1
Introduction: RNA interference, the "Breakthrough of the Year 2002"

When in 2001, with the sequencing of the human genome, the sequencing projects of many organisms reached a summit, there was no doubt among the scientific community that nothing in the near future would be as spectacular. It was called one of the biggest milestones of the 21st century, and the most important achievement in biology. However, this excitement did not last long. Already in the same year, the discovery of RNA interference (RNAi) in mammals created a similar hype, which is now experiencing explosive growth. When *Science* nominated RNAi as the “Breakthrough of the year 2002” (Couzin 2002), it was already clear that RNAi will revolutionize biomedical research during the next few years.

Although RNAi is being used mainly to unravel the functions of genes by switching them “off” at the post-transcriptional level, it offers a novel approach for disease therapy, by shutting off unwanted genetic activity in a targeted manner. It can be applied to targets ranging from rogue genes in cancer to genes of viruses, such as hepatitis B or C virus or HIV.

With the knowledge of the genome sequence of many species, RNAi can contribute to a more detailed understanding of complicated physiological processes, and also to the development of many more new drugs since it connects genomics, proteomics, and functional genomics (functionomics).

Today, RNAi is known as a common denominator for several post-transcriptional gene silencing (PTGS) processes observed in a variety of eukaryotic organisms (Hannon 2002). It is induced by double-stranded RNA (dsRNA).

It comprises phenomena such as co-suppression (Napoli et al. 1990), quelling (Cogoni and Macino 1997), and transgene-induced silencing (Baulcombe 1999), even if those processes are not completely identical. Some scientists prefer the name RNA silencing rather than RNAi, which is solely specific for invertebrates such as worms and flies or vertebrates. This book will further refer to the phenomenon as RNAi, as the majority of the published reports are using this as a general term to describe dsRNA-induced RNA silencing.

The dsRNA-induced gene-silencing effects were first discovered in plants (Napoli et al. 1990) and *Neurospora crassa* (Cogoni and Macino 1997), where they serve as an antiviral defense system. The viruses encoding for the silencing transgenes were known to produce dsRNA during their replication. However, the decisive discovery in RNA silencing was made when Andrew Fire and Craig Mello tried to explain the

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unusually high silencing activity of sense control RNA found in a previously reported antisense experiment in the worm *Caenorhabditis elegans* (Guo and Kemphues 1995). Simultaneous injection of sense and antisense RNA exhibited a tenfold stronger effect than antisense RNA alone, which led to the conclusion that dsRNA triggers an efficient silencing mechanism in which exogenous dsRNA significantly reduces the overall level of target-mRNA. (Fire et al. 1998). This newly discovered phenomenon was termed RNA interference (RNAi) (for a review, see Arenz and Schepers 2003).

1.1 RNAi as a Tool for Functional Genomics

A number of fundamental features were soon caught up by various research groups, who started to develop RNAi as a tool to study gene function and to interfere with pathogenic gene expression in diseases (Schmitz and Schepers 2004)

RNAi is highly selective upon degrading an mRNA target if the exogenously added dsRNA shares sequences of perfect homology with the target. Whereas the transcription of the gene is normal, the translation of the protein is prevented by selective degradation of its encoded mRNA. Further, it turned out that sequences with homology to introns or promoter regions as contained in the DNA sequence showed no effect at all, indicating that the silencing was taking place at the post-transcriptional level (Fire et al. 1998; Montgomery and Fire 1998; Montgomery et al. 1998).

With the full sequence of the human genome and many well-studied model organisms available, it is now possible to choose dsRNAs that selectively degrade the mRNA of a gene of interest, leading to a corresponding loss-of-function phenotype without affecting other or related genes. As a response to substoichiometric amounts of dsRNA, levels of homologous mRNA will be drastically decreased within 2–3 h. In some species, the RNAi phenotype can cross cell boundaries and is inherited to the progeny of the organism (Zamore et al. 2000). The latter observations are referred to as systemic RNAi, and are described in more detail for the nematode *C. elegans* in Chapter 2. Moreover, cultured cells transfected with dsRNA can maintain a loss-of-function phenotype for up to nine cell divisions (Tabara et al. 1998).

This disproportion between input dsRNA and its long lived-effects seen in *C. elegans* and plants (Grishok et al. 2000; Wassenegger and Pelissier 1998) suggests that the mechanism of RNAi is catalytic and does not function by titrating endogenous mRNA, as was proposed for antisense RNA.

Today, RNA silencing including RNAi is assumed to be an ancient self-defense mechanism of eukaryotic cells to combat infection by RNA viruses (Ruiz et al. 1998; Voinnet 2001) and transposons (mobile parasitic stretches of DNA that can be inserted into the host's genome) (Ketting et al. 1999; Tabara et al. 1999). The trigger for this cellular defense mechanism is dsRNA, which occurs during replication of those elements but never from tightly regulated endogenous genes. Intermediate dsRNA will be recognized and degraded by a multipart protein machinery. Furthermore, RNAi is presumed to carry out numerous additional functions in depending

on the organism. There is evidence that it eliminates defective mRNAs by degradation (Plasterk 2002), as there is overlapping activity of *C. elegans* genes for RNAi and Nonsense-mediated mRNA decay (NMD) (Domeier et al. 2000). RNAi is further assumed to tightly regulate protein levels in response to various environmental stimuli, although the extent to which this mechanism is employed by specific cell types remains to be discovered (McManus et al. 2002).

Later, the real RNAi technology arose from the observation that exogenously applied naked dsRNAs induce specific RNA silencing in plants and *C. elegans*, when the nucleotide sequence of the dsRNA is homologous to the respective mRNA.

1.2 Mechanism of RNAi

Since its discovery, much progress has been made towards the identification and characterization of the genes implicated in the RNAi events in *C. elegans* (Qiao et al. 1995; Smardon et al. 2000), *Arabidopsis* (Mourrain et al. 2000), *N. crassa* (Cogoni and Macino 1997, 1999), *Drosophila*, and mammals. Most of the important mechanistic steps and molecular components were discovered in *C. elegans*, *D. melanogaster*, and in plants. Far from being understood, RNAi has emerged as a more complex mechanism than expected, as it involves several different proteins and small RNAs. Even if it shares common features with established dsRNA-induced RNA silencing phenomena such as "co-suppression" in plants and "quelling" in fungi, it is not known if RNAi uses identical mechanisms.

