the generation of DSBs to chromosome rearrangements in permissive cells, and hence to oncogenic progression [6,7]. The molecular characterization of such alternative DNA repair pathways should be an important goal of future research.

References

1 Kuehl, M. *et al.* (2002) Multiple myeloma: evolving genetic events and host interactions. *Nat. Rev. Cancer* 2, 175–187
2 Ferguson, D.O. and Alt, F.W. (2001) DNA double strand break repair and chromosomal translocation: lessons from animal models. *Oncogene* 20, 5572–5579
3 Pipiras, E. *et al.* (1998) Interstitial deletions and intrachromosomal amplification initiated from a double-strand break targeted to a mammalian chromosome. *EMBO J.* 17, 325–333
4 Richardson, C. and Jasin, M. (2000) Frequent chromosomal translocations induced by DNA double-strand breaks. *Nature* 405, 697–700
5 Alt, F.W. *et al.* (1978) Selective multiplication of dihydrofolate reductase genes in methotrexate-resistant variants of cultured murine cells. *J. Biol. Chem.* 253, 1357–1370
6 Zhu, C. *et al.* (2002) Unrepaired breaks in p53-deficient cells lead to oncogenic gene amplification subsequent to translocation. *Cell* 109, 811–821
7 Difilippantonio, M.J. *et al.* (2002) Evidence for replicative repair of DNA double-strand breaks leading to oncogenic translocation and gene amplification. *J. Exp. Med.* 196, 469–480
8 Vanasse, G.J. *et al.* (1999) Genetic pathway to recurrent chromosome translocations in murine lymphoma involves V(D)J recombinase. *J. Clin. Invest.* 103, 1669–1675
9 McClintock, B. (1951) Chromosome organization and gene expression. *Cold Spring Harb. Symp. Quant. Biol.* 16, 13–47
10 Toledo, F. *et al.* (1993) The origin of chromosome rearrangements at early stages of *AMPD2* gene amplification in Chinese hamster cells. *Curr. Biol.* 3, 255–264
11 Sutherland, G.R. *et al.* (1998) Fragile sites still breaking. *Trends Genet.* 14, 501–506
12 Richards, R.I. (2001) Fragile and unstable chromosomes in cancer: causes and consequences. *Trends Genet.* 17, 339–345
13 Coquelle, A. *et al.* (1997) Expression of fragile sites triggers intrachromosomal mammalian gene amplification and sets boundaries to early amplicons. *Cell* 89, 215–225
14 Debatisse, M. *et al.* (1998) Gene amplification mechanisms: the role of fragile sites. *Recent Results Cancer Res.* 154, 216–226
15 Hellman, A. *et al.* (2002) A role for common fragile sites induction in amplification of human oncogenes. *Cancer Cell* 2, 89–97
16 Ciullo, M. *et al.* (2002) Initiation of the breakage-fusion-bridge mechanism through common fragile site activation in human breast cancer cells: the model of PIP gene duplication from a break at FRA7I. *Hum. Mol. Genet.* (in press)
17 Turner, B.C. *et al.* (2002) The fragile histidine triad/common chromosome fragile site 3B locus and repair-deficient cancers. *Cancer Res.* 62, 4054–4060
18 Mai, A. *et al.* (1996) Genomic instability in MycER-activated Rat1-MycER cells. *Chromosome Res.* 4, 365–371
19 Felsher, D.W. and Bishop, J.M. (1999) Transient excess of Myc activity can elicit genomic instability and tumorigenesis. *Proc. Natl. Acad. Sci. U. S. A.* 96, 3940–3944
20 Vafa, O. *et al.* (2002) c-Myc can induce DNA damage, increase reactive oxygen species and mitigate p53 function : a mechanism for oncogene-induced genetic instability. *Mol. Cell* 9, 1031–1044

Michelle Debatisse
Institut Curie, Section de Recherche, Pavillon Trouillet-Rossignol, 26 Rue d'Ulm, 75428 Paris, France.

RNA interference: a promising approach to antiviral therapy?

