5 Subramaniam, R. *et al.* (2001) Direct visualization of protein interactions in plant cells. *Nat. Biotechnol.* 19, 769–772
6 Walhout, A.J. *et al.* (2000) Protein interaction mapping in *C. elegans* using proteins involved in vulval development. *Science* 287, 116–122
7 Uetz, P. *et al.* (2000) A comprehensive analysis of protein–protein interactions in *Saccharomyces cerevisiae*. *Nature* 403, 623–627
8 Rain, J.C. *et al.* (2001) The protein–protein interaction map of *Helicobacter pylori*. *Nature* 409, 211–215
9 Delaney, T.P. (1997) Genetic dissection of acquired resistance to disease. *Plant Physiol.* 113, 5–12
10 Ryals, J.A. *et al.* (1996) Systemic acquired resistance. *Plant Cell* 8, 1809–1819
11 Delaney, T.P. *et al.* (1994) A central role of salicylic acid in plant disease resistance. *Science* 266, 1247–1250
12 Klessig, D.F. and Malamy, J. (1994) The salicylic acid signal in plants. *Plant Mol. Biol.* 26, 1439–1458
13 Friedrich, L. *et al.* (1996) Benzothiadiazole induces systemic acquired resistance in tobacco. *Plant J.* 10, 61–70
14 Vernooij, B. *et al.* (1995) 2,6-dichloroisonicotinic acid-induced resistance to pathogens without the accumulation of salicylic acid. *Mol. Plant–Microbe Interact.* 8, 228–234
15 Cao, H. *et al.* (1997) The *arabidopsis* NPR1 gene that controls systemic acquired resistance encodes a novel protein containing ankyrin repeats. *Cell* 88, 57–63
16 Ryals, J. *et al.* (1997) The *Arabidopsis* NIM1 protein shows homology to the mammalian transcription factor inhibitor I kappa B. *Plant Cell* 9, 425–439
17Després, C. *et al.* (2000) The *Arabidopsis* NPR1/NIM1 protein enhances the DNA binding activity of a subgroup of the TGA family of bZIP transcription factors. *Plant Cell* 12, 279–290
18 Zhang, Y. *et al.* (1999) Interaction of NPR1 with basic leucine zipper protein transcription factors that bind sequences required for salicylic acid induction of the PR-1 gene. *Proc. Natl. Acad. Sci. U. S. A.* 96, 6523–6528
19 Zhou, J.M. *et al.* (2000) NPR1 differentially interacts with members of the TGA/OBF family of transcription factors that bind an element of the PR-1 gene required for induction by salicylic acid. *Mol. Plant–Microbe Interact.* 13, 191–202
20 Enyedi, A.J. *et al.* (1992) Localization, conjugation, and function of salicylic acid in tobacco during the hypersensitive reaction to tobacco mosaic virus. *Proc. Natl. Acad. Sci. U. S. A.* 89, 2480–2484

Ingrid Remy
Université de Montréal, Dépt de Biochimie, C.P. 6128, Succursale centre-ville, Montréal, Québec, H3C 3J7, Canada.
e-mail: ingrid.remy@umontreal.ca

A new approach to the inhibition of gene expression

Natasha J. Caplen

The induction of a specific down-regulation in gene expression is an important research tool. Recently, several related processes including post-transcriptional gene silencing (PTGS) and RNA interference (RNAi) have been identified that generate sequence-specific inhibition of gene expression. Although the physiological function of PTGS and RNAi is still being elucidated, these pathways have been used to rapidly determine gene function. The recent observation of RNAi in mammalian cells extends the possible applications of these mechanisms.

