RNA interference: advances and questions

Elisabetta Ullu^{1,2*}, Appolinaire Djikeng¹, Huafang Shi¹
and Christian Tschudi¹

¹Department of Internal Medicine, and ²Department of Cell Biology, Yale Medical School, 333 Cedar Street, New Haven, CT 06520-8022, USA

In animals and protozoa gene-specific double-stranded RNA triggers the degradation of homologous cellular RNAs, the phenomenon of RNA interference (RNAi). RNAi has been shown to represent a novel paradigm in eukaryotic biology and a powerful method for studying gene function. Here we discuss RNAi in terms of its mechanism, its relationship to other post-transcriptional gene silencing phenomena in plants and fungi, its connection to retroposon silencing and possibly to translation, and its biological role. Among the organisms where RNAi has been demonstrated the protozoan parasite *Trypanosoma brucei* represents the most ancient branch of the eukaryotic lineage. We provide a synopsis of what is currently known about RNAi in *T. brucei* and outline the recent advances that make RNAi the method of choice to disrupt gene function in these organisms.

Keywords: double-stranded RNA; RNA interference; genomics; *Trypanosoma brucei*

1. THE DISCOVERY OF RNAI

In a 1998 issue of *Nature* (19 February) the laboratories of Fire and Mello described the unexpected effects of injecting dsRNA into the nematode *Caenorhabditis elegans*: dsRNA induced gene silencing in a gene-specific manner, and some of the resulting animals had phenotypes of corresponding null mutants (Fire *et al.* 1998). Strikingly, the silencing activity of dsRNA spread from the cells at the site of injection to most of the cells in the worm and could be passed on to the next generation. The term RNAi was then coined to indicate genetic interference by dsRNA (Fire *et al.* 1998). In these and other experiments it was further shown that for dsRNA to be an effective trigger of RNAi its sequence needs to be homologous to the mRNA coding region of the gene of interest and should be a few hundred base pairs in length (Montgomery *et al.* 1998). While this work was in progress we independently discovered that in the parasitic protozoa *Trypanosoma brucei* transient expression or electroporation of dsRNA homologous to α -tubulin mRNA led to downregulation of α -tubulin synthesis, because the corresponding mRNA was degraded (Ngo *et al.* 1998). The silencing effect was not specific to α -tubulin mRNA but could be obtained with dsRNA homologous to other mRNAs. Within a short time thereafter several laboratories simultaneously reported RNAi in a variety of other organisms, including *Drosophila*, *Planaria* and *Hydra*, and, subsequently, RNAi was described in mouse embryos and zebrafish (for a review, see Sharp 1999). Thus, at an early stage, it was established that gene silencing by dsRNA is widespread throughout the eukaryotic lineage. Considering that trypanosomatid protozoa separated relatively early from the main eukaryotic lineage, the ability of dsRNA to induce

gene silencing represents an ancient function of eukaryotic cells. However, to everybody's disappointment, to date, RNAi has not been reported in somatic cells of mammals. This is probably because, in these cells, dsRNA activates the interferon response, a cascade of antiviral defence mechanisms that are initiated by binding of dsRNA to the PKR (Williams 1999).

In plants, and in the fungus *Neurospora crassa*, it has been known for quite some time that there exist PTGS phenomena induced by transgenes in the form of inverted repeats, viruses or viroids (for a review, see Depicker & Montagu 1997). In these instances the term co-suppression is commonly used to indicate PTGS of homologous genes induced by the genetic elements described above. In *N. crassa* co-suppression is referred to as quelling (Cogoni & Macino 1997). Genetic analysis of RNAi in *C. elegans* (Ketting *et al.* 1999; Tabara *et al.* 1999), PTGS in plants (Kooter *et al.* 1999; Dalmay *et al.* 2000; Fagard & Vaucheret 2000; Mourrain *et al.* 2000; Plasterk & Ketting 2000) and quelling in *Neurospora* (for a review, see Cogoni & Macino 1999) has identified a subset of genes shared by these gene silencing phenomena, thus establishing a relationship between RNAi, PTGS and quelling. Whereas in plants PTGS/RNAi functions both at the transcriptional and post-transcriptional levels, in animals and protozoa only post-transcriptional RNAi has so far been detected.

