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The Antiviral Potential of Mammalian RNA Silencing

by

Leonid Gitlin

DISSERTATION

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DOCTOR OF PHILOSOPHY

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of the

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To my parents

ACKNOWLEDGEMENTS

When I started graduate school, the last place I expected to find myself was a virology laboratory. Yet, two years into it, I remember being told by the Neuroscience faculty that, while I am not the first UCSF Neuroscience student to do his graduate work in a non-neuroscience lab, I do seem to be the first one to work in a non-neuroscience lab on a non-neuroscience thesis. Given this circuitous (and at times, treacherous) path towards completing my thesis, I am grateful to the people in the Neuroscience program who have helped me navigate it. Specifically, I thank John Rubenstein, Marc Tessier-Lavigne, and Jenny LaVail for their encouragement and advice, and Louis Reichardt and especially Cori Bargmann for their tolerance and support of a dissident within the program's ranks. Incidentally, I continue to enjoy neuroscience, and I certainly hope to participate in the field at some point in the future – I still believe that this is where much of the future lies.

In the meanwhile, however, I have come across virology, and which has proven quite contagious. Largely due to Raul, I've discovered the vast possibilities hidden in this discipline. Raul's role during my time here, however, has been infinitely larger than that. First, Raul agreed to

gamble on a black sheep from the Neuroscience program, and was thus a major reason for my remaining in graduate school. Second, while never short on valuable scientific advice, he has allowed me to beat my own path in research, which I think has contributed a great deal to my scientific development. Third, he did not lose his faith in any of us in the lab even during a very difficult time which was a direct outcome of misjudgment or rushed judgement of several lab members including myself. And finally, I cannot help but say that Raul remains forever young – the daring hypotheses that he generates sometimes make me feel ossified: a few weeks ago I found myself in an unlikely role of telling Raul that a certain idea of his was just too crazy to work.

Largely due to Raul, his lab over the years has been a wonderful place to do science and to learn how it is done, and I have not seen many places that would rival it. I will first mention Jens Herold, whose truly outstanding scientific rigor and vision remain the criteria by which I try to measure myself. Of course, Andrea Gamarnik is a most enthusiastic and thoughtful scientist and an excellent experimentalist – I only regret that I did not get a chance to work side by side with her. Oren Beske and Shane Crotty were the two graduate students whose scientific duels during lab meetings I truly miss; it is incredible how much science and everything

related – or unrelated – to it, one could learn from them. And of course, it is impossible to mention Debbie Silvera and Ale Arbetman – as well as Sergio Baranzini – without bringing back the whole atmosphere of the "old" Andino lab and sharing maté around the table.

I owe a lot to Yana Meltzer and especially Sveta Karelsky for volunteering in the lab, and all their hard work that was instrumental to our progress – as well as for being wonderful people to *have* around. Alas, they have been lost to medical school, as was Matias Elijovich, who even sacrificed his computer games for it. In my time here, I have also been lucky to get to know Marina Abramova, who is a truly extraordinary person; I wish her an upturn in her fortunes. Martha Neagu, even though she is now on the opposite coast, is still "partying" in the lab late at night, I hear. Her company is what is missing from our lab on these cold Mission Bay nights now.

I reserve special thanks to my baymate, Doro Vogt. She has managed to adapt to many of my quirks and certainly to my style of expression, which many find weird or even offensive (why?); that is in itself quite a feat. Doro helped me (and others) in many ways, and I simply could not wish for a better baymate. Liz Mathew helped me navigate vaccinia and yellow fever waters, and I hope that these

experiments will further our understanding of RNA silencing and viruses. And of course, the "new" Andino lab is a whole different place. Chanti Polacek must be one of the friendliest people on the planet, and she saved many a rainy day. Carla Saleh and Ronald van Rij are the twins carrying the torch of RNA silencing in the lab – I wish them good luck. One of the latest additions to the lab is Jeff Stone. Thanks to the long and undoubtedly tedious time that he spent in reading this thesis, I finally started to understand some of the finer usage of articles in the English language. More importantly, it was great to work together on catching some of the escape mutants. Some tantalizing hints have already emerged, and I am sure that his steadfastness, interest and hard work will soon lead to great discoveries. Keep it up, Jeff: a rolling Stone gathers no moss!

I am indebted to Alan Frankel, Don Ganem and Lewis Lanier for their advice on both specific experiments and my path as a scientist, and for their continued support. In particular, Don is a tremendous resource in regards to both science and the approaches to doing it, and my experience in graduate school would not have been the same without him. And Lewis is the person whose advice on postdoctoral laboratories was crucial in my decision, and whose scientific precision and breadth of immunology

perspective is something that I can only hope to acquire in my future life as an immunologist.

It would be impossible to list all the people I have met at UCSF over the years; but I hope to keep in touch with them. We were lucky to be neighbors with the Ganem and Lanier labs; they are an incredible group. Remembering UCSF, I will also, of course, remember Annette Bistrup and Mark Singer as my first mentors, Lev Osherovich as a roommate and an insightful scientist, and Christine Mirzayan, Elke Stein, and Sasha Faynboim, who I learned so much from, in and outside the lab.

My wife Eva is the person who must have suffered the most from my love affair with science. I thank her for her love and for standing by me at all times; I cannot imagine what things would be like had we not met. And of course, I thank my parents and my sister for encouraging me to be ambitious and supporting me in all my endeavors.

The work described in this dissertation was conducted in collaboration with other members of the Andino lab. Specifically, Sveta Karelsky has performed measurements of the replicon growth in Chapter 2 and the poliovirus experiment in Chapter 4. Liz Mathew performed titrations of, and infections with, vaccinia and yellow fever viruses in Chapter 4. Jeff Stone has constructed and grown the let-7 (+) and let-7 (-) polio viruses in Chapter 3.

Chapter 2 has been published in Nature (418: 430-434) in 2002; the results described Chapter 3 are being submitted for publication.

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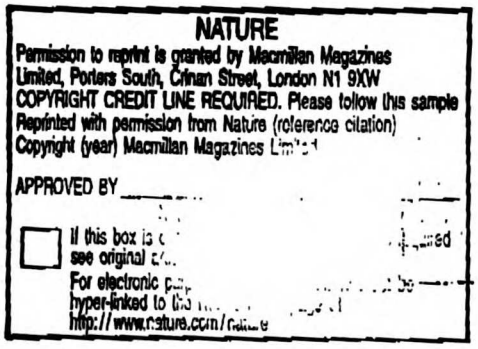

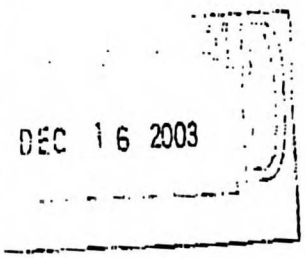

THE ANTIVIRAL POTENTIAL OF MAMMALIAN RNA SILENCING

Leonid Gitlin

Abstract

RNA silencing is a major antiviral defense in plants. Its conservation across eukaryotes suggests that mammalian viruses may also be natural targets of its action. We investigated the relationship between poliovirus and RNA silencing (or RNA interference) pathways, focusing on the potential use of double-stranded RNA as an antiviral therapy. Pre-treatment of mammalian cells with short interfering (si)RNAs against the polioviral genome reduces the progeny viral titer and promotes clearance of the viral genetic material from the majority of the infected cells. The antiviral effect is highly sequence-specific and independent of interferon and the classical mammalian double-stranded RNA response pathways. Analysis of viral mutants escaping from the siRNAs indicates that very limited sequence divergence is sufficient to impede RNA interference. It also suggests that both location and nature of the mismatch between the viral genome and siRNA determine the efficiency of target recognition. We propose that the RISC requires an A-form helix at the center of the siRNA-target duplex, while

recognition at the 5' half of the antisense siRNA strand may depend on the thermodynamic stability of the helix. In order to prevent viral escape, we utilized a population of siRNAs derived from 1 kilobase-long double-stranded RNA corresponding to the viral genome sequence; no escape mutants were observed in this case. Furthermore, analysis of cells infected with poliovirus, yellow fever, vaccinia and herpes simplex viruses indicates that these viruses do not actively suppress siRNA action. These observations suggest that it may be possible to develop double-stranded RNA therapeutics targeted against viral pathogens.

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CHAPTER 1. Introduction

Yet another revolution seems to be underway in molecular biology. It touches on many fields, and virology has been at its core from the beginning. The plant virologists have been reaping the harvest of new developments for years, and in the last several months seeds of change have been sown in animal and, more specifically, mammalian virology.

This revolution has been brought about in large part thanks to the efforts of the plant scientists in understanding and conceptualizing long-standing observations concerning virus-plant interactions. It promises a deeper understanding of host-pathogen relationships, development of powerful and very timely tools for functional genomics, and, very importantly, new approaches to therapies. Even though the field of RNA silencing (or RNA interference), as applied to mammalian viruses, is barely a couple of years old, there are already many exciting results and many more breathtaking possibilities for future discoveries.

The roots of the revolution

The history of RNA silencing (also called post-transcriptional gene silencing, or PTGS) research is deeply rooted in plant virology. The first observations indicating a presence of an adaptive and specific antiviral system operating in plants date back to the 1920's and 30's. It was described then that tobacco plants infected with one strain of tobacco mosaic virus failed to replicate a different strain (49). It was then shown that a mild strain of potato virus X (PVX) protected plants from a virulent strain of PVX, but did not protect against potato virus Y (65). This phenomenon was called cross-protection. Such a phenomenon is indeed reminiscent of the adaptive immune system in mammals. However, the hypothesis that plants possess an adaptive immune system of sorts has not taken hold in the field, despite an early suggestion that cross-protection may be, in fact, aquired immunity (19). Instead, the major hypotheses centered on some kind of dominant negative effects (57). It was thought that either viral proteins or viral RNA from the mild strain interfered with the replication of the incoming pathogenic strain, or else that the mild strain proteins soaked up the host factors required for viral replication. (It has to be noted here that such mechanisms may be sufficient to explain some cases of cross-protection; however, it now seems that many cross-protection phenomena are indeed due to something akin to an adaptive RNA immune system, since replication of RNA viruses interferes not only with homologous viruses, but also with homologous transgenes ((61), and see VIGS below)).

In the early 1980's, the introduction of transgenesis in plants led to a quest for the production of plants resistant to infection by certain viruses. Creating transgenics expressing whole genomes of mild viral strains was obviously not a viable option, and instead transgenics expressing individual viral proteins were made. Plants expressing coat proteins of various viruses successfully resisted infection by the cognate virus (1). In some cases, it was shown that expression of the coat protein in question was required for the resistance phenotype. Yet not all of the many examples of such pathogen-derived resistance (PDR) were due to expression of the desired protein (78); some controls designed to express viral RNA, but not the viral protein, elicited resistance. Eventually, the hypothesis of a plant adaptive immune system based on RNA was advanced (46). In this study, a plant transgenic for a fragment of tobacco etch virus (TEV) coat protein, was shown to restrict TEV replication. More importantly, the transgene RNA was apparently actively degraded upon viral infection. This led the authors to propose that a sequence-specific antiviral system was operative in the plant, whereby a given RNA sequence could be somehow marked as foreign and degraded.

Not only viruses could be subject to the action of this system. The acquired immunity hypothesis could also explain the puzzling effects of transgene expression observed several years prior in petunia (53, 72). Attempts to create transgenic plants expressing extra copies of chalcone synthase or dihydroflavonol-4-reductase not only failed to increase the enzyme levels, but led unexpectedly to a shutdown of the

corresponding endogenous genes. This effect was dubbed co-suppression, and was included in the long list of phenomena associated with gene silencing. Another addition to this list was virus-induced gene silencing (VIGS), whereby viruses were shown to elicit silencing of sequence-related transgenes or endogenous genes (46, 64). In fact, it became clear that both viruses and transgenes can both be the initiators and targets of RNA silencing (Fig. 1). The molecular underpinning of these processes, however, remained a mystery.

The central role of double-stranded RNA

In the mid-1990's, attempts to block gene expression in the worm C. elegans through injection of antisense RNA demonstrated that either the antisense or control sense strand of RNA occasionally phenocopied the expected loss-of-function mutation (37). This remained a puzzling observation until it was suggested that potentially the preparation of one strand could be contaminated with traces of the complementary strand, forming double-stranded RNA (dsRNA), which was hypothesized to be a signal for the degradation of cognate single-stranded RNA (33).

Initially thought to be a peculiarity of the worm, RNA interference, as it came to be called, proved to be astonishingly universal. With the exception of *S. cerevisae*, we now know that every other tested eukaryote exhibits a related response to dsRNA. Besides proving an exceptionally useful technique for gene inactivation, RNA

interference (RNAi), when taken together with the long-standing research by plant virologists, offered a very appealing mechanism for the silencing phenomena.

Double-stranded RNA is a hallmark of viral infection. There are arguments that little if any dsRNA is sensed by the host in the process of viral replication. Nevertheless, even excluding the silencing system described here, there appears to be at least three mammalian dsRNA-interacting proteins that are upregulated by interferon signaling: protein kinase R (PKR), 2-5A synthetase, and double-stranded RNA adenosine deaminase (ADAR) (29). Importantly, upon binding dsRNA, PKR induces interferon gene transcription (20). It is thus not at all surprising that double-stranded RNA would be treated by various organisms as a danger signal. Indeed, as an obligatory intermediate in the replication of RNA viruses, double-stranded RNA is their Achilles' heel. Even DNA viruses produce dsRNA as a byproduct of bidirectional transcription from their genome (21, 44).

Thus, a reason for the existence of a dsRNA recognition system clearly exists. The fact that the material recognized is RNA immediately provides the host with a template which could instruct the RNA silencing machinery as to which mRNAs to degrade. The outcome is an adaptive immune system, which stands ready to recognize and remember a particular nucleic acid sequence as foreign. A much more evolved immune system of mammals, built to recognize foreign proteins, has been well-studied; both it and RNA silencing seem to derive their potency from being able to adapt to a

diversity of invaders. Curiously, no one predicted the existence of a nucleic acid-based immune system, even when data rather suggestive of it started becoming available.

RNA silencing: molecular mechanisms.

RNA silencing is known to be elicited by RNA viruses (46) (64), transgenes (53, 72), and transposons (41, 70). Aside from in the case of viruses (which presumably activate silencing by dsRNA), the induction stage of the process is not understood. Is dsRNA the only nucleic acid able to induce RNA silencing? It is possible that other signals exist. It has been proposed, somewhat loosely (76), that "aberrant" RNAs or overexpressed RNAs in plants serve as an initiator of silencing. In this case, however, it is possible that they are first converted into dsRNA by an RNA-dependent RNA polymerase (RdRP) (66).

The stages following dsRNA appearance are now understood much better due to recent studies of RNA interference in in vitro systems of Drosophila and mammalian cell extracts*. These studies have led to the model schematically presented in Fig. 2. Specifically, dsRNA is processed into 21-25 nucleotide double-stranded short interfering

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* A note on nomenclature: currently, the terms RNA interference and RNA silencing are used interchangably, with silencing preferred by plant biologists, and interference, by animal biologists – due to historical circumstances. However, the split between the terms can be viewed also as a difference in the phenomena studied: most of the animal studies have employed exogenous dsRNA, while the plant research has focused on the viruses and transgenes as the in vivo silencing inducers. Therefore, in this thesis, I will follow the nomenclature where RNA interference denotes an artificial introduction of dsRNA by the

RNAs (siRNAs) by an enzyme complex, which is comprised of Dicer and possibly homologs of C. elegans rde-4, rde-1 and drh-1/2. The siRNAs are then unwound and incorporated into the RISC (RNA-induced silencing complex). RISC monitors the sequence of cytoplasmic RNAs, and upon discovery of a mRNA complementary to the siRNA, proceeds to cleave the mRNA approximately in the middle of the homology region. Members of the Argonaute gene family and a putative nuclease have been identified as RISC components (17, 48).

Another proposed siRNA fate may involve its use as a primer by the RdRP, which could extend it along the targeted RNA, and thus amplify the double-stranded silencing signal. This pathway exists in C. elegans (68) and evidently plants (26, 52), however, its existence in Drosophila is contentious, and it has not been seen in mammalian cell extracts (63, 69). Finally, another pathway, described in plants (75), appears to shunt siRNAs to the nucleus to block transcription of homologous genes. This is accompanied by de novo methylation of homologous sequences. Interestingly, some fungi go as far as introducing mutations into repetitive regions (repeat-induced point mutation, or RIP-(14), or even deletion of sequences homologous to dsRNA (79).

One of the most fascinating aspects of RNA silencing is its ability to spread between cells, as documented in plants and C. elegans (33, 56). The utility of such a

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experimenter (as well as the resulting target RNA knockdown), while RNA silencing is a natural process, initiated by dsRNA or another inducer produced in vivo.

process as an antiviral defense is self-evident. Indeed, in certain cases the spread of the silencing signal in infected plants seems to outpace the spread of the virus itself (38).

Roles of RNA silencing: overview.

Our current picture of the natural roles of RNA silencing is something of a mosaic, owing to the diversity of model organisms studied. The evidence of silencing as an antiviral system has been derived from plant-virus interactions (see below). In *C. elegans* and *D. melanogaster*, evidence was obtained for the involvement of RNA silencing in transposon shutdown. Since certain nematode and fruitfly strains deficient in RNAi also have a mutator phenotype (increased transposon mutagenesis), it was suggested that RNA silencing blocks transposon accumulation in the animal genome (11, 41, 70).

A separate and long-standing line of research in *C. elegans* has implicated short RNAs in the fine temporal control of protein synthesis. Genomically encoded double-stranded hairpins, now termed microRNAs (miRNAs) are expressed at precise times in development (16). They are processed by Dicer, much like long dsRNA would be, but instead of mediating cleavage of the target, they induce a translational shutdown by an enigmatic mechanism (54). The reason for this difference is not well understood, but may have to do with the location of the miRNA-complimentary sites in the message

(there are more than one in the 3'UTR), and the fact that the resulting duplex is not perfect, but is usually predicted to contain a bulge in the middle of the sequence, where RISC is thought to cleave (48). This arrangement apparently provides for the rapid shutoff of the translation of a specific message. Roles for miRNAs in apoptosis and other processes have been proposed (7). It would seem that any process requiring rapid shutdown of a given mRNA's expression (some obvious examples would be found in the nervous system) will likely utilize the capabilities of miRNAs.

RNA silencing vs. viruses in plants and invertebrates

And what of the antiviral role of RNA silencing? It is now generally accepted that PTGS is a major antiviral defense mechanism in plants (5, 31, 61). This concept rests on several lines of evidence, described here and depicted in Fig. 3. First, as described above, natural infection by plant viruses elicits strong gene silencing (46). This observation proves that the viral genomic RNA, or intermediates of replication, are used effectively by silencing machinery to trigger PTGS. Second, viral replication can be efficiently suppressed by experimentally induced RNA silencing (46, 77), demonstrating that viruses can indeed be targeted by PTGS. Third, plant viruses encode a variety of inhibitors of the RNA silencing machinery (8, 13, 40, 73). The fact that viruses devote their scarce genetic resources to interfering with PTGS suggests that, for successful replication in nature, viruses must overcome the double-stranded RNA response. Fourth,

some components of this response, like the RdRp, are upregulated by viroid and virus infection (12, 66). Finally, mutations in genes that encode the silencing machinery, like *sgs2* (also called *sde1*), *sgs3*, and *sde3*, can result in enhanced susceptibility to virus infection (27, 52). Taken together, these observations are proof that RNA silencing is a major antiviral mechanism in plants.

RNA silencing also appears to contribute to antiviral defense in invertebrates. Preinfection of cells or whole mosquitoes with Sindbis virus, carrying dengue virus genome fragments, was shown to inhibit dengue virus replication (2, 34, 55). Although these results are reminiscent of the cross-protection phenomenon seen in plants (67), these experiments could not distinguish whether the inhibition was produced by RNA silencing, RNA antisense activity, or dominant-negative effects such as those induced by defective interfering RNAs. However, a recent study demonstrated that it is indeed possible to inhibit dengue virus production by transfection of dsRNA into mosquito cells; in contrast, a single-stranded RNA control was not effective (15). Furthermore, cells transfected with a plasmid designed to express an inverted-repeat RNA derived from the dengue virus genome were resistant to virus infection and accumulated what appears to be siRNAs (3).