In fact, genetic studies in RNA silencing-deficient mutants of *Arabidopsis* (Mourrain et al. 2000), *N. crassa* (Cogoni and Macino 1997, 1999), and *C. elegans* (Qiao et al. 1995; Smardon et al. 2000) revealed several genes involved in quelling, co-suppression and RNAi-including members of the helicase family, RNaseIII-related nucleases, members of the Argonaute family, and RNA-dependent RNA polymerases (RdRp). So far it is known that, despite all differences and similarities, the process of RNA silencing consists of an initiator step, in which long dsRNA is cleaved into short dsRNA fragments, and an effector step in which these fragments are incorporated into a protein complex, unwound and used as a guiding sequence to recognize homologous mRNA that is subsequently cleaved (Schmitz and Schepers 2004)

1.3 Dicer – the Initiator to "Dice" the dsRNA?

A common characteristic of all RNA silencing pathways initiated by dsRNA is the cleavage of long dsRNA by a double strand-specific RNase called "Dicer" (Bernstein et al. 2001). Dicer cleaves dsRNA into so-called small interfering RNA duplexes (siRNAs) encompassing a length of 21 to 25 nt (Hamilton and Baulcombe 1999; Zamore et al. 2000). Such small dsRNAs, which are complementary to both strands of the silenced gene, have been initially observed by Baulcombe and co-workers in

plants undergoing transgene- or virus-induced post-transcriptional gene silencing or co-suppression. These first experiments in plants revealed that the small dsRNAs – later termed siRNAs – are the active components of the RNA silencing pathway (Hamilton and Baulcombe 1999), leading later on to their discovery in many other species such as *Drosophila* embryos (Yang et al. 2000) and *C. elegans* (Parrish et al. 2000) that were injected with dsRNA, as well as in *Drosophila* Schneider 2 (S2) cells that were transfected with long dsRNA (Hammond et al. 2000). Surprisingly, endogenously expressed siRNAs have not been observed in mammals, indicating that there are slightly modified mechanisms for different species.

The mechanism by which these siRNAs mediate the cleavage and degradation of RNA has been thoroughly investigated by several groups. Various studies have shown that this process is restricted to the cytosol (Hutvagner and Zamore 2002 a; Kawasaki et al. 2003; Zeng and Cullen 2002) facilitating the experimental set-up. Based on these results, processing of long dsRNAs to 21–23-nt RNAs was repeated in vitro, using RNase III enzyme from *Drosophila* extract *(*cytosol).

Precise studies of these so-called "short interfering RNAs" (siRNAs) revealed characteristic 3′-overhangs of two nucleotides on both strands (Hamilton and Baulcombe 1999; Parrish et al. 2000), and unphosphorylated hydroxyl groups (Elbashir et al. 2001 b) that play a crucial role in the recognition by the other RNAi components. The specific features of siRNA resemble the characteristic cleavage pattern of nucleases of the RNase III family that specifically cuts dsRNAs (Bernstein et al. 2001; Billy et al. 2001; Robertson et al. 1968) and leaves them with staggered cuts on each side of the RNA (Zamore 2001). The RNase III family is divided into three classes, depending on their domain organization. While members of class I from bacteria and yeasts contain only one conserved RNase III domain and an adjacent dsRNA-binding domain (dsRBD), class II enzymes have tandem RNase III domains and one dsRBD, as well as an extended amino-terminal domain of unknown function (Filippov et al. 2000; Fortin et al. 2002; Lee et al. 2003).

Beside the already characterized classes of RNase III enzymes such as the regular canonical RNase III (Filippov et al. 2000) and Drosha – a member of the class II enzymes localized to the nucleus (Wu et al. 2000) – homology screens of genomic data from *Drosophila* revealed many new candidate genes carrying RNase III-like domains. Among those candidates, Hannon and colleagues (Bernstein et al. 2001) identified a nuclease with 2249 amino acids predicted from *Drosophila* sequence data containing two RNase III domains (Mian 1997; Rotondo and Frendewey 1996), a dsRNA-binding motif (DSRM) (Aravind and Koonin 2001), an amino-terminal DexH/DEAH RNA helicase/ATPase domain, and a motif called "PAZ domain" (Cerutti et al. 2000) – all properties that characterize class III of the large noncanonical ribonucleases (RNase) III family. Due to the capability of producing fragments from long dsRNA that comprise a uniform size, the newly discovered enzyme was called Dicer (Bernstein et al. 2001; Ketting et al. 2001 b). So far, it is loosely associated with ribosomes in the cytoplasm (endoplasmic reticulum–cytosol interface) (Billy et al. 2001; Provost et al. 2002).

Usually, bacterial RNase III-type enzymes cleave dsRNA by building a dimeric structure comprising two active centers that embrace a cleft in the protein structure