The discovery of RNAi has brought to the scientific community not only a new and exciting biological paradigm but also an incredibly potent tool for addressing questions of gene function in an easy and high-throughput fashion. This discovery has recently been exploited by two groups for the analysis of gene function in *C. elegans* (Piano *et al.* 2000; Bargmann 2001). Here, the beauties of the system are that: (i) delivery of dsRNA to the worms can be achieved by soaking the animals in dsRNA solutions (Tabara *et al.* 1998) or even by simply feeding the

*Author for correspondence (elisabetta.ullu@yale.edu).

Figure 1. A hypothetical model for RNAi adapted from Bass (2000). In the model outlined here dsRNA is first recognized by the RNAi nuclease, which contains helicase and RNase III domains (Bernstein *et al.* 2001) and degraded to siRNAs that remain attached to the complex. In the second step, siRNAs target the cleavage of mRNA which we hypothesized is bound to polyribosomes. The RNAi nuclease is depicted as interacting with the ribosome; however, at present there is no evidence for this interaction. In the third step, there is strand exchange of the antisense strand of the siRNA with the mRNA via the helicase domain of the RNAi complex. Once the mRNA is associated with the active site of the enzyme, cleavage will occur.

organisms bacteria expressing dsRNA from plasmids equipped with opposing T7 RNA polymerase promoters (Timmons & Fire 1998); (ii) the silencing effect of RNAi spreads to most tissues of the animal (Fire *et al.* 1998); and (iii) the RNAi response is long lasting (Fire *et al.* 1998). Unfortunately, in all other organisms in which RNAi has been described to date, delivery of dsRNA is more labour intensive in that it can only be achieved by microinjection, electroporation or by transfecting organisms with appropriate expression constructs producing dsRNA.

2. THE MECHANISM OF RNAI

Mechanistically RNAi can be divided into two steps (Bass 2000). During the first step dsRNA is processed by a nuclease to produce 21–25 nucleotide small RNAs, termed small interfering RNAs or siRNAs (Elbashir *et al.* 2001), which act as guide sequences to target mRNA degradation and are now recognized as the hallmark for RNAi (figure 1). SiRNAs have both the sense and antisense polarity, but it is not known whether the two strands are paired *in vivo*. SiRNAs were originally identified in association with co-suppression in plants (Hamilton & Baulcombe 1999) and since then have been detected in *Drosophila* (Hammond *et al.* 2000; Yang *et al.* 2000; Zamore *et al.* 2000) and in *C. elegans* (Parrish *et al.* 2000) upon expression of transgenes, microinjection of synthetic dsRNAs or incubation of dsRNA in cell extracts competent for RNAi. Importantly, 21–22 nucleotide RNAs with short 3' extensions are able to induce RNAi in an *in vitro Drosophila* extract (Elbashir *et al.* 2001), thus establishing that siRNAs are the active species that guide degradation of target RNA. A candidate gene coding for the

dsRNA nuclease has been identified in *Drosophila* and has been termed 'Dicer' for its ability to digest dsRNA to homogeneous-size pieces, the siRNAs. The protein contains multiple domains, a helicase domain close to the amino terminus, followed by a PAZ domain, two adjacent RNase III domains and a dsRNA binding motif (Bernstein *et al.* 2001). At present it is not known whether all the various domains function solely during the first step of RNAi, namely digestion of dsRNA to siRNAs, or whether the same protein is involved in destroying the target mRNA. Importantly, homologous genes have been found in the genomes of *C. elegans*, *Arabidopsis*, mammals and *Schizosaccharomyces pombe*. The human homologue expressed *in vitro* is capable of generating siRNA-like fragments from dsRNA substrates, which indicates that this protein shares similar biochemical properties with 'Dicer' (Bernstein *et al.* 2001). Time and experimentation will tell whether the human protein functions in the RNAi pathway, and which type of cells might be endowed with the pathway.