Jose M. Silva, Scott M. Hammond and Gregory J. Hannon

RNA interference (RNAi) has developed into a powerful tool for probing gene function. Recently, several reports have demonstrated the use of RNAi for attenuation of viral infection and replication in cultured cells. Furthermore, RNAi has been shown to suppress gene expression in adult animals. Although these results suggest that RNAi might become a novel therapeutic approach, significant hurdles must still be overcome before RNAi can be exploited in the fight against human disease.

The phenomenon of RNA interference (RNAi) was first described by Fire *et al.* approximately four years ago [1]. They observed that the response to double-stranded RNA (dsRNA) in the nematode, *Caenorhabditis elegans,* resulted in potent sequence-specific gene silencing at the post-transcriptional level. The RNAi pathway has since been recognized as a conserved biological module, and numerous experimental models have contributed to the understanding of it.

Mechanistically, RNAi is a two-step process (Fig. 1). In the first step, the dsRNA that triggers the silencing response is cleaved into small interfering RNAs (siRNAs) [2,3] of 21–23 nucleotides. This is accomplished by Dicer, an RNase-III-family nuclease [4]. In the second step, siRNAs are incorporated into a targeting complex, known as RISC (RNA-induced silencing complex), which destroys mRNAs that are homologous to the integral siRNA [3]. The net result is a suppression of gene expression.

RNAi as an RNA-based cellular 'immune system'

The complete spectrum of biological processes in which the RNAi machinery acts is far from clear. However, both biochemical and genetic studies have shown that RNAi is an important means of combating viral infection in plants (reviewed in [5]). Single-stranded-RNA viruses produce dsRNA as a replication intermediate. Exploiting this 'endogenous' dsRNA trigger, the plant cell uses RNAi to target viral genomic RNAs, breaking the replication cycle of the virus and preventing its systemic spread. Interestingly, plant viruses have evolved mechanisms to suppress the RNAi response, in much the same way that many animal viruses have developed methods to suppress antiviral immune responses. Many plant viruses express specific proteins that interfere with the RNAi machinery. Although these viral inhibitors of gene silencing are normally essential for pathogenesis, they become dispensable in plants with genetic lesions in RNAi pathways (reviewed in [5]). This phenomenon has recently been observed in an animal system; flock-house virus, an insect virus of the nodavirus family, contains a virulence determinant – similar to those found in some plant viruses – that is not required in *Drosophila* cells in which the RNAi response has been attenuated [6]. Furthermore, some elements of RNAi pathways are required for suppressing the movement of transposons in *C. elegans* [7,8].

It has been suggested that the RNAi response might be an evolutionarily conserved mechanism for combating