that can accommodate a dsRNA substrate. The presence of divalent cations, including Mg^{2+}, has significant impact on crystal packing, intermolecular interactions, thermal stability, and the formation of two RNA-cuffing sites within each active center for catalysis (Blaszczyk et al. 2001; Zamore et al. 2000). Modeling and comparison of a RNase III structure led to the proposal of a working model of Dicer. The first assumption was that the enzyme presumably aggregates as an anti-parallel homodimer, in which only two of four catalytic actives sites are involved in dsRNA cleavage leading to ~22-mers, whereas the activity of all four sites would lead to the production of 11-mers (Blaszczyk et al. 2001). The central pair of active sites should be then replaced by a noncanonical motif making it inactive, whereas the 5′- and 3′-site remains active (Blaszczyk et al. 2001). Another working model proposes a monomeric action of Dicer in a semireciprocal fashion, cleaving the dsRNA during translocation of the enzyme down its substrate (Bernstein et al. 2001). Thus, the helicase/ATPase domain of Dicer is supposed to either induce structural rearrangement of the dsRNA template or to drive movement of the enzyme along the dsRNA in an ATP-dependent manner (Bernstein et al. 2001; Hutvagner et al. 2001; Ketting et al. 2001 b; Myers et al. 2003; Provost et al. 2002; Zhang et al. 2002). The unwinding of such an RNA-duplex by a helicase homologue would require at least a temporary energy-consuming step as a prerequisite. Studies performed with *Drosophila* and *C. elegans* Dicer indicated that generation of siRNAs from dsRNA is ATP-dependent (Bernstein et al. 2001; Ketting et al. 2001 a; Nykänen et al. 2001; Zamore et al. 2000). It has been shown that Dicer from enriched fractions of *Drosophila* extracts could be inactivated by ATP withdrawal (Zamore et al. 2000). However, experiments with purified recombinant human Dicer recently showed that Dicer is preferentially cleaving dsRNAs at their termini into 22 nt-long siRNAs, which is clearly an ATP-independent process (Zhang et al. 2002). Testing the human recombinant Dicer in the presence of human cell extracts without ATP revealed the same nuclease activity as in the presence of ATP, but compared to the *Drosophila* Dicer the catalytic efficiency is much lower. Since common RNasesIII do not show an ATP requirement, it might be specific for the RNAi mechanism in *Drosophila*. The results from mammalian Dicer suggest that direct dsRNA cleavage by Dicer may not involve ATP, but do not exclude the necessity of an ATP-dependent catalytic activity in the RNAi pathway (Provost et al. 2002). Further results suggest that, if ATP is necessary for the Dicer cleavage reaction, it might be involved in the siRNA release – a process which is also Mg^{2+}-regulated (Zhang et al. 2002).

Evolutionarily conserved homologues of Dicer exist in *C. elegans* (Grishok et al. 2001; Hutvagner et al. 2001), *Arabidopsis thaliana* (Jacobsen et al. 1999), mammals, and *Schizosaccharomyces pombe* (Volpe et al. 2002), where they might share similar biochemical functions. Recently, the cDNAs of murine and human Dicer were identified. The mouse cDNA spans 6.13 kilobases (kb), and encodes for a polypeptide of 1906 amino acids (Figure 5), which shares 92% sequence homology with the human orthologue (Nicholson and Nicholson 2002).

In *Arabidopsis*, two species of siRNAs have been detected, of which the shorter 21-mers appears to be responsible for mRNA degradation, while the longer 24- to 25-nt species are held responsible for the systemic spreading of the effect (Hamilton

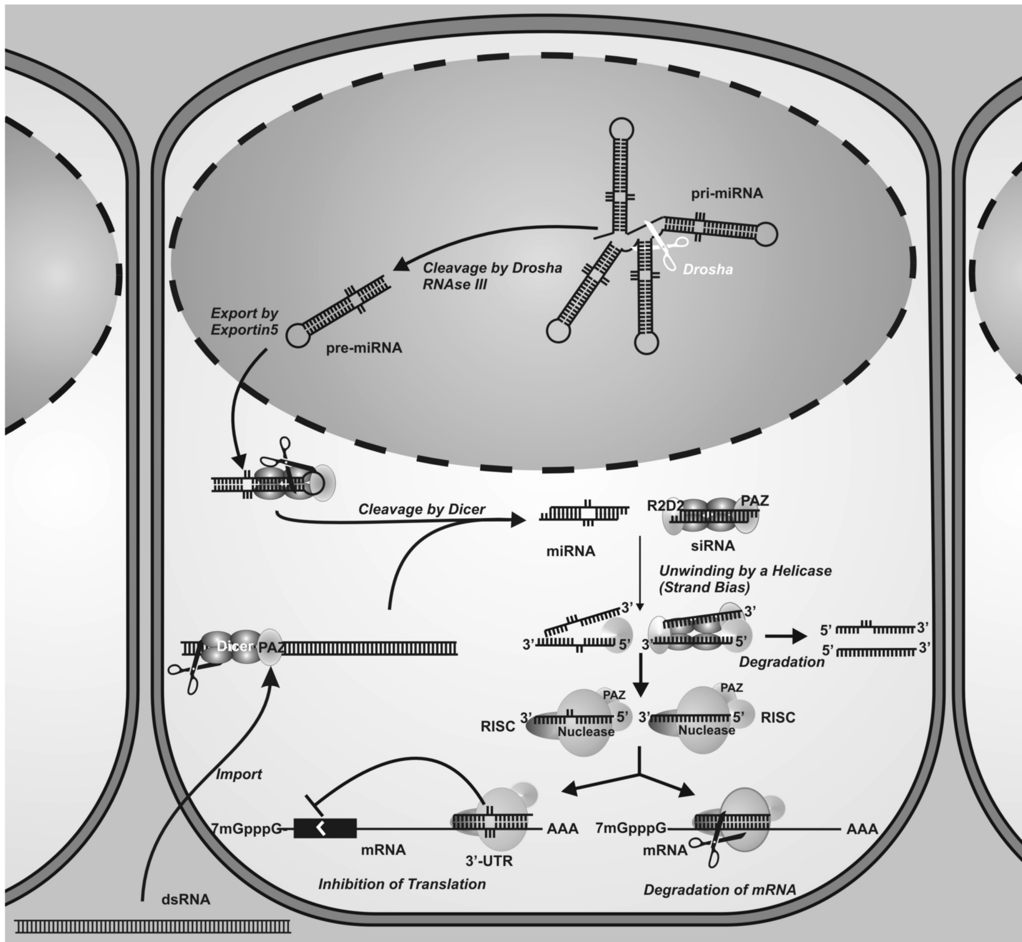

Fig. 1.1 Schematic view of siRNA- and miRNA-induced gene silencing.

et al. 2002). Mutants deficient of the CARPEL/FACTORY gene are deficient of siRNA production, indicating that the plant homologue of Dicer is encoded by this locus. Studies in wheat germ extracts led to the assumption that the two species of siRNAs might originate from the action of two distinct Dicer orthologues, one favoring the production of 21-mers from exogenous dsRNA, the other being responsible for the production of 24–25-mers from dsRNA derived from transgenic mRNA (Tang et al. 2003)

Although only one Dicer enzyme is found in *C. elegans* and humans, two Dicer homologues, DCR-1 and DCR-2, have been identified in *Drosophila* (Bernstein et al. 2001). In fact, recent findings clearly demonstrate that Dicer is involved in more processes than cleavage of dsRNA after a viral attack.

