pathogenesis by RNAi

(A–F) Embryos were electroporated 2 days after fertilization with an RSV proviral DNA and the indicated siRNAs. A total of 36 hr later, the embryos were sectioned and stained with antibodies recognizing the indicated proteins (mpm2, kip1, or gag; [A–F]). The mpm2 marker detects tyrosine phosphorylation characteristic of mitosis, and kip1 detects the kip1 protein that inhibits cell cycle progression and so marks postmitotic cells. (A, C, and E) No siRNA; (B, D, and F) siRSV-gag-2.

(G) Quantitation of abnormal cells stained with the mpm2 marker, comparing staining on the electroporated (+) and control (−) sides of the embryo. Cells outside the normal axial zone of proliferation were summed over four slides. Note that the mpm2 marker detects cells in a specific phase of the proliferative cycle and so stains relatively low numbers in any given section.

(H) Quantitation of cells stained with the kip1 marker, comparing staining on the electroporated (+) and control (−) sides of the neural tube. Cells were counted in four sections for each bar graph.

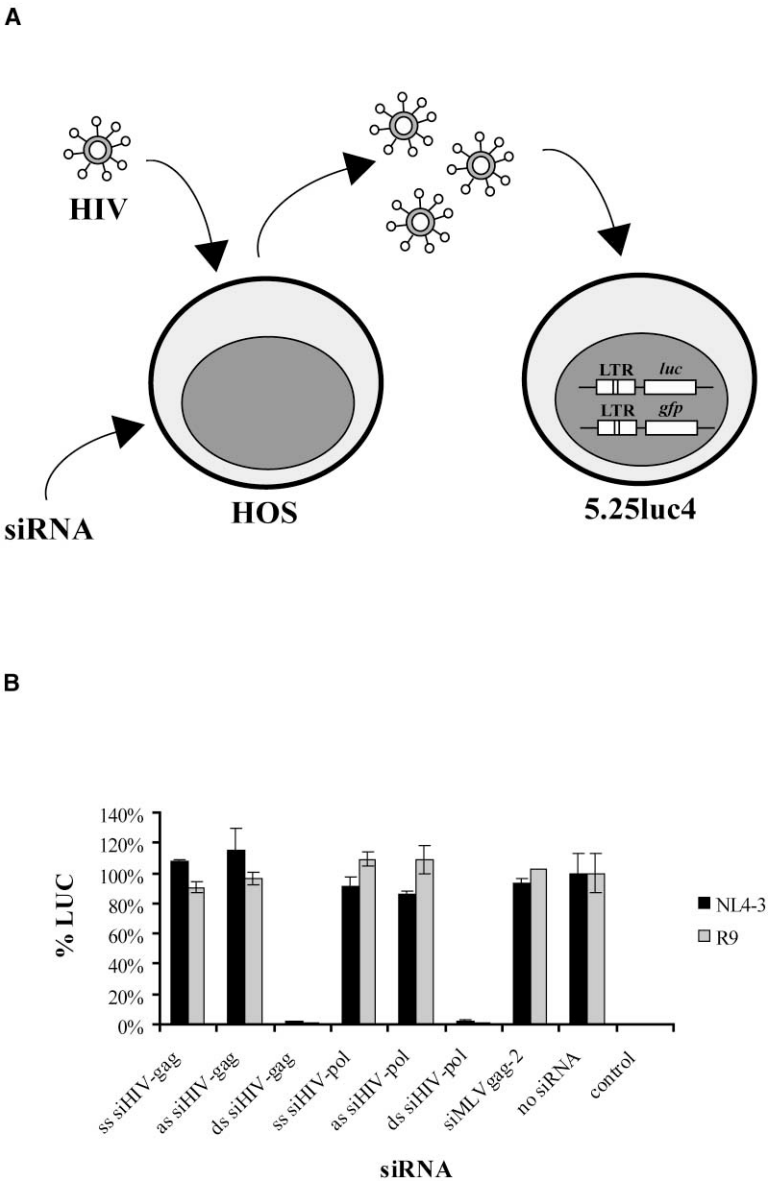


Figure 4. Inhibition of HIV-1 Replication by RNAi

(A) A diagram of the assay for siRNA inhibition of HIV-1 replication. HOS.T4.CXCR4 cells were transfected with siRNAs and were then infected with HIV-1 2 hr later. Two days later, culture supernatants were harvested and applied to 5.25luc4 indicator cells. Integration of HIV-1 cDNA followed by synthesis of Tat protein activates the production of luciferase and green fluorescent protein from the integrated HIV-1 LTR-*luc* and LTR-*gfp* reporter genes.