Important evidence supporting a physiological antiviral role for RNAi in invertebrates was obtained by studies of flock house virus (FHV) and its interaction with the RNA silencing machinery in *Drosophila* cells (45). This nodavirus infects insects but

can also replicate in plant and mammalian cells. FHV infection results in accumulation of siRNAs specific for the viral genome. These siRNAs are able to promote specific degradation of an artificially introduced viral RNA segment. Interestingly, FHV protein B2 can block RNA silencing in both plant and invertebrate cells. B2 can functionally (and mnemonically) replace the 2b protein of cucumber mosaic virus, which is an established suppressor of RNA silencing. Notably, FHV-induced RNA silencing was prevented by expression of B2 or by depletion of AGO2 (a putative RISC component of the Argonaute family). These experiments argue for the role of RNA silencing as an adaptive antiviral defense in invertebrates. However, there are currently no studies in mammalian systems which suggest that RNA silencing in mammals is directed against viral invaders. While any data falling into the five categories outlined above and in Fig. 3 for plant-virus interactions would strongly suggest a similar role for RNA silencing in mammals, no direct evidence for any of them has yet emerged.

Poliovirus as a model RNA virus

Motivated by this lack of data on mammalian RNA silencing-virus interaction, we decided to study the effects of RNA interference on the replication of poliovirus. Poliovirus (or, more affectionately, polio) is a model RNA virus, and its contributions to the fields of virology and molecular biology are numerous. They range from the developoment of animal virus culture techniques (32) to the demonstration of viral

synthesis in a cell-free system (51); they include insights into the monocistronic nature of eukaryotic mRNAs (39) and a demonstration of cap-independent translation (18, 58).

Polio initiates infection by attaching to the poliovirus receptor (PVR) on the cell surface and releasing its positive-sense RNA genome into the cytoplasm; indeed, naked polio RNA is infectious (6, 22). Viral RNA replication takes place in the cytoplasm and does not require nuclear functions – (23). The viral polymerase 3D amplifies the polio genome in an exponential process of RNA-dependent RNA polymerization. This process must involve dsRNA intermediates; however, the extent to which viral RNA is double-stranded inside the infected cell has not been examined satisfactorily. It is possible that no more than a few base pairs between complementary strands are actually formed at any given time. However, even if the base-pairing is more extensive, the replication intermediates are unlikely to be exposed to the cytosol, as replication takes place on the surface of intracellular membranes inside replication complexes formed by an interaction between the many 3D polymerase subunits (47, 59). Many more positive strands than negative strands are eventually made in HeLa cells (10), and these positive strands are encapsidated, creating the progeny virus.

The viral RNA is polyadenylated and possesses an internal ribosome entry site (IRES), which promotes translation from its long open reading frame (ORF) (42, 58, 60, 80). The polio polyprotein is co- and post-translationally processed into several polypeptides (39). These polypeptides fall into two classes: the structural, or capsid,

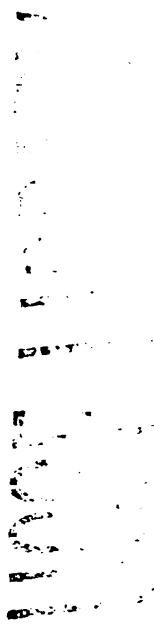
proteins, encoded by the N-terminal P1 region, and the non-structural proteins, encoded by the P2 and P3 regions (see Fig. 4). The structural proteins are not required for RNA replication, and thus have been successfully substituted for the reporter gene firefly luciferase (9). The resulting RNA, called a replicon, amplifies itself in the cell, while the luciferase activity faithfully reports the level of viral protein expression. Obviously, the amplified replicon RNA is not encapsidated and fails to exit the cell.

The virus, not surprisingly, modifies the cellular environment radically in the course of replication. It blocks transcription and translation (28, 81); the latter effect is accomplished through the viral proteinase 2A. 2A cleaves the translation initiation factors eIF4GI and II, preventing translation of capped cellular mRNAs (35). Viral RNA translation is further enhanced by virtue of the removal of competition for the translation apparatus from the cellular mRNA. The virus sets an apoptotic process in motion, but blocks it at about 2.5 hours post-infection (hpi) (4). Infection in most cell lines used to study replication, such as HeLa/HeLa S3 or HEK293 cells, results in cell lysis within 6-9 hpi. While non-primate mammalian cells are resistant to the virus, this resistance is due to the lack of a functional poliovirus receptor, and can be overcome by expression of the PVR (50); in fact, mouse models expressing PVR have been created to study poliovirus pathogenesis (25, 43, 62).

For a poliovirologist, there are not many good options for inhibition of the virus once its genome has entered the cell. Mutagens that lead the virus into error catastrophe

(24) delay the cytopathic effect (CPE); unfortunately, at higher concentrations they are also cytopathic (in fact, their toxicity limits their use in the clinic). Other drugs block viral replication by virtue of blocking vital cellular processes (30). Conventional antisense oligodeoxynucleotides have not proved particularly successful against a related enterovirus as they required high and very toxic concentrations of the agent and led to relatively modest viral inhibition (74). Curiously, there is one report in the literature (71) which claims that anti-poliovirus antibodies block an already established infection and prevent CPE in SK-N-MC neural cell line (although not in HeLa); this report, however, has not been followed by further publications.

We reasoned that treatment of the cell with dsRNA to poliovirus may provide the cell with an almost perfectly selective antiviral agent, and set us on a road leading to the answer of whether dsRNA is naturally utilized by mammalian cells in an antiviral manner. These considerations guided us in the research presented below.



Scope of this thesis

A simple-minded pre-treatment of HeLa cells with siRNAs against poliovirus leads to a strong reduction of the titer of progeny virus. Chapter 2 builds on this observation to investigate the mechanism and sequence-specificity of this reduction. We find that siRNA in our system acts early in the viral life cycle, proves extremely sequence-specific and independent of interferon and the interferon effectors PKR and

RNase L. Furthermore, the siRNA leads to effective clearance of the viral genomes from infected cells, thus suggesting that RNA silencing may be a mechanism underlying non-cytopathic viral genome clearance observed in certain infectious diseases (36).

Despite its effectiveness againt viruses such as polio, siRNA remains very far from the clinic. For example, delivery of siRNAs could prove a major hurdle. However, the problem especially highlighted by our research has been viral escape. It is obvious that an RNA virus will produce enough variants to evade any given siRNA; this is exactly what we see in tissue culture. Chapter 3 of this thesis deals with analysis of escape mutants emerging from cells transfected with two anti-polio siRNAs. We find that in the case of one siRNA, viruses bearing one mutation, approximately in the center of the siRNA complementarity region, dominated the population. In case of the other siRNA, there seemed to be less of an emphasis on the center; instead, efficient escape required at least two separate mutations in the complementarity region. It is possible that in the latter case, the 5'-half of the antisense siRNA strand is an important element of the recognition function of the RISC.

We then investigate two possible strategies for preventing or limiting the viral escape from the siRNAs. The first one relies on targeting siRNAs to the most conserved genomic regions of the virus. Only two such regions exist in poliovirus, both inside the IRES, and siRNAs against them are inactive. Another approach is based on the generation of a wide array of siRNAs, which is most easily done by RNase III-mediated

processing of long dsRNA derived from the viral genome. In vitro processed 1 kilobase-long dsRNA allows for suppression of viral replication, and prevents the appearance of escape mutants over the course of three consecutive viral passages. In cells capable of processing dsRNA by Dicer, the same result can be achieved by treatment with long dsRNA.

Thus, chapters 2 and 3 focus on the potential therapeutic use of dsRNA. As mentioned above, one hypothesized natural role of RNA silencing is antiviral defense; indeed, the difficulty that poliovirus has escaping a diverse array of siRNAs stresses how effective naturally induced RNA silencing would be in this capacity. Chapter 4 begins to investigate this hypothesis by positing that some viruses would evolve to block any process that serves to defend the host against them. Thus, mammalian viruses could be expected to block RNA silencing. This possibility was addressed by setting up RNA interference against an unrelated target (luciferase) in cells previously infected with several viruses. Unfortunatly, only the first part of this investigation was carried out, where siRNAs were used to silence luciferase; the second planned part, where long anti-luciferase dsRNA was to be used, was not completed in the course of this research. No anti-siRNA activity was found to be encoded by poliovirus, yellow fever virus, vaccinia virus and herpes simplex type 1 virus. I hope that this research will be continued, and I remain optimistic regarding our chances of finding a virally encoded blocker of mammalian RNA silencing.

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Figure Legends

Fig. 1. Relationship between RNA silencing inducers, targets, and the corresponding silencing-related phenomena. Replication of viruses leads to a block in replication of sequence-related viruses (cross-protection) and in the expression of nucleus-encoded genes (VIGS). Some transgenes, or endogenous genes producing dsRNA, will block expression of related genes (co-suppression) and replication of related viruses (PDR).

Fig. 2. The central dogma of RNA silencing. The process can be triggered by viral infection, transposons, or aberrant RNAs. Long dsRNA precursors are processed into siRNAs by Dicer. siRNAs associate with the RISC, which in turn directs degradation of specific RNAs. The siRNAs can be amplified by a cellular RdRp, or shunted to the nucleus to trigger a transcriptional shutdown of homologous DNA sequences.

Fig. 3. Is RNA silencing a natural antiviral defense system in mammals? Establishing it as such requires addressing the five critical questions which are schematically presented in the figure. See text for details.

Fig. 4. Poliovirus genome, polyprotein expression, and replicon structure. A) The poliovirus genome is a 7.5 kb messenger RNA that is covalently linked to VPg, a viral

peptide, on its 5'-end. B) Its translation yields a polyprotein which is proteolytically processed into several polypeptides. C. The replicons are RNA molecules in which the P1 region of the viral genome has been substituted with luciferase, either firefly or renilla.

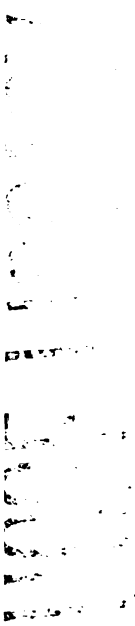


Figure 1

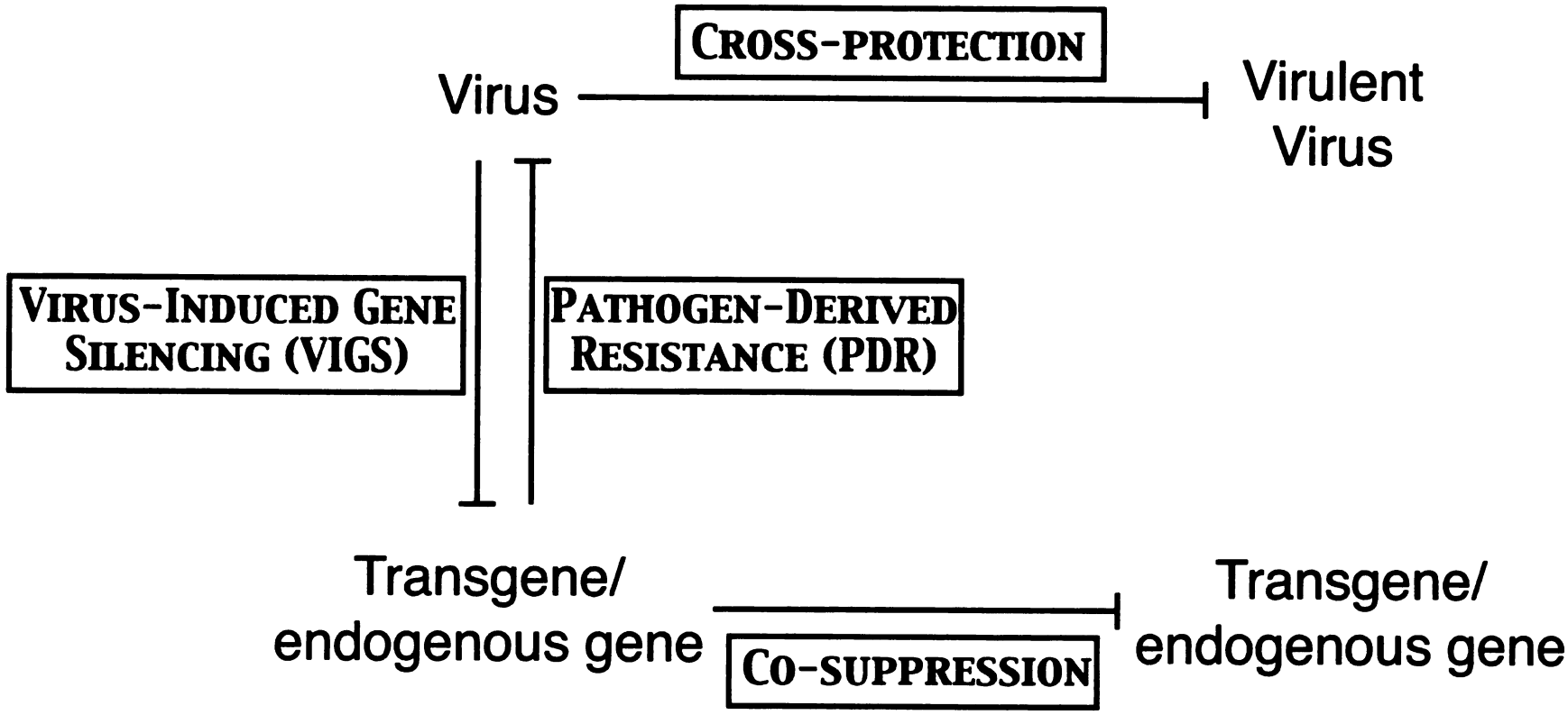
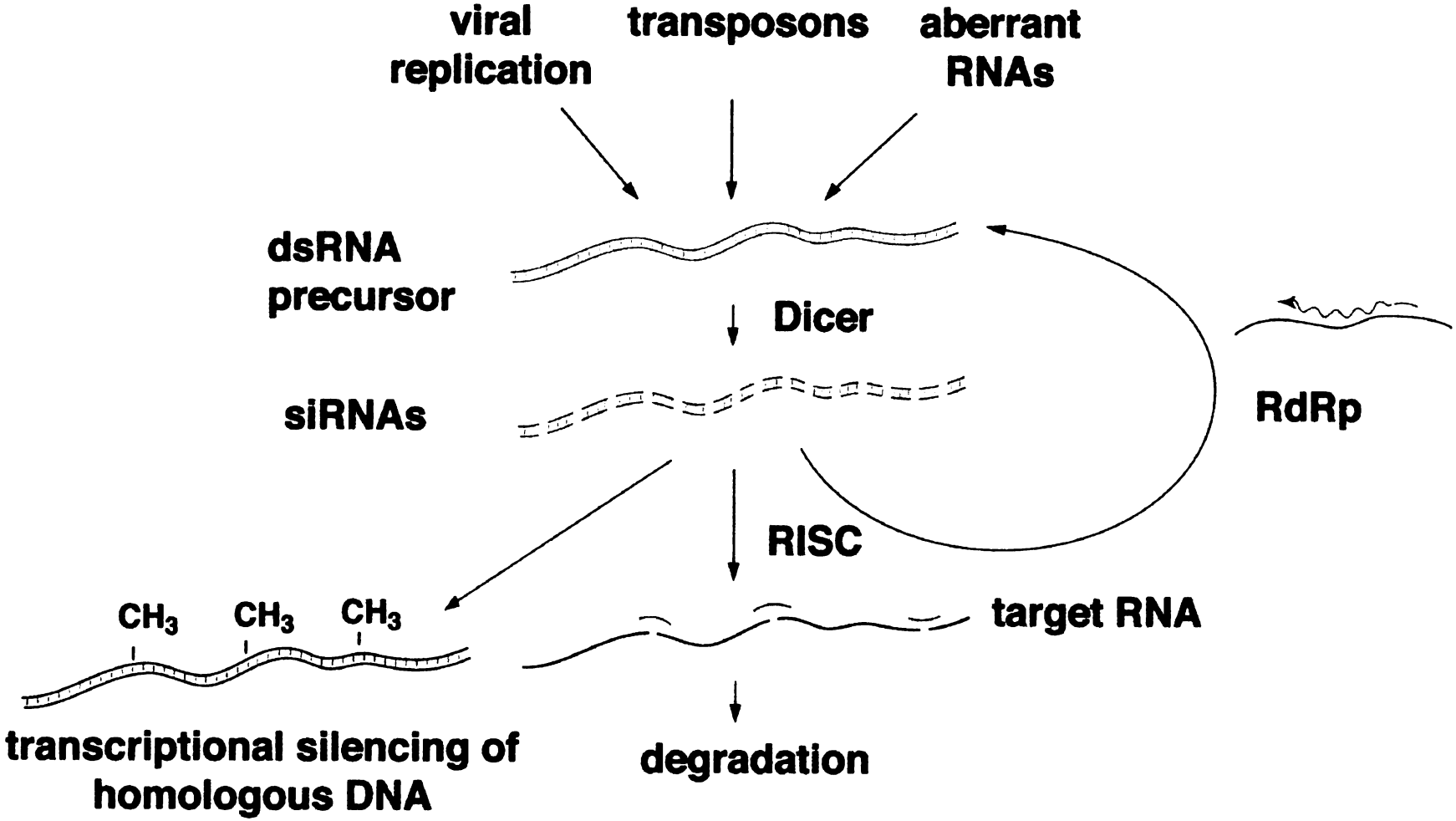

CROSS-PROTECTION
Virus
Virulent Virus
VIRUS-INDUCED GENE SILENCING (VIGS)
PATHOGEN-DERIVED RESISTANCE (PDR)
Transgene/ endogenous gene
CO-SUPPRESSION
Transgene/ endogenous gene

Figure 2


viral replication
transposons
aberrant RNAs
dsRNA precursor
Dicer
siRNAs
RdRp
RISC
target RNA
CH3
CH3
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transcriptional silencing of homologous DNA
degradation