1.4 miRNAs versus siRNAs: Two Classes of Small RNAs Using the RNAi Pathway?

During mechanistic studies of RNAi in *C. elegans*, another species of small RNAs was discovered which resembled the cleavage pattern of an RNaseIII. lin-4 (lineage-abnormal-4) and let-7 (lethal-7) RNAs are expressed as 22-nt RNAs, having been processed from a ~70-nt precursor hairpin RNA. Additionally, Dicer-1-deficient (Dcr-1) *C. elegans* mutants show deficiencies in development, fertility and in RNAi (Grishok et al. 2001; Ketting et al. 2001 a; Knight 2001). The phenotypes resembled the ones observed with let-7- and lin-4-deficient worms that exhibit heterochronicity (Reinhart et al. 2000) and affect larval transition (Lee et al. 1993). More remarkably, the Dcr-1 phenotype could be rescued by the application of short RNA transcripts encoded by the let-7 and lin-4 loci (Hutvagner et al. 2001). It was assumed that inside the nucleus a longer precursor is encoding the ~70-nt hairpin RNAs that form by folding back to a stem-loop structure bearing one or two mismatches in the double-stranded region (Lee and Ambros 2001). Besides its role in initiating RNAi, Dicer also cleaves these 70-nt precursor RNA stem-loop structures known as small temporal RNAs (stRNAs) or others known as microRNAs (miRNAs) derived from larger stem-loop precursors into single-stranded 21- to 23-nt RNAs during germline development of *C. elegans* (Grishok et al. 2001; Hutvagner et al. 2001; Ketting et al. 2001 b; Reinhart and Bartel 2002). They do not trigger RNA degradation, but rather bind with partially complementary binding sites at the 3′-UTR of the mRNA to inhibit the translation of specific genes (Olsen and Ambros 1999; Seggerson et al. 2002; Slack et al. 2000).

Hundreds of small RNAs of miRNAs have been discovered recently in animals and plants (Lagos-Quintana et al. 2001, 2002, 2003; Lau et al. 2001; Lee and Ambros 2001; Lee et al. 1993; Mourelatos et al. 2002; Park et al. 2002; Reinhart et al. 2000, 2002). Although some of their functions are being unraveled (Brennecke et al. 2003; Kawasaki and Taira 2003 a, b; Llave et al. 2002; Tang et al. 2003), their mechanism of biogenesis remains poorly understood. The generation of miRNAs occurs via sequential processing and maturation of long primary transcripts (pri-miRNAs). A preprocessing happens in the nucleus, where the pri-miRNAs are cleaved into stem-loop precursors of ~70 nt (pre-miRNAs), which are eventually exported into the cytosol by Exportin 5 (Lund et al. 2003). As reported recently, Exportin 5 specifically binds correctly processed pre-miRNAs, while interacting only weakly with extended pri-miRNAs (Lund et al. 2003). Dicer is now mediating the final cleavage of the pre-miRNAs into mature miRNAs (Grishok et al. 2001; Hutvagner et al. 2001; Ketting et al. 2001 b; Knight and Bass 2001; Lee et al. 2002). It was previously speculated that Dicer is also actively involved in the processing of pri-miRNAs in the nucleus, since in-silico analysis of the various Dicer orthologues identified several nuclear localization signals (NLS) within each sequence (Nicholson and Nicholson 2002). Although experimental evidence indicates that RNAi operates in the cytoplasm, the predicted NLS suggest possible additional functions for Dicer in the nucleus.

In-vitro digestion of pri-miRNAs using Dicer as a nuclease revealed an incorrect miRNA processing (Lund et al. 2003), leading to the speculation that the nuclear processing enzyme is different from Dicer. Recently, human Drosha – another

RNase III type nuclease, which localizes predominantly to the nucleus (Wu et al. 2000) – was found to be the core nuclease that executes the initiation step of miRNA processing in the nucleus (Lee et al. 2003). Immunopurified Drosha cleaves pri-miRNA to release pre-miRNA in vitro. Furthermore, RNA interference of Drosha resulted in the strong accumulation of pri-miRNA and the reduction of pre-miRNA and mature miRNA in vivo, showing a collaboration of Drosha and Dicer in miRNA processing (Lee et al. 2003).

Like siRNAs, miRNAs show the Dicer-specific staggered cut and bear 5′-monophosphate and 3′-hydroxyl groups (Elbashir et al. 2001 a, b; Hutvagner et al. 2001). However, miRNAs seem to recognize their targets by imperfect base pairing, with the exception of those occurring in plants, where it has been shown that miRNAs with high complementarity direct RNAi by guiding an endonuclease to cleave efficiently mRNA for correct plant development. The imperfect base pairing occurring in animals makes it very difficult to locate their targets and to predict the miRNA function. *Drosophila* lacks the miRNA precursor completely, but it can process the transgenic precursor to mature miRNA, supporting the idea of Dicer being the responsible factor.

However, siRNAs and miRNAs were found to be functionally interchangeable. If synthetic siRNAs bear a sufficiently low degree of complementary bases, target translation will be inhibited without degradation (Ambros et al. 2003 b), whereas miRNAs will lead to mRNA degradation if a target with perfect complementarity is provided (Doench et al. 2003; Hutvagner and Zamore 2002 a; Zeng and Cullen 2003).

In human cell extracts, the miRNA *let*-7 naturally enters the RNAi pathway, suggesting that only the degree of complementarity between an miRNA and its RNA target determines its function (Hutvagner and Zamore 2002 a).

Very early on, it was assumed that the distinction of the two mechanisms could be conveyed by the presence of wobble-base pairs resulting from mismatches in the homology region of miRNAs and their targets (Ha et al. 1996).