(B) Inhibition of HIV replication assayed with the LTR-*luc* reporter. All values were normalized to the no siRNA sample. "ss" indicates sense strand only of the siRNA, "as" indicates antisense strand only, and "ds" indicates the complete double-stranded siRNA. siHIV-1 matches HIV *gag*, siHIV-2 matches HIV *pol*, and siMLV matches Moloney murine leukemia virus *gag* and is active against MLV in cell culture (data not shown).

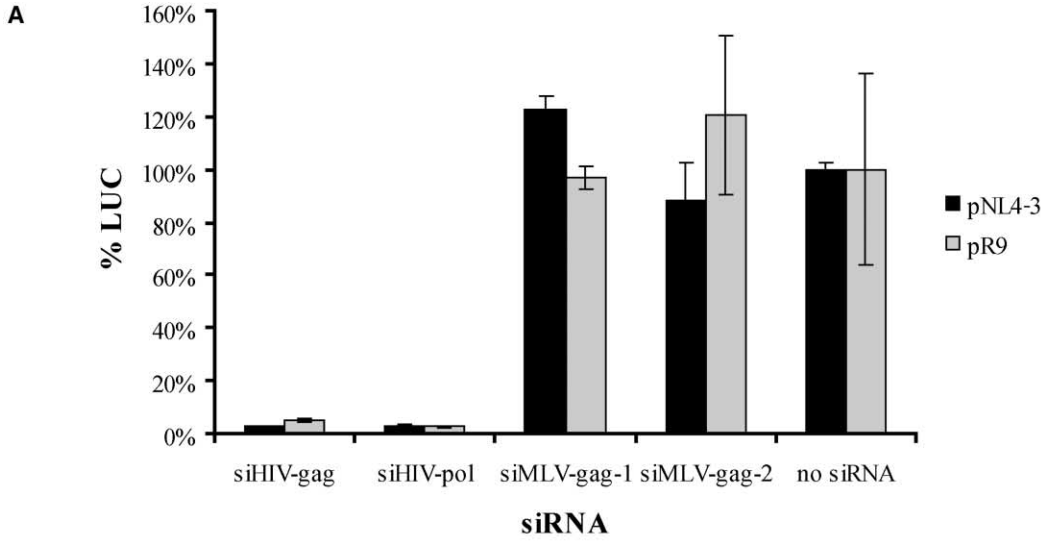
RNA (Figure 2A), Gag staining could be seen in one half of the spinal cord (red signal). The lumen of the neural tube and the unilateral electroporation method restricted the infection to one half of the cord; however, the replication-competent virus did spread into the surrounding mesenchyme due to secondary cell infection.

After electroporation of the RSV genome with either of the siRSVs, infection was only evident in a few cells (Figures 2B and 2C), indicating efficient inhibition of viral replication. Much less viral spread was evident at the time point shown (2 days postelectroporation). There was little virus evident in the nonelectroporated half of the neural tube, apparently because the lumen of the tube and surrounding membranes formed a barrier to viral spread. Quantitation of the intensity of the Gag signal (Figure 2D) suggested that siRSV-gag-2 is a somewhat more effective inhibitor than siRSV-gag-1. Electroporation of single strands of the siRNAs did not inhibit viral replication (data not shown). These findings indicate that RNAi can suppress RSV replication efficiently in chick embryos.

Inhibition of RSV Pathogenesis by RNAi

We next investigated whether RNAi could inhibit pathogenesis by RSV in the chick embryo model. RSV was electroporated into 2-day-old embryos, and the effects were assessed after another 3 days of incubation. All embryos electroporated with RSV only or RSV plus control siRNA were dead by this time (16/16 and 12/12 assayed, respectively). In contrast, 7/12 embryos treated with siRSV-gag-2 survived, indicating inhibition of the lethal effect by RNAi.