Figure 3

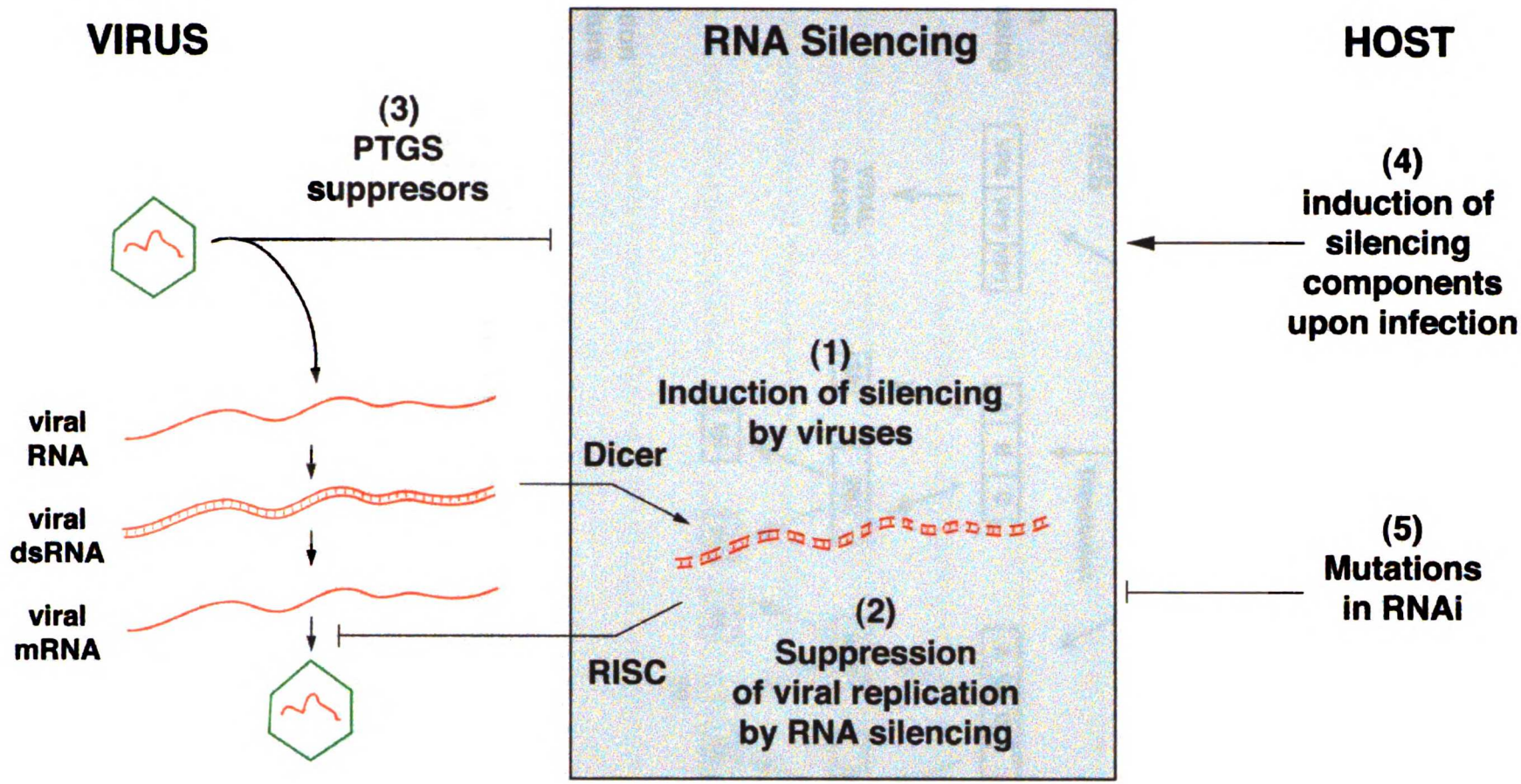
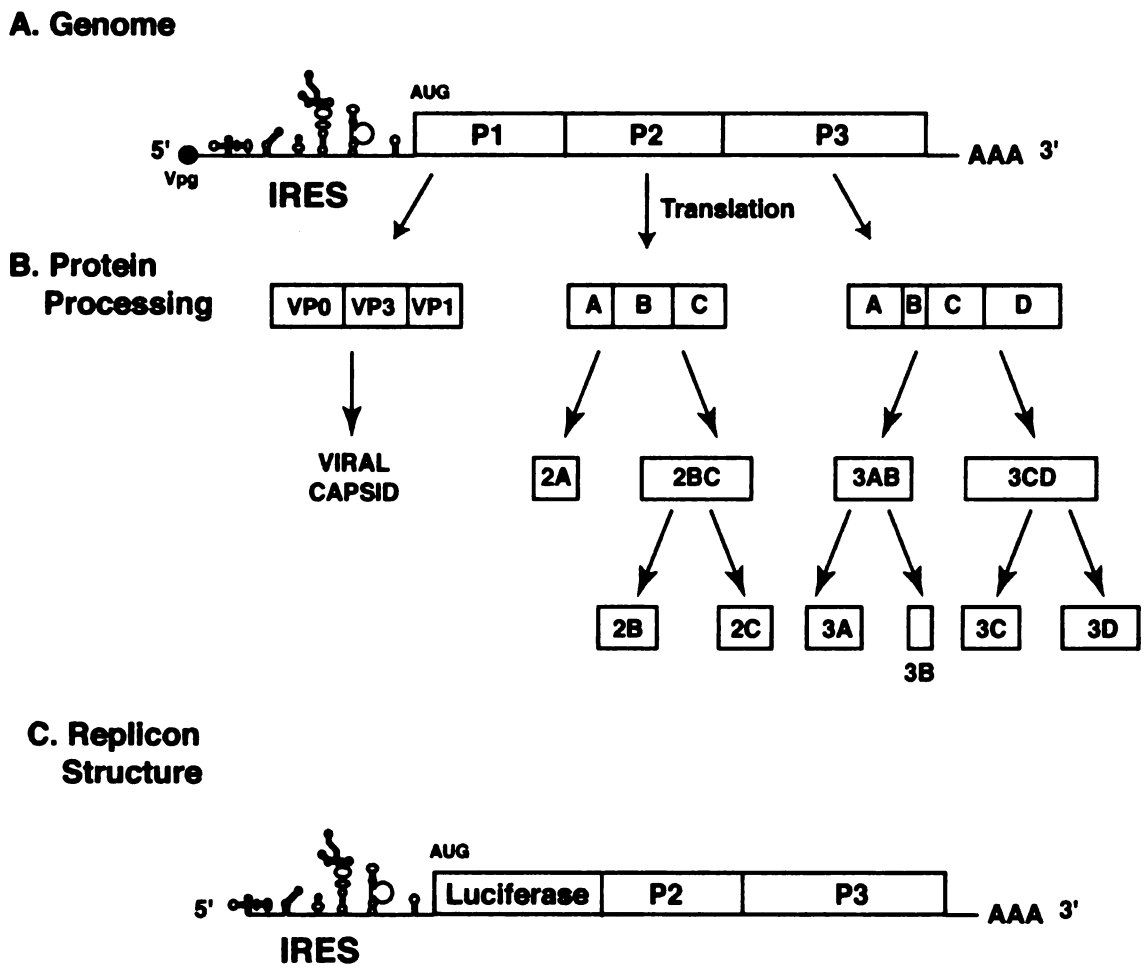


Figure 4


A. Genome
AUG
P1
P2
P3
5'
Vpg
AAA 3'
IRES
Translation
B. Protein Processing
VP0
VP3
VP1
A
B
C
A
B
C
D
VIRAL CAPSID
2A
2BC
3AB
3CD
2B
2C
3A
3B
3C
3D
C. Replicon Structure
AUG
Luciferase
P2
P3
5'
AAA 3'
IRES

CHAPTER 2. Short-interfering RNA confers mammalian intracellular immunity to viral infection

Double stranded RNA (dsRNA)-mediated gene silencing is highly conserved in eukaryotes. In plants, it appears to serve as an antiviral defense mechanism. Mammalian cells also possess this machinery but its specific function is still unclear. Here we demonstrate that dsRNA can effectively protect cells against infection by a rapidly replicating and highly cytolytic human RNA virus. Pretreatment of human and mouse cells with double-stranded, short interfering RNAs (siRNAs) to the poliovirus genome drastically reduces the titer of virus progeny and promotes clearance of the virus from most infected cells. The antiviral effect is sequence-specific and not attributable to either classical antisense mechanisms or to interferon and the interferon response effectors PKR and RNaseL. Importantly, protection is the result of direct targeting of the viral genome by siRNA as sequence analysis of escape virus, resistant to siRNAs, reveals one nucleotide substitution in the middle of the targeted sequence. Thus, siRNAs elicit specific intracellular antiviral resistance that could provide a therapeutic strategy against human lytic viruses.

Controlling infection by viral pathogens is a major challenge for all multicellular organisms. Mammals have developed a highly sophisticated immune system that protects them against a large variety of pathogens. Both innate and acquired immunity play an important role in clearing viral infections. A frequent strategy of these responses is the destruction of infected cells. However, recent observations suggest the existence of mechanisms of immunity that reach inside infected cells, inhibiting viral replication and enabling cell survival. For example, hepatitis B virus (HBV) infects large numbers of cells but is cleared from most of them without significant cytopathology (11). Similarly, non-cytolytic virus clearance has been observed in neurons infected with Sindbis virus (17). This intracellular protection against viruses has been attributed to activation of interferon-mediated responses, central components of innate immunity.

A nucleic acid-based, intracellular defense mechanism has been well documented in plants (18, 26, 27). This exquisitely sequence-specific system uses dsRNA to silence gene expression, both at the level of transcription (transcriptional gene silencing or TGS) and RNA stability (post-transcriptional gene silencing or PTGS) (21). TGS and PTGS have also been described in animals (2, 8, 10, 14, 20, 22, 28). In invertebrates, gene silencing can protect against mobilization of endogenous transposons (13, 15, 23). Interestingly, a 21-nucleotide dsRNA intermediate, siRNA, has been shown to specifically downregulate cellular as well as viral gene expression in human and mouse

cells, suggesting that the phenomenon of dsRNA interference (RNAi) is conserved in mammals (4, 5, 9). These findings raise the question of whether gene silencing can be an effective mechanism of intracellular immunity, promoting viral clearance and cell survival.

To determine whether RNAi can protect against a highly cytopathic virus we studied the effects of siRNA on viral replication using poliovirus as a model. This positive-stranded RNA virus replicates in the cytoplasm of infected cells with rapid kinetics. Cells are lysed and viral progeny released within 6-8 hours post-infection. In addition, virally induced apoptosis appears to be initiated soon after infection, resulting in rapid cell death even in the absence of ongoing replication (24).

The effect of RNAi on viral replication was examined using two polio-specific siRNAs, one corresponding to a capsid sequence (siC) and another to a viral polymerase sequence (siP) (Fig. 1a). An unrelated siRNA corresponding to a firefly luciferase coding sequence (siL, Fig. 2b) was used to control for non-specific dsRNA effects. Additional controls included the individual strands of siC [ssC(+) and ssC(-)] and double-stranded DNA (C-DNA) of identical sequence to siC, which is unable to elicit RNA interference (10).

Strikingly, transfection of siC prior to infection with the virulent poliovirus Mahoney strain completely inhibited virus plaque formation in both human (Fig. 1) and mouse cells (data not shown). In contrast, none of the control transfections affected plaque formation (Fig 1b). One-step growth curves indicated that cells treated with siC (Fig. 1c) or siP (not shown) produced only 1-3% of the virus titer observed in control treated cells.

To examine the effect of siRNA on viral protein and RNA production, cells were infected with high multiplicity-of-infection (MOI=10). Although this treatment ensures infection of every cell, production of the viral polymerase ($3D^{pol}$) and its precursors was greatly impaired by treatment with siC and siP (Fig. 1d). Likewise, accumulation of poliovirus RNA in siC-treated cells was drastically reduced (Fig. 1e). Our results indicate that siRNA interferes with viral replication early after infection.

The effect of RNAi on viral replication was further investigated using poliovirus replicons, in which the capsid protein coding sequences have been replaced by either firefly (FLuc) or renilla (RLuc) luciferase reporter genes (Fig. 2a). Cells were transfected with a 1-to-1 mixture of both replicons, plus siRNAs (Fig. 2c). Although siL had no effect on virus production (Fig 1c), co-transfection of replicons with siL resulted in a specific reduction of firefly, but not renilla, luciferase produced (Fig. 2c). Conversely, siC, which inhibits viral production (Fig. 1), did not affect FLuc luciferase expression and

replicon RNA replication (Fig. 2c). Furthermore, siL had no inhibitory effect on FLuc^{mut}, a firefly luciferase replicon bearing 5 silent point mutations within the siL target sequence (Fig. 2d). Thus, the inhibitory effect requires a perfect match between the siRNA and the target RNA sequences.

It is in principle possible that inhibition of viral replication is caused by an interferon-mediated response, which can be induced by dsRNA (16). We thus examined whether interferon or other secreted factors mediate the siRNA-induced inhibition and found that supernatants from siC-transfected cells have no effect on poliovirus replication (not shown). Furthermore, dsRNA activates two interferon-induced enzymes, PKR and RNaseL, which inhibit gene expression in a non-specific manner. These two proteins have also been suggested to participate in activation of sequence-specific gene silencing systems (7, 25). However, a robust inhibition of FLuc expression was observed in response to siL in mouse fibroblasts deficient in PKR and RNaseL (PKR/RNaseL double KO) (Fig. 2e). We conclude that neither interferon nor its dsRNA-activated effectors PKR and RNaseL are required for suppression of poliovirus replication by siRNA.

The siRNA-mediated inhibition of poliovirus replication must involve mammalian post-transcriptional gene silencing (PTGS) mechanisms, since the virus replicates in the cytoplasm through RNA intermediates. There are three possible modes for this PTGS action: it may interfere with viral RNA synthesis, block translation of the

viral genome as observed for stRNAs (19), or destabilize the viral RNA, as observed for RNAi in Drosophila extracts (29). Since a non-replicating RNA expressing firefly luciferase (FLuc mRNA, Fig. 3a) is also susceptible to inhibition by siL (Fig. 3b), we concluded that RNA replication is not required for siRNA inhibition. We then examined the stability of FLuc mRNA in cells transfected with either siC or siL (Fig. 3c). Treatment with siL induced a reduction in the levels of FLuc mRNA, this effect was most evident 1 hour post-transfection (Fig 3c compare lanes 5 and 6). Interestingly, a weak band corresponding in size to the expected 3' cleavage product, presumably an intermediate of degradation, was detectable at 0.5 hours post-transfection (Fig. 3c, lane 4). These experiments indicate that RNA interference by siRNAs in mammalian cells is mediated, at least in part, by mRNA degradation.

The 100-fold reduction in viral titer caused by siRNA treatment could result from a reduction in virus production per infected cell or alternatively from a reduction in the number of productively infected cells. As the high MOI used in our experiments ensures that all cells are infected, the latter possibility would indicate that RNAi is capable of aborting viral infection. The number of cells expressing viral proteins at 6 hours post-infection was determined by immunofluorescent staining and FACS analysis. Strikingly, over 90% of cells did not express detectable amounts of viral proteins (Fig. 4a, b).

siRNA-treated cells, negative for viral antigen, may be completely cleared of infection or may carry viral genomes expressing low amounts of viral proteins. Thus, we cloned, by limiting dilution, cells infected with polio (MOI=10) and treated with siC to detect the presence of latent virus. In contrast to controls, siC- and siP-transfected cells reproducibly formed colonies in at least half of the wells (not shown). Importantly, we were unable to re-isolate virus from these cultures and a highly sensitive RT-PCR analysis failed to detect viral RNA indicating that the cloned cells are not persistently infected (Fig. 4c). Although protected from the original infection, the cloned cells were fully susceptible to reinfection with poliovirus 21 days after siRNA treatment (not shown). Taken together, these data indicate that dsRNA can induce effective antiviral intracellular immunity that clears most cells of infection.

We next evaluated the protective effect of siRNAs over a time-course of poliovirus infection. At 24 hours post-infection, cells transfected with siC prior to infection show no signs of cytopathic effect, while control cultures are completely lysed by 8 hours (Fig. 5a). About 75% of siC- or siP-transfected cells were viable 26 hours after infection; notably, a combination of siC+siP extended the protection to 54 hours (Fig. 5b). However, despite the initial protective effect, prolonged incubation led to cell lysis, even after siC RNA treatment (Fig. 5b). We thus considered two possible reasons for loss of protection: waning of siRNA activity or emergence of siRNA-resistant viruses.

To address these possibilities, cells were treated with siC or siP (at time=0) and infected with poliovirus at 0, 1, 2, 4, or 5 days post-transfection. Effective inhibition of viral production was observed as late as 5 days after transfection (Fig. 5b). Thus, waning of siRNA activity over time does not appear to account for the observed monolayer destruction. However, the virus isolated from siC-transfected cells was barely, if at all, sensitive to siC (not shown). Sequencing analysis revealed silent point mutations in the majority of clones analyzed (19 clones in total). There were two types of mutants (siC^r-1 and siC^r-2), carrying U->C transitions in the middle of the siC-target region (Fig. 5d). Thus, it seems that a small sequence divergence allowed escape from protection through siRNA. These results underscore the importance of sequence identity of siRNA to the target sequence for effective antiviral activity. Importantly, these escape mutants rarely emerged when infecting cells with lower MOIs (not shown). This, together with the fact that many infected cells are completely free of viral RNA (Fig. 4c) suggests that the siRNA-resistant variants were present in the initial viral population and did not emerge from the siRNA-treated cells. We conclude that the PTGS apparatus interacts directly with viral sequences and is not dependent on activation of other, intermediary antiviral mechanisms.

The present work indicates that the mammalian PTGS machinery can be programmed with siRNAs corresponding to viral sequences to induce an effective

antiviral response. It is possible that, as observed in plants, gene silencing functions as an adaptive, nucleic acid-based defense system in mammalian cells. However, additional experiments are necessary to determine whether PTGS mechanisms function as a natural intracellular response to viral infection, without the need for pretreatment with artificial siRNAs. Even though our results indicate that poliovirus is susceptible to siRNA-mediated gene silencing, it is possible that the virus neutralizes the PTGS pathway by inhibiting the processing of long, viral dsRNA to siRNA. Nonetheless, our demonstration of viral genome clearance from mammalian cells infected by an otherwise lytic virus is evidence that sterilizing antiviral immunity may be achieved without the destruction of cells harboring virus.

It was previously proposed that intracellular immunization may be achieved by expression of proteins or RNAs that confer resistance to virus infection (3). Double-stranded RNA is a promising vehicle for induction of intracellular immunity. Unlike classical antisense techniques, double-stranded RNA taps into existing, powerful gene silencing pathways, which may facilitate its therapeutic potential. Our results also predict that attempts to use siRNA therapeutically against viruses, especially RNA viruses, will have to contend with their variability due to high mutation rates. However, the emergence of escape variants resistant to siRNA can be minimized by employing dsRNA directed against multiple RNA target sequences. In addition, this model system can be exploited

to further examine the mechanism of action of PTGS in mammalian cells and the ability of RNA viruses to neutralize the inhibitory effect.

Methods

Molecular biology procedures

RNA oligonucleotides were purchased from Dharmacon Research (Lafayette, CO), and resuspended in 1 mM Na citrate at 100 _M. For generation of double stranded siRNA, individual strands were mixed, heated to 100°C for 3 min and allowed to cool to room temperature; final concentration of the siRNAs was 40 _M in 100 mM potassium acetate, 30 mM HEPES pH 7.4, 2 mM magnesium acetate. DNA oligonucleotides were purchased from Operon Technologies (Alameda, CA), and treated similarly where used as siRNA controls. Cytoplasmic RNA was isolated from cells in accordance with RNeasy protocol (Qiagen). Reverse transcriptions were done with SuperScript™ RT (Invitrogen) using oligo d(T) primer. PCRs were run with Elongase™ (Invitrogen) for detection of viral/tubulin-__RNA or PfuTurbo™ (Stratagene) for amplification and cloning of cDNA derived from resistant viruses, according to manufacturers' instructions. PCR primers used were: tubulin-_:5'-GATGGAGCCCTGAATGTTGA and 5'-TGATGTTAATGACTTTACTTTGAGATATG; Mahoney detection:5'-TATGATGCATCTCTCAGCCCT and 5'-GCGAACGTGATCCTGAGTGTT; Mahoney cloning: 5'-GCTAGACACCGTGTCTTGGA and 5'-GGACTGTGTTGTCAATCATGCT

Northern blotting hybridizations were done at 42°C in 50% formamide as described (1), with probes prepared using RediPrime kit (Amersham-Pharmacia). The probes corresponded to nucleotides 1894-2408 of the poliovirus genome, 1109-1601 of the luciferase coding region and 732-1385 of __tubulin_coding region.

Western blotting was performed by standard protocols with ECL System reagents (Amersham-Pharmacia), using purified rabbit polyclonal antibody direct against a 3D N-terminus peptide. Cells were grown in 24-well plates, lysed in 50 _l of 1% NP40 in 140mM NaCl, 5 mM KCl, 10 mM Tris pH7.5, and 5 μl of each sample loaded on a denaturing 12% polyacrylamide gel.

Cell culture and virus

HeLa S3 and mouse embryonic fibroblasts were cultured in DMEM/F12 supplemented with 10% FBS, 2 mM glutamine, 100 units/ml of penicillin, and 100 μg/ml streptomycin (Invitrogen).

Immortalized PKR/RNase L-deficient cells were established from mouse embryonic fibroblasts (30) by transfecting them with a plasmid encoding SV40 large T-antigen. Typically, cells were seeded at $5x10^4$/well the day before and transfected with 2 μl LipofectAMINE 2000 (Invitrogen) combined with 3 μl of 40 μM siRNA. Transfection

mixtures were left on cells overnight and washed off immediately before viral infections, unless indicated otherwise.

Mahoney strain of poliovirus was produced from a cDNA clone by T7 transcription and amplified in HeLa S3 cells. For infections, virus was diluted in PBS or culture medium and adsorbed onto cells for 20-40 minutes. Incubation were at 37°C. For plaque reduction assays, the monolayers were covered with 1% semi-solid agar overlay and stained with crystal violet after 2 days. For titer determination, cells were collected from the wells, lysated by 3 freeze-thaw cycles and the virus titered by plaque assays (6).