1.5
RISC – the Effector to "Slice" the mRNA?

In vivo, Dicer is part of a protein complex. Today, even if the molecular mechanism of Dicer-mediated dsRNA cleavage is partially unraveled, it is still not fully clear how the initiator step is connected to the effector step of the RNAi process, since Dicer is not directly involved in the target cleavage process (Martinez et al. 2002). During the past two years, several protein factors have been identified that seem to play a role as interaction partners or even RNAi signal transporters.

During the early mechanistic studies on RNAi it was assumed that the newly generated siRNAs form a ribonucleotide protein complex (RNP) with some unknown proteins. This promotes unwinding of the RNA duplex, presumably in an ATP-dependent manner, and leads to the final activation of the RNA-induced silencing complex called RISC. Eventually, this complex presents the antisense strand of the siRNA to the target mRNA and guides mRNA degradation (Zamore et al. 2000).

Several recent studies have shown that Dicer and several components of the RISC could be co-purified, suggesting an association between the initiation and effector complex, although Dicer is not required for the final target cleavage. The connection between the two reaction steps is the transfer of siRNA, which are not assumed to move freely throughout the cytoplasm.

To date, the RISC complex is barely characterized, but it appears that RISC from *Drosophila* is a ~500 kDa complex bound to ribosomes in cell-free extracts (Nykänen et al. 2001). Closer studies of the protein complex revealed that RISC contains a DEAD-box helicase and an elusive nuclease. These constituents seem to be conserved in *Drosophila*, *C. elegans*, and mammals, although the overlap is not complete (Carmell et al. 2002).

The helicase domain is probably required to unwind the siRNA, as the tight binding of the complementary strands would prevent any specific target recognition. This is achieved in an ATP-dependent step that leads to the remodeling of the complex into its active form referred to as RISC* (Hammond et al. 2000; Nykänen et al. 2001). The antisense strand then serves as a template for the recognition of homologous mRNA (Martinez et al. 2002; Tijsterman et al. 2002) which, upon binding to RISC*, is cleaved in the center of the recognition sequence 10 nt from the 5′-end of the siRNA antisense strand (Hutvagner and Zamore 2002 b) by the nuclease activity of the complex. The two fragments are subject to degradation by unspecific exonucleases. The template siRNA is not affected by this reaction, so that the RISC can undergo numerous cycles of mRNA cleavage that comprise the high efficiency of RNAi. Recently, a nuclease was purified in association with the RISC complex. This was an evolutionarily conserved 103-kDa protein comprising five repeats of a nuclease domain usually found in *Staphylococcus* bacteria. While four of the five RNase domains remain active, the fifth is fused to a Tudor domain which is involved in the binding of modified amino acids, which gives the nuclease its name, Tudor-staphylococcal nuclease (Tudor-SN) (Caudy et al. 2003). The nuclease has been shown to be conserved in plants (Ponting 1997), *C. elegans*, *Drosophila* (Callebaut and Mornon 1997; Caudy et al. 2003; Ponting 1997), and mammals (Callebaut and Mornon 1997), but is rather responsible for the unspecific degradation of the mRNA remainder than for the siRNA-targeted specific mRNA cleavage (Caudy et al. 2003).

Further compounds of RISC are siRNAs and proteins, one of which was identified as Argonaute-2 (Hammond et al. 2001). Like Dicer, Argonaute-2 contains a PAZ domain and appears to be essential for the nuclease activity of RISC (Hammond et al. 2001). Moreover, using affinity-tagged siRNAs, Tuschl and colleagues showed that single-stranded siRNA resides in the RISC together with mammalian homologues of Argonaute proteins Ago-2, eIF2C1 and/or eIF2C2 (Martinez et al. 2002), which contain two characteristic domains, PAZ and PIWI. The PAZ domain plays an essential role in RNAi, since a mutation in the PAZ domain of the *C. elegans* RDE-1 gene correlates with an RNAi-deficient phenotype (Cerutti et al. 2000). It is highly conserved and is found only in Argonaute proteins and Dicer. Structural analysis revealed highly conserved structural residues, suggesting that PAZ domains in all members of the Argonaute and Dicer families adopt a similar fold with a nucleic-acid binding function (Lingel et al. 2003). Even though the binding affinity for nu-

cleic acids is usually low, PAZ domains exhibit enhanced affinity siRNA binding, most likely interacting with the extended 3′ ends or the 5′-phosphorylated ends of siRNAs for their specific incorporation into the RNAi pathway (Song et al. 2003; Yan et al. 2003). Recently, several reports proposed the atomic structure of the PAZ domain to contain a six-stranded β-barrel with an additional appendage, to bind both single- and double-stranded RNA in a sequence-independent manner (Lingel et al. 2003; Song et al. 2003; Yan et al. 2003). This revealed a nucleic acid-binding fold that is stabilized by conserved hydrophobic residues. NMR studies on the PAZ-siRNA complex suggest two modes of possible binding mechanisms: The lack of sequence specificity suggests either multiple PAZ domain molecules binding to a single RNA molecule, forming a complex analogous to "protein beads on an RNA string" (Yan et al. 2003), or a single PAZ domain is engaged in different modes of interactions with a single RNA molecule such as "sliding" through the RNA sequence, resulting in the co-existence of different complex species. RISC, which can be separated from the Dicer fraction by centrifugation of *Drosophila* S2 lysates at 100 000 *g*, is not able to cleave dsRNA. There was a speculation that Dicer and the RISC complex physically interact between the two PAZ domains of Argonaute-2 and Dicer, facilitating incorporation of siRNAs out of the Dicer complex into RISC (Hammond et al. 2001). In parallel to the solution of the PAZ structure, another protein – R2D2 – was found which is probably the key player in the Dicer-RISC interaction. Wang and coworkers termed this the 36-kDa small protein with tandem dsRNA binding domains (R2) and a *Drosophila* Dicer-2 binding domain (D2) R2D2 (Liu et al. 2003). Like its putative *C. elegans* homologue RDE-4 (Grishok et al. 2000; Tabara et al. 2002), it forms a stable complex with the nascent siRNAs, and has been shown to be essential for transfer of the siRNA from the initiator complex Dicer to the molecular components responsible for the effector step (Liu et al. 2003). While Dicer alone is sufficient to cleave dsRNA, it needs R2D2 to bind not only the nascent siRNAs but also synthetic siRNAs.