Although PTGS (first identified in plants and fungi) and RNAi (first observed in *Caenorhabditis elegans* and *Drosophila*) were identified independently, genetic and biochemical analyses suggest a strong evolutionary link between these multi-step pathways [1–3]. These pathways share some common elements, including that they are triggered by double stranded RNA (dsRNA) and that the trigger dsRNA is enzymatically processed into small fragments of 20 to 25 nucleotides (nts) in length – the fragments appear to mediate the sequence-specific recognition of the target single stranded RNA (ssRNA) [4,5]. The enzyme responsible for the cleavage of the triggering dsRNA has been identified as a dsRNA-specific RNase, called Dicer, that generates dsRNA breakdown products resembling those generated by RNase III cleavage with a 5′ phosphate group, a 3′ hydroxyl group and two or three nt 3′ overhangs [6]. Evidence that these small dsRNAs or short interfering RNAs (siRNAs) act as guides for the enzymatic complex required for the degradation of the target ssRNA includes the cleavage of the target mRNA at regular intervals of ~21–23 nts in the region corresponding to the input dsRNA [5]. Dicer, which also has a potential helicase activity and an additional domain of undetermined function, has been shown to be essential for RNAi in *Drosophila* and *C. elegans* [6,7]. In addition, Dicer has a role in the processing of transcripts required for normal development [8,9]. So far, only one protein component of the multi-subunit complex or RNA-induced silencing complex (RISC) required for the cleavage of the target RNA has been identified. This protein, Argonaute2, is a member of a large family of proteins involved in germ cell and stem cell production that contain conserved domains known as PAZ and PIWI domains [10]. Several genes with PAZ or PIWI domains have been linked with RNAi, including Dicer but, as yet, it is unclear how most of these proteins are involved in PTGS and RNAi [11]. Figure 1 shows a model of some aspects of dsRNA mediated gene silencing. In some species, there is evidence for the role of a RNA-dependent RNA polymerase in PTGS and RNAi [12,13], processes that induce systemic spread of the gene silencing

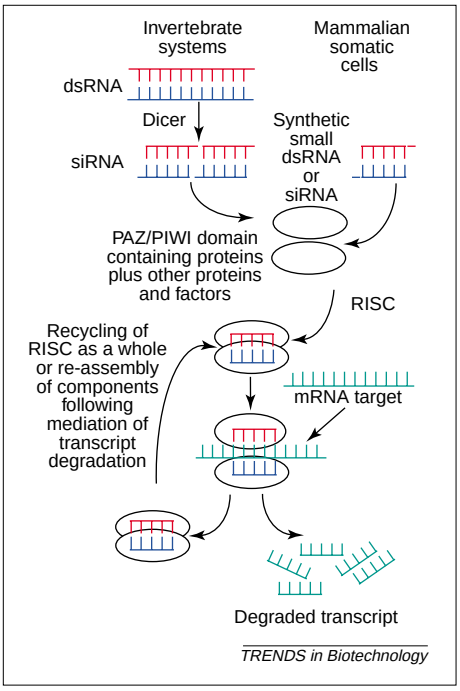

Fig. 1. A model of parts of the proposed RNAi pathway in invertebrate and mammalian somatic cells. Processed or synthesized dsRNAs of ~20–23 nucleotides interact with several proteins and factors to generate a complex that mediates sequence-specific degradation of the target transcript. It is currently unclear as to what extent the final structure of the dsRNAs that mediate optimal inhibition in somatic mammalian cells will resemble the naturally processed siRNAs (short interfering RNAs) identified in plants and invertebrates and so these structures have been referred to as both small dsRNAs and siRNAs. Abbreviation: RISC, RNA interfering silencing complex.