Currently, only siRNAs produced from transgenes, viruses or synthetic dsRNAs have been characterized, and it is not known whether siRNAs are naturally produced in cells from endogenous dsRNAs to silence specific transcripts. However, we feel it is highly probable that this will be the case, simply because it is difficult to imagine that RNAi does not fulfill some important biological role beside offering us, the investigators, a powerful tool to analyse gene function. Once we know the identity of endogenous siRNAs, it will be possible to identify what transcripts are naturally targeted by RNAi and to clarify the biological role of the RNAi response.

The second step of RNAi involves recognition and targeting of the homologous RNA, which in animals and

protozoa is the mature mRNA. At the present we know very little about where in the cell this step takes place, except that the activity degrading mRNA in *Drosophila* cells is present in a multicomponent complex that contains siRNAs and can be largely cleared from extracts by centrifugation at 100 000g for 60 min (Bernstein *et al.* 2001). Interestingly, these centrifugation conditions are those that are usually employed to enrich for polyribosomes. One exciting area of investigation is the possible association between the RNAi and the translational machineries. There are two hints that there might be an association. First, in *C. elegans* a genetic screen for RNAi-deficient organisms has revealed that the *RDE-1* gene is highly similar to rabbit eIF2C, a translation factor of unknown function (Tabara *et al.* 1999). Second, worms that are deficient in the *SMG2* gene, a helicase that is involved in nonsense-mediated mRNA decay (NMD), and is present on polyribosomes, are capable of escaping silencing by RNAi, i.e. the RNAi response in these animals is only transient (Domeier *et al.* 2000). From a purely speculative viewpoint the association between the RNAi and translational machineries would be advantageous in that most mRNAs interact, at some point in their lifespan, with ribosomes. Of course the exceptions are the mRNAs that are stored for later use, as occurs, for instance, during oogenesis. Interestingly, stored mRNAs can be targeted by RNAi (Svoboda *et al.* 2000). However, this does not exclude the possibility that RNAi of abundant and polysome-associated mRNAs does occur in association with the translational apparatus. We have some indications that this might be the case in *T. brucei*. In *T. brucei* most mRNAs are found on polyribosomes (figure 2), indicating that the target for RNAi is mRNA while it is being translated. Ribosomes are equipped for numerous activities, for instance, the ability to recognize premature termination codons in mRNAs and to specifically target these aberrant mRNAs for degradation through the NMD pathway (Ruiz-Echevarria *et al.* 1998). We believe that RNAi fulfils an important biological role as a mechanism of quality control for mRNA surveillance in order to ensure degradation of deleterious transcripts, and the translational machinery would be ideally positioned to exert this control.

3. THE BIOLOGICAL ROLE OF RNAI

Do dsRNAs homologous to naturally occurring transcripts occur in cells, and how do cells deal with them? One class of genes that potentially can generate transcripts of both strands is the transposable elements which inhabit the genome of most eukaryotic organisms (Haren *et al.* 1999). Usually, transposons can be inserted in either orientation relative to cellular genes and they often carry internal promoter sequences. If they happen to land in the genome in the reverse orientation relative to a cellular promoter it is not difficult to imagine how antisense transcripts of these elements are generated. Thus, the possibility exists that sense and antisense transcripts are synthesized, and that they anneal to form dsRNA. Interestingly, once a new transposon is inserted into a naïve *Drosophila* strain its rate of transposition declines as soon as the copy number of the element reaches a certain limit, which the organism is able to sense in some way (Jensen

Figure 2. Association of mRNAs with polyribosomes in *T. brucei*. (a) Polyribosome profile of procyclic form *T. brucei* cells stably expressing a GFP transgene. A cytoplasmic extract was prepared from 5×10^8 cells in the presence of $100 \mu\text{g ml}^{-1}$ of cycloheximide (Brecht & Parsons 1998). The cytoplasmic extract was layered onto a 15–50% sucrose density gradient. After centrifugation at 180 000g in a SW41 rotor for 90 min, 0.5 ml fractions were collected using an ISCO density gradient fractionator. The OD₂₅₄ was recorded and the profile is depicted. The 40S and 60S subunits, 80S monosomes and polyribosomes are indicated. (b) Localization of α -tubulin and GFP mRNAs in polyribosome gradients. Odd-numbered fractions, as indicated at the bottom of the panel, were processed for Northern blot analysis and fractionated through a 1.2% agarose-formaldehyde gel. The gel was blotted onto a nylon filter and hybridized sequentially with a GFP and an α -tubulin DNA probe.

