


Nobelförsamlingen

The Nobel Assembly at Karolinska Institutet



RNA INTERFERENCE

This year's Nobel Prize in Physiology or Medicine is shared by Professor Andrew Z. Fire at Stanford University, California, USA, and Professor Craig C. Mello at the University of Massachusetts Medical School in Worcester, USA. They receive the prize for their discovery that double-stranded RNA triggers suppression of gene activity in a homology-dependent manner, a process named RNA interference (RNAi). Their discovery revealed a new mechanism for gene regulation, and the biochemical machinery involved plays a key role in many essential cellular processes. Double-stranded RNA synthesized within the cell can reduce or abolish gene activity by RNAi-like mechanisms. This control system for gene expression has proven to be important for both the development of an organism and the physiological functions of cells and tissues. Furthermore, RNAi protects against RNA virus infections, especially in plants and invertebrate animals, and secures genome stability by keeping mobile elements silent. Today, double-stranded RNA is used as a powerful tool to experimentally elucidate the function of essentially any gene in a cell. The discovery of RNAi has already had an immense impact on biomedical research and will most likely lead to novel medical applications in the future.

Introduction

The gene expression process is of fundamental importance for all living organisms. Most genes reside in the chromosomes located in the cell nucleus and express themselves via proteins synthesised in the cytoplasm. The genetic material was identified as deoxyribonucleic acid (DNA) in 1944 (ref. 1) and the double-helical nature of DNA was revealed in 1953 (by Francis Crick, James Watson and Maurice Wilkins; Nobel Prize in Physiology or Medicine in 1962). At that time, the main problem outstanding was how DNA in the cell nucleus could govern protein synthesis in the cytoplasm. It was proposed that another nucleic acid, single-stranded ribonucleic acid (RNA), acts as an intermediary in the process, and the so-called Central Dogma was formulated, i.e. the idea that the genetic information is transcribed from DNA to RNA and then translated from RNA into protein. The RNA carrying the genetic information was first believed to be the RNA in ribosomes; for several years the hypothesis was formulated as "one gene-one ribosome-one protein". In 1961, Francois Jacob and Jacques Monod presented a visionary gene control model, for which they received the Nobel Prize in Physiology or Medicine in 1965 together with André Lwoff. In their model, they proposed that the gene is transcribed into a specific RNA species, messenger RNA (mRNA). Soon afterwards it was demonstrated that a short-lived, non-ribosomal RNA directs the synthesis of proteins. Subsequently, Marshall Nirenberg and Gobind Khorana broke the genetic code and could assign code words (codons; triplets of nucleotides) to the twenty amino acids (they received the Nobel Prize in Physiology or Medicine in 1968 together with Robert Holley). Francis Crick predicted that an RNA molecule could act as an adaptor between mRNA and the amino acid, and a short, stable RNA, transfer RNA (tRNA) was soon identified as the predicted adaptor.

For many years, messenger RNA was believed to correspond to an uninterrupted nucleotide sequence in the DNA. It therefore came as a complete surprise when Phillip Sharp and Richard Roberts showed in 1977 that the mRNA sequence could be distributed discontinuously in the genome (the split gene concept; Nobel Prize in 1993). It was known that long RNA molecules (pre-mRNA, heterogeneous nuclear RNA) are trimmed to much shorter mature mRNAs, and Sharp and Roberts therefore suggested that the mRNA sequences, the exons, are likely to be cut out from the primary transcript and spliced, while the intervening sequences, the introns, are degraded. It was immediately realised that the discontinuous arrangement of the mRNA sequences in DNA had important evolutionary implications. In addition, the RNA splicing process could generate different mRNAs, and more than one protein can emanate from the primary transcript (alternative splicing).

The discovery that RNA can act as a catalyst gave a radically new perspective on the roles of RNA (Nobel Prize in Chemistry to Sidney Altman and Thomas Cech in 1989). It was soon revealed that RNA is able to catalyse its own replication and the synthesis of other RNA molecules (the ribozyme concept), which led to the idea that RNA was the first genetic material on earth. An “RNA world” is believed to have existed before DNA took over the role of being the key genetic material, and RNA was relegated to the role of messenger between DNA and protein. Not only did the discovery of catalytic RNA have evolutionary implications, but it also suggested that RNA could play a more active role in gene expression than earlier realised. It is now well established that ribosomal RNA catalyses peptide bond formation during translation.