To assay pathogenesis in more detail, embryos were sectioned 36 hr after infection and were stained with markers for mitotic cells (mpm2), nonproliferative cells (kip1), and RSV Gag (Figure 3). In the absence of specific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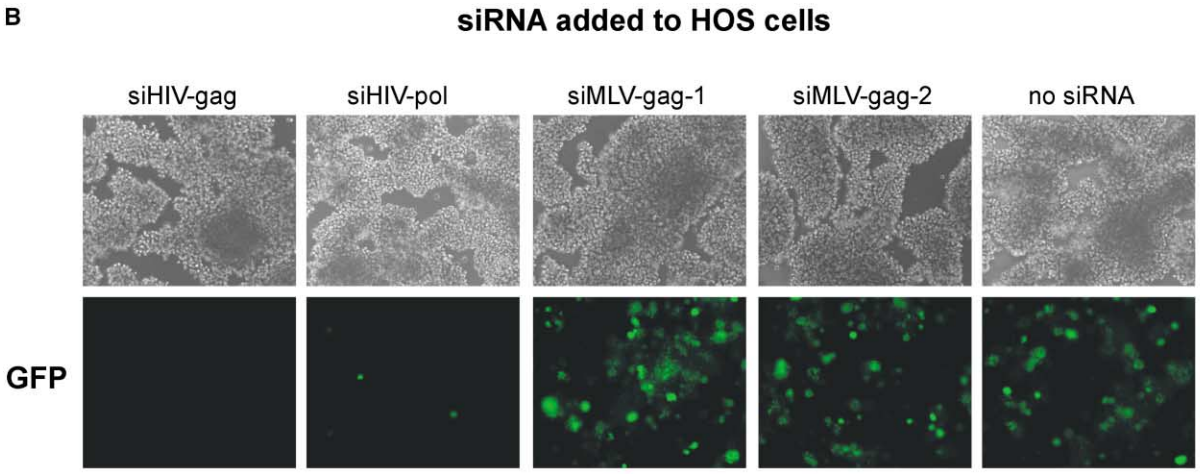
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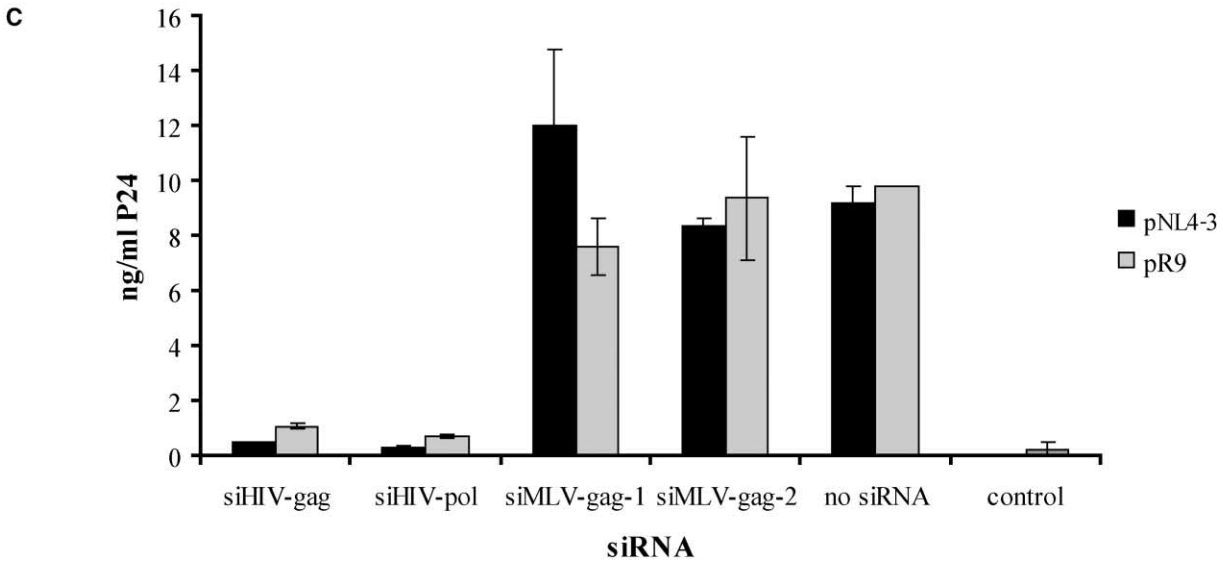
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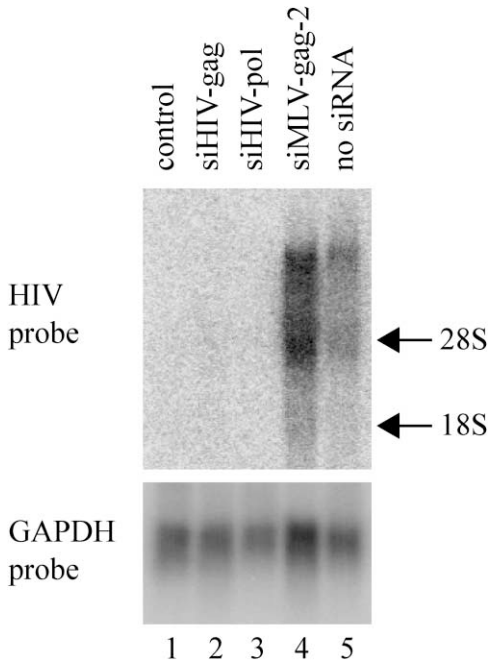

Figure 6. RNAi Inhibits Viral RNA Accumulation

RNAs produced in control and siRNA-treated cells were assayed by Northern blotting. HOS.T4.CXCR4 cells were transfected with pR9 and the indicated siRNAs. Two days later, total RNA was harvested, separated by electrophoresis, and probed with a fragment of pR9 complementary to the integrase region.