et al. 1999). This phenomenon is analogous to co-suppression and it is probable that RNAi is involved in transposon silencing in *Drosophila*. RNAi in *C. elegans* is certainly implicated in transposon silencing, since mutations in a subset of genes involved in RNAi led to transposon mobilization in the germ line (Ketting *et al.* 1999; Tabara *et al.* 1999). This established a genetic connection between RNAi and transposon mobilization. How the pathways intersect at the molecular level is at present unknown. One gene, *MUT-7*, has been identified that is common to both pathways (Ketting *et al.* 1999). *MUT-7* shows some similarity to the human Werner's syndrome gene, has been predicted to have RNase activity and is similar to the *QDE-3* gene of *Neurospora* that is required for quelling. It has also been shown to have the characteristics of a RecQ DNA helicase (Cogoni & Macino 1999). The specific substrate of *MUT-7* and *QDE-3*, whether it is DNA, RNA or both, is of course of great interest but, as yet, is unidentified. Thus, in this light RNAi can be considered a defence mechanism against harmful transcripts (Ketting *et al.* 1999; Tabara *et al.* 1999). This concept is not a new one, since PTGS in plants has long been known to be an antiviral defence mechanism (Ratcliff *et al.* 1999; Mourrain *et al.* 2000). Furthermore, although

the results have not been described in this paper, sequencing of 20–22 nucleotide small RNAs from a *Drosophila* extract also revealed sequences derived from endogenous retroposons (Elbashir *et al.* 2001). Whether these RNAs represent siRNAs has not yet been established. However, it will be important to determine whether siRNAs corresponding to transposon sequences are present in all organisms that harbour them. In particular, the mammalian genome is rich in short- and long-interspersed sequence elements that are known to be transcribed, both in the sense and antisense orientation. Old data on reassociation kinetics of heterogeneous nuclear RNA indicated that a considerable proportion is rapidly reassociating, suggesting a high prevalence of inverted repeats. If these transcripts were to be dealt with by the RNAi machinery, the machinery would probably be overloaded. However, most of these transcripts are confined to the nucleus and it may also be possible that dsRNAs are prevented from forming *in vivo*, perhaps through protein binding. Another aspect of dsRNA biology in higher eukaryotes worth mentioning is the presence of enzymes, termed ADARs, that specifically deaminate adenosine to inosine in dsRNA (Bass 1997). The introduction of inosine has a destabilizing effect on dsRNA and could lead to unwinding of the two strands, thus effectively removing dsRNA from the cells (Liu *et al.* 1998). ADARs are present in *C. elegans*, where they act upon a number of transcripts including some containing extensive double-helical regions (Morse & Bass 1999). However, it is clear that the presence of ADARs in this organism does not prevent activation of the RNAi pathway.

4. ARE THERE OTHER CELLULAR TRANSCRIPTS BESIDE TRANSPOSON TRANSCRIPTS THAT ARE SILENCED BY THE RNAi RESPONSE?

This is obviously an area of active research, but at present there is no answer to this question. One important requirement for certain genes to be naturally silenced by the RNAi response is that endogenous transcripts need to contain or acquire dsRNA regions. One possible reason for this occurring is when inverted repeats are present in the mRNA sequence itself. Because the RNAi response requires dsRNA regions that are at least 20–25 nucleotides in length, one way to identify potential natural targets for RNAi would be to search for RNA transcripts predicted to form 20–25 nucleotide long stem structures. However, as far as we know, perfectly matched dsRNA helices of this length are not common in naturally occurring mRNAs. Nevertheless as it is known that they exist in *C. elegans* (Morse & Bass 1999) it would be worthwhile to search for genes with such stem structures, given the availability of several completed eukaryotic genomes. The potential to form such a structure is by no means evidence that the same structure will form in the cell. For instance, one can imagine a scenario in which folding into dsRNA helices might be prevented or perhaps regulated by specific RNA-binding proteins. Another possibility for mRNA to acquire dsRNA regions is via antisense transcription, which occurs in a variety of systems and could thus potentially activate the RNAi response. The advantage here would be that sense and antisense transcripts will be produced in close proximity, and this would enhance the potential for for-

ming dsRNA helices *in vivo*. In fact, we, and other workers, have shown that in *T. brucei* simultaneous expression of sense and antisense transcripts from opposing promoters located on the same plasmid (Shi *et al.* 2000), or in the chromosome from adjacent transcription units, triggers a potent RNAi response (Bastin *et al.* 1999), whereas transcription of sense and antisense RNAs from unlinked transcription units is less efficient at triggering RNAi (Shi *et al.* 2000; E. Ullu, unpublished observations).