A large number of small RNA molecules work in conjunction with proteins in ribonucleoprotein (RNP) complexes. There are non-coding RNA molecules that affect transcription (e.g. human 7SK snRNA bound to elongation factors), translation (e.g. SRP RNA in the signal recognition particle), replication (e.g. telomerase RNA) and chromosome structure (e.g. XIST RNA causing X chromosome inactivation). Others regulate RNA processing (e.g. M1 RNA in RNase P, snRNAs and snoRNAs) and RNA editing (guide RNAs). These various RNP particles are now being extensively investigated to understand their specific roles in the cell.

In the early 1980s it was revealed in *Escherichia coli* that small RNA molecules (about 100 nucleotides in length) can bind to a complementary sequence in mRNA and inhibit translation^{2,3}. Today, about 25 cases of regulatory trans-acting antisense RNAs are known in *E. coli*⁴. Regulation of translation by antisense RNA also occurs in eukaryotes as was first demonstrated in 1993 when genes governing the development of the worm *Caenorhabditis elegans* were studied^{5,6}. For many years, this thoroughly documented case of posttranscriptional regulation was regarded as an oddity. The mechanism received more attention when a second example of a small regulatory RNA was found in *C. elegans*⁷, because in this case similar sequences were also present in other species. However, the situation changed drastically when a large number of small RNA molecules, called microRNAs (miRNAs), were revealed in 2001 (ref. 8-10).

Prior to the discovery of RNA interference, a phenomenon called gene (or RNA) silencing was described in plants. It was noted in experiments around 1990 that a cloned gene incorporated into the genome (a transgene) could not only induce or stimulate gene activity but could also inhibit the expression of homologous sequences, a phenomenon called homology-dependent gene silencing. The inhibition of gene activity could take place at the transcriptional level (transcriptional gene silencing, TGS)¹¹⁻¹³, or at the posttranscriptional level (posttranscriptional gene silencing, PTGS)¹⁴⁻¹⁸. A PTGS-like process called quelling was also established in the fungus *Neurospora crassa*¹⁹. Analyses of viral infection in plants gave further insight into the mechanism of PTGS²⁰⁻²¹. However, although it was evident that RNA played a key role in gene silencing, the phenomenon remained enigmatic until the discovery of RNA interference provided a most unexpected explanation with many profound consequences.

The discovery of RNA interference

Andrew Fire and Craig Mello published their break-through study on the mechanism of RNA interference in *Nature* in 1998 (ref. 22). It was earlier known that antisense RNA²³, but remarkably also sense RNA²⁴ could silence genes, but the results were inconsistent and the effects usually modest. However, due to the fact that both sense and antisense RNA could cause silencing Mello argued that the mechanism could not just be a pairing of antisense RNA to mRNA, and he coined the term RNA interference for the unknown mechanism²⁵.

In their *Nature* paper, Fire and Mello tested the phenotypic effect of RNA injected into the worm *C. elegans*. They established that annealed sense/antisense RNA, but neither antisense nor sense RNA alone, caused the predicted phenotype (Fig. 1). Furthermore, only injection of double-stranded RNA (dsRNA) led to an efficient loss of the target mRNA (Fig. 2). Fire and Mello could present a series of straightforward conclusions in their study. The main results can be summed up as follows: First, silencing was triggered efficiently by injected dsRNA, but weakly or not at all by sense or antisense single-stranded RNAs. Second, silencing was specific for an mRNA homologous to the dsRNA; other mRNAs were unaffected. Third, the dsRNA had to correspond to the mature mRNA sequence; neither intron nor promoter sequences triggered a response. This indicated a posttranscriptional, presumably cytoplasmic mechanism. Fourth, the targeted mRNA disappeared suggesting that it was degraded. Fifth, only a few dsRNA molecules per cell were sufficient to accomplish full silencing. This indicated that the dsRNA was amplified and/or acted catalytically rather than stoichiometrically. Sixth, the dsRNA effect could spread between tissues and even to the progeny, suggesting a transmission of the effect between cells. Furthermore, Fire and Mello made the remark that RNAi could provide an explanation for a phenomenon studied in plants for several years: posttranscriptional gene silencing (PTGS). Finally, they ended their paper by speculating about the possibility that “dsRNA could be used by the organism for physiological gene silencing”.