 1471-4914/02/$ – see front matter PII: S1471-4914(02)02421-8

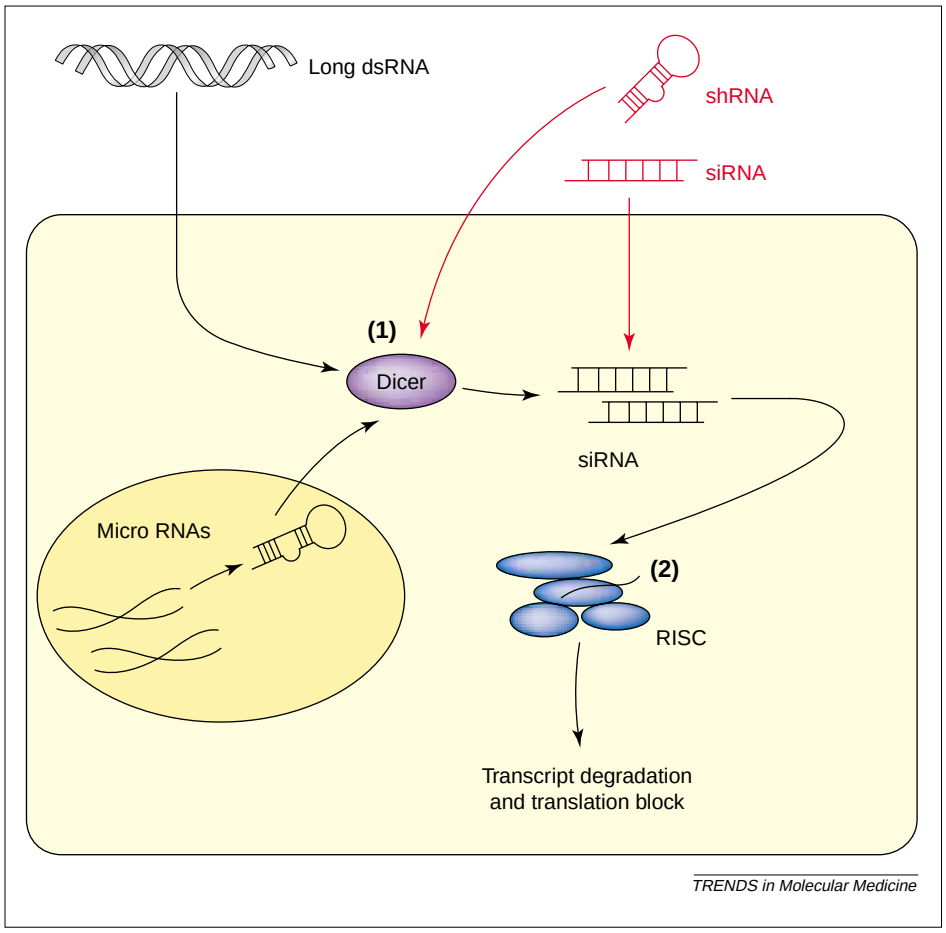

Fig. 1. The RNA interference (RNAi) pathway. RNAi is a two-step process that results in the degradation of targeted mRNAs. In stage (1), exogenous double-stranded RNA >21–23 bp (dsRNA) is cleaved into small interfering RNAs (siRNAs) by Dicer. In stage (2), siRNAs are incorporated into an RNA-induced silencing complex (RISC), which destroys mRNAs that are homologous to the integral siRNA. Short hairpin RNAs (shRNAs) transcribed *in vivo* from expression constructs or chemically synthesized siRNAs (both red) can be used to artificially stimulate this pathway.

viruses or parasitic endogenous genetic elements. However, mammals, with their adaptive immune systems, have clearly evolved alternative, or perhaps additional, defense mechanisms. Nevertheless, it is clear that RNAi pathways, which are conserved in mammalian cells, might be exploited therapeutically as an antiviral defense to augment the adaptive immune system. However, the use of RNAi as an experimental or therapeutic tool in mammalian systems was initially problematic owing, ironically, to the presence of an endogenous antiviral response. In most mammalian somatic cells, dsRNA of >30 bp activates a set of pathways that ultimately induce cell death. These mechanisms are enhanced by the presence of interferons, but even in the absence of these immune modulators, dsRNA activates Protein Kinase R (PKR) and RNase L pathways, leading to a generalized suppression of gene expression followed by apoptosis.

RNAi in mammalian cells

Recently, two approaches have been developed that avoid nonspecific dsRNA responses and that permit the exploitation of RNAi for sequence-specific silencing in mammalian somatic cells. The first is the use of synthetically produced siRNAs of 21 bp in length that are essentially chemically synthesized mimics of Dicer cleavage products (Fig. 1). These small RNAs have been shown to induce sequence-specific gene silencing when transiently transfected into mammalian cells [9], and they have become a powerful experimental tool for probing gene function. A second strategy uses expression constructs harboring a 19–29 bp inverted repeat that forms a short hairpin (shRNA) when transcribed *in vivo*. It reproduces the secondary structure of endogenous interfering RNAs (micro RNAs) [10,11]. The use of shRNAs extends the utility of RNAi in mammalian cells because hairpin RNAs can be expressed stably from integrated vectors.