5. RNAi IN *TRYPANOSOMA BRUCEI*

As mentioned above, we first reported the effect of dsRNA silencing for α -tubulin mRNA in the autumn of 1998. However, we had observed the phenomenon almost two years earlier, when we serendipitously observed the appearance of multinucleated trypanosomes, termed FAT cells, following a control transfection of an expression plasmid that we were assembling for a promoter trap experiment. It took quite a long time to realize what was happening. The breakthrough came when we decided to bite the bullet and to electroporate synthetic α -tubulin dsRNA into cells. To our delight we were able to reproduce the phenomenon we had observed by transfecting plasmids containing a portion of α -tubulin mRNA as an inverted repeat (Ngo *et al.* 1998). Subsequently, great progress has been made in understanding some of the principles of RNAi in *T. brucei* and in constructing vectors that can be used for expressing dsRNA in cells. Although dsRNA transfection was very useful for observing phenotypes by downregulation of tubulin mRNA, we soon realized that the RNAi response triggered by transfecting dsRNA in *T. brucei* was only transient and lasted for approximately one cell cycle (Ngo *et al.* 1998). This is in contrast to what has been observed in *C. elegans* and *Drosophila* and most probably results from the instability of dsRNA in *T. brucei*. The identification of the enzyme that degrades dsRNA could be very useful for generating transgenic parasites that would be able to maintain a sustained RNAi response, and perhaps would make it possible to use dsRNA transfection as a high-throughput method for genome-wide analysis of gene function. At the present there are two vectors that the authors, and others, have constructed for triggering a persistent RNAi response in *T. brucei* (figure 3). The first vector uses the tetracycline (tet)-inducible promoter from the PARP genes (Wirtz *et al.* 1999; Bastin *et al.* 2000; Shi *et al.* 2000) to drive expression of hairpin RNAs. In the absence of tetracycline, expression from this promoter is almost negligible allowing cloning of toxic products, whereas under full induction conditions high levels of expression can be achieved. In our experience a strong promoter is required for triggering RNAi. One drawback of the hairpin RNA strategy is that generation of the appropriate constructs requires three cloning steps, and thus is time consuming. The second vector is equipped with opposing tet-inducible T7 RNA polymerase promoters (Wirtz *et al.* 1999; LaCount *et al.* 2000; Wang *et al.* 2000). In this case, a simple cloning step is required to insert a portion of the gene of interest in between the two promoters for producing dsRNA *in vivo*. Although this is a wonderful system, the major problem is that a considerable level of dsRNA is produced, even in the absence of tetracycline (Wang *et*

Figure 3. Vectors for achieving stable RNAi responses in *T. brucei*. Hairpin vector: this vector utilizes the *pLew79* vector backbone (Wirtz *et al.* 1999) in which the luciferase gene is substituted by a cassette consisting of two inverted repeats of the mRNA target sequence separated by a stuffer sequence. RNA transcription is driven by the tetracycline-inducible PARP promoter and produces a hairpin-like RNA molecule (Shi *et al.* 2000). Double promoter vector: in this vector two opposing tet-inducible T7 RNA polymerase promoters flank the mRNA target sequence (Shi *et al.* 2000). PARP arrow, tetracycline-inducible PARP promoter; filled boxes, tetracycline operator; TT, T7 transcription terminators; T7 arrow, T7 promoter; rDNA, ribosomal DNA non-transcribed spacer; p(A), poly(A) addition site; BLE, PHLEO-resistance gene; SAS, splice-site acceptor.

al. 2000). This is because binding of the tet-repressor to the tet-operators does not completely shut off the T7 promoters (Wirtz *et al.* 1999). Thus, in certain instances one should use caution in using this particular vector. However, it is clear that the double T7 promoter system is ideally suited for genome-wide analysis of gene function. Another application of this system is forward genetic studies. This would involve constructing a library of genomic fragments in this vector, generating a library of transgenic trypanosomes and then screening for mutant phenotypes of interest. As with all genetic screens the difficulty is not in generating the mutants but in setting up the appropriate screening procedure. However, it can be foreseen that this experimental method will soon be attempted by several investigators.