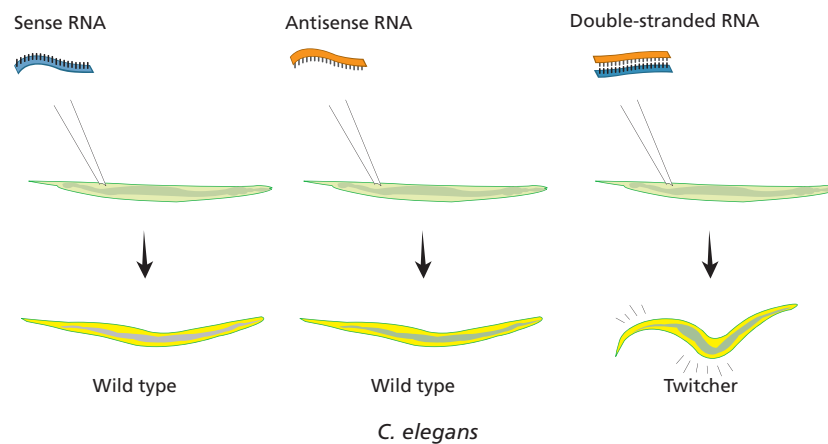


Figure 1. Phenotypic effect after injection of single-stranded or double-stranded *unc-22* RNA into the gonad of *C. elegans*. The *unc-22* gene encodes a myofilament protein. Decrease in *unc-22* activity is known to produce severe twitching movements. Injected double-stranded RNA, but not single-stranded RNA, induced the twitching phenotype in the progeny.

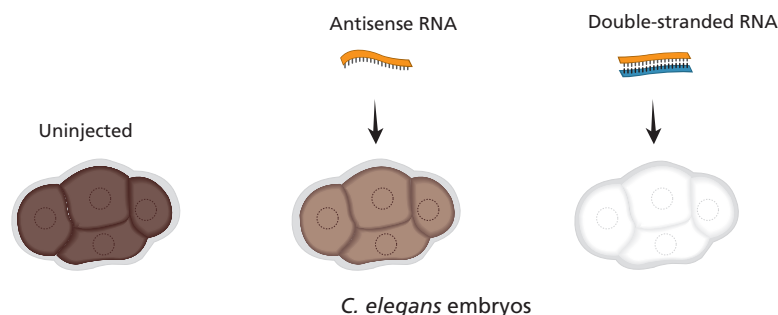


Figure 2. The effect on *mex-3* mRNA content in embryos after injection of single-stranded or double-stranded *mex-3* RNA into the gonad of *C. elegans*. *mex-3* mRNA is abundant in the gonads and early embryos. The mRNA was lost after injection of double-stranded RNA, while injection of antisense RNA only reduced the content of mRNA to some extent. The extent of brown colour reflects the amount of mRNA present.

In their *Nature* paper, Fire and Mello did not take a firm stand on the issue of whether dsRNA acts via a transcriptional or posttranscriptional mechanism. However, in a follow-up study published in *PNAS* the same year, Fire provided good evidence for the view that mRNA is the target for dsRNA (recognition via complementary strands), and that the targeted mRNA is degraded prior to translation, i.e. dsRNA exerts its effect at the posttranscriptional level²⁶. He also presented a specific model showing how *double-stranded* RNA could function in a *catalytic* manner to target homologous mRNAs for degradation. This model differed strikingly from the simple antisense model of that time, which only predicted interaction between an interfering single-stranded RNA molecule and mRNA. It can be added that in this *PNAS* paper, Fire also indicated the possibility that the RNAi mechanism could be a specific “tactical” approach to viral defence in lower organisms (to be compared with the global interferon response in mammals).