RNA electroporations were done essentially as described (12), but were modified to contain 10 μg of 1:1 firefly:renilla replicon mixture +/- 5 μl of 40 μM siRNA; for PKR/RNase L knockouts, ~$4x10^6$ cells were used per electroporation. Luciferase assays were performed using the Dual Luciferase Assay System (Reporter kit, Promega).

Microscopy, immunofluorescence, and FACS

Cells were observed on Nikon Eclipse TE200 microscope and photographed using Spot CCD camera. For immunofluorescence, cells were grown on coverslips and fixed in 4% PFA for 20 min at room temperature, then washed in PBS, permeabilized with 0.1% TritonX-100, and stained with anti-N-terminus poliovirus 2C rabbit antiserum diluted 1:500 into 1% BSA/PBS; Alexa Fluor 488-conjugated goat anti-rabbit secondary was

used (Molecular Probes) at 1:300, and coverslips mounted in VectaShield medium containing DAPI (Vector). Pictures were taken on Leica DMLB fluorescent microscope with ScionImage software. For FACS, cells were permeabelized with 0.02% saponin and stained with 1:1500 dilution of anti-C-terminus 2C rabbit serum, then treated with PE-conjugated donkey anti-rabbit secondary (Jackson Labs). FACS was performed with with 25,000 cells per condition, using FACSCalibur and CellQuest software (Becton-Dickinson).

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Figure Legends

Fig. 1. Poliovirus growth is suppressed by siRNA. **a,** Schematic representation of poliovirus genome and relative locations of siC and siP. **b,** Plaque reduction assays: HeLa monolayers were transfected with siC or controls. After removing transfection mixtures, monolayers were infected with poliovirus (~30 plaque-forming units). **c,** One-step poliovirus growth curve using siRNA pre-treated cells. **d,** Western blot analysis with anti-$3D^{pol}$ of cells transfected with buffer, siL, siC or siP and infected with poliovirus (MOI=10). **e,** Northern blot analysis of poliovirus RNA at 6 and 8 hours post-infection; cells were pre-transfected with siRNA as indicated. Alpha-tubulin served as the loading control.

Fig. 2. siRNA inhibits replication of poliovirus replicon in a sequence-dependent manner. **a,** Schematic representation of poliovirus firefly, renilla luciferase replicons; target sequences in $FLuc^{wt}$ and $FLuc^{mut}$. **b,** Firefly luciferase-specific siRNA. **c,** Firefly (solid) and renilla (dotted) luciferase activity of replicons co-electroporated into HeLa with siRNAs or L-DNA. Rightmost panel represents firefly/renilla ratios normalized to siC, (at each timepoint siC=1.0); this data is the mean ratio values for 3 experiments +/- standard

deviations. **d,** Firefly luciferase expression by mutant ($FLuc^{mut}$) and wildtype ($FLuc^{wt}$) replicons in siL-transfected HeLa, normalized as in c. **e,** Inhibition of firefly luciferase production by siL in PKR/RNaseL double-knockout cells, normalized as in c.

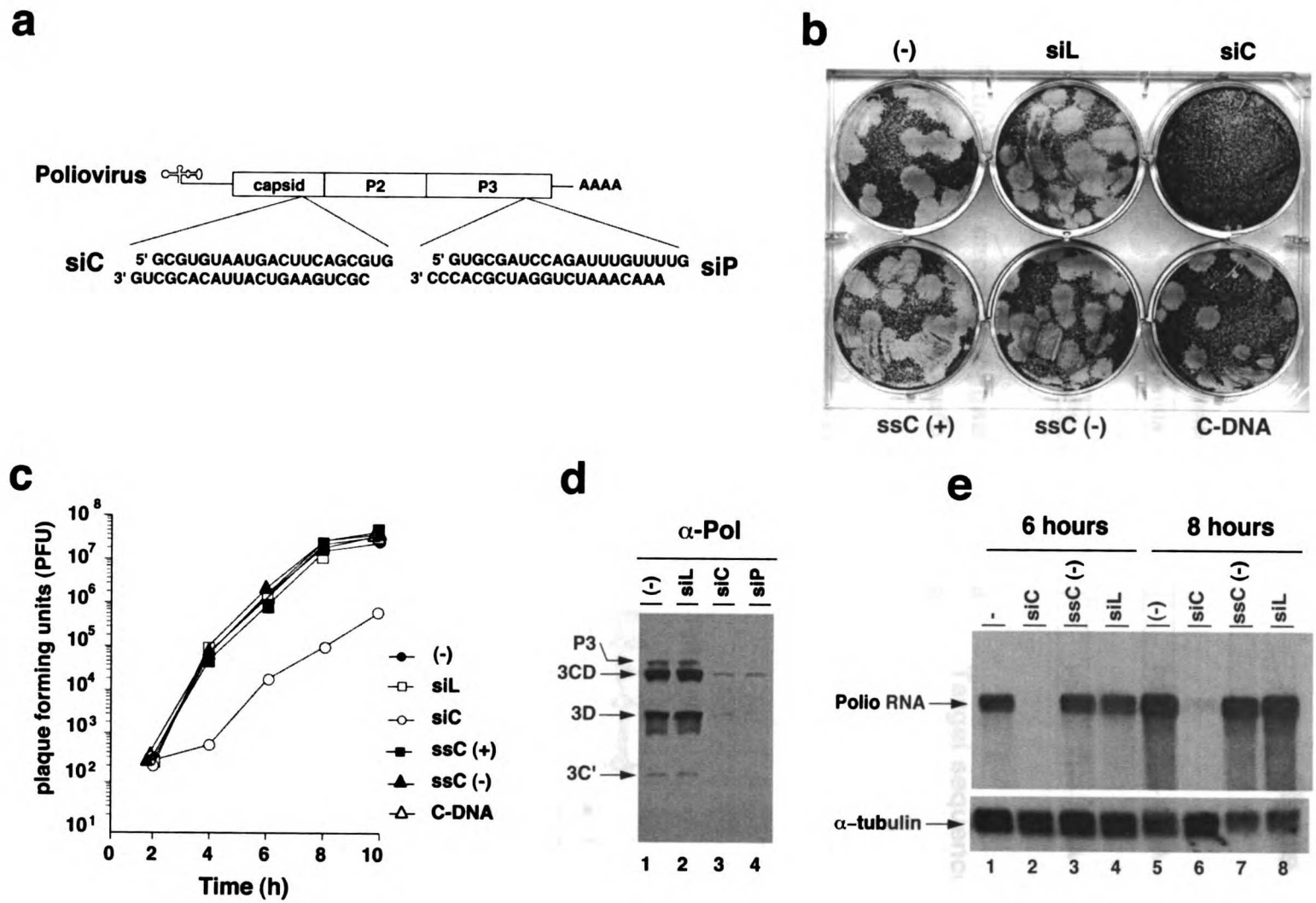
Fig. 3. Gene silencing is rapid, post-transcriptional, and not dependent on viral replication. **a,** Schematic representation of non-replicating firefly luciferase mRNA (FLuc mRNA). **b,** FLuc mRNA was electroporated together with various siRNAs. Results shown as firefly:renilla ratios (as in Fig. 2). **c,** Northern blot of firefly luciferase mRNA was electroporated with siC or siL at various times post-infection. The lower arrow indicates a putative 3' cleavage intermediate derived from Luc mRNA. Alpha-tubulin served as the loading control. The amount of radioactivity in each band was determined by phosphoimaging. Ratios of FLuc to tubulin mRNA are included at the bottom of the figure.

Fig. 4. Most cells treated with siC clear infection and do not harbor viral genomes. **a,** Immunofluorescence of siL/siC-treated cells, infected at MOI=10. The poliovirus-infected cells were distinguished using an anti-2C antibody and nuclei were stained by DAPI. **b,** FACS profile of cells transfected with buffer (red), siL (green), siC (orange), or

siP (blue), then infected (MOI=10); or control uninfected cells (solid purple). **c,** RT-PCR of viral RNA from 6 independent cell clones obtained from infected, siC-transfected cultures (lanes 2-7). Positive control: RT-PCR from cells infected at low MOI (0.1, 0.01, 0.001) for 1 hour (lanes 8-10). At MOI of 0.001, <1000 cells are expected to be infected, indicating that the assay is very sensitive. Alpha-tubulin served as loading control.

Fig. 5. Infected cells survive more than a day, but are eventually lysed by emerging siRNA-resistant virus. **a,** Bright-field micrographs of cells pre-transfected with buffer, siC, and siL, taken 24 hours post-infection (MOI=10). **b,** Cell viability in presence of siRNA, determined by trypan blue staining at various times post-infection. **c**, HeLa were transfected with siRNAs (siL, siC and siP) and infected 0, 1, 2, 4, or 5 days post-transfection (scheme). Virus titers were determined 24 hours post infection. The effect of siC and siP is presented as % titer reduction with respect to siL negative control (0% reduction). **d,** siC target sequences of sensitive (wild type) and siC-resistant (siC^r) virus genomic RNAs.

Figure 1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siP
b
(-)
siL
siC
ssC (+)
ssC (-)
C-DNA
c
plaque forming units (PFU)
Time (h)
(-)
siL
siC
ssC (+)
ssC (-)
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α-Pol
(-)
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siC
siP
P3
3CD
3D
3C'
1 2 3 4
e
6 hours
8 hours
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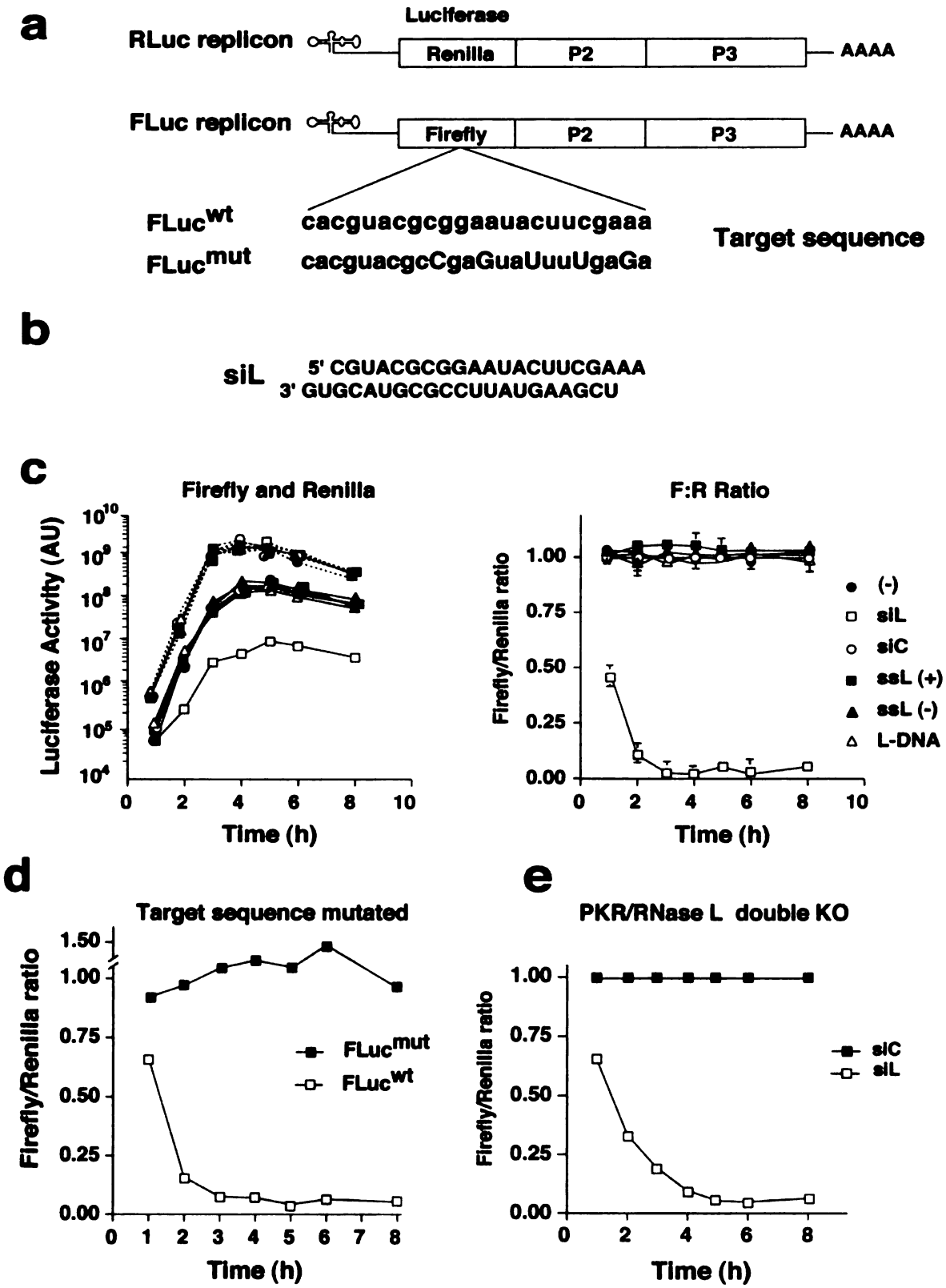
Figure 2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F:R Ratio
Firefly/Renilla ratio
Time (h)
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siC
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ssL (-)
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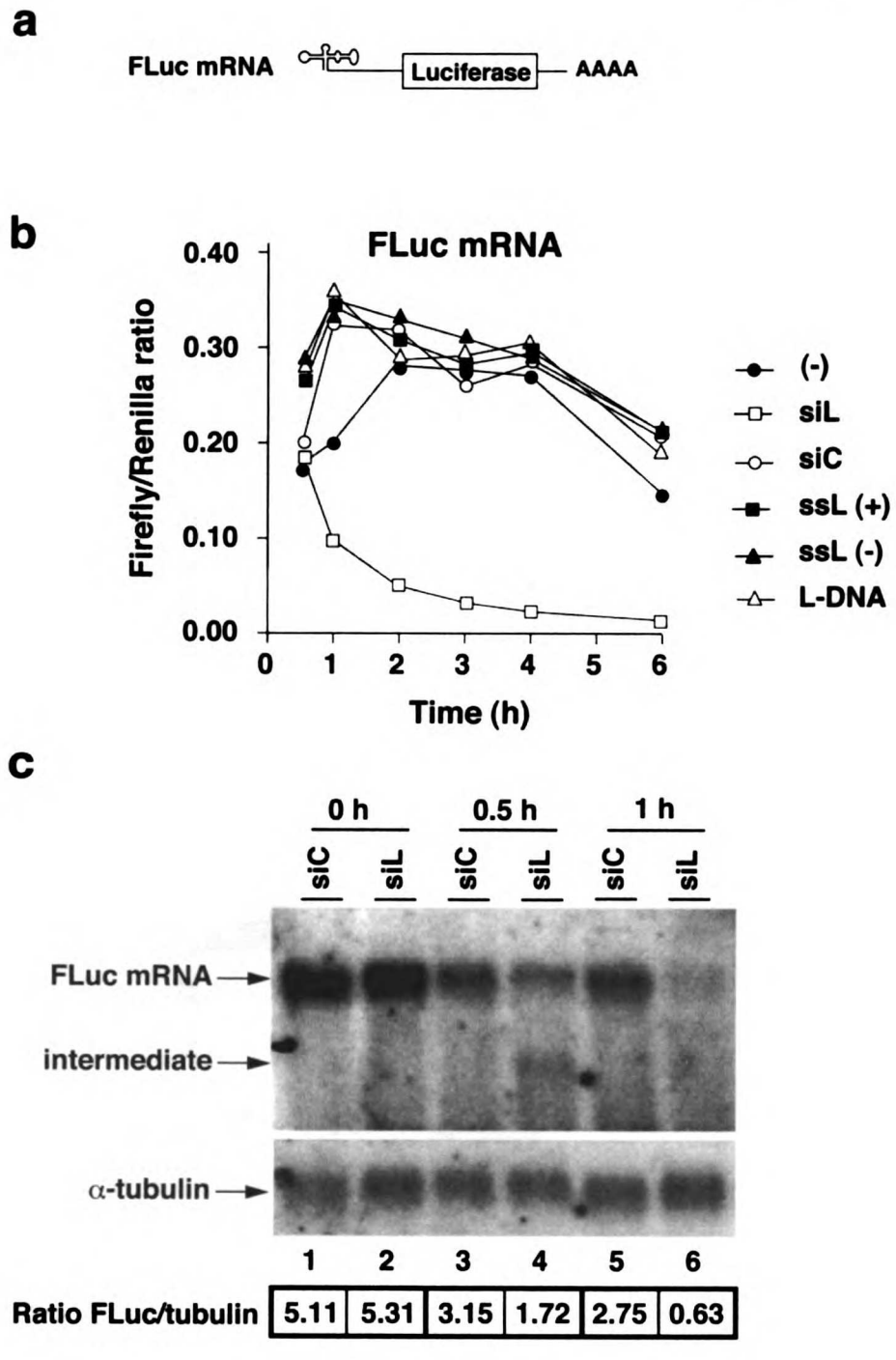
Figure 3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3
4
5
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(-)
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siC
ssL (+)
ssL (-)
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0.5 h
1 h
siC
siL
siC
siL
siC
siL
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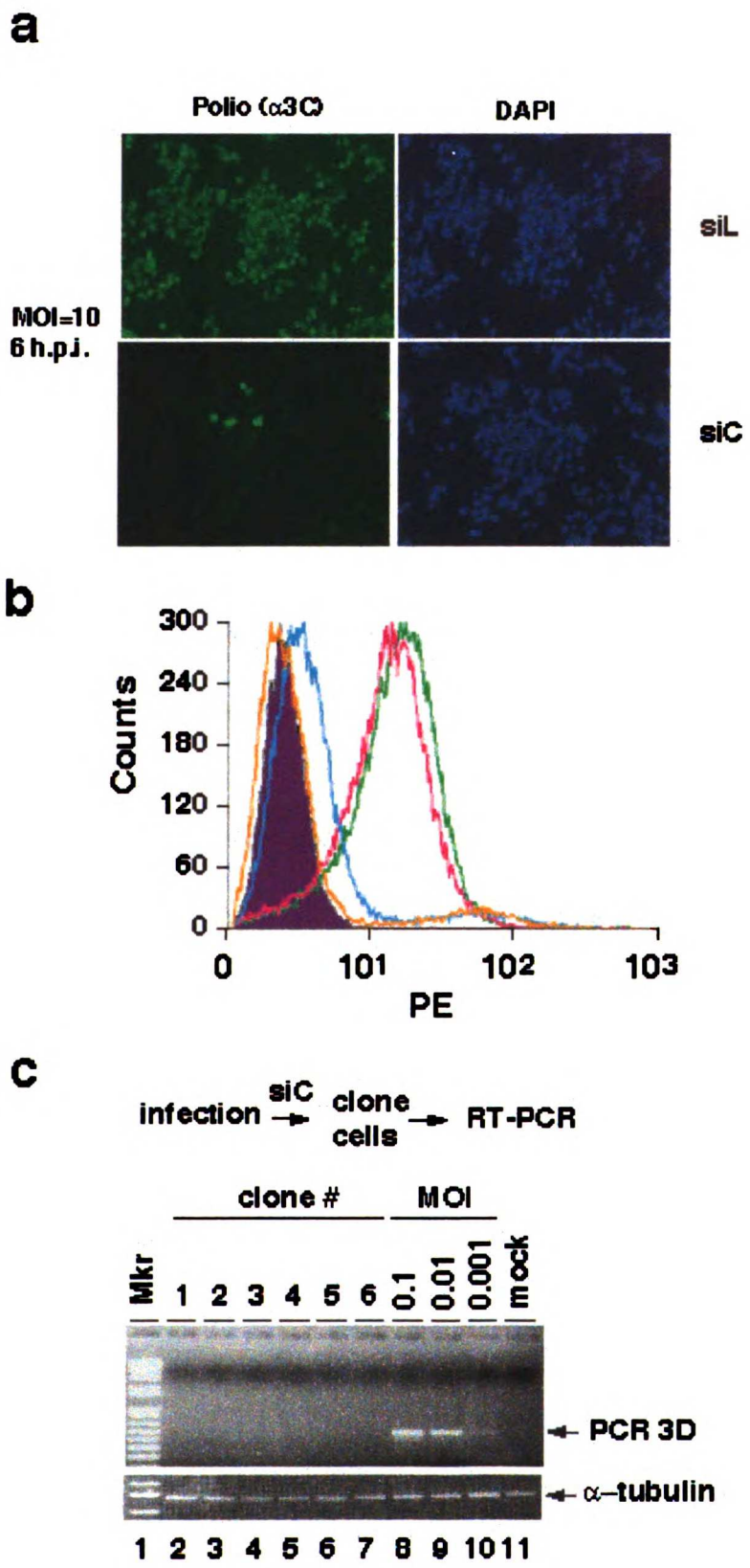
Figure 4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0
101
102
103
PE
c
infection
siC
clone cells
RT-PCR
clone #
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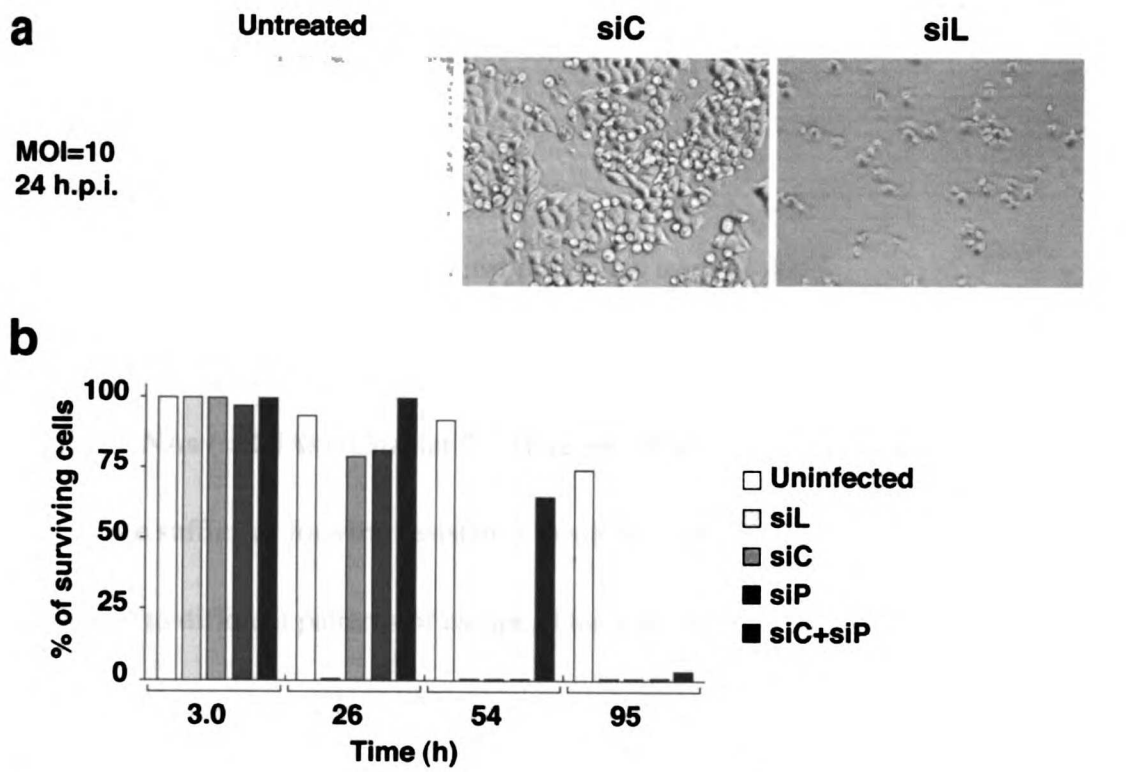
Figure 5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siL
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24 h.p.i.
b
% of surviving cells
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75
50
25
0
3.0
26
54
95
Time (h)
Uninfected
siL
siC
siP
siC+siP
c
siRNA transfection → infection → titration
(t = 0)
(t = 0 to 5 days)
% of Inhibition
100
75
50
25
0
0 1 2 3 4 5
Days
siC
siP
d
wild type
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siC^r-1
5' CAGCGUGUAACGACUUCAGCGUG
siC^r-2
5' CAGCGUGCAAUGACUUCAGCGUG