So far, we have used RNAi for downregulating expression of a variety of mRNAs. The size of the dsRNA trigger should be a few hundred nucleotides, although we have shown that for α - and β -tubulin mRNAs the size of the dsRNA region can be as short as 50 nucleotides (E. Ullu, unpublished results). In our hands downregulation of mRNA by gene-specific dsRNA has never failed. However, in about 50% of the cases the observation of a phenotype failed, and this has also been the experience of other laboratories. The problem remains because no matter how long the dsRNA sequence, there is always a residual amount of mRNA that remains in the cell. Hence, the synthesis of the gene product of interest is never completely abolished, and in many cases it appears that trypanosomes can survive even when little mRNA is there. At this point, it would be to everybody's advantage to be able to improve the system, to make it more reliable and, in particular, to establish why certain constructs work better than others.

In particular, one aspect of RNAi in *T. brucei* that we feel needs to be taken into consideration is the compartment in which RNAi operates. At an early stage, it was shown that RNAi targets mature mRNA for degradation and that pre-mRNA is not a substrate for RNAi (Ngo *et al.* 1998). Furthermore, transient expression of plasmids equipped with opposing T7 RNA polymerase promoters

led to 99% transfection efficiency as assessed by the appearance of FAT cells (Shi *et al.* 2000). These two pieces of evidence suggest that the RNAi machinery resides in the cytoplasm. Thus, if dsRNA is expressed from a chromosomal location it needs to travel to the cytoplasm for instructing the RNAi machinery for mRNA targeting. It is probable that over-expression of dsRNA in the nucleus from the extremely strong PARP or T7 promoters results in a proportion of dsRNA being translocated to the cytoplasm, however it would be desirable to be able to improve this step. For instance, it is known that the HIV virus produces the rev protein that binds to the rev response elements present in unspliced or partially spliced mRNA, to ensure that these mRNAs, which are usually retained in the nucleus, are delivered to the cytoplasm for translation (Cullen 1992). One could possibly adapt this system to trypanosomes. In principle, however, the best strategy would be to generate an RNA replicon that resides in the cytoplasm where it would produce large amounts of dsRNA. In this instance the replication system of alpha viruses could be used as a paradigm to contemplate engineering an RNA replicon in trypanosomes.

At the time of writing of this review *T. brucei* still remains the only trypanosomatid protozoa, or for that matter the only parasitic protozoa, in which the RNAi response has been shown to be functional. It is self-evident that the identification of an RNAi response in other important human pathogens, like the apicomplexan parasites, *Leishmanias* and *T. cruzi* would be a major step forward for functional genomics and for the validation of drug targets.

This work was supported by National Institutes of Health grant RO1-AI28798 and by a Scholar Award in Molecular Parasitology to E.U. C.T. is a recipient of a Burroughs Wellcome New Investigator Award in Molecular Parasitology.

REFERENCES

- Bargmann, C. I. 2001 High-throughput reverse genetics: RNAi screens in *Caenorhabditis elegans*. *Genome Biol.* **2**, 1005.1–1005.3.