Within a year, the presence of RNAi had been documented in many other organisms, including fruit flies, trypanosomes, plants, planaria, hydra and zebrafish²⁷. In initial experiments with mammalian cultured cells, it was not possible to elicit a potent and specific RNAi response because of a predominant nonspecific physiological reaction of these cells to long dsRNA. However, when the cells were exposed to a short, 21 nucleotide long, dsRNA, an efficient targeted silencing was also obtained in these cells²⁸. Thus, the generality of the RNAi phenomenon among eukaryotes was proven very rapidly; a remarkable exception is the budding yeast, *Saccharomyces cerevisiae*.

The revelation of the RNAi mechanism

Shortly after the discovery of RNAi, it was shown that PTGS in plants is correlated to the presence of a population of small RNAs (about 25 nucleotides long), and that this RNA contains both sense and antisense RNA sequences²⁹. It was proposed that this RNA is the determinant of PTGS. Earlier, but subsequent to the discovery of RNAi in animal cells, it had been shown that RNA in double-stranded form also initiates PTGS in plants³⁰.

The biochemistry of RNAi was further elucidated in an *in vitro* system based on *Drosophila* embryo extracts²⁷. It could be shown that dsRNA is processed to 21-23 nucleotide long dsRNA fragments³¹, which was in good agreement with the data for PTGS in plants²⁹. It was proposed that this short dsRNA, siRNA (small interfering RNA), guided the cleavage of mRNA. Subsequently, Fire and Mello were able to follow the process *in vivo*³². They established that long dsRNA is cleaved to small RNA (about 25 nucleotides long), and that antisense RNA triggers degradation of mRNA via base-pairing to mRNA. Thus, the trigger, dsRNA, had then been connected to the low molecular weight effector.

The molecular machinery involved in RNAi was subsequently revealed (Fig. 3). In an *in vitro* system, built on *Drosophila* cultured cells, it was demonstrated that a large complex, called RISC (RNA-induced silencing complex) is targeted to the mRNA via a short antisense RNA, and that mRNA is cleaved and subsequently degraded³³. Subsequently, it has been shown that RISC contains at least one member of the argonaute protein family, which is likely to act as an endonuclease and cut the mRNA (nowadays often referred to as the Slicer function). It was also demonstrated that a ribonuclease III-like nuclease, called Dicer, is responsible for the processing of dsRNA to short RNA³⁴. In certain systems, in particular plants, worms and fungi, an RNA dependent RNA polymerase (RdRP) plays an important role in generating and/or amplifying siRNA³⁵.

Thus, in just a few years a vast amount of information accumulated on the specific proteins and protein complexes involved in RNAi and on the molecular details of the particular steps in the process³⁶⁻³⁹.

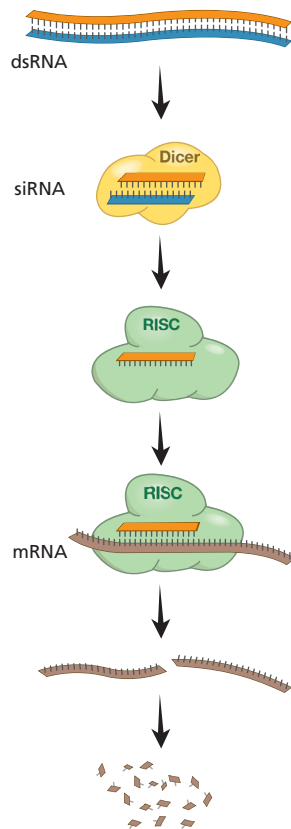


Figure 3. The RNA interference process and the biochemical machinery involved. Double-stranded RNA is cut into short pieces (siRNA) by the endonuclease Dicer. The antisense strand is loaded into the RISC complex and links the complex to the mRNA strand by base-pairing. The RISC complex cuts the mRNA strand, and the mRNA is subsequently degraded.

Significance of the discovery of RNAi

It was evident from the very beginning that the significance of the discovery of RNAi would be exceptional. The far-reaching consequences of the discovery can be summed up as follows (Fig. 4):

1. RNAi protects against viral infections: The finding of Fire and Mello that cells can process injected dsRNA and eliminate homologous single-stranded RNA suggested that RNAi could constitute a defence mechanism against viral attacks. It had earlier been shown that plant cells have an efficient defence against viruses based on the PTGS phenomenon^{40,41}. When it became apparent that PTGS is the plant equivalent to RNAi, this early work in plants supported the proposition that RNAi is involved in protecting cells from viral attacks. Today, we know that this anti-viral mechanism is at work in plants, worms and flies, whereas it is still unclear how relevant it is for vertebrates, including man.