 0167-7799/02/$ – see front matter PII: S0167-7799(01)01900-X

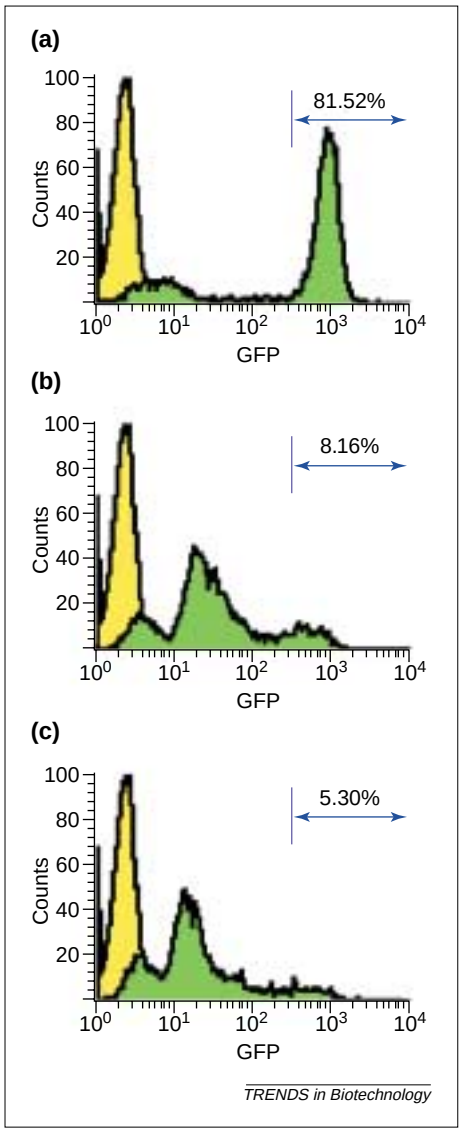

Fig. 2. Small dsRNA mediated inhibition of gene expression in human cells. (a–c) Representative histograms of fluorescent activated cell sorting (FACS) analysis (FacsCaliber, Becton Dickinson). HeLa cells stably expressing a destabilized version of enhanced green fluorescent protein (d2EGFP) with a half-life of 2 hours (HeLa/d2EGFP cells) transfected with (a) 22 nucleotide [nt] dsRNA corresponding to a portion of the *LacZ* gene (sense 5′ PO_4 r(GGUGGCGCUGGAUGGUAAGCCG) 3′ OH, antisense 5′ PO_4 r(GCUUACCAUCCAGCGCCACCAU) 3′ OH), (b) 20 nt *egfp* dsRNA (5′ PO_4 r(CAUCUUCUUCAAGGACGACG) 3′ OH, antisense 5′ PO_4 r(UCGUCCUUGAAGAAGAUGGU) 3′ OH) and (c) 22 nt *egfp* dsRNA (sense 5′ PO_4 r(GCAAGCUGACCCUGAAGUUCAU) 3′ OH, antisense 5′ PO_4 r(GAACUUCAGGGUCAGCUUGCCG) 3′ OH). All cells were transfected with 2 μg dsRNA using a cationic lipid carrier (Lipofectin; Invitrogen, Gaithersburg, MD, USA) and were assayed by FACS analysis 48 hours after transfection. For FACS analysis HeLa cells transfected with 2 μg *egfp* 22nt dsRNA were used as a control for non-GFP expressing cells (shown in yellow in each histogram), 10 000 non-gated events were acquired. Abbreviation: GFP, green fluorescent protein.

effect [3] and links with mechanisms that generate long-term maintenance of gene silencing, for example, DNA methylation in plants [14].

dsRNA triggered gene silencing in somatic mammalian cells

dsRNA-triggered gene silencing has been identified in a wide range of species of plants, fungi and invertebrates but the demonstration of an RNAi-like response in somatic mammalian cells has been hampered by the presence of several dsRNA-triggered pathways that mediate non-specific effects on gene-expression in vertebrate cells [15]. The two best-defined mammalian pathways activated by dsRNA are (1) the dsRNA-dependent protein kinase (PKR) and interferon pathway, which causes a generalized suppression of protein synthesis and apoptosis, and (2) the dsRNA-induced synthesis of 2′–5′ polyadenylic acid, which leads to the activation of a non-specific RNase, RNaseL. However, PKR is not activated by dsRNA of less than 30 nts, and thus we, and other groups, have recently examined whether small synthetic dsRNAs or siRNAs could avoid this non-specific pathway and trigger a sequence-specific inhibition of gene expression in mammalian cells [16,17].

In these first studies, the principal method used was the co-transfection (using lipofection) of plasmids expressing marker genes and synthetic dsRNAs corresponding to the target marker gene or control sequences. The small dsRNAs (21–23 nts) tested in these two studies were broadly similar to each other and consisted of chemically synthesized and annealed single stranded sense and antisense RNAs with 2 nt 3′ overhangs. Several somatic mammalian cell lines were tested including murine, non-human primate and human-derived cells. All these cell lines showed evidence for sequence-specific inhibition of the target gene when the appropriate small dsRNA was transfected. In addition, small dsRNAs (21–23 nts) did not activate PKR *in vitro* [17]. Elbashir and colleagues extended their study to investigate the ability of dsRNA to down-regulate the expression of an endogenously expressed protein. Double stranded RNAs corresponding to the nuclear envelope protein lamin A/C induced an estimated 90% reduction in protein levels 40–45 hours after initiation of transfection [16]. Figure 2 shows an example of the decrease in gene expression induced by small dsRNAs in a human cell line.

