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RNA Interference: A New Way to Analyze Protein Function

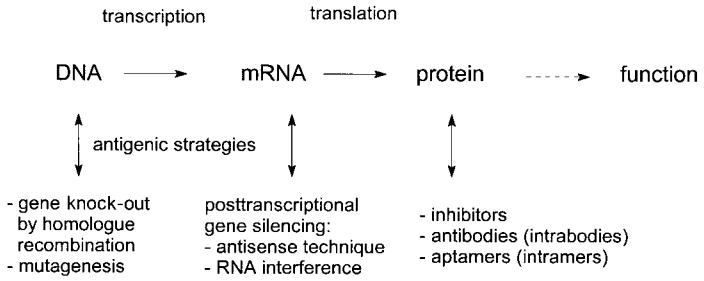
Ute Schepers and Thomas Kolter*

Introduction

Within the last few years a tremendous amount of work has been done to identify the genetic background of different species. Even the human genome has been sequenced. However, the data obtained create a need for additional information that will allow us to understand the determinants of biochemical, biological, and pharmacological processes. In particular, more information is needed to enable us to assign functions to the gene products, mostly proteins, encoded by the genome of a species (functional genomics). Recently, a novel technique has been described which promotes a much faster and simplified analysis of protein function. This technique uses gene-specific double-stranded RNA (dsRNA) to disrupt gene expression at the level of messenger RNA (mRNA) in tissue culture and whole organisms (Scheme 1).[1]

Function of Proteins

Researchers have been looking for methods to study the function of proteins for a long time. In the past various experimental procedures have been used but have often turned out to be very laborious and time consuming. A common method to investigate the function of proteins and other biological molecules is the knock-out experiment.



Scheme 1. Examples of methods for the disruption of protein function.

For this purpose, the phenotype of tissue cultures or organisms that lack a specific protein due to mutations or alterations is analyzed. In humans, inherited diseases were used as models for these knock-out experiments.

One of the most promising new techniques for the study of protein function seems to be the RNA interference (RNAi) method. It is based on the degradation of selected mRNA by administration of double-stranded RNA. RNAi offers strong advantages over other antigenic strategies[2] such as gene knock-out by homologue recombination, antisense oligonucleotides, or ribozymes.[3]

History

RNAi was discovered in 1995 in the nematode *Caenorhabditis elegans*, a model organism for biological experiments,[4] when researchers attempted to use the antisense RNA approach to inactivate the expression of a single gene. *C. elegans* was injected with RNA complementary to a target

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mRNA (antisense RNA), in order to capture this mRNA and to prevent the production of the encoded protein. Surprisingly, simultaneous injection of sense and antisense RNA was much more effective in suppression of specific gene expression than antisense RNA alone.[1] This finding was the basis for a simple method of disabling the expression of selected proteins, through the introduction of exogenous dsRNAs.

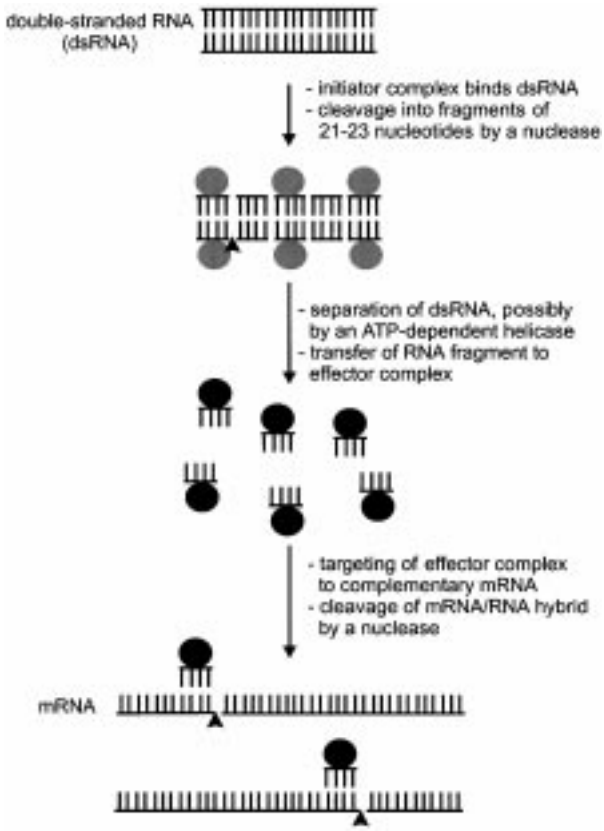
RNAi falls into the category of post-transcriptional gene silencing (PTGS). PTGS was observed in several organisms in the early 1990s and was subsequently named as "cosuppression" in plants, "quelling" in fungi, and "RNA interference" in nematodes and insects.[5, 6] It is thought to be an ancient self-defense mechanism of cells, to combat infection by RNA viruses and transposons (mobile parasitic stretches of DNA that can be inserted into the host's genome). During the process of copying themselves these viruses and transposons generate dsRNA that is recognized and degraded by the host cell. This self-defense mechanism has previously been described in a number of organisms, such as trypanosomes,[7] insects,[8, 9] plants,[10] and recently in mouse embryonic stem cells and oocytes.[11] The latter result is of considerable interest for future applications. RNAi involves this mechanism through the introduction of exogenous dsRNA. If this trigger mimics a part of the (single-stranded) host-cell mRNA sequence, the homologous mRNA will be degraded as well as the exogenous RNA.