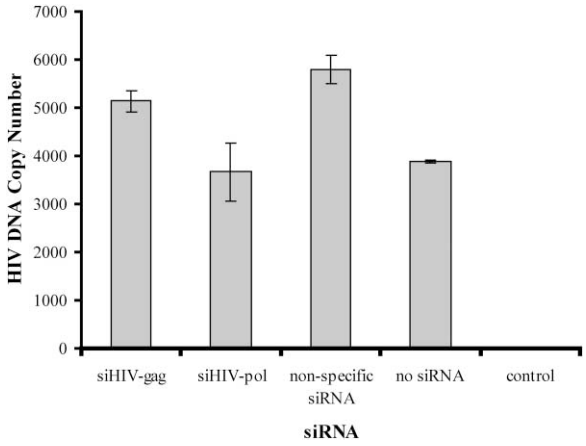

Figure 7. RNAi Does Not Act Early against Incoming Viral RNA Genomes

The HIV-1 cDNA copy number in HOS.T4.CXCR4 cells was assayed after infection with HIV R9. Cells were transfected with the indicated siRNA and were then infected with HIV 2 hr later. A total of 12 hr after that, DNA was harvested and the total HIV-1 cDNA genomes were quantified by fluorescence-monitored PCR as described [24]. The PCR primers that were used required that the second jump of reverse transcription be completed for amplification to take place.

siRNA, the neural tube was disorganized, with misplaced proliferative cells and abnormal masses of nonproliferative cells evident (Figures 3A, 3C, and 3E). In the presence of siRSV-gag-2, the embryos were indistinguishable from embryos that were not infected with RSV (Figures 3B, 3D, and 3F; quantitated in 3G and 3H, and data not shown). These data confirm that RSV causes abnormal proliferation and tissue disorganization in embryos, and this effect can be reversed with RNAi.

RNAi Inhibits Accumulation of Retroviral mRNAs but Does Not Attack Incoming Viral Genomes

We next asked which step(s) in the viral life cycle were interrupted by RNAi. Initially, we used HIV as a model due to its clinical importance and the availability of efficient assays to dissect the viral life cycle. siRNAs were designed against sequences in the HIV-1 gag (siHIV-gag) or integrase (siHIV-pol) regions. To test for RNAi inhibition of HIV-1 replication, HOS.T4.CXCR4 cells [23] were transfected with siRNAs and were then infected with HIV-1 (Figure 4A). Cells were washed, then incubated for 3 days to allow virus production. Virus in culture supernatants was then quantified by infection of 5.25luc4 indicator cells, which contain LTR-*luc* and LTR-*gfp* reporters that are activated by Tat protein supplied by an integrated HIV provirus (Figure 4A, N. Landau, personal communication). Thus, assay of luciferase or GFP provides a quantitative measure of virus production. As a control, a nonspecific siRNA was compared with siHIV-gag and siHIV-pol for inhibition of HIV-1 infection. The controls shown used siRNAs matching sequences from Moloney murine leukemia virus (MLV), but similar data were obtained with several other nonspecific siRNAs (not shown).

HIV-1 production was inhibited >90% by siHIV-gag or siHIV-pol, as assayed by the LTR-*luc* reporter (Figure 4B). siMLV-gag-2, which inhibits MLV replication (data not shown), was not inhibitory. Each of the siHIV-gag or siHIV-pol RNA strands tested alone showed no inhibition. The effect of siRNA could also be monitored by assaying the LTR-*gfp* reporter in 5.25luc4 indicator cells or by assaying the accumulation of HIV-1 p24 capsid antigen in the supernatant of infected HOS.T4.CXCR4 cells. In both cases, strong inhibition was seen by the specific siRNAs, but not by the control siRNAs (data not shown; see below).

We next used this system to determine the step in HIV replication that is affected by the siRNAs. Inhibition

Figure 5. RNAi Blocks Retroviral Gene Expression Late during Infection

(A) Cotransfection of siRNAs and HIV-1-encoding plasmids into HOS.T4.CXCR4 cells, followed by an assay of virus output with 5.25luc4 indicator cells (*luc* marker). Two different HIV-encoding plasmids were tested, pNL4-3 and pR9. The nonspecific siRNAs tested include siGFP-1 (not shown) and siMLV-1 and siMLV-2, which also had no effect (shown). Values were normalized to the no siRNA control.