Because effective silencing can now be achieved using 'classical' DNA expression vectors, it should be possible to adapt RNAi to adult mammals by its incorporation into established gene-therapy vehicles. Retroviral delivery of shRNA expression constructs into mammalian cells has been achieved [12], and several investigators are currently evaluating the efficacy of adenoviral transduction. Induction of RNAi following hydrodynamic transfection of shRNA expression vectors or siRNA into the liver and other somatic tissues has been reported [13,14].

'…it is clear that RNAi pathways, which are conserved in mammalian cells, might be exploited therapeutically as an antiviral defense…'

RNAi as an antiviral therapy

Following the discovery that RNAi pathways exist in mammalian cells, it has been proposed that this biological response might be exploited for therapeutic purposes. Partly because of the natural role of RNAi as an antiviral defense in plants, pathogenic human viruses were deemed a good starting point for evaluating the therapeutic potential of RNAi. Recently, several groups have explored the use of RNAi to limit infection by viruses in cultured cells, initially focusing on HIV [15–17] and poliovirus [18]. Several different strategies were used, and all the studies yielded encouraging results.

For example, Jacque *et al.* directed siRNAs against several regions of the HIV-1 genome, including the viral long terminal repeat (LTR) and the accessory genes, *vif* and *nef* [15]. Using Magi cells (CD4-positive HeLa cells) as a model system, they demonstrated a sequence-specific reduction of >95% in viral infection after cotransfection of siRNAs with an HIV-1 proviral DNA (Fig. 2). When the same assay was done in primary peripheral blood lymphocytes, which are natural targets for HIV-1, the frequency of infected cells was also substantially reduced.

In this study, transfection of cells with the infectious HIV-1 DNA clone reproduced late events in the viral life cycle. One potential concern was that retroviral genomes, tightly associated with nucleocapsid proteins, might be resistant to siRNA-induced destruction. However,

Jacque and colleagues reported that 1 h after infection, genomic viral RNA was undetectable in cells previously transfected with homologous siRNAs, and that the synthesis of viral cDNA intermediates was greatly inhibited [15]. The authors also examined whether shRNAs directed against *vif* produced inhibition similar to that seen with siRNAs. Cotransfection experiments in Magi cells revealed a suppression of ~95%. Similar results were obtained by Novina and colleagues in experiments targeting the HIV *gag* gene [17].

In a separate study, Lee *et al.* used a construct that produced an *in-vivo*-transcribed siRNA targeting the HIV-1 nonstructural protein, *rev* [16]. They showed that cotransfection of HIV-1 proviral DNA with this construct in 293 cells reduced the level of viral antigen in viral supernatants by four orders of magnitude, with a corresponding loss of viral integration.

Additional support for the potential of RNAi as an antiviral therapy has come from studies of other pathogenic viruses. For example, Gitlin *et al.* attenuated infection by poliovirus after transfection with siRNAs that targeted either a capsid-protein mRNA or the viral polymerase mRNA [18]. They also showed that mutation of the viral genome within the sequence targeted by siRNAs led to the production of a resistant variant. Similarly, RNAi has been used to attenuate infection by Rous Sarcoma Virus in chick embryos [19], and sequences within the Hepatitis-C virus have been successfully targeted in living mice when present as a fusion with a reporter construct [13].

'…several groups have explored the use of RNAi to limit infection by viruses in cultured cells…and all these studies yielded encouraging results.'

Targeting of viral versus host mRNAs

Unfortunately, the targeting of viral mRNAs using RNAi might suffer from three significant disadvantages. First, viral genomes might not be freely accessible in the context of the nucleocapsid, although success in targeting early steps of the HIV life cycle suggest that this might not be as problematic as had been anticipated [15]. Second, individual isolates of many viruses contain sequence variations that could complicate the design of inhibitory RNAs. Third, reverse transcriptases and RNA replicases are notoriously error-prone, increasing the likelihood of the emergence of mutant, resistant variants. An alternative approach is therefore to target host mRNAs whose protein products are essential for the viral life cycle. For example, Novina *et al.* used this method to suppress CD4 expression on host cells, thus preventing HIV entry and subsequent replication (Fig. 2) [17]. Variations on this approach might lend themselves to *ex vivo* manipulation of stem cells to create virus-resistant populations for autologous transplantation.