For a long time RNAi has not been applied to mammals because these organisms were thought to have evolved a different response to RNA viruses and transposons. Extremely small amounts of dsRNA can trigger a signal cascade, part of which is the activation of dsRNA responsive protein kinase R (PKR). PKR phosphorylates and inactivates the translation factor EIF2α leading to a global suppression of protein biosynthesis and subsequently to programmed cell death (apoptosis). However, it seems that recent RNAi experiments in mouse embryonic stem cells have been successful.[11] It is possible that the interferon response has not yet developed in these early embryonic mouse cells, and there may be difficulties in using RNAi in later developmental stages. Nevertheless, there will be many efforts to develop this technique for application in differentiated mammalian cells.

Mechanism

To date, the mechanism underlying RNAi is still unclear. One current model that has been proposed is based on experiments with *C. elegans* and other species (Scheme 2). In response to substoichiometric amounts of dsRNA, levels of homologous mRNA sequences are drastically decreased within 2–3 h.[12] This degradation happens in two steps: First, exogenous dsRNA is cleaved into fragments containing 21–23 nucleotides and, second, these fragments trigger the degradation of the homologous endogenous mRNA.

Genetic screening with *C. elegans* and insects has identified some gene families required for PTGS. They encode for proteins which are assembled in complexes. These complexes contain 3′–5′ exonucleases, helicases, RNA polymerases, and RNA binding proteins. In *Drosophila* it has been recently discovered that a nuclease of the RNAse III family is involved



Scheme 2. Proposed mechanism of PTGS by RNAi (modified from ref. [12] after consideration of ref. [13]).

during initiation in the cleavage of dsRNA into fragments of 21–23 nucleotides.[12] The identity of this nuclease has been demonstrated in *Drosophila*.[13] It is thought, at least in *Neurospora*[14] and presumably also in *C. elegans*,[13] that these fragments of dsRNA will be amplified by dsRNA-dependent RNA polymerases to enhance the alarm signal in the cell. The newly generated fragments will be separated by an ATP-dependent helicase[12] and subsequently targeted to the complementary mRNA by RNA binding proteins. Further nucleases will be activated to degrade the newly created dsRNA derived from the fragment and the complementary mRNA.

With this mechanism, addition of exogenous dsRNA can be used to knock out a specific protein. Since RNAi is happening posttranscriptionally, the exogenous dsRNA has to be complementary to exon sequences of the gene that is to be knocked out. Double-stranded RNA sequences complementary to nontranscribed sequences, like introns and promoter regions, are ineffective.[1] Meanwhile, expression of a number of proteins with highly conserved (homologous) domains can be disrupted by addition of just one dsRNA encoding this region.[1, 8]

Application and Outlook

As with intracellular antibodies (intrabodies) or aptamers (intramers),[15] a remarkable feature of RNAi is the disruption of protein expression without manipulation of the genetic

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material. Compared to the generation of traditional knock-out animals by homologue recombination, the method is simple and fast.

Antisense techniques, where a 1:1 hybridization of endogenous mRNA with antisense RNA is necessary, often fail to exhibit full gene silencing, whereas the application of catalytic amounts of dsRNA promotes the complete degradation of homologous mRNA. With antisense DNA instead of antisense RNA, degradation of mRNA/DNA hybrids will be triggered by RNase H.[3]

RNAi allows the disruption of protein expression not only in tissue cultures and organisms, but also during different developmental stages of the organism. This offers a strong advantage over the competing gene knock-out method in animals, since eliminating the gene of interest is often lethal for the embryo. Although it is already possible to generate inducible and tissue-specific knock-out/transgenic animals using the Cre/loxP technique,[16] this approach is still very laborious. At present researchers are attempting to apply RNAi to mammals, which would reduce time-consuming steps like homologous recombination and site-directed mutagenesis.

An absolute requirement for RNAi is the knowledge of the nucleic acid sequence for about 200 – 1000 bases. Transient phenotypes can be created using modern transfection technology such as lipid transfer, micro-injection, gene-gun methods, or feeding to introduce dsRNA into tissue culture or organisms, as described for *C. elegans*.[1]. Thereby, simultaneous and parallel knock-out of a number of proteins offers a new way to analyze the function of either known or unknown proteins in single experiments or with high-throughput screening (*C. elegans*).[17, 18]

To generate inheritable and inducible RNAi phenotypes in tissue culture or organisms, one has to manipulate the genetic background. The resulting transgenic cell lines or organisms carry plasmids that contain a stretch of DNA encoding for about 1000 bases of the gene to be silenced in a sense and antisense orientation (inverted repeat). During transcription of the inverted repeat sequence an RNA molecule is formed that is supposed to fold back into a hairpin-like structure by intramolecular hybridization. The resulting RNA is then effectively double stranded. RNAi phenotypes can be induced in a variety of tissues as well as in different developmental stages, through the use of tissue-specific or drug-inducible promoters to express the hairpin dsRNA. Among many other advantages there is one which is of common interest. RNAi can be used to disrupt the expression of viral and parasitic proteins as a defense against pathogens. So far, it has been reported that transformation of tobacco plants with a hairpin DNA encoding a protease of the potato virus Y lead to a complete immunization against the virus.[19]

It is very impressive how many insights researchers have gained on the function of proteins using a variety of methods. Nevertheless, despite the tremendous success of knock-out or gene-silencing experiments, one has to be critical in judging data obtained in cell cultures. The absence of the appropriate multicellular surroundings or the artificial conditions can lead to discrepancies between results obtained in cell cultures and in whole organisms.[20] Even the analysis of knock-out animals can be misleading; for example, knock-out mice developed as models for severe human diseases like Tay – Sachs disease[21] or Lesch – Nyhan syndrome[20] do not show the expected phenotypes. In this respect, one also has to be concerned about the analysis of protein functions derived from RNAi experiments.

There are still plenty of mysteries concerning the mechanism and the application of RNAi but the number of researchers focussing on RNAi is growing rapidly and promises increasing developments in this field.[22]

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