(B) The effects of cotransfection of siRNAs and HIV-1-encoding plasmids revealed by activation of the LTR-*gfp* reporter in 5.25luc4 indicator cells.

(C) Output of HIV-1 from infected HOS.T4.CXCR4 cells scored by quantitating HIV-1 p24 (capsid antigen) in the culture supernatant. "Control" indicates no virus in the initial infection.

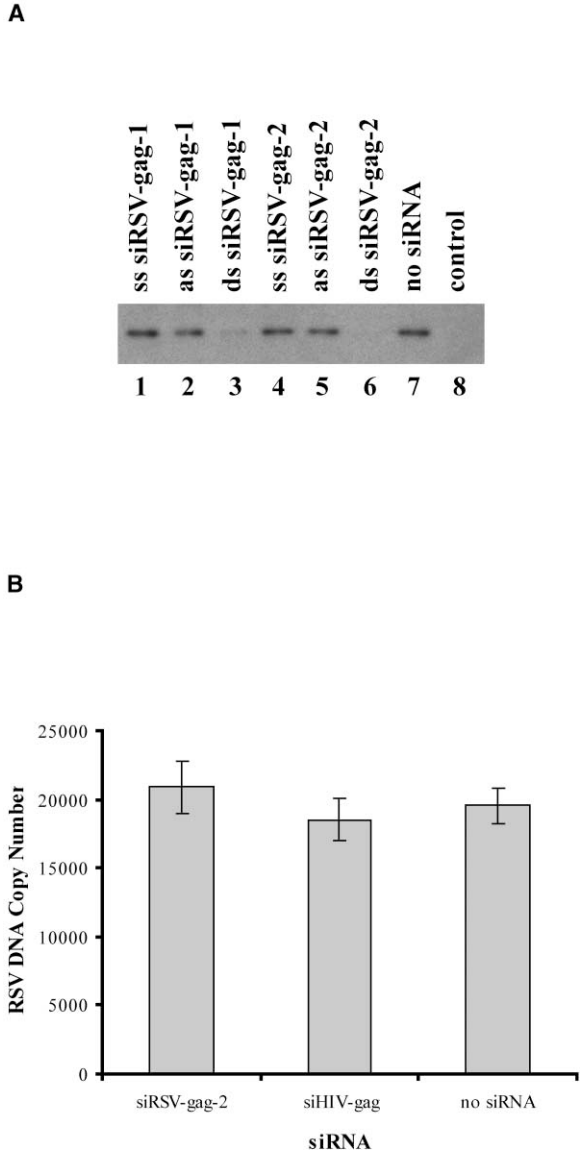

Figure 8. RNAi Inhibits RCASBP(B) by Blocking Late Accumulation of Viral Proteins but Not by Degrading Incoming Viral Genomes

(A) Inhibition of RSV production by RNAi. RCASBP(B) genomes were transfected into DF-1 cells along with the indicated siRNAs. A total of 48 hr later, culture supernatants were harvested, virions were recovered by centrifugation, and viral proteins were quantified by a Western blot probed with an anti-RSV p19 antibody.

(B) RNAi does not inhibit the accumulation of viral cDNA early after infection. DF-1 cells were infected with RCASBP(B), then total DNA was harvested 12 hr later. The number of viral genomes was quantitated by using real-time PCR (Taqman).

of late viral transcription could be assayed by cotransfecting siRNAs with a cloned HIV-1 provirus. Introducing the HIV genome in this way bypasses the early steps of entry, reverse transcription, and integration and allows the effects on the late steps to be analyzed in isolation. The effects on two different HIV-1-encoding plasmids were compared (pR9 and pNL4-3). Viral particles produced from transfected HOS.T4.CXCR4 cells were analyzed by infecting 5.25luc4 indicator cells (Figures 5A and 5B) or by quantifying p24 capsid production (Figure 5C). siRNAs against HIV-1 were highly inhibitory in this setting (>95%), while control siRNAs (siMLV-gag-1 and siMLV-gag-2) did not inhibit virus production. Analysis by Northern blot revealed that siHIV-1 and siHIV-2 specifically reduced accumulation of the viral genomic RNA (Figure 6).