CHAPTER 3. Poliovirus rapidly escapes defined siRNAs, but not an siRNA population

Abstract

RNA interference (RNAi) against poliovirus leads to rapid emergence of viruses with point mutations in the regions targeted by short interfering RNAs (siRNAs) (Chapter 2). Here we show that these mutations are sufficient for viral resistance to siRNA, and that different siRNAs lead to different patterns of escape. One siRNA led to the appearance of viruses mismatched in the center of the si-targeted region, while the mutants elicited by the other siRNA were preferentially located either in the center or in the 3' half of the targeted sequence. We propose that an A-form helix, and not thermodynamic stability, is required in the central region of the siRNA-mRNA duplex for efficient RISC recognition. The recognition of the 3' target region, however, either varies depending on the siRNA, or hinges on the thermodynamic properties of the helix. Most escape mutations are silent, but missense mutations can be readily generated, thus enabling a selection procedure for the generation of mutants in a short, defined region of a replicating RNA. Escape is efficient, since two mismatches can result in a virus which is insensitive to

an siRNA. Despite the ease of escape, selection of viral mutants can be prevented by employing a population of siRNAs, produced either *in vivo* from long dsRNA, or enzymatically by treating dsRNA with RNase III. This finding advances RNAi as a viable therapy against viruses.

Introduction

The recent discovery of RNA silencing and rapid progress in understanding its mechanism (18) hold promise for basic research as well as for the clinic. RNA silencing is a eukaryotic mechanism which recognizes double-stranded RNA (dsRNA), digests it into ~21 nucleotide pieces through the action of Dicer, a member of the RNase III enzyme family, and employs the resulting small interfering RNAs (siRNAs) to direct sequence-specific degradation of complementary mRNAs. A cytoplasmic RNA-induced silencing complex (RISC), possibly containing a staphylococcal nuclease-like enzyme and one strand of an siRNA serving as a guide (7, 33), is thought to scan the mRNAs and catalyze the cleavage of the complementary target mRNA. In plants, RNA silencing is a major antiviral mechanism (47). Its role in mammals is less well defined. The initial investigations of viral susceptibility to siRNAs have established that most mammalian virus families are susceptible to RNAi (5, 15, 25, 26, 44), thus paving the way for a possible use of RNAi in the clinic. However, several problems will need to be resolved for this to happen: dsRNA delivery, dsRNA stability (or persistence) *in vivo*, and viral escape (reviewed in ref. (16)). Our discovery of the escape mutations (Chapter 2) has highlighted the difficulty which awaits potential siRNA-

based antiviral therapies, and led us to investigate this phenomenon in more depth.

It is not clear yet how much, or what kind of sequence divergence between the viral genome and siRNA will lead to abrogation of RNA interference. In preliminary experiments, our escape mutants appeared to replicate relatively efficiently while containing only one mutation (Chapter 2 of this thesis). In the case of anti-HIV siRNA, single-nucleotide mismatches appeared less significant (25). However, a recent study suggested that the virus can still escape with one mutation in the center of the targeted nucleotide stretch (6). Many studies have addressed the specificity of RNA interference in non-viral systems. No clear consensus has been reached, suggesting that it may depend to a large extent on the system being used. Soon after application of siRNAs in *Drosophila* it became clear that a centrally located nucleotide in siRNA, if not complementary to the target, can abolish silencing (13) – presumably because the cleavage of the target strand happens between the central (11^{th} and 12^{th}) positions, counting from the 5'-most nucleotide targeted by the 21-nucleotide long antisense siRNA strand. Similar results have been reported for the centrally located mismatches in mammals, with more peripherally placed mismatches not influencing RNAi as much (19, 34).

Others, however, have found less of a requirement for siRNA-target complementarity in the center of the duplex, with a double mismatch at positions 9 and 10 from the 5' end of the antisense siRNA strand still being partially active, as were duplexes mismatched at the 3' half of the antisense siRNA strand (3, 10). However, mutations in the 5' half of the antisense strand abolished RNAi. Still others described a shRNA which was very sensitive to any mismatch across its span, except at the three 5'-most base pairs; the most important positions were 4-6, 11-13, and 18-19 of the antisense strand (38). The argument continued with gene expression profiling. Some studies found relatively little, if any, nonspecific effects of siRNAs (8, 43). In stark contrast, another group reported that extensive non-specific targeting by siRNA can take place (24); the unintended targets fell roughly into two classes: one was largely complementary to the siRNA, while the other was only complementary to the 5' half of the antisense siRNA strand. Recent target prediction and validation analyses for microRNAs (miRNAs) (14, 31, 45) have indicated that it is complementarity to positions 2-8 of the miRNA (i.e., antisense) strand that leads to the recognition of the mRNA; other positions appeared rather insignificant.

We reasoned that poliovirus escaping siRNA presents an excellent model system in which selection for the most fit viruses may reveal important features of siRNA-target recognition. Furthermore, as a model RNA virus, poliovirus provides a perfect testing ground for the strategies to limit emergence of escape mutants. While poliovirus was the first virus documented to escape from siRNA (17), any virus is expected to behave in a similar fashion, as has recently been observed for HIV (6).

One strategy for limiting viral escape is to provide the cell with a complex mixture of siRNAs directed against the viral genome. Indeed, this would mimic the natural antiviral RNA silencing response seen in plants (Chapter 1), which is initiated by long viral dsRNA that is subsequently processed into siRNAs. Transgenic plants expressing long dsRNA derived from viral genomes are resistant to the corresponding pathogens, and so far, no viruses have been noted to circumvent this protection (20). Consequently, we expected that long dsRNA, processed by mammalian Dicer, or *in vitro* (by bacterial RNase III), would be effective at minimizing viral escape. Indeed, poliovirus did not manage to evade the siRNA mixture in the course of our experiments.

Materials and Methods

Cells and viruses. HeLa S3 cells were cultured as described (17). P19 mouse embryonic carcinoma was obtained from the UCSF cell culture facility and grown in MEM alpha supplemented with nucleosides, 10% fetal bovine serum (FBS), 2 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin (UCSF cell culture facility). We used pRib(+)XpA plasmid (21) to generate wildtype Mahoney strain of poliovirus. All mutations were cloned back into pRib(+)XpA backbone. For viral production, 10 μg of *in vitro*-transcribed viral RNA was electroporated into HeLa (21), and cells were left overnight until complete lysis. Plasmid encoding the Leon polioviral strain was a kind gift of D. J. Evans; the virus was produced similarly to Mahoney. Virus was titered according to standard procedures (12). For competition assays, viruses were mixed in equal proportions unless indicated otherwise, and propagated for two passages (a passage concluded with full lysis) on cells transfected with siRNAs (transfections were done as in Chapter 2); MOI's are indicated in the text. Plaque reduction assays were done as described (Chapter 2); cells were allowed to grow for one day between the siRNA removal and

infection. P19 transfections were done by combining 0.5 μg of pPVR plasmid with 0.3 μl of 1 μg/μl dsRNA or 0.3 μl of 40 μM siRNA, and transfecting the mixture with 1 μl of Lipofectamine2000 per ~$1x10^5$ cells in one well of a 24-well plate overnight in the presence of 10% serum.

Molecular biology. RNA oligos were bought from Dharmacon and were treated as before (17); the sequence of siRh strands is as follows: 5'-AAUACCAGAACACCAACUGGC-3' and 5'-CAGUUGGUGUUCUGGUAUUAC-3'. siC region of the viral genome was amplified by isolating cytoplasmic RNA from HeLa cells at 6 hpi with Rneasy kit (Qiagen), reverse transcription using random hexamers and SuperScriptII enzyme (Invitrogen), and PCR with PfuTurbo (Strategene) and primers: 5'-GCTAGACACCGTGTCTTGGA-3' and 5'-GGACTGTGTTGTCAATCATGCT-3'. PCR products were sequenced using the primer 5'-AGATGATAGTTTCACCGAAGG-3'. For cloning individual mutants, each PCR fragment was digested with NruI and NheI, and swapped for the wildtype fragment in pRib(+)XpA. Analogous procedure for isolation of the siP region used the primers 5'-GGTGAAATCCAGTGGATGAGA-3' and 5'-

GCGAACGTGATCCTGAGTGTT-3' for amplification, 5'-AGGAAGCAATTACATCATCACC-3', for sequencing, and AvrII and XbaI enzyme sites for cloning the fragment into a shuttle vector. For producing point mutants in the siP region, the following DNA stretch was used in a QuikChange protocol (Stratagene): 5'-CAGCAGTGGGGTGCGATCCAGATTTGTTTTGGAGCAAAATTC-3', with corresponding mutations introduced in the primers. PCRs were run from a plasmid containing BglII-EcoRI fragment of pRib(+)XpA, and the mutant BglII-EcoRI fragments were moved back into pRib(+)XpA. Cloning of the let(+) virus was accomplished by using 5'-TGAGGTAGTAGGTTGTATAGTTACAATTTCAACAGTTATTTCAATCAGAC-3' and 5'-CCTCAGTGCATCAGGCAACT-3' primers in one PCR, and 5'-AACTATACAACCTACTACCTCACTTAGAGTAAACACACTCAATGG-3' and 5'-AGAAGCCCAGTACCACCTCG-3' primers in a parallel PCR, both of which were consequently purified, mixed and extended again for 15 cycles with Pfu polymerase. The product was digested with BlpI and AatII and moved into the polioviral backbone, introducing the sequence identical to let-7a (UGAGGUAGUAGGUUGUAUAGUU) or complementary to it (30).

1-kb long regions of Mahoney and firefly luciferase were amplified with primer pairs 5'-ATGATGGAATTGGCAGAAATC-3' and 5'-TAAGGCATGCCCATTGTTAGT-3', and 5'-AGAACTGCCTGCGTGAGATT-3' and 5'-TTTCTTGCGTCGAGTTTTCC-3'; one of the primers in each reaction contained a T7 transcription start site. Each strand was then transcribed with T7 RNA polymerase (NEB), the RNA annealed and treated for 2 hrs at 37°C with RNase III (a gift of Dun Yang and J. M. Bishop), followed by a purification on Qiagen's PN columns and ethanol precipitation of the flowthrough fraction.

Results

Analysis of escape mutants

Treatment of HeLa cells with siC, a siRNA against a stretch of RNA in the capsid region of the polioviral genome, results in the appearance of viruses that contain silent mutations in the sequence complementary to siC (17) within 30 hours post-infection (hpi). To find out whether these mutations were sufficient for escape, we cloned the naturally arising mutations U11C, U8C, and C20U (fig. 1A) into the polioviral backbone, thus ensuring that no other mutations were present. Viruses produced from these plasmids (called cU9C, cU6C, and cC18U, 'c' denoting the location of the mutations in the siC-targeted region) grew normally and did not appear to be impaired in any way. We then used these mutants and the wildtype parental poliovirus at a MOI of 10 to infect siC-transfected HeLa monolayers, and measured titers of viral progeny after 8 hpi. The titers achieved by the mutants (Fig. 1B) correlated with the location of the mutation relative to the center of the siRNA: cC20U, which is the most 'peripheral' mutant, was barely distinguishable from the wildtype virus, and replicated poorly in the presence of siC. In contrast, cU9C and cU6C grew well under siC, demonstrating that one mutation

inside the siRNA complementarity region is sufficient to largely block the recognition of viral RNA by the RISC. We note that the mutants studied here do not alter the RNA interference pathway or viral interaction with it, as all the mutants remained fully susceptible to a siRNA against a different region of the viral RNA (siP) (Fig. 1B).

A plaque reduction assay (Fig. 1C) confirmed and extended these results. While both plaque size and plaquing efficiency of cC18U were markedly decreased on siC-transfected monolayers, the plaque size of cU9C and cU6C were not affected significantly. However, their plaquing efficiency was decreased by about three-fold. The block to viral RNA recognition by RISC is therefore not complete. Considering this result, we set up a competition assay between cU9C and cU6C. In a span of two passages (Fig. 2), cU6C mutant dropped from 50% of the population to less than 10%. When the mutant mixture contained 90% cU6C and 10% cU9C, their proportions after two passages were roughly reversed. These results are consistent with those studies showing the importance of the siRNA-target complementarity at the central duplex positions for efficient RISC activity (13, 19, 33).

Tracing escape from the other antiviral siRNA, siP (to the polymerase region), revealed a more complicated picture. Population

sequencing of the virus arising from the very first passage (at 50 hpi) demonstrated that two mutations were rather prevalent in three parallel cultures (Fig. 3A). Further passaging, in the presence or absence of siP, did not result in one obvious mutant taking over the population, but rather a varying mixture, where the centermost (closest to the center of the siRNA-target RNA duplex) A->G mutation was detectable only in some populations, but not others (data not shown). Therefore, to understand what the endpoint of siP escape would be, we performed ten blind passages of poliovirus in the presence of siP or a control siRNA directed against rhinovirus (siRh). One viral stock was used to infect three HeLa cultures, and these independent viral populations were subsequently grown in parallel. The first two rounds of infection were done at a MOI of 1. Then each virus population was split in two, and eight subsequent rounds were done at MOI's estimated to be either around 0.1 or 10, resulting in six different viral populations. We then cloned and sequenced individual viral sequences from these passage 10 populations. The results are shown in Fig. 3B.

Examination of these mutants led to several interesting conclusions. The first stems from the fact that, like the siC escape mutants, the mutations in siP tend to be found at the third nucleotides of

the codons, as expected from a RNA sequence constrained by the protein coding requirements. However, this is only true of the 5' half of the target sequence. In the 3' half of the target, there appears to be a hotspot of mutation selection, spanning nucleotides 16-19. The second conclusion is connected to the fact that a very significant number of mutations in the same hotspot are transversions. It is accepted that poliovirus replication leads predominantly to transition mutations (11, 29), likely due to the intrinsic misincorporation frequencies of the polio polymerase (29). It was thus surprising to find many transversions among the siP escape mutations. It is possible that transversions, at least those leading to a purine-purine mismatch, lead to a more distorted siRNA-target duplex and therefore more efficient escape (9); however, the possibility of a thermodynamic escape is no less likely. Importantly, one consequence of the high transversion rate is a high frequency of missense mutations. We conclude that siP selection leads to viral variants whose protein sequences have been diversified in this short defined stretch. Indeed, most of the amino acid changes present in our mutant collection have not been described by phylogenetic comparison of enteroviral strains (A. Palmenberg, personal communication).

The most striking result of the ten-passage selection scheme lies in the fact that there does not seem to be one predominant mutation, central or otherwise. Instead, two mutations appear to be required for efficient escape (Fig. 3B). In contrast, similar blind passaging of wildtype virus under the siC pressure does not yield mutations beyond the "centermost" cU11C (not shown). These results suggest that the centermost mutations in the siP region (i.e., closest to the center) may simply fail to provide sufficient protection from siP, and therefore require additional siRNA-target mismatches for efficient escape. Alternatively, secondary or tertiary structure of the polio genome may favor an adenosine at the central position of the siP region, such that a centermost mutation, while providing an optimal siP escape potential, lowers the viral viability, thus failing to achieve prevalence in the viral population.

To differentiate between these possibilities, we wished to examine viability and escape efficiency of defined single-nucleotide mutants. We constructed nine mutant polioviral plasmids, including one of the passage 10 double mutants among them (Fig. 3C). These mutants were chosen to represent all 3 (silent) permutations of the centermost position, as well as several naturally arising mutations, one of them missense, pU19A. (Note that 'p' indicates the mutant's location in the siP-targeted region). Viruses

containing these point mutations were produced from the plasmids. They reached wildtype or near-wildtype titers in cells transfected with control siRNA (siRh, solid bars in Fig. 3C). In the presence of siP (open bars), however, it became evident that pA12G, pU16C, pG18A and pU19A escape much more efficiently than pC6U, pU9C, pA12U or pA12C. Therefore, it appears that the RNA structure in the siP region is not strongly constrained, and that the escape potential of the central mutant is simply not sufficiently high compared to the other defined single mutants. We also note that the double mutant pU6C/pG18C reached essentially wildtype levels under siP, demonstrating that two mutations in the target region can suffice for a complete escape from an siRNA.