This model is supported by previous studies, in which it was shown that if the dsRNA was processed from the 5′ → 3′ direction of the sense strand, it would generate RISC that can mediate degradation of the sense but not antisense target mRNA, and vice versa (Elbashir et al. 2001 b). Since synthetic siRNAs do not need a cleavage process by Dicer rather than a binding by Dicer/R2D2 complex, they can be transferred to RISC in an nonoriented fashion, leading to the degradation of either sense or antisense target mRNA. This suggests that newly synthesized symmetric siRNA generated from a longer dsRNA is not released from the complex, but rather is retained by DCR-2/R2D2 in a fixed orientation, which is determined by the direction of dsRNA processing. Then, only the antisense strand can become the guiding RNA for RISC (Liu et al. 2003).

If this mechanism is homologous to *C. elegans*, where RDE-4 also interacts with RDE-1, an AGO2 homologue and a RISC component, it can be proposed that R2D2 play a similar role in bridging the initiation and effector steps of the *Drosophila* (Liu et al. 2003; Tabara et al. 2002).

In contrast to the results in *Drosophila*, the human RISC is found in the 100 000 *g* fraction of HeLa cells (Hutvagner and Zamore 2002 b), revealing a slightly different

localization of the complex and a variation of the mechanism. Dicer, as well as the human RISC, are both localized in the cytosol. Determination of the RNAi mechanism in invertebrates does not necessarily imply that it is the same in humans.

Studies with chemically synthesized short dsRNAs showed that only siRNAs with lengths between 20 and 23 base pairs are able to integrate into the RISC and to guide this complex to its substrate mRNA by conventional base-pairing (Hammond et al. 2000). Recognition of mRNAs by RISC eventually triggers their destruction. Common models of RNAi propose that only the antisense strand of siRNAs is part of the RISC complex, thus provoking the question of whether ssRNA of appropriate size can mediate RNAi.

An important result which has emerged from recent studies is that single-stranded antisense RNA ranging from 19 to 29 nt can also enter the RNAi pathway, albeit less efficiently, than the double-stranded siRNA (Martinez et al. 2002; Schwarz et al. 2002). Zamore and colleagues showed that with siRNA there is a profound strand bias in the mRNA targeting and cleavage. Even if the separate antisense and sense strand of a distinct siRNA reveal a similar intrinsic efficacy in targeting the mRNA, they show different activities when hybridized to a duplex siRNA. The stability of the 5′-end determines which strand enters into RISC, whereas the other strand is degraded (Schwarz et al. 2003). 5′-ends starting with an A-U base pairing are preferred over those beginning with G-C, the hypothesis being that a less stable 5′-end will be preferentially accepted by RISC. Even an energy difference corresponding to a single hydrogen bond can largely favor the incorporation of one strand over the other (Schwartz et al. 2003). Statistical analysis of the internal energies of a vast number of naturally occurring siRNAs and synthetic siRNAs has recently revealed a decreased stability at the 5′-ends of the functional duplexes and a slightly decreased stability between base pairs 9–14 counting from the 5′-terminus (Khvorova et al. 2003). The 5′-instability is assumed to facilitate duplex unwinding by the DEAD box helicase. Mutational analysis of the siRNA strands revealed that the decreased stability between base pairs 9–14 might also facilitate the dissociation–association reaction observed for the DEAD box helicase (Nykänen et al. 2001). It is also likely to play a role in target cleavage that takes place between the 9th and 10th base pair from the 5′-end of the guiding ssRNA strand, or in the release of the mRNA fragments. From siRNAs isolated from cytosolic extracts it was concluded that the natural selection of siRNAs is based on thermodynamic properties rather than mere function. Those studies raised the question whether the asymmetry found in the miRNA strand selection of RISC is closely related to the asymmetric incorporation of siRNA strands. Very early on, it was assumed that the distinction of the two mechanisms could be conveyed by the presence of wobble-base pairs resulting from mismatches in the homology region of miRNAs and their targets (Ha et al. 1996). This goes along with studies showing that duplex unwinding plays a crucial role in the processing of siRNAs and miRNAs and their incorporation into RISC (Bernstein et al. 2001; Nicholson and Nicholson 2002). Mature miRNAs are usually unstable at their 5′-end and present a lower stability near their center.

For miRNAs, it is the miRNA strand of a short-lived, siRNA duplex-like intermediate that assembles into a RISC complex, causing miRNAs to accumulate in vivo as

single-stranded RNAs. Alternatively, both strands of siRNA could be integrated into RISC and form a triple helix with the target mRNA.

From this, it was concluded that the effector complexes containing siRNAs and miRNAs are related, but function by different mechanisms. Exogenously supplied siRNAs and shRNAs with single mismatches fail to repress the translation of their target gene (Elbashir et al. 2001 a; Paddison et al. 2002). However, siRNAs and miRNAs were found to be functionally interchangeable. If synthetic siRNAs bear a sufficiently low degree of complementarity, target translation will be inhibited without degradation (Ambros et al. 2003 a, b), whereas miRNAs will lead to mRNA degradation if a target with perfect complementarity is provided (Doench et al. 2003; Hutvagner and Zamore 2002 a; Zeng and Cullen 2003).

1.6 Are RNA-Dependent RNA Polymerases (RdRps) Responsible for the Catalytic Nature of RNAi?

Considering the high efficiency of RNA degradation, as was first observed in *C. elegans* (Fire 1994), the question arises as to whether this is due to a form of catalysis or to an amplification mechanism.

