RNA INTERFERENCE MEDIATING SMALL RNA MOLECULES

CROSS REFERENCE TO RELATED APPLICATION

This application is a divisional of Ser. No. 10/433,050 filed Jul. 26, 2004, which is a 35 USC § 371 National Phase Entry Application from PCT/EP01/13968, filed Nov. 29, 2001, and designating the U.S., which claims the benefit of provisional application No. 60/279,661, Mar. 30, 2001. All of these applications are incorporated herein by reference.

DESCRIPTION

The present invention relates to sequence and structural features of double-stranded (ds)RNA molecules required to mediate target-specific nucleic acid modifications such as RNA-interference and/or DNA methylation.

The term "RNA interference" (RNAi) was coined after the discovery that injection of dsRNA into the nematode *C. elegans* leads to specific silencing of genes highly homologous in sequence to the delivered dsRNA (Fire et al., 1998). RNAi was subsequently also observed in insects, frogs (Oelgeschlager et al., 2000), and other animals including mice (Svoboda et al., 2000; Wianny and Zernicka-Goetz, 2000) and is likely to also exist in human. RNAi is closely linked to the post-transcriptional gene-silencing (PTGS) mechanism of co-suppression in plants and quelling in fungi (Catalanotto et al., 2000; Cogoni and Macino, 1999; Dalmay et al., 2000, Ketting and Plasterk, 2000; Mourrain et al., 2000; Smardon et al., 2000) and some components of the RNAi machinery are also necessary for post-transcriptional silencing by co-suppression (Catalanotto et al., 2000; Dernburg et al., 2000; Ketting and Plasterk, 2000). The topic has also been reviewed recently (Bass, 2000; Bosher and Labouesse, 2000; Fire, 1999; Plasterk and. Ketting, 2000; Sharp, 1999; Sijen and Kooter, 2000), see also the entire issue of Plant Molecular Biology, vol. 43, issue 2/3, (2000).

In plants, in addition to PTGS, introduced transgenes can also lead to transcriptional gene silencing via RNA-directed DNA methylation of cytosines (see references in Wassenegger, 2000). Genomic targets as short as 30 bp are methylated in plants in an RNA-directed manner (Pelissier, 2000). DNA methylation is also present in mammals.

The natural function of RNAi and co-suppression appears to be protection of the genome against invasion by mobile genetic elements such as retrotransposons and viruses which produce aberrant RNA or dsRNA in the host cell when they become active (Jensen et al, 1999; Ketting et al., 1999; Ratcliff et al., 1999; Tabara et al., 1999). Specific mRNA degradation prevents transposon and virus replication although some viruses are able to overcome or prevent this process by expressing proteins that suppress PTGS (Lucy et al.; 2000; Voinnet et al., 2000).

DsRNA triggers the specific degradation of homologous RNAs only within the region of identity with the dsRNA (Zamore et al., 2000). The dsRNA is processed to 21–23 nt RNA fragments and the target RNA cleavage sites are regularly spaced 21–23 nt apart. It has therefore been suggested that the 21–23 nt fragments are the guide RNAs for target recognition (Zamore et al., 2000). These short RNAs were also detected in extracts prepared from *D. melanogaster* Schneider 2 cells which were transfected with dsRNA prior to cell lysis (Hammond et al. 2000), however, the fractions that displayed sequence-specific nuclease activity also contained a large fraction of residual dsRNA.

The role of the 21–23 nt fragments in guiding mRNA cleavage is further supported by the observation that 21–23 nt fragments isolated from processed dsRNA are able, to some extent, to mediate specific mRNA degradation (Zamore et al., 2000). RNA molecules of similar size also accumulate in plant tissue that exhibits PTGS (Hamilton and Baulcombe, 1999).

Here, we use the established *Drosophila* in vitro system (Tuschl et al., 1999; Zamore et al., 2000) to further explore the mechanism of RNAi. We demonstrate that short 21 and 22 nt RNAs, when base-paired with 3' overhanging ends, act as the guide RNAs for sequence-specific mRNA degradation. Short 30 bp dsRNAs are unable to mediate RNAi in this system because they are no longer processed to 21 and 22 nt RNAs. Furthermore, we defined the target RNA cleavage sites relative to the 21 and 22 nt short interfering RNAs (siRNAs) and provide evidence that the direction of dsRNA processing determines whether a sense or an antisense target RNA can be cleaved by the produced siRNP endonuclease complex. Further, the siRNAs may also be important tools for transcriptional modulating, e.g. silencing of mammalian genes by guiding DNA methylation.

Further experiments in human in vivo cell culture systems (HeLa cells) show that double-stranded RNA molecules having a length of preferably from 19–25 nucleotides have RNAi activity. Thus, in contrast to the results from *Drosophila* also 24 and 25 nt long double-stranded RNA molecules are efficient for RNAi.

The object underlying the present invention is to provide novel agents capable of mediating target-specific RNA interference or other target-specific nucleic acid modifications such as DNA methylation, said agents having an improved efficacy and safety compared to prior art agents.

The solution of this problem is provided by an isolated double-stranded RNA molecule, wherein each RNA strand has a length from 19–25, particularly from 19–23 nucleotides, wherein said RNA molecule is capable of mediating target-specific nucleic acid modifications, particularly RNA interference and/or DNA methylation. Preferably at least one strand has a 3'-overhang from 1–5 nucleotides, more preferably from 1–3 nucleotides and most preferably 2 nucleotides. The other strand may be blunt-ended or has up to 6 nucleotides 3' overhang. Also, if both strands of the dsRNA are exactly 21 or 22 nt, it is possible to observe some RNA interference when both ends are blunt (0 nt overhang). The RNA molecule is preferably a synthetic RNA molecule which is substantially free from contaminants occurring in cell extracts, e.g. from *Drosophila* embryos. Further, the RNA molecule is preferably substantially free from any non-target-specific contaminants, particularly non-target-specific RNA molecules e.g. from contaminants occuring in cell extracts.

Further, the invention relates to the use of isolated double-stranded RNA molecules, wherein each RNA strand has a length from 19–25 nucleotides, for mediating, target-specific nucleic acid modifications, particularly RNAi, in mammalian cells, particularly in human cells.

Surprisingly, it was found that synthetic short double-stranded RNA molecules particularly with overhanging 3'-ends are sequence-specific mediators of RNAi and mediate efficient target-RNA cleavage, wherein the cleavage site is located near the center of the region spanned by the guiding short RNA.

Preferably, each strand of the RNA molecule has a length from 20–22 nucleotides (or 20–25 nucleotides in mammalian cells), wherein the length of each strand May be the same or different. Preferably, the length of the 3'-overhang