To assay the effect of siRNA on the early steps of infection, HOS.T4.CXCR4 cells were infected with HIV-1 and the accumulation of reverse transcription products was quantified. Efficient reverse transcription requires an intact RNA template, so possible action of RNAi should result in reduced accumulation of viral cDNA. DNA samples were harvested from cells 12 hr after infection, a time at which reverse transcription products are known to peak in abundance [24]. Viral cDNA copies were quantified by fluorescence-monitored quantitative PCR with primers that require the two template transfers of reverse transcription to be completed for the amplicon to be produced [24]. No significant differences were detected between siRNA-treated and nontreated cells, and this reveals that viral RNA genomes packaged in early replication complexes were not efficient substrates for RNAi (Figure 7). Thus, RNAi was active against mRNAs transcribed late during infection from proviral DNA but did not attack RNA genomes early after entry.

Inhibition of the late steps of RSV replication by RNAi was also tested (Figure 8A). Chicken DF-1 cells were transfected with siRSV-gag-1, siRSV-gag-2, or nonspecific siRNAs, and then infection was initiated by transfection with a plasmid encoding RCASBP(B). Two days later, RSV particles were harvested from cell supernatants and were analyzed by Western blot for accumulation of RSV capsid proteins. siRSV-gag-1 and siRSV-gag-2 showed inhibition in both settings. The single strands of siRSV-1 or siRSV-2 were not inhibitory (Figure 8). Inhibition of RSV replication by RNAi could also be documented in experiments in which RSV genomes were introduced by infection rather than transfection (data not shown).

To assess the effects of siRSV on the early steps of infection, DF-1 cells were treated with siRSV-gag-2 or siHIV-gag as a control and were infected with RCASBP(B), and reverse transcription was measured 12 hr later (Figure 8B). As with HIV, there was no significant inhibition of cDNA accumulation by specific siRNAs. These data indicate that RNAi acts primarily on RSV messages produced late during infection and, together with data on HIV, suggests that this may be generally true of retroviruses.

Discussion

Here, we report that RNAi can act as an effective antiviral system in vertebrates. We found that chick embryos supported efficient RNAi using in ovo electroporation to introduce nucleic acids. RNAi targeted against RSV could inhibit retroviral replication and pathogenesis in this model, and RNAi could also block retroviral replication in cell culture. These findings provide a convenient vertebrate animal model for studies of RNAi, and they open a wide range of possibilities for the use of RNAi against viral diseases.

Table 1. siRNAs Used in This Study

siRNA	Sequence	Target Gene	nt Coordinates
siLUC-1	5′-CUUACGCUGAGUACUUCGAAA-3′ 3′-GUGAAUGCGACUCAUGAAGCU-5′	firefly *luc*	156–178
siGFP-1	5′-GCAAGCUGACCCUGAAGUUCAU-3′ 3′-GCCGUUCGACUGGGACUUCAAG-5′	*egfp*	120–143
siHIV-gag	5′-GCAUUGGGACCAGGAGCGACA-3′ 3′-UUCGUAACCCUGGUCCUCGCU-5′	HIV *gag*	1793–1815 (NL4-3)
siHIV-pol	5′-GGGGCAGUAGUAAUACAAGAU-3′ 3′-UUCCCCGUCAUCAUUAUGUUC-5′	HIV *pol*	4966–4988 (NL4-3)
siMLV-gag-1	5′-UACUGGCCGUUCUCCUCUUTT-3′ 3′-TTAUGACCGGCAAGAGGAGAA-5′	MLV *gag*	1033–1055 (AF033811)
siMLV-gag-2	5′-CCACCUAGUCCACUAUCGCTT-3′ 3′-TTGGUGGAUCAGGUGAUAGCG-5′	MLV *gag*	1338–1360 (AF033811)
siRSV-gag-1	5′-GGGUUGCUUAUGUCUCCCUCA-3′ 3′-UUCCCAACGAAUACAGAGGGA-5′	RSV *gag*	478–500 (RCASBP[B])
siRSV-gag-2	5′-CGCUAAACAGUGUAGGAAGCG-3′ 3′-UUGCGAUUUGUCACAUCCUUC-5′	RSV *gag*	2007–2029 (RCASBP[B])