- Bass, B. L. 1997 RNA editing and hypermutation by adenosine deamination. *Trends Biochem. Sci.* **22**, 157–162.
- Bass, B. L. 2000 Double-stranded RNA as a template for gene silencing. *Cell* **101**, 235–238.
- Bastin, P., Pullen, T. J., Sherwin, T. & Gull, K. 1999 Protein transport and flagellum assembly dynamics revealed by analysis of the paralysed trypanosome mutant *snl-1*. *J. Cell Sci.* **112**, 3769–3777.
- Bastin, P., Ellis, K., Kohl, L. & Gull, K. 2000 Flagellum ontogeny in trypanosomes studied via an inherited and regulated RNA interference system. *J. Cell Sci.* **113**, 3321–3328.
- Bernstein, E., Caudy, A. A., Hammond, S. M. & Hannon, G. J. 2001 Role for a bidentate ribonuclease in the initiation step of RNA interference. *Nature* **409**, 363–366.
- Brecht, M. & Parsons, M. 1998 Changes in polysome profiles accompany trypanosome development. *Mol. Biochem. Parasitol.* **97**, 189–198.
- Cogoni, C. & Macino, G. 1997 Isolation of quelling-defective (qde) mutants impaired in post-transcriptional transgene-induced gene silencing in *Neurospora crassa*. *Proc. Natl Acad. Sci. USA* **94**, 10 233–10 238.
- Cogoni, C. & Macino, G. 1999 Post-transcriptional gene silencing in *Neurospora* by a RecQ DNA helicase. *Science* **286**, 2342–2344.
- Cullen, B. R. 1992 Mechanism of action of regulatory proteins encoded by complex retroviruses. *Microbiol. Rev.* **56**, 375–394.
- Dalmay, T., Hamilton, A., Mueller, E. & Baulcombe, D. C. 2000 Potato virus X amplicons in Arabidopsis mediate genetic and epigenetic gene silencing. *Plant Cell* **12**, 369–379.
- Depicker, A. & Montagu, M. V. 1997 Post-transcriptional gene silencing in plants. *Curr. Opin. Cell Biol.* **9**, 373–382.
- Domeier, M. E., Morse, D. P., Knight, S. W., Portereiko, M., Bass, B. L. & Mango, S. E. 2000 A link between RNA interference and nonsense-mediated decay in *Caenorhabditis elegans*. *Science* **289**, 1928–1931.
- Elbashir, S. M., Lendeckel, W. & Tuschl, T. 2001 RNA interference is mediated by 21- and 22-nucleotide RNAs. *Genes Dev.* **15**, 188–200.
- Fagard, M. & Vaucheret, H. 2000 Systemic silencing signal(s). *Plant Mol. Biol.* **43**, 285–293.
- Fire, A., Xu, S., Montgomery, M. K., Kostas, S. A., Driver, S. E. & Mello, C. C. 1998 Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. *Nature* **391**, 806–811.
- Hamilton, A. J. & Baulcombe, D. C. 1999 A species of small antisense RNA in post-transcriptional gene silencing in plants. *Science* **286**, 950–952.
- Hammond, S. M., Bernstein, E., Beach, D. & Hannon, G. J. 2000 An RNA-directed nuclease mediates post-transcriptional gene silencing in *Drosophila* cells. *Nature* **404**, 293–296.
- Haren, L., Ton-Hoang, B. & Chandler, M. 1999 Integrating DNA: transposases and retroviral integrases. *A. Rev. Microbiol.* **53**, 245–281.
- Jensen, S., Gassama, M. P. & Heidmann, T. 1999 Taming of transposable elements by homology-dependent gene silencing. *Nat. Genet.* **21**, 209–212.
- Ketting, R. F., Haverkamp, T. H., van Luenen, H. G. & Plasterk, R. H. 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.
- Kooter, J. M., Matzke, M. A. & Meyer, P. 1999 Listening to the silent genes: transgene silencing, gene regulation and pathogen control. *Trends Plant Sci.* **4**, 340–347.
- LaCount, D. J., Bruse, S., Hill, K. L. & Donelson, J. E. 2000 Double-stranded RNA interference in *Trypanosoma brucei* using head-to-head promoters. *Mol. Biochem. Parasitol.* **111**, 67–76.