2. RNAi secures genome stability by keeping mobile elements silent: It was proposed early on that RNAi/PTGS in *C. elegans* and plants could block the action of transposons (mobile elements in the genome). Subsequently, it could be shown that when components of the RNAi machinery are mutated in *C. elegans*, transposons are activated and the mobile elements cause disturbances in the function of the genome^{42,43}. It has been proposed that in transposon-containing regions of the genome both DNA strands are transcribed, dsRNA is formed, and the RNAi process eliminates these undesirable products. As short dsRNAs can also operate directly on chromatin and suppress transcription (see below), this would be another mode to keep transposons inactive (see below under 4). Even if the mechanisms are not yet fully revealed, it is clear that if the RNAi machinery is not efficient, the transposons are not kept under control and can start to jump and cause deleterious effects in the genome.

soon revealed in several organisms⁴⁶. The small miRNAs are processed from larger hairpin-like precursors by an RNAi-like machinery^{47,48} (Fig. 4). The miRNAs can regulate gene expression by base-pairing to mRNA, which results in either degradation of the mRNA or suppression of translation. Today, it is estimated that there are about 500 miRNAs in mammalian cells, and that about 30% of all genes are regulated by miRNAs. It is known that miRNAs play an important role during development in plants, *C. elegans* and mammals. Thus, the miRNA-dependent control of gene expression represents a new major principle of gene regulation. However, the full significance of small regulatory RNAs is probably still not apparent.

4. RNAi-like mechanisms keep chromatin condensed and suppress transcription: It was known from work in plants that gene silencing could take place at the transcriptional level (TGS). After the discovery of RNAi, it was soon shown that TGS in plants operates via RNAi-like mechanisms^{49,50}. In the fission yeast *Schizosaccharomyces pombe*^{51,52}, and later on in *Drosophila* and vertebrates, it was found that similar processes keep heterochromatic regions condensed and transcriptionally suppressed. In addition, the RNAi-like machinery regulates the activity of genes in the immediate vicinity of the condensed blocks of chromatin. The phenomenon is still not understood at the molecular level although histone modifications, binding of specific chromatin condensing proteins (HP1), and DNA methylation all play important roles⁴⁶. It is, however, evident that this action on chromatin is most important for proper functioning of the genome and for maintenance of genome integrity.

5. RNAi offers a new experimental tool to repress genes specifically. The targeted action of RNAi immediately suggested that this phenomenon could be utilized as a general method to suppress specific genes and look for the resulting phenotypic effect. It was also soon evident that this could be accomplished in such an efficient manner that essentially any gene in an organism could be studied functionally. After the initial work in *C. elegans*, this technical approach could be applied to cells from almost all organisms, including mammalian cells. This targeted gene silencing by RNAi has already had a tremendous impact in studies of the function of individual genes. It is now exploited not only in cultured cells but also in transgenic organisms. DNA constructs are introduced into the organisms under appropriate promoter control, and dsRNA hairpin structures are produced and further processed to achieve specific effects on gene regulation.

6. RNAi might be a useful approach in future gene therapy. The possibility to achieve RNAi-governed gene regulation in transgenic organisms has stimulated many explorations of whether this would be a useful option for medical therapy^{53,54}. Promising results have been reported in several animal models⁵⁵⁻⁵⁸ and even in recent clinical trials, but it is too early to predict the outcome of these challenging efforts.

Conclusions

The discovery that cells have a special mechanism for suppressing the expression of homologous genes by recognizing and processing double-stranded RNA was totally unexpected and has dramatically expanded our knowledge of gene control. Remarkably, the RNAi machinery can handle double-stranded RNA entering the cell as well as double-stranded RNA generated within the cell. The development of an organism and proper function of its cells and tissues are dependent on an intact RNAi machinery. Infection by RNA viruses can be blocked by RNAi, especially in plants and lower animals, and foreign elements in the genome (viruses and transposons) can be kept silent. Finally, the discovery of RNAi has not only provided us with a powerful new experimental tool to study the function of genes but also raises expectations about future applications of RNAi in medicine.

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