To understand the escape in molecular terms, it was important to establish which strand of the poliovirus is targeted by the RISC. Both strands of siP and siC may enter the RISC, making it difficult to pinpoint which strand(s) is active against the virus. However, endogenous microRNAs, although initially expressed as largely double-stranded precursors, are later processed in an asymmetrical fashion. Only one strand of the precursor is incorporated into the RISC complex (22). We therefore designed polioviruses which incorporated 22-nt sequences complementary to the let-7 microRNA in either their positive (let(-)

poliovirus) or the negative (let(+) poliovirus) strand. These sequences were inserted in the hypervariable region between the IRES and the first AUG of the open reading frame, which was previously shown to tolerate an insertion of 70-nt long foreign sequence (29).

let-7 controls translation of endogenous mRNAs by forming an imperfect duplex with the 3' noncoding region of target mRNAs. However, let-7 can act as a siRNA, directing cleavage of a perfectly complementary target RNA (23). Given that HeLa express let-7 (22), we tested whether the insertion of the let-7 complementarity sequence within the 5' noncoding region of the poliovirus genome would inhibit viral replication. Following the electroporation of the recombinant viral RNAs into HeLa, cell lysis took 24 hours for let(+) and 40 hours for let(-) virus. While let(+) virus sequence was unchanged, let(-) virus populations, cloned after an additional round of replication in HeLa, were found to have an A->G transition, resulting in a G:U base pair between the viral RNA and let-7 at position 13 of the let-7 miRNA, which is equivalent to position 9 on the viral sense strand. Other mutations were also evident; strikingly, all of them appeared to be A->G. While the mutation at position 9 is not as central as one at position 11 or 12 (the viral RNA would be predicted to be cut between positions 11 and 12), its abundance

may be a result of the predominance, or even exclusivity, of the A->G mutations in this region.

The susceptibility of let-7(-), but not let-7(+) virus to the miRNA led us to conclude that only the positive virus strand is successfully targeted by RISC. The apparently dominant escape mutation at position 9 of the let-7 complementarity region again emphasized the earlier observation that the escape pattern varies from one si (mi) RNA to the next. This suggested that the mode of recognition of the targets by siRNAs can vary from one siRNA to the next. However, as in the case of the peculiar mutation spectrum of the let(-) virus, a possibility remained that the difference in escape in various cases is due to the relatively low mutation frequency at some positions. Specifically, we have not seen mutants at positions 16-19 of the siC complementarity region, which is a hotspot of siP escape.

Thus, to compare the siP escape and siC escape, we constructed cC17U, a silent transition predicted to form a G:U mismatch between the 5' half of the siC antisense strand and the mutant viral RNA. We then composed profiles of the siC and siP escape by conducting several competition assays (as in Fig. 2A) with viruses which were singly mutated in either region (Fig. 4). Each competition assay was scored as 1 for a

virus when the virus' proportion increased under the siRNA in question, 0.5 when the proportion was unchanged, or 0 when it decreased (Fig 4A and 4C). Calculating the sum of these scores allowed us to quantitate the relative escape potential (P_{esc}) of each mutant and graph P_{esc} along the axis representing the mutant's position along the siRNA (Fig 4B and 4D). This quantification demonstrates that there is a region in the 3' half of the siRNA target sequence of siP (corresponding to the 5' half of the antisense siP strand) which is very sensitive to mismatches, perhaps more so than the central region. In contrast, the 5' half of the siC target sequence appears to be more sensitive to mismatches than the 3' half.

Minimizing escape capacity

Regardless of the specifics of escape from a given siRNA, it evidently takes only two mutations for the virus to become insensitive to it (Fig. 3C). We therefore searched for the most conserved regions in the genome. There are two such sequences in the 5' non-coding region; their variation is very minimal, and even most rhinoviruses contain identical nucleotide stretches. Unfortunately, siRNAs designed against these regions (Fig. 5A) failed to produce antiviral effects (Fig. 5B). It is possible that the target region is buried inside the IRES structure and thus

rendered inaccessible to the RISC. Alternatively, it has been recently found that unwinding of the siRNAs is very inefficient in the cell if their GC content, especially at the ends, is too high (27, 42); since these 2 siRNAs are rather GC-rich, they may not be processed efficiently. In this case they may be rescued by changing some nucleotides in one of the strands to weaken the interaction between the siRNA strands (42).

However, even targeting such highly conserved regions may not prevent viral escape. We reasoned that it would be much more difficult to escape from a large population of different siRNAs through a very limited number of mutations. Indeed, in plants, the natural RNA silencing response to viruses results in long portions of viral RNA becoming substrates for the silencing machinery (47). We began to address this by using P19 mouse embryonic carcinoma, which are capable of channeling long dsRNA efficiently into the RNA interference pathway (4). P19 cells were co-transfected with 1-kb long dsRNA corresponding to the polioviral P1 capsid region, and a plasmid encoding PVR, which enables polioviral infection of the mouse cells. Subsequent infection of P19 with Mahoney strain of poliovirus demonstrated that Mahoney titers are very strongly suppressed by the capsid dsRNA (Fig. 5C), while control dsRNA exerted no effect. Control infection with poliovirus type 3 Leon strain, which

shares only 54% nucleotide identity with Mahoney in the capsid region, proved that polioviral replication as such remains intact, and is not affected in any way by the capsid dsRNA. Unlike siP, which slowed the replication at first but lost its antiviral effect by 33 hpi (Fig. 5C), dsRNA to the capsid retained its antiviral capacity up to 60 hpi.

Most cell types tested to date, however, express PKR and RNase L, which are thought to stop translation when activated by dsRNA (40). This suggests that an efficient therapeutic strategy against viruses may have to rely on siRNAs instead of long dsRNA. To begin to address this challenge, we decided to process long dsRNA *in vitro* to produce a complex mixture of enzymatically prepared siRNAs (esiRNAs, (49)), and examine the effect of this mixture on poliovirus replication in HeLa cells. 1-kb long dsRNAs from the Mahoney strain genome and from the luciferase gene were treated *in vitro* with E. coli RNase III, and the resulting short RNAs transfected into HeLa. The capsid esiRNA-treated cells were protected from Mahoney (but not Leon), while luciferase esiRNA-treated cells remained susceptible to both viral strains (Fig. 5D). Surprisingly, the antiviral effect of Mahoney esiRNA was lost at about the same time as that of siP control (~30 hpi), since cells were lysed. To examine whether this is due to appearance of esiRNA-resistant mutants,

we performed two more rounds of infection on fresh HeLa cells transfected with the same preparations of esiRNAs. While the cell lysis ensued at about 30 hpi at each passage, we observed no diminution of the antiviral effect with each subsequent passage (as measured by progeny titers at 10 hpi, Fig. 5D, or following lysis at 48 hpi (not shown)). Thus, while potency of esiRNA antiviral effect seems to wane at 30 hpi, this is not due to emergence of esiRNA escape mutants. This was directly confirmed by sequencing the 1-kb region in question from viruses following their third passage under capsid esiRNA (esiM) or luciferase esiRNA (esiF). We observed 1 mutation appearing in one of 6 clones of esiM-passaged viruses, and no mutations in 3 clones of esiF-passaged viruses (data not shown). An entirely unexpected finding was made in one of the esiM clones: it appeared that no less than 9 A->G mutations were found in it, all in the 400 base pair region 5' of the esiM-targeted sequence (and none in the esiM target region itself). Of these mutations, 3 were predicted to be silent, and 6, missense. Their locations were as follows: nucleotides 1549, 1568, 1576, 1584, 1615, 1634, 1743, 1824, and 1916. No other clones harbored such mutations.

Discussion

Viruses often protect their genetic material by association with membranes or viral proteins (2, 16); it is less clear from what they protect it. Our results in this chapter suggest that the RISC complex, which may play an antiviral role in mammals as it does in plants and invertebrates (Chapter 1), can cleave the positive, but not the negative strand of a replicating poliovirus genome. Poliovirus replicates in membrane-associated complexes inside the cell, which are presently not well defined (1). It is possible that the reason for the negative strand's resistance to RISC is its constant association with the newly synthesized positive strand. However, the fact that at least one strand of the RNA is inaccessible to RISC inside the cell is not inconsistent with the idea that viruses can evade a dsRNA response. It will be interesting to find out whether the positive strand of the virus is susceptible to RISC once the replication process commences. The positive strand may only be vulnerable during the first few minutes in the cell, before the replication complexes have been formed.

Our studies of RNAi-virus interactions have indicated early on that poliovirus escapes an siRNA in a very predictable fashion (17). A more extensive investigation of this escape, reported here, reveals that the two

siRNAs that we used elicit different escape patterns. siC results mostly in a viral population with one silent transition cU11C, at nucleotide 11 of the sequence complementary to the antisense strand of siC. Protection afforded by this mutation is evidently sufficient for successful replication through ten consecutive passages without having to acquire further mutations. On the other hand, the centermost mutation in the siP region, pA12G, does not seem to provide as efficient a protection. Instead, there appear to be two major escape hotspots, one being central, and the other, approximately in the middle of the 3' half of the target (Fig. 4D). Additionally, it appears that two mutations in the siP homology region are required for efficient escape (Fig. 3B).

We are thus confronted with three differences between siP and siC escape, which may or may not be related. First, two mutations are required for siP, but one suffices for siC; second, the centermost mutant in siP is not nearly as efficient as in siC; and finally, the 3' hotspot of siP escape is apparently not present at the equivalent location in the siC homology region. It was possible to suggest at first that the differences in the escape pattern are due to certain mutations either never arising because of polymerase misincorporation frequency or being deleterious for the virus and thus never selected for. However, building defined point

mutations into the backbone, producing viruses and competing them against the wildtype in the presence or absence of the siRNA pressure failed to identify any evidence for that (Figs. 3C and 4). Therefore, the simplest explanation for the observed differences is that the escape pattern differs from one siRNA to the next. This can be due to local variation in the siRNA melting temperature (specifically, note the U-rich region at the 3' end of the viral siP-homology region). Alternatively, this can be due to a difference in the RISC complex assembly, perhaps as a result of different location of the siRNA target on the mRNA (a possibility, given that it has been claimed that 3' regions of a mRNA can be targeted by a miRNA-charged RISC leading to a translation block (41)).

The other explanation for the differences assumes that the RISC is indeed the same, and that siRNAs are treated by the cell in a relatively uniform manner. If this hypothesis is true, we have to postulate, first of all, that position 12 of the target, in the siP region (equivalent to the 10^{th} position on the antisense strand of the siRNA) is a less efficient escape location than position 11 of the target (equivalent to the 11^{th} position on the antisense siRNA strand), in the siC region. This will account for the siC/siP escape difference vis-a-vis the centermost escape efficiency. Secondly, the same line of reasoning suggests that a second mutation may

be required for the siP escape, explaining the propensity for a second mutation within the siP homology region. Lastly, the hypothesis would have to take into account the presence of the escape hotspot in the 3' end of the siP homology region. This can be done by assuming that the profiles in Fig. 4 are entirely superimposable, and the differences are due entirely to the fact that not all positions have been queried in both targets (in fact, there is no correspondence between them, owing to the constraints placed by the amino acid conservation). This explanation does not seem very likely, but the other way to take the 3' differences into account is by carefully examining the mismatches that result from the mutations tested here. Both cC17U and cC20U lead to a G:U mismatch between the viral target and the siRNA. On the other hand, pU16C and pG18A lead to a C:A mismatch, while pU19A leads to an A:A mismatch, both of which are much less thermodynamically stable than a G:U base pair (28). Therefore, if the 3' hotspot of escape is determined thermodynamically, it is possible that we have missed it in the siC region. This hypothesis predicts that cA18U silent mutation, which was not produced in this study, would lead to a thermodynamically unstable base pair (U:U) and a high escape potential (P_{esc}).

In fact, we can say with certainty that the precise nature of the mismatch is important for specificity. We find that pA12G, which leads to a G:U mismatch, is less permissive for RNAi than pA12C or pA12U, which lead to C:U and U:U mismatches, respectively. Ironically, in this case the results are the opposite of those predicted above, since thermodynamically, a G:U mismatch is much more stable than most other mismatches in dsRNA (28). Accordingly, if the RISC detected local thermodynamic instability of the helix, a G:U mismatch would have led to a relatively inefficient escape. However, if the RISC detects distortion of the regular A-form dsRNA helix, it may preferentially exclude G:U and A:C mismatches over U:U and C:U mismatches, since both G:U and A:C may lead to more significant deviations from the perfect A-form helix (37, 46, 48). Therefore, we predict that, near the cleavage site, the RISC ensures fidelity of target mRNA recognition through steric, but not thermodynamic, mechanisms. It has been argued previously that an A-form helix is important as a recognition element (9), since siRNA activity was blocked by an incorporation of a two-nucleotide bulge; however, such a bulge is both a thermodynamic (32) and a very bulky steric obstacle to recognition. In contrast, we observe that even single and relatively subtle mismatches such as G:U lead to a steric impediment in the RISC activity.

Furthermore, it appears that most double mutants escape siP efficiently, regardless of what the P_{esc} of individual mutations is, suggesting that, altogether, the RISC is extremely sensitive to mismatches along most of the length of the RNA duplex. How such sensitivity and apparently cooperativity in mismatch detection is achieved is a puzzle left for the future.

The rules of the siRNA-target recognition are being addressed in different systems. While the importance of the central position(s) in specificity of the process was noted early on (13), other more recent results tend to suggest that the identity of the 5' half, but not the 3' half, of the antisense strand to its target is also very significant (3, 10, 24). Our data supports the crucial role of the central nucleotide, since cU11C was the dominant escape variant in the siC region and pA12G possessed a strong escape capacity against siP (Figs. 1A and 3C). In addition, depending on the siRNA or on its interaction with the target, we see different patterns of escape. Perhaps, then, some of the discrepancies in the literature are a result of using different siRNAs, and not a result of using different systems. Even if the recognition mechanism is the same, the nature of the mismatch may influence the outcome, as discussed above.

Our system provides a novel approach to study RNAi specificity by virtue of its ability to rapidly generate a spectrum of mutant targets. Since most studies on the influence of mismatches on siRNA-target recognition have utilized the same target paired with different siRNAs (3, 38), the differences in the initial processing of various siRNAs (unwinding, RISC incorporation) were inseparable from the effects of siRNA-target recognition. Our system combines the use of one given siRNA with the benefit of studying naturally arising mutations, and can thus help decipher the rules of RNAi specificity. The caveat of this system is the fact that the final mutant spectrum is molded not only by the selection pressure exerted by siRNA at these positions, but also by mutation frequencies of the nucleotides in question and the general viability of the virus containing given mutations. However, the latter two of these variables can be readily controlled, through use of the competition assays (Figs 2 and 4C). Indeed, the competition assays are an important advantage of this system, as they provide a sensitive *in vivo* test for comparison of relative tolerance of RNAi to siRNA-target mismatches. Another caveat of the system we used is the severely restricted number of nucleotides which can mutate without an adverse effect on the protein sequence, as evidenced in the distribution of mutations that we observed

(Figs. 1A, 3B). This caveat seems surmountable, given the effectiveness of the miRNA let-7 against the let-7-complementary insert in the 5'-untranslated region (UTR). It follows that nearly any test sequence can be inserted into the 5' UTR, and the escape mutants selected.

Let(+) virus was further examined with regard to the escape mutations following an electroporation of the *in vitro* transcribed RNA into HeLa. To our surprise, all of the let-7 escape mutations observed in four separate viral population sequences were A->G transitions (on the positive poliovirus strand). Even more surprising, they were not limited to the region of let-7 homology, but extended for at least 9 nucleotides in the 5' direction. In this, they were reminiscent of the one clone from the esiM-treated cells, which harbored at least nine separate A->G mutations along a 400-nt stretch, all of them 5' of the targeted region. Clearly, more research is required before any conclusions can be drawn. However, a speculative hypothesis to guide this research is that we are observing an outcome of the action of an adenosine deaminase (such as ADAR1), which may interact with the RISC. If so, this will be among the first pieces of evidence to support an antiviral function of the RISC.

Our results from the investigation reported here have implications for design of siRNAs and interpretation of experimental results. First of

all, it has become clear that, while a single mismatch can impair siRNA-target recognition, at least two mismatches should be ideally allowed (Fig. 3B). Also, mismatches at the extreme ends of the siRNA do not seem sufficient for abrogation of siRNA activity (Figs 1B and 3B). Furthermore, we demonstrate here that siRNA can be used as an efficient selection agent to obtain point mutations in a defined stretch of replicating RNAs. We have observed frequent transversions, numerous coding mutations, and thus probed conservation of not only RNA, but also of protein sequence in a given region of the virus. Unexpectedly, some of the amino acid substitutions, which we discovered in a very conserved region of the viral polymerase (Fig. 3B), have not been seen in a phylogenetic analysis (A. Palmenberg, personal communication).

The above data practically exclude the possibility of using defined siRNA in the clinic against viral pathogens. Enormous pre-existing virus variation, coupled with very high mutation rates, guarantees that any given siRNA can be obviated, and the fact that so few mutations are sufficient for escape makes it unlikely that even a highly conserved region will prove to be a good target. What will it take to solve this problem? One promising approach is targeting host factors required for viral replication, such as CD4 and CCR5 in the case of HIV (35, 36, 39). However, it is not

clear that such a strategy will succeed (for HIV, CD4 is obviously not a viable therapeutic target, while CCR5 may only be useful prophylactically, but not after infection).

Furthermore, most antiviral drugs are costly in development and yet, similar to defined anti-virus genome siRNAs, they act against one specific viral target. We propose the use of virus-specific long dsRNAs, which may be pre-processed into siRNAs *in vitro*. Theoretically, employing 1 kb of the viral sequence could result in 979 (1000-21) different siRNAs. This strategy is unlikely to result in significant off-target damage because the concentration of any individual siRNA will be very low, and only a target that contains long stretches of homology should be recognized efficiently by RNA interference machinery. We show here that this strategy is efficacious against poliovirus *in vitro*: we did not observe viral escape, either phenotypically or by sequencing viral genomes. While it remains possible that, given sufficient passaging, the virus will evolve around dsRNAs, experience in plants suggests that it will not (20). In case escape proves to be a problem, it is even possible to envision a strategy where viral sequences are periodically amplified from a patient, transcribed, and processed into siRNAs that can later be introduced back into the patient.

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Figure Legends

Fig. 1. Escape from siC. A) Names and sequences of the escape mutants in the siC region. B) Titers of wt and the mutants following infection of siC- (black bars), siP- (dark gray bars), and siL (light gray bars)-transfected HeLa. Infections were done at a MOI of 10, and progeny virus collected at 8 hpi. C) siC and siL-transfected plates were infected with approximately 50-100 pfu of wt and each mutant. Plates were covered with agar and plaques allowed to develop. Ratio of plaques on the siC monolayer to plaques on the siL monolayer determines the plaquing efficiency.

Fig. 2. Centermost mutant in siC wins the competition assay. cU11C and cU7C were mixed in either 50:50 (top half) or 10:90 (bottom half) ratios, and passaged twice on siRh- or siC-transfected cells. For determination of the mutants' proportions in the population, HeLa were infected with the viral populations, and their RNA extracted at 6 hpi. Sequencing traces of the RT-PCR products are shown.

Fig. 3. Escape from siP. A) Population sequencing of the viral siP region after one passage in siP-transfected cells. Arrows: newly arising mutations. B) Sequences of individual viral genomes in the siP region, appearing after 10 passages. Only nucleotides different from the wt sequence (shown on top) are spelled out. If a sequence was isolated more than once, then the number of isolations is given in the column second from left. Six viral populations (1-6, numbered on the left) were sequenced. Populations 1-3 are derived from passaging virus at a MOI of 10; populations 4-6, at a MOI of 0.1. Pairs of populations: 1 and 4, 2 and 5, and 3 and 6 were related, as each pair was derived from one viral stock following 2 initial passages under siP. C) Names, sequences, and titers of cloned point mutants at 8 hpi under siP (open bars, means +/- standard deviation based on 3 samples) and under control RNA siRh (closed bars).