The main question of why RNAi is so much more powerful than the antisense approach led to investigations of RNA-dependent RNA polymerases (RdRps). In 2000, Dalmay reported that the suppression of transgenes in *Arabidopsis* is disrupted in sgs2/sde2 mutants. This locus encodes a putative RNA-dependent RNA polymerase (RdRp) (Dalmay et al. 2000; Mourrain et al. 2000). Since then, various RdRp proteins have been identified in a number of organisms, such as EGO-1, RRF-1 and RRF-2 in *C. elegans* (Sijen et al. 2001; Simmer et al. 2002; Smardon et al. 2000) and QDE-1 in *Neurospora* (Cogoni and Macino 1999).

RdRps are enzymes that are characteristically involved in RNA-virus replication by synthesizing complementary RNA molecules using RNA as a template.

In cells displaying RNAi, RdRp is assumed to convert the single-stranded target mRNA to dsRNA using the antisense strands of primary siRNAs as primers (Lipardi et al. 2001; Sijen et al. 2001). After Dicer-mediated cleavage of dsRNA, the resulting primary siRNAs are proposed to bind to their complementary target mRNA and to be extended by nucleotide addition in a target-dependent manner to form dsRNA. The resulting dsRNA can then be cleaved by Dicer to form siRNAs that lead to degradation of the mRNA. Since RdRp should be capable of transforming all targeted mRNA to dsRNA, the nuclease activity of Dicer would be sufficient to completely cleave trigger-dsRNA and also the target-mRNA.

In the course of this process – termed random degradative PCR (Lipardi et al. 2001) – the regions upstream of the primary dsRNA sequence are also amplified, leading to a set of secondary siRNAs that mediate cleavage of sequences that do not show homology to the primary dsRNA sequence. Notably, such a mechanism would not necessarily include the RNA-induced silencing complex (RISC) as an additional nuclease. The model is supported by a report of an antisense RNA ranging from 19

to 40 nt effectively triggering germline RNAi in *C. elegans* in the presence of Dicer (Tijsterman et al. 2002). In several organisms such transitive RNAi has been observed, in which siRNAs of a sequence beyond the targeted region of homology are detected (Sijen et al. 2001).

So far, the role of various RdRps remains to be clarified. Mutation studies in *C. elegans* showed that the RdRp rff-1 is essential for RNAi (Sijen et al. 2001), suggesting that primary siRNAs are neither quantitatively nor qualitatively sufficient for RNAi, and that RdRp plays an additional role for RNAi distinct from synthesis of secondary siRNAs.

Further studies in plants revealed that transitive RNAi was found to proceed in both $5' \rightarrow 3'$ or $3' \rightarrow 5'$ directions, pointing out that aberrant mRNAs from altered chromatin structures serve as substrates for RdRps. Experiments with wheat germ extracts have shown that ssRNA originating from transgenes is amplified by an RdRP, albeit no corresponding siRNAs are present (Tang et al. 2003). However, previous reports from experiments in *Drosophila* indicated an mRNA cleavage only within the homology sequence that it shares with the siRNA (Nykänen et al. 2001; Schwarz et al. 2002). In flies and mammals, no cellular RdRp for the generation of secondary siRNAs has been found (Stein et al. 2003). Thus, the high efficiency of RNAi can only be accounted for by the catalytic nature of RISC.

In these organisms, RISC is assumed to turn over many times, thereby presenting evidence for its catalytic nature. Again, it should be mentioned that different mechanisms have apparently evolved in different species for amplification of the silencing effect.

1.7
Is RNAi Involved in the Regulation of Gene Expression?

A few years after the discovery of co-suppression, it was found that in plant dsRNA also leads to methylation of genomic DNA (Wassenegger et al. 1994). No effect on transcription was observed if stretches of the coding sequence were affected, but it was terminated upon the methylation of the promoter sequence (Mette et al. 2000). This so-called transcriptional gene silencing (TGS) is not only stable but also heritable (Jones et al. 2001; Pal-Bhadra et al. 2002). The findings that mutations in methyltransferases (MET1) and chromatin remodeling complexes (DDM1) in *Arabidopsis* have an influence on the efficiency and stability of post-transcriptional gene silencing (PTGS) (Jones et al. 2001) suggested a link between dsRNA-induced gene silencing and gene regulation in plants. Studies in *C. elegans* revealed that this connection also exists in animals. Mutations in mut-7 and rde-2 release the repression of transgenes that are otherwise silenced on the level of transcription due to the reorganization of chromatin by polycomb proteins (Tabara et al. 1999). Adding to this finding, it has recently been reported that proteins of the same family are required for RNAi under some conditions (Dudley et al. 2002; Hannon 2002).

Now, more recent studies – especially in *Schizosaccharomyces pombe* (Volpe et al. 2002, 2003) and *Tetrahymena* (Mochizuki et al. 2002; Taverna et al. 2002) – revealed

functional small RNAs complementary to centromeric repeats directly interacting with the chromatin remodeling (Reinhart and Bartel 2002) and chromosome dynamics (Hall et al. 2003).

In these organisms, these small RNAs seem to be part of mechanism that is responsible for the methylation of histone H3 at lysine 9 (H3K9) on genes corresponding to the small RNA. Thus far, H3K9 methylation triggers the formation of heterochromatin, leading to the repression of gene expression at this site. The generation of small RNAs and the H3K9 methylation require Dicer and putative RdRps. This observation suggests an implication of the RNAi pathway in the regulation of gene expression. Presumably, RdRp is recruited to chromatin by priming itself with small RNAs or siRNAs: If RdRp is primed while the repeats are being transcribed, coupling of RNA-dependent and DNA-dependent transcription would tether RdRP to the chromatin (Martienssen 2003). To date, some working models have been proposed, one of which is the idea that RISC-bound small RNAs are guiding a H3K9 methyltransferase to the respective DNA via their associated RdRps.

Nevertheless, a direct interaction between the small RNAs and the putative binding domains on the H3K9-methyltransferase was not observed (Hall et al. 2003).

Additionally, RNAi itself is not needed for the maintenance and inheritance of heterochromatin domains (Hall et al. 2002), suggesting a different mechanism of RISC in the nucleus. A further link between chromatin remodeling and RNAi was also found in *Drosophila* where mutations of PIWI, that is related to one of the RISC components, reduces the degree of silencing on both the transcriptional and post-transcriptional level (Pal-Bhadra et al. 2002).