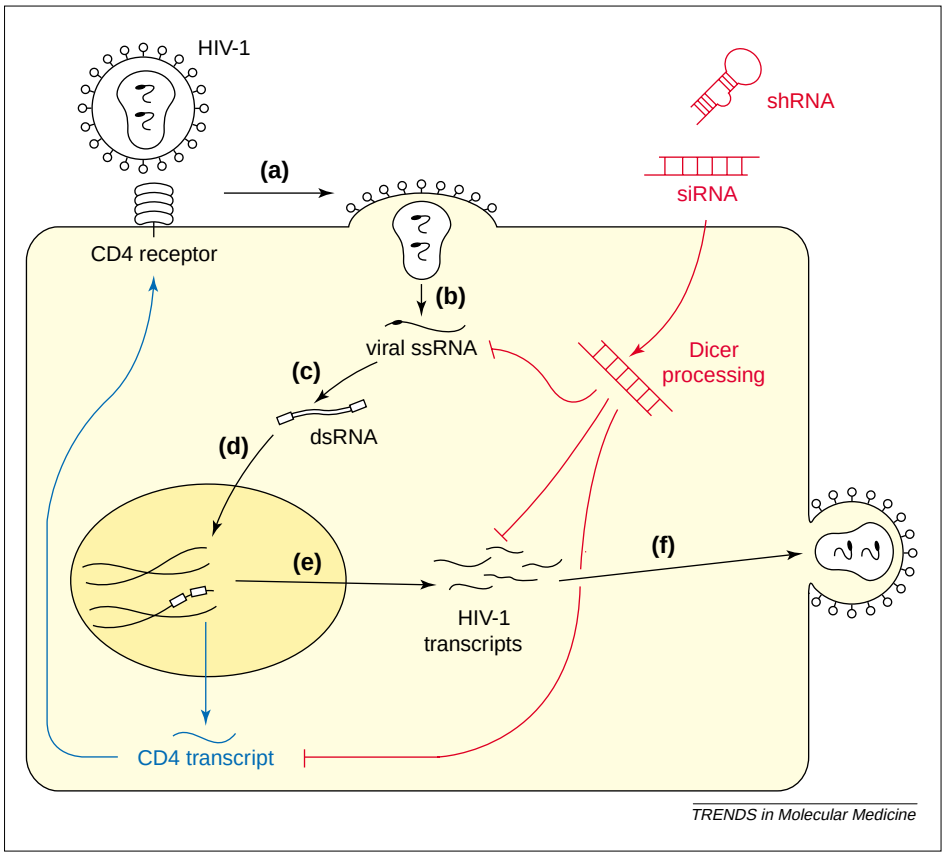

Fig. 2. Strategies for targeting viruses in mammalian cells. The lifecycle of the HIV virus: (a) infection of a cell expressing the CD4 receptor, (b) release of single-stranded RNA (ssRNA), (c) reverse transcription to double-stranded DNA (dsDNA), (d) integration into the host genome, (e) transcription of viral genes, and (f) progeny-virus production and release. HIV infection and replication have been targeted by RNA interference (RNAi) using either short hairpin RNAs (shRNAs) transcribed *in vivo* from expression constructs, or chemically synthesized small interfering RNAs (siRNAs) (both red). RNAi has also been used to suppress CD4 expression in host cells (blue), thus preventing HIV entry and subsequent replication.

Conclusions

Several recent reports have provided proof-of-concept that RNAi can be used to intervene at multiple points in the virus life cycle. However, many questions need to be addressed, and many problems still need to be solved, before RNAi can realize its potential as an antiviral therapy. For example, all the experiments described above were performed using cell-culture models. Although siRNAs and shRNAs have been shown to function in adult mammals [13,14], as with all nucleic-acid-based therapies, efficiently delivery *in vivo* remains the most significant hurdle.