RNAi in Chick Embryos

The chick embryo has served as a classical system for studying the cellular interactions that control development [20, 25, 26]. Though many tools are in place for studying this system [27, 28], what has been lacking is the ability to inhibit gene expression. This paper provides evidence that chick embryos, in particular the cells in the developing neural tube that give rise to the spinal cord, contain the machinery to use siRNAs as a substrate for the targeted degradation of mRNAs. In this study, electroporation with siRNAs did not block development by causing, for example, a general block to translation via the interferon pathway. RNAi was specific, since endogenous genes *Isl1/2* were not affected by the siRNAs tested and no other nonspecific effects were seen. The methods described in this report offer great promise for studies of the neural tube, a powerful model for understanding how the nervous system is assembled [29]. It is now reasonable to expect that gene "knockdowns" can be performed in a tissue-specific manner through targeted electroporations, that multiple genes can be targeted simultaneously by combining multiple siRNAs, and that the timing of the knockdown can be controlled by selecting the appropriate stage of development to introduce the siRNA. Optimal use of this method would require inducing RNAi in a large fraction of the cells present. This may potentially be challenging to achieve by electroporating siRNAs alone, but we note that RNAi can be induced by suitably engineered DNA hairpins that can be transcribed to yield double-stranded RNA, and these may be delivered incorporated in a RCAS vector. This thereby allows for spreading after electroporation [6, 30].

Table 2. DNA Oligonucleotides Used in This Study

Primer	Sequence (5′ to 3′)
RSV-src-SalI	GAGAGCGTCGACAGCACACAAGGTAGTT
RSV-src-ClaI	CCATCGATGAAGCAGCGCAAAACGCCTAAC
HIV-F	TGTGTGCCCGTCTGTTGTGT
HIV-R	GAGTCCTGCGTCGAGAGAGC
HIV-probe	(FAM)-CAGTGGCGCCCGAACAGGGA-(TAMRA)
RSV-F	CCCCGACGTGATAGTTAGGG
RSV-R	CGAGACGGATGGAGACAGGA
RSV-probe	(FAM)-TCGGCCACAGACGGCGTGG-(TAMNph)

RNAi against Eukaryotic Viruses

We have surveyed siRNAs against four viruses, RSV, HIV, MLV, and adenovirus (this work and W.-Y.H., F.D.B., and M. Weitzman, unpublished data), and found that each could be specifically inhibited, suggesting that RNAi will be widely effective at inhibiting viral replication. As yet, no vertebrate virus has been reported to be insensitive to RNAi. This is in contrast to plant viruses such as potyvirus and cucumber mosaic virus, which encode proteins that inhibit RNAi and permit escape of the antiviral effect [11, 15–17]. We note that the experiments reported here employed transfection of short double-stranded RNAs, so any viral-encoded inhibitors of earlier steps in the pathway would not have been detected. It will be of great interest to determine whether any such functions are present in eukaryotic viruses.

It is as yet unclear whether any eukaryotic viruses produce double-stranded RNAs that can elicit RNAi during normal infection. Many viruses are inhibited by the interferon response, which is activated in part by double-stranded RNA and suggests that double-stranded RNA is produced in many viral infections. Double-stranded RNA viruses would appear to be particularly at risk for activating RNAi. Further studies should soon clarify the extent to which RNAi normally contributes to the control of viral infection in vertebrates.

Action of RNAi in the Viral Life Cycle

Studies reported here reveal that RNAi inhibited HIV-1 or RSV replication primarily by reducing accumulation of viral messenger RNAs late during the infection cycle. RNAi did not detectably degrade the genomic RNA of HIV or RSV early after viral entry, as measured by quantifying late products of reverse transcription. Early during infection, the incoming genomic RNA may be protected by association with capsid, reverse transcriptase, and other proteins associated with the viral core. Another possibility is that the incoming genomic RNA does not traffic through a cellular compartment that is required for association with the RNAi machinery. It has been

suggested that RNAi complexes may capture target messages as they exit the nuclear pore or bind to the ribosome (reviewed in [17]), so trafficking of early retroviral replication complexes may be unfavorable for engaging RNAi.

Applications of RNAi in Treating Infectious Disease

The demonstration of RNAi activity against retroviral pathogenesis suggests diverse possible applications in the prevention and therapy of disease. Practical application of RNAi technology will be facilitated by the use of engineered DNAs containing inverted repeat sequences, which can produce hairpin RNAs that are processed by the dicer nuclease to yield active siRNAs [6, 30]. If such molecules are well tolerated by cells, it may be possible to stably incorporate siRNA-producing DNA molecules in vertebrate cells to inhibit viral replication.

Farm animals might be engineered to resist economically important infections. For example, several viral diseases of chickens might be targeted, including fowl pox, chicken flu virus, and chicken anemia virus. Our finding that RNAi is highly active in chicken embryos suggests that it may be possible to block the replication of these viruses by introducing genes producing inhibitory siRNAs into the chicken germline [31–33]. Many other applications in animal husbandry can be also envisioned.