All of these findings indicate that genomic DNA is affected by the cell's response to dsRNA. It is assumed that a nuclear variant of RISC exists that bears a chromatin remodeling complex instead of a nuclease activity.

The latest findings indicate that tandem repeats play a role in heterochromatin silencing (Martienssen 2003). It has been shown that tandemly repeated genes form heterochromatin and are prone to epigenetic silencing in many organisms (Dorer and Henikoff 1994). If those tandem repeats are transcribed and subsequently amplified by RdRps, dsRNA is generated that is cleaved into siRNAs by Dicer (Matzke et al. 2001; Mourrain et al. 2000). These siRNAs then not only lead to the degradation of the transcripts but also serve as primers to the RdRp so that a pool of siRNAs covering the full sequence is maintained and the stretch of heterochromatin remains silenced.

1.8 RNAi in Mammals

The interesting features of RNAi in *C. elegans* and *Drosophila* led to many research projects, which focused on adapting this technique to mammalian and human cell lines.

It appears that mammals have developed different pathways to combat parasitic and viral dsRNA, however. In mammalian cells, dsRNA causes an interferon response, which leads to the activation of RNase H degrading all mRNA transcripts in

Fig. 1.2 Schematic view of the proposed mode of small RNA action in the nucleus (modified from Finnegan and Matzke 2003).

an unspecific fashion. At the same time, γ-interferon triggers the activation of protein kinase R (PKR), which phosphorylates and thus inactivates transcription factor EIF2α. This leads to a global shutdown of protein biosynthesis and, as a result, to apoptosis (Clemens 1997; Clemens and Elia 1997). For this reason it was believed that RNAi could not be induced in mammalian cells.

Despite the arguments that RNAi would not function in mammalian cells, several independent groups proved the existence of mammalian RNAi pathways by the in-

troduction of dsRNA or vectors producing dsRNA into cell lines lacking the interferon machinery; examples were mice oocytes or mice embryonic cancer cell lines (Billy et al. 2001; Wianny and Zernicka-Goetz 2000). However, in most somatic mammalian cells this approach provokes a strong cytotoxic response. Unlike plants and nematodes, RNAi in mammalian cell lines underlies some serious limitations, and in most mammalian cells the approach of transient introduction of large dsRNAs is not feasible. The decisive breakthrough in acquiring the new RNAi technique in the field of mammalian functional genomics was the studies by Tuschl and co-workers (Elbashir et al. 2001 a). This group found that transiently applied siRNAs of 21–23 nt are able to trigger the RNA interference machinery in cultured mammalian cells, without initiating the programmed cell death response. Although these siRNAs are probably too short to trigger the interferon response, they are able to direct sequence-specific cleavage of homologous mRNAs in mammalian cells (Hutvagner 2000). Clearly, the siRNAs produced from long dsRNA by the enzyme Dicer are too short for the activation of PKR. Further studies by Tuschl and co-workers showed that dsRNAs shorter than 21 bp and longer than 25 bp are inefficient in initiating RNAi (Elbashir et al. 2001 c) as well as siRNAs with blunt ends. Only short dsRNAs with a 2-nt 3′-overhang, which resembles the naturally active products of Dicer, are efficient mediators of RNAi. With this technology even somatic primary neurons have been successfully treated to produce knock-down RNAi phenotypes (Krichevsky and Kosik 2002).

Until now, efforts to synthesize modified siRNAs more potent in inducing RNAi have failed. The replacement of siRNA 3′ two ribouridine overhangs at the 3′-end by two deoxy-thymidine overhangs resulted in a decreased induction of RNAi. Furthermore, complete replacement of either sense or antisense strand of siRNAs by DNA resulted in the reduction or complete loss of RNAi activity (Hohjoh 2002).

Several groups have investigated the minimal chemical requirements for siRNAs to function in RNAi by altering the 3′-end of the antisense strand with either 2‘,3′-dideoxy cytidine, amino modifier (Schwarz et al. 2002), puromycin, or biotin (Chiu and Rana 2002; Martinez et al. 2002). This does not inhibit siRNA action either in vivo or in vitro in *Drosophila* and human systems. However, data from Zamore's group revealed an absolute requirement for a 5′-phosphate residue for siRNAs to direct target-RNA cleavage in *Drosophila* embryo lysates, which is thought to be necessary for the so-called authentication in the assembly of the RISC by building noncovalent interactions with other components of the RNAi (Schwarz et al. 2002). Nonetheless, the 5′-phosphate requirement might also reflect a requirement for the phosphate group in covalent interactions, such as the ligation of multiple siRNAs to generate cRNA (Nishikura 2001).

The striking results from Philipp Zamore and co-workers of the siRNA strand bias will also help with the design of synthetic siRNAs with a high degree of silencing efficiency. In this context, the design of synthetic siRNAs that more closely resemble these double-stranded miRNA intermediates reveals highly functional siRNAs, even when targeting mRNA sequences apparently refractory to cleavage by siRNAs selected by conventional siRNA design rules (Schwarz et al. 2003).

1.9 Practical Approaches

RNAi procedures are much more rapid and straightforward than traditional genomics approaches, such as the generation of knock-out animal models or the study of inherited diseases (Arenz and Schepers 2003).

Beyond its biological relevance, PTGS is emerging as a powerful tool to study the function of individual proteins or sets of proteins. User-friendly technologies for introducing siRNA into cells, in culture or in vivo, to achieve a selective reduction of single or multiple proteins of interest are rapidly evolving.

This chapter has focused mainly on the mechanism and cellular requirements of RNAi. Further details – especially on the practical aspects derived from many recent publications – will be provided elsewhere in this book in order to avoid duplication. This comprises topics from systemic gene silencing and high-throughput applications in *C. elegans* to endogenous expression of short hairpin RNAs (shRNAs) in mammals. This book is intended as a laboratory manual and will provide useful protocols and working notes, not only for those who are novices in the RNAi field but also for those experts and users who might discover some new tricks.

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