Acknowledgements

We thank Jim Duffy for preparation of the figures. J.M.S. is supported by a fellowship from the U.S. Army Prostate Cancer Research Program (DAMD17-01-1-0827). G.J.H. is supported by an Innovator Award from the U.S. Army Breast Cancer Research Program and is a Rita Allen Scholar. This work was supported in part by a grant from the NIH (G.J.H).

References

1 Fire, A. *et al.* (1998) Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. *Nature* 391, 806–811

2 Zamore, P.D. *et al.* (2000) RNAi: double-stranded RNA directs the ATP-dependent cleavage of mRNA at 21 to 23 nucleotide intervals. *Cell* 101, 25–33

3 Hammond, S.M. *et al.* (2000) An RNA-directed nuclease mediates post-transcriptional gene silencing in *Drosophila* cells. *Nature* 404, 293–296
4 Bernstein, E. *et al.* (2001) Role for a bidentate ribonuclease in the initiation step of RNA interference. *Nature* 409, 363–366
5 Waterhouse, P.M. *et al.* (2001) Gene silencing as an adaptive defence against viruses. *Nature* 411, 834–842
6 Li, H. *et al.* (2002) Induction and suppression of RNA silencing by an animal virus. *Science* 296, 1319–1321
7 Tabara, H. *et al.* (1999) The *rde-1* gene, RNA interference, and transposon silencing in *C. elegans*. *Cell* 99, 123–132
8 Ketting, R.F. *et al.* (1999) *Mut-7* of *C. elegans*, required for transposon silencing and RNA interference, is a homolog of Werner syndrome helicase and RNaseD. *Cell* 99, 133–141
9 Elbashir, S.M. *et al.* (2001) Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. *Nature* 411, 494–498
10 Brummelkamp, T.R. *et al.* (2002) A system for stable expression of short interfering RNAs in mammalian cells. *Science* 296, 550–553
11 Paddison, P.J. *et al.* (2002) Short hairpin RNAs (shRNAs) induce sequence-specific silencing in mammalian cells. *Genes Dev.* 16, 948–958
12 Paddison, P.J. and Hannon, G.J. (2002) RNA interference: the new somatic cell genetics? *Cancer Cell* 2, 17–23
13 McCaffrey, A.P. *et al.* (2002) RNA interference in adult mice. *Nature* 418, 38–39
14 Lewis, D.L. *et al.* (2002) Efficient delivery of siRNA for inhibition of gene expression in postnatal mice. *Nat. Genet.* 32, 107–108
15 Jacque, J.M. *et al.* (2002) Modulation of HIV-1 replication by RNA interference. *Nature* 418, 435–438
16 Lee, N.S. *et al.* (2002) Expression of small interfering RNAs targeted against HIV-1 *rev* transcripts in human cells. *Nat. Biotechnol.* 20, 500–505
17 Novina, C.D. *et al.* (2002) siRNA-directed inhibition of HIV-1 infection. *Nat. Med.* 8, 681–686
18 Gitlin, L. *et al.* (2002) Short interfering RNA confers intracellular antiviral immunity in human cells. *Nature* 418, 430–434
19 Hu, W. *et al.* (2002) Inhibition of retroviral pathogenesis by RNA interference. *Curr. Biol.* 12, 1301–1311

Jose M. Silva
Scott M. Hammond
Gregory J. Hannon*
Cold Spring Harbor Laboratory, Watson School of Biological Sciences, 1 Bungtown Road, Cold Spring Harbor, NY 11724, USA.
*e-mail: hannon@cshl.org

Editorial policy

The majority of articles published in *Trends in Molecular Medicine* (*TMM*) are commissioned by the Editor. However, authors who wish to contribute to any section of the journal should contact the Editor with the names of all authors and a point-by-point outline of the proposed article, including 10–12 key references. Completed manuscripts submitted without liaison with the *TMM* Editorial Office will not be considered. *TMM* readers who wish to propose that a particular topic be covered are also invited to send their suggestions to the Editor.

tmm@current-trends.com