- Liu, Y., Herbert, A., Rich, A. & Samuel, C. E. 1998 Double-stranded RNA-specific adenosine deaminase: nucleic acid binding properties. *Methods* **15**, 199–205.
- Montgomery, M. K., Xu, S. & Fire, A. 1998 RNA as a target of double-stranded RNA-mediated genetic interference in *Caenorhabditis elegans*. *Proc. Natl Acad. Sci. USA* **95**, 15502–15507.
- Morse, D. P. & Bass, B. L. 1999 Long RNA hairpins that contain inosine are present in *Caenorhabditis elegans* poly(A)+ RNA. *Proc. Natl Acad. Sci. USA* **96**, 6048–6053.
- Mourrain, P. (and 13 others). 2000 Arabidopsis SGS2 and SGS3 genes are required for posttranscriptional gene silencing and natural virus resistance. *Cell* **101**, 533–542.
- Ngo, H., Tschudi, C., Gull, K. & Ullu, E. 1998 Double-stranded RNA induces mRNA degradation in *Trypanosoma brucei*. *Proc. Natl Acad. Sci. USA* **95**, 14687–14692.
- Parrish, S., Fleenor, J., Xu, S., Mello, C. & Fire, A. 2000 Functional anatomy of a dsRNA trigger. Differential requirement for the two trigger strands in RNA interference. *Mol. Cell* **6**, 1077–1087.
- Piano, F., Schetterdagger, A. J., Mangone, M., Stein, L. & Kempthues, K. J. 2000 RNAi analysis of genes expressed in the ovary of *Caenorhabditis elegans*. *Curr. Biol.* **10**, 1619–1622.
- Plasterk, R. H. & Ketting, R. F. 2000 The silence of the genes. *Curr. Opin. Genet. Dev.* **10**, 562–567.
- Ratcliff, F. G., MacFarlane, S. A. & Baulcombe, D. C. 1999 Gene silencing without DNA. RNA-mediated cross-protection between viruses. *Plant Cell* **11**, 1207–1216.
- Ruiz-Echevarria, M. J., Gonzalez, C. I. & Peltz, S. W. 1998 Identifying the right stop: determining how the surveillance complex recognizes and degrades an aberrant mRNA. *EMBO J.* **17**, 575–589.
- Sharp, P. A. 1999 RNAi and double-strand RNA. *Genes Dev.* **13**, 139–141.
- Shi, H., Djikeng, A., Mark, T., Wirtz, E., Tschudi, C. & Ullu, E. 2000 Genetic interference in *Trypanosoma brucei* by heritable and inducible double-stranded RNA. *RNA* **6**, 1069–1076.
- Svoboda, P., Stein, P., Hayashi, H. & Schultz, R. M. 2000 Selective reduction of dormant maternal mRNAs in mouse oocytes by RNA interference. *Development* **127**, 4147–4156.
- Tabara, H., Grishok, A. & Mello, C. C. 1998 RNAi in *C. elegans*: soaking in the genome sequence. *Science* **282**, 430–431.
- Tabara, H., Sarkissian, M., Kelly, W. G., Fleenor, J., Grishok, A., Timmons, L., Fire, A. & Mello, C. C. 1999 The rde-1 gene, RNA interference, and transposon silencing in *C. elegans*. *Cell* **99**, 123–132.
- Timmons, L. & Fire, A. 1998 Specific interference by ingested dsRNA. *Nature* **395**, 854.
- Wang, Z., Morris, J. C., Drew, M. E. & Englund, P. T. 2000 Inhibition of *Trypanosoma brucei* gene expression by RNA interference using an integratable vector with opposing T7 promoters. *J. Biol. Chem.* **275**, 40 174–40 179.
- Williams, B. R. 1999 PKR; a sentinel kinase for cellular stress. *Oncogene* **18**, 6112–6120.
- Wirtz, E., Leal, S., Ochatt, C. & Cross, G. A. 1999 A tightly regulated inducible expression system for conditional gene knock-outs and dominant-negative genetics in *Trypanosoma brucei*. *Mol. Biochem. Parasitol.* **99**, 89–101.
- Yang, D., Lu, H. & Erickson, J. W. 2000 Evidence that processed small dsRNAs may mediate sequence-specific mRNA degradation during RNAi in *Drosophila* embryos. *Curr. Biol.* **10**, 1191–1200.
- Zamore, P. D., Tuschl, T., Sharp, P. A. & Bartel, D. P. 2000 RNAi: double-stranded RNA directs the ATP-dependent cleavage of mRNA at 21 to 23 nucleotide intervals. *Cell* **101**, 25–33.