RNAi as a reverse genetics tool and its potential as a therapeutic approach

For a long time, the inhibition of the expression of a particular gene has been an important means of establishing the function of a gene. With the identification of RNAi, biologists have a new technology with which to achieve this. Exploitation of RNAi as a reverse genetics tool has required the development of slightly different methods for each of the organisms under study, principally because different techniques are required for the transfer of dsRNA. In *C. elegans*, in which RNAi has been most widely adapted as a reverse genetics tool, the dsRNA is usually administered using one of three methods: (1) direct injection into the gonads of worms, (2) soaking worms in a solution containing dsRNA, or (3) feeding worms with bacteria carrying plasmids that express either complementary ssRNA transcript or an inverted repeat hairpin structure. In the past 18 months, the direct injection of dsRNA into *C. elegans* has been used to establish or confirm the function of numerous genes. However, perhaps more importantly, RNAi in *C. elegans* has been used to assign phenotypic effects to many genes that were previously only identified as computer-predicted open reading frames [18,19].

The direct injection of dsRNA into *Drosophila* embryos has been used to study the function of several genes expressed during development and to achieve stable silencing in adult flies strategies based on an integrative structure expressing an inverted repeat-hairpin structure have been developed [20]. It is probably too early to predict how widely RNAi will be used in vertebrate cells because it is unclear whether all mammalian cell types can support RNAi and work is still required to determine the key parameters that will generate consistent RNAi against any given RNA target. However, two reports using small dsRNA triggered RNAi in human 293 cells to determine gene function have been published recently [21,22].

A therapeutic approach to many diseases could be to inhibit the production of proteins involved in initiation or progression of disease, for example proteins involved in virus or parasite–host interactions, pathogen replication, oncogenesis or cell toxicity. Many invertebrate species are important

vectors or mediators of disease. RNAi occurs in the protozoan parasite *Trypanosoma brucei* where it is being actively used as a reverse genetics tool [23] but there is also the potential that the same strategy could be adapted to down-regulate genes involved in the replication and/or maturation of this organism so blocking its natural life cycle. In mammalian cells we have used small dsRNA to rescue the cellular toxicity induced by plasmids expressing transcripts encoding an expanded polyglutamine tract, a defect associated with several dominant genetic disorders [24].

These studies only hint at the ways that dsRNA-triggered gene silencing could be used to block gene expression, but as our understanding of PTGS and RNAi improves it is probable that methods that use these pathways will prove to be versatile reverse genetics tools in a wide range of species and potentially a novel means of treating disease.