Fig. 4. Comparison of the profiles of escape from siC and siP. (A and C) Competition Tables. Each row denotes a mutant and its scores in the competition assays. For competition assays, viruses, mixed in equal proportions, were allowed to replicate for two passages in siC- (A) or siP- (C) transfected cells. Resulting viral populations were sequenced and compared to those replicating in control cells. An increase in the mutant's

proportion is scored as 1, a decrease as 0.5, and no change as 0. The sum of these scores, shown on the right, is the "escape potential" (P_{esc}) of the mutant. (B and D) P_{esc} graphed according to the mutants' position along the siRNA homology region.

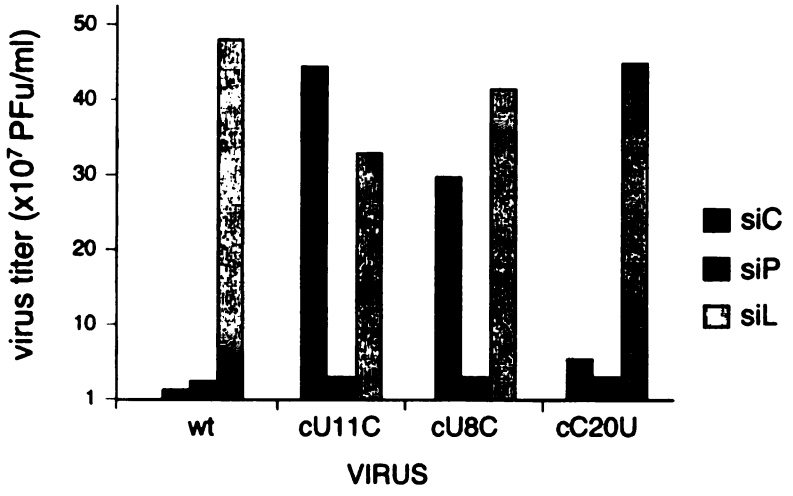
Fig. 5. A counter-escape strategy. A) Sequences of siRNAs in the conserved regions and B) titers of viruses in cells transfected with these siRNAs. C) Titers of Mahoney and Leon strains grown in P19 cells transfected with 1 kb-long dsRNA to the Mahoney genome (dsM), dsRNA to firefly luciferase (dsF), or si RNAs siP or siRh. D) Susceptibility of the Mahoney strain to siRNAs siP and siRh, or to esiRNAs prepared from dsM (esiM) and dsF (esiF). A scheme of the experiment is shown on top. Titers of virus from three consecutive passages are on the bottom.

Figure 1

A.

```
wt:      caGCGUGUAAUGACUUCAGCGug
cU11C:   caGCGUGUAACGACUUCAGCGug
cU8C:    caGCGUGCAAUGACUUCAGCGug
cC20U:   caGCGUGUAAUGACUUCAGUGug
```

B.



C.

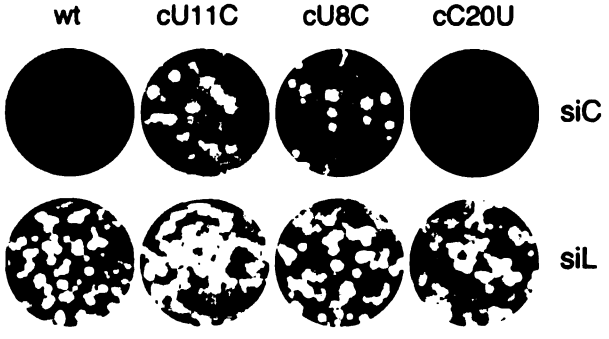


Figure 2

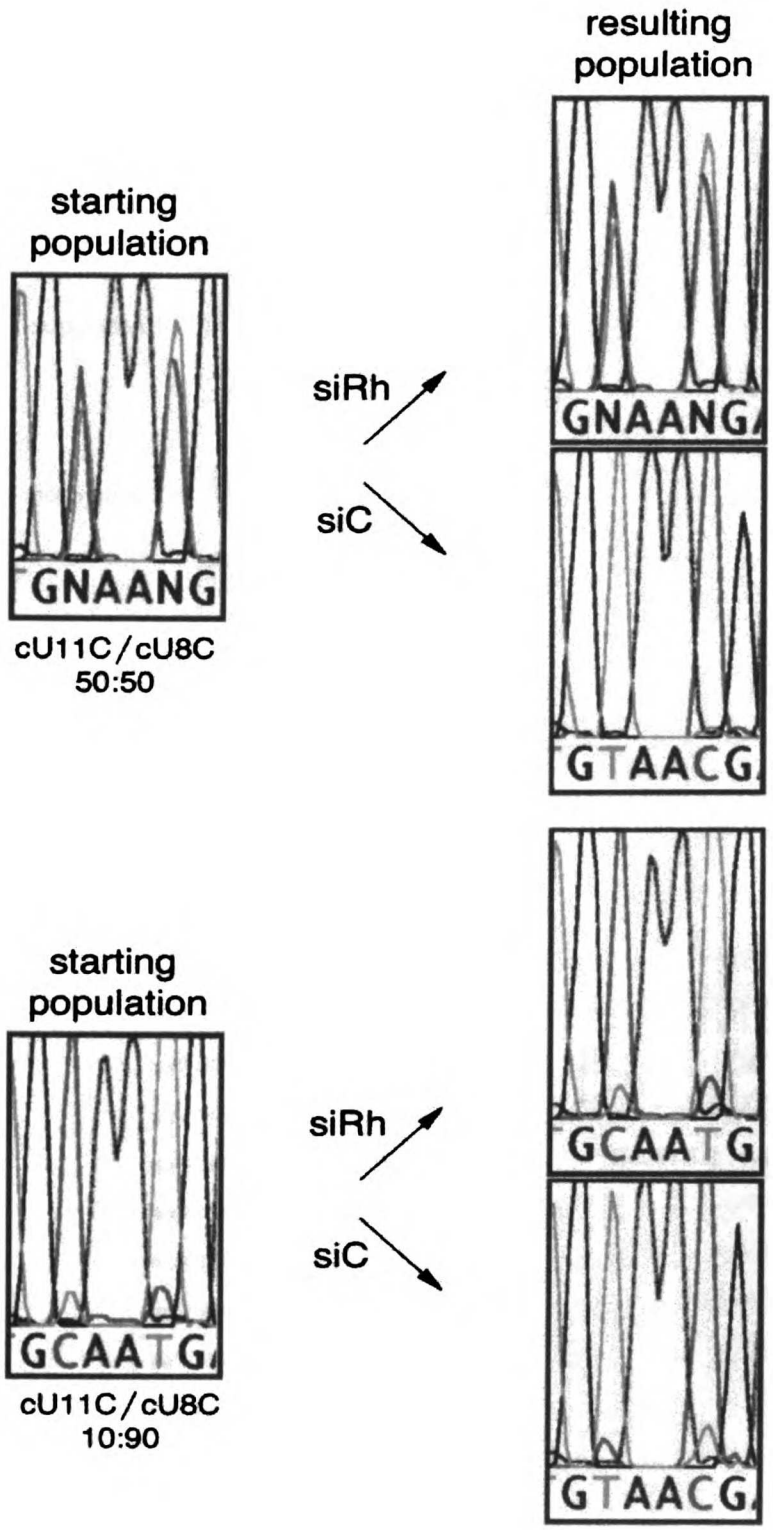
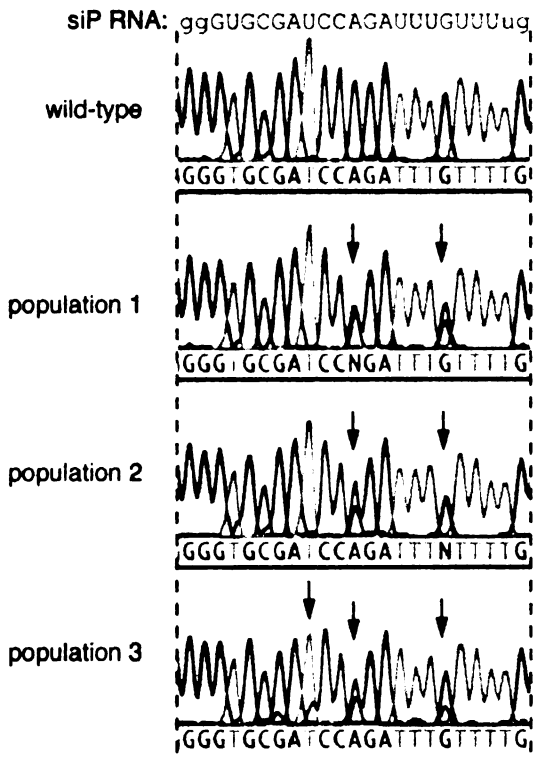
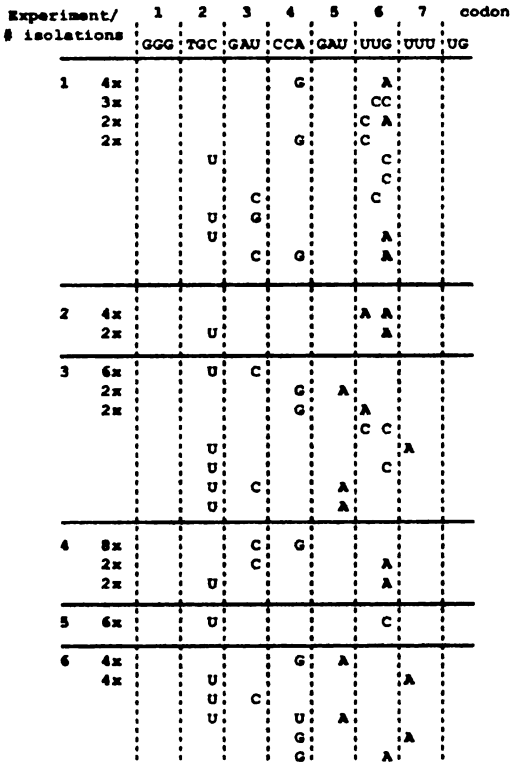

resulting population
starting population
GNAANG
cU11C/cU8C
50:50
siRh
siC
GNAANG
GTAACG
starting population
GCAATG
cU11C/cU8C
10:90
siRh
siC
GCAATG
GTAACG

Figure 3

A.


siP RNA: ggGUGCGAUCCAGAUUUGUUUUg
wild-type
population 1
population 2
population 3

B.


Experiment/ # isolations
codon
1 2 3 4 5 6 7
GGG TGC GAU CCA GAU UUG UUU UG

C.

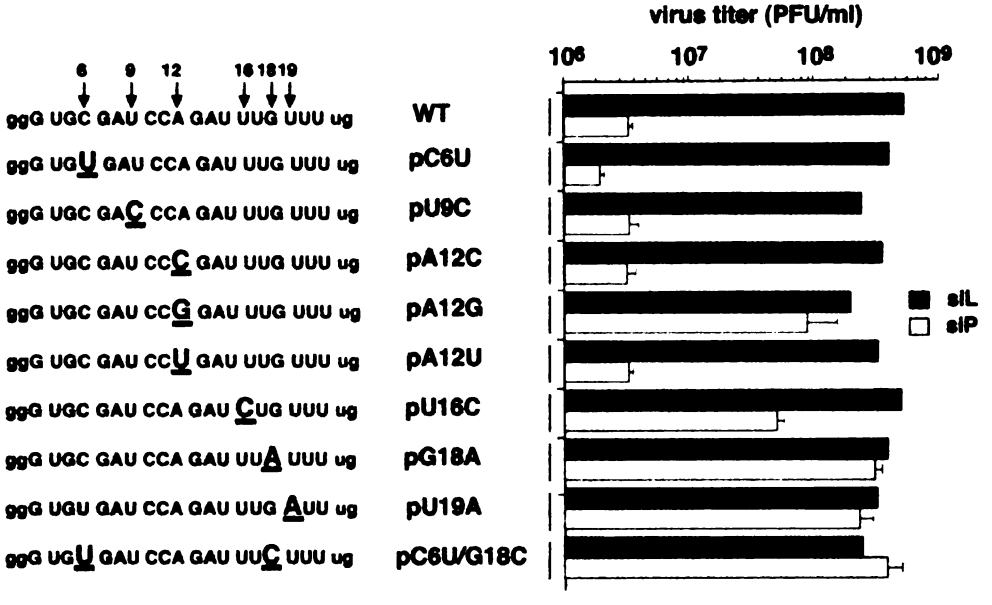
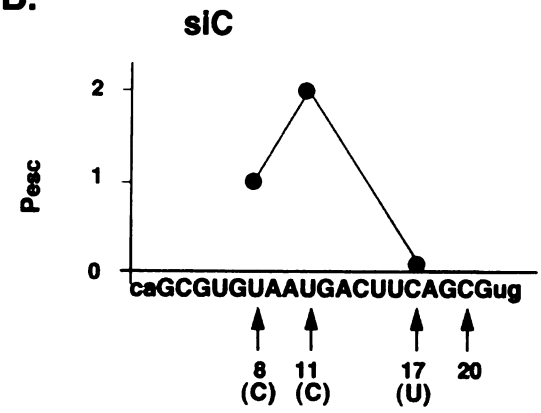

6 9 12 16 18 19
ggG UGC GAU CCA GAU UUG UUU ug
WT
pC6U
pU9C
pA12C
pA12G
pA12U
pU16C
pG18A
pU19A
pC6U/G18C
virus titer (PFU/ml)
10⁶ 10⁷ 10⁸ 10⁹
siL
siP

Figure 4

A.

VIRUS	COMPETING VIRUS cU8C	cU11C	cC17U	P_{esc}
cU8C		0	1	1
cU11C	1		1	2
cC17U	0	0		0

B.



C.

VIRUS	COMPETING VIRUS pU9C	pU12G	pU16C	pG18A	pU19A	P_{esc}
pU9C		0	0	0	0	0.0
pA12G	1		1	0	0.5	2.5
pU16C	1	0		0	0	1.0
pG18A	1	1	1		1	4.0
pU19A	1	0.5	1	0		2.5

D.

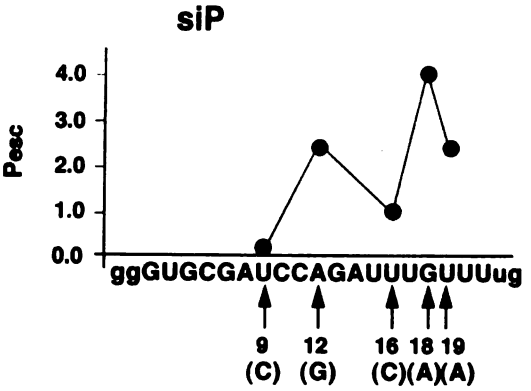


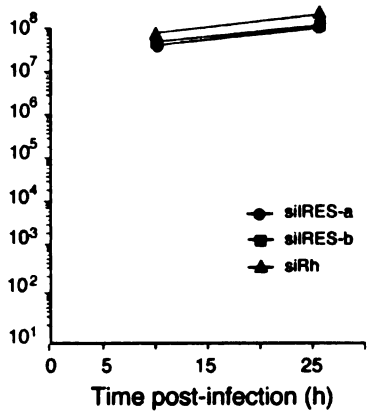
Figure 5

A.

siIRES-A:
CGACUACUUUGGGUGUCCGUG
UGGCUGAUGAAACCCACAGGC

siIRES-B:
CCGGCCCCUGAAUGCGGCUAA
GAGGCCGGGGACUUACGCCGA

B.



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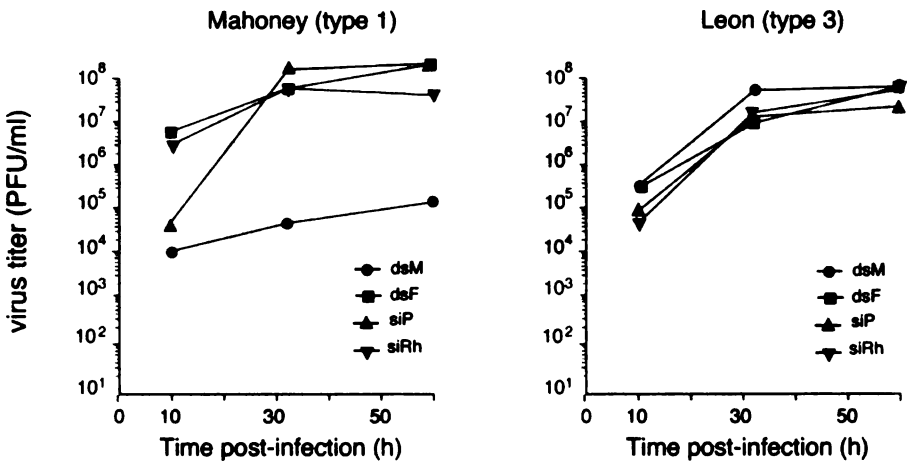
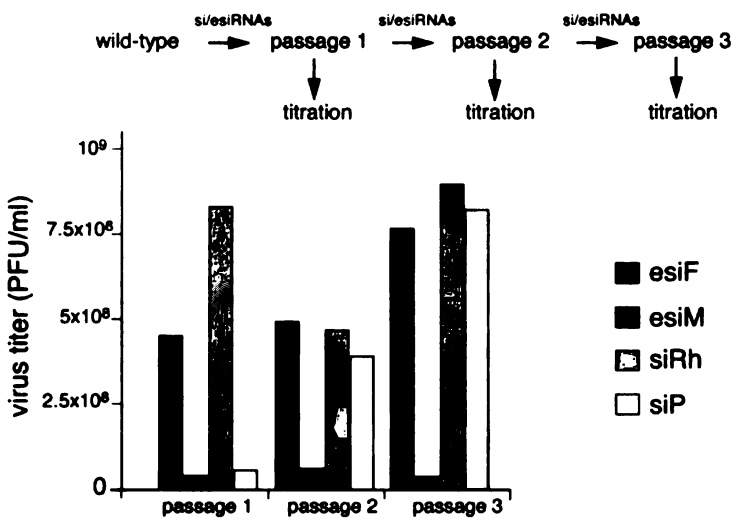


D.



CHAPTER 4. A search for anti-RISC functions of mammalian viruses

Abstract

An assay is presented here to detect virally encoded or mediated RISC (RNA-induced silencing complex)-suppressing activity. A test of poliomyelitis, vaccinia, yellow fever, and herpes simplex viruses has suggested that they do not block the RISC activity in HeLa cells. This assay can be readily applied to other viruses as well. Furthermore, the assay can be adapted to P19 cells in order to test the role of the Dicer step in RNA silencing, which is directly upstream of RISC.

Introduction

There are many ways to approach the question of RNA silencing's putative role as a mammalian antiviral system, detailed in the Introduction to this dissertation. One of the arguments which emphasized not just the existence, but the major importance of RNA silencing as an antiviral mechanism in plants, is based on the fact that most plant RNA viruses encode suppressors of silencing. It is presumed that viruses would not devote their limited genetic resources to functions that are not central to the success of their replication.

Likewise, if the same hypothesis is to hold true in mammalian systems, then we ought to be able to discover viral anti-silencing functions there. Are we likely to have missed such activities encoded by various viruses? The lessons of the virally encoded suppressors of plant PTGS suggest so. Over the last several years, many different anti-silencing activities have been discovered by plant virologists (9). These activities had previously been described only as "pathogenicity determinants" of their respective viruses. Taking a look at the mammalian viruses, we cannot help but notice how little is known about the functions of their non-structural gene products. For example, the polymerase 3D of poliovirus is the only non-structural protein for which both function and the mode of action are more or less understood. 3B, a short peptide, is known to be coupled to the 5' end of the genomic RNA, but the reason for this is unclear. 2A and 3C are proteases which process the polio polyprotein, but only some of their other activities

(notably cleaving the translation initiation factors eIF4G's (4, 5)) are characterized. Finally, viral proteins 2C, 2B and 3A are described as an ATPase and two membrane-binding proteins, respectively (1). The situation is not much different for many other viruses. Many, if not most, viral gene products are poorly characterized, and some of them may, as is the case in plants, contribute to a block of RNA silencing.