More speculatively, siRNA molecules might be used directly to treat viral diseases in humans. A pivotal question in assessing the feasibility will be determining whether RNAi amplifies within cells and spreads between cells in vertebrates, as has been reported in plants and worms. The electroporation method described here should allow these key questions to be addressed in a vertebrate model.

Experimental Procedures

Materials

Lipofectamine 2000 (LF2000) and Opti-MEM (OMEM) were purchased from Invitrogen. siRNAs were designed according to [34] and were purchased from Dharmacom. The siRNAs used in this study are shown in Table 1. Deprotection and annealing of siRNA was carried out as described by the manufacturer's protocol and [34]. All siRNAs tested showed inhibitory effects, though the levels of inhibition obtained with different siRNAs did vary. The *gfp*-encoding plasmid used was p156RRLsinPPTCMVGFPPRE [35]. The RCASBP(B) plasmid and RSV strain SRA-2 were obtained from S. Hughes [22]. The pR9 plasmid is described in [36]; pNL4-3 was obtained from the National Institute of Health AIDS Research and Reference Reagent Program.

The RSV proviral clone used was constructed by amplifying the *src* gene from SRA-2 with primers RSV-src-SalI and RSV-src-ClaI (Table2) and cloning into the SalI and ClaI sites of RCASBP(B).

In Ovo Electroporation

Chick embryos (SPAFAS, McIntyre Farms) were incubated in a humidified chamber and were staged according to Hamburger and Hamilton (H.H.) [20]. H.H. stage 11 chick embryos were windowed, DNA (0.5–3.0 μg/ul) and siRNA (0.1–1.5 μg/ul) were pipetted into the lumen of the neural tube, and the electrodes were placed on either side of the neural tube over the vitelline membrane. A square wave electroporator (BTX) was used to administer five pulses of current at 25V for 50 ms each, as described in [18]. Eggs were sealed, and the embryos were allowed to develop to H.H. stage 23 and were prepared for immunocytochemical analysis. Whole-mount GFP-transfected chick embryos were photographed with a Zeiss Stemi SV fluorescent dissecting scope.

Cell Culture and siRNA Transfection

293T, HOS.T4.CXCR4, and DF-1 cells were cultured in DMEM plus 10% fetal bovine serum (FBS). 5.25luc4 cells (which are CEMX174 cells transfected with LTR-*gfp* and LTR-*luc* reporters, a gift of N. Landau) were cultured in RPMI medium plus 10% FBS. All media also contained penicillin, streptomycin, and glutamine (1× PSG; GIBCO).

LF2000 transfections were carried out as described in the manufacture's protocol (Invitrogen). A total of 1 μl LF2000 was mixed with 50 ml OMEM and incubated for 5 min at room temperature before being added to 50 μl OMEM containing 10 nM siRNA and 0.5 μg DNA. The 100 μl mixture was then incubated for 20 min at room temperature. The siRNA-containing mixture was added to 5×10^4–1×10^5 cells in DMEM (with FBS and without antibiotics) to a final volume of 0.6 ml in either 12- or 24-well plates. The mixture was incubated for 2–4 hr, then the medium was replaced with 1–2 ml complete medium.

Retrovirus Infection

HIV-1 stocks were generated by transfection of 293T cells by using the LF2000 technique [37]. After 72 hr, supernatants were collected and filtered through 0.45-μm filters. Infections of siRNA-treated HOS.T4.CXCR4 cells were carried out at a multiplicity of infection of 0.5–1 (180 ng p24 per 10^5 cells in 1 ml volume in a 12-well plate). After 12 hr, cells were washed and 2 ml media was added. 5.25luc4 indicator cells were infected with 0.2–0.5 ml viral supernatant in 24-well plates (1 ml final volume per well).

For analysis of RSV infection in DF-1 cells, cells were transfected with siRNA and RCASBP(B)-encoding DNA. A total of 48 hr after infection, 1 ml viral supernatant was collected and centrifuged at 14,000 rpm in an Eppendorf table top centrifuge for 1 hr at 4°C, then the supernatant was removed and the pellet was analyzed by Western blot essentially as described [38]. Antibody against RSV Gag was obtained from the Developmental Studies Hybridoma Bank (University of Iowa).

Quantitative PCR Assays of Viral cDNA Synthesis

The total DNA was purified at 12 hr after infection with the QIAGEN DNeasy kit and was suspended in 25 ng/μl final concentration. Quantitative PCR was carried out as described [24]. The primers and probes used for quantitative PCR are listed in Table 2.

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Note Added in Proof

After this paper was submitted, several reports appeared documenting inhibition of viral replication by RNAi in cell culture:

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