References

1 Matzke, M.A. *et al.* (2001) RNA-based silencing strategies in plants. *Curr. Opin. Genet. Dev.* 11, 221–227
2 Cogoni, C. (2001) Homology-dependent gene silencing mechanisms in fungi. *Annu. Rev. Microbiol.* 55, 381–406
3 Fire, A. *et al.* (1998) Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. *Nature* 391, 806–811
4 Hamilton, A.J. *et al.* (1999) A species of small antisense RNA in posttranscriptional gene silencing in plants. *Science* 286, 950–952
5 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
6 Bernstein, E. *et al.* (2001) Role for a bidentate ribonuclease in the initiation step of RNA interference. *Nature* 409, 363–366
7 Knight, S.W. *et al.* (2001) A role for the RNase III enzyme DCR-1 in RNA interference and germ line development in *C. elegans*. *Science* 293, 2269–2271
8 Grishok, A. *et al.* (2001) Genes and mechanisms related to RNA interference regulate expression of the small temporal RNAs that control *C. elegans* developmental timing. *Cell* 106, 23–34
9 Hutvagner, G. *et al.* (2001) A cellular function for the RNA-interference enzyme Dicer in the maturation of the let-7 small temporal RNA. *Science* 293, 834–838
10 Hammond, S.M. *et al.* (2001) Argonaute2, a link between genetic and biochemical analyses of RNAi. *Science* 293, 1146–1150
11 Cerutti, L. *et al.* (2000) Domains in gene silencing and cell differentiation proteins: the novel PAZ domain and redefinition of the Piwi domain. *Trends Biochem. Sci.* 25, 481–482
12 Cogoni, C. *et al.* (1999) Gene silencing in *Neurospora crassa* requires a protein homologous to RNA-dependent RNA polymerase. *Nature* 399, 166–169
13 Smardon, A. *et al.* (2000) EGO-1 is related to RNA-directed RNA polymerase and functions in germ-line development and RNA interference in *C. elegans*. *Curr. Biol.* 10, 169–178
14 Morel, J. *et al.* (2000) DNA methylation and chromatin structure affect transcriptional and post-transcriptional transgene silencing in *Arabidopsis*. *Curr. Biol.* 10, 1591–1594
15 Caplen, N.J. *et al.* (2000) dsRNA-mediated gene silencing in cultured *Drosophila* cells: a tissue culture model for the analysis of RNA interference. *Gene* 252, 95–105
16 Elbashir, S.M. *et al.* (2001) Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. *Nature* 411, 494–498
17 Caplen, N.J. *et al.* (2001) Specific inhibition of gene expression by small double-stranded RNAs in invertebrate and vertebrate systems. *Proc. Natl. Acad. Sci. U. S. A.* 98, 9742–9747
18 Fraser, A.G. *et al.* (2000) Functional genomic analysis of *C. elegans* chromosome I by systematic RNA interference. *Nature* 408, 325–330
19 Gonczy, P. *et al.* (2000) Functional genomic analysis of cell division in *C. elegans* using RNAi of genes on chromosome III. *Nature* 408, 331–336
20 Kennerdell, J.R. *et al.* (2000) Heritable gene silencing in *Drosophila* using double-stranded RNA. *Nat. Biotechnol.* 18, 896–898
21 Koepp, D.M. *et al.* (2001) Phosphorylation-dependent ubiquitination of cyclin E by the SCFFbw7 ubiquitin ligase. *Science* 294, 173–177
22 Garrus, J.E. *et al.* (2001) Tsg101 and the vacuolar protein sorting pathway are essential for HIV-1 budding. *Cell* 107, 55–65
23 Ngo, H. *et al.* (1998) Double-stranded RNA induces mRNA degradation in *Trypanosoma brucei*. *Proc. Natl. Acad. Sci. U. S. A.* 95, 14687–14692
24 Caplen, N.J. *et al.* Rescue of polyglutamine mediated cytotoxicity by double-stranded RNA-mediated RNA interference. *Hum. Mol. Genet.* (in press)

Natasha J. Caplen
Medical Genetics Branch, National Human Genome Research Institute, National Institutes of Health, 10 Center Drive, 10C103, Bethesda, MD 20892 USA.
e-mail: ncaplen@nhgri.nih.gov

Meeting Report

Genes, technology and public dialogue in Tartu, Estonia

Andres Metspalu

The Gene Technology Forum 2001 was held in Tartu, Estonia, 13–15 September 2001.

The success of the implementation of gene technologies in improving everyday life, including healthcare and food production, depends on one single and major issue – whether the general public will love (or hate) it. The key to this issue is to continue the dialog between the scientific community and the general public. Advances in human genetics and pharmacogenetics, directed either to gene discovery or drug trials, straightforwardly depend on large-scale population-based studies. Estonia has launched the Estonian Genome Project (see http://www.genomics.ee) with the major goal of creating the largest health database, and including genetic data of the participating individuals. As a part of public dialog and education devoted to human genetics and the development of gene technologies, Tartu has been hosting international meetings since 1999.

This year, about 400 medical doctors, scientists, students and specialists with diverse backgrounds gathered and the meeting started with a minute of silence to remember those who perished in the World Trade Center and the Pentagon.

The keynote speaker of the conference, Klaus Lindpaintner (Roche Genetics, the genetics division of F. Hoffmann-La Roche AG, Basel, Switzerland) offered his interpretation of genomics- and genetics-based healthcare. Elaborating on the increasing importance of genetics in healthcare, he explained how individualized, more efficient medicines developed on the basis of gene technology will help to reduce healthcare costs and how genome research will permit the prevention of diseases earlier and on a larger scale than at present. He emphasized that we should handle genetics as any other big advance in medicine and not mystify it – genomic approaches are not going to change the paradigm of how medicine is or will be practiced, they will just provide new tools

 0167-7799/02/$ – see front matter PII: S0167-7799(01)01883-2