Our initial investigation centered on four viruses: poliovirus, herpes simplex, vaccinia, and yellow fever viruses. Despite the fact that poliovirus RNA is a substrate for the silencing machinery, it is important to note that in the experiments described in Chapters 2 and 3, the siRNAs were transfected into the cells well in advance of the viral infection. For technical reasons, it is difficult to examine a 3-hr transfection process and the 6-hr replication cycle of poliovirus in the same experiment. Therefore, if the virus does encode some anti-silencing activity, this activity would be pre-empted by the transfected siRNAs. As for the other viruses in this study, no data on their interaction with the RNA silencing mechanisms have been published. We were interested in yellow fever as an RNA virus, in vaccinia as a DNA virus that nevertheless produces dsRNA, and in herpes simplex as a DNA virus that, in fact, is not known to produce dsRNA to a significant extent.

Methods

Cells and Viruses. Herpes simplex virus 1, strain 17, was obtained from Bruce Banfield (Department of Microbiology, University of Colorado Health Sciences Center, Denver, CO), amplified in Vero cells in Dulbecco's Modified Eagle's Medium (DMEM) with 2% heat-inactivated FCS and 2 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin (Invitrogen); it was plaque-assayed in the same medium, but containing 4% heat-inactivated FCS and 0.1% human IgG. Vaccinia virus strain WR was obtained from Rafael Blasco (Departamento de Biotecnología – INIA, Madrid, Spain) and titered on BS-C-40 African green monkey kidney cells in MEM with Earle's BSS medium containing 2% FCS and standard glutamine and antibiotics supplements. We used 17D strain of yellow fever virus, grown and plaque-assayed on BHK cells in the same medium as used for BS-C-40 cells. The procedures for poliovirus were as described in chapters 2 and 3.

Electroporations. HeLa cells were grown overnight in 10-cm dishes (as in Chapter 2). Infections were at moi of 10, or moi of 5 for yellow fever; viruses were kept on cells for 1 hour in 2% (HSV, yellow fever) or 2.5% (vaccinia) heat-inactivated FBS, or 10% FBS (polio), and supplements as above. Cells from each dish (i.e., each infection timepoint) were then trypsinized, split into two cuvettes, and electroporated as described (6), using a

mixture of 5 μg firefly, 5 μg renilla mRNAs, and 5 μl of either siRNA (40 μM). They were kept on ice throughout the procedure; owing to the number of samples, the electroporation step took anywhere from 1 hr 45 min to 2.5 hrs. Timepoints of luciferase expression were measured from the time the samples were put into the incubator.

Results

The experimental system to address the question of active viral anti-silencing mechanisms was designed along the following guidelines. siRNA-mediated RNAi against a model target (luciferase) was set up in cells that had been pre-infected with a given virus for a certain length of time. A virus that interferes with either induction or maintenance of RNA silencing at the RISC step would be expected to prevent the decrease in the luciferase activity imparted by RNAi. It was important to ensure that the great majority of cells in the dish would be infected to improve the dynamic range of the assay; thus viral infection preceded siRNA and target transfection. This setup also allowed us to query different timepoints of viral infection.

This assay, which was first developed for poliovirus, had to be designed with the following limitation in mind. Many viruses, especially those actively studied in cell culture systems, possess host shutoff functions. This shutoff can target any of the different steps in the pathway of host gene expression, from transcription to RNA transport to translation. Poliovirus, for example, blocks cap-dependent host translation. Such shutoff functions could have proven problematic in terms of interpreting data. The assay, therefore, had to utilize an siRNA target which would not depend on cap-dependent translation, namely an IRES-driven luciferase mRNA. Additionally, introduction of the siRNA and the luciferase mRNA would have to be rapid and

synchronous across cells, which demanded electroporation rather than chemical transfection.

In accordance with these guidelines, our experimental setup involved infection of cultured HeLa at high moi (5-10), followed by electroporation at specific times post-infection with a mixture of siRNA against firefly luciferase and firefly luciferase mRNA containing a polio IRES and a poly(A) tail. An mRNA in which the firefly coding sequence was substituted with the Renilla luciferase gene, was included as an internal control. The negative control was a mixture which included an unrelated siRNA.

The outline of the assay is presented in Fig. 1. This basic outline did not change from one virus to the next. Our initial experiments were conducted with poliovirus. Cells were tested at 0, 1, and 3 hours post-infection; the results are shown in Fig. 2A. No appreciable increase in the level of luciferase activity was found. This suggests that poliovirus does not block RNA interference at the level of siRNA/RISC activity.

Herpes simplex virus type 1, which was expected to behave as a negative control, did not show any anti-siRNA function after 1,3,6, and 18 hours after infection at a moi of 10 (Fig. 2D). We verified that the cells were successfully infected by observing the progress of the infection; all cells died at approximately 24-30 hpi.

Cells infected with vaccinia virus at a moi of 10 were electroporated with the mRNA/siRNA mixture at 1,2,3, and 6 hpi (Fig. 2C). In what seemed a very reproducible assay, we did not observe any recovery of luciferase levels, only the expected decrease in

relative terms, suggesting that vaccinia does not block siRNA-mediated silencing. Vaccinia also led to cell death.

Similar results were obtained with yellow fever virus, used at a moi of 5, at 1,3,6, and 18 hpi (Fig. 2B). Again, the assay appeared to be very reproducible from one infection timepoint to the next. However, in this case we have not verified the extent of HeLa cell infection by the virus; that will require an indirect immunofluorescence experiment.

Discussion

Establishing RNAi as a natural antiviral defense in mammals will require addressing several critical questions that are schematically described in Fig. 3 of Chapter 1 of this thesis. The questions to be answered are:

-Are siRNAs generated during the course of a natural infection? A demonstration of such natural siRNA generation in tandem with its effectiveness at reducing viral replication would directly show the antiviral role of RNA silencing.

-Are silencing components upregulated during viral infection? Such an upregulation would put RNA silencing in a physiological antiviral context.

-Do mutations or deletions in mammalian homologues of RNAi components render cells or animals more susceptible to viral infection? Ultimately, these experiments will have to be carried out to test the true significance of RNA silencing in antiviral defense.

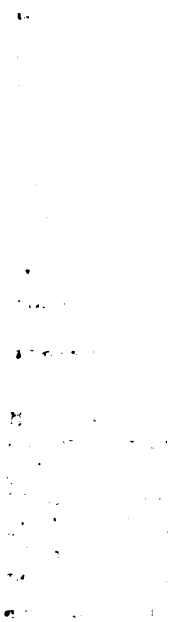
-Can dsRNA in one infected cell trigger a systemic sequence-specific response? Even though formally not a component of the proof (and thus omitted from Fig. 3 in Chapter 1), a systemic RNA silencing signal would likely exist if it is protective against rapidly spreading viruses.

-Finally, and as emphasized in this chapter, have viruses evolved mechanisms to suppress or escape a silencing response? Two types of mechanisms of RNA silencing evasion can be envisioned. First, it is possible that viruses protect their RNAs passively

by sequestering them in viral particles or replication complexes. Second, viruses may actively block RNA silencing by producing, or inducing, a protein or RNA which would interfere with the silencing machinery.

It was likely that poliovirus can passively evade the RISC and perhaps Dicer; indeed, our own results in Chapter 3 demonstrate that the negative strand of poliovirus, as opposed to the positive strand, is not susceptible to let-7 action as a siRNA. Moreover, we do not know yet at which stage in replication the positive strands are susceptible; it is even possible that the RISC has access to viral RNA only during the first round of translation, before the replication complexes have formed. In this scenario, all later rounds of translation happen in the context of the replication complexes, and the progeny viral RNA is encapsidated without coming into extensive contact with the cytosol. It has also been stated regarding the respiratory syncytial virus that its mRNAs are targets of siRNAs, but its genomic RNA is not (2); unfortunately, the experiments that would support this statement were not described in the paper.

Just as passive avoidance of RISC may be a property of RNA viruses, passive avoidance of Dicer seems to be a property of hepatitis delta virus (3). The delta genome is a largely self-complementary RNA; however, this RNA complementarity is regularly interrupted, and apparently does not allow for Dicer ribonucleolytic action. Thus, the currently available (little) data is consistent with the hypothesis of a passive viral evasion of RNA silencing.

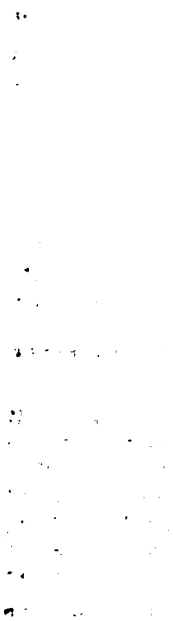


However, while sequestering the replication complexes of RNA viruses may serve the purpose of shielding them against the RNA silencing machinery, it is not possible to make that connection yet, since compartmentalization could, and most certainly does, have many other roles (such as concentrating the replication machinery, for example). Therefore, in this chapter, we consider a second, active type of evasion mechanism, which could rely on virus-encoded or induced gene products that directly inhibit specific steps of RNA silencing.

Diverse plant viruses have been shown to possess such active mechanisms (9). Even assuming that various passive evasion mechanisms are at play, it is easy to rationalize the presence of the active ones. Suppose that the viral replication complexes protect the replicative intermediates against Dicer. However, the Dicer step in the pathway may need very little dsRNA to initiate a silencing cascade, and the triggering dsRNA could either leak from the shielded compartment inside the infected cells or even from the remnants of cells lysed by the virus. Following the Dicer processing, the siRNAs can be utilized against viral mRNAs, and even spread to surrounding cells to block the infection much like the siRNAs used in our work do (see Chapter 1 on the discussion of dsRNA spread). Therefore, it is likely that mammalian viruses, like their plant counterparts, have evolved an active anti-silencing mechanism. Here, we attempted to search for it.

Our assay, as summarized in Fig. 1, was applied to poliovirus, herpes simplex, vaccinia, and yellow fever viruses. No block of siRNA-mediated luciferase activity downregulation was apparent in any experiments (Fig 2A-D). This result suggests that these viruses do not possess an active mechanism to suppress the RISC step of RNA silencing in HeLa cells. It is possible that some of these viruses may block this step in other cell types, or cells derived from other species; alternatively, they may block a different step in the RNA silencing pathway. (Indeed, given that at least some components of RISC may be shared between the siRNA-induced complex and miRNA-induced complex (8), it may be disadvantageous for at least some viruses to block all RISC activity). Another possibility is that the infected cell becomes compartmentalized in such a way that the viral translation or replication complexes block the RISC locally, while RISC activity on other messenger RNAs remains intact. However, it should be kept in mind that only four viruses have been tested here.

The assay is flexible enough, however, to be extended to a number of other viruses that infect HeLa cells. Assuming that none of the other viruses are found to block RISC, the next step in this project should involve testing viruses for inhibition of the Dicer step in the silencing pathway. Initial experiments have been done in a P19, a cell type that is capable of processing dsRNA through the Dicer pathway. Poliovirus IRES proved to be functioning poorly in P19 mouse embryonic carcinoma cells; however, new constructs, which incorporated the encephalomyocarditis virus (EMCV) IRES instead of



the polio IRES, gave a sufficiently strong signal. Unfortunately, this project has not been carried through to completion, and no viruses have been tested in this assay in P19 cells. Yet it is ready to be expanded to P19 cells, just as it is ready to be expanded to other viruses. Only if these experiments prove negative will it become possible to speculate that mammalian viruses do not possess active anti-silencing mechanisms.

However, I consider it very likely that some block of Dicer activity will be observed, if only because of viral blockade of other dsRNA pathways, such as PKR (with, for example, a dsRNA-binding vaccinia protein E3L (7)). If indeed certain viruses lead to the inhibition of RNA silencing, further experiments should be concerned with understanding the relationship between viral activities targeting the non-specific dsRNA pathways and those targeting the sequence-specific ones. If separate viral activities are required, the case for RNA silencing being an antiviral defense will be greatly strengthened. In case these activities are found to be identical, further research in this line of experimentation may involve infections of mice deficient in the different dsRNA pathways. It is possible that a mouse deficient in PKR or RNase L may display a certain (or none) susceptibility phenotype to a given virus, while a mouse deficient in a RNA silencing component will present a different susceptibility profile. Such experiments will provide us with a foothold in understanding RNA silencing as an antiviral defense.

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Figure Legends

Fig. 1. Outline of the virus assay. See text for details. Either siP or siR served as the control siRNA. Figures 2A-D detail the siRNAs used as controls and the exact times used in each experiment.

Fig. 2. Viruses do not block siRNA action in HeLa. Firefly and Renilla luciferase activity of mRNAs co-electroporated into infected cells with siL or control siRNA were measured, their ratio (F:R) computed and normalized to the control siRNA-containing electroporations. The ratios of electroporations involving control siRNA are assigned the value of 1.00 (black open squares in each graph). Each line represents values of electroporations started at a certain time post-infection (hpi) or in a mock infection, as detailed in the inset. Cells were infected with poliovirus (A), herpes simplex type 1 (B), vaccinia (C), and yellow fever (D).

Figure 1

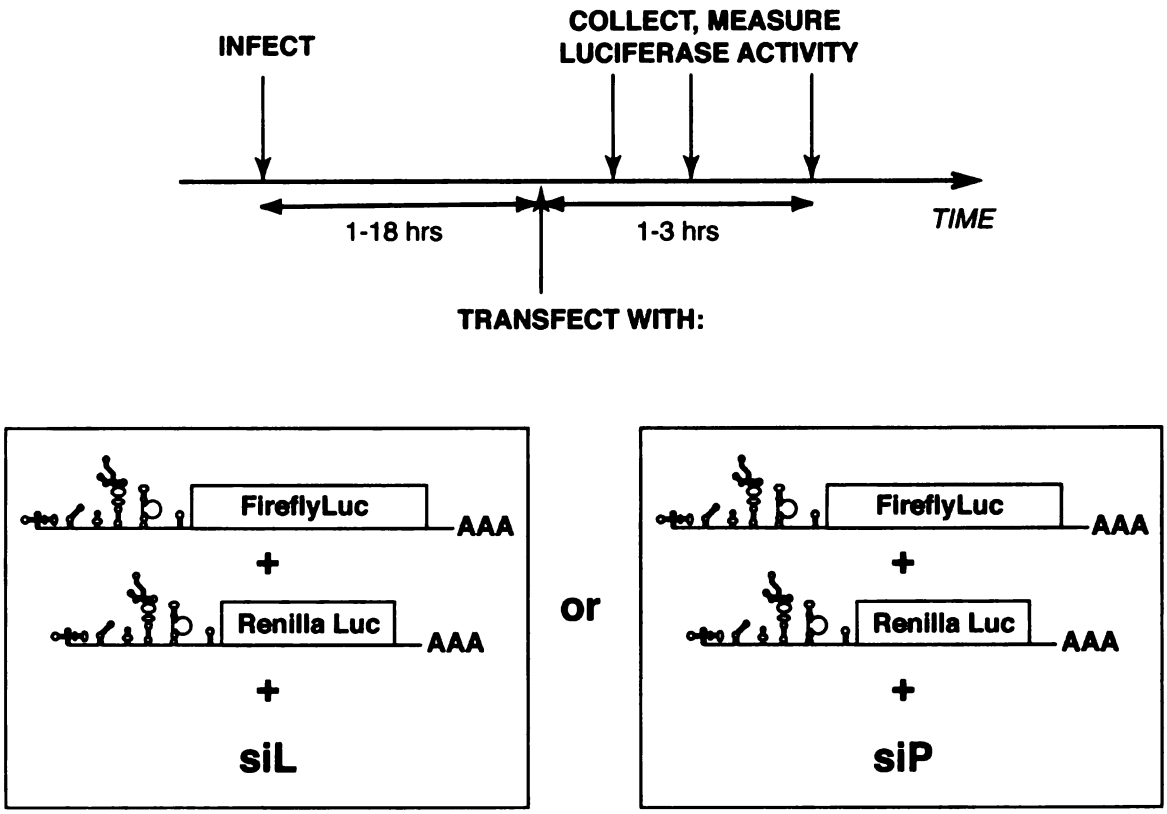
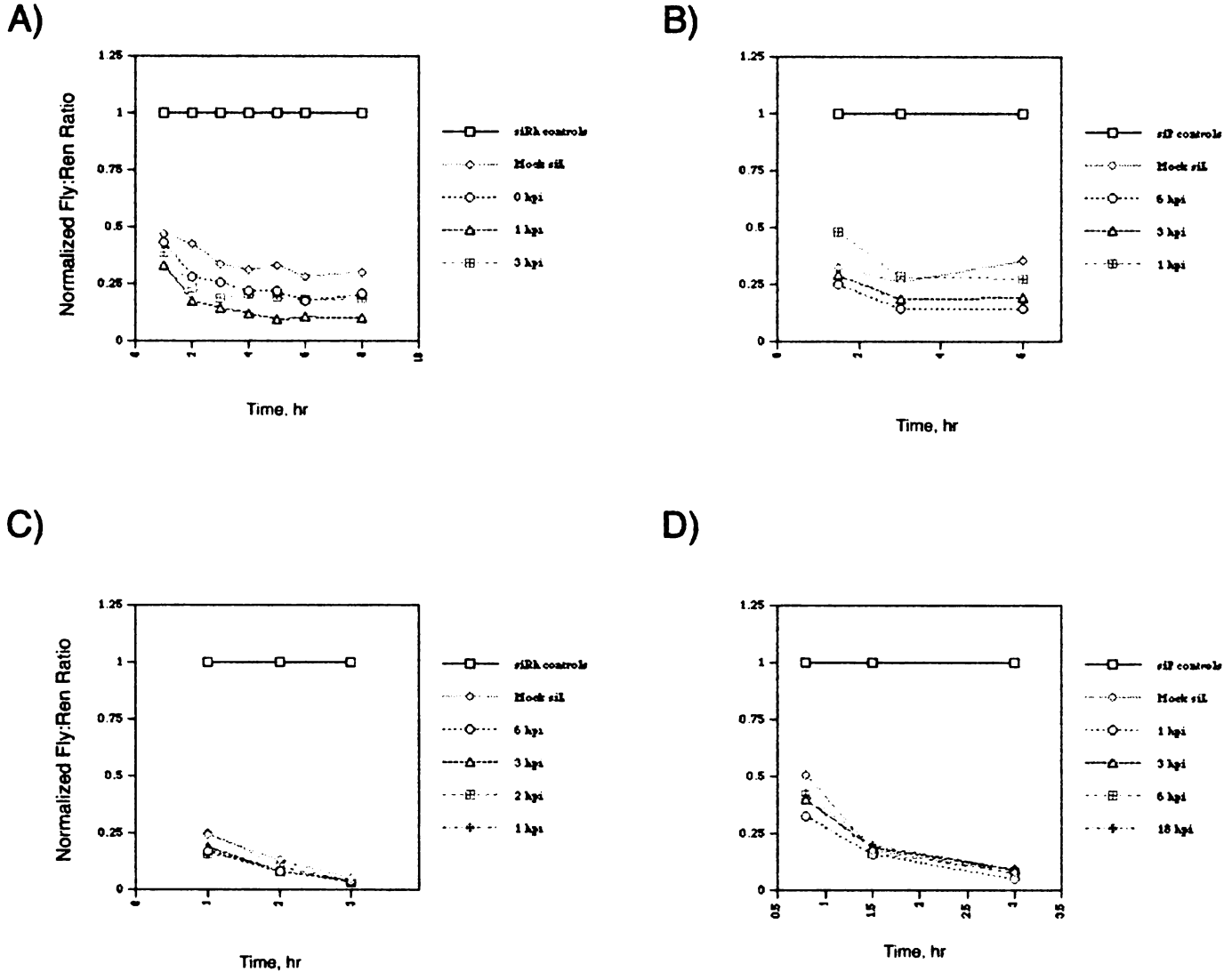

INFECT
COLLECT, MEASURE
LUCIFERASE ACTIVITY
1-18 hrs
1-3 hrs
TIME
TRANSFECT WITH:
FireflyLuc
AAA
+
Renilla Luc
AAA
+
siL
or
FireflyLuc
AAA
+
Renilla Luc
AAA
+
siP

Figure 2


A)
B)
C)
D)
Normalized Fly:Ren Ratio
Time, hr
siRh controls
Mock siL
0 hpi
1 hpi
3 hpi
siI2 controls
6 hpi
2 hpi
18 hpi